Original Article

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Overexpression of C35 in breast carcinomas is associated with tumor progression and lymphnode metastasis

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Summary **To investigate C35 protein expression in breast carcinoma and to investigate its clinicopathological significance, a total of 68 cases of breast carcinoma and 20 cases of normal breast tissue samples were obtained from the clinic. Protein expression of C35, ER, PR and HER-2 were determined using immunohistochemistry. The correlations between C35 expression and clinicopathological parameters were analyzed on the basis of individual clinicopathologic records. Overexpression of C35 was detected in 56 of 68 (82.35%) breast carcinoma samples and only 3 of 20 (15%) normal breast tissue samples, and frequency of C35 expression was significantly associated with clinical Tumor Node Metastasis staging and Scarff-Bloom-Richardson grade ($p < 0.05$), but was not related to patients age, menstrual status and tumor diameter. C35 expression was positively related with the expression of HER-2 ($r = 0.207$), whereas negatively related with the expression of ER and PR. Further, C35 was prevalent in all four molecular subtypes of breast carcinoma with no significant difference of expression frequency. However, they have significant differences in lymphatic metastasis cases compared to the non-metastasis cases ($p < 0.05$). Since C35 protein was extensively expressed in all stages of breast carcinoma, and was closely associated with tumor progression and lymph node metastasis, it might be used as a reliable biomarker or therapeutic target for diagnosis and treatment.**

Keywords: C35, breast carcinoma, biomarker, expression, clinicopathologic correlation

1. Introduction

Breast carcinoma (BC) is the most common malignant cancer in women living in China at present. According to the statistics from the Fan *et a*l. report published in 2014, the incidence of BC in China is increasing rapidly, more than 1.6 million people were diagnosed and 1.2 million people died of BC each year, and the newly diagnosed cases account for 12.2% (*1*). The increasing incidence of BC is associated with the residents increasing socioeconomic status and unique reproductive patterns. Additionally, higher incidence and younger patients occur in high-income cities such as Beijing, Shanghai and Tianjin (*2*). Notably, delays in diagnosis and inherent subjectivity involved in histopathology lead to a more advanced stage of this disease, thus effective biomarkers are introduced into the clinic to improve tumor classification and diagnosis of this disease.

Currently, three molecular biomarkers progesterone (PR), estrogen (ER) receptors, and human epidermal growth factor receptor-2 (HER-2) are systematically assessed and applied in clinical practice (*3*). However, all these traditional biomarkers have suboptimal effects on the treatment of BC, since the disease is caused by the accumulation of multiple molecular alterations (*4*). C35 is a newly identified biomarker of BC, also termed C17orf37, is located within the minus region of human chromosome 17q12 bounded by the oncogene *ERBB2* and the growth factor receptor-bound protein 7 (*GRB7*) genes, and encodes a 12kDa membrane-anchored protein (*5*). By comparing 10 kinds of human mammary carcinoma cell lines and 38 kinds of normal tissue cells, Evans *et al*. reported that C35 was highly expressed in 7 kinds of carcinoma cell lines but barely expressed in normal cells except for Leydig cells (*5*). Overexpression of C35 was also detected in other cancer cells and tissues

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such as colorectal cancer and prostate cancer (*6,7*). The conserved canonical immunoreceptor tyrosine-based activation (ITAM) motif located in its C-terminal end and the last four amino acids CVIL of C35 were proved to have an important role in cancer progression and metastasis (*7,8*). These studies suggested that C35 functions as an oncogene and highlight biomarker in breast cancer cell lines and has a potential application value in BC therapies. In this study, the extensive detection of C35 protein expression in 68 breast cancer tissues and 20 normal tissues was executed to investigate the clinicopathologic correlation, including differences of tumor types and age groups. The expression of C35 was also compared with the expression of the hormone receptor ER, PR and HER-2, in order to demonstrate the expression relationships among the genes; in addition, analysis of C35 expression pattern in different BC molecular subtypes was also evaluated.

