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Clinical correlations and prognostic relevance of Fn14 expression in breast carcinoma

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Summary. The aim of the present study was to clarify the expression of fibroblast growth factor-inducible 14 (Fn14), a type I transmembrane protein, in breast carcinoma and its correlation with clinicopathological features. We examined the expression of Fn14 in normal breast epithelial cells as well as in breast carcinoma cells, and in 12 cases of breast carcinoma tissues and the paired normal breast tissues by RT-PCR and Western blot analysis. In addition, we analyzed Fn14 protein expression in 171 clinicopathologically characterized breast carcinoma cases by immunohistochemistry. Statistical analyses were applied to test for prognostic and diagnostic associations. The results show that the level of Fn14 mRNA and protein were higher in the cancer cell lines and most cancer tissues than in normal control tissues. Immunohistochemistry showed that Fn14 was expressed in 148 of 171 cases (86.5%). Statistical analysis of cases showed that there was a significant difference of Fn14 expression in patients categorized according to HER-2 expression, lymph node metastasis and clinical stage. Our results suggest that Fn14 protein is a valuable marker of breast carcinoma progression. Fn14 might be used as a valuable prognostic marker for breast carcinoma patients.

Key words: Fn14, Breast cancer, Prognosis

Introduction

Breast cancer is the most common carcinoma in females and the second most common cause of cancer related mortality in women (Bombonati and Sgroi, 2006), with more than 1,000,000 new cases and 370,000 deaths yearly worldwide. Although numerous proteins have been identified as regulators of breast cancer invasive and metastatic capacity (Minn et al., 2005), the identification of additional proinvasive and/or prometastatic molecules is required to further define the malignant cell phenotype.

TNFRSF12A (tumor necrosis factor receptor superfamily, member 12A), also known as FGF-inducible 14 (Fibroblast Growth Factor-Inducible-14/Fn14), has been reported to stimulate cell proliferation (Donohue et al., 2003), migration (Harada et al., 2002) and differentiation (Polek et al., 2003). Fn14 is the smallest member of the TNFR superfamily described so far, and it appears to signal via recruitment of several different TNFR-associated factors (Brown et al., 2003). This molecule has been reported to be expressed in variety of organs including the heart (Chorianopoulos et al., 2010; Mustonen et al., 2010), kidney (Weinberg, 2011), and lung (Xu et al., 2004). Additionally, Fn14, which is overexpressed in advanced esophageal (Wang et al., 2006; Watts et al., 2007) and brain (Tran et al., 2003, 2006) tumors, may regulate tumor cell motility (Watts et al., 2007).

Therefore, further studies are needed to explore the expression of Fn14 in primary breast cancer and its correlation with the clinicopathological and biological characteristics of breast cancer. In the present study, the expression of Fn14 was investigated by immunohistochemistry using tissue microarray according to immunohistochemical phenotypes and the correlationships between Fn14 expression and the clinicopathological data. We demonstrated a possibility of its predictive role in chemotherapy in breast cancer.

Materials and methods

Patients and tissue samples

We retrieved tissue samples from patients with

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breast carcinoma in the Department of Pathology of the First Affiliated Hospital of China Medical University during July 1999 through December 2008. Formalin-fixed and paraffinembedded tissue specimens from 171 patients with primary breast cancer were included. All archival hematoxylin and eosin (H&E)-stained slides for each patient were reviewed by two pathologists. For the usage of the clinical materials for research purposes, prior patient consent and approval from the Institutional Research Ethics Committee were obtained. All the diagnoses were made following the Pathology and Genetics of Tumors of Breast of the World Health Organization Classification of Tumors. Clinico-pathologic classification and staging were determined according to the American Joint Committee on Cancer criteria. The histological grade was assessed using the Nottingham grading system, and nuclear grade was evaluated according to modified Black's nuclear grade. Histological parameters such as histological subtype, nuclear grade and histological grade were evaluated according to H&E-stained slides. Clinical parameters included patients' age, tumor size, lymph node status, clinical stage and biological markers (ER, PR, HER2 and ki67 et al).

Immunohistochemistry (IHC)

The streptavidin-peroxidase-biotin (SP) immuno-histochemical method was utilized to study the expression of Fn14 in 171 paraffin-embedded breast tissues. In brief, paraffin-embedded specimens were cut into 4 μm sections and baked at 60°C for 60 min. The sections were deparaffinized with xylenes and rehydrated. Then, sections were submerged in EDTA antigenic retrieval buffer in a pressure cooker for 10 minutes and then cooled at room temperature for 20 minutes. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with normal serum to block nonspecific binding. Monoclonal antibody was incubated with the sections overnight at 4°C; the second antibody was from an SP reagent kit (Zhongshan Biotechnology Company, Beijing, China). After washing, the tissue sections were treated with biotinylated anti-mouse secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex for 20 mins. Stained with diaminobenzidine (DAB), the sections were counter-stained with hematoxylin. For negative controls, the antibody was replaced with PBS.

