Detection of TGIF1 homeobox gene in oral squamous cell carcinoma according to histologic grading

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Objective. TGIF1 homeobox gene involvement in oral cancer has not yet been investigated. This study analyzed the expression of TGIF1 transcripts and protein in oral squamous cell carcinoma (OSCC).

Study design. Snap-frozen samples from 16 patients were taken from both OSCC and nontumoral adjacent epithelium (NT) for in situ hybridization (ISH). Forty-six paraffin-embedded samples of OSCC were submitted to immunohistochemistry (IHC). A descriptive analysis of the transcript signal detection was accomplished, and TGIF1 immunoexpression was carried out considering protein levels, localization, and cellular differentiation.

Results. ISH reactions showed TGIF1 transcripts with a signal that was frequently intense in NT, and generally weak in OSCC, and that had stronger transcript signal in well-differentiated areas of OSCC when compared with poorly differentiated ones. IHC reactions had poorly differentiated cases associated with TGIF1 protein expression in both the nucleus and cytoplasm (P = .05, Fisher test).

Conclusions. TGIF1 gain or loss of function might possibly play a role in oral cancer cell differentiation. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:218-224)

Biomarkers that could predict cancer behavior have been extensively searched for.1,2 The study of genes that are responsible for embryonic development can provide insights into processes that differ between normal and neoplastic cells, such as cellular communication, migration, growth, differentiation, and apoptosis, as many of the molecular pathways that underlie carcinogenesis are aberrations of the normal process.3,4 Homeobox, a family of developmental genes, has been progressively associated with skin, colon, prostate, breast, ovary, kidney, lung, thyroid, and esophagus cancer and leukemia.5-7 The participation of homeobox genes in oral squamous cell carcinoma (OSCC) has been previously reported in 2 articles.8,9 TG-interacting factor 1 (TGIF1) belongs to the homeobox family of transcription factors (http://ncbi.nlm.nih.gov. Gene ID 7,050). This gene is a member of the 3-amino acid loop extension superclass of atypical homeodomains and appears to act in multiple transcriptional regulatory pathways, either as a DNA binding repressor or as a corepressor in association with other DNA binding proteins.10 TGIF1 has been implicated in holoprosencephaly etiology and has been found to be expressed in several cancers, such as esophagus, stomach, liver, and leukemia.7,11-13

TGIF1 is located at 18p11.3, a region that is commonly involved with head and neck squamous cell carcinoma.14,15 A previous study of the Brazilian head and neck cancer project found that transcripts of this gene were expressed when it researchers carried out a detailed genome mapping analysis of 213,636 expressed sequence tags derived from tumoral and nontumoral tissues.16 These findings, together with the fact that there are no previous reports on the expression of this gene in OSCC, prompted us to analyze the expression of its transcripts and protein in this neoplasm.

MATERIAL AND METHODS

Patients and tissue specimens
Snap-frozen tissue samples from non-necrotic OSCC tissues and nontumoral adjacent epithelium (NT) were
removed from 22 patients with OSCC at the Head and Neck Surgery Department (Clinical Hospital of University of São Paulo [USP]), and processed for in situ hybridization. The diagnosis of OSCC was confirmed and NT was reported as “tumor-free” by pathologists from the Clinical Hospital and from the Department of Oral Pathology.

Formalin-fixed, paraffin-embedded tissue samples from different patients, totaling 46 OSCCs of the tongue and the floor of the mouth, were retrieved from files of the Oral Pathology Department at USP, and submitted to immunohistochemistry for the detection of TGIF1 protein. Clinical data, such as age, gender, and site of occurrence are described in Table I.

