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Abstract. High expression of high mobility group protein A2 (HMGA2) is correlated with the invasiveness of gastric cancer and is an independent prognostic factor. The reason may be that HMGA2 promotes epithelial-mesenchymal transition (EMT) and the acquisition of tumor stem cell properties, yet the mechanism remains unclear. In this study, immunohistochemistry and western blot analysis revealed that the expression of HMGA2 and Twist-related protein 1 (TWIST1) in gastric carcinoma tissues was higher than that in the peritumoral tissues and that the expression levels of these two proteins were positively correlated. The protein expression levels of HMGA2 and TWIST1 were high in the poorly differentiated gastric cancer MKN-45 cells and were low in the moderately differentiated SGC-7901 cells. TWIST1 was inhibited after HMGA2 interference and was significantly increased after overexpression of HMGA2. Luciferase experiments showed that TWIST1 was a direct downstream target gene of HMGA2. The simultaneous interference of HMGA2 expression and the overexpression of TWIST1 in MKN-45 cells reversed the inhibitory effect of HMGA2 interference on the invasion and migration of gastric cancer cells, EMT and the expression of stemness markers. However, the simultaneous overexpression of HMGA2 and the interference of TWIST1 expression in the SGC-7901 cells reversed the promoter effect of HMGA2 overexpression on the invasiveness and migration of gastric cancer cells, EMT and the expression of stemness markers. In addition, animal experiments showed that TWIST1 overexpression reversed the inhibition of HMGA2 interference on the metastasis of MKN-45 cells. Therefore, HMGA2 regulates the EMT of gastric cancer cells and the acquisition of tumor stem cell properties through direct regulation of the downstream target gene TWIST1.

Introduction

Gastric cancer is one of the most common gastrointestinal tumors. Although its mechanism of development is unclear, the invasive behavior and metastasis of cancer cells are the main cause of death in patients with gastric cancer. In recent years, many studies have suggested that epithelial-mesenchymal transition (EMT) in epithelial tumors such as gastric cancer is an important step in tumor metastasis (1). During the EMT process, cell invasiveness and migration are enhanced, and tumor cells acquire stem cell-like characteristics, which promote tumor invasion and metastasis (2-4).

High mobility group protein A2 (HMGA2) is a non-histone chromosomal protein, which plays a structural role as a transcription factor primarily by binding the AT-rich regions in the DNA sequence of target genes; this protein thus affects embryogenesis, tissue development and tumorigenesis (5). In recent years, HMGA2 has been reported to be highly expressed in tissues derived from thyroid (6), colon (7) and gastric cancer (5). It has also been reported that HMGA2 can induce tumor cells to undergo EMT via interference of the cell cycle (8-11), that HMGA2 can help tumor cells acquire stem cell properties, and that it can promote tumor metastasis (12). Recent studies have demonstrated that HMGA2 may promote EMT in gastric cancer (13) and promote an increase in the expression of several tumor markers involved in breast cancer (14). HMGA2 serves as a specific downstream target of Lin28b, which regulates the self-renewal of mouse hematopoietic stem cells (15). HMGA2 has been suggested to mediate breast tumor metastasis via the promotion of the expression of LOX and syndecan-2 (16). Previous studies, including ours, have shown that HMGA2 may be responsible for the invasiveness and metastasis of gastric cancer and for its poor prognosis (5,13,17), yet the related mechanism remains unclear.

Recently, TWIST proteins have been re-established as important transcription factors associated with EMT (18,19); Twist-related protein 1 (TWIST1) is most closely related to tumor metastasis and angiogenic mimicry formation. Some studies have reported that the potential combination
of TWIST1 and E-box, which is an E-cadherin promoter, negatively regulates the transcription of the E-cadherin gene. This in turn induces EMT and the acquisition of tumor stem cell properties, which enhances invasiveness, metastasis and the angiogenic capacity of breast cancer cells (20-22). One study revealed that TWIST1 enhanced cell migration beyond EMT, as TWIST1 induced the activation of Rac1 (23). Yang et al (24) confirmed that TWIST1 may be incorporated into the intron region of the BMI1 gene and that it can generate a stem cell-like phenotype and promote tumor initiation. TWIST1 overexpression and poor tumor prognosis are associated with a high rate of tumor invasion and metastasis (25). Research has shown that TWIST1 is involved in the malignant progression of colorectal cancer (26), but no evidence has indicated its involvement in gastric cancer.

