Evaluation of *MAGE A1* in oral squamous cell carcinoma

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Abstract. *MAGE A1* is a cancer testis antigen (CTA) described in a variety of human cancers. CTAs exhibit a highly restricted tissue expression and by virtue of their immunogenic potential, these genes are promising target molecules for cancer vaccines. DNA hypomethylation is associated with gene regulation in several types of tumours. The aim of this project was to identify the presence of *MAGE A1* in oral squamous cell carcinoma (OSCC) samples and to investigate the hypomethylation profile of CpG islands situated in the promoter region of this gene. The expression of *MAGE A1* in OSCC and healthy oral mucosal samples was determined by real-time quantitative and conventional endpoint PCR and also by immunohistochemistry staining. In addition, to investigate the hypomethylation profile of promoter *MAGE A1* CpG islands, we performed bisulphite sequencing. Real-time quantitative and endpoint PCR assays demonstrated a lower level of *MAGE A1* transcription. Endpoint PCR showed expression of *MAGE A1* in 10% (2/20) of OSCCs. Sodium bisulphite sequencing analysis of *MAGE A1* CpG islands did not reveal a difference between OSCC and normal oral mucosal samples. We further assessed MAGE A1 protein immunoexpression and found 80% (16/20) of immunopositivity in OSCCs. We did not observe a correlation between the presence of MAGE A1 protein and lower levels of transcripts. Identification of MAGE A1 protein in OSCCs and absence of immunoexpression in normal oral mucosa support the idea that this protein can be used as a biomarker for detection of OSCC; however, it is not associated with hypomethylation or high expression of the *MAGE A1* gene.

Introduction

Cancer of the head and neck, including oral squamous cell carcinoma (OSCC), is the sixth most common malignancy worldwide, being one of the major causes of morbidity and mortality due to cancer (1,2). Five-year survival of oral cancer varies from 81% for patients with localised disease to 42% for those with regional disease and to 17% when distant metastases are present (2). Despite advances observed with treatment, recurrences in OSCC are common, and an effective treatment is required. There is a clear need for identification of new molecular targets and signalling pathways, which are necessary to design an appropriate therapeutic strategy (3).

Aberrant DNA methylation on promoter sites has been previously associated with inactivation of tumour suppressor genes in oral carcinogenesis (4-6). Methylation is a frequent epigenetic event and occurs by the addition of a methyl radical ($-CH_3$) to a cytosine (C) situated at a 5' position of a guanine (G) in CpG dinucleotides of superior eukaryotic cell DNA (7,8). Genetic and epigenetic events can confer competitive advantages to a cell, leading to a cancer phenotype (9).

Cancer testis antigens (CTAs) are immunogenic proteins normally expressed in germ cells and in trophoblasts, being aberrantly expressed in various cancer types (10). Those that are epigenetically activated in many human cancers (11) and exhibit high tissue-restricted expression, are considered promising target molecules for cancer vaccines (12).

About 44 CTA gene families have been described and contain multiple members (e.g. *MAGE A, GAGE1*). Forty-one CTA families were found associated with malignant tumours, including oesophageal cancer, gastric cancer, head and neck cancer, ovarian cancer, and hepatocellular carcinoma (12-15).

The first CTA identified was observed on the surface of melanoma cells and received the melanoma-associated antigen 1 denomination (*MAGE A1*) (16). *MAGE A1* belongs to a family of 12 genes located in the q28 region of chromosome X (17). Expression of *MAGE A1* was detected in melanomas, breast cancer, head and neck squamous cell carcinoma (HNSCC), and oesophageal carcinoma, but not in normal tissues other than testicles (15,18,19). *MAGE A1* has been described to act as a potent transcriptional repressor by binding and recruiting histone deacetylase 1 (HDAC1) and by binding to Ski-interacting protein (SKIP) (a transcriptional regulator involved in many signalling pathways) (20).

In the present study, we assessed *MAGE A1* expression in OSCC by quantitative and qualitative analysis and investigated the presence of MAGE A1 protein by immunohistochemistry in OSCC. In an attempt to establish a participation of epigenetic

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events in the regulation of this gene, we also investigated *MAGE A1* promoter CpG profile by bisulphite sequencing. This is the first research showing the methylation profile of *MAGE A1* in OSCC.

