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ORIGINAL ARTICLE

The expression of melanoma-associated antigen A (MAGE-A) in oral squamous cell carcinoma: an evaluation of the significance for tumor prognosis

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Abstract

Objectives Melanoma-associated antigens A had been detected repeatedly in oral squamous cell carcinoma, but not in healthy mucosa. Additionally, patients with MAGE-A expressing cancers are regarded to have a worse survival prognosis, so that MAGE-A are supposed to be part of carcinogenesis. Which role these antigens fulfill within OSCC is still, up today, largely unknown. This study examines the hypothesis that MAGE-A is being produced in OSCC but not in mucosa tissue and if MAGE-A has any correlation to clinical patient's parameters like tumor size, lymph node metastasis, distant metastasis, overall survival, and recurrence.

Materials and methods For this purpose, 50 tumor samples and 39 mucosa samples were analyzed by means of PCR and immunohistochemical staining with the antibody 6C1.

Results Forty of 41 stained tumor samples showed a positive antibody reaction with a maximum staining rate of 53%. Sixteen mucosa samples showed a mild positive reaction. The PCR revealed a linear expression pattern of MAGE-A in which the genes are proportionally expressed in OSCC. We did not find any relationship between MAGE-A and tumor size, overall survival, or recurrence. There was also no connection between MAGE-A and tumor parameters Hif-1 and LDH. Their expression was detected tendentially in tumors with higher staging, advanced lymph node metastasis, and rising age of the patients. The genes MAGE-A3+6 and MAGE-A4 had a statistically significant correlation with lymph node metastasis ($p = 0.007$ and $p = 0.004$). Patients got distant metastasis and influence of MAGE-A on metastatic behavior could not be verified. The genes MAGE-A3 and -A4 are consequently qualified as tumor markers in the field of diagnosis and follow-up of OSCC.

Conclusions and clinical relevance Two genes have great potential as target proteins in immunotherapy. The genes MAGE-A3+6 and MAGE-A4 had a statistically significant correlation with lymph node metastasis.

Keywords Squamous cell carcinoma · Cancer · Tumor · Tumor predictor · MAGE

Introduction

In spite of numerous developments in the diagnosis and treatment of oral squamous cell carcinoma, the survival rate of these patients remains 50% [1]. Concurrently, the number of newly diagnosed cases increases with a rising tendency [2, 3]. One of the most challenging parts is the duly recognition of the malignancy. Most of the OSCC are diagnosed in an advanced stadium by which the survival rate can decline to 30% and lower [4, 5]. The standard treatment is the surgical resection of the tumor. Advanced diseases in stadium III or IV require a combined-modality treatment consisting of resection, radiation, and chemotherapy [6, 7]. This approach enhances the overall survival rate compared to a surgical intervention only, but this progress is still not decisive [8]. The more important it seems to develop reliable methods for the identification of early-stage OSCC and to improve the therapy approach of the advanced diseases for a better overall prognosis. Recently, there are a number of examinations concerning the immunological features of tumors, especially the use of

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tumor markers and antibodies, for instance, to develop an OSCC-specific non-invasive screening of the saliva for early cancer detection [9]. Another retrospective study demonstrated that patients with advanced and metastatic oral cancer, who were treated with cetuximab additional to radiation and platinum-based chemotherapy, had a better survival outcome than without the use of antibodies [10]. So, the research of the tumor immunology of oral cancer, in particular, the determination of tumor-specific marker, could provide possible new therapeutic approaches for optimized treatment and a more favorable survival outcome of oral cancer.

Cancer/testis (CT) antigens are a group of proteins whose expression in healthy human tissue is restricted to the testis and the placenta only. Furthermore, they were detected in various solid tumors like the melanoma, the NSCLC, the OSCC, and in tumors of the breast, prostate, and bladder [11–13]. Because of their specific expression profile, these antigens are of particular interest for antitumor immunotherapy. CT antigens are categorized into two subclasses depending on the chromosomal localization of their encoding genes: the chromosome X-encoded and the autosome-encoded CTAs [14]. The antigens of the first subclass are generally highly germ cell–specific and immunogenic. The MAGE-A gene family, the first ever discovered CT antigen, is a chromosome X-encoded gene cluster containing 12 members called MAGE-A1–MAGE-A12. All the 12 subtypes have the same MAGE homology domain in common [15–17]. MAGE-A is supposed to interact with the tumor suppressor gene p53 and the enzyme E3 ubiquitin ligase and thereby to promote the proliferation and growth of malignant cells [18–24]. However, MAGE-A also seems to be involved in the tumor progression and metastasis and to have features of epithelial-mesenchymal transition [25–29]. Based on these attributes, the MAGE-A gene family represents a potential target for the diagnosis and immunotherapy of cancer. This study has examined the qualification and the prognostic value of MAGE-A as a specific marker for oral squamous cell carcinoma.

