EXPRESSION OF CANCER-TESTIS ANTIGENS MAGE-A4 AND MAGE-C1 IN ORAL SQUAMOUS CELL CARCINOMA

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Abstract: *Background.* Tumor markers are genes or their products expressed exclusively or preferentially in tumor cells and cancer-testis antigens (CTAs) form a group of genes with a typical expression pattern expressed in a variety of malignant neoplasms. CTAs are considered potential targets for cancer vaccines. It is possible that the CTA *MAGE-A4* (melanoma antigen) and *MAGE-C1* are expressed in carcinoma of the oral cavity and are related with survival.

Methods. This study involved immunohistochemical analysis of 23 patients with oral squamous cell carcinoma (SCC) and was carried out using antibodies for *MAGE-A4* and *MAGE-C1*. Fisher's exact test and log-rank test were used to evaluate the results.

Results. The expression of the *MAGE-A4* and *MAGE-C1* were 56.5% and 47.8% without statistical difference in studied variables and survival.

Conclusion. The expression of at least 1 CTA was present in 78.3% of the patients, however, without correlation with clinicopathologic variables and survival. © 2011 Wiley Periodicals, Inc. *Head Neck* **00:** 000–000, 2011

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Oral carcinoma has become 1 of the most prevalent malignant tumors in South America and in Brazil and ranks as the fifth and seventh most common cancer in men and women, respectively.[1,2] In spite of advances both in diagnosis and treatment,[3] there is an increase in the oral cancer mortality rate in the last decades and additional treatment options are sought.

Tumor markers may be genes or their products that are expressed exclusively or preferentially in tumor cells, and which may be used for their distinction from normal cells.[4] Cancer-testis antigens (CTAs) form a group of genes and gene families with a typical expression pattern as they are expressed in a variety of malignant neoplasms but which are not present in any normal adult tissues except in germ cells of the testis.[5] The prototypical CTA *MAGE-1* (now *MAGE-A1*) was identified by autologous T-cell epitope cloning in a patient with metastatic melanoma. Subsequently, with the use of other techniques such as serological analysis of recombinant cDNA expression libraries (SEREX), database mining, and serial analysis of gene expression (SAGE), various other genes and gene families were identified.[6,7] To date, approximately 100 CTAs have been identified and classical CTAs which map to chromosome X are distinguished from nonclassical mapping to other chromosomes.[8] Many CTAs were shown to elicit cellular or serological immune responses in the autologous host.[9]

Due to its almost exclusive presence in various types of malignant tumors, and their capacity to induce autologous immune responses, CTAs are considered potential targets for cancer vaccines. Several

Table 1. Frequency of staining of the antibodies 57B and CT7-33.

	Number of patients (%)	
Staining	57B	CT7-33
0	10 (43.5)	12 (52.2)
1+	3 (13)	4 (17.4)
2+	2 (8.7)	3 (13)
3+	3 (13)	0 (0)
4+	5 (21.8)	4 (17.4)

clinical trials have been undertaken or are being conducted using CTAs as vaccine targets to stimulate autologous immune responses in patients with tumors. Although some of them have rendered promising results.[10] Because of the importance of CTAs as targets for cancer vaccines, detailed studies of their expression in human cancers is a necessity.

Although the expression of CTA has been studied in carcinomas of the head and neck, there exists few studies specifying their primary site. Moreover, specific analyses of carcinomas of the oral cavity are missing/rare in the literature.[11]

The purpose of this study was to analyze the expression of 2 CTAs, *MAGE-A4* and *MAGE-C1*, in carcinomas of oral cavity, analyzing their potential association with sociodemographic variables, histological characteristics of the tumor, as well as the overall survival.

MATERIALS AND METHODS

Patients. The present study was part of the project GENCAPO (Head and Neck Genome Project of the State of São Paulo, Brazil), approved by the ethics committee of the Faculty of Medicine of the University of São Paulo, Ribeirão Preto, Brazil (process number 11677/2009). Twenty-three patients who were diagnosed with squamous cell carcinoma (SCC) of the oral cavity and who underwent a surgical procedure from January 2001 to January 2006 at the Head and Neck Service of the University Hospital, Ribeirão Preto, were included in this study. Patients with lip cancer, and/or with secondary involvement of the oral cavity, and patients who missed clinical follow-up were excluded from the study. All patients were required to give informed consent to participate in the study. Sociodemographic, clinical, and anatomopathological data were collected and transferred to the data base of the Clinical Genomics Project.

