p53-Independent Negative Regulation of p21/Cyclin-Dependent Kinase–Interacting Protein 1 by the Sonic Hedgehog-Glioma-Associated Oncogene 1 Pathway in Gastric Carcinoma Cells

Miki Ohta, Keisuke Tateishi, Fumihiko Kanai, Hirotsugu Watabe, Shintaro Kondo, Bayasi Guleng, Yasuo Tanaka, Yoshinori Asaoka, Amarsanaa Jazag, Jun Imamura, Hideaki Iijichi, Tsuneo Ikenoue, Masataka Sata, Makoto Miyagishi, Kazunari Taira, Minoru Tada, Takao Kawabe, and Masao Omata

Departments of 1Gastroenterology and 2Cardiovascular Medicine, Graduate School of Medicine and 3Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo; 4Department of Endoscopy and Endoscopic Surgery and Clinical Research Center, University of Tokyo Hospital, Tokyo, Japan; and 5Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

Abstract

The activation of Hedgehog (Hh) signaling has been implicated in the growth of various tumor types, including gastric carcinoma. However, the precise mechanisms of Hh activation and suppression of tumor growth by the blockade of Hh signaling in gastric carcinoma cells remain unknown. The aim of this study was to elucidate the mechanism of abnormal Hh signaling and the key molecules contributing to dysregulated growth of gastric carcinoma. The Sonic hedgehog (Shh) ligand and its receptor Patched were expressed in all five gastric carcinoma cell lines examined (MKN1, MKN7, MKN45, MKN74, and AGS cells). The blockade of Hh signaling with anti-Shh antibody inhibited the growth of all five gastric carcinoma cell lines. Shh was overexpressed (mean, 12.8-fold) in 8 of 14 (57.0%) cancerous tissue samples from patients with gastric carcinoma as compared with expression in the surrounding noncancerous tissues. The disruption of glioma-associated oncogene 1 (Gli1) by small interfering RNA induced an interferon RNA induced an increase in p21/cyclin-dependent kinase–interacting protein 1 (CIP1), interfered with the G1-S transition, and suppressed cell proliferation. The stimulation or inhibition of Hh signaling did not affect p53 activity and the induction of p21/CIP1 expression and the G1 arrest by inhibition of Hh signaling were not affected by the p53 status. These findings suggest that the overexpression of Shh contributes to constitutive Hh activation and that this signaling pathway negatively regulates p21/CIP1 through a Gli1-dependent and p53-independent mechanism in gastric carcinoma cells.

Introduction

The Hedgehog (Hh) signaling pathway has indispensable roles in organized cell growth and differentiation in a variety of embryonic tissues, including limbs, the nervous system, and the digestive tract (1–5). Recent findings have implicated Hh signaling in the growth of various tumors (8, 9), such as basal cell carcinoma (10–12), medulloblastoma (13, 14), small-cell lung cancer (15), digestive tract tumors (16, 17), prostate carcinoma (18, 19), and breast cancer (20).

Hh signaling is transduced by a seven-transmembrane-spanning protein, Smo (10, 22) cause abnormal activation of Hh signaling in a ligand-independent manner in basal cell carcinoma and brain tumors. By contrast, Hh ligand–dependent abnormal activation has also been reported in some digestive tumors and prostate carcinoma (17, 18). Hh signaling is also activated in gastric carcinoma cells (17, 23); however, the molecular mechanisms underlying this abnormal activation remain unclear. Cyclopamine, a steroidal alkaloid that interacts directly with Smo to inhibit Hh signaling, effectively retards the growth of various tumors, including gastric carcinoma, indicating that Hh signaling is involved in tumor growth (15–17, 20). Nevertheless, it remains unclear how the blockade of Hh signaling leads to tumor growth suppression in gastric cancer.

