European Review for Medical and Pharmacological Sciences 2020; 24: 8001-8007

TRPP2 promotes the proliferation of nasopharyngeal carcinoma through upregulating Skp2/c-Myc

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Abstract. – OBJECTIVE: To elucidate the promotive role of TRPP2 in nasopharyngeal carcinoma (NPC) proliferation by targeting Skp2/c-Myc, thus accelerating the malignant progression.

PATIENTS AND METHODS: TRPP2 levels in NPC patients with different T stages were detected. Correlation between TRPP2 level and clinical features of NPC patients was analyzed. Kaplan-Meier curves were depicted for assessing the prognostic value of TRPP2 in NPC. Subsequently, regulatory effects of TRPP2 on viability and 5-ethynyl-2'-deoxyuridine (EdU)-positive ratio were determined by cell counting kit-8 (CCK-8) and EdU assay, respectively. Relative levels of Skp2 and c-Myc in NPC cells transfected with si-TRPP2 were examined. At last, the involvement of c-Myc in TRPP2-regulated proliferative ability of NPC was evaluated by performing rescue experiments.

RESULTS: TRPP2 was upregulated in NPC tissues. TRPP2 level was higher in NPC patients with T3+T4 than those with T1+T2. Worse survival was observed in NPC patients expressing high level of TRPP2. TRPP2 level was correlated to T stage, N stage, M stage, and locoregional failure of NPC patients. Knockdown of TRPP2 reduced viability and EdU-positive ratio in NPC cells. In addition, relative levels of Skp2 and c-Myc in NPC cells transfected with si-TRPP2 were downregulated. Overexpression of c-Myc could partially reverse the regulatory effects of TRPP2 on NPC proliferation.

CONCLUSIONS: TRPP2 stimulates NPC cells to proliferate by upregulating expressions of Skp2/c-Myc, thus deteriorating the development of NPC.

Key Words:
Nasopharyngeal carcinoma, TRPP2, Skp2/c-Myc.

Introduction

Nasopharyngeal carcinoma (NPC) originates from nasopharyngeal mucosal epithelium. It is characterized as insidious growth, atypical symptoms, and high rate of lymphatic metastasis. The development of NPC involves dysregulation of multiple factors and pathways. Imbalanced oncogenes and tumor-suppressor genes are responsible for the tumorigenesis of NPC[1-3]. It is urgent to clarify the pathogenesis of NPC and search for effective biomarkers.

TRPP2, also known as Polycystin-2 (PKD2), contains 6 transmembrane regions and abundant extracellular environment. TRPP2 locates on human chromosome 4q21-22, consists of 15 exons and spans 70 kDa. The mRNA transcribed by TRPP2 is 5.1 kb, which mainly encodes PC2, a non-selective cation channel distributed in endoplasm enriched with membranes and plasma membranes with high Ca^{2+} permeability[4,5]. Functionally, TRPP2 is involved in the regulation of smooth muscle contraction, cell proliferation, fertilization, spouse behavior, mechanical sensation, and asymmetric expressions of genes[6]. It is reported that TRPP2 triggers metastasis of laryngeal squamous cell carcinoma through mediating EMT[7]. Its potential function in NPC, however, is rarely reported.

Disordered cell cycle progression is a key event during tumor development. S-phase kinase associated protein 2 (Skp2) is a F-box protein involved in cell cycle progression. Skp2 contributes to the formation of SCF-Skp2 complex alongside Skp1, Cullin, and Rocl/Rbxl, and this complex specifically targets p27, a negative regulator in cell cycle[8]. The interaction between Skp2 and c-Myc enhances S phase transformation and activates downstream genes of c-Myc[9]. Qu et al[10] demonstrated that activation of c-Myc could downregulate protein level of p27. In melanoma, Skp2/c-Myc contributes to the deterioration of disease. In this paper, we mainly discuss the role of TRPP2 in influencing the progression of NPC by upregulating Skp2/c-Myc.

