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Reduced lncRNA Aim enhances the malignant invasion of triple-negative breast cancer cells mainly by activating Wnt/β-catenin/mTOR/PI3K signaling

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Increasing evidence has suggested the important role of lncRNAs in the progression of triple-negative breast cancer (TNBC). In the current study, we first demonstrated that the expression of Airn was reduced in TNBC tissues and cells. Our data showed that the level of Airn was reduced in TNBC tissues and cell lines compared with that of normal control. Furthermore, silencing of Airn markedly enhanced MDA-MB-231 cell migration. Meanwhile, knockdown of Airn significantly increased MDA-MB-231 cell invasion. Western blot analysis showed that knockdown of Airn markedly enhanced the activation of Wnt/β-catenin/mTOR/PI3K in both MDA-MB-231 cells. Moreover, real time PCR analysis showed that the mRNA level of IGF2R was significantly enhanced when Airn was silenced in MDA-MB-231 cells. In addition, overexpression of IGF2R significantly increased MDA-MB-231 cell migration and invasion. To further explore whether Airn activated Wnt/β-catenin/mTOR/PI3K signaling independent of IGF-2R, a specific siRNA targeting IGF2R was selected. Western blot analysis showed that Wnt/β-catenin/mTOR/PI3K signaling could be largely activated in MDA-MB-231 cells transfected with siRNA targeting Airn, even when the protein level of IGF2R was silenced. In summary, decreased expression of lncRNA Aim enhanced the malignant invasion of triple-negative breast cancer cells mainly by activating Wnt/β-catenin/mTOR/PI3K signaling independent of Igf2R.

1. Introduction

Triple-negative breast cancer (TNBC) cells are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) negative, which leads to early recurrence, visceral metastasis and poor prognosis (Bilir et al. 2013; Ma et al. 2017). Therefore, the search for TNBC pathological molecular mechanisms is important.

The human genome project has shown that only 2% of RNAs are transcribed into proteins, such as mRNA (Augoff et al. 2012), the remaining 98% are non-coding RNAs (ncRNA). According to the different length of the transcripts, ncRNAs can be divided into short chain and long chain non-coding RNAs (LncRNA) (Augoff et al. 2012; Jiang et al. 2016). LncRNA is a transcript with longer than 200 bp nucleotides, which was previously mistaken for the "noise" of gene transcription (Lin et al. 2016). However, recent studies show that lncRNA could regulate gene expression at genetic, transcriptional and post transcriptional regulation levels (Jiang et al. 2016; Lin et al. 2016; Wang et al. 2017). Many biological processes, such as embryonic development, cell proliferation, differentiation, apoptosis, and various diseases including the occurrence and development of diabetes, are closely related to the abnormal expression of lncRNAs (Jiang et al. 2016; Lin et al. 2016; Wang et al. 2017). Therefore, an in-depth study of lncRNA will help to reveal the mechanism of lncRNA in TNBC, so as to provide new ideas for the treatment of TNBC.

Antisense to IGF-2R non-coding RNA (Aim), is a IGF-2 receptor mRNA antisense non imprinted gene encoding RNA (Marcho et al. 2015). Its transcription overlaps the transcription of IGF-2R in a reverse direction and induces silencing of imprinted gene IGF-2 receptor (Farmer et al. 2013). Previous results suggest that lncRNAs may play an important role in the proliferation of pancreatic islet B cells and the pathophysiology of diabetes mellitus (Latos et al. 2012; Santoro et al. 2013). However, the role of Airn in the progression of TNBC is largely unknown so far. In the current study, we first demonstrated that the expression of Airn was reduced in TNBC tissues and cells. Further study indicated that Airn enhanced the malignancies of TNBC mainly by triggering Wnt/β-catenin/mTOR/PI3K signaling.

2. Investigations, results and discussion

2.1. Decreased level of Airn in the TNBC tissues and breast cancer cells

Firstly, we evaluated the expression of Airn in the TNBC tissues and breast cancer cells. Compared with normal control, the level of Airn was significantly decreased in TNBC tissue (Fig. 1A). Meanwhile, the level of Airn was significantly reduced in the BT549, MDA-MB-231, HCC70 cells compared to that of MCF10A cells (Fig. 1B).