Our previous study found that HMGA2 induced EMT in gastric cancer cells and that this phenomenon was related to the activation of the Wnt/b-catenin signaling pathway. Using ChIP-on-chip by chromatin immunoprecipitation (ChIP) technology, we also screened TWIST genes that may be associated with EMT in gastric cancer cells (13,27). We speculated that HMGA2 may regulate EMT and the acquisition of tumor stem cell properties via the regulation of the downstream target gene TWIST1. Therefore, the aim of the present study was to investigate the relationship between HMGA2 and TWIST1 in gastric cancer and to determine whether HMGA2 can regulate EMT in gastric cancer cells, as well as the acquisition of tumor stem cell properties, through the regulation of TWIST1.

Materials and methods

Ethics statement. This study was approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University. All of the procedures that involved animals were conducted as indicated in the Guidelines of the National Institutes of Health (NIH) for Animal Care (Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services, NIH Publication no. 86-23, revised 1985). We made every effort to minimize suffering and to minimize the number of animals used.

Patient collection. In total, 172 paraffin-embedded tissue specimens (142 gastric cancer tissues and 30 peritumoral tissues) were collected from the archives of the Department of Pathology, Chongqing Medical University. These tissue samples were subjected to the aminbiotin-peroxidase method for immunohistochemical analysis. An additional 72 fresh surgical specimens (36 gastric cancer tissues and 36 corresponding peritumoral tissues) were obtained from the Department of Gastrointestinal Surgery, First Affiliated Hospital of Chongqing Medical University. All specimens were frozen in liquid nitrogen immediately after surgical resection and were maintained at -80°C until protein extraction was performed. None of the patients had received preoperative treatment, such as radiation or neoadjuvant chemotherapy.

Immunohistochemical analysis. Tissue sections were deparaffinized in two changes of xylene, and antigen retrieval was performed by heating the sections in 0.01 M sodium citrate buffer in a microwave oven at 95°C for 15 min. The slides were incubated in 3% hydrogen peroxide for 15 min and were then incubated in 0.5% Triton-X-100 (Beyotime Biotech, Jiangsu, China) for 15 min. The slides were blocked with 5% BSA for 30 min and were incubated with rabbit primary antibodies against human HMGA2 (1:100; Cell Signaling Technology, Danvers, MA, USA) and TWIST1 (1:100, Proteintech Group, Inc., Wuhan, China) overnight at 4°C. Negative controls were treated identically but were not incubated with the primary antibodies. Tissue sections were washed in PBS (3x5 min), incubated with the appropriate secondary antibody for 30 min, washed in PBS (3x5 min) and incubated for 30 min with streptavidin-HRP (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). Immunoreactivity was visualized using a DAB kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.); the slides were counterstained with hematoxylin and mounted with PVP. The percentage of positive cells was scored according to the following criteria: 0, <10%; 1, 10-30%; 2, >30-50%; and 3, >50% (28). The patients were subsequently categorized into either a positive expression group (score of 1-3) or a negative expression group (score of 0).

Cell culture. MKN-45, MKN-28 and SGC-7901 human gastric carcinoma cell lines were obtained from the Key Laboratory of General Surgery at the First Affiliated Hospital of Chongqing Medical University. TheGES-1 human immortalized gastric epithelial cell line was purchased from the Chinese Type Culture Collection. The cell lines were cultured in RPMI-1640 medium (HyClone, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 2% penicillin-streptomycin (Beyotime) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were cultured until a confluence of 80% was reached, at which point they were passaged by trypsinization.

Western blot analysis. The cells were lysed in buffer containing 1% NP40, 1 mmol/l EDTA, 50 mmol/l Tris-HCl (pH 7.5), and 150 mmol/l NaCl supplemented with a complete protease inhibitor mixture (Sangon Biotech, Shanghai, China). Total proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then blocked in 5% non-fat milk in TBS for 2 h and probed with antibodies to HMGA2 (1:1,000; Cell Signaling Technology), TWIST1 (1:1,000; Proteintech Group), E-cadherin (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), N-cadherin (1:1,000) and vimentin (1:1,000; both from eBioscience, Inc., San Diego, CA, USA), Oct4 (1:1,000; San Ying Biotechnology, Wuhan, China), CD44 (1:1,000; San Ying Biotechnology) and GAPDH (1:500; Millipore). After the membranes were washed, they were incubated with the appropriate secondary antibodies for 1 h at 37°C. The results were visualized using an enhanced chemiluminescence kit (Beyotime Biotech). Each band was quantified using ImageJ software and normalized to GAPDH (Beyotime Biotech).

