Cancer Letters 355 (2014) 130–140

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

HMGA2 induces transcription factor Slug expression to promote epithelial-to-mesenchymal transition and contributes to colon cancer progression

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ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form 3 September 2014
Accepted 4 September 2014

Keywords:
HMGA2
Epithelial-to-mesenchymal transition (EMT)
Proliferation
Metastasis
Slug

ABSTRACT

Epithelial-to-mesenchymal transition (EMT) is considered to play an essential role in progression and metastasis. This study aims to investigate the expression and underlying molecular targets of high-mobility group AT-hook 2 (HMGA2) in the progression of colon cancer. The expression of HMGA2 is upregulated by both active extracellular signal-regulated kinase (ERK)1/2 and TGF-β signaling in colon cancer cells through a series of lentiviral infection and pharmacological assays. HMGA2 knockdown by specific shRNAs attenuates proliferation, motility and invasion of colon cancer cells *in vitro* and *in vivo*. Besides, exogenous HMGA2 expression caused EMT in colon cancer cells, which was confirmed by the downregulation of the epithelial markers and the upregulation of the mesenchymal markers. Moreover, HMGA2 positively regulates the Slug expression by directly binding to the regulatory region in *Slug* promoter. Importantly, the knockdown of Slug could reverse the HMGA2-induced EMT and decrease the migration and invasion ability of colon cancer cells. Taken together, our results reveal a critical role for HMGA2 in promoting EMT, migration, invasion, and proliferation of colon cancer cells, suggesting HMGA2 as a potential molecular target to prevent colon cancer progression.

Introduction

Colon cancer is the third most commonly diagnosed malignant tumor in males and the second in females, and ranks second in accordance with cancer-related deaths worldwide [1,2]. Although the overall incidence of colon cancer had declined in the United States, the incidence rates remain high in Australia, Europe, and Eastern Asia [1]. Despite advances in treatments, the current therapies available to colon cancer patients in locally advanced or metastatic stage play a minor role to improve the survival rate, as evidenced by the poor 5-year survival rate [3,4]. To provide insight that will enable the development of new therapeutic strategies, it is crucial to illuminate the molecular mechanisms promoting the metastatic properties of colon cancer cells.

Recent studies have shown that a morphologic conversion, known as epithelial-to-mesenchymal transition (EMT), is related to the acquisition of malignant characteristics in colon cancer [5–8]. EMT and EMT-like processes are regarded as a fundamental program in the metastatic cascade by regulating motility, invasion, and anoikis resistance [9,10]. The EMT process is associated with the downregulation of epithelial markers like E-cadherin and zonula occludens 1 (ZO-1) and aberrant upregulation of mesenchymal markers such as vimentin and N-cadherin, which are representative molecular hallmark of EMT [11,12]. Previous studies revealed that EMT is governed by a wide variety of regulatory networks, such as active extracellular signal-regulated kinase (ERK)1/2, TGF-β, PDGF, and EGF signaling [11–13]. Hyperactivation of ERK1/2 signaling is common in multiple cancer types including colon cancer, where mutations in K-RAS (35%–42%) or B-RAF (10%–20%) are prevalent [14,15]. Mutant B-RAF, especially B-RAF^{V600E}, predisposes to inhibition of apoptosis, increases invasiveness [16], and occurs earlier during colorectal carcinogenesis [17]. However, the factors inducing progression in colon cancer downstream of RAS-RAF-MEK-ERK1/2 are currently unclear.

In addition to above signaling pathways, transcription factors, for instance Snail, Slug, ZEB1, ZEB2, and Twist, have been

Abbreviations: ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transition; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HMGA2, high mobility group AT-hook 2; HOXA, homeobox A; IGF2BP2, insulin-like growth factor 2 mRNA-binding protein 2; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; pRb, retinoblastoma protein; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; shRNA, short hairpin RNA; Slug^{BR}, the binding region of *Slug* promoter; Slug^{DR}, the distal region of *Slug* promoter; TET1, ten-eleven translocation 1; TGFβ1, transforming growth factor beta 1; ZO-1, zonula occludens 1.

