Expression of MAGE-A1-A12 subgroups in the invasive tumor front and tumor center in oral squamous cell carcinoma

M. BRISAM[1], S. RAUTHE[2], S. HARTMANN[1], C. LINZ[1], R.C. BRANDS[1],
A.C. KÜBLER[1], A. ROSENWALD[2] and U.D. MÜLLER-RICHTER[1]

[1]Department of Oral and Maxillofacial Plastic Surgery, University Hospital Würzburg, D-97070 Würzburg;
[2]Institute of Pathology, University Würzburg, D-97080 Würzburg, Germany

Abstract. MAGE-A proteins are highly expressed in oral squamous cell carcinoma (OSCC) and are promising targets for cancer immunotherapy. This study examined the presence of MAGE-A expression within the tumor center (TC) and tumor invasive front (TIF) and evaluated its relationship to poor prognosis. The expression rate of each MAGE-A subtype, A1-A12, was examined in 68 OSCCs at the TIF and TC. Slides (1-μm) of tissue microarrays (diameter =0.6 mm) were immunohistochemically stained, and the findings were correlated to clinical data. Approximately 95% of the tumors had MAGE-A expression. Higher expression in the TC was shown significantly for MAGE-A1, -A5, -A6, -A9 and -A12 (P<0.05). MAGE-A2 and -A3 exhibited the opposite behavior (not significant, P>0.05). Age, tumor size, grade and survival time were not associated with the expression of certain MAGE-A subgroups. When expression in the whole tumor tissue was considered, only MAGE-A1 was expressed at a significantly higher rate in male patients (P=0.034). At the TIF, MAGE-A9 and the UICC disease stage were significantly correlated (P=0.0263), and MAGE-A6 and the UICC disease stage exhibited a strong trend (P=0.0596). The expression of MAGE-A3, -A4, -A5, -A9 and -A11 was significantly associated with lymph node metastasis, while MAGE-A4 was expressed in all regions of the tumors (TIF and TC). This study showed that higher expression of most MAGE-A antigens occurred at the TC rather than at the TIF. MAGE-A1, -A3, -A4, -A5, -A9 and -A11 were significantly associated with clinically advanced stages of disease and seem to be of particular interest.

Correspondence to: Dr U.D. Müller-Richter, Department of Oral and Maxillofacial Plastic Surgery, University Hospital Würzburg, Pleicherwall 2, D-97070 Würzburg, Germany
E-mail: mueller_u2@ukw.de

Introduction

Oral squamous cell carcinoma (OSCC) has a poor prognosis and outcome. It is one of the six most common types of cancer, with an annual incidence of >300,000 cases worldwide (1-3). Although cancer therapy has improved within the last three decades (particularly advanced radiotherapy with or without chemotherapy and enhanced surgical procedures), the prognosis remains poor, with an average 5-year survival rate of 55% (1,4,5). Promising immunotherapies that use specific antigen-antibody interactions may diminish this disappointing outcome. MAGE-A antigens are only expressed in certain cancer cells, fetal tissue, placenta and testis but not in healthy tissue, except in the thyroid where it is expressed only to a small degree (6). MAGE-A antigens belong to MAGE antigen group I, a type of cancer/testis antigen (CTA) that is localized on the X chromosome and appears to play a key role in tumorigenesis in many types of cancer, including those of the head and neck (7,8). MAGE-A1, which is the first type of antigen recognized by cytotoxic T lymphocytes, marks the first of these subtypes. The remaining subtypes A2-A12 (with the exception of MAGE-A7, which presents itself as a pseudogene) all appear to be interesting immunogenic markers that could be used for cancer therapy (8). Their expression has been described for several types of tumors, such as breast carcinoma, non-small cell lung cancer and squamous cell carcinoma of the head and neck (9-11). Several authors have suggested that MAGE-A antigens are associated with the development and maintenance of aggressive characteristics in tumors (12,13). Therefore, these antigens could represent tools to improve the diagnosis, therapy and prognosis of OSCC. Recently, a cocktail of more than five MAGE-A subgroups (stained by the antibody MAGE57B) was shown to have interesting expression rates in OSCC (14-19). Therefore, the detailed expression of each subtype is now of particular interest and should be determined. The rates for each MAGE-A subtype in OSCC were investigated in this study. Furthermore, the differences in the tumor center and invasive front were examined. Previous studies have described different characteristics of cells in the center and at the invasive front of the same tumor. These studies showed that there were higher mutation rates of p53 or kinase-inhibitor p21 and a higher rate of cell proliferation at the invasive front (20-22). Expression rates of MAGE-A antigens