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Patients and Methods

Sample Collection

A total of 58 NPC patients treated in Affiliated Hospital of Guizhou Medical University from January 2016 to December 2018 were enrolled. None of NPC patients had preoperative anti-tumor treatments. Their matched NPC tissues and adjacent ones were surgically resected, and samples were immediately frozen in liquid nitrogen. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of Affiliated Hospital of Guizhou Medical University. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

NPC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified using a spectrometer. RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. Primer sequences were listed as follows: TRPP2, forward: 5'-CCAACTTCGCTCACGTCTCA-3' and reverse: 5'-CTTGTCCACCAGGAACTCCACAG-3'; Skp2, forward: 5'-GGCTGAAGCGCAAAGGGAGT-3', and reverse: 5'-GGCAATCACCCCTTGAGACA-3'; c-Myc, forward: 5′-CAAGAGGCGAACACACAACG-3' and reverse: 5′-GTCGTTTCCGCAACAAGTCC-3'; GAPDH, forward: 5′-CAATGACCCCTTCATTGACC-3′ and reverse: 5′-GACAAGCTTCCCGTTCTCAG-3′.

Cell Transfection

Cells in logarithmic growth period were cultured to 70-80% confluence and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, 1 μg siRNA and 3 μL of Lipofectamine 2000 were respectively diluted in serum-free DMEM. The mixture was maintained at room temperature for 20 min and applied in each well. Sequences of TRPP2 siRNA were 5′-GAGUUUGAGUACCUGAAGCUG-3′; sequences of siRNA negative control were 5′-UUCUCCGAACGUGUCACGUTT-3′.

Cell Counting Kit-8 (CCK-8) Assay

The cells were inoculated in the 96-well plate (2×10^3 cells per well) and reacted with 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent per well for 4 h culture. Absorbance (A) at 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

200 μL of diluted EdU (1:1000 in DMEM; Sigma-Aldrich, St. Louis, MO, USA) was applied in each well of a 6-well plate and incubated in the dark for 1 h. After phosphate-buffered saline (PBS) washing twice, with 5 min each, 200 μL of Hoechst was applied and cultured in the dark for 30 min. Finally, EdU-labeled and Hoechst-stained cells were captured.

Western Blot

Cellular protein was isolated using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After preparation of protein samples, 40 μg protein per sample was electrophoresed and loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± SD (standard deviation). Differences between two groups were analyzed by the *t*-test. Kaplan-Meier curves were introduced for assessing the prognostic potential of TRPP2 in NPC. $p<0.05$ indicated the significant difference.

Results

TRPP2 was Highly Expressed in NPC

Compared with adjacent normal tissues, TRPP2 was highly expressed in NPC tissues (Figure 1A, 1B). In particular, NPC patients with

