Disease Markers 27 (2009) 75–84
DOI 10.3233/DMA-2009-0652
IOS Press

A novel multiple-marker method for the early diagnosis of oral squamous cell carcinoma

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Abstract. *Objective*: Melanoma associated antigens-A (MAGE-A) expression is highly specific to cancer cells. Thus, they can be the most suitable targets for the diagnosis of malignancy. The aim of this study was to evaluate the sensitivity of multiple MAGE-A expression analysis for the diagnosis of oral squamous cell carcinoma (OSCC).

Methods: Total of 70 OSSC and 20 normal oral mucosal (NOM) samples of otherwise healthy volunteers were examined for the expression of 10 different single antigens out of 12 different MAGE-A subtypes by highly sensitive reverse transcriptase polymerase chain reaction (RT-PCR) methods. The results were correlated to clinicopathological parameters of tumor samples.

Results: Expression of MAGE-A was restricted to OSCC. The expression frequency of single antigen was between 10% and 55%. However, expression rate was increased up to 93% by the elevated number of genes examined. A significant correlation was found between the expression of MAGE-A and malignancy ($p = 0.0001$). In addition, multiple MAGE-A detection has also correlated to the incidence of lymph node metastasis, grading and advanced clinical stages.

Conclusions: Analysis of multiple MAGE-A expression is more sensitive than the analysis of a single MAGE-A for the diagnostic evaluation of OSCC. Multiple MAGE-A expression analysis may be a very sensitive method to be used for the diagnosis even in the early stage of OSCC.

Keywords: Oral squamous cell carcinoma, MAGE-A expression, multiple markers, RT-PCR, diagnosis

1. Introduction

Oral squamous cell carcinoma (OSCC) is the 6th most frequent occurring cancer world wide [1]. Although, early stage of OSCC is often curable, the prognosis of advanced clinical stages generally remains poor. Despite the extensive researches on treatment modalities for OSCC five year survival rate has been reported to be approximately 50% and has not been improved over the last four decades [2,3]. The major contributing factors for un-improvement in the survival rate might be the late diagnosis of primary tumors and the high incidence of local recurrences due to occurrence of occult cancer cells in tumor margins [4,5]. Furthermore, OSCC is known to exhibit "field cancerization" which may result in a second primary tumor [6]. Additionally, early dissemination of tumor cells into bone marrow or peripheral blood during the surgery may also cause recurrence and metastasis, which is usually overlooked. To increase the survival rate of patients with OSCC, highly sensitive and specific molecular diagnos-

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ISSN 0278-0240/09/$17.00

tic techniques to discriminate malignant from benign lesions and to detect residual or disseminated tumor cells (DTC) using multiple molecular tumor markers might be effective.

Melanoma associated antigens-A (MAGE-A) are suitable markers to be used in the early molecular diagnosis of cancer. The MAGE-A family consists of 12 subtypes including MAGE-A1 to -A12 [7–10]. Based on the reverse transcriptase polymerase chain reaction (RT-PCR) typing results for MAGE-A genes, except MAGE-A7 which is not found to be not transcribed, MAGE-A is expressed almost exclusively in various tumor types and testis [9–19]. Expression of single MAGE-A genes has already been extensively examined for the diagnostic application of different malignancies. However, tumor detection by a single antigen was not sensitive enough for the diagnostic application due to its low expression frequency and heterogeneous expression pattern, although they have a high specificity to cancer cells. Nevertheless, expression frequency was higher when multiple MAGE-A was applied. Previous studies reported that multiple MAGE-A analysis does increase the sensitivity of tumor cell detection in solid tumors including OSCC compared to single MAGE-A analysis [15–17,20–24].

There are several reports regarding to establishment of molecular diagnostic techniques for the early diagnosis of primary tumors, and also detection of rare occult tumor cells or DTC's using multiple molecular markers with definitive accuracy to malignancies including the one based on MAGE-A expression pattern [15,23–28]. Therefore, in order to increase the sensitivity of the previously established methods, it was aimed to investigate the expression rate of mRNA transcripts of 10 different MAGE-A subtypes using RT-PCR in a large number of OSCC samples to determine whether multiple expression analysis of MAGE-A is a more sensitive application for the detection of OSCC.

