

Maspin suppresses growth, proliferation and invasion in cutaneous squamous cell carcinoma cells

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Abstract. Cutaneous squamous cell carcinoma (cSCC) is a common malignant tumor. Mammary serine protease inhibitor (Maspin), a member of serpin family, has been reported as a tumor suppressor in various carcinomas. In this study, we detected the expression level of Maspin in cSCC tissues by real-time PCR and western blotting, and found that Maspin was downregulated in the cSCC tissues compared with the adjacent normal tissues. Moreover, Maspin was stably overexpressed in A431 cells, and CCK-8 assay, colony formation assay, Transwell assay, Hoechst staining and western blotting were carried out to detect the growth, proliferation, invasion, cell cycle and apoptosis of A431 cells. The results revealed that overexpression of Maspin inhibited growth, proliferation, invasion and cell cycle G1/S/G2 transition and enhanced apoptosis of A431 cells. The pro-apoptotic protein cleaved caspase-3, poly(ADP-ribose) polymerase (PARP) and Bax increased, and the anti-apoptotic protein Bcl-2 decreased after Maspin overexpression. Therefore, we demonstrated that Maspin suppressed growth, proliferation and invasion by delaying cell cycle transition and promoting apoptosis in cSCC cells, which may provide new insights for the clinical diagnosis and therapy of cSCC.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common cutaneous malignant tumor, following cutaneous basal cell carcinoma (cBCC) (1,2). Pathogenesis of cSCC is related with sun exposure, genodermatosis and immunosuppression (3,4). Compared with cBCC, cSCC shows more potential to metastasize to lymph nodes or distant organs (5),

and 5-year survival rate of the patients with local metastasis is <30% (6,7). Currently, the main treatment is surgery, supplemented with radiotherapy and/or chemotherapy, however, this may leave serious sequelae (8). Therefore, it is of important clinical significance to study the pathogenesis of cSCC and to develop more effective diagnoses and treatments.

Mammary serine protease inhibitor (Maspin) belongs to serpin family, and its encoding gene is located in 18q21 in *Homo sapiens* (9). Since the subtle differences between Maspin and other members of serpin family in secondary structure, there is no protease inhibited by Maspin via the classical serpin pathway to date (10-13). Initially Maspin is expressed in human mammary epithelial tissue and breast carcinoma tissue with an antitumor function (14). Subsequently, a large number of studies reported that Maspin was expressed in multiple normal tissues, including breast, prostate, placenta and epidermal keratinocytes, while downregulated or could not be detected in various cancers. Maspin inhibits epidermal growth factor (EGF)-induced epithelial-mesenchymal transition (EMT) of esophageal carcinoma cells, suppresses proliferation in soft agar, migration and invasion, even induces transition of tumor cells into benign cells (15). Maspin inhibits vasculogenic mimicry in non-small cell lung cancer (NSCLC) cells, and its expression level is positively correlated with prognostic implication in NSCLC patients (16). Maspin promotes tumor cells apoptosis in breast and prostate cancer (17,18). Maspin suppresses prostate tumor growth, invasion and metastasis by inhibiting histone deacetylation, and activates host neutrophil- and B cell-dependent antitumor immune response (19). Therefore, Maspin could be an effective marker for diagnosis and therapy of cancers. Recently, some reports found that Maspin is expressed in detected normal skin tissue, but partly expressed in cSCC and cBCC (20,21), and the expression level of Maspin is positively correlated with prognosis of cSCC patients (16), suggesting that Maspin may participate in cSCC progress. However, the role of Maspin in cSCC carcinogenesis and development is not clear.

In this study, we examined the expression level of Maspin in cSCC tissue, and found that it was obviously downregulated compared with normal cutaneous tissues. Subsequently, by establishing Maspin stably expressed cell line, we demonstrated that Maspin inhibited growth, proliferation, invasion by delaying cell cycle transition and enhancing apoptosis in

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cSCC cells. These findings may provide new insights into carcinogenesis, diagnosis and therapy of cSCC.

Materials and methods

Human tissue specimens. Seventeen pairs of clinical cSCC samples were collected from cSCC patients operated from September 2012 to April 2015 in Department of Dermatology, The First Affiliated Hospital of China Medical University. Each sample pair contains a cSCC tissue, an adjacent tissue 0.5 cm from cSCC tissue and an adjacent tissue 1 cm from cSCC tissue. The patients had not been treated with radiotherapy or chemotherapy. Informed consent was obtained from the patients, and our collection and treatment procedures were in line with standards of the ethics committee of The First Affiliated Hospital of China Medical University.

RNA extraction, reverse transcription and real-time PCR. The total RNA was extracted by total RNA rapid extraction kit (BioTeke, Beijing, China) from tissues or cells, and detailed procedure referred to the manufacturer's protocol. The obtained RNA samples were reverse transcribed into cDNA by Super M-MLV reverse transcriptase (BioTeke), with the oligo(dT) and random primers. All instruments in this section were treated by RNase Eraseol (Tiandz, Beijing, China), and all reagents were RNase-free.

