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REVIEW

Diet, *H pylori* infection and gastric cancer: Evidence and controversies

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Abstract

Despite decreasing incidence and mortality rates, gastric cancer (GC) still remains the fourth most common cancer and the second most common cause of cancer-related deaths worldwide. Due to the limited treatment options, at present, prevention is likely to be the only effective means of controlling this disease. The success of a prevention strategy depends upon the understanding of etiological and pathogenic mechanisms underlying gastric carcinogenesis. The etiology of GC is multi-factorial, however, in the recent years, mounting evidence suggests that environmental factors play a key role. The most important environmental factors implicated in the pathogenesis of GC are diet and *H pylori* infection. Thus, modifications in lifestyle and dietary habit associated with eradication of *H pylori* infection could hypothetically represent the most promising potential targets for GC prevention. In this review we will address the evidence and the controversies on the role of these agents in non-cardia GC by focusing on retrospective and prospective observational studies and interventional trials.

Key words: Gastric cancer; *H pylori*; Diet; Observational studies; Interventional dietary trials

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INTRODUCTION

Despite the decreasing incidence and mortality rates observed worldwide over the last 50 years, gastric cancer (GC) still ranks as one of the most frequent and lethal cancers worldwide[1]. Today, GC is the fourth leading cancer type in incidence accounting for almost a million new cases diagnosed annually (International Agency for the Research on Cancer-IARC 2002)[2]. At present, primary or secondary prevention are likely to be the most effective means of reducing the incidence of and mortality from this disease. However, to be successful, this strategy depends upon knowledge of the etiologic factors involved in gastric carcinogenesis.

Topographically, GC may arise in the cardia of the stomach or more distally (non-cardia cancer)[3]. Besides the individual genetic susceptibility, epidemiological data suggest that environmental factors are the predominant cause of this disease even if the etiology and possibly the pathogenesis of these two types of cancer may be completely different[2,3].

The most important factors thought to be responsible for non-cardia GC development are diet and *H pylori* infection. In this review we will address the evidence of and the controversies on the role of these agents in non-cardia GC by focusing on retrospective and prospective observational studies and interventional trials.

DIET AND GASTRIC CANCER

The relationship between diet and cancer has been clearly demonstrated since the 1930s, in a series of experimental classical studies in which severe caloric restriction markedly reduced the occurrence of cancer in rodents[4]. In 1982 the World Health Organization (Food & Agriculture Organization) stated that eating habits were the main factor involved in GC risk.

Numerous epidemiology studies aimed at evaluating the role of diet in gastric carcinogenesis have been carried out both in high- and low-risk geographic areas (Tables 1-3). Despite the lack of homogeneity of age, ethnicity, socio-economic status of the populations studied as well as the different methodological approaches, one of the most remarkable features emerging from these studies is the consistency with which certain foods are reported as being important in the modulation of risk of developing GC.

Observational epidemiology studies

The majority of the case-control epidemiological studies[5-20] have shown that high intake of salted, pickled or smoked foods, as well as dried fish and meat and refined carbohydrates significantly increases the risk of developing

Table 1 Epidemiological studies (population-based case-control) on dietary factors and gastric cancer

Author	Yr	Geographic area	Case/Control *n*	Increased risk	Decreased risk
Risch HA[5]	1985	Canada	246/146	Nitrite, chocolate, carbohydrates	Fiber, Vit. C
Buiatti E[6]	1990	Italy	1016/1159	Nitrites, protein	Vit. C, β-carotene, α-tocopherol, vegetable fat
Graham S[7]	1990	USA	293/293	Sodium, fat, retinol	β-Carotene, raw vegetables, onions, cucumbers
Ramon JM[8]	1993	Spain	117/234	--	Vit. A, Vit. C
Kaaks R[9]	1998	Belgium	301/2851	Vit. A, Vit. B12, mono, disaccharides	Polyunsaturated fat, Vit. C/B1-B2-B6, C/A
Lopez-Carrillo L[10]	1999	Mexico	220/752	Protein, saturated fat, cholesterol	Polyunsaturated fat, fiber, Vit. E
Mathew A[11]	2000	India	194/305	Rice, spicy foods, chili, high-temperature food	--
Palli D[12]	2001	Italy	382/561	Protein, nitrite, sodium	Vit. C/B6, β-carotene, α-tocopherol, nitrates
Mayne ST[13]	2001	USA	352/687	Animal protein, cholesterol, Vit. B12, nitrite	Fiber, β-carotene, folate, Vit. C
Jedrychowski W[14]	2001	Poland	80/--	Carbohydrates	Vit. E, β-carotene
Hamada GS[15]	2002	Brazil	97/192	Beef	Fruits
Chen H[16]	2002	Nebraska	124/449	Saturated fat	Fiber, Vit. C
Hara M[17]	2003	Japan	149/287	--	Cruciferous vegatables, mushrooms
Nomura AM[18]	2003	Hawaii	300/446	Processed meat, bacon	β-carotene, Vit. C, Vit. E, folate
Lagiou P[19]	2004	Greece	110/100	--	Flavanone
De Stefani E[20]	2004	Uruguay	240/960	Salted-stewed meat, rice, tuber	Vegetables, legumes, fruit, black tea

Table 2 Epidemiological prospective cohort studies on association between dietary factors and GC (1990-2004)

Author	Yr	Geographic area	Subjects *n*	FU yr	Increased risk	Decreased risk	No effect
Chyou PH[21]	1990	USA (Hawaii)	8006	18	--	Green/ cruciferous vegetables, fruit	--
Kneller RW[22]	1991	USA	17633	20	Carbohydrates, salted-fish, bacon, cooked cereals, milk	--	--
Kato I[23]	1992	Japan	9753	6	Alcohol, broiling meat	Fruit	--
Nomura A[24]	1995	USA (Hawaii)	8006	25	--	Fruit, vegetables	Alcohol
Dorant E[25]	1996	The Netherlands	120852	3.3	--	Onions	Leek, garlic
Goldbohm RA[26]	1996	The Netherlands	120852	4.3	--	--	Black tea
Ocke MC[27]	1998	The Netherlands	12763	25	--	Vegetables, fruit, fiber-rich cereals	--
Terry P[28]	1998	Sweden	11946	25	--	Fruit, vegetables	--
Galanis DJ[29]	1998	USA (Hawai)	11907	14.8	Coffee	Fruit, raw vegetables	Pickled vegetables, dried/salted fish
Knekt P[30]	1999	Finland	9985	24	--	--	Nitrates, nitrites, NDMA
Jansen MC[31]	1999	Netherlands	12000	25	Refined grains	Fruit	Vegetables Whole grain
Botterweck AA[32]	2000	The Netherlands	120852	6.3	Retinol, carotene	Vit. C	Folate, Vit. E, carotene, lycopene, fibers, Vit. A, BHA, BHT
Tsubono Y[33]	2001	Japan	26311	8	--	--	Green tea
McCullough ML[34]	2001	USA	1200000	14	Vegetables[1]	Vegetables, citrus fruit, whole grain[2]	--
Nagata C[35]	2002	Japan	33304	7	--	Soy products	--
Ngoan LT[36]	2002	Japan	13000	10	Processed meat, cooking oil, pickled food, soup	Green/yellow vegetables, fruit, cuttle-fish, tofu, potatoes	--
Kobayashi M[37]	2002	Japan	39993	10	--	Fruit, vegetables	--
Masaki M[38]	2003	Japan	5765	10	Meat pattern Rice/snack pattern	Vegetable and fruit pattern Western breakfast pattern	--
Khan MM[39]	2004	Japan	3158	18	Carbonated drink/juice[1]	Miso soup[2]	--
Kim MK[40]	2004	Japan	42112	10	Traditional dietary pattern	Healthy dietary pattern[1]	--
Sasazuki S[41]	2004	Japan	72743	11	--	Green tea[1]	--
[1]EPIC[42-44]	2006	Europe	521457	6.6	Red and processed meat, ENOC	Plasma vitamin C Total vegetable intake Onion, garlic	Dietary Vitamin C

[1]Effect limited to women; [2]Effect limited to men; FU: follow-up; NDMA: N-nitrosodimethylamine. EPIC: European prospective investigation into cancer and nutrition study; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene (cooking fats, oils, mayonnaise, creamy salad dressing, dried soup); ENOC: endogenous nitroso compounds.

GC while fiber, fresh vegetables and fruits were found to be inversely associated with GC risk (Table 1).

High consumption of refined carbohydrates has been shown to be associated with a significant increased risk of developing GC with an estimated odds ratio (OR) ranging from 1.5[5] to 8.73/100 mg of daily intake[14]. The increased trend in risk appeared particularly high in females (OR highest quartile of consumption frequency [Q4] *vs* lowest quartile [Q1] 14.8)[28]. High consumption of saturated fat and cholesterol enhanced the risk of cancer for intestinal

Table 3 Randomised controlled dietary intervention trials for prevention of stomach cancer

Author	Yr	Geographic area	Subjects *n*	Dietary intervention	Intervention (yr)	FU (yr)	Results
Wang GQ[67]	1994	China	29584	retinol/zinc; riboflavin/niacin; Vit. C/molybdenum; carotene/Vit.E/selenium	5.25	5.25	↓ gastric cancer mortality
Varis K[68]	1998	Finland	29133	α-tocopherol 50 mg/d; β-carotene 20 mg/d;	5	5	= gastric cancer incidence
Malila N[69]	2002	Finland	29133	α-tocopherol 50 mg/d; β-carotene 20 mg/d Folate 20 mg/d + Vit. B12 1 mg/mo	5-8	8	= gastric cancer incidence No change cancer incidence
Zhu S[70]	2003	China	216	Natural β-carotene 30 mg/d Synthetic β-carotene 30 mg/d	2	8	↓ precancerous lesions
Li H[71]	2004	China	2526	Synthetic allitridum 200 mg + selenium 100 mg	2	5	No change cancer incidence

FU: follow-up.

type GC (OR Q4 *vs* Q1 4.37; 95% CI 1.89-10.12 for saturated fat and OR Q4 *vs* Q1 2.39; 95% CI 1.23-4.64 for cholesterol)[10].

The analysis of dietary micronutrients (vitamin C, vitamin E, carotenoids, fiber, flavonoids and selenium) commonly held to be protective against GC yielded conflicting results. While evidence on the protective effect of beta-carotene has been very consistent, the approximate halving risk associated with vitamin C intake, reported in some studies (OR ranging from 0.3; 95% CI 0.1-0.8 to 0.60; CI 0.41-0.88)[8,9,12,13] has not been confirmed in others[5,18,19].

Epidemiological approaches of case-control design could, in part, account for these contrasting results. Indeed, observational case-control studies are biased by the retrospective assessment of exposure to dietary risk factors: the onset of the symptoms affects the dietary habit and it is difficult to determine it following the diagnosis of cancer ("recall-bias").

Observational cohort studies, in which the evaluation of diet is unaffected by symptoms, should ideally provide much more reliable evidence. Analysis of the data obtained in 21 studies involving a total of 1 651 231 individuals, followed for periods ranging between 3.3 and 25 years[21-44], substantially confirmed the significant increased risk of developing GC due to high intake of total carbohydrates, salted fish, processed meat, refined grains and saturated fat[22,31,36].

Two Japanese studies based on the analysis of dietary patterns failed to demonstrate an increased risk of GC in middle-aged males with a "meat" or "rice" prevalent diet (relative risk [RR] 1.00; 95% CI 0.55-1.10 and RR 1.00; 95% CI 0.52-1.19, respectively)[38] while the "traditional pattern" was a risk factor for both genders (RR 2.88; 95% CI 1.76-4.72 for males and RR 2.40; 95% CI 1.32-4.35 for females)[40]. A large prospective study on diet and cancer carried out on 521 457 individuals aged 35-70 years recruited in 10 European countries (EPIC-European Prospective Investigation into Cancer and Nutrition study), by analyzing 314 incident cases of GC that had occurred after 6.6 average years of follow-up, reported a significant increase of non-cardia cancer risk associated with intake of total meat (calibrated HR per 100 g/d increase 3.52; 95% CI 1.96-6.34), red meat (calibrated HR per 50 g/d increase 1.73; 95% CI 1.03-2.88), and processed meat (calibrated HR per 50-g/d increase 2.45; 95% CI 1.43-4.21). The risk of developing GC was particularly high in *H pylori* antibody-positive subjects[42]. Similar results were obtained for the endogenous formation of nitroso compounds (ENOC). ENOC was significantly associated with non-cardia cancer risk (HR 1.42; 95% CI 1.14-1.78 for an increase of 40 mg/d) especially in those cases with *H pylori* infection (P for interaction = 0.09)[43].

Data on the protective role of fresh fruit and vegetables against stomach cancer were somewhat controversial. The analysis of 11 546 individuals included in the Swedish Twin Registry demonstrated that the lowest compared to the highest fruit and vegetable intake had a RR of developing GC of 5.5 (95% CI 1.7-18.3) with a statistically significant dose-risk trend ($P < 0.05$)[28]. The Japan-Hawaii Cancer Study on 8006 Hawaiian men of Japanese ancestry reported that all types of vegetables were protective against GC. Subjects in the group of highest vegetable consumption ($\geqslant$ 80 g/d) had a RR of developing GC of 0.6 (95% CI 0.3-0.9) compared to non-consumers[21,24]. Green and yellow vegetables showed the highest protective effect against GC (RR 0.4; 95% CI 0.2-0.9 and 0.64; 95% CI 0.45-0.92, respectively)[36,37].

On the other hand, the Seven Countries Study Research Group found no association between total vegetable intake and GC risk[31]. Finally, the Cancer Prevention Study, on a cohort of 1.2 million United States individuals, demonstrated a reduced risk in males (RR 0.79; 95% CI, 0.67-0.93) and an unexpected increased risk in females (RR 1.25; 95 CI 0.99-1.58)[34].

Data from EPIC study analysing the association of plasma and dietary vitamin C levels with the risk of GC, after adjustment by body mass index, total energy intake, smoking (status, duration and intensity) and *H pylori* status demonstrated no association with GC risk for dietary vitamin C. In contrast an inverse GC risk was observed in the highest versus lowest quartile of plasma vitamin C (OR 0.55 95% CI 0.31-0.97). The inverse association was more pronounced in subjects consuming higher levels of red and processed meats, a factor that may increase endogenous N-nitroso compound production. The protective effect of plasma vitamin C was independent of GC anatomical sub-site (cardia *vs* non-cardia) or histological sub-type (diffuse *vs* intestinal) or presence of *H pylori* infection[44].

Several epidemiology studies specifically addressed the association of garlic consumption and risk of stomach cancer. Six case-control studies analyzing on the whole 3209 GC cases and 7600 controls, suggested a protective effect of high intake of raw and/or cooked garlic for

GC (OR ranging from 0.3 to 0.89; 95% CI 0.12-0.77 and 0.64-1.24, respectively)[6,45-49]. Only one cohort study (based on a case-cohort approach) compared the intake of garlic supplements of 152 subjects who developed GC during a 3.3 years follow-up with that of a random sample from the entire cohort who did not developed any type of cancer. Beside the expectative, garlic supplements slightly increased the risk of developing GC (RR 1.27; 95% CI 0.6-2.6)[25].

Tea is one of the most popular beverages in the world and the consumption of tea has been hypothesized to be associated with a decreased risk of GC[50]. The catechins and their strong antioxidant and anti-angiogenic activity as well as their potential to inhibit cell proliferation and modulate carcinogen metabolism could be responsible for the biological benefits of tea[51,52].

However, epidemiological studies analyzing the relationship between tea and GC risk yielded conflicting results[47,50,53-62]. Among the case-control studies, eight showed that high consumers of green tea (> 10 cups/d) had a statistically significant reduction of the risk of developing GC[47,50,53-58], three studies failed to demonstrate any significant decrease of the GC risk[59-61] and the remaining showed an opposite result[62]. The majority of the prospective studies did not find an inverse association between tea consumption and the risk of GC[26,33,63,64]. In contrast, three studies[41,65,66] confirmed the protective role of tea against GC particularly for non-cardia GC (OR 0.51 95% CI 0.30-0.86) in the highest category of green tea consumption ($\geqslant$ 5 cups/d *vs* $\leqslant$ 1 cup/d)[41]. On the basis of this epidemiological evidence no convincing claims can be made with regard to the protective effect of garlic and tea on GC. However, low study power, variability in consumption categorization within studies and poor adjustment for potential confounders may limit the reliability of any conclusion regarding garlic and tea supplementation.

Interventional dietary trials for prevention of gastric cancer

Randomized clinical trials provide one of the most scientifically rigorous approaches for testing hypotheses emerging from epidemiological and experimental studies and represent the ideal strategic approach to evaluate inhibition of cancer development by preventive measures.

The most relevant finding reported by the observational studies analyzing the role of diet in GC development concerned the inverse association between fruit and vegetable intake and GC risk. These foods contain phytochemicals endowed with anticancer and anti-inflammatory properties and are rich in ascorbic acid, beta-carotene and other carotenoids offering many health benefits. Dietary interventional trials for stomach cancer prevention have, therefore, been based mainly on long-term supplementation with anti-oxidant micronutrients given alone or in combination (beta-carotene, vitamin A, vitamin C, vitamin E, selenium)[67-71]. However, all interventional studies but one[70] failed to demonstrate any significant change in the risk of GC in subjects receiving anti-oxidant supplementation (Table 3). The most important study, the "General Population Trial" involving 29 584 subjects residing in Linxian, China, and followed for 5.25 years, demonstrated no statistically significant reduction in the prevalence of GC for any of the interventional arms, even though, a reduction in total mortality, total cancer mortality and stomach cancer mortality was found among those receiving beta-carotene, vitamin E and selenium[67]. Similar results were obtained in the Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention Study conducted in Southwest Finland and involving 29 133 middle-aged male smokers observed between 1985 and 1993[68,69]. Long-term supplementation with alpha-tocopherol (50 mg/d) and/or beta-carotene (20 mg/d), both at five- and six-year follow-up, had no significant effect on the overall incidence of GC (RR 1.21 95% CI 0.85-1.74 for alpha-tocopherol and RR 1.26 95% CI 0.88-1.80 for beta-carotene). Paradoxically, a subgroup analysis according to histological type suggested an increased risk for beta-carotene on intestinal type cancer (RR 1.59 95% CI 0.99-2.56)[68,69]. Finally, another study involving 216 atrophic gastritis patients treated with folic acid and/or beta-carotene supplementation and followed for a period of 8 years failed to demonstrate any significant reduction in the incidence of GC. However, folic acid significantly improved gastric mucosa lesions by reversing gastric atrophy, inflammation and intestinal metaplasia and dysplasia at the end of follow-up[70].

On the other hand, a double-blinded interventional study involving 2526 subjects at risk of developing GC and 2507 controls from a Chinese province, demonstrated, in the first five years of follow-up, a significant reduction in the morbidity rates of malignant gastric tumours in the intervention group treated with large doses of synthetic allitridum associated with microdoses of selenium for a period of 3 years (RR 0.48; 95% CI 0.21-1.06 for the entire population and RR 0.36; 95% CI 0.14-0.92 for male group)[71].

H PYLORI INFECTION AND GC RISK

Since the incidental discovery in 1983, the association of *H pylori* with GC has become a hot topic of gastroenterological studies. Just a decade later, a large cross-sectional study (the EUROGAST study) involving 17 populations from 13 different countries (Unites States, Japan and 11 European countries), concluded that *H pylori*-infected patients had six-fold increased risk of GC compared with uninfected subjects[72]. In 1994, despite some controversial opinion, the International Agency for Research on Cancer declared *H pylori* to be a group I human carcinogen for gastric adenocarcinoma[73]. The statement was mainly based on epidemiological investigations since no experimental studies had been performed at that time to prove the causal link between *H pylori* and GC. Currently, although substantial evidence supports the role of *H pylori* infection in GC development, the magnitude of the risk of GC associated with infection remains unclear.

Many epidemiological studies have been conducted in an attempt to address this issue (Tables 4 and 5). Retrospective case-control studies analyzing on the whole 8306 GC cases and 15 884 controls reported an increased risk of developing GC for patients with *H pylori* infection

Table 4 Epidemiological studies (case/control) on association between H. pylori infection and GC risk (1990-2005)

Author	Yr	Geographic area	Case/Control *n*	OR (95% CI)	Detection of infection
Loffeld RJ[74]	1990	The Netherland	91/401	2.04 (1.07-3.91)	Serology
Caruso ML[75]	1990	Italy	44/22	4.72 (1.32-19.04)	Histology
Talley NJ[76]	1991	USA	69/252	1.63 (0.79-3.37)	Serology
Sipponen P[77]	1992	Finland	54/84	2.21 (1.01-4.91)	Serology, histology
Kuipers EJ[78]	1993	The Netherlands	116/116	0.86 (0.44-1.68)	Serology
Estevens J[79]	1993	Portugal	80/80	0.54 (0.24-1.19)	Serology
Blaser MJ[80]	1993	Japan	29/58	2.14(0.72-6.40)	Serology
Tatsuta M[81]	1993	Japan	41/19	2.42 (0.69-8.66)	Biopsy culture
Buruk F[82]	1993	Turkey	46/40	1.89 (0.69-5.21)	Serology
Hansson LE[83]	1993	Sweden	112/103	2.60 (1.35-5.02)	Serology
Archimandritis A[84]	1993	Greece	47/50	1.23 (0.51-2.95)	Serology
Lin JT[85]	1993	China, Taiwan	143/823	1.42 (0.97-2.08)	Serology
Hu PJ[86]	1994	China	51/102	5.10 (1.70-15.5)	Serology, histology
Sipponen P[87]	1994	Finland	243/1408	1.31 (0.99-1.74)	Histology
Asaka M[88]	1994	Japan	213/213	2.55 (1.48-4.44)	Serology
Kikuchi S[89]	1995	Japan	105/102	13.3 (5.3-35.6)	Serology
Rudi J[90]	1995	Germany	111/111	1.39 (0.82-2.36)	Serology
Fukuda H[91]	1995	Japan	282/767	1.13 (0.81-1.58)	Serology
Menegatti M[92]	1995	Italy	307/162	3.66 (2.33-5.74)	Serology, histology
Asaka M[93]	1995	Japan	109/109	2.40 (1.20-4.80)	Serology
Hatz RA[94]	1996	Germany	95/93	2.03 (1.05-3.92)	Serology
Shibata T[95]	1996	Japan	50/50	1.10 (0.43-2.86)	Histology
Kato S[96]	1996	Japan	82/151	1.12 (0.60-2.07)	Serology
Kokkola A[97]	1996	Finland	50/22	3.27 (1.42-7.52)	Histology
Menegatti M[98]	1996	Italy	148/54	4.02 (1.99-8.17)	Serology, histology
Sivaprakash R[99]	1996	India	75/75	1.91 (1.00-3.67)	Serology, biopsy culture
Kim HY[100]	1997	Korea	160/160	1.39 (0.89-2.17)	Histology
Miehlke S[101]	1997	Germany	215/215	16.7 (CI 9.6-29.1)	Histology, ^{13}C-UBT
Shi Y[102]	1997	China	110/125	3.30 (1.90-5.9)	Serology
Barreto-Zuniga R[103]	1997	Japan	55/75	3.00 (1.69-5.33)	Serology
Martin-de-Argila C[104]	1997	Spain	48/50	3.01 (1.02-8.86)	Serology
Azuma T[105]	1998	Japan	82/167	0.97 (0.54-1.75)	Serology
Komoto K[106]	1998	Japan	105/105	5.60 (2.33-13.4)	Serology, histology
Wu MS[107]	1998	Taiwan	135/135	2.43 (1.29-4.65)	Serology
Whiting JL[108]	1998	UK	154/154	1.67 (1.01-2.75)	Serology
Lee BM[109]	1998	Korea	175/113	5.20 (3.10-8.70)	CLO test
Kikuchi S[110]	1999	Japan	103/101	15.0 (6.4, 35.2)	Serology
Zhang ZF[111]	1999	USA	134/65	11.2 (2.5-50.3)	Histology
Cai L[112]	2000	China	101/101	3.45 (0.90-13.2)	Serology
Enroth H[113]	2000	Sweden	72/324	2.1 (1.1-3.9)	Serology, histology
Chang WK[114]	2001	Korea	136/136	1.82 (1.10-3.00)	Serology
Ekstrom AM[115]	2001	Sweden	298/244	5.0 (1.10-23.6)	Serology
Fujioka N[116]	2001	Brazil	93/186[1]	0.80 (0.47-1.36)	Serology
			228/226[2]	0.84 (0.54-1.30)	
Konturek SJ[117]	2002	Poland	337/337	2.59 (1.61-4.22)	Serology
Sriamporn S[118]	2002	Thailand	111/232	0.60 (0.40-1.0)	Serology
Wu AH[119]	2003	USA	127/356	1.85 (1.03-3.32)	Serology
Brenner H[120]	2004	Germany	68/360	18.3 (2.4-136.7)	Serology
Machida-Montani A[121]	2004	Japan	122/235	8.20 (3.70-18.2)	Serology
Kato M[122]	2004	Japan	2503/6578	2.47 (2.19-2.79)	Serology
Nomura AM[123]	2005	Hawaii	299/336	4.86 (5.90-8.13)	Serology

[1]Japanese Brazilian; [2]non-Japanese Brazilian.

(OR ranging from 1.10; 95% CI 0.43-2.86 to 18.3; 95% CI 2.4-136.7)[74-123]. However, five studies failed to demonstrate any significant risk associated to previous or concurrent *H pylori* infection[78,79,105,116,118]. Retrospective case-control studies are limited "per se" by several biases. In GC patients (cases) *H pylori* infection is usually assessed after the development of cancer, but advanced gastric diseases can be characterized by the loss of infection resulting in a fall of the circulating anti-*H pylori* antibodies. In addition, the type of control population and the absence of adjustment for confounding factors (age, sex, smoking, and dietary habit) can hamper the statistical evaluation leading, to over- or underestimation of the real risk linked to *H pylori* infection.

Prospective studies, by contrast, should be more informative because they use internal control "nested" within a cohort. The infection is assessed by examining blood samples taken years before the onset of clinical disease, so that the enrollment of the studied population did not suffer of selection bias. All cohort studies[124-143] reported an increased risk of developing GC associated to *H pylori* infection (OR ranging from 1.06; 95% CI 0.80-1.40 to 6.0; 95% CI 2.1-17.3) (Table 5). Only one study conducted in a high-risk population from Shanghai,

Table 5 Epidemiological studies (cohort nested case-control study) on association between *H pylori* infection and GC risk

Author	Yr	Geographic area	Case/Control *n*	OR (95% CI)	Mean follow-up (yr)
Nomura AM[124]	1991	USA	109/109	6.0 (2.1-17.3)	12
Parsonnet J[125]	1991	USA	109/109	3.6 (1.8-7.3)	14.2
Forman D[126]	1991	England	116/484	2.7 (1.0-7.9)	15
Parsonnet J[127]	1993	USA	136/136	2.62 (1.47-4.69)	21
Blaser MJ[128]	1995	USA	102/102	1.45 (0.76-2.80)	3
Lin JT[129]	1995	China, Taiwan	29/220	1.13 (0.81-1.58)	13
Aromaa A[130]	1996	Finland	80/146	1.50 (0.70-3.22)	6
Webb PM[131]	1996	China	87/261	0.93 (0.57-1.54)	40
Siman JH[132]	1997	Sweden	56/224	5.00 (2.20-11.5)	5.7
Watanabe Y[133]	1997	Japan	45/225	1.84 (1.54-5.72)	8
[1]Yuan JM°[134]	1999	China	188/548	1.84 (1.08-3.11)	12
Hansen S[135]	1999	Norvay	208/208	5.15 (2.83-9.37)	13
You WC[136]	2000	China	34/2594	1.8 (1.20-2.60)	4.5
Tulinius H[137]	2001	Iceland	23/128	1.16 (1.05-1.28)	20
Siman JH[138]	2001	Sweden	56/224	5.0 (2.2-11.2)	5.7
Limburg P[139]	2001	China	92/192	2.29 (1.26-4.14)	15
Nomura AM[140]	2002	Hawaii	261/261	2.70 (1.30-5.6)	25
Kosunen TU[141]	2005	Finland	363/4854	2.49 (1.86-3.34)	24
Shin A[142]	2005	Korea	86/344	1.06 (0.80-1.40)	2.6
Knekt P[143]	2006	Finland	225/435	3.12 (1.97-4.95)	15

[1]Re-evaluation of the Webb study with ELISA developed and validated among Shanghai residents.

China, failed to demonstrate an association between *H pylori* infection and the subsequent risk of GC[131]. However, an update of the results at longer follow-up and by using an enzyme-linked immunosorbent assay (ELISA) based on strains validated among the Shanghai residents showed a statistically significant association between *H pylori* seropositivity and GC risk (OR 1.84; 95% CI, 1.08-3.11 raising to 3.74; 95% CI 1.51-9.30 among subjects followed for 5 or more years after enrolment)[134].

A meta-analysis of cohort and case-control studies evaluated that the summary OR for GC in *H pylori* infected patients was 1.92 (95% CI 1.32-2.78), 2.24 (95% CI 1.15-4.4), and 1.81 (95% CI, 1.16-2.84) for all studies, cohort, and case-control studies, respectively. The risk of developing GC was greatest in younger patients (OR 9.29 at age < 29 years) and was equally associated with the intestinal or diffuse type GC[144]. A combined analysis of 12 case-control studies (6 from Europe, 4 from Asia, 2 from the United States) nested with prospective cohorts and involving 1228 GC cases and 3406 controls, revealed that the association of *H pylori* infection with GC was restricted to non-cardia cancers (OR 2.97; 95% CI 2.3-3.7), and was stronger when blood samples for *H pylori* serology were collected ten years or more before cancer diagnosis (OR 5.9; 95% CI 3.4-10.3)[145]. However, the most powerful evidence comes from a prospective study on 1526 Japanese patients followed for approximately 7.8 years. GC developed in 36 out of 1246 *H pylori*-positive patients (2.9%) in contrast to none of the 280 non-infected subjects[146].

Infection with cagA-positive strains further increases the risk of developing GC. According to a recent meta-analysis of 2284 cases and 2770 controls, infection with cag-A-positive strains increased the risk of developing GC up to 1.64-fold (95% CI 1.21-2.24) for all sites GC and 2.01-fold (95% CI 1.21-3.32) for non-cardia GC[147].

The close relationship between *H pylori* infection and GC leads to the critical question of whether antimicrobial therapy can be considered for GC chemoprevention. A prospective, randomized, placebo-controlled, population study carried out in a high-risk area of China involving 1630 subjects observed from 1994 to 2002 reported a comparable incidence of GC in the subjects receiving *H pylori* eradication treatment and those receiving placebo. However, eradication of *H pylori* significantly decreased the development of GC in a subgroup of *H pylori* carriers not presenting precancerous lesions[148]. On the other hand, a randomized, controlled chemoprevention trial conducted in subjects with confirmed histological diagnoses of multifocal, non-metaplastic atrophy and/or intestinal metaplasia, assigned to receive anti-*H pylori* triple therapy and/or dietary supplementation (ascorbic acid, beta-carotene, or their corresponding placebos), demonstrated a significant regression rate of the lesions for all three basic interventions (RR 4.8 95% CI 1.6-14.2 for anti-*H pylori* treatment; 5.1, 95% CI 1.7-15.0 for beta-carotene treatment, and 5.0; 95% CI 1.7-14.4 for ascorbic acid treatment in subjects with atrophy and 3.1; 95% CI 1.0-9.3; 3.4; 95% CI 1.1-9.8, and 3.3; 95% CI 1.1-9.5 in subjects with intestinal metaplasia)[149].

INTERPLAY BETWEEN *H PYLORI* INFECTION AND DIET

A synergistic interaction between *H pylori* infection and diet in GC has been suggested[150]. One possible mechanism by which *H pylori* exerts its "carcinogenic" potential is the greater likelihood of malignant transformation due to inflammatory responses of the gastric epithelium. The generation of reactive oxygen species (ROS) and the increased level of nitric oxide (NO) synthase associated with the mucosal colonization by *H pylori* cause DNA mutations which may be the initial step in the genetic alterations of gastric epithelial cells[151-153]. Another possible explanation is that the *H pylori*-related inflammation

Table 6 Epidemiological studies (hospital-based case-control) on association between dietary factors and H pylori infection and gastric cancer risk

Author	Yr	Geographic area	Case/Control *n*	Increased risk	Decreased risk	*H pylori* risk
Sriamporn S[118]	2002	Thailand	131/262	Salt, fermented foods	Vegetables, fruit	Independent
Lee SA[156]	2003	Korea	69/199	Salt, kimchi, salt-fermented fish	Vegetables, fruit, soybean curds, broth	Increased
Lopez-Carrillo L[157]	2003	Mexico	234/468	Capsaicin	--	Independent
Machida-Montani A[121]	2004	Japan	122/235	Fermented soy bean, rice	--	Independent

induces predisposing morphological changes in the gastric mucosa such as atrophy and intestinal metaplasia[154]. These latter conditions decrease the acidity in the stomach increasing the endogenous formation of nitrosamides, the main subset of N-nitroso compounds[155]. Nitrosamides, spontaneously formed in the stomach from the nitrite and amides, do not require enzymes but depend on the presence of nitrites and are favored by a high pH. Thus, the ability of the host to reduce nitrate to nitrite and the dietary intake of nitrate and amine are critical for the onset of the gastric carcinogenic process. This hypothesis links the theory of "N-nitroso compounds-mediated GC risk" with that of the "*H pylori*-related GC risk" suggesting an "integrated model" of gastric carcinogenesis. However, even if the synergistic interaction between diet and *H pylori* infection is biologically plausible, only a few epidemiological studies have simultaneously evaluated the role of *H pylori* infection and dietary habits in relation to GC risk. Furthermore, the results of these studies were conflicting (Table 6)[118,121,156,157]. A case-control study conducted in Thailand analyzing both the effect of dietary pattern and *H pylori* infection found an increased risk of GC associated with a high intake of salt (OR 1.8; 95% CI 1.1-3.0) and fermented foods (OR 1.9; 95% CI 1.1-3.3)[118]. In contrast, a weak negative association was found between GC risk and vegetable and fruit intake and no association between *H pylori* infection and GC risk (OR 0.6; 95% CI 0.4-1.0)[118]. Likewise, a study evaluating the role of *H pylori* infection and capsaicin consumption on the risk of GC demonstrated an increased risk (OR 1.71; 95% CI: 0.76-3.88) in high-level consumers of capsaicin (90-200 mg/d) as compared to low-consumers (0-29.9 mg/d). However, this effect was independent of *H pylori* status and was higher for diffuse type GC (OR 3.64; 95% CI 1.09-12.2) compared to the intestinal type (OR 1.36; 95% CI 0.31-5.89)[157]. Lastly, Machida-Montani *et al*[121] found a close correlation between GC and *H pylori* infection (OR 8.2; 95% CI 3.7-18.2), frequent intake of fermented soy bean soup (OR 2.1; 95% CI 0.9-5.1), and rice (OR 2.5; 95% CI 1.0-6.1) but no significant interaction between diet and *H pylori* infection. In contrast, in a Korean hospital-based case-control study, subjects with *H pylori* infection and high salt intake had a 10-fold higher risk of developing GC than subjects without *H pylori* infection and low salt intake ($P = 0.047$)[156].

DISCUSSION

GC develops through a multistage process which may span $\geqslant$ 20 years[154]. The long latency period hypothetically provides wide opportunities for intervention to prevent cancer development. However, several questions need to be answered before the results of epidemiological and interventional studies can be extended to the clinical setting.

Firstly, GC comprises at least two main entities, the intestinal and the diffuse type, which differ considerably from an epidemiological, clinical and molecular point of view[158]. Based on epidemiological evidence, the intestinal type, preceded by precancerous lesions, seems more closely influenced by environmental factors while the latter recognizes mainly a "genetic" substrate. However, only a few studies have focused on the nutritional pattern in relation to the histotype of GC[6,159-161]. Even hampered by the small number of cases studied, the results strongly suggest that the dietary risk factors are common to both types of GC while the protective factors play a more important role in preventing the intestinal type. Secondly, trials directly evaluating cancer development as target require very large numbers of subjects to be followed for decades. Trials with smaller groups of subjects followed for shorter periods and focusing on the intermediate steps of the gastric carcinogenic process may hypothetically obtain information on the possible inhibition of cancer development. However, only the "intestinal type" cancer recognizes a precancerous "cascade" of events and only a small subset of patients with precancerous lesions develop GC[154]. Thus, very large number of subjects for many years would need to be followed to obtain conclusive results. Finally, due to the" synergistic" interplay between diet and *H pylori* infection, *H pylori* should always be properly considered.

In conclusion, although GC is a disease of genes, mainly triggered by *H pylori*-related mucosal inflammation, overwhelming evidence suggest that diet and lifestyle factors are important causes leading to cancer. Indeed, the progressive decline in GC incidence observed between 1930s and 1980s, before the discovery of *H pylori*, can be, without doubt, related to improvement of diet and spread use of refrigerators. On the other hand, data suggesting that *H pylori* eradication may reduce the risk of developing GC need still to be confirmed by large-scale population studies[162]. One study that economically modelled the cost of screening per year of life saved estimated that in selected populations such as Japanese American, serological screening for *H pylori* at age 50 years was more beneficial than breast cancer screening[163]. However, there are insufficient data to recommend general screening for *H pylori* of asymptomatic patients to prevent GC. The decision to screen should be based on individual risk factors such as race, and family history of GC[164].

At present, even if foods and food components acting as risk or protective factors for GC still remain to be fully

defined, a diet rich in fruit, vegetables and cereals and poor in meat, fat and salt has a good prophylactic potential for cancer and many other chronic diseases of lifestyle i.e. coronary heart disease, hypertension, obesity and diabetes. Thus, "diet for cancer prevention" can be proposed as a general role of well-being and can represent the basis for a rational health policy.

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Useful detection of CD147 (EMMPRIN) for pathological diagnosis of early hepatocellular carcinoma in needle biopsy samples

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Abstract

AIM: To make clear whether CD147 (EMMPRIN) expression in pathological tumor samples with a fine-needle aspiration biopsy is useful for pathological diagnosis of early hepatocellular carcinoma (HCC).

METHODS: Twenty-two patients (15 men and 7 women; median age 68 years, range 56-81 years) underwent a liver tissue biopsy in order to make a diagnosis of HCC. Paraffin-embedded liver biopsy tissue samples from 22 patients were stained with anti-CD147 antibody, murine monoclonal antibody 12C3 (MAb12C3) for immunohistochemical analysis. An immunohistochemical analysis of CD147 was performed and the degree of staining compared between tumor and non-tumor tissue. In addition, the degree of staining within tumor tissue was compared according to a number of clinicopathological variables.

RESULTS: The degree of staining of CD147 was significantly higher in tumor tissues than non-tumor tissues, even in tumors less than 15 mm in diameter. The expression of this protein was significantly elevated in HCC tissue specimens from patients with a low value of serum AST and γ-GTP.

CONCLUSION: CD147 serves potentially as a pathological target for cancer detection of early HCC.

Key words: CD147; Hepatocellular carcinoma; Needle biopsy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, involving more than 500 000 new cases yearly, with an age-adjusted incidence of 5.5-14.9 per 100 000 people[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death[2]. The incidence of HCC is also increasing in Europe and the United States[3]. The early detection of tumors and development of therapies for HCC is likely to improve the prognosis[4]. Nevertheless, despite improvements in both diagnostic modalities and therapy, in many cases an accurate diagnosis still cannot be confirmed even with diagnostic imaging and the recognition of tumor markers in the serum. Particularly, hypovascular HCC which is often difficult to recognize by computed tomography (CT) requires ultrasound (US) examination for a definitive diagnosis. Tumor biopsy is an important method of evaluation in these cases, particularly in small tumors, less than 15 mm in diameter. Therefore, more sensitive tumor markers for pathological diagnosis are required.

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin, is a transmembrane glycoprotein with two immunoglobulin-like

domains. This is part of a family of proteins that includes embigin and neuroplastin[5]. Tumor cell CD147 triggers the production or release of matrix metalloproteinases in the surrounding mesenchymal cells and tumor cells, thereby contributing to tumor invasion[6-9]. A very high incidence of CD147 expression (> 80% of CD147-positive cases) is detected by immunohistochemical staining in HCC[10].

A previous paper reported a murine monoclonal antibody (MAb12C3), specific to human ovarian carcinomas was generated by immunizing mice with the human ovarian germinoma cell line (JOHY-2)[11]. In further research, using phage display libraries, MAb12C3 hybridized with the extracellular region of CD147[12]. The MAb12C3 reacted with 67.7% (21 of 31 cases) of epithelial ovarian carcinomas, but not with any of benign epithelial ovarian adenomas tested[11].

Despite extensive studies on small early stage HCCs, the morphological criteria for definite diagnosis of well-differentiated, small HCCs are still questionable[13]. This study considered whether the use of MAb12C3 against CD147 protein could recognize early stage HCC. MAb12C3 was used for an examination of antigen expression in early HCC tissue specimens and to identify any correlations between the immunohistochemical findings and the clinicopathologic characteristics of the tumors. In this study, small biopsy samples from HCC were examined with immunohistochemical staining. If significant differences are recognized between HCC with non-tumor liver tissues, CD147 may therefore be effective as a diagnostic and therapeutic target in early stage HCC.

MATERIALS AND METHODS

Patients

The study population included 22 patients (15 men and 7 women; median age 68 years, range 56-81 years) who underwent tumor and non-tumor liver tissue biopsy between January 2003 and December 2005, in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm a diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Communittee Institutional Review Broad.

Pathologic specimens

Tumor specimens were obtained by a tumor biopsy with a 21 G fine-needle aspiration kit. Non-tumorous liver tissue specimens were obtained by an 18-20 G needle liver biopsy concurrently. Formalin-fixed, paraffin-embedded specimens of liver tumors and non-tumor liver tissues were processed for conventional histologic assessment by hematoxylin and eosin (HE) staining. The tumors were histologically graded (well or moderately differentiated).

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were dewaxed and used. The specimens were stained using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system (Ventana Japan, Yokohama, Japan). A murine monoclonal antibody against CD147 protein, MAb12C3[12], was used as the primary antibody (manufactured at Department of Biochemistry 1, Jikei University School of Medicine, Japan). The antigen retrieval procedure was performed with a microwave oven in DAKO antigen retrieval solution for 30 min at 95℃ to efficiently stain the sample. The immunohistochemical staining was stronges, when performed in a microwave oven in DAKO antigen retrieval solution (Figure 1). The

Table 1 Characteristics of patients undergoing tumor biopsies (n = 22)

Features	Median value
Age	68 (56-81)
Sex (Male/Female)	15/7
Plt (× 10^3/μL)	10.0 (5.1-24.5)
AST (IU/L)	68 (21-147)
ALT (IU/L)	61.5 (6-214)
T-Bil (mg/dL)	0.8 (0.4-2.3)
γ-GTP (IU/L)	48 (18-665)
AFP (ng/mL)	21.5 (3-444)
HBs Ag/HCV Ab/Others	3/18/1
Tumor size (mm)	14.5 (8-23)
Cirrhosis (positive/negative)	5/17
Differentiation (well/moderate)	15/7

Data values are expressed as the medians with ranges in parentheses unless indicated otherwise. Normal ranges: Plt (platelet count), 15-35 × 10^3/L; AST (aspartate aminotransferase), 10-33 IU/L; ALT (alanine aminotransferase), 6-35 IU/L; T-Bil (total bilirubin), 0.2-1.2 mg/dL; γ-GTP (γ-glutamyl transferase), 10-50 IU/L; AFP (α-fetoprotein), > 20 ng/mL.

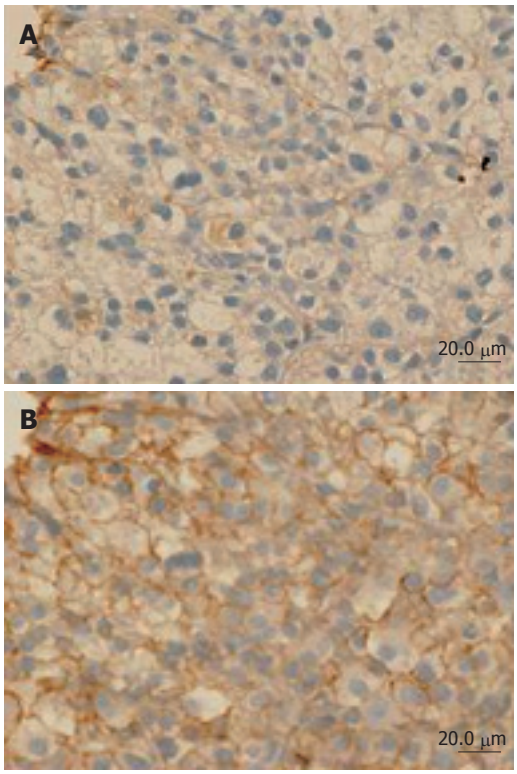

Figure 1 CD147 protein expression of HCC tissues with microwave-stimulated processing. **A**: 10 min of 10 mmol/L citrate buffer (pH 6.0); **B**: 30 min of DAKO antigen retrieval solution.

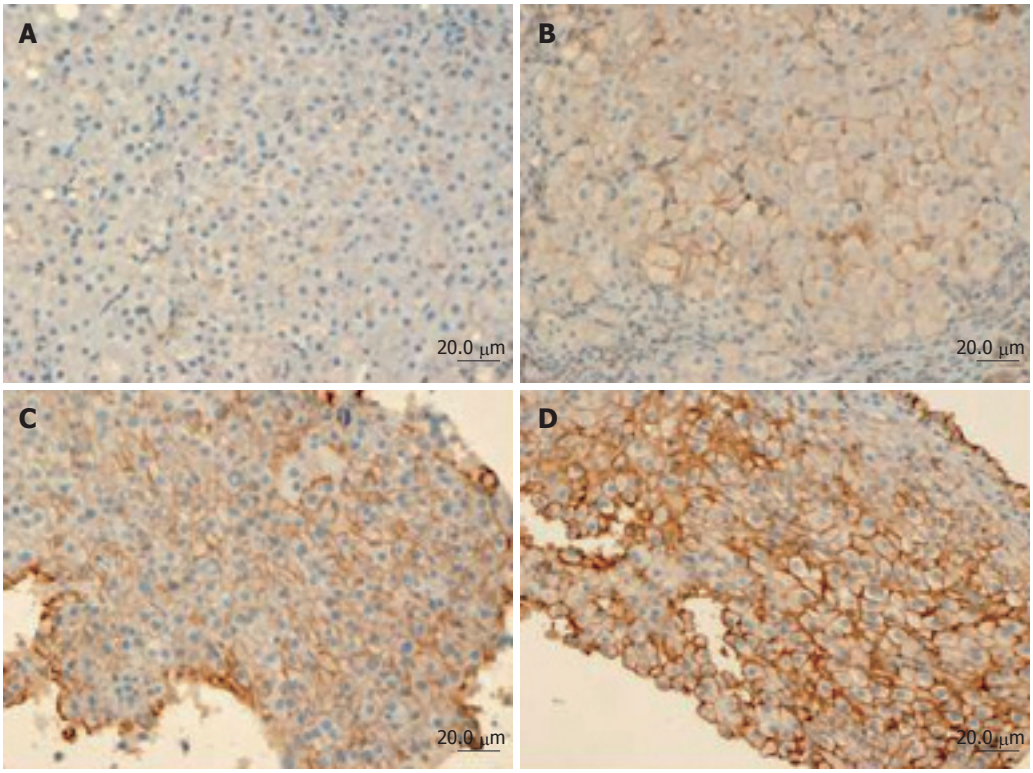

Figure 2 CD147 protein expression of non-tumor liver tissue and HCC tissue specimens. **A**: Non-tumor tissue specimen, very weak expression; **B**: Non-tumor tissue specimen, weak expression; **C**: HCC tissue, moderate expression; **D**: HCC tissue, strong expression.

Table 2 Immunohistochemical scales of tumor and non-tumor biopsy specimens

4-step scales	Tumor tissues *n* (%)	Non-tumor tissues *n* (%)	*P*
Very weak (0)	1 (4.5)	7 (31.8)	< 0.01
Weak (1)	6 (27.3)	6 (27.3)	
Moderate (2)	3 (13.6)	6 (27.3)	
Strong (3)	12 (54.5)	3 (13.6)	
Total	22 (100)	22 (100)	

sections (DAKO Cytomation, Glostrup, Denmark) were developed with 3, 3'-diaminobenzidine with 0.3% H_2O_2 and counterstained with hemotoxylin.

For each tissue sample, the fraction of the immunostained cells was recorded, and the staining intensity was estimated using a 4-step scale (0, 1, 2, 3). The tissue specimens were then initially categorized according to arbitrarily predefined criteria into 4 groups, including completely very weakly positive, strongly positive, and 2 intermediate groups. The exact criteria for these groups were as follows: very weak (1+ staining in some cells) (Scale 0); weak (1+ staining in cells) (Scale 1); moderate (2+ staining in cells) (Scale 2); strong (3+ staining in cells) (Scale 3). The examiners were blinded to patients' clinical and histological (HE staining) profile. Two investigators (H.H. and K.N.) evaluated the staining levels independently, after which any discordant evaluations were adjusted by connected microscopes and scored jointly.

Statistical analysis

Statistical analyses were performed by the Wilcoxon signed-rank test and two-sample Wilcoxon rank-sum (Mann-Whitney) test. *P*-Values < 0.05 were considered statistically significant. All these analyses were performed using STATA 9.1 (STATA Corporation, College Station, Texas, USA).

RESULTS

CD147 expression in HCC and non-tumorous liver tissue

Among all 44 tissues (22 HCC and 22 non tumorous liver tissues), CD147 immunoreactivity was detected on all cell membranes. As shown in Figure 2, CD147 was positively but weakly stained on most non-tumor liver tissues, beacause the antigenicity was activated by microwave-stimulated processing with 30 min treatment of DAKO retrieval solution (Figure 2B). However, a significant difference was observed in CD147 expression between HCC and non-tumor liver tissues (Table 2, Figure 2). In fact, there was significantly greater expression of CD147 in the carcinoma tissue specimens than in non-tumorous liver tissue specimens, including small tumors measuring less than 15 mm in size ($P < 0.05$).

CD147 expression in tumour aspirates correlates with clinical variables

Twenty-two HCC biopsy specimens were categorized into two groups for each clinical variable, above or below the median value. In these two groups, the CD147 intensity was compared. As illustrated in Figure 3, with regard to tumor size, CD147 was highly expressed in large tumors. In contrast, in the detection of serum AST and γ-GTP level, CD147 was more significant in low value groups. No significant differences were observed by other clinical parameters, such as serum AFP level. In addition, although the CD147 intensity was compared between tumor HCC

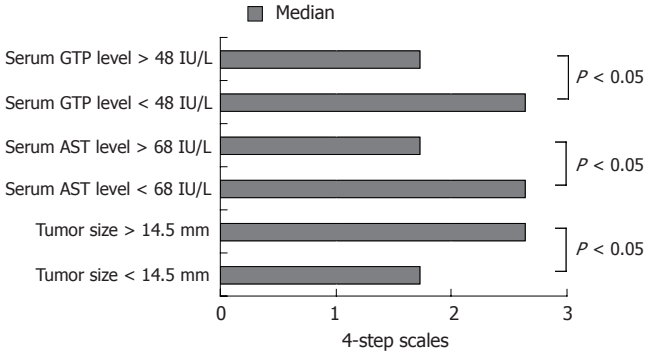

Figure 3 Comparison of CD147 intensity between categories of clinicopathological valiables in tumor biopsies.

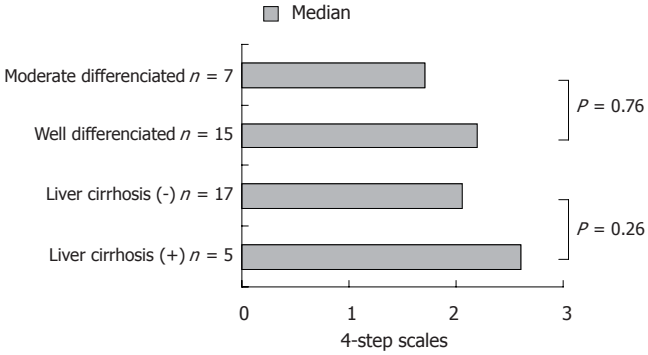

Figure 4 Comparison of the CD147 intensity in tumor biopsy specimens: cirrhosis and tumor differentiation.

associated with and without cirrhosis and between well-differentiated and moderately differentiated HCC, no significant difference was observed (Figure 4).

DISCUSSION

CD147 is an adhesion molecule that binds to endothelial cells and fibroblasts, and stimulates the expression of several matrix metalloproteinases associated with the invasiveness of HCC[14-16]. In a resent study, a significantly positive correlation was identified between the CD147 immunostaining intensity and the histological grading and clinical stage of HCC[17]. Moreover, this marker may be valuable to differentiate between benign liver nodules and HCC, especially in small lesions with good differentiation[17]. Most previous reports were based on the analyses of surgically removed tissues. This study observed tumor nodules obtained by the 21G fine-needle aspiration kit before treatment. To enhance the diagnostic efficacy, the most sensitive detection of CD147 in HCC tissues was also investigated. Therefore, the tissue specimens were stained using microwave-stimulated processing with DAKO antigen retrieval solution. The antigenicity of the CD147 protein was activated with microwave-stimulated processing which resulted in the expression of CD147 in some non-tumor tissues. This result showed that the peritumoral tissues, pathologicaly diagnosed as the non-tumor tissue, may be related to the biological development of the malignant phenotype with CD147. Nevertheless, the CD147 expression in tumor lesions was significantly stronger, even in small tumors. Furthermore, the non-tumorous normal liver tissue and tumor tissue demonstrated significant differences in the expression of the antigen, even in small tumors measuring less than 15 mm in size. This indicates that this marker is therefore useful in the diagnosis of small HCC and also when considering the most appropriate treatment.

Previous studies have demonstrated that CD147 can potentially serve as a target for antitumor therapy. They showed that the CD147 expression could frequently be detected in the vast majority of human malignancies as well as in a subset of benign tumors. Nevertheless, there are significant differences both in the intensity and distribution of CD147 staining among different malignant tumors as well as benign lesions[10]. In fact, tumors expressing high levels of CD147 compared to their normal counterparts include carcinomas of the urinary bladder[18], breast, lung[19,20], oral cavity[21], esophagus[12], skin[22], malignant lymphomas[23,24], and malignant peripheral nerve sheath tumors[25].

In this study, the staining intensity of CD147 was confined to cell membrane expression of this antigen. CD147 expressed on the tumor cell surface and stimulates nearby fibroblasts and endothelial cells[26-28]. CD147 has been shown to be an important mediator of tumor-stroma cross-talk, based on the findings that it mediates not only MMP production but also angiogenesis via the stimulation of vascular endothelial growth factor (VEGF)[29], and anchorage-independent growth and multi-drug resistance in a hyaluronan-dependent fashion[7,30,31]. Further investigation is necessary to examine the expression of CD147 associated with fibrosis.

In this study, expression of CD147 in HCC biopsies was much stronger than the peritumoral tissue. This result may illustrate the intensity of CD147 expression in a tumor biopsy is rare in peritumoral tissues.

The CD147 expression was higher in the HCC specimens from patients with lower levels of serum AST and γ-GTP. This result may indicate that the CD147 expression of HCC can thus be determined even when the liver function is weak.

In this study, the HCC tissue biopsy specimens were small in size, therefore we could not use an automated method to objectively evaluate the expression of CD147. We therefore require further examinations be used to evaluat the automated method.

In conclusion, HCC tissue biopsy specimens, even from small tumors, expressed CD147 protein at significantly higher levels than non-tumorous liver tissue. The immunohistochemical analysis of the murine monoclonal antibody, MAb12C3, is very useful for the detection of HCC in even needle biopsy specimens. Therefore, CD147 can potentially serve as a useful target for cancer detection in HCC.

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VIRAL HEPATITIS

Ultrastructure of oval cells in children with chronic hepatitis B, with special emphasis on the stage of liver fibrosis: The first pediatric study

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Abstract

AIM: To investigate the ultrastructure of oval cells in children with chronic hepatitis B, with special emphasis on their location in areas of collagen fibroplasia.

METHODS: Morphological investigations were conducted on biopsy material obtained from 40 children, aged 3-16 years with chronic hepatitis B. The stage of fibrosis was assessed histologically using the arbitrary semiquantitative numerical scoring system proposed by Ishak *et al*. The material for ultrastructural investigation was fixed in glutaraldehyde and paraformaldehyde and processed for transmission–electron microscopic analysis.

RESULTS: Ultrastructural examination of biopsy specimens obtained from children with chronic hepatitis B showed the presence of two types of oval cells, the hepatic progenitor cells and intermediate hepatic-like cells. These cells were present in the parenchyma and were seen most commonly in areas of intense periportal fibrosis (at least stage 2 according to Ishak *et al*) and in the vicinity of the limiting plate of the lobule. The activated nonparenchymal hepatic cells, i.e. transformed hepatic stellate cells and Kupffer cells were seen in close proximity to the intermediate hepatic-like cells.

CONCLUSION: We found a distinct relationship between the prevalence of oval cells (hepatic progenitor cells and intermediate hepatocyte-like cells) and fibrosis stage in pediatric patients with chronic hepatitis B.

Key words: Pediatric patients; Oval cells; Ultrastructural study; Chronic hepatitis B; Liver biopsy; Fibrosis

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INTRODUCTION

Several authors have suggested that oval cells (syn. liver progenitor/oval cells) play an essential role in the development of liver regeneration and carcinogenesis. These cells have been investigated extensively, using both experimental material[1-6] as well as human biopsy specimens, obtained mainly from adult patients[7-12].

It has been proposed that the major approach to compensate for the loss of liver mass (e.g. after viral infection, hepatectomy, *etc*) involves the proliferation and differentiation in to a fully mature liver of primary, low-differentiated, multipotential cells of bone marrow origin, known as the progenitor cells or stem cells. Although, it is still a matter of dispute, these cells are believed to act as precursor cells of intrahepatic progenitor cells, i.e. oval cells[2,6,7,12-15].

It is believed that oval cells constitute a heterogenic cell population which account for 1%-3% of the normal liver cell pool. These cells are located in the portal and periportal spaces, with the nucleus serving as the common morphological feature shared by these cells. The oval cells are not easy to recognize. They have many features in common, both structural and functional, with hepatoblasts of the embryonic and fetal period[12,16,17]. The oval cells are thought to constitute a reserve compartment that is activated only when hepatocytes fail to proliferate[18].

The most common pathological process involving oval cells occurs in chronic hepatitis, especially during the phase of acute necrosis, when the oval cells are found in the areas of regeneration/proliferation, as well as during the phase of fibrosis and structural reorganization of hepatic parenchyma (liver cirrhosis)[7,4,10,11,19-21].

