

# Adipocyte-derived SFRP5 inhibits breast cancer cells migration and invasion through Wnt and epithelial-mesenchymal transition signaling pathways

Wenzhong Zhou<sup>1,2\*</sup>, Chunmiao Ye<sup>1,2\*</sup>, Liang Li<sup>2,3</sup>, Liyuan Liu<sup>2,3</sup>, Fei Wang<sup>2,3</sup>, Lixiang Yu<sup>2,3</sup>, Fei Zhou<sup>2,3</sup>, Yujuan Xiang<sup>2,3</sup>, Yongjiu Wang<sup>2,3</sup>, Gengshen Yin<sup>1,2</sup>, Zhongbing Ma<sup>2,3</sup>, Qinye Fu<sup>2,3</sup>, Qiang Zhang<sup>2,3</sup>, Dezong Gao<sup>2,3</sup>, Shuya Huang<sup>2,3</sup>, Zhigang Yu<sup>2,3</sup>

<sup>1</sup>School of Medicine, Cheeloo College of Medicine, Shandong University, Jinan 250012, China; <sup>2</sup>Department of Breast Surgery, the Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250033, China; <sup>3</sup>Institute of Translational Medicine of Breast Disease Prevention and Treatment, Shandong University, Jinan 250033, China

\*These authors contributed equally to this work.

Correspondence to: Zhigang Yu, PhD, MD. Department of Breast Surgery, the Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250033, China. Email: yzg@medmail.com.cn; Shuya Huang, PhD. Department of Breast Surgery, the Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250033, China. Email: huangsy@sdu.edu.cn.

## Abstract

**Objective:** Obesity is closely associated with metastasis in breast cancer patients. Secreted frizzled-related protein 5 (SFRP5), one of the novel adipokines with anti-inflammatory properties, is associated with obesity. This study aims to study the role of SFRP5 in the crosstalk between obesity and breast cancer metastasis and identify the underlying mechanism.

**Methods:** 3T3-L1 pre-adipocytes were differentiated to mature adipocytes and a hypertrophic adipocyte model was induced with palmitic acid (PA). Cell motility was measured in MDA-MB-231 and MCF-7 breast cancer cells co-cultured with adipocytes conditioned medium (CM) with or without SFRP5 protein. Wnt and epithelial-mesenchymal transition (EMT) signal pathways were investigated by western blot. Circulating SFRP5 level in 218 breast cancer patients and the association with clinicopathologic characteristics of breast cancer were further determined. Online databases ENCORI and PREDICT Plus were used to exam the link between SFRP5 and prognosis.

**Results:** Reduced SFRP5 level was detected in the hypertrophic adipocyte model. Recombinant SFRP5 protein inhibited MDA-MB-231 and MCF-7 cells invasion and migration induced by PA-treated adipocyte CM, and SFRP5 inhibition by specific antibody reversed the effect of SFRP5. Furthermore, SFRP5 significantly inhibited Wnt and downstream EMT in breast cancer cells. Low circulating SFRP5 level correlated with body mass index (BMI), lymph node (LN) metastasis, TNM stage and high Ki67 expression in breast cancer patients. Increased SFRP5 level was associated with favorable predicted survival. Kaplan-Meier curves showed high SFRP5 level in tumor tissue was associated with better outcome of breast cancer patients.

**Conclusions:** Our findings demonstrated SFRP5 is a vital adipokine that mediates the crosslink between obesity and the metastatic potential of breast cancer. Promotion of SFRP5 expression in the adipose microenvironment may represent a novel approach for preventing breast cancer metastasis.

**Keywords:** Obesity; adipocytes; breast cancer metastasis; SFRP5; EMT

Submitted Dec 20, 2019. Accepted for publication Mar 21, 2020.

doi: 10.21147/j.issn.1000-9604.2020.03.06

View this article at: <https://doi.org/10.21147/j.issn.1000-9604.2020.03.06>

## Introduction

A growing body of epidemiological and clinical evidence supports a strong relationship between obesity and breast cancer risk, metastasis and outcome (1,2). Obesity promotes breast cancer progression by altering tumor microenvironment to facilitate metastatic dissemination or metabolic reprogramming. In the breast, adipocytes represents the most abundant cell type of the breast cancer microenvironment (3-5).

Previous studies have shown that adipocytes can act as an endocrine organ, secreting signaling molecules, such as metabolic substrates, adipokines and cytokines. Adipokines such as leptin and adiponectin have been shown to exert critical roles in breast cancer cell behaviors. Leptin promotes breast cancer cells proliferation and epithelial-mesenchymal transition (EMT), whereas adiponectin stimulates cell apoptosis in some breast cancer subtypes (6,7). There are significant differences in adipocyte biology between lean and obese individuals, but a limited number of studies have focused on the differential effects of lean and obese adipocytes on cancer cell behavior. Notably, adipokine balance is altered in obese humans (8). Many pro-inflammatory cytokines are elevated during obesity, including interleukine (IL)-6, IL-8, chemokine (C-C motif) ligand 2 (CCL2) and CCL5, and aberrant levels of these factors are associated with increased cancer stage and poor prognosis (9,10). In contrast, lower levels of anti-inflammatory adiponectin are associated with obesity (11). Except for adiponectin, few of these adipokines play a positive role in regulating obesity-related breast cancer progression.

Secreted frizzled-related protein 5 (SFRP5), a member of SFRP family, is a novel adipocytokine that belongs to the category of anti-inflammatory adipokines. Plasma SFRP5 levels are significantly decreased in obese patients and patients with related diseases, such as diabetes and coronary artery disease (12,13). SFRP5 expression is also downregulated in a variety of tumor tissues, including ovarian cancer, lung cancer, and hepatocellular carcinoma, and promoter hypermethylation of SFRP5 is a frequent alteration that is associated with poor prognosis in breast cancer (14). SFRP5 contains a cysteine-rich domain in its N-terminal that is homologous to that of Frizzled proteins (Fz), a receptor for Wnt. Therefore, SFRP5 prevents the binding of Wnt and Frizzled proteins through its interaction with the Wnt protein, thus blockading Wnt signaling (15). Decreased expression of SFRP5 activates

Wnt signaling and leads to a pro-inflammatory state in visceral adipose tissue, promoting the progression of obesity-associated diseases (16). However, the interaction between SFRP5 and Wnt, the role of circulating SFRP5 in breast cancer and whether secreted SFRP5 from adipocytes can mediate the link between obesity and breast cancer development have not been explored.

In this study, we investigated the role of SFRP5 in breast cancer cell migration and invasion and explored the association of SFRP5 level with clinicopathological factors and outcomes of breast cancer patients.

