

Original Article

TBL1XR1 is a potential prognostic biomarker for tongue squamous cell carcinoma

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Abstract: Background: The transducin (β)-like 1 X-linked receptor (TBL1XR1) is an F-box/WD40-repeat containing protein that has been implicated in the development and progression of several cancers. The aim of this study is to investigate the expression and prognostic significance of TBL1XR1 in tongue cancer. Methods: TBL1XR1 expression was examined by Real-time PCR in 10 pairs of surgically resected tongue cancer and adjacent non-tumorous tissues. In addition, immunohistochemistry was performed to examine TBL1XR1 expression in 71 paraffin-embedded tongue squamous cell carcinoma (TSCC) samples. Associations of TBL1XR1 expression with clinicopathological factors and prognosis in TSCC patients were analyzed. Results: Upregulated TBL1XR1 mRNA expression was observed in tongue cancer tissues comparing with that in adjacent non-tumorous tissues. Overexpression of TBL1XR1 significantly associated with tumor stage ($P = 0.028$), pathological grade ($P = 0.003$), and cervical nodal metastasis ($P = 0.017$). The Kaplan-Meier survival analysis showed that high TBL1XR1 expression had a reduced overall survival ($P = 0.004$) in TSCC patients. The Cox regression survival analyses identified TBL1XR1 as an important independent predictor for patients' overall survival ($P = 0.042$). Conclusion: Our results suggest that an increased TBL1XR1 expression is involved in tumorigenesis and tumor development of tongue cancer, and that TBL1XR1 represents a novel potential molecular therapeutic target for TSCC.

Keywords: TBL1XR1, TSCC, tumorigenesis, prognosis

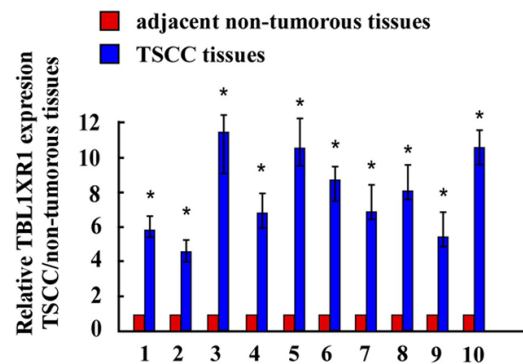
Introduction

Tongue cancer is the most common cancer diagnosed within the oral cavity, with 13,590 new cases and 2150 deaths in the United States alone in 2014 [1]. Tongue squamous cell carcinoma (TSCC) is the most common type of tongue cancer, which is notorious for its aggressive malignance with rapid growth rate and high chance of regional and distant metastasis [2]. The prognosis of TSCC has been improved substantially in these decades as the great advances achieved in surgery and in chemoradiotherapy technology. However, patients with TSCC still have a high risk of developing secondary or recurrent tumors [3]. Unfortunately, the 5-year overall survival rate in patients with lymph nodes spread does not exceed 50% [4]. Therefore, there is an urgent need to identification of novel and improved markers for the diagnosis and treatment of tongue cancer.

Transducin (β)-like 1 X-linked receptor (TBL1XR1), a member of a small family of proteins that include at least two closely related isoforms, X- and Y linked proteins, was initially identified as a component of an SMRT/N-CoR corepressor complex [5]. The TBL1XR1 gene is located at chromosome 3q26.32, encodes an F-box/beta-transducin (WD-40) repeat-containing protein, which contains a lissence-phalytype-1-like homology motif (LisH domain) in its N-terminal region and 8 WD-40 repeats in its C-terminal region [6]. TBL1XR1 functions as an E3 ubiquitin ligase in the recruitment of the UbcH5 ubiquitin conjugating enzymes/19S proteasome, thereby resulting in proteasomal degradation, and the subsequent exchange and transcriptional activation of the SMRT/N-CoR corepressor complex [7]. In addition, it has been implicated to play important roles in the activation of Wnt- β -catenin and NF- κ B signaling pathways [8]. Previous studies have reported

Table 1. Associations of TBL1XR1 expression with clinicopathological characteristics in TSCC

