

# The Hedgehog signaling pathway is associated with poor prognosis in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype

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**Abstract.** Cancer stem cells (CSCs) have been suggested to serve an important role in tumor recurrence and metastasis in breast cancer. The hedgehog (Hh) signaling pathway is essential for the maintenance of breast CSCs. The present study used immunohistochemistry to investigate the expression of Patched (PTCH) and Gli1, which are the main components of the Hh signaling pathway, as well as the expression of cluster of differentiation (CD)44/CD24, which are markers for breast CSCs, in 266 patients with breast cancer. The combined expression of PTCH and Gli1 was significantly associated with larger tumors (>2.0 cm; P=0.001), lymph node metastasis (P=0.003), invasive lobular carcinoma (P=0.016) and Grade II-III tumors (P<0.001). In addition, PTCH and Gli1 expression was associated with lymph node metastasis (P=0.005 and P=0.001) and Grade II-II tumors (P=0.020 and P=0.033) in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. The expression of PTCH and Gli1 was also associated with significantly shorter overall survival and disease-free survival (DFS) in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. Multivariate Cox regression analysis demonstrated that PTCH expression and the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype were independent prognostic factors for decreased DFS in patients with breast cancer. These findings suggest that the Hh signaling pathway in breast CSCs may contribute to the poor outcome of patients with breast cancer.

## Introduction

Cancer stem cells (CSCs) are a small population of cells present in tumors, which exhibit stem cell-like properties, including self-renewal and multi-lineage differentiation potential (1,2). CSCs have been reported to serve an important role in tumor recurrence, metastasis and chemotherapeutic resistance in breast cancer (3,4). Targeting CSCs is considered a promising therapeutic strategy for the treatment of breast cancer (5), and identification of the signaling pathways that regulate breast CSCs may facilitate the development of therapeutic agents that target breast CSCs.

The hedgehog (Hh) signaling pathway is known to regulate cell proliferation and self-renewal in normal stem cells during embryonic development, as well as in malignant stem cells (6-8). The Hh signaling pathway is activated by binding of Hh ligands, including sonic hedgehog, desert hedgehog and Indian hedgehog, to the Patched (PTCH) receptor. PTCH receptor activation subsequently results in Smoothened activation, which eventually leads to regulation of the expression of Gli transcription factors that are responsible for cancer cell proliferation, apoptosis and invasion (9). Previous studies have demonstrated that Hh signaling regulates CSCs in several types of human cancer, including breast cancer (10), glioblastoma (11), glioma (12) and myeloid leukemia (13). In breast CSCs, the Hh signaling pathway has an important role in maintaining the cluster of differentiation (CD)44<sup>+</sup>/CD24<sup>-</sup> subpopulation and the side population of breast cancer cells (14). Activation of the Hh signaling pathway by Hh ligands and Gli1 or Gli2 overexpression promotes self-renewal of breast CSCs via modulation of Bmi-1 expression (10). However, it remains to be elucidated as to whether Hh signaling activation regulates breast CSCs and contributes to clinical outcomes in patients with breast cancer.

The cell adhesion molecules CD44 and CD24 are expressed on breast cancer cells, and are associated with cell adhesion, tumor initiation, development and metastasis (15). The CD44<sup>+</sup>/CD24<sup>-</sup> phenotype is often used as a marker to isolate breast CSCs from solid tumors (16). Breast cancer cells with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype exhibit stem cell-like properties (16), and are associated with enhanced invasion and metastasis (17,18). Furthermore, it has been reported that

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the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype contributes to relapse and poor prognosis in patients with breast cancer (19,20). It has previously been demonstrated that components of the Hh signaling pathway are highly upregulated in breast cancer cells with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, and that the Hh signaling pathway is essential for maintaining this population of breast cancer cells (14). However, the role of the Hh signaling pathway in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype remains to be determined.

The present study used immunohistochemistry to investigate the expression of PTCH, Gli1 and CD44/CD24 in 266 patients with breast cancer. The aim of the present study was to investigate the association between the expression of PTCH and Gli1, which are the main components of the Hh signaling pathway, in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, and to analyze the correlation of their expression with clinicopathological features and prognosis of breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype.

## Materials and methods

**Patients and tissue samples.** The Medical Ethics Committee of China Medical University (Shenyang, China) approved this retrospective study. Due to the retrospective nature of the present study, the Medical Ethics Committee waived the requirement for written informed consent by the patients. Human breast tissues were obtained from 266 female patients with sporadic breast cancer, who underwent surgery at the First Hospital of China Medical University between 2006 and 2010. The diagnosis of breast cancer was confirmed by pathological staining. A total of 232 patients had invasive ductal carcinoma, and 34 patients had invasive lobular carcinoma. The histological grade of the cancer was determined according to the World Health Organization grading system (21). Clinicopathological data, including patient age, menopausal status, tumor size and lymph node metastasis were retrospectively retrieved from medical records. None of the patients underwent radiation therapy or chemotherapy prior to surgery. Following surgery, 195 patients were followed up for 48-77 months. The chemotherapy regimens of these patients included CEF (cyclophosphamide + epirubicin + fluorouracil, n=151), CAF (cyclophosphamide + Adriamycin + fluorouracil, n=18) and CET (cyclophosphamide + epirubicin + taxol, n=26).

