sFRP3 inhibits the progression of invasive ductal breast carcinoma through negative regulation of Wnt/β-catenin signaling

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Received February 26, 2017; Accepted March 23, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: sFRP3 is an antagonist of Wnt/β-catenin signaling. However, its detailed mechanism involved in the progression of invasive ductal breast carcinoma (IDC) is still unclear. We detected the expressions of sFRP3 and correlated factors in 147 IDC samples by immunohistochemistry and examined the detailed mechanism in MDA-MB-231 and MCF-7 cell lines. sFRP3 expression was reduced in IDC samples, which was negatively correlated with nuclear positive expression of β-catenin (R = -0.183, P<0.05), cyclin D1 (R = -0.174, P<0.05) or c-myc (R = -0.259, P<0.05). sFRP3 reduced the activity of Wnt/β-catenin signaling and inhibited cell invasive and proliferative capacity in MDA-MB-231 and MCF-7 cell lines. Above results suggested that sFRP3 was negatively correlated with the progression of IDC. It might influence the progression of IDC through inhibiting Wnt/β-catenin pathway.

Keywords: sFRP3, β-catenin, c-myc, cyclin D1, invasive ductal breast carcinoma

Introduction

Wnt signaling takes part in a number of cell biological pathways in normal development and tumorigenesis [1, 2]. Wnt signal modulates these biological activities by interacting with cell surface frizzled receptors so as to affect downstream signaling pathway, including β-catenin nuclear translocation, and transcription of downstream target genes such as cyclin D1 and c-myc [3, 4].

Frizzled family proteins were involved in the development of tissue polarity [5]. The cystein rich domain (CRD) near the NH2 terminus of frizzled is the putative binding site for Wnt ligands [6]. Related to Wnt pathway, a secreted protein has been identified in a number of organisms [7, 8], containing a region highly homologous to the CRD of the frizzled protein [9]. The name of this protein is secreted frizzled-related protein (sFRP). sFRP functioned as a secreted antagonist of Wnt pathway [10].

sFRP expression is generally observed in modulating the malignant phenotype of various tumors. The sFRP family contains five members: sFRP1, sFRP2, sFRP3, sFRP4 and sFRP5. sFRP1 interacts with thrombospondin-1 and inhibits its stimulatory effects on cell adhesion and motility of breast cancer [11]. sFRP4 is a useful serum marker of the patients with chronic hepatitis B-related hepatocellular carcinoma [12]. sFRP3 expression is reduced in non-small cell lung cancer compared with normal lung tissue [13]. However, the clinical significance of sFRP3 in invasive ductal breast carcinoma (IDC) is still unclear.

In this work, we detected the expression of sFRP3 in IDC tissue and analyses its relationship with clinicopathological factors; in addition, we also sought to identify its involved mechanisms in vitro.

Materials and methods

Tissue samples

We collected 147 samples from IDC patients who underwent complete cutting operation in the First Affiliated Hospital of China Medical
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University between 2005 and 2009. All resected tumor tissues of these patients were obtained in accordance with Human Subject Research Protocols approved by the ethics committees of First Affiliated Hospital of China Medical University. Resected IDC tissues were obtained with written informed consent from adult patients. These patients had not received preoperative radiotherapy or chemotherapy before operation. All of the patients were women. The survival of the patient was defined as the time from the surgery day to the death day attributed to migration or recurrence or to the end of the follow-up period. Formalin-fixed paraffin-embedded sections of tissues were stained with hematoxylin and eosin staining, and reviewed independently by three well-experienced pathologists, using the World Health Organization criteria [WHO Classification of Tumors of the Breast. Fourth edition]. Therefore, the grade of IDC was categorized into three groups: I (low grade), II (moderate grade) and III (high grade). The clinicopathological factors are listed in Table 1. We also collected 30 cases of adjacent normal tissue samples from the same patients as negative control.

Immunohistochemical staining and evaluation

The steps of immunohistochemistry staining were the same as the previous study [14]. Primary antibodies: sFRP3 (1:100; Santa Cruz Biotechnology, Dallas, USA), cyclin D1 (1:150; Santa Cruz), c-myc (1:100; Santa Cruz) and β-catenin (1:100; Santa Cruz).

