Amyloid β precursor protein silencing attenuates epithelial-mesenchymal transition of nasopharyngeal carcinoma cells via inhibition of the MAPK pathway

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Abstract. Advances in the treatment of nasopharyngeal carcinoma (NPC) have significantly improved the local control rate; however, distant metastasis remains a principal cause of mortality. Previous studies have demonstrated that the expression levels of amyloid β precursor protein (APP) are increased in NPC. The present study aimed to investigate the association between APP and the development of NPC. In order to knockdown APP expression, an APP-small interfering RNA vector was synthesized and transfected into SUNE-1 cells. Cell Counting Kit-8 assay was performed to assess cell viability. The migratory and invasive abilities of SUNE-1 cells were examined by wound healing and Transwell assays, respectively. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to measure the mRNA and protein expression levels of APP, and additional factors involved in epithelial-mesenchymal transition (EMT) and in the mitogen-activated protein kinase (MAPK) signaling pathway. APP silencing significantly suppressed cell viability, migration and invasion. In addition, APP interference downregulated the expression levels of metastasis-associated 1, matrix metalloproteinase (MMP)-2 and MMP-9; however, knockdown of APP led to upregulation of tissue inhibitor of metalloproteinases 2 and inhibited EMT. The phosphorylation levels of p38, extracellular signal-regulated kinases 1/2 and c-Jun N-terminal kinases 1/2 were decreased following downregulation of APP. The present results suggested that APP knockdown may significantly inhibit the development of NPC by suppressing cell viability, migration and invasion, and by inhibiting the EMT process via downregulation of the MAPK signaling pathway. Therefore, APP may facilitate the development of a novel gene therapy for the treatment of NPC.

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Introduction

The incidence of nasopharyngeal carcinoma (NPC) is particularly high in Southern China, and radiotherapy and chemotherapy are effective therapeutic strategies for its treatment (1). Recent advances in the treatment of NPC have led to an improvement in the local control rate; however, the distant metastasis rate of NPC remains high (2,3). Notably, >60% of unsuccessful treatments are due to distant metastasis (4,5). Therefore, numerous studies have aimed to investigate the mechanism of distant metastasis, in order to improve the treatment of locally advanced NPC. Epithelial-mesenchymal transition (EMT) is one of the processes involved in distant metastasis of tumor cells, which may promote uncontrolled growth, migration and invasion of epithelial cancer cells (6-8). Various extracellular signaling molecules promote EMT, including transforming growth factor-β (TGF-β), fibroblast growth factor and epidermal growth factor, via a series of cascade reactions (9-11). The mitogen-activated protein kinase (MAPK) signaling pathway is a signal transduction pathway that is able to induce transcriptional alterations downstream to the aforementioned extracellular signals (12). Additionally, hyperactivation of the MAPK signaling pathway may induce metastasis in human breast cancer (13).

Amyloid β precursor protein (APP) is a key gene involved in Alzheimer's disease (14,15). A previous study identified the upregulation of APP expression in various types of tumor cells (16). Proteomic profiling of TGF-β1-treated NPC CNE-2 cells identified an increase in the protein expression levels of APP (17). Bioinformatics analyses demonstrated that APP is differentially expressed in NPC tissues compared with healthy tissues, and it exhibits druggable domains (18). Hérard *et al* (19) reported that transfection of cells with a small interfering RNA (siRNA) targeting APP significantly decreases the protein expression levels of presynaptic APP and amyloid β precursor-like protein 2. Additionally, siRNA targeting APP decreases glucose metabolism in neurons in the superior colliculus (19). In order to further investigate

the mechanism underlying APP function, previous studies established an APP-siRNA plasmid vector, which laid the foundation for further studies examining the role of APP *in vitro* (20,21). APP silencing is able to effectively suppress the proliferation, migration and invasion of tumor cells (22). However, whether APP silencing may inhibit NPC development remains unclear.

In the present study, an APP-siRNA plasmid was designed and successfully transfected into NPC cells. The expression levels of EMT-associated factors were investigated in NPC cells, and the molecular effects of APP silencing on NPC cells were investigated.

Materials and methods

Cell culture. Human NPC cell lines C666-1, 6-10B, HNE3 and SUNE-1, and the normal nasopharyngeal-derived epithelial cell line NP69, were purchased from Xiangya Medical College Cell Bank (Changsha, China). Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/F-12; cat. no. 11320082; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.). All cells were maintained at 37˚C in a humidified incubator containing 5% CO_2.

