IncRNA ZFPM2-AS1 promotes retinoblastoma progression by targeting microRNA miR-511-3p/paired box protein 6 (PAX6) axis

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ABSTRACT
Long non-coding RNAs (lncRNAs) have been shown to play crucial roles in retinoblastoma progression. In this study, we aimed to investigate the mechanism of lncRNA ZFPM2-AS1 (ZFPM2-AS1) in retinoblastoma progression. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and Western blotting assays were performed to determine the expression of lncRNA, microRNA (miRNA), mRNA, and protein. The changes in cell proliferation, apoptosis, and cell migration were assessed by functional experiments. The interaction between ZFPM2-AS1, miR-511-3p, and paired box protein 6 (PAX6) was confirmed by a luciferase assay. Our study found that ZFPM2-AS1 and PAX6 were upregulated, whereas miR-511-3p was downregulated in retinoblastoma. ZFPM2-AS1 inhibition decreased the viability and migration of retinoblastoma cells. We also found that ZFPM2-AS1 targets miR-511-3p to upregulate PAX6 in Y79 and SO-RB50 cells. Moreover, we demonstrated that inhibiting miR-511-3p reversed the negative effects of silencing ZFPM2-AS1 and PAX6 on retinoblastoma cell viability and migration. In conclusion, retinoblastoma development is regulated by the ZFPM2-AS1/511-3p/PAX6 axis.

Introduction
Retinoblastoma (RB), the most common intraocular childhood cancer has an incidence of approximately 1:14,000 to 1:22,000 in newborn [1]. Although the cure rates of RB are encouraging, it remains a serious problem in low- and middle-income countries [2]. In Asia and Africa, the mortality rate of RB in children is between 40% and 70% [2]. Moreover, previous studies have revealed that RB is initiated by inactivation of the RB transcriptional corepressor 1 (RB1) gene [3], mutations in the RB1 gene alone do not fully explain the mechanism of genomic changes in RB cells [2]. Accumulated data indicate that many genetic or epigenetic changes in oncogenes and tumor suppressor genes are involved in the occurrence and development of RB [4]. Therefore, exploring the key regulatory factors involving long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and mRNAs is crucial for RB therapy.

Transcriptome changes in diseases are not limited to the production of abnormal levels of protein-coding RNA, but also include dysregulation of the expression of several non-coding members that comprise the human genome [5]. LncRNAs are a family of non-coding RNAs that are longer than 200 nucleotides in length. LncRNAs have the capacity to interact with proteins, DNA, and RNA, and are thus involved in the regulation of cell processes and disease [2]. In cancers, lncRNAs act as oncogenic and tumor suppressor lncRNAs to regulate different cancers. Among them, several lncRNAs have been demonstrated to promote the progression of RB, such as BANCR, AFAP1-AS1, and NEAT among others [6–8]. ZFPM2-AS1 is a novel lncRNA that was first discovered at high levels in gastric cancer [9]. In recent studies, ZFPM2-AS1 has been found to regulate several cancers such as lung adenocarcinoma [10,11] gastric carcinogenesis [9], cutaneous malignant melanoma [3], esophageal squamous cell carcinoma [12], thyroid cancer [13], as well as RB [14]. Lv et al. found that ZFPM2-AS1 was significantly upregulated in RB, and the down-regulation of ZFPM2-AS1 restrained the development of RB by regulating miR-515/homeobox A1
(HOXA1)/Wnt/β-Catenin pathway [14]. However, whether ZFPM2-AS1 is involved in RB progression through other regulatory networks remains unclear.

Here, we investigated the effect of the ZFPM2-AS1/miR-511-3p/paired box protein 6 (PAX6) axis in RB using bioinformatics analysis and cell functional experiments. We hypothesized that ZFPM2-AS1 promotes RB cell proliferation, migration, and apoptosis by sponging miR-511-3p and releasing PAX6. Our findings may provide novel targets for the regulation of RB, which may be useful for RB therapy.

Materials and methods

Bioinformatics analysis

StarBase was used to predict the miRNA binding to ZFPM2-AS1, and TargetScan was used to predict the target genes of miRNAs. GSE97508 from the gene expression omnibus (GEO) DataSets was used to screen differentially expressed genes (DEGs) with an adjusted P < 0.05. Protein-protein interaction network of the top 100 upregulated genes among the DEGs was constructed using the STRING database.