## Materials and methods

### *Cell culture, differentiation and induction of 3T3-L1 pre-adipocytes*

MDA-MB-231 and MCF-7 human breast cancer cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China). The cell lines were authenticated by short tandem repeat profiles analysis before use. MDA-MB-231 cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum (NCS, Sigma, USA) and 100 unit/mL penicillin/streptomycin. MCF-7 cells were cultured in MEM medium supplemented 0.01 mg/mL rh-insulin, 10% fetal bovine serum (FBS, Biological Industries, USA) and 100 unit/mL penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

3T3-L1 pre-adipocytes were purchased from Institute of Biochemical and Cell Biology, Shanghai branch of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured and differentiated in accordance with standard procedures (17). Briefly, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Corning, USA) supplemented with 10% FBS and 100 unit/mL penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. The medium was changed every 2 d. At 2 d post-confluence (referred to as d 0), the cells were incubated with 10 mg/L insulin, 0.5 mmol/L isobutylmethyl-xanthine and 1 mmol/L dexamethasone. On d 2, the medium was replaced by fresh medium supplemented with only insulin. On d 4, the medium was removed and fresh media with 10% FBS, but with no additional hormones, was added. The medium was changed every other day. On d 10, nearly 95% of the cells showed mature adipocyte phenotype.

To promote adipocyte hypertrophy *in vitro*, as described

by Gornicka *et al.* (18), at the end of the differentiation period, differentiated mature 3T3-L1 adipocytes were exposed to palmitic acid (PA, 500  $\mu\text{mol/L}$ ) in medium containing 1% free fatty acid bovine serum albumin (BSA) for up to 24 h; cells treated only with 1% free fatty acid BSA were used as control. Cells were then maintained with DMEM without FBS and the conditioned medium (CM) was collected after 24 h.

### **Oil red O dye staining**

Differentiated 3T3-L1 adipocyte cells (10 days) were collected, washed twice in phosphate buffer saline (PBS), fixed with 4% formaldehyde for 20 min, and washed twice in water. Oil red O dye was dissolved in dimethyl carbinol and filtered. The cells were stained with Oil red O dye solution for 1 h and then washed with water until the background became transparent. Photographs of stained sections were taken with an optical microscope.

### **Cell motility assay**

Cell invasiveness ability was examined using a reconstituted extracellular matrix (1.5 mg/mL Matrigel; BD Biosciences, Lac franklin, USA) coated on polycarbonate membranes in the upper compartment of a 24-well transwell system (8  $\mu\text{m}$  pore size, Millipore). The MDA-MB-231 cells ( $8 \times 10^4$  cells/well) or MCF-7 cells ( $2 \times 10^5$  cells/well) were seeded on the matrigel-coated membrane. CM from control or PA-treated adipocytes, with/without SFRP5 recombinant protein (RD system, Minneapolis, USA) and SFRP5 antibody (RD system), was placed in the bottom of the transwell system. The neutralization dose of antibody was based on the manufacturer's recommended protocol (about 5  $\mu\text{g/mL}$  antibody in the presence of 5  $\mu\text{g/mL}$  recombinant human SFRP5). Normal mouse IgG antibody (Proteintech, China) was used as control. After incubation for 24 h, the upper surface of the membrane was swiped to remove the attached cells. The cells that had invaded to the lower side of the membrane were fixed with 4% paraformaldehyde for 30 min, washed three times with PBS, and stained with 0.1% crystal violet for 30 min at room temperature. The results are reported as mean numbers of stained cells counted in five random fields (200 $\times$  magnification) under a light microscope.

Cell migration was evaluated using a similar system. MDA-MB-231 cells ( $6 \times 10^4$  cells/well) or MCF-7 cells ( $1.5 \times 10^5$  cells/well) were seeded on the upper membrane of the Transwell system without Matrigel coating. Other

conditions and procedures were same to cell invasion assay.

### **Western blot**

Protein lysates were collected using phosphorylated protein extraction reagent (Solarbio, Beijing, China). Protein concentrations were quantified with a Protein BCA Assay Kit (Beyotime, China). Proteins lysates were resolved on SDS-PAGE gels, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA), and immunoblotted with anti-human antibodies to non-phosphorylated (active)  $\beta$ -catenin, phosphorylated c-Jun N-terminal kinase (JNK), E-cadherin, Vimentin, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), phosphorylated glycogen synthase kinase 3 $\beta$  (Ser9), Snail,  $\beta$ -actin (Cell Signaling Technology), or  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Beyotime). The blots were visualized with enhanced chemiluminescence (Amersham Biosciences) and exposed X-ray films. Protein bands were normalized to  $\beta$ -actin blotting for normalization.

### **Study population**

Chinese female breast cancer inpatients who were newly diagnosed and confirmed by needle core biopsy were recruited in the Department of Breast Surgery of the Second Hospital of Shandong University from January 2016 to June 2018. The enrollment and written informed consent were conducted by attending physicians. There were 290 patients who were willing to participate in the study. A total of 218 patients were finally included and 72 patients were excluded. Exclusion criteria were as follows: 1) patients without histopathological evaluation; 2) diagnosed with recurrent or metastasis breast cancer; 3) diagnosed with other concurrent malignancies; 4) a prior history of other cancers; 5) performed neoadjuvant chemotherapy; or 6) no peripheral blood sample pre-operation and pre-therapeutic intervention. Written informed consent was obtained from all study participants, and the study protocols and procedures were approved by the Institutional Review Board at the Second Hospital of Shandong University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### **Data collection**

In-person interviews and record linkages were conducted to collect information on demographics based on a self-

designed structured table by specially trained post-graduates. Current weight and standing height were measured when patients were hospitalized and body mass index (BMI) was computed from the obtained measurements. Medical and pathology records from the hospital were reviewed to obtain intact information on pathological results. Pathological results were primarily determined by immunohistochemically following the American Society of Clinical Oncology (ASCO)/College of American Pathologists (2010) recommendations, with a few blanks that could not find. In addition, all specimens were characterized for all routinely diagnostic immunophenotypic parameters.

Peripheral blood samples were obtained early in the morning after fasting at least 8 h. Blood samples were obtained pre-operation and pre-therapeutic intervention. The blood samples were centrifuged, and the supernatant serum was carefully collected to exclude cell components. All samples with hemolysis or clotting were discarded. Serum samples were stored at  $-80^{\circ}\text{C}$  until use.

#### ***Enzyme-linked immunosorbent assay (ELISA)***

SFRP5 expression in the CM, adipocyte cell lysate and serum were determined by using a quantitative ELISA kit (Cusabio, Wuhan, China). The average value was calculated as the final concentration. No samples were below the detection limits. All analyses were performed according to the manufacturer's recommended protocols.

#### ***Statistical analysis***

Continuous data was first tested for normal distribution. The P-values of continuous variables were determined by rank sum test and one-way analysis of variance (ANOVA). The P-values of categorical variables were determined by Chi-square tests. The odds ratio (OR) and 95% confidence interval (95% CI) were obtained using single binary logistic regression analyses. We also extended our analysis by stratifying the results according to BMI category.

The cut-off values for SFRP5 were assessed for the overall and corresponding subgroup by receiver operating characteristic (ROC) curve analysis, in which the sensitivity (SE) was plotted as a function of 1-specificity (1-SP). The Youden index (J) can be formally defined as  $J = \max_c [SE(c) + SP(c) - 1]$ . The cutoff value that achieves this maximum is referred to as the optimal cut-off point that optimizes the biomarker's discriminating power.

SFRP5 expression levels in breast cancer tissues and

adjacent normal tissues were obtained from the Cancer Genome Atlas (TCGA) dataset and ENCORI website (<http://starbase.sysu.edu.cn>) (19). The 5-year and 10-year predicted overall survival (OS) were calculated using updated PREDICT Plus model ([www.predict.nhs.uk](http://www.predict.nhs.uk)) (20) for each patient after entry of patient age, menopausal status, tumor size, number of positive nodes, tumor grade, estrogen receptor (ER) status, human epidermal growth receptor 2 (HER2) status, Ki67 status, mode of detection, and adjuvant systemic therapy information.