Materials and methods

OSCC specimens and control samples. Tissue samples of 20 primary OSCC patients submitted to tumour resection at a local hospital, who received neither radiotherapy nor chemotherapy prior to surgery, and 10 normal oral mucosal controls (N) of healthy donors submitted for third molar extraction at the Department of Oral Surgery of the Universidade Federal de Minas Gerais were investigated. Written informed consent was obtained from all patients at the time of enrolment, and the local ethics committee approved all aspects of this investigation. Each sample was divided into 2 pieces, one was paraffin-embedded and the other was immediately snap frozen and stored at -80˚C and destined to nucleic acid examination. Testis cDNA generously donated by Dr Andre Vettore, UNIFESP, São Paulo) was used as a positive control for real-time quantitative and endpoint PCR.

Immunostaining. Immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded tissue sections mounted on glass slides (SuperFrost Plus, Menzel, Braunschweig, Germany). The sections were deparaffinized, rehydrated, and stained with haematoxylin using standard histological techniques. After deparaffinization in xylene, the slides were rehydrated in decreasing concentrations of ethanol. The sections were then submitted to antigen retrieval with EDTA pH 9.0, followed by incubation of the rabbit polyclonal antibody anti-MAGE A1 (Abcam, Cambridge, UK; 1:100, ab53131). This was followed by incubation with the indirect dextran polymer detection system (EnVision, Dako, Carpinteria, CA, USA). Staining was completed by incubation with 3,3'-diaminobenzidine tetrachloride (DAB). The specimens were then lightly counterstained with Mayer's haematoxylin, dehydrated, and mounted with a glass coverslip and xylene-based mounting medium. Negative controls were achieved by substituting primary specific antibodies with bovine serum albumin. The positive control consisted of 1 OSCC sample known to be positive to antibody anti-MAGE A1.

RNA extraction and cDNA synthesis. Total cellular RNA from normal and tumour samples was isolated using the Tri-Phasis reagent (BioAgency, São Paulo, SP, Brazil) according to the manufacturer's protocol with some modifications. The isolated RNA was quantified by spectrophotometry using BioSpec-mini (Shimadzu, Kyoto, Japan). The quality of the RNA was assessed by 1% agarose gel electrophoresis and GelRed™ nucleic acid 10.000X (Biotium, Hayward, CA, USA) staining. To prevent contamination of genomic DNA, 1.5 μg of RNA was subsequently treated with 1 U DNase I (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using SuperScript™ II First Strand Synthesis kit (Invitrogen). The quality of cDNAs synthesised was evaluated through polymerase chain reaction (PCR) amplifications of *ACTB* (NM_001101) forward primer 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3' and reverse primer 5'-AACCAATAAAACCTACTCCTCCCTTAA-3'. Reactions were carried out under the following conditions: 94˚C for 2 min, followed by 35 cycles at 94˚C for 30 sec, 57˚C for 45 sec, 72˚C for 1 min, and 72˚C for 7 min. PCR products were loaded onto 6.5% polyacrylamide gels and visualised by silver staining.

Real-time quantitative PCR (qRT-PCR) analysis. Quantitative PCR (TaqMan) analysis of *MAGE A1* expression in 15 cases of OSCC and 10 normal oral samples was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expression was evaluated by qRT-PCR using gene-specific FAM/MGB commercial assays Hs00607097_m1 for *MAGE A1* (Applied Biosystems). Expression values of the target gene were normalised by expression of endogenous control RPLPO TaqMan FAM/MGB assay (Applied Biosystems) and normalised on the basis of the expression of testis, a reference sample (calibrator). Reactions were carried out in a total volume of 10 μl containing 1 μl of cDNA solution, 3.5 μl of water, 5 μl of 2X PCR Master mix ABI (Applied Biosystems), and 0.5 μl of TaqMan. The cycling program was 50˚C for 2 min, 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Reactions were performed in duplicates.

Endpoint PCR. Expression level of *MAGE A1* was evaluated by endpoint PCR in 20 OSCCs and 10 normal oral mucosal specimens. Primer pairs of *MAGE A1* have been previously described (21). The amplification parameters for these PCR assays and primers were as follows: 94˚C for 2 min and 35 cycles of 94˚C for 30 sec, 68˚C for 30 sec, and 72˚C for 30 sec. The primers were 5'-CGGCCGAAGGAACCTGACCCAG-3' and 5'-GCTGGAACCCTCACTGGGTTGCC-3'. Cycling was concluded with a final extension step at 72˚C for 7 min.

