Transcriptional activation of FN1 and IL11 by HMGA2 promotes the malignant behavior of colorectal cancer

Jingjing Wu†, Yuhong Wang†, Xi Xu†, Hui Cao, Sana Sahengbieke, Hongqiang Sheng, Qiong Huang and Maode Lai*

Department of Pathology, Key Laboratory of Disease Proteomics of Zhejiang Province, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China

*To whom correspondence should be addressed. Tel/Fax: +86 571 8820 8198; Email: lmp@zju.edu.cn

†These authors contributed equally to this work.

Abstract

Colorectal cancer (CRC) is the second leading cause of cancer deaths worldwide, and metastasis is the principle reason for its poor prognosis. Overexpression of high-mobility gene group A2 (HMGA2) contributes to the aggressiveness of CRC. However, the underlying molecular mechanism of its overexpression is still elusive. In this study, we showed that ectopic expression of HMGA2 significantly enhanced cell migration and invasion in vitro and promoted tumor growth and distant metastasis in vivo. In contrast, the silencing of HMGA2 produced the opposite effects in vitro and in vivo. Chromatin immunoprecipitation-PCR and luciferase assays revealed that HMGA2 bound directly to the promoters of FN1 and IL11 and significantly induced their transcriptional activities. Moreover, as the direct downstream target of HMGA2, IL11 modulated cell migration and invasion through a pSTAT3-dependent signaling pathway. Furthermore, a strong positive correlation between HMGA2 and IL11 expression was identified in 122 CRC tissues. High IL11 expression was associated with poor differentiation, a large tumor size, lymph node metastasis and low overall survival in CRC patients. Collectively, our data reveal novel insights into the molecular mechanisms underlying HMGA2-mediated CRC metastasis and highlight the possibility of targeting HMGA2 and IL11 for treating CRC patients with metastasis.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality, with an estimated 132,700 new cases and 49,700 deaths in the USA in 2015 (1). As approximately two-third of CRC patients are diagnosed at an advanced stage, local invasion and distant metastasis account for the majority of cancer mortalities (2). Unfortunately, the reasons for the aggressiveness of advanced stage colon cancer are still not well understood. Therefore, it is crucial to elucidate the molecular mechanisms promoting the metastatic properties of CRC cells.

High-mobility gene group A2 (HMGA2), an architectural transcription factor, modulates the transcription of multiple genes through protein–DNA or protein–protein interactions (3–5). HMGA2 plays a crucial role in cancer metastasis and stem cell reprogramming by upregulating the expression of lysyl oxidase and syndecan-2 in breast cancer (6), inducing SOX2 expression in oral cancer (7) and modulating the Wnt/β-catenin pathway in gastric cancer (8). As an oncogenic protein, HMGA2 was found to be upregulated in a variety of cancers, including ovarian (9,10), pancreatic (11), lung (12–14) and CRC (15), and was associated with a metastatic phenotype, aggressive behavior and poor prognosis. Wang et al. (15) reported that higher HMGA2 expression was associated with more advanced stages of cancer and a higher risk of distant metastasis in CRC patients. Overall survival (OS) and progression-free survival analyses revealed that overexpression of HMGA2 was significantly related to poor prognosis in stages III–IV CRC patients. Consistent with their study, we also found that HMGA2 was statistically correlated with lymph node metastasis and advanced stage. HMGA2 could be used to predict CRCs, as the elevated expression of HMGA2 indicated
ChIP samples were then amplified, labeled and hybridized to Nimblegen human 720K RefSeq promoter arrays (Roche Nimblegen). The arrays consisted of ~17 000 genes and covered ~2500 to ~500 nts surrounding the transcriptional start site. The significant peak regions with false discovery rate ≤0.05 were mapped to the nearest genes. Gene ontology and pathway analysis were also performed.

**Chromatin immunoprecipitation**

ChIP was performed using the ChIP-IT Express Kit (Active Motif). In brief, cells were cross-linked with 1% formaldehyde, lysed and sonicated. Sheared chromatin was immunoprecipitated with anti-HMGA2 (Santa Cruz) or normal IgG followed by end-point or real-time PCR with primers designed to amplify specific promoters (primer sequences are listed in Supplementary Table 6D, available at Carcinogenesis Online). IgG was used as the negative control antibody, whereas the chromatin extract without any antibody treatment was used as the positive control. All of the experiments were repeated at least three times.

