

Original Article

Overexpression of LncRNA-HOTAIR promotes chemoresistance in acute leukemia cells

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Abstract: Chemotherapy treatment of acute myeloid leukemia (AML) can be compromised due to the multidrug resistance (MDR) of leukemia cells. HOTAIR, a long noncoding RNA (LncRNA), is involved in MDR development of various solid tumors. However, whether it functions in MDR development of leukemia remains unclear. In this study, expressions of HOTAIR in leukemia cell line K562/A02 and bone marrow samples from 10 patients with refractory and relapsed AML were detected by qRT-PCR. The apoptosis, proliferation, and susceptibility of K562/A02 cells to Adriamycin (ADR) were analyzed by flow cytometry and CCK8 assay, respectively. The expression of cell cycle regulator P21 and Notch1 in the K562/A02 cells was examined by qRT-PCR. The accumulation of total AKT and the phosphorylated AKT (pAKT^{S473}) were detected by western blotting. We found that the expression of HOTAIR in drug-resistant cells and patient samples was increased. Inhibition of HOTAIR expression could suppress the proliferation, increase the apoptosis, and promote the doxorubicin sensitivity of K562/A02 cells. Moreover, inhibiting expression of HOTAIR could attenuate the expression of P21 and Notch1 and inhibit the phosphorylation of AKT in drug-resistant cells. In conclusion, our results demonstrated that LncRNA-HOTAIR is involved in MDR development of leukemia cells by regulating the expression of P21 and the AKT/Notch1 signaling pathway.

Keywords: Acute myeloid leukemia, LncRNA-HOTAIR, chemoresistance mechanism

Introduction

Acute myeloid leukemia (AML) is a hematopoietic disease, characterized by accumulation of immature hematopoietic cells in bone marrow and insufficient production of normal blood cells [1]. Currently, anthracycline-based combined chemotherapy is still the main choice of acute myeloid leukemia (AML). 60-80% of patients with AML achieve hematologic remission through chemotherapy, and some patients at low and moderate risk can obtain long-term survival [2]. Multidrug resistance (MDR) of leukemia cells is an important cause of chemotherapy failure and death during the treatment of leukemia [3, 4]. Therefore, it is of great value to investigate the mechanism of drug resistance of AML cells and find the biologic markers of multidrug resistance of leukemia cells in order to improve the therapeutic effect of AML.

Long non-coding RNA (LncRNA) is a type of non-coding RNA. Numerous studies have shown

that LncRNA plays an important role in gene expression, such as RNA processing, transcription, post-transcriptional modification, regulation of protein activity, and mediation of information transmission. HOX transcript antisense RNA (HOTAIR) is the earliest found and most thoroughly studied LncRNA, with a length of 2158 kb, located on the antisense chain of HOXC loci [5]. Many studies have shown that HOTAIR regulates the development of chemoresistance in various solid tumors such as colorectal cancer, non-small cell lung cancer (NSCLC), and gastric cancer by a variety of pathways [6-8]. However, whether HOTAIR is involved in the development of chemoresistance in AML has not been reported.

In this study, the role of HOTAIR in the development of drug resistance in AML was investigated using the drug-resistant leukemia cell line K562/A02 and validated in clinical samples to explore the correlation between HOTAIR ex-

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pression in AML cells and clinical efficacy, and find targets for overcoming drug resistance in AML.

Materials and methods

Cell line and cell culture

Human myeloid leukemia cell line K562 and its corresponding drug-resistant cell line K562/A02 were purchased from Tianjin Institute of Hematology (Tianjin, China). Adriamycin (ADR) was added to K562/A02 cell culture medium at a final concentration of 1 µg/mL to maintain cell resistance. The culture medium without ADR was replaced two weeks before each experiment. The two cell lines were cultured in RPMI-1640 complete medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum and inoculated into a 10 cm culture dish. The cells were cultured in an incubator at 37°C, 5% CO₂ and saturated humidity. All cells used in the experiment were taken from logarithmic growth phase.