Cell culture

Human normal breast cell lines MCF-10A, Human breast cancer cell lines MDA-MB-231 and MCF-7 were maintained in Dulbcco's Modifed Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Gibco,USA), 100 units/ml streptomycin, and 100 units/ml penicillin in a humidified 5% v/v CO_2 atmosphere until 75% confluent.

RNA isolation and reverse transcriptase-PCR

Total RNA was isolated using TRIZOL (Invitrogen, USA) according to the manufacturer's instructions. The primer sequences are: Fn14, forward 5'-CTC GCC CAC TCA TCA TTC ATT CAT C-3', reverse 5'-GCG TGA GGC TCC CTT TCT GTT CT-3'; ß-actin, forward 5'-AAATCGTGCGTGACATTAA-3' and reverse 5'-CTCGTCATACTCCTGCTTG-3'.The PCR products for Fn14(210 bp) and ß-actin (455 bp) were amplified with 30 PCR cycles (1 min at 95°C; 1 min at 53°C, 1 min at 72°C), and visualised by ethidium bromide stainingafter agarose gel electrophoresis.

Western blot analysis

Total protein was extracted with lysis buffer (150 mM NaCl, 1%v/v NP-40, 0.1% v/v SDS, 2 μg/ml aprotinin, 1 mM PMSF), and 60 μg of protein lysates were separated on a 12% v/v SDS-poly-acrylamide electrophoresis gel, transferred to Polyvinylidene Fluoride (PVDF) membranes. Proteins were visualised with horse-radish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Zhongshan,Beijing,China) followed by DAB. Subsequently, densitometric analyses of the bands were performed.

Statistic alanalysis

All values are expressed as mean±standard deviation (SD). The t test was used to analyze all results. All analyses were performed using the statistical software package SPSS14.0 (SPSS Incorporated, Chicago). For all the tests, $p<0.05$ was considered statistically significant

Results

Expression of Fn14 in breast carcinoma cell lines

To investigate the expression levels of Fn14 transcripts and protein in breast cancer cell lines, RT-

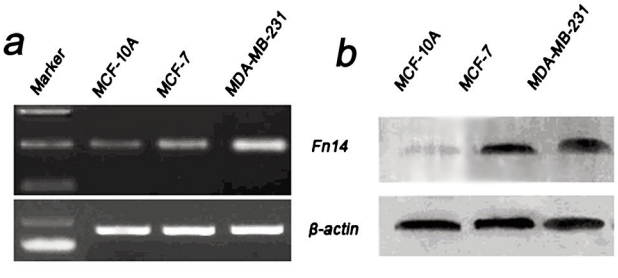

Fig. 1. Expression of Fn14 in breast carcinoma cell lines. **a.** RT-PCR shows that expression of Fn14 mRNA was detected and had different density in normal breast cell and breast cancer cell lines with an internal control of ß-actin. **b.** Western blot shows that expression of Fn14 protein was detected and had different density in normal breast cell and breast cancer cell lines with an internal control of ß-actin.

PCR analysis and Western blotting analysis were done in MCF-10A, MCF-7 and MDA-MB-231 cell lines. The breast cancer cell lines showed higher level expression of Fn14 mRNA in comparison with the normal breast cell line (Fig. 1a). Western blotting analysis showed that Fn14 protein was highly expressed in all cell lines, whereas it was weakly detected in normal breast cell line (Fig. 1b).

Expression of Fn14 in paired breast cancer and nontumorous tissues

As we detected Fn14 overexpression in breast carcinoma cell lines, we were interested in investigating the status of Fn14 expression in breast carcinoma. We initially did RT-PCR analysis on 12 breast tumor tissues (T) versus normal breast tissues (N) obtained from the same patients. As shown in Fig. 2, the expression levels of Fn14 mRNA in most cancer tissues (6/12) was higher than in normal tissues, and quantitative analysis showed that there was a significant difference in Fn14 expression between cancer tissues and normal tissues.