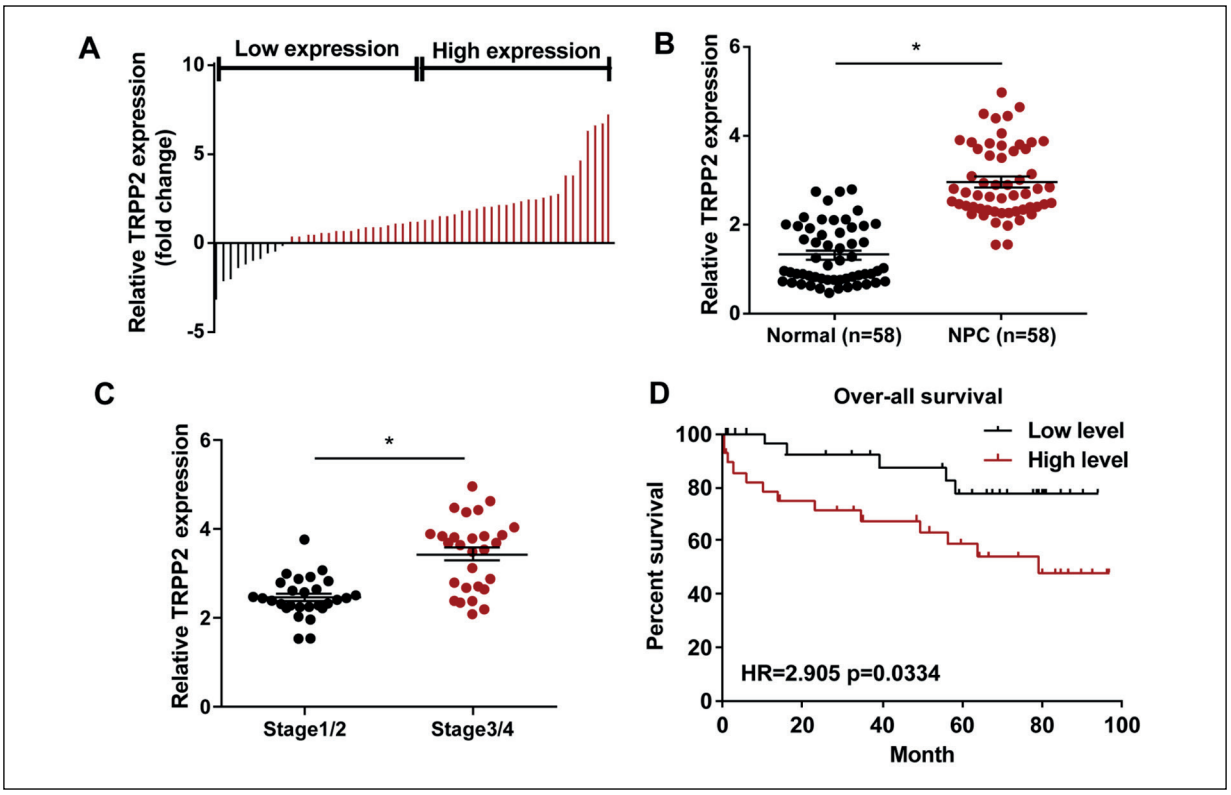

Figure 1. TRPP2 was highly expressed in NPC. **A, B,** TRPP2 levels in 58 matched NPC tissues and adjacent normal tissues. **C,** TRPP2 levels in NPC patients with T1+T2 or T3+T4. **D,** Overall survival in NPC patients expressing high or low level of TRPP2.

T3+T4 expressed higher abundance of TRPP2 than those with T1+T2 (Figure 1C). Kaplan-Meier curves revealed that NPC patients expressing high level of TRPP2 suffered worse overall survival (HR=2.905, p=0.0334, Figure 1D). Hence, TRPP2 was unfavorable to the prognosis of NPC.

Correlation Between TRPP2 Level and Clinical Features of NPC Patients

Based on the median level of TRPP2 in enrolled 58 NPC patients, they were assigned into high-level and low-level group. Correlation between TRPP2 level and clinical features of them was analyzed. It is shown that TRPP2 level was correlated to T stage, N stage, M stage, and locoregional failure in NPC patients, rather than age and sex (p>0.05, Table I).

Knockdown of TRPP2 Suppressed Proliferative Ability in NPC

To clarify the biological function of TRPP2 in NPC, transfection efficacy of si-TRPP2 was first verified in HNE1 and CNE1 cells. Transfection of si-TRPP2 markedly decreased mRNA and protein levels of TRPP2 in NPC cell lines (Figure 2A, 2B). Knockdown of TRPP2 greatly decreased viability in HNE1 and CNE1 cells (Figure 2C). Similarly, EdU-positive ratio was reduced after knockdown of TRPP2 (Figure 2D). It is suggested that TRPP2 was able to promote NPC cells to proliferate.

Knockdown of TRPP2 Downregulated Expressions of Skp2/c-Myc

Interestingly, transfection of si-TRPP2 markedly downregulated mRNA levels of Skp2 and c-Myc in HNE1 and CNE1 cells (Figure 3A, 3C). Protein levels of Skp2 and c-Myc were identically downregulated in NPC cells transfected with si-TRPP2 (Figure 3B, 3D).

