Transcription Factor Sp1 Expression Is a Significant Predictor of Survival in Human Gastric Cancer

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ABSTRACT

The transcription factor Sp1 regulates the expression of multiple genes. However, its expression and role in human tumor development and progression remain unclear. Using immunohistochemistry, we investigated Sp1 expression patterns in 86 cases of human gastric cancer having various clinicopathologic characteristics, 57 normal gastric tissue specimens, and 53 lymph node metastases. We found that Sp1 protein was expressed predominantly in the nuclei of cells located in the mucous neck region, whereas Sp1 expression was not detected either in the cells located toward the gastric pit (foveolar differentiation) or cells of the glandular epithelium (glandular differentiation). In sharp contrast, strong Sp1 expression was detected in tumor cells, whereas no or very weak Sp1 expression was detected in stromal cells and normal glandular cells surrounding or within the tumors.

We also evaluated the effect of Sp1 expression on the survival of patients who have undergone surgical resection. The median survival duration in patients who had a tumor with negative, weak, and strong Sp1 expression was 43, 37, and 8 months, respectively (P = 0.0075). Next, Sp1 expression, stage, completeness of resection, age, and sex were entered into a Cox proportional hazard model. In multivariate analysis, Sp1 (P = 0.003) and stage (P < 0.001) were independently prognostic of survival. Therefore, normal and malignant gastric tissues have unique Sp1 expression patterns. Given the importance of Sp1 in the expression of multiple molecules key to tumor cell survival, growth, and angiogenesis, its disregulated expression and activation may play important roles in gastric cancer development and progression.

INTRODUCTION

Although the incidence of gastric cancer declined in the West from the 1940s to the 1980s, it remains a major public health problem throughout the world (1). In Asia and parts of South America in particular, it is the most common epithelial malignancy and leading cause of cancer-related deaths. In fact, gastric cancer remains the second most frequently diagnosed malignancy worldwide and cause of 12% of all cancer-related deaths each year (1, 2). Advances in treatment of this disease are likely to come from a fuller understanding of its biology and behavior.

The aggressive nature of human metastatic gastric carcinoma is related to mutations of various oncoproteins and tumor suppressor genes (3–6), and abnormalities in several growth factors and their receptors (4, 5). These abnormalities affect the downstream signal transduction pathways involved in the control of cell growth and differentiation. Specifically, these perturbations confer a tremendous survival and growth advantage to gastric cancer cells (4–6). Previous studies indicated the role of several growth factor families in gastric cancer development and progression, including basic fibroblast growth factor, transforming growth factor, nitric oxide synthase, matrix metalloproteinase-2, epidermal growth factor receptor, insulin-like growth factor receptor, interleukin 8, and vascular endothelial growth factor (VEGF; Refs. 7–11). However, the molecular mechanisms behind the abnormal expression of multiple molecules remain unclear. Our recent study of human pancreatic cancer suggested that abnormal Sp1 activation augments the angiogenic and metastatic capacity of tumor cells through overexpression of multiple Sp1 downstream genes, including the key angiogenic factor VEGF (12).

Sp1 is a sequence-specific DNA-binding protein that is important in the transcription of many cellular and viral genes that contain GC boxes in their promoter (13). Additional transcription factors (Sp2, Sp3, and Sp4) that are similar to Sp1 in their structural and transcriptional properties have been cloned, thus forming a Sp1 multigene family (14–20). Because Sp1 is an essential transcription factor for many genes that are key to the regulation of multiple aspects of tumor cell survival, growth, and angiogenesis, abnormal Sp1 expression and activation may contribute to gastric cancer development and progression. For example, Sp1 mRNA and Sp1 and Sp3 DNA-binding activity are increased in epithelial tumors when compared with that in papillomas in skin (21). Interestingly, interference of Sp1 function has been shown to inhibit cell growth (22). Consistent with these findings, we have shown that Sp1 is constitutively overactivated in human pancreatic cancer (12). However, it is un-
known whether and how Sp1 signaling pathways contribute to tumor development and progression.

In the present study, we examined Sp1 expression in human gastric cancer. We found that elevated Sp1 activation occurred in human gastric cancer and was inversely correlated with patient survival, suggesting that abnormally activated Sp1 expression may represent a potential molecular marker for poor prognosis and directly contribute to gastric cancer development and progression.

