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Original Article

Role of HOXB7 in promoting gastric cancer progression and oxaliplatin (L-OHP) resistance

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Abstract: Background and Aim: Our study aimed to investigate the ways by which HOXB7 affects gastric cancer progression and oxaliplatin (L-OHP) resistance. Methods: First, the expression of HOXB7 in paired cancer and para-cancerous tissues of L-OHP-sensitive and L-OHP-resistant gastric cancer patients was qualitatively and quantitatively analyzed by immunohistochemistry. Then, the expression of HOXB7 in these tissues was further quantitatively analyzed at protein and transcriptional levels. The expression of HOXB7 in the SGC-7901 L-OHP-resistant gastric cancer cell line was further verified by immunofluorescence, western blot, and RT-qPCR. In addition, by transfecting the SGC-7901 cell line, control (sh-con) and HOXB-7-silenced (sh-HOXB7) gastric cancer cell lines were created. Subsequently, the migratory and invasive abilities of these cells were determined by the transwell assay. The proliferation rate of both control and HOXB-7-silenced cells induced by varying concentrations of L-OHP was detected by the CCK-8 assay, while the degree of apoptosis in the same cells induced by 60 μM L-OHP was detected by flow cytometry. Results and conclusion: Results suggested that HOXB7 was overexpressed in both the tissues of L-OHP-resistant gastric cancer patients and the SGC-7901 gastric cancer cell line. Moreover, HOXB7 promoted the migratory and invasive abilities of gastric cancer cells. By silencing HOXB7 protein expression, the proliferation rate of L-OHP-resistant gastric cancer cells decreased considerably, while their degree of apoptosis increased significantly. These results showed that HOXB7 promoted gastric cancer progression and L-OHP resistance.

Keywords: Chemotherapy, drug resistance, homeobox B7, HOXB7, gastric cancer, oxaliplatin, L-OHP

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide, and China is one of the countries exhibiting the highest gastric cancer rates, with incidence and mortality of this type of cancer being the second among all malignancies diagnosed in the country [1]. Approximately 1 million new cases of gastric cancer are diagnosed annually worldwide, of which China accounts for 40% of the total, with a morbidity and mortality twice the world average [2]. In recent years, incidence of gastric cancer has trended up in young people, highlighting the need for increased attention towards the prevention and treatment of the disease. Although prevention and treatment strategies for gastric cancer have developed rapidly in the past decades, prognosis of patients with advanced and recurrent gastric cancer remains poor, with a 10-20% one-year survival rate reported [3]. As is widely accepted, the biologic and clinical behavior of cancer is influenced by a variety of molecular pathways mainly controlled by transcription factors [4]. Therefore, a thorough study of how transcription factors act on the biologic behavior of cancer might be able to promote a greater depth of understanding of the mechanisms of cancer, as well as the discovery of new therapeutic strategies.

HOX genes, a highly conserved subgroup of the homeobox superfamily, have crucial roles in development, regulating numerous processes, including apoptosis, receptor signaling, differentiation, motility, and angiogenesis [5]. Abnormal expression of HOX genes has been shown to promote occurrence and development of malignancies [6]. Expression of homeobox B7 (HOXB7) was upregulated in many malignancies, such as in hepatocellular cancer [7], lung cancer [8], and cervical cancer [9]. However,

the mechanism of action of HOXB7 in gastric cancer remains unclear, and needs to be further studied.

Gastric cancer cells are sensitive to chemotherapeutic drugs. Chemotherapy has been widely used in the clinical treatment of GC in recent years [10]. Oxaliplatin (trans-/-diaminocyclohexane oxalatoplatinum; L-OHP) is the most commonly used chemotherapeutic drug in clinical practice. Most patients with GC in the early stage can prolong their survival through L-OHP chemotherapy. However, drug resistance often severely hinders the efficacy of L-OHP and treatment progress [11]. A small minority of GC patients are inherently resistant to L-OHP, while most cancer patients will gradually develop L-OHP resistance during their treatments [12, 13]. Therefore, L-OHP resistance remains a major obstacle in the long-term treatment of GC patients. However, the molecular mechanisms involved in L-OHP resistance are still unknown.

Previous studies found that HOXB7 promoted L-OHP resistance through the EGFR-dependent pathway [14, 15]. Therefore, we investigated the expression of HOXB7 in cancer tissues of both L-OHP-sensitive and L-OHP-resistant gastric cancer patients, as well as in the L-OHP-resistant SGC-7901 gastric cancer cell line. We further examined the ways by which L-OHP resistance in SGC-7901 cells is affected by silencing of HOXB7.