All *in vitro* experiments were repeated independently at least three times. Data are expressed as  $\bar{x} \pm s$  and were analyzed by one-way ANOVA.  $P < 0.05$  was considered significant. Statistical analyses were performed by IBM SPSS Statistics (Version 21.0; IBM Corp., New York, USA) and GraphPad Prism 8.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA).

## **Results**

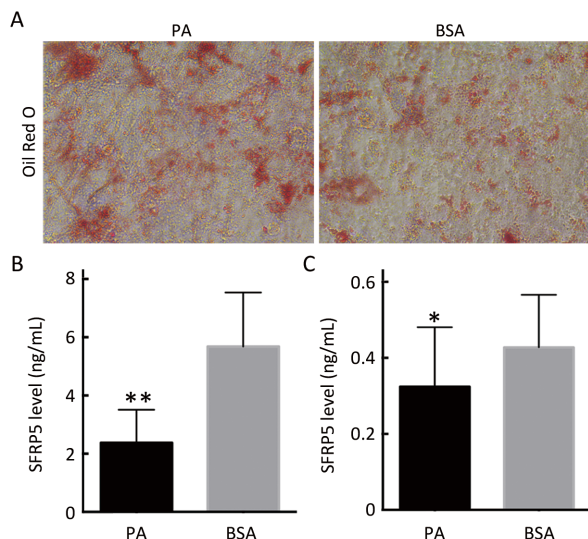
### ***SFRP5 is down-regulated during adipocytes hypertrophy***

We first investigated the expression level of SFRP5 during adipocyte hypertrophy. Previous studies showed that treatment with the free fatty acid PA resulted in significant lipid accumulation and hypertrophy in mature 3T3-L1 adipocytes (18). Therefore, we used PA treatment to mimic adipocyte hypertrophy in a cell model and confirmed the lipid accumulation in mature adipocytes after PA treatment (*Figure 1A*). SFRP5 levels in adipocyte supernatants and lysates were assessed by ELISA. The results showed that, SFRP5 level was significantly reduced in the supernatant of adipocytes treated with PA compared with control group ( $2.37 \pm 1.14$  ng/mL vs.  $5.68 \pm 1.85$  ng/mL,  $P < 0.01$ ; *Figure 1B*). Similar results were observed in adipocytes lysates ( $P < 0.05$ , *Figure 1C*). Notably, SFRP5 level in adipocyte lysate was much lower than that in supernatant, indicating SFRP5 is mostly present as a secretory protein in adipocytes.

### ***SFRP5 inhibits breast cancer cells motility stimulated by adipocytes CM***

To investigate the impact of adipocytes CM on breast cancer cell motility, we next used a cell co-culture model. Compared with CM from BSA, adipocytes CM treated with PA significantly promoted cell invasion and migration of MDA-MB-231 and MCF-7 cells (*Figure 2*).

We next determined the effect of adipocyte-secreted



**Figure 1** SFRP5 downregulation in hypertrophic adipocyte culture medium and lysate. (A) 3T3-L1 adipocytes were differentiated to mature adipocytes and induced with PA (500  $\mu\text{mol/L}$ ) in 0.1% fatty acid free BSA for 24 h. Mature adipocytes treated with 0.1% fatty acid free BSA were used as control. Cells were stained with oil red O and observed by using a microscope. The levels of SFRP5 in culture medium (B) and cell lysate (C) of mature adipocytes treated with PA and BSA were evaluated by ELISA.  $n=3$ . SFRP5, secreted frizzled-related protein 5; PA, palmitic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; \*,  $P<0.05$ ; \*\*,  $P<0.01$ .

SFRP5 on breast cancer cell motility. The mouse and human protein sequences of SFRP5 showed conservation with 95.8% homology. Therefore, we used recombinant human SFRP5 protein for experiments and incubated recombinant protein with breast cancer cells together with adipocyte CM. The numbers of invaded and migrated cells were obviously reduced in the presence of recombinant SFRP5 protein in both MDA-MB-231 and MCF-7 cells. We further used SFRP5 antibody to bind and neutralize SFRP5 and found that SFRP5 antibody reversed the effect of SFRP5 on breast cancer cell migration and invasion, while no effect was observed by using normal mouse IgG control (Figure 2, Supplementary Figure S1).

#### **SFRP5 inhibits Wnt and EMT pathways in breast cancer cells**

Previous studies showed that SFRP5 blocks Wnt signaling by binding with Wnt protein (15). To examine the mechanism underlying the effects of secreted SFRP5 on breast cancer cells, we evaluated the expression of Wnt

signaling proteins in MDA-MB-231 and MCF-7 cells incubated with adipocytes CM treated with PA with or without SFRP5. The indicators of canonical Wnt signaling including active  $\beta$ -catenin and phosphorylated GSK3 $\beta$ , and phosphorylated JNK, as the target of non-canonical Wnt signaling were detected. The levels of these markers were all significantly increased in both MDA-MB-231 and MCF-7 cells co-cultured with adipocyte CM treated with PA compared with controls. Moreover, the expression of all Wnt signaling proteins were inhibited in cells cultures with SFRP5 compared with PA alone (Figure 3A,B).