Patients and methods

Patient cohort Tissue samples from 58 patients were collected intraoperatively at the Department of Oral and Maxillofacial Surgery of Mainz University Hospital. Fifty of these samples came from patients with OSCC, thereof 31 accompanying tumor and mucosa samples from the same patient and 19 tumor samples. The remaining 8 samples were mucosa tissue from 8 healthy patients as a control group. This results in a total of 50 tumor samples and 39 mucosa samples. The mean age of the tumor patients was 72 years, none of them had distant metastasis. The tumor patient group consisted of 33 male and 17 female patients (relation ≈ 2:1). Furthermore, we divided the patients with OSCC into two groups depending on the progress of their disease. Group 1 included 20 patients with OSCC stadium I or II, group 2 included 30 patients with OSCC stadium III or IV.

Immunohistochemistry After the extraction, the tissue was snap-frozen in 196 °C liquid nitrogen and stored in − 80 °C. In a cryomicrotome, the tissue was sliced in 8–10-μm-thin sections and fixed in acetone on a microscope slide, the temperature during this process was − 20 °C. MAGE-A protein staining was performed according to the LSAB-method using the monoclonal antibody 6C1 (Invitrogen, CA, USA) which is specific to seven members of the MAGE-A gene family (-A1, -A2, -A3, -A4, -A6, -A10, and -A12, as the genes -A3 and -A6 have almost identical sequences and many antibodies or primers are not able to distinguish between them, these two genes have been listed as one gene -A3/-A6). The sections were unfrozen at room temperature, washed in phosphate-buffered saline (Sigma, Germany), and incubated for 1 h with Roti-Block (Carl Roth, Germany) to avoid unspecific binding of the antibody. Afterwards, the tissue was incubated for 1 h with the antibody 6C1 (1:10 dilution), washed in PBS, treated with 3% H2O2, and washed again in PBS. The following incubation with horseradish peroxidase-conjugated secondary antibody was performed with the DAKO Real Detection System. The sections were again washed in PBS, counterstained with hematoxylin, and visualized under the microscope. Samples were considered positive for MAGE-A when showing brownish nuclear and/or cytoplasmic antibody signal. Negative samples were absent of any antibody reaction. Samples with double Roti-Block incubation instead of the antibody were used as a negative control in each staining process (Fig. 1).

Microscopic evaluation The stained tissue sections were analyzed with the BZ 9000E microscope (Keyence, Germany) by first scanning them under × 20 magnification and then evaluating them with the "Hybrid Cell Count" function (color tolerance:50 for tumor samples because of the various brown shades and standard color tolerance:20 for mucosa samples). This technique enabled an objective evaluation of the immunohistochemical tissue staining.

Cell lines In order to establish reference values for the following PCR, we cultured three cell lines. The tumor cell line PCI-13 was kindly provided by the Pittsburg Cancer Institute and was used as positive control for MAGE-A as all 12 members of MAGE-A had been detected in this tumor cells. The cell lines HOK (Provitro, Germany) and HGEP (CellNTec, Switzerland), human oral keratinocytes, and human gingival epithelial cells were used as negative control in PCR. The cells were cultured in proliferation medium, PCI-13 was supplemented with Dulbecco's Modified Eagle Medium (Gibco), 10% Fetal Calf Serum (Life Technologies), 1% PSN

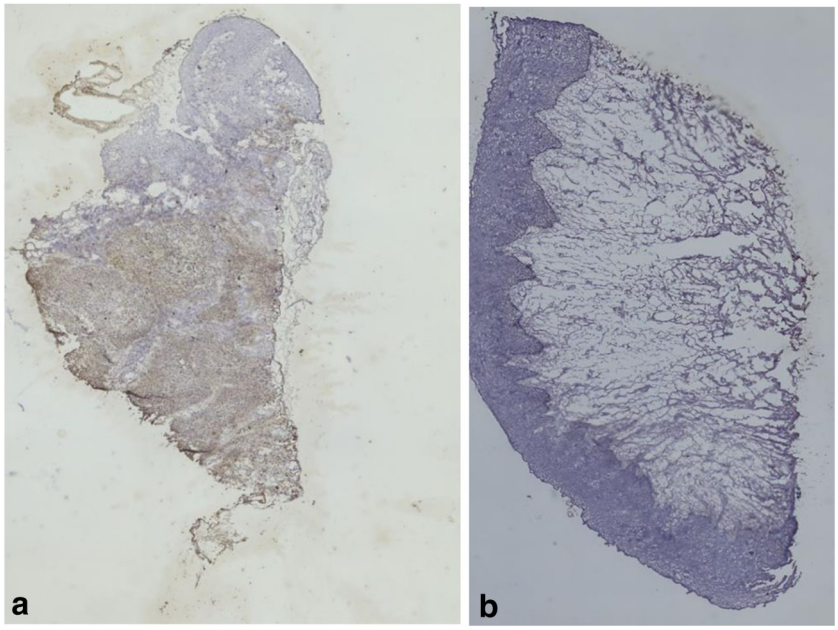

Fig. 1 Immunohistochemical staining with the antibody 6C1. **a** Tumor tissue sample after successful staining with 6C1. The brown areas mark the presence of MAGE-A antigens in the tumor. **b** Mucosa tissue after successful staining with 6C1. The lack of reaction shows the absence of MAGE-A antigens

