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Original Article

miR-21 inhibitor suppresses proliferation and migration of nasopharyngeal carcinoma cells through down-regulation of BCL2 expression

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Abstract: This study is to investigate the expression of miR-21 in nasopharyngeal carcinoma (NPC) cells, and the effect of miR-21 in the biological behavior and expression of B-cell lymphoma 2 (BCL2) in NPC cells. Paired NPC and adjacent non-tumor tissues were obtained from 53 patients who underwent primary surgical resection of NPC tissues. Luciferase reporter assay was performed to test whether BCL2 is a direct target of miR-21. Methylthiazolyl blue tetrazolium assay and colony assay were used to evaluate the effect of miR-21 on NPC cell proliferation. Transwell and wound-healing assays were carried out to test the effect of low expression of miR-21 on cancer cell migration and invasion. QRT-PCR and Western blotting were used to measure the levels of mRNA and protein expression, respectively. Tumor tissues showed a positive correlation between the levels of miR-21 and BCL2 protein expression. Cells transfected with miR-21 inhibitor healed slower compared the control ($P < 0.05$). In addition, cell migration was notably inhibited by the down-regulation of miR-21 *in vitro* ($P < 0.05$). The reduction in miR-21 expression showed a remarkable effect on the biological behavior of NPC cell clone formation ($P < 0.05$). Low expression of miR-21 by transfection with miRNA expression plasmid led to a decrease in BCL2 expression, which was accompanied by reduced migration and proliferation of the cancer cells. Our results demonstrated that miR-21 inhibitor down-regulated BCL2 expression level, suggesting that BCL2 might be a target gene for the initiation and development of NPC cells.

Keywords: MicroRNA, miR-21, nasopharyngeal carcinoma, B-cell lymphoma 2

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common diseases in China. NPC is closely associated with Epstein-Barr virus (EBV) infection, primarily due to the latent membrane protein 1 oncogene of EBV. It has been shown that NPC is sensitive to radiotherapy and chemotherapy, with a cure rate of approximately 70% [1, 2]. EBV-encoded RNA signal is present in all NPC cells, and early diagnosis of the disease is possible through the detection of raised antibodies against EBV. However, a few genes were reported to contribute to the risk of NPC according to studies on genetic linkage and association [3, 4].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at the posttranscriptional level by degrading their target mRNAs or by translational repression [5]. microRNAs play important roles in numerous biological processes, such as development, differentiation, and cellular stress response [6-8]. Recently, miRNA (miR)-21 has been highlighted, because it is among the most up-regulated miRNAs in diverse cancers including colorectal cancer [9], renal cancer [10], breast cancer [11], colon cancer [12], gastric cancer [13], hepatocellular carcinoma [14], oral cancer [15], and liver cancer [16]. miR-21 has a strong effect on cancer cell proliferation and is a putative oncogene [17].

It has been discovered that the transcription factors that regulate miRNA are involved in many cellular processes, such as cell differentiation [18], proliferation and apoptosis [19]. Previous study reported that miR-21 negatively regulates several targets, and thus impacts

angioplasty restenosis [20]. However, the exact mechanism of action of miR-21 in NPC cell line is poorly understood, and there is currently no direct evidence for a correlation between miR-21 function and nasopharyngeal carcinoma. As an oncogene, B-cell lymphoma 2 (BCL2) gene was implicated in cell migration and invasion. It has been reported that overexpression of a 27-nt miRNA significantly suppressed endothelial nitric oxide synthase expression and endothelial cell proliferation via the inhibition of signal transducer and activator of transcription 3 signaling [21], suggesting that some nuclear transcription factors (TFs) might be involved in the regulation of gene expression by the miRNA. However, BCL2-like 12 is a new member of the apoptosis-related BCL2 gene family. Members of this family are implicated in various malignancies. Recently, we have showed that *BCL2* has a significant prognostic value in NPC. Therefore, we hypothesize that miR-21 might play important roles in the metastasis of NPC and the regulation of BCL2. In this study, we investigate whether miR-21 significantly promotes the migration and invasion of NPC cells, and regulates the expression of BCL2.

Materials and methods

Patients and tissue samples

This study was approved by the Human Research Ethics Committee of Guangxi Medical University (China). Paired NPC and adjacent non-tumor tissues were obtained from 53 patients who underwent primary surgical resection of NPC tissues with informed consent between October 2012 and August 2013 at The First Affiliated Hospital, Guangxi Medical University (China). Tissue samples were immediately frozen in liquid nitrogen after resection and stored at -80°C until use. The clinical stage was defined according to the revised International Staging System. Both tumor and non-tumor tissues were identified by pathological examination. Pathological stage, grade, and lymph nodal status were defined independently by three experienced pathologists. The clinical characteristics of the patients, including age, gender, pathology, and tumor-node-metastasis staging, were collected and analyzed.

Cell culture and transfection

NPC cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified environment at 37°C in the presence of 5% CO_2. NPC cell line was obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China).

