Clinical Significance of High Mobility Group A2 in Human Gastric Cancer and Its Relationship to let-7 MicroRNA Family

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Abstract

Purpose: The high mobility group A2 (HMGA2) nonhistone chromosomal protein can modulate transcription by altering chromatin architecture. HMGA2 is highly expressed during embryogenesis and in various benign and malignant tumors. Recent studies report that HMGA2 is negatively regulated by the let-7 microRNA (miRNA) family. However, no studies have examined the clinical significance of HMGA2 and its relationship to the let-7 miRNA family in gastric cancer.

Experimental Design: Using quantitative real-time reverse transcription–PCR, we analyzed HMGA2 expression with respect to various clinicopathologic factors in 110 patients with gastric cancer. We also did an association study comparing HMGA2 expression and let-7 miRNA family expression in gastric cancer.

Results: Expression of HMGA2 in cancerous tissues was significantly higher than in noncancerous tissues (P < 0.05). Elevated HMGA2 expression was significantly correlated with serosal invasion (P < 0.05) and poor clinical prognosis (P < 0.05). A multivariate analysis showed that HMGA2 expression status was an independent prognostic factor (P < 0.05). An inverse correlation between HMGA2 and let-7a was found in gastric cancer cell lines (P = 0.08). The expressions of let-7a, let-7b, and let-7c in gastric cancer patients with low HMGA2 expression were significantly higher than those with high HMGA2 expression (P < 0.05).

Conclusions: High expression of HMGA2 in gastric cancer correlates with tumor invasiveness and is an independent prognostic factor. Furthermore, our findings suggest that HMGA2 is negatively regulated by the let-7 miRNA family in human gastric cancer.

The high mobility group A2 (HMGA2; also called HMGI-C) protein is a small nonhistone chromosomal protein that has no intrinsic transcriptional activity but can modulate transcription by altering chromatin architecture (1, 2). HMGA2 is abundantly expressed during embryogenesis, but it is either undetectable or its expression remains at low levels in normal adult tissues, suggesting that HMGA2 plays a critical role in cell proliferation and/or differentiation during embryonic development (3, 4). Knocking out the HMGA2 gene in mice leads to the so-called pygmy phenotype with characteristic hypoplasia of mesenchymal tissue, which confirms the important role of HMGA2 in mammalian growth and development (5). A recent study indicated that common variation in the HMGA2 gene is associated with adult height with very high levels of statistical confidence (6).

HMGA2 overexpression is a hallmark of various benign and malignant tumors. Disruption of the gene by chromosomal rearrangements at chr12q13-15 and attendant overexpression of the protein results in several benign mesenchymal tumors, such as lipoma and uterine leiomyoma (7–9). Although the deletion and/or fusion partners were originally thought to be important for tumorigenesis, overexpression of full-length HMGA2 protein was found to be sufficient to cause a neoplastic transformation of mesenchymal cells (10). Recent studies report that HMGA2-mediated E2F1 activation is a crucial event in onset of pituitary adenomas in transgenic mice (11, 12). These observations suggest that overexpression of HMGA2 is tumorigenic in differentiated tissues where HMGA2 expression is normally undetectable.

HMGA2 is also elevated in various human epithelial-type neoplasias, including breast cancers (13), lung cancers (14, 15), pancreatic carcinomas (16), oral squamous cell carcinomas (17), and thyroid carcinomas (18). Overexpression of HMGA2 is a poor prognostic factor in lung cancers (14), oral squamous cell carcinomas (17), ovarian cancers (19), and metastatic breast cancers (20).

The microRNAs (miRNA), a family of mature noncoding small RNA 21 to 25 length nucleotides, have been shown to play a role in a variety of biological processes, including...
development, differentiation, proliferation, and cell death. They regulate gene expression at the posttranscription level by direct cleavage of a target mRNA using interference machinery (mRNA cleavage) or by inhibition of protein synthesis (translational repression; refs. 21, 22). Recent evidence has shown that miRNA mutations or misexpression correlate with various human cancers and indicate that some miRNAs can function as oncogenes or tumor suppressors (22). One of the founding members of the miRNA family, let-7, was first identified in Caenorhabditis elegans (23). In humans, like C. elegans, the expression of let-7 is barely detectable in embryonic stages but increases after differentiation and in mature tissues (24). Previous studies indicate that let-7 acts as a tumor suppressor for lung and colon cancer, in part, through targeting of RAS (25–28). Recent studies report that HMGA2 is negatively regulated by the let-7 miRNA family in a mouse model system (29), in head and neck cancers (30), in uterine leiomyomas (31), in lung cancer cell lines (32), and in ovarian cancers (19). Recently, Fusco and Fedele made comprehensive review on HMGA, and they focused on the role of the HMGA proteins in