The percentage of positively immunohistochemical staining cells was calculated by counting 400 tumor cells. For sFRP3, the scoring criteria were defined as follows: the proportion of positively staining cells (0 = absent; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; 4 = more than 75%); the staining intensity (0 = negative; 1 = weak; 2 = moderate; 3 = strong). The product of above two scores was the final result. Positive expression of sFRP3 was identified as the score no less than 3; negative expression was identified as the score less than 3. For c-myc and cyclin D1, the positive staining localized in the nucleus. For β-catenin, the scoring criteria were the same as the previous report [15].

Cell culture and transfection

The human breast cancer cell lines MDA-MB-231 (with low level of sFRP3) and MCF-7 (with high level of sFRP3) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37°C with 5% CO$_2$. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used to transfect the plasmid or siRNA into cells. sFRP3 cDNA was purchased from OriGene (Rockville, USA). sFRP3 siRNA was purchased from Santa Cruz.

Western blotting

We used RIPA lysis buffer to extract total protein. Nuclear and cytoplasmic extracts were prepared by using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech, China). The steps of Western blot were the same as the previous report [16]. The primary antibodies are as follows: sFRP3 (1:200; Santa Cruz), c-myc (1:200; Santa Cruz), cyclin D1 (1:300; Santa Cruz), β-catenin (1:200; Santa Cruz), LaminB1 (1:500, Santa Cruz), α-tubulin (1:500, Santa Cruz).

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</table>
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MTT assay

We plated cells in 96-well plates (3000 cells/well) and cultured them in medium containing 10% fetal bovine serum for 4 days. For demonstrating the survival status of cells, we added 20 μl of 5 mg/ml MTT (thiazolyl blue) solution to every well and incubated it for 4 h at 37°C. Then it was replaced by the resultant MTT formazan solubilized in DMSO (150 μl). We obtained the final results by the way of spectrophotometer (490 nm) under microplate reader (Bio-Rad, Hercules, CA).

Dual-luciferase assay

Cells were plated in 24-well plates for 24 h and then transfected with TOPFlash or FOPFlash plasmids (Addgene, Cambridge, USA). We used Dual-Luciferase Assay System (Promega, Madison, WI) to examine the expression of reporter gene after incubation for 30 h at 37°C. The activity of Tcf-mediated gene transcription was determined by the ratio of TOPFlash to FOPFlash luciferase activity normalized to Renilla luciferase activity from the control plasmid pRL-TK. All experiments were performed by three times. sFRP3 cDNA was co-transfected with TOPFlash or FOPFlash for analyzing the effect of sFRP3 on Wnt/β-catenin signaling.

Statistical analysis

SPSS 17.0 was performed for this study. We applied Chi-squared test to evaluate the correlation with clinicopathological characteristics. The Spearman correlation test was applied to assay the correlations among the expression of sFRP3, c-myc, cyclin D1 and β-catenin. Kaplan-Meier analysis was selected to compare survival time among various IDC patients. A two-tailed P<0.05 was thought to be statistically significant.

Results

*sFRP3 is reduced expressed in IDC, which is negatively associated with poor prognosis of patients*

We selected 147 IDC samples and 30 normal breast tissues for this study. Immunohisto-
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The correlation between sFRP3 and Wnt/β-catenin signaling

sFRP3 is an antagonist of Wnt/β-catenin signaling [17]. β-catenin nuclear translocation is an important marker of the activation of Wnt/β-catenin signaling [18]. c-myc and cyclin D1 are important downstream target genes of Wnt/β-catenin signaling [3, 4]. Based on above reports, we detected the expressions of β-catenin, c-myc and cyclin D1 in IDC and analyzed their correlations with sFRP3. sFRP3 reduced expression were negatively correlated with β-catenin nuclear positive expression (R = -0.183, P<0.05), as well as cyclin D1 nuclear positive expression (R = -0.174, P<0.05) and c-myc nuclear positive expression (R = -0.259, P<0.05, Table 2) (Figure 3).