Cell transfection. Human HMGA2 and TWIST1 cDNAs were cloned into pLV-Ubc-IRE52-EGFP via homologous recombination [SKE (Shanghai) Bio-pharmaceutical, Shanghai, China]. The HMGA2 shRNA sequences, the TWIST1 shRNA sequences and the scrambled RNA were synthesized by Ribobio.
Corporation (Guangzhou, China). The TWIST1 shRNA sequences and the scrambled RNA were cloned into the hU6-Neomycin Vector by S&E (Shanghai) Bio-pharmaceutical. The cells were transiently transfected with TWIST1 shRNA or scrambled RNA (con) using Lipofectamine 2000 (Invitrogen Life Technologies, Shanghai, China) according to the manufacturer's instructions. The TWIST1 overexpression vector was purchased from S&E (Shanghai) Bio-pharmaceutical. The sequences of the HMGA2- and TWIST1-specific shRNAs used in these experiments were as follows: control-shRNA, 5'-GAC GAGCCGCCACGTGCACATT-3'; HMGA2-shRNA, 5'-CCGC CAAGAGGCAAGACCTATT-3'; TWIST1-shRNA, 5'-CGGC AAAGCTGAAGAAAGCCAAATC-3'. The cells were plated in 6-well clusters and transfected for 24 h or 48 h. Transfected cells were used in further assays or for RNA/protein extraction.

Luciferase reporter assay. The ChIP-on-chip experiments showed that HMGA2 could bind to the 5650-5900 bp region before the TWIST1 transcriptional start site; therefore, this region was amplified by PCR and inserted into the pGL3-Promoter luciferase reporter vector (Promega, Madison, WI, USA). All constructs were verified by DNA sequencing. SGC-7901 cells were plated in 96-well clusters and then co-transfected with 100-ng constructs with or without the HMGA2 overexpression plasmid. At 48 h after transfection, the luciferase activity was detected using a dual-luciferase reporter assay system (Promega) and was normalized to Renilla luciferase activity. All primers were purchased from Genewiz Biotechnologies, Inc. (Suzhou, China) and are as follows: forward, 5'-TCTATCGATAGGTACCAGGAAAA AAGCTGAGCAAGATTC-3'; reverse, 5'-GATCGCAGATCGAAGAGGCAGACCTATT-3'; TWIST1-shRNA, 5'-GATCGCAGATCGAAGAGGCAGACCTATT-3'; HMGA2-shRNA, 5'-CGGC AAAGCTGAAGAAAGCCAAATC-3'. The cells were transiently transfected with TWIST1 shRNA or scrambled RNA (con) using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) with RPMI-1640 medium with 2% FBS. The bottom chamber was filled with 600 µl RPMI-1640 medium with 10% FBS. After 48 h of incubation, cells on the lower surface of the membrane were stained with crystal violet. The number of cells was counted in three random fields under a light microscope.

Wound healing assay. HepG2 and SMMC7721 cells were transfected with the following: HMGA2 shRNA or HMGA2 overexpression vectors alone; HMGA2 shRNA combined with the TWIST1 overexpression clone; or the HMGA2 overexpression clone with TWIST1 shRNA. The transfected cells were seeded in 6-well plates and allowed to grow to 100% confluence; afterwards, a scratch was made across the cell monolayer. The cells were rinsed with PBS and fresh growth medium was added. The cells were incubated for 24 h and then imaged using a phase-contrast microscope.

Cell invasion assay. For the cell invasion assay, 1x10^4 cells were seeded on Matrigel-coated Transwell cell-culture inserts (Invitrogen Life Technologies, Carlsbad, CA, USA) with RPMI-I640 medium with 2% FBS. The bottom chamber was filled with 600 µl RPMI-I640 medium with 10% FBS. After 48 h of incubation, cells on the lower surface of the membrane were stained with crystal violet. The number of cells was counted in three random fields under a light microscope.

Animal experiment. Female BALB/c mice at 6 weeks of age were obtained from the National Biological Industry Base, Laboratory Animal Center of Chongqing Medical University. The mice were randomly divided into four groups (five mice per group) and were maintained under pathogen-free conditions. The mice were intravenously injected with MKN-45 cells, and the mice were dissected four weeks after inoculation to observe MKN-45 cell metastasis.

Statistical analysis. All experimental data are presented as the means ± SD, and single comparisons between two groups were evaluated by Student's t-test using SPSS 20.0. Associations between the expression levels of HMGA2 and TWIST1 were analyzed by the Pearson correlation coefficient. P<0.05 was considered statistically significant.