**Luciferase reporter assay**

Promoter fragments of IL11 (−2400/+1, −2400−300 and −300/+1) or FN1 (−300/+323, −800/+323 and −800/−300) were subcloned into the XhoI/HindIII sites of the pGL3 vector (Promega). The constructs of mutant IL11 (−2400−300 MUT) or FN1 (−300/+323 MUT), which carried a replacement of several nucleotides within the binding sites, were generated through site-directed mutagenesis (Stratagene). The primer details are listed in Supplementary Table 6B and C, available at Carcinogenesis Online. HEK293T cells were transiently cotransfected with pGL3-wt or pGL3-mut and pcDNA3.1–HMGA2. The pRL-TK vector was cotransfected in each experiment as an internal control. Forty-eight hours after transfection, the cells were harvested and analyzed using the Dual-Luciferase Reporter Assay Kit (Promega). All of the experiments were performed in triplicate.

**Animal experiments**

For the in vivo tumorigenesis assays, 1 × 10⁶ cells from each cell line were subcutaneously injected into the bilateral flanks of 4 week old female BALB/c nude mice. The tumor sizes were monitored every 5 days, and the tumor volume was calculated according to the formula: volume (in cubic millimeter) = (width)² × length/2.

For the experimental metastasis assays, luciferase-tagged cells (1 × 10⁶) were injected into the lateral tail veins of 6 week old female BALB/c nude mice. Six weeks later, the mice were i.p. injected with 100 μg/ml of luciferin (15 mg/ml in phosphate-buffered saline, PerkinElmer) and anesthetized with isoflurane. Fifteen minutes after injection, bioluminescence was imaged using the IVIS Spectrum Imaging System (Caliper Life Sciences). Then, the mice were sacrificed and grossly examined at necropsy for the presence of metastases. Lungs and bones were fixed in formalin and embedded in paraffin. Hematoxylin–eosin staining and immunohistochemistry (IHC) were subsequently performed. All of the experiments were approved by the Animal Care and Use Committee of Zhejiang University.

**Statistical analysis**

The results are expressed as the mean of the triplicates ± SD. Student’s t-test (for two groups) and analysis of variance (for three groups) were used where appropriate. Pearson’s correlation test was performed to assess the relationship between the expression status and clinicopathological parameters. OS curves were carried out by Kaplan–Meier analysis and compared to the log-rank test. Univariate and multivariate Cox hazard proportional models were used to identify the relevant prognostic factors. Significance was assigned at P < 0.05.

**Results**

HMGA2 facilitates the migratory and invasive capacities of CRC cells in vivo

We first evaluated the expression of HMGA2 in colon cancer cell lines, including HT29, SW620, SW480, RKO, LoVo, CW2 and HCT116. Substantially higher levels of HMGA2 protein and

**Abbreviations**

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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CRC</td>
<td>colorectal cancer</td>
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<td>EMT</td>
<td>epithelial–mesenchymal transition</td>
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<td>FN1</td>
<td>fibronectin 1</td>
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<td>GI</td>
<td>gastrointestinal</td>
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<td>HMGA2</td>
<td>high-mobility gene group A2</td>
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<td>HR</td>
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<td>OS</td>
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an unfavorable prognosis in the distal and low-stage CRC subgroups. However, the exact mechanism by which HMGA2 is regulated and the functional role of HMGA2 in metastasis are still not well understood in CRC.

Here, we report the oncogenic role of HMGA2 in promoting CRC metastasis and epithelial–mesenchymal transition (EMT) in vitro and in vivo. Chromatin immunoprecipitation (ChIP)-PCR and luciferase assays showed that HMGA2 functioned as a transcriptional activator through the direct binding of HMGA2 to the +175/+196 region of FN1 and the −2147/−2132 region of the IL11 promoter. This was accompanied by a significant induction of STAT3 phosphorylation. Moreover, a study of a large cohort of CRC patients revealed that the expression of HMGA2 and IL11 is strongly correlated and that elevated IL11 expression is associated with a high histological grade, large tumor size, lymph node metastasis and unfavorable OS in CRC patients. Collectively, our findings revealed that HMGA2 promoted CRC metastasis and EMT via activation of the FN1 and IL11/STAT3 signaling pathways. Thus, HMGA2 could be a potential target for combating CRC metastasis.

