

TEAD1 Silencing Regulates Cell Proliferation and Resistance to 5-Fluorouracil in Cutaneous Squamous Cell Carcinoma

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Purpose: Cutaneous squamous cell carcinoma (cSCC) is a skin malignant tumor account for approximately one-third of all nonmelanoma skin cancers. Studies have shown that TEA domain transcription factor 1 (TEAD1) is discovered to be involved in the pathogenesis of some human cancers, but to our knowledge its role in cSCC has not been reported.

Patients and Methods: Samples from 16 cSCC patients and 27 healthy individuals were obtained for immunohistochemical staining of TEAD1. The expressions of TEAD1 in SCL-1, HSC-1 cells compared with the primary neonatal human epithelial keratinocytes were detected by Western blot and RT-qPCR. Proliferation and cell cycle of TEAD1 knockdown in cSCC cell lines were examined by MTT and flow cytometry analysis. Annexin V/PI and JC-1 staining were used to determine the cell apoptosis.

Results: The expression of TEAD1 decreased significantly in cSCC compared to its expression in normal skin tissues and cell lines. Down-regulation of TEAD1 in cSCC cell lines promoted cell growth via regulation of the G2/M progression. Additionally, silence of TEAD1 also protected cells against 5-Fluorouracil-induced apoptosis and decreased the expression of apoptosis-related protein (p53).

Conclusion: Our results suggested that TEAD1 expression is down-regulated and functioned as a tumor suppressor in cSCC and that it may serve as a biomarker or therapeutic target of cSCC.

Keywords: TEAD1, cutaneous squamous cell carcinomas, proliferation, apoptosis, p53

Introduction

Cutaneous squamous cell carcinomas (cSCC) are originated from the keratinocytes located in the epidermis or adnexal structures. They account for approximately one-third of all nonmelanoma skin cancers (NMSCs), with its incidence rising steeply for decades.¹ About 2% of cSCCs metastasize and their 5-year disease-specific survival was 79.1%.² More than two-thirds of patients with cSCC die from local invasion or nodal metastases, rather than distant organ metastases.³

In unresectable and metastatic cases, the treatment remains challenging. Targeted therapy with anti-EGFR (epidermal growth factor receptor) antibodies and PD-1 immune checkpoint inhibitors have been used in advanced cSCC.⁴ However, each of these methods has its own limitations. EGFR inhibitors have activity only in a subset of patients. Primary or secondary resistance is a problem that cannot be ignored for PD-1 immune checkpoint inhibitors.⁵ Further studies are needed to search for more predictive biomarkers and better therapeutic targets.

Hippo pathway is an important regulator of tumorigenesis.⁶ TEAD1 (TEA domain transcription factor 1) proteins belong to a family of 4 transcription factors (TEAD1–4), which are major downstream nuclear effectors of the Hippo signaling pathway.⁷ Previous research reported TEAD1 enhanced cell proliferation in colorectal cancer⁸ and mediated oncogenic activities in osteosarcoma.⁹ Nevertheless, to our knowledge, the role of TEAD family in cSCC has not been investigated. In this study, we examined TEAD1 expression in cSCC, as well as the biological function of TEAD1 by a knockdown strategy.

Materials and Methods

Patient Samples

Patient samples were acquired from the tissue bank of the Department of Dermatology at the Second Affiliated Hospital of Xi'an Jiaotong University. There were a total of 43 samples collected, including 16 cSCCs (9 males and 7 females, 49 to 83 years) and 27 normal skin tissues (17 males and 10 females, 44 to 80 years) obtained from cosmetic surgery. All biopsies were done under local anesthesia. Prior to the study, written informed consent for organizational procurement was obtained from all patients, and ethics approval was received from the Institutional Ethics Committee of Xi'an Jiaotong University. The studies were conducted in accordance with Declaration of Helsinki guidelines.

