Frequent activation of the hedgehog pathway in advanced gastric adenocarcinomas

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Introduction

The hedgehog pathway plays a critical role in the development of the foregut. Recent studies indicate that the hedgehog pathway activation occurs in the stomach and other gastrointestinal cancers. However, the association of hedgehog pathway activation with tumor stage, differentiation and tumor subtype is not well documented. Here, we report our findings that the elevated expression of hedgehog target genes human patched gene 1 (PTCH1) or Gli1 occurs in 63 of the 99 primary gastric cancers. Activation of the hedgehog pathway is associated with poorly differentiated and more aggressive tumors. The sonic hedgehog (Shh) transcript is localized to the cancer tissue, whereas expression of Gli1 and PTCH1 is observed both in the cancer and in the surrounding stroma. Treatment of gastric cancer cells with KAAD-cyclopamine, a hedgehog signaling inhibitor, decreases expression of Gli1 and PTCH1, resulting in cell growth inhibition and apoptosis. Overexpression of Gli1 under the control of the cytomegalovirus (CMV) promoter renders these cells resistant to cyclopamine-induced apoptosis. Thus, our analysis of in vivo tissues indicates that the hedgehog pathway is frequently activated in advanced gastric adenocarcinomas; our in vitro studies suggest that hedgehog signaling contributes to gastric cancer cell growth. These data predict that targeted inhibition of the hedgehog pathway may be effective in the prevention and treatment of advanced gastric adenocarcinomas.

Materials and methods

Tumor samples
A total of 117 specimens of gastric tissues were used in our study (99 cancer specimens and 18 normal or inflamed gastric tissues). From the Surgery Department at the Shan Dong Qi Lu Hospital, Jinan, China, or from the UTMB Surgical Pathology, 54 specimens were received as discarded materials with the approval from the Institutional Review Board (IRB). In addition, we purchased a tissue microarray, which contains 63 specimens of gastric cancer, from Chaoying Biotechnology Co., Ltd (Xi’an, China). Pathological reports, H and E staining of each specimen were reviewed to determine the nature of the disease and the tumor histology. In addition, immunohistochemistry with keratin antibodies was used to confirm the tumor pathology. The randomly sorted samples with masked identity were evaluated by at least two independent certified pathologists. Gastric cancers were divided into three major subtypes according to the WHO guideline (16): tubular adenocarcinoma, papillary adenocarcinoma and signet-ring cell carcinomas. This study also includes specimens for normal stomach tissues (n = 11), gastritis (n = 4) and other stomach tissues (n = 3) (see Supplementary Table 1A for more information).

In situ hybridization
Gli1 (X07384) was cloned into pBluescript M13+KS, the direction of insert is HindIII-5’ and XbaI-3’. The plasmid was digested with NotI to generate the of hedgehog signaling, through loss-of-function mutations of PTCH1 or activated mutations of SMO, occurs frequently in human basal cell carcinomas (BCCs) and medulloblastomas (3–7). More recently, abnormal activation of the sonic hedgehog pathway has been reported in subsets of small cell lung cancer, pancreatic cancer, prostate cancer and gastrointestinal (GI) cancers (8–14).

Gastric cancer is the second most common cancer worldwide in terms of incidence and mortality (15). Patients with gastric cancer usually present at late stages and have a poor prognosis. Thus, identification of specific drug targets in the tumor is an essential step to reduce the mortality. A previous study indicated that activation of the hedgehog pathway occurred in all nine primary gastric cancers (10). To determine if hedgehog signaling activation can be utilized for the diagnosis and treatment of gastric cancer, we performed a comprehensive study to assess the hedgehog pathway activation in 99 primary gastric cancers using in situ hybridization, real-time PCR and immunohistochemistry.

