

High expression of Sonic Hedgehog signaling pathway genes indicates a risk of recurrence of breast carcinoma

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Background: Abnormal activation of the Sonic Hedgehog (*SHH*) signaling pathway contributing to carcinogenesis of some organs has been reported in the literature. We hypothesize that activation of the *SHH* pathway contributes to the recurrence of breast carcinoma.

Methods: Fifty consecutive patients with invasive breast carcinoma following curative resection were enrolled in this prospective study. The ratios of messenger RNA (mRNA) expression for Sonic Hedgehog (*SHH*), patched homolog-1 (*PTCH-1*), glioma-associated oncogene-1 (*GLI-1*), and smoothened (*SMO*) were measured between breast carcinoma tissue and paired noncancerous breast tissue. These ratios were compared with their clinicopathologic characteristics. These factors and the mRNA ratios were compared between patients with recurrence and those without recurrence.

Results: The size of the invasive cancer correlated significantly with the ratio of *SHH* mRNA ($P=0.001$), that of *PTCH-1* mRNA ($P=0.005$), and that of *SMO* mRNA ($P=0.021$). Lymph node involvement correlated significantly with the ratio of *SMO* mRNA ($P=0.041$). The correlation between Her-2 neu and the ratio of *GLI-1* mRNA was statistically significant ($P=0.012$). Each ratio of mRNA of *SHH*, *PTCH-1*, *GLI-1*, and *SMO* correlated significantly with cancer recurrence ($P<0.001$ for each).

Conclusion: We suggest that high expression of *SHH* mRNA, *PTCH-1* mRNA, *GLI-1* mRNA, and *SMO* mRNA in breast cancer tissue correlates with invasiveness and is a potential biomarker to predict postoperative recurrence.

Keywords: *SHH* pathway, breast carcinoma, prediction, recurrence

Introduction

Rates of breast cancer, a leading cause of death in Taiwan and many other countries, have increased rapidly in recent times.¹ In spite of advances in diagnostic tools and surgical techniques, and chemotherapy, radiotherapy, and targeted therapy, the potential for cancer recurrence remains.² Early prediction of those who are likely to experience postoperative recurrence is a challenge. For those with a high potential of recurrence, postoperative adjuvant therapies have to be introduced early. This makes it important to identify new biomarkers in predicting patient prognosis.

The Sonic Hedgehog (*SHH*) signaling pathway plays a critical role in organizing cell growth and differentiation during embryonic tissue patterning,³⁻⁵ and is important in mouse mammary gland development.⁶ Disruption of the *Patched homolog-1* (*PTCH-1*) or *glioma-associated oncogene-2* (*GLI-2*) gene results in severe defects in ductal morphogenesis, such as ductal dysplasia, similar to human breasts.^{6,7} In vitro research shows that disruptions of these genes also occur in breast carcinoma.⁷ These implicate the potential role of the *SHH* pathway in breast oncogenesis. However,

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whether the *SHH* pathway contributes to cancer recurrence remains unknown.

We hypothesize that *SHH* activation contributes to the recurrence of breast carcinoma. This prospective study was designed to assess the correlation between mRNA expression of *SHH*, *PTCH-1*, *glioma-associated oncogene-1 (GLI-1)*, and *smoothened (SMOH)* and postoperative outcomes.

Materials and methods

Eligibility and exclusion criteria

Sixty patients with invasive ductal carcinoma of the breast undergoing surgery were screened prospectively for entry into this institutional review board (Far Eastern Memorial Hospital Research Ethics Review Committee) approved study between September 2008 and December 2009. Exclusion criteria included preoperative neoadjuvant therapies, previous mastectomy (recurrent category), a nearest resection margin of less than 5 mm, carcinoma in situ, refusal to participate, and lack of attendance for regular postoperative follow-up. After exclusion, 50 consecutive female patients of mean age 54.61 ± 10.25 years were enrolled.

Operative procedures and methods

Surgical procedures included 20 modified radical mastectomies and 30 partial mastectomies plus axillary lymph node dissection. At the end of each operation, we did a 0.5×0.5 cm sized tissue biopsy from both cancerous and noncancerous portions of the resected specimens. The noncancerous biopsy site was at least 2 cm away from the cancer margin. The instruments were changed between biopsy procedures to avoid transfer of cancer cells into noncancerous tissues.