**Immunohistochemistry.** Immunohistochemical staining was performed as previously described (22). Briefly, sections (4 µm) were obtained from formalin-fixed and paraffin-embedded tissue blocks. Sections were deparaffinized with xylene, rehydrated in a graded alcohol series, and heated in citrate buffer solution (pH 6) for 10 min to retrieve antigens. To suppress endogenous peroxidase activity, the sections were treated with 3% H<sub>2</sub>O<sub>2</sub> at 37°C for 20 min. To block nonspecific protein binding sites, sections were incubated in 10% normal goat serum at 37°C for 30 min. For immunohistochemical staining of PTCH and Gli1, the sections were incubated with primary antibodies against PTCH (rabbit anti-human polyclonal antibodies; 1:100 dilution; cat. no. ab39266; Abcam, Cambridge, UK)

Table I. Clinicopathological characteristics of 266 patients with breast cancer.

| Clinicopathological feature     | Number | %    |
|---------------------------------|--------|------|
| Age                             |        |      |
| ≤50 years                       | 138    | 51.9 |
| >50 years                       | 128    | 48.1 |
| Menopausal status               |        |      |
| Premenopausal                   | 148    | 55.6 |
| Postmenopausal                  | 118    | 44.4 |
| Histologic type                 |        |      |
| Invasive ductal carcinoma       | 232    | 87.2 |
| Invasive lobular carcinoma      | 34     | 12.8 |
| Tumor size <sup>a</sup>         |        |      |
| ≤2.0 cm                         | 145    | 61.4 |
| >2.0, ≤5.0 cm                   | 91     | 38.6 |
| Lymph node metastasis           |        |      |
| Negative                        | 167    | 62.8 |
| Positive                        | 99     | 37.2 |
| Histological grade <sup>b</sup> |        |      |
| I                               | 42     | 21.2 |
| II                              | 124    | 62.6 |
| III                             | 32     | 16.2 |

<sup>a</sup>Tumor size was determined in 236 patients; <sup>b</sup>Histological grade was determined in 198 patients.

or Gli1 (rabbit anti-human polyclonal antibodies; 1:200 dilution; cat. no. ab92611; Abcam) overnight at 4°C. For double immunohistochemical staining of CD44 and CD24, sections were incubated with primary antibodies against CD44 (clone 156-3C11; mouse anti-human monoclonal antibodies; 1:800 dilution; cat. no. MA5-13890; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and CD24 (Clone SN3b; mouse anti-human monoclonal antibodies; 1:400 dilution; cat. no. MA5-11828; Thermo Fisher Scientific, Inc.) overnight at 4°C. Sections in which primary antibodies were replaced with PBS were used as a negative control. Sections were subsequently incubated with biotinylated secondary antibodies (1:1,000 dilution) for 30 min at 37°C, followed by incubation with streptavidin-horseradish peroxidase for an additional 20 min (LSAB kit; Dako, Glostrup, Denmark). For PTCH and Gli1, sections were stained with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and counterstained with hematoxylin. For CD44 and CD24 staining, CD24 was detected with Permanent Red (from the Double SP kit; Maixin Biotech. Co., Ltd., Fuzhou, China) and CD44 with DAB. Subsequently, the sections were dehydrated and mounted. Images from each section were captured using a Digital Sight digital camera under a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan).

**Evaluation of immunohistochemistry.** Immunoreactivity was evaluated by two independent investigators blinded to

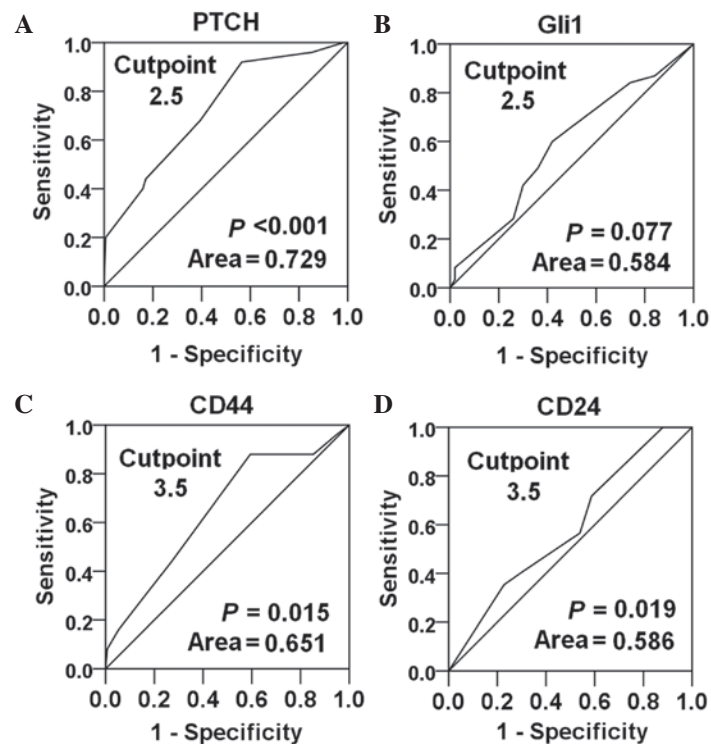


Figure 1. Receiver operating characteristic curves were used to determine the cutoff score for the expression of (A) PTCH, (B) Gli1, (C) CD44 and (D) CD24 in patients with breast cancer. The sensitivity and specificity for OS, DFS, OS and lymph node metastasis were plotted for PTCH, Gli1, CD44 and CD24 expression, respectively. The areas under the curve and P-values are indicated. PTCH, Patched; CD, cluster of differentiation; OS, overall survival; DFS, disease-free survival.

