

## Original Article

# Downregulation of the transcription factor, FoxD3, is associated with lymph node metastases in invasive ductal carcinomas of the breast

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**Abstract:** FoxD3 is a transcription factor of the forkhead gene family. We investigated its expression in invasive ductal carcinomas (IDC) of the breast and its association with metastasis. The expression of FoxD3, human epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER), progesterone receptor (PR) and Ki67 was examined by immunohistochemistry in samples from 121 patients with IDC. Non-tumorous breast adenosis tissues served as controls. HER2 expression was confirmed by fluorescence *in situ* hybridization (FISH). The expression levels of FoxD3 in IDC tissues and the breast cancer cell lines MCF-7 and MDA-MB-231 were additionally measured by western blotting. A greater percentage of total IDC patients and patients with lymph node metastases showed reduced FoxD3 expression compared to adenosis controls ( $p < 0.05$ ). Overall, FoxD3 was associated with metastatic status of IDC but not with age, pathological or clinical staging, or status of HER-2, ER, or PR. In particular, FoxD3 protein expression was down-regulated in the tumor epithelia of IDC samples from patients with metastases. Furthermore, FoxD3 protein expression was decreased in the metastatic MDA-MB-231 breast cancer cell line relative to the non-metastatic cell line, MCF-7. A greater number of patients with invasive, triple-negative breast cancer were also negative for FoxD3 expression than in other, non-triple-negative tumor types. These results suggest an inverse relationship between FoxD3 expression and tumor metastasis and warrants further investigation.

**Keywords:** Breast cancer, invasive ductal carcinomas, FoxD3, HER-2

## Introduction

Breast cancer is one of the leading causes of cancer-related deaths among adult females in the world [1]. Metastasis and breast cancer recurrence are major events that are responsible for death. However, the molecular mechanisms underlying these processes remain elusive due to the heterogeneous nature of breast cancer and the involvement of multiple genes. Mounting evidence suggests stem cells may be the cells of origin for cancer and share many features with adenocarcinomas, including expression of specific gene markers. For instance, breast carcinomas also express human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3, indicating embryonic stem cell genes may have a role in carcinogenesis [2].

FoxD3 is a member of the forkhead box transcription factor family which is important for maintaining the pluripotency and self-renewal capacity of embryonic stem cells [3-5]. FoxD3 is also required for cell maintenance and regulation of lineage specification [6-8]. FoxD3 regulates NANOG, a regulator of cell pluripotency, LIF, and BMP4 [9]. Both LIF and BMP4 have been implicated in the carcinogenesis and metastasis of breast cancer. Therefore, we hypothesized that FoxD3 may be also involved in the metastasis of breast cancer.

Invasive ductal carcinoma (IDC), sometimes called infiltrating ductal carcinoma, is the most common type of breast cancer. About 80% of all breast cancers are invasive ductal carcinomas and share similar epidemiological profiles. The aim of the present study was to investigate the

## FoxD3 in breast cancer

**Table 1.** Summary of clinical and histological characteristics of IDC samples

Clinical profile	<i>n</i>
Age in (years)	
<50	53
≥50	68
Pathological staging	
I-II	67
III	54
Clinical staging	
I-II	96
III	25
HER-2	
Negative	63
Positive	58
ER, PR status	
Negative	49
Positive	72
Lymph nodes	
Non-metastatic	66
Metastatic	55

expression of FoxD3 in IDC and its possible association with metastasis.

### Materials and methods

#### *Ethics statement*

This study was approved by the Ethics Committee of Wuxi Maternity and Child Health Hospital of Nanjing Medical University, China. All patient-derived tissues were obtained with written informed consent.

