GATA6 predicts prognosis and hepatic metastasis of colorectal cancer

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Abstract. Liver metastasis is a major cause of mortality for colorectal cancer (CRC). However, the underlying mechanisms remain largely unknown. GATA binding protein 6 (GATA6), a zinc-finger transcription factor, is expressed in the colorectal epithelium. We investigated the clinical significance of GATA6 and its role in invasion and metastasis in CRC. Expression of GATA6 in 89 cancerous, 35 adjacent normal and 39 liver metastatic samples from 89 CRC patients undergoing surgical resection was detected by immunohistochemical (IHC) methods. The effect of GATA6 on invasion and metastasis was assessed in CRC cells by shRNA lentivirus or expressed-plasmid transfection. We found that GATA6 expression was significantly higher in liver metastatic tissues compared with adjacent normal tissues. Aberrant GATA6 expression in CRC was associated with liver metastasis. Kaplan-Meier analysis showed GATA6 expression correlated with poor overall survival (OS) in CRC. The cell invasion and migration of established CRC cell lines were decreased by GATA6 knock-down and enhanced by GATA6 overexpression *in vitro*. Thus, aberrant expression of GATA6 correlates with poor prognosis and liver metastasis in CRC.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide (1) and has a high propensity for liver metastasis (2). The primary cause of mortality in patients with CRC is liver metastasis (3), and the 5-year overall survival (OS) is only 25-40% (3,4). The molecular mechanisms underlying CRC metastasis are not completely understood. Early treatment targeting CRC liver metastatic foci may be important for improving patient survival. Therefore, there is an urgent need to identify molecules that facilitate the metastasis of CRC to the liver, which would be potential therapeutic targets for treating patients with CRC and liver metastases.

The GATA factors belong to an evolutionarily conserved family of C2-type zinc finger proteins. There are six members of the vertebrate GATA family. GATA1, 2 and 3 are mainly expressed in the hematopoietic lineages and GATA4, 5 and 6 are expressed in endodermally derived tissues, such as the gut, liver and lungs (5). In the adult small intestine, GATA4, 5 and 6 are expressed in a partially overlapping pattern along the crypt/villus axis (6,7). GATA4, 5 and 6 regulate various differentiation marker genes expressed in gastrointestinal tissue by binding to the WGATAR sequences within the regulatory regions of these genes and interacting with other ubiquitous and tissue-enriched transcriptional regulators (8-10). In addition to differentiation, GATA4, 5 and 6 have been associated with cell survival, cell proliferation, and neoplastic transformation of various cell types (11-15). Among gastrointestinal cancers, GATA4 is amplified in up to 10% of esophageal adenocarcinomas and Barrett metaplasia (16). The transition from normal esophageal epithelium to Barrett metaplasia to adenocarcinoma is associated with upregulation of GATA6 (17). Furthermore, the GATA6 gene is amplified in pancreaticobiliary cancer (18). A strong expression of GATA6 in the proliferative crypt compartment of the intestine has suggested that GATA6 may be associated with cellular proliferation. In support of this, GATA6 is strongly expressed in CRC-derived cell lines and is upregulated in CRC (19). However, the roles of GATA6 in the prognosis and the metastasis of CRC to the liver have yet to be explored.

In the present study, we first investigated the expression of GATA6 in a series of CRC patients and its correlations with patient prognosis and liver metastasis. Then, the role of GATA6 in CRC invasion and metastasis was studied in CRC cells.

Materials and methods

CRC patient samples. A total of 89 CRC patients undergoing surgical resection between January 1, 2004 and October 1, 2010, in Guangzhou First People's Hospital, Guangzhou Medical College, were included. One hundred and six cancerous samples, 35 adjacent normal, 39 liver metastatic samples from 89 CRC patients were collected. The median age of the patients was 54 years at operation, range 28-81 years.

