HMGA2–FOXL2 Axis Regulates Metastases and Epithelial-to-Mesenchymal Transition of Chemoresistant Gastric Cancer

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Abstract

Purpose: Chemoresistance is the main cause of treatment failure in cancer and is associated with distant metastases and epithelial-to-mesenchymal transition (EMT). This study was aimed to explore the mechanism of metastases and EMT in chemoresistant gastric cancer.

Experimental Design: A key molecular pathway was identified via gene profiling and a bioinformatic analysis in a chemoresistant gastric cancer model. The roles of FOXL2, HMGA2, and ITGA2 were validated via loss-of-function and gain-of-function experiments in vitro and in an orthotopic gastric cancer animal model.

Results: HMGA2 and FOXL2 directly regulated the metastasis and EMT of chemoresistant gastric cancer. The interaction between HMGA2 and pRb facilitated the transactivation of FOXL2 by E2F1, and ITGA2 was the downstream effector of the HMGA2–FOXL2 pathway. HMGA2, FOXL2, and ITGA2 were associated with the TNM classification and staging of gastric cancer and were increased in metastatic lymph nodes and distant metastases. Increased HMGA2, FOXL2, and ITGA2 levels were associated with reduced overall survival periods of patients with gastric cancer.

Conclusions: This study demonstrated that the transactivation of FOXL2 driven by interactions between HMGA2 and pRb might exert critical effects on the metastases and EMT of chemoresistant gastric cancer. Blocking the HMGA2–FOXL2–ITGA2 pathway could serve as a new strategy for gastric cancer treatment.

Chemotherapy is the primary treatment for late-stage cancer. Its therapeutic effects have been confirmed on the basis of its demonstrated ability to reduce the tumor burden and prolong the life span of patients (1). However, most patients ultimately exhibit chemoresistance, which is defined as a reduced response to chemotherapy during the treatment, and this phenomenon could result in local recurrence and distant metastasis (1, 2).

Recent studies have shown that the phenotype of epithelial-to-mesenchymal transition (EMT) can be observed in gemcitabine-resistant pancreatic cancer cells, oxaliplatin-resistant colorectal cancer cells, paclitaxel-resistant breast cancer cells (3–6). Chemoresistant cancer cells might gain cancer stem cell (CSC)-like properties through EMT (7). Mani and colleagues described the gain of stem cell–like properties in immortalized human mammary epithelial cells (HMLE) ectopically expressing Twist or Snail (6). Most of these mesenchymal-like cells generated by EMT acquired an antigenic phenotype typical of neoplastic mammary stem cells (8, 9). As a well-known process in initiating and promoting metastasis, EMT might also help chemoresistant cancer cells acquire enhanced motility (10, 11). However, this hypothesis has been challenged by recent studies in mouse cancer models, which showed that the regulation of EMT by miR-200 or E-cadherin repressors was not associated with distant metastasis but did contribute to the development and reversion of chemoresistance (12, 13).

The forkhead box (FOX) family of proteins are a large group of master transcriptional factors with important roles in chemoresistance and EMT. FOXC2 played critical roles in the transition of epithelial cells to CSC-like cells through EMT (8). FOXC1 and FOXQ1 promoted hepatocellular carcinoma metastasis through the regulation of critical EMT-associated molecules (14, 15). FOX1 was reported to modulate EMT, cell stemness, and chemoresistance in ovarian cancer (16). FOXL2, another member of the FOX protein family, played critical roles in ovary differentiation and maintenance, and its dysregulation could lead to premature ovarian failure (17). FOXL2 was also found to be upregulated in progressive and recurrent granulosa cell tumors that were resistant to conventional chemotherapy (18, 19). However, the effects of FOXL2 on EMT and malignant phenotypes of other types of cancer remain unclear.

