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MiRNA-409-5p dysregulation promotes imatinib resistance and disease progression in children with chronic myeloid leukemia

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Abstract. – OBJECTIVE: To elucidate the role of miRNA-409-5p in the pathogenesis of child chronic myeloid leukemia (CML) and its potential mechanism.

PATIENTS AND METHODS: Expression levels of miRNA-409-5p and NUP43 in peripheral blood of CML children and healthy controls were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK-8) and flow cytometry were conducted to evaluate the regulatory effect of miRNA-409-5p on proliferative potential and cell cycle progression of CML cells. Protein levels of PCNA, c-Myc, and cyclin D1 in CML were examined by Western blot. Dual-luciferase reporter gene assay verified the binding of target gene NUP43 to miRNA-409-5p. Finally, the potential effect of miRNA-409-5p on Imatinib resistance in CML was elucidated.

RESULTS: Compared with healthy children, miRNA-409-5p expression in peripheral blood of CML children markedly decreased. Similarly, miRNA-409-5p expression was lower in CML cells. Contrary to the expression pattern of miRNA-409-5p, NUP43 was highly expressed in CML. The miRNA-409-5p overexpression remarkably inhibited proliferative potential and arrested cell cycle in the G0/G1 phase. Protein levels of PCNA, c-Myc, and cyclin D1 were downregulated in CML cells overexpressing miRNA-409-5p. The knockdown of miRNA-409-5p obtained the opposite trends. NUP43 was proved to be the target gene of miRNA-409-5p and negatively regulated by miRNA-409-5p. After overexpression of miRNA-409-5p, Imatinib treatment elevated proliferation inhibition and cell cycle arrest of K562 and KG-1a cells.

CONCLUSIONS: MiRNA-409-5p is lowly expressed in child CML, which inhibits proliferative potential and cell cycle progression by upregulating NUP43 expression. In addition, miRNA-409-5p overexpression enhances Imatinib resistance in CML.

Key Words:
CML, MiRNA-409-5p, NUP43, Cell cycle, Proliferation, Imatinib.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease caused by malignant transformation of hematopoietic stem cells[1]. Translocation of t(9;22)(q34;q11) leads to the formation of Philadelphia (Ph) chromosome containing the BCR-ABL1 gene[2]. Outbreak crisis is the final stage of CML, manifesting as acute onset, rapid progress, and short survival time[3]. Imatinib is generally applied as the first-line treatment for CML, which prolongs the survival of most CML patients[4]. Compared with adult CML, Imatinib treatment in child CML is less applied. Since the clinical application of the first-generation TKI, a small number of CML patients have experienced Imatinib resistance or recurrence due to genetic mutations and poor medication compliance[5]. Therefore, it is urgent to study the mechanism of Imatinib resistance in CML.

MicroRNA (miRNA) is a small RNA with 16-25 bases in size and widely present in eukaryotes. MiRNA exerts its biological effects through binding to the 3'untranslated regions (3'UTR) of target mRNA[6, 7]. The regulatory effects of miRNA are achieved at the post-translational level, which inhibits protein translation and promotes mRNA degradation[8-10]. MiRNA exerts vital roles in regulating cellular behaviors, embryonic development and tumorigenesis. In 2002, Calin *et al*[11] first reported the role of miRNAs in tumors. It is found that miR-15 and miR-16 are downregulated in chronic lymphocytic leukemia. Specific roles of miRNA in tumors have been well concerned and widely explored in recent years.

Studies have shown that miRNA-409-3p is involved in tumor development, serving as a tumor-suppressor gene in gastric cancer[12], colon cancer[13, 14] and bladder cancer[15]. On the contrary, miRNA-409-5p is upregulated in breast cancer

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and promotes the development of breast cancer by inhibiting RSU1 expression[16]. In this paper, we mainly studied the role of miRNA-409-5p in child CML, and initially explored its mechanism.

