Hsa_circ_0084904 drives cervical cancer advancement by modulating the miR- 578/AURAK pathway

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Abstract

Cervical cancer (CC) is associated with elevated prevalent rates. Circular RNAs (circRNAs) play vital functions in the pathogenesis of various cancers, such as CC. Here, we evaluated the function and associated molecular mechanisms of hsa_circ_0084904 (circKIAA1429) in CC progression. Analysis of dataset GSE102686 showed that hsa_circ_0084904 expressions were elevated. Function assays found that hsa_circ_0084904 knockdown repressed cell proliferation, formation of colonies, as well as invasion capacities *in vitro*. *In vivo*, it reduced tumor progression. Furthermore, in CC cells, hsa_circ_0084904 was shown to upregulate AURKA by sponging miR-578. MiR-578 silencing or AURKA overexpression reversed the effects associated with hsa_circ_0084904 silencing. In conclusion, hsa_circ_0084904 is a potential regulator of CC tumorigenesis via modulation of miR-578/AURKA axis and highlight a therapeutic potential for hsa_circ_0084904 against CC.

Highlights

1. hsa_circ_0084904 upregulated in CC
2. hsa_circ_0084904 promoted CC progression
3. hsa_circ_0084904/miR-578/AURKA axis in CC

Introduction

Globally, cervical cancer (CC) is the 4th commonest malignancy among women [1, 2]. Human papillomavirus (HPV), a double stranded DNA virus, is a predisposing factor for CC [3]. Despite significant progress in the understanding of CC pathogenesis, tumor metastases have led to poor 5-year overall survival outcomes [4, 5]. Thus, elucidation of the molecular mechanisms associated with CC will aid in identification of effective biomarkers and therapeutic targets.

Circular RNAs (circRNAs) are products of non-canonical back splicing of pre-messenger RNA into a closed-loop structure lacking polarity [6, 7]. circRNAs have been implicated in the occurrence as well as progression of various cancer types, including CC [8]. For example, *in vitro,* circRNA_102231 upregulation has been shown to enhance lung cancer cell multiplication, migration, as well as tissue invasion [9]. By activating the Wnt/β-catenin pathway, circRNA_069718 enhances triple-negative breast cancer cell proliferation as well as invasion [10], while hsa_circ_0042666 suppresses laryngeal squamous cell carcinoma cell multiplication, migration as well as tissue invasion by controlling the miR-223/TGFBR3 axis [11]. However, circRNA functions and mechanisms in tumor occurrence, progression and invasion are still largely unclear.

CircRNAs act as miRNA “sponges”, thereby altering miRNAs activity [12, 13]. circPRRC2A enhances renal cell carcinoma cell progression as well as invasion by regulating the miR-514a-5p/miR-6776-5p/TRPM3 axis [14]. In liver cancer, hsa_circ_0039053 has been proposed to be a competing endogenous RNA (ceRNA) that is involved in positive modulation of USP21 expression, when combined with miR-637 [15],

and circ_SMAD2 influences colorectal cancer cell progression by targeting the miR-1258/RPN2 signaling pathway [16]. However, the association between hsa_circ_0084904, miR-578 and AURKA in CC progression is unclear.

Here, we detected a new circular RNA hsa_circ_0084904 (circKIAA1429) in CC tissues and found that its upregulation in CC cells promotes tumor proliferation and invasion. Furthermore, hsa_circ_0084904 upregulated the expression levels of AURKA by sponging miR-578 in CC cells, thereby facilitating CC progression, indicating that hsa_circ_0084904 is oncogenic in CC and that it is a prospective treatment target for CC.

Materials And Methods

Clinical samples

Forty-seven pairs of CC and their corresponding normal tissues were collected from CC patients undergoing surgical resection at the Department of Gynecology, South China Hospital of Shenzhen University. Obtained tissues were kept at -80°C for further analyses. The study participants signed written informed consents declaring their volunteer-ship. Ethical Committee of South China Hospital of Shenzhen University's approved this study.

Cell culture and transfection

Human cervical epithelial immortalized cells (H8) as well as CC cells (CaSki, HeLa, C-33A, SiHa and MS751) were procured from the Chinese Academy of Sciences' cell bank (Shanghai, China). Cell cultures in DMEM (Gibco, Carlsbad, CA, USA) with 10% FBS (Gibco) were incubated at 37°C, 5% CO_2.

SiRNAs against hsa_circ_0084904 (si-hsa_circ_0084904#1, si-hsa_circ_0084904#2, and si-hsa_circ_0084904#3), normal control siRNA (si-NC), miR-578 mimics (miR-578), negative control (miR-NC), miR-578 inhibitors (anti-miR-578) and its negative control (anti-miR-NC), were bought from GenePharma (Shanghai, China). The lipofectamine™2000 reagent (Invitrogen, USA) was employed in transfection assays, as instructed by the manufacturer.

Stimulation with RNase R

Hsa_circ_0084904 and KIAA1429 mRNA levels determined by RT-qPCR. The RNase R (Solarbio) was treated with 2μg of RNA for 30 min. Then, mRNA expressions of hsa_circ_0084904 and KIAA1429 were assessed via RT-qPCR.

