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Original Article

Association of ultrasonographic features with NGX6 expression and prognosis in invasive ductal breast carcinoma

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Abstract: Introduction: Nasopharyngeal carcinoma-associated gene 6 (NGX6) is a newly discovered tumor suppressor gene. It contains one epidermal growth factor (EGF)-like domain. Many studies have shown that proteins contain (EGF)-like domain structure affect a variety of biological actions. However, little is known about the relationships between NGX6 expression and biological behaviors in invasive ductal breast carcinoma (IDBC). The study discussed the association of ultrasonographic features with NGX6 expression and prognosis in IDBC. Methods: Ultrasonographic feature and clinical data in 122 patients with IDBC were retrospectively analyzed. NGX6 expression of IDBC was measured using immunohistochemistry methods. Results: The incidence of the burr sign, lymph node metastasis and abundance blood flow in NGX6 expression negative groups were higher than those in positive groups. Kaplan-Meier analysis showed that the association between NGX6 positive expression and higher disease-free survival (DFS) or higher overall survival (OS); Lymph node metastasis is associated with lower DFS or lower OS; Lower blood flow grade is associated with higher DFS. In univariate and multivariate survival analysis, NGX6 expression, lymph node metastasis, TNM and the blood flow grade were the independent prognostic factors for DFS and OS of IDBC. Conclusions: ultrasonographic features are associated with NGX6 expression in IDBC. NGX6 is involved in the invasion and metastasis activity of IDBC. Our results suggest that NGX6 may be employed as a promising prognostic factor and useful therapeutic target for IDBC. Combination of ultrasonic findings and NGX6 detection may yield clinically useful information for IDBC prognosis.

Keywords: Invasive ductal breast carcinoma (IDBC), ultrasonography, NGX6, prognosis

Introduction

Breast cancer is the most prevalent cancer among women. The mortality of breast cancer continually rose over the last few years. Ultrasound is one of the most effective tumor diagnostic techniques. Biological characteristics of tumor such as growth, invasion and metastasis can be observed, non-invasively and accurately, using ultrasonic technique [1-3]. NGX6 is a newly discovered tumor suppressor gene (GenBank accession no. AF188239). NGX6 gene contains one epidermal growth factor (EGF)-like domain, and that is one of the most important biological characteristics. Many studies have shown that proteins contain (EGF)-like domain structure affect a variety of biological actions, such as tumor growth, invasion and metastasis [4-7]. However, little is known about the relationships between NGX6 expression and biological behaviors in breast cancer. The study discussed the association of ultrasonographic features with expression of NGX6 proteins and prognosis in IDBC in order to provide valuable information for clinical prognosis.

Materials and methods

Patients

This study was approved by the Ethics Committee of Third Xiangya Hospital. From January 2009 to April 2011, 130 patients scheduled for surgery took preoperative ultrasound and were confirmed to be IDBC with pathological examination. 8 women who were pregnant or nursing were also excluded. 122

