MicroRNA-215 promoted the progression of nasopharyngeal carcinoma through targeting RB1 and activating Wnt/β-catenin pathway

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Summary

Purpose: MicroRNA-215 (miR-215) has been reported to show different effects in human cancers. However, the function of miR-215 remains unclear in nasopharyngeal carcinoma (NPC). Hence, this research was designed to investigate the effect of miR-215 on the development of NPC.

Methods: The expression levels of miR-215 and RB1 were examined in NPC via the qRT-PCR assay. The protein expression was observed through immunocytochemical assay and western blot. MTT (methyl thiazolyl tetrazolium) and Transwell assays were employed to explore the effect of miR-215. The relationship between miR-215 and retinoblastoma (RB1) was assessed by dual luciferase assay.

Results: Upregulation of miR-215 was identified in NPC tissues and predicted worse prognosis of NPC. Cell proliferation, migration and invasion were promoted by overexpression of miR-215 in NPC. Furthermore, miR-215 directly targeted RB1 which was downregulated in NPC. MiR-215 promoted the progression of NPC through targeting RB1. In particular, miR-215 promoted EMT (epithelial-mesenchymal transition) and activated Wnt/β-catenin pathway in NPC.

Conclusion: MiR-215 promoted the development of NPC through suppressing RB1 and activating Wnt/β-catenin pathway.

Key words: miR-215, RB1, nasopharyngeal carcinoma, Wnt/β-catenin pathway

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor occurring at the top and side walls of the nasopharyngeal cavity [1]. In China, the incidence of NPC is the highest in otorhinolaryngology [2] and has obvious regional differences, showing a trend of high in the south and low in the north [3]. The etiology of NPC is not clear and it is currently considered to be a polygenic disease. Relatively certain pathogenic factors are: Epstein-Barr (EB) virus infection, chemical carcinogenic factors or environmental factors, genetic factors and others [4]. Furthermore, the distant metastasis rate of NPC is high, which is one of the main causes of death. NPC is sensitive to radiation, and radiotherapy is currently recognized as an effective radical treatment for NPC. Radiotherapy alone is usually used for NPC patients at early stage and radiotherapy and chemotherapy are mainly used NPC patients at late stage [5]. However, surgical resection and chemotherapy are also indispensable for patients with highly differentiated NPC.

Because NPC is prone to recurrence and early metastasis, the prognosis is poor [6]. The 5-year survival rate of NPC is 0-30%. Moreover, early di-
agnosis has an important influence on the prognosis of NPC. Therefore, it is urgent to develop effective biomarkers to timely diagnose NPC.

MicroRNAs (miRs) are well-known to act as important regulators of tumor formation [7]. Recently, abnormal expressions of miRs were identified in human NPC. Some miRs act as oncogenes and others functions as tumor suppressors in NPC. For instance, miR-92a promoted metastasis of NPC by targeting the PTEN/AKT pathway [8]. On the contrary, miR-328 inhibited migration and epithelial–mesenchymal transition (EMT) by targeting CD44 in NPC [9]. The aberrant miR-215 expression has been identified in several human cancers, which exhibited different effects. Li et al reported that miR-215 exhibited its suppressive effect in human pancreatic cancer via targeting ZEB2 [10]. Zang et al proposed that cell migration and invasion were promoted by miR-215 in gastric cancer through regulating FOXO1 [11]. Moreover, it was reported that miR-215 could be used as indicator for the prognosis of cervical cancer [12]. However, the function of miR-215 is little known in NPC.

Retinoblastoma 1 (RB1) had been reported to participate in tumor angiogenesis and metastasis [13]. RB1 was identified as a negative regulator of the cell cycle [14]. Ren et al demonstrated that RB1 was associated with tumorigenesis of human osteosarcoma acting as a suppressive gene [15]. RB1 had been reported to be expressed at low levels and act as tumor suppressor in the development of human cancers [16]. In particular, miR-215 acted as carcinogenic in high-grade glioma via inhibiting RB1 [17]. But the effect of miR-215/RB1 axis on EMT and Wnt/β-catenin pathway remains unknown in NPC and still need to be explored.

Therefore, the aim of this research was to detect the alternative aspects of miR-215 and confirm its function in NPC by investigating its underlying mechanisms. MiR-215 may become a biomarker for the diagnosis of NPC.

**Methods**

In this study the C666-1 cell line was mainly used in the evaluation of miR-215 as its expression was more obvious.