IHC

Immunohistochemical staining was performed according to standard protocol.¹⁰ Based on the following scoring system, two independent pathologists assessed and quantified the staining results under microscopes. Firstly, a positive rate score was estimated by the positive cell percentage (5% $\frac{1}{4}$ 0, 5%~25% $\frac{1}{4}$ 1, 25% ~50% $\frac{1}{4}$ 2, 50%~75% $\frac{1}{4}$ 3, >75% $\frac{1}{4}$ 4). Next, a score of staining intensity was given by objective colorless (colorless $\frac{1}{4}$ 0, yellow $\frac{1}{4}$ 1, yellowish brown $\frac{1}{4}$ 2, chocolate brown $\frac{1}{4}$ 3). Finally, the total score is added by aforementioned two scores. The average score of five fields was taken as the final score of TEAD1 expression for each slide.

Cell Culture

The human cSCC cell line SCL-1¹¹ and HSC-1¹² cells were from Beijing Beinachuanglian Biotechnology Research Institute (Beijing, China). The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 mg/mL). The cells were conventionally cultured at 37°C and 5% CO₂ in a humidified incubator. Lysates of primary neonatal human epithelial keratinocytes (HEKn cells) were kindly provided by Dr. Hao Wang at Xi'an Jiaotong University.

Transient Transfection of TEAD1 siRNA

The following siRNA oligonucleotides were purchased from GenePharma (Shanghai, China): TEAD1-1 siRNA (5'-CCACUGCCAUAUCAUACAATT-3'), TEAD1-2 siRNA (5'-CUGCAGAUAAAGCCAAUUGATT-3'). Cells were transfected with siRNA according to the procedure recommended by IMAX transfection reagent (Invitrogen, Carlsbad, CA). 100,000/well cSCC cells were plated into 6 well plate 24 hours before transfection.

MTT Assay

Cell proliferation was detected by MTT assay. Cells were seeded in 96-well plates with a density of 4×10^3 cells/well and transfected with siRNA 24 hours later. Then, relative cell numbers were measured every 24 hours by incubating cells with 0.4 mg/mL MTT followed by optical density (OD) reading. Average readings from three independent experiments were plotted at each time point. Six technical replicates were performed for each data point.

Cell Cycle Analysis

Cells were harvested and fixed with 75% ethanol at 4°C overnight. For the analysis, PI staining solution (50 mg/mL PI and 100 mg/mL ribonuclease A) was added to the cells and incubated for 30 minutes in darkness at 37°C. The cells were analyzed using NovoCyte flow cytometry (ACEA Biosciences). Three independent experiments were conducted.

Annexin V/PI Staining

The siRNA-transfected cells were treated with 5 μ g/mL of 5-Fu for 48 hours before harvest. For the analysis, cells were stained with the annexin V-fluorescein isothiocyanate/PI Apoptosis Detection Kit (BD Pharmingen), following the manufacturer's instructions. Stained cells were analyzed by NovoCyte flow cytometry (ACEA Biosciences). Three independent experiments were performed.

JC-1 Staining

The loss of MMP ($\Delta\Psi_m$), a hallmark of apoptosis, was measured using the Mitochondrial membrane potential assay kit with JC-1 from Beyotime (Shanghai, China) according to the manufacturer's instructions. After 5 $\mu\text{g/mL}$ of 5-Fu treatment of 48 hours, cells were treated with JC-1 probe (2 $\mu\text{g/mL}$) for 30 min at 37°C and measured at an excitation wavelength of 490 nm and an emission wavelength of 530/590 nm by a spectrophotometer (FLUOstar Omega, Labtech). Then, the $\Delta\Psi_m$ was displayed by a change in the ratio of red (aggregated JC-1) and green (monomeric JC-1) fluorescence. Three independent experiments were performed.

Western Blot Analysis

Protein expression levels were analyzed by standard Western blotting protocols. The following antibodies were used in this study: TEAD1 (ab133533) was purchased from Abcam (Cambridge, UK). Cyclin B1 (sc-595) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). P53 (10442-1-AP) was from Proteintech (Chicago, IL, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AC001) was from Abclonal (China).

