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Original Article

Expression of transient receptor potential canonical 1 (TRPC1) in tongue squamous cell carcinoma and correlations with clinicopathological features and outcomes

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Abstract: Since transient receptor potential canonical 1 (TRPC1) is involved in cancer biology, this study aimed to investigate the expression of TRPC1 in tongue squamous cell carcinoma (TSCC) and to evaluate associations between tumor angiogenesis and clinical outcomes. TRPC1 mRNA and protein levels were measured in different cell lines. Immunohistochemical staining was conducted to detect TRPC1 protein expression in 72 primary TSCC specimens and 17 specimens of normal tongue mucosa. The correlations among TRPC1 and clinicopathological parameters were evaluated, and microvessel density was calculated in TSCC samples. Results demonstrated that TRPC1 was significantly overexpressed in both TSCC cell lines and tissue samples, compared with normal control. In addition, protein expression of TRPC1 was significantly correlated with EphA2, ephrinA1, e-NOS, VEGF-A expression and microvessel density of TSCC specimens. Unfavorable clinicopathological features and outcomes were observed in patients with high TRPC1 expression. Our results revealed that TRPC1 to certain extent is linked to angiogenesis and malignity of TSCC, indicating TRPC1 is a potential target for TSCC treatment.

Keywords: TRPC1, tongue squamous cell carcinoma, angiogenesis, clinicopathological features and outcomes

Introduction

Tongue squamous cell carcinoma (TSCC), arising from epithelial tissue of tongue, is a common neoplasm in head and neck region. Despite recent therapeutic advances, the five-year overall survival rate of TSCC patients remains unsatisfactory. The low survival is due to the development of local recurrence and distant metastases [1-3]. Therefore, understanding the pathogenesis of TSCC development and identification of new potential treatment targets are important for promoting therapeutic strategies.

Intracellular Ca^{2+} signaling is important for endothelial remodeling and endothelial progenitor cell activation [4, 5]. The transient receptor potential (TRP) channel is a superfamily of non-selective cation channels that participate in the regulation of tumor growth and progression by modulating Ca^{2+} influx and downstream signaling cascades [6, 7]. The TRP canonical (TRPC) subfamily is expressed in endothelial cells in the vascular system and involved in many fundamental roles in blood vessels [8, 9]. It has been noted that, as a member of the TRPC subfamily, TRPC1 is expressed in various kinds of human cancers both in tumor and endothelial cells. In addition, evidence has shown that inhibition or overexpression of TRPC1 channels can regulate cancer cell proliferation and survival, as well as migratory and invasive abilities [10-12]. TRPC1 may act as a potential therapeutic target in cancer biology [13].

Studies have shown that TRPCs are downstream effectors of the axon guidance molecules netrin-1 and brain-derived neurotrophic factor and are required for axon guidance triggered by these cues [14, 15]. A recent study has revealed that calcium channels can regulate ephrinA/EPH receptor A (EphA) expression, which are the axon guidance molecules [16]. We previously reported that overexpression of EphA2 in oral cancer was significantly correlated with angiogenesis [17] and that ephrin-A1 is upregulated by hypoxia in cancer cells and promotes angiogenesis through cross-talk with endothelial nitric oxide synthase (e-NOS) [18]. It has been demonstrated that TRPC1 plays an important role in regulating angiogenesis both *in vitro* and *in vivo* [19-21]. It was also indicated that TRPC1 might be alternative targets for anti-angiogenic therapy [22]. Furthermore, based on these known roles of EphA2, ephrinA1, e-NOS and vascular endothelial growth factor A (VEGF-A) in angiogenesis process, we speculated that these variables might be related to TRPC1 expression. However, the literature on the expression and functions of TRPC1 in TSCC is limited.

Here, we analyzed the expression levels of TRPC1 in TSSC cell lines and tissue specimens from TSCC patients. We further examined the correlations between the expression levels of TRPC1 and those factors associated with tumor angiogenesis, including microvessel density (MVD), EphA2, ephrinA1, VEGF-A and e-NOS. The relationships among parameters with clinical outcomes in terms of TRPC1 expression were also analyzed. Our results suggest that TRPC1 may perform important functions in TSCC angiogenesis and tumor development, which could serve as a potential biomarker or target for TSCC treatment.

