Hedgehog Signaling Pathway is a New Therapeutic Target for Patients with Breast Cancer

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ABSTRACT

The Hedgehog (Hh) signaling pathway functions as an organizer in embryonic development. Genetic analysis has demonstrated a critical role for the Hh pathway in mammalian gland morphogenesis. Disruption of Patched1, a component of the Hh pathway, results in abnormal growth of mammary duct. Recent studies have shown constitutive activation of the Hh pathway in various types of malignancies. However, it remains unclear whether this pathway is activated in human breast cancer. Here, we determined the expression of the components, including Sonic Hh, Patched1, and Gli1, of the Hh pathway by immunohistochemical staining in a series of 52 human breast carcinomas. All of 52 tumors display staining of high intensity for Gli1 when compared with adjacent normal tissue. The nuclear staining ratio of Gli1 correlates with expression of estrogen receptor and histologic type. Exposure to cycloamine, a steroidal alkaloid that blocks the Hh pathway, suppresses expression of Gli1 and the growth of the Hh pathway-activated breast carcinoma cells. These data indicate that the Hh pathway is a new candidate for therapeutic target of breast cancer.

INTRODUCTION

The Hedgehog (Hh) signaling pathway is crucial to the growth and patterning during embryonic development (1). This pathway is a highly coordinated and orchestrated network involving inhibition of the twelve transmembrane protein Patched1 (Pch1) by binding Hh protein, activation of the seven transmembrane protein Smoothened (Smo), release of a five-zinc finger transcription factor Gli from a large protein complex, nuclear translocation of Gli, and transcription of target genes (1–3). In the absence of Sonic Hh (Shh), Glis form a large protein complex with Costal2, Fused, and Suppressor Fused and are sequestered in cytoplasm (1–3). In the presence of Shh, a full-length Gli3 released from the large protein complex is transported into the nucleus to activate Hh target genes (1–3). Gli1 is one of target genes of Gli3 (4). Therefore, Gli1 is a marker of the Hh pathway activation (1, 4, 5).

Recent data have shown an association between the Hh pathway activation and the initiation of human tumors (6, 7). Constitutive activation of the Hh pathway has been found in several types of tumors. In a small subset of tumors in the brain, skin, and muscles, mutations in Pch1 or Smo trigger ligand-independent activation of the Hh pathway (8, 9). On the other hand, ligand-dependent activation of the Hh pathway has been shown in small-cell lung carcinoma and digestive tract tumors such as esophageal carcinoma, gastric carcinoma, and pancreatic carcinoma. The fact that the Hh pathway is constitutively activated in such wide range of tumors suggests a significance of the Hh pathway as novel diagnostic and therapeutic targets. In fact, it has been shown that the plant-derived teratogenic steroidal alkaloid cycloamine, which inhibits the Hh pathway by antagonizing Smo (10, 11), suppressed the growth of cancer cells showing the Hh pathway activation both in vitro and in vivo (12, 13).

The Hh pathway has also been established as an important signaling system in mouse normal mammary gland development (14, 15). Disruption of Pch1 or Gli2 gene results in severe defects in ductal morphogenesis such as ductal dysplasias that closely resemble some hyperplasias of the human breast (16). In addition, it has been reported that breast carcinoma cells have disruption of these genes (17). Thus, these data implicate a potential role of the Hh pathway in mammary oncogenesis. However, biological significance of the Hh pathway in human breast cancer has not been clarified yet. Here, we analyzed the Hh pathway using relatively large number of breast carcinomas and revealed for the first time that the Hh pathway is constitutively activated in most breast carcinomas. In addition, data shown in this study suggest that inhibition of the Hh pathway may be a valuable therapeutic strategy for patients with breast carcinoma.

MATERIALS AND METHODS

Clinical Samples. Fifty-two patients with primary breast carcinoma underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan), between 2002 and 2003. All 52 patients gave informed consent before surgical treatment and were entered into the present study. The primary breast carcinoma surgical specimens were fixed with 10% formalin, embedded in paraffin, examined histopathologically, and classified using Tumor-Node-Metastasis classification. Histopathological examination revealed that the specimens consist of 46 invasive ductal carcinomas, 5 intraductal ductal carcinomas, and 1 invasive lobular carcinoma (Table 1).

