Sphingosine Kinase 1 Is Associated with Gastric Cancer Progression and Poor Survival of Patients

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Abstract

Purpose: The present study was to investigate the clinical significance of sphingosine kinase 1 (SPHK1), an oncoenzyme, in the development and progression of gastric cancer.

Experimental Design: mRNA and protein levels of SPHK1 expression in normal gastric epithelial cells, gastric cancer cell lines, and paired gastric cancer lesions and the adjacent noncancerous tissues were examined using reverse transcription-PCR and Western blotting. Immunohistochemistry was employed to analyze SPHK1 expression in 175 clinicopathologically characterized gastric cancer cases. Statistical analyses were applied to derive prognostic and diagnostic associations.

Results: Levels of SPHK1 mRNA and protein were higher in gastric cancer cell lines than in normal gastric epithelial cells. SPHK1 protein level was up-regulated in gastric cancer lesions compared with that in the paired adjacent noncancerous tissues. Gastric cancer tissues from 115 of 175 (65.7%) patients revealed high level of SPHK1 protein expression in contrast to the undetectable or marginally detectable expression of SPHK1 in the adjacent noncancerous gastric tissues. Significantly different expression levels of SPHK1 were found in patients at different clinical stages (P = 0.003), T classification (P = 0.035), and M classification (P = 0.020). Patients with higher SPHK1 expression had shorter overall survival time, whereas those with lower SPHK1 expression survived longer. Further multivariate analysis suggested that SPHK1 up-regulation was an independent prognostic indicator for the disease.

Conclusions: SPHK1 protein could be a useful marker for the prognosis of gastric cancer. Further study on the potential use of SPHK1 as a therapeutic target is also warranted.

Gastric cancer is the fourth most commonly diagnosed cancer and the second major cause of cancer-related death in the world (1–3). In many cases, gastric cancer spreads before it is detected; hence, the majority of patients suffering from gastric cancer are diagnosed at advanced stages (3–5). Although considerable improvements have been made in high-resolution imaging, surgery techniques, chemotherapy, and radiotherapy, patients with advanced gastric cancer still face poor prognosis (6). It has been reported that, in the United States, the 1- and 5-year survival rates of stage I/II patients were 93% and 67%, respectively. Rates for stage III patients decreased to 31% and 8%, respectively, despite that most late-stage patients receive adjuvant therapies such as chemotherapy and radiation therapy (7).

Investigation of the molecular and biological changes, including gene amplification and activations that occur during carcinogenesis and progression, can provide new insights into the pathology of the disease and may add biological factors that can be used as new prognostic markers. It is widely accepted that the pathogenesis of gastric carcinomas is multifactorial and includes genetic predisposition and environmental factors. Genetic predisposition has been found to be accompanied by several genetic alternations including tumor suppressor genes, oncogenes, cell adhesion molecules, growth factors, and genetic stability (5). However, little is known about the exact molecular
Translational Relevance

In the current study, we show for the first time that the characterization of sphingosine kinase 1 (SPHK1) expression in human gastric cancer tissues and their correlation with the clinicopathologic grading of the disease. We found that SPHK1 expression correlated with clinical staging, T classification, and M classification of gastric cancer. The effectiveness of SPHK1 as a prognostic factor was assessed using multivariate analysis. It has been reported that SPHK1 is an oncogenic enzyme and that activation of SPHK1 is closely associated with transformation, proliferation, and survival of tumor cells. The oncogenic feature of SPHK1 has also been associated with abrogation of the apoptotic pathway, which is linked to the failure of clinical cancer therapies such as chemotherapy and radiotherapy. Furthermore, it has been shown that inhibiting or down-regulating SPHK1 could decrease cell proliferation and arrest cell cycle in glioblastoma cells and breast cancer cells and that a dominant-negative form of SPHK1 could decrease tumor formation in nude mice. Thus, our study also suggests that SPHK1 could be a potential target for gastric cancer therapy.

