

# CircAHNAK1 inhibits proliferation and metastasis of triple-negative breast cancer by modulating miR-421 and RASA1

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## ABSTRACT

**Background:** There is increasing evidence that circular RNAs (circRNAs) participate in regulating cancer progression. However, the function and potential molecular mechanisms of circRNA in triple negative breast cancer (TNBC) are currently largely unclear.

**Results:** We found that circAHNAK1 was significantly down-regulated in TNBC, and its expression was negatively associated with RFS and OS. Overexpression of circAHNAK1 can inhibit TNBC proliferation, migration and invasion in vitro. In vivo studies confirmed that circAHNAK1 inhibited TNBC tumor growth and metastasis. Mechanistic analysis indicated that circAHNAK1 acted as a miR-421 ceRNA (competitive endogenous RNA) to attenuate the inhibitory effect of miR-421 on its target gene RASA1.

**Conclusions:** In conclusion, CircAHNAK1 inhibits proliferation and metastasis of TNBC by modulating miR-421 and RASA1.

**Methods:** CircRNA microarrays were used to screen for differential circRNA expression profiles. qRT-PCR was used to detect the expression levels of circRNAs. The effect of circAHNAK1 on recurrence-free survival (RFS) and overall survival (OS) in patients with TNBC was subsequently analyzed. The role of circAHNAK1 in the progression of TNBC was further evaluated by multiple in vivo and in vitro assays. Finally, we focused on the regulation of circAHNAK1 on miR-421 and its targeted gene RASA1 in TNBC.

## INTRODUCTION

Triple-negative breast cancer (TNBC) is one of the most deadly types of breast cancer. TNBC featured with highly invasive and highly metastatic, resulting in a poor prognosis [1]. The 5-year mortality rate for TNBC is still relatively high due to difficulties in curbing recurrence and metastasis and poor response to various treatments [2]. Although recent research on non-TNBC treatment has made great progress, the treatment of TNBC is still limited [3]. Non-coding RNA plays an important regulatory role in the biological properties of cancer and is also considered

a potential therapeutic target for TNBC. Some endogenous non-coding RNAs such as circRNA are closely related to tumor cell proliferation, apoptosis and metastasis by regulating a large number of signaling pathways in cancer [4, 5]. In particular, circRNA can act as a microRNA sponge to regulate gene expression [6, 7]. The study of dysregulated circRNAs and their function in TNBC has attracted increasing attention [8–10].

Here, we reanalyzed the circRNA expression profiles of the TNBC tissue and the matched para-cancer normal tissue we performed in our previous study [11]. We

found a circRNA derived from the AHNAK gene and named it circAHNAK1, which was significantly lower in TNBC tissues than in normal tissues, and that low expression of circAHNAK1 was associated with shortened survival. Subsequently, we conducted a variety of experiments to further confirm that circAHNAK1 played an important role in the development of TNBC. In vitro, down-regulation of circAHNAK1 promoted the proliferation and invasion of TNBC cells. Decreased expression of circAHNAK1 in TNBC cells increased tumor proliferation, migration and invasion. In terms of mechanism, circAHNAK1 may promote the expression of the tumor suppressor gene RASA1 through sponging miR-421, thereby exerting its regulatory function in TNBC. Therefore, this study suggests that circAHNAK1 is a prognostic biomarker for TNBC and a therapeutic target that can be used for TNBC patient.

## RESULTS

### CircAHNAK1 is down-regulated in TNBC patients and significantly affects the prognosis of TNBC

To study the function and role of circRNAs in TNBC, the circRNA microarrays performed in our previous study were reanalyzed by us. qRT-PCR was used to verify the expression level of one of the most obvious down-regulated circRNAs, hsa\_circ: chr11:62297840-62298224(hsa\_circ\_0000320), in TNBC tissues and cell lines. According to the information provided by the human reference genome (GRCh37/hg19), hsa\_circ\_0000320 is derived from the AHNAK gene according to predictions. Therefore, circRNA hsa\_circ\_0000320 is named “circAHNAK1” by us. In order to verify the circular structure of circAHNAK1, we performed RNase R digestion test and the actinomycin D test. Resistance to RNase R exonuclease further confirmed that the RNA is a circular structure (Supplementary Figure 1A). In addition, actinomycin D analysis indicated that the half-life of the circular RNA circAHNAK1 was longer, indicating the stability of circAHNAK1 (Supplementary Figure 1B). According to the results of qRT-PCR, the expression level of circAHNAK1 in the TNBC cell line was significantly lower than that of the Non-TNBC cell line (Figure 1A). Then, we evaluated its expression in TNBC tissue and adjacent normal tissues. Expression of circAHNAK1 was also found to be significantly down-regulated in TNBC tissues (Figure 1B). ROC analysis suggested that circAHNAK1 can be used as a diagnostic indicator for distinguishing between TNBC and normal breast tissue (Supplementary Figure 1C). Our result also suggested that circRNA is significantly down-regulated in stage III–IV TNBC tissues compared to stage I–II (Supplementary Figure 1D).

To investigate the clinical significance of circAHNAK1 in TNBC, we analyzed the expression of circAHNAK1 in 136 TNBC patients. We used the median value of the expression as the cutoff value and classified all TNBC patients as “circAHNAK1-high” and “circAHNAK1-low” groups. We found that circAHNAK1 expression was negatively correlated with larger tumors ( $P=0.045$ ), positive lymph node metastasis ( $P=0.001$ ), and late TNM stage ( $P<0.001$ , Table 1), suggesting that circAHNAK1 may play an important role in the malignant progression of TNBC. Subsequently, we performed survival analysis and found that low expression of circAHNAK1 in TNBC patients predicted poor OS and DFS (Figure 1C and 1D).

