The Hedgehog signaling pathway is associated with poor prognosis in breast cancer patients with the CD44+/CD24- 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+/CD24- 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+/CD24- phenotype. Multivariate Cox regression analysis demonstrated that PTCH expression and the CD44+/CD24- 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.

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

the $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ phenotype, and to analyze the correlation of their expression with clinicopathological features and prognosis of breast cancer patients with the $CD44^+/CD24^-$ 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 + epimbicin + fluorouracil, n=151), CAF (cyclophosphamide + Adriamycin + fluorouracil, n=18) and CET (cyclophosphamide + epimbicin + taxol, n=26).

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[a]		
≤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[b]		
I	42	21.2
II	124	62.6
III	32	16.2

[a]Tumor size was determined in 236 patients; [b]Histological grade was determined in 198 patients.

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_2O_2 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) 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

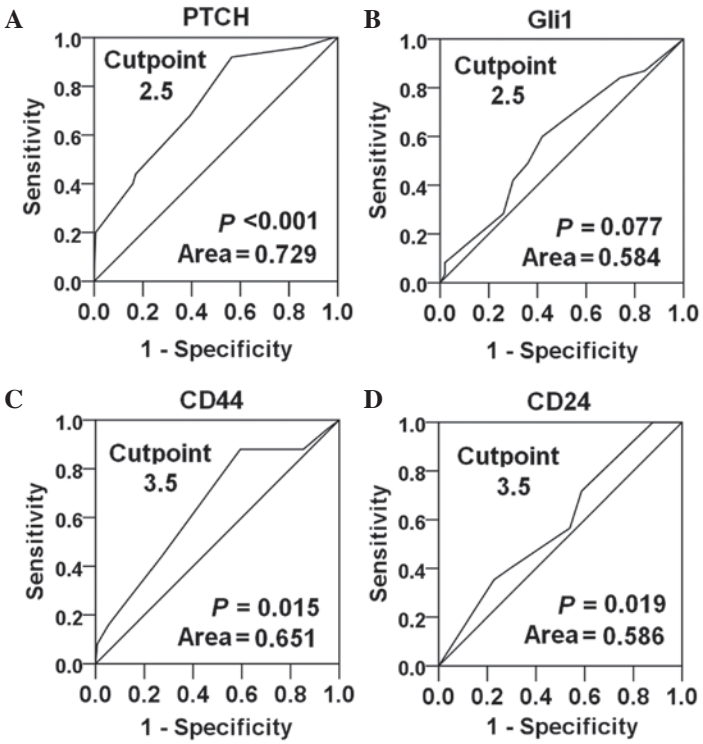

Figure 1. Receiver operating characteristic curves were used to determine the cutoff score for the expression of (A) PTCH, (B) Gli1, (C) CD44 and (D) CD24 in patients with breast cancer. The sensitivity and specificity for OS, DFS, OS and lymph node metastasis were plotted for PTCH, Gli1, CD44 and CD24 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.

