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Expression of MAGE-A12 in oral squamous cell carcinoma

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Abstract. Melanoma associated-A antigens (MAGE-A) are silent in normal tissues except testis. However, they are activated in a variety of different tumors. Thus, their expression is highly specific to cancer cells. Reverse transcription-nested polymerase chain reaction (RT-nPCR) is a highly sensitive technique that has been used successfully for the detection of MAGE genes in tissue samples. The aim of the study is to analyze the expression rate of MAGE-A12 in oral squamous cell carcinoma (OSCC) using a high sensitive RT-nPCR. Total of 57 tissue samples obtained from patients with OSCC and 20 normal oral mucosal (NOM) probes of otherwise healthy volunteers were included to this study. No expression of MAGE-A12 was observed in the non-neoplastic NOM tissues. MAGE-A12 was expressed in 49.1% of the investigated tumor samples. The correlation between malignant lesion and MAGE-A12 detection was significant ($p < 0.001$). It is concluded that results of this study may indicate MAGE-A12 as a useful additional diagnostic marker especially for the early detection of OSCC distinguishing neoplastic transformation and detection of occult and/or rare disseminated cancer cells. In addition, MAGE-A12 expression in OSCC may also determine a new immunotherapeutic target and might be warranted to develop vaccine for OSCC.

Keywords: Nested RT-PCR, MAGE-A12 gene expression, oral squamous cell carcinoma, diagnostic, therapy

1. Introduction

Oral squamous cell carcinoma (OSCC) is the 6^{th} most common cancer worldwide accounting for 3–5% of all malignancies [13]. Although, the early stage of OSCC is often curable, the prognosis of advanced cases generally remains poor. Oral cancer has been known to exhibit "field cancerization" that may cause a secondary primary tumor varying between 4.3% and 30% [6]. The local recurrence of OSSC might have been seen due to the existence of occult cancer cells in tumor margins. In such cases, early detection and/or treatment of oral cancer can significantly improve the survival rate. Therefore, estimation of recurrency or the premalignant stage of a secondary primary tumor using a high specific tumor associated marker to differentiate malignant from a benign might be of important. Despite the diagnostic and therapeutic advances in combination therapy including surgery, radiation and chemotherapy, the prognosis of OSCC remains still 50%–60% in five year survival analysis and has not been changed significantly during the last three decades [23]. New therapeutic strategies are actively pursued one of the most popular immunotherapy. Active immunotherapy approaching malignant cells by using vaccines derived from defined antigens appeared to be especially attractive to treat OSCC. Prerequisites for the development of specific vaccines are the existence and identification

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of genes that are exclusively or preferentially expressed in malignant tissues compared to normal tissues.

Melanoma associated-A antigens (MAGE-A) are expressed in a variety of different tumors and their detection is highly specific to cancer cells. The human MAGE-A gene family consists of 12 members including MAGE-A1-A12 and encodes products that can be recognized by autologous cytotoxic T (CT) cells. These genes are silent in normal tissues except testis but are activated in a variety of neoplastic lesions. These neoplastic and immunogenic features made MAGE an attractive target for cancer detection and also for immunotherapy [3,7,11]. It has been previously reported that MAGE-A12 is expressed in esophageal, cervical and breast cancer [1,10,17].

Reverse transcription-nested polymerase chain reaction (RT-nPCR) used for the detection of MAGE-A12 is a highly sensitive technique developed by Kufer et al. [24]. This method has been successfully utilized in detection of disseminated tumor cells in blood and bone marrow when used in combination with the detection of other tumor-associated antigens in patients suffering from breast, lung, colorectal and prostate cancer. Furthermore, there are numerous studies on the expression of the MAGE-A gene family members in head and neck carcinomas and oral cancer [11,12,16]. However, there has been only one study that has been reported the expression of MAGE-A12 in HNSCC including relatively small amount of samples (n=29) including only 15 OSCC specimens [5]. Therefore, we aimed to analyze the expression frequency and pattern of MAGE-A12 only in relatively larger amount of OSCC samples using a high sensitive RT-nPCR.