Fig. 1. Immunohistochemistry of HMGA2 expression in representative samples of gastric cancer. Note that no positive staining is observed in the normal gastric epithelium (bottom right corner, A). A, well-differentiated adenocarcinoma; magnification, 40×. B, moderately differentiated adenocarcinoma; magnification, 40×. C, poorly differentiated adenocarcinoma; magnification, 40× and 100× (c). D, signet ring cell adenocarcinoma; magnification, 40× and 100× (d). E, cancer cells at invasive front are strongly stained for HMGA2; magnification, 40×. F, noninvasive front; magnification, 100×. G, invasive front; magnification, 100×.
human neoplastic diseases, the mechanisms by which they contribute to carcinogenesis, and therapeutic strategies based on targeting HMGA proteins (33). To our knowledge, there have been no reports investigating a possible correlation between HMGA2 and the let-7 miRNA family in gastric cancers.

In this study, we investigated HMGA2 expression in gastric cancer to determine its clinicopathologic and prognostic value. We also investigated possible associations between HMGA2 expression and let-7 miRNA family member expressions in gastric cancer.

Materials and Methods

Cell lines and tissue samples. Cell lines derived from human gastric cancer, including MKN1, MKN28, MKN45, MKN74, KATOIII, and AZ521, were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University). One hundred ten gastric tumor samples and matched control samples were frozen in liquid nitrogen immediately after surgical resection and were kept at -90°C. One hundred ten gastric tumor samples and matched control samples were kept at -4°C. Immunohistochemical studies of HMGA2 expression in representative clinical samples. These include well-differentiated

electrophoresed on a 2% agarose gel containing ethidium bromide. To ensure that RNA was of sufficient purity for reverse transcription–PCR (RT-PCR), a PCR assay with primers specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was carried out for 22 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C. The primers for GAPDH were as follows: sense primer 5'-TGGTTATGCCT-GAAGGACTCA-3' and antisense primer 5'-TGTCATCATATTTGG-CAGGTT-3' (36).

Quantitative real-time RT-PCR. PCR amplifications for quantification of HMGA2 and GAPDH mRNA in clinical samples were done in a LightCycler system (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). In brief, a master mixture was prepared on ice, containing 1 μL of complementary DNA, 2 μL of LC DNA Master SYBR Green I mix, 50 ng of primers, and 2.4 μL of 25 mmol/L MgCl2. The amplification conditions for 40 cycles consisted of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. The products were then subjected to a temperature gradient from 68 to 95°C at 0.1°C/s, with continuous fluorescence monitoring to produce melting curves of the products. The expression levels were normalized to GAPDH mRNA expression (37).

Quantitative real-time RT-PCR for miRNA. Total RNA was extracted from cell lines and tissue samples of gastric cancer using Trizol total RNA isolation reagent (Invitrogen) as per the manufacturer's protocol. The let-7 miRNA family (let-7a, let-7b, and let-7c) and RNU6B (as an internal control) – specific complementary DNA were synthesized from total RNA using gene-specific primers according to the TaqMan miRNA assays protocol (Applied Biosystems). Reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/L stem-loop RT primer, 1× RT buffer, 0.25 mmol/L each of deoxynucleotide triphosphate (dNTP), 3.33 units/μL MultiScribe reverse transcriptase, and 0.25 units/μL RNase inhibitor. The 7.5-μL reaction volumes were incubated in BIO-RAD i-Cycler (Bio-Rad Laboratories) in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C.

Real-time PCR was done using an Applied Biosystems 7500 real-time PCR system. The 10-μL PCR included 0.67 μL of RT products, 1× TaqMan Universal PCR master mix, and 1 μL of primers and probe mix of the TaqMan miRNA assays. The reactions were incubated in 96-well optical plates at 95°C for 10 min and followed by 45 cycles of 95°C for 15 s and 60°C for 10 min. Relative quantification of miRNA expression was calculated by using the 2(-ΔΔCt) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B, and relative to a calibrator sample.

