Keratin 6A gene silencing suppresses cell invasion and metastasis of nasopharyngeal carcinoma via the β-catenin cascade

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Abstract. Nasopharyngeal carcinoma (NPC) is a type of head and neck cancer. This study aimed to study the mechanisms of ectopic keratin 6A (KRT6A) in NPC. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were performed to detect KRT6A levels in NPC cell lines (C666-1, 5-8F and SUNE-1) and a nasopharyngeal epithelial cell line (NP69, as a control). After SUNE-1 NPC cells had been silenced by KRT6A, cell viability, metastasis and invasion were determined using Cell Counting Kit-8, wound healing and Transwell assays, respectively. KRT6A levels, metastasis-associated factors and the Wnt/β-catenin pathway were measured using RT-qPCR and western blotting. It was demonstrated that KRT6A was upregulated in all detected NPC cells, among which KRT6A was the highest in SUNE-1 cells. In SUNE-1 cells, cell viability was inhibited at 24 and 48 h, and that cell metastasis and invasion were demonstrated to be suppressed by KRT6A silencing. Both the mRNA and protein levels of KRT6A, matrix metalloproteinase (MMP)-2, MMP-9, β-catenin, lymphoid enhancer binding factor 1 and T-cell specific factor 4 were reduced in the small interfering (si)KRT6A group. However, the results demonstrated that the levels of epithelial-cadherin and tissue inhibitor of metalloproteinase-2 (TIMP-2) were promoted in the siKRT6A group. The activation of the Wnt/β-catenin pathway by lithium chloride reversed the effect of si-KRT6A by modulating the expression of MMP-2/9 and TIMP2. It was observed that KRT6A silencing suppressed cell invasion and metastasis of NPC via the β-catenin cascade. Together these results provide important insights into a novel approach for the diagnosis and treatment of NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is a type of head and neck cancer (1,2). The incidence of NPC is the highest near the parts of or in the ear, nose and throat malignancies. The incidence of NPC has obvious regional clusters and certain ethnic groups are likely to experience a higher incidence of NPC than others. Incidence is low in most areas of the world, generally below $1/10^5$ (3). However, in China NPC is mainly distributed in southern China and Southeast Asia (4). The onset ages of NPC are mostly between 40-60 years old, with males having a higher incidence compared with their female counterparts (5). The etiology of NPC includes viral infections, genetic factors, environmental and dietary factors as well as the interaction of various oncogenes and tumor suppressor genes (6). Almost 70% of NPC patients are diagnosed at the advanced stages and the five-year survival rates are lower than 10-40% (7). The main factors contributing to the failure of treatment are the characteristics of tumor recurrence and distant metastasis of NPC (8). Therefore, it is becoming increasingly significant to study how to reduce distant metastasis so as to improve the outcome of treating NPC (9).

The epithelial-mesenchymal transition (EMT) process is prevalent during tumor metastasis. EMT is characterized by weakened intercellular adhesion and increased metastasis associated factors, for example, matrix metalloproteinases (MMPs) (10,11). Tumor cells with EMT frequently manifest invasive phenotypes, which are associated with difficulty treating the tumor (12,13). Notably, a couple of studies have focused on how biomarkers regulate EMT in NPC (14,15). In addition, the aberrant activation of the Wnt/β-catenin pathway is commonly reported to be involved in the EMT process of NPC (16).

In recent years, research on NPC in association with tumor biomarkers has attracted much attention and such studies aid early diagnosis, treatment efficacy and prognosis of NPC (17-20). Keratin is a major component of the intermediate filament protein family and is also a major structural protein of epidermal, and hair keratinocytes (21). The expression of keratin is mainly in epithelial tissues (22,23). Understanding the expression of keratin genes and their normal function are the basis for maintaining the stability and normal differentiation of epidermal cells (24). A previous study demonstrated that keratin may also function to regulate cell growth and apoptosis as well as protecting cells from impairment and

non-mechanical stress (25). Keratin 6A (KRT6A) is a family member of keratin proteins and can lead to epidermalization of squamous epithelium (26,27). KRT6A is a biomarker that is unique to squamous cells (28), while NPC is an undifferentiated squamous cell carcinoma (29,30). However, the correlation of KRT6A and NPC still remains unclear.

In the present study, the KRT6A silencing cell model was constructed in order to investigate the association between KRT6A and EMT in NPC. Taken from the perspective of tumor metastasis, this study help further understand the formation of NPC, aiming to improve the diagnostic compliance rate and survival rate of NPC.

Materials and methods

Cell culture. Human nasopharyngeal epithelial cell line (NP69, as a control) and NPC cell lines (C666-1, 5-8F and SUNE-1) were purchased from the American Type Culture Collection (Manassas, MA, USA), and cultured in 5% CO_2 at 37˚C. Dulbecco's modified Eagle's medium (DMEM) was used as basic culture medium. 10% fetal bovine serum (FBS), 100 U/ml streptomycin and 100 μg/ml penicillin were then added in to the medium. DMEM, FBS, streptomycin and penicillin were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Lithium chloride (LiCl; 20 mM; incubation time 24 h) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was used to activate the Wnt signaling pathway as previously described (31).

