miR-154 inhibits EMT by targeting HMGA2 in prostate cancer cells

Chen Zhu · Jie Li · Gong Cheng · Hai Zhou · Liangjun Tao · Hongzhou Cai · Pu Li · Qiang Cao · Xiaobing Ju · Xiaoxin Meng · Meilin Wang · Zhengdong Zhang · Chao Qin · Lixin Hua · Changjun Yin · Pengfei Shao

Received: 23 September 2012 / Accepted: 21 March 2013 / Published online: 17 April 2013 © Springer Science+Business Media New York 2013

Abstract Epithelial–mesenchymal transition (EMT) is a crucial process that plays an important role in the invasion and metastasis of human cancers. High-mobility group AT-hook 2 (HMGA2) has been found to be involved in the EMT program, with its aberrant expression having been observed in a variety of malignant tumors. However, the mechanisms regulating HMGA2 expression remain incompletely understood. The objective of this study was to investigate whether miR-154 plays a critical role in EMT by regulating HMGA2. The expression levels of HMGA2 were examined in four samples of prostate cancer (PCa) tissue and adjacent non-tumorous tissue by Western blot analysis. The effects of forced expression of miR-154 or HMGA2 knockdown on PCa cells were evaluated by cell migration and invasion assays and Western blot analysis. HMGA2 was upregulated in the PCa tissue samples compared with the adjacent normal ones. Forced expression of miR-154 or HMGA2 knockdown significantly reduced the migratory and invasive capabilities of PCa cells in vitro and inhibited EMT gene expression, increased the levels of E-cadherin, an epithelial marker, and decreased the levels of vimentin, a mesenchymal marker. HMGA2 is a direct target gene of miR-154 by dual-luciferase reporter assay. Our findings suggest that miR-154 plays a role in regulating EMT by targeting HMGA2. Understanding the targets and regulating pathways of miR-154 may provide new insights into the underlying pathogenesis of PCa.

Keywords Prostate cancer · miR-154 · EMT

Introduction

Epithelial–mesenchymal transition (EMT) is a crucial process wherein epithelial cells lose their polarity and convert into more motile and invasive mesenchymal phenotypes. It plays an important role in the invasion and metastasis of human cancers [1]. However, the factors underlying this transformation remain unclear.

Recent studies have suggested that microRNA (miRNA) expression is associated with EMT by regulating the target genes [2, 3]. miRNAs are a class of small, 20–22 nt non-coding RNAs that regulate the expression of different genes by binding to the 3′-untranslated region (3′-UTR) of their target genes. Emerging evidence indicates that miRNAs are involved in the regulation of gene expression for development, differentiation, proliferation, and apoptosis [4]. Prostate cancer (PCa) is the most common malignancy in men and the leading cause of cancer-related death in American men [5]. Previous studies have shown that miRNA expression is dysregulated in prostate tumor samples compared with benign samples [6]. We have previously demonstrated that the expression level of miR-154 is downregulated in PCa compared with normal tissue [7].

High-mobility group AT-hook 2 (HMGA2) is a potential target gene for miR-154 based on bioinformatics analysis.
It is a non-histone chromatin binding protein that belongs to the HMGA protein family, which is composed of HMGA1a, HMGA1b, HMGA1c, and HMGA2 in humans, each of which contains several highly conserved DNA binding motifs named AT-hooks that preferentially bind the minor groove of AT-rich DNA sequences [8]. Research has shown that HMGA2 is overexpressed during embryonic development but rarely expressed or even undetectable in most normal adult tissues [8–10]. Aberrant expression of HMGA2 has also been observed in a variety of malignant tumors [11], and its overexpression is a potential prognostic factor for colorectal cancer [12], lung cancer [13], bladder cancer [14], ovarian cancer [15], and breast cancer [16]. One study reported that HMGA2 expression levels in carcinoma were higher than those of non-malignant tissues in a canine model of PCa [17]. HMGA2 has also been found to be involved in EMT [18, 19]. In spite of this wealth of data, the mechanisms regulating HMGA2 expression remain incompletely understood. Recent studies have reported post-transcriptional control of HMGA2 gene expression by miRNAs [20, 21] and that ectopic expression of let-7 reduced HMGA2 expression in lung cancer cells [22].

In this study, we used in vitro approaches to investigate whether HMGA2 is a direct target of mir-154 and if mir-154 plays a critical role in EMT by regulating HMGA2.