### Overexpression of circAHNAK1 inhibits TNBC proliferation and metastasis

To investigate the function of circAHNAK1 in TNBC, we transfected the overexpression vector of circAHNAK1 in MDA-MB-231 and BT-549 cells, for subsequent studies (Figure 2A). CCK-8 assay suggested that overexpression of circAHNAK1 significantly inhibits proliferation of TNBC cells (Figure 2B). Overexpression of circAHNAK1 also inhibited the colony formation (Figure 2C and 2D). The invasive ability of TNBC cells after circAHNAK1 overexpression was found to be significantly reduced according to the invasion assay (Figure 2E and 2F). Migration assays suggested that circAHNAK1 overexpression significantly inhibited migration potential compared to controls (Figures 2G and 2H). Subsequently, we established a mouse xenograft model to investigate the role of circAHNAK1 in vivo. Overexpression of circAHNAK1 significantly reduced tumor volume and weight (Figure 2I and 2J). Moreover, the size and number of lung metastases were also significantly inhibited by overexpression of circAHNAK1 (Figure 2K–2M), indicating circAHNAK1 can inhibit the malignant progression of TNBC cells.

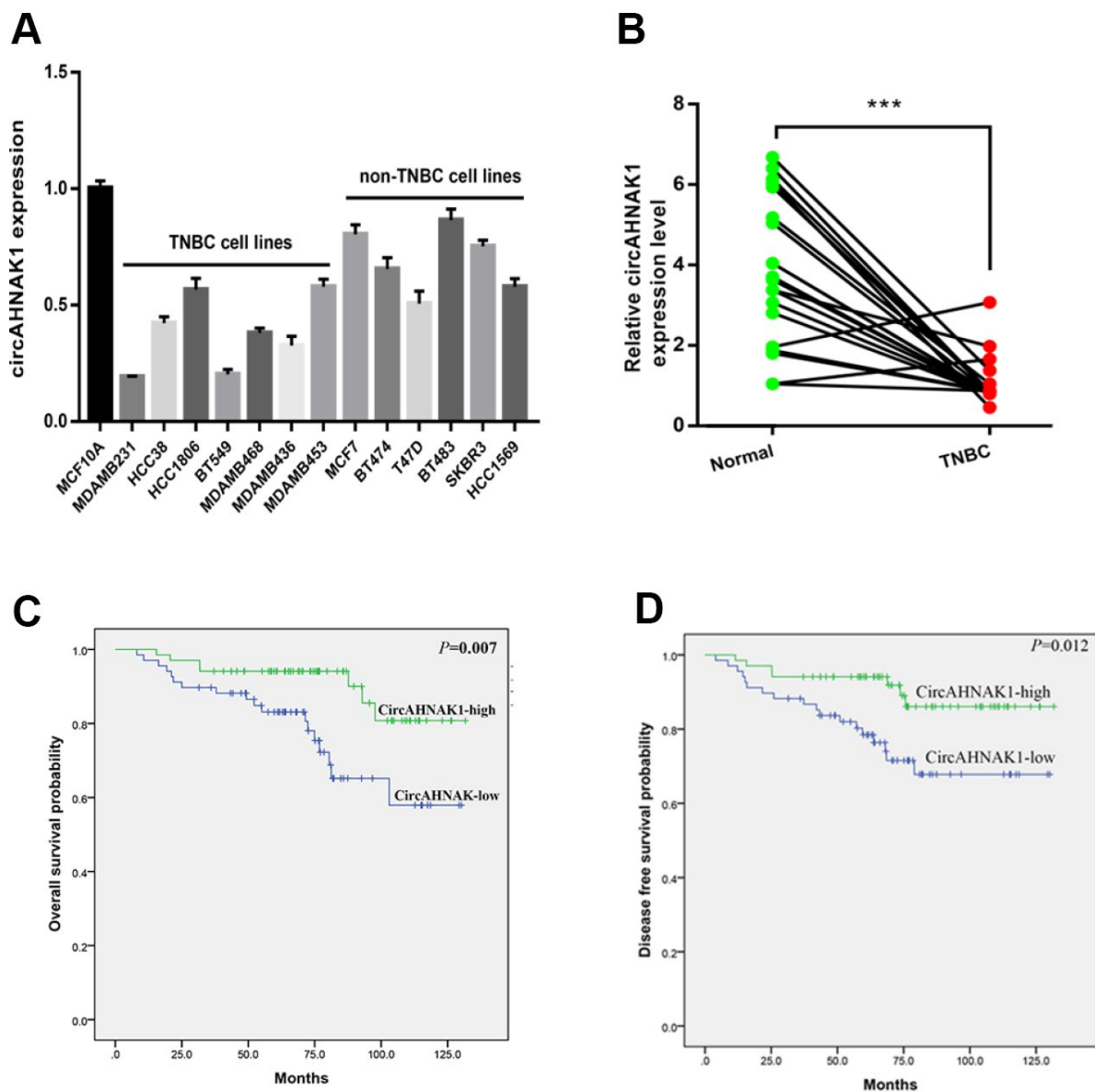
### circAHNAK1 sponges miR-421 in TNBC

We found that circAHNAK1 is mainly distributed in the cytoplasm in cells (Figure 3A), suggesting that it may have a role through sponging miRNA. In order to predict potential miRNAs that bind to circAHNAK1, Circular RNA Interactome was utilized in this study (<https://circinteractome.nia.nih.gov/>). We found two binding sites for miR-421 in the circAHNAK1 sequence (Figure 3B). Using qRT-PCR, we found that miR-421 was upregulated in TNBC cell lines (Figure 3C). We then observed that co-transfection of miR-421 mimics and the wild-type vector significantly reduced luciferase activity, but no similar phenomenon was observed for transfection of the mutant luciferase reporter (Figure 3D). To confirm that circAHNAK1 interacts directly with miR-421, we

