

# HMGA2 Participates in Transformation in Human Lung Cancer

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## Abstract

Although previous studies have established a prominent role for *HMGA1* (formerly *HMG-I/Y*) in aggressive human cancers, the role of *HMGA2* (formerly *HMGI-C*) in malignant transformation has not been clearly defined. The *HMGA* gene family includes *HMGA1*, which encodes the HMGA1a and HMGA1b protein isoforms, and *HMGA2*, which encodes HMGA2. These chromatin-binding proteins function in transcriptional regulation and recent studies also suggest a role in cellular senescence. HMGA1 proteins also appear to participate in cell cycle regulation and malignant transformation, whereas HMGA2 has been implicated primarily in the pathogenesis of benign, mesenchymal tumors. Here, we show that overexpression of *HMGA2* leads to a transformed phenotype in cultured lung cells derived from normal tissue. Conversely, inhibiting *HMGA2* expression blocks the transformed phenotype in metastatic human non-small cell lung cancer cells. Moreover, we show that *HMGA2* mRNA and protein are overexpressed in primary human lung cancers compared with normal tissue or indolent tumors. In addition, there is a statistically significant correlation between HMGA2 protein staining by immunohistochemical analysis and tumor grade ( $P < 0.001$ ). Our results indicate that *HMGA2* is an oncogene important in the pathogenesis of human lung cancer. Although additional studies with animal models

are needed, these findings suggest that targeting HMGA2 could be therapeutically beneficial in lung cancer and other cancers characterized by increased HMGA2 expression. (Mol Cancer Res 2008;6(5):743–50)

## Introduction

Although previous studies have established a role for high-mobility group A2 or *HMGA2* gene (formerly *HMGI-C*) in slow-growing, benign, mesenchymal tumors (1-6), its function in malignant transformation has not been clearly defined. The *HMGA* genes encode a subfamily of the high-mobility group (HMG) proteins, which were discovered >25 years ago as abundant, nonhistone chromatin-binding proteins, and named based on their electrophoretic mobility in polyacrylamide gels (6-8). The HMGA protein subfamily (8) includes HMGA1a (formerly HMG-I), HMGA1b (formerly HMG-Y), and HMGA2 (formerly HMGI-C). HMGA1a and HMGA1b protein isoforms result from alternative splicing of the *HMGA1* mRNA (9, 10), whereas HMGA2 is encoded by the separate but related gene, *HMGA2* (1, 2). All HMGA proteins contain AT-hook DNA-binding motifs that mediate binding to the minor groove of chromosomal DNA. After binding to DNA, HMGA proteins induce a conformational change and facilitate DNA transcription factor interactions. Previous studies indicate that HMGA proteins function in regulating gene expression (reviewed in ref. 11); more recent studies also suggest a role in cellular senescence (12).

Increasing evidence implicates HMGA proteins in normal and neoplastic cell growth. *HMGA1* and *HMGA2* are expressed as delayed-early genes after growth factor stimulation and their expression is increased in cellular proliferation (13). *HMGA1* expression is also increased in neoplastic transformation (11). Several independent laboratories have shown that overexpression of HMGA1a and HMGA1b result in a transformed phenotype in cultured cells (14-18). In addition, two different transgenic mouse models overexpressing HMGA1 develop lymphoid malignancies and other tumors (18-20). Antisense experiments show that decreasing HMGA1 proteins block the transformed phenotype (15-17, 20, 21). Moreover, increased levels of *HMGA1* mRNA or protein correlate with more advanced disease in some tumors (22).

The role of HMGA2 in normal and neoplastic cell growth is less well established. *In vitro* studies show that HMGA2 chimeric proteins bind to DNA and alter the transcription of reporter genes (23). HMGA2 seems to be involved in the

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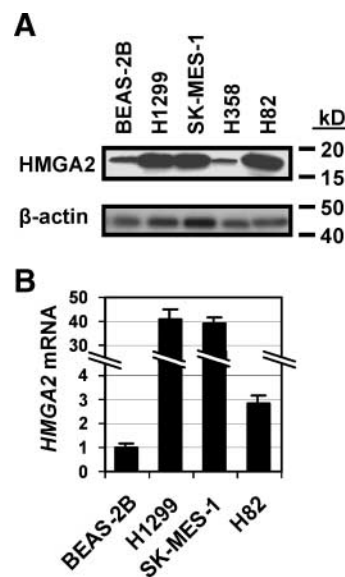
pathogenesis of benign, solid human tumors (1-6). Specifically, the HMGA2 AT-hooks have been identified in chimeric proteins associated with lipomas and other benign, mesenchymal tumors (2-5). These chimeras are thought to function by binding to DNA via the AT-hooks and altering gene expression, possibly through the potential transcriptional regulatory domains acquired in their rearrangement with HMGA2 (3). The HMGA2 chimeras or a truncated HMGA2 with three AT-hook DNA-binding domains, induce anchorage-independent cell growth in NIH3T3 murine fibroblasts (24), whereas full-length HMGA2 confers a transformed phenotype in cultured, rat fibroblasts and human lymphocytes (14). In addition, transgenic mice expressing a truncated *HMGA2* transgene develop increased fat, gigantism, and lipomas (25, 26). Transgenic mice expressing full-length, wild-type *HMGA2* driven by the cytomegalovirus promoter develop benign epithelial tumors (27), including pituitary adenomas (85% of females and 40% of males) as well as micropolycystic kidney disease (~50% of all mice). At lower frequencies, these mice also develop lipomatosis (20-29%), large cell lymphomas (<25%), and lung adenomas (<25%). In another transgenic model with *HMGA2* driven by a differentiated mesenchymal cell (adipocyte)-specific promoter, mice developed mesenchymal tumors, including fibroadenomas of the breast and salivary glands (28). Interestingly, this same group also generated transgenic mice expressing a truncated version of HMGA2 containing only the three DNA-binding domains which produced similar mesenchymal tumors (28). Both HMGA2 transgenic mouse models resulted in mice with predominantly benign, slow-growing tumors, similar to the types of human tumors with mutations leading to the expression of abnormal HMGA2 fusion proteins. Interestingly, the *HMGA2* (*HMGI-C*) knockout mouse displayed a pygmy phenotype (29). More recently, increased expression of *HMGA2* was identified in a significant percentage of malignant, primary human breast epithelial tumors (30) and lung cancers (31, 32), which indicates a potential role for HMGA2 in the pathogenesis of epithelial cancers in addition to benign mesenchymal tumors.