2. Patients and methods

2.1. Patients and sample collection

Total of 70 tumor samples obtained from patients with OSCC and 20 normal oral mucosa (NOM) samples were included to this study. All NOM tissues were obtained from retromolar area of otherwise healthy volunteers during prophylactic lower third molar removal. Sampling areas for NOM were all free of inflammation and infection. This side also was the most resembling area where all tumor samples were collected. Patients' informed consent and the approval of the local ethical committee was taken. Prior to biopsy or tumor resection, tumor patients received neither radiotherapy nor chemotherapy. Each sample taken was divided into two pieces. One piece was histologically examined and the second piece was immediately snap frozen and stored at -80°C until the molecular examination. For RT-PCR analysis, RNA of normal colon and testis has been used as negative and positive controls respectively.

2.2. Histopathology and staging

Clinical staging and TNM classification were done for each tumor patient according to UICC [29]. All biopsies were evaluated by the same pathologist to ensure the consistent results. The OSCC were also classified according to WHO [30] for loss of differentiation as G1, G2 and G3 for the well, moderately and poorly differentiation respectively. Additional clinical information including age, gender and clinical staging (stage I to IV) were also recorded. In addition, tumor samples were classified according to early (including stage I and II) and late (including stage III and IV) clinical stages and also into two groups as T1 & T2 and T3 & T4 displaying the early and advanced tumor staging. Moreover, state of lymph nodes was recorded as $N = 0$ and $N>0$ for negative and positive lymph node existence respectively.

2.3. Detection of MAGE-A expression by RT-PCR

Total RNA from frozen tissues was isolated using RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden). Total of 200 ng RNA was used for the detection of MAGE-A expression. All primers used for amplification steps are summarized in Table 1. High sensitive RT-nested PCR methods (nPCR) were used for the detection of MAGE-A1-A6 (MAGE-A1-6 Assay) and A12 (RT-nPCR) expressing tissues [15,24,25].

MAGE-A1-6 Assay: Reverse transcription and the first PCR reaction were carried out using One Step RT-PCR Kit (Qiagen, Hilden, Germany) and multiple MAGE-A recognizing primers (MMRP1/2). These primers can bind to the sequences of MAGE-A1, A2, A3, A4, A5 and A-6 together and allow a simultaneous detection of all subtypes by the same method as described previously [15,23]. For nPCR, 1 μl of first PCR product was used as a template for the second PCR. For detection of simultaneous gene expression of MAGE-

Table 1
Primer for amplification of GAPDH and MAGE-A members. S = sense; as = antisense; MMRP = Multiple MAGE-A 1 to A6 recognizing primers; W = A or T; M1- M6 = specific primers for MAGE-A1 to A6; o = outer primer of nested RT-PCR; I = inner primer of nested RT-PCR; *detection of two transcript variants due to different splicing

Primer	Primer sequence	Cycles	Size (bp)	Annealing (°C)
GAPDH s	GCAGGGGGGAGCCAAAAGGG	35	567	60
GAPDH as	TGCCAGCCCCAGCGTCAAAG			
MAGE-A1-6 Assay				
MMRP1	CTGAAGGAGAAGATCTGCC	30	831 to 855	60
MMRP2	CTCCAGGTAGTTTTCCTGCAC			
MMRP3	CTGAAGGAGAAGATCTGCCWGTG	30	496 to 493	60
MMRP4	5-CCAGCATTTCTGCCTTTGTGA-3			
M1	CGGAACAAGGACTCCAGGATACAA	30	375	60
M2	GAAAGAAGTCCTGGCAATTTCTGAG	30	522	60
M3	CCAAAGACCAGCTGCAAGGAACT	30	568	60
M4	CGTCAATGCCAAAGATCATCTTCAG	30	577	60
M5	CCTTTGTGACCAGCTCCTTGACTTA	30	510	60
M6	CCAGGCAGGTGGCAAAGATGTACAC	30	628	60
MAGE-A8				
A8 s	GGAGGCAAGGTTCGCAGAGA	40	299	60
A8 as	GGAGGACTTGGTGACCCAGA			
MAGE-A9				
A9 s	GACCTTGAAGCCCAAGGAGA	35	300	58
A9 as	GACCTTGAAGCCCAAGGAGA			
MAGE-A10*				
A10 s	GGAACCCCTCTTTTCTACAGAC	40	411 501	60
A10 as	TCCTCTGGGGTGCTTGGTATTA			
RT-nPCR				
MAGE-A12os	TCCGTGAGGAGGCAAGGTTC	30	341	60
MAGEA12oas	ATCGGATTGACTCCAGAGAGTA			
MAGE-A12is	TCCGTGAGGAGGCAAGGTTC	30	181	58
MAGEA12ias	GAGCCTGCGCACCCACCAA			