Cloning and bisulphite genomic sequencing. Genomic DNA was obtained using the Tri-Phasis reagent according to the manufacturer's protocol and 4 M guanidinium isothiocyanate, 50 mM of sodium citrate, and 1 M Tris, buffer pH 8.0. After centrifugation, the DNA precipitate was washed with 100% isopropanol and 70% ethanol and resuspended in 50 μl of water and stored at -20˚C. DNA integrity was evaluated through PCR amplification of the β-globin using 5'-CAACTTCATCCACGTTCACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3' primers. Sodium bisulphite conversion of unmethylated cytosine residues to uracil was performed using a modification of a previously described method (22). Briefly, 2 μg of genomic DNA and 1 μl of salmon sperm DNA (1 μg/ml) were denatured with NaOH (final concentration 0.3 M) to a final volume reaction of 20 μl followed by incubation for 20 min at 50˚C. A volume of 500 μl freshly made bisulphite solution pH 5.0 (2 M sodium metabisulphite, 125 mM hydroquinone, and 350 mM NaOH) was added to each denaturation reaction, and the mixture was incubated at 70˚C for 3 h in the dark. The resulting bisulphite-converted DNA was then purified using Wizard DNA purification resin (Promega Corp. Madison, WI, USA) according to the manufacturer's instructions. The bisulphite-modified DNA was resuspended in 20 μl of water. Identification and primer

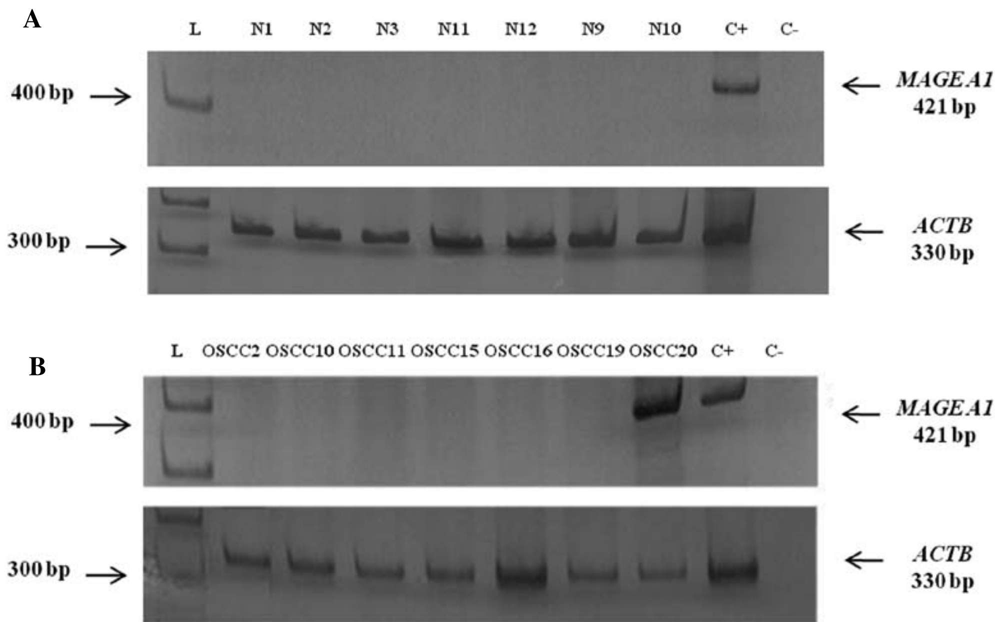

Figure 1. Analysis of *MAGE A1* gene expression. (A) *MAGE A1* expression in normal oral mucosal samples (N). (B) *MAGE A1* expression in oral squamous cell carcinoma samples (OSCC). RNAm *ACTB* was used to evaluate quantity in each endpoint PCR reaction. L, 100-bp DNA ladder; bp, base pairs; C+, positive control (testicle RNA); C-, negative control (without cDNA).