Here, we show that *HMGA2* gene and protein expression are increased in human lung cancer cell lines and primary lung tumors compared with benign tumors or normal lung tissue. We also show that *HMGA2* is both necessary and sufficient for transformation in cultured lung cells. Moreover, our findings specifically implicate *HMGA2* in the pathogenesis of human lung cancers.

## Results

### *HMGA2* Expression Is Increased in Cultured Human Lung Cancer Cells

To determine if HMGA2 protein levels are increased in lung cancer cells derived from malignant neoplasms compared with normal lung tissue or benign tumors, we first surveyed cultured human lung cancer cell lines for HMGA2 protein levels and compared them to cultured cells derived from normal lung tissue (Fig. 1A). For control cells, we used BEAS-2B cells, which were isolated from normal human bronchial epithelium (33). We found that HMGA2 protein levels were increased in all metastatic lung cancer cell lines compared with control cells,



**FIGURE 1.** *HMGA2* expression is increased in lung cancer cell lines from metastatic disease. **A.** Western blot analysis shows increased HMGA2 in the human lung cancer cell lines H1299 (metastatic non-small cell carcinoma), SK-MES-1 (metastatic squamous cell carcinoma), and H82 (metastatic small cell carcinoma) compared with the normal human bronchial epithelium cell line BEAS-2B. In H358 cells derived from nonmetastatic, non-small cell bronchioloalveolar carcinoma, HMGA2 protein levels are similar to that of the BEAS-2B immortalized control cell line. **B.** Quantitative RT-PCR analysis shows increased *HMGA2* mRNA in H1299, SK-MES-1, and H82 metastatic lung cancer cells compared with BEAS-2B cells, consistent with the Western blot analysis (**A**). Fold differences of RNA expression compared with BEAS-2B cells (arbitrarily assigned a value of 1); columns, mean from two independent experiments done in triplicate; bars, SD.

including H1299 metastatic non-small cell carcinoma cells, SK-MES-1 metastatic squamous cell carcinoma cells, and H82 metastatic small cell carcinoma cells. H358 cells, which were derived from a localized non-small cell bronchioloalveolar carcinoma, had HMGA2 levels similar to the BEAS-2B cells. Interestingly, bronchioloalveolar carcinomas tend to be localized and lacking in parenchymal invasion (34, 35). Moreover, patients with bronchioloalveolar carcinomas have a 1-year survival rate of 64.9% (34), which is significantly better than most lung cancers (35).

We also analyzed *HMGA2* expression by quantitative reverse transcription-PCR (RT-PCR) in the H1299, SK-MES-1, and H82 lung cancer cell lines (Fig. 1B). Consistent with our Western results, we found high levels of *HMGA2* mRNA in the cells derived from lung cancers compared with normal lung cells (BEAS-2B).

### *Inhibiting HMGA2* Expression Blocks Transformation in Lung Cancer Cells

To determine if HMGA2 is required for transformation in lung cancer, HMGA2 protein was decreased in the H1299 lung cancer cells (derived from the metastatic, non-small cell lung cancer) using an RNA interference approach with a ribozyme antisense vector (15). Two polyclonal cell lines (HMGA2-AS-1 and 2) were independently generated by transfecting H1299 with the antisense construct HMGA2-AS. Both cell lines