Materials and methods

Antibodies

Primary antibody against TRPC1 (ACC-010) was purchased from Alomone Labs (Jerusalem, Israel), while antibodies against EphA2 (ab5386), ephrinA1 (ab199697), and VEGF-A (ab46154) were obtained from Abcam (Cambridge, MA, USA). CD34 (ZA-0550), e-NOS (TA336799) and GAPDH (TA336768) antibodies were acquired from ZSGB-BIO (Beijing, China).

Cell lines and cell culture

Three established TSCC cell lines (SCC-9, SCC-25 and Cal-27) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Human immortalized oral epithelial cells (HIOEC) (Shanghai Key Laboratory of Stomatology, Shanghai, China) [23-25], was obtained as gift for research from Professor Zhiyuan Zhang, were used as a normal control. Cal-27 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). SCC-9 and SCC-25 cells were cultivated in DMEM-F12 (Hyclone) supplemented with 10% FBS. HIOEC was cultivated in keratinocyte cell basal medium (Lonza, Walkersville, MD, USA) with 10% FBS. All cells were cultured in an incubator maintained at 37°C and supplied with 5% CO_2.

Real-time reverse transcriptase (RT)-polymerase chain reaction

Total RNA was extracted from the cells by using TRIzol (Invitrogen, Carlsbad, CA, USA). The following gene-specific primers were used for cDNA synthesis: TRPC1, forward: 5′-ATGTATACAACCAGCTCTATCTTG-3′ and reverse: 5′-AGTCTTTGGTGAGGGAATGATG-3′; and GAPDH forward: 5′-CTCCTGCACCACCAACTGCT-3′ and reverse: 5′-GGGCCATCCACAGTCTTCTG-3′ (Sangon Biotech, Shanghai, China). The relative expression of each gene was determined following the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal standard. Real-time RT-PCR was conducted in three times, separately. Data were quantified to GAPDH control and gene expression was calculated based on the standard curve.

Western blot analysis

Western blot was conducted following protocols described in a previous study [26]. The protein expression levels of TRPC1, EphA2, ephrinA1, e-NOS and VEGF-A in the above cell lines were detected by Western blot analysis. Western blot analysis was repeated at least 3 times to test reproducibility. GAPDH served as the reference protein for normalization.

Patients and tissue specimens

The study subjects consisted of 72 primary TSCC patients treated in the Hospital of Sto-

