

# Deletion of C9orf53 promotes the growth of head and neck squamous cell carcinoma and is associated with poor prognosis of patients with head and neck squamous cell carcinoma

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**Abstract.** Head and neck squamous cell carcinoma (HNSCC) is the fifth most common carcinoma worldwide, and accounts for ~600,000 new cases every year. The information on the molecular carcinogenesis of HNSCC is very limited. In the present study, the role of C9orf53 in HNSCC was investigated. The levels of C9orf53 were assayed by reverse transcription-quantitative polymerase chain reaction. The levels of C9orf53 in cells were overexpressed by overexpression plasmid and inhibited by small-interfering RNA. Cell proliferation was assayed by MTT, and cell apoptosis was assessed by FACS analysis. It was demonstrated that C9orf53 deletion was associated with a decreased survival of patients. The level of C9orf53 in HNSCC tissues was lower compared with the matched normal tissues adjacent to tumors. A lower expression of C9orf53 promoted cell proliferation, and the overexpression of C9orf53 induced cell apoptosis. In conclusion, a low level of C9orf53 in HNSCC promoted the growth of HNSCC cells, which might be associated with the low survival rate of patients with HNSCC.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) encompasses a heterogeneous group of tumors with aggressive nature and is the fifth most common carcinoma worldwide (1,2).

HNSCC accounts for ~4% of all malignancies worldwide and 5% of all cancer mortalities (3).

The choice of cancer treatment depends on the site of the primary tumor, the stage of the disease, treatment toxicities and the expected oncological and functional outcomes. One approach to improve treatment efficacy is to add novel molecular targeted agents to the classical treatment regimens (4).

Monoclonal antibodies targeting the epidermal growth factor receptor have shown clinical benefits in palliative and curative settings (5). However, only a minority of patients presenting with current or metastatic HNSCC exhibited tumor regression with these agents, and the majority of patients develop acquired tumor resistance following several months of treatments (4). Therefore in order to develop agents that target novel proteins, the identification of novel genes in HNSCC is required.

In the present study, the mutated genes in HNSCC were screened by searching the Cancer Genome Atlas (TCGA). The TCGA research network has profiled and analyzed large numbers of human tumors in order to identify molecular aberrations at DNA, RNA, protein and epigenetic levels (6-13). It was identified that there were many genes with amplification and deletion in HNSCC (Table I), including C9orf53. C9orf53 is a protein-coding gene. C9orf53 (also named CDKN2A-AS1) is associated with Alzheimer's disease (14). The present study focused on the role of C9orf53 in HNSCC, and it was found that the main type of C9orf53 mutation in HNSCC is deletion. Importantly, it was observed that deletions in C9orf53 are associated with lower patient survival rates. In the cell experiments, the role of C9orf53 in HNSCC was investigated *in vitro* by overexpressing or inhibiting C9orf53 expression. In conclusion, the present study revealed the role of C9orf53 in HNSCC, and the study may provide a novel therapeutic target for future investigation.

## Materials and methods

**Frequency of gene alteration and patient survival analysis for cancer.** The data on the frequency of C9orf53 alteration, mRNA level analysis and patient survival were obtained from

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TCGA via cBioportal for Cancer Genomics (<http://www.cbioportal.org/public-portal/index.do>) (15,16).

**Patients.** Surgical specimens from 19 patients with HNSCC (age range: 36-75 years; mean age: 54.3±7.4 years; Sex ratio: Male to Female 1.7:1) and matched normal tissues adjacent to tumors were obtained postoperatively in June, 2009 from the Department of Head and Neck Surgery, Changhai Hospital, Second Military Medical University (Shanghai, China). All patients gave signed, informed consent for their tissues to be used for scientific research. Ethics approval for the present study was obtained from Changhai Hospital, Second Military Medical University (Shanghai, China). All diagnoses were based on pathological and/or cytological findings. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria (17). The tissues were obtained prior to chemotherapy and radiotherapy. The samples were immediately frozen and stored at -80°C prior to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.

**Cell culture.** 293, SCC-5 and SCC-9 cells were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). 293, SCC-5 and SCC-9 cells were cultured in 37°C, 5%CO<sub>2</sub>, and in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 2 mM L-glutamine and 100 µg/ml penicillin/streptomycin (Sangon Biotech Co., Ltd., Shanghai, China) as described in previous studies (18-20).