Immunohistochemistry. Single-antibody detection was accomplished as previously described (18), with the following protocol modifications: endogenous peroxidase activity was blocked using 3% H2O2 in methanol for 30 minutes at room temperature. Antigen retrieval was achieved by boiling tissue in 0.01 mol/L sodium citrate (pH 6.0) for 5 minutes. All primary antibodies were incubated overnight at 4°C. Primary antibodies used were as follows: Shh (N-19, sc-1194); Pch1 (H-267, sc-9016); and Glic (N-16, sc-6155) (1:250; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies (Shh and Glis, rabbit anti-rabbit immunoglobulin; Pch1, goat anti-rabbit immunoglobulin; Nichirei Co., Ltd., Tokyo, Japan) were applied for 1 hour at room temperature. Detection of protein was visualized by brown peroxidase via standard 3,3'-diaminobenzidine protocol. Slides were lightly counterstained with hematoxylin. One-hundred carcinoma cells or adjacent normal breast epithelial cells were counted for each section. When cells >50% were stained, we judged it to be a positive. A ratio of Gli1-nuclear staining carcinoma cells to total carcinoma cells was expressed as percentage of nuclear staining of Gli1.

Immunostaining of Cell Lines. Four human breast adenocarcinoma cell lines (BT-474, SK-BR-3, MDA-MB-231, and MCF-7) and a human colonic adenocarcinoma cell line (DLD-1) were maintained as monolayer cultures in complete medium composed of RPMI 1640 and 10% fetal bovine serum (Sigma Chemical, St. Louis, MO). Cells (2 to 4 x 10⁵ cells/well) were incubated on an 8-well CultureSlide (BICOAT; Becton Dickinson, San Jose, CA) for 4 hours at 37°C. The slides were air-dried and immersed in 100% methanol for 5 minutes at −30°C. The later protocol is same to the immuno-histochemistry described above.
**Table 1**  Clinicopathological features and expression of Hh pathway-related molecules in the 52 breast carcinoma specimens

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>No. of positive specimens</th>
<th>% nuclear staining of Gli1*</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
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<td></td>
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<tr>
<td>&lt;40</td>
<td>3</td>
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<td>76.00 ± 14.53</td>
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<td>40-70</td>
<td>44</td>
<td>42</td>
<td>37.16 ± 20.82</td>
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<tr>
<td>&gt;70</td>
<td>5</td>
<td>5</td>
<td>59.20 ± 11.73</td>
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<tr>
<td>T classification‡</td>
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<td></td>
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</tr>
<tr>
<td>pT1</td>
<td>31</td>
<td>29</td>
<td>39.16 ± 20.82</td>
</tr>
<tr>
<td>pT2</td>
<td>21</td>
<td>21</td>
<td>44.95 ± 20.77</td>
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<td>Histologic type</td>
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<tr>
<td>Intraductal carcinoma</td>
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<td>5</td>
<td>26.60 ± 11.67</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>46</td>
<td>44</td>
<td>43.39 ± 21.02</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>1</td>
<td>1</td>
<td>29.00</td>
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<tr>
<td>Estrogen receptor status</td>
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<td></td>
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<tr>
<td>Negative</td>
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<td>26.31 ± 18.01</td>
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<tr>
<td>Positive</td>
<td>39</td>
<td>38</td>
<td>46.56 ± 19.30</td>
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<td>Lymph node involvement</td>
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<td>pTNM stage†</td>
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<tr>
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<td>22</td>
<td>38.52 ± 21.35</td>
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<td>II</td>
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<tr>
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<td>51.14 ± 22.59</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>50</td>
<td>40.37 ± 20.71</td>
</tr>
</tbody>
</table>

* (No. of Gli1-nuclear-staining carcinoma cells/100 carcinoma cells) × 100. The data represent mean % ± SD.
† Kruskal-Wallis test.
‡ According to the TNM classification system of International Union against Cancer.
§ Mann-Whitney U test; n.s., not significant.
Abbreviation: pTNM, pathological Tumor-Node-Metastasis.