Materials and methods

Cell lines and tissues

The human PCa cell lines DU145 and PC-3 were obtained from KeyGene Biotech (Nanjing, China) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. All tissue samples used in this study were obtained from patients who underwent radical prostatectomy at the urology department of the First Affiliated Hospital of Nanjing Medical University in Nanjing, China. All samples were taken after surgery, immediately frozen in liquid nitrogen, and stored at −80 °C for further analysis.

Transfection

The human PCa cell lines were seeded in six-well plates and cultured to 70% confluence 1 day before transfection. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were incubated in a transfection mixture for 6 h, and the culture medium was replaced with RPMI-1640 containing FBS. The sense and anti-sense sequences of the miR-154 mimics were 5'-UAGGUUAUCCGUUGUGCCU CG-3’ and 5’-AAGGCAACAGUAACCUAUU-3’, respectively. RNA with no homology to any human genomic sequence was used as negative control (NC): sense, 5’-UCCUCGAACGUUGUCAGUTT-3’; anti-sense, 5’-ACGUGACCUCCGUAGAATT-3’. The sequences of mim-154 inhibitor were: 5’-CGAAGGGAACCGGAUAAACCUA-3’; the sequences of NC inhibitor were: 5’-CAGUACUUUUGUGUAGUAA’.

Western blot analysis

Cell lines and human PCa specimens were lysed in RIPA buffer (KeyGene Biotech) supplemented with protease inhibitors at 4 °C for 1 h. The protein samples were electrohoresed in 10% SDS–PAGE gels, transferred onto PVDF membranes (Millipore), and then blocked for 1 h with 5% non-fat milk at room temperature. After being incubated with primary antibodies at 4°C overnight, the PVDF membranes were washed thrice with TBST (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.01% Tween-20) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for 2 h. The related antibodies we used included anti-GAPDH (Bioworld), rabbit anti-HMGA2 (Cell Signaling Technology), anti-vimentin (Bioworld), anti-N-cadherin (Bioworld), and anti-E-cadherin (Bioworld). The blots were detected using chemiluminescence (Thermo Scientific). Protein levels were determined by normalizing to GAPDH.

Total RNA extraction and real-time RT-PCR

Total RNA was extracted from prostate cell lines using Trizol (Invitrogen) according to the manufacturer’s protocol. RNA was reverse-transcribed into cDNA using PrimeScript™ One-Step RT-PCR Kit (TAKARA, Dalian, China) following the manufacturer’s instructions. Real-time PCR was performed using a standard SYBR Green PCR kit (TAKARA) protocol on an Applied Biosystems 7300 Real-Time PCR System. The forward and reverse primers for HMGA2 were 5’-CAGCCGTCCACTTCAGC-3’ and 5’-TGCTTTTGGGTTCTTCCC-3’, respectively; those for GAPDH were 5’-GAAGGTGAAGGTCGGAGTC-3’ and 5’-GAAGATGTTGATGGAGATTTTCC-3’, respectively. The reaction conditions used for mRNA detection were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C...
for 30 s. All reactions were performed in triplicate and normalized by the internal control products of GAPDH.

Cell migration and invasion assays

For the migration assays, cells were harvested 48 h post-transfection and \(5 \times 10^4\) cells with 200 \(\mu\)l of serum-free medium were seeded into the upper chamber of the transwell (pore size, 8 mm; Millpore). For the invasion assays, cells with 200 \(\mu\)l of serum-free medium were placed into the upper chamber of an insert coated with Matrigel (BD Bioscience) according to the manufacturer’s protocol. Media containing 10 % FBS were added to the lower chamber. After 24 h of incubation, the cells remaining on the upper membrane were removed with cotton swabs, whereas those that had migrated or invaded through the membrane were fixed in 90 % alcohol and stained with 0.1 % crystal violet. Five random high-power microscopic fields (200× magnification) per filter were photographed, and the number of cells was counted. Experiments were independently repeated three times.

Plasmid construction and dual-luciferase assay

A fragment of the HMGA2 3'-UTR and a mutated 3'-UTR of HMGA2 that contained the putative miR-154 binding sites was prepared to construct reporter plasmids containing the 3'-UTR regions of HMGA2. DNA fragments were cloned into the downstream of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen). For luciferase assay, cells were seeded into 24-well plates and cultured for 24 h. Then, cells were co-transfected with pGL3-3'-UTR and control reporter plasmid, miR-154 or NC mimics and miR-154 inhibitor or NC inhibitor. 2 days later, cells were harvested and lysed in passive lysis buffer and reporter activity was measured using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well.