2. Materials and methods

2.1. Tissues and cell lines

Total of 57 tissue samples obtained from patients with OSCC and 20 normal oral mucosal (NOM) probes of otherwise healthy volunteers were included to this study following the approval by the Ethics Committee of Friedrich Alexander University Erlangen-Nuremberg, Germany. All the OSCC samples were obtained from surgical specimens collected during tumor surgery. Prior to our investigation tumor staging has been done for all the samples according to TNM classification of International Union Against Cancer (UICC) by both clinically and pathologically [25]. Patients have received neither radiotherapy nor chemotherapy prior to biopsy or tumor resection. Collection and storage of the tissue samples were done carefully in the same manner. Each sample was divided into two pieces. First piece was used for histological examination. The second piece was immediately snap frozen and stored at -80°C until examination. Patients were followed up clinically in regular intervals. For RT-nPCR analysis, RNA extracted from normal colon and human foreskin fibroblasts (HFF) were used as negative controls. In addition, human melanoma cell line (Gerl 43) (G) and tissue of testis (T) were used as positive controls (Fig. 1).

2.2. RT-nPCR for MAGE A12

A high sensitive RT-nPCR for detection of MAGE-A12-expressing tissues was used. In summary, total RNA from frozen tissues and cells were isolated using Rneasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden).

2.3. Reverse transcription and first PCR amplification step

200ng of total RNA were used for the detection of the expression of MAGE-A12 gene. Reverse transcription and the first PCR reaction were carried out using One Step RT-PCR Kit (Qiagen, Hilden) using outer MAGE primers (Table 1). Cycling conditions were as follows: Reverse transcription was done at 50°C for 30 min. Initial PCR activation step was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72 °C for 45 s. The final extension incubation was performed at 72°C for 10 min.

2.4. Nested PCR

For the second round of PCR, 1 μl of the first reaction, 1 μl of 10x PCR buffer [100 mM Tris (pH 8.3), 500 mM KCl, 10 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate], 0.4 μM each of the inner MAGE primers (Table 1), 5 μg of BSA (Roche), and 0.6 units of *Taq* DNA Polymerase were run according to the following cycle profile: an initial denaturation at 94°C for 10 min to activate the enzyme, followed by 94°C for 40 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s for 30 cycles and a final extension at 72°C for 2 min. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The size of the fragment amplificated by RT-PCR is 181 bp (Fig. 1) (Table 1). Only the amplificated fragments in

Table 1
Primer amplification of MAGE-A12

Primer	Primer sequence	Cycle number number	Size product bp.	Annealing temperature
MAGE-A12os	TCCGTGAGGAGGCAAGGTTC	30	341	60
MAGEA12oas	ATCGGATTGACTCCAGAGAGTA			
MAGE-A12is	TCCGTGAGGAGGCAAGGTTC	30	181	58
MAGEA12ias	GAGCCTGCGCACCCACCAA			