The hedgehog pathway may be effective in the prevention and treatment of advanced gastric adenocarcinomas.
sense fragment (412 bp) and with Ndel to generate the antisense fragment (682 bp). PTCH1 (U59464) (cloned into XbaI3 and ClaI3 of PRK3) was digested with DraIII to generate a small cDNA fragment (590 bp). Gli3 (M57609) was initially cloned into Srf (5') and Srf (3') of pBluescript-SK. SnaBI was used to generate the sense fragment (552 bp) and PshAI to generate the antisense fragment (770 bp). Sense and antisense probes were obtained by T3 and T7 in vitro transcription using a DIG RNA labeling kit from Roche (Mannheim, Germany). Tissue sections (6 μm thick) were mounted onto poly-l-lysine slides (19). Following deparaffinization, the tissue sections were rehydrated in a series of dilutions of ethanol. To enhance the signal and facilitate probe penetration, sections were immersed in 0.3% Triton X-100 solution for 15 min at room temperature and in proteinase K (20 μg/ml) solution for 20 min at 37°C, respectively. The sections were then incubated with 4% (v/v) paraformaldehyde/phosphate-buffered saline (PBS) for 5 min at 4°C. After washing with PBS and 0.1 M triethanolamine, the slides were incubated with prehybridization solution (50% formamide, 50% 4× SSC) for 2 h at 37°C. The probe was added to each tissue section at a concentration of 1 μg/ml and hybridized overnight at 42°C. After high stringency washing (2× SSC twice, 1× standard saline citrate twice and 0.5× SSC twice at 37°C) the sections were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody, which catalyzed a color reaction with the NBT/BCIP (Nitro-Blue-Tetrazoium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Roche, Mannheim, Germany). Blue indicated strong hybridization. As negative controls, sense probes were used in all hybridizations and no positive signal was observed.

RNA isolation and quantitative PCR
Total RNA of the cells was extracted using an RNA extraction kit from Promega (Madison, WI). For this analysis, we selected only tumors in which 70% of the tissue mass was actually tumor tissue. For quantitative PCR analyses, we detected transcripts of sonic hedgehog, Gli1 and PTCH1 using the Applied Biosystems' assays-by-demand assay mixtures (the sequences for human Gli1, HIP and PTCH1 have been patented by Applied Biosystems, Foster City, CA) and assays-by-demand assay mixtures (the sequences for human Gli1, HIP and PTCH1 using the Applied Biosystems' PD 40 32/40 (80%) 34/40 (85%) 11/11 (100%) assays). The primers were designed to span exon-exon junctions so as not to detect genomic DNA, and the primers and probe sequences were searched against the Celera database to confirm specificity. To obtain the relative quantitation of gene expression, a validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount versus ΔCt were <0.1. Separate tubes (single-plex) one-step RT-PCR was performed with 20 ng RNA for both target genes and the endogenous control using TaqMan one-step RT–PCR master mix reagent (P/N 4319413E) as an internal control. The primers were designed to span exon-exon junctions so as not to detect genomic DNA, and the primers and probe sequences were searched against the Celera database to confirm specificity. To obtain the relative quantitation of gene expression, a validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount versus ΔCt were <0.1. Separate tubes (single-plex) one-step RT–PCR were reverse transcription at 48°C for 30 min, AmpliTaq activation at 95°C for 10 min, denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min (repeat 40 times) on AB17000. Triplicate Ct values were analyzed in Microsoft Excel using the comparative Ct (ΔΔCt) method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target (2^−ΔΔCt) was obtained by normalization to an endogenous reference (18S RNA) and relative to a calibrator.

Immunohistochemistry
Representative formalin fixed and paraffin embedded tissue sections (6 μm thickness) were used for immunohistochemistry with specific antibodies to human Shh and PTCH1 (Cat. no. 9024 for Shh and Cat. no. 6149 for PTCH1, Santa Cruz Biotechnology Inc., Santa Cruz, CA). First, tissue sections were deparaffinized, followed by rehydration with decreased concentrations of ethanol, and immersed in 3% H2O2 (in distilled H2O) for 10 min to inhibit endogenous peroxidase activity. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with normal goat serum to block nonspecific antibody binding (20 min at room temperature). The sections were then incubated with primary antibodies (at 1:200 dilution) at 37°C in humid chambers for 2 h. After washing with PBS three times, the sections were incubated with the biotinylated secondary antibody (goat anti-rabbit IgG or monkey anti-goat IgG) and streptavidin conjugated to horseradish peroxidase for 20 min at 37°C, followed by PBS wash. The sections were incubated with the DAB substrate for less than 30 min. Haematoxylin was used for counterstaining. Negative controls were performed in all cases by omitting the first antibodies. All primary antibodies have been previously tested for immunohistostaining (9,11,18).