After diagnostic confirmation, OSCCs were histologically graded as well, moderately, and poorly differentiated17 by 2 blinded examiners. Well-differentiated squamous cell carcinoma (SCC) consists of sheets and nests of tumoral cells that are generally large with a distinct intercellular bridge and are highly keratinized. Moderately differentiated SCC contains a more evident nuclear pleomorphism and mitotic activity, less keratinization, and infiltrating solid cords and nests. In poorly differentiated SCC, immature cells predominate, with minimal keratinization, and marked and widespread cellular dissociation in small groups of cells (small nests) or in single cells. It is important to consider that the same tumor may show different degrees of differentiation in varying areas, and histologic features, such as nests and cords may be present in different histologic gradings.

Informed consent was obtained from all individuals, and the study was approved by the Bioethics in Research Committee from the Clinical Hospital, School of Medicine, USP and from the School of Dentistry, USP.

**Table I. TGIF immunolocalization distribution regarding gender, age, histologic grading, and site of OSCC according to cell compartment (nuclear or cytoplasmic)**

<table>
<thead>
<tr>
<th>TGIF immunolocalization</th>
<th>Gender</th>
<th>Age</th>
<th>Histologic grading</th>
<th>Site</th>
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<tr>
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<td>N</td>
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<td>Range</td>
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<td>Nuclear only</td>
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<td>8</td>
<td>5</td>
<td>40-83</td>
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<tr>
<td></td>
<td>61.2</td>
<td>7*</td>
<td>3</td>
<td>7</td>
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<tr>
<td>Cytoplasmic and nuclear</td>
<td>29</td>
<td>27</td>
<td>2</td>
<td>39-80*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>17*</td>
<td>10</td>
</tr>
<tr>
<td>Cytoplasmic only</td>
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<td>52-69</td>
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<td></td>
<td>0</td>
<td>2</td>
<td>1</td>
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<td>57</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

WD, well-differentiated squamous cell carcinoma (SCC); MD, moderately differentiated SCC; PD, poorly differentiated SCC.

*P < .05.

†Two cases with missing data.

crodissected to remove nonepithelial or neoplastic tissues. Total RNA was extracted after homogenization with TRIzol according to the manufacturer’s instructions. RNA was quantified by absorbance reading at 260 nm and integrity was evaluated on 0.7% agarose gel with formol and stained with ethidium bromide. The cDNA synthesis was carried out for 50 minutes at 42°C using enzyme reverse transcriptase in the presence of oligoDT in a 20-µL reaction mixture containing 1 µg of total RNA, and treated with RNase-free DNase (1 U).

Reverse-transcriptase polymerase chain reaction. After cDNA synthesis, polymerase chain reaction (PCR) amplification was carried out using primers for TGIF1 (sense: ggctcctcctgcctcttg and antisense: tgcaacatcactagaagctg, nucleotides 1049-1545, product size 497 base pairs (bp), accession number NM_170695.2) in well-controlled conditions and 3 independent reactions. The primers were designed to amplify a region that is present in all splicing variants of TGIF1, and with high similarity between human and mice sequences, using the software Gene Tool 2.0 and Blast 2 sequences (Biotools, Alberta, Canada).

For all reactions, we used a solution containing a mixture of 10× PCR buffer, MgCl₂, dNTP, primers, Taq DNA polymerase, and cDNA in an optimized condition, and a final volume of 25 µL. The PCR reaction was optimized and consisted of denaturation pretreatment at 94°C for 3 minutes, followed by 35 to 45 cycles of denaturation at 94°C for 1 minute, annealing temperature of 58 to 65°C for 50 seconds, and extension at 72°C for 50 seconds followed by an additional 7 minutes at 72°C.

Positive (*Mus musculus* E9-E10 embryo heads) and negative (omission of target cDNA) controls were used. PCR products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. In situ hybridization (ISH) was carried out in 16 samples that were selected based on the amplification of TGIF1 transcripts (data not shown).