The cDNA was used to perform real-time PCR with 2X power Taq PCR MasterMix (BioTeke) and SYBR Green (Solarbio, Beijing, China) to test Maspin mRNA level, with β -actin as the internal control. The PCR procedure was set as follows: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec, and finally 4°C for 5 min. The data were analyzed by Exicycler™ 96 (Bioneer, Daejeon, Korea), calculated by $2^{-\Delta\Delta C_t}$ method (cells) or $2^{-\Delta C_t}$ method (tissues) (22). Sequences of real-time PCR primers (Sangon, Shanghai, China) are shown in Table I.

Western blotting. The total protein was extracted from cells or tissues with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China). After measuring the concentration, the protein sample was denatured by boiling, separated by SDS-PAGE (density of gel dependent on the protein size), and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). After blocking with 5% skim milk (YILI, Hohhot, Inner Mongolia, China) for 1 h, the PVDF membrane was incubated with the following antibodies at 4°C overnight: polyclonal rabbit anti-Maspin (Abcam, Cambridge, UK) (1:1,000), polyclonal rabbit anti-cleaved caspase-3 (Abcam) (1:1,000), monoclonal rabbit anti-cleaved poly(ADP-ribose) polymerase (PARP) (Abcam) (1:1,000), polyclonal rabbit anti-Bcl-2 (Boster, Wuhan, Hubei, China) (1:400), and polyclonal rabbit anti-Bax (Boster) (1:400). After rinsing with TBST, the PVDF membrane was incubated with goat anti-rabbit IgG-HRP (Beyotime) (1:5,000) at room temperature for 45 min, and exposed with ECL reagent (7 sea, Shanghai, China). After antibodies were removed by stripping buffer (Beyotime), the PVDF membrane was incubated with monoclonal mouse anti- β -actin (Santa Cruz, CA, USA) (1:1,000) and goat anti-mouse IgG-HRP (Beyotime) (1:5,000) to test the internal control, β -actin.

Plasmid construction. To construct Maspin overexpression plasmid, Maspin coding sequence (CDS) was obtained by PCR from human cDNA, and amplified by TA cloning using UltraPower pUM-T rapid cloning kit (BioTeke). After sequencing, the Maspin CDS fragment was inserted into pcDNA3.1 vector (*XhoI*+*HindIII*), and the overexpression plasmid pcDNA3.1-Maspin was gained. The sequences of PCR primers are shown in Table II.

Cell culture, transfection and monoclonal cell line. Three cSCC cell lines A431, SCL-1 and SCC12, were preserved in our laboratory. A431 cells and SCL-1 cells were cultured in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone) (SCC12 cells with 20% FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin (Beyotime) at 37°C incubator (Lishen, Tianjin, China) with 5% CO₂. The overexpression plasmid pcDNA3.1-Maspin and the control pcDNA3.1 were transfected into A431 cells by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) with serum-free medium, 24 h later, G418 (Invitrogen) was added into medium with 100 μ g/ml to screen the integrated cells, and the medium was refreshed every 2-3 days until the monoclonal cell lines were obtained.

CCK-8 assay. Cell counting kit-8 (CCK-8) assay was performed to test the cell viability. A431 cells were seeded into 96-well plates with 3×10^3 per pore beforehand. After cells adhered, CCK-8 reagent (Beyotime) was added into the 96-well plate with 10 μ l per pore to incubate for 1 h, and the optical density of the solution was tested at 450 nm with a microplate reader (BioTek, VT, USA). This OD₄₅₀ value was the datum at 0 h, and the data in 24 h, 48 h, 72 h and 96 h were detected later, respectively.

Colony formation assay. Colony formation assay was performed to detect the colony formation ability of A431 cells. A431 cells were seeded into a 35-mm petri dish at 400/well, and cultured in 5% CO₂ at 37°C, medium was refreshed every 3 days. Approximately 14 days later, most of the clones had formed. The cells were fixed with 4% paraformaldehyde (Sinopharm, Beijing, China) for 20 min, stained with Wrings-Giemsa stain (Jiancheng, Nanjing, Jiangsu, China) for 5-8 min, and the clone number was counted with an inverted phase contrast microscope (Motic, Xiamen, Fujian, China). The clones with ≥ 50 cells were considered positive.