**RNA extraction and RT-qPCR.** RNA was extracted with Trizol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The cDNA synthesis and RT-qPCR were subsequently performed using the Qiagen system as described previously (21). RT-qPCR analysis was performed using standard protocols on Applied Biosystem 7500 HT sequence Detection system. The relative mRNA levels of C9orf53 were normalized to levels of the housekeeping gene GAPDH and calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (22). The primers used are as follows: GAPDH forward, 5'-CCATGTTTCGTCATGGG-TGTGAACCA-3' and reverse, 5'-GCCAGTAGAGGCAGGGATGATGTTG-3' and C9orf53 forward, 5'-AAGAATTTCGGCAGGAGGGTT-3' and reverse, 5'-CTCTGCCACAGTGGGATTGT-3'.

**MTT assay.** For MTT assay, 5x10<sup>3</sup> cells per well were seeded in triplicate in a 96-well plate with complete growth medium. The cells were counted over 5 days using the MTT assay (Promega Corporation, Madison, WI, USA) as described previously (19,20). The data was measured using the Microtiter plate reader (Promega Corporation) at 570 nm.

**Transfection of C9orf53 overexpression plasmid and small-interfering (si)RNA.** C9orf53 overexpression plasmid (pcDNA3.1-C9orf53;), C9orf53-siRNA (5'-GTGTGATTTTCGTAAACAGATA-3') and control-siRNA were designed and constructed by Sangon Biotech Company (Shanghai, China). Untransfected cells were used as a blank

control. Transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. SCC-5 and SCC-9 cells were seeded into 24-well plates at a density of 5x10<sup>4</sup> cells/well and were allowed to culture overnight. DNA Plasmid (500 ng) with Lipofectamine® 2000 and siRNA (600 ng) with Lipofectamine® 2000 were mixed for transfection. Then DNA-Lipofectamine® 2000 complexes were added into wells at 37°C for 24 h prior to subsequent experimentation.

**Apoptosis assay.** SCC-5 and SCC-9 cells were labeled with Annexin V-fluorescein isothiocyanate and propidium iodide (PI) using the apoptosis detecting kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Then samples were analyzed by fluorescence-activated cell sorting (FACS) assay with a flow cytometer (BD FACSVerse™ flow cytometer, with BD FACSuite™ software v1.0.6, BD Biosciences, San Jose, CA, USA) (23).

**Statistical analysis.** Data are presented as the mean ± standard deviation from at least three independent experiments. The difference between groups was analyzed using two-tailed, paired Student's t-test when only two groups were compared. The Wilcoxon matched-pairs signed rank test was used to determine if there was a statistically significant difference in the expression of C9orf53 between matched pairs. The difference between groups was analyzed using one-way ANOVA with post hoc contrasts by Student-Newman-Keuls test, when three or more than three groups were compared. Correlation analysis was performed using two-tailed Person's correlation coefficient analysis. Patient survival was determined by Kaplan-Meier analysis (with log-rank test). Statistical analyses were performed using SPSS software (version 17.0; IBM Corp., Armonk, NY, USA). P<0.05 was considered significantly different.

## Results

**Screening of novel genes amplified or deleted in HNSCC.** To identify the potential target genes of HNSCC, the genes that are amplified or deleted in HNSCC were screened. It was observed that there were a number of genes amplified or deleted in HNSCC (Table I). To date, to the best of our knowledge, there is no study on the role of C9orf53 in HNSCC. Therefore, in the present study, the role of C9orf53 in HNSCC was investigated. Initially, mutations in C9orf53 were investigated in various types of cancer and mutations were identified in 302 HNSCC tissue samples. There were 27.5% HNSCC tissues with C9orf53 deletions (Fig. 1A). Next, it was observed that a low expression of C9orf53 mRNA was associated with C9orf53 deletions (Fig. 1B). Importantly, these data indicated that C9orf53 deletion was associated with a decreased survival rate (Fig. 1C).

**Reduced expression of C9orf53 in HNSCC tissues compared with normal tissues.** Next, 19 surgical specimens and matched tumor-adjacent normal tissues were obtained from patients with HNSCC. The levels of C9orf53 mRNA were assayed by RT-qPCR. It was identified that 17 of the 19 HNSCC tissues exhibited lower C9orf53 expression compared with

Table I. Expression of genes that are deleted or amplified in HNSCC.

