RETRACTED ARTICLE: Silence of IncRNA ANRIL represses cell growth and promotes apoptosis in retinoblastoma cells through regulating miR-99a and c-Myc

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\textbf{ABSTRACT}

Retinoblastoma is a rare cancer of the immature retina. This study designed to see the function of the IncRNA ANRIL in retinoblastoma Y79 cells. ANRIL, miR-99a and c-Myc expression in Y79 cells was altered by transfection and then trypan blue, transwell assay and flow cytometry were carried out to evaluate the changes of cell phenotype. The connection between ANRIL, miR-99a and c-Myc was measured by luciferase reporter assay and RNA immunoprecipitation analysis. As a result, ANRIL expression was highly expressed in human retinoblastoma tissue as relative to the adjacent noncancerous tissues. ANRIL suppression inhibited Y79 cells viability, migration, invasion, while promoted apoptosis. ANRIL negatively regulated miR-99a by binding to miR-99a. Silence of miR-99a reversed the ANRIL-knockdown effects on Y79 cells. miR-99a overexpression suppressed Y79 cell viability, migration, invasion, and enhanced apoptosis through downregulating c-Myc. Meanwhile, we found that miR-99a inhibited JAK/STAT and PI3K/AKT pathways. To conclude, it seems that ANRIL suppression inhibits cell growth and metastasis in retinoblastoma Y79 cells by regulating miR-99a and c-Myc.

\textbf{Introduction}

Retinoblastoma, a rare cancer of the retina, develops from the immature cells of the retina. Globally, retinoblastoma is diagnosed in approximately 8000 children annually [1]. According to statistics, in developing countries, mortality of retinoblastoma in children is considered high, approximately 50\%–70\% of all cases [2]. Retinoblastoma is divided into two clinical forms: unilateral, nonhereditary form that accounts for 75\% of all cases; and bilateral or multifocal, hereditary form that accounts for 25\% of all cases and characterized by germ-line mutations of the retinoblastoma gene (RB1) [3]. If not treated timely, retinoblastoma can lead to metastasis, trilateral retinoblastoma or second primary tumours [4]. The standard management of retinoblastoma includes chemoreduction as the primary option; enucleation is done only in few cases [5,6].

Long noncoding RNAs (lncRNAs) are a sort of RNAs without coding capacities and longer than 200 nt [7]. lncRNAs have low sequence conservation and mediate gene expression at post-transcriptional level through interaction with nucleic acids and proteins. And lncRNAs broadly participate in varied biological aspects, such as cell growth, differentiation, chromosome inactivation and even tumorigenesis [8–11]. In regard of cancer, an increasing amount of lncRNAs have been found to function as oncogenes or antioncogenes [12]. Moreover, some of them are aberrant expressed in cancer [8]. Thus, lncRNAs are supposed to be novel targets for diagnose and treatment of cancers.

It has been reported that several lncRNAs are associated with retinoblastoma, such as BRAF-activated noncoding RNA (BANCR), brain-derived neurotrophic factor antisense (BDNF-AS) and maternally expressed gene 3 (MEG3). Studies have shown that overexpression of MEG3 and BDNF-AS, and knockdown of BANCR suppressed the progression of retinoblastoma cells [13–15]. The lncRNA ANRIL (antisense noncoding RNA in the INK4 locus) is transcribed as a 3.8-kb IncRNA from the INK4B-ARF-INK4A gene cluster in the opposite direction [16]. It has been reported that ANRIL is correlated with coronary artery disease, intracranial aneurysm, type 2 diabetes mellitus and cancer [17]. Yap et al. showed that ANRIL is expressed in higher levels of prostate cancer [16]. Nonetheless, less was known regarding ANRIL in retinoblastoma. Herein, we designed to see the functions of ANRIL in retinoblastoma Y79 cells and revealed one of the underlined mechanisms.

\textbf{Methods}

\textbf{Cell culture and treatment}

Retinoblastoma Y79 cells (ATCC, Catalogue No: HTB-18, Manassas, VA) were cultivated in RPMI-1640 plus 15\% FBS

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(Invitrogen, Carlsbad, CA) and were subcultured grown at 37 °C under a damp environment with 5% CO₂.

**Collection of human retinoblastoma tissues**

The retinoblastoma and adjacent noncancerous tissues were derived from 20 patients with retinoblastoma during surgery at The First Affiliated Hospital of Xinxiang Medical University (Henan, China). Before tissue collection, written informed consent was signed and the procedure was authorized by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University. Before surgery, none of the volunteers received other therapies. The specimens were preserved in liquid nitrogen immediately for subsequent testing.

