Clinical implication of *vascular endothelial growth factor T-460C* polymorphism in the risk and progression of prostate cancer

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Abstract. Vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, is suggested to play a crucial role in tumor neovascularization and is associated with tumor progression and metastasis in prostate cancer. This study evaluated the significance of the *VEGF T-460C* polymorphism in the risk and the progression of prostate cancer. In a case-control experiment, 270 patients with prostate cancer and 252 male controls were investigated to assess the association of the *VEGF T-460C* polymorphism with the risk of prostate cancer. Prostate-specific antigen (PSA) recurrence in 95 patients who underwent radical prostatectomy and survival in 99 patients with metastases at diagnosis were analyzed to evaluate the influence of the polymorphism in cancer progression. The *CC* and *TC* genotypes of the polymorphism were associated with significantly higher rates of PSA recurrence after radical prostatectomy than the *TT* genotype and were independent predictors of PSA recurrence (P=0.011) in a multivariate analysis. In contrast, metastatic prostate cancer patients with the *TT* genotype showed significantly worse survival as compared to the *CC* and *TC* genotypes. In a multivariate analysis, the *TT* genotype was an independent predictor of cancer-specific survival (P=0.006). The *VEGF T-460C* polymorphism may have a substantial impact on both PSA recurrence after radical prostatectomy and survival in advanced prostate cancer. The molecular mechanisms of the polymorphism on the differing status in prostate cancer should be elucidated in further studies.

Introduction

Neovascularization is inevitable in tumor progression and metastasis, since the tumor growth requires the development and remodeling of the blood vessels to ensure the supply of oxygen and nutrients (1). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a crucial role in tumor neovascularization through a paracrine mechanism and in tumor growth through an autocrine mechanism (1,2). Many studies have observed that an over-expression of the VEGF in cancer cells is associated with tumor progression and metastasis in various types of cancers (3-5). An increased level of plasma and urinary VEGF in cancer patients is suggested to be a possible diagnostic and prognostic predictor (6,7). In prostate cancer, a higher expression level of VEGF protein in human prostate cancer cells than benign prostate hyperplasia or normal prostate cells was confirmed immunohistochemically (8,9). The immunohistochemical analysis of human prostate specimens and LNCaP and its metastatic derivatives demonstrated a high VEGF expression in association with cellular dedifferentiation and metastatic potential, respectively (3,10). Meanwhile, an increased microvessel density (MVD) of cancer lesions is shown to be associated with tumor aggressiveness and prognosis (11-13). The MVD of prostate cancer lesions is reported to increase with the progression of the tumor and is a useful marker for predicting pathological stage and malignant potential (13-15).

The *VEGF* gene, which is mapped on 6p21, has several important single nucleotide polymorphisms (SNPs) in the promoter, 5'- and 3'-untranslated regions (UTR). Some of these SNPs are reportedly implicated in the risk of various types of cancers such as breast, prostate, bladder cancer and malignant melanoma (16-19) and associations of the SNPs with plasma and urinary VEGF expression levels were demonstrated (20-23). Genetic polymorphisms have also shown to affect the risk of cancer and the survival of cancer patients suggesting that polymorphisms as host genetic factors are partially involved in the progression and response to cancer treatments (24). In previous studies, significant associations were observed between the polymorphisms in the 5'- flanking region of the *VEGF* promoter, which is highly polymorphic, and various types of cancers and other diseases (22,25). Among the polymorphisms in the promoter region, the *VEGF T-460C* polymorphism was reported as a useful genetic marker for prostate cancer and demonstrated the impact on the survival of patients with breast cancer (16,17).

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In this study, we evaluated the significance of the *VEGF T-460C* polymorphism in the risk, clinical phenotypes and prognosis of prostate cancer in the Japanese population. The association between the polymorphism and VEGF protein expression and MVD was also examined using prostate specimens obtained at radical prostatectomy.