Formalin-fixed, paraffin-embedded sections of obtained tissues were stained with hematoxylin for histology according to World Health Organization breast carcinoma histologic classification criteria.⁸ The clinical stage was categorized as the American Joint Committee on Cancer TNM classification (7th edition).⁸ Clinicopathologic characteristics regarding age, invasive tumor size, lymph node metastasis, lymphovascular invasion, and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 (Her-2neu) status were obtained from clinical and pathology reports (Table 1). All patients agreed to participate in this study and signed an informed consent before surgery.

Detection of human mRNA for *SHH*, *PTCH-1*, *GLI-1*, and *SMOH*

The examination included extraction of RNA and reverse transcription and amplification of cDNA for *SHH*,

Table 1 Clinicopathologic characteristics of patients (n=50)

Parameters	Number of Patients (%)
Right or left side	
Right	24 (48.0%)
Invasive tumor size	
pT1 <2 cm	27 (54.0%)
pT2 2–5 cm	18 (36.0%)
pT3 >5 cm	5 (10.0%)
Lymph node involvement	
pN0	26 (52.0%)
pN1	12 (24.0%)
pN2	7 (14.0%)
pN3	5 (10.0%)
Vascular-lymphatic invasion	
Present	22 (44.0%)
Perineural invasion	
Present	22 (44.0%)
Estrogen receptor	
>40%	24 (48.0%)
Progesterone receptor	
>40%	13 (26.0%)
Her-2 neu	
0	9 (18.0%)
1+	11 (22.0%)
2+	10 (20.0%)
3+	20 (40.0%)
Recurrence	2 (4.0%)
Recurrence-related death	0 (0.0%)
Follow-up, months, mean \pm SD	30.2 \pm 14.8

Notes: Age (mean \pm SD) = 54.61 ± 10.25 ; pT1–T3, pN0–N3 according to *AJCC Cancer Staging Manual* (7th edition).⁸

Abbreviation: SD, standard deviation.

PTCH-1, *GLI-1*, *SMOH*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by real-time polymerase chain reaction (PCR).

Extraction of RNA and reverse transcription PCR

RNA was extracted from tumor tissue and noncancerous tissue using the innuPREP RNA mini kit (Analytik, Jena AG, Jena, Germany). All the tissues were homogenized in lysis buffer at room temperature for 5 minutes, and then centrifuged at 12,000 rpm for 2 minutes. Each sample was mixed with 70% alcohol and then centrifuged. We added a first wash buffer and centrifuged for one minute, then a second wash buffer and repeated the procedure. After removing the ethanol completely, the samples were centrifuged at 13,000 rpm for 3 minutes. To elute the RNA, we added RNase-free water, incubated the samples for 3 minutes, and then centrifuged them again.

cDNA was synthesized from 1 μ g of mRNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription reaction

solution consisted of 2.0 μL 10 \times reverse transcription buffer, 0.8 μL 100 mM dNTP mix, 2.0 μL 10 \times reverse transcription random primers, and 1.0 μL of MultiScribe reverse transcriptase (Applied Biosystems). The RNA solution was mixed with reverse transcription solution and incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds.

Quantification of gene expression by real-time PCR

Real-time PCR was carried out on a LightCycler 480 (Roche, Mannheim, Germany) using SYBR Green PCR mix (Clontech, Palo Alto, CA, USA). The specific primer sequences were: *SHH* (forward) 5'-GAA AGC AGA GAA CTC GGT GG-3' and (reverse) 5'-GGA AAG TGA GGA AGT CGC TG-3'; *PTCH-1* (forward) 5'-CTC CCA AGC AAA TGT ACG AGC A-3' and (reverse) 5'-TGA GTG GAG TTC TGT GCG ACA C-3'; *GLI-1* (forward) 5'-CTC CCG AAG GAC AGG TAT GTA AC-3' and (reverse) 5'-CCC TAC TCT TTA GGC ACT AGA GTT G-3'; *SMOH* (forward) 5'-GGG AGG CTA CTT CCT CAT CC-3' and (reverse) 5'-GGC AGC TGA AGG TAA TGA GC-3'; and *GAPDH* (forward) 5'-CAC CAC CAA CTG CTT AG-3' and (reverse) 5'-CTT CAC CAC CTT CTT GAT G-3'. The housekeeping gene *GAPDH* was used as a loading control. PCR conditions were as follows: one cycle at 95°C for one minute followed by 40 cycles at 95°C for 10 seconds, 58°C for 5 seconds, and 72°C for 20 seconds. The specificity of the PCR products was tested by dissociation curves. The crossing points of primer probes were normalized to *GAPDH*. Relative values of transcripts were calculated using the equation $2^{-\Delta\Delta\text{Ct}}$, where ΔCt is equal to the difference in crossing point for target and reference and relative quantization according to the following equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{control-sample})}}$$