at different tumor sites could have clinical value as indicators of high progressive tumor growth, guiding the switch to more radical therapeutic strategies or the generation of individual patient protocols. Hartmann *et al* have already investigated the efficacy of chemotherapeutics on tumor cell lines that expressed several MAGE-A types (23) and also evaluated the use of monoclonal antibodies such as cetuximab and panitu-mumab (24). Their investigations clarified the need to even more precisely identify the particular MAGE-A subtypes that influence the failure of chemotherapy and immune therapy in OSCC.

Materials and methods

Patients and tissue microarrays. The pathological samples for this study were selected from the archives of the Department of Pathology of the University of Würzburg, Germany. Sixty-eight paraffin-embedded OSCCs with clear evidence of invasive fronts at the deep surface of the biopsy were chosen. The anatomic source of the biopsy specimens included the tongue, lip, tonsil, cheek, palate, oropharynx and other sites of the oral cavity, but no sites with respiratory epithelia. The mean age of the patients at diagnosis was 56.85 years (SD: 13.0 years), and the study sample included 51 males and 17 females. Twenty-seven tumors were stage T1, 17 were stage T2 and 21 cancers were stage T3 or higher. The staging for 3 samples was not evaluable (Table I).

From each case, a representative block was retrieved, and tissue microarrays (TMAs) of the tumor invasive front and the tumor center were constructed such that each case was represented by three 0.6 mm cores (Fig. 1). One-micron-thick sections were cut and mounted on saline-coated slides.

Table I. The patient specimens: Separate examination of the different clinical parameters was performed due to the hetero-geneous retrospective data.

	T1	T2	T3	T4
n=65	27	17	9	12
	N0	N1	N2	
n=52	36	9	7	
	UICC 1	UICC 2	UICC 3	UICC 4
n=65	25	13	12	15

n, number of valid cases; T1-4, T-stages; N0-N2, lymph node status; UICC1-4, disease stage.

Immunohistochemical staining. As a positive control for immunohistochemical staining, normal adult testis tissue was used. As negative controls, either healthy lung tissue or testis without the use of secondary antibodies was used. One-micron-thick sections of the TMAs were cut and mounted on saline-coated slides. Phosphate-buffered saline (PBS) was used as the diluent for washing and rinsing steps throughout the immunohistochemistry protocol, except for the purposes of antigen retrieval. The antibodies of MAGE-A1-A12 were diluted as shown in Table II.

The TMA sections were deparaffinized, rehydrated and subjected to antigen retrieval by autoclaving for 15 min in individually tested buffer saline as shown in the Table II. The sections were incubated with primary antibody for 1 h at room
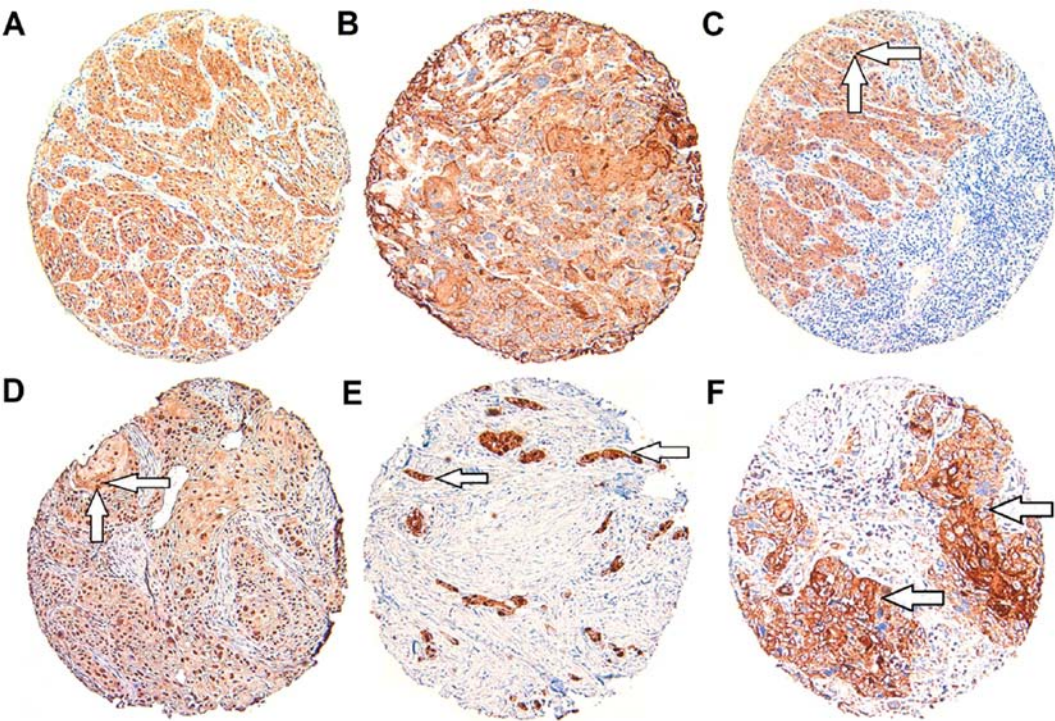

