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Hypoxia-inducible factor-1α polymorphisms associated with enhanced transactivation capacity, implying clinical significance

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Hypoxia-inducible factor-1 (HIF-1) is a pivotal factor that regulates cellular responses to hypoxia and is presumably linked to regulation of angiogenesis and tumor growth. We assessed the difference in transcription activity of two HIF-1α polymorphic variants (P582S and A588T), along with molecular epidemiological study among head and neck squamous cell carcinoma (HNSCC) patients. Both HIF-1α variants revealed significantly higher transcription activity than wild-type (WT) did, under normoxic and hypoxic conditions ($P < 0.02$). Furthermore, tumors from HNSCC patients with heterozygous alleles having P582S or A588T had significantly increased numbers of microvessels compared with those with homozygous WT ($P = 0.02$). In addition, all patients with tumors of T1 (below 2 cm diameter) were WT, while 14 of 47 patients with tumors of ≥T2 were heterozygous. The elevated transactivation capacity of variant forms of HIF-1α implies a role of HIF-1α polymorphisms in generating individually different tumor progression.

Introduction

Hypoxia-inducible factor-1α (HIF-1α) is a key regulator of cellular response to hypoxia and has been suggested as playing an important role in tumor progression and metastasis through activation of various genes that are linked to regulation of angiogenesis, erythropoiesis, energy metabolism, vasomotor function and apoptotic/proliferative responses (1–5). Enhanced expression levels of HIF-1α have recently been reported in human malignancies including colon, breast, stomach, pancreas, prostate, kidney and esophagus (6–8). HIF-1α protein rapidly degrades in cells under normoxic conditions but is strikingly induced in hypoxic cells (9), which are often found in tumor mass (10). HIF-1α protein levels are regulated by the conditional interaction of HIF-1α with the von Hippel-Lindau tumor suppressor protein (pVHL), which functions as an E3 ubiquitin ligase predominantly targeting the minimal N-terminal transactivation domain (N-TAD) within the oxygen-dependent degradation domain (ODD) of HIF-1α (11–13). The affinity of pVHL for this degradation domain is determined by oxygen-sensitive hydroxylation of a critical proline residue within the N-TAD (14,15). Hypoxic stabilization of HIF-1α protein leads to multiple-step activation of HIF-1α function involving its nuclear translocation, and heterodimerization with HIF-1β (also called aryl hydrocarbon receptor nuclear translocator, Arnt) to form transcription factor HIF-1. Subsequently, HIF-1 interacts with cognate hypoxia-response elements of target promoters, followed by recruitment of transcriptional coactivators (2,5,9,16).

Very recently, two polymorphisms found in human *HIF-1α* gene were shown to cause amino acid substitutions within or near the N-TAD, although the functional significance of these polymorphisms was not studied at the time, and no difference in genotype distribution was found between renal cell carcinoma patients and controls (17). In our study, we elucidated the functional significance of these two polymorphisms by *in vitro* assay, and examined the impact on tumor progression in Japanese head and neck squamous cell carcinoma (HNSCC) patients by using molecular epidemiological analysis.

Materials and methods

DNA extraction and PCR

Genomic DNA was isolated from peripheral mononuclear cells as described previously (18). PCR was performed to amplify the 178-bp fragment of human *HIF-1α* gene using a primer set, HIFE12 U (forward 5′-CAT GTA TTT GCT GTT TTA AAG-3′) and HIFE12L (reverse 5′-GAG TCT GCT GGA ATA CTG TAA CTG-3′) under the following conditions: 30 cycles of denaturing at 95°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s.

Denaturing high-performance liquid chromatography analysis

Denaturing high-performance liquid chromatography (DHPLC) analyses of the 178-bp amplicons were performed using the DNA-RP column 3.5 μm, 4.6 × 33 mm and the DNA Screen™ (Shimazu Co., Kyoto, Japan) following manufacturer's instructions. Heteroduplexes were detected at 60°C, which was proved to be optimal among multiple settings.