Cell culture, MTT assay and TUNEL assay
Cell lines (AGS, SIIA and RKO) were cultured in F12 Medium with 10% FBS (AGS and SIIA) or DMEM with 10% FBS (RKO), respectively. Colorimetric MTT assay was performed according to our published protocol in the presence of 0.5% FBS (19). Cells were treated with 2 μM of Keto-β-aminophenylacrypoxydihydronorcanonamolv-cyclophosphamide (KAAD-cyclophosphamide) for specific times (see Figure 6 for details). Etopic expression of Gli1, under the control of the cytomegalovirus (CMV) promoter, in AGS cells was achieved by the transient transfection with LipofectAmine 2000 (20,21), and Gli1 was detected by immunofluorescent staining with the Myc tag antibody 9e10 (Sigma, St Louis, MO) (19). TUNEL assay was performed using a kit from Roche according to the manufacturer’s instructions (19,21).

Statistical analysis
Student’s t-test for two samples was performed for the difference between the tumor groups: P < 0.05 was considered statistically significant. For example (Table 1), the difference between Stage I and Stage III tumors for expression of Shh and PTCH1 was statistically significant with a P value of 0.01923. Similarly, the difference between well-differentiated (WD) and poorly differentiated (PD) tumors for expression of Shh and PTCH1 was significantly

Table 1. Summary of gastric specimens: clinical pathological data and the hedgehog pathway activation

<table>
<thead>
<tr>
<th>Tumor number</th>
<th>Expression of Shh</th>
<th>Expression of PTCH1</th>
<th>Expression of Gli1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>99</td>
<td>64/99 (~65%)</td>
<td>63/99 (~64%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>77</td>
<td>50/77 (~65%)</td>
<td>47/77 (~61%)</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>14/22 (~64%)</td>
<td>16/22 (~73%)</td>
</tr>
<tr>
<td>Stage*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>22</td>
<td>12/22 (~55%)</td>
<td>11/22 (50%)</td>
</tr>
<tr>
<td>II</td>
<td>22</td>
<td>17/22 (~77%)</td>
<td>15/22 (~68%)</td>
</tr>
<tr>
<td>III</td>
<td>41</td>
<td>30/41 (~73%)</td>
<td>32/41 (~78%)</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>WHO classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>90</td>
<td>64/90 (~71%)</td>
<td>63/90 (70%)</td>
</tr>
<tr>
<td>Tubular</td>
<td>43</td>
<td>27/43 (~63%)</td>
<td>25/43 (~58%)</td>
</tr>
<tr>
<td>Papillary</td>
<td>7</td>
<td>5/7</td>
<td>4/7</td>
</tr>
<tr>
<td>PD</td>
<td>40</td>
<td>32/40 (80%)</td>
<td>34/40 (85%)</td>
</tr>
<tr>
<td>Signet-ring cell carcinomas</td>
<td>9</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Differentiation*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated (WD)</td>
<td>18</td>
<td>7/18 (~39%)</td>
<td>7/18 (~39%)</td>
</tr>
<tr>
<td>Moderately differentiated (MD)</td>
<td>25</td>
<td>20/25 (80%)</td>
<td>18/25 (72%)</td>
</tr>
<tr>
<td>Poorly differentiated (PD)</td>
<td>40</td>
<td>32/40 (80%)</td>
<td>34/40 (85%)</td>
</tr>
</tbody>
</table>

*Statistical analysis was performed using student’s t-test for two samples: P < 0.05 was considered statistically significant (see Materials and methods for details).
*Signet-ring cell carcinomas were excluded from this analysis.
*Only tubular adenocarcinomas were used in this analysis.

Hedgehog pathway activation in gastric adenocarcinomas
Thus, activation of hedgehog signaling appears to be inversely associated with tumor differentiation. As poor differentiation of the tumor is often associated with prognosis, our findings suggest that activation of the hedgehog pathway may serve as a valuable prognostic biomarker for gastric cancer. This hypothesis predicts that activation of the hedgehog pathway may be more common in advanced stages of gastric cancer. Indeed, 11 out of the 22 Stage I gastric adenocarcinomas (50%) had elevated levels of the target gene transcripts, 78% of Stage III tumors had detectable expression of the target genes (Table 1).

To confirm the in situ hybridization data, we performed real-time PCR analyses in selected tumors, in which >70% of the tissue mass was actually tumor tissue, to detect the levels of Gli1 and PTCH1 expression. In the tumors with elevated Gli1 and PTCH1 transcripts, we found an approximate 5- to 20-fold increase in levels of Gli1 and PTCH1 expression compared with the matched normal tissues (Figure 2), indicating that the in situ hybridization results are in agreement with real-time PCR data. Furthermore, we detected the PTCH1 protein by immunohistochemistry (11). We found that PTCH1 protein was detected in primary gastric cancer, but not in the normal tissues (Figure 3). The data from the immunohistochemical analysis are consistent with the results from real-time PCR and in situ hybridization analyses. Taken together, these data indicate that the hedgehog pathway is frequently activated in advanced gastric adenocarcinomas.

