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Original Article

Insulin receptor is implicated in triple-negative breast cancer by decreasing cell mobility

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Abstract

Triple-negative breast cancer (TNBC) has a poor prognosis and typically earlier onset of metastasis in comparison with other breast cancer subtypes. It has been reported that insulin receptor (INSR) is downregulated in TNBC, however, its clinical significance and functions in TNBC remain to be elucidated. In this study, we found that INSR expression was significantly downregulated in TNBC, and overexpression of INSR suppressed cell migration and invasion in TNBC. In addition, the survival rate of breast cancer patients with low INSR expression was lower than that of patients with high INSR expression. INSR expression was significantly correlated with lymph node metastasis, clinical tumor stages, ER status, PR status, and the proliferation index Ki-67 expression. In summary, our study suggests that INSR may serve as a biomarker for breast cancer prognosis and it may be a potential target for TNBC treatment.

Keywords: insulin receptor, triple-negative breast cancer, migration, invasion, prognosis

Introduction

Triple-negative breast cancer (TNBC) represents approximately 10% to 15% of all breast cancers and is a highly aggressive subtype of tumors that lack estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) gene amplification[1–2]. Although the prognosis of most breast cancer is greatly improved due to earlier detection and application of new treatment approaches, such as target therapy, the prognosis of TNBC remains poor[3–4]. In comparison with other breast cancer subtypes, TNBC has an earlier onset of metastasis. Currently, no effective marker is available to serve as a therapeutic target for the systemic treatment of TNBC[5–6].

Insulin receptor (INSR) is a dimeric protein that plays important roles in controlling glucose homeostasis, regulating the metabolism of lipid, protein, and carbohydrate[7–8]. INSR dysfunctions have been involved in many diseases, including diabetes, cancer, and Alzheimer's disease[8–9]. INSR can be activated by insulin or insulin-like growth factor II, which plays a key role in the regulation of growth and metabolism as well as in the initiation and maintenance of breast tumors[10]. It has been reported

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that INSR is downregulated in TNBC[11]. However, the roles and clinical significance of INSR in TNBC are still not well known.

In this study, we investigated the clinical significance of INSR expression in TNBC and breast cancer through database and tissue microarray analysis. We found that the expression of INSR was significantly downregulated in TNBC. Wound healing and transwell assays indicated that overexpression of INSR significantly inhibited cell migration and invasion in TNBC. Moreover, we found that breast cancer patients with higher INSR expression had a longer survival time than patients with lower INSR expression. These results suggest that INSR may be a potential prognostic and therapeutic target for TNBC.

Materials and methods

Cell culture and transfection

Human TNBC cell line MDA-MB-231 was purchased from American Type Culture Collection (USA). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Invitrogen, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were authenticated by STR profiling and tested for mycoplasma contamination. The INSR expression plasmid pCMV3-INSR-Flag and empty vector were from Sino Biological (China). Cells were plated in 6-well plates one day before transfection. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Total RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen), and complementary DNA was synthesized from 1 μg of RNA using the PrimeScript RT Reagent (Takara, Japan) following the manufacturer's instructions. The quantitative RT-PCR (qRT-PCR) was performed using FastStart Universal SYBR Green Master (Yeasen, China) in a Roche LightCycler 96 qRT-PCR System. The mRNA expression values were normalized to that of the *ACTB* gene. The primer sequences were listed in ***Table 1***.