Sequence analysis

PCR products were directly sequenced using HIFE12U primer. When heteromeric nucleotides were observed, PCR products were subcloned into pGEM-T Easy™ vector (Promega, Madison, WI) to confirm the nucleotide sequence. Sequencing analyses were carried out using Big Dye Terminator Cycle Sequencing Kit™ and ABI PRISM 310 Genetic Analyzer™ (Applied Biosystems, Foster City, CA).

Cell culture, plasmid constructs and reporter assays

COS7 cells (obtained from ATCC) were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum plus penicillin (50 IU/ml) and streptomycin (50 μg/ml). pFLAG-CMV2-wild-type HIF-1α expression plasmid vectors have been described elsewhere (19). Mutated

Abbreviations: HIF-1, hypoxia-inducible factor-1; HNSCC, head and neck squamous cell carcinoma; N-TAD, N-terminal transactivation domain; pVHL, von Hippel-Lindau tumor suppressor protein; VEGF, vascular endothelial growth factor; WT, wild-type.

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forms of HIF-1α were generated using QuikChange site-directed mutagenesis kit™ (Stratagene, La Jolla, CA) with pFLAG-CMV2-wild-type HIF-1α as template, and confirmed by sequencing. The transcription activity of wild-type (WT) or mutant-type HIF-1α (0.2 or 0.5 μg of expression vectors/15-mm well) was analyzed in a co-transfection assay using the FuGENE6™ Transfection Reagent (Roche Diagnostics Co., Indianapolis, IN) with a luciferase reporter gene under the control of thymidine kinase minimal promoter, three tandem copies of hypoxia-response element (HRE-Luc) (19) (0.5 μg/15-mm well) and a Renilla-luciferase vector (pRL-TK™) (Promega) (0.01 μg/15-mm well) as an internal control. After 6 h of transfection, cells were incubated for 36 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions prior to analysis of reporter gene activity.

Study subjects

Fifty-five patients with head and neck squamous cell carcinoma, 41 men and 14 women, participated in this study with the approval of the Genetic and Medical Ethics Commission, Hiroshima University. They had been diagnosed at the Department of Oral and Maxillofacial Surgery, Hiroshima University Dental Hospital in 1990–1995 (18). The tumors were staged according to the TNM classification of malignant tumors defined by UICC (1987). The subsites of tumors were tongue ($n = 20$), gingiva (16), oral floor (10), buccal mucosa (4), oropharynx (2) and maxillary sinus (3). Controls were chosen from a prospective cohort study among a Japanese general population and individually matched to the patients with respect to gender and age (in 2-year age units). Two controls were randomly selected for each of the patients within the matching conditions.

Immunohistochemical analysis for microvessels in tumors

Twelve tumor tissues of the 14 patients with rare alleles were found to be available for analysis. Of 41 patients with predominant alleles, we randomly chose 12 patients who matched the 12 patients with rare alleles in terms of T classification for comparison. Immunohistochemistry was performed on formalin-fixed paraffin-embedded biopsy specimens that were obtained before treatment, such as chemotherapy or radiation therapy. Immunoglobulin enzyme bridge technique (ABC method) was employed as described previously (20) with some modifications, and anti-CD34 antibody (Nichirei, Tokyo, Japan) was used as primary antibody. Intra-tumoral CD34-positive microvessels were counted on ×400 fields. Three areas (per slide) were randomly chosen, and final vessel number was calculated as mean value. Mann–Whitney's *U*-test was used to determine the *P*-value.

Results

Two polymorphisms in exon 12 of HIF-1α gene encoding the N-TAD

We examined polymorphisms in exon 12 of human *HIF-1α* gene, which encodes N-TAD (Figure 1). Using PCR fragments amplified from peripheral mononuclear cell DNA of 55 patients with primary HNSCC, we carried out a DHPLC analysis in exon 12. We found that 14 of 55 patients had mismatched heteroduplex patterns, indicating the existence of polymorphisms in the N-TAD of *HIF-1α* (Figure 2A). To identify and confirm the polymorphisms, sequencing of the PCR fragments of all patients was performed: these fragments identified a base change of C to T at 1772, or G to A at 1790, resulting in the substitution of proline for serine at codon 582, or alanine for threonine at 588, respectively, as recently reported (17) (Figures 1 and 2B). No other polymorphisms were found in exon 12, nor was any homozygous nucleotide substitution identified.

