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Original Article

STMN1 overexpression correlates with biological behavior in human cutaneous squamous cell carcinoma

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ABSTRACT

Stathmin 1 (STMN1) is an important molecule in regulating cellular microtubule dynamics and promoting microtubule depolymerization in interphase and late mitosis. Evidences showed that STMN1 was up-regulated in many cancers, but there was no report about the roles of STMN1 in human cutaneous squamous cell carcinoma (cSCC). Here, we confirmed significant upregulation of STMN1 in cSCC tissues and cell lines compared with non-tumor counterparts. STMN1 upregulation was associated with the proliferation, migration, invasion and apoptosis of cSCC cells. The results suggested that STMN1 may play an important role in the development and tumor progression of cSCC.

Introduction

Nonmelanoma skin cancers (NMSCs), including cutaneous basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC), are the most common cancer in the world [1]. cSCC is less common than BCC [2], but it is a malignant lethal tumor with potential of recurrence and metastasis. Metastatic cSCC has a poor prognosis, and the ten-year survival rates are less than 10% [3]. The most important risk factor of cSCC is DNA damage induced by the exposure to UV irradiation [4]. Genetic alterations, including mutations in RAS, EGFR, CDKN2A [5], p53, NF-κB and AP-1 [6], underlines the molecular mechanisms resulting in cSCC. These genes may be used as prognostic markers and therapeutic targets for cSCC.

The phosphoprotein stathmin 1 (STMN1), as known as oncoprotein 18 (OP18), is a 17-kDa cytoplasmic protein. As a ubiquitous microtubule destabilizing protein, STMN1 is an important key in preventing microtubule polymerization and promoting microtubule catastrophe in interphase and late mitosis [7]. STMN1 is overexpressed across many different human cancers such as Merkel cell carcinomas of the skin [8]. Besides that, STMN1 is positive in all invasive squamous cell carcinoma [9]. Recently, Chen et al. [10] report that STMN1 was up-regulated during the progression of melanoma, and STMN1 was directly regulated by miR-193b. But there were no studies revealing the relationship between STMN1 and non-melanoma skin cancer such as cSCC. In this regard, we hypothesized that STMN1 might behave as an oncogene which would induce cSCC cell proliferation.

However, STMN1 was not studied in cSCC, and the relationship between STMN1 expression and clinicopathological characteristics in cSCC was not elucidated yet. Besides that, the mechanisms of how STMN1 promoted the cSCC progress remained elusive. Whether STMN1 expression status was an independent prognostic factor for cSCC and/or as a target for the cSCC therapy remained unknown.

In this study, we evaluated the expression level of STMN1 in cSCC and compared it with that in the normal tissue. Furthermore, we explored the biological functions, such as proliferation, migration, invasion and apoptosis of STMN1 in cSCC by silencing the expression of STMN1.

Materials and methods

Human tissue specimens and immunohistochemical (IHC) staining

Biopsy samples from a total of 52 cases cSCC tissues were obtained from the dermatological department in the Affiliated

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Table 1
Sequences of siRNAs targeting STMN1.

siRNA names	Sequence (5′–3′)
STMN1_siR1	Sense: GCUUCAGAAGGCAAUAGAAdTdT Antisense: UUCUAUUGCCUUCUGAAGCdTdT
STMN1_siR2	Sense: GGAGGAAAUUCAGAAGAAAdTdT Antisense: UUUCUUCUGAAUUUCCUCCdTdT
STMN1_siR3	Sense: GCACGAGAAAGAAGUGCUUdTdT Antisense: AAGCACUUCUUUCUCGUGCdTdT
STMN1_siR4	Sense: GAACAACAACUUCAGUAAAdTdT Antisense: UUUACUGAAGUUGUUGUUCdTdT
NC_siR	Sense: UUCUCCGAACGUGUCACGUdTdT Antisense: ACGUGACACGUUCGGAGAAdTdT

Hospital of Nantong University. And 10 cases of normal skin tissues were obtained as normal controls.

All resected samples were routinely processed, fixed in formaldehyde solution and embedded in paraffin. IHC staining was performed using the Envision Plus/Horseradish Peroxidase system (DAKO, USA), and 4 μm sections were incubated in 0.3% hydrogen peroxide in absolute methanol to block endogenous peroxidase activity. Subsequently, they were pressure-cooked in sodium citrate buffer (10 mM sodium-citrate monohydrate, pH 6.0) for antigen retrieval. The sections were then incubated with rabbit anti-STMN1 polyclonal antibody (Abcam, USA, dilution 1:100), overnight at 4 °C. After washing with Tris-buffered saline, the tissue was incubated using the Envision Plus secondary antibody for 30 min, followed by diaminobenzidine for 5 min. Appropriate positive and negative controls (incubation with secondary antibody only) were stained in parallel.

