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Original Article

Predictive value of CD44 and CD24 for prognosis and chemotherapy response in invasive breast ductal carcinoma

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Abstract: Objective: Cells with unique phenotypes and stem cell-like properties have been found to exist in breast cancer. The aim of the present study was to study the relationship of CD24, CD44, CD44(+)/CD24(-/low) and CD44(-)/CD24(+) tumor phenotypes' with clinico-pathological features, chemotherapy response and with prognosis. Methods: The study included paraffin-embedded tissues of 140 primary and secondary invasive ductal carcinoma samples. All the patients received routine chemotherapy. Expression of CD24, CD44, ER, PR, and Her2 were assayed immunohistochemically. We applied double-staining immunohistochemistry for the detection of CD44(+)/CD24(-/low), CD44(+)/CD24 (+), CD44(-)/CD24(-) and CD44(-)/CD24(+) cells. The association between the proportions of CD44(+)/CD24(-/low) and CD44(-)/CD24(+) and clinicopathological features, chemotherapy response and with prognosis of these patients was evaluated. Results: CD24 expression was not significantly associated with tumor characteristics, but was significantly associated with poor prognostic variables including ER-, PR-, HER2(+) and triple negative (TN) phenotype; There was no association of CD44 with nodal status, age or HER2 expression. In the correlation analysis, CD24 expression was positively associated with chemotherapy response (P = 0.018), however, CD44 expression was not associated with pathological response to chemotherapy When both markers are considered, the CD44(+)/CD24(-) phenotype had the poor prognosis. The proportion of $CD44^+/CD24^-$ tumor cells was significantly associated with lymph node involvement, recurrent or metastatic tumors and ER/PR status. High CD44(+)/CD24(-) phenotype had poor response to chemotherapy. The median disease-free survival (DFS) of patients with and without CD44(+)/CD24(-/low) tumor cells were 19.8 ± 2.6 months and 31.7 ± 4.2 months, and the median overall survival (OS) of patients with and without CD44(+)/CD24(-/low) tumor cells were 33.5 ± 2.8 months and 51.4 ± 3.9 months, respectively, and with both univariate and multivariate analyses showing that the proportion of CD44(+)/CD24(-/low) tumor cells was strongly correlated with DFS and OS. However, the CD44(-)/CD24(+), CD44(+)/CD24(+), CD44(-)/CD24 (-) phenotype had no relation with prognosis. Conclusion: There was significant correlation between CD44(+)/CD24(-/low) tumor cell prevalence and tumor metastasis, prognosis and chemotherapy response. The CD44(+)/CD24(-) phenotype may be an important factor for malignant relapse following surgical resection and chemotherapy in patients with invasive ductal carcinoma.

Keywords: Breast cancer, CD24, CD44, prognosis, chemotherapy

Introduction

The leading cause of cancer-related death in women is invasive ductal carcinoma, which is the most common breast malignancy worldwide [1]. The breast cancer has obvious propensity to bread at an early stage and the acquired resistance to a wide range of anticancer agents. Although systemic treatment of breast cancer, such as cytotoxic, hormonal, and immunotherapeutic agents, the patients with advanced stage invasive ductal carcinoma may in the end emerge tumor recurrence or metastasis within several years after treatment [2]. Therefore, selecting sensitive diagnostic markers and prognostic markers in the early stage is particular importance.

CD24, a membrane glycoprotein with unusual lipid-like, is overexpressed in various tumor types such as ovarian, breast, pancreatic carcinomas, non-small lung, hepatocellular carcinoma, renal cell carcinoma and B-cell lymphoma [3-6]. Additionally, CD24 expression is correlated with tumorigenesis and tumor progression [4-6]. Recently, it has reported that CD24 could