Western blot analysis

All tissues were homogenized. After evaporation of liquid nitrogen, lysis buffer containing protease inhibitor was added. The samples were placed on ice for 10 minutes before centrifugation. The supernatants containing proteins were harvested, and protein concentration was determined using the bicinchoninic acid assay. Protein samples were diluted in buffer, boiled for 10 minutes, loaded onto gels, and electrophoresed. Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). These membranes

were then blocked with 5% milk and 0.1% Tween-20 (Sigma-Aldrich, St Louis, MO, USA) in Tris-buffered saline at pH 7.4 for one hour prior to overnight incubation with the primary antibody. The antibodies included a rabbit polyclonal antibody against human *SHH* (1843-1, 1:1,000, Epitomics, Burlingame, CA, USA), human *PTCH-1* (NBP1-71662, 1:500, Novus Biologicals, Littleton, CO, USA), human *GLI-1* (AB3444, 1:1000), and human *SMOH* (NBP1-01011, 1:500, Novus Biologicals), respectively, and a mouse monoclonal antibody against human actin (AC-15, 1:1,000). Blots were washed and incubated with the appropriate peroxidase-conjugated secondary antibody. Immunorecognition was done using enhanced chemiluminescence. Images were captured and analyzed densitometrically using MultiGauge version 3.0 software (Fuji Photo Film Co. Ltd., Tokyo, Japan). The grayscale values of the bands for *SHH*, *PTCH-1*, *GLI-1*, and *SMOH* were normalized to determine the protein level. The experiments were repeated three times independently.

Ratio measurement

After examination, the ratios of mRNA expression in cancer tissue and in noncancerous tissue were measured for *SHH*, *PTCH-1*, *GLI-1*, and *SMOH*.

Follow-up

After discharge, the patients were followed up regularly at the outpatient clinic (mean 30.2 \pm 14.8 months, range 36–48 months) for periodic assessment, including breast ultrasonography, abdominal ultrasonography, serum CA153 and carcinoembryonic antigen (every 3 months during the first 3 years, and every 6 months thereafter), chest X-ray, and a whole body bone scan (every 6 months for one year, then annually). Magnetic resonance imaging or positron emission tomography-computed tomography (PET-CT) scan was done selectively if there was suspicion of recurrence.

Confirmation of recurrence depends upon two points. One is from the imaging studies, including ultrasonography, chest X-ray, whole body bone scan, magnetic resonance imaging, or PET-CT scan. The other is the tissue proof. Biopsy is a key procedure to establish the pathologic diagnosis, if it is feasible. The pathologist usually performs the immunohistochemical stain, estrogen receptor, progesterone receptor and the Her-2 neu for the detected lesion between recurrence and a second primary breast tumor.

Outcome measures

Irrespective of location, detection of a tumor on any imaging was defined as recurrence.

Statistical analysis

The correlation between recurrence, clinicopathologic variables, and the ratio of each mRNA was analyzed. Comparisons between groups were performed using the chi-square test (or Fisher's exact test) for continuous variables. Statistical analysis was performed by analysis of variance using Statistical Package for the Social Sciences version 10 software (SPSS Inc, Chicago, IL, USA). Statistical significance was considered at $P < 0.05$.

Results

All patients survived, but morbidity was noted in two patients (seroma, subsided after conservative treatment). Two patients (4%) had recurrence during follow-up. Table 2 shows the mean values for *SHH* mRNA, *PTCH-1* mRNA, *SMOH* mRNA, and *GLI-1* mRNA detected in cancer tissue and non-cancerous tissue. The mean ratios of *SHH* mRNA, *PTCH-1* mRNA, *GLI-1* mRNA, and *SMOH* mRNA between cancer tissue and noncancerous tissue were 2.79 ± 2.50 , 4.21 ± 7.55 , 1.88 ± 1.69 , and 2.91 ± 3.97 , respectively.

