High-mobility-group A2 overexpression provokes a poor prognosis of gastric cancer through the epithelial-mesenchymal transition

JUNHYUN LEE[1], SHINJUNG HA[1], CHAN-KWON JUNG[2] and HAN HONG LEE[1]

[1]Division of Gastrointestinal Surgery, Department of Surgery, [2]Department of Pathology, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

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Abstract. Tumor metastases are the ultimate target in cancer therapy. In epithelial malignancies, the expression of high-mobility-group A2 (HMGA2) is associated with disease progression and the epithelial-mesenchymal transition (EMT), which is involved in the metastatic process. The present study assessed the clinical and molecular effects of HMGA2 with the malignant tissues of 170 patients with gastric cancer and gastric cancer cells expressing HMGA2. HMGA2 expression was determined using immunohistochemistry and analyzed with respect to the clinicopathological data of patients with this tumor. In the gastric cancer cell line MKN28, in which HMGA2 was knocked down by two different short-hairpin RNAs, Transwell migration and invasion assays were conducted and western blotting was used to detect the altered expression of EMT markers. In patients with gastric cancer, HMGA2 overexpression correlated with tumor progression and was indicative of a significantly worse overall survival. Migration and invasion assays using HMGA2-knocked down MKN28 cells showed a reduction in cell migration and invasion. The upregulation of E-cadherin, an epithelial marker, and the downregulation of N-cadherin, a mesenchymal marker were observed in HMGA2-knocked down cells. In addition, expression of the transcriptional factors Snail and Zeb1 and of the EMT-pathway molecule β-catenin were decreased. HMGA2 overexpression, through its relationship to EMT, thus seems to aggravate invasion and metastasis in gastric cancer. It may therefore serve as a predictive marker in determining the clinical outcome of patients with gastric cancer and offer a promising therapeutic target.

Correspondence to: Dr Han Hong Lee, Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 271 Cheonbo-ro, Uijeongbu-si, Gyenggi-do 480-717, Republic of Korea
E-mail: painkiller9@catholic.ac.kr

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Introduction

Metastases of primary tumors are the main cause of cancer-related death, such that the mechanism of their development is an important area of investigation (1). Metastasis has been shown to involve several distinct steps: detachment and migration from the primary tumor, penetration of the basement membrane, entry into the blood or lymphatic stream, exit from the blood or lymphatic stream, and, finally, the formation of a metastatic nodule (2). The first of these steps, detachment and migration from the primary tumor, involves epithelial-mesenchymal transition (EMT), which is controlled by multiple molecules and signaling pathways (1,3).

High-mobility-group A2 (HMGA2) is a small nonhistone chromosomal protein that binds through its three AT-hook DNA-binding motifs to AT-rich sequences in the minor groove of DNA strands (4). Although HMGA2 has no intrinsic transcriptional activity, it modulates transcription by altering the architecture of chromatin (5,6). Since HMGA2 is highly expressed during embryogenesis but is absent or present only at low levels in normal adult tissues, it seems to play a critical role in cell proliferation and differentiation during embryonic development (7,8). However, according to several studies, HMGA2 is also expressed in the development of malignancy and, through its relationship to EMT, participates in tumor metastasis (9,10). Yet, while a correlation between HMGA2 overexpression and malignancy has been reported (11,12), the exact mechanism is not fully explained. In gastric cancer, for example, there are few studies on the consequences of HMAG2 overexpression in these tumors.

The aim of the present study was to use surgical gastric cancer specimens and patient clinicopathological data to investigate the clinical significance of HMGA2 overexpression in gastric cancer. In addition, the mechanism by which HMGA2 expression acts on the gastric cancer and the association between that mechanism and EMT were analyzed in an *in vitro* study using a gastric cancer cell line.

Materials and methods

Patients and samples. A series of 170 consecutive gastric cancer patients who underwent gastrectomy at Uijeongbu

St. Mary's Hospital (Uijeongbu, Gyeonggi-Do, Korea) from 2001 to 2005 were enrolled in the study. Clinicopathological parameters including operative details were collected retrospectively from the hospital's Gastric Cancer Patients Registry. Cancer stage was determined based on the TNM classification of the Seventh American Joint Cancer Committee (AJCC).

The 123 male and 47 female patients ranged in age from 29 to 89 years (median 61.5 years). In this group, 90% underwent lymph node dissection level D2 and greater. Distal and total gastrectomy was performed in 110 and 60 patients, respectively. According to the final pathological diagnosis, 23 patients had stage I disease, 51 stage II, 94 stage III, and 2 stage IV (Table I). Adjuvant chemotherapy was administered to stage II and stage III patients according to institutional guidelines. Fluorouracil (5-FU)- or cisplatin-based systemic chemotherapy was administered with adjuvant intention to all patients. None of the patients received neoadjuvant chemotherapy.

