The C1772T genetic polymorphism in human HIF-1α gene associates with expression of HIF-1α protein in breast cancer

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Abstract. Hypoxia-inducible factor 1 (HIF-1) is an important genetic component involved in the cellular response to hypoxia. HIF-1 is also linked to the regulation of tumor development and growth. In previous studies, the C1772T (P582S) or the G1790A (A588T) polymorphisms of the HIF-1α gene have been identified in renal cell carcinoma, head and neck and esophageal squamous cell carcinomas as well as colorectal and prostate cancers. In our study, we investigated whether polymorphisms of the HIF-1α gene may account for the expression patterns of HIF-1α protein and impact of clinical progression in breast cancer. We also examined the impact of prognosis of HIF-1α gene polymorphism and protein expression in the prediction of biological behavior. We performed polymerase chain reaction and direct sequencing to detect polymorphisms in exon 12 of HIF-1α from 90 breast cancer patients and 102 healthy controls. The expression of HIF-1α was measured in paraffin-embedded specimens from patients by immunohistochemistry. We associated its expression with known prognostic factors. The frequency of the T allele for C1772T in breast cancer patients and healthy controls was 5.6 vs. 4.4%, whereas, the frequency of the A allele for G1790A was 1.7 vs. 4.4%. HIF-1α was overexpressed in 56.7% (51 of 90) of the patients. Its overexpression associated with the T1772 polymorphic allele (p=0.04). Elevated levels of HIF-1α protein were found in cases of breast cancer with lymph node metastasis (p=0.041), high histological grade (p=0.001) and increased Ki-67 index (p=0.031). These results suggest the potential use of C1772T (P582S) polymorphism and expression analysis in providing a new prognostic factor for unfavorable disease outcomes and may help for clinical decision-making in the treatment of breast cancer patients.

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Introduction

Hypoxia is defined as an inadequacy of oxygen reaching the body's tissues and is common in many solid tumors, including breast cancer (1,2). A hypoxic microenvironment initiates multiple cellular responses, such as proliferation and angiogenesis, resulting in tumor growth and progression (3). Hypoxia inducible factor-1 (HIF-1) consists of HIF-1α and HIF-1ß subunits and acts as a transcription activator to mediate cellular responses to hypoxia (4-6). HIF-1 activity is regulated by the oxygen-dependent expression of the HIF-1α subunit (7). Under normoxic conditions, hydroxylation of Pro402 and Pro564 within the oxygen-dependent degradation domain (ODD) of the HIF-1α protein leads to the binding of ODD with the von Hippel-Lindau tumor suppressor protein (pVHL). Thereafter, the HIF-1α protein is rapidly degraded through the pVHL-mediated ubiquitin-proteasome pathway. Under hypoxic conditions, however, the rate of hydroxylation of Pro402 and Pro564 decreases and the HIF-1α protein accumulates. The HIF-1α then translocates to the nuclear compartment and activates the transcription of hypoxia-inducible gene after binding with HIF-1ß (8).

Previously, two polymorphisms (SNPs designated as C1772T, G1790A) in the ODD domain of the HIF-1α gene were identified as a base change from C to T at 1772, or G to A at 1790 (C1772T, G1790A), giving rise to Pro/Ser variation at codon 582 or Ala/Thr variation at codon 588 (P582S, A588T) (9). These missense polymorphisms revealed higher transcription activities than wild-type under normoxic or hypoxic conditions (10,11). Moreover, in the instance of androgen-independent prostate cancer in Caucasians and African-Americans, there is a significant increase in the frequency of the CT or TT genotypes in patients compared to

healthy controls (12). In contrast, there apparently is no significant genotype difference between patients and controls in colorectal cancer in Japan and Sweden (13,14) and esophageal cancer in China (15). Thus, we have explored the frequency difference of HIF-1α polymorphism between patients and healthy controls regarding breast cancer in Korea.