Table 3 indicates the correlation between clinicopathologic characteristics and the ratio of each mRNA. The size of invasive cancer correlated significantly with mRNA ratios for *SHH* ($P=0.001$), *PTCH-1* ($P=0.005$), and *SMOH* ($P=0.021$), respectively (Figure 1). Lymph node involvement correlated significantly with the ratio of *SMOH* mRNA ($P=0.041$) while Her-2 neu correlated significantly with the ratio of *GLI-1* mRNA ($P=0.012$).

Compared with the mRNA values, the amounts of protein detected by Western blot were relatively small. The mean values for the tumors were 5.26 for *SHH*, 1.80 for *PTCH-1*, and 3.16 for *GLI-1*, whereas those for noncancerous tissue were 3.80 for *SHH*, 1.55 for *PTCH-1*, and 1.97 for *GLI-1*. The amounts of protein detected for *SMOH* were too small to measure, but were higher in tumors, albeit not significantly so (Figure 2), while the ratio of cancer/non-cancerous tissue of protein for *GLI-1* correlated significantly with expression of Her-2 neu ($P=0.012$).

Table 2 Mean of ratios of mRNA for different genes in the Sonic Hedgehog pathway between cancerous tissue and noncancerous tissue by real-time polymerase chain reaction

Mean	<i>SHH</i>	<i>PTCH-1</i>	<i>GLI-1</i>	<i>SMOH</i>
Cancer tissue	15.76	12.81	6.9	2.51
Non-cancerous tissue	7.12	6.83	2.47	1.18
Ratio (mean \pm SD)	2.79 ± 2.50	4.21 ± 7.55	1.88 ± 1.69	2.91 ± 3.97

Abbreviations: *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1; *GLI-1*, glioma-associated oncogene homolog-1; *SMOH*, smoothened; SD, standard deviation; mRNA, messenger RNA.

Table 4 shows a comparison of the ratios of mRNA between those with recurrence and those without recurrence. Each ratio is statistically significant (both $P < 0.001$). Table 5 shows the significant factors affecting recurrence, including invasive tumor size ($P=0.024$), and mRNA ratios for *SHH*, *PTCH-1*, *GLI-1*, and *SMOH* ($P < 0.001$ for each).

Discussion

Our study shows that, compared with paired noncancerous tissue, a higher expression of *SHH* mRNA, *PTCH-1* mRNA, *GLI-1* mRNA, and *SMOH* mRNA in breast cancer tissue is associated with an increased risk of recurrence (Tables 4 and 5). Excluding *GLI-1*, all correlated significantly with the size of the invasive cancer (Table 2). In addition, *SMOH* mRNA correlated with lymph node involvement (Table 2). Both tumor size and lymph node involvement are invasive characteristics and significant prognostic determinants.⁹ As Souzaki et al have mentioned, the *SHH* pathway mediates progression from noninvasive cancer to invasive cancer.¹⁰

The mRNA for each gene was expressed in over 98% of cancer tissue. This high expression is similar to that reported by Kubo et al¹¹ and Cui et al,¹² but different from that reported by Mukherjee et al.¹³ We attribute this discrepancy to two possible factors. One is the different examination methods used. Real-time PCR is more sensitive than conventional PCR or Western blotting.¹⁴ The other reason is the different clinicopathologic stages of the patients. All of our patients had invasive carcinoma of variable size (T₁, 54%, Table 1), similar to those in the studies of Kubo et al and Cui et al,^{11,12} whereas the stage of patients in the study by Mukherjee et al was more advanced.¹³

GLI-1 is a well known target gene in the *SHH* pathway.^{15,16} The expression of nuclear *GLI-1* is positively associated with *SHH* in breast tissues.¹² Aberrant activation of the *SHH* pathway leads *GLI-1* into the nucleus, promoting gene transcription and maintaining the biological behavior of cancer.^{15,16} *GLI-1* contributes to the proliferation, survival, and migration of inflammatory breast cancer.¹⁷

