Downregulation of CD9 protein expression is associated with aggressive behavior of oral squamous cell carcinoma ☆

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ARTICLE INFO

SUMMARY

Squamous cell carcinoma of the oral cavity (OSCC) is a malignancy characterized by a high degree of local aggression and metastasis to cervical lymph nodes. Tetraspanins are proteins with functional roles in a wide array of cellular processes and are reported to be associated with tumor progression. The present study investigated the expression of the CD9, CD37, CD63, CD81 and CD82 tetraspanins in OSCC using immunohistochemistry (IHC) and quantitative Real Time-PCR (qRT-PCR). Tissue microarray (TMA) analysis of samples from 179 cases of OSCC and 10 normal samples oral mucosa were evaluated immunomorphologically. We analyzed CD9 and CD82 expression by qRT-PCR in 66 OSCC cases and 4 normal samples of oral mucosa. Expression of CD63, CD37 and CD81 was not detected in the samples studied. CD82 was downregulated or negative in 127 of 179 (80%) specimens; no correlation was observed between CD82 expression, clinicopathological parameters, disease-free survival and 5-year overall survival. CD9 expression was downregulated or negative in 75 of 129 (42%) OSCC samples. Loss of CD9 expression in OSCC samples correlated with the incidence of lymph node metastasis ($p = 0.017$). Disease-free survival and the 5-year overall survival of patients with downregulated or negative CD9 expression were significantly lower than in patients with positive CD9 expression ($p = 0.010$ and $p = 0.071$, respectively). No correlation was found between CD9 or CD82 expression and clinicopathological parameters by qRT-PCR. Our results suggest that the downregulation or lack of expression of the CD9 protein might indicate a more aggressive of OSCC.

Introduction

One of the clinical characteristics of squamous cell carcinoma of the oral cavity (OSCC) is its capacity to invade locally and metastasize to regional lymph nodes. Identification of cervical lymph node metastasis is the main prognostic factor of patients with OSCC and has a major impact on treatment selection.[1]

Tetraspanins have been implicated in a wide array of cellular processes including cell adhesion, motility, intracellular signaling, cell matrix adhesion and proliferation.[2–5] These molecules have also been considered as suppressors of metastasis in solid tumors.[3,6]

Reduced expression of tetraspanin proteins has been identified in many different types of carcinoma. Additionally, lack of expression of these proteins has been frequently reported in metastatic lesions, and patients with tumors that lack tetraspanins tend to have poorer prognoses and survival rates.[7–11]

The aim of this study was to analyze the patterns of expression of CD9, CD37, CD63, CD81 and CD82 antigens in OSCC and normal oral tissue by immunohistochemistry (IHC) and correlate this information with clinicopathological parameters. Based on the results from IHC, where OSCC expressed CD9 and CD82 exclusively, we also investigated the patterns of CD9 and CD82 gene expression by quantitative Real Time-polymerase chain reaction (qRT-PCR).

Materials and methods

Tissue samples and patient characteristics

Surgically resected primary OSCC were collected at the Department of Head and Neck Surgery, Hospital A.C. Camargo, São Paulo,

Brazil, between 1992 and 2004. None of the patients had received radiotherapy or chemotherapy before surgical excision. Paraffin-embedded specimens from 179 patients were sampled and used to construct two tissue microarray (TMA) blocks. These blocks were then used for IHC tetraspanins analyses. Additionally, qRT-PCR was performed in 66 cases (from these, 50 cases were present in the TMA blocks and 16 were additional cases included in the study). Paraffin-embedded tissue from the 16 additional cases was not accessible for IHC analysis. Follow-up information was not available from these 66 samples.

Fourteen samples of normal mucosa were used as controls, ten samples for IHC analysis, and four samples for the qRT-PCR experiments.

The studies were performed according to local ethical committee approval (Study No. 986/07). The patients ranged in age from 30 to 90 years old (mean age of 57 years). The clinicopathological characteristics of patients with OSCC are summarized in Table 1.

TMA and immunohistochemistry

The selected blocks of each sample of tumor and normal tissue were used for the construction of the TMA using a manual arraying instrument (Manual Tissue Arrayer 1, Beecher Instruments Microarray Technology, Silver Spring, MI, USA). The sampling consisted of 2 mm cores from different areas of the tumor from a single case of OSCC, placed at a specified coordinated place. Each case and tissue of normal mucosa was spotted in duplicate.