**Clinical tissues**

Seventy-two human NPC tissues and normal tissues were acquired from the Jining no.1 People’s Hospital. None of the patients with NPC received other treatment except the operation. These tissues were frozen in liquid nitrogen and then stored at a -80°C refrigerator. All the patients provided written informed consent and this experiment was approved by the Institutional Ethics Committee of Jining no.1 People’s Hospital.

**Cell culture**

The SUNE-1, C666-1, 5-8F NPC cell lines and NP69 normal nasopharyngeal epithelial cell line, were used for in this study. These cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Then these cell lines were seeded in RPMI-1640 medium with 10% fetal bovine serum (FBS) and cultured for 24h at 37°C in an atmosphere with 5% CO₂.

**Cell transfection**

The miR-215 mimics or inhibitor and negative control (NC) were obtained from Genecopoeia (Guangzhou). RB1 plasmid was purchased from Promega Corporation (WI, USA). Then, they were transferred into C666-1 cells respectively with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) based on the manufacturer’s protocols.

**Quantitative real-time polymerase chain reaction**

TRIzol reagent (TaKaRa Bio, USA) was applied to extract total RNA in NPC. The synthesis of cDNA was performed using iScript cDNA synthesis kit (TaKaRa Bio). Then, they were transferred into C666-1 cells respectively with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) based on the manufacturer’s protocols. We conducted real-time quantitative polymerase chain reaction (RT-qPCR) by using SYBR PrimeScript miRNA RT-PCR kit (TaKaRa Bio) on ABI 7500 Fast system (Applied Biosystems, CA, USA). U6 or GAPDH were used as control for miR-215 or RB1. Their expressions were calculated using the 2−ΔΔct method.

**Figure 1.** Upregulation of miR-215 was identified in NPC tissues. A: The expressions of miR-215 in NPC tissues detected via qRT-PCR. B: High miR-215 expression was correlated with shorter overall survival of NPC patients. *p<0.05, **p<0.01.
Western blot analysis

The protein samples were obtained using RIPA (ristocetin-induced platelet agglutination) lysis buffer. Then, the proteins were separated through a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and incubated with 5% skim milk in PVDF (polyvinylidene fluoride) membranes at room temperature. Next, the membranes were incubated overnight at 4°C with EMT markers (E-cadherin, N-cadherin, vimentin), Wnt/β-catenin pathway markers (β-catenin, p-β-catenin), RB1 and GAPDH antibodies. After washing with TBST for 30 min, they were incubated with corresponding secondary antibodies for 2 h at room temperature. Then, the protein expression levels were measured by electrochemical luminescence (ECL) (Pierce Biotechnology, USA).

MTT assay

C666-1 cells transfected with miR-215 mimics/inhibitor or negative control (NC) were incubated at a density of 5×10^3 cells in 96-well plates. At 24, 48, 72 and 96 h after the transfection, the cells were incubated at 37°C in a serum-free medium with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. Finally, they were dissolved in 100 μl of dimethyl sulfoxide after removing the MTT solution and absorbance was read at 570 on a microplate reader (Molecular Devices, Sunnyvale, USA).

Transwell assay

Transwell chambers (8-μm pore size membranes) were employed to perform cell migration and invasion assays. C666-1 cells transfected with miR-215 mimics/inhibitor or negative control (NC) were incubated with a serum-free medium for 24 h. Next, 1×10^4 C666-1 cells were put into the upper chamber and a medium with 10% FBS was put into the lower chamber. After 24 h at 37°C, the non-migrating cells were completely removed. Migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The invasion assay was similar to migration assay, except the upper chamber was coated with 1 mg/ml Matrigel (BD Biosciences, San Jose, CA, USA). Finally, the number of removed cells was counted using a microscope.

Dual luciferase assay

The wild or mutant type of 3’-UTR of RB1 was inserted into the pmirGLO luciferase vector (Promega, Madison, USA) to perform luciferase reporter experiments. Then, wild or mutant type of 3’-UTR of RB1 and miR-215 mimic were transfected into C666-1 cells. Subsequently, the luciferase activity was measured through dual luciferase assay system (Promega, USA).

Statistics

The data are shown as mean±SD. The difference between the groups was calculated through χ² test or Tukey’s one-way ANOVA by using SPSS 19.0 and Graphpad Prism 6. The survival curves were drawn by Kaplan-Meier method, and log-rank test was used to compare the survival differences. Significant difference was set at p<0.05.

