**miR-96-5p promotes breast cancer migration by activating MEK/ERK signaling**

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**ABSTRACT**

**Background**: Breast cancer is the leading cause of cancer deaths in women worldwide. Many microRNAs play a vital role in breast cancer migration. The purpose of the current study was to investigate the potential role of miR-96-5p in breast cancer.

**Methods**: Breast cancer tissues and matched para-cancerous tissues were collected. The expression of microRNA-96-5p (miR-96-5p) and arginine kinase 3 (AK3) was detected by quantitative real-time PCR (qRT-PCR). The correlation between miR-96-5p and AK3 was calculated by Pearson’s Chi-square test. Moreover, mimics or inhibitors of miR-96-5p were applied to explore whether miR-96-5p influences the migration capacity in a transwell or wound healing assay. Bioinformatics analysis was performed to identify the target genes of miR-96-5p through the TargetScan, miRDB and miRanda databases. A luciferase reporter assay was performed to verify AK3 as a downstream target gene of miR-96-5p.

**Results**: The expression of miR-96-5p was significantly increased in BC tissue and BC cell lines compared with para-cancerous tissue or a breast cell line, respectively. The expression of miR-96-5p negatively correlates with AK3 gene expression. AK3 was demonstrated to be a direct mRNA target of miR-96-5p. AK3 is positively associated with the overall survival of breast cancer patients. Kaplan-Meier curve and log rank test analyses revealed that decreased AK3 levels are significantly associated with reduced overall survival. miR-96-5p was shown to promote the migration of breast cancer cells through the MEK/ERK signaling pathway, as demonstrated by the enhanced expression of p-MEK and p-ERK.

**Conclusion**: Our results identify the role of miR-96-5p in promoting breast cancer cell migration by activating MEK/ERK signaling via targeting AK3.

**Key words:** miR-96-5p; AK3; Breast cancer; Proliferation; Migration

**Background**

Breast cancer is one of the most prevalent female cancers and usually has a poor prognosis[1-3](#_ENREF_1). Recently, the occurrence of breast cancer has progressively increased, and the age of onset has gradually become younger[4](#_ENREF_4). Breast cancer formation is a complex biological process that includes hormones, miRNAs and the environment[5](#_ENREF_5).

MicroRNAs (miRNAs), small noncoding RNAs with 19-22 nucleotides, could cause protein degradation. A host of studies have clearly elaborated that miRNA could directly repress the target messenger RNA (mRNA) through binding to the 3’UTR of mRNA[6](#_ENREF_6). Several previous studies have revealed that miRNAs have roles in regulating cholesterol metabolism[7](#_ENREF_7), embryo implantation[8](#_ENREF_8), insulin resistance[9](#_ENREF_9), and hematopoiesis[10](#_ENREF_10). Iwai et al.[11](#_ENREF_11) reported that miR-96-5p, which is frequently upregulated in hepatocellular carcinoma, inhibits apoptosis by targeting CASP9. The arginine kinase 3 (AK3) gene is located on chromosome 9 and mainly functions in the mitochondrial matrix. The tumor suppressor function of AK3 has been demonstrated. For example, Melle et al.[12](#_ENREF_12) reported that AK3 was identified as a specific marker for hepatocellular carcinoma.

Previously, we performed a miRNA microarray between breast cancer and matched adjacent noncancerous tissues. Differentially expressed miRNA arrays showed that miR-96-5p was one of the most dramatically increased miRNAs (log fold change=2.859, P=0.000). However, the possible effects and mechanism of miR-96-5p on breast cancer cell migration have not been elucidated.

Therefore, we reported the extract role of miR-96-5p and its underlying mechanism in promoting the migration of breast cancer cells.

**Materials and methods**

**Patient tissues**

Breast cancer and matched para-cancerous tissues were collected from 66 female patients with breast cancer between August 2016 and August 2019 at The Second Affiliated Hospital of Soochow University. All of the tissues were pathologically diagnosed as invasive ductal carcinoma. Current research received ethical approval from the ethics committee of The Second Affiliated Hospital of Soochow University. All subjects involved in this study signed informed consent forms.

