INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

The Genotypes of IL-1 beta and MMP-3 are Associated with the Prognosis of HCV-related Hepatocellular Carcinoma

Kinya Okamoto[1], Chihiro Ishida[1], Yuichiro Ikebuchi[1], Mari Mandai[1], Kenichi Mimura[1], Yoshikazu Murawaki[1] and Isao Yuasa[2]

Abstract

Background and Aim Cytokines and matrix metalloproteinases (MMPs) are involved in tumor growth, invasion, and remote metastasis in various cancers. Recently, functional gene polymorphisms in these cytokines and MMPs have been found, and some reports have revealed an association between these polymorphisms and the prognosis of various cancers. In this study, we examined the relationship between the gene polymorphisms of interleukin 1 beta (IL-1b), IL-1 receptor antagonist (IL-1 RN), transforming growth factor beta 1 (TGF-b1), MMP-1, MMP-3, and MMP-9 and the prognosis of hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

Methods We enrolled 92 HCV-related HCC patients in the study, and gene polymorphisms of IL-1b -31 C/T, IL-1 RN variable number of tandem repeats (VNTR), TGF-b1 +869 C/T, MMP-1 -1,607 1G/2G, MMP-3 -1,171 5A/6A, and MMP-9 -1,562 C/T were analyzed.

Results In HCC clinical features, TGF-b1 C carriers and MMP-3 5A carriers had significantly larger HCC diameters than TGF-b1 T and MMP-3 6A homozygotes. In HCC prognosis, IL-1b T homozygotes and MMP-3 5A carriers had a significantly poorer prognosis than IL-1b C carriers and MMP-3 6A homozygotes. Those with a combination of IL-1b T homozygosity and MMP-3 5A had synergistically poorer HCC prognosis.

Conclusion The IL-1b -31 T allele and MMP-3 5A allele are cooperative risk factors for poor prognosis in HCC patients, suggesting that these gene polymorphisms might be potential markers for predicting the prognosis of HCC patients.

Key words: interleukin-1 beta, MMP-3, gene polymorphism, hepatocellular carcinoma, prognosis, hepatitis virus C

(Inter Med 49: 887-895, 2010)
(DOI: 10.2169/internalmedicine.49.3268)

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world (1). Many approaches have been developed for HCC therapy, including percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), and transarterial chemoembolization (TACE). However, the total prognosis of HCC patients is still poor because of the high rate of recurrence (2).

Many intracellular and extracellular factors participate in carcinogenesis, cancer growth and metastasis. Recently, numerous functional gene polymorphisms among pro-inflammatory and pro-fibrogenetic factors and their inhibitors have been found, and some of them are thought to influence carcinogenesis and tumor progression (3, 4).

IL-1b is one of the potent pro-inflammatory cytokines and has a wide array of biological functions, including cell sur-

[1]The Second Department of Internal Medicine, Tottori University School of Medicine, Yonago and [2]Department of Legal Medicine, Tottori University School of Medicine, Yonago
Received for publication December 15, 2009; Accepted for publication January 28, 2010
Correspondence to Dr. Kinya Okamoto, okamotka@grape.med.tottori-u.ac.jp

vival and proliferation (5). Some single nucleotide polymorphisms (SNPs) have been reported in IL-1b, as well as cytosine (C)/ thymidine (T) base transitions at -31 bp from the transcriptional start site. It has been shown that the -31T allele enhances IL-1b transcriptional activity (6).

The IL-1 receptor antagonist (IL-1RN) variable 86-bp sequence repeats (variable number tandem repeat, VNTR) in intron 2. Some reports have shown that allele 2 (two repeats) enhances IL-1b production (7, 8). Several studies have examined the relationship between IL-1b gene polymorphisms and various cancer features including HCC, but the results are controversial (9-11).

Transforming growth factor-beta 1 (TGF-b1) is one of the potent inducers of hepatic fibrogenesis (12). Indeed, liver TGF-b1 mRNA and plasma TGF-b1 levels are increased in patients with chronic hepatitis, and correlated with the degree of liver fibrosis (13). In the human TGF-b1 gene, eight polymorphisms have been identified (14). Among them, two functional SNPs are located in the leader sequence at +869 T/C and +915C/G resulting in changed amino acid sequences (leucine 10 proline and arginine 25 proline, respectively). The +869T homozygote is reported to have higher expression levels of TGF-b1 and to be associated with some risk and prognosis of carcinomas (15, 16). +915C/G polymorphisms have not been detected in East Asian populations such as Koreans and Japanese (15, 17).

Degradation of ECM is thought to be a prerequisite for cancer invasion, metastasis, and angiogenesis. Matrix metalloproteinases (MMPs) directly participate in ECM degradation (18). Since HCC tumors develop in fibrotic livers, MMP activity in particular is necessary for growth, invasion and metastasis. Indeed, the levels of MMPs are frequently increased in HCC tumor tissues, and some reports have suggested that intratumoral MMP overexpression is closely correlated with the potential for HCC invasion and metastasis (19, 20). Recently, gene polymorphisms in the promoter regions of MMP genes, MMP-1 (interstitial collagenase-1), MMP-3 (stromelysine-1), and MMP-9 (gelatinase B) have been found (21-23).