Clinical tissues

We collected RB and adjacent normal retinal tissues from 34 patients diagnosed with RB in our hospital between May 2020 and April 2021. Inclusion criteria: Patients met the diagnostic assessment requirements of RB proposed by the European Retinoblastoma Imaging Collaboration group [15], and were diagnosed with RB by imaging and pathology. The exclusion criteria were patients with severe infectious diseases, severe renal insufficiency, other malignancies, and previous radiotherapy or chemotherapy. All the patients signed the written consent form, and the ethical committee of our hospital approved our study (Approval number: 武三医伦LW2019-013). Supplementary Table S1 shows the clinico-pathological features of the 34 patients.

Cell culture and transfection

All the cells were provided by the American Type Culture Collection, including human retinal epithelial cells ARPE-19 and human RB cell lines (SO-RB50, WERI-Rb1, Y79, and HXO-RB44). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with 95% air and 5% CO₂. The culture medium was replaced every three days.

ZFPM2-AS1 was downregulated using three small interfering RNAs (siRNAs) obtained from Tsingke Biotechnology Co. Ltd. (Wuhan, Hubei, China), whereas PAX6 was downregulated using si-PAX6 obtained from Tsingke Biotechnology Co. Ltd. siRNA vector with nonspecific RNA oligonucleotide was used as a negative control (si-NC). miR-511-3p mimic/inhibitor, mimic-NC, and inhibitor-NC were provided by Tsingke Biotechnology Co. Ltd. For transfection, 3 × 10⁵ cells were resuspended in RPMI-1640 medium with 10% FBS and placed in a 6-cm dish, and transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The sequences used in this study are listed in Supplementary Table S2.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Cells were collected in 1.5 μL tubes and RNA was extracted with Trizol® (Sigma, St. Louis, MO, USA) reagent. cDNA was subsequently generated using the Quantscript RT Kit (Tiangen, Beijing, China) for RT-qPCR. qRT-PCR was conducted using Taq Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Relative expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Uracil 6 (U6) as internal references. The relative expression levels of ZFPM2-AS1, miR-511-3, and PAX6 were calculated using the 2⁻ΔΔCt method [16], and three technical replicates were used for each sample. The primers used are listed in Table 1.

Subcellular fractionation

Nuclear and cytoplasmic RNA was extracted using nuclear and cytoplasmic extraction reagents (Thermo Fisher, Waltham, MA, USA). ZFPM2-AS1 expression in the nucleus and cytoplasm was quantified by qRT-PCR. U6 and GAPDH were used as nuclear and cytoplasmic controls, respectively [17].
Detection of cell proliferation

The transfected cells were seeded in 96-well plates at a density of $3 \times 10^4$ cells/well. At 0, 24, 48, and 72 h, 10 μL of Cell Counting Kit-8 (CCK-8) reagent was added to each well and incubated for 2 h at 37°C. The absorbance was measured at 450 nm using a microplate analyzer (Thermo Fisher) [18].

Detection of caspase-3 activity

Caspase-3 activity assay was performed as previously described [19]. Cell apoptosis was confirmed by detecting caspase-3 activity using a Caspase-3 Assay Kit (ab39401, Abcam, Cambridge, UK). After transfection, the cells were lysed using 50 μL of cell lysis buffer to isolate proteins. Then, the protein was added to 50 μL reaction buffer containing 5 μL DEVE-p-NA and 0.5 μL dithiothreitol for 60 min at 37°C. The absorbance was measured at 405 nm using a microplate analyzer.

Cell migration assay

A 24-well Transwell (BD Biosciences, San Jose, CA, USA) with 8 μm pores was used to analyze cell migration. Briefly, transfected cells were placed in the upper chambers with serum-free medium. The bottom chamber was supplemented with 500 μL of RPMI-1640 medium containing 10% FBS. After 24 h, the cells that migrated to the lower membrane surface were immobilized with 4% paraformaldehyde, and then stained with 1% crystal violet for 5 min. The migrated cells attached to the lower membrane were counted using a microscope (Olympus, Tokyo, Japan). The cells migrating to the bottom of the well were counted using a blood cell counting plate [20].

Luciferase assay

Wild-type (Wt) ZFPM2-AS1, WT PAX6 3’UTR, mutant-type (Mut) ZFPM2-AS1, and MUT PAX6 3’UTR were purchased from Tsingke Biotechnology Co. Ltd., and cloned into the pGL3 luciferase reporter (Promega, Madison, WA, USA). These reporter plasmids were then co-transfected with miR-511-3p mimic or mimic-NC into SO-RB50 and Y79 cells. The Dual-Luciferase® Reporter Assay System (Promega) was used to detect luciferase activity in the cells after 72 h of incubation [21].