The unfavorable prognosis of many tumors is associated with concentration of the HIF-1α protein (16-18). For breast cancer, an upregulation of HIF-1α protein was associated with the increased proliferation and poor differentiation, showing an association of HIF-1α overexpression in the cytoplasm and nuclei of 40-80% with the more aggressive phenotype (2,19). Although the effect of HIF-1α polymorphism of expression was specifically studied in an invasive breast cancer patient group in The Netherlands, there was a failure to identify the effect due to an absence of polymorphisms in HIF-1α overexpressing breast cancer patients (20).

We have evaluated the correlation between HIF-1α (polymorphisms, expression levels) and clinicopathological features or biomarkers in breast cancer patients in Korea to assess whether HIF-1α plays a role in the development of breast cancer or serves as a prognostic marker.

Materials and methods

Patients and healthy subjects. The present study included 90 women who were diagnosed with breast cancer and 102 healthy controls of the same ethnic background as the patients during the years 2000-2007 at the Department of Pathology, Keimyung University Hospital, Daegu and Kyunghee University Hospital, Seoul, Korea. Breast cancer and paired normal tissue samples from the respective patients were collected at hospitalization. Tumors were fixed in formalin and embedded in paraffin. Hematoxylin- and eosin-stained histological sections of each case were evaluated for confirmation of the diagnosis. Clinicopathological data included histological type, cancer grade, lymph node status and several biomarkers (ER, PR, HER2, p53 and Ki-67). All patients and healthy controls agreed to genetic testing as approved by the Hospital Institutional Review Board.

Genomic DNA extraction and PCR amplification. Genomic DNA was extracted from solid tissue and blood samples to assess polymorphisms of HIF-1α. The sliced tissue samples were lysed overnight at 56˚C in a solution containing 20 mM Tris-Cl (pH 8.0), 5 mM EDTA, 400 mM NaCl, 1% SDS and 20 mg/ml proteinase K and the DNA extracted using phenol: chloroform:isoamyl alcohol (25:24:1). The Core-One™ blood genomic DNA isolation kits (Core-Bio System, Seoul, Korea) were used for DNA extraction of case-matched healthy control blood samples according to the manufacturer's instructions. The polymerase chain reaction (PCR) was carried out in a 20 μl reaction volume containing 1.5 mmol/l $MgCl_2$, 40 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 250 μmol/l dNTP, 1 unit Taq DNA polymerase (BIONEER, Daejeon, Korea) and 50 ng genomic DNA in distilled water. PCR was performed to amplify exon 12 of HIF-1α gene (400 bp fragment) using a primer set. The primer sets were as follows: forward 5'-AAGACACAGAAGCAAAGAAC CCA-3'; reverse 5'-CCATACGGTCTTTTGTCCTGTTT-3. The conditions for the PCR reaction was denaturation at 95˚C for 10 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 57˚C for 30 sec and extension at 72˚C for 30 sec, followed by a final extension at 72˚C for 10 min.

Sequencing. PCR products purified by 95% ethyl alcohol were used as template DNA for cycle sequencing. The PCR for sequencing was performed using Big Dye Terminator (ver 3.1) cycle sequencer and analyzed using an ABI Prism® 3730 Automated DNA sequencer (Applied Biosystems, Foster City, CA).

Tissue microarray construction. To assess the immuno-expression of HIF-1α, we examined tissue microarrays (TMA) which were constructed from specimens of 90 breast cancer patients. Paraffin wax blocks were punched with a 3.0-mm diameter from the selected area in the donor blocks and transferred and arrayed in the recipient blocks using a manual tissue microarray device (UNITMA, Seoul, Korea). The presence of cancer in the arrayed samples was verified on hematoxylin- and eosin-stained sections. Breast cancer tissue samples with strong expression of the protein examined were used as positive controls.

Immunohistochemistry. Immunostaining was performed on 4-μm sections. After deparaffination and rehydration, sections were immersed for 10 min in methanol containing 3% hydrogen peroxide to block endogenous peroxidase activity. All slides were pretreated with a citrate buffer (10 mM; pH 6.0) for antigen retrieval by heating the slides in a microwave oven at 97˚C for 15 min. A cooling period of 20 min preceded the incubation of the monoclonal anti-HIF-1α antibody (clone monoclonal antibody H1a67, NB 100-105; Novus Biologicals, Littleton, CO, USA) diluted 1:200 at 4˚C for overnight. Thereafter, the EnVision™ systems (Dako, Glostrup, Denmark) were used for HIF-1α staining according to the manufacturer's instructions. All staining procedures were developed with diaminobenzidine. Before the slides were mounted, all sections were counterstained for 30 sec with hematoxylin and dehydrated in alcohol and xylene.