The oval cells also share some features with the cells that appear in the mature organ in the process of

hepatocarcinogenesis, giving rise to hepatocellular carcinoma and cholangio-cellular type neoplasms[8,9,12,16]. Parent *et al*[8] reporting on hepatic progenitor cells in liver pathology, attached special emphasis to their role in carcinogenesis seen in human chronic liver diseases.

Several authors agree that the oval cells have a bipotent nature (and can therefore be called *bipotent small epithelial cells*, *bipotent oval cells*, *bipotent liver progenitor cells*), i.e. these cells exhibit a two-directional differentiating ability, which during regeneration and hepatocarcinogenesis, constitutes a major source of precursor cells both for hepatocytes and for epithelial cells of bile ductules[2,6,8,11,12,19,20].

Among the numerous reports on the role of oval cells in various liver pathologies, the one published by Novikoff *et al*[13] deserves special attention. Using an experimental model of carcinogenesis, the authors identified a population of non-differentiated cells in the liver, termed small blast-like cells (syn. small non-epithelial cells). These cells give rise to two morphologically and phenotypically different groups of oval cells. One group contains non-polarized basic ductal blast-like cells. The other comprises two types of polarized transitional epithelial cells-the oval/bile ductule epithelial cells and hepatocytes.

Roskams *et al*[22], have identified three categories of oval cells in humans, which ultrustructurally and phenotypically do not differ much from those described earlier by Novikoff *et al*[13]. However, they treated the putative progenitor cells as category I oval cells and not as a separate group. Under category Ⅱ, these researchers included intermediate bile-duct like cells, and in category Ⅲ-the intermediate hepatocyte-like cells[22]. As noted in the experimental model, the "progenitor cells" exhibit immunoreactivity for a panel of bile ductular cell markers, including rat oval cell marker OV6, cytokeratin 7, cytokeratin 19 and chromogranin A[22].

There is no agreement with regard to the morphogenesis and role of oval cells in different liver pathologies, which is reflected in a number of names used to describe this cell population. Moreover, we have found no reports on this subject in children with chronic viral infections.

Therefore, the objective of the current study was the ultrastructural assessment of oval cells in children with chronic hepatitis B, especially in the areas of collagen fibroplasia of varying intensity. The current study is a continuation of our morphological research on liver fibrosis in pediatric patients with chronic inflammation of the liver, including chronic hepatitis B[23-26].

MATERIALS AND METHODS

Patients

Histological and ultrastructural assessment was made of liver specimens of children with biopsy proven chronic hepatitis B (HBs/+/, HBe/+/ and HBV DNA/+/), before administration of antiviral treatment. The study was carried out in the Department of Clinical Pathomorphology, Medical University of Bialystok. Retrospective evaluation of the stage of liver fibrosis was made on material obtained by needle biopsy in 40 children, aged 3-16 years (mean age 8.5 years; 25 boys and 15 girls). Patients with autoimmune hepatitis, liver cirrhosis (including incomplete cirrhosis) and HCV co-infection were excluded from the study. None of the children were treated with antiviral or immunomodulating drugs during the 12-mo-period before enrolment in the study.

Histological analysis

The liver biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin. Histological stains used in the analysis included hematoxylin and eosin, Azan method, Masson's trichrome, Masson's-Goldner and reticulum stain according to Gomori. Fibrosis stage (S) was assessed in a blind fashion by a single pathologist using the semiquantitative scoring system proposed by Ishak *et al*[27]. In the group of 40 children, we identified 10 patients with advanced fibrosis, 10 with mild and 20 with moderate liver fibrosis. The material was also subjected to ultrastructural analysis.

Ultrastructural analysis

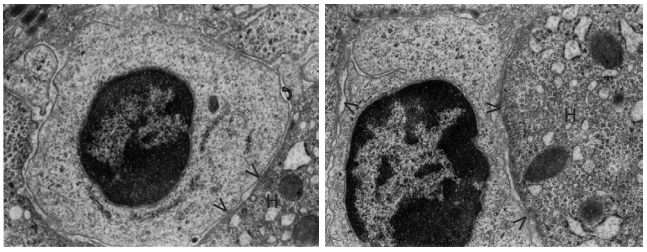
For ultrastructural examination, fresh liver blocks (1 mm^3) were fixed in a solution containing 2.5 glutaraldehyde, 2% paraformaldehyde, and 0.1 mol/L cacodylate buffer at pH 7.4. The specimens were post fixed in 2% OsO4, dehydrated in ethanol and propylene oxide, embedded in Epon 812 and sectioned on an ultramicrotom (Reichert) to obtain semithin sections (0.5-1 μm thick) which were stained with 1% methylene blue in 1% sodium borate and examined under a light microscope. Ultrathin sections prepared from selected specimens were double stained with uranyl acetate and lead citrate, and examined using an Opton 900 PC transmission electron microscope (Zeiss, Oberkochen, West Germany). Assessment of oval cells was made by an investigator who was blinded to the clinical information. The study was approved by the Local Ethical Committee at the Medical University of Bialystok.

RESULTS

In the group of children (10) with Ishak's fibrosis stage (S) 0-1, ultrastructural analyses revealed either no oval cells or only sporadic presence of these cells. The cells were found in one patient with S-0 and in 3 patients with S-1 (i.e. in 4 cases out of 10). Oval cells were more common in patients with S-2 fibrosis (7 cases out of 10). In patients with advanced liver fibrosis (S-3 or 4), the number of oval cells, although still not very high, showed a two to three-fold increase in all cases (10 patients).

The oval cells were seen mainly in areas of periportal and portal fibrosis, especially in areas close to the limiting plate of the lobule, where they were squeezed in the intercellular spaces, being enclosed by hepatocytes, and in the vicinity to bile ductules (Figures 1-3).

At times the cells were observed in dilated perisinusoidal spaces of Disse, usually accompanied with collagen fiber bundles (Figure 4A and B). Activated nonparenchymal hepatocytes were seen in the vicinity of the intermediate hepatocyte-like cells. These cells transformed into hepatic stellate cells, i.e. transitional Ito-fibroblast/myofibroblast cells and Kupffer cells. Sometimes the activated nonparenchymal cells were found to adhere to the oval cells (Figures 3 and 4).



Figures 1 The view of hepatic progenitor cells in hepatic intracellular spaces. The nuclei contain dense heterochromatin clumped under nuclear envelope and sparse euchromatin. The cytoplasm shows rare poorly developed cell organelles. Between progenitor cells and the neighboring mature hepatic cells (H) point desmosomes are present (>) (× 12 000).

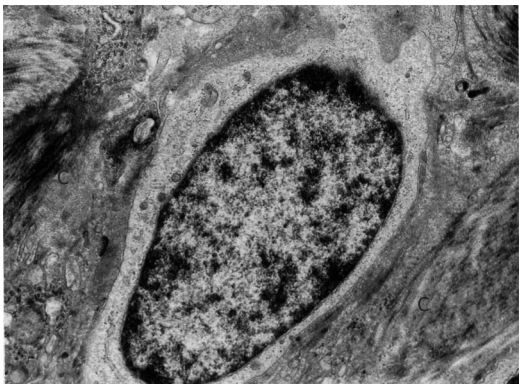


Figure 2 In the field of massive periportal fibrosis, an intermediate hepatocyte-like cell bigger than hepatic progenitor cell, with the electron-lighter cytoplasm and the nucleus with low heterochromatin content and resembling the hepatocyte nucleus. Cell organelles: a small number and poorly developed. C: collagen fiber bundles (× 7000).

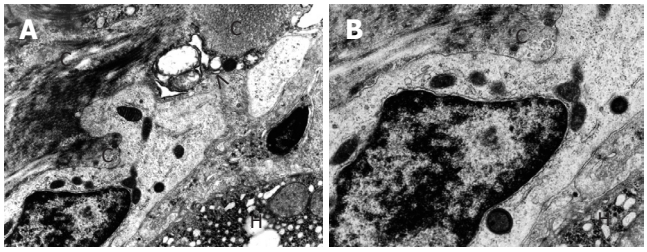
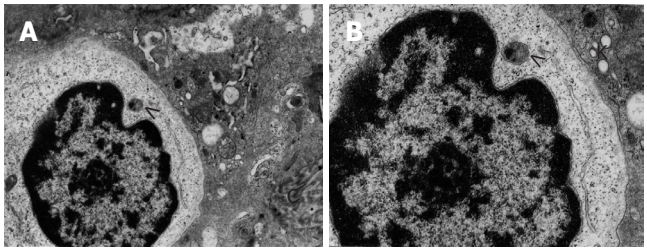


Figure 3 In a fibrotic field of the periportal space; an intermediate hepatic-like cell and transformed Ito cell. A thick bundle of collagen fibers (C) exerts a pressure on the cell from the outside, causing its focal narrowing; the electron-light cytoplasm contains relatively well developed dark mitochondria and elements of endoplasmic reticulum. Transformed Ito cell (>) adhering to intermediate hepatic-like cell surrounded by collagen deposits (C); H: hepatocyte of the limiting plate of the lobule. **A**: × 7000; **B**: × 12 000.



Figures 4 The distended perisinusoidal space of Disse shows an intermediate hepatocyte-like cell with adhering Kupffer cell (K); the IHC nucleus has less nuclear heterochromatin than the HPC nucleus and contains the nucleolus; the low electron dense cytoplasm with a distinct structure that resembles a peroxysome currently being formed (>) and with granular endoplasmic reticulum profiles. C: a bundle of collagen fibers. **A**: × 7000; **B**: × 12 000.

Ultrastructural examination allowed us to distinguish two types of oval cells: Ⅰ-hepatic progenitor cells (HPCs) and Ⅱ-intermediate hepatocyte-like cells (IHCs). In patients with S-4 fibrosis, intermediate bile duct-like cells were found, but because of their sporadic occurrence they are not discussed any further.

Hepatic progenitor cells were small (usually not exceeding 5 microns) and oval or nearly oval in shape. They had a large nucleus containing dense and highly clumped heterochromatin, accumulated distinctly under the nuclear envelope, and less abundant euchromatin (Figure 1). The cytoplasm was relatively scarce and slightly brighter than in the surrounding hepatocytes. As a result, the nucleus to the cytoplasm ratio was very high. The number of cytoplasmic structures was very small and these were only minimally differentiated (Figure 1). The cytoplasm contained tonofilaments. Some of the progenitor cells had intercellular junctions (point desmosomes), which helped connect these cells to the adjacent fully mature hepatocytes (Figure 1).

Intermediate hepatocyte-like cells varied in size, and were twice as large as the hepatic progenitor cells, whereas their diameter did not exceed one-half of the diameter of the mature hepatocytes. The nuclei were less abundant in heterochromatin compared to the progenitor cell nuclei, and occasionally contained nucleoli (Figures 4A and B). Frequently, these nuclei resembled the nuclei of mature hepatocytes.

The IHC cytoplasm showed much lower electron density compared to that of HPCs and contained better developed cell organelles, mainly mitochondria and elements of the endoplasmic reticulum, in which channels of the granular endoplasmic reticulum prevailed (Figures 2-4). The organelles accumulated in the vicinity of one of the nuclear poles (ultrastructural polarization) or were irregularly scattered throughout the cytoplasm. Among the intracellular organelles, small structures were seen that could correspond to newly formed peroxisomes (Figures 4A and B). Occasionally, the cells showed apical alterations in the form of well developed or newly formed capillary bile canaliculus.

DISCUSSION

Our study of the ultrastructure of liver specimens obtained from children with chronic hepatitis B showed the presence of small cells with an oval nucleus in the parenchyma, especially in areas of intense periportal fibrosis (at least stage 3 according to classification of Ishak *et al*[27]).

These cells corresponded to two types of submicroscopic oval cells, previously described by Roskams *et al*[22], i.e. the hepatic progenitor cells and intermediate hepatocyte-like cells. Intermediate bile duct-like cells were

seen sporadically, which may be related to the absence of regenerative nodules that are characteristic of liver cirrhosis.

In the vicinity of some intermediate hepatocyte-like cells, especially those cells lying close to collagen fiber bundles we observed activated nonparenchymal hepatocytes-transformed hepatic stellate cells, i.e. transitional Ito-fibroblast/myofibroblasts and Kupffer cells.

To the best of our knowledge, this is the first report on the electron microscopic study of oval cells in children with chronic viral infection of the liver associated with pronounced hepatic fibrosis.

In the present study, the ultrastructural appearance of the oval cells in children with chronic hepatitis B was very similar to that observed by other authors in adult patients, including those suffering from chronic viral hepatitis B and hepatitis C[10,11,20,22], as well as in various experimental models of liver damage[2,6,13,14,20]. These cells did not exhibit any significant morphological specificity related to the type and duration of liver damage and the patient's age.

It is worth noting, that our results regarding the location of oval cells are consistent with the observations made with the light microscope by Fotiadu *et al*[7] in chronic hepatitis B and chronic hepatitis C in adults. These authors performed a semiquantitative evaluation of the liver progenitor cells stained for cytokeratin 7, and found an increase in the number of oval cells parallel to the grade and stage of the disease in both types of hepatitis. These workers suggested that the proliferating liver progenitor cells may play a role in hepatic regeneration that occurs in the setting of viral hepatitis[7].

Xiao *et al*[10,11] conducted some very interesting ultrastructural and immunohistochemical studies on the hepatic progenitor cells in liver cirrhosis. These researchers found a small number of progenitor cells mainly at the sites of intensive collagen fibrosis-on the margins of regenerative nodules, across the fibrous span and within the proliferating bile ductules. The cells exhibited immunoreactivity to cytokeratin 7 and albumin[10,11].

In our pediatric patient population also, the oval cells, both the hepatic progenitor cells and the intermediate hepatocyte-like cells, were observed mainly in the areas of intense liver fibrosis, i.e. at least in patients with stage S-2.

It is believed that the proliferation of oval cells, their gradual migration in the organ and differentiation into hepatocytes or cholangiocytes is controlled by the nonparenchymal cells, especially the activated hepatic stellate cells, and by a number of growth factors and cytokines[1,19,28,29].

It should be mentioned that activated Ito cells, also found in the vicinity of the oval cells in our study, assume phenotypic features of fibroblasts by producing desmine, alpha-actin and specific laminin chains and play a key role in the morphogenesis of collagen fibroplasia[28,29].

On the other hand, in the course of chronic inflammatory diseases it is the oval cells that by synthesizing and releasing numerous growth factors (transforming growth factor alpha, transforming growth factor beta, acid fibroblast growth factor, insulin-like growth factor, stem cell factor) and cytokines may exert a significant effect on the environment and stimulate (especially through the release of transforming growth factor beta) hepatic extracellular matrix synthesis[4,19,20,31,32].

Finally, oval cells form a compartment of the so called "cell reserve", which are activated when the regenerative/proliferative properties of hepatocytes are inhibited. Therefore, some researchers treat these cells as a highly effective "third" protective system, especially in relation to the process of hepatocyte regeneration, which may open the way to cell-based therapy for liver diseases[12,19].

In conclusion, our study shows that there is a substantial correlation between the prevalence of oval hepatic progenitor cells and intermediate hepatocyte-like cells, and the stage of fibrosis in pediatric patients with chronic hepatitis B. Since the activated nonparenchymal cells were observed in the vicinity of the oval cells, it can be assumed that an interaction between these cells, especially between the transformed Ito cells, and the growth factors and cytokines secreted by them may play an essential role in the development of fibrosis in chronic hepatitis B. The present ultrastructural study provides interesting material for studies on the morphogenesis and differentiation of oval cells, and fibrosis progression in chronic hepatitis B in children.

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CagA+ *H pylori* infection is associated with polarization of T helper cell immune responses in gastric carcinogenesis

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Abstract

AIM: To characterize the immune responses including local and systemic immunity induced by infection with *H pylori*, especially with CagA+ *H pylori* strains and the underlying immunopathogenesis.

METHODS: A total of 711 patients with different gastric lesions were recruited to determine the presence of *H pylori* infection and cytotoxin associated protein A (CagA), the presence of T helper (Th) cells and regulatory T (Treg) cells in peripheral blood mononuclear cells (PBMCs), expression of plasma cytokines, and RNA and protein expression of IFN-γ and IL-4 in gastric biopsies and PBMCs were determined by rapid urease test, urea [^{14}C] breath test, immunoblotting test, flow cytometry , real time RT-PCR and immunohistochemistry.

RESULTS: Of the patients, 629 (88.47%) were infected with *H pylori*; 506 (71.16%) with CagA+ and 123 (17.30%) with CagA- strains. Among patients infected with CagA+ *H pylori* strains, Th1-mediated cellular immunity was associated with earlier stages of gastric carcinogenesis, while Th2-mediated humoral immunity dominated the advanced stages and was negatively associated with an abundance of Treg cells. However, there was no such tendency in Th1/Th2 polarization in patients infected with CagA- *H pylori* strains and those without *H pylori* infection.

CONCLUSION: Polarization of Th cell immune responses occurs in patients with CagA+ *H pylori* infection, which is associated with the stage and severity of gastric pathology during the progression of gastric carcinogenesis. This finding provides further evidence for a causal role of CagA+ *H pylori* infection in the immunopathogenesis of gastric cancer.

Key words: *H pylori*; CagA; Gastric carcinogenesis; T helper cells; Regulatory T cells; Immune response

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INTRODUCTION

In 1994, epidemiological surveys by WHO showed that more than half of the world's population was infected with *H pylori* and *H pylori* infection was closely related to chronic gastritis, gastric ulcer and gastric adenocarcinoma. These results led to the conclusion that *H pylori* is a first class risk factor (a definite carcinogen) for gastric adenocarcinomas[1]. It has been generally accepted that *H pylori* infection is involved in all stages of gastric carcinogenesis which progresses from chronic gastritis (CG) to gastric atrophy (GA), intestinal metaplasia (IM), dysplasia(DP) and ultimately gastric cancer (GC)[2-4]. However, the functional interaction of *H pylori* infection with distinct members of the immune compartment, especially T cell immune responses, in gastric carcinogenesis has not been fully elucidated. T helper (Th) cells can be divided into two subsets, Th1 cells and Th2 cells. Th1 cells mediate cellular immunity mainly by producing interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-β, while Th2 cells primarily mediate humoral immunity by secreting IL-4, IL-5, IL-6, IL-10 and IL-13. Previous studies have reported the differential expression of cytokines between *H pylori* positive and *H pylori* negative patients[5-7] or between gastritis and gastric cancer patients[8]. However, these studies did not examine the cellular role of *H pylori* infection in the immune responses during the progression of gastric pathology. Under normal homeostasis, the cytokines produced by one Th subset reciprocally inhibit the development of the other to keep

the balance of Th1 and Th2. Moreover, regulatory T (Treg) cells, which are a low abundance cell subset, help mediate the balance of Th1 and Th2, Treg cells inhibit the proliferation of CD4+ CD25-T lymphocytes, CD8+ T lymphocytes, immune memory cells, and antigen presenting cells (APCs) by recognizing inner and outer antigens. These immune responses result in the decrease of various cytokine secretion and the weakening of cellular immune function *in vitro*. The aim of the present study was to characterize the immune responses including local and systemic immunity induced by infection with *H pylori*, especially with CagA+ *H pylori* strains and the underlying immunopathogenesis, by analyzing the populations of T cells present in a range of progressive gastric pathologies during gastric carcinogenesis.

MATERIALS AND METHODS

Patients

Candidates with gastric discomfort, who had an endoscopy during October 2004 to May 2006, were recruited into this study based upon three clinical screening tests for *H pylori* infection. These tests were rapid urease test (RUT) (Lizhu Company, Zhuhai, China) of gastric tissue, urea [^{14}C] breath test (UBT) (Syncor Medicine Company Ltd., Shanghai, China) and the immunoblotting test (Yuangu company Ltd., Shanghai, China). The RUT and UBT tests have been reported to have sensitivity of 80%-99% and 95%-99% and specificity of 92%-100% and 77%-99%, respectively[9,10]. The immunoblotting test was designed to detect four major antigens including vacuolating cytotoxin (VacA, 95K), the cytotoxin associated protein A (CagA, 128K), urease A, urease B, and has been shown to be high sensitive and specific[11]. Patients were considered to be *H pylori*-positive when more than two tests were positive and to be negative when all three tests were negative, while those with two negative results were excluded from this study. In addition, two gastric biopsies taken from the antrum during the upper endoscopy were used for histological examination, real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. Histological examination was performed by experienced pathologists who were blinded to the patients' clinical diagnosis according to the updated Sydney system[11]. Patients with serious diseases or immune diseases were excluded from the study. None of the current study participants received surgery, radiotherapy, chemotherapy, or any other medical interventions before this study and all provided written informed consent after consultation. All the protocols and patient inclusion and exclusion criteria were approved by the Committee for Human Use and Institutional Review Board of Nanjing Medical University affiliated Nanjing first hospital for human subject studies.

Detection of IFN-γ, IL-4 expression and Treg cells

Heparinized venous blood (5 mL) taken from each patient was used to detect the expression of IFN-γ and IL-4. Treg cells were identified with a FACSCalibur flow cytometer (FCM, BDIS Biosciences, Franklin Lakes, USA). All reagents for FCM were provided by Caltag laboratories (Burlingame CA, USA). Cells in the blood were stimulated as described by Morita *et al*[12]. Briefly, heparinized venous blood was incubated with a combination of 25 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, Saint Louis MO, USA) and 1 μg/mL of the calcium ionophore, ionomycin (Sigma), for 5 h. After cells were cultured in RPMI 1640 for 1 h, 10 μg/mL Brefeldin A (Sigma) was added to enhance flow cytometric analysis of intracellular cytokine staining. After stimulation, the cells were incubated with peridinin chlorophyll (PerCP) mouse anti-human CD3 (CD3-PC) and fluorescein isothiocyanate (FITC) mouse anti-human CD8 (CD8-FITC) for 15-20 min. After fixation and permeabilization, corresponding antibodies [mouse IgG1-phycoerythrin (PE), PE conjugated mouse anti-human IFN-γ (IFN-γ-PE), Rat IgG1-PE, PE-conjugated Rat anti-human IL-4 monoclonal antibody (IL-4-PE)] were added and incubated for 15 min. The cells were then detected by FCM. Data from at least 50 000 cells in one sample were acquired and analyzed by Cell Quest software (BDIS Biosciences). As CD4 expression is known to be down-regulated after stimulation with PMA[13], the CD4+ lymphocytes were analyzed indirectly by gating the CD3+ CD8-lymphocytes. For the detection of Treg cells, whole blood (100 μL) that was incubated with 5 μL CD3-PC, 5 μL CD4-FITC, and 5 μL mouse IgG1-PE for 15-20 min and was used as a control tube, while whole blood (100 μL) that was incubated with 5 μL CD3-TC, 5 μL CD4-FITC and 5 μL CD25-PE,and lysed with 2 mL red blood cell (RBC)-lysis buffer for 5-10 min was used as the detection tube. The precipitates were analyzed by Cell Quest software of FCM.

Detection of cytokines in plasma by ELISA

Enzyme-linked immunosorbent assay (ELISA) kits for quantitative detection of soluble human IL-4 and IFN-γ were purchased from Bender MedsystemsTM (Vienna, Austria), and of IL-2, 6, 10, and 12 from R&D Systems Inc. (Minneapolis, USA). The assays were performed in accordance with the manufacturers' instructions.

Quantification of IFN-γ and IL-4 mRNA in PBMCs and gastric biopsies by real-time RT-PCR

To analyze IFN-γ and IL-4 mRNA, total RNA was extracted with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) from PBMCs and gastric biopsy specimens according to the manufacturer's recommendations and then reverse transcribed into cDNA by PTC-200 DNA Engine (Bio-Rad, Hercules, CA,USA).

PCR primers for human IFN-γ, IL-4 and β-action were designed by Takara Biotechnology Company (Dalian, China, Table 1). The amount of the PCR product was monitored by the SYBR Premix Ex Taq™ (Takara Biotechnology Company) on a Lightcycler system (Roche Molecular Biochemicals, Indianapolis, USA). The PCR mixture contained 20 μL reaction solution, 2.0 μL cDNA, 10 μL 2 × SYBR Premix Ex Taq™ and 250 nM of the primer. PCR amplification was performed according to the temperature profile: 95℃ for 10 s, followed by 45 cycles of 95℃ for 5 s, annealing and extension at 62℃ for 25 s. Data analysis was performed by the Light cycler software. All data were normalized by β-actin. The up-or down-

Table 1 Sequences of cDNA primers for human IFN-γ, IL-4 and β-actin

Target	Sequences (5′→3′)	Length of amplicon (bp)
IFN-γ	Forward: CTTTAAAGATGACCAGAGCATCCAA	189 (372-560 NM000619.2)
	Reverse: GGCGACAGTTCAGCCATCAC	
IL-4	Forward: GACTGTGCTCCGGCAGTTCTA	182 (589-770 NM000589)
	Reverse: CCAACGTACTCTGGTTGGCTTC	
β-actin	Forward: ATTGCCGACAGGATGCAGA	89 (991-1079 CR609136.1)
	Reverse: GAGTACTTGCGCTCAGGAGGA	

regulation (F) of cytokines were calculated by the formula $F = 2^{-\Delta\Delta Ct}$[14].

Immunohistochemistry for the detection of IFN-γ and IL-4 in antral biopsies

Immunohistochemical staining was performed on 8-μm-thick frozen sections mounted on glass slides as described previously[15]. Briefly, sections were fixed in 2% paraformaldehyde, air dried, and frozen at -20℃ for at least 1 h. After permeabilization and blocking, the sections were incubated with the cytokine-specific monoclonal antibodies (MAbs) (Caltag Laboratories, South San Francisco, CA, USA) at 4℃ overnight. They were then treated with 1% normal goat serum and subsequently incubated with 1:300 biotinylated goat anti-mouse IgG1 (Caltag Laboratories) and avidin-biotin horseradish peroxidase complex (Vector Laboratories Inc. Burlingame, CA, USA). The sections were developed with 3, 3-diaminobenzidine (Vector Laboratories Inc.) and counterstained with Mayer's hematoxylin (Histolab, Goteborg, Sweden). After dehydration, they were mounted with Mountex (Histolab). The tissue sections were analyzed by PAS-9000 Pathological Report system (Logene- I Biotech, Wuxi, China). The positively stained mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) per high power were counted. Only cells with a distinct cytoplasmic staining were included. For each sample, numbers of IL-4 and IFN-γ positive cells from 200 PMNs and MNCs were counted from five randomly selected fields and averaged. The positive rate was used to grade the expression levels: negative: 0%; +: 1%-25%; ++: 26%-50%; +++: 51%-100%.

Statistical analysis

One-way analysis of variance (ANOVA) and Pearson Correlation was performed to determine the difference and association by using Statistical Product and Service Solutions (SPSS, version 11.5, Chicago, IL, USA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

The prevalence of H pylori infection and CagA classification

Among 711 patients, 61 had normal gastric mucosa (normal mucosa, NM), 268 suffered from CG, 114 from GA, 104 from IM, 71 from dysplasia and 93 from GC (Table 2**).** Overall, 629 (88.47%) were infected with *H pylori*; 506 (71.16%) with CagA+ *H pylori* and 123 with CagA- *H pylori* (Table 2)**.** Following the progression of gastric lesions, the prevalence of CagA+ *H pylori* infection in NM, CG, GA, IM, DP and GC groups increased and the rate of CagA+ *H pylori* was positively associated with the severity of gastric pathologies ($r = 0.896$, $P = 0.016$), while the prevalence of CagA-*H pylori* infection was not significantly associated with the severity of gastric pathologies ($r = -0.794$, $P = 0.059$).

Systemic immune response in patients with CagA+ H pylori infection

Because the data of Th1/Th2 did not follow a Gaussian distribution, analysis of variance (ANOVA) was performed after the data were transferred by logarithm. As shown in Figure 1, there was no significant difference in IFN-γ among the different gastric pathologies, while there was an increasing tendency of IL-4 expression ($P = 0.022$) following the progression of gastric pathologies, leading to a gradual decrease in Th1/Th2 ratio in CagA+ *H pylori* infected patients. However, there was no such a tendency both in patients with CagA- *H pylori* infection and those without *H pylori* infection for both IFN-γ and IL-4, indicating that there was no significant difference in Th1/Th2 ratios in these patients. As for Treg cells, there was an increasing trend according to the progression of gastric pathologies, which was strongly correlated with CagA+ *H pylori* infection, but not with CagA- *H pylori* infection. There was a significant negative association between Th1/Th2 and the expression of Treg cells in gastric patients' PBMCs in patients with CagA+ *H pylori* infection ($r = -0.321$, $P < 0.001$), however, no such association was found in patients with CagA- *H pylori* infection and those without *H pylori* infection.

Following the progression of gastric pathologies associated with CagA+ *H pylori* infection, the levels of IL-2 and IL-12 gradually decreased, but the levels of IL-6, IL-10 gradually increased, which indicated that there were some changes in the patterns of cellular to humoral immunity. However, there was no significant difference for the key representative cytokines of Th1 and Th2 cells in all the groups (IFN-γ and IL-4, respectively) (Table 3). The patterns of six cytokines in the patients with CagA- *H pylori* infection and those without *H pylori* infection were similar to that in CagA+ *H pylori* infected patients (Tables 4 and 5), indicating there was no correlation between *H pylori* infection and cytokine levels.

Following the progression of the CagA+ *H pylori* associated gastric pathologies, the expression of IFN-γ mRNA showed a decreasing tendency ($P = 0.006$) while the expression of IL-4 mRNA increased ($P = 0.006$) (Figure 2). To check the specificity of the experiment, the molecular weight of the amplicon was confirmed by agarose gel electrophoresis (Figure 3). The expression of IFN-γ mRNA was significantly higher in CG than in IM, DP, GC ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), and was significantly higher in the GA than in DP and GC groups ($P < 0.001$, and $P < 0.001$), but there was no significant difference in the expression among the IM, DP and GC. On the contrary, the expression of IL-4 mRNA was significantly lower in CG than in IM, DP, and

Table 2 The prevalence of *H pylori* infection and CagA classification

Pathological diagnosis	Gender		Age	*H pylori* positive			*H pylori* negative
	Male	Female	(yr, mean ± SD)	Overall (%)	CagA+ (%)	CagA- (%)	
Normal mucosa (n = 61)	31	30	44.62 ± 12.41	48 (78.69)	37 (60.65)	11 (18.03)	13 (21.31)
Chronic gastritis (n = 268)	121	147	44.53 ± 14.37	232 (86.57)	170 (63.43)	62 (23.13)	36 (13.43)
Gastric atrophy (n = 114)	73	41	51.60 ± 12.39	104 (91.23)	88 (77.19)	16 (14.03)	10 (8.77)
Intestinal metaplasia (n = 104)	47	57	50.69 ± 12.81	95 (91.35)	80 (76.92)	15 (14.42)	9 (8.65)
Dysplasia (n = 71)	45	26	54.23 ± 12.25	65 (91.55)	57 (80.28)	8 (11.26)	6 (8.45)
Gastric cancer (n = 93)	70	23	64.70 ± 11.33	85 (91.40)	74 (79.57)	11 (11.83)	8 (8.60)
Total (n = 711)	387	324	50.18 ± 14.65	629 (88.47)	506 (71.16)	123 (17.30)	82 (11.53)

The prevalence of CagA+ *H pylori* was significantly associated with the progression of gastric pathology (r = 0.896, P = 0.016).

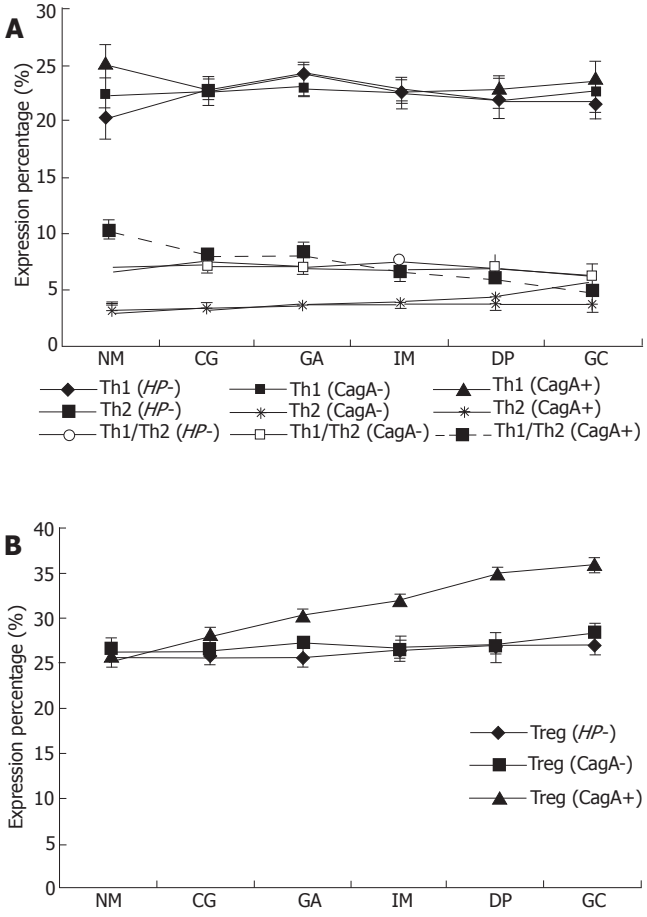


Figure 1 Expression of peripheral blood Th1, Th2 and Th1/Th2 ratio (**A**) and Treg cells (**B**) in patients with CagA+ and CagA- *H pylori* infection and in those without *H pylori* infection (Hp-) as determined by FCM. In CagA+ *H pylori* infected subjects, there is no significant difference in the percentage of Th1 cells in all groups, while the percentage of Th2 cells increases along the progression of gastric pathology, with significant difference between gastric cancer (GC) and chronic gastritis (CG) (P < 0.001), gastric atrophy (GA) (P < 0.001), and intestinal metaplasia (IM) (P < 0.001), respectively. There is a significant difference in the Th1/Th2 ratio between CG and dyspepsia (DP), GC (P = 0.033, P < 0.001), and between GA and GC (P < 0.001) (**A**). Treg cell expression increases along the progression with significant difference between CG and IM, DP, and GC (P < 0.001, P < 0.001, and P < 0.001, respectively), between GA and DP, and GC (P = 0.002, and P < 0.001, respectively), and between IM and GC (P = 0.012) (**B**). There is no difference in the expression of Th1, Th2, Treg cells and Th1/Th2 ratio between the subjects with CagA- *H pylori* infection and those without *H pylori* infection among all the groups.

GC (P < 0.001, P < 0.001, and P < 0.001, respectively), and in GA than in DP and GC (P < 0.001 and P < 0.001), but there was no significant difference in the expression between DP and GC. In patients with CagA- *H pylori* infection and those without *H pylori* infection, there was no significant difference in the expression of IFN-γ and IL-4 mRNAs along the progression of gastric pathologies (Figure 2).

Local immune response in patients with CagA+ H pylori infection

As shown in Figure 2, the expression of IFN-γ mRNA in gastric biopsies of patients with CagA+ *H pylori* infection decreased gradually along the progression of gastric pathologies from CG to GC; there was a significant difference in the expression of IFN-γ mRNA between CG and DP, GC (P = 0.013, P = 0.001), between GA and DP, GC (P = 0.043, P = 0.004). However, the gastric expression of IFN-γ mRNA was constant from CG to GC in patients with CagA- *H pylori* infection and those without *H pylori* infection. On the contrary, there was an increase in gastric IL-4 mRNA expression in patients with CagA+ *H pylori* infection along the progression of gastric pathologies from CG to GC (P = 0.004). The expression of IL-4 mRNA was significantly lower in CG and GA than in IM, DP and GC. However, the gastric IL-4 mRNA in patients with CagA- *H pylori* infection and those without *H pylori* infection was expressed at low levels and remained constant in all the gastric pathologies.

Those results indicated that IFN-γ mRNA expression showed a decreasing tendency while the IL-4 mRNA expression showed an increasing tendency following the progression of the CagA+ *H pylori* associated pathologies. Pearson correlation analysis indicated that there was a significant positive correlation in IFN-γ mRNA expression (r = 0.201, P < 0.001), and IL-4 mRNA expression (r = 0.212, P < 0.001) between gastric biopsy and PBMCs. Compared with the expression of IFN-γ mRNA and IL-4 mRNA in PBMCs, changes in the biopsy specimens were more conspicuous, especially in IM, DP and GC (Figure 2).

As shown in Figure 4A, gastric IFN-γ expression from CG to GC in patients with CagA+ *H pylori* infection showed a decreasing tendency with the percentage of cases with IFN-γ expression of "+++" decreasing from 45.9% in CG patients to 18.9% in GC patients. IFN-γ expression with "+++" predominated in CG, while the percentage of cases with IFN-γ expression with "+" increased significantly in GC. On the other hand, the percentage of cases with IL-4 expression with "+"

Table 3 Plasma cytokine contents in patients with CagA+ *H pylori* infection (mean ± SE, pg/mL)

Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 37)	27.46 ± 1.05	10.86 ± 0.62	81.42 ± 1.89	66.92 ± 2.15	47.96 ± 1.99	54.17 ± 1.09
Chronic gastritis (CG, *n* = 170)	28.94 ± 0.59	11.22 ± 0.45	72.49 ± 0.93	80.65 ± 0.99	63.40 ± 1.57	45.95 ± 0.49
Gastric atrophy (GA, *n* = 88)	30.48 ± 0.75	11.23 ± 0.33	53.29 ± 0.79	100.80 ± 1.69	94.10 ± 2.57	39.61 ± 0.69
Intestinal metaplasia (IM, *n* = 80)	31.71 ± 1.02	12.08 ± 0.59	52.18 ± 0.94	104.18 ± 1.90	112.03 ± 2.64	34.07 ± 0.54
Dysplasia (DP, *n* = 57)	30.14 ± 1.04	10.92 ± 0.39	43.30 ± 1.10	157.72 ± 2.64	156.74 ± 3.66	32.71 ± 0.81
Gastric cancer (GC, *n* = 74)	31.00 ± 0.84	11.03 ± 0.29	39.10 ± 0.89	196.65 ± 1.83	159.32 ± 2.57	28.39 ± 0.65

There was no difference in the expression of IFN-γ and IL-4 in the plasma of CagA+ *H pylori* subjects among all the groups. There were significant differences for IL-12 among all the groups except between GA and IM (P = 1.000), DP and GC (P = 0.237). There were significant differences for IL-10 between all groups except between GA and IM (P = 1.000). For IL-6, there were significant differences among all the groups except between DP and GC (P = 1.000). For IL-2, there were significant differences among all the groups except between IM and GA, DP (P = 1.000, P = 1.000).

Table 4 Plasma cytokine contents in patients with CagA- *H pylori* infection (mean ± SE, pg/mL)

Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 11)	29.28 ± 0.54	11.07 ± 0.89	82.17 ± 4.59	67.29 ± 3.26	48.37 ± 4.01	54.11 ± 2.87
Chronic gastritis (CG, *n* = 62)	29.19 ± 1.18	10.89 ± 0.27	64.59 ± 3.10	80.25 ± 1.87	57.99 ± 2.61	53.14 ± 1.25
Gastric atrophy (GA, *n* = 16)	30.25 ± 2.01	10.67 ± 0.67	49.67 ± 3.28	91.28 ± 3.19	92.56 ± 5.79	42.33 ± 2.32
Intestinal metaplasia (IM, *n* = 15)	28.91 ± 2.23	11.08 ± 0.74	48.19 ± 2.99	98.11 ± 4.08	98.20 ± 5.92	40.98 ± 2.38
Dysplasia (DP, *n* = 8)	31.08 ± 2.19	11.01 ± 1.27	42.64 ± 5.49	157.09 ± 6.81	147.65 ± 6.89	34.79 ± 3.05
Gastric cancer (GC, *n* = 11)	28.06 ± 2.45	12.07 ± 0.91	38.97 ± 3.29	185.29 ± 9.38	154.68 ± 6.78	30.29 ± 2.11

There was no difference in the expression of IFN-γ and IL-4 in plasma of subjects with CagA- *H pylori* infection among all the groups (P_{all} > 0.05). For IL-12, there were significant differences between CG and GA, IM, DP, and GC (P < 0.05, P < 0.05, P < 0.05, P < 0.001). For IL-10, there were significant differences between all the groups except between GA and CG and IM (P_{all} > 0.05). For IL-6, there were significant differences among all the groups except between GA and IM (P = 1.000) and DP and GC (P = 1.000). For IL-2, there were significant differences between CG and GA, IM, DP, and GC (P_{all} < 0.001) and between IM and GC (P < 0.05).

Table 5 Plasma cytokine contents in patients without *H pylori* infection (mean ± SE, pg/mL)

Pathological diagnosis	IFN-γ	IL-4	IL-12	IL-10	IL-6	IL-2
Normal mucosa (NM, *n* = 13)	31.83 ± 0.17	10.57 ± 0.68	80.84 ± 4.81	68.56 ± 3.87	49.53 ± 3.94	53.13 ± 1.48
Chronic gastritis (CG, *n* = 36)	29.10 ± 1.27	10.37 ± 0.30	68.57 ± 2.43	79.03 ± 1.64	57.66 ± 2.56	52.54 ± 1.41
Gastric atrophy (GA, *n* = 10)	29.06 ± 1.93	10.49 ± 0.58	51.14 ± 3.47	90.94 ± 3.63	91.08 ± 6.11	41.39 ± 2.18
Intestinal metaplasia (IM, *n* = 9)	28.57 ± 2.72	11.75 ± 0.61	50.97 ± 1.54	97.60 ± 3.53	96.35 ± 5.19	41.54 ± 0.99
Dysplasia (DP, *n* = 8)	31.59 ± 1.29	11.46 ± 0.58	43.10 ± 5.02	153.34 ± 6.12	149.92 ± 6.23	35.00 ± 2.04
Gastric cancer (GC, *n* = 6)	27.04 ± 2.30	11.91 ± 1.26	40.06 ± 2.08	181.36 ± 9.02	153.04 ± 6.63	30.95 ± 1.91

There was no difference in the expression of IFN-γ and IL-4 in plasma of subjects without *H pylori* infection among all the groups (P_{all} > 0.05). For IL-12, there were significant differences between CG and GA, IM, DP, and GC (P = 0.006, P = 0.009, P = 0.001, P < 0.001). For IL-10, there were significant differences between all the groups except between GA and CG and IM (P = 0.219, P = 1.000). For IL-6, there were significant differences among all the groups except between GA and IM (P = 1.000) and DP and GC (P = 1.000). For IL-2, there were significant differences between CG and GA, IM, DP, and GC (P_{all} < 0.001) and between IM and GC (P = 0.037).

decreased gradually from 68.2% in CG patients to 35.1% in GC patients, but the rate of IL-4 expression with "+++" increased gradually from 0% in CG patients to 28.4% in GC patients. In patients with CagA- *H pylori* infection and those without *H pylori* infection, there was no difference between IFN-γ and IL-4 expression among the different gastric pathologies; IFN-γ expression with "++" was always dominant, while IL-4 expression with "+" was always dominant (Figure 4B and C). The representative immunohistochemistry staining results in the gastric biopsies of with CagA+, CagA- *H pylori* infected patients and uninfected patients with different gastric pathologies are shown in Figure 5A-C.

DISCUSSION

H pylori is a small, curved, highly motile, Gram-negative bacterium that colonizes the epithelium of the human stomach. Previous studies have shown that *H pylori* infection is associated with the development of chronic gastritis and gastric cancer[3,4,16] Several factors including virulence among different *H pylori* strains have been attributed to the diversity in clinical outcome[17]. Until now, very few papers have reported the prevalence of *H pylori* infection in China, especially the subtypes of the organism[18,19]. Our study showed that the prevalence of *H pylori* infection in our outpatients is 88.47% (629/711) and the majority (80.45%, 506/629) of the infected subjects is infected with CagA+ *H pylori*. This confirms that CagA+ *H pylori* infected subjects are predominant in the Chinese *H pylori*-infected population[19]. Moreover, we observed that the subtypes of *H pylori* infection may play different role in the progression of gastric carcinogenesis; CagA+ *H pylori* infection was positively associated with the severity of gastric pathologies, especially gastric cancer, whereas CagA- *H pylori* infection was not associated with the

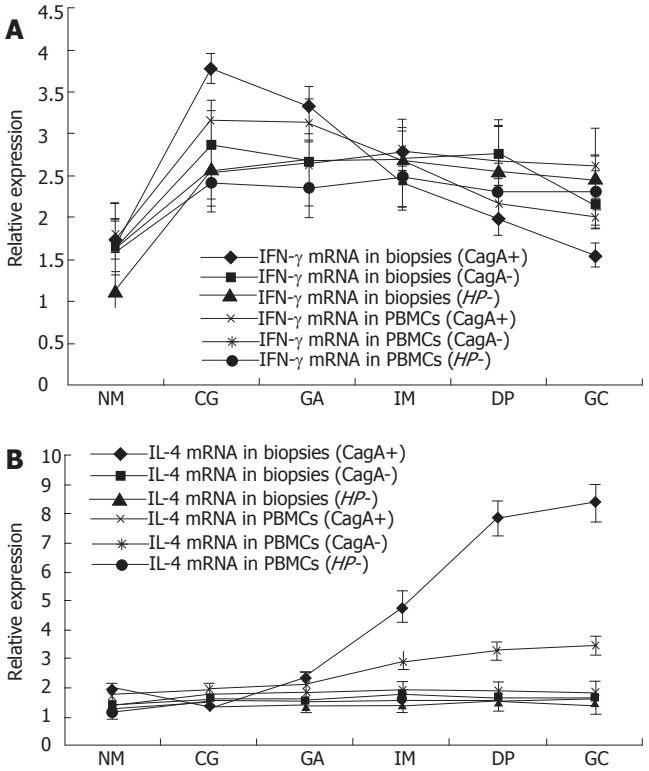


Figure 2 The relative expression of IFN-γ (**A**) and IL-4 (**B**) mRNA in peripheral blood mononuclear cells (PBMCs) and gastric biopsies in patients with CagA+ and CagA- *H pylori* infection and those without *H pylori* infection as determined by real time RT-PCR. The relative expression of IFN-γ mRNA decreases from CG to GC, while the relative expression of IL-4 mRNA increases from chronic gastritis (CG) to gastric cancer (GC) in PBMCs and biopsies of CagA+ *H pylori* infected subjects. However, the magnitudes of relative expression of IFN-γ mRNA and IL-4 mRNA in biopsies are obviously larger than those in PBMCs. There is no difference in the expression of IFN-γ and IL-4 mRNA in PBMCs and local biopsies of subjects with CagA- *H pylori* infection and of those without *H pylori* infection.

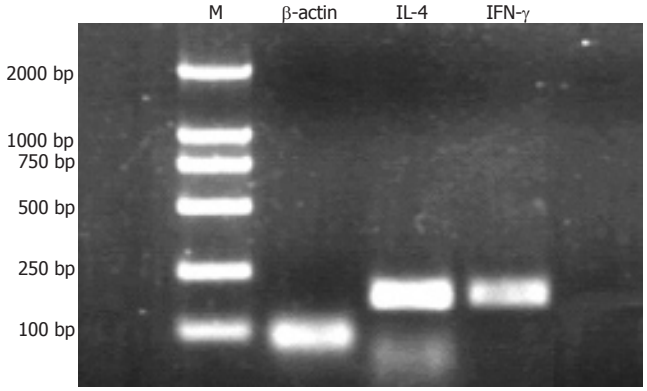


Figure 3 A representative of real time RT-PCR for IFN-γ and IL-4 mRNA expression in PBMCs by agarose gel electrophoresis. Using the real time PCR products to run agarose gel electrophoresis is to confirm the size of every fragment. M: DNA marker, and the molecular weight of IL-4, IFN-γ and β-actin was 189 bp, 182 bp and 89 bp, respectively.

severity of gastric pathologies.

Previous epidemiological studies have demonstrated that severe clinical manifestations do not frequently occur in most individuals with *H pylori* infection and the different clinical manifestations caused by this bacterium could be a consequence of interactions among microorganism characteristics, environmental influences, and the host immune response[20-22]. In the present study, 711 patients with different gastric pathologies were recruited to explore the interaction between bacterial virulence and host immune responses including systemic immunity and local immunity. We observed that in patients with CagA+ *H pylori* infection, the expression of IFN-γ remained unchanged, while IL-4 increased gradually in PBMCs during the progression of gastric pathologies, resulting in decreases of Th1/Th2 ratios, which was negatively correlated to the increased expression of Treg cells. During the development of gastric pathologies, IFN-γ mRNA expression gradually decreased, while IL-4 mRNA expression gradually increased, which was consistent with Th1/Th2 results in PBMCs. In addition, IL-2 and IL-12 protein expression decreased gradually while IL-6 and IL-10 increased gradually following the progression of gastric pathologies, although IFN-γ and IL-4, the representative cytokines of Th1 and Th2 cells, did not change. However, the ratios of Th1/Th2 in patients with CagA- *H pylori* infection and those without *H pylori* infection remained unchanged at both the cellular level and the mRNA level. Therefore, we postulate, based on our observations, that following the progression of the gastric pathologies, Th1 cells change slightly but Th2 cells are gradually increased, resulting in a gradual decrease in the ratio of Th1/Th2. Indeed, our systemic immunity analysis demonstrated that there was a shift from Th1 response to Th2 response during the progression of CagA+ *H pylori* infection associated gastric pathologies. However, it is noticed that IFN-γ and IL-4 expression in the plasma did not show any tendency of the Th1/Th2 shift, which may be due the fact that the levels of cytokines in plasma are affected by many factors other than *H pylori* infection[23,24], which may also explain the observation that IFN-γ and IL-4 expression were similar among patients with CagA+, or CagA- *H pylori* infection and those without *H pylori* infection.

Real-time RT-PCR and immunohistochemistry analysis were employed in the preset study to compare the local immunity of *H pylori* infected subjects, including mRNAs and protein expression of IFN-γ and IL-4 in gastric mucosa of patients with *H pylori* infection, with that in patients without *H pylori* infection. We observed that IFN-γ mRNA expression decreased gradually, while IL-4 mRNA expression increased during the development of gastric pathologies and the changing magnitude of IFN-γ mRNA and IL-4 mRNA expression in gastric mucosa were more obvious than those in PBMCs. Moreover, during the progression of gastric pathologies associated with CagA+ *H pylori* infection, IFN-γ expression primarily decreased from grade "+++" in CG patients to grade "+" in GC patients, whereas IL-4 expression increased from "+" in CG patients to "+++" in GC patients. On the contrary, in patients with CagA- *H pylori* infection and those without *H pylori* infection, IFN-γ and IL-4 expression remained unchanged during the progression of gastric pathologies; IFN-γ expression remained with grade "++" and IL-4 expression with grade "+". These findings demonstrate that there is a shift from Th1 mediated immune response

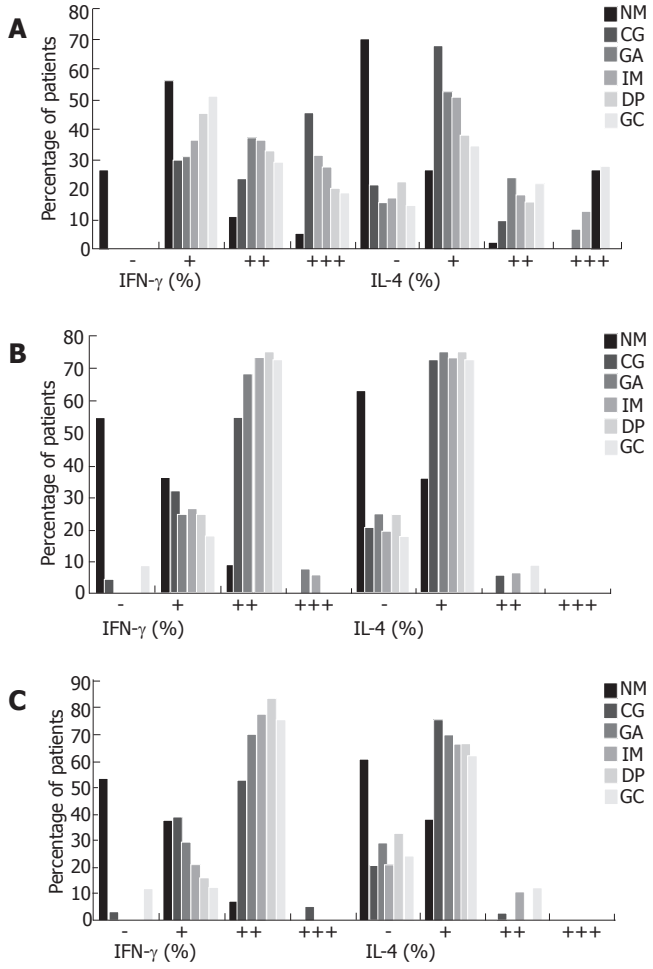
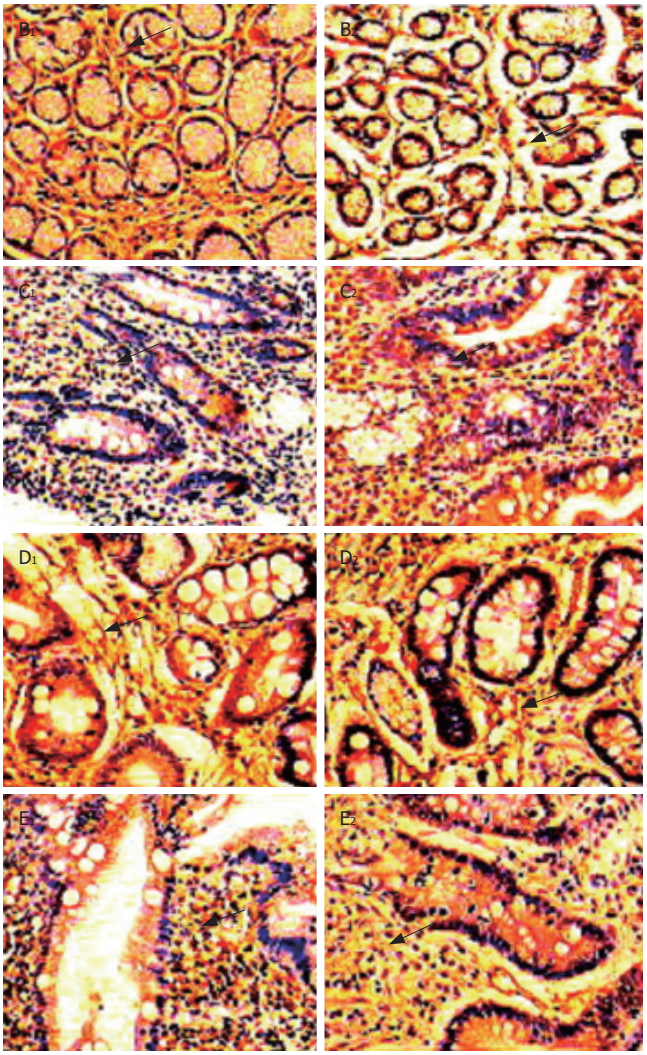
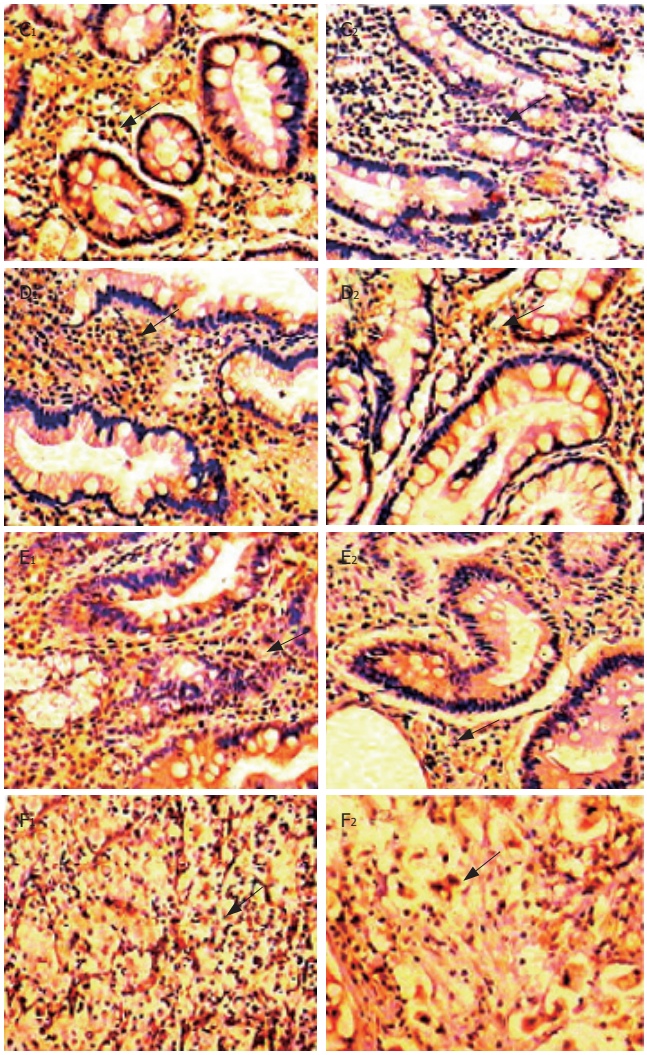


Figure 4 Local immunity responses in patients with CagA+ (**A**) , CagA- *H pylori* (**B**) infection and those without *H pylori* infection (**C**) as determined by immunohistochemistry. Numbers of IFN-γ and IL-4 positive cells from 200 polymorphonuclear cells (PMNs) and mononuclear cells (MNCs) were counted from five randomly chosen fields and averaged. The positive rate was used to grade the expression levels: negative: 0%; +: 1%-25%; ++ 26%-50%; +++, 51%-100%. **A**: IFN-γ expression with "+++" predominates in chronic gastritis (CG), while IFN-γ expression with "+" becomes the majority when the disease develops to gastric cancer (GC). On the contrary, IL-4 expression with "+" decreases gradually when the disease develops from CG to GC, but the rate of IL-4 expression with "+++" increases gradually following the progression; **B**: Following the progression of gastric lesions, there was no difference of IFN-γ and IL-4 expression in that the former was always with "++", while the latter was always with "+" in all the groups; **C**: Following the progression of gastric lesions, IFN-γ expression with "++" were at all dominant, while IL-4 expression with "+" was in the main in all the groups.

to Th2 mediated immune response within gastric mucosa during the progression of CagA+ *H pylori* infection associated gastric carcinogenesis, which is more significant than the Th1/Th2 shift in the systemic immunity.