Events leading to its development and progression. Therefore, it is of great clinical value to further understand the molecular mechanism of gastric cancer and find valuable diagnostic markers as well as novel therapeutic strategies.

Sphingosine 1-phosphate is a bioactive lipid mediator that plays a vital role in regulating various biological processes during tumorigenesis (8–13). Generation of sphingosine 1-phosphate is catalyzed by enzyme sphingosine kinase (SPHK; refs. 14, 15). Two functional SPHK isoenzymes, SPHK1 and SPHK2, have been identified in mammalian cells and tissues (16–18). Multiple lines of evidence indicate that SPHK1 is an oncogenic enzyme and that activation of SPHK1 is closely associated with antiapoptosis, transformation, proliferation, and survival of tumor cells (19–23). It has been reported that ectopic expression of SPHK1 in NIH3T3 fibroblasts could increase cell proliferation and promote a transformed phenotype as determined by focus formation, colony growth in soft agar, and the ability to form tumors in NOD/SCID mice (24). Furthermore, a dominant-negative form of SPHK1 has been shown to decrease tumor formation in nude mice possibly by inhibiting estrogen-mediated mitogenic signaling in MCF-7 cells (25). The oncogenic feature of SPHK1 has also been associated with abrogation of the apoptotic pathway, which is linked to the failure of clinical cancer therapies such as chemotherapy and radiotherapy. It has been shown that up-regulation of SPHK1 could regulate apoptosis and caspase activation in PC-12 cells and the effectiveness of chemotherapy could be impaired by overexpression of SPHK1 in human PC-3 and LNCaP prostate cancer cell lines (26, 27). Moreover, tumor cell migration could be regulated by SPHK1. SPHK1 inhibitors, camptothecin and docetaxel, have been found to suppress tumor growth and reduce the occurrence and number of metastases in nude mice (28, 29). The expression level of SPHK1 is frequently up-regulated in various tumor types, including prostate cancer, glioblastoma multiforme, intestinal adenoma, acute erythroleukemia, and colon cancer (19–23). Studies have also shown the involvement of SPHK1 in oncogenic H-Ras-mediated signaling (24, 29) as well as vascular endothelial growth factor-induced Ras activation in bladder cancer cells by favoring inactivation of Ras-GAP (30). In this study, we report for the first time characterization of SPHK1 expression in human gastric cancer tissues and their correlation with clinicopathologic grading. We found that SPHK1 expression correlated with clinical staging, T classification, and M classification of the disease. The effectiveness of SPHK1 as an independent prognostic factor was assessed using multivariate analysis. Our results strongly suggest that SPHK1 could be a potentially promising biomarker for predicting the prognosis of patients with gastric cancer and might be a potential target for gastric cancer therapy.

Materials and Methods

Cell lines. Primary cultures of human gastric epithelial cells were established from gastric biopsies taken during upper gastrointestinal endoscopy and cultured as described previously (31). The gastric cancer cell lines, including SGC7901, AGS, MKN45, MKN28, and MGC803, were obtained from the American Type Culture Collection and kept in our laboratory. All cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone).

Patients and tissue specimens. Ten human normal gastric samples were obtained from partial gastrectomy of adjacent gastric ulcer tissues. Primary gastric cancer tissue samples of 175 patients diagnosed with gastric cancer were obtained from absolute curative gastrectomy, which had been formalin-fixed, paraffin-embedded, and clinically and histopathologically diagnosed at the Departments of Gastrointestinal Surgery and Pathology, The First Affiliated Hospital, Sun Yat-sen University from 1997 to 2001. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained. H&E staining on the 175 gastric cancers tissues was done to determine the depth of cancer invasion and histopathologic features. Clinicopathologic tumor-node-metastasis staging was determined by the extent of tumor invasion in the stomach wall and lymphatic and venous invasion status according to the criteria proposed by the American Joint Committee on Cancer and International Union Against Cancer criteria (32). Routine chemotherapy had been given to the patients with advanced-stage disease after operation, but no radiation treatment was done in any of the patients included in our study. Clinical information of the samples is summarized in Supplementary Table S1. Percentage tumor purity in sections adjacent to regions used for RNA extraction was estimated during routine histopathologic analysis to assure that the sections did contain major cancer lesions.

