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Full Length Research Paper

Clinical and genetic aspects of Turkish hepatocellular carcinoma patients: Results of a single center study

M. C. Kazimi[1], S. Nalbantoglu[2]*, M. Kiliç[1] and A. Berdeli[2]

[1]Hospital of Kent, Center of Transplantation, İzmir, Turkey.
[2]Ege University, Faculty of Medicine, Child Hospital, Molecular Medicine Laboratory 35100, Bornova, İzmir, Turkey.

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Hepatocellular carcinoma is one of the most progressive and aggressive cancer kinds worldwide. In various populations, the pathogenetic link between genetic polymorphisms of matrix-metalloproteinases, vascular endothelial growth factor and hepatocellular carcinoma is variable and limited. The aim of the present study was to retrospectively evaluate the clinical and genetic post-transplantation results of Turkish hepatocellular carcinoma patients treated with orthotopic liver transplantation. Genotypic distributions of the vascular endothelial growth factor -141A/C, -460 C/T and +405 C/G; matrix-metalloproteinase-*2* -735 C/T and matrix-metalloproteinase-*1* -1607 1G/2G polymorphisms were in Hardy–Weinberg equilibrium in patient and control groups (P > 0.05). In case-control analysis, the distribution of genotypes and allele frequencies did not differ from those in the control group (P > 0.05). In genotype-phenotype correlation analysis; for matrix-metalloproteinase-*1*-1607 1G/2G polymorphism, 2G/2G genotype was associated with portal ven invasion (P < 0.02). The post-transplantation findings indicated a 4-year survival in 77.3% and a post-transplantation overall survival without recurrence in 96.2% of the patients group.

Key words: Hepatocellular carcinoma, orthotopic liver transplantation, vascular endothelial growth factor, matrix-metalloproteinase, single nucleotide polymorphism.

INTRODUCTION

Hepatocellular carcinoma is one of the most frequent human cancers, with 0.25 - 1 million of newly diagnosed cases each year (Llovet et al., 2003). The hepatocellular carcinoma incidence was 0.83/100 000 according to 2003 Ministry of Health report in Turkey and the annual incidence of Hepatocellular carcinoma (HCC) in Turkey was similar between 2000 and 2003 (0.80/100 000 at 2000, 0.87/100 000 at 2001, 1.1/100 000 at 2002 and 0.87/100 000 at 2003) (Alacacioglu et al., 2008). HCC is characterized by a propensity for vascular invasion and a high metastatic potential, thus leading to a high incidence of early postoperative recurrence and poor survival. The conventional therapies, including resection, chemoembolization, alcohol injection and thermoablation for hepatic malignancies have not achieved satisfactory results. In recent years, progress has been made in liver transplantation for HCC in patients with viral hepatitis and cirrhosis, and long-term survival has been achieved in those patients who meet the Milan criteria (Milani et al., 1994). As liver transplantation removes the whole liver, HCC recurrence after liver transplantation derives from extrahepatic dissemination before or during transplantation, which displays highly aggressive tumor biology. Interactions between cancer cells and the surrounding microenvironment, which may allow the tumor to invade into the adjacent organs and trigger the recurrence and metastasis through vascular vessels, are critical to the occurrence of extrahepatic dissemination. Currently, biomarkers involved in the process of HCC invasion and metastasis mainly include DNA ploidy, cell cycle regulators, tumor promoter genes, cell cycle controllers, proteinases, adhesion molecules and angiogenic factors. In these markers, matrix metalloproteinases that can degrade extracellular matrix (ECM) are believed to play some pivotal roles in promoting the invasion and

*Corresponding author. E-mail: nalbantoglusinem@gmail.com. Tel: +90(232) 3901015. Fax: +90 (232) 2537682

metastasis HCC. One of these markers, the susceptible gene in physiological and pathological angiogenesis, vascular endothelial growth factor (VEGF*)*, has been cloned to chromosome 6; location at 6p12 involving eight exons/seven introns, and by alternative splicing of its pre-mRNA constitutes the family of VEGF proteins (six VEGF isoforms) and belongs to the PDGF/VEGF growth factor superfamily. The VEGF growth factor is considered to be active in angiogenesis, vasculogenesis and endothelial cell growth thereby inducing endothelial cell proliferation, promoting cell migration, inhibiting apoptosis, and inducing permeabilization of blood vessels (Mattei et al., 1996; Tischer et al., 1991). As well as VEGF family, matrix metalloproteinase (MMP) protein family also have pivoral roles in the breakdown of ECM in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases, and alternative splicing of this gene results in multiple transcript variants. MMP1 gene encodes a secreted enzyme which breaks down the interstitial collagens, types I, II, and III. The gene is part of a cluster of MMP genes which localize to chromosome 11q22.3 with 11 exons/10 introns. MMP2 gene (chromosomally located to 16q13-q21 with 14 exons/13 introns) encodes an enzyme which degrades type IV collagen, the major structural component of basement membranes. The enzyme plays a role in endometrial menstrual breakdown, regulation of vascularization and the inflammatory response.

Polymorphisms in VEGF and MMP genes, influence the level of protein production of these genes and, are subjected to genetic susceptibility of several disorders. Previous case-control studies were presented with/without correlation between MMP/VEGF genotypes and various disease associations. A number of studies have searched the association between common functional VEGF single nucleotide polymorphisms (SNPs) (460C/T, 1154G/A, 2578C/A, 936C/T) and angiogenesis mediated diseases such as diabetic microvascular complications with type 1 diabetes mellitus, acute rejection in renal transplant recipients, prostate cancer, cutaneous malignant melanoma and invasive breast cancer (McCarron et al., 2002; Shahbazi et al., 2002; Howell et al., 2002; Krippl et al.,2003; Lin et al., 2003; Liu et al., 2009), diabetic retinopathy (Awata et al., 2002), renal cell carcinoma (Abe et al., 2002). Renner et al. (2000) reported novel C702T, C936T, and G1612A polymorphisms in the 3'UTR region and found lower protein plasma levels in T allele of *VEGF* 936C/T carriers, and Krippl et al. (2003) reported this allele with decreased breast cancer risk. VEGF 2578C/A and 1154G/A polymorphisms and risk of breast cancer was also not associated (Awata et al., 2002). In 2005, two different groups reported different results in breast cancer studies. Jin et al. (2005) reported that the VEGF polymorphisms 2578C/A, 1154G/A, and 936C/T were not associated with breast cancer risk_in consistence with Smith et al's results (Smith, 2004) and suggested that the -634CC genotype and the -2578/-634 CC haplotype were associated with tumor aggressiveness in Polish, German, and Swedish populations. Whereas, in Chinese, Lu et al. reported (2005) that the -1498C and -634G except for +936 alleles were associated with poorer survival in accordance with the genotype frequency results of Kong et al. (2007). Jin and Lu, studying on different ethnic populations, reported contrast findings relative to the effect of the -634 genotype on breast cancer prognosis; and while -634CC genotype frequency was 9.1% in European, it was 17.5% in Chinese populations, suggesting that the effect of genotype or haplotype on disease prognosis differs according to ethnicity. Tzanakis et al. (2006) found significant association of the 634CC genotype ($P = 0.042$) with increased risk for gastric cancer development and decreased overall survival rates (46.67%). Also, a strong association between the -2578AA ($P = 0.025$), -634CC ($P = 0.013$), +936CT ($P = 0.028$), +936TT ($P = 0.0001$) genotypes and a larger tumor size was observed, while the 2578AA and -634CC genotypes were strongly correlated to poor differentiation ($P = 0.01$) and advanced stage of disease ($P = 0.039$), respectively.