**qRT-PCR**

Trizol (Invitrogen) was executed for RNA extraction. ANRIL expression was checked by One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa, Dalian, China). For the expression levels of miR-99a, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II (TaKaRa) were used. For expression levels of c-Myc, RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa) was used. qPCR was performed according to following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. GAPDH acted as an internal control for ANRIL and c-Myc, and U6 was used for miR-99a. Data was calculated by $2^{-\Delta\Delta C_T}$ cycle threshold method. The primers used were as follows. LncRNA ANRIL: forward 5'-TGCTCATTGGCCAATCTAGG-3’, reverse 5'-GGGCTCTAGTGGCAGCATAC-3’. miR-99a: forward 5'-ACACTCCAGCTGGGCAAGCTGGTCTTATG-3’, reverse 5'-TGTTGCTGGGACGCTGG-3’. c-Myc: forward 5'-CAGCTGCTAGACGCTGGATT-3’, reverse 5'-CTGAGGAAATACGGCTGCACCGA-3’. GAPDH: forward 5'-GTGGCACATACC-3’, reverse 5'-CACGCTGCTTAGACGCTGGATT-3’. miR-99a mimics were co-transfected into the cells. Luciferase activity assay was carried out via adopting Dual Luciferase Reporter Assay System (Promega) in line with the specification.

**Cell transfection**

shRNAs directed against the human ANRIL was inserted into the U6/Neo plasmid (GenePharma, Shanghai, China) and named as sh-ANRIL. Full-length c-Myc sequences and c-Myc shRNAs were constituted into pEX-2 and pGPU6 plasmids (GenePharma), respectively, and were referred as pEX-c-Myc and sh-c-Myc. miR-99a mimics, inhibitor and the peculiar NC were constituted (Invitrogen) and transfected into cells. Lipofectamine 3000 (Invitrogen) was used for transfection, which was lasted for 48 h.

**Cell viability, migration and invasion assays**

For cell viability, 1 × 10⁵ cells in duplicate in 60 mm dishes were stained by trypan blue and cell viability was calculated by counting living cells rate.

For cell migration, a two-chamber transwell system which with an 8-µm pore (Costar, New York) was utilized. After the indicated transfection, cells suspended in 200 µL of serum-free medium were placed into the upper side. and the completed medium was placed into the lower one. Forty-eight hours later, cells in the lower side were stained by crystal violet and counted.

For cell invasion, a 24-well Millicell® Hanging Cell Culture Inserts (Millipore, Bedford, MA) was used. Cell invasion was tested as the same with migration assay.

**Apoptosis assay**

Following the indicated transfection, apoptosis cell rate was analysed by Annexin V-FITC/PI double staining kit (Solorbio, Beijing, China). Briefly, cells were collected into a centrifuge tube on the basis of the operating steps of PI dyeing. After centrifugation, cells were rinsed with cold PBS and suspended in Binding buffer containing Annexin V-FITC and PI and reacted for 30 min with the absence of light. FACScan (Beckman Coulter, Fullerton, CA) was utilized to test the percentage of apoptosis cells.

**Luciferase reporter assay**

A reporter plasmid ANRIL-wild-type (ANRIL-wt) was made by inserting pmirGLO Vector (Promega, Madison, WI) with ANRIL sequences, which contains the target site of miR-99a. The target site was mutated and inserted to form a negative control for reporter plasmid, which was named as ANRIL-mutated-type (ANRIL-mt). The reporter vectors of c-Myc-wt and c-Myc-mt were constructed by the same way. The vectors and miR-99a mimics were co-transfected into the cells. Luciferase activity assay was carried out via adopting Dual Luciferase Reporter Assay System (Promega) in line with the specification.

**RNA immunoprecipitation (RIP)**

RIP was implemented by using EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) to pull down endogenous miR-99a associated with ANRIL in retinoblastoma Y79 cell line. Y79 cells were lysed by RIP lysis buffer, and 100 µL of cell lysate was incubated with RIP buffer containing magnetic beads conjugated with human Argonaute2 (Ago2) antibody or normal mouse IgG (both from Millipore). Proteinase K buffer was then added, and target RNA was extracted for qRT-PCR analysis.

