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Original Article

Long noncoding RNA HOTAIR promotes cisplatin resistance in gastric cancer through promoting WIF-1 promoter methylation

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Abstract: Background and objective: Increasing evidence has illustrated that long noncoding RNAs (lncRNAs) could function as oncogenes and tumor suppressors in human carcinogenesis. However, the roles of lncRNAs in chemoresistance of human gastric cancer (GC) remain largely undefined. The current study aimed to verify the correlation between lncRNA HOTAIR expression and cisplatin resistance of GC. Methods: Cisplatin-resistant BGC823/DDP cells were established from the parental GC BGC823 cells. The expression levels of HOTAIR and WIF-1 mRNA in cells were investigated via qRT-PCR, and WIF-1 protein expression was detected by western blot analysis. CCK-8 assay was performed to explore the cell viability of the BGC823 and BGC823/DDP GC cells to cisplatin. The apoptotic rates to cisplatin of the GC cell lines were investigated via a flow cytometer, and the wound healing assay was conducted to assess the cell mobility to cisplatin of the GC cells. Results: In BGC823/DDP cells, HOTAIR expression were greatly up-regulated, while the WIF-1 promoter methylation level was significant increased and the mRNA and protein expression levels of WIF-1 were obviously reduced (all $P<0.05$). Silence of HOTAIR significantly suppressed WIF-1 promoter methylation and up-regulated WIF-1 expressions both at mRNA and protein levels (all $P<0.05$). The IC_{50} value of cisplatin in BGC823/DDP cells, remarkably higher compared to that of BGC823 cells (all $P<0.05$), could be dramatically down-regulated by silence of HOTAIR (all $P<0.05$). The apoptotic rate to cisplatin of BGC823/DDP cells was significantly down-regulated, while the cell mobility to cisplatin of BGC823/DDP cells was remarkably suppressed than that of BGC823 cells (all $P<0.05$); down-regulation of HOTAIR obviously facilitated the apoptosis of BGC823/DDP cells and inhibited the cell mobility to cisplatin ($P<0.05$). Conclusions: Our results shed novel light on the roles of HOTAIR/WIF-1 signaling axis in cisplatin resistance, and HOTAIR might be considered as a potential therapeutic approach to reverse the cisplatin resistance in GC.

Keywords: Gastric cancer, cisplatin, chemoresistance, HOTAIR, WIF-1

Introduction

Gastric cancer (GC) is one of most common malignant neoplasms, which has approximately one million new cases diagnosed annually [1]. With the rapid development of medical science and technology, there are a series of treatment methods applied in the clinical, including surgery, radiotherapy and chemotherapy, biotherapy and immunotherapy. However, the morbidity and mortality of GC still remain high in the world, especially in China. Resistance of chemotherapeutic drugs, especially cisplatin (cis-diamminedichloroplatinum, DDP) resistance, is one of the major factors to limit the improvement of treatment effects and reduction of mortality rate in GC patients. To date, however, the underlying mechanisms of chemotherapeutic drugs resistance still remain largely unclarified. Therefore, it is quite crucial for us to explore and find a potential mechanism leading to chemotherapeutic drugs resistance and provide a novel and effective treatment strategy for GC patients in the near future.

Long noncoding RNA (lncRNA) is a group of RNAs, which are highly conserved in their secondary and tertiary structures and commonly longer than 200 nt [2]. In human, through regulating expression of target genes at transcriptional, post-transcriptional and epigenetic levels, lncRNAs were widely involved in a number

of physiological processes, including neural development [3], cell cycle regulation [4] and cell protection [5]. Recently, increasing investigations demonstrated that aberrant lncRNAs expression exerted an important effect on human carcinogenesis, including prostate cancer [6], lung cancer [7], breast cancer [8], bladder cancer [9] and GC [10].

Hox transcript antisense intergenic RNA (HOTAIR), a member of lncRNA family, is transcribed from the HOXC locus in chromosome 12 [11]. To date, in a variety of cancers, HOTAIR has been considered to be a poor prognostic factor. It was detected to be involved in the process of gastric tumorigenesis and metastasis. Teschendorff et al. [12] demonstrated that HOTAIR and its methylation status were significantly associated with resistance to carboplatin. However, little was known about the relationship between HOTAIR and cisplatin resistance in cancer patients, and its detailed mechanisms remained to be elucidated.

