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Downregulating HMGA2 attenuates epithelial-mesenchymal transition-induced invasion and migration in nasopharyngeal cancer cells

You-you Xia [a, b, 1], Li Yin [c, 1], Ning Jiang [c, 1], Wen-Jie Guo [c], Hao Tian [b], Xue-Song Jiang [c], Jing Wu [b], Meng Chen [b], Jian-Zhong Wu [d], Xia He [c, *]

[a] Department of Radiation Oncology, Lianyungang First People's Hospital, Lianyungang, Jiangsu, China
[b] The Fourth Clinical Medical College of Nanjing Medical University, Nanjing, Jiangsu, China
[c] Department of Radiation Oncology, The Affiliated Jiangsu Cancer Hospital of Nanjing Medical University, Nanjing, Jiangsu, China
[d] Research Center of Clinical Oncology, The Affiliated Jiangsu Cancer Hospital of Nanjing Medical University, Nanjing, Jiangsu, China

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ABSTRACT

Background: Epithelial-mesenchymal transition (EMT) is associated with invasion and metastasis of cancer cells. High-mobility group AT-hook 2 (HMGA2) has been found to play a critical role in EMT in a number of malignant tumors. However, whether HMGA2 regulates the EMT in human nasopharyngeal carcinoma (NPC) is unclear.

Objective: The aim of this study was to investigate the effect and mechanism of HMGA2 in inducing invasion and migration in NPC.

Methods: In NPC tissues samples, the association of HMGA2 mRNA expression with clinicopathological characteristics were estimated by real-time quantitative RT-PCR(qRT-PCR). In vitro, following the silencing of HMGA2 in CNE-1 and CNE-2 cell lines, the viability and metastatic ability were analyzed using Cell Counting Kit-8 (CCK8), colony formation assay, and transwell assay. EMT and transforming growth factor-beta (TGFβ)/Smad3 signaling pathway-related protein expression changes were evaluated using western blot.

Results: HMGA2 was upregulated in NPC cell lines and clinical specimens ($P < 0.01$), and HMGA2 expression correlated significantly with metastasis ($P = 0.02$) and disease-free survival of NPC (hazard ratio: 3.52; 95% confidence interval: 1.34–7.79; $P = 0.01$). In addition, following in vitro knockdown of HMGA2, the aggressiveness of cells was markedly inhibited, Vimentin and Snail level was downregulated and E-cadherin expression was upregulated. Moreover, the expression of key proteins TGFβRII and p-Smad3 of the TGFβ/Smad3 signaling pathway was inhibited by the downregulation of HMGA2.

Conclusion: HMGA2 might maintain EMT-induced invasion and migration through the TGFβ/Smad3 signaling pathway in NPC cell lines.

1. Introduction

Nasopharyngeal carcinoma (NPC) is highly prevalent in southern China and Southeast Asia with remarkably distinct ethnic and geographic distributions [1]. Although the standard combination of radiotherapy and chemotherapy has improved the therapeutic efficacy, local recurrence and early metastasis are still the common causes of mortality in patients with NPC [2]. So far, the precise molecular changes underlying the invasive and metastatic properties of NPC remain unknown.

Currently, it is believed that epithelial-to-mesenchymal transition (EMT) may play an important role in the biological progression and acquisition of metastatic characteristics of carcinoma [3,4]. EMT is a developmental process that leads the phenotypic shift from an epithelial morphology to a motile morphology [5]. The significant change that characterizes the process of EMT is downregulation of epithelial protein E-cadherin and upregulation of the

* Corresponding author. Department of Radiation Oncology, The Affiliated Jiangsu Cancer Hospital of Nanjing Medical University, 42 Bai Zi Ting Road, Nanjing, Jiangsu, China.
E-mail address: he_xia_2003@163.com (X. He).
[1] These authors contributed equally to this work.

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acquisition of a mesenchymal phenotype such as Vimentin [6,7]. With regard to the upstream molecular mechanism of EMT, transforming growth factor-beta (TGFβ) has been identified as a key driver of EMT in carcinoma [8,9]. The TGFβ signal brings together type I and type II receptors. Following binding ligands, active type II receptor phosphorylates the type I receptor, which leads to the activation of Smad3 [10].

