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RESEARCH ARTICLE

Study on the skip metastasis of axillary lymph nodes in breast cancer and their relation with Gli1 expression

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Abstract The skip metastasis (SM) of axillary lymph nodes (ALN) in breast cancer is an important phenomenon which is crucial to determine the correct choice of surgical resection. The mechanism of SM of ALN is unclear. Gli1 protein is a core epithelial-to-mesenchymal transition (EMT) regulatory factor that plays essential roles in both development and disease processes and has been associated with metastasis in carcinomas. The aim of this study was to investigate the clinicopathological characteristics of SM and evaluate the significance of Gli1 expression in breast cancer patients with metastasis of ALN. Clinicopathological data from 1,037 female breast cancer patients who underwent radical mastectomy were retrospectively reviewed. In this study, an SM was defined as level I absence but level II and/or level III involvement. The expression of Gli1 was evaluated by immunohistochemistry in 102 non-SM cases with positive nodes and 33 SM cases. In univariate analysis, we found that pN category, TNM stage, intrinsic subtypes and Gli1 expression was significant risk factor of SM. Further logistic regression analysis revealed that luminal A cases had a lower risk of SM relative to luminal B 1 (HER2 negative) cases. Further multivariate analysis revealed that Gli1 expression and numbers of positive lymph nodes were the independent factors which associated with SM. Collectively, Breast cancer with SM of ALN associated with the intrinsic subtype of the luminal B1. Gli1 expression related with the procession of breast cancer with SM, which can be used as a predictor of SM of ALN in breast cancer.

Keywords Gli1 · Skip metastasis · Axillary lymph nodes · Breast cancer

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Introduction

Breast cancer is the leading form of cancer in women both in the developed and the developing world; it poses a serious harmful threat on women's health. The incidence of breast cancer is increasing in the developing world due to increased life expectancy, increased urbanization and adoption of Western lifestyles. When a person is diagnosed to have breast cancer, surgery is most commonly the first plan of action. Decisions about surgery being the appropriate breast cancer treatment is never taken lightly; they are dependent on many factors such as the stage of the cancer, the type of breast cancer, what are the acceptable surgeries in terms of the needs and wants of the breast cancer sufferer, and the most appropriate breast cancer treatment. Only the correct choice of surgical resection could effectively improve the postoperative quality of life of the patient. Traditional surgery includes the whole breast and axillary lymph node resection (ALND), and for patients without lymph node metastasis, ALND is considered to be an unnecessary overtreatment. In recent years, sentinel lymph node biopsy (SLNB) has been proven to be a valid method of assessing ALN status in early breast cancer patients, and has been accepted as a standard of care for early breast cancer patients. The ALND can be omitted when sentinel lymph nodes (SLNs) are negative. Skip metastasis (SM) of ALN in breast cancer poses a challenge in regard to the safety of this surgical technique, and the incidence of SM is about 1.5 % to 19.2 % [1–4]. When ALN involvement skips the first station and is detected in the high-level station, the results

obtained via SLNB may be false negative. The positive lymph nodes are not resected by surgery, and may result in postoperative local recurrence and distant metastasis. The mechanism of SM of ALN is unclear; some routine clinicopathological parameters could not yet accurately predict the occurrence of SM [5]. Therefore, the purpose of this paper is based on some exploratory research; we could find some new clinical indicators that may be used to assess the degree of risk of ALN SM.

Recent studies showed that the Hedgehog signaling pathway can regulate the occurrence and development of breast cancer through a variety of ways, and in particular plays an important role in the signaling pathway for epithelial-to-mesenchymal transition (EMT) [6, 7]. EMT is characterized by the acquisition of a mesenchymal, motile phenotype and is accompanied by characteristic molecular changes. It plays vital roles in both physiological responses to injuries in adults and in the onset of pathological conditions such as organ fibrosis and, more importantly, metastasis of cancers [8]. Breast cancers that exhibit properties of EMT are highly aggressive and resistant to therapy [9]. We hypothesized that breast cancer has high risk of ALN SM with the extensive transformation of EMT.

In this study, based on this hypothesis, the works included two parts. In part one, the records of breast cancer patients who underwent radical mastectomy in the First Affiliated Hospital of China Medical University from 2005 to 2009 were retrospectively analyzed in order to access the clinicopathological significance of SM in breast cancer. Gli1 is an early target of Hedgehog signaling and functions exclusively as a transcriptional activator. Hedgehog signaling shapes the transcriptional response of a cell by altering the ratio of activator and repressor functions of the Gli proteins [10, 11]. Thus, in the second part, we investigated the association between Gli1 protein expression and SM, in order to explore the significance and influence of Hedgehog signaling pathway on SM.