MMP-1's promoter gene polymorphism is the insertion/deletion of a guanine (G) at position -1,607, and has two alleles, one with a single guanine (1G) and the other with two (2G). *In vitro* assays have also demonstrated that the 2G allelic promoter has higher transcriptional activity than the 1G allelic promoter (24). The polymorphism of the MMP-3 promoter region is the insertion/deletion of an adenosine (A) at position -1,171 and has two alleles, one having a run of six adenosines (6A) and the other five (5A) (21). *In vitro* studies have shown that the 5A allelic promoter has a 2-fold greater transcriptional activity than the 6A allelic promoter (25). A 9-bp sequence containing the -1,562 C/T polymorphic site in MMP-9 is recognized as an important regulatory element and is a binding site for a transcription repressor protein and T-allelic variant has higher promoter activity (23). Many investigators have examined the association of MMP-1, -3, and -9 gene polymorphisms with the prognosis of various cancers. Consequently, some reports have shown a positive relationship between MMP-1, MMP-3, or MMP-9 gene polymorphisms and the prognosis of malignant neoplasms (24, 26-28).

In this study, we examined the association of IL-1b, TGF-b1, MMP-1, MMP-3, and MMP-9 gene polymorphisms with the prognosis of hepatitis virus C (HCV)-related HCC patients.

Methods

Patients

The subjects of this study were 95 patients with HCV-related HCC (mean age 67±8, 57 males) and 83 HCV-related chronic liver disease (CLD) patients without HCC (mean age 63±11, 46 males). All participants were Japanese and positive for serum HCV RNA. Patients who had chronic hepatitis B virus infection, alcoholism, primary biliary cirrhosis, or autoimmune liver disease were excluded. The diagnosis of HCC was defined by image diagnosis: angiography and computer tomography (CT). Forty-six cases also had liver histological examinations. HCC histologic differentiation grades were determined according to Edmondson and Steiner's classification. Clinicopathological features [gender, age, tumor diameter size and morphology, liver functional tests, serum tumor marker alpha fetoprotein (AFP), and CLIP score (The Cancer of the Liver Italian Program: prognostic scoring system for HCC patients)] of patients were referred to as the first diagnosis of HCC. In multiple HCCs, the largest lesions were measured as the HCC diameter. After the first diagnosis of HCC, all HCC patients had been administered mono or combination therapy of TACE, PEI, and RFA, and had been followed up regularly every month. The mean follow-up term was 3.9±2.5 years. This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects of the Medical Faculty of the Tottori University, and written informed consent was obtained from each subject before blood was collected.

DNA extraction

Genomic DNA samples were extracted from peripheral white blood cells using a DNA extracting kit (DnaQuick II; Dainipponseiyaku, Osaka, Japan) according to the manufacturer's instructions.

Genotype analysis

Gene polymorphisms were analyzed using the polymerase chain reaction (PCR) followed by the restriction fragment length polymorphism (RFLP) method according to previous reports (8, 29-33) (Table 1).

Statistical analyses

Values were expressed as the mean ± S.D. Differences in genotype distributions from those expected for Hardy-Weinberg equilibrium were tested by the Chi-square test. We

Table 1. The Primer Sequences and Protocols of PCR-RFLP for Genotyping the IL-1b -31C/T, IL-1 RN VNTR, TGF-b1 +869 C/T, MMP-1 -1607 1G/2G, MMP-3 -1171 5A/6A and MMP-9 -1562 C/T Gene Polymorphisms

	Primer sequences (F=forward, R=reverse)	PCR protocols				RFLP conditions	Lengths of products
IL-1b -31 T/C	F: 5′-AGAAGCTTCCAC-CAATACTC-3′ R: 5′-AGAAGCTTCCA-CCAATACTC-3′	95°C 1 min.	36 cycles of 94°C 45 sec. 54°C 50 sec. 72°C 1 min.			Alu I (Takara) 37°C Overnight	C allele: 344, 79, 20 and 5 bp T allele: 247, 97, 79, 20 and 5 bp
IL-1 RN VNTR	F: 5′-CCCCTCAGCAAC-ACTCC-3′ R: 5′-GGTCAGAAGGGC-AGAGA-3′	95°C 10 min.	5 cycles of 94°C 45 sec. 65°C 30 sec. 72°C 30 sec.	30 cycles of 94°C 30 sec. 60°C 30 sec. 72°C 30 sec.	5 cycles of 94°C 30 sec. 55°C 30 sec. 72°C 30 sec	Direct base Pair measuring	Allele 1 (4 repeats): 442 bp Allele 2 (2 repeats): 272 bp Allele 3 (3 repeats): 357 bp Allele 4 (5 repeats): 532 bp Allele 5 (6 repeats) 627 bp
TGF-1b +869 T/C	F: 5′-TTCCCTCGAGGC-CCTCCTA-3′ R: 5′-GCCGCAGCTTGG-ACAGGATC-3′	96°C 10 min.	30 cycles of 96°C 1 min. 62°C 1 min. 73°C 1 min.	73°C 5 min.		MspA1-I (Takara) 37°C Overnight	T allele: 161 bp C allele: 149 and 12 bp
MMP-1 -1607 1G/2G	F: 5'-TCGTGAGAATGT-CTTCCCATT-3′ R: 5'-TCTTGGATTGAT-TTGAGATAAGTGAAATC-3'	95°C 1 min.	35 cycles of 95°C 1 min. 55°C 30 sec. 72°C 30 sec.			Xmn I (Takara) 37°C Overnight	2G allele: 117 bp 1G allele: 28 and 89 bp
MMP-3 -1171 5A/6A	F: 5′-GGTTCTCCATTC-CTTTGATGGGGGGAAAgA-3' R: 5'-CTTCCTGGAATT-CACATCACTGCCACCACT-3'	95°C 5 min.	30 cycles of 94°C 30 sec. 67°C 30 sec. 72°C 45 sec.			Tth111 I (Takara) 65°C 3 hours	6A allele:130 bp 5A allele: 97 and 33 bp
MMP-9 -1562 C/T	F: 5'-GCCTGGCACATA-GTAGGCCC-3' R: 5'-CTTCCTAGCCAG-CCGGCATC-3'	95°C 2 min.	30 cycles of 95°C 45 sec. 67°C 45 sec. 72°C 45 sec.			Sph I (Toyobo) 37°C 3 hours	C Allele: 435 bp T Allele: 247 and 188 bp