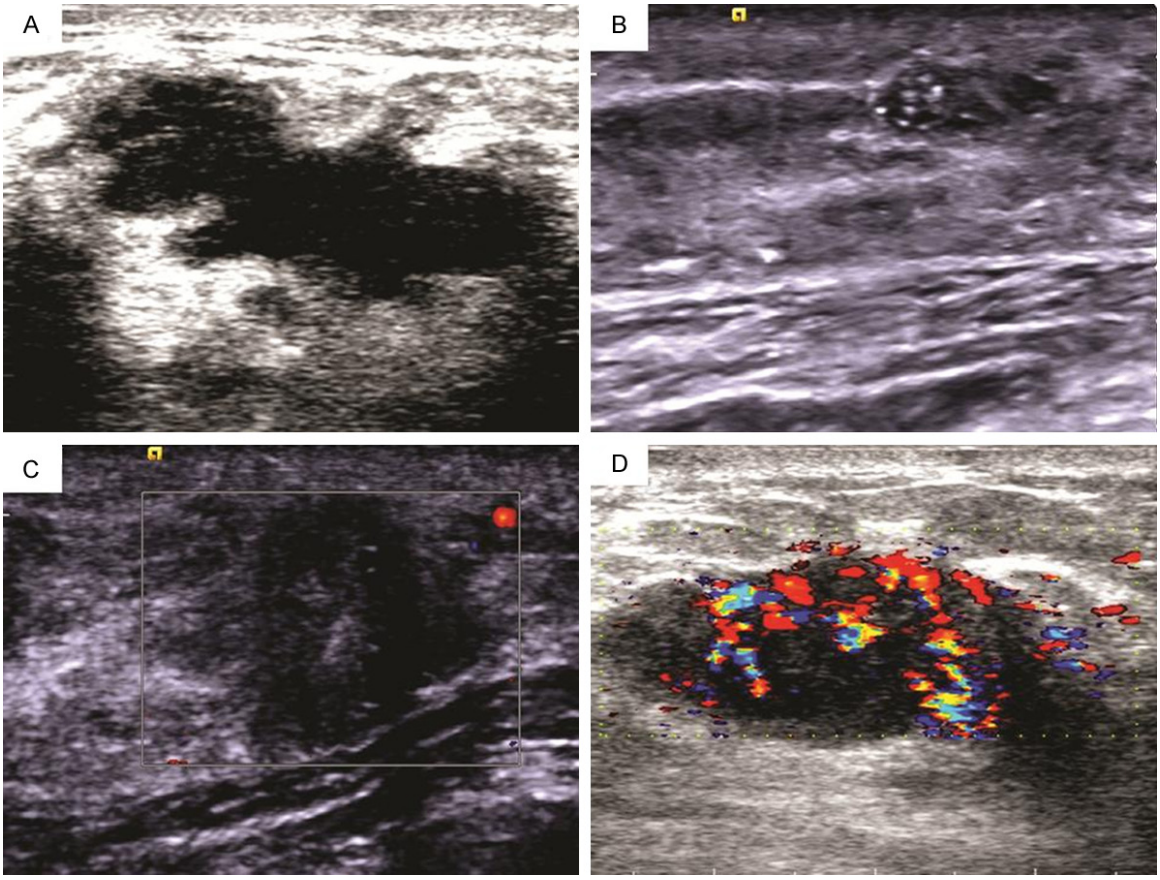

Figure 1. Ultrasonographic features in patients with IDBC. A. Edge burr sign in IDBC. B. Microcalcification in IDBC. C. Blood flow I-II grade in IDBC. D. Blood flow III grade in IDBC.

patients presented with single tumor were included in the study. All patients were female with a mean age 51.8 years ranging from 27 to 76 years. Data on the patients' medical history, tumor features, histologic classification and treatment modalities were recorded. All of the patients undergoing breast surgery received post-operative radiotherapy. Adjuvant chemotherapy was administered depending on the risk of recurrence in accordance with the National Comprehensive Cancer Network guidelines [8]. Dates and causes of death were obtained from the medical records. Survival assessment was last performed in March 2014. All the subjects signed the informed consent.

Ultrasound examination

Conventional ultrasound imaging was performed in patients one week before surgery with using a scanner (Siemens, ACUSON, S2000, Germany) with a multi-frequency linear 9 MHz transducer. Breast scanning was in clockwise direction along the center of the nipple. The content observed included the morphology of mass, size, border, internal echo, internal microcalcifications, axillary lymph node and blood supply. Blood flow signal was graded by Adler classification method [9].

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections (4 μm in thickness) were deparaffinized and subsequently hydrated with an ethanol gradient. Antigen retrieval was performed in a 10 mmol/L sodium citrate (pH 6.0) for 5 min with a high pressure. The tissue sections were immersed in 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. 10% goat serum was added to the tissue sections and incubated for 30 min at 37°C. The sections were incubated with Rabbit anti-NGX6 monoclonal antibodies (1:200 dilution, Abcam, USA) overnight at 4°C, and then incubated at 37°C for 30 min with a

