Prognostic Value of Sonic Hedgehog Protein Expression in Gastric Cancer

Ju-Yeon Kim1, Gyung Hyuck Ko2, Young-Joon Lee1, Woo-Song Ha1, Sang-Kyung Choi1, Eun-Jung Jung1, Chi-Young Jeong1, Young-Tae Ju1, Sang-Ho Jeong1 and Soon-Chan Hong1,*

1Department of Surgery, Gyeongnam Regional Cancer Center and 2Department of Pathology, Gyeongsang National University, Jinju, Republic of Korea

*For reprints and all correspondence: Soon-Chan Hong, Department of Surgery, Gyeongsang National University, 79 Gangnam-ro, Jinju, Gyeongsang South Province 660-702, Republic of Korea. E-mail: hongsoonchangnu@gmail.com; Sang-Ho Jeong, Department of Surgery, Gyeongsang National University, 79 Gangnam-ro, Jinju, Gyeongsang South Province 660-702, Republic of Korea. E-mail: shjeong@gnu.ac.kr

Received June 1, 2012; accepted July 31, 2012

Objective: Sonic hedgehog is produced in gastric epithelial cells and plays a crucial role in parietal cell function and the regulation of gastric epithelial cell differentiation. Emerging evidence suggests that the sonic hedgehog pathway is not only involved in the development of cancers but also in their progression and aggressiveness.

Methods: To assess its prognostic value in gastric cancer, sonic hedgehog protein expression was measured by immunohistochemistry in a clinically annotated tissue microarray comprising 319 human gastric cancer specimens. Cytoplasmic sonic hedgehog expression was scored from 0 to 4, reflecting the percentage of sonic hedgehog-positive cells.

Results: Specimens were classified into two groups according to their sonic hedgehog score: those with a score ranging from 0 to 3 were considered low expressers and those with a score of 4 were considered overexpressers. The sonic hedgehog overexpression group included more patients with early gastric cancer than the low sonic hedgehog expression group (25.9 vs. 74.1%, \( P = 0.000 \)). Sonic hedgehog expression was lower in patients with lymph node metastasis than in patients without lymph node metastasis (31.4 vs. 68.4%, \( P = 0.02 \)). Similarly, patients with a lower TNM stage showed significantly higher sonic hedgehog expression. In addition, the survival time of patients with sonic hedgehog overexpression was significantly prolonged (69.27 ± 1.39 months) compared with that of patients with low sonic hedgehog expression (61.23 ± 2.04 months, log-rank test, \( P = 0.03 \)).

Conclusions: These results indicate that sonic hedgehog overexpression may be a marker of good prognosis in gastric cancer.

Key words: stomach neoplasm – hedgehog pathway – sonic hedgehog – immunohistochemistry – prognosis

INTRODUCTION

Despite technological advance in early diagnosis and treatment, gastric cancer remains the second leading cause of cancer-related deaths worldwide (1). The current efforts have focused on understanding gastric cancer protein and gene expression to develop novel targeted therapies.

Ramalho-Santos et al. (2) reported abnormal stomachs in newborn sonic hedgehog (Shh)-null mice, generating interest in understanding the role of Hedgehog (Hh) signaling in the luminal gastrointestinal tract. Although three Hh family members are founded in the mammalian gastric epithelium (Sonic, Indian, Desert), Shh is most frequently...
studied owing to the gastric phenotype observed in the Shh−/− mice.

In the stomach, Shh is produced in epithelial cells and exerts its effect on the mesenchyme. Emerging evidence indicated that Shh plays a crucial role in parietal cell function and in the regulation of gastric epithelial cell differentiation (3–7). Loss of Shh is associated with cytokine-induced inhibition of acid secretion, which is itself associated with impaired gastric function and subsequent parietal cell atrophy, both of which precede gastric carcinogenesis (8).

Although the association between Shh and gastric cancer development is well established, the mechanism underlying atrophy, both of which precede gastric carcinogenesis (8). impaired gastric function and subsequent parietal cell inhibition of acid secretion, which is itself associated with gastric adenocarcinomas (9–13), the present study was associated with poorly differentiated and aggressive types of microenvironment and the biological significance of Shh pathway in gastric cancer remain largely unknown.

Given recent evidence that Hh pathway activation is associated with poorly differentiated and aggressive types of gastric adenocarcinomas (9–13), the present study was designed to assess the relationship between Shh expression and clinicopathological features in a human gastric cancer tissue microarray (TMA). Results show that Shh overexpression is associated with early-stage and node-negative gastric cancer and with longer survival than low Shh expression.