Hypoxia-dependent transactivation of polymorphic HIF-1α

We next generated, by site-directed mutagenesis, expression vectors encoding the two polymorphic variant forms of HIF-1α: one encoding serine at codon 582 (P582S), the other encoding threonine at 588 (A588T). The transcription activity of WT and P582S or A588T HIF-1α was assessed by co-transfection with an HRE-driven luciferase reporter gene in COS7 cells under normoxic or hypoxic conditions (Figure 3). The reference (WT) HIF-1α showed about a 3–6-fold hypoxia-dependent increase in transcription activity, depending on the amount of transfected plasmid (Figure 3). Under normoxic conditions, A588T variant showed 6.8 or 5.6 times higher transactivation capacity than WT did, when using 0.2 or 0.5 μg of expression vectors, respectively ($P < 0.02$, *t*-test). P582S variant also showed significantly higher transactivation capacity than WT in these conditions ($P < 0.02$, *t*-test). This enhanced transactivation capacity of both A588T and P582S variants was observed also under hypoxic conditions ($P < 0.01$, *t*-test), thereby maintaining the hypoxia-dependent induction response.

Molecular epidemiological study of HIF-1α polymorphisms within N-TAD

HIF-1 directly regulates the expression of several genes involved in angiogenesis, such as vascular endothelial growth factor (*VEGF*), a VEGF receptor (*FLT1*) and plasminogen

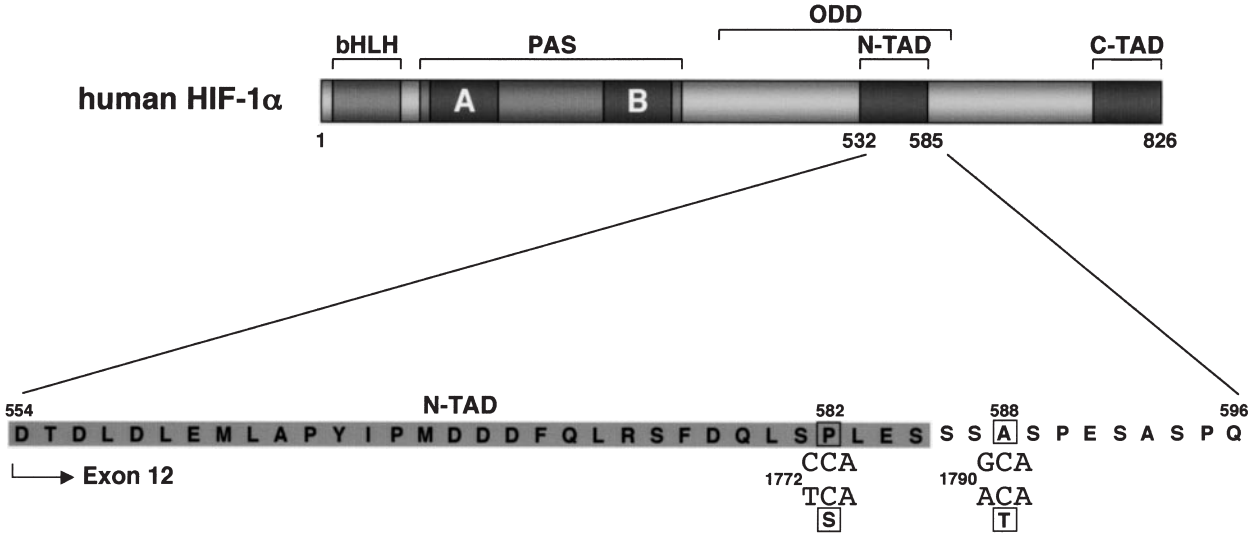

Fig. 1. Structure of HIF-1α. (Upper panel) Organization of functional domains of the HIF-1α protein. bHLH, basic-helix–loop–helix domain; PAS, Per-Arnt-Sim domain; ODD, oxygen-dependent degradation domain; N- and C-TAD, N- and C-terminal transactivation domains. (Bottom panel) Amino acid sequence encoded in exon 12. (Shaded box) N-TAD. (Open boxes) Positions of amino acid substitutions caused by single-nucleotide polymorphisms. Numbers indicate positions of nucleotides or amino acids, respectively.