Experimental grouping. Cells were randomly divided into the following three groups: i) Untransfected SUNE-1 cells (control group), ii) SUNE-1 cells transfected with empty vector (empty vector group) and SUNE-1 cells transfected with APP-siRNA vector (APP-siRNA group).

Cell transfection. The pSUPER vector (Oligoengine, Seattle, WA, USA) containing APP-siRNA (siRNA sequence: 5'-GAUCCAUCAGGGACCAAAACC-3') was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). SUNE-1 cells were seeded in 6-well plates ($3x10^6$ cells/well) and transfected with APP-siRNA (100 nM) and empty vector (100 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The mixture containing APP-siRNA/empty vector, SUNE-1 cells and transfection reagents was incubated at 37˚C for 6 h, and then the whole mixture was transferred to DMEM/F12 medium supplemented with 10% FBS. After 48 h, the cells were harvested and the transfection efficiency of APP-siRNA was examined.

Cell viability. Cell Counting Kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to measure cell viability. Cells were seeded in 96-well plates at a density of $1x10^3$ cells/well. The cells were cultured in a humidified incubator and were subsequently harvested at 12, 24 and 48 h. Subsequently, CCK-8 solution (10 μl) was added to the culture medium and incubated at 37˚C for 2 h. Optical density (OD) was measured at 450 nm using a microplate reader (Cany Precision Instruments Co., Ltd., Shanghai, China). The blank control group comprised culture medium and CCK-8 solution without cells. The relative cell viability was measured as follows: Cell viability=$(OD_{transfected\ cells}-OD_{blank})/(OD_{control}-OD_{blank})$.

Wound healing assay. Migration of SUNE-1 cells was quantified using a wound healing assay. Cells ($1x10^6$ cells/well) were seeded in 6-well plates with complete medium. The cells were starved in serum-free complete medium for 6-8 h, and a 200-μl pipette tip was used to create a straight wound in the cell monolayers. Cell migration was assessed by measuring the relative size of the wounds at 12 and 24 h compared with at 0 h post-wounding using a light microscope (Nikon Corporation, Tokyo, Japan).

Transwell assay. Transwell chambers were used to assess cell invasion. Briefly, $1x10^5$ cells were resuspended in serum-free medium and plated into the upper chamber containing polycarbonate membranes (pore size, 8 μm; Corning Inc., Corning, NY, USA) coated with Matrigel® (BD Biosciences, San Jose, CA, USA). DMEM/F12 containing 10% FBS was added to the lower chamber and cells were incubated at 37˚C for 24 h. Cells remaining on the surface of the upper chamber were removed with a cotton swab. The invading cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and stained with 0.1% crystal violet solution for 20 min at room temperature. To assess cell invasion, three randomly selected fields of view were observed under a light microscope (magnification, x200). The invasion rate was calculated by counting the number of invading cells at 0 and 24 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from SUNE-1 cells ($2x10^4$ cells/well in 6-well plates) using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Extracted RNA was treated with RNase-free DNase (Takara Biotechnology Co., Ltd., Dalian, China). Prime Script first strand cDNA synthesis kit (Takara Biotechnology Co., Ltd.) was used to reverse transcribe the RNA to cDNA, as previously described (23). Briefly, total RNA (2 μg) was used as template, and the RT reaction was performed at 65˚C for 5 min, followed by incubation at 30˚C for 6 min and 50˚C for 60 min. Primer sequences are listed in Table I. The ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for examining the expression levels of EMT- and MAPK-associated genes in SUNE-1 cells using a SYBR Green master mix (Fermentas; Thermo Fisher Scientific, Inc.). The RT-qPCR reaction mixture (20 μl) contained 1 μl forward and 1 μl reverse primers (concentration, 10 μM), 10 μl SYBR Green master mix, 2 μl cDNA and RNase-free dH_2O. RT-qPCR was performed using the following thermocycling conditions: Initial denaturation at 94˚C for 3 min, followed by 40 cycles at 94˚C for 30 sec, 56˚C for 30 sec and 72˚C for 2 min, with a final extension at 72˚C for 10 min. GAPDH was used as the internal control. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (24).

