Original paper

ANRIL regulates retinoblastoma progression *via* targeting autophagy by miR-328-3p/TSC1/ULK signaling

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Retinoblastoma is the most common primary intraocular malignancy of childhood. The aim of our study was to investigate the role and regulatory mechanism of the long non-coding RNA ANRIL in retinoblastoma.

Here, our data demonstrated that ANRIL overexpression inhibited miR-328-3p expression, but promoted expression of autophagy-related proteins (LC3B, ATG5, and BECN1). Then we predicted the binding sites for ANRIL with miR-328-3p, and for miR-328-3p with TSC1/ULK2 3′-UTR, and confirmed the combination of miR-328-3p and ANRIL and TSC1/ULK2 3′-UTR.

Importantly, the data showed that ANRIL overexpression promoted TSC1 and ULK2 expression, and inhibited the phosphorylation of mTOR. Finally, our results indicated that ANRIL overexpression facilitated Y79 cell proliferation and cisplatin-induced apoptosis.

Our results indicated that ANRIL promoted the proliferation and cisplatin resistance of Y79 cells through activating autophagy by promoting TSC1/ULK2 expression *via* acting as a miR-328-3p sponge.

Key words: retinoblastoma, competing endogenous RNA, long non-coding RNA, cisplatin resistance, proliferation.

Introduction

Retinoblastoma is the most common neoplasm of the eye in children, and is the only central nervous system tumour readily observed without specialized equipment [1]. Generally, retinoblastoma is diagnosed at a very early stage of a child's life. The therapeutic approaches for cancer need to consider curing the disease as well as preserving vision with minimal long-term side effects [2]. At present, the common therapeutic methods for retinoblastoma involve intravenous chemoreduction, focal therapy for tumour consolidation, local administration routes of chemotherapy, external beam radiotherapy, and surgical enucleation [3–5]. Exploring the pathogenesis and identifying new therapeutic targets of retinoblastoma are still crucial.

Most of the human genome encodes RNAs that do not code for proteins, called non-coding RNAs, which include microRNA, circular RNA, siRNA, and long non-coding RNAs (lncRNAs). Among the non-coding RNAs, the length of lncRNAs is greater than 200 nucleotides [6]. In recent years, growing evidence has shown that the abnormally expressed lncRNA prolife participates in the pathogenesis of many human diseases, for example, malignant tumours [7, 8]. LncRNAs are widely considered as a promising therapeutic target for many disorders [9]. Gao *et al*. reported that

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lncRNA MALAT1 and NKILA are downregulated in retinoblastoma tissues and cell lines (Weri-Rb1 and Y79 cells), facilitate retinoblastoma cell apoptosis, and suppress cell proliferation [10]. He *et al*. reported that lncRNA RBAT1 is upregulated in bladder cancer tumour tissues and retinoblastoma tissues. RBAT1 could boost the progression of retinoblastoma and the growth of the tumour *via* targeting E2F3 [11]. Recently, more and more retinoblastoma-related lncRNA have been identified. Some lncRNAs could regulate the progression of retinoblastoma through a competing endogenous RNA (ceRNA) mechanism. LncRNA competes with the target of the miRNA for binding, hence upregulating expression of the miRNA target and inhibiting expression of the miRNA [12, 13]. ANRIL is an lncRNA at the *CDKN2A/B* genomic locus [14]. In multiple cancers such as acute myeloid leukemia, gastric cancer as well as retinoblastoma, the level of ANRIL gene expression was reported to be upregulated [15–17]. ANRIL could regulate the proliferation and apoptosis of retinoblastoma cells, and the resistance of retinoblastoma cells to cisplatin through acting as a miRNA sponge and by a ceRNA mechanism [17]. Here, we explored the mechanism of action of ANRIL in regulation of retinoblastoma cell apoptosis, proliferation, and cisplatin resistance through another pathway.