High-mobility group AT-hook (HMGA2), which is a member of the high-mobility group family, is almost expressed highly during embryogenesis [11]. HMGA2 was also found overexpressed in a number of tumors [12–15]. The oncogenic properties of HMGA2 are shown to be involved in the aggressive growth of tumor [16,17], self-renewal of stem cell [18], and EMT [19,20]. A recent study showed high expression of HMGA2 elicits EMT and plays an important role in the progression of gastric cancer cells [21]. However, no study has elucidated the functions and molecular mechanisms of HMGA2 in the process of EMT in NPC.

Our previous study demonstrated that HMGA2 was overexpressed in NPC samples, and the association between HMGA2 and EMT-related markers was significant [22]. Based on this information, for the first time, it was revealed that HMGA2 is involved in EMT in NPC cell lines and might maintain EMT-induced invasion and migration via TGFβ/Smad signaling pathway.

2. Materials and methods

2.1. Clinical NPC specimens

In total, 72 paraffin-embedded primary NPC tissues and 20 nontumoral inflammatory nasopharyngeal tissues through biopsies were collected retrospectively from the Affiliated Jiangsu Cancer Hospital, Nanjing Medical University, Nanjing, China, between May 2006 and May 2011. All patients were selected based on (1) the histopathological diagnosis of NPC, (2) the absence of any anti-tumor treatment before biopsy, (3) the availability of data from the original medical records and complete follow-up, and (4) the absence of distant metastasis at the time of diagnosis. All patients were staged according to the tumor-node-metastasis (TNM) classification of Union for International Cancer Control (2010). The study was approved by the Ethics Committee of the Jiangsu Cancer Hospital.

2.2. Cell culture

The human NPC cell lines CNE-1, CNE-2, 5–8F, 6–10B, and human immortalized nasopharyngeal epithelial cell line NP69 were purchased from Cell Center of Hsiang-Ya Medical College (Changsha, Hunan, China). The human immortalized nasopharyngeal epithelial cell line NP69 was maintained in keratinocyte/serum-free medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract (BD Biosciences, Bedford, MA). The human NPC cell lines CNE-1, CNE-2, 5–8F, and 6–10B were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Western blot assay

Total cellular proteins were extracted from human NPC cell lines using radioimmunoprecipitation buffer (Beyotime, Jiangsu, China). Cell lysates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Subsequently, the membranes were incubated with primary antibodies against human HMGA2 (1:1000; Abcam, Cambridge, UK), anti-TGFβ type II receptor (1:500; Abcam), β-actin (1:5000; Abcam), phospho-Smad3 antibody (1:1000; Cell Signaling Technology, Danvers, MA), anti-human Smad3 antibody (1:1000; Cell Signaling Technology), anti-Snail antibody (1:1000; Abcam), E-cadherin, and Vimentin (1:1000; Proteintech, Chicago, IL) overnight at 4 °C. Membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Protein expression was detected using a chemiluminescence system (Amersham, Arlington Heights, IL). The β-actin signal was used as a control. The representative data from individual experiments, which were repeated at least twice, are shown.

2.4. Isolation of RNA and real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from tumor-rich areas of paraffin-embedded tissues and cells using Ambion RecoverAll™ Total Nucleic Acid Isolation (Ambion, Austin, TX) and TRIzol reagent (Invitrogen) according to the manufacturers' instructions. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed according to the manufacturer's instructions (SYBR-Green miScript PCR Starter kit; Qiagen, Valencia, CA). Primer of HMGA2 was also purchased from Qiagen (PPH24203A). The reaction mixtures of HMGA2 and β-actin were incubated at thermal cycling conditions at 95 °C for 15 min, and 40 cycles at 94 °C for 15 s, 55 °C for 30 s followed by 70 °C for 34 s. All NPC samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$) [23]. Each polymerase chain reaction (PCR) was performed in triplicate.

