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

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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 and is important in mouse mammary gland development.4 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.5,6 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,
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 oncopGene-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).9 Clinicalopathologic characteristics regarding age, invasive tumor size, lymph node metastasis, lymphovascular invasion, and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 (Her-2 neu) 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, 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 µg 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 × reverse transcription buffer, 0.8 μL 100 mM dNTP mix, 2.0 μL 10 × 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: 5′-GAA AGC AGA GAA CTC GGT GG-3′ and (reverse) 3′-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 AGT GAT 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 CAT 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 − ∆∆Ct, where ∆Ct is equal to the difference in crossing point for target and reference and relative quantification according to the following equation:

\[
\text{Ratio} = \frac{E_{\text{target}}^{Ct \text{-target}} - E_{\text{control}}^{Ct \text{-control}}}{E_{\text{ref}}^{Ct \text{-ref}} - E_{\text{ref}}^{Ct \text{-control}}}
\]

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±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 noncancerous 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 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. As Souzaki et al have mentioned, the SHH pathway mediates progression from noninvasive cancer to invasive cancer.

The mRNA for each gene was expressed in over 98% of cancer tissue. This high expression is similar to that reported by Kubo et al and Cui et al, but different from that reported by Mukherjee et al. 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. 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, whereas the stage of patients in the study by Mukherjee et al was more advanced. GLI-1 is a well known target gene in the SHH pathway.

The expression of nuclear GLI-1 is positively associated with SHH in breast tissues. Aberrant activation of the SHH pathway leads GLI-1 into the nucleus, promoting gene transcription and maintaining the biological behavior of cancer. GLI-1 contributes to the proliferation, survival, and migration of inflammatory breast cancer.

According to ten Haaf et al, GLI-1 expression is associated with an unfavorable outcome of invasive cancer. 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.

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

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**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

<table>
<thead>
<tr>
<th>Mean</th>
<th>SHH</th>
<th>PTCH-1</th>
<th>GLI-1</th>
<th>SMOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer tissue</td>
<td>15.76</td>
<td>12.81</td>
<td>6.9</td>
<td>2.51</td>
</tr>
<tr>
<td>Non-cancerous tissue</td>
<td>7.12</td>
<td>6.83</td>
<td>2.47</td>
<td>1.18</td>
</tr>
</tbody>
</table>

**Ratio (mean ± SD)**

<table>
<thead>
<tr>
<th>SHH</th>
<th>PTCH-1</th>
<th>GLI-1</th>
<th>SMOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.79±2.50</td>
<td>4.21±7.55</td>
<td>1.88±1.69</td>
<td>2.91±3.97</td>
</tr>
</tbody>
</table>

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

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**Table 3** 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.
did not correlate with estrogen receptor or progesterone receptor status, but correlated with Her-2 neu.

**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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Ratio of mRNA, mean ± SD (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>SHH</strong></td>
<td><strong>PTCH-1</strong></td>
</tr>
<tr>
<td>Right or left side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>0.59±0.67</td>
<td>0.98±1.31</td>
</tr>
<tr>
<td>Left</td>
<td>1.32±2.26</td>
<td>1.39±2.69</td>
</tr>
<tr>
<td>Invasive size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1 &lt; 2 cm</td>
<td>0.62±0.68</td>
<td>0.85±1.19</td>
</tr>
<tr>
<td>pT2 &gt; 5 cm</td>
<td>0.84±0.96</td>
<td>1.16±1.97</td>
</tr>
<tr>
<td>pT3 &gt; 5 cm</td>
<td>3.96±4.54</td>
<td>4.70±5.03</td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>0.63±0.62</td>
<td>0.81±0.76</td>
</tr>
<tr>
<td>pN1</td>
<td>1.42±2.50</td>
<td>1.33±2.44</td>
</tr>
<tr>
<td>pN2</td>
<td>0.88±1.17</td>
<td>1.04±1.97</td>
</tr>
<tr>
<td>pN3</td>
<td>1.99±3.81</td>
<td>3.79±4.82</td>
</tr>
<tr>
<td>Vascular-lymphatic invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0.62±0.74</td>
<td>1.34±1.68</td>
</tr>
<tr>
<td>Present</td>
<td>1.04±1.95</td>
<td>1.22±2.08</td>
</tr>
<tr>
<td>PR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40%</td>
<td>1.15±2.11</td>
<td>1.39±3.00</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>0.61±0.72</td>
<td>0.85±1.37</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40%</td>
<td>0.73±0.91</td>
<td>0.44±0.29</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>0.91±1.78</td>
<td>1.26±2.53</td>
</tr>
<tr>
<td>Peri-N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1.07±2.01</td>
<td>0.96±1.36</td>
</tr>
<tr>
<td>Present</td>
<td>0.72±0.82</td>
<td>1.18±2.08</td>
</tr>
<tr>
<td>Her-2 neu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.06±0.76</td>
<td>3.34±4.90</td>
</tr>
<tr>
<td>1</td>
<td>0.99±1.27</td>
<td>1.02±1.01</td>
</tr>
<tr>
<td>2</td>
<td>0.85±0.48</td>
<td>1.24±2.15</td>
</tr>
<tr>
<td>3</td>
<td>0.58±0.37</td>
<td>0.64±0.45</td>
</tr>
</tbody>
</table>

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.

The **SHH** pathway is activated mainly in cancer stem cells and not in each cancer cell. Cancer stem cells capable of initiating and sustaining the cancer are usually highly proliferative and invasive. Their presence has been reported in various malignancies, including those of the breast. However, from the perspective of amount, cancer stem cells are only a small population among all cancer cells. Whether activation of the **SHH** pathway occurs early or late in carcinogenesis remains a matter of debate. Some research has demonstrated that increased **SHH** is associated with early pTNM stage, indicating that its upregulation may occur early. Whereas **SHH** also activates in late or recurrent stages in other malignancies. 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
progression and recurrence. MDA-MB-231 cells expressing SHH enhance chemotactic migration and the degradation/invasion of extracellular matrix proteins.\(^5\)

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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recurrence</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA expression ratio*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHH</td>
<td>0.35</td>
<td>30.70</td>
</tr>
<tr>
<td>(0.48–1.42)</td>
<td>(–351.06–412.45)</td>
<td></td>
</tr>
<tr>
<td>PTCH-1</td>
<td>0.39</td>
<td>313.51</td>
</tr>
<tr>
<td>(0.54–1.67)</td>
<td>(–3605.57–4232.59)</td>
<td></td>
</tr>
<tr>
<td>GLI-1</td>
<td>0.33</td>
<td>813.59</td>
</tr>
<tr>
<td>(0.54–1.26)</td>
<td>(–9475.62–11102.8)</td>
<td></td>
</tr>
<tr>
<td>SMOH</td>
<td>0.665</td>
<td>647.82</td>
</tr>
<tr>
<td>(0.66–2.17)</td>
<td>(–7500.92–8796.55)</td>
<td></td>
</tr>
</tbody>
</table>

**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,
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.

References
