Role of *VEGF-C* Gene Polymorphisms in Susceptibility to Hepatocellular Carcinoma and Its Pathological Development

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Background: Vascular endothelial growth factor C (VEGF-C), an angiogenic/lymphangiogenic factor with high expression levels in tumor tissues, plays important roles in the development of several malignancies including hepatocellular carcinoma (HCC). The purpose of this study was to examine whether *VEGF-C* gene polymorphisms are associated with susceptibility to HCC and its clinicopathological development. *Methods:* Genetic polymorphisms of *VEGF-C* of 135 patients with HCC and 520 noncancer controls were analyzed by a real-time polymerase chain reaction (PCR). *Results:* We found that a significantly ($P = 0.021$) higher risk for HCC was shown in individuals with the *VEGF-C* rs1485766 A/A genotype compared to those with wild-type homozygotes; a high frequency of an advanced stage and a low frequency of being positive for cirrhosis were respectively shown in HCC patients with the *VEGF-C* rs7664413 CT/TT and rs3775194 GC/CC genotypes. Moreover, we found that the *GGACA, GACTG, CGATG,* and *GGCTG* haplotypes of five *VEGF-C* single-nucleotide polymorphisms (SNPs) combined were also related to the risk of HCC. *Conclusions:* Our results suggest that the *VEGF-C* rs1485766 SNP and either of five haplotypes combined might contribute to a prediction of susceptibility to HCC. The genetic polymorphism of *VEGF-C* rs7664413 might be a predictive factor for advanced-stage HCC. J. Clin. Lab. Anal. 28:237–244, 2014. © 2014 Wiley Periodicals, Inc.

Key words: vascular endothelial growth factor C; hepatocellular carcinoma; single-nucleotide polymorphism

INTRODUCTION

Genetic factors were found to be vital variants for mediating the susceptibility of an individual to cancer.

When a single nucleotide in the shared sequence of a gene differs between members of a species or paired chromosomes, such as a single-nucleotide polymorphism (SNP), certain diseases may occur or develop in that individual (1). Cancer is a familiar disease involving genetic mutations or variations; genotyping related SNPs and analyzing their distribution frequencies in a community is a strategy often used to predict the risk and prognosis of cancers.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the second leading cause of cancer-related deaths in Taiwan (2, 3). It is known that multiple risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, cirrhosis, carcinogen exposure, excessive alcohol consumption, and a variety of SNPs, contribute to hepatocarcinogenesis (3–6). Although the development of HCC may take 20∼50 years, early detection of this cancer is seldom available because of the lack of reliable markers (7). Numerous studies found that several SNPs in selected candidate genes, such as *insulin-like growth factor 2 (IGF-2), IGF-2R, interleukin-8, reversion-inducing cysteine-rich protein with Kazal motifs,* and *survivin*, are associated with HCC cancer risks and might be appropriate to serve as predictive factors for assessing the risk of HCC (3, 8–10). Since aberrations in some genes might be responsible for the risk and certain clinical features of HCC, exploring and identifying SNPs in certain genes related to HCC should be continued to establish advanced diagnostic markers for HCC (11).

Vascular endothelial growth factor C (VEGF-C) belongs to the platelet-derived growth factor family. VEGF-C is a ligand for both tyrosine kinase receptors, VEGF-R3 and VEGF-R2, but has a higher affinity for VEGF-R3 (12). VEGF-R3 is mainly expressed by lymphatic endothelial cells but is also expressed by certain nonendothelial cells such as osteoblasts (13). The major physiological function of VEGF-C is generally accepted to be the induction of lymphangiogenesis (14). However, it was recently shown that VEGF-R3 may also drive angiogenesis (15, 16).

Accumulating data indicated that the VEGF-C/VEGF-R3 axis is also expressed in a variety of human malignancies (17–19), and this phenomenon was reported to be a possible predictive factor in determining the clinical approach, because it is correlated with lymph node metastasis or a poor prognosis in patients with gastric cancer, HCC, and nonsmall cell lung carcinoma (17–19). Others and we showed that activation of VEGF-C/VEGF-R3 signaling in cancer cells enhances cell mobility and invasiveness and contributes to the promotion of cancer-cell metastasis (17, 20). These findings, taken together, indicate the importance of VEGF-C signaling in tumor progression (growth, invasion, and metastasis) by acting directly on tumor cells.