2.5. Small inhibitory RNA-mediated downregulation of HMGA2 expression

At 24 h prior to transfection, CNE-1 and CNE-2 cells were plated onto six-well plates (2×10^5 cells per well). Later cells were transiently transfected with 100 nM small inhibitory RNA (siRNA) against HMGA2 and the negative control siRNA (Ribobio Corporation, Guangzhou, China) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The HMGA2 siRNA sequences were as follows: 5′-CAAGAGGCAGACCUAGGAA dTdT-3′, 3′-dTdT GUUCUCCGUCUGGA UCC UU-5′. The effectiveness of gene silencing was determined using real-time PCR.

2.6. Cell proliferation assay

Cells were seeded onto 96-well plates (2×10^3 cells per well) 48 h after transfection. The Cell Counting Kit-8 (CCK8) assay (KeyGen, Nanjing, China) was used to test the cell viability at 1, 2, 3, and 4 days according to the manufacturer's instructions; 10 μL of CCK8 was added to each well, followed by incubation for 2 h. The optical density in each well was measured at 450 nm by a microplate reader. For colony formation assay, CNE-1 and CNE-2 cells were plated at 200 cells per well in six-well plates after transfection, and cultured for 10 days. Colonies were fixed with paraformaldehyde, stained with crystal violet, and counted under the inverted microscope. The data shown are the mean of three repeated experiments.

2.7. Cell migration and invasion assay

Cell invasion assay with a Matrigel-coated (BD Biosciences) membrane and migration assay with no Matrigel membrane were performed using transwell inserts with a pore diameter of 8 μm (Corning, Acton, MA). The cells were harvested and resuspended

(5×10^5 cells/mL) in serum-free medium, and then seeded in the upper chamber. RPMI 1640 medium plus 10% fetal bovine serum was placed in the underneath chambers. After incubation for 24 h, the invasive cells were fixed with 4% paraformaldehyde, then stained with crystal violet, and counted under a light microscope at a magnification of × 200. The results presented are the mean of three independent assays.

2.8. Immunofluorescence

Cells grown on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde for 30 min. Later, cells were incubated with anti-E-cadherin (1:100; Proteintech) and anti-Vimentin (1:100; Boster, Wuhan, China) antibodies at 37 °C for 2 h. Lastly, the cells were stained with a mixture of two secondary antibodies conjugated to fluorescein isothiocyanate and tetramethylrhodamine for 1 h after they were washed with PBS. Nuclei were labeled with 4′,6-diamidino-2-phenylindole and observations were performed with a microscope (Olympus Corporation, Tokyo, Japan) at a magnification of × 200.

2.9. Statistical analyses

All statistical analyses were performed using SPSS 16.0 software package (SPSS, Chicago, IL). The data are shown as the mean ± standard deviation from three independent assays. The Chi-square test was used to analyze the relationship between the clinicopathological features and the expression of HMGA2 mRNA. The Student's *t*-test was used for comparisons between groups. Mann–Whitney U-test was used to compare medians of non-normal distributions groups. The Kaplan–Meier method was used to estimate disease-free survival (DFS). Cox regression was used for univariate analysis. $P < 0.05$ was considered significant.

3. Results

3.1. HMGA2 was upregulated in NPC cell lines and clinical specimens

To determine the expression of HMGA2 in NPC, qRT-PCR and western blot to detect mRNA and protein levels of several common human NPC cell lines (6–10B, 5–8F, CNE-1, CNE-2) and a normal nasopharyngeal epithelial cell line (NP69) were performed. It was found that the relative expression level of HMGA2 in CNE-2 cell line was remarkably higher than those in other NPC cell lines and normal nasopharyngeal epithelial cell line (Fig. 1A), which was consistent with the protein level of HMGA2 in these cell lines (Fig. 1C). The HMGA2 mRNA expression was also detected in 72 formalin-fixed paraffin-embedded NPC biopsy samples and 20 nontumor inflammatory nasopharyngeal tissues using quantitative RT-PCR. It was found that the relative mRNA expression in NPC tissues was much higher than those in nontumor tissues (Fig. 1B).

Table 1
Comparison of several clinicopathological parameters and expression levels of HMGA2 in NPC.