In vitro Transwell assay. To measure the invasion potential of A431 cells, the Transwell assay in the presence of Matrigel (BD, Franklin Lakes, NJ, USA) was performed in Transwell chambers with 8- μ m aperture polycarbonate membrane (Corning, NY, USA). Beforehand 40 μ l of 2.5 mg/ml Matrigel was added into Transwell upper chamber to coat the polycarbonate membrane, and preheated at 37°C for 2 h to solidify. Then 200 μ m serum-free cell suspension (2×10^4 cells) were added into Transwell upper chamber, and 800 μ m DMEM with 20% FBS was added in the lower chamber. After culturing for 24 h, the Transwell chamber was fixed in 4% paraformaldehyde (Sinopharm) for 20 min, and stained by 0.5% crystal violet for 5 min. Then the cells in upper chamber were wiped off, and the cells on reverse side of polycarbonate membrane

Table I. The real-time PCR data in 17 pairs of clinical cutaneous squamous cell carcinoma samples.

Sample no.	2 ^{-ΔCt} mean	Sample no.	2 ^{-ΔCt} mean	Sample no.	2 ^{-ΔCt} mean
1A	0.000598	1B	0.000448	1C	0.000799
2A	0.001075	2B	0.000848	2C	0.001307
3A	0.000963	3B	0.000776	3C	0.001453
4A	0.002595	4B	0.002737	4C	0.004600
5A	0.000446	5B	0.000537	5C	0.000865
6A	0.001572	6B	0.001942	6C	0.002049
7A	0.002300	7B	0.002198	7C	0.002537
8A	0.002489	8B	0.004308	8C	0.006680
9A	0.000755	9B	0.000957	9C	0.001874
10A	0.000842	10B	0.001102	10C	0.001078
11A	0.003457	11B	0.003549	11C	0.009217
12A	0.001512	12B	0.001421	12C	0.002616
13A	0.000944	13B	0.001162	13C	0.003862
14A	0.001632	14B	0.002303	14C	0.006408
15A	0.000317	15B	0.000313	15C	0.000820
16A	0.000431	16B	0.001106	16C	0.001471
17A	0.000451	17B	0.000665	17C	0.001570

Each sample pair contains three samples, A represents the cutaneous squamous cell carcinoma (cSCC) tissue, B represents the adjacent tissue 0.5 cm from cSCC tissue, and C represents the adjacent tissue 1 cm from cSCC tissue. The mRNA of each tissue was tested in triplicate by real-time PCR, the data were analyzed by 2^{-ΔCt} method, with respective β-actin as the internal control. The mean of data in each tissue was calculated by Excel software.

Table II. Sequences of primers used in this study.

Name	Sequence (5'-3')
Maspin F	5'-TTGTGGTTAATGCTGCCTAC-3'
Maspin R	5'-CCAAGCCTGTGGACTCATC-3'
β-actin F	5'-CTTAGTTGCGTTACACCCTTTCTTG-3'
β-actin R	5'-CTGTCACCTTCACCGTTCCAGTTT-3'
Maspin-CDS F	5'-CTCAAAGCTTATGGATGCCCTGCAACTA-3'
Maspin-CDS R	5'-CGACCTCGAGCACTTAAGGAGAACAGAAAT-3'

Maspin, mammary serine protease inhibitor; F, forward; R, reverse; CDS, coding sequence.

were counted with an inverted phase contrast microscope (Motic) at x200 magnification.

Hoechst staining. In order to observe the nuclear morphology change, Hoechst staining was performed. A431 cells were seeded onto glass slides in 12-well plates with 1x10⁵/pore. When the confluence reached 80%, the cells were treated with Hoechst Staining kit (Beyotime), observed with a fluorescence microscope (Olympus, Tokyo, Japan) at x400 magnification.

Flow cytometry. Flow cytometry was performed to test apoptosis and cell cycle of A431 cells. A431 cells cultured in 6-well plates were collected when the confluence reached 90%. After rinsing with PBS (Double-helix, Shanghai, China) twice, the

cells were treated with Annexin V-FITC/PI cell apoptosis test kit (Wanleibio, Shenyang, Liaoning, China) and detected using FACSCalibur (BD) according to the manufacturer's protocol.

For the cell cycle analysis, the collected cells were treated by Cell Cycle Analysis kit (Beyotime), and detected using FACSCalibur (BD) according to the specification. Proliferation index (PI) = (percentage of cells in S phase + G2/M phase) / percentage of cells in G1 phase (23).

Statistical analysis. The data in this study are presented as mean ± standard deviation (SD) with three individual experiments, and analyzed by one-way ANOVA test or paired Student's t-test. It was considered statistically significant at P<0.05, P<0.01, or P<0.001; NS, not significant).

Results

Maspin is downregulated in cSCC tissues. First we detected the expression level of Maspin in 17 pairs of cSCC and adjacent tissues by real-time PCR and western blotting. As shown in Fig. 1, A, B and C groups represented the cSCC tissues, the adjacent tissues 0.5 cm from cSCC and the adjacent tissues 1 cm from cSCC, respectively. The results showed that the mRNA level of Maspin in cSCC tissues decreased by 55% (Fig. 1A and Table II), and the protein level decreased by 49% compared to the adjacent tissue 1 cm from cSCC tissues (Fig. 1B and C), respectively.

