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LncRNA HAND2-AS1 inhibits proliferation and promotes apoptosis of chronic myeloid leukemia cells by sponging with micRNA-1275

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Abstract. – OBJECTIVE: The long noncoding RNA (lnc) HAND2-AS1 is down-regulated and microRNA-1275 (miR-1275) is up-regulated in many types of cancers. However, their expressions and the relationship between HAND2-AS1 and miR-1275 in chronic myeloid leukemia (CML) remain unknown and to be further investigated.

PATIENTS AND METHODS: Bone marrow samples from 30 CML patients and 10 healthy donors were collected; HAND2-AS1 and miR-1275 were detected by RT-PCR. The correlation between HAND2-AS1 and miR-1275 was analyzed. After lentiviral (LV) HAND2-AS1 and miR-1275 inhibitors were respectively transfected into KCL22 and K562 cells, the expressions of HAND2-AS1 and miR-1275 were detected by RT-PCR. MTT assay was performed to evaluate cell viability and mRNA and proteins levels of Bcl-2, Caspase-3, MMP-2, MMP-9 were detected by RT-PCR and Western blot (WB). Luciferase reporter assay was performed to explore the binding site of HAND2-AS1 and miR-1275.

RESULTS: Results showed that HAND2-AS1 was significantly downregulated than healthy control ($p<0.05$), and HAND2-AS1 expressions on stages of AP and BP were much lower than that of CP ($p<0.05$). The miR-1275 expression was significantly upregulated than healthy control ($p<0.05$), and the expressions in stages of accelerated phase (AP) and blast phase (BP) were much higher than that in the stage of chronic phase (CP) in CML ($p<0.05$). Furthermore, HAND2-AS1 was negatively correlated with miR-1275 in CML, but not in healthy control ($p<0.05$). After lentiviral HAND2-AS1 transfection, the HAND2-AS1 expression was significantly up-regulated while miR-1275 was significantly down-regulated ($p<0.05$). Then, the cell proliferation was inhibited after 72 h. Furthermore, the mRNA and protein levels of Bcl-2, MMP-2 and MMP-9 were significantly down-regulated ($p<0.05$), while the expression of Caspase-3 was significantly up-regulated ($p<0.05$). Luciferase reporter assay showed that HAND2-AS1 was a target gene of miR-1275. And after treating with miR-1275 inhibitor, HAND2-AS1 was significantly upregulated ($p<0.01$) and cell proliferation was inhibited ($p<0.01$). Furthermore, the expressions of Bcl-2, MMP-2, and MMP-9 were significantly decreased, while Caspase-3 was significantly increased ($p<0.01$).

CONCLUSIONS: HAND2-AS1 was downregulated and miR-1275 was upregulated in CML, and HAND2-AS1 inhibited proliferation and promoted apoptosis of CML cells by sponging with microRNA-1275, which might be a novel therapeutic target for CML.

Key Words

Chronic myeloid leukemia, HAND2-AS1, miR-1275, bcl-2, Caspase-3.

Introduction

Chronic myeloid leukemia (CML) is a kind of leukemia closely related to the Philadelphia chromosome. The myeloid cells used to be unregulated, increased and accumulated in the bone marrow and blood [1-3]. Generally, CML is divided into three stages based on the number of leukemia cells in the bone marrow and the severity of the symptoms: chronic phase (CP), accelerated phase (AP), and blast phase (BP) [4,5]. Tyrosine kinase inhibitors (TKIs) could improve the 5-year survival rate of CML patients in CP, while the prognosis is quite poor for CML patients on stages of AP and BP [6]. However, their mechanisms are not fully understood, so it is quite important to clarify the molecular targets and biological mechanisms of CML, and it is of great significance to find new therapeutic targets for CML on stages of AP and BP.

It was reported that long non-coding RNAs (lncRNAs) are key regulators of important biological processes and play a role in specific cancers and other diseases[7]. LncRNAs were differentially expressed in some solid tumors and hematopoietic tumors[8,9]. Studies had found that the lncRNA HAND2-AS1 was associated with many cancers, such as hepatocellular carcinoma, endometrioid endometrial carcinoma, papillary thyroid cancer,

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osteosarcoma, colorectal cancer and so on [10-15]. Recently, researchers found that HAND2-AS1 inhibited cancer cell proliferation, migration and invasion in esophagus squamous cell carcinoma [16]. Our previous results showed that HAND2-AS1 was also downregulated in CML, but the functions and molecular biological mechanisms between HAND2-AS1 and CML were unclear.