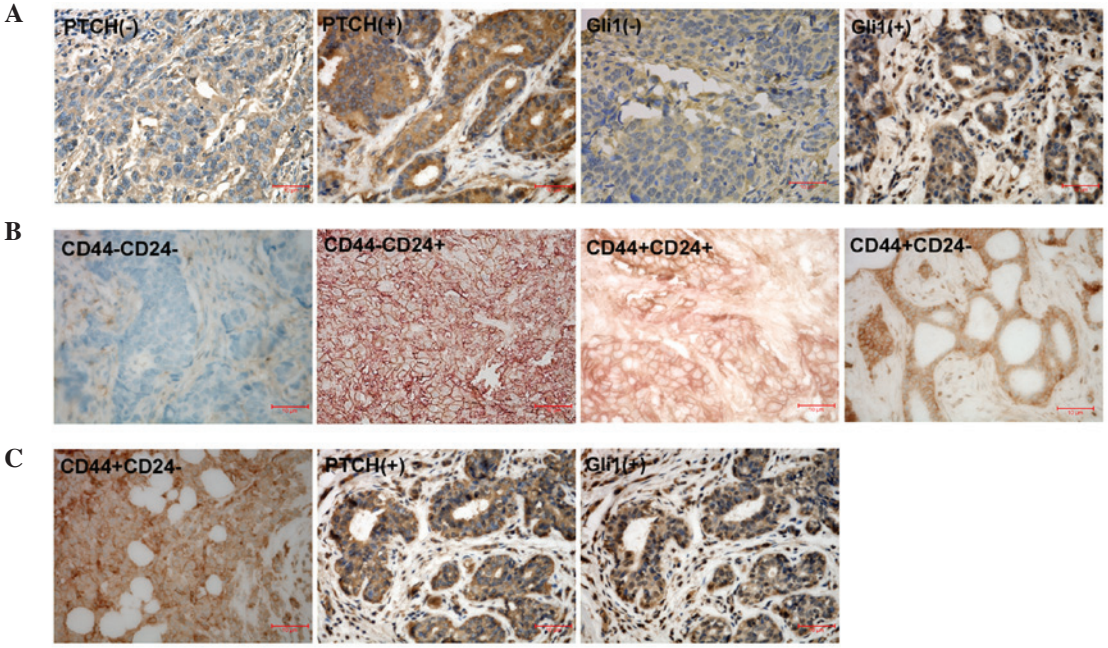

Figure 2. Representative immunohistochemical micrographs of PTCH, Gli1 and CD44/CD24 staining in breast cancer samples. (A) Representative micrographs of $PTCH^-$, $PTCH^+$, $Gli1^-$ and $Gli1^+$ staining. (B) Representative double immunohistochemical staining of $CD44^-/CD24^-$, $CD44^-/CD24^+$, $CD44^+/CD24^+$ and $CD44^+/CD24^-$. (C) Representative $PTCH^+$ and Gli^+ immunohistochemical staining in a $CD44^+/CD24^-$ breast cancer sample. Magnification, x400. Scale bars, 10 μm. 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 $CD44^+/CD24^-$ in 266 breast cancer tissues.

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

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

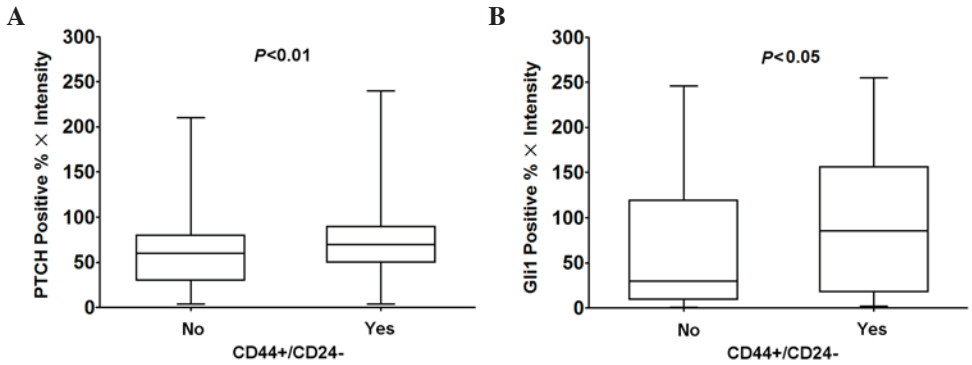

Figure 3. (A) PTCH and (B) Gli1 expression in breast cancer patients with or without $CD44^+/CD24^-$ phenotype. Data were analyzed by Mann-Whitney U test. PTCH, Patched; CD, cluster of differentiation.

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 χ^2 or Fisher's exact probability tests were used to evaluate the association between PTCH, Gli1 and $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$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 $CD44^+/CD2^-$ phenotype with non-$CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ phenotype.