Overexpression of c-Myc Partially Reversed the Inhibitory Effect of Silenced TRPP2 on Proliferative Ability of NPC Cells

Knockdown of TRPP2 decreased viability in NPC cells, and the decreased trend was partially abolished by overexpression of c-Myc (Figure

Table I. Correlation between TRPP2 level and clinical features of NPC patients (n=58).

Clinicopathologic features	No. of cases	TRPP2 expression		*p*-value
		Low (n = 28)	High (n = 28)	
Age (years)				0.7891
≤ 50	27	13	14	
> 50	29	15	14	
Gender				0.7881
Male	25	12	13	
Female	31	16	15	
T stage				0.0321*
T1-T2	26	17	9	
T2-T3	30	11	19	
N stage				0.0033*
N0-N1	26	20	9	
N2-N3	30	8	19	
M stage				0.0156*
M0-M1	25	17	8	
M2-M3	31	11	20	
Locoregional failure				
Yes	29	19	10	0.0161*
No	27	9	18	

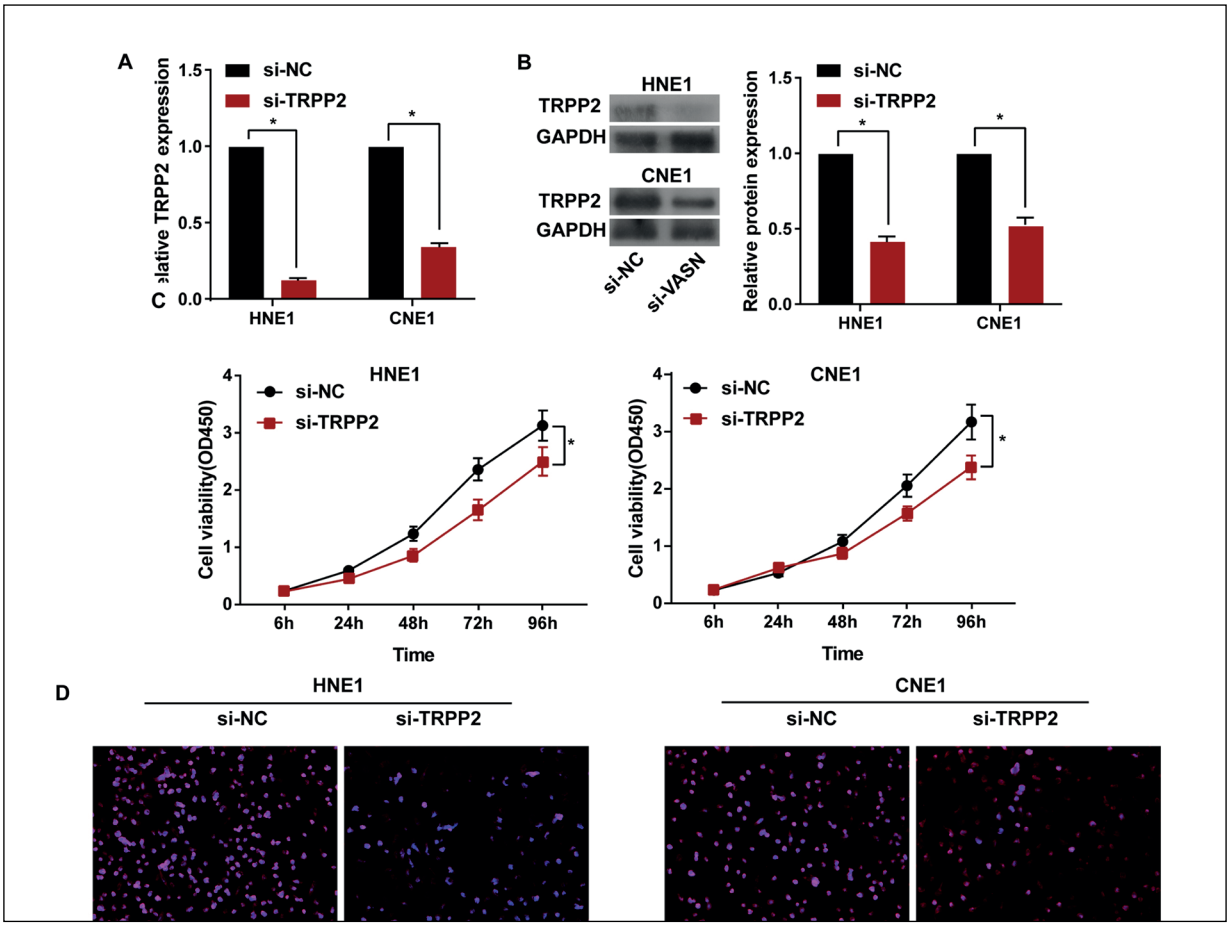

Figure 2. Knockdown of TRPP2 suppressed proliferative ability in NPC. **A, B,** The mRNA (**A**) and protein levels (**B**) of TRPP2 in HNE1 and CNE1 cells transfected with si-NC or si-TRPP2. **C,** Viability in HNE1 and CNE1 cells transfected with si-NC or si-TRPP2. **D,** EdU-positive cells in HNE1 and CNE1 cells transfected with si-NC or si-TRPP2 (magnification 40×).