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Follow-up and clinicopathological characteristics including tumor location, stage, and differentiation were recorded. OS was calculated from the date of surgery until the date of last contact. This study was approved by the Ethics Committee of the Guangzhou First People's Hospital, Guangzhou Medical College.

Immunohistochemical analysis (IHC). Surgical samples containing primary tumors, adjacent normal tissues and liver metastatic tumors were collected in 10% buffered formalin. The details of deparaffinization and IHC were previously described (20). Briefly, following deparaffinization, the endogenous peroxidase activity was blocked with 3% H_2O_2. The array slides were incubated with normal goat serum for 20 min, and then applied with primary antibody for 20 min at room temperature. After 7 min of H_2O_2 treatment, the array slides were incubated with horseradish peroxidase-labeled polymer conjugated with corresponding antibodies for 30 min. Each slide was counterstained with hematoxylin (Dako, Carpinteria, CA, USA). PBS was used as a negative control.

The rabbit anti-human GATA6 monoclonal antibody (Sigma-Aldrich, USA) was used for IHC staining (1:100 dilution). To reduce the image reader bias, an automated imaging system was employed to obtain digital images of the stained sections for subsequent quantitative analyses. In addition, each sample was evaluated by two independent investigators in a double-blind manner. Investigators reviewed and assessed the subcellular localization (cytoplasm vs. nucleus), staining intensity (integrated optical density), and/or percentage of stained cells (total area or percentage of cells positive) for each image. Discrepancies in samples were resolved after joint review by the readers.

CRC cell culture and transfection. The two CRC cell lines (Colo-205 and SW-480 cells) were obtained from the American Type Culture Collection and maintained in RPMI-1640 with 10% calf serum (HyClone) at 37˚C with 5% CO_2.

Cells were transfected with lentiviral vectors encoding short hairpin RNA targeting human GATA6 for GATA6 knockdown (shGATA6) or a scrambled shRNA as control (shControl) (Sigma-Aldrich). Multiplicity of infection was 10. Cells were cultured for 72 h after transfection. Cells were grown to 80% confluency in 60-mm dishes. GATA6 (10 lg) plasmid (ExGATA6) or empty plasmid (ExControl) was transfected using Lipofectamine Reagent (Invitrogen). Cells were cultured for 48 h after transfection.

Cell invasion assay. Cells ($2x10^5$) were suspended in 400 μl serum-free RPMI-1640 medium and seeded in the top chamber that had been coated with a layer of extracellular matrix (BD Biosciences, USA). The complete medium with serum (500 μl) was added to the bottom chamber. After 48 h of incubation, the cells which had invaded through the extracellular matrix layer to the lower surface of the filters were stained. Images of three randomly selected fields of the fixed cells were captured, and cells were counted. Experiments were repeated independently three times.

Cell migration assay. Cells were seeded in a 6-well plate, grown until confluence and then starved for 24 h. A linear wound was made by scraping a pipette tip. The cell motility in terms of wound closure was measured by photographing at three random fields 72 h after wounding. Experiments were repeated independently three times.

Real-time PCR. Total RNA was extracted from CRC cells using RNA Extraction kit (Qiagen, China). The cDNA synthesis was performed according to the manufacturer's instructions (SYBR-Green PCR kit; Qiagen). Quantitative PCR was performed by SYBR-Green PCR kit (Qiagen) using a LightCycler system. PCR reaction conditions for all assays were 94˚C for 30 sec, followed by 40 cycles of amplification (94˚C for 5 sec, 58˚C for 30 sec and 72˚C for 30 sec). β-actin mRNA was used to normalize RNA. Primer sequences were: GATA6 forward, CCAACTTCCACCTCTTCTAAC and reverse, TTGACCCGAATACTTGAGC; β-actin forward, CCATGTACGTTGCTATCCAGG and reverse, TCTCCTT AATGTCACGCACGA.