Materials and methods

Data acquisition

The records of 1,037 female breast cancer patients who underwent radical mastectomy in the First Affiliated Hospital of China Medical University between 2005 and 2009 were prospectively analyzed. None of the patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. Patients' ages ranged from 24 to 84, with an average age of 50.2 years old. The axilla lymph is divided into three levels based on the pectoralis minor muscle: level I is lateral and inferior to the pectoralis minor muscle; level II is posterior to the pectoralis minor muscle; level III is medial and superior to the pectoralis minor muscle. We reviewed the distribution of ALN metastases with the pathology of the complete axillary dissection specimens of 1,037 patients. We categorized the ALN positive patients into SM groups and non-SM groups according to their ALN examinations. Independent parameters were compared between SM and non-SM groups: tumor location, tumor size, age, histopathology type, estrogen and progesterone receptor status, etc. The clinical stage of patients was determined by the American Joint Committee on Cancer (AJCC) staging system. The study was approved by the regional ethics committee at China Medical University.

Immunohistochemistry, fluorescence in situ hybridization and biological sub-types classification

Formalin-fixed and paraffin-embedded specimens of the breast tumor were cut into 4-μm-thick sections, which were subsequently de-waxed and hydrated. Immunohistochemical staining for Ki67 (MAB-0129, Maixin, ready-to-use), ERα (sc-542; Santa Cruz, 1:200), Erb-B2 (MAB-0198; Maixin, ready-to-use) and p53 (MAB-0142; Maixin, ready-to-use) were performed using UltraSensitive™ S-P kits (Maixin-Bio, China) according to the manufacturer's instructions and using the reagent supplied within the kit. For the negative control, phosphate-buffered saline (PBS) was used in place of the primary antibodies. A total of 102 non-SM cases with positive ALN metastasis during 2009, and 33 SM cases were studied further for Gli1 immunohistochemical analysis. Gli1 was expressed in both cytoplasm and nucleus. We also adopted the German semi-quantitative scoring system in considering the staining intensity and area extent, which has been widely accepted and used in previous studies [12, 13]. Every lesion was given a score according to the intensity of the nucleic staining (no staining=0, weak staining=1, moderate staining=2, strong staining=3) and the extent of stained cells (0 %=0, 1–10 %=1, 11–50 %=2, 51–80 %=3, 81–100 %=4; negative means 0 % area staining, focally positive means 1–80 % area staining, diffusely positive means 81–100 % area staining). The final immunoreactive score was determined by multiplying the intensity scores with the extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12 for ER, PR, p53, BRCA-1 and Gli1 [12, 13]. Slides were independently examined by two pathologists (Chui-Feng Fan and Min Song) as previously noted; however, if there was a discrepancy in individual scores both pathologists reevaluated the scores until they reach a consensus before combining the individual scores. To obtain statistical results, a final score equal to or less

than 1 was considered negative, while scores of 2 or more were considered positive. Positive ER, PR and p53 staining were seen in nuclear. A positive BRCA-1 stain was recorded only if immunostaining was observed within the nuclei of cancer cells. Erb-B2 membrane expression was scored using HercepTest™ (DAKO A/S, Glostrup, Denmark) with strict adherence to the manufacturer's instructions [14]. The patients with Erb-B2 (−) and (+) were thought to be HER-2 gene non-amplified, and the patients with Erb-B2 (++) and (+++) were studied further to detect HER-2 status by fluorescence in situ hybridization (FISH) as previously described [14, 15]. Based on the results of immunohistochemistry and FISH, the cases of ALN metastasis were classified to "intrinsic subtypes" of breast cancer: Luminal A, Luminal B 1 (HER2 negative), Luminal B 2 (HER2 positive), Erb-B2 overexpression and Basal-like breast cancer; the criteria were adopted from the 12th St Gallen International Breast Cancer Conference [16] (Table 1).

Statistical analysis

Fisher's exact tests and Pearson's chi-square tests were used for single parameter statistical analysis and correlation analysis between two groups. Independent samples *t*-test was used for comparing means. Binary Logistic Regression was used for multiple parameters statistical analysis. All statistical analyses were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered statistically significant.