Genes	P-value
<b>Amplification</b>	
FADD	P<0.001
USP13	P<0.001
DCUN1D1	P<0.001
EGFR	P<0.001
CCND1	P<0.001
FGF3	P<0.001
MIR548K	P<0.001
PPFIA1	P<0.001
POU5F1B	P<0.001
CASC8	P<0.001
CCAT1	4.82x10 <sup>-22</sup>
FGFR1	1.15x10 <sup>-18</sup>
RGP1	1.24x10 <sup>-13</sup>
LINC00393	1.34x10 <sup>-11</sup>
MYRFL	5.81x10 <sup>-11</sup>
MLANA	0.147
CDK6	0.259
SAMD9L	0.259
SNAI2	9.67x10 <sup>-9</sup>
NFIB	2.38x10 <sup>-8</sup>
E2F1	9.36x10 <sup>-6</sup>
ANKRD39	5.09x10 <sup>-5</sup>
LRRC14B	7.44x10 <sup>-5</sup>
ERBB2	1.16x10 <sup>-4</sup>
TINAG	2.37x10 <sup>-4</sup>
CD44	5.72x10 <sup>-4</sup>
LINC00452	2.99x10 <sup>-3</sup>
PTP4A1	0.037
<b>Deletion</b>	
C9ORF53	P<0.001
ZNF532	P<0.001
CDKN2A	P<0.001
RN7SL5P	1.42x10 <sup>-39</sup>
RNY3P4	3.13x10 <sup>-23</sup>
STK11	1.77x10 <sup>-19</sup>
FAM72C	4.76x10 <sup>-19</sup>
PDE4D	6.60x10 <sup>-18</sup>
RB1	4.14x10 <sup>-17</sup>
PARD3	1.80x10 <sup>-14</sup>
LINC00971	0.101
KIAA0825	1.25x10 <sup>-9</sup>
MIR3182	4.80x10 <sup>-9</sup>
CBWD3	2.74x10 <sup>-7</sup>
PTPRG	1.29x10 <sup>-6</sup>
GPN2	1.62x10 <sup>-6</sup>
ZNF750	2.67x10 <sup>-6</sup>
KCNIP4	1.51x10 <sup>-5</sup>
RNA5SP431	1.61x10 <sup>-5</sup>
RAB24	1.09x10 <sup>-4</sup>
C9ORF163	1.24x10 <sup>-3</sup>

Table I. Continued.

Genes	P-value
LINC00645	7.31x10 <sup>-3</sup>
LRRN1	0.0239

The expression data were acquired from The Cancer Genome Atlas, where there were 302 HNSCC samples analyzed. The mean expression of these genes in tumor tissues and normal tissues were compared by paired t-test. HNSCC, Head and neck squamous cell carcinoma.

matched normal tissues that were adjacent to tumors (Fig. 2A). Furthermore, the median C9orf53 expression in HNSCC tissues was lower compared with normal tissues that were adjacent to tumors, and the standard deviation of C9orf53 expression in HNSCC was higher compared with normal tissues (Fig. 2B).

