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Association of *VEGF* genotype with mRNA level in colorectal adenocarcinomas

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Abstract

The mRNA expression of vascular endothelial growth factor (VEGF) was evaluated in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues in 18 Japanese patients. The expression was confirmed to be up-regulated in the colorectal adenocarcinomas, when compared with the noncancerous tissues. Twelve genotypes of *VEGF*: six positions in the promoter region, two in the 5′UTR, and four in the 3′UTR, and their association with the expression of VEGF mRNA were evaluated. While G-1877A, T-1455C, G-1154A, C702T, and G1612A were not detected, C-2578A, T-1498C, G-1190A, C-634G, C-7T, C936T, and C1451T were found at allele frequencies of 4/36, 15/36, 15/36, 20/36, 8/36, 6/36, and 6/36, respectively, suggesting that C-2578A, G-1154A, and G1612A were associated with a decreased risk for colorectal adenocarcinoma. T-1498C (G-1190A) and C-7T were found to be associated with higher levels of VEGF mRNA, and may be a risk factor for the development of liver metastasis and/or prognosis of colorectal adenocarcinoma.

Keywords: VEGF; Genotype; mRNA expression; Colorectal adenocarcinoma

The prognosis for colorectal cancer remains poor, mainly due to liver metastasis. Although direct observation and biopsy sampling by endoscope have enabled us to understand the natural history of the disease, it is still difficult to predict the development of liver metastasis.

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The liver metastasis depends on the stage of colorectal cancer, and its cascade is a complex multi-step process involving several genetic alterations, angiogenesis, and tissue proteolysis. A number of molecular markers have been examined to predict the development of liver metastasis and/or prognosis of colorectal cancer, including oncogenes, tumor suppressor genes, apoptosis-related proteins, cytokines, growth factors, adhesion molecules, and matrix metalloproteinases [1,2], but a diagnostic methodology with excellent predictability has not yet been established.

Vascular endothelial growth factor (VEGF), first termed vascular permeability factor (VPF), was discovered in the 1980s [3–8]. VEGF is now recognized as an endothelial cell-specific mitogen and survival factor, and is expected to be involved in the pathogenesis of cancer metastasis, retinopathy, age-related macular degeneration, rheumatoid arthritis, and psoriasis [9–11]. Clinical observations have confirmed that VEGF expression is closely associated with the extent of vascularization and prognosis in many solid tumors, and is predictive of resistance to radiotherapy, chemotherapy, and endocrine therapy [9–11]. As for colorectal cancer, it has been recently demonstrated that the VEGF expression is associated with disease progression, microvessel density, venous invasion, lymph node and/or liver metastasis, and prognosis [12–16]. Although reports have not always provided similar conclusions [17,18], VEGF may be a key molecule for predicting the prognosis of colorectal cancer.

The *VEGF* gene is located on chromosome 6p21.3 and comprises a 14-kb coding region with 8 exons and 7 introns, and alternative exon splicing results in the production of four major isoforms of $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, and minor isoforms [9–11]. The promoter region, 5′ untranslated region (UTR), and 3′UTR have been screened for polymorphisms, and the genetically controlled variation in VEGF production was examined in peripheral blood mononuclear cells (PBMCs) or plasma [19–22]. C-2578A and G-1154A in the promoter region and C936T in the 3′UTR have recently been suggested to be associated with a decrease in prostate or breast cancer risk [22,23] and reduction in the invasive potential of malignant melanomas [24]. These polymorphisms were found to result in lower levels of VEGF production [20–22], suggesting that cancer develops via regulation of the antitumor immune response and/or tumor angiogenesis by VEGF [25]. In the present study, the effect of *VEGF* genotype on the expression level of VEGF mRNA was examined in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues, which were obtained as surgical samples from Japanese patients. Herein, the effects of 12 genotypes of VEGF: C-2578A, G-1877A, T-1498C, T-1455C, G-1190A, and G-1154A in the promoter region, C-634G and C-7T in the 5′UTR, and C702T, C936T, C1451T, and G1612A in the 3′UTR, were examined. These polymorphisms were investigated in termspoint of their association with various types of disease or clinical situation, i.e., acute renal allograft rejection [20], survival of peritoneal dialysis patients [26], diabetic retinopathy [27], spontaneous preterm delivery [28], giant cell arteritis [29], and pre-eclampsia [30].