A1 to A6, a primer set recognizing MAGE-A was applied (MMRP3 and MMRP4). To assign the individual MAGE isotype, a combination of MMRP3 and one of MAGE isotype specific primers M1 to M6 were used. PCR products were separated on 1% agarose gels and stained with ethidium bromide in order to identify the different amplification products by size (Fig. 1).

RT-nPCR for MAGE-A12: Reverse transcription and the first PCR reaction was carried out using One Step RT-PCR Kit (Qiagen, Hilden) and outer MAGE-A12 specific primers that were previously described. 1 μl of the first PCR amplification was applied to the second PCR using inner MAGE-A12 primers (Table 1) [25,31]. Polymerase PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The size of the fragment amplificated by RT-PCR was 181bp (Fig. 1).

For the other members of MAGE-A, only one amplification step was performed using One Step RT-PCR Kit (Qiagen, Hilden, Germany). Primer sets, number of cycles, sizes of amplification products as well as annealing temperature have been summarized in Table 1. Cycling conditions for all RT-PCR steps were equal for all reactions: Reverse transcription was done at 50°C for 30 minutes; initial PCR activation step was performed at 95°C for 5 minutes; denaturation happened at 95°C for 30 seconds; elongation was done at 72°C for 45 seconds with a final extension incubation at 72°C for 10 minutes.

To exclude false negative results, quality of RNA was assessed by One Step RT-PCR Kit amplifying cDNA using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers for the reaction and analysing the GAPDH-PCR products by a 2% agarose gel (Fig. 1). To exclude false positive results generated by amplification of genomic DNA sequences that were not totally eliminated by DNAs digestion, 1 μl of purified RNAs from each specimen were tested for amplification of genomic GAPDH using the specific primers for PCR. Only RNA isolations showing no visible band in