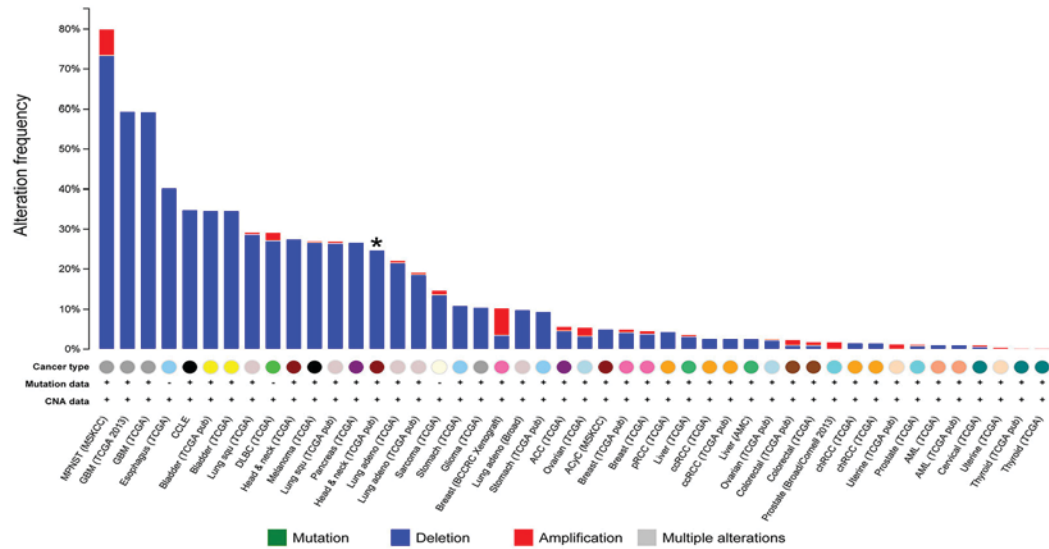
*Reduced expression of C9orf53 promotes proliferation and overexpression of C9orf53-induced apoptosis.* Next, the role of C9orf53 *in vitro* was investigated. The levels of C9orf53 in HNSCC cell lines (SCC-5 and SCC-9) were analyzed, and 293 cells were used as a control. It was observed that the levels of C9orf53 in SCC-5 and SCC-9 were lower compared with 293 cells (Fig. 3A). As SCC-5 cells exhibited lower C9orf53 levels compared with SCC-9 cells, C9orf53 was overexpressed in SCC-5 cells by transfection with pcDNA3.1-C9orf53. C9orf53 expression was suppressed in SCC-9 cells by transfection with si-C9orf53. The levels of C9orf53 were determined by RT-qPCR, 48 h following transfection. It was observed that the level of C9orf53 was successfully increased following transfection of pcDNA3.1-C9orf53 in SCC-5 cells and that C9orf53 expression was successfully inhibited following transfection with si-C9orf53 in SCC-9 cells (Fig. 3B). As transfection with si-C9orf53-1 resulted in the greatest decrease in C9orf53 expression (Fig. 3B), si-C9orf53-1 was selected for subsequent experiments. Following the overexpression or inhibition of C9orf53, proliferation was examined by MTT assay. It was found that overexpressing C9orf53 inhibited proliferation, and suppressing C9orf53 promoted proliferation (Fig. 3C). Next, the cell apoptosis was examined by FACS assay. The overexpression of C9orf53 increased the rate of cell apoptosis (Fig. 3D).

## Discussion

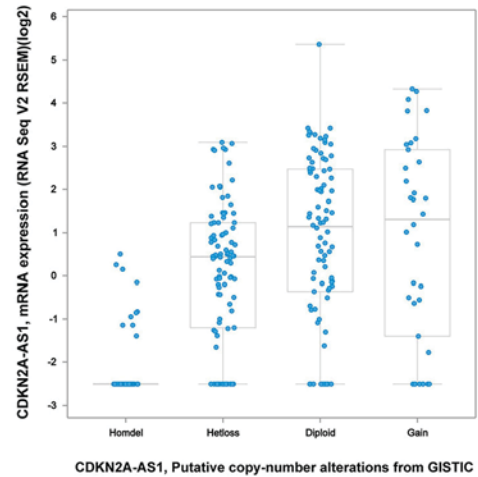
In the present study, the role of C9orf53 in HNSCC was studied. It was identified that C9orf53 was deleted in HNSCC, and the levels of C9orf53 in HNSCC tissues were lower in tumor tissues compared with matched normal tissues that were adjacent to tumor tissues. Notably, the deletion of C9orf53 and a lower expression level of C9orf53 were associated with the rate of patient survival. Subsequently, the role of C9orf53 was confirmed by overexpressing and suppressing C9orf53 expression *in vitro*. The suppression of C9orf53 was able to promote proliferation

A

C9orf53 deletion



B



C

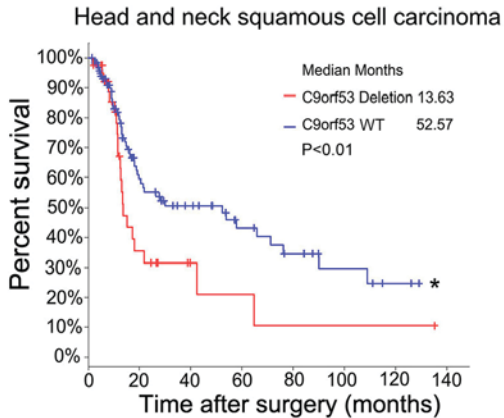


Figure 1. Low C9orf53 level in HNSCC tissues is associated with low survival rates. Data on the alteration frequency of C9orf53 and mRNA levels were obtained from TCGA via cBioportal for Cancer Genomics (<http://www.cbioportal.org/public-portal/index.do>). (A) Alterations in C9orf53 were visualized by cBioPortal for Cancer Genomics. Mutation, deletion, amplification and multiple alterations are shown in different colors. \*Indicating head and neck tumor. (B) Comparison of C9orf53 mRNA level with the copy number of C9orf53 in HNSCC. (C) C9orf53 deletion was correlated with lower patient survival rates. The median months of survival for patients with C9orf53 deletion mutation was 13.63 months. \*P<0.05. C9ORF53, CDKN2A antisense RNA 1; HNSCC, Head and neck squamous cell carcinoma; homdel, homozygous deletion; hetloss, heterozygous loss; TCGA, The Cancer Genome Atlas; WT, wild type.

and induce apoptosis and apoptosis. To the best of our best knowledge, this might be the first report of the role of C9orf53 in cancer.