Materials and methods

Human colorectal adenocarcinomas and adjacent noncancerous colorectal tissues. Colorectal adenocarcinomas were obtained with adjacent noncancerous colorectal tissues as surgical samples from 18 unrelated patients (9 men and 9 women) with primary colorectal adenocarcinoma diagnosed at Kobe University Hospital, Japan. The patients had not undergone cancer chemotherapy. The samples were obtained immediately after resection, quickly stripped of connective tissue, divided into colorectal adenocarcinomas and adjacent noncancerous colorectal tissues, snap-frozen, and stored at −80 °C prior to processing. The expression levels of VEGF mRNA in both samples were evaluated by real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) method and *VEGF* genotypes were evaluated using both samples as described below. The age range was 29–79 years with an average of 65.0 ± 11.0. Informed consent was obtained from all subjects prior to their participation in the study. The protocol was approved by the Institutional Review Board of Kobe University Hospital, Kobe University, Japan.

VEGF mRNA levels in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues. VEGF mRNA expression was determined by real time quantitative RT-PCR method as described before [31,32]. Briefly, total RNA was extracted from the samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and an RNase-Free DNase Set (Qiagen) according to the manufacturer's directions. In each run of the assay, the mRNA levels of VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed using samples serially diluted 4-fold from the authentic sample, and the levels of VEGF mRNA were expressed relative to the concentration of GAPDH mRNA. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. The primer pairs and TaqMan probe for VEGF mRNA (GenBank Accession No. M27281), designed using the Primer Express 1.0 program (Applied Biosystems, Foster City, CA, USA), were as follows: the forward primer: 5′-CCA CTG AGG AGT CCA ACA TCA C-3′; the reverse primer: 5′-CAT CTC TCC TAT GTG CTG GCC T-3′; and the TaqMan probe: 5′-TGC AGA TTA TGC GGA TCA AAC CTC ACC-3′. The primers and TaqMan probe were synthesized by Hokkaido System Science (Sapporo, Japan). The primers and TaqMan probe for GAPDH were purchased from Applied Biosystems (TaqMan GAPDH Control Reagent Kit).

VEGF genotyping. VEGF genotypes were evaluated by a direct sequencing method for C-2578A, G-1877A, T-1498C, T-1455C, G-1190A, and G-1154A in the promoter region, C-634G and C-7T in the 5′UTR, and C702T, C936T, C1451T, and G1612A in the 3′UTR. Twenty-five milligrams of tissue sample cut up into small pieces was placed in a 1.5-ml microcentrifuge tube, and then genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's directions. Each fragment containing the polymorphic site was amplified by PCR with a Gene Amp PCR Reagent Kit (Takara Shuzo, Kyoto, Japan). Table 1 shows the primer pairs which were designed using the Primer Express 1.0 program (Applied Biosystems). The primers were synthesized by Hokkaido System Science. PCR amplification was carried out in a 20 μl reaction mixture using approximately 500 ng of genomic DNA. Each PCR consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of 94 °C for 20 s,

Table 1
Sequences of PCR primers used for the determination of genetic polymorphisms of the *VEGF* gene