MicroRNAs (miRNAs), which did not encode proteins, are about 20 nt in length and participate in many essential cellular processes such as proliferation, apoptosis, development and differentiation[17]. Recent studies[18,19] showed that miRNAs were involved in tumorigenesis and played important roles in inhibiting tumor genes or oncogenes . It was reported that miR-1275 was involved in the progression of bladder cancer, colorectal cancer, non-small cell lung cancer, hepatocellular carcinoma, acute myeloblastic leukemia (AML), etc. It was reported that miR-1275 promoted cell proliferation, invasion and metastasis in these solid tumors and AML [20-25]. Our previous results showed that it was upregulated in CML patients; however, the roles and functions of miR-1275 in the progression of CML were still unknown. Therefore, in this study, we explored the functions of HAND2-AS1 and miR-1275 in CML, also investigating the relationship between these two molecules.

Patients and Methods

Bone Marrow Samples

Bone marrow samples were collected from 30 CML patients who were admitted in our hospital from July 2015 to June 2017, including 10 patients on stage of AP, 10 patients on BP and 10 patients on CP. No chemotherapy was performed before the sample was collected. Bone marrow samples from 10 healthy donors were selected as control. After isolation of lymphocytes, mononuclear cells were obtained from bone marrow samples. This study was approved by the Faculty of Medicine's Ethics Committee of our hospital, and all patients signed the informed consent.

Cell Culture and Cell Transfection

Two human CML cell lines KCL22 and K562 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). KCL22 cells were cultured in Iscove modified Dulbecco's medium (IMDM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). K562 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 culture incubator. KCL22 and K562 cells were seeded in 6-well plates (5×10^6/well) and the EC50 concentration of 5-azacytidine (ApexBio, Houston, TX, USA) was measured by MTT assay. Treatment of 5-azacytidine in K562 and KCL22 cells at concentrations of 60, 80 and 100 μmol/l was carried out according to their respective EC50 values for 48 h. KCL22 and K562 cells were seeded in 6-well plates (5×10^5/well) and then transfected with lentiviral HAND2-AS1 (LV HAND2-AS1) and LV-NC, purchased from Gene Pharma (Shanghai Gene Pharma, Shanghai, China). And miR-1275 NC and miR-1275 inhibitor were transfected into KCL22 and K562 cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to its instructions.

MTT Assay

KCL22 and K562 cells were seeded in 96-well plates (1×10^3/well) and cultured in IMDM and 1640 medium at 37°C and 5% CO_2. Afterward, 10 μL 5 g/l of MTT (Sigma-Aldrich, St. Louis, MO, USA) were added to each well for 0 h, 24 h, 48 h, 72 h and 96 h, and proliferation of KCL22 and K562 cells was measured by MTT assay. The absorbance (OD) value of each well was measured at 490 nm with microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and this experiment was repeated for three times.

RNA Extraction and Quantitative Real-Time PCR

Total RNA of bone marrow samples and cultured cells was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was reversed into the first-strand cDNA by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). PCR primers were synthesized by Gene Pharma (Shanghai, China) and miR-1275 and U6 primers were synthesized by RiBo Biotech (GuangZhou, China); the sequence was shown in Table I. And the relative mRNA expressions were detected with SYBR Premix Ex Taq II (TaKaRa, Dalian, China). The mRNA expressions were normalized to β-actin or U6, and $2^{-\Delta\Delta CT}$ method was used to detect the relative gene expression.

Protein Extraction and Western Blot

Total protein was extracted from bone marrow samples and cells by using a RIPA lysis buffer

(Biyuntian, Shanghai, China) and the protein concentration was detected by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). 40 μg proteins were added to 8-15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Shanghai, China), which were blocked at room temperature for 1 h. Next, membranes were incubated with primary antibodies overnight at 4°C. Bcl-2 (1:3000), Caspase-3 (1:1000), MMP-2 (1:1000), MMP-9 (1:3000) and β-actin (1:5000) were bought from Abcam (Abcam, Cambridge, MA, USA), and subsequently incubated with matched secondary antibodies (1:50000) for 1 h. Protein bands were detected by Pierce ECL Western blot substrate (Thermo Fisher Scientific, Waltham, MA, USA) with ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Assays

Total RNA was extracted from KCL22 cells, which was reverse transcribed into cDNA. The cDNA was used as a template to amplify the 3'UTR and CDS regions of β-catenin. The potential binding site of pmiR-HAND2-AS1-WT and mutant sequence pmiR-HAND2-AS1-MT were synthesized into pmiR-GLO (Promega, Madison, WI, USA). After that, miR-1275 mimics and miR-1275 negative control (NC) were co-transfected into KCL22 cells with pmiR-GLO for 24 h. Plasmids with 200 ng were mixed with Lipofectamine 3000 and DMEM medium for 20 mins, then the mixtures were added into KCL22 cells for 24 h. The cells were lysed and the activity of firefly luciferase and Renilla luciferase was measured by using a Promega Luciferase Assay (Madison, WI, USA). The ratio of these two revealed the relative activity of luciferase.