conducted a GFP-MS2-RIP assay. The results showed that the enrichment of miR-421 was mainly in the MS2-circAHNAK1-WT group, but not the MS2-circAHNAK1-Mut group (Figure 3E) compared to the negative control. This result further indicated that circAHNAK1 could directly bind to miR-421

To investigate whether circAHNAK1 exerts its effects via sponge miR-421, we conducted multiple in vivo and in vitro studies. CCK-8 and colony formation assays showed that miR-421 mimics could partially reverse the inhibition of cell proliferation induced by

circAHNAK1 overexpression (Figure 3F–3H). Invasion and migration assays showed that treatment with miR-421 mimics also partially reverse the decreased cell migration capacity caused by circAHNAK1 overexpression (Figure 3I–3L). Experiments using a xenograft mouse model showed that treatment with miR-421 mimics also partially reverse the reduction in tumor growth and lung metastasis after circAHNAK1 overexpression (Figure 3M–3Q). All these findings indicate that circAHNAK1 plays a role in TNBC by sponging miR-421. Together, these results provide evidence that circAHNAK1 functions in TNBC via sponging miR-421.



**Figure 1. circAHNAK1 is down-regulated and associated with malignant progression and poor prognosis of TNBC.** (A) Expression of circAHNAK1 in breast cancer cell lines. (B) Expression of circAHNAK1 in breast cancer tissues and normal adjacent tissues. (C, D) The effect of circAHNAK1 expression on OS and DFS in patients with TNBC. \*\*\*  $P < 0.001$ .

**Table1. Relationship between circAHNAK1 and clinical-pathological features of TNBC.**

Variables	Cases(n=136)	circAHNAK1		P value
		Low No.(N=68)	High No.(N=68)	
<b>Age(years)</b>				
<40	35	15(42.9%)	20(57.1%)	0.393
40-60	87	44(50.6%)	43(49.4%)	
>60	14	9(64.3%)	5(35.7%)	
<b>Menopause</b>				
No	82	41(50.0%)	41(50.0%)	1.000
Yes	54	27(50.0%)	27(50.0%)	
<b>T stage</b>				
T1-T2	122	57(46.7%)	65(53.3%)	0.045*
T3-T4	14	11(78.6%)	3(21.4%)	
<b>N stage</b>				
N0	75	27(36.0%)	48(64.0%)	0.001*
N1-3	61	41(67.2%)	20(32.8%)	
<b>TNM stage</b>				
I-II	107	43(40.2%)	64(59.8%)	<0.001*
III-IV	29	25(86.2%)	4(13.8%)	

\*P < 0.05, statistically significant

### circAHNAK1 sponges miR-421 to regulate RASA1 expression

To investigate whether circAHNAK1 regulates its downstream target genes via sponging miR-421, we searched potential target genes for miR-421 in TargetScan and predicted RASA1 (Figure 4A). We then found that luciferase activity was decreased after co-transfection of miR-421 mimics and wild-type luciferase by luciferase reporter assay, whereas co-transfection with mutant luciferase reporter had no similar effect (Figure 4B). In addition, after co-transfection of the miR-421 inhibitor and wild-type luciferase reporter, we observed a significant increase in luciferase activity (Figure 4B). Furthermore, miR-421 mimics inhibited the expression of RASA1, while the miR-421 inhibitor increased the expression of RASA1 (Figure 4C), indicating that RASA1 could be regulated by miR-421.

In addition, circAHNAK1 overexpression upregulated the expression of RASA1 by both immunofluorescence and Western blot (Figure 4D, 4E). The results of correlation analysis suggested that there is a positive correlation between the expression of circAHNAK1 and RASA1 in TNBC ( $r=0.4884$ ,  $P<0.01$ ; Supplementary Figure 1E). The Anti-Ago2-RIP assay indicated that circAHNAK1, RASA1 and miR-421 were

predominantly enriched to Ago2 compared to control immunoglobulin G (IgG) antibodies (Figure 4F), indicating that circAHNAK1 and RASA1 were recruited to Ago2-related RNA-induced Silencing Complex (RISC), where they can interact with miR-421. Then, we observed that overexpression of circAHNAK1 increased circAHNAK1 enrichment in Ago2-RIPs and reduced RASA1 enrichment in Ago2-RIPs (Figure 4G), indicating that circAHNAK1 acts as a ceRNA and competes with RASA1 for binding to miRNA. Furthermore, transfection with miR-421 resulted in reduced expression of RASA1, whereas overexpression of circAHNAK1 rescued the reduction (Figure 4H and 4I), indicating that circAHNAK1 could promote RASA1 expression by sponging miR-421.

