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miRNA-21 sensitizes gastrointestinal stromal tumors (GISTs) cells to Imatinib via targeting B-cell lymphoma 2 (Bcl-2)

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Abstract. – OBJECTIVE: miRNA-21 (miRNA-21) has recently been recognized to tumor suppressive in various types of cancers. However, the role of miRNA-21 in gastrointestinal stromal tumors (GISTs) is still ambiguous. In this study, we investigated the regulation by miRNA-21 on the sensitivity of gastrointestinal stromal tumors (GISTs) cells to Imatinib.

MATERIALS AND METHODS: We examined the expression of miRNA-21 and B-cell lymphoma 2 (Bcl-2) in GIST specimens by the real-time quantitative PCR assay (RT-qPCR). Then we explored the regulation by miRNA-21 on the Bcl-2 expression by the RT-qPCR assay, Western blotting assay and the luciferase assay in GIST-T1 cells. In addition, we examined the influence of miRNA-21 on the sensitivity to Imatinib of GIST-T1 cells with colony forming assay and apoptotic assay.

RESULTS: Results indicated that miRNA-21 expression was suppressed in GIST tissues. And we identified putative miRNA-21 binding sites within the 3′-untranslated region (3′-UTR) of the human Bcl-2 gene. Transient transfection of miRNA-21 mimics into human GIST GIST-T1 cell line significantly downregulated the Bcl-2 expression in both mRNA and protein levels. Moreover, the miRNA-21 mimics transfection markedly aggravated the Imatinib-mediated growth inhibition and apoptosis induction in GIST-T1 cells.

CONCLUSIONS: Our results demonstrated that miRNA-21 suppressed Bcl-2 expression in GIST cells and could function as a potent tumor suppressor in GIST. And the miRNA-21 promotion could sensitize GIST cells to Imatinib. It implies a potential role in the GIST treatment.

Key Words:
miRNA-21, Gastrointestinal stromal tumors (GISTs), Imatinib, Bcl2.

Introduction

Gastrointestinal stromal tumors (GISTs) are most common primary gastrointestinal mesenchymal tumors, accounting for 2% of all gastrointestinal tumors[1]. GIST patients had a very poor prognosis because of their poor response to conventional chemotherapy and radiotherapy or because of the limited surgical options[2,3]. Mutations of *KIT* or *PDGFRA*, both of which are tyrosine kinase receptor genes, occur in about 70-80% GIST cases, and are recognized to be the most known molecular event in GIST pathogenesis and development[4,5]. The downstream molecular pathways upon to the KIT mutation include PI3 kinase-AKT, Src family kinase, Ras-ERK, and JAK-STAT[6]. Activation of these molecular pathways, in response to KIT activation, results in GIST tumorigenesis through cell proliferation activation and apoptotic signal inhibition[7,8].

Imatinib, a small molecule inhibitor of both KIT and PDGFRA, has improved the clinical outcome of these patients significantly, with a median overall survival (OS) of 4-5 years in the metastatic phase expected[2]. Imatinib suppresses KIT by its binding to ATP-binding pocket to inhibit the KIT activation and blocks the activation of MAP kinase and PI3 kinase-AKT pathways[9,10]. However, the frequently-occurred mutations in highly conserved positions on several kinases promote the Imatinib resistance and reduce its inhibitory effects[11,12]. And the observed shift towards the active form of these kinases would allow ATP to outcompete the inhibitor[13-15]. Therefore, alternative strategies are

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needed to inhibit the KIT activity, such as approaches via posttranscriptional and posttranslational mechanisms.

microRNAs (miRNAs) are a class of endogenous small non-coding RNAs regulating gene expression in a wide range of cellular processes of various organisms[16-18]. Numerous miRNAs have been recognized to target the 3' untranslated regions (UTRs) of oncogenic or tumor suppressive genes, and thus regulate the cancer tumorigenesis. miRNA expression has been recently reported to exert a relevant role in the GIST biological processes such as tumorigenesis, progression, prognosis and drug resistance[19-21]. Overexpressed levels of miR-125a-5p and miR-107 have been indicated to be associated with Imatinib resistance in GIST via regulating the expression of PTPN18[22]. miRNA-218 negatively regulates KIT expression and inhibits the GIST cell proliferation and invasion, and thus regulates the Imatinib sensitivity of GIST cells through PI3K/AKT pathway[23]. Deregulated miRNA-21 has been recognized in various types of malignant tumors, such as lymphoma[24] and hepatocellular carcinoma (HCC)[25]. However, the role of it in GIST has not been reported.