Statistical Analysis

All data were analyzed by SPSS version 20.0 (SPSS Inc., Armonk, NY, USA). Data were presented by means±SD. Data were analyzed by one-way ANOVA or Student's *t*-test, and multiple comparisons between the groups were performed by SNK method. If $p<0.05$, the difference was statistically significant.

Results

HAND2-AS1 and miR-1275 Expressions in Bone Marrow Samples from Different Stages of CML Patients and Healthy Donors

30 bone marrow samples from CML patients and 10 samples from healthy donors were extracted and the expressions of HAND2-AS1 and miR-1275 were detected by RT-PCR. Results showed that HAND2-AS1 was significantly downregulated in CML patients ($p<0.05$) (Figure 1A), and HAND2-AS1 expressions on stages of AP and BP were much lower than that on the stage of CP in CML ($p<0.05$) (Figure 1B). The miR-1275 expression was significantly upregulated in CML ($p<0.05$) (Figure 1C), and the expressions on stages of AP and BP were much higher, compared to that on the stage of CP in CML ($p<0.05$) (Figure 1D). These results showed that HAND2-AS1 and miR-1275 might play some roles in the progression of CML, which should be further explored.

Table I. Sequences of primers for RT-PCR.

Genes	Primer sequences
HAND2-AS1	Forward: 5'- GGAGTCACAGGCAGTCGTAGA -3' Forward: 5'- GAAGGCACAGATCATTCATGG -3'
Caspase-3	Forward: 5'- CTCTGGTTTTCGGTGGGTGT-3' Forward: 5'- GCTTTGGTTCCCGCAAAACT-3'
Bcl-2	Forward: 5'- CTGCTTTAGTGAACCTTTTGCA -3' Forward: 5'- TTGAATACTCCTGGCTGTCTC -3'
MMP-2	Forward: 5'- AACTACAACAAGAACCCTCGCAA -3' Forward: 5'- CAAAGGCGCCATCCACTGTCTCT -3'
MMP-9	Forward: 5'- CCACCCTTGTGCTCTTCCCTG -3' Forward: 5'- TCTGCCACCCGAGTGTAACCA -3'
β-actin	Forward: 5'- GAGCTACGAGCTGCCTGAC -3' Forward: 5'- GGTAGTTTCGTGGATGCCACAG -3'

