TGIF1 splicing variant 8 is overexpressed in oral squamous cell carcinoma and is related to pathologic and clinical behavior

Tatiana Nayara Libório, DDS, PhD,[a,g] Elisa Napolitano Ferreira, PhD,[b] Flávia Caló Aquino Xavier, DDS, PhD,[c] Dirce Maria Carraro, PhD,[d] Luiz Paulo Kowalski, MD, PhD,[e] Fernando Augusto Soares, MD, PhD,[f] and Fabio Daumas Nunes, DDS, PhD[g]

A.C. Camargo Hospital, and National Institute of Oncogenomics (INCITO); University of São Paulo, São Paulo, São Paulo; Federal University of Amazonas, Manaus, Amazonas; and Federal University of Bahia, Salvador, Bahia, Brazil

Objective. Possible differences in splicing variants of *TGIF1* in oral squamous cell carcinoma (OSCC) have not yet been reported. This study analyzed the expression levels of different splicing variants of the *TGIF1* gene in OSCC compared with nontumoral epithelium (NT) and the relationship with clinical-pathologic features of tumors.

Study Design. Forty-eight frozen samples of OSCC and 17 of NT were analyzed using quantitative reverse transcription polymerase chain reaction.

Results. *TGIF1*v2 and v8 are overexpressed in OSCC, whereas *TGIF1*v5 is underexpressed when compared with NT. Low *TGIF1*v8 expression was correlated with lower cellular differentiation, positive blood vascular invasion, advanced pathologic stage, and positive vascular lymphatic invasion of OSCC. *TGIF1*v8 is also related to overall survival over time, with lower values associated with an increased risk of cancer-related death.

Conclusions. These data suggest that alternative splicing of *TGIF1* is deregulated in OSCC, with overexpression of some splicing variants, especially *TGIF1*v8, which is associated with advanced stages of OSCC. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;116:614-625)

Squamous cell carcinoma is one of the most common cancers of the oral cavity, accounting for at least 90% of all oral malignancies.[1] It is a universal, aggressive disease that usually affects smokers and alcohol drinkers. Despite improved diagnostic and therapeutic methods over the past 20 years, oral squamous cell carcinoma (OSCC) still has high morbidity and mortality rates.[2-3]

Unfortunately, there is no genetic profile for OSCC, and the mechanisms for carcinogenesis are not yet fully understood. The last decade has seen significant progress in understanding the molecular alterations that lead to oncogenic transformation, and there has been an extensive search for biomarkers that could predict the clinical behavior of this cancer.[4-7] Several research groups are dedicated to studying genes related to embryo development, which are also important in carcinogenesis. These genes can provide insights into processes that differ between normal and neoplastic cells, such as cellular communication, migration, growth, differentiation, and apoptosis, because many of the molecular pathways that underlie carcinogenesis are aberrations of normal developmental processes.[8,9] A family of genes closely related to embryonic development is the homeobox family. Several studies have found that homeobox genes have an altered expression in many solid cancers, including skin, colon, prostate, breast, ovary, kidney, lung, thyroid, and esophageal cancer, and also in non-solid cancers such as leukemia.[10] Recent publications have described the participation of homeobox genes in OSCC.[11-15]

A previous study found that *TGIF1* transcripts were expressed differently in OSCC according to histologic grading.[14] *TGIF1* belongs to the homeobox family of

Statement of Clinical Relevance

This study adds information pertinent to cancer genetics and biomarkers of oral squamous cell carcinoma, because it highlights *TGIF1* splicing variants possible role in oral carcinogenesis.

This work was supported by FAPESP grants 01/13644-6, CAPES and CNPq.

Preliminary data from this research was presented in the 87th General Session of the International Association for Dental Research (IADR), 2009, Miami, FL, USA.

[a]Professor of Oral Pathology, Pathology and Legal Medicine Department, Federal University of Amazonas, Manaus, Amazonas.

[b]Laboratory of Genomics and Molecular Biology, International Center of Research and Teaching, A.C. Camargo Hospital, São Paulo, São Paulo.

[c]School of Dentistry, Federal University of Bahia, Salvador, Bahia.

[d]Laboratory of Genomics and Molecular Biology, International Center of Research and Teaching, A.C. Camargo Hospital, National Institute of Oncogenomics (INCITO), São Paulo, São Paulo.

[e]Director, Head and Neck Surgery and Otorhinolaryngology Department, A.C. Camargo Hospital, National Institute of Oncogenomics (INCITO), São Paulo, São Paulo.

[f]Director, Anatomic Pathology Department, A.C. Camargo Hospital, National Institute of Oncogenomics (INCITO), São Paulo, São Paulo.

[g]Associate Professor of Oral Pathology, School of Dentistry, University of São Paulo, São Paulo, São Paulo.

Received for publication Mar 19, 2013; returned for revision Jul 14, 2013; accepted for publication Jul 21, 2013.

2212-4403/$ - see front matter

http://dx.doi.org/10.1016/j.oooo.2013.07.014

transcription factors and is alternatively spliced into 8 different splicing variants, encoding 4 distinct protein isoforms (http://ncbi.nlm.nih.gov, Gene ID 7050). However, Hamid et al.[10] suggested that the *TGIF1* gene has 12 splice isoforms. This gene is a member of the 3-amino-acid loop extension superclass of atypical homeodomains and seems to act in multiple transcriptional regulatory pathways, either as a DNA binding repressor or as a corepressor in association with other DNA binding proteins.[16] *TGIF1* has been implicated in the etiology of holoprosencephaly and is expressed in esophageal, gastric, and liver cancer and leukemia.[17-20]

Alternative splicing is a process by which exons from the same gene can be combined in different ways, resulting in different messenger ribonucleic acid (mRNA) variants, increasing transcriptome diversity and proteome complexity.[21] Alterations of the alternative splicing process have been associated with many human diseases, especially cancer.[22-26] The identification of splicing variants associated with cancer may improve the understanding of cancer biology, contribute to the development of diagnostic protocols and prognosis estimation, and suggest therapeutic targets.

This study reports the expression of *TGIF1* splicing variants in OSCC compared with nontumoral epithelium (NT) and describes TGIF1 protein expression in the same group of patients.

MATERIALS AND METHODS

Samples collection and preparation

This study was approved by the Human Research Ethics Committee of the A.C. Camargo Hospital, São Paulo, Brazil. Fresh tissue samples were obtained from 48 patients with OSCC after surgical resection at the A.C. Camargo Hospital's Head and Neck Surgery and Otorhinolaryngology Department. Seventeen NT samples were also obtained from morphologically normal surgical margins of patients with OSCC, selected by gross examination, and confirmed by microscopic analysis. Samples were snap-frozen in liquid nitrogen immediately after surgical excision. After histologic confirmation, fresh-frozen tissue from each sample was microdissected, after cryostat sectioning and toluidine blue staining, to ensure the removal of at least 70% to 80% of epithelial tumor cells. An expert pathologist reported the corresponding surgical margin as "tumor-free." For all cases, the following information was recorded: age, gender, site of primary tumor, histologic grading, extent of infiltration, lymphatic and blood invasion, treatment, and follow-up. The criteria for differentiating lymphatics from blood capillaries were based on microscopic examination using hematoxylin-eosin staining. The histologic grading was in line with World Health Organization guidelines[27]: well-differentiated OSCC (sheets and nests of neoplastic cells highly keratinized); moderately differentiated OSCC (less keratinized, and infiltrating solid cords and nests); and poorly differentiated OSCC (minimal keratinization, and small groups of cells or single cells). The clinical-pathologic data and follow-up (time from diagnosis until death or latest information) are summarized in Table I.