Cells that have undergone EMT could acquire certain mesenchymal characteristics. HMGA2, a member of the high mobility group (HMG) protein family, is predominantly expressed in...
Translational Relevance

In this study, we revealed the important function of HMGA2–FOXL2 pathway in regulating multiple malignant behavior of gastric cancer, and confirmed its association with EMT, metastatic status, and survival of patients with gastric cancer. The adequate histologic and IHC analyses of endoscopic and surgical tissue specimen are the key steps to the accurate diagnosis and treatment. On the basis of our data from clinical specimen and public sequencing database, HMGA2 and FOXL2 could be helpful to evaluate the risk of metastasis and disease survival by immunostaining analysis in tissues. Also, we have revealed that manipulating the expression of HMGA2 or FOXL2 could directly affect the metastatic behavior of gastric cancer cells, and thus it raises the assumption that HMGA2 and FOXL2 could serve as potential therapeutic targets in gastric cancer management.

Materials and Methods

Cell culture

Cell lines MKN45, AGS, and MKN28 were purchased from China Infrastructure of Cell Line Resources in September 2013. BGC823, SGC7901, and chemoresistant cell model SGC7901/ADR were maintained in our laboratory. All cell lines were maintained in RPMI1640 or DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO₂.

All cells were tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza). The latest test was performed in November, 2015. Cells with negative detection results were passaged 3 days after thawing and then frozen for long-term storage. All experiments involving the cell lines were performed within five passages.

mRNA microarray hybridization and analysis

Total RNA from SGC7901 cells overexpressing FOXL2 or FOXL2-silenced SGC7901/ADR cells was extracted and checked for its RNA integrity number (RIN) to examine RNA integration using an Agilent Bioanalyzer 2100 (Agilent Technologies). The mRNA was labeled, and 600 ng of Cy3-labeled RNA was hybridized to slides using a Gene Expression Hybridization Kit. After hybridization, the slides were washed in staining dishes (Thermo Shandon) with a Gene Expression Wash Buffer Kit. Then, the slides were scanned using an Agilent Microarray Scanner on the default settings. Raw data were normalized using the Quantile Algorithm in Gene Spring Software 11.0.

Western blot analysis

Protein samples were prepared using a RIPA lysis buffer [25 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% TritonX-100] containing protease inhibitor cocktail tablet (Roche Applied Science). Proteins were separated via SDS-PAGE and were transferred to a nitrocellulose membrane. After blocking with Tris-buffered saline containing 5% skimmed milk and 0.1% Tween-20 for 1 hour at room temperature, the membrane was incubated with anti-HMGA2, anti-FOXL2, anti-ITGA2, or anti-actin antibody at 4°C overnight. The next day, the membrane was washed and incubated with a goat anti-mouse or goat anti-rabbit secondary antibody (Boster) for 1 hour at room temperature, and enhanced chemiluminescence was then used to visualize the protein bands in a Bio-Rad ChemiDoc XR5 Imaging System. Antibodies include anti-HMGA2 (#5269, Cell Signaling Technology Inc.), anti-FOXL2 (#19672-1-AP, Proteintech), anti-FOXL2 (#2387, Boster, for IHC only), anti-ITGA2 (#ab133557, Abcam), anti-E-cadherin (#14472, clone 4A2, Cell Signaling Technology Inc.), anti-vimentin (#5741, clone D21H3, Cell Signaling Technology Inc.), anti-Rb (#9309, clone 4H1, Cell Signaling Technology Inc.), anti-pRb (#8516, clone D20B12, Cell Signaling Technology Inc.), anti-vimentin (#5741, clone D21H3, Cell Signaling Technology Inc.), anti-E-cadherin (#14472, clone 4A2, Cell Signaling Technology Inc.), and anti-actin (#A2228, clone AC-74, Sigma-Aldrich).

qRT-PCR

Total RNA was extracted using TRIZol (Invitrogen), and reverse transcription was performed using the Advantage RT-for-PCR Kit (Takara Bio) according to the manufacturer’s instructions. For the real-time PCR analysis, dsDNA were amplified using a SYBR Green PCR Kit (Takara Bio). The cycling parameters were as follows: 95°C for 1 minute, followed by 45 cycles of 95°C for 10 seconds and 55°C–60°C for 30 seconds. A melting curve analysis was then performed. The Ct was measured during the exponential amplification phase, and the amplification plots were analyzed using CFX96 software (Bio-Rad). Expression levels were normalized to the fold change that was detected in the corresponding control cells, which was defined as 1.0. All reactions were performed in triplicate. The primer sequences are listed in Supplementary Table S1.