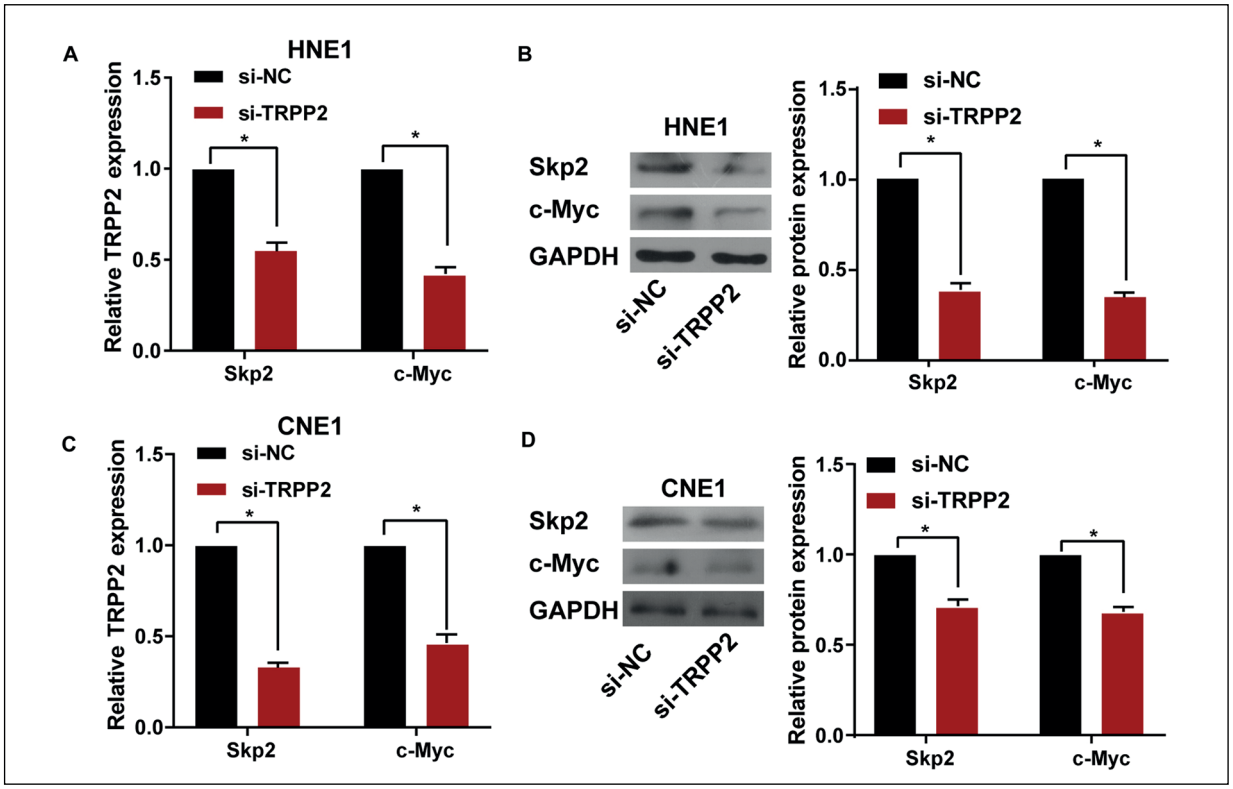

Figure 3. Knockdown of TRPP2 downregulated expressions of Skp2/c-Myc. **A, B,** The mRNA (**A**) and protein levels (**B**) of Skp2 and c-Myc in HNE1 cells transfected with si-NC or si-TRPP2. **C, D,** The mRNA (**C**) and protein levels (**D**) of Skp2 and c-Myc in CNE1 cells transfected with si-NC or si-TRPP2.

4A). Besides, decreased EdU-positive ratio in NPC cells with TRPP2 knockdown was slightly enhanced after co-transfection of pcDNA-c-Myc (Figure 4B). Hence, TRPP2 stimulated NPC cells to proliferate through upregulating Skp2/c-Myc.

Discussion

NPC is a malignant tumor originating from nasopharyngeal mucosa. It mainly affects men than women. NPC is highly prevalent in the middle-aged population[11]. As an invasive malignancy, NPC is able to invade into deep anatomic location in early phase. Currently, radiotherapy is preferred for NPC, although it could damage normal cells and result in severe systematical and local reactions[12]. It is urgent to develop effective biomarkers and therapeutic targets, thus improving the prognosis of NPC.

The interaction between TRPP2 and PKD1 in plasma membrane serves a functional ion channel for cell adhesion and mechanical sensation[13]. By coupling to EGFR, TRPP2 is activated by EGF in renal epithelial cell line LLC-PK1[14]. In addition, TRPP2 contributes to myogenic response of cerebral artery[15]. Endoplasmic reticulum retained TRPP2 reduces Ca^{2+} release from the endoplasmic reticulum, thereby protecting cells from apoptosis damage[16]. The complex formed by TRPP2 and PKD1 is able to regulate proliferation, differentiation, and apoptosis of renal epithelial cells by controlling intracellular Ca^{2+}[17]. TRPP2 is identified as a potential mutation target responsible for autosomal dominant polycystic kidney disease[7]. Besides, TRPP2 is also involved in tumor progression[18,19].

Skp2 locates on human chromosome 5p13, which is closely linked to tumor development[20]. Skp2 leads to excessive proliferation by triggering cell cycle progression from G1 phase to S phase[21]. It is reported that SKp2 is an independent marker predicting the prognosis of hepatocellular carcinoma[22]. The proto-oncogene c-Myc at transcription and translation levels are closely linked to cell cycle progression. c-Myc extensively participates in cell differentiation