Statistical analysis

Data were expressed as mean ± SEM. All statistical calculations were performed using SPSS 11.0. Differences between groups were analyzed using Student’s \(t\) test. \(P < 0.05\) was considered statistically significant.

Results

HMGA2 protein expression in PCa clinical specimens

Total protein was isolated from four PCa and adjacent non-tumorous tissue samples. Western blot analysis revealed that HMGA2 protein expression was significantly higher in the tumor tissue samples than in the adjacent normal ones (Fig. 1).

Inhibition of HMGA2 expression and EMT after transfection of miR-154

To investigate the relationship between miR-154 and HMGA2, we transfected PC-3 and DU145 cells with miR-154 mimics or control miRNA and analyzed them using Western blot analysis and qRT-PCR. After transfection, the expression level of miR-154 in PC-3 and DU145 cells transfected with miR-154 mimics was over a thousand-fold than that transfected with control microRNA (Fig. 2). The expression of HMGA2 protein decreased significantly in cells transfected with miR-154 mimics compared with the cells transfected with control miRNA at 72 h post-transfection. These results indicate that miR-154 might negatively regulate the HMGA2 protein. Moreover, forced expression of miR-154 inhibited EMT gene expression; the expressions of vimentin and N-cadherin protein decreased significantly in cells transfected with miR-154 mimics compared with those transfected with control miRNA; and the expression of E-cadherin protein increased in cells transfected with miR-154 mimics (Fig. 3a). These results...
suggest that miR-154 may serve as a tumor suppressor by inhibiting EMT.

miR-154 affects cell migration and invasion in vitro

To determine whether miR-154 is involved in PCa cell migration and invasion, cell migration and invasion assays were performed. The number of migrated cells transfected with miR-154 was significantly lower than that of the NC (P < 0.05; Fig. 3b). A significant downregulation of invasion into Matrigel was observed in miR-154-transfected cells (P < 0.05; Fig. 3b). These results indicate that miR-154 has an important role in inhibiting the migration and invasion of PCa cells.

Fig. 3 Forced expression of miR-154 inhibited HMGA2 expression, EMT gene expression, as well as cell migration and invasion in vitro. 

(a) HMGA2 expression and EMT suppressed by miR-154 transfection at protein levels in PCa cell lines PC-3 and DU145. 
(b) Ectopic expression of miR-154 inhibited migration and invasion in the PCa cell lines. Data are the mean ± SEM of at least three independent experiments. *P < 0.05. Original magnification ×200

Fig. 4 HMGA2 and EMT expressions after transfection with si-HMGA2. 

(a) HMGA2 protein expression and EMT gene expression after 72 h of transfection with si-HMGA2 or NC. GAPDH was used as a loading control. 
(b) HMGA2 mRNA expression after 72 h of transfection with si-HMGA2 or NC. GAPDH was used as a loading control. The relative expression of HMGA2 compared with that of GAPDH was calculated using 2^-ΔΔCt. 
(c) Effects of HMGA2 knockdown on cell migration and invasion in PCa cell lines. Data are the mean ± SEM of at least three independent experiments. *P < 0.05. Original magnification ×200
Effects of HMGA2 knockdown on PCa cell lines

To determine whether deregulation of HMGA2 is involved in the regulation of cell migration and invasion by miR-154, HMGA2 was knocked down in PCa cells by introducing siRNA and analyzed the level of silencing by Western blot analysis and real-time RT-PCR. Si-HMGA2 successfully reduced the expression level of HMGA2 protein at 72 h post-transfection (Fig. 4a). After 48 h of transfection, the expression level of HMGA2 mRNA significantly decreased in cells transfected with si-HMGA2 compared with that of cells transfected with control siRNA (Fig. 4b). The number of migrated and invaded cells transfected with si-HMGA2 was significantly lower than that of cells transfected with NC (Fig. 4c). Consistent with previous studies, inhibition of HMGA2 expression in the PCa cell lines had an impact on EMT gene expression, including vimentin, N-cadherin, and E-cadherin (Fig. 4a). These findings provide further evidence that HMGA2 promotes prostate tumorigenesis through the regulation of EMT in PCa.

