MicroRNA-708 suppresses the proliferation, migration, and invasion of human retinoblastoma cells by targeting RAP2B, a member of the RAS oncogene family

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Retinoblastoma generally affects children and causes permanent vision failure or even death. MicroRNAs (miRs) have recently gained much attention during recent years. The miR-708 acts as a tumor suppressor in several human cancers, but the former has not been functionally characterized in human retinoblastoma. The present study was designed to investigate the role of miR-708 in human retinoblastoma. The results showed that miR-708 is significantly ($P<0.05$) downregulated in retinoblastoma cell lines. MiR-708 overexpression significantly ($P<0.05$) inhibited retinoblastoma cell growth and proliferation by inducing apoptosis. Furthermore, retinoblastoma cells overexpressing miR-708 exhibited a markedly lower migratory rate and invasiveness compared to negative control cells. The bioinformatics and dual luciferase assay revealed a RAS oncogene family protein, RAP2B, which acts as the regulatory target and functional mediator of the molecular role of miR-708 in retinoblastoma. Together, the present study revealed the tumor suppressor role of miR-708 and pointed to the therapeutic implications of miR-708/RAP2B in the treatment of retinoblastoma.

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Abbreviations: DMEM, Dulbecco's modified Eagles medium; miRs, microRNAs; FBS, fetal bovine serum; MTT, 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide

INTRODUCTION

Retinoblastoma is the neoplastic disorder that frequently affects intraocular tissue among infants and children (Munier *et al.*, 2019). The life-threatening disorder of retinoblastoma originates from the ancient nuclear stem cells of the human retina (Stenfelt *et al.*, 2017). This malignancy is highly aggressive and is reported to account for up to 4% of all pediatric disorders (Darwich *et al.*, 2019). Retinoblastoma can lead to permanent loss of vision or even death in advanced stages (Chen *et al.*, 2019). Advanced stages of retinoblastoma are more commonly reported in young people belonging to the age group of less than 6 years (Sadykova *et al.*, 2020). Orally applied chemo-therapy practice has been shown to exhibit very little success against retinoblastoma, while combination therapy, including focal surgery and chemotherapy, is comparatively more efficient and has become the curative procedure of choice (Rao & Honavar, 2017). Several genetic mutations have been shown to be associated with the initiation of retinoblastoma, such as the Retinoblastoma gene (Rb1), the p53 gene family, and the epithelial cell adhesion molecule (Stenfelt *et al.*, 2017; Singh *et al.*, 2016). However, an in-depth understanding of the underlying cellular and molecular irregularities is elusive, and the investigation of the same will not only increase our knowledge about the pathogenesis of retinoblastoma, but could also lead to the identification of possible molecular regulators and therapeutic targets against retinoblastoma.

There is growing support that microRNAs (miRs) are crucial regulators of human carcinogenesis (McCubrey *et al.*, 2017). By definition, miRs are referred to as RNA transcripts with sizes ranging between 20-25 nucleotides, which are localized to the cytosol or nucleus and lack protein coding ability (Michelini *et al.*, 2018). Apparently, miRs perform regulatory functions which they execute by binding mainly to 3'-UTRs of target gene mRNAs at the post-transcriptional level, resulting in degradation or translational silencing of target transcripts (Oliveto *et al.*, 2017; Michlewski & Cáceres, 2019). A large number of studies have indicated that miRs regulate various physiological and cellular processes (Pradhan *et al.*, 2017). It is noteworthy that human cancers have been shown to often express aberrantly higher or lower transcript levels of different miRs (Xu *et al.*, 2019; Li *et al.*, 2016; Sha *et al.*, 2017). Of the various miRs discovered during recent times, miR-708 (miR-708-5p in the actual sense) has been reflected to exhibit misexpression in human diseases such as neurodegenerative and cardiovascular disorders and more commonly in human cancer (Monteleone & Lutz, 2017). MiR-708 most commonly exhibits tumor suppressor molecular function in human cancers such as breast cancer and lung cancer (Tan *et al.*, 2019; Monteleone & Lutz, 2020). However, the regulatory role exercised by miR-708 in controlling retinoblastoma growth and metastasis has not been studied and therefore was the primary focus of the present study. The results showed that miR-708 targets the RAS oncogene family protein, RAP2B, to negatively regulate the growth, migration, and invasion of human retinoblastoma cells.