Patients underwent surgical resection and postoperative treatment was done according to established treatment standards.

Immunohistochemical Analysis. For immunohistochemical analysis, a paraffin block containing a representative tumor area was retrieved from the archives of the Department of Pathology. Sequential 4-micron sections were cut and mounted on slides with a solution of 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, MO). The following primary antibodies were used: *MAGE-A4* (57B, 1:4.000 in 1 mM EDTA buffer, pH 8.0; Ludwig Institute, NY) and *MAGE-C1* (CT 7-33, 1:32.000, 10 mM citric acid, pH 6.0; Ludwig Institute, NY). To avoid background reactivity due to the presence of endogenous biotin and avidin, a dextran polymer secondary (NovoLink, Novocastra, United Kingdom) was used. For antigen retrieval, slides were heated for 30 minutes in a household vegetable steamer immersed in a buffer solution.

Endogenous peroxidase activity was blocked with H_2O_2 at 6%. Incubation with primary antibody was done in a humidity chamber at 4°C for 12 hours. Incubation with secondary antibody diluted in phosphate buffer saline was made for 30 minutes at 37°C. Finally, tissue sections were washed in running water for 10 minutes, counterstained with Harris hematoxylin for 10 seconds, washed again in running water, dehydrated in ethanol, and cleared in xylene. Slides were mounted in Permount resin (Fisher Scientific, Fair Lawn, NJ). Testicular tissue with preserved spermatogenesis was used as a positive control; slides with omission of the primary antibody replaced by phosphate buffer saline were used as negative controls.

The expression was graded semiquantitatively based on the number of immunopositive tumor cells as follows: 0, up to 5% of the tumor cells stained; 1+, 6% to 25% of the tumor cells stained; 2+, 26% to 50% of the tumor cells stained; 3+, 51% to 75% of the tumor cells stained; 4+, >75% of the tumor cells stained.

Statistical Analysis. Fisher's exact test was used to evaluate the relationship of each variable to *MAGE-A4* and *MAGE-C1* expressions. The Kaplan–Meier method was used to estimate survival curves for the group that expressed the antigen as well as for the group that did not express it and the log-rank test was used to compare them.[12] A p value $< .05$ was considered statistically significant.

RESULTS

Of the 23 patients selected in the study, 87% were men, with an average age of 55.3 years old, the youngest was 29 years old and the oldest was 72 years old. It was also observed in this sample that 91.3% of the patients were smokers, 74% were drinkers, and 74% of the patients were both smokers and drinkers. In the studied sample, the preferred site was the floor of the mouth (47.8%), followed by the tongue (39.1%), gingiva (8.7%), and retromolar trigone (4.4%). Regarding stage, the most part was T2 (56.4%), followed by T3 and T4, both with 17.4% and T1 (8.8%). Of these patients, 65.2% had lymph node metastasis and 47.8% were at clinical stage IVa, 34.8% were at clinical stage III, 13% at clinical stage II, and only 4.4% were at clinical stage I.

The immunohistochemical analysis showed that the expression of *MAGE-A4* by the antibody 57B and *MAGE-C1* by antibody CT7-33 is distributed according to Table 1.