Polymorphism	Primer	Sequence	Flanking sequence of the variant			GeneBank accession number (ID in dbSNP database)
C-2578A	Forward	5′-TCT CAG TCC ATG CCT CCA CA-3′	CTGCAGACCCTGGCA	C/A	GATCTGGGTGGATAA	AF095785
	Reverse	5′-GAA GAT GTG GAG AGT TGG AGG AA-3′				(rs699947)
G-1877A	Forward	5′-TGC CAA ATT CTT CTC CCC TG-3′	GGACACTTCCCAAAG	G/A	ACCCCAGTCACTCCA	AF095785
	Reverse	5′-GAT GGC ACA TTG TCA GAG GGA-3′				(unknown)
T-1498C	Forward	5′-TTC GAG AGT GAG GAC GTG TG-3′	GTGTGGGGTTGAGGG	T/C	GTTGGAGCGGGGAGA	AF095785
	Reverse	5′-CAA AGA GGG AAC GGC TCT CA-3′				(rs833061)
T-1455C	Forward	5′-TTC GAG AGT GAG GAC GTG TG-3′	ACTCCAGGATTCCAA	T/C	AGATCTGTGTGTCCC	AF095785
	Reverse	5′-CAA AGA GGG AAC GGC TCT CA-3′				(rs833062)
G-1190A	Forward	5′-CAA AGA GGG AAC GGC TCT CA-3′	GGCCAGGCTTCACTG	G/A	GCGTCCGCAGAGCCC	AF095785
	Reverse	5′-TTT AAA AGT CGG CTG GTA GCG-3′				(unknown)
G-1154A	Forward	5′-CAA AGA GGG AAC GGC TCT CA-3′	CGAGCCGCGTGTGGA	G/A	GGGCTGAGGCTCGCC	AF095785
	Reverse	5′-TTT AAA AGT CGG CTG GTA GCG-3′				(rs1570360)
C-634G	Forward	5′-GGA AAC CAG CAG AAA GAG GAA A-3′	TGCGAGCAGCGAAAG	C/G	GACAGGGGCAAAGT	AF095785
	Reverse	5′-CTG ACG GAC AGA CAG ACA GAC AC-3′				(rs201963)
C-7T	Forward	5′-GAG GAA GAG TAG CTC GCC GA-3′	CCCCGGTCGGGCCTC	C/T	GAAACCATGAACTTT	AF095785
	Reverse	5′-CCT CCA CCA TGC CAA GGT A-3′				(rs25648)
C702T	Forward	5′-CTG CTC TTA TGG TGC CGG A-3′	GCCGAGGCGGTGAGC	C/T	GGGCAGGAGGAAGG	S85192
	Reverse	5′-CAG ATC TCT CAC CAG GAA AGA CTG-3′				(unknown)
C936T	Forward	5′-AAA TGA AGG AAG AGG AGA CTC TGC-3′	GCGGGTGACCCAGCA	C/T	GGTCCCTCTTGGAAT	AF024710
	Reverse	5′-TTT CTG GGA TTC CTG TAG ACA CAC-3′				(rs3025039)
C1451T	Forward	5′-AGA CGG ACA GAA AGA CAG ATC ACA-3′	CAGGGATGAGGACAC	C/T	GGCTCTGACCAGGAG	AF024710
	Reverse	5′-TGT TGG AAG AAG CAG CCC A-3′				(rs3025040)
G1612A	Forward	5′-AGA CGG ACA GAA AGA CAG ATC ACA-3′	TGAGTTGCCCAGGAG	G/A	CCACTGGCAGATGTC	AF024710
	Reverse	5′-TGT TGG AAG AAG CAG CCC A-3′				(rs10434)

58–60 °C for 20 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The temperature was controlled by a programmable heat block (GeneAmp PCR System 9700, Applied Biosystems). The PCR products were directly sequenced using an automatic ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical analysis. Values are given as means ± standard deviation. The statistical significance of differences between mean values was calculated using Wilcoxon's signed rank test or Mann–Whitney test. *P* values less than 0.05 (two-tailed) were considered significant.

Results

Fig. 1 shows the relative concentrations of VEGF mRNA in 18 sets of colorectal adenocarcinomas and adjacent noncancerous colorectal tissues. VEGF mRNA expression in colorectal adenocarcinomas (VEGF mRNA/GAPDH mRNA: 0.313 ± 0.641) was significantly up-regulated, when compared with levels in adjacent noncancerous colorectal tissues (VEGF mRNA/GAPDH mRNA: 0.042 ± 0.044, $P < 0.05$).

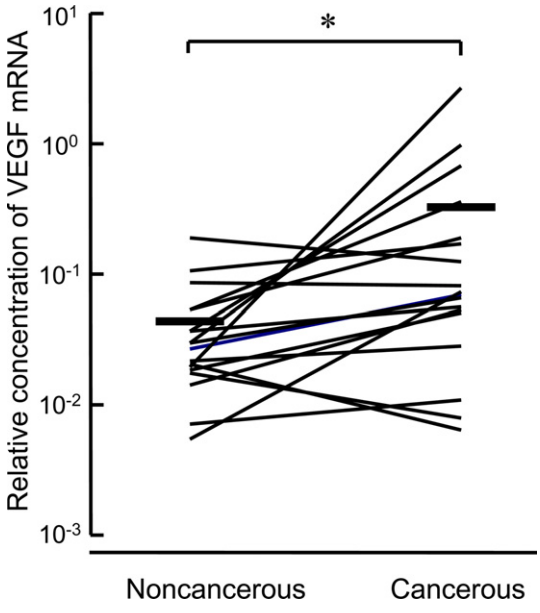

Fig. 1. Relative concentrations of VEGF mRNA in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues obtained from 18 Japanese patients. VEGF mRNA expression was determined by real time quantitative PCR with GAPDH as an internal control. Each bar represents the mean value. *$P < 0.05$, significantly different from the adjacent noncancerous colorectal tissues.