Collection of bone marrow samples from patients with AML

Bone marrow samples were collected from patients with AML in the Department of Hematology, Zhongshan Affiliated Hospital of Xiamen University from June 2016 to June 2018. The diagnostic criteria were in line with the consensus diagnosis and treatment standards of adult acute myeloid leukemia in China. Samples from a total of 10 relapsed and refractory patients, 11 newly treated patients and 13 patients with iron deficiency anemia as control were collected. 3-5 mL of bone marrow samples were collected from patients before chemotherapy in an EDTA anticoagulant tube. Leukemia cells were extracted from bone marrow samples using human lymphocyte separation medium (Human) (Solarbio Life Sciences, China) and red blood cells were lysed with human erythrocyte lysate (China). The cell samples were washed with PBS 2-3 times after lysis and then stored at -80°C.

RNA interference and transfection

RFP fluorescent labeled lentiviral specific small interfering RNA (siRNA) targeting HOTAIR mRNA (si-HOTAIR, 5'-GCAGCACAGAGCAACTCT-ATA-3') and negative control (siR-NC) were synthesized by GenaPharma (Shanghai, China),

and transfected using Polybrene (GenaPharma Shanghai, China) according to the manufacturer's protocol. A total of 2×10⁵ K562/A02 cells were seeded into 6-well plates and divided into two groups as follows: cells transfected with the negative control (si-NC group); cells transfected with si-HOTAIR (si-HOTAIR group); After 48 h, the efficacy of transfection was validated by fluorescence microscope and efficacy of gene silencing was validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Real-time fluorescence quantitative PCR

RNA was extracted from cultured cells and bone marrow sample cells by Trizol (TransGen Biotech, China) lysis. All operations were carried out according to the instructions. cDNA was synthesized using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR reverse transcription kit (TransGen Biotech, China). TransScript® II Green Two-Step, qRT-PCR SuperMix kit (TransGen Biotech, China) was adopted for the qRT-PCR system. The sequences of primers used were as follows: HOTAIR, 5'-GGAAGCGAAGGGGTTGTGTA-3' (forward) and 5'-GGCTAGGGCTGGTTTCAC-3' (reverse); GAS5, 5'-CGACTCCTGTGAGGT-ATGGTG-3' (forward) and 5'-ATCCTTCCTGG-GGACACAAC-3' (reverse); H19, 5'-CAAAGCCT-CCACGACTCTGT-3' (forward) and 5'-ACTCAG-CACACTCGTACTG-3' (reverse); PVT1, 5'-GCCA-TAGATCCTGCCCTGTT3' (forward) and 5'-TCTT-GGTGGGGCTTGTGAAT-3' (reverse); β-actin, 5'-AGCGAGCATCCCCAAAGTT-3' (forward) and 5'-GGGCACGAAGGCTCATCATT-3' (reverse); GAPDH, 5'-CAGGAGGCATTGCTGATGAT-3' (forward) and 5'-GAAGGCTGGGGCTCATT-3' (reverse). The amplification reaction and fluorescence signal acquisition were achieved by ABI Prism 7500 PCR (USA), and the C_T values of each target gene were obtained by SDSv1.4.0 software. The relative expression of the target gene was calculated by 2^{-ΔΔC_T} formula, and GAPDH was used as internal reference.

Western blotting

Cell proteins were extracted by RIPA lysate (Thermo Fisher Science, USA) and added with broad-spectrum protease inhibitor or phosphatase inhibitor (Thermo Fisher Science, USA) according to different experiments. BCA protein detection kit (Thermo Fisher Science, USA) was used for quantitative analysis. The loading

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quantity of protein samples was 15 µg. After electrophoresis, the sample protein was separated and transferred to PVDF membranes, and then blocked with 5% nonfat milk at room temperature for 1 h. Then the samples were incubated with primary antibodies overnight at 4°C: anti-Notch1 (1:1000, Abcam); anti-P21 (1:1000, Cell Signaling Technology); anti-AKT (1:1000, Cell Signaling Technology); anti-Phospho-AKT^{S473} (1:1000, Cell Signaling Technology); anti-β-actinin (1:5000, Applygen, China). Membranes were washed with TBST 2-3 times and then incubated with secondary antibody Horseradish peroxidase labeled goat anti-rabbit IgG (Multi Sciences, China) for 1 h at room temperature. The membranes were then washed with TBST 2-3 times and exposed in dark room using ECL kit (Sigma Aldrich, USA) according to the instructions.

Proliferation and cytotoxicity analysis by CCK8

Cell proliferation assays: cells in logarithmic growth phase were collected and inoculated into a 96-well plate with 3.5×10^3 cells/well. Culture medium was used as blank. According to the instructions, CCK8 solution was added at 0, 24, 48, 72 and 96 h, respectively. The absorbance at 450 nm was determined by enzyme labeling after incubation for 2 to 4 h in an incubator.