**Materials and methods**

**Tissue samples**

This study was based on a cohort of 122 patients diagnosed with CRC at Second Affiliated Hospital of Zhejiang University. The clinical characteristics of these cases are presented in Supplementary Table 2, available at Carcinogenesis Online. All samples were obtained with approval from the Institutional Ethics Committee.

**Cell culture**

Human colon cancer cell lines HT29, SW620, SW480, HCT116 and RKO were obtained from American Type Culture Collection. All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Lentiviral vector construction, viral packaging and cell infection**

To generate the HMGA2 expression vector, pHBLV-Luc lentiviral constructs encoding luciferase and HMGA2 were prepared as described previously [16]. Human HMGA2 short-hairpin RNA (shRNA) in pGIPZ was purchased from Open Biosystems. Lentivirus expressing HMGA2 or shHMGA2 was produced in HEK293T cells (from American Type Culture Collection) and packaged by pMD2G and psPAX2. Subsequently, cells were infected with a mixture of viral supernatant and fresh medium at a 1:1 ratio with the help of 8 μg/ml polybrene (Sigma) for 48 h. After puromycin selection (2 μg/ml) g/mL), the luciferase reporter assay was performed. The results are expressed as the mean of the triplicates ± SD. Student’s t-test (for two groups) and analysis of variance (for three groups) were used where appropriate. Pearson’s correlation test was performed to assess the relationship between the expression status and clinicopathological parameters. OS curves were carried out by Kaplan–Meier analysis and compared to the log-rank test. Univariate and multivariate Cox hazard proportional models were used to identify the relevant prognostic factors. Significance was assigned at P < 0.05.
mRNA were found in SW480, RKO and HCT116 cells compared to the other cell lines (Figure 1A). To determine the potential roles of HMGA2 in colon cancer metastasis, we ectopically expressed it in SW620 and HT29 cells using HMGA2-expressing lentiviral constructs and knocked it down in HCT116, RKO and SW480 cells using lentiviral shRNA. Western blot analysis was performed to examine the transfection efficiency (Figure 1B).

To assess the contribution of HMGA2 to the migratory and invasive phenotypes of cancer cells, we carried out Transwell migration and Matrigel invasion assays in both HMGA2 gain-of-function and loss-of-function cell lines. It was shown that overexpression of HMGA2 significantly increased the number of migrated and invaded SW620 and HT29 cells (P < 0.01, Figure 1D). Conversely, knockdown of HMGA2 in HCT116 and RKO cells dramatically impaired the migratory and invasive abilities of these cells compared to the controls (P < 0.001, Figure 1D).

To investigate the effect of HMGA2 in tumorigenesis, colony formation and soft agar-based clonogenic assays were performed. Overexpression of HMGA2 in SW620 and HT29 cells led to the significant promotion of clonogenicity (P < 0.001, Figure 1E) and anchorage-independent growth (P < 0.001, Figure 1F) compared with control cells. In contrast, HMGA2 depletion in HCT116 and RKO cells remarkably decreased the number of colonies formed in the colony formation (P < 0.01, Figure 1D) and soft agar assays (P < 0.001, Figure 1F). We also found that HMGA2 played a role in cell proliferation. As shown in Supplementary Figure 1, available at Carcinogenesis Online, HMGA2 could induce a decrease in the G1 and an increase in the S population in CRC cells.

This observation was further confirmed by alterations in the protein expression patterns of epithelial and mesenchymal markers. Consistent with the induction of metastasis, HMGA2 overexpression promoted EMT, as evidenced by the suppression of E-cadherin and the upregulation of N-cadherin/MMP9 in SW620 and HT29 cells. Conversely, HCT116-shHMGA2 cells reverted to an epithelial phenotype compared with control cells. We found that the knockdown of HMGA2 increased the expression of the epithelial cell marker (E-cadherin) and blocked that of the mesenchymal markers (N-cadherin and MMP9) in HCT116.