Table 2. Demographic Characteristics of the Enrolled Patients CLIP Score: The Scores from the Cancer of the Liver Italian Program for Staging Hepatocellular Carcinoma. All Interferon Treatments Had Resulted in No Response. *: p<0.05

Clinicopathological features		HCC (n=95)	CLD (n=83)
Age		67 ± 8	63 ± 11
Gender	Male Female	57 (60%) 38 (40%)	46 (55%) 37 (45%)
Number of liver cirrhosis patients		66 (69%)*	17 (20%)
Past history of interferon therapy		20 (21%)	18 (22%)
HCC size	(cm)	2.7 ± 1.4	-
CLIP	score	1.1 ± 1.0	-
Tumor formation	Uni Multi Massive	40% 57% 3%	-
Portal thrombus	+ -	3% 97%	-
AFP	< 400 >= 400	84% 16%	100% 0%
Child-Pugh stage	A B C	77% 23% 0%	80% 15% 5%
Histological differentiation (n=46)	I II and III	28 (60%) 18 (40%)	-

performed Chi-square tests to examine the differences in genotype and allele frequencies between the two subject groups. The difference of data among groups was assessed by analysis of variance (ANOVA) and Scheffe's test. We performed a Breslow-Gehan-Wilcoxon test to examine differences in the overall survival rate by the Kaplan-Meier method. A p-value of<0.05 was regarded as statistically significant.

Results

The characteristics of the enrolled patients are shown in Table 2; 66 (69%) of HCC and 17 (20%) of CLD group were diagnosed as liver cirrhosis by clinical or histological tests. However, the hepatic reserve function did not significantly change between the two groups (p=0.44). There were no significant differences among HCC and CLD groups in

Table 3. Genotype Distributions and Allele Frequencies between CLD and HCC Patients *: p<0.05

	Genotype / Allele	CLD (n=83)	HCC (n=92)	p value
IL-1b -31	T homo	20 (25.0%)	24 (27.9%)	
	hetero	39 (48.8%)	42 (48.8%)	0.868
	C homo	21 (26.3%)	20 (23.3%)	
Allele frequency	Allele T	0.49	0.52	0.672
IL-1 RN VNTR	1/1	70 (85.4%)	77 (86.5%)	
	2/1	10	9	
	2/4	1	1	0.861
	1/4, 1/3	1, 1	1, 0	
Allele frequency	Allele 2	0.07	0.06	0.937
TGF-b1 +869	T homo	29 (34.9%)	22 (24.7%)	
	hetero	32 (38.6%)	47 (52.8%)	0.151
	C homo	22 (26.5%)	20 (22.5%)	
Allele frequency	Allele C	0.54	0.49	0.194
MMP-1 -1607	1G homo	3 (3.7%)	10 (10.9%)	
	hetero	49 (59.8%)	37 (40.7%)	0.002*
	2G homo	30 (36.6%)	44 (48%)	
Allele frequency	Allele 2G	0.65	0.69	0.572
MMP-3 -1171	6A homo	55 (64.0%)	60 (65.2%)	
	hetero	27 (31.4%)	29 (31.5%)	0.892
	5A homo	4 (4.7%)	3 (3.3%)	
Allele frequency	Allele 5A	0.20	0.19	0.877
MMP-9 -1562	C homo	54 (65.1%)	70 (76.1%)	
	hetero	26 (31.3%)	20 (21.7%)	0.274
	T homo	3 (3.6%)	2 (2.2%)	
Allele frequency	Allele T	0.19	0.13	0.144

age, gender, past history of interferon therapy. About 20% of HCC and CLD group (20 cases and 18 cases, respectively) had undergone IFN therapy before enrollment in this study. All cases were non-responders. HCC size and CLIP score were relatively small and had good indications for TACE, PEI, and RFA.

Table 3 shows the genotype distributions and allele frequencies in IL-1b -31 C/T, IL-1RN VNTR, TGF-b1 +869 C/T, MMP-1 -1,607 1G/2G, MMP-3 -1,171 5A/6A, and MMP-9 -1,562 C/T in HCC patients and CLD patients. All biallelic genotype distributions were consistent with the Hardy-Weinberg principle (p>0.05, data not shown). Concerning the presence of HCC, the distribution of MMP-1 was different between HCC and CLD patients, and the MMP-1 2G homozygote was more frequent in HCC patients (Table 3). Genotype distributions of IL-1b, IL-1RN, TGF-b 1, MMP-3, and MMP-9 were not significantly different between HCC and CLD (Table 3).

Next we analyzed the clinical parameters of HCC patients at the first diagnosis of HCC. All genotypes were divided into groups of two, such as IL-1b C carriers and T homozygotes, IL-1RN allele 2 carrier and others, TGF-b1 C carriers and T homozygotes, MMP-1 1G carriers and 2G homozygotes, MMP-3 5A carriers and 6A homozygotes, and MMP-9 T carriers and C homozygotes (Table 4). Child-Pugh patients were compared to IL-1b C carriers (p=0.024, Table 4). As for HCC diameters at first diagnosis, TGF-b1 C carriers and MMP-3 -1,171 5A carriers had significantly larger tumors than TGF-b1 +869 T homozygotes and MMP-3 6A homozygotes (p=0.016 and 0.024, respectively) (Table 4). The HCC histological grades of IL-1 T homozygotes and MMP-9 T carriers were more frequently classified as moderately and poorly differentiated than IL-1 C carriers and MMP-9 C homozygotes (p=0.024 and 0.031, respectively) (Table 4). Statistical significance was not found for other clinical features (age, gender, CLIP score, and some of the parameters of CLIP, such as tumor formation, the presence of portal thrombus, and an AFP value of over 400 mAU/mL).