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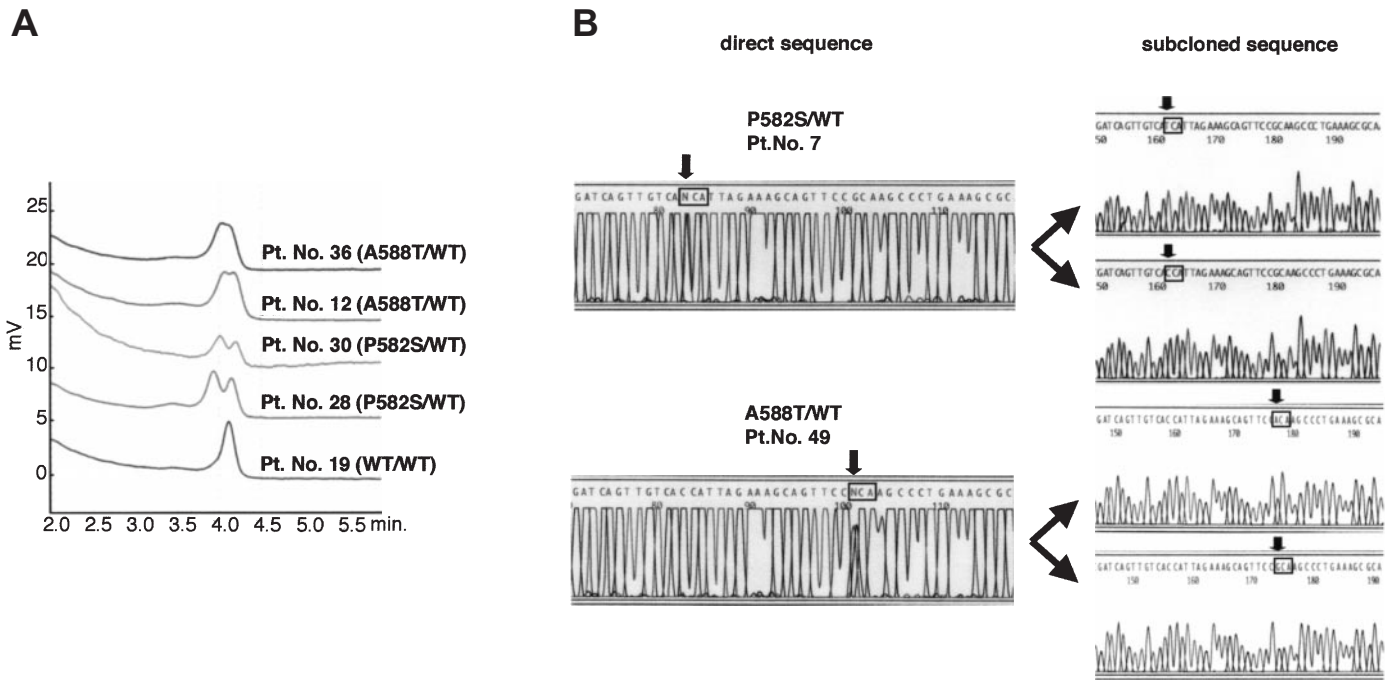

Fig. 2. Genetic polymorphisms of the *HIF-1α* gene. (**A**) Typical complex chromatograms of DHPLC analyses. Exon 12 of the *HIF-1α* gene encoding the N-TAD was amplified using specific primers. After re-annealing of PCR amplicons at optimal temperature, the samples were loaded onto the DNA column and eluted using an acetonitrile gradient. The heteroduplexes were detected at 60°C by UV absorbance at 260 nm. Heterozygous with P582S or A588T show typical hetero-duplex patterns. (**B**) Detection of single-nucleotide polymorphisms by sequencing analyses. A hetero-nucleotide signal 'N' was detected by direct-sequencing analyses (left panels). Sequences of subcloned PCR fragments were comfirmed as hetero-type polymorphisms (right panels).