### RASA1 is negatively correlated with poor survival of TNBC

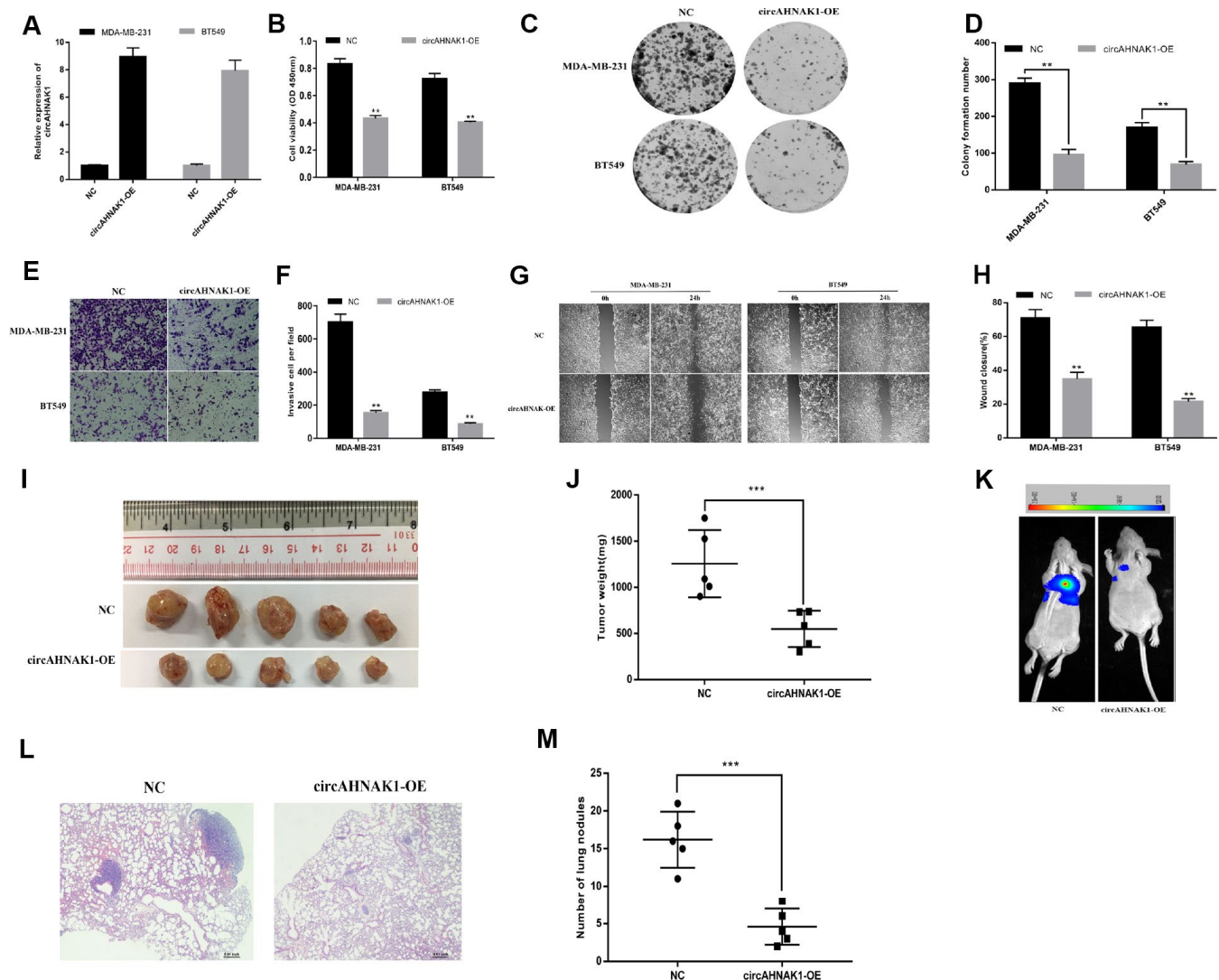
We then examined the expression of RASA1 in the TNBC cell line and found that RASA1 was lowly expressed in the TNBC cell lines (Figure 5A and 5B). We also studied the clinical impact of RASA1 expression on TNBC patients, demonstrating that low expression of RASA1 predicted later TNM stage and a higher rate of lymph node metastasis (Figure 5C, Table 2), indicating an important role for RASA1 in the malignant progression of TNBC. Survival analysis also

indicated that lower *RASA1* expression in TNBC predicted poor OS and DFS (Figure 5D and 5E).

## DISCUSSION

Circular RNAs are a new class of non-coding RNAs that are gaining more and more attention in the regulation of human cancers. In the current study, we evaluated the function of circ-AHNAK1, which is a novel circRNA derived from the AHNAK locus. We found that circAHNAK1 is an important circRNA that is frequently

down-regulated in TNBC tissues, whereas low expression of circAHNAK is positively correlated with malignant progression and poor survival in TNBC patients. Secondly, through mechanism studies, we found that overexpression of circAHNAK1 increased the sponge of miR-421 and up-regulated the expression of the tumor suppressor gene *RASA1*, thereby inhibiting the proliferation and metastasis of TNBC cells. In view of these findings, circAHNAK1 may be used as a potential biomarker or therapeutic target for human TNBC.