RNA isolation and complementary DNA synthesis

Total RNA was extracted from frozen samples using the TRIzol method (Invitrogen, Gaithersburg, MD, USA), according to the manufacturer's instructions. RNA was quantified by absorbance reading at 260 nm, and the integrity was evaluated on agarose gel stained with ethidium bromide. Complementary DNA (cDNA) synthesis was carried out using 1 μg of total RNA in the presence of Oligo-dT (a short sequence of deoxythymine nucleotides) (0.5 μg/μL) by reverse transcriptase enzymes SuperScript II (Invitrogen) or ImProm-II (Promega, Madison, WI, USA), according to the manufacturer's instructions, in a 20-μL reaction mixture.

Quantitative reverse transcription polymerase chain reaction

For the amplification reactions, the study designed specific primers for the splicing variants 1, 2, 5, 7, and 8 (*TGIF1*v1, v2, v5, v7, and v8, respectively) and a generic primer (*TGIF1*) amplifying a region common to all splicing variants (Table II); the amplicon was then confirmed through sequencing. For normalization, the GAPDH housekeeping gene (which encodes for glyceraldehyde 3-phosphate dehydrogenase) was used. Primers were designed to amplify interexon regions, using Gene Tool 2.0 software (Bio Tools Inc.: http://genetool.software.informer.com/) (Figure 1).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). All reactions were duplicated and contained 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), 10 to 40 ng of cDNA, and 200 to 800 mM of each primer at a final volume of 20 μL. The RT-qPCR reactions were optimized for each pair of primers according to the manufacturer's recommendations. The standard thermal cycle (denaturation at 95°C for 10 minutes) was used, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C to 64°C for 60 seconds. The amplification efficiency (E) was calculated for each gene using data collected from a standard curve with the following formula: $E = 10^{(-1/\text{slope})}$. The slope was obtained from the data collected during the exponential

Table I. Clinical-pathologic data and follow-up time of patients with OSCC

Clinical-pathologic features	*No. of cases*	*Percentage*
Gender		
Male	40	83.3%
Female	8	16.7%
Age		
40-60 years	28	58.3%
>60 years	20	41.7%
Alcohol (*)		
Yes	37	84.1%
No	7	15.9%
Smoke (*)		
Yes	43	91.5%
No	4	8.5%
Tumor location		
Tongue	20	41.7%
Floor of mouth	13	27.1%
Gingiva	4	8.3%
Retromolar region	5	10.4%
Palate	4	8.3%
Buccal mucosa	2	4.2%
pN (nodal metastasis) classification (*)		
N+	25	53.2%
N−	22	46.8%
pT (tumor size) classification (*)		
T1/T2 (≤4 cm)	33	70.2%
T3/T4 (>4 cm)	14	29.8%
Pathologic TNM stage (*)		
I/II (early)	17	36.2%
III/IV (advanced)	30	63.8%
Histologic grading (*)		
Well differentiated	26	57.8%
Moderately differentiated	19	42.2%
Poorly differentiated	2[†]	
Perineural invasion (PI) (*)		
PI+	22	47.8%
PI−	24	52.2%
Vascular invasion (VI) (*)		
VI+	4	9.1%
VI−	40	90.9%
Lymphatic invasion (LI) (*)		
LI+	10	22.2%
LI−	35	77.8%
Death/cause		
Yes/cancer	18	37.5%
Yes/comorbidity	5	10.4%
No	25	52.1%
Status/follow-up		
Death from cancer (*) ≤12 months	10	21.3%
>12 months	7	14.9%
Death from comorbidity, 79.5 months	5	10.6%
Alive ≤41 months	9	19.2%
>41 months	16	34%

*Cases with missing data.
[†]Disregarded.

phase of RT-qPCR carried out with 5 serial dilutions of reference cDNA (100, 20, 4, 0.8, and 0.16 ng) for each gene.

Total RNA from a human breast cell line (HB4aC5.2) was used as a calibrated sample for all reactions, and the relative fold was calculated as described by Pfaffl[28] using the mean value of the replicates. Specifications of primer and cDNA concentration, melting temperature, and efficiency of primer for each set of primers are available on request. The number of cases varied for each set of primers: *TGIF1* (OSCC 48/48; NT 16/17), *TGIF1*v1 (OSCC 45/48; NT 17/17), *TGIF1*v2 (OSCC 42/48; NT 16/17), *TGIF1*v5 (OSCC 40/48; NT 9/17), *TGIF1*v7 (OSCC 39/48; NT 11/17), *TGIF1*v8 (OSCC 23/48; NT 8/17), and GAPDH (OSCC 48/48; NT 17/17).

Immunohistochemistry

Tissue microarray construction. The specimens used for tissue microarray (TMA) were obtained from the same patients whose tissue were subject to mRNA analysis. Twenty-four formalin-fixed paraffin-embedded tissues from 48 OSCC surgical specimens and 9 from 17 NT specimens were used. In brief, a TMA paraffin receptor block (Beecher Instruments, Silver Spring, MD, USA) was constructed from the samples collected from the original donor block using a 0.6-mm-diameter needle (TMArrayer Punch; Beecher Instruments) after previous selection and circular marking of the representative area of the tumor on the original hematoxylin-eosin−stained slide. The cases were placed on the receptor block in ascending numerical order. Serial 3-μm thick histologic cuts were made and fixed on glass slides with adhesive film (Microsystems Inc, Downers Grove, IL, USA). The TMA was verified on a hematoxylin-eosin−stained section after construction.

Immunohistochemical assay and the quantitation method. Immunohistochemistry was carried out following the polymer-based immunohistochemistry method using goat polyclonal antibody TGIF1 (H-172; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a fragment corresponding to amino acids 100-272 mapping to the C-terminus of TGIF1; this antibody recognizes all TGIF1 isoforms. Initially, sections were 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, and then antigen retrieval was carried out using a water bath (95°C) treatment for 30 minutes in 10 mM of ethylenediaminetetraacetic acid solution (pH 8.0). After washing twice with Tris-HCl buffer (pH 7.4), sections were preincubated with a swine serum (X0912; DAKO, Glostrup, Denmark) for 30 minutes at room temperature to prevent nonspecific protein binding. Sections were incubated overnight at 4°C in a moist chamber with primary antibody diluted at 1:300. Afterward, the secondary reaction was carried out using EnVision + Dual Link System Peroxidase

Table II. Primers used for generic and specific splicing variants of *TGIF1*, according to access number, orientation, and product size