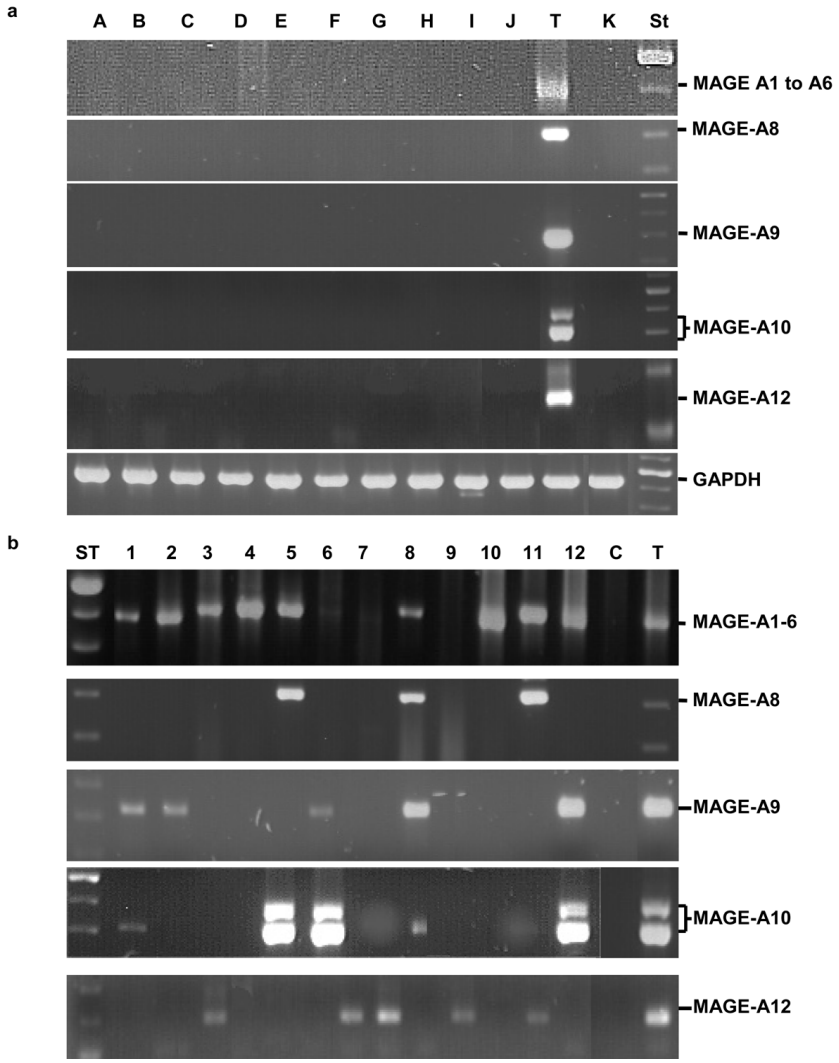

Fig. 1. Expression analysis of MAGE-A in normal oral mucosa (NOM) and OSCC. T = Testis; C = Colon; ST = Standard. A) Analysis of MAGE-A expression in NOM (A-J). The quality of RNA was assessed amplifying cDNA using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) specific primers for PCR. The expression of MAGE-A was not found in NOM. B) Simultaneous detection of MAGE-A1 to A6, −A8, −A9, −A10 and −A12 in samples of OSCC (1–12) are displayed respectively

2% agarose gel were analyzed for the subsequent procedures. Each RT-PCR experiment was performed in triplicate using the same RNA sample and appropriate controls.

2.4. Statistical analysis

Statistical analysis was performed using the statistical software package of SPSS 16 (Inc., Chicago, USA). Association between detection of at least one of the MAGE-A and clinicopathological parameters were analysed by χ^2 test. The prevalence of MAGE–A expression in OSCC and NOM was also compared by χ^2 test, and p value less than 0.05 was considered as an indication of a statistical significance.

3. Results

Fifty-seven of 70 tumor patients were male and 13 of them were female. The mean age was 58.5 years (Min: 34 and Max: 91). Regarding to tumor size 21 patients were at stage T1, 13 at T2, 5 at T3, 27 at T4. In addition, there were 31 patients with no incidence of lymph node metastasis. Moreover, 18 patients were clinically at stage I, nine at stage II, seven at stage III, and 32 were

Table 2
Association between the expression of at least one gene out of nine MAGE-A applied and the clinicopathological parameters of patients suffer from OSCC

	No. of cases	(+)	(−)	Positive %	P value
Diagnosis					
SCC	70	65	5	93	0.0001
NOM	20	0	20	0	
Differentiation degree					
No. of valid cases*	67	62	5	92.5	
G1	3	2	1	66.7	0.031
G2	46	45	1	97.8	
G3	18	15	3	83.3	
Tumor size					
No. of valid cases*	66	61	5	92.4	
1	21	17	4	81	0.87
2	13	12	1	92.3	
3	5	5	0	100	
4	27	27	0	100	
grouped					
T1 & T2	34	29	5	85.3	0.024
T3 & T4	32	32	0	100	
State of lymph node (N)					
No. of valid cases*	64	60	4	93.8	
Nx*	3	3	0	100	0.126
0	31	27	4	87.1	
1	10	10	0	100	
2**	20	20	0	100	
N 0	31	27	4	87.1	0.042
N 1 +2	30	30	0	100	
Stage					
No. of valid cases*	66	61	5	92.4	
Early (I, II)	27	22	5	81.5	0.005
Late (III, IV)	39	39	0	100	
I	18	15	3	83.3	0.044
II	9	7	2	77.8	
III	7	7	0	100	
IV	32	32	0	100	

Valid cases*: number of cases for which the clinico-pathological characteristics (G, T, N) were determined. Cases without these determination were excluded from statistical analysis.
Nx** Lymph node could not be classified;
2*** covers stages 2a, 2b and 2c.

at stage IV. Among all the cases three were well differentiated OSCC, 46 were moderately differentiated, and 18 were poorly differentiated (Table 2).

3.1. *Expression of a single MAGE-A in OSCC and NOM*

Expression of MAGE-A was restricted to OSCC (Fig. 1). None of them was expressed in NOM (Fig. 1a). However, expression frequencies of each gene varied largely in tumor tissues (Fig. 1b; Fig. 2a). Additionally, the number and type of expressed genes varied. No common occurrence of a single MAGE-A has been detected between the different individual subtypes (Fig. 1b).