#### *Patient samples*

Samples were collected from 121 patients with breast IDC from the Maternal and Child Health Hospital of Wuxi (China) from January 2009 to August 2013. Mammary gland samples taken from 21 patients with non-tumorous adenosis were collected as controls. The average age of patients was 53 years (range, 32-70 years) and 51 years (range, 21-65 years) for IDC and adenosis patients, respectively. None of the patients received radiation or chemotherapy. Pathological diagnosis and staging were followed using the World Health Organization guidelines for breast cancer staging [10]. The clinical and pathological characteristics of patient breast cancer samples are summarized in **Table 1**. All samples were fixed in 10% neu-

tral formalin, dehydrated and paraffin-embedded before sectioning.

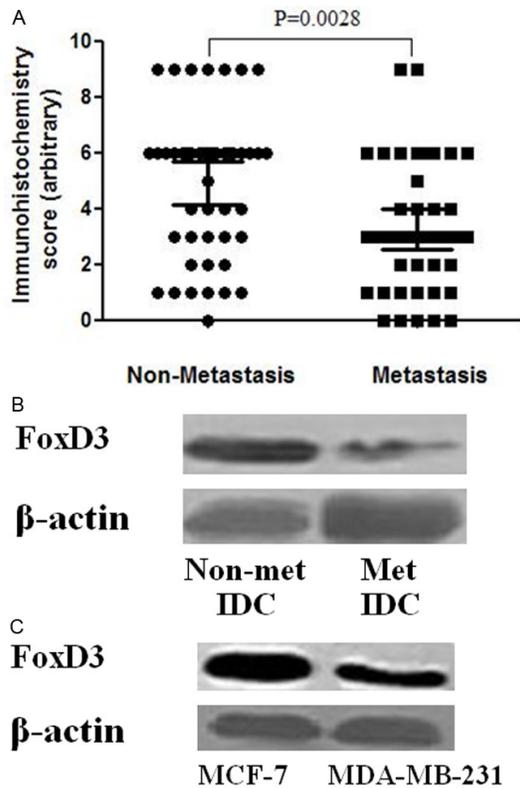
#### *Immunohistochemistry*

Anti-FoxD3 antibody was purchased from Abcam (Cambridge, USA). Anti-estrogen receptor (ER), anti-progesterone receptor (PR), and anti-human epidermal growth factor receptor-2 (HER-2) antibodies were purchased from Jingqiao (Beijing, China). Anti-Ki-67 was purchased from Maixin (Fuzhou, China). Antigen retrieval was performed by treatment with citric acid (pH 6.0) for 15 minutes. Non-specific antibody binding was blocked by incubating with 10% fetal calf serum for 20 minutes. Tissue samples were incubated with rabbit anti-human FoxD3 polyclonal antibody (1:200), rabbit anti-human ER monoclonal antibody (1:100), rabbit anti-human PR monoclonal antibody (1:100), or rabbit anti-human HER-2 polyclonal antibody (1:100) for 1 hour at room temperature. Sections were then washed with PBS and incubated with HRP-labeled anti-mouse or anti-rabbit IgG (Maxvision™2 kit, Maxim. BIO, Fuzhou China) for 15 min. The antigen-antibody complexes were visualized using diaminobenzidine (DAB) and counterstained with haematoxylin.

Cervical cancer tissue served as the positive control for FoxD3 staining and haematoxylin and eosin staining served as a histology control. Each slide was evaluated blindly by two persons. FoxD3-positive cells showed brown staining in the cytoplasm. The expression was assessed based on the semi-quantitative measurements of color and percentage of color-stained area in the cells. Grading of expression intensity was based on color as follows: colorless, light yellow, brown and sepia were graded as 0, 1, 2 and 3, respectively. Grading of area percentage of positively-stained cells was defined as follows: ≤10%, 11%-50%, 51%-75% and >75% as 0, 1, 2 and 3, respectively. The raw data were converted by multiplying the quantity and staining intensity scores. Negative controls were generated by performing no prior incubation and directly staining with the primary antibody.

Positive expression of ER and PR was observed as nuclear staining in tumor or ductal epithelial cells. Normal ductal epithelial cells served as internal controls and staining ≥1% was defined as positive staining [11].