MATERIALS AND METHODS

Culture and transfection of cell lines

Four different human retinoblastoma cell lines (Y79, RB335, WERI-Rb-1, and C-33A) as well as RPE, normal human retinal epithelial cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cell lines were cultured and maintained in Dulbecco's modified Eagles medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (both from Hyclone) at 37°C with 5% CO_2 in a humidified incubator. Synthetic oligos of miR-708 mimics or negative control (miR-NC), small interfering RNAs of si-RAP2B or negative control (si-NC), luciferase reporter plasmid carrying wild-type (WT) or mutant (MUT) 3'-UTR of RAP2B in psiCHECK 2 and RAP2B overexpression plasmid (pcDNA-RAP2B) or its negative control (pcDNA3.1 vector alone) were designed by RiboBio Co. Ltd. (Guangzhou, China). These were transfected with the help of Lipofectamine® 2000 (Invitrogen) into RB335 according to the manufacturer's instructions in 6-well plates. Transfected cells were cultured overnight and harvested at 80–90% confluence.

RNA extraction and qRT-PCR

Total RNA was isolated from cultured cell lines with the help of Trizol reagent (Invitrogen) according to the manufacturer's guidelines. Following evaluation of its quantity and purity using the spectrophotometric method, 2.5 μg RNA was reverse transcribed into cDNA with the help of the Prime-Script RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. Relative miRNA708 transcript levels were examined using a SYBR PrimeScript miRNA RT PCR kit (Takara), while PowerSYBR Green PCR master mix (Thermo Fisher Scientific, VA, USA) was used for the relative expression analysis of RAP2B. qRT-PCR was performed on an Applied Biosystems 7900 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The snRNA U6 and GADPH were used as corresponding internal controls for the expression study of miR-708 and RAP2B, respectively. The primers used in the present study are listed in Table 1. Relative expression levels were quantified with the help of $2^{-\Delta\Delta Ct}$ method.

MTT assays

To assess their proliferation, transfected RB335 cells were propagated at 37°C in 96-well plates with an initial

Table 1. List of primers used in the study.

Primer	Direction	Sequence
miR-708	Forward	5'-CCGCACGAAGGAGCTTACAAT-3'
	Reverse	5'-GTGCAGGGTCCGAGGTATTC-3'
RAP2B	Forward	5'-ATGATTGTAGGAAAAGACCGGGA-3'
	Reverse	5'-GCAGTCACCATCGACGTATAGAC-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
GADPH	Forward	5'-GGCTGTAGACCAGGATGAAG-3'
	Reverse	5'-TTGGAGGGATCTCGCTCCT-3'

inoculation density of 10^4 cells per well. At 0, 12, 24, 48 and 96 h of cell culture. Each well was added with 0.5% 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, USA) and cells were again incubated for 4 h at 37°C Next, 125 μL of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals. The absorbance of each well was detected at 570 nm with the help of a spectrophotometer.

EdU assay

The 5-ethynyl-2-deoxyuridine labeling kit (EdU) (Ribobio, Guangzhou, China) was used to analyze the proliferative vigor of transfected cells. In summary, cells were cultured in 24-well plates at 2.5×10^4 cells per well. After 24 h of transfected cells at 37°C, 0.05 mM EdU labeling solution was inoculated into each well. Cells were again incubated for 2.5 h at 37°C with 5% CO_2. Cells were then harvested and fixed with 4% paraformaldehyde and 1% Triton X-100. At this point, the anti-EdU working solution was used to label the cells. 4',6-diamidino-2-phenylindole (DAPI) was used as counter stain to label cell nuclei. The EdU-positive cells were analyzed under fluorescent microscopy.

