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Original Article

Overexpression of Rsf-1 correlates with pathological type, p53 status and survival in primary breast cancer

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Abstract: Aim: The incidence of breast cancer in developing countries still increasing, to identify novel molecular markers associated with carcinogenesis and prognosis of breast cancer still being implemented. The largest subunit of Remodeling and spacing factor (RSF), Rsf-1, mediates ATPase-dependent chromatin remodeling. Its oncogenic properties have been demonstrated in certain carcinomas. The aim of this study was to examine the prognostic value of Rsf-1 in patients with primary breast carcinoma. Methods: A total of 537 patients with primary breast cancer, and 54 with benign breast hyperplasia, were performed resection surgery in the same period were enrolled. Rsf-1 immunoexpression was retrospectively assessed by immunohistochemistry (IHC). As well as, it relationship with clinicopathological factors and patient survival (LRFS, DFS and OS) was investigated. Results: Compared with benign breast hyperplasia tissues, higher percentage of Rsf-1 positive expression was detected in malignant breast carcinomas. Based on IHC staining extent × intensity scores and ROC analysis, 278 of 526 cancers (52.9%) had high-expression (cut-off values 2.5) of Rsf-1, which correlated significantly to pathologic subtypes of breast cancer (DCIS vs. IDC, $P < 0.001$; ILC vs. IDC, $P = 0.036$), bigger tumor size ($P = 0.030$), higher TNM stage ($P = 0.044$), and p53-positive expression. In addition, there was a trend that high-expression of Rsf-1 associated with younger age ($P = 0.053$). We further prove that combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) was correlated with the bigger tumor size ($P = 0.018$), and higher TNM stage ($P = 0.024$). Kaplan–Meier survival analysis showed that Rsf-1 high-expression and combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) exhibited a significant correlation with poor overall survival of patients with primary breast cancer, and no association has been identified in relation to LRFS or DFS. Especially, Univariate and multivariate survival analysis demonstrated Rsf-1 expression is an independent prognostic parameter for the overall survival of patients with breast cancer. Conclusions: High-expression of Rsf-1 is associated with pathologic subtypes of breast cancer, aggressive phenotype, p53 positive and poor clinical outcome, which confers tumor aggressiveness through chromatin remodeling, and targeting Rsf-1 gene and the pathway it related may provide new therapeutic avenues for treating breast cancer.

Keywords: Rsf-1, p53, breast cancer, chromatin remodeling, prognosis

Introduction

Breast cancer is the most commonly diagnosed cancer in female worldwide. Together with the increased incidence of breast cancer in developing countries [1], the research to identify novel molecular markers to be associated with tumorigenessis and prognosis of breast cancer still being implemented.

Recent studies have shown that defects in, or aberrant expression of, chromatin remodeling proteins associated with various developmental disorders and cancer [2]. Remodeling and spacing factor complex Rsf-1, is a member of ATP-dependent chromatin remodeling factors, interact with its partner, sucrose non-fermenting protein 2 homologous (hSNF2H), to form the RSF-1/hSNF2H complex. This complex belonging to the ISWI chromatin remodelling family mediates nucleosome deposition and generates regularly spaced nucleosome arrays [3, 4]. Such nucleosome remodeling is essential for transcriptional regulation, DNA replication, and

cell cycle progression [5-7]. Oncogenic properties of Rsf-1 have been demonstrated in high-grade serous ovarian carcinoma, in which patients with Rsf-1 amplification detected by FISH have a significantly shorter overall survival than those without amplification [8-10]. Although, the 11q13.5 amplicon that harbors Rsf-1 gene was reported in breast carcinomas [11], the clinical role of Rsf-1 expression in primary breast carcinomas is still unclear.

Davidson reports that Rsf-1 expression is down-regulated in breast carcinoma effusions compared with primary tumors and lymph node metastases, and has no prognostic role at this anatomic site, who get the conclusion by analyzed for Rsf-1 expression in forty-seven effusions from 30 patients with breast cancer [12]. This prompted us to assess systematically the immunoexpression of Rsf-1, and it clinicopathologic implication and prognostic significance in patients with primary breast carcinoma.

Materials and methods

Patients and tissue specimens

A total of 547 patients with histologically confirmed breast carcinomas, treated with modified radical mastectomy at the First Affiliated Hospital of China Medical University Between May 2008 and Aug. 2013, were enrolled in this study. Meanwhile, 54 cases of benign breast hyperplasia, which were performed resection surgery in the same period, were enrolled as controls. The acquisition of archival anonymous tissues was approved by the Regional Institute Research Medical Ethics Committee of China Medical University. For cases with breast carcinoma, electronic medical records were reviewed to extract data on clinicopathological characteristics, including the age of patients at diagnosis, menopausal status, number of lymph nodes positive, TNM stage, histological type and estrogen receptor (ER) status, progesterone receptor (PR) status, Her2/neu (human epidermal growth factor receptor 2) amplification, et al. T and N stages of all patients were documented staged according to the 7th American Joint Committee on Cancer (AJCC) TNM staging system [13].

Immunohistochemical analysis

Rsf-1 expression status was detected in formalin-fixed and paraffin-embedded breast tissues and using the Rabbit monoclonal antibody (ab109002; Abcam, Cambridge, UK; dilution 1:250). Briefly, Tissue sections from breast biopsies were cut onto precoated slides from paraffin-embedded tissues blocked at 4 μm thickness. Paraffin-embedded sections were deparaffinized and re-hydrated. For antigen retrieval, slides were heated treatment by high-pressure in a 10 mM citrate buffer (pH 6) for 10 min. After blocking endogenous peroxidase activity and non-specific binding, the sections were incubated with the primary antibody at 4°C overnight in a humidified chamber. Secondary antibody and streptavidin biotin-peroxidase were from SP immunohistochemical (IHC) staining kit (Fuzhou Maxim Biotechnological Co.) and were each applied for 30 minutes at 37°C. Antibody complexes were stained with 3, 3'-diaminobenzidine hydrochloride (DAB) and then counterstained with Gill's hematoxylin. An ovarian high-grade serous adenocarcinoma previously known to express Rsf-1 was used as a positive control. Normal rabbit serum IgG was used to replace primary antibody as a negative control. Both of which were run with each batch to ensure consistency of staining.

