直腸癌術後補助療法のエビデンスをそのまま国内の臨床に当てはめることには問題がある。したがって日本独自のレジメを確立していくことが重要と考えている。

上記のようにN・SAS-CCで直腸癌において，UFTはStage III直腸癌の治癒切除例に対する術後補助化学療法として，明らかに予後を改善することが判明したこと，また，五つの無作為化比較試験のmeta-analysisでUFTが有意に無再発生存率を改善させていることから，日本においてUFTは直腸癌術後補助化学療法の標準治療の一つとなり得ると考えている。

またS-1はUFTと同じ作用機序を有するDIF製剤(dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine)であるが，配合されているgimeracilにより5-FUの血中濃度を高く維持し，持続静注療法と同様な薬物動態を示すことが知られており，UFTを上回る効果が得られる可能性が期待される。

以上のことから，治癒切除を受けたStage IIA（T 3, N 0, M 0), Stage IIB(T 4, N 0, M 0), Stage IIIA(T 1-2, N 1, M 0), Stage IIIB(T 3-4, N 1, M 0)およびStage IIIC（any T, N 2, M 0）（TNM分類，UICC第6版，2002年）の直腸癌（Rsを除く）症例を対象に，術後補助化学療法としてのS-1療法の有用性を比較評価する目的で，本比較試験が計画された。今回の試験ではStage II，Stage III直腸癌を対象としており，Stage IIIのみを対象としているN・SAS-CCと異なる。しかしmeta-analysisではDukes BでもUFTの有効性が認められていることや，最終的にはStage IIIのサブセット解析も可能であることから登録スピードも考慮してStage II，Stage IIIを対象としている。

おわりに

UFTに関しては近年，肺癌，乳癌，胃癌，直腸癌に対して術後補助化学療法としての有用性を示す報告が行われている。一方S-1は胃癌，大腸癌などに対して高い奏効率を示して承認されたが，術後補助化学療法としての有用性に関してはまだ報告されていない。本研究により，S-1術後補助化学療法としての高いエビデンスを世界に発信できることを期待している。

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GASTROENTEROLOGY

Impact of loss of heterozygosity of encoding phosphate and tensin homolog on the prognosis of gastric cancer

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Abstract

Background and Aim: Encoding phosphate and tensin homolog (PTEN) is a cancer suppressor gene and it has been assumed that gene mutation and loss of heterozygosity (LOH) occurs frequently in various types of carcinoma. However, the role of LOH of PTEN and its outcome variables in gastric cancer have not been well established. In the present study, we investigated the roles of PTEN, LOH and their outcomes.

Methods: Fresh frozen tumor samples from 119 gastric cancer patients with a primary diagnosis of gastric carcinoma were evaluated for LOH of PTEN using an automated sequencer. Results were compared with pathological parameters. The median follow-up period was 559 days.

Results: Loss of heterozygosity of PTEN was observed in 17.1% of patients (13/76) diagnosed with gastric cancer. No particular relationship was found with any clinicopathological factor. However, the prognosis of patients with LOH of PTEN was significantly poor. Multivariate analyses revealed that vascular invasion, invasion depth, LOH of PTEN, histology and lymph node metastasis were correlated with survival of the patient.

Conclusions: Even though mutation of PTEN in gastric cancer has rarely been reported, according to our findings, LOH of PTEN frequently occurs in gastric cancers and is correlated with disease-related deaths. The LOH of PTEN is an independent prognostic factor and PTEN is a candidate as a haploinsufficient tumor suppressor in gastric cancers.

Key words: Akt, encoding phosphate and tensin homolog, gastric cancer, loss of heterozygosity.

INTRODUCTION

The encoding phosphate and tensin homolog (PTEN) gene, a tumor suppressor candidate, is located on chromosome 10q23 and has an extensive homology with the cytoskeletal proteins auxilin and tensin.[1,2] Mutations of PTEN have been observed frequently in various neoplasms, including glioblastoma, melanoma, prostate cancer and breast cancer.[1–7] In glioblastoma, melanoma and prostate cancer, PTEN mutations and allelic deletions are observed during the late stages, whereas in thyroid and endometrial cancers, PTEN mutations/alterations are found during the early stages and include endometrial hyperplasia and benign thyroid tumors.[3–6,8,9] Germline mutations of PTEN are found in patients with Coden syndrome, a familial syndrome associated with predisposition for multiple benign hamartomas, and malignant thyroid and breast neoplasms.[10]

The PTEN gene encodes an enzyme with phosphatase activity towards acidic protein substrates and the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3).[11] The phosphatase activity of PTEN is crucial in controlling the phosphatidylinositol

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3-kinase (PI3-K) signal transduction pathway and in the activation of the protein kinase B (PKB/Akt) proto-oncogene. This indicates that PTEN exerts its tumor-suppressor function by negatively regulating the anti-apoptotic PI3-K/AKT signaling pathway.[12,13] The role of PTEN as a tumor-suppressor gene has been attributed to its ability to modulate cell cycle progression and cell motility.[14] It has been demonstrated that PTEN can dephosphorylate focal adhesion kinase (FAK) and inhibit the mitogen-activated protein kinase pathway.[15,16]

Although gastric cancer is one of the leading causes of cancer-related deaths in Japan,[17] its pathogenesis and progression are not yet clearly understood. Deletion or downregulation of tumor-suppressing genes play important roles in the multiple steps of tumorigenesis and the progression of gastric carcinoma. Because *p53* is the most frequently mutated tumor suppressor gene in gastric cancer, most studies on the relationship between the development of gastric cancer and abnormalities of tumor suppressor genes have mainly focused on *p53*.[18] Owing to frequent gene mutation and loss of heterozygosity (LOH), PTEN has been reported as one of the new tumor suppressor genes in various types of cancer. Previous reports have shown protein loss and promoter methylation of PTEN in gastric cancers.[19–22] However, the prognostic significance of the loss of PTEN in gastric cancer has not been well established. In the present study, we provide information on the LOH of PTEN, which is correlated with disease-related deaths of gastric cancer patients.

METHODS

Tissue samples

Pairs of primary gastric carcinoma tissue and corresponding normal mucosa were obtained consecutively from 119 patients who underwent surgery in the Department of Surgery II at Kyushu University Hospital from 1996 to 2000. Informed consent was obtained from all patients prior to their inclusion in the study. In all cases, the histopathological type of the tumor was adenocarcinoma. Cancer tissues and well-separated normal gastric mucosa obtained by gastrectomy were snap frozen immediately and kept in liquid nitrogen. Genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction, which was followed by ethanol precipitation.

Loss of heterozygosity analysis

Loss of heterozygosity was analyzed using a DNA sequencer with microsatellite markers. The oligonucleotide primers for D10S796 were synthesized and purified by HPLC. The sequences of the primers used for the polymerase chain reaction (PCR) are as follows: D10S1765-forward, 5′-CAATGGAACCAAATGTG GTC; D10S1765-reverse, 5′-AGTCCGATAATGC CAGGATG. The PCR reactions using genomic DNA were performed using a TAKARA GeneAmp PCR Reagent Kit (Takara, Tokyo, Japan) and run on the Perkin-Elmer GeneAmp PCR system 9700 (Norwalk, CT, USA). The thermal conditions of the system were as follows: one cycle at 95°C for 4 min; 35 cycles at 95°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.5 min; and one cycle at 72°C for 10 min. The DNA derived from cancer tissues was amplified with 6-carboxy-x-rhodamine (ROX)-labeled 5′ primer and cold 3′ primer, whereas DNA from normal tissues was amplified with 6-carboxy-24,44,74,4,7-hexachloro-fluorescein (HEX)-labeled 5′ primer and cold 3′ primer. The PCR reactions and running conditions with the Perkin-Elmer Genetic Analyzer 310 were as described previously.[23,24]

Statistical methods

Clinicopathological data were stored in an IBM 3090 mainframe computer (IBM, Armonk, NY, USA). Biomedical computer programs (BMDP; Statistical Solutions, Saugus, MA, USA) were used for all statistical analyses. The BMDP programs P4F and P3S were used for Chi-squared and Mann–Whitney *U*-tests to compare characteristics among groups. The BMDP program P1L was used for Kaplan–Meier analysis of survival rates and the Mantel-Cox test was used to test the equality of survival curves. The median follow-up period was 559 days. The BMDP program P2L was used for simultaneous multivariate adjustments of all covariates in Cox regression analysis. The level of statistical significance was set at $P < 0.05$.