2.2. Reduced Airn enhanced MDA-MB-231 cell migration and invasion

Then, two specific siRNAs targeting Airn were selected and real time PCR analysis demonstrated that the siRNAs could markedly suppress the expression of Airn in MDA-MB-231 cells (Fig. 2A). To further explore the effects of Airn on breast cancer cell migration and invasion, HCC70 and MCF-7 cells were transfected with siRNAs targeting Airn. As shown in Fig. 2B, silencing of Airn markedly enhanced MDA-MB-231 cell migration and invasion. Furthermore, we constructed adenovirus vectors overexpressing Airn. Real time PCR analysis validated that the level of Airn was significantly enhanced after transfection of Ad-Airn into MDA-MB-231 cells for 48 h (Fig. 2C). In contrast, overexpression

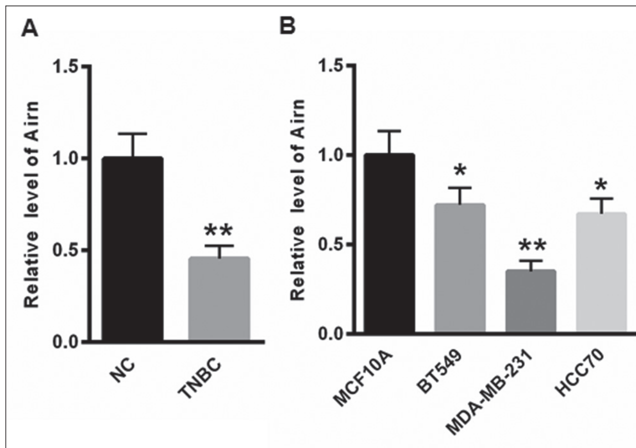

Fig. 1: The level of Airn was reduced in the TNBC tissues and breast cancer cells. (A) Compared with normal control, the level of Airn was significantly decreased in the level of TNBC tissues. (B) The level of Airn was significantly reduced in the BT549, MDA-MB-231, HCC70 cells than that of MCF10A cells. *P<0.05, **P<0.01.

of Airn significantly increased MDA-MB-231 cell migration and invasion (Fig. 2D). These data indicated a tumor suppressor role of Airn in breast cancer cell lines.

2.3. Airn suppressed the activation of Wnt/β-catenin/mTOR/PI3K

To evaluate the potential role of Airn in breast cancer cell malignancies, we evaluated the activation of Wnt/β-catenin/mTOR/PI3K. Western blot analysis showed that knockdown of Airn markedly enhanced the activation of Wnt/β-catenin/mTOR/PI3K in both MDA-MB-231 cells.

2.4. Airn activated Wnt/β-catenin/mTOR/PI3K signaling independent of IGF-2R

Previous studies have shown that Airn mainly serves as an antisense lncRNA against IGF-2R. Then, we evaluated the effects of Airn on IGF-2R expression (Marcho et al. 2015). Real time PCR analysis showed that the mRNA level of IGF2R was significantly enhanced when Airn was silenced in MDA-MB-231 cells (Fig. 4A). In addition, overexpression of IGF2R significantly increased MDA-MB-231 cell migration and invasion (Fig. 4B). To further explore whether Airn activated Wnt/β-catenin/mTOR/PI3K signaling independent of IGF-2R, a specific siRNA targeting Airn was selected (Fig. 4C). Western blot analysis showed that Wnt/β-catenin/mTOR/PI3K signaling could be largely activated in MDA-MB-231 cells transfected with siRNA targeting Airn, even when the protein level of IGF2R was silenced (Fig. 4D). Together, these data showed that Airn activated Wnt/β-catenin/mTOR/PI3K signaling independent of IGF-2R.