In this study, the expression of HOXB7 in cancer tissues of both L-OHP-sensitive and L-OHP-resistant gastric cancer patients was first qualitatively and quantitatively detected. Subsequently, a gastric cancer cell line silenced for HOXB7 was successfully created, demonstrating the effect of HOXB7 on the migratory and invasive abilities of gastric cancer cells, as well as on their resistance to L-OHP.

Materials and methods

Tissue samples

Cancer and paracancerous tissues (less than 3 cm away from cancer tissues) were obtained from 30 pairs of both L-OHP-sensitive and L-OHP-resistant gastric cancer patients. A total of 60 gastric cancer samples were collected from Department of Pathology, affiliated Hospital of Guilin Medical College from March 1, 2017 to March 1, 2019. 37 patients were male and 23 female, aged 33-89, with a median age of 61 years. All patients received traditional radical gastrectomy, and L-OHP chemotherapy before the operation. Half of the samples were frozen in liquid nitrogen and stored at -80°C for ensuing RNA or protein extraction. The other half were immobilized immediately with 10% neutral formalin solution and embedded in paraffin wax for subsequent immunohistochemical staining. This study and all of its protocols were examined and approved by the Institutional Review Board of affiliated Hospital of Guilin Medical College.

The sh-con and sh-HOXB7 plasmid and DNA transfection

The SGC-7901 gastric cancer cell line was purchased from the National Institute of Biological Sciences, (Beijing, China). Cells were cultured in a modified Dulbecco's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Both the sh-HOXB7 plasmid and the empty expression control vector (sh-con) were obtained from Dr. Xiaowei Dong (Origimed, Shanghai, China). All transfections were performed using the LipoFiter™ Liposomal Transfection reagent (Hanbio Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Briefly, cells were plated (6×10^6 cells) in 100 mm dishes in fresh RPMI-1640 medium containing 10% FBS. Cells were transfected with 4.6 μg of the expression vector using 4.8 μl LipoFiter™ reagent. Media was replaced 6 h following transfection and transfected cells were incubated at 37°C for 48 h. These transfected cells were used in all subsequent experiments.

Immunohistochemistry

Four μm thick sections were prepared from paraffin-embedded tissues for immunohistochemical analysis using the PV-6000 polymer detection system. Sections were deparaffinized in xylene, hydrated in a series of gradient ethanol solutions (100%, 90%, 80%, each for 2 min), and then boiled in EDTA buffer (pH 9.0) for 2.5 min in a high-pressure pot. Consequently, sections were combined and incubated at 4°C overnight with anti-HOXB7 antibody (1:50, mouse monoclonal antibody; Cell Signaling Technology, Beverly, MA, USA) and anti-β-catenin antibody (1:50, mouse monoclonal antibody; Beijing

Zhongshan Biotechnology Co., Ltd., Beijing, China). Then, 3,3'-diaminobenzidine (DAB) peroxidase was used for color detection. The PV-6000 system, secondary antibody, and DAB were all purchased from Beijing Zhongshan Biotechnology. There was no antibody staining in sections used as negative controls.

Immunohistochemistry assessment

We randomly examined 100 gastric tumor cells and healthy gastric cells, respectively, and cells with positive HOXB7 staining were scored. Healthy gastric cells were examined as either negative or positive controls in the same field. Experiments were performed at least 3 times to ensure reproducibility of results. HOXB7 staining was scored as follows: 0, no staining or staining observed in < 10% tumor cells; 1+, faint/barely perceptible staining detected in ≥ 10% tumor cells; 2+ and 3+, moderate and strong staining, respectively, observed in ≥ 10% tumor cells. A score of 0 or 1+ was considered negative and a score of 2+ or 3+ was considered positive. Immunostained slides were evaluated independently by 2 pathologists in a blind manner. In most cases, the evaluation of both pathologists was identical; discrepancies were resolved by reexamination and consensus.

Real-time quantitative PCR

Based on manufacturer's instructions, total RNA was extracted from samples using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Following cDNA synthesis using the All-in-One First-Strand cDNA Synthesis kit (GeneCopoeia Inc, Santa Cruz, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed on an ABI 7500HT system using the All-in-One qPCR Mix (GeneCopoeia) and HOXB7 primers. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 20 sec and 72°C for 27 sec. Melting curve analysis was used to analyze and verify specificity for each RT-qPCR reaction. The β-actin gene was used as an internal control in repeated reactions for each sample. Relative changes of gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method. All experiments for each sample were conducted in duplicate.