Small interfering (si)RNA transfection. The well-differentiated SUNE-1 cells were evenly plated in 6-well plates at the initial concentration of $6x10^4$ cells/well. After being cultured for 12 h, the cells reached 60-70% confluence. The cells were cultured in DMEM without FBS. A mixture of 50 pmol KRT6A-siRNA (Shanghai GeneChem Co., Ltd., Shanghai, China), with the sequence of sense 5'-CCAGCA GGAAGAGCUAUA-3', and antisense 3'-GGUCGUCCU UCUCGAUAUU, and transfection reagent Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (2:1), or 50 pmol scrambled siRNA (as control; Shanghai GeneChem Co., Ltd.), with the sequence of sense: 5'-UUCUCCGAACGUGUCACGUTT-3', and antisense: 5'-ACGUGACACGUUCGGAGAA-3' in mixture with Lipofectamine® 3000 was respectively added to the cells, and cultured for 8 h at 37˚C. The transfected cells were accordingly named the siKRT6A group and the mock group. Cells with non-treatment were named the Cotl group and treated as the control. The cells were then cultured in DMEM with FBS for another 48 h before further measurement of activity.

Cell viability assay. The effect of KRT6A silencing on cell viability of NPC SUNE-1 cells was measured using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) assay. To be more specific, SUNE-1 cells transfected with KRT6A-siRNA or scrambled-siRNA, or untreated SUNE-1 cells were respectively seeded in 96-well plates at an initial density of $2x10^3$ cells/well and incubated at 37˚C for 12, 24, and 48 h. Next, 20 μl CCK-8 reagent was added to each well. Then the plate was incubated at 37˚C for 3 h. Finally, optical density values were read at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The ratios of cell viabilities to Cotl group at 0 h were compared.

Cell scratch wound healing assay. The effect of KRT6A silencing on cell metastasis ability of NPC SUNE-1 cells was measured using wound healing assay. To be more specific, SUNE-1 cells transfected with KRT6A-siRNA or scrambled-siRNA, or SUNE-1 cells without transfection were respectively seeded in 6-well plates at an initial density of $6x10^4$ cells/well. After reaching 80% confluence, a wound was created using pipette tip scratching. After being cultured for 24 h, the wound closure size images in various fields were captured and measured by light microscope.

Transwell assay. Cell invasion abilities were measured using Transwell plates with 8-μm pore filter (Corning, Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells ($6x10^4$ cells/well) suspended in serum-free medium were seeded in the upper-chambers, while medium with 20% FBS was added to the lower-chambers in order to induce cell invasion. After being cultured at 37˚C for 24 h, the cells were stained with crystal violet (Beyotime Institute of Biotechnology) for 15 min at room temperature. Finally, cells images were captured and invaded cell numbers were counted under a light microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA expression levels of KRT6A in NP69 nasopharyngeal epithelial cells and different NPC cell lines (C666-1, 5-8F and SUNE-1) were determined. After conducting KRT6A silencing, levels of KRT6A, E-cadherin, tissue inhibitor of metalloproteinase-2 (TIMP-2), MMP-2, MMP-9, β-catenin, lymphoid enhancer binding factor 1 (LEF-1) and T-cell specific factor 4 (TCF-4) were determined in the Cotl, mock and siKRT6A cell groups. Primers used are listed in Table I and GAPDH was treated as an internal control. The process was performed as follows: Total RNA was first extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed to cDNA using Transcriptor Universal cDNA Master (Roche Diagnostics, Indianapolis, IN, USA), with the protocol of 70˚C for 5 min and 42˚C for 60 min. Then, the PCR amplification process was conducted using LightCycler® Multiplex Masters (Roche Diagnostics) with LightCycler® 480II System (Roche Diagnostics) under the conditions as follows: Pre-denaturation at 95˚C for 2 min, 36 cycles (denaturation at 95˚C for 25 sec, annealing at 60˚C for 25 sec, extension at 72˚C for 30 sec) and a final extension at 72˚C for 10 min. The changes were calculated by the $2^{-\Delta\Delta Cq}$ method (32).

Western blotting. The protein levels of KRT6A were detected in NP69 nasopharyngeal epithelial cells and in the different NPC cell lines (C666-1, 5-8F and SUNE-1). After conducting KRT6A silencing, levels of KRT6A, E-cadherin, TIMP-2, MMP-2, MMP-9, β-catenin, LEF-1 and TCF-4 were measured in Cotl, mock and siKRT6A cell groups. The cells were lysed on ice using radio immunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China)

Table I. Primer sequences used in the reverse transcription-quantitative polymerase chain reaction.