(Penicillin/Streptomycin/Nystatin, Sigma), and 1% L-Glutamine (Sigma). The cell culture flasks were stored at 37 °C with 5% CO2 in a humidified atmosphere. The medium was changed every 48 h and the cells were viewed daily under the microscope. As soon as the adherent growing monolayer reached a confluence of 75–80% the cells were split.

Real-time PCR We extracted RNA from the frozen tumor and mucosa tissues as well as from PCI-13, HOK, and HGEP cells according to the manufacturer's instructions with the RNeasy Mini Kit, the QIAshredder, and the RNase-free DNase Set (Quiagen, Germany). Next, a quantitative rating of the RNA was done using the NanoDrop ND-1000 spectrophotometer (Peqlab, Germany). Depending on the calculated RNA concentration an individual mastermix for each sample was produced with the BioRad cDNA Kit (Bio-Rad Laboratories GmbH, Germany), and the RNA was transformed into cDNA in the peqSTAR 96X Universal Thermocycler (Peqlab, Germany) by means of reverse transcription. The final cDNA samples were stored at − 20 °C. To gain an expression profile of MAGE-A, a real-time PCR was performed using the iQ5 Thermocycler (Bio-Rad Laboratories GmbH, Germany). The matching primer sequences for MAGE-A1, -A2, -A3+6, -A4, -A10, and -A12 were created with the software NCBI Primer-BLAST (National Center for Biotechnology Information, USA) and are listed in Table 1. With a PCR Kit from Bio-Rad, a mastermix was created and dispensed in a 96-well microtiter plate containing 12.5 µl iScript, 8.5 µl H2O, 1 µl Primer sense, and 1 µl Primer antisense per each well. Finally, 2 µl cDNA of the respective gene was added and the PCR cycle was started. Initial incubation was performed at 95 °C for 10 min, the amplification was performed for 39 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72° for 30 s. The housekeeping genes Aktin and RPL-5 were included in each PCR as an intern reference for real-time PCR quantification. PCI-13 functioned as positive control in every PCR, HOK, and HGEP as a negative control. The measured PCR values of HOK and HGEP are illustrated in Table 2. Only

Table 1 Primer sequences used for polymerase chain reaction analysis. A sense and antisense primer was created for each gene

Primer name	Sequence (von 5′-3′)
MAGE-A1 s	5′-CCC AGA GGA CAG GAT TCC CT-3′
MAGE-A1 as	5′-GGA CTC TGG GGA GGA TCT GT-3′
MAGE-A3/6 s	5′-GCC CTG AGC AAC GAG CGA-3′
MAGE-A3/6 as	5′-AGA ACC TTG CCT CCT CAC CG-3′
MAGE-A4 s	5′-AGA CAA GCG AGC TTC TGC G-3′
MAGE-A4 as	5′-TCC TTA TAG AGC CTG GGC GG-3′
MAGE-A10 s	5′-GAG GCA AGG GAG GTG AGA AC-3′
MAGE-A10 as	5′-AAC CTT GAC TCC TGG CAC TG-3′
MAGE-A12 s	5′-AGG GGA AGA CTT CTC AGG CT-3′
MAGE-A12 as	5′-CCC CTG TGT TGA CCT GAG TC-3′
Aktin s	5′-GGA GCA ATG ATC TTG ATC TT-3′
Aktin as	5′-CCT TCC TGG GCA TGG AGT CCT-3′
RPL-5 s	5′-GCG CAG CGT ATG CAC ACG AAC-3′
RPL-5 as	5′-CAG CTC CCT TCA GGG CAC CA-3′

Table 2 PCR measurements of HOK and HGEP for each MAGE antigen were set as threshold values. The measurements represent the "background noise" during amplification

	MAGE-A1	MAGE-A3+6	MAGE-A4	MAGE-A10	MAGE-A12
HOK	0.63	0.10	2.07	0.83	1.26
HGEP	0.16	0.09	4.21	2.53	0.11

measurements higher than this threshold value were allowed for our PCR analysis.