Table 1 Surrogate definitions of intrinsic subtypes of breast cancer

Intrinsic subtype	Clinicopathologic definition
Luminal A	'Luminal A'
	ER and/or PR positive
	HER2 negative
	Ki-67 low (<14 %)
Luminal B	'Luminal B1 (HER2 negative)'
	ER and/or PR positive
	HER2 negative
	Ki-67 high
	'Luminal B2 (HER2 positive)'
	ER and/or PR positive
	Any Ki-67
	HER2 overexpressed or amplified
Erb-B2 overexpression	'HER2 positive (non luminal)'
	HER2 overexpressed or amplified
	ER and PR absent
'Basal-like'	'Triple negative (ductal)'
	ER and PR absent
	HER2 negative

Results

Incidence of skip metastasis

In this study, we defined the patients who had level I absence but level II and/or level III involvement as SM groups. Pathological ALN metastasis involvement was found in 438 out of 1,037 patients (42.2 %). An SM was found in 3.2 % of all patients (33/1,037) and 7.5 % of those with ALN metastasis (33/438). The most common type of SM was the SM with skipping level I to II, which accounted for 97 % (32/33) of breast cancer patients with SM of ALN (Table 2).

Univariate analysis on the clinicopathological characteristics of SM

We retroactively compared the common clinicopathological characteristics between the SM and non-SM groups with ALN. As shown in Table 3, the mean tumor size of SM was 2.54±0.96, and that in non-SM was 3.32±1.88. Independent sample test showed that the tumor size of SM group was smaller than that of the non-SM group (F=8.304, p=0.004). Age, pT category, location and pathological type were similar in patients with or without SM. SM had no significant correlation with phenotypic expression patterns of ER, PR, Erb-B2, p53 and BRCA-1. Furthermore, we found a strong correlation between numbers of positive nodes with SM cases, so as with the pN category of pathology (regional lymph nodes). Overall, we observed 87.9 % SM cases (29/33) with pN1 stage vs. 48.1 % non-SM cases (195/405) with pN1 stage (p<0.001). Also, TNM stage II cases (28/33, p<0.001) and Luminal B1 cases (17/33, p=0.009) accounted for a higher percentage of patients with SM, and univariate analysis confirmed that Gli1 expression (26/33, p<0.001) was strongly related to SM (Tables 3 and 4). Phenotypic expression patterns of Gli1, BRCA1, p53, ER, PR and Erb-B2 protein in SM group are shown in Fig. 1.

Multivariate analysis of clinicopathological features correlated with SM

In the multivariate analysis of clinicopathological features correlated with SM (Table 5), Gli1 positive expression (p<0.001, odds ratio [OR]=20.177) and numbers of

Table 2 Incidence of skip metastasis in axillary lymph nodes

Classification	Pathway	Cases	Incidence
Skipping level I to II	I(−) II(+) III(−)	32/33	97 %
Skipping level I to II and III	I(−) II(+) III(+)	0	0
Skipping level I and II to III	I(−) II(−) III(+)	1/33	3 %

Table 3 Association of SM with clinicopathological parameters of breast cancer

Characteristics	*N*	Patterns of ALN involvement		χ^2	*p* value
		Non-SM group (*n*=405)	SM group (*n*=33)		
Age					
≦50 years	222	204 (50.4 %)	18 (51.5 %)	0.213	0.719
>50 years	216	201 (49.6 %)	15 (48.5 %)		
Menopausal status					
Premenopausal	213	197 (48.7 %)	16 (48.5 %)	0	1
Postmenopausal	225	208 (51.4 %)	17 (51.5 %)		
Tumor size (cm)					
Mean ± SD		3.32±1.88	2.54±0.96		0.004[a]
pT category					
T1 (0<*t*≦2)	132	119 (29.4 %)	13 (39.4 %)	2.655	0.231
T2 (2<*t*≦5)	261	242 (59.8 %)	19 (57.6 %)		
T3 (*t*>5)	45	44 (10.9 %)	1 (3.0 %)		
Tumor location					
Upper outer	222	199 (49.1 %)	23 (69.7 %)	5.644	0.196
Lower outer	38	35 (8.6 %)	3 (9.1 %)		
Upper inner	55	53 (13.1 %)	2 (6.1 %)		
Lower inner	16	15 (3.7 %)	1 (3.0 %)		
Middle and others	107	103 (25.4 %)	4 (12.1 %)		
ER status					
Positive	303	280 (69.1 %)	23 (69.7 %)	0.005	1
Negative	135	125 (30.9 %)	10 (30.3 %)		
PR status					
Positive	308	285 (70.4 %)	23 (69.7 %)	0.007	1
Negative	130	120 (29.6 %)	10 (30.3 %)		
HER-2 status					
Amplified	119	111 (27.4 %)	8 (24.2 %)	0.154	0.839
Non-amplified	319	294 (72.6 %)	25 (75.8 %)		
P53 status					
Positive	214	197 (48.6 %)	17 (51.5 %)	0.101	0.857
Negative	224	208 (51.4 %)	16 (48.5 %)		
BRCA1 status					
Positive	291	265 (65.4 %)	26 (78.8 %)	2.441	0.129
Negative	147	140 (34.6 %)	7 (21.2 %)		
Pathological type					
Infiltrating ductal	387	355 (87.7 %)	32 (97.0 %)	2.574	0.156
Others	51	50 (12.3 %)	1 (3.0 %)		
pN category					
N1 (1–3)	224	195 (48.1 %)	29 (87.9 %)	21.864	<0.001
N2 (4–9)	129	125 (30.9 %)	4 (12.1 %)		
N3 (>10)	85	85 (21.0 %)	0		
TNM stage					
II	208	180 (44.4 %)	28 (84.8 %)	19.976	<0.001
III+IV	230	225 (55.6 %)	5 (15.2 %)		