Western blotting. Total protein was extracted from SUNE-1 cells ($2x10^4$ cells/well in 6-well plates) using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.), and the protein concentration was determined using a bicinchoninic acid protein assay kit (Takara Biotechnology Co., Ltd.). Proteins (30 μg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene symbol	Primer sequence
APP	F: 5'-TGGCCCTGGAGAACTACATC-3'
	R: 5'-AATCACACGGAGGTGTGTCA-3'
MTA-1	F: 5'-AGCTACGAGCAGCACAACGGGGT-3'
	R: 5'-CACGCTTGGTTTCCGAGGAT-3'
TIMP-2	F: 5'-CCAAAGCAGTGAGCGAGAA-3'
	R: 5'-CATCCAGAGGCACTCATCC-3
MMP-2	F: 5'-GGAGGCACGATTGGTCTG-3'
	R: 5'-TTGGTTTCCGCATGGTCT-3
MMP-9	F: 5'-TTGACAGCGACAAGAAGTGG-3'
	R: 5'-GGCACAGTAGTGGCCGTAG-3
GAPDH	F: 5'-ACCACAGTCCATGAAATCAC-3'
	R: 5'-AGGTTTCTCCAGGCGGCATG-3

APP, amyloid β precursor protein; F, forward; MMP, matrix metalloproteinase; MTA-1, metastasis-associated 1; R, reverse; TIMP-2, tissue inhibitor of metalloproteinases 2.

(Thermo Fisher Scientific, Inc.). Subsequently, the PVDF membranes were placed in blocking buffer (1X TBS with 0.1% Tween-20 and 5% non-fat dry milk) for 1 h at room temperature. All primary antibodies were diluted to 1:1,000 with blocking buffer, and the membranes were incubated with these primary antibodies at 4˚C overnight. All primary and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primary antibodies used were as follows: Anti-GAPDH (cat. no. 5174), anti-APP (cat. no. 2452), anti-metastasis-associated 1 (MTA-1; cat. no. 5646), anti-tissue inhibitor of metalloproteinases 2 (TIMP-2; cat. no. 5738), anti-matrix metalloproteinase (MMP)-2 (cat. no. 4022), anti-MMP-9 (cat. no. 3852), anti-phosphorylated (p)-p38 (cat. no. 9211), anti-p38 (cat. no. 9212), anti-p-extracellular signal-regulated kinases 1/2 (ERK1/2; cat. no. 9102), anti-ERK1/2 (cat. no. 9101), anti-p-c-Jun N-terminal kinases 1/2 (JNK; cat. no. 9251) and anti-JNK (cat. no. 9252). Following incubation with primary antibodies, the PVDF membranes were incubated with the appropriate secondary antibody for 2 h at room temperature. The secondary antibodies used were as follows: Anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody (cat. no. 7074; 1:2,000). The signals were detected using Pierce ECL Western Blotting Substrate (cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc.). Optical band density was semi-quantified using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the internal control.

Statistical analysis. Data are presented as the means ± standard error of the mean. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

APP-siRNA inhibits viability of SUNE-1 cells. The expression levels of APP were significantly increased in NPC cells compared with in NP69 cells (Fig. 1A and B). The mRNA expression levels of APP were highest in SUNE-1 cells among all of the NPC cell lines tested; therefore, SUNE-1 cells were selected for further experiments. Post-transfection with APP-siRNA, the mRNA and protein expression levels of APP were assessed by RT-qPCR and western blotting, respectively (Fig. 1C and D). The results revealed that the expression levels of APP were efficiently decreased following APP-siRNA transfection compared with in the control groups. Cell viability was assessed using the CCK-8 assay, and the results suggested that APP-siRNA significantly inhibited cell viability at 48 h; in addition, a slight effect was observed at 24 h (Fig. 1E).

APP-siRNA suppresses migration and invasion of SUNE-1 cells. Wound healing and Transwell assays were used to investigate the effects of APP-siRNA on migration and invasion of SUNE-1 cells, respectively. The present data suggested that APP-siRNA was able to decrease the migratory and invasive abilities of SUNE-1 cells compared with in the control groups (Fig. 2A and B). Collectively, the present results indicated that APP may be involved in migration and invasion of SUNE-1 cells.

APP-siRNA suppresses EMT in SUNE-1 cells. EMT serves an important role in the invasion and metastasis of tumor cells (25). To investigate the effects of APP-siRNA on EMT in SUNE-1 cells, the expression levels of MTA-1, TIMP-2, MMP-2 and -9 were assessed using RT-qPCR (Fig. 3A-D) and western blotting (Fig. 3E). Compared with the control groups, APP knockdown led to a significant decrease in the expression levels of MTA-1, MMP-2 and -9; however, APP silencing increased the mRNA and protein expression levels of TIMP-2. The present results suggested that APP-siRNA may inhibit EMT by regulating the expression levels of associated factors.