Table 1. Correlation of CD44 and CD24 expression with tumor characteristics

		CD24			CD44		
Characteristic	All tumors (n = 120)	-	+	*P*-value	-	+	*P*-value
Age				0.462			0.368
< 50	62	16	46		13	49	
> 50	58	18	40		8	50	
Tumor size				0.138			0.127
< 2 cm	34	19	15		13	21	
> 2 cm	86	15	71		8	78	
Lymph node metastasis				0.069			0.058
No	35	15	20		15	20	
Yes	85	19	66		6	79	
Distant metastasis				0.087			0.076
No	94	27	67		14	80	
Yes	26	7	19		7	19	
TNM stage				0.073			0.064
I + II	59	23	36		17	42	
III + IV	61	11	50		4	57	
PR				0.034			0.026
Negative	66	9	57		6	60	
Positive	54	25	29		15	39	
ER				0.025			0.045
Negative	77	17	60		9	68	
Positive	43	17	26		12	31	
HER2				0.023			0.073
Negative	58	22	36		8	50	
Positive	62	12	50		13	49	
Triple negative phenotype				0.012			0.065
No	99	33	66		11	88	
Yes	21	1	20		10	11	
Recurrence				0.072			0.064
No	72	16	56		7	65	
Yes	48	18	30		14	34	
Metastasis (Post-operation)				0.063			0.058
No	73	22	51		13	60	
Yes	47	12	35		8	39	
Chemotherapy response				0.018			0.074
Well	34	3	31		15	19	
Poor	63	25	38		13	50	

be a target toward treating osteosarcoma, gastric cancer, breast cancer and enhance chemosensitivity in these cells [7-9].

CD44 is a member of the adhesion molecule families. It is closely associated with cancer cell metastasis and chemotherapy resistance [10-14]. Previous report has demonstrated that the overexpression of CD44 is correlated with poor prognosis in human breast cancer [15]. Also, CD44 is overexpressed in trastuzumab-resistant breast cancer cells, and CD44 contributes to trastuzumab resistance independently of its role as a putative breast cancer stem cell (CSC) marker [16].

The subpopulation $CD44^+/CD24^-$ was found to be the putative stem cells in human breast tissue. These cells have been shown to survive cytotoxic therapies, which results in treatment failures and recurrences [17]. More importantly, patients with high expression of $CD44^+/$