Establishment of Maspin stably expressed cell line. Because of the low expression level of Maspin in cSCC tissues, among

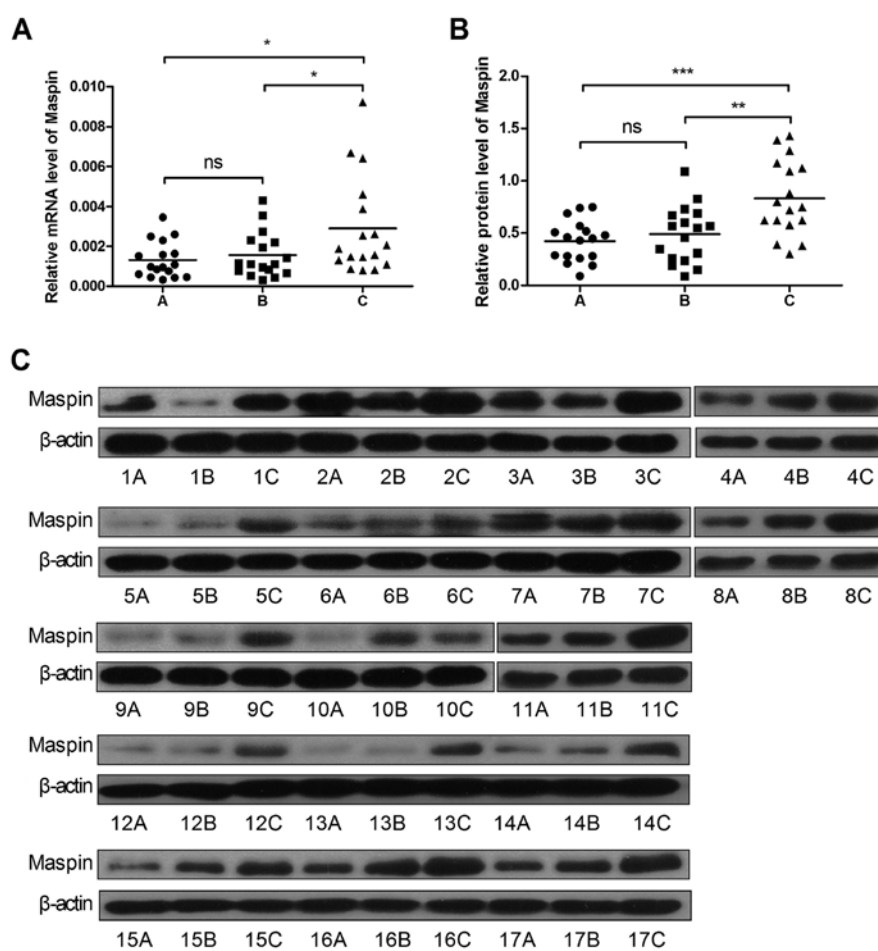


Figure 1. Maspin is downregulated in cSCC tissues. (A) The mRNA level of mammary serine protease inhibitor (Maspin) in cutaneous squamous cell carcinoma (cSCC) and adjacent tissues was detected by real-time PCR. (B) The protein level of Maspin in cSCC and adjacent tissues was detected by western blotting. (C) The protein bands in each tissue. A, cSCC tissues; B, adjacent tissues 0.5 cm from cSCC; C, adjacent tissues 1 cm from cSCC (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, not significant).

three cSCC cell lines SCL-1, SCC12 and A431, we selected A431 cells, in which the Maspin expression level was the lowest (Fig. 2A), to measure the effect of Maspin on cSCC cells. To stably express Maspin, the overexpression plasmid pcDNA3.1-Maspin was constructed, and transfected into A431 cells. By screening with G418, monoclonal cells of pcDNA3.1-Maspin were obtained, and the pcDNA3.1 vector monoclonal cells were gained as the control. Real-time PCR and western blotting were performed to verify the expression efficiency of pcDNA3.1-Maspin. The results showed that the mRNA level increased by 3.78-fold (Fig. 2B), and the protein level increased by 3.86-fold (Fig. 2C) in contrast to the pcDNA3.1 group. Hence Maspin overexpression cell line was used for the subsequent experiments.

Maspin inhibits growth, proliferation and invasion in cSCC cells. CCK-8 assay, colony formation assay and Transwell assay supplemented with Matrigel *in vitro* were performed to detect the cell viability, colony formation ability and invasion potential of A431 cells. The results showed that A431 cell viability decreased by 22%, 19%, 20% and 22% at 24 h, 48 h, 72 h and 96 h, respectively (Fig. 3A), the colony formation rate declined by 45% (Fig. 3B), and the invasion potential descended by 39% as a result of elevation of Maspin (Fig. 3C).

Collectively, the results demonstrated that Maspin inhibited growth, proliferation and invasion in A431 cells.