APP-siRNA suppresses activation of the MAPK signaling pathway. The present study investigated the effects of APP-siRNA on MAPK pathway. The protein expression levels of ERK1/2, p38 and JNK1/2, and their corresponding phosphorylated forms, were measured by western blotting (Fig. 4A). The present results suggested that, compared with the control groups, APP silencing did not affect the protein expression levels of total ERK1/2, p38 and JNK1/2; however, it did significantly decrease their phosphorylation levels (Fig. 4B-D). Collectively, the present results suggested that APP-siRNA was able to decrease the phosphorylation of MAPK signaling factors.

Discussion

Previous studies have demonstrated that APP is a membrane-bound protein present in multiple cell types; notably, the expression levels of APP are increased in various types of cancer cells (16,26). A previous study reported that the proliferation, migration and invasion of CNE-2 cells treated with anti-APP antibody are significantly decreased compared

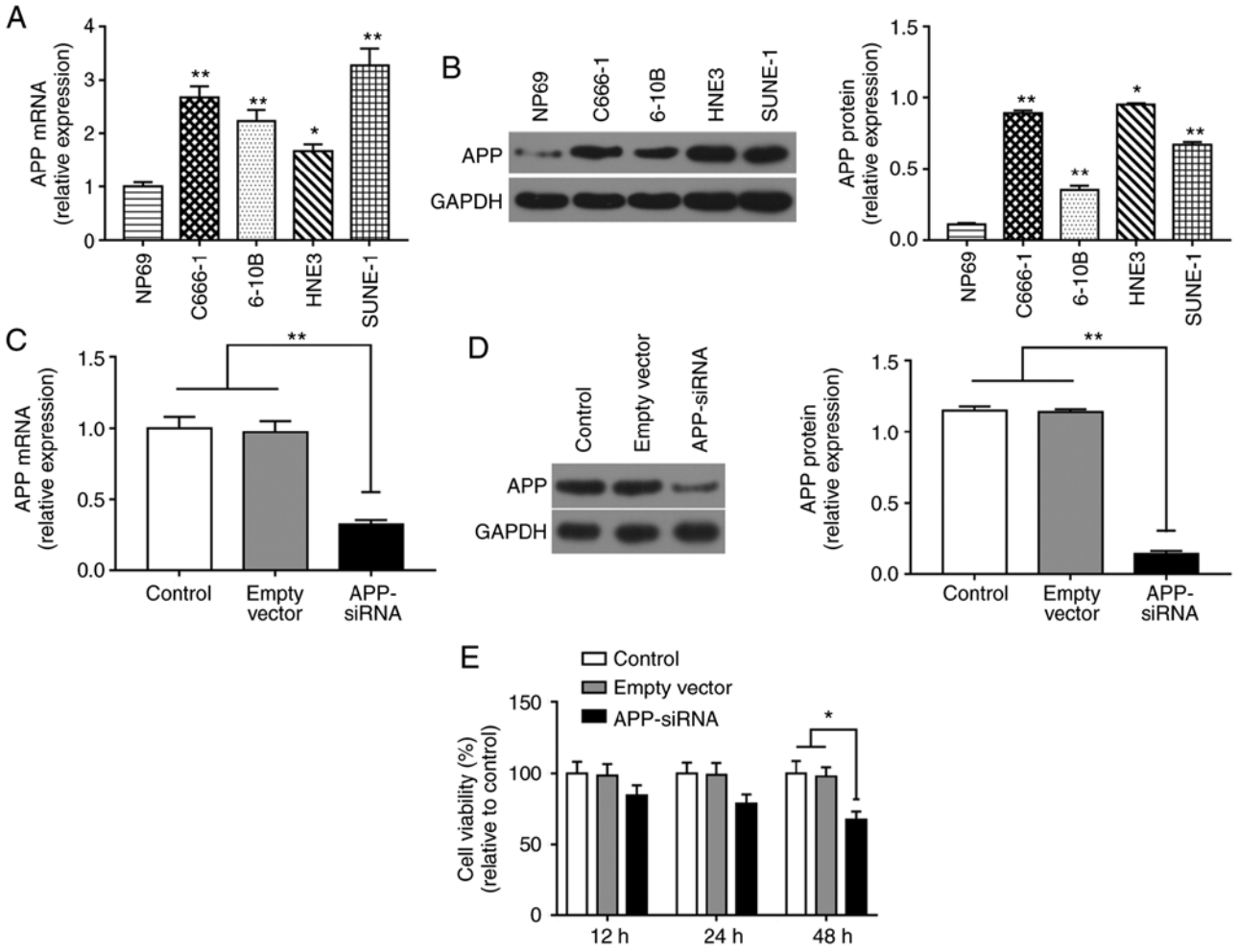

Figure 1. APP-siRNA inhibits the viability of SUNE-1 cells. (A) mRNA expression levels of APP in the four NPC cell lines C666-1, 6-10B, HNE-3 and SUNE-1, and in the normal nasopharyngeal-derived epithelial cell line NP69, as assessed by RT-qPCR. (B) Protein expression levels of APP in NP69, C666-1, 6-10B, HNE-3 and SUNE-1 cells, as assessed by western blotting. *P<0.05, **P<0.01 vs. NP69 cells. (C) mRNA expression levels of APP following APP knockdown, as assessed by RT-qPCR. (D) Protein expression levels of APP following APP knockdown, as assessed by western blotting. (E) Cell Counting Kit-8 analysis was performed to investigate cell viability following APP knockdown at 12, 24 and 48 h. *P<0.05, **P<0.01. GAPDH was used as the internal control for western blotting. Data are presented as the means ± standard error of the mean (n=3). APP, amyloid β precursor protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