We also examined the prognosis of HCC patients between the paired genotype groups mentioned previously. In the Kaplan-Meier analysis, IL-1b T homozygotes and MMP-3 5 A carriers had a significantly poorer prognosis than those of C carriers and 6A homozygotes (p=0.028 and 0.043, respectively) (Fig. 1A, B). The 3- and 5-year survival rates of IL-1 b C carriers were 86.6% and 64.9%, and those of T homozygotes were 69.8% and 45.9%, respectively. The 3- and 5-year survival rates of MMP-3 6A homozygotes were 91.3% and 66.3%, and those of 5A carriers were 64.9% and 53.4%, respectively. No clear relationship was observed between the prognosis of HCC patients and IL-1RN VNTR, TGF-b1 C/T, MMP-1 1G/2G, and MMP-9 C/T genotypes (Fig. 1C, D, E, F).

Furthermore, we examined combination groups of IL-1b and MMP-3 genotypes. In clinicopathological features, no significant differences were found between four combinations: IL-1b C carriers and MMP-3 6A homozygotes (C/6 A), IL-1b C carriers and MMP-3 5A carriers (C/5A), IL-1b

Table 4. The Clinicopathological Parameters of HCC Patients at First HCC Diagnosis. *: p<0.05

		Age	Gender	CLIP Score	Tumor formation	Portal thrombus	AFP (mAU/mL)	Child-Pugh grade	HCC size	Grade of HCC differentiation
			M / F (%)		Uni / Multi / Massive (%)	- / + (%)	< 400 / ≥ 400 (%)	A / B (%)	(cm)	I / II, III (%)
IL-1b -31	C carrier	66 ± 9	60 / 40	1.2 ± 1.0	42 / 56 / 2	98 / 2	81 / 19	*65 / 35*	2.6 ± 1.4	*73 / 27*
	T homo	68 ± 8	54 / 46	0.8 ± 0.6	37 / 63 / 0	100 / 0	89 / 11	*94 / 6**	2.7 ± 1.4	*30 / 70**
IL1 RN VNTR	2 carrier	64 ±10	36 / 64	1.1 ± 0.8	33 / 67 / 0	100 / 0	67 / 30	89 / 11	2.6 ± 1.0	67 / 33
	Others	66 ± 9	60 / 40	1.0 ± 1.0	42 / 58 / 0	98 / 2	87 / 13	73 / 27	2.7 ± 1.4	60 / 40
TGF-b1 +869	C carrier	66 ± 8	61 / 39	1.2 ± 1.1	38 / 57 / 5	98 / 2	81 / 19	70 / 30	*2.9 ± 1.6**	61 / 39
	T homo	70 ± 8	50 / 50	0.8 ± 1.1	41 / 59 / 0	95 / 5	88 / 12	82 / 12	*2.0 ± 0.6*	67 / 33
MMP-1 -1607	1G carrier	67 ± 8	60 / 40	0.9 ± 0.8	48 / 52 / 0	100 / 0	88 / 12	70 / 30	3.0 ± 1.5	45 / 55
	2G homo	67 ± 8	60 / 40	1.2 ± 1.1	31 / 63 / 6	94 / 6	84 / 16	81 / 19	2.5 ± 1.3	73 / 27
MMP-3 -1171	5A carrier	68 ± 8	69 / 31	1.3 ± 1.1	55 / 40 / 5	96 / 4	70 / 30	60 / 40	*3.2 ± 1.7**	*53 / 47*
	6A homo	65 ± 9	53 / 47	1.0 ± 0.9	34 / 64 / 2	98 / 2	91 / 9	80 / 20	*2.5 ± 1.2*	*68 / 32*
MMP-9 -1562	C homo	67 ± 8	61 / 39	1.0 ± 1.0	43 / 55 / 2	98 / 2	86 / 14	76 / 24	2.8 ± 1.5	*50 / 50*
	T carrier	67 ± 8	59 / 41	1.2 ± 1.0	33 / 60 / 7	94 / 6	80 / 20	81 / 19	2.4 ± 1.3	*10* / 90*

T homozygotes and MMP-3 6A homozygotes (T/6A), and IL-1b T homozygotes and MMP-3 5A carriers (T/5A). As for survival rates, T/5A, the higher transcriptional genotype combination, had a significantly poorer prognosis in HCC patients than all other combination groups ($p \leq 0.0001$ vs. all other combination groups) (Fig. 2). The 3- and 5-year survival rates of T/5A were 38.3% and 19.0%, respectively.

Discussion

In this study, we investigated the relationship of gene polymorphisms IL-1b -31 C/T, IL-1RN VNTR, TGF-b1 + 869 C/T, MMP-1 -1,607 1G/2G, MMP-3 -1,171 5A/6A, and MMP-9 -1,562 C/T with the prognosis of HCV-related HCC patients. The distribution rates and allele frequencies in our study are similar to those from previous reports from Japan (9, 11, 17, 34, 35). Among these genotypes, the IL-1b, TGF-b1, MMP-1, and MMP-9 genotype distributions in the Japanese population are similar to those in Caucasians, whereas the allele frequencies of the IL1-RN allele 2 and MMP-3 5A allele in Japanese and other Eastern Asian populations are lower than those in Caucasians (10, 26).