Autophagy is an ancient mechanism controlled by a series of proteins including autophagy-related genes (ATGs), LC3, rapamycin (mTOR), and BECN1 [18]. It was reported that autophagy plays a crucial role in the progression of cancer. Recently, Yao *et al*. reported that lncRNA XIST silencing could suppress the proliferation and autophagy of retinoblastoma cells, and strengthen the sensitivity of the cancer cells to vincristine by targeting miR-204-5p and its downstream target [19]. It is not clear that whether ANRIL regulates the progression of retinoblastoma through targeting autophagy. Our data verified that ANRIL could regulate the proliferation and cisplatin resistance of retinoblastoma cells through activating cell autophagy by promoting the expression of tuberous sclerosis complex 1 (TSC1) and unc-51 like autophagy activating kinase 2 (ULK2) *via* acting as a miR-328-3p sponge. Our data may provide new evidence that ANRIL acts as a potential target for retinoblastoma treatment.

Materials and methods

Cell culture and treatment

Retinoblastoma cell line Y79 was obtained from American Type Culture Collection (ATCC; USA). The lentivirus packaging with pcDNA-ANRIL or an empty vector was purchased from WZ Biosciences Inc. (Shandong, China). The pcDNA-ANRIL vector is an ANRIL overexpression vector. Y79 cells (1×10^5 cells *per* well) were planted into 6-well plates, and were infected with the ANRIL overexpression lentivirus (MOI = 50) for 48 hours. All cells were cultured in RPMI-1640 medium (HyClone, UT, USA) supplemented with 20% fetal bovine serum (FBS; Gibco, CA, USA) and 1% penicillin-streptomycin (Gibco, CA, USA) in an incubator with 5% CO_2 and 37°C atmosphere.

Quantitative real-time polymerase chain reaction assay

The total RNA in retinoblastoma cells was isolated using TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Subsequently, cDNA was synthesized using PrimeScript Master Mix (Takara, Otsu, Shiga, Japan). Next, quantitative real-time polymerase chain reaction (PCR) was carried out using a SYBR Green PCR kit (Takara). All processes were carried out according to the instructions of the kits. Here, *GAPDH* served as the internal reference. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The total protein in retinoblastoma cells was extracted using RIPA buffer (Solarbio, Beijing, China), and then the concentration of the proteins was measured using a BCA kit (Solarbio). After that, total protein samples were separated through a 12% SDS-PEGF gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane. Subsequently, the PVDF membranes were maintained with 5% non-fat milk for 1 hour at room temperature followed by primary antibodies, including anti-LC3B (1 : 2000, Abcam), anti-ATG5 (1 : 2000, Abcam), anti-BECN1 (1 : 2000, Abcam), anti-TSC1 (1 : 1000, Abcam), anti-ULK2 (1 : 2000, Abcam) and p-mTOR (1 : 1000, Abcam), at 4°C overnight. Then, the PVDF membranes were incubated with secondary antibodies for 1 hour at room temperature. Finally, the protein bands were visualized using an ECL kit (Solarbio). Here, β-actin served as an internal reference.

Luciferase activity assay

Y79 cells were co-transfected with a pGL3-based vector expressing TSC1 3′-UTR, ULK2 3′-UTR or the sequence which binds with miR-328-3p with miR-328-3p mimic or negative control mimic usage Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells were collected, lysed, and then utilized for the analysis of the firefly and Renilla luciferase activities through the Dual-Luciferase Reporter Assay System (Promega) at 48 hours after cell transfection. The activity of Renilla luciferase was used as an internal reference.

CCK-8 assay

CCK-8 assay was performed to determine the proliferation of Y79 cells using a CCK-8 kit (Solarbio). Y79 cells were plated into 96-well plates at the density of 4×10^4 cells *per* well, and were infected with the ANRIL overexpression lentivirus for 24, 48, 72, and 96 hours. After that, 10 μl of CCK-8 reagent was added to each well. Y79 cells were incubated with the reagent for another 4 hours. Finally, the optical density of the cells at 450 nm wavelength was determined on a microplate reader.

Flow cytometry

Y79 cells were planted into 96-well plates at the density of 4×10^4 cells *per* well. Lentivirus infection was accomplished according to the experimental purpose, and then the cells were treated with 0, 5, 10, and 15 μmol/l cisplatin for 24 hours. Then, according to the manufacturer's protocol, cell apoptosis was detected through a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA). The horizontal coordinate FITC in the flow cytometry results represents that FITC-Annexin V selectively binds phosphatidylserine and thus detects apoptosis. Longitudinal coordinate propidium iodide is a DNA-binding dye that detects cell necrosis.