with in a control group (27). In addition, a previous study compared 10 control and 31 NPC cell lines, and observed that APP is differentially expressed in NPC cells, thus suggesting an association between APP and the occurrence and development of NPC (18). In the present study, the mRNA and protein expression levels of APP were significantly upregulated in NPC cells. The discrepancy between mRNA and protein expression levels in various cell lines may be due to the post-transcriptional regulation of APP, which remains unclear (28). In the present study, the protein and mRNA expression levels of APP were increased in SUNE-1 cells compared with in normal NP69 cells. Therefore, SUNE-1 cells were selected for further experiments. In the present study, the effects of APP-siRNA on cell viability, migration and invasion were investigated. APP knockdown was able to significantly inhibit the viability, migration and invasion of SUNE-1 cells. Furthermore, knockdown of APP increased the expression levels of TIMP-2; however, APP-siRNA decreased the mRNA expression levels of MTA-1, MMP-2 and -9. Additionally, APP knockdown significantly decreased the phosphorylation levels of MAPK-associated factors. Collectively, the present data suggested that APP-siRNA may suppress the occurrence, development and metastasis of human NPC.

EMT is a necessary process underlying distant metastasis of tumor cells (29). The present findings suggested an association between APP and EMT in SUNE-1 cells. In the present study, the protein expression levels of factors involved in EMT were decreased in the APP-siRNA group compared with in the control group. The EMT process involves various proteins. β-catenin interacts with E-cadherin to form complexes that promote intercellular adhesion. β-catenin is able to enter the nucleus and to induce the expression of T-cell-factor/lymphoid enhancer binding factor 1, thus increasing the protein expression levels of vimentin and MMPs, promoting EMT (30,31). Previous studies have demonstrated that TIMP-2 inhibits the activity of MMP-2 and -9, thus decreasing degradation of the extracellular matrix (ECM), and inhibiting tumor invasion and metastasis (32,33). In addition, MTA-1 has been identified to be associated with tumorigenesis, tumor invasion and metastasis (34-36). The present results suggested that APP-siRNA