Cell viability assay

Briefly, cells cultures were done in 96-well plates (5×10^3 cells/well) followed by incubation for 24, 48, and 72 h. Then, 10 uL of the CCK-8 reagent was added followed by 4 h of incubation at 37℃. Absorbance (450 nm) was determined by a microplate reader.

Colony formation tests

CC cells (1000 cells/well) were inoculated in 6-well plates followed by incubation for 2 weeks. Fixation was done using 4% PFA (Sigma), staining of colonies was done using crystal violet (0.1%; Solarbio) after which colonies with >50 cells were counted and imaged by inverted microscopy (Nikon, Japan).

Transwell assay

For assessment of cell invasion abilities, the upper chamber of 6-well plates (8 μm pore, Millipore, Billerica, MA, USA) was pre-coated with Matrigel and 200μL of serum-free media containing 1×10^5 CC cells seeded. After 24 h, fixation of invading cells was done using 4% PFA (Sigma), followed by staining with crystal violet (0.1%), counting and imaging on an inverted microscope (Nikon, Japan).

Gene expression measurement with RT-qPCR)

Total RNA were obtained from cells and tissues by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples were transformed into cDNA which was subjected to RT-PCR on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) using a SYBR Premix Ex Taq (TaKaRa) with GAPDH as a reference gene for circRNA and AURKA and U6 as reference gene for miR-578. The levels of target genes were calculated with $2^{-\Delta\Delta Ct}$ method.

Dual-luciferase reporter assay

The luciferase reporter assay kit (Promega, Madison, WI, USA) was utilized for this assay. psiCHECK-2 vectors (Promega) were used to construct hsa_circ_0084904-wile type (wt), hsa_circ_0084904-mutant (mut), AURKA-wt, or AURKA-mut reporters. When the cells reached 80% confluence, luciferase vectors containing hsa_circ_0084904-wt, hsa_circ_0084904-mut, AURKA-wt, or AURKA-mut were co-transfected into CC cells with miR-578 or miR-NC. After incubation (48 h), luciferase activities were determined.

RNA immunoprecipitation (RIP) assay

Cell lysis was with RIP kit buffer. The lysate was incubated in the presence of magnetic beads coupled to anti-Ago2 and anti-IgG. Next, protein was eliminated by protease K digestion and immunoprecipitated RNA extracted, followed by RT-qPCR quantification of hsa_circ_0084904 and miR-578 levels.

Statistical analysis

Data are presented as the mean ± SD. SPSS 21.0 (IBM) was used for analyses. Student's t-test compared differences between 2 groups. For comparison of means among groups, one-way ANOVA, followed by post hoc Student's was selected. Significance was set at $p \leq 0.05$.

Results

Hsa_circ_0084904 is highly expressed in CC

To investigate the roles of circRNAs in CC, circRNA microarray expression profiles (GSE102686) were investigated with a focus on the upregulated circRNAs (hsa_circ_0084904) (Figure 1A and 1B). hsa_circ_0084904 levels in CC cell lines as well as tissues were assessed by RT-qPCR. hsa_circ_0084904 was markedly elevated in CC cell lines as well as tissues, relative to adjacent non-tumor tissue and cells (Figure 1C and 1D).

Hsa_circ_0084904 (1346 nt) is located on chr8:95518727-95524317 and is spliced from the KIAA1429 gene (Figure 2A). Hsa_circ_0084904 features were characterized using RNase R assays. RT-qPCR revealed that hsa_circ_0084904, but not KIAA1429 mRNA in CC cells, was resistant to RNase R (Figure 2B and 2C). Moreover, FISH assay showed that hsa_circ_0084904 was found to be majorly localized in the cytoplasm (Figure 2D).

To investigate the mechanisms involved in CC progression, GO and KEGG pathway analyses were conducted on hsa_circ_0084904. GO analysis revealed that hsa_circ_0084904 is related to cell motility (Figure 3A). KEGG pathway analysis indicated hsa_circ_0084904 is related to tumor progression (Figure 3B). These results indicated that hsa_circ_0084904 was stable and may influence CC carcinogenesis.

Hsa_circ_0084904 silencing suppresses CC proliferation and invasion

To determine its function in CC, hsa_circ_0084904 in CC cells were knocked down using siRNA (Figure 4A). Then, CCK-8 as well as colony formation assays showed that proliferative capacities of CC cells were suppressed after hsa_circ_0084904 knockdown (Figure 4B-D). Transwell assay revealed that hsa_circ_0084904 silencing significantly suppressed CC cells metastasis potential *in vitro* relative to controls (Figure 4E). These findings imply that hsa_circ_0084904 knockdown suppresses CC progression.