[a]Continuous variable, independent samples *t*-test of mean ± SD

positive lymph nodes (p=0.030, OR=0.379) were found to be independent risk factors associated with SM. Logistic regression analysis also revealed that Luminal A (p=0.003, OR=0.139) cases had a lower risk of SM relative to Luminal B1 cases. Other clinicopathologcial features such as TNM stage showed no statistically significant in the multivariate analysis.

Table 4 Association of SM with Gli-1 and intrinsic subtypes of breast cancer

Characteristics	*N*	Patterns of ALN involvement		χ^2	*p* value
		Non-SM group (*n*=102)	SM group *n*=33		
Ki67 labeling index					
High (>14 %)	87	54 (52.9 %)	23 (69.7 %)	2.857	0.108
Low (≦14 %)	48	48 (47.1 %)	10 (30.3 %)		
Gli-1 status					
Positive	44	18 (17.6 %)	26 (78.8 %)	42.424	<0.001
Negative	91	84 (82.4 %)	7 (21.2 %)		
Intrinsic subtypes					
Luminal A	31	38 (37.3 %)	3 (9.1 %)	12.860	0.009
Luminal B1 (HER-2 -)	57	30 (29.4 %)	17 (51.5 %)		
Luminal B2 (HER-2 +)	12	7 (6.9 %)	5 (15.2 %)		
Erb-B2 overexpression	13	10 (9.8 %)	3 (9.1 %)		
Basal-like (3-negtive)	22	17 (16.7 %)	5 (15.2 %)		

Discussion

ALN status has been the most important prognostic factor for breast cancer throughout the past century. SLN biopsy has largely replaced ALN dissection as the preferred method of lymph node staging for breast cancer at many centers in the US, China and worldwide. The literature in merit has since reported that less than half of all patients who have undergone ALND following a positive SLNB have shown additional metastases [17]. SM of ALN in breast cancer is a challenge to the safety of this surgical technique. In our study, the overall incidence of SM was 3.2 %, accounting for 7.5 % of the patients with positive ALN. Although the frequency of SM in our hospital is relatively rare, it disrupts the process of finding the appropriate choice for the comprehensive treatment and impedes the accurate evaluation of prognosis. How about the mechanism of the SM? Can we predict it according to current research on metastasis?

According to current clinical treatment guidelines, there are no clear indicators that can be used for preoperative assessment of the occurrence of SM of ALN. Sun et al. [5] reported that routine clinicopathological parameters such as age, tumor size, location, or pathological types were similar in patients with or without SM. Gaglia et al. [18] indicated that SM is associated with the number of positive lymph nodes. They noted that SM was more likely to occur when there was only one to three lymph nodes positive, where the incidence of SM is 26.3 %, and when the number of positive lymph nodes was larger than 4, the incidence of SM was 13.1 % [18]. Msuya and Hartveit [2] had a similar conclusion after studying the relationship between SM and micrometastasis; they opined that the separate micrometastasis and SM are found in the early stage of axillary metastasis. In this study, level I negative patients with SM