Tumor cell immunoreactivity was scored according to both the extent of nuclear staining (relative number of HIF-1α positive cells) and the intensity of the stain reaction by two independent observers (J.H. Lee and S.S. Lee). Only cells with completely darkened nuclear staining were regarded as positive; such nuclear staining was interpreted as an increased levels. The following schema was applied: -, not detected; +, ≤5% positive cells; ++, 5-10% weakly to moderately stained cells; +++, 5-10% intensively stained cells or 10-30% weakly stained cells. For statistical analysis, the four grades of staining were reduced to the two grades of negative, (-/+) and positive, (++/+++).

Statistical analysis. The Chi-square test (SPSS for windows version 11.5) was used to access correlations of clinicopathological feature (using grade, lymph node status, biomarkers) and HIF-1α (using polymorphism and expression). P-values of <0.05 were regarded as statistically significant. All statistical tests were two-sided.

Table I. Polymorphism of C1772T and G1790A in breast cancer patients (n=90) and control subjects (n=102).

Nucleotide	Amino acid	Genotype	Patients (%)	Controls (%)
C1772T (rs11549465)	P582S	CC	81 (90%)	93 (91.2%)
		CT + TT	9 (10%)	9 (8.8%)
		Allele	Patients (%)	Controls (%)
		C	170 (94.4%)	195 (95.6%)
		T	10 (5.6%)	9 (4.4%)
Nucleotide	Amino acid	Genotype	Patients (%)	Controls (%)
G1790A (rs11549467)	A588T	GG	87 (96.7%)	94 (92.1%)
		GA + AA	3 (3.3%)	8 (7.9%)
		Allele	Patients (%)	Controls (%)
		G	177 (98.3%)	195 (95.6%)
		A	3 (1.7%)	9 (4.4%)

Table II. Frequency of HIF-1α polymorphism (C1772T) in a variety of cancers.

Type of cancer	Genotype	Patients	Controls	Racial group
Breast cancer (our result)	CC	81 (90%)	93 (91.2%)	Korean
	CT + TT	9 (10%)	9 (8.8%)	
HNSCC[a] (10)	CC	45 (81.8%)	98 (89.1%)	Japanese
	CT + TT	10 (18.2%)	12 (10.9%)	
CRC[b] (13)	CC	100 (100%)	89 (89%)	Japanese
	CT + TT	0 (0%)	11 (11%)	
ESCC[c] (15)	CC	84 (88.4%)	93 (89.4%)	Chinese
	CT + TT	11 (11.6%)	11 (10.6%)	
Prostate cancer (12)	CC	161 (82.1%)	179 (91.3%)	Caucasian and African-American
	CT + TT	35 (17.9%)	17 (8.6%)	
CRC[b] (14)	CC	167 (84%)	213 (82%)	Swedish
	CT + TT	31 (16%)	45 (18%)	
RCC[d] (21)	CC	16 (10%)	1 (0.7%)	English
	CT + TT	144 (90%)	161 (99.3%)	

[a]HNSCC, head and neck squamous cell carcinoma; [b]CRC, colorectal carcinoma; [c]ESCC, esophageal squamous cell carcinoma and [d]RCC, renal cell carcinoma.

