

Prediction of recurrence using exfoliative cytology and melanoma-associated antigen-A mRNA analysis following wide excision of oral squamous cell carcinoma: short report

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Background: Oral squamous cell carcinoma (OSCC) is the sixth most common cancer. The local recurrence of OSSC might result from the existence of occult cancer cells around tumour margins. Exfoliative cytology has lately gained great importance as a method for obtaining RNA samples from suspicious oral mucosal lesions in order to carry out molecular diagnosis. In addition, melanoma associated-A antigens (MAGE-A) are expressed in various tumours and their detection is a highly accurate sign that cancer cells are present.

Objective: The prediction of a recurrence using MAGE-A mRNA expression analysis to follow-up OSCC cases using a newly established molecular diagnostic technique applied to cytological materials.

Methods: RNA was extracted from three recurrent OSCC cases and from 20 healthy volunteers as a control group using a cytobrush. The expression of MAGE-A3, A4, A6, A10 and A12 was investigated in these specimens using quantitative real-time (RT-PCR).

Results: There was no expression of MAGE-A in the specimens of normal oral mucosa. However, the expression analysis of five different MAGE-A genes indicated a high potential for malignant change in biopsy-proven recurrent OSCC cases. Except for MAGE-A10, the rest of the genes were expressed in different ratios by the three recurrent cases, which had been determined on histopathology to be OSCC or carcinoma *in situ*.

Conclusion: It is suggested that analysis of MAGE-A expression may be used as a risk prediction method in the diagnosis of recurrence after wide excision of OSCC to enhance the accuracy of exfoliative cytology, which has limitations due to false negative and false positive results.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide, accounting for 3–5% of all malignancies.¹ Although the early stage of OSCC is often curable, the prognosis of advanced cases generally remains poor. Local recurrence of OSSC

may be seen due to the existence of occult cancer cells around tumour margins. In such cases, early detection and/or treatment of recurrent oral cancer can significantly improve the survival rate. Therefore, the estimation of recurrence or the premalignant stage of a secondary primary tumour using a highly specific tumour-associated marker to differentiate malignancy from benign condition may be of importance.

Moreover, premalignant lesions such as leukoplakia and erosive lichen planus may also develop into OSCC over time. They constitute highly suspicious oral lesions in the oral cavity. Malignant changes take place at a molecular level before they are visible under

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the microscope or before any clinical changes occur. Therefore, exfoliative cytology has lately gained great importance as a rapid, painless and simple method for obtaining RNA samples from such suspicious mucosal lesions in order to carry out highly sensitive molecular diagnostic methods.^{2,3} Early diagnosis of high-risk oral premalignant lesions can reduce the mortality, morbidity and also the cost of treatment. However, biopsy still remains the gold standard for obtaining a definitive diagnosis. Nevertheless, it is an invasive surgical technique that may have some limitations, especially when the lesion is large and multiple.⁴⁻⁶

Exfoliative cytology can be performed simply and rapidly, in a non-aggressive and relatively painless way using a cytobrush. Therefore, it is easily accepted by patients, and it can be used effectively and routinely to follow-up suspected oral lesions as well as resected tumour cases with a high recurrence rate. Using this technique multiple sampling is always possible when required. Furthermore, melanoma associated-A antigens (MAGE-A) are expressed in a variety of different tumours and their detection is a highly accurate sign that cancer cells are present. The human MAGE-A gene family consists of 12 members including MAGE-A1 to A12. These genes are silent in normal tissues except the testis but are activated in a variety of neoplastic lesions including OSCC. This neoplastic feature makes MAGE-A, an attractive target for cancer detection.⁷⁻⁹

In this study, the establishment of a novel non-invasive molecular diagnostic technique applied to cytological material is presented using expression analysis of MAGE-A3, A4, A6, A10 and A12 mRNA in the diagnosis of primary recurrence that developed following a wide tumour excision in three different OSCC recurrence cases.

