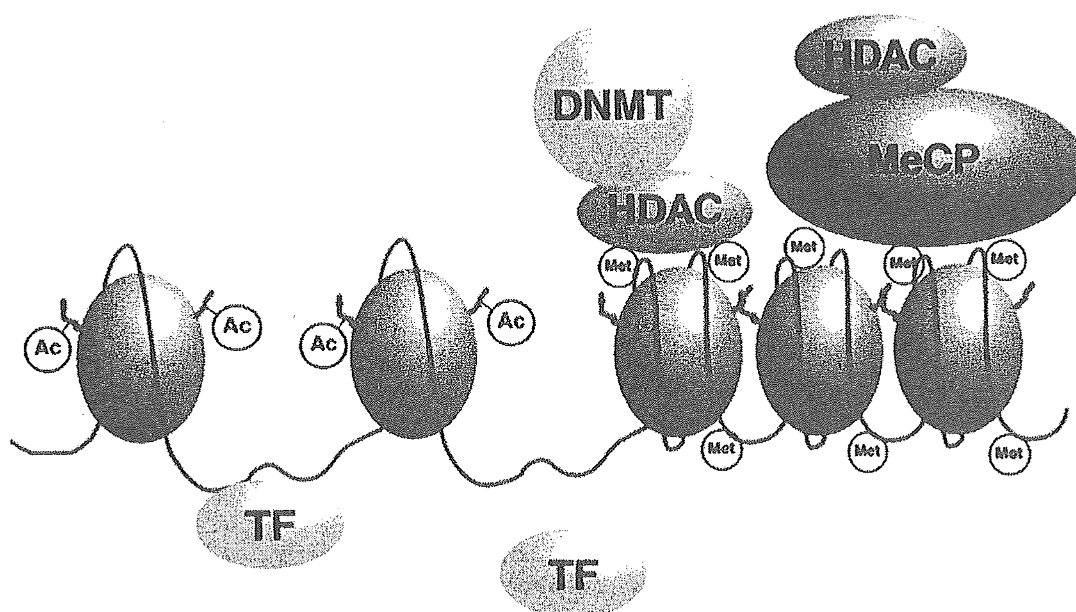

FIGURE 1. Schematic illustration of the regulation of gene expression by histone acetylation and CpG methylation. Ac, acetylation of histone tail; Met, methylation of CpG island; TF, transcription factor; DNMT, DNA methyltransferase; HDAC, histone deacetylase; MeCP, methyl-CpG binding proteins.

terminal tails of histones are subjected to acetylation and methylation, which recruit downstream regulatory factors, influence chromatin structure, and are critical determinants of transcription.[5] Relaxed chromatin resulting from histone acetylation is generally associated with transcriptional activation.[6] There is a close association between histone acetylation and DNA methylation.[7,8] Histone deacetylase-1 (HDAC1) can form a complex with both methyl-CpG–binding proteins (MeCP) and DNA methyltransferase-1 (DNMT1) to silence the gene expression.[9] In contrast, methylation of histone tails is linked to either activation or repression, depending on the residue methylated.[10] However, the global acetylation status of histones in cancer and its relation to the biological behavior of cancer cells still remains unclear.

On the other hand, cancer morbidity results in large part from metastases, and a majority of advanced cancer patients die due to complications resulting from metastases, not to the primary tumor. To produce a metastasis, tumor cells must complete a multistep progression through a series of sequential and selective events.[11] Dr. Isaiah Fidler has alluded to the metastatic tumor cell as a champion among decathlon athletes.[11] The metastatic process consists of detachment, local invasion, motility, angiogenesis, vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in different organs.[11] In each step, causative molecules have been identified; these include cell adhesion molecules, various growth factors, matrix degradation enzymes, motility factors, and so on. In this article, by summarizing a series of our studies, we will describe the importance of altered histone acetylation in gastrointestinal carcinogenesis, especially in relation to invasion and metastasis. Finally, we will propose that histone acetylation might be an important target of interventions aimed against invasive and metastatic disease.

HYPOACETYLATION STATUS OF HISTONE IN GASTROINTESTINAL CANCER

The global acetylation status of histones during carcinogenesis was studied by examining the expression of acetylated histone H4 by Western blotting in nonneoplastic gastric mucosa and various stages of gastric tumors.[12] By using antiacetylated histone H4 antibody, the level of acetylated histone H4 expression was shown to be reduced in 70% of gastric carcinomas in comparison with nonneoplastic mucosa, while the total amount of histone did not differ significantly between tumor and normal tissues, indicating global hypoacetylation in gastric cancer. In immunohistochemistry studies using the same antibody, acetylated histone H4 was uniformly expressed in the nuclei of nonneoplastic gastric mucosa in both epithelial cells and stromal cells including lymphocytes and fibroblasts (FIG. 2). Acetylated histone H4 was localized in the nuclei of these cells. The expression of acetylated histone H4 was reduced in about 70% of gastric cancers and 45% of gastric adenomas, respectively. Reduced histone H4 acetylation was also found in some gastric lesions exhibiting intestinal metaplasia, a condition predisposing to gastric cancer. We then analyzed the relationship between reduced histone acetylation and clinicopathological findings of gastric cancer. Reduced expression of acetylated histone H4 correlated well with advanced tumor stage, deep tumor invasion, and lymph node metastasis (TABLE 1). Thus, low levels of global histone acetylation are closely associated with tumorigenesis as well as invasion and metastasis of gastric cancer.

A similar reduction of histone H4 acetylation is observed in colorectal cancer (Ono and Yasui, unpublished observation). The levels of histone H4 acetylation are heterogeneous or reduced in about 80% of cancers and in 39% of adenomas of the colorectum, respectively. Reduced acetylation of histone H4 is significantly correlated with advanced tumor stage and depth of tumor invasion. Furthermore, in advanced cancer cases, there is a tendency for the superficial part of the tumor to express a high level of acetylated histone H4, whereas the deeply invasive part of the tumor reveals reduced histone acetylation. From these observations, at least in gastrointestinal cancers, it appears possible that reduced levels of global histone acetylation may participate not only in cancer development, but also in invasion and metastasis.

INHIBITION OF GROWTH AND INVASION BY INDUCED HISTONE ACETYLATION

Histone deacetylase (HDAC) reduces acetylation of histone tails. Trichostatin A (TSA), originally used as an antifungal drug, inhibits HDAC activity, induces histone acetylation, and, in general, enhances gene expression.[13,14] HDAC inhibitors induce growth arrest, apoptosis, and differentiation of a variety of tumor cells.[15–17] Therefore, HDAC inhibitors are believed to be one of the most promising class of new anticancer agents.[14] TSA actually induces growth arrest of all the eight gastric adenocarcinoma cell lines and the three oral squamous cell carcinoma cell lines examined.[18] TSA triggers such morphological characteristics of apoptosis as smaller size and rounder shape. Apoptotic cell death is confirmed by apoptosis ladder formation and induction of a cleaved form of poly(ADP-ribose) polymerase (PARP) in-