Materials and methods

Patients. In a case control experiment, 522 males, comprising of 270 patients with prostate cancer diagnosed at Akita University Hospital, its related community hospitals, or Kyoto University Hospital and 252 male controls attending medical check-ups at community hospitals, were registered. Blood specimens were collected from April 1997 to December 2003 for the prostate cancer patients and from March 1998 to September 2001 for the controls. In a survival analysis of localized prostate cancer, 95 consecutive patients who underwent radical prostatectomy at Akita University Hospital were enrolled in the experiment from July 1989 to December 2003. Patients who underwent neoadjuvant or advent hormone therapy were excluded. In a survival analysis of advanced prostate cancer, 99 patients who had prostate cancer with bone metastasis at diagnosis were enrolled from July 1980 to July 2003. Prostate needle biopsy specimens provided material for pathological diagnosis and metastases were identified using X-ray, CT scan and bone scintigraphy. All the patients had metastatic prostate cancer without any previous treatment for the disease and underwent surgical castration or treatment with LH-RH analogues with or without antiandrogens as the initial hormone therapy after diagnosis. Other optional therapies, such as estrogen, antiandrogen agents, steroids, palliative radiation and combinations of these, were added to or replaced by the preceding therapies when treatment failure was noted. The controls comprised of 252 male volunteers without any apparent voiding symptoms. They were tested for serum total prostate-specific antigen (PSA) levels (the Tandem-R assay) and those with abnormal levels (≥4.0 ng/ml) were omitted from the study. A written informed consent was obtained from all the subjects in both of the studies. The present study was approved by the Institutional Review Board (the Ethics Committee) of the Akita University School of Medicine and the Kyoto University Graduate School of Medicine.

Histological evaluation of specimens obtained by transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms was done for all prostate cancer patients. The clinical or pathological stage of prostate cancer at the time of diagnosis was determined by reviewing the medical records based on the tumor-node-metastasis (TNM) system. Prostate cancer was classified into stage A (T1a-bN0M0), stage B (T1c-2N0M0), stage C (T3-4N0M0) and stage D (T1-4N1M0-1 or T1-4N0-1M1) by the modified Whitmore-Jewett system. The final pathological stage was applied for patients who underwent radical prostatectomy and the clinical stage was applied for those who did not undergo radical prostatectomy. Pathological grading was determined according to the General Rule for Clinical and Pathological Studies on Prostate Cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is mainly based on the WHO criteria and the Gleason score. Well, moderately, and poorly differentiated carcinomas generally correspond to Gleason scores of 2-4, 5-7 and 8-10, respectively. In the present study, because the two grading systems were used by local pathologists, the tumor grade system was categorized as follows: low-grade cancer included well-differentiated or Gleason 2-4 carcinomas, intermediate-grade cancer included moderately differentiated or Gleason 5-7 carcinomas and high-grade cancer included poorly differentiated or Gleason 8-10 carcinomas.

Genotyping of VEGF T-460C polymorphism. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to detect *VEGF T-460C* polymorphisms. The primer sets were as follows: forward, 5'-TGAGTGTGTGCGTGTGGGGTTGAGCG -3'; reverse, 5'-AGAGCCGTTCCCTCTTTGCTAG -3'. In the forward primer, guanine was substituted for cytosine (underlined) to create a digestion site for *HinP1* I. PCR was carried out on a 15-μl aliquot containing ~25 ng of genomic DNA, 12 pmol of each primer, 2.5 μl of 10x buffer solution, 20 nmol/μl each of dATP, dCTP, dGTP and dTTP and 1U of Taq polymerase (Ampli-Taq Gold DNA polymerase, PE Applied Biosystems, Branchburg, NJ, USA). Initial denaturation at 94˚C for 10 min was followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C and extension at 72˚C for 30 sec, with a final extension at 72˚C for 7 min. The products obtained by overnight digestion at 37˚C with *HinP1* I were electrophoresed on 3.0% agarose gels. Digestion of the 162 bp PCR products resulted in two fragments of 138 and 24 bp where the C allele was present. Several samples were directly sequenced using PCR primers and a Dye Terminator Sequencing Kit version 1.0 (PE Applied Biosystems) on an ABI prism 310 auto-sequencer to confirm the results of PCR-RFLP for each polymorphism.

Immunohistochemical analysis. Fifty-eight prostate specimens obtained at radical prostatectomy were subjected to immunohistochemical analysis. The pathological T stage of the cancers was T2 in 29 patients, T3 in 22 and T4 in 7. The Gleason score of the cancers was <7 in 11, 7 in 27 and >7 in 20 specimens. The specimens were fixed in 10% buffered formalin and embedded in paraffin. A paraffin block containing cancer lesions with representative Gleason scores was selected from each specimen. Tissue sections of 5 μm were deparaffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. To retrieve the antigen, the sections were boiled in 0.01 M citric acid (pH 6.0) and non-specific binding was blocked with 5% goat serum for 10 min. After washing, rabbit anti-VEGF monoclonal antibody (A-20; Santa Cruz, CA, USA) diluted 1:200 was applied and incubated at 4˚C overnight. After washing in PBS, secondary antibody conjugated with horseradish peroxidase (Histofine Simplestain MAX PO, Nichirei) was applied, followed by incubation at room temperature for 30 min. After washing in PBS again, tissue sections were developed with diamino-benzidine (DAB; Nichirei) and counterstained with hematoxylin. Similarly for CD34 immunostaining, mouse anti-CD34 monoclonal antibody (NU-4A1; Nichirei,