Wnt/β-catenin signaling pathway, highly conserved on evolution, plays a critical role in normal tissue homeostasis. Mounting studies showed that aberrant activation of Wnt signaling is associated with a number of diseases, including systemic sclerosis [13], kidney injury and repair [14], airway diseases [15], diabetes mellitus [16] and neoplasms [17, 18]. Wnt inhibitory factor-1 (WIF-1), initially detected in the human retina, is considered as an antagonist of Wnt signal pathway, which can inhibit Wnt canonal pathway and noncanonal pathway [19]. Recent articles revealed that WIF-1 is a tumor suppressor, which is down-regulated along with hypermethylation in a majority of cancers [20]. Moreover, Jiang et al. [21] indicated that inhibition of HOTAIR can increase the radiosensitivity of pancreatic ductal adenocarcinoma cells by up-regulating the expression of WIF-1. However, it is not clear whether WIF-1 could affect the chemosensitivity of chemotherapy drugs in GC.

In the present study, we aimed to explore the correlation between HOTAIR and cisplatin resistance and its underlying mechanism. In summary, our data verified that HOTAIR might contribute to cisplatin resistance via down-regulating WIF-1, which could provide novel therapeutic strategies for GC.

Materials and methods

Cell culture

Human GC cell line BGC823, purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), were grown in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 units/ml of penicillin and 100 mg/ml of streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2, and the culture medium was routinely changed every 3 days. To establish the cisplatin-resistant BGC823/DDP cells, the parental BGC823 cells were maintained to persistent gradient exposure to cisplatin (DDP) (Qilu Pharmaceutical Co., Ltd., Jinan, China) for 1 year, by rising cisplatin concentration from 0.05 μg/ml to 1 μg/ml. Prior to the further assays, the BGC823/DDP cells were cultured without DDP for 2 weeks.

SiRNA transfection

siRNAs were synthesized by Jima Biotech (Shanghai, China). BGC823/DDP cells were grown on six-well plates to confluency and transfected with 50 nM siRNA targeting HOTAIR (5'-CAUAUUAUAGAGUUGCUCUGUGCUG-3') or siRNA/WIF-1 (5'-GAGUACUCAUAGGAUUUGA dT-dT-3') or negative scrambled siRNA (5'-GAACGGAGCGAGCAGACCUUU-3') using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations; thus HOTAIR and WIF-1 mRNA expression was investigated by qRT-PCR 48 h after transfection.

RNA extraction and qRT-PCR

Total RNA was isolated from BGC823/DDP and parental BGC823 cells using Invitrogen TRIzol® reagent (Thermo Fisher Scientific). RNA was reverse transcribed to cDNA from 1.0 μg of total RNA through using a Reverse Transcription Kit (Takara, Shiga, Japan). qRT-PCR analysis was performed using the Power SYBR Green (Takara, Shiga, Japan) on an ABI 7500 thermocycler (Thermo Fisher Scientific). All protocols were carried out according to the instructions provided by the manufacturer. The primers for quantitative PCR in this study, synthetized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, were listed in **Table 1**. Glyceraldehyde-3-phosphate dehydro-

Table 1. The sequences of primers used for qRT-PCR in this study

Gene name	Primer sequences
HOTAIR	
Forward	5'-CAGTGGGGAACTCTGACTCG-3'
Reverse	5'-GTGCCTGGTGCTCTCTTACC-3'
WIF-1	
Forward	5'-TATGGATCGATGCTCACCAG-3'
Reverse	5'-CAGAGGGACATTGACGGTTG-3'
GAPDH	
Forward	5'-GTCAACGGATTTGGTCTGTATT-3'
Reverse	5'-AGTCTTCTGGGTGGCAGTGAT-3'

genase (GAPDH) was used as internal controls in this study. All expression levels were calculated through using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

BGC823/DDP and parental BGC823 cells were washed by phosphate-buffered saline twice, harvested and lysed in CelLytic buffer (Sigma-Aldrich) with protease inhibitors. Cell lysates containing 20 μg of protein were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to NC membrane (Amersham Bioscience, Buckinghamshire, UK), and probed with the antibodies of rabbit anti-human WIF-1 antibody (1:1000, Abcam, Cambridge, MA, USA) and rabbit anti-human GAPDH antibody (1:1000, Abcam). Primary antibodies were detected with the horseradish peroxidase-conjugated secondary antibodies of goat anti-rabbit (Cell Signaling Technology, Boston, USA). Protein bands were visualized through using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). GAPDH was applied as the internal control.