Immunofluorescence analysis

Cells were seeded in 4-well chamber slides, and then washed with sterile PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then, they were permeabilized with 1% Triton X-100 in 50 mmol/L Tris–HCl (pH 7.4) containing...
samples were washed in PBS three times and incubated with Cy3-conjugated secondary antibody (1:500) for 1 hour. Immunofluorescence images were captured using an Olympus Fluoview FV1000 confocal microscope.

**Plasmid construction and transfection**

FOX2L, HMG2A, and ITGA2 plasmids, and all the lentiviral vectors (full-length molecules and shRNAs) were obtained from GeneCopoeia. siRNA for FOX2L, HMG2A, and ITGA2 were purchased from Ribobio Co., Ltd, and the sequences of siRNA are listed in Supplementary Table S2. Target cells were transfected with oligonucleotides using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

**In vitro migration and metastasis assays**

A total of 1 × 105 cells were seeded in media without FBS into the top chamber of each transwell, which was precoated with Matrigel. Medium with 20% FBS was placed in the bottom chamber. Twenty-four hours after seeding, noninvasive cells in the top chamber were removed with a cotton swab, and the cells on the lower surface of the membrane were fixed and stained with crystal violet and photographed at 200× magnification using an Olympus BX51 microscope. Photographs of three random fields from triplicate wells were recorded, and the number of cells was counted. For the migration assays, all procedures were the same as in invasion assays except that 5 × 104 cells were seeded in each chamber without Matrigel coating.

**Orthotopic mouse model and in vivo luciferase imaging**

Nude mice (6 to 8 weeks old) were obtained from Vital River Laboratories (Beijing, China). First, 3 × 106 cells were subcutaneously injected into the flanks of the mice to generate the subcutaneous tumors used for tissue transplantation. Two weeks later, isolated subcutaneous tumors were cut into pieces (2 mm³) and kept on ice prior to orthotopic implantation surgery. Before performing surgery under sterilized condition, mice were anesthetized with isoflurane via inhalation. A 2-cm incision was made on the left abdominal flank and the stomach was isolated and fixed in position. A partial-thickness cut in the stomach wall was made using a fine needle, and a piece of the extracted tumor was sutured into the incision. After 6 weeks, mice were injected intraperitoneally with α-luciferin (Caliper Life Sciences) and allowed to move freely for 5 minutes to promote the absorption of the substrate. After anesthetization, whole-body live images were captured using the IVIS Imaging System (Caliper Life Sciences). As the strong signals from the orthotopic tumor masked the much weaker lung and liver metastatic signals, mice were then immediately sacrificed, and whole lungs and livers were harvested for the detection of bioluminescence signals. Finally, organs and tumor tissues were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

**Immunoprecipitation**

A/G-Agarose (Santa Cruz Biotechnology) was washed with RIPA lysis buffer three times (5,000 rpm for 5 minutes) and then incubated with BSA (1 mg/mL) for 30 minutes. After this incubation, A/G-Agarose was washed with RIPA lysis buffer three times (5,000 rpm for 5 minutes). Cell lysis was centrifuged for 10 minutes at 12,000 rpm and 500 µL of supernatants were transferred to another tube. Three micrograms of IgG (Santa Cruz Biotechnology) and primary antibodies, together with 20 µL A/G-Agarose were added into cell lysis and incubated at 4°C for 12 hours. After this incubation, the samples were centrifuged, and the beads were washed with RIPA buffer three times (5,000 rpm for 5 minutes). The remaining beads were added to 30 µL of RIPA buffer with 5× sample buffer, denatured at 90°C for 10 minutes, and used for Western blot assays.

**Luciferase reporter assay**

Plasmids carrying the wild-type FOXL2 promoter sequence Luc-FOXL2 and the mutant Luc-FOXIL2-mu were synthesized (GeneCopoeia). Luciferase activity was detected using the Dual Luciferase Assay kit (GeneCopoeia) according to manufacturer’s instructions. Briefly, 1 × 105 cells/well were plated in a 24-well plate. After 12–24 hours, the cells were cotransfected with 0.6 µg of the expression vector plasmids, 0.18 µg of the promoter reporter plasmids, and 0.02 µg of the pRL-TK plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Eight hours later, the transfected cells were lysed in culture dishes containing a lysis buffer, and the resulting lysates were centrifuged at maximum speed for 1 minute in an Eppendorf microcentrifuge. The relative luciferase activity of the samples was determined using a Modulus TD20/20 Luminometer (Turner Biosystems), and the transfection efficiencies were normalized according to their Renilla activity.