RESULTS

One hundred and nineteen cases were analyzed in the present study and data were processed using GeneScan software (ABI; Applied Biosystems, Foster City, CA, USA). However, in 43 cases, the lengths of the microsatellite sequences of the paternal and maternal alleles were almost the same and, hence, it was difficult to discriminate between these alleles. These 43 cases were excluded from the diagnoses because the analysis of LOH, which was assumed for the declination of one allele, was theoretically impossible (Fig. 1b). Thus, LOH of PTEN was observed in 17.1% of the remaining 76 cases (13/76), where any case in whom the peak value of one gene locus diminished more than 30% in the carcinoma was judged as having LOH (Fig. 1a). However, clinicopathological differences due to the presence of LOH of PTEN were not evident (Table 1).

In the present study, we also investigated the relationship between LOH of PTEN and a patient's survival. The Kaplan–Meier overall survival curve (Fig. 2) demonstrates that patients in whom PTEN showed LOH had a significantly lower survival rate than patients whose tumors had biallelic PTEN (retention of heterozygosity (ROH)). After 5 years, only 22.0% of patients were alive in the LOH group; however, 50.9% patients were alive in the ROH group.

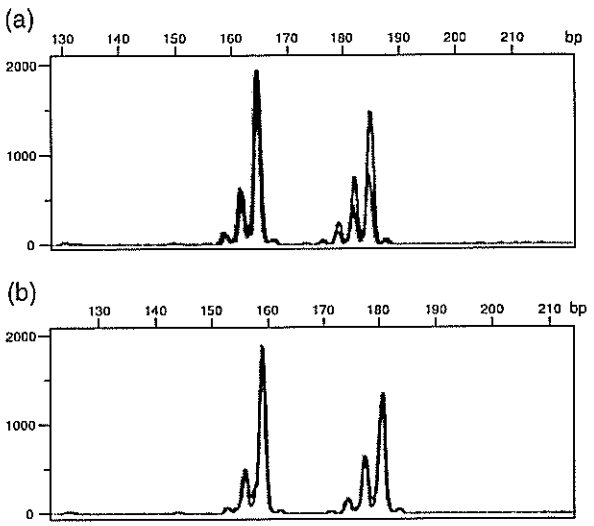

Figure 1 Genomic DNA was extracted from each tumor specimen and from each corresponding normal tissue. Each pair of DNA samples was subjected to loss of heterozygosity (LOH) analysis. Electrophoretic profiles judged to be (a) LOH and (b) ROH (retention of heterozygosity) are shown. The green line represents normal tissues, whereas the red lines represent cancer tissue.

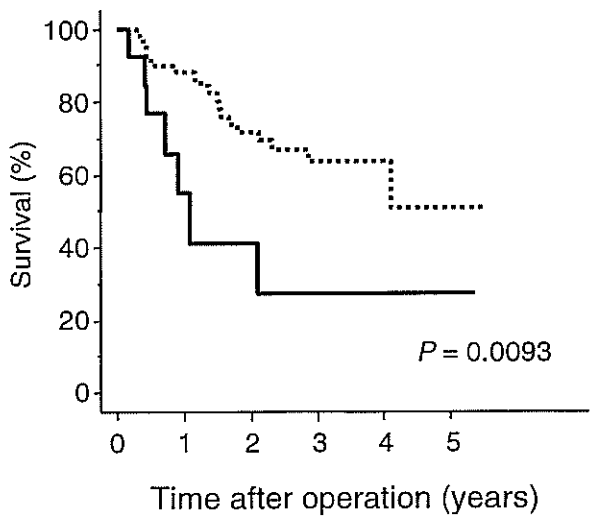

Figure 2 Kaplan–Meier overall survival curves. The solid line is for patients who had loss of heterozygosity (LOH) of encoding phosphate and tensin homolog (PTEN). The dotted line is the survival curve of patients whose tumors had biallelic PTEN.

To search for an independent prognostic factor for gastric cancer, we conducted multivariate Cox's regression analysis. The factors examined included gender, age, tumor size, macroscopic appearance, histological type, depth of invasion, lymphatic or vessel invasion, lymph node metastasis, liver metastasis, peritoneal dissemination and LOH of PTEN. As shown in Table 2, vessel invasion, depth of invasion, LOH of PTEN, histological difference and lymph node metastasis were proven to be independent covariates.

Table 1 Clinicopathological features of gastric cancers and loss of heterozygosity of encoding phosphate and tensin homolog

Variable	PTEN LOH ($n = 13$)	PTEN ROH ($n = 63$)	P
Gender			
No. males	11	49	NS
No. females	2	14	
Age (years)	66.8	62.5	NS
Histology			
Intestinal (n)	4	28	NS
Diffused (n)	8	35	
Serosal invasion			
Negative (n)	5	28	NS
Positive (n)	8	35	
Histological lymph node metastasis			
Negative (n)	4	25	NS
Positive (n)	9	38	
Vascular involvement			
Negative (n)	5	32	NS
Positive (n)	8	31	
Peritoneal dissemination			
Negative (n)	12	61	NS
Positive (n)	1	2	
Liver metastasis			
Negative (n)	11	60	NS
Positive (n)	2	3	
Stage			
I + II (n)	4	29	NS
III + IV (n)	9	34	

PTEN, encoding phosphate and tensin homolog; LOH, loss of heterozygosity; ROH, retention of heterozygosity; NS, not significant.

DISCUSSION

In gastric carcinoma, deletion or downregulation of tumor suppressor genes play important roles in the development of tumors. The role of PTEN, identified as a novel tumor suppressor gene, has been investigated in a variety of cancers. Loss of heterozygosity and mutation of PTEN have been reported in melanoma, glioblastoma, renal cancer, lung cancer and breast cancer.[1,2,6] It has been reported that PTEN mutations re rarely observed in gastric cancers, but diminished protein expression or LOH is often detected.[19–22]

In the present study, LOH of PTEN was investigated in 119 gastric cancer patients using the high-resolution microsatellite analysis method. Our method to determine LOH is based on high-resolution fluorescent microsatellite analysis developed previously.[23] In this system, electrophoretic profiles of PCR-amplified microsatellite sequences are markedly improved and

Table 2 Cox regression analysis of data of patients with gastric cancer

Explanatory variables (observed value)	*P*	Relative risk (95% CI)
Vascular invasion (negative, positive)	0.0004	9.93824 (2.3380, 39.7374)
Serosal invasion (negative, positive)	0.0004	6.2879 (2.1446, 18.4349)
LOH of PTEN (ROH, LOH)	0.0008	8.065 (0.0377, 0.4076)
Histology (intestinal, diffuse)	0.0059	4.458 (0.0788, 0.6384)
Lymph node metastasis (negative, positive)	0.0077	7.7977 (1.0121, 60.0713)

PTEN, encoding phosphate and tensin homolog; LOH, loss of heterozygosity; ROH, retention of heterozygosity; CI, confidence interval.