Figure 1. Expression of MAGE-A antigens in invasive oral squamous cell carcinoma. Examples of positive staining (A-F). For most MAGE-A antigens in this study, mainly cytoplasmic staining is shown (C), although nuclear and cytoplasmic staining together can also be observed (D). Although squamous cell carcinomas often have diffuse expression of special antigens within a tumor, focal expression is not uncommon (E and F). (Magnification, x50).

Table II. Origin, dilution and source of the MAGE-A antibodies used in the study.

Antigen	Origin	Code	Heat buffer	Dilution	Source
MAGE A1	Rabbit	Cat # RP 144, 144-05, Lot # 066N, ABIN125659	CA	1:100	Antibodies online (Aachen, Germany)
MAGE A2	Rabbit	(N-18) sc-130164 Lot # D0209	TR	1:20	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
MAGE A3	Rabbit	NBP-1-02506 Lot # 20930	CA	1:100	Novus Biologicals (LLC, CO, USA)
MAGE A4	Rabbit	Cat # PAB 4746, Lot # SH030122L	TR	1:40	Abnova, Taipei City, Taiwan
MAGE A5	Rabbit	Cat # PAB-10795 Lot # RB 2085	CA	1:100	Antibodies online (Aachen, Germany), www.allele.biotech.com
MAGE A6	Rabbit	ABIN303468, Lot # 20945	CA	1:100	Antibodies online (Aachen, Germany)
MAGE A8	Rabbit	(v-25), sc-102016, Lot # G0708	TR	1:30	Santa Cruz Biotechnology, Inc.
MAGE A9	Rabbit	(G-24), sc-130811, Lot # H2010	TR	1:10	Santa Cruz Biotechnology, Inc.
MAGE A10	Rabbit	Cat # PAB4741, Lot # SH030124Q	TR	1:10	Abnova
MAGE A11	Rabbit	(N-16), sc-130162, Lot # I1809	TR	1:40	Santa Cruz Biotechnology, Inc.
MAGE A12	Rabbit	Cat # PAB4743, Lot # SA110311AH	TR	1:10	Abnova

CA, citric acid (pH 6.0); TR, target retrieval® (pH 6.1).
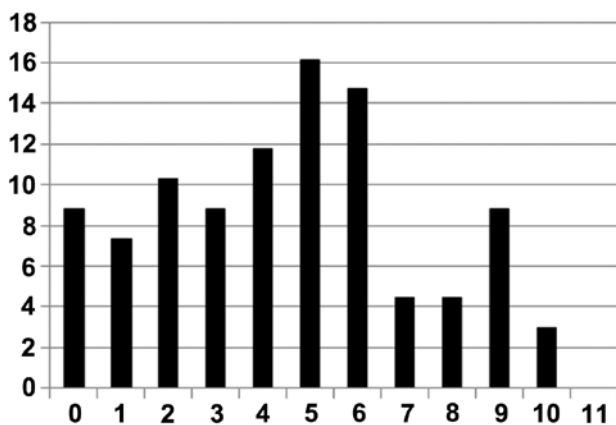

Figure 2. The x-axis shows the number of expressed MAGE-A subtypes within a single tumor specimen. The ordinate represents the percentage of specimens.

temperature, followed by detection using the DAKO Advance+ detection system (DakoCytomation, Pathology Products Dako Deutschland GmbH, Hamburg, Germany) and DAB as the chromogen. The slides were counterstained with hematoxylin and evaluated.