Immunohistochemistry. Immunohistochemical studies of HMGA2 were done on surgical specimens from 33 selected gastric cancer patients using the avidin-biotin-peroxidase method (LSAB2 kit; Dako) on formalin-fixed, paraffin-embedded tissues. After deparaffinization and blocking, the antigen-antibody reaction was carried out overnight at 4°C. The LSAB2 kit was applied to detect the signal of the HMGA2 antigen-antibody reaction. The goat polyclonal antibody against human HMGA2 (R&D Systems) was used at 10 μg/mL.

Statistical analysis. Bistatistical analyses were done with JMP 5.0.1a for Windows software (SAS Institute). Possible differences between groups were analyzed using Student's t test and χ2 test. The association between expression levels of HMGA2 mRNA and let-7 miRNA family was analyzed by Pearson correlation coefficient. Survival curves were obtained by the Kaplan-Meier method (38); comparison between curves was made by log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards model). A probability level of 0.05 was chosen for statistical significance.

Results

Immunohistochemistry. Figure 1 shows the results of immunohistochemical studies of HMGA2 expression in representative clinical samples. These include well-differentiated
were strongly stained for HMGA2 (Fig. 1E-G). Cytoplasm of the cancer cells. Cancer cells at invasive front expression of HMGA2 was localized in the nucleus and cells and none in the normal gastric epithelium (Fig. 1A). The HMGA2 expression was predominantly observed in the cancer (Fig. 1C), and signet ring cell adenocarcinoma (Fig. 1D).

Expression of HMGA2 mRNA in clinical tissue specimens. With regard to HMGA2 mRNA expression in clinical samples, 83 of 110 (75.4%) showed a higher expression level of HMGA2 mRNA in tumor than in nontumor tissues by quantitative real-time RT-PCR. As shown in Fig. 2, after normalization to GAPDH gene expression levels, the mean (±SD) expression level of HMGA2 mRNA was higher in tumor tissues (15.8 ± 40.7) than in nontumor tissues (3.6 ± 6.8; P < 0.05). We examined HMGA2 mRNA expression in seven representative cases by RT-PCR (data not shown; GAPDH mRNA expression is also shown for reference).

High HMGA2 expression correlates with clinicopathologic variables and poor prognosis. Based upon the median HMGA2 mRNA expression level, the 110 clinical cases were divided into two groups: high HMGA2 expression (n = 55) and low HMGA2 expression (n = 55). Table 1 shows the expression of HMGA2 mRNA in cancer samples and the clinicopathologic data of the 110 gastric cancer patients. The incidence of serosal invasion was significantly greater in the high-expression group than in the low-expression group (P < 0.05). The high-expression group also had inclinations of more lymphatic invasion (P = 0.09) and venous invasion (P = 0.08) than the low-expression group. No significant differences were observed with respect to sex, size, histologic cell type, lymph node metastasis, liver metastasis, peritoneal dissemination, and stage. The overall survival rate was significantly lower in patients with high HMGA2 mRNA expression than in those with low HMGA2 expression (P < 0.05; Fig. 3).

Univariate and multivariate analyses of survival. From Table 2, univariate analysis showed that the following factors were significantly related to postoperative survival: tumor size, serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion, liver metastasis, peritoneal dissemination, and high HMGA2 expression (P < 0.05). Multivariate analysis, using Cox proportional hazards model, indicated that high HMGA2 expression, lymph node metastasis, and peritoneal dissemination were found to be significantly independent prognostic factors for the patients with gastric cancer (P < 0.05). For overall survival, high HMGA2 expression was an independent predictor of reduced survival with a hazard ratio of 2.00 and a 95% confidence interval ranging from 1.32 to 3.15.

HMGA2 expression inversely correlates with expression of let-7 miRNA family. To evaluate the correlation between HMGA2 mRNA and the let-7 miRNA family, we analyzed HMGA2 mRNA and three variants of the let-7 miRNA family (let-7a, let-7b, and let-7c) in gastric cancer cell lines (MKN1, MKN28, MKN45, MKN74, KATOIII, and AZ521). As shown in Fig. 4, we found an inverse correlation between HMGA2 mRNA and let-7a in gastric cancer cell lines (r = -0.75, P = 0.08). No significant correlations between HMGA2 mRNA and let-7b or let-7c were observed.
Next, we selected nine gastric cancer cases with high expression of HMGA2 mRNA and 10 cases with low-expression levels. We analyzed the expressions of let-7a, let-7b, and let-7c in each of these 19 “high” and “low” cases by quantitative real-time RT-PCR. As shown in Fig. 5, the expressions of let-7a, let-7b, and let-7c in the HMGA2 low-expression group were significantly higher than in the HMGA2 high-expression group (P < 0.05).