Western blot analysis

Whole cell protein extracts were collected and analyzed using western blotting. Briefly, 30 μg of proteins were separated on 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with HOXB7 antibody (1:1000 dilution, Genetex, San Antonio, TX, USA) at 4°C overnight. After incubation, the membrane was washed with PBST (PBS containing TW20; Hyclone, Logan, UT, USA), and was subsequently incubated with HRP-labeled goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The β-actin protein at 1:1000 dilution (Proteintech Group) was used as internal control. Each experiment was repeated 3 times.

Immunofluorescence

The SGC-7901 gastric cancer cell line was inoculated into a 6 well plate, incubated for 48 h, and then inoculated into a 12 well plate (Corning, New York, NY, USA) and further incubated for a day. Cells were fixed with 4% polyformaldehyde solution for 15 min and permeated with 0.3% Triton X-100. Consecutively, cells were incubated with HOXB7 antibody (1:100 dilution, Novus, Littleton, CO, USA) at 4°C overnight. Cells were washed 3 times with PBST and incubated with Alexa Fluor 594 goat anti-rabbit IgG (H + L) (1:2000 dilution, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:500 dilution, Cell Signaling Technology) secondary antibodies for 50 min away from light. Finally, 4',6-diamidino-2-phenylindole was used for counterstain. Images were captured using the Leica DFC 450 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Measurement of cell migration and invasion

To measure the migratory and invasive abilities of transfected cells, the BD BioCoat Matrigel transwell chamber and control inserts (BD Biosciences) were used. The transwell chamber had a polyethylene terephthalate membrane with an 8 mm pore. The SGC-7901 gastric cancer cell line was transfected with 30 nmol/l of sh-con or sh-HOXB7, respectively and incubated at 37°C with 5% CO_2. Twenty-four hours after transfection, 1.56×10^5 cells were reinoculated in each chamber supplemented with low serum containing medium (1% FBS). The transwell chamber was then placed in a 24 well plate, containing high serum (20% FBS) medium added to each well as chemical attractant. Cells were incubated at 37°C for 48 h with 5%

Table 1. HOXB7 expression in cancer and paracancerous tissues of L-OHP-sensitive gastric cancer patients

Group	HOXB7 negative expression	HOXB7 positive expression	χ^2	P
L-OHP-sensitive group	6	24	15.1	0.0001
Paracancerous tissues	22	8		

Table 2. HOXB7 expression in cancer and paracancerous tissues of L-OHP-resistant gastric cancer patients

Group	HOXB7 negative expression	HOXB7 positive expression	χ^2	P
L-OHP-resistant group	4	26	26.7	< 0.0001
Paracancerous tissues	25	5		

CO_2, then washed, stained and mounted on slides. The number of migrating or invading cells was calculated using optical microscopy. Relative migration was calculated by comparing transfected cells to negative control cells. Percentage of invasion was calculated as the number of cells that invaded the Matrigel matrix divided by the total number of implanted cells.

CCK-8 assay for cell viability

SGC-7901 cells were collected at the logarithmic growth stage and inoculated in a 96 well plate at a density of 3×10^3 per well. After 24 h of culture, 100 μl of varying concentrations of L-OHP (0, 15, 30, 45, and 60 μM) were added to each well. Blank wells were used for calibration. After 24 h of incubation, a total of 10 μl of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well. Following incubation of cells with the reagent for 2 h, the optical density (OD) of each well was measured at 450 nm wavelength using the enzyme labeling instrument (Bio-Rad, Hercules, CA, USA). The experiment was repeated 3 times.

Detection of apoptosis

Both control (sh-con; negative-control) and HOXB7-silenced (sh-HOXB7) SGC-7901 cells, were treated with 60 μM L-OHP. After 24 h of culture, cells were washed twice with 1 × PBS and digested with 0.25% trypsin in 1 × PBS. Cells were then collected in microcentrifuge tubes and centrifuged at 1000 rpm for 5 min at 4°C. Supernatant was removed and cells were collected as pellet. The procedure was conducted according to the instructions of the FITC/Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Cells were resuspended in 500 μl of a 1 × buffer, and then stained with 5 μl of Annexin V and 50 μg/ml of propidium iodide (PI) for 20 min away from light at 37°C. Cell apoptosis was detected in each sample by FACSAriaTM flow cytometry and analysis of data was performed using the Win MDI 2.9 software.