Name	Type	Sequence (5'-3')
GAPDH	Forward	CCATCTTCCAGGAGCGAGAT
	Reverse	TGCTGATGATCTTGAGGCTG
KRT6A	Forward	TCACCGTCAACCAGAGTCTC
	Reverse	GAACCTTGTTCTGCTGCTCC
E-cadherin	Forward	ACGCATTGCCACATACACTC
	Reverse	GGTGTTCACATCATCGTCCG
TIMP-2	Forward	TTCAAAGGGCCTGAGAAGGA
	Reverse	TCAGGCTCTTCTTCTGGGTG
MMP-2	Forward	CAGCCCTGCAAGTTTCCATT
	Reverse	GTTGCCCAGGAAAGTGAAGG
MMP-9	Forward	GAGACTCTACACCCAGGACG
	Reverse	GAAAGTGAAGGGGAAGACGC
β-Catenin	Forward	CTTGTGCGTACTGTCCTTCG
	Reverse	AGTGGGATGGTGGGTGTAAG
LEF-1	Forward	ACGAGCACTTTTCTCCAGGA
	Reverse	ATAGCTGGATGAGGGATGCC
TCF-4	Forward	TCCTCCGATGTCCACTTTCC
	Reverse	CCTGCTGAGAGAGATGGAGG

MMP, matrix metalloproteinase; E, epithelial; TIMP2, tissue inhibitor of metalloproteinase-2; LEF-1, lymphoid enhancer binding factor 1; KRT6A, keratin 6A; TCF-4, T-cell specific factor 4.

for 30 min and centrifuged at $1x10^4$ x g at 4˚C for 20 min. Next, the proteins in supernatant were quantified using BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 20 μg protein was first loaded to each well that contained 12% SDS-PAGE and then separated and transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific, Inc.). Next, the membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and incubated with specific primary antibodies overnight at 4˚C. Finally, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase: Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 7074, 1:5,000) for 1 h at room temperature. GAPDH was treated as a loading control. The proteins were detected by enhanced chemiluminescence detection reagents (Pierce; Thermo Fisher Scientific, Inc.) and analyzed by Bio-Rad ChemiDoc XRS densitometry with Image Lab™ Software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary antibodies used were as follows: Rabbit anti-KRT6A (Abcam, Cambridge, UK; cat. no. ab18586; 1:200), E-cadherin (Abcam; cat. no. ab15148; 1:500), TIMP-2 (Abcam; cat. no. ab180630; 1:1,000), MMP-2 (Abcam; cat. no. ab92536; 1:1,000), MMP-9 (Abcam; cat. no. ab38898; 1:1,000), β-catenin (Abcam; cat. no. ab16051; 1:5,000), LEF-1 (Abcam; cat. no. ab22884; 1:500), TCF-4 (Abcam; cat. no. ab185736; 1:1,000) and GAPDH (Abcam; cat. no. ab9485; 1:2,000).

Statistical analysis. SPSS software (version 20.0; IBM Corps, Armonk, NY, USA) was used for data analysis. Three replications were conducted in each assay. Data were expressed with the mean ± standard deviation. The data were analyzed by one-way analysis of variance and Dunnett t's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

KRT6A is upregulated in NPC cells. The expression levels of KRT6A in the nasopharyngeal epithelial cell line (NP69, as control) and NPC cell lines (C666-1, 5-8F and SUNE-1) were measured. The results of RT-qPCR and western blotting demonstrated that the mRNA and protein levels of KRT6A were significantly upregulated in all detected NPC cells, among which the value was the highest in the SUNE-1 cells ($P<0.01$; Fig. 1). Therefore, SUNE-1 cells were selected for the following gene modification experiments.

KRT6A silencing inhibits the cell viability of NPC cells. KRT6A-siRNA was transfected into NPC SUNE-1 cells using the liposome method. RT-qPCR and western blot assays analysis demonstrated that the mRNA and protein levels of KRT6A were significantly decreased in the siKRT6A group, compared with those of the Cotl and mock groups ($P<0.01$; Fig. 2A-C). CCK-8 assay results demonstrated that cell viabilities in the siKRT6A group was inhibited compared with the two groups, Cotl and mock ($P<0.01$; Fig. 2D).

KRT6A silencing inhibits cell metastasis and invasion of NPC cells. Cell metastasis and invasive abilities of SUNE-1 NPC cells prior to and following performing KRT6A silencing were measured by scratch wound healing and Transwell assays, respectively following 24 h of cell growth. The wound distance siKRT6A group were significantly increased compared with the Cotl and mock groups, indicating that KRT6A silencing inhibited cell metastasis of SUNE-1 NPC cells ($P<0.01$; Fig. 3A and B). In addition, the invasion rates of SUNE-1 NPC cells significantly decreased in the siKRT6A group, suggesting that KRT6A silencing inhibited cell invasion of SUNE-1 NPC cells ($P<0.01$; Fig. 3C and D).