Table 1 **Primers for quantitative RT-PCR**

Primer name	Primer sequence 5′→3′
Human-*INSR*-Forward primer	GAACTACAGCGTGCGAATCC
Human-*INSR*-Reverse primer	GAAAGACAAAGATGAGGGGG
Human-*ACTB*-Forward primer	AGATGTGATCAGCAAGCAG
Human-*ACTB*-Reverse primer	GCGCAAGTTAGGTTTTGTCA

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and harvested in radioimmunoprecipitation assay buffer (Beyotime, China) supplemented with a protease inhibitor cocktail (Beyotime). Equal amounts of protein were loaded on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membrane was probed with primary antibodies overnight at 4 °C and then incubated with secondary antibodies at room temperature for 1 hour. Immunoblots were developed with the enhanced chemiluminescence Western blotting substrate kit (Millipore). The following primary antibodies were used: β-actin (Cat. No. bs-0061R, Bioss, China; 1:2000 dilution) and INSR (Cat. No. ab137747, Abcam, UK; 1:500 dilution).

Immunohistochemical assay

Tumor tissue sections were deparaffinized with 100% xylene, followed by rehydration using gradient ethanol (100%, 95%, 70%, 30%, and 0%). Antigen retrieval was performed by microwaving the slides in citrate buffer for 15 minutes after endogenous peroxidase activity was blocked with 3% H_2O_2/methanol for 10 minutes. After blocking with 3% BSA, the tumor tissue sections were incubated with primary antibodies against INSR (Cat. No. ab137747, Abcam; 1:100 dilution), and then with HRP-labeled anti-rabbit IgG antibody (Cat. No. SA00001-2, Proteintech, USA; 1:500 dilution). The specimens were counterstained with hematoxylin. Both positive and negative controls were performed throughout the procedure.

INSR immunostaining was analyzed by evaluation of the percentage of stained cells and staining intensity, allowing assessment of H-score performed independently by two pathologists who were blinded to the clinical data. For each sample, 10 high power fields (400×) were assessed. Staining intensity on a scale of 0 to 3 (0=negative, 1=weak, 2=moderate, 3=strong) was determined for the cells in each field[12]. H-scores were calculated as the sum of the percentage of cells at each intensity multiplied by staining intensity, with a range from 0 to 300[12].

Human tissue microarray

The breast cancer tissue microarrays containing 63 cases of TNBC tissue samples (Cat. No. HBreD075Bc01) and 63 cases of non-TNBC tissue samples (Cat. No. HBreD080CS01) were purchased from Outdo Biotech (China). None of the patients had

received preoperative radiotherapy or chemotherapy. All pathologic diagnoses were included with the manufacturer's instructions.

All clinicopathological parameters of tissue microarrays were shown in ***Table 2***. The age range was 27 to 91 years in the total patient cohort with a median age of 58 years. In total, 112 tumors were diagnosed as invasive ductal carcinomas and 2 carcinomas were classified as invasive lobular subtype. Special subtypes such as medullary carcinoma, secretory carcinoma and others were diagnosed in a separate category, which contained 12 cases. This information was provided by the pathology report.

Grading was available in 126 cases according to the modified Bloom-Richardson grading system. Of the 126 cases, 64 cases were poorly differentiated (G3: TNBC, *n*=38; non-TNBC, *n*=26), 57 cases were moderately differentiated (G2: TNBC, *n*=21; non-TNBC, *n*=36) and 5 cases were well-differentiated (G1: TNBC, *n*=4; non-TNBC, *n*=1).

Wound healing assay

Cells were seeded in 6-well plates and grown to 90% confluence, and then a linear wound was scratched in the cell monolayer with a pipette tip. Separated cells were washed out using PBS. Wounded cultures were incubated in a serum-free medium, and the edges of the scratch were photographed. Random migration was evaluated by measuring the area of occupancy with Image-Pro Plus (Media Cybernetics, USA).

Table 2 **Clinicopathological parameters of the breast cancer cohort**

Parameters	TNBC (*n*)	Non-TNBC (*n*)
Histological type		
Invasive-ductal	56	56
Invasive-lobular	0	2
Other	7	5
Grading*		
G1	4	1
G2	21	36
G3	38	26
pT stage		
pT1	22	10
pT2	36	43
pT3	2	8
pT4	3	2
Nodal status		
pN0	59	21
pN+	4	42

*Tumors were graded according to the modified Bloom-Richardson grading system. TNBC: triple-negative breast cancer.