Figure 1. Effect of HMGA2 expression on cell migration and invasion in vitro. (A) Real-time PCR (upper panel) and western blot analysis (lower panel) of HMGA2 expression in colon cancer cells. (B) Western blot analysis of stable HMGA2 overexpression in SW620/HT29 cells transfected with an HMGA2 expression vector or a control vector, and western blot analysis of HMGA2 knockdown in HCT116/RKO/SW480 cells expressing HMGA2-targeting or control shRNAs. (C) Western blot analysis of E-cadherin, N-cadherin, MMP9 and HMGA2 expression in the indicated cells. Protein band intensities were measured by Image J and normalized to β-actin. Data were expressed as a fold change relative to control. (D–F) Transwell migration and Matrigel invasion assays (D), clonogenic assay (E) and anchorage-independent growth assay (F) were performed in SW620/HT29 cells with or without HMGA2 overexpression and in HCT116/RKO cells expressing scrambled or HMGA2-targeting shRNA. Representative images (upper panel) and quantification (lower panel) are shown. Data are presented as the mean ± SD of three independent experiments. **P < 0.01, ***P < 0.001.
cells (Figure 1C). Together, these observations demonstrated that HMGA2 is a positive regulator of migration, invasion and EMT in colon cancer.

**HMGA2 promotes tumorigenesis and distant metastasis in vivo**

To further explore whether HMGA2 had an impact on tumorigenesis in vivo, HMGA2-overexpressing cells (SW620-A2) and their corresponding controls (SW620-NC), or HMGA2-knockdown cells (HCT116-shA2) and their corresponding controls (HCT116-shNC), were subcutaneously injected as xenografts into nude mice. As expected, ectopic expression of HMGA2 significantly increased the sizes of SW620 xenograft tumors (Figure 2A). However, HMGA2-silenced HCT116 tumors were smaller and grew more slowly than control tumors (Figure 2B). Immunoblotting showed that HMGA2 overexpression decreased the expression of the corresponding controls (SW620-NC), or HMGA2-knockdown cells (HCT116-shA2) and their corresponding controls (HCT116-shNC), were subcutaneously injected as xenografts into nude mice. As expected, ectopic expression of HMGA2 significantly increased the sizes of SW620 xenograft tumors (Figure 2A). However, HMGA2-silenced HCT116 tumors were smaller and grew more slowly than control tumors (Figure 2B). Immunoblotting showed that HMGA2 overexpression decreased the expression of the

![Figure 2. Effect of HMGA2 expression on CRC tumorigenesis and metastasis in vivo. (A) Left panel: representative images of SW620-NC and SW620-A2 xenograft tumors in nude mice. Right panel: growth curves of tumor volumes measured from day 9 to day 44. (B) Left panel: representative images of HCT116-shNC and HCT116-shA2 xenograft tumors in nude mice. Right panel: growth curves of tumor volumes measured from day 16 to day 32. Bars represent the mean ± SD. (C) Western blot analysis of E-cadherin, Vimentin, HMGA2 and β-actin in xenograft tumors. Protein band intensities were measured by Image J and normalized to β-actin. Data were expressed as a fold change relative to control. (D–G) Luciferase-tagged SW620-NC and SW620-A2 cells were injected into the lateral tail veins of nude mice. Representative bioluminescent images of the lungs (D) and bones (F) in tumor-harboring mice at week 8 are shown. Bioluminescent intensity was quantified and normalized, and photon flux is presented as the mean ± SD for each group. **P < 0.01. Metastatic lesions in the lungs (E) and bones (G) were subjected to hematoxylin–eosin staining and IHC to detect HMGA2 and Ki67 expression.](https://academic.oup.com/carcin/article-abstract/37/5/511/1744636?term=514)
epithelial marker (E-cadherin) and increased the level of the mesenchymal marker (Vimentin); the knockdown of HMGA2 had the opposite effect (Figure 2C).

To further explore whether HMGA2 could promote colon cancer cell metastasis in vivo, SW620 cells expressing luciferase with or without HMGA2 were injected into the lateral tail veins of nude mice. Metastasis formation was measured by quantita
tive bioluminescence imaging after 8 weeks. Luciferase signals in the lungs were 5-fold stronger in the HMGA2-overexpressing group compared with the control group (Figure 2D). As expected, there was an increase in the number and size of metastatic foci in the lungs of mice inoculated with HMGA2-overexpressing cells compared with controls (Figure 2D). Interestingly, we also found that the bone metastasis seeding of tumor cells were greatly enhanced when HMGA2 was overexpressed (Figure 2F).