**MATERIALS AND METHODS**

**Human Tissue Specimens and Patient Information.** We used human gastric cancer tissue specimens preserved in the Gastric Cancer Tissue Bank, and information about the patients was obtained from what was recorded in its comprehensive database at The University of Texas M. D. Anderson Cancer Center. Primary gastric cancer in these patients was diagnosed and treated at M. D. Anderson Cancer Center from 1985 to 1998. The patients had well-documented clinical histories and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. Eighty-six patients with primary gastric cancer were randomly selected for this study. All of the patients had undergone gastrectomy with lymph node dissection. All of the patients also were observed at M. D. Anderson through the end of 1999. The median follow-up period for the 86 patients was 25.7 months. At the end of 1999, 30 patients were still alive, whereas 56 patients had died. Fifty-three lymph node metastasis specimens and 57 normal gastric tissue specimens obtained from patients without gastric cancer were also included in this study.

**Western Blot Analysis.** Whole cell lysates were prepared from human normal and gastric cancer tissue specimens. Standard Western blotting was performed using a polyclonal rabbit antibody against human Sp1 (clone PEP2; Santa Cruz Biotechnology, Santa Cruz, CA) and antirabbit IgG, ahorse displacement peroxidase-linked F(ab')2 fragment obtained from a donkey (Amersham Life Sciences, Arlington Heights, IL). Equal protein sample loading was monitored by incubating the same membrane filter with an anti-β-actin antibody (12). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer’s instructions.

**Immunohistochemistry.** Sections (5-μm thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with 0.05% saponin for 30 min at room temperature. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated for 20 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated at 4°C in a 1:200 dilution of rabbit polyclonal antibody against human Sp1 (clone PEP2). The samples were then rinsed and incubated for 1 h at room temperature with peroxidase-conjugated antirabbit IgG. Next, the slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were washed three times with distilled water, counterstained with Mayer’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed once each with distilled water and PBS. Afterward, the slides were mounted using Universal Mount (Research Genetics) and examined using a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the nuclei (23, 24). Besides clone PEP2, other specific antihuman Sp1 antibodies were used, including a polyclonal rabbit antihuman Sp1 NH2 terminus peptide antibody (clone H-225; Santa Cruz Biotechnology) and monoclonal mouse antihuman Sp1 amino acid 520–538 peptide antibody (clone 1C6; Santa Cruz Biotechnology). All three of the antibodies were used on formalin-fixed, paraffin-embedded tumor sections. A positive reaction was indicated by a reddish-brown precipitate in the nuclei (23, 24).

Depending on the percentage of positive cells and staining intensity, Sp1 staining was classified into three groups: negative, weak positive, and strong positive. Specifically, the percentage of positive cells was divided into five grades (percentage scores): <10% (0), 10–25% (1), 25–50% (2), 50–75% (3), and >75% (4). The intensity of staining was divided into four grades (intensity scores): no staining (0), light brown (1), brown (2), and dark brown (3). Sp1 staining positivity was determined by the formula: overall scores = percentage score × intensity score. The overall score of ≤3 was defined as negative, of >3 - ≤6 as weak positive, and of >6 as strong positive.

**Electrophoretic Mobility Shift Analysis and Supershift Assay.** Nuclear protein extracts from normal stomach and gastric cancer tissues were prepared by lysing cells in a high-salt buffer (20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na2HPO4, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min; the freezing and thawing procedures were performed twice. The supernatant was then centrifuged and harvested. Protein concentrations were determined using the Coomassie Plus Protein Assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s protocol. Protein extracts (10 μg) were incubated in a final 20-μl volume of 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, and 100 μg/ml poly(ethyleneimine-deoxytocid acid) using electrophoretic mobility shift analysis with the radiolabeled double-stranded Sp1 consensus-binding motif 5’-ATTGATCGGGGCAGGCAGC-3’ (Santa Cruz Biotechnology) or as indicated for 20 min at room temperature. For supershift analysis, the nuclear extracts were preincubated with specific antibodies against Sp1 (Santa Cruz Biotechnology). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature. The results were autoradiographed (12).