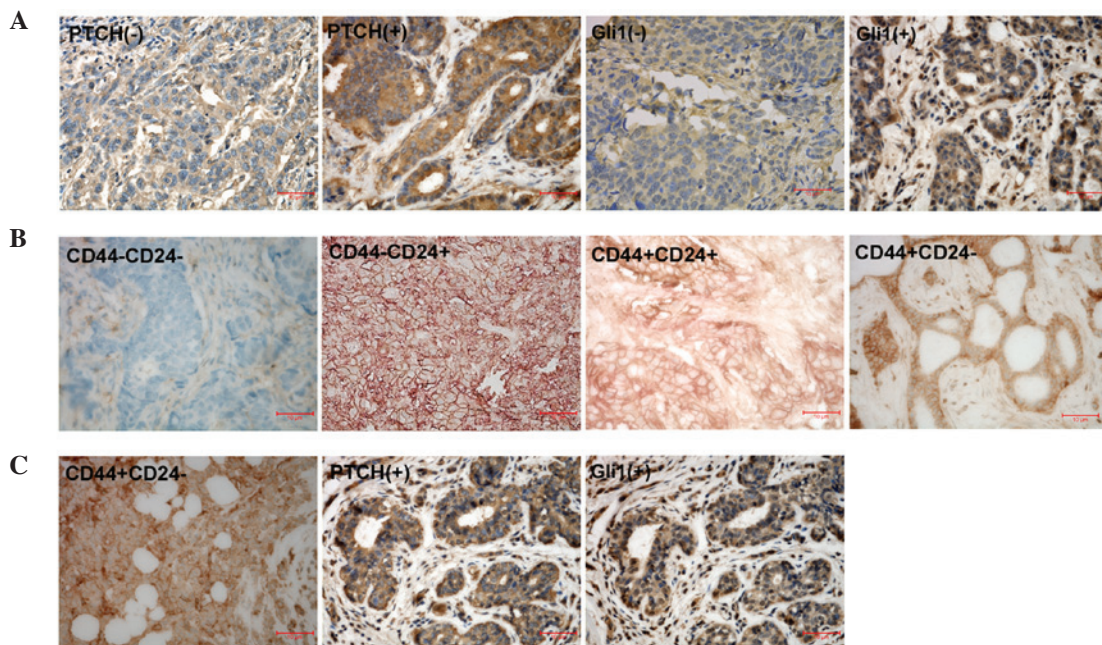


Figure 2. Representative immunohistochemical micrographs of PTCH, Gli1 and CD44/CD24 staining in breast cancer samples. (A) Representative micrographs of PTCH<sup>-</sup>, PTCH<sup>+</sup>, Gli1<sup>-</sup> and Gli1<sup>+</sup> staining. (B) Representative double immunohistochemical staining of CD44<sup>-</sup>/CD24<sup>-</sup>, CD44<sup>-</sup>/CD24<sup>+</sup>, CD44<sup>+</sup>/CD24<sup>+</sup> and CD44<sup>+</sup>/CD24<sup>-</sup>. (C) Representative PTCH<sup>+</sup> and Gli1<sup>+</sup> immunohistochemical staining in a CD44<sup>+</sup>/CD24<sup>-</sup> breast cancer sample. Magnification, x400. Scale bars, 10  $\mu$ m. PTCH, Patched; CD, cluster of differentiation.

the patients' clinicopathological characteristics, according to the percentage of stained cells and the intensity of immunoreactivity (23,24). Immunoreactive intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of stained cells was

scored as follows: 0, <5% stained cells; 1, 5-25% stained cells; 2, 26-50% stained cells; 3, 51-75% stained cells; and 4, >75% stained cells. The final immunoreactive score was calculated by multiplying the intensity score with the score for the percentage of stained cells, and was used to generate the



Table II. Association of the expression of PTCH or Gli1 with the expression of CD44<sup>+</sup>/CD24<sup>-</sup> in 266 breast cancer tissues.

| Clinicopathological feature | No. of cases (%) | CD44 <sup>+</sup> /CD24 <sup>-</sup> |           | P-value      |
|-----------------------------|------------------|--------------------------------------|-----------|--------------|
|                             |                  | Yes (%)                              | No (%)    |              |
| PTCH                        |                  |                                      |           |              |
| Negative                    | 109 (41.0)       | 30 (30.3)                            | 79 (47.3) | <b>0.006</b> |
| Positive                    | 157 (59.0)       | 69 (69.7)                            | 88 (52.7) |              |
| Gli1                        |                  |                                      |           |              |
| Negative                    | 126 (47.4)       | 35 (35.4)                            | 91 (54.5) | <b>0.003</b> |
| Positive                    | 140 (52.6)       | 64 (64.6)                            | 76 (45.5) |              |

P-values were obtained from Pearson  $\chi^2$  test. PTCH, Patched; CD, cluster of differentiation.

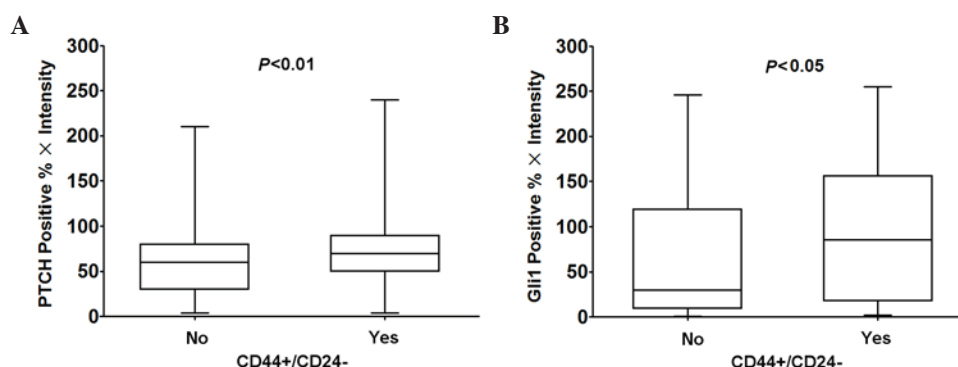


Figure 3. (A) PTCH and (B) Gli1 expression in breast cancer patients with or without CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. Data were analyzed by Mann-Whitney U test. PTCH, Patched; CD, cluster of differentiation.

receiver operating characteristic (ROC) curve analysis. The ROC was used to determine the cutoff value for discriminating tumors with positive expression of PTCH, Gli1, CD44 and CD24, from those with negative expression, as previously described by Kim *et al* (25).