All sections were blindly analyzed by two experienced pathologists. The immunohistochemistry scoring results for STMN1 were based on the frequency of distribution of immunopositive cells. The percentage of positive cells was determined in 5 fields per section at high magnification and was graded as: (−), <5%; (+), 5–50%; (++), 50–75%; (+++), >75%.

This study was approved by the Institute Research Ethics Committees of the Affiliated Hospital of Nantong University.

Cell culture and siRNA transfection

Human cSCC cell line A431 and normal skin cell line HaCaT were all purchased from Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. Two cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C and 5% CO_2.

To silence the expression of STMN1 (Accession No. NM_203401 form NCBI GenBank), A431 cells were transfected with four siRNAs which were designed and obtained from Biomics Biotechnologies Co., Ltd. (China). The sequence alignment (BLAST) was carried out to ensure no homology with others genes in human. And the negative control siRNA (NC_siR) was also designed. The targeted sequences are shown in Table 1.

Real-time quantity PCR (RT-qPCR)

Total RNA were extracted using a TRIzol® reagent (Life Technologies, USA) according to the manufacturer's procedure and reverse transcribed into cDNA samples using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). Then 480 SYBR Green I Master (Roche, Germany) was used for RT-qPCR. The primers used were obtained from Biomics Biotechnologies Co., Ltd. (Nantong, China) and were as follows: STMN1 forward: 5′-TGGAGAAGCGTGCCTCAG-3′; reverse: 5′-TTCATGGGACTTGCGTCTTTC-3′; GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse: 5′-GAAGATGGTGATGGGATTTC-3′. GAPDH served as the reference gene. RNAs were reverse transcribed to first strand cDNA at 42 °C for 30 min, and then amplified for 2 min at 94 °C for pre-denature, followed by 45 cycles of denature at 94 °C for 20 s, annealing at 58 °C for 20 s and extension at 72 °C for 30 s. The experiment was performed in triplicate. The results were analyzed by $2^{-\Delta\Delta Ct}$ method [11].

Western blot analysis

The cells were lysed in ice-cold cell RIPA buffer (Pierce, USA) and then centrifuged at 10,000 rpm for 20 min at 4 °C. Protein concentrations were determined with BCA protein assay kit (Pierce, USA). The supernatant was diluted in 5× SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris–HCl (pH 6.8), 10% (w/v) SDS, 0.5% (w/v) bromphenol blue, 50% (v/v) glycerol, 5% (w/v) β-mercaptoethanol) and boiled. 20 μg of total cell lysates per lane were separated with SDS-PAGE and electroblotted onto polyvinylidine difluoride filter (PVDF) membranes (Millipore, USA), followed by blocking with 5% skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) for 2 h at room temperature. The membrane was incubated with primary antibody of Rabbit monoclonal to STMN1 (Abcam, USA, 1:500 dilution), and mouse-anti-human β-actin (Santa Cruz, USA, 1:200 dilution) as internal control. After being washed with TBST, the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) (goat anti-rabbit IgG-HRP with 1:1000 dilution for STMN1; goat anti-mouse IgG-HRP with 1:2000 dilution for β-actin) for about 1.5 h at room temperature, and then washed for three times in TBST for 5 min. The specific proteins were detected with ECL plus kit (ZSbio, China).

Cell proliferation assay

The proliferation of cells was measured using the Cell Counting Kit-8 (CCK-8) kit (Beyotime, China). A431 cells (5×10^3 cells/well) were plated into a 96-well plate before transfection and were grown to 70–80% confluence for 24 h. After treatment with siRNAs for 0, 24, 48, 72 and 96 h, 10 μL CCK-8 reagents were added to each well of a 96-well plate containing 100 μL culture medium and the plate was incubated for 2 h at 37 °C. Then the optical density (OD) was measured at 450 nm using a Fluorescence Spectrophotometer (HITACHI, Japan). 5 wells were used for each experimental condition and all the experiments were independently repeated 3 times.

Cell migration assay

The migration of A431 cells was detected by wound-healing assay. A431 cells were plated and transfected with siRNAs as described above in a 12-well plate at the density of 3×10^5 cells/well. After cells confluence reached about 80%, the monolayer cells were wounded by scraping off the cells using a 100-μL pipette tip and cells were washed with PBS and then DMEM medium with no FBS. Photographs of cells were taken at 0, 24, 48, and 72 h to monitor cell movements.

Cell invasion assay

A431 cells were treated with siRNAs for 48 h, and then cells were treated with 0.25% trypsin and suspended in DMEM medium with