Statistical analysis

All experiments were run in triplicates and the results were presented as mean ± SD, and were analyzed using GraphPad Prism 7 software. The p-values were calculated by Student's t-test. $P < 0.05$ was deemed statistically significant.

Results

ANRIL overexpression facilitated autophagy of retinoblastoma cells

ANRIL has been reported to facilitate malignant behaviors, including migration, proliferation and invasion, of retinoblastoma cells *via* some typical signaling pathways [20]. Here, we explored the influence of ANRIL on the autophagy of retinoblastoma cells. The expression of ANRIL was markedly increased by infection of ANRIL overexpression lentivirus (Fig. 1A). Nevertheless, the level of miR-328-3p was downregulated by the infection of ANRIL overexpression lentivirus (Fig. 1B). The protein levels of LC3B, ATG5 and BECN1 were also upregulated by ANRIL overexpression (Fig. 1C–F). In accordance with the initial inspection results, ANRIL inhibited the miR-328-3p expression and promoted retinoblastoma cell autophagy.

ANRIL completes miR-328-3p with TSC1 mRNA 3′-UTR

To elucidate the regulatory mechanism of ANRIL in retinoblastoma cell autophagy, we obtained the binding sites between ANRIL and miR-328-3p through the starBase v2.0 database. The luciferase reporter assay showed that ANRIL-WT could downregulate the luciferase activity of the retinoblastoma cells treated with miR-328-3p mimic, while ANRIL-MUT had no effect on the transfected cells (Fig. 2A). Then, we predicted the binding sites between miR-328-3p and TSC1 3′-UTR through the TargetScan Human and miRanda databases, and detected the combination of miR-328-3p and TSC1 3′-UTR. As shown in Figure 2B, TSC1-WT significantly downregulated the luciferase activity of the retinoblastoma cells treated with miR-328-3p mimic (Fig. 2B). Moreover, we predicted the binding sites between miR-328-3p and ULK2 3′-UTR using TargetScan Human and miRanda databases. Our results indicated that ULK2-WT significantly downregulated the luciferase activity of the retinoblastoma cells treated with miR-328-3p mimic (Fig. 2C). Overall, the above results suggested the combination of ANRIL and miR-328-3p, and proved that TSC1 and ULK2 were two downstream targets of miR-328-3p.

ANRIL promoted retinoblastoma cell autophagy through increasing TSC1/ULK2

To explore whether ANRIL regulates the autophagy of retinoblastoma cells by targeting TSC1 and ULK2, we detected the expression of TSC1 and ULK2 in the retinoblastoma cells infected with ANRIL overexpression lentivirus. As shown in Figure 3A, ANRIL facilitated TSC1 and ULK2 mRNA expression in retinoblastoma cells. Consistently, ANRIL overexpression promoted the expression of TSC1 and ULK2 proteins in retinoblastoma cells. Importantly, ANRIL overexpression inhibited the expression of phosphorylated mTOR protein (Fig. 3B, C). It was reported that mTOR is an inhibitor of autophagy [21]. Our data showed that ANRIL may promote the autophagy of retinoblastoma cells through increasing TCS1 and ULK2.

ANRIL promoted retinoblastoma cell proliferation and resistance to cisplatin

To investigate the regulatory effect of ANRIL on retinoblastoma cell behavior, we detected the proliferation of retinoblastoma cells and the resistance of them to cisplatin. As shown in Figure 4A, the cell proliferation was inhibited by ANRIL overexpression. Then, the retinoblastoma cells infected with ANRIL overexpression lentivirus were treated with 0, 5, 10, and 15 μg/l cisplatin for 24 hours. Our data indicated that ANRIL overexpression suppressed cisplatin-induced retinoblastoma cell apoptosis (Fig. 4B, C). In summary, ANRIL overexpression promoted retinoblastoma cell proliferation and resistance to cisplatin.