Immunoblotting Analysis. Immunoblotting analysis was performed as described previously (19). Briefly, cultured cells were processed in buffer containing 62.5 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% (wt/vol) SDS, and 10% glycerol, applied for SDS-PAGE, and transferred to nitrocellulose membrane. Protein signals were visualized using the following antibodies: anti-Gli1 antibodies (N-16, sc-6153) and horseradish peroxidase-conjugated antigoat antibodies (KPL, Gaithersburg, MD). Signals were visualized by Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA).

Proliferation Assay. Cyclopamine and tomatidine were purchased from Toronto Research Chemicals (North York, Ontario, Canada) and Sigma Chemical, respectively. These agents were dissolved in 100% methanol and added in vitro at concentrations indicated in the results; appropriate additions of methanol were made in control samples. To test for cyclopamine responsiveness, human breast carcinoma cells were grown for 4 days in control medium containing tomatidine or methanol alone or experimental medium containing cyclopamine. We changed the medium every 2 days. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (20).

Statistical Analysis. All statistical analyses were performed with SAS Statistical Software (Release 6.12) on a UNIX workstation. The correlation between mean staining rate of Gli1 and the clinical features listed in Table 1 was analyzed by Kruskal-Wallis test or Mann-Whitney U test via the SAS/STAT CORP procedure. P < 0.05 was considered significant.

**RESULTS AND DISCUSSION**

We show, for the first time, the constitutive activation of the Hh pathway in human breast carcinoma using 52 resected specimens (Table 1). In addition, we also show that the Hh pathway is a candidate of the novel therapeutic target for patients with breast carcinoma (Fig. 3A).

Although the Hh pathway activation has been shown in various types of malignancies (6, 7), there are only few reports showing it using human carcinoma specimens. To our knowledge, constitutive activation of the Hh pathway in relative large number of specimens has been reported only in pancreatic carcinoma (13) and gastric carcinoma (21). We used 52 paraffin-embedded carcinoma specimens resected for breast carcinoma, which consist of 47 invasive carcinomas and 5 intraductal carcinomas (Table 1). Part of normal breast epithelium was found in the 52 resected specimens. Expressions of functional components, Shh, Ptch1, and Gli1, of the Hh pathway were determined using immunohistochemistry repeated on at least two different days. Carcinoma cells overexpress Shh, Ptch1, and Gli1 in 52, 50, and 52 of the 52 breast cancer specimens, respectively (Fig. 1, bottom panel). In contrast, adjacent normal breast epithelia do not express detectable levels of these proteins (Fig. 1, top panel). Histologic type of both Ptch1-negative cases is invasive ductal carcinoma. Staining intensity of Shh of these specimens seems to be weaker compared with that in the 50 Ptch1-positive specimens (data not shown). However, such a difference is not clear in Gli1 expression. Ptch1 and Gli1 but not Shh are target genes of the Hh pathway (1–4). Taken together, there is a possibility that these two cases may have mutations of Ptch1, which the antibody used here cannot recognize, because mutations of Ptch1 have been reported in human breast carcinoma (17). However, we have now no definite answer against this question. Nevertheless, the present data show that constitutive activation of the Hh pathway is a common phenomenon in breast carcinoma because expression of Gli1 is an acceptable marker of the Hh pathway activation (1, 4, 5).

Nuclear staining of Gli1 is found in all of the 52 specimens (Table 1 and Fig. 1) but not in adjacent normal breast epithelium (Fig. 1). A ratio of carcinoma cells showing nuclear staining of Gli1 to total carcinoma cells (percentage of nuclear staining of Gli1) is various, ranging from 2 to 95% with mean of 40.87% (Table 1). Because it has been shown that Gli1 functions only as a transactivator (4) and that Gli1 can drive the production of basal cell carcinoma in the mouse when overexpressed in the epidermis (22), we analyzed the correlation between nuclear staining of Gli1 and traditional histopathologic findings. Strikingly, a positive cor-
relation is seen between percentage of nuclear staining of Gli1 and expression of estrogen receptor or histologic type, i.e., invasive type (Table 1). There is an interesting article that suggests a possibility of the involvement of the Hh pathway in hormone-induced development of breast carcinoma (23).