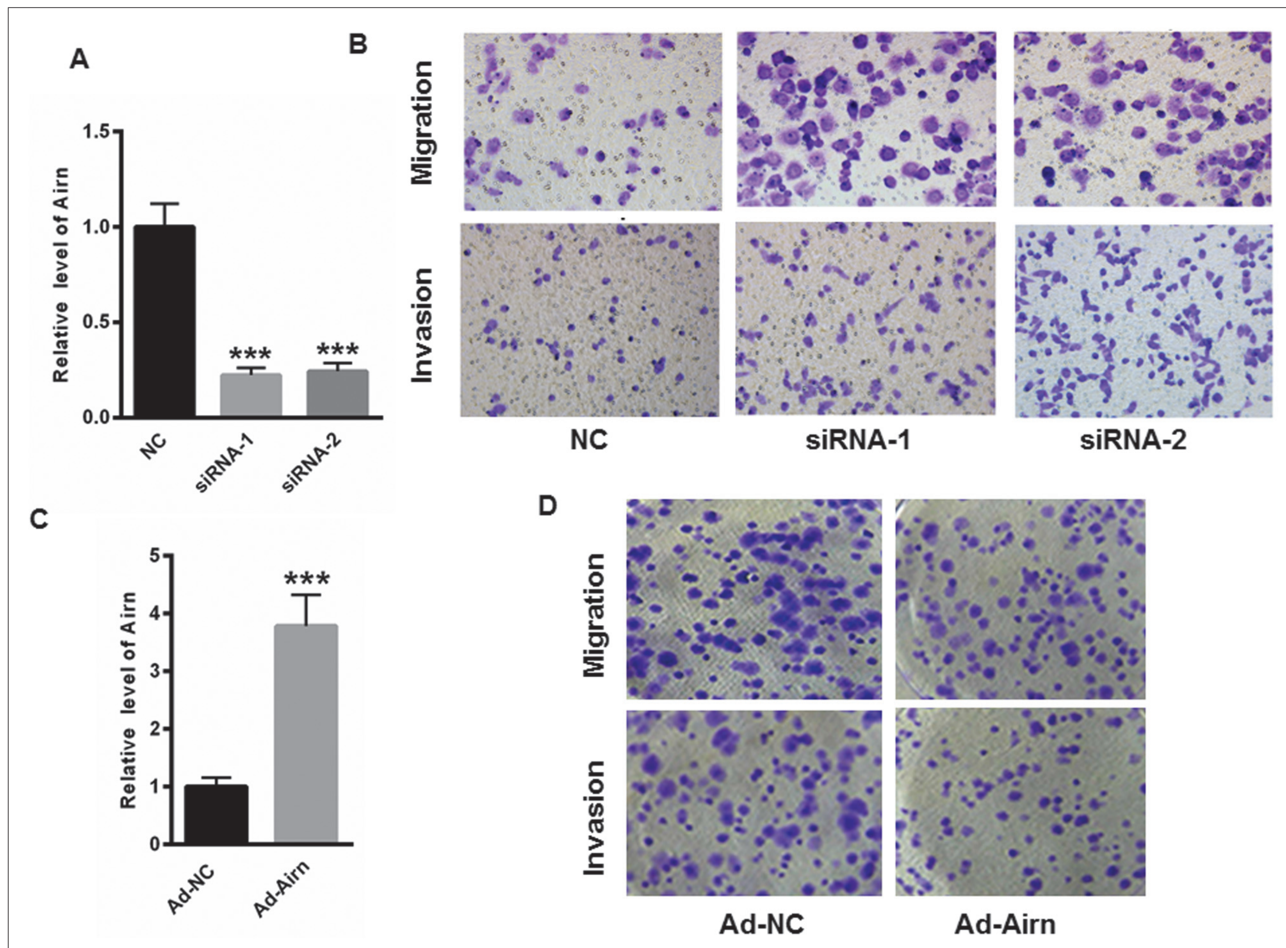

Fig. 2: Reduced Airn enhanced MDA-MB-231 cell migration and invasion. (A) Real time PCR analysis demonstrated that the siRNAs could markedly suppressed the expression of Airn in MDA-MB-231 cells. (B) Silencing of Airn markedly enhanced MDA-MB-231 cell migration and invasion. (C) Real time PCR analysis validated that the level of Airn was significantly enhanced after transfection of Ad-Airn into MDA-MB-231 cells for 48h. (D) Overexpression of Airn significantly increased MDA-MB-231 cell migration and invasion. *P<0.05, **P<0.01.