Western blot analysis. Nuclear protein or total proteins of CRC cells were isolated using RIPA (Cell Signaling Technology). For immunoblotting, equal amounts of proteins were separated on 5-8% SDS-PAGE and were electrophoretically transferred onto nitrocellulose membranes (Millipore), which were blocked in TBST containing 5% milk for 2 h at room temperature and blotted with antibody overnight at 4˚C: anti-GATA6 (1:1,000; Abcam) and anti-histone H3 (1:500; Cell Signaling Technology). After washing with TBST and incubating with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 2 h at room temperature, immunocomplexes were visualized using the chemiluminescence (GE Healthcare Life Sciences, USA) following the manufacturer's protocol.

Statistical analysis. Data were analyzed using SPSS 17.0. Continuous data were measured by the t-test. For categorical data, Chi-square analysis or Fisher's exact test was used. Multivariate logistic regression was used to adjust for covariate effects on the odds ratio (OR). Kaplan-Meier was applied for OS. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GATA6 expression in primary CRC, metastatic liver lesions and normal tissues. We examined GATA6 protein expression in metastatic CRC by IHC. In general, GATA6 was predominantly nuclear staining in IHC. Some perinuclear granulation in cytoplasm was also observed. GATA6 expression was quantified using a visual grading system based on the extent of staining. Only immunoreactivity in the nucleus was evaluated. GATA6 was defined as: negative nuclear staining (-), <5%; positive (+), ≥5% and <50% or strong positive (++), >50% positive nuclear staining from CRC cells. The GATA6 IHC standards of negative (-), weak positive (+) and strong positive (++) are displayed in Fig. 1A-C, respectively. GATA6 expression was predominantly observed in the primary cancer cells, but not in the adjacent normal colorectal epithelium (Fig. 1D-F). Meanwhile, strong positive GATA6 was also observed in metastatic CRC to liver (Fig. 1G-I).