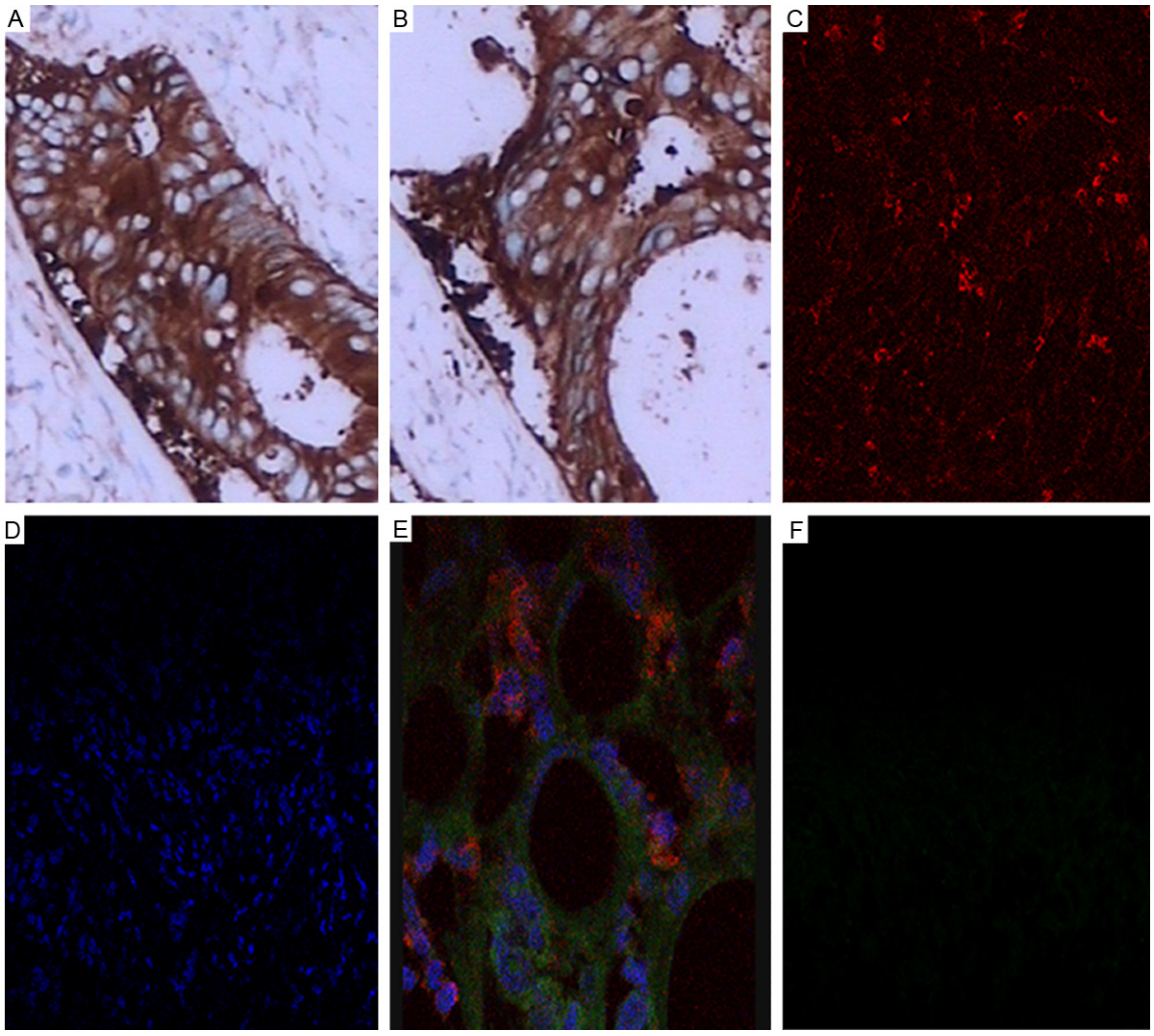

Figure 1. Immunohistochemical staining of CD44 and CD24 in breast tumor tissue sections. A (magnification x100). A. Standard immunohistochemical analysis of CD24 in breast tumor tissue sections; B. Standard immunohistochemical analysis of CD44 in breast tumor tissue sections. C. Area abundant of fuchsin-stained $CD44^{+}/CD24^{-/low}$ tumor cells by Double-staining immunohistochemistry. D. Area abundant of fuchsin-stained $CD44^{-}/CD24^{+}$ tumor cells by Double-staining immunohistochemistry. E. Area abundant of fuchsin-stained $CD44^{+}/CD24^{+}$ tumor cells Double-staining immunohistochemistry. F. Area abundant of fuchsin-stained $CD44^{-}/CD24^{-}$ tumor cells by Double-staining immunohistochemistry.

$CD24^{-}$ breast cancer cells has poor prognosis [18]. Furthermore, $CD44^{+}/CD24^{-}$ cells were often negative for erythroblastic leukemia viral oncogene homolog 2 (erbB2) receptor and estrogen receptor (ER) [19] Ahmed et al. [20] has reported although CD24 and CD44 expression could individually yield prognostic data in breast cancer, when both CD24 and CD44 are considered, the CD44(+)/CD24(-) phenotype had the best prognosis, while the CD44(-)/CD24(+) phenotype had the worst prognosis.

In the present study, we first investigated the expression of CD24, CD44, and CD44/CD24 subpopulation in surgical specimens of breast cancer using immunohistochemical and double immunohistochemical staining methods. Then we investigated the relation between CD24, CD44, and CD44/CD24 with clinicopathological features and chemotherapy response.