Immunohistochemical evaluation

The immunostained sections were evaluated and scored independently by two pathologists (Xiao-Yi Mi, and En-Hua Wang), who were blinded to the patients' clinicopathological characteristics and follow-up information, after randomly counting approximately 500 tumor cells from 5 different high-power fields (×400) within 1 specimen. For discordant cases, a multiheaded microscope was used to reach a consensus.

In this study, nuclear localization was interpreted as positive staining. Firstly, the percentage of tumor cells with Rsf-1 immunoexpression for each specimen was classified into five groups of various expression levels from 0 to 4+, denoting none, 1%~24%, 25%~49%, 50%~74% and 75%~100% of tumor cells with moderate to strong nuclear reactivity, respectively. Beside the staining extent, the intensity of staining was categorized as 0 (no staining), 1+ (weak), 2+ (moderate) and 3+ (strong). A final IHC score from 0 to 12 was calculated by multiplying the staining extent and intensity of nuclear staining. The immunohistochemical cut-off for high expression of Rsf-1 was determined through the receiver operating characteristic (ROC)

Table 1. Patients and tumors characteristics

Variable	Total no. of patients	
	(n)	%
No. of patients	537	
Age at diagnosis, years		
Median (mean ± SD)	51.3 ± 10.7	
≤ 35	28	5.2
36-49	223	41.5
≥ 50	286	53.3
Menopausal status		
Pre-	293	54.6
Post-	244	45.4
Histologic type		
DCIS[a]	25	4.7
IDC[b]	433	80.6
ILC[c]	62	11.5
Medullary	5	0.9
Mucinous	9	1.7
Paget's disease	3	0.6
Tumor size		
T_1	189	35.2
T_2	292	54.4
T_3	56	10.4
Lymph node metastasis		
N_0	290	54.0
$N_{(+)}$	247	46.0
TNM stage		
I	119	22.2
II	278	51.8
III	140	26.1
ER status		
Negative	163	30.4
Positive	374	69.6
PR status		
Negative	183	34.1
Positive	354	65.9
HER2 status		
Negative	388	72.3
Positive	149	27.7
IHC molecular subtype		
Luminal A	302	56.2
Luminal B	115	21.4
HER-2-overexpressed	42	7.8
Triple-negative[d]	78	14.5

[a]DCIS, Ductal carcinoma in situ; [b]IDC, Invasive ductal carcinoma; [c]ILC, Invasive lobular carcinoma. [d]Triple-negative, ER-PgR-Her2-.

curve analysis. The sensitivity and specificity for discriminating death or alive was plotted at each IHC score, thus generating a ROC curve. The cut-off value was established to be the point on the ROC curve where the sum of sensitivity and specificity was maximized [14, 15]. Cancers with scores above the obtained cutoff value were considered to have high Rsf-1 expression, which led to the greatest number of cancers classified, based on the presence or absence of a clinical outcome.

The expression of ER, PR, Her2 and p53 were also determined by IHC. In accordance with previous studies [16, 17], tumors were considered positive for hormone receptors (ER/PR) if at least 10% of the tumor cells showed staining in the nucleus. For HER2, the staining intensities scored from 0 to 3. HER2 expression was considered as positive when the staining intensity was score 3 (Strong complete membrane staining in more than 10% of tumor cells), if staining score was 0 to 2 (No staining, weak to moderate complete membrane staining in more than 10% of tumor cells) as negative. For p53, nuclear labeling was scored, labeling of > 10% of nuclei was considered aberrant overexpression, which was closed but not perfectly correlated with p53 mutations [18].

Survival analysis

All patients with primary breast cancer in this study were treated by mastectomy. Follow-up was determined from the date of surgery, and were followed up until Dec. 2013. All patients who are not reviewed in the last consultation were contacted again by telephone. The clinical endpoints of this study were locoregional recurrence-free survival (LRFS), disease-free survival (DFS), and overall survival (OS). Locoregional recurrence (LRR) was referred to as recurrent breast cancer in the ipsilateral chest wall, skin, axilla, internal mammary, or supraclavicular lymph nodes. LRFS, DFS, and OS were defined as the time from the date of surgery to the last visit or the date of the first evidence of LRR, recurrence or metastasis, or date of breast cancer related death, respectively.

Statistical analysis

All analyses were carried out by using the SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). ROC curve analysis was applied to determine the cutoff value for Rsf-1 high-expression by the 0, 1-criterion,

and the areas under curves (AUCs) were calculated. The Chi square test or Fisher's exact test was used to analyze associations between Rsf-1 expression status and tumor clinicaopathologic parameters. Survival curves (LRFS, DFS and OS) were constructed using the Kaplan-Meier method and statistical significance of differences between the survival rates was evaluated by the log-rank test.

The relationship between each of the explanatory variables and outcome of patients (LRFS, DFS and OS) was assessed in turn using univariate Cox's regression analysis. The variables associated with the value of $P < 0.20$ in univariate analyses were then put into the multivariate analysis. For all statistical associations, a P value of less than 0.05 was considered to be statistically significant.

Results

Chinicopathological characteristics of patients with primary breast carcinoma

Patient demographics and tumor characteristics are summarized in **Table 1**. Briefly, median age at diagnosis was 51 years, ranging from 20 to 82 years old. 28 patients (5.2%) were younger than age 36, and 286 patients (53.3%) were older than age 51. Tumor histology was Ductal carcinoma in situ (DCIS) in 4.7%, Invasive ductal carcinoma (IDC) in 80.6%, Invasive lobular carcinoma (ILC) in 11.5%, medullary in 0.9%, Mucinous in 1.7%, and Paget's disease of breast in 0.6%. Presenting stage was as follows: TNM stage I 22.2%, stage II 51.8%, and stage III 26.1%, which was pathologic stage for all patients who had surgery as the first intervention. ER positive expression was found in 69.6%, PR in 65.9% tumors and 27.7% patients had tumors that over-expressed HER2. Furthermore, the distribution of molecular subtypes was as follows: luminal A: 56.2%, luminal B: 21.4%, HER2-overexpressed: 7.8% and Triple-negative: 14.5%.