The highest expressed antigen was MAGE-A4 (55%) followed by MAGE-A9 (50%) and A12 (50%). The rest was as follows A3 (46%), A6 (44%), A10 (41%), A1 (36%), A2 (28%) and A5 (25%). Only 10% of the all OSCC cases expressed MAGE-A8 (Fig. 2a). These expression ratios were obtained when the genes were applied as a single gene.

3.2. *Expression frequencies in OSCC by multiple MAGE-A analysis*

Among all OSCC samples, 93% expressed at least one antigen when all ten of the MAGE-A genes were applied as multiple gene markers. In the early clinical stages (Stage I and II) 81% of the tumor cases expressed

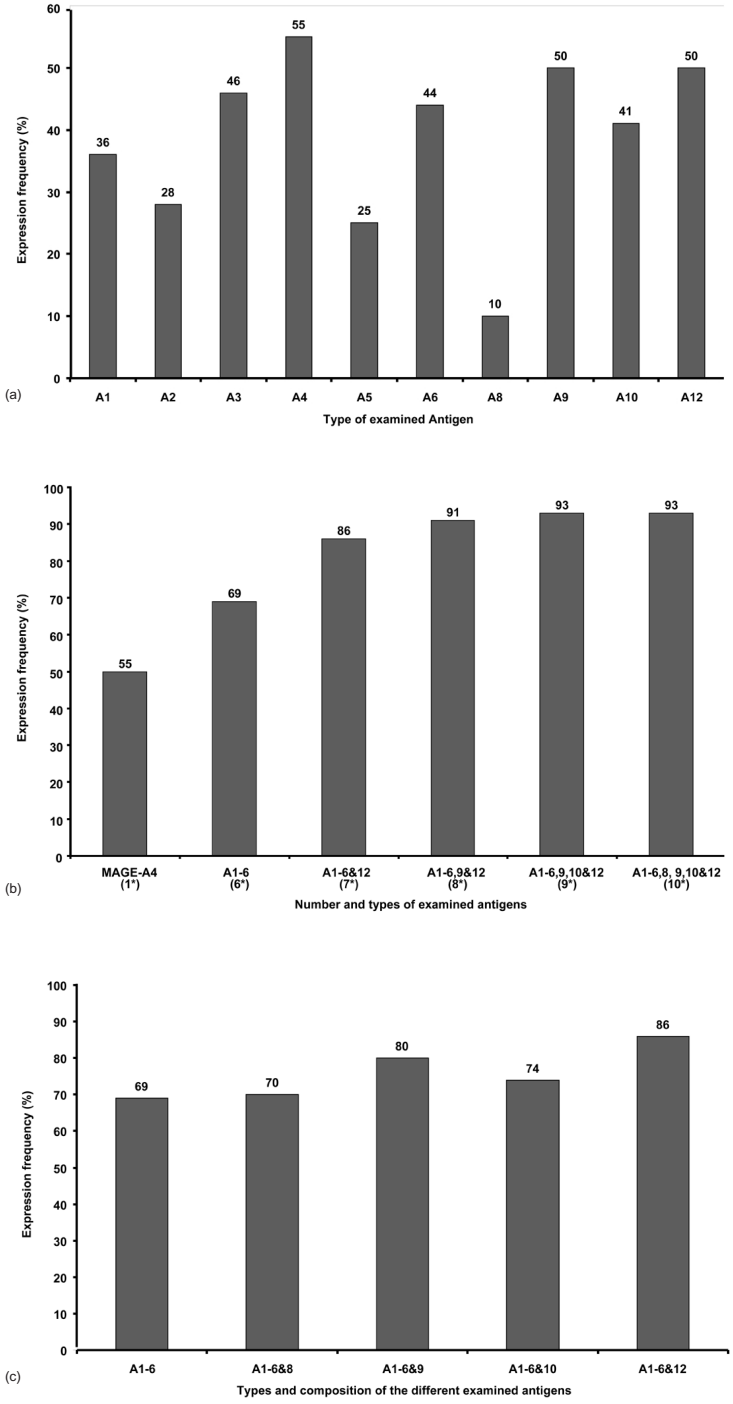

Fig. 2. Expression frequencies of single MAGE-A and at least one out of 10 investigated antigens in OSSC. (a) Expression frequencies of single antigen. (b) Expression frequency of at least