The miR-21 mimics and miR-21 inhibitor were synthesized by Tiangen Biochemical Technology Co., Ltd. (Beijing, China). For transfection, cells were grown to 90% confluence, and transfected with miR-21 mimics or its inhibitor using Lipofectamine 2000 (Invitrogen, USA) by incubation in Opti-Mem I media for 4 h. The cells were then transferred into fresh RPMI-1640 containing 10% FBS. After incubation for 24 h, the culture medium was replaced, and fluorescent images were utilized to monitor transfection efficiency. After 48 h, cells were harvested for analysis.

Luciferase reporter assay

Cells cultured in 6-well plates were transfected with 1 μg of either pMIR/BCL2 vector or pMIR/BCL2/mut vector containing firefly luciferase, or 0.05 μg of the pRL-TK vector (Promega, USA) containing renilla luciferase together with 30 nM miR-21 inhibitor or negative control. Cells cultured in 24-well plates were transfected with wild-type (WT) or mutant reporter plasmid using Lipofectamine 2000. After transfection for 6 h, cells were transfected again with miR-21 inhibitor or negative control. Luciferase activity was measured using dual luciferase assay system (Promega, USA) after incubation for 36 h.

Methylthiazolyl blue tetrazolium (MTT) assay

Cell growth was determined using MTT (Sigma-Aldrich, USA) spectrophotometric dye assay. At 24 h post-transfection with miR-21 inhibitor or negative control, cells were seeded into 96-well plates (2×10^3 cells/well), and cell proliferation was recorded every 24 h for 4 days. The number of viable cells was assessed by measuring the absorbance at 450 nm using a microplate reader.

Colony formation assay

The cells were transfected with 50 nmol/l miR-21 mimics or its inhibitor, and cultured in media containing 10% FBS. After incubation for 15 days, the cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. Triplicate wells were measured for each group.

Table 1. Primers for PCR

miR-21	Stem-loop RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3′
	Forward: 5′-GCCCGCTAGCTTATCAGACTGATG-3′
	Reverse: 5′-GTGCAGGGTCCGAGGT-3′
U6	RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATG-3′
	Forward: 5′-GCGCGTCGTGAAGCGTTC-3′
	Reverse: 5′-GTGCAGGGTCCGAGGT-3′
BCL2 mRNA	Forward: 5′-CTGTGCTGCTATCCTGC-3′
	Reverse: 5′-TGCAGCCACAATACTGT-3′
β-actin	Forward: 5′-TCACCCACACTGTGCCCCATCTACGA-3′
	Reverse: 5′-CAGCGGAACCGCTCATTGCCAATGG-3′