designs to amplify CpG islands situated in the promoter site of the *MAGE A1* gene was performed using *in silico* tools: Nucleotide (NCBI), Blat UCSC (www.genome.ucsc.edu), Methprimer (http://www.urogene.org/methprimer/index1.html) sites and GeneRunner software (www.generunner.net/); according to CpG island criteria 100 bp, %GC >50 and the observed (Obs) to expected (Exp) CpG ratio, Obs_{CpG}/Exp_{CpG} >0.6. The pair of primers used for PCR amplification of bisulphite-converted DNA did not contain any CpG nucleotide; in this way, both methylated and unmethylated samples were amplified. The PCR conditions for *MAGE A1* CpG island amplification and primers sequences were: 94˚C for 2 min and 30 cycles of 94˚C for 30 sec, 66˚C for 45 sec, 72˚C for 1 min and a final extension of 72˚C for 7 min. The primers used were 5'-GGAAGGGTTGTTTAGGAGAGGGTAG-3' and 5'-TCAATCCTCCCTCAACCTCTCAC-3'. Amplified products were electrophoresed on 1.5% agarose gels and purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Waukesha, WI, USA). After purification, products were cloned into the pCR4-TOPO™ vector using the TOPO-TA Cloning kit for Sequencing (Invitrogen). Preparation of electrocompetent cells was performed according to the Sambrook and Russel protocol (23). All products of transformation were electroporated into competent *Escherichia coli* MC1061 with 2500 V using Electroporator 2510 (Eppendorf, Hamburg, Germany). Plasmid DNAs from ampicillin-resistant colonies were extracted. We used 400 ng of each plasmid extraction in 10 μl sequencing reaction mixture using the ABI Prism BigDye Terminator cycle sequence kit (Applied Biosystems) and M13 primers 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' and 5'-TCACACAGGAAACAGCTATGAC-3'. Sequences were resolved on an ABI Prism™ 310 Genetic Analyser and analysed with Chromas software (http://www.technelysium.com.au/chromas.html).

Results

Absence of MAGE A1 transcripts in OSCC. To evaluate the expression level of *MAGE A1* in OSCC, we analysed 15 OSCC samples and 10 normal oral mucosal samples by qRT-PCR. *MAGE A1* transcript levels were undetectable in most OSCC and were absent in normal mucosal samples. We found evidence of *MAGE A1* expression in testis RNA used as a positive control and in only 1 OSCC sample, but this expression was on a small level near the detection limit of 38 cycles (data not shown). We also observed similar results when 10 normal oral mucosal samples and 20 OSCC samples were evaluated by endpoint PCR. We found, in agreement with the qRT-PCR data, low *MAGE A1* transcript levels. *MAGE A1* transcripts were absent in normal oral mucosal samples (Fig. 1A). Higher expression of this gene was detected in control and at least in 1 sample of OSCC (sample OSCC20) (Fig. 1B) (this sample was not evaluated by qRT-PCR assay).

Expression of *MAGE A1* was also visualised in OSCC17 sample at 40 cycles of endpoint PCR (Fig. 2). This sample was the one that demonstrated amplification near the detection limit of 38 cycles by qRT-PCR assay. *MAGE A1* was not detectable in any remaining OSCC samples or normal samples (data not shown).

MAGE A1 promoter methylation status. We studied the methylation profile of 16 CpGs situated in the promoter region of *MAGE A1* in clones produced from 3 OSCC samples and 3 normal mucosal samples. As the mechanism of *MAGE A1* gene activation is promoter hypomethylation, we performed a bisulphite DNA sequencing analysis to verify hypomethylation status in the promoter region of this gene. The *MAGE A1* CpG island, defined according to CpG island criteria previously described, spanned from –117 to +42, where the initiation nucleotide was defined as

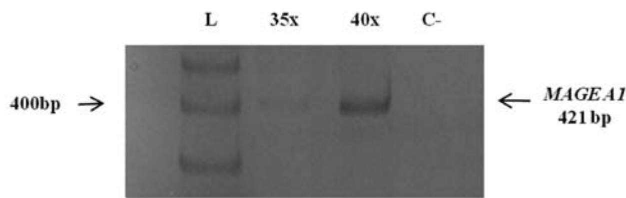

Figure 2. Expression of *MAGE A1* on oral squamous cell carcinoma case (sample OSCC17) using endpoint PCR analysis. 35x, 35 cycles of PCR amplification; 40x, 40 cycles of PCR amplification PCR; L, 100-bp DNA ladder. bp, base pairs; C-, negative control (without cDNA).