Western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma) containing the protease inhibitor cocktail (Sigma) to isolate total protein, and the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Following blocking with 5% skim milk for 2 h, the membranes were incubated with anti-PAX6 (1:1000, ab195045, Abcam) and anti-GAPDH antibodies (1:2000, ab8245, Abcam) at 4°C overnight. After washing, the membranes were incubated with the secondary antibody (Abcam) conjugated with horseradish peroxidase for 2 h at room temperature. Enhanced chemiluminescence (ECL) substrate (Bio-Rad) was used to visualize the protein bands [22].

Statistical analysis

The data in this study are shown as the mean ± standard deviation (SD) from three independent experiments. GraphPad Prism 7.0, was used to analyze the statistical significance using Student’s $t$-test for comparing two groups and one- or two-way ANOVA with post-hoc Dunnett’s or Tukey’s multiple comparison for comparing multiple groups. Statistical significance was set at $P < 0.05$. Pearson analysis was used to analyze the correlation between ZFPM2-AS1 and miR-511-3p or miR-511-3p and PAX6 in RB tissues.
Results

The objective of this study was to investigate the effects and mechanisms of ZFPM2-AS1 in RB. We conducted a series of in vitro studies and found that ZFPM2-AS1 promotes RB cell proliferation, migration, and apoptosis by sponging miR-511-3p and releasing PAX6. Therefore, for the first time, we demonstrate the role of the FPM2-AS1/miR-511-3p/PAX6 axis in RB, which provides a valuable theoretical basis for the diagnosis and treatment of RB.

**PAX6 and miR-511-3p are potentially critical downstream effectors of ZFPM2-AS1 in RB**

Because ZFPM2-AS1 was reported to be an oncogene in RB, we used starBase to predict the miRNAs sponged by ZFPM2-AS1, and identified a total of ten potential target miRNAs. Among these miRNAs, miR-511-3p was found to provide the strongest experimental evidence; hence, miR-511-3p was chosen as the target miRNA for the subsequent experiments. By intersecting the top 100 significantly upregulated genes in RB according to GSE97508 profiling and the predicted targets of miR-511-3p using the TargetScan algorithm, we identified 26 mRNAs (Figure 1a). By uploading the 26 genes to the STRING database, three interaction networks were enriched (Figure 1b). Among them, PAX6 showed the most interactions in the largest network; thus, we chose to focus on PAX6 in the study.

**ZFPM2-AS1 is high expressed in RB**

To investigate the potential role of ZFPM2-AS1 in RB, we analyzed the expression of ZFPM2-AS1 by qRT-PCR. ZFPM2-AS1 expression was significantly increased in RB samples compared to that in normal samples (Figure 2a). The correlation between ZFPM2-AS1 expression and clinicopathological features is shown in Supplementary Table S1. ZFPM2-AS1 expression is closely associated with tumor size, choroidal invasion, and optic nerve invasion. However, there was no obvious association between AFAP1-AS1 expression and age, sex, laterality or pathologic grade. Moreover, we found that ZFPM2-AS1 was highly expressed in the RB cell lines, SO-RB50, Y79 and HXO-Rb44 (Figure 2b). To examine whether ZFPM2-AS1 functions post-transcriptionally, we used qRT-PCR to confirm the localization of ZFPM2-AS1 in the cytosol or nucleus. We observed that ZFPM2-AS1 was mostly present in the cytosol in SO-RB50 and Y79 (Figure 2c) cells, supporting the post-transcriptional function of ZFPM2-AS1. To assess the role of ZFPM2-AS1 in RB development, we designed three siRNAs against ZFPM2-AS1, and found that si-ZFPM2-AS1-1 showed the strongest inhibition (Figure 2d). Therefore, si-ZFPM2-AS1-1 was selected for subsequent inhibition experiments with ZFPM2-AS1. Using the CCK-8 assay, we found that inhibition of ZFPM2-AS1 significantly decreased the viability of Y79 and SO-RB50 (Figure 2e) cells. Moreover, we found that ZFPM2-AS1 inhibition increased the

![Figure 1. PAX6 and miR-511-3p can be potentially critical downstream effectors of ZFPM2-AS1 in retinoblastoma.](image-url)
apoptosis marker Caspase-3 activity (figure 2f). Transwell assays showed that the number of migrating cells was significantly decreased when ZFPM2-AS1 was inhibited by si-ZFPM2-AS1 (Figure 2g). Our results show that inhibition of ZFPM2-AS1 increases apoptosis and suppresses cell viability and migration capacity of RB.