2. Materials and Methods

2.1. *Source of sampless*

Cancer tissue samples and normal breast tissues were collected from the Second People's Hospital of Jining City in Shandong Province with the informed consent of patients, and were excised between July 2012 and January 2015. All of the 68 cancer samples were obtained from female inpatients and ranged in age from 34 to 81 years, with a mean and median age of 49 years. Normal tissue samples were derived from 20 cases of hyperplasia of mammary gland patients and mammary gland fibroma excision patients. Female patient age range was 17 to 38 years, with a median and mean age of 26.67 years. All of these samples were subjected to immunohistochemistry.

2.2. *Clinicopathologic characteristics of patients*

Inpatients involved in this study included 45 premenopausal patients and 23 postmenopausal patients. Samples ranged in degree of lymph node involvement, and included 43 axillary lymph node metastasis cases and 25 no metastasis cases. Of all the samples, 43 were invasive ductal carcinoma (IDC), 17 were invasive lobular carcinoma (ILC) and 8 were other carcinomas. Diameter of tumors ranged from 2.0 to 6.0 cm, 19 cases were less than 2 cm, 35 cases were 2~5cm and 14 cases were greater than 5 cm.

According to the clinical Tumor Node Metastasis (TNM) staging and confirmed by two qualified pathologists individually, samples were staged as 12 cases of stage I, 32 cases of stage II and 24 cases of stage III to IV. Samples were graded according to a modified Scarff-Bloom-Richardson (SBR) system by Elston Ellis, of which 14 were classified to Grade I (low malignant), 41 were classified to Grade II (moderate malignant), 13 were Grade III (highly malignant).

2.3. *Sample selection criteria*

Samples were selected according to four criteria: *1*) Have complete clinical and pathological records; *2*) All samples have been confirmed to be the primary breast cancer after surgery; *3*) None of the patients received preoperative treatment such as neoadjuvant chemotherapy, endocrinotherapy and radiotherapy; *4*) Surgical procedures included radical mastectomy, modified radical mastectomy and breast-conserving therpy plus axillary lymph node dissection. The study protocol was approved by the Hospital Ethics Review Committee.

Samples were excluded according to the following two criteria: *1*) Abandon bilateral primary breast cancer cases and complicated patients with other malignant tumor; *2*) According to National Comprehensive Cancer Network (NCCN) guidelines, abandon patients who have been undergoing postoperative treatment such as chemotherapy, radiotherapy and endocrine therapy in terms of their tumor stage and hormone receptor level.

2.4. *Immunohistochemistry*

Samples were fixed with 10% paraformaldehyde and made into 4 μm paraffin embedded serial sections. Immunohistochemical streptavidin-biotin complex (SABC) staining was performed according to the instruction of SABC immunohistochemical detection kit (Boster Biological Technology, Wuhan, China) rabbit anti-C35 polyclonal antibody (ZSGB-BIO, Beijing, China) at 1:100 dilution and rabbit anti-ER, PR and HER-2 monoclonal antibodies (Boster, Wuhan, China) at 1:20 dilution were used in this study.

ER and PR tumors were defined as positive only when the brownish yellow granules appeared in the cell nucleus. C35 positive staining was defined as when the brown or brownish yellow granules appeared in cell membrane. A final manual immunohistochemistry grade was devised by adding the staining score and positive proportion score together. The staining scores were evaluated according to the staining intensity as follows: 0 for unstained cells, 1 for cells stained pale yellow, 2 for cells stained brownish yellow, 3 for cells stained brown. Scores of 0 were considered negative, scores of 1 were considered weakly positive, scores of 2 were considered moderately positive, and scores of 3 were considered strongly positive. The positive proportion of stained cells was calculated according to semi-ration standard using cell counting estimation, and the method was as follows: first the highest intensity of tissue staining was found at low magnification, then a total of 100 cells were counted under five high power fields (HPF for 400×) to calculate the proportion of positive cells, and the positive proportion score was established on the basis of the following criteria: 1 for 0-10%, 2 for 10-25%, 3 for 26-50%, and 4 for > 50%

cell staining. The final positive grades were shown as 0~2 for (−), 3 for (+), 4~5 for (++) and 6 for (+++).