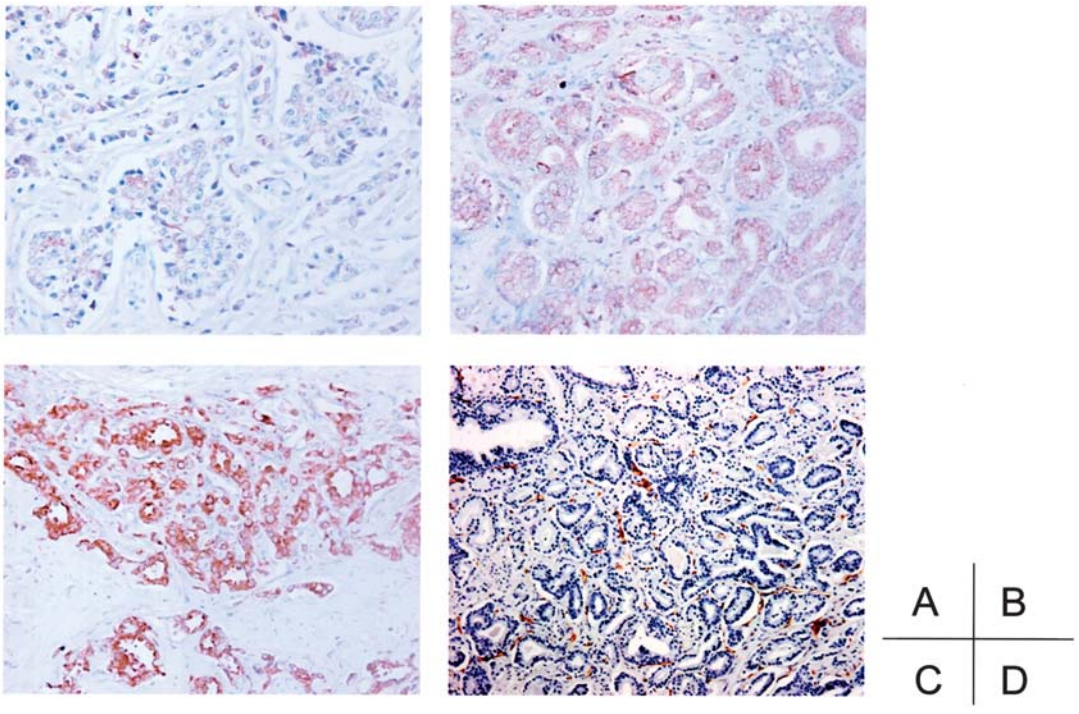

Figure 1. Representative immunohistochemical stainings of vascular endothelial growth factor (VEGF). Prostate cancer lesions with weak, moderate and strong stainings are shown in A, B and C, respectively. A representative immunohistochemical staining of CD34 in a prostate cancer lesion is shown in D.

Tokyo, Japan) diluted 1:100 was used as primary antibody and incubated at 4˚C overnight.

Assessment of VEGF expression and measurement of microvessel density. The expression of VEGF was assessed using a modified scoring system based on a method previously reported (26). Briefly, VEGF expression was classified into four categories according to the sum of staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong staining) and percentile quadrants of positive cells (0=0%, 1=1-25%, 2=26-50%, 3=>50%). Weak, intermediate, and strong expressions correspond to the sum of the two scores: 0-2, 3-4 and 5-6, respectively (Fig. 1A, B and C).

MVD was assessed by counting the number of microvessels with CD34-positive endothelial cells in four separate high-power fields at x200 that had the highest vascularization (hot spot) by scanning at x40 in cancer lesions of each section (Fig. 1D). The mean MVD of the four fields was calculated (27). Each assessment was performed by two independent observers (H.F and N.T.) unaware of clinical information.