one antigen which gradually increases by the addition of several antigens. (c-e) Increase of expression frequencies depending on the antigen taken into to account as a multiple marker system. (c) A highest frequency of expression when MAGE-A12 was applied as the 7th marker to the multiple marker system including MAGE-A1- A6 (86%). (d) A highest frequency of expression when eight antigens which are MAGE-A1 to A6, MAGE-A9 and A12 were applied (91%). (e) Highest number of frequency reached by the analysis of MAGE-A1 to A6, A9, A10 and A12 (93%). When MAGE-A8 was added as the 10^{th} gene, no increase was found.

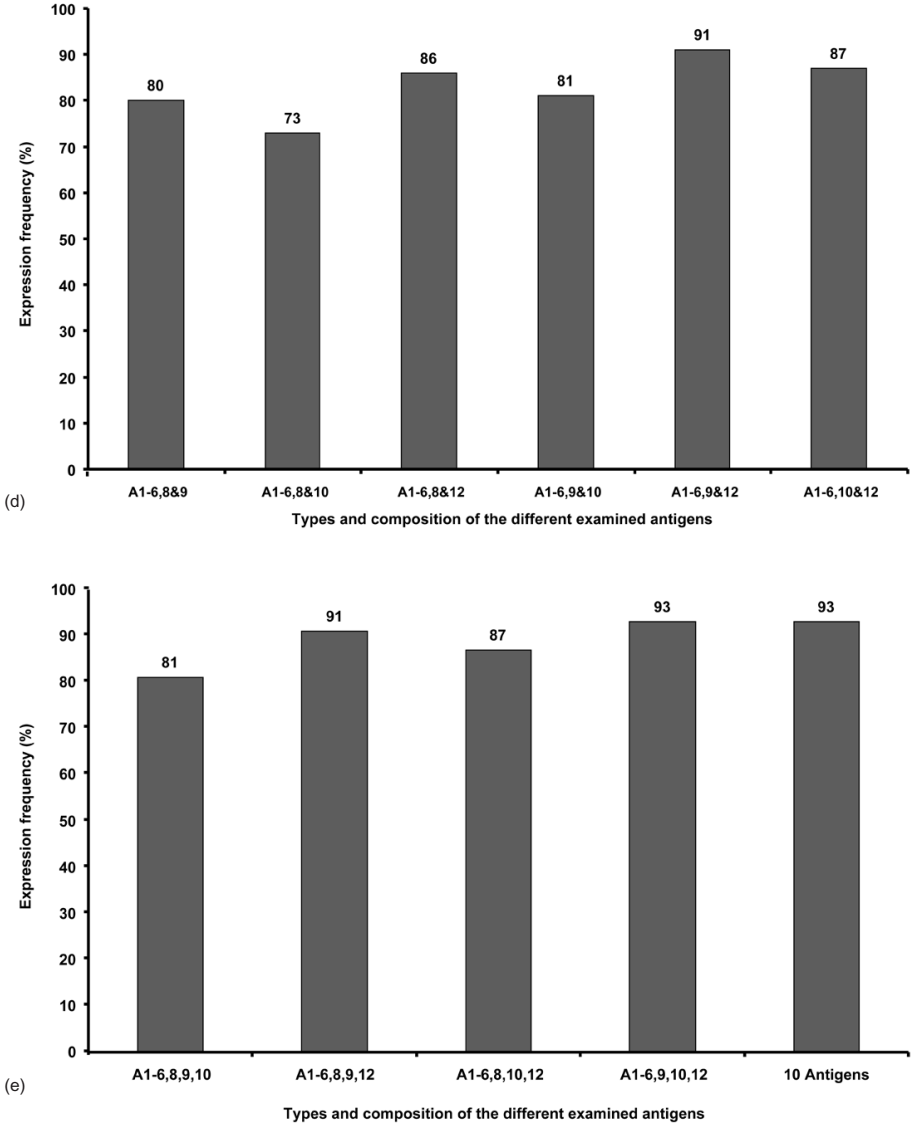

Fig. 2. continued.

at least one molecular marker. In the late clinical stages (Stage III and IV), the occurrence of frequency of at least one antigen was 100% (Table 2).