**Statistical Analysis.** The two-tailed chi-squared test was performed to determine the significance of the difference between the covariates. Survival durations were calculated using the Kaplan-Meier method. The log rank test was used to compare cumulative survival of patient groups. Cox proportional hazard model was used to provide univariate and multivariate hazard ratios for the study parameters. The level of Sp1 expression, age, sex, Lauren’s histology type, stage (American Joint Committee on Cancer), and completeness of surgical resection (R0 versus
RESULTS

Unique Sp1 Expression Pattern in Human Normal Gastric and Tumor Tissue. To localize Sp1 protein expression in both human normal gastric and tumor tissue specimens, we performed immunohistochemical staining of paraffin-embedded tissue specimens using an antibody against Sp1 protein (clone PEP2). There was either weak or negative expression in most of the cell nuclei in the gastric tissue specimens obtained from patients who did not have cancer. Positive staining was observed in the nuclei of cells localized predominantly in the mucous neck region, whereas Sp1 expression was not detected in the cells located toward the gastric pit (foveolar differentiation) or cells of the glandular epithelium (glandular differentiation; Fig. 1). In sharp contrast, strong Sp1 staining was seen in the tumor-cell nuclei of various types of gastric cancer, whereas no or very weak Sp1 expression was detected in the stromal cells and normal glandular cells surrounding or within the tumors (Fig. 2). These Sp1 expression patterns were additionally confirmed using three different anti-Sp1 antibodies (data not shown). These results suggested that Sp1 is rarely expressed in normal human gastric cells but commonly expressed in human gastric cancer cells.
Sp1 DNA-Binding Activity in Normal and Tumor Tissues. To determine the functional level of Sp1 expression in both human normal gastric and tumor tissue cells, whole cell and nuclear protein extracts were prepared from specimens obtained from 8 patients with known Sp1 expression as detected via immunostaining. Sp1 protein expression was determined using Western blot analysis with 10-μg whole cell protein extracts (Fig. 3A), whereas Sp1 DNA-binding activity was de-

Fig. 2 Sp1 protein expression in human gastric cancer tissue. Tissue sections were prepared from formalin-fixed, paraffin-embedded specimens of various types of gastric cancers (86 cases): A, diffuse; B, papillary; C, tubular; D, tubular with adjacent normal; E, mucinous; and F, signet ring. Immunohistochemical staining was performed using a specific anti-Sp1 antibody (clone PEP2). Of note is that the majority of the tumor cells were positive for Sp1 in the nuclei, whereas the majority of the normal gland (A), stromal (C), and adjacent tissue cells (D), and infiltration lymphocytes were negative for Sp1. Insets in A and F are representative areas from A and F, respectively, with higher magnifications. Calibration bar, 50 μm (A–F), and 20 μm (insets).
termined via electrophoretic mobility shift analysis using 10-μg nuclear protein extracts and a 32P-labeled oligonucleotide probe containing a consensus-binding motif for Sp1 (Fig. 3B). It was apparent that cells in the tumor tissue specimens had a significantly higher level of Sp1 activity than those in the normal tissue specimens did, which was consistent with the level of Sp1 protein expression determined via immunostaining.

**Sp1 Expression in Primary Tumor and Nodal Metastases of Gastric Cancer.** We analyzed Sp1 expression in 86 gastric tumor, 53 lymph node metastasis, and 57 normal human gastric tissue specimens. Overall, lymph node metastasis specimens exhibited the highest level of Sp1 expression (54.7% strong positive; \( P < 0.001 \)), and tumor tissue specimens had higher levels of Sp1 expression than normal tissue specimens (24.4% versus 0% strong positive; \( P < 0.001 \); Table 1; Fig. 4A).

**Sp1 Expression and Survival in Gastric Cancer Patients.** The median survival duration in patients who had a tumor with negative, weak, and strong Sp1 expression was 43, 37, and 8 months, respectively. The elevated Sp1 expression was associated with inferior survival duration (\( P = 0.0075 \); Fig. 4B). Other parameters that affected survival in univariate analyses included stage (\( P < 0.0001 \)) and completeness of resection (\( P < 0.0002 \)). Age at diagnosis (as a continuous variable by univariate Cox proportional hazard analysis), sex, and Lauren’s histology type did not have a statistically significant affect on survival.

Next, Sp1 expression, stage, completeness of resection, age, and sex were entered into a Cox proportional hazard model for multivariate analysis (Table 2). Adjust for the effect of covariates, high Sp1 expression (\( P = 0.003 \)) and advanced stage (\( P < 0.001 \)) were independent predictors of poor survival. The hazard ratio for the group with strong Sp1 expression (hazard ratio, 4.5; 95% confidence interval, 1.8–11.2) was significantly higher than that of the Sp1-negative group (referent). Completeness of resection, age at diagnosis, sex, and Lauren’s histology type did not have a statistically significant affect on survival in multivariate analyses.