Results

HIF-1α polymorphisms in cancer patients and health controls. The frequencies of C1772T and G1790A polymorphisms in breast cancer patients and case-matched healthy controls are shown in Table I. The genotype distribution of the C1772T in the breast cancer patients (C/C=90%, C/T=8.9%, T/T=1.1%), controls (C/C=91.2%, C/T=8.8%) and the G1790A in the patients (G/G=96.7%, G/A=3.3%), controls (G/G=92.1%, G/A=6.9%, A/A=1%) are noted. The allele distribution of C1772T in the breast cancer patients (C=94.4%, T=5.6%), healthy controls (C=95.6%, T=4.4%) and the G1790A in the patients (G=98.3%, A=1.7%) and healthy controls (G=95.6%, A=4.4%) was detected as indicated. We observed the genotype, allele distribution of the two polymorphisms (C1772T, G1790A) to not be different between breast cancer patients and healthy controls. Two polymorphisms of HIF-1α were confirmed by germline origin of normal tissue within the same patients. Frequencies of C1772T or G1790A polymorphisms of HIF-1α in various cancer patients and healthy controls are shown in Table II and Table III. In six other reported cancer studies of C1772T polymorphism (10,12-15,21), two patterns were observed; a) patient groups with a CT or TT genotype are more elevated than in control

Table III. Frequency of HIF-1α polymorphism (G1790A) in a variety of cancers.

Type of cancer	Genotype	Patients	Controls	Racial group
Breast cancer (our result)	GG	87 (96.7%)	94 (92.1%)	Korean
	GA + AA	3 (3.3%)	8 (7.9%)	
HNSCC (10)	GG	51 (92.7%)	101 (91.8%)	Japanese
	GA + AA	4 (7.3%)	9 (8.2%)	
CRC (14)	GG	189 (95%)	247 (96%)	Swedish
	GA + AA	9 (5%)	9 (4%)	
RCC (21)	GG	65 (44.5%)	239 (83%)	English
	GA + AA	81 (55.5%)	49 (17%)	

Table IV. Association of HIF-1α expression with clinicopathological features, biomarkers in patients with breast cancer (n=90).

	HIF-1α expression		
Parameter	≤5% (%)	>5% (%)	P-value
Histological type			
Ductal	32 (82.1%)	45 (88.2%)	
Non-ductal	7 (17.9%)	6 (11.8%)	NA[a]
Histological grade			
I	13 (33.3%)	3 (5.9%)	
II	4 (10.3%)	14 (27.5%)	
III	22 (56.4%)	34 (66.7%)	**0.001**
Lymph node metastasis			
Negative	23 (59%)	19 (37.3%)	
Positive	16 (41%)	32 (62.7%)	**0.041**
ER[b]			
Negative	15 (38.5%)	22 (43.1%)	
Positive	24 (61.5%)	29 (56.9%)	0.655
PR[c]			
Negative	23 (59.0%)	27 (52.9%)	
Positive	16 (41.0%)	24 (47.1%)	0.568
HER2[d]			
Negative	22 (56.4%)	29 (56.9%)	
Positive	17 (43.6%)	22 (43.1%)	0.966
p53			
Negative	28 (71.8%)	30 (58.8%)	
Positive	11 (28.2%)	21 (41.2%)	0.203
Ki-67			
≤10	25 (64.1%)	21 (41.2%)	
>10%	14 (35.9%)	30 (58.8%)	**0.031**

Using the Chi-square test, p-values <0.05 were regarded as significant. P-value <0.05 is shown in bold; [a]NA, not applicable; [b]ER, estrogen receptor; [c]PR, progesterone receptor and [d]HER2, epidermal growth factor receptor type 2.

groups (head and neck, esophagus, prostate cancer) and b) the CC genotype is more elevated in patient groups (renal, colorectal cancer). Based on our investigations in breast cancer patients, the CT or TT genotypes were more frequent in the patient than control group (Table II). In three reported studies of G1790A polymorphism (10,14,21), GA or AA genotype in renal and colorectal cancers showed a higher frequency in patient than control groups. In contrast, the GG genotype in head and neck cancer showed a higher frequency in patient group. In our analysis in breast cancer, more GA or AA genotype were observed in the control group (Table III).

