Overexpression of TMOD1 is associated with enhanced regional lymph node metastasis in human oral cancer

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Abstract. Tropomodulin1 (TMOD1), which regulates the length and depolymerization of actin filaments by binding to the pointed end of the actin filament, has been reported to be a powerful diagnostic marker for ALK-negative anaplastic large-cell lymphoma; however, little is known about the relevance of TMOD1 in the behavior of oral squamous cell carcinoma (OSCC). We evaluated TMOD1 expression in OSCC-derived cell lines and primary OSCC samples (n=200) using quantitative reverse transcriptase-polymerase chain reaction, immunoblotting and semi-quantitative immunohistochemistry. We also analyzed the clinical correlation between TMOD1 expression status and clinical parameters in patients with OSCC and performed a prospective study using 40 primary OSCC samples. TMOD1 expression was upregulated significantly (P<0.05) in OSCC in vitro and in vivo compared with normal counterparts. TMOD1 expression was also correlated significantly (P=0.0199 and P=0.0064, respectively) with regional lymph node metastasis (RLNM) and 5-year survival rates. This prospective study also showed that high TMOD1 expression was seen in 12 (75%) of 16 cases in RLNM-positive patients and 9 (37.5%) of 24 cases in RLNM-negative patients. The current data provide the first evidence that TMOD1 expression is a critical biomarker for RLNM and prognosis of patients with OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is a frequently occurring neoplasm that is usually aggressive and has a poor prognosis (1). OSCC accounts for >50% of all head and neck SCC. The prognosis in advanced cases is poor, and the 5-year survival rates of OSCC are below 50% (2,3). The 5-year survival rate is 90% for patients without metastasis, but <40% for patients with metastasis, suggesting that the regional lymph node metastasis (RLNM) is one of the most adverse prognostic factors (4-10). However, the mechanisms of metastasis are poorly understood (11). Therefore, molecular changes in a number of oncogenes and tumor suppressor genes associated with development of OSCC may be important clues for preventing this disease, and elucidating the molecular mechanisms involved in cancer metastasis is needed (3,4).

The tropomodulin family (TMOD1-4) is expressed differentially in a tissue-specific manner and is involved in regulating actin filament architecture in diverse cellular types (12). TMOD1-4 are 70% similar in amino acid sequence with TMOD1-4, inhibit elongation and depolymerization of actin filaments by binding to the pointed end of the actin filament (16-18). Among them, TMOD1 has two actin-binding regions and two tropomyosin-binding regions (19-22).

Recent studies have reported that TMOD1 is a diagnostic marker for triple-negative breast cancers and ALK-negative anaplastic large-cell lymphoma (23,24); however, the role of TMOD1 in OSCC remains unknown. We present the results of measurements of TMOD1 levels in OSCC that are clinically and functionally linked to RLNM.

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Key words: tropomodulin1, oral squamous cell carcinoma, metastasis

Materials and methods

Ethics statement. The Ethics Committee of the Graduate School of Medicine, Chiba University, Chiba, Japan (approval number, 236) approved the study protocol, which was performed in accordance with the tenets of the Declaration of Helsinki. All patients provided written informed consent.

OSCC-derived cell lines and tissue specimens. Human OSCC-derived cell lines (HSC-2, HSC-3, HSC-4, Sa3, Ca9-22, Ho-1-u-1, Ho-1-N-1, KOSC-2 and SAS) were obtained from the Human Science Research Resources Bank (Osaka, Japan) or the RIKEN BioResource Center (Ibaraki, Japan)
I. Clinical classification in OSCCs from 200 patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at surgery (years)</td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>111 55.5</td>
</tr>
<tr>
<td>≥70</td>
<td>89 44.5</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>132 66</td>
</tr>
<tr>
<td>Female</td>
<td>68 34</td>
</tr>
<tr>
<td>T-primary tumor</td>
<td></td>
</tr>
<tr>
<td>T1 + T2</td>
<td>116 58</td>
</tr>
<tr>
<td>T3 + T4</td>
<td>84 42</td>
</tr>
<tr>
<td>N-regional lymph node</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>120 60</td>
</tr>
<tr>
<td>Positive</td>
<td>80 40</td>
</tr>
<tr>
<td>Histopathological type</td>
<td></td>
</tr>
<tr>
<td>Well and moderately differentiated</td>
<td>190 95</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>10 5</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>149 74.5</td>
</tr>
<tr>
<td>Positive</td>
<td>51 25.5</td>
</tr>
</tbody>
</table>

Table I. Clinical classification in OSCCs from 200 patients.

mRNA expression analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. cDNA was generated using ReverTra Ace qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in a 20-µl reaction volume using the LightCycler 480 apparatus (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. The general amplification conditions were performed as previously described (31-33). Primers and universal probes were designed using the Universal ProbeLibrary Assay Design Center (Roche Diagnostics), which specifies the most suitable set. The primer sequences used for qRT-PCR were: TMOD1, forward, 5'-AGCTGAGGACCTGGAAAAAT-3' and reverse, 5'-GCAGGACAGGATTGGATTAT-3'; and universal probe #42, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward, 5'-CATCTCTGCCCCCTCTGCTGA-3' and reverse, 5'-GGATGACCTTTGCCCCAGACTGCT-3'; and universal probe #60. The transcript amount for TMOD1 was estimated from the respective standard curves and normalized to the GAPDH transcript amount determined in corresponding samples.