Statistical analysis Statistical analysis was performed using SPSS software (version 14.1). The Wilcoxon test was used to compare the staining of tumor and mucosa tissue as well as to compare PCR results of tumor and mucosa samples. The Mann–Whitney *U* test was performed to evaluate differences between the patient groups 1 and 2. The Pearson test was applied to demonstrate if there is any correlation between MAGE-A antigens and tumor size, lymph node metastasis, distant metastasis, age of the patients, overall survival, recurrence, and the tumor parameters LDH and Hif-1. $P < 0.05$ for Wilcoxon and Mann–Whitney *U* tests and $p < 0.01$ for the Pearson test were considered to indicate a statistically significant difference. Boxplots were created to visualize the results (Fig. 2).

Results

Evaluation of immunohistochemical staining A total of 50 tumor and 39 mucosa samples had been stained with the antibody 6C1. The staining of 9 tumor and 1 mucosa samples were invalidated. Because of the little amount of tissue, the experiment could not be repeated for these samples. So, results for 41 tumor and 38 mucosa samples were available. In 40 of 41 tumor samples, a positive reaction was detected, which means a staining rate of 97.6%. The average stained portion per sample was 14.05%, the highest stained portion per sample was 53% whereas the lowest was 0%. Twenty of 38 mucosa samples had a completely negative staining reaction, 16 mucosa samples showed staining ≤ 5% and 2 mucosa samples showed staining ≥ 5%. The average mucosa staining was 1.13% with a maximum staining portion of 8%. Tumor samples showed a significantly higher staining rate than mucosa samples ($p \leq 0.001$). A difference between patient groups 1 and 2 could not be observed ($p = 0.533$).

Evaluation of real-time PCR For the analysis of the MAGE-A PCR, 39 tumor samples and 24 mucosa samples could be used. For all of the 24 mucosa samples, the matching tumor sample from the same patient was available for comparative analysis. According to the threshold values from PCR with HOK and HGEP, a minimum expression level for MAGE-A was set: 0.63 for MAGE-A1, 0.1 for MAGE-A3+6, 4.21 for MAGE-A4, 2.53 for MAGE-A10, and 1.26 for MAGE-A12. After checking through the PCR data, 3 samples of MAGE-A4, 15 samples of MAGE-A10, and 6 samples of MAGE-A12 had to be excluded as their expression level was lower than threshold value. The highest expression rate of 390.22 was identified for MAGE-A10, the lowest expression rate of 0.14 for MAGE-A3+6. At the same time, MAGE-A10 had the largest standard deviation of 99.06. In general, the PCR values for MAGE-A were relatively wide diversified with large standard deviation so the median was used to describe the average expression level instead of the mean. In descending sequence, the detected average expression of MAGE-A is MAGE-A4 with 12.04, MAGE-A10 with 9.29, MAGE-A1 with 5.40, MAGE-A12 with 2.89, and MAGE-A3+6 with 0.81. There was no significant difference for none of the antigens between tumor and mucosa expression (Table 3). With regard to the presence of MAGE-A in patient groups 1 and 2, we could not identify a statistically proved difference but MAGE-A1 and MAGE-A3+6 showed a tendency for higher occurrence in advanced tumors ($p = 0.085$ and $p = 0.053$). A correlation between the tumor size T and MAGE-A was not observed. Whereas the lymph node metastasis *N* correlated with a rising expression of MAGE-A3+6 and MAGE-A4 ($p = 0.007$ and $p = 0.004$). The genes MAGE-A1, MAGE-A10, and MAGE-A12 were also tendentially more present in tissues with high lymph node metastasis. Based on our patient cohort, we could not make any statement about MAGE-A and tumor metastasis *M* as none of our patients had distant metastasis. However, older patients tended to have a more frequent expression of MAGE-A3+6, MAGE-A4, and MAGE-A10 ($p = 0.013$, $p = 0.002$, and $p = 0.025$). Expression analysis of MAGE-A and the overall survival, recurrence, LDH, and Hif-1 values revealed no interrelation between MAGE-A and these parameters. Though we observed a linear association between the MAGE-A genes among themselves. The examined genes MAGE-A1, MAGE-A3+6, MAGE-A4, and MAGE-A10 were detected in the tissue in a proportional relationship (p between ≤ 0.001 and 0.01). MAGE-A12 was proportional to MAGE-A1 only ($p \leq 0.001$) but not to the other genes. We calculated the factor of proportionality for each gene pair as shown down below in Table 4 with standard deviation from 0.24 (MAGE-A3+6/MAGE-A1) to16.60 (MAGE-A4/MAGE-A3+6) (Fig. 3).