RT-qPCR

Total RNA was extracted from the cells using the Trizol reagent (Invitrogen) and then reverse transcribed into complementary DNA. The primer oligonucleotide sequences were as follows: human TEAD1: forward 5'-TTCGCCACGTGTGTTTGTTC-3' and reverse 5'-ACGCTTACATCACAAGCCCA-3', human GAPDH: forward 5'-CTCCTCCACCTTTGACGCTG-3' and reverse 5'-TCCTCTGTGCTCTTGCTGG-3'. GAPDH was used as an internal control, and data were analyzed by the $2^{-\Delta\Delta\text{CT}}$ method.

Statistical Analysis

All data were expressed by the standard error of the mean (SEM). SPSS statistical software package 26 (SPSS, Chicago, IL, USA) was used for data analysis. The groups were compared by chi-square tests (χ^2) or independent-samples *t*-tests (*t*). $P < 0.05$ was considered statistically significant.

Results

TEAD1 Expression is Down-Regulated in cSCC

Immunohistochemistry was used to investigate TEAD1 protein expression in tissue samples from patients, including normal skin tissues ($n = 27$), cSCC ($n = 16$). The results showed cytoplasmic staining of TEAD1 (Figure 1A and B). The positive TEAD1 staining was significantly lower in the cSCC group than the control group ($\chi^2 = 8.159$, $P \leq 0.01$) (Table 1, Figure 1C). In addition, in contrast to the primary neonatal human epithelial keratinocytes (Figure 1D and E), TEAD1 RNA and protein expression were down-regulated in two cSCC cell lines, HSC-1, and SCL-1.

Si-TEAD1 Promotes cSCC Cell Growth via Regulation of the G2/M Progression

As for decreased expression of TEAD1 in cSCC skin lesions, we speculated that knockdown of TEAD1 may promote development of cSCC. Small-interfering RNAs (siRNAs) were used to knockdown TEAD1 in two cSCC cell lines, SCL-1 and HSC-1 (Figure 2A and B). Next, we determined cell proliferation and cell cycle by MTT and propidium iodide (PI) staining, respectively. The results showed that depletion of TEAD1 promoted cell growth in both cell lines (Figure 2C). In addition, TEAD1 knockdown prompted cell growth via the G2/M stage transition (Figure 2D), with the significant elevation of cell cycle regulator cyclin B1 (Figure 2E).

Si-TEAD1 Protects cSCC Cells Against Apoptosis

We explored the influence of TEAD1 on regulation of apoptosis, via annexin V/PI and JC-1 staining. Considering the low basal apoptosis levels, we firstly used 5-fluorouracil (5-Fu) to induce cell apoptosis. As expected, 5-Fu treatment increased apoptosis rate of SCL-1 cells. Then, we found that, compared to the control group, knockdown of TEAD1 reduced the apoptosis rate, which was promoted by 5-Fu (Figure 3A) and inhibited the activation of p53 (Figure 3C) in