**Figure 2.** Semi-quantitative analysis of immunohistochemistry indicated there was significantly lower expression of FoxD3 in IDC with lymphatic metastasis than in IDC without lymphatic metastasis (A,  $p=0.0028$ ). Protein levels of FoxD3 detected by western blotting in IDC samples without (left lane) or with (right lane) lymphatic metastasis,  $\beta$ -actin served as a loading control (B). Protein levels of FoxD3 in the metastatic breast cancer cell line, MDA-MB-231, and the non-metastatic breast cancer cell line, MCF-7, as detected by western blotting.  $\beta$ -actin served as a loading control (C).

100 U/ml penicillin and 10  $\mu$ g/ml streptomycin in a 5% CO<sub>2</sub> environment at 37°C. Cells growing in log phase were used for the experiments.

#### Western blotting

Tissues from invasive ductal carcinomas and mammary adenocarcinoma cell lines were lysed and centrifuged at 12,000 rpm for 30 minutes to remove cellular debris. Thirty micrograms of protein was loaded per lane for SDS-PAGE and then transferred onto a PVDF membrane (Millipore, Billerica, MA). After blocking with 5% non-fat milk containing 0.5% Tween 20 for 1 hour at room temperature, the membrane was incubated with rabbit polyclonal anti-human FoxD3 antibody (1:1000) overnight at 4°C. The membrane was then incubated with

HRP-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Protein was analysed by ECL (Perkin Elmer, Waltham, MA) with  $\beta$ -actin as a loading control. Western blotting was repeated with five different invasive ductal carcinomas.

#### Fluorescence in situ hybridization (FISH) assay

GLP HER-2 (17q11.2-q12) and CSP17 probes (Jingpujia, China) were used to label HER-2 and the centromere of chromosome 17, respectively. HER-2 positive breast cancer samples were used as positive controls and normal epithelial cells from the same sample were used as negative controls.

The DNA probe for the chromosome 17 centromere was visualized using green fluorescence and the DNA probe for HER-2 was visualized using red fluorescence. Nuclei were stained with DAPI. From each tumor site, 30 cells were randomly selected to record red and green signals and signal ratios were calculated. A ratio of less than 1.8 was defined as negative, indicating no amplification of HER-2, whereas a ratio greater than 2.2 was defined as positive, indicating amplification of HER-2.

#### Statistical analysis

Semi-quantitative data of FoxD3 are presented as mean  $\pm$  SD. Statistical significance of the results was evaluated by the Mann-Whitney test. Correlation of FoxD3 and ki-67 results was assessed by the Spearman's rank correlation coefficient. All statistical analyses were performed by the SPSS software suite (version 16.0; SPSS Inc., Chicago, IL). A  $p$ -value of  $<0.05$  was considered to indicate statistical significance.

## Results

#### Expression of FoxD3 in adenosis and IDC patient samples

Immunohistochemical analysis demonstrated that FoxD3 was more highly expressed in adenosis samples. Compared to adenosis controls, FoxD3 was down-regulated in IDC ( $p<0.05$ , **Figure 1**).

#### FoxD3 expression and pathological characteristics

No association was found between FoxD3 and age, pathological staging, clinical staging,