KRT6A silencing attenuates EMT in NPC cells. EMT is the major mechanism of cell metastasis and invasion, Therefore, the epithelial marker E-cadherin and matrix degrading enzyme-associated factors were measured. RT-qPCR demonstrated though the mRNA and protein expression levels of E-cadherin and TIMP-2 were significantly promoted in the siKRT6A group ($P<0.01$), the mRNA and protein expression levels of MMP-2 and MMP-9 were significantly reduced compared with those in both the Cotl and mock groups ($P<0.01$; Fig. 4).

KRT6A silencing inhibits the Wnt/β-catenin pathway in NPC cells. As the Wnt/β-catenin pathway strongly affects tumorigenesis, the effect of KRT6A silencing on the Wnt/β-catenin pathway was investigated using RT-qPCR and western blot assays. The results demonstrated that both the mRNA and protein levels of β-catenin, LEF-1 and TCF-4 were significantly reduced in the siKRT6A group, compared with those in both the Cotl and mock groups ($P<0.01$; Fig. 5).

Activation of the Wnt/β-catenin pathway reverses the effect of si-KRT6A. LiCl was used to activate the Wnt/β-catenin

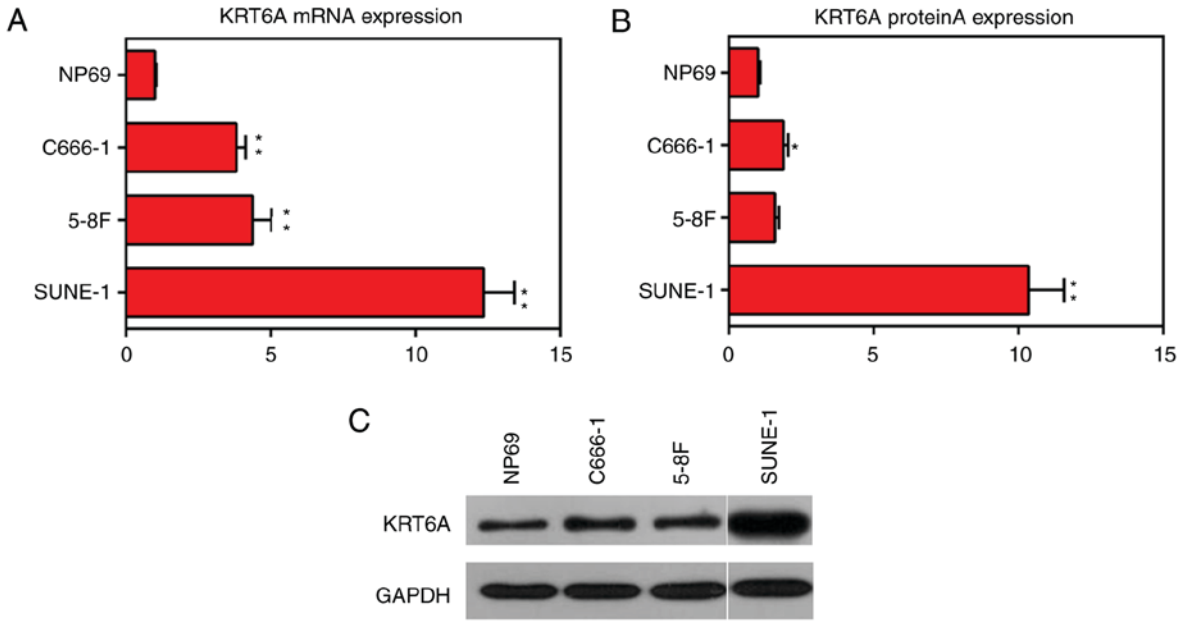

Figure 1. KRT6A is upregulated in NPC cells. (A) The mRNA expression levels of KRT6A were significantly upregulated in NPC cells (C666-1, 5-8F and SUNE-1), compared with normal nasopharyngeal epithelial cell line (NP69). (B) The protein levels of KRT6A were significantly upregulated in NPC cells and (C) and the western blot is presented. **P<0.01 vs. the NP69 group. KRT6A, keratin 6A; NPC, nasopharyngeal carcinoma.