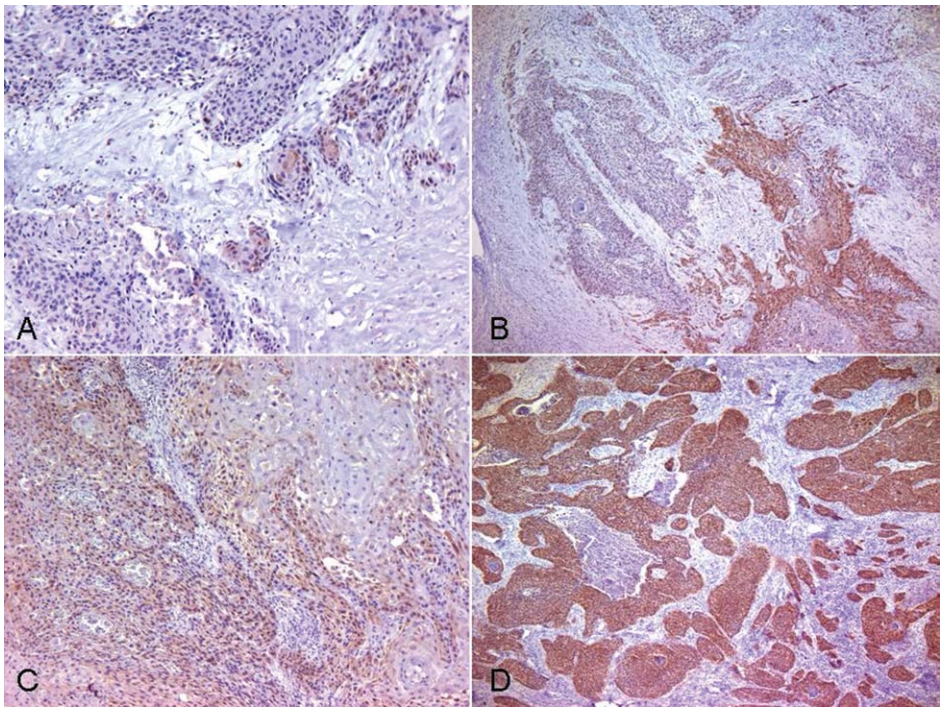

FIGURE 1. Expressions of the MAGE A4. **(A)** Expression 1+. **(B)** Expression 2+. **(C)** Expression 3+. **(D)** Expression 4+.

Considering the positive expression for the antibody 57B staining higher than 5% of the cells, the expression of the *MAGE-A4* in oral carcinoma was 56.5% (Figure 1) and the expression of *MAGE-C1* in oral carcinoma was 47.8% (Figure 2).

It was observed that the *MAGE-A4* and *MAGE-C1* expressions did not have statistical significance to

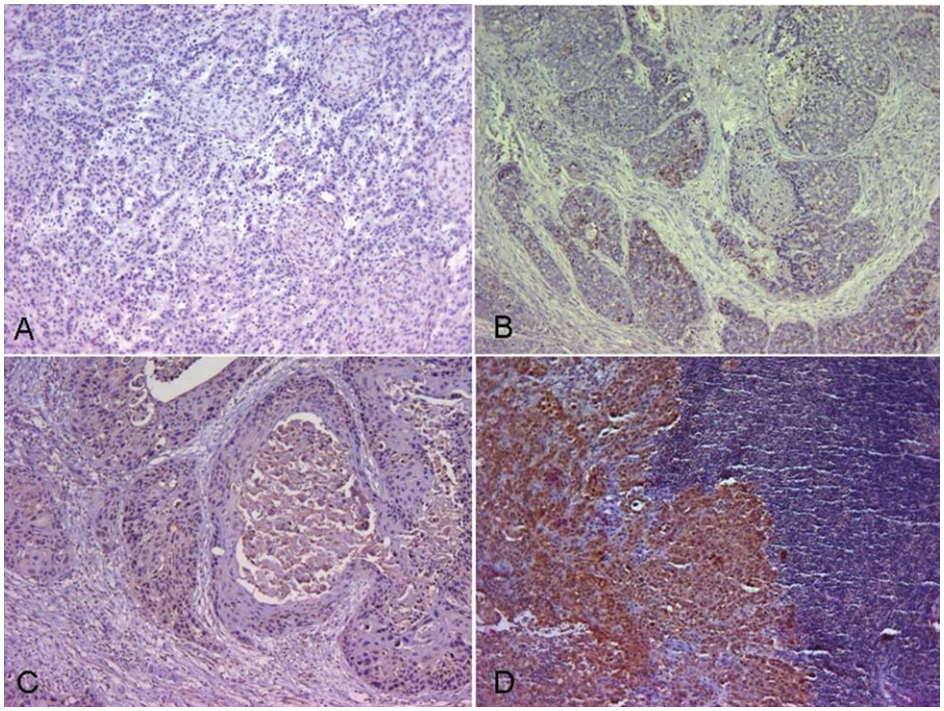

FIGURE 2. Expressions of the MAGE C1. **(A)** Expression 0. **(B)** Expression 1+. **(C)** Expression 2+. **(D)** Expression 4+.