Clinicopathological feature	No. of cases (%)	PTCH(+) Positive n (%)[a]	PTCH(+) P[b]	Gli1(+) Positive n (%)[a]	Gli1(+) P[b]	$CD44^+/CD24^-$ Positive n (%)[a]	$CD44^+/CD24^-$ P[b]	PTCH(+)/Gli1(+) Positive n (%)[a]	PTCH(+)/Gli1(+) P[b]	PTCH(+)/$CD44^+/CD24^-$ Positive n (%)[a]	PTCH(+)/$CD44^+/CD24^-$ P[b]	Gli1(+)/$CD44^+/CD24^-$ Positive n (%)[a]	Gli1(+)/$CD44^+/CD24^-$ P[b]
Age													
≤50 years	138 (51.9)	82 (59.4)	0.891	70 (50.7)	0.518	61 (44.2)	**0.014**	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)	**0.011**	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)	**0.002**	68 (46.9)	**0.028**	52 (35.9)	0.816	43 (29.7)	**0.001**	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)	**0.003**	79 (47.3)	**0.024**	54 (32.3)	**0.032**	51 (30.5)	**0.003**	33 (19.8)	**0.005**	29 (17.4)	**0.001**
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)	**0.003**	84 (36.2)	0.373	80 (34.5)	**0.016**	56 (24.1)	0.164	51 (22.0)	**0.038**
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)	**0.012**	14 (33.3)	**0.001**	13 (31.0)	0.300	7 (16.7)	**<0.001**	5 (11.9)	**0.020**	5 (11.9)	**0.033**
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)	

[a]Numbers in parentheses are percentage. [b]P-value obtained from Pearson χ^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.

	DFS		OS	
Clinicopathological feature	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/				
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)	**0.028**	0.650 (0.254~1.661)	0.368
Lymph node metastasis				
No/yes	1.248 (0.893~1.744)	0.194	3.081 (1.360~6975)	**0.007**
Histologic type				
Invasive ductal carcinoma/				
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)	**0.016**	2.117 (1.000~4.478)	**0.046**
PTCH expression				
Positive/negative	1.466 (1.046~2.056)	**0.027**	8.827 (2.080~37.460)	**0.003**
Gli1 expression				
Positive/negative	1.564 (1.119~2.186)	**0.009**	2.692 (1.075~6.742)	**0.034**
CD44$^+$/CD24$^-$				
Yes/no	1.501 (1.079~2.088)	**0.016**	2.709 (1.196~6.138)	**0.017**
CD44$^+$/CD24$^-$ patient group				
PTCH (positive/negative)	2.286 (1.311~3.984)	**0.004**	42.080 (0.607~2917)	0.084
Gli1 (positive/negative)	2.177 (1.221~3.881)	**0.008**	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-postive immunoreactivity was detected in 140 (52.6%) out of 266 breast cancer samples (P<0.001). The CD44$^+$/CD24$^-$ 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$^+$/CD24$^-$ 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$^+$/CD24$^-$ phenotype, PTCH and Gli1 expression was significantly increased in breast cancer tissues with the CD44$^+$/CD24$^-$ 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$^+$/CD24$^-$ expression was associated with age (≤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.

	DFS		OS	
Clinicopathological feature	RR (95% CI)	P	RR (95% CI)	P
Age, years				
≤50/>50	0.895 (0.450~1.780)	0.751	1.796 (0.158~20.473)	0.637
Menopausal state				
Pre-menopause/				
Post-menopause	1.659 (0.832~3.306)	0.150	2.280 (0.225~23.130)	0.486
Tumor size, cm				
≤2.0/>2.0	0.905 (0.528~1.554)	0.718	0.249 (0.050~1.235)	0.089
Lymph node metastasis				
No/yes	0.615 (0.344~1.101)	0.102	0.706 (0.121~4.107)	0.698
Histologic type				
Invasive ductal carcinoma/				
Invasive lobular carcinoma	1.478 (0.731~2.985)	0.277	1.796 (0.158~20.473)	0.494
Histological grade				
I/II/III	1.637 (0.958~2.796)	0.071	2.822 (0.700~11.372)	0.145
PTCH expression				
Positive/negative	2.018 (1.164~3.499)	**0.012**	4.417 (0.701~27.810)	0.114
Gli1 expression				
Positive/negative	1.061 (0.612~1.842)	0.832	1.783 (0.349~9.123)	0.487
$CD44^+/CD24^-$				
Yes/no	1.888 (1.140~3.126)	**0.014**	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 $CD44^+/CD24^-$ phenotype. In tumors with the $CD44^+/CD24^-$ 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). $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ phenotype (Table IV). Furthermore, multivariate Cox regression analysis demonstrated that the expression of PTCH and the $CD44^+/CD24^-$ phenotype were independent prognostic factors for a shorter DFS in patients with breast cancer (Table V).

