Increased lncRNA HOTAIR expression promotes the chemoresistance of multiple myeloma to dexamethasone by regulating cell viability and apoptosis by mediating the JAK2/STAT3 signaling pathway

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Abstract. Multiple studies have shown that HOX antisense intergenic RNA (HOTAIR), an oncogenic long non-coding RNA (lncRNA), is dysregulated in leukemia and is involved in tumor progression. The aim of the present study was to determine whether HOTAIR could serve as a novel biomarker for the diagnosis of multiple myeloma (MM), and to investigate its role in regards to MM cell viability and chemoresistance to dexamethasone (DEX). The results revealed that the expression of HOTAIR was significantly upregulated in serum, bone marrow and primary CD138+ cells from MM patients compared with those from normal controls as determined by qPCR. HOTAIR expression was obviously increased in MM cell lines compared to that in normal plasma cells. ROC curve analysis showed that the serum level of HOTAIR exhibited a higher diagnostic value for MM. Furthermore, loss-of-functional assays indicated that HOTAIR inhibition suppressed MM cell viability by arresting the cell cycle at the G0/G1 phase as determined by cell viability assay and flow cytometry. An in-depth study revealed that knockdown of HOTAIR led to decreased chemoresistance of DEX in MM cells by regulating the JAK2/STAT3 signaling pathway. Taken together, our findings suggest that upregulation of serum HOTAIR may prove to be a novel biomarker for the diagnosis of MM. HOTAIR promoted MM cell viability and increased chemoresistance of MM to DEX via the JAK2/STAT3 signaling pathway, indicating HOTAIR may also serve as a potential therapeutic target for MM.

Introduction

Long non-coding RNAs (lncRNAs) are a newly reported type of non-coding RNAs, which are greater than 200 nucleotides in length and are unable to encode proteins. lncRNAs are involved in cellular development, genomic imprinting and homeostasis, and numerous studies suggest that dysregulated expression of lncRNAs occurs in various diseases and contributes to tumor biology, including multiple myeloma (MM). For example, Wang et al (3) showed that lncRNA PRAL plays a critical role in the progression of MM, and PRAL could become a novel diagnostic, prognostic and therapeutic candidate for MM patients. Moreover, Zhang et al (4) reported that lncRNA UCA1 promotes MM cell growth by targeting TGF-β, and overexpression of TGF-β partially reverses the effects of UCA1 knockdown. Chen et al (5), identified that lncRNA CCAT1 exerts an oncogenic role in MM by promoting tumor progression, and CCAT1 could serve as a novel diagnostic marker and therapeutic target for MM. However, the associations between others lncRNAs and MM remain unclear, and related reports are lacking.

MM is a type of malignant plasma-cell disorder, which originates from plasma cells of the bone marrow leading to the overproduction of immunoglobulins. MM is the second most common hematological malignant tumor, with approximately 80,000 newly diagnosed cases annually worldwide. Despite the significant progress in elucidation of the molecular biology of MM and therapeutic options over the past few decades, MM remains incurable, and its 5-year survival rate is approximately 40% (8,9). Currently, the etiologies and pathogenesis of MM remain poorly understood, and therapies are usually complicated and have poor efficacy. Dexamethasone (DEX) is a key front-line chemotherapeutic agent used for B-cell malignant MM, yet MM cells often become DEX-resistant (10). Therefore, it is urgent to explore the novel functional mechanism of MM and identify new biomarkers to provide effective therapeutic targets for MM.
In 2007, Rinn et al (11) first identified an lncRNA that regulated gene expression in the trans rather than cis at the HOXC gene locus on chromosome 12, and named the lncRNA as HOX antisense intergenic RNA (HOTAIR). Gupta et al (12), subsequently found that HOTAIR expression is higher in breast cancer, and is associated with tumor metastasis and poor prognosis. Further research showed that HOTAIR is upregulated and plays a significant role in lung cancer, colorectal cancer, hepatocellular carcinoma, nasopharyngeal carcinoma and pancreatic cancer (13). More recently, HOTAIR has been shown to play an oncogenic role in acute myeloid leukemia due to its ability to mediate the migration and invasion of leukemia cells (14). Although HOTAIR plays key roles in different solid cancers and acute myeloid leukemia, little is known concerning its specific function and molecular mechanism in MM.