Histology of the lungs (Figure 2E) and bones (Figure 2G) using hematoxylin–eosin staining further confirmed the biolumines
cent and macroscopic findings. The IHC of metastatic lesions in the lungs (Figure 2E) and bones (Figure 2G) showed that SW620-A2 had increased immunoreactivity toward HMGA2 and Ki67 compared with control SW620-NC. All of these results demonstrated that HMGA2 promoted tumor growth and metastasis in vivo.

HMGA2 binds directly to the promoters of FN1 and IL11

To identify target genes with promoters that were specifically bound by HMGA2 in colon cancer, a ChIP-on-chip assay was employed in SW620–HMGA2 cells using Nimblegen human 720K ReSeq promoter arrays. A total of 29 promoters, including FN1 and IL11, were enriched by ChIP using an HMGA2 antibody (false discovery rate < 0.05, Supplementary Table 1, available at Carcinogenesis Online). They had known functions in cancer metastasis, invasion, cell cycle and epigenetics.

Next, we carried out ChIP with control IgG or an antibody against HMGA2 to investigate whether HMGA2 binds directly to these promoters. To determine which genes mediated HMGA2-induced metastasis in colon cancer, we focused on the EMT-related genes (FN1 and IL11) that were identified from the ChIP-on-chip assays and further confirmed by real-time PCR. FN1 and IL11 were shown to be positively regulated by HMGA2 in CRC cells following HMGA2 overexpression or knockdown compared to the controls (Figure 3A). To test whether these HMGA2-binding regions were functional, we truncated the FN1 promoter to remove the regions with which HMGA2 might interact. Serial deletion constructs of the FN1 promoter (~300/+323, ~800/+323 and ~800/~300) were also generated. We found that the luciferase activity from the constructs containing the FN1 promoter regions of ~300/+323 and ~800/~323, but not from that of ~800/~300, was strongly stimulated when luciferase was cotransfected with HMGA2 (Figure 3B). This demonstrated that the region of ~300/+323 was the essential HMGA2 response element in the FN1 promoter. We then performed ChIP-PCR and ChIP-qPCR assays using two pairs of primers flanking the promoter segments of FN1 (+6/+278 and +186/+46). As shown in Figure 3C, there was a significant amount of HMGA2 bound to the +6 to +278bp promoter region of FN1. In addition, mutation of the HMGA2-binding site (from ATTTTT to ATGCCT) in the +175/+196 FN1 promoter significantly abolished luciferase activity induced by HMGA2 (Figure 3B). All of these results suggested that HMGA2 bound directly to the +175/+196 region of the FN1 promoter to induce its transcription.

As illustrated in Figure 3D, we also constructed various pGL3 plasmids containing different fragments of the human IL11 promoter, including ~2400/~1, ~2400/~300 and ~300/~1, and tested them in the luciferase reporter assay. We found that the regions of ~2400/~1 and ~2400/~300 exhibited strong luciferase activity, which was higher than that produced by the region of ~300/~1, indicating that the fragment ~2400/~300 within the IL11 promoter contains the HMGA2-binding site (Figure 3D). We then performed ChIP-PCR and ChIP-qPCR using eight primers flanking the promoter segments of IL11 (~536/~266, ~806/~536, ~1076/~806, ~1346/~1076, ~1616/~1346, ~1886/~1616, ~2154/~1886 and ~2400/~2154). The schematic structure of the IL11 promoter and the primers for the ChIP experiments are shown in Figure 3E. We found that HMGA2 was recruited to site VII (~2147/–1886) within the IL11 promoter, but not to the other sites (Figure 3E). In agreement with the findings from the ChIP-qPCR analysis, mutation of site VII (~2147/–1886) from ATGGCTTGGTA to AAGGCTTGGCA significantly reduced the reporter activity in cells overexpressing HMGA2 (Figure 3D).

All of these results suggested that HMGA2 directly promoted the transcription of FN1 and IL11 and that the binding sites between +175 and +196 of the human FN1 promoter and between ~2147 and ~2132 of the human IL11 promoter contributed to the activity of HMGA2-mediated transcription.