RNA extraction and reverse transcription-PCR. Total RNA from cells and primary tumor samples was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was pretreated with RNase-free DNase, and 2 μg RNA from each sample was used for cDNA synthesis primed with random hexamers. For PCR-mediated amplification of SPHK1 cDNA, an initial amplification using SPHK1-specific primers was done with a denaturation step at 95°C for 10 min followed by 30 denaturation cycles at 95°C for 60 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 30 s. On completion of the cycling steps, a final extension at 72°C for 5 min was carried out before the reaction was stopped and stored at 4°C. Real-time PCR was then employed to determine the fold increase of SPHK1 mRNA in each of the primary gastric tumors relative to the paired noncancerous gastric tissues, to determine the fold increase of SPHK1 mRNA in each of the primary gastric tumors relative to the paired noncancerous gastric tissues, with each pair taken from the same patient. Expression data were normalized to the geometric mean of the housekeeping GAPDH gene to control the variability in expression levels. Reverse transcription-PCR and real-time PCR primers were designed using the Primer Express
Western blotting. Cells were harvested in sampling buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol]. All fresh tissues were grounded to powder in liquid nitrogen and then lysed with the sampling buffer. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories). Equal amounts of proteins were applied to 9% polyacrylamide SDS gels (SDS-PAGE), separated electrophoretically, and transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech). The membrane was incubated with anti-SPHK1 rabbit antibody (1:1,000; Abgent). SPHK1 expression was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Amersham Pharmacia Biotech) and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Anti-α-tubulin antibody (1:1,000 dilution; Sigma) was used as the loading control.

Immunohistochemistry. Immunohistochemical analysis was done to study altered protein expression in 10 noncancerous human gastric tissue controls and 175 human gastric cancer tissues. In brief, paraffin-embedded specimens were cut into 4 μm sections and baked at 60°C for 2 h followed by deparaffinization with xylene and rehydrated. The sections were submerged into EDTA antigenic retrieval buffer and microwaved for antigenic retrieval, after which they were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with rabbit anti-SPHK1 (1:1,000; Abgent) overnight at 4°C. Normal goat serum was used as a negative control. After washing, tissue sections were treated with biotinylated anti-rabbit secondary antibody (Zymed) followed by further incubation with streptavidin-horseradish peroxidase complex (Zymed). Tissue sections were then immersed in 3,3-diaminobenzidine and counterstained with 10% Mayer’s hematoxylin, dehydrated, and mounted.

The degree of immunostaining was reviewed and scored independently by two observers based on the proportion of positively stained tumor cells and intensity of staining (33–35). Tumor cell proportion was scored as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10-35% positive tumor cells), 3 (35-70% positive tumor cells), and 4 (>70% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3.
(strong staining = brown). Staining index was calculated as the product of staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated SPHK1 expression in benign gastric epithelia and malignant lesions by determining the staining index, with scores of 0, 1, 2, 3, 4, 5, 6, 7, 8, or 12. The cutoff value for high and low expression level was chosen based on a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: a staining index score of ≥6 was used to define tumors with high SPHK1 expression and a staining index score of ≤4 was used to indicate low SPHK1 expression.

**Statistical analysis.** All statistical analyses were carried out using the SPSS 13.0 statistical software package. The $\chi^2$ and Fisher’s exact tests were used to analyze the relationship between SPHK1 expression and clinicopathologic characteristics. Bivariate correlations between study variables were calculated by Spearman’s rank correlation coefficients. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. $P < 0.05$ in all cases was considered statistically significant.