This study examined the mechanism of abnormal Hh signaling in gastric carcinoma cells and sought to identify the key molecules that contribute to tumor cell growth regulated by Hh signaling. We showed that the abnormal Hh signaling in gastric carcinoma cells is caused mainly by the constitutive overexpression of the Sonic hedgehog (Shh) ligand in the cancer cells themselves. Furthermore, our data indicate that the Hh signaling pathway negatively regulates the expression of the cyclin-dependent kinase (CDK) inhibitor p21/CDK-interacting protein 1 (CIP1) in a glioma-associated oncogene 1 (Gli1)–dependent and p53-independent manner. Our findings suggest that Shh-Gli1 signaling contributes to the acceleration of tumor growth through the negative regulation of p21/CIP1 expression in gastric carcinoma cells.

Materials and Methods

Cell lines and human gastric tissue samples. Five human gastric carcinoma cell lines (AGS, MKN1, MKN7, MKN45, and MKN74) and an embryonic kidney cell line (HEK 293T) were purchased from American Type Culture Collection (Manassas, VA) or Japanese Riken Cell Bank (Tsukuba, Japan). Tissue specimens from 14 patients with gastric carcinoma who
underwent gastrectomies were obtained from the archives of Motojima General Hospital, Gunma, Japan, after approval from the medical ethics committee and acquisition of informed consent. The samples of cancerous and noncancerous gastric tissues (the normal tissue surrounding the tumors) had been collected immediately after gastrectomy, frozen in liquid nitrogen, and stored at −80°C. Formalin-fixed, paraffin-embedded sections were examined using H&E staining and immunohistochemistry.

**Reagents and blocking antibody.** The Smo-specific inhibitor cycloamine (Toronto Research Chemicals, North York, Ontario, Canada; refs. 16, 24) was dissolved in DMSO. Mouse anti-Shh blocking antibody (5E1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and control mouse immunoglobulin G (IgG; Sigma-Aldrich, St. Louis, MO) were used at the concentrations indicated in the text.

**Transfection constructs.** The Gli1 expression plasmid pcDNA3/Gli1 (25, 26) and the pS3 expression plasmid pCXN2/pS3 (27) and the respective control plasmids was used. The wild-type expression plasmid was also obtained and the mutated Smo (pGEM/Sm-o539), which activates the Hh signal pathway without stimulation by Hh ligand, was generated (21, 28). The p53 reporter plasmid containing the Photinus pyralis (firefly) luciferase gene was purchased from Stratagene (La Jolla, CA). The pRL-TK control plasmid that expresses the Renilla reniformis (sea pansy) luciferase gene was driven by the Herpes simplex virus thymidine kinase promoter was also used (Toyo Ink, Tokyo, Japan). The transfection assay was done using FuGene transfection reagent (Roche, Penzberg, Germany) as previously described (19).

**Construction and transfection of the vector for Gli1 RNA interference.** A plasmid expressing a double-stranded small interfering RNA targeting the Gli1 gene was generated from the pcPUR-U6i cassette vector (pPUR) as previously described (29, 30). Briefly, a sequence targeting the Gli1 gene was selected and sense and antisense oligonucleotides (5′-CACCAGTACCGGTTTCATTTGAACGGAT-CAAAAGTCTGTGCTTCTT-3′ and 5′-GACTATAAAGCAAGACGATTTGGATCT-TAAACGGACACGACGTGAAATGATCAACGTCTATC-3′, respectively) were designed to generate a short hairpin RNA. The two oligonucleotides were annealed to each other and inserted into the pcPUR-U6i cassette vector to generate the Gli1 knockdown vector (pc-PUR/siGli1; siGli1). A control pc-PUR vector that produces RNA interference against the green fluorescent protein (GFP) gene (pc-PUR/siGFP; siGFP) was also used (29). For transfection, the cells were seeded onto 10-cm dishes and siGli1 or the control vector was added to each dish 24 hours later. The transfected cells were cultured for 24 hours in the medium containing 2 μg/mL puromycin (Wako, Osaka, Japan) followed by an additional 24 hours in medium without puromycin. Transfected cells were used for proliferation assays, cell cycle analysis, extraction of total cell lysates for immunoblot analysis, and preparation of total RNA for reverse transcription-PCR (RT-PCR) and cDNA arrays.