According to ten Haaf et al, *GLI-1* expression is associated with an unfavorable outcome of invasive cancer.¹⁸ Similarly, our recurrent cases had a higher expression of *GLI-1* mRNA (Tables 4 and 5). Overexpression of *GLI-1* in other cancers also adversely affects the risk of recurrence.^{19,20}

Kubo et al reported that nuclear *GLI-1* correlates with estrogen receptor status.¹¹ Koga et al mentioned a link between the *SHH* pathway and estrogen receptor alpha.²¹ Kameda et al suggested this pathway as a therapeutic target for those with estrogen receptor-negative carcinoma.²² In our cases, *GLI-1*

Table 3 Correlation of clinicopathologic factors of breast cancer and the ratios of *SHH* mRNA, *PTCH-1* mRNA, *GLI-1* mRNA, and *SMOH* mRNA between cancerous and noncancerous tissue

Factors	Ratio of mRNA, mean ± SD (95% CI)				P-value			
	<i>SHH</i>	<i>PTCH-1</i>	<i>GLI-1</i>	<i>SMOH</i>	<i>SHH</i>	<i>PTCH-1</i>	<i>GLI-1</i>	<i>SMOH</i>
Right or left side								
Right	0.59±0.67	0.98±1.31	1.19±1.75	1.49±2.72	0.133	0.502	0.345	0.917
Left	1.32±2.26	1.39±2.69	0.82±1.02	1.41±2.73				
Invasive size								
pT1 <2 cm	0.62±0.68	0.85±1.19	0.86±1.04	1.18±1.49	0.001	0.005	0.358	0.021
pT2 2–5 cm	0.84±0.96	1.16±1.97	1.03±1.70	1.45±2.87				
pT3 >5 cm	3.96±4.54	4.70±5.03	2.00±1.68	5.41±5.74				
Lymph node involvement								
pN0	0.63±0.62	0.81±0.76	0.83±0.85	1.20±1.56	0.460	0.087	0.664	0.041
pN1	1.42±2.50	1.33±2.44	0.83±1.00	0.94±0.92				
pN2	0.88±1.17	1.04±1.97	0.88±1.62	1.00±1.23				
pN3	1.99±3.81	3.79±4.82	1.53±1.76	4.35±5.51				
Vascular-lymphatic invasion								
Absent	0.62±0.74	1.34±1.68	1.41±2.29	2.14±3.77	0.486	0.862	0.536	0.359
Present	1.04±1.95	1.22±2.08	1.05±1.16	1.23±1.53				
PR								
≤40%	1.15±2.21	1.39±3.00	1.36±1.96	2.26±4.45	0.409	0.557	0.236	0.362
>40%	0.61±0.72	0.85±1.37	0.63±1.00	0.99±1.77				
ER								
≤40%	0.73±0.91	0.44±0.29	0.55±0.49	0.52±0.36	0.817	0.441	0.465	0.382
>40%	0.91±1.78	1.26±2.53	1.08±1.72	1.91±3.78				
Peri-N								
Absent	1.07±2.01	0.96±1.36	1.00±1.81	1.49±2.86	0.453	0.694	0.877	0.692
Present	0.72±0.82	1.18±2.08	1.07±1.14	1.20±1.51				
Her-2 neu								
0	1.06±0.76	3.34±4.90	2.84±3.53	0.45±0.35	0.414	0.068	0.012	0.875
1	0.99±1.27	1.02±1.01	0.96±1.07	1.88±3.99				
2	0.85±0.48	1.24±2.15	0.46±0.28	1.59±2.19				
3	0.58±0.37	0.64±0.45	0.67±0.71	1.66±2.95				

Notes: Ratio indicates mRNA of cancerous tissue/mRNA of noncancerous tissue. The bold values indicate the P-value <0.05 is statistically significant.

Abbreviations: *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1; *GLI-1*, glioma-associated oncogene homolog-1; *SMOH*, smoothened; ER, estrogen receptor; PR, progesterone receptor; CI, confidence interval; SD, standard deviation; mRNA, messenger RNA; Peri-N, perineural involvement.

did not correlate with estrogen receptor or progesterone receptor status, but correlated with Her-2 neu.

