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Original Article

Prognostic significance of Flotillin1 expression in clinically N0 tongue squamous cell cancer

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Abstract: Purpose: The present study aimed to investigate the clinical and prognostic significance of Flotillin1 (FLOT1) in clinically N0 tongue squamous cell cancer (cN0 TSCC). Methods: Real-time PCR and Western blotting analyses were carried out to examine FLOT1 expression in four tongue squamous cell cancer cell lines, primary cultured normal tongue epithelial cells, and eight matched pairs of oral tongue cancer samples and adjacent non-cancerous tissue samples from the same patient. Immunohistochemistry was performed to examine FLOT1 protein expression in paraffin-embedded tissues from 181 cN0 TSCC patients. Statistical analyses evaluated the diagnostic value and the associations of FLOT1 expression with clinical parameters. Results: *FLOT1* mRNA and protein levels were upregulated in tongue squamous cell cancer cell lines and cancerous tissues compared with that in TEC and adjacent non-cancerous tissue samples. The level of FLOT1 protein was positively correlated with clinical stage ($P = 0.001$), T classification ($P = 0.009$), N classification ($P = 0.001$) and recurrence ($P = 0.018$). Patients with higher FLOT1 expression had shorter overall survival times. Conclusion: Our results suggest that overexpression of FLOT1 can be found in patients with higher pathological stage, T classification, N classification or recurrence. FLOT1 expression is associated with cN0 TSCC progression and may be valuable for the prognostic evaluation of cN0 TSCC.

Keywords: FLOT1, Flotillin1, prognosis, clinically N0 tongue squamous cell cancer

Introduction

Tongue squamous cell cancer (TSCC) is the most common cancer diagnosed in the oral cavity [1]. As a result of its highly invasive nature, tongue cancer frequently leads to severe defects in speech, mastication and deglutition, as well as cancer-related death. Despite modern treatment, the 5-year overall survival (OS) rate for oral squamous cell cancer remains poor [2, 3]. The long-term survival rate for patients with tongue cancer has not improved substantially, and the tongue remains among the worst sites in terms of prognosis for all cancers [4]. In clinical practice, although head and neck surgeons mostly depend on the TNM classification system for planning treatment strategy, no consensus exists on the optimal treatment of the neck in cN0 oral tongue cancer patients. Therefore, it is important to elucidate the etiology of TSCC and identify valuable diagnostic and prognostic markers and novel therapeutic strategies. This is particularly relevant as high-risk patients may benefit from more aggressive primary surgery or adjuvant treatment following surgery.

Since the proposal of the 'lipid raft' hypothesis by Simons and Ikonen [5], the biochemical and structural definition of these compartments in cellular membranes has been the subject of intense debate. Lipid rafts have recently been suggested as new therapeutic targets and intervention strategies against cancer [6, 7]. Flotillin proteins comprise the major protein

Table 1. Clinical characteristics and FLOT1 expression of patients with cN0 tongue squamous cell cancer of the study cohort (n = 181)

Characteristics	Number of cases (%)
Gender	
Male	109 (60.2)
Female	72 (39.8)
Age (years)	
< 53	91 (50.2)
≥ 53	90 (49.8)
Pathologic stage	
I	82 (45.3)
II	67 (37.0)
III	17 (9.4)
IV	15 (8.3)
T classification	
T1	89 (49.2)
T2	87 (48.1)
T3	5 (2.7)
N classification	
N0	151 (83.4)
N1	15 (8.8)
N2	15 (8.8)
Nodal status	
N0	151 (83.4)
N+ (N1 and N2)	30 (16.6)
Pathologic differentiation	
Well	138 (76.2)
Moderately	36 (19.9)
Poorly	7 (3.9)
Recurrence	
No	121 (66.9)
Yes	60 (33.1)
Vital status (at follow-up)	
Alive	124 (68.6)
Dead	57 (31.4)
Expression of FLOT1	
Low or none expression	93 (51.4)
High expression	88 (48.6)

family isolated from lipid rafts [8, 9]. The flotillin family of proteins (also known as the Reggie family) contains two homologous isoforms: flotillin-1 (FLOT1) and flotillin-2 (FLOT2) [8, 10]. Flotillin proteins physically interact with each other to form heterooligomeric complexes that play important roles in various cellular processes, such as cell adhesion, actin cytoskeleton reorganization, endocytosis, phagocytosis and the transduction of cellular signals [11].