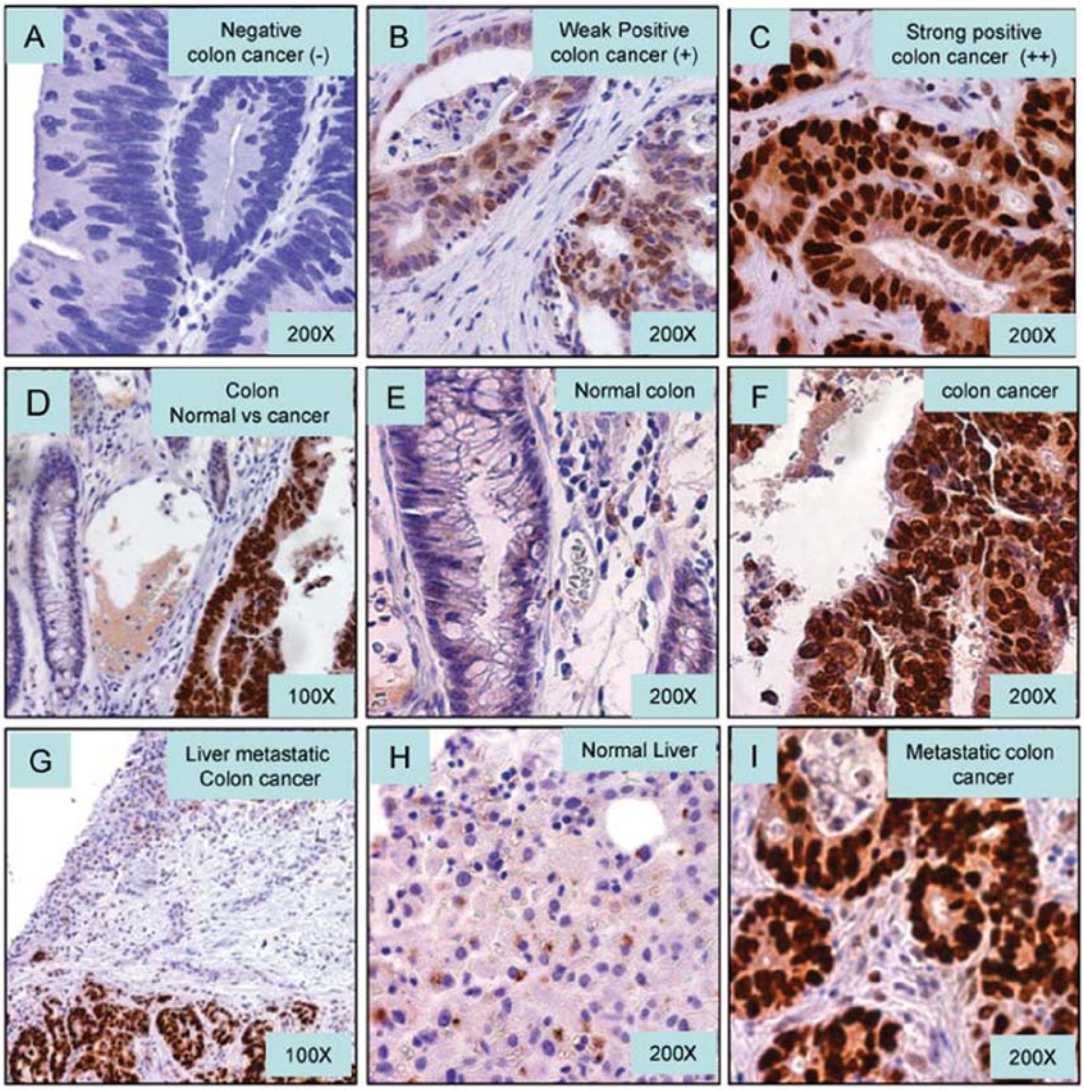

Figure 1. Immunohistochemical (IHC) staining of GATA6. Upper panels (A-C) show GATA6 IHC staining with negative (-), weak positive (+) and strong positive (++). Middle panels (D-F) display GATA6 staining in colorectal tumor, adjacent normal and cancer section. Lower panels (G-I) indicate GATA6 expression in metastatic colorectal cancer (CRC) to liver, normal liver and metastatic CRC. The magnification (x100 and x200) is indicated in each panel.

Expression of GATA6 is associated with hepatic metastasis of CRC. Based on the IHC staining of GATA6, 32 of 89 CRC samples were defined as GATA6 nuclear positive staining [including weak positive (+) and strong positive (++)]. The TNM stage of CRC was based on clinical diagnosis. According to the univariate analysis results, the GATA6 staining was positively and significantly associated with hepatic metastasis ($P<0.01$) and tumor invasion ($P<0.05$) of CRC, but not with age, gender, tumor location and lymph node involvement (Table I).

To validate this finding, non-conditional logistic analysis was employed for univariate and multivariate analyses. The GATA6 positive and negative were stratified as unfavorable and favorable subsets, respectively. Tumor invasion, lymph node involvement, and hepatic metastasis were considered as the endpoint in logistic analysis. The expression of GATA6 significantly impacted the risk of hepatic metastasis but not tumor invasion and lymph node involvement according to either univariate or multivariate analysis. After adjusting for age and gender, the OR of GATA6-positive for hepatic metastasis was 3.53 (95% CI, 1.37-9.70) (Table II). Therefore, our analyses revealed that GATA6 significantly affected hepatic metastasis of CRC, suggesting that GATA6 may be used to prognosticate CRC.

Positive GATA6 expression correlates with poor prognosis in CRC. The prognostic significance of GATA6 was determined by GATA6 staining and the corresponding clinical follow-up records. Kaplan-Meier survival analysis revealed a correlation between higher GATA6 expression levels and shortened OS times (Fig. 2). The Log-rank test indicated that the positive GATA6 expression was significantly related to OS ($P<0.05$). The strong positive GATA6 (++) exhibited a more significantly reduced survivability ($P<0.001$). Taken together, these observations indicate that overexpression of GATA6 is significantly associated with CRC liver metastasis and poor prognosis in patients with CRC.