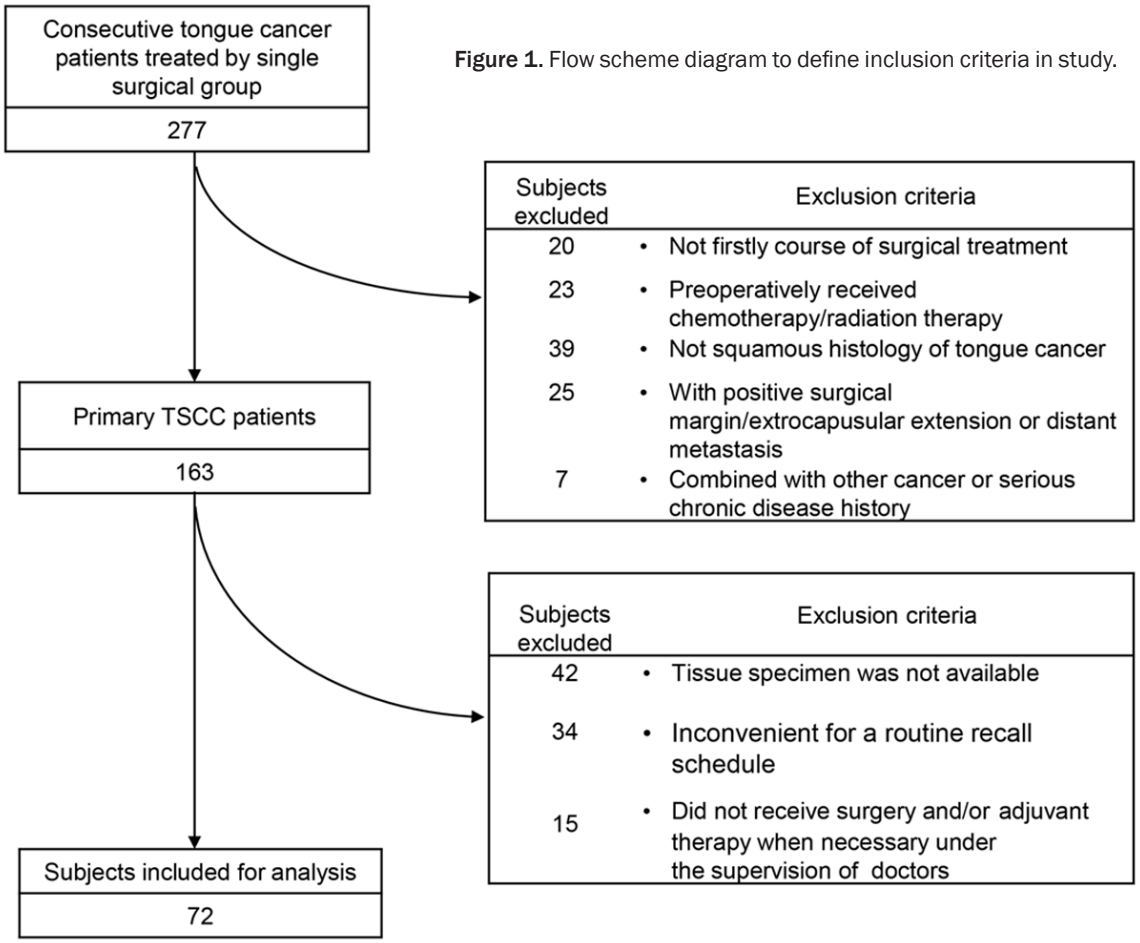

Figure 1. Flow scheme diagram to define inclusion criteria in study.

matology, Wuhan University, between February 2006 and July 2011. Patients were only included on premise that they were treated consecutively in a single surgical group. Inclusion criteria were strict in our study in order to control bias in terms of evaluating correlations between clinical outcomes. Inclusion criteria are shown in detail in **Figure 1**. Seventeen normal tongue mucosa specimens were obtained in our hospital as healthy controls.

Haematoxylin-eosin (HE) stained tissue sections of all samples were again assessed to confirm the correct diagnosis of TSCC. TNM classification and clinical stages of patients were evaluated using the 2002 criteria of the International Union Against Cancer [27]. A routine recall schedule combined with periodical phone calls was applied to obtain follow-up data. Written informed consent was provided to all subjects included, and the study was approved by the Medical Ethics Committee of School and Hospital of Stomatology, Wuhan University (Approval number: 2013LUNSHENZI103).

Immunohistochemical staining and evaluation

Immunohistochemical staining was conducted as described in a previous study [26]. Sections were incubated at 4°C overnight with primary antibodies (TRPC1, 1:200 dilution; EphA2 1:250 dilution; ephrinA1, 1:200 dilution; e-NOs, 1:300 dilution; CD34, 1:200 dilution; and VEGF-A, 1:200 dilution) and secondary antibodies for 1 h at room temperature. Negative controls (without primary antibody incubation) were used in each immunostaining step.

Typical fields were chosen randomly at a magnification of 200 and immunohistochemical staining was blindly evaluated and scored by two independent researchers, without prior knowledge of patient data, based on relative color area and intensity of the brown 3. 3'-diamino-