Variable	Categories	No.	TBL1XR1		P
			High	Low	
Age (y)	≤60	43	14	29	0.784
	>60	28	10	18	
Sex	Male	41	13	28	0.663
	Female	30	11	19	
Tumor Stage	I-II	45	11	34	0.028
	III-IV	26	13	13	
Tumor Grade	I	38	7	31	0.003
	II-III	33	17	16	
Cervical Node	-	46	11	35	0.017
	+	25	13	12	
Local Invasion	No	56	18	38	0.568
	Yes	15	6	9	

**Figure 1.** Expression of TBL1XR1 is increased in tongue cancer tissues compared with non-tumorous tissues through qRT-PCR.

that overexpression of TBL1XR1 could be detected in several malignancies such as lung squamous cell carcinoma, cervical cancer, and breast cancer [9-11]. However, the biological function and clinical significance of TBL1XR1 in the development of TSCC remain unknown.

In the present study, we performed a systematic analysis of the TBL1XR1 gene for its role in TSCC development and its prognostic role in patients with tongue cancer. We found that TBL1XR1 was overexpressed in TSCC tissues compared with that in adjacent non-tumorous tissues. Furthermore, we showed that TBL1XR1 expression was associated with several clinicopathological factors and clinical prognosis. Our data point to the possibility that TBL1XR1 may be used as a biomarker of prognosis and a potential therapeutic target in tongue cancer.

Materials and methods

Patients

The study group included a retrospective cohort of 71 cases of primary TSCC who were diagnosed at the Department of Stomatology in Foshan Hospital of Chinese Traditional Medicine between January 2001 and December 2009. For the use of these clinical materials for research purposes, informed consent from all patients and approval from the Institute Research Ethics Committee were obtained in accordance with our institutional guidelines. The patient characteristics are presented in **Table 1**. All patients underwent surgery, and the surgical specimens were examined and histologically confirmed as TSCC at the Department of Pathology in Foshan Hospital of Chinese Traditional Medicine. Patients with prior chemotherapy or radiotherapy were not included in this study. Overall survival was calculated as the time interval between the date of surgery and the last follow-up or the date of death. Disease-free survival was defined from the date of treatment until the time of recurrence or metastasis.

RNA extraction and real-time PCR

Total RNA from 10 pairs of fresh tongue cancer and adjacent non-tumorous tissues were extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was pretreated with RNase-free DNase, and about 1 mg RNA from each sample was used for cDNA synthesis. The cDNAs were amplified and quantified in ABI Prism 7500 Sequence Detection System (Applied Biosystems). The primer sequences are: TBL1XR1, forward primer: 5'-GAATTCCT-TGTGCC TCCAT-3', reverse primer: 5'-TGCAAC-TGAATATCCGGTCA-3'; GAPDH, forward primer: 5'-GACTCATGACCACAGTCCATGC-3', reverse primer: 5'-AGAGGCAGGGATGATGTTCTG-3'.

Immunohistochemistry

Immunohistochemical staining was performed on formalin fixed paraffin-embedded biopsy specimens that were obtained in the pre-treatment period and preserved in the departmental archives. In brief, paraffin-embedded specimens were cut into 5 μm sections and baked at 60°C for 3 h followed by deparaffinization with xylene and rehydrated. Antigen retrieval