PATIENTS AND METHODS

CLINICAL DATA COLLECTION

Surgically resected gastric cancer tissue specimens were obtained from 319 patients who underwent gastrectomy at the Gyeongsang National University Hospital between 1 October 2004 and 31 December 2007. Medical charts and pathological reports were reviewed to assess clinicopathological parameters such as age, gender, histological subtype, presence of lymphatic invasion, invasion depth, presence of lymph node (LN) or distant metastasis and pathological TNM stages.

In cases of death, the cause of death was determined by consulting the National Statistical Office of the Republic of Korea. Ninety-eight percent of the patients had undergone a curative resection (R0), performed according to the guidelines of the International Union Against Cancer. Clinical outcome was evaluated from the date of surgery to the time of death, or until 31 January 2011. Cases lost to follow-up and non-gastric cancer-related deaths were regarded as censored data in the survival analysis. The study was approved by the Institutional Review Board of Gyeongsang National University Hospital.

TMA ANALYSIS

Core tissue biopsy specimens (diameter: 2 mm) were obtained from individual formalin-fixed, paraffin-embedded archived tissues (donor blocks). The specimens were arrayed into new recipient paraffin blocks using a trephine apparatus (Quick-RAYTM, Unitma Co., Seoul, Korea). We obtained the core tissues from the area near the invasive front. The subtypes of these tissues indicated the histolopathological subtype of the entire tumor. Because we obtained and analyzed the core tissues, there was less diversity in the subtypes. We believed that it is important to analyze the invasive front of a tumor, which reflects the tumor prognosis. TMA blocks were constructed for all 319 patients.

IMMUNOHISTOCHEMISTRY

Specimens fixed in formalin and embedded in paraffin were cut into 4 μm sections, dewaxed, dehydrated and mounted on glass slides. The slides were then incubated in 3% H₂O₂ for 10 min to reduce non-specific background staining due to the presence of endogenous peroxidase. For epitope retrieval, the specimens were heated for 20 min in 10 mmol/l citrate buffer (pH 6.0) in a microwave oven (700 W). Background staining was blocked by incubation in Ultra V Block solution (Lab Vision Corporation, Fremont, CA, USA) for 7 min at room temperature. The slides were then incubated for 1 h at room temperature with a rabbit polyclonal antibody specific for Shh (1:100; Epitomics, Inc., CA, USA). Antibody binding was detected using the Ultra Vision LP detection system (Lab Vision Corporation) in accordance with manufacturer’s recommendations. Color was developed with 3,3′-diaminobenzidine and the slides were counterstained with hematoxylin. The expression of Shh was scored by a pathologist blinded to the clinicopathological data. Shh expression was cytoplasmic. We have defined immunohistochemistry-positive cells as those that usually showed an intensity different from the background intensity. Scores were based on the percentage of Shh-positive cells as follows: 0 (0–4%), 1+ (5–24%), 2+ (25–49%), 3+ (50–74%), and 4+ (75–100%) (Fig. 1).

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS software (version 15.0 for windows, SPSS Inc., Chicago, IL, USA). A χ² test and Student’s t-test were used to analyze the relationship between Shh scores and clinical and pathological features. Survival was analyzed using the Kaplan–Meier method and log-rank test. A P value of <0.05 was considered significant.

RESULTS

Among the 319 patients included in the study, 166 (52.0%) had early gastric cancer and 210 (65.9%) did not show any LN metastasis. Shh expression was cytoplasmic. Four patients (1.2%) had a score of 0, 28 (8.7%) had a score of 1, 37 (11.5%) had a score of 2, 46 (14.4%) had a score of 3 and 204 (63.9%) had a score of 4 (Table 1).

Specimens were classified according to their Shh score: those with a score ranging from 0 to 3 were considered low
expressers and those with a score of 4 were considered over-expressers. According to the WHO classification, the low expression group included a greater number of poorly differentiated carcinomas (38.9 vs. 25.9%) and signet ring cell carcinomas (14.2 vs. 8.0%) than the overexpression group \((P = 0.003)\). The Shh overexpression group included more patients with early gastric cancer than the low Shh expression group \((25.9 \text{ vs. } 74.1\%, \ P = 0.000)\). The two groups had a similar mean tumor size; yet, early gastric cancer was more frequent in the overexpression group \((37.4 \text{ vs. } 60.3\%, \ P = 0.000)\). Similarly, the incidence of metastasis to LNs was statistically higher in the low expression group \((42.6 \text{ vs. } 29.4\%, \ P = 0.02)\). As a result, the overexpression group showed a lower overall TNM stage (Table 2).