We next examined whether the Hh pathway can become a therapeutic target in breast carcinoma. To investigate this possibility, we used four human breast carcinoma cell lines. All of the four cell lines show increased expressions of Shh, Ptch1, and Gli1 (Fig. 2). We used a colonic cell line, DLD-1, as negative control, because it has been reported that the Hh pathway is not activated in colonic carcinomas (21). Nuclear staining of Gli1 was clearly found in three cell lines, except for MDA-MB231. Because Gli1 is one of indicator of the Hh pathway activation, our data suggest a constitutive activation of the Hh pathway in these cell lines. Two main mechanisms of the Hh pathway activation in malignant cells have been proposed. One is a Hh ligand-dependent mechanism. Binding of Hh ligands such as Shh to Ptc1 activates proto-oncogenic Smo through suppression of Ptc1 tumor suppressor function. The other is Hh ligand-independent mechanism. Mutations of Ptc1 or Smo are frequently associated with this type of the Hh pathway activation. It has been proposed that cyclopamine could inhibit Hh ligand-dependent and independent Hh pathway activation because cyclopamine inhibits the Hh pathway through direct interaction with Smo (11, 12). In this study, cyclopamine suppresses the proliferation of three breast carcinoma cells except for MCF-7 in a dose- and time-dependent manner (data not shown). The reason why cyclopamine cannot induce the growth inhibition in MCF-7 is unclear. Recently, it has been shown that monoclonal antibodies against Shh could not inhibit the growth of MCF-7 (24). When carcinoma cells are treated with 10 μmol/L cyclopamine for 4 days, significant decrease in cell density is found in three breast carcinoma cell lines (Fig. 3A). In sharp contrast with cyclopamine, no significant changes in cell density are found in carcinoma cells treated with 10 μmol/L tomatidine, an inactive cyclopamine analogue. Although 1-day treatment with 10 μmol/L cyclopamine does not induce a significant change in cell density, it decreases both cytoplasmic expression and nuclear staining of Gli1 in BT-474 and SK-BR-3, indicating the inhibition of the Hh pathway by cyclopamine (Fig. 3B). These cyclopamine effects on Hh signaling activity were additionally confirmed by immunoblotting (Fig. 3C). Gli1 expression in BT-474 and SK-BR-3 is attenuated by cyclopamine treatment. However, 10 μmol/L cyclopamine did not decrease the level of Gli1 signal in MCF-7 because immunohistochemistry consistent with that same molar of cyclopamine has no inhibitory effect on proliferation. However, cyclopamine suppressed the proliferation and nuclear staining level of Gli1 in MCF-7 in a dose-dependent manner between 20 and 100 μmol/L (data not shown). These results suggest that the Hh pathway contributes to cell proliferation, even in MCF-7. However, it remains unclear why MCF-7 are more resistant against cyclopamine than other Hh pathway-activating cells such as BT-474 and SK-BR-3. Although Gli1 functions as a transcription factor in nuclei, MDA-MB231 has tiny nuclear Gli1 (Fig. 2), indicating Gli1 may not functional. However, immunostaining and immunoblotting experiments show that MDA-MB231 has a relatively higher amount of cytoplasmic Gli1. Cyclopamine treatment decreases the amount of cellular Gli1. These data suggest that the Hh signaling pathway is partially activated in MDA-MB231, and we presently could only speculate that the Hh signaling pathway is not fully activated in MDA-MB231 as in the cells with nuclear Gli1 through unknown inhibition resulted in vacancy in nuclei and relatively lower cyclopamine effect. We conclude that the Hh pathway is constitutively activated in most breast carcinomas and that the Hh pathway is a potential therapeutic target for patients with breast carcinoma.
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**REFERENCES**

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