PTCH-1 promotes tumor formation by inhibiting expression of *Fas*, which mediates apoptosis.^{15,16,23} *PTCH-1* regulates cell cycle progression and predisposes cells to proliferative and expansive behavior.^{15,16,24} Our patients with recurrence showed overexpression of *PTCH-1*. High *PTCH-1* also affects metastasis of other cancers.^{19,25} *SMOH* encoding of a transmembrane protein acting in a receptor complex was affected by *PTCH-1*.^{15,16,18,23} Tao et al studied patients having triple-negative breast cancer by immunohistochemical analysis of factors in the SHH pathway.²⁶ They observed that expression of *PTCH-1* was significantly decreased in breast cancer compared with mammary hyperplasia. They hypothesized that continuous activation of mutated Sonic Hedgehog signaling may transform breast stem cells to oncogenesis and malignant progression.²⁶

The *SHH* pathway is activated mainly in cancer stem cells and not in each cancer cell.²⁷ Cancer stem cells capable of initiating and sustaining the cancer are usually highly proliferative and invasive.^{28–33} Their presence has been reported in various malignancies, including those of the breast.^{28–35} However, from the perspective of amount, cancer stem cells are only a small population among all cancer cells.^{27–33} Whether activation of the *SHH* pathway occurs early or late in carcinogenesis remains a matter of debate.^{12,36,37} Some research has demonstrated that increased *SHH* is associated with early pTNM stage, indicating that its upregulation may occur early.^{33,36,37} Whereas *SHH* also activates in late or recurrent stages in other malignancies.^{19,20,37–39} We found that overexpression of these mRNA genes correlates with larger tumor size and recurrence (Tables 3 and 4). We believe that this pathway activates not only early on in cancer stem cells to enhance growth, but also later on to accelerate

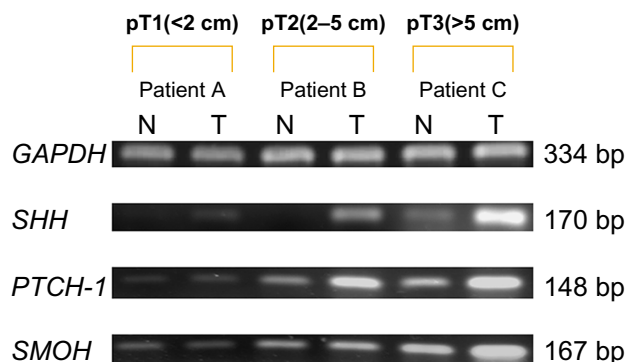


Figure 1 PCR expression of mRNA for *SHH*, *PTCH-1*, and *SMOH*. Real-time PCR parameters were cycled as follows: hot start at 95°C for one minute, followed by 30 cycles of denaturing at 95°C for 10 seconds, annealing at 58°C for 5 seconds, and extension at 72°C for 20 seconds. PCR products were detected using 2% agarose gel to confirm the expected sizes. N indicates breast tissue of the nontumor portion. T indicates tumor tissue. Patients A and B had no postoperative recurrence. Patient C had postoperative recurrence. PCR expression of *SHH*, *PTCH-1*, and *SMOH* showed a significant difference between this and the size of invasive carcinoma. Patient C had a significantly higher ratio of mRNA for *SHH*, *PTCH-1*, and *SMOH*. The ratios of T/N for *SHH* in patient A, patient B, and patient C were 1.91, 3.98, and 8.06, respectively. The ratios of T/N for *PTCH-1* in patient A, patient B, and patient C were 1.21, 1.34 and 1.40, respectively. The ratios of T/N for *SMOH* in patient A, patient B, and patient C were 0.83, 1.07, and 1.18, respectively.
Abbreviations: PCR, polymerase chain reaction; *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1; *SMOH*, smoothened; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

progression and recurrence. MDA-MB-231 cells expressing *SHH* enhance chemotactic migration and the degradation/invasion of extracellular matrix proteins.³³

In both in vivo and in vitro studies, blockade of *SHH* pathway may inhibit tumor growth.⁴⁰ Cancer recurrence is complicated and contributed to by circulating cancer cells detached from the primary tumor, the molecular signature of the cancer, and the susceptibility of the microenvironment of the remnant tissues.^{41,42} Cui et al showed that the *SHH* gene is important in vascular formation, thus contributing to recurrence.¹² Upregulation of the *SHH* pathway in a tumor may impact the microenvironment.