Figure 2 shows the Kaplan–Meier survival curves based on Shh expression. The overall survival of patients in the Shh overexpression group was significantly longer \((69.27 \pm 1.39 \text{ months})\) than that of patients in the low Shh expression group \((61.23 \pm 2.04 \text{ months}, \text{log-rank test, } P = 0.03)\).

**DISCUSSION**

The aim of the study was to investigate whether Shh expression may have a prognostic value in gastric cancer. The results show that the Shh overexpression group had a higher incidence of early-stage and node-negative gastric cancer, and longer survival than the low Shh expression group, suggesting that Shh overexpression is associated with a good prognosis.

The Hh pathway plays a critical role in the growth, patterning and morphogenesis of multiple organs, including the gastrointestinal tract. In mammalian cells, three Hh ligands have been identified, namely Shh, Indian Hh (Ihh) and desert Hh (Dhh) \((14)\). Shh is a peptide morphogen produced by gastric epithelial cells, such as surface pit, parietal, mucous neck and zymogenic cells \((15–18)\). Stromal cells express Shh to some extent, but unlike the gastric epithelium, they also robustly express signal transduction components, including the 12-transmembrane receptors Patched-1 (Ptch-1) and

![Image of Shh expression in gastric carcinoma tissue](image_url)
Smoothened (Smo), the 7-transmembrane receptor and the Gli transcription factor family (19). In the absence of Shh, Ptch-1 inhibits the activity of another transmembrane protein, Smo, resulting in inactivation of Hh signaling. Binding of Shh to Ptch-1 abrogates the inhibitory effect of Ptch-1, thereby activating Smo and subsequently leading to the transcriptional activation of Gli1 and Gli2, and decreasing the expression of the repressor, Gli3. Therefore, Hh signaling increases the expression of activator Gli and decreases the expression of repressor Gli (20).

Recently, the Shh pathway was found to be involved not only in the development of multiple cancers but also in their progression and aggressiveness. Furthermore, most studies using clinical data have suggested that Shh expression in tumors is correlated with tumor aggressiveness (21–25). Although studies on gastric cancer are limited, similar results have been obtained (9–13). Yanai et al. (11) assessed Gli nuclear staining in 58 gastric cancer tissue specimens and found that Gli nuclear staining was higher in patients with advanced gastric cancer than in patients with early gastric cancer (T1: T2–4 = 27.1 ± 4.4%; 41.8 ± 5.2%, P = 0.02). Furthermore, the percentage of cells with Gli nuclear staining positively correlated with LN metastasis and lymphatic invasion. In addition, Yoo et al. (13) showed that patients with LN metastasis or TNM stage II–IV had higher expression of Shh than patients without LN metastasis or TNM stage I and concluded that Shh overexpression correlated with poor prognosis in patients with gastric cancer.

In contrast, the present study shows that Shh overexpression is predictive of good prognosis. One plausible explanation for this discrepancy may be epithelial-to-mesenchymal transition (EMT). EMT, the process by which cells lose epithelial characteristics and acquire a migratory mesenchymal phenotype is a key step in cancer metastasis (26, 27). Recently, a study of pancreatic cancer cells identified Gli1 as an important positive regulator of epithelial differentiation and offered an explanation for how decreased levels of Gli1 may contribute to a highly metastatic phenotype: although this was not established in gastric cancer, low Shh expression decreased Gli1, which may be associated with the induction of EMT and poor clinical outcome (28).