**DISCUSSION**

In the present study, we provided evidence of distinct Sp1 expression patterns in normal gastric and gastric tumor tissues.
Sp1 and Gastric Cancer Progression

Specifically, we found that Sp1 protein was expressed predominantly in the nuclei of cells located in the mucous neck region where stem cells reside, suggesting that dividing cells express Sp1, whereas Sp1 expression was not detected in either cells located toward the gastric pit (foveolar differentiation) or cells of the glandular epithelium (glandular differentiation), suggesting that terminally differentiated cells lost their Sp1 expression. In contrast, significantly elevated Sp1 activity was observed in gastric tumor specimens and inversely correlated with patient survival, suggesting that Sp1 is an independent prognostic marker for gastric cancer and that the Sp1 signaling pathway may play an important role in gastric cancer development and progression.

The notion that abnormal Sp1 expression and activation contribute to gastric cancer development and progression is well supported not only by our study of pancreatic cancer (12) but also by other lines of evidence showing that Sp1 may regulate many aspects of cancer biology, including cell growth, survival, invasion, and angiogenesis. Early studies indicated that Sp1 regulates multiple growth-regulated genes, arguing that Sp1 may be important for cell-growth regulation. Direct evidence of the ability of Sp1 to regulate transcription during changes in cell growth came with the demonstration that Sp1 affects serum stimulation of quiescent cells at the rep3a promoter (25), as well as at the hamster dihydrofolate reductase (26, 27) and ornithine decarboxylase promoters (21). More recently, several studies established the ability of Sp1 to mediate the growth induction of a variety of promoters, including those of the genes encoding FGFR1, epidermal growth factor receptor, insulin-like growth factor receptor 1 (28–31), insulin-like growth factor-binding protein 2 (32), VEGF (12, 33), thymidine kinase (34), and serum response factor (35). In addition to altered growth regulation, tumor progression requires resistance to apoptosis and stimulation of angiogenesis (36). Furthermore, Sp1 has been shown to regulate the promoters of many apoptosis-related genes, including Bcl-2 and Bcl-x (37, 38), survivin (39), and TGF-β and its receptors (40, 41). Thus, increasing evidence indicates that Sp1 is involved in the regulation of apoptosis in numerous systems (42–45). Moreover, members of the Sp1 family regulate (positively or negatively) the expression of VEGF and basic fibroblast growth factor, two major signaling molecules key to tumor angiogenesis (12, 46–48). Accumulating evidence has also suggested that Sp1 family members regulate multiple aspects of angiogenesis and that this involvement appears to extend to all of the cell types involved in vessel formation (49, 50). Furthermore, evidence has shown that Sp1 also up-regulates matrix metalloproteinase-2 (51, 52) and urokinase-type plasminogen activator (53), which play very important roles in tumor invasion and metastasis.

Given the demonstrated role of the Sp1 factors in growth regulation, cell survival, angiogenesis, and metastasis, it is conceivable that members of the Sp1 family have been implicated in tumor development and progression. In fact, Sp1-mediated transcription is involved in many signal transduction pathways linked to cancer; this role has been shown to directly impinge upon transformation (54). In addition, Sp1 mRNA, and Sp1 and Sp3 DNA-binding activity are increased in epithelial tumors when compared with papillomas, indicating that increased activity of these factors contributes to tumor progression in skin (21). Interestingly, a dominant-negative Sp1 (a truncated COOH-terminal Sp1, which contains the zinc finger DNA-binding domain but not the major transactivating domains of the protein) has been shown to inhibit the growth of HeLa cells (22). Targeting Sp1 using Sp1 decoy oligonucleotides or small inhibitory RNA blocks cell cycle progression and suppresses cell growth (55, 56). In fact, nuclear levels of Sp1 protein have been

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The two-tailed χ² test was performed using SPSS software.