**Expression of sonic hedgehog in gastric cancer**

It is reported that overexpression of sonic hedgehog may be responsible for the activation of the hedgehog pathway in pancreatic cancer, subsets of small cell lung cancer, prostate cancer and several primary gastric cancers (8–14). To test this possibility, we examined expression of sonic hedgehog in gastric specimens by in situ hybridization. Many cancers had a high level of sonic hedgehog transcript (Figure 4, Supplementary Figure C and Supplementary Table 1A). In most of the cases, elevated levels of Shh were consistent with elevated levels of PTCH1 and Gli1 expression (see Supplementary Table 1A and B). We found that elevated sonic hedgehog expression is associated with poor differentiation of the tumor and higher tumor grades (Table 1). Also in agreement with lack of PTCH1 and Gli1 expression in signet-ring cell carcinomas, there was no detectable expression of sonic hedgehog in any of the signet-ring cell carcinomas analyzed (Table 1). In contrast, sonic hedgehog expression was undetectable or very low in normal and inflamed gastric tissues (Figure 4). To confirm the in situ hybridization data, we detected the sonic hedgehog protein by immunohistochemistry (9,11). In agreement with the in situ hybridization data, we found that tumors with increased sonic hedgehog mRNA expression had high levels of the sonic hedgehog protein (Figure 5). The correlation of sonic hedgehog expression with Gli1 and PTCH1 transcripts indicates an important role of sonic hedgehog in the activation of the hedgehog pathway in gastric adenocarcinomas.

**Hedgehog signaling and cellular functions of gastric cancers**

If hedgehog pathway activation is required for gastric cancer development, gastric cancer cells should be susceptible to the treatment using the SMO antagonist, cyclopamine. All available gastric cancer cell lines have elevated hedgehog signaling (10), we chose a GI cancer cell line RKO as the negative control [RKO cells do not have elevated levels of Gli1 and
PTCH1, unpublished data and (10). In this experiment, we used two gastric cancer cell lines (AGS and SIIA) to test KAAD-cyclopamine sensitivity. The addition of KAAD-cyclopamine (2 μM) greatly decreased the levels of Gli1 and PTCH1 mRNA expression in both the cell lines (Figure 6A shows the data for Gli1 expression in SIIA and RKO cells), indicating inhibition of the hedgehog pathway by KAAD-cyclopamine. The closely related compound tomatidine, 

Fig. 1. Detection of Gli1 and PTCH1 expression in primary gastric cancers by in situ hybridization. In situ hybridization detection of PTCH1 expression (denoted by arrows) in gastric cancers (A and B) and normal gastric tissue (C). D is the sense probe control of B. Expression of Gli1 was similar to PTCH1 in gastric cancer (E and F) and normal gastric tissue (G) (denoted by arrows). H is the sense probe control of F. The pattern of Gli1 and PTCH1 expression is very similar in the same tumor (comparing A and E), further affirming that the hedgehog pathway is activated in the tumor. See online Supplementary material for a color version of this figure.
which does not affect SMO signaling and thus served as a negative control, had little discernible effect on these target genes. As expected, we found that cell growth of SIIA and AGS (Figure 6B) cells was inhibited by KAAD-cyclopamine (2 μM). In addition, we detected apoptosis in AGS cells following the treatment with cyclopamine (Figure 6C). Tomatidine did not induce apoptosis in AGS cells (Figure 6C). Similar data were also observed in SIIA cells (data not shown). In contrast, RKO cells, which do not have active hedgehog signaling, were not affected by KAAD-cyclopamine treatment (Figure 6B), with no detectable apoptosis (Figure 6C). These data indicate that inhibition of the hedgehog pathway by KAAD-cyclopamine dramatically decreases cell growth, and induces apoptosis in gastric cancer cells.

Our model predicts that overexpression of Gli1 in AGS cells under the control of a strong promoter (such as the CMV promoter) would constitutively activate the hedgehog pathway, which could render these cancer cells resistant to cyclopamine treatment. Indeed, cyclopamine did not induce apoptosis in constitutive Gli1-expressing AGS cells, as indicated by lack of TUNEL staining in all Gli1 positive cells (n = 500) (Figure 6D). Thus, ectopic expression of Gli1 under the control of the CMV promoter prevents KAAD-cyclopamine-induced apoptosis in gastric cancer cells.