Immunoblotting analysis. The cells were washed three times with cold phosphate-buffered saline (PBS) and briefly centrifuged gently. The cellular pellets were incubated at 4°C for 30 min in a lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 10 mM Tris; pH 7.4) with a protease inhibitor cocktail (Roche Diagnostics). The total protein concentration was measured using a dye-binding method based on the Bradford assay with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA).

Protein extracts were electrophoresed on 4-12% Bis-Tris gel and transferred to nitrocellulose membranes (Invitrogen) and blocked for 1 h at room temperature with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan). The membranes were washed three times with 0.1% Tween-20 in Tris-buffered saline (TBS-T) and incubated with affinity-purified rabbit anti-TMOD1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-GAPDH monoclonal antibody overnight at 4°C. The membrane was washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as a secondary antibody (Promega Corp., Madison, WI, USA), for 1 h at room temperature. Finally, the membranes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA), and immunoblotting was visualized by exposing the membranes to the ChemiDoc XRS Plus system (Bio-Rad Laboratories). The signal intensities were quantitated using the Image Lab system (Bio-Rad Laboratories). Densitometric TMOD1 protein data were normalized to GAPDH protein levels.

Semi-quantitative IHC. Semi-quantitative IHC (sq-IHC) of 4-µm sections of paraffin-embedded OSCC clinical specimens was performed. Briefly, after paraffinization, hydration, activation of antigen, hydrogen peroxide quenching, and blocking, the clinical sections were incubated with rabbit anti-TMOD1 monoclonal antibody (Santa Cruz Biotechnology) at 4°C in a moist chamber overnight. Upon incubation with the primary antibody, the specimens were washed three times with PBS and treated with EnVision reagent (Dako, Carpinteria, CA, USA) followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako). The slides were counterstained lightly with hematoxylin, dehydrated with ethanol, cleaned with xylene and mounted.

To quantify the status of the TMOD1 protein expression in clinical samples, we used the sq-IHC scoring systems previously described (28,34-37). The mean percentages...
of positive tumoral cells were determined in at least three random fields in each section; the intensities of the TMOD1-immunoreactions were scored as follows: 0+, none; 1+, weak; 2+, moderate; and 3+, intense. The staining intensity and the numbers were multiplied to produce a TMOD1 sq-IHC score.

To determine the cut-off points of the TMOD1 sq-IHC scores, we analyzed the OSCCs sq-IHC scores of 200 patients using receiver operating characteristic (ROC) curves. Two independent pathologists from Chiba University Hospital, neither of whom had knowledge of the clinical status of the patients, made these judgments. To calculate the 5-year survival rate, we followed-up each patient, until death.

Prospective study. To evaluate the effect of the cut-off value from RLNM by ROC curve analysis, we performed a prospective study using 40 primary OSCC specimens at Chiba University Hospital. We randomly selected 40 primary OSCC specimens and analyzed the correlation between RLNM and TMOD1 expression using sq-IHC.

Statistical analysis. To compare the TMOD1 expression levels, statistical significance was evaluated using the Mann-Whitney U test. The relationships between the TMOD1 sq-IHC scores and clinicopathological profiles were evaluated using the Student’s t-test and the Mann-Whitney U test. The 5-year survival rate was evaluated using the log-rank test. P<0.05 was considered statistically significant. The data are expressed as the mean ± the standard error of the mean.

Results

Upregulation of TMOD1 in OSCC-derived cell lines. To investigate the expression status of TMOD1, we performed qRT-PCR and immunoblotting analyses using 9 OSCC-derived cell lines (HSC-2, HSC-3, HSC-4, Sa3, Ca9-22, Ho-1-u-1, Ho-1-N-1, KOSC-2 and SAS) and HNOKs. TMOD1 mRNA was upregulated significantly (p<0.05) in all OSCC-derived cell lines compared with the HNOKs (Fig. 1A). We also performed immunoblotting analysis to investigate the TMOD1 protein expression in the OSCC-derived cell lines compared with that in the HNOKs. Densitometric TMOD1 protein data are normalized to the GAPDH protein levels. The values are expressed as percentages of the HNOKs (B).