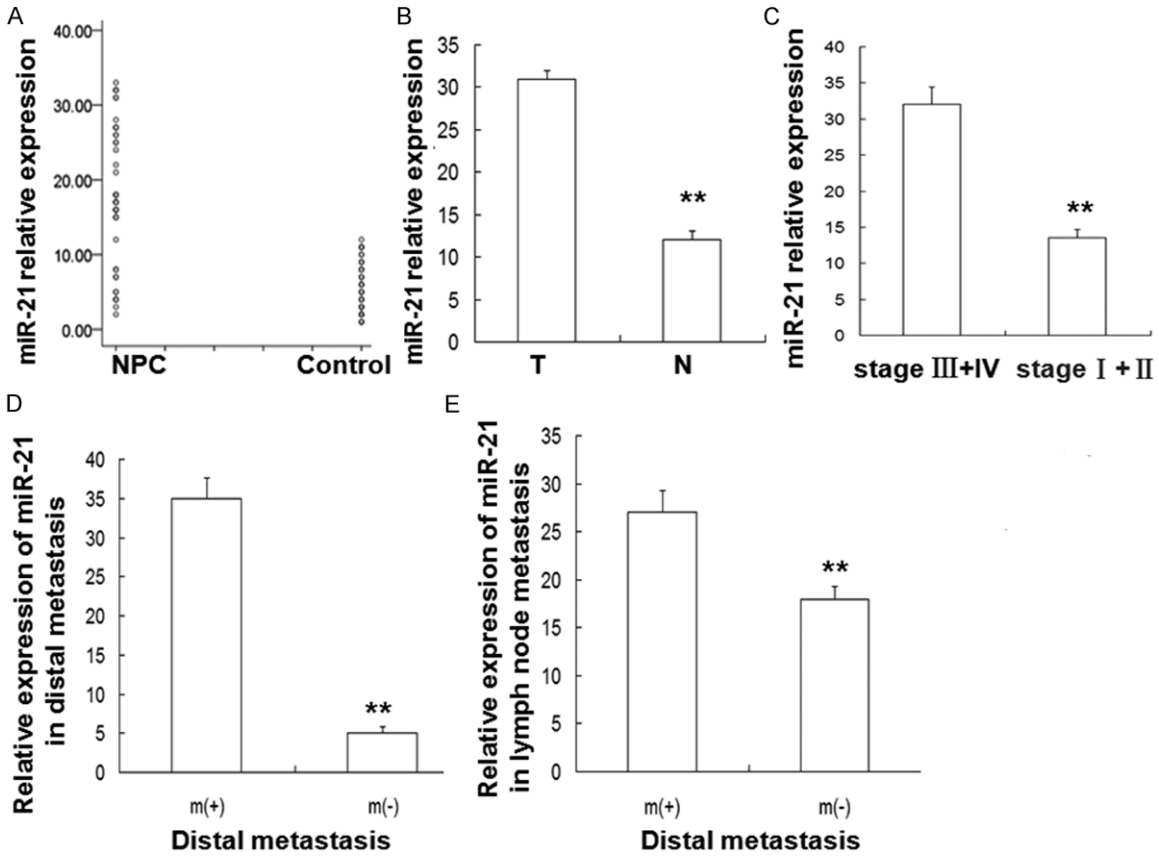

Figure 1. Overexpression of miR-21 in NPC tissues and its clinical significance. Paired NPC tissues and adjacent non-tumor tissues were obtained from 45 patients who underwent primary surgical resection for NPC. The miR-21 expression was detected by qRT-PCR, and was normalized to U6 expression. A: Relative expression of miR-21 in NPC tissues (n = 45) in contrast to matched non-tumor tissues (n = 45). NPC, tumor tissues; Control, non-tumor tissues. B: Relative expression of miR-21 in tumor tissues and adjacent non-tumor tissues. T, tumor tissues; N, adjacent non-tumor tissues. **, $P < 0.01$. C: miR-21 expression in patients with stages III+IV and those with stages I+II. **, $P < 0.01$. D: miR-21 expression in patients with [m (+)] and without [m (-)] distal metastasis. **, $P < 0.01$. E: miR-21 expression in patients with [m (+)] and without [m (-)] lymph node metastasis. **, $P < 0.01$.

Wound-healing assay

The cultured cells were transfected with 50 nM miR-21 inhibitor or negative control. At 24 h post-transfection, the cells were allowed to reach confluence before dragging a 1-ml sterile pipette tip through the mono-layer. The cells were then washed and allowed to migrate for

Table 2. Characteristics of nasopharyngeal carcinoma patients

Characteristics	No. of patients (%)
Median age (range), in years	48 (30-75)
Gender (Male/Female)	33/45 (73.3); 12/45 (26.7)
Histological type (Squamous cell/Adenocarcinoma/others)	12/45 (26.7); 30/45 (66.7); 3/45 (6.7)
TNM stage (I+II/III+IV)	12/45 (26.7); 33/45 (73.3)
Lymph node metastasis (Positive/Negative)	26/45 (57.8); 19/45 (42.2)
Differentiation (Well/Moderate/Poor)	6/45 (13.3); 13/45 (28.9); 26/45 (57.8)
Tumor size (cm) (≤ 5/> 5)	19/45 (42.2); 26/45 (57.8)
Distal metastasis (Positive/Negative)	37/45 (82.2); 5/45 (17.8)

Note: TNM, tumor-node-metastasis.

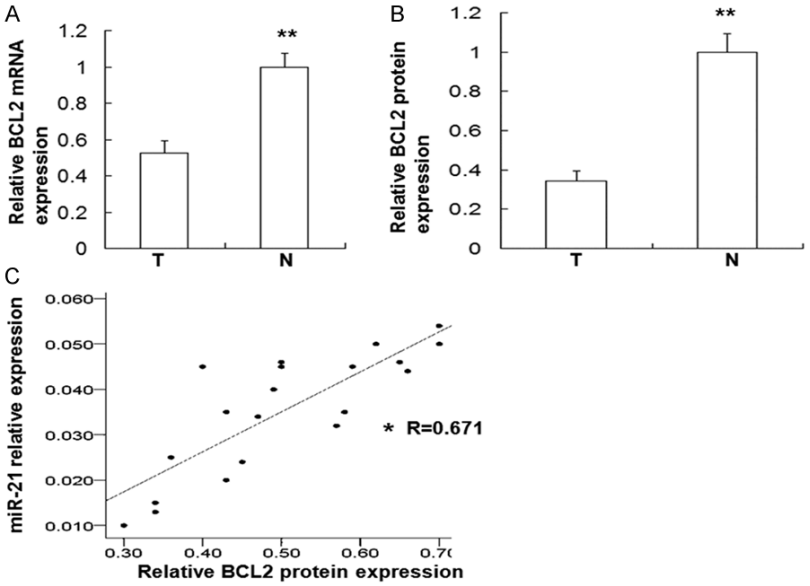

Figure 2. Expression levels of BCL2 mRNA and protein in NPC tissues. A: Relative expression of BCL2 mRNA in NPC tissues (n = 20) compared with matched non-tumor tissues (n = 20). T, tumor tissue; N, non-tumor tissue. **, $P < 0.01$. B: BCL2 protein expression detected by Western blotting and normalized to β-actin protein expression. The results were normalized with β-actin mRNA levels and were presented as relative BCL2 mRNA expression. T, tumor tissue; N, non-tumor tissue. **, $P < 0.01$. C: Correlation between miR-21 expression and BCL2 protein levels in NPC (Pearson correlation = 0.671, *, $P < 0.05$).

12 or 24 h. At 0, 12 and 24 h post-wounding, images were captured. Cell motility was determined according to the percentage of the repaired area. The experiment was performed in triplicate.