Table 2. Frequency distribution of variables with the expression *MAGE-A4* and *p* values.

Variable	Number of patients (%)			
	MAGE-A4 negative	*MAGE-A4* positive	Total	*p* value
Sex				
Females	2 (66.7)	1 (33.3)	3	
Males	8 (40)	12 (60)	20	.56
Age				
<60 y	7 (41.2)	10 (58.8)	17	
≥60 y	3 (50)	3 (50)	6	1.0
Smoking				
No	2 (100)	0 (0)	2	
Yes	8 (38.1)	13 (61.9)	21	.178
Consumption of alcohol				
No	3 (50)	3 (50)	6	
Yes	7 (41.2)	10 (58.8)	17	1.0
T classification				
1	1 (50)	1 (50)	2	
2	6 (46.2)	7 (53.8)	13	
3	2 (50)	2 (50)	4	
4	1 (25)	3 (75)	4	.919
Nodal involvement				
Presence	6 (40)	9 (60)	15	
Absence	4 (50)	4 (50)	8	.685
Differentiation				
Well	5 (41.7)	7 (58.3)	12	
Moderately	5 (50)	5 (50)	10	
Poorly	0 (0)	1 (100)	1	1.00
Vascular invasion				
Absence	9 (47.4)	10 (52.6)	19	
Presence	1 (25)	3 (75)	4	.604
Lymphatic invasion				
Absence	10 (52.6)	9 (47.4)	19	
Presence	0 (0)	4 (100)	4	.104
Neural spread				
Absence	9 (45)	11 (55)	20	
Presence	1 (33.3)	2 (67.3)	3	1.00
Lymphoplasmacytic infiltration				
Intense	1 (50)	1 (50)	2	
Moderate	3 (42.9)	4 (57.1)	7	
Absent	6 (42.9)	8 (57.1)	14	1.00
Clinical stage				
I or II	3 (75)	1 (25)	4	
III	4 (50)	4 (50)	8	
IV	3 (27.3)	8 (72.7)	11	.249
Total	10 (43.5)	13 (56.3)	23	

Fisher's exact test was used to check the statistical significance between the expression of the cancer-testis antigens and the variables.

any of the studied variables as shown in Tables 2 and 3.

Only the relationship of *MAGE-C1* to the age variable ($p = .069$) and neural spread ($p = 0.093$) showed results that are close to the significance ($\alpha = 0.050$).

Survival in 5 years was 38% in the studied patients. Or the other hand, the survival in 5 years of patients who expressed *MAGE-A4* was not significantly different compared to those who did not express it ($p = .330$; Figure 3). The same occurred in terms of *MAGE-C1* because the survival in 5 years of patients who expressed this gene was not significantly different compared to those who did not express it ($p = .43$; Figure 4).

DISCUSSION

In view of its pattern of expression, CTA have attracted attention as potential targets for tumor immunotherapy,[11] but few reports are available in the literature about their expression, especially regarding the MAGE family in head and neck cancers.

In a previous study, through the reverse transcription polymerase chain reaction (RT-PCR) analysis in a sample of 33 head and neck SCCs from several sites, Figueiredo et al[11] observed the expression of *MAGE-A4* in 48.5% of the cases. Ries et al[14] also identified the expression of *MAGE-A4* in these levels when they studied 21 oral cavity SCCs by RT-PCR (57% of gene expression was found). These

Table 3. Frequency distribution of variables with the expression *MAGE-C1* and *p* values.