Patients and Methods

Sample Collection

This study enrolled 42 CML patients with 2-12 years and 40 healthy children in The First People's Hospital of Hefei City from September 2010 to September 2015. The obtained peripheral blood samples from subjects were anticoagulated with Ethylene Diamine Tetraacetic Acid (EDTA). Peripheral blood mononuclear cells (PBMCs) were isolated according to Ficoll-Hypaque density gradient separation method. This experiment was approved by the hospital Ethics Committee, and all subjects signed informed consent form.

Cell Culture and Transfection

CML cell lines K562 and KG-1a were cultured in Roswell Park Memorial Institute-1640 (PRIM-1640) medium (containing 10% fetal bovine serum (FBS)) (Gibco, Rockville, MD, USA) at 37°C, 5% CO_2. Culture medium was daily changed. K562 and KG-1a cells were treated with 267 nM and 65 nM Imatinib for 24 h, respectively.

One day prior to transfection, K562 and KG-1a cells were inoculated in the 6-well plate at 1×10^6 cells/mL. Until 50%-60% of confluence, cells were transfected with miRNA-409-5p mimic, miRNA-409-5p inhibitor or negative control using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Complete medium was replaced at 6 h, and cells were harvested at 48 h.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were washed with phosphate buffered saline (PBS) twice and extracted for RNA using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were examined. We selected qualified RNA samples with the concentration of 100-500 ng/μL and A260/280 nm of 1.9-2.1 for reverse transcription. The obtained complementary deoxyribose nucleic acid (cDNA) was subjected to qRT-PCR using SYBR Green method. Three replicate wells were set to ensure that the Ct difference < 0.5. QRT-PCR conditions were: Pre-denaturation at 95°C for 5 min; 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, for a total of 35 cycles. Relative level was calculated as $2^{-\Delta\Delta Ct}$. Primer sequences were as follows: MiRNA-409-5p, F: GAATGTTGCTCGGTGA, R: GTGCAGGGTCCGAGGT; U6, F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; NUP43, F: TGCCTCCGGGAAGTTTACAGA, R: TCTCCTTCAAACCCTCCATCA; GAPDH, F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cell Proliferation Assay

After transfection for 48 h, cells were cultured in 96-well plates with 5×10^3 cells per well. Each sample set 5 replicate wells. After incubation for 0 h, 24 h, 48 h and 72 h, 10 μL of cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was added each well. Absorbance was recorded at 450 nm with a microplate reader for plotting the growth curve.

Cell Cycle Assay

Transfected cells for 48 hours were fixed in 75% ice-cold ethanol overnight. At the other day, cells were washed with 1 mL of pre-cold PBS twice, resuspended in 150 μL of RNase A and digested in 37°C water bath for 30 min. Subsequently, cells were subjected to 100 μL of Propidium Iodide (PI) staining for 30 min in dark. Finally, cell cycle progression was determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Western blot

Total protein from cells or tissues was extracted using radioimmunoprecipitation assay (RIPA), quantified by bicinchoninic acid (BCA) and loaded for electrophoresis (Beyotime, Shanghai, China). After transferring on a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 300 mA for 100 minutes, the membrane was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by ImageJ Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

Luciferase reporter vectors (NUP43-WT and NUP43-MT) containing the wild-type or mutant-type NUP43 3'UTR were constructed. K562 and KG-1a cells were seeded in 12-well plates, co-transfected with miRNA-409-5p mimic or NC