Hsa_circ_0084904 is a miR-578 sponge

Various studies show that circRNAs are miRNA sponges [6, 12]. To establish the mechanisms of hsa_circ_0084904 in CC, we estimated which miRNAs may possess site complementarity to hsa_circ_0084904 using circInteractome and CSCD (Figure 5A-B). miR-578 had complementary sites to hsa_circ_0084904 (Figure 5C). Moreover, miR-578 mimics suppressed the luciferase activities of the hsa_circ_0084904-wt reporter in CC cells (Figure.5D and 5E). RIP assay revealed miR-578 and hsa_circ_0084904 co-immunoprecipitation with Anti-Ago2 in CC cells (Figure 5F). These results imply that hsa_circ_0084904 sponges miR-578 in CC cells.

miR-578 targets AURKA

To establish the specific mechanism through which hsa_circ_0084904/miR-578 influences CC, we searched for potential miR-578 target genes expression (Figure 6A and 6B) in TCGA datasets, and focused on AURKA, which is reported to be involved in tumorigenesis [17]. Our data revealed AURKA upregulation in CC tissues, which correlated inversely with overall survival in CC patients (Figure 6C and 6D). Dual-luciferase reporter assays showed that miR-578 overexpression markedly suppressed the WT-AURKA -3'UTR luciferase activity (Figure 6E and 6F). In addition, RT-qPCR investigation revealed that

overexpression of miR-578 significantly suppressed AURKA expression in CC cell lines (Figure.6G). These data suggest AURKA as a miR-578 target in CC progression.

Hsa_circ_0084904 promoted CC progression via miR-578/AURKA

We further confirmed the function of hsa_circ_0084904/miR-578/AURKA axis in the progression of CC. hsa_circ_0084904 suppression significantly down-regulated AURKA expression levels while miR-578 inhibitors rescued the effects of si-circ_0084904 on AURKA levels in CC cells (Figure 7A). *In vitro,* EdU as well as transwell assays revealed that miR-578 inhibition or AURKA upregulation alleviated the effects of hsa_circ_0084904 silencing on CC cells (Figure 7B and 7C). Therefore, hsa_circ_0084904 acts as an oncogene in CC via the miR-578/AURKA axis.

Hsa_circ_0084904 silencing reduced tumor progression *in vivo*

To validate the roles of hsa_circ_0084904 *in vivo*, xenograft assays were performed, and it was found that hsa_circ_0084904 silencing markedly inhibited tumor growth (Figure 7D-7F). We also found that hsa_circ_0084904 and AURKA expression levels decreased, while miR-578 increased in xenograft tumors in the sh-hsa_circ_0084904 group, relative to the sh-NC group (Figure 7G). These data show that hsa_circ_0084904 silencing blocked tumorigenesis *in vivo*.

Discussion

Numerous studies show that circRNAs influence cancer development and progression [4, 13] and that circRNAs have diagnostic and therapeutic potential in CC. For example, hsa_circ_0000745 enhances CC cell proliferation, migration, as well as invasion by downregulating E-cadherin expression [18], circAMOTL1 promotes AMOTL1 expression to facilitate CC growth [19], and circ_0067934 overexpression enhances CC growth via the miR-545/EIF3C axis [20]. Here, we identified hsa_circ_0084904 as a novel circRNA and confirmed its stability by Rnase R digestion. Analysis of hsa_circ_0084904 function showed that hsa_circ_0084904 is oncogenic in CC.

Mounting evidence indicate that circRNAs exert either oncogenic or tumor suppressive effects by acting as competing endogenous RNAs (ceRNAs) for miRNAs [21]. Predictive analysis of the potential miRNA target for hsa_circ_0084904 indicated that it may sponge MiR-578. MiR-578 is a tumor repressor in cancers. Chen et al showed that circZFR silencing suppressed breast cancer malignant progression *in vitro* by regulating the miR-578/HIF1A axis [22]. Ji et al showed that circ_001621 enhances osteosarcoma cell proliferation, migration as well as invasion by sponging miR-578 and regulating the expression of VEGF [23]. However, the functions of miR-578 in CC have not been established. Here, we detected reduced miR-578 expression in CC tissues. MiR-578 downregulation rescued the interference of si-hsa_circ_0084904 on CC cells proliferation and invasion. Therefore, hsa_circ_0084904 modulates the progression of CC by sponging miR-578.

Aurora kinase A (AURKA), a sequence-specific transcriptional promoter is implicated in many cancers. For example, Chen et al found that AURKA enhances hepatocellular carcinoma metastasis by regulating EMT and cancer stem cell properties [24]. Wang-Bishop et al. found that AURKA suppression inhibited gastrointestinal cancer cells with activated KRAS by preventing activation of RPS6KB1 [25]. Zhen et al suggested that lncRNA00958 enhances bladder cancer cell migration and invasion through the miR-490-3p/AURKA axis [26]. Mounting evidence show that miRNAs regulate gene expression levels by binding the 3'UTR of mRNA [27]. Here, we showed that AURKA is a miR-578 target and AURKA overexpression rescues CC progression by silencing hsa_circ_0084904. Indicating that hsa_circ_008490 enhances CC cell proliferation and invasion by modulating the miR-578/AURKA axis.

Conclusion

Hsa_circ_0084904 promotes CC development by elevating AURKA expression through sponging miR-578. Therefore, hsa_circ_0084904 is a latent target for CC treatment.

Abbreviations

circRNAs: Circular RNAs

CCK-8: Cell Counting Kit-8

qRT-PCR: Quantitative real time Polymerase Chain Reaction

cDNA: complementary DNA

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

AHW and YW designed the research and revised the manuscript; **YW, LXL, CHJ** performed the experiments and drafted the manuscript; **JJY and XYT** collected the data and did the analysis; All authors read and

approved the final manuscript.