optimized for judgment of LOH and microsatellite instability.[23] However, 43 cases were excluded from analysis in the present study because the sequence lengths of their paternal and maternal microsatellite alleles were completely matched and could not be separated from the band pattern. Therefore, the rate of false positives is very low and, consequently, accurate positive rates are provided. In the present study, LOH of PTEN was found in 17.6% of the 76 patients analyzed. This percentage is not high when compared with results of previous reports.[19,21,22]

Consequently, there were no specific differences in the clinicopathological factors with the LOH of PTEN. However, there was a significant correlation between a patient's prognosis and LOH of PTEN. On multivariate analysis, LOH of PTEN was proved to be an independent covariate of patient survival. It is still not clear why the PTEN gene mutation is rarely observed in gastric cancer and why LOH is so frequently detected. One possible explanation is that PTEN may be a haploinsufficient tumor suppressor gene in gastric cancer, similar to mouse lymphoma, in which PTEN has been proven to be a haploinsufficient tumor suppressor.[25] It is known that PTEN is important not only as a tumor suppressor, but also as a signal transduction molecule. It is also possible that the dephosphorylattion of PI3-K becomes difficult when PTEN is aberrant. Therefore, AKT located downstream of PI3K is continuously phosphorylated and AKT conveys a resistant signal for apoptosis. As a result, a cell becomes resistant to various treatments, such as chemotherapy. In Japan, many patients with gastric cancers at more advanced stages than Stage II receive adjuvant chemotherapy postoperatively. It is speculated that the presence of LOH of PTEN reflects the chemotherapeutic effect. In those cases, a deficiency of PTEN is connected with significant hyposensitivity to postoperative treatment rather than gastric carcinogenesis. In brief, because patients with LOH of PTEN are hyposensitive to anticancer drugs, careful follow up is necessary in the clinical situation. However, in our analysis, no significant findings were obtained when the prognoses were compared between cases with postoperative chemotherapeutic treatment and cases with operation only (data not shown).

Previous reports indicated that mutations of PTEN were found in breast and prostate cancers more frequently than in gastrointestinal cancer. Poor prognoses and resistance to chemotherapy have also been reported.[26–29] For gastric cancers, there have been reports published on the correlation between the diminished protein expression of PTEN and lymph node metastasis or tumor infiltration. Yang *et al.* reported that low expression of PTEN protein was related to clinicopathological stage and the metastasis of gastric cancers.[20] It has also been reported that the LOH rate was significantly higher in advanced tumors than in early stage tumors and that LOH occurs more frequently in poorly differentiated tumors than in well- or moderately differentiated tumors. Byun *et al.* reported that 47% of gastric cancer patients had LOH of PTEN.[19] However, no report has clarified relationship between LOH of PTEN and the outcome of gastric cancer. The results of the present study indicate the significant importance of PTEN for tumor development and the treatment of gastric cancers. Further extensive studies on PTEN in gastric cancer patients are necessary.

In conclusion, LOH of PTEN occurs in 17.1% of gastric cancers and is correlated with disease-related deaths. According to the findings of the present study, LOH of PTEN is an independent prognostic factor and PTEN is a candidate haploinsufficient tumor suppressor in gastric cancers. The present study has shed light on the significant importance of PTEN for the treatment of gastric cancers in addition to *p53*.

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Relationship of hypoxia-inducible factor 1α and p21$^{WAF1/CIP1}$ expression to cell apoptosis and clinical outcome in patients with gastric cancer

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Abstract

Background: Hypoxia-inducible factor-1α (HIF-1α) plays an essential role in oxygen homeostasis. The expression of HIF-1α-inducible genes is associated with tumor progression. p21 mediates cell cycle arrest and is one of the downstream genes targeted by HIF-1.

Patients and methods: We examined the relationship between HIF-1α and p21 expression, apoptosis and tumor progression using tissue specimens obtained surgically from 126 patients with gastric cancer.

Results: Immunohistochemical analysis indicated that loss of p21 expression correlated positively with patient age and tumor size. Lymph node metastasis was significantly more frequent in tumors with loss of p21 expression ($P = 0.022$). HIF-1α-positive/p21-negative tumors had a lower apoptotic index than any other tumor samples, and patients with HIF-1α-positive/p21-negative tumors also had a significantly poorer prognosis than the other patient populations.

Conclusion: These results suggest that loss of HIF-1α-dependent p21 expression results in decreased apoptosis, increased cell survival and more aggressive tumors.

Background

The unregulated growth of cancer cells often results in hypoxic conditions in tumor cell masses. Tumor hypoxia results from an imbalance between elevated consumption of oxygen in the rapidly cycling tumor cells and insufficient oxygen supply due to the lack of a physiological vascular network. Multicellular organisms have evolved cellular mechanisms that mediate a cascade of adaptive molecular responses to hypoxia. HIF-1α is a transcription factor that activates gene expression by binding to the hypoxia responsive element (HRE), a cis-acting DNA sequence present upstream of several genes essential for the cellular response to hypoxia [1]. HIF-1α-responsive genes also function in the glycolysis pathway and in hematopoiesis and angiogenesis, through all of which cells acquire an hypoxia-adapted metabolism and increased oxygen supply [2]. Recently, HIF-1α has emerged as a key regulator in the growth of gastric cancer [3].

Apoptosis is an evolutionarily conserved cell death mechanism that also occurs in the adaptive cellular response to hypoxic stress. Apoptosis, too, is an important safeguard against tumor development. Tumors that exhibit loss of

the p53 tumor-suppressor gene exhibit reduced levels of hypoxia-induced cell death and an associated increase in tumor progression [4]. The p21 gene (WAF1) was cloned in a genetic screen for downstream effectors of p53 and separately in a screen for upstream regulators of cyclin-dependent kinases (CDKs) as CDK-interacting protein (CIP1) [5]. The p21 promoter can be transactivated by HIF-1 in a human prostate cancer cell line, indicating that p21 is an HIF-1 target gene [6]. Furthermore, hypoxia-induced p21 expression was abrogated in cells lacking HIF-1α, but not in parental cells [7].

HIF-1α may therefore promote both cell survival and growth arrest through the induction of hypoxia-responsive genes. In the present study, we examined the role of HIF-1α in hypoxic control of tumor progression, by examining the relationship between HIF-1α expression, p21 expression and apoptosis in tissue specimens from patients with gastric cancer.

Materials and methods

Clinical materials

Subjects were 126 patients with gastric cancer (85 men, 41 women; age range, 27 to 88 years; mean age, 65.2 years) who underwent gastrectomy at our institution in 1994. Curative resection was performed in 77 patients and non-curative resection in 49. Resected tissue specimens were fixed in a 10% formaldehyde solution and embedded in paraffin. Sections (4 μm thickness) were mounted on glass slides. All samples were examined macroscopically and histologically, based on criteria proposed by the General Rules for the Gastric Cancer Study [8]. Histological examination was carried out on tissue preparations stained with hematoxylin and eosin (H&E). In the current study, tumors were divided into two histological types: differentiated type, comprising papillary adenocarcinoma and tubular adenocarcinoma, and undifferentiated type, comprising poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma. Two paraffin blocks were prepared for all patients" one containing both tumor tissue and adjacent normal tissue, and the other containing tumor tissue invading to the deepest level of the stomach wall.