Regular follow-up was conducted according to our standard protocol (every 3 and 6 months in advanced and early gastric cancer, respectively, for the first 3 years, every 12 months thereafter) and included an evaluation of tumor markers, abdominal CT, and endoscopic examination. The mean follow-up period of the 170 enrolled patients was 71.4±50.5 months (range, 0-152 months). The survival results were confirmed by using the registration data of the Korea National Statistical Office (KNSO) and the patients' medical records.

From the 170 participants in the study, 76 gastric cancer samples and the corresponding control samples were used for reverse transcription-polymerase chain reaction (RT-PCR) and western blot. All samples had been frozen immediately after surgical resection and stored in liquid nitrogen of -90˚C. Immunohistochemistry was performed on 170 tissue samples, obtained as paraffin-embedded resected gastric specimens, after histopathological diagnosis.

This study was approved by the Institutional Review Board of the Ethics Committee of the College of Medicine, Catholic University of Korea (UC13SISI0008).

Table I. Patient characteristics.

Variable	n=170
Gender	
Male	123
Female	47
Age in years	
Median (range)	61.5 (29-89)
Extent of resection	
Subtotal	110
Total	60
Lymph node dissection	
$D1^+$	17
D2	88
More than D2	65
Retrieved lymph nodes	
Mean ± SD (range)	29.2±13.9 (3-76)
Reconstruction	
Billroth-I	12
Billroth-II	98
Roux-en-Y	60
Pathological stage (7th AJCC)	
I	23
II	51
III	94
IV	2

SD, standard deviation; AJCC, American Joint Cancer Committee.

Immunohistochemistry. Formalin-fixed and paraffin-embedded human gastric tumor tissues were sectioned at a thickness of 4 μm and then immunohistochemically stained using a standard avidin-biotin peroxidase complex method (13). The slides were deparaffinized in xylene three times for 10 min, rehydrated through a graded ethanol series to distilled water, and then incubated for 10 min with 3% hydrogen peroxidase-methanol to inhibit endogenous peroxidase activity. For antigen retrieval, the slides were treated with 10 mM citrate buffer (pH 6.0) at 98˚C for 15 min in a microwave oven and allowed to cool for 1 h at room temperature. After incubation of the sections for 10 min in a blocking solution (Histo-Plus kit, Zymed, San Francisco, CA, USA) containing 10% normal serum in phosphate-buffered saline (PBS), they were treated with a primary rabbit polyclonal anti-HMGA2 antibody (Abcom, Cambridge, MA, USA) diluted 1:100 in blocking solution and then incubated overnight at 4˚C in a humidified chamber. The primary antibodies were detected with a secondary antibody (Histo-Plus, Zymed) used together with biotin, incubating the slides for 10 min at 45˚C. The sections were rinsed three times in PBS and then incubated for 10 min with streptavidin-horseradish peroxidase complex (Histo-Plus, Zymed). Antigens were localized using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen followed by counterstaining with hematoxylin.

Evaluation of immunoscores. The HMGA2 positivity of gastric tumor glands was defined as nuclear staining with the corresponding antigen. The additional presence of HMGA2 deposits in the cytoplasm was also considered positive. The extent and the intensity of immunopositivity were considered when scoring the expression of HMGA2 protein. The intensity of positive staining was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong, and the extent of positive staining according to the percentage of positive cells in the respective lesions: 0, 0%; 1, 1-10%; 2, 11-25%; 3, 26-50%; 4, 51-75%; 5, 76-90%; and 6, >90%. The final score was obtained by multiplying the positivity and intensity scores, yielding a range from 0 to 18. HMGA2 expression was considered positive when the final score was ≥9.

Cell culture and transfection. MKN28 cells, obtained from the Korean Cell Line Bank, were maintained in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% calf serum (Hyclone) and incubated at 37˚C in a 5% CO_2 humidified atmosphere. Two pairs of oligonucleotides encoding the

Table II. Precursor sequences of miRNA-HMGA2.