Also, there was no significant association between 936C/T polymorphism and risk of cutaneous malignant melanoma, and renal cell carcinoma (Abe et al., 2002; Howell et al., 2002). VEGF −1154 SNP was found to influence tumour invasion in cutaneous malignant melanoma and (Howell et al., 2002), VEGF −1154 GG genotype was found as the 'high expression' genotype and −1154 AA was the 'low expression' genotype in patients. According to Maltese et al's VEGF SNP results (2009) −2578A, −460T, and +405G was a protective haplotype and −2578A,−460C, and +405G and −2578C, −460C, and +405C were the high-risk haplotypes in the etiology of colorectal cancer disease. For the risk of endometriosis development, while Hsieh et al. (2004) showed a significant association and susceptibility with -460C/T polymorphism, Bhanoori et al. (2005), Kim et al. (2005), Ikuhashi et al. (2007) and Zhao et al. (2008) did not. In 2009, Liu et al. showed the association of VEGF 460/1154/2578 TGC, CAA, TAA and TAC haplotypes with endometriosis, and suggested decreased *VEGF* promoter activity of 1154A and 2578A alleles with low protein production as protective factors against endometriosis.

Several studies were also performed on the SNPs and level of expression rates of MMP genes (Rutter et al., 1998; Wyatt et al., 2002; Biondi et al., 2000; Ye et al., 1995). MMP1 rs1799750 was associated with an increased risk of developing lung, ovarian, colorectal, head and neck cancers (Zhu et al., 2001; Kanamori et al., 1999; Ghilardi et al., 2001), and in both normal

fibroblasts and melanoma cells, MMP1 transcription was reported to be effected by MMP1 rs1799750 1G/2G promoter polymorphism (Rutter et al., 1998), contrary to the findings of Murray et al. (1996, 1998) and Ito et al. (1999). Also, in renal cell carcinoma cases, Hirata et al. (2003) reported an increased frequency of the rs1799750 2G variant. The connection between MMP2 promoter polymorphism and MMP1 2G/2G genotype was reported for the risk and increased susceptibility to lung cancer (Zhu et al., 2001; Yu et al., 2002). MMP3 K45E (rs679620) variant was associated with differences in MMP3 activity and has been linked to cancer susceptibility in some studies (Zhang et al., 2004; Zinzindhoue et al., 2004; Krippl et al., 2004). MMP1 and MMP3 SNPs were associated with the risk of renal cell carcinoma (Hirata et al., 2003), colorectal cancer (Hinoda et al., 2002), breast cancer (Ghilardi et al., 2002), and the invasion of cutaneous malignant melanoma (Ye et al., 2001), ovarian cancer (Kanamori et al., 1999), and colorectal cancer (Ghilardi et al., 2001). Fang et al.(2005) showed the combined effect of the MMP1 and MMP3 alleles on transcription and expression of MMPs, and found that, MMP1 and MMP3 1G/5A haplotype had more than 3-fold increased risk to have lymphatic metastasis than the presence of a single SNP, compared with the 2G/6A haplotype in lung cancer, suggesting that 1G/5A haplotype requires a more active treatment. However, MMP1 1G/2G and MMP3 Glu45Lys SNPs association studies within a MMP gene cluster at 11q22.3 showed strong linkage disequilibrium between rs1799750 and rs679620. The strongest link with renal cell carcinoma was associated with the MMP1 rs1799750 and MMP3 rs679620 haplotype in contrast to the effects of SNPs alone (Ricketts et al., 2009). In Vairaktarisa's study (2008), MMP-9 (–1562C/T) was found to contribute to the risk of oral carcinoma out of MMP-1 (–1607 1G/2G), MMP-3 (–1171 5A*/6A*), and VEGF (+936C/T) SNPs related to angiogenesis, inflammation and thrombosis in a European population. Ricketts et al. (2009) found an association between renal carcinoma and MMP1 (rs1799750), MMP3 (rs679620) functional polymorphisms, but not VEGF gene polymorphism (rs1570360).

For HCC, Kong et al. (2007) reported that VEGF -634CC variant was associated with increased overall survival than -634 GG or GC carriers, and VEGF gene promoter polymorphisms were associated with aggressive clinical features. In 2009, Wu et al investigated association of VEGF genomic polymorphisms with risk for developing HCC and tumor recurrence after Liver Transplantation (LT). To date, VEGF gene polymorphisms were associated with an adverse outcome in various malignancies including HCC treated with resection. Only in Wu's study, VEGF polymorphisms were searched for HCC treated with LT. Seven polymorphisms in the VEGF gene (rs699947, rs1570360, rs2010963, rs3024997, rs3025010, rs3025035, rs3025039) were examined in 93 HCC patients treated with LT. Significant association was seen only between rs3025035 and recurrence; rs3025035 CT genotype was independently associated with a shortened recurrence-free survival and suggested as a potential genetic marker for HCC recurrence in LT patients. The aim of the present study was to retrospectively evaluate the clinical and genetic post-transplantation results of hepatocellular carcinoma patients treated with liver transplantation relative to suspicious genes of hepatocellular carcinoma; VEGF and MMP. Due to association with invasion and angiogenesis in cancer metastasis, the MMP and VEGF are candidate genes for predisposition to hepatocellular carcinoma. In various populations, gene polymorphism susceptibility studies for hepatocelular carcinoma disease are rare and unsatisfactory, and the pathogenetic link between MMPs-VEGF and hepatocellular carcinoma is variable and limited. Nevertheless, the relationship between VEGF-MMP polymorphisms and risk of HCC in Turkish patients has not been reported, hence the present study was designed to investigate the association of these two loci with the risk of HCC.