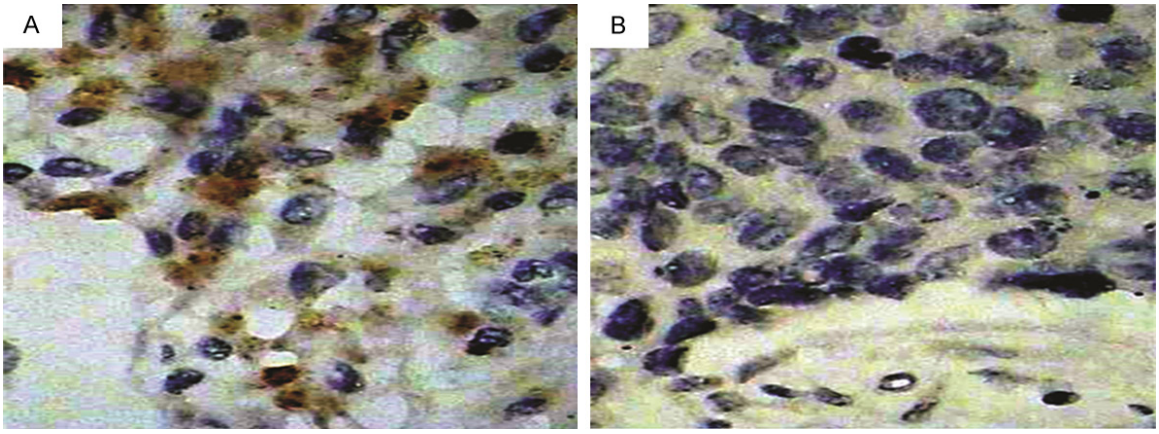

Figure 2. Representative immunohistochemical staining of NGX6 expression in IDBC tissues. A. Positive expression of NGX6; B. Negative expression of NGX6. Representative images are shown at × 400 magnifications.

Table 1. The relationship between Ultrasonographic features and NGX6 expression in IDBC

Ultrasonographic features	n	NGX6 (+)	NGX6 (-)	χ^2	*P*
Tumor size					
≥ 2 cm	68	31	37	0.016	0.900
< 2 cm	54	24	30		
Edge burr sign					
Present	76	27	49	7.434	0.006
Absent	46	28	18		
Microcalcification					
Present	74	33	41	0.018	0.893
Absent	48	22	26		
Lymph node metastasis					
Present	69	25	44	5.025	0.025
Absent	53	30	23		
Blood flow grade					
I-II	45	26	19	4.642	0.031
III	77	29	48		

secondary antibody against rabbit and mouse immunoglobulins (EnVision, DAKO, Denmark). Afterwards, the sections were stained with DAB for 5 min. The cytoplasm and cell membrane stained in yellow brown were the positive pattern. Five areas representative were randomly observed in the perspective of 400 times microscope. The number of positive cells counts was not less than 200 in each observed area. Classification is done according to the strength of cells staining and the proportion of the positive cell. The number of positive cells < 10% was defined as negative, and 10% or higher was defined as positive [10]. All immunohistochemical analyses were evaluated by two pathologists blindly and independently.

Statistical analysis

The data was processed by SPSS13.0 statistical analysis package, the measurement data used variance test, counting information using chi-square test. The Kaplan-Meier method was used to estimate the survival outcomes and groups were compared using the log-rank test. The Cox proportional hazards model was used for multivariate analysis. The significance level was set at $P < 0.05$ (both sides).

Results

Ultrasonographic features in patients with IDBC

Among 122 patients with IDBC, there were 68 (55.7%) cases with the tumor diameter greater than 2.0 cm and 54 (44.3%) cases with diameter less than 2.0 cm; 76 (62.3%) cases with mass around a burr (**Figure 1A**) and 74 (60.7%) cases with mass with microcalcifications (**Figure 1B**). Blood flow signal in tumors were graded with following: I-II level in 45 (36.9%) cases (**Figure 1C**), III level in 77 (63.1%) cases (**Figure 1D**) lymph node metastasis in 69 (56.6%) cases.

NGX6 expression in IDBC tissue

Among 122 cases of IDBC, positive expression was found in 55 (45.1%) cases (**Figure 2A**) and negative expression was found in 67 (54.9%) cases (**Figure 2B**).