The aim of the present study was to investigate the expression of miRNA-21 in GIST specimens, and explore the association of miRNA-21 with Imatinib response, clinic-pathological features of GIST patients. We, then, explored the functional and potential role of miRNA-21 in Imatinib sensitivity of GIST-T1 cells.

Materials and Methods

GIST Tissue Samples, GIST-T1 Cell Culture and Treatment

The 31 GIST specimens included in this study were identified in the Department of Gastroenterology at the Affiliated Hospital of Inner Mongolia Medical University between June 2012 and August 2014 for molecular marker studies. Authorization to use these tissues for research purpose was obtained from the Institutional Review Board of Affiliated Hospital of Inner Mongolia Medical University. All 31 specimens were permitted with written consent from each patient for scientific research. Clinic-pathologic characteristics including were indicated in Table I.

GIST-T1 cell line was purchased from Cosmo Bio Co. Ltd (Tokyo, Japan) and was cultured in the Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% (v/v) penicillin-streptomycin solution (Ameresco, Framingham, MA, USA), and was incubated at 37°C in a humid incubator under 5% CO_2. For the Imatinib treatment, GIST-T1 cells with approximately 85%-confluence were updated with the DMEM medium which was supplemented with 2% FBS and Imatinib (Sigma-Aldrich, St. Louis, MO, USA) (to a final concentration of 5 μM). For the miRNA mimics transfection, appropriate miRIDIAN miRNA mimics (nontargeting miRNA or miRNA-21, Thermo Scientific, Rockford, IL, USA) were transfected into the 85%-confluent GIST-T1 cells with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA).

RNA Preparation and TaqMan miRNA Assay

Total mRNA samples from the GIST/control gastric specimens or from GIST-T1 cells were extracted with the TRIzol reagent (Life Technologies, Grand Island, NY, USA) and were added with 1μl SUPERase•In™ RNase Inhibitor (Thermo Scientific, Rockford, IL, USA). The quantitative analysis of Bcl-2 mRNA level was performed with Takara One Step RT-PCT kit (Takara, Otsu, Shiga, Japan). And the RT-qPCR was performed at 42 °C for 5 min, and then at 95 °C for 10 sec for the reverse transcription, at 95

Table I. Clinico-pathological characteristics of GIST patients.

Variable	Numbers
Gender	
Male	16
Female	15
Age (years)	
Median (range)	55 (23-72)
Clinical presentation (N)	
Symptomatic	19
Incidental	12
Diameter	
≤ 5 cm	8
> 5 cm	23
Site	
Stomach	12
Small bowel	10
Others	9
Depth of invasion	
Mucosa	4
Muscular	6
Serous	19
Adjacent tissue	2

°C for 5 sec and at 60 °C for 20 sec for the PCR reaction, with 40 cycles. The miRNA samples were isolated from GIST-T1 cells with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). The expression of miRNA-21 was quantified by the mirVana™ qRT-PCR miRNA Detection Kit (Thermo Scientific, Rockford, IL, USA) on the Applied Biosystems 7300 Real-Time PCR system. Fold changes of Bcl-2 or miRNA-21 were calculated, with β-actin or U6 as internal control, using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta C_t$ is $\Delta Ct_{(stimulus)} - \Delta C_{t(solvent)}$, ΔC_t is $Ct_{(target\ gene)} - C_{t(control\ gene)}$ and the Ct is the cycle, at which the threshold is crossed. Basal expression levels were calculated using the formula $2^{-(\Delta Ct)}$.