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http://dx.doi.org/10.1016/j.canlet.2014.09.007
0304-3835/

confirmed to play critical roles in promoting EMT [18]. Slug, also named Snail2, incorporates five zinc finger domains, which play a pivotal role in the patterning during vertebrate embryo development and mesenchymal tumorigenesis [19]. Upregulation of Slug correlates with E-cadherin downregulation, lymph node metastasis, and shorter survival in various cancers [20,21]. The expression of Slug had been implicated to be involved in cancer cell migration regulated by ERK1/2 signaling [22].

HMGA2, a member of the high mobility group family, is a small non-histone chromatin protein. It contains three AT-hook domains that enable to combine the minor groove of A:T-rich DNA sequences to serve as architectural transcription factor or organize assembly on enhancers of a variety of genes [23]. HMGA2 is almost omnipresently expressed in relatively high levels during embryogenesis, whereas it is only found in uterine myoblasts, testicular cells and proliferating preadipocytes of adult tissues [24]. HMGA2 plays a crucial part in proliferation and differentiation during the development of the embryo. The deletion of HMGA2 in mice induces the pygmy phenotype with characteristic hypoplasia of mesenchymal tissue [25]. Interestingly, it is reported that the *HMGA2* gene variant correlated with adult and childhood heights with a significantly statistical confidence [26]. HMGA2 was also found amplified and overexpressed in a variety of epithelial and mesenchymal tumors. The overexpression of HMGA2 is associated with late stage of Dukes' classification in colon cancer [27] and high histological grading of invasive ductal type in breast cancer [28]. Besides, HMGA2 high level was related to decreased survival rates in breast cancer [29], colorectal cancer [30], and lung cancer patients [31]. Despite the expression profile of HMGA2 which indicates that this gene might play an essential role in the progression of various cancers, only limited studies have been reported which detailed the mechanisms downstream of HMGA2 in cancer cells [24,32–34].

In this study, we found that HMGA2 deletion in colon cancer cells suppresses the proliferation, migration, and invasion *in vitro* and *in vivo*. HMGA2 overexpression was able to activate EMT program, and increase the invasion property in colon cancer cells. Moreover, we revealed that ectopic HMGA2 expression induces EMT by HMGA2/Slug axis downstream of active ERK1/2 and TGF-β signaling.

Materials and methods

Cell culture

Human colon cancer cell lines HT-29 [American Type Culture Collection (ATCC) #HTB-38], COLO 205 (ATCC #CCL-222), LS 174T (ATCC #CL-188), SW480 (ATCC #CCL-228), SW620 (ATCC #CCL-227), and human normal colon epithelial cell line CCD-18Co (ATCC #CRL-1459) were provided by the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Human embryonic kidney cell line 293FT was a gift from Dr. W. Zhang (Chinese Academy of Sciences, Shanghai, China). HT-29 cells were cultured in McCoy's 5A medium. COLO 205 cells were cultured in RPMI 1640 medium. LS 174T, CCD-18Co, and 293FT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). SW480 and SW620 cells were cultured in L-15 medium. All medium contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Reagents

Recombinant human TGF-β1 was purchased from R&D Systems. B-RAF^{V600E} inhibitor PLX4032, MEK1/2 inhibitor U0126, and TβRI inhibitor SB525334 were purchased from Selleck Chemicals (Houston, TX, USA). Puromycin was bought from Gibco. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Cell Signaling Technology.

Plasmids and construction of the recombinant lentiviral vector

For the overexpression experiments, the coding sequence (CDS) of human *HMGA2* (NM_003483.4) and *Smad4* (BC002379.2) genes was synthesized by Shanghai GenePharma Co., Ltd. and inserted into the pENTR/SD/D-TOPO vector (Invitrogen). The amplified fragment was subcloned into the pLenti6.2/V5-DEST lentiviral expression vector (Invitrogen). For knockdown experiments, DNA oligonucleotides (Sigma) against human HMGA2, Slug, B-RAF, mutant B-RAF were annealed and ligated into the vector pLKO.1-puro according to the manufacturer's protocol (Addgene). The targeting sequences of shRNA constructs are listed in Supplementary Table S1.