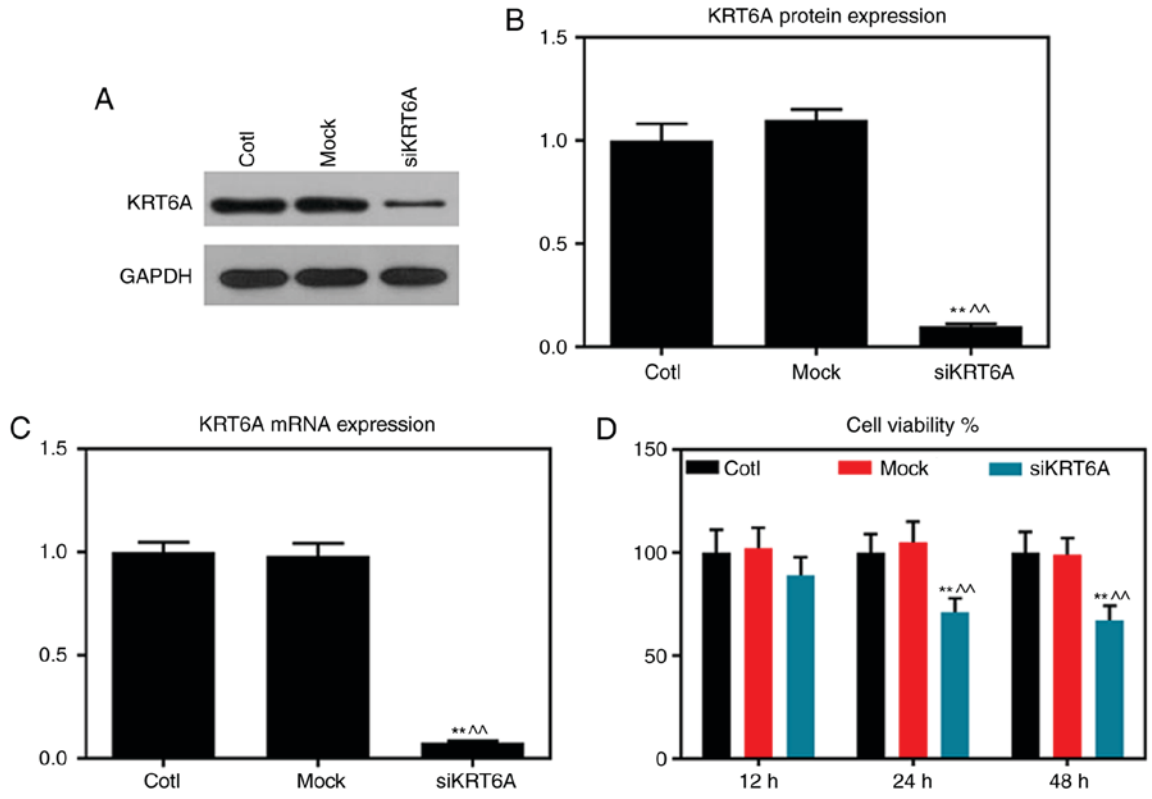

Figure 2. KRT6A silencing inhibits the cell viability of NPC cells. (A) The western blot is demonstrated and (B) the protein levels of KRT6A were demonstrated to decrease in the siKRT6A group. (C) The mRNA levels of KRT6A decreased in the siKRT6A group. (D) Cell viabilities in the siKRT6A group were inhibited at 24 and 48 h. **P<0.01 vs. the Cotl group and ^^P<0.01 vs. the mock group. KRT6A, keratin 6A; NPC, nasopharyngeal carcinoma; si, small interfering; Cotl, nontreatment group.

pathway and determined the association between the Wnt/β-catenin pathway and si-KRT6A. It was observed that decreased expression of MMP2/9 caused by si-KRT6A was then rescued by LiCl. By contrast, the expression of TIMP2 was significantly decreased in LiCl+si-KRT6A group compared with the si-KRT6A group (P<0.05; Fig. 6). This suggested that the effect of si-KRT6A on NPC cells may be largely dependent on the inactivation of the Wnt/β-catenin pathway.

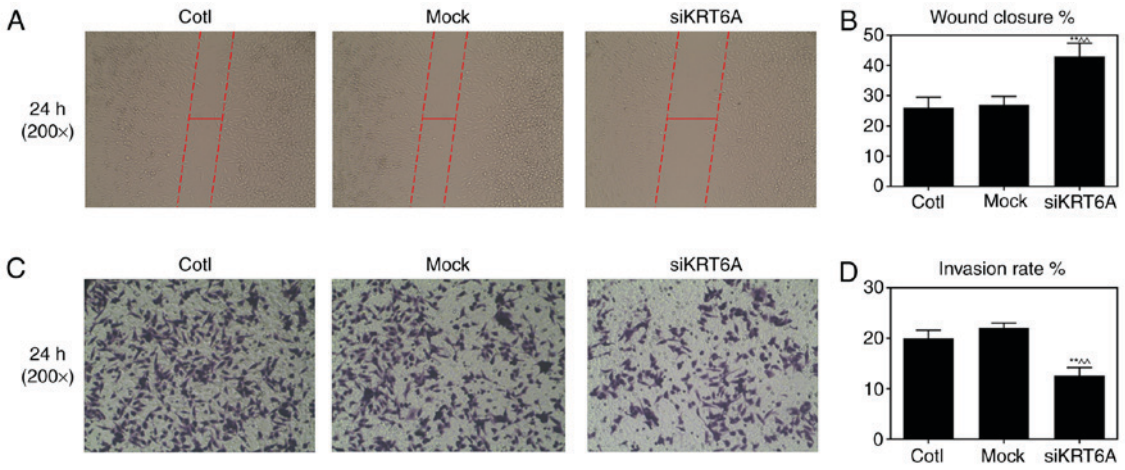

Figure 3. KRT6A silencing inhibits cell metastasis and invasion of NPC cells. KRT6A silencing inhibited cell metastasis of SUNE-1 NPC cells as detected by (A) a scratch wound healing assay and (B) the statistical analysis is presented. KRT6A silencing inhibited cell invasion of SUNE-1 NPC cells, detected by (C) a Transwell assay and (D) the statistical analysis is presented. **P<0.01 vs. the Cotl group and $^{\wedge\wedge}$P<0.01 vs. the mock group. KRT6A, keratin 6A; NPC, nasopharyngeal carcinoma; Cotl, nontreatment group.