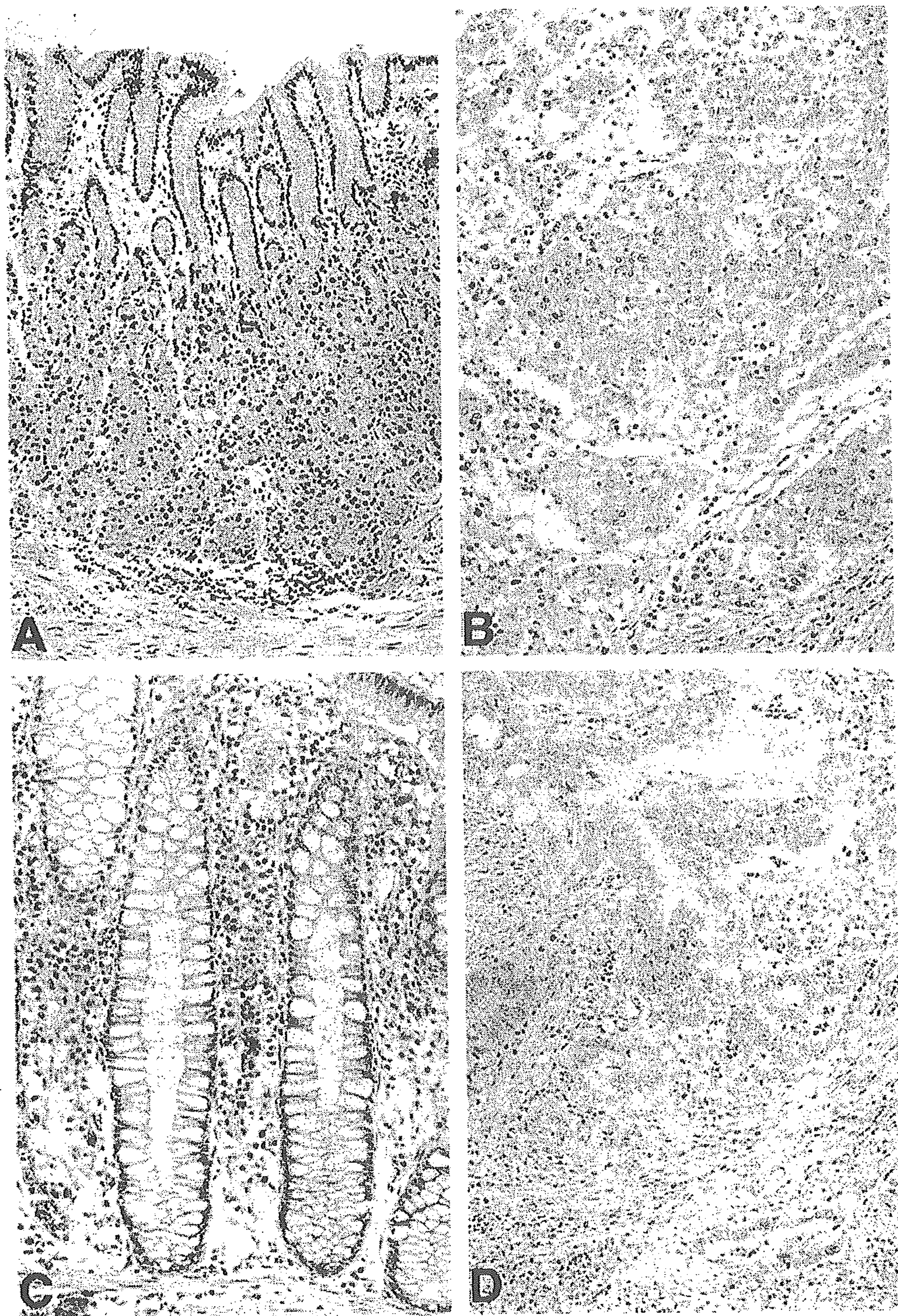

FIGURE 2. Immunohistochemical analysis of histone acetylation in normal gastric mucosa **(A)**, gastric carcinoma **(B)**, normal colon mucosa **(C)**, and colon carcinoma **(D)**. Tissue sections were immunostained using anti-acetylated histone H4 antibodies. Positive reactions were observed as a *dark color* in the nuclei of the cells. Acetylated histone H4 is well preserved in both epithelial and stromal cells of normal mucosa **(A, C)**, whereas it is markedly reduced in carcinoma cells **(B, D)**.

TABLE 1. Expression of acetylated histone H4 in gastric mucosa, adenomas, and adenocarcinomas, and its correlation with clinicopathological parameters

	Number of cases	Expression of acetylated histone H4[a]			P value[b]
		Preserved	Reduced	Markedly reduced	
Normal mucosa	65	65 (100%)	0	0	<0.0001
Adenoma	13	7 (54%)	5 (38%)	1 (8%)	
Adenocarcinoma	57	19 (33%)	23 (40%)	15 (26%)	
Histology					0.2465
Well differentiated	40	16 (40%)	15 (38%)	9 (23%)	
Poorly differentiated	17	3 (18%)	8 (47%)	6 (35%)	
Stage					0.0240
1	37	14 (38%)	19 (51%)	4 (11%)	
2	2	1 (50%)	0	1 (50%)	
3	12	3 (25%)	2 (17%)	7 (58%)	
4	6	1 (17%)	2 (33%)	3 (50%)	
Depth of invasion					0.0072
m, sm	34	14 (41%)	17 (50%)	3 (9%)	
mp, ss	8	1 (13%)	3 (38%)	4 (50%)	
se, si	15	4 (27%)	3 (20%)	8 (53%)	
Lymph node metastasis					0.0006
Negative	38	15 (39%)	19 (50%)	4 (11%)	
Positive	19	4 (21%)	4 (21%)	11 (58%)	

[a]Grades of acetylated histone H4 expression were classified as preserved, reduced, and markedly reduced according to the staining intensity and number of stained cells. Preserved: most cells express acetylated histone H4 at levels equal to stromal cells such as lymphocytes and fibroblasts. Reduced: 5–30% of cells express at lower levels than stromal cells. Markedly reduced: over 30% of cells express at lower levels than stromal cells.

[b]Correlation was analyzed by chi-square, and *P* values are shown.

duction in all the cell lines. Following TSA treatment, the expression of growth- and apoptosis-related molecules is altered. TSA induces the expression of *p21*^{WAF1}, *Bak,* and *Bax*, while it reduces the expression of *E2F-1, E2F-4,* and the phosphorylated form of Rb protein. Induction of *p21*^{WAF1} and *Bax* must be *p53* independent because it is found in cell lines regardless of the presence of *p53* mutations. Moreover, HDAC-1 is reduced, whereas CBP (CREB binding protein), a histone acetyltransferase, is induced. These findings indicate that histone deacetylation participates in unbridling cell proliferation by modifying gene expression of cell cycle regulators and apoptosis-related molecules.

We then asked whether histone acetylation affects the invasion ability of cancer cells. We performed an invasion assay using two gastric cancer cell lines, MKN-28 and MKN-1. Both of them possess mutated *p53* genes, and their growth is suppressed by TSA. We used two concentrations of TSA (200 nM and 1 μM), and-both clearly suppressed invasion of MKN-28 but not MKN-1. Because TSA inhibited cell growth of the two cell lines monitored by MTT [3(4,5-dimethylthiazol-2-yl)-2,5-

diphenyl tetrazoliumbromide] assay, we normalized the numbers of invading cells by taking growth inhibition into account, and the percent inhibition of invasion in MKN-28 cells was found to be 60–70%. Therefore, although induction of histone acetylation by TSA inhibits cell growth and induces apoptosis of all the cancer cell lines, inhibition of tumor invasion by TSA varies with the individual cancer.

MOLECULAR BASIS OF TSA INHIBITION OF TUMOR INVASION

Systematic analysis of gene expression profiles is a powerful tool to elucidate the molecular mechanism of cancer development and progression. To identify genes participating in TSA inhibition of tumor invasion through histone acetylation, we treated the two gastric cancer cell lines MKN-1 and MKN–28 and studied the alterations of the gene expression profile using cDNA microarrays. TSA treatment upregulates or downregulates many genes, including those possibly related to invasion and metastasis. However, the categories of genes whose expression is changed by TSA treatment are not always the same in the two cell lines. For instance, amphiregulin, a bifunctional growth factor,[19] is induced in both cell lines, while *TIMP-1*, *-3*, *nm23H1,* and *nm23H2* are induced in MKN-28, but not in MKN-1, cells. *Nm23H1/H2* are the genes that participate in suppression of cell motility and metastasis of various tumors, including melanoma, breast cancer, and gastric cancer.[20–22] *TIMP-1* and *TIMP-3* might function as suppressors of tumor invasion through inhibition of extracellular matrix degradation.[23] These differential alterations of gene expression by TSA treatment coincide with the difference in inhibition of tumor invasion between the two cell lines.