Statistical analysis

Experimental data were analyzed using the SPSS18.0 software, and expressed as mean value ± standard deviation (mean ± SD) in the graphs drawn. SD values were obtained from experiments that were repeated at least 3 times. The t-test was used for comparison between 2 groups, and one-way ANOVA for comparison among multiple groups. A result with $P < 0.05$ was considered significant.

Results

Expression of HOXB7 in cancer tissues of L-OHP-sensitive and L-OHP-resistant gastric cancer patients

The expression of HOXB7 in cancer and paracancerous tissues of 30 L-OHP-sensitive and 30 L-OHP-resistant patients (**Tables 1** and **2**) was analyzed by immunohistochemistry. Of 30 L-OHP-sensitive specimens, 24 were positive for HOXB7, whereas 6 were negative for HOXB7 expression. Concomitantly, of 30 L-OHP-resistant specimens, 26 were positive, whereas 4 were negative for HOXB7 expression. Moreover, our results showed that HOXB7 was weakly expressed in L-OHP-sensitive gastric cancer tissues, whereas in tissues of L-OHP-resistant gastric cancer, HOXB7 was more strongly expressed and diffuse in cancer cell membranes (**Figure 1A**). Next, HOXB7 expression was quantitatively analyzed in these specimens by western blotting and RT-qPCR analysis. Results showed that expression of HOXB7 protein in L-OHP-resistant tissues was significantly higher than that in L-OHP-sensitive tissues (**Figure 1B**). Similarly, the expression of HOXB7 mRNA in L-OHP-resistant tissues was significantly

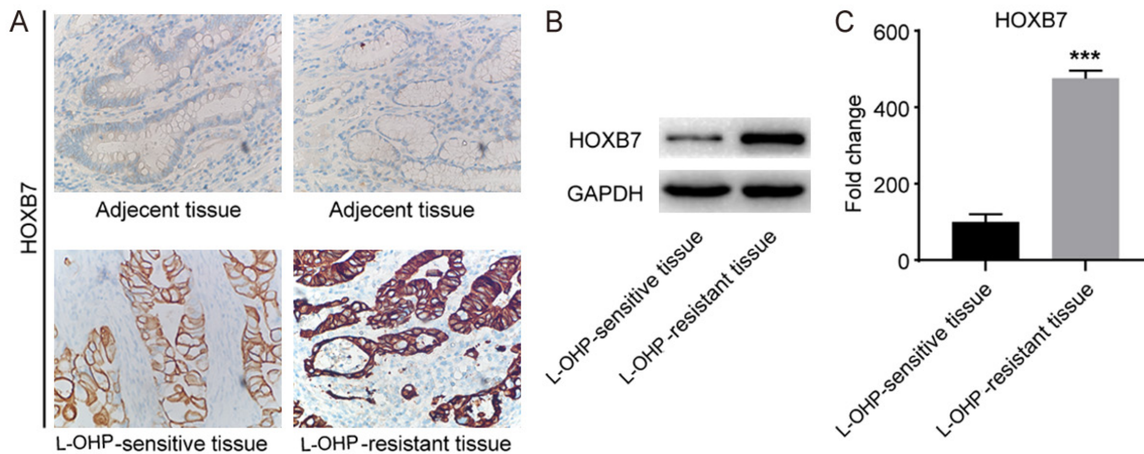

Figure 1. HOXB7 expression in tissues of oxaliplatin (L-OHP)-sensitive and oxaliplatin (L-OHP)-resistant gastric cancer patients. A. Negative membranous staining (left) representing loss of expression of HOXB7 in oxaliplatin-sensitive tissues; intense membranous staining (right) representing high expression of HOXB7 in oxaliplatin-resistant tissues (× 400). B. HOXB7 protein expression in the L-OHP-sensitive tissue and L-OHP-resistant tissue in western blot test. C. HOXB7 protein expression was higher in the L-OHP-resistant tissue than in the L-OHP-sensitive tissue (***$P<0.05$, compared with the L-OHP-sensitive tissue).

higher than that in L-OHP-sensitive gastric cancer tissues (**Figure 1C**, $P < 0.001$), suggesting that HOXB7 was overexpressed in cancer tissues of L-OHP-resistant gastric cancer patients.