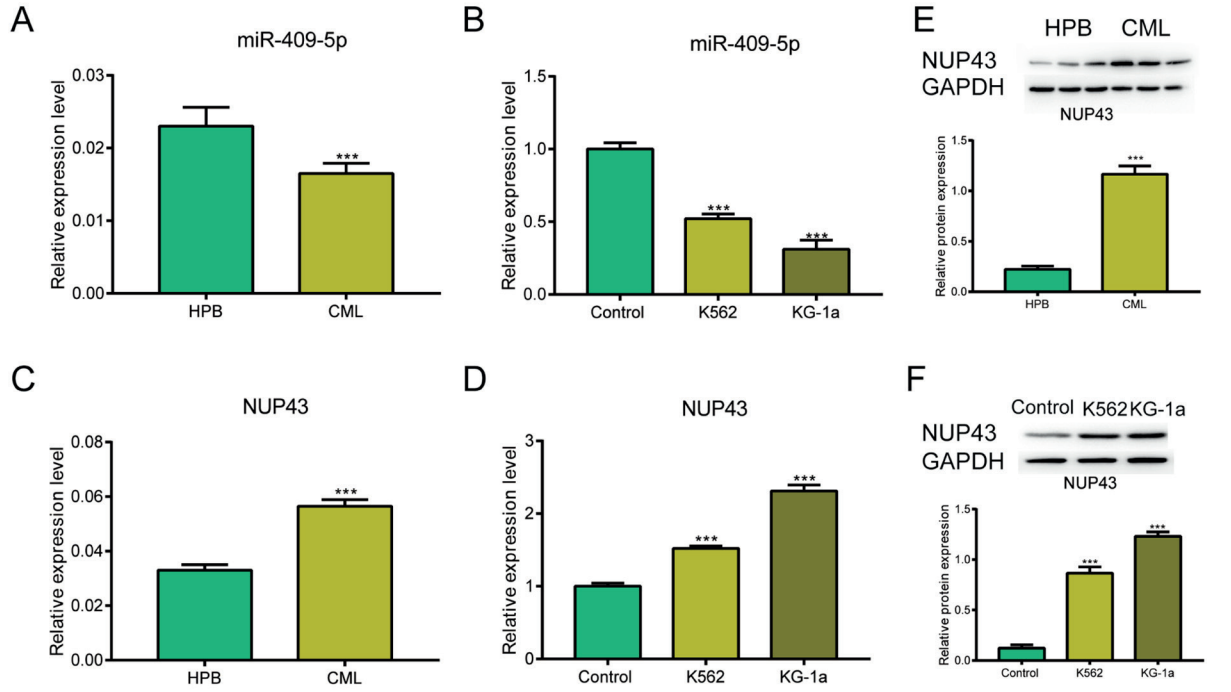

Figure 1. MiR-409-5p expression was low in child CML. **A,** MiR-409-5p expression was lower in peripheral blood of 42 CML children than those of 40 healthy children (HPB). **B,** MiR-409-5p expression was lower in CML cell lines (K562 and KG-1a) than healthy peripheral blood cells. **C,** NUP43 expression was higher in peripheral blood of 42 CML children than those of HPB. **D,** NUP43 expression was higher in K562 and KG-1a cells than healthy peripheral blood cells. (E,) Protein level of NUP43 was higher in CML than HPB. **F,** Protein level of NUP43 was higher in K562 and KG-1a cells than healthy peripheral blood cells.

and NUP43-WT or NUP43-MUT, respectively. Medium was replaced at 6 h of transfection. Luciferase activity was detected at 48 h after co-transfection. Relative luciferase activity = Firefly luciferase activity / Renilla luciferase activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± SD (standard deviation). The *t*-test was used for comparing differences between two groups. $p<0.05$ indicated the significant difference.

Results

MiRNA-409-5p Expression was Low in Child CML

We first examined miRNA-409-5p expression in CML children and healthy controls. MiRNA-409-5p expression remained lower in peripheral blood of 42 CML children relative to controls (Figure 1A). Similarly, miRNA-409-5p expression was identically lower in human CML cell lines K562 and KG-1a compared with controls (Figure 1B). Through bioinformatics prediction, NUP43 was searched as the target gene of miRNA-409-5p. Both protein and mRNA levels of NUP43 were higher in CML patients than controls (Figure 1C, 1E). Similar expression pattern was observed in CML cells as well (Figure 1D, 1F).