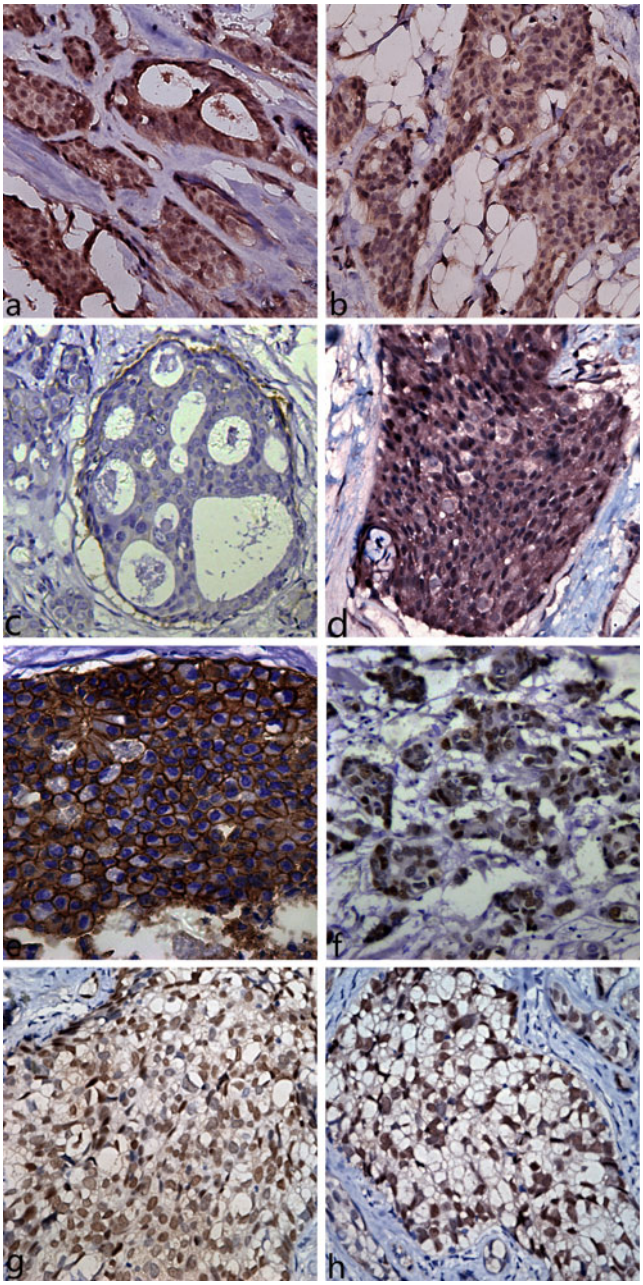

Fig. 1 Immunohistochemical staining for Gli1, BRCA1, p53, ER, PR and Erb-B2 in the primary foci of breast cancer which had SM in ALN metastases. **a** Gli1 positive staining in cytoplasm and nuclear of breast cancer cells by IHC. **b** Gli1 positive staining in cytoplasm and nuclear of breast cancer cells by IHC. **c** Gli1 negative staining in breast cancer cells by IHC. **d** BRCA2 staining was found in cytoplasm and nuclear in cancer cells by IHC. **e** Entire membrane staining with cytoplasm staining of Erb-B2 in breast cancer cells by IHC. **f** p53 nuclear accumulation in breast cancer cells by IHC. **g** ER positive staining in nuclear in breast cancer cells by IHC. **h** PR positive staining in nuclear in breast cancer cells by IHC, which was similar to that of ER by IHC

Table 5 Logistic regression analysis of clinicopathologic features correlated with SM with level I negative

Factor	Odds ratio	95 % confidence interval	*p* value
Gli-1 positivity	20.177	4.790–84.986	<0.001
Nodes positive	0.379	0.158–0.908	0.030
TNM stage	0.108	0.006–2.097	0.141
Tumor size	0.577	0.325–1.025	0.061
Tumor location	1.475	0.318–6.840	0.619
Age	1.063	0.961–1.175	0.234
Menopausal status	0.320	0.041–2.477	0.275
Ki67 labeling index	1.059	0.134–8.397	0.957
ER status	1.414	0.134–14.981	0.773
PR status	2.673	0.332–21.538	0.356
HER-2 status	0	0	0.999
P53 status	0.967	0.263–3.556	0.960
BRCA1 status	6.602	0.683–63.763	0.103
Pathologic types	0	0	0.999
Intrinsic subtypes			
Luminal B1	1	Reference	
Luminal A	0.139	0.037–0.520	0.003
Luminal B2	1.261	0.346–4.592	0.726
Erb-B2 overexpression	0.529	0.128–2.192	0.380
Basal-like	0.519	0.163–1.658	0.268