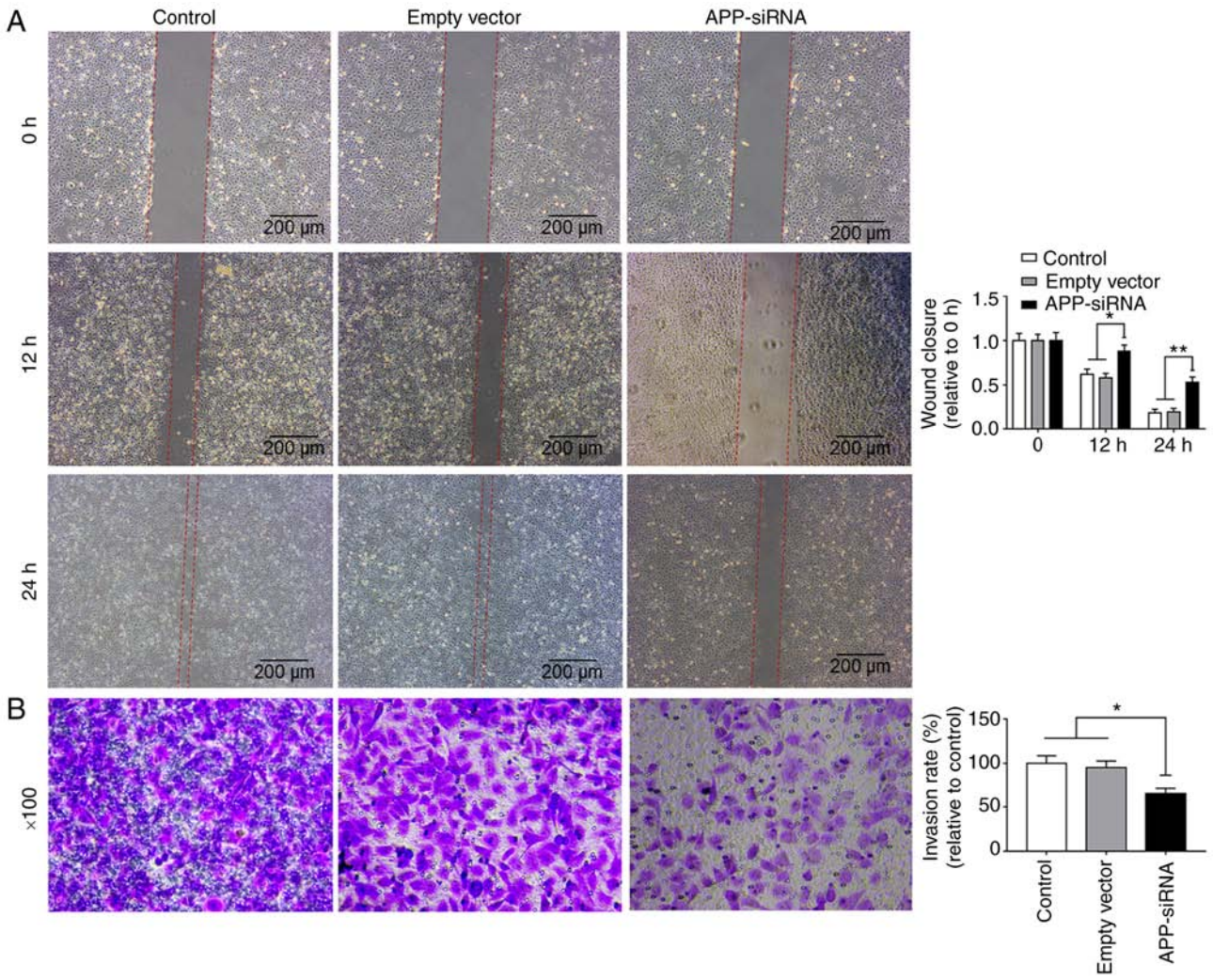

Figure 2. APP-siRNA suppresses the migration and invasion of SUNE-1 cells. (A) Cell migration in control, empty vector and APP-siRNA groups at 0, 12 and 24 h following wounding. (B) Effects of APP silencing on cell invasion was measured using Transwell assay. Data are presented as the means ± standard error of the mean (n=3). *P<0.05, **P<0.01. APP, amyloid β precursor protein; siRNA, small interfering RNA.