Discussion

Cancer/testis antigens were first discovered and described in 1991. They are expressed in HLA-I-negative male germ cells,

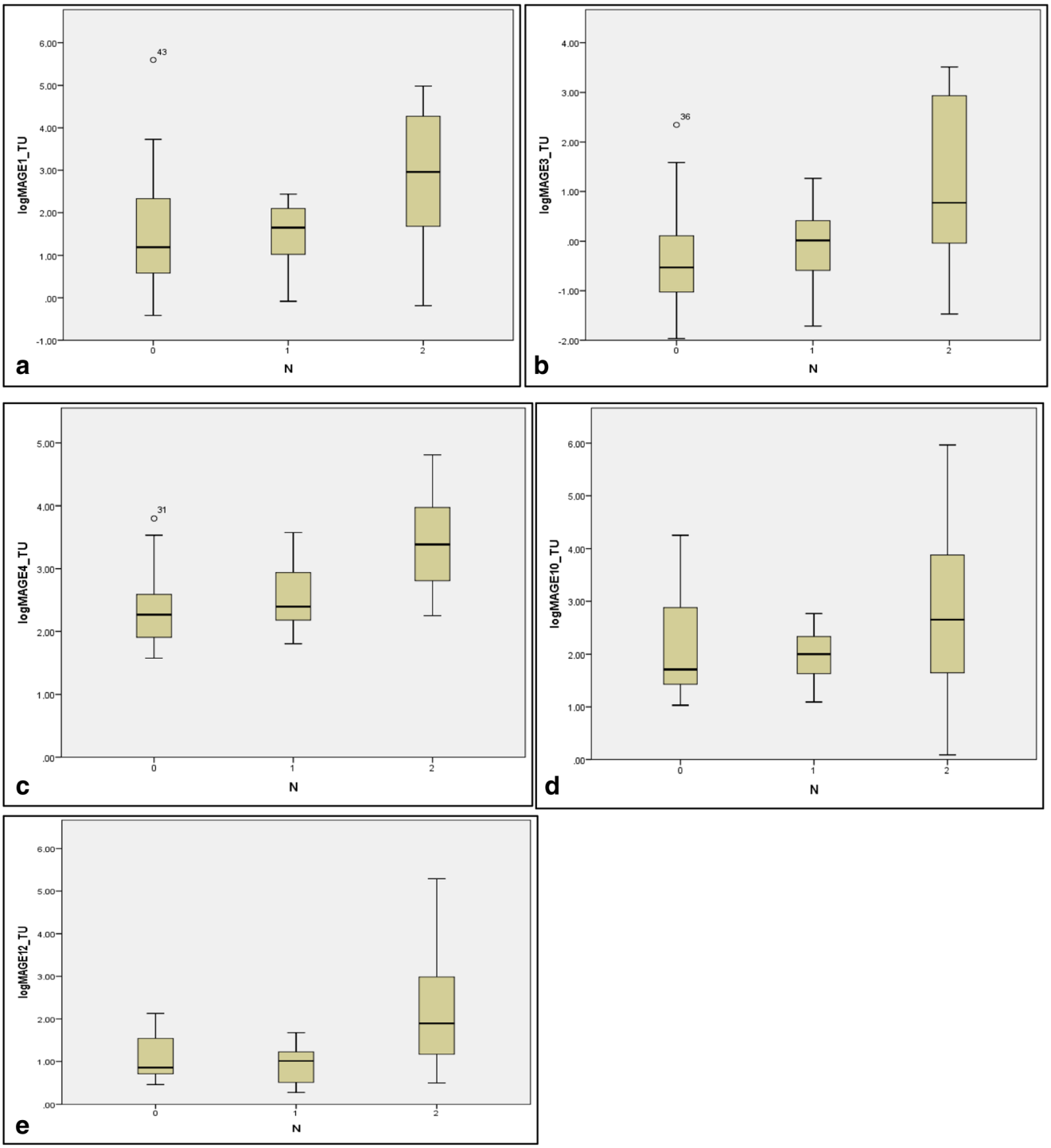

Fig. 2 Boxplots of the expression of MAGE-A in relation to lymph node metastasis N. MAGE-A3+6 and MAGE-A4 are statistically associated with a high lymph node metastasis. **a** MAGE-A1, **b** MAGE-A3+6, **c** MAGE-A4, **d** MAGE-A10, **e** MAGE-A12

in the placenta, and in fetal oral keratinocytes [11, 12]. Besides, they were detected in a series of malignancies, so that they shifted into the focus of tumor diagnosis and immunotherapy because of their special expression profile [30]. Especially the MAGE family, a subgroup of cancer/testis antigens, seems to play a key role within squamous cell carcinoma and their precancerous lesions [30–34]. When choosing a suitable marker for immunotherapy and the diagnosis of tumors, it is important to know the frequency and the pattern of expression of these target molecules within the corresponding malignancy to be able to use and interpret them efficiently. At the present time, there is still not enough

Table 3 Wilcoxon test to examine the tumor and mucosa PCR data. No statistically significant difference could be found

Wilcoxon test	MAGE-A1	MAGE-A3+6	MAGE-A4	MAGE-A10	MAGE-A12
p	0.903	0.879	0.794	0.937	0.332

evidence-based information about the expression of MAGE-A antigens in cancers of the head and neck, especially in OSCC, so that the function of MAGE-A within these entities and their possible influence on clinical parameters remain largely unclear. On this account, this study is dedicated to the analysis of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12 in oral squamous cell carcinoma.