		Primers (5′3′)		
Gene	*Accession No.*	*Sense*	*Antisense*	*Amplicon (bp)*
TGIF1	NM_170695	cttcgggattggctgtatga	ggcgggaaattgtgaactg	190
*TGIF1*v1	NM_170695	gccgactcctggaaacaatga	agccagcggatgaagaaaggt	105
*TGIF1*v2	NM_173207	agtgcctcgccagctttaac	ccagcggatgaagaaaggtc	169
*TGIF1*v5	NM_173209	cccgagggacgagtgacagc	agccagcggatgaagaaaggt	135
*TGIF1*v7	NM_173211	accctccccaccgccacatt	agccagcggatgaagaaaggt	108
*TGIF1*v8	NM_174886	cctccacttccacattccag	gagccagcggatgaagaaag	120
GAPDH	NM_002046.3	gaaggtgaaggtcgga	gggtcattgatggcaac	102

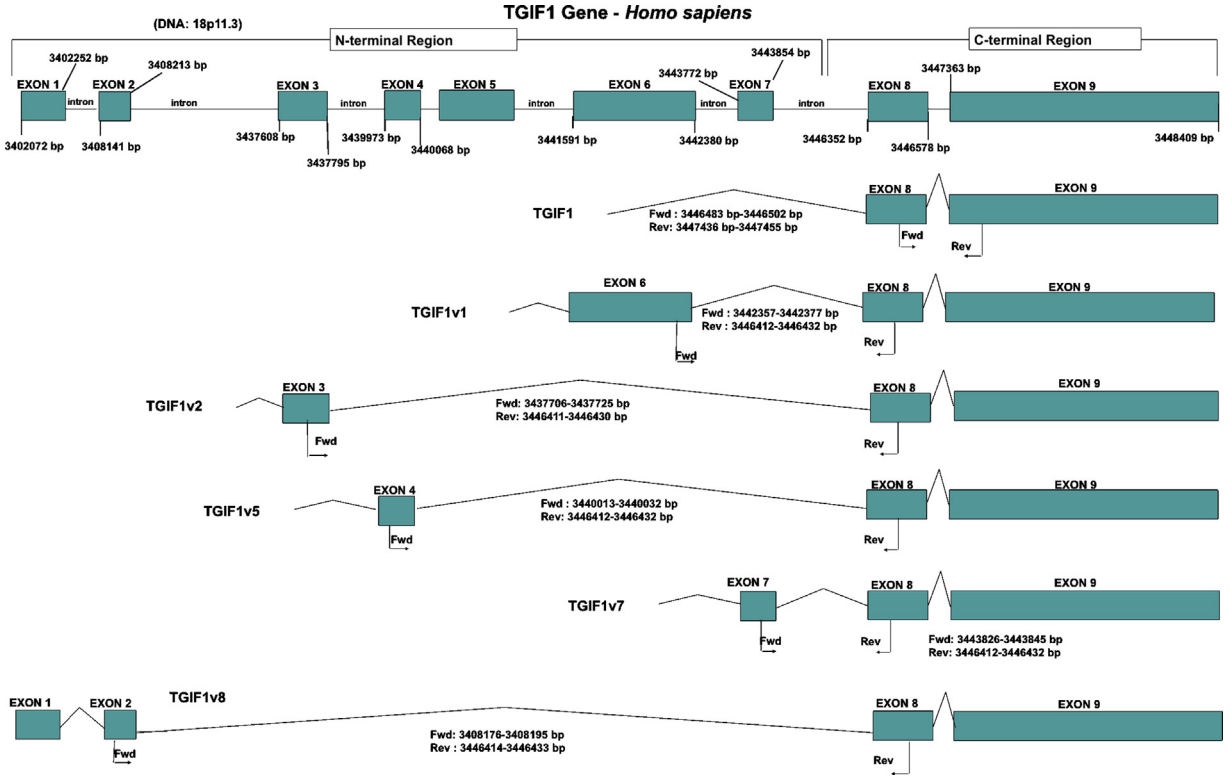

Fig. 1. N- and C-terminal regions of the TGIF1 splicing variant (Gene ID:7050). The C-terminal region is similar for all splicing variants. The discrimination between splicing variants depends on its N-terminal region. Observe the genomic region for each pair of primers on interexon positions (*arrows*).

(K4061, DAKO), and 3,3′-diaminobenzidine tetrahydrochloride was used as a chromogen (K3468; DAKO). The slides were rinsed and counterstained with Mayer's hematoxylin. Internal positive controls consisted of normal plasma cells of the surrounding stroma. Immunohistochemical staining was evaluated quantitatively using the ACIS III (Automated Cellular Imaging System; Dako, Carpinteria, CA, USA).

The ACIS system consisted of an automated robotic bright-field microscope module, a computer, and a Windows NT−based software interface. The robotic microscope module scanned the immunohistochemically stained slides, and a computer monitor displayed the digitized tissue images; it was possible to detect, count, and classify cells based on color, shape, and size. The ACIS recognized 256 levels of immunohistochemical staining intensity and converted these to fractional scores for the selected individual areas. A mean score for all selected areas was also calculated. To analyze the TGIF1 immunoexpression, a nuclear analysis application program was used, to obtain the nuclear component percentage. In addition, a cytoplasmic analysis

application program was used to measure the intensity of the cytoplasmic signal. The ACIS III analysis took place at the Department of Pathology and Anatomy, A.C. Camargo Hospital.

Statistical analysis

The Mann-Whitney test, which is a nonparametric test used in situations with inexistence of normal distribution of data, was used to compare the OSCC and NT samples for mRNA *TGIF1* splicing variants and for protein expression. The Mann-Whitney test was also used to assess the association between selected splicing variants and protein regarding clinical-pathologic features such as gender, nodal metastasis (pN), tumor size (pT), pathologic tumor-nodes-metastasis (TNM) stage (early, I/II; advanced, III/IV), histologic grading (well-differentiated, moderately differentiated, and poorly differentiated), perineural invasion, vascular invasion, and lymphatic invasion. The Kruskal-Wallis test was used to assess the anatomic site regarding selected splicing variants and protein.

Correlations were assessed using the Spearman test for mRNA and protein expression, age, and follow-up in the OSCC samples. The parameters used were the Spearman correlation coefficient (r), with values ranging from -1 to 1, and the P value hypothesis test: H0: $r = 0$ and H1: $r \neq 0$. The closer $|r|$ is to 1, the better the correlation between the 2 measurements assessed. A significant correlation ($\neq 0$) is present when H0 is rejected. To evaluate the correlation grade, the following criteria were considered: $|r| < 0.4$, weak correlation; $0.4 \leq |r| < 0.7$, moderate correlation; $0.7 \leq |r| < 0.9$, strong correlation; and $0.9 \leq |r| \leq 1$, very strong correlation. A negative value indicates a decrease in a measurement as the other increases, and a positive value indicates an increase in a measurement as the other also increases.

The overall survival of patients, defined as time from surgery to the day of death or last follow-up, was calculated using the Kaplan-Meier product-limit estimation with the log-rank test, the Breslow method, and the Cox regression model (multivariate analysis). A commercially available software package (SPSS, version 16.0; SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Statistical significance was determined at $P \leq .05$.