No.	miRNA-HMGA2 precursor sequence
HMGA2i-1	
Sense	5'-TGCTGCTTGGTAGTAGATTGTCCCATGTTTTGGCCACTGACTGACATGGGACACTACTACCAAG-3'
Antisense	5'-CCTGCTTGGTAGTAGTGTCCCATGTCAGTCAGTGGCCAAAACATGGGACAATCTACTACCAAGC-3'
HMGA2i-2	
Sense	5'-TGCTGTTTGGTACTGTTTCCCAGAGAGTTTTGGCCACTGACTGACTCTCTGGGACAGTACCAAA-3'
Antisense	5'-CCTGTTTGGTACTGTCCCAGAGAGTCAGTCAGTGGCCAAAACTCTCTGGGAAACAGTACCAAAC-3'

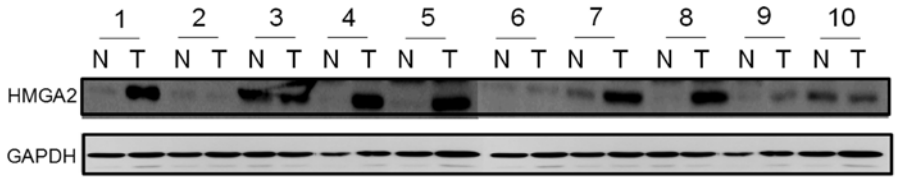

Figure 1. HMGA2 protein expression in 10 gastric cancer tissues and matched normal gastric mucosa. Western blotting shows an increase in HMGA2 protein levels in six of the cancer tissues (1, 4, 5, 7, 8 and 9).

pre-micro-RNA (miRNA) were synthesized, annealed, and then cloned into plasmid pcDNATM6.2-GW/EmGFP-miR (Invitrogen, Carlsbad, CA, USA), carrying a gene encoding the selectable marker blasticidin. MKN28 cells were seeded in six-well plates in RPMI-1640 containing 10% fetal bovine serum (FBS) without antibiotics for 24 h, then transfected with 400 ng of either the purified pcDNATM6.2-GW/EmGFP-miR expression vector, containing the HMGA2 insert HMGA2i-1 or HMGA2i-2, or the negative control (HMGA2i-NC) obtained from Invitrogen. The inserts were designed to different coding regions of human HMGA2 mRNA sequence (NM_003484.1) using the BLOCK-iT RNAi express search engine (http://rnaidesigner.lifetechnologies.com/rnaiexpress/rnaiExpress.jsp). The sequence information for the HMGA2 RNAi duplexes is provided in Table II. Effectin transfection reagents (Qiagen, Hilden, Germany) were used according to the manufacturer's instructions. The efficiency of transfection was determined by fluorescence microscopy after 24 h. Stable cell lines were selected using blasticidin, a translational inhibitor, and were harvested for the evaluation of gene expression or for use in functional assays.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNeasey mini kit (Qiagen) according to the manufacturer's protocol. Total RNA (3 μg) were reverse-transcribed into cDNA using the Superscript III first-strand synthesis system (Invitrogen). The resultant cDNA was used for PCR amplification with Quick Taq HS dye mix (Toyobo, Osaka, Japan). The primers for HMGA2 were: sense 5'-CAG CCGTCCACTTCAGC-3' and antisense 5'-TGCCTTTGG GTCTTCC-3'. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: sense 5'-GAAGGTGA AGGTCGGAGTC-3' and antisense 5'-GAAGATGGTGA TGGGATTTC-3'. GAPDH was amplified in parallel as the internal control. PCR was performed at 94˚C for 2 min, followed by 35 cycles of 94˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 30 sec, and finally one cycle of 72˚C for 10 min. The PCR products were then separated on 2% agarose gels and visualized by ethidium bromide staining. For the detection of HMGA2 mRNA expression, real-time quantitative PCR (QPCR) analysis was performed using iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA). cDNA (2 μl) was combined with the HMGA2 or GAPDH primers and the SYBR Green master mix in a final volume of 20 μl in a 96-well RT-PCR plate (iCycleriQ PCR plates; Bio-Rad). QPCR was carried out in an iCycler (Bio-Rad) for 35 cycles. The experiments were performed in duplicate for each set of primers.

Western blotting. The cells were collected and washed with PBS. Proteins were extracted using the NE-PER nuclear and cytoplasmic protein extraction kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Approximately 10 mg of frozen tissue were homogenized in 600 μl of Pro-Prep protein extraction solution (iNtRON Biotechnology, Korea) after which cell lysis was induced by incubation of the homogenates for 30 min on ice. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of proteins were separated on 8% or 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 1 h, incubated overnight at 4˚C with primary antibodies against HMGA2 (1:1000; Abcom), N-cadherin (1:1000; Cell Signaling Technology, Beverly, MA, USA), E-cadherin (1:1000; Thermo Scientific), Snail (1:1000; Abcam), β-catenin (1:1000; Invitrogen), and Zeb1 (1:1000, Abcam), and washed extensively with TBST. Lamin B1 (1:10000; Abcam) antibody served as the loading control. The immune complexes were detected autoradiographically using the appropriate horseradish-peroxidase-labeled secondary antibodies (Bio-Rad) at