HMGA2-mediated IL11 signaling promotes migration and invasion in a pSTAT3-dependent manner in CRC cells

We then asked whether IL11 promotes metastasis in colon cancer. To address this question, Transwell and Matrigel assays were used to examine the effect of IL11 on the migration and invasion potential of CRC cells. As shown in Figure 4A, treatment with IL11 (60 ng/ml) significantly induced migration and invasion in SW620, HT29, HCT116 and RKO cells compared with untreated cells (P < 0.001).

Further studies concentrated on validating IL11-stimulated STAT3 phosphorylation (Tyr705) in CRC cells. Immunoblot analysis showed that IL11 treatment led to the robust phosphorylation of STAT3 (Tyr705) beginning at the 24 h time point in HT29 and HCT116 cells, whereas total STAT3 remained unchanged (Figure 4B). Decreased expression of an epithelial marker (E-cadherin) was also observed after phosphorylation of STAT3 (Tyr705) at the 48 h time point in HT29 and HCT116 cells (Figure 4B).

To investigate the role of HMGA2 in IL11-regulated invasion in CRC cells, Matrigel invasion assays were performed. As shown in Figure 4C, IL11 induced invasion in both HMGA2-knockdown cells and in their corresponding control cells (P < 0.001). However, IL11 treatment partially rescued the reduced invasion caused by HMGA2 knockdown in SW480, HCT116 and RKO cells (P < 0.001, Figure 4C). All of these data demonstrated that IL11-stimulated STAT3 phosphorylation (Tyr705) induced cell invasion in an HMGA2-dependent manner in CRC cells.

High levels of HMGA2 and IL11 expression correlate with tumor aggressiveness and poor clinical outcome in CRC patients

To further define the role of HMGA2 and IL11 in colorectal tumorigenesis, we evaluated their expression in 122 tissues by IHC. Statistical analysis revealed a significant positive correlation when the expression level of HMGA2 in the 122 tissues was plotted against that of IL11 (P < 0.001, Figure 5A). These findings suggested that HMGA2 and IL11 might be coregulated in CRC. And the clinical and pathological features are described in Supplementary Table 2, available at Carcinogenesis Online. The available information on the cohort included age, gender, tumor...
location, histological grade, tumor size, TNM stage, clinical stage and follow-up time. The patients were followed for 1–97 months (median follow-up time was 58.8 months). Next, we determined the potential clinicopathologic implications of altered HMGA2 and IL11 expression. The IL11 levels were significantly higher in patients with poor differentiation ($P = 0.018$, Figure 5B–C and Supplementary Table 2, available at Carcinogenesis Online), a large tumor size ($P = 0.034$, Figure 5D and Supplementary Table 2, available at Carcinogenesis Online) and lymph node metastasis ($P = 0.028$, Figure 5E and Supplementary Table 2, available at Carcinogenesis Online). In addition, increased HMGA2 levels were correlated with lymph node involvement ($P = 0.006$) and advanced clinical stage ($P = 0.011$, Supplementary Table 3, available at Carcinogenesis Online).
To assess the association between HMGA2 or IL11 expression and patient survival, we performed a Kaplan–Meier survival analysis. The patients with high HMGA2 ($P = 0.015$, Figure 6A) and IL11 ($P = 0.023$, Figure 6B) levels had a shorter OS than those with low levels. Stratified according to tumor location, the IL11 high tumors showed a trend toward reduced OS in the distal subgroup ($P = 0.001$, Figure 6C). When stratified into low-grade and high-grade subtypes, IL11 showed a significantly worse outcome in terms of OS in the low-grade subgroup ($P = 0.045$, Figure 6D). It also showed a prognostic effect on survival in the tumor size <5 cm subgroup ($P = 0.007$, Figure 6E). Patients with low-level HMGA2 expression had a survival advantage in the distal ($P = 0.005$, Supplementary Figure 2A, available at Carcinogenesis Online) and low-grade ($P = 0.045$, Supplementary Figure 2B, available at Carcinogenesis Online) CRC subgroups. Subgroup univariate analysis also revealed that a high level of
IL11 indicated an unfavorable prognosis in the distal (P = 0.003), low-grade (P = 0.049) and tumor size <5 cm (P = 0.010) subgroups. The same held true for HMGA2 in the distal (P = 0.007), low-grade (P = 0.049) and tumor size <5 cm subgroups (P = 0.043, Supplementary Table 4, available at Carcinogenesis Online).