For investigation, the dual system of Remmele and Stegner (25) that was first used in breast cancer was used to evaluate the levels of antigen expression and generate a score [amount of staining (SI): 0, no reaction, 1, weak reaction, 2, moderately high reaction, 3, strong reaction; number of positive cells (PP): 0, negative, 1, <10% positive cells, 2, 10-50% positive cells, 3, 21-80% positive cells, 4, >80% positive cells]: SI x PP = Immunoreactive score (IRS).

As different punches of the tumor center and the tumor invasive front were prepared, an IRS (C) for the center, an IRS (F) for the front and an IRS (T) were evaluated. The IRS (T) represents the whole tumor tissue in this study, and its value was determined through summation as follows: IRS (T) = IRS (total) = IRS (C) + IRS (F).

For graphical presentation, IRS (T) has been simplified into four score groups of staining grade (0, negative, 1, weak, 2, medium and 3, high).

Statistical analysis of expression rates and clinical parameters was performed with the help of the Department of the Mathematical Branch of the University of Würzburg using Statistica 10® (StatSoft, Tulsa, OK, USA) and SPSS 20® (IBM, Armonk, NY, USA). Investigation included using the Spearman's correlation, Mann-Whitney U test, Kruskal-Wallis test and Gehan's test. For additional investigation of detailed information within the groups, the post hoc test was applied.

Results

General expression rates of MAGE-A. Among 68 tumors, 62 (91.17%) had antigen expression of several individual MAGE-A subtypes, and 6 tumors (8.82%) had no staining.

Table III. Comparison of expression in the tumor center and tumor front.

	A1	A2	A3	A4	A5	A6	A8	A9	A10	A11	A12
P-value	0.01	0.13	0.12	0.46	0.01	0.02	0.11	0.05	0.54	0.15	0.01
Valid cases	60[a]	68	68	68	68	68	68	68	68	68	68

For MAGE-A1, a decreased set of 60 cases were microscopically evaluated due to the first cut of the tissue microarray block, which caused a moderate loss of material during the washing process of the staining procedure.
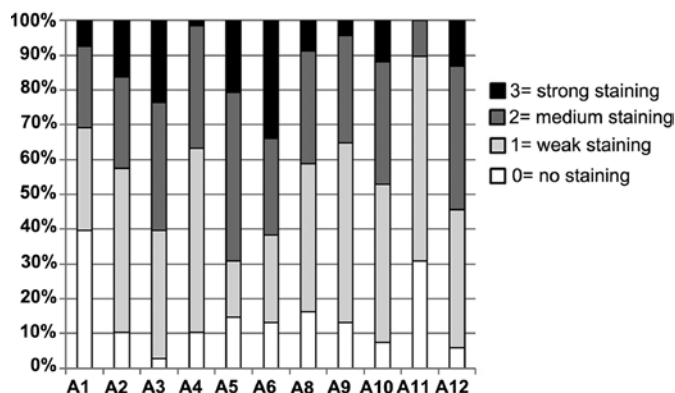

Figure 3. Overall MAGE-A expression in both tumor regions (IRS C + IRS F). The ordinate shows the percentage of stained specimens represented by the respective staining score.

As shown in Fig. 2, 36 tumors (52.94%) had simultaneous expression of five or more MAGE-A subtypes. Expression of one to four MAGE-A antigens was observed in 26 (38.24%) specimens. Six tumors (8.82%) had no staining for any of the MAGE-A subtypes, as shown in the first column of Fig. 2.

The highest expression rates (summarized through scoring grade 1, 2 and 3) were observed for MAGE-A2 (90%), -A3 (97%), -A4 (90%), -A10 (93%) and -A12 (94%).

Statistical investigation of the correlation between expression of five or more antigen subtypes in a single tumor revealed no significant differences in any clinical parameters (P>0.05).

Expression in the tumor center and tumor invasive front. For MAGE-A1, -A4, -A5, -A6, -A8, -A9, -A10, -A11 and -A12, there was higher expression in the tumor center than in the tumor invasive front. Only MAGE-A2 and -A3 clearly exhibited the opposite behavior as shown in Fig. 4.

Statistical investigation using immunoreactive score [IRS (C)/IRS (F)] showed a significant difference in the expression between the tumor invasive front and tumor center for the MAGE-A subtypes A1, A5, A6, A9 and A12 as shown in Table III. This difference occurred for all of the subtypes with stronger expression in the tumor center. For the other MAGE-A subgroups, the results were not significant.

