Diagnostic and Therapeutic Relevance of NY-ESO-1 Expression in Oral Squamous Cell Carcinoma

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Abstract. *Background: Cancer/testis antigen 1B (NY-ESO-1) is exclusively expressed in various types of tumor but not in healthy normal tissue, except testis, and induces strong cellular and humoral immune responses. Therefore, it represents an ideal target for diagnostic and immunotherapeutic applications. The aim of the study was to investigate the expression of NY-ESO-1 in oral squamous cell carcinoma (OSCC) to determine its impact as a diagnostic parameter or a therapeutic target for oral cancer. Patients and Methods: A total of 65 OSCC and 20 normal oral mucosal samples of otherwise healthy volunteers were included in this study. Expression of NY-ESO-1 was determined using reverse transcriptase polymerase chain reaction (RT-PCR). The results were correlated to diagnosis and clinicopathological parameters. Results: NY-ESO-1 was expressed in 27.7% of the investigated tumor samples, but not in normal oral mucosal. The correlation between NY-ESO-1 expression and malignancy was significant (p=0.008). The prevalence of NY-ESO-1 expression was significantly associated with tumor size (p=0.033), but not with histological grading, positive lymph node status or clinical stage of disease. Conclusion: NY-ESO-1 expression is restricted to OSCC, clearly indicating malignancy. However, the expression rate of this antigen is too low for clinical application but it might be a useful additional biomarker within a multiple marker system for the diagnosis of OSCC. In addition, NY-ESO-1 might be a candidate for immunotherapy and polyvaccination in patients suffering from OSCC.*

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Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide, accounting for 3-5% of all malignancies. Although early-stage OSCC is often curable, the prognosis for advanced cases generally remains poor (1, 2). OSCC has been known to exhibit field cancerization that may cause second primary tumor and recurrences in 30% of the patients who underwent surgery (3). The local recurrence of OSSC may be seen due to the existence of occult cancer cells in tumor margins. In such cases, early detection and/or treatment can significantly improve the survival rate. Therefore, the estimation of recurrence or the detection of premalignant stage of a second primary tumor using high specific tumor-associated markers to differentiate malignant from benign tissue might be of importance.

Despite the diagnostic and therapeutic advances in combined therapy, the prognosis of OSCC still remains 50%-60% in five-year survival analysis and has not changed significantly during the last three decades (2). Therefore, new therapeutic strategies are being actively pursued. The most popular ones are immunotherapeutic approaches employing the immune system by tumor-associated antigens recognized by cellular or humoral effectors of the immune system (4-6). Among tumor antigens, those such as NY-ESO-1 have received particular attention as diagnostic markers and potential targets for vaccine-based immunotherapy in cancer because of their unique expression pattern (5, 6). While gene expression profile of melanoma associated antigen family-A (MAGE-A) has already been well investigated in OSCC (7-9), the expression profile of Cancer/testis antigen 1B (NY-ESO-1) in lesions of oral cavity has not been evaluated yet.

NY-ESO-1 is a 22-kDa cytoplasmic protein encoded on chromosome Xq28. It was first identified in esophageal SCC. Its expression has also been determined in melanoma, breast, bladder, lung and prostate cancer (5, 10). However, NY-ESO-1 is absent from healthy normal adult tissues, except the germ cells of adult testis (10, 11). Furthermore, NY-ESO-1 is one of the most immunogenic proteins described in human cancer which can induce simultaneous antibody and CD8+