**Immunoblotting and immunohistochemistry.** Immunoblotting was done as previously described (31). Mouse anti-Shh (1:1,000), rabbit anti-Gli1 (1:250; Chemicon, Temecula, CA), mouse anti-p21/CIP1 (1:1,000; Transduction Laboratories, Lexington, KY), mouse anti-p53/kip1 (1:1,000; Transduction Laboratories), rabbit anti-p53 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-j-actin (1:5,000, Sigma-Aldrich) antibodies were used as the primary labeling antibodies and the appropriate horseradish peroxidase–conjugated antibodies (1:2,000; Amersham, Uppsala, Sweden) were used as secondary antibodies. An enhanced chemiluminescence detection system (ECL-Plus, Amersham) was used for detection.

**Immunohistochemistry was done as previously described (16) using anti-Shh (1:100) and anti-Gli1 (1:200) antibodies. For the second antibody, Histofine Simple Stain MAX-PO (Nichirei, Tokyo, Japan), which is an amino acid polymer coated with goat immunoglobulin (Fab')2 and peroxidase, was used. Endogenous peroxidase activity was blocked using 3% H2O2 for 10 minutes at room temperature. Antigen retrieval was achieved by boiling the tissue in 0.01 mol/L sodium citrate (pH 6.0) for 10 minutes. All of the primary antibodies were incubated overnight at 4°C. The protein was visualized by the brown peroxidase reaction using the standard 3,3′-diaminobenzidine protocol. All sections were counterstained with hematoxylin to visualize nuclei and tissue structure.

**Reverse transcription-PCR and quantitative reverse transcription-PCR analyses.** Total RNA was extracted from cultured cells and from frozen gastric tissue specimens using ISOGEN reagent (NipponGene, Tokyo, Japan). The extracted RNA was treated with DNase I (Roche) and purified using the RNaseasy MiniElute Cleanup kit (Qiagen, Tokyo, Japan). The purified RNA was reverse transcribed and amplified by RT-PCR using the ImProm-II Reverse Transcription system (Promega, Madison, WI). The amplifications were done by denaturation at 95°C for 5 minutes, followed by 32 cycles of 60 seconds each at 95°C, 60°C, and 72°C. The following primer pairs were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GACAATCAAGAAGTGTTGGA-3′ and 5′-TGTGATACGAGAATGAC-3′; Gli1, 5′-TGCTGGTACCCCTCCCGA-3′ and 5′-GCCGATCTGTGGTATGAGTA-3′; Shh, 5′-ATGCTGCTGTGCGGAAGATCTGTGGTCTA-3′ and 5′-TCAACGTGGTACCCCGCTGACGGGCGG-3′; Smo, 5′-CTTACGCTGCGACCTTACGT-3′ and 5′-AGACGGATATCTCCTGGGGCCAGATG-3′; Ptch, 5′-TTCTTCAACACCTGCGAAACC-3′ and 5′-CTGCGACTCTAATGACCTTCCACCTG-3′.

The quantitative RT-PCR analysis was done also with a PCR mixture containing 1 μmol/L of each primer and SYBR Green master mix (Applied Biosystems, Foster City, CA). The amplifications were conducted at 95°C for 10 seconds and 60°C for 60 seconds using the ABI PRISM 7000 Quantitative PCR system (Applied Biosystems; ref. 32). Each sample was examined in triplicate and the amounts of the PCR products were normalized with respect to the GAPDH internal control. The following primer pairs were used: GAPDH, 5′-TGGAGATTTCCATTTGAGACAAG-3′ and 5′-CCACCACAAATCTCCAGAT-3′; Gli1, 5′-GCCGCTGACCGATTCTAAGA-3′ and 5′-TGTCGCCGTGGTGGGCGG-3′; Shh, 5′-GAGGGCAAGCCTGATGACT-3′ and 5′-CTCTGCCACGTCTAC-3′.

**Analysis of gene expression using cDNA array assays.** AGS cells were seeded onto 10-cm dishes, transfected with the siGli1 or control plasmid, and then plated and cultured with puromycin for 24 hours. Seventy-two hours after transfection, the total RNA was isolated and used for cDNA array analysis as previously described (33). The cDNA array membranes contained 96 genes that are closely related to the cell cycle (Human Cell Cycle Gene Array, SuperArray Bioscience, Frederick, MD).