**Discussion**

In this study, we show that HMGA2 is highly expressed in gastric cancer tissues compared with corresponding normal tissues, as illustrated in Figs. 1A and 2. Previous studies have reported that HMGA2 is highly expressed in various human epithelial-type neoplasias, such as breast cancers (13), lung cancers (14, 15), pancreatic carcinomas (16), oral squamous cell carcinomas (17), and thyroid carcinomas (18). Using array CGH and microarray analysis, Yang et al. reported that HMGA2 was 1 of 30 up-regulated genes showing a high degree of correlation between DNA copy number and mRNA expression in gastric cancers (39). However, to our knowledge, there are no reports concerning the clinical significance of increased HMGA2 expression in gastric cancer.

We studied HMGA2 mRNA expression with respect to various clinicopathologic factors in 110 gastric cancer patients. Our findings show that overexpression of HMGA2 mRNA was significantly associated with serosal invasion (P < 0.05) and that the high HMGA2 expression group had inclinations of more lymphatic invasion (P = 0.09) and venous invasion (P = 0.08) than the low-expression group (Table 1). Two prior studies reported that a strong correlation existed between HMGA2 and tumor cell invasiveness in breast cancer (13, 40). Rogalla et al. reported that HMGA2 expression was mainly observed in invasive ductal tumors with high histologic grade: 17 of 21 breast cancer samples with histologic grade 3, but only 3 of 16 samples with histologic grades 1 or 2 showed expression of HMGA2 (13). Fabjani et al. reported that the degree of HMGA2 mRNA expression in 13 invasive breast cancer cell lines was significantly higher than that in 13 noninvasive cell lines (40). Epithelial-mesenchymal transition converts epithelial cells to motile mesenchymal cells and triggers carcinoma invasiveness (41, 42). Two prior studies showed that HMGA2 is involved in epithelial-mesenchymal transition (17, 43). Miyazawa et al. found that strong staining of HMGA2 and loss of E-cadherin expression were observed at the invasive front of oral squamous cell carcinoma and that HMGA2 might be associated with epithelial-mesenchymal transition.

**Table 2. Univariate and multivariate analysis of clinicopathologic factors affecting survival rate**

<table>
<thead>
<tr>
<th>Clinicopathologic variables</th>
<th>No. patients</th>
<th>5-y survival rate (%)</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>Relative risk (confidence interval)</td>
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<tr>
<td>Age (y)</td>
<td>48</td>
<td>47.9</td>
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<tr>
<td>&gt;65</td>
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<td>51.5</td>
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<td>Sex</td>
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<td></td>
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<tr>
<td>Male</td>
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<td></td>
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<tr>
<td>&gt;3 cm</td>
<td>23</td>
<td>64.0</td>
<td>0.04*</td>
<td>1.17</td>
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<tr>
<td>≤3 cm</td>
<td>87</td>
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<td>Histologic cell type</td>
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<td>Well and moderately</td>
<td>55</td>
<td>51.4</td>
<td>0.39</td>
<td></td>
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<tr>
<td>Poorly and others</td>
<td>55</td>
<td>47.1</td>
<td></td>
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<td>Serosal invasion</td>
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<tr>
<td>Absent</td>
<td>67</td>
<td>69.0</td>
<td>&lt;0.0001*</td>
<td>1.33</td>
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<tr>
<td>Present</td>
<td>43</td>
<td>21.3</td>
<td></td>
<td>(0.91-1.98)</td>
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<td>Lymph node metastasis</td>
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</tr>
<tr>
<td>Absent</td>
<td>40</td>
<td>92.3</td>
<td>&lt;0.0001*</td>
<td>3.88</td>
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<tr>
<td>Present</td>
<td>70</td>
<td>27.5</td>
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<td>(1.94-10.25)</td>
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<td>Lymphatic invasion</td>
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<tr>
<td>Absent</td>
<td>32</td>
<td>91.1</td>
<td>0.0001*</td>
<td>0.95</td>
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<tr>
<td>Present</td>
<td>78</td>
<td>35.5</td>
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<td>(0.45-2.55)</td>
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<td>Venous invasion</td>
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<tr>
<td>Absent</td>
<td>80</td>
<td>63.0</td>
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<td>1.02</td>
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<tr>
<td>Present</td>
<td>30</td>
<td>12.2</td>
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<td>(0.68-1.50)</td>
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<td>Liver metastasis</td>
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<tr>
<td>Absent</td>
<td>105</td>
<td>51.2</td>
<td>0.0004*</td>
<td>1.93</td>
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<tr>
<td>Present</td>
<td>5</td>
<td>0</td>
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<td>(0.89-3.59)</td>
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<tr>
<td>Peritoneal dissemination</td>
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</tr>
<tr>
<td>Absent</td>
<td>93</td>
<td>59.9</td>
<td>&lt;0.0001*</td>
<td>3.09</td>
</tr>
<tr>
<td>Present</td>
<td>17</td>
<td>0</td>
<td></td>
<td>(1.79-5.46)</td>
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<tr>
<td>HMGA2 expression</td>
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<td></td>
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<tr>
<td>High</td>
<td>55</td>
<td>36.2</td>
<td>0.01*</td>
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<tr>
<td>Low</td>
<td>55</td>
<td>62.8</td>
<td></td>
<td>(1.32-3.15)</td>
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</table>