To confirm whether histone acetylation occurs in the promoter regions of the genes whose expression is induced by TSA treatment, we studied the time course of the effect of TSA on the expression of these genes. For this purpose, we used RT-PCR (reverse-transcription polymerase chain reaction) and chromatin immunoprecipitation, using antibodies to acetylated histone H3 or acetylated histone H4. As expected, the expression of amphiregulin was enhanced by TSA, and histone acetylation in the amphiregulin promoter was induced in both cell lines. Acetylation of histones H3 and H4 was induced to the same extent and with the same timing. In the eight cancer cell lines examined, the constitutive level of amphiregulin expression was directly associated with histone acetylation in the promoter, suggesting that expression of the amphiregulin gene is tightly regulated by promoter acetylation. Furthermore, the expression of genes related to invasion and metastasis, such as *nm23H1/H2* and *TIMP-3*, was also confirmed to be regulated by histone acetylation. At 24 hours after TSA treatment, peak induction of acetylation of histone H4 in the *nm23* promoter was detected, while expression of *nm23* was at its highest level in MKN-28 cells at 24 hours. However, no such induction in *nm23* expression and histone acetylation was detected in MKN-1 cells. Expression of *TIMP-3* and histone acetylation in the *TIMP-3* promoter were also induced by TSA in MKN-28, but not in MKN-1 cells. These findings indicate that, although response to TSA in terms of tumor invasion differs depending on individual cancers, inhibition of tumor invasion is closely associated with altered expression of genes related to invasion and metastasis via TSA-induced histone acetylation in their promoter regions.

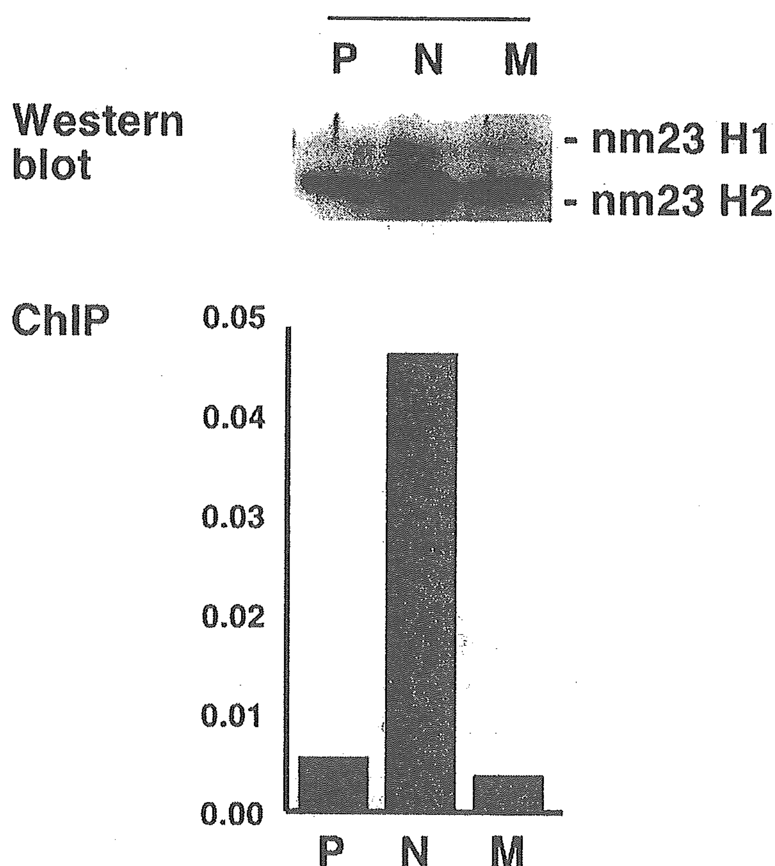

FIGURE 3. Tight association between the expression of nm23 and histone acetylation in the nm23 promoter in a gastric carcinoma case with nodal metastasis. The expression of nm23 proteins was examined by Western blotting using an anti-nm23 antibody. Acetylation status of *nm23* promoter was analyzed by chromatin immunoprecipitation using anti-acetylated histone H4. N, normal mucosa; P, primary tumor; M, lymph node metastasis; ChIP, chromatin immunoprecipitation.

To verify the close association between metastasis and histone acetylation in the *in vivo* setting, we examined histone acetylation of the *nm23H1* promoter by chromatin immunoprecipitation and compared it with the levels of *nm23* expression in normal gastric mucosa, primary gastric cancer, and metastatic tumor from the same patient. Chromatin immunoprecipitation revealed that acetylation of histone H4 in the *nm23* promoter is well preserved in normal tissue, but reduced in primary tumor and the corresponding metastasis. Parallel studies of the same tissues showed that expression of *nm23* was suppressed in the primary and metastatic tumors (FIG. 3). Experimental animal models of metastasis are expected to demonstrate the effect of global histone acetylation on metastatic ability.

NOVEL METASTASIS-RELATED GENES IDENTIFIED THROUGH SERIAL ANALYSIS OF GENE EXPRESSION

To understand the relationship between histone acetylation and gastrointestinal carcinogenesis in more detail, a search for novel genes involved in cancer development, invasion, and metastasis must be carried out. For this purpose, we are evaluat-

ing global gene expression profiles by means of serial analysis of gene expression (SAGE). SAGE analyzes about 10- to 12-base pair tags derived from a defined position, near the polyA tails of cDNAs, just downstream of the CATG sequence.[24] The SAGE tag quantities directly reflect the abundance of the messenger RNAs (mRNAs); therefore, SAGE data are highly accurate and quantitative. The Human Genome Project has facilitated the mapping of specific genes to individual tags detected by SAGE.[25] A large number of normal and tumor tissues and cells have been analyzed by SAGE, creating large databases. Over a hundred SAGE libraries are now online and available to the public.[26,27] We analyzed the global gene expression profiles of two types of primary gastric cancer using SAGE and compared these with SAGE libraries of other normal and tumor tissues in the database. We have identified sets of genes and tags that are highly expressed in well-differentiated or poorly differentiated primary gastric cancers. Since the database contains accurate information about the distribution and frequency of the genes and tags, the gene expression in gastric cancer can be compared with that in other cells and tissues by virtual Northern analysis using the online SAGE tag-to-gene mapping and virtual Northern functions.[28] Typical examples are shown in FIGURE 4. Tags for genes A, B, C, and EST-1 are frequently found in the well-differentiated type of gastric cancer, while they are infrequent in poorly differentiated gastric cancer or other SAGE libraries including those for cancers of the breast, colon, pancreas, brain, and prostate. Therefore, these genes may participate in the development of the well-differentiated type