Characteristic	N	HMGA2		*P*
		Low, n (%)	High, n (%)	
Gender				
Male	55	26 (72)	29 (81)	0.41
Female	17	10 (28)	7 (19)	
Age (years)				
<50	33	19 (53)	14 (39)	0.24
≥50	39	17 (47)	22 (61)	
Histologic type				
NKC	6	4 (11)	2 (6)	0.39
KSCC	66	32 (89)	34 (94)	
T stage				
T1–2	41	24 (67)	17 (47)	0.10
T3–4	31	12 (33)	19 (53)	
N stage				
N0–1	43	25 (69)	18 (50)	0.09
N2–3	29	11 (31)	18 (50)	
TNM stage				
I–II	30	18 (50)	12 (33)	0.15
III–IV	42	18 (50)	24 (67)	
Metastasis[a]				
No	58	33 (92)	25 (69)	0.02
Yes	14	3 (8)	11 (31)	

Abbreviations: HMGA2, high-mobility group protein 2; KSCC, keratinizing squamous cell carcinoma; NKC, non-keratinizing carcinoma; NPC, nasopharyngeal carcinoma; TNM, tumor-node-metastasis.
[a] Metastasis within 2 years after treatment.

3.2. Metastasis and DFS of NPC were associated with HMGA2 expression in tumor tissues

To evaluate the clinical relevance of HMGA2 expression, qPCR of HMGA2 in a cohort of 72 formalin-fixed paraffin-embedded NPC tissues was performed. As summarized in Table 1, interestingly among several clinicopathological features only metastasis (within 2 years after treatment) was associated with HMGA2 expression ($P = 0.02$), whereas the expression level of HMGA2 was not

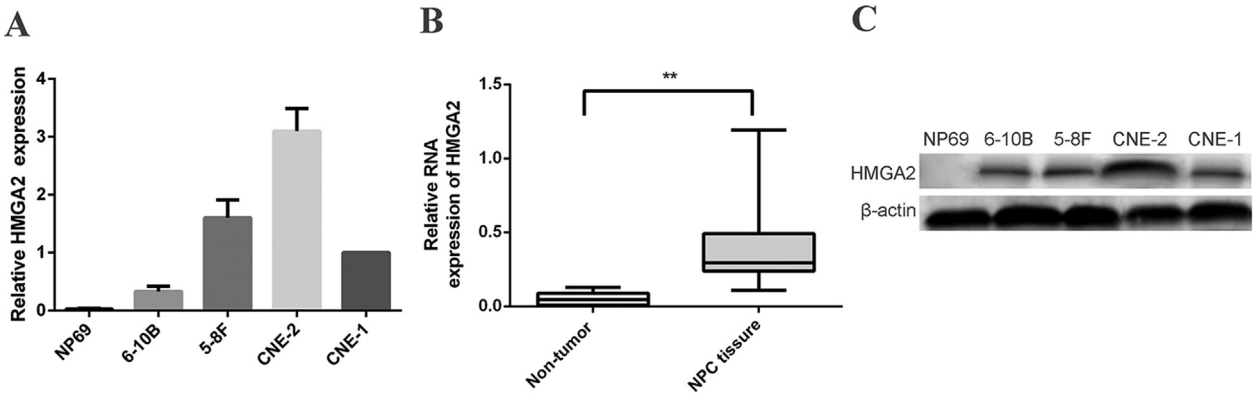

Fig. 1. HMGA2 was upregulated in NPC cell lines and clinical specimens. (A) Relative HMGA2 mRNA expression in normal nasopharyngeal epithelial cell line NP69 and NPC cell lines. (B) Relative HMGA2 mRNA expression in nontumor tissues (n = 20) and NPC tissues (n = 72). (C) HMGA2 protein levels in NP69 and NPC cell lines. Data are presented as mean ± SD or median. β-actin was used as an internal control and P-values were calculated using the one-way ANOVA or Mann–Whitney U test. $^{**}P < 0.01$. ANOVA, analysis of variance; HMGA2, high-mobility group AT-hook 2; NPC, nasopharyngeal carcinoma; SD, standard deviation.