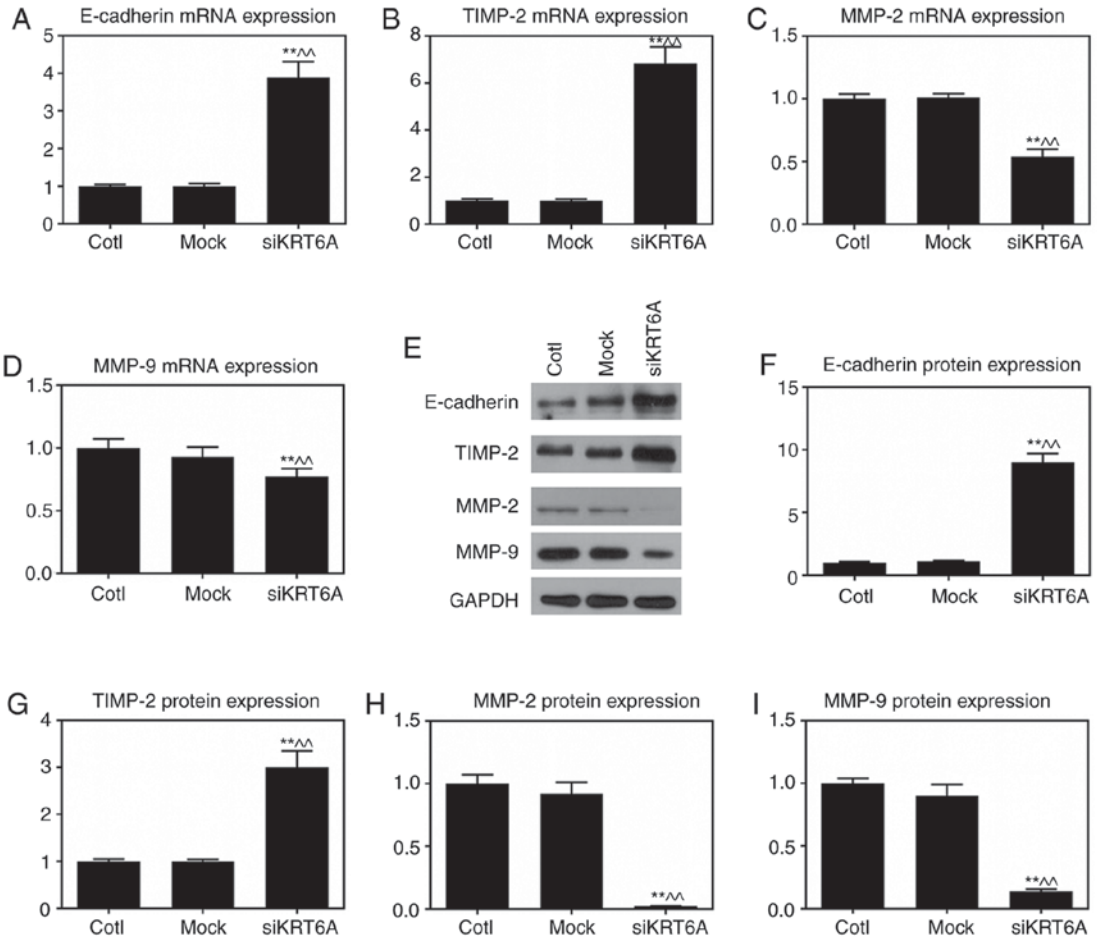

Figure 4. KRT6A silencing attenuates epithelial mesenchymal transition in NPC cells. (D) The mRNA expression levels of (A) E-cadherin and (B) TIMP-2 were significantly promoted in the siKRT6A group, while those of (C) MMP-2 and (D) MMP-9 were markedly reduced. (E) The protein levels of (F) E-cadherin and (G) TIMP-2 were significantly promoted in siKRT6A group, while those of (H) MMP-2 and (I) MMP-9 were markedly reduced. **P<0.01 vs. the Cotl group and $^{\wedge\wedge}$P<0.01 vs. the mock group. MMP, matrix metalloproteinase; E, epithelial; TIMP2, tissue inhibitor of metalloproteinase-2; NPC, nasopharyngeal carcinoma; si, small interfering; KRT6A, keratin 6A; Cotl, nontreatment group.