Quantitative reverse transcriptase-PCR

For the gene expression at the mRNA level, the extraction of total RNA was performed using TRIzol reagent (TaKaRa, Dalian, China). Total RNA was converted to cDNA through iScript cDNA Synthesis Kit (Bio-Rad). Quantitative reverse transcriptase (qRT)-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad). Relative fold changes in mRNA levels were calculated after normalization to *GAPDH* using the comparative C_t method in all the experiments. The primers were listed in Supplementary Table S1.

Immunoblotting

Cells were lysed with Laemmli sample buffer and boiled for 5 minutes. Protein lysates were resolved by SDS–polyacrylamide electrophoresis (SDS–PAGE) gels and transferred to PVDF membranes, and then the following primary antibodies and dilutions were used: HMGA2 (1:1000, Abcam), GAPDH (1:1000, Abcam), E-cadherin (1:1000, Cell Signaling Technology), N-cadherin (1:1000, Cell Signaling Technology), ZO-1 (1:2000, Thermo-Pierce), vimentin (1:1000, Cell Signaling Technology), Slug (1:2000, Cell Signaling Technology), Snail (1:1000, Cell Signaling Technology), ZEB-1 (1:1000, Cell Signaling Technology), ZEB-2 (1:1000, Santa Cruz Biotechnology), Twist (1:1000, Cell Signaling Technology), ERK1/2 (1:1000, Cell Signaling Technology), pERK1/2 (1:2000, Cell Signaling Technology), B-RAF (1:1000, Cell Signaling Technology).

Immunofluorescence

Cells were seeded onto glass cover slips in 24-well plates, fixed in 4% paraformaldehyde and treated with 0.2% Triton X-100/phosphate-buffered saline (PBS). Cover slips were detected with anti-E-cadherin antibody (1:200, Cell Signaling Technology), vimentin (1:100, Cell Signaling Technology), ZO-1 (1:100, Thermo-Pierce), and N-cadherin (1:200, Cell Signaling Technology) overnight at 4 °C and Alexa Fluor 647-labeled secondary antibody (1:500, Cell Signal Technology) for 1 h at 37 °C and stained with DAPI. Finally, cover slips were observed under a confocal fluorescence microscope (TCS SP5, Leica, Mannheim, Germany).

Cell proliferation assays

Cells were seeded into 96-well plates at a density of 2×10^3/well for culture, and cell proliferation was measured by the WST-8 assays utilizing Cell Counting Kit-8 (Dojindo, Shanghai, China). The staining intensity in the medium was documented every 24 h for 6 days. Each experiment was repeated 3 times and 6 wells were used for each time point per group.

Transwell invasion and migration assays

The 24-well Transwell chambers with a pore size of 8 μm (6.5 mm diameter; Corning Costar Corporation, Cambridge, MA, USA) were utilized for *in vitro* cell migration and invasion assays. For cell migration, 2.5×10^4 cells were seeded into the upper compartments. For invasion assays, 4.0×10^4 cells were added to the upper compartments coated with Matrigel (1:3 dilution; BD Biosciences, Franklin Lakes, NJ, USA). The medium supplemented with 20% FBS was added into the lower compartment as chemoattractant. After 24–48 h incubation, the filter membrane was washed with PBS and stained with 0.25% crystal violet. Five random fields per membrane were counted using Image J software in three independent inserts.

Scratch wound-healing assays

Cells were plated in six-well plates in complete medium and grown to reach ~70–80% confluency. A linear wound was generated by scraping the middle cell monolayer with a sterilized pipette tip. The migration of cells to cover the wound space was examined and photographed at indicated time points (0, 24, 36 hours) in five random microscopic regions using a light microscope at 4× (Nikon, Japan) and were processed by Adobe Photoshop CS5. The migrated distance was analyzed by measuring the wound space at the above time points, and subtracted the wound width at time 0. The traveling distance values were indicated as a migration percentage, and the wound blank width at 0 hours was set as 0%. Each independent experiment was repeated 3 times.

Soft agar assays

The test cells (1×10^3) were suspended in 1 mL of medium containing 0.3% agarose with low gelling temperature (SIGMA-ALDRICH) and seeded onto a base layer of 1.4 mL