Expression of Rsf-1 and its association with clinicopathological characteristics of primary breast cancer

Due to the complexity of sectioning, staining, as well as heterogeneity of the samples, 526 of BC samples and 54 cases of breast fibroadenoma could be interpreted for Rsf-1 expression. Base on the percentage of tumor cells with Rsf-1 immunoexpression, beside in 41 cases (7.8%) Rsf-1 negative expression, Rsf-1 was detected and successfully scored in 485 cases of breast cancer with a wide range of positively stained tumor nuclei, varying from 15% to 95% (median, 55%). In which, 223 (42.4%) cases that displayed strong or moderate nuclear immunostaining in more than 50% of tumor cells, score 4+ or 3+ (**Figure 1A**, **1B**). While, stained less than 50% of tumor cells were detected in 262 (49.8%) cases, score 2+ or 1+ (**Figure 1C**, **1D**), more detail showed in **Table 2**.

In contrast, only 24 cases detected less expression of Rsf-1 (score 2+ or 1+) (**Figure 1F**), and more than half of cases (55.6%, 30/54) Rsf-1 negative expression in benign breast hyperplasia tissues (**Table 2**). Thus, compared with benign breast hyperplasia tissues, Rsf-1 protein was significantly higher-expressed in malignant breast carcinomas ($P < 0.001$).

As the immunostaining results of Rsf-1 (staining extent, 0 to 4+) in differing histopathological types of breast cancer were shown in **Table 2**. It was shown less Rsf-1 expression (4 cases, negative; 12 cases, 1+; and 9 cases, 2+) in breast ductal carcinoma in situ (DCIS) (**Figure 2A**, **2B**). However, high level of Rsf-1 expression (extent 3+ and 4+) was seen predominantly in IDC (198/424 cases), 33.3% (3/9 cases) Mucinous (**Figure 2E**, **2F**), and Paget's disease (all of 3 cases) (**Figure 2G**, **2H**). Compared to the immunoexpression of Rsf-1 in IDC, it was significantly lower in DCIS and ILC (**Figure 2C**, **2D**) ($P < 0.001$, and $P = 0.024$, respectively).

Moreover, based on IHC staining extent × intensity scores, a ROC analysis was plotted to investigate the optimal cut-off values that maximized the sum of sensitivity and specificity. Rsf-1 showed statistically significant AUCs with 0.598 ($P = 0.018$), a threshold value of 2.5 was the optimal point for maximum sensitivity and specificity, and selected as the cut-off score. As shown in **Table 3**, The 526 cases of breast cancer were then categorized into high and low Rsf-1 expression groups. 278 of 526 cancers (52.9%) had high expression of Rsf-1, which correlated significantly to pathologic subtypes of breast cancer (DCIS vs. IDC, $P < 0.001$; ILC vs. IDC, $P = 0.036$), tumor size ($P = 0.030$) and TNM stage ($P = 0.044$). In addition, there was a trend that high-expression of Rsf-1 associated

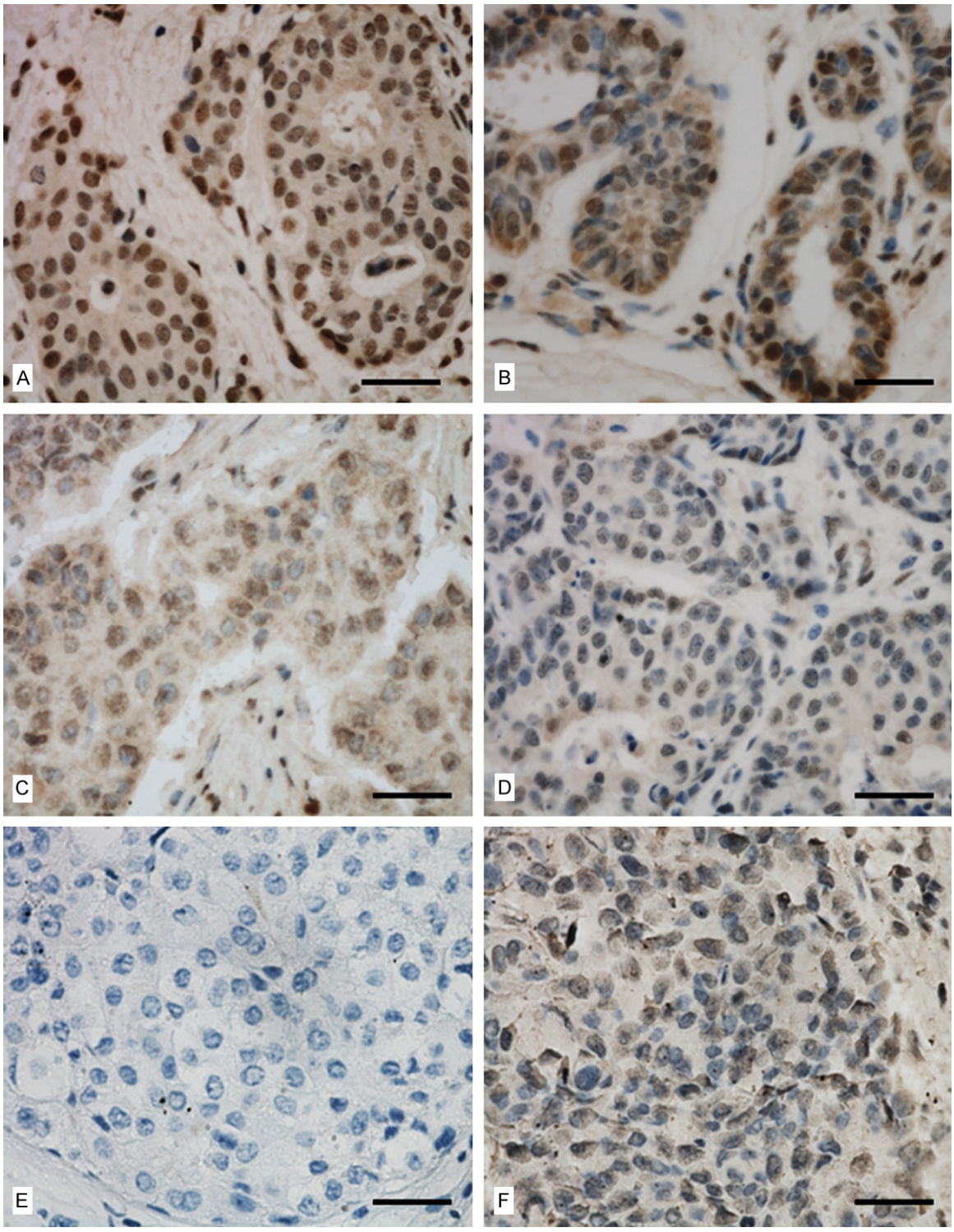

Figure 1. Representative immunohistochemical staining of Rsf-1 expression in breast invasive ductal carcinomas and benign breast hyperplasia tissues (magnification ×400). Examples of the immunointensity extent scores 4+ to 1+ of Rsf-1 expression in IDC (A to D), negative control (E) and immunointensity extent scores 1+ of Rsf-1 expression in benign breast hyperplasia tissues (F) are shown. Scale bar: 50 μm.