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Not Applicable

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Figures

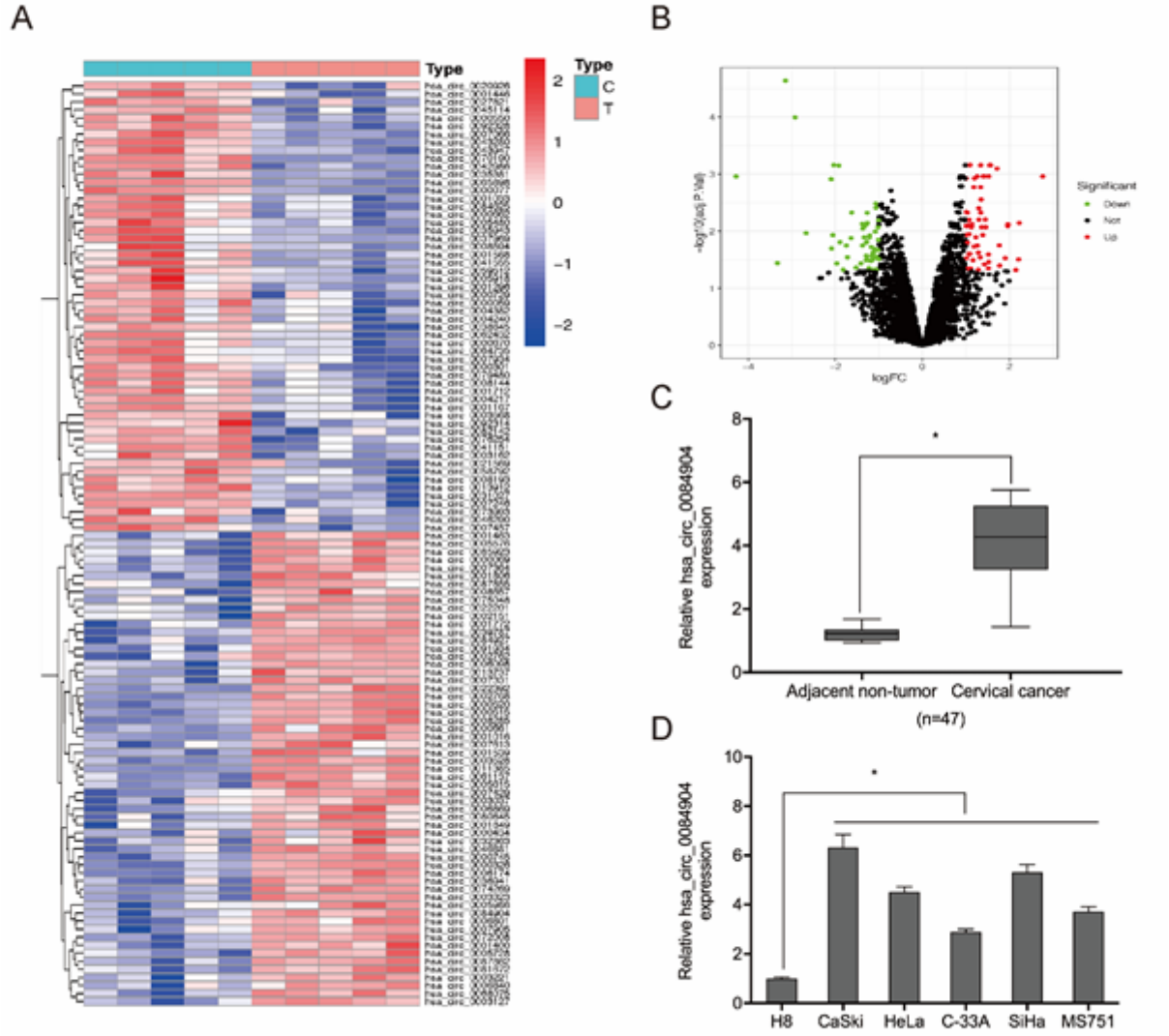

Figure 1

Hsa_circ_0084904 is highly expressed in CC. (A) Cluster heatmaps of the 20 most increased and decreased circRNAs. (B) Volcano plot visualization of circRNA expression in CC vs adjacent non-tumor tissues. (C, D) Hsa_circ_0084904 expression levels in CC tissues and cell lines were determined by RT-qPCR. *P=<0.05.