Evaluation of TMOD1 expression in primary OSCCs. To investigate the expression status of TMOD1 in primary OSCCs and the relation to the clinicopathological characteristics, we analyzed the TMOD1 protein expression in primary OSCCs (n=200) and normal counterparts by the sq-IHC scoring system. The TMOD1 sq-IHC scores for OSCCs and normal oral tissues range from 204.44 to 7.00 (median, 100.00) and from 105.50 to 4.00 (median, 35.83), respectively. TMOD1 protein expression levels in OSCCs are significantly higher (p<0.05, Student’s t-test) than in normal oral tissue (C).
Evaluation of TMOD1 expression in primary OSCCs by age at surgery, gender, primary tumoral size, histologic type and vascular invasiveness. We did not find differences between TMOD1 protein expression and the clinical parameters...
Prospective study of TMOD1 expression in primary OSCCs from 40 patients.

<table>
<thead>
<tr>
<th>Relative expression</th>
<th>RLNM (n=40)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- (%)</td>
<td>+ (%)</td>
</tr>
<tr>
<td>High TMOD1</td>
<td>9 (37.5)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>Low TMOD1</td>
<td>15 (62.5)</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Total</td>
<td>24 (100)</td>
<td>16 (100)</td>
</tr>
</tbody>
</table>

High TMOD1 expression is seen in 12 (75%) of 16 RLNM-positive patients and 9 (37.5%) of 24 RLNM-negative patients. Low TMOD1 expression is seen in 4 (25%) of 16 RLNM-positive patients and 15 (62.5%) of 24 RLNM-negative patients.

Evaluation of TMOD1 expression in primary OSCCs by RLNM. The ROC curve analysis showed that the area under the curve (AUC) was 0.608 [95% confidence interval (CI), 0.527-0.688; sensitivity, 65.8%; specificity, 57.5%] and the cut-off value was 100.00 (Fig. 4A). The TMOD1 sq-IHC scores of the RLNM-negative patients and RLNM-positive patients ranged from 204.44 to 12.50 (median, 85.48) and from 201.17 to 7.00 (median, 112.16), respectively. TMOD1 protein expression of primary OSCCs with RLNM was significantly (P=0.0199) higher than without RLNM (Fig. 4B).

Evaluation of TMOD1 expression in primary OSCCs with 5-year survival. Using the cut-off value from RLNM from ROC curve analysis, the 5-year survival rates in the TMOD1-positive OSCCs (n=103) and the TMOD1-negative OSCCs (n=97) were 60.4 and 79.9%, respectively. The survival rates in the TMOD1-positive group were significantly (P=0.0064) lower than those in the TMOD1-negative group (Fig. 5).

Prospective study of TMOD1 expression in primary OSCCs. To determine if the cut-off value of the TMOD1 IHC scores from RLNM (Fig. 4) are useful as a clinical indicator, we prospectively assessed the correlation between RLNM and TMOD1 expression in 40 patients with OSCC. High TMOD1 expression was seen in 12 (75%) of 16 RLNM-positive patients and 9 (37.5%) of 24 RLNM-negative patients. Thus, TMOD1 expression was significantly (P=0.027) higher in the RLNM-positive patients (Table II).

Discussion

We found that TMOD1 was overexpressed frequently in OSCC in vitro and in vivo (P<0.05; Figs. 1 and 2), and that TMOD1 expression in RLNM-positive patients with OSCC was significantly (P<0.05) greater than in RLNM-negative patients (Fig. 4). In addition, the survival rates in the TMOD1-positive patients were significantly lower than in the TMOD1-negative patients (Fig. 5). In the prospective study, high TMOD1 expression was seen in 12 (75%) of 16 RLNM-positive patients and 9 (37.5%) of 24 RLNM-negative patients (Table II).

OSCCs are characterized by a high degree of local invasiveness and a high rate of RLNM in an early phase (38). A study reported recently that 37% of patients with OSCC had RLNM (39). The 5-year survival rate in RLNM-negative patients was 81%, whereas that in RLNM-positive patients was 57% (39). Metastasis represents a highly organized, non-random, organ-specific and multistep process (40). Although many molecules, such as integrins and matrix metalloproteinases (MMPs), play key roles in cancer cell invasiveness and metastasis (41-43), the precise factors and mechanisms affecting its preferred migration and invasion into the regional lymph nodes are poorly understood. Overexpression of TMOD1, a novel target of NF-κB, induces the translocation of β-catenin to nucleus, leading to activation of MMPs in triple-negative breast cancer samples (23). Since NF-κB signaling also relates to RLNM and tumor-induced lymphangiogenesis (44), our hypothesis is that TMOD1 may contribute to the cellular invasiveness and metastasis in OSCCs through the NF-κB signaling.

In conclusion, the current results indicated that TMOD1 is overexpressed frequently in human oral cancer. TMOD1 overexpression is associated with RLNM and the 5-year survival rate. The prospective study also confirmed the correlation between TMOD1 expression and RLNM. While further studies are needed to study the NF-κB-TM0D1 axis in the cancer microenvironment, TMOD1 overexpression may directly affect tumoral metastasis in OSCCs, and TMOD1 may be a critical biomarker of RLNM.

Acknowledgements

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References