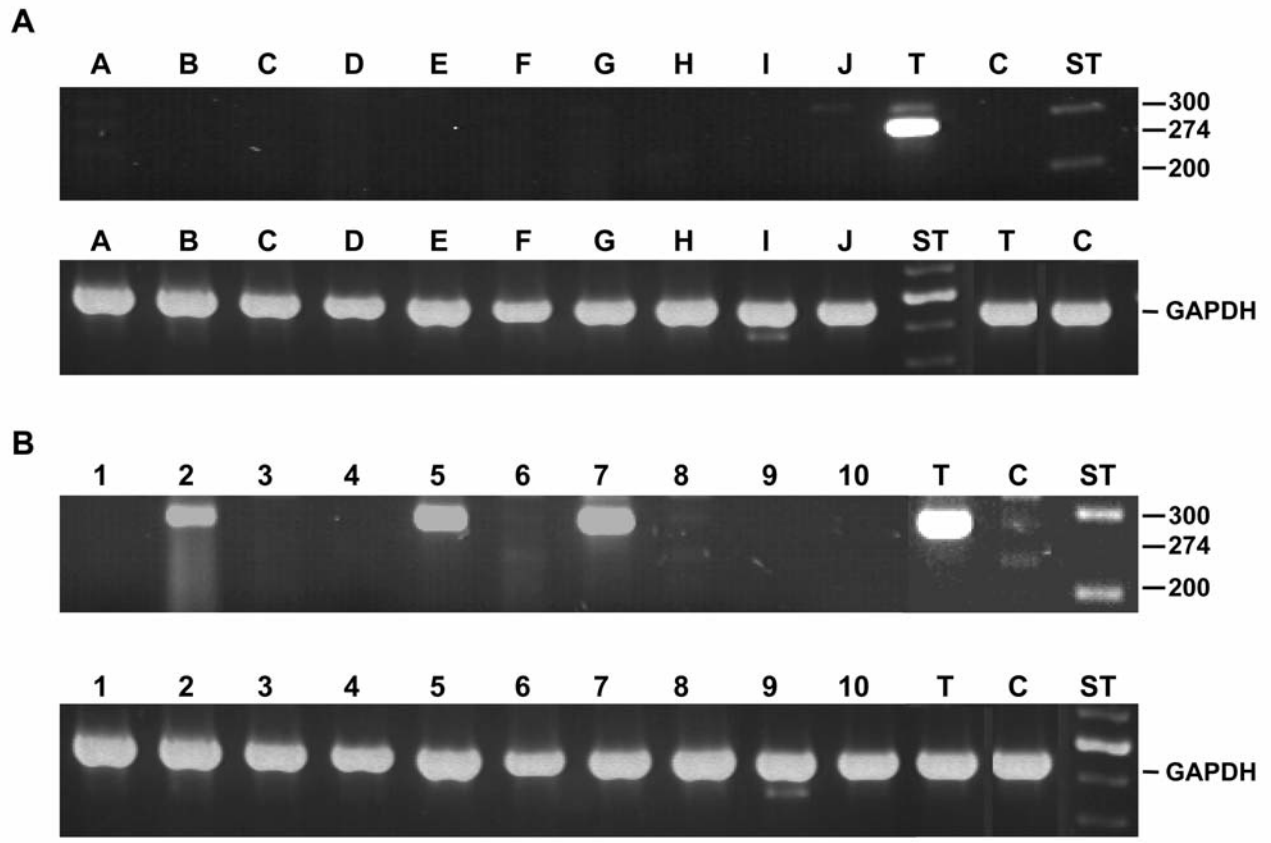

Figure 1. *Expression analysis of NY-ESO-1 and GAPDH by RT-PCR. A: Expression in normal tissue (samples A-J were obtained from otherwise healthy volunteers), B: expression in OSCC (samples 1-12 were obtained from patients with OSCC). C, Colon tissue; T, testis tissue; ST, molecular weight standard.*

T-cell responses *in vivo* (12, 13). In addition, human leukocyte antigen (HLA) class II restricted epitopes of NY-ESO-1 have also been identified, and the first studies on the status of natural $CD4^+$ T-cell responses in cancer patients have already been carried out (5, 12, 14). Due to these immunogenetic features, NY-ESO-1 might be considered as a target for immunotherapy in OSCC. Moreover, like the members of MAGE-A family, it may also have a great impact in the early diagnosis of OSCC via the detection of residual, occult cancer cells due to its gene expression being restricted to malignant tissue. Therefore, the aim of this study was to investigate the expression rate of NY-ESO-1 (CTAG1B) in OSCC using reverse transcriptase-polymerase chain reaction (RT-PCR) to determine its possible diagnostic and therapeutic relevance.

Patients and Methods

Patients and tissues. A total of 65 tumor tissue samples obtained from patients suffering from primary OSCC and 20 normal oral mucosal tissues of otherwise healthy volunteers were included in this study following the approval by the Ethics Committee of University of Erlangen, Germany. All the OSCC samples were obtained from surgical specimens removed during tumor excision. Patients had received neither radiotherapy nor chemotherapy prior to biopsy or tumor resection. Collection and storage of the tissue samples were carried out carefully in the same manner. Each sample was divided into two pieces. One piece was used for histological examination; the second piece was immediately snap frozen and stored at –80˚C until the molecular examination. Patients were followed up clinically at regular intervals after tumor excision. In earlier work, the expression pattern of 10 MAGE-A genes were already examined in all samples. For the RT-PCR analysis, RNA extracted from normal colon (C) and testis (T) were used as negative and positive control respectively (Figure 1).