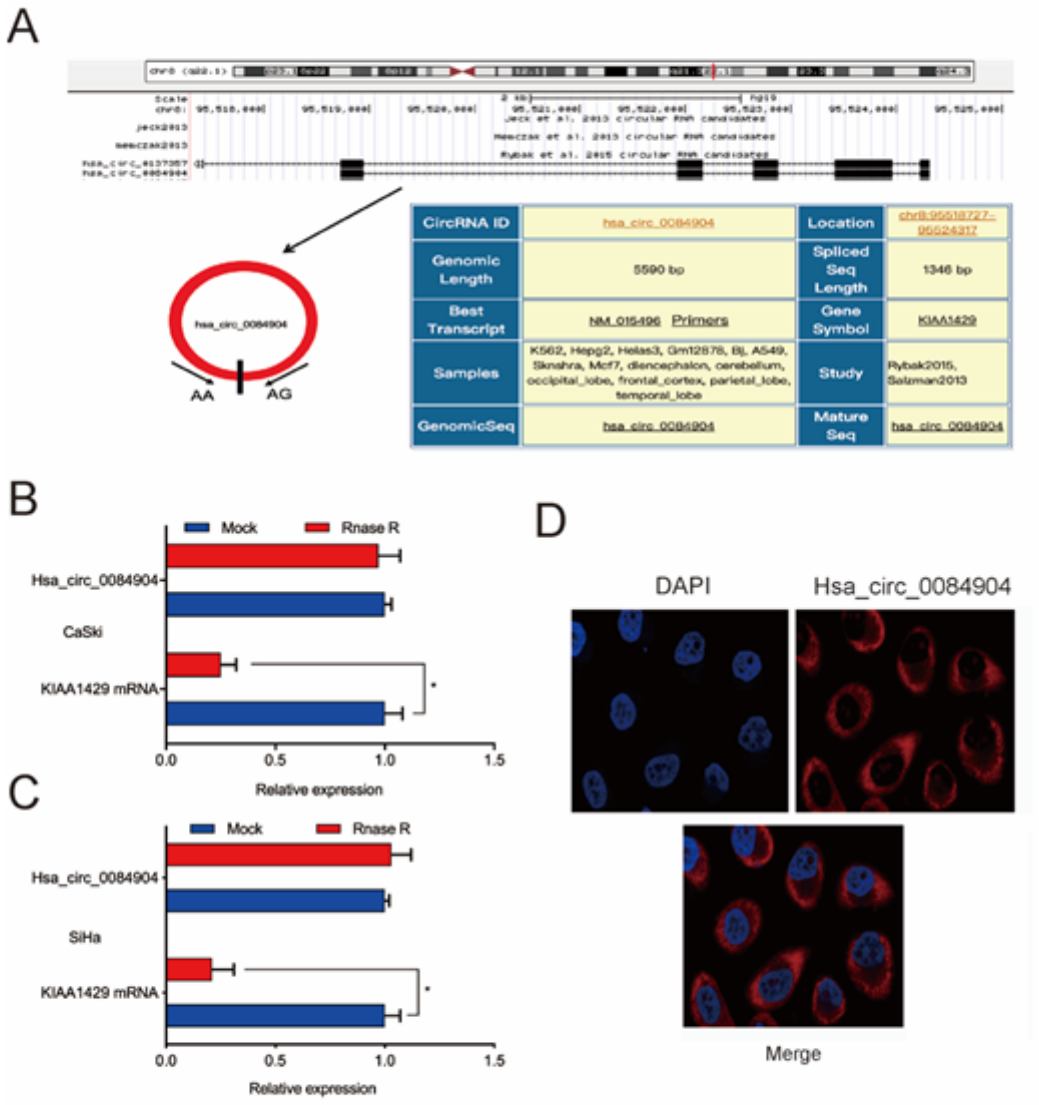

Figure 2

Characteristics of hsa_circ_0084904. (A) Schematic of hsa_circ_0084904. (B, C) Hsa_circ_0084904 and KIAA1429 mRNA expression levels in CC cells under RNase R treatment. (D) Subcellular fractionation revealed the predominant location of hsa_circ_008490 in CC cells. *P=<0.05.

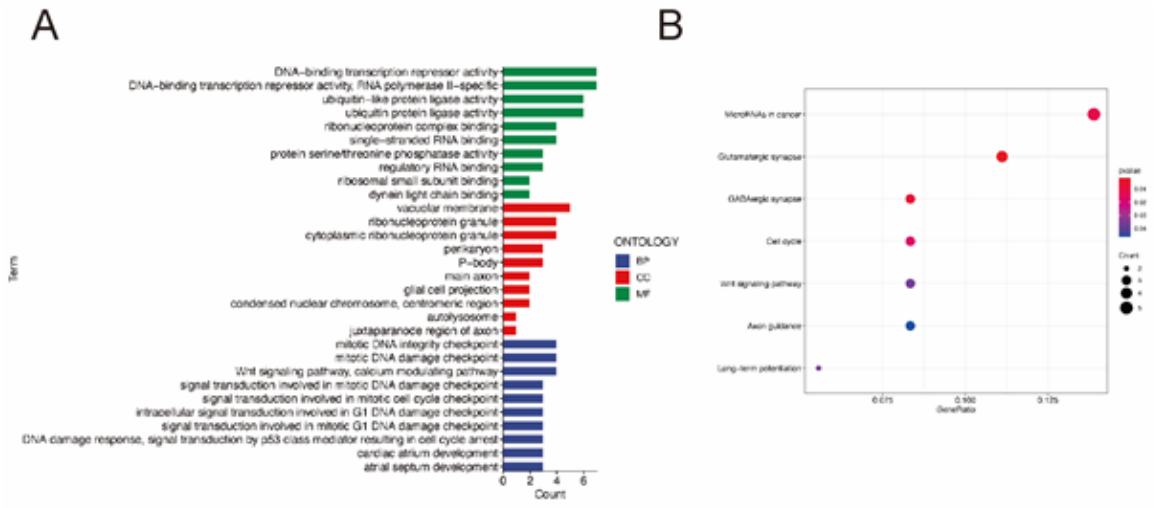

Figure 3

GO and KEEG pathway analysis of hsa_circ_0084904 in CC. (A) Top 10 GO processes of CircTXNDC11 enrichment in molecular function (MF), biological process (BP), as well as cellular component (CC). (B) Top 10 pathways in which hsa_circ_0084904 was enriched, as predicted by KEGG analysis.