Methylation-specific PCR (MSP)

Genomic DNA obtained from BGC823/DDP and parental BGC823 cells was modified with bisulfite reagents using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, China). 2 μg of genomic DNA, diluted in C to T conversion reagent (bisulfite), was denatured and converted at 98°C for 10 minutes and 64°C for 2.5 hours. Bisulfite-treated DNA was then cleaned and desulphonated using a QIAquick Gel Extraction Kit (Qiagen NV, Venlo, the Netherlands). The PCR amplification of bisulfite modified DNA was performed using a pair of methylation specific primers (MSP, forward: 5'-GGGCGTTTTATTGGGCGTAT-3'; reverse: 5'-AAACCAACAATCAACGAAC-3') and unmethylation specific primers (UMSP, forward: 5'GGGTGTTTTATTGGGTGTAT-3'; reverse: 5'-AAACCAACAATCAACAAAAC-3') [22].

Cell viability assay

One day prior to treatment, BGC823/DDP and parental BGC823 cells was plated in 96-well plates at a density of 5000 cells per well. The cells were subjected to different concentrations of cisplatin (0, 0.05, 0.1, 0.5, 1, 5, 10 and 20 μg/ml). After 48 h, the cell viability was investigated through using Cell Counting Kit (CCK)-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Absorbance was detected at a wavelength of 450 nm and was expressed as the viability percentages of the cells in comparison to the controls. The cell variable curves were plotted and the IC_{50} values were assessed through non-linear regression analysis using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Resistance Index (RI) was calculated using the following equation: Resistance Index = IC_{50} value of resistant cells/ IC_{50} value of parental cells.

Cell apoptosis assay

BGC823/DDP and parental BGC823 cells for apoptotic analysis were double stained with Annexin V-FITC and propidium iodide (PI) 48 h after treatment with 0.5 μg/ml cisplatin and analyzed using a flow cytometer (FACScan; BD Biosciences, Shanghai, China) equipped with Cell Quest software (BD Biosciences). Cells were classified as viable, dead, early apoptotic, or apoptotic. The percentage of early apoptotic cells was counted and compared between cells receiving different treatment.

Cell mobility assay

Cell mobility was measured through a wound healing assay. BGC823/DDP and parental BGC823 cells were seeded into six-well plates 48 h after treatment with 0.5 μg/ml cisplatin and grown to 70% confluence, which were subsequently wounded using a sterile micropipette tip and any cellular debris was removed by washing several times with PBS. The wounded monolayers were then incubated for 24 h. Photos were captured at 0 and 24 h after wounding to calculate healing percentages.

Statistical analysis

All statistical analysis of this research was performed using SPSS 21.0 software (SPSS Inc.,