We then utilized a Cox proportional hazards regression model to assess the association between IL11 and HMGA2 expression and prognosis. In the univariate analysis, poor OS in patients was associated with a high-level of HMGA2 expression [hazard ratio (HR) = 1.809, P = 0.017], a high-level of IL11 expression (HR = 1.792, P = 0.025), advanced T stage (HR = 2.699, P = 0.033), lymph node involvement (HR = 2.014, P = 0.004), distant metastasis (HR = 2.825, P = 0.003) and advanced clinical stage (HR = 2.255, P = 0.001, Supplementary Table 5, available at Carcinogenesis Online). After multivariate analysis, advanced stage remained a significant independent predictor for poor OS (HR = 2.255, P = 0.001, Supplementary Table 5, available at Carcinogenesis Online). All of these data supported the finding that HMGA2-induced IL11 overexpression was the crucial molecular event that was strongly associated with a high risk of tumor metastasis and poor clinical outcome.

Discussion

HMGA2 is an architectural transcription factor that positively or negatively regulates the transcriptional activity of multiple genes (17–19), and influences many biological processes, ranging from embryonic development to carcinogenesis (20–23). Shell et al. (24) identified HMGA2 and let-7 as good candidates to distinguish type I (mesenchymal) from type II (epithelioid) carcinomas. They stated that HMGA2 and let-7 are better markers for advanced cancer than established markers, such as E-cadherin and Vimentin. Wang et al. (15) found that higher HMGA2 expression was associated with a more advanced stage and worse survival in CRC. However, the underlying molecular mechanism remains unknown. For this reason, it is important to understand the mechanisms of HMGA2-mediated cell invasion and metastasis in CRC.

Our present study provides a new mechanistic insight into the function of HMGA2 in CRC metastasis. We found that overexpression of HMGA2 induced EMT, cell migration and invasion, whereas its silencing produced the opposite effects in CRC in vitro and in vivo. To our knowledge, we have, for the first time,
characterized the involvement of FN1 and IL11 in the transcriptional activity of HMGA2. Using ChIP and dual luciferase reporter gene assays (truncation and mutation), we mapped a highly active promoter region located between +175/+196 of FN1 and −2147/−2132 of IL11. Further analysis of IHC staining in CRC tissues indicated that IL11 expression was significantly correlated with HMGA2 expression. Furthermore, IL11-stimulated STAT3<sup>Y705</sup> phosphorylation largely contributed to the metastasis-enhancing potential of CRC cells in an HMGA2-dependent manner. To investigate the correlation between HMGA2 or IL11 and the clinical features and prognosis, a large cohort of 122 CRC cases was enrolled in this study. It was found that increased IL11 expression was correlated with an advanced histological grade, large tumor size and lymph node metastasis. Patients with high IL11 expression have a shorter OS than those with low levels. In addition, IL11 expression is associated with poor prognosis in the distal, low-grade CRC and tumor size <5 cm subgroups.

Figure 6. Prognostic significance of HMGA2 and IL11 expression in CRC. (A) Kaplan-Meier OS curves for all patients comparing high and low HMGA2 expression. N = 122, P = 0.015. (B) Kaplan-Meier OS curves for all patients comparing high and low IL11 expression. N = 122, P = 0.023. (C-E) Kaplan-Meier OS curves for patients with distal CRCs (C, N = 68, P = 0.001), low-grade CRCs (D, N = 76, P = 0.045) and a tumor size <5 cm (E, N = 58, P = 0.007). (F) Graphic model as discussed in the text. Transcriptional activations of IL11 and FN1 by HMGA2 were able to promote the EMT process of CRC through a pSTAT3-dependent pathway.
FN1, fibronectin 1, is a glycoprotein that plays critical roles in metastasis, differentiation, carcinogenesis and embryogenesis (25). Stronger FN1 staining was associated with more advanced stages and a higher metastatic potential in patients with renal cancer (26,27). FN1 was shown to activate MMP9 via the PI3K/Akt pathway to promote metastasis in ovarian cancer (28). In breast cancer, FN1 stimulated the epithelial-to-mesenchymal transition by activating the STAT3 signaling pathway (29). In colon cancer, FN1 played an important role in the modulation of cell invasion by activating the phosphorylation of FAK at Tyr-397 (30). Kida et al. (31) found that a single-nucleotide polymorphism in FN1 influenced the tumor shape and incidence of lymphatic permeation in colon cancer. In summary, FN1 acted as a biologically significant oncogene that was responsible for the aggressiveness of and poor prognosis of colon cancer.