**Figure 2. Overexpression of circAHNAK1 inhibits proliferation and metastasis of TNBC.** (A) Successfully established two breast cancer cell lines that overexpress circAHNAK1; (B) CCK-8 assay to evaluate the effect of circAHNAK1 on cell proliferation; (C) Colony formation assay to evaluate the effect of circAHNAK1 on cell colony forming ability; (D) Number of clones quantified by ImageJ; (E) Transwell invasion assay to evaluate the effect of circAHNAK1 on cell invasion; (F) ImageJ quantifies the number of invading cells; (G) Wound healing assay evaluates the effect of circAHNAK1 on wound closure; (H) ImageJ quantifies the extent of wound healing; (I) Xenograft model to evaluate the effect of circAHNAK1 on tumor proliferation in vivo; (J) Effect of circAHNAK1 on proliferation in vivo by tumor weight; (K) Representative images of luciferase signaling to assess the effects of circAHNAK1 on lung metastasis in vivo; (L) Representative images of HE staining of lung metastatic nodule sections; (M) Quantification of the number of lung metastatic nodules.



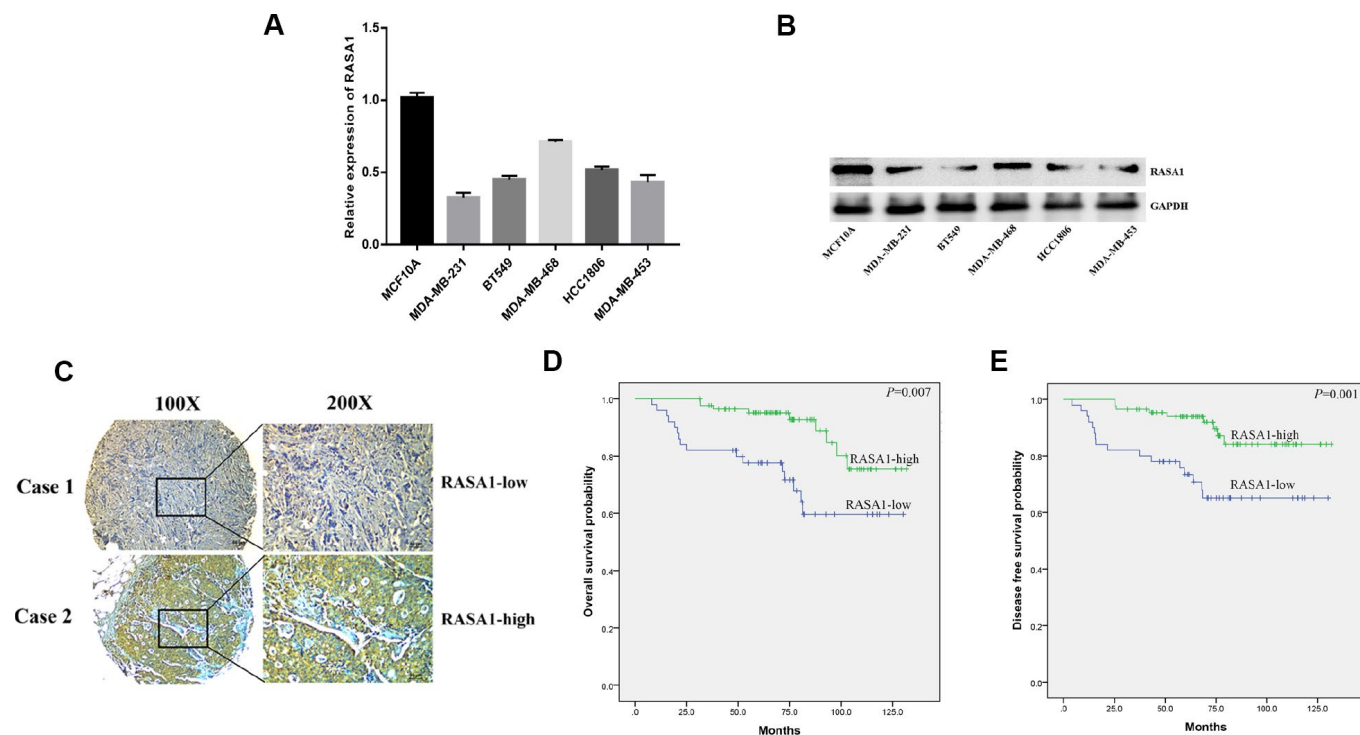


and promoted the growth of gastric cancer cells [16]. miR-421 was found to be upregulated in breast cancer tissues and inhibits apoptosis by inhibiting caspase-10 expression [17]. The up-regulated expression of miR-421 is associated with poor prognosis in non-small cell lung cancer [18]. Overexpression of miR-421 led to down-regulation of ATM and is associated with poor prognosis of breast cancer [19]. In prostatic cancer, miR-421 was also found to inhibit ATM expression and promote prostate cancer metastasis and treatment resistance [20].

RASA1 is a member of the RAS GTPase Activating Protein (RAS-GAP) family. The well-known oncoprotein RAS can be inactivated by binding to RAS-GAP members. Some studies have shown that mutation or loss of function of RASA1 leads to activation of the RAS-MAPK cascade in malignant tumors. RASA1 has been identified as a tumor suppressor gene which is underexpressed in multiple malignancies. RASA1 expression is also remarkably negatively associated with survival outcome in a variety of malignancies. The dysregulation of RASA1 plays an important role in the progression of malignant tumors, so it may become a predictor for prognosis and therapeutic target. Suárez-Cabrera C et al. confirmed by immunohistochemical analysis of human breast tumor tissues that low

expression of RASA1 is common in basal (triple negative) and estrogen receptor negative tumors [21]. It has also been found in colorectal cancer that RASA1 mutation or loss of function leads to activation of the RAS-MAPK cascade [21–23]. In non-small cell lung cancer, RASA1 has also been found to be associated with tumor progression and mediated MEK inhibition [24]. In hepatocellular carcinoma, it has also been found that down-regulation of RASA1 promotes angiogenesis under hypoxic conditions [25]. Consistent with previous studies, we also demonstrated that RASA1 is under-expressed in TNBC compared to normal tissues, and the expression of RASA1 is negatively correlated with RFS and OS.