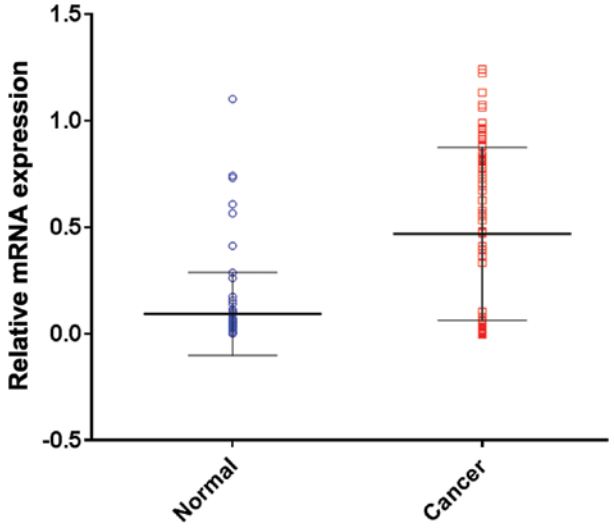

Figure 2. Comparison of HMGA2 mRNA expression in gastric cancer and the corresponding normal tissue. HMGA2 mRNA expression is significantly higher in cancer tissues than normal tissues (P<0.001).

1:2000 dilutions and the enhanced chemiluminescence detection reagent ECL (Thermo Scientific).

Cell migration and invasion assay. The Transwell migration assay was performed in 6.5-mm diameter Boyden chambers with a pore size of 8.0 μm (Corning Inc., Corning, NY, USA). Cells ($2x10^5$) were resuspended in migration medium (serum-free RPMI-1640 containing 5% bovine serum albumin, BSA) and placed in the upper compartment of the Transwell chambers. The lower compartment was filled with 600 μl of RPMI-1640 medium containing 10% FBS. After incubation of the chambers for 24 h at 37˚C, cells on the lower surface of the filter were stained with 0.1% crystal violet, rinsed with distilled water, and eluted with 10% acetic acid for 15 min. Optical densities were measured on a VersaMax spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) at 590 nM. For invasion assays, the cells were plated in 24-well Matrigel-coated invasion chambers, in which the lower chambers contained 600 μl of RPMI-1640 and 10% FBS as a chemoattractant. A suspension of $2x10^5$ cells in 200 μl of serum-free RPMI-1640 containing 5% BSA was added to the upper chamber. The cells were incubated for 38 h at 37˚C in a humidified incubator with 5% CO_2, during which time the invasive cells attached to the lower surface of the membrane insert. The staining of the invasive cell and measuring of the optical densities procedure for the invasion assay were similar to the migration assay. The migration and invasion assays were repeated three times.

Statistical analysis. Differences between groups were analyzed using the t-test for continuous variables and the χ^2 test or Fisher's exact test for proportions. Survival was analyzed using Kaplan-Meier methods with a log-rank test for univariate analysis. Multivariate analysis for survival was carried out using the Cox proportional hazards model with the 'Backward LR' method. Statistical analyses were performed with SPSS (Statistical Package for Social Science, Chicago, IL, USA), version 13.0. P<0.05 indicated statistical significance.

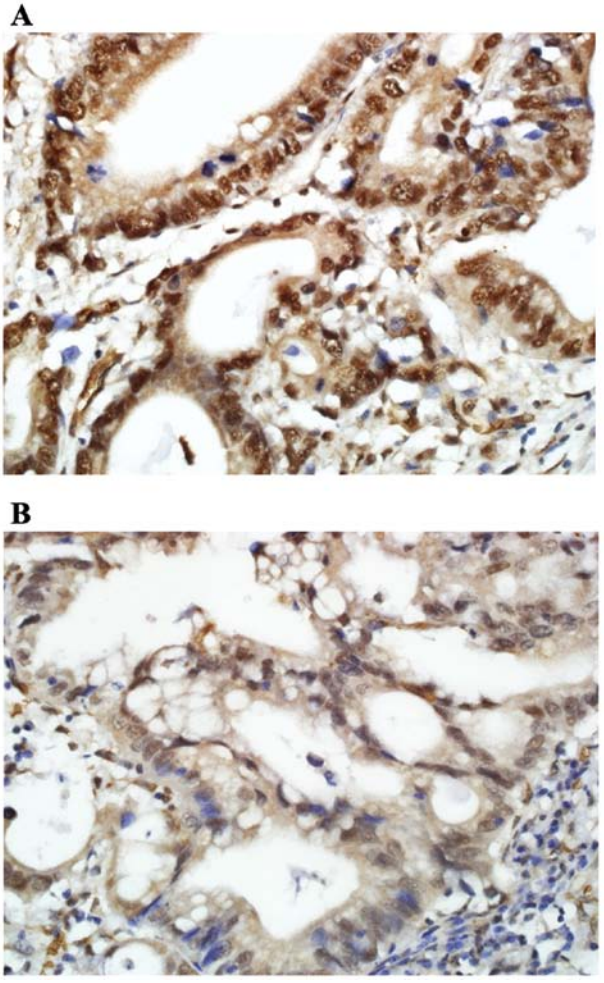

Figure 3. Immunohistochemical staining of HMGA2 in gastric cancer. (A) High expression (x400), (B) low expression (x400).