Immunohistochemical staining

All specimens were immunostained with a monoclonal antibody against $p21^{WAF1/CIP1}$ (SX118, diluted 1:50, DAKO, Glostrup, Denmark), p53 (Do-7, diluted 1:50, DAKO, Glostrup, Denmark), and HIF-1α (NB 100–105, diluted 1:100, Novus Biologicals, Littleton, CO, USA)[9]. After deparaffinization and rehydration, the slides for p21 and p53 immunostaining were autoclaved in citrate buffer (0.01 M, pH 6.0) at 120°C for 10 minutes; 0.001 M EDTA (pH 8.0) was used for HIF-1αimmunostaining to facilitate reactivity of the fixed embedded tissue antigen with the antibody. Endogenous peroxidase was blocked by incubating the samples in methanol containing 0.3% hydrogen peroxide for 10 minutes. Samples were then rinsed in phosphate-buffered saline (PBS) and incubated with normal rabbit serum for 30 minutes. Sections were incubated with the aforementioned primary antibodies for 2 hours at room temperature, then rinsed three times in PBS. For detection, we used a Histofine SAB-PO (M) kit (Nichirei Corp., Tokyo, Japan). The sections were then incubated with biotinylated rabbit anti-mouse immunogloblin (Ig; IgG, IgA, and IgM; Nicchirei Corp) for 10 minutes, washed three times in PBS and treated with peroxidase-conjugated streptavidin for 10 minutes. After a final washing in PBS, the peroxidase label was detected by incubating the sections in diamino-benzidine tetrahydrochloride (DAB) for 3 minutes. Nuclear counter-staining was done using Mayer's hematoxylin solution. For negative controls, primary antibodies were replaced with non-immune, normal serum. Automated immunohistochemistry was also carried out to support the immunostaining described above, using a Ventana Discovery™ System (Ventana Medical Systems, Inc., Tucson, AZ, USA).

Evaluation of immunostaining

p21 protein was present in the nuclei of tumor cells (Figure 1A). In some cases, normal gastric mucosa expressed p21 protein, and nuclear staining could be detected in the superficial reaches of the tumor, but not in the deeper regions. We counted the number of p21-positive cells in the whole tumor section, and evaluated the number of p21-postive cells according to the depth of layers. The pattern of HIF-1α immunostaining in the tumor was nuclear and/or cytoplasmic (Figure 1B). Nuclear staining of HIF-1α was absent in normal tissue excluding cytoplasmic staining. In all samples, p53 protein was undetectable in normal tissue, and present in the nuclei of tumor cells (Figure 1C). A cell with nuclear immunostaining for p21, p53 or HIF-1α (weak or strong) was scored as positive. Based on these criteria, areas of focal staining with the highest percentage of p53- and p21-positive nuclear staining within deep tumor tissue were estimated. A tumor was scored as p21- or p53-positive when more than 10% of the tumor cells had nuclear staining, in keeping with previous reports [10]. HIF-1α expression was frequently evident in regions around the invading edges of the tumor, and necrotic areas such as those close to a deep ulcer. We scored tumors as positive for HIF-1α overexpression if nuclear staining was detected in more than 10% of the tumor cells, irrespective of cytoplasmic reactivity at any level [9,11].

Evaluation for apoptosis

We scored apoptotic cells as those showing the typical characteristics of apoptosis when tissue specimens were stained with H&E (Figure 1D). Apoptotic cells were iden-

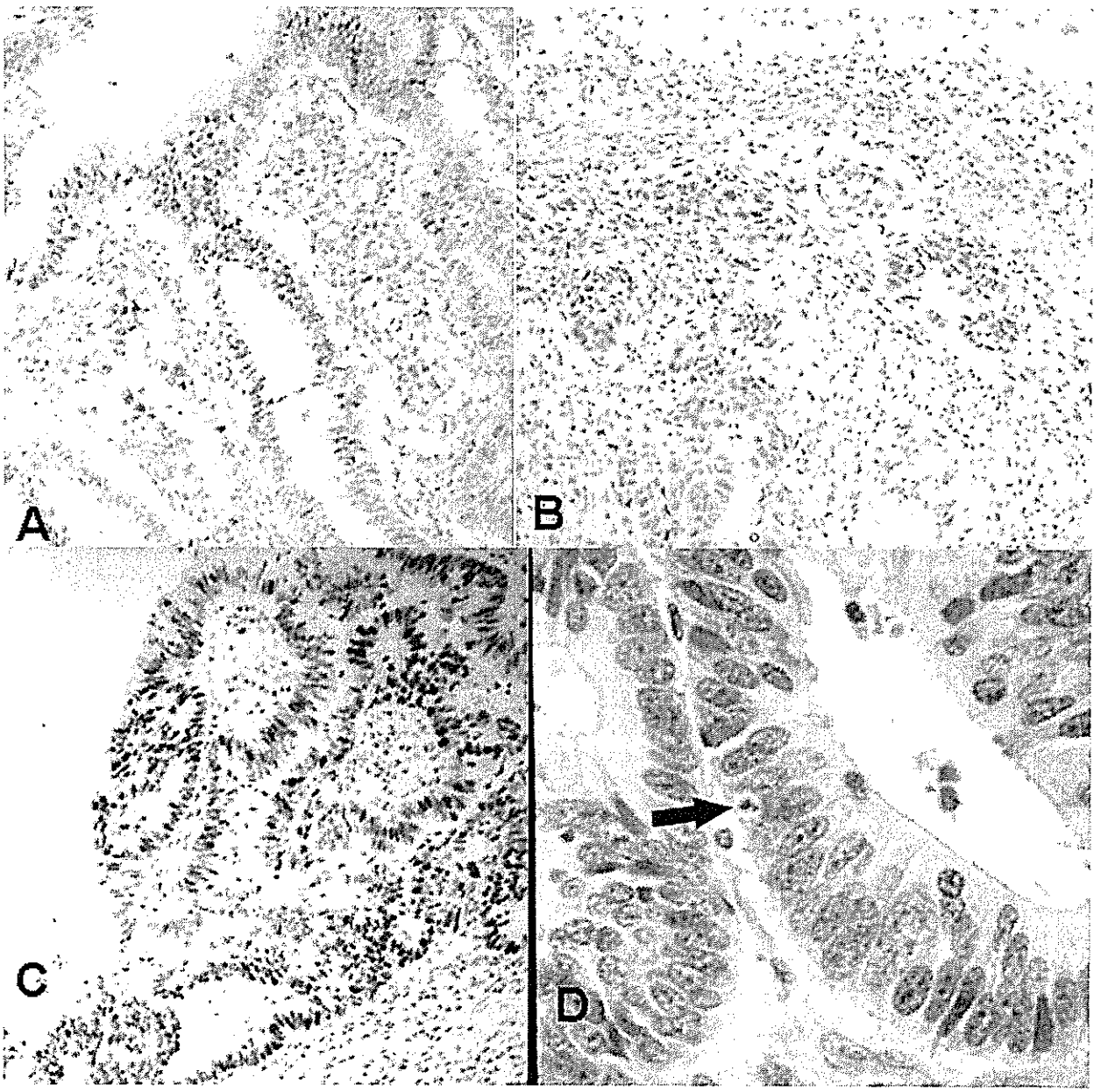

Figure 1
A) p21 expression in adjacent tumor tissue. Nuclear immunostaining is evident (original magnification, 100×). B) HIF-1α expression in invading regions of tumor. Variability in intensity of nuclear immunostaining is evident, accompanied by cytoplasmic staining (original magnification, 100×). C) Nuclear immunostaining of p53 in tumor tissue (original magnification, 100×). D) Tumor tissue stained with H&E (original magnification, 200×). Tumor tissue contains cells (arrow) with characteristic features of apoptosis.

tified based on the characteristic features of apoptosis: compaction and migration of nuclear and cell outlines, nuclear fragmentation and protuberance, and the presence of apoptotic bodies [12]. Apoptotic cells were counted in tissue specimens from all patients. Five high-power fields (×400) with the most abundant distribution of tumor cells were selected for counts and between 1000 and 1500 tumor cells were counted. The apoptotic index

was then calculated as the percentage of apoptotic cells. Areas with extensive necrosis were avoided. A single pathologist at each institution reviewed the slides and counted apoptotic cells according to the criteria described above.