Results

Upregulation of miR-215 was identified in NPC tissues

The miR-215 expressions were observed in NPC tissues via RT-qPCR assay and miR-215 was obviously upregulated in NPC tissues in contrast to the normal tissues (Figure 1A). Moreover, abnormal miR-215 expression was closely correlated

<table>
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<tr>
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Statistical analyses were performed by the χ² test. *p<0.05 was considered significant.
with distant metastasis (p=0.048) and TNM stage (p=0.017, Table 1). Besides, high expression of miR-215 predicted worse prognosis of NPC (p=0.0444, Figure 1B). We considered that miR-215 might be related to tumorigenesis and prognosis of NPC.

**Upregulation of miR-215 promoted the proliferation, migration and invasion of NPC cells**

Next, miR-215 expression levels were examined in SUNE-1, C666-1, 5-8F and NP69 cell lines. MiR-215 was also upregulated in SUNE-1, C666-1 and 5-8F cell lines in comparison with NP69 cells (Figure 2A). In order to investigate the role of miR-215 in NPC, miR-215 mimics or inhibitor were transfected into C666-1 cells. The transfection efficiency of miR-215 was measured via RT-qPCR assay as shown in Figure 2B. After transfection, MTT and Transwell assays were performed to detect the miR-215 function. We found that miR-215 overexpression promoted the proliferation in C666-1 cells (Figure 2C). Inversely, the knockout of miR-215 suppressed the proliferation in NPC cells (Figure 2D). Moreover, upregulation of miR-215 also promoted cell migration whereas knockout of miR-215 repressed the migration of C666-1 cells (Figure 2E). Similarly, the same function of miR-215 was observed in NPC cells.

**Figure 2.** MiR-215 overexpression promoted cell proliferation, migration and invasion in NPC. **A:** The miR-215 expression in SUNE-1, C666-1, 5-8F and NP69 cell lines. **B:** The miR-215 expressions were examined in C666-1 cells containing miR-215 mimics or inhibitor via qRT-PCR. **C, D:** The cell proliferation was measured in cells containing miR-215 mimics or inhibitor via MTT assay. **E, F:** Cell migration and invasion analysis in C666-1 cells containing miR-215 mimics or inhibitor was examined by Transwell assay. *p<0.05, **p<0.01.
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MiR-215 was identified for cell invasion in C666-1 cells (Figure 2F). In brief, the carcinogenic effect of miR-215 was involved in the progression of NPC.

**RB1 was a direct target of miR-215**

Subsequently, RB1 was predicted as a target gene of miR-215 which showed binding sites with miR-215 in TargetScan (http://www.targetscan.org/) (Figure 3A). Then, we performed luciferase reporter assay to verify that prediction. The results showed that luciferase activity of Wt-RB1 was distantly declined by miR-215 mimics. However, miR-215 mimics was not found to affect the luciferase activity of Mut-RB1 (Figure 3B). Moreover, negative association between miR-215 and RB1 was identified in NPC tissues (p<0.001, R²=0.4772; Figure 3C). Next, the expression levels of RB1 regulated by miR-215 mimics or inhibitor were measured in C666-1 cells. The RT-qPCR suggested that the RB1 expression was reduced by overexpression of miR-215 (Figure 3D) and the knockout of miR-215 enhanced RB1 expression in C666-1 cells (Figure 3E) indicating that miR-215 directly targets RB1 which had negative association with miR-215 in NPC.

Then, the abnormal expression of RB1 was evaluated in NPC. Downregulation of RB1 was identified in NPC tissues and cell lines (Figure 4A, 4B). In order to further confirm the interaction between miR-215 and RB1, miR-215 mimics and RB1 vector were co-transfected into C666-1 cells. What we found was that the decreased RB1 expression induced by miR-215 mimics was recovered by RB1 vector in C666-1 cells (Figure 4C). Moreover, the facilitation of miR-215 for cell proliferation was weakened by upregulation of RB1 in C666-1 cells (Figure 4D) and the inversion of RB1 for miR-215 function was also identified for the migration (Figure 4E) and invasion (Figure 4F) of NPC cells. These results implied that cell proliferation, migration and invasion were promoted by miR-215 through targeting RB1 in NPC.