Several points should be considered when explaining the discrepancy between our results and previously published results. The first point is that many studies of the effects of Shh on cancer aggressiveness were performed in cancer cell lines (9–13); in contrast, we used gastric cancer surgical specimens. The second point is that definitions of Shh overexpression and of clinical and pathological features vary between studies. For example, Yoo et al. (13) analyzed clinical data from 178 patients and classified patients based on

<table>
<thead>
<tr>
<th>Pathological variables</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO classification</td>
<td>WD/MD/PD/Mucinous/SRC 69/109/96/8/32</td>
</tr>
<tr>
<td>Lauren classification</td>
<td>Intestinal/diffuse/mixed 176/59/7</td>
</tr>
<tr>
<td>Tumor size and T stages</td>
<td>Mean tumor size 4.2 ± 2.7 T1/T2/T3/T4 166/38/87/28</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>Mean involved LN 2.3 ± 5.3 NO/N1/N2/N3 210/33/34/42</td>
</tr>
<tr>
<td>TNM stages</td>
<td>Stage I/Stage II/Stage III 188/57/74</td>
</tr>
<tr>
<td>Shh expression status</td>
<td>0/1/2/3/4 4/28/37/46/204</td>
</tr>
</tbody>
</table>

| WHO, World Health Organization; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; Shh, sonic hedgehog protein; SRC, signet ring cell carcinoma; LN, lymph node.

Table 1. Clinicopathological data of the patients in the tissue microarray experiment

<table>
<thead>
<tr>
<th>Levels of Shh expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underexpression (0–3+)</td>
<td>Overexpression (4+)</td>
</tr>
<tr>
<td>WHO classification</td>
<td></td>
</tr>
<tr>
<td>W/D</td>
<td>16 (14.2%)</td>
</tr>
<tr>
<td>M/D</td>
<td>37 (32.7%)</td>
</tr>
<tr>
<td>P/D</td>
<td>44 (38.9%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>SRC</td>
<td>16 (14.2%)</td>
</tr>
<tr>
<td>Lauren classification</td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>53 (63.9%)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>26 (31.3%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>4 (4.8%)</td>
</tr>
<tr>
<td>Mean tumor size</td>
<td>4.45 ± 2.45</td>
</tr>
<tr>
<td>Mean involved LN</td>
<td>3.04 ± 6.47</td>
</tr>
<tr>
<td>Tumor invasion</td>
<td></td>
</tr>
<tr>
<td>EGC(T1)</td>
<td>43 (37.4%)</td>
</tr>
<tr>
<td>AGC(T2,3,4)</td>
<td>72 (62.6%)</td>
</tr>
<tr>
<td>LN metastasis</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>66 (57.4%)</td>
</tr>
<tr>
<td>Present (&gt;1)</td>
<td>49 (42.6%)</td>
</tr>
<tr>
<td>pTNM stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>55 (47.8%)</td>
</tr>
<tr>
<td>II</td>
<td>25 (21.7%)</td>
</tr>
<tr>
<td>III–IV</td>
<td>35 (30.4%)</td>
</tr>
</tbody>
</table>

EGC, early gastric cancer; AGC, advanced gastric cancer.

Table 2. Comparison of the clinicopathological features in the Shh underexpression and overexpression groups
Shh expression in a positive group and a negative group; however, the criterion for classification into the two groups was not clearly defined, precluding a direct comparison with our results. Furthermore, Yoo et al. showed that 17.4% of the patients in their study had Stage I disease; however, the exclusion of patients with TNM Stage I from the survival analysis, which relied on the Kaplan–Meier survival estimates to assess the difference between the Shh-negative and -positive groups, might have artificially inflated the survival rate of the Shh-negative group. In contrast, in our study, 58.9% (188) of the patients had Stage I disease; among them, 70.7% (133) showed Shh overexpression, defined by a score of 4.

In this study, Shh scores were assorted based on the percentage of Shh-positive cells from 0 to 4+, which were then divided into underexpression and overexpression groups. We used a variety of categorizations of Shh scores to find out the cut-off value to reflect the clinicopathological features of gastric cancer. As a result, a score of 4+ was identified as a surrogate marker for a good prognosis.

Our study may be the first to suggest that Shh signaling may predict a good gastric cancer prognosis. Since our study relied on clinical data and TMAs, the exact mechanism underlying this association cannot be explained. Another limitation is that the fact that it was a retrospective study with patients from a single institution having also an observational bias because this estimation was conducted by only one pathologist. Further studies are necessary to elucidate this mechanism, requiring clinical and survival data from a larger number of gastric cancer patients.

Funding
This study was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family affairs, Republic of Korea (0820050).

Conflict of interest statement
None declared.

References

Figure 2. The Kaplan–Meier survival analysis was performed to compare the outcomes of patients overexpression or under-expressing Shh. The Shh overexpression group (69.27 ± 1.39 months) showed longer survival times than the underexpression group (61.23 ± 2.04 months). The difference between the two groups was significant (log-rank test, \( P = 0.03 \)).