RESULTS

Expression of *TGIF1* splicing variants by RT-qPCR

Initially, expression of *TGIF1* gene (using the generic primer that may potentially amplify any splicing variant) was assessed in OSCC samples and their respective nontumoral margins, and no statistical difference ($P = .20$) was found. However, when the *TGIF1* splicing variants were individually assessed, using specific pairs of primers, the splicing variants *TGIF1*v2 and v8 were found to be overexpressed in OSCC when compared with NT ($P = .001$ and $P = .03$, respectively). Conversely, *TGIF1*v5 was underexpressed in OSCC when compared with NT ($P = .034$) (Figure 2). *TGIF1*v1 and v7 expressions were not statistically different between OSCC and NT ($P = .40$ and $P = .99$, respectively).

There was a strongly positive correlation between the transcript expression of *TGIF1*v2 and v8 ($R = 0.72$, $P = .0002$) in the OSCC (Figure 3). A moderately positive correlation was found between *TGIF1* (all transcripts) and *TGIF1*v8 ($R = 0.61$, $P = .0020$) in the OSCC. Furthermore, a low positive correlation was found between *TGIF1* (all transcripts) and *TGIF1*v5 ($R = 0.26$, $P = .10$). *TGIF1*v2 and v5 had a low negative transcript expression correlation ($R = -0.30$, $P = .072$) (Table III).

Correlation between the expression of *TGIF1*, *TGIF1*v8, the clinical-pathologic features, and disease outcome in OSCC

To establish the relevance of *TGIF1* and its splicing variants' expression in OSCC, the correlation with clinical-pathologic features and disease outcome was assessed. There was an association between low expression of *TGIF1* (all transcripts) and both positive nodal metastasis (pN+, $P = .013$) and advanced pathologic TNM stage (III/IV, $P = .002$) (Figure 4). Evidence of low expression of *TGIF1* in positive vascular invasion was also observed ($P = .094$).

Moreover, there was a correlation between a low expression of *TGIF1*v8 and both moderate differentiation ($P = .005$) and positive vascular invasion ($P = .028$) (Figure 5). There was also a borderline correlation between a low expression of *TGIF1*v8 and both advanced pathologic TNM stage (III/IV, $P = .059$) and positive lymphatic invasion ($P = .053$).

The univariate analysis revealed that *TGIF1* (all transcripts), *TGIF1*v2, *TGIF1*v5, and *TGIF1*v8 were not associated with overall survival rates (log-rank test; $P = .20$, $P = .40$, $P = .54$ and $P = .16$, respectively). As expected, a lower overall survival rate was observed in cases with advanced pathologic TNM stage (III/IV) compared with cases with an early pathologic TNM stage (I/II) ($P = .004$). A lower overall survival rate was also observed in positive vascular invasion compared with negative vascular invasion ($P < .0001$) (data not shown).

Multivariate analysis also revealed that *TGIF1*v8 seems to be related to the overall survival over time, considering tumor size, pathologic TNM stage, histologic grading, perineural invasion, vascular invasion,

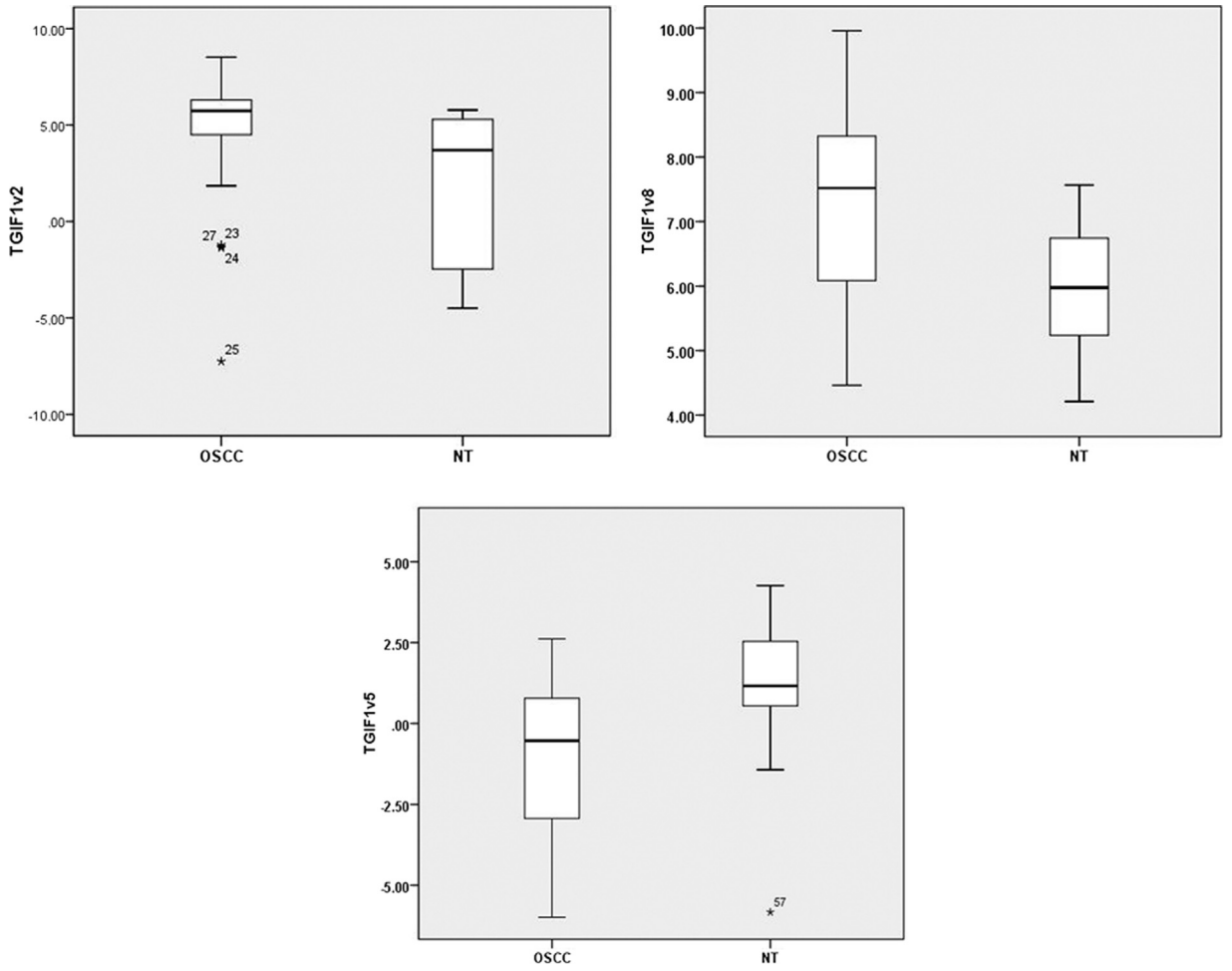

Fig. 2. Expression of TGIF1 splicing variants in OSCC and NT groups using RT-qPCR. TGIF1v2 and v8 are overexpressed in OSCC compared with the nontumoral margin (NT) ($P = .001$ and $P = .03$, respectively), whereas TGIF1v5 is underexpressed in OSCC compared with NT ($P = .034$, Mann-Whitney Test).

and lymphatic invasion, with lower values associated with an increased risk of cancer-related death (Cox, $P = .066$). In this case, an increase of each unit of *TGIF1*v8 decreases the chance of cancer-related death by 41.1% (1-0.59) (Table IV).