Expression of HOXB7 in the SGC-7901 L-OHP-resistant gastric cancer cell line

We wished to further validate the expression profile of HOXB7 in the SGC-7901 gastric cancer cell line. To that end, both the regular SGC-7901 and a constructed L-OHP-resistant SGC-7901 cell lines were used. Results of protein levels, immunofluorescence and western blot analysis showed that expression of HOXB7 in the L-OHP-resistant cells was significantly higher than that in regular SGC-7901 cells (**Figure 2A** and **2B**). Concurrently, RT-qPCR results showed that the amount of HOXB7 mRNA in L-OHP-resistant cells was significantly higher than that in regular SGC-7901 cells (**Figure 2C**, $P < 0.001$). The above results suggested that HOXB7 was also overexpressed in the L-OHP-resistant SGC-7901 gastric cancer cell line. In conclusion, abnormal overexpression of HOXB7 may be closely correlated to L-OHP resistance in gastric cancer cells.

Effect of HOXB7 on the migratory and invasive abilities of the SGC-7901 gastric cancer cell line

First, we transfected the SGC-7901 gastric cancer cell line with either the sh-HOXB7 or the sh-con plasmid to obtain a cell line silenced for HOXB7 (sh-HOXB7 group) and a control cell line (sh-con group), respectively. Next, successful creation of the 2 cell lines was verified at both the protein and mRNA levels. Results of protein levels, immunofluorescence and western blot analysis revealed that expression of HOXB7 protein in the HOXB7-silenced cells (sh-HOXB7 group) was significantly lower compared to that in the control (sh-con group) (**Figure 3A** and **3B**). Similarly, RT-qPCR results demonstrated that the amount of HOXB7 mRNA in the HOXB7-silenced cells (sh-HOXB7 group) was significantly lower than that in the control (sh-con group) (**Figure 3B**, $P < 0.001$). As such, the above results suggested that we successfully created the HOXB7-silenced SGC-7901 gastric cancer cell line. The migratory and invasive abilities of the HOXB7-silenced cells were evaluated using the Matrigel transwell assay, and were shown to be significantly lower than those in the control (sh-con group) (**Figure 3C** and **3D**, $P < 0.001$). In conclusion, our results revealed that expression of HOXB7 promoted gastric cancer progression.

Effect of HOXB7 on L-OHP resistance in the SGC-7901 gastric cancer cell line

The effects of L-OHP on the proliferation and apoptosis of the HOXB7-silenced gastric cancer cells were studied by performing the CCK-8 assay and flow cytometry. Results of CCK-8 analysis showed that the proliferation rate of the HOXB7-silenced cells (sh-HOXB7 group) was significantly lower relative to that in con-

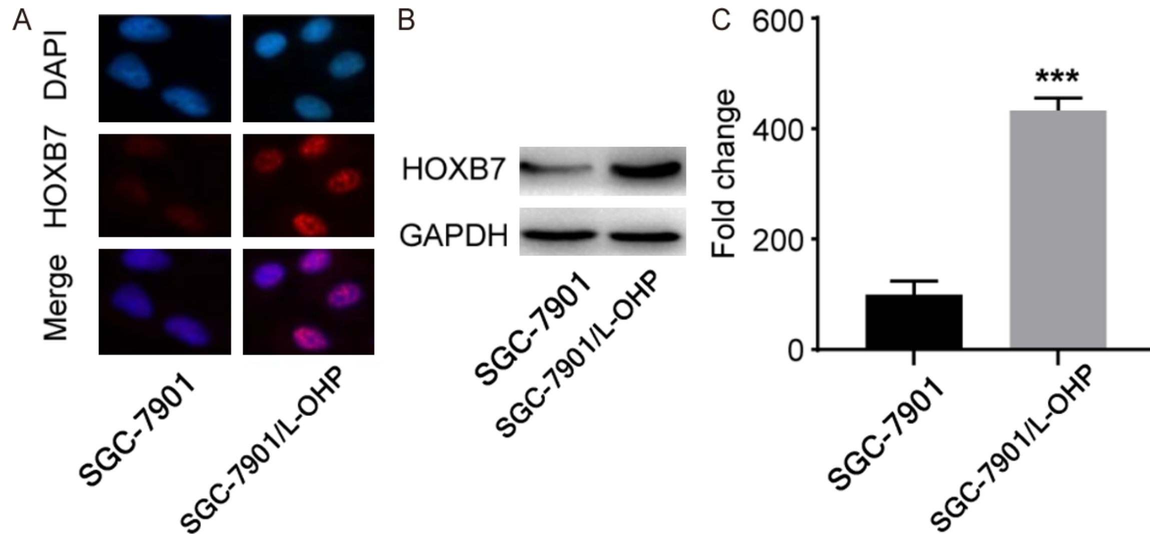

Figure 2. Expression of HOXB7 in regular and oxaliplatin (L-OHP)-resistant SGC-7901 gastric cancer cell lines. Expression of HOXB7 in the 2 cell lines, at protein (A and B) and mRNA (C) levels. ***P < 0.001, SGC-7901/L-OHP vs. SGC-7901.