Discussion

NPC is widely regarded as an undifferentiated squamous cell carcinoma that is characterized by distant metastasis (17,33). KRT6A is a biomarker unique to squamous cells and the correct expression of keratin genes is the basis for maintaining

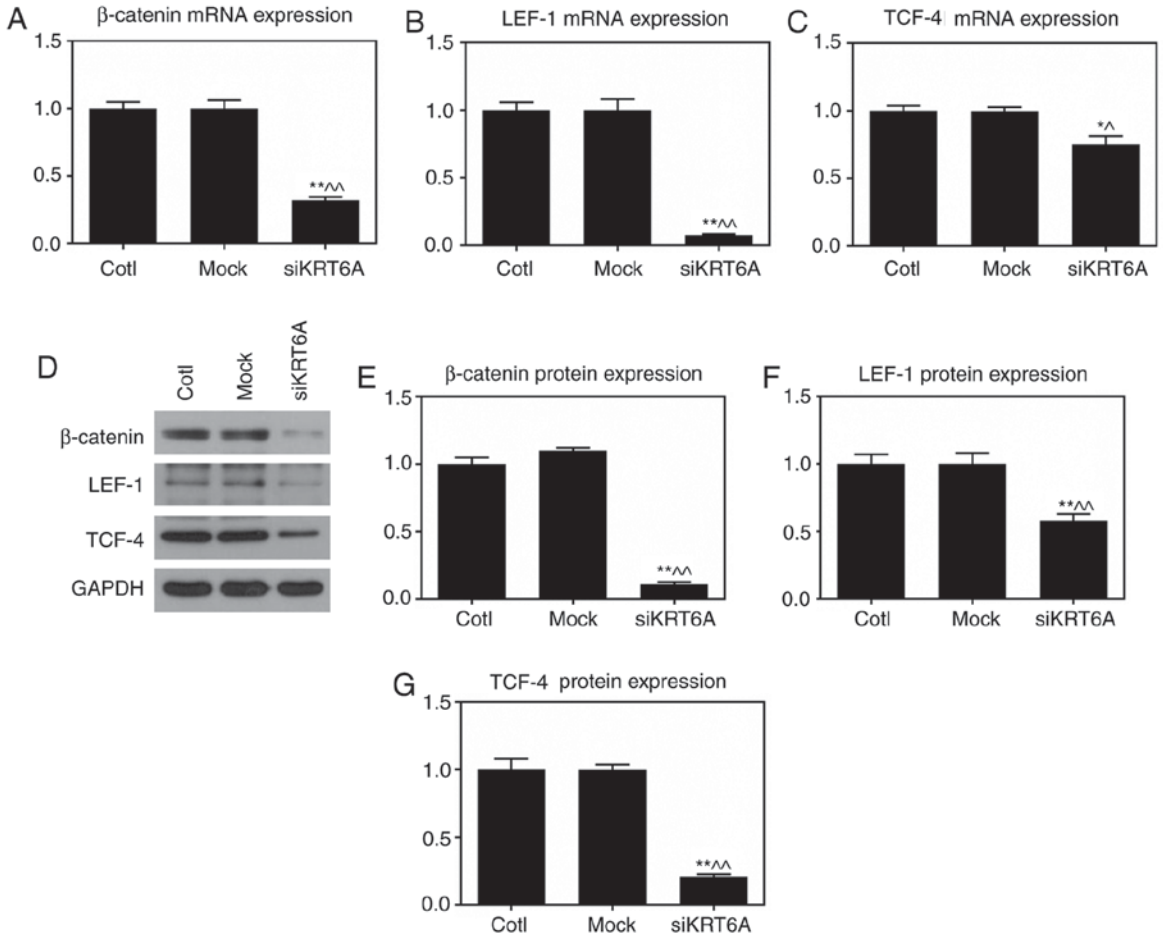

Figure 5. KRT6A silencing inhibits the Wnt/β-catenin pathway in NPC cells. The mRNA expression levels of (A) β-catenin, (B) LEF-1 and (C) TCF-4 were significantly reduced in the siKRT6A group. (D) The protein levels of (E) β-catenin, (F) LEF-1 and (G) TCF-4 were significantly reduced in the siKRT6A group. **P<0.01 vs. the Cotl group and ^^P<0.01 vs. the mock group. TCF-4, T-cell specific factor 4; LEF-1, lymphoid enhancer binding factor 1; NPC, nasopharyngeal carcinoma; si, small interfering; KRT6A, keratin 6A; Cotl, nontreatment group.