To determine the levels of Fn14 protein expression, we performed Western blot analysis with protein extracts from matched samples of tumor (T) and adjacent normal tissue (N). As shown in Fig. 3, Fn14 was found to be

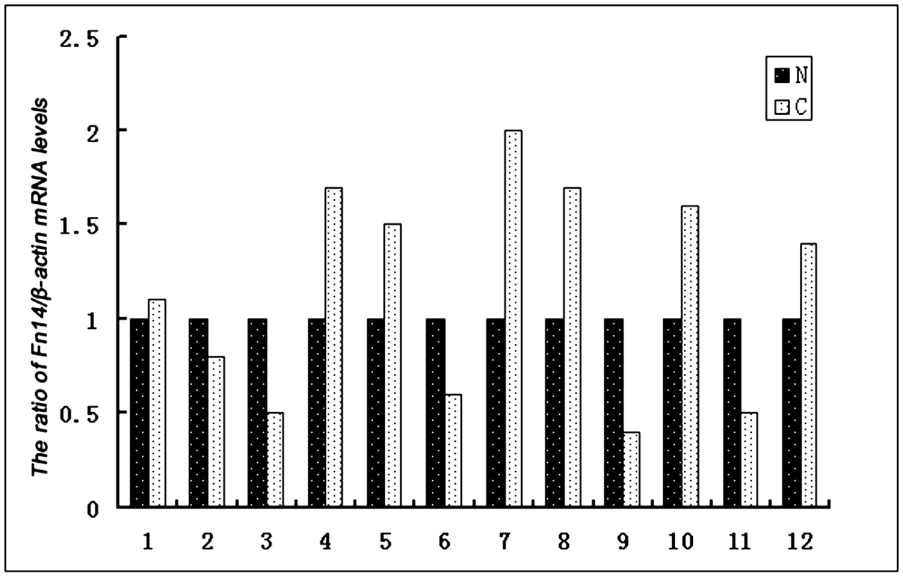

Fig. 2. Fn14 mRNA expression in breast cancer tissue and matched normal tissue by real-time RT-PCR. Fn14 mRNA expression level was higher in breast cancer than paired normal by real-time RT-PCR.

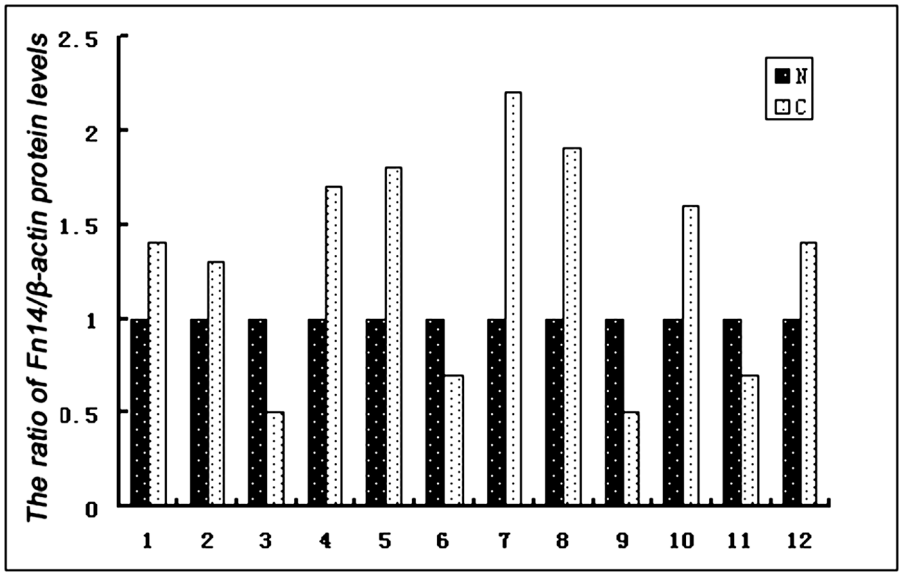

Fig. 3. Fn14 protein expression in breast cancer tissue and matched normal tissue by western blot. Fn14 protein expression level was higher in breast cancer than paired normal by western blot.

greatly overexpressed in 8 of 12 cases of primary breast carcinoma, whereas only faint Fn14 expression was found in the normal breast tissues, with at least twofold overexpression of Fn14 in cancer tissues compared with normal tissues in these 8 cases (Fig. 3). Taken together, these data demonstrate that Fn14 is highly expressed at both mRNA and protein levels in most of the breast cancer tissues.

Expression of Fn14 in archival breast cancer tissues

The specificity of the immunodetection was confirmed by using the monoclonal antibodies. A positive stain for Fn14 was defined as a brown stain observed in the cytoplasm and cytomembrane. Positive staining of vascular endothelium serves as an internal positive control.No staining of lymphoid cells was seen.