Cells stained brown or brownish yellow in plasma membrane or partly stained in cytoplasm were defined as HER-2 positive, while only cytoplasm staining should be defined as nonspecific staining. Positive immunohistological grades of HER-2 cells was assessed by the following standard: (−) for negative or nonspecific staining; (+) for > 10% discontinuous membrane coloration; (++) for > 10% with moderate intensity of membrane coloration consecutively; (+++) for > 10% with strong intensity of membrane coloration consecutively.

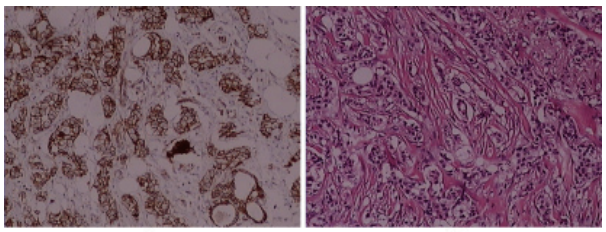

Figure 1. Immunohistochemistry of tissues derived from breast carcinomas and normal breast epithelia. C35 expression profile can be observed by SABC staining. (A) Breast carcinomas tissues stained intensely with a brownish yellow granules pattern for strong positivity of C35. **(B)** Negative expression of C35 in normal breast tissues. Magnification, ×100.

2.5. *Statistical analysis*

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 18.0 (USA). A possible correlation between C35 expression and clinicopathological parameters was assessed using a chi-square (χ^2) test or Fisher's exact test. Differences with a p value less than 0.05 were considered to be statistically significant.

3. Results

3.1. *Expression of C35 protein in breast Carcinoma*

The overexpression of C35 protein in human breast carcinoma specimens was detected by immunohistochemical SABC staining of 68 breast cancers and 20 normal breast tissues. Results indicated that expression of C35 was mainly localized at the plasma membrane of breast cancer cells, with profiles of brown or brownish yellow granules, as shown in Figure 1A. At the same time, no positive staining existed in mesenchymal cells. For normal breast epithelium tissue, in contrast, only a minimal number of cells showed weakly positive staining, as shown in Figure 1B. The positive proportion of C35 expression in breast carcinomas and normal breast tissues was 82.35% (56/68) and 15.00% (3/20) respectively, with a statistically significant difference between the two

Table 1. Correlation between C35 positive expression and clinicopathological parameters

Clinicopathological parameters	C35, *n*	C35 (+)	C35 (−)	Positive rate (%)	χ^2	*p* value[a]
Age (year)						
≤ 50	36	31	5	86.1	0.744	0.389
> 50	32	25	7	78.1		
Menstrual status						
Regular status	45	39	6	86.7	0.939	0.333
Pausimenia	23	17	6	73.9		
Tumor diameter (cm)						
≤ 2 cm	19	15	4	78.9	0.562	0.755
2-5 cm	35	30	5	85.7		
> 5cm	14	11	3	78.6		
TNM stage[b]						
I	12	5	7	41.7	16.608	0.000**
II	32	29	3	90.6		
III + IV	24	22	2	91.7		
SBR grade[c]						
I	14	7	7	50.0	14.947	0.001**
II	41	39	2	95.1		
III	13	10	3	76.9		
Pathological type						
IDC	43	37	6	86.0	4.485	0.106
ILC	17	11	6	64.7		
Other	8	5	3	62.5		
Armpit Lymph node metastasis						
Yes	43	39	4	90.7	4.151	0.042*
No	25	17	8	68.0		

[a]), *$p < 0.05$; **$p < 0.01$. [b]), C35 (+) in the TNM stage columns: the differences between I and II, I and III + IV stages with *p* values less than 0.05, the difference between II and III + IV stages with a *p* value less than 0.01. [c]), C35 (+) in the SBR grade columns: the differences between I and II, I and III grades with *p* values less than 0.05, the difference between II and III + IV stages with a *p* value more than 0.05.