Annexin V-FITC/PI staining

The apoptosis of transfected RB335 retinoblastoma cells was analyzed using Annexin V-FITC/PI double staining procedure. Cells were seeded at a density of 2.5×10^4 cells into each well of 6-well plates and incubated at 37°C for 24 h. Afterwards, the cells were harvested, washed three times with phosphate buffered saline (PBS), carefully resuspended in binding buffer from the Annexin V-FITC/PI double staining kit (7Sea Biotech, Shanghai, China). This was followed by the addition of 7.5 μL Annexin V-FITC and 15 μL propidium iodide (PI) and dark incubation for 20 min. Cell apoptosis levels were finally examined using an Accuri C6 flow cytometer (BD Biosciences, CA, USA) Flow Cytometer.

Western blot

For the extraction of total proteins from transfected cells, the latter were lysed with RIPA lysis buffer (Thermo Fisher Scientific). Proteins were then separated on 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Amersham, Munich, Germany). The membranes were then blocked with 5% skim milk for 2 h at room temperature. The PVDF membranes were then incubated with primary antibodies overnight at 4°C TBS-T was used to wash the membranes three times, which were then incubated with horseradish peroxidase conjugated secondary antibodies for 2.5 h. Membranes were again washed with TBS-T and immunoreactive protein bands were detected with the help of an enhanced chemiluminescence substrate (ECL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) substrate.

Migration and invasion assays

Around 2×10^5 RB335 transfected cells suspended in serum-free DMEM were added to the upper chamber of the transwell chamber (8 μm pore size, Millipore) without or with Matrigel (Sigma-Aldrich, St Louis, MO, USA) to examine their migration and invasion, respectively. The underlying chamber was seeded with DME containing 10% FBS. After 24 h of incubation at 37°C cells that migrated or invaded the lower chamber were fixed in 70% ethanol and then stained with 0.2% crystal violet (Sigma-Aldrich). Photographs of cells on the lower surface of the

membrane were obtained and five random fields were used to measure the relative migration or invasion of cells.

MiR target analysis

Online bioinformatics analysis was performed through TargetScan Human 7.2 (http://www.targets-can.org/vert_72/) to predict the potential regulatory target of miR-708. For the examination of the direct interaction between miR 708 and the RAP2B gene, a luciferase reporter assay was performed. In summary, RiboBio Co. obtained the luciferase reporter plasmid of the 3' 'untranslated region (3'UTR) of RAP2B carrying the wild-type (WT) or mutant (MUT) miR-708 binding site. Ltd., Guangzhou, China. This followed the cotransfection of reporter plasmids (WT or MUT) with miR-708 mimics or negative control, miR-NC, into RB335 cancer cells. After 48 h, transfected cells were lysed and their luciferase activity was quantified with the help of the Dual Luciferase Reporting Assay System (Promega Corp., USA). The luciferase activities were normalized to the firefly luciferase activity.

Statistical analysis

All statistical analyzes were performed with the help of SPSS software version 19.0 (IBM, Chicago, IL, USA). Data are presented as mean ±S.D. Differences were considered significant at $P<0.05$ obtained by performing the Student's t-test or one-way ANNOVA.
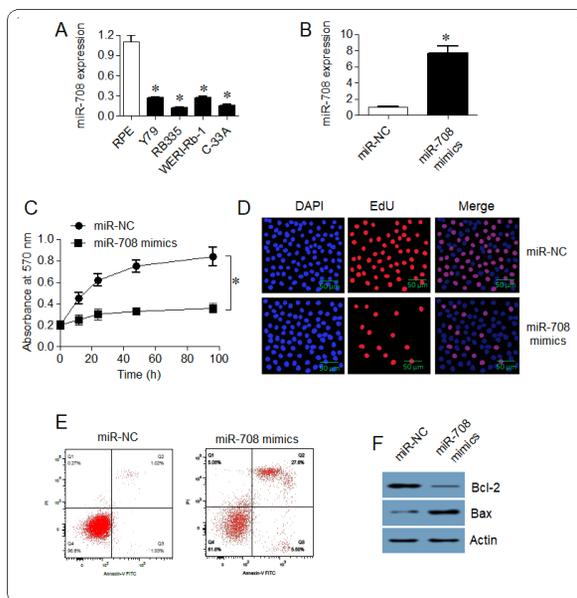