MATERIALS AND METHODS

Characterization of Subjects

We evaluated our results of liver transplantation for patients with HCC. The retrospective study comprised of 73 HCC patients with preoperatively or incidentally diagnosed by biopsy, tomography and AFP levels that were confirmed by pathological examination after operation underwent Orthotopic Liver Transplantation (OLT) at Ege University, Center of Transplantation from November, 1999 to April, 2009. Mean follow-up in the post-OLT period was 5,1887 +/- 1,9 (range, 3-10) years. The median patient age was 55 years (55, 4932 ± 9,665) and 60 patients (82%) were men. For healthy control group who do not have any liver disease or systemic or chronic syndrome; 60, and 137 healthy individuals were enrolled for MMP1 -1607 1G/2G and MMP2 -735 C/T polymorphism studies, respectively, and 62, 88, and 90 healthy individuals were enrolled for VEGF -460 C/T, -141A/C, +405G/C polymorphism studies, respectively. The diagnosis was made according to the diagnostic criteria of United Network for Organ Sharing (UNOS). In this study, 17 of the 73 patients (23%) had undergone deceased LT while 56 of them (77%) were treated with living donor transplantation. Of the 73 patients, in 6 patients, tumors exceeded the Milan criteria which were present in the group of living related donor recipients.

The study procedures followed were in accordance with the standards of the Ethical Committee of Ege University School of Medicine. Blood samples and consent to genotyping and to taking medical histories were obtained through physicians-in-care. Every patient was informed about the study and a written informed consent was signed by all individuals for blood sampling.

Patient characteristics and prognostic risk factors for hepatocellular carcinoma

Clinicopathological parameters and patient characteristics in 73 HCC patients who had undergone OLT are shown in Table 1. All liver organs were exposed to pathological examination after being removed during OLT. The major prognostic factors were age at

Table 1. Clinicopathological parameters and patient characteristics.

Factor	No of data (%)
Demographic data	
Age (y)	55. 4932 ± 9.665 (Mean±SD; Ranges from 2 years to 65)
Gender	
Male	60 (82)
Female	13 (18)
Clinical data	
Etiology	
Cirrhosis – causes	73 (100)
HBV status	44 (60.2)
HCV status	9 (12.3)
GDH (Glycogen storage disease)	3 (4.1)
PBS (primary biliary cirrhosis)	3 (4.1)
Alcohol	3 (4.1)
Cryptogenic	5 (6.8)
HBV+HDV	3 (4.1)
Tyrosinemia	3 (4.1)
Noncirrhosis	0
Tumor data	
Number and dimensions of nodules	
Single, ≤5 cm	48
≤3 nodules, ≤3 cm	19
≤3 nodules, <10 cm	6
Portal vein invasion	
Absent	31 (42.5)
Present	42 (57.5)
Histopathological grade differentiation	
G1	20
G2	37
G3-4	16
pTNM Grading	
T1	5
T2	20
T3-4	48

diagnosis, etiology, size of tumor, number of tumors, portal vein invasion and histopathological grade differentiation. The etiology was classified as follows; a total of 73 patients were grouped as viral and non-viral. Among the total, 20 patients constituted the non-viral patient group, and 53 patients were subgrouped into HBV (n=44) and HCV (n=9) originated patient groups. In addition, HBV originated HCC (44) and heterogeneous originated HCC (non-HBV) (29) patients were taken into genotype-phenotype correlation analysis including the phenotypic and genotypic factors; size/number of tumors, portal vein invasion and histopathological grade differentiation in comparison with the VEGF and MMP SNP genotypes in each of the groups.

HCC were histopathologically graded as Grade I, II, and II-IV according to the grading criteria of Edmondson and Steiner (1954), and HCC phasing was performed according to pTNM classification by International Union Against Cancer. Of the 73 HCC specimens, 20 were graded as Grade I, 37 were Grade II, 16 were Grade III-IV. 92% of the study group consisted one single tumor ≤5 cm or no more than three tumors each of which no larger than 3 cm. Portal vein invasion was absent in 31 patients (42.5%) and present in 42 patients (57.5%). Prior to OLT, HCC was treated with routine immunosuppression included a calcineurin inhibitor, mycophenolate mofetil, and methylprednisolone. After OLT, tumor recurrence was determined by liver ultrasonography and AFP levels during the first year of OLT in every 3 months and after the first year in every 6 months. CT and bone scintigraphy were performed for

establishing recurrence, and the suspicious lesions were biopsied to confirm the diagnosis.

Molecular Genetic Analysis

Selection of VEGF and MMP Single Nucleotide Polymorphisms

VEGF and MMP candidate polymorphisms, each of which located in the promoter, coding or noncoding regions of the genes, were selected considering the different biological effects of each SNP from the nucleotide level to the protein processing level (from genotype to the phenotype), the reports of functionality and haplotype association, susceptibility rates to diseases reported by gene expression levels in transcriptional or translational state in various literature, probability value, and having a minor allele frequency of at least 6%.

Genomic DNA Preparation and Quantitation

Genomic DNA (gDNA) from 2 ml of peripheral blood samples which were collected into ethylenediaminetetraacetic acid (EDTA)–anticoagulated tubes by the standard venipuncture method was extracted using the QIAmp blood DNA Isolation kit following manufacturer's instructions. DNA concentration was determined by using Thermo Scientific Nanodrop apparatus.