Luciferase Reporter Assay

For the Luciferase reporting assay, the pGL3-luciferase vector (Promega, Madison, WI, USA) was inserted with three copies of miRNA-21-targeted sites in 3' UTR of *Bcl-2* (for Bcl-2 Reporter) or with three copies of mutant 3' UTR of *Bcl-2* sequence (for Bcl-2^{mut} Reporter) after the luciferase coding sequence. The Bcl-2 Reporter or the Bcl-2^{mut} Reporter was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into the 85%-confluent GIST-T1 cells, which were also transfected with 30 or 60 nM miRNA-21 or control mimics. After the inoculation for 24 hours, the luciferase activity was assayed with the dual luciferase reporter assay kit (Promega, Madison, WI, USA).

Cell Proliferation Assay, MTT Assay and Apoptosis Assay

The GIST-T1 cells were seeded in a 12-well plate (300 cells per well), 12 hours later, cells were treated with 0 or 5 μM Imatinib and were transfected with 60 nM nontargeting miRNA or miRNA-21. Then cells were incubated at 37 °C containing 5% CO_2, for 96 hours. Then the GIST-T1 cells were stained with crystal violet (0.005%) for 30 minutes and the colony numbers were counted. MTT assay was performed to examine the cellular viability of GIST-T1 cells after treatment. In brief, GIST-T1 cells were seeded in 96-well plates to 85% confluence and, then, were treated with 0 or 5 μM Imatinib and were transfected with 60 nM nontargeting miRNA or miRNA-21 for 0, 24 or 48 hours. Then cells were updated with 1× MTT solution for 2-hour incubation at 37 °C. The optical density was then measured at 450 nm using a spectrophotometer. The cell viability was expressed as relatively viable cells (%) to control GIST-T1 cells. Apoptosis induction in GIST-T1 cells after Imatinib treatment or (and) miRNA transfection was examined by a flow cytometry. GIST-T1 cells were firstly stained with the Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the product's manual and, then, were assayed with a flow cytometry.

Results

Downregulated miRNA-21, in Association with an Upregulated Bcl-2 mRNA Level in GIST Specimens

The 31 human GIST specimens from patients were analyzed in this study. Clinical characteristics of these GIST patients were presented in Table I. The mean relative miRNA-21 level to U6 was 0.6729 ± 0.07632 in the GIST specimens, markedly lower than 1.0000 ± 0.1085 in the normal gastric tissues ($p = 0.0129$, Figure 1A). However, the mRNA level of Bcl-2 was significantly upregulated in these GIST specimens to 1.500 ± 0.1484, compared to 1.0000 ± 0.1193 in the control groups ($p = 0.0103$, Figure 1B). Also, we correlated miRNA-21 level with Bcl-2 mRNA level in the specimens, as shown in Figure 1C, there was a significant negative correlation between the Bcl-2 mRNA level and the miRNA-21 level ($R^2 = 0.2450$, $p = 0.0046$). Taken together, miRNA-21 was downregulated, in an association with upregulated Bcl-2, in GIST specimens.

miRNA-21 Downregulates Bcl-2 via Targeting the 3′ UTR of Bcl-2 in GIST-T1 Cells

To further explore the correlation between the miRNA-21 downregulation and the Bcl-2 upregulation in GISTs, we transiently transfected miRNA-21 mimics into GIST-T1 cells, and then examined the Bcl-2 expression. Figure 2A indicated that there was a marked upregulation of miRNA-21 level in the GIST-T1 cells, by the miRNA-21 mimics transfection ($p<0.001$ or $p<0.0001$ for 30 or 60 nM), compared with the control miRNA-transfected cells. In contrast, the Bcl-2 mRNA level was significantly downregulated by the miRNA-21 mimics transfection ($p<0.05$ or $p<0.01$ for the 30 or 60 nM, Figure 2B). Moreover, the Bcl-2 downregulation was reconfirmed in protein level by the miRNA-21 mimics transfection ($p<0.05$ or $p<0.01$, Figure 2C and 2D).