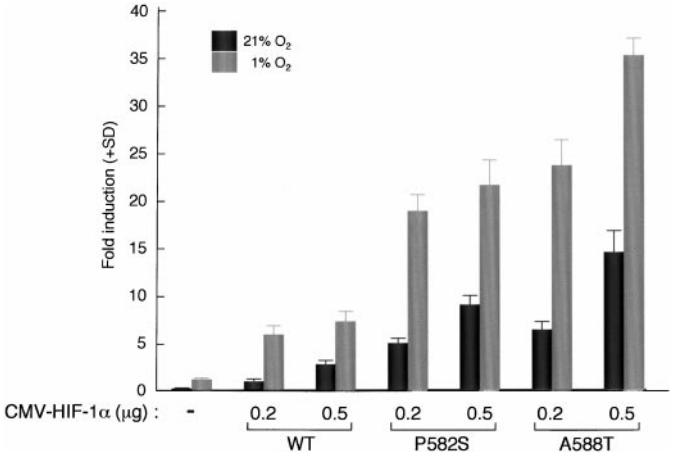

Fig. 3. The transactivation capacity of variant forms of HIF-1α. Transcription activity of vector (−), wild-type (WT), P582S or A588T HIF-1α (0.2 or 0.5 μg of expression vector/15-mm well) was analyzed in a co-transfection assay using reporter plasmid HRE-Luc (0.5 μg/15-mm well) and a Renilla-luciferase (0.01 μg/15-mm well) as internal control. Relative transcription activities were calculated as fold induction relative to the activity of a low amount of the wild-type protein at normoxia, showing the average of three independent experiments (bars: +SD).

activator inhibitor 1 (*PAI1*) (21). We therefore examined the relation between polymorphisms of *HIF-1α* gene and microvessel formation in tumors among 24 patients with HNSCC. Specifically, the number of microvessels was assessed for the T-classification-matched 12 pairs of tumors from patients with rare allele (P582S/WT or A588T/WT) and those with predominant homozygous alleles (WT/WT) in terms of immunohistochemical analysis. We found that tumors with rare allele (P582S/WT or A588T/WT) had significantly higher numbers of microvessels (median = 9.7) than those with predominant alleles (WT/WT) did (median = 5.3) ($P = 0.02$, Mann–Whitney's *U*-test; Figure 4A and B).

We also compared the frequencies of genotypes of *C1772T* (P582S) and *G1790A* (A588T) polymorphisms of *HIF-1α* in 55 patients with primary HNSCC, and 110 healthy controls. We found two genotypes [*C*/*C* and *C*/*T*] of *C1772T* polymorphism and [*G*/*G* and *G*/*A*] of *G1790A* polymorphism, but none of the subjects had a homozygous genotype *T*/*T* or *A*/*A*. The genotype frequencies observed in the patients and controls were 18.2 (10/55) versus 10.9% (12/110) for genotype *C*/*T*, and 7.3 (4/55) versus 8.2% (9/110) for genotype *G*/*A* (Table I). The genotype distribution among controls showed a good agreement with the Hardy–Weinberg equilibrium. There was no linkage disequilibrium between these two polymorphisms, and we found no subject with combined genotypes of *C*/*T* and *G*/*A* (data not shown).

The association between the polymorphisms and clinicopathological characteristics including TNM classification, clinical stage and disease-free survival was studied, showing that all tumors of below 2 cm diameter (T1) had predominant homozygous alleles (no rare alleles), while 14 of 47 tumors of T2 had rare alleles ($P = 0.08$, Fisher's exact probability test, Table II).