Variable	Number of patients (%)			
	MAGE-C1 negative	*MAGE-C1* positive	Total	*p* value
Sex				
Female	0 (0)	3 (100)	3	
Males	12 (60)	8 (40)	20	.093
Age				
<60 y	11 (64.7)	6 (35.3)	17	
≥60 y	1 (16.7)	5 (83.3)	6	.069
Smoking				
No	11 (64.7)	6 (35.3)	2	
Yes	1 (16.7)	5 (83.3)	21	1.000
Consumption of alcohol				
No	2 (33.3)	4 (66.7)	6	
Yes	10 (58.8)	7 (41.2)	17	.371
T classification				
1	1 (50)	1 (50)	2	
2	6 (46.2)	7 (53.8)	13	
3	2 (50)	2 (50)	4	
4	3 (75)	1 (25)	4	.909
Nodal involvement				
Presence	8 (53.3)	7 (46.7)	15	
Absence	4 (50)	4 (50)	8	1.000
Differentiation				
Well	6 (50)	6 (50)	12	
Moderately	6 (60)	4 (40)	10	
Poorly	0 (0)	1 (100)	1	.680
Vascular invasion				
Absence	11 (57.9)	8 (42.1)	19	
Presence	1 (25)	3 (75)	4	.317
Lymphatic invasion				
Absence	11 (57.9)	8 (42.1)	19	
Presence	1 (25)	3 (75)	4	.317
Neural spread				
Absence	12 (60)	8 (40)	20	
Presence	0 (0)	3 (100)	3	.093
Lymphoplasmacytic infiltration				
Intense	2 (100)	0 (0)	2	
Moderate	4 (57.1)	3 (42.9)	7	
Absent	6 (42.9)	8 (57.1)	14	1.485
Clinical stage				
I or II	2 (50)	2 (50)	4	
III	2 (25)	6 (75)	8	
IV	8 (72.7)	3 (27.3)	11	.154
Total	12 (52.2)	11 (47.8)	23	

Fisher's exact test was used to check the statistical significance between the expression of the cancer-testis antigens and the variables.

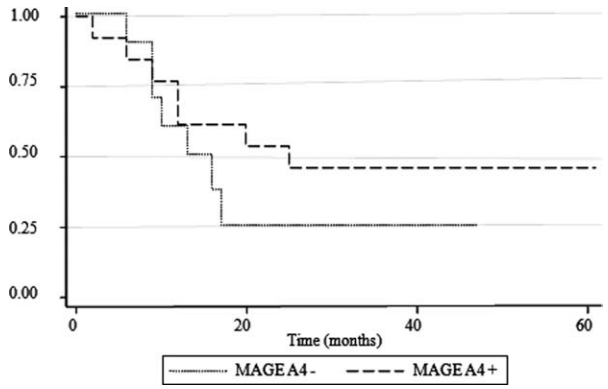

FIGURE 3. Survival curves of the expressions negative and positive of the MAGE A4

results are similar to those found in this research in which the expression of *MAGE-A4* was 56.5% despite the study methods being different (immunohistochemical analysis).

MAGE-C1 had also been evaluated previously through RT-PCR. When Atanackovic et al[15] studied a series of CTA in 51 specimens of head and neck SCCs, they found the expression of *MAGE-C1* in 28% of the cases, a smaller expression than the expression found in this study, which was 47.8%. This expression difference of *MAGE-C1* should not be laid on the immunohistochemistry and RT-PCR study methods, as Sharma et al[16] came to the conclusion that the expressions were similar in both methods when they compared the expression of 9 CTAs in urothelial carcinoma analyzed by RT-PCR and immunohistochemistry. The difference is probably due to the fact that expressions of the gene proteins were heterogeneous among the different sites of the tumor.

There are several studies in the literature analyzing the correlation of CTAs, clinicopathologic variables, and survival, and each 1 presents different results.[17–20] Some show that these antigens are related to aspects of the worst prognosis, some to aspects of a better prognosis, and others yet do not identify any relationship to the effect of tumor suppression.[17–20] In this study, we did not find a significant correlation of the expression of CTAs to the studied clinicopathologic variables.