RT-PCR. Total RNA from frozen tissues was isolated using RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A total of 200 ng RNA was used for the detection of NY-ESO-1 expression. Reverse transcription and the PCR reaction were carried out using One Step RT-PCR Kit (Qiagen) and NY-ESO-1-specific primers (sense: 5'-CCCCACCGCTTCCCGTG-3', antisense: 5'-CTGGCCACTCGTGCTGGG-3'). Cycling conditions were as follows. Reverse transcription was carried out at 50˚C for 30 min. Initial PCR activation was performed at 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 s, 58˚C for 45 s, and 72˚C for 60

s. The final extension incubation was performed at 72˚C for 10 min. Polymerase PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The size of the fragment amplified by RT-PCR was 274 bp (Figure 1).

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 reaction and analysing the GAPDH-PCR products on a 1.5% agarose gel (Figure 1). To exclude false-positive results generated by amplification of genomic DNA sequences that were not totally eliminated by DNAse digestion, 1 μl of purified RNA from each specimen were tested for amplification of genomic GAPDH using the specific primers for PCR. Only RNA isolations showing no visible band in 1.5% agarose gel were analyzed for subsequent procedures. Each RT-PCR experiment was performed in triplicate using the same RNA sample and the appropriate controls.

Histopathology and staging. All biopsies were evaluated by the same pathologist to ensure consistent results. Clinical staging and TNM classification were carried out for each tumor patient according to International Union Against Cancer (15). The OSCC were also characterized according to the World Health Organisation for loss of differentiation (G1, G2 and G3 for well, moderately and poorly differentiated tumors, respectively). Additional information including age, gender and clinical stage (I–IV) was also noted. In addition, tumors were also grouped as early (including stage I and II) and late (including stage III and IV) clinical stages. Furthermore, the state of lymph nodes was grouped as N=0 and N>0 in order to indicate cases with negative and positive lymph nodes, respectively.

Statistical analysis. Statistical analysis was performed using the statistical software package SPSS 16 (SPSS, Chicago, USA). Association between NY-ESO-1 expression and clinicopathological parameters, in addition, the prevalence of NY-ESO-1 mRNA expression in cancer and normal tissue samples were tested using χ^2 test after Pearson. A *p*-value less than 0.05 was considered as an indication of a statistical significance.

Results

In this study, 10 out of 65 OSCC patients were female and 55 of them were male, with a mean age of 59.5 years (min: 34 and max: 91). NY-ESO-1 expression was restricted to malignancy (Figure 1). The expression rate was 27.7%, and the correlation between malignancy and NY-ESO-1 detection was statistically significant (p=0.008) (Table I). The comparison with the expression rates of 7 particular MAGE-A which were previously determined in the same tissue samples, showed up that NY-ES0-1 expression is lowest within these genes. However detection of gene expression was increased by the analysis of multiple antigens. Ninety-three percent of all investigated tumor samples expressed at least one of these antigens (Figure 2).

Tumor classification, staging, grading and lymph node status of the 65 specimens from OSCC are displayed in Table I. Among the 65 tumor cases studied, three had histological characteristics of well-differentiated tumor, 42 were moderately differentiated, 17 were poorly differentiated and three of them could not be classified. No statistical correlation was observed between gene expression and grading (p=0.568).

Table I. *Association between expression of NY-ESO-1 and clinicohistopathological parameters.*

	No. of cases	+	–	% Positive cases	*p*-Value
Diagnosis[1]					
OSCC	65	18	47	27.7	0.008
Normal mucosa	20	0	20	0	
Differentiation					
No. of valid cases	62	17	45	27.4	
G1	3	1	2	33.3	0.568
G2	42	13	29	31.0	
G3	17	3	14	17.6	
Tumor size (T)					
No. of valid cases	61	17	44	28	
1	19	1	18	5.3	0.033
2	12	6	6	50.0	
3	5	1	4	20.0	
4	25	9	11	36.0	
Lymph node status (N)					
No. of valid cases	58	17	41	29.3	
Nx[2]	3	1	2	33.3	0.456
0	25	6	19	24.0	
1	10	5	5	50.0	
2[3]	20	5	10	25.0	
N0	25	6	19	24.0	0.742
N>0	30	10	20	33.3	
Stage					
No. of valid cases	61	17	44	27.9	
Early (I,II)	24	4	20	16.7	0.116
Late (III,IV)	37	13	24	35.1	
I	16	1	15	6.3	0.155
II	8	3	5	37.5	
III	7	2	5	28.6	
IV	30	11	19	36.7	

[1]Analyzed by Fisher's exact test. The rest have been analysed by Chi-square test. [2]Lymph node not classified. [3]Includes stages 2a, 2b and 2c.