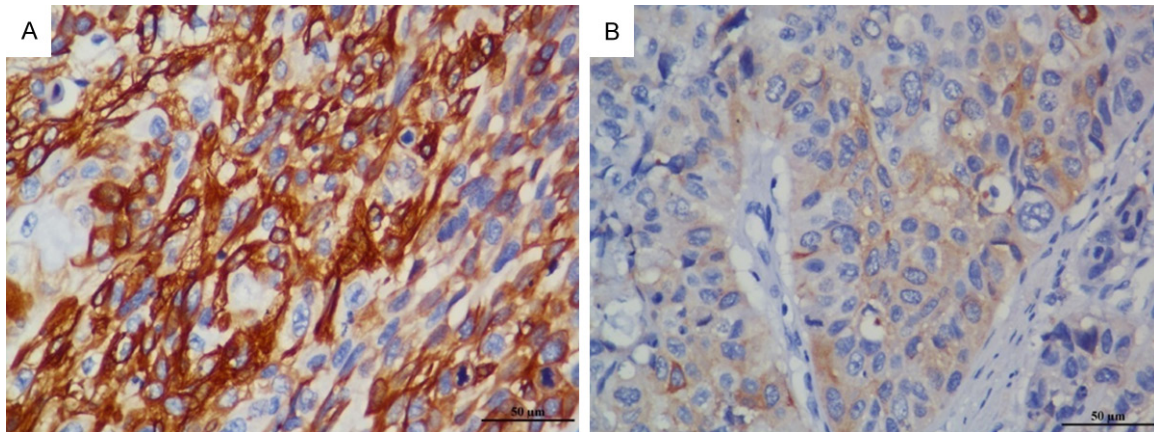


Figure 2. Representative immunostainings for high (A) and low (B) TBL1XR1 expression in TSCC tissues.

was performed by heating slides for 30 min in citrate buffer (pH = 6.0) at 90°C, and then washing. Endogenous peroxidase was quenched with methanol and 3% H₂O₂ for 10 min, followed by incubation with 1% bovine serum albumin to decrease nonspecific binding. Sections were incubated with polyclonal rabbit anti-TBL1XR1 antibody (1:200; Abcam) for 1 h, at room temperature (37°C), at a dilution 1:200. After washing with PBS, the slides were incubated with prediluted secondary antibody, followed by further incubation with diaminobenzidine (DAB). Sections were counterstained with Harris' hematoxylin and mounted in Entellan.

Evaluation of immunohistochemistry

Two investigators without prior knowledge of the clinical data assessed the degree of immunostaining based on the proportion of positively stained tumor cells and intensity of staining. The staining results were scored based on the following criteria: percentage of positive tumor cells in the tumor tissue: 0 (0%), 1 (1-30%), 2 (31-50%), 3 (51-80%) and 4 (81-100%); staining intensity: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The SI was calculated as staining intensity score and the proportion of positive tumor cells. An optimal cutoff value was identified: the SI >6 was used to define as high TBL1XR1 expression while SI ≤6 as low TBL1XR1 expression.

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for Social Sciences),

Version 16.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was applied for measuring the association between the clinical parameters and the immunohistochemical results of TBL1XR1. Kaplan-Meier product limit method and log-rank test were used for survival analysis. Cox regression analysis was applied to find out the association between the score and the event after adjusting for the other co-variants. P<0.05 in all cases was considered statistically significant.

Results

TBL1XR1 is overexpressed in tongue cancer tissues

To initially explore the TBL1XR1 expression in tongue cancer, we first evaluated both mRNA and protein levels of TBL1XR1 in 10 pairs of tongue cancer and adjacent non-tumorous tissues. As shown in **Figure 1**, TBL1XR1 mRNA levels in the cancerous tissues were significantly higher than that in non-cancerous tissues as assessed by Real-time PCR assay. The results indicate that TBL1XR1 is aberrantly overexpressed in tongue cancer tissues analyzed here. The numbers in bold indicates statistically significant.

Clinicopathological characteristics of TSCC patients and TBL1XR1 expression pattern

Our initial results of TBL1XR1 overexpression in tongue cancer tissues prompted us to hypothesize that TBL1XR1 may be a novel biomarker for tongue cancer with key oncogenic roles and clinical significance. To address this, we next evaluated TBL1XR1 expression levels by immu-