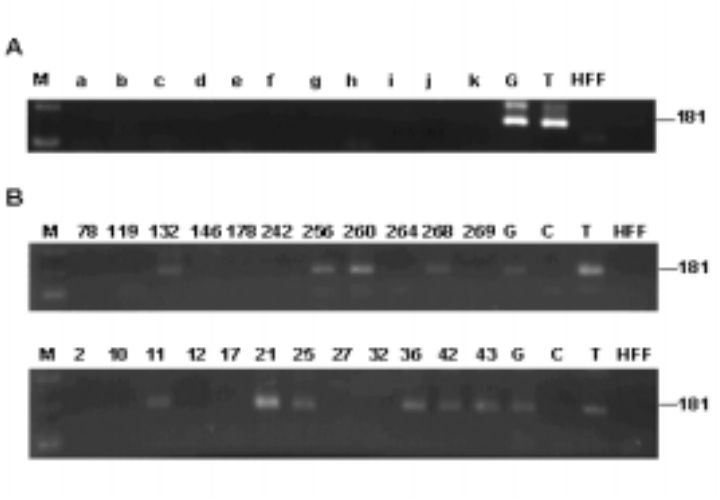

Fig. 1. Expression analysis of MAGE-A12 by RT-nPCR. A: Expression in normal tissue (samples a-k are all obtained from otherwise healthy volunteers); B: Expression in OSCC (samples 2-269 are all obtained from patients with OSCC); (+) and (−) controls: **G**: human melanoma cell line (+), **T**: Testis (−); **HFF**: human foreskin fibroblasts (−); **M**: Marker.

size of 181 bp have been estimated as positive. The positive and negative results were confirmed by running the assay for both results with a second aliquot of each original total RNA sample.

2.5. Statistical analysis

The statistical software package SPSS 14.0 (Inc., Chicago, USA) was used. The prevalence of MAGE-A12 mRNA expression in cancers and normal tissues was compared by Chi Square test. The association between MAGE-A12 mRNA expression in tumors and various clinicopathological variables was also examined by Chi Square test and Fisher's exact test. P value less than 0.05 indicated statistical significance.

3. Results

Total of 57 specimens from OSCC to determine the MAGE-A12 expression profile and 20 specimens from NOM taken from otherwise healthy volunteers were analysed. MAGE-A12 expression was restricted to neoplastic specimens. Fourty-eight of 57 OSCC patients were female and nine of them were female with a mean age of 58.5 years old (Min:34 and Max:91). The percentage of the positive expression of tumor samples is 49.1%. The correlation between malignant lesions and MAGE-A12 detection was statistically significant ($p < 0.001$) (Table 2). Tumor classification, staging, grading and lymph node status of the 57 specimens from OSCC were displayed in Table 2. The relationship between the expression of the analysed MAGE-A12 and different clinicopathological factors were statistically evaluated. Statistical differences between the MAGE-A12 expression and these parameters of OSCC were not statistically significant ($p > 0.05$) (Table 2).

Of the 57 tumor cases studied, 16 (37.5%) had histological characteristics of well-differentiated tumors, 35 (60%) were moderately differentiated, and three (33.3%) were poorly differentiated. No correlation was observed between gene expressions and grading of tumor (Table 2).

Staging of tumors and the expression of MAGE-A12 were as follows; T4 displayed 45% expression of MAGE-A12 in OSCC samples, T3 displayed 75%, T2

Table 2
Correlation between expression of MAGE-A12 and clinicohistopathological parameters (p value related to diagnosis)

	Total	(+)	(−)	expression (%)	p-value
Nr. of cases	57	28	29	49.1	$<$ **0.001**
Grading					0.264
G1	3	1	2	33.3	
G2	35	21	14	60	
G3	16	6	10	37.5	
Tumor size					0.528
1	18	10	8	55.6	
2	11	4	7	36.4	
3	4	3	1	75	
4	20	9	11	45	
State of lymph node					0.144
0	27	17	10	63	
1	8	2	6	25	
2	12	4	8	33.3	
State of lymph node					**0.039***
N0	27	17	10	63	
N $>$ 0	20	6	14	58.3	
Stage					
Early (I, II)	25	13	12	52	0.685
Late (III, IV)	28	13	15	46.4	
I	17	11	6	64.7	0.257
II	8	2	6	25	
III	5	3	2	60	
IV	23	10	13	43.5	

*Analysed by Fisher's exact test. The rest have been analysed by Chi Square test.
The number of missing cases for Grading: 3; Tumor size: 4; State of lymph node: 10; Stage: 4.

displayed 36.4% and T1 displayed 55.6% respectively. Expression rate was highest in stage T3. However, there was no statistical significance found between staging and expression of MAGE-A12 (Table 2). In addition, no correlation was observed between gene expressions and state of lymph node when N0 was compared by N1 and N2 separately ($p > 0.05$). However, there was a statistical significance when N0 was compared by N1 + N2 together (N0 $<$ N1 + N2) ($p < 0.05$) (Table 2).