In the present study, the expression and function of HOTAIR were investigated in MM. Our data showed that the expression levels of HOTAIR were significantly upregulated in serum, bone marrow and primary CD138+ cells from MM patients compared with those from normal controls, and increased serum HOTAIR may be used as an ideal biomarker for MM detection. Functional assays showed that knockdown of HOTAIR suppressed MM cell viability and arrested the cell cycle in the G0/G1 phase. Furthermore, it was found that HOTAIR increased the chemoresistance of MM to DEX by regulating the JAK2/STAT3 signaling pathway. These data may provide novel theoretical bases for the application of lncRNA HOTAIR in the diagnosis and therapy of MM.

Materials and methods

**MM samples.** Peripheral blood and bone marrow samples were collected from 118 MM patients with a mean age of 54.18 years (51 males and 67 females) who were hospitalized at the Department of Hematology, Daqing Oilfield General Hospital from August 2009 to April 2015. Seventy-eight healthy volunteers with a mean age of 56.05 years (36 males and 42 females) were collected as normal controls. All patients were free from other coexisting malignant diseases. Patients with extramedullary myeloma were not enrolled in the present study. No local or systemic treatment had been conducted before operation. For the use of clinical samples for research, the study was approved by the Ethics Committee of Daqing Oilfield General Hospital and performed in accordance with the Declaration of Helsinki (2000). Written informed consent was obtained from all participants. Samples were freshly frozen in liquid nitrogen, and then were stored at -80°C until RNA extraction.

**Cell lines and cell culture.** MM cell lines (MM.1S, MM.1R, JIN-3 and U266) and normal plasma cells (nPCs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were routinely cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% (vol/vol) FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin in a 5% CO₂ atmosphere at 37°C. Primary CD138+ cells were isolated and purified from bone marrow of MM patients and healthy volunteers by using CD138-coated magnetic beads (Miltenyi Biotec, Paris, France). Primary CD138+ cells were freshly frozen in liquid nitrogen, and then were stored at -80°C for further use.

**Quantitative PCR (qPCR).** Total RNA was extracted from cells or samples using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A TaqMan® MicroRNA Reverse Transcription kit (cat. no. 4366597, Thermo Fisher Scientific, Inc.) was used to reversely transcribe RNA into cDNA. qPCR was performed with a Mir-X™ miRNA qRT-PCR TB Green™ kit (cat. no. 638314; Takara, Beijing, China) on the Applied Biosystems 7500 Sequence Detection system (Thermo Fisher Scientific, Inc.) in triplicate with the following cycling conditions: 95°C for 10 min (initial denaturation), and then 40 cycles of 95°C for 10 sec, 60°C for 30 sec. The specific primers for HOTAIR, GAPDH, JAK2 and STAT3 were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). GAPDH was used as an internal control. The primer sequences were as follows: HOTAIR forward, 5′-CCCTCAGGTCCCTAATA-3′ and reverse, 5′-CCGGCGG TCTGTAACCT-3′; GAPDH forward, 5′-CCACTCTCCAC CTTTG-3′ and reverse, 5′-ACACCCCTGTTCGTG-3′; JAK2 forward, 5′-ACCTGTGAACTGCCC-3′ and reverse, 5′-CCA ATCACCCGATAAA-3′; STAT3 forward, 5′-AGACCCACT CTTGCC-3′ and reverse, 5′-GCTCAGTCTCGTTT-3′. Results were normalized to the expression of GAPDH, and the 2ΔΔct method (15) was used for analysis of the quantitative changes in expression of each gene.