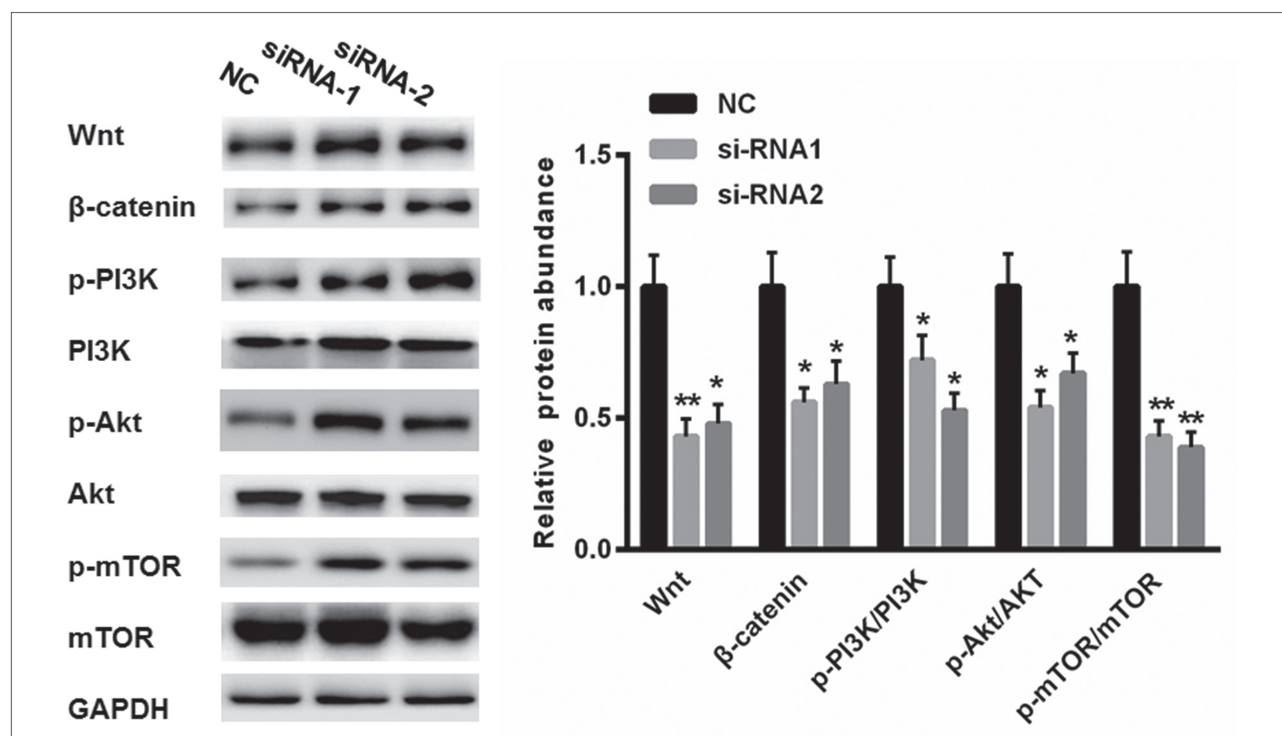

Fig. 3: Knockdown of Airn markedly enhanced the activation of Wnt/β-catenin/mTOR/PI3K in both MDA-MB-231 cells. *P<0.05, **P<0.01.

TNBC is a breast cancer characterized by the loss of ER, PR and HER2 expression (Taipaleenmaki et al. 2016). Although the treatment has made great progress, the prognosis of patients is characterized by poor prognosis, high recurrence and low overall survival rate (Yang et al. 2014; Yin et al. 2016). TNBC accounts for about 15% of all breast cancers, and its biological characteristics are similar to those of basal like breast cancer, but there are some differences in gene expression and immunophenotype (Sulaiman et al. 2016; Taipaleenmaki et al. 2016). Therefore, it is an urgent problem to find and develop effective targets for TNBC.