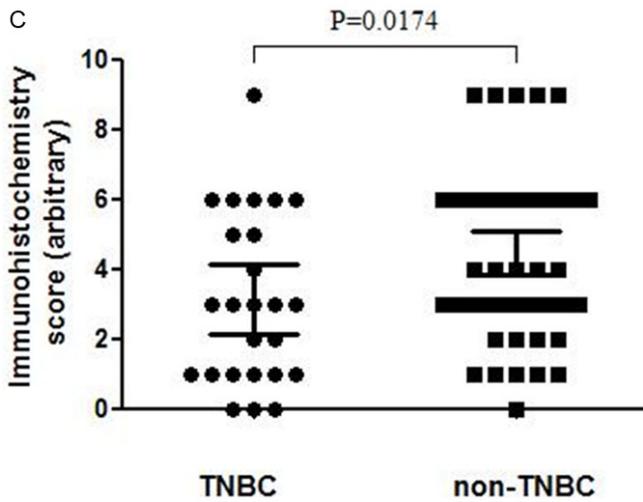
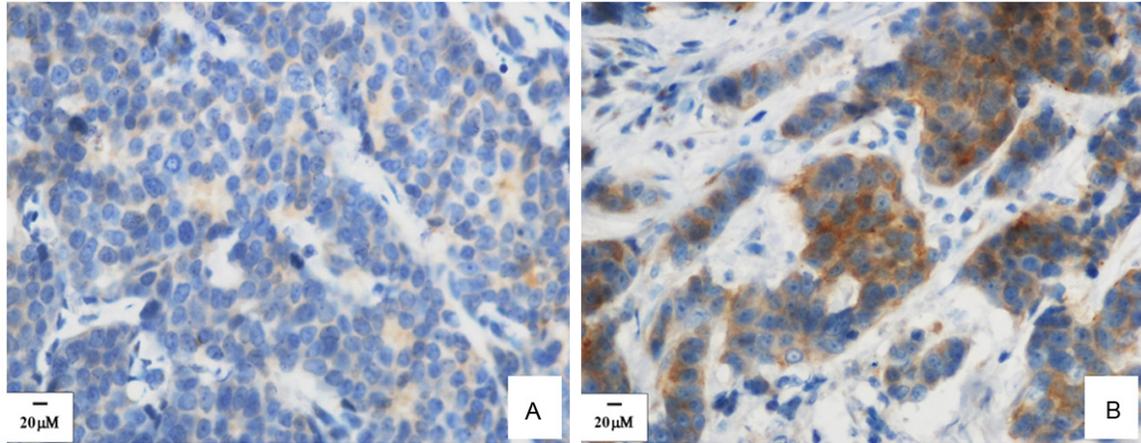


Figure 3. Representative immunohistochemistry images of FoxD3 protein staining in TNBC (A) and non-TNBC invasive ductal breast cancer (B) tissues (200 × magnification, scale bar=20 μm). Semi-quantitative analysis of immunohistochemistry indicated there was significantly lower expression of FoxD3 in TNBC than in non-TNBC, invasive ductal breast cancer tissues (C, p=0.0174).

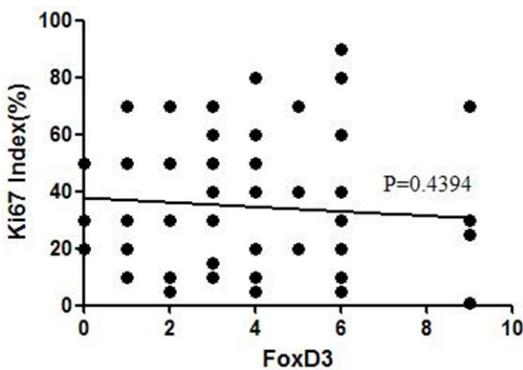


Figure 4. Semi-quantitative analysis showed that the expression of FoxD3 was not significantly correlated with Ki67 index in IDC ( $r=-0.108$ ,  $p=0.4394$ ).

HER-2 amplification, or PR or ER expression in the analyzed tissue samples (Table 1). FoxD3 protein expression was significantly reduced in breast cancer tissues with lymph node metastases compared to breast cancer tissues without lymph node metastases as measured by

semi-quantitative immunohistochemistry ( $p < 0.05$ , Figure 2A).

Western blot analysis confirmed the pattern of protein expression observed by immunohistochemistry. FoxD3 expression was also strikingly downregulated in metastatic protein lysates relative to non-metastatic lysates (Figure 2B). These results were further confirmed in the established non-metastatic and metastatic breast cancer cell lines, MCF-7 and MDA-MB-231. Again, FoxD3 expression was much lower in metastatic MDA-MB-231 cells than in non-metastatic MCF-7 cells (Figure 2C). However, FoxD3 expression was significantly lower in triple-negative breast cancer (TNBC) tissues than in non-TNBC, invasive ductal breast cancer tissues ( $p < 0.05$ , Figure 3).