Maspin delays cell cycle transition and enhances apoptosis in cSCC cells. We confirmed that Maspin expression was low in cSCC tissues, and inhibited growth, proliferation and invasion in cSCC cells, hence we were interested in how Maspin affects the phenotype of cSCC cells. In order to explore the underlying mechanism, we tested whether Maspin affected cell cycle transition and apoptosis in cSCC cells. Flow cytometry results showed that Maspin overexpression delayed cell cycle G1/S/G2 transition (Fig. 4A), and proliferation index (PI) decreased by 60% in A431 cells (Fig. 4B).

To detect the effect of Maspin on apoptosis of A431 cells, Hoechst staining, flow cytometry and western blotting were performed. In the Hoechst staining results, Maspin overexpressed A431 cells showed more intense fluorescence than parental and pcDNA3.1 groups (Fig. 5A), which is characteristic of apoptotic cells. Flow cytometry results showed that Maspin increased cell apoptosis rate of A431 cells 7-fold (Fig. 5B and C). In addition, we detected several apoptosis related genes. As shown in Fig. 5, cleaved caspase-3 increased 2-fold, cleaved PARP was upregulated 2.5-fold, Bcl-2 descended by 48%, and Bax was elevated 1.6-fold (Fig. 5D).

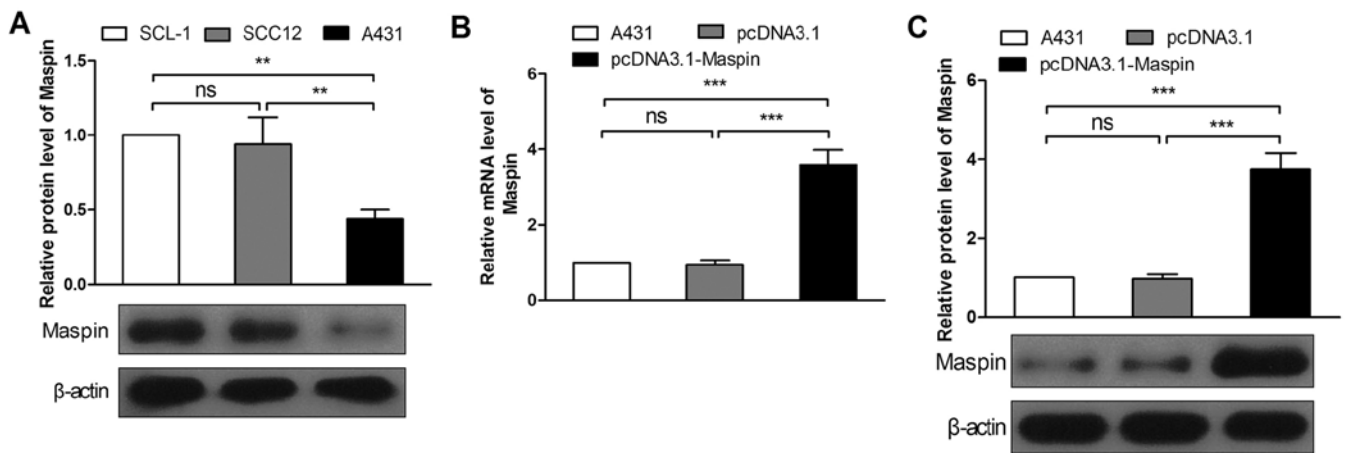


Figure 2. Establishment of Maspin stably expressed cell line. (A) The expression level of Maspin in SCL-1 cells, SCC12 cells and A431 cells detected by western blotting. (B and C) Real-time PCR and western blotting were used to confirm the expression efficiency of pcDNA3.1-Maspin in A431 cells (** $P < 0.01$, *** $P < 0.001$; NS, not significant).