**Results**

**Up-regulation of SPHK1 in gastric cell lines.** Overexpression of SPHK1 has been reported in many human cancers. Its expression status in gastric cancer, however, remains unclear. To determine SPHK1 protein expression, Western blotting analysis was conducted on protein samples derived from normal human gastric epithelial cells (HGEC) and several gastric cancer cell lines. All cancer cell lines expressed high levels of SPHK1 protein compared with the NGEC (Fig. 1A). To investigate whether SPHK1 up-regulation was at the transcription level, mRNA of SPHK1 in gastric cancer cell lines was quantified using reverse transcription-PCR analysis, whose result was subsequently confirmed by real-time PCR (data not shown). We found that all gastric cancer cell lines revealed higher SPHK1 expression at both mRNA and protein levels compared with those in HGEC (Fig. 1B).

**Expression of SPHK1 is up-regulated in gastric cancer lesions.** To determine whether SPHK1 up-regulation in gastric cancer cell lines could clinically correlate with gastric cancer progression, reverse transcription-PCR analysis and Western blotting analysis were done in paired gastric cancer tissues and noncancerous tissues adjacent to cancer lesions, with each pair taken from the same patient. SPHK1 was found to be differentially overexpressed at both mRNA and protein levels in all four examined human primary gastric cancer samples paired with tissues adjacent to tumors from the same patients (Fig. 2A and B). Importantly, all four tumors displayed >2-fold increase of SPHK1 protein compared with tissues adjacent to the tumors by protein quantification (data not shown). In addition to the Western blotting, four tumor samples were further detected for SPHK1 expression by immunohistochemical analysis. In agreement with the result of Western blotting assay, immunohistochemical analysis also showed SPHK1 overexpression in all four tumors in comparison with the paired noncancerous adjacent tissues (Fig. 2B).

**Overexpression of SPHK1 in archived gastric tissue samples.** To further examine whether SPHK1 protein up-regulation is linked to clinical progression of gastric cancer, the following samples were subjected to immunohistochemical staining with a human SPHK1 antibody: 10 paraffin-embedded, archived noncancerous human gastric tissues; and 175 paraffin-embedded, archived gastric cancer tissue samples, including 38 cases of stage I/II and 137 cases of stage III/IV tumors; and 27 cases of matched lung or hepatic metastases derived from gastric cancer relapse patients. Immunohistochemical results are summarized in Table 1. SPHK1 protein was detected in 161 of 175 (92%) human gastric cancer cases. Strong cytoplasmic staining of SPHK1 protein was detected in 115 (65.7%) tumors. Weak or negative staining was detected in 60 (34.3%) tumors. As shown in Fig. 3, SPHK1 was found to be overexpressed in gastric tumors (Fig. 3C and D). In contrast, SPHK1 was either undetectable or only marginally detectable in the noncancerous gastric tissues in the adjacent section regions (Fig. 3A and B). SPHK1 was mainly localized in the cytoplasm of primary cancer cells, consistent with previous reports on SPHK1 expression in other cancer types (refs. 36–38; Fig. 3C and D). Taken together, these observations suggest that high levels of SPHK1 expression are associated with clinical development of primary gastric cancer.

**Relationship of SPHK1 up-regulation with the clinical features of gastric cancer.** Immunohistochemical staining of SPHK1 levels was statistically analyzed to determine their relationship with the clinical features of gastric cancer. As shown in Table 1, SPHK1 expression strongly correlated with clinical staging.