**Cell proliferation assay.** The cells were seeded onto six-well plates at a density of 2 × 104 cells per well. Cyclopamine dissolved in DMSO or DMSO alone was added to some wells at 0 hours. The number of viable cells was determined in triplicate wells at 24, 48, and 72 hours using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, ref. 34, 35).

**Reporter assay.** The p53 reporter assay was done using AGS and MKN1 cells as previously described (27). Briefly, 1 × 105 cells were plated onto six-well tissue culture plate 24 hours before transfection. The transfection complexes containing a total of 0.6 μg of plasmid DNA (0.29 μg of the p53 reporter plasmid, 0.01 μg of pRL-TK, and a total of 0.3 μg of the pS3 and Gli1 expression plasmids or the empty plasmids) and the FuGene transfection reagent were then added to each well. Cyclopamine dissolved in DMSO or DMSO alone was added 24 hours after transfection. The cells were harvested 48 hours after transfection and luciferase assays were carried out using the PicaGene Dual Sea Pansy system (Toyo Ink). The firefly and sea pansy luciferase activities were measured as relative light units with a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). The firefly luciferase activity was normalized to that of the sea pansy luciferase to control for transfection efficiency. All assays were done at least in triplicate.

**Cell cycle analysis.** AGS cells were seeded onto 10-cm dishes and were cultured for 72 hours under the conditions indicated in the text. The cells were harvested by trypsinization, fixed with 70% ethanol, and stained with propidium iodide before analysis of the DNA content by flow cytometry (36). The cell cycle phases were analyzed using MultiCycle software (Beckman Coulter, Fullerton, CA).

**Statistics.** The results are presented as mean ± SE. Comparisons were made using two-tailed Student’s t test. P < 0.05 was considered statistically significant.

**Sonic Hedgehog-Gli1 Signaling Regulates p21/CIP1 in Gastric Carcinoma Cells**

Sonic Hedgehog-Gli1 Signaling Regulates p21/CIP1 in Gastric Carcinoma Cells.
Results

The Sonic hedgehog ligand is constitutively expressed and has an essential role in the proliferation of gastric carcinoma cells cultured in vitro. The expression of Shh ligand mRNA was detected by RT-PCR in all five gastric carcinoma cell lines examined, as was the expression of Ptch, Smo, and Gli1 (Fig. 1A). The expression of Shh protein was also confirmed in all five gastric carcinoma cell lines but not in HEK 293T cells derived from embryonic kidneys (Fig. 1B). By contrast, the expression of Indian hedgehog protein was barely detectable in gastric carcinoma cells (data not shown). Treatment of AGS cells with anti-Shh blocking antibody inhibited cell proliferation in a dose-dependent manner as did treatment with cyclopamine, a specific inhibitor of Smo, as previously reported (17); conversely, the overexpression of Gli1 accelerated AGS cell growth (Fig. 1C). The quantitative RT-PCR analysis showed that the anti-Shh blocking antibody reduced the

![Figure 1. Constitutive expression of Shh induces cell proliferation in gastric carcinoma cell lines. A, RT-PCR analysis of the expressions of major molecules involved in Hh signaling, Shh, Ptch, Smo, and Gli1, in the five gastric carcinoma cell lines. GAPDH gene expression served as an internal control. Control, samples of total RNA from gastric carcinoma cells without reverse transcription reaction. B, immunoblot of Shh expression in the five gastric carcinoma cell lines. C, effects of treatment with anti-Shh blocking antibody or cyclopamine on the proliferation of AGS cells transfected with either a Gli1 expression vector or empty vector as determined by MTT assays at 72 hours after seeding. Results of MTT assays were independently normalized to the appropriate DMSO, IgG, or empty vector controls. Columns, mean fold changes relative to the appropriate DMSO, IgG, or empty vector controls. **, P < 0.01.](http://cancerres.aacrjournals.org/content/cr/65/23/10824/F1.large.jpg)