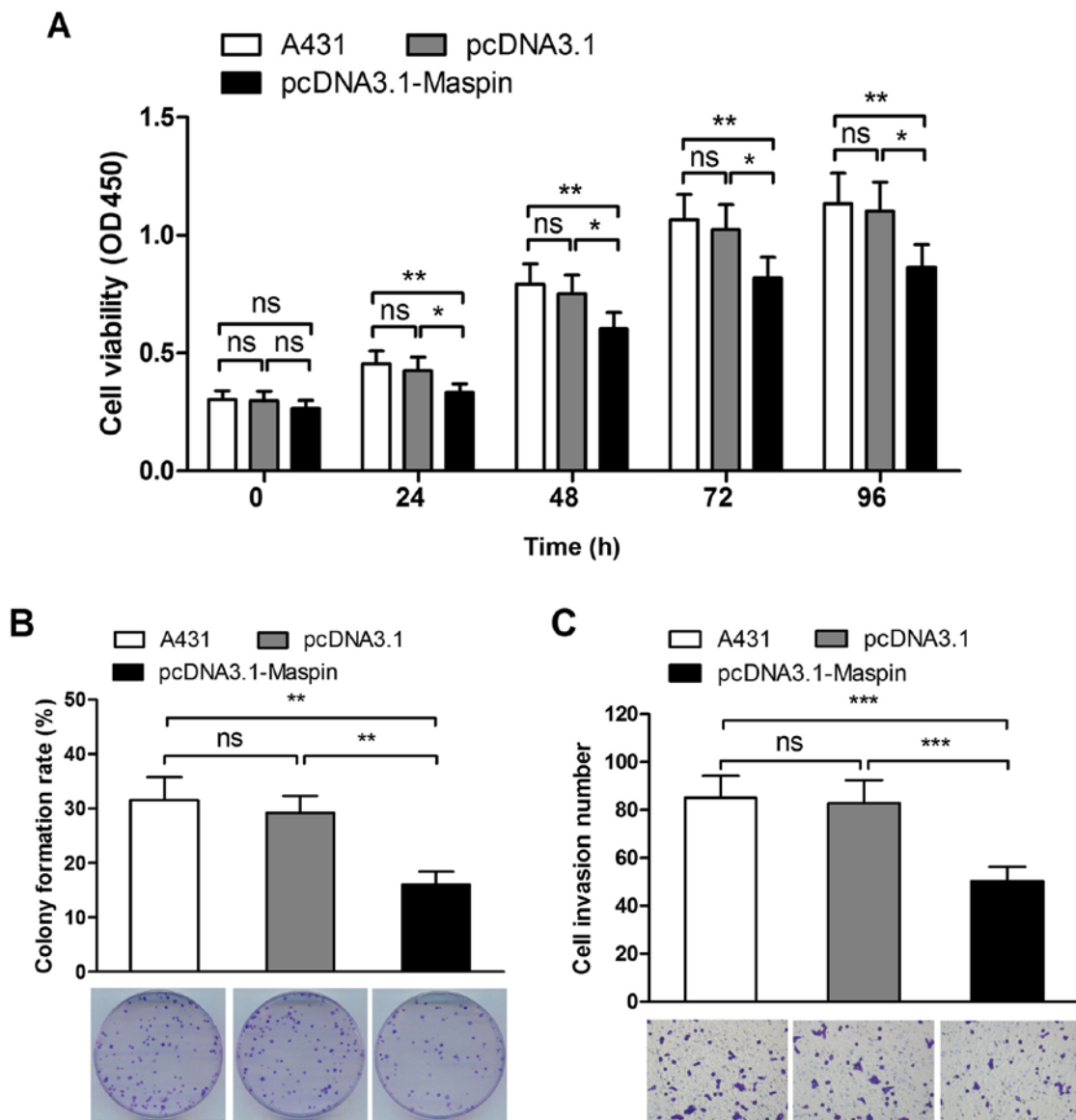


Figure 3. Maspin inhibits growth, proliferation and invasion in A431 cells. (A) Cell viability of A431 cells was detected by CCK-8 assay in different time-points. (B) Colony formation ability of A431 cells was tested by plate colony formation assay. (C) Invasion potential of A431 cells was measured by Transwell assay in the presence of Matrigel *in vitro* (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, not significant).