Methods

This study concerns three patients who had been previously operated on in 2006 for squamous cell carcinoma on the floor of the mouth. Patients were closely and routinely followed-up. However, the practitioner noticed oral mucosal changes around the operation site and immediately referred the patients to the University of Erlangen, Department of Oral and Maxillofacial Surgery, at the beginning of 2007. The patients were examined and the suspected areas were sampled by cytobrush prior to incisional biopsy following the signing of a consent form. All the cell samples collected by cytobrushes were treated as a

material for the molecular analysis of MAGE-A gene expression, and the results were compared by the histopathological assessments. Clinico-pathological parameters such as age, gender, tumour size, regional lymph node metastasis, and histopathological grading according to the World Health Organisation (WHO) criteria¹⁰, were obtained from the Cancer Center of the University of Erlangen-Nuremberg. All the haematoxylin-eosin (H&E) stained slides were re-evaluated under a light microscope and scored according to the standard criteria of the WHO international histological classification of tumours. Clinico-pathological parameters of these three recurrent lesions along with the expression level of MAGE-A genes are shown in Table 1; one was clinical stage IV, one stage I and one carcinoma *in situ* (CIS). The control group included cell samples collected with a cytobrush from normal buccal mucosa of 20 healthy volunteers. MAGE-A gene expression revealed that none of the control samples expressed any of the MAGE-A genes, in contrast to recurrent cases.

Isolation of RNA

RNA was extracted from cells harvested by cytobrush using the RNA-Bee extraction method according to the manufacturer's protocol (AMS Biotechnology, Europe, Abington UK). In addition, the RNA quality and quantity was assessed by the 'Lab-on-a-Chip' method (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA, USA) according to the instructions provided by the manufacturer.

The quality of the RNA was determined by the use of the OneStep RT-PCR Kit (Qiagen, Hilden, Germany), which amplifies the cDNA using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers. The GAPDH-PCR products were then analysed on a 2% agarose gel. To exclude false positive results generated by amplification of genomic DNA sequences that were not totally eliminated by DNA digestion, purified RNA from each specimen was tested for the amplification of genomic GAPDH using the specific primers for PCR. The GAPDH-PCR products were analysed on a 2% agarose gel. Only the RNA isolations showing the specific amplification product in RT-PCR and no visible band for genomic amplifications were analysed for the subsequent procedures. All the incisional biopsies taken from patients were investigated histologically at the same place: the University of Erlangen's Department of Pathology.

Table 1. Histological results and MAGE-A expression analysis in normal oral mucosa, two patients with recurrence of oral squamous cell carcinoma and one of carcinoma *in situ*

Patient	Primary recurrence	Region	MAGE-A expression				
			A3	A4	A6	A10	A12
10 volunteers	Negative control	Buccal mucosa	-	-	-	-	-
Patient 1	OSCC: T4-N0-MX-G2-clinical stage IV	Right root of tongue	+	-	+	-	-
Patient 2	OSCC: T1-N0-MX-G2-clinical stage I	Right floor of mouth	+	+	-	-	+
Patient 3	CIS	Left retromolar area	+	+	+	-	+

OSCC, oral squamous cell carcinoma; CIS, carcinoma *in situ*.

Reverse transcription and real time PCR

The cDNA from the RNA was synthesized using the High-Capacity cDNA Archive Kit (Cat. 4322171; Applied Biosystem, Carlsbad, CA, USA) according to the manufacturer's protocol. A real-time RT-PCR analysis was performed using a QuantiTect Primer Assay kit (Qiagen; (Hs_QT01841224 QuantiTect Primer Assay) for MAGE-A3 and (Hs_QT01841225 QuantiTect Primer Assay) for MAGE-A4). For normalization, GAPDH was used (Hs_GAPDH_QT000792471 QuantiTect Primer Assay). The Detection of mRNA was performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The QuantiTect TM SYBR® green PCR kit (Cat. 204143; Qiagen) was used for PCR amplification. In brief: 50 ng of cDNA was used for each PCR reaction out of a total volume of 25 µl. The cycling conditions used for real-time PCR were as follows: the initial denaturation/enzyme activation took 15 minutes at 95 °C followed by 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds and elongation at 72 °C for 34 seconds. The production of a single product was confirmed by the use of the melting curve analysis. A Ct value of less than 33 was recommended by the manufacturer as positive for the expression of the target gene. All reactions were run in triplicate and verified by a second analysis. Results are displayed in Figure 1. The formation of undesired side products during PCR that contribute to fluorescence was assessed by the use of the melting curve analysis after PCR.

