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 mammary 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 cyclopamine, 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), Gli3 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, activation of the Hh pathway has been found in several 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 cyclopamine, 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.

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 Gli1 (N-16, sc-6153) (1:250; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies (Shh and Gli1, rabbit antigoat immunoglobulin; Pch1, goat antirabbit immunoglobulin; Nichirei Co., Ltd., Tokyo, Japan) were applied for 1 hour at room temperature. Detection of protein was visualized by brown pigmentation via standard 3,3′-diaminobenzidine protocol. Slides were lightly counterstained with hema-toxylin. One-hundred carcinoma cells or adjacent normal breast epithelial cells were counted for each section. When cells >50% were dyed, we judged it to be 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 colon 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 × 105 cells/well) were incubated on an 8-well CultureSlide (BIOCOAT; 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 immunohistochemistry described above.
**Table 1** Clinicopathological features and expression of Hh pathway-related molecules in the 52 breast carcinoma specimens

<table>
<thead>
<tr>
<th>Clinicalopathological features</th>
<th>No. of positive specimens</th>
<th>% nuclear staining of Gli1*</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>3</td>
<td>3</td>
<td>76.00 ± 14.53 0.0039†</td>
</tr>
<tr>
<td>40-70</td>
<td>44</td>
<td>44</td>
<td>37.16 ± 20.82</td>
</tr>
<tr>
<td>&gt;70</td>
<td>5</td>
<td>5</td>
<td>59.20 ± 11.73</td>
</tr>
<tr>
<td>T classification‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>31</td>
<td>21</td>
<td>39.16 ± 20.82 n.s.‡</td>
</tr>
<tr>
<td>pT2</td>
<td>21</td>
<td>21</td>
<td>44.95 ± 20.77</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
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<tr>
<td>Intraductal carcinoma</td>
<td>5</td>
<td>5</td>
<td>26.60 ± 11.67 0.02168</td>
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<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>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>13</td>
<td>13</td>
<td>26.31 ± 18.01 0.00368</td>
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<tr>
<td>Positive</td>
<td>39</td>
<td>39</td>
<td>46.56 ± 19.30</td>
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<td>Lymph node involvement</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>37</td>
<td>37</td>
<td>39.60 ± 20.43 n.s.‡</td>
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<tr>
<td>Positive</td>
<td>15</td>
<td>15</td>
<td>45.20 ± 21.64</td>
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<tr>
<td>pTNM stage†</td>
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<tr>
<td>I</td>
<td>23</td>
<td>23</td>
<td>38.52 ± 21.35 n.s.†</td>
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<tr>
<td>II</td>
<td>22</td>
<td>22</td>
<td>40.84 ± 19.37</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>7</td>
<td>52.14 ± 22.59</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>52</td>
<td>40.97 ± 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 mMTris-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.** Cyclopane 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 cyclopane responsiveness, human breast carcinoma cells were grown for 4 days in control medium containing tomatidine or methanol alone or experimental medium containing cyclopane. 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 epithilia do not express detectable levels of these proteins (Fig. 1, top panel). Histologic type of both Ptc1-negative cases is invasive ductal carcinoma. Staining intensity of Shh of these specimens seems to be weaker compared with that in the 50 Ptc1-positive specimens (data not shown). However, such a difference is not clear in Gli1 expression. Ptc1 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 Ptc1, which the antibody used here cannot recognize, because mutations of Ptc1 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-

![Fig. 1. The Hh pathway is activated in human breast carcinoma. Fifty-two human breast carcinoma specimens were subjected for immunohistochemistry with antibodies against indicated proteins, Shh, Ptch1, and Gli1 (brown). Nuclei were counterstained with hematoxylin (purple). Breast carcinoma cells express high levels of Shh, Ptch1, and Gli1 (bottom panel), whereas adjacent normal breast epithelia do not express detectable levels of indicated proteins (top panel). In addition, carcinoma cells show extensive nuclear staining for Gli1 (arrow). Pictures show a representative specimen (×400). Bars, 10 μm.](cancersres.aacrjournals.org)
A significant change in cell density is found in three breast carcinoma cell lines treated with 10 μmol/L cyclopamine. This result indicates the inhibition of the Hh pathway by cyclopamine (Fig. 3B). These findings suggest that the Hh pathway is a potential therapeutic target for patients with breast carcinoma.

Immunoblotting shows almost the same result as immunostaining. Nuclei were counterstained with hematoxylin (C bottom panel). BT-474 and SK-BR-3 treated with cyclopamine (B, top panel) compared with cells treated with tomatidine (A, bottom panel). MCF-7 and DLD-1 were treated with 10 mM tomatidine or 10 mM cyclopamine at 37°C for 24 h. After the treatment, cells were applied for immunostaining (B) and immunoblotting (C) with anti-Gli1 antibodies. Nuclei were counterstained with hematoxylin (purple). BT-474 and SK-BR-3 treated with cyclopamine (B, bottom panel) showed decreased Gli1 expression compared with cells treated with tomatidine (B, top panel). MCF-7 and DLD-1 show no difference between before and after cyclopamine treatment (×400). Bars, 10 µm. C. Immunoblotting shows almost the same result as immunostaining.

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REFERENCES


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