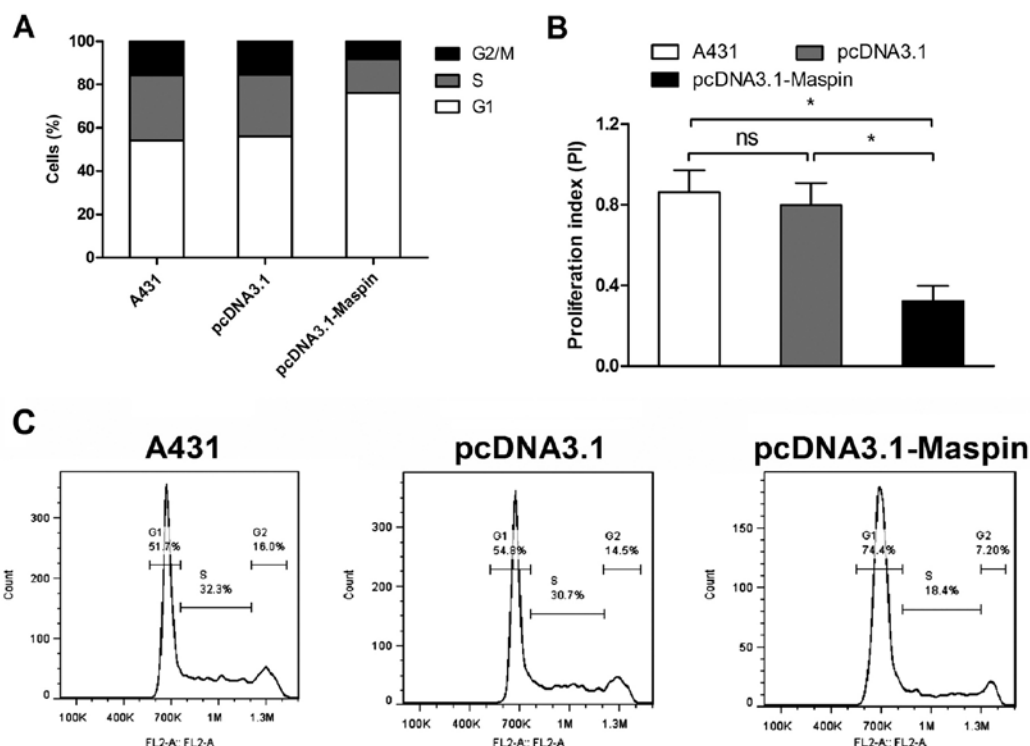


Figure 4. Maspin delayed cell cycle transition in A431 cells. (A) Distribution of A431 cells in three phases of cell cycle (G1 phase, S phase and G2/M phase) detected by flow cytometry. (B) Proliferation index (PI) of A431 cells in each group. PI = (percentage of cells in S phase + G2/M phase) / percentage of cells in G1 phase. (C) Images of cell cycle detection of flow cytometry (* $P < 0.05$; NS, not significant).

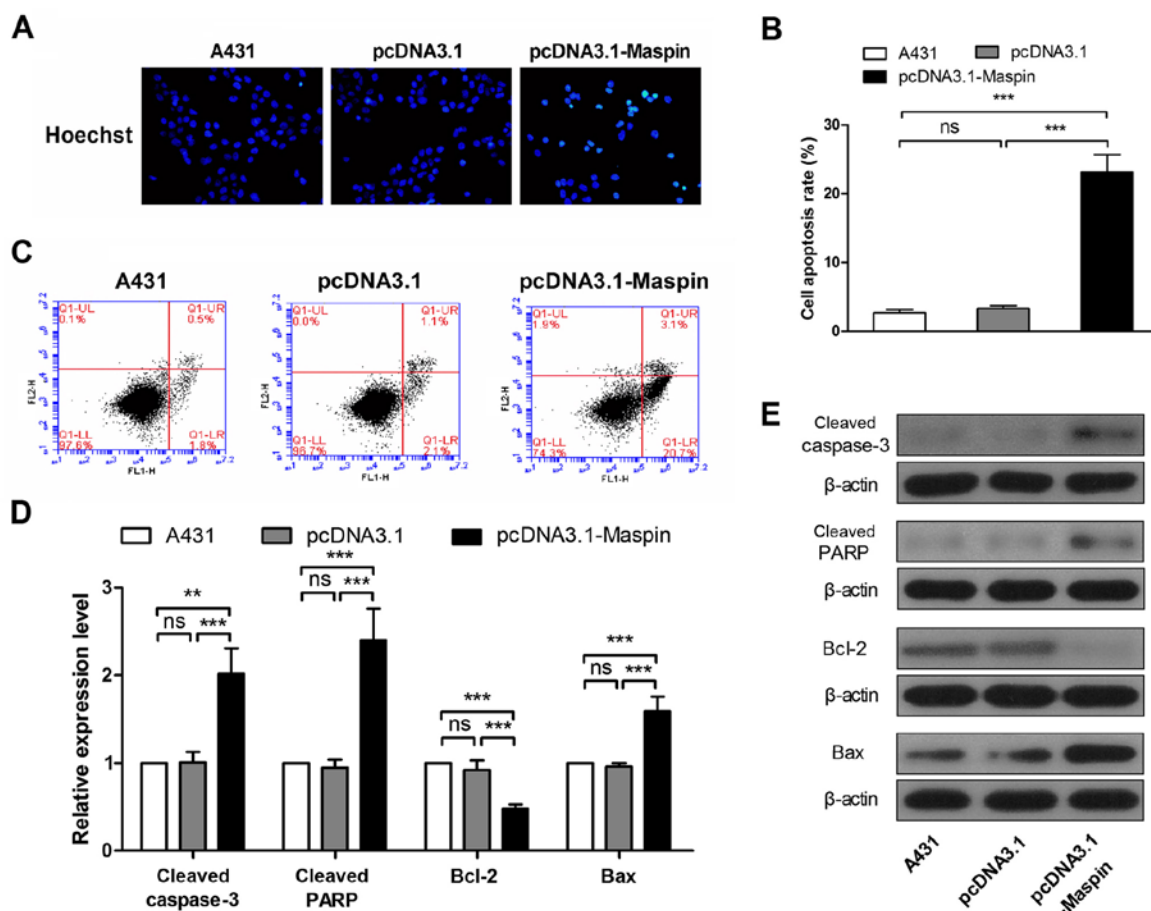


Figure 5. Maspin enhanced apoptosis in A431 cells. (A) Hoechst training was performed to detect apoptosis of A431 cells. (B) Apoptosis rate of A431 cells in each group was detected by flow cytometry. (C) Images of flow cytometry detection of apoptosis. (D and E) The expression levels of cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), Bcl-2 and Bax assessed by western blotting (** $P < 0.01$, *** $P < 0.001$; NS, not significant).

and E). These data suggested that Maspin enhanced apoptosis in A431 cells.