A total of 12 genotypes of the *VEGF* gene were also evaluated using colorectal adenocarcinomas, but G-1877A, T-1455C, G-1154A, C702T, and G1612A were not detected (Table 2). C-2578A, T-1498C, G-1190A, C-634G, C-7T, C936T, and C1451T were found at allele frequencies of 4/36, 15/36, 15/36, 20/36, 8/36, 6/36, and 6/36, respectively, and linkage was found between T-1498C and G-1190A, and C936T and C1451T (Table 2). The genotypes were also determined in adjacent noncancerous colorectal tissues, but were the same as those in corresponding colorectal adenocarcinomas.

Fig. 2 shows the effects of *VEGF* T-1498C (G-1190A), C-634G, C-7T, and C936T (C1451T) on VEGF mRNA expression in colorectal adenocarcinoma. The effect of C-2578A was not assessed due to a relatively low frequency (Table 2). T-1498C (G-1190A) and C-7T resulted in higher levels of VEGF mRNA; TT^{-1498} (GG^{-1190}), 0.412 ± 0.737 for TC^{-1498} (GA^{-1190}) + CC^{-1498} (AA^{-1190}) ($p < 0.05$), and 0.059 ± 0.050 for CC^{-7} and 0.712 ± 0.925 for $CT^{-7} + TT^{-7}$ ($P < 0.05$). C-634G tended to increase the expression, but C936T (C1451T) had no effect. The effects of these polymorphisms on VEGF mRNA expression were also examined in adjacent noncancerous colorectal tissues, but the expression was independent of these polymorphisms (Fig. 3).

Discussion

In the present study, the level of VEGF mRNA was examined in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues, which were obtained as surgical samples from Japanese patients. It was con-

Table 2
VEGF genotypes in 18 Japanese patients with colorectal adenocarcinoma

Position	Subject No.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
C-2578A	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	A/A	–/–	A/A	–/–	–/–	–/–	–/–	–/–
G-1877A	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
T-1498C	–/–	–/–	–/C	–/C	–/C	–/C	–/C	–/C	–/C	–/C	C/C	–/–	C/C	–/C	–/–	–/C	–/C	–/–
T-1455C	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
G-1190A	–/–	–/–	–/A	–/A	–/A	–/A	–/A	–/A	–/A	–/A	A/A	–/–	A/A	–/A	–/–	–/A	–/A	–/–
G-1154A	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
C-634G	–/–	–/G	–/G	–/G	–/G	G/G	–/G	–/G	G/G	–/G	G/G	–/–	G/G	–/G	–/–	–/G	G/G	–/G
C-7T	–/–	–/–	–/T	–/–	–/T	–/–	–/–	–/T	–/T	–/T	–/T	–/–	T/T	–/–	–/–	–/–	–/–	–/–
C702T	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
C936T	–/–	–/–	–/T	–/–	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/T
C1451T	–/–	–/–	–/T	–/–	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/–	–/T	–/–	–/T
G1612A	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–

– Indicates the wild-type allele.