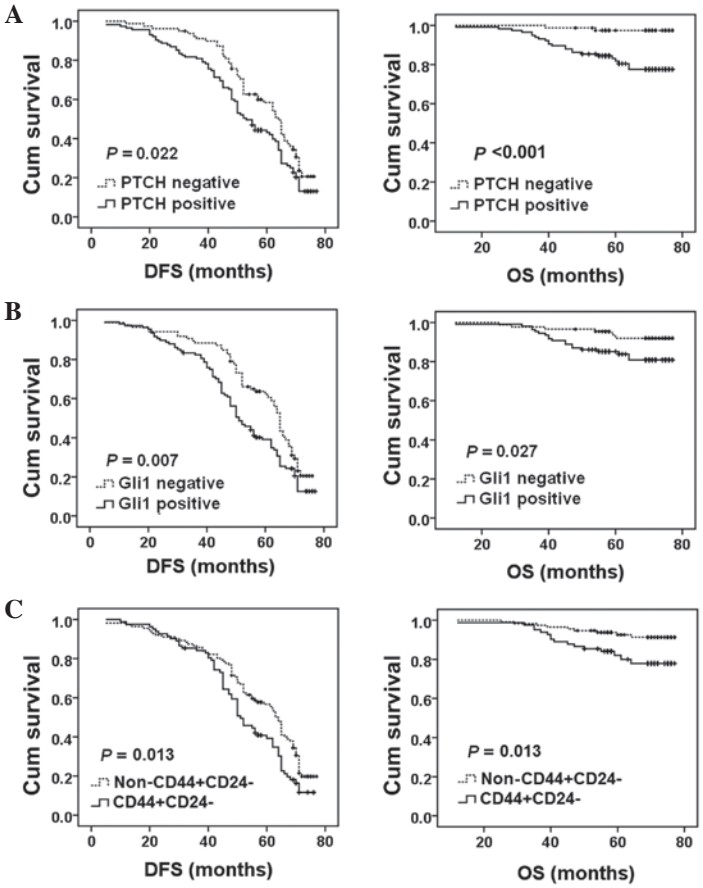

Figure 4. Kaplan-Meier survival analysis of PTCH, Gli1 and CD44+/CD24- expression in patients with breast cancer. The log-rank test was performed to determine statistical significance. Survival curves demonstrate the association between the expression of (A) PTCH, (B) Gli1 and (C) CD44+/CD24- and cumulative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