Correlation of MAGE-A expression with clinical parameters. For correlation of expression with clinical parameters, the overall MAGE-A expression for each subtype was determined and the additional values, represented by IRS (T), were used for statistical analysis.

Age. The study group was divided into 3 groups according to age (1, 35-50 years, 2, 51-70 years and 3, 71-85 years). Within these groups, MAGE-A subtypes were represented by staining score (0, 1, 2 and 3). The highest scores occurred in group 2 (51-70 years), with the exception of MAGE-A10, for which group 3 (71-85 years) had the highest staining scores. Statistical investigation using Spearman's correlation showed no significant results (P>0.05) for any of the MAGE-A subtypes.

Gender. For MAGE-A1, -A2, -A5, -A6, -A8, -A10 and -A12, higher staining and therefore higher expression occurred in specimens from men, while MAGE-A3, -A4, -A9 and -A11 exhibited the opposite behavior. This result must be regarded differentially because of the small number of female specimens (n=17) in the study. Only MAGE-A1 had significantly higher expression in the whole tumor of male specimens as evaluated by the Mann-Whitney U test (P=0.034). In the tumor center, only a trend in the expression of MAGE-A1 for higher grade male specimens was observed (P=0.05). No other results were significant (P>0.05).

Tumor size. In this study, we found that for all MAGE-A antigens, the tumors in stage T3 showed the highest expression. Tumors in stage T1 had less expression than those in stage T2. However, a difference in expression rates was not observed between T3 and T4. Nevertheless, statistical investigation using Kruskal-Wallis test did not show any significant difference between the stages for any of the tested MAGE-A antigens (P>0.05).

Lymph node status. For MAGE-A1, -A2, -A6, -A8, -A10, -A12, no significant differences were observed between the groups N0, N1 and N2. For MAGE-A3, there was significantly higher expression in the tumor center of tumors with associated lymph node metastasis (N1) compared with those with N0 status (P=0.04). Investigation of the overall expression in both regions of the tumor (center and front) revealed significant differences in MAGE-A4 (P=0.02), MAGE-A5 (P=0.02) and MAGE-A9 (P=0.02) with regards to lymph node stages. For MAGE-A11, a significant difference was only observed for the tumor invasive front (P=0.04).

Grading. Regarding the grading stage of the tumors, no significant differences between the stage groups and any of the tested MAGE-A antigens were found (P>0.05).

UICC (Union International Contre le Cancer) stage (disease stage). Only MAGE-A9 expression significantly differed in the invasive tumor front region of the tumors with regards to
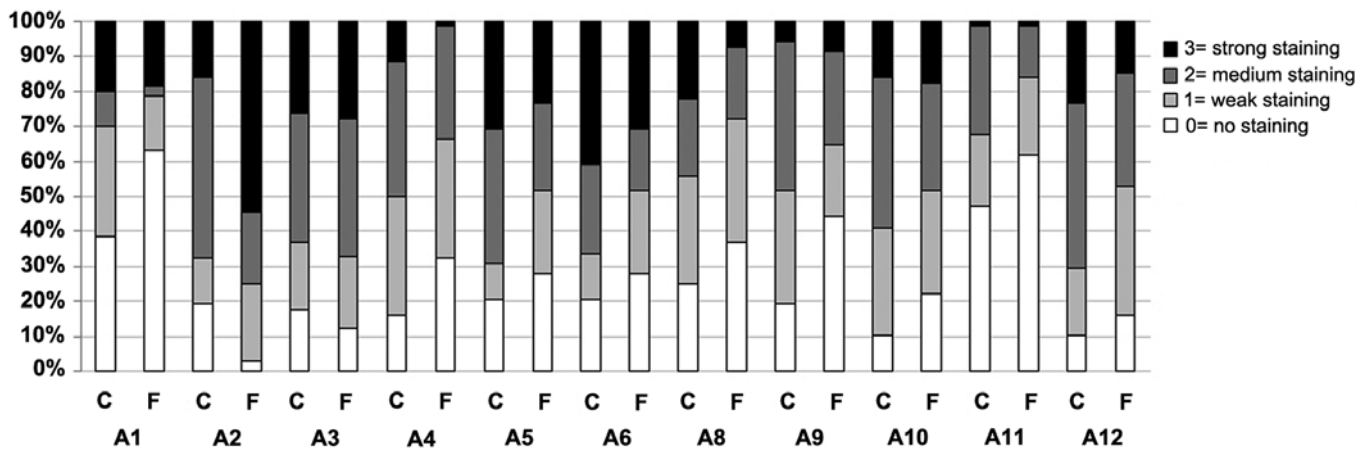
Figure 4. Expression rates for each subtype compared in the tumor center and invasive front (C, center; F, invasive front). The fractions of specimens are shown as percentages.