position +1 (Fig. 3). We found 23.4% (15/64), 11.1% (16/144), and 23.75% (38/160), respectively, hypomethylated CpGs in normal samples (Fig. 3A) as compared to 22.5% (36/160), 7.3% (7/96), and 12.5% (6/48) of hypomethylated CpGs in OSCC samples (Fig. 3B). The CpGs at –263, –231, –112, –151, and +14 were hypomethylated in both OSCC and normal mucosal samples. Thus, the *MAGE A1* CpG island was heavily methylated, and we did not observe differences between the promoter CpGs hypomethylation in normal mucosal samples and OSCC samples.

Presence of MAGE A1 antigen in OSCC. To determine the presence of *MAGE A1* protein, we used anti-MAGE A1 antibodies in immunohistochemical evaluation. Immunopositivity to MAGE A1 was found in 80% (16/20) OSCCs (Fig. 4), and MAGE A1 was negative in 100% (10/10) of normal oral mucosal (data not shown). MAGE A1 protein was distributed in the cytoplasm of OSCC cells.

Discussion

Despite the advances observed in OSCC therapy research, an improvement in the 5-year survival has not been observed (24). This situation may be related to an inadequate TNM classification or the absence of an effective therapy (25).

Immunotherapy is becoming an attractive treatment in HNSCC (26). However, an adequate antigen candidate to immunotherapy is one whose expression is specific and stable, crucial to survival, and absent in normal cells (18). Based on these characteristics, CTA proteins are considered ideal targets for cancer immunotherapy by virtue of their highly tissue-restricted expression (12).

In the present study, we evaluated *MAGE A1* expression and its promoter methylation profile in OSCC samples. The *MAGE A1* gene is a member of a large gene family, which shares a common domain (27). The *MAGE A1* gene was reported to be transcriptionally reactivated in many tumours such as melanomas, breast carcinoma, oesophageal tumours and HNSCC (15,18,19).

Evaluation by qRT-PCR in our study demonstrated the absence of *MAGE A1* transcription in normal oral mucosal samples and a lower expression in OSCCs. This lower expression in OSCC is not in accordance with a previous study (28) that observed a significant increase in the expression