TBL1XR1 is a prognostic marker for TSCC

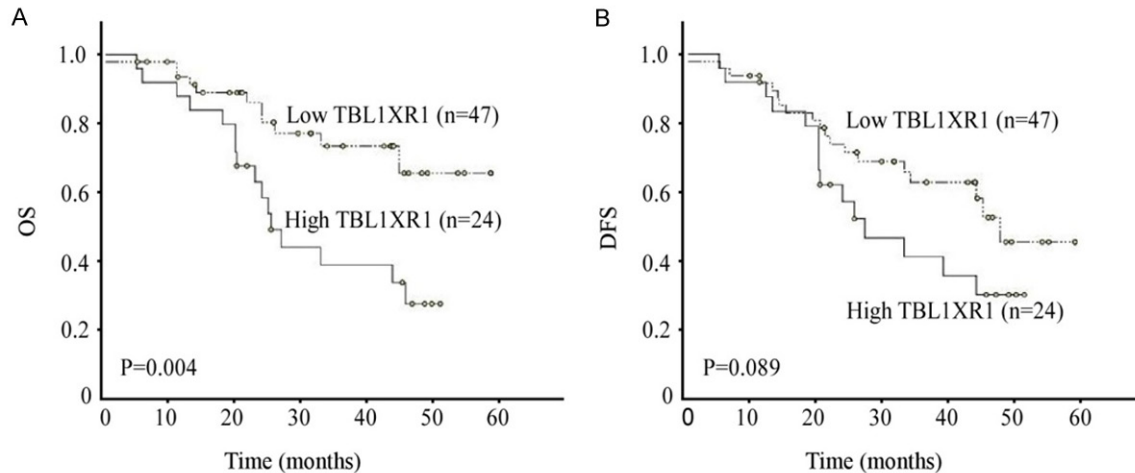


Figure 3. Kaplan-Meier survival analysis stratified according to TBL1XR1 expression in TSCC patients. A. TBL1XR1 expression and patients' overall survival. B. TBL1XR1 expression and patients' disease-free survival.

Table 2. Multivariate survival analysis of OS and DFS for patients with TSCC

Variables	Overall Survival		Disease-free Survival	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (>60 vs ≤60)	1.972 (0.409-5.220)	0.568	1.554 (0.355-4.303)	0.503
Tumor Stage (III-IV vs I-II)	1.747 (0.638-5.049)	0.046	1.527 (0.484-4.450)	0.256
Tumor Grade (II-III vs I)	2.905 (0.419-7.319)	0.119	2.629 (0.312-7.015)	0.160
Cervical Node (+ vs -)	2.573 (1.250-6.313)	0.034	2.921 (1.049-5.671)	0.038
Local Invasion (+ vs -)	2.601 (1.516-7.348)	0.027	2.577 (1.421-7.261)	0.058
TBL1XR1 expression (high vs low)	2.709 (1.064-8.076)	0.042	2.908 (1.237-8.339)	0.064

nohistochemical staining in a retrospective cohort of 71 samples from TSCC patients. The representative immunostaining of TBL1XR1 in TSCC was shown in **Figure 2**. In accordance with our immunohistochemistry scoring, the correlation of TBL1XR1 expression pattern and clinicopathological characteristics of TSCC was summarized in **Table 1**. The data showed that TBL1XR1 expression tended to be positively correlated with tumor stage ($P = 0.028$), pathological grade ($P = 0.003$), and cervical nodal metastasis ($P = 0.017$). No significant differences were found between TBL1XR1 expression and other clinicopathological findings such as age, gender, or local invasion.

TBL1XR1 expression associated with TSCC patients' clinical survival

To determine potential relationship between TBL1XR1 expression and TSCC patients' prognosis, we then evaluate the correlations between TBL1XR1 expression and clinical outcomes. As shown in **Figure 3**, patients with

higher TBL1XR1 expression in cancer tissues had worse overall survival ($P = 0.004$) than those with lower TBL1XR1 expression. Multivariate analysis indicated that TBL1XR1 expression was an independent prognostic factor of patient's overall survival ($P = 0.042$), as shown in **Table 2**.

Discussion

In the present study, we found that the expression of TBL1XR1 was increased in TSCC samples in comparison with samples of normal adjacent tissues. High TBL1XR1 expression was positively correlated with clinical stage and tumor metastasis, but was not associated with other clinicopathological variables. Moreover, the expression of TBL1XR1 was significantly associated with the clinical prognosis of patients with TSCC. Our study is the first to demonstrate the roles of TBL1XR1 in the diagnosis and prognostic assessment of TSCC patients.