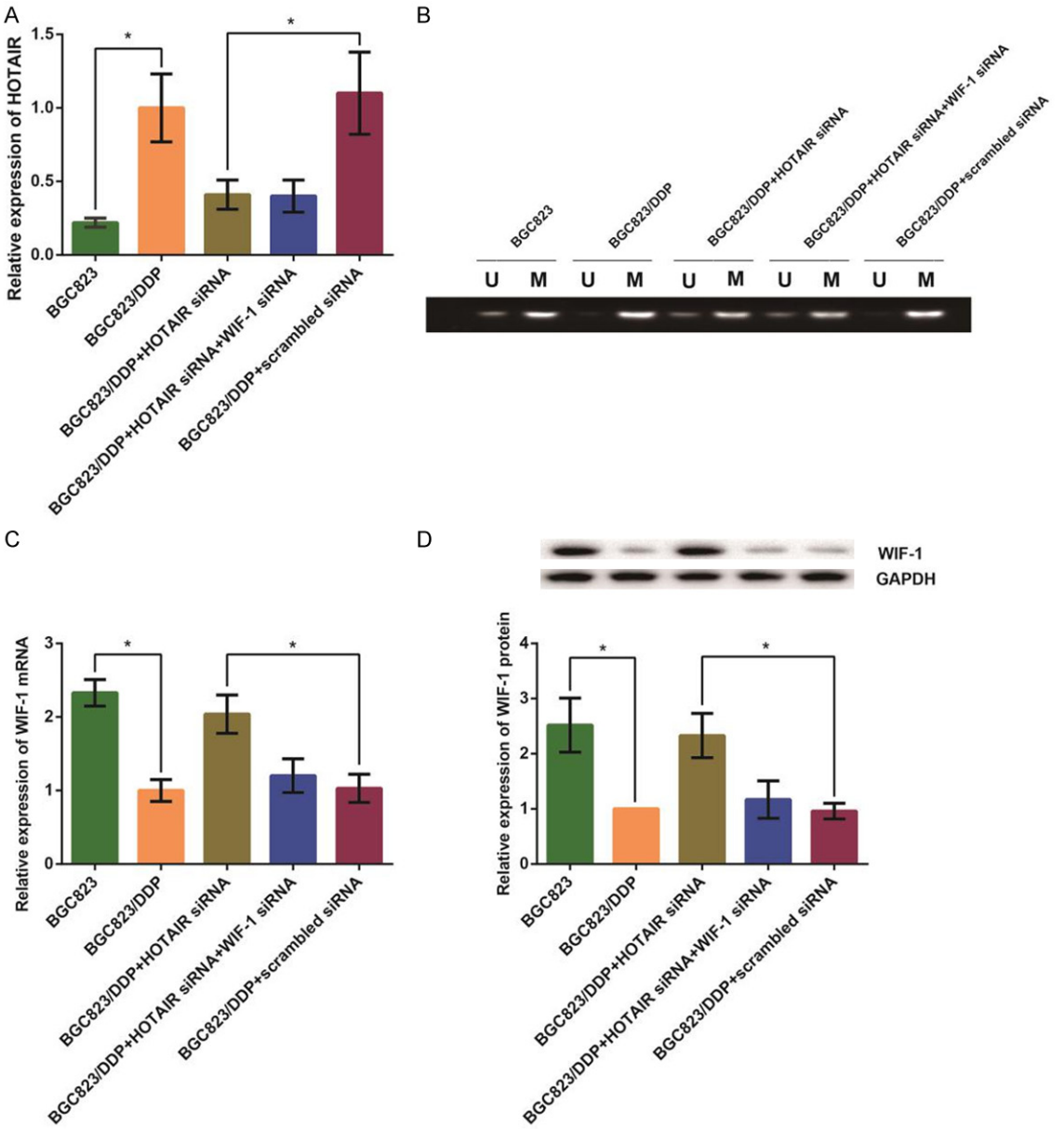

Figure 1. HOTAIR inhibits WIF-1 in the cisplatin-resistant GC cell lines. A. HOTAIR expression in the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was analyzed by qRT-PCR. B. WIF-1 promoter methylation level was investigated by MSP. C. WIF-1 mRNA expression in the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was analyzed by qRT-PCR. D. WIF-1 protein expression in the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was analyzed by western blotting. Data are expressed as mean ± standard deviation from at least three independent experiments.

Chicago, USA) and presented by Graph PAD prism software (GraphPad Software, Inc., US). Results were calculated as mean ± standard deviation. Statistical differences were investigated using Student's *t*-test (two-tailed) or Chi-square test if appropriate, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

HOTAIR inhibits WIF-1 in the cisplatin-resistant GC cell lines

HOTAIR expression were greatly up-regulated in BGC823/DDP cells in comparison to the BGC823 cells ($P<0.05$; **Figure 1A**), thus sug-