Our results suggest that the IL-1b -31 genotype has no relationship with HCC diagnosis, but that a higher transcriptional genotype, the -31 T homozygote, has a poorer prognosis than C allele carriers among HCC patients. To our knowledge, this is the first study to reveal a relationship between IL-1b -31 gene polymorphisms and HCV-related HCC prognosis. Concerning HCC development, several studies have been published, but their conclusions are controversial (9, 10, 33). Most information on the role of IL-1b in malignancy concerns the effects of IL-1b on the invasiveness of already existing malignant cells (4). Microenvironmental IL-1b is mainly secreted from stromal cells (e.g., fibroblasts, endothelial cells, and inflammatory cells) and some tumor cells, and induces inflammatory responses in a paracrine and endocrine manner. The secreted form of IL-1b activates nuclear genes through intracellular signal pathways, including NF-κB, and up-regulates growth factors, angiogenesis factors, and matrix metalloproteinases which relate to both inflammation and tumor growth, invasion and metastasis (4, 5, 36, 37). The IL-1b higher transcriptional -31T allele may affect microenvironmental IL-1b levels in HCC and result in a poorer prognosis.

In our results, the HCC patients with the worse prognostic factor IL-1b T homozygotes are more frequently classified into better hepatic reserve function (Child-Pugh stage A) at the first HCC diagnosis. Regardless of Child-Pugh stage, these HCC patients have been taking a similar treatment by PEI, RFA and TACE, which are relatively tolerable therapies for poorer hepatic reserve function. As with this explanation, therefore there is a possibility that the higher IL-1b transcriptional activity could result in HCC development in earlier stage of liver cirrhosis.

Our results also suggest a positive relationship between the -31T homozygote and poorer HCC histological grades. It has been thoroughly demonstrated that higher cell proliferation and vascular invasion play important roles in the lowering of the differentiation grade of HCC (38). The functional gene polymorphism at IL-1b -31 may influence tumor tissue differentiation through up-regulating the microenvironmental IL-1b level.

As for the IL-1 RN VNTR polymorphism, some reports which have examined Caucasian groups have indicated a possible relationship between allele 2 and HCC, but this has not been found in Asian populations, including in the present study (9-11). This may be due to the low frequency of allele 2 in Asian populations.

In this study, carriers of the lower transcriptional allele TGF-b1 +869 C had significantly larger HCC diameters at

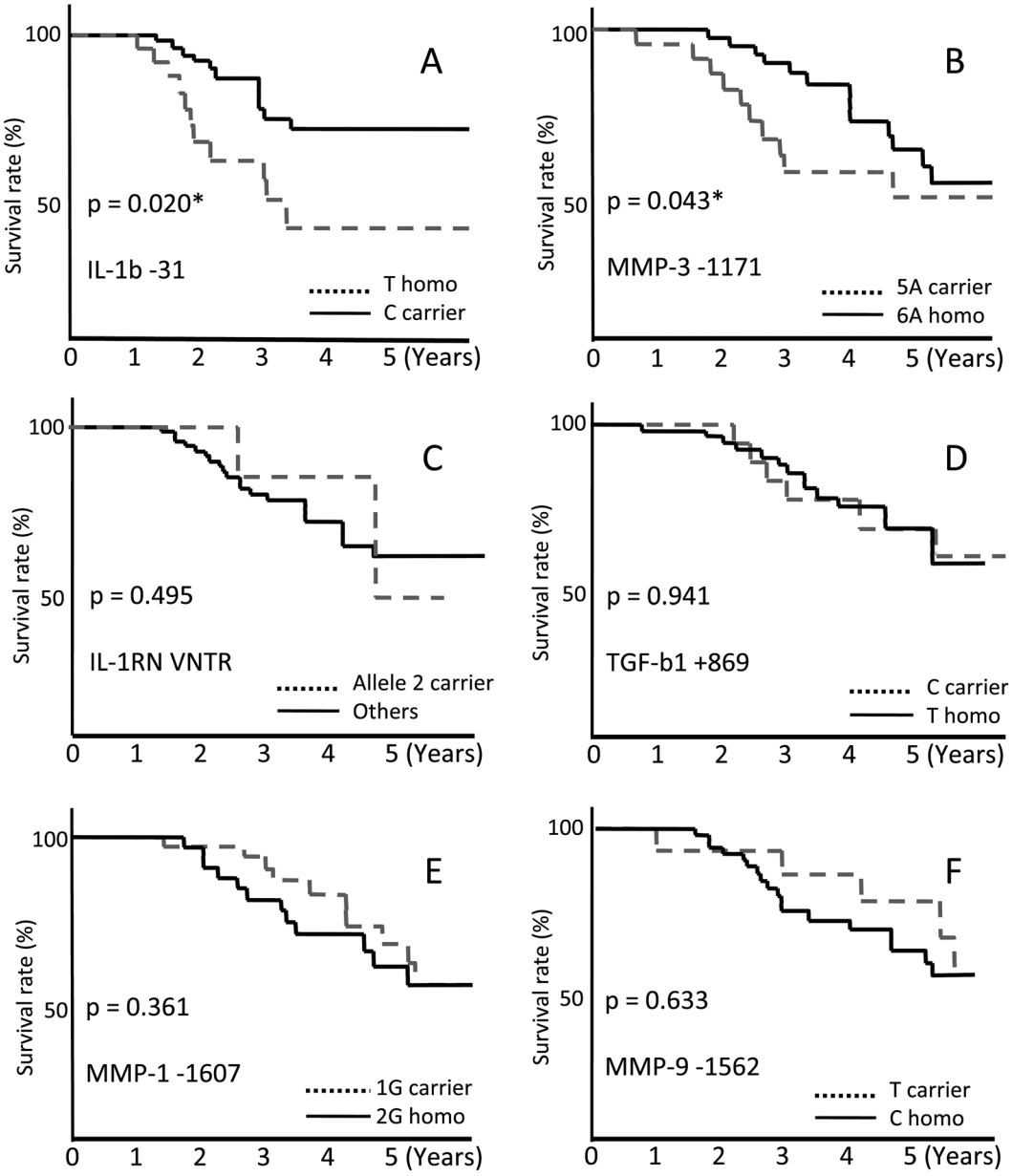

Figure 1. **The survival rates of HCC patients according to genotype. A: IL-1b C carriers and T homozygotes, B: MMP-3 5A carriers and 6A homozygotes, C: IL-1RN allele 2 carrier and other IL-1 RN genotypes, D: TGF-b1 C carriers and T homozygotes, E: MMP-1 1G carriers and 2G homozygotes, and F: MMP-9 T carriers and C homozygotes. *: p<0.05.**