Figure 1. miR-708 inhibits the growth of retinoblastoma cells through the induction of apoptosis.
(**A**) qRT-PCR showing the expression of miR-708 in retinoblastoma cell lines (Y79, RB335, WERI-Rb-1 and C-33A) and normal RPE, retinal pigment epithelial cells (**B**) RB335 cells transfected with miR-708 mimics showed significant upregulation of miR-708 with reference to miR-NC transfected negative control cells (**C**) MTT assay showing overexpression of miR-708 significantly inhibited the proliferation of host RB335 cells, *in vitro* (**D**) EdU assay showing that miR-708 overexpressing RB335 cells incorporated lower EdU levels than the corresponding negative control cells (**E**) Annexin V/PI staining showing induction of apoptosis in RB335 cells overexpressing miR-708 (**F**) Western blots showing that miR-708 overexpression increased Bax expression while it decreases Bcl-2 protein levels in host RB335 cells. The experiments carried three independent replicates and the results were considered significant only when *$P<0.05$.

RESULTS

MiR-708 inhibits the growth of retinoblastoma cells through apoptosis

To obtain a primary view of the probable regulatory functionality of miR-708 in retinoblastoma, the relative expression of the latter was evaluated in four different human retinoblastoma cell lines (Y79, RB335, WERI-Rb-1, and C-33A) with reference to the RPE normal human retinal pigment cell line. The results showed that the retinoblastoma cell lines expressed surprisingly lower ($P<0.05$) miR-708 transcripts than the RPE cells, the expression being the least in RB335 cells among the retinoblastoma cell lines (Fig. 1A). Therefore, the RB335 cell line was used for further experimentation. Synthetic oligos of miR-708 mimics were transfected into RB335 cells to induce miR-708 overexpression and it was found that it was found that the latter was found to be 7.5-fold upregulated in miR-708 mimics compared to the corresponding negative control cells (Fig. 1B). The MTT assay showed that RB335 cells overexpressing miR-708 exhibited lower *in vitro* proliferation than the corresponding negative control cells (Fig. 1C). The cells overexpressing MiR-708 4 further showed lower relative EdU staining than the negative control cells (Fig. 1D). The flow cytometric analysis of doubly stained RB335 cells with Annexin V-FITC and PI showed that apoptosis was significantly higher ($P<0.05$) for miR-708 overexpressing cells compared to the respective negative control cells (Fig. 1E). Furthermore, miR-708 overexpression was shown to considerably increase the expression of the Bax protein while it markedly decreased the expression of the Bcl-2 protein (Fig. 1F). Collectively, the results reveal that overexpression of miR-708 induced apoptosis in RB335 retinoblastoma cells to inhibit their proliferation *in vitro*.

MiR-708 suppresses the migration and invasion of retinoblastoma cells

To confirm whether miR-708 regulates retinoblastoma cell migration and invasion, transwell assays were performed to analyze the effect of miR-708 on the migration potential and invasiveness of RB335 cancer cells.
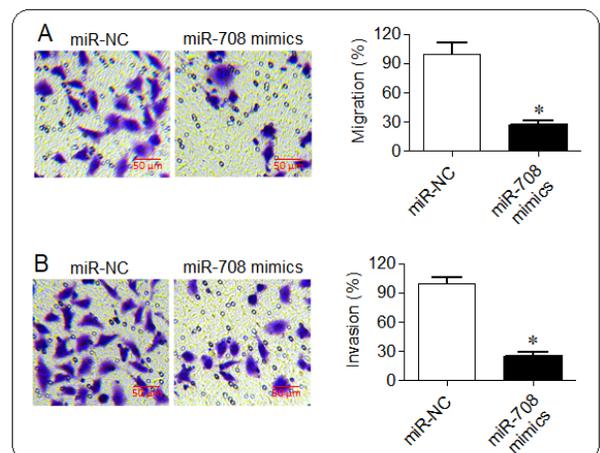