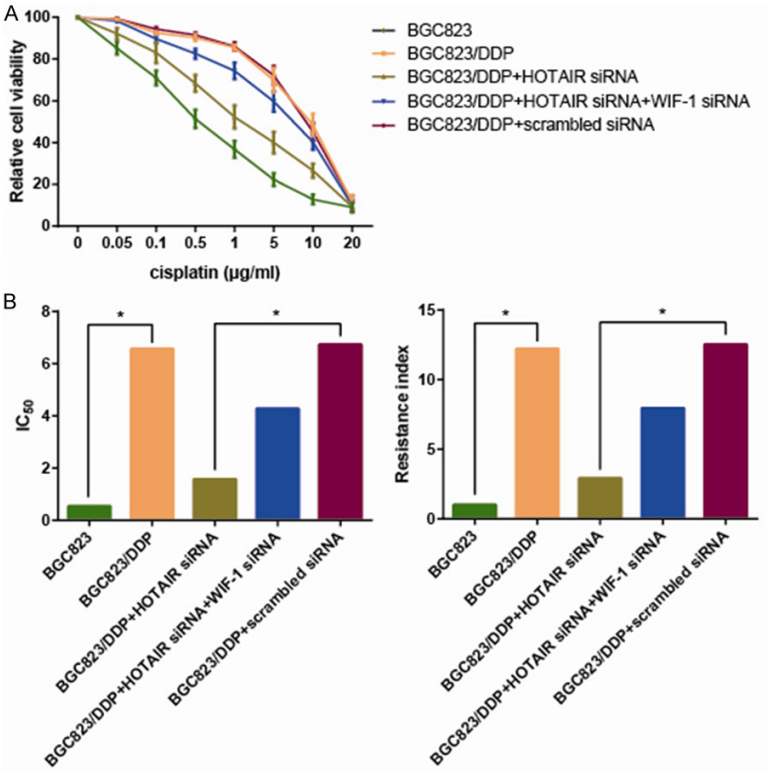

Figure 2. Down-regulation of HOTAIR promotes cisplatin-sensitivity of the cisplatin-resistant GC cell lines. A. The cell viability of the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was determined by CCK-8 assay. B. IC_{50} and RI (Resistance Index) for the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells. Data are expressed as mean ± standard deviation from at least three independent experiments.

gesting that the up-regulation of HOTAIR might suppress the sensitivity of human GC cells to treatment with cisplatin. Analysis of methylation status of CpG islands in the WIF-1 promoter region was carried out in BGC823/DDP and parental BGC823 cells. As showed in **Figure 1B**, the WIF-1 promoter methylation level was obviously decreased after transfection with HOTAIR siRNA in BGC823/DDP cells. In addition, as illustrated in **Figure 1C** and **1D**, the mRNA and protein expression levels of WIF-1 were obviously reduced in BGC823/DDP cells in comparison to BGC823 cells (all $P<0.05$), and silence of HOTAIR significantly up-regulated WIF-1 expressions both at mRNA and protein levels (all $P<0.05$), indicating that HOTAIR might restrain the expression of WIF-1 in the cisplatin-resistant GC cell lines.

Down-regulation of HOTAIR promotes cisplatin-sensitivity of the cisplatin-resistant GC cell lines

The cell viability of BGC823 and BGC823/DDP GC cells to cisplatin were investigated through CCK-8 assay. As demonstrated in **Figure 2A** and **2B**, the IC_{50} value and the calculated resistance index (RI) of cisplatin in BGC823/DDP cells was all significantly higher than that of their parental BGC823 cells (all $P<0.05$), and silence of HOTAIR remarkably down-regulated the IC_{50} value and the RI of cisplatin in BGC823/DDP cells (all $P<0.05$). Intriguing, co-silence of WIF-1 could reverse the downregulated IC_{50} value and RI of cisplatin in BGC823/DDP cells transfected with HOTAIR siRNA.

Down-regulation of HOTAIR promotes apoptosis and inhibits mobility of the cisplatin-resistant GC cell lines in treatment with cisplatin

The apoptotic rate to cisplatin of BGC823 and BGC823/DDP GC cells were investigated via a flow cytometer. As demonstrated in **Figure 3**, the apoptotic rate to cisplatin of BGC823/DDP cells was significantly suppressed than that of their parental BGC823 cells ($P<0.05$), and down-regulation of HOTAIR obviously facilitated the apoptosis of BGC823/DDP cells to cisplatin ($P<0.05$). Besides, co-silence of WIF-1 could reverse the relatively high apoptotic rate to cisplatin of BGC823/DDP cells transfected with HOTAIR siRNA.

The wound healing assay was conducted to assess the cell mobility to cisplatin of the BGC823 and BGC823/DDP GC cells. As demonstrated in **Figure 4**, the cell mobility to cisplatin of BGC823/DDP cells was remarkably suppressed than that of their parental BGC823 cells ($P<0.05$), and silence of HOTAIR significantly inhibited the cell mobility to cisplatin of BGC823/DDP cells ($P<0.05$). Moreover, co-silence of WIF-1 could reverse the relatively down-regulated cell mobility to cisplatin of BGC823/DDP cells transfected with HOTAIR siRNA.