*P < 0.05.
transition and contribute to tumor invasion (17). Thuault et al. reported that HMGA2 regulates the transcription factors, such as Snail, Slug, Twist, and inhibitor of differentiation 2, and that HMGA2 is an effector of transforming growth factor-β that causes epithelial-mesenchymal transition (43). These observations indicate that HMGA2 plays a key role in tumor cell invasiveness, supporting our findings that HMGA2 overexpression is related to clinicopathologic factors, such as serosal invasion, lymphatic invasion, and venous invasion, and that cancer cells at invasive front were strongly stained for HMGA2 in gastric cancer.

We also found that the overexpression of HMGA2 mRNA in gastric cancer patients was significantly associated with poor prognosis and low overall survival (Fig. 3). Furthermore, a multivariate analysis showed that high HMGA2 mRNA expression was an independent prognostic factor associated with overall survival rate (Table 2). A strong association with high expression of HMGA2 and carcinoma invasiveness has led us to speculate that HMGA2 overexpression results in poor prognosis in patients with gastric cancer. Increased nuclear expression of HMGA2 correlated with poor survival for 49 lung adenocarcinoma patients (14). In oral squamous cell carcinomas, HMGA2-positive staining was correlated to long-term survival of patients and an independent prognostic value for disease-specific overall survival (17). High expression of HMGA2 correlated significantly with an adverse prognosis both for progression-free and overall survival of 100 ovarian cancer patients (19). HMGA2 mRNA expression in the peripheral blood of patients with metastatic breast cancer is an independent indicator of poor overall survival (20). These observations support our findings that HMGA2 overexpression is significantly related to poor prognosis in gastric cancer and suggests that HMGA2 might be a critical indicator for the prediction of survival in patients with gastric cancer.

Recently, there have been a number of reports of HMGA2 as a target of the let-7 miRNA family (19, 29–32). Thus, we investigated whether HMGA2 mRNA was negatively regulated by the let-7 miRNA family in gastric cancer. As shown in Fig. 4, we found an inverse relationship between HMGA2 mRNA and let-7a in gastric cancer cell lines. Additionally, expression of the let-7 miRNA family (let-7a, let-7b, and let-7c) in a low-expression group of HMGA2 mRNA is significantly higher than that in a high-expression group of HMGA2 mRNA, as illustrated in Fig. 5. miRNAs generally regulate gene expression at the posttranscription level in two different ways: (a) direct cleavage of the target mRNA using interference machinery (mRNA cleavage) and (b) inhibition of protein synthesis (translational repression; ref. 22). miRNA-mRNA cleavage is commonly found in plants, but most animal miRNAs are thought to use a second mechanism of gene regulation (translational repression; ref. 22). However, Shell et al. (19) and Lee et al. (32) recently showed that the let-7 miRNA family caused degradation of HMGA2 mRNA in several human cancer cell lines, including H1299, Tera-2, HeLa, and HepG2. These observations support our results that HMGA2 mRNA was negatively regulated by the let-7 miRNA family in human gastric cancer. To the best of our knowledge, this is the first
report showing a correlation between HMGA2 mRNA and the let-7 miRNA family in clinical samples of human cancer.

In conclusion, our results show that increased HMGA2 expression is associated with tumor cell invasiveness and poor prognosis in gastric cancer. Furthermore, high HMGA2 expression status is an independent prognostic factor for patients with gastric cancer. Our findings indicate that HMGA2 is negatively regulated by the let-7 miRNA family in human gastric cancer.

Acknowledgments

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References

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