![Figure 2. Expressions of Shh and Gli1 in gastric carcinoma tissues. Representative H&E staining (A) and expressions of Shh (B and C) and Gli1 (D) in gastric cancer tissues. A and B, right, cancer tissue; left, noncancerous tissue. The Shh ligand was expressed mainly in the cytoplasm (C) and Gli1 was strongly expressed in both the cytoplasm and nuclei (D) of gastric carcinoma cells. All sections were counterstained with hematoxylin to visualize nuclei and tissue structure. Magnifications: ×40 (A, B), ×100 (C), and ×200 (D).](http://cancerres.aacrjournals.org/content/cr/65/23/10824/F2.large.jpg)
expression of Gli1 and Ptc (Fig. 1D), both of which are well-known target genes regulated by Hh signaling (18). We also confirmed that the Shh blocking antibody did not affect the growth of HEK 293T embryonic kidney cells (data not shown), which did not express Shh. These findings suggest that the Shh-Smo-Gli1 pathway has a pivotal role in the growth of gastric cancer cells and that stimulation of Hh signaling by Shh ligand occurs in an autocrine/paracrine manner in gastric cancer cells.

**Sonic hedgehog is overexpressed in human gastric carcinoma tissues.** To analyze the expression of Shh ligand in normal and gastric carcinoma tumor tissues collected from patients who had undergone gastrectomy, sections of cancerous or noncancerous tissues were stained by immunohistochemistry using anti-Shh antibody. Compared with the expression level in normal tissues, Shh was constitutively overexpressed in gastric tumors (Fig. 2B and C). Gli1 was up-regulated and imported into the nucleus by activated Hh signaling (20, 37–39); we detected high levels of Gli1 in the nuclei of gastric carcinoma cells, as well as in the cytoplasm (Fig. 2D), suggesting that activated Hh signaling occurs in these tumors. The quantitative RT-PCR analysis of Shh mRNA expression revealed that Shh was overexpressed 12.8-fold (range, 0.3- to 78.8-fold) in 57% of gastric carcinoma tissues as compared with the level in the surrounding noncancerous gastric tissue (Table 1). There was no correlation between the level of Shh mRNA expression and the pathologic or clinical features of the tumor, including tumor type, size, location, and stage.

**Decreased glioma-associated oncogene 1 expression by RNA interference inhibits the growth of gastric carcinoma cells.** Gli1, a downstream transcriptional factor of Shh signal, is commonly expressed in gastric carcinoma cells and tumor tissues. To elucidate whether Gli1 is involved in the proliferation of cancer cells downstream from the Hh pathway, Gli1 was stably knocked down in cultured AGS cells using a Gli1-knockdown vector (siGli1). The reduction of endogenous Gli1 expression in AGS cells was confirmed by RT-PCR and immunoblot assays (Fig. 3A and B). The proliferation assay revealed that Gli1 knockdown markedly repressed AGS cell growth (41.0 ± 8.0% after 72 hours; Fig. 3D). Moreover, Gli1 knockdown led to a significant increase in the proportion of cells in G1 phase (58.2 ± 1.1% versus 43.2 ± 0.9%, P < 0.01; Fig. 3E) and a significant decrease in the proportion of cells in S phase (14.4 ± 1.6% versus 30.9 ± 1.6%, P < 0.01) as compared with the control AGS cells. Similar results were found when AGS cells transfected with the empty vector were used as the negative control (data not shown). For further confirmation that this growth suppression effect of Gli1 knockdown is attributable to the blockade of Hh signaling, the growth rate under the coexpression of siGli1 and active Smo-W395L was also examined in AGS cells. The MTT assay showed that active Smo expression did not antagonize the tumor growth suppression caused by Gli1 knockdown (Fig. 3D). This result indicates that the growth effect gained by activated Hh signaling is mainly through the Gli1 protein. These findings suggest that Gli1 plays a pivotal role in cell proliferation through regulation of the G1-S transition and that Gli1 is implicated in the Shh-dependent autocrine loop that accelerates the proliferation of gastric carcinoma cells.