Discussion

To date, GC remains the second major reason of cancer-associated mortality globally, with a

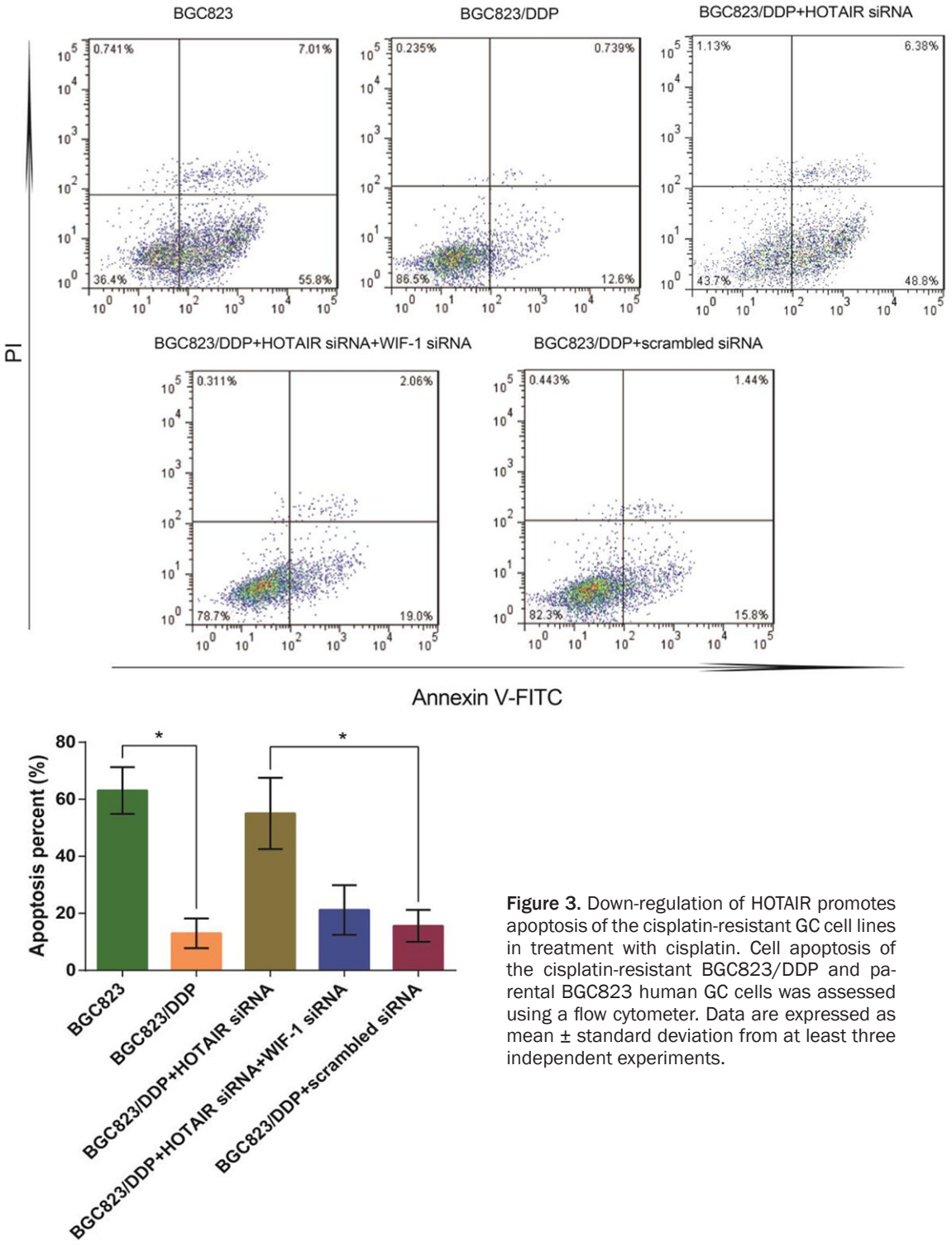

Figure 3. Down-regulation of HOTAIR promotes apoptosis of the cisplatin-resistant GC cell lines in treatment with cisplatin. Cell apoptosis of the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was assessed using a flow cytometer. Data are expressed as mean ± standard deviation from at least three independent experiments.