Statistical analysis. The age of each group was presented as mean ± SD and the difference between the groups was analyzed by an unpaired Student's t-test. Hardy-Weinberg equilibrium analyses were performed to compare the observed genotype frequencies with the expected frequencies using the χ^2 test. MVDs between groups were compared using ANOVA and the relationship between each genotype and the VEGF expression score determined by IHC was analyzed by the χ^2 test. The age-adjusted odds ratio (aOR) and 95% confidence interval (CI) for the relative risk in each genotype were calculated by multiple logistic regression analysis with the inclusion of a factor of age. To compare PSA recurrence-free survival, patients were dichotomized by median preoperative PSA (≥11.3 vs <11.3 ng/ml), pathological T status (T2 vs T3-4), surgical margin status (positive vs negative), Gleason score (≥8 vs <8), *VEGF T*-460C genotype (*TC* or *CC* vs *TT*), VEGF expression score (≥4 vs <4) and median MVD (≥27.0 vs <27.0). PSA recurrence was defined as the persistence of a postoperative serum PSA level >0.4 ng/ml. Similarly, to compare cancer-specific survival, patients were dichotomized by median age (≥73 vs <73 years), tumor grade (high vs low/intermediate), pretreatment PSA (≥186 vs <186 ng/ml), pretreatment hemoglobin (<10.5 vs ≥10.5 g/dl), alkaline phosphatase (increased vs normal), lactate dehydrogenase (increased vs normal) and *VEGF T-460C* genotype (*TT* vs *TC* or *CC*). The survival time was calculated from the date of prostate cancer diagnosis to the day of PSA recurrence, death from prostate cancer, or death from any cause, for PSA recurrence-free, cancer-specific and overall survival, respectively. Survival was evaluated using the Kaplan-Meier method and the statistical difference in survival between groups was evaluated by a log-rank test. The median follow-up time was computed among all cases. Hazard ratios (HRs) and 95% CIs for PSA recurrence and cancer death were tested using the Cox proportional hazard regression model. All statistical analyses were performed using SPSS software version 14.0 (SPSS Inc.) and two-sided P-values of <0.05 were considered to indicate statistical significance.

Results

Association between the VEGF polymorphism and the risk of prostate cancer. The mean age of controls and prostate cancer

Table I. Genotype frequencies of the *VEGF T-460C* polymorphism and age-adjusted odds ratio against controls.

	Prostate cancer		Control			
Genotype	n	(%)	n	(%)	aOR[a] (95% CI[b])	P-value
VEGF T-460C	270		252			
TT	143	(53.0)	132	(52.4)	ref.	
TC	103	(38.1)	97	(38.5)	0.976 (0.677-1.404)	0.893
CC	24	(8.9)	23	(9.1)	0.980 (0.719-1.036)	0.900

[a]Age-adjusted odds ratio; [b]95% confidence interval.

Table II. Genotype frequencies of the *VEGF T-460C* polymorphism in prostate cancer subgroups and age-adjusted odds ratio against controls.

VEGF T-460C Genotype	Stage[a]		aOR[c] (95% CI[d])	P-value
	Localized	Metastatic		
TT	62 (54.9)	81 (51.6)	ref	
TC	41 (36.3)	62 (39.5)	1.170 (0.697-1.961)	0.554
CC	10 (8.8)	14 (8.9)	1.035 (0.668-1.605)	0.876
TC+CC	51 (45.1)	76 (48.4)	1.145 (0.705-1.862)	0.381
TT+TC[5]	103 (91.2)	143 (91.1)	0.999 (0.426-2.342)	0.999
VEGF T-460C Genotype	Grade[b]		aOR[c] (95% CI[d])	P-value
	Low+Intermidiate	High		
TT	87 (53.4)	55 (52.4)	ref	
TC	63 (38.6)	39 (37.1)	0.991 (0.585 -1.676)	0.972
CC	13 (8.0)	11 (10.5)	1.156 (0.748 -1.787)	0.514
TC+CC	76 (46.6)	50 (47.6)	1.048 (0.640 -1.716)	0.853
TT+TC[e]	150 (92.0)	94 (89.5)	0.750 (0.321 -1.748)	0.505

[a]Localized, stage A-C; metastatic, stage D; [b]Low, well-differenciated or Gleason score 2-4; intermediate, moderately differenciated or Gleason score 5-7; High, poorly differenciated or Gleason score 8-10; [c]age-adjusted odds ratio; [d]95% confidence interval, [e] vs CC genotype.

patients were 71.2±8.0 and 71.7±7.9 years, respectively. There was no significant difference in the mean age between the groups (P=0.838). Tumors were classified as stage A, B, C and D such as 26, 88, 56 and 100 patients, respectively. Low, intermediate and high-grade tumors were observed in 42, 121 and 105 patients, respectively and the pathological grade was unknown in two patients due to inadequate records of local pathologists. The observed genotype frequency of the polymorphism did not differ from the expected frequency according to the Hardy-Weinberg equilibrium in the control group (data not shown).