Table 2. Univariate Cox regression analysis of potential prognostic parameters associated with DFS and OS in breast cancer patients

Variable	DFS		OS	
	HR (95% CI)	*P* value	HR (95% CI)	*P*
Ages, years				
≥ 35 years vs. < 35 years	0.734 (0.244-1.369)	0.235	0.858 (0.436-1.653)	0.421
Family history				
Yes vs. No	0.838 (0.452-1.495)	0.476	0.941 (0.658-1.254)	0.256
Menopause status				
Pro vs. Pre	0.987 (0.364-1.883)	0.172	1.575 (1.269-1.786)	0.195
Tumor size				
≥ 2 cm vs. < 2 cm	2.144 (1.234-3.389)	0.127	1.867 (0.988-3.742)	0.248
Edge burr sign				
Present vs. absent	1.035 (0.934-1.367)	0.295	5.864 (2.986-8.467)	0.342
Microcalcification				
Present vs. absent	3.547 (1.838-5.374)	0.176	4.859 (2.743-6.789)	0.273
Lmph node metastasis				
Present vs. absent	4.562 (2.258-7.355)	0.012	6.538 (3.987-11.563)	0.005
Blood flow grade				
I-II vs.III	1.735 (1.234-2.361)	0.033	3.436 (2.658-4.237)	0.015
ER status				
Positive vs. Negative	1.170 (0.725-1.845)	0.520	1.236 (0.748-1.554)	0.132
PR status				
Positive vs. Negative	0.717 (0.439-1.173)	0.232	2.867 (1.752-3.789)	0.432
HER2 status				
Positive vs. Negative	1.934 (1.072-3.236)	0.084	2.909 (1.253-3.745)	0.106
NGX6 status				
Positive vs. Negative	2.531 (1.832-3.347)	0.002	1.653 (1.257-2.172)	0.008
Adjuvant chemotherapy				
Yes vs. No	1.494 (0.554-2.471)	0.166	2.867 (1.732-3.789)	0.093
TNM stage				
I-II vs. III	2.489 (1.597-3.275)	0.018	8.985 (7.773-12.075)	0.023

Relationship between Ultrasonographic features and NGX6 expression in IDBC

Mass edge burr sign, blood flow grade and lymph node metastasis in IDBC were associated with NGX6 expression. Tumor size and microcalcification were not associated with NGX6 expression, as shown in **Table 1**.

Survival analysis

In the **Table 2**, univariate Cox regression analysis revealed that lymph nodal status, TNM stage, blood flow grade and NGX6 expression were significantly associated with the DFS and OS.

Kaplan-Meier analysis of DFS and OS in a subgroup of IDBC, according to the status of NGX6 expression, Lymph node metastasis and blood flow grade, and Kaplan-Meier curves of survival are shown below. This reveals the association between NGX6 positive expression and higher DFS (P = 0.023, log rank test) or higher OS (P = 0.018, log rank test) (**Figure 3**).

Lymph node metastasis is associated with lower DFS (P = 0.045, log rank test) and lower OS (P = 0.026, log rank test) (**Figure 4**).

Lower blood flow grade is associated with higher DFS (P = 0.035, log rank test). However, no significant association has been identified in relation to OS (P = 0.093, log rank test) (**Figure 5**).

Multivariate Cox regression analysis of DFS and OS in breast cancer patients, NGX6 expression,