In conclusion, circAHNAK1 is down-regulated in TNBC and its expression is negatively correlated with survival outcome in patients with TNBC. circAHNAK1 regulates TNBC cell proliferation and invasion, and the mechanism may be related to the expression of the tumor suppressor gene RASA1 by sponge miR-421, thereby promoting the progression of TNBC. The circAHNAK1-miR-421-RASA1 axis participates in TNBC progression through a competitive ceRNA mechanism. Therefore, circAHNAK1 can be used as a predictor of TNBC prognosis and a potential molecular target.



**Figure 5. RASA1 is down-regulated and associated with poor prognosis of TNBC.** (A) qRT-PCR detected mRNA expression of RASA1 in breast cancer cell lines;(B)Western blots detected RASA1 expression in breast cancer cell lines;(C) Representative IHC images of high and low RASA1 expression in TNBC tissues;(D, E)The effect of RASA1 expression on OS and DFS in patients with TNBC.



**Table 2. Relationship between RASA1 and clinical-pathological features of TNBC.**

Variables	Cases(n=136)	RASA1		P value
		Low (n=50)	High(n=86)	
<b>Age (years)</b>				
<40	35	13(37.1%)	22(62.9%)	0.230
40-60	87	29(33.3%)	58(66.7%)	
>60	14	8(57.1%)	6(42.9%)	
<b>Menopause status</b>				
No	82	28(34.1%)	54(65.9%)	0.274
Yes	54	22(40.7%)	32(59.3%)	
<b>T stage</b>				
T1-T2	122	39(32.0%)	83(68.0%)	0.001*
T3-T4	14	11(78.6%)	3(21.4%)	
<b>N stage</b>				
N0	75	15(20.0%)	60(80.0%)	<0.001*
N1-3	61	35(57.4%)	26(42.6%)	
<b>TNM stage</b>				
I-II	107	29(27.1%)	78(72.9%)	<0.001*
III-IV	29	21(72.4%)	8(27.6%)	

\*P < 0.05, statistically significant

## MATERIALS AND METHODS

### Cell culture and transfection

The cell lines used in this study included human normal breast cell (MCF10A), Non-TNBC cells (BT474, T47D, BT483, SKBR3, MCF-7 ,HCC1569) and TNBC cells (HCC38, HCC1806, BT549, MDA-MB-468, MDA-MB-453, MDA-MB-436, MDA-MB-231) were obtained from the American Type Culture Collection. All cell lines were free of mycoplasma infection and identified by STR (short tandem repeat) to ensure cell reliability. Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection. The overexpression vector of circAHNAK1 was synthesized by IGEbio (China). Inhibitors and mimics of miR-421 were obtained from GeneCopoeia (USA).

### qRT-PCR

Extraction of total RNA, separation of cytoplasm and nuclear RNA was performed as described in the previous study [8]. Real-time PCR analysis of mRNA and circRNA was performed using SYBR Premix Ex Taq™ (Takara, Japan) and Bio-Rad CFX96 detection system (USA). Real-time PCR analysis of miRNA was performed using the All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia) and the Bio-Rad CFX96 detection system (USA). Primers for detection were obtained from GENEray (Supplementary Table 1).

### RNase R treatment and Actinomycin D assay

RNA extracted from MDA-MB-231 cells was divided into two parts on average: one for RNase R (Epicentre Technologies, USA) and the other for buffer treatment for control. In the experimental group, 2 µg of total RNA was incubated with RNase R (3 U/ug) for 20 minutes at 37 °C. β-actin was used as an internal control.

For Actinomycin D assay, 1 x 10<sup>5</sup> cells were seeded into 6-well plates and treated with actinomycin D (2 mg/L; Sigma, USA). Subsequently, the treated cells were collected at 8, 16 and 24 hours, respectively, for qRT-PCR analysis of circAHNAK18 and AHNAK mRNA.

### Cell viability assay

1 x 10<sup>3</sup> breast cancer cells were cultured into well plates (96-well), and 10 µl of CCK-8 solution (Dojindo Laboratories, Japan) was added 48 hours after transfection. Subsequently, the incubation continued for 2 hours at 37°C. Finally, their absorbance at 450 nm was measured by using a microtiter plate reader (Bio-Tek EPOCH2, USA).

### Colony formation assay

1 x 10<sup>3</sup> MDA-MB-231 and BT-549 cells were cultured into well plates(6-well), and cultured in a cell culture

incubator under normal conditions. The culture was terminated after 10 days, and the clone was fixed with anhydrous methanol, followed by staining with crystal violet solution (0.1%, Sigma) for 30 min at 25°C.

### Migration assay

MDM-MB-231 and BT-549 cells were cultured in six-well plates and scraped with a fine end of a 200 µl pipette tip (time 0 hours). Cell migration was photographed using an inverted microscope at 0 and 24 hours after injury.