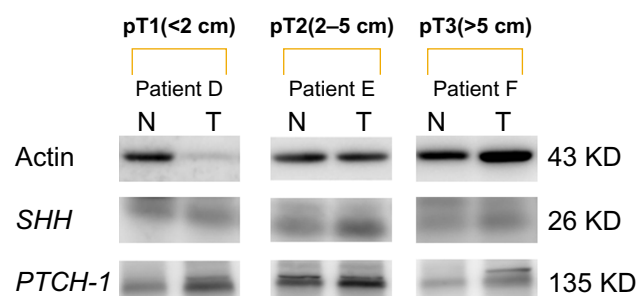


Figure 2 Protein expression of *SHH* and *PTCH-1* by Western blot. N indicates breast tissue from the nontumor portion. T indicates tumor tissue. Tumor size was pT1 (<2 cm) for patient D, pT2 (2–5 cm) for patient E, and pT3 (>5 cm) for patient F. Expression of *SHH* and *PTCH-1* in cancerous tissue was higher than in noncancerous tissue in these three patients with different tumor sizes.
Abbreviations: *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1.

Table 4 Comparison of the ratio of mRNA expression in tumor/nontumor tissue between patients with recurrence and those without recurrence

Parameters	Recurrence		P-values
	No (n=48)	Yes (n=2)	
mRNA expression ratio*			
<i>SHH</i>	0.35 (0.48–1.42)	30.70 (–351.06–412.45)	<0.001
<i>PTCH-1</i>	0.39 (0.54–1.67)	313.51 (–3,605.57–4,232.59)	<0.001
<i>GLI-1</i>	0.33 (0.54–1.26)	813.59 (–9,475.62–11,102.8)	<0.001
<i>SMOH</i>	0.665 (0.66–2.17)	647.82 (–7,500.92–8,796.55)	<0.001

Note: *Indicates median values; (parentheses) indicate 95% confidence interval.
Abbreviations: *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1; *GLI-1*, glioma-associated oncogene homolog-1; *SMOH*, smoothened; mRNA, messenger RNA.

For further clinical application, we suggest an mRNA study of *PTCH-1* and *GLI-1* from core needle biopsy tissue (if feasible) as a reference for prognostication. This would provide useful information for both surgeons and patients before selecting a treatment option. In addition, using specific *SHH* pathway inhibitors to mitigate cancer recurrence is potentially a therapeutic strategy.^{40,43}

Only two patients had cancer recurrence. We attribute to this to two reasons. One is that the follow-up period is not long, and the other is that the number of study patients is not large, and these are the limitations of this study. However, it does give us information on activation of *SHH* in the recurrence pathway. From this study, we suggest that overexpression of *SHH* mRNA, *PTCH-1* mRNA, *GLI-1* mRNA,

Table 5 Comparison of clinicopathologic characteristics between patients with and without recurrence

Parameters	P-values
Age (≤40 vs >40 years)	0.853
Estrogen receptor (≤40% vs >40%)	0.287
Progesterone receptor (≤40% vs >40%)	0.777
Vascular lymphatic invasion (Absent vs present)	0.317
Perineural invasion (Absent vs present)	0.926
Invasive size (pT1 vs pT2 vs pT3)	0.024
Lymph node involvement (pN0 vs pN1 vs pN2 vs pN3)	0.461
Her-2 neu	0.179
Right or left side	0.307
Ratio of <i>SHH</i> mRNA	<0.001
Ratio of <i>GLI-1</i> mRNA	<0.001
Ratio of <i>PTCH-1</i> mRNA	<0.001
Ratio of <i>SMOH</i> mRNA	<0.001

Note: Ratio indicates mRNA of cancerous tissue/mRNA of noncancerous tissue.
Abbreviations: *SHH*, Sonic Hedgehog; *PTCH-1*, patched homolog-1; *GLI-1*, glioma-associated oncogene homolog-1; *SMOH*, smoothened; mRNA, messenger RNA; vs, versus.

and *SMOH* mRNA in breast cancer tissues is a potential biomarker for prediction of postoperative recurrence.

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Disclosure

The authors declare that they have no conflicts of interest to report in this work.

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