MiR-204, down-regulated in retinoblastoma, regulates proliferation and invasion of human retinoblastoma cells by targeting CyclinD2 and MMP-9

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Aberrant expression of miR-204 had been frequently reported in cancer studies; however, the mechanism of its function in retinoblastoma remained unknown. Here, we reported that miR-204 was frequently downregulated in retinoblastoma tissues and cell lines. Enforced expression of miR-204 inhibited retinoblastoma cells’ proliferation and invasion. In vivo study indicated that restoration of miR-204 inhibited tumor growth. CyclinD2 and MMP-9 were identified as potential targets of miR-204. In addition, a reverse correlation between miR-204 and CyclinD2 or MMP-9 expression was noted in retinoblastoma tissues. Taken together, our results identified a crucial tumor suppressive role of miR-204 in the progression of retinoblastoma.

1. Introduction

Retinoblastoma (Rb), a deadly pediatric eye cancer, is the most common intraocular tumor in children [1]. The mortality rate among children diagnosed with Rb is 50–70% in the underdeveloped countries [2]. Children with Rb are at risk for three life-threatening problems, including metastasis of Rb, intracranial neuroblastic malignancy (trilateral Rb), and second primary tumors. In order to improve the therapeutic outcome of patients with retinoblastoma, there is an urgent need to further study the biology and molecular mechanisms of retinoblastoma and identify the specific biomarkers that cause tumor progression.

MicroRNAs (miRNAs), which are single-stranded 19–25 nucleotide short RNAs, function as negative posttranscriptional regulators of target genes [3]. MiRNAs play significant role in lots of physiological and pathological process, such as differentiation, proliferation, metabolism, and death [4]. In addition, accumulating data suggests that miRNAs are frequently dysregulated and play critical roles in various types of cancers through acting as potent oncopgenes or tumor suppressor genes [5]. Interestingly, recent studies show that miRNAs were involved in the development of retinoblastoma. For example, Mu et al. report reduced expression of the tumor suppressor microRNA let-7 in retinoblastoma [6]. Conkrite et al. showed that overexpression of miR-17–92 is important in the formation of a cohort of retinoblastomas [7]. The miR-34 family, which expression is usually downregulated in many cancer cell lines, is variably expressed in retinoblastomas. Restoration of its expression can inhibit the proliferation of retinoblastoma cells [8]. In addition, some plasma miRNA levels are found to be of value in RB diagnosis [9]. These findings suggest that targeting the miRNAs may potentially lead to a novel strategy of diagnosis and therapy to retinoblastomas.

A number of studies have demonstrated that deregulation of miR-204 expression is involved in the initiation and progression of cancer by affecting tumor cell function, including growth, invasion and metastasis [10,11]. However, the expression of miR-204 in human retinoblastoma patients, and its functions in human retinoblastoma cells, as well as the molecular mechanisms by which miR-204 exerts its functions, has not been fully understood. In the present study, we sought to investigate the potential role of miR-204 in the development and progression of retinoblastoma. We identified that miR-204 could regulate the proliferation and

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invasion of retinoblastoma cells by directly targeting the CyclinD2 and MMP-9 genes.

2. Materials and methods

2.1. Cell lines and patient samples

Human retinoblastoma cell lines were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The identities of the cell lines were validated by STR profiling. The STR profiling of SO-RB50 cell line was presented in Supplement Table 2. All cell lines were maintained in a humid wet atmosphere containing 5% CO2 at 37 °C in RPMI-1640 medium supplemented with 10% newborn bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Retinoblastoma tissues and normal retina tissues were obtained from the Center of Ophthalmology, Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong, China. This study was approved by the institute research ethics committee of Affiliated Hospital of Guangdong Medical College.

2.2. RNA isolation and quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, California, USA). For qPCR, RNA was reverse transcribed to cDNA from 1 µg of total RNA using a Reverse Transcription Kit (Takara). Real-time PCR analyses were conducted with Power SYBR Green (Life Technologies). Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System with SYBR Premix Ex Taq (TaKaRa). Results were normalized to the expression of U6 or GAPDH. Melt curve analysis was used to assess qPCR amplicon length (TaKaRa). For the Ct method, the CT value of the endogenous controls was compared with Ct values of the target genes. The relative expression level of the miRNA was calculated and normalized using the 2-ΔΔCT method.