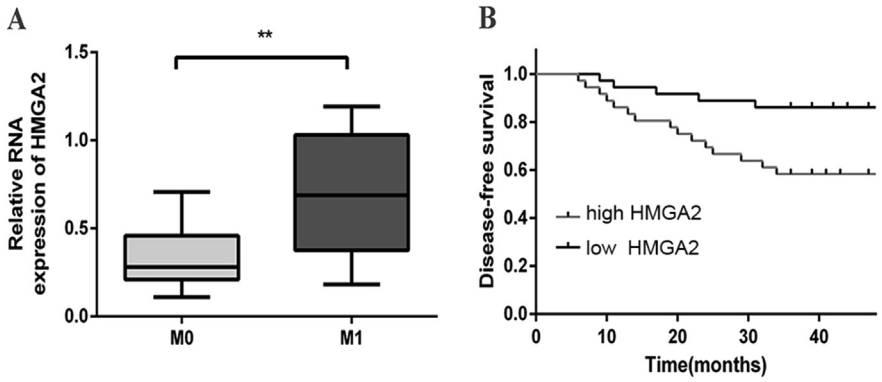

Fig. 2. Upregulation of HMGA2 was associated with tumor metastasis and worse survival in NPC specimens. (A) Expression levels of HMGA2 in metastatic and non-metastatic NPC specimens were measured using qPCR. One of the tumor specimens' transcripts was used for normalization. β-actin was used as the internal control. (B) Expression of HMGA2 was associated with disease-free survival using Kaplan–Meier method. HMGA2, high-mobility group protein 2; qPCR, quantitative polymerase chain reaction; NPC, nasopharyngeal carcinoma; P-values were calculated using the Mann–Whitney U-test. $^{**}P < 0.01$.

associated with clinical variables such as gender, age, histologic type, T stage, N stage, and TNM stage. HMGA2 expression was defined with a cutoff value of the median of 72 patients (high vs low expression). Similarly, there was a significantly higher relative mRNA expression of HMGA2 in metastastic patients than that in patients without metastasis (Fig. 2A, $P < 0.01$). Furthermore, Kaplan–Meier analysis and log-rank test were used to detect the prognostic impacts of HMGA2 expression on patients' DFS. Patients with high HMGA2 expression had significantly reduced DFS (hazard ratio: 3.52; 95% confidence interval: 1.34–7.79; $P = 0.01$) compared with those with low HMGA2 expression group (Fig. 2B).

3.3. Si-HMGA2 suppressed the growth and invasion of NPC cell in vitro

At 48 h after siRNA transfection, HMGA2 expression levels were significantly reduced by qPCR and western blot for CNE-1 and CNE-2 cells, respectively (Fig. 3A and B). To evaluate whether