Expression of TGIF1 protein by immunohistochemistry. After selection of the representative area of the tumor and NT samples, the immunohistochemical feature of TGIF1 was assessed on a TMA with the 24 OSCC and 9 NT. Most cases of OSCC were well differentiated and had sheets, nests, and highly keratinized tumoral cells. TGIF1 protein was found in all samples of both groups (OSCC and NT), in both the cytoplasmic and the nuclear compartments. However, there was a statistically significant score between groups (discussed later).

The ACIS immunohistochemical scores in the nuclei compartment ranged from 95.86% to 99.93% of the tumoral cells. The mean score in the nuclei compartment of NT epithelium was 88.24% (Figure 6, *A*). There was a higher nuclear score in tumoral cells when compared with NT epithelium ($P = .004$). Also, the cytoplasmic scores for TGIF1 protein had a higher positive expression in tumoral cells when compared with NT epithelium ($P = .001$).

In the OSCC samples, a moderate positive correlation was found between nuclear and cytoplasmic staining ($P = .0021$) (see Table III; see Figure 6, *B*). There was no association between nuclear or cytoplasm proteins expression and the overall patient survival rate (log-rank test; $P = .79$ and $P = .85$, respectively).

DISCUSSION

Based on our findings, *TGIF1*v8 may act as an oncogenic splicing variant during oral carcinogenesis. However, during the development and invasion of OSCC, it might change its behavior by decreasing its expression as the tumor progresses. Our study found

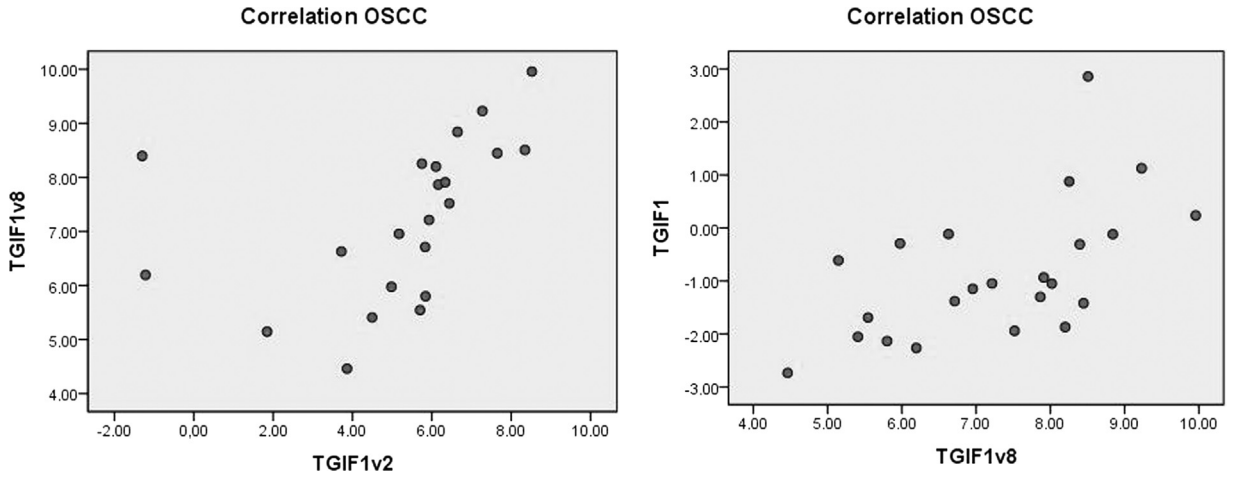

Fig. 3. Correlation of TGIF1 splicing variants expression using RT-qPCR. Dispersion graphic showing the correlation of TGIF1v2 with TGIF1v8 (R = 0.72, $P = .0002$) and TGIF1 (all transcripts) with TGIF1v8 (R = 0.61, $P = .0020$) in OSCC.

Table III. Spearman correlation (*P* value and correlation coefficient r) among age, follow up, nuclear staining, and variants themselves

OSCC	*Follow-up duration (mo)*	*TGIF1*	*TGIF1 V2*	*TGIF1 V5*	*TGIF1 V8*	*Nuclear staining (%)*	*Cytoplasmic staining*
Age (y)							
r	−0.21	0.073	−0.24	0.092	−0.19	0.091	0.30
P	0.16	0.62	0.12	0.57	0.38	0.67	0.15
Follow-up Duration (mo)							
r		0.13	0.18	−0.29	0.24	−0.14	−0.30
P		0.40	0.26	**0.069**†	0.26	0.52	0.15
TGIF1							
r			0.25	0.26	0.61	−0.27	−0.21
P			0.11	**0.10**†	**0.0020***	0.19	0.32
*TGIF1*v2							
r				−0.30	0.7199	−0.062	−0.040
P				**0.072**†	**0.0002***	0.78	0.86
*TGIF1*v5							
r					−0.016	−0.21	−0.22
P					0.95	0.35	0.33
*TGIF1*v8							
r						0.16	−0.41
P						0.61	0.17
Nuclear staining (%)							
r							0.60
P							**0.0021***

*Strong positive correlation for *TGIF1*v2 with *TGIF1*v8; moderate positive correlation for *TGIF1* with *TGIF1*v8 and also for nuclear and cytoplasmic staining in the OSCC group.
†Weak evidence of low correlation for follow-up duration with *TGIF1*v5, for *TGIF1* with *TGIF1*v5, and also for *TGIF1*v2 with *TGIF1*v5.

that in addition to *TGIF1*v8, both *TGIF1*v2 and v5 were also differentially expressed in OSCC compared with NT tissues. However, this study focused on *TGIF1*v8, because it was the only splicing variant among the aforementioned 3 that had a correlation with the clinical-pathologic characteristics of OSCC using both a univariate and a multivariate analysis, and it was particularly associated with advanced stages. These analyses showed that a low *TGIF1*v8 expression was significantly associated with a lower cellular differentiation (in our samples, moderate differentiation) and positive vascular invasion. We also found a weak association of low *TGIF1*v8 expression with advanced pathologic TNM stage and positive lymphatic invasion. The multivariate analysis showed that lower *TGIF1*v8 values were associated with an increased risk of cancer related-death, which reinforces the argument that *TGIF1*v8 is associated with advanced