Discussion

There are different types of polymorphisms related to cancer: some are associated with occurrence of cancer, others, with malignant development of cancer. It is therefore essential to choose different approaches to analyzing each type. For the

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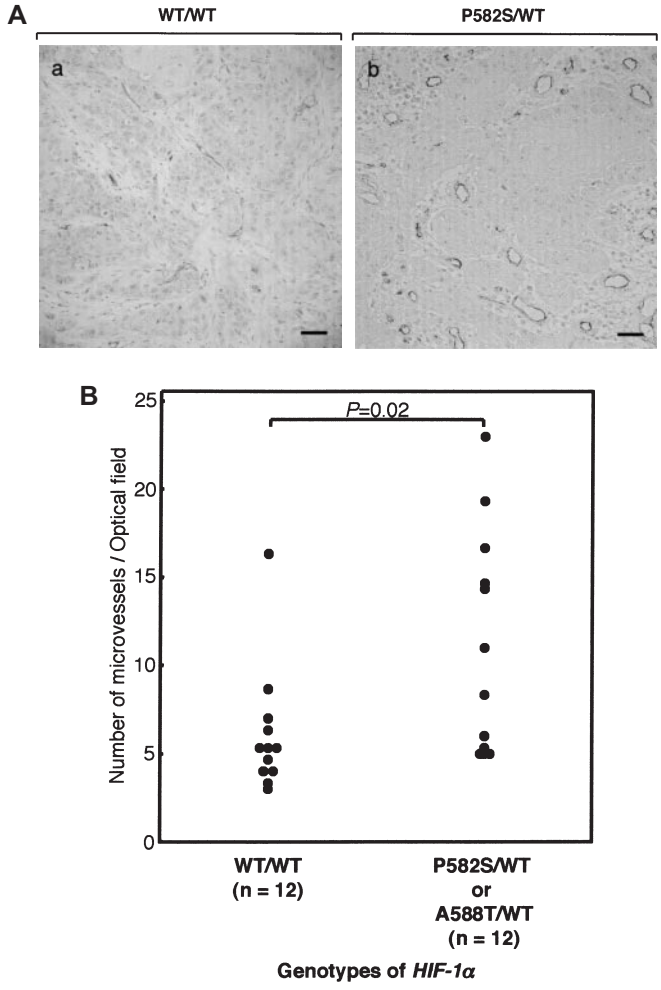

Fig. 4. Polymorphisms of HIF-1α associate with tumor angiogenesis. Twenty-four specimens were subjected to immunohistochemical analysis using anti-CD34 antibody. (**A**) Representative immunostained tumor sections. Tumor with predominant homozygous alleles WT (a), and that with rare allele P582S (b). Magnifications are ×400. Scale bar = 50 μm. (**B**) Intratumoral CD34-positive microvessels were microscopically counted on ×400 fields. Three areas (per slide) were randomly chosen and the final vessel number was calculated as the mean value for each section. Mann–Whitney's *U*-test was used to determine the *P*-value.

latter type, it is necessary to follow patients for a long time in order to evaluate the relevance between identified polymorphisms and prognosis of diseases. In our study, we used a model of molecular epidemiological study of polymorphisms associated with prognostic surrogate markers, namely angiogenesis and tumor growth, combined with *in vitro* assay. Specifically, we for the first time assessed the difference in transcription activities between two HIF-1α polymorphic variants, P582S, A588T and WT, which were recently reported by Clifford *et al*. (17). These HIF-1α variants demonstrated significantly enhanced transcription activities under both normoxic and hypoxic conditions, maintaining the hypoxia-dependent induction response, when compared with WT (Figure 3). Since HIF-1α is activated by a multiple-step pathway, it is possible to speculate on several mechanisms of the enhanced transactivation. Since these substituted amino acids are located within or near the N-TAD, interacting with E3 ubiquitin ligase pVHL, one possible mechanism for the observed enhancement of transactivation capacity may be the alteration of protein stability of these variant proteins.