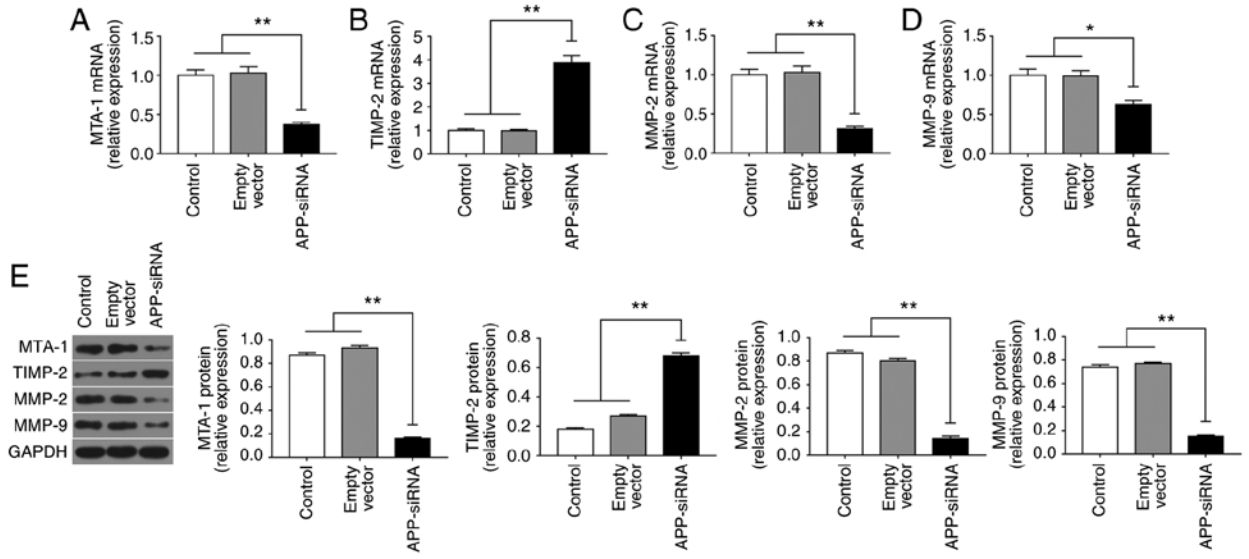

Figure 3. APP-siRNA suppresses the expression of MTA-1 and EMT-associated markers in SUNE-1 cells. mRNA expression levels of (A) MTA-1, (B) TIMP-2, (C) MMP-2 and (D) MMP-9 were assessed using reverse transcription-quantitative polymerase chain reaction. (E) Protein expression levels of EMT-associated factors were assessed using western blotting in control, empty vector and APP-siRNA groups. GAPDH was used as the internal control for western blotting. Data are presented as the means ± standard error of the mean (n=3). *P<0.05, **P<0.01. APP, amyloid β precursor protein; EMT, epithelial-mesenchymal transition; MMP, matrix metalloproteinase; MTA-1, metastasis-associated 1; siRNA, small interfering RNA; TIMP-2, tissue inhibitor of metalloproteinases 2.