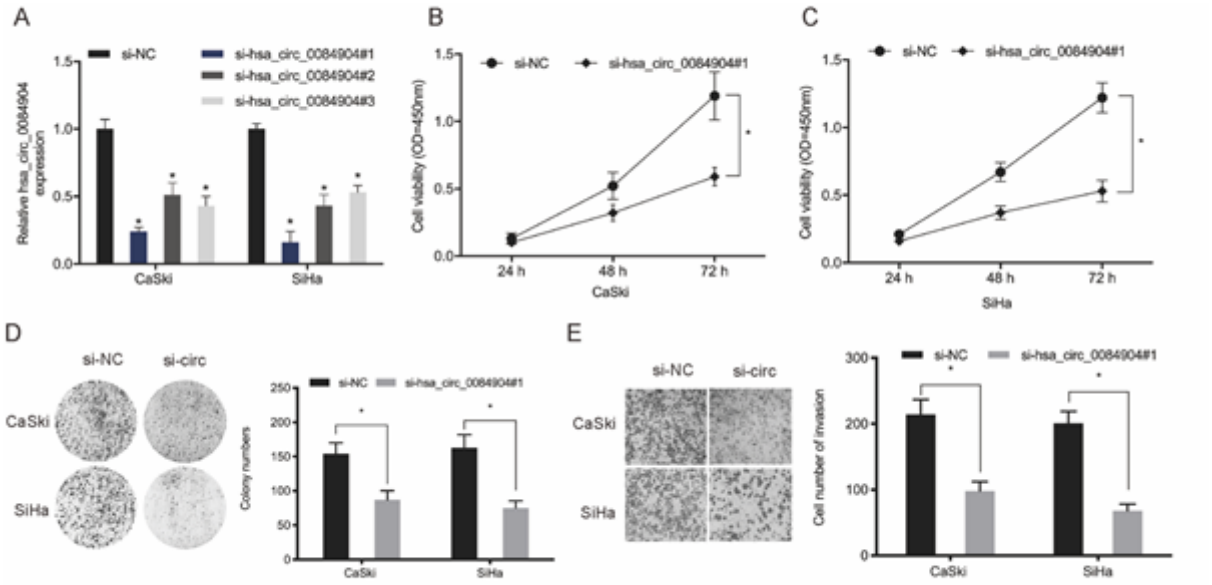

Figure 4

Hsa_circ_0084904 knockdown suppressed CC cell proliferation as well as metastasis *in vitro*. CaSki and SiHa cells were transfected with si-NC or si-circ_0084904. (A) Si-circ_0084904 knockdown efficiency was assessed by RT-qPCR analysis. (B-D) Cell viabilities were determined by CCK-8 as well as colony formation assays. (E) Cell invasiveness were assessed by Transwell assays. *P=<0.05.