Impacts of VEGF-C on human cancer metastasis and prognoses are well documented, but the roles of SNPs of the *VEGF-C* gene in HCC susceptibility and clinical features remain poorly investigated. In this research, a case–control study was performed on five SNPs, which are located in the intron or downstream of the *VEGF-C* gene, and we further analyzed the impact of *VEGF-C* gene polymorphisms on the susceptibility to HCC and its pathological development among a Taiwanese population.

MATERIALS AND METHODS

Subjects and Specimen Collection

The present hospital-based case–control study recruited 135 HCC patients between 2007 and 2010 at Chung Shan Medical University Hospital, Taichung, Taiwan. The diagnosis of HCC was based on characteristic criteria of national guidelines for HCC, such as liver tumor tissue diagnosed by histology or cytology irrespective of α-fetoprotein (AFP) titer where imaging data, either computed tomography or magnetic resonance imaging, showed one of the following three cases: (1) one or more liver masses of ≥2 cm in diameter, (2) one imaging study with early enhancement and a high level of AFP of ≥400 ng/ml, or (3) one imaging study with early arterial phase-contrast enhancement plus early venous phase-contrast washout. Meanwhile, during the same study period, 520 race-matched and ethnic group matched individuals were enrolled as the controls that entered the physical examination at the same hospital. These control groups had no self-reported history of cancer of any sites. Personal information and characteristics collected from the study subjects using interviewer-administered questionnaires contained questions involving demographic characteristics and the status of cigarette smoking and alcohol drinking. Nonsmokers were defined as individuals who had never smoked or had smoked for less than 1 year, and others were defined as smokers. Nondrinkers were defined as those who had never drank or had drank less than once per week and/or for less than 1 year, and others were defined as alcohol drinkers. Before conducting this study, approval from the Institutional Review Board of Chung Shan Medical University Hospital was obtained, and informed written consent was obtained from each individual. The whole blood specimens collected from controls and HCC patients were placed in tubes containing ethylenediaminetetraacetic acid (EDTA), and were immediately centrifuged and then stored at −80°C.
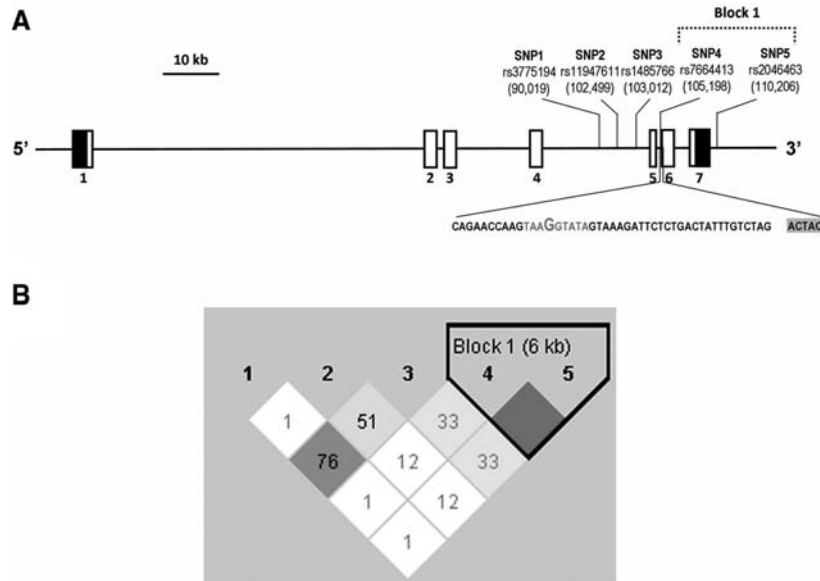
Fig. 1. *VEGF-C* gene, locations of the genotyped variants, and their pairwise LD patterns. Schematic presentation of the *VEGF-C* (gene ID: 7424); (A) indicating locations of the analyzed variants (rs3775194, rs11947611, rs1485766, rs7664413, and rs2046463); (B) the one observed haploblock, and the pairwise LD measure, D′. Black box, untranslated region; white box, coding region. The red color reveals the PESS sequence in intron 5 predicted by the PESXs server. The shading indicates the exon sequence.