Discussion

LncRNAs are potential therapeutic targets for many cancers. Numerous studies have suggested

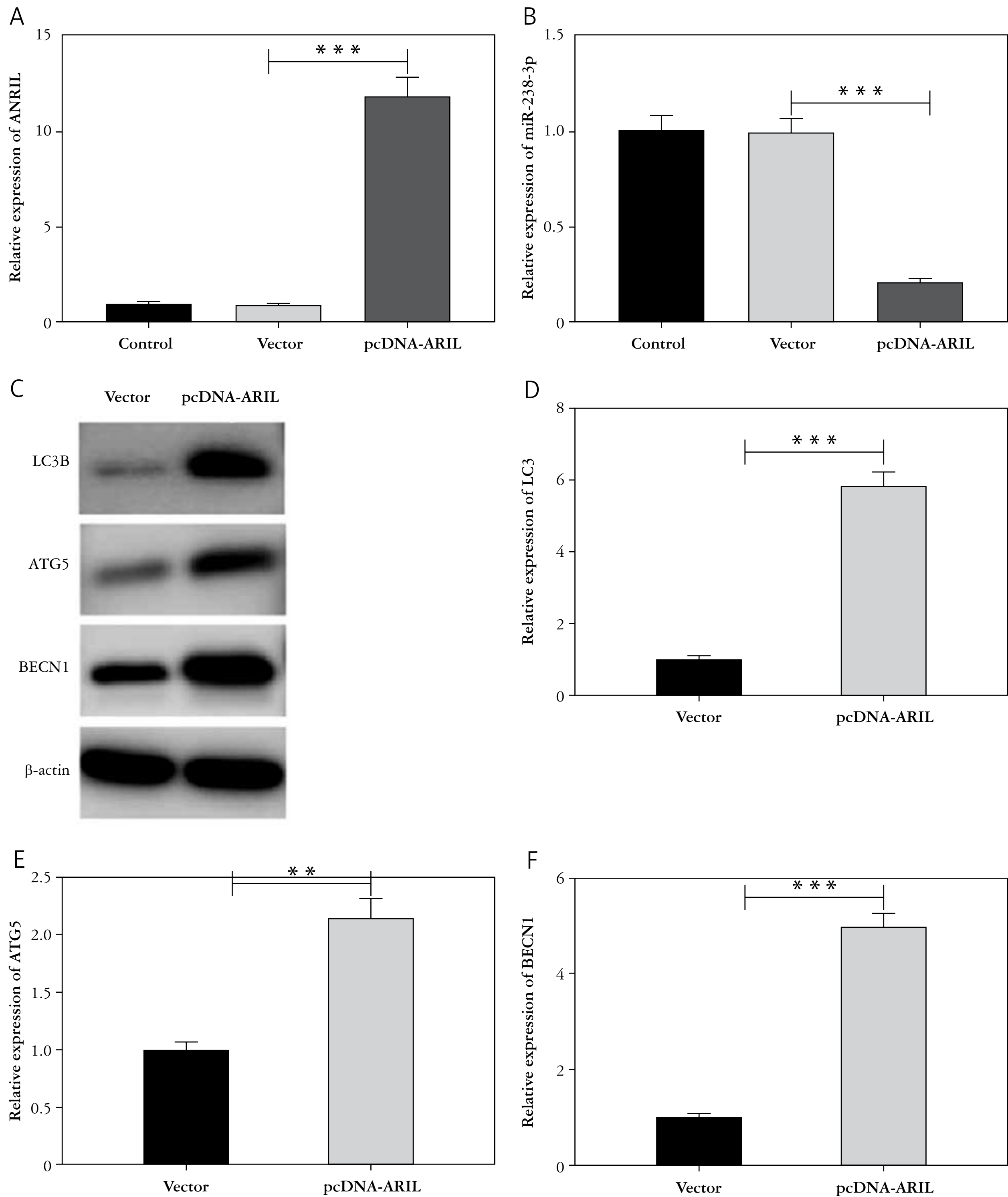

Fig. 1. Effect of ANRIL on miR-328-3p and autophagy-related proteins' expression. Retinoblastoma cells were infected with the lentivirus expressing ANRIL and the control lentivirus. **A**) Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect ANRIL expression in the cells to verify the infection efficiency. **B**) miR-328-3p expression in the cancer cells also was examined through qRT-PCR. **C–F**) Autophagy-related proteins' expression, including LC3B, ATG5 as well as BECN1, was determined using Western blot