The data from the present study indicated that the effects of C9orf53 on proliferation and apoptosis were relative minor. However, the difference in survival between patients with C9orf53 deletion and wild-type C9orf53 was significant. It is surprising that these minor influences were able to cause an effect on patient survival. It was hypothesized that although the effects of C9orf53 on cell proliferation and apoptosis were rather minor, the cells proliferated exponentially *in vivo*. Therefore, minor differences in C9orf53 were able to cause a long-lasting effect on patient survival (130 months).

Recently, a comprehensive landscape of somatic genomic alteration of HNSCC was provided by TCGA (24). It was indicated that human-papillomavirus-associated tumors are dominated by helical domain mutations of the oncogene phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$ . Smoking-related HNSCCs demonstrated near universal loss-of-function tumor protein p53 mutations and CDKN2A inactivation. Whether the role of C9orf53 in HNSCC is associated with human papillomavirus infection or smoking will be investigated in further studies.

In conclusion, the present study investigated the role of C9orf53 in HNSCC. The data indicated that the deletion



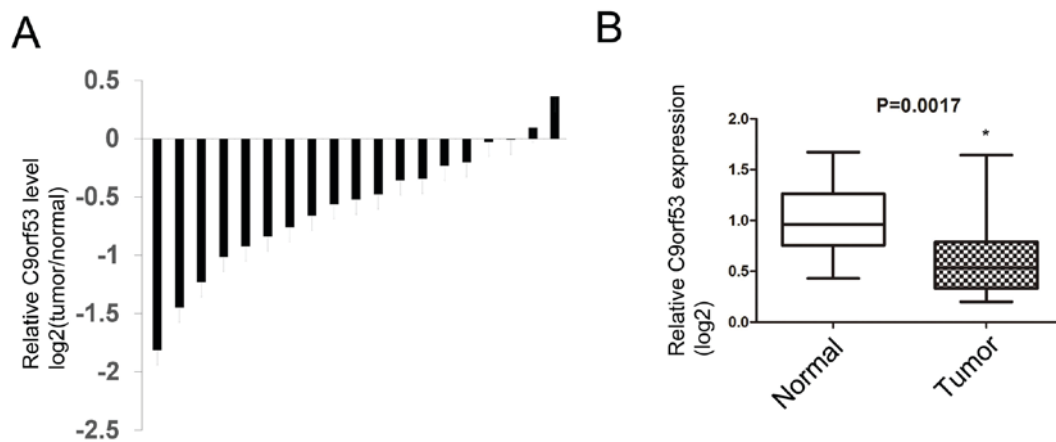


Figure 2. C9orf53 expression in HNSCC tissues is lower compared with normal tissues. The levels of C9orf53 in 19 surgical specimens of patients with HNSCC were assayed by RT-qPCR. The data are expressed in the form of  $\log_2(\text{tumor/normal})$ . (A) The levels of C9orf53 in 19 surgical specimens and in matched tumor-adjacent normal tissues. (B) All RT-qPCR experiments were performed in triplicate, and results are displayed in a box-plot. The difference between the groups was analyzed using t-test. \* $P < 0.05$ . C9ORF53, CDKN2A antisense RNA 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