A constant increase of the expression frequencies was found by the inclusion of different MAGE-A to the analysis (Fig. 2). The expression frequency was 55% in maximum (MAGE-A4) when single antigen was applied (Fig. 2a, b). By simultaneous analysis of six MAGE-A (A1 to A6), the expression frequency increases to 69% (Fig. 2b). Depending upon the combination of the examined genes the expression frequency was elevated from 69% to 86% when seven genes were enrolled into the analysis. The highest increase of positive frequency (86%) was found when MAGE-A12 was considered as a seventh candidate for the multiple markers. When MAGE-A8, A9 and A10 were considered as a 7th, the frequency of a positive cases increased up to 70%, 80% and 74% respectively (Fig. 2c). However, when eight genes were enrolled into the analysis, the highest frequency was found by the MAGE-A1-6, A9 and A12 genes (91%). With other configurations, the frequencies range between 73% and 87% (Fig. 2d). When nine genes were enrolled into the analysis, the highest frequency was found with the combination of MAGE-A1-6, A9, A10 and A12 (93%) (Fig. 2e). The number of positive cases rose

up to different levels by other antigen combinations. An exception was, when MAGE-A8 was included as the 9th antigene to the combination of A1-A6, A9 and A10. In this case, expression frequency remains at 91% (Fig. 2d, e). Moreover, when MAGE-A8 was enrolled to the multiple marker expression analysis as the 10th candidate, no increase of expression frequency was detected (Fig. 2e). Hence, the highest number of positive cases displaying the highest sensitivity for tumor cell detection was found by multiple expression analysis of MAGE-A1-6, A9, A10 & A12 (93%).

3.3. Association between MAGE-A mRNA detection and clinicopathological parameters

The relationship between the different histological and clinicopathological parameters and the expression of al least one out of 10 MAGE-A was statistically evaluated. The results are summarized in Table 2. The expression of al least one antigen by the multiple expression analysis was strictly correlated to malignancy ($p < 0.001$). The expression frequency of at least one of the multiple markers applied was 100% when correlated to the late clinical stage (stage III and IV together) ($p = 0.005$). The expression frequencies were various and still significantly correlated when compared to stage I to IV separately ($p = 0.044$). Moreover, there was no significance between expression of MAGE-A and tumor size ($p = 0.87$). However, the frequency of multiple MAGE-A expression was significantly associated to larger tumor size when tumor classification was grouped together as T1 & T2 and T3 & T4 ($p = 0.024$). In addition, no correlation was observed between gene expressions and state of lymph node when N0 was compared with N1 and N2 separately ($p > 0.05$). However, there was a statistical significance when N0 was compared with N1+N2 together (N0<N1+N2) ($p < 0.05$). It was also determined that all the tumor samples accompanied by the lymph node metastasis expressed at least one of the examined antigens. Additionally, there was a significant association between the multiple antigen expression and the histo-pathological differentiation degree in tumor cases ($p = 0.031$) (Table 2).

4. Discussion

In this study, expression of 10 MAGE-A was analyzed all together in number of OSSC samples in order to increase the sensitivity for the accuracy. According to previous studies, expression frequencies of the single subtypes were low and heterogeneous [16,23,24]. Thus, analysis of a single MAGE-A seems not to be quite sensitive for the diagnostic evaluation of OSCC. Elevated number of MAGE-A analysis does increase gradually the detection of sensitivity for OSCC cells when a certain combination was taken into account, especially when the amount of sample is not sufficient to perform analysis for each markers separately.

This study pointed out that MAGE-A1 to A6 should preferably be used for the detection of malignancy in OSCC, because these six genes can be detected concurrently even with a small amount of cells by the MAGE-A1 to A6 assay [15,16,23,24]. It has also previously been determined that the expression frequency of MAGE-A can increase when MAGE-A12 is used as an additional 7th marker [24]. However, the main analysis can be gradually developed by using other antigens in order to reach the optimal sensitivity. In this study, the best result was determined by the analysis of seven genes when MAGE-A12 is included as the additional gene (85%) [24]. Furthermore, the sensitivity was even higher when eight genes, A1-6, A9 and A12, were added in multiple marker combination (91%). The highest sensitivity in the detection of malignancy was achieved by the analyses of following nine antigens which were MAGE-A1-A6, A9, A10 and A12 (93%). Additional analysis of MAGE-A8 did not increase the sensitivity level. Therefore a multiple marker system which covers only nine antigenes, seems to be sufficient enough to reach the maximum sensitivity for the diagnosis of OSCC.