Materials and methods

Patients and specimens

We analyzed paraffin-embedded tumor tissues of 140 patients with invasive ductal carcinoma samples who underwent breast surgery between 2004 and 2014 at Women and Children's hospital of Qingdao. Clinical information was acquired by reviewing preoperative and perioperative medical records, or by telephone or writ-

Table 2. Correlation of $CD44^+/CD24^{-/low}$ status with tumor characteristics

Characteristic	All tumors (n = 120)	$CD44^+/CD24^{-/low}$		
		-	+	*P*-value
Lymph node metastasis				0.016
No	35	23	12	
Yes	85	44	41	
Distant metastasis				0.001
No	94	64	30	
Yes	26	3	23	
PR				0.02
Negative	66	30	36	
Positive	54	37	17	
ER				0.037
Negative	77	37	40	
Positive	43	30	13	
Recurrence				0.013
No	72	53	19	
Yes	48	14	34	
Metastasis (Post-operation)				0.014
No	73	53	20	
Yes	47	14	33	
Chemotherapy response				0.001
Well	34	20	14	
Poor	63	24	39	

ten correspondence. Patient clinical characteristics are shown in **Table 1**. Postsurgery systemic treatment was included cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), anthracycline, tamoxifen, or radiotherapy, with many patients having received multiple treatments. Patients were staged based on the tumor-node-metastases (TNM) classification of the International Union against Cancer, revised in 2007. Overall, survival included as an event all deaths, whatever the cause. Event-free survival was defined as the time elapsed between excision of the primary tumor to the manifestation of local or distant metastasis or death.

Immunohistochemical staining

Formalin-fixed and paraffin-embedded sections were dewaxed in xylene, rehydrated through graded alcohol and placed in an endogenous peroxide block for 15 min. Antigen retrieval was carried out by microwave in 10 mM citrate buffer. Nonspecific staining was blocked by treating sections with 10% goat serum in phosphate-buffered saline (pH 6.0) for 10 min. Immunohistochemical staining was carried out in a DAKO Autostainer Plus (Dako, Glostrup, Denmark) using an LSAB detection kit (Dako). The following primary antibodies were used: CD44, CD24, ER, PR and HER2. The proportions of each antibody positive tumor cells were counted semiquantitatively.

Double immunohistochemical staining for CD44 and CD24

Double immunostaining with antibodies to detect CD44 and CD24 was studied with EnVision G|2 Doublestain System Rabbit/Mouse (diamlnobenzidinet (DABt)/Permanent Red) (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. CD44 was detected with DAB and CD24 with Permanent Red. We confirmed the accuracy of the double immunostaining by comparing it with single immunostaining for CD44 and CD24, separately. The proportions of $CD44^+/CD24^-$ or $CD44^-/CD24^+$ tumour cells was counted semiquantitatively and scored in 5% increments.

Statistical analyses

Statistical analysis was done using SPSS .11 software (Chicago, IL). Associations between prevalence of CD44, CD24, $CD44^+/CD24^{-/low}$ or $CD44^-/CD24^+$ tumor cells and clinical parameters were assessed by x^2 test, except for age where the Mann-Whitney U test was used. Multivariate analysis was performed using Cox proportional hazards regression to determine the prognostic effect on disease-free survival (DFS) and overall survival (OS). Hazard ratios (HR) and their corresponding 95% confidence intervals (CI) were computed to provide quantitative information about the relevance of the results of statistical analysis. Survival analysis was with the Kaplan-Meier method. All tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

The baseline characteristics of the study population were shown in **Table 1**. All patients were with a mean ± standard deviation (SD) age of 49.8 ± 13.9 years (range, 12.4 to 78.6 years) and a mean ± SD tumor size of 2.4 ± 1.1 cm (range, 0.6 to 6.8 cm). 85 (70.8%) patients were with lymph node involvement. According to TNM classification, 59 (49%) were stage I +

Table 3. Univariate and multivariate analyses of the relationship of $CD44^+/CD24^{-/low}$ tumor cells to DFS