In addition to the reported functions of flotillins in cellular and organelle membranes, FLOT1 has been reported to be involved in tumorigenesis. Santamaria et al. reported that the overexpression of FLOT1 enhanced the expression and activity of Aurora B kinase, which promotes the incorrect attachment of microtubules to kinetochores, this implies that FLOT1 could contribute to genomic instability [11]. Previously, we have reported that the silencing of FLOT1 inhibited the proliferation and tumorigenicity of breast cancer cells both in vitro and in vivo, which was further shown to be mechanistically associated with the suppression of Akt activity, the enhanced transcriptional activity of FOXO-3a, the upregulation of cyclin-dependent kinase inhibitor $p21^{Cip1}$ and $p27^{Kip1}$, and the downregulation of the CDK regulator, cyclin D1 [12, 13]. These findings suggest that FLOT1 may play a dominant positive role in the PI3K/AKT/FOXO3a signaling pathway. However, whether the dysregulation of FLOT1 also occurs in the pathogenesis of TSCC remains unclear.

The aim of this paper is to investigate the expression of FLOT1 in TSCC tissues and cell lines. Furthermore, we evaluated its prognostic significance by correlating the level of FLOT1 expression with clinicopathological features and survival outcomes in 181 archived cN0 TSCC biopsy samples.

Materials and methods

Patients and tissue specimens

The specimens were used with prior written consent from each patient, and the study was approved by the Institutional Research Ethics Committee of the Sun Yat-sen University Cancer Center, China. A total of 189 tissue specimens (eight matched pairs of tumor tissues and adjacent non-cancerous tissue samples, in addition to 181 individual paraffin-embedded cN0 tongue cancer samples) were taken from patients with cN0 tongue cancer, none of whom had received radiotherapy or chemotherapy prior to surgery. For RT-PCR and western blotting analyses, eight matched pairs of tumor tissues and adjacent non-cancerous tissue samples were obtained from glossectomy specimens of patients diagnosed with cN0 TSCC immediately after surgery and stored at -80°C until use. The percentage of tumor tissue within these biopsies was established by the histopathological