Riboprobe synthesis. Conventional reverse-transcriptase (RT)-PCR product for the generic primer of TGIF1 was ligated into pCR II-TOPO vector accord-
ing to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The plasmid vectors were transfected into OneShot chemically competent Escherichia coli DH5α-T1R (Invitrogen) by heat-shock. Plasmids were subjected to sequencing analysis to ensure that PCR product was a specific fragment for the gene. Plasmids were linearized with specific restriction enzymes EcoRV (antisense), BamHI (sense); and the probes molecules were generated using RNA polymerases (antisense: SP6; sense: T7) with mediated incorporation of digoxigenin-11-UTP–labeled nucleotides. Probes were maintained at –80°C until use.

### In situ hybridization of frozen sections

OSCC and NT frozen tissues were processed for in situ hybridization as previously described with changes as follows19: tissues were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) overnight, dehydrated to 30% sucrose, embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA), frozen at –80°C, and serial sections at 10 μm were collected on silane-coated glass slides. Sections were stored at –80°C and allowed to equilibrate at room temperature before postfixation. Slides were post-fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 15 minutes. Sections were permeabilized with 10 μg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN) for 2 minutes. Hybridizations were carried out in heat-sealed bags, overnight at 70°C, each bag containing 2 to 4 slides in 5 mL of hybridization solution with a probe concentration of approximately 0.2 μg/mL. Hybridization solution was 50% formamide, 5× SSC (pH 4.5), heparin 50 μg/mL, yeast RNA 50 μg/mL, 1% (sodium dodecyl sulfate) and water to 50 mL, prewarmed at 70°C. Washes were as follows: three 15-minute changes of 50% formamide, 30% 20× SSC (pH 4.5), and 10% 10× SDS at 70°C, and three 15-minute changes of 50% formamide and 12% 20× SSC (pH 4.5) at 65°C. Detection of bound probe was performed using anti-digoxigenin antibody and nitroblue tetrazolium chloride/5-bromo-4-chloro-3’-indolyrophosphate p-toluidine as color substrate.

Slides were examined using a Nikon SMZ-2T microscope (Tokyo, Japan) to analyze the distribution and cellular location of signal detection. The intensity of signal detection was not considered. Digital photographs were taken with an Axioskop 2 Zeiss microscope and a 3CCD MTI camera (Thornwood, NY, USA). Images were captured and stored on a computer using Adobe Photoshop CS3 software (San Jose, CA, USA).

### Immunohistochemistry

Immunohistochemistry was carried out for a polymer-based staining method (EnVision, DAKO, Carpinteria, CA, USA) using a goat polyclonal antibody TGIF1 (H-172; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were initially deparaffinized in xylene and rehydrated through a graded ethanol series. To quench endogenous peroxidase, sections were incubated with 3% hydrogen peroxide in methanol for 30 minutes at room temperature. Antigen retrieval was carried out using a water bath (95°C) treatment for 30 minutes in 10 mM EDTA solution (pH 8.0). After rinsing twice with Tris-HCl buffer (pH 7.4), sections were preincubated with Swine serum (X0912, DAKO) for 30 minutes at room temperature to prevent non-specific protein binding. The sections were incubated overnight at 4°C in a humid chamber with primary antibody diluted at 1:300. After the secondary reaction was carried out using EnVision + Dual Link System Peroxidase (K4061, DAKO), 3,3’-diaminobenzidine tetrahydrochloride was used as chromogen (K3468; DAKO). The slides were rinsed and counterstained with Mayer’s hematoxylin. Internal positive controls consisted of normal plasma cells in the surrounding stroma. Negative control was carried out by substituting the primary antibody for nonimmune goat (X0907; DAKO) serum.

