Up-regulation of Hypoxia-inducible Factor-1 Alpha and VEGF mRNAs in Peritoneal Dissemination of Patients with Gastric Cancer

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Abstract. Background: Gastric cancer often metastasizes to the peritoneal cavity in which tumor cells are exposed to hypoxia without systemic circulation. Hypoxia-inducible factor-1 (HIF-1) and its target gene, vascular endothelial growth factor (VEGF), may play a role in the development of peritoneal metastases. Materials and Methods: The mRNA levels of HIF-1 alpha and VEGF were examined in 21 normal gastric mucosa, 158 primary tumors and 18 peritoneal metastases by quantitative RT-PCR. Results: HIF-1 alpha and VEGF were significantly up-regulated in the peritoneal metastases compared with those of the normal mucosa and the primary tumors. A positive correlation between HIF-1 alpha and VEGF was observed in the peritoneal dissemination. Conclusion: These results suggest that HIF-1 alpha and VEGF were up-regulated in the peritoneal metastases compared with those of the normal mucosa and the primary tumors. A positive correlation between HIF-1 alpha and VEGF was observed in the peritoneal dissemination.

Gastric carcinoma remains the most frequent malignancy in Japan and the second leading cause of cancer death in the world (1, 2). In patients with gastric cancer, peritoneal dissemination is the most common mode of the distant metastases (3). Although new chemotherapy has been developed, peritoneal dissemination rarely responds to these new regimens (4, 5).

Mechanisms of peritoneal metastases involve isolation from the gastric wall and the growth with neovascularization at the peritoneum. Sugarbaker et al. reported that the peritoneal cavity was a sanctuary, because tumor cells in the peritoneal cavity were not exposed to the systemic circulation involving the immune response (5, 6). Oxygen is not supplied by the systemic circulation in the peritoneal cavity, therefore, tumor cells are exposed to hypoxic conditions.

Hypoxia-inducible factor 1 (HIF-1) consists of HIF-1 alpha and HIF-1 beta subunits (7, 8). HIF-1 beta is constitutively expressed, while the expression of HIF-1 alpha is tightly regulated by cellular O\(_2\) (8). Under normoxic conditions, HIF-1 alpha is maintained at low levels due to continuous degradation via the ubiquitin-dependent proteosome pathway. This pathway is inhibited by hypoxia, leading to stabilization of the HIF-1 alpha protein levels (9, 10). In contrast, many investigators observed that HIF-1 alpha mRNA levels increased following prolonged hypoxia (11-14). Activation of signal transduction pathway also induced HIF-1 alpha mRNA (15-17). Thus, HIF-1 alpha is regulated by proteins levels but also by mRNA levels. HIF-1 activates the transcription of genes encoding erythropoietin, glucose transporters, vascular endothelial growth factor (VEGF) and other genes whose protein products increase O\(_2\) delivery or facilitate metabolic adaptation to hypoxia (18). In many human cancers, HIF-1 alpha protein is overexpressed and is correlated with microvessel density, treatment failure and survival (19-25).

Based on these reports, we hypothesized that HIF-1 played a role in the development of peritoneal metastases. In this study, HIF-1 alpha and VEGF mRNA levels were measured in the normal gastric mucosa, the primary tumors of the stomach and the intraperitoneal tumors obtained from patients with gastric cancer.

Abbreviations: HIF-1, hypoxia inducible factor-1; VEGF, vascular endothelial growth factor.

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Key Words: Hypoxia-inducible factor-1, vascular endothelial growth factor, peritoneal dissemination, gastric cancer.
Materials and Methods

Tissue samples. Eligibility included resectable tumors without distant metastases or the tumors with peritoneal metastases which were suspected by CT scan and required pathological confirmation. Written informed consent was obtained for the molecular analysis. The primary tumor tissues and the normal gastric mucosa were collected immediately after curative gastrectomy. Samples of the peritoneal dissemination were obtained from disseminated tumors or malignant ascites. This study was approved by IRB committees of the Kanagawa Cancer Center Hospital and St. Marianna University, Japan.