Results

HMGA2 expression is increased in human gastric cancer tissues. HMGA2 protein expression was evaluated by western blotting in 10 paired human gastric cancer tissues and corresponding normal gastric mucosa. In six of the cancer tissues, HMGA2 protein levels were higher than in normal tissues whereas in the remaining four samples the expression levels were similar (Fig. 1). Similarly, HMGA2 mRNA expression levels, examined in the paired human gastric cancer tissue and corresponding normal tissue of 76 patients using real-time PCR, were significantly higher in malignant than in normal tissues (P<0.001) (Fig. 2).

Correlation between HMGA2 expression and clinicopathological features in gastric cancer patients. Immunohistochemical staining revealed HMGA2 expression mainly in the nucleus and only partly in the cytoplasm of tumor cells (Fig. 3). Among 170 gastric cancer patients, 39 (22.9%) had high expression of HMGA2 in their tumor tissues. HMGA2 expression in gastric cancer was significantly correlated with the depth of invasion (P=0.015) and lymph node metastasis (P=0.047). In addition, a significant association was determined between pathological stage, evaluated according to the seventh AJCC system, and HMGA2 expression (P=0.003) (Table III).

Table III. Correlation between HMGA2 expression and clinicopathological characteristics.

Variable	HMGA2 expression		P-value
	Positive n=39	Negative n=131	
Age, years (mean ± SD)	59.3±10.6	59.3±11.6	0.967
Gender, n (%)			0.467
Male	30 (24.4)	93 (75.6)	
Female	9 (19.1)	38 (80.9)	
Size, cm (mean ± SD)	5.48±2.7	5.18±2.7	0.546
Histological type, n (%)			0.499
Differentiated	10 (19.6)	41 (80.4)	
Undifferentiated	29 (24.4)	90 (75.6)	
Lauren classification, n (%)			0.232
Intestinal type	12 (20.7)	46 (79.3)	
Diffuse type	24 (27.6)	63 (72.4)	
Mixed type	3 (12.0)	22 (88.0)	
Lymphatic invasion, n (%)			0.083
Present	35 (25.7)	101 (74.3)	
Absent	4 (11.8)	30 (88.2)	
Vascular invasion, n (%)			0.773
Present	5 (26.3)	14 (73.7)	
Absent	34 (22.5)	117 (77.5)	
Perineural invasion, n (%)			0.073
Present	26 (28.3)	66 (71.7)	
Absent	13 (16.7)	65 (83.3)	
Depth of invasion, n (%)			0.015
T1	1 (33.3)	2 (66.7)	
T2	2 (6.3)	30 (93.8)	
T3	1 (7.7)	12 (92.3)	
T4	35 (28.7)	87 (71.3)	
Lymph node metastasis, n (%)			0.047
N0	11 (16.9)	54 (83.1)	
N1	9 (32.1)	19 (67.9)	
N2	4 (12.1)	29 (87.9)	
N3	15 (34.1)	29 (65.9)	
Pathological stage, n (%)			0.003
I	1 (4.3)	22 (95.7)	
II	9 (17.6)	42 (82.4)	
III	27 (28.7)	67 (71.3)	
IV	2 (100.0)	0 (0.0)	

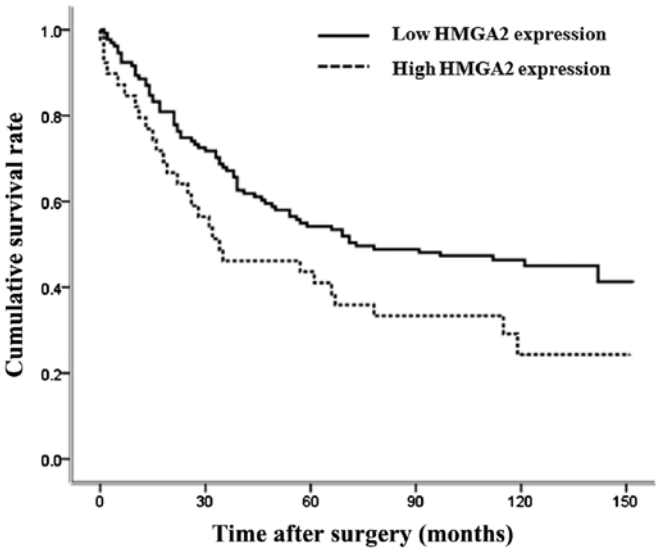

Figure 4. Survival curves of patients with high and low HMGA2 expression. Patients with high-level expression have a significantly worse 5-year overall survival rate than those with low-level expression (43.6% vs. 54.2%; P=0.028).