The genotype distribution of the *VEGF T-460C* polymorphism is summarized in Table I. The frequencies of the *TT*, *TC* and *CC* genotypes in the control group were 52.4, 38.5 and 9.1%, respectively. The frequencies of these genotypes in the prostate cancer group were 53.0, 38.1 and 8.9%, respectively. There was no significant difference in the genotype distribution between the two groups. Age-adjusted logistic regression analysis showed no association between the genotype of the polymorphism and the risk of prostate cancer. When the patients were classified according to tumor stage and grade, there were no significant differences in genotype distribution either between the high-grade cancer and the low-grade cancer groups, or between stage A + B + C (non-metastatic) patients and stage D (metastatic) patients (Table II). For the age at the onset of prostate cancer, the subjects were divided into two groups using the median age at diagnosis in prostate cancer patients (72 years). There were no significant differences in the three genotype frequencies between patients aged 72 years or over and those under 72 and in the mean age of diagnosis of prostate cancer between the three genotype groups (data not shown).

Association of the VEGF polymorphism with expression of VEGF and microvessel density. There was no association between the *VEGF T-460C* genotypes and VEGF protein expression score (P=0.199). When the same analysis was performed in each subgroup and divided according to pathological T stage (T2 or T3-4) or Gleason score (≤7 or >7), no association between the genotypes and VEGF expression level was observed (Table III). Mean MVD in the prostate cancer lesions reported no association with *VEGF T-*

Table III. Association of genotypes of the *VEGF T-460C* with VEGF expression level and microvessel density.

VEGF T-460C Genotype	VEGF expression[a] according to IHC[b]			P-value[c]	Microvessel density[d]	P-value[e]
	weak	intermediate	strong			
TT	7	12	11		29.6±14.4	
TC	7	6	12		28.6±16.3	
CC	0	3	0	0.199	34.3±24.1	0.833

[a]Weak, intermediate and strong correspond to expression score 0-2, 3-4 and 5-7, respectively; [b]Immunohistochemistry; [c]χ^2-square test; [d]Mean ± SD, [e]ANOVA test.

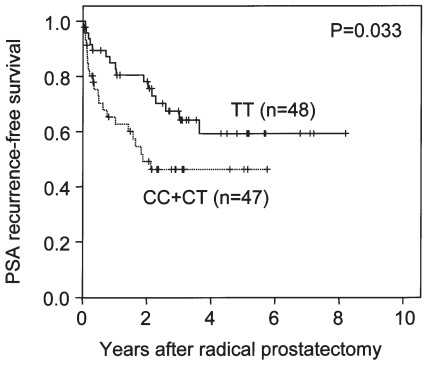

Figure 2. Kaplan-Meier curves of PSA recurrence-free survival stratified by genotype of the *VEGF T-460C* polymorphism in prostate cancer patients who underwent radical prostatectomy.

460C genotypes (*P*=0.833). In each subgroup divided according to pathological T stage (T2 or T3-4) or Gleason score (≤7 or >7), no statistical difference in the mean MVD was observed between the genotypes (Table III).

The association between the VEGF polymorphism and progression after radical prostatectomy. The mean age (± SD) of the 95 patients who underwent radical prostatectomy was 70.2±5.1 years (range, 55-83; median, 71 years). The mean follow-up period was 26.3±22.8 months (range, 1-98; median, 25 months). The mean preoperative serum PSA was 17.0±14.9 ng/ ml (range 2.8-65.0 ng/ml). The distribution of clinical stage was T1, T2, and T3 in 45 (47.4%), 36 (37.9%) and 14 (14.7%), respectively. The distribution of pathological stage was T2, T3 and T4 in 51 (53.7%), 41 (43.2%), and 3 (4.2%), respectively and positive surgical margin was observed in 45 (47.4%). Gleason sum score of <7, 7 and >7 were present in 18 (19.0%), 44 (46.3%) and 33 (34.7%, respectively. The 3- and 5-year PSA recurrence-free survival rates were 56.8% and 51.6%, respectively, with a median survival time of 58.1 months. Kaplan-Meier survival curves stratified by *VEGF* genotype showed that patients with the *TC* or *CC* genotype had a significantly higher rate of PSA recurrence compared with the *TT* genotype (P=0.033, log-rank test, Fig. 2). The VEGF expression score (P=0.929) and MVD (P=0.303) were not influential factors in the survival. Univariate analysis of the PSA recurrence-free survival stratified by dichotomized groups in each factor showed that PSA ≥11.3 (P=0.003), pathological T status ≥T3 (P<0.001), positive surgical margin (P=0.011), Gleason sum score ≥8 (P<0.001) and the *TC* or *CC* genotype (P=0.037) were associated with poor survival (Table IV). In multivariate analysis, higher pathological T status (T3-4), higher Gleason sum score (≥8) and the presence of the *TC* or *CC* genotype were independent risk factors predicting PSA recurrence after radical prostatectomy, with HRs of 2.789 (95% CI, 1.292-6.022; P=0.009), 3.274 (95% CI, 1.641-6.534; P=0.001) and 2.463 (95% CI, 1.233-4.926; P=0.011), respectively (Table IV).