<table>
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<tr>
<th>Characteristics</th>
<th>SPHK1</th>
<th>$\chi^2$ test $P$</th>
<th>Fisher’s exact test $P$</th>
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<tr>
<td>Age (y)</td>
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<td></td>
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<tr>
<td>≥60</td>
<td>29 (48.3)</td>
<td>54 (47.0)</td>
<td>0.863</td>
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<tr>
<td>&lt;60</td>
<td>31 (51.7)</td>
<td>61 (53.0)</td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>38 (63.3)</td>
<td>87 (75.7)</td>
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</tr>
<tr>
<td>Female</td>
<td>22 (36.7)</td>
<td>28 (24.3)</td>
<td>0.063</td>
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<td>Clinical stage</td>
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<tr>
<td>I</td>
<td>5 (8.3)</td>
<td>0 (0.0)</td>
<td>0.003</td>
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<tr>
<td>II</td>
<td>12 (20.0)</td>
<td>21 (18.3)</td>
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<td>III</td>
<td>39 (65.0)</td>
<td>71 (61.7)</td>
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<td>IV</td>
<td>4 (6.7)</td>
<td>23 (20.0)</td>
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<td>T1</td>
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<td>T2</td>
<td>17 (28.4)</td>
<td>20 (17.4)</td>
<td>0.848</td>
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<tr>
<td>T3</td>
<td>9 (15.0)</td>
<td>17 (14.8)</td>
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</tr>
<tr>
<td>T4</td>
<td>29 (48.3)</td>
<td>76 (66.1)</td>
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<tr>
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<tr>
<td>N0</td>
<td>19 (31.7)</td>
<td>22 (19.1)</td>
<td>0.099</td>
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<tr>
<td>N1</td>
<td>15 (25.0)</td>
<td>39 (33.9)</td>
<td>0.068</td>
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<td>N2</td>
<td>26 (43.3)</td>
<td>49 (42.6)</td>
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<td>N3</td>
<td>0 (0.0)</td>
<td>5 (4.3)</td>
<td>0.020</td>
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<td>92 (80.0)</td>
<td>0.020</td>
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<tr>
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<td>4 (6.7)</td>
<td>23 (20.0)</td>
<td>0.020</td>
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<td>111 (96.5)</td>
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<td>60 (100.0)</td>
<td>107 (93.0)</td>
<td>0.037</td>
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<td>Yes</td>
<td>0 (0.0)</td>
<td>8 (7.0)</td>
<td>0.848</td>
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<td>Lymphatic invasion</td>
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<td></td>
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<tr>
<td>No</td>
<td>20 (33.3)</td>
<td>40 (34.8)</td>
<td>0.848</td>
</tr>
<tr>
<td>Yes</td>
<td>40 (66.7)</td>
<td>75 (65.2)</td>
<td>0.848</td>
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</table>

**Table 1.** Correlation between SPHK1 expression and clinicopathologic characteristics of gastric cancer patients
(P = 0.003), T classification (P = 0.035), and M classification (P = 0.020) of patients with gastric cancer, whereas it was not associated with age, gender, or N classification. Spearman correlation analysis was further done to confirm the correlation between SPHK1 expression and clinicopathologic features. As shown in Supplementary Table S3, Spearman correlations of SPHK1 expression levels to clinical staging, T classification, and distal metastasis were 0.196 (P = 0.010), 0.199 (P = 0.008), and 0.175 (P = 0.020), respectively. Taken together, our data revealed a fact that the expression of SPHK1 increases as gastric cancer clinically progresses, with the exception that nodal status does not seem to be correlated with the level of SPHK1 expression in the samples included in the present study.

**Association between SPHK1 expression and patient survival.** Patient survival analysis indicated a clear inverse correlation between SPHK1 protein expression level and the overall survival time of gastric cancer patients (P = 0.009), with a correlation coefficient of -0.203. The effects of clinicopathologic characteristics, including age, gender, histologic type, clinical stage, T classification, N classification, and distant metastasis, in conjunction with SPHK1 protein expression, on patient survival, were evaluated with Kaplan-Meier analysis and the log-rank test. As shown in Fig. 4, the length of survival time was significantly different between patients with low and high SPHK1 expression (P = 0.0019), with the low SPHK1 group having a longer overall survival time. Furthermore, the cumulative 5-year survival rate was 49.66% (95% confidence interval, 0.2707-0.5225) in the low SPHK1 expression group, whereas it was only 23.85% (95% confidence interval, 0.1585-0.3185) in the high SPHK1 expression group (Fig. 4).