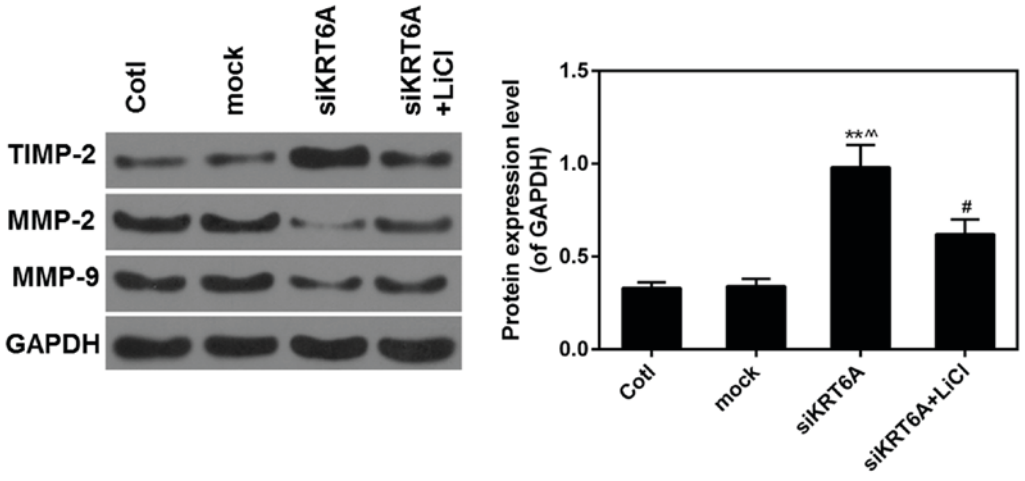

Figure 6. Wnt/β-catenin pathway on the effect of KRT6A silencing. The expression of TIMP-2, MMP-2 and MMP-9 was detected using western blotting. **P<0.01 vs. the Cotl group, ^^P<0.01 vs. the mock group and #P<0.05 vs. the siKRT6A group. MMP, matrix metalloproteinase; TIMP2, tissue inhibitor of metalloproteinase-2; Cotl, nontreatment group; si, small interfering; KRT6A, keratin 6A.

the stability, and normal differentiation of epidermal cells (28). Therefore, it is interesting to know whether KRT6A affects cell growth and differentiation as well as distant metastasis in NPC. Therefore, this study focused on investigating the mechanisms of ectopic KRT6A in NPC. The expression levels of KRT6A in multiple NPC cell lines, which include C666-1, 5-8F and SUNE-1 cells were first measured. It was demonstrated that the expression of KRT6A was high in SUNE-1.

This may be explained by the fact that SUNE-1 is a type of poorly differentiated carcinoma cell. The poorly differentiated carcinoma cells have a high malignancy rate and can invade into distant organs and tissues. Therefore, KRT6A silencing was conducted in SUNE-1 cells and the effects of KRT6A silencing on cell viability, metastasis, and invasion of SUNE-1 NPC cells were determined.

As the process of EMT is critical to tumor metastasis and it has been reported to be involved in the modification process of numerous biomarkers (34), the expression levels of certain critical factors were detected in the EMT process. Being responsible for cell-cell adhesion in epithelial cells, E-cadherin is one of the most critical hallmarks of EMT (35,36). Decrease of E-cadherin induces weakened epithelial characteristics and transition to the mesenchymal phenotype (37). In the present study, upregulated E-cadherin was tested when KRT6A was silenced in NPC cells. MMPs are the main proteolytic enzymes in the process of tumor metastasis, during which MMPs destroy the histological barrier for cell invasion (38-40). Type IV collagenases, including MMP-2 and MMP-9, are important in affecting tumor metastasis (10,41,42). The present study demonstrated that KRT6A silencing suppressed the expression of MMP-2 and MMP-9, as well as facilitated the expression of TIMP-2 in NPC cells.

The Wnt/β-catenin pathway is correlated with the EMT process in cancer. During the EMT process the stabilization and nuclear translocation of β-catenin is the critical events in Wnt/β-catenin pathway (43-46). LEF-1 and TCF-4, two key members of TCF family, mediate the regulation of β-catenin-dependent transcription factors (47-50). To investigate the effect of KRT6A on the canonical β-catenin/TCF pathway in NPC, alterations of the expression of β-catenin, LEF-1 and TCF-4 were identified when KRT6A was silenced in NPC cells. The results proved that KRT6A silencing inhibited the expression of β-catenin, LEF-1 and TCF-4 so as to inactivate β-catenin/TCF pathway. In addition, the activation of Wnt/β-catenin pathway, which was caused by LiCl, reversed the effect of KRT6A silencing by regulating the expression of TIMP2 and MMP2/9. These results suggested that KRT6A silencing may produce its anti-tumor effect largely by inhibiting the Wnt/β-catenin pathway.

The present study demonstrated the anti-tumor role of KRT6A silencing in NPC cells. However, it is equally significant to further investigate the effect of KRT6A through upregulating its expression, as well as to confirm the role of KRT6A in animal experiments.

In conclusion, KRT6A silencing suppressed cell viability, invasion and metastasis of NPC cells via β-catenin/TCF pathway. Therefore, KRT6A may be a novel biomarker in the diagnosis and treatment of NPC. Further research *in vivo* is required in order to provide more evidence that illustrates the role of KRT6A.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CC performed all the experiments. HS contributed to the conception of the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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