Results

In this pilot study, there was a total of three patients, one male and two female, who displayed a recurrence

following previous treatment for OSCC. The mean age of the patients was 60.3 years (range 49–70 years). All the previous OSCCs were located in the floor of the mouth and the primary recurrences were around the area of previous excision. The expression of MAGE-A3, A4, A6, A10 and A12 was restricted to malignant specimens. The expression of MAGE-A genes was positive for MAGE-A3 in all three cases, in contrast to A10. However, there was one negative expression among the three cases for the expression of MAGE-A4, A6 and A12. The location of the recurrences, the expression analysis for the five MAGE-A genes, tumour classification and the staging, grading and lymph node status of the patients are all displayed in Table 1. All the samples taken from the healthy volunteers were negative for the expression of MAGE-A3, A4, A6, A10 and A12.

Discussion

The 5-year survival rate for patients with OSCC remains 50%.² The main reason for the lack of an increase in the survival rate could be the late diagnosis of the primary and/or recurrences, or occult cancer cells remaining around the excised tumour margins. The prognosis for early-treated OSCC patients is always better than for those in an advanced stage and the 5-year survival rate can rise to 80% as curative measures are less complex and aggressive.¹¹ Therefore, risk prediction for cases with a probability of high recurrence is of major importance.

There are certain risk factors known to trigger malignant transformation such as tobacco and alcohol abuse. In such patients, identification or intervention at the early premalignant stage can help to decrease the rate of mortality and morbidity and consequently the cost of treatment associated with OSCC, while