HIF-1α expression, clinicopathological features and biomarkers. HIF-1α was stained and assessed in breast cancer tissue of 90 patients. According to expression patterns, we subdivided staining distribution into positive and negative categories based on a 5% cutoff value and observed 56.7% (51 of 90) positive and 43.3% (39 of 90) negative cases (Table IV). The association between expression of HIF-1α and clinicopathological features or biomarkers is shown in Table IV. For patients with breast cancer, elevated levels of HIF-1α expression were significantly associated with high histological grade (p=0.001) and lymph node metastasis (p=0.041). In addition, the correlation between several biomarkers (ER, PR, HER2, p53, Ki-67), which are known to associate with patient prognosis in breast cancer and HIF-1α, are also presented in Table IV. Ki-67 expression levels (p=0.031) were positively associated with increased levels of HIF-1α; ER (p=0.655), PR (p=0.568), HER2 (p=0.966) and p53 (p=0.230), however, bore no relationship.

C1772T polymorphism, clinicopathological features and HIF-1α expression. In the subjects heterozygous or homozygous for the T allele (n=9), increased levels of HIF-1α in 88.9% (8 of 9), a high histological grade (III) in 77.8% (7 of 9) and the presence of lymph node metastasis in 33.3% (3 of 9) were observed. The HIF-1α overexpression (p=0.040) and accumulation of p53 (p=0.040) were significantly associated with the T1772 polymorphic allele (Table V), but ER (p=0.830), PR (p=0.157), HER2 (p=0.523) and Ki-67 (p=0.779) were not.

Discussion

The purpose of this study was to determine if the coding SNPs (C1772T and G1790A) of the HIF-1α gene have frequency differences between patients and healthy controls and whether these SNPs may affect expression of HIF-1α protein.

Table V. Association of HIF-1α polymorphism with clinicopathological features, biomarker, HIF-1α expression in patients with breast cancer (n=90).

Parameter	CC (%)	CT + TT (%)	P-value
Histological grade			
I	15 (18.5%)	1 (11.1%)	
II	17 (21.0%)	1 (11.1%)	
III	49 (60.5%)	7 (77.8%)	0.596
Lymph node metastasis			
Negative	36 (44.4%)	6 (66.7%)	
Positive	45 (55.6%)	3 (33.3%)	0.205
HIF-1α expression			
≤5%	38 (46.9%)	1 (11.1%)	
>5%	43 (53.1%)	8 (88.9%)	**0.040**
ER			
Negative	33 (40.7%)	4 (44.4%)	
Positive	48 (59.3%)	5 (55.6%)	0.830
PR			
Negative	47 (58.0%)	3 (33.3%)	
Positive	34 (42.0%)	6 (66.7%)	0.157
HER			
Negative	45 (55.6%)	6 (66.7%)	
Positive	36 (44.4%)	3 (33.3%)	0.523
p53			
Negative	55 (67.9%)	3 (33.3%)	
Positive	26 (32.1%)	6 (66.7%)	**0.040**
Ki-67			
≤10%	41 (50.6%)	5 (55.6%)	
>10%	40 (49.4%)	4 (44.4%)	0.779

The role of HIF-1α in cancer has been demonstrated through clarification of the structural and functional relationships between HIF-1α and pVHL (22). Both Pro402 and Pro564 have been shown to be key amino acids in oxygen-dependent regulation of HIF-1α protein stability (23). Polymorphisms of C1772T (P582S) and G1790A (A588T) adjacent to Pro564, as identified for the human HIF-1α gene, have been shown to have significantly higher transcriptional activities and involved in enhanced angiogenesis when compared to wild-type HIF-1α (10). It was also determined that the increased transcriptional activity reflects increased protein expression in cells that have been transfected with P582S mutants (11).