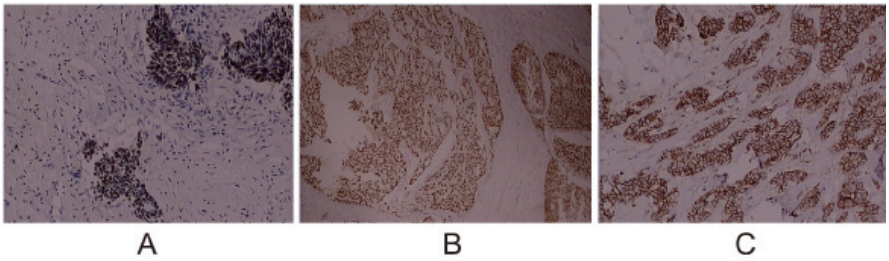

Figure 2. Expression of the other three biomarkers as determined by immunohistochemical SABC staining of the same BC samples used in C35 detection. (A) Positivity of ER and **(B)** PR tissues was stained with brownish yellow granules in cell nucleus. (C) Positivity of HER-2 tissues was stained with brownish yellow granules in plasma membrane or partly stained in cytoplasm. Magnification, ×100.

Table 2. Correlation between ER, PR and HER-2 positive expression and clinicopathological parameters

Clinicopathological parameters	*n*	ER(+)	χ^2	*p* value[a]	PR(+)	χ^2	*p* value[a]	HER-2(+)	χ^2	*p* value[a]
Age (year)										
≤ 50	36	17	0.082	0.774	16	0.337	0.561	26	2.658	0.103
> 50	32	14			12			17		
Menstrual status										
Regular status	45	23	1.636	0.201	15	3.379	0.066	35	0.002	0.964
Pausimenia	23	8			13			18		
Tumor diameter (cm)										
≤ 2 cm	19	11	1.816	0.403	9	0.493	0.782	8	6.784	0.034*
2-5 cm	35	15			14			23		
> 5cm	14	5			5			12		
TNM stage[b]										
I	12	9	10.847	0.004**	6	9.300	0.010*	6	6.526	0.038*
II	32	17			18			26		
III + IV	24	5			4			11		
SBR grade[c]										
I	14	11	9.077	0.011*	10	7.405	0.025*	6	9.275	0.010*
II	41	17			15			31		
III	13	3			3			6		
Pathological type										
IDC	43	24	6.070	0.048*	19	5.485	0.065	27	1.002	0.606
ILC	17	6			7			11		
Other	8	1			2			5		
Armpit Lymph node metastasis										
Yes	43	14	8.006	0.005**	13	5.783	0.016*	32	6.292	0.012**
No	25	17			15			11		

[a]), $*p < 0.05$; $**p < 0.01$. [b]), ER, PR and HER-2 (+) in the TNM stage columns: the differences between I and II, I and III + IV stages with *p* values less than 0.05, the difference between II and III + IV stages with a *p* value more than 0.05. [c]), ER, PR and HER-2 (+) in the SBR grade columns: the differences between I and II, I and III grades with *p* values less than 0.05, the difference between II and III + IV stages with a *p* value more than 0.05.

groups ($\chi^2 = 31.731$, $p < 0.01$).