In the study of Figueiredo et al[11] a statistically significant association of CTA expression to the size of head and neck SCC and tobacco smoking was found. Chitale et al[17] and Krüger et al[18] observed that the expression of *MAGE-C1* had a significant correlation to the tumor grade and a more aggressive behavior in ovary and breast tumors. In tumors of the urinary bladder and vulva, the expression *MAGE-A4* was significantly associated to an advanced stage, metastatic lymph nodes, and high-grade tumors.[19,20] This study found no correlation of the expression of CTA to the studied clinicopathologic variables.

However, when Kienstra et al[21] analyzed the CTA *NY-ESO-1*, *MAGE-A1*, and *MAGE-A3* by RT-PCR and immunohistochemistry, in head and neck SCC, they showed that there was no relationship of the expression of CTA to clinicopathologic data as it occurred in this study. In SCC of the penis and the prostate, the analyzes of the CTAs *MAGE-A1*, *MAGE-A3/4*, and *NY-ESO-1* did not show relationship to clinicopathologic characteristics either.[22,23]

A possible tumor suppression activity was observed by Figueiredo et al[11] when they checked the relationship of the expression of *MAGE-A4* in the primary tumor with lower incidence of metastatic lymph nodes. In other studies, *MAGE-A4* was capable of inducing suppression of tumor activity in hepatocellular carcinomas and of promoting apoptosis in the non-small cell lung cancer.[24,25] We found no relationship between expression of CTA and overall survival.

The studies show that the antigen expression may influence survival positively, negatively, or may not influence survival at all as observed in this study. Bellati et al[20] obtained similar results analyzing 45 cases of vulvar cancer when they studied the CTA *MAGE-A1*, *MAGE-A4*, and *NYESO-1*. Whereas Kocher et al[19] and Yakirevich et al[26] showed that the expression of *MAGE-A4* was related to the worsening of the overall and disease-specific survival in urinary bladder and ovarian cancer, respectively. On the other hand, other studies show that the expression of CTA is related to a better prognosis as in the studies of Krüger et al[16] (regarding *MAGE-C1* in breast cancer) and Sharma et al[18] (regarding CT 10 in urothelial carcinoma).

The question is whether the very advanced tumors are the ones which manifest these antigens showing their relationship to the tumor progression or whether the tumors that express these antigens develop an immunological response of the organism to the tumor because of their characteristic of showing a T-cell response which shows a relationship of these antigens to the tumor suppression.

In our sample, the expression of the studied CTA was frequent, as in 78.3% of the patients at least 1 of

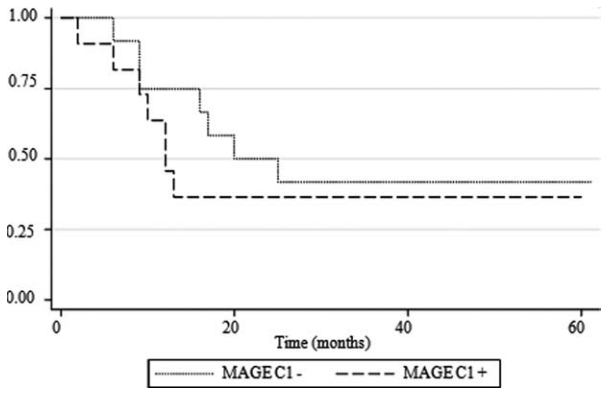

FIGURE 4. Survival curves of the expressions negative and positive of the MAGE C1

the antigens was expressed. Figueiredo et al[11] came to a similar result when they found the expression of at least 1 CTA in 66.6% of the cases and, in the same year, Atanackovic et al[15] observed that this expression could be as high as 86%. These results show that it is possible to use antigen-specific immunotherapy in patients suffering from oral SCC, because the expression increases as the number of studied antigens increases. Therefore, the more antigens involved, the bigger the number of patients that will express at least 1 of them. This shows that an immunological vaccine against oral SCC is feasible, especially if this vaccine involves more than 1 CTA.

CONCLUSION

The expression of at least 1 CTA in oral SCC was present in 78.3% of the patients, however, without correlation with clinicopathologic variables and survival.

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