**Statistical analysis.** Analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Pearson  $\chi^2$  or Fisher's exact probability tests were used to evaluate the association between PTCH, Gli1 and CD44<sup>+</sup>/CD24<sup>-</sup> expression, and the clinicopathological characteristics of the patients with breast cancer. Spearman rank correlation analysis was used to assess the association between PTCH and Gli1 expression and CD44<sup>+</sup>/CD24<sup>-</sup> expression. Survival probabilities were estimated using the Kaplan-Meier method and were assessed by a log-rank test. Disease-free survival (DFS) was calculated as the time between the first day of diagnosis and the occurrence of local recurrence or distant metastasis. Overall survival (OS) was calculated as the time between the first day of diagnosis and disease-related mortality. Univariate and multivariate Cox proportional hazards regression models were used for assessing the association between potential confounding variables and prognosis (OS or DFS). Mann-Whitney U test was used to compared the expression of PTCH and Gli1 in breast cancer with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype with non-CD44<sup>+</sup>/CD24<sup>-</sup> phenotype.  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**Clinicopathological characteristics.** Table I summarizes the clinicopathological characteristics of the 266 patients with breast cancer. The average age of the patients was 50.8 years (range, 29-74 years). The majority of these patients had a tumor that was diagnosed as invasive ductal carcinoma (87.2%), was <2 cm in size (61.4%) and was graded as histological Grade II (62.6%). Lymph node metastasis occurred in 99 (37.2%) of the 266 patients. Follow-up information was available for 195 patients with breast cancer. Relapses occurred in 144 cases and breast cancer-associated mortality occurred in 25 cases. The 5-year survival rate was 84.8%. The mean OS and DFS were 72.5 and 55.3 months, respectively.

**Expression of PTCH, Gli1, CD44 and CD24 in breast cancer tissues.** The expression of PTCH, Gli1, CD44 and CD24 was detected in 266 breast cancer tissues using immunohistochemistry. A ROC curve analysis was performed to determine an optimal cutoff score for the expression of PTCH, Gli1, CD44 and CD24 in breast cancer samples, based on the sensitivity and specificity for each clinicopathological parameter. The parameter with the biggest area under the curve was selected. According to the criteria, OS, DFS, OS and lymph node metastasis were selected to determine the cutoff values for PTCH, Gli1, CD44 and CD24, respectively. Cutoff scores of 2.5, 2.5, 3.5 and 3.5 were determined for PTCH, Gli1, CD44

Table III. Association of the expression of Gli1 and PTCH with the clinicopathological features in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype.