MiRNA-409-5p Inhibited the Proliferative Potential of CML Cells

To explore the role of miRNA-409-5p in child CML, we first constructed miRNA-409-5p mimic and inhibitor. Transfection efficacy of miRNA-409-5p mimic and inhibitor in K562 and KG-1a cells was verified by qRT-PCR (Figure 2A, 2B). CCK-8 assay showed that miRNA-409-5p overexpression markedly inhibited viability of CML cells, and miRNA-409-5p knockdown conversely accelerated cell viability (Figure 2C, 2D). K562 and KG-1a cells were arrested in G0/G1 phase after overexpression of miRNA-409-5p. Conversely, miRNA-409-5p knockdown prolonged the S phase of CML cells, indicating the accelerated cell cycle progression (Figure 2E, 2F). We con-

sidered that miRNA-409-5p may influence cell cycle progression of CML. Western blot analyses showed downregulated protein levels of PCNA, c-Myc and cyclin D1 in CML cells overexpressing miRNA-409-5p. On the contrary, miRNA-409-5p knockdown greatly upregulated their protein levels (Figure 2G, 2H).

NUP43 was the Target Gene of miRNA-409-5p

To explore the mechanism of miRNA-409-5p in CML, we predicted the target gene of miRNA-409-5p online and found the binding sites between NUP43 and miRNA-409-5p (Figure 3A). Dual-luciferase reporter gene assay was conducted and revealed the decreased luciferase activity in wild-type group. However, mutant-type group did not show a remarkable change in luciferase activity, suggesting that miRNA-409-5p could bind to NUP43 (Figure 3B, 3C). Meanwhile, overexpression of miRNA-409-5p decreased protein level of NUP43 in K562 and KG-1a cells. Conversely, miRNA-409-5p knockdown increased protein level of NUP43 in CML cells (Figure 3D, 3E).

MiRNA-409-5p Alleviated Imatinib Resistance in CML

Imatinib resistance is commonly seen in CML patients, which severely restricts the therapeutic efficacy. We speculated whether