Using Immunohistochemistry, we found that Fn14 (4/30,13.33%) was weakly or not expressed in the duct epithelial cells from 30 normal breast tissues (Fig. 4A). The positive expression of Fn14 in DCIS (ductal carcinoma in situ) 83.33% (30/36) (Fig. 4B), and 87.40% (118/135) in IDC (invasive ductal carcinoma) (Fig. 4C), are significantly higher than that in normal breast tissues.

There was no significant correlation between the expression level of Fn14 and biological factors such as patients' age ($p>0.05$), histology type ($p>0.05$), histology-grade($p>0.05$), tumour size ($p>0.05$), ER ($p>0.05$), PR ($p>0.05$)and Ki67 ($p>0.05$). In contrast, statistical analyses indicated that Fn14 expression was positively related with HER-2 expression and the correlation was statistically significant ($p<0.05$); meanwhile, the correlation between Fn14 expression and lymph node metastasis/clinical stage was significant ($p<0.05$). The results of these analyses are summarized in Table 1.

Follow-up information was available on 171 patients with breast carcinoma and period ranged from 0.9 months to 12.1 years (median=66.2 months). Kaplan-Meier analysis indicated that there was a significant difference between cumulative survival rate of the patients with negative and positive Fn14 expression ($p<0.05$, Fig. 5).

Discussion

Here, we present that levels of Fn14 mRNA and

Table 1. The correlation between the expression of Fn14 and the clinicopathological parameter.

	Patient n(%)	Fn14 expression		2 value
		negative	positive	
Age				
≤50years	70	9	61	0.04
>50years	101	14	87	
Histology-type				
IDC	135	17	118	0.13
DCIS	36	6	30	
Histology-grade				
G1/G2	124	17	107	0.03
G3	47	6	41	
Tumor size				
≤2.0cm	84	12	72	0.10
>2.0cm	87	11	76	
LNM				
-	90	7	83	5.25*
+	81	16	65	
ER				
-/+	84	12	72	0.10
++/+++	87	11	76	
PR				
-/+	111	16	95	0.25
++/+++	60	7	53	
HER-2				
-/+	120	21	99	5.67*
++/+++	51	2	49	
Ki67				
+≤50%	134	18	116	0.07
+>50%	37	5	32	
Clinical stage				
Stage	52	3	49	7.81*
Stage a/ b	75	9	66	
Stage a/ b	44	11	33	

*: P<0.05.

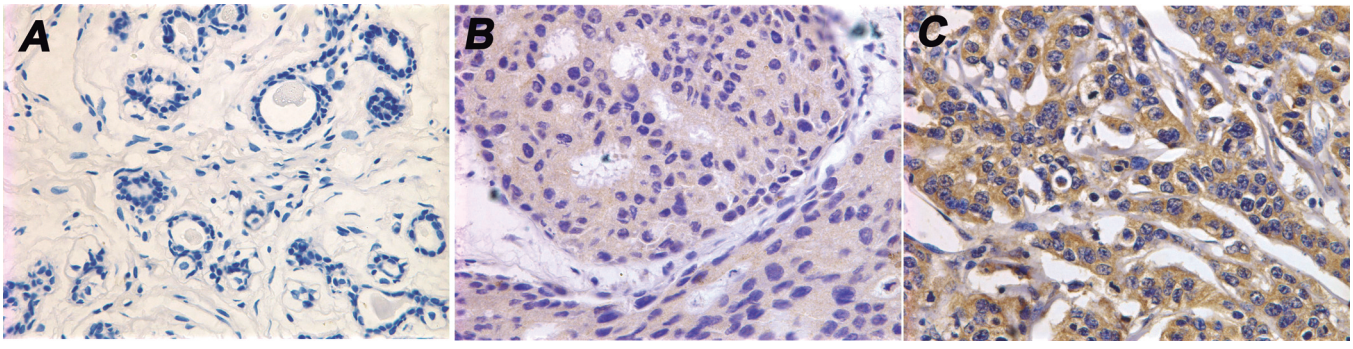

Fig. 4. Immunostaining examination in breast tissue. Fn14 immunoreactivity was detectable in normal breast tissue **(A)**, DCIS **(B)**, and IDC **(C)**. Fn14 protein was localized in the cytoplasm and cytomembrane. x 200

