Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ZKSCAN3 promotes breast cancer cell proliferation, migration and invasion

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ARTICLE INFO

Article history:
Received 2 July 2018
Accepted 5 July 2018
Available online xxx

Keywords:
ZKSCAN3
Breast cancer
Tumor progression
Akt
mTOR

ABSTRACT

ZKSCAN3, a zinc-finger transcription factor, which has been shown to be upregulated in several human cancer. However, the expression level, function and mechanism of ZKSCAN3 in breast cancer remains unknown. In the current study, immunohistochemistry, western blot and quantitative real time polymerase chain reaction (qRT-PCR) results showed that ZKSCAN3 was overexpressed in breast cancer tissue compared with normal breast tissue. Through analyzing the clinicopathological characteristics, we demonstrated that positive ZKSCAN3 expression predicted poor prognosis of patients with breast cancer. The expression level of ZKSCAN3 protein/mRNA in breast cancer cells (MCF-7 and MDA-MB-231) was higher than its expression in normal breast cells (HBL-100). Knocking down ZKSCAN3 via its short hairpin RNA (shRNA) in MCF-7 and MDA-MB-231 inhibited cell viability, migration and invasion. Western blot analysis showed that ZKSCAN3 silencing lead to significant decreases in the expression of Cyclin D1, B-cell lymphoma-2 (Bcl-2), and matrix metalloproteinase (MMP)-2/MMP-9, as well as increases in the expression of Bcl2 Associated X Protein (Bax) in breast cancer cells. Additionally, ZKSCAN3-shRNA expression markedly suppressed tumor growth in breast cancer xenograft mice. Finally, we demonstrated that silencing of ZKSCAN3 was able to inhibit Akt/mTOR signaling pathway by blocking p-Akt and p-mTOR protein expression in breast cancer cells. These results demonstrate that ZKSCAN3 plays a significant role in the progression of breast cancer. Therefore, ZKSCAN3 is a potential therapeutic target for breast cancer.

1. Introduction

Breast cancer is one of the most common malignancies and seriously threatens women's life safety [1,2]. At present, the treatment of breast cancer is mainly based on comprehensive treatment such as surgery, chemotherapy, radiotherapy, and hormone therapy. With the further study of targeted therapy for cancer, the search for the target of breast cancer has become the new direction.

ZKSCAN3 is a member of the zinc finger transcription factors family [3]. ZKSCAN3 regulates a variety of biological functions, including cell proliferation, apoptosis, and autophagy [4–8]. Resent studies found that ZKSCAN3 promoted the development and progression of a few malignancies, and had an influence on the cell growth, migration and invasion of cancer cells [9–12]. A report demonstrated that ZKSCAN3 overexpression activated Akt signaling in colorectal cancer [13]. Akt/mTOR signaling pathway plays an important role in the regulation of cancer cells growth and metastasis [14,15]. Abnormally activated Akt/mTOR pathway is usually related to tumorigenesis, including breast cancer [16].

MMPs play a crucial role in the progression of tumors. It has been reported that the concentration of MMP-2/MMP-9 was related to tumor grade, invasion and metastasis in human tumors [17,18]. Moreover, the role of MMP-2/MMP-9 in tumor invasion has been further studied in melanoma and breast cancer [19,20].

However, the expression level of ZKSCAN3 in breast cancer and its role in breast cancer progression remain unclear. In this study, we found that ZKSCAN3 was upregulated in breast cancer and positive ZKSCAN3 expression was related to poor prognosis in

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https://doi.org/10.1016/j.bbrc.2018.07.019
0006-291X/

breast cancer patients. Furthermore, we confirmed that ZKSCAN3 promoted cell proliferation, migration and invasion, and enhanced cell growth via activating Akt/mTOR signaling pathway in breast cancer.

2. Materials and methods

2.1. Clinical tissue samples

A total of 150 cases of fresh breast cancer tissues and paired adjacent non-tumor breast tissues were acquired from patients with pathologically verified breast cancer who received surgery in First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). This study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

2.2. Cell culture and transfection

Normal breast cell line (HBL-100), breast cancer cell lines (MDA-MB-231 and MCF-7) and 293 T cells were purchased from the Chinese Academy of Sciences Type Culture Collection.