Polymerase Chain Reaction-Restriction Fragment Lenght Polymorphism (PCR-RFLP) Analysis

Polymerase Chain Reaction (PCR) Amplification: For the studied VEGF A gene, the genbank data were as follows: NCBI Reference Sequence: NM_001025366.1 (NC_000006.11; NP_001020537); definition, homo sapiens VEGF A, transcript variant 1, mRNA, 3665 bp mRNA and 232 amino acid sequence lenght. For the studied MMP1 gene, the genbank data were as follows: NCBI Reference Sequence: NM_002421.2 (NC_000011.9; NP_002412.1); definition, matrix metalloproteinase 1 isoform 1 preproprotein 1973 bp mRNA and 469 amino acid sequence lenght; for MMP2 gene, the genbank data were as follows: NCBI Reference Sequence: NM_004530.4 (NC_000016.9; NP_004521.1); definition, transcript variant 1, mRNA matrix metalloproteinase 2 isoform a preproprotein 3549 bp mRNA and 660 amino acid sequence lenght. Amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City,CA) in a 25 µl reaction mixture in 0.2 ml thin-wall PCR strip tubes (Axygen Scientific,Inc., CA) containing 100 ng genomic DNA, GeneAmp Gold Buffer(15 mmol/l Tris-HCl,pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 1.5 mmol $MgCl_2$, 50 µmol/l each of the dGTP,dATp,dTTP and dCTP (Promega, Madison,WI), 5 pmol of each forward and reverse primers and 1.0 U AmpliTaq Gold polymerase (PE Applied Biosystems) (oligonucleotide pairs are available upon request). The cycling conditions comprised an initial denaturation step (5 min at 94°C), samples were subjected to 30 rounds of PCR at 94°C for 30 s (–460 C/T) or 1 min (+405 C/G), 60°C (–460 C/T) or 62°C (+405 C/G) for 30 s (–460 C/T) or 1 min (+405 C/G), and 72°C for 60 s with a final extension time of 5 min at 72°C. The yielded PCR products were separated on a 2% agarose gel stained with ethidium bromide and visualised in the UV illumination.

Restriction Fragment Lenght Polymorphism (RFLP) Analysis

Enzyme Digestion Conditions: Amplified PCR product (15 µL) was digested in a total 25 µL final reaction volume consisting of 2 µL 10 X NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 Mm dithiothreitol, pH 7.9), 0.2 µL BSA 810 mg/ml), 0.3 µL *Xmnl* (20 unit/ml) (New England Biolabs, Inc., Beverly, MA, USA) for MMP-1 -1607 1G/2G polymorphism; *HinfI* for MMP-*2* C-735T polymorphism; *BstU1* for the –460 C/T polymorphism; *BsmFI* for the +405 C/G polymorphism, *HhaI* for the -141 A/C polymorphism and 2.5 µl deionise H_2O at 37 and 60°C for overnight incubation.

The enzyme digested products were analyzed on a 3.0% agarose gel prestained with ethidium bromide (0.5 µg/ ml) for visualization under ultraviolet light. Gels (20 x 20 cm) were run at 100 mV in 1xTBE buffer for 60 min. Three genotypes were distinguished for MMP-2 C-735T polymorphism; CC wild-type nondigested (300 bp), TT homozygote digested (254+46 bp) and heterozygotes CT (300+254 +46 bp); for MMP-*1* -1607 1G/2G polymorphism; 1G/1G wild-type nondigested (118 bp), 2G/2G homozygote digested (89+29 bp) and heterozygotes 1G/2G (118+89+29 bp). The –460C allele (175 bp product size) was cut into two fragments of 155 and 20 bp, while the –460T allele remained uncut (175 bp). The +405G allele (304 bp product size) was cut into two fragments of 193 and 111 bp, while the +405C allele remained uncut (304 bp). For the 141A/C polymorphism (263 bp PCR product size); AA genotype was present with 159 bp and 104 bp fragments; AC genotype was 35, 104, 124, and 159 bp fragments; CC genotype was 35, 104, and 124 bp fragments.

Statistical Analysis

Statistical analysis was performed using SPSS statistical package (V13.0). The genotypic distribution amongst subjects was tested for Chi-Square test. The allele ratios and genotype distributions in the cases and controls were analysed with Chi-Square test. Odds ratios were calculated together with their 95% confidence intervals (CI). All P-values were two-tailed and 95% confidence intervals (CI) were given.

RESULTS

Case-Control Analysis

To investigate the possible role of VEGF and MMP gene polymorphisms for HCC risk and prognosis, we analysed two polymorphisms in the MMP gene and three polymorphisms in the VEGF gene in a case–control study. Genotypic distributions of the VEGF -141A/C, -460 C/T and +405 C/G; MMP2 -735 C/T and MMP1 -1607 1G/2G polymorphisms were in Hardy–Weinberg equilibrium in patients and control groups ($P > 0.05$). In case-control analysis, the distribution of genotypes and allele frequencies did not differ from those in the control group ($P > 0.05$) (Tables 2 and 3).

For VEGF polymorphism, the -460 genotype frequencies amongst the 73 cases were CC = 24.7%, CT = 38.4% and TT = 37.0%; the C and T allele frequencies were 43.8 and 56.2%, respectively. The -460 genotype frequencies amongst the 62 controls were CC = 32.3%, CT = 38.7% and TT = 29.0%; the C and T allele frequencies were 51.6 and 48.4%, respectively. There was no statistically significant difference in the genotype distribu-tions ($\chi^2_{(2;0,05)} = 1.325$ $P = 0.515$) or allele frequencies ($\chi^2_{(2;0,05)} = 1.627$ $P = 0.202$) of VEGF -460 between cases and controls. The +405 genotype frequencies amongst the 73 cases and 90 controls were GG = 32.9 and 44.4%,

Table 2. Genotype distributions and allele frequencies for the studied VEGF gene polymorphisms in patients and controls.

Polymorphism	Genotype distribution	Patients group (n: 73)		Control group (n: 62, 88, 90)		$\chi^2_{(2;0,05)}$	p	OR	95% CI
			n	n	%				
-141A/C	AA	60	82.2	76	86.4	0.529	0.467 NS	1	Ref
	AC	13	17.8	12	13.6			1.372	0.584- 3.225
	CC	-	-	-	-			-	-
	Allele frequency								
	A	133	91.1	164	93.2	0.485	0.486 NS	1	Ref
	C	13	8.9	12	6.8			1.336	0.590- 3.025
-460 C/T	**Genotype distribution**								
	CC	18	24.7	20	32.3	1.325	0.515 NS	1	Ref
	CT	28	38.4	24	38.7			1.296	0.561- 2.998
	TT	27	37.0	18	29.0			1.667	0.697- 3.988
	Allele frequency								
	C	64	43.8	64	51.6	1.627	0.202 NS	1	Ref
	T	82	56.2	60	48.4			1.367	0.845- 2.210
+405 C/G	**Genotype distribution**								
	CC	14	19.2	9	10,0	3.829	.147 NS	1	Ref
	CG	35	47.9	41	45.6			1.423	0.722- 2.802
	GG	24	32.9	40	44.4			2.593	0.975- 6.897
	Allele frequency								
	C	63	43.2	59	32.8	3.704	.054 NS	1	Ref
	G	83	56.8	121	67.2			1.557	0.991- 2.446

*Ref, reference value. A p value was considered significant when <0.05.