**Glioma-associated oncogene 1 knockdown induces an increase in p21/cyclin-dependent kinase–interacting protein 1 expression in gastric carcinoma cells.** To unravel the details of the molecular systems that are regulated by Hh-Gli1 signaling and that contribute to cell proliferation in gastric carcinoma cells, we used a cell cycle gene cDNA array to examine the expression profile of genes involved in the difference in the cell cycle of AGS cells transfected with the siGli1 or siGFP control vector. We noted that the expression of p21/CIP1 mRNA was up-regulated in cells in which Gli1 expression was decreased whereas the expression levels of other CKD inhibitors were not significantly different (Fig. 4A). Analysis using quantitative RT-PCR and immunoblot assays confirmed that p21/CIP1 expression

---

**Table 1. Clinical features of 14 patients with gastric carcinoma and levels of Shh mRNA expression in the lesions compared with the levels in noncancerous tissues**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Differentiation*</th>
<th>Vascular invasion</th>
<th>Tumor-node-metastasis classification</th>
<th>Location †</th>
<th>Tumor size (cm)</th>
<th>Shh expression ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48/M</td>
<td>Mod</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>C</td>
<td>4 × 3</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>70/M</td>
<td>Por</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>A</td>
<td>3 × 2</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>69/M</td>
<td>Pap</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>6 × 5</td>
<td>8.2</td>
</tr>
<tr>
<td>4</td>
<td>70/M</td>
<td>Muc</td>
<td>–</td>
<td>T₁N₁M₀</td>
<td>B</td>
<td>3 × 3</td>
<td>24.2</td>
</tr>
<tr>
<td>5</td>
<td>69/F</td>
<td>Muc</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>5 × 5</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>42/M</td>
<td>Sig</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>2 × 2</td>
<td>0.3</td>
</tr>
<tr>
<td>7</td>
<td>60/M</td>
<td>Por</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>B, A</td>
<td>8 × 7</td>
<td>1.1</td>
</tr>
<tr>
<td>8</td>
<td>69/M</td>
<td>Por</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>C</td>
<td>5 × 5</td>
<td>10.0</td>
</tr>
<tr>
<td>9</td>
<td>54/M</td>
<td>Por</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>4 × 4</td>
<td>78.7</td>
</tr>
<tr>
<td>10</td>
<td>81/M</td>
<td>Mod</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>C</td>
<td>4 × 3</td>
<td>17.1</td>
</tr>
<tr>
<td>11</td>
<td>74/F</td>
<td>Mod</td>
<td>–</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>3 × 3</td>
<td>19.5</td>
</tr>
<tr>
<td>12</td>
<td>55/M</td>
<td>Mod</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>B</td>
<td>5 × 5</td>
<td>1.8</td>
</tr>
<tr>
<td>13</td>
<td>48/M</td>
<td>Sig</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>B, A</td>
<td>15 × 13</td>
<td>0.6</td>
</tr>
<tr>
<td>14</td>
<td>68/M</td>
<td>Mod</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>A</td>
<td>7 × 6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Mod, moderately differentiated; Muc, mucinous; Pap, papillary; Por, poorly differentiated; Sig, signet ring.
†Location: C, cardiac; B, body; A, antrum.
‡Relative expression ratio of the level of Shh mRNA in the cancerous lesion compared with the level in the surrounding noncancerous tissue as determined by quantitative RT-PCR analysis.
The up-regulation of p21/cyclin-dependent kinase–interacting protein 1 and the cell cycle arrest induced by the blockade of Hedgehog signaling are p53 independent. The tumor suppressor p53 is a major transcription factor involved in the regulation of p21/CIP1 expression. We examined the contribution of p53 to the observed up-regulation of p21/CIP1 by the blockade of Hh signaling in gastric carcinoma cells. Both AGS cells and MKN45 cells express wild-type p53 (40, 41) whereas MKN1 and MKN74 cells express mutated p53 proteins (V143A substitution in MKN1 and I251L and E271A substitutions in MKN74; ref. 41).