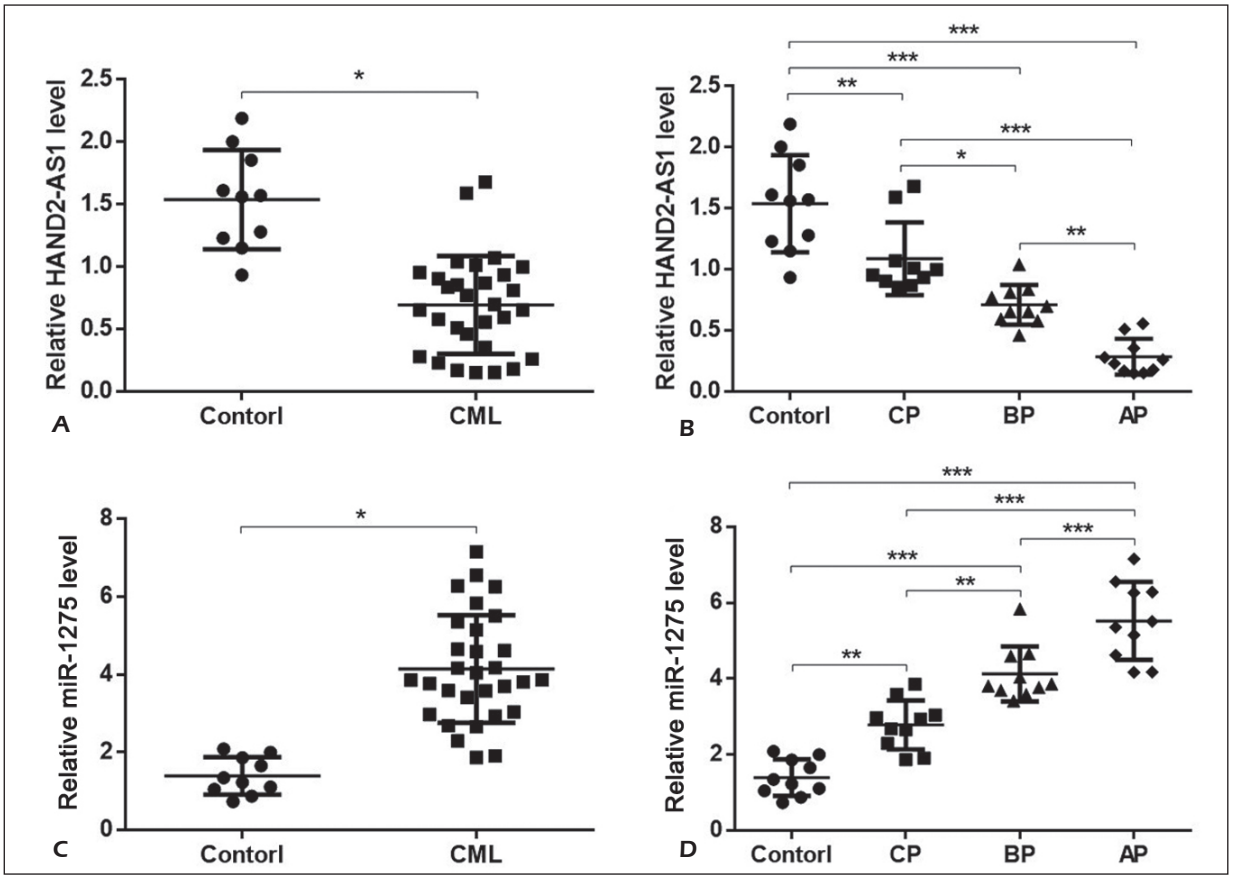

Figure 1. HAND2-AS1 and miR-1275 expressions in bone marrow samples from different stages of CML patients and healthy donors. **A-C**, The mRNA levels of HAND2-AS1 and miR-1275 in CML patients and healthy donors were detected by RT-PCR. **B-D**, The levels of HAND2-AS1 and miR-1275 in CML on different stages were detected by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01, ***p<0.001.

HAND2-AS1 Was Negatively Correlated with miR-1275 in CML

To explore the relationship between HAND2-AS1 and miR-1275, the correlation analysis was performed. Results showed that HAND2-AS1 was negatively correlated with miR-1275 in CML but not in healthy control (p<0.05) (Figure 2A,B). These results indicated that HAND2-AS1 might be regulated or interacted with miR-1275 in CML, but the detailed mechanisms needed to be explored.

Upregulation of HAND2-AS1 Inhibited the Cell Proliferation, Invasion and Promoted Cell Apoptosis in K562 and KCL22 Cells

To explore the functions of HAND2-AS1 in CML, lentiviral HAND2-AS1 was produced and transfected into K562 and KCL22 cells, two kinds of widely used CML cells. After LV HAND2-AS1 transfection, the HAND2-AS1 expression was significantly increased (p<0.05) (Figure 3A). The cell proliferation was inhibited after 72 h and 96 h in both K562 and KCL22 cells (p<0.05) (Figure 3 B,C). Furthermore, the mRNA and protein levels of Bcl-2, MMP-2 and MMP-9 were significantly down-regulated (p<0.05), while the expression of Caspase-3 was significantly up-regulated in both two cells (p<0.05) (Figure 3 D-I). These results suggested that HAND2-AS1 inhibited the cell proliferation, invasion and promoted cell apoptosis in CML.

HAND2-AS1 Interacted with miR-1275 in CML Cells

To explore the relationship between HAND2-AS1 and miR-1275, LV HAND2-AS1 and miR-1275 inhibitor were respectively transfected into CML cells. Results showed that the upregulation of HAND2-AS1 repressed miR-1275 expression, and the inhibition of miR-1275 reversely increased HAND2-AS1 expression (p<0.05) (Figure 4A,B).

