

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 (Ptch1) 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 *Ptch1* 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 cyclopamine, 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 *Ptch1* 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); Ptch1 (H-267, sc-9016); and Gli1 (N-16, sc-6153) (1:250; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies (Shh and Gli1, rabbit anti-goat immunoglobulin; Ptch1, goat anti-rabbit 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 hematoxylin. 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-MB231, 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 × 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.

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Table 1 Clinicopathological features and expression of Hh pathway-related molecules in the 52 breast carcinoma specimens

Clinicopathological features	No. of specimens	No. of positive specimens			% nuclear staining of Gli1*	P
		Shh	Ptch	Gli1		
Age (yrs)						
<40	3	3	3	3	76.00 ± 14.53	0.0039†
40–70	44	44	42	44	37.16 ± 20.82	
>70	5	5	5	5	59.20 ± 11.73	
T classification‡						
pT1	31	31	29	31	39.16 ± 20.82	n.s.§
pT2	21	21	21	21	44.95 ± 20.77	
Histologic type						
Intraductal carcinoma	5	5	5	5	26.60 ± 11.67	0.0216§
Invasive ductal carcinoma	46	46	44	46	43.39 ± 21.02	
Invasive lobular carcinoma	1	1	1	1	29.00	
Estrogen receptor status						
Negative	13	13	12	13	26.31 ± 18.01	0.0036§
Positive	39	39	38	39	46.56 ± 19.30	
Lymph node involvement						
Negative	37	37	36	37	39.60 ± 20.43	n.s.§
Positive	15	15	14	15	45.20 ± 21.64	
pTNM stage‡						
I	23	23	22	23	38.52 ± 21.35	n.s.†
IIA	22	22	21	22	40.84 ± 19.37	
IIIB	7	7	7	7	52.14 ± 22.59	
Total	52	52	50	52	40.87 ± 20.71	

* (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 mmol/L Tris-HCl (pH 6.8), 100 mmol/L 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 anti-goat 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-

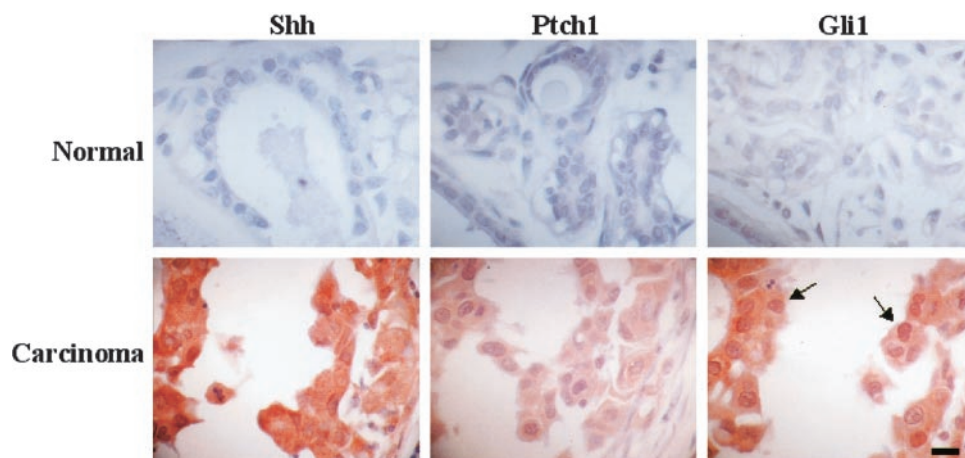


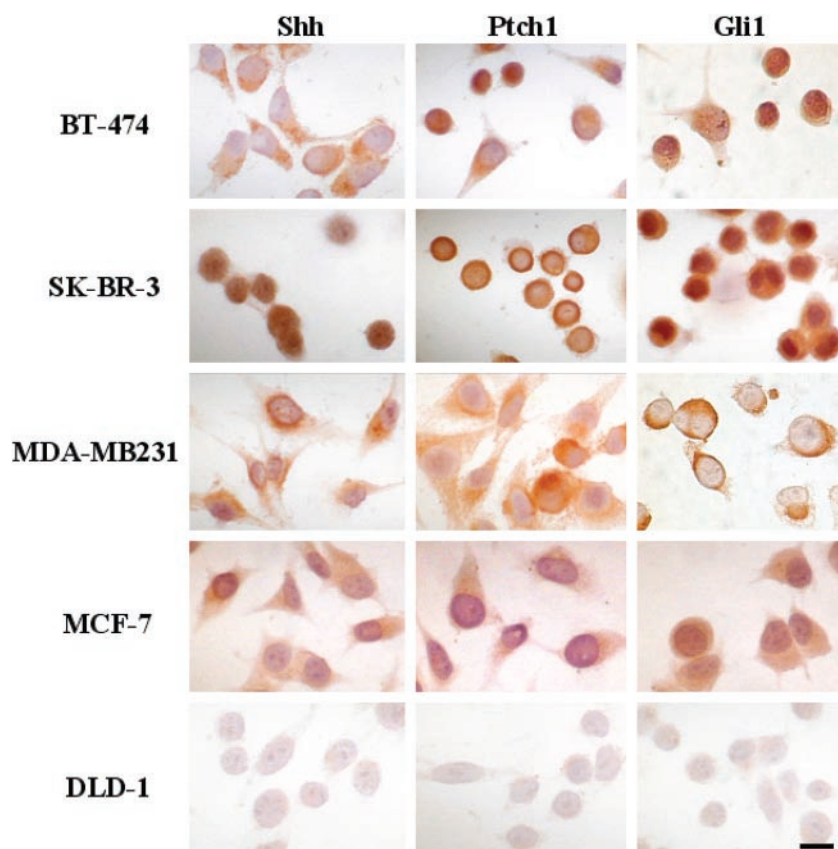
Fig. 1. The Hh pathway is activated in human breast carcinoma. Fifty-two human breast carcinoma specimens were subjected for immunohistochemistry with antibodies against indicated proteins, Shh, Ptch1, and Gli1 (brown). Nuclei were counterstained with hematoxylin (purple). Breast carcinoma cells express 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.