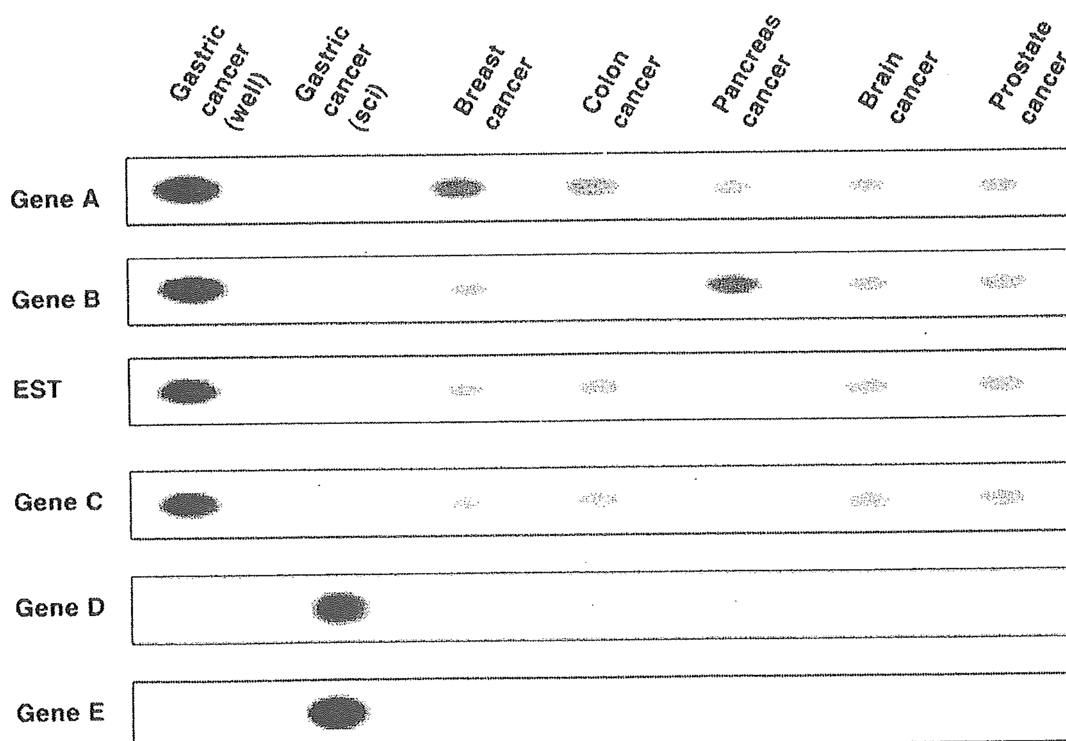

FIGURE 4. Virtual Northern analysis of genes differentially expressed in well-differentiated and poorly differentiated types of gastric carcinomas by serial analysis of gene expression. Genes A, B, and C and EST were preferentially expressed in the well-differentiated type (well), while the expression of genes D and E was almost specific to the poorly differentiated type (sci).

of gastric cancer. Tags for genes D and E may be responsible for carcinogenesis of the poorly differentiated scirrhous type of gastric cancer.

The same strategy is applicable to the identification of genes involved in metastasis. We have analyzed the gene expression profiles of a primary gastric cancer and the metastatic tumor from the same patient. By comparing the expression levels between primary and metastatic tumors, sets of genes and tags whose expression is markedly reduced or lost in the metastatic tumor were identified, indicating that they may be candidate suppressors of tumor metastasis. Genes and tags lost in metastasis must be interesting, because the expression of metastasis suppressors including *nm23* and *TIMP* is induced by histone acetylation. Among them, we have focused

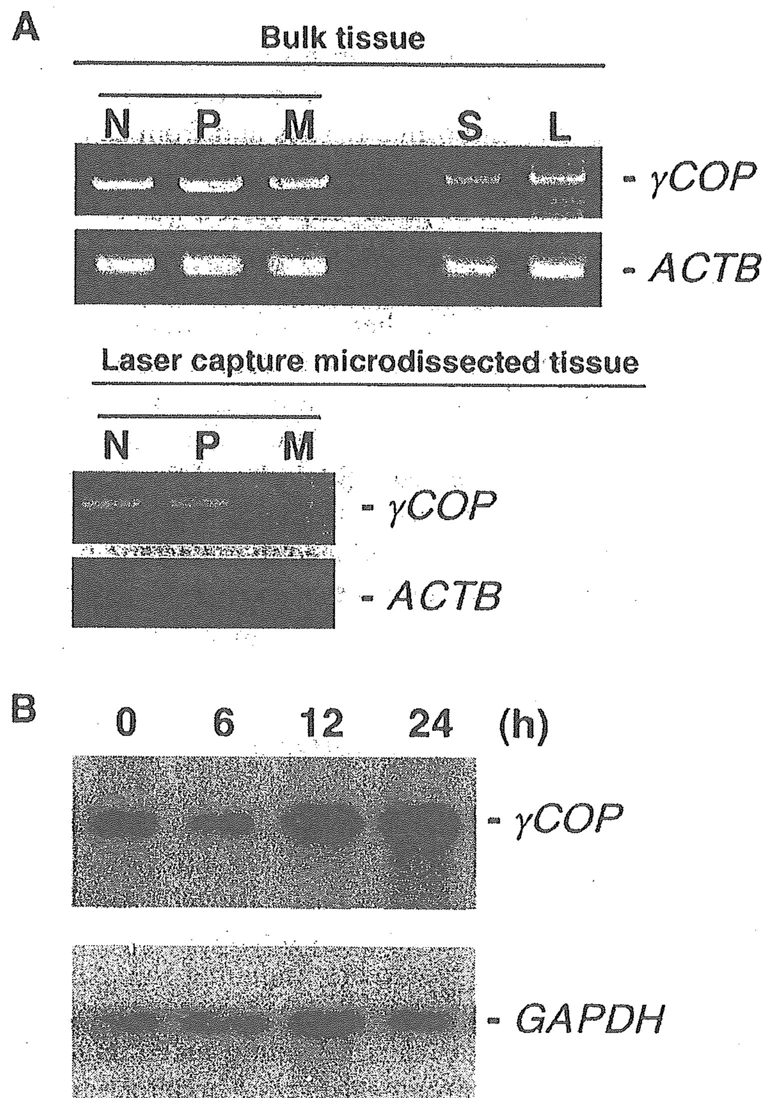

FIGURE 5. Expression of *γCOP* in a primary gastric carcinoma and its metastasis and TSA induction of *γCOP* expression in gastric cancer cell line. **(A)** After laser capture microdissection, loss of *γCOP* expression in metastatic tumor was demonstrated (RT-PCR method). N, normal mucosa; P, primary tumor; M, lymph node metastasis; S, normal spleen; and L, normal lymph node. **(B)** Northern blot analysis was used to examine *γCOP* expression in the gastric cancer cell line, MKN-28, following treatment with TSA. Treatment with TSA clearly induced *γCOP* expression in MKN-28 cells.

on the *γCOP* (coat protein γ) gene, which is expressed at high levels in the primary tumor, but exhibits no expression in the metastasis. *γCOP* is detected at certain levels in SAGE libraries of other primary tumors in the database. *γCOP* is one of the seven subunits forming the COPI coat complex. (This complex, which contains the four COP coat proteins, is also called the Golgi coat promoter, or the "coatomer.")[29] COPI was originally discovered on buds and vesicles derived from *cis* and *medial* Golgi membranes; it mediates transport between the Golgi and the endoplasmic reticulum.[30] The COPI is recruited to the membrane following activation of the small GTPase Arf1. The only information about the relationship of *γCOP* to cancer is that *γCOP* suppresses mutant *cdc42* (ras-related GTP-binding protein), which mediates cellular transformation of NIH3T3 cells.[31] When we examined the expression of *γCOP* by RT-PCR in normal gastric tissue, primary tumor, and metastatic tumor using RNA from bulk tissues, no difference in expression was noticed in these three samples (FIG. 5). *γCOP* is also expressed in normal spleen and lymph nodes. We then enriched target cells by using laser capture microdissection and obtained RNA samples from epithelial cells from normal tissues and from cancer cells, with minimal contamination by stromal cells such as lymphocytes located within tumor tissues. Finally, an obvious reduction in *γCOP* expression was confirmed in metastatic tumors, suggesting that *γCOP* is a novel candidate for a metastasis suppressor gene. As mentioned before, acetylation status is generally reduced in gastric cancer with lymph-node metastases (TABLE 1). We then asked the question whether *γCOP* expression is affected by histone acetylation. We treated the gastric cancer cell line MKN-28 with TSA, to increase histone acetylation, and examined the expression of *γCOP* by Northern blotting and histone acetylation status in the promoter by chromatin immunoprecipitation. As expected, TSA induced the expression of *γCOP* and histone acetylation in the promoter. The CDK inhibitor $p21^{WAF1}$ exhibits a similar situation in that the expression of *p21* is reduced in metastatic tumor and TSA treatment induces *p21* expression and histone acetylation in the promoter in gastric cancer cells.