particular high occurrence rate in China [23]. Surgical resection is often regarded as a leading therapeutic selection for patients with early-stage cancer. However, many GC patients are diagnosed at the stage when the tumor is almost unresectable. Application of chemotherapeutic drugs singly or in combination is an ideal option for the treatment of these cancer patients. Platinum-based antineoplastic agents (e.g., cisplatin, oxaliplatin), paclitaxels, 5-FU

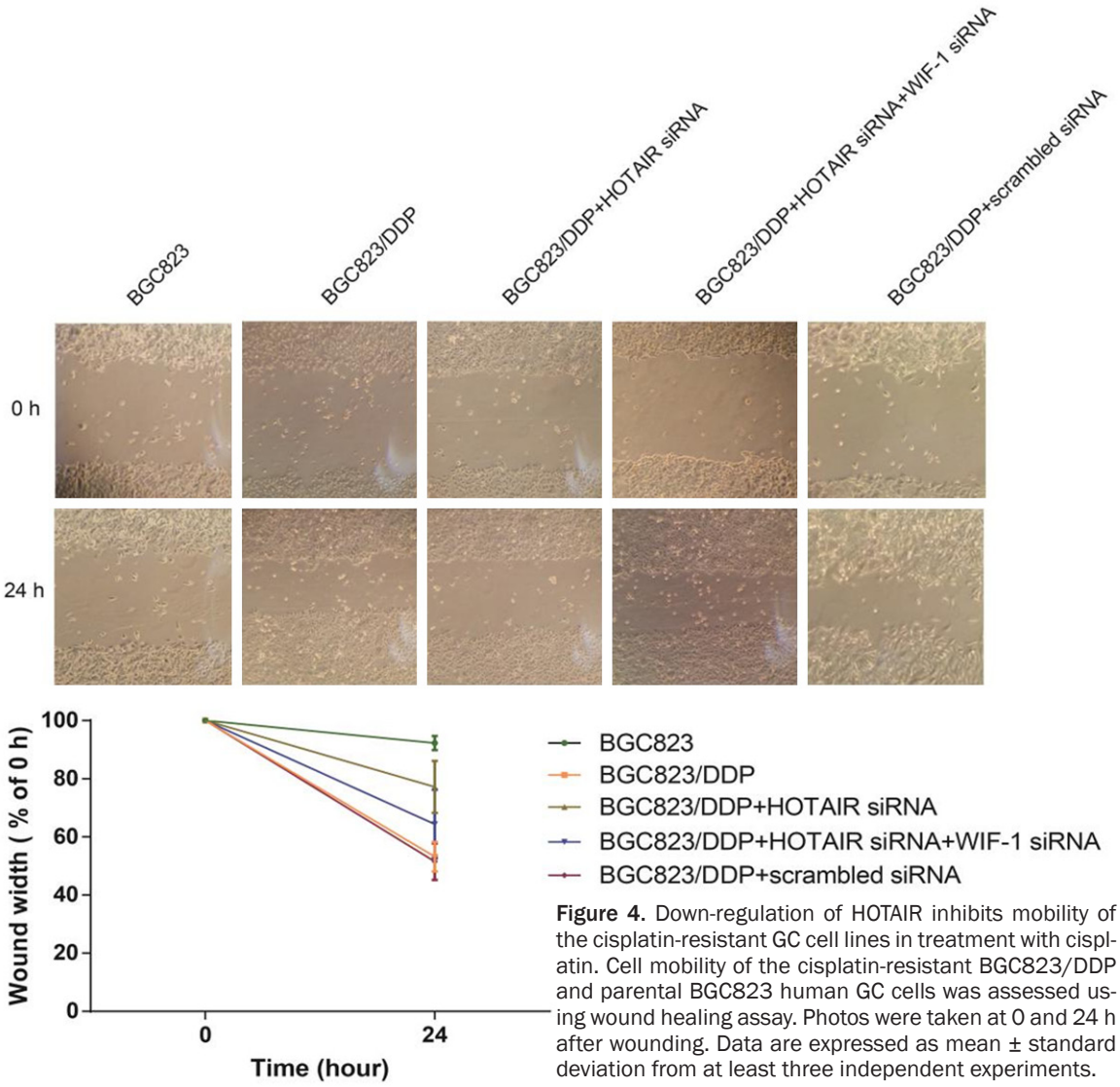

Figure 4. Down-regulation of HOTAIR inhibits mobility of the cisplatin-resistant GC cell lines in treatment with cisplatin. Cell mobility of the cisplatin-resistant BGC823/DDP and parental BGC823 human GC cells was assessed using wound healing assay. Photos were taken at 0 and 24 h after wounding. Data are expressed as mean ± standard deviation from at least three independent experiments.