**miR-511-3p is sponged by ZFPM2-AS1 in RB**

To evaluate whether miR-511-3p acts downstream of ZFPM2-AS1 in RB, we used qRT-PCR to determine miR-511-3p expression. We observed a significant decrease in miR-511-3p expression in RB tissues (Figure 3a), and miR-511-3p expression was negatively correlated with ZFPM2-AS1 expression
(Figure 3b). We also found a decrease in miR-511-3p expression in RB cells (Figure 3c). The region of ZFPM2-AS1 that sponges miR-511-3p was predicted by miRDB (Figure 3d). Luciferase assay demonstrated that the miR-511-3p mimic was specifically inhibited in the ZFPM2-AS1-WT group (Figure 3e), and si-ZFPM2-AS1 increased miR-511-3p expression in Y79 and SO-RB50 cells (Figure 3f). Transfection of miR-511-3p mimic or miR-511-3p inhibitor together with si-ZFPM2-AS1 in Y79 and SO-RB50 cells, revealed that miR-511-3p mimic increased miR-511-3p expression by more than 5-fold, whereas miR-511-3p inhibitor suppressed miR-511-3p expression by 70% (Figure 3g). Moreover, co-transfection of miR-511-3p inhibitor and si-ZFPM2-AS1 did not cause a significant difference in miR-511-3p expression. Together, these results indicate that ZFPM2-AS1 sponges miR-511-3p in RB cells.

ZFPM2-AS1 relieved the effect of miR-511-3p in RB cells

To identify the effects of ZFPM2-AS1 and miR-511-3p in the development of RB, we first analyzed the viability of Y79 and SO-RB50 cells treated with miR-511-3p mimic/inhibitor and si-ZFPM2-AS1-1. The CCK-8 assay showed a decrease in cell viability following transfection of miR-511-3p mimic and an increase in cell viability following transfection of miR-511-3p inhibitor (Figure 4a). si-ZFPM2-AS1 combined with miR-511-3p inhibitor relieved the positive effect of the miR-511-3p inhibitor on cell viability. Next, we analyzed the activity of caspase-3 in Y79 and SO-RB50 cells and found that the activity of caspase-3 was increased when cells were transfected with miR-511-3p mimic, whereas it was decreased when cells were transfected with miR-511-3p inhibitor (Figure 4b). Co-transfection of miR-511-3p inhibitor and si-ZFPM2-AS1 also relieved the positive role of the miR-511-3p inhibitor on the activity of caspase-3. Using the Transwell assay, we found that the upregulation of miR-511-3p decreased the migration of Y79 and SO-RB50 cells: miR-511-3p inhibition increased the migration capability of these cells, whereas si-ZFPM2-AS1 and miR-511-3p inhibitor blocked the increased migration capability caused by miR-511-3p inhibitor (Figure 4c). These data indicate that ZFPM2-AS1 participates in RB development by regulating miR-511-3p.

miR-511-3p and PAX6 are located downstream of ZFPM2-AS1

Using TargetScan, miR-511-3p was predicted to target two positions in the 3’UTR of PAX6 (Figure 5a). To determine whether PAX6 was specifically inhibited by miR-511-3p, we performed luciferase assay and observed that the miR-511-3p mimic significantly decreased the luciferase activity in WT, MUT-1, or MUT-2 PAX6, whereas co-MUT PAX6 did not affect luciferase activity (Figure 5b). Compared to normal samples, the expression of PAX6 was increased in RB samples (Figure 5c), and we also found a negative linear relationship between PAX6 and miR-511-3p (Figure 5d). Moreover, the results from qRT-PCR and Western blot assays suggested that the expression of PAX6 mRNA and protein was inhibited when Y79 and SO-RB50 were treated with si-ZFPM2-AS1 (Figure 5e,f). Here, we also found that miR-511-3p mimic inhibited whereas miR-511-3p inhibitor promoted PAX6 expression, but PAX6 expression remained unaffected by co-transfection with miR-511-3p inhibitor and si-ZFPM2-AS1 (Figure 5g,h). These data suggest that miR-511-3p targeting PAX6 is downstream of ZFPM2-AS1 in Y79 and SO-RB50 cells.