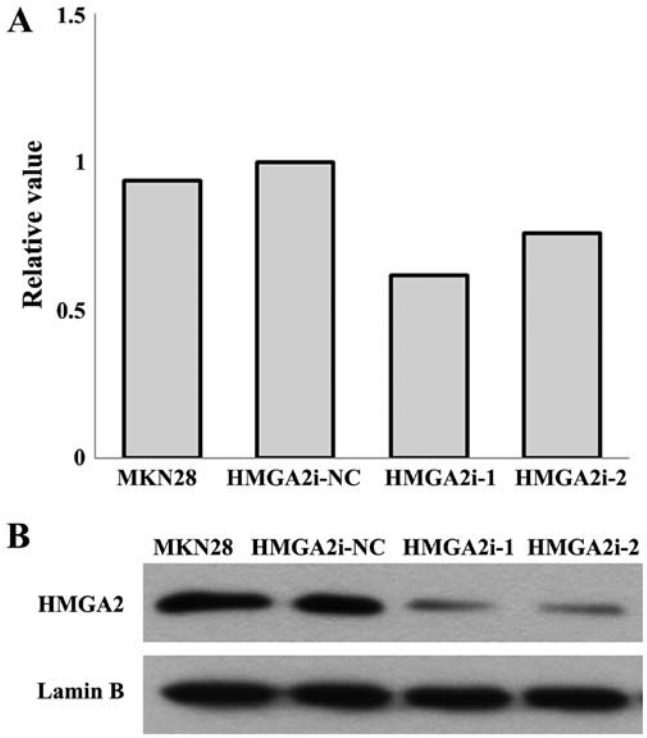

Figure 5. HMGA2 knockdown in MKN28 gastric cancer cells using two kinds of shRNA (HMGA2i-1 and HMGA2i-2). (A) Quantitative real-time PCR and (B) western blot show decreases in HMGA2 mRNA and protein expression in both knockdown models.

Patients with high HMGA2 expression had a significantly worse overall survival outcome than those with low expression of the protein (P=0.028, Fig. 4). However, in multivariate survival analysis using a Cox proportional hazards regression model, HMGA2 expression was not an independent prognostic factor for gastric cancer.

HMGA2 knockdown in gastric cancer cells by short-hairpin RNA (shRNA). Based on a literature search, MKN28 was selected as the HMGA2-expressing gastric cancer cell line to investigate the functional role of HMGA2 in gastric cancer. In these cells, knockdown of HMGA2 was achieved using shRNA. The expression levels of HMGA2 in the knockdown cells were measured by QPCR and western blotting. These