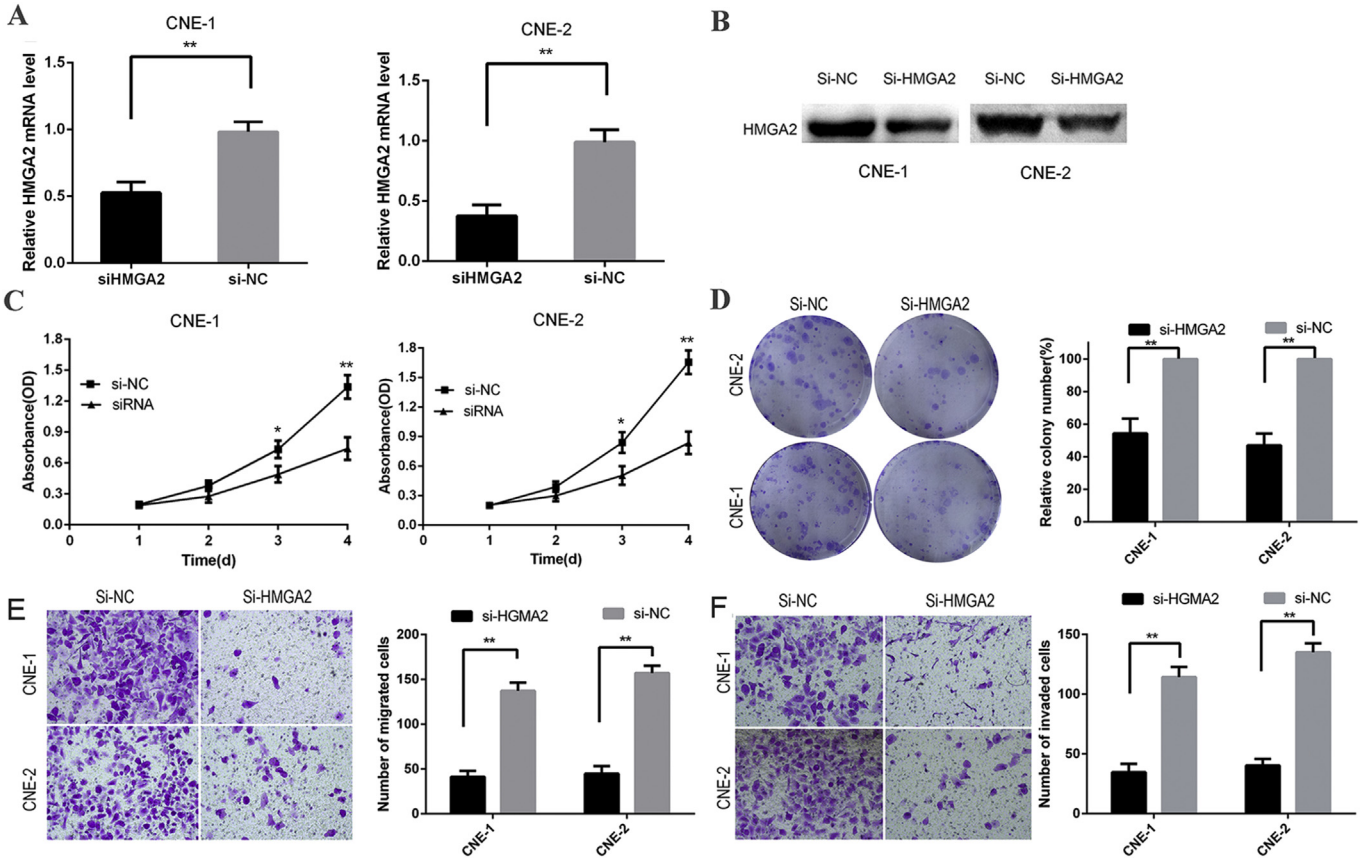

Fig. 3. HMGA2 suppressed the viability, proliferation, migration, and invasion of NPC cell. (A) Silencing efficacy of HMGA2 siRNA was determined using qPCR (B) and western blot in CNE-1 and CNE-2 cells. (C) CCK8 assay was used to analyze cell viability of CNE-1 and CNE-2 cell lines. (D) Representative results show colony formation, (E) migration, and (F) and invasive ability of CNE-1 and CNE-2 cell lines transfected with Si-HMGA2 or Si-NC. Data are presented as mean ± SD. compared with control using the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$. CCK8, Cell Counting Kit-8; HMGA2, high-mobility group AT-hook 2; NPC, nasopharyngeal carcinoma; qPCR, quantitative polymerase chain reaction; SD, standard deviation; siRNA, small inhibitory RNA.

downregulation of HMGA2 affects cell viability and proliferation ability, CCK8 assay and colony formation assay were performed after transient transfection with Si-HMGA2 or Si-NC using CNE-1 and CNE-2 cells. It was found that cell growth was reduced in Si-HMGA2 cells compared to the Si-NC (Fig. 3C, $P < 0.01$). In addition, cell transfection with Si-HMGA2 showed much fewer colonies compared with controls (Fig. 3D, $P < 0.01$). To demonstrate whether HMGA2 has a decreasing effect on the cell migration and invasive ability, CNE-1 and CNE-2 cells were transiently transfected with Si-HMGA2 or the corresponding negative controls. The Si-HMGA2 groups displayed lower capability for invasion and migration than control groups (Fig. 3E and F; $P < 0.01$).