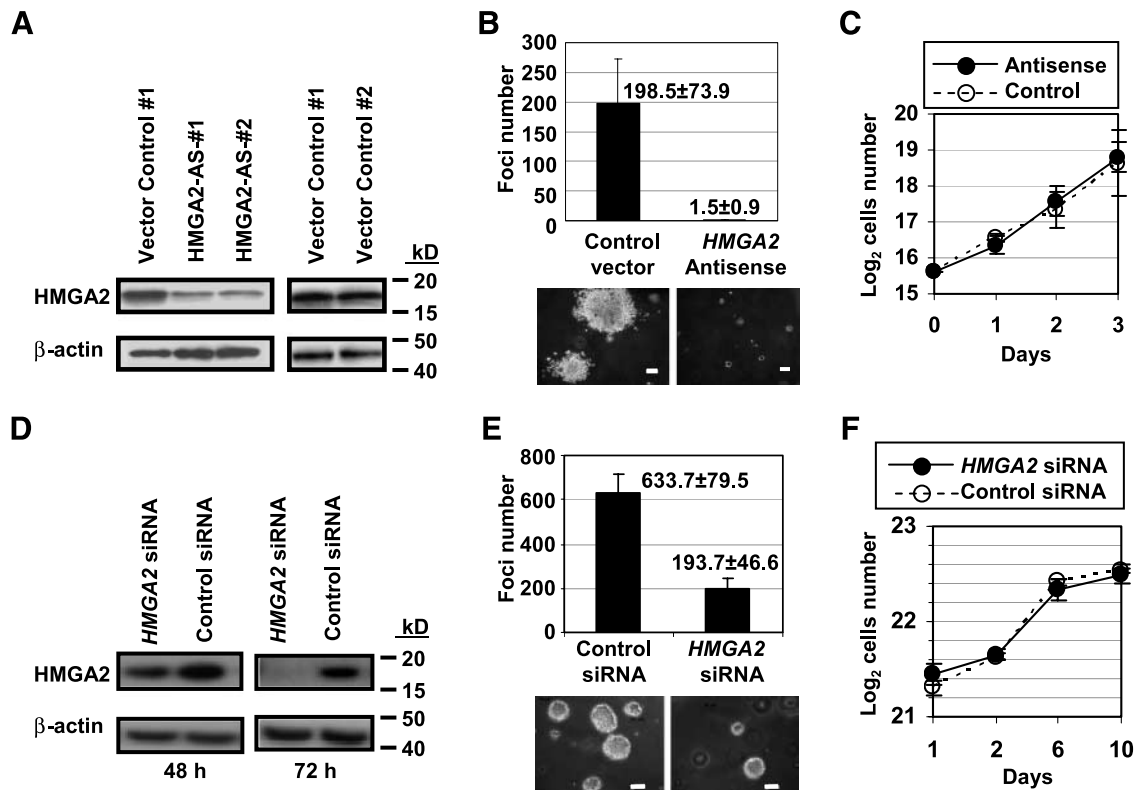
exhibited significantly reduced HMGA2 proteins (Fig. 2A). The related proteins, HMGA1a and HMGA1b, were unaffected (data not shown), indicating that the antisense vectors caused a specific, significant decrease in HMGA2 proteins. We observed that blocking HMGA2 expression strongly impaired anchorage-independent cell growth on soft agar, demonstrating that *HMGA2* is required for this transformed phenotype in these metastatic human lung cancer cells (Fig. 2B). The growth rates of the control and antisense cells were similar, indicating that the difference in foci formation was not due to a general inhibition of cell growth or toxic effect that impairs proliferation (Fig. 2C).

To further investigate the role of HMGA2 in advanced-stage, metastatic non-small cell lung cancer cells, we used a short inhibitory RNA interference oligonucleotide (siRNA; Dharmacon) to knock down *HMGA2* expression in another lung cancer cell line. For these experiments, we used SK-MES-1 metastatic squamous cell lung carcinoma cells which express high levels

of *HMGA2* mRNA and protein. This siRNA approach effectively silences *HMGA2*, both at the protein (Fig. 2D) and mRNA level for up to 6 days (Supplementary Fig. S1). Similar to our results with H1299 cells, we found that knocking down *HMGA2* expression also blocks foci formation in these lung cancer cells (Fig. 2E). Cellular proliferation was not affected by the siRNAs in standard culture conditions, indicating that the siRNAs were not toxic to cell growth at the concentrations used (Fig. 2F). Taken together, our results show that inhibiting *HMGA2* expression in metastatic lung cancer cells blocks anchorage-independent cell growth and suggests an important role for HMGA2 in this cancer phenotype.

*Increased Expression of HMGA2 in Normal Lung Cells Leads to a Transformed Phenotype*

Because our antisense knockdown experiments showed that HMGA2 proteins are necessary for the transformed phenotype



**FIGURE 2.** Interfering with *HMGA2* expression blocks transformation in metastatic lung cancer cells. **A.** Western blot analysis shows decreased HMGA2 in H1299 lung cancer cells transfected with the *HMGA2* antisense constructs (HMGA2-AS-1 and HMGA2-AS-2) compared with control cells transfected with vector alone. The blot was probed with the HMGA2 antibody as well as an antibody to  $\beta$ -actin to control for protein loading. **B.** Anchorage-independent growth on soft agar is inhibited in H1299 lung cancer cells transfected with the *HMGA2* antisense construct compared with control cells transfected with vector alone. The number of foci formed by H1299 cells transfected with the antisense construct is decreased compared with the control cell transfected with vector control. The experiment was done in duplicate and repeated; results are expressed as an average  $\pm$  SD from two independent experiments. Photograph of representative foci; bars, 100  $\mu$ m. **C.** The growth rates of the control and antisense cells were similar, indicating that the difference in foci formation was not due to inhibition of growth in general. Points, averages from duplicate plates; bars, SD. - - -  $\circ$  - - -, control; —●—, *HMGA2* antisense. **D.** Western blot analysis shows decreased HMGA2 in SK-MES-1 lung cancer cells transfected with the *HMGA2* siRNA compared with cells transfected with the control siRNA (siCONTROL nontargeting siRNA pool; Dharmacon). The blot was probed with the HMGA2 antibody as well as an antibody to  $\beta$ -actin to control for protein loading. **E.** Anchorage-independent growth on soft agar is inhibited in SK-MES-1 lung cancer cells transfected with the *HMGA2* siRNA compared with cells transfected with the control siRNA. The number of foci formed by SK-MES-1 cells transfected with the *HMGA2* siRNA was decreased compared with cells transfected with the control siRNA. The experiment was done in duplicate and repeated; results are expressed as an average  $\pm$  SD from two independent experiments. Photograph of representative foci; bars, 100  $\mu$ m. **F.** The growth rates of SK-MES-1 cells transfected with either *HMGA2* siRNA or control siRNA were similar, indicating that the difference in foci formation was not due to a generalized inhibition of growth or a toxic effect. Points, averages from duplicate plates; bars, SD. - - -  $\circ$  - - -, control siRNA; —●—, *HMGA2* siRNA.