Association between the VEGF polymorphism and survival of metastatic prostate cancer. The mean age (± SD) of the 99 patients with bone metastasis at diagnosis was 72.6±8.5 years (range, 53-89; median, 73 years). The mean follow-up period was 53.3±38.9 months (range, 2-184; median, 45 months). The 5- and 10-year overall survival rates were 52.2 and 25.8%, respectively, with a median survival time of 64.8 months. Survival was compared between the two groups divided according to the genotype of the *VEGF* polymorphisms, i.e., patients with the *TT* genotype (n=52) and with the *TC* or *CC* genotype (n=47). The *TT* genotype of the *VEGF T-460C* polymorphism was associated with significantly worse cancer-specific and overall survival compared with the *TC* or *CC* genotype (P=0.036 and P=0.030, respectively; Fig. 3A and B). The 5-year overall survival rates were 45.3 and 60.2% and the 10-year overall survival rates were 7.4 and 40.6% for patients with the *TT* genotype and the *TC* or *CC* genotype, respectively. The median cancer-specific survival rates of patients with the *TT* and the *TC* or *CC* genotype were 46.9 and 81.6 months, respectively and the median overall survival rates of patients with the *TT* and the *TC* or *CC* genotype were 46.9 and 76.9 months, respectively. In a univariate analysis, tumor grade (P<0.001), pretreatment PSA (P=0.025), hemoglobin (P=0.009), alkaline phosphatase (P<0.001) and VEGF polymorphism (P=0.025) were significantly associated with cancer-specific survival. A multivariate analysis revealed that the VEGF polymorphism (P=0.006) and tumor grade (P=0.039), hemoglobin (P=0.007) and alkaline phosphatase (P=0.009) were independent predictors of cancer-specific survival (Table V).

Discussion

Most of the *VEGF* polymorphisms previously examined are located in the 5'-flanking region and some are considered to affect the transcription activity of the gene, resulting in inter-

Table IV. Cox proportional hazard regression analysis of predicting factors for PSA progression-free survival after radical prostatectomy.

Variable	Category for statistical analysis	PSA progression-freec survival		
		HR[a]	95% CI[b]	P-value
Univariate analysis				
Preoperative PSA	≥11.3 vs <11.3 (ng/ml)	2.769	1.419-5.405	0.003
Pathological T status	T3-4 vs T2	3.683	1.886-7.193	<0.001
Surgical margin status	Positive vs Negative	2.373	1.217-4.626	0.011
Gleason score	≥8 vs <8	3.177	1.680-6.009	<0.001
VEGF T-460C polymorphism	*TC/CC* vs *TT*	1.996	1.042-3.817	0.037
VEGF expression score	≥4 vs <4	1.040	0.436-2.480	0.929
Microvascular density	≥27.0 vs <27.0	1.459	0.708-3.006	0.303
Multivariate analysis				
Preoperative PSA	≥11.3 vs <11.3 (ng/ml)	1.841	0.884-3.834	0.103
Pathological T status	T3-4 vs T2	2.789	1.292-6.022	0.009
Surgical margin status	Positive vs Negative	1.831	0.881-3.802	0.105
Gleason score	≥8 vs <8	3.274	1.641-6.534	0.001
VEGF T-460C polymorphism	*TC/CC* vs *TT*	2.463	1.233-4.926	0.011

[a]Hazard ratio; [b]95% confidence interval.