Clinical staging of OSCC and the expression of NY-ESO-1 were as follows: 36.7% of the cases expressed NY-ESO-1 in stage IV, 28.6% in stage III, 37.5% in stage II and 6.3% in stage I OSCC samples. Although the expression rate was highest in stage II, there was no statistical significance between the expression of NY-ESO-1 and staging when stages were taken separately (p=0.155) or grouped as early (I+II) and late (III+IV) stages (p=0.116). In addition, there was no significant correlation between the gene expression and lymph node status when N0 was compared with N1 and N2 separately (p=0.456) or with N1+N2 together as a group (p=0.742). However, there was a statistically significant association between NY-ESO-1 expression and tumor size (p=0.033; Table I).

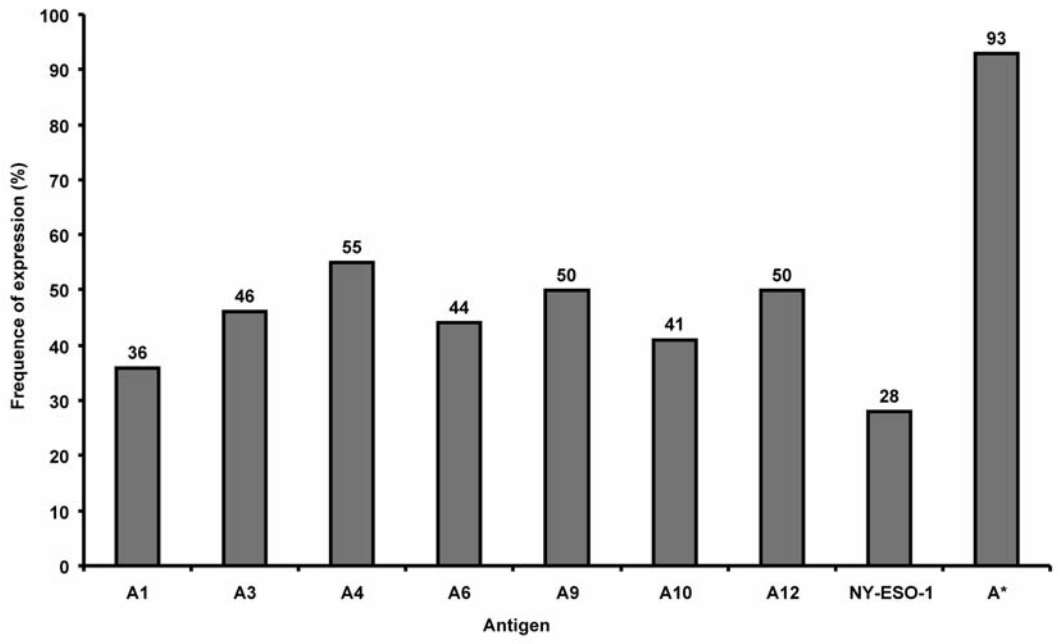

Figure 2. *Expression frequencies of MAGE-A and NY-ESO-1 in OSCC. *Expression of at least one antigen.*

Discussion

The poor prognosis of OSCC patients has hardly been improved even by the recent advances in surgical resection, radiation and chemotherapy. This might be because of the failure in early diagnosis and early detection of recurrences (16). Almost one third of all patients develop a recurrence, even following a complete resection, due to local residual or systemic disseminated tumor cells (17). Therefore, there is an urgent need for suitable biomarkers to be used in diagnostic application. Furthermore, additional effective treatment methods are required to improve the survival rate. Nowadays, many of the new therapeutic strategies are focusing on the immunotherapeutic approach.

Form the diagnostic and immunotherapeutic point of view, the appropriate tumor antigen candidate should be one that is only expressed by OSCC and not by normal tissue. This can allow for an exact distinction between normal and malignant tissue. In addition, it can also allow for high specific elimination of tumor cells without attacking and damaging the normal cells. Cancer/testis antigens represent a group of proteins which meet these criteria. They are expressed in tumors as well as in germ cells, but not in normal tissues (4).