**Mitochondrial membrane potential (ΔΨm) analysis**

ΔΨm was tested by using JC-1 mitochondrial membrane potential detection kit (BestBio, Shanghai, China). Briefly, after the indicated transfection, 2 × 10⁵ cells per sample were collected and resuspended in 500 µL JC-1 solution, and then incubated at 37 °C for 15 min. The cells were finally analysed by a Microplate Reader (Bio-Rad, Hercules, CA) at 490/540 and 540/590 nm.
Western blot

RIPA lysis buffer (Beyotime, Shanghai, China) was utilized for protein extraction. The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI). Primary antibodies against Bcl-2 (ab238041), Bax (ab104156), pro-Caspase-3 (ab32150), cleaved-Caspase-3 (ab32042), pro-Caspase-9 (ab185719), cleaved-Caspase-9 (ab23324), cytochrome c (ab90529), c-Myc (ab39688), PI3K (ab86714), p-PI3K (ab182651), AKT (ab18785), p-AKT (ab38449), JAK1 (ab47435), p-JAK1 (ab203784), STAT3 (ab119352), p-STAT3 (ab76315) and GAPDH (ab181602) were all from Abcam (Cambridge, MA). Following incubation with the secondary antibody, the bands were enhanced by adding 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore). The intensity was analysed by exploiting Image Lab™ Software (Bio-Rad).

Statistics

Graphpad 6.0 software was adopted to figure out the figure statistically from this research, and these data emerged as mean ± standard deviation. p Values were calculated using ANOVA with Tukey post-hoc. p of <.05 was set as differences processed conspicuousness.

Results

Suppression of ANRIL supresses Y79 cell growth, migration and invasion

Twenty pairs of retinoblastoma and adjacent noncancerous tissues were collected and ANRIL expression was detected. As seen, ANRIL expression was remarkably elevated in human retinoblastoma tissues as relative to noncancerous tissues (p < .001, Figure 1(A)). Besides that, we measured the effect of ANRIL on Y79 cells phenotype. Results showed that both sh-ANRIL #1 and sh-ANRIL #2 significantly decreased ANRIL expression as relative to sh-NC (p < .001 and p < .01; Figure 1(B)). As maximum suppression was found out with sh-ANRIL #1, it was used in the further experiments. Suppression of ANRIL using sh-ANRIL #1 notably declined viability (p < .05; Figure 1(C)), migration (p < .05; Figure 1(D)) and invasion (p < .05; Figure 1(E)), while enhanced apoptotic cell rate (p < .001; Figure 1(F)) in Y79 cells. Western blot demonstrated that the suppression of ANRIL gained protein accumulation of Bax, cleaved caspase-3, cleaved caspase-9 and cytochrome c, and downturned protein accumulation of Bcl-2 in Y79 cells (Figure 1(G–H)). Besides that, silencing ANRIL induced a significant drop in ΔΨm (p < .05; Figure 1(I)).

miR-99a is negatively regulated by ANRIL

Next, by using qRT-PCR, we found that suppression of ANRIL notably raised miR-99a level as relative to sh-NC (p < .001, Figure 2(A)), saying that ANRIL negatively regulates miR-99a. Next, whether ANRIL negative regulated miR-99a via binding effects was analysed. The predicted binding sites between miR-99a and ANRIL are shown in Figure 2(B). As seen, luciferase activity was declined by miR-99a and ANRIL-wt (p < .05), and no such changes were found with ANRIL-mt (Figure 2(B)). Furthermore, RIP was performed on Y79 cell extracts to verify the direct association between ANRIL and miR-99a by using antibodies against Ago2. Figure 2(C–D) revealed that, ANRIL and miR-99a were enriched in Ago2 pellets as compared to control IgG (p < .05). The findings revealed that ANRIL works as a sponge for decreasing miR-99a expression.

miR-99a contributes to ANRIL-knockdown effects on Y79 cells

As ANRIL negatively regulated miR-99a, we explored the impacts of miR-99a inhibition on Y79 cells phenotype. Y79 cells were transfected with shNC + siNC, sh-ANRIL + siNC or sh-ANRIL + si-miR-99a; untransfected cells were used as control. As compared to the only ANRIL-knockdown effects, silence of both miR-99a and ANRIL remarkably promoted viability (p < .05; Figure 3(A)), migration (p < .05; Figure 3(B)) and invasion (p < .05; Figure 3(C)), while declined apoptosis (p < .01; Figure 3(D)) in Y79 cells. Likewise, silence of both miR-99a and ANRIL declined the protein accumulation of Bax, cleaved caspase-3, cleaved caspase-9 and cytochrome c and enhanced the accumulation of Bcl-2 in Y79 cells (Figure 3(E–F)). Moreover, silence of both miR-99a and ANRIL increased ΔΨm as relative to silence ANRIL alone (p < .05; Figure 3(G)). Therefore, miR-99a expression might be essential for ANRIL’s effect on Y79 cells.