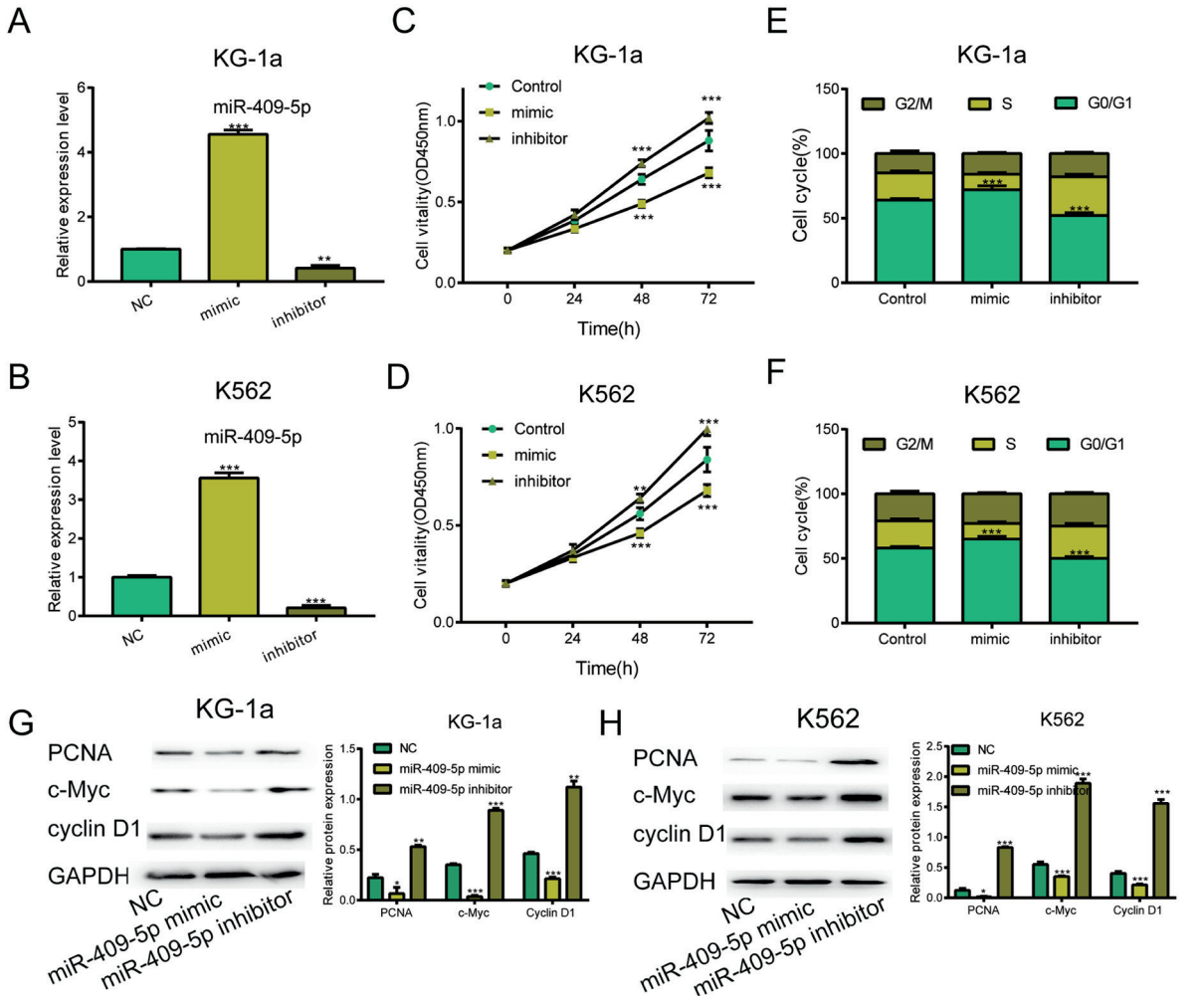

Figure 2. MiR-409-5p inhibited the proliferative potential of CML cells. **A-B,** Transfection efficacy of miR-409-5p mimic and inhibitor in K562 (**A**) and KG-1a cells **(B)** was verified by qRT-PCR. **C-D,** CCK-8 assay showed that miR-409-5p overexpression markedly inhibited viability of CML cells, and miR-409-5p knockdown conversely accelerated cell viability. **E-F,** K562 and KG-1a cells were arrested in G0/G1 phase after overexpression of miR-409-5p. Conversely, miR-409-5p knockdown prolonged the S phase of CML cells, indicating the accelerated cell cycle progression. **G-H,** Western blot analyses showed downregulated protein levels of PCNA, c-Myc and cyclin D1 in CML cells overexpressing miR-409-5p. On the contrary, miR-409-5p knockdown greatly upregulated their protein levels (**G-H**).