Selection of VEGF-C Polymorphisms

In dbSNP database, more than 60 SNPs have been documented in the intron or downstream of the *VEGF-C* gene region. To obtain adequate power for evaluating the potential association, we investigated rs3775194, rs11947611, rs1485766, rs7664413, and rs2046463 (Fig. 1A), those with minor allele frequencies ≥5%. Furthermore, these SNPs of *VEGF-C* gene were selected in this study since these SNPs were found in the cancer patients (21).

Genomic DNA Extraction

Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Valencia, CA) following the manufacturer's instructions. We dissolved DNA in TE buffer (10 mM Tris, 1 mM EDTA; pH 7.8) and then quantified it by measuring the OD260. The final preparation was stored at −20°C and was used to create templates for the polymerase chain reaction (PCR, (22)).

Real-time PCR

Allelic discrimination of the rs3775194, rs11947611, rs1485766, rs7664413, and rs2046463 polymorphisms of the *VEGF-C* gene was assessed with the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and analyzed using SDS version 3.0 software (Applied Biosystems), with the TaqMan assay. The primer sequences and probes for analysis of the *VEGF-C* gene

TABLE 1. TaqMan Primer Sets for *VEGF-C* Genotyped SNPs

SNP	Probe
VEGF-C rs3775194	VIC-5′- AATTTAGCACTATTAAGTTCAAG
	FAM-5′- ATTTAGCACTATTAACTTCAAG
VEGF-C rs11947611	VIC-5′-CTTACTTTTGAAAATGTCA
	FAM-5′-TTACTTTTGAGAATGTCA
VEGF-C rs1485766	VIC-5′-CTTTTTGATTGCCGTGTTA
	FAM-5′-CTTTTTGATTGCAGTGTTA
VEGF-C rs7664413	VIC-5′-CTTTACTATACCTTACTTGG
	FAM-5′-CTTTACTATACTTTACTTGG
VEGF-C rs2046463	VIC-5′- TGTTTAGCACACAGTTTAGT
	FAM-5′- TTTAGCACACGGTTTAGT

polymorphisms are described in Table 1. The final volume for each reaction was 5 µl, containing 2.5 µl TaqMan Genotyping Master Mix, 0.125 µl TaqMan probe mix, and 10 ng genomic DNA. The real-time PCR included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each assay, appropriate controls (nontemplate and known genotype) were included in each typing run to monitor reagent contamination and as a quality control. To validate results from real-time PCR, around 10% of assays were repeated and several cases of each genotype were confirmed by the DNA sequence analysis.

Statistical Analyses

Differences between groups were considered significant for P-values <0.05. Hardy–Weinberg equilibrium (HWE)

was assessed using a goodness-of-fit χ^2 test for biallelic markers. The Mann–Whitney U test and Fisher's exact test were used to compare differences in demographic characteristic distributions between the healthy control group and HCC patients. The adjusted odds ratios (AORs) and 95% confidence intervals (CIs) of the association of genotype frequencies with the risk and clinicopathological characteristics were estimated using multiple logistic regression models after controlling for other covariates. We analyzed all data with Statistical Analytic System (SAS Institute, Cary, NC) software (version 9.1, 2005) for Windows.

RESULTS

The statistical analysis of demographic characteristics is shown in Table 2. There were significantly different distribution of gender ($P < 0.001$) and age distribution ($P < 0.001$) between healthy controls and HCC patients. Hence, the AORs with 95% CIs were estimated by multiple logistic regression models after controlling for these cofounders in each comparison.