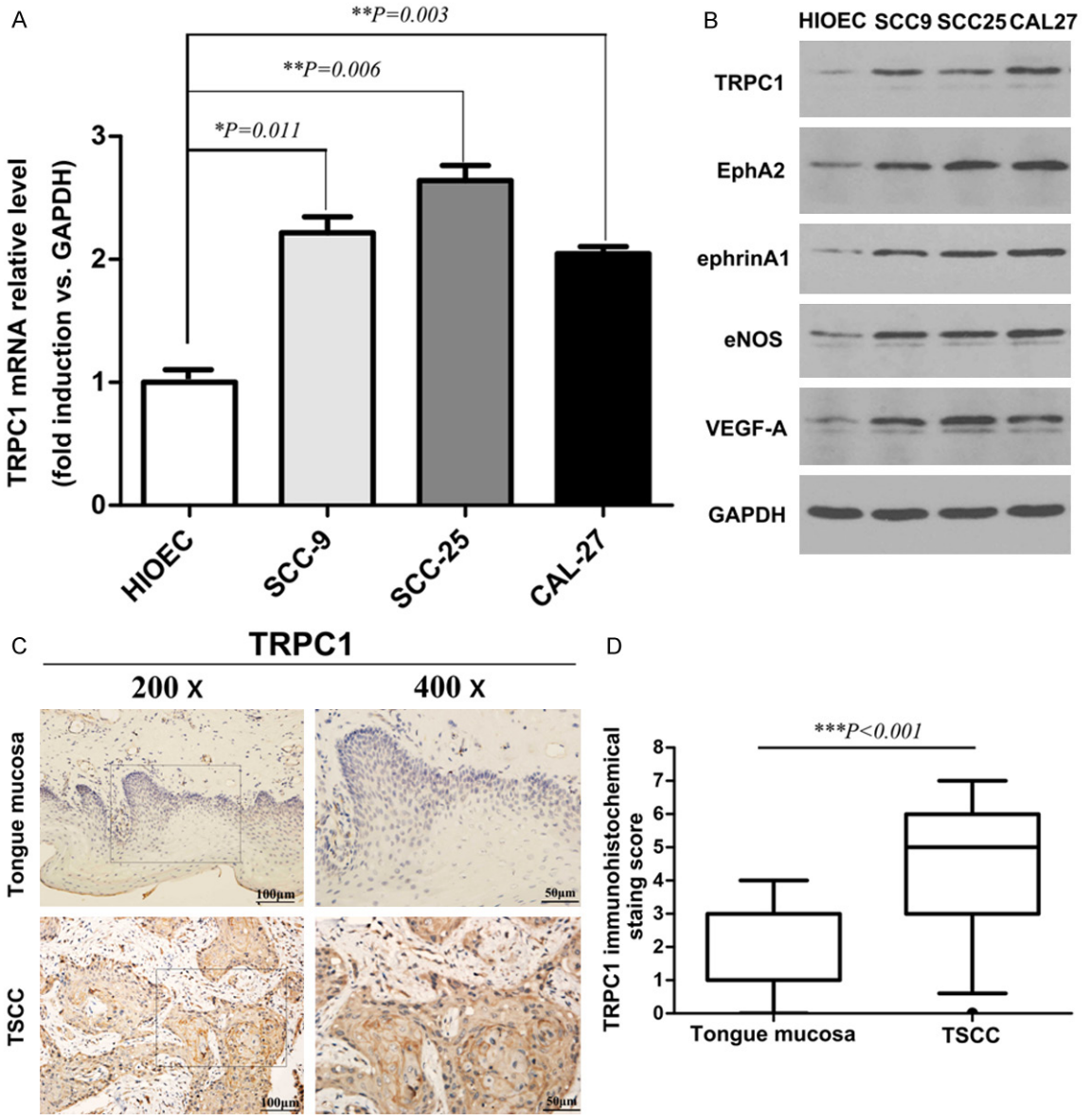

Figure 2. Different expression levels of TRPC1 in cell lines and tissue samples. A. Real-time RT-PCR showing TRPC1 levels in four cell lines. (Values are showed in mean ± SD in three independent experiments, **P*<0.05; ***P*<0.01 versus HIOEC by Student-t test). B. TRPC1, EphA2, ephrinA1, e-NOS and VEGF-A protein levels in four cell lines were detected by western blot analysis (GAPDH served as a reference protein). C. Representative immunohistochemical staining of TRPC1 in normal tongue mucosa and TSCC tissues (200 and 400× magnification). D. Different expression levels of TRPC1 in 17 samples of normal tongue mucosa and 72 samples of TSCC according to immunohistochemistry staining score. TRPC1 expression levels of TSCC are significantly higher than that of normal tongue mucosa (values are showed in box with 2.5-97.5 percentile whiskers, ****P*<0.001 versus normal tongue mucosa tissues by Mann Whitney test).

benzidine (DAB) signal for each section. A minimum of five 5 different fields were analyzed in each section. Staining intensity was scored as follows: 0, absence of staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The proportion of staining was evaluated as follows: 0, no staining of cells in microscopic field; 1, <25% of cells positive; 2, 25%-50% of cells positive; 3, 50%-75% of cells positive; and 4, >75% of cells positive. By adding both scores together, the final immunostaining score of each specimen was finally defined as an average score (0-7) of fields observed, three groups based on the final score were divided: 0-3, negative staining; 4-5, low staining; and 6-7, strong staining.

Table 1. TRPC-1 expression in normal tongue mucosa and TSCC samples

	TRPC1 expression			*P*
	Negative (score: 0-3)	Low (score: 4-5)	High (score: 6-7)	
Normal tongue mucosa	15	2	0	<0.001***
TSCC	18	33	21	

TSCC: Tongue squamous cell carcinoma; the value marked with *** mean statistically significant (*P*<0.001).