**MiR-215 promoted EMT and activated Wnt/β-catenin pathway in NPC**

The effect of miR-215 on EMT and Wnt/β-catenin pathway was investigated to further ex-

![Figure 3. RB1 was a direct target of miR-215. A: RB1 had binding sites with miR-215. B: Luciferase reporter assay. C: MiR-215 had negative correlation with RB1. D,E: The expression of RB1 was observed in C666-1 cells containing miR-215 mimics or inhibitor. **p<0.01.](image-url)
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plain the regulatory mechanism of miR-215 in NPC. Western blot assay indicated that upregulation of miR-215 suppressed E-cadherin expression and promoted N-cadherin and Vimentin expressions in C666-1 cells (Figure 5). And the knock-out of miR-215 had opposite effect on these three markers (Figure 5). Moreover, Wnt/β-catenin pathway had been reported to regulate EMT. Hence, we presumed that miR-215 could activate Wnt/β-catenin pathway in the progression of NPC. As we predicted, miR-215 overexpression was identified to promote p-β-catenin expression (Figure 5) while downregulation of miR-215 reduced p-β-catenin expression (Figure 5). However, the expression of β-catenin was not affected by miR-215 mimics or inhibitor in C666-1 cells. Taken together, miR-215 acts as an oncogene in NPC through promoting EMT and activating Wnt/β-catenin pathway.

Discussion

As potential biomarkers, miRs should be used in various cancers which are challenges and opportunities for the strategies of human cancer treatment [18]. In particular, numerous carcinogenic or suppressive miRs have been identified in NPC

![Figure 4. MiR-215 promoted the progression of NPC through targeting RB1. A: The expressions of RB1 in NPC tissues detected via qRT-PCR. B: The RB1 expression in SUNE-1, C666-1, 5-8F and NP69 cell lines. C: The RB1 expression was measured in C666-1 cells with RB1 vector and miR-215. D: The cell proliferation was measured in C666-1 cells with RB1 vector and miR-215 via MTT. E,F: The cell migration and invasion in C666-1 cells with RB1 vector and miR-215 was measured by Transwell assay. *p<0.05, **p<0.01.](image)
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As a tumor suppressor, the downregulation of miR-215 had been reported in several human cancers, such as epithelial ovarian cancer [21], colon cancer [22] and non-small cell lung cancer [23]. Conversely, miR-215 also exhibited its carcinogenic effect in other tumors. Li et al demonstrated that upregulation of miR-215 promoted the development of gastric cancer through regulating RUNX1 [24]. In this study, miR-215 expression was obviously increased in NPC and cell proliferation, migration and invasion were promoted by miR-215 overexpression in NPC. Similarly, it was also reported that miR-215 promoted EMT and proliferation by regulating LEFTY2 in endometrial cancer [25]. EMT is closely correlated with the progression and metastasis of human cancers, including NPC [26]. Here, we implied that miR-215 promoted the metastasis of NPC cells through promoting EMT. It was suggested that miR-215 was a regulator of EMT in NPC. Besides, miR-215 was found to promote cell migration and invasion via targeting RB1 in gastric cancer [27]. Consistent with previous results, miR-215 also promoted the progression of NPC through targeting RB1.

The critical role of RB1 has been demonstrated to be its participation in the occurrence and progression of cancers [28]. In the current research, downregulation of RB1 was identified in NPC and RB1 was negatively associated with miR-215 expression in NPC tissues. More importantly, the upregulation of RB1 impaired the promotion of cell metastasis and proliferation induced by miR-215, suggesting that RB1 is as a tumor suppressor in NPC. Consistently, RB1 was reported to inhibit the metastasis of osteosarcoma [15]. Valente et al also proposed that overexpression of RB1 suppressor gene inhibited the progression of metastatic melanoma [29]. Additionally, Zhou et al revealed that RB1 induced osteogenic differentiation by Wnt/β-catenin signaling activation [30]. Therefore, the effect of miR-215/RB1 axis on Wnt/β-catenin pathway was investigated in present study.

Previous studies showed that the Wnt/β-catenin signaling pathway was involved in several biological activities, including differentiation, invasion, and proliferation [31,32]. In NPC, the role of Wnt/β-catenin signaling pathway was also reported in previous studies. For instance, YPEL3 suppressed EMT and cell metastasis through regulating Wnt/β-catenin pathway in NPC [33]. Moreover, Wang et al demonstrated that ZNRF3 inhibited the invasion and tumorigenesis in NPC by inactivating the Wnt/β-catenin pathway [34]. These results are in good agreement with our findings. Activating Wnt/β-catenin pathway promoted metastasis and proliferation in NPC. Furthermore, we confirmed that miR-215 could activate the Wnt/β-catenin pathway in NPC. Taken together, miR-215 activated Wnt/β-catenin signaling pathway via targeting RB1 to promote the development of NPC.

Conclusion

In conclusion, miR-215 was upregulated in NPC which was related to poor prognosis of NPC patient. Moreover, miR-215 promoted cell proliferation, migration and invasion through targeting RB1 and activated Wnt/β-catenin pathway in NPC. Taken together, miR-215 can be used as a biomarker for the diagnosis and prognosis of NPC.

Conflict of interests

The authors declare no conflict of interests.

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