Transwell migration and invasion assays

Transwell chambers (BD Biosciences, USA) were coated (for invasion assays) or uncoated (for migration assays) with Matrigel (BD Biosciences). A total of 2×10^3 cells were plated onto the upper chamber for migration assays, and 5×10^3 cells were added to the upper chamber for invasion assays. The total culture medium containing 10% FBS was added to the lower chamber. After incubation for 16 or 24 hours, cells that had migrated and invaded through the membrane to the lower surface were fixed with methanol and stained with crystal violet. The stained cells were photographed and counted under light microscopy in four randomly selected fields per membrane.

Bioinformatics analysis

The UALCAN online database was used to analyze INSR expression in breast cancer (UALCAN, http://ualcan.path.uab.edu/index.html)[13]. A total of 1211 tissue samples were collected and 114 of them were derived from paired normal tissues. The Kaplan-Meier plotter database was used to predict survival value. The KM plotter is available at: http://kmplot.com/analysis/index.php?p=background[14].

Statistical analysis

Statistical significance was calculated by two-tailed Student's *t*-test, Chi-square test accordingly. Data are presented as mean±SD. Graphpad Prism 6 (GraphPad Software, USA) and SPSS 19.0 (SPSS Software, USA) were used to generate graphs and statistical analyses.

Ethics statement

All participants provided written informed consent before participating in the study. The ethics committee of Outdo Biotech approved this study.

Results

INSR expression was associated with clinico-pathologic grading in breast cancer

Correlation between INSR and histopathological parameters from tissue microarrays were shown in ***Table 3***. As shown in ***Table 3***, INSR expression was

significantly correlated with lymph node metastasis (LNM), clinical tumor stages, ER status, PR status, and the proliferation index Ki-67 expression in breast cancer. However, there was no obvious correlation between INSR expression and patients' age, tumor size, or HER2 status (***Table 3***).

INSR expression is correlated with prognostic in breast cancer patients

To investigate the prognostic value of INSR expression in breast cancer, we analyzed overall survival of breast cancer patients using the Kaplan Meier plotter database. As shown in ***Fig. 1***, breast cancer patients with lower INSR expression had a poorer survival rate than patients with higher INSR expression (hazard ratio =0.66, log-rank $P<0.05$), suggesting that INSR may be a positive prognostic factor for breast cancer.

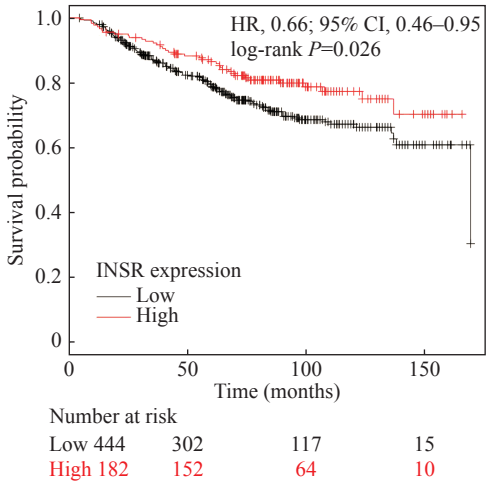

Fig. 1 **Association of INSR expression with breast cancer patients' survival.** Bioinformatic analysis showed that patients with higher INSR expression had a longer survival time than patients with lower INSR expression ($P=0.026$). The Kaplan Meier plotter database was used to estimate survival time, and the log-rank test was performed to analyze survival differences. INSR: insulin receptor; HR: hazard ratio; CI: confidence interval.