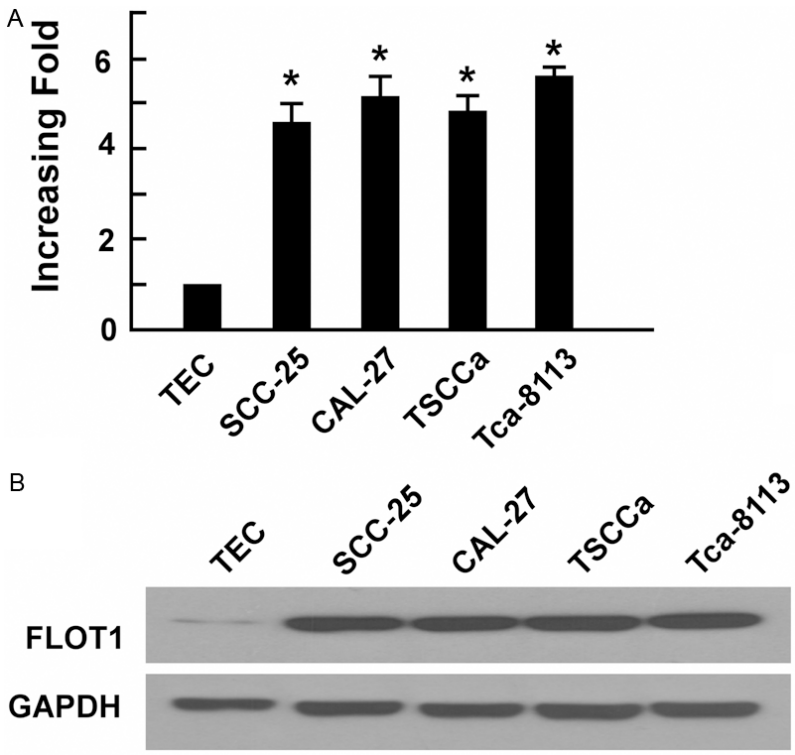

Figure 1. Overexpression of FLOT1 mRNA and protein in tongue squamous cell cancer cell lines, the expression of FLOT1 mRNA and protein in tongue squamous cell cancer cell lines (SCC-9, SCC-25, CAL-27, TSCCa, Tca-8113) and primary cultured normal tongue epithelial cells (TEC) were examined by RT-PCR (A) and Western blotting (B). Expression levels were normalized to GAPDH. Error bars represent standard deviation of the mean (SD) calculated from three parallel experiments.

analysis of adjacent sections prior to RNA and protein analysis.

A total of 181 individual paraffin-embedded cN0 TSCC samples were obtained from 109 male and 72 female patients with a median age of 53 years (range, 20-87 years), who were diagnosed between 1998 and 2005 using standard clinical and histopathological methods at the Department of Head and Neck Surgery and Department of Pathology, Sun Yat-Sen University Cancer Center. Clinical follow-up data for all patients was available for a minimum of 5 years or until death. Clinicopathological and immunohistochemical analyses of these samples were performed to determine the prognostic significance of FLOT1 expression. All patients received standard therapy based on the clinical stage of their tumor according to the practice guideline from National Comprehensive Cancer Network. Patient progress was followed for 78.3 ± 42.1 months (mean ± SD). The clinical information of this patient cohort is summarized in **Table 1**.

Cell lines

Four human tongue squamous cell cancer cell lines were purchased from the American Type Culture Collection (SCC-25 and CAL-27) and the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (TSCCa and Tca-8113). SCC-25 and CAL-27 cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). TSCCa and Tca8113 cells were cultured in RPMI-1640 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (HyClone). Primary cultured normal tongue epithelial cells (TEC) were established from tissue obtained during a glossectomy for a benign lesion and maintained in keratinocyte-SFM (Gibco). All cells were grown in 5% CO_2 in a humidified atmosphere at 37°C.

RNA extraction and real-time PCR

Total RNA from cultured cells and tissue samples was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed according to standard methods, as described previously [22]. The primers selected for *FLOT1* were as follows: forward 5'-CCCATCTCAGTCACTGGCATT-3' and reverse 5'-CCGCCAACAT-CTCCTTGTTC-3'. Expression data were normalized to the geometric mean of housekeeping gene, *GAPDH* (forward: 5'-ACCACAGTCCATGCCATCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3'), to control the variability in expression levels. Fold changes were calculated according to the $2^{-([Ct\ of\ gene] - [Ct\ of\ GAPDH])}$ method, where C_t represents the threshold cycle for each transcript.