Statistical analysis

The BMDP Statistical Package program (BMDP, Los Angeles, CA) for IBM (Armonk, NY) 3090 mainframe computers were used for all statistical analyses [13]. Data sets were compared by chi-square and Student's t-tests using the BMDP 4F and 3S programs. The BMDP 1L program was used to analyze survival time using the Kaplan-Meier method. Statistical significance was set at the $P < 0.05$ level.

Results

p21 expression and clinico-pathologic factors

Table 1 shows the correlation of the expression or loss of expression of p21 with several clinico-pathologic factors. Overall, 71 (56.3%) of the 126 tumor specimens were negative for p21 expression. In tumors from patients under 65 years of age, loss of p21 expression was significantly more frequent than in tumors from patients over the age of 65 ($P = 0.026$). Loss of p21 expression was significantly more frequent in tumors with large size ($P = 0.03$), as compared to smaller tumors. With regard to metastases, tumors with loss of p21 expression tended to show increased frequency of lymphatic invasion ($P = 0.089$, Table 1) and a significantly higher frequency of lymph node metastasis ($P = 0.022$, Table 2) than p21-expressing tumors. Table 3 shows the relationship between p21 expression, and the expression of p53 and HIF-1α. Overexpression of p53 was detected in 57 (45.2%) tumors, and HIF-1α overexpression was detected in 49 (38.9%) tumors. Immunohistochemical analysis did not reveal a correlation between p21 expression and p53 expression, or p21 and HIF-1α overexpression ($P = 0.444$ and 0.609, respectively).

Apoptosis associated with HIF-1α and p21 expression

The mean apoptotic index of all 126 tumors was 8.95 ± 6.24 (range 0–37). Tumors were divided into four different populations based on HIF-1α and p21 expression and the mean apoptotic index for each group was calculated (Table 4). Tumors that were HIF-1α-positive/p21-negative had the lowest apoptotic index. There was a significant difference between tumors that were HIF-1α-positive/p21-negative, and those that were HIF-1α-negative/p21-negative ($P = 0.037$).

Clinical outcome associated with HIF-1α and p21 expression

The mean follow-up time for patients was 55 ± 28 (± 1 S.D.) months (range, 1–82 months). The 5-year survival rate of patients with p21-negative tumors was lower than that of p21-positive tumors, but the difference was not statistically significant (data not shown). The 126 patients were again divided into four populations based on HIF-1α expression and p21 expression, and we examined the relationship between HIF-1α expression and prognosis in p21-positive or -negative tumor samples. Table 5 shows the 1-, 3- and 5-year survival rates for patients and the correlation with HIF-1α and p21 expression. Patients with HIF-1α-positive/p21-negative tumors had a significantly poorer prognosis than the other study populations. In particular, in patients with HIF-1α-positive tumors, those who had lost expression of p21 had a significantly poorer prognosis than those with p21 expression ($P = 0.042$).

Discussion

Our findings show that loss of p21 expression correlated positively with younger patient age, and larger tumor. Moreover, many patients with p21-negative tumors had lymph node metastasis when compared to those with p21-positive tumors, at a significantly higher frequency. These results suggest that the loss of expression of p21 is involved in the processes of tumor growth and metastasis, in agreement with previously reports [14,15].

HIF-1α overexpression has been linked to a poor clinical outcome in some types of human cancers [16-18]. However, some reports have suggested that tumor expression of HIF-1α does not confer a survival advantage [18,19]. Although most of the HIF-1 target genes can promote tumor growth through their enhanced expression, HIF-1-activated genes, including p21, also have the potential to inhibit growth under hypoxic conditions [20,21]. Ectopic expression of HIF-1α in endothelial cells resulted in up-regulation of p21, reduction of CDK activities, cell cycle arrest at the G_0/G_1 check point, and subsequent apoptosis [22]. In the current study, we found that apoptotic cells were under-represented in HIF-1α-positive/p21-negative tumors. Under hypoxic conditions, HIF-1α may inhibit tumor proliferation through p21-mediated cell cycle control, resulting in the selection of cells that are resistant to apoptosis and anti-cancer treatments. Most tumor cells retain the ability to undergo apoptosis in response to hypoxic stress [23]. When the apoptotic response to hypoxia is lost, emerging tumor cells may be more resistant to treatment and may therefore contribute to subsequent tumor relapse [24]. The mechanisms by which hypoxia selects for cells resistant to apoptosis is unclear, but the involvement of the p53 mutation has been examined [25]. Reports have shown that hypoxia inhibits cell growth, and may cause apoptosis through a p53-dependent pathway [26]. HIF-1α has also been shown to promote p53-dependent apoptosis [27], but other studies have shown that growth arrest in response to hypoxia is p53-independent [26]. In the current study, we found no

Table 1: Relationship between p21 expression and clinico-pathologic factors

Factors	p21(+) (n = 55)	p21(-) (n = 71)	P value
Age (years)			
<65	20	40	**0.026**
65≦	35	31	
Gender			
Male	38	47	0.731
Female	17	24	
Depth of invasion			
t1	33	35	0.231
t2,3,4	22	36	
Histology			
Differentiated	35	39	0.325
Undifferentiated	20	32	
Tumor size			
<3 cm	23	22	**0.03**
3 cm≦	22	49	
Lymphatic invasion			
Negative	26	23	0.089
Positive	29	48	
Venous invasion			
Negative	42	57	0.595
Positive	13	14	

evidence of a relationship between p53 and p21 expression. We also evaluated the relationship between HIF-1α and p53 expression to cell apoptosis, but found no statistical significance between HIF-1α and p53 expression (data not shown). Our previous study showed that the combination of HIF-1α overexpression with nonfunctional p53 tended to indicate a dismal prognosis [28].

In patients with HIF-1α-positive tumors, the correlation between loss of p21 expression and poor clinical outcome may reflect a physiological difference in the ability of p21-positive versus p21-negative tumors to survive under hypoxic conditions. Although HIF-1α-dependent transcriptional activation has been associated with tumor growth, our results suggest that concomitant expression of p21 and HIF-1α may retard tumor growth to some degree.

The molecular mechanism underlying HIF-1α expression in cancer warrants particular attention [29]. The widespread occurrence of upregulated HIF-1α in common cancers and the involvement of hypoxia pathways in tumor angiogenesis certainly argue for its importance and wide applicability. Chemotherapy and radiation that target HIF-1α may be effective and realistic, and in fact, this approach has been reported [30]. However, the qualitative and quantitative differences in the hypoxic response of different cell types are not well known [31]. Further research is therefore required in order to evaluate the effects of HIF-1-mediated pathways on cell proliferation and apoptosis in human cancers under hypoxic microenvironments.

In the present study, we showed that HIF-1α overexpression and loss of p21expression in gastric cancers correlated with poor patient prognosis, compared to tumors

Table 2: Relationship between p21 expression and metastasis

	p21-negative tumor (%)	P value
Lymph node metastasis		
Negative	36/75(48.0)	**0.022**
Positive	35/51(68.6)	
Liver metastasis		
Negative	68/122(55.7)	0.447
Positive	3/4(75.0)	
Peritoneal dissemination		
Negative	62/112(55.4)	0.525
Positive	9/14(64.3)	

Table 3: Relationship between p21 and p53, and HIF-1α expression

		p21		
		Positive (n = 55)	Negative (n = 71)	P value
p53	Positive (n = 57)	27	30	0.444
	Negative(n = 69)	28	41	
HIF-1α	Positive (n = 49)	20	29	0.608
	Negative(n = 77)	35	42	

Table 4: Apoptosis associated with HIF-1α and p21 expression

Factor	Apoptotic index
HIF-1α (-), p21(+) n = 35	9.6 ± 7.33***
HIF-1α (-), p21(-) n = 42	9.83 ± 6.96**,***
HIF-1α (+), p21(+) n = 20	8.85 ± 4.04*,**,***
HIF-1α (+), p21(-) n = 29	6.97 ± 4.22*,**,***

* P = 0.129 ** P = 0.037 *** P = 0.078 (mean ± S.D.)