Transwell assay

For the invasion assay, 2×10^5 cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; 8-μm pore size; BD Biosciences, USA). For the migration assay, 5×10^4 cells were plated in the top chamber with a non-coated membrane (24-well insert; 8-μm pore size; BD Biosciences, USA). In both assays, cells were plated in medium without serum, and medium supplemented with 10% serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 to 36 h. Cells that did not migrate or invade through the pores, were removed by a cotton swab. Filters were fixed with 90% ethanol, stained with 0.1% crystal violet, and photographed. Cell numbers were counted.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, USA) for both miR-21 and BCL2 mRNA analyses. For the detection of miR-21 expression, stem-loop qRT-PCR was performed using SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer's protocol. Relative expression was evaluated by comparative computed tomography method and normalized to the expression of U6 small RNA. For the detection of BCL2 mRNA expression, qRT-PCR was performed using Quantities' SYBR Green PCR Kit (Qiagen, Germany). β-actin was used to normalize BCL2 mRNA expression levels. The primers are listed in **Table 1**. All qRT-

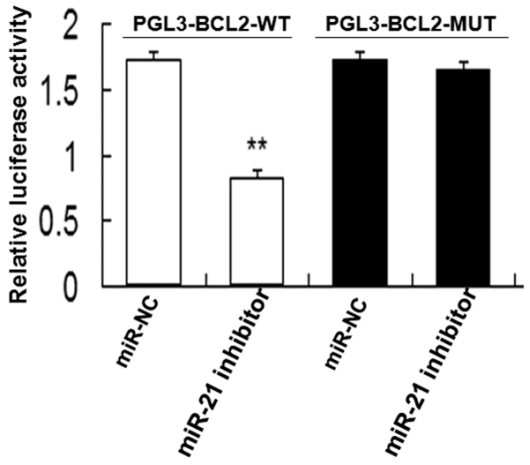

Figure 3. The levels of WT or mutant BCL2 in NPC cells in the presence of miR-21 inhibitor or negative control. The miR-21 reporter plasmid was cotransfected into NPC cells with miR-21 inhibitor or negative control (miR-NC). Luciferase activity in the control group was set as relative luciferase activity. All data are representative of three independent experiments. **, $P < 0.01$ compared with miR-NC.

PCR experiments were performed in triplicates.

Western blotting analysis

Western blotting analysis was performed as previously described. miR-21 was extracted from transfected group, miR-21 mimics transfected cells total protein. The lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gels after protein quantification using bicinchoninic acid kit (Bioteke, Beijing, China), followed by transfer to polyvinylidene difluoride membranes (Invitrogen, USA). The membrane was then blocked for 1 h with blocking solution (Invitrogen, USA) at room temperature with agitation, and incubated with the primary antibody at 4°C for 12 h. After blocking with 5% skimmed milk in Tris Buffered Saline with Tween-20 at 37°C for 1 h, the membrane was incubated with primary antibodies for BCL2 with dilution factor of 1:1000, using β-actin (1:1000) as an internal reference. After incubation with 5% skimmed milk in Tris Buffered Saline with Tween-20 at 4°C overnight, the membrane was washed for three times of 10 min. Then, the polyvinylidene difluoride membrane was incubated with secondary antibodies (Invitrogen, USA) at room temperature for 1 h. The blots were visualized using the electrochemiluminescence detection system (Invitrogen, USA).

Statistical analysis

All data quantification and statistical analysis were performed using SPSS 17.0 software (Chicago, USA). Values were expressed as means ± SEM. Statistical significance was determined by one-way ANOVA or two-tailed unpaired Student's t-test. Differences were considered statistically significant when P value was < 0.05.

Results

miR-21 up-regulation is positively correlated with the advanced tumor-node-metastasis stage of NPC

To evaluate the expression level of miR-21, 45 paired nasopharyngeal carcinoma tissues and adjacent non-tumor tissues were analyzed by qRT-PCR. miR-21 was up-regulated in 35 tumor tissues compared with that in the matched non-tumor tissues (**Figure 1A**). Specifically, up-regulation of miR-21 in NPC tissues was found in 28 out of 30 patients with stage III or IV tumors. The difference of miR-21 expression between tumor and non-tumor tissues was statistically significant (**Figure 1B**). Furthermore, investigation of the association of miR-21 with clinicopathologic factors (**Table 2**) in tumor tissues revealed that miR-21 overexpression was positively associated with advanced clinical tumor-node-metastasis stage, distal metastasis and lymph node metastasis (**Table 2**, **Figure 1C-E**). These data suggested that miR-21 up-regulation was positively correlated with the advanced tumor-node-metastasis stage of NPC.