| Clinicopathological feature | No. of cases (%) | PTCH(+)                     |                | Gli1(+)                     |                | CD44 <sup>+</sup> /CD24 <sup>-</sup> |                | PTCH(+)/Gli1(+)             |                  | PTCH(+)/CD44 <sup>+</sup> /CD24 <sup>-</sup> |                | Gli1(+)/CD44 <sup>+</sup> /CD24 <sup>-</sup> |                |
|-----------------------------|------------------|-----------------------------|----------------|-----------------------------|----------------|--------------------------------------|----------------|-----------------------------|------------------|--|----------------|--|----------------|
|                             |                  | Positive n (%) <sup>a</sup> | P <sup>b</sup> | Positive n (%) <sup>a</sup> | P <sup>b</sup> | Positive n (%) <sup>a</sup>          | P <sup>b</sup> | Positive n (%) <sup>a</sup> | P <sup>b</sup>   | Positive n (%) <sup>a</sup>                  | P <sup>b</sup> | Positive n (%) <sup>a</sup>                  | P <sup>b</sup> |
| Age                         |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| ≤50 years                   | 138 (51.9)       | 82 (59.4)                   | 0.891          | 70 (50.7)                   | 0.518          | 61 (44.2)                            | <b>0.014</b>   | 51 (37.0)                   | 0.927            | 40 (29.0)                                    | 0.184          | 36 (26.1)                                    | 0.422          |
| >50 years                   | 128 (48.1)       | 75 (58.6)                   |                | 70 (54.7)                   |                | 38 (29.7)                            |                | 48 (37.5)                   |                  | 28 (21.9)                                    |                | 28 (21.9)                                    |                |
| Menopausal state            |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| Premenopausal               | 148 (55.6)       | 85 (57.4)                   | 0.555          | 79 (53.4)                   | 0.785          | 65 (43.9)                            | <b>0.011</b>   | 52 (35.1)                   | 0.431            | 40 (27.0)                                    | 0.540          | 40 (27.0)                                    | 0.205          |
| Postmenopausal              | 118 (44.4)       | 72 (61.0)                   |                | 61 (51.7)                   |                | 34 (28.8)                            |                | 47 (39.8)                   |                  | 28 (23.7)                                    |                | 24 (20.3)                                    |                |
| Tumor size                  |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| ≤2.0 cm                     | 145 (61.4)       | 77 (53.1)                   | <b>0.002</b>   | 68 (46.9)                   | <b>0.028</b>   | 52 (35.9)                            | 0.816          | 43 (29.7)                   | <b>0.001</b>     | 33 (22.8)                                    | 0.122          | 31 (21.4)                                    | 0.209          |
| >2.0 cm                     | 91 (38.6)        | 67 (73.6)                   |                | 56 (61.5)                   |                | 34 (37.4)                            |                | 46 (50.5)                   |                  | 29 (31.9)                                    |                | 26 (28.6)                                    |                |
| Lymph node metastasis       |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| Negative                    | 167 (62.8)       | 87 (52.1)                   | <b>0.003</b>   | 79 (47.3)                   | <b>0.024</b>   | 54 (32.3)                            | <b>0.032</b>   | 51 (30.5)                   | <b>0.003</b>     | 33 (19.8)                                    | <b>0.005</b>   | 29 (17.4)                                    | <b>0.001</b>   |
| Positive                    | 99 (37.2)        | 70 (70.7)                   |                | 61 (61.6)                   |                | 45 (45.5)                            |                | 48 (48.5)                   |                  | 35 (35.4)                                    |                | 35 (35.4)                                    |                |
| Histologic type             |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| Invasive ductal carcinoma   | 232 (87.2)       | 134 (57.8)                  | 0.274          | 114 (49.1)                  | <b>0.003</b>   | 84 (36.2)                            | 0.373          | 80 (34.5)                   | <b>0.016</b>     | 56 (24.1)                                    | 0.164          | 51 (22.0)                                    | <b>0.038</b>   |
| Invasive lobular carcinoma  | 34 (12.8)        | 23 (67.6)                   |                | 26 (76.5)                   |                | 15 (44.1)                            |                | 19 (55.9)                   |                  | 12 (35.3)                                    |                | 13 (38.2)                                    |                |
| Histological grade          |                  |                             |                |                             |                |                                      |                |                             |                  |  |                |  |                |
| I                           | 42 (21.2)        | 18 (42.9)                   | <b>0.012</b>   | 14 (33.3)                   | <b>0.001</b>   | 13 (31.0)                            | 0.300          | 7 (16.7)                    | <b>&lt;0.001</b> | 5 (11.9)                                     | <b>0.020</b>   | 5 (11.9)                                     | <b>0.033</b>   |
| II                          | 124 (62.6)       | 82 (66.1)                   |                | 66 (53.2)                   |                | 55 (44.4)                            |                | 51 (41.1)                   |                  | 42 (33.9)                                    |                | 35 (28.2)                                    |                |
| III                         | 32 (16.2)        | 23 (71.9)                   |                | 25 (78.1)                   |                | 14 (43.8)                            |                | 20 (62.5)                   |                  | 11 (34.4)                                    |                | 12 (37.5)                                    |                |

<sup>a</sup>Numbers in parentheses are percentage. <sup>b</sup>P-value obtained from Pearson  $\chi^2$  or Fisher's exact tests. PTCH, Patched; CD, cluster of differentiation.

Table IV. Univariate Cox regression analysis of the association between clinicopathological features and DFS and OS in 195 patients with breast cancer treated with chemotherapy.

| Clinicopathological feature                              | DFS                 |              | OS                   |              |
|--|---------------------|--------------|----------------------|--------------|
|  | RR (95% CI)         | P            | RR (95% CI)          | P            |
| Age, years   |                     |              |                      |              |
| ≤50/>50  | 1.046 (0.753~1.453) | 0.788        | 1.623 (0.737~3.576)  | 0.229        |
| Menopausal state   |                     |              |                      |              |
| Pre-menopause/<br>Post-menopause                         | 1.151 (0.827~1.603) | 0.404        | 1.902 (0.863~4.191)  | 0.111        |
| Tumor size, cm   |                     |              |                      |              |
| ≤2.0/>2.0  | 1.486 (1.045~2.115) | <b>0.028</b> | 0.650 (0.254~1.661)  | 0.368        |
| Lymph node metastasis                                    |                     |              |                      |              |
| No/yes   | 1.248 (0.893~1.744) | 0.194        | 3.081 (1.360~6.975)  | <b>0.007</b> |
| Histologic type  |                     |              |                      |              |
| Invasive ductal carcinoma/<br>Invasive lobular carcinoma | 1.208 (0.767~1.903) | 0.416        | 2.060 (0.822~5.157)  | 0.123        |
| Histological grade                                       |                     |              |                      |              |
| I/II/III   | 1.529 (1.082~2.162) | <b>0.016</b> | 2.117 (1.000~4.478)  | <b>0.046</b> |
| PTCH expression  |                     |              |                      |              |
| Positive/negative  | 1.466 (1.046~2.056) | <b>0.027</b> | 8.827 (2.080~37.460) | <b>0.003</b> |
| Gli1 expression  |                     |              |                      |              |
| Positive/negative  | 1.564 (1.119~2.186) | <b>0.009</b> | 2.692 (1.075~6.742)  | <b>0.034</b> |
| CD44 <sup>+</sup> /CD24 <sup>-</sup>                     |                     |              |                      |              |
| Yes/no   | 1.501 (1.079~2.088) | <b>0.016</b> | 2.709 (1.196~6.138)  | <b>0.017</b> |
| CD44 <sup>+</sup> /CD24 <sup>-</sup> patient group       |                     |              |                      |              |
| PTCH (positive/negative)                                 | 2.286 (1.311~3.984) | <b>0.004</b> | 42.080 (0.607~2917)  | 0.084        |
| Gli1 (positive/negative)                                 | 2.177 (1.221~3.881) | <b>0.008</b> | 6.928 (0.915~52.466) | 0.061        |

RR, relative risk; 95% CI, 95% confidence interval; RR and 95% CI were assessed using univariate Cox regression analysis. PTCH, Patched; CD, cluster of differentiation; DFS, disease-free survival; OS, overall survival.

and CD24 expression, respectively (Fig. 1). Since the final immunoreactive scores were integers, negative and positive immunoreactivity were defined by a final score of <3 and ≥3 for PTCH and Gli1, and <4 and ≥4 for CD44 and CD24.