In conclusion, there is a shift from Th1 mediated cellular immunity to Th2 mediated hormonal immunity in the immune response during the progression of gastric carcinogenesis in patients with CagA+ *H pylori* infection, which is negatively associated with the expression of Treg cells. Moreover, among the immune responses associated with CagA+ *H pylori* infection, local immunity is predominant over systemic immunity and the polarization of Th cells mediated immune response is associated with the chronicity and progression of gastric pathologies,





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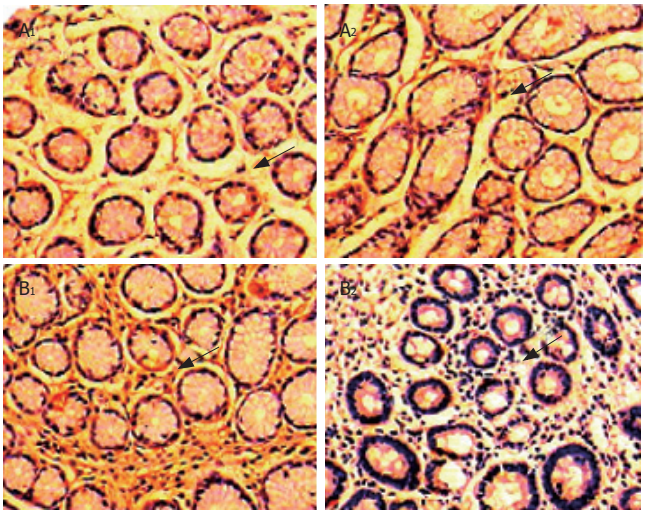

Figure 5 Representatives of immunohistochemistry staining for IFN-γ and IL-4 in gastric biopsies of CagA+ *H. pylori* (**A**), CagA- *H pylori* (**B**) and those without *H pylori* infected patients(**C**) with normal gastric mucosa or with different gastric pathologies (DAB, ×200, Arrowhead shows the representative positive cells, which are stained brown). A_1, B_1, C_1, D_1, E_1 and F_1 represent IFN-γ expression in normal mucosa (NM), chronic gastritis (CG), gastric atrophy (GA), intestinal metaplasia (IM) and gastric cancer (GC), respectively, and A_2, B_2, C_2, D_2, E_2 and F_2 represent IL-4 expression in NM, CG,GA,IM, DP AND GC, respectively. **A**: The rates of IFN-γ+ cells are great in the CG, GA, and IM groups relative to the DP and GC groups. Additionally, the rates of IL-4+ cells are less in the NC and CG groups, but increased along the progression; **B**: The rates of IFN-γ+ cells and IL-4+ cells were constant in all the groups, and IFN-γ expression with "++" was dominant, while IL-4 expression with "+" was in the majority of all the groups; **C**: The rates of IFN-γ+ cells and IL-4+ cells were constant in all the groups, and IFN-γ expression with "++" was dominant, while IL-4 expression with "+" was in the majority of all the groups.

especially gastric cancer. These findings provide clinical evidence for exploring the immunopathogenesis of gastric cancer associated with CagA+ *H pylori* infection.

COMMENTS

Background

Studies have shown that *H pylori* infection is involved in all stages of gastric carcinogenesis which progresses from chronic gastritis to gastric atrophy, intestinal

metaplasia, dysplasia and gastric cancer. However, the functional interaction of *H pylori* infection with immune components, especially T cell immune responses, has not been fully elucidated.

Research frontiers

Previous studies have reported the differential expression of cytokines between *H pylori* positive and *H pylori* negative patients or between gastritis and gastric cancer patients, but these studies did not examine the cellular role of *H pylori*, especially CagA+ *H pylori*, infection in the immune responses during the progression of gastric pathology.

Innovations and breakthroughs

Among patients infected with CagA+ *H pylori* infected, Th1-mediated cellular immunity was associated with earlier stages of gastric carcinogenesis, while Th2-mediated humoral immunity dominated the advanced stages and was negatively associated with an abundance of Treg cells. However, there was no such tendency in Th1/Th2 polarization in patients infected with CagA- *H pylori* strains and those without *H pylori* infection.

Applications

Polarization of Th cell immune responses occurs in patients with CagA+ *H pylori* infection, which is associated with the stage and severity of gastric pathology during the progression of gastric carcinogenesis, will provide further evidence for a causal role of CagA+ *H pylori* infection in the immunopathogenesis of gastric cancer.

Terminology

Immune response: Actions of the body's immune system that come into play to control infection or disease; T helper cells: can be divided into two subsets, Th1 cells and Th2 cells. Th1 cells mediate cellular immunity mainly by producing interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-β, while Th2 cells primarily mediate humoral immunity by secreting IL-4, IL-5, IL-6, IL-10 and IL-13; regulatory T cells: a low abundance cell subset, help mediate the balance of Th1 and Th2, Treg cells inhibit the proliferation of CD4 + CD25-T lymphocytes, CD8 + T lymphocytes, immune memory cells, and antigen presenting cells (APCs) by recognizing inner and outer antigens; Immunopathogenesis: the process of development of a disease in which an immune response or the products of an immune reaction are involved.

Peer review

This is an excellent work that I think has to be accepted as it is. It is well written, well done and reaches original findings.

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BASIC RESEARCH

Leptin treatment ameliorates acute lung injury in rats with cerulein-induced acute pancreatitis

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Abstract

AIM: To determine the effect of exogenous leptin on acute lung injury (ALI) in cerulein-induced acute pancreatitis (AP).

METHODS: Forty-eight rats were randomly divided into 3 groups. AP was induced by intraperitoneal (i.p.) injection of cerulein (50 μg/kg) four times, at 1 h intervals. The rats received a single i.p. injection of 10 μg/kg leptin (leptin group) or 2 mL saline (AP group) after cerulein injections. In the sham group, animals were given a single i.p. injection of 2 mL saline. Experimental samples were collected for biochemical and histological evaluations at 24 h and 48 h after the induction of AP or saline administration. Blood samples were obtained for the determination of amylase, lipase, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, macrophage inflammatory peptide (MIP)-2 and soluble intercellular adhesion molecule (sICAM)-1 levels, while pancreatic and lung tissues were removed for myeloperoxidase (MPO) activity, nitric oxide (NOx) level, CD40 expression and histological evaluation.

RESULTS: Cerulein injection caused severe AP, confirmed by an increase in serum amylase and lipase levels, histopathological findings of severe AP, and pancreatic MPO activity, compared to the values obtained in the sham group. In the leptin group, serum levels of MIP-2, sICMA-1, TNF-α, and IL-1β, pancreatic MPO activity, CD40 expression in pancreas and lung tissues, and NOx level in the lung tissue were lower compared to those in the AP group. Histologically, pancreatic and lung damage was less severe following leptin administration.

CONCLUSION: Exogenous leptin attenuates inflammatory changes, and reduces pro-inflammatory cytokines, nitric oxide levels, and CD40 expression in cerulein-induced AP and may be protective in AP associated ALI.

Key words: Leptin; Acute pancreatitis; Lung injury; CD40; Cytokines

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INTRODUCTION

Acute pancreatitis (AP) is a non-infectious inflammatory reaction of the pancreas, associated with autodigestion of the organ[1]. Lung injury is the most important manifestation of extra abdominal organ dysfunction in acute pancreatitis[2]. Approximately one-third of patients develop acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) accounts for 60% of all deaths in the first week[3]. Apart from mechanical ventilatory support, few therapies have shown any clinical benefit. New agents such as anti-cytokines and anti-nuclear factor kappa are still being evaluated in experimental models of pancreatitis[1,4]. ALI is characterized by an increase in pulmonary microvascular permeability, with protein-rich transudate spilling into the alveolar spaces, resulting in decreased lung compliance. Such physicochemical alterations are mediated by the local release of cytotoxic and vasoactive substances. Recent studies have shown that cytokines and adhesion molecules such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), macrophage inflammatory peptide-2 (MIP-2), intercellular adhesion molecule-1 (ICAM-1) and CD40 (50-kDa protein expressed on the membranes of B lymphocytes, monocytes, dendritic cells, and biliary epithelial cells) as well as neutrophil activation and adhesion contribute to the development and severity of AP and ALI[2,5-7].

Leptin is a peptide hormone that is produced

predominantly by white adipose cells[8]. The mature protein, encoded by the obese (*ob*) gene, is localized on human chromosome 7 and mouse chromosome 6[9]. In addition to metabolic and endocrine functions, leptin also plays a regulatory role in hematopoiesis, immunity and inflammation[10,11]. Alterations in immune and inflammatory responses are present in leptin or leptin-receptor-deficient animals, as well as during starvation and malnutrition. Both conditions are characterized by low levels of circulating leptin. Leptin is believed to play a role in immune function, as it is a member of the helical cytokine family with a structure resembling the hematopoietic cytokines IL-2, IL-6 and IL-15[10]. The leptin receptors (Ob-R) show amino acid sequence homology to hematopoietic (class 1) cytokine receptors[12]. Besides fat tissues, Ob-R is also present in other tissues such as liver, pancreas, lung, and kidney[13]. Leptin exerts proliferative and anti-apoptotic activity in a variety of cell types including T lymphocytes, leukemia cells, and hematopoietic progenitors. It also affects cytokine production, the activation of monocytes/macrophages, wound healing and angiogenesis. Moreover, leptin production increases acutely during infection, inflammation, and pancreatitis[10,14]. Recent studies have demonstrated that AP in rats and humans is accompanied by an increase in plasma levels of leptin[15,16]. Furthermore, the elevation of leptin mRNA expression in the pancreas during AP suggests that the increased level of this hormone in serum originates from the pancreas[16]. Another study showed that exogenous leptin administration was associated with a reduction in the severity of AP, presumably through a decrease in the production of plasma pro-inflammatory cytokines and NO[17]. Whether exogenous leptin has any effect on AP-associated lung injury remains unclear, however, its anti-inflammatory and anti-apoptotic actions would suggest that it may reduce the occurrence of extra-abdominal organ dysfunctions such as ALI. In the present study, we examined the effects of exogenous leptin on lung injury in a cerulein model of AP.

MATERIALS AND METHODS

Experimental animal models and Study groups

Forty eight female Wistar rats weighing 230-260 g were used in the study. The experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and was approved by the Committee for Research and Animal Ethics of Gazi University. The rats were maintained at 23℃ in a 12 h light dark cycle, with free access to water and standard rat chow. Prior to the start of the experiments, the rats were deprived of food while drinking water was available ad libitum. Three experimental groups were established: sham group (n = 16), acute pancreatitis (AP) group (n = 16) and leptin group (n = 16). AP was induced by intraperitoneal (i.p.) injection of cerulein (Sigma-Aldrich Chemical, Steinheim, Germany) diluted in saline and administered at the dose of 50 μg/kg, four times, at 1h intervals[18]. At the end of cerulein injections the rats were given 2 mL i.p. saline in the AP group. The leptin group, received leptin (mouse recombinant leptin, Sigma, Saint-Louis, Missouri, USA) at a dose of 10 μg/kg i.p. dissolved in 1 mL of saline followed by cerulein injections. The sham group was injected with 2 mL 0.9% NaCl solution i.p. All groups were randomly subdivided into 24 h and 48 h groups, each group containing 8 rats. Frossard *et al*[2] have demonstrated that administration of supramaximal dose of cerulein to rats led to AP associated ALI; the severity of ALI was time-dependent, with maximal injury occurring at 24 h and 48 h after the start of cerulein injections. Therefore, in our study, the animals were sacrificed at 24 h and 48 h after the cerulein or saline administration.

Following cessation of treatment, animals were anesthetized by an intramuscular injection of 40 mg/kg ketamine (Ketalar®, Parke Davis, Eczacıbasi, Istanbul, Turkey) and 5 mg/kg xylazine (Rompum®, Bayer AG, Leverkusen, Germany). The abdominal and thoracic cavities were opened and blood, pancreatic and lung samples were obtained. The blood samples were stored at -80℃ for biochemical analysis, which was run in duplicates. Random cross-sections of the pancreatic head, body and tail, and of the right lung were fixed in a 40 g/L solution of formaldehyde, in 0.1 mol/L phosphate-buffed saline (pH 7.4), and embedded in paraffin. Samples of pancreas and lung tissues were stored at -70℃ for subsequent biochemical measurements.

Serum amylase and lipase measurements

Serum amylase and lipase were determined with a Beckman Coulter LX-20® System analyzer (Fullerton, CA, USA) using Beckman kits (Fullerton, CA, USA), according to the supplier's specifications.

Serum TNF-α, IL-1β, MIP-2 and Soluble ICAM-1 measurements

Serum levels of TNF-α, IL-1β, MIP-2 (Biosource International, Camarillo, CA, USA) and soluble ICAM-1 (sICAM-1, Quantikine, R&D Systems, MN, USA) were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA). TNF-α, IL-1β, MIP-2 and sICAM-1 levels were determined from a standard curve for the combination of these cytokines. The concentrations were expressed as pg/mL.

Measurement of tissue myeloperoxidase activity

Pancreas and lung tissues were homogenized in TRIS buffer. Sequestration of neutrophils within the pancreas and lung was evaluated by quantitation of tissue myeloperoxidase (MPO) activity by the ELISA method (Hbt Hycult Mouse MPO ELISA kit, Uden, Netherlands).

Measurement of NOx level

Total nitrate/nitrite (NOx) concentration was measured in a simple two-step process by using the Cayman Chemical NOx Assay Kit. The first step was the conversion of nitrate to nitrite, utilizing nitrate reductase. The second step was the addition of the Griess reagents, which converted nitrite into a deep-purple azo-compound. Photometric measurement of the absorbance due to this azo-chromophore accurately determined nitrite

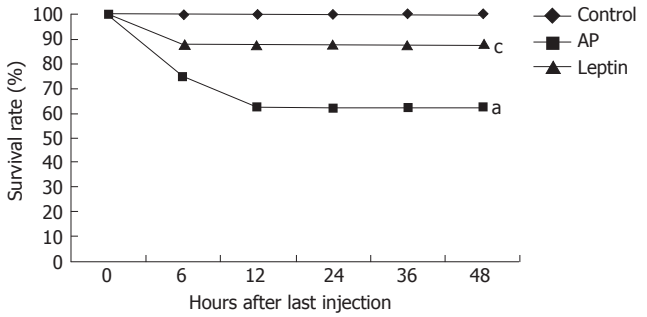

Figure 1 Effects of leptin treatment on survival rate. ($^aP < 0.001$ *vs* sham group, $^cP < 0.05$ *vs* AP group).

concentration. Concentrations were expressed as nmol/mg protein[19].

Histological examination

Sections of the pancreas and lung (4 μm thick) were stained with hematoxylin-eosin (HE) and examined with a light microscope as described previously[20,21].

Immunohistochemical assessment of CD40

Specimens were fixed in a 40 g/L solution of formaldehyde in 0.1 mol/L phosphate-buffed saline (pH 7.4), and embedded in paraffin wax from which four-μm-thick sections were taken on a slide coated with poly-L-lysine in order to be de-waxed for immunohistochemical assessment. The specimens were treated with 3% hydrogen peroxidase, following 10 min of endogenous peroxidase blockage. Antigen retrieval was performed with EDTA. The samples were incubated at room temperature for 45 min, with a 1:50 dilution of anti-CD40 primary antibody (clone: 3/23, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following the processing of the samples with a streptavidin biotin peroxidase complex, 3-amino-9-ethylcarbazole was applied as a chromogen, and hematoxylin was used for floor staining. CD40 is expressed on the surface of B lymphocytes. Tonsil tissue was used as a positive control since the tonsils are lymphoid organs where B lymphocytes are commonly present in the lymphoid follicles[22]. CD40 staining was graded from 0 to 4; the number of cells which revealed CD40-positive staining obtained from five × 400 magnified fields was divided by the total cell number. According to these observations, less than 50% of the stained cell population was considered focally stained, and more than 50% of the stained cell population was considered diffusely stained. Grade 0: no staining; grade 1: slight cytoplasmic or membranous staining in < 50% of the cell population (focal); grade 2: slight cytoplasmic or membranous staining in > 50% of the cell population (diffuse); grade 3: strong cytoplasmic or membranous staining in < 50% of the cell population (focal); grade 4: strong cytoplasmic or membranous staining in > 50% of the cell population (diffuse).

Statistical analysis

All values were expressed as mean ± SE and the results were compared by analysis of variance (ANOVA) with post hoc analysis using the Bonferroni test. The log rank test was used for survival analysis. $P < 0.05$ was considered statistically significant. Statistical evaluation was carried out using the SPSS 12.0 software (SPSS, Chicago, IL, USA).

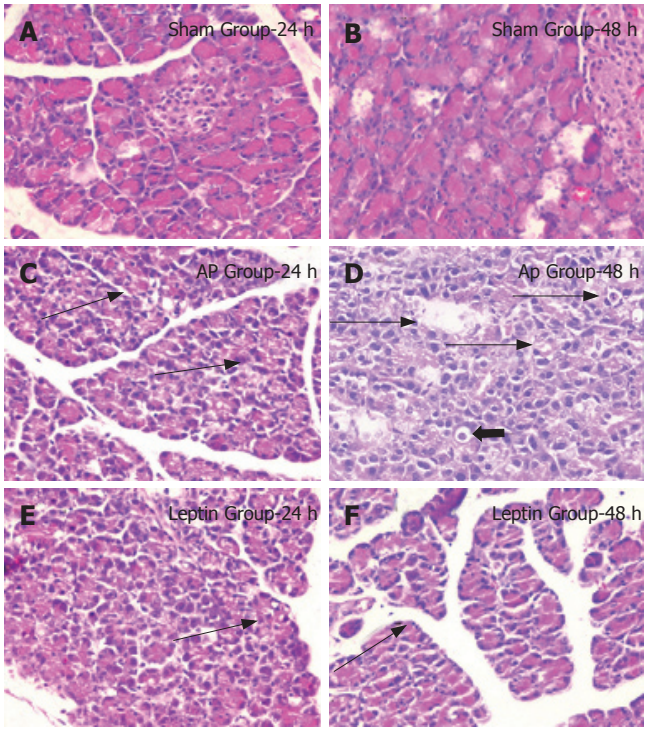

Figure 2 Light microscopy showing normal pancreatic tissue in the sham group (**A**, **B**) (HE, × 200), broad single cell necrosis (arrow) and significant increase in vacuolization (bold arrow) in the AP group at 24 h and 48 h (**C**, **D**) (HE, × 400), attenuated necrosis and vacuolization after leptin treatment at 24 h (**E**) (HE, × 400). Mild edema was observed at 48 h in leptin-treated rats (**F**, arrow).

RESULTS

Kaplan-Meier survival curves (Figure 1) showed absence of mortality after the sham operation (100% survival), while the survival rate was reduced to 62.5% in the AP group ($P < 0.001$ *vs* sham). Treatment with leptin was associated with significant improvement in the survival rate (87.5%) throughout the 48 h observation period ($P < 0.05$).

Effects of exogenous leptin on pancreatic injury in AP

Supramaximal doses of cerulein injections resulted in severe AP in the animals. This was confirmed by an increase in serum amylase and lipase levels ($P < 0.001$, Tables 1 and 2), histopathological findings of severe AP ($P < 0.05$, Table 3, Figures 2C and D), and pancreatic MPO activity, as a measurement of neutrophil infiltration ($P = 0.004$, Tables 1 and 2) in the cerulein injected rats compared to saline administered rats. Serum amylase levels and pancreatic MPO activity in rats treated with leptin were markedly lower at 24 h compared to cerulein-only injected rats ($P < 0.001$, Tables 1 and 2). However, there was no significant difference in serum lipase levels between the AP group and the leptin treated group (Tables 1 and 2). Histopathological examination of the pancreas confirmed the beneficial effect of exogenous leptin treatment on AP (Table 3). In the pancreatic tissue from the AP group,

Table 1 Biochemical markers in the blood, and pancreatic and lung tissues at 24 h mean ± SE

Groups	Amylase (U/L)	Lipase (U/L)	TNF-α (pg/mL)	IL-1b (pg/mL)	MIP (pg/mL)	sICAM (pg/mL)	Pancreas MPO (U/gr)	Lung MPO (U/gr)	Lung NOx (nmol/mg protein)
Sham Group	44.2 ± 5.8	17.2 ± 1.0	9.5 ± 0.7	6.4 ± 0.8	5.7 ± 8.5	11132 ± 626	0.17 ± 0.02	2.5 ± 0.6	8.1 ± 1.1
AP Group	1913 ± 205[a]	47.1 ± 9.0	114.6 ± 16.5[a]	107.9 ± 5.1[a]	128.3 ±5.1[a]	27828 ± 1483[a]	25.0 ± 11.1[a]	95.8 ± 17.0[a]	26.6 ± 0.8[a]
Leptin Group	1419 ± 87	39.4 ± 36.3	67.2 ± 7.0	67.2 ± 7.0	45.0 ± 10.2	21042 ± 1676	4.1 ± 1.6	38.2 ± 6.3	19.3 ± 0.7

[a]$P < 0.05$ *vs* the leptin group.

Table 2 Biochemical markers in the blood, and pancreatic and lung tissues at 48h mean ± SE

Group	Amylase (U/L)	Lipase (U/L)	TNF-α (pg/mL)	IL-1b (pg/mL)	MIP (pg/mL)	sICAM (pg/mL)	Pancreas MPO (U/gr)	Lung MPO (U/gr)	Lung NOx (nmol/mg protein)
Sham group	37.3 ± 14.3	15.3 ± 2.1	5.5 ± 0.8	8.6 ± 3.7	8.5 ±3.8	14216 ± 1148	0.51 ± 0.29	6.2 ± 2.3	6.1 ± 0.8
AP group	1078 ± 77	44.2 ± 4.5	91.2 ± 2.2[a]	94.8 ± 7.5[a]	78.1 ± 9.7[a]	28416 ± 1321[a]	11.4 ± 2.5	97.6 ± 15.1[a]	21.9 ± 0.7
Leptin group	1233 ± 128	36.3 ± 1.7	53.6 ± 3.8	37.0 ± 6.1	28.9 ± 2.3	16712 ± 1054	5.0 ± 1.7	29.7 ± 2.9	15.3 ± 0.8

[a]$P < 0.05$ *vs* the leptin group.

Table 3 Histological scores of pancreatic injury mean ± SE

Group	Edema		Inflammation		Vacuolization		Necrosis	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Sham group	0	0	0	0	0	0	0	0
AP group	2.57 ± 0.20[a]	2.37 ± 0.18[a]	1.28 ± 0.28	1.75 ± 0.46a	2.28 ± 0.35[a]	1.50 ± 0.16[a]	0.71 ± 0.18	1.00 ± 0.00[a]
Leptin group	1.14 ± 0.14	1.12 ± 0.12	0.57 ± 0.20	0.50 ± 0.18	0.42 ± 0.20	0.50 ± 0.18	0.28 ± 0.18	0.50 ± 0.14

[a]$P < 0.05$ *vs* the leptin group.

Table 4 Histological scores of pulmonary injury mean ± SE

Group	Alveolar edema		Alveolar distention		PMNC infiltration		Alveolar wall thickening	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Sham group	0	0	0	0	0	0	0	0
AP group	2.28 ± 0.35[a]	1.62 ± 0.18[a]	1.42 ± 0.36	1.25 ± 0.16	2.71 ± 0.18[a]	2.21 ± 0.12[a]	0.85 ± 0.40	2.00 ± 0.18[a]
Leptin group	1.28 ± 0.18	0.37 ± 0.18	1.25 ± 0.16	0.62 ± 0.51	0.57 ± 0.29	0.75 ± 0.16	0.42 ± 0.29	0.87 ± 0.22

[a]$P < 0.05$ *vs* the leptin group.

pancreatic acinar and inflammatory cells stained strongly with CD40 (Figure 3C and D). However, there was no such staining in the pancreatic tissue of leptin treated (Figure 3E and F) and sham treated rats (Figure 3A and B). The leptin treated group showed a significant reduction in CD40 expression ($P < 0.001$, Table 5).

Effects of exogenous leptin on ALI associated with AP

Tissue MPO activities and NOx levels in lung tissues increased significantly in cerulein administered rats compared with rats that received saline, indicating that increased neutrophil infiltration and microvascular permeability were the result of pancreatitis ($P < 0.001$, Tables 1 and 2). Treatment of exogenous leptin significantly reduced lung MPO activities and NOx levels in cerulein injected animals ($P < 0.001$, Tables 1 and 2). Histopathologically, leptin treatment significantly decreased alveolar edema, PMNC infiltration and alveolar wall thickening in ALI associated with cerulein-induced AP (Figure 2). Exogenous leptin attenuated the lung injury in cerulein injected animals ($P < 0.001$, Table 4). There was more intense cytoplasmic staining with CD40 in fibroblasts in the alveolar wall and inflammatory cells in the AP group (Figures 4C and D) compared with the sham group ($P < 0.001$, Figure 4A and B). In rats treated with leptin, there was a significant decrease in the lung CD40 expression at 24h and 48h compared with the AP group ($P < 0.001$, Table 5, Figure 4E and F).

Effects of exogenous leptin on cytokines in AP

Serum cytokines and sICAM levels were markedly higher in both AP and leptin groups compared to the sham group ($P < 0.001$, Tables 1 and 2). Serum TNF-α, IL-1β and MIP-2 levels increased at 24 h after cerulein injection and tended to decrease at 48 h. sICAM levels remained high during the 48 h study period. Treatment with leptin decreased TNF-α, IL-1β, MIP-2, and sICAM levels in cerulein administered rats ($P < 0.001$, Tables 1 and 2). The

Table 5 Comparison of pancreatic and pulmonary CD40 staining levels in different study groups

Group	Pancreas		Lung	
	24 h	48h	24 h	48 h
Sham group	0	0	0	0
AP group	2.6 ± 0.8[a]	3.4 ± 0.4[a]	2.6 ± 0.8[a]	3.1 ± 0.6[a]
Leptin group	1.5 ± 0.6	2.1 ± 0.3	1.5 ± 0.6	1.9 ± 0.5

[a]$P < 0.05$ *vs* the leptin group.

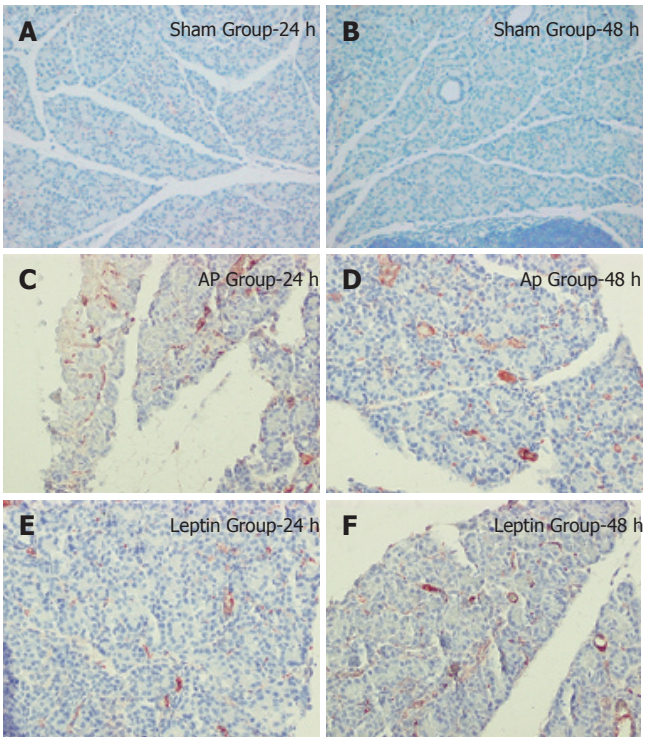

Figure 3 Immunohistochemical localization of CD40 expression in the pancreas in the sham group (**A**, **B**), AP group (**C**, **D**) and leptin group (**E**, **F**). (Divisions **A**, **C**, **E** on the figure represent pulmonary sections at 24 h and **B**, **D**, **F** represent pulmonary sections at 48 h).

decrease in cytokines and sICAM levels in the leptin group may contribute to the attenuation of lung injury associated with cerulean-induced AP.

DISCUSSION

To our knowledge, the present study is the first to demonstrate that exogenous leptin administration significantly reduces ALI in rats with cerulein induced AP. The exogenous leptin treatment reduced cerulein induced pancreatitis, and pancreatitis-associated lung injury, with reduction in the serum concentrations of TNF-α, IL-1β, MIP-2 and sICAM-1, decrease in the pancreatic and pulmonary neutrophil activation, and in MPO activity, and NOx, and CD40 levels. In addition, the exogenous leptin treatment significantly reduced mortality rates in rats with AP induced by supramaximal doses of cerulein.

Histological findings of ALI were more prominent after injection of supramaximal doses of cerulein compared to those seen in the sham group at 24 h and

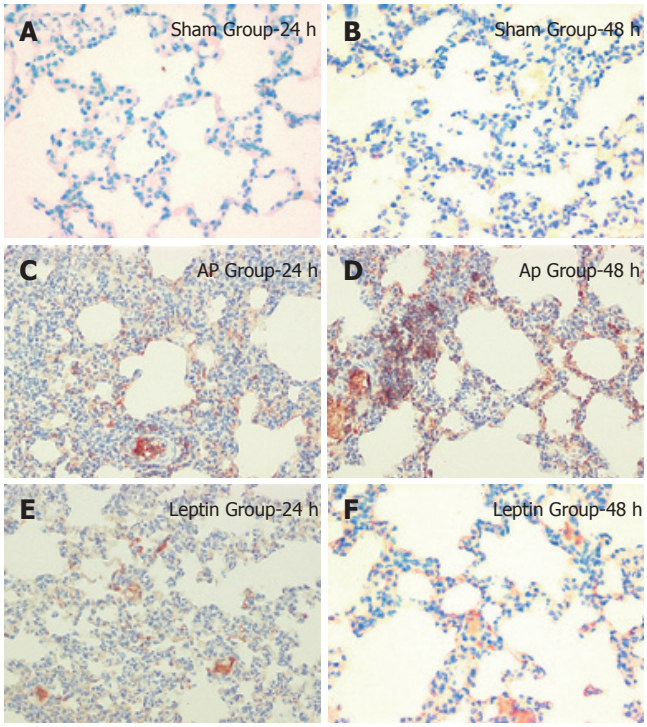

Figure 4 Immunohistochemical localization of CD40 expression in lung tissues. There was no staining in the sham group (**A**, **B**), whereas marked staining was observed in the AP group (**C**, **D**). CD40 staining was significantly reduced in leptin-treated rats (**E**, **F**). (Divisions **A**, **C**, **E** on the figure represent pulmonary sections at 24 h and **B**, **D**, **F** represent pulmonary sections at 48 h).

48 h; maximum improvements in histological features of ALI were observed at 48 h after leptin administration. During AP, lung injury was associated with accumulation of neutrophils in the interstitial and alveolar spaces, a common finding in both clinical and experimental studies[1,2,5,6]. It has been shown that reduction in circulating serum neutrophils, and strategies which interfere with neutrophil recruitment, such as ICAM-1 blocking antibodies are accompanied with decrease in neutrophil infiltration and in pancreatic and distant organ damage in AP[3,23]. In our study, induction of AP was associated with a significant increase in pancreatic and lung MPO activity, indicating that neutrophils were sequestered in both organs. Exogenous leptin treatment markedly decreased the MPO activity. Jaworek *et al*[15] have shown that exogenous leptin treatment reduced neutrophil infiltration. However, an exact explanation of the effects of leptin on neutrophil activation remains unknown. ICAM-1 and MIP-2 play an important role in the activation and the adhesion of neutrophils[23,24]. A better understanding of the effects of leptin on these factors may help in defining their effects on neutrophil activation.

ICAM-1 is an inducible protein expressed on the surface of endothelial cells. ICAM-1 plays an important role in neutrophil adhesion and neutrophil mediated lung injury during AP. Serum ICAM-1 concentrations peak at 24 h after cerulein administration[23]. In the present study, exogenous leptin significantly reduced serum ICAM-1 concentrations compared with the sham group. These findings suggest that neutrophil adhesion in the lung may be ameliorated by leptin administration.

During AP, MIP-2 is involved in neutrophil activation and sequestration in the pancreas and lungs[25]. MIP-2 is a potent rodent chemokine, homologous to GRO-β, which binds to the C-X-C chemokine receptor-2[26]. We found that cerulein induced AP was associated with a significant increase in serum MIP-2 concentrations, and that leptin treatment substantially decreased the MIP-2 concentration. The effect of leptin administration on serum MIP-2 concentration is not clearly established. To the best of our knowledge, this is the first study demonstrating the effect of leptin on serum MIP-2 concentration. A decrease in the concentration of MIP-2 may therefore play an important role in reducing neutrophil adhesion and sequestration. Moreover, Ob-R is present in the pancreas and lungs[12,13]. Therefore, leptin may also reduce pancreatic neutrophil activation by affecting its lung receptors. Several *in vivo* studies have shown that endogenous leptin increases neutrophil activation[27,28]. However, administration of a higher dose of exogenous leptin compared to the baseline circulating level may result in neutrophil inhibition.

Since leptin is a pleiotropic hormone, it is expected that its effects at supraphysiological dose may be similar to the effects in normal doses. In contrast, Konturek *et al*[16] and Warzecha *et al*[17] indicated that high doses of exogenous leptin had greater effect, and markedly attenuated pancreatic damage in both cerulein-induced and ischemia/reperfusion-induced pancreatitis models. We found similar effects with exogenous leptin in this study, where high doses of leptin exhibited the protective effects in AP and in AP associated lung injury. Nevertheless, these dichotomous effects arising from differing doses of leptin remain poorly understood and require further investigation.

TNF-α, and IL-1β are derived predominantly from activated macrophages and act via specific cell membrane-bound receptors in inflammatory situations such as AP and AP associated ALI. The severity of pancreatitis has been shown to correlate with TNF-α and IL-1β levels[2,5,7]. After the onset of pancreatitis, cytokine production from lung parenchyma increases significantly, and large quantities of chemokines, including TNF-α and IL-1β are released from macrophages via the p38 mitogen activated protein-kinase pathway[1,2,5]. Specific treatments that target the reduction of TNF-α and IL-1β levels reduce the severity of ALI in AP. Konturek *et al*[16] have shown that exogenous leptin treatment reduced plasma TNF-α level and pancreatic IL-4 expression in rats with AP. In another study, Warzecha *et al*[17] concluded that leptin treatment reduced plasma IL-1β levels. *Ob-R* is a member of the class I cytokine receptor superfamily. These receptors are membrane-spanning glycoproteins[13]. Leptin is recognized as a pro-inflammatory hormone, and it shares structural similarities to cytokines IL-6, IL-15 and granulocyte colony-stimulating factor[10]. However, our understanding of the role of leptin in inflammation is incomplete. Endogenous leptin protects against TNF-mediated toxicity. *Ob/ob* mice and *db/db* mice, as well as mice treated with leptin-receptor antagonist displayed increased sensitivity to the lethal effects of TNF. The addition of exogenous leptin protected against TNF-mediated toxicity in *ob/ob* mice, but did not increase the protective effect of endogenous leptin in wild-type mice[29]. Our study showed that AP was accompanied with increased plasma levels of TNF-α and IL-1β compared to the sham group, and exogenous leptin reduced these cytokine levels at 24 h and 48 h following cerulein injection. Overstimulation of *Ob-R* might also result in a decrease in cytokine levels. Taken together, exogenous leptin treatment appears to play an important role in the reduction of pancreatic damage during AP, through a reduction in the pro-inflammatory cytokines.

Leinder *et al*[7] have shown that the expression of iNOS correlated with apoptotic changes in the lung, suggesting that NO overproduction is an important factor in the development of a systemic inflammatory reaction in response to severe pancreatitis. In the present study, the NOx concentration in lung tissues was considerably higher in rats with cerulein-induced AP, compared with controls. However, a significant decrease in the NOx level was observed with leptin treatment. Leptin was able to reduce ALI by decreasing the NOx level, which resulted in the reduction of inflammation and neutrophil activation.

CD40, a protein member of the TNF receptor superfamily, is expressed on the membrane of a variety of cells, including B lymphocytes, monocytes, biliary and acinar cells[30,31]. CD40 binds to its ligand CD40L, a membrane glycoprotein, and mediates major immunoregulatory signals involved in auto-immune disease, inflammatory bowel disease and acute experimental pancreatitis[32]. Daoussis *et al*[32] have demonstrated that the CD40 ligation on antigen-presenting cells is associated with the following: (1) enhanced cell survival; (2) secretion of cytokines (TNF-α, IL-1β, IL-6, MIP); (3) enhanced monocyte activity, and (4) NO synthesis. In a recent study, Frossard *et al*[31] concluded that elevation of serum CD40 levels is a prognostic factor for AP. In our study, pancreatic and pulmonary CD40 staining level in the AP group was significantly higher than the sham group, and exogenous leptin treatment reduced both pancreatic and pulmonary CD40 staining. Exogenous leptin may contribute to the reduction in MPO activity by decreasing lung CD40 levels. However, whether lung CD40 levels are suppressed by leptin is unclear. Leptin directly reduces pancreatic CD40 levels and inhibits monocyte activation, which plays an important role in acute lung injury.

In conclusion, supramaximal doses of exogenous leptin treatment significantly abrogated ALI in cerulein-induced AP. Exogenous leptin markedly reduced both neutrophil activation and the levels of soluble pro-inflammatory factors such as cytokines, chemokines, NOx and CD40 that are known to be involved in the development of ALI. However, the pathophysiological role of exogenous leptin in AP and pancreatitis-associated lung injury requires further investigation.

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CLINICAL RESEARCH

Clinical significance of mucosal suppressors of cytokine signaling 3 expression in ulcerative colitis

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Abstract

AIM: To investigate the clinical significance of mucosal expression of suppressors of cytokine signaling 1 (SOCS1) and SOCS3 in human ulcerative colitis (UC).

METHODS: Biopsy specimens for histological analysis and mRNA detection were obtained endoscopically from the rectum of 62 patients with UC (36 men; age 13-76 years). The patients were classified endoscopically according to Matts' grade (grade 1 to 4). Expression of SOCS1 and SOCS3 mRNAs was quantified in samples by competitive reverse transcription-polymerase chain reaction (RT-PCR). GAPDH was used as an internal control for efficiency of RT-PCR and amount of RNA.

RESULTS: SOCS3 mRNA expression was significantly higher in inflamed mucosa of UC than in inactive mucosa. The level of expression was well correlated with the degree of both endoscopic and histologic inflammation. Interestingly, among the patients in remission, the group with relatively low expression of SOCS3 showed a higher rate of remission maintenance over a 12-mo period. In contrast, SOCS1 mRNA was expressed in both inflamed and non-inflamed colonic mucosa and was not correlated with the activity of colonic mucosa or prognosis.

CONCLUSION: These observations suggest that increased expression of mucosal SOCS3, but not of SOCS1, may play a critical role in the development of the colonic inflammation of UC.

Key words: Suppressors of cytokine signaling; Ulcerative colitis

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INTRODUCTION

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease characterized by a dysregulated mucosal immune response[1]. Many cytokines are involved in the immunopathogenesis of UC, but the importance of cytokine signaling in UC is not fully understood.

The suppressors of cytokine signaling (SOCS) are a family of proteins that regulates the strength and duration of the cytokine signaling cascade. There are eight members of the SOCS protein family: the cytokine-inducible SH2 domain-containing protein (CIS) and SOCS1 through SOCS7[2-7]. Accumulated evidence shows that SOCS proteins can potently block the Jak/STAT pathway in the pathogenesis of various inflammatory diseases[8]. The functions of SOCS1 and SOCS3 have been well documented in mouse colitis models. In a dextran sulfate-induced mouse model of colitis, SOCS3 expression was increased at day 5 and remained high for 2 wk[9]. Transgenic mice expressing a dominant-negative mutant of SOCS3 showed increased phosphorylation of STAT3 and suffered a more severe colitis than did wild-type control mice[9]. In a 2, 4, 6-Trinitrobenzene sulphonic acid-induced mouse model of colitis, SOCS1 expression was induced in intestinal mucosal lymphocytes, and SOCS1 transgenic mice developed colitis spontaneously with age[10]. It has been already reported that both SOCS1 and SOCS3 are expressed at high levels in the inflamed colonic mucosa of humans with UC[9,10], but functional significance of local SOCS expression remains unclear. The present study was undertaken to investigate the relation between levels of SOCS mRNAs and degree of inflammation in the colonic mucosa of UC patients.

MATERIALS AND METHODS

Subjects

Our subjects were 62 patients with UC who underwent colonoscopy. The age of the patients ranged from 13 to

Table 1 Description of the criteria of Matts for endoscopic and histologic findings

Endoscopic Matts' grades
Grade 1---normal
Grade 2---mild granularity of the mucosa, with mild contact bleeding
Grade 3---marked granularity and edema of the mucosa, contact bleeding, and spontaneous bleeding
Grade 4---severe ulceration of mucosa with hemorrhage
Histologic Matts' grades
Grade 1---normal appearance
Grade 2---some infiltration of the mucosa or lamina propria with either round cells or polymorphs
Grade 3---much cellular infiltration of the mucosa, lamina propria, and submucosa
Grade 4---presence of crypt abscesses, with much infiltration of all layers of the mucosa
Grade 5---ulceration, erosion, or necrosis of the mucosa, with cellular infiltration of some or all of its layers

Table 2 Characteristics of UC patients

Characteristics		Endoscopic Matts' grade			
		1	2	3	4
n	62	19	15	26	2
Sex (male/female)	36/26	11/8	10/5	13/13	2/0
Mean age (in years) (range)	38.6 (13-76)	36.2 (13-61)	39.1 (18-65)	40.1 (18-76)	37.0 (25-49)
Duration (in years) (range)	6.0 (0.1-29)	4.7 (0.2-10)	8.2 (0.1-20)	5.6 (0.1-29)	5.0 (1-9)
Type (total/left hemi/rectal)	42/10/10	14/1/4	10/4/1	17/4/5	1/1/0
Tx					
Steroid	24	5	7	10	2
Azathioprine	7	3	1	2	1
Leukocytapheresis	9	3	2	3	1

76 years (mean ± SD, 38.6 ± 13.3 years). Rectal lesions were classified macroscopically with endoscopic Matts' classification (Table 1)[11]. Patient characteristics are shown in Table 2.

After endoscopic observation by colonoscopy (450ZH; Fuji Photo Optical Co., Ltd, Saitama, Japan, and/or 240Q; Olympus Co., Ltd, Tokyo, Japan), biopsy specimens were obtained endoscopically from these patients for histologic analysis and reverse transcription-polymerase chain reaction (RT-PCR) assay. The biopsy specimens were fixed routinely in 10% buffered formalin, stained with hematoxylin and eosin, and diagnosed histologically according to histologic Matts' classification (Table 1)[11].

Oligonucleotides

For the amplification of SOCS1, a pair of PCR primers was synthesized. The sequences were 5'-CCTTCCCCTTCCAGATTTGA-3' for the 5' primer and 5'-TCCTGGCTCCAGATACAGTT-3' for the 3' primer. For the amplification of SOCS3, the sequences of the primers were 5'-TCACCCACAGCAAGTTTCCCGC-3' for the 5' primer and 5'-GTTGACGGTCTTCCGACAGAGATGC-3' for the 3' primer. For the amplification of GAPDH, the sequences were 5'-AACATCATCCCTGCCTCTAC-3' for the 5' primer and 5'-TGGCAGGTTTTTCTAGACGG-3' for the 3' primer.

Semi-quantitative RT-PCR

Total RNA from biopsy specimens was isolated with an Rneasy Mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA with random 9-mers in a 20-μL total reaction volume. Before cDNA symthesis, the RNA sample was treated with Rnase-Free Dnase (Qiagen) to eliminate possible false positives due to residual genomic DNA. PCR was performed in triplicate in 10 μL reactions. GAPDH was used as an internal control for efficiency of RT and amount of RNA. Amplification conditions consisted of denaturation for 4 min at 94℃, annealing for 1 min at 65℃, and a final extension for 7 min at 72℃. The samples were amplified for 35 cycles. PCR products were run on 2% agarose gels containing 1 × TAE. After electrophoresis, RT-PCR products of SOCS-1 (277 bp), SOCS-3 (590 bp), and GAPDH (148 bp) were analyzed and quantified with a UV Transilluminator (Toyobo, Tokyo, Japan) and NIH image software. Each sample was investigated in triplicate.

Statistical analysis

Data were analyzed with StatView software (Japanese version, Hulinks, Tokyo, Japan) on a Macintosh Computer (Apple Computer, Cupertino, CA). Groups were compared with Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. Time-to-relapse curves were derived with the Kaplan-Meier method, and statistical significance was determined with the log-rank test.

RESULTS

Expression of SOCS3 mRNA in the colonic mucosa in patients with UC

SOCS3 mRNA was detected in the colonic mucosa of patients with UC (Figure 1A), consistent with previous reports[12]. Upper level shows 4 cases of endoscopic Matts 1, and lower level shows 4 cases of Matts 3 and 4. Cases 1 to 4 with Matts 1 had low expression of SOCS3, and cases 5 to 8 with Matts 3 and 4 had high levels of SOCS3, as compared to expression of GAPDH. Quantitative analysis revealed that expression of SOCS3 mRNA was significantly higher in inflamed mucosa of UC than in inactive mucosa (Figure 2A). SOCS3 mRNA levels was also significantly correlated with histological Matts' grade (Figure 2B).

Expression of SOCS1 mRNA in colonic mucosa in patients with UC

SOCS1 mRNA was expressed in both inflamed and uninflamed UC mucosa (Figure 1B); however, the level did not differ significantly (Figure 3A). The SOCS1 mRNA expression was all equally expressed compared to GAPDH. Quantitative analysis showed that there was no correlation between SOCS1 mRNA expression in the colonic mucosa and histologic Matts' grade (Figure 3B).

Relation between SOCS1 and SOCS3 mRNA expression in patients with UC

Although SOCS1 and SOCS3 are regulated by many cytokines, the relation between SOCS1 and SOCS3 remains un-

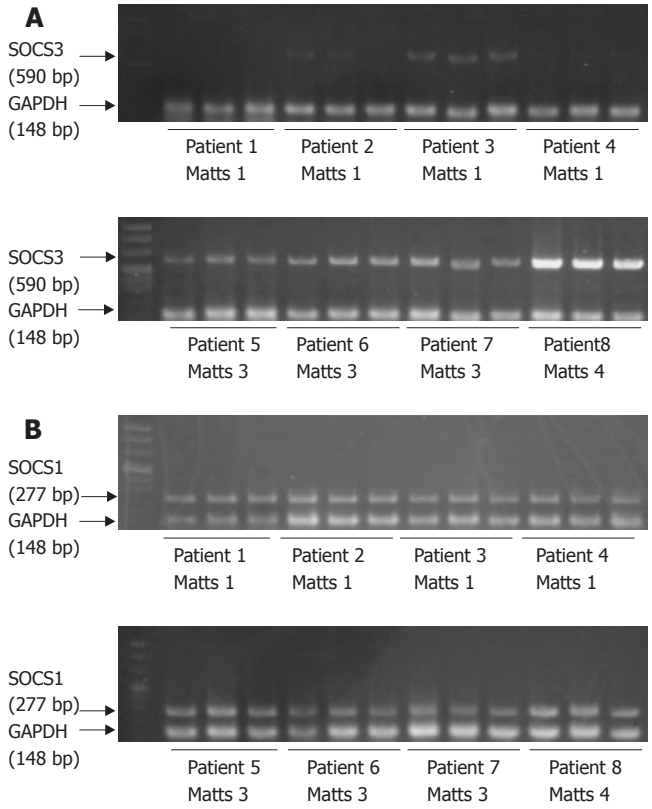

Figure 1 Expression of SOCS3 (**A**) and SOCS1 (**B**) mRNA in the colonic mucosa with UC. PCR was performed in triplicate for each sample. GAPDH was included as an internal control for efficiency of RT and amount of RNA. The colonic mucosae in cases 1 to 4 were shown endoscopically to be in remission and those in cases 5 to 8 were shown endoscopically to be active.

clear. Therefore, we evaluated the relation between expression of SOCS3 and SOCS1 mRNA and found that there was no correlation between SOCS1 and SOCS3 mRNA expression in each colonic biopsy specimen (Figure 4).

Relation between expression of SOCS mRNAs and steroid use

Because treatment with steroids is known to influence several immunological responses, we investigated the effect of steroid use on SOCS expression. There was a tendency for expression of both SOCS1 and SOCS3 to decrease in response to steroid treatment, but this difference was not statistically significant (Figure 5).

Relation between clinical activity of UC and the level of SOCS

We examined the relation between the clinical activity and expression of SOCS mRNAs. The clinical activity of UC was evaluated according to the clinical activity index (CAI), which is calculated as the sum of each parameter[13]. We found that SOCS3 mRNA expression correlated well with CAI, whereas there was no correlation between SOCS1 mRNA and CAI (Figure 6).

Expression of SOCS mRNAs in predicting relapse of UC

To determine if evaluation of SOCS expression is useful for prediction of prognosis for UC, we checked SOCS mRNA levels and CAI over a 12-mo period. Eighteen patients with UC in remission (CAI = 1 or 2) were analyzed

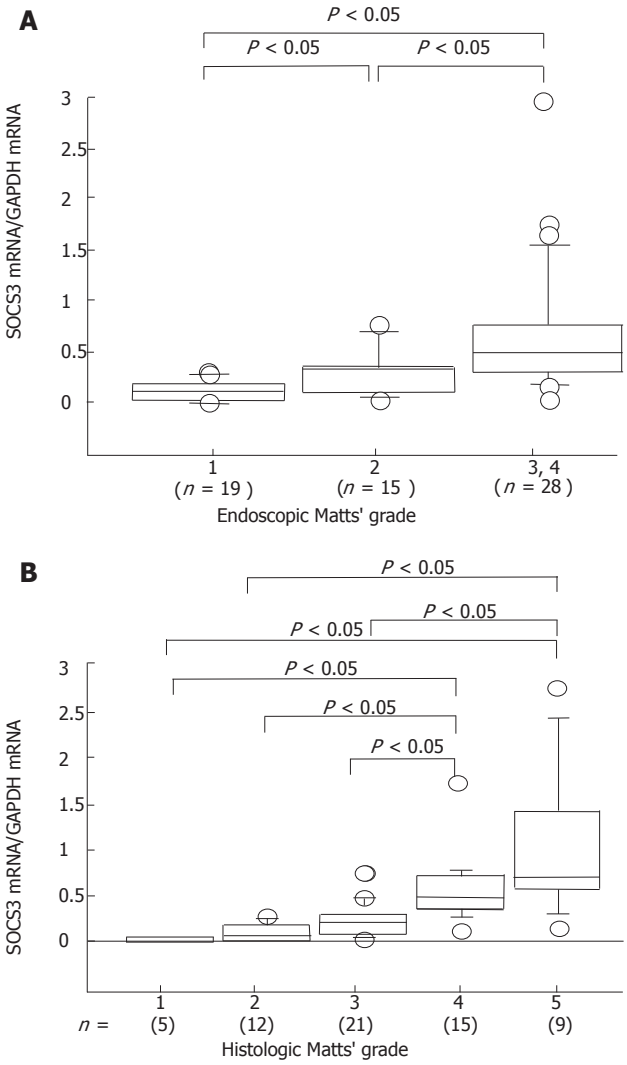

Figure 2 Relation between the degree of endoscopic (**A**) and histologic (**B**) inflammation and levels of SOCS3. The levels of SOCS3 mRNA were determined by quantitative RT-PCR. Box graphic top, bottom, and middle correspond to 75th, 25th, and 50th percentiles (median), respectively. Bar shows 5th and 95th percentiles. The mean + SD of endoscopic Matts' grade was 0.116 + 0.094 for Matts'1, 0.280 + 0.219 for Matts'2, and 0.673 + 0.599 for Matts'3 and 4. The mean + SD of histologic Matts' grade was 0.038 + 0.048 for Matts'1, 0.124 + 0.102 for Matts'2, 0.250 + 0.174 for Matts'3, 0.597 + 0.371 for Matts'4, and 1.039 + 0.821 for Matts'5. There was a significant correlation between SOCS3 expression in the colonic mucosa and Matts' grade.

for rectal SOCS expression and followed up. The patients were assessed when relapse occurred during the 12-mo period. A relapse was defined as CAI > 2. No change in treatment was made during the study. These patients were classified into two groups, low and high, according to SOCS expression levels. High SOCS3 was a SOCS3-to-GAPDH ratio of > 0.3, whereas low SOCS3 was a SOCS3-to-GAPDH ratio of ≤ 0.3. High SOCS1 was a SOCS1-to-GAPDH ratio of > 0.6, and low SOCS1 was a SOCS1-to-GAPDH ratio of ≤ 0.6. Patients who had low levels of SOCS3 (n = 14) remained in remission for the12-mo period, whereas patients with high levels of SOCS3 (n = 4) had low rate of maintaining an existing remission over the 12-mo period (Figure 7). There was no correlation between SOCS1 level and remission rate. No differences were found between the high and low SOCS3 groups in

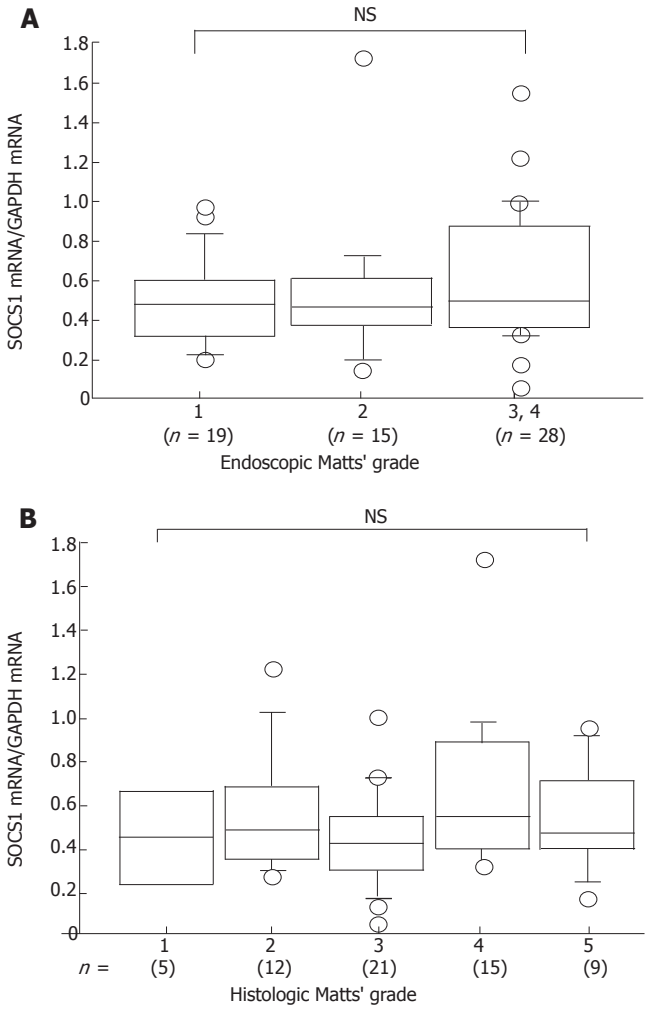

Figure 3 Relation between the degree of endoscopic (**A**) and histologic (**B**) inflammation and expression of SOCS1. Levels of SOCS1 mRNA were determined by quantitative RT-PCR. There was no correlation between the degree of inflammation and mucosal SOCS1 mRNA level.

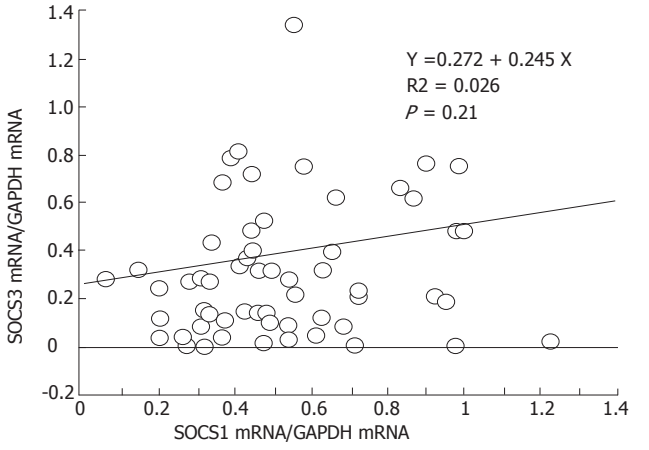

Figure 4 Relation between expression of SOCS1 and SOCS3 mRNAs. SOCS1 mRNA expression was not correlated with SOCS3 mRNA expression in colonic biopsy specimens.

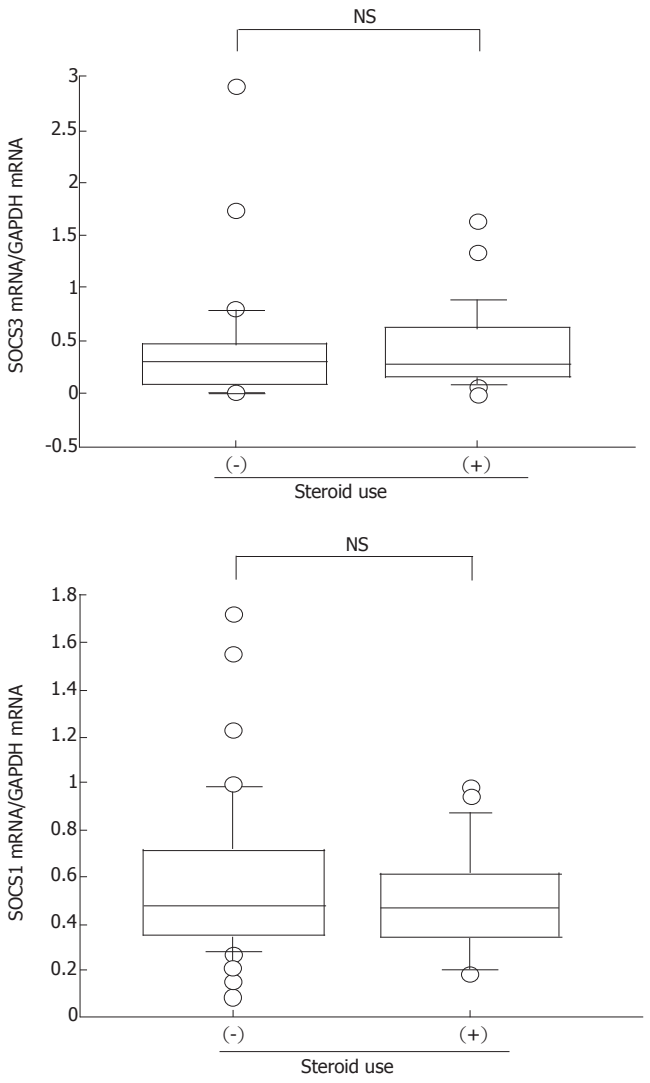

Figure 5 Effect of steroid treatment on expression of SOCS mRNAs. SOCS1 and SOCS3 levels tended to decrease in response to steroid treatment. NS: not significant.

histologic score, concomitant medications, length of remission prior to inclusion in study, or history of frequency of relapses for the individual patients. These data suggest that mucosal SOCS3 expression may be a useful prognostic marker for the maintenance of remission in UC.

DISCUSSION

In the present study, we found that expression of SOCS3 mRNA is increased according to the degree of mucosal inflammation in UC and that the level of SOCS3 may be a useful prognostic marker for patients with UC. These findings suggest that SOCS3 has a significant role in UC.