Four micrometer sections were deparaffinized and rehydrated. Antigen retrieval was performed with citrate buffer solution of sodium citrate (pH 6.0) and boiled in pressure cooker for 15 min.

Slides were placed in 3% hydrogen peroxide 10 V three times for 5 min. The sections were blocked with protein block serum-free (Dako, Carpinteria, USA) at room temperature for 20 min.

The sections were incubated with the primary antibodies (all from NeoMarkers-Lab Vision-Corporation, USA) at room temperature for 2 h. After being washed with phosphate buffered solution three times for 5 min each; sections were then incubated for 1 h with the indirect dextran polymer detection system (Novocastra Laboratories Ltd., Newcastle, UK). Staining was performed by incubating the slides in 3,3′-diaminobenzidine tetrachloride (Dako, Carpinteria, USA). The slides were then lightly counterstained with hematoxylin, dehydrated in absolute ethanol and xylene, and then mounted with coverslips using a permanent mounting medium and observed using an optical light microscope.

All IHC reactions were carried out in quadruplicate (two cores per slide, with two stained slides).

The semi-quantitative analysis of the results evaluated the average area of staining in all cores (four cores each case). We considered negative or downregulated cases up to 10% of tumor cells positively staining (downregulated/negative). Positive cases were identified where over 10% of the tumor cells were stained with the marker.

Isolation of RNA and qRT-PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and first-strand cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA), according to the protocol provided by the manufacturer.

Sixty-six OSCC samples and four samples of normal control oral mucosal tissue (grouped into one pool) were evaluated by qRT-PCR. The CD9 (Genbank Accession Number NM_001769.2) and CD82 (Genbank Accession Number NM_002231) sequences were used as a template for the construction of the primer pairs using the 3.0 version of the PrimerExpress software (Applied Biosystems, Foster City, CA). The PCR primer set sequences were designed as follows: CD9 forward primer 5′-GCA TTG CCG TGG TCA TGA T-3′ and CD9 reverse primer 5′-TGC GGA TAG CAC AGC ACA AG-3′; CD82 forward primer 5′-CGT GGG TGT GGC CAT CAT-3′ and CD82 reverse primer 5′-TTG CTG TAG TCT TCG AAT GG-3′; the internal control was the β-actin forward primer 5′- GCA CCC AGC ACA ATG AAG-3′ and the reverse primer 5′-CTT GCT GAT CCA CAT CTG C-3′.

PCR amplification was performed in duplicate in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reactions were performed in 20 μL volumes with 10 ng of the cDNA sample, 0.2 μM of each of the primers and 1X of the Syber-Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA). The amplification program consisted of one cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The same PCR conditions were used to amplify the β-actin gene.

The relative amount of PCR product generated from each primer set was determined on the basis of the Ct value and relative quantification was calculated by a mathematical model, previously described by Pfaffl.[12]

Statistical analysis

Association between protein expression and clinicopathological parameters were assessed by the χ^2-test. The disease-free survival and the overall survival curves were constructed according to the Kaplan–Meier statistical method. The Mann–Whitney test was used to determine the association between the expression of the CD9 and CD82 genes and the pathological parameters.

The relationship between protein levels and genes expression was evaluated using the Mann-Whitney test. All *p*-values were based on two-tailed statistical analysis and *p* = 0.05 was considered to be statistically significant. Statistical analysis was performed using the SPSS 13.0 statistical package (SPSS Inc, Chicago, IL).

Table 1
Clinicopathological characteristics of patients with OSCC for IHC and qRT-PCR analysis.

Clinicopathological parameters	IHC	qRT-PCR
	Total (*n*) (%)	Total (*n*) (%)
Sex		
Male	145 (81)	52 (79)
Female	34 (19)	14 (21)
Clinical stage		
I and II	53 (30)	30 (45)
III and IV	126 (70)	36 (55)
Lymph nodes		
N0	73 (44)	27 (45)
N1–3	92 (56)	32 (54)
No lymph nodes dissection	14	7
Perineural invasion		
No	91 (55)	33 (55)
Yes	74 (45)	27 (45)
No available	14	6
Vascular embolization		
No	109 (65)	53 (84)
Yes	58 (35)	10 (16)
Not available	12	3
Histological grade		
Well-differentiated	127 (72)	39 (59)
Poorly/moderately differentiated	49 (28)	27 (41)
Not available	03	