GC = 47.9 and 45.6%, CC = 19.2 and 10.0%; respectively. The allele frequencies were G = 56.8% (cases) and 67.2% (controls) and C=43.2% (cases) and 32.8% (controls). There was no statistically significant difference in the genotype distributions ($\chi^2_{(2;0,05)}$= 3,829 P = 0.147) or allele frequencies ($\chi^2_{(2;0,05)}$= 3.704 P= 0.054) of VEGF +405 between cases and controls. The -141 genotype frequencies amongst the 73 cases and 88 controls were AA = 82.2 and 86.4%, AC = 17.8 and 13.6% CC=0; respectively. The allele frequencies were A = 91.1% (cases) and 93.2% (controls) and C= 8.9% (cases) and 6.8% (controls). There was no statistically significant difference in the genotype distributions ($\chi^2_{(2;0,05)}$= 0.529 P= 0.467) or allele frequencies ($\chi^2_{(2;0,05)}$=0 .485 P=0.486) of VEGF -141 between cases and controls. For MMP1 polymorphism, the -1607 genotype frequencies amongst the 73 cases were 1G/1G = 9.6%, 1G/2G = 45.2% and 2G/2G = 45.2%; the 1G and 2G allele frequencies were 32.2 and 67.8%, respectively; for the controls including 60 individuals; 1G/1G=11.7%, 1G/2G = 55% and 2G/2G = 33.3%; the 1G and 2G allele frequencies were 39.2 and 60.8%, respectively. There was no statistically significant difference in the genotype distributions ($\chi^2_{(2;0,05)}$ = 1.937 P = 0.38) or allele frequencies ($\chi^2_{(2;0,05)}$=1.402 P = 0.236) of MMP1 -1607 1G/2G between cases and controls. Genotype and allele frequencies for the MMP2 -735 C/T polymorphism were strafied as cases followed by controls, respectively; CC: 60.3%, CT:38.4%, TT:1.4%, C:79.5%, T:20.5%; for control group including 137 individuals CC: 55.5%, CT:37.2%, TT:7.3%, C: 74.1%, T:25.9%. There was no statistically significant difference in the genotype distributions ($\chi^2_{(2;0,05)}$ = 3.405 P = 0.182) or allele frequencies ($\chi^2_{(2;0,05)}$=1.501 P=0.221) of MMP2 -735 C/T between cases and controls.

Genotype frequencies for the VEGF -460, +405, -141, and MMP2 -735 C/T and MMP1 -1607 1G/2G polymorphisms were further analysed based on the size and number of nodules, differentiation and portal vein invasion. In the patients group, no statistically significant difference was found in the distributions of genotypes and allele frequencies of the studied polymorphisms for clinicopathological parameters of HCC (P > 0.05) (Table 4). Only MMP1 -1607 1G/2G polymorphism (2G/2G genotype) was found out to be associated with portal ven invasion in patients which is completely in consistence with Milan Criteria (P < 0.02).

Post-Transplantation Analysis: Survival Versus MMP-Invasion; VEGF-Recurrence

Mean follow-up in the post-OLT period was 5,1887 +/- 1,9

Table 3. Genotype distributions and allele frequencies for the studied MMP gene polymorphisms in patients and controls.

Polymorphism	Genotype distribution	Patients Group (n = 73)		Control group (n=60-137)		$\chi^2_{(2;0,05)}$	p	OR	95% CI
		n	%	n	%				
MMP1-1607 1G/2G	1G/1G	7	9.6	7	11.7	1.937	0.38 NS	Ref	Ref
	1G/2G	33	45.2	33	55			1.000	0.316- 3.169
	2G/2G	33	45.2	20	33.3			1.650	0.504-5.401
	Allele frequency								
	1G	47	32.2	47	39.2	1.402	0.236 NS	Ref	Ref
	2G	99	67.8	73	60.8			1.356	0.819-2.247
MMP2 -735 C/T	**Genotype distribution**								
	CC	44	60.3	76	55.5	3.405	0.182 NS	Ref	Ref
	CT	28	38.4	51	37.2			0.948	0.525-1.714
	TT	1	1.4	10	7.3			0.173	0.021-1.395
	Allele frequency								
	C	116	79.5	203	74.1	1.501	0.221 NS	Ref	Ref
	T	30	20.5	71	25.9			0.739	0.456-1.200

(range, 3-10) years. Mean survival time was 9.518 ± 0.268 (m±SD); (95% CI; 8.992-10.044), the median value was 5 ±1.8. In patients group, the observed survival times were as follows: 1 year 100%, 3 years 95%, and more than 5 years up to 10 years was 39.6%. Patients with a tumor ≤5 cm or no more than three tumors each no larger than 3 cm (selection of patients in consistence with Milan Criteria) consists of 92% of the study group. Results of this group indicated a 4-year survival in 77.3% and a posttransplantation overall survival without recurrence in 96.2% of the patients group. 2 years after OLT, in 2 patients (2.7%), tumor recurrence was obtained. Two (2.7%) patients died without recurrence from non-tumor related diseases (one patient due to acute myocardial infarction 5 years after OLT and one patient due to sepsis 3 years after OLT). No patients received post-transplantation adjuvant chemotherapy or radiotherapy. It was suggested that, because of non-HCC related death events in two patients, Kaplan Meier survival analysis could not carried out. Nonetheless, since recurrence was obtained in only 2 patients of the 73, regression analysis showing association between recurrence-VEGF and MMP-invasion was not obtained.