Table 1  Clinical characteristics of gastric cancer patients and Sp1 expression
Fig. 4 Correlation of elevated Sp1 expression with reduced survival in gastric cancer patients. A, Sp1 expression was determined in 86 gastric cancer, 53 metastatic lymph node, and 57 normal human gastric tissue specimens. Overall, lymph node metastasis specimens exhibited the highest level of Sp1 expression ($P < 0.001$), and tumor tissue specimens had a much higher level of Sp1 expression than adjacent normal tissue specimens did ($P < 0.001$). B, Kaplan-Meier plots of overall survival in patients who had a tumor with negative, weak, or strong Sp1 expression. The survival curve for 17 patients who had a tumor with negative Sp1 expression was not statistically different from that for 48 patients who had a tumor with weak Sp1 expression ($P = 0.89$). However, the survival curve for 21 patients who had a tumor with strong Sp1 expression was significantly worse than that for the 17 patients with negative Sp1 expression ($P < 0.001$) as well as that for the 48 patients with weak Sp1 expression ($P < 0.001$).
shown to correlate directly with cell proliferation and predominate in the G1 phase of the cell cycle through a proteasome-dependent degradation mechanism (57). Presumably, a high level of cell proliferation results in reduced Sp1 proteolysis, whereas reduced Sp1 degradation and enhanced Sp1 DNA binding may additionally augment the proliferation by overexpressing Sp1-responsive genes, including ODC and cyclin D1 (57). We have consistently shown that Sp1 is constitutively overactivated in and directly correlates with the angiogenic potential of human pancreatic cancer (12). The results of the current study may indicate that abnormally activated Sp1 contributes directly to gastric cancer progression. However, additional studies are clearly needed to test whether Sp1 is a reliable tumor progression marker and/or effective therapeutic target for gastric cancer.

On the other hand, the underlying mechanism that results in Sp1 overactivation is currently unknown. During tumor development and progression, Sp1 can be overactivated through several potential mechanisms. A recent study showed that Sp1 protein positively regulates its own promoter (58), although post-translational modification and degradation of Sp1 protein directly regulate its transcriptional activity (13). Aberrant Sp1 activity may also be due to an altered Sp1:Sp3 ratio (18), methylation status of Sp1-binding sites (19), and potentially inhibitory proteins that bind to Sp1/Sp3 (20). The involvement of altered oncogenes and suppressor genes is particularly important. Tumor suppressors such as p53 (59), p73 (60), von Hippel-Landau (61), and retinoblastoma protein (19, 62) can physically interact with Sp1, form a complex, and block Sp1 binding to the promoter of Sp1-regulated genes. For example, tumor cells harboring the inactivated von Hippel-Landau gene are associated with increased VEGF expression and angiogenesis (63–65). Also, von Hippel-Landau-mediated repression of VEGF expression has convincingly been shown to be mediated by transcriptional and post-transcriptional mechanisms (49, 62). At the transcriptional level, von Hippel-Landau forms a complex with the Sp1 transcription factor and inhibits Sp1-mediated VEGF expression (66). Whether oncogenes and/or tumor suppressor genes contribute to constitutive Sp1 activation in human cancer cells and how their status affects that activation are not fully elucidated. It appears that activated oncogenes, including Ras, Src, and Raf, enhance the transcription activity of Sp1 through constitutive activation of the p42/p44 mitogen-activated protein kinase pathway, which has often been observed in many human tumors. In fact, p42/p44 mitogen-activated protein kinase directly phosphorylates Sp1 on threonines 453 and 739, and increases the DNA-binding ability of Sp1, thus activating many Sp1-regulated genes implicated in tumor growth and progression (66, 67).

Other factors may also be involved in Sp1 overactivation. For example, development of stressful tumor microenvironment may be important, especially at advanced tumor stages. It has been shown that Sp1 activity can be modulated by stress factors such as hypoxia (68, 69) and overproduction of free radicals, e.g., reactive oxygen species and nitric oxide (70–72), which are prominent in the tumor microenvironment. Several lines of evidence additionally indicated that such stress factors can activate the p42/p42 mitogen-activated protein kinase pathway and, to a lesser extent, c-Jun NH2-terminal kinase-related signaling pathways, which may be responsible for Sp1 overactivation (66). Therefore, both genetic and epigenetic factors can cause Sp1 overactivation, which leads to altered expression of multiple Sp1 downstream genes and contributes to tumor growth and metastasis.

In summary, we discovered unique Sp1 expression in normal gastric tissue and various gastric cancer tissues. The level of Sp1 expression was closely related to the postoperative prognosis of gastric cancer. This study demonstrated that abnormally activated Sp1 expression represents a potential risk for poor prognosis and directly contributes to gastric cancer development and progression. Therefore, preoperative determination of Sp1 activity may be useful in deciding the modality and extent of postoperative therapy. We are currently investigating the molecular mechanism by which the Sp1 signaling pathway regu-
lates gastric cancer development and progression, and whether this Sp1 pathway is a potential therapeutic target for controlling gastric cancer growth and metastasis.

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