2.3. Plasmid, microRNA mimic, lentivirus production and infection

Full-length CyclinD2 and MPP-9 cDNA entirely lacking the 3′-UTR was subcloned into the eukaryotic expression vector pcDNA3.1(+)(Invitrogen). The CyclinD2 and MPP-9 3′UTR target site for miR-204 were amplified by PCR cloned into the XbaI site of pGL3 control (Promega, Madison, USA). This vector was sequenced and named WT 3′UTR. The primer sequences of miR-204 mimic control were 5′-GTCAGGCTTCGCTCTCT-3′ (forward) and 5′-CTCGCTTCGGCAGCAGG-3′ (reverse). The primers for GAPDH were 5′-AAGGTGTGATCCATGACT-3′ (forward) and 5′-TAGGCAATTTGTCGCTTGA-3′ (reverse). The primers for CyclinD2 were 5′-ACCTTCGAGACGCTGACCTC-3′ (forward) and 5′-GACACCTCAAGAAAGCCGTC-3′ (reverse). The primers for MMP9 were 5′-TTGACCCGTTATGTTAAGTCACTGGC-3′ (forward) and 5′-GACACCTCAAGAAAGCCGTC-3′ (reverse). The TaqMan PCR assay was used to measure the mature miR-204 and the primers were used as previously described [12]. The relative expression of each gene was calculated and normalized using the 2-ΔΔCT method relative to U6 snRNA or GAPDH.

2.4. Luciferase reporter assay

For the reporter assays, WT or MT 3′UTR vector and the control vector pRL-CMV ((cytomegalovirus) coding for Renilla luciferase, Promega) were cotransfected. We performed the luciferase assays using Y79 cells transiently transfected with Renilla constructs (as an internal control) or plasmids pMIRGLO-CyclinD2-3′UTR-WT or pMIRGLO-CyclinD2-3′UTR-MT with or without miR-204 mimic or miR-204 mimic control using the Dual Luciferase Assay system following the manufacturer’s instructions (Promega, Madison, WI). Luciferase activity was measured 36 h after transfection. All luciferase activity readings were normalized relative to the activity of the Renilla luciferase control and the results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC). All experiments were performed in triplicate at least 3 times independently.

2.5. MTT, colony formation, migration and invasion assays

The MTT assay and colony formation assays were carried out as described previously [13,14]. Cell migration and invasion assays were carried out according to previous description [15]. For cell migration assays, 1 × 10^6 cells in 100 ml PRMI-1640 medium without FBS were seeded on a fibronectincoated polycarbonate membrane insert in a transwell apparatus (Costar, MA). In the lower chamber, 600 ml PRMI-1640 medium with 10% FBS was added as a chemoattractant. After the cells were incubated for 8 h at 37 °C in a 5% CO2 atmosphere, the insert was washed with PBS, and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with Giemsa solution and counted under a microscope in 5 predetermined fields (200 ×). For cell invasion assays, the procedure was similar to the cell migration assay, except transwell membranes were precoated with 24 µg/µl Matrigel (BD Systems, USA). Cells adhering to the lower surface were counted the same way as the cell migration assay.

2.6. Western blot assay

Protein samples were resolved by 10% SDS–PAGE and then transferred to PVDF membranes. The membranes were blocked and probed with antibodies against CyclinD2 (C-17, sc-81, Santa Cruz Biotechnology, Inc.), MMP-9 (2c3, sc-21733, Santa Cruz Biotechnology, Inc.) and GAPDH (C-9, sc-365062, Santa Cruz Biotechnology, Inc.), followed by probed with the secondary antibodies accordingly. The dilution of the primary antibodies were 1:500, while the dilution of the secondary antibodies were 1:1000. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (ECL; Pierce Biotechnology, Inc.).

2.7. In vivo growth assay

Five animals for each group were used for these experiments. For the in vivo tumor growth studies, 1 × 10^6 Y79 cells, stably expressing miR-204, or the control vector, were injected subcutaneously in the upper back of BALB/C-nu/nu athymic nude mice.
After 21 days, tumor samples were carefully removed and weighed. All animal procedures were performed in accordance with institutional guidelines.

2.8. Statistical analysis

Statistical analysis was performed using a SPSS software package (SPSS Standard version 16.0, SPSS Inc). Data were expressed as the mean ± S.D. from at least three independent experiments. Student’s t-test was used for comparison between two groups, and one-way ANOVA was used for comparison among multiple groups. Correlation was analyzed by Spearman test. p Values < 0.05 were considered significant.

3. Results

3.1. miR-204 expression is down-regulated in primary retinoblastoma tissues and cell lines

Real-time PCR was performed to examine the expression levels of miR-204 in a subset of 26 primary human retinoblastoma tissues as compared to 8 normal pediatric retinas (patient demographics can be found in Supplementary Table 1). As shown in Fig. 1A, the expression levels of miR-204 were significantly decreased in retinoblastoma tissues compared to normal pediatric retinas (p < 0.05). Next, we evaluated the expression of miR-204 in three human retinoblastoma cell lines (Y79, WERI-RB-1, and SO-RB50). In comparison to 8 normal pediatric retina controls, miR-204 was down-regulated in these cell lines (Fig. 1B). We also detected the mature miR-204 by TaqMan PCR in the human retinoblastoma tissues. We found that there was a positive correlation between pre-miR-204 and mature miR-204 (Supplement Fig. 3).