in cultured lung cancer cells, we next sought to determine if overexpression of *HMGA2* in normal cultured lung cells results in a transformed phenotype. BEAS-2B cells were transfected with an *HMGA2* expression construct or the control vector. Western analysis showed that the *HMGA2* protein was overexpressed in the cells transfected with *HMGA2* compared with control cells transfected with vector alone (Fig. 3A). We found that both BEAS-2B cells exhibited anchorage-independent cell growth and formed foci on soft agar when transfected to overexpress *HMGA2* (Fig. 3B). Growth rates in standard tissue culture conditions were not significantly affected by *HMGA2* transfection in these cells (Fig. 3C), indicating that the observed phenotypes did not occur simply by enhancing cell growth rates. These studies show that overexpression of *HMGA2* leads to a transformed phenotype in cultured cells derived from normal lung tissue and suggest that *HMGA2* induces transformation by activating transformation-specific pathways independent of cellular proliferation rates.

#### Increased Expression of *HMGA2* in Human Lung Cancers

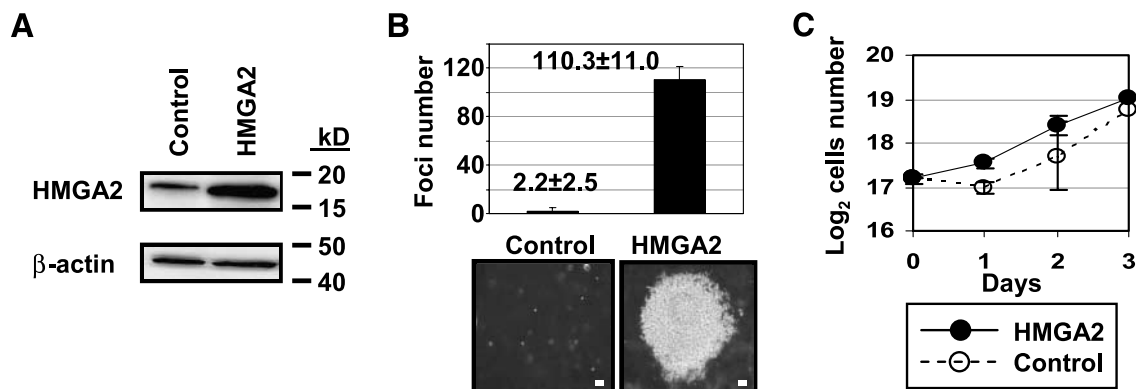
To determine if our studies with cultured cells are relevant to human lung cancers, we evaluated the expression of *HMGA2* by quantitative RT-PCR in primary human lung tumors, including adenocarcinomas, squamous cell carcinomas, and carcinoid tumors (Fig. 4, Supplementary Table S1). In 20 of 26 (77%) primary lung tumors, *HMGA2* expression was increased by 2-fold or more compared with that of normal controls (Clontech). The control RNA was derived from pooled, lung tissue from three previously healthy adults with ages ranging from 19 to 50 years, who had died from causes other than lung cancer. In most cases, *HMGA2* expression was increased by 15-fold or more (Fig. 4). Most of the non-small cell lung cancers had elevated *HMGA2* expression, including all squamous cell carcinomas (9 of 9) and most adenocarcinomas (11 of 15). Both of these tumor types are associated with smoking and a poor outcome with only a 10% overall 5-year

survival rate (35). There was a history of smoking in most of these cases (Supplementary Table S1). Adenocarcinomas are slightly more common lung cancers, accounting for ~40% of all lung cancers, whereas squamous cell carcinomas make up ~20% to 30% of all lung cancers (35). Of note, the tumor with the lowest *HMGA2* mRNA was taken from the only patient with grade 1 histology (well-differentiated). For squamous cell carcinomas, the levels of *HMGA2* mRNA were more than 2-fold above the pooled, normal control lung RNA for tumors designated as grade 2 (moderately differentiated) or grade 3 (poorly differentiated). For adenocarcinoma tumors with *HMGA2* mRNA levels  $\geq 40$ -fold above the control, two of three tumors were grade 3, with the remaining case being grade 2. Interestingly, *HMGA2* expression was not increased in the slow-growing carcinoid tumors. These tumors are typically localized and have a 5-year survival rate of 96% (35). Thus, *HMGA2* expression was increased in most (20 of 24; 83%) of the malignant lung neoplasms, but not in the slow-growing carcinoid tumors (0 of 2). These findings suggest that it may be a marker for malignant lung tumors.