CONCLUSIONS

The expression of acetylated histone H4 is reduced in a majority of gastric and colorectal cancers, suggesting that there is a low level of global histone acetylation in tumor cells. Reduced histone acetylation is significantly associated with invasion and metastasis of gastrointestinal cancers. Treatment with TSA is followed by increased histone acetylation in the promoters, inducing the expression not only of negative cell cycle regulators and apoptosis-related molecules but also of many suppressor genes of invasion and metastasis, including *nm23* and *γCOP*, which were identified by SAGE. TSA induced growth arrest and apoptosis and suppressed invasion of gastric cancer cells. These results suggest that histone deacetylation may participate in invasion and metastasis by modifying expression of a variety of genes. Therefore, histone acetylation should be a promising target for cancer therapy, especially against invasive and metastatic disease. It is important to note that the cellular response to TSA, reflecting the role of histone acetylation, depends on cell type; that is, histone acetylation affects metastasis and invasion ability in certain cancer cells, while it suppresses growth and induces apoptosis of all the cells examined. Never-

theless, this is the first demonstration that histone acetylation is tightly linked to invasion and metastasis of human cancers. Since a tight association has been shown between histone acetylation and DNA methylation, the role of DNA methylation and its relation to histone acetylation in invasion and metastasis should be clarified in the near future.

ACKNOWLEDGMENTS

Our project is supported, in part, by Grants-in-Aid from the Ministry of Education, Culture, Sports, and Technology of Japan, and the Ministry of Health, Labor, and Welfare of Japan.

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Int. J. Cancer: **107**, 593–596 (2003)
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Publication of the International Union Against Cancer

A SINGLE NUCLEOTIDE POLYMORPHISM IN THE TRANSMEMBRANE DOMAIN CODING REGION OF *HER-2* IS ASSOCIATED WITH DEVELOPMENT AND MALIGNANT PHENOTYPE OF GASTRIC CANCER

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Alterations of the *HER-2 (erbB-2/neu)* proto-oncogene have been associated with carcinogenesis and poor prognosis of certain cancers. A single nucleotide polymorphism (*Ile/Val*, A/G) in the transmembrane domain was reported to be associated with a risk of breast cancer. In our study, we examined the association between the *HER-2* polymorphism and gastric carcinoma. The *Ile/Ile*, *Ile/Val* and *Val/Val* genotypes were found in 146 (68.9%), 56 (26.4%) and 10 (4.7%) of 212 gastric cancer patients and in 234 (81.5%), 48 (16.7%) and 5 (1.8%) of 287 control subjects, respectively. The *Ile/Val* or *Val/Val* genotype was significantly more frequent in patients than in controls ($p = 0.005$ and 0.033, respectively). The OR of *Val/Val* genotype then revealed a significantly enhanced risk of 3.25 (95% CI 1.09–9.70) compared to *Ile/Ile* genotype; heterozygous *Ile/Val* genotype showed an intermediate risk of 1.97 (1.27–3.06). In patients, carcinomas of advanced stage were significantly more frequent in patients with *Ile/Val* or *Val/Val* genotype than those with *Ile/Ile* genotype ($p < 0.001$). The logistic regression analysis for tumor invasion, lymph node metastasis and distant metastasis revealed that lymph node metastasis was most closely associated with the *HER-2* genotype. These results suggest that this nucleotide polymorphism in the transmembrane domain-coding region of *HER-2* could be associated with development of gastric carcinoma and may serve as a predictor of risk for a malignant phenotype of gastric cancer. The association of *HER-2* genotype with clinicopathologic characteristics of gastric cancer was also suggested, which has to be confirmed with a larger sample size.
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Key words: *single nucleotide polymorphism;* HER-2; *transmembrane domain coding region; gastric carcinoma; case-control study*

HER-2 (also known as *erbB-2* or *neu*) proto-oncogene, a member of the epidermal growth factor (EGF) receptor family, located at chromosome 17q21, encodes a transmembrane glycoprotein (p185) with tyrosine kinase activity.[1–5] Dimerization of the HER-2 leads to tyrosine kinase activation and subsequent downstream signaling events.[6–9] Amplification of *HER-2* gene has been found in some human cancers including carcinomas of the breast, ovary and stomach.[10,11] In breast cancer, *HER-2* amplification and/or overexpression has been associated with steroid hormone receptor-negative tumors, increased tumor aneuploidy, high growth rate, reduced response to chemotherapy and hormonal therapy and poor prognosis.[12] In gastric cancer, overexpression of HER-2 caused by amplification has been closely related to liver metastasis and poor prognosis.[13,14] HER-2 is now being paid more attention because recently, trastuzumab, a humanized murine monoclonal antibody directed against the extracellular domain of HER-2, was introduced for the treatment of patients with HER-2-overexpressing advanced breast cancer.[15–17] Point mutations in the *HER-2* gene have not been identified,[18–20] and a major mechanism of HER-2 activation is thought to be gene amplification.

Single nucleotide polymorphism (SNP) in the transmembrane coding region of the *HER-2* gene at codon 655, encoding either isoleucine (Ile: ATC) or valine (Val: GTC), has been identified.[21] Xie *et al.*[22] first reported that this *Ile/Val* SNP is associated with significantly increased risk of breast cancer development. However, several studies have shown that this association is controversial. Positive correlation between the *Ile/Val* SNP and breast cancer risk was reported to be associated with stage of disease,[23] whereas no association has been found in breast cancer among the British,[24] German[25] and Japanese[26] populations. In colorectal cancer, *Ile/Val* SNP was not associated with cancer risk in Caucasians.[27] The mechanistic role of this SNP in possible involvement of tumorigenesis has not been fully understood. Fleishmann *et al.*[28] recently reported that the *Val* allele enhanced active dimeric conformations of HER-2, resulting in increased autophosphorylation, tyrosine kinase activation and cell transformation, even under conditions of HER-2 overexpression. Although a role of HER-2 in gastric cancer has been acknowledged, there have been no studies done on the correlation between *HER-2* SNP and gastric cancer. In our present study, we investigated whether the *Ile/Val* SNP of *HER-2* is associated with the development and malignant phenotype of gastric cancer.

MATERIAL AND METHODS

Study subjects

The 287 controls we analyzed were randomly selected from those visiting hospitals in Hiroshima for regular health checks or symptoms such as appetite loss or epigastralgia. They were proven to be free from malignancy by medical examination with gastric endoscope and biopsy. Representative biopsy samples of mucosa confirmed histologically to be benign were used for genotype analysis. We analyzed 212 patients with primary gastric cancer, who underwent surgical operation at Hiroshima University Hospital in 1990–2001, at Hiroshima Memorial Hospital in 1998–2000 or at Hofu Institute of Gastroenterology in 2000–2001. We confirmed microscopically that all gastric cancer patients have gastric adenocarcinomas, and the corresponding nonneoplastic mucosae did not exhibit any tumor-cell invasion or show signifi-

Abbreviations: A, adenine; CI, confidence interval; G, guanine; Ile, isoleucine; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; Val, valine.

Grant sponsor: Ministry of Education, Culture, Sports, and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Received 31 March 2003; Revised 21 May, 22 June 2003; Accepted 8 July 2003

DOI 10.1002/ijc.11450
Published online 29 August 2003 in Wiley InterScience (www.interscience.wiley.com).

cant inflammatory involvement. The clinicopathologic staging and histologic classification were made according to the criteria of the TNM classification (UICC), the 5th edition, 1997, stomach (ICD-O C16). The demographic characteristics of 212 gastric cancer patients and 287 controls are summarized in Table I. There were no significant differences in gender and age at recruitment between the patients and controls. Because written informed consent was not obtained, for strict privacy protection, all samples were unidentified before correlation with genotype. This procedure is in accordance with Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and approved by the Ethical Review Committee of the Hiroshima University School of Medicine.