3.2. miR-204 regulates cell proliferation, migration and invasion in vitro and in vivo

First, miR-204 mimic and miR control were transiently transfected into Y79 cells. qRT-PCR was used to confirm miR-204 overexpression (Fig. 2A). The MTT and colony formation assays were performed to investigate the effect of miR-204 in retinoblastoma cell proliferation. The results showed that restoration of miR-204 significantly inhibited the proliferation of Y79 cells (Fig. 2B). Interestingly, silencing of CyclinD2 mimicked the effect of miR-204 on cell growth in vitro (Supplementary Fig. 1A–C). To examine the effect of miR-204 on cell migration, Y79 cells transfected with miR-204 mimic were cultured on transwell coated with matrigel, we determined changes in cell invasiveness. When compared with the control group, Y79 cells transfected with miR-204 mimic exhibited significantly decreased invasiveness (Fig. 2D). Interestingly, SO-RB50 cells transfected with miR-204 also exhibited significantly decreased proliferation and invasiveness (Supplementary Fig. 2A–C).

Moreover, we investigated the effects of miR-204 on growth and invasion of Y79 cells in vivo. We initially infected Y79 cells with lentiviral vectors expression miR-204 stably (LV-miR-204) or the negative control (LV-Ctrl). Stable transfection of miR-204 into Y79 cells resulted in decreased growth and tumor weight of subcutaneous xenograft tumors in nude mice, when compared with those stably transfected with empty vector (Fig. 2E). These results suggested that miR-204 could inhibit the growth of retinoblastoma cells in vivo.

3.3. CyclinD2 and MMP-9 are potential direct targets of miR-204 in retinoblastoma cells

It is generally accepted that miRNAs exert their function by regulating expression of their downstream target genes. Thus, putative miR-204 targets were predicted by using target prediction programs, miRanda and TargetScan. Our analysis revealed that CyclinD2 and MMP-9 were two potential targets of miR-204. The 3’-UTR of CyclinD2 and MMP-9 mRNA contains a complementary site for the seed region of miR-204 (Fig. 3A).

To verify whether or not CyclinD2 and MMP-9 are direct targets of miR-204, CyclinD2 and MMP-9 wild-type 3’-UTRs and mutants containing the miR-204 binding sites were cloned downstream of the luciferase open reading frame. The results showed that miR-204 significantly inhibited the luciferase activity of the CyclinD2 and MMP-9 WT 3’-UTR but not that of the mutant in Y79 cells (Fig. 3B). To directly assessed the effect of miR-204 on CyclinD2 and MMP-9 expression, we transfected miR-204 into Y79 cells and found that overexpression of miR-204 reduced the CyclinD2 and MMP-9 mRNA and protein levels (Fig. 3C and D). Taken together, these results indicated that CyclinD2 and MMP-9 were direct targets of miR-204 in retinoblastoma cells.

3.4. miR-204 levels are inversely correlated with mRNA expression of CyclinD2 and MMP-9 in retinoblastoma tissues

We further examined the mRNA expression of CyclinD2 and MMP-9 in the 26 cases of retinoblastoma tissues used before. As shown in Fig. 4A and B, when CyclinD2 or MMP-9 mRNA levels significantly less in miR-204-mimics transfected group, when compared with the control group (Fig. 2C). Using a boyden chamber coated with matrigel, we determined changes in cell invasiveness. When compared with the control group, Y79 cells transfected with miR-204 mimic exhibited significantly decreased invasiveness (Fig. 2D). Interestingly, SO-RB50 cells transfected with miR-204 also exhibited significantly decreased proliferation and invasiveness (Supplementary Fig. 2A–C).

Moreover, we investigated the effects of miR-204 on growth and invasion of Y79 cells in vivo. We initially infected Y79 cells with lentiviral vectors expression miR-204 stably (LV-miR-204) or the negative control (LV-Ctrl). Stable transfection of miR-204 into Y79 cells resulted in decreased growth and tumor weight of subcutaneous xenograft tumors in nude mice, when compared with those stably transfected with empty vector (Fig. 2E). These results suggested that miR-204 could inhibit the growth of retinoblastoma cells in vivo.
were plotted against miR-204 expression, a significant inverse correlation was observed (2-tailed Spearman’s correlation, \( p = 0.000 \) and 0.002, respectively).