Taken together, our findings indicate that activation of the hedgehog pathway is quite common in advanced gastric adenocarcinomas, and this activation differs among different subtypes of gastric cancer. Elevated expression of Gli1 and PTCH1 is associated with decreased tumor differentiation and more advanced tumor stages in tubular adenocarcinomas. Inhibition of the hedgehog pathway in gastric cancer cell lines, however, decreases cell growth and induces apoptosis. Thus, our data indicate that activation of the hedgehog pathway may be an important event in the progression of gastric adenocarcinomas.

Discussion

Activation of the hedgehog pathway in gastric cancer

Hedgehog signaling pathway regulates cell proliferation, tissue polarity and cell differentiation during normal development. Abnormal signaling of this pathway has been reported in a variety of human cancers, including BCCs, medulloblastomas, small cell lung cancer, pancreatic cancer, prostate cancer and

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**Fig. 2.** Real-time PCR analysis of Shh, Gli1 and PTCH1 transcripts in primary gastric cancers. Total RNAs were extracted from the primary tumors in which 70% of the tissue mass was actually tumor tissue. The levels of Shh, Gli1 and PTCH1 were measured in our Real-time PCR Core Facility (see Materials and methods for details), and the experiment was repeated twice with similar results. The relative amount of target was obtained by normalization to an endogenous reference (18S RNA) and relative to a calibrator. T59 stands for tumor from patient no. 59 and N59 stands for the matched normal control from patient no. 59. The data from this analysis are consistent with those from in situ hybridization.

**Fig. 3.** Detection of PTCH1 protein in gastric tissues. Ptch1 protein was detected by immunohistostaining in gastric cancer (A) and normal tissue (B). See online Supplementary material for a color version of this figure.
several primary gastric tumors (3–14). Our findings in this report indicate that activation of the hedgehog pathway occurs frequently in advanced gastric adenocarcinomas. We have detected high levels of hedgehog targets PTCH1 and Gli1 in > 60% of gastric cancers. Further analyses of our data indicate an association of the hedgehog pathway activation with poor differentiation of gastric tumors. In a previous report, hedgehog signaling activation was detected in all nine primary

Fig. 4. Expression of Shh in primary gastric cancers. *In situ* hybridization was performed to detect Shh expression in gastric cancers (A and B) and normal gastric tissue (C). (D) The sense probe control for the sample shown in (B) and did not reveal any positive signals. See online Supplementary material for a color version of this figure.

Fig. 5. Protein expression of Shh in primary gastric cancers. *In situ* hybridization was confirmed by immunohistostaining in gastric cancers (A–C) and normal gastric tissue (D). See online Supplementary material for a color version of this figure.
tumors using real-time PCR analysis (10). This discrepancy may be owing to specimen selection. As we have indicated in our study, hedgehog signaling activation is associated with poorly differentiated tumors (tubular adenocarcinoma subtype). Another factor may be the sample size. Since signet-ring carcinomas are not a common subtype of gastric cancer, a large number of tumor specimens may be necessary to verify the results in our study.

In all the tumors with elevated levels of Gli1 and PTCH1 we detected expression of Shh, thus suggesting that overexpression of Shh may be responsible for elevated expression of Gli1 and PTCH1 in gastric cancer. However, a high level of sonic hedgehog expression was not always accompanied by elevated Gli1 and PTCH1 expression in tumors (Table I and Supplementary Table 1A), suggesting additional regulatory mechanisms for the hedgehog pathway activation. We speculate that, in addition to the hedgehog overexpression, other genetic alterations are required to activate the hedgehog pathway in gastric cancers.

Therapeutic perspective of gastric cancer through targeted inhibition of the hedgehog pathway

Our studies using gastric cancer cells indicate that the SMO antagonist, cyclopamine, may be effective in the future treatment of gastric cancers. We further demonstrate that overexpression of Gli1 under the control of the CMV promoter prevents cyclopamine-mediated apoptosis, further supporting the specificity of cyclopamine. Our recent studies indicate that chronic oral administration of cyclopamine to Ptch1+/− mice did not affect the overall survival of the mice (21), which
provides a possibility of clinical trials using cyclopamine for gastric cancers. Thus, in the future, it may be possible to treat the subsets of gastric cancer in which the hedgehog pathway is activated with a specific SMO antagonist (e.g. cyclopamine).

Supplementary material

Supplementary material can be found at: http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References