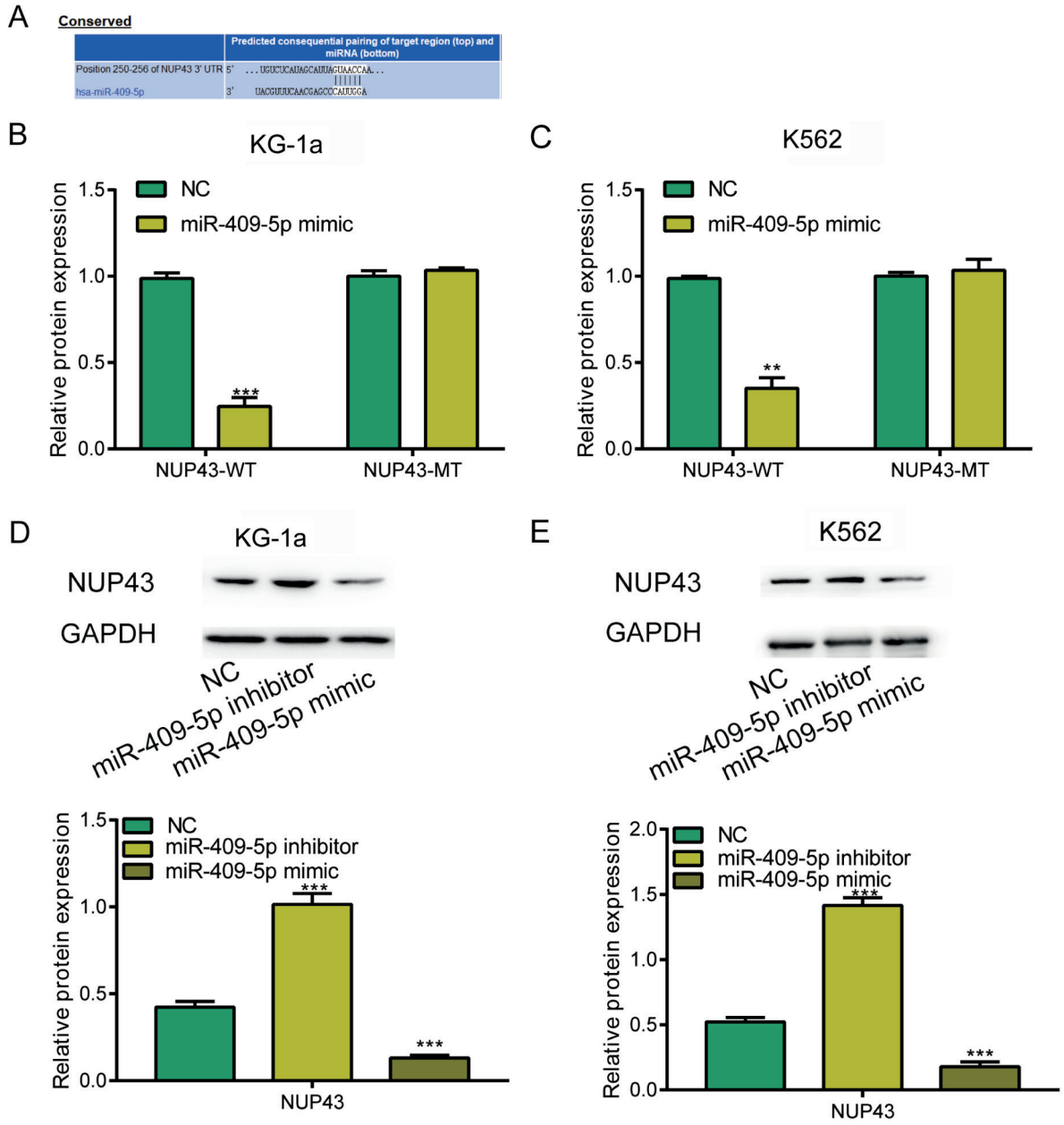

Figure 3. NUP43 was the target gene of miR-409-5p. **A,** Binding sites between NUP43 and miR-409-5p. **B-C,** Dual-luciferase reporter gene assay was conducted and revealed the decreased luciferase activity in wild-type group. However, mutant-type group did not show remarkable change in luciferase activity. **D-E,** Overexpression of miR-409-5p decreased protein level of NUP43 in K562 and KG-1a cells. Conversely, miR-409-5p knockdown increased protein level of NUP43 protein in CML cells.

miRNA-409-5p was capable of protecting Imatinib resistance in CML cells. Here, we examined the potential effect of miRNA-409-5p on Imatinib-induced changes in proliferation and cell cycle progression. CCK-8 assay revealed that Imatinib treatment remarkably inhibited viability of CML cells, which was more pronounced by miRNA-409-5p overexpression (Figure 4A, 4B). Moreover, miRNA-409-5p overexpression enhanced the inhibitory effect of Imatinib on cell cycle progression (Figure 4C, 4D).