We further analyzed the correlation between FoxD3 expression and Ki-67, an indicator of cellular proliferation. Analysis determined no sig-

nificant correlation between FoxD3 and Ki67 staining ( $p > 0.05$ , **Figure 4**).

### Discussions

Cancer stem cells are defined as those cells within a tumor that can self-renew and drive tumorigenesis. Somatic and cancer stem cells have been isolated from a number of human tumors, including breast cancer [12]. The cancer stem cell theory hypothesis states that tumors originate in mammary stem or progenitor cells as a result of dysregulation of the normally tightly regulated process of self-renewal, potentially driving tumorigenesis and differentiation that contributes to cellular heterogeneity [12]. Mounting evidence has also shown that markers of embryonic stem cells, such as NANOG and OCT4, are expressed in various tumors and may be involved in tumorigenesis [13-17]. Another such transcription factor, FoxD3 of the forkhead family, was originally found in embryonic stem cells (ES cells) and also in embryonal carcinoma cells.

The aim of this study was to determine whether there was a correlation between the expression of FoxD3 in IDC of the breast and its possible association with metastasis. The results of this study revealed that FoxD3 expression was down-regulated in IDC with lymph node metastases compared to IDC with metastasis-free lymph nodes. Consistent with the downregulation of FoxD3 in samples with lymph nodes metastasis, FoxD3 expression was significantly lower in the metastatic breast cancer cell line, MBA-MD-231, compared to the non-metastatic, MCF-7 cell line. Also, FoxD3 expression was lower in IDC samples compared to non-tumorigenic, mammary adenosis samples. FoxD3 expression in the TNBC samples was much lower than that detected in the non-TNBC samples; this result was consistent with the higher invasive and metastatic potential of TNBC relative to HER2<sup>+</sup> or ER<sup>+</sup> breast cancers [18]. A negative correlation between FoxD3 expression and metastasis in breast cancer is consistent with a previously reported tumor suppressor role of FoxD3 in gastric cancer [19]. However, FoxD3 expression was independent of age, pathological and clinical staging, and ER or PR expression in IDC.

The role of FoxD3 and its mechanism of action in carcinogenesis and metastasis remains elu-

sive. A recent report showed FoxD3 inhibited migration and infiltration of melanoma cells by regulating the RhoA-ROCK signaling pathway [20]. Interestingly, expression of RhoA, ROCK1 and ROCK2 are abundant in metastatic breast cancer [21, 22]. FoxD3 has also been shown to serve as a negative regulator of cell cycle by inhibiting B-Raf expression [23]. Therefore, it is reasonable to speculate that FoxD3 can influence tumor metastasis by inhibiting RhoA-ROCK signaling and negative regulation of the cell cycle. FoxD3 may affect breast cancer cell metastasis by regulating N-cadherin, a gene required for tumor infiltration and metastasis in IDC [24, 25]. It has previously been shown that BMP4 is required for tumor migration and infiltration [26], and a FoxD3-NANOG complex can regulate BMP4 to affect the tumor cell metastasis.

Although our results suggest expression of Ki-67, a widely used cell proliferation marker, was higher in metastatic than non-metastatic samples, there was no significant association between Ki-67 and FoxD3 expression levels. These results may indicate that cell proliferation and FoxD3 function independently, although analysis of a larger sample cohort may be necessary to determine whether a true relationship exists.

Overall, our study provides evidence of a possible role for FoxD3 in breast cancer metastasis and warrants further investigation into its mechanism of action in this specific tumor type. These results suggest that FoxD3 could serve as a prognostic marker to predict metastasis in breast cancer and may be helpful for highly invasive breast cancer, such as TNBC.

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### Disclosure of conflict of interest

None.

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