**Tissue microarrays**

Tissue microarrays HStm-Ade076Met-1 and HStm-Ade120-Lym-01 were purchased from Shanghai Outdo Biotech. HStm-Ade076Met-1 contained 28 cases of primary gastric cancer and corresponding metastasis tissues, and HStm-Ade120Lym-01 contained 32 cases of primary gastric cancer and corresponding metastatic lymph nodes. Another two microarrays (T14-501, containing 166 gastric cancer cases; T13-385, containing 83 gastric cancer cases) with complete pathologic and survival information were provided by Xijing Hospital of Digestive Diseases, Fourth Military Medical University (Xi'an, Shaanxi, China).

**IHC**

Briefly, the sections were dewaxed in xylene and rehydrated in ethanol and PBS. Endogenous peroxidase was inactivated using 3% H2O2, and antigen retrieval was performed using the Tris-EDTA antigen retrieval buffer for HMG2A and FOXL2 staining and the Citrate Antigen Retrieval Solution for ITGA2 staining. Sections were blocked with 10% normal goat serum at room temperature for 15 minutes and incubated with primary antibodies against HMG2A, FOXL2, or ITGA2 at 4°C for 12 hours. Sections were then incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (Dako) at room temperature for 30 minutes. The staining was visualized using a DAB kit (Zhongshan Golden Bridge Biotechnology Company).

All sections were examined and scored independently by two investigators in a double-blinded manner. Staining intensity was determined according to a histologic scoring method. Briefly, the proportion of positive cells in each specimen was quantitatively evaluated and scored as follows: 0, staining in 0% of the cells examined; 1, 0.01%–25%; 2, 25.01%–50%; 3, 50.01%–75%; and 4, >75%. The staining intensity was graded as follows: 0, no
enhanced migration and invasion of cells. The results suggested that SGC7901/ADR cells displayed increased cell motility and EMT markers using qRT-PCR showed that the expression levels of EMT-promoting transcriptional factors increased in SGC7901/ADR cells while the expression of the mesenchymal marker vimentin decreased, indicating that FOXL2 antagonized the effects of HMGA2 knockdown on cell motility. To further validate the functional association between HMGA2 and FOXL2, we transfected FOXL2 into chemoresistant gastric cancer cells and observed that ectopic FOXL2 expression promoted the functional association between HMGA2 and FOXL2 in vitro.

### Results

**HMGA2 and FOXL2 are associated with metastasis and EMT of chemoresistant gastric cancer**

A chemoresistant cell model (SGC7901/ADR) was established from the human gastric adenocarcinoma cell line SGC7901 through serial Adriamycin (ADR) induction. We compared cell motility between the chemoresistant and parental gastric cancer cells. The results suggested that SGC7901/ADR cells displayed enhanced migration and invasion in vitro. An increased incidence of metastasis and a greater number of lung metastases were also observed in nude mice with chemoresistant cells injected into their tail veins. We also established an orthotopic model for observing tumor in situ and metastases, which were then isolated and used for the establishment of secondary model. After 3-week administration of 5-fluorouracil (5-FU), tumors derived from lung metastasis exhibited stronger signals than those derived from in situ tumors, indicating that cells in the metastasis site showed resistance to 5-FU treatment.

**HMGA2 regulates cell motility and EMT markers by targeting FOXL2**

First, we examined the roles of HMGA2 in regulating cell motility and EMT in gastric cancer. Similar to the results for FOXL2, knockdown of HMGA2 suppressed cell motility in vitro and in vivo and reversed EMT-like molecular changes in E-cadherin, vimentin, and Snail1 expression. Conversely, the ectopic expression of HMGA2 promoted cell motility and induced EMT. To further validate the functional association between HMGA2 and FOXL2, we transfected FOXL2 into chemoresistant gastric cancer cells and observed that ectopic FOXL2 expression antagonized the effects of HMGA2 knockdown on cell motility and increased vimentin expression in cells in which HMGA2 had been knocked down.