trols (sh-con group) (**Figure 4A**, P < 0.001). Similarly, flow cytometry results revealed that the degree of apoptosis in HOXB7-silenced cells (sh-HOXB7 group) was significantly higher compared to that in controls (sh-con group) (**Figure 4B**, P < 0.01). In conclusion, overexpression of HOXB7 promoted L-OHP resistance in gastric cancer cells.

Discussion

Gastric cancer is a lethal malignancy affecting the digestive tract. Oxaliplatin (L-OHP) has been one of the most effective drugs in the chemoradiotherapy treatment of this type of cancer [14]. However, therapeutic resistance has been observed in gastric cancer cells, especially in those patients with metastatic, recurrent, and advanced forms of the disease [10]. In our study, we first found that HOXB7 was selectively expressed in cancer tissues of both L-OHP-sensitive and L-OHP-resistant gastric cancer patients compared with corresponding paracancerous tissues. However, compared to L-OHP-sensitive tissues, HOXB7 was more strongly expressed and diffuse in cell membranes of L-OHP-resistant cancer tissue. Also, the expression of both HOXB7 mRNA and protein was significantly higher in L-OHP-resistant gastric cancer tissues compared to that in L-OHP-sensitive specimens. Next, to create an L-OHP resistant cell line, we treated the SGC-7901 gastric cancer cell line with a high concentration (60 μM) of L-OHP, thus making them resistant to L-OHP. Successful creation of these cell lines was verified by immunofluorescence, western blotting, and gene analysis. Similarly, the expression of both HOXB7 mRNA and protein was significantly increased in the L-OHP-resistant gastric cancer cell line. In conclusion, HOXB7 was demonstrated to be closely correlated to L-OHP resistance in gastric cancer patients.

Some studies have found that HOXB7 was overexpressed in both cancer cell lines and cancer tissues of gastric cancer patients. It has been reported that overexpression of HOXB7 can promote the migratory and invasive abilities of tumors, such as those of glioma, lymphoma, skin squamous cell carcinoma, and many more [15-20]. However, the role of HOXB7 overexpression in gastric cancer was still unknown. Therefore, we used functional assays to address and explore it. First, we transfected the SGC-7901 gastric cancer cell line with either sh-HOXB7 or sh-con plasmids to create a HOXB7-silenced (sh-HOXB7) and a negative control (sh-con) gastric cancer cell line, respectively. Immunofluorescence, western blot analysis and RT-qPCR were used to verify successful creation of both cell lines. The expression of both the HOXB7 mRNA and protein in the HOXB7-silenced cancer cells was significantly lower than that in the control, proving that the HOXB7-silenced cell line was successfully cre-