GATA6 promotes CRC cell migration, invasion and metastasis. As clinical data showed positive correlation between GATA6

Table I. Clinicopathological characteristics and GATA6 expression of CRC.

Characteristics	No. of cases	No. of positive GATA6 (%)	P-value
Age			
<40	4	0 (0.0)	
40-49	5	2 (40.0)	
50-59	20	7 (35.0)	
60-69	30	13 (43.3)	
70-79	23	7 (30.4)	
>80	7	3 (42.9)	0.567
Gender			
Male	42	18 (42.9)	
Female	47	14 (29.8)	0.2
Location			
Rectum	7	5 (71.4)	
Colon	82	27 (32.9)	
Proximal	53	17 (32.1)	
Distal	36	15 (41.7)	0.158
Tumor invasion			
Within propria	20	8 (40)	
Out propria	69	24 (34.8)	0.027[a]
Lymph node			
Negative	38	15 (39.5)	
Positive	51	17 (33.3)	0.55
Hepatic metastasis			
No	50	13 (26.0)	
Yes	39	19 (48.7)	0.001[a]

Proximal colorectal includes hepatic flexure, transverse, cecum, appendix, ascending and splenic flexure. Distal colorectal includes sigmoid and descending colon. Statistical significance, [a]P<0.05. CRC, colorectal cancer.

Table II. Univariate and multivariate logistic analysis for GATA6 and TNM stage of CRC.

	n=89	
	OR (95% CI)	Adjusted OR[a] (95% CI)
Tumor invasion		
T0-2	Reference	Reference
T3-4	0.91 (0.32-2.74)	0.74 (0.24-2.35)
Lymph node involvement[b]		
N0	Reference	Reference
N1 or N2	0.77 (0.32-1.84)	0.48 (0.17-1.31)
Hepatic metastasis[c]		
M0	Reference	Reference
M1	2.70 (1.12-6.72)[d]	3.83 (1.14-14.14)[d]

[a]OR, odds ratio; adjusted OR, adjusted by gender and age at diagnosis, tumor location, chemotherapy and radiotherapy in multivariate logistic analysis. [b]Lympho node involoment: N0, no regional lymph node metastasis; N1, metastasis in 1 to 3 regional lymph nodes; N2, metastasis in 4 or more regional lymph nodes. [c]Metastasis: M0, no hepatic metastasis; M1, hepatic metastasis present. [d]Statistical significance, P<0.05. [e]Tumor invasion: T0, *in situ*; T1, tumor invades submucosa; T2, tumor invades muscularis propria; T3, tumor invades through the muscularis propria into the subserosa, or into the pericolic or perirectal tissues; T4, tumor directly invades other organs or structures, and/or perforates. CRC, colorectal cancer.

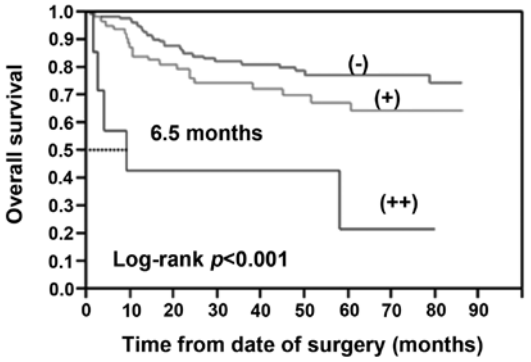

Figure 2. Kaplan-Meier curves of patients with colorectal cancer (CRC) with GATA6 staining. GATA6 overexpression correlates with poor prognosis in patients with CRC. GATA6 negative (-), weak positive (+) and strong positive (++).