Table 3 **Correlation of INSR expression with clinicopathological characteristics in BRCA patients**

Characteristics	INSR			*P*-value
	Weak	Moderate	Strong	
Age (years)				0.558
≥50	56	25	13	
<50	17	8	7	
Tumor size (cm)				0.197
≥2	60	26	19	
<2	16	4	1	
LNM status				5.051E−6****
Positive	15	17	14	
Negative	61	13	6	
Clinical tumor stages				0.026*
Ⅰ–Ⅱ	29	20	13	
Ⅲ	46	11	7	
ER				2.819E−4***
Positive	3	7	7	
Negative	73	23	13	
PR				2.818E−6****
Positive	5	7	11	
Negative	71	23	9	
HER2				0.319
Positive	4	3	3	
Negative	72	27	17	
Ki-67				1.237E−4***
High	53	11	5	
Low	23	19	15	

Statistical significance was calculated by Chi-square test. *$P<0.05$, ***$P<0.001$, ****$P<0.0001$. INSR: insulin receptor; LNM: lymph node metastasis; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; Ki-67: proliferation index with a cut off value of 14%.

INSR expression was significantly downregulated in TNBC

We analyzed the Cancer Genome Atlas (TCGA) transcriptome data using the UALCAN database. A total of 1211 tissue samples were collected and 114 of them were derived from paired normal tissues. The results showed that the expression of INSR was significantly downregulated in breast cancer compared with that in normal tissues (***Fig. 2A***).

Further, INSR was detected by immunohistochemistry analysis using tissue microarrays which contained 63 TNBC tissues and 63 non-TNBC tissues. The results showed that INSR expression was markedly downregulated in TNBC tissues compared with that in non-TNBC tissues (***Fig. 2B–D***). Consistent with the immunohistochemistry result, the UALCAN database analysis showed that INSR was also highly expressed in non-TNBC tissues, and weakly expressed in TNBC tissues (***Fig. 2E***).

INSR suppressed TNBC cell migration and invasion

To investigate the biological functions of INSR in TNBC, we performed wound healing, transwell migration, and invasion assays in MDA-MB-231 cells. As shown in ***Fig. 3A***, INSR was markedly upregulated in MDA-MB-231 cells transfected with