DISCUSSION

In the present retrospective case–control study, the role of VEGF and MMP gene polymorphisms for HCC risk was investigated underlying the prognostic effect of three VEGF and two MMP gene polymorphisms in 73 Turkish HCC patients who had undergone OLT. According to the results of the current study, no significant association was determined between HCC susceptibility and VEGF -141A/C, -460 C/T, +405 C/G, MMP2 -735 C/T and MMP1 -1607 1G/2G gene polymorphisms in Turkish HCC patients. We have found no significant differences in allele frequencies and genotype distributions between HCC patients and control subjects suggesting that the genotypes examined do not contribute to the HCC risk. Furthermore, in examining genotype frequencies of VEGF and MMP polymorphisms in Turkish HCC patients (n=73) based on clinicopathologic

Table 4. Genotype frequencies of VEGF – MMP polymorphisms in Turkish HCC patients (n = 73) based on clinicopathological parameters of carcinoma.

VEGF – MMP polymorphisms	Clinicopathological parameter								
	Number and dimension of tumor			Differentiation			Portal vein invasion		Healthy control
	Single ≤5 cm	≤3 nodules. each ≤3 cm	≤3 nodules. each <10 cm	GI	GII	GIII-IV	Present	Absent	n= 60[a]-137[b]
MMP1-1607 1G/2G									
1G/1G (%)	4 (10.5)	2(9.5)	1(7.1)	2(9.5)	2(6.2)	3(15)	5(11.3)	2(6.8)	7(11.6)
1G/2G (%)	16(42.1)	10(47.6)	7(50)	9(42.8)	14(43.7)	10(50)	18(40.9)	15(51.7)	33(55)
2G/2G (%)	18(47.3)	9(42.8)	6(42.8)	10(47.6)	16(50)	7(35)	21(47.7)	12(27.2)	20(33.3)
p* value		NS			NS		0.029	NS	
MMP2 -735 C/T									
CC (%)	19(54.2)	15(62.5)	10(71.4)	15(65.2)	19(57.5)	10(62.5)	24(57.1)	20(66.6)	76(59.8)
CT (%)	15(42.8)	9(37.5)	4(28.5)	8(34.7)	14(42.4)	6(37.5)	18(42.8)	10(33.3)	51(40.1)
TT (%)	1(2.8)	-	-	-	-	-	-	-	10
p* value		NS			NS		NS		
VEGF -141A/C									
AA (%)	18(81.8)	19(86.6)	23(79.3)	19(79.1)	19(82.6)	22(84.6)	29(78.3)	31(86.1)	76(86.3)
AC (%)	4(18.1)	3(13.6)	6(20.6)	5(20.8)	4(17.3)	4(15.3)	8(21.6)	5(13.8)	12(13.6)
CC	-	-	-	-	-	-	-	-	-
p* value		NS			NS		NS		
VEGF -460 C/T									
CC (%)	7(25)	8(33.3)	3(17.6)	6(25)	5(22.7)	7(25.9)	11(25)	7(24.1)	20(32.2)
CT (%)	10(35.7)	7(29.1)	7(41.1)	9(37.5)	9(40.9)	10(37)	16(36.3)	12(41.3)	24(38.7)
TT (%)	11(39.2)	9(37.5)	7(41.1)	9(37.5)	8(36.3)	10(37)	17(38.6)	10(34.4)	18(29)
p* value		NS			NS		NS		
VEGF +405 C/G									
CC (%)	4(17.3)	5(19.2)	3(13.6)	3(13.6)	6(23)	5(20)	8(20.5)	6(17.6)	9(10)
CG (%)	11(47.8)	12(46.1)	12(4.5)	9(40.9)	13(50)	13(52)	20(51.2)	15(44.1)	41(45.5)
GG (%)	8(34.7)	9(34.6)	7(31.8)	10(45.4)	7(26.9)	7(28)	11(28.2)	13(38.2)	40(44.4)
p* value		NS			NS		NS		

parameters of carcinoma, it was found that VEGF and MMP genotypes were not significantly associated with clinicopathological parameters like tumor histological differentiation grade, dimension-number of tumor and portal vein invasion except for the MMP1 -1607 1G/2G polymorphism 2G/2G genotype association with portal vein invasion ($P < 0.02$).

Polymorphisms in VEGF and MMP genes, influence the level of protein production of these genes and, are subjected to genetic susceptibility of several disorders. For the last decade, in order to obtain a pathogenic link with various diseases, special efforts were put on searching for the functional SNPs in coding and non-coding segments of VEGF and MMP genes. These were all associated with regulated differential protein expression primarily occurring at the transcriptional level *in vitro* (Renner et al., 2000; Watson et al., 2000; Awata et al., 2002; Shahbazi et al., 2002; Krippl et al., 2003). To date, several SNPs through the VEGF and MMP loci, have been examined in various cancer specimens in order to assess the impact of polymorphisms on cancer risk (Table 5). Previously, association has been reported in case–control studies between VEGF polymorphisms and diseases such as diabetic retinopathy (Ray et al., 2004), melanoma (Howell et al., 2002), lung (Lee et al., 2005), prostate cancer (Lin et al., 2003) and breast cancer (Krippl et al., 2003). Also, an important point was that, while some of them were reported to be the certain

Table 5. Genotyping results of VEGF +405C/G, –460C>T and -141A>C; MMP2 -735 C/T and MMP1 -1607 1G/2G polymorphisms with full or lack of association in several cancer disease susceptibility.

Cancer study	Reference	Polymorphisms analyzed	Number of patients	Association	No association
Cutaneous malignant melanoma	Howell et al., 2002	VEGF+405C/G	152	√	
Endometriosis South Indian women	Bhanoori et al., 2005	VEGF+405C/G	215	√	
Endometriosis Korean women	Kim et al., 2005	VEGF+405C/G	215	√	
Endometriosis South Indian women	Bhanoori et al., 2005	VEGF–460C>T	215		√
Endometriosis Korean women	Kim et al., 2005	VEGF–460C>T	215		√
Endometriosis North Chinese women	Liu et al., 2009	VEGF–460C>T	344	√	
Leiomyoma	Hsieh et al., 2004	VEGF–460C>T	159	√	
Prostate	Lin et al., 2003	VEGF–460C>T	96	√	
Breast	Kataoka et al., 2006	VEGF+405C/G VEGF–460C>T	1093		√
Colorectal	Maltese et al., 2009	VEGF+405C/G VEGF–460C>T	302	√	
Gastric	Kim et al., 2007	VEGF+405C/G VEGF–460C>T	503		√
HCC	Zhai et al., 2007	MMP-1 -1607 1G/2G	434		√
HCC	Wu et al., 2009	MMP-2 C-735T	93		√

risk factors, it was obvious that ethnic origin and size of the samples had a direct effect on the results.