3.2. *Correlations between C35 expression and clinicopathological parameters*

As the positive C35 staining results show in Table 1, C35 expression increases with tumor TNM stage and SBR grade progression. The expression of C35 among different clinical stages ($\chi^2 = 16.608$, $p < 0.01$) was significantly different, as well as in different histopathological grades ($\chi^2 = 14.947$, $p < 0.01$). In addition, increasing C35 was more prevalent among BC patients with regional armpit lymph node metastasis (90.7%) than in BC patients without lymph node metastasis (68.0%) ($\chi^2 = 4.151$, $p < 0.05$). However, a similar expression frequency of C35 was detected both in IDC and ILC, with no statistically significant difference ($\chi^2 = 4.485$, $p = 0.106$). Similarly, there was no significant association between C35 expression and other clinicopathological parameters such as age, menstrual status and tumor diameter ($p > 0.05$).

3.3. *Expression of ER, PR and HER-2 in Breast Carcinoma*

Expression of ER, PR and HER-2 detected by SABC staining are shown as Figure 2. The positive rate of ER, PR and HER-2 were 45.59 % (31/68), 41.18 % (28/68) and 63.24% (43/68) of total BC specimens respectively. Expression of ER, PR and HER-2 were analogously

Table 3. Relationships between C35 expression and expression of ER, PR and HER-2 in breast carcinoma

Groups	*n*	C35 (+)	C35 (-)	χ^2	*p* value	*r* value
ER						
positive	31	21	10	12.800	0.000	-0.035
negative	37	35	2			
PR						
positive	28	22	6	19.600	0.000	-0.083
negative	40	34	6			
HER-2						
positive	43	38	5	7.348	0.007	0.207
negative	25	18	7			

Table 4. Relationships between C35 expression and molecular subtypes of breast carcinoma

Molecular subtype	*n*	C35 (+)	C35 (-)	χ^2	*p* value
Luminal A subtype	26	21	5	2.922	0.404
Luminal B subtype	22	17	5		
Basal-like subtype	9	7	2		
HER-2 subtype	11	11	0		

associated with tumor SBR grade, TNM stage and lymph node metastasis, with a significant difference ($p < 0.05$) respectively. The increasing positive rate of HER-2 also can be seen in the advanced TNM stage and SBR grade, as well as in larger size of tumors ($p < 0.05$). When compared with different pathological types of BC, however, the expression of HER-2 was concordant in IDC and ILC ($p > 0.05$) whereas a significant difference was observed in ER paralleling tests ($p < 0.05$). Likewise, the expression of those three biomarkers show also no significant difference with patients age and menstrual status ($p > 0.05$), as shown in Table 2.

3.4. *Relationships between expression of C35 and other biomarkers*

Table 3 shows the relationships between expression of C35 and the other three biomarkers ER, PR and HER-2. Correlation analysis showed that the expression of C35 in BC samples has a positive correlation to the expression of HER-2 ($\gamma = 0.207$), whereas has negative correlations to the expressions of hormone receptors ER ($\gamma = -0.035$) and PR ($\gamma = -0.083$).

3.5. *Relationships between C35 expression and molecular subtypes of breast carcinoma*

To detect relationships between C35 and BC molecular subtypes, all of the 68 samples were divided into four main subtypes by immunohistochemistry (*9*). The molecular subtype criterion were as follows: *1*) Luminal A subtype: HER-2 negative but ER and/or PR positive (HER-2−/ER+ or HER-2−/PR+); *2*) Luminal B subtype: HER-2 positive, ER and/or PR positive (HER-2+/ER+/PR+ or HER-2+/ER−/PR+ or HER-2+/ER+/PR−); *3*) Basal-like subtype: ER, PR and HER-2 all negative (HER-2−/ER−/PR−); *4*) HER-2 subtype: only HER-2 positive (HER-2+/ER−/PR−). Table 4 shows that there was no significant difference between multiple molecular subtypes of BC and C35 positive expression according to chi square test result ($\chi^2 = 2.922$, $p > 0.05$).