UICC stage (P=0.02). Detailed post hoc testing showed that the highest significance was between groups I and IV (P=0.04), which suggests increased malignancy when the expression is elevated.

Survival time. Neither MAGE-A expression nor the expression of five or more antigens were significantly correlated to the survival time. Because of the heterogeneity of the available data, a connection between expression and decreased survival time should not be generally disregarded.

Discussion

This is the first study to use the expression rates of all MAGE-A subtypes to compare the tumor center and tumor front in OSCC via detailed immunohistochemistry. The study showed that there was expression of MAGE-A antigens in 62 of 68 specimens (91.17%). Furthermore, 36 of 68 (52.94%) tumors expressed five or more of the eleven tested antigens. This rate is higher than those previously reported in other studies (14-18).

Comparison of the expression in the tumor center and the invasive front revealed that MAGE-A1, -A4, -A5, -A6, -A8, -A9, -A10, -A11 and -A12 were more highly expressed in the center and that the results were statistically significant for MAGE-A1, -A5, -A6, -A8 and -A12. In contrast, expression was higher in the invasive front for MAGE-A2 and -A3, but this difference was not statistically significant.

Because the invasive front is regarded as the site associated with malignant characteristics such as a higher incidence of p53 mutation, higher levels of Ki-67, more proliferating cells and high epithelial-mesenchymal transition (EMT), our findings are peculiar (20-22). The transition from the somatic cell type to cells with mesenchymal characteristics includes loss of apical-basal polarity, impairment of important cell-cell interactions and therefore higher fragility, higher cell mortality and destabilization of the basal membrane layer (26,27). Because EMT is related to de-differentiation and MAGE-A expressing cells can also exhibit de-differentiation, a relationship between MAGE-A expression and EMT may exist (28,29). Because

our results appear to be contrary to those of previous studies, further investigation is needed to clarify the association between EMT and MAGE-A expression at the tumor front and center.

Promoter demethylation and histone deacetylation are regarded as the key steps for inducing MAGE-A expression in tumor cells (13,30-33). However, several authors have also described the absence of CTA expression in tumors with general DNA hypomethylation (34,35).

Moreover, there is an assumption that a necessary 'switching-on' of a gameto-genetic program exists that is derived from germ cell development and leads somatic cells to express CTAs (30,36).

Higher expression of MAGE-A antigens in the tumor center of the specimens in this study group could be explained by this process of reverting back to a developmental stage and augmenting the life span of tumor cells.

However, Aprelikova *et al* showed a connection between downregulation of MAGE-A11 and a negative influence on hypoxia-inducible factor HIF-1α in OSCC; HIF-1α is believed to play a key role in malignant behavior (e.g., resistance to radiotherapy or chemotherapy) of tumor cells with hypoxic surroundings, which are typically found at the center of tumors (37). Moreover, Hartmann *et al* investigated the negative impact on the effectiveness of therapy for the chemotherapeutics diamindichloridoplatin (DDP) and 5-fluorouracil when high levels of MAGE-A11 were present and found that expression of MAGE-A5 and -A8 resulted in a negative effect on anti-neoplastic therapy including panitumumab treatment (23,24). Interestingly, in our study, the same subgroups have higher expression in the center of the tumor, particularly MAGE-A5. Because the tumor center is the location where so-called 'cancer stem cells' originate and typically proliferate slowly, it is possible that these cells are less practical targets for chemotherapy, which clarifies the frequent occurrence of refractory lapses of the disease (38). These findings clarify the influence of malignant characteristics through the effects of MAGE-A expression on development.

The correlation between patient age and MAGE-A expression was not significant. Nevertheless, the study group with

the highest staining rates was the patients between 51 and 70 years of age. These findings can be explained by the fact that genomic hypomethylation increases the probability of MAGE-A expression and increasing age promotes this condition (39,40). However, in terms of oral cancer, patients of this age group often present with typical risk factors such as smoking and simultaneous alcoholism, which suggests the potential of a relationship or even a direct interaction between MAGE-A expression with the presence of risk factors.