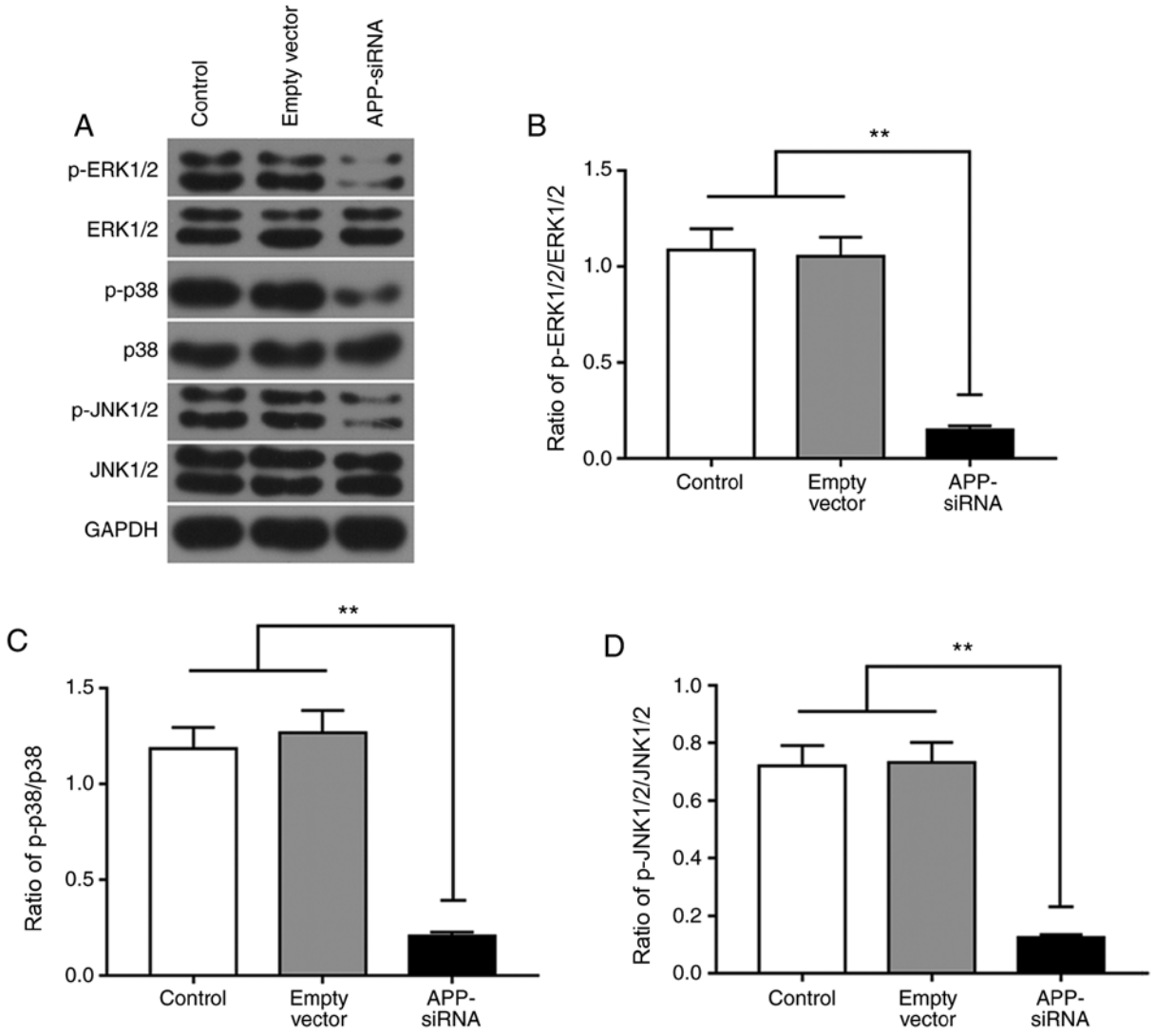

Figure 4. APP-siRNA suppresses the activation of the MAPK signaling pathway. Protein expression levels of (A) ERK1/2, p38 and JNK1/2, and their corresponding phosphorylated forms, were detected by western blotting in SUNE-1 cells. The ratio of (B) p-ERK1/2/ERK1/2, (C) p-p38/p38 and (D) p-JNK1/2/JNK1/2 protein expression levels. GAPDH was used as the internal control for western blotting. Data are presented as the means ± standard error of the mean (n=3). **P<0.01. APP, amyloid β precursor protein; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; p-, phosphorylated; siRNA, small interfering RNA.

was able to increase the expression level of TIMP-2, and to decrease the expression levels of MMP-2 and -9. These findings suggested that the balance between TIMP-2 and MMPs may be gradually restored, which may facilitate recovery of the dynamic equilibrium in ECM and decrease migration and invasion of SUNE-1 cells. In addition, the expression levels of MTA-1 were decreased following APP-siRNA transfection, indicating that APP-siRNA inhibited the metastasis of SUNE-1 cells by downregulating the expression of MTA-1.

The present results suggested that APP-siRNA was able to simultaneously suppress multiple factors involved in EMT, thus regulating tumor cell migration and invasion. Additionally, the mechanism underlying this effect may involve an upstream signaling pathway. A previous study observed that overexpression of aurora kinase A promotes hyperactivation of the MAPK signaling pathway, thus inducing EMT and invasion of NPC cells (37). The present results suggested that APP exhibited a similar effect, and may increase EMT and the activity of the MAPK signaling pathway. Previous studies have demonstrated that activation of p38 and JNK is involved in EMT in response to advanced glycation end products. Additionally, EMT may be induced by activated ERK signaling in renal tubular cell lines and mouse mammary gland epithelial cells *in vitro* (38-40). The present results suggested that APP knockdown decreased the protein expression levels of p-ERK1/2, p-p38 and p-JNK1/2, thus decreasing the activity of the MAPK signaling pathway. A large number of studies have demonstrated that p-ERK1/2 (41-43), p-JNK (44-46) and p-p38 (40,47,48) serve an important role in regulating EMT. Taken together, downregulation of the protein expression levels of p-ERK1/2, p-p38 and p-JNK1/2 in the APP-siRNA group suggested that APP silencing may inhibit the EMT process by suppressing the MAPK signaling pathway in SUNE-1 cells. However, the present study was performed using only one cell line, and further studies are required to confirm the role of APP in multiple NPC cells and *in vivo*.

In conclusion, the present results suggested that APP knockdown decreased the viability, migration and invasion of

SUNE-1 cells. APP silencing increased the expression levels of TIMP-2; however, it decreased the expression levels of MTA-1, MMP-2 and -9, thus suggesting that EMT was inhibited in SUNE-1 cells. Notably, APP silencing may suppress cell migration, invasion and EMT by inhibiting the MAPK signaling pathway. Therefore, APP may be considered a novel biomarker for NPC surveillance, and as a therapeutic target to treat patients with NPC. The present findings may improve the understanding of NPC and may facilitate the development of a novel gene therapy for the treatment of NPC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JX made substantial contributions to the conception and design of the study. YYi, GX, LL, QW and YYa were involved in data acquisition, analysis and interpretation. All authors were involved in drafting or critically revising the manuscript, and approved of the final version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

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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