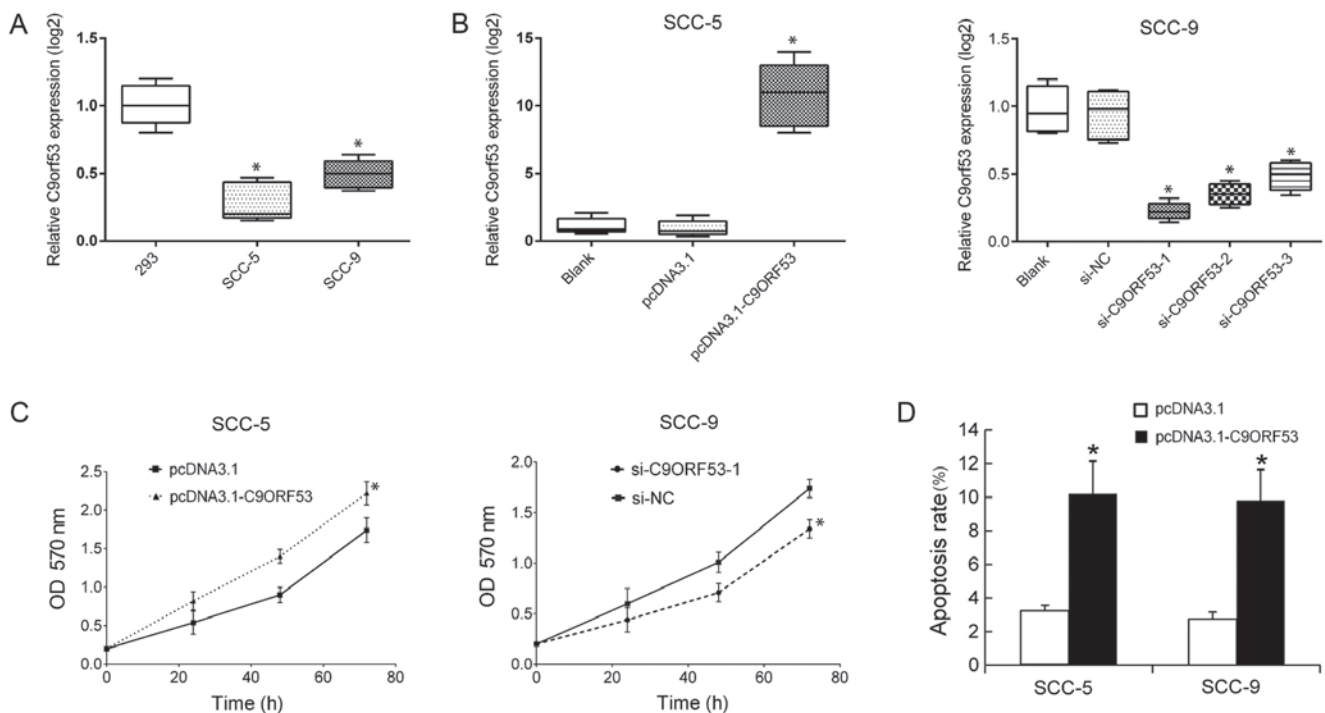


Figure 3. Suppression of C9orf53 promotes proliferation and induces apoptosis. The levels of C9orf53 in 293, SCC-5 and SCC-9 cells were assayed by RT-qPCR. (A) Data on C9orf53 expression are displayed in box plots. \* $P < 0.05$  vs. 293 cells. (B) The levels of C9orf53 in SCC-5 cells were assayed by RT-qPCR 48 h following transfection of pcDNA3.1-C9orf53. All data on C9orf53 expression are displayed in box plots. \* $P < 0.05$  pcDNA3.1-C9orf53 vs. control. (C) Following the transfection of pcDNA3.1-C9orf53 or siC9orf53-1, cell proliferation was analyzed by MTT assay at the indicated time points. (D) Cell apoptosis was analyzed by FACS, 48 h following the transfection of pcDNA3.1-C9orf53 or siC9orf53-1. All experiments were repeated four times. The differences between two groups were analyzed using two-tailed Student's t-test, and the differences between groups were analyzed using one-way ANOVA when three or more groups were compared. \* $P < 0.05$  vs. pcDNA3.1-transfected cells. C9ORF53, CDKN2A antisense RNA 1; FACS, fluorescence-activated cell sorting analysis; NC, negative control; OD, optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small-interfering RNA.

and decreased level of C9orf53 might promote the growth of HNSCC cells. The present study might provide a novel therapeutic target for further investigations.

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#### Availability of data and materials

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

#### Authors' contributions

GW, HJ and YL collected patient data and performed cell experiments. YW and XC performed transfection and apoptosis analysis. YZ and DW contributed to study design and manuscript writing.

#### Ethics approval and consent to participate

The present study was approved by Ethics Committee of Second Military Medical University (Shanghai, China).

#### Patient consent for publication

All patients gave informed consent for the use of their tissues and publication of the data.

#### Competing interests

The authors declare that they have no competing interests.

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