with younger age at diagnosis (≤ 35 year) (P = 0.053).

In addition, it was observed that, compared with 60.2% (109/181) p53-positive tumors

Table 2. Expression of Rsf-1 in malignant and benign breast tissues

Histological type	Total cases	Immunoreactivity for Rsf-1 (staining extent)					*P*-value
		0	1+	2+	3+	4+	
Malignant	526	41 (7.8)	74 (14.1)	188 (35.7)	186 (35.4)	37 (7.0)	
DCIS	25	4 (16.0)	12 (48.0)	9 (36.0)	0	0	< 0.001[a,*]
IDC	426	28 (6.6)	50 (11.7)	150 (35.2)	162 (38.0)	36 (8.5)	
ILC	58	9 (15.5)	10 (17.2)	22 (37.9)	16 (27.6)	1 (1.7)	0.024[a,*]
Medullary	5	0	1 (20.0)	2 (40.0)	2 (40.0)	0	0.898
Mucinous	9	0	1 (11.1)	5 (55.6)	3 (33.3)	0	0.667
Paget's disease	3	0	0	0	3 (100.0)	0	0.305
Benign							
Hyperplasia	54	30 (55.6)	21 (38.9)	3 (5.5)	0	0	< 0.001[a,*]

Values were numbers (percentage); DCIS, Ductal carcinoma in situ; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; [a]indicates a significant difference of Rsf-1 expression between differing histologically groups and IDC or between hyperplasia and malignant breast tissues, [*]*P*-value < 0.05.

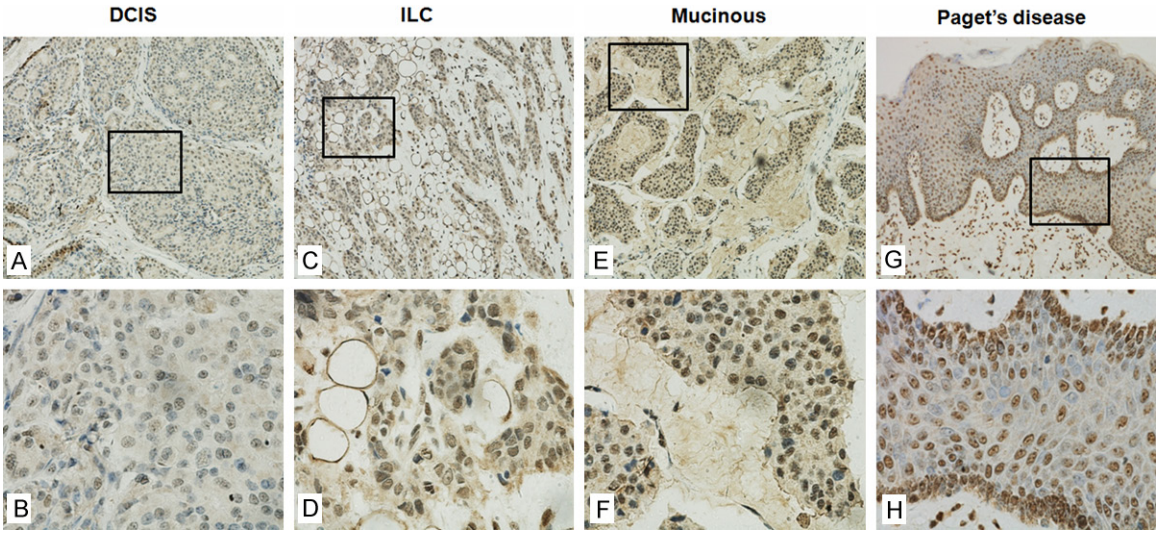

Figure 2. Representative immunohistochemical stainings of Rsf-1 expression in differing histopathologic type breast cancers, including in DCIS, ILC, Mucinous, and Paget's disease. The staining for Rsf-1 was weak in DCIS (A, ×100), moderately positive in ILC (C, ×100) and Mucinous (E, ×100), and comparably strongly positive Paget's disease (G, ×100). The bottom panels (B, D, F, and H) indicate the higher magnification (×400) from the area of the black boxes in the top images, respectively.

showing Rsf-1 high-expression, 49.0% (169/345) p53-negative tumors had Rsf-1 high-expression. The Spearman's rank correlation analysis showed a positive correlation between the expressions of Rsf-1 and p53 ($P = 0.017$). No significant correlation was identified between Rsf-1 high-expression and other clinicopathological variables, including Menopausal status, Lymph node metastasis status, HER-2, hormone receptor status or molecular subtypes ($P > 0.05$).

We further investigated the association of the combined expression of Rsf-1 and p53 with clinicopathological characteristics of breast cancer (**Table 4**). Compared with the double-negative expression of Rsf-1 and p53 (Rsf-1 (-)/p53 (-)) or the expression of either Rsf-1 or p53 (Rsf-1 (+)/p53 (-) and Rsf-1 (-)/p53 (+)), the combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) was more correlated with the bigger tumor size ($P = 0.018$), and higher TNM stage ($P = 0.024$).