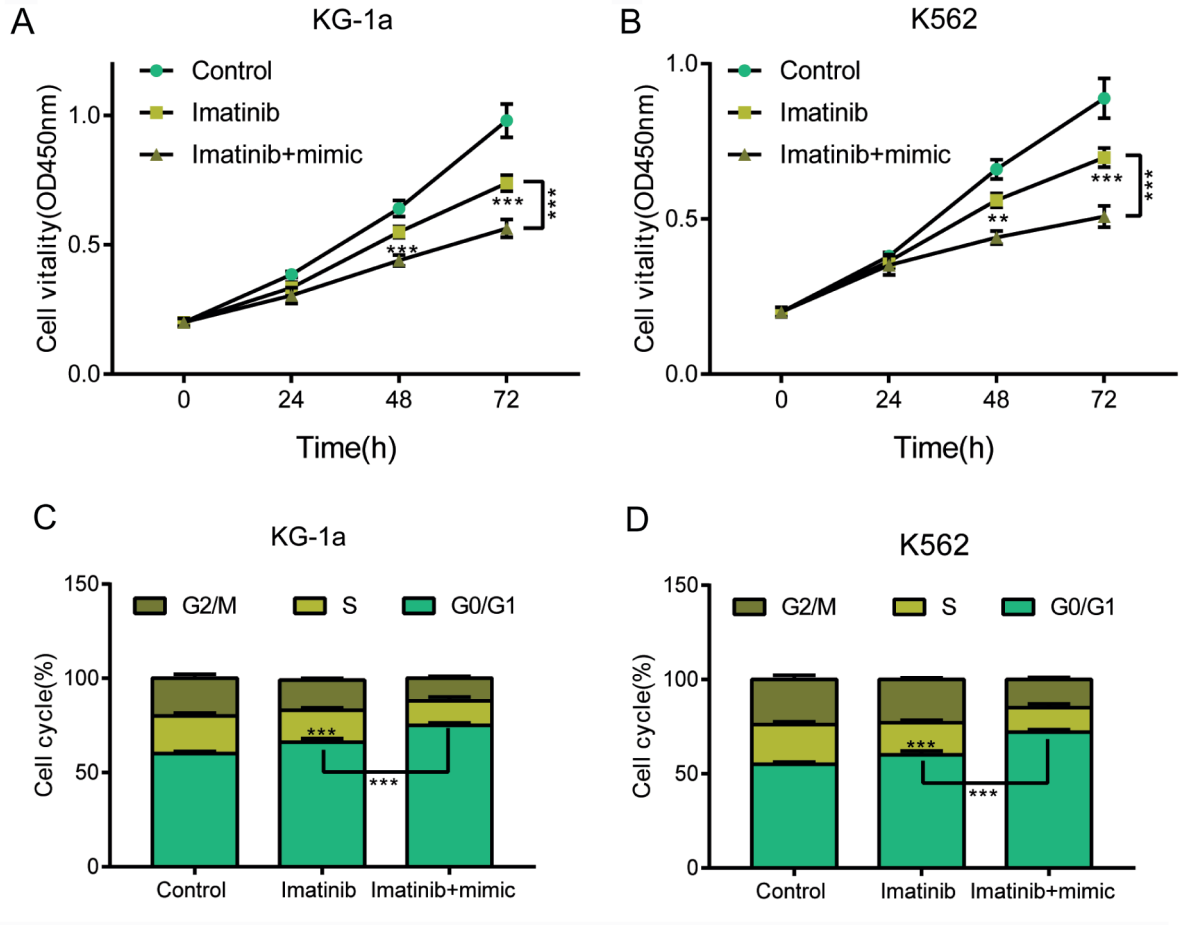

Figure 4. MiR-409-5p alleviated Imatinib resistance in CML. **A-B,** CCK-8 assay revealed that Imatinib treatment remarkably inhibited viability of CML cells, which was more pronounced by miR-409-5p overexpression. **C-D,** MiR-409-5p overexpression enhanced the inhibitory effect of Imatinib on cell cycle arrest.