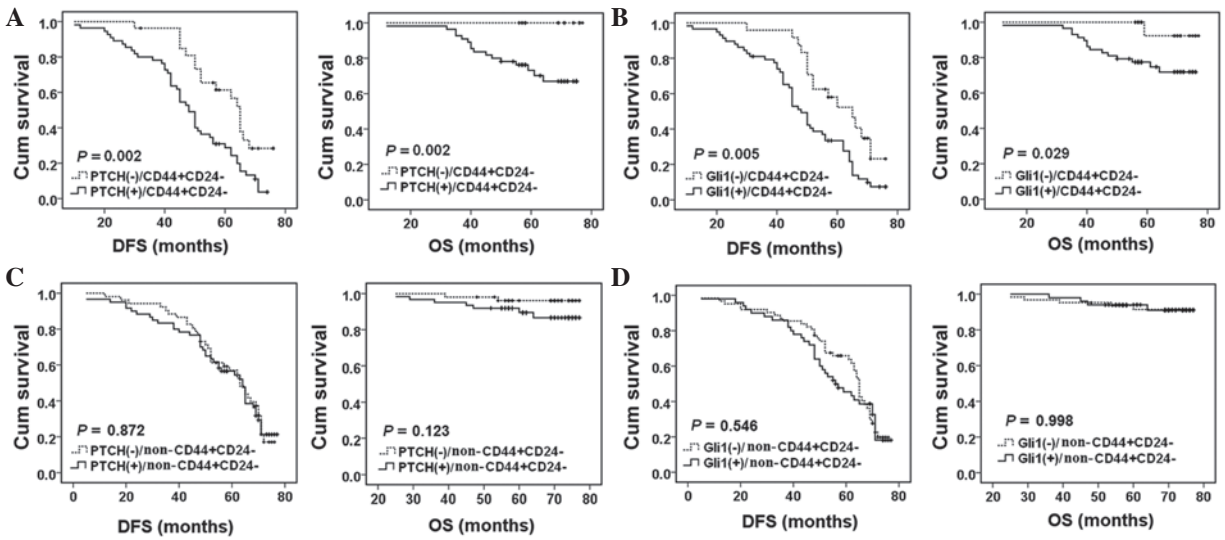

Figure 5. Kaplan-Meier survival analysis of PTCH and Gli1 expression in breast cancer patients with various CD44/CD24 phenotypes. The log-rank test was performed to determine statistical significance. Survival curves demonstrate the association between the expression of PTCH or Gli1 and cumulative DFS or OS in breast cancer patients with (A and B) CD44+/CD24- and (C and D) non-CD44+/CD24- 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 $CD44^+/CD24^-$ phenotype, as compared with those with a non-$CD44^+/CD24^-$ phenotype. The expression of PTCH and Gli1 was positively correlated with $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ phenotype, but not in patients without the $CD44^+/CD24^-$ phenotype. It has been reported that the $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ was associated with poor prognosis of patients with breast cancer. The multivariate analysis identified that PTCH expression and the $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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 $CD44^+/CD24^-$ 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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References

1. Al-Hajj M and Clarke MF: Self-renewal and solid tumor stem cells. Oncogene 23: 7274-7282, 2004.
2. Sampieri K and Fodde R: Cancer stem cells and metastasis. Semin Cancer Biol 22: 187-193, 2012.
3. Ishii H, Iwatsuki M, Ieta K, Ohta D, Haraguchi N, Mimori K and Mori M: Cancer stem cells and chemoradiation resistance. Cancer Sci 99: 1871-1877, 2008.
4. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, *et al*: Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 100: 672-679, 2008.
5. McDermott SP and Wicha MS: Targeting breast cancer stem cells. Mol Oncol 4: 404-419, 2010.
6. Ingham PW and McMahon AP: Hedgehog signaling in animal development: Paradigms and principles. Genes Dev 15: 3059-3087, 2001.
7. Taipale J and Beachy PA: The Hedgehog and Wnt signalling pathways in cancer. Nature 411: 349-354, 2001.
8. Jiang J and Hui CC: Hedgehog signaling in development and cancer. Dev Cell 15: 801-812, 2008.
9. Briscoe J and Thérond PP: The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol 14: 416-429, 2013.
10. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P and Wicha MS: Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 66: 6063-6071, 2006.
11. Bar EE, Chaudhry A, Lin A, Fan X, Schreck K, Matsui W, Piccirillo S, Vescovi AL, DiMeco F, Olivi A and Eberhart CG: Cyclopamine-mediated hedgehog pathway inhibition depletes stem-like cancer cells in glioblastoma. Stem Cells 25: 2524-2533, 2007.
12. Clement V, Sanchez P, de Tribolet N, Radovanovic I and Ruiz i Altaba A: HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 17: 165-172, 2007.
13. Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J, Kwon HY, Kim J, Chute JP, Rizzieri D, *et al*: Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 458: 776-779, 2009.