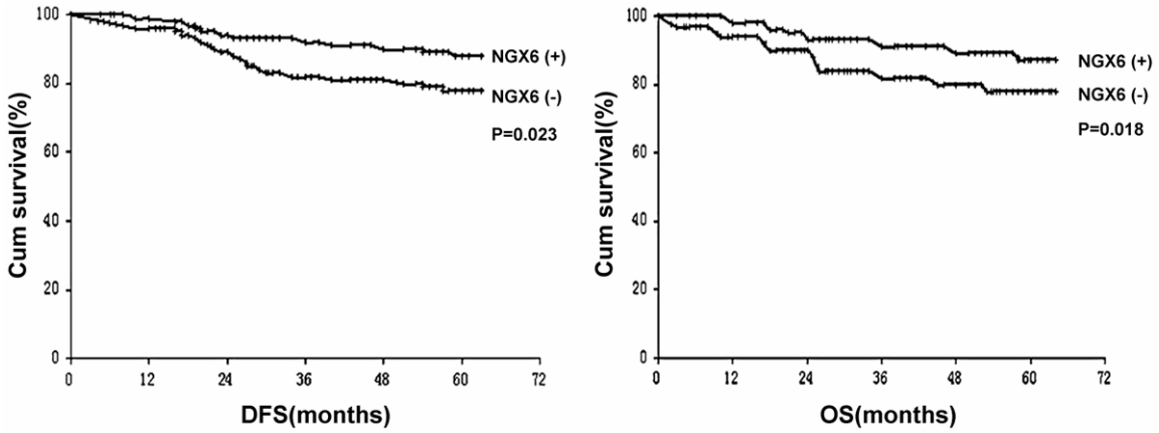

Figure 3. Kaplan-Meier analysis of DFS, OS in patients with IDBC, according to the status of NGX6 expression (positive or negative). *P*-value obtained from log-rank test, n = 122. It's showing the association between of NGX6 positive expression and higher DFS or higher OS in patients with IDBC. NGX6 (+): NGX6 expression positive group; NGX6 (-): NGX6 expression negative group.

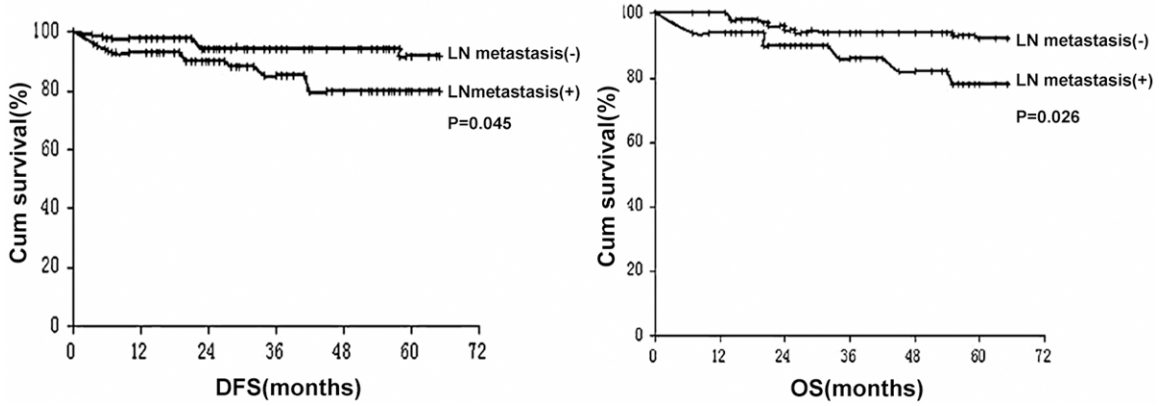

Figure 4. Kaplan-Meier analysis of DFS, OS in patients with IDBC, according to the status of lymph node metastasis (positive or negative). *P*-value obtained from log-rank test, n = 122. It's showing the association between of lymph node metastasis is associated with lower DFS and lower OS in patients with IDBC. LN metastasis (+): Lymph node metastasis positive group; LN metastasis (-): Lymph node metastasis negative group.

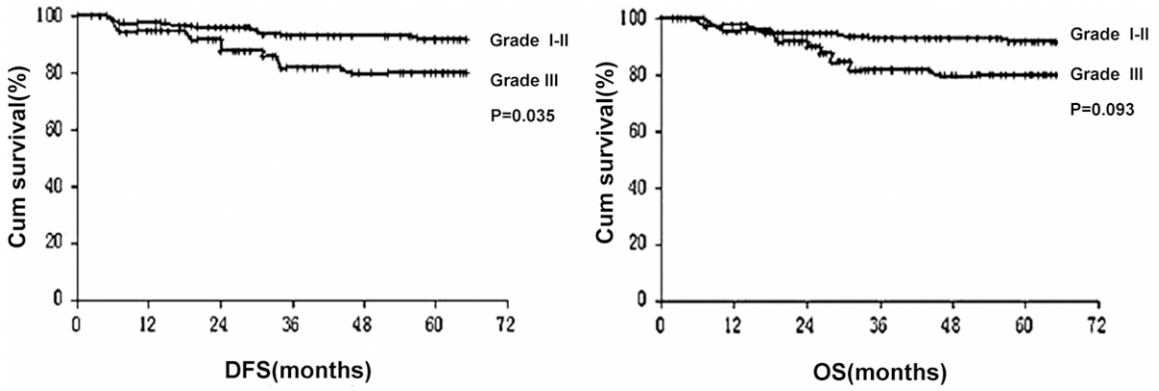

Figure 5. Kaplan-Meier analysis of DFS, OS in patients with IDBC, according to the status of blood flow grade (I-II or III) as determined by ultrasonography. *P*-value obtained from log-rank test, n = 122. It's showing the association between of lower blood flow grade and higher DFS in patients with IDBC.