Evaluation of tumor angiogenesis

Signs of tumor angiogenesis were scored in all tissue sections by 2 investigators independently from each other, as described previously [17, 28]. Briefly, any single immunoreactive endothelial cell or endothelial cell cluster with CD-34 positive immunostaining was recognized as a countable microvessel. All microvessels were counted at 200× magnification. The average number of microvessels in at least 5 fields was recorded as the intratumor microvessel density (MVD).

Statistical analysis

Statistical comparisons of mRNA and protein levels in fourcell lines were performed by Student's t-test, and HIOEC was compared as control. Differences in continuous and categorical variables were examined by Chi square-test or Fisher's exact test as appropriate. Immunostaining scores were compared using Mann Whitney U Test in different groups indicated. Linear tendency and Spearman rank correlation test were used for correlations among indicated parameters. Logistic regression analysis was conducted for analyzing multiple clinicopathological parameters associated with TPRC1 expression levels. Survival rate was obtained by the Kaplan-Meier method and compared by log-rank tests. The total follow-up period was defined as 5 years after initial diagnosis. Overall survival (OS) time was defined from the point of initial diagnosis to the date of death; disease-free survival (DFS) time was confirmed as the time from the point of initial diagnosis to the date of TSCC relapse (recurrence or post-surgical metastasis). The missing data were censored by log-rank tests. The GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) statistical package or SPSS version 19.0 (Armonk, NY, USA) was used to perform statistical data analysis.

Results

Expression of TRPC1 in cell lines and TSCC tissues

Higher expression level of TRPC1 mRNA was found in the three TSCC cell lines compared to HIOEC by real-time RT-PCR (**Figure 2A**). In addition, western blot analysis revealed increases in the protein level of TRPC1 (**Figure 2B**) in TSCC cell lines compared to HIOEC.

Immunochemical staining was performed to investigate the distribution and expression level of TRPC1 in 72 primary TSCC tissues and 17 normal tongue mucosa tissues. TRPC1 immunoreactivity was homogeneous in TSCC tissues, and the intracellular distribution of TRPC1 was similarly diffuse, with both membrane and cytoplasmic staining (**Figure 2C**). TRPC1 proteins showed positive staining in 54 of 72 TSCC tissue samples. The mean score of TRPC1 in TSCC specimens was 4.47±1.583 (mean ± SD). In the normal tongue mucosa group, positive TRPC1 staining cells were mainly observed in basal/parabasal cells if any. The score of TRPC1 staining of normal mucosa was 2.15±1.085 (mean ± SD). TRPC1 staining was significantly stronger in TSCC compared to normal mucosa tissues (**Figure 2D**; **Table 1**). Only 2 specimens of normal tongue mucosa were detected with low TRPC1 staining (**Table 1**).

Correlations between TPRC1 and tumor angiogenesis

Anti-CD34 antibody was used to mark vascular endothelial cells to calculate the MVD (**Figure 3A**) within tumor sites, and the value of MVD was 18.3±7.7 (mean ± SD). Spearman correlation analysis was then conducted to quantify the relationship between TRPC1 and MVD, and they were correlated significantly (**Figure 3C**). Thus, it indicated that TRPC1 expression was positively related with tumor angiogenesis of TSCC.

Levels of EphA2, ephrinA1, VEGF-A and eNOS, which are indicative markers of angiogenesis up-regulation, were also elevated in TSCC cell lines by western blot analysis (**Figure 2B**). In addition, 51 serial sections were prepared to detect variables in similar areas in TSCC tissues. Representative serial section for immu-