The patients with reduced sFRP3 and nuclear positive β-catenin expressions had significantly shorter postoperative survival than others (43.240±5.613 vs 80.017±2.828 months, P<0.05, Figure 2B). Together, above results indicated that sFRP3 might have negative effect on the IDC progression through inhibiting Wnt/β-catenin signaling.

sFRP3 inhibits activation of Wnt/β-catenin signaling in IDC cells

Above results suggested that sFRP3 might inhibit the progression of IDC through negatively regulation of Wnt/β-catenin signaling. We selected MDA-MB-231 and MCF-7 cells to further illustrate this hypothesis. In MDA-MB-231 cell, sFRP3 transfection inhibited the expression of c-myc and cyclin D1; in addition, β-catenin nuclear translocation was negatively regulated by sFRP3. In MCF-7 cell, sFRP3-siR-
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The mechanism by which sFRP3 is involved in IDC progression?

The aberrant activation Wnt signaling is important for tumorigenesis [24]. Wnt pathway is composed of two types: canonical (β-catenin-dependent) and noncanonical (β-catenin-independent) pathways. The canonical Wnt/β-

The effect of sFRP3 in biological behavior of IDC cells

Transwell and MTT were used to test the above-mentioned effects of sFRP3 in the biological behavior of IDC cells. In both MCF-7 and MDA-MB-231 cells, sFRP3 significantly inhibited cell invasion and proliferation compared to the control group (Figure 6). The result suggested that sFRP3 might inhibit cell invasion and proliferation through its downregulation of Wnt/β-catenin signaling.

Discussion

sFRP plays an important role in a number of signaling pathways during embryo development and tumorigenesis [19, 20], which is thought to be an antagonist of Wnt signaling [21]. As a member of sFRP family, sFRP3 takes part in bone regeneration and osteoblastic differentiation in a calvarial bone defect [22]. Recent study reported that sFRP3 inhibited the progression of lung adenocarcinoma through regulating canonical Wnt signaling [23]. In the present work, immunohistochemistry assay showed that sFRP3 exhibited lower expression in IDC tissues, compared with normal duct epithelium. In vitro assay indicated that sFRP3 inhibited the capacity of invasion and proliferation of IDC cells. It raises the question for us: what is the mechanism by which sFRP3 is involved in IDC progression?

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Figure 6. sFRP3 inhibits IDC cell proliferation (A) and invasion (B, C). sFRP3 transfection decreases cell proliferation and invasion in MDA-MB-231 cells; siRNA-sFRP3 (Si-sFRP3) transfection increases cell proliferation and invasion in MCF-7 cells. The graph in (B, C) shows the number of invading cells; *, P<0.05 compared with negative control. NC, negative control; Si-NC, scrambled siRNA for control.

catenin signaling is most extensively studied. In activated state of this signaling cascade, Wnt ligands bind to frizzled receptors, causing β-catenin nuclear translocation to promote the transcription of downstream genes such as cyclin D1 and c-myc [3, 4]. As an antagonist of Wnt, sFRP3 competes with Wnt ligands for binding to frizzled receptors so as to inhibit the activity of Wnt/β-catenin signaling [25]. Our results showed a strong negative correlation between sFRP3 and β-catenin expression in IDC, as well as cyclin D1 and c-myc. sFRP3 transfection inhibited the expression of β-catenin nuclear translocation in IDC cells, as well as the expressions of c-myc and cyclin D1; while sFRP3-siRNA had opposing effect. These results were in accordance with previous reports.

In conclusion, sFRP3 is negatively correlated with the progression of IDC. It might influence the progression of IDC through inhibiting Wnt/β-catenin pathway. Therefore, inhibition the Wnt/β-catenin signaling by sFRP3 might be an effective strategy for the prevention and treatment of breast cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81602022 to Huanyu Zhao and No. 81301930 to Lianhe Yang), and the General Project of Education Department of Liaoning Province (No. L2015595 to Lianhe Yang).

Disclosure of conflict of interest

None.

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