*** and *** p-value lower than 0.01*

that lncRNAs play a crucial role in the regulation of retinoblastoma cell proliferation, migration, invasion, tube formation, and drug resistance, as well as the growth of retinoblastoma tumour [22, 23]. For instance, lncRNA ELFN1-AS1 was highly expressed in retinoblastoma tissues and cell lines. High expression of ELFN1-AS1 was positively correlated with the progression and poor prognosis of the disease,

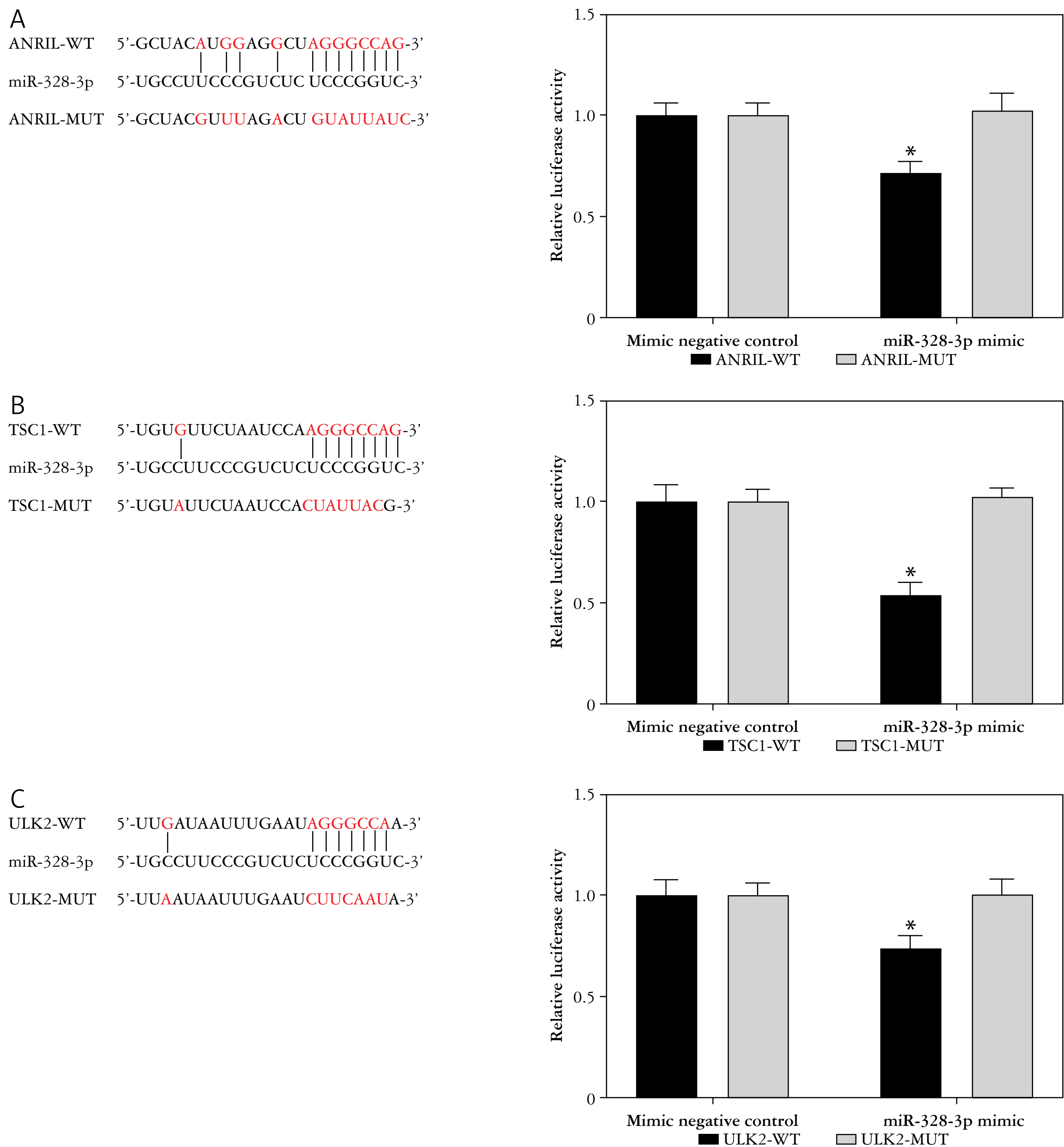

Fig. 2. Detection of the combination of ANRIL and TSC1/ULK2 3′-UTR with miR-328-3p. **A)** StarBase v2.0 was utilized to analyze the binding sites of ANRIL with miR-328-3p, and the combination of them was identified using luciferase reporter assay. **B)** TargetScan Human and miRanda databases were utilized to analyze the binding sites of TSC1 3′-UTR with miR-328-3p, and the combination of them was identified using luciferase reporter assay. **C)** TargetScan Human and miRanda databases were utilized for analysis of the binding sites of ULK2 3′-UTR with miR-328-3p, and the combination of them was identified using luciferase reporter assay

facilitated retinoblastoma cell lines proliferation, and contributed to migration and invasion [24]. Moreover, lncRNA HOTTIP was increased in the retinoblastoma tissues and cell lines. It was reported that HOTTIP knockdown could suppress the proliferation and facilitate the apoptosis of retinoblastoma cells *via* acting as a miR-101-3p sponge [25]. In this study, our results indicated that lncRNA ANRIL could facilitate retinoblastoma cell proliferation, cisplatin resistance and autophagy through promoting TSC1 and ULK2 expression by sponging miR-328-3p.

It was previously reported that miR-328-3p was underexpressed in breast cancer, and participates in regulation of the expression of proliferation-related proteins in breast cancer [26]. Pan *et al*. observed that