Correlation between RSF-1 expression and survival of patients with primary breast cancer

Of all 537 cases, 530 were successfully followed up at a rate of 98.7%. The 5-year cumulative incidence of LRR-free survival (LRFS), dis-

Table 3. The relationship between Rsf-1 expression and clinicopathological parameters in breast cancer

Variables	Total cases	Rsf-1 expression (ROC, cut-off value: 2.5)		*P*-value
		Low	High	
Age at diagnosis, years	526			
≤ 35	28	8 (28.6)	20 (71.4)	0.053
> 35	498	240 (48.2)	258 (51.8)	
Menopausal status				
Pre-	286	133 (46.5)	153 (53.5)	0.793
Post-	240	115 (47.9)	125 (52.1)	
Histologic type				
DCIS	25	21 (84.0)	4 (16.0)	< 0.001[a,*]
IDC	426	187 (43.9)	239 (56.1)	
ILC	58	34 (58.6)	24 (41.4)	0.036[a,*]
Medullary	5	3 (60.0)	2 (40.0)	0.658
Mucinous	9	3 (33.3)	6 (66.7)	0.737
Paget's disease	3	0 (0.0)	3 (100.0)	0.261
Tumor size				0.030[*]
T_1	186	102 (54.8)	84 (45.2)	
T_2	285	121 (42.5)	164 (57.5)	
T_3	55	25 (45.5)	30 (54.5)	
Lymph node metastasis status				0.189
N_0	285	142 (49.8)	143 (50.2)	
$N_{(+)}$	241	106 (44.0)	135 (56.0)	
TNM stage				0.044[*]
I	117	67 (57.3)	50 (42.7)	
II	271	121 (44.6)	150 (55.4)	
III	138	60 (43.5)	78 (56.5)	
ER status				0.776
Negative	161	74 (46.0)	87 (54.0)	
Positive	365	174 (47.7)	191 (52.3)	
PR status				0.582
Negative	180	88 (48.9)	92 (51.1)	
Positive	346	160 (46.2)	186 (53.8)	
HER2 status				0.435
Negative	381	184 (48.3)	197 (51.7)	
Positive	145	64 (44.1)	81 (55.9)	
Molecular subtypes				0.596
Luminal A	300	147 (49.0)	153 (51.0)	
Luminal B	108	45 (41.7)	63 (58.3)	
HER-2-overexpressed	42	21 (50.0)	21 (50.0)	
Triple negative	76	35 (46.1)	41 (53.9)	
Triple negative subtype				0.836
No	450	213 (47.3)	237 (52.7)	
Yes	76	35 (46.1)	41 (53.9)	
P53 status				0.017[*]
Negative	345	176 (51.0)	169 (49.0)	
Positive	181	72 (39.8)	109 (60.2)	

Values were numbers (percentage); DCIS, Ductal carcinoma in situ; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. [a]indicates a significant difference of Rsf-1 expression between differing histologically groups and IDC, [*]*P*-value < 0.05.

Table 4. Association of the co-expression of Rsf-1 and p53 with tumor size, lymph node metastasis, and TNM stage in primary breast cancer

Features		Rsf-1 (-)/p53 (-)	Rsf-1 (-)/p53 (+) and Rsf-1 (+)/p53 (-)	Rsf-1 (+)/p53 (+)	*P*-value
		n (%)	n (%)	n (%)	
Tumor size	526				0.018*
T_1	186	74 (39.8)	87 (46.8)	25 (13.4)	
T_2	285	83 (29.1)	129 (45.3)	73 (25.6)	
T_3	55	19 (34.5)	25 (45.5)	11 (20.0)	
Lymph node metastasis status					0.390
N_0	285	100 (35.1)	132 (46.3)	53 (18.6)	
$N_{(+)}$	241	76 (31.5)	109 (45.2)	56 (23.2)	
TNM stage					0.024*
I	117	51 (43.6)	52 (44.4)	14 (12.0)	
II	271	80 (29.5)	130 (48.0)	61 (22.5)	
III	138	45 (32.6)	59 (42.8)	34 (24.6)	

Note: Values were numbers (percentage), *P*-value obtained from Pearson Chi-Square or Fisher's exact test, *P-value < 0.05.