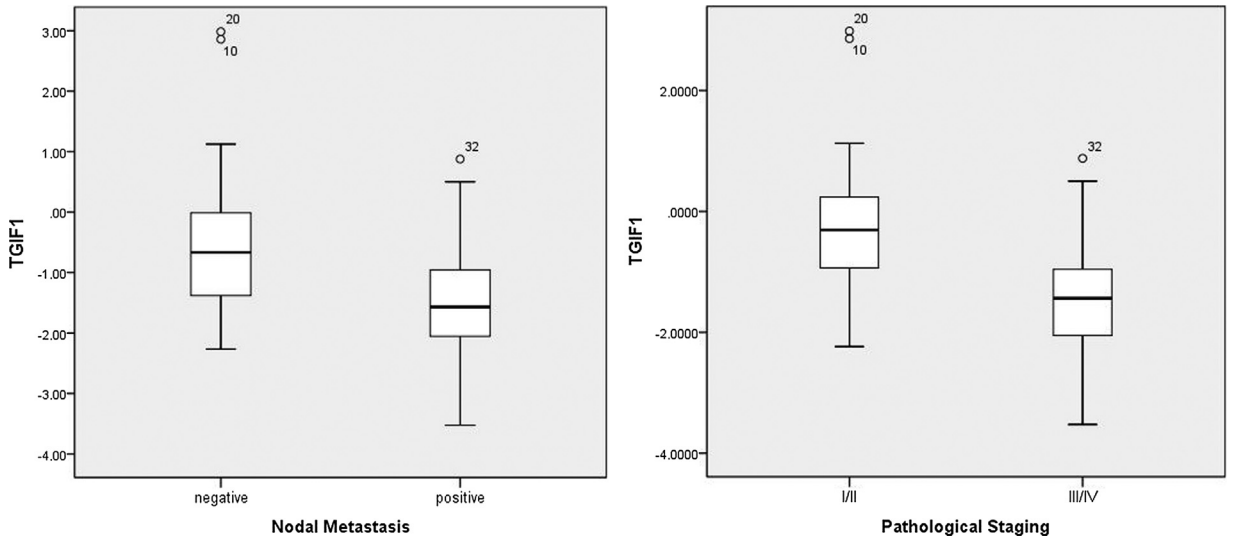

Fig. 4. Correlation between TGIF expression and clinical-pathologic features in OSCC. Low expression of TGIF1 (all transcripts) in the group with positive pathologic lymph node involvement (pN+, $P = .013$) and also in the group of advanced pathologic TNM stage (III/IV) ($P = .002$).

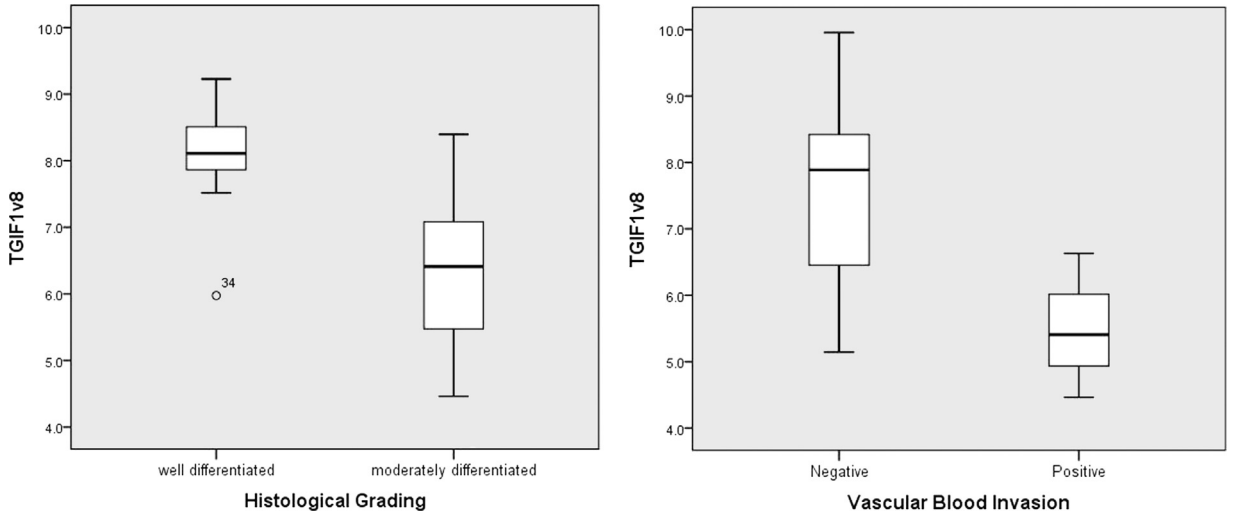

Fig. 5. Correlation between TGIF1v8 expression with clinical-pathologic features of OSCC. Low expression of TGIF1v8 in the group with moderate differentiation ($P = .005$) and also in the group with positive vascular invasion ($P = .028$).

stages of OSCC. In this case, an increase of each unit of *TGIF1*v8 reduces the chance of cancer-related death by 41.1%.

*TGIF1*v2, v5, and v8 are differentially expressed in OSCC when compared with NT

TGIF1, a transcription factor of the homeobox family, has been implicated in a number of distinct pathways. This study shows for the first time that *TGIF1*v2, v5, and v8 were differentially expressed in OSCC when compared with NT. *TGIF1*v2 and v8 may act as oncogenic variants, because they were overexpressed in OSCC, whereas *TGIF1*v5 possibly acts as a tumor suppressor, because it was underexpressed in OSCC in relation to NT samples. Also, *TGIF1*v2 and v8 might play similar roles in oral carcinogenesis, because a strong positive correlation between them was found. However, *TGIF1*v8 might represent, within our samples, the putative splicing variant that has the most relevant expression in tumoral cells, owing to its moderate correlation to *TGIF1*.

A previous in situ hybridization study showed that *TGIF1* transcripts had a signal that was frequently intense in NT, and generally weak in OSCC,[14] suggesting that *TGIF1* expression is higher in NT

Table IV. Results of the Cox model set in the presence of TGIF1v8

	Effect	*Standard error*	*P value*	*Odds ratio*	*CI 95%, OR*
Initial model					
pT	2.85	1.80	0.11	17.32	0.51, 589.05
pTNM	−2.24	1.73	0.20	0.11	0, 3.15
Histologic grading	0.049	1.27	0.97	1.050	0.090, 12.71
Perineural invasion	−0.29	0.93	0.76	0.75	0.12, 4.60
Vascular invasion	1.37	1.32	0.30	3.95	0.30, 52.48
Lymphatic invasion	−2.0	1.50	0.18	0.14	0.010, 2.56
TGIF1_v8	−0.70	0.50	0.16	0.50	0.19, 1.31
Final model					
TGIF1_v8_tumor	−0.53	0.29	0.066	0.59	0.34, 1.040

pT, tumor size; *pTNM*, pathologic TNM stage; *CI*, confidence interval; *OR*, odds ratio.

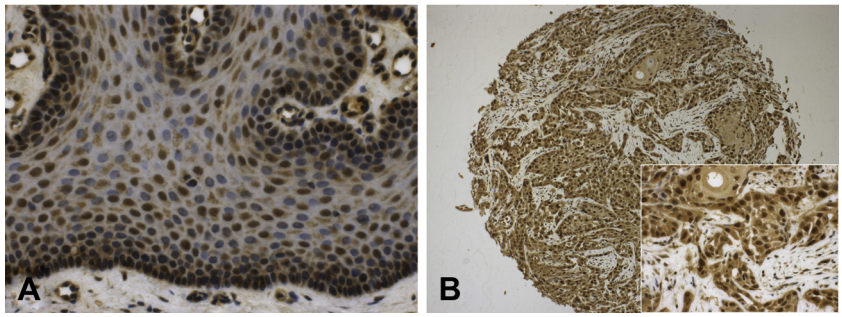

Fig. 6. The TGIF1 protein was found in all OSCC and NT samples, in both the cytoplasmic and the nuclear compartments, although a statistically significant score difference between groups was found. **A**, TGIF1 expression in NT epithelium in both the cytoplasmic and the nuclear compartments (×100). **B**, TGIF1 expression in OSCC in both the cytoplasmic and the nuclear compartments (×100; inset, ×400).

compared with OSCC. Conversely, by using RT-qPCR, we found no differences regarding *TGIF1* expression in OSCC when compared with NT, although an imbalance of specific splicing variants (*TGIF1*v2, *TGIF1*v5, and *TGIF1*v8) was found, which suggests that alternative splicing of *TGIF1* may be deregulated in OSCC. The lack of difference between OSCC and NT samples, when amplifying all *TGIF1* transcripts, is probably because of the interference of imbalanced splicing variants.