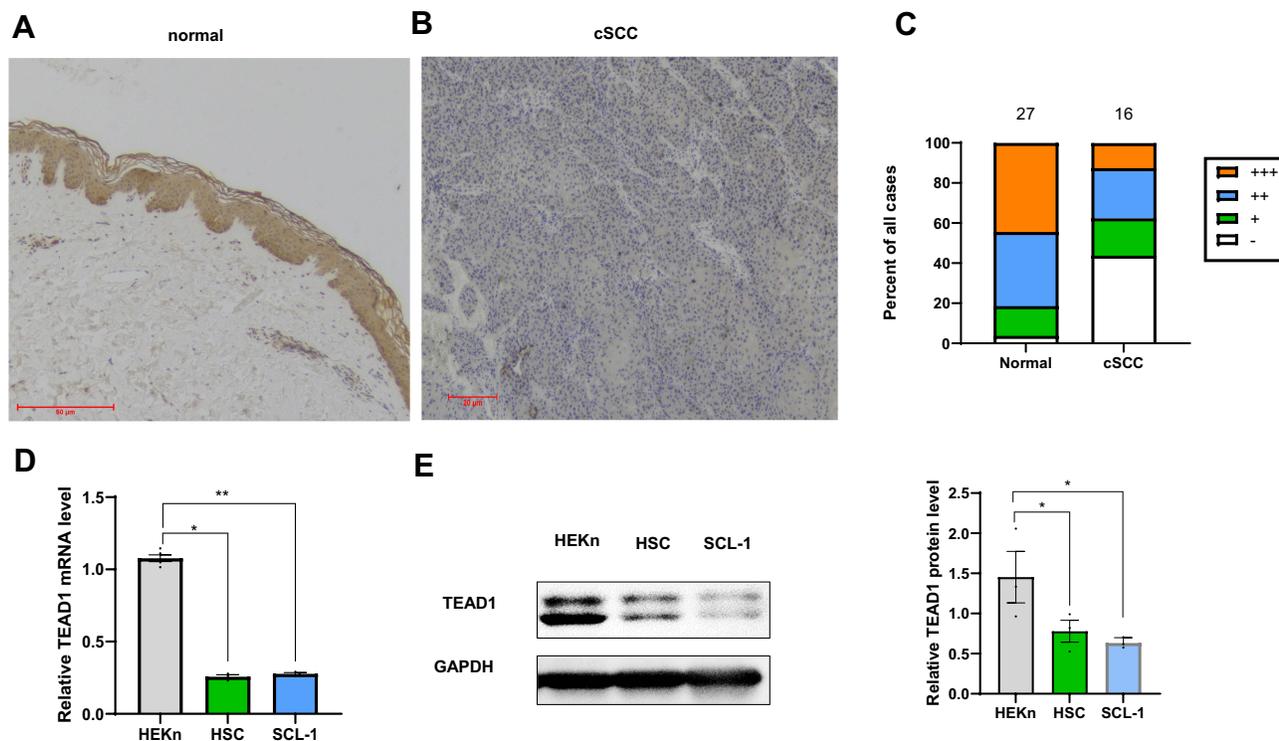


Figure 1 Analysis of TEAD1 expression in different tissues and cells. **(A)** Normal skin (n=27). Bar length =50µm. **(B)** cSCC (n =16). Bar length =20µm. **(C)** Semiquantitative analysis of TEAD1 staining. **(D)** qPCR analysis of TEAD1 RNA expression in HEK293n and two cSCC cell lines: HSC-1 and SCL-1. **(E)** Western blot analysis of TEAD1 protein expression in HEK293n and two cSCC cell lines: HSC-1 and SCL-1. All the quantitative data are presented as mean standard error of the mean (SEM). *P < 0.05, **P < 0.01. **Abbreviations:** cSCC, cutaneous squamous cell carcinoma; HEK293n, human epidermal keratinocyte-neonatal.

SCL-1 cells. JC-1 dye was adopted to detect the depolarization of the inner mitochondrial membrane, which was a signal of apoptosis initiation. Similarly, si-TEAD1 increased mitochondrial membrane potential against 5-Fu induced apoptosis compared with the control group (Figure 3B). Therefore, si-TEAD1 protected cSCC cells against 5-Fu induced apoptosis.

Discussion

TEAD1 is discovered to be involved in the pathogenesis of several kinds of human cancers.^{8,9,13,14} But its role in cSCC is still unclear. Here, we investigated the functional role of TEAD1 in cSCC. As a result, the TEAD1 was proved to be down-regulated in cSCC patient samples and cell lines. Knockdown of TEAD1 cSCC promotes cell survival and resistance to 5-Fu.