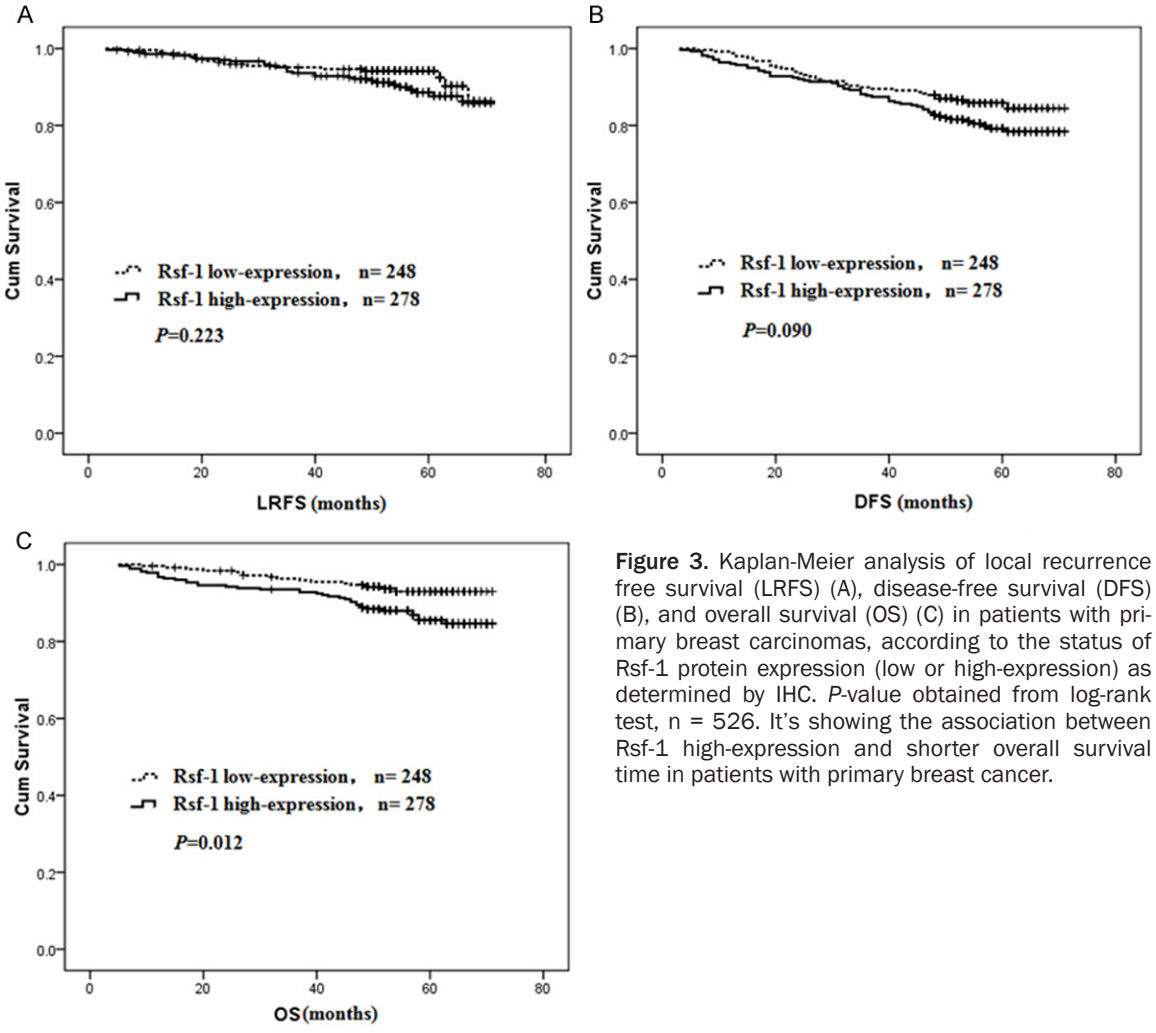

Figure 3. Kaplan-Meier analysis of local recurrence free survival (LRFS) (A), disease-free survival (DFS) (B), and overall survival (OS) (C) in patients with primary breast carcinomas, according to the status of Rsf-1 protein expression (low or high-expression) as determined by IHC. *P*-value obtained from log-rank test, n = 526. It's showing the association between Rsf-1 high-expression and shorter overall survival time in patients with primary breast cancer.

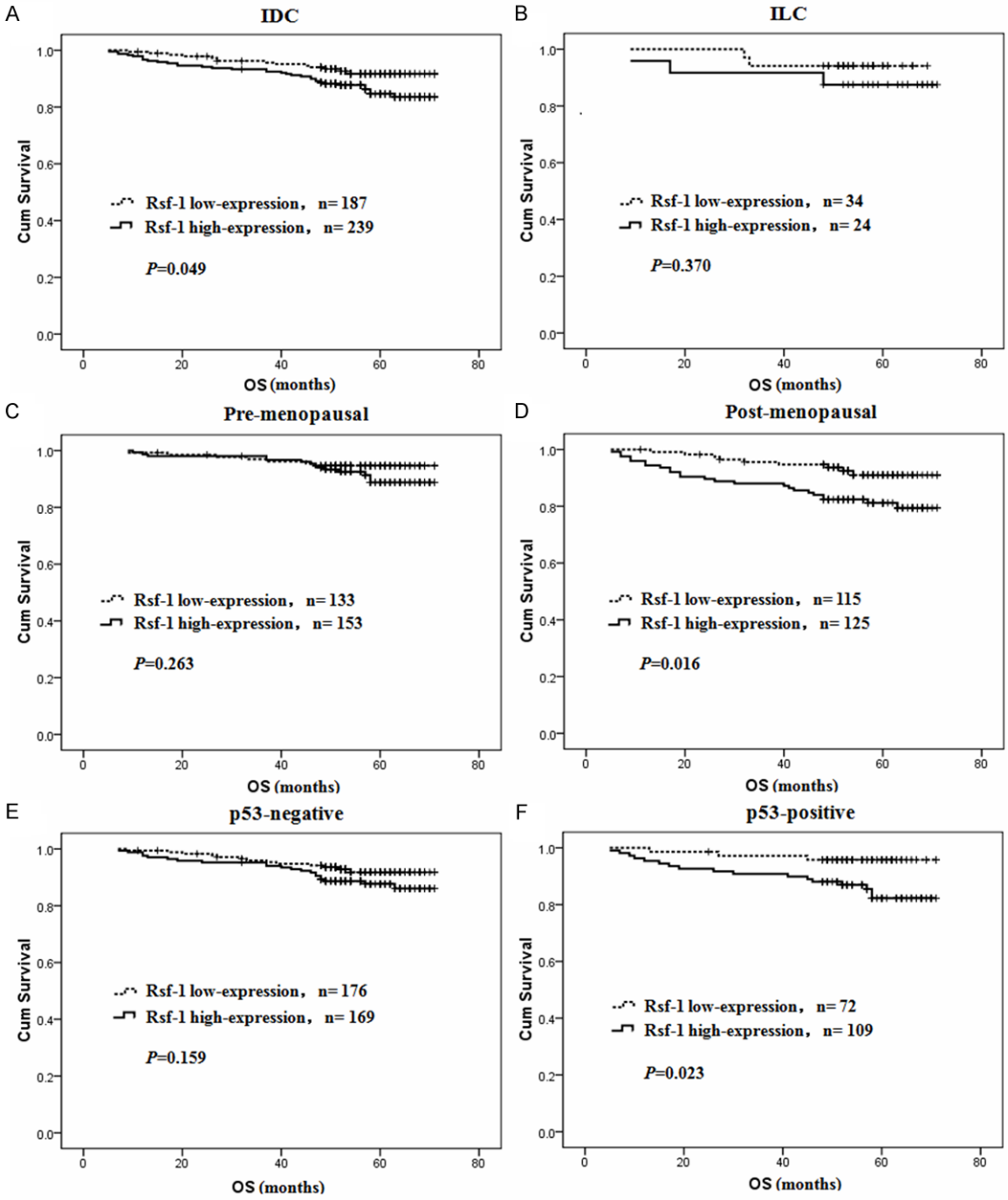

Figure 4. Kaplan-Meier analysis of overall survival in a subgroup of breast cancers, according to the status of Rsf-1 protein expression, which evaluated by IHC extant, including (A) histopathological type IDC, (B) ILC, (C) pre-menopausal, (D) post-menopausal, (E) p53-negative and (F) p53-positive subgroups. Rsf-1 high-expression was associated with OS in IDC, post-menopausal, or p53-positive subgroups (P = 0.049, P = 0.016 and P = 0.023, respectively).