TBL1XR1 has been found as a core component of the nuclear receptor corepressor complex, which contains TBL1X, GPS2, IR10, and HDAC3, and is responsible for repression of many different transcription factors, including AR and other nuclear hormone receptors (NHR) [12]. In addition, TBL1XR1 has two putative phosphorylation sites and one SUMO modification site, and is susceptible for posttranslational modifications, including phosphorylation and sumoylation [13]. Recent evidence suggested that TBL1XR1 may play a crucial role in carcinogenesis, tumor invasion, metastasis, and developing chemo/radioresistance. TBL1X can bind to T cell factor/lymphoid enhancing factor and play roles for the recruitment of β -catenin to Wnt target gene promoters, which is crucial for β -catenin mediated oncogenesis [14]. Likewise, TBL1XR1 can promote tumor cell proliferation and tumorigenicity through the activation of Wnt/ β -catenin signaling pathway in breast cancer, while depletion of TBL1XR1 by shRNA reduces the recruitment of β -catenin to its target gene promoters [9]. Moreover, TBL1XR1 can directly interact with BCL-3 and is involved in NF- κ B-mediated tumorigenesis [15]. Chen et al. demonstrated that upregulation of TBL1XR1 leads to resistance of the tumor cells to cisplatin by activating the NF- κ B pathway in NPC cells [15], they were supported by Wang et al., who reported that TBL1XR1 promotes invasion by inducing epithelial-mesenchymal transition through the activation of NF- κ B in cervical cancer cell lines [10]. By using quantitative real-time PCR analysis, we in this study found that TBL1XR1 mRNA is upregulated in tongue cancer tissues comparing with that in normal adjacent tissues, indicating an oncogenic role of TBL1XR1 in tongue cancer.

Accumulating studies have shown that TBL1XR1 is positively correlated with aggressive behavior of tumors. Liu et al. revealed that TBL1XR1 promotes lymphangiogenesis and lymphatic metastasis in esophageal squamous cell carcinoma by inducing the expression of VEGF-C [14]. Li et al. proposed that TBL1XR1 functions as a coactivator of androgen receptor (AR) in prostate cancer cells and the activation is dependent on both phosphorylation and 19S proteasome machinery, and that it physically occupies the androgen-response elements of the affected AR target genes in an

androgen dependent manner [16]. Ectopic nuclear expression of TBL1XR1 selectively activates androgen-regulated genes associated with differentiation and growth suppression, which lead to androgen-dependent growth suppression of prostate cancer cells. Moreover, amplification of TBL1XR1 gene has been detected in invasive prostate cancer, which associates with tumor progression [12]. In the current study, we found that TBL1XR1 expression was positively correlated with tumor stage. The patients with higher levels of TBL1XR1 expression have more advanced tumors, while patients with lower levels of TBL1XR1 expression have relatively early-stage tumors.

It is worth noting that patients with high expression of TBL1XR1 have shorter overall and recurrent-free survival compared to those with lower levels of expression. Multivariate analysis also showed that TBL1XR1 high expression is an independent prognostic indicator for the survival of TSCC patients. Recent studies indicate that TBL1XR1 is a prognostic biomarker in several human cancers. Kuang et al. demonstrated that the disease-free survival and overall survival of hepatocellular carcinoma patients with high expression of TBL1XR1 were significantly shorter than that with low expression [13]. Liu reported that TBL1XR1 expression is positively correlated with patient survival in esophageal squamous cell carcinoma [14]. Chen suggested that upregulation of TBL1XR1 leads to resistance of the NPC cells and is associated with poor prognosis in patients with nasopharyngeal carcinoma [15]. Thus, these findings suggest a function of TBL1XR1 as a valuable prognostic factor for human cancers.

In conclusion, the upregulation of TBL1XR1 may be involved in tumorigenesis and tumor development of TSCC. TBL1XR1 may function as an oncogene and may play an important role in the prognosis of TSCC patients, suggesting that TBL1XR1 represents a novel potential molecular therapeutic target for tongue cancer.

Disclosure of conflict of interest

None.

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