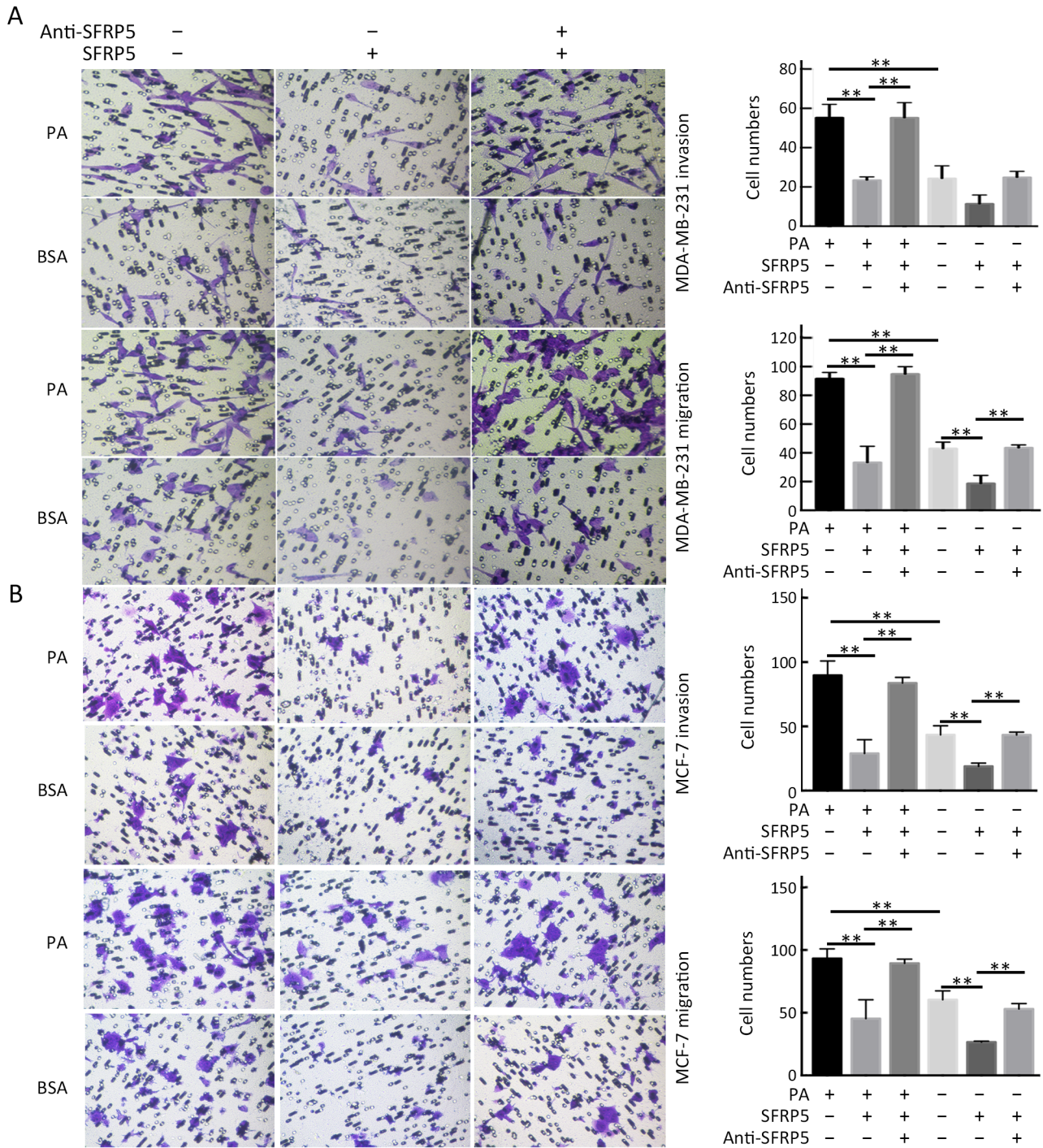
EMT is a major process involved in promoting metastasis of breast cancer and is closely associated with the Wnt/ $\beta$ -catenin pathway (21). We further investigated whether EMT was involved in the effects of SFRP5 on breast cancer cells. We detected increased mesenchymal markers Snail, Vimentin, and  $\alpha$ -SMA and decreased epithelial marker E-cadherin were shown in both MDA-MB-231 and MCF-7 cells co-cultured with adipocytes CM treated with PA compared with controls. Furthermore, SFRP5 significantly decreased Snail, Vimentin and  $\alpha$ -SMA expression and increased E-cadherin level compared with PA treatment alone (Figure 3C,D).

#### **Correlation of circulating SFRP5 level with clinicopathological parameters of breast cancer patients**

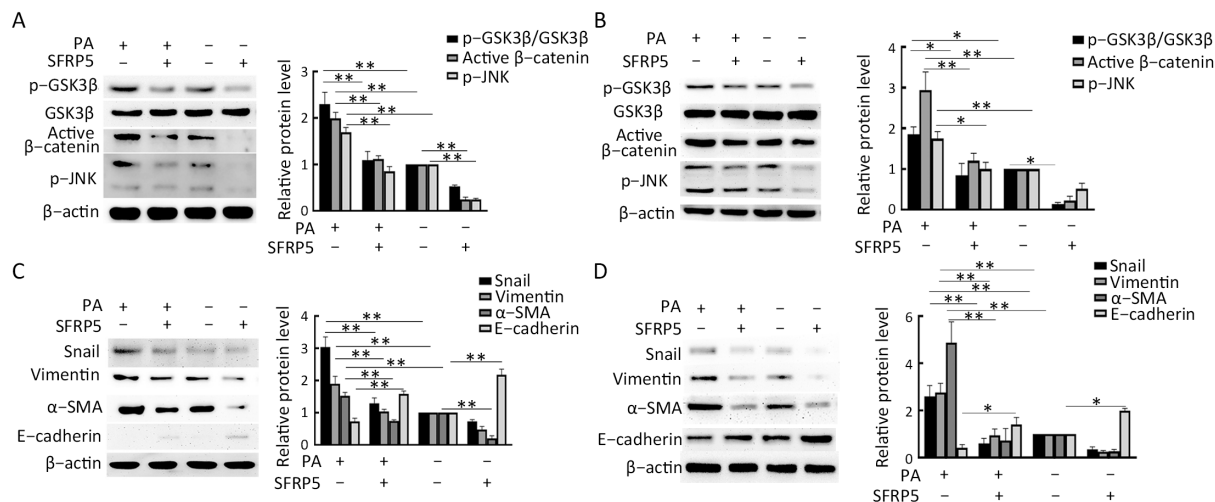
We next explored the association between circulating SFRP5 level and clinicopathological characteristics of breast cancer patients (Table 1). We assessed the plasma level of SFRP5 in 218 primary breast cancer patients using ELISA. The results showed that SFRP5 level was significantly lower in patients with  $\text{BMI} \geq 24.0 \text{ kg/m}^2$  compared with those with  $\text{BMI} < 24.0 \text{ kg/m}^2$  (30.46 vs. 55.67 ng/mL,  $P<0.001$ ). SFRP5 level was also significantly lower in patients with age of menarche  $>15$  (49.21 vs. 29.43 ng/mL,  $P=0.002$ ). Breast cancer patients with higher number of lymph nodes (LN) infiltration ( $\geq 4$ ) also showed lower circulating SFRP5 level (16.33 vs. 43.65 ng/mL,  $P=0.007$ ). Low SFRP5 level was also observed in patients with higher tumor stage and higher Ki67 expression. No significant relationships were found between SFRP5 level and other characteristics (Table 1).

#### **Circulating SFRP5 level was inversely associated with BMI, LN infiltration, TNM stage and Ki67 expression**

To further examine the association of SFRP5 with BMI, ROC curve was performed to obtain the best cutoff value and the effectiveness of SFRP5 on BMI was evaluated by



**Figure 2** SFRP5 inhibited MDA-MB-231 and MCF-7 cell motility induced by hypertrophic adipocyte culture medium. MDA-MB-231 (A) and MCF-7 cells (B) were seeded in the upper chamber with (invasion assay) or without (migration assay) Matrigel-coated transwell culture system, and adipocytes CM treated with PA or BSA was added in the lower chamber. Co-cultured cells were incubated with or without 300 ng/mL SFRP5 recombinant protein. Equal amounts of SFRP5 antibody (anti-SFRP5) were added in the presence of SFRP5 protein. After 24 h, cells that migrated across the membrane were fixed and examined by crystal violet staining. The results are reported as mean numbers of stained cells in five random views from three independent experiments (200×). SFRP5, secreted frizzled-related protein 5; CM, conditioned medium; PA, palmitic acid; BSA, bovine serum albumin; \*\*, P<0.01.