Sample preparation. Tissue samples were dissected and stored immediately under stringent sterile conditions in RNA later solution (Ambion, Austin, TX, USA) at 4°C. Cells floating in the ascites were precipitated by centrifugation. The precipitated cells were also stored immediately under stringent sterile conditions in RNA later solution (Promega). The copy number of each transcribed RNA was calculated from the amount of RNA (evaluated by spectrophotometry) and the molecular weight was deduced from the nucleotide sequence of the transcript. In vitro transcribed RNA was used as a standard for copy number of each target mRNA in real time PCR experiments. The standard RNA solution was prepared in 25 ng/µl of unrelated viral MS2 RNA (Roche) solution to mimic the condition of RNA samples.

Table I. Primer and hybridization probe sequences and labels for each target gene. Nucleotide sequences used to design PCR primers and hybridization probes were in GenBank under accession numbers NM_001530 for HIF-1 alpha, XM_052681 for VEGF and X04217 for PBGD.

<table>
<thead>
<tr>
<th>Primer primers</th>
<th>Hybridization probes</th>
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<tbody>
<tr>
<td>HIF-1 alpha forward</td>
<td>ACTAGCTTTGCGACAGATGTAATG</td>
</tr>
<tr>
<td>HIF-1 alpha reverse</td>
<td>TCGAGCGCAGGAAAAGGAGTCA</td>
</tr>
<tr>
<td>VEGF forward</td>
<td>GCTACTGCCATCCAATCGAG</td>
</tr>
<tr>
<td>VEGF reverse</td>
<td>AGTAGCTGCATGATCGTCTG</td>
</tr>
<tr>
<td>PBGD forward</td>
<td>CAGAGCAGGAAAAGGAGTCA</td>
</tr>
<tr>
<td>PBGD reverse</td>
<td>GCTACTGCCATCCAATCGAG</td>
</tr>
</tbody>
</table>

Establishment of standards to assess copy numbers of target mRNAs. Each PCR product for real-time PCR of HIF-1 alpha, VEGF or PBGD was recovered and subcloned into a plasmid vector pGem-T-easy (Promega, Madison, WI, USA), which contains both T7 and T3 promoters outside of the cloning site to generate RNA corresponding to the cloned sequence. The cloned plasmid was confirmed by nucleotide sequencing. Sense RNA for the cloned amplicon was transcribed from the plasmid with T7 or T3 RNA polymerase by using Riboprobes in vitro Transcription System (Promega). The copy number of each transcribed RNA was calculated from the amount of RNA (evaluated by spectrophotometry) and the molecular weight was deduced from the nucleotide sequence of the transcript. In vitro transcribed RNA was used as a standard for copy number of each target mRNA in real time PCR experiments. The standard RNA solution was prepared in 25 ng/µl of unrelated viral MS2 RNA (Roche) solution to mimic the condition of RNA samples.

Data analyses. With the LightCycler software (version 3), crossing points were assessed and plotted versus the molar concentrations of the standards. The relative mRNA amount in each sample was expressed as ratio marker copy numbers of HIF-1 alpha or VEGF mRNA/PBGD mRNA in 200 ng of total RNA. The crossing points (beginning of the PCR exponential phase) for each reaction were determined by the Fit points algorithm.

Statistical analyses. Data were expressed as mean±sd. Comparisons between the paired data were examined by the paired Mann-Whitney U-test. Comparisons among the three were examined by Mann-Whitney U-test and adjusted by Bonferroni’s method. Correlations were analyzed by Sperman’s test. All statistics were calculated by SPSS ver.11.0 software for Windows (Chicago, IL, USA).

Results

Samples collected. Twenty-one normal gastric mucosa, 158 primary tumors and 18 peritoneal metastases were collected from patients with gastric cancer.