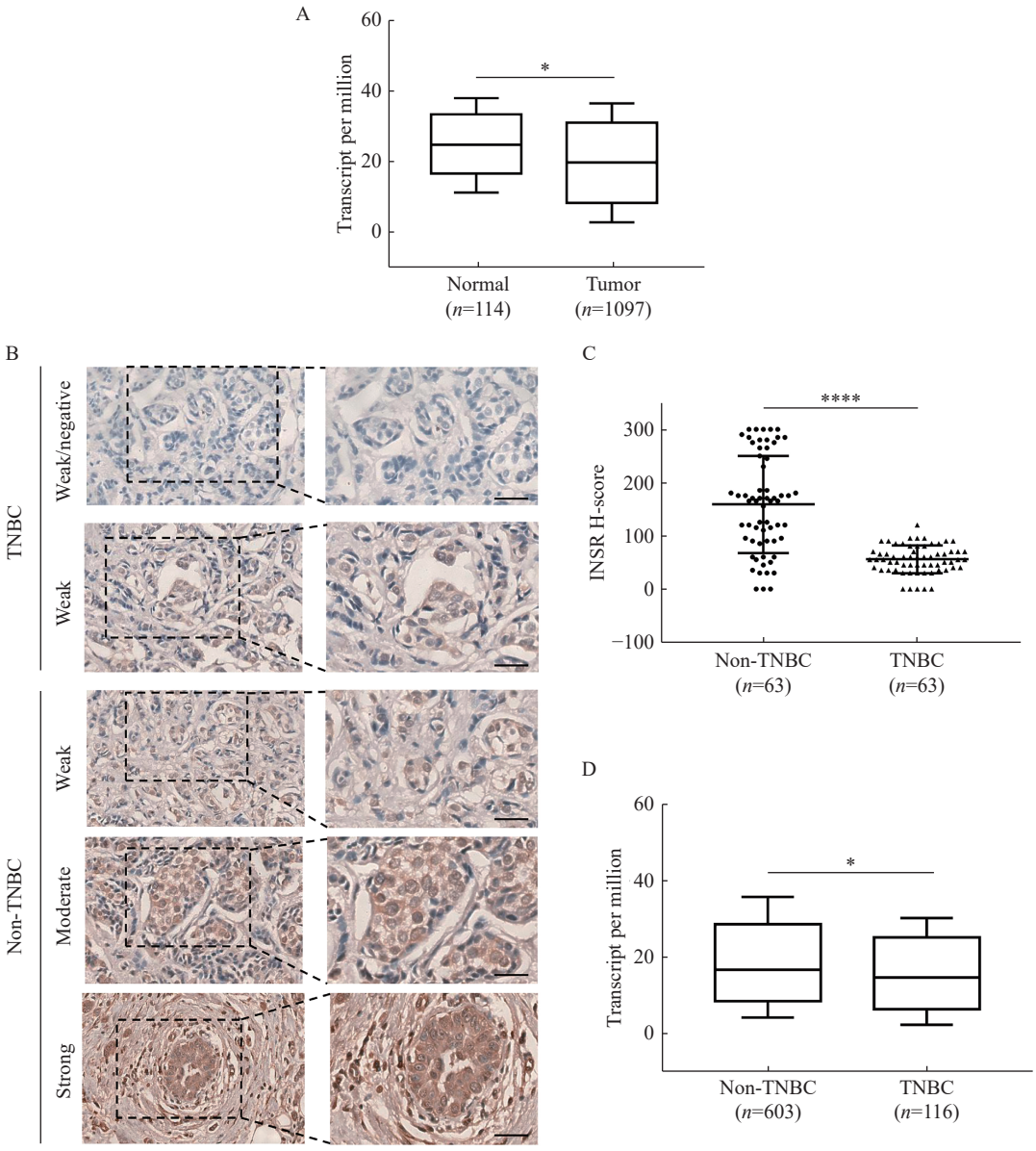

Fig. 2 **INSR was significantly downregulated in TNBC and correlated with breast cancer progression.** A: Relative expression of INSR in breast cancer tissues (tumor) compared with non-tumor samples (normal) was analyzed using the UALCAN database and TCGA transcriptome data. B: Representative pictures of immunohistochemical analysis of INSR expression using tissue microarrays. Scale bar, 20 μm. C: H-score of INSR according to intensity of the staining multiplied by the percentage of positive cells. D: Expression of INSR in TNBC and non-TNBC tissues were analyzed from the UALCAN database using TCGA transcriptome data. Data are presented as mean±SD. Comparisons between two groups were analyzed using a two-sided Student's *t*-test. $^{*}P<0.05$, $^{****}P<0.0001$. TNBC: triple-negative breast cancer; INSR: insulin receptor.

INSR expression plasmids. INSR overexpression significantly decreased the migratory and invasive abilities of MDA-MB-231 cells (***Fig. 3B*** and ***C***). These results suggest that INSR may serve as a suppressor in TNBC metastasis.

Discussion

Over the last decades, intensive efforts have been made to explore the underlying mechanisms of TNBC and identify therapeutic targets[15–16]. It is widely accepted that dysregulation of gene expression[17], copy number variation[18–19], and DNA methylation are involved in the initiation and progression of TNBC[20–21]. Multiple molecules have been identified as biomarkers and therapeutic targets for TNBC. Ectopic expression and therapeutic delivery of the secreted protein tubulointerstitial nephritis antigen-like 1 (Tinagl1) suppress TNBC progression and metastasis through direct binding to integrin α5β1, αvβ1, and epidermal growth factor receptor (EGFR), and subsequent simultaneous inhibition of focal