Results

Protein expression of HMGA2 and TWIST1 is high in gastric cancer according to immunohistochemistry and western blot analysis. To investigate the association between HMGA2 and TWIST1 expression in gastric cancer, we performed immunohistochemical staining for these proteins in tissue specimens from 142 gastric cancer patients. The staining for HMGA2 and TWIST1 in representative clinical samples is shown in Fig. 1A. Among the 142 gastric cancer specimens, 75 (52.8%) and 65 (45.8%) cases were positive for HMGA2 and TWIST1 expression, respectively. In addition, immunohistochemical analysis showed that the expression of HMGA2 and that of TWIST1 were positively correlated (P<0.01; Table I). HMGA2 and TWIST1 were largely expressed in the nucleus of gastric cancer cells. The expression levels of HMGA2 and TWIST1 were significantly higher in gastric cancer tissues compared with those in the peritumoral tissues (P<0.01). Additionally, we tested the protein expression levels of HMGA2 and TWIST1 in 36 paired tissue samples via western blot analysis. As expected, the protein expression levels of HMGA2 and TWIST1 were significantly higher in the gastric cancer tissues than these levels in the peritumoral tissues (P<0.05; Fig. 1B).

Expression of HMGA2 and TWIST1 in gastric cancer cell lines. To investigate whether HMGA2 and TWIST1 levels are also increased in gastric cancer cell lines compared with immortalized gastric epithelial cells, we assessed the HMGA2 and TWIST1 protein levels in the human gastric cancer cell lines MKN-45, MKN-28, and SGC-7901, as well as in the human immortalized gastric epithelial cell line GES-1, by western blot analysis. The HMGA2 and TWIST1 protein levels were significantly higher in the poorly differentiated gastric cancer cell line (MKN-45), the moderately differentiated cell

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Figure 1. HMGA2 and TWIST1 were highly expressed in gastric cancer tissues and gastric cancer cells. (A) Expression of HMGA2 and TWIST1 in gastric cancer tissues and adjacent normal tissues was detected by immunohistochemistry. (B) Expression of HMGA2 and TWIST1 in gastric cancer tissues and normal adjacent tissues was detected by western blot analysis. N, adjacent normal tissues; T, tumor tissues; 10 gastric cancer tissues were randomly tested. (C) Expression of HMGA2 and TWIST1 was detected by western blot analysis in the gastric cancer cell lines MKN-45, MKN-28, and SGC-7901 and in the normal gastric cell line GES-1. The assay was repeated three times. *P<0.05, **P<0.01. HMGA2, high mobility group protein A2.

Figure 2. Expression of HMGA2 is regulated by the upstream sequence in the TWIST1 coding region. (A) HMGA2 expression was inhibited in MKN-45 cells, and HMGA2 was overexpressed in SGC-7901 cells. The protein expression of HMGA2 and TWIST1 was then detected by western blot analysis. The experiment was repeated three times. (B) Construction of the luciferase reporter vector plasmids of the upstream sequence of the TWIST1 coding region, the co-transfection of SGC-7901 cells with the HMGA2 expression plasmid, and the detection of the luciferase activity of the reporter vector. The experiment was repeated three times. *P<0.05. HMGA2, high mobility group protein A2.
TWIST1 is a direct target of HMGA2 in gastric cancer cells. HMGA2 and TWIST1 protein expression levels in various gastric epithelial cell lines such as MKN-45-con shRNA, MKN-45-HMGA2 shRNA, SGC-7901-NC, and SGC-7901, in which HMGA2 was originally overexpressed, were detected by western blot analysis. It was found that after the application of HMGA2 shRNA in the MKN-45 cells, the expression levels of HMGA2 and TWIST1 were significantly decreased (P<0.05; Fig. 2A). After HMGA2
HMGA2 regulates the invasion and migration of gastric cancer cells via TWIST1. The inhibition of HMGA2 suppressed the invasion and migration of MKN-45 cells, and the simultaneous overexpression of TWIST1 reversed the inhibitory effect of HMGA2 expression on the invasion and migration of gastric cancer cells (Fig. 3A and B). The overexpression of HMGA2 significantly promoted the invasion and migration of SGC-7901 cells, whereas the simultaneous inhibition of TWIST1 expression reversed the promotive effect of HMGA2 overexpression on the invasion and migration of gastric cancer cells (Fig. 3A and B).

Overexpression of TWIST1 reverses the inhibitory effect of HMGA2 abrogation on the metastasis of gastric cancer cells in nude mice. To further investigate whether HMGA2 regulates the invasion and migration of gastric cancer cells via TWIST1, we injected MKN-45 cells into the tail veins of mice to detect the in vivo metastasis of MKN-45 cells after the application of HMGA2 shRNA. The results showed that the metastatic ability of MKN-45 cells was significantly decreased after the abrogation of HMGA2 expression, whereas the simultaneous overexpression of TWIST1 reversed the inhibitory effect of HMGA2 interference on the metastasis of gastric cancer cells in nude mice (Fig. 4).