the first HCC diagnosis point; however, this SNP did not affect HCV-related HCC incidence and survival rates. No previous reports have studied the TGF-b1 +869 C/T gene polymorphism in HCV-related chronic liver disease patients only. Concerning HBV patients or etiologically mixed patients, several reports have suggested that being a carrier for +869T is a HCC risk factor (15, 16, 34). This inconsistency may be due to these double-faced functions whose ratio is affected by the etiology of background liver disease. TGF-b1 serves as either an epithelial cell growth inhibitor or a tumor promoter, depending on the ECM context (39). As for the tumor suppressing aspect, TGF-b1 inhibits cell-cycle progression through enhanced expression of cyclin-dependent kinase inhibitors such as p15INK4B and p21CIP1, and induces apoptosis through smad signal pathways in hepatocytes and some cancer cells (40, 41). Indeed, previous studies have shown that TGF-b1 inhibits carcinogenesis, tumor progression, and therapeutic resistance in several cancers, including HCC (39, 42). On the other hand, it has been reported that TGF-b1 up-regulates the expression of vascular endothelial growth factor and matrix metalloproteinases, resulting in accelerated tumor angiogenesis and metastasis (43).

In the present study, the MMP-1 -1,607 2G homozygote tended to relate to the incidence of HCC patients; however, a clear relationship has not been found. A positive association of -1,607 1G/2G genotypes with the prognosis of cancers has been reported (25). The authors have explained that

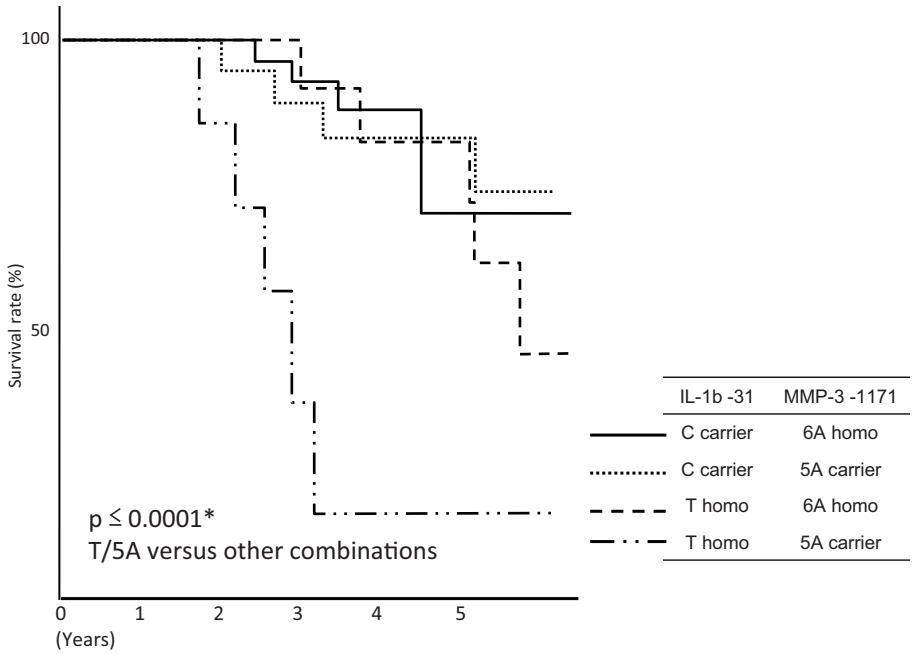

Figure 2. The survival rates of HCC patients with combinations of IL-1b -31 C/T and MMP-3 -1171 5A/6A genotypes.

the higher transcriptional activity of the 2G allele causes MMP-1 overexpression in host tissues, which increases the ability of the tumor for cell invasion. However, Hettiaratchi et al have shown that 2G homozygosity is associated with a favorable prognosis of colorectal carcinoma patients (44). Regarding HCC histology, Okazaki et al reported that MMP-1 protein and mRNA are detected in only the early stage of HCC but not in advanced HCC (20). Taken together, the role of the MMP-1 -1,607 1G/2G gene polymorphism in cancer progression is still controversial.

MMP-3 is mainly secreted from activated fibroblasts and degrades not only collagens, but also many non-collagenous matrix components and activates interstitial procollagenases. Bodey et al reported that strong expression of MMP-3 is found in HCC tissue, especially in the ECM adjacent to blood vessels (45). Our results showed that the MMP-3 -1,171 5A allele influenced HCC growth and is related to a poorer prognosis in HCC patients. The 5A allele has higher transcriptional activity than the 6A allele due to preferential binding of a transcriptional repressor (25). There are no previous reports concerning the relationship between MMP-3 polymorphism and HCC prognosis. In other carcinomas, Ghilardi et al have shown that the presence of the 5A allele represents an unfavorable prognostic feature (26). Holliday et al have reported that the fibroblasts derived from breast cancer patients who are 5A homozygous demonstrate significantly higher MMP-3 release and have more aggressive invasion-promoting capacity than 6A carriers or those with normal fibroblasts with the same genotype, and suggested a possible interaction between the MMP-3 -1,171 gene polymorphism and the tumor-stroma cross-talking (46).