DNA extraction

DNA was extracted from freshly frozen nonneoplastic gastric mucosae of 84 patients and also from paraffin-embedded mucosae of 128 patients using a genomic DNA purification kit (Promega, Madison, WI) and proteinase K as described previously, respectively.[29] DNA was extracted from paraffin-embedded gastric mucosae of all controls using proteinase K.

PCR/restriction fragment length polymorphism (PCR-RFLP)-based assay

Genotypes of the *HER-2* gene were analyzed by PCR-RFLP as previously described using the DNA extracted from nonneoplastic gastric mucosae.[22] PCR fragments were generated from 10–20 ng of genomic DNA in a 25 μl reaction mixture containing 0.75 units Ampli Taq Gold (Perkin Elmer, Norwalk, CT), 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 200 μM of each deoxynucleotide triphosphate (dNTP). PCR was performed at 94°C for 30 sec followed by 35 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR primers used, based on the published sequence of human complementary DNA of the *HER-2* gene,[22] were 5′-AGAGCGCCAGCCCTCTGACGTCCAT-3′ (HER-2/U) and 5′-TCCGTTTCCTGCAGCAGTCTCCGCA-3′ (HER-2/L). The 148 bp of PCR products (7 ′l) were digested with *BsmAI* (New England BioLabs, Beverly, MA) at 55°C for 2 hr in a total reaction volume of 10 μl followed by heat inactivation at 80°C for 20 min. *BsmAI* gives 116 bp and 32 bp fragments for the *Val* (GTC) allele and a single 148 bp fragment for the *Ile* (ATC) allele.[22] Fragments digested with *BsmAI* (6 μl) were subjected to electrophoresis with 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. The genotyping was made by 2 investigators (K.K. and S.M.) without knowledge of case-control status. About 10% of the samples were randomly selected for repeated assays.

Statistical analysis

Fisher's exact test was used to test whether the distribution of *HER-2* genotypes was significantly different between gastric cancer patients and controls. In gastric cancer patients, correlation between the genotypes and clinicopathologic characteristics was also examined by Fisher's exact test (InStat Ver. 2.01, GraphPad Software, San Diego, CA). The logistic regression model calculated odds ratios for the genotypes, adjusting for age and gender; the logistic regression analysis was performed for the association between the genotypes and clinicopathologic characteristics (SPSS software, Ver .11.0).

TABLE I – CHARACTERISTICS OF STUDY SUBJECTS

	Patients (n = 212)	Controls (n = 287)
Gender[1]		
Male	152 (72.2%)	193 (67.2%)
Female	60 (27.8%)	94 (32.8%)
Age (years, ± SD)	66.0 ± 12.0	64.2 ± 11.8

[1]p = 0.3 for differences between patients and controls.

RESULTS

Risk of gastric cancer by HER-2 *genotyping*

Representative PCR-RFLP patterns of *HER-2* genotypes are shown in Figure 1. Digestion of PCR product (148 bp) with *BsmAI* resulted in a single fragment of 148 bp for the *Ile* allele or 2 fragments of 116 bp and 32 bp for the *Val* allele, as reported previously.[22] We confirmed that each PCR product had no nonspecific bands corresponding to these fragments before digestion with *BsmAI* by polyacrylamide gel electrophoresis. Genotypes *Ile/Ile, Ile/Val* and *Val/Val* were found in 146 (68.9%), 56 (26.4%) and 10 (4.7%) of 212 gastric cancer patients and in 234 (81.5%), 48 (16.7%) and 5 (1.8%) of 287 controls, respectively (Table II). The genotype distribution among controls was in good agreement with Hardy-Weinberg equilibrium ($p < 0.05$). Genotypes *Ile/Val* and *Val/Val* were more frequent in gastric cancer patients than those in controls ($p = 0.005$ and 0.033, respectively); *Val* allele frequencies were 0.179 and 0.101 in patients and controls, respectively ($p < 0.001$). The OR of the *Val/Val* genotype then revealed a significantly enhanced risk of 3.25 (95% CI 1.09–9.70) compared to the *Ile/Ile* genotype; the heterozygous *Ile/Val* genotype showed an intermediate risk of 1.97 (1.27–3.06). Adjustment for age and gender did not make a substantial change.

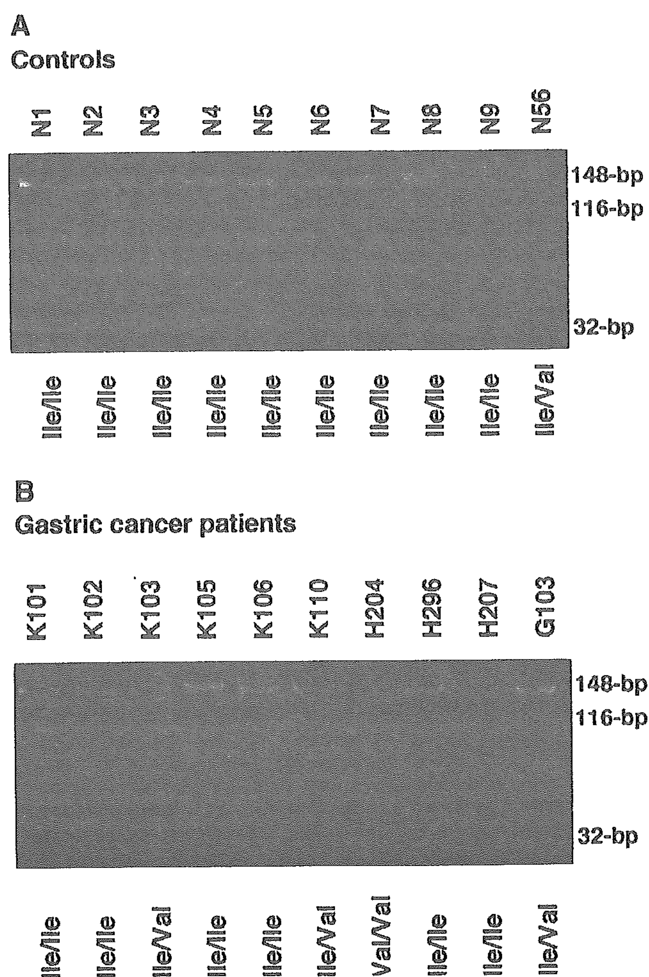

FIGURE 1 – Genotype of the *HER-2* transmembrane domain coding region at codon 655 analyzed by PCR-RFLP in healthy control subjects *(a)* and in gastric carcinoma cases *(b)*. Numbers above the panel are case numbers. Genotypes are shown below each panel.

TABLE II – *HER-2* GENOTYPE DISTRIBUTION OF STUDY SUBJECTS

	Patients (n = 212)	Controls[1] (n = 287)	P^2	OR (95% CI)	
				Crude	Adjusted[3]
Ile/Ile	146 (68.9%)	234 (81.5%)		1 (ref.)	1 (ref.)
Ile/Val	56 (26.4%)	48 (16.7%)	0.005	1.97 (1.27–3.06)	2.00 (1.28–3.10)
Val/Val	10 (4.7%)	5 (1.8%)	0.033	3.25 (1.09–9.70)	3.25 (1.08–9.76)
Allele frequencies					
Ile	0.821	0.899	<0.001		
Val	0.179	0.101			

[1]The observed genotype distribution of controls was in agreement with Hardy-Weinberg equilibrium.–[2]*P*-values were for the difference in genotype frequencies between patients and controls.–[3]ORs were adjusted for age and gender.