Interleukin (IL)-11 is a member of the IL6 cytokine family, which is defined by the shared use of the gp130 receptor subunit. The signal transduction of IL11 is mediated by the binding of IL11 to the IL11-Ra and gp130 receptor complex (32,33). It plays an important role in the development and progression of epithelial neoplasms, including colorectal (34), endometrial (35), breast (36,37), bladder (38) and prostate cancers (39). Putoczki et al. (40) found that compared with IL6, IL11 had a higher positive correlation with enhanced STAT3 phosphorylation and acted as a more potent driver during the progression of sporadic and inflammation-associated gastrointestinal (GI) cancers. The absence of IL11/STAT3 signaling delayed tumor onset in mice. Pharmacologic inhibition of IL11 signaling suppressed tumor progression and reduced tumor growth in mouse models of spontaneous GI cancer and human tumor cell line xenografts. All of these data demonstrated that IL11 played crucial roles in GI tumorigenesis and suggested that a therapy based on the suppression of IL11 could represent a promising approach for the treatment of GI cancer.

Our findings established a prominent role for HMGA2 in the pathogenesis of human CRC, as it directly activates the transcription of FN1, a classical mesenchymal marker, and IL11, an immune-associated cytokine. This study showed that IL11 is an independent predictor of OS for CRC patients. All of our data suggest that HMGA2 can confer powerful oncogenic properties during inflammation in the early stages of carcinogenesis. Besides IL11 and FN1, HMGA2 could also directly modulate transcriptions of multiple target genes in the pathogenesis of cancer metastasis, including Slug (41), Snail (42), Twist (43) and IGFBP2 (44). The metastatic process consists of a series of sequential steps, including loss of cellular adhesion, increased motility, EMT, entry and survival in the blood and/or lymphatic system, colonization at a distant site and vascularization of the metastatic tumor (45). During the metastatic cascade, loss of cell polarity, increased cell motility and remodeling of cytoskeleton are attributed to the process of EMT that facilitate cancer cells disseminate from the primary site and migrate to distant organs. In this study, our finding demonstrated that HMGA2 promoted CRC metastasis through EMT by regulation of IL11/STAT3/E-cadherin pathway. In addition, study conducted by Li et al. (46) confirmed that IL11 could promote cancer cell motility by increasing the expression of intercellular adhesion molecule-1. Based on that, our future studies may be focused on understanding how HMGA2 and IL11 disrupted intercellular adhesion in the proinflammatory microenvironment of CRC. Although there is emerging knowledge regarding HMGA2, a significant number of issues regarding the process of CRC carcinogenesis deserve further investigation in the future. Because HMGA2 is an architectural transcriptional factor, we proposed that HMGA2-mediated tumor transformation may require additional genetic alteration or partner proteins. Future characterization of the functional and mechanistic relationship between HMGA2 and other genetic events remains to be explored. Galdiero et al. (47) found that circulating HMGA2 mRNA could be detected in the plasma of ovarian cancer patients and that it significantly correlated with the corresponding expression of HMGA2 protein from cancer tissues. In contrast, no HMGA2 mRNA was found in the healthy controls. All of these studies will help us expand our understanding of HMGA2 and develop new HMGA2-based therapeutic approaches for CRC patients.

In summary, our data revealed that the oncogenic properties of HMGA2 in CRC were mediated through directly binding to the promoters of IL11 and FN1, transcriptionally activating their expressions and inducing EMT process through a pSTAT3-dependent pathway (Figure 6F). HMGA2 and IL11 may serve as potential biomarkers for predicting worse prognosis of CRC and become attractive therapeutic candidates in future.

Supplementary Material
Supplementary Figures 1–2 and Supplementary Tables 1–6 can be found at http://carcin.oxfordjournals.org/

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