Figure 2. miR-708 inhibits the migration and invasion of retinoblastoma cells.
Transwell assays showing (**A**) migration and (**B**) invasion of miR-NC and miR-708 mimics transfected RB335 cells. The experiments carried three independent replicates and the results were considered significant at *$P<0.05$.
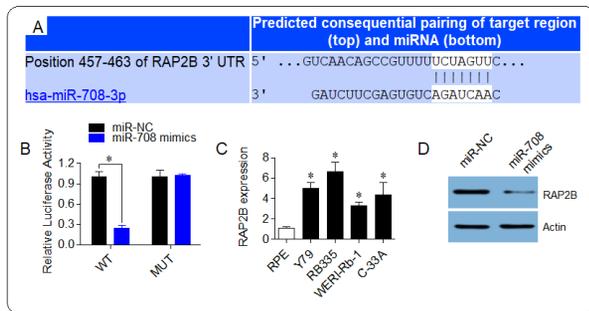
Figure 3. miR-708 targets RAP2B in retinoblastoma.
(**A**) TargetScan analysis showing miR-708 and its potential binding site in 3'-UTR of RAP2B mRNA (**B**) Dual luciferase assay showing interaction between miR-708 and RAP2B (**C**) qRT-PCR showing retinoblastoma cell lines (Y79, RB335, WERI-Rb-1 and C-33A) express higher levels of miR-708 transcript than RPE, normal human retinal pigment epithelial cells (**D**) Western blots showing that overexpression of miR-708 decreases levels of RAP2B protein in host RB335 retinoblastoma cells. The experiments carried three independent replicates and the results were considered significant at *$P<0.05$.

The overexpression of miR-708 was shown to significantly reduce ($P<0.05$) both the migration and invasion of RB335 cells (Fig. 2A and 2B). MiR-708-overexpressing RB335 retinoblastoma cells were found to exhibit only 27% and 25% relative percent migration and invasion, respectively, with respect to the corresponding negative control cells. Thus, the results show that miR-708 inhibits the migration and invasion of retinoblastoma cells.

RAP2B acts as the regulatory target of miR-708 in retinoblastoma

Online bioinformatic analysis was used to predict the potential target of miR-708. The results indicate that miR-708 possibly interacts with the RAP2B mRNA transcripts by binding to a specific binding site at its 3'-UTR, posttranscriptionally (Fig. 2A). To validate the direct interaction of miR-708 with RAP2B mRNA, the luciferase reporter constructs of 3'-UTR of the latter with wild type (WT) or mutant (MUT) were cotransfected with miR-708 mimics or its negative control, miR-NC. Regarding miR-708 mimics and cotransfection of the MUT reporter plasmid, cotransfection of miR-708 mimics with the WT reporter plasmid led to a significant decrease ($P<0.05$) in the luciferase activity of host RB335 cancer cells (Fig. 3B). Furthermore, retinoblastoma cell lines (Y79, RB335, WERI-Rb-1 and C-33A) were shown to possess significantly higher ($P<0.05$) relative transcript levels ($P<0.05$) of RAP2B compared to normal RPE cells, thus negatively correlated with the miR-708 expression pattern (Fig. 3C). Additionally, miR-708 overexpression significantly repressed RAP2B protein levels ($P<0.05$) in RB335 cells (Fig. 3D). Therefore, the results are conclusive that miR-708 targets RAP2B for posttranscriptional suppression in retinoblastoma.