**Cell culture.**

Four kinds of cell lines were applied in this study, including three triple-negative breast cancer cell lines (BT549, HS578T and MDA-MB-231) and a normal breast cell line (MCF10A). The above cell lines were all obtained from Procell Life Science & Technology Co. (Wuhan, Hubei Province, China). BT549, HS578T, MDA-MB-231 and MCF-10A cells were maintained in RPMI 1640 medium and DMEM, respectively. The media were all supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich. St Louis, MO, USA) and cultured to confluence in a 37 ˚C cell culture incubator with 5% CO2.

**Plasmids and transfection**

MiR-96-5p mimic/miR-96-5p inhibitor and corresponding control single stranded oligonucleotides (miR-mimic NC/miR-inhibitor-NC) were synthesized by GenePharma (Shanghai, China) and are listed in **Table 1**. According to the manufacturer’s instructions, MDA-MB-231 cells (1×106/well) were seeded in 6-well culture plates and transfected with miR-96-5p mimics (50 nM)/miR-96-5p inhibitor (50 nM) or the corresponding control (50 nM) by using Lipofectamine 2000 reagent (Invitrogen, CA, USA) after the cells reached 70-80% confluence.

**Cell migration assessment.**

MDA-MB-231 cells were plated onto 6-well culture plates and grown to 90% confluence. The cell monolayers were scratched by micropipette tip and cultured in FBS-free media with different treatments (scram, vector, miR-96-5p mimic and miR-96-5p inhibitor). Photomicrographs were taken at 0 and 24 hours after scratching (Olympus, Japan). Each wound healing assay was independently repeated three times.

**Migration assay**

Cell migration assays were performed using Transwell migration chambers (8 μm pore size, 24 wells, Corning-Costar, New York, USA). In brief, 5x104 cells with different treatments (scram, vector, miR-96-5p mimic and miR-96-5p inhibitor) were suspended in 100 μL of serum-free medium. Equal numbers of cells were added to the upper chamber with serum-free medium. The bottom chamber was filled with standard media for different treatments. Cells were incubated at 37 °C for 24 h. Cells that did not migrate through the membrane were removed by swab. Then, the membrane was fixed with 4% paraformaldehyde and stained with 0.2% crystal violet (Solarbio, Beijing, China). The numbers of migrant cells from five random areas of the membrane were counted using light microscopy. All of the experiments were biologically repeated more than three times independently.

**PCR**

Total RNA in the different groups was extracted with TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) as previously described[13](#_ENREF_13). RNA concentration and purity were measured, and cDNAs were synthetized by reverse transcription using oligo(dT) with RNA samples (1 μg) by PrimeScript RT reagent kit (Takara Biotechnology Co., Japan). Expression levels of target genes were assessed by real-time PCR (qRT-PCR) using SYBR® Green PCR Master Mix (TaKaRa, Dalian, China) in a LightCycler 480II Real-Time PCR System. Specific primers for AK3 and GAPDH were designed and purchased from AUGCT Company (Beijing, China), and the sequences are shown in **Table 2**.

**Western blot analysis**

Protein was extracted from breast cancer cells by radioimmunoprecipitation assay (RIPA, Beyotime, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF). Protein samples (20-100 μg) were added equally into SDS-polyacrylamide gel electrophoresis as described previously[14](#_ENREF_14). After proteins were electrotransferred onto polyvinylidene difluoride membranes (PVDF), the membranes were blocked with 5% nonfat milk at room temperature for 1.5 h. Then, the membranes were incubated with primary antibodies against AK3 (Proteintech, 1:1000, USA), MEK (Abcam, 1:1000, UK), p-MEK (Abcam, 1:1000, UK), ERK (Proteintech, 1:1000, USA), and p-ERK (Proteintech, 1:1000, USA) at 4 °C overnight. After washing three times with TBS Tween 0.1% (TBST), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime, Shanghai, China) for 60 min at 37°C. The target protein was visualized by a SuperSignal West Pico Trial Kit (Thermo).