Dysregulated VEGF and MMP gene expressions were linked to a number of pathologies, involving tumour growth and metastasis (Claffey and Robinson, 1996), rheumatoid arthritis (Koch et al., 1994), cancer, diabetes, diabetic retinopathy (Miller et al., 1997), ischaemic heart disease and proteinuric nephropathies, and used as useful genetic markers. Nucleotide variations, seen in MMP and VEGF genes, as spesific genotypes, initiated by different physiological stimuli, may alter protein production/activity, thereby causing interindividual differences with high or low levels of protein production in the occuring of the disease and predisposing patients to VEGF/MMP mediated pathologies. For the first time, in the promoter and 5'UTR region of VEGF gene, fifteen novel polymorphisms were analysed for correlation with variation in VEGF protein production by Watson et al in 2000, and significant correlation was observed between only C+405G polymorphism and VEGF protein production; lowest VEGF protein production observed for CC homozygotes and highest production for GG homozygotes, and, presence of a C allele was suggested to decrease VEGF gene transcription and protein production (Watson et al., 2000). In contrast, Awata et al. (2002) reported +405 C/C genotype linked with higher serum VEGF level than those with other genotypes, and with an increased risk of diabetic retinopathy. In an *in vitro* study of Stevens et al. (2003), it was proved that -460C/+405G haplotype as higher basal VEGF promoter activity than 460T/+405C haplotype and a haplotype containing the –1498C/-634G polymorphism was found to significantly increase basal VEGF promoter activity. Kim et al found no correlation between the level of VEGF or VEGF-C protein expression and four common functional VEGF SNPs (-460T>C, -116G>A, +405G>C, and +936C>T) (Kim et al., 2007). In contrast, lower VEGF plasma levels were correlated with 936T allele (Renner et al., 2000; Krippl et al., 2003). Koukourakis et al. (2004) reported 2578 C/C, 634 G/G, and 1154 A/A and G/A genotypes with low VEGF protein expression in non-small cell lung cancer.

According to previous literature, it is controversial that the measurement should be carried out in serum or plasma, especially in cancer patients (Banks et al., 1998; George et al., 2000; Adams et al., 2000; Poon et al., 2003). Kong's study found that, although plasma VEGF levels were associated with some tumor characteristics, the serum VEGF level may be a more useful marker in HCC patients as it was higher than the plasma VEGF level and was correlated with many clinical features. Yuan et al. (2000) correlated the percentage of tumor tissue that showed positive VEGF protein expression with VEGF mRNA quantified by RTQ RT-PCR, and found good correlation between the VEGF mRNA expression and VEGF protein expression in the tumors. In another study of Yuan et al. (2000), total mRNA from resected lung tissue was analyzed. VEGF mRNA expression correlated strongly and positively with VEGF protein expression (linear regression, $r = 0.91$, $P < 0.001$). Tumoral VEGF mRNA levels correlated strongly with the VEGF protein staining score and microvessel count. The expression levels of total VEGF mRNA and protein in NSCLC are strongly associated with histologic type, tumor angiogenesis, survival and timing of relapse. However, Lichtinghagen et al. (2002) studied mRNA and protein expression patterns of MMP-2, MMP-9, and TIMP-1 in cancerous and noncancerous parts of 17 prostates removed by radical prostatectomy, and found that, mRNA and protein expression of MMP-2, MMP-9 and TIMP-1, respectively, did not show any significant relationships. In these reports, Yuan and Kong reported contrast findings on VEGF pattern; at transcriptional level mRNA is detected generally from the tissue, as well as the immunohistochemical protein detection system from the tissue. Only Kong et al reported serum/plasma/tissue expression levels. Future studies are needed to confirm this issue in Turkish HCC patients. VEGF and MMP single nucleotide polymorphism, haplotype association, and gene expression studies in hepatocelular carcinoma have been limited.

Yamamoto et al. (1999) showed an association between enhanced secretion of active matrix metallo-proteinases (MMPs; gelatinase A and matrilysin) and early recurrence in HCC, and overexpression of MMP mRNA was associated with portal invasion, intrahepatic metastasis and recurrence within the first postoperative year ($P < 0.05$). Guo et al. (2006) found that (90 patients who underwent curative hepatic resection for HCC) the expression of MMP-2, MMP-9 and VEGF was correlated to the recurrence of HCC patients. The positive correlations were found between MMP-2 and VEGF; MMP-9 and VEGF. In an immunohistochemical analysis of tissue samples from 82 HCC patients with cirrhosis who had undergone LT, expressions of MMP-2 ($P = 0.0312$) and *MMP-9* ($P=0.0280$) in stromal compart-ment were reported as significant predictors in predicting HCC recurrence, while VEGF, MMP-2 and MMP-9 in tumor compartment were not significantly associated with poor prognosis (Zhang et al., 2006). The expression of MMP-2 and VEGF in cancer tissue was related to the recurrence in primary HCC patients who underwent tumor resection (Cui et al., 2004). So far, except for expression analysis, only one VEGF polymorphism study was carried out on HCC, indicating -2578/-1203/-1190/-1179/-1154/-634/-7/+936 polymorphisms of VEGF gene as significant prognostic indicators for HCC patients who received thetreatments other than OLT (Kong et al., 2007). However, no association between circulating VEGF levels (serum or plasma), tumor VEGF expression or MVD assessed by immunostaining on 63 specimens and any VEGF gene polymorphism were found, and as an explanation, it was linked to the complex effect of many clinical factors such as tumor burden on VEGF

concentration together with the certain molecules that regulate VEGF levels.