In this study, we found a close correlation between SOCS3 expression and the severity of both macroscopic and histologic inflammation of UC. In contrast, SOCS1 expression did not correlate with the severity of colonic inflammation. Expression of both SOCS1 and SOCS3 is induced by a wide variety of inflammatory and anti-inflammatory cytokines, including IL-6, IL-12, IFN-γ, and IL-10[8]. We also found that there was no correlation between the levels of SOCS1 and SOCS3. Thus, high expression of SOCS3 may not be a secondary effect of mucosal cytokine induction due to inflammatory responses.

The pathogenesis of UC is still unknown, but there

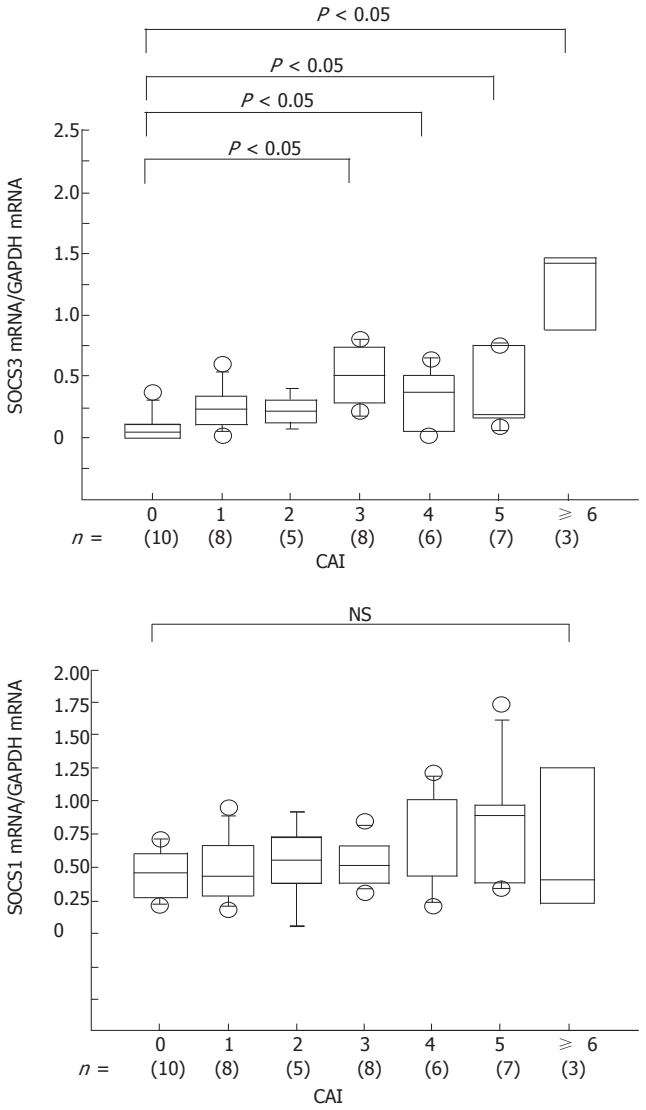

Figure 6 Relation between expression of SOCS mRNAs and CAI. SOCS3 expression is well correlated with CAI. There was no correlation between SOCS1 expression and CAI. NS: not significant.

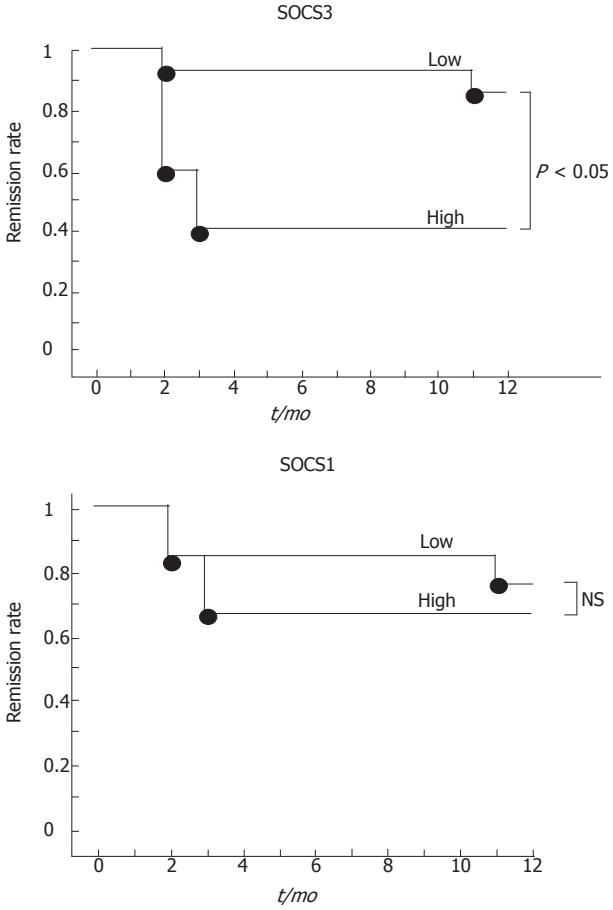

Figure 7 Kaplan-Meier time to relapse curves for patients with UC in relation to the expression of SOCS mRNAs. There is a significant difference ($P < 0.05$, log rank) in the proportion of patients who relapsed over a 12-mo period with respect to the levels of mucosal SOCS3 (> or < 0.3) at time of inclusion in the study. There was no correlation between SOCS1 and remission rate. NS: not significant.

is accumulating evidence that T-helper 2 (T_H2)-skewing immune dysregulation may be crucial in UC. Fuss *et al*[14] reported that lamina propria lymphocytes from UC secrete large amounts of IL-5 compared to those from healthy controls and Crohn's disease patients. Recently, it was reported that lamina propria mononuclear cells from patients with UC produce large amounts of IL-13, much more than those from control subjects or patients with Crohn's disease[15]. Moreover, it was also reported that IL-13 is the key effector T_H2 cytokine in UC that affects epithelial tight junctions, apoptosis, and cell restitution[16]. Taken together, these data all suggest that the T_H2-type immune response plays a crucial role in the pathogenesis of UC.

SOCS3 is expressed predominantly by T_H2 cells[17]. SOCS3 negatively regulates the IL-12 to STAT4 Th1 pathways and is possibly required for mediating T_H2 responses[18]. Interestingly, Seki *et al*[19] described a strong correlation between SOCS3 expression and the pathology of asthma and atopic dermatitis, well-known T_H2-type diseases, as well as serum IgE levels in allergic human patients. They also showed that SOCS3 transgenic mice have increased T_H2 responses in an airway hyperresponsibility model system[19]. Thus, SOCS3 has an important role in regulating the onset and maintenance of T_H2-mediated immune diseases. Recently, it was reported that overexpression of SOCS3 in lung through adenovirus SOCS3 gene transfer enhances IgG immune complex-induced lung injury[20]. SOCS3 expressed at high levels in inflamed mucosa may therefore have a pathologic role in UC.

We have not examined the cellular localization of mucosal SOCS3 expression. In mice, it has been confirmed that SOCS3 mRNA is expressed mainly in hyperplastic epithelial cells and lamina propria mononuclear cells in DSS-treated inflamed colon[9]. Han *et al*[21] confirmed that SOCS3 protein is increased and localized primarily in lamina propria lymphocytes with a lower level of expression in crypt epithelial cells in a mouse model of colitis. According to our data, expression of SOCS3 mRNA is well correlated with the degree of histologic inflammation. Therefore, accumulation of inflammatory lamina propria immune cells may be one of the main sources of increased SOCS3 mRNA. The correlation between SOCS3 expression and the severity of UC raises the possibility that high SOCS3 expression in patients may be attributable to the accumulation of T_H2 cells in the lamina propria, resulting in exacerbation of mucosal

inflammation. Further studies of the cellular localization of SOCS3 are needed.

To explore the impact of high expression of SOCS3, we examined the relation between the period of remission and SOCS3 expression. The rate of remission maintenance was significantly higher in the low SOCS3 group than in the high SOCS3 group for 1 year. This finding also suggests that mucosal SOCS3 expression is not merely the result of inflammatory change. SOCS3 may be involved in the progression of the inflammation in UC.

Treatment with steroids is known to affect cellular immune responses. We found that the patients treated with steroids showed lower levels of both SOCS1 and SOCS3 than did untreated patients. It has been reported that rat SOCS3 gene in hepatocytes is down-regulated by glucocorticoids[22]. In contrast, removal of adrenal steroids by adrenalectomy reduces SOCS3 mRNA and protein levels[23]. It has been reported that SOCS1 is involved in the response of leukemia cells to glucocorticoids[24]. These raise the possibility that steroids may directly regulate SOCS expression. Targeting SOCS3 may provide a novel strategy to UC.

In summary, our present observations suggest that mucosal SOCS3 expression may play a critical role in the development of colonic inflammation associated with UC. Monitoring of rectal SOCS3 expression may be a useful means to evaluate prognosis of patients with UC.

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CLINICAL RESEARCH

Impact of preoperative chemoradiotherapy on survival in patients with resectable pancreatic cancer

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Abstract

AIM: To explore whether preoperative chemoradiation therapy improves survival of patients with pancreatic cancer undergoing resectional surgery.

METHODS: Forty-seven patients with a malignant pancreatic tumor localized in the head or uncinate process of the pancreas underwent radical pancreaticoduodenectomy. Twenty-two received chemoradiation therapy (gemcitabine and radiation dose 50.4 Gy) before surgery (CRR) and 25 patients underwent surgery only (RO). The study was non-randomised. Patients were identified from a prospective database.

RESULTS: The median survival time was 30.2 mo in the CRR group and 35.9 mo in the RO group. No statistically significant differences were found in subclasses according to lymph node involvement, TNM stages, tumor size, or perineural invasion. The one, three and five year survival rates were 81%, 33% and 33%, respectively, in the CRR group and 72%, 47% and 23%, respectively, in the RO group. In ductal adenocarcinoma, the median survival time was 27 mo in the CRR group and 20 mo in the RO group. No statistically significant differences were found in the above subclasses. The one, three and five year survival rates were 79%, 21% and 21%, respectively, in the CRR group and 64%, 50% and 14%, respectively, in the RO group. The overall hospital mortality rate was 2%. The morbidity rate was 45% in the CRR group and 32% (NS) in the RO group.

CONCLUSION: Major multicenter randomized studies are needed to conclusively assess the impact of neoadjuvant treatment in the management of pancreatic cancer.

Key words: Pancreatic cancer; Neoadjuvant; Chemoradiation; Gemcitabine

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INTRODUCTION

Pancreatic carcinoma is the sixth most common cause of cancer death worldwide. In 2004, 878 new pancreatic cancers were diagnosed in Finland, and in 2003, 915 patients died of pancreatic cancer (www.cancerregistry.fi). The prognosis for patients with pancreatic carcinoma remains extremely poor, with only 0.4% of patients without surgical treatment surviving five years[1]. Between 1990 and 1996, the Finnish Cancer Registry recorded 4922 pancreatic cancer patients, 89 of whom survived for at least five years. Reviewing this series of patients revealed that 45 (49%) of the surviving patients did not have pancreatic ductal adenocarcinoma (PDAC) and, further, 18 patients had no histological verification of their disease. In 26 patients recorded as having histologically proven PDAC, re-evaluation of histological specimens confirmed PDAC in only 10 patients. Therefore, the five year survival rate for PDAC was thus far less than 1%[2].

Only 20% of patients with early stage pancreatic carcinoma are amenable to resection treatment. However, even then the risk for relapse is high, with only 0% to 24% of patients surviving for 5 years[3-5]. Yet, surgery is the only curative treatment for pancreatic cancer. In a recent study, adjuvant chemotherapy yielded survival benefit after surgery[6], whereas adjuvant chemoradiotherapy had an adverse effect on survival[7]. In another study, postoperative gemcitabine significantly delayed the development of recurrent disease after complete resection of pancreatic cancer compared with observation alone, but there was no difference in overall survival between the adjuvant chemotherapy with gemcitabine and the observation group[8].

Neoadjuvant (preoperative) chemoradiation offers several advantages as compared with adjuvant chemoradiation[9,10]: (1) radiotherapy is more effective with intact vascularization, (2) preoperative chemoradiotherapy may reduce cancer cell seeding during tumor manipulation, (3) the potential

retardation of postoperative recovery will not postpone neoadjuvant therapy, and (4) the effect of neoadjuvant therapy is identifiable in histopathological examination of the operative specimen.

In a randomized study, gemcitabine improved survival in inoperable pancreatic cancer in comparison with 5-fluorouracil (5-FU)[11]. Gemcitabine has also been shown to exert an effect in 5-FU-refractory pancreatic cancer[12].

We have already reported our first results on gemcitabine and concomitant irradiation in patients undergoing pancreaticoduodenectomy for locally advanced pancreatic cancer[13]. In this study, we compare the outcome of radically operated patients receiving neoadjuvant therapy (gemcitabine-radiotherapy) with that of patients surgically treated without neoadjuvant therapy during same time period.

MATERIALS AND METHODS

The data were collected from 47 consecutive patients who underwent pancreaticoduodenectomy for cure with extended lymphadenectomy for pancreatic carcinoma between January 1999 and January 2002 at the Helsinki University Central Hospital, Helsinki, Finland.

Among the 47 patients, 22 received chemoradiation therapy (CRT) before surgery and these patients formed the chemoradiaton and resection (CRR) group. During the same period 25 patients were surgically treated without neoadjuvant therapy forming the resection only (RO) group. The study was non-randomised. The preoperative chemoradiotherapy was offered to patients living in the Helsinki metropolitan area, but patients who lived in areas further away, where neoadjuvant chemoradiotherapy was not available were operated on directly without neoadjuant therapy. The independent, hospital Committee of Ethics approved the chemoradiation protocol. The patients received both oral and written information about the trial and patients signed informed consent forms before enrollment in the study. Patients were studied with whole body CT with 2 mm slices, with magnetic resonance imaging and with endoscopical ultrasound before the neoadjuvant therapy. Endoscopic retrograde cholangiopancreatography (ERCP) was performed for all patients in the CRR group and for 22/25 patients in the RO group. Percutaneous transhepatic cholangiography (PTC) was done once in both groups in combination with ERCP. Insertion of a biliary stent was done when indicated. A cytological specimen was also obtained using brush or needle aspiration.

CRR

The inclusion criteria for neoadjuvant therapy were: (1) adenocarcinoma located in the head or uncinate process of the pancreas or in the ampullary region, with a diameter of less than 5 cm and without evidence of local or metastatic spread of the disease, (2) WHO performance status less than 2, (3) good co-operation, and (4) no other malignancy or serious illness. The radiotherapy dose was constant whereas gemcitabine was given as a 30-min infusion twice weekly before irradiation at three dose levels, which were 20, 50 and 100 mg/m^2. The targeted irradiation volume included the tumor, possible surrounding oedema, and 1 cm margin. The tumor radiation dose in planning target volume was 50.4 Gy (ICRU). The therapy was given in 28 fractions of 1.8 Gy per day, five days per week. The median number of gemcitabine-cycles was 10 and everyone received the whole planned radiotherapy. During the study the maximal tolerated dose of gemcitabine was found to be 50 mg/m^2. Dose reduction of gemcitabine was needed in 65% of patients. Toxicity and tolerance of concomitant gemcitabine and radiotherapy treatment in these patients has been reported previously[12].

Following CRT, patients were given a 4-wk break for recovery of blood counts and nutrition, and new staging CT and MRI scans were performed.

The CT or MRI criteria for unresecability of tumor were metastatic disease or tumor encircling the superior mesenteric artery. Portal vein resection and reconstruction due to tumor invasion was performed when needed.

RO

Patients underwent pancreaticoduodenectomy without neoadjuvant therapy. Preoperative CT and MRI scans were performed, and a biliary stent was inserted if the patient was jaundiced.

Surgical treatment

In both groups, the operation was begun with a diagnostic laparoscopy to rule out metastatic or locally advanced disease. Radical pancreaticoduodenectomy was performed with extended lymphadenectomy and removal of retroperitoneal tissue. In the case of tumor infiltration into the portal vein, resection of the vein and reconstruction with autologous venal graft was performed. All operations were performed by one of two surgeons, however, the vast majority of the patients were operated on by surgeon T.K.

Pathological diagnosis

Frozen sections of the resection margins of the pancreas and common hepatic duct were collected during operations. All specimens were delivered as fresh, the resection margins were stained and the specimen was fixed in formalin for 1-2 d. The entire tumor area was sectioned in 2-3 mm slides. One macro slide was taken from the largest macroscopically identified tumor area. Lymph nodes and resection margins were also sectioned. All tissues were embedded in paraffin and stained according to the Herovici-van Gieson and Alcian blue-PAS methods. One pathologist (P.K.) examined all specimens. The tumor stage was defined according to the AJCC 2002[14].

Follow-up

After radical surgery, the patients were followed using a routine follow-up program. Liver tests and CA19-9 were measured. Radiological examinations were performed only if the patients developed symptoms, such as pain or ascites, or elevation of the CA19-9 value was observed. In the case of recurrence of the disease, an oncologist was consulted and the patient was offered chemotherapy.

Statistical analysis

Fishers exact test (*F*) was used to test differences between

Table 1 Patients characteristics. Data shown are number (%) or median (range)

Characteristic	*CRR*	*RO*	*P*
n	22	25	NS
Age	65 (49-83)	63 (43-76)	NS
Sex (male)	13 (59%)	12 (48%)	NS
Tumor site			NS
Head	20 (91%)	20 (80%)	
Body		3 (12%)	
Tail			
Diffuse	2 (9%)	2 (8%)	
Tumor size (mm)	24 (10-41)	29 (10-150)	NS
Tumor type			NS
Ampullary cancer	4 (18%)	4 (16%)	
Ductal adenocarcinoma	15 (68%)	14 (56%)	
Cystadenocarcinoma	2 (9%)	4 (16%)	
Invasive IPMT	1 (5%)	3 (12%)	
Tumor stage			NS
Ⅰ A	4 (18%)	0	
Ⅰ B	4 (18%)	5 (20%)	
Ⅱ A	7 (32%)	9 (36%)	
Ⅱ B	7 (32%)	11 (44%)	
Lymph node positive	7 (32%)	11 (44%)	NS

CRR: chemoradiation and resection; RO: resection only.

categorical variables and Student's *t*-test or the Wilcoxon-Mann-Whitney test (*W*) was used to assess differences between means of continuous variables. Normality of continuous variables was tested with the Kolmogorov-Smirnov test. Kaplan-Meier analyses and log rank tests were used to assess survival. This study was designed to detect 25 mo increase in median survival time, assuming 20 patients in each group, power of 0.80, significance level of 0.05 and median survival time of 15 mo in the surgically treated only group. Accrual time was 48 mo and follow-up time after the end of the recruitment was 48 mo. $P < 0.05$ was considered statistically significant (NS: not significant).

RESULTS

Clinical data

Of the 47 patients enrolled in the study, 22 (9 females 13 males) belonged to the CRR group. Their mean age was 65 (49-83) years. 25 patients (13 females and 12 males) belonged to the RO group. Their mean age was 63 (43-76) years. There were no differences between the groups regarding age and sex, status of lymph nodes, tumor stage or tumor size. After chemoradiotherapy, 8 patients were judged as inoperable for laparotomy: 4 patients had metastases to the liver, 2 patients had a poor general condition and 1 patient had a fractured femur and died from a pulmonary embolism before the operation. In addition, one patient refused the operation. All these patients were excluded from the series.

The CRR group included 15 ductal adenocarcinomas, 4 ampullary adenocarcinomas, 2 cystadenocarcinomas and one invasive malignant intraductal papillary mucinous tumor (IPMT) of the pancreas. Thirteen patients with ductal adenocarcinoma were treated by pancreticoduodenectomy. For two patients, total pancreatectomy was performed due to positive resection margins after partial resection. One portal resection was performed due to tumor invasion to the portal vein. The two patients with cystadenocarcinoma were also treated with pancreaticoduodenectomy. In the patient with IPMT, total pancreaticoduodenectomy was performed. Ampullary tumors were treated with pancreaticoduodenectomy.

The RO group included 14 patients with ductal adenocarcinomas, 4 with ampullary adenocarcinomas, 4 with cystadenocarcinomas and 3 with invasive malignant IPMTs. All patients with ductal adenocarcinomas and ampullary tumors were treated with pancreaticoduodenectomy. One portal resection was performed due to tumor invasion to the portal vein. One patient with cystadenocarcinoma was treated with pancreaticoduodenectomy, and three patients with total pancreatectomy. One patient with IPMT underwent pancreaticoduodenectomy. However, after investigation of the paraffin specimens, an intraluminal noninvasive component was found close to the resection border and the operation was completed with total pancreatectomy six months later. The two remaining patients with IPMT were treated with pancreaticoduodenectomy. Table 1 gives the distribution of the stages of tumors for both groups.

Mortality and morbidity

One patient in the CRR group died of multiorgan failure on the 43rd postoperative day (overall hospital mortality rate 2%). In this patient, duodenal perforation with abscess formation due to a biliary stent was found in the primary operation. The patient was reoperated on the same day because of bleeding, and again on the 10th postoperative day because of leakage of the gastrojejunal anastomosis and peritonitis.

The overall morbidity rate was 38%. In the CRR group, ten patients (45%) had complications. There were five cases of intra-abdominal infections, which were treated conservatively, and one case of pneumonia. Intra-abdominal infection was recorded if the CRP had increased and the broad spectrum antibiotic had been changed after the 5th postoperative day and pneumonia was excluded. Two patients suffered from ileus. The central venous catheter of one patient was accidentally inserted into the pleural space. In the RO group, eight patients (32%) had complications. Two patients were reoperated because of bleeding with gastrointestinal anastomosis. Three patients had minor wound infections and one had pneumonia. In one patient, rupture of the intima of the common hepatic artery was suspected due to lack of pulse and immediate reconstruction with venous graft was performed. One patient suffered a stroke during the postoperative convalescence period. Information on complications is presented in Table 2.

The length of hospital stay was 15 ± 1.6 d (mean +SE) in the CRR group and 11.8 ± 0.9 d (NS) in the RO group. Blood loss during operation was 2200 ± 300 mL (range 700-4300 mL) in the CRR group and 2400 ± 400 mL (range 300-8500 mL; NS) in the RO group.

Survival

Survival rates were calculated for all types of pancreatic cancers and for the subgroups of ductal adenocarcinoma.

Table 2 Complications

	CRR	RO
Infection		
Wound infection/prolonged antibiotic therapy	[1,2]5	[1]3
Pneumonia	1	1
Ileus/delayed gastric emptying	2	0
Postoperative bleeding and reoperation	[2]1	2
Postoperative intestinal haemorraghia: no reoperation	1	1
Central venous catheter in pleural place	1	0
Stroke during the postoperative time	0	[1]1
Vascular injury: occlusion of hepatic artery: immediate reconstruction	0	1
Reoperation: broken drain	[1]1	0
Leakage of gastroenteral anastomosis	[2]1	0
Death	[2]1	0
Complicated patients rate	10/22	8/25

1 or 2 same patient; CRR = chemoradiation and resection; RO = resection only.

The median survival time was 30.2 mo (95% CI 25.46-34.94) in the CRR group (n = 22), and 35.9 mo (95% CI 10.51-61.29) in the RO group (n = 25). No statistical differences were found by log Rank analyses. 1-y survival was 81% ± 8.6 (SE) in the CRR group and 72% ± 9.0 in the RO group, 3-year survival rates were 33 ± 10 and 47% ± 10 and 5 year survival rates were 33% ± 10.3 and 23% ± 9.0, respectively (Figure 1A).

The median survival time of LN negative patients in the CRR group (n = 15) was 30.2 mo (95% CI 17.98-42.42), and in the RO group (n = 14) 37.4 mo (95% CI 7.79-67). The respective values of LN positive patients in the CRR group (n = 7) were 27.0 mo (95% CI 0.0-55.2), and in LN positive patients in the RO group (n = 11) 20.7 mo (95% CI 6.75-34.65). The 1-year survival rate for LN negative patients was 79% ± 11 in the CRR group and 79% ± 11.0 in the RO group, 3-year survival rates were 43% ± 13.2 and 57% ± 13.2, and 5-year survival rates were 43% ± 13.2 and 41% ± 13.6, respectively. In LN positive patients, the 1-year survival rate was 86% ± 13.2 in the CRR group and 64% ± 14.5 in the RO group, 3-year survival rates were 14% ± 13.2 and 33% ± 15 and 5-year survival rates were and 14% ± 13.2 and 0%, respectively. There were no significant differences between the groups (Figure 2). Median survival times for different cancers are given in Table 3.

When the CRR and RO groups were divided according T classes (T1, T2, and T3), no statistical differences were revealed between the groups (data not shown).

The survival calculations were also made according to tumor size, tumor stage and perineural invasion, but no statistically significant differences between the CRR and RO groups were found in these subgroups.

Ductal adenocarcinoma

The median survival time was 27.00 mo (95% CI 5.55-48.45) in the CRR group (n = 15) and 20.20 (95% CI 0-52) in the RO group (n = 14, Table 3). The 1-year survival rate was 78.6% ± 11.0 in the CCR group and 64.3 % ± 12.8 in the RO group. The respective 3-year survival rates were 21.4% ± 11.0 and 50.0% ± 13.4 and

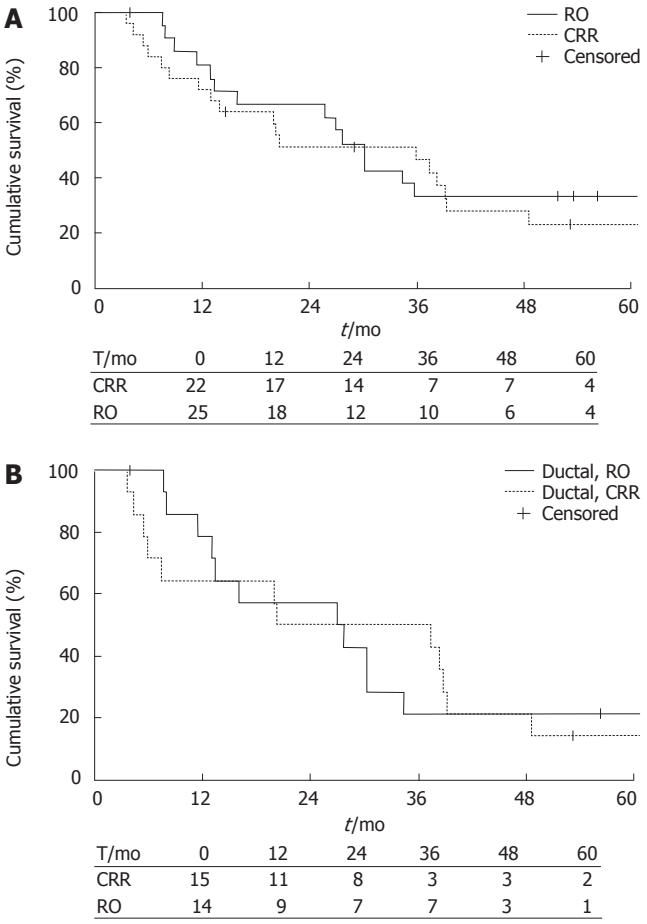

Figure 1 **A**: Survival rates of all pancreatic cancers; **B**: Survival rates of ductal adenocarcinomas. CRR: chemoradiation and resection; RO: resection only.

5-year survival rates were 21.4% ± 11 and 14.3 % ± 9.4. No statistical differences were found between the groups. (Figure 1B).

No difference was found between the CRR and RO groups regarding lymph node status. The median survival time of LN negative patients in the CRR group (n = 8) was 30.2 mo (95% CI 9.94-50.46) and in the RO group (n = 9) 20.2 mo (95% CI 19.62-20.78) (Figure 2B). The median survival time of LN positive patients in the CRR group (n = 7) was 27.0 mo (95% CI 0.00-55.23) and in the RO group (n = 5) the median survival time was 38.8 mo (95% CI 0.00-110.51). The 1-year survival rates in LN negative patients were 71% in the CRR group and 67% in the RO group, 3-year survival rates were 29% and 44% and 5-year survival rates were 29% and 22%, respectively. In LN positive patients, the rates were 86% *vs* 60%, 14% *vs* 60% and 14% *vs* 0%, respectively. There were no statistical differences between the groups (Figure 3).

Survival calculations were also made according to tumor size, perineural invasion and tumor stage, but no statistical differences were found between the CRR and RO groups for any subgroup.

At the end of the follow up, two patients with ductal adenocarcinoma in the CRR group were disease free. Their follow up times were 71 and 57 mo and tumor stages were T1N0M0 and T3N0M0, respectively. One patient (T3N1M0) was followed 71 mo and recurrence of disease

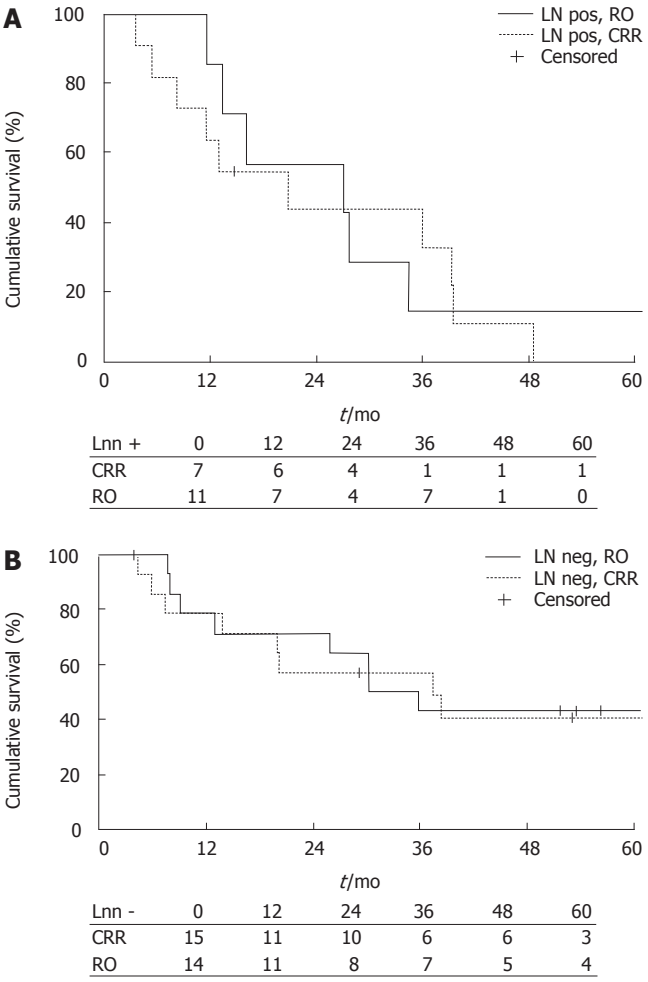

Figure 2 Survival rates of pancreatic cancers. **A**: with nodal involvement; **B**: without nodal involvement. CRR: chemoradiation and resection; RO: resection only; LN: lymph node.

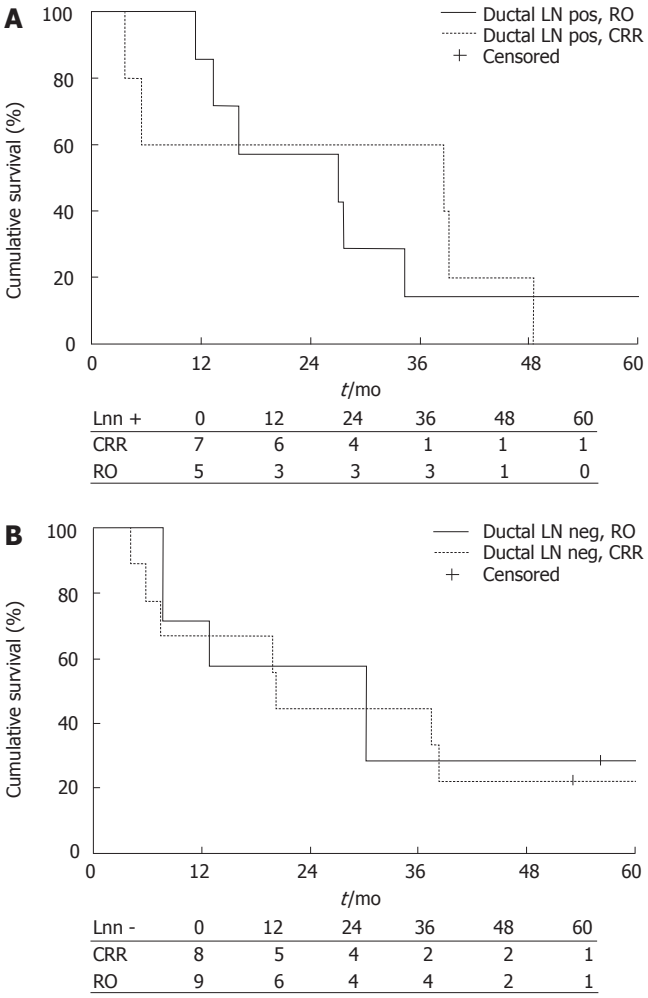

Figure 3 Survival rates of ductal adenocarcinomas. **A**: with nodal involvement; **B**: without nodal involvement. CRR: chemoradiation and resection; RO: resection only; LN: lymph node.

Table 3 Median survival time in different cancers

	CRR **median months (95% CI)**	***RO*** **median months (95% CI)**
All cancers	30.2 (25.5, 34.9)	35.9 (10.5, 61.3)
LNN negative	30.2 (17.9, 42.4)	37.4 (7.8, 6.7)
LNN positive	27.0 (0, 55.2)	20.7 (16.7, 34.6)
Ductal adenocancers	27.0 (5.5, 48.4)	20.2 (0, 52.0)
LNN negative	30.0 (9.9, 50.5)	20.2 (19.6, 20.3)
LNN positive	27.0 (0, 55.2)	39.0 (0, 110.5)
Ampullar cancers	39.4 (5.9, 72.8)	
Cystadenocancers	20.7 (00, 48.2)	

CRR: chemoradiation and resection, RO: resection only; LN: lymph node.

was detected two months before the end of follow-up. Two of the 14 patients with ductal adenocarcinoma in the RO group were alive, but only one was disease free (follow up 53 mo, T2N0M0). The other (T3N0M0) had local recurrence at 46 mo with the total follow up time being 61 mo.

Patients with adenocarcinoma originating from the ampullary region had a median survival time of 39.4 mo (95% CI 5.95-72.85). In this group, the one year survival rate was 88%, the three year survival rate was 60% and the five year survival rate was 45%. In patients with cystadenocarcinoma (n = 6), the median survival time was 20.70 (95% CI 0.0-48.2), and the one year survival rate was 83%, the three year survival rate was 33%, and the five year survival rate was 33%.

DISCUSSION

In the present study, the survival time for pancreatic cancer treated by surgery only is comparable to earlier reports[15-18]. We did not show, however, any statistical survival benefit with preoperative chemoradiation therapy. Although chemoradiation is associated with improved overall survival in locally advanced disease, it rarely leads to surgical "downstaging" with consequent potential curative pancreatic resection[18]. In both groups, more than 70% (73% in the CRR group and 71% in the RO group) of the tumors were T3 tumors, i.e. locally advanced tumors, which extend beyond the pancreatic capsule, but do not involve the celiac axis or superior mesenteric artery. In patients with ductal adenocarcinoma, the median survival time in the CRR group (27 mo) was clearly longer than that in the RO group (20 mo), however again, the difference was not statistically significant, perhaps because

of the small number of patients. On the other hand, when all cancers were included, neoadjuvant chemoradiation seemed to shorten, albeit not significantly, the survival time (30 *vs* 36 mo).

We reported earlier acceptable toxicity for preoperative twice-weekly gemcitabine and concomitant irradiation therapy[13]. Others have reported late toxicity in some patients receiving a neoadjuvant gemcitabine-radiotherapy[20,21] regimen, and the finding that gemcitabine increases radiosensitivity of both normal and malignant tissue, including pancreatic cancer, has also been reported[22-24].

Preoperative chemoradiation therapy seemed to lengthen hospital stay after the operation, but the difference was not statistically significant. The rate of complications due to infection was similar, but the infections in the RO group were only minor wound infections, whereas in the CRR group there were five cases of abdominal sepsis and one case of pneumonia, which were treated by antibiotics. This suggests that preoperative chemoradiation therapy may enhance susceptibility to infection. The length of time to carry endobiliary stents was significantly longer in the CRR group than in the RO group, which might predispose to infection, although any major disadvantages of biliary drainage have not been reported[25-28]. Only one patient died of complications after pancreaticoduodenectomy. The trigger for this complication was doubtlessly perforation of the duodenal wall by the biliary stent into the retroperitoneal space with resultant abscess formation. Although the neoadjuvant therapy was generally well-tolerated[13], the patients might have been more fragile and susceptible to complications, with compromised postoperative capacity to recovery.

After operation, no adjuvant therapy was given, but if a recurrence was found, the possibility of chemotherapy or systemic oncological treatment was offered and the patients also consented. The possible effect of the oncological therapy after relapse was not documented.

White *et al*[29] reported a median survival time of 23 mo after neoadjuvant (5-fluorouracil and radiation) therapy and a 3-year survival rate of 37% (n = 70) and an estimated 5-year survival rate of 23%. These figures are rather similar to our results of 27 mo and 20%. Breslin *et al*[30] reported a 21 mo survival time (5-fluorouracil, paclitaxel, or gemcitabine and radiation therapy; n = 132), but the median follow up time was only 14 mo. We followed our patients from 48 to 72 mo.

To our knowledge, the present study is the only one in which chemoradiaton neoadjuvant therapy is compared to operation only without neoadjuvant therapy, performed by the same two surgeons during the same period of time. No benefit of neoadjuvat therapy was observed in this study.

The behavior and prognosis of pancreatic malignancies varies and it is most important to know the histological diagnosis of tumors when the results are reported. Therefore, in our study, a reliable survival time could only be reported for ductal adenocarcinomas. The other groups (ampullary carcinomas, cystadenocarcinomas and IMPT) were too small for accurate and reliable calculations.

The weakness of this study was its unrandomized nature. However, the groups were analyzed and patients with ductal adenocarcinoma did not differ regarding tumor size, age, lymph node involvement, perineural invasion, or sex. The surgical team was the same and the patients were operated on during the same period of time. Also, the number of patients was too small for definitive conclusions. Consequently, major multicenter randomized studies are needed to assess conclusively the impact of neoadjuvant treatment in the management of pancreatic cancer.

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RAPID COMMUNICATION

Efficacy of transcatheter embolization/chemoembolization (TAE/TACE) for the treatment of single hepatocellular carcinoma

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Abstract

AIM: To investigate the efficacy of transcatheter embolization/chemoembolization (TAE/TACE) in cirrhotic patients with single hepatocellular carcinoma (HCC) not suitable for surgical resection and percutaneous ablation therapy.

METHODS: A cohort of 176 consecutive cirrhotic patients with single HCC undergoing TAE/TACE was reviewed; 162 patients had at least one image examination (helical CT scan or triphasic contrast-enhanced MRI) after treatment and were included into the study. TAE was performed with Lipiodol followed by Gelfoam embolization; TACE was performed with Farmorubicin prepared in sterile drip at a dose of 50 mg/m^2, infused over 30 min using a peristaltic pump, and followed by Lipiodol and Gelfoam embolization.

RESULTS: Patients characteristics were: mean age, 62 years; male/female 117/45; Child-Pugh score 6.2 ± 1.1; MELD 8.7 ± 2.3; mean HCC size, 3.6 (range 1.0-12.0) cm. HCC size class was ⩽ 2.0 cm, *n* = 51; 2.1-3.0 cm, *n* = 35; 3.1-4.0 cm, *n* = 29; 4.1-5.0 cm, *n* = 22; 5.1-6.0 cm, *n* = 11; and > 6.0 cm, *n* = 14. Patients received a total of 368 TAE/TACE (mean 2.4 ± 1.7). Complete tumor necrosis was obtained in 94 patients (58%), massive (90%-99%) necrosis in 16 patients (10%), partial (50%-89%) necrosis in 18 patients (11%) and poor (< 50%) necrosis in the remaining 34 patients (21%). The rate of complete necrosis according to the HCC size class was: 69%, 69%, 52%, 68%, 50% and, 13% for lesions of ⩽ 2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5.1-6.0, and > 6.0 cm, respectively. Kaplan-Mayer survival at 24-mo was 88%, 68%, 59%, 59%, 45%, and 53% for lesions of ⩽ 2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5.1-6.0, and > 6.0 cm, respectively.

CONCLUSION: Our study showed that in cirrhotic patients with single HCC smaller than 6.0 cm, TAE/TACE produces complete local control of tumor in a significant proportion of patients. TAE/TACE is an effective therapeutic option in patients with single HCC not suitable for surgical resection or percutaneous ablation therapies. Further studies should investigate if the new available embolization agents or drug eluting beads may improve the effect on tumor necrosis.

Key words: Transcatheter embolization/chemoembolization; Hepatocellular carcinoma

Miraglia R, Pietrosi G, Maruzzelli L, Petridis I, Caruso S, Marrone G, Mamone G, Vizzini G, Luca A, Gridelli B. Efficacy of transcatheter embolization/chemoembolization (TAE/TACE) for the treatment of single hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(21): 2952-2955

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INTRODUCTION

The intra-arterial treatment using transarterial chemoembolization (TACE), transarterial oily chemoembolization (TOCE) and transarterial embolization (TAE) is considered a palliative therapy for multifocal HCC not suitable for surgical resection or percutaneous ablation therapies[1-3]. Recent meta-analysis showed an improvement of overall survival in patients with well preserved liver function treated with intra-arterial treatment[4,5]. The efficacy of intra-arterial treatment in patients with single HCC is not well defined. The aim of this study was to evaluate the efficacy of intra-arterial therapy in a large series of cirrhotic patients with single HCC.

MATERIALS AND METHODS

This study is a retrospective cohort study based on the analysis of 176 consecutive cirrhotic patients with single HCC, treated with intra-arterial therapy and evaluated with

follow-up imaging at a single transplant centre. One hundred and sixty-two patients had at least one image examination (helical CT scan or triphasic contrast-enhanced MRI) after treatment and were included into the study. Diagnosis of HCC was based on radiological findings, alfa-fetoprotein level and biopsy according to the Barcelona criteria[6]. The mean age (range) of patients were 62 (35-80) years, male to female ratio was 117/45, the Child-Pugg score (mean ± SD) was 6.1 ± 1.1, MELD score (mean ± SD) was 8.7 ± 2.3, Mean diameter of HCC was 3.6 (1.0-12) cm, number of HCC in the right lobe/left lobe was 133/29.

Informed consent was not specifically required for the study, although written informed consent was obtained for every diagnostic and interventional radiology procedure.

Intra-arterial treatment was performed in patients with single non-resectable HCC and contraindications to radiofrequency thermal ablation (RFA). In our centre, contraindications to RFA are the: (1) size of the lesion that is greater than 4 cm, (2) lesion near to vital organs as gallbladder, stomach, and colon, (3) lesion adjacent to a big portal or hepatic vein branches at risk of bleeding, (4) subphrenic lesion not easily accessible for RFA and (5) lesion in subcapsular position at high risk of tumor seeding[7].

Inclusion criteria for intra-arterial treatments of HCC are as follows: Absence of extrahepatic tumor; HCC < 50% of hepatic volume; absence of complete thrombosis of main portal vein; transaminases < 300 U/L, serum bilirubin < 0.3 mg/L; serum creatinine < 0.18 mg/L; white blood cell > $2.5 \times 10^3/\mu L$; platelets > $35 \times 10^3/\mu L$; no refractory ascites; performance status < 3.

Exclusion criteria for intra-arterial treatments of HCC are as follows: extrahepatic tumor spread; ascites not controlled by diuretics; encephalopathy; active or recent (4 wk) gastrointestinal bleeding; biliary obstruction; severe debilitation; active infection or sepsis; pregnancy; failure (ejection fraction less than 50%); severe pulmonary dysfunction; serum creatinine > 0.2 mg/L; serum bilirubin > 0.3 mg/L; Hb level < 8 g/dL; White blood cells < $2.5 \times 10^3/\mu L$; platelets < $35 \times 10^5/\mu L$.

The intra-arterial treatments were performed by two radiologists with 15 and 5 years of experience in interventional radiology, respectively. TACE was performed using Epirubicine at a dose of 50 mg/m^2 of body surface; the dose was reduced to 50% if serum bilirubin level was > 0.12 mg/L and < 0.2 mg/L and/or white blood cell count (WBC) was 3-4 × $10^3/\mu L$; a dose reduction to 25% was performed if bilirubin was > 0.2 mg/L and/or WBC 2.5-3 × $10^3/\mu L$. Epirubicine was prepared in sterile drip and infused over 30 min using a peristaltic pump. Afterwards, the embolization was performed using Gelfoam (Pfitzer, Belgium) till a stagnation flow was visualized at the fluoroscopy. In patients with good liver function and superselective catherization of the hepatic artery, 2-10 mL of Lipiodol (Lipiodol Ultrafluid, Guebert, Italy) were infused, by hand, before the Gelfoam embolization (TOCE). No chemotherapeutic agent (TAE) was used in presence of WBC less than 2.5 × $10^3/\mu L$, previous episodes of neutropenia (< 500/mL), positive HBsAg and HBV DNA[8] and ejection fraction ⩽ 45%. In these patients the treatment was performed using Lipiodol and/or Gelfoam. Superselective catheterization of the artery supplying the lesion was performed whenever possible. In the other cases the treatment was performed in the branch of the right hepatic artery or in the branch of the left hepatic artery supplying the lesion. Discharge from the hospital was the day after the procedure. The intra-arterial treatment was repeated every 6-12 wk according to the tumor response based on the CT follow-up imaging and on clinical assessment.

All CT scan studies were performed with a 16-slice multidetector CT (Light speed, General Electric Medical Systems, USA) 4-6 wk after the intra-arterial treatment. Images of the liver were acquired in cranium-caudal direction, during a single breath-hold acquisition, with slice thickness 1.25 mm, collimation 2.5 mm and table speed 7.5 mm per gantry rotation. Quadruple-phases protocol was used (unenhanced phase, arterial phase, portal venous phase and late phase). Iopromide (Ultravist 370 mg I/L, Schering, Germany) contrast was injected, using a power injector (Stellant 2, Medrad) with a dose of 1.8 mL/kg of body weight at a rate of 5 mL/s. If the serum creatinine was > 0.15 mg/L, Iodixanolo (Visipaque 320 mg I/L, Amersham Healt, Italy) was used as contrast medium. Before the study, patients received 500 mL of water as oral contrast agent. The test bolus technique (10 mL of contrast material at 5 mL/s) was used to calculate the correct time of the arterial phase. Arterial scanning was performed with a delay of 8 s from the peak time of the aortic enhancement obtained at the celiac axis level. The portal venous phase andlate phase acquisitions were performed after 60 s and 180 s from the beginning of contrast injection, respectively. Imaging analysis was performed by three radiologists experienced in liver imaging. The presence of arterial enhancement at the CT imaging was considered as viable tumor. In patients that underwent TOCE, the complete necrosis was considered only if the lesion had homogeneous Lipiodol uptake without contrast enhancement in arterial phase. In case of an unclear result, MRI studies with gadobenate dimeglumine (Gd-BOPTA), using a 1.5T MR Scan (General Electric Medical Systems, USA) was performed. The efficacy of intra-arterial treatment was defined according to the amount of tumor necrosis valuable on CT follow-up imaging and following the WHO recommendations[9]: complete response was defined the absence of any contrast enhancement in arterial phase, massive response as a necrosis involving 90%-99% of the lesion, partial response as a necrosis involving 50%-89% of the lesion, and poor response as a necrosis involving less than 50% of the lesion.

Patients were followed up monthly in outpatient clinics. We considered a complication due to the treatment if it occurred within 6 wk from TAE/TACE.

Statistical analysis

All analyses were performed with SPSS program. The results are expressed as mean ± SD. Survival curves were modeled using the Kaplan-Meier method.

Table 1 Rate of complete tumor necrosis according to the size of HCC

Size of HCC (cm)	*n*	Complete necrosis (% of patients)	Number of treatments (mean ± SD)
≤ 2.0	51	69	2.0 ± 1.5
2.1-3.0	35	69	2.2 ± 1.7
3.1-4.0	29	52	2.7 ± 2.0
4.1-5.0	22	68	2.8 ± 1.6
5.1-6.0	11	50	2.0 ± 1.3
> 6.0	14	13	3.3 ± 2.1

Table 2 Kaplan-Meier survival analysis according to the size of HCC

Size of HCC (cm)	12 mo survival (%)	24 mo survival (%)	36 mo survival (%)
≤ 2.0	87	87	79
2.1-3.0	85	67	54
3.1-4.0	72	58	50
4.1-5.0	88	59	49
5.1-6.0	78	45	30
> 6.0	77	53	26

RESULTS

Patients received a total of 368 sessions of TAE/TACE. The mean number of treatment sessions was 2.4 ± 1.7 per patient, range 1-9. In 69 (43%) of 162 patients a single procedure was sufficient to induce a complete tumor necrosis. Technical success was achieved in all the TAE/TACE performed. No major, life-threatening complications occurred without any perioperative mortality. As minor, reversible, complications 3 (1.8%) of 162 patients had transitory neutropenia ($< 0.5 \times 10^3/\mu L$), 2 (1.2%) of 162 patients had partial dissection of intrahepatic artery and 4 (2.4%) of 162 patients had a transitory liver failure (defined as occurrence of encephalopathy, ascites, increased bilirubin level and/or coagulopaty). The results of treatments on HCC necrosis are summarized in Table 1. On imaging analysis overall complete necrosis was obtained in 94 lesions (58%), massive necrosis was obtained in 16 lesions (10%), partial necrosis was obtained in 18 lesions (11%), and poor necrosis was obtained in the remaining 34 lesions (21%). According to the size of HCC complete necrosis was obtained in 69% of lesions ≤ 2.0 cm, 69% of lesions between 2.1-3.0 cm, 52% of lesions between 3.1-4.0 cm, 68% of lesions between 4.1-5.0 cm, 50% in lesions between 5.1-6.0 cm and 13% in lesions > 6 cm. The cumulative survival rates were 81%, 61% and 48% at 12, 24 and 36 mo respectively. Table 2 shows Kaplan-Meier survival analysis according to the size of HCC. The survival rates at 12, 24 and 36 mo were respectively: 87%, 87% and 79% for lesions ≤ 2.0 cm, 85%, 67% and 54% for lesions between 2.1-3.0 cm, 72%, 58% and 50% for lesions between 3.1-4.0 cm, 88%, 59% and 49% for lesions between 4.1-5.0 cm, 78%, 45% and 30% for lesions between 5.1-6.0 cm, 77%, 53% and 26% for lesions > 6 cm.

DISCUSSION

HCC is the fifth most common cancer worldwide. Surgical therapy, as a curative option, is indicated only in patients with single HCC without portal hypertension and preserved liver function[1]. Hepatic resection of HCC in patients with cirrhosis is associated with significant perioperative mortality and morbidity[10,11]. Cirrhotic patients with non-resectable HCC have a poor prognosis influenced by hepatic reserve function and tumor staging. TAE/TACE are the most used treatment for HCC, which are non-resectable or that can not be treated with percutaneous interventions, with proven improvement on survival in selected patient with well preserved liver function[4,5] Progression of HCC is related to neoangiogenic activity. Rationale for intra-arterial treatments for HCC is the almost complete arterial blood supply of the tumor (90%-100%) compared to normal liver parenchyma where the arterial flow is only 25% and the portal flow is responsible of the 75% of the inflow[12]. The goal of TAE/TACE is to deliver a high dose of chemotherapeutic drug and/or embolizing agent in the HCC, causing tumor necrosis and tumor control, preserving as much normal liver parenchyma. TAE/TACE is considered a palliative therapy for multifocal HCC but the efficacy in patients with single HCC is not well defined. It has been reported by Liem *et al*[13] that morbidity, mortality and short-term and intermediate-term survival data after TACE for HCC eligible for radiofrequency ablation are comparable to those reported after radiofrequency ablation in literature. In our series of patients, in 94 patients (58%) a complete necrosis was achieved. Interestingly, According to the lesion size, complete necrosis was achieved in 68% of patients with lesion between 4.1-5.0 cm and in 50% of patients with lesion between 5.1-6.0 cm, a very poor rate of complete response, 13%, was obtained in lesions > 6 cm. Livraghi *et al*[14] report a complete necrosis after radiofrequency ablation, detected by computed tomography follow-up, in 71% of cases with non-infiltrating HCC between 3.1-5.0 cm in diameter and in 25% of lesions > 5 cm. Cabassa *et al*[15] report a complete necrosis after radiofrequency ablation, detected by computed tomography follow-up, in 53% of lesions between 3.1-5.0 cm and in 20% of lesions > 5 cm. Our results after TAE/TACE in medium size, single, HCC in causing tumor necrosis are comparable to data reported after radiofrequency ablation, in same size lesions, by Livraghi and Cabassa.

Our data on survival are similar to data reported by Takayasu *et al*[16] in a recent large, prospective, cohort study of transarterial chemoembolization for non-resectable HCC, the cumulative survival rate of 3648 patients with single HCC was 87% at one year and 57% at 3 years; according to tumor size the survival reported was 83% at one year and 43% at 3 years for lesions between 3.0-5.0 cm and 63% at one year and 30% at 3 years for lesions > 5 cm. Our data on overall survival rate are also comparable with data reported by Teh *et al*[10] who reports an overall survival rate of patients with cirrhosis undergoing hepatic resection of 50% at 3 years but with a reported perioperative mortality of 16%. In a recent paper of

Benzoni *et al*[11], 7% of perioperative mortality and 47% of postoperative morbidity (including the rising of ascites, hepatic insufficiency, biliary fistulas, hepatic abscess, hemoperitoneum and pleural effusion) is reported after hepatic resection for HCC in cirrhotic patients.

The absence of major complication and the low rate of minor complication of our study could be explained by an accurate clinical patients' selection. In fact no patient with Child Pugg class C was treated. The use of segmental or subsegmental treatments is in our opinion mandatory, limiting the injury to the surrounding non-tumoral parenchyma and reducing the adverse effects of single or repeated intra-arterial treatments on liver function as reported in previous studies[17,18]. At the same time the systematic use of micro-catheters to selectively catheterize the feeding artery of the lesion increases the amount of chemotherapeutic drug and/or of embolizing agents in the target HCC increasing the amount of necrosis. Super selective treatment should be recommended especially in patients with Child Pugg class B or MELD score $\geqslant 9$.

The use and dosage of Epirubicine not with a fixed dose but according to patients' body surface, severity of liver disease and white blood cells count could have an important rule in the low incidence of complications reported. All Patients received antibiotic prophylaxis before TAE/TACE and no cases of sepsis or hepatic abscesses happened in the follow up. Patients were well hydrated before and after procedure to avoid renal injuries. If creatinine was > 0.15 mg/L, a less nephrotoxic contrast dye was used for intra-arterial treatments and for CT scan and no cases of renal impairment were recorded.

The use of more efficient chemotherapeutic drug and embolizing agents, associated with systematic selective catheterization of the feeding artery with micro-catheter, could further improve our results. As limitation of our study we did not analyze the relationship between the dosage of chemotherapeutic drug used and the dose of the Lipiodol used with the tumoral necrosis and the survival rate obtained because in our protocol the dosages of Epirubicine and Lipiodol used were not fixed, but dependent on clinical characteristic of the patient the same day of the procedure, so in the same patient it was possible to have different dosages of chemotherapeutic drug and Lipiodol in repeated treatments.

In conclusion our study showed that in cirrhotic patients with single HCC $\leqslant$ 6 cm, TAE/TACE can be considerate a safe and effective therapeutic option producing complete local control of tumor in a significant proportion of patients. In single HCC between 3 and 5 cm the effect on necrosis induced by intra-arterial treatments is not different from the data reported using RFA. Accurate patients' selection and factors procedure-related (use of micro-catheters, selective treatments, sterile drip for cytotoxic drug and antibiotic prophylaxis) reduce the possible complications increasing tumor necrosis. At our opinion an important aspect is the needs to standardize the dose of cytotoxic drug on the basis of the clinical characteristics of the patients to avoid liver failure.

Further studies should investigate if the new available embolization agents or drug eluting beads may improve the effect on tumor necrosis.

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RAPID COMMUNICATION

Frequency of primary iron overload and HFE gene mutations (C282Y, H63D and S65C) in chronic liver disease patients in north India

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Abstract

AIM: To identify the frequency of iron overload and study the three mutations in the HFE gene (C282Y, H63D, and S65C) in patients with chronic liver disorders (CLD) and controls.

METHODS: To identify patients with iron overload (transferrin saturation > 45% in females and > 50% in males and serum ferritin > 1000 ng/mL) we evaluated 236 patients with CLD, including 59 with non-alcoholic steatohepatitis (NASH), 22 with alcoholic liver disease (ALD), 19 of cirrhosis due to viruses (HBV, HCV), and 136 with cryptogenic cirrhosis. Mutations of the HFE gene were analyzed by PCR-RE. hundred controls were screened for iron status and the mutations.

RESULTS: Seventeen patients with CLD showed evidence of iron overload. Fifteen cases of iron overload had cryptogenic cirrhosis and two had ALD. None of the controls showed iron overload. We did not find any individual with 282Y or 65C either in the cases or in the controls. The prevalence of H63D heterozygosity was 12% in normal individuals, 14.8% in 236 patients (16.9% in NASH, 13.6% in ALD, 26.3% in viral and 12.5% in cryptogenic cirrhosis) and the overall prevalence was 13.98%. Only two of the 17 patients with primary iron overload were heterozygous for H63D. One patient with NASH and one normal individual who were homozygous for H63D showed no iron overload.

CONCLUSION: Primary iron overload in Indians is non-HFE type, which is different from that in Europeans and further molecular studies are required to determine the defect in various iron regulatory genes.

Key words: HFE gene mutations; C282Y; H63D; S65C; Population genetics

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INTRODUCTION

Hereditary hemochromatosis (HH) is one of the most common genetic disorders encountered in the Northern European population. Its inheritance is autosomal recessive and results in disordered iron metabolism leading to enhanced iron absorption and progressive iron deposition in parenchymal organs, most notably in liver[1]. Excess iron may cause damage to parenchymal organs, with an increased risk of developing diabetes mellitus, arthropathy, liver cirrhosis and ultimately hepatocellular carcinoma[2,3]. The HFE gene encodes a protein, which is highly similar to HLA class 1 molecules. Two missense mutations (C282Y, H63D) have been described on the HFE gene in patients suffering from HH on the basis of phenotypic data. The predominant mutation in the Caucasians, C282Y, is a G-A transition at nucleotide 845 of the open reading frame that changes the amino acid cysteine to tyrosine[4].

H63D is a C-G transition at nucleotide 187 of the HFE gene which results in a histidine to aspartic acid substitution. It has been found to be present with a frequency of 3.3%-15.2% in the general population across the world[5-7]. A third mutation in the HFE gene, S65C, has been found in eight French HH patients[7]. However, its clinical importance remains controversial as the S65C variant is associated with increased percent transferrin saturation in healthy Canadian blood donors.