Overexpression of miR-21 up-regulates the expression of BCL2 protein in NPC

To investigate the expression of oncogene BCL2, 20 pairs were randomly selected from the 45 pairs of matched NPC tissues for the analysis of BCL2 mRNA using qRT-PCR and BCL2 protein using Western blotting. In comparison to the non-tumor counterparts, tumor tissues expressed significantly higher levels of BCL2 mRNA (1.000 ± 0.075 vs 0.524 ± 0.067, $P < 0.01$) (**Figure 2A**). Western blotting showed that BCL2 protein expression in tumor tissues was significantly higher (1.000 ± 0.905) than that in non-tumor tissues (0.342 ± 0.053) (**Figure 2B**). In addition, a statistically significant inverse correlation was observed between

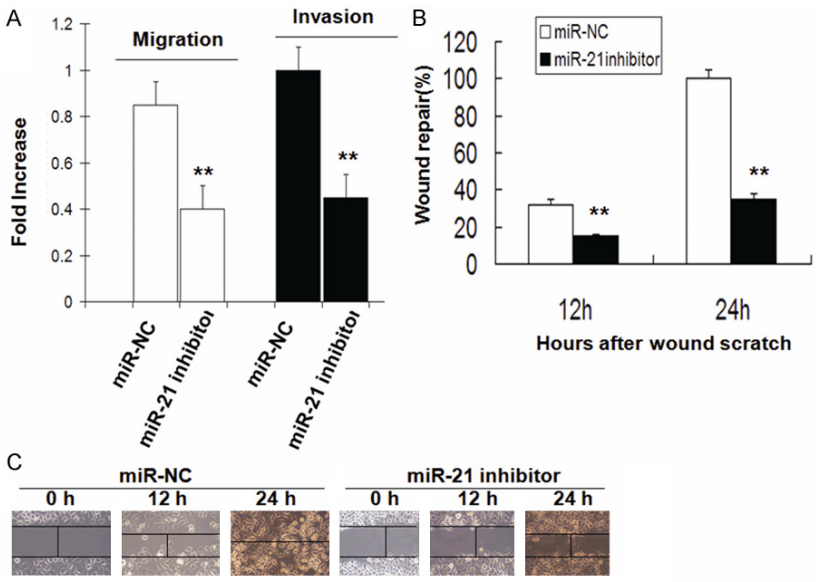

Figure 4. A: Migration and invasion of NPC cells transfected with miR-21 inhibitor or negative control determined in Transwell migration and invasion assays. Data are shown as means ± SD. **, $P < 0.01$ compared with miR-NC. B: Quantification of wound repair at 12 and 24 h after wound scratch in wound-healing assay. Data are shown as means ± SD. **, $P < 0.01$ compared with miR-NC. C: Representative images of NPC cells transfected with miR-21 inhibitor or negative control at 0, 12 and 24 h after wound scratch in wound-healing assay.

miR-21 and BCL2 protein (**Figure 2C**). These results suggested that miR-21 up-regulated the expression of BCL2 protein.

miR-21 modulates BCL2 expression by directly targeting BCL2 mRNA

To test whether BCL2 is a direct target of miR-21, WT or mutant reporter plasmids was cotransfected into NPC cells along with miR-21 inhibitor or negative control. Data showed that miR-21 inhibitor significantly decreased the activity when cotransfected with WT reporter plasmid, compared with control (0.83 ± 0.05 vs 1.72 ± 0.07, $P < 0.01$). Meanwhile, when transfected with negative control miRNA, there was no significant difference between WT or mutant vector (1.72 ± 0.07 vs 1.65 ± 0.06, $P > 0.05$) (**Figure 3**). These results indicated that miR-21 modulated BCL2 expression by directly targeting BCL2 mRNA.

Low expression of miR 21 inhibits cell migration and invasion in NPC cells

To test whether low expression of miR-21 affects the ability of cancer cell migration and invasion, Transwell and wound-healing assays were performed. Transwell assay demonstrated that low expression of miR-21 significantly reduced the migration and invasion capacity of NPC cells (**Figure 4A**). Meanwhile, *in vitro* wound-healing assay showed that wound repair in NPC cells transfected with miR-21 inhibitor was delayed when compared with cells transfected with the negative control. Compared with the control at 24 h after wound scratch, low expression of miR-21 inhibited NPC cells migration by up to 65% (**Figure 4B** and **4C**). These data demonstrated that low expression of miR-21 suppressed migration and invasion in NPC cell lines.

Low expression of miR-21 inhibits the proliferation of NPC cells

In order to explore the biological function of miR-21 in NPC, we down-regulated the expression level of miR-21 by transfecting the cells with 60 nM inhibitor and performed MTT assay. MTT assay showed that low expression of miR-21 significantly inhibited the growth of NPC cells. By contrast, high expression of miR-21 resulted in more rapid proliferation compared with the control (**Figure 5A**). Furthermore, inhibition of miR-21 in NPC cells suppressed colony formation on soft agar compared with the control. In comparison, transfection with miR-21 resulted in significantly increased colony formation (**Figure 5B**). These results indicated that low expression of miR-21 inhibited NPC cell proliferation *in vitro*.