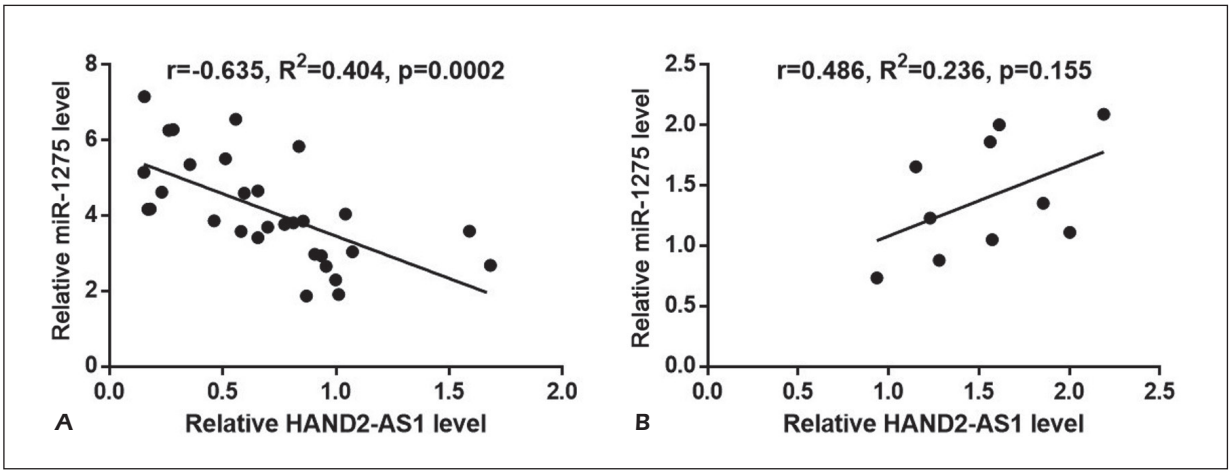

Figure 2. HAND2-AS1 was negatively correlated with miR-1275 in CML. **A-B**, Correlation analysis was performed to explore the relation between HAND2-AS1 and miR-1275 in CML patients and healthy control. *p<0.05.

As a result, we assumed that HAND2-AS1 might interact with miR-1275. The potential binding site was predicted by using bioinformatics method (Figure 4C) and luciferase reporter assay was performed. The potential binding sequence was synthesized into pmiR-GLO vector, which was named as pmiR- HAND2-AS1-WT. The mutant binding sequence was also synthesized into pmiR-GLO vector, which was named as pmiR-HAND2-AS1-MT. After miR-1275 mimics and miR-1275 NC were transfected into KCL22 cells for 24 h, relative luciferase activity was detected. Results showed that the luciferase activity of cells transfected with pmiR-HAND2-AS1-WT was significantly decreased, compared to that in cells transfected with miR-1275 mimics and pmiR-GLO vector (p<0.01). And the luciferase activity in pmiR-HAND2-AS1-MT was reversely increased, compared to pmiR-HAND2-AS1-WT (p<0.01) (Figure 4D). These results indicated that HAND2-AS1 was a target gene of miR-1275, which acted as an endogenous "sponge" that regulated its target genes by competing with miR-1275 and inhibited their functions. But the roles and functions of miR-1275 were still unknown in CML.

Inhibition of miR-1275 Inhibited Cell Proliferation, Invasion and Promoted Cell Apoptosis in CML Cells

To explore the roles of miR-1275 in CML cell lines, miR-1275 inhibitor was transfected into KCL22 and K562 cells, then MTT assay was performed to measure the cell viability. Results showed that cell proliferation was significantly inhibited after treating with miR-1275 inhibitor in both two cells, compared to the control (p<0.01) (Figure 5A,E). To further show the role of miR-1275 in cell proliferation and apoptosis, MMP-2, Caspase-3, bcl-2 and MMP-9 were detected by RT-PCR and WB after transfection with miR-1275 inhibitor. Results showed the mRNA and protein levels of bcl-2, MMP-2 and MMP-9 were significantly decreased in the miR-1275 inhibitor group, and Caspase-3 mRNA and protein levels were significantly increased (p<0.01) (Figure 5B-D,F-H). These results indicated that the inhibition of miR-1275 inhibited cell proliferation, invasion and promoted cell apoptosis in CML cells.

Discussion

CML is a hematological malignancy accounting for more than 10% of leukemia and its incidence is recently increasing[26]. TKIs had been found to be widely used in treating with leukemia; however, the prognosis for CML patients with on the stages of AP and BP was quite poor, and the detailed mechanisms are yet not fully understood [6]. Therefore, it was of great significance to clarify and find the molecular targets and biological mechanisms of CML on stages of AP and BP. HAND2-AS1 had been reported as a tumor suppressor in some cancers[10-15]. And researchers recently found that HAND2-AS1 inhibited cancer cell proliferation, migration and invasion in esophagus squamous cell carcinoma[16]. Our previous results showed that HAND2-AS1 was also downregulated in CML, but the functions and mo-