**Figure 3** SFRP5 inhibited non-canonical and canonical Wnt signal pathway and downstream EMT process. MDA-MB-231 cells (A,C) and MCF-7 (B,D) cells were exposed to PA (500 μmol/L) or BSA induced adipocyte CM with or without SFRP5 for 24 h. The protein levels of active β-catenin, p-GSK3β, and p-JNK were detected by western blot and quantified from at least three independent experiments (A,B); The expressions of EMT markers, including Snail, Vimentin, E-cadherin and α-SMA were analyzed by western blot and quantified from at least three independent experiments (C,D). SFRP5, secreted frizzled-related protein 5; EMT, epithelial-mesenchymal transition; PA, palmitic acid; BSA, bovine serum albumin; CM, conditioned medium; \*, P<0.05; \*\*, P<0.01.

logistic regression. The AUC was 0.625 and the cutoff value 17.64 ng/mL of SFRP5 significantly discriminated BMI. SFRP5 level was inversely correlated with BMI in breast cancer patients (Table 2, P<0.001, OR=0.318, 95% CI: 0.181–0.560).

Furthermore, using the ROC curve to confirm the cutoff value, we found that reduced SFPR5 level was also associated with increased LN infiltration, Ki67 expression and TNM stage in all cases (Table 3). Considering the intrinsic link between BMI and SFRP5, subgroup analysis was further performed. We found that lower SFPR5 level was inversely associated with higher LN infiltration number in both patients with BMI<24.0 kg/m<sup>2</sup> (OR=0.159, 95% CI: 0.028–0.892) and BMI≥24.0 kg/m<sup>2</sup> (OR=0.250, 95% CI: 0.085–0.735) subgroups. The association between SFRP5 level and Ki67 status was more evident in the BMI≥24.0 kg/m<sup>2</sup> subgroup (OR=0.088, 95% CI: 0.020–0.396), whereas a negative correlation of SFRP5 level with TNM stage was only observed in the BMI<24.0 kg/m<sup>2</sup> subgroup (OR=0.105, 95% CI: 0.016–0.700) (Table 3).

**Higher SFRP5 level correlates with favorable survival in breast cancer patients**

Because of the short follow-up period of the patients recruited in current study, we used the online tool PREDICT (www.predict.nhs.uk) to estimate 5-year and

10-year predicted OS for 186 breast cancer patients with detailed information. The data showed that higher circulating SFRP5 level increased 10-year predicted OS rate in patients of BMI≤24 kg/m<sup>2</sup> (Table 4).

We compared SFRP5 expression levels in 1,104 breast invasive carcinoma tissue samples with 103 normal breast tissues using existing data from The Cancer Genome Atlas (TCGA) posted on the Encyclopedia of RNA Interaction (ENCORI) (<http://starbase.sysu.edu.cn>). The expression of SFRP5 was significantly lower in breast invasive carcinoma tissues compared with normal tissues (fold change=0.91, P<0.001, Figure 4A). The relationship between SFRP5 mRNA expression and the clinical outcome of breast cancer patients was further evaluated by ENCORI. The results showed that higher SFRP5 mRNA expression strongly correlated with favorable OS in breast cancer patients (n=1,082, Figure 4B).

**Discussion**

Adipocytes are a major component of breast tissue and secrete several factors, including adipokines and cytokines that affect breast cancer progression (22,23). An interaction between breast cancer cells and adipocytes has been reported in previous studies, but the adipocyte metabolic alterations associated with obesity and the effect on breast cancer have not been examined. In this study, we

**Table 1** Characteristics and plasma SFRP5 levels of breast cancer patients

Characteristics	Cases (N=218)		P
	n	Mean (ng/mL)	
Numbers of births			0.586
0-1	112	42.44	
≥2	103	37.08	
N/A	3	95.24	
Age of menarche (year)			0.002
≤15	120	49.21	
>15	78	29.43	
N/A	20	32.90	
BMI (kg/m <sup>2</sup> )			<0.001
<24.0	88	55.67	
≥24.0	130	30.46	
Menopause status			0.570
Yes	116	35.63	
No	102	46.33	
Family history of breast cancer			0.661
Yes	16	22.35	
No	198	41.42	
N/A	4	74.72	
Histological grade			0.559
I-II	134	45.25	
III	76	34.03	
N/A	8	26.00	
TNM stage			0.047
I-II	188	43.83	
III	25	18.30	
N/A	5	32.36	
ER status			0.962
Positive	182	43.43	
Negative	35	27.00	
N/A	1	9.81	
PR status			0.941
Positive	172	41.41	
Negative	46	37.73	
HER2 status			0.558
Positive	49	40.02	
Negative	146	34.62	
N/A	23	80.14	
Ki67 status			0.011
<14%	26	84.49	
≥14%	192	34.70	
Numbers of LN infiltration			0.007
0-3	191	43.65	
≥4	22	16.33	
N/A	5	32.36	
Molecular subtype			0.610
Luminal A	20	51.41	
Luminal B	162	39.02	
HER2	10	38.36	
Basal like	19	18.79	
N/A	7	109.81	

SFRP5, secreted frizzled-related protein 5; BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth receptor 2; LN, lymph node; N/A, unknown.



**Table 2** Association between circulating SFRP5 level and BMI in breast cancer patients

Factors	Univariate model		
	OR	95% CI	P
Menopause (no = reference)	1.490	0.862–2.573	0.153
Family history of breast cancer (no = reference)	0.872	0.312–2.436	0.793
Numbers of birth ( $\leq 1$ = reference)	1.330	0.770–2.300	0.307
Age at menarche ( $\leq 15$ years old is reference)	1.494	0.829–2.694	0.182
SFRP5* ( $\leq$ cut-off value is reference)	0.318	0.181–0.560	<0.001

SFRP5, secreted frizzled-related protein 5; BMI, body mass index; OR, odds ratio; 95% CI, 95% confidence interval; \*, the cut-off value for SFRP5 is 17.64 ng/mL.

investigated the effect of the novel anti-inflammatory adipokine SFRP5 on breast cancer cell motility during obesity. We used an adipocyte and breast cancer cell co-culture system and confirmed that hypertrophic adipocyte CM could promote cell migration and invasion *in vitro*. The SFRP5 level was decreased in hypertrophic adipocytes, and higher SFRP5 level could attenuate hypertrophic adipocyte-induced breast cancer cell motility through inhibiting Wnt and the EMT process. *In vivo* results showed that circulating SFRP5 level was negatively related with obesity and LN infiltration in breast cancer patients, and higher SFRP5 level was associated with a favorable outcome of breast cancer patients.

Several studies reported that hyperplasia of adipose tissue occurs only in certain periods of life. Adipocyte hypertrophy is the main process connected with obesity in adults (23,24). Our findings showed that both ER+ MCF-7 and triple-negative MDA-MB-231 cells displayed enhanced migration and invasion after treatment with PA-exposed adipocyte CM (Figure 2). These data suggested that obesity induced alterations of adipocyte-derived cytokines and further promoted breast cancer cell motility.

SFRP5 is an anti-inflammatory adipokines that may exhibit specific roles in obesity-related breast cancer progression. However, current data on the biological role of SFRP5 in breast cancer are very limited. Our results revealed that SFRP5 secretion was reduced in hypertrophic adipocytes and that circulating SFRP5 level was inversely associated with BMI in breast cancer patients. In line with our findings, circulating SFRP5 level was also negatively correlated with obesity and related diabetes mellitus (25), metabolic syndrome (26), and cardiometabolic risk factors (27). Decreased SFRP5 was also investigated in an insulin resistance model of 3T3-L1 adipocytes (28). Hyper-methylation of the SFRP5 promoter has been reported in various tumor tissues, and SFRP5 overexpression significantly inhibited cancer metastasis in several cancer

types (29-31). However, the association between circulating SFRP5 level and tumor characteristics has not been explored.