The allelic frequency of S65C is 0.6%-1.95% in Caucasian population[8].

There are only a few studies from India on the frequency of the known HFE gene mutations[9-12]. We attempted to study the prevalence of these mutations in patients with various liver disorders and healthy controls. We also determined the frequency of primary iron over load in patients with various liver disorders.

MATERIALS AND METHODS

A prospective study was undertaken in the Department of Haematology of the Postgraduate Institute Medical Education & Research, Chandigarh, a referral hospital in North India by studying cases of chronic liver disease (CLD) from the Hepatology Department, for identifying subjects with primary iron overload based on iron studies and liver biopsy wherever possible.

hundred controls were screened for iron status and the three known HFE gene mutations, namely C282Y, H63D and S65C. The controls were unrelated individuals from the indigenous population of north India without any disease or biochemical abnormality and willing to enter the study. Two hundred and thirty-six patients with various types of liver disorders were screened for iron status, including 59 cases of non-alcoholic steatoheptitis (NASH), 22 cases of alcoholic liver disease (ALD), 19 cases of cirrhosis due to viral etiology (HBV, HCV), and 136 cases of cryptogenic cirrhosis.

Collection of samples

A complete history was taken with special emphasis on the duration of illness, disease activity, and complications such as loss of blood. The patients were excluded if they had a history of multiple blood transfusions and hemoglobinopathy or if they were on iron supplements. Overnight fasting blood samples were collected for iron study, 6-8 mL blood sample was taken in iron free tube and 5 mL was taken in liquid EDTA for DNA extraction. Institutional ethical clearance and informed consent from all the patients and controls were obtained for the study.

Iron studies

Serum iron and total iron binding capacity (TIBC) were measured by the colorimetric method with ferrozine chromogen as described by Dacie *et al*[13] and percentage transferrin saturation was calculated. Serum ferritin was measured using an enzyme immunoassay kit (Orgentec diagnotika GmbH, Germany). Percentage transferrin saturation (%TS) of > 55% in males and postmenopausal females and > 45% in premenopausal females, serum ferritin > 1000 μg/mL were taken as biochemical criteria for the diagnosis of primary iron overload. Liver biopsy was performed for confirmation of parenchymal iron overload by haematoxylin and eosin stain and Perls Prussian blue stain.

DNA analysis

Genomic DNA was extracted from the peripheral blood leucocytes by standard phenol chloroform method. HFE gene mutations (C282Y, H63D and S65C) were determined by specific polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described previously[4,7]. The PCR products were digested with restriction enzymes *Rsa*- I , *Bcl*- I and *Hinf*- I to identify the C282Y, H63D and S65C variants respectively[4,7].

Table 1 Prevalence and allelic frequencies of the H63D mutation in controls and chronic liver disorder patients

Group	Prevalence				Allele frequency
	-/-	-/+	+/+	%	
Controls (*n* = 100)	88	11	1	12	6.5
Cryptogenic (*n* = 136)	119	17	0	12.5	6.2
NASH (n =59)	49	9	1	16.9	9.3
Viral (*n* = 19)	14	5	0	26.3	13.1
ALD (*n* = 22)	19	3	0	13.6	6.8
Total (*n* = 336)	289	45	2	13.98	7.29

RESULTS

No case of iron overload was encountered in the 100 controls (58 males and 42 females). Iron deficiency was found in 26 individuals, of them six were males (10.3%) and 20 (47.6%) were females. Two hundred and thirty-six patients with liver disorder were analyzed for iron parameters, namely percentage transferrin saturation (%TS) and serum ferritin as well as the HFE genotypes, namely, C282Y, H63D and S65C. Out of the 236 chronic liver disease patients, 17 showed biochemical iron overload. The clinical features of the 17 cases were consistent with a diagnosis of primary iron overload. Fifteen out of the seventeen patients had cryptogenic cirrhosis and two had alcoholic cirrhosis. The overall percentage of iron overload in cryptogenic cirrhosis was 11% (15/136). Four patients presented with associated diabetes mellitus. A positive family history with more than one family member affected was found in only one patient. Liver biopsy could be performed in 12 cases and could not be carried out in 5 cases because of their low platelet counts. All the biopsies showed 3+ to 4+ parenchymal deposition of iron on Perls' staining. There were 14 males and 3 females (M:F = 4.6:1) and the mean age of the males was 48 years and that of the 3 females was 68, 54 and 49 years, respectively.

All the controls and patient group were detected to have the wild type of C282Y and S65C. Hundred normal controls showed the prevalence of H63D (11 individuals were heterozygous and one individual was homozygous). Out of the 236 patients with liver disorders, 34 were heterozygous and one was homozygous for the H63D mutation. The prevalence of this mutation in the patients with liver disorders was 14.8% (normals = 12%, NASH = 16.9% viral = 26.3%, ALD = 13.6%, cryptogenic = 12.5%) and the overall frequency was 13.98%. The Odd' s ratio was found to be 1.27 (95% confidence interval was 0.63-2.57). The prevalence and allelic frequencies of the H63D in the normals and chronic liver disorder patients are summarized in Table 1. Only two individuals, one in the control and one with NASH were found to be homozygous for H63D. However, these two individuals

did not show iron overload. Overall, in the 17 patients with primary iron overload only two showed heterozygosity for H63D.

DISCUSSION

Primary iron overload is uncommonly encountered in Indians and happens to be common in the Caucasians of North Europe. In the west, the C282Y mutation of the HFE gene is associated with HH in majority of cases. Variations in prevalence of the HFE gene mutations (C282Y and H63D) have been established in many European populations and descent (United States, Canada, Australia, South Africa). Few studies are available from India on the prevalence of these mutations in the general population[9-12].

We attempted to study the prevalence of iron overload in different types of liver disorders and the frequency of the three known point mutations of the HFE gene. Our study shows that of all the groups of chronic liver disorder, cryptogenic cirrhosis showed the highest frequency (15/136, 11%) of iron overload. Two patients with alcoholic cirrhosis showed iron overload. The other groups of NASH and viral cirrhosis did not reveal iron overload. We have shown that iron does not have any etiological role in the development of NASH[14]. The lower frequency of primary iron overload in our population may be due to a high frequency of iron deficiency anemia encountered in Indians.

On screening for the HFE gene mutations (C282Y, H63D and S65C) in 672 alleles of our population, no case of C282Y and S65C was identified. No C282Y mutation has been identified in Asian people including Hong Kong Chinese, Taiwanese aboriginals and Indonesians[15]. In addition, a study involving 252 Japanese subjects confirmed complete absence of the C282Y[16]. The prevalence of HH seems to be low in people of Asian origin. Few HH patients of Japanese origin, a small series of Chinese patients[17] and single Chinese women with marked iron overload were negative for the HFE C282Y mutation[18]. Therefore, absence of the Hfe C282Y mutation supports the hypothetical existence of non-Caucasian haemochromatosis, which seems to be non-HLA linked. However, it remains to be defined at the genetic level.

In the present study, the overall prevalence of H63D was 13.98% (12% in normal and 14.8% in patients with chronic liver disease). Thirty-four of the 236 patients with liver disease and 11 normal subjects were H63D heterozygous, and one in each group was homozygous. Neither the patients with NASH nor the normal individuals who were homozygous for H63D showed iron overload. A pilot study was previously conducted by us in 58 normal subjects, 154 subjects with beta thalassemia trait (BTT) and 9 with HH for the HFE mutations (C282Y and H63D). No individual was found to be positive for the C282Y and the prevalence of H63D mutation in the 212 subjects (normal and BTT) was 16.5%[9]. Since primary iron overload exists in our population and there is paucity of molecular information in these patients, more studies to delineate this defect are warranted in our population. These frequencies are similar to those found for this genotype in liver disorder subjects and controls[19].

In conclusion, primary iron overload is uncommonly encountered in our population. Of the known HFE gene polymorphisms, both C282Y and S65C are absent in our population. H63D is present in a frequency of 13.9% in our population but is not associated with iron overload even in the homozygous state. Our study reiterates the fact that primary iron overload in Indians is the non-HFE type and further molecular studies are required to determine the exact defect in various iron regulatory genes, like the transferrin receptor 2, hepcidin, ferroportin, ferritin and hemojuvelin.

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RAPID COMMUNICATION

Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-α single nucleotide gene polymorphisms in inflammatory bowel disease

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Abstract

AIM: To study the (functional) relevance of single nucleotide polymorphisms (SNPs) in genes encoding matrix metalloproteinases (MMP)-1, -2, -3, -9, tissue inhibitors of metalloproteinases (TIMP)-1, -2 and tumor necrosis factor (TNF)-α in the etiopathogenesis of inflammatory bowel diseases (IBD), that may enhance susceptibility and/or disease severity.

METHODS: Genomic DNA from 134 Crohn's disease (CD), 111 ulcerative colitis (UC) patients and 248 control subjects was isolated from resected intestinal tissue or blood. Allelic composition at SNP loci was determined by PCR-RFLP or tetra primer ARMS PCR.

RESULTS: The TIMP-1 genotype TT in women and T in men at SNP +372 T/C was found to increase CD susceptibility (39% *vs* 23.8%, $P = 0.018$ and 67.9% *vs* 51.6%, $P = 0.055$, respectively), while women with this genotype were less prone to development of fistulae during follow-up (41.4% *vs* 68.3%, $P = 0.025$). Male IBD or CD patients carrying the TIMP-1 +372 T-allele expressed lower levels of TIMP-1 in surgically resected macroscopically inflamed tissue ($0.065 < P < 0.01$). The 5T5T genotype at MMP-3 SNP -1613 5T/6T increased the chance of stenotic complications in CD during follow-up (91.2% *vs* 71.8%, $P = 0.022$) but seemed to protect against colonic involvement of this disease at first endoscopic/radiologic examination (35.3% *vs* 59.5%, $P = 0.017$).

CONCLUSION: Allelic composition at the examined SNPs in genes coding for TIMP-1 and MMP-3 affect CD susceptibility and/or phenotype, i.e., fistulizing disease, stricture pathogenesis and first disease localisation. These findings reinforce the important role of these proteins in IBD.

Key words: Crohn's disease; Ulcerative colitis; Matrix metalloproteinases; Inhibitors of matrix metalloproteinases; Single nucleotide gene polymorphisms

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INTRODUCTION

Crohn's disease (CD) is characterised by chronic, patchy, transmural inflammation of the gastrointestinal tract, predominantly in the ileocecal area, while ulcerative colitis (UC) is manifested by chronic, continuous, rather superficial inflammation of the mucosal layers of the colon[1,2]. The incidence and prevalence of both CD and UC have increased in the Western population since the second World War[3,4], and lately also increased in developing industrialising countries. Although there has been much controversy regarding etiology and pathogenesis of both forms of inflammatory bowel disease (IBD), recent evidence points to an exaggerated immune response to enteric bacterial flora in genetically susceptible individuals. Based on a higher disease concordance in monozygotic *vs* dizygotic twins[5], a higher frequency of IBD in certain families and ethnic groups[6,7], the association of IBD with genetic disorders like Turner's and Hermansky-Pudlak syndrome[8,9], the presence of a genetic component in IBD is evident. Indeed, large-scale genome-wide linkage studies have mapped several regions of the human genome to IBD, i.e., 16q12 (IBD1),

12q13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 19p13 (IBD5), 5q31-q33 (IBD6) and Xq21.3[10-15] and subsequent research has identified several CD predisposing mutations in the IBD1 gene encoding NOD2[16]. However, the different chromosomal locations found to be associated with IBD in these studies suggest disease heterogeneity: different sets of disease predisposing mutations may lead to a similar clinical outcome. This is corroborated by evidence obtained from animal models, where distinct genetic manipulations, for instance deletion of the DNA encoding TCRα, IL-10 or TNF-α 3'UTR AU repeat motifs, all lead to ileitis and/or colitis[17-19]. Therefore, genes on other loci, not identified in the studies mentioned above, may also contribute to IBD susceptibility and worthy considering in this respect are the matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The MMPs constitute a group of neutral, Ca- and Zn-activated endoproteinases and are involved in physiological matrix turnover during embryogenesis, angiogenesis, *etc*[20]. Production is tightly regulated at the transcriptional and post-transcriptional levels, and excessive MMP-mediated tissue destruction is prevented by strictly regulated activation mechanisms of the latent pro-enzyme and inhibition of the active enzyme in a 1:1 stoichiometry by TIMPs. Recently, several functional single nucleotide polymorphisms (SNPs) in the genes encoding MMPs and TIMPs have been described. The insertion of an additional guanosine residue at -1607 in the promoter of MMP-1 creates a PEA3 consensus sequence next to an AP-1 binding site up-regulating promoter activity, while the insertion of an additional thymidine at -1613 of the MMP-3 promoter results in decreased mRNA transcription[21,22]. The -1306 C/T transition in the promoter of MMP-2 results in decreased binding affinity for stimulating protein Sp1, leading to decreased mRNA transcription[23]. In contrast, the -1562 C/T transition in the promoter of MMP-9 results in the removal of a binding site for an unknown repressor protein, thus elevating transcription[24]. In TIMP-1 and -2 SNPs have been found in the exon part of the genes (+372 T/C and +303 G/A, respectively). Although no effect on transcriptional activity/mRNA stability was observed, these SNPs might serve as markers in association studies[25]. Both MMP and TIMP expression are affected by TNF-α and this pro-inflammatory cytokine is known to play a pivotal role in IBD, particularly CD but also UC, as demonstrated by impressive clinical improvement following anti-TNF-α antibody infliximab administration[26,27]. The G/A transition at -308 in the TNF-α promoter might result in increased levels of circulating TNF-α protein[28], thus inducing extra MMPs and/or TIMPs. Of note, the gene encoding TNF-α is mapped to the 6p21 IBD3 region, while the MMP and TIMP genes have not been mapped to any known IBD region (MMP-1, -3: 11q22-q23; MMP-2: 16q13; MMP-9: 20q11.2-q13.1; TIMP-1: Xp11.3-p11.23 and TIMP-2: 17q25). Conceivably, direct and indirect SNP-linked overproduction of MMPs and/or down-regulation of TIMPs, would result in net destruction of tissue, impairment of intestinal barrier function, influx of bacteria and consequently excessive immune response, thus predisposing to or worsening IBD. Therefore, we analysed the genotype distributions at these SNP loci of the genes encoding MMP-1, -2, -3, -9, TIMP-1, -2 and TNF-α in CD, UC and controls. Recently, we measured MMP and TIMP protein/activity levels in a large group of resected intestinal IBD tissues (Meijer *et al*[32], submitted) and here the expression data in a subgroup of which we also had DNA, are correlated to MMP, TIMP and TNF genotypes.

MATERIALS AND METHODS

Study design

Surgically resected intestinal mucosa from predominantly Dutch Caucasian patients with CD (n = 134, 40% male, median age at surgery 36.3 years, range 11.6-78.7 years) or UC [n = 111, 42% male, 37.8 (15.9-81.9) years], was collected in the period 1983-2002 at the department of Pathology, LUMC and stored at -70℃. The control group consisted of 79 patients with colorectal carcinoma [CRC, macroscopically normal tissue obtained at least 10 cm away from evident neoplasia, 43% male, median age at surgery 56.4 (19.0-85.0) years] and 169 healthy volunteers [37% male, age at blood collection date 33.3 (18.2-72.9) years], recruited among spouses of patients from the out-patient clinic and through advertisement. Informed consent from participants and approval of the LUMC ethics committee for the study protocol was obtained[29]. In both the IBD and control groups, more than 95% of the participants were of Caucasian origin. Resected tissue was homogenised with a Turrax device, blood was centrifuged and genomic DNA was isolated using the salting out method[30] and reconstituted to 10 ng/μL in 0.01 mol/L Tris/0.1 mmol/L EDTA, pH = 7.5. Differential diagnosis of CD or UC was established by routine clinical, radiological and histological findings. Age at onset, localisation at first endoscopy/radiology and development of fistulae and stenotic processes in a subset of CD patients (n = 123) were recorded in medical files. The measurement of myeloperoxidase was according to the procedure described by Kruidenier *et al*[31], while the MMP and TIMP protein/activity levels in IBD and CRC control tissue were measured previously by our group (Meijer *et al*[32] and submitted). In brief, homogenates obtained from surgically resected tissue were appropriately diluted. The MMPs and TIMPs antigen levels were measured by ELISAs (MMP-2, -9, TIMP-1, -2) or by highly sensitive bio-immuno activity assays (BIA) involving the conversion of chromogenic peptide S-2444 by MMP-activated pro-urokinase (MMP-1, -3), with all BIAs performed in the presence of APMA to account for total MMP antigen levels. Allelic composition at the SNPs of interest was determined by PCR-RFLP (MMP-1, -3, -9, TIMP-1, -2) or tetra primer ARMS PCR (MMP-2), as described previously[32,33]. Differences between groups were assessed by Chi-square, Kruskal-Wallis or Mann-Whitney U tests, as indicated. Statistical significance was reached if two-tailed P value $\leqslant 0.05$.

RESULTS

Allelic composition at SNP loci of MMP, TIMP and TNF genes

Between IBD and controls, no significant differences in genotype distribution were found at -1607 1G/2G and

Table 1 Genotype distributions at SNP loci in IBD patients compared to controls

Protein	SNP	Genotype	IBD (% of patients)	Controls (% of individuals)	Allele	IBD (% of total)	Controls (% of total)
MMP-1	-1607 1G/2G	1G1G	31	26.6	1G	55.4	51.4
	11q22-q23	1G2G	49	49.6	2G	44.6	48.6
		2G2G	20.1	23.8			
MMP-2	-1306 C/T	CC	57.7	61.9	C	75.5	76.8
	16q13	CT	35.6	29.9	T	24.5	23.2
		TT	6.7	8.2			
MMP-3	-1613 5T/6T	5T5T	29.2	27.9	5T	52.3	52
	11q23	5T6T	46.3	48.4	6T	47.7	48
		6T6T	24.6	23.8			
MMP-9	-1562 C/T	CC	72.4	69.7	C	85.6	84
	20q11.2-q13.1	CT + TT	27.6	30.3	T	14.4	16
♂, TIMP-1[1]	+372 T/C	T	61.6	51.6	T	61.6	51.6
	Xp11.3-p11.23	C	38.4	48.4	C	38.4	48.4
♀, TIMP-1[1]	+372 T/C	TT	31.2	23.8	T	52.8	50.7
	Xp11.3-p11.23	TC	43.3	53.6	C	47.2	49.3
		CC	25.5	22.5			
TIMP-2	+303 G/A	GG	77.5	78.7	G	88.3	89.1
	17q25	GA + AA	22.5	21.3	A	11.7	10.9
TNF-α	-308 G/A	GG	68.8	69.7	G	82.0	83.0
	6p21	GA + AA	31.4	30.3	A	18.0	17.0

n = 239-240/245 IBD patients *vs* *n* = 244/248 controls with successful genotype determinations. [1]For TIMP-1, ♂ these numbers are 99/101 *vs* 93/96 and for TIMP-1, ♀ 141/144 *vs* 151/152. Note: for MMP-9, TNF-α and TIMP-2 frequency of the homozygote mutant genotype (TT, AA and AA, respectively), was below 5% and these groups were combined with the corresponding heterozygote group, solely for the purpose of accurate statistical analysis. No statistically significant differences (Chi-Square testing) in genotype distributions or allele frequencies were found between IBD and controls.

Table 2 TIMP-1 genotype distribution at SNP + 372 C/T in CD patients compared to controls

Protein	Genotype	CD (% of patients)	Controls (% of individuals)	*P*[1]	Allele	CD (% of total)	Controls (% of total)	*P*-value
TIMP-1, ♂	T	67.9	51.6	0.055	T	67.9	51.6	0.055
	C	32.1	48.4		C	32.1	48.4	
TIMP-1, ♀	TT	39	23.8	0.018	T	56.5	50.7	0.238
	TC	35.1	53.6		C	43.5	49.3	
	CC	26	22.5					

♂: *n* = 53/54 *vs* 93/96; ♀: *n* = 77/80 *vs* 151/152 successful genotype determinations, respectively. [1]*P*-value, Chi-Square test.

-1306 C/T of MMP-1 and -2 promoters, respectively (Table 1). Also, 1G MMP-1 (55.4 *vs* 51.4%) and C wild-type (75.5 *vs* 76.8%) MMP-2 allelic frequencies were similar in both groups. The MMP-3 and MMP-9 genotype distribution at -1613 5T/6T and -1562 C/T, respectively, were also similar. The TIMP-1 gene is located on the X-chromosome, thus the results are presented according to gender. In both men and women the T (T) genotype seems relatively abundant in IBD (men T 61.6 *vs* 51.6%; women TT 31.2 *vs* 23.8%) and especially in CD (men T 67.9 *vs* 51.6%, $P = 0.055$; women TT 39.0 *vs* 23.8%, $P = 0.018$, Table 2). No differences in genotype distribution were observed for TIMP-2 and TNF-α at +303 G/A and -308 G/A, respectively (Table 1). For all SNPs, genotype frequencies in the control group are similar to what was expected from the Hardy-Weinberg equilibrium, except for MMP-2 (CC, CT, TT: 61.9, 29.9, 8.2 observed *vs* 59.0, 35.7, 5.3% expected, $\chi^2 = 6.36$, $P < 0.05$). Genotype and allelic frequencies for all SNPs examined were similar in CD *vs* UC and also in the healthy volunteers *vs* the carcinoma controls. As MMPs and TIMPs are involved in cancer and metastasis, all analyses were repeated with a control group consisting only of the healthy volunteers ($n = 169$), yielding similar results as mentioned above.

Effect of MMP and TNF-α SNPs on CD phenotype

The median age at onset of disease in 123 CD patients with a full medical record was 21.5 (range 0.3-61.5) years. Patients stratified according to genotype at the SNPs examined had similar ages at onset (Table 3). At first endoscopic/radiologic examination, in 53.3% of the patients colonic w/wo ileal involvement was evident. The MMP-3 genotype was associated with disease localisation ($P = 0.04$ for all three groups) and further analysis revealed a lower chance of colonic involvement at first endoscopy/radiology in patients with the 5T5T MMP-3 genotype ($P = 0.017$, 5T5T *vs* 5T6T and 6T6T combined). However, this genotype also conferred a major risk to development of stenotic complications: 91.2% of patients carrying the 5T5T genotype suffered from stenotic complications compared to 71.8% for the other genotypes ($P = 0.022$). The allelic polymorphisms at other SNP loci were not associated with disease localisztion or stricture involvement. Of all CD patients, 80/123 or 65.0%

Table 3 Effect of MMP, TIMP and TNF-α allelic SNP composition on age at onset of disease, colon involvement at first endoscopic/radiologic examination and development of fistulae or stenotic strictures during follow-up [median 24.7 (range 3.3-58.5) yr] in CD

Protein	SNP	Genotype	Number of patients	Age (yr) at onset of disease, median (range)	Colonic involvement at first examination (% of patients)	Fistulae during follow-up (% of patients)	Strictures during follow-up (% of patients)
MMP-1	-1607 1G/2G	1G1G	38	20.7 (0.3-45.6)	52.6	71.1	84.2
		1G2G	54	22.4 (7.0-61.5)	50	63	75.9
		2G2G	26-27	21.0 (3.4-40.2)	57.7	63	70.4
MMP-2	-1306 C/T	CC	66-67	20.1 (5.8-61.5)	57.6	64.2	74.6
		CT+TT	52[1]	22.4 (0.3-50.1)	46.2	67.3	80.8
MMP-3	-1613 5T/6T	5T5T	34	22.4 (0.3-53.6)	35.3[a]	70.6	91.2[a]
		5T6T	54	19.3 (3.4-61.5)	63	66.7	74.1
		6T6T	30-31	23.5 (7.2-49.1)	53.3	58.1	67.7
MMP-9	-1562 C/T	CC	88-89	21.0 (0.3-61.5)	53.4	68.5	75.3
		CT+TT	30[1]	22.7 (7.2-45.5)	50	56.7	83.3
TIMP-1, ♂	+372 T/C	T	32	18.9 (0.3-48.3)	53.1	78.1	87.5
		C	17	18.5 (5.9-61.5)	52.9	76.5	94.1
TIMP-1, ♀	+372 T/C	TT	29	24.3 (7.0-56.8)	55.2	41.4[c]	69.0
		TC	22-23	23.5 (3.4-39.6)	45.5	69.6	78.3
		CC	18	22.2 (10.4-49.1)	55.6	66.7	55.6
TIMP-2	+303 G/A	GG	91-92	21.5 (0.3-61.5)	56.0	68.5	77.2
		GA+AA	27[1]	22.4 (3.4-56.8)	40.7	55.6	77.8
TNF-α	-308 G/A	GG	79-80	22.6 (0.3-61.5)	50.6	68.8	78.8
		GA+AA	39[1]	18.9 (3.4-45.5)	56.4	59.0	74.4

[1]Only 7, 1, 2 and 4 patients were carrying the MMP-2, -9, TIMP-2 or TNF-α mutant genotype, respectively, and were combined with the heterozygote group for statistical purposes. Differences in phenotype between genotypes were tested for statistical significance by Kruskal-Wallis/Mann-Whitney *U* (onset) or Chi-Square test (colon involvement/fistulae and stricture development). [a]$P \leq 0.03$ *vs* 5T6T and 6T6T combined, [c]$P = 0.025$ *vs* TC and CC combined.

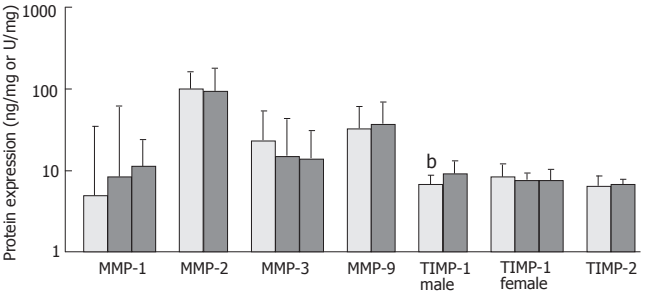

Figure 1 Protein expression in inflamed IBD tissue stratified to genotype. Values represent the median + 75th percentile and are in ng/mg (MMP-2, -9, TIMP-1, -2) or in arbitrary units/mg (MMP-1, -3). From left to right; MMP-1: 1G1G, 1G2G, 2G2G (n = 6, 17, 5); MMP-2: CC, CT + TT (n = 99, 77); MMP-3: 5T5T, 5T6T, 6T6T (n = 21, 33, 16); MMP-9: CC, CT + TT (n = 129, 46); TIMP-1 male: T, C (n = 47, 24); TIMP-1 female: TT, TC, CC (n = 42, 37, 26); TIMP-2: GG, GA + AA (n = 135, 41). [b]$P < 0.01$, Mann-Whitney *U* test.

developed peri-anal, entero-entero or entero-cutaneous fistulae during follow-up. Female patients with the TT genotype at +372 SNP of TIMP-1 appeared less prone to develop fistulae ($P = 0.08$), and when the TT group was compared with the combined TC and CC group, this result was statistically significant ($P = 0.025$).

Effect of SNPs on protein expression

Male IBD patients carrying the T allele at SNP +372 expressed lower levels of TIMP-1 in inflamed tissue compared to those carrying the C allele, $P = 0.009$ (Figure 1), with similar MPO levels in both groups [median 24.2 (range 9.1-80.4) *vs* 28.6 (2.5-75.9) U/g, $P = 0.194$]. In male CD patients a similar pattern in TIMP-1 expression was observed [6.8 (1.7-18.6) *vs* 9.2 (1.8-19.9) ng/mg TIMP-1, $n = 46$ *vs* 19, T *vs* C allele, respectively], although not longer statistically significant ($P = 0.065$). However, female IBD or CD patients carrying the TT, TC or CC genotype expressed similar levels of TIMP-1 in inflamed tissue. The respective protein expression was not affected by genotype at other MMP and TIMP SNPs in inflamed intestinal tissue. In non-inflamed IBD and control CRC tissue, no differences in protein levels were observed between patients stratified to genotype. Finally, allelic composition at TNF-α-308 G/A was not associated with higher or lower levels of MMPs or TIMPs in inflamed and non-inflamed IBD or control tissue.

DISCUSSION

In this study we found increased susceptibility to CD in men and women carrying the T and TT genotype, respectively, at TIMP-1 SNP +372. The X chromosome region p11.3-p11.23 might thus represent a novel linkage marker in IBD, extending the results obtained in previous genome-wide linkage studies[14,34]. Women with this genotype also appear less prone to the development of fistulae. The direct or indirect involvement of the X-chromosome in CD etiopathogenesis is further corroborated by a higher incidence of CD in women compared to men[35], the association of CD with X-linked Turner's syndrome[8] and the higher incidence of extra-intestinal complications and surgery recurrence rates in female compared to male CD patients[36]. Importantly, in men the T allele at SNP +372 was accompanied with a lower TIMP-1 protein expression in inflamed tissue. The lower TIMP-1 protein levels relative to MMP in susceptible individuals might shift the balance to a more proteolytic mucosal Crohn's disease phenotype. The TIMP-1 SNP might also be linked to other markers on

the X-chromosome increasing CD susceptibility and conferring protection against fistulae pathogenesis thus explaining the observed results in women. We observed no association between allelic composition at MMP-3 SNP-1613 and susceptibility to IBD. Our findings in UC confirm previous publications on primary sclerosing cholangitis and UC[22,37], but those on CD are different from the results obtained by the group of Pender *et al*[38], who noted increased susceptibility to sporadic, but not familial CD in individuals carrying the 5T allele. These contrasting results might arise from a different proportion of sporadic versus familial cases in our study. We also found a decreased chance of colonic involvement at first endoscopic/radiologic examination and a higher incidence of stenotic complications in patients carrying the 5T5T MMP-3 genotype at SNP-1613. Previously, over transmission of the 5T allele was associated with ileal localisation and stenosis in CD CARD15 mutation carriers[38] and the group of Warnaar *et al*[39] reported increased levels of MMP-3 in stenotic and pre-stenotic resected CD ileum, pointing to an MMP-3 mediated altered clinical course of CD patients by an, as yet, unidentified mechanism. The 5T5T genotype was reported to both increase[40,41] and decrease[42] MMP-3 protein expression, but in our study patients stratified according to MMP-3 genotype expressed similar MMP-3 total activity. Previously, the A allele at TNF-α SNP-308 was reported to increase susceptibility to UC[43], CD[44] and the incidence of fistulae in CD[45], possibly mediated by an increased promoter activity[46,47]. In contrast, we found no effect of allelic composition at this SNP on disease risk and phenotype, in line with other reports[48,49], adding further complexity to this matter. As mentioned before, the patient populations might differ dependent on the genetic (ethnic) background, thus explaining the contrasting results. We could not demonstrate an association of MMP-1, -2, -9 and TIMP-2 SNPs with disease susceptibility or clinical course of disease, in line with previous (genome-wide) linkage reports[11-13,37,50]. As other studies have clearly shown the involvement of these proteins in IBD pathology[51,52], it seems that they primarily function as mediators/effectors instead of initiators during IBD etiopathogenesis. However, the regulation of these proteins by immuno-suppressive medication, such as infliximab, might be dependent on the allelic composition at the SNPs examined, as previously shown by *ex vivo* explant studies from our group[32]. In principle, enhanced MMP expression might also be associated with SNPs in other genes, for instance with those encoding cytokines regulating MMP expression, e.g., IL-1β[44] and TNF-α. Dependent on the presence of relevant cis-acting elements in the promoter sequence, especially MMP-1 and MMP-9 would be affected[53,54], but we found no effect.

In summary, several studies reported associations between SNPs in diverse genes and IBD[16,48,55-59]. We have focused on the SNPs in genes coding for matrix remodeling proteins, i.e., MMPs and TIMPs, and believe the T allele at SNP +372 T/C in TIMP-1 might be involved in CD susceptibility in both sexes and in men by down-regulating TIMP-1 expression, while the 5T5T genotype at MMP-3-1613 might protect for colonic disease localization but also confers a major risk to stenotic complications. These findings reinforce the potential role of MMP and TIMPs in IBD and should be confirmed in larger prospective follow-up studies.

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RAPID COMMUNICATION

Optimal length of triple therapy for *H pylori* eradication in a population with high prevalence of infection in Chile

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Abstract

AIM: To compare the efficacy of 7-d versus 14-d triple therapy for the treatment of *H pylori* infection in Chile, with a prevalence of 73% in general population.

METHODS: *H pylori*-infected patients diagnosed by rapid urease test, with non-ulcer dyspepsia or peptic ulcer disease were randomized to receive omeprazole 20 mg bid, amoxicillin 1 g bid, and clarithromycin 500 mg bid for 7 (OAC7) or 14 (OAC14) d. Primary outcome was eradication rate 6 wk after the treatment. Subgroup analysis was carried out considering the eradication rate among patients with or without peptic ulcer disease and eradication rate among smokers or non-smokers.

RESULTS: One hundred and thirty-one patients were randomized to OAC7 (n = 69) or OAC14 (n = 62). The overall eradication rate (intention-to-treat) was 78.3% in OAC7 and 85.5% in OAC14 groups, without a significant difference (P =0.37). No significant difference in the eradication rate was found among the patients with peptic ulcer disease (n = 31) between the OAC7 group (85.7%) and OAC14 group (87.5%). However, smokers had an obviously lower eradication rate compared to non-smokers, particularly in the OAC7 group (57.1% in smokers *vs* 83.6% in non-smokers; P = 0.06). Adverse effects rate were similar between both groups.

CONCLUSION: Short-term efficacy of triple therapy with OAC for 7 d is comparable to 14 d in this high-prevalence population. Longer follow-up, and studies focused to some subgroups of patients (smokers and non-ulcer patients) are necessary to support widespread use of 7-d instead of 10-14-d triple therapy in a developing country like Chile.

Key words: *Helicobacter pylori*; Antibiotic treatment; Omeprazole; Amoxicillin; Clarithromycin; Quasi-randomized controlled trial

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INTRODUCTION

H pylori causes chronic gastritis and is associated with a higher incidence of peptic ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma[1].

Peptic ulcer disease and localized, low-grade MALT lymphoma are undisputed indications for treatment[2]. In other conditions, such as gastric cancer among first-degree relatives, patients with atrophic gastritis and dyspeptic patients with proven infection, there is a general consensus that *H pylori* eradication is also indicated. Even asymptomatic subjects with *H pylori* infection for any reason should be informed about risk of infection-related complications, and cost-benefit of treatment discussed in a case-by-case analysis[3,4].

Although several different treatment regimens have been proposed for *H pylori* eradication, triple therapy with a proton pump inhibitor (PPI), clarithromycin and amoxicillin is the most accepted therapy worldwide[5,6]. The eradication rate obtained with this combination is rather variable, ranging from less than 50%[7] to more than 95%[8]. There are important regional or geographical differences in success rates that have not been completely understood[6]; length of treatment, antibiotic dosage, bacterial resistance and other factors could be related to this variability.

In Europe, short (7 d) and low-dose antibiotic

treatment (clarithromycin 250 mg bid plus amoxicillin 500 mg bid) is usually recommended[5,9,10] while in the United States, 10 to 14 d of treatment is still preferred[11,12]. In 1997, the Asia Pacific Consensus Conference on the management of *H pylori* recommended a number of 7-d regimens[13]. The Latin American Consensus recommended 7 to 14-d treatment, with 10-d as the preferred regimen[14]. However, some recent studies from Asia have reported the eradication rates as low as 40.8%, even using triple therapy with PPI and antibiotics for 14 d[7].

Many recent studies have compared PPI-based triple therapies in short (7 d or less) and extended (10-14 d) schedules, and few have found significant differences in eradication rates although a trend towards better results with longer therapies is observed. However, as the number of patients is reduced, a B-type error may be present[8,15-17]. A recent meta-analysis on this topic showed that prolonging PPI-based triple therapy beyond 7 d improved treatment cure rates, and significant differences were found when 14-d therapies were compared to 7-d schedules[18]. Calvet *et al*[18] highlighted the importance of studies evaluating the cost-effectiveness of different lengths of therapy and suggested that geographical differences must be taken into account. To our knowledge, none of the included reports came from developing or high-prevalence countries. Because developing countries represent most of the infected population in the world, experiences coming from these areas are very important.

The scarce available information suggests that results obtained in developing countries usually are worse than those obtained in developed countries with the same therapy[19,20], but to our knowledge, there are no published systematic reviews on this particular topic.

Chile has a 73% (95% CI: 70%-76%) prevalence of *H pylori* infection in adult population and 79.5% in areas with high risk of gastric cancer[21]. This study was carried out in an urban area of Santiago with a high risk of gastric cancer and a frequency of *H pylori* infection among symptomatic patients of 78.7%[22]. *In vitro* antibiotic resistance was studied in 91 *H pylori* strains in Chile. All strains were susceptible to amoxicillin and only two strains (2.2%) were resistant to clarithromycin. Forty-two percent of strains were resistant to metronidazole and 13% were resistant to bismuth subcitrate[23]. Therefore, amoxicillin and clarithromycin plus proton pump inhibitors are recommended to eradicate *H pylori* in Chile. A randomized-controlled trial evaluated the efficacy of a short-term triple therapy among Chilean patients with peptic ulcer disease, with 3 d of azithromycin (500 mg OD) and 7 d of amoxicillin (750 mg tid) and a high (40 mg bid) or low (20 mg bid) dose of omeprazole; and the eradication rates were 57% and 61%, respectively[24], suggesting that short-term triple therapy with azithromycin has poor efficacy. However, there are no studies comparing short-term and extended triple therapy schedules with clarithromycin in a Chilean population.

This study aimed to compare the efficacy of 7-d versus 14-d course of omeprazole, amoxicillin and clarithromycin to eradicate *H pylori* infection in symptomatic patients from an urban area with high prevalence of *H pylori* infection.

MATERIALS AND METHODS

Protocol

The study was a quasi-randomized, open, comparative trial with two parallel treatment arms, performed in an outpatient care setting. Hispanic patients from an urban area of Santiago, with high prevalence of *H pylori* infection[21,22] and high risk of gastric cancer among patients with dyspepsia or abdominal pain[25], who were referred by gastroenterologists or general practitioners to an outpatient clinic with the indication of an upper gastro-intestinal (UGI) endoscopy to be performed in the endoscopy unit of the outpatient clinic of the Catholic University of Chile, were invited to participate in this study if *H pylori* infection was found. *H pylori* infection was assessed by a positive rapid urease test (RUT) (ProntoDry™, Medical Instruments Corporation, Brignais, France) on two or more antral biopsies[26,27]. Patients older than 18 years with positive RUT on antral biopsy were considered elegible. They were interviewed to check for inclusion criteria. After taking informed consent, the patients were randomized to receive either 7 or 14 d of omeprazole 20 mg bid, amoxicillin 1 g bid, and clarithromycin 500 mg bid. Exclusion criteria were: (1) Previous attempt of *H pylori* eradication; (2) concomitant or recent (within 3 mo) use of PPI, antibiotics or non-steroidal anti-inflammatory drugs; (3) total or partial gastrectomy; (4) gastric cancer suspected or demonstrated; and (5) known allergies to any of the drugs included in this study. Previous antibiotic exposure, defined as antibiotic usage more than 3 and less than 24 mo before exclusion, was registered at the first interview.

At least 6 wk after the end of therapy, all patients underwent a second UGI endoscopy, and RUT on antral biopsies was performed again in order to document *H pylori* eradication. Those patients not eradicated at this time received a second-line treatment beyond the study protocol. Primary outcome was *H pylori* eradication. Secondary outcome was the occurrence of adverse effects. Subgroup analyses were also performed.

The endoscopic findings were categorized as: (1) Peptic ulcer disease, when a discrete mucosal defect, at least 5 mm wide or with perceptible depth was observed in the gastric or duodenal mucosa; (2) erosive non-ulcer disease, when multiple (more than three) small (less than 5 mm) superficial mucosal defects, with a flat edge and no depressed base were observed in the gastric or duodenal mucosa, or esophageal mucosal breaks were seen, according to Los Angeles classification[26]; (3) normal endoscopy, when no evident lesions were observed. Non-erosive, non-specific changes on the esophageal, gastric or duodenal mucosa were classified as normal.

Recruitment of patients was performed from January 2003 to July 2004. The study was performed in accordance with the principles of good clinical research practice and the Declaration of Helsinki.

Assignment

Randomization was open, by means of the last digit of the identification number: odd numbers were assigned to the OAC7 group and even numbers to the OAC14 group.

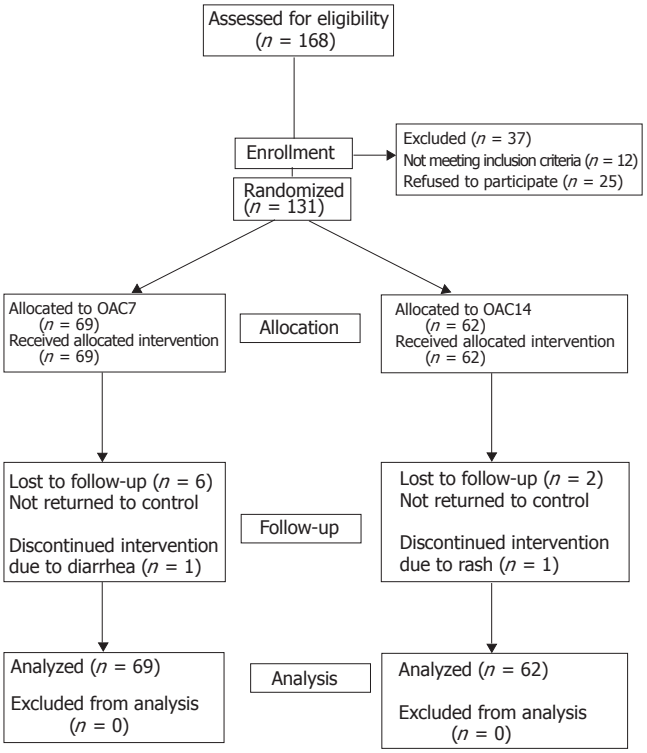

Figure 1 Study design and flow chart.

Compliance was assessed by the pill count method during a second interview after treatment. Adverse effects were registered and graded throughout and after treatment by the investigators as follows: none; mild (aware of symptoms, but easily tolerated); moderate (discomfort sufficient to cause interference with normal activities); and severe (incapacitating, with inability to perform normal activities).

Clinical characteristics of the groups

Patient flow chart, considering eligibility, randomization process and allocation of patients to OAC7 or OAC14 and follow-up of patients from both groups is described in detail in Figure 1.

Statistical analysis

Statistical effect size estimation was done. A sample size of 112 patients was estimated considering an effect size of 15% and a confidence interval ranging from 9% to 23% (with 80% power and 95% of confidence). The demographic and clinical characteristics of the patients were compared with Student's *t*-test for independent samples or Fisher's exact test for discrete variables (sex, adverse effects and presence or absence of eradication). Eradication was analyzed both on an intention-to-treat and on a per-protocol basis. Results were expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS version 10.0 (Standard version, SPSS Inc.).

RESULTS

One hundred and sixty-eight patients were considered eligible and 37 of them were excluded from the study for the following reasons. Twenty-five were excluded because they refused to consent, 4 with suspected or proven gastric cancer, 6 because of previous adverse reactions to amoxicillin or penicillin and 2 patients because of recent use of antibiotics. Finally, 131 patients were included.

Table 1 Baseline characteristics of patients *n* (%)

	OAC7 (*n* = 69)	OAC14 (*n* = 62)
Age (yr)	47.5 ± 16	45 ± 15
Male	27 (39.1)	18 (29)
Smokers	14 (20.3)	21 (33.9)
Previous antibiotic exposure		
Betalactamics	56 (81.2)	51 (82.3)
Clarithromycin	13 (18.8)	18 (29)
Metronidazol	13 (18.8)	12 (19.4)
Tetracyclines	32 (46.4)	26 (41.9)
Endoscopic diagnosis		
Ulcer disease	14 (20.3)	24 (38.7)
Non-ulcer disease	44 (63.8)	30 (48.4)
Normal	11 (15.9)	8 (12.9)

Table 2 Eradication rate after treatment and subgroup analyses *n* (%)

Eradication rate according to:	OAC7 (*n* = 69)	OAC14 (*n* = 62)	*P*
Length of treatment	54 (78.3)	53 (85.5)	0.37
Smoking status			
Smoker	8 (57.1)	17 (81)	0.15
Non smoker	46 (83.6)	36 (87.8)	0.88
Endoscopic diagnosis			
Ulcer disease	12 (85.7)	21 (87.5)	1.0
Non-ulcer disease	35 (79.5)	25 (83.3)	0.77
Normal	7 (63.6)	7 (87.5)	0.34
Previous antibiotic exposure			
Betalactamics	46 (82.1)	43 (84.3)	0.8
Clarithromycin	10 (76.9)	16 (88.9)	0.63
Metronidazol	9 (69.2)	10 (83.3)	0.64
Tetracycline	25 (78.1)	20 (76.9)	1.0

Sixty-nine patients were randomized to the OAC7 group and 62 patients to the OAC14 group. Demographic characteristics at baseline were similar between both groups (Table 1), including previous antibiotic exposure and endoscopic diagnosis.

On a per-protocol analysis, eradication of *H pylori* infection was achieved in 54 of 63 patients (85.7%) in OAC7 group and 53 of 60 patients (88.3%) in OAC14 group. On an intention-to-treat analysis, eradication was achieved in 54 of 69 patients (78.3%) in OAC7 group and 53 of 62 patients (85.5%) in OAC14 group. The differences did not reach statistical significance ($P = 0.37$) (Table 2). Most treatment failures ($n = 16$) received a second-line treatment beyond the study protocol.

Sub-group analysis

Eradication rate for subgroups of patients is presented in Table 2. Smokers showed a lower eradication rate compared to non-smokers. Interestingly, the difference was more evident in the OAC7 group, with 57.1% and 83.6% eradication rate in smokers and non-smokers, respectively

($P = 0.06$), almost reaching a statistical significance. Although the non-ulcer patients had a lower eradication rate compared to the ulcer patients, the difference did not reached statistical significance.

In addition, eradication rates between groups were not significantly affected by previous antibiotic exposure. The patients previously exposed to penicillin had eradication rates of 82.1% and 84.3% in OAC7 and OAC14 groups, respectively ($P = 0.8$), while the patients previously exposed to clarithromycin had eradication rates of 76.9% and 88.9% in OAC7 and OAC14 groups, respectively ($P = 0.63$).

Adverse effects and compliance

Abdominal pain, nausea and diarrhea were the most common adverse effects in both groups (Table 3). Most were mild. Only 2 patients discontinued treatment because of adverse effects (diarrhea and rash), after taking 71% and 90% of scheduled medication, respectively. Frequency of adverse effects was similar in both groups (29% in OAC7 versus 37% in OAC14 groups).

Eight patiens did not return to be evaluated for the eradication of *H pylori*, 6 from the OAC7 group and 2 from the OAC14 group. As shown in Figure 1, complete follow-up was achieved in 123 patients (93.9%).

Table 3 Adverse events during treatment *n* (%)

	OAC7 (*n* = 69)	OAC14 (*n* = 62)	Total (*n* = 131)
Abdominal pain	10 (14.5)	8 (12.9)	18 (13.7)
Nausea	7 (10.1)	11 (17.7)	18 (13.7)
Diarrhea	9 (13.0)	5 (8)	14 (10.7)
Taste disturbance	2 (2.9)	7 (11.3)	9 (6.9)
Headache	2 (2.9)	5 (8.1)	7 (5.3)
Vomiting	1 (1.58)	2 (3.2)	3 (2.3)
Loss of appetite	2 (2.9)	1 (1.6)	3 (2.3)
Rash	0	2 (3.2)	2 (1.5)
Discolored faeces	1 (1.4)	1 (1.6)	2 (1.5)
Tongue discoloration	1 (1.4)	0	1 (0.76)
Total	20 (29)	23 (37)	43 (33)

DISCUSSION

The ideal regimen for *H pylori* eradication is far from settled, and the search is ongoing[27,28]. The current standard triple therapy with two antibiotics and a PPI is being challenged by quadruple therapy (bismuth, PPI and two antibiotics) and lately by the so-called "sequential therapy" (PPI plus three antibiotics)[29,30]. The length of treatment is an important factor because it influences cost of treatment, and it may be related to eradication rates, adverse effects and compliance. The search for effective, but shorter therapies is totally justified. However, information about this topic has come almost exclusively from developed countries with low-prevalence of infection, and there are evidences to suggest that regional or geographical differences in the efficacy of therapy against *H pylori* could be related to socio-economic status[31].

The published trend toward similar results of two-week versus one-week triple therapy for *H pylori* eradication may not hold true for developing, high-prevalence countries. Where there is higher antibiotic resistance and bacterial load, this might compromise the outcome of shorter treatments. A recent report, coming from Alaska, showed an eradication rate of 34%, even using 14-d triple therapy[32]. Validated local information is very important to define standard therapy for *H pylori* infection in different geographical areas.

This is the first quasi-randomized controlled trial comparing 7-d versus 14-d triple therapy for *H pylori* eradication in this high-prevalence Hispanic population. This study seems to reproduce the results obtained in similar studies from developed countries, showing a slight (7.2%) but non-statistically significant improvement in eradication rate when PPI-based triple therapy is extended from 7 to 14 d. This is below the 15% conventionally defined as clinically significant (see Methods) and suggests that, at least in this population, one or two-week triple therapies are comparable in terms of eradication rate, with clear cost-advantages for the one-week regimen.

The subgroup analyses, although underpowered to detect significant differences, also confirmed previous reports, showing that smokers[33,34] seem to be more resistant to eradication therapy for *H pylori*. With respect to non-ulcer patients, some but not all reports identify this condition as a significant risk factor for treatment failure[35,36]. A recent single study[37] and also a systematic review found a trend to a worse response to treatment only when using 7-d regimen[38]. If confirmed by adequately designed studies, smokers and non-ulcer patients might constitute a subgroup of patients that should be treated for 10 or 14 d instead of 7 d[37].

These patients had not a long-term follow-up after treatment. Besides eradication rate, reinfection or recrudescence of infection are other critical factors to determine final effectiveness of therapy. As expected, there is a marked paucity of information coming from developing, high-prevalence countries. A recent report from Vietnam showed a 23.5% reinfection rate one year after treatment, with most strains being identical to the pre-treatment isolates[39]. Another report from Iran showed a 20.4% reinfection rate 3 years after treatment[40]. It has been reported that patients with duodenal ulcer from the same population have a 13% reinfection rate after 3 years of a 14-d triple therapy, with most cases occurring during the first year[41], very similar to the 10% reinfection rate found in Bangladesh, 3 to 18 mo after treatment[42]. Available information suggests that recrudescence accounts for the majority of early recurrences after treatment[43]. It has been convincingly demonstrated that early reinfection rate is inversely related to the initial eradication rate obtained with therapy[44], and it is possible that the small difference (7.2%) in short-term eradication rate between 7 and 14-d regimens may later determine a higher than expected "reinfection" rate in the OAC7 group. We are not aware of any published study comparing long-term reinfection rate after 7 and 14-d regimens. Longer follow-up of this cohort of patients may help to answer this question.

In summary, this quasi-randomized comparative trial showed that 7-d and 14-d PPI-based triple therapies

are comparable in eradicating *H pylori* in a population with a high prevalence of infection, at least in the short-term follow-up. It is possible that smokers and non-ulcer patients might be candidates for a longer period of treatment. Because of the potentially higher reinfection risk in these sub-groups of the population and the demonstrated inverse relationship between initial eradication rate and recrudescence of infection, it is reasonable to prolong the follow-up of this cohort of patients for at least one year, before to recommend a 7-d triple therapy as the standard treatment for *H pylori* infection in this population.

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RAPID COMMUNICATION

Comparison of postpolypectomy bleeding between epinephrine and saline submucosal injection for large colon polyps by conventional polypectomy: A prospective randomized, multicenter study

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Abstract

AIM: To evaluate and compare the clinical outcomes of prophylactic submucosal saline-epinephrine injection and saline injection alone for large colon polyps by conventional polypectomy.

METHODS: A prospective study was conducted from July 2003 to July 2004 at 11 tertiary endoscopic centers. Large colon polyps (> 10 mm in diameter) were randomized to undergo endoscopic polypectomy with submucosal saline-epinephrine injection (epinephrine group) or normal saline injection (saline group). Endoscopic polypectomy was performed by the conventional snare method, and early (< 12 h) and late bleeding complications (12 h-30 d) were observed.

RESULTS: A total of 561 polyps in 486 patients were resected by endoscopic polypectomy. Overall, bleeding complications occurred in 7.6% (37/486) of the patients, including 4.9% (12/244) in the epinephrine group, and 10.3% (25/242) in the saline group. Early and late postpolypectomy bleeding (PPB) occurred in 6.6% (32/486) and 1% (5/486) of the patients, respectively, including 4.5% (11/244), 0.4% (1/244) in the epinephrine group, and 8.7% (21/242), 1.7% (4/242) in the saline group. No significant differences in the rates of overall, early and late PPB were observed between the 2 groups. Multivariate stepwise logistic regression analysis revealed that large size (> 2 cm) and neoplastic polyps were independently and significantly associated with the presence of PPB.

CONCLUSION: The prophylactic submucosal injection of diluted epinephrine does not appear to provide an additional advantage over the saline injection alone for the prevention of PPB.

Key words: Colonoscopic polypectomy; Bleeding; submucosal injection; Saline; Epinephrine

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INTRODUCTION

Colonoscopic polypectomy effectively reduces the risk of colorectal cancer[1-3]. The major complication of this procedure is hemorrhage, the occurrence of which varies between 0.3% and 6.1%, following perforation and problems with premedication[1-5]. It was reported that a submucosal saline solution injection is used for at least some polyps by 82% of physicians[6]. This submucosal saline cushion has been used to reduce postpolypectomy complications and enhance complete resection[7,8]. Local epinephrine has also been used to minimize mucosal bleeding due to its hemostatic effect, but its clinical benefit may not be clear. One prospective comparative study reported that epinephrine in the submucosal injection fluid could reduce the risk of immediate bleeding but not delayed bleeding[9]. In an American Society for Gastrointestinal Endoscopy (ASGE) editorial, because the overall risk of immediate bleeding is low and the immediate bleeding can generally be treated successfully by experienced endoscopists, there is no mandate to include epinephrine in the injection fluid[10]. The purpose of the current prospective multicenter study was to evaluate and compare the clinical outcomes of prophylactic submucosal saline-epinephrine injection and saline injection alone in conventional colon polypectomy.

MATERIALS AND METHODS

Between July 2003 and May 2004, patients diagnosed with colon polyps with a diameter > 10 mm were randomized to receive either a submucosal saline-epinephrine injection (epinephrine group) or a normal saline injection (saline group) before conventional polypectomy. The following exclusion criteria were used in the study: (1) diameter of polyp < 1 cm, (2) diameter of polyp larger than the size of a polypectomy snare requiring the submucosal dissection method, (3) patients taking anticoagulants, (4) disease impairing normal blood clotting, (5) abnormal coagulogram (platelet count, INR, APTT), (6) patients unwilling to give written informed consent, (7) age < 18 years. The trial profile is shown in Figure 1. All colonoscopies were performed with an Olympus CF-230 or CF-240 video colonoscope, after careful preparatory cleansing of the bowel using a polyethylene glycol-electrolyte solution. Midazolam was given intravenously only if needed. Patients were randomized using computerized randomization. The result of the randomization was kept blind from the endoscopist and the assistant. A submucosal injection solution was made in advance by the 2nd assistant. The Korean Association for the Study of Intestinal Diseases (KASID) approved the design of the trial. Informed consent was obtained from every enrolled patient before each procedure. If a patient had more than one polyp, all the polyps fulfilling the inclusion criteria were selected for prophylactic injections according to randomization.

In each group, injections of 0.01% epinephrine or normal saline were administered into the polyp stalk or base using a flexible needle injector, before resection with a standard snare. A total of 2-25 mL of solution was injected in this study. The injection volume was determined by observation of tissue elevation sufficient to perform polypectomy safely and completely. Polypectomy was executed according to the conventional method. The polyps were snared with a diathermic snare, linked to an electrocoagulator. The polyps were removed by bipolar electrocauterization, using a blended current (setting power: 30-40 Watts) only. If the remaining tissue was under suspicion, the procedure was repeated until resection was complete. After colonoscopic polypectomy, patients were nonhospitalized, or hospitalized for observation for 24 h. Early bleeding was defined as hematochezia within 12 h of the procedure, and late bleeding as bleeding occurring 12 h-30 d after the endoscopic procedure.

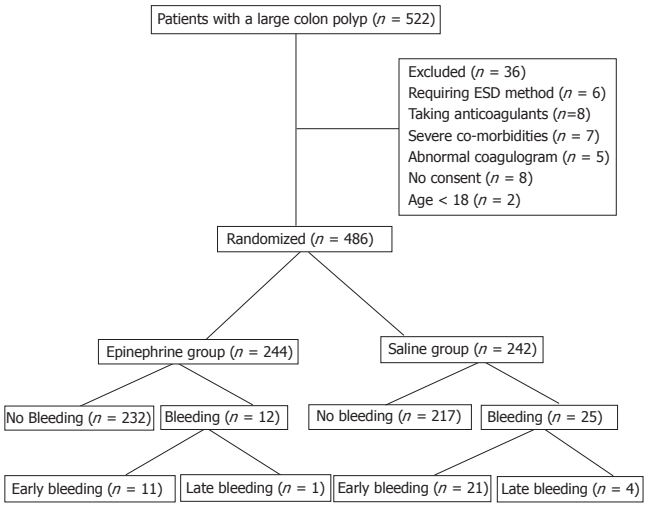

Figure 1 Flow of study participants.

The data were analyzed in several subgroups within each main group, according to the size, shape (protruding type: sessile, semipedunculated, pedunculated or superficial elevated type), and distribution of polyp (left colon, right colon or both).

Histologic studies were performed on all removed polyps. The resected polyps were fixed and embedded in paraffin. Serial sections, perpendicular to the mucosal surface, were obtained and stained with hematoxylin and eosin. The data were analyzed by their histologic features as nonneoplastic or neoplastic (dysplasia, high grade dysplasia or carcinoma *in situ*, cancer). We determined our sample size by assuming that submucosal saline-epinephrine injection solution would reduce the bleeding rate by 10%-20%, based on various reports of the rates of PPB. Given $\alpha = 0.05$, a power of 80%, we required a sample size of 450 patients. Data were analyzed by using Statistical Package for the Social Sciences program (version 6.12, SAS Institute Inc, Cary, NC). Categorical variables were compared by using the chi-square test. When two variables were dichotomous, the Fisher's exact test was used. To evaluate the continuous variables, Student's *t* test was used. Multivariate logistic regression analyses were performed for the risk factor of PPB. $P < 0.05$ was considered statistically significant.