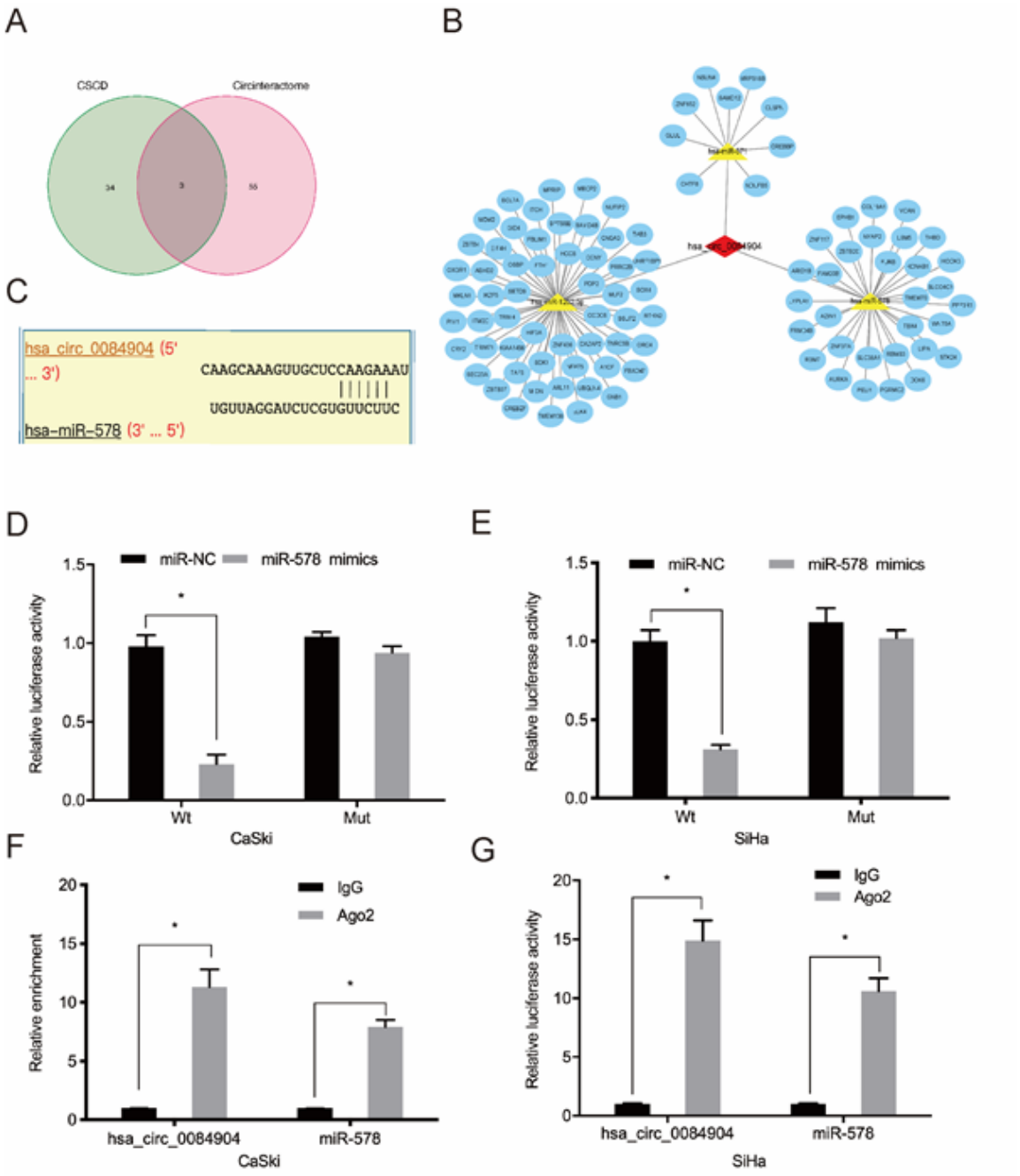

Figure 5

Hsa_circ_0084904 sponges miR-578 in CC cells. (A, B) 3 miRNAs predicted by circInteractome and CSCD to bind hsa_circ_0084904. (C) Predicted binding sites between hsa_circ_0084904 and miR-578. (D-G) The interaction between hsa_circ_0084904 and miR-578 were explored by luciferase activities assay and RIP assay. *P=<0.05.