### Transwell assay

The invasion test was performed in a 24-well Transwell chamber. 8-µm well inserts were coated with 30 µg matrigel (BD Biosciences).  $5 \times 10^4$  breast cancer cells (200 µl of serum-free medium) were added to the coated upper chamber. 700 µl of medium containing 20% FBS (fetal calf serum) was added as a chemoattractant to the lower chamber. After an appropriate time in an incubator at 37 °C, the cells migrated through the filter were fixed with methanol, stained with 0.5% crystal violet, and counted on three random regions.

### Immunofluorescence staining

The cells were fixed using 4% paraformaldehyde for 20 minutes. 0.5% Triton X-100 was used for permeabilization for 10 minutes and then blocked with 5% BSA (bovine serum albumin) for 1 hour. Next, it was incubated with the RASA1 primary antibody (1:100, ZEN Bio, China) overnight at 4 °C. The secondary antibody conjugated to Dylight (1:200, Abbkine, China) was then added for incubation for 1 hour at room temperature. Finally, the nucleus was stained by adding Hoechst solution (Beyotime, China), and the stained cells were photographed.

### Western blots

We prepared cell lysates using RIPA buffer (Thermo Scientific). Then, a BCA Protein Assay Kit (Pierce, Thermo Scientific) was used to determine the concentration of the extracted protein. It was separated by 8% SDS-PAGE electrophoresis and transferred to a PVDF membrane (Millipore). It was then blocked with 5% skim milk for 1 hour at room temperature and incubated overnight at 4 °C with primary anti-RASA1 (1:1000, ZEN Bio, China). The HRP-labeled secondary antibody (CST) was then incubated for 1 hour at room temperature. Finally, chemiluminescence methods were used for protein detection. The internal reference used anti-GAPDH antibody (1:1,000, Affinity, USA).

### Immunohistochemical staining

Tissue immunohistochemistry (IHC) staining was performed on formalin-fixed, paraffin-embedded tissue sections using an IHC test kit (Kangwei shiji Biotechnology). Paraffin sections were deparaffinized, antigen was repaired with sodium citrate buffer, and then incubated with 3% hydrogen peroxide for 15 minutes at room temperature to block endogenous peroxidase and then blocked with 10% goat serum for 30 minutes. The RASA1 antibody (1:100, ZEN Bio, China) was incubated overnight at 4 °C in a humid chamber and then incubated with the secondary antibody for 1 hour at room temperature. The color reaction was carried out by DAB, and once browned, the reaction was terminated by immersing the tissue sections in PBS. Tissue sections were counterstained with hematoxylin, dehydrated and fixed in gradient ethanol. IHC staining is based on the percentage of positively stained tumor cells, 0 means less than 5% of tumor cells stain positive, 1 means 5-30%, 2 means 31-50%, 3 means 51-80%, 4 means > 80% positive staining. The expression of RASA1 in the TNBC specimen was divided into a low expression group (0-1 points) and a high expression group (2-4 points) according to the score.

### Animal experiments

Breast cancer cells ( $5 \times 10^6$ ) were injected subcutaneously into female nude mice. The mice were sacrificed 4 weeks later and the tumor was excised and the tumor weight was measured. An animal model of lung metastasis was constructed by injecting breast cancer cells ( $1 \times 10^6$ ) into nude mice through the tail vein. Detection of lung metastases was performed under anesthesia by a small animal in vivo imaging system (Bruke MI, USA). After 6 weeks, the mice were sacrificed and the lungs were removed and the number of lung metastatic nodules was counted. It was confirmed by microscopy after staining with hematoxylin and eosin (HE). Animal experiments were approved by the Sun Yat-sen University Cancer Center Animal Experimental Ethics Committee and conducted at the Sun Yat-sen University Cancer Center Animal Experimental Center.

### RNA immunoprecipitation (RIP) assay

For the GFP-MS2-RIP assay, MS2bs-circAHNAK1, MS2bs-circAHNAK1mt or MS2bs-Rluc and MS2bp-GFP were used to co-transfect cells. After 48 hours, RIP assays were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) to determine if the RNA complex contained circAHNAK1 and its potentially binding miR-421. The immunoprecipitated RNA was subsequently quantitatively analyzed by qRT-PCR. For Ago2-RIP

assay, anti-Ago2 antibody (Millipore) was used for immunoprecipitation. RNA complex contained miR-421 and its potential binding circAHNAK1 or RASA1 mRNA were then purified and quantified by qRT-PCR.

### Luciferase reporter assay

The circAHNAK1 sequence containing the miR-421 binding site (AGGACCCAAGGUGGA-UGUUGAAG) was synthesized and cloned into the luciferase vector pGL3 (Promega). Mutant miR-421 binding sites were performed using the Fast SiteDirected Mutagenesis Kit (TIANGEN, China). The wild type and mutant 3'UTR (UUUCUCUUGCUGGAACUGUUGAA) of RASA1 containing the miR-421 binding site was synthesized and cloned into the luciferase vector. The vectors constructed above were then co-transfected with the miR-421 mimics or inhibitor. Luciferase activity was detected by the dual luciferase reporter kit (Promega) 48 hours after transfection.

### Statistical analysis

Statistical analysis was performed using SPSS 21.0 statistical software. Measurement data were determined statistically by Student's t-test (two-tailed) ( $P < 0.05$ ), while count data was assessed by chi-square test. Differences in survival were assessed using Kaplan-Meier plots and log-rank tests.