Table 5: Year survival rate associated with HIF-1α and p21 expression

Factor	1-year survival	3-year survival	5-year survival
HIF-1αg(-), p21(+) n = 35	94.1	82.0	82.0***
HIF-1α (-), p21(-) n = 42	92.7	80.0	80.0**,***
HIF-1α (+), p21(+) n = 20	95.0	85.0	70.0*,**,***
HIF-1α (+), p21(-) n = 29	68.1	57.4	45.9*,**,***

* P = 0.042 ** P = 0.003 *** P = 0.003 (%)

that retained p21 expression, or had lost HIF-1α expression. A potential mechanism for this was suggested by the finding that apoptotic cells were under-represented in HIF-1α-positive/p21-negative tumors. Aggressive tumors that fail to induce p21 in an HIF-1α – dependent manner may have increased cell survival without apoptosis, and contribute to a poor prognosis for patients.

Conflict of interest

The author(s) declare that they have no competing interests.

Authors' contributions

KM carried out the immunohistochemical study and performed the statistical analysis, and drafted the manuscript.

YK conceived the study, and participated in its design and coordination and helped to draft the manuscript.

SO involved in drafting the manuscript and revising it critically for important intellectual content.

YM gave final approval of the version to be published.

All authors read and approved the final manuscript.

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Clinicopathologic Significance of Hypoxia-Inducible Factor 1α Overexpression in Gastric Carcinomas

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Background: Hypoxia-inducible factor 1α (HIF-1α) plays a key role in responses to hypoxia and expression of HIF-1α downstream genes leads to both an adapted metabolism and increased oxygen supply. We investigated the clinical significance of HIF-1α expression in gastric carcinoma.

Methods: We examined HIF-1α, vascular endothelial growth factor (VEGF), and insulin-like growth factor-2 (IGF-2) expression patterns immunohistochemically in 126 specimens of gastric carcinoma. CD34 antigen levels were also examined by immunohistochemistry to determine microvessel density (MVD) within tumors and correlations between HIF-1α expression, clinicopathological features, and survival were examined.

Results: HIF-1α expression correlated with tumor size ($P < 0.005$), depth of invasion ($P = 0.018$), VEGF expression ($P = 0.03$), and intra-tumor MVD ($P < 0.005$). IGF-2 expression was more prevalent in HIF-1α positive than in HIF-1α negative tumors and the 5-year survival rate was 58.4% for HIF-1α positive patients and 81.5% for HIF-1α negative patients ($P = 0.009$). HIF-1α expression is an independent prognostic factor in gastric carcinoma ($P = 0.032$).

Conclusions: Overexpression of HIF-1α in gastric carcinomas may upregulate its downstream gene products leading to VEGF-mediated angiogenesis, and resulting in a poor prognosis for patients.

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KEY WORDS: **gastric carcinoma; HIF-1α; VEGF; microvessel density; IGF-2; prognosis**

INTRODUCTION

Dysregulated, unlimited growth of tumors perturbs homeostasis and creates a physiological disruption in the surrounding tissues as well as the tumor interior. Oxygen tension also often decreases dramatically in tumors, and this hypoxia is the result of an imbalance between an elevated consumption of oxygen in tumor cells and an insufficient oxygen supply due to a lack of vascular networks in the tumor tissue. The adaptation of tumor cells to hypoxia is associated with tumor progression and metastasis, as well as resistance to both chemotherapy and radiotherapy, and there is an inverse correlation between tumor oxygenation and clinical outcome [1,2]. At the cellular level, hypoxia induces expression of a group of physiologically important genes including erythropoietin, vascular endothelial growth factor (VEGF), glycolytic enzymes, and glucose transporters, many of which are upregulated in some types of human carcinomas. Each of these genes is under the control of HIF-1, a transcriptional regulator that binds to specific hypoxia-response elements (HRE). Hypoxia-inducible factor 1α (HIF-1α) is a component of the HIF-1 complex,

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and its accumulation in the nucleus is a rate limiting determinant of the functional activity of HIF-1 [3]. HIF-1α is both strongly induced and stabilized under hypoxic conditions, under which it can then translocate from the cytoplasm into the nucleus, and its target genes promote cell proliferation and viability, angiogenesis, and also metabolic adaptations to hypoxia [4]. HIF-1α acts as a tumor-promoting factor by inducting expression of VEGF, probably the most potent angiogenic factor known [5]. VEGF mRNA is not induced by hypoxia in HIF-1α-deficient embryonic stem (ES) cells, and dramatic vascular regression occurs in HIF-1α-null mouse embryos [6]. Furthermore, hypoxia induces expression of various growth factors that are known to promote cell proliferation. HIF-1α induces production of insulin-like growth factor-2 (IGF-2) and expression and secretion of this growth factor may lead to an acceleration of tumor growth [7–9].

In various human carcinomas HIF-1α expression has been examined immunohistochemically, and shown to correlate with both angiogenesis and a poor prognosis for patients. The aim of this study was therefore to investigate the clinical significance of HIF-1α expression in gastric carcinoma. Our data show that HIF-1α appears to be an important factor in determining the behavior and outcome of gastric carcinomas.

MATERIALS AND METHODS

Patients

This study included 126 randomly selected Japanese patients with primary gastric carcinomas, all of whom underwent a gastrectomy procedure in the Department of Surgery, Saga Prefectual Hospital Koseikan, Saga, Japan in 1994. There were 85 men and 41 women between the ages of 27 and 88 years old (mean, 65.2 years). Curative resections were performed on 77 patients and 49 underwent non-curative surgical procedures. A thorough pathological examination was undertaken using hematoxylin and eosin-stained tissue preparations, and pathological diagnosis and classification of the resected gastric carcinoma tissues was performed according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan [10]. Tumors were collected and fixed in 10% formalin and embedded in paraffin. Each paraffin block contained both tumor and adjacent non-tumor tissue and for each patient the tumor tissue invading to the deepest level of the stomach wall was selected for this study.

Immunohistochemistry

A streptavidin-biotin-based immunohistochemical method was used to determine HIF-1α expression. Briefly, 4-μm thick sections prepared from paraffin-embedded blocks were deparaffinized in xylene and rehydrated using a graded series of ethanol solutions. Slides were autoclaved in 1 mM EDTA (pH 8.0) at 120°C for 10 min and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 10 min. The sections were incubated for 2 hr at room temperature with a monoclonal antibody against HIF-1α (IgG2b, clone MAb H1α67, NB 100-105, Novus Biologicals, Littleton, CO), at a dilution of 1:100 [11]. The sections were washed three times with phosphate-buffered saline (PBS) and the sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin (Histofine SAB-PO (M) kit, Ig; IgG, IgA, and IgM; Nichirei Corp.) for 10 min followed by three washes in PBS. The slides were treated with peroxidase-conjugated streptavidin for 10 min and following PBS washes, peroxidase labeling was developed by incubating the sections in diamino-benzidine tetrahydrochloride (DAB) for 3 min. Finally, Mayer hematoxylin solution was used for nuclear counter-staining. For negative controls, we omitted the primary antibody. HIF-1α protein expression in the cytoplasm and the nucleus of tumor cells was judged as positive-staining if there was nuclear staining in more than 10% of tumor cells and was accompanied by weak or strong cytoplasmic staining, as described in previous reports [12].