and metastatic behavior in CRC, the roles of GATA6 in invasion and metastasis were investigated in two human CRC cell lines, Colo-205 and SW-480 cells. Both mRNA and nuclear protein levels of GATA6 were significantly higher in SW-480 cells than in Colo-205 cells (Fig. 3A and B), and therefore, knockdown of GATA6 was performed in SW-480 cells by GATA6-shRNA lentivirus transfection and overexpression of GATA6 was performed in Colo-205 cells by GATA6 plasmid transfection, respectively. The GATA6 nuclear protein levels after transfection were also validated (Fig. 3C). By transwell assay, the number of invading cells was markedly reduced by knockdown of GATA6 in SW-480 cells and was markedly increased by overexpression of GATA6 in Colo-205 cells (Fig. 4A). Consistently, cell migration was significantly reduced by GATA6 knockdown and enhanced by GATA6 overexpression by wound-healing assay (Fig. 4B). Collectively, the data suggested a role of GATA6 in promoting CRC cell invasion and metastasis.

Discussion

In the present study, we examined the expression level of GATA6 in CRC to assess its potential role as a prognostic or predictive marker. Our findings revealed that the positive expression of GATA6 was significantly associated with the hepatic metastasis. The adjusted ORs of GATA6 for the risk of hepatic metastasis were 3.53 (95% CI, 1.37-9.70). We also found

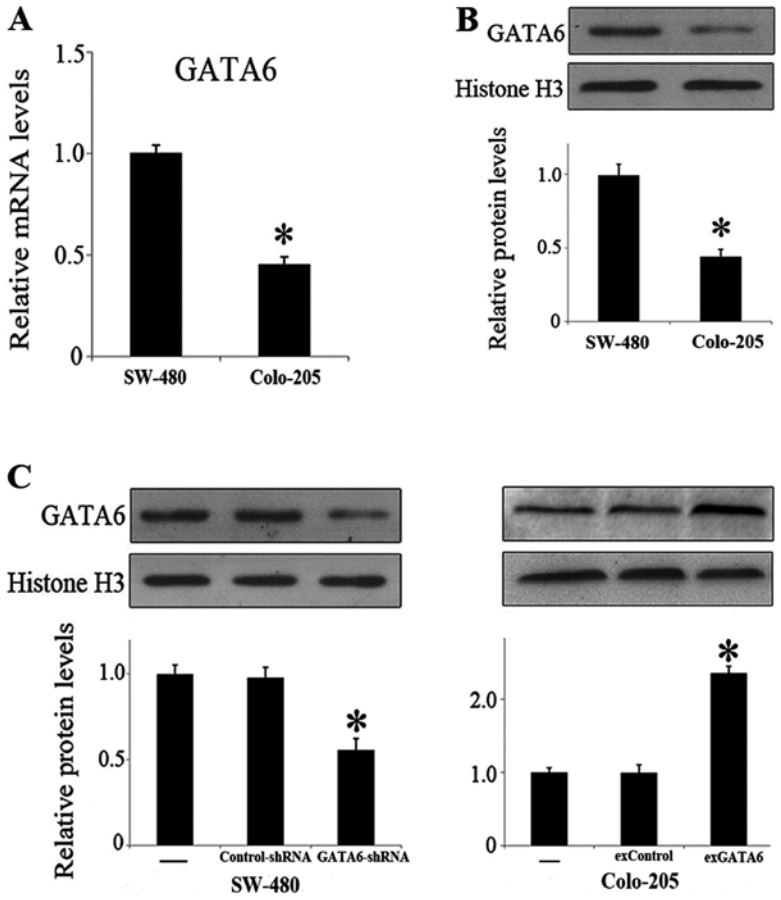

Figure 3. GATA6 expression in colorectal cancer (CRC) cells. (A) The mRNA levels of GATA6 between two CRC cell lines, SW-480 and Colo-205, by real-time PCR analysis. (B) Nuclear protein levels of GATA6 between SW-480 and Colo-205 cells by western blot analysis. (C) Validation of GATA6 nuclear protein levels after transfection. shRNA, shRNA lentivirus; ex, transfected with plasmid. *P<0.01.