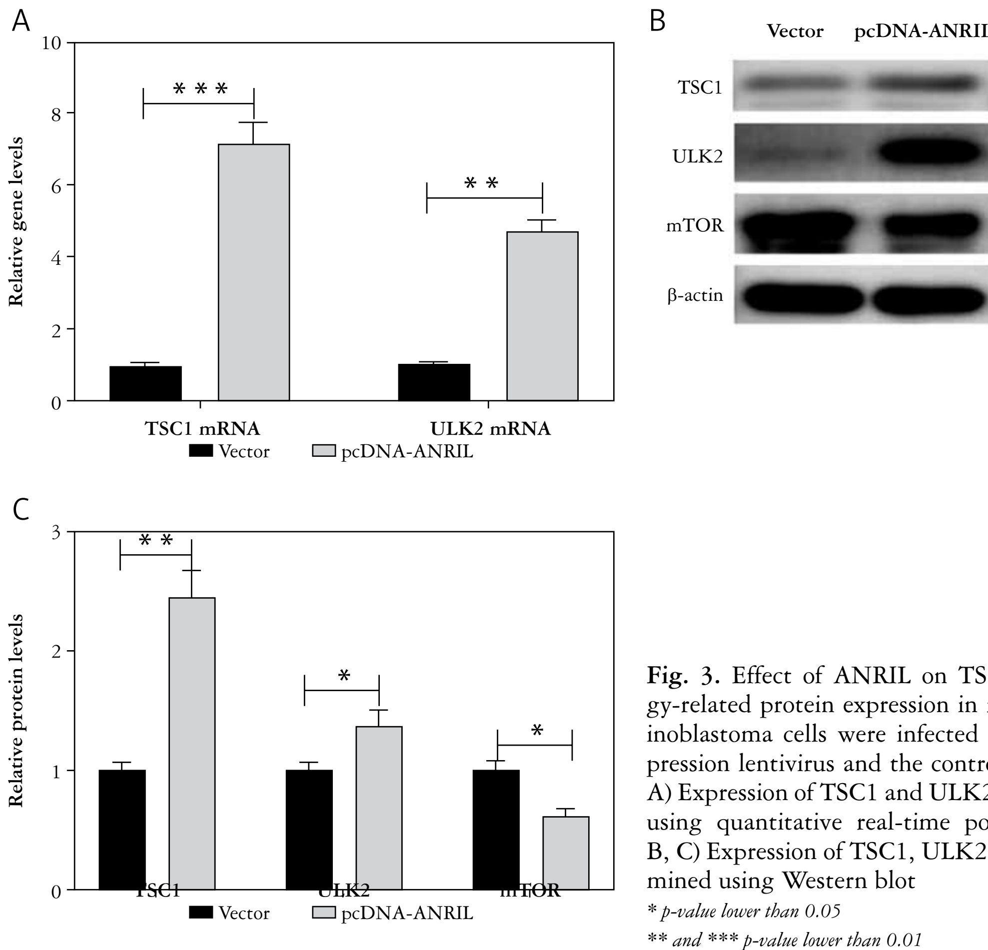

Fig. 3. Effect of ANRIL on TSC1, ULK2 and autophagy-related protein expression in retinoblastoma cells. Retinoblastoma cells were infected with the ANRIL overexpression lentivirus and the control lentivirus for 48 hours. A) Expression of TSC1 and ULK2 mRNAs was determined using quantitative real-time polymerase chain reaction. B, C) Expression of TSC1, ULK2, and p-mTOR was determined using Western blot

** p-value lower than 0.05*

*** and *** p-value lower than 0.01*

overexpression of miR-328-3p in colorectal cancer could inhibit the malignant phenotypes of the cancer cells through PI3K/AKT signaling [27]. In another study, increased levels of miR-328-3p inhibited the malignant behaviors of liver cancer cells, and limited the growth of liver cancer tumours through the PI3K/AKT/mTOR signaling pathway [28]. More and more studies are suggesting that miR-328-3p plays an inhibitory role in cancer development. However, the role of miR-328-3p in retinoblastoma progression is still unclear. Here, our results proved that ANRIL overexpression could inhibit the expression of miR-328-3p in Y79 retinoblastoma cells. We also demonstrated the combination of ANRIL and miR-328-3p.