derivatives (e.g., Xeloda) and S-1 have been wide applied for the treatment of GC in clinic. Cisplatin, as one of the first-line platinum-based chemotherapeutic drug widely applied in cancer treatment, could lead to cell apoptosis through promoting DNA damage by formatting an intra-strand cross-linking on DNA [24]. But cancer cells could often develop various mechanisms to suppress cisplatin-induced DNA damage and apoptosis, thus causing cisplatin resistance [25, 26], which is considered as a major clinic obstacle the successful treatment of GC. Accordingly, investigation of the molecular mechanisms involved in chemotherapy resistance is in urgent needed.

The mechanisms of cisplatin resistance have been extensively explored at the levels of genes and proteins. Previous articles have revealed multiple molecular mechanisms involved in cisplatin resistance, such as decreased drug accumulation [22], restored DNA damage repair [27] and inactivated apoptotic signaling [28]. LncRNAs have been demonstrated to regulate a series of biological programs, such as cell proliferation, apoptosis and carcinogenesis. By investigating the expression profile in a total of 60 tumoral and non-tumoral gastric specimens, Emadi-Andani E et al. determined that HOTAIR displayed a greater than 2-fold expression difference between GC tissues and normal gastric tissues [29]. Previous studies have also demonstrated that lncRNAs might be correlated to the development of resistance in various cancer cells to chemotherapeutics. For instance, lncRNA AK126698 might regulate

cisplatin resistance of non-small-cell lung cancer cells via the canonical Wnt pathway [30]. In this research, we also observed that overexpressed HOTAIR was closely correlated to the development of cisplatin resistance in GC cells, and down-regulation of HOTAIR might enhance the cisplatin-sensitivity of GC cells. However, at present, little is known about their functional role in chemoresistance.

To our knowledge, the present article might be the first study to reveal a relationship between HOTAIR levels and the cisplatin-sensitivity of GC cells. As we all known, dysregulated cell proliferation and apoptosis might contribute to the development of multiple cancers. And in accordance to this view, cisplatin-resistance might also be associated to the imbalance of cell proliferation and apoptosis. Wnt/β-catenin signaling pathway, engaged in regulating several biological and pathological responses in numerous kinds of cancers, including cell viability and apoptosis, was also found to augment resistance to cisplatin, docetaxel, and radiotherapy [31, 32]. The article of Li et al. indicated that HOTAIR could induce cisplatin resistance through activating Wnt/β-catenin signaling pathway, which could be reversed via pre-treatment with XAV939, a Wnt/β-catenin inhibitor, in human ovarian cancer [33].

Furthermore, WIF-1 silencing through promoter methylation of WIF-1, a secreted antagonist that can directly bind to Wnt proteins and suppress Wnt signaling pathway, could be regarded as an early epigenetically pathological event and serves a critical role in tumor development and progression of many cancer types [34, 35]. Paluszczak J et al. observed that the methylation of WIF-1 was associated to shorter overall survival of the patients with oral and oropharyngeal squamous cell carcinoma [36]. In this article, we observed that WIF-1 promoter methylation, which was closely associated to the down-regulation of WIF-1 mRNA and protein expression, was found to be significantly enhanced in BGC823/DDP GC cells, and WIF-1 silencing could contribute to decreased apoptotic rate and up-regulated cell mobility of BGC823/DDP GC cells to cisplatin.

In conclusion, the present study illustrated that HOTAIR expression levels were remarkably up-regulated in the cisplatin-resistant BGC823/DDP human GC cell line in comparison to BGC823 cell line. Furthermore, down-regulation of HOTAIR was able to promote the cisplatin-sensitivity of the BGC823/DDP cells to cisplatin through inhibiting Wnt pathway; thus suggesting that HOTAIR could be applied as a novel therapeutic biomarker for unfavorable response to cisplatin, and a combination of cisplatin administration, alongside HOTAIR silence, might be considered a promising strategy for the treatment of patients with cisplatin-resistant GC in the near future.

Disclosure of conflict of interest

None.

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