miR-511-3p relieved the effect of PAX6 on RB cells

To determine whether miR-511-3p promotes RB development through PAX6, we transfected si-PAX6 and miR-511-3p inhibitor in Y79 and SO-RB50 cells. Results from qRT-PCR and Western blot assays showed a decrease in PAX6 expression when cells were treated with si-PAX6, and an elevation in PAX6 expression following transfection of miR-511-3p inhibitor (Figure 6a,b). In addition, PAX6 expression was restored in cells transfected with si-PAX6 and miR-511-3p inhibitors. Using the CCK8 assay, we confirmed that the cell viability was decreased after treatment with si-PAX6, but cell viability was restored when cells were treated with miR-511-3p inhibitor together with si-PAX6 (Figure 6c). We also found that caspase-3 activity was increased when cells were treated with si-PAX6, but miR-511-3p inhibitor and
si-PAX6 co-transfected cells recovered caspase-3 activity (Figure 6d). Furthermore, we observed that cell migration was inhibited when PAX6 was suppressed, but miR-511-3p restored the inhibitory effect of si-PAX6 on cell migration (Figure 6e). These data suggest that the inhibitory effect of si-PAX6 on RB cells is reversed by the miR-511-3p inhibitor.

**Discussion**

Our current study demonstrated that lncRNA ZFPM2-AS1 plays a role in RB progression by targeting the microRNA miR-511-3p and PAX6. Specifically, ZFPM2-AS1 and PAX6 are overexpressed in RB, and silencing ZFPM2-AS1 or...
PAX6 decreased cell viability and migration of Y79 and SO-RB50 cells. In addition, miR-511-3p restored the effect of ZFPM2-AS1 and PAX6 on the malignancy of RB cells by targeting PAX6.

ZFPM2-AS1 is involved in the pathogenesis of several tumors. For instance, ZFPM2-AS1 promotes gastric cancer through the macrophage migration inhibitory factor/p53 signaling axis [9]. Significant upregulation of ZFPM2-AS1 was observed in lung adenocarcinoma cell lines [11]. ZFPM2-AS1 promotes cell invasion in hepatocellular carcinoma [23]. ZFPM2-AS1 is highly expressed in gliomas, and its silencing inhibits the survival of cancer cells in vitro and in vivo [24]. In RB, Lyv et al. [14] revealed that knockdown of ZFPM2-AS1 inhibited the growth and metastasis of RB in vivo and in vitro by sponging miR-515. Similarly, we found that ZFPM2-AS1 is overexpressed in RB, and showed that silencing ZFPM2-AS1 inhibited cell viability and migration. However, we found that ZFPM2-AS1 sponges miR-511-3p to participate in the development of RB, which is different from the results of Lyv et al.

LncRNAs can competitively bind microRNAs with transcripts, and thereby regulate genes post-transcriptionally [4]. Our study found that

Figure 4. ZFPM2-AS1 relieved the effect of miR-511-3p on retinoblastoma cells. (a) ZFPM2-AS1 relieved the effect of miR-511-3p on cell viability. (b) ZFPM2-AS1 relieved the effect of miR-511-3p on caspase-3 activity. (c) ZFPM2-AS1 relieved the effect of miR-511-3p on cell migration. mimic, miR-511-3p mimic. inhibitor, miR-511-3p inhibitor. **P < 0.001 compared with mimic-NC. ##P < 0.001 compared with inhibitor-NC. &P < 0.001 compared with inhibitor-NC.
Figure 5. miR-511-3p and PAX6 locate in the downstream of ZFPM2-AS1. (a) Target sequences between PAX6 and miR-511-3p. (b) Luciferase assay proved the target relationship between PAX6 and miR-511-3p in SO-RB50 and Y79 cells. **P < 0.001 compared with mimic-NC. (c) Expression of PAX6 in tumor and normal samples. **P < 0.001. (d) The correlation of expression between PAX6 and miR-511-3p in tumor samples was analyzed by Pearson analysis. E-F. si-ZFPM2-AS1 upregulated the expression of PAX6 mRNA (e) and protein (f) in SO-RB50 and Y79 cells. **P < 0.001 compared with si-NC. G-H. The effects of miR-511-3p mimic, miR-511-3p inhibitor, si-ZFPM2-AS1 on the expression of PAX6 mRNA (g) and protein (h) in SO-RB50 and Y79 cells. mimic, miR-511-3p mimic. inhibitor, miR-511-3p inhibitor. **P < 0.001 compared with mimic-NC. ##P < 0.001 compared with inhibitor-NC. &P < 0.001 compared with inhibitor-NC.
ZFPM2-AS1 was overexpressed in the cytoplasm of RB cells, suggesting the post-transcriptional regulation of ZFPM2-AS1. We then showed that ZFPM2-AS1 inhibited miR-511-3p binding to the PAX6 3’UTR, leading to the upregulation of PAX6. According to the starBase software, a total of ten potential target miRNAs were predicted to be sponged by ZFPM2-AS1. Among these, miR-511-3p was predicted to provide the strongest experimental evidence. Previous studies have
demonstrated that miR-511-3p is involved in cancer development. For example, inhibition of miR-511-3p accelerates lung adenocarcinoma progression [11]. In human prostate cancer, miR-511-3p acts as a tumor suppressor by targeting AKT3 to inhibit tumor growth in vivo and in vitro [25]. miR-511-3p sponged by LINC02163 restrains the malignant properties of breast cancer by targeting HMGA2 [26]. These studies suggest that miR-511-3p may play an inhibitory role in RB, although its role has not been explored in RB. Our findings revealed that downregulation of miR-511-3p inhibited the malignant properties of RB cells, which enriched the function of miR-511-3p in cancers.