3.4. Silencing HMGA2 blocks EMT in NPC cell lines through TGFβ/Smad3 pathway

To indicate whether depletion of HMGA2 expression can induce the molecular changes consistent with EMT, western blot was performed on CNE-1 and CNE-2 cell lines by silencing HMGA2. Cells transfected with HMGA2-siRNA had significantly increased the expression of E-cadherin in comparison with siRNA-negative control, while levels of Snail and Vimentin were decreased in si-HMGA2 group compared with the control group (Fig. 4B). Further investigation by immunofluorescence also showed that si-HMGA2 reduced Vimentin expression but increased the levels of E-cadherin in the same area of cell glass coverslip (Fig. 4A).

To investigate whether silencing HMGA2 alters key molecules of the TGFβ/Smad3 signaling pathway, western blot was performed to confirm the effect of HMGA2 protein on the TGFβRII and Smad3 expression. The results showed that total Smad3 expression levels were unchanged in HMGA2 siRNA-treated NPC cell compared with control; however, phospho-Smad3 protein expression was significantly decreased in si-HMGA2 cell group. In addition, depletion of HMGA2 resulted in the suppression of TGFβRII protein expression (Fig. 4C).

4. Discussion

In the present study, it was found that HMGA2 was overexpressed in NPC cell lines and human NPC tissues. And HMGA2 mRNA level was closely linked with cancer metastasis and progression-free survival in patients with NPC. Additionally, downregulation of HMGA2 inhibited the proliferation and migration of NPC cell lines. Importantly, EMT-related markers and downstream key proteins of the TGFβ/Smad3 pathway also have been significantly changed following the inhibition of HMGA2. These results suggest that HMGA2 may be involved in EMT-induced invasion and migration of NPC cell lines through the TGFβ/Smad3 signaling pathway.

Recently, it was reported that HMGA2 mRNA and protein were overexpressed in many malignant neoplasms [24,25]. Some studies suggested that HMGA2 plays an important role in carcinogenesis [26], progression, and prognosis [16,27–29] of different types of tumors. However, little is known about the biological function of HMGA2 in the progression of NPC. The present study showed that HMGA2 was upregulated in NPC cell lines and NPC paraffin-embedded samples; thus, the results indicated that HMGA2 expression is likely related to the occurrence of NPC. Moreover, the in vitro data also demonstrated the important role of siHMGA2 in NPC cell lines in the control of cell proliferation and metastasis. In agreement with these findings, the knockdown of HMGA2 by specific shRNAs in colon cancer cells attenuates proliferation,