14. Tanaka H, Nakamura M, Kameda C, Kubo M, Sato N, Kuroki S, Tanaka M and Katano M: The Hedgehog signaling pathway plays an essential role in maintaining the $CD44^+CD24^-$/low subpopulation and the side population of breast cancer cells. Anticancer Res 29: 2147-2157, 2009.
15. Jaggupilli A and Elkord E: Significance of CD44 and CD24 as cancer stem cell markers: An enduring ambiguity. Clin Dev Immunol 2012: 708036, 2012.
16. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
17. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M and Brauch H: Prevalence of $CD44^+/CD24^-$/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin Cancer Res 11: 1154-1159, 2005.
18. Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R Jr, Badve S and Nakshatri H: $CD44^+/CD24^-$ breast cancer cells exhibit enhanced invasive properties: An early step necessary for metastasis. Breast Cancer Res 8: R59, 2006.
19. Lin Y, Zhong Y, Guan H, Zhang X and Sun Q: $CD44^+/CD24^-$ phenotype contributes to malignant relapse following surgical resection and chemotherapy in patients with invasive ductal carcinoma. J Exp Clin Cancer Res 31: 59, 2012.
20. Lee HE, Kim JH, Kim YJ, Choi SY, Kim SW, Kang E, Chung IY, Kim IA, Kim EJ, Choi Y, *et al*: An increase in cancer stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer. Br J Cancer 104: 1730-1738, 2011.
21. Tavassoli FA and Devilee P (eds.): World Health Organization Classification of Tumours Pathology and Genetics Tumours of the Breast and Female Genital Organs. IARC Press, Lyon, 2003.
22. Bai X, Song Z, Fu Y, Yu Z, Zhao L, Zhao H, Yao W, Huang D, Mi X, Wang E, *et al*: Clinicopathological significance and prognostic value of DNA methyltransferase 1, 3a, and 3b expressions in sporadic epithelial ovarian cancer. PLoS One 7: e40024, 2012.
23. Kim TJ, Lee JY, Hwang TK, Kang CS and Choi YJ: Hedgehog signaling protein expression and its association with prognostic parameters in prostate cancer: A retrospective study from the view point of new 2010 anatomic stage/prognostic groups. J Surg Oncol 104: 472-479, 2011.
24. He HC, Chen JH, Chen XB, Qin GQ, Cai C, Liang YX, Han ZD, Dai QS, Chen YR, Zeng GH, *et al*: Expression of hedgehog pathway components is associated with bladder cancer progression and clinical outcome. Pathol Oncol Res 18: 349-355, 2012.
25. Kim BW, Cho H, Chung JY, Conway C, Ylaya K, Kim JH and Hewitt SM: Prognostic assessment of hypoxia and metabolic markers in cervical cancer using automated digital image analysis of immunohistochemistry. J Transl Med 11: 185, 2013.
26. Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, Wen S, Chang YF, Bachmann MH, Shimono Y, *et al*: Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. Proc Natl Acad Sci USA 107: 18115-18120, 2010.
27. Song Z, Yue W, Wei B, Wang N, Li T, Guan L, Shi S, Zeng Q, Pei X and Chen L: Sonic hedgehog pathway is essential for maintenance of cancer stem-like cells in human gastric cancer. PLoS One 6: e17687, 2011.
28. Kubo M, Nakamura M, Tasaki A, Yamanaka N, Nakashima H, Nomura M, Kuroki S and Katano M: Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. Cancer Res 64: 6071-6074, 2004.
29. Im S, Choi HJ, Yoo C, Jung JH, Jeon YW, Suh YJ and Kang CS: Hedgehog related protein expression in breast cancer: Gli-2 is associated with poor overall survival. Korean J Pathol 47: 116-123, 2013.
30. Tao Y, Mao J, Zhang Q and Li L: Overexpression of Hedgehog signaling molecules and its involvement in triple-negative breast cancer. Oncol Lett 2: 995-1001, 2011.
31. Xuan Y and Lin Z: Expression of Indian Hedgehog signaling molecules in breast cancer. J Cancer Res Clin Oncol 135: 235-240, 2009.
32. Liu R, Wang X, Chen GY, Dalerba P, Gurney A, Hoey T, Sherlock G, Lewicki J, Shedden K and Clarke MF: The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med 356: 217-226, 2007.
33. Charafe-Jauffret E, Ginestier C and Birnbaum D: Breast cancer stem cells: Tools and models to rely on. BMC Cancer 9: 202, 2009.
34. Ramaswamy B, Lu Y, Teng KY, Nuovo G, Li X, Shapiro CL and Majumder S: Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. Cancer Res 72: 5048-5059, 2012.