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
$CD44^+/CD24^{-/low}$ tumor cells			0.0018			0.015
Low	1.000			1.000		
High	2.263	1.426-3.652		1.890	1.217-3.464	
PR status			0.0028			0.001
Negative	1.000			1.000		
Positive	0.57	0.335-0.870		0.236	0.13-0.527	
ER status			0.359			0.185
Negative	1.000			1.000		
Positive	0.763	0.562-1.467		1.360	0.628-2.834	
Her2 status						0.329
Negative	1.000		0.784	1.000		
Positive	0.983	0.628-1.630		0.681	0.326-1.634	
TNM stage			0.031			0.035
I + II	1.000			1.000		
III + IV	2.042	0.940-2.956		1.985	1.074-3.241	
Lymph node involvement			0.23			0.445
Yes	1.000			1.000		
No	0.762	0.483-1.57		1.152	0.672-2.250	

II, and 61 (51%) were stage III + IV. Of the 120 patients, 43 (35.8%) were positive for ER expression, 54 (45%) were positive for PR, 58 (48%) were positive for Her2. Of the 120 patients, 97 (80.8%) were followed adjuvant chemotherapy and 75 (62.6%) were followed agents targeted against estrogen receptor. Median follow-up time was 21.6 months (range, 1.8 to 93 months), during which 48 patients (40%) experienced tumor recurrence and 47 (39%) developed metastases.

Immunohistochemical analysis for CD44 and CD24

Standard immunohistochemical analysis CD24 and CD44 was predominantly expressed on the cell membrane of breast tumor epithelial cells (**Figure 1A** and **1B**), and 71.6% (86/120) of cases were positive for CD24 expression, and 82.5% (99/120) of cases were positive for CD44 expression.

CD44 and CD24 by Double-Staining immunohistochemical analysis showed the $CD44^+/CD24^{-/low}$ cells were found in 44% (53/120) tumors (**Figure 1C**), $CD44^-/CD24^+$ cells were found in 20.8% (25/120) tumors (**Figure 1D**), CD44(+)/CD24 (+) were found in 20% (24/120) tumors (**Figure 1E**), and CD44(-)/CD24(-) were found in 15.8% (19/120) tumors (**Figure 1F**).

Correlation of CD44 and CD24 expression with tumor characteristics

The correlation of CD24 and CD44 expression with tumor characteristics were shown in **Table 1**. CD24 expression was not significantly associated with age, tumor size, lymph node status, TNM stage, distant metastasis and recurrent, et al, but was significantly associated with poor prognostic variables including ER-, PR-, HER2(+) and triple negative (TN) phenotype; There was no association of CD44 with nodal status, age or HER2 expression. In the correlation analysis, CD24 expression was positively associated with chemotherapy response (P = 0.018), however, CD44 expression was not associated with pathological response to chemotherapy.

Clinical significance of the prevalence of $CD44^+/CD24^{-/low}$ tumor cells

The proportion of $CD44^+/CD24^-$ tumor cells was significantly associated with lymph node involvement, recurrent, metastatic tumors, ER and PR status. High CD44(+)/CD24(-) phenotype had poor response to chemotherapy