When univariate and multivariate analyses were done to determine whether SPHK1 expression level is an independent prognostic factor of patient outcomes, as Supplementary Table S4 shows, SPHK1 expression, as well as clinical stage and N classification, was recognized as independent prognostic factors. Furthermore, the prognostic value of SPHK1 expression in selective patient subgroups was evaluated according to the clinical staging. Despite that the difference in patient overall survival times between the low and the high SPHK1 expression groups was not different in the early clinical subgroups (stages I and II, n = 36; log-rank, P = 0.186; Fig. 5A), in the advanced disease group (stages III and IV), patients with tumors exhibiting high SPHK1 expression had significantly lowered overall survival rates compared with those with low level expression of SPHK1 (n = 131; log-rank, P = 0.004; Fig. 5B). Taken together, our data suggest that SPHK1 might represent a novel and potentially useful independent biomarker for the prognosis of patients with gastric cancer.

**Discussion**

The current study has, for the first time, revealed that SPHK1 is up-regulated in gastric cancer cell lines and clinical tumors at both mRNA and protein levels in comparison with those in normal gastric cells and gastric tissues. SPHK1 protein expression levels were found to significantly correlate with the prognosis of gastric cancer, as high level of SPHK1 protein expression in gastric cancer lesions is closely associated with advanced clinical staging, higher T and M classifications, and shorter survival times of the patients. Our study suggests that
overexpression of SPHK1 is a common feature in gastric cancer and might represent a novel predictive marker for the clinical outcome of the disease.

Lipid mediators such as sphingosine, ceramide, sphingosine 1-phosphate, and ceramide-1-phosphate are believed to play important roles in cell death, stress responses, and metabolism in multicellular eukaryotes (8–13, 24). Numerous recent reports have shown that cellular sphingolipid expression levels are highly associated with cancer and cancer therapy. However, the molecular mechanism underlying the biological significance of sphingolipids in cancer development and progression remains obscure. Sphingosine 1-phosphate is produced through sphingosine phosphorylation catalyzed by SPHK, which has been identified as an oncoenzyme. Indeed, SPHK1 overexpression has been found to correlate with malignant phenotypes in cancers (19–23). It has been reported that SPHK1 is overexpressed in human colorectal cancer and rodent colon cancer (23). Breast cancer patients whose tumors display low SPHK1 have lower metastasis risk (39). Our study has provided evidence that SPHK1 up-regulation might play an important role in the progression of gastric cancer. Up-regulation of SPHK1 in gastric cancer was identified by our study and confirmed by several lines of evidence, including assessment of SPHK1 mRNA and protein expression in gastric cancer cell lines in comparison with those in HGEC, comparative determination of SPHK1 expressions in paired gastric cancer tissues and noncancerous gastric tissues, and a clear demonstration of generally high level of SPHK1 expression in a relatively large number of gastric cancer lesions. The importance of SPHK1 up-regulation in gastric cancer is further highlighted by our finding of its correlation with the advanced staging of the disease and poorer patient prognosis. These results not only suggest a potentially promising usefulness of SPHK1 as a prognostic and survival indicator but also warrant further studies on a possible link between the biological function of SPHK1 and the pathogenesis of gastric cancer, which is already being conducted in our laboratory. In this respect, investigating in more detail the phenotypic changes in SPHK1-overexpressing and knockdown models will provide valuable mechanistic data toward a better understanding of the role of SPHK1 in the development and progression of gastric cancer, which might eventually lead to the development of a new anti-gastric cancer strategy.

It is of note that we also comparatively examined the expression SPHK1 in the superficial versus deeper layers within each individual case to investigate whether SPHK1 expression is also related to the tumor depth. Interestingly, no difference was found (data not shown). Whether this result suggests a lack of heterogeneity of SPHK1 expression in different layers of gastric wall of gastric is of interest for further study.

Further translational research on the clinical use of SPHK1 will be needed to improve the molecular diagnostic methodology.
for quantifying SPHK1 in clinical samples and to establish a practically applicable variable system and criteria for the evaluation of SPHK1 levels. In addition, it will be of great interest to investigate whether such an important marker is also detectable in other forms of patient samples, such as blood and gastric fluid, in addition to biopsy or surgical tissues. Nevertheless, our study has provided a basis for the development of a novel biomarker for the diagnosis and prognosis of gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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