---

**Fig. 5** miR-154 suppresses HMGA2 expression by binding to the HMGA2 3′-UTR.

A. Schematic description of the HMGA2 3′-UTR with two putative binding sites for miR-154. B, C. Dual-luciferase assay results for PC-3 cells suggesting that HMGA2 is a target gene of miR-154. *P < 0.05 by t test
HMGA2 is a direct target of miR-154

Analysis of the 3′-UTR sequence of HMGA2 using TargetScan revealed two putative binding sites for miR-154: one is located from nucleotides 163 to 169, and the other is located from nucleotides 296 to 302 (Fig. 5a). To further validate the predicted target, we fused the predicted HMGA2 3′-UTR target site, including binding sites 1 or 2 for miR-154, to the downstream of the firefly luciferase gene. The reporter plasmids were co-transfected into PC-3 cells along with miR-154 mimics or NC. miR-154 mimics significantly suppressed the luciferase activity compared with co-transfection with NC of the wild-type reporter but not that of the mutant one. In contrast, inhibition of endogenous miR-154 with the NC of the wild-type reporter did not affect luciferase activity compared with NC inhibitor of the wild-type reporter (Fig. 5b, c). Taken together, our results suggest that HMGA2 is a direct target gene of miR-154.

Discussion

This is the first study to show that HMGA2 is negatively regulated by miR-154 at the post-transcriptional level through two specific target sites within the 3′-UTR. We also showed that miR-154 inhibited EMT by regulating HMGA2 in PCa cells.

MiRNA expression is involved in crucial biological processes, including invasion and metastasis [4]. One critical step in metastasis is the process of EMT through which epithelial cells lose their epithelial marker, E-cadherin, and gain the mesenchymal marker vimentin. EMT is regulated by a variety of signaling pathways, including TGFB, EGF, and HGF [23]. In recent years, aberrantly expressed miRNAs were reported to be likely implicated in regulating EMT. For example, miR-1 and miR-200 inhibit EMT through regulating Slug [2], and the miR-200 family suppresses EMT by targeting the SIRT1 3′-UTR in breast cancer [24]. Computational analysis predicted potential binding sites for miR-154 in the 3′-UTR of the HMGA2 gene. In this study, we found that forced expression of miR-154 inhibited HMGA2 gene expression; we also determined that HMGA2 is a direct target of miR-154 by dual-luciferase assay. The HMGA2 gene is located on chromosome 12q14 and encodes a 109-amino acid protein. Overexpression of HMGA2 has been observed in a variety of malignant tumors [11]. Moreover, we demonstrated that HMGA2 protein expression was significantly higher in tumor tissues compared with adjacent normal tissues, suggesting that aberrant expression of HMGA2 is likely implicated in the biology of PCa. Recent studies have indicated that HMGA2 plays a critical role in the EMT program [18, 19, 25].

We thus investigated knockdown of HMGA2 expression with siRNA and found that depression of HMGA2 increased the levels of E-cadherin and decreased those of vimentin. Consistent with previous studies, our data suggest that the inhibition of HMGA2 expression prevents EMT. We went on to utilize cell migration and invasion assays to evaluate migration and invasion responses. Knockdown of HMGA2 significantly decreased the migratory and invasive capabilities of PCa cells in vitro. We also proved that ectopic expression of miR-154 inhibited EMT gene expression as well as migration and invasion of PCa cells in vivo. Taken together, our data show that miR-154 modulates the EMT process by regulating the HMGA2 gene.

In conclusion, our data reveal that miR-154 contributes to the regulation of the EMT program by targeting HMGA2 at the post-transcriptional level in PCa cell lines. We have identified HMGA2 as an EMT regulator in PCa and determined that ectopic expression of miR-154 decreases the migratory and invasive capabilities of PCa cells in vitro. miR-154 is clearly a promising molecular target for the treatment of PCa.

Acknowledgments This work was supported by the Program for Development of Innovative Research Team in the First Affiliated Hospital of Nanjing Medical University, Provincial Initiative Program for Excellency Disciplines of Jiangsu Province, by the National Natural Science Foundation of China (Grant numbers 81171963, 81102089, and 81201998) and the Natural Science Foundation of Jiangsu Province (Grant number BK2011773).

Conflict of interest The authors have no financial conflict of interest.

References

cancer cell proliferation by targeting CCND2. Urol Oncol. doi: 10.1016/j.urolonc.2012.11.013


学霸图书馆
www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：
图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具