Table I. Genotype distribution of *HIF-1α* gene in head and neck squamous cell carcinoma (HNSCC) patients and controls

Nucleotide	Amino acids	Genotypes	Patients (%)	Controls (%)
C1772T	P582S	*C/C*	45 (81.8)	98 (89.1)
		C/T	10 (18.2)	12 (10.9)
		T/T	0 (0.0)	0 (0.0)
G1790A	A588T	*G/G*	51 (92.7)	101 (91.8)
		G/A	4 (7.3)	9 (8.2)
		A/A	0 (0.0)	0 (0.0)
		Total	55 (100)	110 (100)

Table II. Tumor size of HNSCC by genotyping of *HIF-1α* gene

Tumor size	*C1772T* and *G1790A*		Total
	C/C and *G/G* (%)	*C/T* or *G/A* (%)	
≤2 cm (T1)	8 (14.5)	0 (0.0)	8 (14.5)
>2 cm (T2–T4)	33 (60.0)	14 (25.5)	47 (85.5)
Total	41 (74.5)	14 (25.5)	55 (100)

However, our preliminary examination found no difference in protein degradation between WT and its variants in the presence of pVHL (data not shown). Another possible explanation may be enhanced recruitment of transcriptional cofactors such as CBP/p300 and SRC-1 that interact with HIF-1α (16), by the variant forms via conformational changes caused by amino acid substitution. Further mechanistic investigations will be required.

HIF-1 has three dozen target genes to mediate the adaptive response to hypoxia, including *VEGF*, *FLT1* and *PAI1*, which are involved in angiogenesis (21). We found that tumors of HNSCC patients with rare alleles encoding variant HIF-1α proteins had significantly increased numbers of microvessels compared with those with WT (Figure 4). We further found that all patients with rare alleles had tumors of ≥ T2, indicating possible involvement of these *HIF-1α* variants in tumor growth (Table II). It is notable that both P582S and A588T HIF-1α proteins showed higher transactivation capacity *in vitro* as compared with WT (Figure 3). Hence, one of the most plausible interpretations for Figure 4 is that these variant forms may be associated with increased expression levels of HIF-1α-regulated genes contributing to enhanced angiogenesis.

Tumor–stroma interaction should be considered in tumor angiogenesis, specifically the secretion of angiogenic factors including VEGF from surrounding tissue. In mice xenograft experiments, disruption of *HIF-1α* gene revealed the importance of HIF-1α in tumor vascularization: *HIF-1α*$^{-/-}$ tumors lacked medium- and large-sized vessels and had more avascular zones than *HIF-1α*$^{+/+}$ tumors (22). Furthermore, HIF-1 activation has been shown to be a major influence on the angiogenesis and growth of a tumor xenograft of a HIF-1β deficient hepatoma cell line (23). These reports indicated that tumors lacking the genes encoding components of HIF-1 transcription factor changed their phenotypes in terms of angiogenesis and cell growth. On the other hand, when transplanted into SCID mice, tumors derived from *VEGF*$^{-/-}$ ES cells showed

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substantial amounts of VEGF, indicating the supplement of VEGF from stromal cells (24). Furthermore, very recently HIF-1α expression in human tumor-associated macrophages has been reported (25), suggesting a role of HIF-1α in stromal cells. In patients with *HIF-1α* variants, angiogenic factors, such as VEGF, could be up-regulated not only in tumor cells but also in stromal cells via the enhancement of HIF-1α transactivation. Taken together, polymorphic variant forms of HIF-1α may comprehensively promote tumor angiogenesis in terms of tumor–stroma interactions. It has so far proved difficult to extrapolate the results of knockout experiments of HIF-1α or angiogenic factors to genetic polymorphisms, since genetic polymorphisms influence all the cells of the body, including tumor and stromal cells.

Although we found no individuals with the rare homozygotic genotype *A*/*A* or *T*/*T* among the study subjects, it is expected that 0.2–0.3% of the general population have this genotype. Individuals with genotype *A*/*A* or *T*/*T* may be characterized by higher transcriptional activity of HIF-1α than those with the heterozygous genotype *G*/*A* or *C*/*T*. Extended molecular epidemiological studies focusing on the prognosis of various cancers are therefore warranted in terms of these polymorphisms of *HIF-1α*.

In summary, we have shown here the elevated transactivation capacity of variant forms of HIF-1α that implies a role of HIF-1α polymorphisms in generating individually different tumor progression potential by molecular epidemiological study tightly combined with *in vitro* functional assay.

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