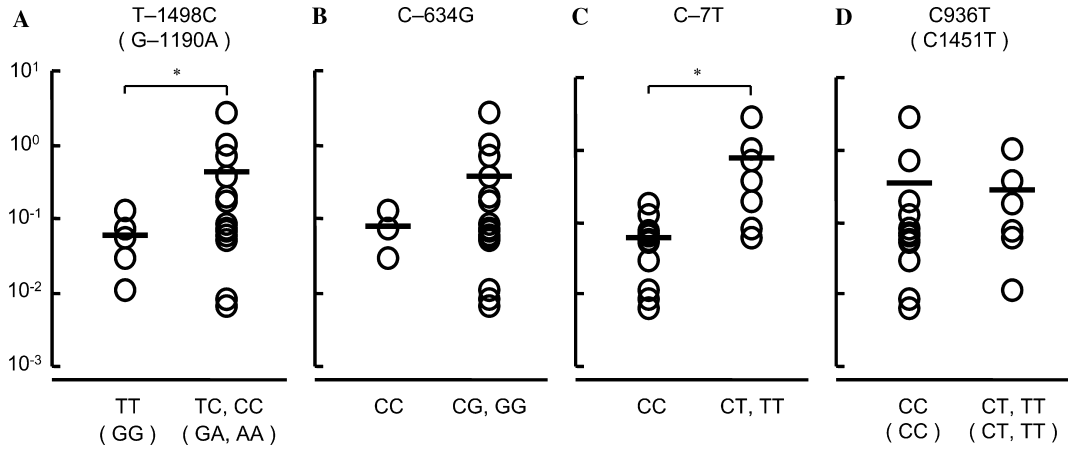

Fig. 2. Effects of *VEGF* genotypes of (A) T-1498C (G-1190A), (B) C-634G, (C) C-7T, and (D) C936T (C1451T) on the relative concentrations of VEGF mRNA in colorectal adenocarcinomas. *VEGF* genotypes were assessed by direct sequencing. Each bar represents the mean of the respective relative concentrations of mRNA. $*P < 0.05$, significantly different from homozygous wild type.

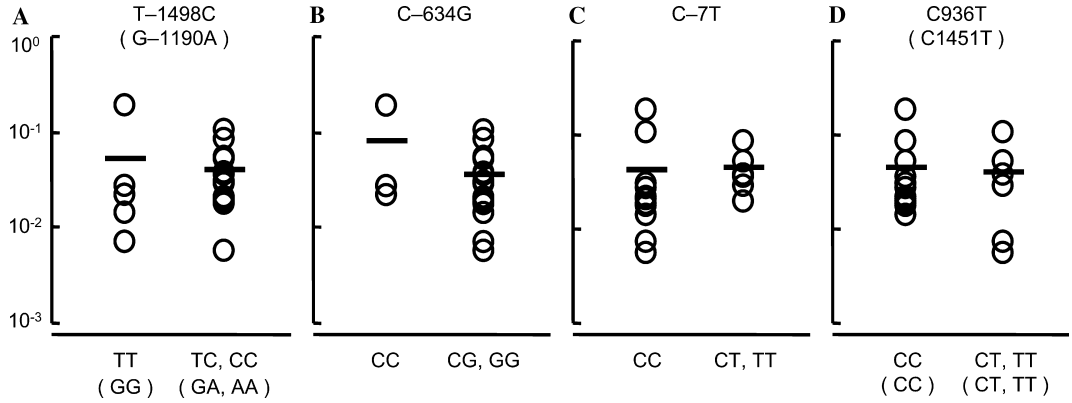

Fig. 3. Effects of *VEGF* genotypes of (A) T-1498C (G-1190A), (B) C-634G, (C) C-7T, and (D) C936T (C1451T) on the relative concentrations of VEGF mRNA in adjacent noncancerous colorectal tissues. *VEGF* genotypes were assessed by direct sequencing. Each bar represents the mean of the respective relative concentrations of mRNA.

firmed that the expression of VEGF mRNA was up-regulated in colorectal adenocarcinomas, when compared with the noncancerous tissues (Fig. 1). Nakasaki et al. [12] showed that VEGF antigen was positive in 64% of colorectal cancers. Ishigami et al. [13] confirmed the expression of VEGF in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues by Northern blot analysis, and reported that the ratio of VEGF mRNA in colorectal adenocarcinomas to that in adjacent noncancerous colorectal tissues ranged from 0.57 to 23.85, but was more than unity in 86.7% of cases. These reports and our present finding suggested that VEGF plays an important role in the development of colorectal adenocarcinomas. Although the association with pathological alterations of tissue was not examined herein, several reports have suggested that VEGF expression is associated with disease progression, microvessel density, venous invasion, lymph node and/or liver metastasis, and prognosis in case of colorectal adenocarcinomas [12–16].

The effect of VEGF genotype on the expression of VEGF mRNA was also evaluated. A total of 12 genotypes of *VEGF*: C-2578A, G-1877A, T-1498C, T-1455C, G-1190A, and G-1154A in the promoter region, C-634G and C-7T in the 5′UTR, and C702T, C936T, C1451T, and G1612A in the 3′UTR, were examined. G-1877A, T-1455C, G-1154A, C702T, and G1612A were not detected (Table 2). C-2578A, T-1498C, G-1190A, C-634G, C-7T, C936T, and C1451T were found at allele frequencies of 4/36, 15/36, 15/36, 20/36, 8/36, 6/36, and 6/36, respectively (Table 2). Howell et al. [24] reported that CC^{-2578}, CA^{-2578}, and AA^{-2578} were found at 0.308, 0.432, and 0.259 in 266 healthy Caucasians, respectively. McCarron et al. [23] and Howell et al. [24] also reported the genotype frequency to be 0.456, 0.414, and 0.129 for GG^{-1154}, GA^{-1154}, and AA^{-1154} in 263 healthy Caucasians, respectively. As for the 3′UTR, Renner et al. [21] reported that C702T, C936T, and G1612A were found at an allele frequency of 0.017, 0.160, and 0.471 in 119 healthy subjects,