Our data investigated the association between lower circulating SFRP5 levels and increased LN infiltration numbers and we found that addition of SFRP5 protein inhibited breast cancer cell migration and invasion induced by hypertrophic adipocyte CM (Figure 2). Our results showed that Wnt and downstream EMT signaling pathway were suppressed by SFRP5 in breast cancer cells (Figure 3). Dysregulation of Wnt signaling has been reported in various cancers, including breast cancer. Previous studies indicated that both canonical and non-canonical Wnt activate EMT, and SFRP5 was shown to inhibit both canonical and non-canonical Wnt5a-JNK activation (15,32,33). Our results confirmed that SFRP5 may be an antagonist of both canonical and non-canonical Wnt signaling pathways and that SFRP5 inhibits downstream EMT process in breast cancer.

The TNM staging system uses tumor size, LN status, and distant metastatic status to determine disease stage and help estimate outcomes of breast cancer patients. ER, PR expression and HER2 status also exhibit critical impacts on treatment recommendations, prognosis, and recurrence. Higher expression of Ki67, a nuclear protein associated with cellular proliferation, also indicates worse outcome (34). Our results showed that lower SFRP5 level was detected in breast cancer patients with LN metastasis, higher Ki67 expression and TNM stage (Table 1), all of which indicated for poorer prognosis. Logistic regression analyses indicated a significant negative association between circulating SFRP5 level and higher numbers of LN infiltration, Ki67 expression and TNM stage (Table 3). However, we could not evaluate the effect of SFRP5 on actual outcomes in this dataset because of short follow-up period. Therefore, we used prognostication prediction model PREDICT Plus to investigate the association of

**Table 3** Association between circulating SFRP5 level and numbers of LN infiltration, Ki67 status, TNM stage of breast cancer patients

Factors	Numbers of LN infiltration* [n (%)]		P	Ki67 status** [n (%)]		P	TNM stage*** [n (%)]		P	OR	95% CI		
	0-3	≥4		N/A	<14% ≥14%		I-II	III				N/A	
					OR								95% CI
All cases	5	5	0.001	<0.001	5	0.003							
SFRP5≤ cut-off value	38 (19.9)	12 (54.5)		ref	17 (65.4)	177 (92.2)	38 (20.2)	12 (48.0)		ref			
SFRP5> cut-off value	153 (80.1)	10 (45.5)		0.207	9 (34.6)	15 (7.8)	150 (79.8)	13 (52.0)		0.160	0.061-0.420		
BMI<24.0 kg/m <sup>2</sup>	2	2	0.037		9	64	11	3	0.052		0.105-0.700		
SFRP5≤ cut-off value	11 (13.8)	3 (50.0)		ref	9 (64.3)	64 (86.5)	11 (13.6)	3 (60.0)		ref			
SFRP5> cut-off value	69 (86.2)	3 (50.0)		0.159	5 (35.7)	10 (13.5)	70 (86.4)	2 (40.0)		0.281	0.078-1.012		
BMI≥24.0 kg/m <sup>2</sup>	3	3	0.012		8	113	27	9	0.002		0.154-1.102		
SFRP5≤ cut-off value	27 (24.3)	9 (56.3)		ref	8 (66.7)	113 (95.8)	27 (25.2)	9 (45.0)		ref			
SFRP5> cut-off value	84 (75.7)	7 (43.7)		0.250	4 (33.3)	5 (4.2)	80 (74.8)	11 (55.0)		0.088	0.020-0.396		

SFRP5, secreted frizzled-related protein 5; LN, lymph node; BMI, body mass index; OR, odds ratio; 95% CI, 95% confidence interval; N/A, unknown; \*, \*\*, \*\*\*, the cut-off value of SFRP5 are 9.36, 89.53 and 9.36 ng/mL, respectively.

SFRP5 level and the predicted survival in the current study. The PREDICT Plus model was originally derived from cancer registry information on 5,694 women treated in East Anglia from 1999 to 2003. Breast cancer mortality models for ER-positive and ER-negative tumors were constructed using Cox proportional hazards, adjusted for known prognostic factors and mode of detection (version 1.0) (35). The model was updated in October 2011 to include HER2 status based on an analysis of 10,179 cases collected by the Breast Cancer Association Consortium (BCAC) (version 1.1) (36). Ki67 status was added using a data set from Nottingham of 1,274 women diagnosed in 1989–1998 and followed for 10 years. The addition of Ki67 led to a small improvement in calibration and discrimination in 1,274 patients with ER positive disease with the area under the ROC curve improved from 0.7611 to 0.7676 (version 1.2) (37). The model was further re-fitted by taking into account age at diagnosis, tumor size and node status to improve the accuracy for ER-negative disease and extended the prediction to 15 years. PREDICT Plus provides reliable survival data, especially for long-term (10-year) outcomes of breast cancer patients, which have been validated in several studies (20,38,39). Using predicted survival data, our results showed that higher circulating SFRP5 level increased the 10-year survival rate in patients of BMI<24.0 kg/m<sup>2</sup> (Table 4). We plan to investigate the association of SFRP5 level and realistic patient outcome in a future study and also validate the prediction accuracy of PREDICT Plus model in Chinese patients. We further used TCGA data and found that higher SFRP5 level in

tumor tissue showed better OS in breast cancer patients (Figure 4), which confirmed SFRP5 as a favorable prognosis marker in breast cancer.

**Conclusions**

Our findings provide new evidences that obesity-induced hypertrophic adipocytes promote breast cancer metastasis through inhibition of SFRP5 secretion. Addition of SFRP5 into hypertrophic adipocyte CM inhibited breast cancer cell migration and invasion through downregulating the Wnt and downstream EMT process. Further investigation revealed a negative association between SFRP5 and BMI, LN infiltration, TNM stage and higher Ki67 expression in breast cancer patients. Finally, high SFRP5 levels were associated with improved outcomes in breast cancer patients. Together, our findings confirmed SFRP5 as a vital adipokine that mediates the crosslink between obesity and breast cancer metastasis and further emphasizes the importance of weight control for improving breast cancer prognosis.

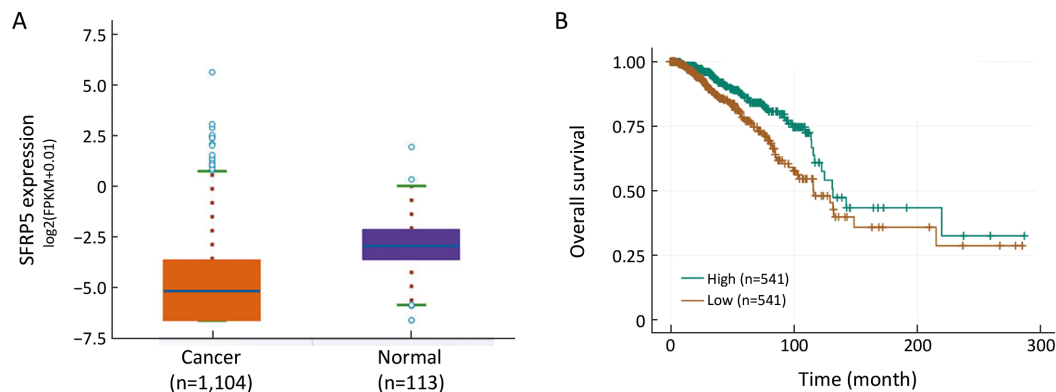
**Acknowledgements**

This study was supported by the Major Scientific and Technological Innovation Project of Shandong Province (No. 2017CXGC1212), National Natural Science Foundation of China (No. 31701258), and the National Key Research and Development Program of China (No. 2016YFC0901300).