Cytotoxicity test: cells in the logarithmic growth phase were collected and inoculated into a 96-well culture plate with 3.5×10^3 cells/well. ADR at different concentrations was added to K562 cells (0.02, 0.04, 0.08, 0.16, 0.32, 0.64 µg/mL) and K562/A02 cells (2, 4, 8, 16, 32, 64 µg/mL). The untreated cells were set as the control group, and the medium was set as the blank group. After 48 h of incubation, the absorbance value was determined at 450 nm according to the instructions of CCK8 kit. Cell growth inhibition rate was calculated as follows: $(\text{control group absorbance} - \text{experimental group absorbance}) / (\text{control group absorbance} - \text{blank group absorbance}) \times 100\%$. The median inhibitory concentration (IC_{50}) of adriamycin was calculated by SPSS software.

Statistical analysis

Excel, SPSS 17.0 software and Graphpad Prism 5.0 were used for statistical analysis. t-test was used for comparison of measurement data between the two groups. One-way

ANOVA was used for comparison of measurement data among multiple groups. $P < 0.05$ was considered significant.

Results

High expression of HOTAIR in drug-resistant cell line K562/A02

First, CCK8 kit was used to detect the toxicity of ADR to drug-resistant cell line K562/A02 and sensitive cell line K562. The results showed that the IC_{50} of K562/A02 was 170 times higher than that of K562 (0.4 ± 0.08 µg/mL), which confirmed the quality of K562 and K562/A02 cell lines. Next, based on related literature reports, the expressions of four lncRNAs including HOTAIR, H19, GAS5 and PVT1 were detected by qRT-PCR. It was found that the expression of HOTAIR and H19 in K562/A02 cells was significantly higher than that in K562 cells (**Figure 1A, 1B**), while the expression of GAS5 was lower than that in K562 cells ($P = 0.0223$) (**Figure 1C**), and the expression of PVT1 showed no significant difference between the two cell lines ($P = 0.3255$) (**Figure 1D**). The results suggested that HOTAIR and H19 might be associated with the development of drug resistance in leukemic cells.

Targeted inhibition of HOTAIR expression suppressed cell proliferation and increased drug sensitivity of K562/A02 cells

In order to find out whether HOTAIR is essential for AML cell survival, we established HOTAIR knockdown cell lines by transfecting a specific siRNA targeting HOTAIR (siHOTAIR) into K562/A02 cells. The results of qRT-PCR showed that the expression of HOTAIR in si-HOTAIR K562/A02 group decreased by about 72% with a significant difference (**Figure 2A, 2B**). Results of CCK8 assays showed that targeted inhibition of HOTAIR expressions significantly decreased the proliferation rate of cells in the si-HOTAIR group and increased the sensitivity to ADR with the IC_{50} of 45 ± 1.9 µg/mL for the si-HOTAIR group and 65 ± 2.3 µg/mL for the si-NC group (**Figure 2C, 2D**).

Targeted inhibition of HOTAIR promoted P21 gene expression and inhibited AKT phosphorylation in K562/A02 cells

To explore the mechanism underlying the involvement of HOTAIR in the proliferation of

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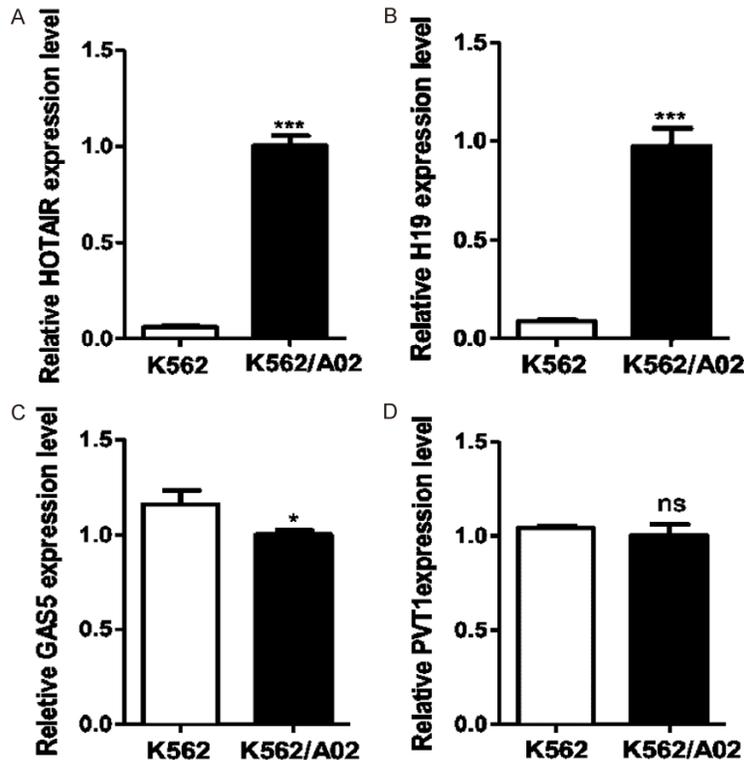