TABLE III – ASSOCIATION BETWEEN GENOTYPING OF HER-2 AND CLINICOPATHOLOGIC CHARACTERISTICS

	Genotypes		p
	Ile/Ile (n = 146)	*Ile/Val* or *Val/Val* (n = 66)	
TNM classification[1]			
T			
Tis or T1	54 (37.0%)	14 (21.2%)	0.026
T2, 3 or 4	92 (63.0%)	52 (78.8%)	
N			
N0	87 (59.6%)	23 (34.8%)	0.001
N1, 2 or 3	59 (40.4%)	43 (65.2%)	
M			
M0	142 (97.3%)	60 (90.9%)	0.074
M1	4 (2.7%)	6 (9.1%)	
Stage			
0 or I	80 (54.8%)	17 (25.8%)	<0.001
II, III or IV	66 (45.2%)	49 (74.2%)	
Histopathologic grading			
Well	46 (31.5%)	16 (24.2%)	0.068
Moderately	45 (30.8%)	15 (22.7%)	
Poorly	55 (37.7%)	35 (53.1%)	

[1]TNM classification: T, primary tumor; N, regional lymph node metastasis; M, distant metastasis.

Association between HER-2 *genotyping and clinicopathologic characteristics*

We analyzed the association between the *HER-2* genotypes and clinicopathologic characteristics in gastric cancer patients. Patients with *Ile/Val* or *Val/Val* genotype showed deeper invasion over T2 (p = 0.026) and more lymph node metastasis (p = 0.001) than those with *Ile/Ile* genotype (Table III). Carcinomas of advanced stage were significantly more frequent in patients with the *Ile/Val* or *Val/Val* genotype than those with the *Ile/Ile* genotype ($p <$ 0.001). Moreover, poorly differentiated adenocarcinoma tended to be more frequently found in patients with the *Ile/Val* or *Val/Val* genotype than those with the *Ile/Ile* genotype (p = 0.068). The logistic regression analysis then revealed that clinicopathologic staging was significantly associated with the *HER-2* genotype (p = 0.004), but histopathologic grading was not (p = 0.6); a subsequent analysis for T, N and M showed that N was most closely associated with the genotype (p = 0.054).

DISCUSSION

In our study, we examined whether the risk of gastric cancer is associated with the *Ile/Val* SNP of *HER-2* transmembrane domain coding region at codon 655. We found significant differences in genotype distribution between gastric cancer patients and controls, suggesting that the individuals with *Val/Val* or *Ile/Val* genotype, which may account for about 3% or 21%, respectively, in a Japanese general population, have an enhanced risk of gastric cancer development, with an OR of 1.97 or 3.25, respectively. Furthermore, this genotyping was associated with invasion, lymph node metastasis and poor differentiation in gastric cancer patients. These observations imply that this *HER-2* SNP may participate in not only development but also progression of gastric cancer.

However, the molecular mechanism of the association between the *Ile/Val* SNP at codon 655 and cancer has not been fully clarified. Several studies showed that a missense point mutation (Val664Glu) in the transmembrane domain of the *neu* proto-oncogene (*HER-2* human homologue) greatly enhanced its kinase activity and cell transformation properties.[7,30,31] It has been proposed that tyrosine kinase activity of HER-2 protein was stimulated by reorientation of the cytoplasmic domain within receptor dimers, resulting in increased transautophosphorylation and enzymatic activity, and the conformations of transmembrane domain affected this dimerization.[30,32] Recently, Fleishmann *et al.*[28] found 2 stable conformations, active or inactive, of the HER-2 transmembrane domain, using a computational exploration of conformation space of the transmembrane segments of a HER-2 homodimer; the *Val* allele was associated with active dimeric conformations of the HER-2 transmembrane domain, resulting in increased autophosphorylation, tyrosine kinase activation and cell transformation. We recently examined the association between this *HER-2* genotyping and autophosphorylation levels of HER-2 protein and found that the *Val* allele did not show higher kinase activity than the *Ile* allele in human gastric cell lines (data not shown). The *Ile/Val* genotyping may influence the ability of HER-2 protein to promote cell proliferation and transformation through other mechanisms than autophosphorylation such as dimerization capacity with other EGFR families and interactions with tumor-specific human leukocyte antigen (HLA)-A2-restricted CTLs.[33] Activation of the HER-2 signal transduction pathway is known to result in subsequent activation of the mitogen-activated protein kinase (MAPK) signaling pathway.[34] Stress-activated protein kinase-2 (SAPK2/p38), one of MAPKs, was reported to play an important role in cancer metastasis.[35] In our present study, the logistic regression analysis for tumor invasion, lymph node metastasis and distant metastasis revealed that lymph node metastasis was most closely associated with the *HER-2* genotype, suggesting that this SNP may affect the interaction between HER-2 and SAPK2/p38.

This SNP has been reported to be associated with significantly increased risk of breast cancer development.[22,23] Our present study also showed the association of this SNP with development and progression of gastric carcinoma. However, no association of this SNP with cancer development was found among other populations. These conflicting reports might be attributed partly to the small number of subjects with the homozygous *Val* genotype, leading to a decreased statistical power to detect the association between the SNP and cancer risk. Another possibility is difference of environmental factor for cancer etiology in different populations. In addition, the distribution of this *HER-2* polymorphism has been reported to vary considerably between ethnic groups. The *Val* allele has a frequency of 13% in Japanese (this study), 20% in Caucasians and 24% in African-Americans but was not detected in an African population.[36] Another HER-2 polymorphism (A to G, A23275G, where the positions are numbered from the translation initiation site; Genebank accession no. AC087491) at the intron 3′ to the transmembrane domain-coding region may also affect cancer risk.[37]

In conclusion, our study suggests that this nucleotide polymorphism in the transmembrane domain-coding region of *HER-2*

could be associated with development of gastric carcinoma and may serve as a predictor of risk of malignant phenotype of gastric cancer. Although the association of the *HER-2* genotype with clinicopathologic characteristics, especially with malignant phenotype, was also suggested, this has to be confirmed with a larger sample size.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. M. Takatani, Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, and the staff members at the Pathology Division, Hiroshima City Medical Association Clinical Laboratory for skillful technical assistance.

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Carcinogenesis vol.24 no.11 pp.1779–1783, 2003
DOI: 10.1093/carcin/bgg132

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).

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.

†*Declaration of interest*: L.Poellinger holds stock in AngioGenetics Ltd.

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

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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

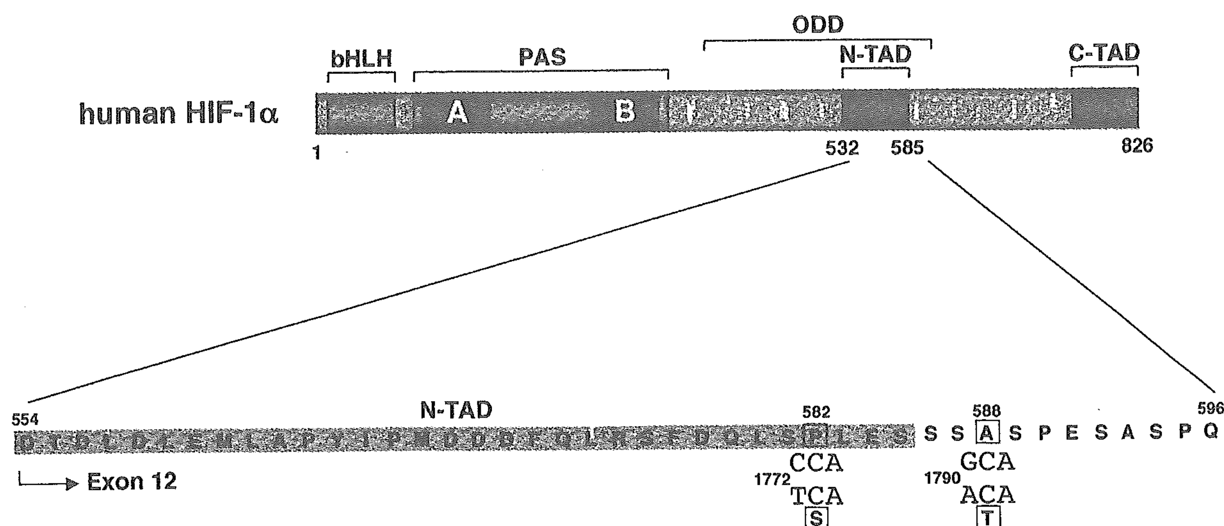

Fig. 1. Structure of HIF-1α. (Upper panel) Organization of functional domains of the HIF-1α protein. bHLH, basic-helix–loop–helix domain; PAS, Per-Arnt-Sim domain; ODD, oxygen-dependent degradation domain; N- and C-TAD, N- and C-terminal transactivation domains. (Bottom panel) Amino acid sequence encoded 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.