### Scoring of immunohistochemical analysis

Immunohistochemical staining for TGIF1 was evaluated semi-quantitatively by considering protein levels and cell compartment immunolocalization (nuclear or cytoplasmic staining). Scoring was as follows: negative (0, not detectable), 1 (detectable but less than 50% of tumoral or atypical cells stained), 2 (labeling of more than 50% and less than 75% of tumoral or atypical cells), and 3 (widely and highly expressed in more than 75% of the tumoral or atypical cells) as described by Ogbureke et al.20

### Statistical analysis of immunohistochemistry

Data were tabulated and statistical analysis was carried out using Fisher’s exact test with a 5% significance level (P ≤ .05), using SPSS software for Windows v. 12.0 (SPSS Inc, Chicago, IL, USA).
RESULTS
Localization of TGIF1 transcripts in NT and OSCC specimens

In morphologically NT tissues, TGIF1 expression was present in all samples, but there were different patterns of expression among cases. In 50% of cases, transcripts were present only in basal and suprabasal layers (Fig. 1, A), and in 50% of cases TGIF1 signal was dispersed through all the epithelial layers (Fig. 1, B). No signal was observed on parakeratinized or keratinized layers. The ISH sense probe resulted in no signal (Fig. 1, C).

According to World Health Organization (WHO) grading systems, 4 OSCCs (25%) were classified as well differentiated, 8 (50%) as moderately differentiated, and 4 (25%) as poorly differentiated. The expression patterns of the TGIF1 gene in OSCC samples were evaluated according to the histologic characteristics of SCC (described previously). In the well-differentiated cases, the TGIF1 signal was evident in tumoral cells...
inside sheets and cords (Fig. 1, D). With moderately differentiated OSCC, TGIF1 signal was more evident at the nest peripheral cells (Fig. 1, E). All keratin pearls were negative for TGIF1 expression, peripheral cells showed evident signal (Fig. 1, D, arrows; Fig. 1, F), and focal cells exhibited a stronger signal (Fig. 1, F). When morphologically normal superficial epithelium was present in OSCC samples, the signal was weaker in small nests and in isolated cells when compared with the overlying epithelium (Fig. 1, F).

**Detection of TGIF1 protein in OSCC specimens**

According to WHO grading systems, 13 (28.3%) cases were well-differentiated OSCC, 12 (26.1%) moderately differentiated, and 21 (45.6%) poorly differentiated. Immunohistochemistry showed that 27 (58.7%) cases exhibited TGIF1 protein expression in more than 75% of cells (score 3), 14 (30.4%) in 50% to 75% of cells (score 2), 4 (8.7%) in at least 50% of cells (score 1), and 1 (2.2%) case was without protein expression (score 0). There was no statistical association between the score of immunostaining and the WHO grading (Fig. 2, A–C).

When analyzed according to cell compartment, 29 cases (64.4%) exhibited nuclear and cytoplasmic protein expression (Fig. 2, D), only nuclear staining (Fig. 2, E) was seen in 13 (28.9%) cases, and only cytoplasm (Fig. 2, F) in 3 (6.7%) cases (Table I). Well-differentiated
OSCC exhibited a statistically significant level of TGIF1 protein expression in the nucleus compartment, and poorly differentiated cases were associated with TGIF1 protein expression in both the nucleus and cytoplasm compartment (P < .05, Fisher test, Table I). There was no statistical association between TGIF1 immunoexpression and clinical parameters, such as gender, age, or site (Table I).

DISCUSSION

The present study detected both transcripts and protein of TGIF1 homeobox gene in OSCC. TGIF1 transcript expression decreases with the degree of histologic grading and cellular differentiation, as a more intense signal was seen in well-differentiated areas of OSCCs, unlike poorly differentiated ones. Furthermore, TGIF1 protein expression in the nucleus and cytoplasm compartments was also seen in poorly differentiated OSCC. How these 2 findings are related remains to be explained.

Although, TGIF1 is a homeobox transcriptional repressor implicated in several biological and pathologic processes, hardly any information exists regarding both transcripts and protein expression of this gene in human tissues or in cancer.

The role of TGIF1 in carcinogenesis is still unclear. Some studies have suggested that TGIF1 is implicated in tumor development or progression. Hu et al. suggested that TGIF1 may induce differentiation of stomach neoplastic cells. It would be interesting to investigate whether TGIF1 induces differentiation in OSCC cell lineages, as this study found that the ISH signal was more intense in differentiated neoplastic cells.