**Plasmid construction and cell transfection.** Lentivirus for short hairpin RNA (shRNA) directed against HOTAIR (Lv-HOTAIR) and control lentiviral vector (Lv-control) were chemically synthesized from GenePharma Co., Ltd. (Shanghai, China). The sequences of the shRNAs were as follows: 5′-GAA CGGGAGTACAGAGAGA-3′ (Lv-HOTAIR) and 5′-GACTGA TTCGATACGCTATGT-3′ (Lv-control). Cells were plated in 6-well plates and transfected with Lv-HOTAIR or Lv-control using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Twenty-four h post-transfection, the transfected cells were selected with 800 mg/ml G418 for at least one month. After selection and isolation of stably transfected clones, the clones were analyzed by HOTAIR expression using qPCR assay.

**Flow cytometric assay.** For analysis of cell apoptosis, the stably transfected cells were co-cultured with 50 μM DEX (cat. no. D4902, Sigma-Aldrich; Merck KGaA) for 48 h. Then, the cells were harvested and resuspended in fixation fluid. Propidium iodide (PI) (2 μl) and Annexin V-FITC (5 μl) were added to the cell suspension for 30 min at 37°C. The cell suspension was analyzed using FACSCalibur flow cytometry (BD Biosciences, USA). The percentage of early apoptotic cells was counted and compared for the cells receiving different treatments. For analysis of cell cycle distribution, at 48 h post-transfection, the cells were harvested via trypsinization, washed with cold PBS and fixed in 70% ice cold ethanol overnight, followed by staining with 50 mg/ml PI for 30 min. Finally, the samples were detected with FACSCalibur flow cytometry.

**Cell viability assay.** Cell viability was measured using a Cell Counting Kit-8 (CCK-8 kit) (Dojindo, USA), according to the
HOTAIR is upregulated in MM samples and cell lines. To results of predicting MM patients and normal controls. P<0.05 was estimated by Student's t-test or one-way ANOVA followed by Student-Newman-Keuls test. Receiver operating characteristic (ROC) curves were plotted, and the area under the ROC curve was calculated to assess the specificity and sensitivity of predicting MM patients and normal controls. P<0.05 was considered to indicate a statistically significant difference.

Results

**HOTAIR is upregulated in MM samples and cell lines.** To explore whether HOTAIR is detectable and altered in MM, the expression of HOTAIR was examined by qPCR in our collection of peripheral blood from 118 MM patients and 78 healthy volunteers. As presented in Fig. 1A, elevated expression of HOTAIR was observed in the serum of MM patients compared with that noted in the normal controls (P<0.05). We then determined HOTAIR expression in bone marrow samples, and the results revealed that HOTAIR expression in MM patients was significantly higher than that in normal controls (P<0.05; Fig. 1B). To further test the hypothesis that serum HOTAIR was primarily released from tumor cells, the expression level of HOTAIR in primary CD138+ cells was also detected. The results showed that HOTAIR expression was significantly upregulated in the primary CD138+ cells from MM patients compared to those from the normal controls (P<0.05; Fig. 1C). Finally, HOTAIR expression was investigated in MM cell lines including MM.1S, MM.1R, JNJ-3 and U266, and normal plasma cells (nPCs). The results revealed that the HOTAIR level was considerably increased in the four MM cell lines compared to this level in the nPCs cells (P<0.05; Fig. 1D). According to the relatively high levels of HOTAIR in MM.1S, U266 and MM.1R cells, and the fact that these cell lines are suitable models for MM as described previously (16,17), three cell lines were selected for further study in vitro.

**HOTAIR exhibits a higher diagnostic value for MM.** To investigate the characteristic of HOTAIR as a potential diagnostic biomarker for MM patients, ROC curve was performed on data from clinical samples. As presented in Fig. 2, representation of the data showed the AUC of HOTAIR was 0.798 (95% CI, 0.732-0.865), and the sensitivity and specificity were 70.12 and 79.94%, respectively.