Silencing of RAP2B imitated the effects of miR-708 overexpression

To infer whether miR-708 exercise its functional role in retinoblastoma through posttranscriptional repression of RAP2B, RAP2B was transiently silenced in RB335 cells by transfecting them with small interfering RNAs against RAP2B (si-RAP2B). The silencing of RAP2B in RB335 cells was confirmed by qRT-PCR with respect to the corresponding si-NC transfected negative control cells (Fig. 4A). The incorporation assays of MTT and
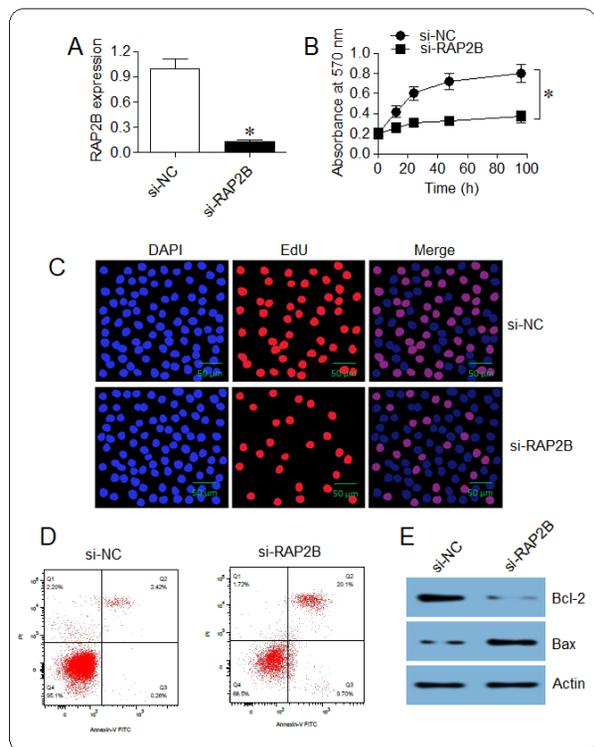

Figure 4. Silencing of RAP2B inhibited the proliferation of retinoblastoma cells through apoptosis.
(**A**) qRT-PCR showing that si-RAP2B transfected RB335 cells exhibit downregulation of RAP2B with reference to si-NC transfected negative control cells (**B**) MTT assay showing that knockdown of RAP2B significantly inhibited the proliferation of host RB335 cells, *in vitro* (**C**) EdU assay showing that RAP2B suppressing RB335 cells incorporated lower EdU levels than the corresponding negative control cells (**D**) annexin V/PI assay showing induction of apoptosis was observed in RB335 cells downregulating RAP2B (**E**) Western blotting showing that silencing of RAP2B increased Bax expression while decreasing Bcl-2 protein levels in host RB335 cells. The experiments carried three independent replicates and the results were considered significant oat *$P<0.05$.

EdU showed that the suppression of RAP2B in RB335 significantly ($P<0.05$) lowered their proliferation and reduced the relative abundance of EdU staining with reference to the corresponding negative control cells (Fig. 4B and 4C). Again, RB335 cancer cells were shown to be inducted with apoptosis by down-regulation of RAP2B, as confirmed by flow cytometry and Western blot analysis of Bax and Bcl-2 marker proteins (Fig. 4D). Therefore, the results indicate that the effects of silencing of RAP2B proliferation were similar to those of miR-708 overexpression.