Previous studies have provided evidence by means of immunohistochemical staining that MAGE-A is present in dissections of OSCC [30, 35, 36]. Laban et al. recorded a staining rate of 18% for MAGE-A1 and 39% for MAGE-A3/-A4 in their patient group. Montoro et al. documented in 56.6% of their patients MAGE-A4. In our immunohistochemical experiment, 97.6% of the tissues reacted positively to the antibody 6C1. This noticeably higher staining rate can be explained by the fact that 6C1 is simultaneously specific to 6 of 12 MAGE-A antigens whereas in the mentioned studies, the experiments were performed with antibodies which are specific to 1 or maximum 2 of the genes. Thus, our result confirms the hypothesis that MAGE-A is expressed in OSCC and is consistent with the statement of Hartmann et al. that at least 1 of 12 MAGE-A antigens is present in OSCC tissue. A contradictory fact is that 16 mucosa samples showed an antibody reaction, thereof 5 samples from healthy patients. The staining rate of these 5 samples was from 1 to 3%. These measurements could be affiliated to cross-reactivity during IHC or an eventual lack of sensitivity of the used antibody, so that a failure quotient of ≈ 3% can be set for this experiment. Nevertheless, the expression in tumor tissue was always higher than in the mucosa. This result suggests that MAGE-A either contributes to the genesis of OSCC or at least is part of its metabolism. This theory is supported by reports that MAGE-A1, -A2, and -A6 interact with the tumor suppressors p53 and p73 and inhibit these genes which are responsible for the control of the cell

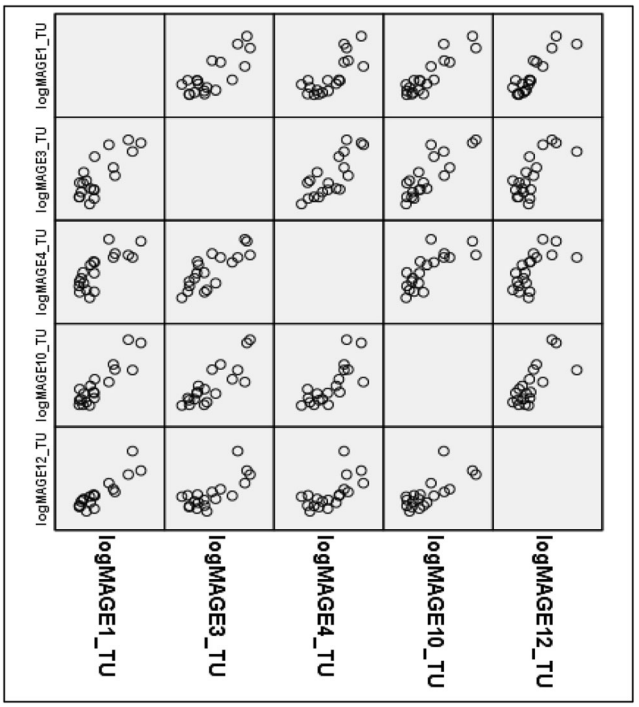

Fig. 3 Scatter plot of the proportional relationship of MAGE-A. Every examined gene is proportionally associated with the others, except for MAGE-A12

cycle and the genome stability [19]. Furthermore, there are hints that oral leukoplakia with following malignant transformation produce MAGE-A in contrast to oral leukoplakia without malignant transformation and that MAGE-A correlates with the malignant transformation potential of these precancerous lesions [31]. By using IHC methods, we proved that MAGE-A antigens are present in the tissue of OSCC but not or rather a small proportion in mucosa tissue. The antibody 6C1 did indeed not allow any conclusion about the exact distribution of the several genes within the tissue. So, in connection, we performed a RT-PCR in order to create an expression profile of MAGE-A. The PCR data turned out not as obvious as the IHC data. We could not observe a significantly higher expression of MAGE-A in tumor tissue compared to mucosa tissue although according to Sharma et al. both methods should provide similar results for the majority of the cases [37]. A possible reason for that might be that the standard experimental setup for PCR was not sensitive

Table 4 Listing of the factors of proportionality for each MAGE-A gene. The abbreviation n.p. means not proportional