4. Discussion

Despite the advancements in treatment options, improvements in retinoblastoma patient survival have been limited owing to lack of early detection. Biomarkers to improve retinoblastoma diagnosis, prognosis and prediction of treatment response therefore represent opportunities to improve patient outcome. In recent years, investigation of epigenetic biomarkers such as miRNA expression, has implicated that these alterations may be enticing translational biomarker candidates in retinoblastoma [16,17]. It is well documented that microRNAs could contribute to cancer development by acting as oncogenes or tumor suppressor genes. Recently, we and other researchers have found that miR-204 functioned as a tumor suppressor in a subset of cancer [11,18–21]. A previous study had demonstrated the significant role of miR-204 in controlling vertebrate eye development. The ablation of miR-204 expression could result in an eye phenotype characterized by microphthalmia, abnormal lens formation, and altered dorsoventral (D-V) patterning of the retina [22]. However, the underlying mechanism of miR-204 in retinoblastoma has not been explored.

In this study, we found that the expression of miR-204 in retinoblastoma specimens was significantly lower than that in normal pediatric retinas. Interestingly, the expression of miR-204 in human retinoblastoma cell lines was also down-regulated. Subsequently, we found that restoration of miR-204 in retinoblastoma cells inhibited cell growth in vitro. In addition, restoration of miR-204 significantly decreased the migration and invasion capacity of retinoblastoma cells. Furthermore, we observed that miR-204 decreased cell growth in vivo. Our results were analogous to an emerging body of literature, which suggested that miR-204 functioned as a tumor suppressor [23–25].

Subsequently, CyclinD2 and MMP-9 were identified as potential functional targets of miR-204. Our results showed that miR-204 bound the complementary sites of 3’-UTR of both CyclinD2 and
MMP-9, and dramatically decreased their expression. In addition, a significant inverse correlation between the levels of miR-204 and mRNA expression of CyclinD2 and MMP-9 was found in human retinoblastoma tissues. These observations provide the first line of evidence, to our knowledge, that miR-204 mechanistically acts via the regulation of CyclinD2 and MMP-9. Elevated CyclinD2 has been observed in various cancers and aberrant expression of CyclinD2 often leads to proliferation of cancer cells [26,27]. In addition, our data suggested that silencing of CyclinD2 inhibited the growth of retinoblastoma cells in vitro. Metastasis is a complex process of tumor cell invasion to new tissues, involving the coordination of several signaling pathways that allow changes in cell morphology, changes in adhesion and migration capabilities between the cells and the extracellular matrix (ECM) and changes in cell–cell interaction [28,29]. Degradation of the ECM by cancer cells is mediated via protease, such as matrix metalloproteinases (MMPs), serine proteinase, and cathepsins [30,31]. MMP-9 is one of the most vital enzymes for the degradation of the main constituent of the basement membrane, and is therefore involved in cancer invasion and metastasis [32]. A previous report found that MMP-9 was significantly higher in retinoblastoma tissue than in normal retina and suggested that MMP-9 could be connected to the invasion and development of retinoblastoma cells [33]. In the present study, we speculated that miR-204 may modulate proliferation and invasion, at least partly, by regulating CyclinD2 and MMP-9 expression in retinoblastoma cells. Interestingly, CyclinD2 and MMP-9 expression may be regulated by other mechanism in retinoblastoma. For instance, CyclinD2 was negatively regulated by let-7 [34], which was often down-regulated in retinoblastoma [6]. Further research is needed for deeper understanding of the

Fig. 3. CyclinD2 and MMP-9 are potential direct targets of miR-204. (A) The 3′-UTR of CyclinD2 and MMP-9 mRNA contains a complementary site for the seed region of miR-204. (The MT sequence, which were indicated by blue color, represent mutants containing the miR-204 binding sites.) (B) miR-204 significantly inhibited the luciferase activity of the CyclinD2 (left panel) and MMP-9 (right panel) WT 3′-UTR but not that of the mutant in Y79 cells. (One-way ANOVA was used for comparison among these groups.) (C) Restoration of miR-204 reduced the CyclinD2 (left panel) and MMP-9 (right panel) mRNA levels (student’s t-test was used and p-values less than 0.05 indicated statistically significant difference). (D) Overexpression of miR-204 reduced the CyclinD2 (left panel) and MMP-9 (right panel) protein levels.

Fig. 4. miR-204 levels are inversely correlated with mRNA expression of CyclinD2 and MMP-9 in retinoblastoma tissues (correlation was analyzed by Spearman test).
biological behavior of miR-204 and other microRNAs in the progression of retinoblastoma. As the limit on the number of retinoblastoma samples and cell types, more elaborate studies will be necessary for further exploration of the potential role of miR-204 in development of retinoblastoma. In addition, the eyes used for comparisons of miR-204 expression were postnatal. To better compare the expression of miR-204 between retinoblastoma and normal eyes, fetal retina should be enrolled in the study. However, our findings on miR-204 are encouraging and suggest that this miRNA could be a potential target for the treatment of retinoblastoma in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.01.030.

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