RAP2B overexpression restored proliferative vigor of retinoblastoma cells

To gather more support for RAP2B being the functional target of miR-708 in retinoblastoma, it was evaluated whether overexpression of RAP2B in retinoblastoma cells reverts the tumor suppressing effects of mi8R-708 overexpression. To induce RAP2B overexpression, RB335 cells were transfected with the pcDNA-RAP2B overexpression plasmid. With respect to negative control RB335 cells transfected with the pcDNA3.1 vector alone, pcDNA-RAP2B transfected cells showed significant ($P<0.05$) upregulation of RAP2B (Fig. 5A). As expected, RAP2B overexpression restored the proliferative vigor of RB335 cancer cells overexpressing miR-708
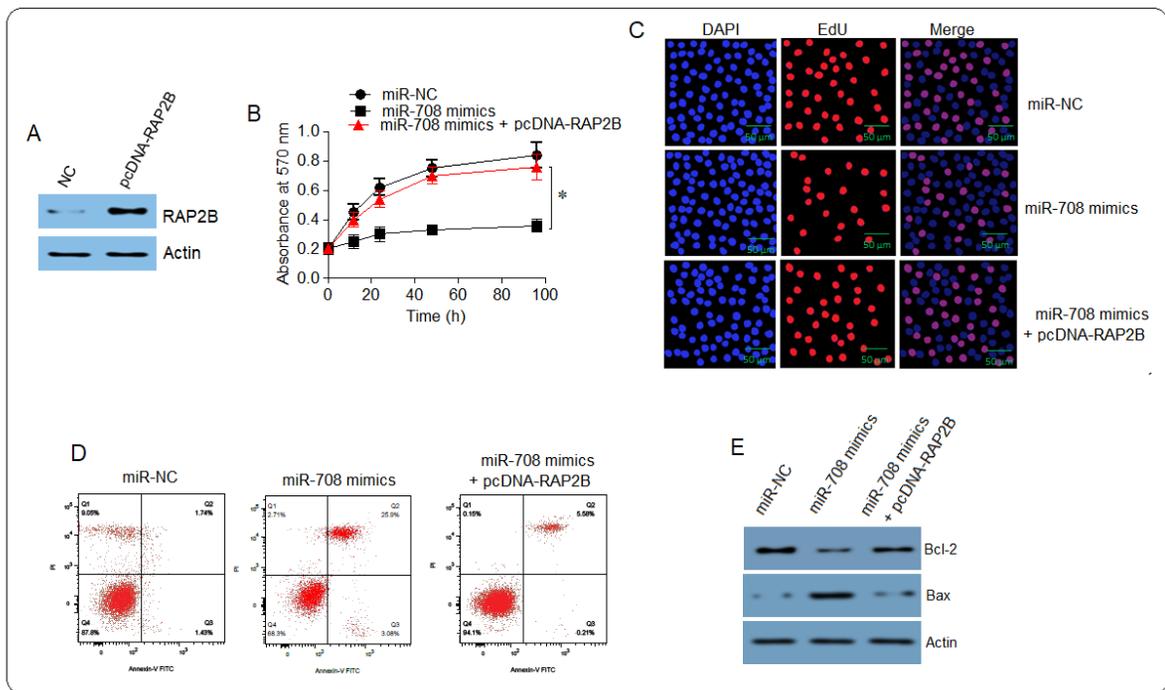
Figure 5. Overexpression of RAP2B inhibited the tumor suppressive effects of miR-708.
(**A**) Western blots showing RB335 cells transfected with pcDNA-RAP2B showed upregulation of RAP2B with reference to pcDNA3.1 vector alone transfected negative control cells (**B**) MTT assay showing the proliferation of pcDNA-RAP2B transfected RB335 cells overexpressing miR-708 was equivalent to that of miR-NC transfected negative control cells (**C**) EdU assay showing the relative abundance of EdU incorporation in pcDNA-RAP2B transfected RB335 cells overexpressing miR-708 was equivalent to that of miR-NC transfected negative control cells (**D**) Annexin V/PI assay showing the apoptosis rate of pcDNA-RAP2B transfected RB335 cells overexpressing miR-708 was equivalent to that of miR-NC transfected negative control cells (**E**) Western blots showing Bax and Bcl-2 protein levels in pcDNA-RAP2B transfected RB335 cells overexpressing The experiments carried three independent replicates and the results were considered significant at *$P<0.05$.