### AUTHORS CONTRIBUTIONS

XMx and HLT designed these experiments. Experiments were conducted with WKX, SQZ and Ytz. WKX, SQZ, Ytz, ALY and XHX analyze and interpret these data. WKX and SQZ are the main contributors to writing manuscripts. The final draft read and approved by all authors.

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### CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

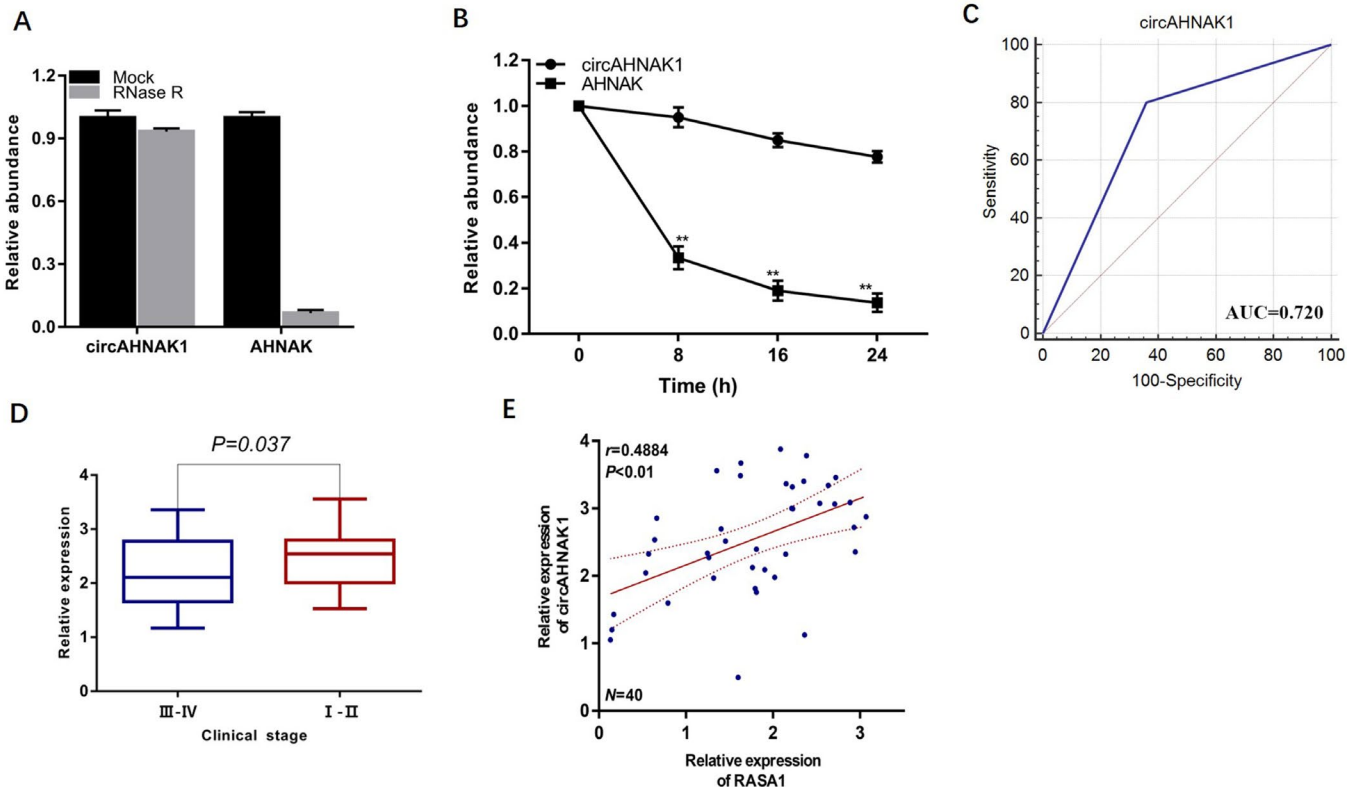
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## SUPPLEMENTARY MATERIALS

### Supplementary Figure



**Supplementary Figure 1. Characterization of circular RNA CircAHNAK1 in TNBC.** (A) Analysis of abundance of circAHNAK1 and AHNAK mRNA in MDA-MB-231 cells treated with RNase R by qRT-PCR; (B) qRT-PCR analysis of the abundance of circAHNAK1 and AHNAK mRNA after actinomycin D treatment of MDA-MB-231 cells; (C) ROC analysis suggested that circAHNAK1 can be used as a diagnostic indicator for distinguishing between TNBC and normal breast tissue; (D) circRNA is significantly down-regulated in stage III-IV TNBC tissues compared to stage I-II; (E) Correlation analysis showed a significant linear correlation between circAHNAK1 and RASA1.

## Supplementary Table

Supplement Table 1. Primer sequences for qRT-PCR used in this study.

Construct	Primers	Sequence (5' - 3')
circAHNAK1	Forward	CATGCCTGATGTGGACCTGA
	Reverse	CAGTCTGGGCCTTGAACCTC
18S	Forward	TTAATTCCGATAACGAACGAGA
	Reverse	CGCTGAGCCAGTCAGTGTAG
$\beta$ -actin	Forward	AGCGAGCATCCCCAAAGTT
	Reverse	GGGCACGAAGGCTCATCATT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG
RASA1	Forward	TGTAGTTGAGGTCAATGAAGGG
	Reverse	TGGCCACCTGTTCTCCTCGTATT