Paired box protein 6 (PAX6) is essential for eye formation and retinal development in vertebrates and invertebrates [27]. PAX6 regulates the expression of a variety of molecules, including transcription factors, cell adhesion and short-term cell signaling molecules, hormones, and structural proteins [28]. PAX6 is also involved in a variety of key biological processes, including cell proliferation, migration, adhesion, signal transduction in normal development and tumorigenesis [29,30]. PAX6 showed the most interactions in the largest network, and has been reported to be a cancer driver in various cancers including lung [31–33] breast [30], colon [34,35], liver [36], pancreatic [36], and thyroid cancers [37]. Studies have shown that forced expression of PAX6 induces the formation of retinal heterotopic tissue [38]. Several studies have demonstrated the function of PAX6 in RB. For instance, Li et al. found that suppressing the expression of PAX6 inhibits the malignancy of RB [39]. The proliferation, migration, and invasion of RB cells were increased when PAX6 was upregulated by the miR-129-5p inhibitor [40]. Our study further confirmed the positive effect of PAX6 in RB cells, however, we are the first to demonstrate the targeting relationship between PAX6 and miR-511-3p, which is different from the results of previous studies on PAX6 in RB.

Although we have identified regulatory mechanisms of the ZFPM2-AS1/miR-511-3p/PAX6 axis in RB progression, the mechanism by which downregulation of PAX6 inhibits tumor progression remains unclear. In lung cancer cells, PAX6 was shown to promote tumor migration through the PI3K/AKT pathway [31]. Therefore, whether PAX6 regulates RB progression via the PI3K/AKT pathway needs to be further explored. Moreover, we did not disrupt the expression of ZFPM2-AS1 in an animal model to confirm the inhibition of ZFPM2-AS1 in RB progression; hence, more experimental evidence is needed in the future.

**Conclusion**

In conclusion, our study demonstrated that ZFPM2-AS1 contributes to RB progression via the miR-511-3p/PAX6 axis. Inhibition of ZFPM2-AS1 and PAX6 or upregulation of miR-511-3p may serve as promising therapeutic strategies for RB.

**Article highlights**

1. ZFPM2-AS1 and PAX6 are upregulated, whereas miR-511-3p is downregulated in retinoblastoma
2. Inhibition of ZFPM2-AS1 or PAX6 decreased cell viability and migration of retinoblastoma cells
3. ZFPM2-AS1 targets miR-511-3p to upregulate PAX6 in retinoblastoma cells
4. Inhibiting miR-511-3p restores the effect of silencing ZFPM2-AS1 and PAX6 on retinoblastoma cell behavior

**Consent to participate**

All patients signed written informed consent

**Consent for publication**

Consent for publication was obtained from the participants.

**Availability of Data and Material**

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

**Ethics approval**

The present study was approved by the Ethics Committee of the Wuhan Third Hospital Guanggu District (Approval No. KY2020-059). The processing of clinical tissue samples is in strict compliance with the ethical standards of the
Declaration of Helsinki. All patients signed written informed consent.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Authors’ contributions
WCN conceived and designed the study. ZL performed the experiments and data analysis. WCN and ZL made the acquisition of data. KA did the analysis and interpretation of data. All authors read and approved the manuscript.