Discussion

In this investigation, miRNA-409-5p was lowly expressed in CML patients. *In vitro* researches showed that miRNA-409-5p inhibited viability, arrested cell cycle in G0/G1 phase, and downregulated cyclin proteins in K562 and KG-1a cells. Through bioinformatics prediction, NUP43 was found to be a target gene of miRNA-409-5p, which was highly expressed in CML. Furthermore, dual-luciferase reporter gene assay showed the binding of NUP43 to miRNA-409-5p, and its expression was inhibited by miRNA-409-5p. More importantly, miRNA-409-5p enhanced the sensitivity of K562 and KG-1A cells to Imatinib.

MiRNA-409 matures include miRNA-409-3p and miRNA-409-5p. MiRNA-409-3p locates on chromosome 14q32.31. It could regulate angiogenesis through interaction with vascular endothelial cells or angiogenic factors, further influencing tumor growth and metastasis[17]. A series of reports identified the tumor-suppressor role of miRNA-409-3p in various tumors. MiRNA-409-3p is downregulated in gastric cancer tissues and cell lines, which promotes the *in vivo* and *in vitro* proliferation and apoptosis of gastric cancer through PHF10[12]. In colon cancer, downregulated miRNA-409-3p inhibits metastasis of tumor cells through targeting GAB1[13, 14]. Xu et al[15] pointed out that the highly expressed miRNA-409-3p in bladder cancer cells markedly suppresses the invasion and metastasis of bladder cancer. So far, researches on miRNA-409-5p are rare. Both miRNA-409-3p and miRNA-409-5p have their own target genes and common target genes, which may share similar functions[18]. RSU1 is a downstream target of miRNA-409-5p in breast cancer[16], while Akt1 is a downstream target of miRNA-409-3p in

breast cancer as Zhang et al[19] indicated. Functional differences between miRNA-409-3p and miRNA-409-5p could be explained by their different target genes, allowing them to be an tumor-suppressor and oncogene even in the same tumor. In this experiment, miRNA-409-5p expression was downregulated in child CML. Overexpression of miRNA-409-5p inhibited the proliferative rate and arrested cell cycle in G0/G1 phase of CML cells, suggesting the tumor-suppressor role of miRNA-409-5p in child CML.

Disorders of NPCs-related genes may be crucial in tumorigenesis. NUP88 promotes the formation of haploids, and its high expression is of significance in the initial stages of various tumors[20]. NUP98 can specifically regulate the nuclear transport of galactoside-3, participating in tumor cell proliferation, adhesion and metastasis[21]. NUP43 is a component of the NUP107-160 complex, which was originally identified as a regulator of cell mitosis[22]. Jagot-Lacoussiere et al[23] have shown that NUP107-160 complex leads to DNA damage by acting on Apaf-1, and its dysregulated expression is associated with tumor metastasis. We believed that NUP203 is involved in the progression of certain tumors. In patients with lumina type A and $HER2^+$ breast cancer, NUP43 presents a high expression and indicates a poor prognosis. In this experiment, NUP43 also showed high expression in child CML.

So far, Imatinib resistance has become a major problem in the treatment of child CML, resulting in numerous gene mutations[24]. Since the tumor-suppressor role of miRNA-409-5p in child CML has been already proved, we further hypothesized whether miRNA-409-5p could against Imatinib resistance. Here, we demonstrated that miRNA-409-5p overexpression enhanced Imatinib-induced proliferation inhibition and cell cycle arrest of K562 and KG-1a cells.

Conclusions

MiRNA-409-5p is lowly expressed in child CML, which inhibits proliferative potential and cell cycle by upregulating NUP43 expression. In addition, miRNA-409-5p overexpression enhances Imatinib resistance in CML. MiRNA-409-5p can be utilized as a potential target in CML treatment.

Conflicts of interest

The authors declare no conflicts of interest.

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