The role of the MMP polymorphisms in HCC, however, has never been specifically investigated. Previous studies have suggested that the functional polymorphisms in the promoters of MMP genes were associated with the risk of cancers, but no study has ever explored these polymorphisms as risk factors for HCC. To date, seven polymorphisms in the promoters of six MMP genes; MMP-1 -1607 1G/2G (rs1799750), MMP-2 C-1306T (rs243865) and C-735T (rs2285053), MMP-3 -1612 5A/6A (rs3025058), MMP-9 C-1562T and six MMP polymorphisms, including four functional polymorphisms in the promoters of MMP-7 (A-181G and C-153T) and MMP-*8* (C-799T and A-381G), and two nonsynonymous polymorphisms in MMP-10 (A180G) and MMP-21 (C572T) were reported with no association in susceptibility to HCC (Zhai et al., 2007). Also, abnormal expression rate and incidence of MMP2 was established to be correlated with HCC recurrence and malignancy (Wu et al., 2008). Recently, functional gene polymorphisms in the promoter regions of MMP-1 1G/2G, MMP-3 5A/6A and MMP-9 C/T have been investigated, and have been reported to be associated with the prognosis of various cancers. In a population based study, unlike MMP-1 and MMP-9 genotypes, MMP-3 5A allele, with higher transcriptional activity, was reported to be a risk factor for the poor prognosis of HCV-related HCC patients (Okamoto et al., 2005).

Apart from the common assessed functional genes, in Marsh's study (2003), tumor suppressor genes (APC, CDKN2A, DCC, MET, MYC1, OGG1, p34, p53, PTEN) were found out as biomarkers for recurrence-free prognostication in HCC patients undergoing liver transplantation. So, microdissection genotyping of hepatocellular carcinoma in liver transplant recipients provides predictive power for determining recurrence-free survival.

In this study, we have also searched for both the significance of viral etilogy and the viral and non-viral relation to HCC. Viral etiology (hepatitis B and C infections) in Turkish population was found to be an important factor in development of the disease as in accordance with the reports of Alacacioglu et al. (2008). Hepatitis B infection was found as the primary risk factor (60.2%) for HCC development, followed by nonviral factors (27.3), and HCV infection (12.3) in the 73 Turkish HCC patients. Possibly due to the small sample sizes and successful post-OLT events involving low recurrence rates (2), together with the low mortal cases (2), a significant correlation was not observed between the clinicopathological parameters, recurrence, survival, VEGF/MMP SNPs and the relation of the HBV or heterogeneous origin of HCC ($P > 0.05$) (data not shown). Kiyici et al. (2008) analyzed the relationship between HBV and HCC recurrence in a large cohort involving two hundred eighty-seven HBV patients with OLT (72 also with HCC) and found that HBV and HCC recurrence were significantly high among the patients with OLT underscoring a powerful association between HBV and HCC recurrence. In a multicenter study consecutively from five hospitals in Turkey, two hundred and twenty-one patients with HCC were analyzed and, hepatitis B infection was determined as the primary risk factor (44.4%) for HCC development in the Turkish population (Alacacioglu et al., 2008). In the epidemiologic studies of Uzunalimoglu et al. (2001), hepatitis B infection was shown as the most important risk factor in Turkey. In both studies, hepatitis C infection has been found to be the secondary risk factor. However, HCV etiology was reported as the most important risk factor for HCC in Europe and the other countries of the Mediterranean (Borzio et al., 2007; Markovic et al., 1998; Stroffolini et al., 1998).

As can be seen, in various reports, a certain correlation was observed between neither the SNPs and the disease susceptibility, nor the SNPs and the protein production, and the results remain inconsistent/controversial due to the effect of genotypes and haplotypes in various ethnicities or sample size divergencies. Different genotypes and different alleles of the different SNPs in the same disease, but in different ethnicities, demonstrate contrast results and risk of developing a certain disease. Functional polymorphisms may affect gene expression regulation and contribute to the differences between individuals in susceptibility to diseases. Enhanced rate of transcription of a specific polymorphic allele compared to non-polymorphic sequences may constitute the association to disease susceptibility. The effect may be caused by a single polymorphism, or by the combination with the any other single nucleotide polymorphisms. Remarkably, the haplotype effect of the alleles may be more significant than a single polymorphism alone in susceptibility to diseases. It is suggested that there might be some differences in the same association results in different cohorts due to the ethnicity, selection criteria, sample size of patients, molecular genetic analysis procedure, sensitive phenotyping and genotyping and the investigated polymorphism which may have important keys in functionality are critical.

Orthotopic liver transplantation is accepted currently as the most effective treatment of HCC in the world by most of the transplantation centers since an entire oncologic resection and certain treatment of end stage liver disease, HCC, are performed. Although, in 42% of the patients portal vein invasion was present, results of this group (92% of the study group consisted one single tumor ≤5 cm or no more than three tumors each of which no larger than 3 cm) indicated a 4-year excellent survival in 77.3% and a posttransplantation overall survival without recurrence in 96.2% of the patients group. In a recent study (Mazzaferro et al., 1996), 48 HCC patients with a single tumor no larger than 5 cm or with up to three

tumors, none larger than 3 cm, underwent transplantation and the results included a 4-year overall survival rate of 75% and a 4-year recurrence-free survival rate of 83%.

Poor association has been seen between the studied polymorphisms of VEGF and MMP genes and invasion, angiogenesis, survival and prediction of early recurrence after OLT in Turkish HCC patients. Also, OLT provided long-term disease-free survival for patients with HCC, even those with locally advanced tumors who had no effective alternative treatment than transplantation. To show the association of polymorphic alleles with portal vein invasion and Milan criteria, bigger sample sizes are needed. Our data confirmed that there was no significant difference relative to survival rates between living-related and deceased donors, and in and exceeding the Milan criteria (data not shown); only living related donors provide a reliable source for the organ pool. OLT provided long term disease free survival in the two different donor type even in patients with locally advanced tumors (8%) who had no effective alternative treatment than transplantation and in patients with HCC that exceeds the Milan criteria.

In conclusion, satisfactory results can be achieved with life-saving procedure, OLT, in patients with HCC by mandatory follow-up involving serial AFP screening and combined radiologic imaging studies. Moreover, the necessities of understanding the effect of gene polymorphisms both on the transcriptional and the translational phases on the clinicopathological phenotype of HCC are growing. Further functional confirmation on the genotype effect in a larger multi-ethnic study is required to determine the roles of the polymorphic alleles of the spesific suspicious gene SNPs on the level of gene transcription and ultimately protein production in PBMCs and in other cell types, however these molecular and post-OLT findings may be useful information for the clinical therapeutic trials targeting MMP and VEGF pathways. Accordingly, further studies on the functional relevance with larger samples are needed to feature the correlation between not only VEGF and MMP gene polymorphisms alone but also the combined haplotypes having LD in the certain gene cluster and blood (serum/plasma)/tissue mRNA and protein expression levels in HCC to confirm our findings.

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