Although the inhibition of Hh signaling by cyclopamine treatment induced an increase in both p21/CIP1 mRNA (Fig. 5A) and protein (Fig. 5B) in AGS, MKN1, MKN45, and MKN74 cells, it did not affect the expression of p53 in these cell lines (Fig. 5B). In addition, the luciferase activity in AGS and MKN1 cells transfected with a luciferase reporter plasmid containing the p53 response element was not affected by cotransfection with a Gli1 expression vector or by cyclopamine.

was up-regulated at both the mRNA and protein levels by the knockdown of Gli1 in AGS cells (Fig. 4B and C). The inhibition of Hh signaling by cyclopamine treatment similarly increased p21/CIP1 expression and the transfection with Gli1 expression vector suppressed the cyclopamine-induced increase in p21/CIP1 (Fig. 4D). By contrast, the knockdown of Gli1 did not alter p27/KIP1 expression (Fig. 4C and D) and p16/INK4a and p19/INK4d could not be detected in AGS cells (data not shown). These results suggest that the negative regulation of p21/CIP1 expression by activated Hh signaling through Gli1 promotes growth in gastric carcinoma cells.

**Figure 3.** Suppression of AGS cell growth induced by Gli1 knockdown. Gli1 expression 72 hours after transfection with the siGli1 vector analyzed by RT-PCR (A) and immunoblotting (B). Proliferation of AGS cells by siGli1 transfection (24, 48, and 72 hours after reseeding; C) and by cotransfection with active Smo expression vector (Smo-W539L) and siGli1 (48 hours after reseeding; D). Columns, mean fold changes relative to the control from three independent experiments; bars, SE. **, P < 0.01. E, AGS cells were transfected with the siGli1 or control siGFP vector, harvested after 72 hours, stained with propidium iodide, and analyzed by flow cytometry. Representative data from one of three independent experiments.

**Figure 4.** Expression of p21/CIP1 was negatively regulated by Hh-Gli1 signaling in AGS cells. A, AGS cells were transfected with the siGli1 or control siGFP vector 72 hours before total RNA extraction and cDNA array analysis. B, p21/CIP1 gene expression level was confirmed by quantitative RT-PCR analysis. Columns, mean of three experiments; bars, SE. *, P < 0.02. C, expression levels of p21/CIP1 and p27/KIP1 examined by immunoblot assay. D, AGS cells were transfected with Gli1 expression vector or empty vector 24 hours before treatment with cyclopamine (10 μmol/L). Levels of p21/CIP1 and p27/KIP1 expressions were examined 72 hours after transfection.
treatment (Fig. 5C). The inhibitory effect of cyclopamine treatment on cell growth was observed in MKN1 cells as well as in AGS cells (Fig. 5D). Cell cycle analysis showed that the blockade of Hh signaling in AGS cells by cyclopamine treatment increased the proportion of cells in G1 phase (54.3 ± 0.2% versus 45.4 ± 0.8%, P < 0.03) and decreased the proportion of cells in S phase (24.3 ± 0.8% versus 37.2 ± 0.8%, P < 0.01; Fig. 5E). Similarly, the proportions of MKN1 cells in G1 and S phase were increased (78.5 ± 0.1% versus 38.6 ± 0.4%, P < 0.01) and decreased (13.0 ± 0.1% versus 34.2 ± 0.2%, P < 0.01), respectively (Fig. 5E). These results suggest that the increased expression of p21/CIP1 and the inhibition of the G1-S cell cycle transition are induced in a p53-independent manner by the blockade of Hh signaling in gastric carcinoma cells.

Discussion
Although some recent studies have indicated that Hh signaling is constitutively activated in gastric carcinoma cells (17, 23), the cancer-specific mechanism of this constitutive activation remains
unclear. Treatment with the Smo inhibitor has been reported to be effective for various cancers; however, the molecules involved in the cyclopamine-induced inhibition of cancer cell growth are also unknown. This study showed that gastric carcinoma cells constitutively produced the Shh ligand, thereby activating downstream signaling both in vitro and in vivo, and that Shh-Gli1 signaling negatively regulated the expression of the CDK inhibitor p21/CIP1 in gastric carcinoma cells.