The reconstructed linkage disequilibrium (LD) plot of the five SNPs is shown in Figure 1B. We determined one observed haploblock in which rs2046463 and rs7664413 showed 100% LD in our study. Genotype distributions and associations between HCC and *VEGF-C* gene polymorphisms are shown in Table 3. In our recruited control group, the frequencies of *VEGF-C* rs3775194 ($P = 0.696$, χ^2 value: 0.153), rs11947611 ($P = 0.092$, χ^2 value: 2.833), rs1485766 ($P = 0.292$, χ^2 value: 1.110), rs7664413 ($P = 0.403$, χ^2 value: 0.699), and rs2046463 ($P = 0.403$, χ^2 value: 0.699) were in HWE, respectively. Alleles with the highest distribution frequencies for the rs3775194, rs11947611, rs1485766, rs7664413, and

rs2046463 genes of *VEGF-C* in both of our recruited HCC patients and healthy control were, respectively, homozygous for G/G, heterozygous for A/G, heterozygous for C/A, homozygous for C/C, and homozygous for A/A. After adjusting for several variables, there was no significant difference in having HCC for individuals with the rs3775194, rs11947611, rs7664413, and rs2046463 polymorphisms of the *VEGF-C* gene compared to wild-type (WT) individuals. However, subjects with the *VEGF-C* polymorphic rs1485766 AA genotype exhibited significantly ($P = 0.021$) higher risks of 2.012-fold (95% CI = 1.150~3.519) of having HCC, compared to their corresponding WT homozygotes. Moreover, we also analyzed the distribution allele frequencies of these five *VEGF-C* SNPs between HCC and control subjects, and no significant difference in the frequency distributions was shown for any allele between patients with HCC and healthy control subjects (data not shown).

Next, we explored the haplotypes to evaluate the combined effect of the five polymorphisms on HCC susceptibility. The distribution frequencies of the *VEGF-C* rs3775194, rs11947611, rs1485766, rs7664413, and rs2046463 haplotypes in our recruited individuals were analyzed. There were five haplotypes with frequencies >5% among all cases, and the most common haplotype in the control was GACCA (38%); therefore, it was chosen as the reference. Compared to the reference, three *VEGF-C* haplotypes, GGACA, GACTG, and CGATG, showed significantly ($P < 0.05$) increased risks for HCC of 1.598-fold (95% CI = 1.105~2.310), 1.696-fold (95% CI = 1.129~2.548), and 2.13-fold (95% CI: 1.031~4.402), respectively (Table 4). Contrarily, subjects with the *VEGF-C* haplotype, GGCTG, exhibited a significantly ($P < 0.05$) lower risk of 0.303-fold (95% CI = 0.092~0.997) of having HCC (Table 4).

The impact of the *VEGF-C* polymorphic genotype on the pathological development of HCC was initially estimated by comparing the frequency distributions of polymorphic genotypic subgroups and WT genotypic subgroups in HCC patients who had progressed to different clinical statuses including tumor, node, and metastases (TNM) clinical staging, primary tumor size, lymph node involvement, distant metastasis, hepatitis B surface antigen, antibody to HCV, and liver cirrhosis. The comparison showed that the WT or polymorphic genotypes of rs3775194, rs11947611, and rs1485766 SNPs in HCC patients were irrelevant to the development of any HCC clinical pathological variable (Tables 5 and 6). However, among HCC patients, those who had the rs7664413 CT/TT SNP had a higher risk of an advanced clinical stage ($P = 0.0027$) and a trend toward elevated distant metastasis than did WT patients ($P = 0.066$, Table 5). Furthermore, patients with HCC with at least one mutated C allele of rs3775194 had a 0.42-fold lower

TABLE 2. Distributions of Demographic Characteristics of 520 Controls and 135 Patients With HCC

Variable	Controls ($N = 520$)	Patients ($N = 135$)	P-value
Age (years)	Mean $\pm$ SD	Mean $\pm$ SD	
	52.43 $\pm$ 14.67	64.24 $\pm$ 11.08	<0.001[*]
Gender	n (%)	n (%)	
Male	426 (81.9)	92 (68.1)	
Female	94 (18.1)	43 (31.9)	<0.001[*]
Alcohol consumption			
No	309 (59.4)	88 (65.2)	
Yes	211 (40.3)	47 (34.8)	0.222
Tobacco consumption			
No	310 (59.6)	80 (59.3)	
Yes	210 (40.4)	55 (40.7)	0.940

Mann–Whitney U test or Fisher's exact test was used between healthy controls and patients with HCC.
*Statistically significant at $P < 0.05$.