Table 3. Multivariate Cox regression analysis of DFS and OS in IDBC patients

Survival	Influencing factors	HR (95% CI)	*P*
DFS	Tumor size	2.465 (1.176-3.708)	0.324
	Lmph node metastasis	3.548 (1.235-5.984)	0.035
	Blood flow grade	2.217 (1.368-3.138)	0.023
	HER2 status	3.354 (2.252-4.258)	0.079
	NGX6 status	1.948 (1.327-2.636)	0.025
	TNM stage	3.469 (1.254-5.578)	0.032
OS	Tumor size	0.852 (0.454-1.326)	0.224
	Lmph node metastasis	5.487 (2.495-9.328)	0.047
	Blood flow grade	3.543 (1.258-6.084)	0.224
	HER2 status	2.514 (1.289-4.038)	0.068
	NGX6 status	2.318 (1.752-2.983)	0.015
	TNM stage	6.568 (3.693-9.165)	0.043

lymph node metastasis, TNM and blood flow grade was the independent prognostic factors for DFS. NGX6 expression, Lymph node metastasis and TNM were the independent prognostic factors for OS. As shown in **Table 3**.

Discussion

The molecular basis of tumor determines biologic behavior, tissue pathological morphology and imaging changes. Many literatures confirmed that there are correlations between tumor imaging and prediction factors, including relevant genetic expression, clinicopathological features [11-15]. Many information obtained from these types of studies contributed to treatment selection and prognostic estimation for a variety of tumors. NGX6 is a newly identified tumor suppressor gene. NGX6 expression level can reflect the progress of tumor invasion and metastasis level [4-8, 16-19]. Combination of ultrasonic findings and NGX6 detection in breast cancer may yield clinically useful information.

In this study, occurrence rate of edge burr, lymph node metastasis in NGX6 negative expression group was higher than NGX6 positive expression group in IDBC. Pathological basis of edge burrs presented as tumor infiltration and promotion to the surrounding tissues [20, 21]. This data showed that NGX6 gene expression was correlated with edge burr and lymph node metastasis. NGX6 gene is likely involved in the invasion and metastasis activity of IDBC.

Color Doppler flow imaging (CDFI) observation showed that the blood flow of breast cancer was richer in the NGX6 express negative group than in the NGX6 positive group. Tumor growth and metastasis are inseparable from the formation of blood vessels. Tumor vessels enlarge and actively engage in sprouting and branching to meet the metabolic demands of adjacent malignancy [22]. NGX6 could influence many angiogenic molecules, such as up-regulating p19 and down-regulating EphB4 [23]. Lian P et al [24] found microvessel density (MVD) of NGX6 transfection tumor model was significantly lower than that of non-transfection tumor model. These suggest that NGX6 expression is associated with the tumor vascular abundance and NGX6 gene may participate in the process of inhibiting angiogenesis.

In univariate and multivariate survival analysis of breast cancer patients, the data showed that DFS and OS are significantly associated with NGX6 expression, lymph node metastasis and TNM. To the best of our knowledge, this is the first study examining the relationship between NGX6 and ultrasonographic features and prognosis in patients with breast cancer. NGX6 expression was demonstrated to be independently associated with ultrasonographic imaging features and clinical outcomes, highlighting that NGX6 may be employed as promising prognostic factor and useful therapeutic targets to improve the survival of breast cancer patients.

It is well known that TNM and lymph node metastasis are prognostic factors for breast cancer and is responsible, at least in part, for the mortality from breast cancer in women. Our data were consistent with these reports [25-29]. Blood flow grade status was a significant predictor of DFS. However, it was not identified as prognostic factor affecting OS. Many other factors including tumor size, ER, PR and HER2 status were also not identified as prognostic factor in our data statistical analysis. One of the reasons is that only a small number of patients were examined in this study, making it difficult to reach statistical significance. We hope to study a greater number of patients in the future.