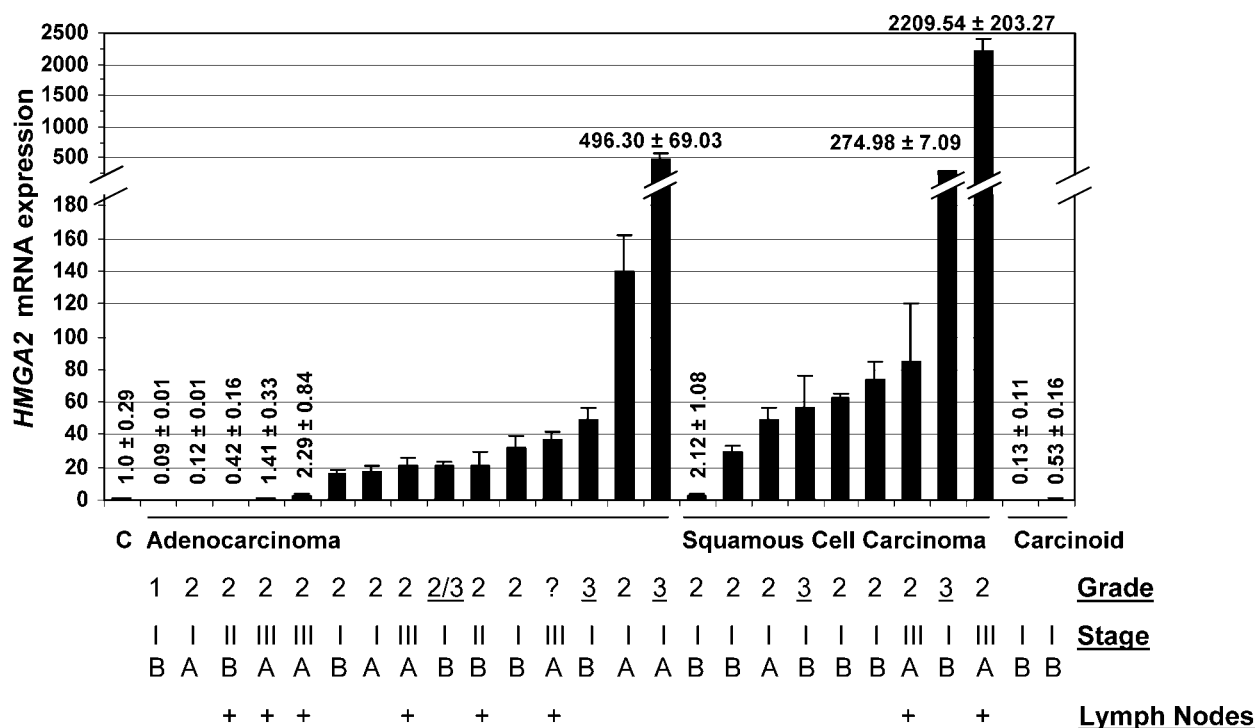
To investigate whether *HMGA2* overexpression was associated with gene amplification, we did a quantitative PCR analysis on genomic DNA extracted from 15 of the 26 lung cancer samples for which sufficient frozen material was available. These included adenocarcinomas ( $n = 10$ ), squamous cell carcinoma ( $n = 3$ ), and carcinoid tumors ( $n = 2$ ), as well as control, normal lung tissue ( $n = 9$ ). None of these samples showed any evidence of amplification.

#### *HMGA2* Protein Expression Correlates with Tumor Grade

To determine if *HMGA2* protein levels are increased in lung cancer, we performed immunohistochemical staining of a lung tissue microarray containing 91 primary lung tumors using a commercial *HMGA2* antibody (Table 1; Fig. 5). We identified *HMGA2* immunoreactivity in 37 of 89 (41.6%) evaluable cases, with scores ranging from 1 to 9 (median 6). Two cases did not contain sufficient tumor for evaluation and were



**FIGURE 3.** Overexpression of *HMGA2* in human lung cells derived from normal tissue leads to a transformed phenotype. **A.** Western blot analysis shows that the BEAS-2B cells transfected with the pSG5-HMG-C (*HMGA2*) construct overexpress *HMGA2* compared with cells transfected with control vector. All lanes were probed with the *HMGA2* antibody as well as the  $\beta$ -actin antibody to control for sample loading. **B.** Transformed foci in BEAS-2B cells transfected with the pSG5-HMG-C (*HMGA2*) construct compared with cells transfected with vector alone. The number of transformed foci formed in the cells overexpressing *HMGA2* or control cells are indicated. The experiments were done in triplicate and repeated twice; columns, mean from independent experiments; bars, SD. Photograph of representative foci; bar, 100  $\mu$ m. **C.** The growth rates of the control and antisense cells were similar, indicating that *HMGA2* overexpression does not affect adherent growth in these cells. Points, averages from duplicate plates; bars, SD. ---○---, control; —●—, *HMGA2* antisense.