TABLE 3. Distribution Frequency of VEGF-C Genotypes in 520 Healthy Controls and 135 Patients With HCC

Variable	Controls ($N = 520$, n (%))	Patients ($N = 135$, n (%))	OR (95% CI)	AOR (95% CI)
rs3775194				
GG	372 (71.5)	105 (77.8)	1.00	1.00
GC	137 (26.4)	30 (22.2)	0.776 (0.494~1.217)	0.879 (0.546~1.415)
CC	11 (2.1)	0 (0.0)	–	–
GC+ CCI	148 (28.5)	30 (22.2)	0.718 (0.459~1.124)	0.826 (0.515~1.326)
rs11947611				
AA	221 (42.5)	59 (43.7)	1.00	1.00
AG	249 (47.9)	60 (44.4)	0.903 (0.604~1.350)	0.813 (0.530~1.248)
GG	50 (9.6)	16 (11.9)	1.199 (0.637~2.255)	1.323 (0.671~2.609)
AG + GG	299 (57.5)	76 (56.3)	0.952 (0.650~1.395)	0.889 (0.592~1.334)
rs1485766				
CC	190 (36.5)	43 (31.9)	1.00	1.00
CA	239 (46.0)	59 (43.7)	1.091 (0.705~1.688)	1.254 (0.788~1.997)
AA	91 (17.5)	33 (24.4)	1.602 (0.955~2.689)	**2.012 (1.150~3.519)**[*]
CA + AA	330 (63.5)	92 (68.1)	1.232 (0.823~1.844)	1.451 (0.943~2.232)
rs7664413				
CC	309 (59.4)	72 (53.3)	1.00	1.00
CT	188 (36.2)	55 (40.7)	1.256 (0.846~1.864)	1.262 (0.829~1.921)
TT	23 (4.4)	8 (5.9)	1.493 (0.642~3.473)	1.734 (0.692~4.347)
CT + TT	211 (40.6)	63 (46.7)	1.281 (0.876~1.875)	1.308 (0.872~1.961)
rs2046463				
AA	309 (59.4)	72 (53.3)	1.00	1.00
AG	188 (36.2)	55 (40.7)	1.256 (0.846~1.864)	1.262 (0.829~1.921)
GG	23 (4.4)	8 (5.9)	1.493 (0.642~3.473)	1.734 (0.692~4.347)
AG + GG	211 (40.6)	63 (46.7)	1.281 (0.876~1.875)	1.308 (0.872~1.961)

The ORs and their 95% CIs were estimated by logistic regression models. The AORs with their 95% CIs were estimated by multiple logistic regression models after controlling for age and gender.
*Statistically significant at $P < 0.05$.

risk (95% CI = 0.177~0.998) of liver cirrhosis compared to patients with the G/G homozygote (Table 6).

AFP, Aspartate aminotransferase (AST), and Alanine transaminase (ALT) are common clinical pathological markers of HCC. To clarify the relationship between the progression of the clinical status and the level of clinical pathological markers in HCC patients, we further analyzed levels of these pathological markers associated with *VEGF-C* genotypic frequencies, and results are shown in Table 7. After comparing data of Table 7 with those in Tables 5 and 6, we concluded that levels of clinicopathological liver-related markers did not significantly differ