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 Ptch1 activates proto-oncogenic Smo through suppression of Ptch1 tumor suppressor function. The other is Hh ligand-independent mechanism. Mutations of *Ptch1* 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ tomatidine, an inactive cyclopamine analogue. Although 1-day treatment with 10 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ (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.

Fig. 2. The Hh pathway is activated in human breast carcinoma cells. Four human breast carcinoma cell lines, BT-474, SK-BR-3, MDA-MB231, and MCF-7, and one colon cancer cell line, DLD-1, are subjected to immunostaining with antibodies against indicated proteins Shh, Ptch1, and Gli1 (brown). Nuclei were counterstained with hematoxylin (purple). All of four breast carcinoma cell lines express high levels of Shh, Ptch1, and Gli1, whereas DLD-1 cells do not express detectable levels of indicated proteins ($\times 400$). Bars, 10 μm .



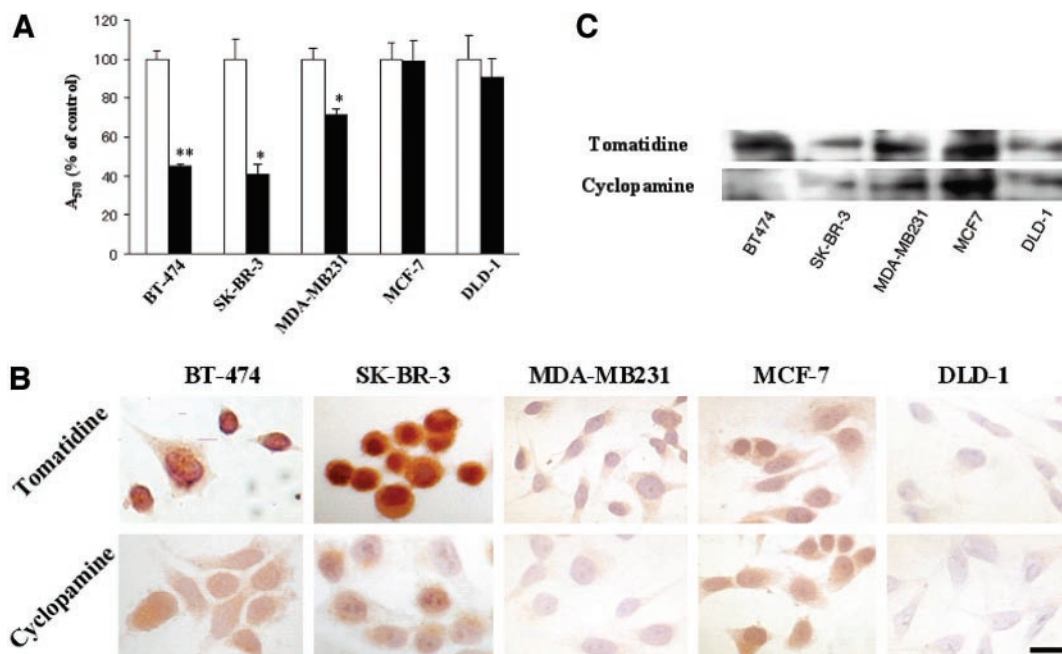


Fig. 3. A. Cyclopamine decreases the cell number of breast cancer cells. Cells were treated with 10 $\mu\text{mol/L}$ tomatidine (\square) or 10 $\mu\text{mol/L}$ cyclopamine (\blacksquare) at 37°C for 4 days as described in Materials and Methods. Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, detected at an absorbance at 570 nm ($A_{570\text{ nm}}$; $n = 3$ independent wells) and expressed as percentage of control (control medium containing methanol alone) \pm SD. Representative data from one of three independent experiments are shown. *, $P < 0.01$; **, $P < 0.001$. B and C. Cyclopamine decreases the expression of Gli1 in BT-474 and SK-BR-3 cells. BT-474, SK-BR-3, MDA-MB231, MCF-7, and DLD-1 were treated with 10 $\mu\text{mol/L}$ tomatidine or 10 $\mu\text{mol/L}$ 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) show decreased Gli1 expression compared with cells treated with tomatidine (B, top panel). MCF-7 and DLD-1 shows no difference between before and after cyclopamine treatment ($\times 400$). Bars, 10 μm . C. Immunoblotting shows almost same result as immunostaining.

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