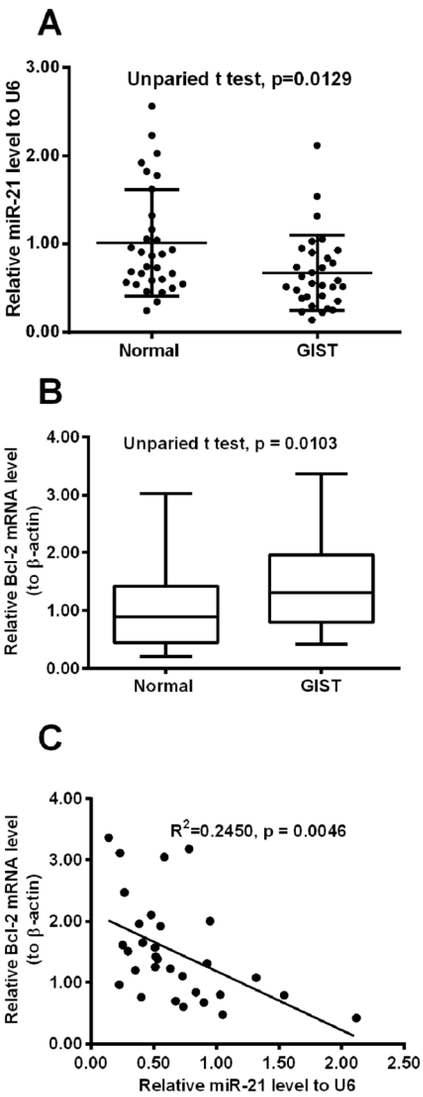

Figure 1. Correlation of decreased miRNA-21 with increased Bcl-2 in gastrointestinal stromal tumor (GIST) specimens. ***A,*** Decreased miRNA-21 level in GIST specimens; ***B,*** Increased Bcl-2 mRNA level (with β-actin as internal reference gene) in the GIST specimens; ***C,*** Correlation of decreased miRNA-21 with increased Bcl-2 mRNA level in the GIST specimens. Statistical significance was considered when $p < 0.05$.

To identify whether the Bcl-2 downregulation by miRNA-21 was mediated by the targeting inhibition 3' UTR of *Bcl-2*, we performed the luciferase reporter assay with the reporter plasmid with the 3' UTR of *Bcl-2* or with the 3' UTR of *Bcl-2* (Bcl-2^{mut} reporter) in GIST-T1 cells, which were transfected with miRNA-21 mimics or control miRNA. The construction of the reporter plasmid or the Bcl-2^{mut} reporter was indicated in Figure 3A. And the reporting assay demonstrated