FKBP12-rapamycin complex-associated protein is a conserved serine/threonine protein kinase, and is also termed as mTOR. mTOR is a member of the PI3K/AKT signaling pathway [21, 29]. It was proved that mTOR is an inhibitor of autophagy [30]. In our present study, the results indicated that ANRIL overexpression inhibited the phosphorylation of mTOR in retinoblastoma cells, but promoted the expression of LC3B, ATG5, and BECN1 expression in the cells. Growth factors, cytokines, and TLR ligands regulate TSC1/TSC2 phosphorylation and inactivation *via* a plethora of upstream protein kinases, thus primarily triggering mTOR activation [31]. In this study, we found the binding sites between miR-328-3p and TSC1 3′-UTR, and determined the combination of them using luciferase activity assay. ULK2 is an evolutionarily conserved serine/threonine kinase ortholog of the yeast ATG family member Atg1, which has a redundant role in the regulation of autophagy [32, 33]. Here, we also found the binding sites between miR-328-3p and ULK2 3′-UTR, and determined the combination of them. Importantly, ANRIL overexpression facilitates the expression of ULK2 in retinoblastoma cells. Furthermore, our data showed that ANRIL overexpression promotes the proliferation and cisplatin resistance of retinoblastoma cells.

Conclusions

Our experiments verified that ANRIL overexpression promotes proliferation and inhibits cisplatin-induced apoptosis through activating autophagy *via* facilitating TSC1/ULK2 expression by acting as a miR-328-3p sponge. ANRIL may be a potential target of retinoblastoma treatment.