**Table 4** Association between circulating SFRP5 level and OS

Factors	5-year OS* [n (%)]		P	OR	95% CI	10-year OS** [n (%)]		P	OR	95% CI
	≤85%	>85%				≤65%	>65%			
All cases			0.131					0.077		
SFRP5≤ cut-off value	27 (77.1)	96 (63.6)		ref		11 (37.9)	35 (22.3)		ref	
SFRP5> cut-off value	8 (22.9)	55 (36.4)		1.934	0.822–4.550	18 (62.1)	122 (77.7)		2.130	0.921–4.929
BMI<24.0 kg/m <sup>2</sup>			0.253					0.044		
SFRP5≤ cut-off value	8 (66.7)	29 (48.3)		ref		4 (40.0)	8 (12.9)		ref	
SFRP5> cut-off value	4 (33.3)	31 (51.7)		2.138	0.581–7.866	6 (60.0)	54 (87.1)		4.500	1.038–19.512
BMI≥24.0 kg/m <sup>2</sup>			0.375					0.466		
SFRP5≤ cut-off value	19 (82.6)	67 (73.6)		ref		7 (36.8)	27 (28.4)		ref	
SFRP5> cut-off value	4 (17.4)	24 (26.4)		1.701	0.526–5.508	12 (63.2)	68 (71.6)		1.469	0.523–4.129

SFRP5, secreted frizzled-related protein 5; OS, overall survival; BMI, body mass index; OR, odds ratio; 95% CI, 95% confidence interval; \*, \*\*, the cut-off values of SFRP5 are 22.71 and 9.36 ng/mL, respectively.



**Figure 4** Higher SFRP5 level correlated with favorable survival in breast cancer patients. (A) SFRP5 mRNA expression levels in 1,104 breast invasive carcinoma tissue samples and 103 normal breast tissues were obtained from TCGA and algorithms available at ENCORI (<http://starbase.sysu.edu.cn>) (fold change=0.91,  $P < 0.001$ ); (B) Kaplan-Meier curves for OS were obtained from TCGA data and algorithms available at ENCORI with 1,082 breast invasive cancer patients classified according to high and low SFRP5 gene expression (HR=0.60, log-rank  $P = 0.016$ ). SFRP5, secreted frizzled-related protein 5; OS, overall survival; TCGA, The Cancer Genome Atlas; ENCORI, the Encyclopedia of RNA Interaction; OS, overall survival; HR, hazard ratio.

## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

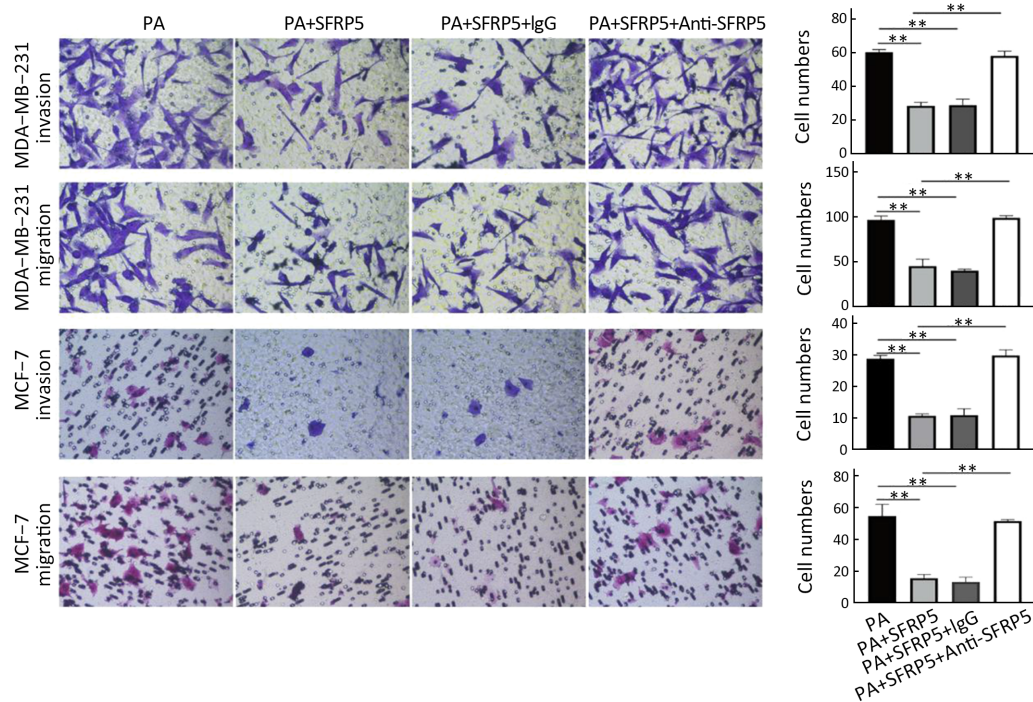
## References

- Calle EE, Rodriguez C, Walker-Thurmond K, et al. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003;348:1625-38.
- Neuhouser ML, Aragaki AK, Prentice RL, et al. Overweight, obesity, and postmenopausal invasive breast cancer risk: A secondary analysis of the women's health initiative randomized clinical trials. *JAMA Oncol* 2015;1:611-21.
- Booth A, Magnuson A, Fouts J, et al. Adipose tissue, obesity and adipokines: role in cancer promotion. *Horm Mol Biol Clin Investig* 2015;21:57-74.
- Wang YY, Lehuédé C, Laurent V, et al. Adipose tissue and breast epithelial cells: a dangerous dynamic duo in breast cancer. *Cancer Lett* 2012;324:142-51.
- Wu Q, Li B, Li Z, et al. Cancer-associated adipocytes: key players in breast cancer progression. *J Hematol Oncol* 2019;12:95.
- Dalamaga M, Diakopoulos KN, Mantzoros CS. The role of adiponectin in cancer: a review of current evidence. *Endocr Rev* 2012;33:547-94.
- Haque I, Ghosh A, Acup S, et al. Leptin-induced ER- $\alpha$ -positive breast cancer cell viability and migration is mediated by suppressing CCN5-signaling via activating JAK/AKT/STAT-pathway. *BMC Cancer* 2018;18:99.
- Quail DF, Dannenberg AJ. The obese adipose tissue microenvironment in cancer development and progression. *Nat Rev Endocrinol* 2019;15:139-54.
- Picon-Ruiz M, Morata-Tarifa C, Valle-Goffin JJ, et al. Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. *CA Cancer J Clin* 2017;67:378-97.
- Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. *Cytokine Growth Factor Rev* 2006;17:325-37.
- Han SH, Quon MJ, Kim JA, et al. Adiponectin and cardiovascular disease: response to therapeutic interventions. *J Am Coll Cardiol* 2007;49:531-8.
- Hu Z, Deng H, Qu H. Plasma SFRP5 levels are decreased in Chinese subjects with obesity and type 2 diabetes and negatively correlated with parameters of insulin resistance. *Diabetes Res Clin Pract* 2013;99:391-5.
- Miyoshi T, Doi M, Usui S, et al. Low serum level of secreted frizzled-related protein 5, an anti-inflammatory adipokine, is associated with coronary artery disease. *Atherosclerosis* 2014;233:454-9.
- Veeck J, Geisler C, Noetzel E, et al. Epigenetic inactivation of the secreted frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated

- with unfavorable prognosis. *Carcinogenesis* 2008;29:991-8.
15. Liu LB, Chen XD, Zhou XY, et al. The Wnt antagonist and secreted frizzled-related protein 5: implications on lipid metabolism, inflammation, and type 2 diabetes mellitus. *Biosci Rep* 2018;38:BSR20180011.
  16. Catalán V, Gómez-Ambrosi J, Rodríguez A, et al. Activation of noncanonical Wnt signaling through WNT5A in visceral adipose tissue of obese subjects is related to inflammation. *J Clin Endocrinol Metab* 2014;99:E1407-17.
  17. Kajimoto K, Terada H, Baba Y, et al. Essential role of citrate export from mitochondria at early differentiation stage of 3T3-L1 cells for their effective differentiation into fat cells, as revealed by studies using specific inhibitors of mitochondrial di- and tricarboxylate carriers. *Mol Genet Metab* 2005;85:46-53.
  18. Gornicka A, Fettig J, Eguchi A, et al. Adipocyte hypertrophy is associated with lysosomal permeability both *in vivo* and *in vitro*: role in adipose tissue inflammation. *Am J Physiol Endocrinol Metab* 2012;303:E597-606.
  19. Li JH, Liu S, Zhou H, et al. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 2014;42(Database issue):D92-7.
  20. Candido Dos Reis FJ, Wishart GC, Dicks EM, et al. An updated PREDICT breast cancer prognostication and treatment benefit prediction model with independent validation. *Breast Cancer Res* 2017;19:58.
  21. Glackin CA. Targeting the Twist and Wnt signaling pathways in metastatic breast cancer. *Maturitas* 2014;79:48-51.
  22. Dirat B, Bochet L, Dabek M, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* 2011;71:2455-65.
  23. Iyengar NM, Gucalp A, Dannenberg AJ, et al. Obesity and cancer mechanisms: tumor micro-environment and inflammation. *J Clin Oncol* 2016;34:4270-6.
  24. Spalding KL, Arner E, Westermark PO, et al. Dynamics of fat cell turnover in humans. *Nature* 2008;453:783-7.
  25. Hu W, Li L, Yang M, et al. Circulating Sfrp5 is a signature of obesity-related metabolic disorders and is regulated by glucose and liraglutide in humans. *J Clin Endocrinol Metab* 2013;98:290-8.
  26. Xu Q, Wang H, Li Y, et al. Plasma Sfrp5 levels correlate with determinants of the metabolic syndrome in Chinese adults. *Diabetes Metab Res Rev* 2017;33.
  27. Carstensen-Kirberg M, Kannenberg JM, Huth C, et al. Inverse associations between serum levels of secreted frizzled-related protein-5 (SFRP5) and multiple cardiometabolic risk factors: KORA F4 study. *Cardiovasc Diabetol* 2017;16:109.
  28. Lv C, Jiang Y, Wang H, et al. Sfrp5 expression and secretion in adipocytes are up-regulated during differentiation and are negatively correlated with insulin resistance. *Cell Biol Int* 2012;36:851-5.
  29. Liang J, Kang X, Halifu Y, et al. Secreted frizzled-related protein promoters are hypermethylated in cutaneous squamous carcinoma compared with normal epidermis. *BMC Cancer* 2015;15:641.
  30. Sheng W, Zhang ZC, Shi DY, et al. Epigenetic silencing of SFRP5 promotes the metastasis and invasion of chondrosarcoma by expression inhibition and Wnt signaling pathway activation. *Chem Biol Interact* 2018;296:1-8.
  31. Yu J, Xie Y, Li M, et al. Association between SFRP promoter hypermethylation and different types of cancer: A systematic review and meta-analysis. *Oncol Lett* 2019;18:3481-92.
  32. Li Y, Rankin SA, Sinner D, et al. Sfrp5 coordinates foregut specification and morphogenesis by antagonizing both canonical and noncanonical Wnt1 signaling. *Genes Dev* 2008;22:3050-63.
  33. Ren D, Minami Y, Nishita M. Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition. *Genes Cells* 2011;16:304-15.
  34. National Health Commission of The People's Republic of China. Chinese guidelines for diagnosis and treatment of breast cancer 2018 (English version). *Chin J Cancer Res* 2019;31:259-77.
  35. Wishart GC, Azzato EM, Greenberg DC, et al. PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. *Breast Cancer Res* 2010;12:R1.

36. Wishart GC, Bajdik CD, Dicks E, et al. PREDICT Plus: development and validation of a prognostic model for early breast cancer that includes HER2. *Br J Cancer* 2012;107:800-7.
37. Wishart GC, Rakha E, Green A, et al. Inclusion of KI67 significantly improves performance of the PREDICT prognostication and prediction model for early breast cancer. *BMC Cancer* 2014;14:908.
38. Maishman T, Copson E, Stanton L, et al. An evaluation of the prognostic model PREDICT using the POSH cohort of women aged  $\leq 40$  years at breast cancer diagnosis. *Br J Cancer* 2015;112:983-91.
39. Gray E, Marti J, Brewster DH, et al. Independent validation of the PREDICT breast cancer prognosis prediction tool in 45,789 patients using Scottish Cancer Registry data. *Br J Cancer* 2018;119:808-14.

**Cite this article as:** Zhou W, Ye C, Li L, Liu L, Wang F, Yu L, Zhou F, Xiang Y, Wang Y, Yin G, Ma Z, Fu Q, Zhang Q, Gao D, Huang S, Yu Z. Adipocyte-derived SFRP5 inhibits breast cancer cells migration and invasion through Wnt and epithelial-mesenchymal transition signaling pathways. *Chin J Cancer Res* 2020;32(3):347-360. doi: 10.21147/j.issn.1000-9604.2020.03.06



**Figure S1** Validation of effect of SFRP5 by using isotype-matched IgG antibody, as control for anti-SFRP5 antibody. Migration and invasion assays were further performed with isotype-matched IgG2B antibody as the control for anti-SFRP5 antibody. Co-cultured cells were incubated with or without 300 ng/mL SFRP5 recombinant protein in adipocyte CM treated with PA. Equal amounts of SFRP5 antibody (anti-SFRP5) or IgG were added in the presence of SFRP5 protein. After 24 h, cells that migrated across the membrane were fixed and examined by crystal violet staining. The results are reported as mean numbers of stained cells in five random views from three independent experiments (200×). The data were presented as  $\bar{x} \pm s$ . SFRP5, secreted frizzled-related protein 5; CM, conditioned medium; PA, palmitic acid; \*\*,  $P < 0.01$ .