TABLE 4. Distribution Frequency of VEGF-C Haplotype in Controls and HCC Patients

Variable								
rs3775194 G/C	rs11947611 A/G	rs1485766 C/A	rs7664413 C/T	rs2046463 A/G	Controls ($N = 1040$, n (%))	Patients ($N = 270$, n (%))	OR (95% CI)	P-value
G	A	C	C	A	395 (38.0)	89 (33.0)	Reference	
G	G	A	C	A	175 (16.8)	63 (23.3)	**1.598 (1.105~2.310)**	**0.012**
G	A	C	T	G	123 (11.8)	47 (17.4)	**1.696 (1.129~2.548)**	**0.011**
C	A	A	C	A	86 (8.3)	14 (5.2)	0.722 (0.393~1.330)	0.295
G	A	A	C	A	77 (7.4)	27 (10.0)	1.556 (0.949~2.553)	0.078
G	G	C	C	A	39 (3.8)	4 (1.5)	0.455 (0.159~1.307)	0.134
G	G	C	T	G	44 (4.2)	3 (1.1)	**0.303 (0.092~0.997)**	**0.038**
C	G	A	C	A	29 (2.8)	2 (0.7)	0.306 (0.072~1.306)	0.091
C	G	A	T	G	25 (2.4)	12 (4.4)	**2.130 (1.031~4.402)**	**0.037**
G	G	A	T	G	24 (2.3)	6 (2.2)	1.110 (0.441~2.794)	0.825
C	G	C	T	G	13 (1.3)	2 (0.7)	0.683 (0.151~3.080)	0.618
C	A	C	C	A	5 (0.5)	0 (0)	—	0.289
G	A	A	T	G	4 (0.4)	1 (0.4)	1.110 (0.123~10.047)	0.926
C	A	A	T	G	1 (0.1)	0 (0)	—	0.637

Bold values refer $P < 0.05$.

TABLE 5. Clinical TNM Staging and VEG Factor-C Genotypic Frequencies in 135 HCC Patients

Variable	rs3775194			rs11947611			rs1485766			rs7664413		
	GG (N = 105) n (%)	GC + CC (N = 30) n (%)	P-value	AA (N = 59) n (%)	AG + GG (N = 76) n (%)	P-value	CC (N = 43) n (%)	CA + AA (N = 92) n (%)	P-value	CC (N = 72) n (%)	CT + TT (N = 63) n (%)	P-value
Clinical stage												
Stage I/II	67 (63.8)	17 (56.7)	0.477	35 (59.3)	49 (64.5)	0.540	22 (51.2)	62 (67.4)	0.070	51 (70.8)	33 (52.4)	**0.027***
Stage III/IV	38 (36.2)	13 (43.3)		24 (40.7)	27 (35.5)		21 (48.8)	22 (32.6)		21 (29.2)	30 (47.6)	
Tumor size												
≤T2	70 (66.7)	17 (56.7)	0.313	37 (62.7)	50 (65.8)	0.711	23 (53.5)	64 (69.6)	0.069	51 (70.8)	36 (57.1)	0.097
>T2	35 (33.3)	13 (43.3)		22 (37.3)	26 (34.2)		20 (46.5)	28 (30.4)		21 (29.2)	27 (42.9)	
Lymph node metastasis												
No	99 (94.3)	29 (95.6)	0.604	55 (93.2)	73 (96.1)	0.462	40 (93.0)	88 (95.7)	0.521	69 (95.8)	59 (93.7)	0.568
Yes	6 (5.7)	1 (3.3)		4 (6.8)	3 (3.9)		3 (7.0)	4 (4.3)		3 (4.2)	4 (6.3)	
Distant metastasis												
No	100 (95.2)	29 (96.7)	0.738	55 (93.2)	74 (97.4)	0.246	40 (93.0)	89 (96.7)	0.329	71 (98.6)	58 (92.1)	0.066
Yes	5 (4.8)	1 (3.3)		4 (6.8)	2 (2.6)		3 (7.0)	3 (3.3)		1 (1.4)	5 (7.9)	

*Statistically significant at $P < 0.05$.

between the rs7664413 CC and CT/TT genotypes or between the rs3775194 GG and GC/CC genotypes, although these genotypes presented a significant difference in the HCC clinical status.