Expression of a single MAGE-A has already been detected in precursor lesions of intrahepatic cholangiocarcinoma and dysplastic precancerous tissues of lung cancer [32,33]. In addition, in the present study 81% of OSCC which were in early stage expressed at least one of the multiple antigens applied and it was claimed that analysis of multiple MAGE-A can also provide a high accurate and sensitive tool for the early diagnosis of OSCC. Moreover none of the NOM specimens expressed any MAGE-A indicating the high specificity to malignant cells. Thus, detection of multiple MAGE-A can also be applied as a predictor for the potential malignant transformation of oral pre-malignant lesions allowing the detection of already malignant transformed cells. Nevertheless, carefully designed studies are still needed to determine the expression profiles in mild dysplastic lesions of NOM that seem to have no potential of malignant transformation, and also in carcinoma in situ, , in order to clarify in particular the role of such molecular-biological methods as an additional tool for the cancer risk assessment of oral pre-malignancies.

Furthermore, operated OSCC patients may still suffer from postoperative recurrence or metastases of neck lymph nodes which may indicate residual, occult malignant cells in tumor margins and also early DTCs in peripheral blood, bone marrow or lymph node as an intrinsic path [5]. Prognostic relevance of the tumor cell detection in hematopoietic cell compartments has already been studied in a variety of solid tumors [25, 34–38]. Expression of MAGE-A in the surgical margin and normal tissues adjacent to the oesophageal and lung cancer has been previously reported and was determined to be related to local recurrence. Thus, this may indicate that residual, occult OSCC cells may also be detected around tumor margins by the analysis of multiple expression profile of these genes and the occurrence of local recurrence may easily be predicted [33, 38]. Although, multiple expression analysis of MAGE-A has already been applied to the early detection of lymph node metastasis and DTCs in blood and bone marrow of patients suffer from different solid tumors, carefully designed studies are still needed to determine the expression profiles of NOM at a distant and/or close to tumor margins to evaluate the role of MAGE-A [15, 23–25,28,39]. In addition, the present study determined a significant association between the expression frequency of multiple MAGE-A mRNA analysis and the positive lymph node metastases indicating the local recurrence irrespective of the tumor classification or size ($p = 0.042$). On the other hand in a previous study of Ferris et al. expression ratio of MAGE-A1, A2, A3, A4, A8, A10, A12 on Head and Neck SCC was investigated in tumor and benign lymph nodes in order to elucidate their role for molecular staging of the cervical lymph nodes. They found positive gene expression of MAGE-A, but the lowest or median detection level of single gene expression was at very low or at non-detectable level. Only MAGE-A3 showed detectable expression but display only a low tumor/highest benign node expression ratio and also a relatively low median expression in tumors. Thus this gene was omitted from the screening method, too [40]. However, it can be hypothesized that application of multiple MAGE-A mRNA expression can also be used as an additional prognostic marker for the detection of even a very small or clinically not visible lymph node metastasis because they would express at least one of the multiple antigen applied in a high ratio. Nevertheless, expression pattern of MAGE-A in lymph node, bone marrow or peripheral blood sample of patients with OSCC still remains unclear, and warrants further investigations as well.

In conclusion, expression analysis of multiple MAGE-A is found to be more sensitive for the diagnostic evaluation of OSCC than the detection of a single MAGE-A. Based on the expression analysis of several MAGE-A, OSCC can be diagnosed early, surely with high probability. In addition, it also seems possible to detect residual, occult malignant cells in tumor margins and DTCs. Therefore, it is hypothesized that such a molecular-biological method may also represent an additional prognostic tool for the early diagnosis of OSCC and for the early prediction of recurrences/metastasis to provide a suitable individual therapy for each patient with OSCC.

Acknowledgement

Authors would like to indicate that this study has been supported by the grant of Johannes und Frieda Marohn-Stiftung, Erlangen and also would like to thank Ms. A. Krautheim-Zenk for the technical support.

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