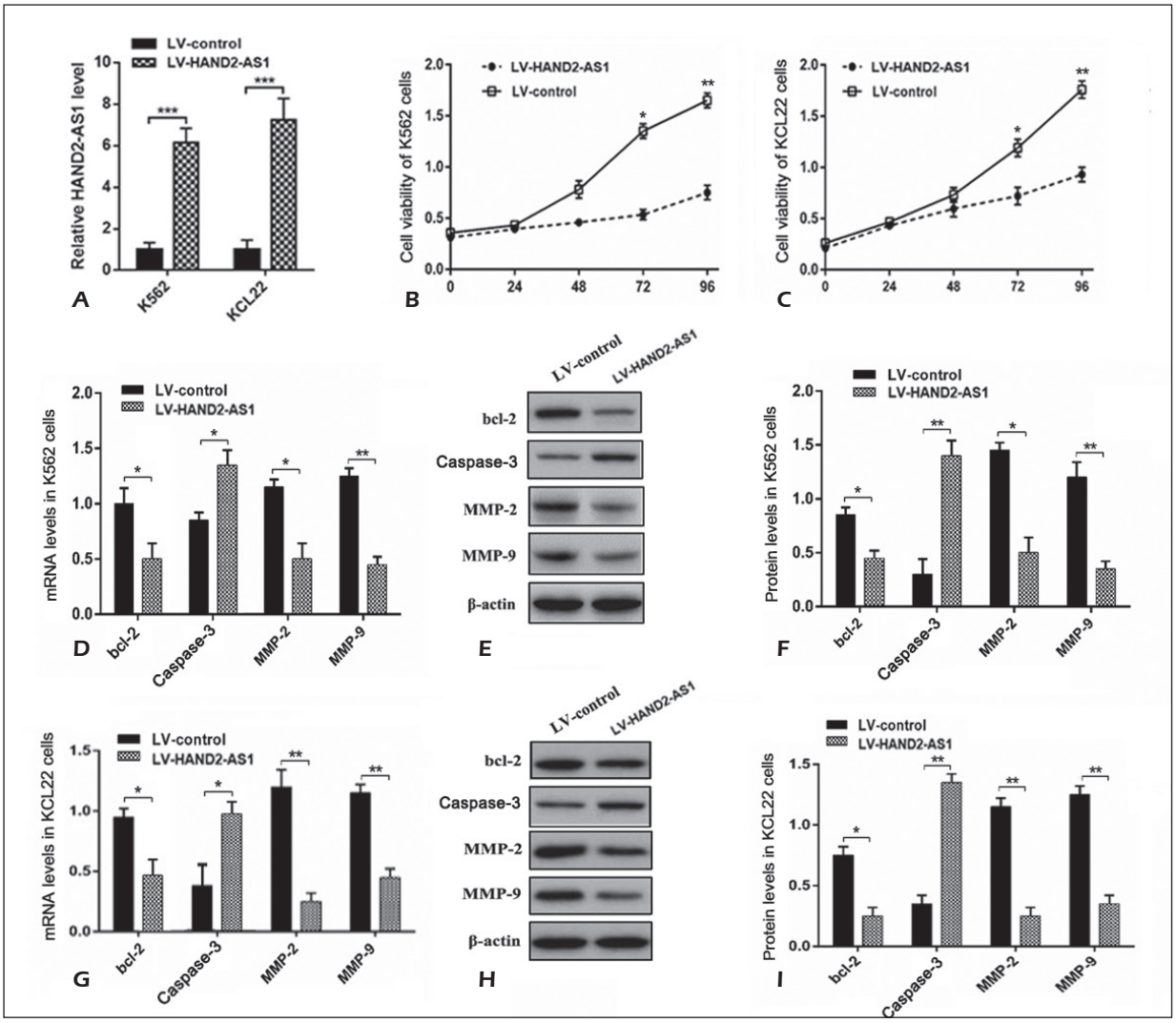

Figure 3. Upregulation of HAND2-AS1 inhibited the cell proliferation, invasion and promoted cell apoptosis in K562 and KCL22 cells. ***A***, LV HAND2-AS1 and control were transfected into K562 and KCL22 cells, and the HAND2-AS1 expression was detected by RT-PCR. ***B***-***C***, After LV HAND2-AS1 transfection, the cell viability was detected by MTT assay. ***D***-***G***, The mRNA levels of Bcl-2, Caspase-3, MMP-2 and MMP-9 were detected by RT-PCR. ***E***, ***F***, ***H***-***J***, The protein levels of Bcl-2, Caspase-3, MMP-2, MMP-9 and β-actin were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, *$p<0.05$, **$p<0.01$.

lecular biological mechanisms between HAND2-AS1 and CML were unclear. It was reported that miR-1275 promoted cell proliferation, invasion and metastasis in these solid tumors and AML [20-25]. And our previous findings showed that it was upregulated in CML patients; however, the roles and functions of miR-1275 in the progression of CML were still unknown. We thus explored the roles and mechanisms of HAND2-AS1 and miR-1275 in CML. In this study, 30 bone marrow samples from CML patients and 10 from healthy donors were extracted and expressions of HAND2-AS1 and miR-1275 were detected by RT-PCR. Results showed that HAND2-AS1 was downregulated while miR-1275 was upregulated in CML patients, especially on stages of AP and BP. And HAND2-AS1 expression was negatively correlated with miR-1275 levels in CML but not in healthy control. These results indicated that the downregulation of HAND2-AS1 and upregulation of miR-1275 might play some roles in CML, especially on stages of AP and BP. Therefore, their functions should be further investigated. To explore the functions of HAND2-AS1 in CML, lentiviral HAND2-AS1 was produced and transfected into K562 and KCL22 cells, two CML cells. After LV