Representative immunohistochemical staining for PTCH, Gli1 and CD44/CD24 in breast cancer samples is presented in Fig. 2. PTCH-positive immunoreactivity was observed in 157 (59.0%) out of 266 breast cancer samples, and Gli1-positive immunoreactivity was detected in 140 (52.6%) out of 266 breast cancer samples ( $P<0.001$ ). The CD44<sup>+</sup>/CD24<sup>-</sup> phenotype was observed in 99 (37.2%) out of 266 breast cancer samples.

**Association of PTCH and Gli1 expression with CD44 and CD24 expression.** Spearman rank correlation analysis was used to analyze the association between PTCH and Gli1 expression in breast cancer. The expression levels of PTCH were positively correlated with those of Gli1 ( $r=0.235$ ,  $P<0.001$ ). In addition, the expression levels of CD44<sup>+</sup>/CD24<sup>-</sup> were positively correlated with those of PTCH ( $r=0.167$ ,  $P=0.006$ ) and Gli1 ( $r=0.185$ ,  $P=0.003$ ) (Table II). Compared with breast cancer with a non-CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, PTCH and Gli1

expression was significantly increased in breast cancer tissues with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype (Mann-Whitney U test;  $P<0.01$ ,  $0.05$ ; Fig. 3).

**Association of the expression of PTCH, Gli1, and CD44/CD24 with clinicopathological characteristics of breast cancer patients.** The present study subsequently examined the association of PTCH, Gli1 and CD44/CD24 expression with the clinicopathological characteristics of patients with breast cancer (Table III). PTCH expression was associated with larger tumors ( $>2.0$  cm;  $P=0.002$ ), lymph node metastasis ( $P=0.003$ ) and Grade II-III tumors ( $P=0.012$ ); Gli1 expression was associated with larger tumors ( $>2.0$  cm;  $P=0.028$ ), lymph node metastasis ( $P=0.024$ ), invasive lobular carcinoma ( $P=0.003$ ) and Grade II-III tumors ( $P=0.001$ ). Combined expression of PTCH and Gli1 was associated with larger tumors ( $>2.0$  cm;  $P=0.001$ ), lymph node metastasis ( $P=0.003$ ), invasive lobular carcinoma ( $P=0.016$ ) and Grade II-III tumors ( $P<0.001$ ). CD44<sup>+</sup>/CD24<sup>-</sup> expression was associated with age ( $\leq 50$  years old;  $P=0.014$ ), premenopausal state ( $P=0.011$ ) and lymph node metastasis ( $P=0.032$ ).

Table V. Multivariate Cox regression analysis of clinicopathological features correlated with DFS and OS in 195 patients with breast cancer treated with chemotherapy.

| Clinicopathological feature   | DFS                 |              | OS                   |       |
|---|---------------------|--------------|----------------------|-------|
|   | RR (95% CI)         | P            | RR (95% CI)          | P     |
| Age, years<br>≤50/>50   | 0.895 (0.450~1.780) | 0.751        | 1.796 (0.158~20.473) | 0.637 |
| Menopausal state<br>Pre-menopause/<br>Post-menopause                        | 1.659 (0.832~3.306) | 0.150        | 2.280 (0.225~23.130) | 0.486 |
| Tumor size, cm<br>≤2.0/>2.0   | 0.905 (0.528~1.554) | 0.718        | 0.249 (0.050~1.235)  | 0.089 |
| Lymph node metastasis<br>No/yes   | 0.615 (0.344~1.101) | 0.102        | 0.706 (0.121~4.107)  | 0.698 |
| Histologic type<br>Invasive ductal carcinoma/<br>Invasive lobular carcinoma | 1.478 (0.731~2.985) | 0.277        | 1.796 (0.158~20.473) | 0.494 |
| Histological grade<br>I/II/III  | 1.637 (0.958~2.796) | 0.071        | 2.822 (0.700~11.372) | 0.145 |
| PTCH expression<br>Positive/negative  | 2.018 (1.164~3.499) | <b>0.012</b> | 4.417 (0.701~27.810) | 0.114 |
| Gli1 expression<br>Positive/negative  | 1.061 (0.612~1.842) | 0.832        | 1.783 (0.349~9.123)  | 0.487 |
| CD44 <sup>+</sup> /CD24 <sup>-</sup><br>Yes/no                              | 1.888 (1.140~3.126) | <b>0.014</b> | 1.909 (0.459~7.942)  | 0.374 |

RR, relative risk; 95% CI, 95% confidence interval; RR and 95% CI were assessed using multivariate Cox regression analysis. PTCH, Patched; CD, cluster of differentiation; DFS, disease-free survival; OS, overall survival.

Table III summarizes the association of PTCH and Gli1 expression with the clinicopathological features in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. In tumors with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, PTCH expression was associated with lymph node metastasis (P=0.005) and Grade II-III tumors (P=0.020) (Table III); and Gli1 expression was associated with invasive lobular carcinoma (P=0.038), lymph node metastasis (P=0.001) and Grade II-III tumors (P=0.033).