Low expression of miR-21 inhibits BCL2 protein expression in NPC cells

To test the effect of miR-21 on BCL2 protein expression, we used cultured NPC cells transfected with miR-21 mimics and inhibitor. Western blotting analysis revealed that miR-21 inhibitor significantly decreased BCL2 protein expression by 66.81% (0.313 ± 0.021) com-

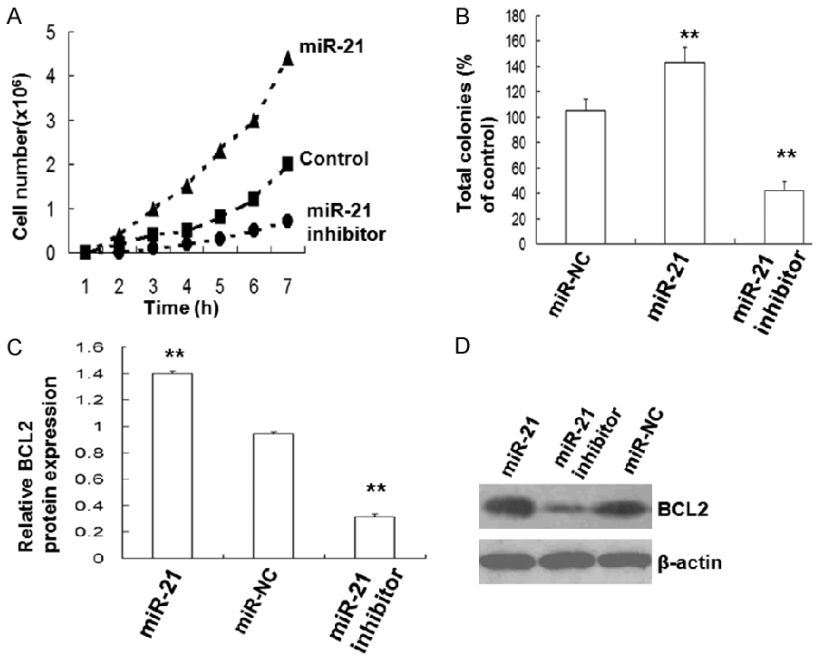

Figure 5. The effect of low expression of miR-21 on NPC cells growth and BCL2 protein expression *in vitro*. A: Proliferation curve of NPC cells stably transfected with empty vector and vectors expressing miR-21 inhibitor or miR-21 mimics. B: Colony formation of NPC cells transfected with miR-21 mimics, miR-21 inhibitor and control mimics. C: Western blotting analysis of BCL2 protein expression in NPC cells transfected with miR-21 mimics, miR-21 inhibitor and control. D: Quantification of BCL2 protein expression in NPC cells transfected with miR-21 mimics, miR-21 inhibitor and control. Data are expressed as means ± SEM. **, $P < 0.01$.

pared with control (0.943 ± 0.015) ($P < 0.01$). By contrast, the miR-21 mimics significantly increased BCL2 protein expression by 48.14% (1.397 ± 0.021) compared with control (0.943 ± 0.015) ($P < 0.01$) (**Figure 5C** and **5D**). These results demonstrated that low expression of miR-21 directly inhibited BCL2 protein expression in NPC cells.

Discussion

miRNAs are a class of naturally occurring small non-coding RNAs that target protein-coding mRNAs by repressing translation or causing mRNA degradation [22, 23]. It has been reported that miRNAs play important roles in regulating diverse cellular processes including proliferation, apoptosis, migration and invasion [24, 25]. Down-regulation of miRNAs affects normal cell growth and development, leading to a variety of disorders including human cancer, diabetes and cardiovascular disease [26-28]. miRNAs can function as tumor suppressors or oncogenes. It has been demonstrated that miR-21 is overexpressed in tumors or tumor cell lines, and miR-21 inhibitor suppresses the expression of BCL2. As a tumor oncogene, BCL2 is implicated in cell migration and invasion [29], suggesting that miR-21 may also have a role in migration and invasion. We predicted that miR-21 inhibitor might target BCL2, but there is no clear evidence to support its correlation with miR-21. Three major findings are described in this study. First, we showed that BCL2 expression was increased in the NPC tissues. Second, we showed that low expression of miR-21 inhibited cell invasion and migration and restrained cell growth in the cultured NPC cells. Moreover, we found that miR-21 expression had a correlation with BCL2 protein levels in NPC tissues. Finally, BCL2 was found to be a direct target of miR-21.

In the past decade, it has been reported that miRNAs are important players involved in carcinogenesis [30, 31]. Recently, miR-21 became well-known in all kinds of cancer cells. However, there is no report indicating the alteration of BCL2 expression in NPC. In the present study, we found that miR-21 expression was significantly up-regulated in NPC tissues. The present study revealed that NPC cells transfected with low levels of miR-21 inhibited cell growth and invasion, which may be due in part to the negative regulation of BCL2 by miR-21. Although evidence indicates that miR-21 expression may be regulated by miRNA-specific hypermethylation and histone deacetylation [32, 33], the precise mechanisms involved in the up-regulation of miR-21 in NPC tissue need to be further investigated, such as whether other transcription factor(s) take part in the role of miR-21 inhibitor in the regulation its target gene(s).