**FOXL2 increases cell motility and induces EMT in chemoresistant gastric cancer cells**

To examine the effects of FOXL2 on cell motility, we performed transwell assays and found that the inhibition of FOXL2 in SGC7901/ADR cells impaired their migratory and invasive potential, while ectopic FOXL2 expression in SGC7901 cells significantly increased the number of cells migrating to the bottom side of membrane. Using the orthotopic model, we observed synchronous but inverse correlations of HMGA2 and FOXL2 with E-cadherin via IHC staining in gastric cancer tissues. Indicating that HMGA2 and FOXL2 should be associated with EMT of gastric cancer.
BGC823 cells were pretreated with ADR (1 and 2 μg/mL), and enhanced cell motility and EMT-like molecular features were also observed (Supplementary Fig. S4A). We also used AGS cell line as another cell model and treated cells with 5-FU (10 μg/mL) for 24, 48, and 72 hours. It was showed that longer exposure to low concentration of 5-FU could increase cell motility and induce EMT.

**Figure 1.**
HMGA2 and FOXL2 were associated with metastasis and EMT of chemoresistant gastric cancer. A and B, Chemoresistant gastric cancer cells (SGC7901/ADR) acquired increased migrating and invasive capacities than chemosensitive gastric cancer cells (SGC7901) in vitro (A) and in vivo (B). *P < 0.01. C, E-cadherin was decreased while Vimentin was increased in chemoresistant gastric cancer cells. Red, E-cadherin; blue, nucleus. D, qRT-PCR showed that Snail1, ZEB1, and ZEB2 were increased in chemoresistant gastric cancer cells. **P < 0.01; ***P < 0.001. E, cDNA array, GO analysis, and survival database search for 8 transcription-associated genes. F, Both HMGA2 and FOXL2 were inversely correlated with E-cadherin in gastric cancer tissues.
The interaction between HMGA2 and pRb facilitates the transcriptional activation of FOXL2 by E2F1

We have observed that knockdown of HMGA2 inhibited FOXL2, and we further confirmed that FOXL2 was increased by ectopic HMGA2 (Fig. 4A). The architectural transcription regulator HMGA2 may facilitate the function of some transcription
factors. One such factor is E2F1, whose transcriptional activity was increased in the drug-resistant gastric cancer cells as indicated by the expression of several signature target genes including CDC2, CCNE1, and TK1 (Supplementary Fig. S1D). Meanwhile, when we measured E2F1 expression via Western blot assays, we detected no elevation in its expression (Supplementary Fig. S1B and S1C), suggesting that it might be an increase in transactivity rather than an increase in expression that accounts for the effects of E2F1 in chemoresistant gastric cancer cells.

To explore the association between E2F1 and FOXL2, we transfected E2F1 into SGC7901 cells and found that FOXL2 and classical E2F1 downstream effectors were increased (Fig. 4B and Supplementary Fig. S2E). Conversely, knockdown of E2F1 led to decreased FOXL2, CDC2, CCNE1, and TK1 (Fig. 4C and Supplementary Fig. S2F). By JASPAR, we found a putative binding site (GAAGGCGCCTGA, /C0 1970 to /C0 1959) of E2F1 in FOXL2 promoter region. By luciferase reporter gene assay, E2F1 could increase the luminescence of the luciferase reporter containing the potential wild-type FOXL2 promoter, while there was no such effect of E2F1 on the reporter gene containing the FOXL2 promoter sequence but lacking the potential E2F1-binding site predicted (Fig. 4D). The effects of E2F1 on FOXL2 transactivity were also revealed by the observations that the overexpression of E2F1 increased the expression signature of this same panel of genes (Supplementary Fig. S2E).