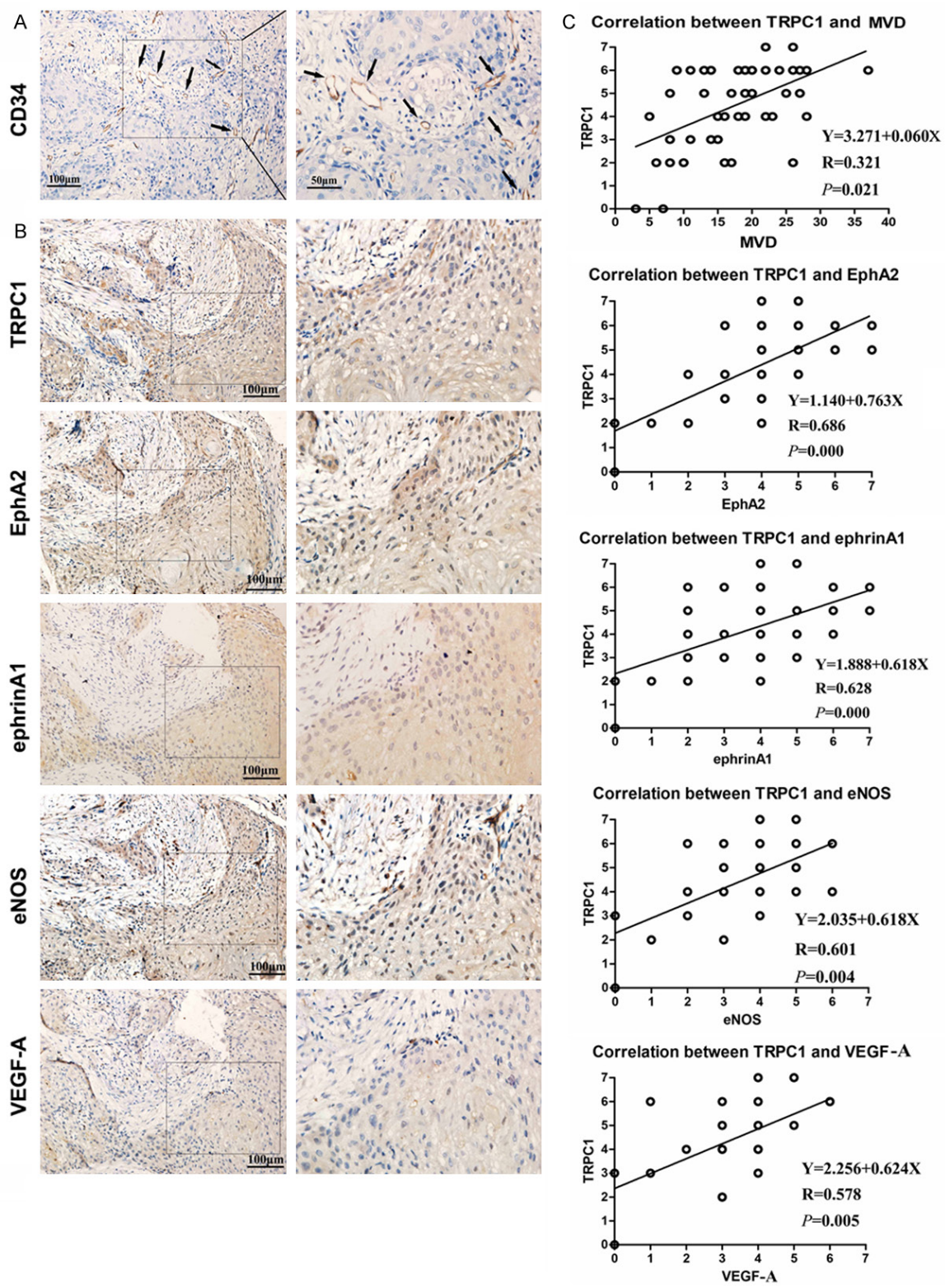

Figure 3. Associations among parameter associated with tumor angiogenesis in TSCC tissue. A. Representative staining of TRPC1, EphA2, ephrinA1, eNOS and VEGF-A is shown in a series section of one TSCC specimen. Local magnification (400×) is indicated by a frame and shown on the right. B. Representative staining of CD34 in TSCC tissues to mark microvessels (200 and 400× magnification). C. Correlation between TRPC1 and EphA2/ephrinA1/eNOS/VEGF-A and microvessel density, and linear tendency *P*-values were shown from Spearman rank correlation test.

Table 2. Clinicopathological variables of 72 TSCC patients stratified by TRPC1 expression levels

Variables	TRPC1 staining score			*P*
	0-3 (n = 18)	4-5 (n = 33)	6-7 (n = 21)	
Age (years)				
<58	8	23	15	0.139
≥58	10	10	6	
Gender				
Male	12	20	13	0.996
Female	6	13	8	
Tumor size (cm)				
<3	11	15	4	0.025*
≥3	7	18	17	
Clinical Stage				
I/II	17	16	8	<0.001***
III/IV	1	17	13	
Lymph node metastasis				
No	16	19	7	0.002**
Yes	2	14	14	
Tumor Differentiation				
Well/moderate	16	18	9	0.012*
Poorly	2	13	12	

The value marked with * mean statistically significant; *P<0.05; **P<0.01; ***P<0.001.

nochemical staining was shown in **Figure 3A**. The expression level of TRPC1 was significantly correlated with EphA2, ephrinA1, e-NOS and VEGF-A expression in TSCC sections (**Figure 3C**).