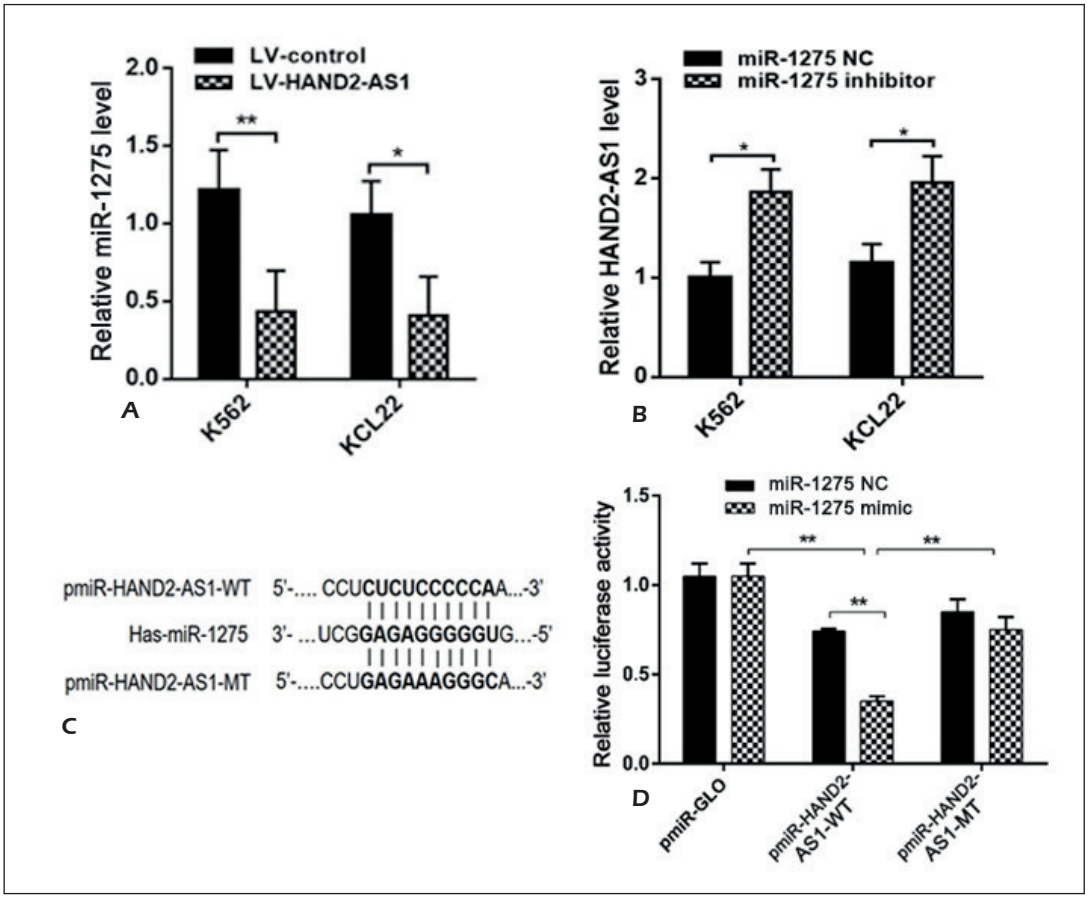

Figure 4. HAND2-AS1 interacted with miR-1275 in CML cells. **A**, miR-1275 expression was detected by RT-PCR after LV HAND2-AS1 transfection. **B**, HAND2-AS1 expression was detected by RT-PCR after miR-1275 inhibitor transfection. **C**, The potential binding site was predicted by using bioinformatics method. **D**, Relative luciferase activity was detected by luciferase reporter assay. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01.

HAND2-AS1 transfection, the HAND2-AS1 expression was significantly increased and the cell proliferation was inhibited after 72 h and 96 h in both two cells. Furthermore, the mRNA and protein levels of Bcl-2, MMP-2 and MMP-9 were significantly down-regulated, while the expression of Caspase-3 was significantly up-regulated in both two cells. These results suggested that HAND2-AS1 played a role in inhibiting cell proliferation, invasion and promoting cell apoptosis in CML. Whether HAND2-AS1 directly interacted with miR-1275 was unknown in CML cells. To explore the relationship between HAND2-AS1 and miR-1275, LV HAND2-AS1 and miR-1275 inhibitor were transfected into CML cells, respectively. Results showed that the upregulation of HAND2-AS1 repressed miR-1275 expression and the inhibition of miR-1275 reversely increased HAND2-AS1 expression, indicating that HAND2-AS1 might interact with miR-1275. To explore whether HAND2-AS1 directly interacted with miR-1275, the potential binding site was predicted by using bioinformatics method, and luciferase reporter assay was performed. Results showed that the luciferase activity of cells transfected with pmiR-HAND2-AS1-WT was significantly decreased, compared to that in cells transfected with miR-1275 mimics and pmiR-GLO, and the luciferase activity in pmiR-HAND2-AS1-MT was reversely increased. These results indicated that HAND2-AS1 directly interacted with miR-1275, which acted as an endogenous "sponge" that regulated its target genes by competing with miR-1275 and inhibited their functions. However, whether miR-1275 played some roles and functions in CML was unknown. To explore the roles of miR-1275 in CML cell lines, miR-1275 inhibitor was transfected into KCL22 and K562 cells. MTT assay results indicated that the cell proliferation was inhibited after treating with miR-1275 inhibitor.