The insulin-like growth factor type 2 receptor (IGF2R) modulates fetal growth by removing IGF2 from circulation (Latos et al. 2012; Santoro et al. 2013). And the expression of the Igf2r gene is only imprinted after implantation and is correlated with expression of the antisense non-coding (nc)RNA, Airn (Santoro and Pauler 2013). However, Airn has hardly been studied in TNBC. For the first time, our data indicated that the level of Airn was reduced in TNBC tissues and cell lines compared with that of normal control. Furthermore, silencing of Airn markedly enhanced MDA-MB-231 cell migration. Meanwhile, knockdown of Airn significantly increased MDA-MB-231 cell invasion. These data indicated a tumor suppressor role of Airn in TNBC cells.

The Wnt/β-catenin pathway plays an important role in the regulation of cell proliferation, metastasis, differentiation and embryonic development (Dey et al. 2013; Dey et al. 2013). Studies have shown that Wnt/ beta -catenin is highly expressed in TNBC cells, and the prognosis of TNBC is related to the Wnt/ beta -catenin pathway (King et al. 2012; Koval et al. 2014). Another study showed that the occurrence and development of Wnt is closely related to the development of TNBC (Dey et al. 2013; Bernemann et al. 2014). Glycogen synthase kinase 3 beta (GSK3β) interacts with tuberous sclerosis complex 2 (TSC2) interacting proteins, thereby connecting Wnt/ beta -catenin and mTOR signal pathway (Koval et al. 2014; Ma et al. 2017). Recent studies have shown that Wnt/beta -catenin and PI3K pathways interact with each other to increase the proliferation of colorectal cancer cells and enhance the resistance of tumor cells to targeted drugs (Shrivastava et al. 2016; Sulaiman et al. 2016). Then, the signaling cascade triggers the malignancies of TNBC. Therefore, how to inhibit the Wnt/β-catenin/mTOR/PI3K signaling pathway is widely investigated, since a single treatment cannot effectively inhibit the proliferation and metastasis of cancer cells (Szuplewski et al. 2017; Tokunaga et al. 2017). In this study, we found that Airn suppressed the activation of Wnt/β-catenin/mTOR/PI3K signaling independent of Igf2R. In contrast, decreased levels of Airn activated Wnt/β-catenin/mTOR/PI3K signaling, thereby enhancing the malignancies of TNBC. This study shows that Airn regulates Wnt/β-catenin/mTOR/PI3K signaling, thereby enhancing TNBC cell proliferation and inducing HCC70 cell resistance.

LncRNA is one of the hot spots in the field of biomedical research, and its important function in cells has been widely recognized (Cui et al. 2017; Liang et al. 2017; Shan et al. 2017). Currently, the function of lncRNA is in the initial stage of breast cancer and the current data is only the tip of the iceberg. There are more lncRNAs functions that need to be excavated. With updated research and development technology, to identify a high tissue specific lncRNA is expected to become a new drug target for the treatment of TNBC.

3. Experimental

3.1. Patient selection

This study was approved by the Hongqi Hospital of Mudanjiang Medical College Review Board. Informed patient consent was obtained from all the participants. Triple negative breast cancer was defined as invasive breast carcinoma with ER and PR staining in less than 1% of the tumor cells by immunohistochemistry and no HER2 overexpression [defined as negative (0 to 1+) or equivocal (2+) staining by immunohistochemistry and no amplification by fluorescence *in situ* hybridization], in accordance with the American Society of Clinical Oncology/College of American Pathologists guidelines (Hammond et al. 2010). Patients receiving neoadjuvant chemotherapy were excluded from the study.

3.2. Cell culture

Three negative breast cancer cell lines BT549, MDA-MB-231, HCC70 cells and human normal breast epithelial cell line MCF10A was purchased from American Type Culture Collection (ATCC, Manassas, VA). BT549, MDA-MB-231, MCF10A cells were cultured in RPMI 1640 medium (Gibco, USA), SKOV3 cells were cultured in McCoy's 5A medium (Gibco, USA). Both media were supplemented with 10% fetal bovine serum (FBS, Gibco, Los Angeles, CA, USA). All cell lines were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were trypsinized with trypsin/EDTA and medium was changed twice a week.