Relations between TPRC1 and clinicopathologic features

Clinicopathologic features of TSCC patients were stratified according to TRPC1 expression levels (**Table 2**). The associations between TRPC1 expression levels and clinicopathologic parameters of TSCC were investigated by logistic regression analyses. TRPC1 showed low/strong staining was identified as a positive event in terms of studying clinicopathologic parameters, and odds ratios (OR) were revealed in **Table 3**. Collectively, TPRC1 expression was positively correlated with clinical stage, lymph node metastasis and tumor differentiation of TSCC.

Relations between TRPC1 expression level and TSCC prognosis

Five patients (6.9%) were lost in the total follow-up period. There were 29 patients alive without relapse (local recurrence and/or regional/distant metastasis), and the disease-free survival rate of total subjects was 40.3%. During total follow-up period, 43 patients showed relapse. Sequential therapies (e.g. secondary surgery and/or adjuvant radiotherapy or chemo-radiotherapy et al.) were conducted for patients according to the suggestions and guidance of the treatment group. Among the patients who showed relapse; there were 13 who were still alive at the end of the follow-up. The other 30 patients passed away or lost during follow-up. The result of Kaplan-Meier survival analysis implied that high TRPC1 expression (score: 6-7) presented a significantly adverse prognostic impact on both patients' overall survival rates and disease-free survival rate, compared with negative and low TRPC1 expression (score: 0-5) group, revealed by the results of the log-rank test (**Figure 4**).

Discussion

Biomarkers in TSCC are worth exploring to improve prognosis and to identify possible therapeutic targets. Intracellular Ca^{2+} signaling ubiquitously regulates physiological and pathological processes, including signaling cascades of tumor genesis. TRPC1 could modulate store-operated calcium entry and then regulate cellular functions such as contraction, proliferation, and migration [6]. TRPC1 is expressed in many kinds of tumors. In non-small cell lung cancer, TRPC1 expression is associated with tumor differentiation [29]. In human colon carcinoma cells, upregulated TRPC1 contributes to enhanced store-operated Ca^{2+} entry, which correlates with increased tumor cell proliferation, invasion and survival characteristics [30]. However, the role of TRPC1 in TSCC has not been yet explored. In our study, it is demonstrated that TRPC1 expression is overexpressed in TSCC cell lines and tissues, compared with normal control.

Table 3. Correlations between positive TRPC1 expression and clinicopathological features of TSCC patients (logistic regression model)

Clinicopathological parameter	Categories compared	TRPC1 expression (0 = staining score <4, 1 = staining score ≥4)	
		OR (95% CI)	*P*
Tumor size (cm)	Ref = <3; 1 = ≥3	2.310 (0.523, 10.211)	0.270
Clinical stage	Ref = I/II; 1 = III/IV	20.010 (2.218, 180.527)	0.008**
Lymph node metastasis	Ref = No; 1 = Yes	6.608 (1.155, 37.811)	0.034*
Tumor differentiation	Ref = Well/moderate; 1 = Poorly	8.286 (1.454, 47.213)	0.017*

The value marked with * mean statistically significant by logistic regression analysis; *P<0.05; **P<0.01; 95% CI, the 95% confidence intervals. Ref, Reference; OR, Odds ratio.