In conclusion, ultrasonographic features are associated with NGX6 expression in IDBC. NGX6 gene expression is likely involved in the invasion and metastasis activity. NGX6 expression, Lymph node metastasis, TNM and blood flow grade are the important independent prognostic factors for IDBC. NGX6 detection and ultrasound findings may complement each other and provide important prognosis information for breast cancer patients.

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Disclosure of conflict of interest

None.

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References

[1] Li YJ, Wen G, Wang Y, Wang DX, Yang L, Deng YJ, Wei HQ, He J, Zhang X, Gu YS. Perfusion heterogeneity in breast tumors for assessment of angiogenesis. J Ultrasound Med 2013; 32: 1145-1155.

[2] Giger ML, Karssemeijer N, Schnabel JA. Breast image analysis for risk assessment, etection, diagnosis, and treatment of cancer. Annu Rev Biomed Eng 2013; 15: 327-357.

[3] Lee B, Lim AK, Krell J, Satchithananda K, Coombes RC, Lewis JS, Stebbing J. The efficacy of axillary ultrasound in the detection of nodal metastasis in breast cancer. AJR Am J Roentgenol 2013; 200: 314-320.

[4] Wang L, Ma J, Li J, Li X, Zhang Q, Peng S, Peng C, Zhou M, Xiong W, Yang J, Zhou J, Fan S, Tan C, Yan Q, Shen S, Li G. NGX6 gene inhibits cell proliferation and plays a negative role in EGFR pathway in nasopharyngeal carcinoma cells. J Cell Biochem 2005; 95: 64-73.

[5] Ma J, Zhou J, Fan S, Wang L, Li X, Yan Q, Zhou M, Liu H, Zhang Q, Zhou H, Gan K, Li Z, Peng C, Xiong W, Tan C, Shen S, Yang J, Li J, Li G. Role of a novel EGF-like domain-containing gene NGX6 in cell adhesion modulation in nasopharyngeal carcinoma cells. Carcinogenesis 2005; 26: 281-291.

[6] Wu M, Li X, Li X, Li G. Signaling transduction network mediated by tumor suppressor/susceptibility genes in NPC. Curr Genomics 2009; 10: 216-222.

[7] Liu M, Peng Y, Wang X, Guo Q, Shen S, Li G. NGX6 gene mediated by promoter methylation as a potential molecular marker in colorectal cancer. BMC Cancer 2010; 27: 160.

[8] Bevers TB, Anderson BO, Bonaccio E, Buys S, Daly MB, Dempsey PJ, Farrar WB, Fleming I, Garber JE, Harris RE, Heerdt AS, Helvie M, Huff JG, Khakpour N, Khan SA, Krontiras H, Lyman G, Rafferty E, Shaw S, Smith ML, Tsangaris TN, Williams C, Yankeelov T; National Comprehensive Cancer Network. National Comprehensive Cancer Network: NCCN clinical practice guidelines in oncology: breast cancer screening and diagnosis. J Natl Compr Canc Netw 2009; 7: 1060-1096.

[9] Adler DD, Carson PL, Rubin JM, Quinn-Reid D. Doppler ultrasound color flow imaging in the study of breast cancer: Prelininary findings. Ultrasound Mes Biol 1990; 16: 553.

[10] Ma F, Ding X, Fan Y, Ying J, Zheng S, Lu N, Xu B. A CLDN1-negative phenotype predicts poor prognosis in triple-negative breast cancer. PLoS One 2014; 9: e112765.

[11] Jiang L, Ma T, Moran MS, Kong X, Li X, Haffty BG, Yang Q. Mammographic features are associated with c1inicopathological characteristics in invasive breast cancer. Anticancer Res 2011; 31: 2327-2334.

[12] Rauch GM, Kuerer HM, Scoggins ME, Fox PS, Benveniste AP, Park YM, Lari SA, Hobbs BP, Adrada BE, Krishnamurthy S, Yang WT. Clinicopathologic, mammographic, and sonographic features in 1,187 patients with pure ductal carcinoma in situ of the breast by estrogen receptor status. Breast Cancer Res Treat 2013; 139: 639-647.