(Fig. 5B). Furthermore, RAP2B overexpression was found to enhance EdU incorporation in RB335 cancer cells overexpressing miR-708, equivalent to negative control cells (Fig. 5C). Furthermore, RB335 retinoblastoma cells overexpressing both miR-708 and RAP2B exhibited significantly ($P<0.05$) lower rate of apoptotic cell death compared to those only overexpressing the miR-708 (Fig. 5D). The reduction in the rate of apoptosis of RB335 cancer cells overexpressing miR-708 was also confirmed by Western blotting of the Bax and Bcl-2 marker proteins (Fig. 5E). Summing up, the results show that miR-708 inserts its regulatory control on proliferation, migration, and invasion of retinoblastoma cells through posttranscriptional suppression of RAP2B.

DISCUSSION

The research interest in microRNAs (miRs) is increasing day by day owing to their regulatory involvement in almost all the crucial physiological, metabolic,, and cellular pathways of the the human body (Li *et al.*, 2014; Rotllan & Fernández-Hernando, 2012). MiRs are frequently dysregulated in pathological conditions, in particular human cancer (Salvi *et al.*, 2013). Several studies have proposed that retinoblastoma is associated with aberrant expression of different types of miRs (Martin *et al.*, 2013; Song *et al.*, 2017; Zhao & Cui, 2019; Montoya *et al.*, 2015). The latter have been shown to tremendously affect the growth and metastatic characteristics of retinoblastoma cells (Hu *et al.*, 2021). In addition, the miRs have been shown to exert their molecular role via multiple regulatory nodes. The latter thus culminate into an intricate down-stream crosstalk fine tuning the over-all cellular behavior underlying the process of tumorigenesis

(Speciale *et al.*, 2020). Therefore, researchers are of the view that exploring the regulatory mechanics of miRs in retinoblastoma might not only improve our knowledge of its pathogenesis but could possibly also lead to the development of novel efficient therapeutic measures against this serious malignancy at the molecular level.

MiR-708 was shown to be aberrantly down-regulated in human retinoblastoma cells in the present study. Previous research instances have made similar inferences about miR-708 expression in different human cancers together with its involvement in the regulation of malignant cell growth (Jang *et al.*, 2012; Song *et al.*, 2013). Taking this into account, miR-708 was overexpressed in retinoblastoma cells, which interestingly curtailed the proliferation rate of host cells *in vitro*. The loss of proliferative vigor in human cancer cells by modulating miR-708 transcript levels is mainly due to induction of apoptosis or insertion of halt in cell division at a specific mitotic stage (Saini *et al.*, 2011). Here, the reduction in retinoblastoma was shown to be a result of induction of apoptosis. MiR-708 has been reported to regulate the migration and invasion of human cancer cells (Ma *et al.*, 2016). Confirming the same, the results of the current study indicated that miR-708 negatively regulates the migration and invasion of retinoblastoma cells, potentially affecting their metastatic behavior. Next, to examine the mechanism of action of miR-708 in regulating the growth, migration, and invasion of retinoblastoma cells, *in silico* analysis was carried out that predicted RAP2B as the functional regulatory target of miR-708. The proposition was validated after further experimentation. Located in the RAS oncogene family of GTP-binding proteins, RAP2B reportedly exhibits higher expression in various human cancers and is important for tumor development

and progression (Di *et al.,* 2016; Di *et al.,* 2015; Peng *et al.,* 2016). Retinoblastoma cells were also shown to express higher levels of RAP2B transcripts, which were deduced in one way as a result of miR-708 downregulation. Therefore, aberrant expression of the miR-708/RAP2B molecular axis might possibly be one of the important molecular irregularities associated with retinoblastoma tumorigenesis that in addition signifies its potential therapeutic value.

CONCLUSIONS

Taken together, miR-708 is aberrantly down-regulated in retinoblastoma cells. MiR-708 negatively regulates the growth, migration, and invasion of retinoblastoma cells and its overexpression induces host cell apoptosis. RAP2B acts as the functional regulatory target of miR-708 in retinoblastoma. The current study revealed the regulatory importance of miR-708/RAP2B molecular axis in retinoblastoma and information on its potential therapeutic usefulness against the same.

Declarations

Conflict of interest. The authors declare that there are no conflicts of interest.

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