**Luciferase assay**

The 3′UTR region in AK3 DNA was amplified by PCR with the following primers: F: 5′ATCGCTCGAGAGACATGGAAATTATTTTAT3′; R: 5′CCAGCGGCCGCTATCTGATTATTGGCTGTGTC3′. Mutations were introduced using the following primers: 5′GTGATATGGATTGGAAACTGAAATTCAGAGAAATTTCGTGTTCAATAGATACCACGAATT3′;R:5′AATTCGTGGTATCTATTGAACACGAAATTTCTCTGAATTTAGTTTCCAATCCATATCAT3′. The enlarged DNA sequence was ligated into the psi-AK3 vector to generate luciferase vectors for AK3 or mutated AK3 3 3′UTR. Using the XhoI/NotI restriction cleavage site, the 3′UTR sites containing the target seed sequences were placed in the vector. Human renal epithelial 293T cells (ATCC No. CRL-11268) contain a repressed SV40 T antigen. For the reporter assay, 293T cells were seeded into a 24-well plate and transfected with miR-96-5p mimic, miR-96-5p inhibitor, or NC (RiboBio, China). The 3′UTR of AK3 containing wild-type or mutant DNA was cotransfected with the psi-CHECK2 vector (2 mg/mL) into 293T cells. Approximately 48 h after transfection, the luciferase activities were measured with the Dual Luciferase® Reporter Assay (GloMax, Promega, Madison, WI, USA).

**Bioinformatic Analysis**

Targetscan (http://www.targetscan.org/vert\_72/), miRDB (http://mirdb.org/) and miRanda (<http://www.microrna.org/microrna/home.do>. ) databases were used to predict the target genes of miR-96-5p. Moreover, a Venn diagram was performed to obtain the overlapping genes of these three databases through VENNY 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>).

**Oncomine Analysis**

Oncomine database analysis Expression levels of AK3 in breast cancer vs. normal tissues were analyzed using the Oncomine database (<https://www.oncomine.org/resource/login.html>). Differences in transcript levels associated with P < 0.05 and log fold change >2 were considered significant.

**GEPIA Dataset**

Gene Expression Profiling Interactive Analysis (GEPIA) is a new web-based tool for analyzing tumors and normal samples from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects using a standard processing pipeline (http://gepia.cancer-pku.cn/)[15](#_ENREF_15). AK3 gene expression correlation analysis and overall survival analysis based on given sets of TCGA expression data were performed by GEPIA.

**Statistical analysis**

All experiments involved at least three biological replications, and the mean ± SD was calculated to evaluate the data. When appropriate, to assess differences between sets, we used Student’s t-test and the Mann-Whitney U test for two-group comparisons. One-way ANOVA followed by Dunnett’s t-test was used for comparisons of multiple groups. P<0.05 was regarded as statistically significant. Data from the experiment will be analysed using SPSS software (version 19, SPSS Inc., City, State, USA).

**Results**

**miR-96-5p expression levels are increased in** **breast cancer cells and tumor tissues**

Compared with MCF10A, the expression of miR-96-5p was significantly upregulated in three TNBC cell lines (BT549, HS578T and MDA‑MB‑231, P<0.05, **Figure 1B). 1 A**). As shown in **Figure. As shown in Figure 1 B**, miR-96-5p was significantly increased in breast cancer tissues compared with matched paracancerous tissues (P<0.05). Moreover, the expression of miR-96-5p had a negative correlation with AK3 expression (r=-0.895, P=0.000, **Figure 4B). 1 C**).

**Downregulation of** **miR-96-5p inhibits cell migration via upregulation of AK3 in breast cancer cells**

Transwell migration assays and wound healing assays were performed to investigate the effect of miR-96-5p on breast cancer cell migration. The results revealed that the migration capabilities (Transwell migration assay (**Figure 1B) were lower than those of the controls. 2 A**) and wound healing (**Figure. 2 B**) were significantly increased in breast cancer cells when miR-96-5p was upregulated.

**MiR-96-5p inhibits AK3 expression and activates the MEK/ERK pathway in breast cancer cells**

Successful miR-96-5p overexpression and inhibition in breast cancer cells was confirmed by qRT-PCR (**Figure 3A). 3A and B**). The upregulation of miR-96-5p in breast cancer cells transfected with miR-96-5p mimic significantly decreased AK3 mRNA expression, while downregulation of miR-96-5p in breast cancer cells transfected with miR-96-5p inhibitor had the opposite trend.