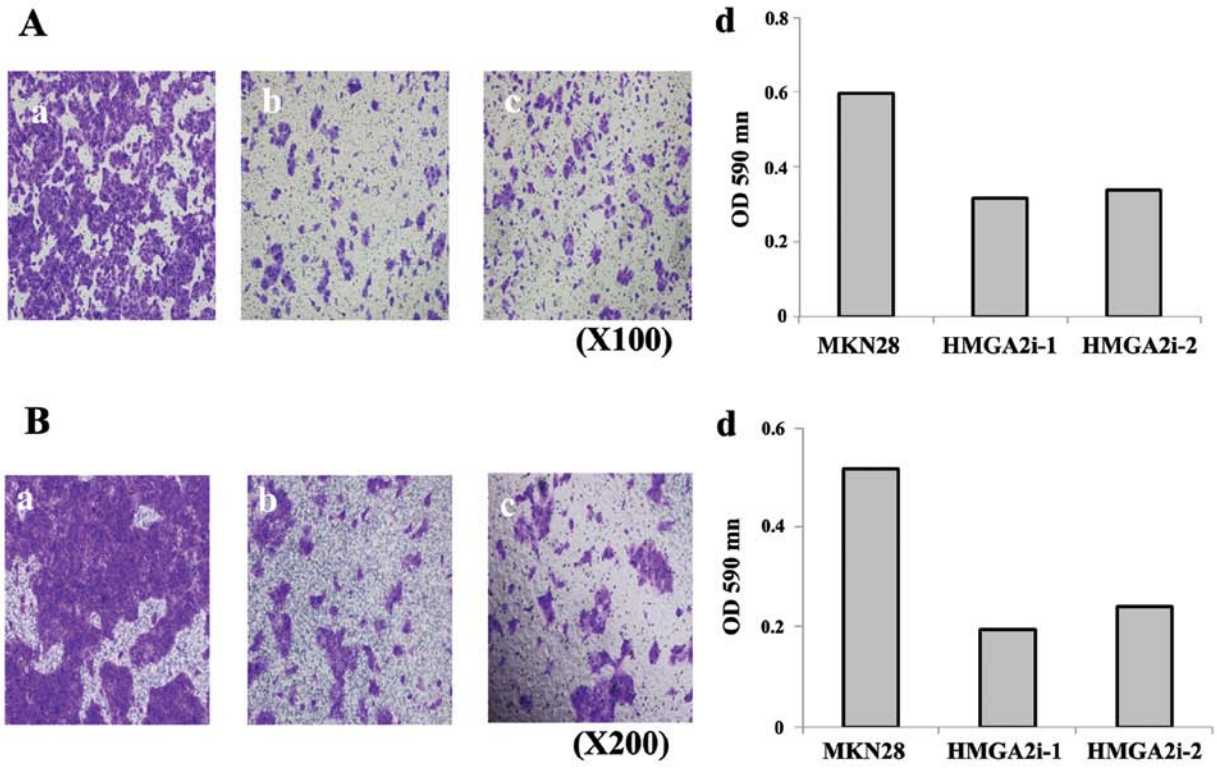

Figure 6. Migration and invasion assays using two HMGA2 knockdown models. (A) Inhibition of cell migration by HMGA2 knockdown (x100). (a) MKN28, (b) HMGA2i-1-MKN28, (c) HMGA2i-2-MKN28 and (d) number of migrating cells based on (a-c). (B) Inhibition of cell invasion by HMGA2 knockdown (x200). (a) MKN28, (b) HMGA2i-1-MKN28, (c) HMGA2i-2-MKN28, and (d) number of invading cells based on (a-c). Columns show the means of three independent experiments.

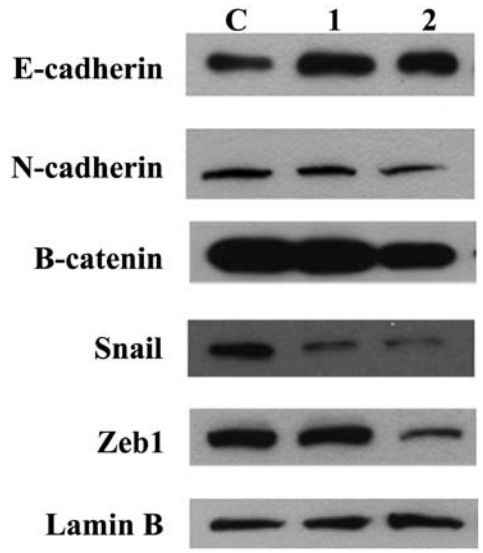

Figure 7. Western blot of EMT-associated protein markers in two HMGA2 knockdown models. Compared to control cells, E-cadherin is upregulated whereas N-cadherin, β-catenin, Snail, and Zeb1 are downregulated in HMGA2i-MKN28 cells (C, MKN28; 1, HMGA2i-1-MKN28; 2, HMGA2i-2-MKN28).

methods confirmed HMGA2 knockdown by shRNA at the mRNA and protein levels, respectively, in MKN28 cells (Fig. 5).

Migration and invasion of gastric cancer cells are inhibited by HMGA2 knockdown. To assess the effect of HMGA2 expression on cancer cell migration and invasion, *in vitro* migration and invasion assays were conducted with the HMGA2 knockdown cells. Expression of either HMGA2i-1 or HMGA2i-2 inhibited MKN28 cell migration (Fig. 6a) and invasion (Fig. 6b).

HMGA2 knockdown represses EMT. Inhibition of cell migration and invasion in MKN28 HMGA2 knockdown cells suggested that HMGA2 participates in EMT and thus in tumor metastasis. To confirm the relevance of HMGA2 in EMT, the expression of established EMT-related proteins in HMGA2 knockdown MKN28 cells was evaluated by western blotting. The results showed increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal marker N-cadherin. In addition, HMGA2 knockdown induced downregulation of Snail and Zeb1, two transcriptional markers of EMT, and of β-catenin, a key molecule in the Wnt/β-catenin signaling pathway, which is known to be involved in this metastasis-related process (Fig. 7).