RESULTS

In a total of 486 patients, 561 polyps were resected by endoscopic polypectomy. The median age of the patients

Table 1 Baseline characteristics of the studied patients

	Epinephrine group (n = 244)	Normal saline group (n = 242)	P
Clinical features			
Age (mean ± SD)	56.0 ± 11.4	56.8 ± 11.3	0.429
Sex (Male%)	195 (70.9%)	184 (64.3%)	0.096
BMI (mean ± SD)	23.0 ± 2.9	23.8 ± 2.7	0.572
Endoscopic features			
Total No. of polyps	275	286	0.587
Mean No. of polyps	1.13 ± 1.6	1.18 ± 2.0	0.149
Mean size of polyps (mm)	14.5 ± 5.7	15.0 ± 6.8	0.318
Macroscopic form of polyps			0.427
Pedunculated (Ip)	62 (22.5%)	66 (23.1%)	
Semipedunculated (Isp)	93 (33.8%)	107 (37.4%)	
Sessile (Is)	70 (25.5%)	80 (28.0%)	
Superficial elevated type (Ⅱa)	50 (18.1%)	33 (11.5%)	
Distribution of polyps			0.809
Left colon (descending-)	131 (53.7%)	126 (52.1%)	
Right colon (-transverse)	51 (20.9%)	48 (19.8%)	
Both	62 (25.4%)	68 (28.1%)	
Pathologic features			
Non-neoplastic	23 (8.4%)	28 (9.8%)	0.557
Neoplastic			
Dysplasia	184 (66.9%)	178 (62.2%)	0.269
HG dysplasia + CIS[1]	44 (16.0%)	51 (17.8%)	0.626
Cancer	24 (8.7%)	29 (10.2%)	0.557
Procedure related features			
Inexpert operator[2]	42 (17.2%)	48 (19.8%)	0.626
En bloc resection	261 (94.9%)	268 (93.7%)	0.070

[1]HG + CIS, high grade dysplasia + carcinoma *in situ*. [2]Inexpert operator, experience in polypectomy < 2 years.

was 56.6 ± 11.3 years, 379 men and 182 women were included in this study. There was no significant difference between the two groups in clinical features and base line characteristics, including the size, distribution, shape of the polyps and the pathological diagnosis (Table 1). The mean size of polyps was 14.5 mm ± 5.7 mm and 15.0 mm ± 6.8 mm in each group, and these polyps were usually present in the left colon (53.7% and 52.1% in each group). The protruding type of polyp was dominant, accounting for 74.5% and 72% in each group. Neoplastic polyps were present in 91.6% and 90.2% of each group, including coexisting cancer (8.7% and 10.2% in each group, respectively). The rate of *en bloc* polypectomy was high in both groups, accounting for 94.9% (261/275) and 93.7% (268/286) respectively.

Postpolypectomy bleeding

The overall rate of postpolypectomy bleeding (PPB) was 4.9% (12/244) in the epinephrine group and 10.3% (25/242) in the saline group. There was no statistical significance in the overall PPB between the two groups. Early PPB showed a tendency to be high in the saline group (4.5 % *vs* 8.7%, P = 0.065), but statistical difference was not proved. Late PPB did not exhibit a statistical difference in the two groups (0.4 % *vs* 1.7%, P = 0.154) (Table 2).

Table 2 Postpolypectomy bleeding, n (%)

	Epinephrine group (n = 244)	Normal saline group (n = 242)	P value
Early bleeding	11 (4.5)	21 (8.7)	0.065
Late bleeding	1 (0.4)	4 (1.7)	0.154

Table 3 Risk factors for postpolypectomy bleeding (multivariable analysis)

Risk factor	Adjusted odds ratio (95% CI)[1]	P
Size (> 2 cm)	1.07 (1.01, 1.14)	0.034
Injection (normal saline only)	1.31 (0.57, 2.99)	0.527
Pathology (neoplastic)	9.88 (1.26, 77.75)	0.029
Morphology (protruding type: Ip, Isp, Is)	0.59 (0.18, 1.95)	0.393
Inexpert operator (< 2 yr)	2.62 (0.98, 7.06)	0.056
Hospitalization (not hospitalized)	2.14 (0.57, 8.04)	0.261

[1]Adjusted odds ratios are calculated from a multivariate logistic regression model except for age, sex and procedure time.

Colonoscopic features of postpolypectomy bleeding

Colonoscopic features were not statistically different between the two groups. There was no statistical difference in the disrtibution of colon polyps and the presence of multiplicity, or the shape of the colon polyp (Table 1). PPB was significantly higher in the case of large sized (> 2 cm) polyps (Table 3).

Histologic and other features of postpolypectomy bleeding

PPB was significantly higher in neoplastic polyps, but there was no statistical significance in the pattern of neoplastic histology (dysplasia, high grade dysplasia or carcinoma *in situ*, cancer). The less experienced endoscopist (< 2 years) observed high PPB in nonhospitalized patients, but a statistical significance was not shown (Table 3). No free perforation was observed in relation to the procedure. In all the patients with postpolypectomy bleeding, endoscopic treatment was successfully performed without the need for surgery or angiography.

DISCUSSION

Several prospective studies have shown that removal of adenomatous polyps is associated with a reduction in the incidence of colorectal cancer[1,2]. Endoscopic polypectomy is a standard method of treatment of polyps in the gastrointestinal tract, but it is associated with substantial complications. Bleeding is the most frequent complication of endoscopic polypectomy. The reported incidence of bleeding after polypectomy ranges from 0.3% to 6.1%[1-5]. To reduce this complication, two aspects of technical development should be considered. One is the type of cutting currents used, such as a pure cutting current, blended current or pure coagulation. The other is the use

of a submucosal cushion injection.

The effect of pure cutting current is to vaporize the cells, whereas coagulation tends to heat-seal blood vessels[11]. Therefore, the risk and pattern of bleeding might be expected to differ depending on the type of currents used. Using a pure cutting or a blended current, the major episode was immediate hemorrhage, in contrast to delayed hemorrhage with a pure coagulation current[12]. Pure cutting current has several advantages over pure coagulation current because it is a faster procedure which provides clearer margins in the resected specimen and reduces the risk of transmural burn and perforation. However, it is generally accepted that if pure cutting current is used for polypectomy, hemostasis would be inadequate, and the risk of bleeding is high[4]. Therefore, the coagulation or blended electrosurgical current is generally preferred, because it is believed to reduce the risk of major hemorrhage. One survey of colonoscopic polypectomy practices among clinical gastroenterologists reported that the electrosurgical current used for polypectomy was pure coagulation current in 46%, blended current in 46%, and pure cutting current in 4%[6]. Because of the risk of transmural burn and delayed bleeding which is more difficult to treat than immediate bleeding, we theoretically believe that the blended current is safer and more effective than the pure coagulation current. In the present study, to preclude interprocedural bias, only one type of electrocoagulation or blended current, was used.

Submucosal saline injection has been demonstrated to be an effective method for a complete endoscopic polypectomy, especially in flat or sessile lesions[13,14]. Elevation of the colorectal polyp far enough from the muscle layer and serosal surface prevents a deep intramural burn as well as perforation[8]. Besides the prevention of perforation, the injection technique might also reduce the bleeding rate after polypectomy[9,14-16]. In one retrospective study[15], among 77 polyps more than 15 mm in diameter, there was no bleeding in the epinephrine injection group (28 polyps). In contrast, 9 of 49 polypectomies (18.4%) without submucosal injection were associated with a bleeding episode. There are 2 prospective studies on the efficacy of prophylactic submucosal saline-epinephrine injection in colonoscopic polypectomy[9,16]. A total of 120 patients with 151 sessile polyps were randomized into the epinephrine injection group or the control group. There was no significant difference in overall PPB, but immediate bleeding occurred significantly less frequently in the epinephrine group than in the control group (1/75 *vs* 7/76, $P = 0.03$)[9]. In another prospective study[16], 100 polyps (more than 10 mm in size) were randomized to receive submucosal injection or no injection, and there were nine episodes of PPB, one in the epinephrine group and eight in the control group (1/50 *vs* 8/50, $P < 0.05$). Although submucosal saline-epinephrine injection has been shown to reduce the risk of PPB, there is no prospective randomized study to compare PPB between submucosal saline injection with epinephrine and saline injection alone. Various submucosal injection materials, such as hyaluronic acid, fibrinogen mixtures and other viscoelastic substances, have been introduced into the conventional polypectomy and extended endoscopic mucosal resection, but these materials may have some side effects[17,18]. Submucosal injection of diluted epinephrine is a simple, effective, and cheap method for endoscopic polypectomy. The proposed method can affect tamponade, vasoconstriction, endarteritis, and possibly has a direct effect on the clotting process at the site of arterial defect. One major concern is the safety of epinephrine injection. However, side effects seem to be very rare in all aspects of therapeutic endoscopy, both epinephrine and mixed injection materials carry unwanted theoretical risks, such as local ischemia and cardiovascular side effects[19].

In the present study, the epinephrine injection did not show superiority over the saline injection in decreasing PPB. There was no statistical significance in overall and delayed PPB between the two groups. As in a prior study[9], early PPB showed a tendency to be high in the saline group (4.5 % *vs* 8.7%), but statistical difference was not proved. Although type-2 error could be influential, this result shows that there is no mandate to include epinephrine in injection fluid for conventional colonoscopic polypectomy.

Immediate bleeding can generally be treated successfully by endoscopic hemostasis. Some efforts have been made to decrease the delayed PPB using mechanical devices and many endoscopists prefer pretreatment of pedunculated polyps with thick stalks by placement of a detachable snare[20,21]. However, the clinical benefit may be marginally significant only for pedunculated polyps. Therefore, the use of detachable snares in clinical practice is not mandated. Another prospective trial showed that prophylactic clip placement does not decrease the occurrence of delayed PPB[22]. In the present study, there was no preventive method for delayed bleeding, and PPB was successfully controlled by endoscopic hemostasis without operation or angiographic embolization.

In general, the risk of PPB increases with the size of polyps and a more proximal colonic location. For polyps larger than 2 cm in diameter, particularly in the proximal colon, bleeding rate may exceed 10%[23,24]. In the present study, a multivariate logistic regression model excluding age, sex and procedure time showed that the size of polyp (> 2 cm) and neoplastic histology were associated with the risk of PPB ($P < 0.05$). In terms of large polyps, possible explanations for the increased risk are that resection of large polyps is technically more difficult, and these large lesions may contain large vessels. In terms of the neoplastic histology, it is difficult to interpret this finding, and to our knowledge, no study has shown such a result. There was no statistical difference in the site of colon polyp. Other possible causal factors for PPB include that the less experienced endoscopist (< 2 years) observed high PPB in nonhospitalized patients. However, these differences did not show a statistical significance.

The main limitation of our study was the relatively small sample size. Because the rate of PPB was less than 5%-10%, larger-scale studies are necessary to confirm our results. Another limitation was the method of polypectomy. Recently, for flat or depressed lesions greater than 20 mm in diameter, endoscopic mucosal resection with precutting or endoscopic submucosal dissection method has been recommended. It is presumed that complication rate due to more complex methods will be

different from that due to conventional snare polypectomy.

In conclusion, prophylactic submucosal injection of diluted epinephrine does not appear to offer a distinct advantage over saline injection for preventing postpolypectomy bleeding. Submucosal injection of normal saline is an adequate method for safe and effective colonoscopic polypectomy using the conventional snare method.

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RAPID COMMUNICATION

Prevalence of IgA-antiendomysial antibody in a patient cohort with idiopathic low bone mineral density

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Abstract

AIM: To investigate the frequency of serum IgA-antiendomysial antibody positivity in patients with low bone mineral density and to assess the risk group for screening of celiac disease.

METHODS: One hundred and thirty-five patients (14 male, 121 female) with idiopathic low bone mineral density were evaluated. The median age was 57.2 years (24-81). Antiendomysial antibody was determined by the immunofluorescence method using a commercial kit (INOVA Diagnostics Inc., CA, USA), which employs a 5 μm thin cryostat section of monkey esophagus as a substrate.

RESULTS: Of the 135 patients evaluated, 13 were found to have positive IgA antiendomysial antibody test (9.6%) response. None of the patients had IgA deficiency. Endoscopic appearance and histological examination were normal in all of these patients. Seropositive patients had significantly lower age (48.9 ± 4.3 *vs* 59.2 ± 6.2, $P < 0.05$), higher ratio of male gender (61.5% *vs* 4.9%, $P < 0.01$) and pre-menopausal status (8.7% *vs* 1.3%, $P < 0.01$). Lumbar spine and femoral neck z-scores, but not *t*-scores were significantly lower in seropositive patients. Seropositive patients had lower serum 25 (OH) vitamin D, calcium and higher serum parathormone levels than seronegative patients.

CONCLUSION: The screening of celiac disease in idiopathic osteoporosis should be restricted to patients without classical risk factors (younger, pre-menopausal, male gender) for osteoporosis. Bone mineral density measurements using z-scores should be considered for identifying risk groups for celiac disease.

Key words: Antiendomysial antibodies; Celiac disease; Osteoporosis

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INTRODUCTION

Celiac disease (CD) is a life-long inflammatory condition of the gastrointestinal tract that affects the small intestine in genetically susceptible individuals[1]. Although originally thought to occur only rarely in childhood, it is now recognized as a common condition that could be diagnosed at any age[2]. There is a wide range of presentations from asymptomatic through fatigue and vague abdominal symptoms, weight loss and diarrhea to frank malabsorption with steatorrhea[1].

Osteomalacia is a well-recognized feature of CD in adults and children[3,4]. This condition improves with calcium and vitamin D supplementation. Bone pain, pseudofractures or deformity may occur and the finding of a raised serum alkaline phosphatase with normal calcium and phosphate levels may be present.

Osteopenia and osteoporosis are also common features. Bone mineral density (BMD) is usually reduced[5]. Osteopenia is the most common complication of CD and its prevalence increases with age at diagnosis. More than 70% of patients with untreated CD have osteopenia[6] and osteoporosis occurs in more than one quarter of all patients[7].

The prevalence of CD in idiopathic osteoporotic patients was investigated in many studies[8-15]. Controversy still exists about the value of screening CD in patients with low BMD. Recent findings indicated an increased prevalence of CD in pre-menopausal women[10]; however, a study on post-menopausal women failed to find a significant difference from the normal population[9].

In order to assess the frequency of CD in patients with low BMD, we have investigated the prevalence of EMA-IgA positivity in this population.

MATERIALS AND METHODS

Patients

Subjects were prospectively recruited from unselected

consecutive patients with a diagnosis of osteoporosis or osteopenia in the Department of Physical Therapy and Rehabilitation at Gazi University Faculty of Medicine between April 2003 and January 2006. Inclusion criteria were idiopathic low BMD (below 1 SD of the mean), normal values of serum calcium, phosphorus, alkaline phosphatase and creatinine. Exclusion criteria were diseases affecting bone metabolism (Cushing's disease, hyperparathyroidism, hyperthyroidism, cholestatic liver diseases, osteogenesis imperfecta, acromegaly, *etc*.), neoplastic diseases or taking drugs known to affect bone metabolism, such as corticosteroid, antiepileptics or heparin or any other condition known to cause secondary osteoporosis were excluded from the study.

Questionnaires

The data on the general health status and lifestyle factors were collected by questionnaires. The questionnaire included demographic features, menarcheal and menopausal age, parity, breast-feeding, consumption of dairy products, smoking, alcohol consumption, medical history including symptoms or signs of CD (diarrhea, dyspepsia, bloating, altered bowel habits resembling irritable bowel syndrome and anemia) and current diseases, physical activity and immobilization history longer than 2 mo and previous fractures.

Bone mineral density measurements

BMD of the proximal femur and lumbar spine was carried out using dual-energy X-ray absorptiometry (Hologic QDR 4500C, Wolthom, MA, USA). BMD was expressed as standard deviation scores, which compare individual BMD determinations to those of young (T) and age/sex-matched (Z) normal populations.

Biochemical measurements

Biochemical parameters including serum calcium, phosphate, alkaline phosphatase and creatinine were measured by standard automated techniques. Serum intact parathyroid hormone (PTH) was measured using the chemiluminescence method (PTH Intact DPC Immulite 2000 autoanalyser). 25-hidroxy-vitamin D were determined by radioimmunoassay (RIA) using a commercial kit (Biosource Inc., USA).

Hematological (hemoglobin, folic acid, vitamin B12, mean corpuscular volume, erythrocyte sedimentation rate, and immunoglobulin) tests were performed using routine methods.

Determination of anti-endomysial antibody

EMA was determined by the immunofluorescence method using a commercial kit (INOVA Diagnostics Inc., CA, USA), which employs a 5 μm thin, cryostat section of monkey esophagus as a substrate. Duplicate serum samples were tested at a dilution of 1:5. Total IgA levels were also determined. Sensitivity and specificity of EMA are 97.4% and 99.6%, respectively[16,17].

Small bowel histology

All small intestinal biopsies are obtained *via* videogastroscope (from distal duodenum). At least three biopsies were obtained and preserved conventionally. Pathologic assessment was done by an experienced pathologist. The histological characteristics of intestinal mucosa were assessed by conventional microscopy.

Diagnosis of celiac disease

A minimal criterion for CD diagnosis was positive serology together with characteristic features of intestinal mucosal changes (villous atrophy, crypt hyperplasia, increased intraepithelial lymphocyte infiltration > 30%).

Statistical analysis

Statistical analyses were performed using the SPSS 15.0 statistical program. Student's unpaired *t*-test was used for comparison of the differences between the EMA positive and negative patients. In EMA positive patients, Pearson's correlation analysis was performed to assess the association between BMD and Ca, PTH, 25(OH) vitamin D levels. Data were expressed as the mean ± standard deviation (SD). Differences were considered significant, if the *P* values were less or equal to a level of 5% and all results are expressed at a 95% confidence level.

RESULTS

One hundred and thirty-five patients (14 male, 121 female) with idiopathic low BMD were evaluated. The median age was 57.2 years (24-81). Upon evaluation of the questionnaires, none of the patients was found to have signs or symptoms of CD such as malabsorption, diarrhea, weight loss or anemia.

Of the 135 patients evaluated, 13 were found to have positive IgA EMA test (9.6%). None of the patients had IgA deficiency. All of the thirteen patients with positive EMA in their sera underwent upper gastrointestinal endoscopy and duodenal biopsy. Endoscopical appearance of duodenal mucosa was normal in all of these patients. The histopathological examination revealed non-specific changes, such as mild lymphocyte infiltration in lamina propria and none of them had findings consistent with CD. We could not detect any patient with celiac disease in this population.

For the statistical analysis, the data obtained from the 13 EMA positive patients were compared with the data of 122 EMA negative patients. The demographic features and BMD values of these patients are shown in Table 1. When EMA positive patients were compared with EMA negative patients, EMA positive patients had significantly lower age (48.9 ± 4.3 *vs* 59.2 ± 6.2, $P < 0.05$), higher ratio of male gender (61.5% *vs* 4.9%, $P < 0.01$) and pre-menopausal status (8.7% *vs* 1.3%, $P < 0.01$).

Other parameters including weight, height, BMI, lumbar spine and femoral neck t-scores were similar between groups. However, lumbar spine and femoral neck z-scores were significantly lower in EMA positive patients (Table 1).

Table 2 shows laboratory findings and comparison between EMA positive and negative patients. EMA positive patients had lower serum 25(OH) vitamin D,

Table 1 Demographic features and BMD values of EMA positive and negative patients

Mean	EMA negative (n = 122)	EMA positive (n = 13)
Age (yr)	59.2 ± 6.2	48.9 ± 4.3[a]
Gender (M/F)	6/116	8/5[b]
Pre-menopausal females (n = 46) (%)	42/46 (91.3%)	4/46 (8.7%)[d]
Post-menopausal females (n = 75) (%)	74/75 (98.7%)	1/75 (1.3%)[d]
Weight (kg)	65 ± 8.4	57 ± 4.1
Height (cm)	158 ± 6.7	157 ± 3.9
BMI (kg/m^2)	26.5 ± 4.2	27.9 ± 3.1
Lumbar spine T-score	-2.8 ± 0.8	-2.9 ± 0.9
Femoral neck T-score	-3.1 ± 1.1	-3.0 ± 0.4
Lumbar spine z-score	-2.0 ± 0.9	-2.7 ± 2.2[a]
Femoral neck z-score	-1.9 ± 2.1	-2.4 ± 1.7[a]

[a]$P < 0.05$ *vs* EMA negative groups (unpaired *t*-test); [b,d]$P < 0.01$ *vs* EMA negative groups (unpaired *t*-test); BMI: Body mass index.

Table 2 Laboratory findings of EMA positive and negative patients

Mean	EMA negative (n = 122)	EMA positive (n = 13)
25(OH) vitamin D (ng/mL)	19.8 ± 4.88	11.6 ± 1.89[b]
Parathormone (pmol/L)	42.7 ± 14.8	59.9 ± 18.6[b]
Hemoglobin (g/dL)	12.3 ± 1.5	11.9 ± 2.3
Mean corpuscular volume (fL)	88.7 ± 4.8	86.5 ± 3.2
Calcium (mg/dL)	9.7 ± 0.9	9.0 ± 0.5[a]
Phosphorus (mg/dL)	3.4 ± 0.4	3.2 ± 0.8
ALP (IU/L)	70.6 ± 21.8	71.4 ± 12.8
Vitamin B12 (pg/mL)	355.6 ± 121.7	302 ± 45.9

[a]$P < 0.05$ *vs* EMA negative groups (unpaired *t*-test); [b]$P < 0.01$ *vs* EMA negative groups (unpaired *t*-test).

calcium and higher serum parathormone levels than EMA negative patients. Other indices, including hemoglobin, mean corpuscular volume, phosphorus and vitamin B12 were similar between groups.

DISCUSSION

Asymptomatic (subclinical and silent) CD manifests with extra-intestinal features[1]. The most frequent extraintestinal marker of subclinical CD is iron-deficiency anemia (27.77%), alopecia and dermatitis herpetiformis (11.36%), osteoporosis (6.81%) and recurrent aphtous stomatitis (5.68%). The most frequent features in silent form of CD are CD history in first-degree relatives (30%), Basedow's disease (25%) and insulin-dependent diabetes (20%).

Clinical diversity and its potential complications are the main logic behind the studies investigating asymptomatic CD. Lindh *et al*[15] reported the first seroprevalance study of CD in idiopathic osteoporosis. They investigated 11 out of 92 seropositive patients (11%). Duodenal biopsy was positive in three of them. Similar findings were reported in 255 osteoporotic women from Italy. The seroprevalance was 9.4% (24 patients) and celiac disease histology was verified in six of them[16].

Mather *et al*[14] reported conflicting results. Seropositivity rate of EMA IgA was 7 out of 96 patients. However, none of these patients had clues of CD in their duodenal histology, nor had they altered intestinal permeability. The authors mentioned the low titers of EMA in this sub-group of patients.

In another study from Argentina, 127 post-menopausal women with osteoporosis were screened for CD. In this study, one of 127 patients was diagnosed as CD compared to six of 747 control group patients. The authors concluded that it is unnecessary and not cost-effective to screen post-menopausal women for CD[9].

EMA is directed against endomysium, a connective tissue protein found between myofibrils in the gastrointestinal tract of primates. Replacement of primate esophagus with human umbilical cord as the substrate for EMA facilitated wider application of this test. The sensitivity and specificity of EMA using primate esophagus or umbilical cord as substrate are reported as high as 97%-100% and 98%-99%, respectively[17,18]. IgA deficiency is frequent in CD patients and reported as high as 2%-5%[19]. For this reason, adjunct total IgA determination must be performed together with EMA. EMA is considered as a "gold standard" in the serological diagnosis of CD and antibody titers fall after strict gluten-free diet[18].

In our study, we have found 13 EMA positive results out of 135 patients (9.6%). None of them exhibited characteristic histological features of CD. These patients did not have any other extra-intestinal manifestations of CD.

Post-menopausal status, advanced age and female gender are the established risk factors for low BMD[20]. O'Leary *et al*[21] from Ireland, studied 371 female subjects attending for bone densitometry, without secondary causes of osteoporosis. Two of 115 (1.7%) female subjects with normal bone density and five of 256 (1.9%) female subjects with sub-normal bone density were positive for EMA.

In our study, EMA positive patients had significantly lower age (48.9 ± 4.3 *vs* 59.2 ± 6.2, $P < 0.05$), higher ratio of male gender (61.5% *vs* 4.9%, $P < 0.01$) and pre-menopausal status (8.7% *vs* 1.3%, $P < 0.01$). These patients have lower risk factors for low BMD. In this sub-group (pre-menopausal, younger patients or males), serological screening led to higher frequency of EMA positive patients.

Our findings also indicated that serum parathormone levels were higher, however, 25(OH) vitamin D and serum Ca levels were lower in the EMA positive group than the EMA negative group (Table 2). These findings were also reported in another study[10]. Armagan *et al*[10] studied 89 premenopausal women with idiopathic osteoporosis. Of the 89 patients evaluated, 17 were found to have positive IgA AGA tests (19%) and 9 were found to be positive for EMA (10.11%). They also reported lower levels of 25(OH) vitamin D and serum Ca in EMA positive patients. However, the main limitation of this study is the lack of endoscopic and histologic evaluation of EMA positive patients.

Chronic gastrointestinal diseases can affect bone remodeling by altering both systemic and local regulatory factors, which means that bone loss can be induced by Ca and phosphate alterations, hormones, and local factors such as growth factors and cytokines. Although decreased bone mass is clearly documented in celiac patients, the underlying pathological mechanisms are still controversial. It seems that at least two main mechanisms should be considered. The first is due to intestinal malabsorption, which can lead to not only Ca and vitamin D deficiency, but also to general malnutrition and a reduced BMI; the second is related to the presence of inflammation[5]. Absence of gastrointestinal and other symptoms of CD is not a prerequisite for the development of bone related complications. Malabsorption and inflammation may not be the sole factors for decreased BMD in CD. Sugai *et al*[22] reported increased prevalence of anti-bone antibodies (51.5%) in CD and these antibodies significantly decreased after gluten-free diet. Thus, biochemical abnormalities, which were observed in our study in EMA positive patients, might be a consequence of pathogenetic mechanisms other than malabsorption in CD.

Controversial results obtained from previous studies might be the result of the study cohort. Most of the studies on post-menopausal women failed to find an increased prevalence of CD in osteoporotic patients[9,14]. However, premenopausal women with low BMD have an increased seroprevalence of EMA[10]. The results should also be evaluated according to the baseline prevalence of CD in the population studied. Such as in Ireland, where the prevalence of CD is high[23], the prevalence of CD among osteoporotic women and controls might be similar[21].

Predominance of older, postmenopausal patients in our study might affect our EMA seroprevalence. However, postmenopausal patients might also have silent CD and this might augment the degree of osteoporosis. For this reason, we have not selected a premenopausal patient cohort in this study.

Prevalence of CD in Turkey is reported in a recent study on school-age children. Although adult prevalence is lacking, prevalence of biopsy-proven CD in Turkey is 1/115 in children[24]. This prevalence approximates the prevalence in many European and North American countries[16]. For this reason, the increased EMA seroprevalence in our study (9.6%) is not augmented by the background general prevalence of the disease.

In EMA positive patients, z-scores but not t-scores were lower than EMA negative patients (Table 1). These two scores are expressed as the number of standard deviations by which BMD deviates from the mean value. The t-score is expressed in relation to the mean value in subjects aged 30 years and the z-score to the mean age-matched value. At the age of 30, the z-scores are close to the t-scores. The t-score is an absolute measure, and as BMD decreases with age, an increasing proportion of subjects will fall below a t-score of -2.5. For this reason, z-score might be more sensitive for detecting changes related to secondary (not post-menopausal bone loss related to age) causes of bone loss, such as CD.

In conclusion, we have detected higher EMA seroprevalence in patients with idiopathic low BMD than the general population. EMA positive patients are younger and of premenopausal status or male gender predominates. These patients have low serum values of Ca, 25(OH) vitamin D, BMD (z-scores) and higher serum PTH. The screening of CD in idiopathic osteoporosis should be restricted to patients without classical risk factors for osteoporosis (advanced age, post-menopausal). A novel finding in our study is that z-scores are more accurate in determining risky groups for CD. However, larger studies are needed for clarifying this issue.

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RAPID COMMUNICATION

H pylori infection in patients with Behcet's disease

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Abstract

AIM: To evaluate endoscopic findings and the prevalence of *H pylori* in patients with Behcet's disease (BD) who have upper gastrointestinal symptoms.

METHODS: The patients with BD diagnosed according to the International Study Group and followed up in the Department of Dermatology and other related departments and who had any upper gastrointestinal complaints, were included in this study. Forty-five patients with BD and 40 patients in the control group were evaluated by upper gastrointestinal endoscopy and two biopsied specimens were taken during endoscopy for *H pylori*. A two-week triple therapy for *H pylori* eradication was administered to *H pylori* positive patients. Two months after the treatment, the patients were evaluated by urea-breath test for eradication control.

RESULTS: Patients with BD had a mean age of 36.2 ± 11.4 years (18-67 years). The mean follow-up time was 35 ± 14 mo (16-84 mo). Aphthous or deep ulcer in esophagus, stomach and duodenum had never been confirmed by endoscopic examination. Most gastric lesions were gastric erosion (40%) and the most duodenal lesions were duodenitis (17.5%) in two groups. *H pylori* was positive in 33 patients (73.3%) with BD. The two-week triple eradication therapy was successful in 75% of the patients. There was no difference between the groups in respect to prevalence of *H pylori* (73.3% *vs* 75%, $P > 0.05$), and eradication rate (75% *vs* 70%, $P > 0.05$).

CONCLUSION: Endoscopic findings, eradication rate and prevalence of *H pylori* were similar in patients with BD and control group.

Key words: *H pylori*; Behcet's disease; Vasculitis; endoscopic findings; aphthous ulcer

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INTRODUCTION

Behcet's disease (BD), which was first described by Hulusi Behcet in 1937[1], is a multisystemic, chronic, relapsing vasculitis of unknown origin that affects nearly all organs and systems of patients. Involvement of the gastrointestinal system is called Entero-Behcet's disease. The frequency of gastrointestinal involvement varies among countries, with a lower frequency in Turkey (2.8%-5%), India (5.2%), and Israel (0%); a moderate frequency in France (14%), England (14%), Kuwait, and the United States (21%); and the highest frequency in Scotland (50%) and Japan (50%-60%)[2]. The most frequent extra-oral sites of gastrointestinal involvement are the ileocecal region and the colon. Compared to the other parts of the gastrointestinal tract, the gastric mucosa appears to be the least frequently involved segment of the gastrointestinal tract. Endoscopic findings are aphthous ulcers, esophagitis, fistulae and stricture in esophagus, aphthous ulcers and erosions in stomach, aphthous ulcers, erosions and bulbitis in duodenum. The most frequent gastric lesions of BD are aphthous ulcers. The most common symptoms are abdominal pain that can be colicky, nausea, vomiting, diarrhea with or without blood in the stool, and constipation. The most common hepatic complication of BD is Budd-Chiari syndrome[3]. In the literature a few clinical trials exist in the investigation of upper gastrointestinal tract involvement.

METHODS

Patients and control group

With the approval by the ethics committee of the Ankara Diskapi Education and Research Hospital, Turkey, a prospective study was conducted in the Department of Internal Medicineand Department of Dermatology. The patients with BD who were followed up in the

Department of Dermatology were sent to the Department of Internal Medicine to have their gastrointestinal symptoms evaluated. They were diagnosed according to the diagnostic criteria by the International Study Group for BD[1]. Forty-five patients with BD aged over 18 years with gastrointestinal complaints were included in this study and were evaluated by endoscopic examination.

Forty patients aged over 18 years with gastrointestinal complaints were included as control group. Informed consent was obtained from all subjects enrolled in the study.

Gastrointestinal complaints

Gastrointestinal complaints were noted before endoscopy and required signed informed consent. Gastrointestinal symptoms include stomach pain, upper abdominal bloating, upper abdominal dull ache, stomach pain before meals, stomach pain when anxious, vomiting, nausea, belching, acid regurgitation, heartburn, feeling of acidity in stomach and loss of appetite. Additional alarm symptoms such as gastrointestinal bleeding (rectal bleeding or melena), dramatic weight loss (dramatic weight loss was defined as weight loss of over 10% of body weight), anemia, severe dysphagia and abdominal mass, family histories of gastric cancer and previous histories of peptic ulcer were noted.

Exclusion criteria

Exclusion criteria include past histories of upper gastrointestinal surgery, pregnancy, severe concomitant illness, and use of *H pylori* eradication therapy (amoxicillin, clarithromycin, metronidazole and tetracycline). Additional proton pump inhibitors, bismuth, antibiotic, aspirin or other nonsteroid anti-inflammatory drugs were stopped for endoscopic examination in the preceding two weeks.

Endoscopic procedure

All endoscopic procedures for the upper gastrointestinal system were performed under appropriate sedation (lidocaine 10 mg/puff for pharyngeal anesthesia and intravenous midazolam 2.5-7.5 mg for premedication) using the same videoendoscope (Olympus GIF Q240). Endoscopic examination was performed by the same gastroenterologist. During endoscopy, two biopsies were taken from the antrum for rapid urease test (RUT) and histological examination. Patients were diagnosed having Hp infection if either of the two tests were positive. If other lesions were found during endoscopic examination, biopsy will be performed. Histopathologic examination was done by the same pathologist.

Therapeutic protocol for H pylori eradication

A two-week eradication therapy with amoxicillin 1 g, clarithromycin 500 mg and lansoprazole 30 mg was administered twice daily to the patients who were *H pylori* positive by RUT or histopathologic examination. Two months after the eradication therapy, all were evaluated by the urea breath test (UBT) for eradication control.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL) (10.0 software package program). Data were analyzed by definitive statistics (mean ± SD, maximum, minimum, and percentage). Data between groups were compared with independent sample *t* test and Fisher's exact Chi-square test. *P* values less than 0.05 were considered statistically significant.

Table 1 Characteristics of patients with Behcet's disease

Age (yr)	36.2 ± 11.4 (18-67)
Female/male ratio	21/24
Mean duration of the disease (mo)	35 ± 14 (16-84)
HLA B5 (+)	89%
Recurrent oral ulcers	100%
Eye lesions	22%
Genital ulcers	98%
Skin lesions	13%
Arthralgia	42%
Deep vein thrombosis	0%
Positive pathergy test	98%

RESULTS

Forty-five patients (24 male, 21 female) with BD and 40 patients (21 male, 19 female) of the control group underwent endoscopic examination. General characteristics of the patients with BD are shown in Table 1. The mean age of the patients with BD was 36.2 ± 11.4 years (range, 18-67 years) and that of the control group was 34.8 ± 12.2 years (range, 20-63 years). The mean follow-up time of BD was 35 ± 14 mo (range, 16-84 mo).

Gastrointestinal complaints of the two groups were similar. The major symptom was stomach pain. Alarm symptoms such as gastrointestinal bleeding, dramatic weight loss, anemia, severe dysphagia and abdominal mass, family histories of gastric cancer, and previous histories of peptic ulcer were not noted in all patients. No patient had a past history of upper gastrointestinal surgery.

The endoscopic findings were also similar between the two groups. Esophageal lesions such as ulceration, ulcer, fistula, luminal stricture, pseudomembrane esophagitis, and "downhill" or classical esophageal varices were not confirmed in the two groups, nor were the gastric lesions such as ulceration and ulcer, but as superficial gastritis, erosive gastritis and gastric erosion. The most common endoscopic gastric lesion was gastric erosion (40.1% in the patients with BD and 40% in control group). Erosive gastritis was the second most frequent after gastric erosion. Duodenal lesions, ulceration and ulcer were not found. On the other hand, duodenitis (17.7% in the patients with BD and 17.5% in control group) was the most common endoscopic duodenal lesion in both groups. Aphthous or deep ulcer in esophagus, stomach and duodenum has not been confirmed by endoscopic examination in this study. The endoscopic findings in patients with and without *H pylori* are summarized in Table 2.

H pylori was positive in 33 (73.3%) patients with BD and 30 (75%) patients in the control group by RUT or histopathologic examination. Those patients were treated by a two-week eradication therapy. Two months after the eradication therapy, the patients were evaluated by UBT for eradication control. *H pylori* was negative in 25 of 33 with BD and 21 of 30 in the control group. The two-week

Table 2 Endoscopic findings in patients with and without *H pylori* infection

		Patients with BD *n*/%		Control group *n*/%	
		Hp (+)	*Hp* (-)	*Hp* (+)	*Hp* (-)
Esophageal lesions[1]		-	-	-	-
	Gastric ulcer	-	-	-	-
Gastric	Superficial gastritis	8/17.7	2/4.4	8/20	2/5
lesions	Erosive gastritis	9/20	2/4.4	8/20	2/5
	Gastric erosion	14/31.2	4/8.9	12/30	4/10
Duodenal lesions	Duodenal ulcer	-	-	-	-
	Duodenitis	7/15.5	1/2.2	6/15	1/2.5
Normal		2/4.4	4/8.9	2/5	2/5
Total		33/73.3	12 26.7	30/75	10/25

BD: Behcet's disease; *Hp*: *H pylori;* [1]Esophageal lesions; ulceration or ulcer, fistula, luminal stricture, pseudomembrane esophagitis, esophageal varices.

triple eradication therapy was successful in 75% in the patients with BD and 70% in the control group. There was no difference between the groups in respect to prevalence of *H pylori* (73.3% *vs* 75%, $P > 0.05$) and eradication rate (75% *vs* 70%, $P > 0.05$).

DISCUSSION

Compared to other parts of the gastrointestinal tract, the gastric mucosa appears to be the least frequently involved segment. Cases of a Dieulafoy's ulcer[4] and a gastric non-Hodgkin's lymphoma associated with BD[5] have been described. Aphthous ulcers can occur in the duodenum. In two large autopsy series, a total of six patients with BD were found to have duodenal ulcers[6,7]. Two cases of duodenal involvement have been reported[8,9]. On the contrary, the prevalence of combined gastric ulcers (3 of 28) or duodenal ulcers (3 of 28), was significantly higher in Chinese patients compared with previous reports[10]. In our study, aphthous or deep ulcer in esophagus, stomach and duodenum was never been confirmed by endoscopic examination. The most common endoscopic gastric lesion was gastric erosion in the two groups.

The patients who had past histories of upper gastrointestinal surgery and who used *H pylori* eradication therapy were excluded from our study and the patients who were included stopped taking proton pump inhibitors, bismuth, antibiotic, aspirin or other nonsteroidal anti-inflammatory drugs for the endoscopic examination in the preceding two weeks, ulcer can not be confirmed by endoscopic examination. In addition, none of the patients had past histories of peptic ulcer and gastrointestinal bleeding, abdominal mass, and family histories of gastric cancer.

Two studies assessing the frequency of *H pylori* infection in Turkish patients with BD have been reported[11,12]. In the first study, urease positivity rate was 65% in 34 cases of BD, being not different from that of a control group[11]. In the second report, a higher prevalence (85%) was noted and the presence of *H pylori* correlated with the disease activity was manifested as the presence of gastrointestinal complaints or endoscopic findings[12]. In a recent report, only one of 28 patients showed evidence of *H pylori* infection[10]. In our study, prevalence of *H pylori* was 73%, which was the same with the control group.

A study[13] reported that eradication rate with a one-week eradication therapy (amoxicillin 1 g, clarithromycin 500 mg and lansoprazole 30 mg take twice daily) was 65%. In our study, the eradication rate with a two-week eradication therapy for *H pylori* was 75% in the patients with BD, which was similar to that in the control group.

In conclusion, endoscopic findings, eradication rate and prevalence of *H pylori* are similar in patients with BD and patients in the control group.

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RAPID COMMUNICATION

Prognostic value of DNA alterations on chromosome 17p13.2 for intrahepatic cholangiocarcinoma

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Abstract

AIM: To characterize and evaluate DNA alterations among intrahepatic cholangiocarcinoma (ICC) patients.

METHODS: DNA from tumor and corresponding normal tissues of 52 patients was amplified with 33 arbitrary primers. The DNA fragment that alters most frequently in ICC was cloned, sequenced, and identified by comparison with known nucleotide sequences in the genome database (www.ncbi.nlm.nih.gov). The DNA copy numbers of the allelic alterations in cholangiocarcinoma were determined by quantitative real-time PCR and interpreted as allelic loss or DNA amplification by comparison with the reference gene. Associations between allelic imbalance and clinicopathological parameters of ICC patients were evaluated by χ^2-test. The Kaplan-Meier method was used to analyze survival rates.

RESULTS: From 33 primers, an altered DNA fragment (518 bp) amplified from BC17 random primer was found frequently in the tumors analyzed and mapped to chromosome 17p13.2. Sixteen of 52 (31%) cases showed DNA amplification, while 7 (13%) showed allelic loss. Interestingly, DNA amplification on chromosome 17p13.2 was associated with a good prognosis, median survival time (wk) of amp *vs* no amp was 44.14 *vs* 24.14, $P = 0.002$; whereas allelic loss of this DNA sequence corresponded with a poor prognosis, median survival time (wk) of loss *vs* no loss was 18.00 *vs* 28.71, $P = 0.019$). Moreover, Kaplan-Meier curves comparing the DNA alterations with survival depicted highly significant separation that the median survival time equal to DNA amplification, allelic loss, and normal was 44.14 wk, 18.00 wk, and 24.29 wk, respectively ($P = 0.005$).

CONCLUSION: Alterations in the DNA sequence on chromosome 17p13.2 may be involved in cholangiocarcinogenesis, and could be used as a prognostic marker in the treatment of ICC patients.

Key words: Intrahepatic cholangiocarcinoma; 17p13.2; Allelic imbalance; Prognosis

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is predominantly found in Thai patients with bile duct cancer[1,2] and its incidence is much higher in the northeastern region of Thailand, where it is closely associated with liver-fluke infestation[3-7] and the consumption of carcinogen-contaminated daily foods[8]. Data from the World Health Organization (WHO) database, and national registries worldwide, show an increase in ICC-related mortality[9,10].

Recent investigations into the underlying molecular mechanisms involved in cholangiocarcinogenesis and tumor growth have contributed greatly to understanding the disease. Significant progress has been made over the past decade in defining molecular alterations associated with cholangiocarcinoma (CCA). Alterations in *p53*[11-13] and *p16INK4α*[12] are frequently detected in CCA and likely contribute to oncogenesis in the biliary tract. Other alterations that seem to occur early in cholangiocarcinogenesis include over-expression of the receptor tyrosine kinases (RTKs), ErbB-2, and Met[14] as well as the up-regulation of COX-2[15]. Likewise, WISP1v expression has been found to be associated with lymphatic and perineural spread of CCA and poor clinical outcome[16]. Human telomerase reverse transcriptase (hTERT) has also been detected in a high percentage of analyzed cases of ICC, irrespective of tumor grade and subtype, as well as heterogeneously in dysplastic lesions,

suggesting that acquired hTERT activity may reflect an early stage leading to CCA development[17]. However, the clinical value of established survival predictors seems limited, since they fail to reliably predict post-resection survival.

With regards to genome scanning method, arbitrarily primed polymerase chain reaction (AP-PCR)[18], also called random amplified polymorphic DNA (RAPD)[19], has enormously wide application, since it can be used to study virtually any nucleic acid entity, whether previously characterized or not. The semi-quantitative native of AP-PCR has proven a promising technique for identifying novel gene alterations in many human cancers[20].

In this study, we evaluated the DNA alteration as a prognostic factor for survival of ICC patients post-resection. The localization of an altered DNA fragment on chromosome 17p13.2, which significantly predicted overall survival of ICC patients is reported here for the first time. Furthermore, the finding of aberrations in this DNA region, correlating with clinicopathological parameters of patients with ICC, was also demonstrated.

MATERIALS AND METHODS

Subjects and tissue samples

Samples from 52 cases with primary ICC (42 males and 10 females) and corresponding normal tissues were obtained from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, during surgical resection. The median age at diagnosis was 56 (range 26-75) years. This work was approved by the Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (HE471214). Samples were frozen after resection and stored at -80℃ until DNA extraction. None of the patients received radiation or chemotherapy before surgery. All patients were residents of northeast Thailand, where liver-fluke infections are highly endemic. Hematoxylin-eosin stained sample sections from each tumor block were examined microscopically to confirm the presence of > 80% cancer cells. Paired normal tissues from the same patient were used as controls and showed normal histological features.

DNA preparation

Genomic DNA was isolated from fresh cancerous and normal counterpart tissues by proteinase K digestion and salting-out method[21], with some modifications. The cancerous and normal tissues were embedded in optimum cutting temperature (OCT) and cryostat tissue sections were performed (10 μm). The tissue sections were washed out 3 times from OCT with normal saline, then incubated in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 400 mmol/L NaCl, 2 mmol/L EDTA), 200 μL of 10% sodium dodecyl sulfate (SDS) and 10 mg/mL proteinase K at 60℃ for 3 h. The solutions were mixed with 6 mmol/L NaCl, shaken and centrifuged at 10000 × *g* for 10 min at 4℃. DNA was precipitated by absolute ethanol and washed 3 times with 70% ethanol. The DNA pellet obtained was dissolved in TE buffer. DNA concentration was determined by spectrophotometry at A260 nm. The paired DNA was equalized by comparison with PCR product amplified from exon 1 of β-globin, a housekeeping gene (110 bp).

AP-PCR analysis

Thirty-three arbitrary decamer primers: AA14, AB19, AD10, AO5, AO10, AO16, AO19, AP19, AT11, AT17, AU1, AY19, AZ2, BB3, BB13, BC17, BF12, BG4, F2, G14, H8, J3, L1, M7, M19, N20, O15, Q7, S3, S13, U8, Y7, and Y19 (Operon, USA) were used in screening the ICC genome for alterations. DNA isolated from ICC and corresponding normal tissues of the same patients was used as template. AP-PCR was performed in a thermal cycler (GeneAmp 9700, Perkin-Elmer, USA) for 45 cycles based on the method described by Williams *et al*[18]. The total volume of 25 μL contained 100 ng of genomic DNA extracted from carcinoma or corresponding normal tissues, 1× PCR buffer, 200 μmol/L each of dATP, dCTP, dGTP, dTTP, 2.5 mmol/L $MgCl_2$, 20 μmol/L of each random ten-mer primer and 1 unit of Taq DNA polymerase (Pharmacia Biotech, USA). Each cycle consisted of denaturation at 95℃ for 1 min, annealing at 36℃ for 1 min, and extension at 72℃ for 2 min. Generally, we adjusted the working DNA concentration at 20 ng/μL. The AP-PCR products were resolved by electrophoresis on 1.4% agarose gels. The gels were stained with ethidium bromide for photography under UV light. By direct visualization, the amplified DNA fragments exhibited presence/absence, or increase/decrease, in ICC sample intensity when compared case-by-case with the rest of the bands. The DNA content in tumor and normal tissues was normalized using *β-globin* gene amplification with primer sequences 5'ACACAACTGTGTTCACTAGCA 3' (forward primer) and 5'GGTGAACGTGGATGAAGTTG 3' (reverse primer).

Cloning and sequencing of aberrant DNA fragment

The altered band (518 bp) employing the primer BC17 was excised from the 0.8% agarose gels and purified with DNA purifying kit (Nucleospin, Machery-Nagel GmbH & Co. KG, Germany). The eluted DNA was confirmed for purity and quantity on 0.8% agarose gels. The 518 bp fragment was cloned using a TA cloning kit (Invitrogen, USA) following the manufacturer's instructions. Plasmid DNA was isolated from each bacterial colony using a QIAGEN plasmid kit (QIAGEN, USA). Restriction analysis of the recombinant plasmid DNA was carried out by *Eco*R I digestion. The plasmid DNA containing DNA fragment insertion was further nucleotide-sequenced with either forward or reverse M13 primer as the sequencing primer (customized by Macrogen Inc., Korea).

Bioinformatic analysis

The nucleotide sequences obtained from each clone were identified by comparison with known nucleotide sequences in the human genome database (http://www.ncbi.nlm.nih.gov/blastn) *via* BLASTn program.

Real-time quantitative PCR

The 3 loci on chromosomes 2q24, 17p13.2, and 18p11 were identified from the altered band. Specific primers

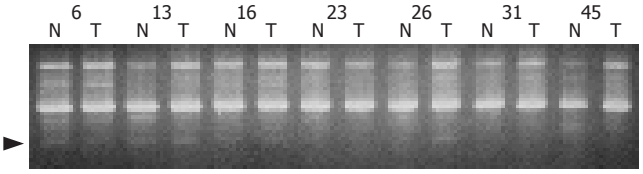

Figure 1 Representative AP-PCR fingerprints of ICC (T) and corresponding normal tissue DNA (N), with primer BC17. Arrowhead indicates the position of altered DNA fragments.

were designed using the GeneFisher program (http://www.genefisher.com), i.e. 5′TGGACAAACAGGCTCCA 3' (forward primer) and 5'CTGGCTTCTCGCGAGA 3' (reverse primer) for DNA alteration on chromosome 2q24, 5'CATGCCAACTGCATCCA 3' (forward primer) and 5′TCTCCAGTGGTTTCCCAA 3' (reverse primer) for detection of allelic imbalance on chromosome 17p13.2 and 5'GAGTTGGACCTTTCCAGA 3' (forward primer) and 5′TGCTTGCACAGATGTGA 3' (reverse primer) for determination of DNA alteration on chromosome 18p11. The DNA alterations on these chromosomal loci were detected by real-time PCR using the specific primers (BioService Unit, Thailand). The *β-globin* gene amplified from DNA extracted from cancerous and corresponding normal tissues was normalized as a reference gene. PCR was performed in a total volume of 20 μL in each LightCycler glass capillary, containing 40 ng genomic DNA, 3 mmol/L $MgCl_2$, 5 μmol/L of each primer and 1 × LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostic, Germany). The PCR condition consisted of an initial denaturation step at 95℃ for 15 s, at 67℃-57℃ (step delay 15 cycles, touchdown PCR) for 5 s, and at 72℃ for 20 s. Thermal cycling and fluorescent monitoring were performed using a LightCycler (Roche Applied Science, Germany). The point at which the PCR product is first detected above the fixed threshold, termed the cycle threshold (Ct), was determined for each sample. The relative concentrations were determined employing Ct values, which are equivalent to the cycle number at which the PCR product is first detected above a fixed threshold. The Ct values obtained from the analyzer were then calculated for DNA copy number utilizing the delta-delta-Ct method[22]. Samples were run in duplicate. In this study, a sample with an amplification ⩾ 1.5 fold was interpreted as having DNA amplification, otherwise ⩽ 0.5 was interpreted as having allelic loss, and the rest was interpreted as no aberration[23].

Statistical analysis

Clinicopathological features of patients with ICC, including patient's age at initial diagnosis, gender, histological type, tumor size, lymph node and/or intrahepatic metastasis, were correlated with the alterations in the distinct region. Results were evaluated by χ^2-test. Survival analysis was carried out with patients who were followed up for at least 200 wk, or until death, after surgery. Three patients who died in the post-operative period were excluded and 4 cases were lost to follow-up. Thus, only 45 patients were available for the survival study. Overall survival distributions were calculated by Kaplan-Meier method and analyzed by log-rank test. $P < 0.05$ was considered statistically significant.

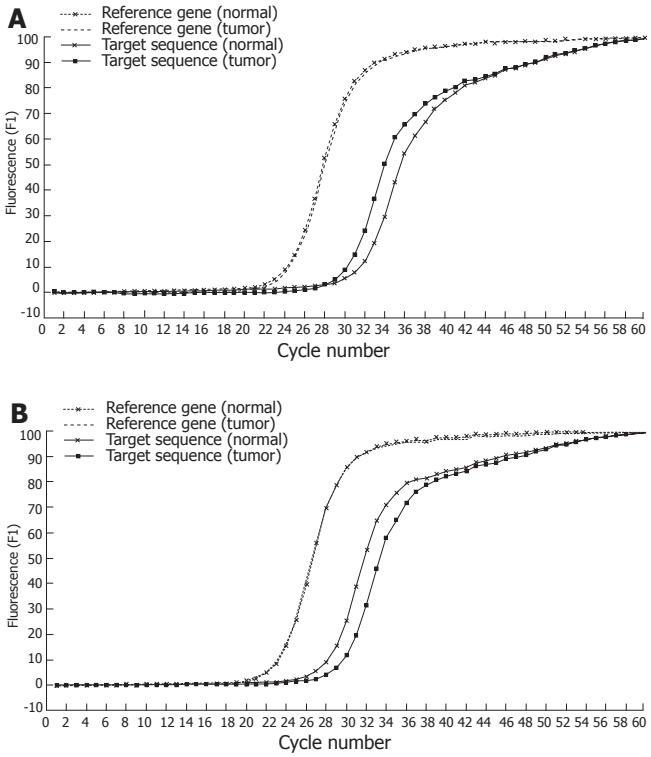

Figure 2 Real-time PCR SYBR Green I fluorescence record versus cycle number of target fragment (518bp) and reference gene (*β-globin*) in tumor DNA and corresponding normal DNA, in case of DNA amplification (**A**) and allelic loss (**B**).

RESULTS

DNA alterations detected by AP-PCR and quantitated by real-time PCR

In this study, all genomic DNAs from 52 Thai patients with ICC were analyzed using 33 different arbitrary primers. Twenty-one of 33 decamer primers revealed genetic aberrations. The highest frequency of altered band (518 bp) was from a DNA fingerprint amplified from the BC17 primer (Figure 1). The discriminative band was cloned, sequenced, and identified by BLASTn program for locus identification. The results revealed 3 different sequences deposited in NCBI, including clone RP11-357L2 on chromosome 2q24 (AC009961.11) and clone RP11-931H21 on chromosome 17p13.2 (AP005900.1) as well as clone RP11-459C13 on chromosome 18p11 (AC067815.5). The changes in DNA copy number at the 3 definite regions were quantitated by real-time PCR with specific primers (Figure 2). Their reliability was confirmed by running amplicons at cycle 33, the mid log phase of fluorescence graph, on 0.8% agarose gel and stained with ethidium bromide (Figure 3). The results indicated that, of all 52 patients with ICC, aberrations in the DNA fragment on chromosome 2q24 were observed in 9 cases (18%), including 6 (12%) with DNA amplification and 3 (6%) with allelic loss; 23 cases (44%) had allelic imbalance on chromosome 17p13.2, of these, 16 (31%) showed DNA amplification and 7 (13%) allelic loss; 27 cases (52%) presented DNA alteration on chromosome 18p11, 25

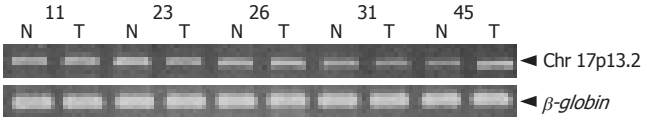

Figure 3 Changes in DNA copy number of ICC (T) and corresponding normal tissue DNA (N) compared with reference gene (*β-globin*). Case 11 showed no change. Cases 23 and 31 showed loss of DNA copy number, whereas cases 26 and 45 showed DNA amplification at chromosome 17p13.2.

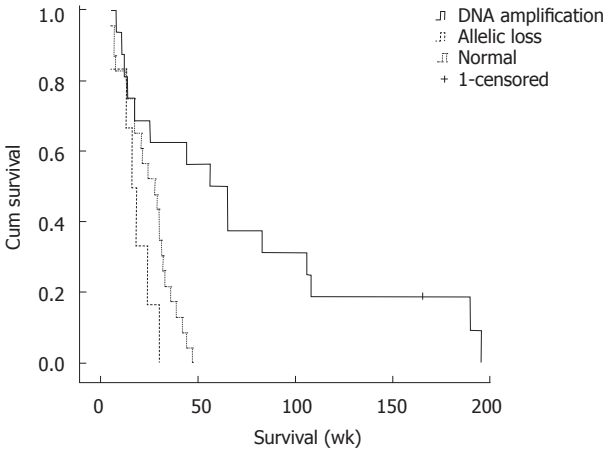

Figure 4 Kaplan-Meier estimated survival rates according to the altered DNA fragment on chromosome 17p13.2.

(48%) had DNA amplification and 2 (4%) allelic loss.

Association between allelic imbalance and patient clinicopathological parameters

Statistical analysis showed that DNA amplification on 17p13.2 was associated with a good prognosis, median survival time (wk) of amp *vs* no amp was 44.14 *vs* 24.14 ($P = 0.002$); whereas allelic loss on this chromosomal region corresponded with a poor prognosis, median survival time (wk) of loss *vs* no loss was 18.00 *vs* 28.71 ($P = 0.019$). Moreover, Kaplan-Meier survival curves showed significant correlation with allelic aberrations on chromosome 17p13.2, the median survival time equal to DNA amplification, allelic loss, and normal was 44.14 wk, 18.00 wk, and 24.29 wk, respectively ($P = 0.005$ for all, by log-rank test) (Figure 4). However, the DNA alterations on chromosome 17p13.2 did not correlate with the clinicopathological data (Tables 1 and 2).

Table 1 Clinocopathological parameters and DNA amplification of the target fragment (518 bp) on chromosome 17p13.2 in ICC patients

Parameters	DNA amplification		*P*	OR (95% CI)
	+ *n* (%)	- *n* (%)		
Age (yr)			0.771	
≤ 50	6 (37.5)	10 (62.5)		1.00 (referent)
> 50	12 (33.3)	24 (66.7)		0.83 (0.24-2.84)
Gender			0.69	
Male	14 (33.3)	28 (66.7)		1.00 (referent)
Female	4 (40.0)	6 (60.0)		1.33 (0.32-5.51)
Histological type			0.61	
Well-differentiated	7 (29.2)	17 (70.8)		1.00 (referent)
Moderately differentiated	7 (50.0)	7 (50.0)		2.43 (0.51-12.04)
Poorly differentiated	5 (45.5)	6 (54.5)		2.02 (0.37-11.45)
Tumor size (cm)			0.843	
≤ 7	3 (20.0)	12 (80.0)		1.00 (referent)
> 7	3 (23.1)	10 (76.9)		1.20 (0.20-7.31)
Metastasis			0.368	
Positive	14 (40.0)	21 (60.0)		1.83 (0.41-8.58)
Negative	4 (26.7)	11 (73.3)		1.00 (referent)
Lymph node metastasis			0.197	
Positive	8 (53.3)	7 (46.7)		2.29 (0.54-9.88)
Negative	10 (33.3)	20 (66.7)		1.00 (referent)

Table 2 Clinocopathological parameters and allelic loss of the target fragment (518 bp) on chromosome 17p13.2 in ICC patients

Parameters	Allelic loss		*P*	OR (95% CI)
	+ *n* (%)	- *n* (%)		
Age (yr)			0.456	
≤ 50	3 (18.8)	13 (81.3)		1.00 (referent)
> 50	4 (11.1)	32 (88.9)		0.54 (0.11-2.76)
Gender			0.721	
Male	6 (14.3)	36 (85.7)		1.00 (referent)
Female	1 (10.0)	9 (90.0)		0.67 (0.07-6.26)
Histological type			0.726	
Well-differentiated	6 (25.0)	18 (75.0)		1.00 (referent)
Moderately differentiated	6 (42.85)	8 (57.15)		2.25 (0.45-11.62)
Poorly differentiated	3 (27.27)	8 (72.73)		1.13 (0.17-7.25)
Tumor size (cm)			0.63	
≤ 7	2 (13.3)	13 (86.7)		1.00 (referent)
> 7	1 (7.7)	12 (92.3)		0.54 (0.04-6.77)
Metastasis			0.447	
Positive	5 (14.3)	30 (85.7)		2.33 (0.22-57.93)
Negative	1 (6.7)	14 (93.3)		1.00 (referent)
Lymph node metastasis			0.737	
Positive	2 (13.3)	13 (86.7)		1.38 (0.14-12.27)
Negative	3 (10.0)	27 (90.0)		1.00 (referent)

DISCUSSION

The modified AP-PCR technique is a useful method for screening genetic alterations in various cancers, such as novel gene alteration on chromosome 11q23.2 in Wilms tumor[24], gene amplification on chromosome 10q24.3 in ovarian cancer[25], and DNA amplification on chromosomes 2p25.3 and 7q11.23 in CCA[26]. In addition, amplification of the DNA on chromosome 2p25.3 has been predominantly observed in poorly differentiated tumors of cholangiocarcinoma patients[26].