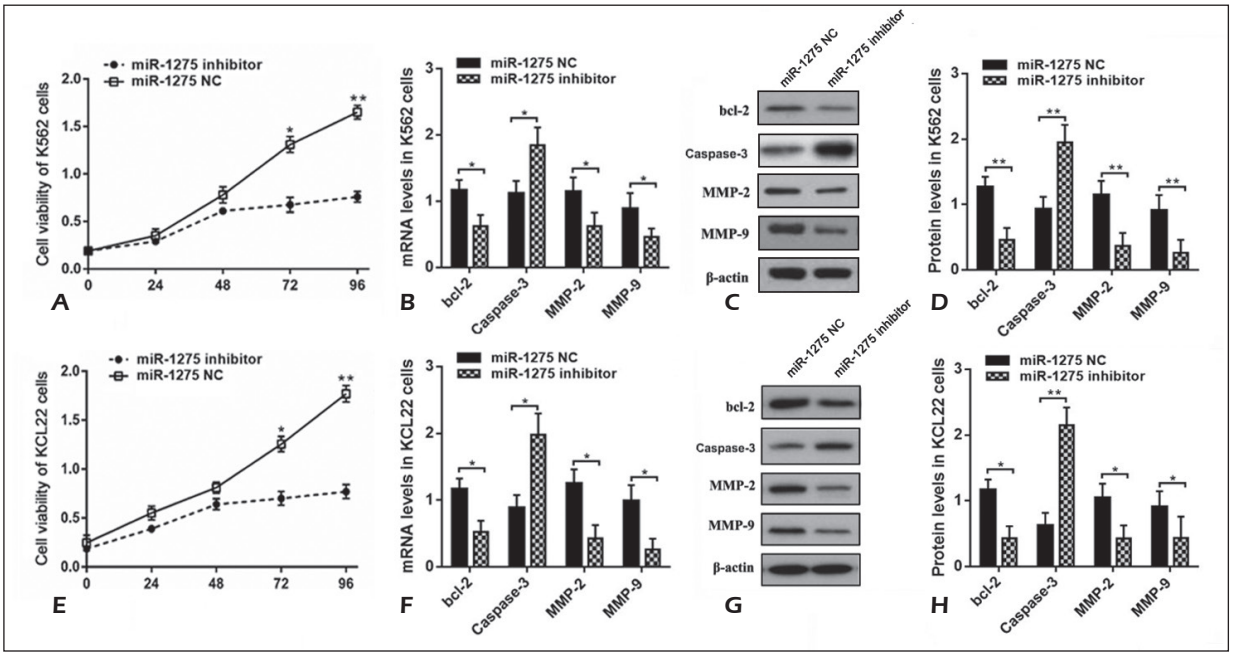

Figure 5. Inhibition of miR-1275 inhibited cell proliferation, invasion and promoted cell apoptosis in CML cells. **A**-**E**, After miR-1275 inhibitor transfection into K562 and KCL22 cells, the cell viability was detected by MTT assay. **B**-**F**, The mRNA levels of Bcl-2, Caspase-3, MMP-2 and MMP-9 were detected by RT-PCR. **C**, **D**, **G**, **H**, The protein levels of Bcl-2, Caspase-3, MMP-2, MMP-9 and β-actin were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01.

To further confirm the role of miR-1275 in cell proliferation and apoptosis, MMP-2, Caspase-3, bcl-2 and MMP-9 were detected after transfection with miR-1275 inhibitor. Results showed the expressions of bcl-2, MMP-2 and MMP-9 were decreased while Caspase-3 levels were increased. These results indicated that the inhibition of miR-1275 inhibited cell proliferation, invasion and promoted cell apoptosis in CML cells, which were consistent with the results of HAND2-AS1.

Conclusions

We detected that HAND2-AS1 was downregulated and miR-1275 was upregulated in CML; HAND2-AS1 inhibited proliferation and promoted apoptosis of CML cells by sponging with micRNA-1275, which might be a novel therapeutic target for CML on the stages of AP and BP.

Acknowledgments

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Conflict of Interests

The authors declare that they have no conflict of interest.

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