Expression of VEGF and IGF-2 was investigated, using the rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA) and the mouse monoclonal antibody W3D9-1 (American Research Product, Belmont, MA), respectively. Specimens were incubated for 2 hr with a 1:50 dilution of the primary antibody solution at room temperature and the degree of reactivity with individual tissue sections was deemed positive if unequivocal staining of the cytoplasm was seen in more than 5% of tumor cells, as previously reported.

A procedure for detecting microvessels using an anti-CD34 monoclonal antibody was also utilized (Dako, Glostrip, Denmark). Briefly, evaluation of microvessel density (MVD) was determined, using the modified technique of Weidner et al. [13]. Each slide was scanned at low magnification (40× or 100×), and the area of the most dense neovascularization (greatest number of capillaries or small venules) was determined. Individual microvessel counts were made on a 200× field (0.739 mm^2 per field) and positive cells or cell clusters, clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements, were counted as a single microvessel. Occasional immunopositive leukocytes were excluded on morphological grounds. The distribution of areas of most intense vascularization was

heterogeneous for each tumor. In each of the samples the mean values for the numbers of microvessels was calculated from five highly vascularized areas "hot spots."

Fully automated staining was also performed to confirm the results obtained from the immunostaining procedures described above, using a Ventana Discovery™ System (Ventana Medical Systems, Inc., Tucson, AZ). All evaluations of immunostaining data were performed by two independent observers (K.M. and Y.K.) without knowledge of the patient's clinical status, and two pathologists reviewed the slides.

Statistical Analysis

The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the IBM 3090 mainframe computer (Armonk, NY) was used for all analyses. The association of factors was evaluated using a chi-square and a Student *t*-test and differences among survival curves, based on HIF-1α expression, were examined using the generalized Wilcoxon test. Survival curves were constructed using the Kaplan–Meier method. A multivariate analysis of prognostic factors related to overall survival was conducted using the Cox proportional hazards model. A value of $P < 0.05$ was considered statistically significant.

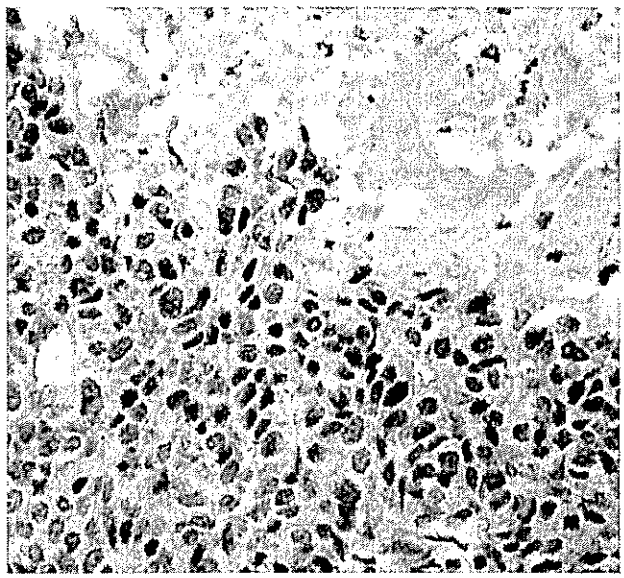

Fig. 1. The nuclear and cytoplasmic staining of HIF-1α in ulcerated regions of an advanced gastric carcinoma is shown (200×).

RESULTS

Expression of HIF-1α in gastric carcinomas was frequently detected at the invading edge of the tumor margin and at the periphery of necrotic regions within the tumor mass (Fig. 1). Of the gastric carcinoma specimens

TABLE I. Relationship of HIF-1α, VEGF, and IGF-2 to Clinicopathologic Factors in Gastric Carcinomas

	HIF-1α (−) n = 77	HIF-1α (+) n = 49	*P*-value	VEGF (−) n = 87	VEGF (+) n = 39	*P*-value	IGF-2 (−) n = 81	IGF-2 (+) n = 45	*P*-value
Age									
<65	31	29	0.038	35	25	0.013	42	18	0.202
≥65	46	20		52	14		39	27	
Gender									
Male	50	35	0.448	58	27	0.776	31	35	<0.005
Female	27	14		29	12		50	10	
Depth of invasion									
t1	48	20	0.018	51	17	0.118	45	23	0.631
t2,3,4	29	29		36	22		36	22	
Histology									
Differentiated	56	18	<0.005	58	16	0.007	49	25	0.59
Undifferentiated	21	31		29	23		32	20	
Tumor size									
<3 cm	35	10	<0.005	33	12	0.438	37	8	<0.005
≥3 cm	42	39		54	27		44	37	
Lymph node metastasis									
Negative	51	24	0.054	57	18	0.041	45	30	<0.005
Positive	26	25		30	21		36	15	
Lymphatic invasion									
Negative	40	9	<0.005	40	9	0.015	31	20	<0.005
Positive	37	40		47	30		50	25	
Venous invasion									
Negative	64	35	0.119	68	31	0.867	64	35	<0.005
Positive	13	14		19	8		17	10	

TABLE II. Relationship of HIF-1α to VEGF and IGF-2 Expression

	HIF-1α (−) (n = 77)	HIF-1α (+) (n = 49)	*P*-value
VEGF			
Negative	58	28	0.03
Positive	19	21	
IGF-2			
Negative	53	28	0.181
Positive	24	21	

TABLE III. Relationship of MVD to HIF-1α, VEGF, and IGF-2

HIF-1α	HIF-1α (−) (n = 77)	HIF-1α (+) (n = 49)	*P*-value
MVD	57.5 ± 29.6	79.5 ± 30.9	<0.005
VEGF	VEGF (−) (n = 87)	VEGF (+) (n = 39)	*P*-value
MVD	66.1 ± 33.1	66.1 ± 29.3	0.996
IGF-2	IGF-2 (−) (n = 81)	IGF-2 (+) (n = 45)	*P*-value
MVD	66.1 ± 33.4	76.8 ± 30.0	<0.005

from 126 patients, 49 (38.9%) were positive for HIF-1α immunoreactivity. The relationships between HIF-1α expression and clinicopathological features of the tumors are shown in Table I. HIF-1α expression was positively correlated with tumor size ($P < 0.005$) and with depth of invasion ($P = 0.018$) and was more frequent in cases of tumors with lymphatic invasion and undifferentiated adenocarcinomas. Tumors with HIF-1α expression at the invading edge were significantly larger than tumors lacking this expression pattern ($P = 0.03$). VEGF expression was negatively correlated with lymph node metastasis ($P = 0.041$) and lymphatic invasion ($P = 0.015$). IGF-2 expression also correlated with increased tumor size, depth of invasion, lymph node metastasis, lymphatic and venous invasion ($P < 0.005$ in all comparisons).

VEGF expression was significantly correlated to HIF-1α expression, being detected in 24.7% of HIF-1α negative and 42.9% of HIF-1α positive (Table II). Additionally, cytoplasmic staining of IGF-2 was observed more frequently in HIF-1α positive tumors (46.7%) than in HIF-1α negative tumors (31.2%). Expression of HIF-1α, VGEF, and IGF-2 proteins was observed in serial sections of several specimens (Fig. 2a–c). The intra-tumor MVD, determined using anti-CD34 antibodies, was significantly higher (79.5 ± 30.9, mean ± SD) in tumors from HIF-1α positive patients than in HIF-1α negative sections (57.5 ± 29.6, mean ± SD) (Table III). Tumor expressing IGF-2 showed a significant increase in MVD, similar to those expressing HIF-1α.