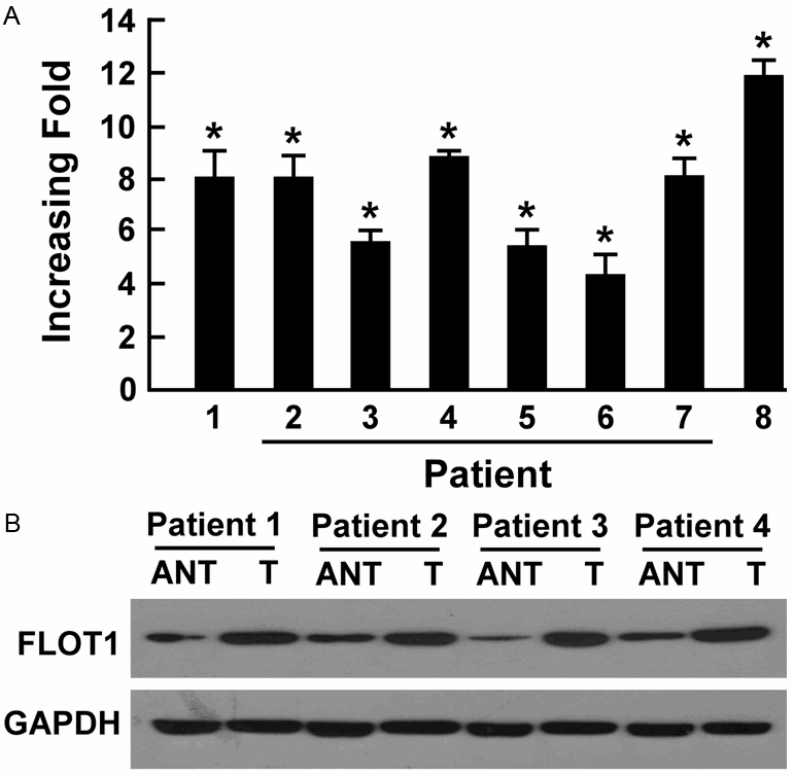

Figure 2. Overexpression of FLOT1 protein in tongue squamous cell cancer specimens, (A) Average T/N ratios of FLOT1 mRNA expression in paired tongue squamous cell cancer (T) and adjacent noncancerous tissues (N) was quantified by RT-PCR and normalized to GAPDH. Error bars represent standard deviation of the mean (SD) calculated from three parallel experiments. (B) Representative western blotting analysis of FLOT1 protein expression in four matched pairs of tongue squamous cell cancer (T) and adjacent noncancerous tissues (N). GAPDH was the loading control.

Western blotting

Western blotting was performed according to standard methods, as described previously [23], using a rabbit anti-FLOT1 polyclonal antibody (1:1,000, Sigma, St. Louis, MO, USA). Blotting membranes were stripped and reprobed with anti-GAPDH antibody (1:1,000, Sigma) as a loading control.

Immunohistochemical (IHC) analysis

IHC analyses were performed in a similar manner to previously described methods [23]. Briefly, tissue sections were incubated with a rabbit anti-FLOT1 polyclonal antibody (1:500; Sigma) overnight at 4°C. For negative controls, the rabbit anti-FLOT1 polyclonal antibody was replaced with normal non-immune serum. The degree of paraffin-embedded sections was reviewed and scored independently by two observers. Scores were based on both the proportion of positively stained tumor cells and the intensity of staining [14]. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10-50% positive tumor cells) and 3 (> 50% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown). The staining index (SI) was calculated as the staining intensity score multiplied by the proportion of positive tumor cells. Using this method of assessment, a score of ≥ 6 was defined as high FLOT1 expression and scores of < 6 were defined as low FLOT1 expression.

Statistical analyses

All statistical analyses were carried out using the SPSS software package (IBM, standard version 17.0). Pearson's χ^2 and Fisher's exact tests were used to analyze the relationship between FLOT1 expression and clinicopathological characteristics. Overall survival (OS) was defined as the time from surgery to death or the last follow-up and progress-free survival (PFS) was defined as the time from surgery to the onset of recurrence (as diagnosed by clinical assessment or imaging). Kaplan–Meier survival curves were plotted and compared using a log-rank test. Multivariate survival analysis was performed for all parameters that were found to be significant in the univariate analysis using the Cox regression model. $P < 0.05$ in all cases was considered statistically significant.

Results

Overexpression of FLOT1 in tongue squamous cell cancer cell lines

The expression levels of *FLOT1* mRNA and protein were compared in four tongue squamous