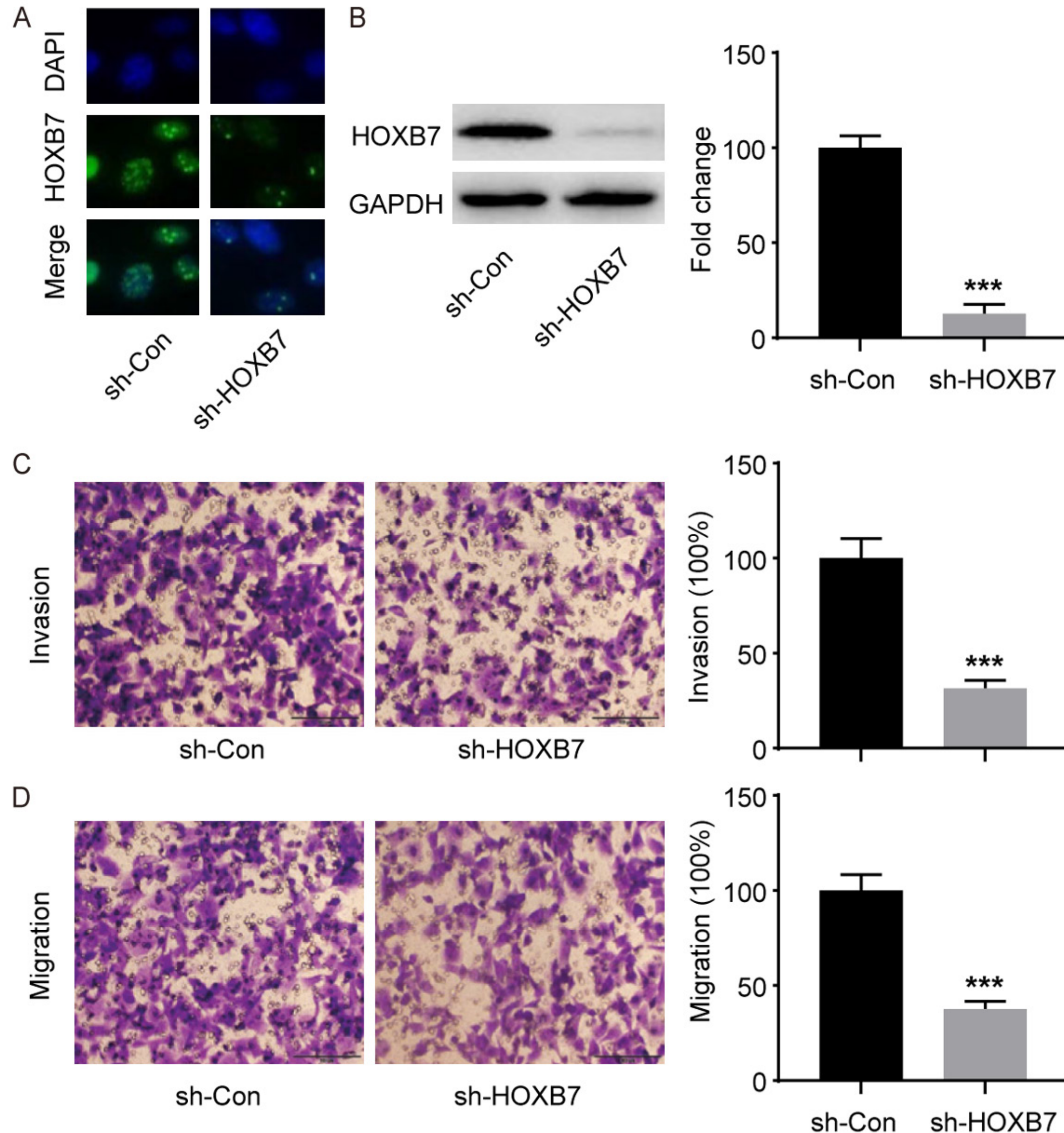

Figure 3. Effect of expression of HOXB7 on migration and invasion abilities of the SGC-7901 gastric cancer cell line. Expression of HOXB7 in control (sh-con) and HOXB7-silenced (sh-HOXB7) cells at protein (A and B, left) and mRNA (A and B, right) levels. (C) Representative images of invasion assay (left); graph of statistical analysis of the invasion assay (right). (D) Representative images of migration assay (left); graph of statistical analysis of the migration assay (right). ***P < 0.001 vs. NC group, n = 3.

ated. Then, we evaluated the migratory and invasive abilities of both the negative control and HOXB7-silenced cells by performing the transwell assay. We found that both the migratory and invasive abilities of the HOXB7-silenced (sh-HOXB7) gastric cancer cells were significantly lower relative to those of the negative control (sh-con). These results suggested that expression of HOXB7 also promoted gastric cancer progression.

Our results have indicated a close correlation of HOXB7 with L-OHP resistance in gastric cancer cells. However, a specific role of HOXB7 in L-OHP resistance of gastric cancer patients has not been previously reported. Therefore, we used the CCK-8 assay to detect the proliferation rate of control and HOXB7-silenced cells treated with varying concentrations of L-OHP and found that HOXB7-silenced cells exhibited a significantly lower proliferation rate compared