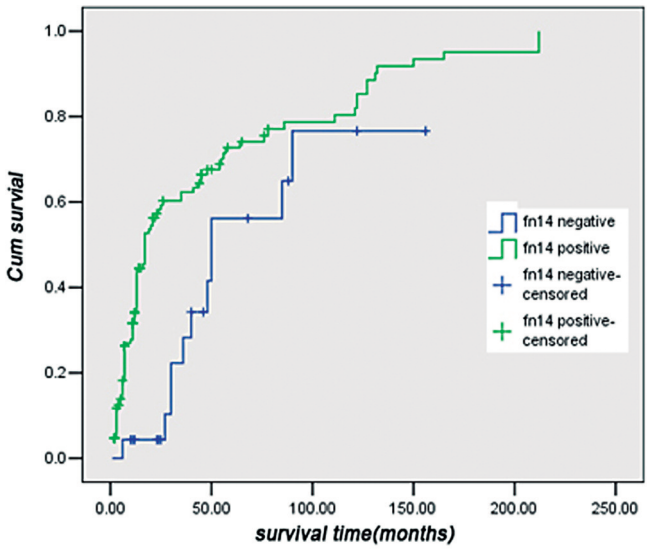

Fig. 5. Correlation between Fn14 expression status and prognosis of breast cancer patients. Kaplan-Meier curves for cumulative survival rate of patients with breast cancer according to Fn14 expression status

protein were higher in the cancer cell lines and most cancer tissues than in normal control cell and tissues. Immunohistochemistry showed that Fn14 was expressed in 148 of 171 cases (86.5%), which was in accordance with the findings in the studies of other cancers (Thompson et al., 1992; Michaelson et al., 2005). We reported that there was a significant difference of Fn14 expression in patients categorized according to HER-2 expression, lymph node metastasis and clinical stage. Taken together, our study suggests that Fn14 might represent a novel indicator for the prognosis of breast cancer patients.

Elevated Fn14 expression has been consistently detected in several human tumor types including liver, esophageal, and brain tumors (Winkles et al., 2007). In a previous report, Fn14 overexpression was detected in a panel of breast tumor specimens; however, no stratification of breast tumor subtype or clinical information was provided (Michaelson et al., 2005). Fn14 expression was also associated with HER-2 expression, lymph node metastasis and clinical stage.

The HER2/neu gene, encoding a member of the epidermal growth factor receptor family of tyrosine kinases, is amplified and overexpressed in 25% of primary breast tumors (Press et al., 2005). HER2 overexpression, which results in constitutive kinase activation, is associated with increased metastatic potential and poor patient survival (Slamon et al., 1989). Our Immunohistochemical results revealed a significant association between Fn14 expression and the HER2+subtype. It is not yet clear if there is a direct mechanistic link between HER2 and Fn14. However, it is interest to note that ectopic HER2 expression can promote NF-κB activation (Pianetti et al., 2002) and HER2+ tumors have constitutive NF-κB pathway activation (Biswas and Iglehart, 2006). Fn14 is a NF-κB–inducible gene (Tran et al., 2006); thus, it is possible that NF-κB activity may be one mechanism leading to activation of Fn14 gene expression in HER2+ tumors in vivo. The correlation we have observed between Fn14 and HER2 expression suggests that new therapeutic agents could target Fn14 or its downstream signaling mediators.

Breast cancer shows a poor prognosis because of the occurrence of systemic metastasis, mainly via the lymphatic node (Minn et al., 2005). Various proteins have been shown to be associated with development and progression of breast cancer, including cyclinD1 (Jiang et al., 2011), Ki-67 (Xiang et al., 2012) and ß-catenin (Xu et al., 2012). We have shown that the expression of Fn14 is closely associated with clinical stage and lymph node metastatic status of breast cancer patients, which strongly suggests that Fn14 can be used as a marker to identify subsets of breast cancer patients with more aggressive features. It suggests that Fn14 protein may play a role in tumor metastasis, especially in lymph node metastasis.

Importantly, patients with positive Fn14 expression had shorter overall survival time, whereas patients with negative Fn14 expression had better survival, and Fn14 expression was identified as a prognostic factor. These results are consistent with a recent report about Fn14 expression in breast cancer by Willis et al. (2008).

In summary, we show here that Fn14 is over-expressed in breast tumors and may contribute to the invasive nature of breast cancer cells. Our findings using breast cancer cells cultured in vitro are consistent with previous studies indicating that Fn14 expression levels can influence glioma (Tran et al., 2006) and esophageal (Watts et al., 2007) cell invasion capacity in vitro. Additionally, there was a significant difference of Fn14 expression in patients categorized according to HER-2 expression, lymph node metastasis and clinical stage. In conclusion, our study suggests that Fn14 might be used as a valuable prognostic marker for breast carcinoma patients.

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