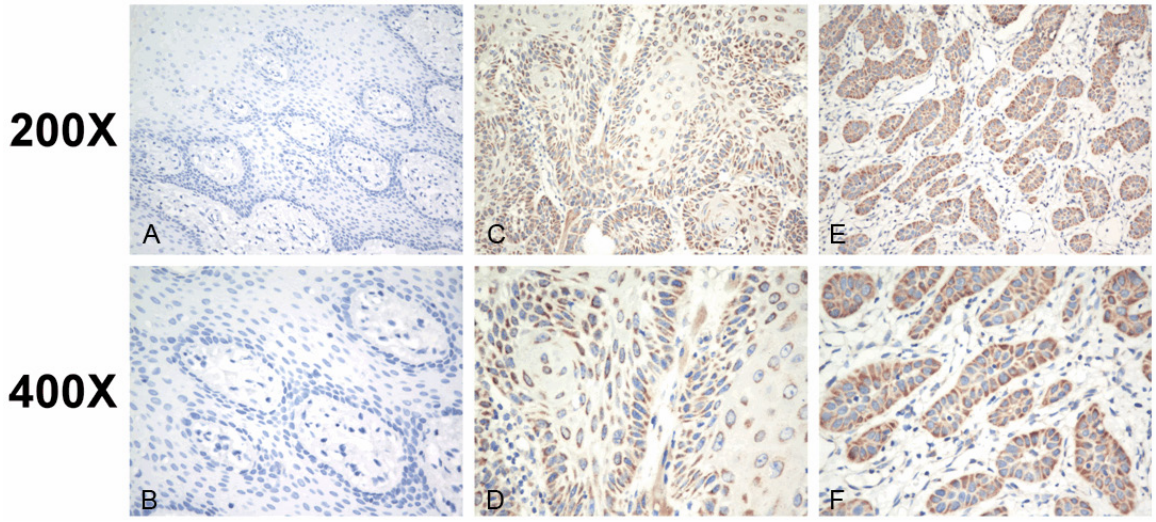

Figure 3. The expression of FLOT1 protein in tongue squamous cell cancer specimens, Representative immunohistochemical images of cN0 tongue squamous cell cancer tissue specimens indicating weak or marginally detectable FLOT1 staining (A, B); moderate FLOT1 staining (C, D); and strong FLOT1 staining (E, F). Magnification is ×200 (A, C, E) or ×400 (B, D, F).

cell cancer cell lines (SCC-25, CAL-27, TSCCa and Tca-8113) and TECs. *FLOT1* mRNA expression was at least 4.5-fold higher in tongue squamous cell cancer cell lines than in TEC (**Figure 1A**), and FLOT1 protein was highly expressed in TSCC cell lines and only weakly expressed in TECs (**Figure 1B**).

Overexpression of FLOT1 in TSCC tissues

RT-PCR and western blotting analyses were performed on eight matched pairs of TSCC samples (T) and adjacent non-cancerous tissue samples (N). *FLOT1* mRNA was expressed at higher levels in all TSCC tissue samples than in adjacent non-cancerous tissues, with a differential expression that ranged from 4.3-fold to 11.8-fold (**Figure 2A**). Consistent with this data, FLOT1 protein was also upregulated in cN0 TSCC samples compared with matched controls (**Figure 2B**).

Association between FLOT1 expression and the clinical characteristics of cN0 TSCC

We further investigated the association between FLOT1 protein expression and the clinicopathological characteristics of TSCC using a panel of 181 archived paraffin-embedded TSCC specimens, which included 82 Stage I tumors, 67 Stage II tumors, 17 Stage III tumors, and 15 Stage IV tumors. FLOT1 expression was analyzed by immunohistochemical staining with an anti-FLOT1 antibody. As shown in **Table 1**, 174 out of the total 181 TSCC samples (96.1%) were positive for FLOT1 based on immunohistochemical staining. High FLOT1 protein expression was detected in 88 samples (48.6%), and weak or negative staining was observed in 93 tumor samples (51.4%, **Figure 3**).

Statistical analysis showed a strong correlation between FLOT1 expression, as determined using immunohistochemical staining, and the clinicopathological characteristics of cN0 TSCC, including pathological stage ($P = 0.001$), T classification ($P = 0.009$), N classification ($P = 0.001$), and recurrence ($P = 0.018$). In contrast, FLOT1 expression was not correlated with age, gender and tumor differentiation (**Table 2**). Furthermore, a Spearman correlation analysis determined that the level of FLOT1 expression correlated with pathological stage ($P = 0.001$), T classification ($P = 0.003$), N classification ($P = 0.001$), recurrence ($P = 0.013$) and vital status ($P = 0.001$). Taken together, our data shows that FLOT1 protein overexpression was positively correlated with pathological stage, T classification, N classification, recurrence and vital status, and FLOT1 overexpression occurs during the increasing clinical progression of TSCC.