TFs and miRNAs are two large families of trans-acting gene regulators in multi-cellular genomes

with extensive interactions on gene regulation [34, 35]. Because most genes in genomes are not controlled by a single trans-acting factor, a model of inter- and intra-combinatorial regulation by TFs and miRNAs has been proposed as a mechanism for regulating gene expression [36]. This dynamic interactive mechanism of gene regulation by TFs and miRNAs has been supported by a few recent observations [34, 37]. The common nuclear locations of both TFs and miR-21 increase the likelihood of their interactions. miR-21 has been shown to be able to enter the nucleus by transfection or via reverse transportation [38]. As predicted, our data showed that low expression of miR-21 substantially suppressed NPC cell expression in association with decreased levels of BCL2. Furthermore, we found that increased BCL2 protein expression was inversely associated with the up-regulation of miR-21. Using luciferase reporter assay, we found that a segment of BCL2 was the direct target of miR-21. Our study indicated that miR-21 plays important roles in the occurrence and development of NPC by regulating BCL2. We predicted that BCL2 and miR-21 might be two factors that keep the regulation of NPC cell expression under normal physiological condition. BCL2 is positive, while miR-21 is negative for NPC cell expression. These findings provided a novel mechanistic paradigm for the regulation of host gene expression by miRNA and TFs.

In summary, we demonstrated that miR-21 inhibitor played an important role in the regulation of BCL2 gene expression. The effect of miRNAs on NPC cell expression occurred at both mRNA and transcription levels, and at least in part through targeting BCL2. However, we emphasize that miR-21 inhibitor may be capable of controlling tumor-specific gene(s), consequently favoring cell growth and migration. Therefore, our study suggests that targeting of miR-21 inhibitor may provide a better strategy to block tumor metastasis.

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Disclosure of conflict of interest

None.

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References

[1] Wei WI, Sham JS. Nasopharyngeal carcinoma. Lancet 2005; 365: 2041-2054.

[2] Cao SM, Simons MJ, Qian CN. The prevalence and prevention of nasopharyngeal carcinoma in China. Chin J Cancer 2011; 30: 114-119.

[3] Feng BJ, Huang W, Shugart YY, Lee MK, Zhang F, Xia JC, Wang HY, Huang TB, Jian SW, Huang P, Feng QS, Huang LX, Yu XJ, Li D, Chen LZ, Jia WH, Fang Y, Huang HM, Zhu JL, Liu XM, Zhao Y, Liu WQ, Deng MQ, Hu WH, Wu SX, Mo HY, Hong MF, King MC, Chen Z, Zeng YX. Genome-wide scan for familial nasopharyngeal carcinoma reveals evidence of linkage to chromosome 4. Nat Genet 2002; 31: 395-399.

[4] Zhou G, Zhai Y, Cui Y, Zhang X, Dong X, Yang H, He Y, Yao K, Zhang H, Zhi L, Yuan X, Qiu W, Zhang X, Shen Y, Qiang B, He F. MDM2 promoter SNP309 is associated with risk of occurrence and advanced lymph node metastasis of nasopharyngeal carcinoma in Chinese population. Clin Cancer Res 2007; 13: 2627-2633.

[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[6] Hornstein E, Mansfield JH, Yekta S, Hu JK, Harfe BD, McManus MT, Baskerville S, Bartel DP, Tabin CJ. The microRNA miR-196 acts upstream of Hoxb8 and Shh in limb development. Nature 2005; 438: 671-674.

[7] Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 2004; 5: R13.

[8] Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 2004; 436: 214-220.

[9] Xu XM, Qian JC, Deng ZL, Cai Z, Tang T, Wang P, Zhang KH, Cai JP. Expression of miR-21, miR-31, miR-96 and miR-135b is correlated with the clinical parameters of colorectal cancer. Oncol Lett 2012; 4: 339-345.

[10] Dey N, Das F, Ghosh-Choudhury N, Mandal CC, Parekh DJ, Block K, Kasinath BS, Abboud HE, Choudhury GG. microRNA-21 governs TORC1

activation in renal cancer cell proliferation and invasion. PLoS One 2012; 7: e37366.

[11] Song B, Wang C, Liu J, Wang X, Lv L, Wei L, Xie L, Zheng Y, Song X. MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression. J Exp Clin Cancer Res 2010; 29: 29.

[12] Yu Y, Sarkar FH, Majumdar AP. Down-regulation of miR-21 induces differentiation of chemoresistant colon cancer cells and enhances susceptibility to therapeutic regimens. Transl Oncol 2013; 6: 180-186.

[13] Yamanaka S, Olaru AV, An F, Luvsanjav D, Jin Z, Agarwal R, Tomuleasa C, Popescu I, Alexandrescu S, Dima S, Chivu-Economescu M, Montgomery EA, Torbenson M, Meltzer SJ, Selaru FM. MicroRNA-21 inhibits Serpini1, a gene with novel tumour suppressive effects in gastric cancer. Dig Liver Dis 2012; 44: 589-596.

[14] Bihrer V, Waidmann O, Friedrich-Rust M, Forestier N, Susser S, Haupenthal J, Welker M, Shi Y, Peveling-Oberhag J, Polta A, von Wagner M, Radeke HH, Sarrazin C, Trojan J, Zeuzem S, Kronenberger B, Piiper A. Serum microRNA-21 as marker for necroinflammation in hepatitis C patients with and without hepatocellular carcinoma. PLoS One 2011; 6: e26971.