**Knockdown of HOTAIR suppresses MM cell viability by altering the cell cycle.** To further investigate the biological roles of HOTAIR in MM cells in vitro, stable inhibition of HOTAIR expression was established in MM.1S and U266 cell lines using Lv-HOTAIR. As confirmed by qPCR analysis, HOTAIR expression in MM.1S and U266 cells was significantly downregulated by Lv-HOTAIR compared with the expression in the Lv-control-treated and untransfected cells (P<0.01; Fig. 3A). These data indicated that cell transfection was successful, and Lv-HOTAIR is a suitable target to knock down HOTAIR in MM.1S and U266 cells. Then, a CCK-8 assay was performed to detect the role of HOTAIR in the cell viability of MM cells. The results showed that knockdown of HOTAIR inhibited cell viability of the MM.1S and U266 cell lines compared to the cell viability of the Lv-control groups (P<0.05; Fig. 3B and C). To determine whether the function of HOTAIR in regards to cell viability was caused by alteration of the cell cycle, flow cytometry was performed. Cell cycle analysis showed that knockdown of HOTAIR induced MM cell cycle arrest at the G0/G1 phase (P<0.05; Fig. 3D).

**Knockdown of HOTAIR decreases MM chemoresistance to DEX by regulating the JAK2/STAT3 signaling pathway.** To investigate whether HOTAIR plays a role in the DEX resistance of MM cells, CCK-8 assay and flow cytometric analysis were carried out to investigate the influence of HOTAIR on the viability and apoptosis of MM cell lines to 50 μM DEX. As shown in Fig. 4A and B, knockdown of HOTAIR significantly decreased the chemoresistance of MM.1R cells to 50 μM DEX by reducing cell viability at 24, 48, 72 and 96 h, and inducing cell apoptosis at 48 h (P<0.05). To confirm whether the effect of HOTAIR on DEX chemoresistance was mediated by the JAK2/STAT3 pathway, qPCR and western blot analysis were performed. The results showed that the mRNA levels of JAK2 and STAT3 in the MM.1R cells demonstrated no significant difference between the Lv-HOTAIR and Lv-control groups (Fig. 5A). Compared with the Lv-control groups, as determined by western blot analysis, expression levels of p-JAK2 and p-STAT3 levels were significantly downregulated in MM.1R cells by HOTAIR inhibition; in contrast, no obvious total protein expression changes in JAK2 and STAT3 were observed between cells receiving different treatments (P<0.05; Fig. 5B). Taken together, these data suggest that JAK2/STAT3 signaling mediated the effect of HOTAIR on MM chemoresistance to DEX.
Multiple myeloma (MM) is a hematological malignancy characterized by abnormal growth of monoclonal plasma cells in the bone marrow leading to various end-organ damage including hypercalcemia, anemia, osteolytic bone disease and renal insufficiency (18). During the past few years, great progress has been achieved in understanding the initiation and tumorigenesis of MM; however, the clinical prognosis of patients with MM still remains highly heterogeneous (8,9). Traditional laboratory biomarkers including \(\beta\)-2 microglobulin, IgG and serum albumin have been used as an objective staging system for MM (19,20). However, these biomarkers have demonstrated limited ability for diagnostic risk stratification. Due to the rapid development of basic medical research, an increasing number of studies have shown that abnormal expression of lncRNAs is detectable in the plasma of cancer patients and therefore may be utilized as a tool for cancer diagnosis (21,22). For example, Shen et al (23) reported that serum lncRNA PCAT-1 is expressed at a higher level in MM patients than that in healthy controls, and may serve
as a valuable biomarker in the auxiliary diagnosis of MM. However, the clinical significance and molecular mechanisms of specific lncRNAs warrant further investigation.