c-Myc is negatively regulated by miR-99a

Next, c-Myc expression was detected following miR-99a dysregulation, since c-Myc has significant role in regulating cell growth and metabolism [18]. qRT-PCR and Western blot data displayed that miR-99a mimic declined the mRNA (p < .05; Figure 4(A)) and protein (p < .05; Figure 4(B)) levels of c-Myc, while si-miR-99a increased c-Myc expression (p < .01). Furthermore, whether miR-99a negatively regulated c-Myc through binding effects was tested. Results from Figure 4(C) showed that luciferase activity was declined in cells co-transfected with miR-99a mimic and c-Myc-wt (p < .05). However, no such decline was seen with c-Myc-mt vector.

miR-99a overexpression suppresses Y79 cell growth, migration and invasion by c-Myc

Y79 cells were transfected with scramble + pEX, miR-99a mimic + pEX or miR-99a mimic + pEX-c-Myc; untransfected cells served as control. qRT-PCR results showed that miR-99a mimic repressed c-Myc expression as relative to scramble (p < .05), whereas pEX-c-Myc increased c-Myc expression as relative to miR-99a mimic (p < .001; Figure 5(A)). Next, the role of c-Myc in Y79 cells was measured. Results displayed that miR-99a mimic declined viability (p < .05; Figure 5(B)), migration (p < .05; Figure 5(C)), invasion (p < .01; Figure 5(D)) and induced apoptosis (p < .001; Figure 5(E–F)) as relative to the cells transfected with scramble + pEX. Conversely, miR-99a mimic plus pEX-c-Myc enhanced cell...
viability (\(p < .05\); Figure 5(B)), migration (\(p < .05\); Figure 5(C)), invasion (\(p < .01\); Figure 5(D)), while repressed apoptosis (\(p < .01\); Figure 5(E–F)) as relative to the cells treated with miR-99a mimic alone. Also, Western blot analysis displayed that miR-99a mimic raised the protein accumulation of Bcl-2, diminished the protein accumulation of Bax but unaffected the caspase-3 and caspase-9 (Figure 5(G–H)). miR-99a mimic plus pEX-c-Myc significantly decreased Bcl-2 accumulation while increased the accumulation of Bax, cleaved caspase-3 and cleaved caspase-9 (Figure 5(G–H)). Moreover, miR-99a mimic declined \(\Delta \Psi \text{m}\) level (\(p < .01\)), and overexpression of c-Myc significantly abolished the declined \(\Delta \Psi \text{m}\) (\(p < .05\); Figure 5(I)). Those phenomena indicated that the modulation of miR-99a overexpression in cell growth and metastasis was reversed by c-Myc upregulation.

**Overexpression of miR-99a inactivates JAK/STAT and PI3K/AKT signal pathways**

Finally, the possible underlying mechanisms of which miR-99a modulated Y79 cells growth and metastasis are studied.

Western blot was utilized to assess the levels of basis factors in JAK/STAT and PI3K/AKT pathways. As seen, miR-99a overexpression decreased the phosphorylated forms of PI3K, AKT, JAK1 and STAT3, while suppression of miR-99a increased these expressions (Figure 6(A–B)). Besides this, suppression of c-Myc reversed the impact of miR-99a inhibition in the regulation of these two pathways, as decreased levels of p-PI3K, p-AKT, p-JAK1 and p-STAT3. The findings illustrate that overexpression of miR-99a inactivates JAK/STAT and PI3K/AKT signal pathways; and the mechanism may be through the regulation of c-Myc.

**Discussion**

Herein, we checked the function of the lncRNA ANRIL in Y79 cells growth and metastasis. We found silence of ANRIL repressed cell viability, migration and invasion and enhanced apoptosis in Y79 cells. Functions of lncRNAs in retinoblastoma have been sporadic studies [13–15]. Cite an instance, Su et al. concluded that the poor prognosis retinoblastoma owned to the dysregulated lncRNA BANCR, which could
regulate tumour growth and metastasis [13]. Through suppressing cell-cycle transition, lncRNA BDNF-AS overexpression was able to inhibit retinoblastoma development [14]. Likewise, lncRNA MEG3 acted as an antitumour lncRNA in the onset and development of retinoblastoma by negatively regulating Wnt/β-catenin pathway [15].

The lncRNA ANRIL has been studied in many diseases, including several types of cancer. Chen et al. demonstrated that ANRIL overexpression accelerated epithelial-mesenchymal transition of pancreatic cancer cells by ATM-E2F1 signalling [19]. Wei et al. showed that ANRIL improved osteosarcoma cell invasion and suppressed apoptosis under hypoxic condition [20]. Zhu et al. illustrated ANRIL was highly expressed in bladder cancer and was capable of regulating tumour cells proliferation and apoptosis [21]. However, literature-studying effects of ANRIL on retinoblastoma cells are not available. To our knowledge, we are the first to evaluate the functions and mechanisms of ANRIL in retinoblastoma cells.