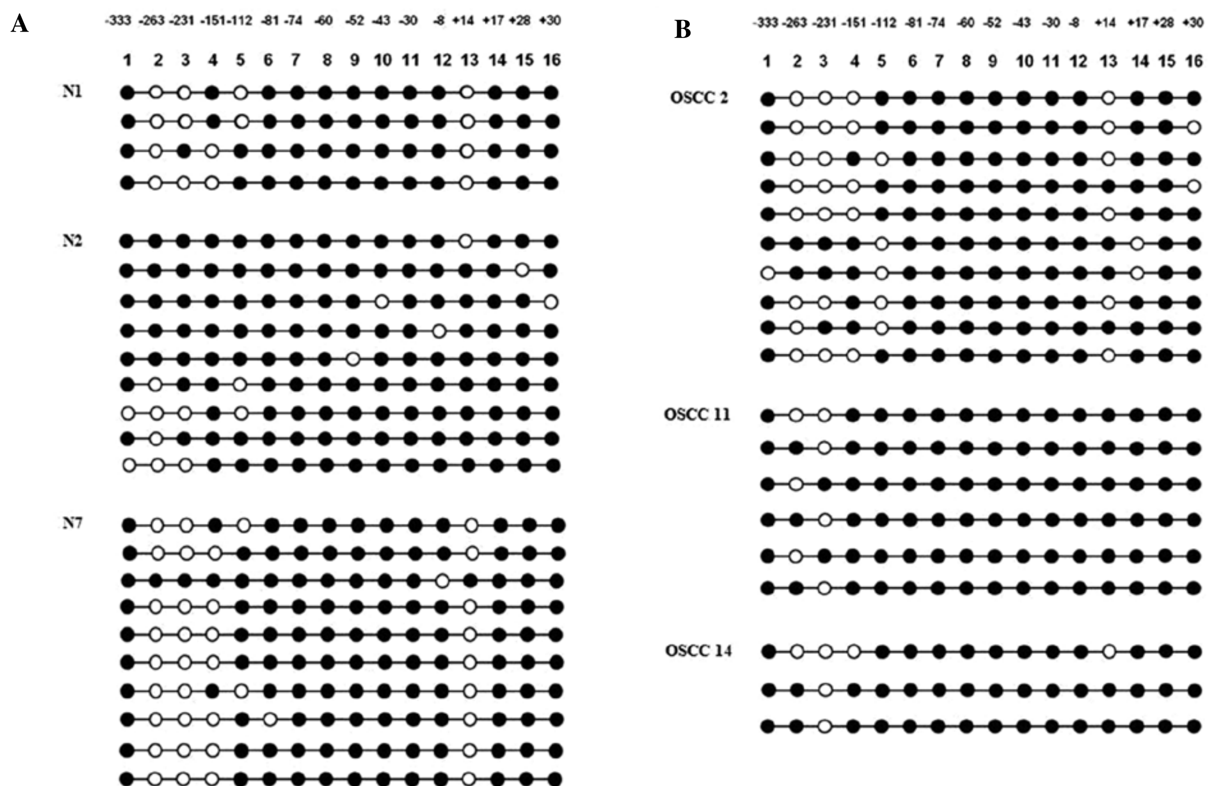

Figure 3. Analysis of *MAGE A1* CpG island methylation status by bisulphite sequencing in normal oral mucosal and oral squamous cell carcinoma samples. (A) Methylation profile of *MAGE A1* CpG island in normal mucosa (N). (B) Methylation profile of *MAGE A1* CpG island in oral squamous cell carcinoma samples (OSCC). Each row of circles represents a single sequenced clone. Open circles represent non-methylated CpGs, while closed circles represent methylated CpGs (16 CpGs).

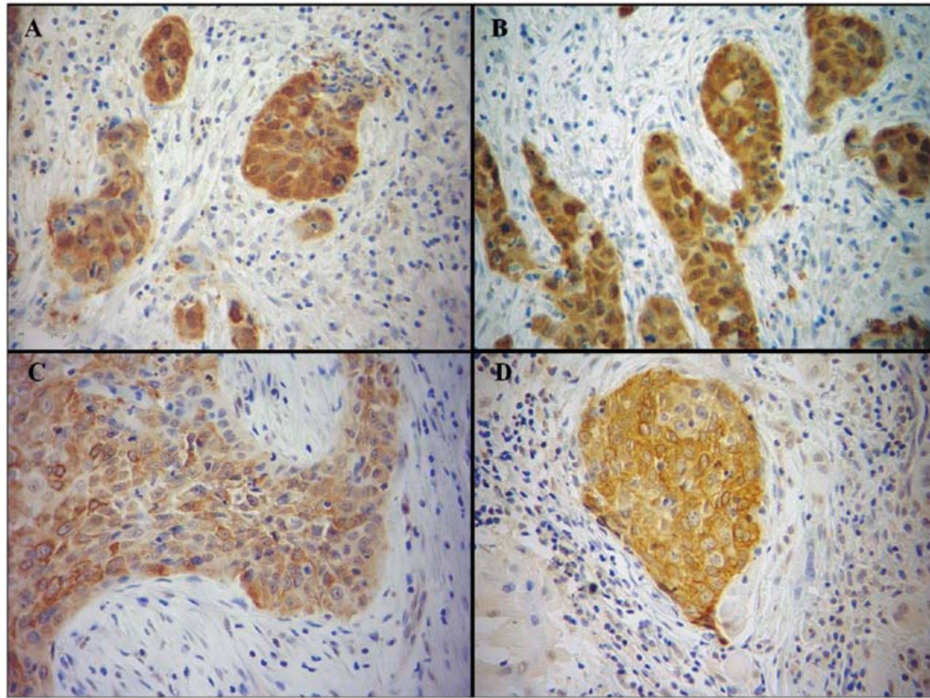

Figure 4. Immunohistochemical of MAGE A1 staining pattern. Cytoplasmic staining was observed. (A and B) oral squamous cell carcinoma sample OSCC 14; (C and D) oral squamous cell carcinoma sample OSCC18.

of *MAGE A* gene family in 5 OSCC cell lines by qRT-PCR normalised to the expression in adult keratinocytes. However, it should be noted that these authors verified *MAGE A* expression in cell lines and not in fresh tissue samples. Unfortunately, there is no literature data available regarding *MAGE A1* quantitative expression evaluation in patients' tissue samples to support our results.