NY-ESO-1 is one of the antigens that are expressed in wide variety of tumor types, but not in normal adult tissues (10). It was expressed in 32% to 33% of esopharyngeal carcinomas, 24% to 44% of melanomas and 17% to 20% of lung, 10% to 30% of breast, 25% of prostate, 32% of urinary tract, 25% of ovarian, 43% of epithelial ovarian and 6% to 7% of head and neck cancer cases (11, 12, 18-24). In the present study, NY-ESO-1 was expressed in 27.7% of all the examined OSCC cases. The expression rate of NY-ESO-1 is not as high as expected and hence could not be used as a diagnostic biological marker. There was a similar circumstance for members of the MAGE-A family as well. Single gene expression frequency of MAGE-A ranged between 10% and 55% in OSCC. Thus, expression analysis of a single gene was also not sufficient for clinical application. However, multiple MAGE-A expression analysis was found to be more sensitive than the single MAGE-A expression for the diagnostic evaluation in OSCC and it is already postulated that analysis of multiple MAGE-A expression can increase the detection sensitivity of OSCC cells (9). By adding investigation of NY-ESO-1 expression to the analysis of multiple antigens the expression rate of at least one antigen in the tumor tissues increased up to 93%. Therefore, it is assumed that expression analysis of NY-ESO-1 may be a useful additional biomarker to be employed within the multiple analysis of biomarkers, especially MAGE-A, to increase the sensitivity of tumor cell detection and to detect the rare neoplastic cells which are undetectable under the microscope. It may also be conceivable that determination of NY-ESO-1 expression around tumor margins may allow detection of residual, occult malignant cells following tumor excision. Thereby, prediction of the occurrence for locoregional recurrence may also be possible.

Due to their restricted expression cancer/testis antigens appear to be ideal targets for immunotherapy. In contrast to many other such antigens, NY-ESO-1 displayed even stronger immunogenicity. Immune responses to NY-ESO-1 have been more frequently observed than to other cancer/testis antigens. Inductions of immune responses which are humoral and cytological were seen in more than 50% of patients with different types of cancer (5, 11, 12, 19). Naturally occurring $CD8^+$ T-cell response to NY-ESO-1 has already been determined in melanoma and oesophageal carcinoma (19, 25). Hence, NY-ESO-1 has been regarded as one of the most immunogenic molecules discovered yet. Additionally, NY-ESO-1-expressing testis germ cells are not affected by immune response because they can be considered as an immunological privileged site due to blood/testis barrier and the lack of major histocompatibility complex (MHC)I/II expression. As a consequence of its special characteristics, NY-ESO-1 has made one of the fastest transitions from molecular, cellular, and immunological description to vaccine and immunotherapy. Candidates for the immunotherapeutic vaccine have already been tested in various formulations in more than 30 clinical trials worldwide. Results elicited spontaneous antibody and T-cell responses in number of cancer patients when vaccination used NY-ESO-1 peptide or the protein (12, 13, 20, 22-24, 26-29). This indicates that NY-ESO-1 is one of the most promising candidates for the vaccination of cancer patients (12, 13).

Immunotherapeutic vaccines may also be applied to patients with OSCC. Thus, patients who suffer from OSCC which display NY-ESO-1 expression can profit from immunotherapeutic strategies based on this antigen as a target. In addition, the gene may be one of the candidates for future polyvaccines. Polyvalent vaccinations using multiple immunogens is important for immunotherapeutic strategies because it increases the probability of inducing a specific immune response and reduces the risk of tumor escape from the immune system by selection of antigen-loss variants. However, immunogenicity of this antigen has not yet been elucidated for OSCC and needs further investigation.

Previous research has already reported that patients with NY-ESO-1 antigen expression and antibody response tend to have advanced-stage cancer. For example, a higher frequency of NY-ESO-1 expression in bladder cancer was correlated to tumor grading (30) and occurrence of early recurrence (31). Additionally NY-ESO-1 antibody response has also been correlated with advanced-stage melanoma and ovarian cancer (21, 22, 32). Moreover, it was also claimed that patients with metastatic melanoma who express NY-ESO-1 may benefit from NY-ESO-1-based immunotherapy (32). In the present study, although there was a lack of correlation between NY-ESO-1 antigen expression and clinicopathological characteristics of OSCC, such as histological grading, lymph node status and clinical staging, there was a significant correlation with tumor size. This finding may suggest that the expression of NY-ESO-1 also correlates with advanced-stage OSCC. In conclusion, NY-ESO-1 may be an useful additional biomarker for the diagnosis of OSCC within multiple marker analysis, particularly MAGE-A. In addition, it may also be used as a target for immunotherapy, particularly as an additional target for polyvaccination in OSCC.

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