References
[1] Bai SW, Li B, Zhang H, et al. Pax6 regulates proliferation and apoptosis of human retinoblastoma cells. Invest Ophthalmol Vis Sci. 2011;52(7):4560–4570.
[2] Fabian ID, Abdallah E, Abdullahi SU, et al. Global retinoblastoma presentation and analysis by national income level. JAMA Oncol. 2020;6(5):685–695.
[3] Liu W, Hu X, Mu X, et al. ZFPM2-AS1 facilitates cell proliferation and migration in cutaneous malignant melanoma through modulating miR-650/NOTCH1 signaling. Dermatol Ther. 2021;34(2):e14751.
[4] Zhao Z, Sun W, Guo Z, et al. Mechanisms of lncRNA/microRNA interactions in angiogenesis. Life Sci. 2020;254:116900.
[5] Liz J, Esteller M. lncRNAs and microRNAs with a role in cancer development. Biochim Biophys Acta. 2016;1859(1):169–176.
[6] Su S, Gao J, Wang T, et al. Long non-coding RNA BANCR regulates growth and metastasis and is associated with poor prognosis in retinoblastoma. Tumour Biol. 2015;36(9):7205–7211.
[7] Hao F, Mou Y, Zhang L, et al. LncRNA AFAP1-AS1 is a prognostic biomarker and serves as oncogenic role in retinoblastoma. Biosci Rep. 2018;38(3).
[8] Luan L, Hu Q, Wang Y, et al. Knockdown of lncRNA NEAT1 expression inhibits cell migration, invasion and EMT by regulating the miR-24-3p/LRG1 axis in retinoblastoma cells. Exp Ther Med. 2021;21(4):367.
[9] Kong F, Deng X, Kong X, et al. ZFPM2-AS1, a novel lncRNA, attenuates the p53 pathway and promotes gastric carcinogenesis by stabilizing MIF. Oncogene. 2018;37(45):5982–5996.
[10] Han S, Cao D, Sha J, et al. LncRNA ZFPM2-AS1 promotes lung adenocarcinoma progression by interacting with UPF1 to destabilize ZFPM2. Mol Oncol. 2020;14(5):1074–1088.
[11] Li J, Ge J, Yang Y, et al. Long noncoding RNA ZFPM2-AS1 is involved in lung adenocarcinoma via miR-511-3p/AF44R pathway. J Cell Biochem. 2020;121(3):2534–2542.
[12] Sun G, Wu C. ZFPM2-AS1 facilitates cell growth in esophageal squamous cell carcinoma via up-regulating TRAF4. Biosci Rep. 2020;40(4).
[13] Ren R, Du Y, Niu X, et al. ZFPM2-AS1 transcriptionally mediated by STAT1 regulates thyroid cancer cell growth, migration and invasion via miR-515-5p/TUSC3. J Cancer. 2021;12(11):3393–3406.
[14] Lyv X, Wu F, Zhang H, et al. Long noncoding RNA ZFPM2-AS1 knockdown restrains the development of retinoblastoma by modulating the MicroRNA-515/FOXA1/Wnt/β-Catenin axis. Invest Ophthalmol Vis Sci. 2020;61(6):41.
[15] de Graaf P, Görick S, Rodjan F, et al. Guidelines for imaging retinoblastoma: imaging principles and MRI standardization. Pediatr Radiol. 2012;42(1):2–14.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–408.
[17] Chen L, Shi J, Wu Y, et al. CircRNA CDR1as promotes hepatoblastoma proliferation and stemness by acting as a miR-7-5p sponge to upregulate KLF4 expression. Aging (Albany NY). 2020;12(19):19233–19253.
[18] Lu J, Lin J, Zhou Y, et al. MiR-328-3p inhibits lung adenocarcinoma-genesis by downregulation PYCR1. Biochem Biophys Res Commun. 2021;550:99–106.
[19] Renema P, Kozhuhkar N, Pastukh V, et al. Exoenzyme Y induces extracellular active caspase-7 accumulation independent from apoptosis: modulation of transmissible cytotoxicity. Am J Physiol Lung Cell Mol Physiol. 2020;319(2):L380–L90.
[20] Guo L, Fu J, Sun S, et al. MicroRNA-143-3p inhibits colorectal cancer metastases by targeting ITGA6 and ASAP3. Cancer Sci. 2019;110(2):805–816.
[21] Song AF, Kang L, Wang YF, et al. MiR-34a-5p inhibits fibroblast-like synoviocytes proliferation via XBPI. Eur Rev Med Pharmacol Sci. 2020;24(22):11675–11682.
[22] Song W, Zhang T, Yang N, et al. Inhibition of micro RNA miR-122-5p prevents lipopolysaccharide-induced myocardial injury by inhibiting oxidative stress, inflammation and apoptosis via targeting GIT1. Bioengineered. 2021;12(1):1902–1915.
[23] He H, Wang Y, Ye P, et al. Long noncoding RNA ZFPM2-AS1 acts as a microRNA sponge and promotes cell invasion through regulation of miR-139/GDF10 in hepatocellular carcinoma. J Exp Clin Cancer Res. 2020;39(1):159.
[24] Zhang Y, Zhang Y, Wang S, et al. SP1-induced lncRNA ZFPM2 antisense RNA I (ZFPM2-AS1) aggravates glioma progression via the miR-515-5p/Superoxide dismutase 2 (SOD2) axis. Bioengineered. 2021;12(1):2299–2310.
[25] Zhang F, Wu Z. Significantly altered expression of miR-511-3p and its target AKT3 has negative prognostic value in human prostate cancer. Biochimie. 2017;140:66–72.