Although the central components of Hippo pathway are well defined, factors modulating Hippo transcriptional outputs remain elusive. Hippo effectors YAP and TAZ were co-activators for the TEAD family of transcription factors (TEAD1-4). YAP and TAZ can act either as tumor suppressors in the cytoplasm or tumor enhancer in the nucleus.¹⁵⁻¹⁹ Based on opposite pro- or anti-cancer YAP activity, cancers can be classified into YAP^{on} or YAP^{off}.²⁰ However, the

Table 1 Positive Expression Rates of TEAD1 (%)

Group	n	TEAD1				Positive Rate%	χ ²
		-	+	++	+++		
Case	16	7	3	4	2	9 (56.25)	8.159
Control	27	1	4	10	12	26 (96.2)**	

Notes: **P ≤ 0.01 compared with control group; χ², chi-square test; +++, strong positive, score 9-12; ++, moderately positive, score 5-8; +, weakly positive, score 1-4; -, negative, score 0.

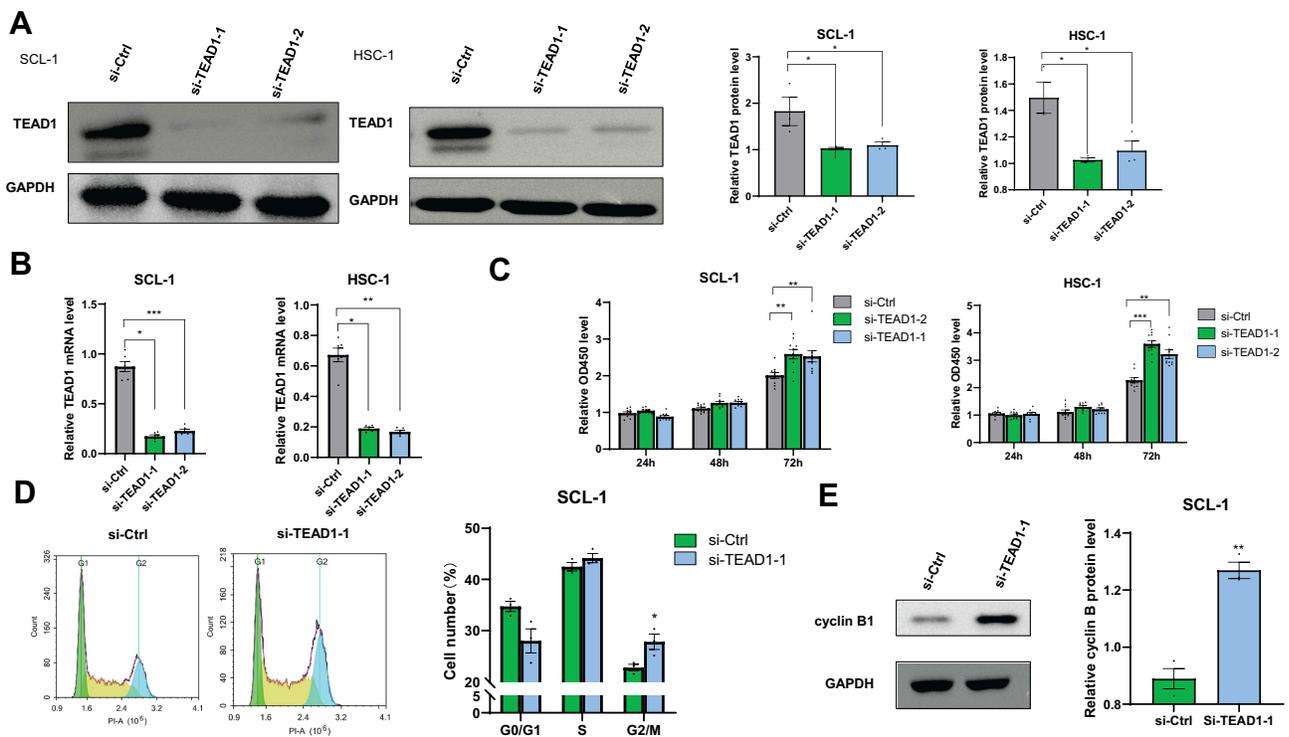


Figure 2 Effect of down-regulation TEAD1 on cell proliferation and cell cycle. **(A and B)** TEAD1 RNA and protein expression (48 hours after transfection) was measured after TEAD1-siRNA interference. **(C)** Cells were transfected with TEAD1 siRNA, and MTT assay was performed after 24, 48, and 72 hours. **(D)** The cell cycle profiles were analyzed 48 hours after siRNA by flow cytometry. **(E)** Cell cycle regulators were analyzed by Western blot analysis after TEAD1 knockdown, with GAPDH being an internal control. All the quantitative data are presented as mean standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: Ctrl, control; h, hours; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PI, propidium iodide; si, small interfering.

expression patterns of YAP and TAZ were not always the same, for example, seven cell lines of small cell lung cancer were YAP^{off} but one of them expressed TAZ.²⁰