	MAGE-A1	MAGE-A3+6	MAGE-A4	MAGE-A10	MAGE-A12
MAGE-A1		0.15	2.58	1.24	0.47
MAGE-A3+6	0.15		13.20	7.34	n.p.
MAGE-A4	2.58	13.20		0.61	n.p.
MAGE-A10	1.24	7.34	0.61		n.p.
MAGE-A12	0.47	n.p.	n.p.	n.p.	

enough. The average Ct-value of MAGE-A PCR was about 26 which marks a very low concentration of the antigen in the tissue. A closer inspection of the PCR data revealed a linear connection of MAGE-A and actually all genes except for -A12 that had a proportional expression frequency. MAGE-A12 was only proportional to MAGE-A1, the other genes correlated on a level from $p \leq 0.001$ and $p = 0.01$. This matter of fact, which to our knowledge had not been mentioned in any other study to date, could help in characterizing the expression pattern of MAGE-A in OSCC and simplifying the detection of each single gene by using the calculated factors of proportionality. MAGE-A4 was, on average, the most frequent expressed antigen in our patient cohort. This observation is consistent with the literature references about MAGE-A, not only about their prevalence in OSCC but also in other malignant entities like mammary or urothelial carcinoma [30, 36–39]. Besides MAGE-A4, these publications describe also a high prevalence of MAGE-A3. However, our patients showed the lowest expression level of MAGE-A3 among the respective genes.

The next aspect of our research question was the relationship between MAGE-A and clinical parameters of the patients, a theme which is discussed in literature with different results and without a unified opinion. The evaluation of our test series revealed no connection between MAGE-A and the tumor size T of the TNM classification. This recognition corresponds with the most published literature, in which the authors could not verify any correlation between MAGE-A and clinical parameters [30, 36, 39–43]. However, a statistically valid context between lymph node metastasis N and the genes MAGE-A3+6 and MAGE-A4 ($p = 0.007$ and $p = 0.004$) became apparent. Patients with lymph node status N2 or N1 had distinctly higher measurements than N0 patients. Laban et al. also pointed out a tendency towards MAGE-A and lymph node status, whereas Chen et al. claimed a certain connection between the expression of MAGE-A3 in NSCLC and lymph node metastasis on a level of significance of $p = 0.012$ [36, 44]. Furthermore, Chen et al. report about increased expression of MAGE-A3 in tumors with advanced stadium III or IV and nominate MAGE-A3 as a possible biomarker for tumor progression in NSCLC. There is no evidence in the current study for a definite correlation between MAGE-A and tumor staging, but we tendentially also found more frequent MAGE-A1 and MAGE-A3+6 measurements in advanced tumors ($p = 0.085$ and $p = 0.053$). In our patient cohort was no distant metastasis for which reason we cannot make any statement about a possibly higher metastasis in presence of MAGE-A. Hartmann et al. analyzed 38 tumor samples from patients with OSCC and discovered a different expression pattern in the tumor center and the tumor front depending on the advancement of the disease. In samples with stadium I or II, MAGE-A was detected mainly in the center. Stadium III or IV samples had a high concentration of MAGE-A in the invasive tumor front with mostly involvement of MAGE-A3 [35]. This could be an indication of MAGE-A, especially MAGE-A3, being part of the invasive potential of OSCC and a reference when describing the malignancy of the current tumor. Moreover, we observed a higher production of the genes -A3+6, -A4, and -A10 with rising age of the patients ($p = 0.013$, $p = 0.002$, and $p = 0.025$). According to our PCR data, MAGE-A had no influence on the overall survival or recurrence, so that we cannot draw a conclusion concerning the direct prognosis. Recent studies did also find no dependency of MAGE-A on recurrence [39, 41, 44], but a context with the overall survival and direct prognosis. Kocher et al. examined 2.317 samples of different carcinoma of the urinary bladder and came to the conclusion that MAGE-A4 was most frequently expressed in squamous cell carcinoma of the urinary bladder and correlated with the staging, grading, and a poorer outcome of the patients [45]. A poorer prognosis and overall survival in connection with the CTAs MAGE-A3, -A4, and -A11 has also been described for HNSCC, NSCLC, and mammary carcinoma. By the coexpression of 4 or more CT antigens, patients with HNSCC had a poorer prognosis and the overall survival was reduced by about 18%. For this reason, the authors suggested MAGE-A3 as an independent prognostic marker for NSCLC and MAGE-A4 for HNSCC [39, 44].