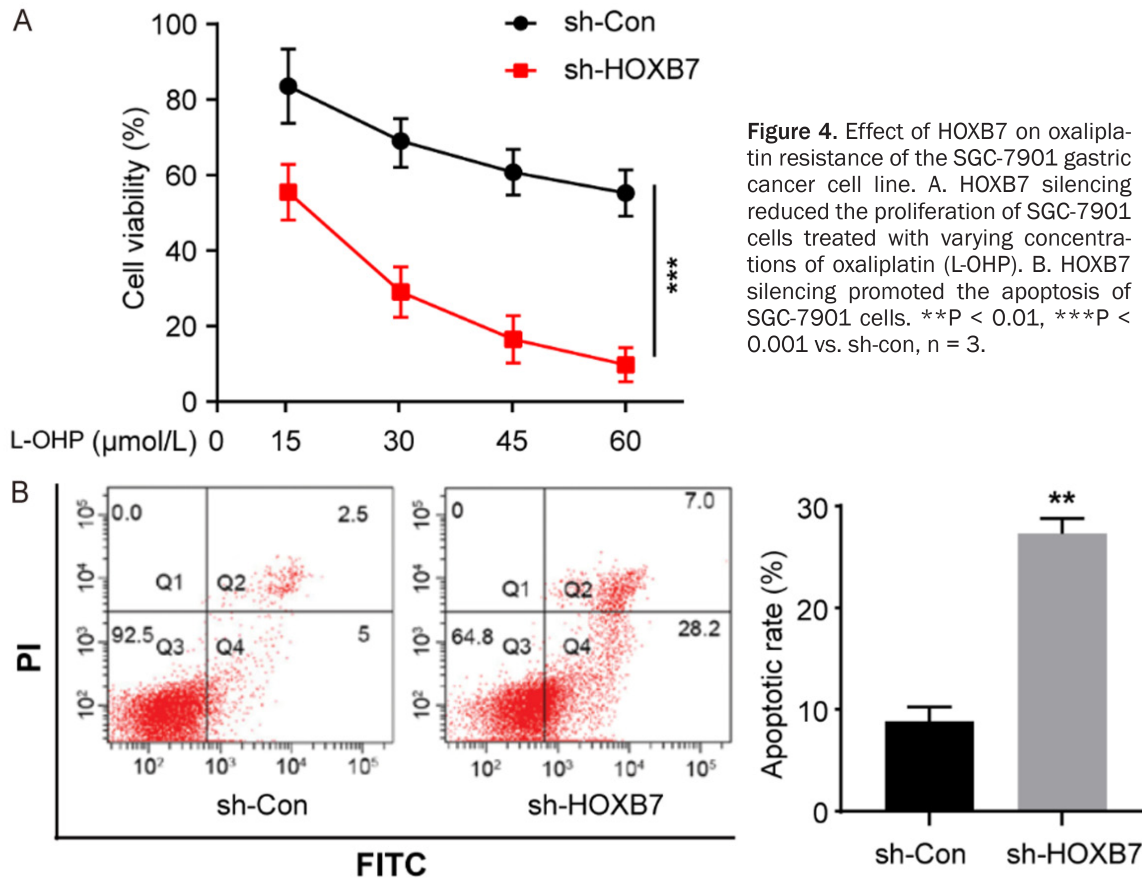

Figure 4. Effect of HOXB7 on oxaliplatin resistance of the SGC-7901 gastric cancer cell line. A. HOXB7 silencing reduced the proliferation of SGC-7901 cells treated with varying concentrations of oxaliplatin (L-OHP). B. HOXB7 silencing promoted the apoptosis of SGC-7901 cells. **$P < 0.01$, ***$P < 0.001$ vs. sh-con, n = 3.

to that of the negative control. Using flow cytometry, we also examined the degree of apoptosis in control and HOXB7-silenced cancer cells treated with a high concentration (60 μM) of L-OHP. As expected, HOXB7-silenced cells displayed a significantly higher degree of apoptosis relative to that of the negative control. These results indicated that silencing of HOXB7 abolished resistance and increased the sensitivity of gastric cancer cells to L-OHP, thus suggesting that expression of HOXB7 promotes L-OHP resistance in gastric cancer cells.

In conclusion, overexpression of HOXB7 promoted the migratory and invasive abilities of gastric cancer cells, as well as the resistance of gastric cancer patients to L-OHP treatment. In this study, HOXB7 was used for the first time as a possible target for abolishing L-OHP resistance in gastric cancer treatment, and it was revealed that L-OHP sensitivity could indeed be restored by silencing HOXB7. It would be of great significance to explore and understand the role of HOXB7 in the chemotherapeutic resistance of cancer, so as to allow for the development of novel and effective approaches in the treatment of gastric cancer. Therefore, further studies are required to investigate and delineate the specific role of HOXB7 in chemical resistance and signal transduction.

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Disclosure of conflict of interest

None.

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