Figure 1. Expressions of four chemoresistance-related LncRNAs in K562/A02 and K562 cell lines were examined. The expressions of HOTAIR and H19 in drug-resistant cell line K562/A02 were significantly higher than those in sensitive cell line K562 (A, B). The expression of GAS5 was lower than that in K562/A02 cells ($P = 0.0223$) (C), and the expression of PVT1 was not significantly different between the two cell lines ($P = 0.3255$) (D).

ADR-resistant K562/A02 cells, we then examined the expression of cell cycle-regulation gene P21 and AKT phosphorylation. The qRT-PCR showed that the expression of P21 gene in K562/A02 cell line increased after targeted inhibition of HOTAIR (Figure 3A, 3B), suggesting that HOTAIR could inhibit apoptosis of K562/A02 cells by inhibiting the expression of P21. At the same time, western blotting showed that targeted inhibition of HOTAIR also decreased the phosphorylation of AKT (Figure 3C). These results indicated that HOTAIR over-expression could influence the expression of cell cycle regulatory genes and the apoptosis of leukemia cells.

Targeted inhibition of HOTAIR inhibited Notch1 gene expression in K562/A02 cells

The expression of Notch1 at mRNA and protein levels in K562/A02 cells was detected by qRT-PCR and western blot, respectively. The results showed that the expression of Notch1

gene and protein in K562/A02 cells decreased after targeted inhibition of HOTAIR ($P = 0.0124$) (Figure 4B), suggesting that HOTAIR may promote the resistance of K562/A02 cells by promoting Notch1 expression.

HOTAIR is highly expressed in refractory-relapsed AML cells

To further verify the association between HOTAIR and the chemoresistance of AML cells, the HOTAIR expression was measured in bone marrow samples of leukemia patients. Results showed that the expression of HOTAIR in bone marrow leukemia cells of patients with newly diagnosed (ND) and refractory-relapsed (RR) AML was significantly higher than that of patients with iron deficiency anemia. In addition, the expression of HOTAIR in leukemia cells of patients with refractory AML was significantly higher than that of patients with newly-diagnosed leukemia, and the

difference was statistically significant (Figure 5). These results indicated that an elevated HOTAIR expression in acute myeloid leukemia cells may be a biologic marker of chemoresistance, which might be used for diagnosis and evaluation of therapeutic efficacy in patients with acute myeloid leukemia.

Discussion

Acute myeloid leukemia (AML) is the most common malignant myeloid disorder of adulthood, and one of the major challenges in AML is high relapse rate after chemotherapy [1]. LncRNA has attracted much attention due to its wide participation in all stages of tumorigenesis and development and has become a new research hotspot of tumor regulatory factors after microRNAs. High expression of HOTAIR, a typical trans-acting LncRNA, is related to the development of drug resistance in various carcinomas. Targeted inhibition of LncRNA HOTAIR expressions sensitized various cancer cells to chemotherapeutic drugs, including gastric cancer [9],