Discussion

HMGA2 plays an important role in EMT and in cancer stem cells (CSCs), but the mechanism by which HMGA2 stimulates downstream target genes to induce EMT and the acquisition of tumor stem cell properties remain unclear. The analysis of downstream regulatory networks of HMGA2 may be an important clue in the exploration of the correlation between EMT and CSCs.

We previously reported that HMGA2 can bind to the TWIST1 promoter region in gastric cancer (13). Therefore, an important regulatory relationship exists between HMGA2 and TWIST1. First, we detected the expression of HMGA2 and TWIST1 in pathological specimens and found that the immunohistochemical and protein expression of HMGA2 and TWIST1 in gastric cancer cells were high and showed a positive correlation compared with that of matched normal adjacent tissues. Furthermore, we detected the correlation between the protein expression of HMGA2 and TWIST1 in gastric epithelial cell lines and found that HMGA2 and TWIST1 were highly expressed in MKN-45 cells and were lowly expressed in SGC-7901 and MKN-28 cells, which was consistent with what has been observed in gastric cancer. To investigate the changes in TWIST1 after its expression was blocked and after the HMGA2 gene was overexpressed, we abrogated HMGA2 expression in MKN-45 cells and overexpressed HMGA2 in SGC-7901 cells. We found that the TWIST1 protein expression level was correspondingly increased and decreased, respectively, in MKN-45 and SGC-7901 cells. To further confirm the regulatory effect of HMGA2 on TWIST1, we constructed a reporter vector of TWIST1 and found that HMGA2 significantly improved the promoter activity of TWIST1. Therefore, both our preliminary results and this experimental result indicate that TWIST1 is a direct downstream target gene of HMGA2.

Previous studies have shown that the overexpression of HMGA2 can significantly promote the invasion and migration of gastric cancer cells (27), whereas TWIST1 overexpression was also found to promote migration and invasion in breast, esophageal and colorectal cancer (26,29-32). However, whether HMGA2 regulates migration and invasion in gastric cancer through TWIST1 has not been confirmed. First, we inhibited the expression of HMGA2 in MKN-45 cells and simultaneously overexpressed TWIST1; we also overexpressed HMGA2 in SGC-7901 cells and simultaneously inhibited...
the expression of TWIST1. We then observed the role of TWIST1 and HMGA2 on invasion and migration, initiation of EMT and acquisition of tumor stem cell properties in gastric cancer cells. The results showed that TWIST1 overexpression reversed the decrease in the invasion and migration abilities and EMT and the expression of stemness markers induced by HMGA2 interference in MKN-45 gastric cancer cells; the results also showed that TWIST1 interference inhibited the increase in HMGA2-mediated invasion and migration, EMT and the expression of stemness markers induced by HMGA2 overexpression in SGC-7901 gastric cancer cells. To further validate the effect of TWIST1 on the metastasis of gastric cancer cells, we performed in vivo tumor metastasis experiments in nude mice. In this model, the expression of HMGA2 was inhibited and TWIST1 was overexpressed in MKN-45 cells. We found that TWIST1 overexpression

Figure 5. HMGA2 affects EMT of gastric cancer cells and the expression of stemness indicators via TWIST1. (A) MKN-45 cells were transfected with con, HMGA2 shRNA, HMGA2 shRNA + NC, or HMGA2 shRNA + TWIST1; and then EMT (E-cadherin, N-cadherin and vimentin expression) and the expression of stemness markers (CD44 and OCT4) in the cells were detected by western blot analysis assay in each group. SGC-7901 cells were transfected with NC, HMGA2, HMGA2 + con, or HMGA2 + TWIST1 shRNA; and then EMT (E-cadherin, N-cadherin and vimentin expression) and the expression of stemness markers (CD44 and OCT4) in the cells were detected by western blot analysis assay in each group. The experiment was repeated three times. (B) Quantification assays of each index of the western blot analysis results. *P<0.05, **P<0.01. HMGA2, high mobility group protein A2.
could reverse the inhibition of HMGA2 interference on the metastasis of MKN-45 cells.

In summary, HMGA2 can directly regulate the expression of TWIST1, which induces migration and invasion, EMT and the acquisition of tumor stem cell properties in gastric cancer cells.

Acknowledgements

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References