Our study has also shown that the higher transcriptional genotype combination, IL-1b -31 T and MMP-3 -1,171 5A, leads to a distinctly poorer prognosis in HCC patients compared to a single genotype analysis. MMP-3 mRNA production is at a low level in normal physiological conditions, but can be markedly induced by IL-1b, TNF alpha, interferon gamma, and some epidermal growth factors under pathological conditions (47). IL-1b up-regulates MMP-3 transcription via activating the ERK and p38 MAPK pathways (37). The functional gene polymorphisms of IL-1 -31 and MMP-3 -1,171 may cooperate by a cascade acceleration of HCC tumor progression and metastasis.

We observed a positive relationship between MMP-9 T carriers and poorer HCC differentiation but could not detect a significant relationship between MMP-9 -1,562 C/T genotypes with either development or prognosis of HCC. Previous reports have shown that MMP-9 mRNA levels in HCC tissue are frequently up-regulated through the activation of PI3K, Akt, and NF-kB pathways and correlate to HCC capsular infiltration (48). It is not yet clear why poorer HCC histological grades, which seem to correlate with the MMP-9 -1,562 T allele, are not correlated with overall survival. However, such a discrepancy is likely to result from different balances of the multiple MMP-9 functions. For example, MMP-9 cleaves plasminogen to generate angiostatin, one of the most potent inhibitors of angiogenesis (49). HCC is a highly vascular solid tumor in which angiogenesis plays an important role, therefore raising the hypothesis that two MMP-9 functions, the degradation of ECM and angiogenesis inhibition, induce a complicated transition in the balance in HCV-related HCC.

In conclusion, the present study suggests that IL-1b -31 T/C and MMP-3 -1,171 5A/6A gene polymorphisms influence the prognosis of HCV-related HCC patients and that a combination of these genotypes has a synergistic effect. A

clinical study should be undertaken in larger populations to investigate the correlation between the alleles of these polymorphic sites and the prognosis of individual HCC.

Acknowledgement

We are indebted to Mr. Yujiro Ikuta for excellent technical assistance.

The authors declare no conflict of interest.

References

1. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology **127**: S5-S16, 2004.

2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet **362**: 1907-1917, 2003.

3. Bataller R, North KE, Brenner DA. Genetic polymorphisms and the progression of liver fibrosis: A critical appraisal. Hepatology **37**: 493-503, 2003.

4. Apte RN, Dotan S, Elkabets M, et al. The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. Cancer Metastasis Rev **25**: 387-408, 2006.

5. Roshak AK, Jackson JR, McGough K, Chabot-Fletcher M, Mochan E, Marshall LA. Manipulation of distinct NF kappaB proteins alters interleukin-1 beta-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. J Biol Chem **271**: 31496-31501, 1996.

6. El-Omer EM, Carrington M, Chow WH, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature **404**: 398-402, 2000.

7. Santtila S, Savinainen K, Hurme M. Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1 b production in vitro. Scand J Immunol **47**: 195-198, 1998.

8. Hwang IR, Kodama T, Kikuchi S, et al. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1 beta production in *Helicobacter pylori* infection. Gastroenterology **123**: 1793-1803, 2002.

9. Tanaka Y, Furuta T, Suzuki S, et al. Impact of interleukin-1 beta genetic polymorphisms on the development of hepatitis C virus-related hepatocellular carcinoma in Japan. J Infect Dis **187**: 1822-1825, 2003.

10. Hirankarn N, Kimkong I, Kummee P, Tangkijvanich P, Poovorawan Y. Interleukin-1 beta gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection. World J Gastroenterol **12**: 776-779, 2006.

11. Migita K, Maeda Y, Abiru S, et al. Polymorphisms of interleukin-1 beta in Japanese patients with hepatitis B virus infection. J Hepatol **46**: 381-386, 2007.

12. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest **115**: 209-218, 2005.

13. Murawaki Y, Nishimura Y, Ikuta Y, Idobe Y, Kitamura Y, Kawasaki H. Plasma transforming growth factor-beta 1 concentration in patients with chronic viral hepatitis. J Gastroenterol Hepatol **13**: 680-684, 1998.

14. Gewaltig J, Mngasser-Stephan K, Gartung C, Biesterfeld S, Gressner AM. Association of polymorphisms of transforming growth factor-b1 gene with the rate of progression of HCV-induced liver fibrosis. Cln Chim Acta **316**: 83-94, 2002.

15. Kim YJ, Lee HS, Im JP, et al. Association of transforming growth factor-beta1 gene polymorphisms with a hepatocellular carcinoma risk in patients with chronic hepatitis B virus infection. Exp Mol Med **35**: 196-202, 2003.

16. Falleti E, Fabris C, Toniutto P, et al. TGF-beta 1 genotypes in cirrhosis: relationship with the occurrence of liver cancer. Cytokine **44**: 256-261, 2008.

17. Suzuki S, Tanaka Y, Orito E, et al. Transforming growth factor-beta-1 genetic polymorphism in Japanese patients with chronic hepatitis C virus infection. J Gastroenterol Hepatol **18**: 1139-1143, 2003.

18. Forget MA, Desrosiers MM, Beliveau R. Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis. Can J Physiol Pharmacol **77**: 465-480, 1999.

19. Yamamoto H, Itoh F, Adachi Y, et al. Relation of enhanced secretion of active matrix metalloproteinases with tumor spread in human hepatocellular carcinoma. Gastroenterology **112**: 1290-1296, 1997.

20. Okazaki I, Wada N, Nakano M, et al. Difference in gene expression for matrix metalloproteinase-1 between early and advanced hepatocellular carcinomas. Hepatology **25**: 580-584, 1997.