Collectively, Maspin was downregulated in cSCC tissues, and overexpression of Maspin inhibited growth, proliferation and invasion by delaying cell cycle transition and enhancing apoptosis in cSCC cells.

Discussion

With a metastatic rate of 5-10% (24,25), it is crucial for cSCC to be prevented and diagnosed in the early stage. Detection of clinical cSCC tissue samples showed that Maspin was significantly lowly expressed in cSCC tissues, suggesting that Maspin may be a practicable biomarker. Considering the infiltration and invasion, we chose the adjacent tissues 1 cm from cSCC tissue as the control to make the results more reliable.

Proliferation and metastasis of tumor cells are the crucial aspects of pathogenesis of malignant tumors. Our study demonstrated that Maspin overexpression inhibited cell growth, proliferation and invasion by delaying cell cycle transition and enhancing apoptosis in cSCC cells. Cells in different interphases could be distinguished by flow cytometry after staining with propidium iodide due to different amounts of DNA. By counting number of cells in different interphases and calculating the proliferation index (PI), we can determine the cell cycle process and compare the cell division speed among various groups. Apoptosis involves many aspects, detected by various methods. When apoptosis occurs, phosphatidylserine (PS) located in the inner cytomembrane moved to the external cytomembrane, which could be specially combined by Annexin V, thus early apoptosis could be discovered by testing PS using flow cytometry (26). With apoptosis further proceeding, and through staining, condensed chromatin, nuclear fragmentation and apoptotic bodies in apoptotic cells exhibited intense fluorescence under a fluorescence microscope (27).

In addition, apoptosis involves a variety of signaling pathways, expression change of genes in these signaling pathways would suggest the occurrence of apoptosis. Cysteiny aspartate specific protease (caspase) is a protease family, including 11 members, with vital functions in apoptosis (28). Caspase family members are classified as initiators (caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12) and executioners (caspase-3, -6, -7 and -14) (29). In normal cells, all caspase members are present as precursors without protease activity. When the extrinsic apoptotic signal enters the cell, the initiator (mainly caspase-8 and -10) is self-cleaved and activated via allosteric cleavage and activates the executioner (mainly caspase-3) (30,31). The cleaved caspase-3 shears and activates PARP, causing apoptosis (32). On the other hand, executioner caspase also could be activated by intrinsic signals, such as DNA damage, growth factor deprivation and endoplasmic reticulum (ER) stress, which are regulated by members of B-cell lymphoma-2 (Bcl-2) family (33). Bcl-2 is one of the most important genes in apoptosis. Twenty-five homologous proteins have been found in Bcl-2 family to date, divided into two categories: anti-apoptotic proteins, including Bcl-2, Bcl-XL, Bcl-W, and Mcl-1; pro-apoptotic proteins, including Bax, Bak, Bim, and Bad (34). Pro-apoptotic protein receives intrinsic signals and

induces downstream mediators (Bax and Bak), leading to the mitochondrial membrane permeability changes and the release of apoptogenic compound such as cytochrome c, which binds APAF-1 and activates caspase-9, further activating caspase-3 (35,36). In this study, we demonstrated that Maspin participated in apoptosis signal pathway to enhance apoptosis, which was consistent with previous reports in other models (17,18).

Since first reported in 1994 (14), Maspin has been studied widely, and its antitumor effect has been proved in many cell carcinoma and adenocarcinoma. Maspin suppresses cell proliferation, invasion and EMT, enhances apoptosis, repairs DNA damage (37), inhibits angiogenesis and elicits host antitumor immunity (19). During these processes, multiple genes play roles, including Bcl-2, Bax (17), p53, phosphatase and tensin homolog deleted on chromosome ten (PTEN) (38) and miRNA (39,40), suggesting that Maspin participates in multiple signaling pathways. Since secreted by keratinocytes, Maspin is closely related to the function and fate of epidermal cells and keratinocytes (20). The role of Maspin in skin diseases has attracted increased attention. It has been reported that the survival rate of Maspin-positive cSCC patients is much higher than Maspin-negative patients (16), but the mechanism is unknown.

Our study found that Maspin was lower expressed in the clinical cSCC tissues than the adjacent normal tissues. We overexpressed Maspin in A431 cells, in which Maspin was downregulated, and demonstrated that the overexpression of Maspin inhibited cell growth, proliferation and invasion by suppressing cell cycle transition and enhancing apoptosis in cSCC cells. These findings may be of significance for the diagnosis and therapy of cSCC.

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