ZKSCAN3 shRNA plasmid and control shRNA plasmid were provided by Genepharma. 1 μg/ml puromycin (Sigma) was used to select stable clones.

2.3. Immunohistochemistry

Breast cancer tissues were paraffin-embedded and sliced into 4-μm thick sections for ZKSCAN3 staining (NOVUS). The staining intensity was evaluated into four degrees: 0, none; 1, weak; 2, moderate; and 3, strong. The proportion of positive staining was separated into four grades: 0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >75%. The score of staining intensity multiplied by the score of proportion of positive staining were used for statistical analysis. A final staining score $\geq$5 was regarded as high ZKSCAN3 expression and <5 as low ZKSCAN3 expression in breast cancer patients.

2.4. Western blot

Cells were lysed with RIPA lysis buffer containing protease inhibitor and phosphatase inhibitor. Total proteins were transferred to a polyvinylidene fluoride membrane (PVDF; Beyotime) by electroblotting. Then the membrane was blocked with 5% skimmed milk powder. The membrane was incubated overnight at 4 °C with primary antibodies: ZKSCAN3 (NOVUS), Bax and bcl2 (Cell Signaling), Cyclin D1 (abcam), MMP-2 and MMP-9 (Abcam), Akt and phosphorylated-Akt (phosphoresce on S473, Sanying); mTOR and phosphorylated mTOR (phosphoresce on S2448, Abcam), rabbit anti-GAPDH and mouse anti-GAPDH (Santa Cruz). Secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG (Affinity) were then incubated with the membrane for 1 h. Signals were detected using the ECL detection reagent (Santa Cruz). GAPDH was used as the loading control. The greyscale values of protein band were analyzed using ImageJ software.

2.5. RNA isolation and qRT–PCR

Total RNA was obtained from clinical samples and cultured cell lines via TRIzol reagent (TaKaRa, Japan) and reverse transcribed using PrimeScript™ RT Master Mix (TaKaRa) on the basis of the manufacturer's instructions. qRT-PCR was performed to determine the levels of GAPDH and ZKSCAN3 using SYBR® Premix Ex Taq™ (Roche). The sequence-specific primers were designed by Sangon (Shanghai, China). ZKSCAN3-F: 5′-CCCAGGGTCACAAAGTAGCC-3′, ZKSCAN3-R: 5′-GGACTCTGGAGTAAGCCTAGAA-3’; GAPDH-F: 5′-CTCCTCCACCTTTGACG CTG-3′, GAPDH-R: 5′-CATACCAGGAAATGAGCTTGACAA-3’.

2.6. Proliferation assay

Cells are passed into 96-well plates at the concentration of 2000 cells/well after transfection. Cell viability was determined by CCK-8 at 12 h, 24 h, 48 h and 72 h. The proliferation capacity of cells was shown by the ratio of absorbance, measured at 450 nm of the test group to that of control cells.

2.7. Cell cycle assay

Flow cytometry to evaluate cell cycle distribution was performed using a cell cycle detection kit (KeyGEN, China) on the basis of instructions. Cells are collected and washed with cold PBS twice. Then cells were centrifuged at 2000 rpm for 5 min, re-suspended

Table 1
Expression of ZKSCAN3 in breast cancer tissues and normal breast tissues, and association between ZKSCAN3 expression and clinicopathological features of breast cancer patients.

Clinic pathology reasons	Cases	Expression of ZKSCAN3		$\chi 2$	P(two sides)
		Negative(−)	Positive(+)		
Tissue				4.339	$P = 0.037$
normal breast tissues	150	129	21		
breast cancer tissues	150	38	112		
Age				0.214	$P = 0.644$
≤50	82	22	60		
>50	68	16	52		
Size of tumor				4.165	$P = 0.041$
T ≤ 2 cm	98	30	68		
T > 2 cm	52	8	44		
Lymph node				4.696	$P = 0.030$
Negative	101	31	70		
Positive	49	7	42		
Differentiation				8.718	$P = 0.013$
Well	33	12	21		
Moderate	69	21	48		
Poor	48	5	43		
Stage				13.707	$P < 0.001$
I+II	97	34	63		
III+IV	53	4	49		

Please cite this article in press as: Y. Chi, et al., ZKSCAN3 promotes breast cancer cell proliferation, migration and invasion, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.07.019