21. Ye S, Watts GF, Mandalia S, Humphries SE, Henney AM. Preliminary report: genetic variation in the human stromelysin promoter is associated with progression of coronary atherosclerosis. Br Heart J **73**: 209-215, 1995.

22. Rutter JL, Mitchell TI, Buttice G, et al. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res **58**: 5321-5325, 1998.

23. Zhang B, Ye S, Herrmann SM, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation **99**: 1788-1794, 1999.

24. Ghilardi G, Biondi ML, Mangoni J, et al. Matrix metalloproteinase-1 promoter polymorphism 1G/2G is correlated with colorectal cancer invasiveness. Clin Cancer Res **7**: 2344-2346, 2001.

25. Ye S, Eriksson P, Hamsten A, Kurkinen M, Humphries SE, Henney AM. Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. J Biol Chem **271**: 13055-13060, 1996.

26. Ghilardi G, Biondi ML, Caputo M, et al. A single nucleotide polymorphism in the matrix metalloproteinase-3 promoter enhances breast cancer susceptibility. Clin Cancer Res **8**: 3820-3823, 2002.

27. Fang S, Jin X, Wang R, et al. Polymorphisms in the MMP1 and MMP3 promoter and non-small cell lung carcinoma in North China. Carcinogenesis **26**: 481-486, 2005.

28. Tu HF, Wu CH, Kao SY, Liu CJ, Liu TY, Lui MT. Functional -1562 C-to-T polymorphism in matrix metalloproteinase-9 (MMP-9) promoter is associated with the risk for oral squamous cell carcinoma in younger male areca users. J Oral Pathol Med **36**: 409-414, 2007.

29. Dunleavey L, Beyyzade L, Ye S. Rapid genotype analysis of the matrix metalloproteinase-1 gene 1G/2G polymorphism that is associated with risk of cancer. Matrix Biol **19**: 175-177, 2000.

30. Gnasso A, Motti C, Irace C, et al. Genetic variation in human stromelysin gene promoter and common carotid geometry in healthy male subjects. Arterioscler Thromb Vasc Biol **20**: 1600-1605, 2000.

31. Nelissen I, Vandenbroeck K, Fiten P, et al. Polymorphism analysis suggests that the gelatinase B gene is not a susceptibility factor for multiple sclerosis. J Neuroimmunol **105**: 58-63, 2000.

32. Wood NAP, Thomson SC, Smith RM, Bidwell JL. Identification of human TGF-b1 signal (leader) sequence polymorphisms by PCR-RFLP. J Immunol Meth **234**: 117-122, 2000.

33. Wang Y, Kato N, Hoshida Y, et al. Interleukin-1 gene polymorphisms associated with hepatocellular carcinoma in hepatitis C virus infection. Hepatology **37**: 65-71, 2003.

34. Migita K, Miyazoe S, Maeda Y, et al. Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection--association between TGF-beta 1 polymorphisms and hepatocellular carcinoma. J Hepatol **42**: 505-510, 2005.

35. Hinoda Y, Okayama N, Takano N, et al. Association of functional polymorphisms of matrix metalloproteinase (MMP-1) and MMP-3 genes with colorectal cancer. Int J Cancer **102**: 526-529, 2002.

36. Song X, Voronov E, Dvorkin T, et al. Differential effects of IL-1 alpha and IL-1 beta on tumorigenicity patterns and invasiveness. J Immunol **171**: 6448-6456, 2003.

37. Thampatty BP, Li H, Im HJ, Wang JH. EP4 receptor regulates collagen type-I, MMP-1, and MMP-3 gene expression in human tendon fibroblasts in response to IL-1β treatment. Gene **386**: 154-161, 2007.

38. Sasaki Y, Imaoka S, Ishiguro S, et al. Clinical features of small hepatocellular carcinomas as assessed by histologic grades. Surgery **119**: 252-260, 1996.

39. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet **29**: 117-129, 2001.

40. Hannon GJ, Beach D. $p15^{INK4B}$ is a potential effector of TGF-b-induced cell cycle arrest. Nature **371**: 257-261, 1994.

41. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci USA **92**: 5545-5549, 1995.

42. Liu P, Menon K, Alvarez E, Lu K, Teicher BA. Transforming growth factor-beta and response to anti-cancer therapies in human liver and gastric tumors in vitro and in vivo. Int J Oncol **16**: 599-610, 2000.

43. Edwards DR, Murphy G, Reynolds JJ, et al. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J **6**: 1899-1904, 1987.

44. Hettiaratchi A, Hawkins NJ, McKenzie G, et al. The collagenase-1 (MMP-1) gene promoter polymorphism - 1607/2G is associated with favourable prognosis in patients with colorectal cancer. Br J Cancer **96**: 783-792, 2007.

45. Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. Immunocytochemical detection of MMP-3 and -10 expression in hepatocellular carcinoma. Anticancer Res **20**: 4585-4590, 2000.

46. Holliday DL, Hughes S, Shaw JA, Walker RA, Jones JL. Intrinsic genetic characteristics determine tumor-modifying capacity of fibroblasts: matrix metalloproteinase-3 5A/5A genotype enhances breast cancer cell invasion. Breast Cancer Res **9**: R67, 2007.

47. Sciavolino PJ, Lee TH, Vilcek J. Interferon-beta induces metalloproteinase mRNA expression in human fibroblasts. Role of activator protein-1. J Biol Chem **269**: 21627-21634, 1994.

48. Chen J-S, Wang Q, Fu X-H, et al. Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. Hepatol Res **39**: 177-186, 2009.

49. Variaktaris E, Vassiliou S, Nkenke E, et al. A metalloproteinase-9 polymorphism which affects its expression is associated with increased risk for oral squamous cell carcinoma. Eur J Surg Oncol **34**: 450-455, 2008.