DISCUSSION

The familiar major etiologies for HCC in Taiwan include infection with HBV or HCV, habitual alcohol consumption, a disease history of liver cirrhosis, and a family history of HCC (23). Increasing evidence revealed that genomic changes might progressively cause the cellular phenotype to evolve from a preneoplastic stage into HCC; many gene polymorphisms and somatic mutations were identified as being associated with the risk of HCC (3, 8–11). The genetic component is therefore affirmed as a pivotal factor affecting the occurrence of HCC. A variety of genetic and molecular aberrations derived from multiple gene alterations (e.g., allelic deletion, insertion, polymorphism, mutation, and methylation change) were detected with HCC (24). Hence, comparisons of genetic information between patients with HCC and healthy subjects without HCC might be a particularly valuable strategy to determine genes for predicting risk and the pathological development of HCC.

The overexpression of VEGF-C was found in many types of cancers, including HCC (17–19). Recently, some *VEGF-C* SNPs (rs7664413 and rs1485766) were reported

TABLE 6. Clinical Status and VEGF-C Genotypic Frequencies in 135 HCC Patients

Variable	rs3775194			rs11947611			rs1485766			rs7664413		
	GG (N = 105) n (%)	GC + CC (N = 30) n (%)	P-value	AA (N = 59) n (%)	AG + GG (N = 76) n (%)	P-value	CC (N = 43) n (%)	CA + AA (N = 92) n (%)	P-value	CC (N = 72) n (%)	CT + TT (N = 63) n (%)	P-value
HBsAg												
Negative	63 (60.0)	18 (60.0)	1.000	31 (52.5)	50 (65.8)	0.119	23 (53.5)	58 (63.0)	0.291	46 (63.9)	35 (55.6)	0.324
Positive	42 (40.0)	12 (40.0)		28 (47.5)	26 (34.2)		20 (46.5)	34 (37.0)		26 (36.1)	28 (44.4)	
Anti-HCV												
Negative	47 (44.8)	16 (53.3)	0.407	31 (52.5)	32 (42.1)	0.228	21 (48.8)	42 (45.7)	0.730	32 (44.4)	31 (49.2)	0.580
Positive	58 (55.2)	14 (46.7)		28 (47.5)	44 (57.9)		22 (51.2)	50 (54.3)		40 (55.6)	32 (50.8)	
Child-Pugh grade												
A	76 (72.4)	23 (76.7)	0.640	44 (74.6)	55 (72.4)	0.774	32 (74.4)	67 (72.8)	0.845	51 (70.8)	43 (76.2)	0.483
B or C	29 (27.6)	7 (23.3)		15 (25.4)	21 (27.6)		11 (25.6)	25 (27.2)		21 (29.2)	16 (23.7)	
Liver cirrhosis												
Negative	23 (21.9)	12 (40.0)	**0.046**	16 (27.1)	19 (25.0)	0.781	13 (30.2)	22 (23.9)	0.435	15 (20.8)	14 (31.7)	0.149
Positive	82 (78.1)	18 (60.0)		43 (72.9)	57 (75.0)		30 (69.8)	70 (76.1)		57 (79.2)	45 (68.3)	

Anti-HCV, antibody to HCV; HBsAg, hepatitis B surface antigen.
Bold values refer $P < 0.05$.

TABLE 7. Association of VEGF-C Genotypic Frequencies With the HCC Laboratory Status

Characteristic	AFP[a] (ng/ml)	AST[a] (IU/l)	ALT[a] (IU/l)	AST/ALT ratio[a]
rs3775194				
GG	2677.2 ± 1115.8	202.4 ± 37.9	152.8 ± 27.7	1.58 ± 0.12
GC/CC	5943.1 ± 4428.4	104.4 ± 28.1	108.2 ± 29.9	1.23 ± 0.11
P-value	0.301	0.180	0.413	0.135
rs11947611				
AA	4062.6 ± 1971.3	198.1 ± 43.3	158.7 ± 28.9	1.44 ± 0.13
AG/GG	2890.9 ± 1755.2	167.1 ± 42.3	130.5 ± 33.3	1.55 ± 0.14
P-value	0.658	0.614	0.538	0.565
rs1485766				
CC	5571.4 ± 3290.4	155.7 ± 39.5	117.2 ± 21.7	1.43 ± 0.16
CA/AA	2389.4 ± 1149.1	192.3 ± 40.6	154.8 ± 31.5	1.54 ± 0.12
P-value	0.258	0.575	0.440	0.612
rs7664413				
CC	2,144.8 ± 872.0	172.6 ± 29.4	156.8 ± 24.2	1.38 ± 0.10
CT/TT	4,840.8 ± 2,618.1	189.8 ± 55.9	127.0 ± 39.7	1.64 ± 0.17
P-value	0.305	0.778	0.512	0.173