[26] Qin C, Jin L, Li J, et al. Long noncoding RNA LINC02163 accelerates malignant tumor behaviors in breast cancer by regulating the MicroRNA-511-3p/HMGA2 axis. Onco Res. 2020;28(5):483–495.

[27] Park JW, Yang J, Xu RH. PAX6 alternative splicing and corneal development. Stem Cells Dev. 2018;27(6):367–377.

[28] Simpson TI, Price DJ. Pax6; a pleiotropic player in development. BioEssays: news and reviews in molecular, cellular and developmental biology. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology. 2002;24(11):1041–1051.

[29] Fang J, Zhang T, Liu Y, et al. PAX6 downregulates miR-124 expression to promote cell migration during embryonic stem cell differentiation. Stem Cells Dev. 2014;23:2297–2310.

[30] Zhang XH, Li BF, Ding J, et al. LncRNA DANC-miR-758-3p-PAX6 molecular network regulates apoptosis and autophagy of breast cancer cells. Cancer Manag Res. 2020;12:4073–4084.

[31] Wu DM, Zhang T, Liu YB, et al. The PAX6-ZEB2 axis promotes metastasis and cisplatin resistance in non-small cell lung cancer through PI3K/AKT signaling. Cell Death Dis. 2019;10(5):349.

[32] Liu Z, Han L, Yu H, et al. LINC01619 promotes non-small cell lung cancer development via regulating PAX6 by suppressing microRNA-129-5p. Am J Transl Res. 2020;12(6):2538–2553.

[33] Jiang Q, Xing W, Cheng J, et al. Knockdown of lncRNA XIST suppresses cell tumorigenicity in human non-small cell lung cancer by regulating miR-142-5p/PAX6 axis. Onco Targets Ther. 2020;13:4919–4929.

[34] Hao C, Gao C, Shang H, et al. MicroRNA-31 inhibits the growth and metastasis and enhances drug sensitivity of the human colon cancer cells by targeting PAX6. J BUON. 2020;25:1860–1865.

[35] Zhang X, Xu J, Zhang H, et al. MicroRNA-758 acts as a tumor inhibitor in colorectal cancer through targeting PAX6 and regulating PI3K/AKT pathway. Oncol Lett. 2020;19(6):3923–3930.

[36] Xu Q, Liu K. MiR-369-3p inhibits tumorigenesis of hepatocellular carcinoma by binding to PAX6. J Biol Regul Homeost Agents. 2020;34(3):917–926.

[37] Zhang S, Wang Q, Li D, et al. MicroRNA-509 targets PAX6 to inhibit cell proliferation and invasion in papillary thyroid carcinoma. Mol Med Rep. 2019;19(2):1403–1409.

[38] Cvekl A, Callaerts P. PAX6: 25th anniversary and more to learn. Exp Eye Res. 2017;156:10–21.

[39] Li X, Yang L, Shuai T, et al. MiR-433 inhibits retinoblastoma malignancy by suppressing Notch1 and PAX6 expression. Biomed Pharmacother. 2016;82:247–255.

[40] Liu Y, Liang G, Wang H, et al. MicroRNA-129-5p suppresses proliferation, migration and invasion of retinoblastoma cells through PI3K/AKT signaling pathway by targeting PAX6. Pathol Res Pract. 2019;215(12):152641.