Our endpoint PCR experiments also showed the absence of *MAGE A1* transcripts in normal mucosa and a lower expression in OSCC samples. Using this same assay, another group (15) evaluated the expression of *MAGE A1, MAGE A4, MAGE A10, MAGE A12, BAGE, GAGE-1, LAGE-1, NYESO-1*, and *PRAM*E in 33 HNSCC patient samples (15, oral cavity; 14, larynx; and 4, pharynx). They reported that 57.1% of the cases expressed at least one of the *MAGE* family members (*MAGE A1, MAGE A4, MAGE A10* and *MAGE A12*), of which *MAGE A1* was expressed in 30.3% of the cases. The expression described by these authors was distributed at different anatomic sites in HNSCC, not only in the oral cavity. Ries *et al* (24) investigated the expression of *MAGE A1-A6* and *MAGE A12* using nested PCR in 55 OSCC samples and 20 normal mucosae, and they noted the absence of expression of these genes in normal mucosa. However, according to these authors, the majority of tumour samples (85.45%) (47/55) of OSCC expressed at least 1 of the 7 examined *MAGE A* subtypes, whereas 60% of them expressed 2 subtypes, supporting the hypothesis that simultaneous detection of *MAGE A* subtypes has been found to be more sensitive and specific for diagnostic and prognostic evaluation of OSCC.

Hypomethylation is extensively described as the main mechanism responsible for the activation of the *MAGE* gene family expression (29-32). In our study, we did not observe any difference between hypomethylation levels in normal oral mucosa and OSCC samples. Despite the presence of hypomethylation detected in several clones, the CpG island situated at the *MAGE A1* promoter site evaluated was found heavily methylated in both normal mucosal and OSCC samples, presenting a small number of demethylated CpGs. New sequencing experiments with a large number of samples can probably help us to better comprehend this mechanism.

Immunohistochemical analysis in this present study revealed 80% of MAGE A1 protein immunopositivity in OSCC and absence of immunopositivity in normal oral mucosa. An immunohistochemical investigation of MAGE A antigens in 47 primary OSCC, was previously conducted by Muller-Richter *et al* (33) and showed that these antigens were present in 55% of samples. Krauss *et al* (34) investigated by immunohistochemistry the presence of MAGE A proteins in benign, precancerous, and cancerous lesions of the oral mucosa and did not detect MAGE A antigens in benign lesions [oral traumatic lesions (ulcers), dental follicles, and epulis]. The staining rate of dysplastic precancerous lesions or malignant lesions ranged from 33 to 65%. According to these authors, MAGE A antigens may facilitate differentiation between precancerous and lesions of the oral mucosal.

The lack of correlation between the presence of proteins and the absence of transcripts of *MAGE* that we found in OSCC can probably be explained by the presence of *MAGE A1* isoforms that we could not detect during transcript analysis. We need to emphasize the fact that the MAGE family members are strongly related. The *MAGE A1* gene presents 64-85% homology of the last coding exon with other *MAGE* genes. MAGE A2-A6 proteins have 57% homology with MAGE A1 protein, and MAGE A8-12 have 77% homology with MAGE

A1 protein (17,35). Dhodapkar *et al* (36) also cited the existence of discrepancy between the RNA and protein levels during the analysis of CTA expression. According to them, this great homology among several CTAs cannot exclude the cross-reaction of CTA reagents; however, until recently, a cross-reaction between these reagents with proteins that do not belong to the CTA families had not been found.

In conclusion, our results show the absence and/or low expression of *MAGE A1* transcripts in OSCC. Presence of hypomethylation at a small level at the promoter site of *MAGE A1* was detected in both OSCC and normal oral mucosa. However, a protein recognised by the anti-MAGE A1 antibody was present in most OSCC samples and absent in all normal oral mucosal samples. Despite the low level of expression observed during the evaluation of *MAGE A1* transcripts in OSCC, our immunohistochemistry results reinforce the necessity of additional investigations. This finding supports previous investigations that suggest the importance of these antigens as potential targets of immunotherapy (15).

Acknowledgements

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