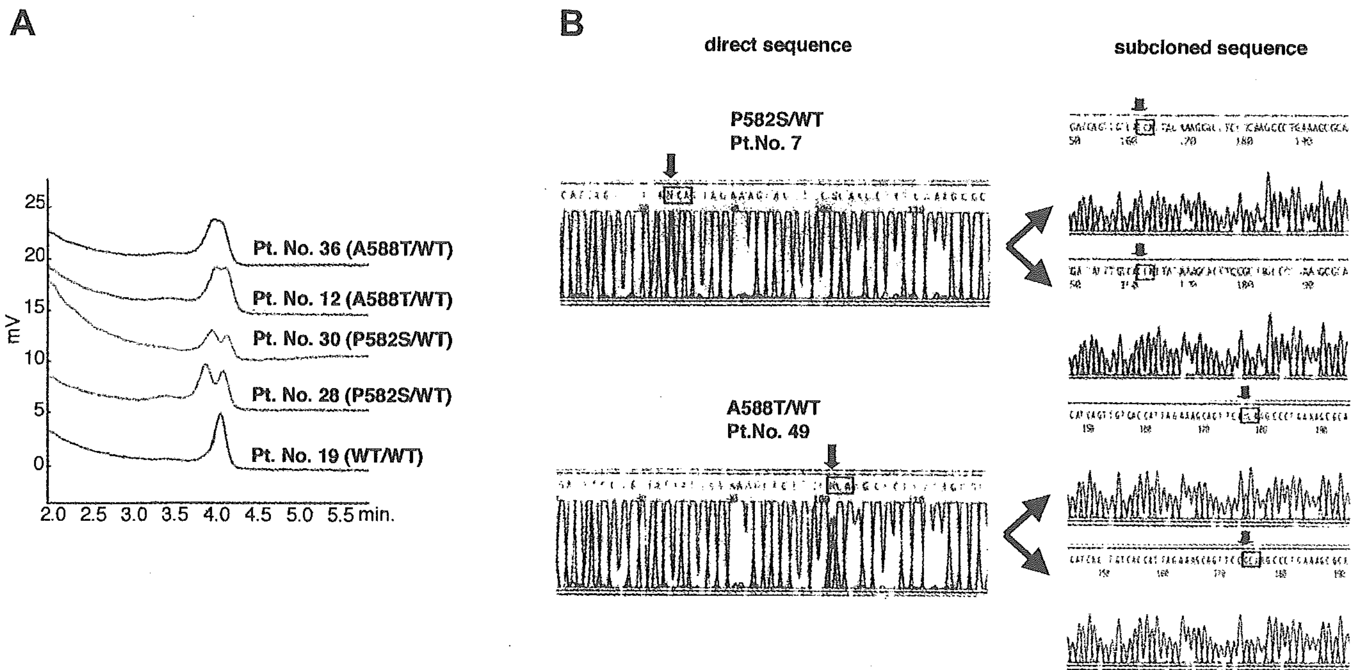

Fig. 2. Genetic polymorphisms of the *HIF-1α* gene. (**A**) Typical complex chromatograms of DHPLC analyses. Exon 12 of the *HIF-1α* gene encoding the N-TAD was amplified using specific primers. After re-annealing of PCR amplicons at optimal temperature, the samples were loaded onto the DNA column and 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).

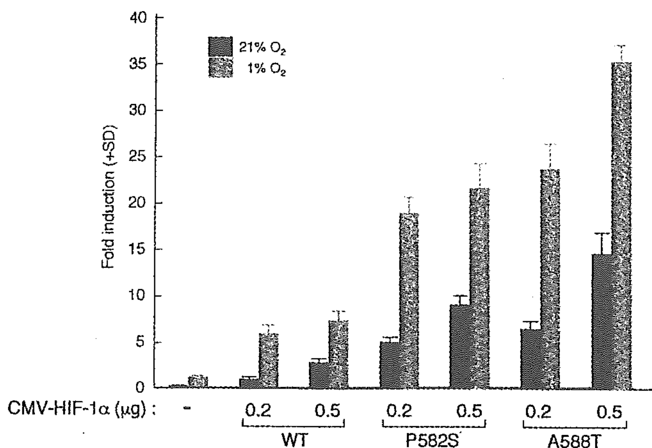

Fig. 3. The transactivation capacity of variant forms of HIF-1α. Transcription activity of vector (−), wild-type (WT), P582S or A588T HIF-1α (0.2 or 0.5 μg of expression vector/15-mm well) was analyzed in a co-transfection assay using reporter plasmid HRE-Luc (0.5 μg/15-mm well) and a Renilla-luciferase (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

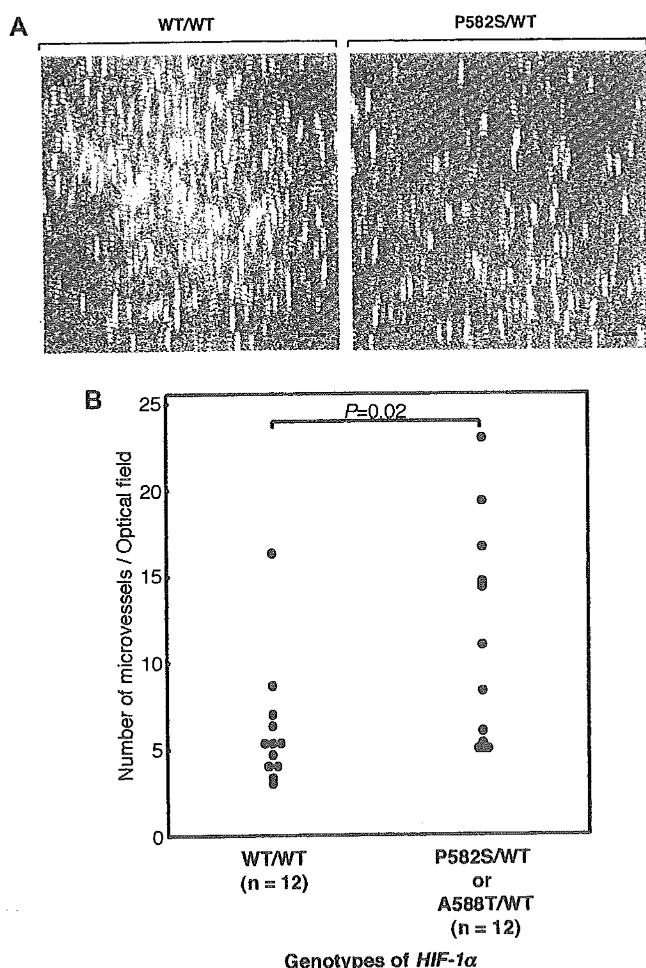

Fig. 4. Polymorphisms of HIF-1α associate with tumor angiogenesis. Twenty-four specimens were subjected to immunohistochemical analysis using anti-CD34 antibody. (A) Representative immunostained tumor sections. Tumor with predominant homozygous alleles WT (a), and that with rare allele P582S (b). Magnifications 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.

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)

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. 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 co-factors 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

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.

Acknowledgements

We thank Dr Ikue Morita-Hayashi for her helpful advice on DHPLC analysis. We also thank Dr Shin-ichi Hayashi, Dr Takeshi Ichikawa, Dr Keiko Hiyama for their helpful discussions in preparing this manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan (to K.Tanimoto, K.Yoshiga and K.Nakachi) and a grant from the Smoking Research Foundation (to K.Nakachi).

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Received March 13, 2003; revised July 14, 2003; accepted July 25, 2003