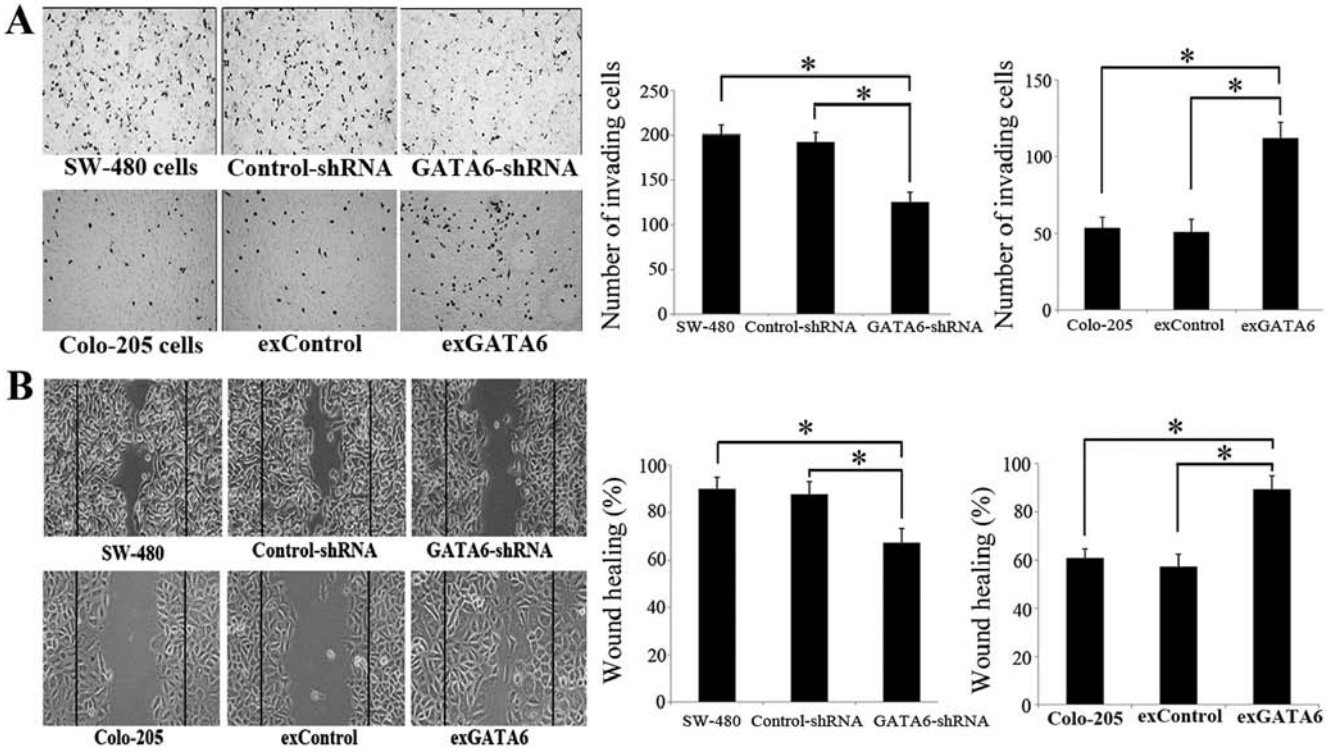

Figure 4. GATA6 promotes colorectal cancer (CRC) cell invasion and metastasis. (A) Impact of intervening GATA6 expression on CRC cell invasion by transwell assay *in vitro*. (B) Impact of intervening GATA6 expression on CRC cell migration by wound-healing assay *in vitro*. shRNA, shRNA lentivirus; ex, transfected with plasmid. *P<0.05.

that the positive expression of GATA6 was an indicator of poor prognosis. Kaplan-Meier survival analysis revealed a correlation between higher GATA6 expression levels and shorter OS. The log-rank test indicated that the positive GATA6 expression was significantly related to OS. *In vitro*, the cell invasion and migration of established CRC cell lines were decreased by GATA6 knockdown and enhanced by GATA6 overexpression. Therefore, our findings suggest that the positive expression of GATA6 is associated with the hepatic metastasis of CRC and a reduced patient survival.