The prognosis of the 126 Japanese cancer patients in this study was monitored for a median follow-up time of 55.4 months. During the observation period, 36 patients died from gastric carcinoma and the 5-year survival rate was 58.4% for HIF-1α positive patients and 81.5% for HIF-1α negative patients. The cumulative overall survival rates for these two populations were determined (Fig. 3) and the prognosis for HIF-1α positive patients was significantly poorer than that of HIF-1α negative patients ($P = 0.009$). A multivariate analysis of all clinicopathological factors and all three markers for all 126 patients showed that depth of invasion, lymph node metastasis, and HIF-1α expression were independent prognostic factors (Table IV).

DISCUSSION

In surgically resected specimens of gastric carcinoma, HIF-1α expression was more frequent in larger, deeply invasive tumors. Gastric carcinomas having either a large size or deep invasiveness grew expansively and contained necrotic regions and deep ulcerations within the tumor tissue. In typical cases, nuclear staining of HIF-1α was detected around the invading edges and necrotic regions, including deep ulcerations. A similar pattern of HIF-1α expression is prominent in glioblastoma multiforme, an extremely aggressive tumor [14]. A tumor with invasive properties can rapidly grow beyond the capacity of available blood supplies,

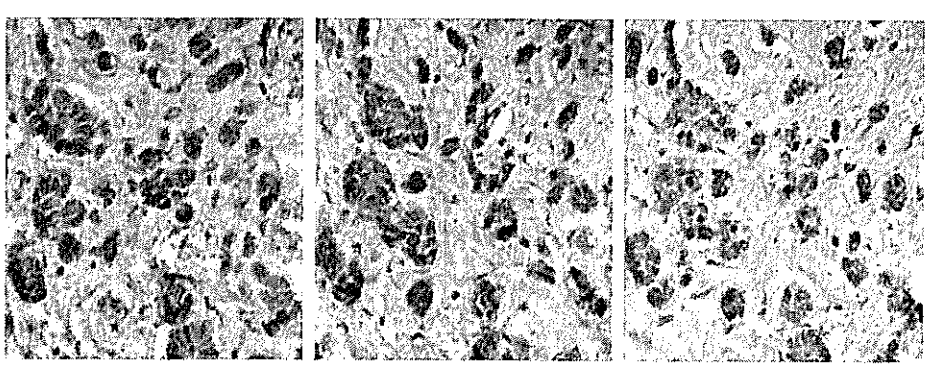

Fig. 2. Immunohistochemical analysis using serial sections showing colocalization of (a) HIF-1α, (b) VEGF, and (c) IGF-2 expression (400×).

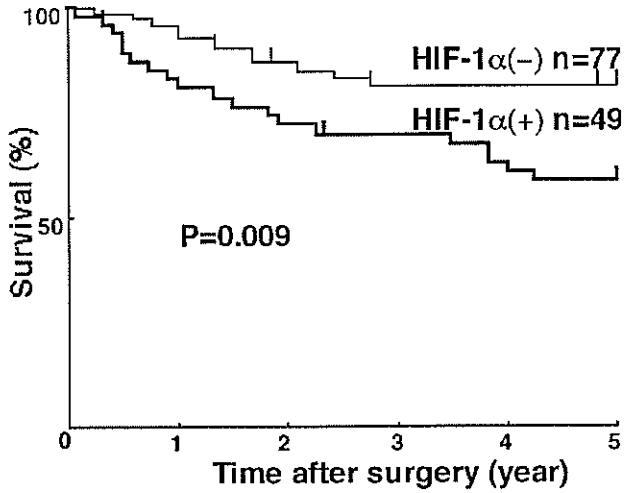

Fig. 3. Cumulative overall survival of two populations, with and without HIF-1α overexpression, respectively, was determined using the Kaplan–Meier method. Differences in survival were statistically significant ($P = 0.009$). (Bold line) HIF-1α positive patients. (Thin line) HIF-1α negative patients.

which results in hypoxia. Since most tumor cells that are distant from blood vessels are exposed to reduced oxygen pressure and nutrients, HIF-1α expression is induced. Conversely, we observed some tumor tissues containing necrotic regions that did not express HIF-1α. Increasing hypoxia may reduce further proliferation in tumors that cannot induce adaptive responses to hypoxic microenvironments. Tumors with necrotic regions or deep ulcerations that were HIF-1α negative tended to correlate with a more favorable prognosis for the patient.

In human gastric cancer models, inhibition of HIF-1α function is associated with the inhibition of gastric tumor growth and angiogenesis [15]. In the current study, HIF-1α expression in gastric carcinomas correlated with VEGF expression and increased intra-tumor MVD. Tumor angiogenesis and neovascularization requires the expression of VEGF and the binding of HIF-1 to HRE in the VEGF promoter is a major pathway leading to induction of VEGF expression under hypoxic conditions [16]. Our findings suggest that in gastric carcinoma HIF-1α induces VEGF and this event leads to formation of vascular networks which supply oxygen and nutrients. Pancreatic cancer cell lines with high constitutive levels of HIF-1α protein produce higher basal levels of VEGF [17]. An aggressive tumor may therefore contain a high vascular density with the concomitant expression of HIF-1α and VEGF during continued growth.

Several studies have shown that IGF-2 is overexpressed in tissues from patients with gastric carcinoma, compared to normal gastric tissues [18,19]. IGF-2 may therefore play an important role in the development and growth of gastric carcinoma and we examined the pattern of IGF-2 expression in gastric carcinomas and compared it to the HIF-1α expression profiles. Although there was no statistically significant correlation, coincident expression of HIF-1α, VGEF, and IGF-2 was observed in serial sections in tumor tissue from several subjects. This finding may indicate that HIF-1α functioned as a transcription factor to upregulate expression of various downstream genes in these regions.

In our study, aggressive gastric carcinomas were suggested to have hypoxic regions due to elevated levels of oxygen consumption, and to induce HIF-1α as an adaptation to hypoxic conditions. HIF-1α then activates transcription of VEGF, which mediates angiogenesis when secreted by the tumor cells. Thus, enhancement of angiogenesis by hypoxia is a prerequisite for progressive growth of gastric carcinomas, and the level of intra-tumor MVD has been previously reported to correlate with patient outcome in subjects with gastric carcinoma [20]. Significant associations between HIF-1α overexpression and patient outcome have been shown in many human carcinomas, including gastrointestinal tumors of the stomach [21]. Our data also suggest that increasing HIF-1α expression plays an important role in tumor progression of gastric carcinomas. Although it is a point of some controversy as to whether the HIF-1α-induced genes can in fact promote malignant tumor growth, disruption of the HIF-1 pathway by genetic or pharmacological means may potentially have anti-tumor effects [22,23].

In summary, HIF-1α overexpression in gastric carcinoma correlates with VEGF expression and increased

TABLE IV. Association of Various Factors With Overall Survival Determined by the Cox Proportional Hazard Model

	Regression coefficient	Standard error	Relative risk (95% CI)	*P*-value
Depth of invasion	3.13	1.05	22.9 (2.96–178)	0.005
Lymph node metastasis	1.77	0.552	5.83 (1.98–17.2)	0.005
HIF-1 expression	0.738	0.348	2.09 (1.08–4.06)	0.032

CI, confidence interval.
The covariates included age, gender, depth of invasion, histology, tumor size, lymph node metastasis, lymphatic invasion, venous invasion, HIF-1α, VEGF expression, IGF-2 expression, MVD.

intra-tumor MVD and was shown to be an independent prognostic factor. Therefore, gastric carcinomas that express HIF-1α may continue to grow by various adaptive responses, especially VEGF-mediated angiogenesis which has a negative impact on the prognosis of patients with gastric carcinoma.

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