Furthermore, the miR-96-5p mimic could significantly increase the relative protein expression of AK3 in MDA-MB-231 cells (**Figure 1B). 3 C**). Moreover, the upregulation of miR-96-5p significantly increased MEK and ERK phosphorylation, while its downregulation significantly decreased this modification (**Figure 4B). 3 C**). Taken together, these results demonstrated that upregulation of miR-96-5p was capable of inhibiting AK3 expression by targeting the MEK/ERK signaling pathway.

**AK3 is a target gene of miR-96-5p in breast cancer cells.**

To analyze the regulation of miR-96-5p-mediated AK3 expression, we performed luciferase assays by cloning a fragment of the 3´-UTR including the miRNA binding sites (**Figure 3A). 4 A**). By cotransfecting MDA-MB-231 and BT549 cell lines with the 3´-UTR controlling the luciferase gene and miR-96-5p overexpression plasmids, we found that miR96-5p expression decreased the activity of luciferase when it was controlled by the 3´-UTR of AK3 (**Figure 7B). 4 B and C**). Together, these results demonstrate the regulation of miR-96-5p in the expression of AK3 in vitro.

**Bioinformatic analysis of miR-96-5p and AK3**

First, we revealed a total of 491 overlapping genes from the TargetScan, miRDB and miRanda databases (**Figure 1). 5 A**). ONCOMINE analysis found that the mRNA expression of AK3 was downregulated in patients with breast cancer (**Figure 1). 5 B**).

The GEPIA dataset also revealed that the expression levels of AK3 were downregulated in patients with breast cancer compared with normal tissue (**Figure 3A). 5 C**). The AK3 group did not significantly differ in tumor stage (P>0.05, **Figure 1). 5 D**).

The Kaplan-Meier curve and log rank test analyses revealed that decreased AK3 levels were significantly associated with reduced overall survival (OS, P < 0.05) (**Figure 2). 5 E**). **Figure. Figure 5 F** presents the gene ontology and KEGG pathway of the target genes of miR-96-5p. We selected the MAPK signaling pathway for further study. Then, AK3 and neighboring genes were constructed, in which nodes were PIK3R1, PAK1, and FYN. (**Figure 1). 5 C**).

**Immunohistochemistry (IHC) of AK3 in breast cancer and normal tissues**

To further compare AK3 protein expression levels in breast cancer and normal breast tissues, we obtained AK3 protein expression from the Human Protein Atlas (https://www.proteinatlas.org/). The results showed an extremely low expression level in breast cancer and positive strong expression in adjacent non-cancerous tissues (**Figure 1A). 6**).

**Discussion**

MiR-96-5p is significantly accumulated in human breast cancer tissue and triple-negative breast cancer cell lines, especially in MDA-MB-231 cells. Moreover, miR-96-5p has a negative correlation with AK3 levels. MiR-96-5p dramatically downregulated the expression of AK3. MiR-96-5p significantly increased breast cancer cell migration by mediating the MEK/ERK signaling pathway. Decreased expression of AK3 was negatively associated with OS in breast cancer.

MiR-96-5p was previously revealed as a promoting factor for gastric cancer[16](#_ENREF_16) and prostate cancer[17](#_ENREF_17). Additionally, miR-96-5p plays different roles in cell viability and migration in different diseases, including thyroid cancer[18](#_ENREF_18), bladder cancer[19](#_ENREF_19), adrenocortical and adrenal medullary tumors[20](#_ENREF_20), head squamous carcinoma[21](#_ENREF_21), and osteosarcoma[22](#_ENREF_22). However, the effects and mechanism of miR-96-5p in breast cancer have not yet been detailed.

We first identified that miR-96-5p was differentially expressed between breast cancer and paracancerous tissues. Migration ability experiments (Transwell and wound healing assays) revealed that the miR-96-5p mimic could significantly increase the migration number and distance. The potential mechanism revealed that MEK/ERK signaling may promote breast cancer cell migration. Li et al.[23](#_ENREF_23) found that activation of MEK/ERK signaling pathways could significantly enhance the aggressiveness of breast cancer cells. Degirmenci et al.[24](#_ENREF_24) summarize a review and found that targeting aberrant MEK/ERK signaling could be administered for cancer therapy.