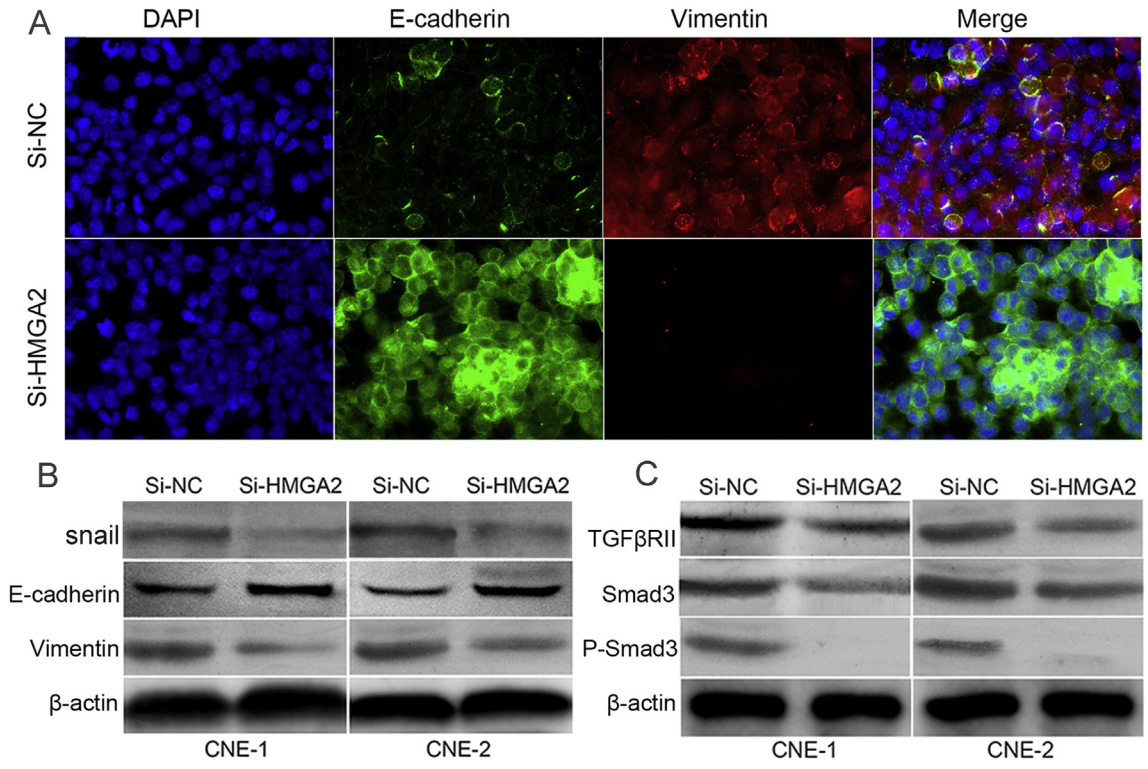

Fig. 4. Suppression of HMGA2 alters EMT-related markers and key signaling molecules in CNE-1 and CNE-2 cells. (A) The cells were stained for the expression of E-cadherin (green) and Vimentin (red) by immunocytofluorescence. Nuclei are labeled by DAPI (blue) (× 400). (B) Western blot were used for analyzing changes in Snail, E-cadherin, and Vimentin protein expression by silencing HMGA2. (C) The representative western blot image shows the effect of HMGA2 suppression on TGFβ/Smad3 pathway key molecules. DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial–mesenchymal transition; HMGA2, high-mobility group AT-hook 2; TGFβ, transforming growth factor-beta. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

motility, and invasion in vitro and in vivo [30]. The depletion of HMGA2 decreased the growth and metastasis of breast cancer cells [31]. Additionally, the clinical data also indicated a role for HMGA2 mRNA in the metastasis in patients with NPC. More importantly, these results provide evidence that upregulation of HMGA2 was significantly associated with reduced DFS in patients. Thus, HMGA2 may be a valuable prognostic biomarker in patients with NPC. Similarly, some studies also indicated a significant association between HMGA2 expression and poor prognosis [25,29].

During EMT, tumor cells acquire more aggressive and metastatic ability. Currently, some findings suggested that overexpression of HMGA2 induced specific molecular changes consistent with EMT [32,33]. Luo et al. [34] found that HMGA2 upregulates the expression of EMT-related markers Snail and Twist in hepatocellular carcinoma cell lines. Similar to these prior results, our previous study indicated that HMGA2 expression was significantly associated with EMT-related proteins in NPC tissues, and this study further showed that silencing HMGA2 in CNE-1 and CNE-2 cells resulted in significant increase in E-cadherin and loss of Vimentin and Snail, which is a characteristic of EMT.

To further explore the molecular mechanism through which HMGA2 induces EMT, some data indicate the existence of multiple regulatory mechanisms in different tumor cells. For instance, Zha et al. [21] found that HMGA2 activated the Wnt/β-catenin pathway and elicited EMT. A study reported that the TGFβ/Smad pathway could induce EMT independently without HMGA2 [35]. By contrast, Thuault et al. [36] observed that HMGA2 cooperates with the TGFβ/Smad pathway in regulating Snail expression, which acts as a major effector downstream of HMGA2 for the induction of EMT. Morishita et al. [11] also showed the mechanism by which HMGA2 mediates the induction of EMT through the TGFβ pathway. In agreement with the above study, our data also demonstrated a significant decrease in TGFβRII and p-Smad3 by the downregulation of HMGA2 in NPC cell lines, which revealed a potential mechanism of HMGA2-induced EMT through the TGFβ/Smad3 pathway.

In conclusion, an important role of HMGA2 in the metastasis and prognosis of NPC is shown. In addition, the knockdown of HMGA2 could significantly contribute to the inhibition of migration and EMT in NPC cell lines, and the TGFβ/Smad3 signaling pathway may be critical in the HMGA2-induced promotion of EMT. The next step involves the validation of these findings in vivo and discover whether this phenomenon can be reversed by blocking the TGFβ/Smad3 pathway.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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