ease-free survival (DFS) and overall survival (OS) were 90.4% (95% CI, 88.9%-91.9%), 81.1% (95% CI, 79.2%-83.0%), and 88.7% (95% CI, 87.1%-90.3%) respectively in this cohort of patients. Kaplan-Meier curves of survival stratified by Rsf-1 expression (high vs. low) were shown in **Figure 3**. It revealed an association between Rsf-1 high-expression and poor overall survival by log-rank tests (P = 0.012, log rank test, **Figure 3C**). However, no significant

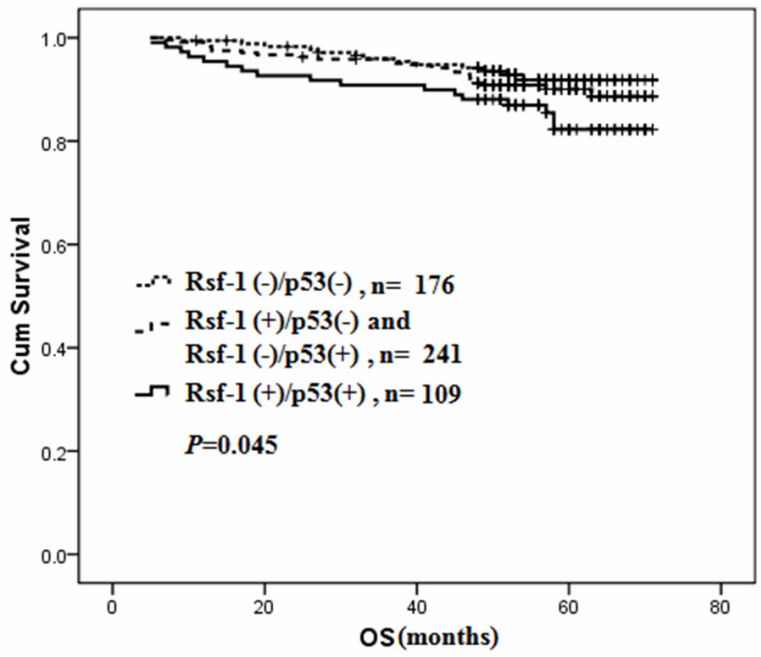

Figure 5. Kaplan-Meier analysis of overall survival in breast cancers, according to the co-expression status of Rsf-1 and p53 protein. The combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) was associated with a significantly shorter OS (P = 0. 045) in patients with breast cancer.

association has been identified in relation to LRFS (P = 0.223, **Figure 3A**) or DFS (P = 0.090, **Figure 3B**).

As well as, Further analysis was performed in subsets of patients with different clinicopathologic parameters, such as age (≤ 35 or > 35), differing molecular subtypes, tumor size (T1, T2 or T3), lymph node status (positive or negative), TNM stage (I, II or III), ER, PR, and HER2 (positive or negative), to investigate the association between Rsf-1 expression and OS. As it was shown in **Figure 4**, subgroup analysis demonstrated that Rsf-1 high-expression was associated with shorter overall survival in the IDC, Post-menopausal, and p53-positive subgroup (P = 0.049, P = 0.016 and P = 0.023, **Figure 4A**, **4D** and **4F**). No significant difference in overall survival was observed between highly expressed Rsf-1 cases and low expression Rsf-1 cases in others subgroup patients (P > 0.05).

We also examined the association of the combined expression of Rsf-1 and p53 with the OS, LRFS and DFS in breast cancer patients. Compared with the double-negative expression of Rsf-1 and p53 (Rsf-1 (-)/p53 (-)) or the expression of either Rsf-1 or p53 (Rsf-1 (+)/p53 (-) and Rsf-1 (-)/p53 (+)), the combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) was associated with a significantly shorter OS (P = 0.045, **Figure 5**) in patients with primary breast cancer. Due to the limited number of patients were examined in the subgroups, further trials are needed to prove the results.

A univariate Cox regression model was performed to estimate the impact of each clinicopathological variable on the LRFS, DFS and OS. As shown in **Table 5**, Univariate Cox regression analysis revealed that lymph nodal status, and TNM stage was significantly associated with the LRFS, DFS and OS of breast cancer patients. The tumor size was significantly associated with the DFS (P = 0.009) and OS (P = 0.042), but not the LRFS of breast cancer patients (P = 0. 058) (**Table 5**). In addition, the expression of Rsf-1 alone or in combination with p53 was significantly associated with the shorter OS (P = 0.018, and P = 0.031, respectively), and HER2 expression was associated with the shorter DFS of breast cancer patients (P = 0.047, **Table 5**). We failed to identify patient's age, ER, and PR expression as valuable prognostic factors.

More importantly, besides, TNM stage was found to have an independent prognostic impact in all survival analyses (LRFS, P = 0.002, HR 2.522; DFS, P < 0.001, HR 2.508; OS, P < 0.001, HR 9.053). Rsf-1 high-expression remained to be a significant prognosticator for overall survival in multivariate analysis (P = 0.013, HR 2.162, **Table 6**).

Discussion

In the recent years, great progress has been made in identifying new ATP-dependent chromatin remodeling factors that play a role in the orchestration of regulating gene transcription, as well as inappropriate chromatin remodeling activity can lead to deregulated gene activation that is associated with carcinogenesis.