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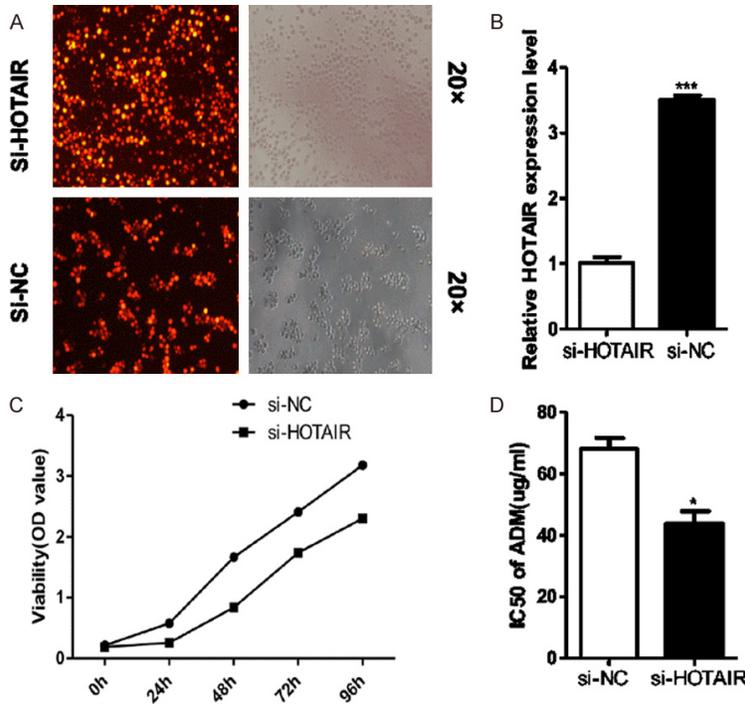


Figure 2. (A) Inhibition of HOTAIR expression reduced drug resistance in K562/A02 cells. (B) After HOTAIR was knocked down by gene transfection, the HOTAIR expression in si-HOTAIR K562/A02 cells was significantly lower than that in si-NC group. The cell proliferation was inhibited (C); and sensitivity to ADM increased (D) in si-HOTAIR K562/A02 cells compared with si-NC cells.

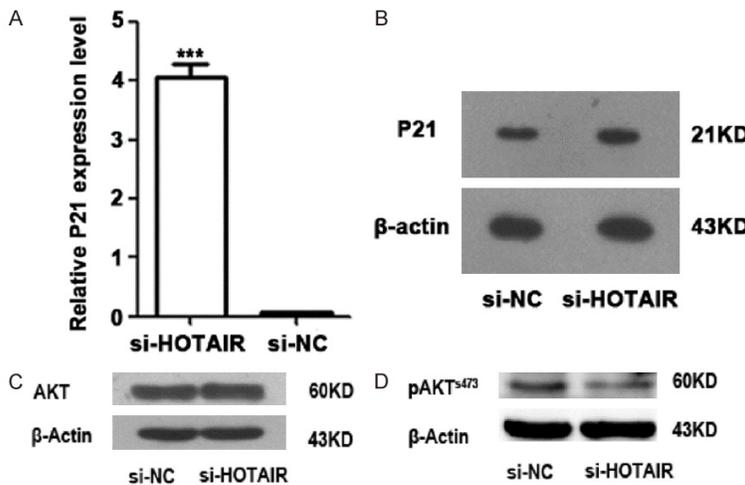


Figure 3. Targeted inhibition of HOTAIR expression affected the expression of cell cycle gene P21 at mRNA level (A) and protein level (B), as well as AKT phosphorylation (C, D) in K562/A02 cells.

breast cancer [10], small-cell lung cancer [11], non-small cell lung cancer [12], colorectal cancer [13] and ovarian cancer [14]. In the context of AML, it was reported that overexpression of HOTAIR predicted a poor prognosis [15]. In this

study, we first confirmed that the HOTAIR expression in the drug-resistant AML cell line was significantly higher than that in the non-resistant one, and this abnormal expression was further confirmed in the clinical refractory-relapsed AML samples, suggesting that HOTAIR overexpression may be a marker of chemoresistance in leukemia cells and involved in the development of multidrug resistance in leukemia cells. It was then found that after targeted inhibition of HOTAIR, the proliferation of K562/A02 cells was inhibited, the proportion of apoptotic cells was increased, and sensitivity to ADM was enhanced. Similar results were reported in the published literature. An *in vitro* study of leukemia cells confirmed that HOTAIR could compete with microRNA-193a in c-KIT as a competitive endogenous RNA (ceRNA), thereby reducing the inhibitory effect of microRNA-193a on c-KIT, and thus protecting leukemia cells from apoptosis [16]. Our results suggested that the high expression level of HOTAIR in leukemia cells may be one of the reasons for the continuous proliferation and development of drug resistance of leukemia cells.