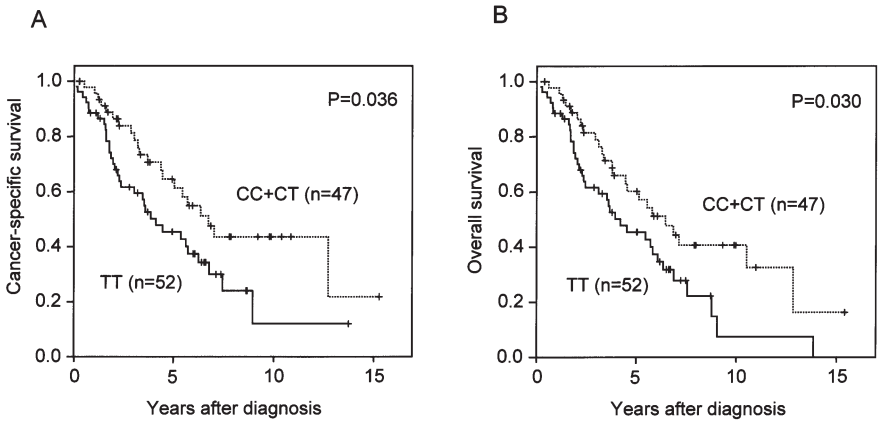

Figure 3. Kaplan-Meier curves of cancer-specific (A) and overall (B) survival stratified by genotype of the *VEGF T-460C* polymorphism in prostate cancer patients with bone metastasis at diagnosis.

individual variations in the concentration of tissue and circulating VEGF protein (22,25). For example, the *C*, *G*, *C*, *C* and *G* alleles of the *C-2578A*, *G-1154A*, *G-634C*, *T-460C* and *G-405C* polymorphisms, respectively, are related to higher VEGF expression levels and men with the T allele of *C936T* reportedly have lower plasma VEGF levels (20-23). The association between the *C* allele of the *C-460T* polymorphism and the risk of disease was demonstrated for cancers of the breast, prostate and oral cavity, psoriasis, end-stage kidney disease and proliferative diabetic retinopathy (16,17,28-31). Conversely, a series of studies from Taiwan showed an association between the *TT* homozygote of the *C-460T* polymorphism and risk of prostate cancer, oral cancer and endometriosis (17,28,32). With regard to the controversial results, since none of the genotype frequencies of the control groups in the studies were in Hardy-Weinberg equilibrium (17,28,32), these results must be attributed to problems of experimental technique or sampling bias of the control subjects.

Several studies have reported that the presence of the *C* allele or the *CC* genotype of the *VEGF C-460T* polymorphism is a risk factor for disease progression in chronic renal disease and for poor survival in breast cancer (16,30). The present study demonstrated that PSA recurrence after radical prostatectomy was significantly associated with the *C* allele of the polymorphism. Since it is known that VEGF is involved in tumor neovascularization through a paracrine mechanism and tumor growth through an autocrine mechanism (1,2), higher VEGF levels in the tumor microenvironment possibly induce more rapid progression and robust survival of residual tumor and may develop metastasis or local recurrence under circumstances of activated tumor growth and abundant tumor vessels. Therefore, our finding that the *C* allele is associated with a higher probability of PSA recurrence after radical

Table V. Cox proportional hazard regression analysis of predicting factors for Cancer-specific survival in prostate cancer patients with bone metastasis at diagnosis.

Variable	Category for statistical analysis	Cancer-specific survival		
		HR[a]	95% CI[b]	P-value
Univariate analysis				
Age	≥73 vs <73 (yrs)	1.252	0.726-2.159	0.420
Tumor grade	nHigh vs Low/Intermediate[c]	3.138	1.644-5.918	<0.001
PSA	≥186 vs<186 (ng/ml)	1.933	1.087-3.438	0.025
Hemoglobin	<11.5 vs ≥11.5 (g/dl)	2.463	1.256-4.831	0.009
Alkaline phosphatase	Increased vs Normal	3.922	2.049-7.519	<0.001
Lactate dehydrogenase	Increased vs Normal	1.267	0.666-2.410	0.471
VEGF T-460C polymorphism	*TT* vs *TC/CC*	1.852	1.079-3.185	0.025
Multivariate analysis				
Tumor grade	High vs Low/Intermediate[c]	2.354	1.043-5.309	0.039
PSA	≥186 vs <186 (ng/ml)	1.184	0.805-4.092	1.151
Hemoglobin	<11.5 vs ≥11.5 (g/dl)	2.882	1.342-6.173	0.007
Alkaline phosphatase	Increased vs Normal	3.175	1.330-7.576	0.009
VEGF T-460C polymorphism	*TT* vs *TC/CC*	2.976	1.364-6.494	0.006

[a]Hazard ratio; [b]95% confidence interval; [c]Low, well-differenciated or Gleason score 2-4; intermediate, moderately differenciated or Gleason score 5-7; High, poorly differenciated or Gleason score 8-10.

prostatectomy is thought to be in line with previous studies that identified the *C* allele of the polymorphism as a risk allele for disease.