#### *Association of the expression of PTCH, Gli1 and CD44/CD24 with the survival of patients with breast cancer.*

The present study performed a Kaplan-Meier analysis to evaluate the association between the expression of PTCH, Gli1 and CD44/CD24 and the DFS or OS in 195 patients with breast cancer that were treated with chemotherapy. PTCH expression was significantly associated with a shorter DFS (P=0.022) and OS (P<0.001) (Fig. 4A). Gli1 expression was significantly associated with a shorter DFS (P=0.007) and OS (P=0.027) (Fig. 4B). CD44<sup>+</sup>/CD24<sup>-</sup> expression was significantly associated with a shorter DFS (P=0.013) and OS (P=0.013) (Fig. 4C).

The present study also investigated the association of the expression of PTCH and Gli1 with the OS or DFS in breast cancer patients with various CD44/CD24 phenotypes. In

patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, PTCH expression was significantly associated with a shorter DFS (P=0.002) and OS (P=0.002) (Fig. 5A). In addition, Gli1 expression was significantly associated with a shorter DFS (P=0.005) and OS (P=0.029) (Fig. 5B). However, in patients without the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, PTCH or Gli1 expression was not significantly associated with OS or DFS (P>0.05, Fig. 5C and D).

Univariate Cox regression analysis was performed to evaluate the impact of each clinicopathological variable on the OS and DFS in 195 patients with breast cancer treated with chemotherapy (Table IV). The univariate analysis identified that tumor size and histological grade were significantly associated with the DFS in patients with breast cancer. Lymph node metastasis and histological grade were significantly associated with the OS in patients with breast cancer. In addition, PTCH, Gli1 and CD44<sup>+</sup>/CD24<sup>-</sup> expression was significantly associated with a shorter DFS and OS in patients with breast cancer. Furthermore, the expression of PTCH or Gli1 was significantly associated with a shorter DFS in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype (Table IV). Furthermore, multivariate Cox regression analysis demonstrated that the expression of PTCH and the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype were independent prognostic factors for a shorter DFS in patients with breast cancer (Table V).

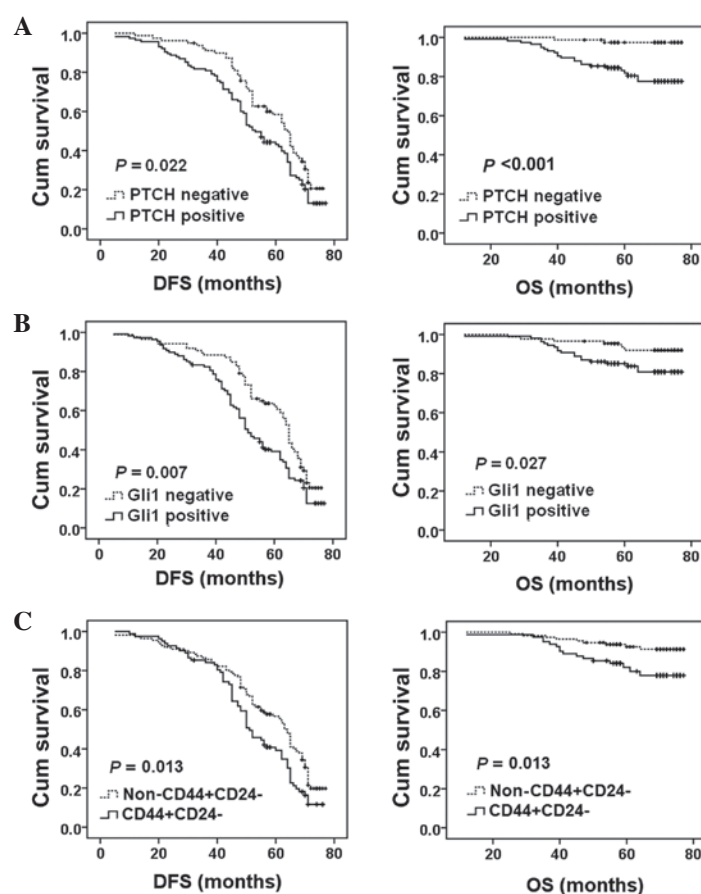


Figure 4. Kaplan-Meier survival analysis of PTCH, Gli1 and CD44<sup>+</sup>/CD24<sup>-</sup> expression in patients with breast cancer. The log-rank test was performed to determine statistical significance. Survival curves demonstrate the association between the expression of (A) PTCH, (B) Gli1 and (C) CD44<sup>+</sup>/CD24<sup>-</sup> and cumulative DFS or OS in 195 patients with breast cancer. PTCH, Patched; CD, cluster of differentiation; DFS, disease-free survival; OS, overall survival.