to be correlated with the risk of preeclampsia (25), osteonecrosis of the femoral head (26), and the survival rate with ovarian cancer (22). However, to our knowledge, there are no reports concerning the roles of the *VEGF-C* SNPs in HCC. This study revealed a significant association between the *VEGF-C* rs1485766 polymorphism and HCC. Data in Table 2 show that individuals with the *VEGF-C* polymorphic rs1485766 AA genotype had a higher risk of HCC compared to the WT genotype. The rs1485766 SNP is located in the intron 4 region of the *VEGF-C* gene (Fig. 1A), and the functional importance of this SNP to VEGF-C expression has not been tested experimentally. A previous report indicated that if the intronic SNP of a gene was detected in the RNA extract, the allelic gene expression with intronic SNPs gives very similar estimates to those obtained with exonic SNPs (27). Several reports also indicated that an intronic SNP can affect gene expressions (28, 29). We assumed that *VEGF-C* mRNA levels might also be affected by this intronic SNP. Higher messenger (m)RNA levels might translate into higher protein levels; but, as yet, we have no proof of this assumption.

The expression level of VEGF-C was reported to be correlated with advancing tumor stages in many cancer types including HCC (19, 30–32), and down-regulation of VEGF-C was demonstrated to decelerate tumor growth in vivo (33). In the present study, individuals carrying the *VEGF-C* rs7664413 CT/TT (Table 5) or rs2046463 AG/GG (data not shown) SNPs had a higher risk of an advanced clinical stage and a trend toward elevated distant metastasis. The rs7664413 SNP is located on the intron 5 flanking region (−33 nt upstream) of the *VEGF-C* gene (Fig. 1A). Many alternative splicing (AS) cis-regulated elements are located in this region (34). We further found that rs7664413 SNP was located in a sequence

of a putative exonic splicing silencer (PESS; TAAG-GTATA; Fig. 1A). PESSs are cis-regulatory elements that inhibit the use of adjacent splice sites by interacting with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and often contribute to AS. PESSs regulate AS by recruiting factors that directly interfere with the splicing machinery (35). For example, hnRNP I/PTB binds many exonic splicing silencers and appears to block access to the splicing machinery through protein multimerization (36). Other evidence supports this observation of two splicing variants (ENST00000280193 and ENST00000507638) reported in the Ensemble database (version GRCh37). One encodes the functional VEGF-C protein (NM_005429, 420 amino acids), while the other only processes transcripts (CF128431, without protein production, EST sourced from a chondrosarcoma lung metastatic cell line). Those data suggest that the rs7664413 SNP might affect *VEGF-C* mRNA splicing. However, further specifically designed studies are needed to verify the effects and underlying mechanism of the polymorphic rs7664413 on premessenger RNA splicing.

In summary, the *VEGF-C* polymorphic rs1485766 AA genotype might increase the risk for HCC. A combination of the *GGACA, GACTG, CGATG,* and *GGCTG* haplotypes of the five *VEGF-C* SNPs (rs3775194, rs11947611, rs1485766, rs7664413, and rs2046463) might also contribute to predicting susceptibility to HCC. After adjusting for other confounding factors, HCC patients with the *VEGF-C* polymorphic rs3775194 GC/CC genotype showed a low frequency for cirrhosis positivity, while patients with the *VEGF-C* polymorphic rs7664413 CT/TT genotype had a high risk for developing clinical stage III or IV compared with those with the C/C homozygote.

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