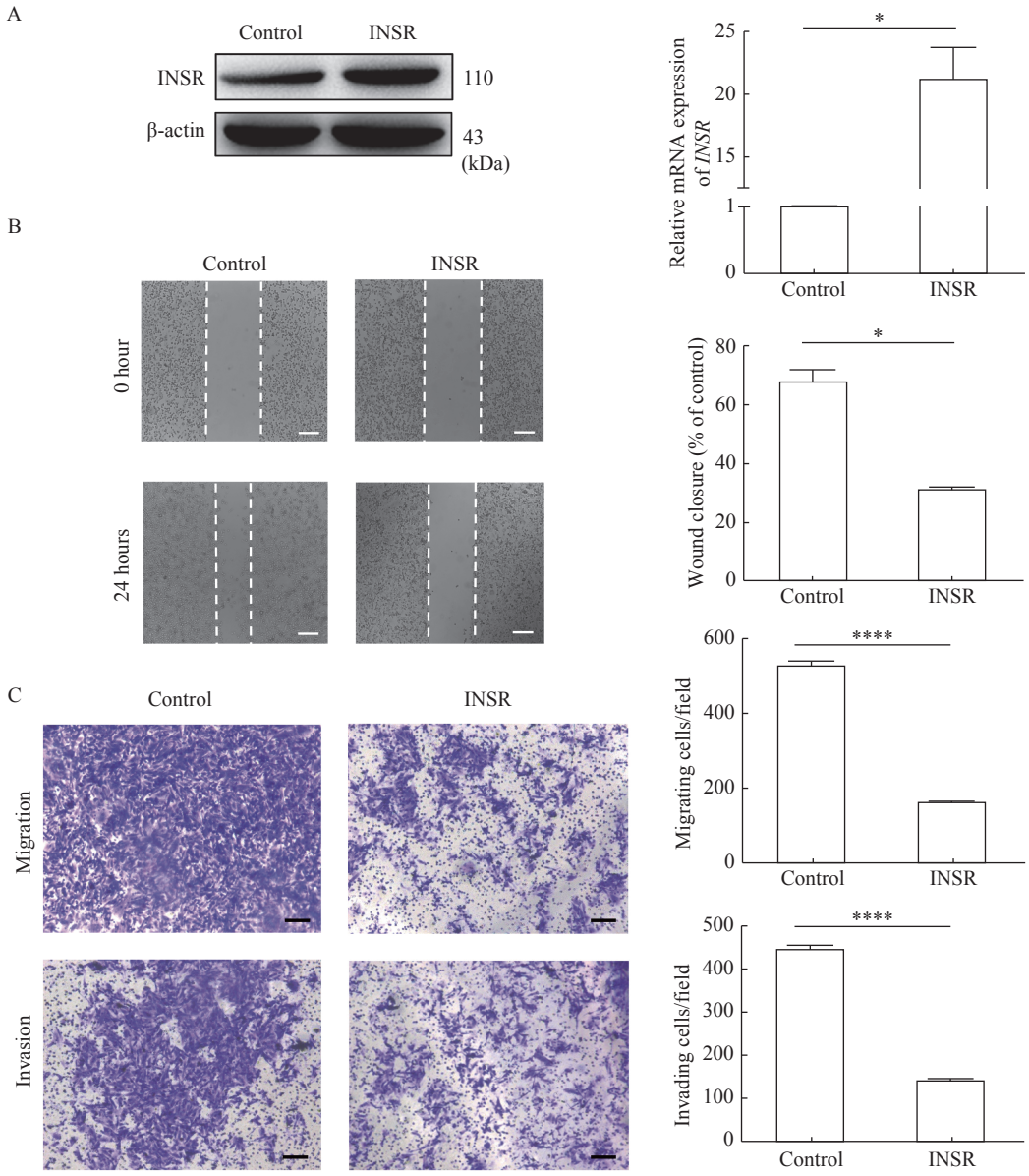

Fig. 3 **INSR suppressed TNBC cell migration and invasion.** A: qRT-PCR and Western blotting detection of INSR expression levels in MDA-MB-231 cells transfected with pCMV3-INSR-Flag plasmids (Control: overexpression control; INSR: overexpression of INSR). B: Wound healing assay of the migration abilities of INSR overexpressing MDA-MB-231 cells. Scale bar, 200 μm. C: Transwell assay of the migratory and invasive abilities of INSR overexpressing MDA-MB-231 cells. The representative images of migrated and invaded cells 24 hours after seeding. Scale bar, 100 μm. Data obtained from three independent experiments are presented as mean±SD. Comparisons between two groups were analyzed using a two-sided Student's *t*-test. $^{*}P<0.05$, $^{****}P<0.0001$. INSR: insulin receptor.

adhesion kinase (FAK) and EGFR signaling pathways. Moreover, Tinagl1 protein level is associated with good prognosis and reversely correlates with FAK and EGFR activation status in TNBC[22]. GGNBP2 expression is decreased in TNBC tissues and is associated with the prognosis of breast cancer patients. Furthermore, overexpression of GGNBP2 in TNBC cells suppresses cell proliferation, migration and invasion, reduces the cancer stem cell subpopulation, and promotes cell apoptosis *in vitro* as well as inhibits tumor growth *in vivo*[23]. Recent evidence from clinical trials and preclinical studies has demonstrated a pivotal role of signal transducer and activator of transcription 3 (STAT3) in the initiation, progression, metastasis, and immune evasion of TNBC. STAT3 is overexpressed and constitutively activated in TNBC cells and contributes to cell survival, proliferation, cell cycle progression, anti-apoptosis, migration, invasion, angiogenesis, chemoresistance, immunosuppression, and stem cells self-renewal and differentiation[24]. In this study, we found that INSR expression is significantly downregulated in TNBC tissues compared with that in non-TNBC tissues. In addition, INSR expression was