[13] Kim MY, Choi N. Mammographic and ultrasonographic features of triple-negative breast cancer: a comparison with other breast cancer subtypes. Acta Radiol 2013; 54: 889-894.

[14] Chang JM, Park IA, Lee SH, Kim WH, Bae MS, Koo HR, Yi A, Kim SJ, Cho N, Moon WK. Stiffness of tumours measured by shear-wave elastography correlated with subtypes of breast cancer. Eur Radiol 2013; 23: 2450-2458.

[15] Wan CF, Du J, Fang H, Li FH, Zhu JS, Liu Q. Enhancement patterns and parameters of breast cancers at contrast-enhanced US: correlation with prognostic factors. Radiology 2012; 262: 450-459.

[16] Wu M, Li X, Li X, Li G. Signaling transduction network mediated by tumor suppressor/susceptibility genes in NPC. Curr Genomics 2009; 10: 216-222.

[17] Liu J, Zhu X, Xu X, Dai D. DNA promoter and histone H3 methylation downregulate NGX6 in gastric cancer cells. Med Oncol 2014; 31: 817.
[18] Eskandari-Nasab E, Hashemi M, Rezaei H, Fazaeli A, Mashhadi MA, Moghaddam SS, Arbabi F, Jahantigh M, Taheri M. Evaluation of UDP-glucuronosyltransferase 2B17(UGT2B17) and dihydrofolate reductase (DHFR) genes deletion and the expression level of NGX6mRNA in breast cancer. Mol Biol Rep 2012; 39: 10531-10539.
[19] Lin ZF, Shen XY, Lu FZ, Ruan Z, Huang HL, Zhen J. Reveals new lung adenocarcinoma cancer genes based on gene expression. Eur Rev Med Pharmacol Sci 2012; 16: 1249-1256.
[20] Jiang L, Song E, Xu X, Ma G, Zheng B. Automated detection of breast mass spiculation levels and evaluation of scheme performance. Acad Radiol 2008; 15: 1534-1544.
[21] Huo Z, Giger ML, Vyborny CJ, Bick U, Lu P, Wolverton DE, Schmidt RA. Analysis of spiculation in the computerized classification of mammographic masses. Med Phys 1995; 22: 1569-1579.
[22] Zhang L, Yang N, Park JW, Katsaros D, Fracchioli S, Cao G, O'Brien-Jenkins A, Randall TC, Rubin SC, Coukos G. Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res 2003; 63: 3403-12.
[23] Wang L, Zhang Q, Ma J. Role of NGX6 in nasopharyngeal carcinoma with high ability of metastasis cell line 5-8F. Progress in Biochemistry and Biophysics (China) 2005; 32: 618-624.
[24] Lian P, Guo Q, Peng Y, Wang L. Nasopharyngeal carcinoma-associated gene 6 inhibits tumor angiogenesis in colon cancer. Shijie Huaren Xiaohua Zazhi (China) 2009; 17: 2577-2584.
[25] Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, Mandelblatt JS, Yakovlev AY, Habbema JD, Feuer EJ; Cancer Intervention and Surveillance Modeling Network (CISNET) Collaborators. Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 2005; 353: 1784-1792.
[26] van der Hage JA, Mieog JS, van de Velde CJ, Putter H, Bartelink H, van de Vijver MJ. Impact of established prognostic factors and molecular subtype in very young breast cancer patients: pooled analysis of four EORTC randomized controlled trials. Breast Cancer Res 2011; 13: 68.
[27] Engin H, Bilir C, Tekin IO. Prognostic significance of peripheral blood flow cytometry parameters in patients with non-metastatic breast cancer. Asian Pac J Cancer Prev 2013; 14: 7645-7649.
[28] Karimi A, Delpisheh A, Sayehmiri K, Saboori H, Rahimi E. Predictive factors of survival time of breast cancer in kurdistan province of Iran between 2006-2014: a cox regression approach. Asian Pac J Cancer Prev 2014; 15: 8483-8488.
[29] Liu Y, Xin T, Huang DY, Shen WX, Li L, Lv YJ, Jin YH, Song XW, Teng C, Jiang QY. Prognosis in very young women with triple-negative breast cancer: retrospective study of 216 cases. Med Oncol 2014; 31: 222.