HOTAIR (HOX antisense intergenic RNA), as a spliced and polyadenylated RNA, has six exons with 2.2-kb nucleotides in length. Accumulating evidence suggests that HOTAIR...
plays a critical role in various areas of human cancer, such as growth, survival, migration, drug resistance and genomic stability (13). Nevertheless, few studies have evaluated the role of serum HOTAIR in the diagnostic value for cancer. A recent study showed that serum HOTAIR could serve as a promising biomarker for diagnosing and monitoring non-small cell lung cancer (24). Elsayed et al. (25) concluded that plasma HOTAIR may serve as a potential non-invasive biomarker for the diagnosis of gastric cancer. Tan et al. (26) demonstrated that serum HOTAIR could be used as a novel prognostic and diagnostic biomarker for glioblastoma multiforme. In addition, Wang et al. (27) reported that serum HOTAIR may serve as a potential biomarker for the diagnosis of esophageal squamous cell carcinoma. However, the diagnostic value and functional role of HOTAIR in MM are still largely unknown. To the best of our knowledge, this is the first study to demonstrate the clinical significance of serum HOTAIR as a diagnostic biomarker for MM. Our results showed a significant upregulation of serum HOTAIR in MM patients compared with that noted in normal controls, and its capability of discriminating between MM patients and healthy volunteers had 70.12% sensitivity and 79.94% specificity. Moreover, it was also determined that HOTAIR expression was increased in bone marrow and primary CD138+ cells from MM patients as well as in MM cell lines. Therefore, these data indicated that upregulation of serum HOTAIR may prove to be a novel biomarker for MM diagnosis.

To further investigate the biological role of HOTAIR in MM cells, CCK-8 assay and flow cytometric analysis were carried out to investigate the influences of HOTAIR on the viability and apoptosis of MM cell lines. Function assays showed that silencing of HOTAIR significantly suppressed MM cell viability by arresting the cell cycle in the G0/G1 phase. Dexmethasone (DEX) is one of the most widely used glucocorticoids, which was introduced as a treatment regimen for MM in the 1970s, and its efficacy was observed in up to 70% of MM patients in clinical trials (28). Recent studies have found that MM cells are resistant to DEX, and some therapeutic strategies for overcoming DEX resistance have been reported (10). For example, miR-221-222 plays a role in DEX-induced drug resistance in MM, and treatment with anti-miR-221-222 was found to partially restore the DEX sensitivity of MM.1R cells (29). NEAT1 inhibition was found to decrease DEX resistance in MM by targeting the miR-193a/Mcl1 pathway (16). Importantly, our results showed that knockdown of HOTAIR significantly decreased the chemoresistance of MM.1R cells to DEX by reducing cell viability and inducing cell apoptosis. These data indicate that inhibition of HOTAIR suppresses MM cell viability and decreases DEX resistance.

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor and is widely expressed in a broad range of cancers including MM, and plays an important role in regulating cellular activities (30). STAT3 was found to be highly expressed in more than 63% of CD138+ bone marrow cells of MM patients and is associated with chemoresistance to DEX therapy (31). Currently, the JAK2/STAT3 signaling pathway has been proposed as a promising target for MM therapy (32). To confirm whether the effect of HOTAIR on DEX chemoresistance was mediated by the JAK2/STAT3 pathway, qPCR and western blot analysis were conducted. The data showed that inhibition of HOTAIR significantly downregulated the expression levels of p-JAK2 and p-STAT3 in MM.1R cells, whereas no obvious changes in the total protein expression of JAK2 and STAT3 were observed after cells were transfected with Lv-HOTAIR. Values are expressed as the mean ± standard deviation of three independent experiments. DEX, dexmethasone; MM, multiple myeloma; lncRNA, long non-coding RNA; HOTAIR, HOX antisense intergenic RNA.
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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

WW designed the study. RG, BF and YP performed the experiments. YL and HL performed the statistical analyses. WW and RG prepared the manuscript. All authors reviewed and edited the final manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethics Board of Daqing Oilfield General Hospital and was performed in accordance with the Declaration of Helsinki (2000). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