Recently, it has been widely accepted that one of lncRNA functions is in acting as competing endogenous RNAs (ceRNAs). LncRNA works as a sponge for miRNA in having miRNA exhausted and thereby prevents mRNA from being decayed by miRNA [22,23]. miR-99a has been identified as an antitumour miRNA in diverse cancers, such as cervical, lung, breast and oral cancers [24–28]. Yang and Mei found that the expression of miR-99a could distinguish the retinoblastoma samples from the healthy samples [29]. Nonetheless, the specific role of miR-99a in retinoblastoma cells is not fully clarified yet. A previous study displayed that silence of ANRIL could elevate miR-99a expression in gastric cancer cells [30]. Considering of that, we hypothesized that ANRIL conferred its functions also via regulating miR-99a in retinoblastoma cell line Y79. As expected, we found that ANRIL worked as a molecular sponge for miR-99a. We additionally found that the silence of miR-99a reversed the effects of ANRIL-knockdown on Y79 cells, which further confirmed that ANRIL exerted its impacts on retinoblastoma cells growth and apoptosis though sponging miR-99a.

We also studied the possible mechanism of which miR-99a functioned to retinoblastoma cells. Towards this goal, we pinpointed c-Myc as a target of miR-99a. c-Myc is an proto-oncogene that products multifunctional c-Myc protein and participates in cell growth, cell cycle, apoptosis, angiogenesis and transformation [31]. c-Myc activation is a common
hallmark of many types of cancer, including cancers with mutations in the retinoblastoma pathway [32]. Chang et al. illustrated down-regulation of miR-99a in high c-Myc state [33], which was in line with our finding. We also showed that miR-99a mimic suppressed Y79 cells growth, migration and invasion. Interestingly, overexpression of c-Myc reversed these effects, which further confirmed that miR-99a’s effect on retinoblastoma cells was dependent on c-Myc.

In further experiments, we studied the effects of miR-99a and c-Myc on JAK/STAT and PI3K/AKT pathways. JAK activation is capable of stimulating cell growth, differentiation, migration and death [34]. STAT3 functions as a transcription factor that modulates gene expression under stimulations. Therefore, STAT3 is critical in tumour cell growth and metastasis or by suppressing cell death [36]. PI3K/AKT is also a significant pathway for cell growth and metastasis [37]. PI3K/AKT signal pathway proteins were frequently altered in cancer, including retinoblastoma [38,39]. We found that miR-99a overexpression inhibited the phosphorylation of proteins associated with JAK/STAT3 and PI3K/AKT signal pathways. However, miR-99a inhibition enhanced the activity of JAK/STAT3 and PI3K/AKT signal pathways. Interestingly, suppression of c-Myc reversed the effect of miR-99a down-regulation on these two signalling pathways.

In short, this study showed that silence of ANRIL repressed retinoblastoma Y79 cells growth, migration and invasion by regulating miR-99a. Interestingly, miR-99a negatively regulated c-Myc through binding effect, and overexpression of c-Myc flattened miR-99a’s effect on Y79 cells phenotype. Our research might allow us to better understand the
Figure 4. c-Myc is negatively regulated by miR-99a. (A) qRT-PCR and (B) Western blot were utilized to see mRNA and protein levels of c-Myc in Y79 cells transfected with nothing, scramble, miR-99a mimic, siNC or si-miR-99a. (C) Luciferase activity of cells transfected with c-Myc-wild-type (c-Myc-wt) and miR-99a mimic. c-Myc-mutated-type (c-Myc-mt) and scramble were transfected as controls. * and ** represent the p values <.05 and .01.

Figure 5. miR-99a overexpression represses cell growth, migration and invasion by c-Myc in Y79 cells. (A) qRT-PCR was utilized to analyse mRNA level of c-Myc in Y79 cells transfected with control, scramble + pEX, miR-99a mimic + pEX or miR-99a mimic + pEX-c-Myc. (B) Viability, (C) migration, (D) invasion and (E-F) apoptotic cells were tested by trypan blue, transwell analysis and flow cytometry. (G-H) Western blot was utilized to see the protein accumulation of Bax, Bcl-2, caspase-3 and caspase-9. (I) ΔΨm was measured by JC-1 probe. * and ** represent the p values <.05 and .01.
pathological process of retinoblastoma and grant novel insight in the management of retinoblastoma.

Acknowledgement
This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Disclosure statement
Authors declare that there is no conflict of interests.

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