4. Discussion

The expression of different subtypes of MAGE-A genes has been extensively studied in adult individual neoplasia arising from epithelial cell like melanoma, lung, bladder, breast, colorectal, gastric, esopharyngial SSC, hepaticellular carcinoma and head & neck cancer [5,9,12,16,21]. However, there are only a few papers reporting the expression of MAGE-A genes in OSCC [5,11]. In the present study, MAGE-A12 was expressed in 49.1% of tumor samples diagnosed as OSCC. In addition, expression of MAGE-A12 was significantly correlated to malignancy which may indicate its diagnostic excellence ($p < 0.001$). Previous studies have also reported positive expression of MAGE-A12 in different types of tumors. The expression of MAGE-A12 was 26.7% in oesophageal tumor, 9% in breast cancer and 9.1% in HNSCC [5,10,17]. Our result indicates that MAGE-A12 is expressed by a higher frequency in OSSCs compared to other types of solid tumors and it might be a promising target for antigen-specific immunotherapy for OSCC. The reasons of high expression frequency of MAGE-A12 in OSCC might because of two reasons. First the oral cavity may express MAGE-A12 more than other types of tumors and second RT-nPCR is more sensitive than RT-PCR so expression frequency would automatically expected to be higher in our samples compared to previous reports [5, 10,17].

The function of MAGE proteins and their involvement in tumor progression have not been well explained yet. MAGE-A12 is reported to be reactivated mostly in early stages of carcinogenesis. Its expression follows demethylation which is an early hallmark in cancer prognosis to distinguish normal from neoplastic tissues [4]. In the present study, MAGE-A12 gene was

expressed 52% in the early clinical stage and 46.4% in the late clinical stage when clinical stages of I and II were combined as early stage and stage III and IV as late stage. The late clinical stage reactivation might display that MAGE-A12 expression rate does not increase by the advancement of tumor stage indicating that expression of MAGE-A12 may be an early event in oral carcinogenesis. In addition, there found to be no significant association between any of the clinical parameter and mRNA expression of MAGE-A12. However, when state of lymph nodes, N1 and N2 grouped together as N, there is a significant difference lying between N0 and N as N $>$ N0. Thus, grouping the lymph node state may be more advisable for MAGE-A12 expression. Furthermore, higher proportion of MAGE-A12 expression among N0 cases may sign, that cases without MAGE-A12 expression may have higher propensity for lymph node metastasis and be short of immuno-recognition which hinders tumor progression.

MAGE genes are currently of great interest for the development of a vaccine against cancer. It has been already established that there are some peptides derived from MAGE-A12 that can induce CT cell immunresponce [14,18]. MAGE gene products are of particular interest in immunotherapy owing to their wide expression in many tumors and their potential to induce HLA class I mediated tumor-specific CTL responses [19,20, 22,26]. It was suggested that autologous dendritic cells vaccination with MAGE-3 peptide is a safe and promising approach in the treatment of specific carcinomas depending on the patient's HLA haplotype (HLA-A2 or A24) [2,8,26]. It has been already known that both MAGE-3 and 12 peptides bind HLA-A2 [16]. Therefore, MAGE-A12 may be employed as a potential target for tumor-specific immunotherapy in OSCC. The successful development of antigen-specific vaccines against cancer depends on the identification of the appropriate target antigens. Results of the present study display a frequent expression of MAGE-A12 in OSCC. This finding may suggest that patients with OSCC can be a potential target for immunotherapy using "cancer vaccines" and/or using antibodies pointed against the antigen that carried specifically by the OSCC cells such as MAGE-A12.

In conclusion, results of this study may indicate MAGE-A12 as a useful additional diagnostic marker especially for the early detection of OSCC distinguishing neoplastic transformation and also detection of occult and/or rare disseminated cancer cells. In addition, monitoring of MAGE-A12 expression in OSCC may also determine a new immunotherapeutic target and might be warranted to develop OSCC vaccine.

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