were more frequently observed in pN1 (χ^2=21.864, p<0.001) and stage II (χ^2=19.976, p<0.001); significant differences could be observed between the groups. Multivariate analysis also confirmed the numbers of positive nodes as an independent high risk factor. Meanwhile, although the pT category could not be confirmed as a risk factor, the *t*-test performed in the SM group showed that the mean size of tumor was less than that in the control group (F=8.304, p=0.004). We also found that age, tumor location, pathological type, estrogen and progesterone receptors, HER-2 status all had no relationship with SM. It is suggested that the SM with level I negative occurred in the early stage of carcinogenesis. And this phenomenon can be covered with extensive metastasis in lymph nodes in the process of breast cancer advancing to stage III or later. This may explain why the data of SM is not high in our research. Further investigation of the mechanism of SM appears to be warranted in breast carcinogenesis.

Molecular profiling has fundamentally changed our understanding of breast cancer in the last 10 years, by identifying several distinct breast cancer subtypes. Perou et al. [19] first studied gene expression of breast cancer in 2000, and they first proposed gene subtypes of breast cancer. After that, during the 12th St Gallen International Breast Cancer Conference (2011), the experts adopted more clinical valued biological subtypes that may be approximated using clinicopathological rather than gene expression array criteria [16]. With the in-depth study on intrinsic biological subtypes of breast cancer, it was found that the different subtypes have different epidemiological, clinical, pathological and prognostic features [20–23]. Van Calster et al. [24] studied the relationship between ALN metastasis and subtypes, found that the luminal B2's incidence of ALN positive was highest, and is significantly higher than that of Erb-B2 overexpression and Basal-like type. Nguyen et al. [25] found that the local recurrence rate of luminal A type was the lowest, and that of the Erb-B2 overexpression and luminal B is relatively higher, while the distant metastasis rate of Luminal B and Basal-like type were higher than luminal A. However, there are only a few studies on the association between ALN skip metastases and subtypes. In the current study, we found SM with level I negative was more frequently observed in the luminal B1 type. The mechanism involved in it may be associated with different subtypes' characteristics of invasion and metastasis. Much research has indicated that the luminal-A type has the weakest capability of invasion and metastasis. Compared with other subtypes, basal-like type is more susceptible to hematogenous metastasis; ALN metastasis of Erb-B2 overexpression type is more severe and earlier to implicate multiple levels of lymph nodes' metastasis. The luminal B-type is the most controversial; this type of metastasis mainly occurs in ALN. Although the prognosis may still be good, clinicians found that some patients with luminal B-type often have an earlier recurrence and metastasis. This may explain why SM tends to occur in luminal B-type.

Previous studies suggested that breast carcinoma cells often activate a trans-differentiation program termed the EMT to acquire the ability to execute the multiple steps of invasion-metastasis cascade [26]. Gli1 protein is a core EMT regulatory factor that plays essential roles in both development and disease processes and has been associated with metastasis in carcinomas. A previous study reported that Gli1 promotes the growth, survival, migration, invasion and metastasis of human breast cancer cell lines MDA-MB-231 and SUM1315 [27]. Gli1 is activated by hedgehog signaling pathway, and in the hedgehog pathway, the interactions of EGF/FGF, WNT and TGF-beta, Notch and of NF-β signaling pathway can inhibit the expression of E-cadherin, and result in the induction of EMT [28, 29]. Hh/Gli signal pathway is involved in breast carcinogenesis and progression. Souzaki et al. [30] studied 149 cases of Gli1 expression in breast cancer and found that Gli1 nuclear expression has a positive correlation with breast cancer's invasion and metastasis. They confirmed that the hedgehog signaling pathway could impact the invasion and metastasis of breast cancer via EMT [30]. O'Toole et al. [31] had a similar finding — that Gli1 and hedgehog ligand expression were related to the risk of metastasis and poor prognosis [31]. In the current study, the expression of Gli1 in primary breast cancer

specimens is an independent risk factor for SM with level I negative. It is suggested that Gli1 expression may play a role in breast cancer with SM. What is the mechanism involved in this? Since research on the molecular predictor of SM of ALN is very limited, this needs to be studied further.

In conclusion, we found SM with level I negative metastasis can occur at the early stage of cancer progression. Positive lymph nodes with SM are often not more than 3. Breast cancer with SM associated with the intrinsic subtypes of the luminal B1. Gli1 expression may be involved in the procession of breast cancer with SM, which can be used as a predictor of SM of ALN in breast cancer. Experimental validation of these possibilities is essential for a better understanding of SM of ALN in breast cancer.

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Conflicts of interest None

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