Table 5. Univariate Cox regression analysis of prognostic parameters associated with locoregional recurrence-free survival, disease-free survival, and overall survival in primary breast cancer patients

Variables	LRFS			DFS			OS		
	HR[a]	95%CI[b]	*P*	HR	95%CI[b]	*P*	HR	95%CI	*P*
Age, years									
> 35 vs. ≤ 35	0.490	1.194~1.240	0.132	0.692	0.320~1.496	0.349	0.712	0.257~1.974	0.514
Menopause State									
Post vs. Pre	0.978	0.550~1.739	0.940	1.537	1.019~2.319	0.240	1.885	1.087~3.270	0.724
Tumor size									
T3 vs. T1&T2	2.020	0.976~4.181	0.058	2.017	1.191~3.415	0.009	2.045	1.027~4.070	0.042*
LN metastasis status									
$N_{(+)}$ vs. N_0	1.982	1.106~3.551	0.021*	2.725	1.762~4.214	< 0.001*	4.996	2.572~9.705	< 0.001*
TNM stage									
III vs. I, II	2.522	1.414~4.500	0.002*	4.200	2.783~6.338	< 0.001*	9.247	5.018~17.038	< 0.001*
ER status									
P vs. N	0.981	0.525~1.833	0.952	0.792	0.516~1.217	0.288	0.590	0.342~1.019	0.158
PgR status									
P vs. N	0.879	0.479~1.613	0.678	0.725	0.478~1.100	0.131	0.632	0.367~1.088	0.298
HER2 status									
P vs. N	1.412	0.778~2.565	0.257	1.535	1.005~2.345	0.047*	1.452	0.828~2.546	0.193
p53 status									
P vs. N	1.174	0.652~2.114	0.594	1.073	0.701~1.643	0.745	1.111	0.639~1.930	0.710
Rsf-1 expression									
High vs. Low	1.450	0.795~2.646	0.225	1.438	0.942~2.194	0.092	2.037	1.133~3.665	0.018*
Co-expression of Rsf-1 and p53									
++/+-, -+/--	1.150	0.884~1.496	0.299	1.110	0.924~1.334	0.264	1.259	0.981~1.616	0.031*

[a]HR, Hazard Ratio; [b]CI, confidence interval; P, positive; N, negative. *$P < 0.05$.

Table 6. Multivariate Cox regression analysis of prognostic parameters associated with locoregional recurrence-free survival (LRFS), disease-free survival (DFS), and overall survival (OS) in breast cancer patients

Variable	LRFS			DFS			OS		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age, years									
> 35 vs. ≤ 35	0.529	0.208~1.345	0.181		NR			NR	
Tumor size									
T_3 vs. $T_{1, 2}$	1.525	0.709~3.281	0.281	1.184	0.682~2.057	0.548	1.027	0.503~2.095	0.798
LN metastasis status									
$N_{(+)}$ vs. N_0	1.276	0.555~2.937	0.566	1.167	0.458~1.928	0.864	0.776	0.215~2.795	0.615
TNM stage									
III vs. I, II	2.522	1.414~4.500	0.002*	2.508	1.802~3.491	< 0.001*	9.053	4.910~16.693	< 0.001*
ER expression									
P vs. N		NR			NR		0.684	0.255~1.835	0.534
PR expression									
P vs. N		NR		0.739	0.450~1.212	0.230		NR	
HER2 expression									
P vs. N		NR		1.339	0.794~2.259	0.274	1.025	0.419~2.510	0.704
Rsf-1 expression									
High vs. Low		NR		1.353	0.878~2.084	0.171	2.162	1.180~3.959	0.013*
Co-expression of Rsf-1 and p53									
++/+-, -+/--		NR			NR		1.025	0.419~2.510	0.704

HR, Hazard Ratio; CI, confidence interval; NR, variable was not included in the resultant model; *$P < 0.05$.

Remodeling and spacing factor Rsf-1, also known as hepatitis B X-antigen associated protein (HBXAP), is a subunit of an ISWI chromatin remodeling complex. Our findings suggest that Rsf-1 high-expression may be a more frequent event in breast carcinoma than previous

reports of Mao et al [8], and high-expression of Rsf-1 correlates with pathologic types of breast carcinomas. Compared with that in IDC, DCIS and ILC were comparably had a significantly less frequent of Rsf-1 high-expression. Breast cancer is an extraordinarily diverse group of diseases in terms of presentation, morphology and molecular profile [19, 20]. It was reported that ILC seems to be more likely multifocal, estrogen receptor positive, HER-2 negative and to have a lower proliferative index compared to IDC [21]. Expression of Rsf-1 in primary breast cancer differentiates ILC and IDC suggested that Rsf-1 protein expression is a biomarker of subtype per se. This scenario in breast cancer is also therefore similar to that observed in ovarian cancer where overexpression of Rsf-1 was only significantly associated with high-grade ovarian serous carcinoma, as compared with other types of ovarian tumors, including ovarian serous borderline tumors, ovarian endometrioid carcinomas, and ovarian mucinous carcinomas [8]. Due to the limited number of special pathologic subtype of breast carcinomas in this study, further trials are needed to prove the Rsf-1 status in differing pathologic subtypes.

Furthermore, we found high-expression of Rsf-1 was associated with the bigger tumor size and higher TNM stage, similar to what was previously reported in other histotypes of cancers [8, 22-26], and tend to associate with younger age of patients. In addition, high-expression of Rsf-1 was associated with p53 expression, and the combined positive-expression of Rsf-1 and p53 (Rsf-1 (+)/p53 (+)) was also significantly correlated with tumor size, and TNM stage. Especially, the combined positive-expression of Rsf-1 and p53, and Rsf-1 high-expression alone was associated with shorter overall survival times. As Shih and colleagues' study demonstrated that expression of Rsf-1 caused DNA double-strand breaks in non-transformed normal cells, which subsequently lead to DNA damage response (DDR) and activation of the ataxia-telangiectasia mutated (ATM) -CHK2-p53-p21 pathway, leading to growth arrest and apoptosis. However, Rsf-1-induced DDR as a selecting barrier that favored outgrowth of cell clones with a Tp53 mutation, thus promote chromosomal instability in cancer cells, allow further genetic alterations and oncogene activation during cancer development [27]. Furthermore, recently, it was reported Tp53 mutations were a prerequisite for tumor-promoting functions of the RSF/cyclin E1 complex [28]. So Rsf-1 maybe collaborates with p53 during cancer development, and the tumor with combined positive-expression of Rsf-1 and p53 showing the aggressive phenotype and poor survival. However, Chromatin remodeling factors are present in large multiprotein complexes whose exact roles warrant further investigation.

In conclusion, this is the first time shown that Rsf-1 highly expressed in primary breast cancers and significantly correlated with the pathologic types of breast cancer and p53 expression. In addition, high-expression of Rsf-1 represents a significant prognosticator of worse prognosis for overall survival, which confers tumor aggressiveness through chromatin remodeling, and targeting Rsf-1 gene and the pathway it related may provide new therapeutic avenues for treating breast cancer.

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Disclosure of conflict of interest

None.

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