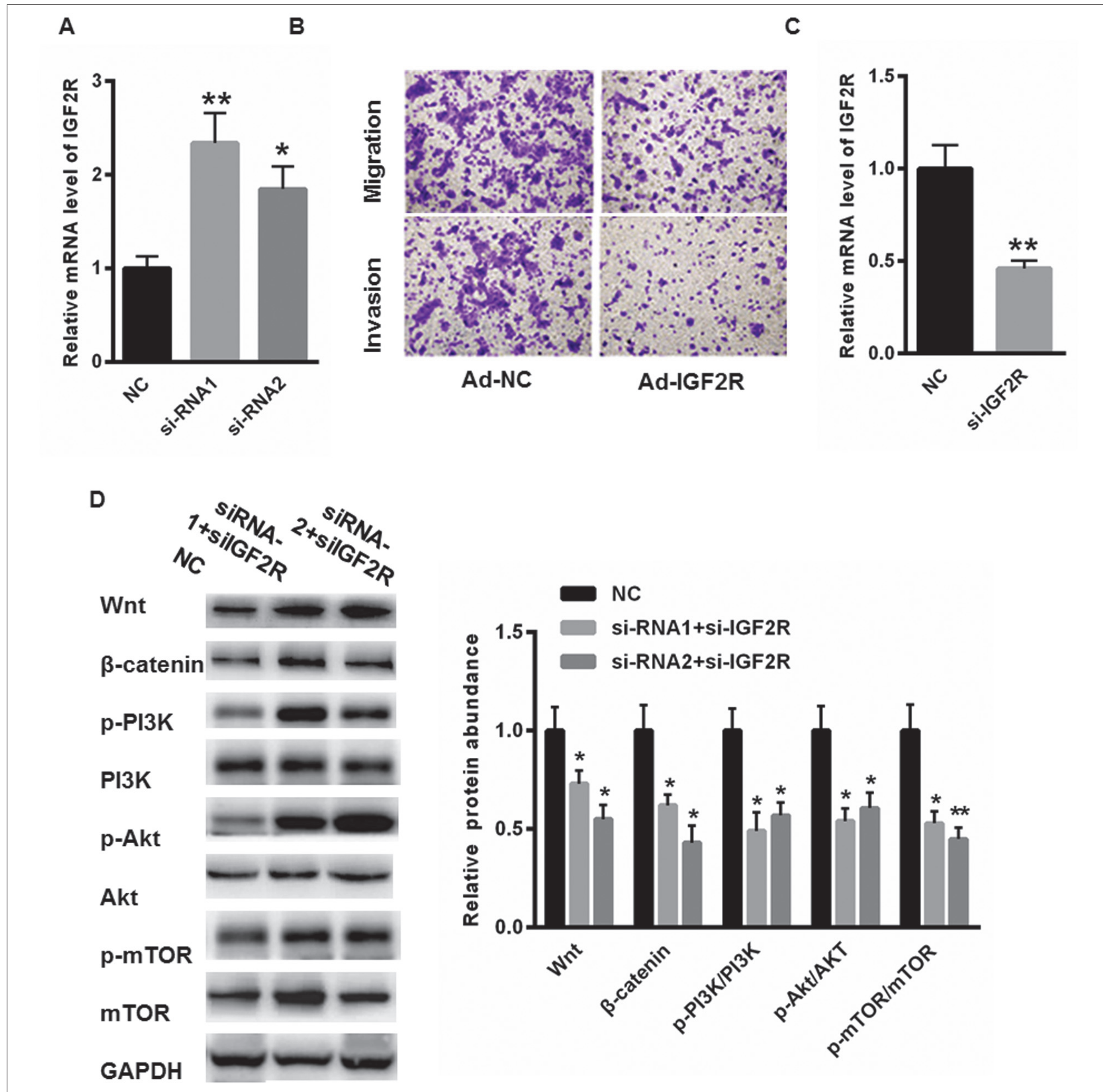

Fig. 4: Airn activated Wnt/β-catenin/mTOR/PI3K signaling independent of IGF-2R. (A) Real time PCR analysis showed that the mRNA level of IGF2R was significantly enhanced when Airn was silenced in MDA-MB-231 cells. (B) Overexpression of IGF2R significantly increased MDA-MB-231 cell migration and invasion. (C) Real time PCR analysis showed that a specific siRNA targeting IGF2R was selected. (D) Wnt/β-catenin/mTOR/PI3K signaling could be largely activated in MDA-MB-231 cells transfected with siRNA targeting Airn, even when the protein level of IGF2R was silenced. *P<0.05, **P<0.01.