Sensitivity and accuracy of measurements of HIF-1 alpha and VEGF mRNAs by quantitative RT-PCR. In vitro transcribed RNA standards for real time PCR experiments were employed accurately to evaluate the amount of target mRNA in samples. Each transcribed RNA in 10-fold dilutions from 1x10² to 1x10⁷ copies was prepared in a reaction of 20 µl containing MS2 RNA as described in “Materials and Methods” section. All of the standard curves for each target mRNA were linear (Figure 1) and we considered that the rage from 100 to 1x10⁷ copies were accurately evaluated in the study.

HIF-1 alpha and VEGF mRNA levels. Expressions of HIF-1 alpha and VEGF mRNA were significantly increased in the peritoneal dissemination compared with those of the normal gastric mucosa and the primary tumors (Figure 2), while those were almost the same between the normal gastric mucosa and the primary tumors. Correlations between HIF-1 alpha and VEGF were examined in the peritoneal dissemination and a
significant positive correlation was found between the two (Figure 3).

**Discussion**

We demonstrated that HIF-1 alpha mRNA levels were increased in the intraperitoneal tumors, but not in the primary tumors of the stomach. Our results suggest that the different microenvironments between the peritoneal cavity and the gastric wall, possibly hypoxia, may influence the mRNA expression of HIF-1 alpha. Moreover, an enhanced expression of VEGF was correlated with HIF-1 alpha in the peritoneal metastases, suggesting that VEGF may be induced by HIF-1. Koshikawa et al. found that high-metastatic potential cells produced a large amount of VEGF through constitutive HIF-1 alpha mRNA up-regulation under hypoxic conditions in human lung cancer cell lines (26). Our study confirmed these findings in the development of peritoneal dissemination of patients with gastric cancer. Increased expression of HIF-1 alpha may be a specific target for the treatment of patients with gastric cancer metastasizing to the peritoneum.

To date few reports have examined HIF-1 alpha mRNA in human gastric carcinoma. Only Zhong et al. investigated HIF-1 alpha protein expression in gastric cancer (27). These authors examined the expression of HIF-1 alpha protein in only two cases of primary tumors and found that both tumors expressed elevated immunostaining of HIF-1 alpha (27). In most human neoplasms, expression of HIF-1 was evaluated by...
protein levels (19-25). Recently, HIF-1 alpha mRNA and protein levels were found to be up-regulated in primary tumors of the urinary bladder and kidney (28, 29). Zhong et al. demonstrated that HIF-1 alpha mRNA was overexpressed in six rat prostate cancer cell lines and that metastatic potential correlated with HIF-1 alpha mRNA levels in those cell lines (30). More recently, Matsuyama et al. reported that HIF-1 alpha mRNA correlated with HIF-1 alpha protein levels (31). Thus, overexpression of HIF-1 alpha in solid tumors was confirmed both in protein and mRNA levels.

HIF-1 alpha activity is regulated by the protein degradation and the transcriptional activity. As an acute reaction for hypoxic conditions, HIF-1 alpha activity is mainly determined by the accumulation of HIF-1 alpha protein by inhibition of proteolytic degradation (9, 10). In contrast, many investigators suggested that the up-regulation of HIF-1 mRNA contributes to the physiological responses \textit{in vivo} during prolonged hypoxia (11-14). Moreover, activation of the signal transduction pathway by the growth factor receptor also induced HIF-1 alpha mRNA (15-17). Besides hypoxia, those mechanisms might be involved in the specific up-regulation of HIF-1 alpha mRNA in peritoneal dissemination.

We examined VEGF mRNA together with HIF-1 alpha mRNA. VEGF is a strong angiogenic factor which is induced by the HIF-1 protein accumulated under hypoxic conditions (16, 18). VEGF plays an important role in tumor growth through the neovascularization of the tumor. Here, VEGF mRNA was up-regulated in the peritoneal metastases of gastric cancer. A positive correlation was observed between VEGF and HIF-1 alpha mRNAs. These results suggest that
VEGF mRNA is induced by augmented transcriptional activity of HIF-1 alpha.

References


11 Nguyen SV and Claycomb WC: Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem Biophys Res Commun 265: 382-386, 1999


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