[15] Jung HM, Phillips BL, Patel RS, Cohen DM, Jakymiw A, Kong WW, Cheng JQ, Chan EK. Keratinization-associated miR-7 and miR-21 regulate tumor suppressor reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in oral cancer. J Biol Chem 2012; 287: 29261-29272.

[16] Zhu M, Wang N, Tsao SW, Yuen MF, Feng Y, Wan TS, Man K, Feng Y. Up-regulation of microRNAs, miR21 and miR23a in human liver cancer cells treated with Coptidis rhizoma aqueous extract. Exp Ther Med 2011; 2: 27-32.

[17] Schramedei K, Mörbt N, Pfeifer G, Läuter J, Rosolowski M, Tomm JM, von Bergen M, Horn F, Brocke-Heidrich K. MicroRNA-21 targets tumor suppressor genes ANP32A and SMARCA4. Oncogene 2011; 30: 2975-2985.

[18] Kawasaki H, Taira K. Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. Nature 2003; 423: 838-842.

[19] Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene 2007; 26: 5017-5022.

[20] Silvestri P, Di Russo C, Rigattieri S, Fedele S, Todaro D, Ferraiuolo G, Altamura G, Loschiavo P. MicroRNAs and ischemic heartdisease: towards a better comprehension of pathogenesis, new diagnostic tools and new therapeutic targets. Recent Pat Cardiovasc Drug Discov 2009; 4: 109-118.

[21] Yan L, Hao H, Elton TS, Liu Z, Ou H. Intronic microRNA suppresses endothelial nitric oxide synthase expression and endothelial cell proliferation via inhibition of STAT3 signaling. Mol Cell Biochem 2011; 357: 9-19.

[22] Pillai RS. MicroRNA function: multiple mechanisms for a tiny RNA? RNA 2005; 11: 1753-1761.

[23] Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. Science 2005; 309: 1519-1524.

[24] Croce CM, Calin GA. miRNAs, cancer, and stem cell division. Cell 2005; 122: 6-7.

[25] Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science 2004; 303: 83-86.

[26] Collares CV, Evangelista AF, Xavier DJ, Rassi DM, Arns T, Foss-Freitas MC, Foss MC, Puthier D, Sakamoto-Hojo ET, Passos GA, Donadi EA. Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. BMC Res Notes 2013; 6: 491.

[27] Sugihara H, Ishimoto T, Watanabe M, Sawayama H, Iwatsuki M, Baba Y, Komohara Y, Takeya M, Baba H. Identification of miR-30e* regulation of Bmi1 expression mediated by tumor-associated macrophages in gastrointestinal cancer. PLoS One 2013; 8: e81839.

[28] Yan L, Kang M, Qin Z, Zhang W, Li Y, Ou H. An intronic miRNA regulates expression of the human endothelial nitric oxide synthase gene and proliferation of endothelial cells by a mechanism related to the transcription factor SP-1. PLoS One 2013; 8: e70658.

[29] Rajnai H, Heyning FH, Koens L, Sebestyén A, Andrikovics H, Hogendoorn PC, Matolcsy A, Szepesi Á. The density of CD8+ T-cell infiltration and expression of BCL2 predicts outcome of primary diffuse large B-cell lymphoma of bone. Virchows Arch 2013; 464: 229-239.

[30] Allegra A, Alonci A, Campo S, Penna G, Petrungaro A, Gerace D, Musolino C. Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer (review). Int J Oncol 2012; 41: 1897-1912.

[31] Garzon R, Marcucci G. Potential of microRNAs for cancer diagnostics, prognostication and therapy. Curr Opin Oncol 2012; 24: 655-659.

[32] Aure MR, Leivonen SK, Fleischer T, Zhu Q, Overgaard J, Alsner J, Tramm T, Louhimo R, Alnæs GI, Perälä M, Busato F, Touleimat N, Tost J, Børresen-Dale AL, Hautaniemi S, Troyanskaya OG, Lingjærde OC, Sahlberg KK, Kristensen VN. Individual and combined effects of DNA methylation and copy number alterations on

miRNA expression in breast tumors. Genome Biol 2013; 14: R126.
[33] Shen R, Pan S, Qi S, Lin X, Cheng S. Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 in gastric cancer. Biochem Biophys Res Commun 2010; 394: 1047-1052.
[34] Hobert O. Gene regulation by transcription factors and microRNAs. Science 2008; 319: 1785-1786.
[35] Makeyev EV, Maniatis T. Multilevel regulation of gene expression by microRNAs. Science 2008; 319: 1789-1790.
[36] Aguda BD, Kim Y, Piper-Hunter MG, Friedman A, Marsh CB. MicroRNA regulation of a cancer network: Consequences of the feedback loops involving miR-17-92, E2F, and Myc. Proc Natl Acad Sci U S A 2008; 105: 19678-19683.
[37] Taguchi A, Yanagisawa K, Tanaka M, Cao K, Matsuyama Y, Goto H, Takahashi T. Identification of hypoxia-inducible factor-1 alpha as a novel target for miR-17-92 microRNA cluster. Cancer Res 2008; 68: 5540-5545.
[38] Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008; 18: 350-359.