Previously, Fedele and colleagues reported that the interaction between HMGA2 and pRb enhanced the activity of E2F1 in pituitary tumorigenesis (25). pRb was a well-known repressor for E2F1 function. By knockdown of Rb by siRNAs, FOXL2 and classical E2F1-targeted molecules were increased (Fig. 4E; Supplementary Fig. S2G). We then performed an immunoprecipitation assay in SGC7901/ADR cells and confirmed the mutual interaction between HMGA2 and pRb (Fig. 4F). We then constructed a truncated Flag-tagged HMGA2 which could not interact with pRb (25), and established SGC7901 subline with stable expression of ectopic HMGA2 (Fig. 4G). FOXL2 promoter and siRNAs for E2F1 were then transfected into SGC7901-stable HMGA2 and SGC7901-stable truncated HMGA2. The luciferase reporter assay showed that knockdown of E2F1 abolished the effects of HMGA2 on FOXL2 transcription (Fig. 4H). In addition, truncated HMGA2 failed to affect the luciferase activity of the FOXL2 promoter reporter gene (Fig. 4H). Taken together, these results showed that the interaction between HMGA2 and pRb facilitated the transcriptional activation of FOXL2 by E2F1.

ITGA2 is a downstream effector of FOXL2

To explore the downstream effectors of FOXL2, we silenced FOXL2 in SGC7901/ADR cells and transfected FOXL2 plasmids into SGC7901 cells. The mRNA profiles of these cells, determined via cDNA microarray, were analyzed, and 6 genes (RGS2, EGFR, PLEK2, TGFb3, ITGA5, and ITGA2) were found to be positively correlated with FOXL2 (Fig. 5A). It was further confirmed that ITGA2 expression was reduced by siRNAs for FOXL2 (Fig. 5B). In addition, silencing ITGA2 significantly reduced the cell motility of chemoresistant gastric cancer cells and reversed EMT (Fig. 5C and E), and its ectopic expression exerted opposite effects (Fig. 5D and F).

To further determine the functional correlation between FOXL2 and ITGA2, we knocked down ITGA2 in SGC7901 cells pretreated with siRNAs for FOXL2. We found that the knockdown of ITGA2 significantly increased E-cadherin expression caused by FOXL2 and had an
antagonistic effect on FOXL2-induced cell motility in vitro and in vivo (Fig. 5G and H).

High HMGA2, FOXL2, and ITGA2 expression is associated with poor prognosis in patients with gastric cancer

To fully evaluate the clinical significance of HMGA2, FOXL2, and ITGA2 in patients with gastric cancer, we analyzed the expression of HMGA2, FOXL2, and ITGA2 in four sets of gastric cancer tissue microarrays via IHC staining. In 32 pairs of primary gastric cancer tissue samples and corresponding lymph node metastases, the staining intensities of HMGA2 (19/32), FOXL2 (20/32), and ITGA2 (18/32) were significantly higher in the lymph node metastases (Fig. 6A; Supplementary Fig. S5A and S5B). In 28 pairs of primary gastric cancer tissue samples and corresponding distant metastases, the staining intensities of HMGA2 (16/28), FOXL2 (16/28), and ITGA2 (15/28) were also significantly higher in the distant metastases (Fig. 6A; Supplementary Fig. S5C and S5D).

Compared with their expression in nonmetastatic primary gastric cancer tissues, HMGA2, FOXL2, and ITGA2 levels were increased in metastatic primary gastric cancer tissues (Fig. 6A; Supplementary Tables S3–S5). In 249 gastric cancer cases with prognostic information, the intensities of HMGA2, FOXL2, and ITGA2 in late-stage gastric cancer (stage III and IV) were stronger than those in relative early-stage gastric cancer (stage I and II), and their expression levels were also correlated with T, N, and M staging separately (Supplementary Table S6). In addition, a Kaplan–Meier survival analysis showed that high expression levels of any of these three proteins was correlated with a poor prognosis: patients with higher expression levels of just one or a combination of these genes exhibited reduced overall survival compared with patients with a reduced expression of these genes. These data suggested the potential roles of the HMGA2–FOXL2–ITGA2 pathway in evaluating the metastatic potential and the prognosis of patients with gastric cancer.