3.3. RNA Isolation and real-time PCR

Total RNA from cells or tissues was extracted with Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. To quantify mRNA, real-time PCR was performed as previously reported. In brief, a total of 1 μg RNA was reverse transcribed using a cDNA synthesis kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. For real-time PCR, 2 μl template cDNA was mixed with 5 μl SYBR Green Supermix, 0.4 μl forward primer, 0.4 μl reverse primer, and 2.2 μl ddH$_2$O. The cycling conditions were held as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The fluorescent signals were analyzed using an ICycler IQ5 detection system (Bio-Rad). The relative level of uc.372 was determined using the 2-delta delta Ct analysis method. β-actin was applied as the endogenous control.

3.4. Transwell migration and invasion assays

Cell migration assays were performed using Boyden chambers with 8-μm-pore filters (Corning, New York, USA). For the cell invasion assay, the upper chamber was pre-coated with matrigel (BD,USA). MDA-MB-231 cells (5×10^3) treated with ad-NC and ad-Airn in RMPI 1640 medium and McCoy's 5A medium without FBS were plated on the upper chamber respectively. 600 μL of medium with 20 % FBS as chemoattractants were plated on the lower chamber of the 24-well plates. Then cells were incubated under standard culture conditions for 48h. Non-migrating and non-invading cells in the upper chamber were completely removed by using cotton swab. Cells that migrated or invaded to the lower surface of the membrane were fixed in methanol for 30 min at 37 °C and stained with 0.5% crystal violet for 1 h. Cells were quantified by counting the number of stained nuclei in five random fields by fluorescence microscopy, in triplicate.

3.5. Construction of adenoviral vectors

Adenoviral vectors overexpressing Airn (Ad-Airn) and a control vector (Ad-NC) were constructed by Genepharma (Shanghai, China).

3.6. Transient transfection

Airn siRNA, Igf2R siRNA and their non-specific siRNA (NC) were constructed by Genepharma (Shanghai, China). Transfection of siRNAs was performed with HiPerFect transfection reagent (Qiagen, Düsseldorf, Germany) according to the instructions. Briefly, 6 × 10^5 cells were seeded in 6-well plates with 2 ml MEM culture medium containing 10% FBS and antibiotics. At the same time, siRNAs or NC were mixed with HiPerFect transfection reagent and incubated at room temperature for 10 min. Then, the complexes were transfected into HepG2 cells for 48 h.

3.7. Western blot

Total cell proteins were extracted in RIPA lysis buffer(Biyuntian, Jiangsu, China) with fresh-added proteinase inhibitor cocktail and phosphatase inhibitor(Sigma, St. Louis, MO, USA) on ice for 15 min and centrifuged at 12,000 rpm for 20 min. Bicinchoninic acid (BCA, Sigma, St. Louis, MO, USA) was used to determine the concentration of total protein of EOC cells. For Western blot analysis, lysates (20 μg/well) were subjected to 10% SDS-PAGE, and then fractionated proteins were transferred to PVDF membrane (Millipore, USA). The membrane was blocked in 5% non-fat skim milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 ℃. The next day, samples were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies for 1h at room temperature. Positive immunoreactive proteins were detected using an ECL kit (Thermo Fisher, USA) to visualize signals and bands. The bands intensity was analyzed using Quantity one analysis software (Bio-Rad, California).

3.8. Statistical analysis

All experiments were repeated three times in triplicate or in quadruplicate. All values were expressed as mean±SEM and analyzed by t-test or chi-squared test. Statistical analysis were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).Statistical differences in protein expression levels were identified by one-way ANOVA followed by Tukey's test. Provability values (P) <0.05 was considered to indicate a statistically significant difference.

Conflicts of interest: Non declared.

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