Consistent with the results of a previous study, miR-96-5p may be a novel oncogenic miRNA in breast cancer cells. Additionally, we further validated that luciferase activity was decreased in the wild-type 3’UTR of AK3 but not in the mutant-type 3’UTR. The AK3 gene is located on chromosome 9 and mainly functions in the mitochondrial matrix. AK3, mainly as a mitochondrial-matrix protein, could regulate the biological function of downstream genes.

A public database (ONCOMINE) was used and found that AK3 was downregulated in patients with breast cancer compared with normal tissue. AK3 is also related to the function of cells resistant to cisplatin[25](#_ENREF_25). Immunohistochemical results further confirmed that AK3 was downregulated in breast cancer tissue compared with normal tissue. Decreased AK3 levels were significantly associated with overall survival.

**Conclusion**

In short, our data support the tumor-promoting role of miR-96-5p resulting in enhanced breast cancer cell migration. Inhibitors of miR-96-5p might eventually become effective molecular therapeutic reagents for breast cancer.

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**Figure legend**

Figure. 1 Relative expression of miR-96-5p in MCF10A, BT549, HS578T and MDA-MB-231 cells, \*\*P<0.01 vs MCF10A cell line (A); B, relative expression of miR-96-5p in nontumor and tumor tissue, \*\*P<0.01 vs nontumor; C, correlation between miR-196-5p and AK3.

Figure. 2 (A) Migratory cells stained with crystal violet 24 h after stimulation with miR-96-5p mimic or inhibitor in the Transwell migration assay. (B) Relative migration distance after stimulation with miR-96-5p mimic or inhibitor in the wound healing assay in MDA‑MB‑231 cells. Data are presented as the mean ± SD, n=3, \*\*\* indicates P<0.001; ### indicates P<0.001. One-way ANOVA.

Figure. 3 (A) Transfection with miR‑96‑5p mimic or inhibitor increased and decreased miR-96-5p expression in MDA-MB-231 cells, respectively; (B) Upregulation and downregulation of miR‑96‑5p decreased and increased AK3 mRNA levels, respectively, in MDA‑MB‑231 cells. (C) Immunoblotting showing the levels of ERK1/2, p-ERK1/2, MEK, p-MEK and AK3 in MDA-MB-231 cells in the miR-96-5p mimic and inhibitor groups. \*\*P<0.01 vs. the respective miR‑NC group. NC, negative control.

Figure. 4 A, Schematic graph showing the binding sites of miR-96-5p on the AK3 transcript. Luciferase assay of pmiR-Target-AK3-3’UTR reporter co-transfection with pcDNA-miR-96-5p in MDA-MB-231 cells (B) and BT549 cells (C). (\*\*p<0.001).

Figure. 5. A, The transcription levels of AK3 in breast cancer (Oncomine); b, Venn diagram revealing the overlapping genes of the miR-96-5p target in the TargetScan, miRDB and miRanda databases; C, Box plot showing the expression of AK3 in breast cancer (GEPIA); D, AK3 relative expression in different breast cancer clinical stages in breast cancer patients (GEPIA database); E, The correlation between AK3 expression and the overall survival of breast cancer patients; F, Gene Ontology (GO) and KEGG pathway enriched terms through the Metascape website sorted by -log10 (P value); G, Protein–protein interaction (PPI) network of the miR-96-5p target genes and eight most significant MCODE models.

Figure. Figure 6 The expression of AK3 in breast cancer and normal tissues (IHC) through the Human Protein Atlas.

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| --- | --- | --- |
| Gene Forward/Reverse |  | Sequence |
| Has-GAPDH | F | 5'-GGAGCGAGATCCCTCCAAAAT-3' |
| Has-GAPDH | R | 5'-GGCTGTTGTCATACTTCTCATGG-3' |
| Has-U6 | F | 5'-CGCGCTTCGGCAGCACA-3' |
| Has-U6 | R | 5'-AAGCGAAGTGCTTAAAGCGT-3' |
| miR-96-5p | F | 5'-ACGATGCACCTGTACGATCA-3' |
| miR-96-5p | R | 5'-TCTTTCAACACGCAG GACAG -3' |
| AK3 | F | 5'-AATGCACTTCAGCCACCATCA-3' |
| AK3 | R | 5'-TCGTAGGTCTGTCCTGGGAGTTC-3' |

Table 1: The following contents are qRT-PCR primers.