4. Discussion

Recently, molecular biomarkers have been introduced to overcome the inherent subjectivity of BC involved in histopathology, such as ER, PR and HER-2 (*10*), and therapists tend to formulate a pointed therapeutic plan on the basis of biomarker expression results. Slamon *et al*. (*11*) reported that HER-2 protein overexpression can merely be detected in 13-20% of IDC patient tissues, and the expression levels rise with the increase of tumor clinical stage and histological grade. Moreover, overexpression of HER-2 was associated with improved clinical outcomes (*12*). Clinical trials have demonstrated that patients with HER-2 (+) BC could achieve a satisfactory therapeutic effect by using a humanized monoclonal antibody against HER-2 (trastuzumab) (*13*). However, the proportion of HER-2 protein overexpression patients is only 20-40 % (*11*), which limited the clinical use of trastuzumab in the majority of patients without HER-2+ BC. At the same time, the cytotoxicity of trastuzumab on the heart narrows the pool of candidates eligible for HER2-directed therapies (*13,14*).

TAM has been established as one of the most effective drugs in estrogen receptor-dependent BC treatment, and has been widely used as the first-line of anti-tumor drugs. Nevertheless, recent findings have shown that the frequent occurrence of drug resistance against TAM substantially reduced its anti-tumor efficacy, and the resistance mechanism of TAM remains unclear (*15*).

Consistent with previous study (5), our results demonstrated that a high level of C35 protein was frequently expressed in BC cells and tissues compared to normal breast cells, and the expression intensity difference was up to 10 times greater, thus it became a new attractive BC biomarker. In this study, the positive rate of C35 protein (82.35%) is higher than the other three biomarkers ER, PR and HER-2 at all stages. Increased expression level of C35 during tumor progression was also detected when correlating with SBR grade and TNM stage. Simultaneously, our results indicated that durative expression of C35 was associated with the clinical and pathological features of BC, since a higher positive rate was found in lymph node metastatic tumor tissues. Our results suggested that C35 expression might play an important role in tumor progression and lymphatic metastasis, in agreement with the conclusion presented previously

(*7,16,17*). Although these features were not independent prognostic factors, they were also associated with poor clinical outcome. At the same time, Dasgupta S *et al*. also indicated that down regulation of C35 results in reduced expression of MMP-9, uPA and VEGF in prostate cancer cells (*7*), which have been reported to be positively correlated with poor prognosis (*18*). Therefore C35 shows potential as a prognostic indicator for BC. In combination with previous results, C35 is involved in cancer cell migration and metastasis by prenylation of its C-terminal CAAX motif (*16*), has a functional role in prostate cancer infiltration (*7*) and drug resistance of ovarian (*17*) cancer, and hence might be a more appropriate and reliable biomarker for tumor diagnosis and prognosis, and is also likely to be a new therapeutic target for cancer treatment.

Our results found that C35 expression was independent of age and menstrual status of patients, but needs further evidence from similar research work using a larger panel of patients. In the present study, the positive rate of C35 expression was significantly higher than previous work (*5*), these differences might be related to clinicopathological factors. A majority of samples in this work occurred in advanced histological grade and clinical stage, or in BC with lymph node metastasis, which may attribute to a higher score for C35 expression.

Based on the previous study (*5*), C35 was strongly associated with the expression of HER-2 protein and inversely associated with clinical pathological features of BC such as pathological grade, clinical stage and lymphatic metastasis, and the frequency of C35 was higher than HER-2 at all stages (*14*), which was consistent with our results. At the same time, the correlation between C35 expression and four BC molecular subtypes was irrelevant ($p > 0.05$) in this study, and the expression of C35 highly overlapped the other three biomarkers, which indicated that C35 was prevalent in all those BC molecular subtypes.

In conclusion, our findings indicate that high frequency of C35 expression could occur in all stages of BC, has a significant correlation with the clinicopathological factors including disease progression and lymphatic metastasis, and is also positively related to traditional biomarker HER-2, suggesting C35 has a great potential to be a reliable diagnostic marker, or to develop a new C35-targeted therapy in the clinical treatment of BC.

Acknowledgements

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