Several growth signals mediate the accelerated growth of cancer cells by autocrine loops involving the expressions of both ligand and receptor by the same cell. The Shh ligand was constitutively expressed in all of the cultured gastric carcinoma cell lines we examined; the growth of these cells was inhibited by anti-Shh blocking antibody as well as by cyclopamine. Moreover, the overexpression of Shh in gastric carcinoma cells was also confirmed in many specimens of human gastric carcinoma tissues collected at gastrectomy. The overexpression of Shh ligand and the resulting autocrine/paracrine loop might partly explain the abnormal activation of the Hh pathway in gastric carcinoma cells. We found no specific correlation between the constitutive expression of Shh in gastric carcinomas and their clinical characteristics; consequently, Hh signaling might be one of the growth regulatory mechanisms involved in the initial stages of malignant cell transformation. To determine the contribution of abnormal Hh signaling to carcinogenesis, it is crucial to examine whether Hh signaling is activated in benign tumors and early-stage cancers, as well as in progressing tumors.

The results suggest that the oncogene Gli1 also has a pivotal role in the proliferation of gastric carcinoma cells. Strikingly, the cDNA arrays showed that the disruption of Gli1 expression by small interfering RNA increased the expression of p21/CIP1 mRNA and protein. p21/CIP1 can induce G1 arrest and block S-phase entry by inactivating CDKs (42) and the overexpression of p21/CIP1 effectively suppresses tumor growth (43). In addition, defects in p21/CIP1 increase susceptibility to chemically induced carcinoma formation (44). These findings imply that Shh-Gli1 signaling accelerates the G1-S transition down-regulating the expression of p21/CIP1, thereby inducing increased proliferation of gastric carcinoma cells.

The regulation of p21/CIP1 by Hh signaling in gastric carcinoma cells was independent of p53. Although the p53 protein is one of the most important transcriptional factors regulating p21/CIP1 expression (45, 46), Hh signaling did not affect the expression or activity of p53 and the inhibition of Hh signaling increased p21/CIP1 expression even in gastric carcinoma cells with mutated p53 genes. It is possible that Hh signaling pathways cross talk with other signaling pathways, such as those involving MYC, SMAD, and FOX regulatory and transcription factors (46–48), which repress the expression of p21/CIP1 in a p53-independent manner in tumor cells. The relationship between abnormal Hh signaling and change-of-function mutations in other genes in gastric cancer should also be examined.

In conclusion, this study showed that Hh signaling may control cell proliferation through the negative regulation of p21/CIP1 expression in gastric carcinoma cells. Although many details of the molecular biology of Hh signaling in the digestive tract remain to be elucidated, a deeper understanding of these pathways may shed light on the mechanisms of carcinogenesis in gastric tumors and may lead to the development of novel therapeutic strategies for gastric carcinomas in the near future.

Acknowledgments

Received 3/6/2005; revised 7/19/2005; accepted 8/12/2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Mitsuko Tsutsumi, Susanne Oyama, M., Dr. H. Yamada (Division of Gastroenterology, Kobe University Graduate School, Kobe, Japan), and the members of the Sata laboratory for their technical assistance; and Drs. T. Motojima and S. Yamada (Division of Abdominal Surgery, Motomiya General Hospital, Guma, Japan), Dr. T. Oyama (Division of Pathology, Motomiya General Hospital), Dr. H. Sasaki (RIKEN Center for Developmental Biology, Kobe, Japan), Dr. P.A. Beachy (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD), Drs. B. Vogelstein and K.W. Kinzler (Howard Hughes Medical Institute, Sidney Kimmel Comprehensive Cancer Center and Program in Human Genetics and Molecular Biology, Johns Hopkins Medical Institutions, Baltimore, MD), and the Developmental Studies Hybridoma Bank for supplying key materials.

References

p53-Independent Negative Regulation of p21/Cyclin-Dependent Kinase–Interacting Protein 1 by the Sonic Hedgehog-Glioma-Associated Oncogene 1 Pathway in Gastric Carcinoma Cells

Miki Ohta, Keisuke Tateishi, Fumihiko Kanai, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/23/10822

Cited articles
This article cites 46 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/23/10822.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/23/10822.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/65/23/10822.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.