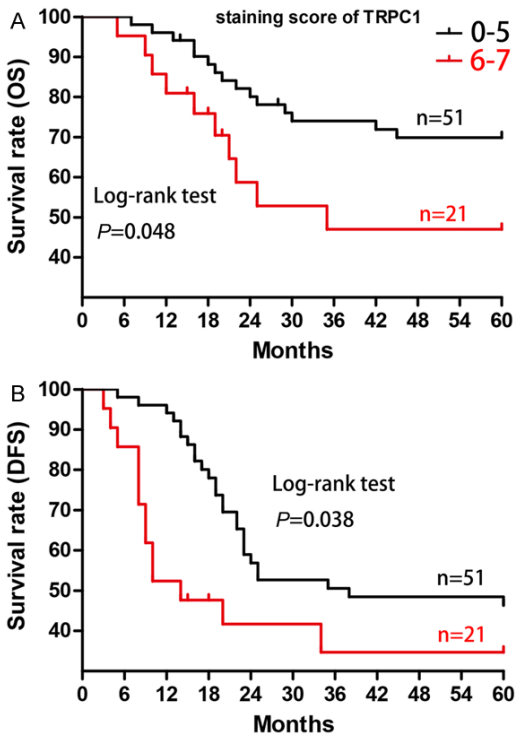

Figure 4. Kaplan-Meier graphs representing the probability of cumulative overall and disease-free survival in patients with TSCC based on TRPC1 expression. Log-rank survival analysis identified the relationship between TRPC1 expression and overall/disease-free survival of TSCC patients. A. High TRPC1 expression in TSCC was associated with poor overall survival. *P*-value from log-rank test is shown. B. High TRPC1 expression was associated with poor disease-free survival in TSCC patients. *P*-value revealed by log-rank test is shown. OS, overall survival rate; DFS, disease-free survival rate.

Growth and metastasis of TSCC are highly dependent on tumor angiogenesis, which is regarded as a complex process that involves many proteins and signaling pathways [31, 32]. TRPC channels are involved in angiogenesis in several respects, such as hypoxia-induced VEGF expression, VEGF-induced elevation of intracellular Ca^{2+} and regulation of vascular permeability [33, 34]. It has been shown that TRPC1 is involved in hypoxia-induced VEGF expression in U-78 MG cells and acts synergistically with VEGF-A in controlling intersegmental vessel growth in zebrafish larvae [19, 33]. In addition, anti-TRPC1 antibody inhibits VEGF-induced Ca^{2+} entry and increases endothelial permeability [34]. TRPC1 also plays a crucial role in regulation for the invasion, migration, and proliferation of thyroid cancer cells by regulating the VEGF receptor expression [12].

In recent years, several studies have also addressed the importance of EphA2/ephrinA1 function in the context of tumor angiogenesis. Tumor vasculature-specific expression of EphA2 and ephrinA1 was first reported in the blood vessels of breast carcinoma and Kaposi's sarcoma xenografts. EphrinA1 is regarded as a chemoattractant for endothelial cells and EphA2 is closely related to endothelial morphology. Furthermore, EphA2/ephrinA1 interaction also plays an important role in regulating angiogenesis [35, 36]. EphrinA1 is closely related to VEGF in endothelial cells [37, 38]. In hypoxic environments, ephrinA1 function can also be regulated by HIF-1α [39]. What's more, ephrin-A1 is overexpressed and promotes angiogenesis in tumor microenvironment through cross-talk with e-NOS and the PI3K/Akt pathway [18].

In our study, we demonstrated that the expression level of TRPC1 was positively correlated with those of EphA2/ephrinA1, VEGF, e-NOS and MVD separately. Our results are taken to suggest that EphA2/ephrinA1 and TRPC1 may share similar regulation mechanisms involving

VEGF, e-NOs and hypoxic environment in tumor angiogenesis. Although the present study didn't establish specific mechanisms, several signaling pathways such as the ERK/RSK and PI3K/AKT pathways may take part in this process [40].

In keeping with demonstrating TRPC1 overexpression in TSCC, up-regulated TRPC1 expression was also correlated with unfavorable clinical and pathological characteristics of TSCC patients. TRPC1 expression showed significant associations with clinical stage, tumor differentiation and regional lymph node metastasis. Furthermore, the survival analyses revealed that high TRPC1 expression (score: 6-7) was a risk factor for both overall survival and disease-free survival rate of TSCC patients. Inclusion criteria were strict in our study in order to control bias in terms of evaluating relationships between TRPC1 expression and clinical outcomes (recurrence, post-surgical metastasis and survival time) since many factors (e.g. surgical techniques, subsequent therapeutic scheme) play roles in prognosis of TSCC patients. All patients were treated in one surgery team with a uniform treatment guidance established. However, our results are limited to some extent. It still could be speculated that a larger sample size is still needed in order to demonstrate more conclusively that TRPC1 is an independent risk factor for unfavorable outcomes of TSCC patients. However, our present data did reveal that TRPC1 is expressed abnormally in TSCC and associated with unfavorable clinicopathological outcomes. To our knowledge, this is the first report on poor prognosis due to TPRC1 up-regulation in TSCC patients.

Taken together, the present study suggests that TRPC1 is overexpressed in TSCC specimens and cell lines. This overexpression seems to be associated with unfavorable clinicopathological features and outcomes, suggesting that it may promote the development of TSCC. Further clinical and basic research studies are still necessary to investigate the possible mechanisms underlying the observed phenomena.

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Disclosure of conflict of interest

None.

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