Previous study has manifested that Hippo pathway is essential for skin homeostasis and epithelial stem cell maintenance. TEAD transcriptional-network, downstream of Hippo pathway, was reported to be down-regulated during keratinocyte differentiation.^{21,22} In primary human keratinocytes, TEAD1 and TEAD3 work crucially in the regulation of human epidermal proliferation and compensate for each other.²³

The expression of TEAD1 decreased significantly in cSCC and was mainly localized in the cytoplasm according to our study. Significantly, these results are consistent with the known tumor suppressor function of its subcellular localization. Recent studies indicated that YAP1 and TAZ both contribute to skin squamous (SCC) and basal (BCC) cell carcinoma formation.^{24,25} YAP1 has been identified as an oncogene in cSCC, which is expressed mainly in the nucleus.²⁶ It is possible that the function of TEAD1 may be independent of YAP1 because of different subcellular localizations. A similar example is that TEAD suppresses IL6 through the mechanism, which is independent of functional TEAD-YAP activity.²⁷ Therefore, there is abundant space for further exploration in investigating whether TEAD1 proteins make cytoplasmic translocation²⁸ in cSCC.

Our data also discovered that si-TEAD1 promoted cSCC cell growth by regulating the G2/M transition, and TEAD1 depletion increased the expression of cyclin B1, the key regulator of the G2/M cell cycle transition.²⁹ Similar regulation of cell proliferation by YAP/TEAD activity has been observed in other studies. For example, expressing YAP/TEAD inhibited 2D or 3D growth in retinoblastoma and reduced tumor load in small-cell lung cancer.²⁰

Mitochondrial membrane potential ($\Delta\psi$ m) is an indicator of mitochondrial state. Loss of $\Delta\psi$ is an early event in apoptosis.³⁰ Our results of JC-1 and Annexin V/PI staining support the anti-apoptotic role of si-TEAD1 against 5-Fu. 5-Fu, a widely used chemotherapy drug, can induce p53 activation and p53-dependent apoptosis.³¹⁻³³ Thus, we further detected p53 expression in TEAD1 knockdown cells with 5-Fu treatment. As expected, Western blot analysis indicates