respectively. Krippl et al. [22] indicated that C936T was found at 0.294 in 500 healthy female subjects. It is noted that the genetic polymorphism was examined using tissue samples obtained from a small number of Japanese subjects herein, but C-2578A, G-1154A, and G1612A were suggested to be associated with a decreased risk for colorectal adenocarcinomas. It has been reported that C-2578A is associated with a lower risk of developing cutaneous malignant melanoma [24], and G-1154A with that of prostate cancer [23] and cutaneous malignant melanoma [24]. Krippl et al. [22] reported that C936T was linked with a lower risk of breast cancer, but no such association was suggested here. A link was found between T-1498C and G-1190A, and between C936T and C1451T (Table 2). The linkage between T-1498C and G-1190A was found in 268 type 2 diabetic patients [27]. Shahbazi et al. [20] suggested a link between C-2578A and G-1154A in 173 renal transplant recipients, but this was not confirmed here. More studies should be conducted using genomic DNA from Japanese.

The effect of *VEGF* genotype on the expression of VEGF mRNA was evaluated for T-1498C (G-1190A), C-634G, C-7T, and C936T (C1451T) (Fig. 2). T-1498C (G-1190A), C-7T, and possibly C-634G might be a risk factor for the development of liver metastasis and/or prognosis of colorectal cancer via an association with higher levels of VEGF mRNA, since these polymorphisms were associated with increased expression of VEGFmRNA in colorectal adenocarcinomas, but not in adjacent noncancerous colorectal tissues. Although little information is available on the association of these genotypes with susceptibility to cancer, C-634G was found more frequently in patients with proliferative than nonproliferative diabetic retinopathy, but T-1498C (G-1190A), C-7T, and C936T were not [27]. C936T was associated with spontaneous preterm delivery [28] or pre-eclampsia [30], and C-634G with susceptibility to developing giant cell artheritis [29].

VEGF genotypes have been investigated for an association with various types of disease, and polymorphisms other than those tested herein have sometimes attracted attention, including G+405C for psoriasis [33]. Recently, additional polymorphisms have been found in exon 3 or exon 4, and implicated in the susceptibility to a certain class of diseases [34]. VEGF is now recognized to be a member of the VEGF gene family, which includes VEGF-B to -D, PlGF, and two VEGF-like proteins. In the new system of nomenclature, VEGF is defined as VEGF-A. Other members are expected to play an important role in the growth and differentiation of vascular and lymphatic endothelial cells [9–11]. Thus, the systemic assessment of genotypes of VEGF and related proteins might be promising for prediction of the development of liver metastasis and/or prognosis of colorectal cancer.

Collectively, the level of VEGF mRNA was evaluated in colorectal adenocarcinomas and adjacent noncancerous colorectal tissues obtained from 18 Japanese patients, and it was confirmed that the expression was up-regulated in colorectal adenocarcinomas, compared with the noncancerous tissues. A total of 12 genotypes of *VEGF*: C-2578A, G-1877A, T-1498C, T-1455C, G-1190A, and G-1154A in the promoter region, C-634G and C-7T in the 5′UTR, and C702T, C936T, C1451T, and G1612A in the 3′UTR, were also evaluated in the tissues and their association with the expression of VEGF mRNA was assessed. Although G-1877A, T-1455C, G-1154A, C702T, and G1612A were not detected, C-2578A, T-1498C, G-1190A, C-634G, C-7T, C936T, and C1451T were found at allele frequencies of 4/36, 15/36, 15/36, 20/36, 8/36, 6/36, and 6/36, respectively, suggesting that C-2578A, G-1154A, and G1612A were associated with a decreased risk for colorectal adenocarcinomas. The effect of *VEGF* genotype on the expression of VEGF mRNA was evaluated for T-1498C (G-1190A), C-634G, C-7T, and C936T (C1451T), and T-1498C (G-1190A) and C-7T were found to be associated with higher levels of VEGF mRNA in colorectal adenocarcinomas, but not in adjacent noncancerous colorectal tissues, and therefore may be a risk factor for the development of liver metastasis and/or prognosis of colorectal cancer.

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