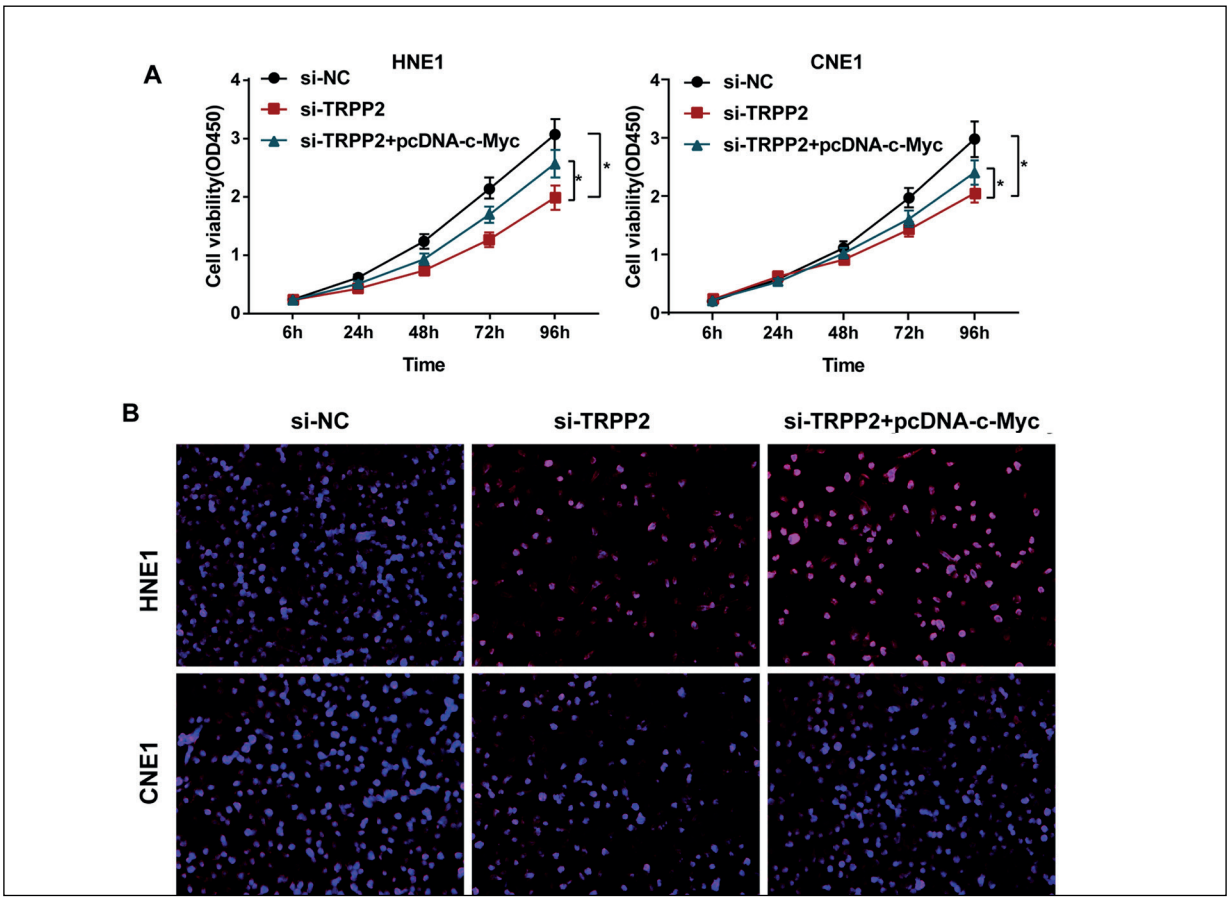

Figure 4. Overexpression of c-Myc partially reversed the inhibitory effect of silenced TRPP2 on proliferative ability of NPC cells. **A,** Viability in HNE1 and CNE1 cells transfected with si-NC, si-TRPP2 or si-TRPP2+pcDNA-c-Myc. **B,** EdU-positive cells in HNE1 and CNE1 cells transfected with si-NC, si-TRPP2 or si-TRPP2+pcDNA-c-Myc (magnification 40×).

and programmed cell death[23,24]. It is estimated that c-Myc is activated in about 20% of human tumors[25]. Abnormally upregulated c-Myc is observed in 70% colorectal cancer cases, 80% breast cancer cases, and 60% stem cell cancer cases. A signaling transduction from TGF-β1 to Skp2 through Akt1 and c-Myc is responsible for the deterioration of melanoma[10].

In this paper, mRNA and protein levels of Skp2 and c-Myc were downregulated in NPC cells after knockdown of TRPP2. Notably, overexpression of c-Myc could partially reverse the inhibitory effect of silenced TRPP2 on proliferative ability of NPC cells. Collectively, TRPP2 stimulated the progression of NPC by upregulating Skp2/c-Myc. The novelty of this study was that we first attempted to explore the role and mechanism of TRPP2 in NPC via the Skp2/c-Myc pathway, providing a new research target for the diagnosis and treatment of NPC.

Conclusions

TRPP2 stimulates NPC cells to proliferate by upregulating expressions of Skp2/c-Myc, thus deteriorating the development of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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