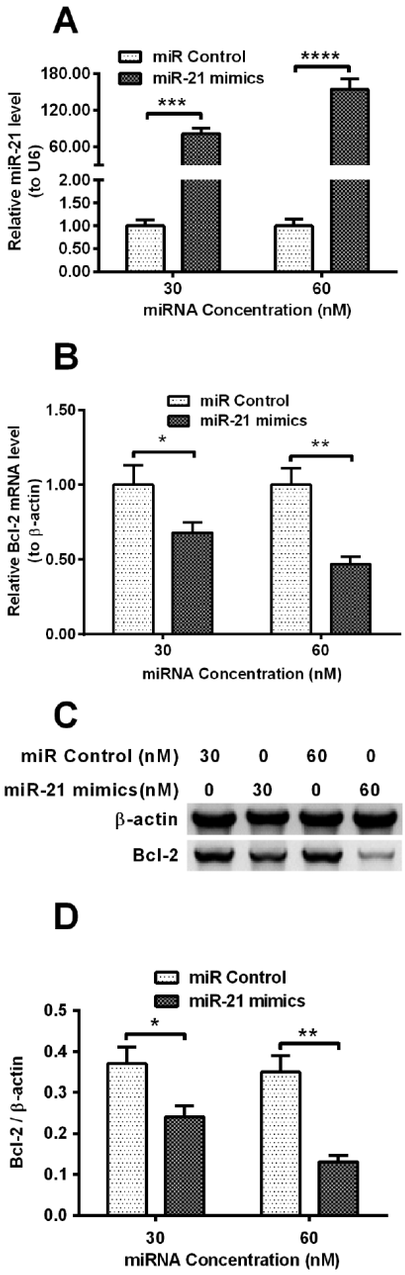

Figure 2. miRNA-21 downregulates Bcl-2 in both mRNA and protein levels in the GIST-T1 cells. ***A,*** Relative miRNA-21 level to U6 in the GIST-T1 cells, post the transfection with 30 or 60 nM miRNA-21 mimics or control miRNA (miR Control) for 8 hours; ***B,*** Relative Bcl-2 mRNA level of to β-actin in the GIST-T1 cells, post the transfection with 30 or 60 nM miRNA-21 mimics or miR Control for 8 hours; ***C,*** Western blotting assay for Bcl-2 in the GIST-T1 cells, post the transfection with 30 or 60 nM miRNA-21 mimics or control miRNA (miR Control) for 24 hours; ***D,*** Relative protein level of Bcl-2 to β-actin in the miRNA-21 mimics- or miR Control-transfected GIST-T1 cells. Results were averaged for triple independent experiments; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ or $^{****}p<0.0001$.

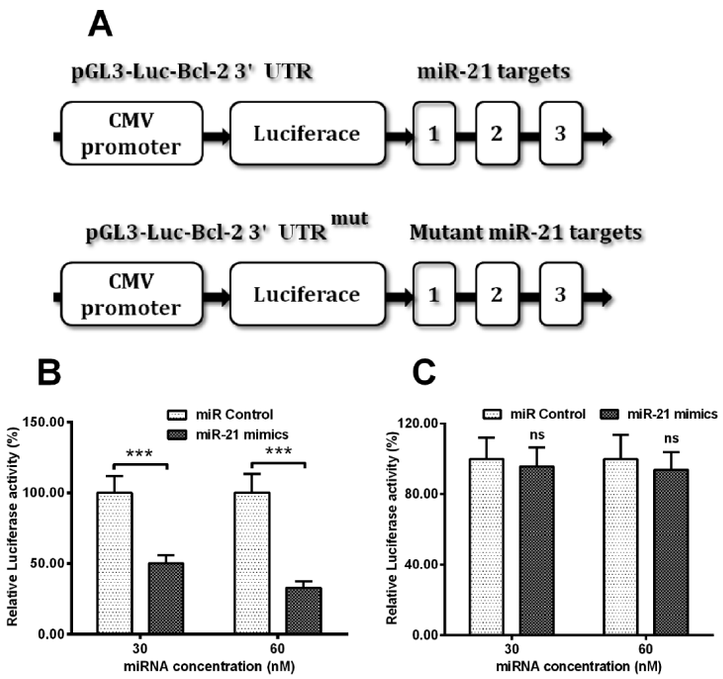

Figure 3. miRNA-21 downregulates the transcriptional activity of *Bcl-2* gene, with a luciferase reporter. ***A,*** Schematic diagram of the luciferase reporter with the 3‘ UTR of *Bcl-2* or with the mutated 3‘ UTR of *Bcl-2*; the 3‘ UTR or the mutant 3‘ UTR of *Bcl-2* was inserted behind the Cytomegalovirus promoter; ***B*** and ***C,*** Relative luciferase activity of the reporter with the 3‘ UTR of *Bcl-2* (B) or the mutated 3‘ UTR of *Bcl-2* (C) in the miRNA-21 mimics- or the miR Control-transfected GIST-T1 cells. Data was indicated as mean ± SEM for triple independent experiments. Statistical significance was shown as $^{***}p < 0.001$ or ns: no significance.

that the transfection with 30 or 60 nM miRNA-21 mimics significantly reduced the luciferase activity than the control miRNA ($p<0.001$ for 30 or 60 nM, Figure 3B). However, there was no such luciferase reduction by miRNA-21 mimics with the Bcl-2^{mut} reporter (Figure 3B). Therefore, we confirmed that the miRNA-21 targeted the 3’ UTR of *Bcl-2* and inhibited Bcl-2 expression in GIST-T1 cells.