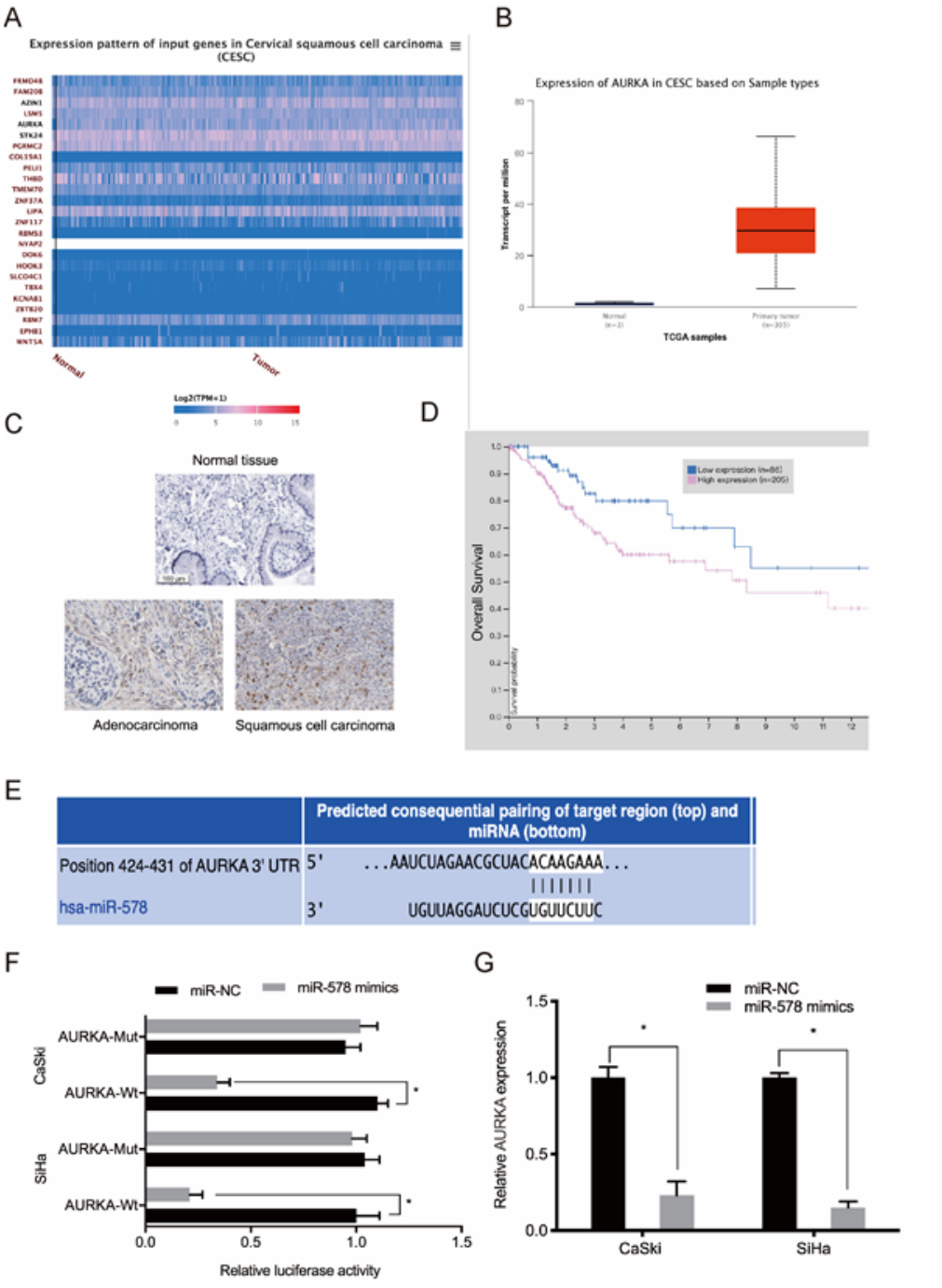

Figure 6

AURKA is a miR-578 target. (A) Potential targets expression was explored in TCGA datasets. (B-D) AURKA expression was upregulated and showed negative correlation with overall survival in CC patients. (E, F) The predicted binding site of AURKA and miR-578. (G) MiR-578 overexpression reduced AURKA expression in CC cell lines. *P=<0.05.

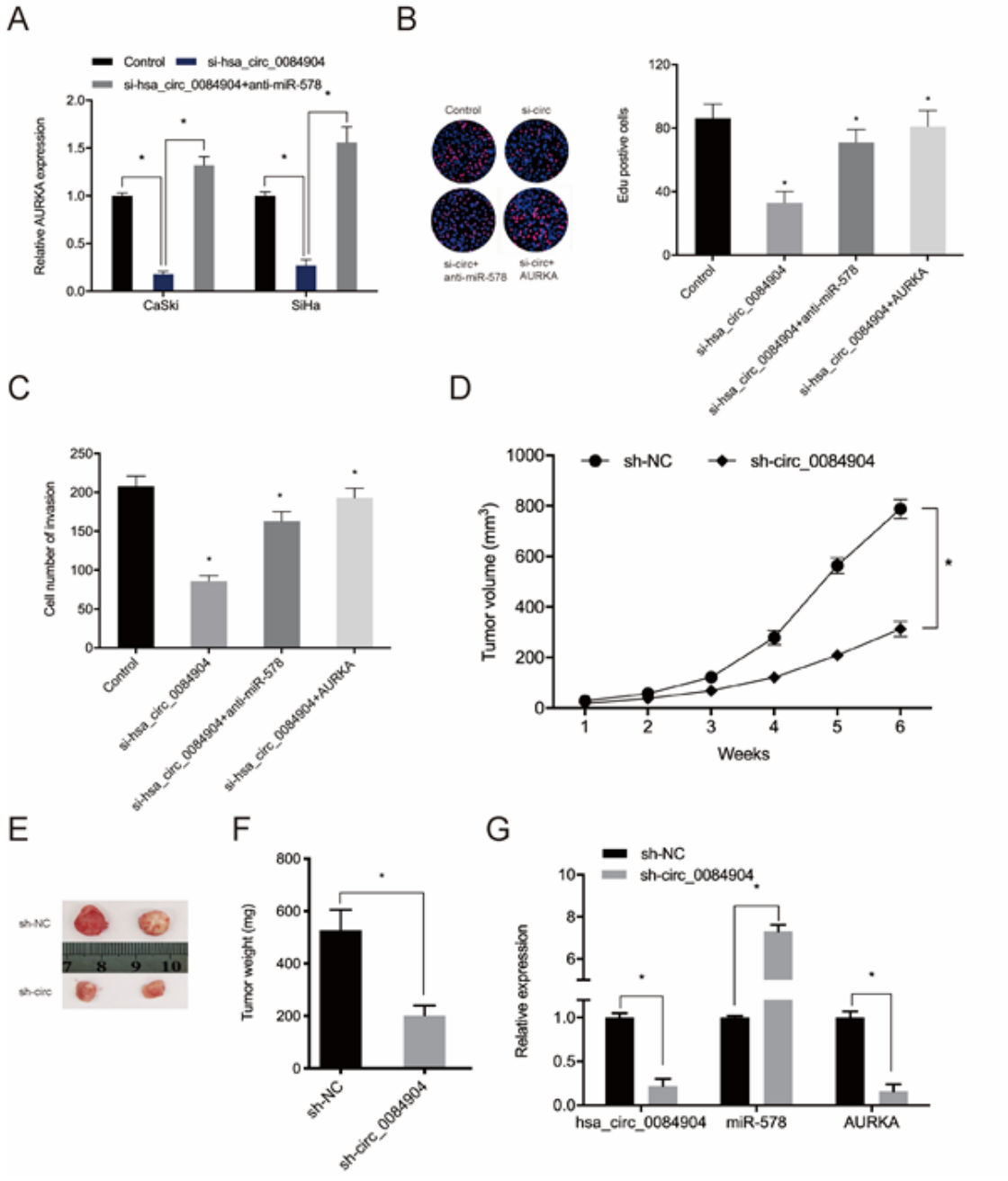

Figure 7

Hsa_circ_0084904/miR-578/AURKA axis in CC. (A) miR-578 inhibitors rescued the effects of si-circ_0084904 on AURKA levels in CC cells. (B, C) MiR-578 inhibition or AURKA upregulation ameliorated the outcomes of hsa_circ_0084904 silencing on CC cells proliferation as well as invasion *in vitro*. (D-F) Tumor volume and weights were examined. (G) hsa_circ_0084904, miR-578, and AURKA levels in xenograft tumors were measured by RT-qPCR. *P=<0.05.