Conclusion

Chemoresistance and metastasis are closely linked phenotypes during malignant tumor progression. In clinical practice, a lack of response to chemotherapy in solid tumors can be evaluated on the basis of the modified Response Evaluation Criteria in Solid Tumors (mRIST) criteria, and this lack of response can be associated with another round of uncontrolled proliferation or
even metastasis. In our study, SGC7901/ADR cells, an ideal cell model for examining acquired chemoresistance in gastric cancer (28–31), exhibited enhanced metastatic capacities with EMT-like morphologic and molecular characteristics. Using gene profiling, bioinformatics, and functional studies, we identified HMGA2–FOXL2–ITGA2 signaling as an important pathway in metastasis and the EMT of chemoresistant gastric cancer. We also examined the patterns and intensities of HMGA2, FOXL2, and ITGA2 expression in four sets of gastric cancer tissue microarrays and found that they were significantly increased in metastases relative to their

Figure 5.
ITGA2 was the effector of FOXL2. A and B, FOXL2 was found to regulate ITGA2 by cDNA array (A) and Western blotting (B); *, P < 0.01. C, Knockdown of ITGA2 by 3 pairs of siRNAs increased E-cadherin and decreased vimentin; †, P < 0.01. D, ITGA2-induced increased vimentin but decreased E-cadherin; ‡, P < 0.01. E, Inhibition of ITGA2 reversed high migrating and invasive potential of SGC7901/ADR cells in vitro and in vivo (n = 6 for each group; •, P < 0.01). F, Ectopic ITGA2 expression increases metastasis of gastric cancer cells; ‡, P < 0.01. G and H, Knockdown of ITGA2 rescued FOXL2-induced migration and invasion in SGC7901 cells and reversed EMT-like E-cadherin expression. Red, E-cadherin; blue, nucleus.
expression in primary gastric cancer tissues. Metastatic primary gastric cancer samples also showed stronger staining of these three proteins than nonmetastatic gastric cancer samples. Individual and combined HMGA2, FOXL2, and ITGA2 levels could serve as potential markers for reduced overall survival in patients with gastric cancer.

Figure 6.
HMGA2–FOXL2–ITGA2 pathway was aberrantly activated in gastric cancer tissues with lymph node or distant metastasis and correlated with overall survival periods in patients with gastric cancer. A, IHC analysis of HMGA2, FOXL2, and ITGA2 in primary gastric cancer and corresponding lymph node or distant metastases. Similar analysis was also performed in primary gastric cancer with different metastatic status. B, The correlation between HMGA2 (FOXL2, ITGA2) and overall survival was individually analyzed in 249 gastric cancer cases. C, The prognosis significance of combined HMGA2, FOXL2, and ITGA2 in gastric cancer cases.
EMT, which is marked by the loss of epithelial markers and the acquisition of mesenchymal markers, has been widely regarded as a process that initiates cancer metastasis (9–11). Meanwhile, a large body of evidence suggests that EMT is associated with drug resistance in cancer (7). In fact, cancer cells that have undergone EMT could acquire the intrinsic chemoresistant properties of CSCs (7). EMT also interacts with various pathways, including NFκB, Wnt, and Notch signaling, which play important roles in antagonizing chemotherapy-induced cell apoptosis (7). The chemoresistant gastric cancer model used in our study exhibited enhanced cell motility and EMT characteristics, but we could not definitively conclude that increased cell motility was driven by EMT. Actually, the most recent studies show that EMT might be essential for chemoresistance but may not be necessary for distant metastasis (12, 13). Therefore, whether changes in cell motility and EMT occur in parallel or have a cause-and-effect relationship remains to be determined.

Various stimuli could enhance the expression of a panel of EMT-promoting transcription factors, such as Snail, Twist, and ZEB, which repress epithelial markers and promote mesenchymal markers (32, 33). The gap between the original stimuli and the expression of EMT transcription factors can be bridged directly or indirectly by multiple signaling pathways, including that of the FOX family of transcription regulators. FOXC1 and FOXQ1 have been reported to directly bind to the promoter of E-cadherin repressors in cancer cells (14, 15). FOXO3 modulates EMT indirectly through the transactivation of miRNA-34b/c, which inhibits β-catenin expression (34). Here, we showed for the first time that FOXL2 directly regulated chemotherapy-associated metastasis and EMT in gastric cancer, and simultaneous alterations in Snail1 and ZEB2 levels were observed when FOXL2 was regulated in gastric cancer cells. Therefore, we speculate that FOXL2 regulates EMT in a similar manner as other FOX family members.