GATA6 functions as a promoter or suppressor according to the tumor origin. GATA6 activates the expression of tumor suppressor, Dab2, and regulates the activity of LKB tumor suppressor protein (21,22). Loss of GATA6 protein function due to epigenetic silencing of GATA6 gene or GATA6 protein exclusion from the nuclei has been reported in ovarian cancer (12,23). Although GATA6 was expressed in normal adrenal cortex, GATA6 expression was downregulated in adrenocortical tumors (15). Furthermore, GATA6 was downregulated in hyperplastic neointimal smooth muscle cells and forced expression of GATA6 restored normoplasia (24). A recent study revealed tumor suppressor activity of GATA6 in astrocytomas (25). In contrast to the tumor-suppressing activity of GATA6 in these tissues, the promoter function of GATA6 is reported mainly in digestive malignancies, such as tumorigenesis in the esophagus, pancreas and intestines (13,17,18,26) indicating that GATA6 may be a digestive-lineage tumor promoter, which, however, requires further elucidation. The studies above are in agreement with our findings, and the present study *in vitro* showed GATA6 induces migratory and invasive behavior, promoting invasion and metastasis, and confers survival advantages.

However, the mechanism by which overexpressed GATA6 participates in the initiation and/or progression of CRC is not known. GATA6 binding sites are present in the regulatory regions of members of the Wnt family of secreted glycoproteins such as Wnt 2, 4, 6, 7b and 8b, and GATA6 regulates the expression of Wnt 2, 7b and 8 and the Wnt receptor Fzd2 (27-30). Dysregulated expression of GATA6 in preneoplastic colorectal lesions may lead to overexpression of target Wnts, which may trigger the Wnt signaling pathway implicated in the pathogenesis of colorectal cancer (31). Continued overexpression of GATA6 in benign and malignant lesions may aid in maintaining the activated Wnt-β-catenin signaling during progression of CRC. In addition to Wnts, GATA6 also regulates the expression of the members of the transforming growth factor-β (TGF-β) family of proteins such as BMP4. The BMP4 promoter contains consensus GATA binding sites, and these sites are essential for GATA6-induced activation of the BMP4 promoter (32). BMP4 is overexpressed in malignant and metastatic CRC compared with benign lesions and normal mucosa (33). A recent genome-wide study showed the association between BMP4 and CRC (34). BMP4 activates the Smad signaling pathway and induces epithelial-mesenchymal transition and uPA production in CRC cells and promotes cell migration and invasion. In support of this, a previous study showed that forced expression of BMP4 in HCT116 CRC cells induced uPA gene expression and enhanced cell migration and invasion (33). GATA6 and the related protein, GATA4, physically and functionally interact with Smads and play an essential role in TGF-β signal transduction (8). Thus, it is conceivable that the overexpression of GATA6 will serve dual functions: it will lead to excessive production of BMP4 and it will participate in the activation of BMP4-induced Smad pathway signaling, leading to cell migration and invasion.

Although results from previous studies suggested that GATA6 functions as an oncogene that is overexpressed in various types of human cancer, it remains unclear how GATA6 promotes cancer invasion and metastasis in these types of cancer. GATA6 may affect CRC cell migratory and invasive properties independently. Multiple extracellular matrix degrading proteases such as matrix metalloproteinases 2 and 9 are regulated by GATA2 in endothelial cells (35). Since GATA6 is expressed in CRC, the expression of these matrix metalloproteinases in colorectal tumors may be regulated by GATA6. GATA6 may also be involved in other pathways independent of proteases to influence the cell migratory properties. GATA6 and the related GATA4 proteins are targeted by the small GTPase RhoA pathway, an evolutionarily conserved pathway that regulates the migratory behavior of normal and cancerous cells (36).

In conclusion, our finding that GATA6 overexpression is an informative biomarker, which is associated with poor prognosis of patients with CRC, has potential implications for CRC survival prediction, choice of treatment regimens and future development of treatment strategies.

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