Table 2. Correlation between FLOT1 expression and clinical characteristics of patients with cN0 tongue squamous cell cancer

Characteristics	*n*	FLOT1 expression		χ^2 test *P*
		Low or none, no. (%)	High, no. (%)	(Fisher's exact test *P*)
Gender				0.560
Male	109	56 (51.4)	53 (48.6)	
Female	72	37 (51.4)	35 (48.6)	
Age (years)				0.413
< 53	91	48 (52.3)	43 (47.7)	
≥ 53	90	45 (50)	45 (50)	
Pathological stage				0.001
I	82	51 (62.2)	31 (37.8)	
II	67	33 (49.3)	34 (50.7)	
III	17	9 (53.0)	8 (47.0)	
IV	15	0 (0)	15 (100)	
T classification				0.009
T1	89	56 (63.0)	33 (37.0)	
T2	87	35 (43.7)	52 (56.3)	
T3	5	2 (40.0)	3 (60.0)	
N classification				0.001
N0	151	84 (55.6)	67 (44.4)	
N1	15	9 (60.0)	6 (40.0)	
N2	15	0 (0)	15 (100)	
Pathologic differentiation				0.556
Well	138	74 (53.4)	64 (46.6)	
Moderately	36	16 (44.4)	20 (55.6)	
Poorly	7	3 (42.9)	4 (57.1)	
Recurrence				0.018
No	121	70 (57.9)	51 (42.1)	
Yes	60	23 (38.3)	37 (61.7)	
Vital status (at follow-up)				0.001
Alive	124	76 (61.3)	48 (38.7)	
Dead	57	17 (29.9)	40 (70.1)	

Association between FLOT1 expression and patient survival

Patient survival analysis showed a clear negative correlation between the level of FLOT1 protein expression and both the OS and progression-free survival durations of patients with cN0 TSCC (*P* = 0.001 and 0.009, respectively; **Figure 4A**, **4B**). The cumulative 5-year OS and PFS rates for patients with high levels of FLOT1 expression were found to be 58% and 54.5%, respectively, compared to 84.7% and 75.3%, respectively, for patients with low or no FLOT1 expression. Cox regression revealed that only N classification (relative risk: 1.689, CI: 1.220-2.338, *P* = 0.002) and FLOT1 overexpression (relative risk: 20386, CI: 1.308-4.352, *P* = 0.005) were independent prognostic factors for poor OS outcomes.

Discussion

Tumorigenesis, characterized by uncontrolled cell growth and tumor formation, is associated with various alterations in genes or proteins that are related to the regulation of cell proliferation, cell death and genomic instability [14]. Thus, the identification of genes and their products which are involved in the molecular events that lead to tumorigenesis is critical to develop effective therapeutic strategies. Although some biomarkers have been found to correlate with the prognosis of TSCC, no reliable prognostic biomarkers are available yet for clinical use. Improved prognostic markers are urgently needed, as survival rates for patients with tumors at the same clinicopathological stage vary considerably. In this study, we present the first evidence of FLOT1 upregulation in TSCC tissues and cell lines at both the mRNA and protein levels compared with adjacent noncancerous tissues and TECs. FLOT1 protein expression was observed in 96.1% of archived TSCC specimens, and the expression level of FLOT1 protein was found to be significantly correlated with pathological stage, T classification, N classification and recurrence, as well as with an unfavorable prognosis in patients with TSCC. Our results suggest an important role for FLOT1 protein in the development and progression of TSCC. These results imply that FLOT1 may function as an oncogenic protein in the development and progression of TSCC. Although the potential oncogenic function of FLOT1 has been demonstrated in prostate and breast cancer cells, the precise mechanisms of its action remain unclear [7, 15].