miRNA-21 Sensitizes GIST-T1 Cells to Imatinib

To further investigate the regulatory role of miRNA-21 on the sensitivity of GIST-T1 cells to Imatinib, we performed the colony assay in GIST-T1 cells. As indicated in Figure 4A, the treatment with 5 μM Imatinib markedly reduced the colonies which were formed by GIST-T1 cells ($p<0.01$, column 3 *vs*. column 1 in Figure 4B). Moreover, such reduction was more significant in the GIST-T1 cells which were transfected with 60 nM miR-21 mimics, than with miR control ($p<0.001$ for column 4 *vs*. column 2, $p<0.05$ for column 4 *vs*. column 3, Figure 4B).

We also examined the viability and apoptosis in the Imatinib-treated GIST-T1 cells which were transfected with 60 nM miR-21 mimics or with miR control. Figure 5A demonstrated that the transfection with 60 nM miR-21 mimics or with miR control posed no regulation on the viability of GIST-T1 cells. However, the cellular viability significantly decreased in the GIST-T1 cells, 24 or 48 hours after the treatment with 5 μM Imatinib ($p<0.05$ or $p<0.01$, Figure 5B). Moreover, such viability reduction was more significant when cells were transfected with 60 nM miR-21 mimics than with miR control ($p<0.05$ for 24 or 48 hours after the treatment, Figure 5B). In addition, Imatinib induced apoptosis in GIST-T1 cells ($p<0.01$ or $p<0.001$, Figure 5C); in addition, the Imatinib-induced apoptosis was also markedly aggravated by the miR-21 mimics transfection ($p<0.05$ for 24 or 48 hours after the treatment, Figure 5C). Therefore, miRNA-21 sensitizes GIST-T1 cells to Imatinib.

Discussion

miRNAs can regulate target genes on the post-transcriptional level, thus affecting signal transduction both directly and indirectly. Recently, miRNAs have been recognized to play major regulatory roles in signal transduction pathways and tumorigenesis[26,27]. Dysregulated miRNA expression has been regulatory in the KIT overexpression and further in the tumorigenesis[28,29]. Previous studies[28-30] identified the regulation by miRNA-494 on KIT expression, with a negative correlation between the miRNA-494 expression

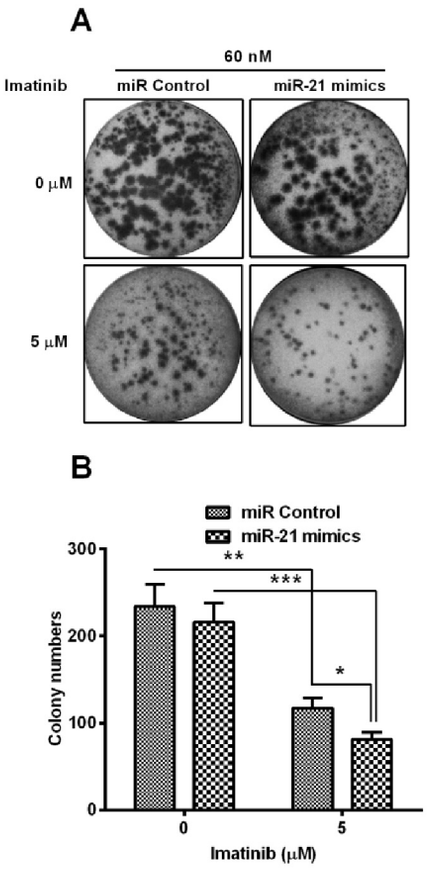

Figure 4. Influence by miR-21 mimics transfection and Imatinib treatment on the colony forming of GIST-T1 cells. ***A,*** Representative images of the colony formed by GIST-T1 cells which were treated with 0 or 5 μM Imatinib and were transfected with 60 nM miR-21 mimics or miR Control; ***B,*** Colony counting in the groups of GIST-T1 cells post the treatment with 0 or 5 μM Imatinib and with the 60 nM transfection with miR-21 mimics or miR Control. Each quantitative data was averaged for triple independent results. Statistical significance was shown as $*p < 0.05$ or $**p < 0.01$.