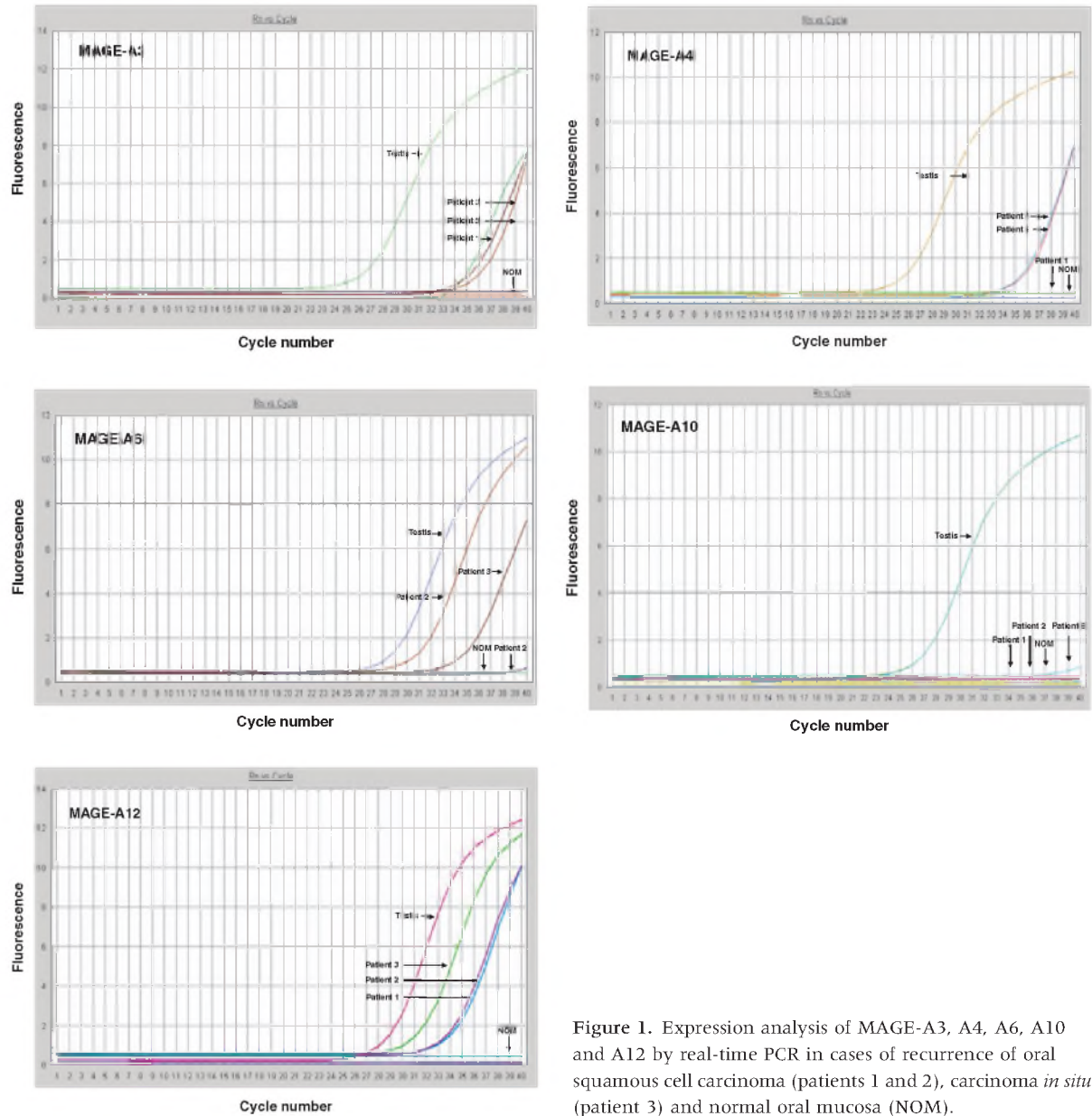


Figure 1. Expression analysis of MAGE-A3, A4, A6, A10 and A12 by real-time PCR in cases of recurrence of oral squamous cell carcinoma (patients 1 and 2), carcinoma *in situ* (patient 3) and normal oral mucosa (NOM).

increasing the quality of life for the patients. Exfoliative cytology is a technique that can be used to sample suspicious mucosal lesions in such patients. The technique has the advantage of being minimally invasive and relatively painless, and allows multiple sampling from different parts of the lesion without the need for a local anaesthetic. However, the technique needs professional training as cell samples should be collected vigorously from the full thickness of squamous epithelium of the oral mucosa. The dysplastic stage of OSCC is first identified in the basal

epithelial layer, and the diagnostic morphological findings may be lost when the cells reach the squamous layer as they mature and produce parakeratin and keratin. Thus, when harvesting cells it is important to collect cells from different layers of the epithelium in order to identify early malignant transformation. In the present study, there were three cases of recurrence, one classified as a tumour in an advanced stage (T4), one classified as a tumour in the initial stage (T1) and the remaining one classified as CIS. These last two cases may indicate that

harvesting cells from different layers is possible using a cytobrush.

Molecular markers have lately become essential in the diagnosis and management of patients with OSCC. Combining information from MAGE-A gene expression on exfoliated cell samples may help to overcome some of the current limitations of exfoliative cytology, such as false negative and false positive results. In this way, diagnosis and staging will be possible before changes in cell morphology occur or become clinically visible.⁵ Therefore, in this study we entirely focused on molecular analysis of the cell samples collected by cytobrush. However, the precise regulatory mechanism of the MAGE-A expression is still not fully understood, but it is already known that there is a link with overall DNA demethylation, especially in the promoter regions.¹² Demethylation is accepted to be an early step in oral carcinogenesis. It causes the early reactivation and the transcription of MAGE-A genes, which may be used to pinpoint the malignant transformation. As a result, running a curve analysis of a MAGE-A expression can be a useful tool for the early diagnosis of malignant transformation. Once the expression of MAGE-A is completed, its expression is retained during tumour progression and will increase along with the progression of malignancy.

The expression of the MAGE-A gene family is known to be restricted to tumours. Specifically, MAGE-A3, A4, A6, A10 and A12 were expressed but not all genes were expressed in each case of OSCC.^{13,14} Therefore, to increase the rate of accuracy of the expression analysis of the MAGE-A gene family, multiple gene expression analysis was applied to the cell samples and it was determined that, except for MAGE-A10, two to four of them were expressed in each of the three cases of recurrence, indicating the possibility of a malignant turnover (Table 1). In addition, except for MAGE-A10, the rest of the four MAGE-A genes were expressed in the CIS case, which is considered to be an early indication of malignant transformation in OSCC.

In conclusion, a non-invasive repeatable technique was established, using MAGE-A3, A4, A6 and A12 mRNA expression analysis applied on cell samples collected by cytobrushes, and found to be efficient in carrying out a risk assessment in OSCC cases that had already been excised and were being closely followed-up. It enables the identification of possible recurrence in order to increase the patients' survival rate and quality of life.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft (DFG)-The Scientific and Technological Research Council of Turkey (TÜBİTAK). In addition, the authors would like to thank Ms A. Krautheim-Zenk for her valuable technical support.

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