**FIGURE 4.** *HMGA2* mRNA expression is increased in primary lung tumor samples. Quantitative RT-PCR shows that *HMGA2* mRNA expression is increased in 20 of 24 primary lung tumors compared with the control pooled, normal lung tissue or slow-growing carcinoid tumors. Fold differences of mRNA expression compared with normal pooled lung mRNA (arbitrarily set at 1); columns, mean from two independent experiments done in triplicate; bars, SD. Bottom, tumor grade and stage. Additional clinical data is available in Supplementary Table S1.

excluded. Of the positive cases, 4 (10.8%) displayed limited reactivity (score <3), 15 (40.5%) displayed moderate reactivity (score 3-6), and 18 (48.6%) displayed strong reactivity (score >6). There was a significant positive correlation between *HMGA2* staining and tumor grade ( $P < 0.001$  by Spearman's rank correlation). The mean *HMGA2* score for tumors with a histologic grade > 2 was  $4.43 \pm 4.14$  compared with  $1.52 \pm 2.61$  for tumors with a histologic grade  $\leq 2$  ( $P = 0.0021$ ; Student's *t* test). Immunoreactivity for *HMGA2* was seen in adjacent nonneoplastic bronchial epithelium, but was not

observed in any other normal tissues. These findings show that *HMGA2* protein levels correlate positively with a more advanced histologic tumor grade.

### Discussion

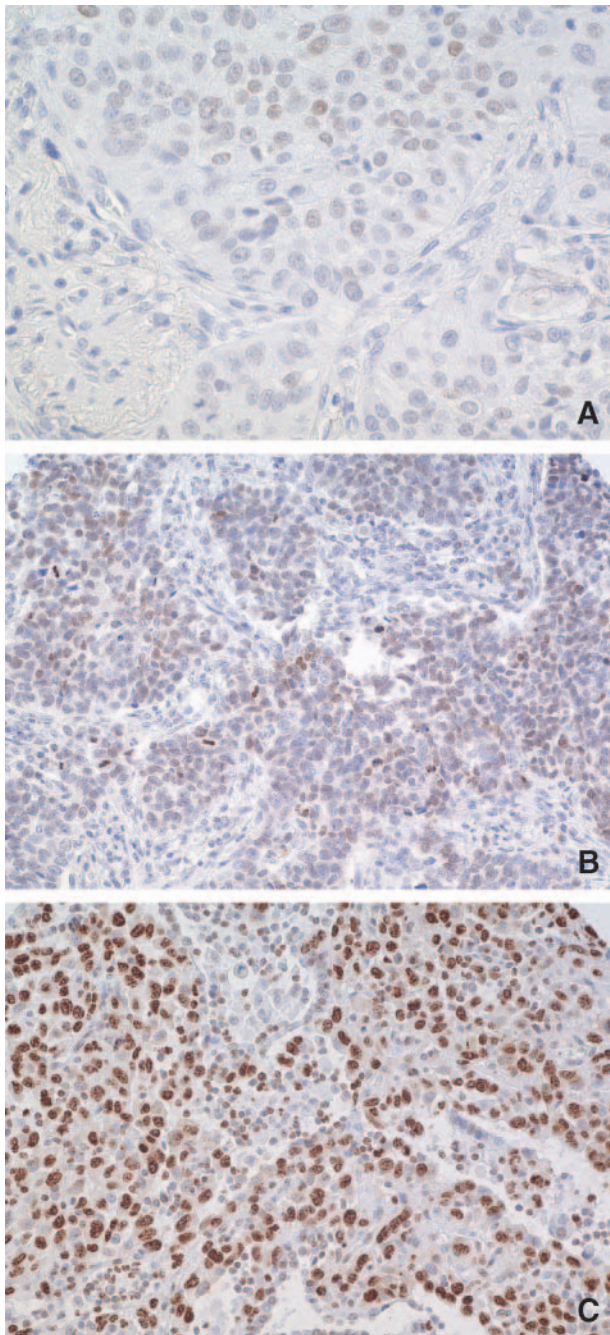
Lung cancer is the leading cause of cancer death worldwide, striking nearly 175,000 individuals with >160,000 deaths each year in the United States alone (35). Approximately 1 in 13 men and 1 in 17 women will develop lung cancer over the course of

**Table 1. HMGA2 Expression by Histologic Type and Clinical Characteristics in Tissue Microarray**

Histologic type	Age, y (mean $\pm$ SD)	Gender (male/female)	Smoking history*	Stage				Death before 5 y	HMGA2		
				I	II	III	IV		<3	3 to 6	>6
AC (n = 29)	66.3 $\pm$ 11.4	9/20	22/25	17	6	1	5	13	24	4	1
SCC (n = 30)	68.1 $\pm$ 8.0	25/5	25/27	18	3	5	4	16	12	10	8
BAL (n = 8)	66.4 $\pm$ 9.7	2/6	5/6	8	0	0	0	2	1	0	0
CA NOS (n = 8)	66.3 $\pm$ 8.4	4/4	7/7	4	1	1	2	4	2	0	6
Carcinoid (n = 3)	61.3 $\pm$ 17.1	1/2	1/3	1	0	0	1	1	3	0	0
LCLC (n = 2)	76.5 $\pm$ 5.0	1/1	2/2	1	1	0	0	1	1	1	0
AGC (n = 1)	68	1/0	1/1	0	0	0	1	1	1	0	0
PSC (n = 1)	63	0/1	1/1	1	0	0	0	0	0	0	1
SCLC (n = 3)	64 $\pm$ 3.0	2/1	3/3	NA	NA	NA	NA	1	3	0	0
Undiff (n = 3)	60 $\pm$ 13.1	1/2	1/3	1	0	1	1	1	1	0	2