Lipid rafts represent specialized dynamic domains of eukaryotic membranes that are characterized by unique chemical composition and physical properties [5, 16]. Flotillins are

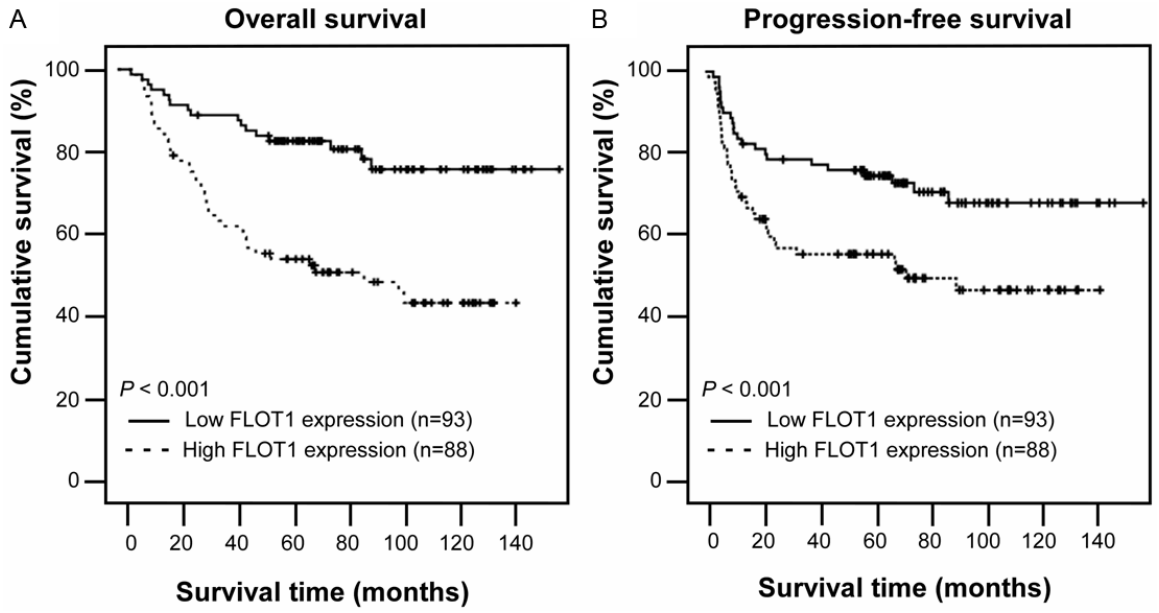

Figure 4. The level of FLOT1 protein expression affects overall survival and disease-free survival. Kaplan–Meier curves with univariate analysis (log-rank) for cN0 tongue squamous cell cancer patients with high FLOT1 expression (n = 88) versus low or no FLOT1 expression (n = 93) for overall survival (A) and disease-free survival (B).

evolutionarily highly conserved proteins [17] hat belong to the SPFH (for stomatins, prohibitins, flotillins, H.K/C) protein superfamily and have a propensity for oligomerization [18, 19]. Flotillins are enriched at the plasma membrane, where they associate with the inner leaflet via hydrophobic amino acid stretches and post-translationally attached acyl groups of palmitic and myristic acids [19-21].

While our studies offer some insights into the function of FLOT1 in TSCC, the underlying mechanism of FLOT1-mediated TSCC progression and the role of FLOT1 generally in malignant transformation and cell growth, in addition to its effects on clinical outcomes, remain to be defined. we found that up-regulation of FLOT1 correlated with poor prognosis and reduced survival of patients with cN0 tongue squamous cell cancer. Multivariate analysis showed that FLOT1 protein levels could be used as an independent prognostic predictor for cN0 tongue squamous cell cancer patients. In conclusion, we have demonstrated an important role for FLOT1 in cN0 tongue carcinogenesis. We further propose that targeting FLOT1 may be a useful strategy for the development of novel therapeutic modalities.

In this study, we found that up-regulation of FLOT1 correlated with poor prognosis and reduced survival of patients with cN0 tongue squamous cell cancer. Multivariate analysis showed that FLOT1 protein levels could be used as an independent prognostic predictor for cN0 tongue squamous cell cancer patients. Thus, testing the FLOT1 protein level may be useful for formulating prognosis and guiding the follow-up schedule in patients with cN0 tongue squamous cell cancer.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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