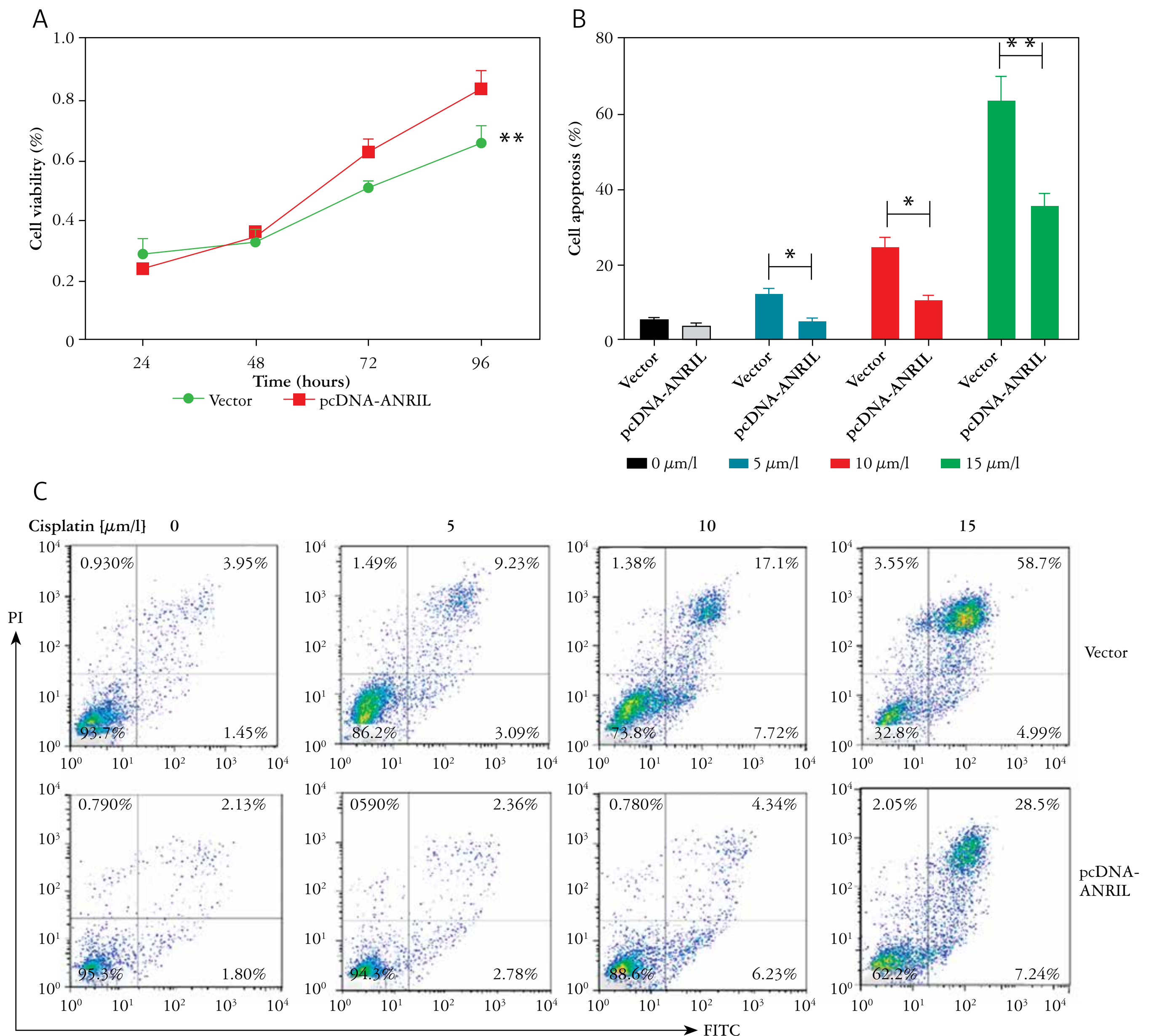

Fig. 4. Effect of ANRIL on retinoblastoma cell proliferation and cisplatin resistance. A) CCK-8 assay was performed to detect proliferation of retinoblastoma cells infected with ANRIL overexpression lentivirus. B) Retinoblastoma cells infected with the ANRIL overexpression lentivirus were treated with cisplatin for 24 hours. C) Cell apoptosis was determined using flow cytometry assay

PI – propidium iodide
** p-value lower than 0.05*
*** p-value lower than 0.01*

Disclosures

1. All protocols were authorized by the Ethics Committee of the Second Affiliated Hospital of Nanchang University.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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