The Lin28B–Let-7 axis contributes to EMT and stem cell self-renewal in cancer. As a direct target of the let-7 family, HMGA2 could function as the effector in the Lin28B–Let-7 axis (35). In a mouse model of breast cancer, HMGA2 has been shown to activate TGFβR2, and their colocalization has been observed in the invasive front of tumors (35, 36). All of these effects could enhance the sensitivity of cancer cells to EMT-associated stimuli. It has recently been noticed that HMGA2 can directly regulate gene transcription. HMGA2 can bind directly to the promoters of FN1 and IL11, which modulates tumor growth and metastasis in colorectal cancer (36). HMGA2 can also directly transactivate Twist, an important E-cadherin repressor (37). Meanwhile, HMGA2 also functions as a transcription coregulator by recruiting other transcription-associated proteins (21, 38). Thuault and colleagues observed that HMGA2 could interact with the TGFβSmad pathway, leading to an increased binding of Smads to the Snail1 promoter (38). HMGA2 has also been reported to facilitate E2F1 transcription through an interaction with the tumor suppressor gene Rb and histone acetyltransferase in pituitary adenocarcinomas. In our study, HMGA2 was found to regulate the expression level of FOXL2, but its underlying mechanism of action remains unclear. Interestingly, FOXL2 is also correlated with pituitary adenoma carcinogenesis (39), and the putative binding sites for E2F1 could be found in the FOXL2 promoter. Therefore, we performed immunoprecipitation and luciferase reporter assays and confirmed that the interaction between HMGA2 and pRb facilitated the transactivation of FOXL2 by E2F1. Thus, a HMGA2–FOXL2–ITGA2 axis was identified in metastasis and EMT of chemoresistant gastric cancer.

Integrins constitute one of the most important adhesion molecules for the proper formation of tissue structures. Aberrant integrin expression has been linked to cancer proliferation, chemoresistance, and metastasis. Several integrin subunits have been reported to participate in EMT, mainly serving as the terminal executor of the EMT process. For example, silencing integrin β3 inhibits the EMT and metastasis of triple-negative breast cancer (40). Integrin α5 has been reported to be a target of ZEB2 and promotes metastasis during EMT (41). The role that ITGA2 plays in different cancers is controversial. Ramirez and colleagues showed that integrin α5β1 suppressed metastasis in human breast cancer and in mouse models (42), while Yoshimura and colleagues showed that ITGA2 contributed to the formation of hepatic metastasis in human colorectal cancer (43). ITGA2 regulation by miRNAs and epigenetic modifications is crucial in metastasis and EMT (44–46). Our data suggest that ITGA2 served as the effector of FOXL2 and that it was associated with metastasis and EMT in chemoresistant gastric cancer cells.

The clinical significance of HMGA2, FOXL2, and ITGA2 were carefully evaluated in comprehensive tissue microarrays. We found that the HMGA2–FOXL2–ITGA2 axis was aberrantly activated in distant metastases and lymph nodes relative to its activity at the primary gastric cancer cancer sites. This phenomenon indicates that patients whose gastric cancer tissues have abundant HMGA2, FOXL2, and ITGA2 levels are at a greater risk for metastasis. In addition, increased expression levels of HMGA2, FOXL2, and ITGA2 were correlated with reduced survival periods in patients with gastric cancer. These data suggest that HMGA, FOXL2, and ITGA2 might be risk factors for metastasis and that they could serve as potential markers for evaluating prognoses in patients with gastric cancer.

In conclusion, we report a novel pathway that functions in the metastasis and EMT associated with chemoresistance in gastric cancer. HMGA2 prompts FOXL2 expression by facilitating E2F1 transactivity, leading to metastasis and EMT, which is executed by the integrin subunit ITGA2. This study identified the HMGA2–FOXL2–ITGA2 axis as a potential therapeutic target of metastasis in cases of gastric cancer with reduced sensitivities to chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Liang, B. Feng, Y. Shang, D. Fan
Development of methodology: G. Ren, Y. Nie
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