Abbreviations: AC, adenocarcinoma; SCC, squamous cell carcinoma; BAL, bronchoalveolar carcinoma; CA NOS, carcinoma, not otherwise specified; LCLC, large cell lung cancer; AGC, anaplastic giant cell carcinoma; PSC, pleomorphic sarcomatoid carcinoma; SCLC, small cell lung carcinoma; Undiff, undifferentiated carcinoma; NA, not available.

\*Smoking history where available.



**FIGURE 5.** Immunohistochemical analysis of the HMGA2 protein expression in lung squamous cell carcinoma. **A.** Grade 2, moderately differentiated squamous cell carcinoma with weak staining (score <3; magnification,  $\times 100$ ). **B.** Grade 2, moderately differentiated squamous cell carcinoma with moderate staining (score 3-6; magnification,  $\times 64$ ). **C.** Grade 3, poorly differentiated SCC with strong staining (score >6; magnification,  $\times 64$ ).

a lifetime and the incidence is rising, particularly in developing nations (35). The overall 5-year survival rate for all lung cancers is only 15%, and it is only 10% for squamous cell carcinoma and adenocarcinoma (35). Thus, research is urgently needed to identify the molecular pathways that lead to lung

carcinogenesis. The inactivation of tumor suppressor genes, such as *p53*, *Rb*, and *p16* is a frequent molecular abnormality in lung cancer, as well as the activation or amplification of proto-oncogenes including *K-ras*, *c-myc*, and *HER2/neu*, *EGFR*, *cyclin D1*, and *BCL2* (36, 37). Telomerase RNA and the hTERT catalytic component are also expressed in most lung cancers, and may provide a mechanism for unlimited cellular replication (36). These genetic alterations are believed to lead to aberrant cell growth and ultimately contribute to the malignant phenotype. Interestingly, HMGA2 has been shown to bind to and inactivate pRB, which could play a role in lung carcinogenesis (38).

HMGA2 has been implicated primarily in benign tumors derived from mesenchymal tissue (2-5), although there is an emerging body of evidence indicating that HMGA2 may also play a role in the pathogenesis of aggressive tumors (30-32). We first showed that overexpression of HMGA2 leads to anchorage-independent cell growth in both rat and human cultured cells (14). There have been limited studies suggesting that *HMGA2* is required for transformation in cancer. An earlier study showed that down-regulating HMGA2 by an antisense approach prevented the transformation of thyroid cells by *v-mos* or *v-ras*-Ki (39). In this study, it was not clear if the antisense vector was specific for *HMGA2* alone, or also led to the down-regulation of HMGA1 proteins. It was later shown that repressing *HMGA2* expression in retinoblastoma cells decreases proliferation in these cells, although anchorage-independent cell growth, tumorigenicity, or other transformation phenotypes were not assessed (39). Suppressing HMGA2 protein synthesis also blocks proliferation and induces apoptosis in well-differentiated liposarcoma cells (40). There have been no prior studies to show that inhibiting HMGA2 expression in metastatic or advanced stage cancer cell lines blocks anchorage-independent cell growth. More recently, nuclear staining for both HMGA1 and HMGA2 proteins was found to be elevated in varied lung cancer subtypes, including adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma, large-cell lung cancer, large-cell neuroendocrine carcinoma, small-cell lung cancer, and pleomorphic carcinoma (31). In that study, increased levels of HMGA2 protein, using a semiquantitative approach with immunohistochemical analysis, also correlated with patient death in primary lung adenocarcinomas from 49 cases. A quantitative RT-PCR assay was used to assess *HMGA2* mRNA in a small subset of these tumors. A more recent study showed similar results with increased *HMGA2* mRNA in lung cancer samples compared with adjacent lung tissue (32). In our study, we extend these studies and show a functional role for *HMGA2* in lung cancer. We show that *HMGA2* mRNA expression (assessed by quantitative RT-PCR) from primary tumors is increased in most primary lung cancers (21 of 24 or 88%), including 12 of 15 adenocarcinoma samples and 9 of 9 squamous cell carcinomas compared with control lung tissue or indolent, carcinoid tumors (2 of 2). We also found that HMGA2 protein levels correlate with increasing tumor grade in a large tissue microarray of primary lung tumors. Our studies also show that ectopic expression of HMGA2 is sufficient for anchorage-independent cell growth and transformation in soft agar in cells derived from normal lung tissue. Moreover, HMGA2 is required for the

transformed phenotype in two different, metastatic non-small cell lung cancer cells (H1299 and SK-MES-1). Taken together, our results indicate that *HMGA2* plays an important role in lung carcinogenesis. Although additional studies are needed, our studies also suggest that *HMGA2* could be a rational therapeutic target in lung and other aggressive cancers characterized by increased *HMGA2* expression.