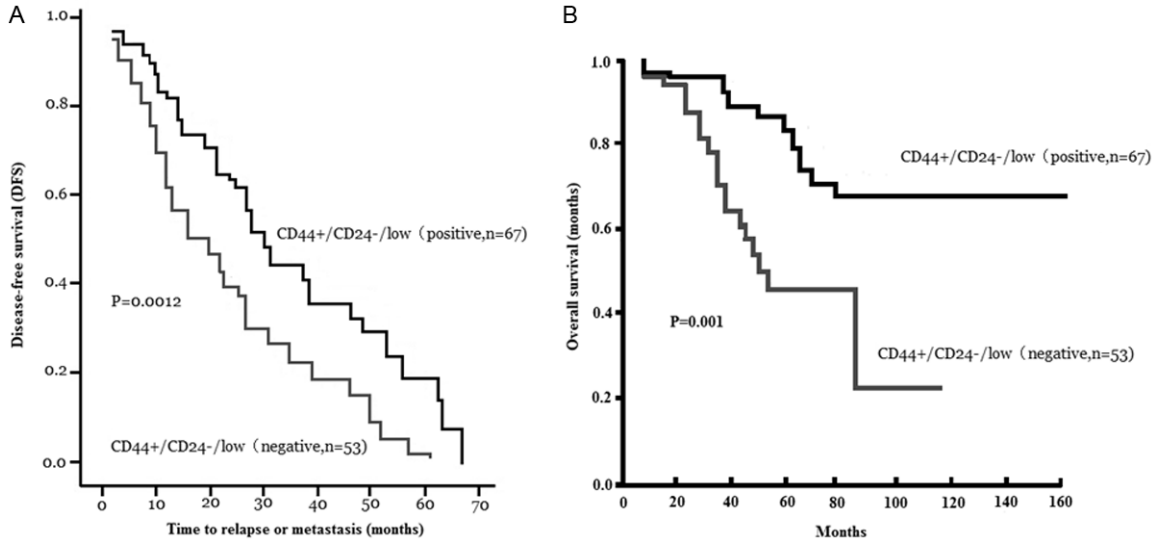

Figure 2. Kaplan-Meier curves for overall survival of DFS and OS. A. The presence of $CD44^{+}/CD24^{-/low}$ tumor cells on disease-free survival (DFS); B. The presence of $CD44^{+}/CD24^{-/low}$ tumor cells on overall survival (OS).

(**Table 2**). No relation was found between CD44(+)/CD24(-) phenotype with HER2, triple negative phenotype, TNM stage and tumor size (data not shown). The presence of $CD44^{-}/CD24^{+}$ tumor cells was solely associated with HER2 staining (P = 0.018) and not with any other tumor characteristics. The presence of double-positive ($CD44^{+}/CD24^{+}$, n = 24) tumor cells and double-negative ($CD44^{-}/CD24^{-}$, n = 19) tumor cells was not associated with any tumor features (data not shown).

Association of $CD44^{+}/CD24^{-/low}$ phenotype with DFS and OS

Univariate analyses showed the results of the relations between each individual predictor and DFS in **Table 3**. The proportion of $CD44^{+}/CD24^{-/low}$ tumor cells, PR status and TNM stage were strongly correlated with DFS. Kaplan-Meier analysis showed that the presence of $CD44^{+}/CD24^{-/low}$ tumor cells was significantly associated with shorter DFS compared with the absence of $CD44^{+}/CD24^{-/low}$ tumor cells (19.8 ± 2.6 months vs 31.7 ± 4.2 months P = 0.0012; **Figure 2A**). Multivariate analysis showed the presence of $CD44^{+}/CD24^{-/low}$ tumor cells, PR status, and TNM stage retained their prognostic significance for DFS (**Table 3**).

Univariate analyses showed the associations between each individual predictor and OS in **Table 4**. Similarly with the relation with DFS, the proportion of $CD44^{+}/CD24^{-/low}$ tumor cells, and TNM stage were strongly correlated with OS. Kaplan-Meier analysis showed that the presence of $CD44^{+}/CD24^{-/low}$ tumor cells was significantly associated with shorter OS compared with the absence of $CD44^{+}/CD24^{-/low}$ tumor cells (P = 0.001, **Figure 2B**); Multivariate analysis showed the presence of $CD44^{+}/CD24^{-/low}$ tumor cells and TNM stage retained their prognostic significance for OS (**Table 4**). However, the CD44(-)/CD24(+), CD44(+)/CD24(+), CD44(-)/CD24(-) phenotype had no relation with prognosis (data not shown).

Discussion

The most common breast malignancy in women is invasive ductal carcinoma. Relapse or metastasis frequently occurred after surgical resection. Additional, resistance to conventional chemotherapies is the important reason for unfavourable prognosis. Therefore, selecting sensitive diagnostic markers, prognostic markers and anticancer agents in the early stage is particular importance.