In our evaluation, MAGE-A3+6 and MAGE-A4 had the lowest standard deviation and so provided the most constant course of expression among the examined genes. These two antigens had also the previously mentioned correlation to clinical parameters as lymph node metastasis and staging. On protein level, MAGE-A demonstrated a reliable specificity with a failure quotient of ≈ 3% presumably due to the experimental setup as literature does not cite any MAGE-A detection in normal tissue. The qualification of MAGE-A as a tumor marker concerning its sensitivity on gene level was slightly restricted as only approximately, since cycle 26, the genes were detected in PCR cycle. Yet in our opinion, MAGE-A can be classified as tumor markers, not exclusively but also for OSCC. MAGE-A3 and MAGE-A4 could be used as markers for the progression of OSCC. An in vitro experiment performed on NSCLC cell line A549 suggests that MAGE-A3 influences the ability of tumor cells to metastasize in lymph nodes and is involved in EMT of malignant cells [44]. So, the antigen -A3 could represent a marker for the classification of the invasive potential of OSCC. In this context, the creation of a "CT high score" would be advisable; as according to Cuffel et al., the survival prognosis of patients with head and neck cancer declines almost a fifth in presence of four or more CTAs [39]. The tumor markers Hif-1 and LDH, which represent a key initiator of angiogenesis activity in tumors and

cellular changes [46, 47], should not be added to this high score because we could find no evidence for a correlation to MAGE-A. A promising candidate for CT screening would be NY-ESO-1, a CT antigen, which is also encoded on the q28 region of the X-chromosome like MAGE-A and has been detected in OSCC in correlation to the tumor size [48]. Together with MAGE-A3 and -A4, this gene could complement the correlation to the TNM classification.

Considering all mentioned aspects, the CTAs MAGE-A3 and MAGE-A4 are based on this study's suitable tumor markers for the diagnosis and early detection of oral squamous cell carcinoma. However, they should not be used alone but together with other CTAs like NY-ESO-1, as on basis of the low concentration within the tissue, a single gene's result would be of only limited informative value. Furthermore, discussion is necessary on whether patients with high MAGE-A expression should be integrated into a stricter therapy concept and shorter recall intervals because of the relationship between MAGE-A and lymph node metastasis. It is also conceivable to use MAGE-A3 and MAGE-A4 in follow-up examinations. A definite prognostic value of MAGE-A for the direct patient's prognosis could not be verified in this study, as the survival of our patients proceeded independently from the respective antigens. MAGE-A are considered by many other authors as oncoproteins, which promote the survival of malignant cells, as MAGE-A are often expressed in chemoresistant tumors and cell lines and can in vitro reduce the efficiency of Cisplatin, 5-fluorouracil, Docetaxel, Paclitaxel, Cetuximab, Panitumumab, Erlotinib, and Gefitinib in squamous cell carcinoma of the head and neck [49–51]. Consequently, MAGE-A3 and -A4 could be used as target proteins in immunotherapy of OSCC. Through blood-testis barrier, they normally have no contact to antigen-presenting cells or HLA-I and can therefore evoke a reaction of CD4-positive and CD8-positive T-cells when being expressed outside of the testis tissue [52]. Currently, 48 clinical studies are available at the Database of clinicaltrials.gov when entering the keyword "MAGE-A," 21 in phase 0 or I, 21 in phase II, 3 in phase III, and 3 finished studies without any information about the phase status (https://clinicaltrials.gov/). All 3 studies, which reached phase III, used MAGE-A3 as target protein but were stopped because the disease-free survival of the test persons was not significantly improved compared to the placebo group [11] (http://www.gsk.com/en-gb/media/press-releases/the-investigational-mage-a3-antigen-specific-cancer-immunotherapeutic-does-not-meet-first-co-primary-endpoint-in-phase-iii-melanoma-clinical-trial/). Another version of immunotherapy with MAGE-A would be by means of transferred T cell receptors directed against MAGE-A3, -A4, and NY-ESO-1 in order to provoke the combating of malignant cells by the immune system in MAGE-A positive OSCC patients. This therapeutic approach is pursued by some ongoing studies with a success from 1 to 2 years clinical remission in patients with multiple myeloma, though with side effects in form of on-target-off-target toxicity [52–54]. In conclusion, MAGE-A3 and MAGE-A4 have great potential as tumor markers for early detection, diagnosis, and follow-up of oral squamous cell carcinoma. The exact function within the tumor and the connection to the clinical parameters should be investigated further in representative studies with a preferably large pool of patients to establish these antigens in the screening and therapy of OSCC.

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Author contributions A.S., T.Z., M.A., P.H., and B.A. performed the experiments and analyzed the data. A.S., T.Z., and F.H. wrote the paper. A.S. and T.Z. provided clinical information about patients and treatments. P.H. and M.A. helped in the discussion. B.A. and T.Z. designed the study, analyzed the data, and guided the research presented.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The local ethics committee (Landesärztekammer Rheinland-Pfalz) approved the following experiments (Ethikvotum 837.837.11 (7924)). This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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