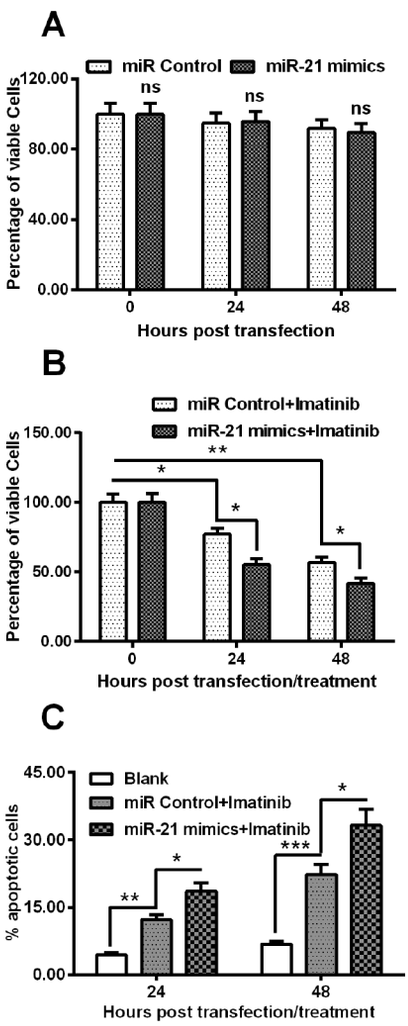

Figure 5. Cellular viability and apoptosis induction in the GIST-T1 cells, post the miR-21 mimics transfection or (and) Imatinib treatment. ***A,*** MTT assay of GIST-T1 cells which were transfected with 60 nM miR-21 mimics or miR Control for 0, 24 or 48 hours; ***B,*** MTT assay of GIST-T1 which were transfected with 60 nM miR-21 mimics or miR Control, post the treatment with 5 μM Imatinib for 0, 24 or 48 hours; ***C,*** Apoptosis induction in the blank GIST-T1, in the GIST-T1 cells, post the transfection with 60 nM miR-21 mimics or miR Control and post the treatment with 5 μM Imatinib for 24 or 48 hours. All experiments were performed in triplicate. ns: no significance, $*p<0.05$ or $**p<0.01$.

and KIT expression in GIST tissues or *in vitro*. In this study, we found that miRNA-21 expression was suppressed in GIST tissues. miRNA-21 has been reported to negatively regulate tumor suppressive by targeting Bcl-2[31]. And work study also indicated that transient transfection of miRNA-21 mimics into human GIST cell line, GIST-T1 cells significantly downregulated the expression of Bcl-2 in both mRNA and protein levels and reduced the luciferase activity of the Bcl-2 reporter with the 3' UTR of *Bcl-2*. Therefore, thesse results confirmed that miRNA-21 suppressed Bcl-2 expression in GIST cells and could function as a potent tumor suppressor in GIST.

Although the tyrosine kinase inhibitor, Imatinib, has been confirmed to considerably improve the outcome of patients, Imatinib resistance still remains a major therapeutic challenge in GIST therapy. Recently, overexpressed miRNA-125a-5p has been recognized in GIST882 cells upon Imatinib treatment, with a suppressed PTPN18 expression; and the silencing of

PTPN18 expression increased the viability of the Imatinib-treated GIST882 cells, highlighting a novel functional role of miR-125a-5p on Imatinib response through PTPN18 regulation in GIST[32]. Our results demonstrated that the miRNA-21 mimics transfection markedly aggravated the Imatinib-mediated growth inhibition and apoptosis induction in GIST-T1 cells. Thus, we observed that the miRNA-21 promotion could sensitize GIST cells to Imatinib, implying miRNA-21 as a potential regulator in the GIST treatment.

Conclusions

Our results demonstrated that miRNA-21 suppressed Bcl-2 expression in GIST cells and could function as a potent tumor suppressor in GIST. And the miRNA-21 promotion could sensitize GIST cells to Imatinib. It implies a potential role in the GIST treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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