In this study, we used this AP-PCR technique to detect and characterize genomic instability in primary ICC. According to the delta-delta-Ct method[22], the DNA copy number could be detected by real-time PCR, calculated and compared with an internal control[27,28]. The result revealed allelic imbalance on chromosome 17p13.2 in 44% of ICC patients and was prognostic for these patients. BLAST analysis showed several candidate genes at this chromosomal region, such as *cyb5d2* (cytochrome b5 domain containing 2), *zzef1* (zinc finger, ZZ-type with EF-hand domain 1), *atp2a3* (ATPase, Ca^{++} transporting, ubiquitous), *p2rx1* (purinergic receptor P2X, ligand-

gated ion channel, 1), and *camkk1* (calcium/calmodulin-dependent protein kinase kinase 1, alpha). Of those, variants of camkk1 gene confer susceptibility to lung cancer[29].

In addition, this DNA fragment was located 3.5 Mbp from *p53* (17p13.1), a generally altered tumor-suppressor gene that controls cell proliferation and survival through several coordinated pathways, and this gene mutation plays a key role in the development of several cancers, including CCA[30-32].

Deletion of the 17p13.2 locus has also been connected to breast cancer[33], brain malignancy[34] and gastric adenocarcinoma[35]. Likewise, the allelic imbalance on chromosome 17p13 presented in various malignancies, such as non-small-cell lung cancer (NSCLC)[36], natural killer (NK) cell lymphomas/leukemias[37], and sporadic breast cancer[38], indicating that the aberration imbalance on chromosome 17p13-13.2 might play an important role in cancer development.

In our series of 45 ICCs, Kaplan-Meier survival analysis (Figure 4) showed that the altered DNA sequence on chromosome 17p13.2 affected patient survival (P = 0.005), with DNA amplification patients having longer lives (44 wk) than those with allelic loss in the particular region (18 wk), showing that DNA alterations on chromosome 17p13.2 could serve as prognostic indicators for cholangiocarcinoma.

In keeping with the theory that pathological parameters are not prognostic for ICC patients, age, gender, tumor size, and metastasis all failed to correlate with outcome. Lymph node metastasis is the strongest factor for ICC after surgery[39-41]. Although previous reports suggested that some types of ICC might be less aggressive, even if lymph node metastasis is present[42-44], some ICC tumors might produce lymph node metastasis at an early stage, suggesting that surgery might control lymph node metastasis in selected ICC patients.

In conclusion, aberrations in DNA amplification on chromosome 17p13.2 provide an independent biological predictor of survival for ICC patients. Thus, assessment of this specific region could help identify patients who might benefit from particularly aggressive surgical strategies, and could also be useful for planning adjuvant therapies during follow-up.

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RAPID COMMUNICATION

Significance of platelet activating factor receptor expression in pancreatic tissues of rats with severe acute pancreatitis and effects of BN52021

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Abstract

AIM: To investigate the dynamic changes and significance of platelet activating factor receptor (PAF-R) mRNA and protein in pancreatic tissues of rats with severe acute pancreatitis (SAP) and effects of BN52021 (Ginkgolide B).

METHODS: Wistar male rats were randomly assigned to the negative control group (NC group), SAP model group (SAP group), and BN52051-remedy group (BN group), and each of the groups was divided into 6 subgroups at different time points after operation (1 h, 2 h, 3 h, 6 h, 12 h, and 24 h) (n = 10 in each). PT-PCR and Western blot methods were used to detect PAF-RmRNA and protein expression in pancreatic tissues of rats respectively. Pathological examination of pancreatic tissues was performed and the serum amylase change was detected.

RESULTS: Serum amylase and pathological results showed the that SAP model was successfully prepared, BN52021 was able to decrease serum amylase, and the pathological ratings in BN group at 3 h, 6 h, and 12 h significantly decreased compared with those in the SAP group (8.85 ± 0.39 *vs* 5.95 ± 0.19, 9.15 ± 0.55 *vs* 5.55 ± 0.36, 10.10 ± 0.65 *vs* 6.72 ± 0.30, $P < 0.05$). The result of PAF-mRNA showed dynamic changes in SAP and BN groups, which increased gradually in early stage, reached a peak at 3 h (0.71 ± 0.14 *vs* 0.54 ± 0.14, 0.69 ± 0.13 *vs* 0.59 ± 0.04, $P < 0.05$), and decreased gradually later. There were significant differences at each time point except 1 h and 2 h, when compared with those in the NC group (0.71 ± 0.14 or 0.69 ± 0.13 *vs* 0.47 ± 0.10, 0.38 ± 0.08 or 0.59 ± 0.04 *vs* 0.47 ± 0.09, 0.25 ± 0.07 or 0.29 ± 0.05 *vs* 0.46 ± 0.10, 0.20 ± 0.06 or 0.20 ± 0.04 *vs* 0.43 ± 0.09, $P < 0.05$), whereas there was no significant difference between BN and SAP groups at each time point. The result of PAF-R protein showed that the change of PAF-R protein in the SAP group and the BN group was consistent with that of PAF-R mRNA. There were significant differences at each time point except 1 h, when compared with those in the NC group (0.90 ± 0.02 or 0.80 ± 0.05 *vs* 0.48 ± 0.02, 1.69 ± 0.06 or 1.58 ± 0.02 *vs* 0.48 ± 0.03, 1.12 ± 0.10 or 0.98 ± 0.03 *vs* 0.49 ± 0.09, 1.04 ± 0.14 or 0.87 ± 0.02 *vs* 0.52 ± 0.08, 0.97 ± 0.16 or 0.90 ± 0.05 *vs* 0.49 ± 0.10, $P < 0.05$), whereas there was no significant difference between the BN group and the SAP group.

CONCLUSION: PAF-R plays an important role in occurrence and development of SAP. BN52021 exerts biological effects through competitively inhibiting the binding of increased both PAF and PAF-R expression rather than through decreasing PAF-R expression in pancreatic tissues.

Key words: Acute pancreatitis; Platelet activating factor receptor; BN52021

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INTRODUCTION

Severe acute pancreatitis (SAP) is a serious condition which has an acute onset and a high death rate. So far, as pathogenesis of SAP has not been clarified, there is no clinically effective therapeutic strategy for it. Therefore, research on its pathogenesis and treatment is quite important. In recent years, people are concerned with the significance of the signal transduction pathway of platelet activating factor (PAF) in the pathogenesis. PAF is an endogenous active substance produced by multiple cells, which has extensively biological effects. Through binding with platelet activating factor receptor (PAF-R), PAF may, through G-protein transduction, activate

phospholipase C, phospholipase A_2, adenylate cyclase, tyrosine protein kinase, etc.,leading to occurrence and development of SAP[1,2]. BN52021 (Ginkgolide B) is a terpinoid of gingko extract[3]. It is a potent antagonist of PAF, and may inhibit platelet aggregation, antagonize inflammation and shock, and protect blood vessels of heart and brain[4]. BN52021 has shown significant effects in treatment of experimental SAP[5]. The present study was aimed to investigate the role of PAF-R and BN52021 in occurrence and development of SAP, and its mechanism of action. The dynamic change of PAF-R mRNA and protein in pancreatic tissues of rats with SAP and the effects of BN52021 were detected using PT-PCR and Western blot analysis.

MATERIALS AND METHODS

Main reagents and apparatus

BN52021 and sodium taurocholate were purchased from Sigma (St. Louis, MO, USA), amylase kit from Beijing Kemei Reagent Co. (Beijing, China), trizol and diethyl pyrocarbonate (DEPC) from Invitrogen (Carlsbad, CA, USA), RT kit from MBI Fermentas (Ontario, Canada), DNTPs and RNA enzyme inhibitor from TaKaRa Dalian Co., Ltd. (Dalian, China), DNA Tag enzyme from Promega (Madison, WI, USA), primary antibody of PAF-R rabbit-anti-rat serum and the enhanced chemiluminescence (ECL) system from Santa Cruz Biotechnology (Santa Cruz, CA, USA), secondary antibody of sheep-anti-rabbit from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China), polyvinylidene fluoride (PVDF film) from Millipore Corp. (Bedford, MA, USA), prestained marker from Beijing Tianwei Time Biotechnology Co., Ltd. (Beijing, China), β-actin from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China), DYY-12 electrophoresis system and electric trans-blot SD from Beijing Liuyi Instrument Factory (Beijing, China), Type-2720 PCR apparatus from ABI (Foster, CA, USA), and gel scanning and imaging system and vertical electrophoresis system from Bio-Rad (Hercules, CA, USA).

Experimental animal grouping and model preparation

One hundred and eighty Wistar male rats (Laboratory Animal Center of PLA Academy of Military Medical Sciences, Beijing, China), aged 6-8 wk, and weighing 200-220 g (Grade Ⅱ, Certificate SCXK 2002-001) are used for this study. All rats were maintained in an environment of controlled "temperature (22-25℃)", humidity (55%-58%), and lighting (12 h light/12 h dark), with free access to tap water and regular chow diet. They were randomly assigned to the negative control group (NC group, n = 60), SAP model group (SAP group, n = 60), and BN52051-remedy group (BN group, n = 60), and each of the above groups was divided into 6 subgroups at different time points after operation (1 h, 2 h, 3 h, 6 h, 12 h, and 24 h) (n = 10). SAP model was established based on the method by Aho, *et al*[6]. Wistar male rats were weighed, marked and fasted for 24 h before the operation, with free access to water. The rats were anesthetized with abdominal injection of 0.4% pentobarbital sodium (40 mg/kg), and fixed in dorsal decubitus. A 2-cm cut was made at the center of the upper belly, entering the abdominal cavity to look for rat's duodenum and pancreaticobiliary duct. The hepatic end of the pancreaticobiliary duct was clipped with a non-invasive vascular clip, pancreaticobiliary duct retrograde centesis was performed with a obtuse needle through duodenum seromuscular layer. Then 5% sodium taurocholate (0.1 mL/100 g) was injected in the retrograde direction of pancreaticobiliary duct with a micro-syringe at an injection rate of 0.20 mL/min. After injection of the drug, the port of pancreaticobiliary duct entering duodenum was clipped with a non-invasive vascular clip and observed for 10 min. After confirming there was no active bleeding in the abdominal cavity, we closed the abdomen in two layers, and covered the cutting wound with sterile gauze. In the NC group, we stirred duodenum and touch pancreas several times after opening the abdomen. In the BN group, BN52021 (5 mg/kg: dissolved with DMSO) was injected intravenously 15 min after the operation. In the NC and the SAP groups, the same volume of physiological saline (0.9% NaCl) was injected through a femoral vein.

Collection and storage of samples

Rats in each group were anaesthetized at all time points after the operation (1 h, 2 h, 3 h, 6 h, 12 h, and 24 h), veinous blood was collected from the right atrium after a 10-min water bath at 37℃, and then centrifuged for 10 min at 3000 g/min. The supernatant was placed into a sterilized EP tube, and stored in a refrigerator at -20℃ for determination of serum amylase. Meanwhile, a portion of pancreatic tissues was placed in liquid nitrogen overnight, and frozen in a refrigerator at -80℃. A portion of pancreatic tissues was fixed with 40 g/L neutral buffer formaldehyde, embedded with paraffin wax, cut into slices and examined with HE staining, and pathological observation and scoring were made.

Determination of serum amylase

Serum amylase was detected with fully automatic biochemical apparatus and amylase kit.

Pathological observation and grading of pancreas

Pancreatic tissue samples were observed pathologically and scored (Table 1): randomly select 10 visual fields under a high-power microscope (HE stain, × 400), and conduct grading and scoring as shown in Table 1.

Primers

PAF-R and β-actin primer series are provided by Invitrogen (Carlsbad, CA, USA): PAF-R: F (Forward primer): 5'-CCGCTGTGGATTGTCTATTA-3', R (Reverse primer): 5'-AGGAGG TGATGAAGATGTGG-3' (377 bp)[7]; β-actin: F: 5'-TCC TAGCACCATGAAGATC-3', R: 5'-AAACGCAGCTCAGTAACAG-3' (190 bp)[8].

Determination of PAF-RmRNA expression by RT-PCR method

Total RNA from pancreatic tissue in each group was extracted first. Afterward, intergrity of the total RNA was checked with agarose electrophoresis, its concentration and purity were determined with an UV spectrophotometer, and the concentration of the total sample RNA was calculated. RNA 5 μg and Oligo DT_{15} 1.25 μg were placed

Table 1 Scoring standard of pathological changes in rat pancreatic tissues with SAP

Pathological grading	Pathological change	Scores
Edema	Inter-lobule local edema, widened pleura	1
	Inter-lobule diffuse edema, widened intra-lobule clearance	2
	Increased intra-lobule clearance, alveolus swollen, and separated	3
Inflammatory	White cells < 20/visual field under high-power microscope	1
Cell infiltration	White cells 20-50/visual field under high-power microscope	2
	White cells > 30/visual field under high-power microscope, or micro-abscess occurs	3
Hemorrhage	Parenchymal hemorrhage < 20%	1
	Parenchymal hemorrhage 20%-50%	2
	Parenchymal hemorrhage > 50%	3
Necrosis	Necrosis area < 20%	1
	Necrosis area 20%-50%	2
	Necrosis area > 50%	3

into a water bath at 70℃ for 5 min, rapidly put into ice for 5 min, and centrifuged just for 15 s. 5 × M-MLV reverse transcription buffer 5 mL, DNTPS 0.05 μmol, RNA enzyme inhibitor 40 U, M-MLV and reverse transcriptase 1 μL were added, then diluted to 25 μL with deionized water treated by DEPC. The whole mixture was placed at 42℃ for 60 min. Reverse transcription was conducted and reverse transcriptase was deactivated at 95℃ for 5 min. Then the mixture was placed at 4℃ for 5 min and preserved at -20℃.The PCR reaction system included: reverse transcription product 50 μL:3 μL, DNTPS 0.01 μmol, 10 × PCR buffer 5 μL, $MgCl_2$ (Magnesium Chloride) 0.075 mol, PAF-R specific sense strand and anti-sense strand 50 pmol each, and β-actin sense strand and anti-sense strand 50 pmol each. Reaction conditions were as follows: pre-denaturizing at 94℃ for 3 min, denaturizing at 94℃ for 45 s, annealing at 58℃ for 45 s, and extending at 72℃ for 45 s, 38 cycles in total; extending at 72℃ for 7 min, and preserving at 4℃. The PCR product was included in the PAF-R gene sequence of rat spleen (gi: 470384), which was performed by Beijing Boya Biotechnology Co., Ltd.. The PCR product was analyzed with gel electrophoresis by 2% agarose, EB staining observation, and a gel imaging system scanning. Gray level of PCR product treated by PAF-R and β-actin was analyzed using the Quantity-One Software, and the change of PAF-R mRNA expression was evaluated semi-quantitatively.

Determination of PAF-R protein with Western blot

The prepared total protein was added to the gel-loading buffer in the ratio of 1:2 and was boil in 100℃ water for 5 min. Electrophoresis with a vertical plate was conducted: 25 μL of the sample was added to two parallel gels, one for staining, the other for transferring membrane. The electrophoresis voltage 80 V was used for 10 min for concentration gel and 120 V for 60 min for isolation of gel. The gel and the membrane were placed between 6 filter papers and 2 foam pads, put into the electric trans-blot containing buffer, and then was added into ice-water mixture to pre-cool for 10 min. Trans-blot was switched on for 4 h at a current of 1 mA/cm^2, staining for the trans-blotted gel was performed, and whether the trans-blotting was complete was checked. The labeled PVDF membrane was put in 0.05% Tween-20 buffer (TBST) and closed for 1 h. PAF-R antibody (diluted in 1:200) was added and was placed at 4℃ overnight. Afterward, the PVDF membrane was washed with 0.05% TBST for 3 times and each lasted 5 min. The washed PVDF membrane was added into IgG (diluted in 1:200) that was labeled by horseradish peroxidase and incubatedt at 4℃ overnight. Subsequently, PVDF membrane was immersed in 3 mL mixture of A and B ECL working solution for 1 min and then taken out with forceps and wrapped well with a charged nylon membrane. After exposure in a dark room for imaging, the PVDF membrane was placed in the desorption solvent and shaken at 50℃ for 30 min. After being closed, β-actin primary antibody (diluted in 1:200) was added and preserved at 4℃ overnight. The above procedures were repeated and the IgG (diluted in 1:200) labeled with horseradish peroxidase was added. Finally, ECL coloration and imaging were performed. The image was scanned into computer and was analyzed with the Gel-imaging System. The optical density value and the the ratio of the value of the former and the latter were calculated.

Table 2 Serum amylase level at different time points in each group after operation (n = 10, mean ± SD, U/L)

Groups	Time phase points (h)					
	1	2	3	6	12	24
NC	1835.6 ± 613.2	1491.5 ± 507.0	1530.4 ± 247.0	1400.2 ± 447.5	2153.2 ± 236.3	1337 ± 243.7
SAP	2560.5 ± 121.5[a]	2810.5 ± 147.2[a]	4799.3 ± 107.0[a]	4919.7 ± 139.6[a]	3486.3 ± 181.8[a]	2283 ± 127.0[a]
BN	2214.5 ± 109.1[a]	3331.7 ± 196.4[a]	4185 ± 147.8[a,b]	3784.7 ± 124.1[a,b]	3454 ± 264.1[a]	1360.4 ± 161.4[b]

[a]$P < 0.05$ *vs* NC group; [b]$P < 0.05$ *vs* SAP group.

Statistical analysis

All the experiments were repeated for 3 times and their average values were used as the final value. All values were expressed with mean ± SD. Comparison of the difference between paired group was performed using t test and one-way ANOVA was used to analyze the differences of multiple samples. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS 11.5 statistical software (SPSS, Chicago, IL, USA).

RESULTS

Serum amylase

We showed that the serum amylase in the SAP group and the BN group was more significantly increased at each time phase point than in the NC group ($P < 0.05$); however, in the BN group it became significantly lower at 3 h, 6 h, and 24 h than in the SAP group ($P < 0.05$) (Table 2).

Pathological observation and scoring

The pathological results showed that there was no obvious abnormality in abdominal cavity at each time

Table 3 Scores of pancreatic tissues at different time points in each group after operation (n = 10, mean ± SD)

Groups	Time phase points (h)					
	1	2	3	6	12	24
NC	0.12 ± 0.05	0.11 ± 0.06	0.12 ± 0.05	0.13 ± 0.04	0.12 ± 0.06	0.13 ± 0.05
SAP	4.82 ± 0.35[a]	7.65 ± 0.40[a]	8.85 ± 0.39[a]	9.15 ± 0.55[a]	10.10 ± 0.65[a]	11.75 ± 0.25[a]
BN	5.55 ± 0.30[a]	6.65 ± 0.42[a]	5.95 ± 0.19[a,b]	5.55 ± 0.36[a,b]	6.72 ± 0.30[a,b]	9.95 ± 0.58[a]

[a]P < 0.05 *vs* NC group; [b]P < 0.05 *vs* SAP group.

Table 4 Absorbance values of PAF-R mRNA in pancreatic tissues at various time points in each group after operation (n = 10, mean ± SD)

Groups	Time phase points (h)					
	1	2	3	6	12	24
NC	0.46 ± 0.10	0.48 ± 0.10	0.47 ± 0.10	0.47 ± 0.09	0.46 ± 0.10	0.43 ± 0.09
SAP	0.49 ± 0.09	0.54 ± 0.14	0.71 ± 0.14[a]	0.38 ± 0.08[a]	0.25 ± 0.07[a]	0.20 ± 0.06[a]
BN	0.49 ± 0.05	0.51 ± 0.07	0.69 ± 0.13[a]	0.59 ± 0.04[a]	0.29 ± 0.05[a]	0.20 ± 0.04[a]

[a]P < 0.05 *vs* NC group.

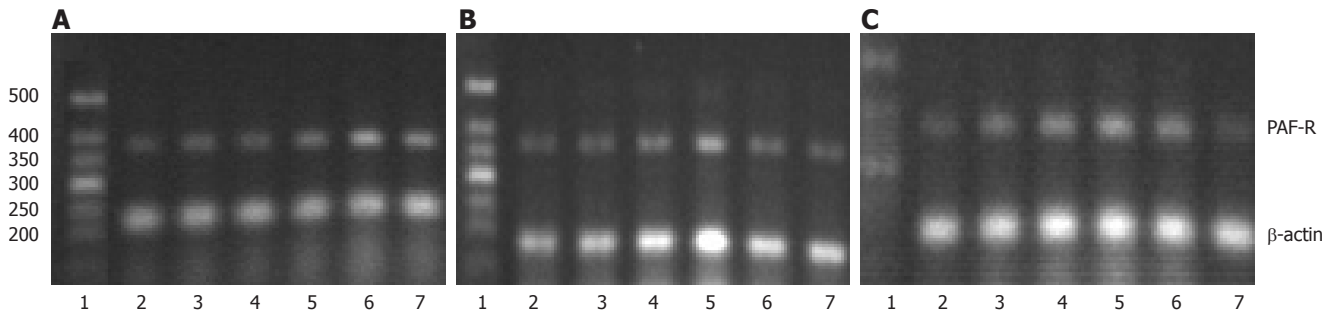

Figure 1 Expression of PAF-R mRNA detected by RT-PCR at various time points in each group after operation. **A**: NC group; **B**: SAP group; **C**: BN group. 1: Marker, 2: 1 h, 3: 2 h, 4: 3 h, 5: 6 h, 6: 12 h, 7: 24 h.

phase point, and the pancreatic structure was normal in the NC group. In the SAP group, hemorrhagic ascites occurred, and necrosis focused in prancreas, a number of saponifying spots were found in mesentery and greater omentum, inflammatory cells infiltrated in pancreatic stroma and glandular lobule, and diffuse bleeding and piecemeal necrosis occurred. With time elapsed later, the pathological changes were exacerbated. In the BN group, the pathological changes were less serious than in the SAP group. The scores in the SAP and the BN groups were significantly higher at each time phase point than in the NC group (P < 0.05); however, the scores in the BN group were significantly lower at 3 h, 6 h, and 24 h than in the SAP group (P < 0.05) (Table 3).

PAF-RmRNA expression in pancreatic tissues and BN52021 effects

PAF-R mRNA expression was changed dynamically in SAP and BN groups. It increased gradually in early stage, reached a peak at 3 h (P < 0.05), and then decreased gradually. There were significant differences at each time point except 1 h and 2 h, when compared with those in NC group (all P < 0.05), whereas there were no significant differences between the BN and SAP groups at each time point (Table 4, Figure 1).

PAF-R protein expression in pancreatic tissues and BN52021 effects

The changes of PAF-R protein in SAP and BN groups were consistent with those of PAF-R mRNA. There were significant differences at each time point except 1h, when compared with those in the NC group (all P < 0.05), whereas there was no significant difference between BN group and SAP group (Table 5, Figure2).

Table 5 Absorbance values of PAF-R protein in pancreatic tissues at various time points in each group after operation (n = 10, mean ± SD)

Groups	Time phase points (h)					
	1	2	3	6	12	24
NC	0.44 ± 0.02	0.48 ± 0.02	0.48 ± 0.03	0.49 ± 0.09	0.52 ± 0.08	0.49 ± 0.10
SAP	0.53 ± 0.03	0.90 ± 0.02[a]	1.69 ± 0.06[a]	1.12 ± 0.10[a]	1.04 ± 0.14[a]	0.97 ± 0.16[a]
BN	0.43 ± 0.06	0.80 ± 0.05[a]	1.58 ± 0.02[a]	0.98 ± 0.03[a]	0.87 ± 0.02[a]	0.90 ± 0.05[a]

[a]P < 0.05 *vs* NC group.

DISCUSSION

At present, the four generally accepted mechanisms associated with SAP are theories of self-digestion of pancreas by pancreatic enzyme, microcirculation disturbance of pancreas, over-activation of leucocytes, and migration of intestinal bacteria in pancreas[9]. The four mechanisms may interact with each other. All the four theories concentrate on the important roles of cytokine and inflammatory mediator in the pathogenesis of SAP and the influence of PAF on SAP has been proved[10]; however, the actual mechanism still needs further investigations.

To date, PAF is the strongest platelet aggregation

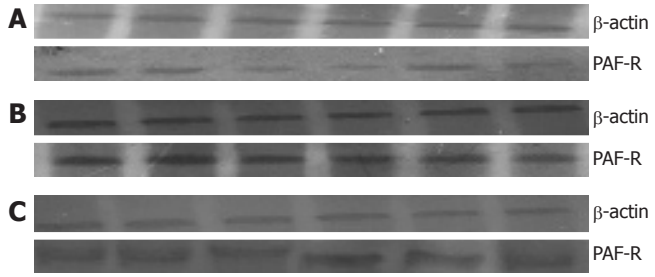

Figure 2 Expression of PAF-R protein at various time points in each group after operation. **A**: NCgroup; **B**: SAP group; **C**: BN group. 1: 1 h, 2: 2 h, 3: 3 h, 4: 6 h, 5: 12 h, 6: 24 h.

agonist and active vascular lipid transmitter known[11,12]. It may activate and aggregate neutrophils, and release free oxygen radicals. It may act on endothelial cells of blood vessel and increase permeability of micro-blood vessels significantly, the effect of which is 1000-10 000 times as great as that of histamine[13]. It may also exert effects through a lot of inflammatory mediators, such as amines (histamine, 5-HT, catecholamine, *etc.*), arachidonic acids metabolites, as well as other activated fluid and cellular substances (free oxygen radicals, lysozyme, cytokines, *etc.*). Current studies have shown that PAF may transmit signals and exert its biological effects through the signal transduction system consisting of PAF-R, G-protein, and membrane effector enzyme[14].

PAF-R was found in the ligand-binding experiment of [^{3}H]-PAF and [^{3}H]-WEB2086, an antagonist of PAF-R[15]. It is a G-protein coupled receptor and it has been proved that PAF-R exists in many cells of human beings and animals, such as platelet, neutrophil, differentiated cell in leukemia, T or B lymphocyte, monocyte, macrophage, Kupffer cell, and smooth muscle cell[16]. Flickinger *et al*[17] found that PAF-R exists in capillaries of rat pancreatic tissues. Our study found that PAF-R also exists in nucleus and cytoplasm of rat pancreatic islets[18]. Therefore, if inflammation occurs in pancreatic tissues, the PAF released from pancreatic cells will increase. A large number of PAF will activate the signal transduction pathway of PAF and PAF-R through bounding with PAF-R in endothelial cells and pancreatic islet cells of pancreatic blood vessel. It will interact with other inflammatory cytokines, resulting in network effects and exacerbating injury to pancreatic tissue. It is important to investigate the dynamic changes of PAF-R in SAP and intervention.

This study showed that dynamic changes occurred in PAF-R mRNA and protein in the SAP model group, with similar trend. They increased gradually in the early stage, reached a peak at 3 h, and then decreased gradually. There were significant differences in the middle and late stages of disease course compared with those in the NC group. This suggested that PAF-R played a role in the pathogenesis of SAP and a significant role in occurrence and development of SAP. At 3 h, infiltration by substantial inflammatory cells can be seen in the pathological slices of pancreatic tissues of rats with SAP, including cells with PAF-R expression, such as leucocyte, macrophage, and monocyte. The results showed that the blood PAF level of SAP increased gradually in the early stage; meanwhile, other inflammatory factors also increased, such as sPLA$_2$ and TNF-α[19]. PLA$_2$ is a significant effector of PAF. PLA$_2$ may decompose phospholipid of cell membrane, release PAF, and form a vicious cycle of the following fashion: PAF synthesis → activation of signal transduction pathway → increased PAF synthesis, to release substantial inflammatory mediators to induce increased expression of PAF-R[20,21]. TNFα may activate NF-κB to induce increased expression of PAF-R in MonoMac-1[22]. A previous study incubated B-lymphocytes with different levels of PAF for 24 h and the results showed PAF-R number substantially increased in such lymphocytes with dose-dependence and without any change of receptor affinity. However, it was reported that, in the early stage, PAF expression decreased in the form of PAF-R mRNA expression in renal mesangial cells when it was incubated, the mechanism of which is still unclear[23].

The pathological results showed that large area necrosis occurred at 6 h in rat pancreatic tissue of SAP. The cells with expression of PAF-R were damaged, and thus PAF-R expression decreased, which may be a key reason for decreased PAF-R expression in the middle and late stages of SAP course. Studies on human monocytes showed that PAF was able to activate PKC. PKC was able to deactivate PAF-R through phosphorylation, leading to decreased PAF-R expression and inhabitation of PAF effects[24]. It was suggested that there was a negative feedback mechanism between deactivated PAF-R and PAF. Low-level endotoxin was able to down-regulate PAF-R expression in human macrophage through inhibiting combination of NF-κB and DNA[12]. Endotoxin produced due to infection in the middle and late stages of SAP course was also able to down-regulate PAF-R expression through NF-κB[25,26].

The expression of PAF-R in rat pancreatic tissues with SAP increased in the early stage and then decreased in the middle and late stages, being consistent with the pathological change of pancreatic tissues. However, there are many influencing factors that need further investigations. It is also suggested that diagnosis and treatment of SAP in the early stage is quite significant, and that further investigation, as well as use of specific PAF-R antagonist in the early stage, is particularly significant. BN52021 is a specific PAF-R antagonist[27,28]. It is able to antagonize binding of PAF and its receptor (PAF-R) competitively, and thus PAF is unable to activate effector enzyme through G-protein transduction to block signal transduction of PAF-R[29]. The results of our early studies showed that Ginkgolide B was able to decrease the PAF level in blood, thus decreasing biological effects of PAF.

The serum amylase and pathological results of this study showed that BN52021 decreased serum amylase and pathological scores of SAP to some extent, thus exerting some therapeutic effects; however, it showed no significant effect for PAF-R expression in SAP course. It is obvious that BN52021 decreases the biological effects of PAF possibly through inhibiting binding of increased PAF with PAF-R with increased expression, other than through decreasing PAF-R expression. It inhibits PAF signal transduction and release and self-activation of pancreatic enzyme, and finally plays a therapeutic role in SAP.

To sum up, many factors induce the increased PAF-R expression in pancreatic tissues in the early stage of SAP, and the increased plasma PAF may bind PAF-R at many sites, which expands its biological effects of PAF, increases inflammatory network effects to a large extent, and exacerbates the injury of pancreatic and non-pancreatic tissues. Regarding treatment of rats with SAP, BN52021 exerts biological effects through competitively inhibiting the binding of increased both PAF and PAF-R expression rather than through decreasing PAF-R expression in pancreatic tissues.

COMMENTS

Background

BN52021 (ginkgolide B) is a specific antagonist to platelet activating factor receptor (PAF-R). In recent years, studies at home and abroad have showed that it has significant physiological activities, such as platelet aggregration inhibition, anti-inflammation, anti-shock, *etc*. BN52021 has significant effects in treatment of animals with severe acute pancreatitis (SAP). But the exact pathogenesis of BN52021 on SAP is unknown. This study was aimed at dynamically investigating the changes and significance of the expression of PAF-R mRNA and its protein in pancreatic tissues and effects of BN52021 in rats with SAP.

Research frontiers

To explore molecule mechanism of BN52021 on SAP.

Innovations and breakthroughs

BN52021 has remarkable curative effect in SAP. But the mechanism of BN52021 needs further studies. This study explored the significance of PAF-R in the molecule mechanism of BN52021 on SAP.

Applications

The results may provide theoretic and experimental evidences for the study and application of BN52021, and new approachs for the treatment of SAP.

Terminology

Platelet activating factor receptor (PAF-R) is a G-protein coupled receptor and it exists in multiple cells of human beings and animals, such as platelet, neutrophil, differentiated cell in leukemia, smooth muscle cell, *etc*. PAF is bound with PAF-R to activate the signal transduction pathway between PAF and PAF-R, and interact with other inflammatory cytokines, forming network effects, and exacerbating injury to various tissues. BN52021, code of ginkgolide B, one of the effective components of Chinese medicine *Ginkgo Biloba* leaf and a strong antagonist against the inflammatory medium of PAF, can not only block the signal transduction of PAF but also decrease the blood content of PAF to exert its biological effects. It has significant physiological activities, such as platelet aggregation inhibition, anti-inflammation, anti-shock, *etc*.

Peer review

In this experimental study, the authors analyzed the effect of BN52021 (Ginkgolide B), a terpinoid of gingko extract, on the severity of acute pancreatitis in rats. The authors tried to find a causal link between the effect of BN52021 on tissue injury and the level of expression of PAF receptor (PAF-R) in pancreatic tissues. The data show that BN52021 attenuates amylase activity in serum as well as tissue damage but does not influence the inflammation-induced PAF-R expression.

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RAPID COMMUNICATION

Comparison scoring model of severe viral hepatitis and model of end stage liver disease for the prognosis of patients with liver failure in China

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Abstract

AIM: To estimate the prognosis of patients with liver failure using a scoring model of severe viral hepatitis (SMSVH) and a model of end stage liver disease (MELD) to provide a scientific basis for clinical decision of treatment.

METHODS: One hundred and twenty patients with liver failure due to severe viral hepatitis were investigated with SMSVH established. Patients with acute, subacute, and chronic liver failure were 40, 46 and 34, respectively. The follow-up time was 6 mo. The survival rates of patients with liver failure in 2 wk, 4 wk, 3 mo and 6 mo were estimated with Kaplan-Meier method. Comparison between SMSVH and MELD was made using ROC statistic analysis.

RESULTS: The survival curves of group A (at low risk, SMSVH score $\leqslant$ 4) and group B (at high risk, SMSVH score $\geqslant$ 5) were significantly different (The 4-wk, 3-mo, 6-mo survival rates were 94.59%, 54.05%, 43.24% in group A, and 51.81%, 20.48%, 12.05% in group B, respectively, $P < 0.001$). The survival curves of group C (SMSVH scores unchanged or increased), group D (SMSVH scores decreased by 1) and group E (SMSVH scores decreased by 2 or more) were significantly different .The survival rates of groups C, D and E were 66.15%, 100%, 100% in 2-wk; 40.0%, 91.18%, 100% in 4-wk; 0%, 58.82%, 80.95% in 3-mo and 0%, 38.24%, 61.90% in 6-mo, respectively, $P < 0.001$). The area under the ROC curve (AUC) of SMSVH scores at baseline and after 2 wk of therapy was significantly higher than that under the ROC curve of MELD scores (0.804 and 0.934 vs 0.689, $P < 0.001$).

CONCLUSION: SMSVH is superior to MELD in the estimation of the prognosis of patients with severe viral hepatitis within 6 mo. SMSVH may be regarded as a criterion for estimation of the efficacy of medical treatment and the decision of clinical treatment.

Key words: Liver failure; Survival analysis; Scoring model

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INTRODUCTION

The prognosis of patients with severe viral hepatitis is a concern of clinicians, patients and their relatives. The factors that may influence the prognosis of patients with severe viral hepatitis are complicated. Many investigators have studied the clinical condition and prognosis of severe viral hepatitis with multiple factor regression analysis, and the results are generally consistent[1-3]. We have previously established a scoring model of severe viral hepatitis (SMSVH) with logistic regression analysis[1]. The survival rate of patients with liver failure in 6 mo was investigated with SMSVH in this study, and the accuracy of SMSVH and model of end stage liver disease (MELD) was compared.

MATERIALS AND METHODS

Subjects

One hundred and twenty patients with liver failure due to severe viral hepatitis admitted to Beijing Youan Hospital in October 2002-January 2004 were enrolled in this study. A diagnostic workup was performed including physical examination, laboratory tests and liver pathology according to the criteria suggested by Chinese Medical Association for Liver Diseases in 2000. In brief, the diagnostic criteria of acute liver failure include significant digestive symptoms, extreme fatigue, hepatic encephalopathy within 2 wk, and prothrombin activity less than 40%. The diagnostic criteria of subacute liver failure include symptoms, physical examination and laboratory test between 15 d and 24 wk.

Table 1 Scoring model of the prognosis of patients with severe viral hepatitis

Variables	Scores			
	0	1	2	3
Clinical type	-	Chronic	Subacute	Acute
HE	-	Grade 1-2	Grade 3	Grade 4-5
PTA (%)	⩾ 80	60-80	40-60	< 40
Serum sodium (mmol/L)	⩾ 135	125-135	120-125	< 120

HE: hepatic encephalopathy; PT: prothrombin time; PTA: prothrombin activity. PTA= (control`PT-8.7) ÷ (patient`s PT-8.7) × 100%.

Table 2 Survival rates of patients in groups A and B

	Group A	Group B	χ^2	*P*
2 wk (N)	97.30% (36/37)	74.70% (62/83)	8.729	0.003
4 wk (N)	94.59% (35/37)	51.81% (43/83)	20.594	< 0.001
3 mo (N)	54.05% (20/37)	20.48% (17/83)	13.525	< 0.001
6 mo (N)	43.24% (16/37)	12.05% (10/83)	14.673	< 0.001

The chronic liver failure diagnostic criteria include a history of chronic liver diseases, clinical manifestations similar to those of subacute liver failure, prothrombin activity lower than 40%, serum total bilirubin greater than 10 times of normal upper limit. Of the 120 subjects, 40 had acute liver failur, 46 had subacute liver failure, and 34 had chronic liver failure. The ratio of males to females was 98/22 and the age ranged from 17 to 74 years, the mean age was 42.5 years. The study was performed in accordance with the Declaration of Helsinki. The study program was explained to the patients and/or their relatives and informed consent was obtained from all patients. The study was approved by the Ethical Committee of Beijing You'an Hospital of Capital Medical University.

Data collection

Blood was drawn from all patients for analysis of prothrombin, albumin, ALT, AST, alkaline phosphatase, gamma glutamyl transferase, bilirubin, cholesterol, createinine, urea nitrogen, serum Na^+, Cl^- and K^+ using OLYMPUS automatic biochemical analyzer. In addition, blood was obtained for markers of hepatitis B or C by ELISA method and hepatitis B DNA by real-time PCR. Complications of liver disease were noted in the patients. The data for blood test were provided by the National Center for Clinical Laboratory.

The scoring model established with 4 independent risk factors is presented in Table 1. The patients were scored with SMSVH at admission and after 2 wk of medical therapy, and divided into group A (at low risk, SMSVH score ⩽ 4) and group B (at high risk, SMSVH score ⩾ 5) according to the cutoff value of the SMSVH score 5 at admission.

The MELD score was calculated using MELD formula[4]: MELDscore = 3.8 × ln (total bilirubin mg/dL) + 11.2 × ln (INR) + 9.6 × ln (creatinine mg/dL) + 6.4 × ln (etiology: coefficient was zero in alcoholic hepatitis patients, or 1 in virus hepatitis). INR = prothrombin time ÷ 12.

Table 3 Survival rates of patients in groups C, D and E

	Group C	Group D	Group E	χ^2	*P*
2 wk (N)	66.15% (43/65)	100% (34/34)	100% (21/21)	22.794	< 0.001
4 wk (N)	40% (26/65)	91.18% (31/34)	100% (21/21)	39.405	< 0.001
3 mo (N)	0% (0/65)	58.82% (20/34)	80.95% (17/21)	66.201	< 0.001
6 mo (N)	0% (0/65)	38.24% (13/34)	61.90% (13/21)	43.512	< 0.001

A case database was established with 6 mo as the follow-up endpoint. The subjects were divided into 3 groups according to the changes in SMSVH scores after 2 wk of medical therapy: group C (SMSVH scores unchanged or increased), group D (SMSVH scores decreased by 1) and group E (SMSVH scores decreased by 2 or more).

Statistical analysis

Statistical analysis was performed using SPSS softaware(version13.0 for Windows). For dichotomous variables, data were analyzed by Fisher's exact test, and chi-square test. For continuous variables, data were evaluated with Student's *t* test. Logistic analysis was used to assess the likelihood of influence of various factors on risk of progressive diseases. Survival curves of patients with liver failure were plotted according to the Kaplan-Meier method. $P < 0.05$ was considered statistically significant. The accuracy of SMSVH and MELD in estimating the prognosis of patients was compared by ROC statistical analysis.

RESULTS

Predicting survival using SMSVH

The 3- and 6-mo survival rate was 54.05% and 43.24%, respectively in group A and 20.48% and 12.05%, respectively in group B ($P < 0.001$, Table 2). The survival curves of the two groups differed significantly ($\chi^2 = 22.858$, $P < 0.001$), and the average survival time of groups A and B was 116.4 d (95% CI: 95.7-137.1) and 53.3 d (95% CI: 40.8-65.8), respectively (Table 3).

Statistical analysis with Kaplan-Meier method showed that the 4-wk survival rate was 40.0% in group C and the 4-wk, 3-and 6-mo survival rate was 91.18%, 58.82% and 38.24%, respectively in group D, and 100%, 80.95% and 61.90%, respectively in group E (Table 3). The survival curves for the 3 groups differed significantly ($\chi^2 = 91.159$, $P < 0.001$). The average survival time of groups C, D and E was 25.8 d (95% CI: 21.6-30.1), 110.4 d (95% CI: 89.1-131.8), and 153.0 d (95% CI: 133.9-172.0), respectively. The survival time of the three groups increased gradually. The 6-mo survival rate of patients with liver failure whose SMSVH scores decreased by 2 or more was 61.90%, significantly higher than that of the patients whose SMSVH scores unchanged or increased after 2 wk of medical therapy ($P < 0.001$). SMSVH score could help estimate the survival time of patients with liver failure within 6 mo and decide clinical treatment and reasonable distribution of medical resources (Figures 1 and 2).

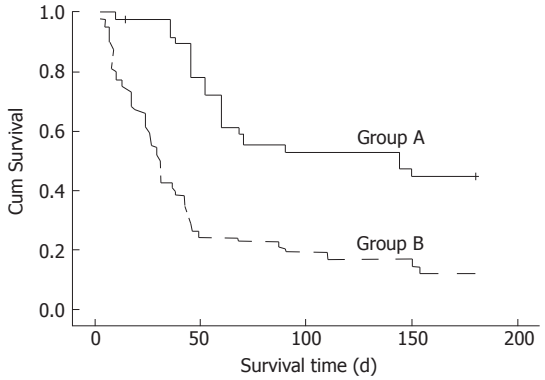

Figure 1 Kaplan-Meier survival curves for group A (at low risk, SMSVH scores ≤ 4) and group B (at high risk, SMSVH scores ≥ 5).

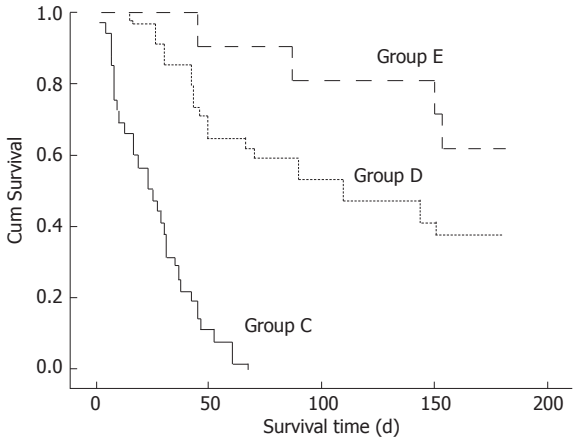

Figure 2 Kaplan-Meier survival curves for group C (SMSVH score unchanged or increased) and group D (SMSVH score decrease by 1) and group E (SMSVH score decreased by 2 or more) after 2 wk of medical therapy.

Comparison of SMSVH and MEL in estimation of survival

The SMSVH scores were analyzed with ROC curve (Figure 3), and the cutoff value for the scores was 5. The sensitivity and specificity were 77.7% and 88.0%, respectively. The area under the ROC curve of SMSVH scores at baseline and after 2 wk of medical therapy was 0.804 (95% CI: 0.708-0.901) and 0.934 (95% CI: 0.883-0.985), respectively. The area under the ROC curve of MELD scores at baseline was 0.689 (95% CI: 0.563-0.814), significantly lower than that under the ROC curve of SMSVH scores at baseline and after 2 wk of medical therapy ($P < 0.001$). The results demonstrated that the SMSVH scores at baseline and after 2 wk of medical therapy were more useful than MELD scores at baseline in estimating the prognosis of patients with liver failure.

DISCUSSION

Liver failure is induced by severe viral hepatitis and a series of complications due to extensive degeneration, necrosis and apoptosis of hepatocytes[2]. Severe viral hepatitis is dangerous, deteriorates rapidly and the case fatality rate is up to 60%-80%[1,5]. The factors that influence the prognosis of patients with severe viral hepatitis are multiple and complicated. Many investigators have studied

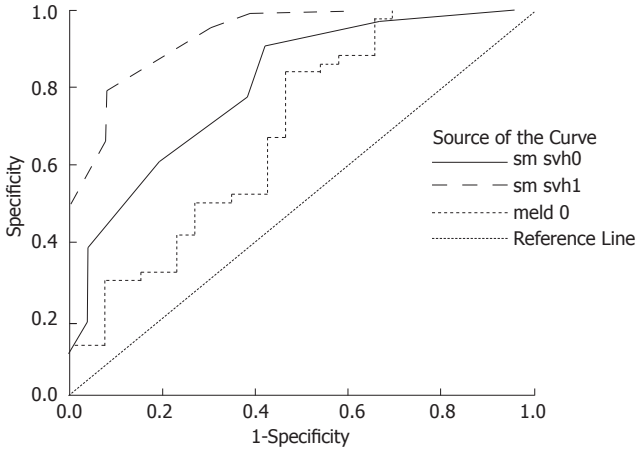

Figure 3 ROC curves for the 6-mo survival rate estimated with MELD and SMSVH at baseline (SMSVH0) and after 2 wk of medical therapy (SMSVH1).

the clinical condition and prognosis of severe hepatitis by multiple factor regression analysis, and the results are generally consistent[1-3]. In our previous study[1], 9 factors including metabolic acidosis, serum sodium, PTA, serum bilirubin, clinical type, hepatic encephalopathy, gastrointestinal bleeding, serum urea nitrogen, and spontaneous peritonitis were found to be associated with the prognosis of severe viral hepatitis, while 4 factors including serum sodium, PTA, clinical type and hepatic encephalopathy could be used as independent risk factors in estimation of the prognosis of patients with severe viral hepatitis, and SMSVH was established according to the four factors. The SMSVH scores of ≥ 5 were taken as the cutoff value in estimating the prognosis of the patients, and its accuracy was 80%[1]. Other factors such as the sex and age of patients, the type and titration of the virus were not significantly related to the prognosis of patients with severe viral hepatitis, which is consistent with the reported results[1,6-8]. A prognostic index consisting of 4 clinical and laboratory features, namely clinical type, low serum sodium and PTA, hepatic encephalopathy, can predict the likelihood of death significantly better than other published models suggesting that disease specific prognostic models and may be of value in patients with severe liver diseases in China.

It was reported that recovery from liver impairment after hepatectomy for hepatocellular carcinoma in cirrhosis starts from postoperative d 3 (POD), increased MELD scores between PODs 3 and 5 may identify patients at risk of liver failure and represents the trigger for beginning intensive treatment or evaluating salvage transplantation[9]. In this study, the survival time of 120 patients with liver failure was investigated with SMSVH, the results showed that SMSVH score of ≥ 5 was the best cutoff value in estimating the prognosis of the patients. The case fatality rate in 3- and 6-mo was 79.52% and 87.95%, respectively, which is similar to the reported results[10]. This study also demonstrated that the 2- and 4-wk survival rate of patients whose SMSVH score had no change or increased after 2 wk of medical therapy was 66.15% and 40.0%, respectively; the 2- and 4-wk, 3- and 6-mo survival rate of patients whose SMSVH score decreased by 1 was 100%,

91.18%, 58.82% and 38.24%, respectively; the 2- and 4-wk, 3- and 6-mo survival rate of patients whose SMSVH score decreased by 2 or more was 100%, 100%, 80.95% and 61.90%, respectively. The survival rates of patients with different SMSVH scores differed were significantly, suggesting that SMSVH is helpful in estimating the survival time of patients with liver failure within 6 mo. Furthermore, in comparison with the the reported survival rates[1], the survival rate of each group in this study was higher, and the reason is that some of the patients in this study administered growth hormone in combination with lactulose. Growth hormones have been demonstrated to be able to increase the survival rate of patients[11].

The Child-Pugh scoring system is the most commonly used model in the assessment of liver reservation function and prognosis of patients with liver cirrhosis[7-8,12]. The classification criteria of Child-Pugh system are strict, but lack of quantification of patients' survival status and the inclusion of objective evaluation parameters such as ascites make the Child-Pugh system easily influenced by clinical treatment. It was reported that that cirrhotics admitted to ICU with > or = 3 failing organ systems have a 90% mortality of 90%. Sequential organ failure assessment (SOFA) and MELD are better predictors than acute physiology and chronic health evaluation (APACHE) Ⅱ or Child-Pugh scores. Salerno *et al*[14] used MELD to estimate the short-term outcome of patients with liver cirrhosis and compared it with the Child-Pugh system, demonstrating that the MELD scoring system is superior to the Child-Pugh scoring system in the estimation of the short-term outcome of patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt (TIPS), but the accuracy of MELD decreases in the long-term estimation[14]. Another study showed that the MELD scoring system is also a reliable method for predicting mortality in patients with AOCH[15].

At present, no easy, objective and effective model is available to estimate the prognosis of patients with severe hepatitis. SMSVH was used in this study and the score of 5 was demonstrated on ROC curve to be the best cutoff value in the estimation of the prognosis of patients with severe hepatitis. The specificity, sensitivity and discrimination power of SMSVH were 88.0%, 77.7% and 0.804 (95% CI: 0.708-0.901), respectively, suggesting that SMSVH is a relatively scientific and objective scoring system in estimation of the prognosis of patients with severe hepatitis, and can be used in clinical practice.

There is no ideal treatment for severe hepatitis so far, and the case fatality rate is up to 80%. Therefore, how to utilize the limited medical resources effectively and reduce unnecessary medical cost is concerned by all levels of government and lots of physicians. SMSVH score may help determine the clinical treatment optimum. SMSVH as a prognostic tool should be considered in predicating the progress of liver failure.

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RAPID COMMUNICATION

Assessment of health-related quality of life in Chinese patients with minimal hepatic encephalopathy

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Abstract

AIM: To evaluate the health-related quality of life (HRQOL) based on the Chinese version of SF-36 and Chronic Liver Disease Questionnaire (CLDQ) in subjects with chronic hepatitis B, liver cirrhosis, including patients with minimal hepatic encephalopathy (MHE).

METHODS: The SF-36 and CLDQ were administered to 160 healthy volunteers, 20 subjects with chronic hepatitis B and 106 patients with cirrhosis (33 cases exhibited MHE). HRQOL scores were compared among the different study groups. The SF-36 includes eight health concepts: physical functioning, role-physical, body pain, general health, vitality, social functioning, role-emotion, and mental health. Six domains of CLDQ were assessed: abdominal symptoms, fatigue, systemic symptoms, activity, emotional function and worry.

RESULTS: Compared with healthy controls (96.9 ± 4.5, 86.6 ± 18.4, 90.1 ± 12.5, 89.0 ± 5.7, 87.5 ± 4.3, 95.8 ± 7.1, 88.5 ± 15.9, 88.7 ± 5.2 in SF-36 and 6.7 ± 0.5, 6.1 ± 0.6, 6.3 ± 0.6, 6.5 ± 0.5, 6.3 ± 0.5, 6.8 ± 0.4 in CLDQ), patients with chronic hepatitis B (86.3 ± 11.0, 68.8 ± 21.3, 78.9 ± 14.4, 60.8 ± 10.5, 70.8 ± 8.6, 76.1 ± 12.6, 50.0 ± 22.9, 72.2 ± 10.6 and 5.5 ± 1.0, 4.5 ± 1.0, 5.2 ± 1.1, 5.3 ± 0.9, 4.8 ± 0.9, 4.9 ± 1.0) and cirrhosis (52.8 ± 17.4, 32.8 ± 27.9, 61.6 ± 18.9, 30.2 ± 18.3, 47.9 ± 20.1, 54.0 ± 19.2, 28.9 ± 26.1, 51.1 ± 17.8 and 4.7 ± 1.2, 3.9 ± 1.2, 4.7 ± 1.2, 4.7 ± 1.3, 4.7 ± 1.0, 4.4 ± 1.1) had lower HRQOL on all scales of the SF-36 and CLDQ ($P < 0.01$ for all). Increasing severity of liver cirrhosis (based on the Child-Pugh score/presence or absence of MHE) was associated with a decrease in most components of SF-36 and CLDQ, especially SF-36.

CONCLUSION: The Chinese version of SF-36 along with CLDQ is a valid and reliable method for testing MHE in patients with liver cirrhosis. Cirrhosis and MHE are associated with decreased HRQOL.

Key words: Minimal hepatic encephalopathy; Liver cirrhosis; Health-related quality of life; Chronic hepatitis B; Chinese

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INTRODUCTION

Minimal hepatic encephalopathy (MHE) is defined as a condition in which patients with liver cirrhosis show several quantifiable neuropsychological defects in the presence of a normal neurological examination[1-3]. In the past two decades, there has been an increasing realization that the traditional assessment of medical outcomes following medical interventions is unsatisfactory. Therefore, health-related quality of life (HRQOL) has gained importance as an outcome measure in clinical and epidemiological studies[4,5].

Generic and specific instruments have been used to measure HRQOL. Generic instruments, such as the widely used Medical Outcomes Study 36-Item Short Form Health Survey (SF-36)[6], provide a global assessment of a given disease and allow comparisons with the general population and other diseases. Generic instruments do not assess disease-specific symptoms, such as pruritus in liver disease, and probably are less responsive to small, yet clinically important, changes. To assess specific aspects of a disease and provide a more responsive instrument for clinical studies, disease-specific instruments have been developed. The disease-specific HRQOL instrument evaluated for different stages of liver diseases is the Chronic Liver Disease Questionnaire (CLDQ)[7].

With the growing interest in monitoring the state of an illness by means of HRQOL instruments, the question arises as to which biological, psychological, and sociodemographic factors may influence HRQOL in

patients with chronic liver diseases. Only a few studies have assessed biological and psychosocial predictors of HRQOL measured by both a generic and a disease-specific HRQOL instrument in a cohort of patients with different causes and severities of liver disease. Therefore, in the present study we used the Chinese version of the SF-36 as a generic instrument and the CLDQ as a disease-specific instrument. The purpose of our study was to identify the most relevant domains of HRQOL impairment in patients with various chronic liver diseases; and to assess predictors of disease severity in patients with various chronic liver diseases, and especially MHE.

MATERIALS AND METHODS

This study was approved by the hospital ethics committee. Education level of all the objects was not less than 9 years.

Patients

All patients gave an informed consent to participation after a full explanation of the study protocol. The diagnosis of chronic hepatitis B and cirrhosis was made according to the criteria revised in 2000 National Symposium in China[8]. The diagnosis of chronic HBV was based on the presence of hepatitis B surface antigen for at least 6 mo, elevated serum alanine aminotransferase (ALT) levels, HBeAg or anti-HBe positive test, and presence or absence of serum HBV DNA as detected by the hybridization method. The diagnosis of cirrhosis was based on clinical finding, laboratory tests, imaging studies, and liver histological examination.

Exclusion criteria were overt hepatic encephalopathy (HE) or a history of overt HE or neurological or mental diseases; alcohol-related liver disease or history of recent (< 4 wk) alcohol intake; history of recent (< 4 wk) use of drugs affecting psychometric performances like sedatives or other psychotropic drugs and antiviral treatment for chronic hepatitis B; a history of liver transplantation or shunt surgery or transjugular intrahepatic portosystemic shunt for portal hypertension; a history of recent (< 4 wk) gastrointestinal bleeding and electrolyte imbalance; severe medical problems such as congestive heart failure, pulmonary disease, cerebrovascular diseases and diabetes mellitus that could influence HRQOL measurement; and inability to perform neuropsychological tests and correct filling of the questionnaires because of poor vision.

The enrollment period extended from December 2003 to February 2006 and the study was conducted at the Renji Hospital and Huadong Hospital. Twenty adult patients with a diagnosis of hepatitis B and 106 patients with cirrhosis confirmed by clinical findings, laboratory tests, imaging studies and liver histological (10 patients) were invited to take part in the study. The Child-Pugh's scores were used to assess the severity of liver cirrhosis.

Comparison groups

One hundred and sixty healthy volunteers who presented for their yearly physical examination and had no specific complaints or illness requiring treatment served as the controls. These individuals also completed the neuropsychological assessment, and SF-36 and CLDQ questionnaires.

Neuropsychological assessment

Number Connection Test-A (NCT-A): This test is a derivative of the Trail Making Test and measures cognitive motor abilities. In the NCT-A, subjects have to connect numbers printed on paper consecutively from 1 to 25, as quickly as possible. Errors are not enumerated, but patients are instructed to return to the preceding correct number and then carry on. The test score is the time the patient needs to perform the test, including the time needed to correct the errors. A low score indicates a good performance.

Symbol digit test (SDT): This is a subtest of the Wechsler Adult Intelligence Scale (WAIS) and measures motor speed and accuracy. The patient is given a list of symbols associated with digits from 1 to 9 and is asked to fill in blanks with numbers that correspond to each symbol. The test score is the total number of correct sequential matching of numbers to symbols in a 90-second interval. A high score indicates a good performance.

After an explanation of each psychometric test, an abbreviated demonstration test was administered to ensure that the patient understood the test properly. Age dependent normal values of NCT-A and DST were determined from the 160 healthy volunteers. Normal values were expressed as mean ± 2 standard deviations[9].