In contrast to previous studies, the present study report that patients with the *TT* genotype, which is considered to be associated with the lower expression of VEGF, showed a worse survival in patients with metastasis at diagnosis. Moreover, in addition to other clinical factors that have been reported to predict survival, the *TT* genotype was an independent predictor. Although we do not know the exact reason for the contradictory results, a similar finding is observed in predicting survival in prostate cancer by pretreatment serum testosterone level. Lower pretreatment serum testosterone levels have been reported to be associated with a lower response to endocrine therapy, leading to worse survival in patients with metastatic prostate cancer (33). Since androgen ablation therapy is a standard and first-line treatment for metastatic prostate cancer, endocrine therapies were administered to most of the patients in a standardized fashion. It is known that testosterone induces VEGF synthesis and prostate cancer cell proliferation and expression of VEGF in prostate cancer is significantly reduced by endocrine therapies, suggesting that advanced prostate cancer with lower VEGF expression possibly shows less response to the endocrine therapies than cancer with higher VEGF expression (34,35). The higher VEGF levels induced by the *C* allele of the *C-460T* polymorphism may positively influence local recurrence or early micro-metastases by facilitating neovascularization, while metastatic prostate cancers with lower VEGF levels, partially detracted by the T allele, may have a poor response to the endocrine therapy. Further analyses with a large number of patients and various clinical factors are needed to validate our preliminary findings and speculations.

In this study, we failed to show an association between the *VEGF C-460T* polymorphism and risk of prostate cancer and clinical phenotype at diagnosis. Prostate cancer is a slow-growing cancer with a long period of time between initiation and clinically significant cancer, suggesting that progression rather than initiation is the rate-limiting factor for the diagnosis of clinical cancer. Various factors, such as angiogenic factors responsible for the tumor microenvironment, are involved in the clinical elicitation of prostate cancer. These factors are known to show inter-individual variation, partially explained by genetic polymorphisms and may act in a coordinated manner with the VEGF polymorphisms on cancer progression. In addition, since there are several significant polymorphisms besides *C-460T*, which may have an association with the risk of prostate cancer independently or cooperatively. Haplotype analyses among those polymorphisms are needed to further understand the significance of the *VEGF* polymorphisms.

The significant association of VEGF protein expression and MVD in prostatectomy specimens with tumor stage, Gleason score and progression after surgery are reported in several studies (36,37), however, conflicting results regarding MVD have also been published (38,39). This study, which is the first attempt to assess the influence of the *VEGF* polymorphism on VEGF expression and MVD in human prostate cancer specimens, demonstrated no significant association. In the previous studies, biological functions of genetic polymorphisms were investigated using a technique in which cultured cells are transfected with plasmids containing polymorphic sequences, or using healthy individuals to measure the serum concentration or activity of products that are expected to be influenced by the polymorphisms (22,40). Since various clinical or pathological factors such as tumor extension and grade possibly have a strong effect on VEGF expression and MVD in radical prostatectomy specimens, it may be difficult to evaluate the effect of the *VEGF* polymorphism in the tissue section of prostatectomy specimens. The present study also report a lack of association of VEGF

expression and MVD with PSA recurrence. These results may be partially attributed to the small number of subjects analyzed and the evaluation methods used. VEGF may play an important role in the tumor microenvironment that facilitates tumor recurrence in other sites in the prostate gland. Furthermore, scoring VEGF expression and measuring MVD using immunostaining are labor-intensive and less objective and there are no definite methods for evaluating multifocal prostate cancer lesions, suggesting that it may be difficult to apply these tests in the clinical setting.

In conclusion, the *VEGF T-460C* polymorphism may be associated with both PSA recurrence after radical prostatectomy and survival in advanced prostate cancer, though the polymorphism did not appear to be involved in the development of prostate cancer. The molecular mechanisms and clinical implications of the contradictory effects of the polymorphism on disease of differing status (e.g. localized vs metastatic) in prostate cancer should be elucidated in further studies.

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