CD24 is overexpressed in various human malignancies [21]. Its high expression is often correlated with poor prognosis and chemoresistance [4-9]. In the present study, we found that CD24 was significantly associated with poor prognostic variables including ER-, PR-, HER2(+), triple negative (TN) phenotype and positively associated with chemotherapy response. Because most of breast cancers is *CD24* positive, which

Table 4. Univariate and multivariate analyses of the relationship of $CD44^{+}/CD24^{-/low}$ tumor cells to OS

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
$CD44^{+}/CD24^{-/low}$ tumor cells			0.002			0.017
Low	1.000			1.000		
High	2.194	1.404-3.452		1.92	1.248-3.586	
PR status			0.342			0.287
Negative	1.000			1.000		
Positive	0.74	0.652-1.427		0.84	0.54-1.05	
ER status			0.284			0.530
Negative	1.000			1.000		
Positive	0.757	0.523-1.246		1.260	0.57-2.46	
Her2 status						0.336
Negative	1.000		0.662	1.000		
Positive	0.968	0.74-1.769		0.78	0.417-1.584	
TNM stage			0.028			0.017
I + II	1.000			1.000		
III + IV	1.893	0.892-3.06		1.794	1.086-3.452	
Lymph node involvement			0.325			0.563
Yes	1.000			1.000		
No	0.876	0.682-1.684		1.380	0.803-2.142	

is especially valuable for patients with *CD24*(-) tumors, who appear to have a very low risk of tumor progression.

CD44 is a member of the adhesion molecule families, which is closely associated with cancer cell metastasis and *chemotherapy* resistance [10-14]. McFarlane et al has reported that CD44 expression was correlated with reduced disease-free survival and distant metastasis in lymph node-positive patients and patients with large tumor size [22]. Klingbeil et al has reported that CD44 expression is especially enriched in ER-negative, PR-negative and/or Her2-negative breast cancers which have the worst clinical prognosis and outcome [23]. However, in our study, we found there was no association of CD44 with survival, nodal status, age, HER2 expression or chemotherapy response. Therefore, a larger number of patients are needed to answer the question of whether CD24 and CD44 is the true prognostic marker of patient outcome or a predictive marker of sensitivity to therapy.

$CD44^{+}/CD24^{-/low}$ breast cancer cells have been reported to have tumor-initiating properties [24, 25]. We first investigated the importance of $CD44^{+}/CD24^{-/low}$ phenotype in the tumor characteristics of invasive ductal carcinoma cells. Abraham et al has reported that the prevalence of CD44(+)/CD24(-/low) tumor cells in breast cancer may not be associated with clinical outcome and survival but may favor distant metastasis [26]. Mylona et al. has found that CD44(+)/CD24(-/low) tumor cells seem to be associated with lack of lymph node metastasis and a tendency toward an increase of the relapse-free survival of the patients [27].

In the present study, $CD44^{+}/CD24^{-/low}$ tumor cells were significantly associated with lymph node involvement, recurrent, metastatic tumors, ER and PR status. High CD44(+)/CD24(-) phenotype had poor response to chemotherapy. Otherwise, the proportion of $CD44^{+}/CD24^{-/low}$ tumor cells was strongly correlated with DFS and OS. This was in line with Giatromanolaki and Lin's study [28, 29]. However, Ahmed et al has found that the CD44(+)/CD24(-) phenotype had the best prognosis, while the CD44(-)/CD24(+) phenotype had the worst prognosis [30]. Our study found that the CD44(-)/CD24(+), CD44(+)/CD24(+), CD44(-)/CD24(-) phenotype had no relation with prognosis of breast cancer. This demonstrats that the correlation between basic cell biology and tumor characteristics is not always

consist and the true clinical impact of breast cancer cells need to befurther investigated.

Conclusion

We observed variations in the prevalence of CD24, CD44 and $CD44^{+}/CD24^{-/low}$ tumor cells in breast cancer cells. CD24 was significantly associated with poor prognostic variables and positively associated with chemotherapy response. The presence of $CD44^{+}/CD24^{-/low}$ tumor cells was associated with lymph node involvement, recurrent, ER and PR status, poor response to chemotherapy and a shorter cumulative DFS and OS, suggesting that the CD24 and CD44+/CD24- phenotype may be an important factor of malignant relapse in patients with surgically resected invasive ductal carcinoma.

Disclosure of conflict of interest

None.

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