Neurophysiological assessment

Electroencephalogram (EEG): The EEG was recorded while the patient lay comfortably in a quiet room using standardized techniques (Harmanie, Stellate Co., Canada). The EEG was considered abnormal if the background frequency showed alpha rhythm abnormity, slowing (< 8 Hz) or disappearing or obvious asymmetry, or appearance of theta waves when compared with the background frequency of normal adults of the same age. All the records were evaluated manually by a single observer to avoid interobserver error.

Diagnosis of MHE

MHE was diagnosed when patients had an abnormal score on at least one of the two psychometric tests (NCT-A and DST), or if the EEG was abnormal[10-13].

Assessment of daily function

The SF-36 is a reliable and valid instrument to measure all domains of health status by means of 36 items[6]. It measures 4 domains in the area of physical health (Physical Functioning, Role limitation-physical, Bodily Pain, and General Health) and 4 domains in the area of mental health (Role Limitation-Emotional, Vitality, Mental Health, and Social Functioning). Responses to the questions in each domain are added to provide 8 scores between 0 and 100, with higher scores reflecting better HRQOL. The component summary scores of the SF-36 were used as generic measures of HRQOL.

The CLDQ is designed to assess all relevant domains of HRQOL in patients with chronic liver disease and has

been recently validated in Chinese-speaking patients[7]. With 29 items on a 7-point Likert scale ranging from 1 (all the time) to 7 (none of the time), 6 subscale scores (abdominal symptoms, fatigue, systemic symptoms, activity, emotional functioning, worry) and a CLDQ overall score can be calculated. By dividing each domain score, CLDQ results can be presented on a scale of 1-7, with 1 indicating the worst and 7 indicating the best HRQOL. Scores of the subscales of the CLDQ were used as specific measures of HRQOL.

Patients were asked to complete the questionnaires of the Chinese version during regular outpatient visits or during a hospital stay, while healthy volunteers completed the questionnaires during their annual physical examinations. Physicians were trained to give instructions when needed, collect the questionnaires, and record clinical data using standardized forms. The SF-36 and CLDQ scores obtained in patients were compared with the scores in 160 healthy individuals recruited from two hospitals in Shanghai.

Statistical analysis

All data were analyzed using SPSS (version 10.0; SPSS, Inc., Chicago, IL). Data derived from descriptive statistical analysis are presented in the form of percentages for categorical variables and mean ± SD for continuous data. Categorical data were compared using chi-square test, and continuous data, Student's *t* test or, if appropriate, nonparametric tests. Stepwise multiple regression analysis was used to study the influence of independent variables on the CLDQ and SF-36 domains while controlling the effect of other variables. A P value < 0.05 was considered statistically significant.

RESULTS

Education level of all the objects was not less than a 9 years old. All 126 patients and 160 healthy subjects completed the two questionnaires. The demographic and clinical data are shown in Table 1. The various causes of cirrhosis were chronic hepatitis B (70 cases), chronic hepatitis C (6 cases), schistosomiasis (11 cases), autoimmune liver disease including primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis (19 cases).

The results of HRQOL in different study groups are shown in Table 2. Reliability refers to the precision or reproducibility of a measure, and validity refers to an instrument's ability to truly measure what it intends to measure[14]. To assess test reliability, 23 subjects completed the questionnaire twice; the interval between the tests was 2 d. Pearson's item-scale correlation coefficients of SF-36 for the results of the two tests ranged from 0.71 to 0.92, and CLDQ from 0.78 to 0.88 (Table 2). Cronbach's alpha was computed to assess SF-36 and CLDQ internal-consistency reliability[15], for all eight scales of SF-36 and six domains of CLDQ. The alpha value exceeded 0.70 (from 0.71 to 0.94) for all the test results with the exception of social-functioning (0.67) in the SF-36 questionnaire (Table 2).

Thirty-three (31.1%) of the 106 patients with cirrhosis had at least one abnormal test result. These 33 patients were considered to have MHE, whereas the remaining 73 patients were not considered to have MHE.

Table 1 Demographic and clinical data of different study groups

	Control subjects (n = 160)	Chronic hepatitis B (n = 20)	Cirrhosis (n = 106)
Age	44.8 ± 7.1	43.2 ± 6.3	45.4 ± 7.2
Gender M/F	115/45	6/14	73/33
Educational level, yr	11.7 ± 2.5	12.3 ± 2.7	11.3 ± 2.3
Child-Pugh class	-	-	A 28/B 64/C 14
MHE (+/-)	-	-	33/73
ALT (U/L)	25.4 ± 8.0	89.4 ± 32.5	48.2 ± 23.6

Compared with healthy controls, patients with chronic hepatitis B and cirrhosis had lower HRQOL on all scales of the SF-36 and CLDQ questionnaires (P < 0.01 for all). Increasing severity of liver cirrhosis (based on the Child-Pugh score/presence or absence of MHE) was associated with a decrease in most components both SF-36 and CLDQ (Table 2). However, patients with Child-Pugh B and C had similar HRQOL score on both SF-36 and CLDQ (P > 0.05), with the exception of role-physical and vitality on SF-36. There was a significant difference between patients with and without MHE on SF-36 score (P < 0.01), but no significant difference (P > 0.05) on CLDQ score except for abdominal symptoms.

DISCUSSION

In 1946, the World Health Organization (WHO) defined health as "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity"[16]. This definition represents a departure from defining health solely in terms of death and disease. Testa and Simonson defined HRQOL as the "physical, psychological and social domains of health, seen as distinct areas that are influenced by a person's experiences, beliefs, expectations and perceptions"[17]. HRQOL cannot be observed and measured directly, the assessment of HRQOL depended on the subjects response to the questionnaires.

Two basic approaches characterize the measurement of HRQOL: generic instruments and specific instruments[18]. Although developed recently relative to other generic measures, the SF-36 is currently the most widely used health status measure, particularly in the gastroenterology literature. Major advantages of generic instruments include dealing with a variety of areas and use in any population, regardless of the underlying condition. Because generic instruments apply to a variety of populations, they allow for broad comparisons of the relative impact of various health care programs. Generic profiles may, however, be unresponsive to changes in specific conditions. In this study, we chose CLDQ, which is a disease-specific scale developed by Younossi and colleagues to measure HRQOL in patients with chronic liver disease. The CLDQ focuses the respondent on the previous 2 wk period, and are in a question format with seven-category response scales. These scales have been used in several countries,

Table 2 Comparison of health related quality of life in different study groups as assessed by the SF-36 and CLDQ questionnaires

Domains	Control (n = 160)	Chronic hepatitis B (n = 20)	Cirrhosis (n = 106)			Cirrhosis (n = 106)		Pearson item-scale correlations (n = 23)	Internal reliability coefficients (Cronbach's α) (n = 106)
			Child-Pugh A (n = 28)	Child-Pugh B (n = 64)	Child-Pugh C (n = 14)	Non-MHE (n = 73)	MHE (n = 33)		
SF-36									
PF	96.9 ± 4.5	86.3 ± 11.0[a]	63.0 ± 15.8[a]	50.4 ± 17.0[a,b]	43.6 ± 13.4[a,b]	59.9 ± 14.2[a]	37.1 ± 13.1[a,d]	0. 92	0.85
RP	86.6 ± 18.4	68.8 ± 21.3[a]	50.9 ± 24.0[a]	28.9 ± 26.1[a,b]	10.7 ± 18.9[a,b,e]	40.8 ± 27.5[a]	15.2 ± 19.7[a,d]	0.83	0.86
BP	90.1 ± 12.5	78.9 ± 14.4[a]	71.0 ± 13.6[a]	58.0 ± 19.7[a,b]	59.5 ± 18.3[a,b]	66.8 ± 16.5[a]	50.2 ± 18.9[a,d]	0.72	0.80
GH	89.0 ± 5.7	60.8 ± 10.5[a]	46.1 ± 16.5[a]	25.9 ± 15.7[a,b]	18.2 ± 13.1[a,b]	34.8 ± 18.8[a]	20.0 ± 12.3[a,d]	0.78	0.73
VT	87.5 ± 4.3	70.8 ± 8.6[a]	61.1 ± 17.4[a]	45.5 ± 19.4[a,b]	32.1 ± 11.6[a,b,e]	55.1 ± 19.1[a]	31.8 ± 11.0[a,d]	0.82	0.71
SF	95.8 ± 7.1	76.1 ± 12.6[a]	67.1 ± 18.0[a]	50.3 ± 17.1[a,b]	44.4 ± 18.5[a,b]	61.5 ± 16.7[a]	37.4 ± 13.0[a,d]	0.71	0.67
RE	88.5 ± 15.9	50.0 ± 22.9[a]	51.2 ± 21.2[a]	22.4 ± 23.8[a,b]	14.3 ± 17.1[a,b]	35.2 ± 26.0[a]	15.2 ± 20.6[a,d]	0.90	0.86
MH	88.7 ± 5.2	72.2 ± 10.6[a]	64.4 ± 11.6[a]	48.3 ± 17.5[a,b]	37.1 ± 13.1[a,b]	56.0 ± 17.5[a]	40.2 ± 13.2[a,d]	0.82	0.78
CLDQ									
AS	6.7 ± 0.5	5.5 ± 1.0[a]	5.2 ± 1.1[a]	4.5 ± 1.2[a,b]	4.1 ± 1.1[a,b]	4.9 ± 1.2[a]	4.1 ± 1.0[a,d]	0.81	0.87
FA	6.1 ± 0.6	4.5 ± 1.0[a]	4.3 ± 1.2[a]	3.8 ± 1.2[a,c]	3.5 ± 1.1[a,b]	4.0 ± 1.2[a]	3.6 ± 1.2[a]	0.83	0.94
SS	6.3 ± 0.6	5.2 ± 1.1[a]	5.2 ± 1.2[a]	4.7 ± 1.3[a]	4.4 ± 1.2[a,c]	4.9 ± 1.3[a]	4.6 ± 1.1[a]	0.87	0.81
AC	6.5 ± 0.5	5.3 ± 0.9[a]	5.0 ± 1.2[a]	4.7 ± 1.3[a]	4.4 ± 1.3[a]	4.9 ± 1.3[a]	4.5 ± 1.2[a]	0.88	0.75
EF	6.3 ± 0.5	4.8 ± 0.9[a]	4.8 ± 1.0[a]	4.7 ± 1.0[a]	4.6 ± 1.0[a]	4.8 ± 1.0[a]	4.6 ± 1.1[a]	0.82	0.89
WO	6.8 ± 0.4	4.9 ± 1.0[a]	4.8 ± 1.0[a]	4.3 ± 1.1[a,c]	4.2 ± 1.1[a]	4.5 ± 1.1[a]	4.2 ± 1.0[a]	0.78	0.88

PF: physical functioning; RP: role-physical; BP: body pain; GH: general health; VT: vitality; SF: social functioning; RE: role-emotion; MH: mental health; AS: abdominal symptoms; FA: fatigue; SS: systemic symptoms; AC: activity; EF: emotional function; WO: worry. [a]$P < 0.01$ *vs* control group; [b]$P < 0.01$, [c]$P < 0.05$ *vs* Child-Pugh A; [e]$P < 0.05$ *vs* Child-Pugh B; [d]$P < 0.01$ *vs* non-MHE.

and their validity, reproducibility, reliability and sensibility have been confirmed[15,19-22].

Chronic hepatitis B and liver cirrhosis are two of the most common diseases in China. It has been observed that 30%-84% patients with cirrhosis have MHE[23-26]. Patients with MHE have no recognizable clinical symptoms of hepatic encephalopathy but do have mild cognitive and psychomotor deficits[12,27-29]. There is a significant reduction in many of the domains related to HRQOL in patients with MHE[30]. Early diagnosis and treatment of MHE is extremely important, because of the high prevalence of liver diseases in China. We found that HRQOL in patients with chronic liver disease is much lower than that of healthy people. The SF-36 and CLDQ have both recently been proposed as useful additions to the clinical armamentarium when investigating HRQOL. Severely ill individuals can find answering detailed questions quite demanding and, therefore, the SF-36 and CLDQ were a logical and appropriate development for use in such situations. The CLDQ contains questions that may be important in patients with hepatic encephalopathy; however, items important in patients with variceal bleed or ascites are lacking. Both SF-36 and CLDQ are short and easy to administer, and correlate with the severity of liver disease as defined by Child-Pugh classification. However, patients with Child's B and C disease had similar HRQOL scores on both the CLDQ and the SF-36, possibly indicating that neither focuses sufficiently on issues of particular concern to patients with the most significant hepatic decompensation. Our data shows that as the liver disease becomes more severe, patients' HRQOL as measured by the SF-36 and the CLDQ deteriorates. This supports the construct validity of the instrument as a cross sectional measure of HRQOL for chronic liver disease. Although scale scores for CLDQ also deteriorate with disease severity, this was not true for all scales. Only the abdominal symptoms scales of CLDQ did capture this change in MHE patients.

The questionnaires are cheap and convenient, can be used in developing countries, and are complementary to the clinical data. More complicated tests would increase the patients' fatigue and mental burden and affect their performance efficiency[31]. These tests do not take much time to complete. The neuropsychological assessment (SDT and NCT) and questionnaires (SF-36 and CLDQ) could be completed within 60 min in the outpatient clinics and on the patients bedside.

There is only limited data on HRQOL in patients with MHE. Most studies in these patients have used generic instruments, mainly the SF-36. The results obtained in the present study demonstrate that the Physical, Psychological and Social domain scores on both the SF-36 and the CLDQ remain relatively stable in the absence of any therapeutic intervention (based on repeat assessment after a 2 d interval in a proportion of subjects with cirrhosis and in the control group).

It is possible that recruiting patients from just two hospitals may have resulted in a selection bias, although our study sample included patients with a wide spectrum of disease severity (non-cirrhotics to Child's C cirrhosis). However, given the nature of the quality of life concerns, a major difference across populations seems unlikely. Finally, we did not investigate the ability of these questionnaires to detect important changes over time, even if that change is small. We are addressing these issues in ongoing studies on patients with MHE.

In summary, the SF-36 and CLDQ appear to be responsive to clinically meaningful change in the Physical, Psychological and Environmental domains. We conclude that patients with MHE have impairment in their daily functioning as assessed by the SF-36 and the CLDQ. In addition to existing biochemical and physiological parameters, the use of HRQOL instruments (both generic and disease-specific) will enhance our ability to measure

comprehensively the delivery of health care to patients with chronic liver disease[32].

COMMENTS

Background

Owing to the high prevalence in liver cirrhosis, clinicians and researchers in increasing numbers are beginning to recognize minimal hepatic encephalopathy (MHE). The importance of MHE is related to several factors: the neuropsychological defects may result in a reduction in the ability to carry out daily activities, may confer an increased risk for road traffic accidents and accidents at the workplace, it could lead to a deterioration in the quality of life, and it could be a marker for clinical hepatic encephalopathy in the future. The rapid economic development in China has resulted in improvement in the quality of life, and traditional indicators such as mortality and objective clinical parameters are no longer sufficient to assess the effect of illness and the outcome of treatment. There is an increasing demand for a valid and acceptable health-related quality of life (HRQOL) measure for Chinese people.

Research frontiers

Generic and specific instruments are used to measuring HRQOL. Although many HRQOL measures have been developed in Western countries, few are applicable to the people in China. The major obstacle is the cultural and language differences between the populations of China and Western countries. The purpose of this study is to evaluate the HRQOL based on SF-36 and Chronic Liver Disease Questionnaire (CLDQ) in subjects with chronic hepatitis B and liver cirrhosis, especially with regard to the status of MHE. Therefore, we used the Chinese version of the SF-36 as a generic instrument and the CLDQ as a disease-specific instrument in a well-characterized sample of patients.

Related publications

The present study is one of a series of studies we have performed which cover the diagnosis, treatment, HRQOL and follow-up assessment of patients with MHE. We have cited several articles from other investigators that provide additional information related to MHE and HRQOL.

Innovations and breakthroughs

Despite the importance of HRQOL, few workers have assessed quality of life and its determinants in patients with chronic liver disease. We evaluated the impact of chronic liver disease on HRQOL, looked for differences in HRQOL by severity of liver disease, and attempted to identify clinical variables with disproportionate effects on HRQOL. Our findings indicate that the Chinese version of SF-36 along with CLDQ are valid and reliable methods for measuring HRQOL in patients with liver cirrhosis. We observed that cirrhosis and MHE are associated with a decrease in the HRQOL.

Applications

HRQOL is important for measuring the clinical impact of chronic disease. Physiologic measures provide useful information to the clinicians, but are of limited interest to patients since they often correlate poorly with functional capacity and well-being, areas in which patients have the most interest. For example, two patients with similar severity of clinical disease often have dramatically different responses to HRQOL. Furthermore, the questionnaires are cheap and convenient, can be applied in developing countries, and provide information that is complementary to the clinical data.

Terminology

The term "subclinical hepatic encephalopathy" is well recognized but has been replaced by the term "minimal HE" because of the potential misleading consequence of the word "subclinical." Subclinical may suggest a different pathogenesis of the problem and may imply a lack of clinical importance to this diagnosis. We used the term "health-related quality of life" because there are various aspects of life that are generally not considered as "health," including income, freedom, and quality of the environment. Clinicians and researchers focus on HRQOL, although when a patient is ill, almost all aspects of life become health related.

Peer review

The present study is a well performed analysis looking at the quality of life in patients with hepatitis B and cirrhosis, with and without MHE. We recommend that future studies should assess PHES (psychometric hepatic encephalopathy score) to determine and quantify the presence of MHE. PHES is a battery of five tests (number connection test A and B, digit symbol test, line tracing test and serial dotting test) that is currently considered as the "gold standard" for the determination of MHE. The purpose of this recommendation is that the same test is used in all studies. This would allow clinicians and researchers to compare data obtained in different parts of the world, which up to now has not been possible. This would accelerate the progress in the understanding of MHE, a condition which cannot be ignored any longer.

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BASIC RESEARCH

Predicting prognosis of rectal cancer patients with total mesorectal excision using molecular markers

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Abstract

AIM: To explore the prognostic variables in rectal cancer patients undergoing curative total mesorectal excision and the effect of postoperative chemotherapy in advanced rectal cancer.

METHODS: A total of 259 consecutive rectal cancer patients treated with curative total mesorectal excision between 1999 and 2004 were collected. p53, p21, PCNA, and CD44v6 were examined using immunohistochemistry (IHC). The correlation between clinicopathological or molecular variables and clinical outcomes, including local recurrence, metastasis, disease-free survival and overall survival, was analyzed.

RESULTS: The median follow-up was 44 mo. Five-year survival rates and 5-year disease free survival rates were 75.43% and 70.32%, respectively. Multi-analysis revealed TNM staging, preoperative CEA, and CD44v6 level were independent risk factors predicting overall survival or disease free survival. The hazard ratio of peroperative CEA was 2.65 (95% CI 1.4-5) and 3.10 (95% CI 1.37-6.54) for disease free survival and overall survival, respectively. The hazard ratio of CD44v6 was 1.93 (95% CI 1.04-3.61) and 2.21 (95% CI 1.01-4.88) for disease free survival and overall survival, respectively. TNM staging was the only risk factor predicting local recurrence. Postoperative chemotherapy without radiotherapy did not improve patients' outcome.

CONCLUSION: TNM staging, preoperative CEA and CD44v6 were independent prognostic factors for rectal cancer patients with total mesorectal excision. Postoperative chemotherapy may be only used together with radiotherapy for rectal cancer patients.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer death in both males and females. Approximately 35% of colorectal cancers are located in the rectum of patients from Western countries. In China the proportion reached approximately 50%.

New advances such as the standardized surgical technique total mesorectal excision (TME), preoprative or post-oprative radiotherapy and adjuvant chemoradiotherapy have reduced the previously high local recurrence rate and improved overall survival time in rectal cancer patients. Despite these advances, about 40% of patients still die from local or distant recurrence. Hence, new prognostic markers are required to help predict the patients who would benefit from adjuvant treatment.

The knowledge regarding the molecular biology of colorectal cancer has facilitated the study of molecular markers in patients with colorectal cancer. Several tumor associated proteins including p53, p21, p27, cyclin D1, PCNA, CD44, Ki67 may be relevant prognostic markers in rectal cancer. These markers were widely studied in many cancers including colorectal cancer, but the results related to prognosis and implications in colorectal cancer remain controversial, especially in rectal cancer. No single molecular marker has been demonstrated to provide consistent prognostic information yet.

Immunohistochemical (IHC) technique, which is easy, stable with experienced pathologists, and fast with commercially available antibody, is widely used in studies for molecular markers. In this study, the protein expression of p53, p21, PCNA and CD44 was examined with immunohistochemical technique to evaluate their prognostic value in rectal cancer patients undergoing

curative total mesorectal excision (TME).

MATERIALS AND METHODS

Clinicopathological information

A total of 343 rectal cancer patients, who underwent total mesorectal excision in Cancer Hospital of Fudan University from January 1999 to June 2004, were collected retrospectively. The median follow-up time is 44 mo, ranging from 1-90 mo. Twenty-one cases (6.5%) who were lost at the beginning of the surveillance were excluded. Sixty-three patients with simultaneous distant metastases or lesions invading other organs (e.g. bladder, vesicle, prostate, posterior of vagina or urethra) were excluded in this study. All the surgeries were performed by experienced colorectal surgeons. Lateral lymphadenectomy was not performed in our series. A total of 259 patients were available after the screening.

The basic clinicopathological information is presented in Table 1. All cases were histologically confirmed adenocarcinoma and reviewed by two pathologists.

Adjuvant treatment

Adjuvant radiation was not routinely given to stage Ⅱ or stage Ⅲ patients with optimal total mesorectal excision with R0 resection before 2005 in our hospital. Only patients with T4 tumors below peritoneal reflex, which invaded other organs (bladder, prostate, vesicle, vagina, *etc.*) would receive postoperative radiotherapy or chemoradiotherapy. Chemotherapy with 5-Fu based regimenswas given to a part of patients with stage Ⅱ or stage Ⅲ disease and prospective observation was carried out to find out its effect in rectal cancer. None of the patients had received preoperative radiotherapy or chemoradiotherapy.

Immunohistochemistry

Two hundred and fifty-nine formalin fixed paraffin embedded tumor specimens were obtained at the department of pathology in the same hospital. These specimens were cut into 4 μm slides, dewaxed with dimethyl benzene and dehydrated in graded acetone. Tissues previously shown to express the antigen of interest were considered positive controls (i.e. colonic adenocarcinoma for p53, CD44v6, breast carcinoma for p21, normal colon for PCNA), and the primary antibody was replaced by TBS in the negative controls. A minimum of eight sections were examined per case, in which every two slides were used for a single marker.

All the 259 colorectal cancer specimens were collected and specific biological markers were analysed with immunohistochemical procedure, using the enVision two-step visualization technique (DAKO) which was described by Ulrike Kämmerer[1] and Schwandner[2]. The monoclonal antibodies, including anti-p53 (Clone DO-7,code no. M7001, DAKO, dilution, 1/50), anti-p21ras (Clone: NCC-RAS-001,code no. M0637, DAKO, dilution, 1/100), anti-PCNA (Clone PC 10,code no. M 0879,DAKO,dilution 1/300), and anti-CD44 variant 6 (Clone VFF-7,code no. M0130, Antibody Diagnostica,dilution 1/50) were used for immunohistochemical examination.

Table 1 Summary of clinicopathological data (n = 259)

Characteristics	Cases (%)
Gender	
Male	146 (56.4)
Female	113 (43.6)
Age (yr)	
Range	18-80
Median	56
Tumor location	
> 10 cm[1]	62 (23.9)
7-10 cm[1]	115 (44.4)
5-7 cm[1]	82 (31.7)
Mean Max diameter (cm)	4.68
Pathology	
Adenocarcinoma	236 (91.1)
Mucinous aden[3]	18 (6.9)
Signet ring ca[3]	5 (2)
T stage	
T1	18 (7.0)
T2	83 (32.0)
T3	85 (32.8)
T4	73 (28.2)
N stage	
N0	147 (56.8)
N1	62 (23.9)
N2	50 (19.3)
TNM stage (AJCC/UICC)	
Ⅰ	80 (30.9)
Ⅱ	67 (25.9)
Ⅲ	112 (43.2)
Lymphovascular invasion	
Yes	33 (12.7)
No	226 (87.3)
Neural invasion	
Yes	21 (8.1)
No	238 (91.9)
Pre-operative CEA[2]	
Positive	48 (18.5)
Negative	211 (81.5)
Adjuvant therapy	
S	167 (64.5)
S + C	92 (35.5)

[1]Distance of the tumor from anal verge; [2]In our hospital lab, CEA > 10 μg/L is considered positive. S: surgery; C: chemotherapy.

Scoring system and statistics

Immunostained tumor sections were analysed by two experienced pathologists without the knowledge of clinicopathological data. Sections immunostained for p53 and p21 were scored semi-quantitaitvely by scanning the entire section to estimate the percentage of tumor cell nuclear staining, and CD44v6 expression was estimated by the percentage of tumor cell membrane staining. The PCNA staining was expressed as a labeling index (LI) defining the positive nuclei of all the nuclei counted. The median value for the PCNA LI in this tumor series(59.5%,)was used as a cut-off point and tumors were classsified as either less than or greater than the median value.

For statistical analysis, p53 and p21 levels were considered to be positive if over 10% of cancer cells were nuclear immunoreactive; and CD44v6 was defined positive if over 10% of cancer cells were membrane

Table 2 Distribution of stage Ⅱ or Ⅲ patients with or without adjuvant chemotherapy

		Adjuvant chemotherapy (n = 179)				*P*
		No		Yes		
		Cases	%	Cases	%	
N staging	N0	114	68.2	33	35.9	< 0.05
	N1	33	19.8	29	31.5	
	N2	20	12	30	32.4	
T staging	T1-2	90	53.9	11	12	< 0.05
	T3	38	22.7	47	51	
	T4	39	23.4	34	37	
Differentiation	High-Medium	145	86.8	77	83.7	> 0.05
	Low	22	13.2	15	16.3	
Lymphovascular invasion	None	19	11.4	14	15.6	> 0.05
	Yes	148	88.6	78	84.4	
Neural invasion	None	14	8.4	7	7.6	> 0.05
	Yes	154	91.6	85	92.4	

immunoreactive.

Association between these proteins and clinicopathological data, and the univariate analysis between these data and prognosis were both performed by Chi-square test. The overall survival, local recurrence and metastasis rates were calculated using life tables. The multivariate analysis of these proteins and clinicopathological data was made using Cox regression. Significance levels were set at $P < 0.05$.

Follow-up

All patients were followedup every 3 to 6 mo at the Colorectal Cancer Center after surgery by their operative team. Follow-up included a full history and physical examinations including digital rectal examination (DRE) at each session. Chest X-ray, CT or ultrasound of abdomen, and lab tests were performed every 6 mo. And colonoscopy was performed every year for the first three years and then every 2 years. All surviving patients were asked to return to the Colorectal Cancer Center for follow-up for the purpose of this study.

RESULTS

Clinicopathological variables

Forty-eight patients exhibited elevated serum CEA levels. The disease stage, lymphnode metastastic status, lymvascular invasion, neural invasion, histopathology and tumor differentiation were not associated with CEA levels.

Among 179 stage Ⅱ or stage Ⅲ patients including 33 in stage Ⅱ (49.3%) and 59 in stage Ⅲ (52.7%), 92 (51.4%) received 5-Fu based adjuvant chemotherapy,. The detailed clincopathological information for these patientsis presented in Table 2.

Of the 259 rectal carcinomas with anterior resection, 45.6% were p53 postitive (n = 118), 80.7% were p21 positive (n = 209), 49.8% were CD44v6 positive (n = 129), and 61.4% were PCNA positive (n = 159). There was no positive association among these four protein expressions.

The association between these markers and clinicopathological variables were analysed using Chi-square test, including tumor location, histopathological type, TNM staging, invasion depth, lymph node metastasis, neural invasion, lymphovascular invasion and preoperative CEA level. There was no significant difference in the distribution of these proteins and different clinicopathological variables, either (Table 3). But p21 expression was found to have significant association with histopathological type (P = 0.067) and invasion depth (P = 0.052).

Patients' outcome

The median follow-up was 44 mo (range 1-90 mo). Thirty-three patients (12.7%) were dead due to tumor progression. Eleven patients (4.24%) had local recurrence, and 35 patients (13.5%) had distant metastases. The outcome of the patients is shown in Figure 1. Our five-year actual survival rate was 75.43% (Figure 1A), and disease free survival rate was 70.32% (Figure 1B). Overall local recurrence rate was 6.73% (Figure 1C).

In stage Ⅱ and stage Ⅲ locally advanced rectal cancer, our 5-year survival rate was 66.9%, disease free survival rate was 61.1%, and overall local recurrence rate was 8.9%.

Association of clinicopathological variables and immunohistochemistry with recurrence, metastasis and survival

Univariate analysis using Chi-square test as a screening method revealed that possible overall survival related risk factors were histopathological type, TNM staging, invasion depth, lymphnode metastasis, preoperative CEA and CD44v6 levels; possible disease free survival related risk factors were gender, histopathological type, TNM staging, invasion depth, lymph node metastasis and CD44v6 and preoperative CEA levels; possible local recurrence related risk factors were histopathological type, TNM staging, lymphnode metastasis; and possible metastasis related risk factors were TNM staging, invasion depth, lymphnode metastasis and preoperative CEA level (Table 4).

In all the 179 stage Ⅱ or stage Ⅲ patients, adjuvant chemotherapy had negative significant association with overall metastasis and disease free survival. But in stratification for each stage, adjuvant chemotherapy did not have any significance with local recurrence, overall metastasis, disease free survival and overall survival. One reason is that patients with more progressive disease were morelikely to receive adjuvant chemotherapy. For multivariate analysis, these possible risk factors screened in 259 rectal cancer patients by univariate analysis were included in Cox regression model. TNM staging, preoperative CEA, and CD44v6 level were independent risk factors predicting overall survival or disease free survival. The hazard ratio of peroperative CEA was 2.65 (95% CI 1.4-5) and 3.10 (95% CI 1.37-6.54) for disease free survival, and overall survival, respectively. The hazard ratio of CD44v6 was 1.93 (95% CI 1.04-3.61) and 2.21 (95% CI 1.01-4.88). TNM staging was the only risk factor predicting local recurrence.

In 179 stage Ⅱ or stage Ⅲ patients, we added the chemotherapy variable to Cox regression model, the results showed that adjuvant chemotherapy did not improve the overall survival, disease free survival or local recurrence in stage Ⅱ or Ⅲ patients.

Table 3 Association between IHC and clinicopathological data

Characteristics		P53 (*n*)			P21 (*n*)			PCNA (*n*)			CD44 (*n*)		
		+	-	*P*	+	-	*P*	+	-	*P*	+	-	*P*
Tumor location	High	29	33	NS	53	9	NS	36	26	NS	32	30	NS
	Median	53	62		93	22		70	45		63	52	
	Low	36	46		63	19		53	29		34	48	
Pathology	Adeno.	109	127	NS	191	45	< 0.05	144	92	NS	117	119	NS
	Muci	8	10		16	2		13	5		11	7	
	Signet	1	4		2	3		2	3		1	4	
TNM stage	Ⅰ	34	46	NS	59	21	NS	47	33	NS	37	43	NS
	Ⅱ	30	37		58	9		42	25		33	34	
	Ⅲ	54	58		92	20		70	42		59	53	
Invasion depth	T1-2	46	55	NS	74	27	0.052	63	38	NS	46	55	NS
	T3	36	49		72	13		55	30		43	42	
	T4	36	37		63	10		41	32		40	33	
Lymphnode meta.	N0	64	83	NS	117	30	NS	89	58	NS	70	77	NS
	N1-2	54	58		92	20		70	42		59	53	
Lymph-vascular invasion	+	17	16	NS	25	8	NS	21	12	NS	19	14	NS
	-	101	125		184	42		138	88		110	116	
Neural invasion	+	11	10	NS	18	3	NS	15	6	NS	11	10	NS
	-	107	131		191	47		44	94		118	120	
Preoperative CEA	+	18	30	NS	41	7	NS	30	18	NS	109	102	NS
	-	100	111		168	42		129	82		20	28	

Adeno: adenocarcinoma; Muci: mucinous adenocarcinoma; Signet: signet ring adenocarcinoma; Meta: metastasis; NS: not significant.

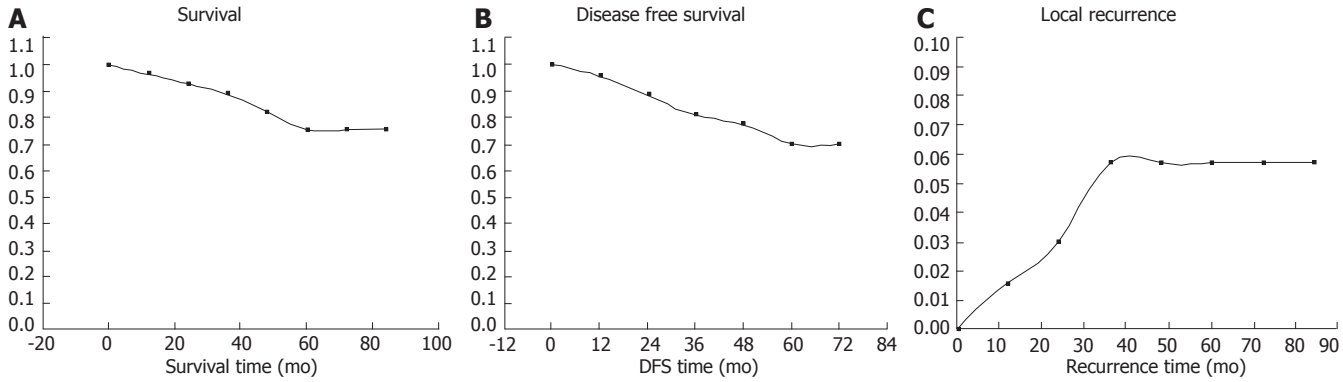

Figure 1 Outcome of 259 rectal cancer patients with TME. **A**: Overall survival curve; **B**: Disease free survival curve; **C**: Local recurrence curve.

DISCUSSION

The surgical management of primary rectal cancer presents unique problems for the surgeon basedlargely on the anatomic constraints of the pelvis. For most of the tumors over 5 cm above the anal verge, anterior resection was increasingly performed in recent years, occupying about 80%-90% of all rectal cancer surgeries in large centers. However, the local and distant recurrence was still challenging. The importance of mesorectum in rectal cancer surgery has been widely recognized. By total mesorectal excision, Heald *et al*[3] and Enker *et al*[4] had reported a lower local recurrence and improved the DFS and overall survival of the patients. In our series, overall local recurrence rate was 6.73%, disease free survival and overall survial were 70.32% and 75.43%, respectively, which was consistent with other studies about TME.

Many factors have been studied in predicting the outcome of the patients with rectal cancer who underwent total mesorectal excision. Whereas the use of clinical and histologic parameters for the determination of prognosis and treatment strategies for patients with rectal cancer is still of great value, they may be distressingly inaccurate in many clinical situations, especially in patients with stage Ⅱ-Ⅲ disease, which may need post- or pre-operative treatment. This may be attributed, at least in part, to differences in the biological behavior of tumors that are determined by altered molecular regulatory mechanisms. Thus, the characterization of molecular changes in colorectal cancer in recent years has been the focus of great interest for both researchers and clinicians, because it may lead to the identification of new prognostic markers more closely resembling the biological nature of the disease. Among the various alterations in gene and protein expression in colorectal cancers, cell-cycle control related genes and proteins (including p53, p21 and PCNA) and cell adhension protein CD44 were widely elucidated in many studies, but the prognostic values were still confusing, and very few studies exclusively focused on rectal cancer patients with anterior resection. In our series, we analyzed the prognostic effect of clinical variables and immnohistochemical markers. TNM staging, preoperative

Table 4 Variables association with overall survival, DFS, recurrence and metastasis

	n	Survival		DFS		Local recurrence		Metastasis	
		%	*P*	%	*P*	%	*P*	%	*P*
Gender									
Male	146	85.6	NS	78.1	< 0.05	5.5	NS	16.4	NS
Female	113	89.4		87.6		2.7		9.7	
Tumor location			NS		NS		NS		
High	62	88.7		85.5		3.2		12.9	
Medium	115	87.8		83.5		6.1		10.4	NS
Low	82	85.4		80.5		2.4		18.3	
Pathology			< 0.05		< 0.05		< 0.05		
Adenocarcinoma	236	87.7		83.9		3.8		13.1	
Mucinous cancer	18	94.4		83.3		5.6		11.1	NS
Signet ring cancer	5	40		40		20		40	
Differentiation									
High-medium	222	87.4	NS	82.9	NS	4.1	NS	13.1	NS
Low	37	86.5		78.4		5.4		16.2	
TNM staging			< 0.05		< 0.05		< 0.05		
I	80	95		92.5		1.3		6.3	
II	67	92.5		89.6		1.5		9.0	< 0.05
III	112	78.6		70.3		8		21.4	
Invasion depth			< 0.05		<0.05		NS		
T1-2	101	95.0		91.1		2		6.9	
T3	85	83.5		78.8		4.7		16.5	< 0.05
T4	73	80.8		75.3		6.8		19.2	
N staging			< 0.05		< 0.05		< 0.05		
N0	147	93.9		91.2		1.4		7.5	< 0.05
N1-2	112	78.6		72.3		8.0		21.4	
Lymphvascular invasion			NS		NS		NS		
+	33	81.8		78.8		6.1		15.2	
-	226	88.1		83.6		4		13.3	NS
Neural invasion					NS		NS		
+	21	85.7	NS	85.7		4.8		14.3	
-	238	87.4		82.8		4.2		13.4	NS
Preoperative CEA			0.06		< 0.05		NS		
+	48	20.8		31.3		6.3		25	
-	211	10.9		14.7		3.8		10.9	< 0.05
P53			NS		NS				
+	118	84.4		80.9		6.4	0.06	13.5	NS
-	141	90.7		85.6		1.7		13.6	
P21			NS		NS		NS		
+	209	87.6		81.8		4.3		13.9	NS
-	50	86		84		4.0		12.0	
PCNA			NS		NS		NS	12.6	
+	159	89.3		84.3		3.1		15.0	NS
-	100	84.0		81.0		6.0			
CD44			< 0.05		< 0.05		NS		
+	129	82.9		76.7		6.2		17.1	0.97
-	130	91.5		87.8		2.3		10.0	

NS: not significant.

CEA and CD44v6 levels were recognized as prognostic factors predicting the disease free survival and overall survival.

Serum CEA level is a common preoperative and follow-up marker in colorectal carcinoma patients. Adenocarcinomas overexpress CEA, which may facilitate metastasis of colorectal carcinoma. Elevated preoperative serum levels are associated with high rates of recurrence and cancer mortality, and it should not be discarded in the current array of prognostic factors. Granell *et al*[5] studied preoperative CEA level and p53 expression in 134 colorectal cancer patients, and found patients with elevated preoperative CEA level were at significant high risk of local recurrence in two years after surgery, whose hazard ratio was 3.26. In our series, preoperative CEA level was an independent prognositic factorin predicting DFS and overall survival, the hazard ratio was 2.65 and 3.10, respectively. Our results suggested that preoperative CEA, like postoperative CEA, may be also a useful prognostic marker for rectal cancer patients.

The expression of specific cell adhesion molecule CD44 splice variants has been shown to be associated with metastasis and poor prognosis in certain human malignancies, such as breast cancer and colorectal cancer, especially the CD44 variant 6 (CD44v6)[6]. In most of these studies, increased levels of CD44 and/or different patterns of splice variants were found in tumors in comparison with their normal counterparts[7,8]. The studies addressing the relationship between CD44 expression at the protein level and clinicopathological variables, such as

tumour grade and stage, have not been uniform. Ishida examined CD44v6 expression in 62 colorectal cancer patients, and the result showed CD44v6 has no correlation with gross type, histology, lymph node involvement, and clinical stage[9]. Bhatavdekar *et al*[10] examined CD44 in 98 Duke's B and C colorectal adenocarcinomas with IHC, and they also found a significantly reduced relapse-free survival in patients with positive CD44. Similary, Yamagnchi *et al*[11] have shown that CD44 is an independent prognostic factor in multivariate analysis. In our study, we did not find any significant association between CD44v6 and clinicopathological parameterseither. But in multivariate analysis, we found CD44v6 was the independent biological prognostic marker for disease free survival and overall survival, and the hazard ratio was 1.93 and 2.21, respectively, suggesting CD44v6 is a valuable molecular marker for rectal cancer prognosis.

P53 was studied in colorectal cancer, but the results of IHC p53 rectal tumor status have been inconsistent. Hilska *et al*[12] studied 363 colorectal cancer patients, including 124 with rectal cancersfrom Duke's stage A to D. The author s used different cut-off values for defining p53 positive, but none of them showed any significance for survival in all colorectal cancer groups. Morgan *et al*[13] studied 171 patients with curative resection of rectal cancer. By immunohistochemical assay for p53 and DCC expression, they found p53 and DCC status of rectal cancers was not associated with other clinical or pathological variables, nor predictive outcomes. The cyclin inhibitors p21 negatively regulates the action of cyclin/CDK complexes, and prevents cell-cycle progression. Lebe *et al* examined IHC p53 p21 and p27 expression in 45 rectal adenocarcinomas, and found p53, p21 and p27 status was not significantly associated with local and distant recurrence. PCNA is an auxiliary factor essential for DNA polymerases activity and exists in a quaternary complex with CDK/cyclin/p21. PCNA is frequently used to measure the proliferative activity of tissues, which was widely studied to evaluate the response of chemotherapy and radiotherapy. PCNA was found associating with improved survival in advanced colorectal cancer by Paradiso *et al*[14]. but several studies discovered no significant association between PCNA expression and prognosis in colorectal cancer[15-17]. In our study, we did not find any association between the three markers and clinicopathological variables. The three markers had no significant prognostic effect for predicting DFS or overall survival, either.

The benefit of adjuvant chemotherapy was of great controversy in rectal cancer patients. The EORTC Radiotherapy Group Trial 22921 found in 253 patients with postoperative chemotherapy, adjuvant chemotherapy was of benefit for local control in T3-4 rectal cancer patients[18]. In that clinical trial, patients were all assigned to receive preoperative radiotherapy or chemoradiotherapy. And the adherance to postoperative chemotherapy was very poor, which made the results accepted. Our patients received adjuvant chemotherapy alone after curative total mesorectal excision. We found in patients with curative excised rectal cancer, postoperative chemotherapy did not improve patients' local control of the tumor or survival. The results suggested that postoperative chemotherapy may only improve the local control by enhancing the effect of radiotherapy. We therefore, do not recommand postoperative chemotherapy for stage Ⅱ or Ⅲ patients without preoperative radiotherapy.

This has been coupled in several series with an improved cancer-specific survival directly attributed to the performance of TME itself[19]. The outcomes are favorable for strictly defined curatively excised rectal cancers with meticulous total mesorectal excision. TNM staging, preoperative CEA, and CD44v6 levels are recognized as independent prognostic facotrs for these patients. And postoperative chemotherapy is not recommanded for curative excised rectal cancer patients without preoperative radiotherapy.

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CASE REPORT

Complete eradication of hepatic metastasis from colorectal cancer by Yttrium-90 SIRT

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Abstract

Yttrium-90 (Y-90) radioembolization, also known as selective internal radiation therapy (SIRT), is a regional hepatic therapy used in the treatment of unresectable colorectal cancer (CRC) liver metastases. In SIRT, Y-90 impregnated microspheres are injected into the VASCULAR SUPPLY of hepatic tumor, leading to selective irradiation and necrosis of tumor TISSUE. While several studies demonstrate improved local control and survival with SIRT, the specific indications for this therapy have yet to be defined. Typically, SIRT is given in combination with chemotherapy as multimodal treatment for unresectable hepatic CRC. However, it HAS ALSO FOUND INCREASING USE as a salvage therapy in chemo-refractory patients. Herein, the authors describe their experience with SIRT as "stand alone" therapy in a surgically-prohibitive, chemotherapy naive patient with hepatic CRC metastasis. The results suggest that Y-90 SIRT may have potential applications beyond its usual role as a palliative or salvage therapy for unresectable hepatic CRC.

Key words: Yttrium-90, SIRT; Radioembolization; Hepatic metastasis; Ablation

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INTRODUCTION

Surgical resection is the only potentially curative strategy in the treatment OF PATIENTS WITH HEPATIC COLORECTAL CANCER (CRC) METASTASIS. Unfortunately, due to advanced liver disease or widespread extrahepatic metastases, only about 10%-15% of patients are candidates for resection. FURTHER, of those who are deemed appropriate surgical candidates and undergo hepatectomy, the overwhelming majority develop recurrent DISEASE that IS not amenable to re-resection[1]. Clearly, other liver-directed therapies are needed to treat unresectable malignancy and to improve the overall response to surgery.

Recently, data have emerged supporting the use of Yttrium-90 selective internal radiation therapy (SIRT) in the treatment of unresectable CRC liver metastases[2-4]. SIRT is a regional, liver-directed therapy based on the principle that hepatic tumors derive their arterial blood supply predominantly from the systemic circulation rather than the portal vein. In SIRT, the pure beta-emitting isotope Y-90 is compounded onto millions of microspheres that are injected into the hepatic artery or one of its branches. The radioactive microspheres deposit in the feeding vasculature of the tumor, resulting in the delivery of intense local radiation to tumor but relative sparing of normal liver parenchyma.

Y-90 SIRT is a safe and effective regional therapy for single or multiple unresectable hepatic colorectal metastases. The principal limitation to SIRT is excessive hepatopulmonary shunt (> 18%), though this occurs rarely in metastatic hepatic disease. In general, SIRT is well-tolerated by patients, with limited duration (24-96 h) side effects inclusive of fatigue, anorexia, nausea, and vomiting. Major complications occur occasionally, and are not SIRT-treatment dependent but RATHER associated with the percutaneous arterial access as with ANY other selective hepatic embolization.

SIRT has been evaluated AS an adjuvant to systemic or hepatic artery chemotherapy and AS salvage therapy in chemo-refractory patients with unresectable hepatic CRC[5,6]. While the data show improved response rates and prolonged survival, there is CURRENTLY no consensus currently on the exact place of Y-90 SIRT in the treatment algorithm for CRC hepatic metastases. The following

report highlights the novel use of Y-90 SIRT as "stand alone" therapy in a chemotherapy naive patient with hepatic CRC metastasis.

CASE REPORT

A 68-year-old man with previously resected hepatic flexure colorectal cancer presented with a single 1.5 cm lesion in medial segment Ⅶ of the liver. The lesion was detected on PET scan performed during routine oncologic follow-up 9 mo after primary colon resection. The patient was referred to the hepatobiliary surgery clinic for consideration of possible resection or ablative therapy. Due to the lesion location and the patient's explicit desire not to receive systemic chemotherapy, treatment options were limited to right hemihepatectomy or RFA ablation. However, the patient had undergone three previous laparotomies and had multiple medical co-morbidities (obesity with BMI > 40, hypertension, and cane-assisted ambulation). Furthermore, his tumor was located in the medial aspect of the right hemi-liver, necessitating a right hemihepatectomy. As the patient did not wish to proceed with this procedure, resection was not an option. Further limit of the potential treatment options was the poor ultrasound visualization of the lesion due to the patients' body habitus, which precluded the use of percutaneous RFA. Consequently, Y-90 SIRT was offered as an alternate treatment strategy, with the recommendation that the patient receive adjuvant systemic chemotherapy after SIRT. The patient agreed to the treatment plan and subsequently underwent pre-therapy imaging.

Celiac and mesenteric angiography was performed from a standard femoral artery approach using a 5 Fr catheter and 3 Fr coaxial microcatheter (Renegade High-Flow; Boston Scientific, Natick, MA). Normal hepatic arterial anatomy was noted and coil embolization of the right gastric and gastroduodenal arteries was performed in order to prevent reflux of microspheres into the gastrointestinal circulation. Following angiography, the patient underwent a technetium-99m labeled macroaggregated albumin (MAA) scanning, which showed an acceptable hepato-pulmonary shunt fraction of only 2.4%.

After completion of pre-therapy planning, the patient underwent Y-90 SIRT. In July 2005, he received a single dose of 1 GBq (about 27 mCi) Y-90 resin microspheres into the right hepatic artery. He tolerated the procedure without complication and was discharged from the hospital the following day. Initial pre-therapy PET scan showed a single 1.5cm focus in the right hepatic lobe with an SUV of 8.04. At 6 wk after treatment, repeated PET scan demonstrated a reduction of SUV to 3.09. PET scan at 12 wk showed complete disappearance of the lesion (Figure 1). The patient remains alive and well at 18 mo of follow-up, with no evidence of hepatic recurrence on repeated PET scan.

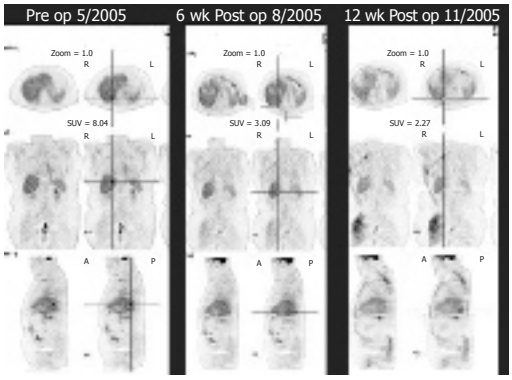

Figure 1 PET studies before SIRT, 6 wk and 12 wk after SIRT.

DISCUSSION

Surgical resection is the treatment of choice in SELECT patients with favorable CRC hepatic tumors. Unfortunately, only 5-10% of patients qualify for resection; moreover, recurrence rates are high among those who undergo surgery[7]. As TRANSPLANTATION is not considered an option in metastatic liver DISEASE, other modalities must be considered.

Neoadjuvant chemotherapy is currently employed in some institutions to downsize hepatic CRC tumors and facilitate subsequent resection[8]. In addition, ablative modalities like RFA are occasionally used to expand the limits of resection in SELECT tumors that are too large to be encompassed by a surgical approach alone[9]. For patients in whom resection is precluded despite the aforementioned strategies, adjuvant systemic chemotherapy has become the mainstay of treatment. Likewise, systemic chemotherapy has become standard treatment for prevention of post-resection recurrence after hepatic metastectomy. Despite the development of new chemotherapeutic regimens, response and survival following systemic treatment ALONE remain dismal.

Recently, hepatic arterial chemotherapy (HAC) has been introduced into the algorithm of hepatic CRC tumor management. Several lines of evidence support the use of HAC alone or in combination with systemic chemotherapy as primary or second-line treatment for unresectable hepatic CRC. A randomized clinical trial comparing HAC to systemic chemotherapy in a previously untreated cohort of patients demonstrated significantly better response, survival, and time to hepatic progression with HAC[10]. Similarly, phase I trials show favorable response rates when HAC is used in combination with systemic chemotherapy in the second-line setting[11,12]. Additionally, combined HAC and systemic chemotherapy results in superior overall and progression-free survival in patients who have previously undergone hepatic metastectomy[13].

Despite these advances, the prognosis of patients with unresectable hepatic CRC remains grim at best. The introduction of Y-90 SIRT, however, represents a significant improvement in the management of unresectable hepatic CRC. Y-90 SIRT is a versatile modality that may be used as both an adjunct to potentiate the effects of chemotherapy and as a stand alone option in chemotherapy-refractory disease. Van Hazel *et al*[2] published a randomized trial in 2004 in which they evaluated the COMBINATION OF SIRT AND THE THEN CURRENT STANDARD OF CHEMOTHERAPY, 5-FU and leucovorin. Twenty-one

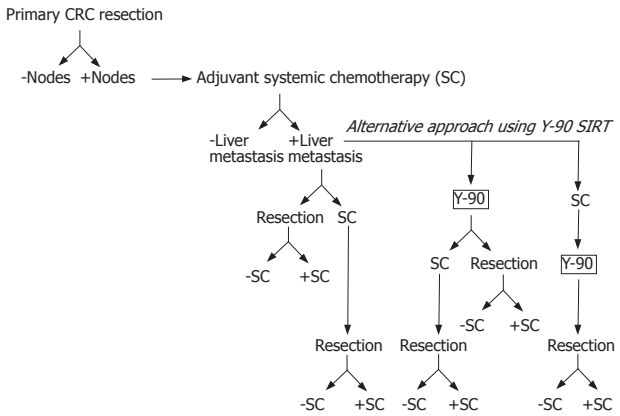

Figure 2 Proposed treatment algorithm for hepatic colorectal cancer.

patients with previously untreated CRC hepatic metastases underwent SIRT with systemic chemotherapy (5-FU/leucovorin) or chemotherapy alone. The combination group had higher response rates and longer time to disease progression than the chemotherapy alone group.

SIRT can also potentiate the effect of regional hepatic arterial chemotherapy. In a randomized trial by Gray *et al*[3], 74 patients with bilobar hepatic CRC metastases received either hepatic arterial chemotherapy with FUDR alone or HAC with a single injection of SIRT. Response rate, time to disease progression, and survival were all significantly increased in the SIRT plus HAC group. While the studies by Van Hazel and Gray employed chemotherapy regimens that are now outdated, their results are still informative as they illustrate the overall efficacy of SIRT as an adjunct to systemic and regional chemotherapy in hepatic CRC. New studies are already in progress to assess the potential of SIRT with modern chemotherapeutic drugs (i.e. oxaliplatin and irinotecan) and biological agents (i.e. Cetuximab, antiepidermal growth factor receptor monoclonal antibody)[6].

Not only does SIRT play a role in multimodal therapy, it may also be used as a salvage modality for chemorefractory metastases[4,6]. Kennedy *et al*[4] recently published the results of a large, multi-institutional series of patients with chemorefractory liver metastases. They noted encouraging tumor response rates based on CT, PET, and CEA levels. Interestingly, the maximum response occurred at approximately 3 mo after treatment; in our patient, complete regression of lesion was also noted at 3 mo of follow-up PET scan.

As SIRT burns no bridges with other modalities, it may actually be effective at multiple points in the algorithm of CRC hepatic tumor management. In addition to palliative and salvage therapy, SIRT may have applicability as a "neoadjuvant" or stand alone treatment in highly selected patients with hepatic CRC. INDEED Y-90 internal radiation has already been explored as a bridge to ablation, resection, or transplantation in patients with primary hepatocellular cancer[14-17]. The limited but promising experience in HCC suggests a need for further investigation of this indication in hepatic metastases.

In this report, treatment of metastatic hepatic CRC with Y-90 radioembolization alone resulted in resolution of a single 1.5 cm lesion in the right hepatic lobe on PET imaging. While only anecdotal, these results support a broader role for SIRT in the management of unresectable hepatic CRC. Y-90 could be considered as a possible stand alone therapy in patients with a small, single hepatic focus of metastatic CRC who are not surgical candidates and do not wish to undergo standard systemic chemotherapy. Alternatively, Y-90 could serve as an effective adjuvant therapy to decrease or stabilize tumor bulk before undergoing standard systemic chemotherapy, lesion ablation, or liver resection (Figure 2). Larger series and formal clinical trials are needed to define the optimal indications for SIRT in hepatic CRC management.

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LETTERS TO THE EDITOR

Hepatitis C virus RNA kinetics: Drug efficacy and the rate of HCV-infected cells loss

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TO THE EDITOR

We read the study by Medeiros-Filho *et al*[1] with much interest. The study shed light on early HCV RNA kinetics in conjunction with liver cirrhosis, different genotypes (gen-1 *vs* gen-3) of HCV and sustained viral response (SVR) rates. In particular, Medeiros-Filho *et al*[1] showed that the HCV RNA first phase decline, under interferon-α (IFN) and ribavirin therapy, which represents the effectiveness (ε) of IFN to block viral production[2,3], was significantly larger in gen-3 cirrhotic patients (mean $\varepsilon = 0.99$) than gen-1 cirrhotic patients (mean $\varepsilon = 0.8$). In addition, in these cirrhotic patients, they found that the HCV RNA second phase decay slope in gen-3 patients was significantly faster than in gen-1 patients, and suggested that the immune response against infected HCV cells in gen-1 patients may be less potent than in gen-3 patients.

We recently introduced the notion of a critical drug efficacy ε_c, such that if the drug efficacy, ε, is higher than the critical drug efficacy, i.e., $\varepsilon > \varepsilon_c$, then viral levels will continually decline on therapy, while if $\varepsilon < \varepsilon_c$, then viral loads will initially decline but ultimately stabilize at a steady state level lower than baseline (i.e., exhibit a flat phase)[4,5]. We have shown that the flat phase may be a simple consequence of liver homeostasis in which proliferation of hepatocytes compensates for the loss of infected cells, hence observing a flat phase does not imply a poor or absent immune response.

In light of these predictions, the interpretation of Medeiros-Filho *et al*[1] on the difference in viral kinetics between gen-1 and gen-3 in cirrhotic patients needs to be further addressed. First, if $\varepsilon < \varepsilon_c$, then following the first phase viral decay, the virus will reach a steady state lower than its baseline viral load very rapidly (i.e., flat phase). However, if ε is close to ε_c (but still $\varepsilon < \varepsilon_c$), then after the rapid viral decay phase a second slower phase of decay is predicted followed by a flat phase. Since in Medeiros-Filho *et al*[1] data was obtained only until d28 one can speculate that the drug efficacy in gen-1 cirrhotic patients, which are known to be difficult to treat, was lower than the critical drug efficacy ($\varepsilon < \varepsilon_c$) and that the 2nd slower phase reflects the flat phase or is just intermediate in an approach to reach a flat phase. Indeed, 4 of 7 gen-1 cirrhotic patients had a second phase slope equal to 0, which represents a flat phase, where the rest had a positive second phase decline slope but one that was lower than the predictive cut-off slope of SVR (i.e., 0.3 log IU/mL per week[1]), that may indicate an intermediate in an approach to reach the aforementioned flat phase.

Second, if $\varepsilon > \varepsilon_c$, then the viral second phase slope represents the death/loss rate of HCV-infected cells only if $\varepsilon \sim 1$[4,5]. Thus, if ε in some gen-1 cirrhotic patients from Medeiros-Filho *et al*[1] was higher then ε_c, then the 2nd slope decay still does not reflect with confidence the actual death/loss rate of HCV-infected cells, since the IFN effectiveness, ε, was < 1 (mean $\varepsilon = 0.8$). However, in gen-3 cirrhotic patients for which the mean value of the IFN effectiveness was close to 1 (mean $\varepsilon = 0.99$), the second phase slope could well reflect the immune-mediated loss rate of HCV-infected cells. Thus, we argue that the mechanisms that lead to different viral kinetics between gen-1 and gen-3 cirrhotic patients may be attributed to different drug effectivenesses and not solely to the immune response against HCV-infected cells.

In conclusion, Medeiros-Filho *et al*[1] made an important step towards understanding why cirrhotic patients have lower SVR rates (see also review on therapy in HCV decompensated cirrhotic patients by Navasa & Forns[6]). However, we suggest that in future studies data sampling longer than d28 needs to be done in order to better capture the viral kinetic profiles in treated cirrhotic patients.

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Present as mean ± SD or mean ± SE.

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