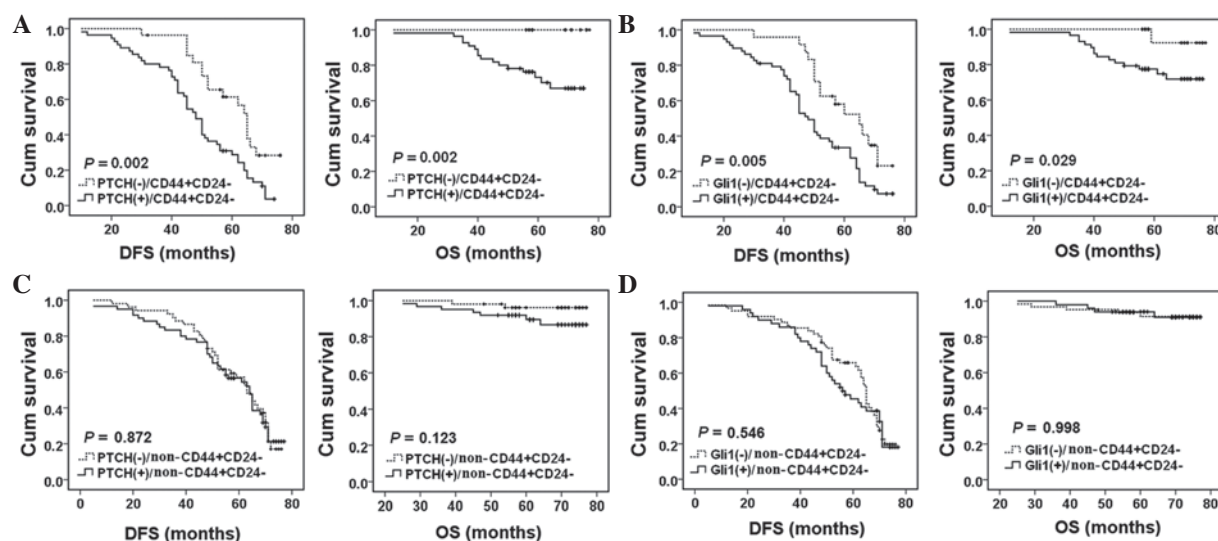


Figure 5. Kaplan-Meier survival analysis of PTCH and Gli1 expression in breast cancer patients with various CD44/CD24 phenotypes. The log-rank test was performed to determine statistical significance. Survival curves demonstrate the association between the expression of PTCH or Gli1 and cumulative DFS or OS in breast cancer patients with (A and B) CD44<sup>+</sup>/CD24<sup>-</sup> and (C and D) non-CD44<sup>+</sup>/CD24<sup>-</sup> phenotypes. PTCH, Patched; CD, cluster of differentiation; DFS, disease-free survival; OS, overall survival.

## Discussion

It is generally believed that breast CSCs contribute to chemoresistance, recurrence and metastasis in breast cancer (4,16,26).

The Hh signaling pathway has been reported to be important for maintaining the stemness of CSCs (10,13,27). In addition, the Hh signaling pathway has been demonstrated to be activated in patients with breast cancer, and inhibition



of Hh signaling reduces the growth of breast cancer cells *in vitro* (28). However, it remains to be elucidated as to whether the Hh signaling pathway affects breast CSCs in patients with breast cancer. In the present study, PTCH, Gli1 and CD44/CD24 expression was detected in samples from 266 patients with breast cancer. The results demonstrated that the expression of PTCH and Gli1, which are the two main components of the Hh signaling pathway, was higher in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, as compared with those with a non-CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. The expression of PTCH and Gli1 was positively correlated with CD44<sup>+</sup>/CD24<sup>-</sup> expression, thus suggesting that the Hh signaling pathway is activated in breast CSCs. Furthermore, the expression of PTCH and Gli1 was associated with poor survival in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. These findings suggested that Hh signaling activation in breast CSCs may contribute to poor outcomes in patients with breast cancer.

It has previously been reported that PTCH expression is associated with lymph node metastasis and a greater histological grade in patients with breast cancer (29). Tao *et al* (30) demonstrated that Gli1 was significantly upregulated in breast cancer patients with lymph node metastasis. Furthermore, Xuan *et al* reported that Gli1 expression was correlated with lymph node metastasis (31). Similarly, the present study demonstrated that the expression of PTCH and Gli1 was associated with lymph node metastasis. These findings suggested that the Hh signaling pathway is important for lymph node metastasis in breast cancer. Furthermore, the expression of PTCH and Gli1 was more positively associated with lymph node metastasis in tumors with a CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, further suggesting that the Hh signaling pathway is important for CSC-mediated metastasis in breast cancer. It has previously been reported that breast cancer cells with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype express high levels of metastasis-associated genes and exhibit enhanced metastasis (17,32,33). In addition, Lin *et al* (19) revealed that the expression of CD44<sup>+</sup>/CD24<sup>-</sup> was associated with lymph node metastasis in breast cancer patients with invasive ductal carcinoma. Since the present study demonstrated that the expression of PTCH and Gli1 was significantly associated with lymph node metastasis in tumors with a CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, it may be suggested that the Hh signaling pathway is essential for CSC-induced lymph node metastasis in patients with breast cancer.

The present study also demonstrated that the expression of PTCH and Gli1 was associated with a shorter DFS and OS in patients with breast cancer, thus suggesting that the Hh signaling pathway contributes to poor outcomes in patients with breast cancer. Consistent with these findings, Ramaswamy *et al* (34) reported that Gli1 overexpression was associated with a shorter DFS and OS in patients with breast cancer. Notably, the present study revealed that the expression of PTCH and Gli1 was significantly associated with a shorter DFS and OS in breast cancer patients with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, but not in patients without the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. It has been reported that the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype contributes to poor prognosis in patients with breast cancer (19,20). The present findings indicated that the Hh signaling pathway in CSCs contributes to poor prognosis of breast cancer.

Furthermore, a univariate Cox regression analysis identified that the expression of PTCH, Gli1 and CD44<sup>+</sup>/CD24<sup>-</sup> was associated with poor prognosis of patients with breast cancer. The multivariate analysis identified that PTCH expression and the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype were independent prognostic factors for poor outcome in patients with breast cancer.

In conclusion, the present study investigated PTCH and Gli1 expression, and the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype in patients with breast cancer, and analyzed the association of their expression with clinicopathological characteristics and prognosis. The results demonstrated that the expression of PTCH and Gli1 was associated with lymph node metastasis and a worse clinical outcome in patients with breast cancer, particularly those with the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. This study suggests that the Hh signaling pathway in CSCs may be associated with a poor prognosis in patients with breast cancer. Therefore, inhibition of the Hh signaling pathway may be an effective therapeutic strategy for the inhibition of breast CSCs, thus preventing breast cancer recurrence and metastasis.

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