## Materials and Methods

### Cell Culture and Transfection

BEAS-2B, H1299, SK-MES-1, H358, and H82 cells were obtained from American Type Culture Collection and grown according to its guidelines. Cells were transfected using lipofectin as described by the manufacturer (Life Technologies). Polyclonal, pooled, resistant cell lines overexpressing *HMGA2* or control vector were selected in medium containing puromycin (0.75 µg/mL). H1299 cells transfected with the *HMGA2* ribozyme antisense or control constructs were selected in medium containing zeocin (75 µg/mL). Two polyclonal, pooled, resistant cell lines generated from two independent transfection experiments, both with decreased *HMGA2* proteins following Western analysis, were used for soft agar analysis.

### Plasmids

The *HMGA2* antisense construct was derived from the vector pU1/RIBOZYME (15) by inserting a synthetic *HMGA2*-specific dsDNA sequence in the *EcoRI/SpeI* cloning site (5'-GAATTCTTTAGAGGGACCTGATGAGTCCGTGAGGACGAACTCTTGTCTTCTGCCTAG-3'). The cloned DNA fragment incorporates an autocatalytic hammerhead ribozyme sequence (underlined). The parent vector pU1/RIBOZYME was used as a control. The plasmid pSG5-HMG-C (*HMGA2*) has been previously described (14).

### *HMGA2* siRNA Knockdown Experiments and Western Analysis

SK-MES-1 cells were transiently transfected with the *HMGA2*-specific siRNA or siCONTROL nontargeting pooled siRNA (20 µmol/L; Dharmacon) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. *HMGA2* and  $\beta$ -actin protein levels were assessed at 2, 3, and 6 days after transfection with the siRNA by Western analysis. mRNA levels were assessed at 1, 2, 3, and 6 days as described under quantitative RT-PCR methods. Western blot analysis of *HMGA* proteins was done as described (14, 15).

### Cellular Growth Rate Determinations

Cellular growth rates were determined as previously described (14, 15), with the following exceptions. Cells were seeded at  $5 \times 10^4$  into six separate 5-cm tissue culture dishes. Duplicate dishes were harvested every 24 h for 3 days and the cells were counted.

### Soft Agar Assays

The soft agar assay was done as previously described (14, 15), except that cells were suspended in 3 mL of 0.4% agarose and poured onto a 5-mL 0.7% agarose bed in 100-mm tissue culture dishes. Colonies >0.1 mm in size were counted after 3 to 4 weeks.

### Quantitative RT-PCR

Quantitative RT-PCR was done by TaqMan chemistry as previously described (19, 20), except that the *HMGA2*-specific primers 5'-AAAGCAGCTCAAAGAAAGCA and 5'-TGTTGTGGCCATTCCTAGGT and the *HMGA2*-specific probe 5'-CACTGGAGAAAACGGCCAAG were used. Gene expression indicated as fold changes compared with control samples was calculated with the Applied Biosystem software using the absolute quantification (standard curve) option and  $\beta$ -actin as housekeeping internal standard as we previously described (20). Control samples were arbitrarily assigned a value of 1.0.

To assess possible gene amplification, quantitative RT-PCR was done on DNA from frozen specimens as previously described (41, 42) using SYBR green chemistry. The primers were 5'-ACCAACCGGTGAGCCCTCTC and 5'-TTGAGC-TGCTTTAGAGGGACTCTTG (for *HMGA2* exon 2), 5'-AAAGCAGAAGCCACTGGAG and 5'-CATTTCCTAGGTCTGCCTC (for *HMGA2* exon 3), and 5'-ACAGATTCGGGCCGAGATG and 5'-GATAGCTCCAGGCCA-GAAAG for  $\beta$ -2 microglobulin exon 1 as an internal control. Standard curves from serial dilutions of cDNA were calculated for each run and primer/probe set.

### Immunohistochemical Analysis

Tissue microarray blocks were created using 1-mm cores taken from 91 archival, routinely processed, paraffin-embedded, lung cancer specimens retrieved from the surgical pathology archives of the Johns Hopkins Medical Institutions, Baltimore, MD. The specimens had been accessioned between 1997 and 2001, and were selected solely based on availability of tumor tissue in the block. Each case was represented by three cores on the tissue microarray. Additionally, each array contained cores from routinely processed, paraffin-embedded normal tissues also taken from the archives, including artery, brain, breast, cecum, duodenum, gallbladder, intestine, kidney, liver, lung, lymph node, ovary, muscle, pancreas, placenta, prostate, salivary gland, skin, stomach, testis, thyroid, and tonsil. Tissue microarrays were sectioned at 4-µm intervals, deparaffinized in xylene, and rehydrated with graded alcohols. The sections were then heated in antigen retrieval solution (Dako) and were immunohistochemically stained for *HMGA2* protein following directions of the manufacturer (*HMGA2*, 0.004 mg/mL; Biocheck, Inc.). Positive staining was identified as nuclear immunoreactivity and was scored using a nine-point scale based on the product of staining intensity (weak, 1; moderate, 2; strong, 3) and staining extent (percentage of positive cells: 5-30%, 1; 30-60%, 2; >60%, 3). The highest score per case was used for subsequent analyses. Tissue microarrays were analyzed microscopically in a blinded fashion by a single investigator (A. Hristov).

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