The analysis of the TGIF1 protein showed that there was a statistically significant difference between the nuclear and cytoplasmic expression when comparing OSCC and NT samples. In line with Matizonkas et al. (2011),[14] we agree that TGIF1 cytoplasmic immunolocalization implies that some isoforms could develop additional functions other than transcription. Furthermore, Lo et al. (2001)[29] revealed that the mitogen-activated protein kinase transducing pathway can phosphorylate TGIF1, prolonging its half-life and consequently raising its protein level, which could also justify the higher levels of TGIF1 protein in the cytoplasmic compartment of OSCC cells. It has previously been found that EGF-Ras-Mek pathway deregulation is associated with OSCC proliferation.[30] Unfortunately, specific antibodies against TGIF1 protein isoforms are not commercially available. Additional studies to examine specific TGIF1 isoforms are necessary to clarify the exact role of each isoform and also the role of such isoforms in oral carcinogenesis.

Correlation between expression of *TGIF1*, *TGIF1*v2, and v8 and the clinical-pathologic features and disease outcome in OSCC

TGIF1 is a homeobox transcriptional repressor that is implicated in several biologic and pathologic processes. Its role in carcinogenesis is still unclear, because there are hardly any studies in the literature regarding *TGIF1* expression in cancers. It has been reported that *TGIF1* is expressed in cancers of the esophagus, stomach, and liver[17-20]; in leukemia[17-20]; and (as more recently reported) in oral cancer.[14] Some of these studies have suggested that *TGIF1* is implicated in tumor development or progression, but none of them found an association between *TGIF1* and prognostic factors. This study presents data that are consistent with a possible role of *TGIF1* in oral carcinogenesis, especially *TGIF1*v8, because this splicing variant was correlated with prognostic factors. Hamid et al.[10,20] found

expression of the *TGIF1* splicing variants in human myeloid leukemia, suggesting that *TGIF1* may regulate the balance between proliferation and differentiation. There are several reports in the literature of alternative splicing variants being potential biomarkers.[22,31] Although *TGIF1*v2 and *TGIF1*v8 overexpression may play an important role in oral carcinogenesis, it is crucial to decipher the mechanisms underlying aberrant splicing in cancer to understand how splicing machinery is controlled and integrated with other cellular processes, in particular transcription and signaling pathways.[32] Furthermore, patterns of alternative splicing can be tissue-specific, stimulus-specific, disease-specific, or a combination of these, and the presence or level of specific splice variants, even without knowledge of their activity, may provide useful biomarkers that can be causative of disease, can be involved in disease development, or can act as surrogate markers. Either way, targeted clinical studies are required to confirm their usefulness in the diagnosis and monitoring of cancer.[22] However, caution is advised, because previous clinical trials for advanced head and neck carcinoma, using bivatuzumab mertansine (a cytotoxic immunoconjugate) specifically targeting CD44v6,[33] had to be discontinued because of the occurrence of skin toxicity.[34]

It has previously been found, by in situ hybridization, that there is a weaker transcript signal of *TGIF1* transcripts in poorly differentiated areas of OSCC when compared with both well-differentiated and adjacent NT.[14] These findings are not in accordance with the absence of difference between OSCC and NT when all transcripts were analyzed using the generic primer for *TGIF1*. This fact may be explained by the different techniques used. In situ hybridization enables an individualized cell to be analyzed, whereas RT-qPCR amplifies mRNA of all cells present in a tumoral area. However, in this study, which used RT-qPCR to target specific splice variants, a low expression of *TGIF1*v8 was correlated with moderately differentiated OSCC when compared with well-differentiated OSCC. The correlation of *TGIF1* with its splicing variant *TGIF1*v8 has been previously discussed.

On the other hand, low expression of *TGIF1* (all transcripts) was related to advanced pathologic TNM stage (III/IV) and node-positive carcinomas of the oral cavity and was probably also related to a low expression of *TGIF1*v2 and v8, because they may act in a similar way, judging by their correlation. However, the multivariate analysis did not show the association between isolated splice variants with clinical-pathologic features and the overall survival, except for *TGIF1*v8, where lower values seem to be associated with an increased risk of cancer-related death. Hamid et al. (2008)[10] showed that the expression of *TGIF1* splicing variants is similar in leukemia, except for isoform 4, which had a very low expression level. The same group (Hamid et al., 2009)[20] reported that reduced *TGIF1* (generic form) expression could lead to quiescence, thus providing progenitor as well as hematopoietic stem cells protection from anticycle agents, and they suggested that *TGIF1* has an important role in myelopoiesis and may regulate the balance between proliferation and differentiation. In general, many studies show that splicing variants are cell specific. Well-known CD44 splicing variants as examples of cancer biomarkers have been reviewed.[22] In brief, antibodies directed against CD44v6 have been shown to efficiently target head and neck squamous cell carcinoma and have been proposed for tumor imaging.[22] CD44v10 expression can apparently differentiate between metastatic and nonmetastatic pancreatic cancer cells; CD44v6 can serve as an indicator of tumor progression in gastric carcinoma; CD44v7 to v9 are overexpressed in prostate cancer tissues; CD44v5 expression correlates with the aggressiveness of thymic epithelial tumors; and decreased levels of CD44v3 and CD44v6 possibly correlate with sputum cytology−negative cases of lung cancer.[22]

Although *TGIF1v8* was found overexpressed in OSCC when compared with NT, this finding is not necessarily relevant regarding tumor aggressiveness. Our findings show that as OSCC progresses, the expression of *TGIF1v8* decreases. One possible explanation is that this gene has a dual role during carcinogenesis, similar to what has been reported for *TGFβ* (transforming growth factor beta).[35]

TGIF1 and *TGFβ* may have both antagonists and similar roles in carcinogenesis. It is known that *TGIF1* acts as a transcriptional corepressor of the signaling pathway activated by *TGFβ*, and it can also be regulated by *TGFβ*, which indicates a negative feedback mechanism.[16,36] The *TGFβ* pathway is complex, especially because it can play a dual role in cancer, acting as a tumor suppressor in early stages and as an oncogene in later stages of carcinogenesis.[35-37] Based on our results, we suggest that *TGIF1*v8 acts as an oncogene in early stages and as a tumor suppressor in advanced stages of carcinogenesis, unlike *TGFβ*. This is probably due to a negative feedback mechanism that *TGFβ* may exert on *TGIF1*v8.

Matizonkas et al. (2011)[14] reported that the simultaneous localization of TGIF1 in both the cytoplasm and the nucleus was correlated with poorly differentiated cases. In our study, there was no distinct difference between the nuclear and cytoplasmic protein staining in relation with clinical-pathologic data, which suggests that the TGIF1 protein is not a marker of OSCC prognosis. The availability of specific antibodies to study the expression of individual TGIF1 isoforms may challenge this concept, as suggested by our *TGIF1*v2 versus v8 data.