Discussion

In the metastasis of a primary tumor, and thus in cancer-related death, EMT is regarded as a key event. Signaling molecules, such as smad and β-catenin, and transcriptional factors, such as Zeb and Snail, have been shown to participate in EMT (14,15), but the mechanism of their involvement has yet to be fully explained. HMGA2 is among the newly identified factors involved in the EMT of malignancies of epithelial

origin. HMGA2 is overexpressed in many epithelial-type malignancies, such as breast cancer (16), lung cancer (17), oral squamous cell carcinoma (18), and pancreatic carcinoma (19). In addition, overexpression is a predictor of poor prognosis in patients with lung cancer (20), oral squamous cell carcinoma (18), ovarian cancer (21), metastatic breast cancer (22), and colorectal cancer (23). In gastric cancer, there is one previous study suggesting that HMGA2 overexpression might be related to the patient prognosis (24). However, the mechanism of the relationship between poor prognosis and HMGA2 overexpression was not ascertained. In another study, although a mechanism for the association between HMGA2 and EMT in gastric cancer was suggested, the clinical role and prognostic value of HMGA2 were poorly explained (12). Thus, the present study was designed to determine the clinical impact of HMGA2 expression and its correlation with EMT in gastric cancer.

Our results showed that HMGA2 was overexpressed in gastric cancer tissue compared to normal epithelium. As determined by western blotting, the expression of HMGA2 protein in gastric cancer tissues was much higher than in corresponding normal gastric mucosa. This finding was supported by the 5-fold increase in HMGA2 mRNA in gastric cancer tissues compared to normal tissues and together with the protein data suggested an association between HMGA2 expression and clinical outcome in gastric cancer. Indeed, the high-level overexpression of HMGA2 was related to a poor prognosis and significantly lower overall survival rates. These findings are consistent with those presented in a previous study (24) and suggested that HMGA2 levels are a significant prognostic factor for poor clinical outcome in gastric cancer.

Several studies have demonstrated a crucial role for EMT in the progression of gastric cancer (25-27). In other epithelial malignancies, HMGA2 was shown to participate in tumor metastasis and disease progression by inducing EMT (28-30), but only one study found evidence of the involvement of HMGA2 in the EMT of gastric cancer (12). Therefore, in the present work we investigated the relationship between HMGA2 and EMT by examining the expression patterns of molecules known to be involved in EMT, including E- and N-cadherin (13,14) and several other transcriptional factors and signal molecules (31-33). The downregulation of E-cadherin and the upregulation of N-cadherin are considered as hallmarks of EMT (27), with a loss of E-cadherin associated with a poor prognosis in gastric cancer (25). To further elucidate the role of HMGA2 during EMT, we conducted an *in vitro* study in which MKN28 cells expressing HMGA2 were transfected with two different shRNAs, resulting in two knockdown models, and the levels of several EMT-related factors were then determined.

The transcriptional factor Zeb1 induces EMT by repressing the expression of E-cadherin, via binding to its E-box. Snail, another transcriptional regulator, also represses E-cadherin transcription directly during EMT (14,15). The present study was based on the hypothesis that the expression of EMT-related factors is altered following the knockdown of HMGA2. As expected, the knockdown of HMGA2 increased the expression of E-cadherin and repressed the expression of N-cadherin, Zeb1, and Snail. Furthermore, in Transwell migration and invasion assays, the expression of HMGA2 significantly influenced the degree of tumor cell migration and invasion, two properties of oncogenesis related to EMT. Specifically, compared with the control group, the number of migrating and invading MKN28 cells was significantly higher in the absence of HMGA2 knockdown. These results provide strong evidence for a correlation between HMGA2 overexpression and EMT. In addition, they support the ability of HMGA2 to promote the metastatic properties of tumor cells and thus, at least in part, explain the poor prognosis of gastric cancer patients with high-level HMGA2 expression.

Additional evidence for a role of HMGA2 in EMT is the nuclear expression of β-catenin. In EMT, β-catenin has been described as the ultimate downregulator of E-cadherin, via the upregulation of Snail (34). To determine whether HMGA2 affects the nuclear expression of β-catenin, we compared control cells with HMGA2 knockdown cells. The results showed significantly lower β-catenin expressions in the two types of knockdown cells than in control cells and the concurrent repression of Snail expression. A possible mechanism underlying this relationship is that HMGA2 overexpression increases the nuclear expression of β-catenin, which leads to an increase in Snail expression and the subsequent repression of E-cadherin. This would explain the stimulation by HMGA2 overexpression of EMT in gastric cancer.

To our knowledge, this is the first study to clarify both the clinical impact of HMGA2 overexpression; i.e., as an indicator of a poor prognosis in gastric cancer, and the molecular mechanism of the association between HMGA2 expression and EMT in these tumors. Thus, in gastric cancer patients with HMGA2 overexpression, an aggressive treatment strategy should be considered. The development of therapies that interfere with HMGA2 expression, thereby reducing its pro-metastatic function via EMT, would initiate a new era of treatment of gastric cancer.

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