Recent research has identified two SNPs (C1772T and G1790A) as valuable candidate tools to diagnose cancer, however, they remain the subject of ongoing debate involving a variety of cancers and involving multiple research groups (Table II and Table III). In a head and neck cancer study, Tanimoto *et al* reported that CT or GA genotypes correlate with significantly elevated transcription activity (10) while in a renal cancer study, Ollerenshaw *et al* reported that either CC or GA genotype of HIF-1α may confer susceptibility to cancer (21). For colorectal cancer, Kuwai *et al* suggest that the CT genotype has no involvement in either cancer progression or metastasis (13) whereas Fransen *et al* reported that for CT or TT, GA genotype displayed a significantly higher risk to developing ulcerative colorectal cancer (14). Previously, in an esophageal cancer report, the CT genotype was associated with larger tumors and higher rates of lymph node metastasis (15). In the case of androgen-independent prostate cancer, Chau *et al* observed that a CT or TT genotype may contribute to the progression or metastasis of cancer (12). According to our analysis of these frequencies as reported by others, we confirmed that our result (C1772T or G1790A) for breast cancer patients is similar to that of head and neck, esophageal cancers. The genotype frequencies of CT or TT observed in the patients and healthy controls were 10 vs. 8.8% (breast cancer; Korean), 11.6 vs. 10.6% (ESCC; Chinese) and 18.2 vs. 10.9% (HNSCC; Japanese) whereas the frequency in renal cancer (English) indicated 90.0 vs. 99.3% (Table II). In addition, the genotype frequencies of GA or AA observed in the patients and healthy controls were 3.3 vs. 7.9% (breast cancer; Korean) and 7.3 vs. 8.2% (HNSCC; Japanese). In contrast, the frequency in renal cancer (English) shows 55.5 vs. 17% (Table III). Based on these results, we concluded that there is a difference of frequency pattern in HIF-1α polymorphisms between Asian and European populations. We suggest, therefore, that HIF-1α genotype distribution is associated with different racial groups.

Induction of HIF-1α expression appears to be a crucial step in a tissues response to hypoxia. The response occurs via increased mRNA expression, protein stabilization, nuclear

localization and augmented activity of its transcriptional activation domains (24). An increase in tissue concentration of HIF-1α has recently been demonstrated in association with a more aggressive phenotype of cancer cells and with progression of human malignant diseases (7,22,25,26). In particular, for breast cancer, overexpression of HIF-1α was associated with increased proliferation and poor differentiation (19). As shown in Table IV, HIF-1α overexpression occurred in 56.7% (51 of 90) of our breast cancer cases according to immunochemical staining. Further, elevated levels of HIF-1α expression were significantly associated with a high histological grade (p=0.001) and metastasis to lymph nodes (p=0.041). We also determined a positive association between expression of HIF-1α and Ki-67 (Table IV). Indeed, Ki-67 overexpression in the S or G1-G2 phase indicates a high proliferation rate and thus poor prognosis (27). In the case of an HIF-1α positive group, there seems to be a significant association with increased levels of Ki-67 as a marker of proliferation (Table IV). These results are consistent with previous reports of HIF-1α staining in breast cancer (19,28-30). Thus, we suggest that HIF-1α is involved in unfavorable prognosis for Korean breast cancer patients.

In the subgroup of patients carrying the polymorphic T1772 allele (n=9), we found a high histological grade (III) in 77.8% (7 of 9). In addition, we determined a significant association between the C1772T polymorphism and expression of HIF-1α (p=0.040) (Table V). These results are consistent with previous findings in lung cancer patients demonstrating that CT or TT cases have an HIF-1α overexpression of 81.8% (9 of 11) (31). Although a significant association between HIF-1α overexpression and p53 accumulation were not found, positive relations were found in accordance with the findings of Bos *et al* and Zhong *et al* in colon and breast cancers (2,29). We also found a significant association between C1772T polymorphism and p53 expression (p=0.04). As a matter of fact, tumor suppressor gene p53 represses HIF-1-stimulated transcription and HIF-1 and wt p53 both are up-regulated in hypoxic condition (32). Although the G1790A polymorphism was studied, the GA genotype seems not to have significant meaning (data not shown). Finally, patients carrying the polymorphic T1772 allele likely result in a poorer prognosis and presumably more aggressive tumors.

In conclusion, our study demonstrated, for the first time, that the polymorphism C1772T of HIF-1α gene increase expression of HIF-1α protein in breast cancer. We have determined that HIF-1α overexpression depends on cancer progression and Ki-67 as a marker of proliferation. Moreover, we suggest that the polymorphic T1772 allele with HIF-1α overexpression may contribute to development of higher grade cancers. We stress the need for C1772T (P582S) polymorphism and expression of HIF-1α relationship be further assessed as a risk factor for cancer development and predictive marker of poor prognosis in breast cancer patients.

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