CONCLUSION

There was no difference between the transcripts of *TGIF1* found in OSCC and NT samples, although there is an imbalance of specific splicing variants, which suggests that alternative splicing of *TGIF1* is deregulated in OSCC. The data presented support a significant association between low expression of *TGIF1*v8 with lower cellular differentiation and positive vascular invasion. A weak association was also found between low *TGIF1*v8 expression and advanced pathologic TNM stage and positive lymphatic invasion. Finally, low *TGIF1* expression is associated with positive nodal metastasis and advanced pathologic TNM staging. Taking these considerations together, our data suggest that *TGIF1*v8 is overexpressed in OSCC and is related to pathologic and clinical behavior.

The authors are thankful to Dr Edward K. Chan for critical reading of this manuscript.

REFERENCES

1. Walker DM, Boey G, Mcdonald LA. The pathology of oral cancer. *Pathology*. 2003;35:376-383.
2. Brinkman BM, Wong DT. Disease mechanism and biomarkers of oral squamous cell carcinoma. *Curr Opin Oncol*. 2006;18:228-233.
3. Kim MM, Califano JA. Molecular pathology of head-and-neck cancer. *Int J Cancer*. 2004;112:545-553.
4. Sotiriou C, Lothaire P, Dequanter D, Cardoso F, Awada A. Molecular profiling of head and neck tumors. *Curr Opin Oncol*. 2004;16:211-214.
5. Jemal A, Murray T, Ward E, et al. Cancer statistics. *CA Cancer J Clin*. 2005;55:10-30.
6. Massano J, Regateiro FS, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2006;102:67-76.
7. Zheng M, Li L, Tang YL, Liang XH. Biomarkers in tongue cancer: understanding the molecular basis and their clinical implications. *Postgrad Med J*. 2010;86:292-298.
8. Grier DG, Thompson A, Kwasniewska A, Mcgonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. *J Pathol*. 2005;205:154-171.
9. Krumlauf R. Hox genes in vertebrate development. *Cell*. 1994;78:191-201.
10. Hamid R, Patterson J, Brandt SJ. Genomic structure, alternative splicing and expression of TG-interacting factor, in human myeloid leukemia blasts and cell lines. *Biochim Biophys Acta*. 2008;1779:347-355.
11. Zhu F, Li J, Li WX, Liu ZC, Long X. Overexpression and clinicopathological significance of homeobox gene Quox-1 in oral squamous cell carcinoma. *J Biochem Mol Biol*. 2004;37:671-675.
12. Hassan NM, Hamada J, Murai T, et al. Aberrant expression of HOX genes in oral dysplasia and squamous cell carcinoma tissues. *Oncol Res*. 2006;16:217-224.
13. De Souza Setubal Destro MF, Bitu CC, Zecchin KG, et al. Overexpression of HOXB7 homeobox gene in oral cancer induces cellular proliferation and is associated with poor prognosis. *Int J Oncol*. 2010;36:141-149.
14. Matizonkas-Antonio LF, Liborio TN, Aquino Xavier FC, Silva-Valenzuela MD, Michaluarte-Júnior P, Nunes FD. Detection of TGIF1 homeobox gene in oral squamous cell carcinoma according to histologic grading. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2011;111:218-224.
15. Liborio TN, Acquafreda T, Matizonkas-Antonio LF, Silva-Valenzuela MD, Michaluarte-Júnior P, Nunes FD. In situ hybridization detection of homeobox genes reveals distinct expression patterns in oral squamous cell carcinomas. *Histopathology*. 2011;58:225-233.
16. Wotton D, Knoepfler PS, Laherty CD, Eisenman RN, Massagué J. The Smad transcriptional corepressor TGIF recruits mSin3. *Cell Growth Differ*. 2001;12:457-463.
17. Nakakuki K, Imoto I, Pimkhaokham A, et al. Novel targets for the 18p11.3 amplification frequently observed in esophageal squamous cell carcinomas. *Carcinogenesis*. 2002;23:19-24.
18. Hu ZL, Wen JF, Xiao DS, Zhen H, Fu CY. Effects of transforming growth interacting factor on biological behaviors of gastric carcinoma cells. *World J Gastroenterol*. 2005;11:84-88.
19. Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. *Oncogene*. 2005;24:1809-1819.
20. Hamid R, Brandt SJ. Transforming growth-interacting factor (TGIF) regulates proliferation and differentiation of human myeloid leukemia cells. *Mol Oncol*. 2009;3:451-463.
21. Venables JP. Aberrant and alternative splicing in cancer. *Cancer Res*. 2004;64:7647-7654.
22. Brinkman BM. Splice variants as cancer biomarkers. *Clin Biochem*. 2004;37:584-594.
23. Bartel F, Taubert H, Harris LC. Alternative and aberrant splicing of MDM2 mRNA in human cancer. *Cancer Cell*. 2002;2:9-15.
24. Jiang Z, Cote J, Kwon JM, Goate AM, Wu JY. Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17. *Mol Cell Biol*. 2000;20:4036-4048.
25. Hyman BT, Augustinack JC, Ingelsson M. Transcriptional and conformational changes of the tau molecule in Alzheimer's disease. *Biochim Biophys Acta*. 2005;1739:150-157.
26. Ferreira EN, Rangel MC, Galante PF, et al. Alternative splicing enriched cDNA libraries identify breast cancer-associated transcripts. *BMC Genomics*. 2010;11(suppl 5):S4.
27. Johnson N, Frenceschi S, Ferlay J, et al. Squamous cell carcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky D, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours*. Lyon, France: IARC Press; 2005:168-175.
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29:e45.
29. Lo RS, Wotton D, Massague J. Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO J*. 2001;20:128-136.
30. Mishima K, Inoue K, Hayashi Y. Overexpression of extracellular-signal regulated kinases on oral squamous cell carcinoma. *Oral Oncol*. 2002;38:468-474.
31. Omenn GS, Yocum AK, Menon R. Alternative splice variants, a new class of protein cancer biomarker candidates: findings in pancreatic cancer and breast cancer with systems biology implications. *Dis Markers*. 2010;28:241-251.
32. Ghigna C, Valacca C, Biamonti G. Alternative splicing and tumor progression. *Curr Genomics*. 2008;9:556-570.
33. Colnot DR, Roos JC, de Bree R, et al. Safety, biodistribution, pharmacokinetics, and immunogenicity of 99mTc-labeled

humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with squamous cell carcinoma of the head and neck. *Cancer Immunol Immunother*. 2003;52:576-582.
34. Pajares MJ, Ezponda T, Catena R, Calvo A, Pio R, Montuenga LM. Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol*. 2007;8:349-357.
35. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res*. 2005;11:937-943.
36. Chen F, Ogawa K, Nagarajan RP, Zhang M, Kuang C, Chen Y. Regulation of TG-interacting factor by transforming growth factor β. *Biochem J*. 2003;371:257-263.
37. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol*. 2005;23:2078-2093.

Reprint requests:

Tatiana Nayara Libório, DDS, PhD
Pathology and Legal Medicine Department
Federal University of Amazonas
Av. Valdemar Pedrosa, 1097
Manaus, Amazonas, 69020-160
Brazil
tliborio@ufam.edu.br; tatiana.liborio@gmail.com