Because homeoproteins are under several levels of regulation, including posttranscriptional controls, nuclear-cytoplasmic transport, and protein-protein interactions, it is important to study TGIF1 expression at the protein level. To understand TGIF1 protein expression in the different histologic gradings of OSCC, an analysis was made regarding its expression according to the immunohistochemical scoring, and to the cell compartment (nuclear or cytoplasmic). Although no statistical correlation was found between histologic grading and immunohistochemical scoring, the simultaneous localization of the protein in both cytoplasm and nucleus was significantly correlated with poorly differentiated cases. On the other hand, well-differentiated cases showed TGIF1 protein expression mostly in the nucleus.

It is accepted that TGIF1 protein is constitutively expressed in the cellular nucleus, and to our knowledge there is no report in the literature showing its cytoplasmic expression. Other homeobox genes, such as HOXA5 and HOXD9 were described mainly in the cytoplasm of human esophageal SCC. As considered by the authors, homeobox proteins are reported to have roles other than as DNA binding transcription factors. HOX proteins bind to CREB binding protein (CBP) through homeodomain and block its acetyltransferase activity, which indicates that HOX proteins modulate the transcriptional activity through interaction with other proteins in a non-DNA binding manner.

In OSCC, the cytoplasmic expression of TGIF1 may be explained by the fact that epidermal growth factor signaling via the ras-MEK pathway causes its phosphorylation, leading to stabilization and an increase in half-life. Moreover, it would explain the fact that TGIF1 transcripts ISH signal was less intense in these areas, but the protein could be detected by immunohistochemistry. It has been shown previously that EGR-Ras-Mek pathway deregulation is associated with proliferation of OSSC.

On the other hand, the nuclear expression of TGIF1 protein in well-differentiated OSCC that was found here may be related to different mechanisms, as homeobox genes bind to the target gene promoting its activation, repression, or even modulation, depending on the signal received from the cell. Also, how this finding relates to the evident mRNA expression in more differentiated cells remains to be explained.

TGIF1 participates in a number of distinct pathways, for instance TGIF1 inhibits the transforming growth factor (TGF)-β signaling pathway. Although the signaling of TGF-β in epithelial cells is very complex, under normal circumstances its signal is transmitted into the cell via cell surface receptors. When activated, these receptors phosphorylate the cytoplasmic signaling proteins Smad2 and Smad3, which then form complexes with Smad4 and translocate into the nucleus, where they function as transcription factors of target genes. In the nucleus, they can form an alternate transcriptional complex, in which coactivators are displaced by a complex of corepressors recruited via a protein, such as TGIF1. In oral cancer, the role of TGF-β is controversial. In some oral carcinoma cell lines Smad4 mRNA expression is markedly reduced, and Smad4 protein is undetectable, which results in loss of TGF-induced growth inhibition. Immunohistochemical analysis of Smad4 expression in OSCC showed an absence in 40% of the samples. In cells with a high level of TGIF1, the transcriptional response to TGF-β will be relatively weak, since the high level of TGIF1 will compete efficiently with coactivators for the formation of Smad transcriptional complexes. Taken together, this information supports the idea that TGIF expression in poorly differentiated OSCC may be related to repression of Smad4.
As suggested previously, several mechanisms may influence expression of TGIF1 in OSCC. Studies regarding the effect of TGIF1 expression under the TGF-β signaling pathway, functional assays, and correlation with prognostic factors would be important to clarify the possible participation of TGIF1 transcripts and proteins in OSCC behavior.

CONCLUSIONS

The simultaneous expression of TGIF1 protein in the nucleus and cytoplasm of cells may be related to poorly differentiated OSCC. TGIF1 transcripts have different expression patterns in OSCC with loss of its expression in poorly differentiated neoplasms. These findings might indicate a possible role of TGIF1 in oral neoplastic cell differentiation.

REFERENCES


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