Notch signaling is a signaling pathway closely related to the occurrence and development of malignant tumors and their response to chemotherapy. It was found that more than 50% of patients with T-cell acute lymphoblastic leukemia (T-ALL) harbored Notch 1 gene abnormalities [17]. Continuous activation of Notch signaling pathway as an oncogene participated in the pathogenesis of T-ALL [18, 19]. Hang *et al.* found that Notch 1 could promote the drug resistance development of gastric cancer cells

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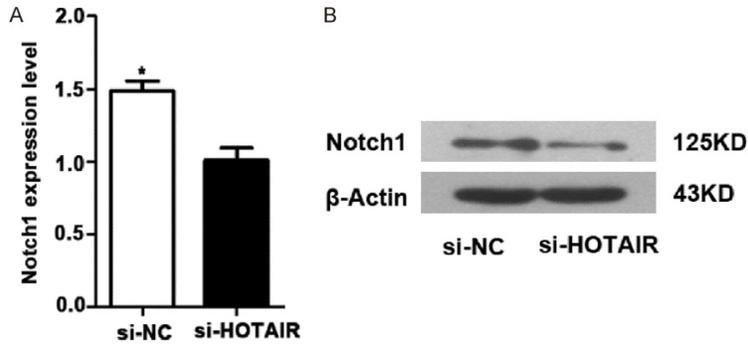


Figure 4. Targeted inhibition of HOTAIR expression inhibited Notch1 expression in K562/A02 cells at mRNA level (A) and protein level (B).

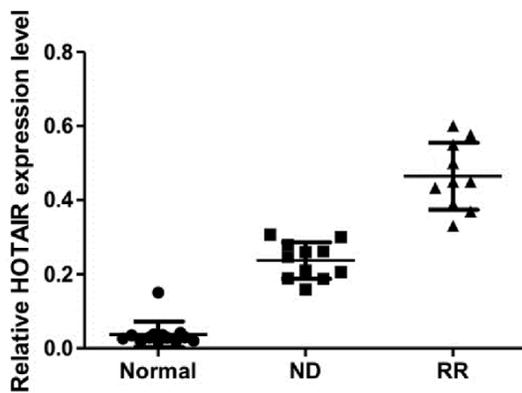


Figure 5. The expression of HOTAIR in leukemia cells of refractory-relapsed leukemia (RR) patients was significantly higher than that of patients with iron deficiency anemia (Normal) and newly diagnosed leukemia (ND). Expression of HOTAIR in RR patients was also significantly higher than that of ND patients.

by up-regulating the expression of LncRNA AK-022798 [20]. Meanwhile, Kamga *et al.* found that Notch signaling played an important role in AML cell survival mediated by bone marrow stromal cells [21]. In our study, it was found that the expression of Notch1 gene was decreased after targeted inhibition of HOTAIR expressions in drug-resistant leukemia cells. These results suggest that the abnormal expression of Notch1 might be involved in the development of chemoresistance induced by high expression of HOTAIR in leukemia cells, which provides a basis for studying the mechanism of chemoresistance of leukemia cells.

In addition, P21 gene is an important member of the family of cyclin-dependent kinase inhibitors (CDKI), which is located in the downstream of p53 gene and plays an important role in the regulation of drug resistance of can-

cer cells along with p53. A study on drug resistance of cervical cancer found that overexpression of HOTAIR inhibited the expression of P21 gene and increased the resistance of cervical cancer cells to radiotherapy, indicating that targeting HOTAIR can be used as therapy for cervical cancer [22]. A study on drug resistance of colon cancer confirmed that microRNA-520g participated in the development of drug resistance by regulating

the expression of P21 [23]. In this study, we confirmed that targeted inhibition of HOTAIR expression promoted the expression of P21 gene in leukemia drug-resistant cells, thereby affecting the proliferation and apoptosis of drug-resistant cells, and sensitized K562/A02 cells to ADR. Therefore, the P21/AKT pathway plays an important role in HOTAIR-mediated drug resistance.

Conclusions

Through *in vitro* cytological studies, we found that HOTAIR overexpression could affect the molecular pathway of sensitivity of leukemia cells to ADR, possibly by regulating the Notch signaling and P21 gene. Therefore, it can be speculated that the overexpression of HOTAIR is a molecular marker of chemoresistance in leukemia cells, and downregulation of HOTAIR expression may be a way to overcome chemoresistance in leukemia cells. Nevertheless, more research is needed to further elucidate the complex regulatory mechanism of HOTAIR in the chemoresistance of AML.

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Disclosure of conflict of interest

None.

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