markedly downregulated in breast cancer tissues compared with that in normal tissues. breast cancer patients with lower INSR expression had a poorer survival rate than patients with higher INSR expression. Together, these results suggest that INSR may serve as a biomarker for breast cancer prognosis and it may be involved in the progression of TNBC.

The insulin-like growth factor (IGF) system is composed of three receptors (INSR, IGF-1 receptor and mannose 6-phosphate receptor [M6P/IGF-2R]), three ligands (insulin, IGF-1, IGF-2), and six known types of circulating IGF-binding proteins (IGFBP1–6) that modulate the bioavailability and bioactivity of the IGFs[25]. IGF-1 signaling is involved in glycolysis[26] and IGF-1/PI3K signaling promotes both mitochondrial biogenesis and mitophagy[27]. Moreover, mutations in *INSR* gene have been found in insulin resistance as well as in several types of obesity-related invasive cancers, such as colorectal cancer, pancreatic cancer, liver cancer and breast cancer[9,28–29]. It has been reported that INSR expression in TNBC tissues is lower than that in normal tissues[11]. However, its biological functions in TNBC are still unclear. In this study, we demonstrated that overexpression of INSR in MDA-MB-231 cells decreased the migration and invasive abilities of the cells. Therefore, INSR may exert an inhibitory function in the metastasis of TNBC. The precise molecular mechanisms by which INSR suppresses TNBC metastasis needs to be further explored.

In summary, we found that INSR expression was significantly downregulated in TNBC patients. Overexpression of INSR markedly inhibited the migration and invasion of MDA-MB-231 cells. Moreover, INSR expression was significantly correlated with tumor size in non-TNBC patients. We found that INSR expression was significantly correlated with LNM, clinical tumor stages, ER status, PR status, and the proliferation index Ki-67 expression in breast cancer. Importantly, breast cancer patients with lower INSR expression had a poorer survival rate than patients with higher INSR expression. These data indicate that INSR may serve as a biomarker for breast cancer prognosis and it may be a potential target for TNBC treatment.

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