As was shown significantly for MAGE-A1 as well as for MAGE-A2, -A5, -A6, -A8, -A10 and -A12, male specimens have a tendency to present higher expression rates of CTAs or rather higher staining rates by immunohistochemistry. Because males are much more frequently heavy smokers and drinkers, a direct connection between these risk factors and augmented MAGE-A expression could also be hypothesized.

In addition, tumor size did not significantly correlate with increased MAGE-A expression, as was described in most previous studies. Nevertheless, Müller-Richter *et al* showed an augmentation of expression rates with the increase of tumor size, and Napoletano *et al* showed a significant correlation between tumor size of cervical cancer and expression of MAGE-A1, -A2,- A3, -A4, -A6, -A12 using a global MAGE57B antibody (40,41).

However, based on their use of this special global antibody, the studies must be compared and regarded differentially.

Our results showed a correlation of MAGE-A expression with the general presence of lymph node metastasis (N$^+$) that was significant for MAGE-A3, -A4, -A5, -A9, and -A11. This correlation did not impact on whether the tumor was stage N1 or N2. Again, the evaluation of Hartmann *et al* (23) is of interest because they clearly specified that MAGE-A5, -A8 and -A11 are negative predictors of OSCC. The presence of lymph node metastasis normally leads to application of adjuvant therapy, which is often combined with additional chemotherapy (42,43). Therefore, the currently discussed MAGE-A types may represent novel indicators that could lead to more aggressive approaches, even in cases with no positive lymph node status but high expression of certain MAGE-A subtypes. The present study is the first time a significant correlation between MAGE-A expression and further lymph node status in OSCC has been investigated (15,18). Similar results have been reported only for other types of tumors, such as ductal breast, colorectal or gastric cancer (44-46).

Here, significant results for all tests regarding lymph node status were obtained, especially for MAGE-A4. In addition, Forghanifard *et al* described similar results in their studies for MAGE-A4 in SCC of the esophagus (47). MAGE-A4 was also one of the most expressed subtypes in the present study, and Ries *et al* (14) showed that MAGE-A4 had the highest expression rates in OSCC.

This difference in expression might represent a reliable tool for the prognosis of OSCC. Moreover, a significantly worse response to the chemotherapeutics docetaxel and paclitaxel occurred for tumors that expressed MAGE-A4, similar to the findings reported by Müller-Richter *et al* (48). For MAGE-A5, significant results were obtained in general and for the tumor center. Other studies have not yet reported such results in OSCC but have done so for other malignancies, such as lung cancer and colorectal cancer, with comparable

findings reported (49). Our non-significant findings regarding tumor grade are in contrast to the results of previous studies by Ries *et al* (14), Figueiredo *et al* (17) and Eura *et al* (11), who described significantly higher malignancy and grading in connection with high MAGE-A expression in their studies of OSCC and head and neck cancers.

Because the 'cancer stem-cell theory' exists and other authors have proposed that only cells with the characteristics of stem cells within a tumor mass are able to express CTAs, de-differentiation could be explained. Literature results regarding the heterogeneity of cells in a tumor and the ability or inability to express antigens should be stressed (50,51).

Correlation studies of UICC stage to expression showed that there was significant expression of MAGE-A9 and a trend for MAGE-A6 in the tumor center. Gu *et al* recently found that in HCC, MAGE-A9 expression is an independent prognostic factor for disease-free survival and overall survival and that high MAGE-A9 expression suggests unfavorable survival outcomes in HCC patients (52). In addition, Xu *et al* recently reported similar results in breast cancer, suggesting that the MAGE-A9 subtype should be regarded as a favorable tool for the evaluation of the prognosis of patients with malignant disease (53). Shigematsu *et al* showed significantly higher expression of MAGE-A4 in stage II-IV non-small cell lung cancer relative to stage I (54). Because these results only applied to the tumor center, this result also supports the often postulated higher prognostic value of the tumor invasive front (20-22,55,56). Several authors have already described significantly lower 5-year survival rates of patients with certain types of cancers (57-59) that express MAGE-A antigens; therefore, the results of this study have to be considered critically. Investigating larger patient groups may allow us to draw more reliable conclusions.

Although cure rates in OSCC are improving slowly with current therapeutic methods, the present study reveals that MAGE-A3, -A4, -A5, -A9, -A11 are factors that are related to metastatic tendencies and therefore could be used as prognostic tools for improving the follow-up care of patients with OSCC.

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