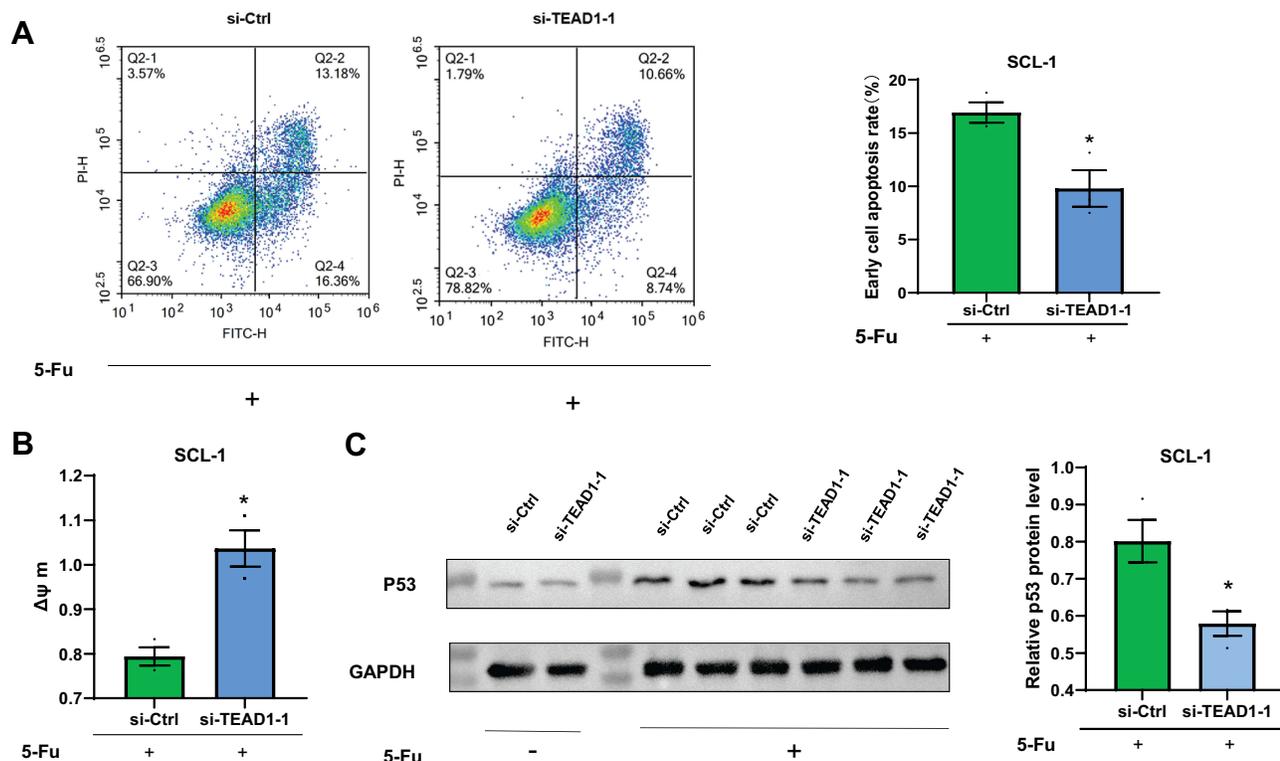


Figure 3 Effect of down-regulation of TEAD1 on apoptosis. **(A)** Cells were transfected with si-TEAD1 for 24 hours before the addition of 5 ug/mL of 5-fluorouracil. After another 48 hours, cell apoptosis was analyzed by annexin V/propidium iodide staining and flow cytometry analysis. **(B)** Cell apoptosis of **(A)** was analyzed by JC-1 ($\Delta\psi m$). **(C)** Western blot analysis on indicated proteins. GAPDH was used as an internal control. All the quantitative data were presented as the standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$.

Abbreviations: Ctrl, control; FITC, fluorescein isothiocyanate; PI, propidium iodide; si, small interfering; 5-Fu, 5-fluorouracil; $\Delta\psi m$, mitochondrial membrane potential.

that si-TEAD1 suppress p53 activation increased by 5-Fu. Interestingly, in addition to inducing apoptosis by TEAD1 in cardiomyocytes,³³ YAP/TAZ-TEAD expression is inversely proportional to apoptosis in existing research.²⁶ Its exact mechanism of action needs to be investigated in further studies.

Conclusion

In conclusion, our study indicated that TEAD1 plays a crucial role in human cSCC. Restoration of protein expression of TEAD1 may reduce tumor cell survival and drug resistance of 5-Fu in cSCC patients. In other words, it sheds light on another carcinogenic mechanism in the development of cSCC as well as novel target for treatment and prognostic purpose.

Acknowledgments

We thank Dr. Hao Wang at Xi'an Jiaotong University for providing the primary neonatal human epithelial keratinocyte lysates. This study was supported by grants from the National Natural Science Foundation of China (81573055, 81773328).

Disclosure

The authors report no conflicts of interest in this work.

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