ORIGINAL ARTICLE

PROGNOSTIC VALUE OF MAGE-A AND NY-ESO-1 EXPRESSION IN PHARYNGEAL CANCER

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Abstract: *Background.* The prognostic value of cancer testis antigens in pharyngeal cancer is understudied.

Methods. We recruited 90 patients who were treated for pharyngeal cancer. Monoclonal antibodies 57B and B9.8.1.1 were used for detection of MAGE-A and NY-ESO-1 genes.

Results. MAGE-A and NY-ESO-1 gene products were detectable in 70.0% and 33.3% of pharyngeal tumors, respectively. No correlation was established between MAGE-A and NY-ESO-1 expression and TNM staging at presentation. Survival analysis showed a trend toward a shorter 5-year disease-free survival in the group of patients with MAGE-A–positive tumors (log-rank test, $p = .122$). In contrast, a trend toward a prolonged 5-year disease-free survival was observed in the group of patients with NY-ESO-1–positive tumors (log-rank test, $p = .219$).

Conclusion. In a large population of patients with pharyngeal cancer and available 5-year survival data, prognosis tended to be poorer with MAGE-A expression and better with NY-ESO-1 expression, but the correlations did not reach statistical significance. © 2009 Wiley Periodicals, Inc. *Head Neck* **32:** 1178–1184, 2010

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Age standardized world incidence rates for pharyngeal cancer range from 0.1 per 100,000 men in China to 10.5 per 100,000 men in France.[1] The overall survival rate for patients with pharyngeal squamous cell cancer (SCC) is low and has not markedly improved in the last few decades.[2–5] While histologic grade has some value in predicting the course of disease,[6] the major independent prognostic factor is the presence of regional lymph node metastases.[7] A high proportion of patients with pharyngeal cancer has regional lymph node metastases at presentation,

without clear correlation between tumor size and the presence of regional nodal disease.[7,8]

Identification of tumor associated antigens (TAAs) has opened new possibilities for the development of targeted cancer immunotherapy.[9–13] Melanoma antigen gene (MAGE)-A and NY-ESO-1 genes belong to the cancer testis antigen (CTA) family, which are expressed in normal spermatogonia and placental cells and in tumors of diverse histologic origin.[14] The T lymphocytes have been shown to be capable of HLA class I and II restricted recognition of CTA derived epitopes expressed on cancer cells.[13,15–19] Furthermore, NY-ESO-1 and MAGE-A gene products may induce spontaneous humoral responses.[10,11,18,20]

MAGE-A antigen is expressed in more than 60% of cutaneous melanomas and in a high proportion of head and neck cancers, lung cancers, synovial sarcomas, urinary bladder carcinomas, and seminomas.[15,21–23] Notably, expression of MAGE-A antigens has been shown to correlate with poor histologic differentiation in melanoma and breast cancer,[22,24] with severe prognosis in urinary bladder carcinoma,[25] and in SCC of the lung.[26]

Only a few previous studies with small sample sizes have analyzed specific gene expression in head and neck squamous cell cancer (HNSCC). These studies show that MAGE-A expression is present in a high proportion of HNSCC but has no clear correlation with the prognosis.[27] MAGE-A gene product was detectable in 43% to 45% of tumors in 3 studies that together included 178 patients[27–29] and in 72% of participants in 1 study that included 51 patients.[30] Eura et al[28] showed that the distribution of MAGE-A expression depended on localization of the primary cancer and histologic grade. No correlation was found with clinical stage of the disease and development of metastases.[27,29]

Although initially discovered in squamous cell esophageal cancer, NY-ESO-1 shows a low expression in HNSCC.[27] Kienstra et al[27] used the real-time reverse transcriptase polymerase chain reaction (RT-PCR) method to analyze 45 HNSCCs, and only 3 showed NY-ESO-1 expression. In that study, patients with NY-ESO-1–positive tumors presented at an advanced stage of the disease with positive cervical lymph nodes and with histologically low-grade tumors. Atanackovic et al[30] detected NY-ESO-1 expression in 3 of 51 analyzed HNSCCs, with positive antibodies in the patients' sera. Only 1 previous study used immunohistochemistry to detect CTA gene products in HNSCC, but due to the low number of cases, no correlation with clinical data could be postulated.[29]

We investigated possible correlations between expression of CTA at the protein level and prognosis in a relatively large group of patients with pharyngeal SCC, in order to assess the prognostic potential of the CTA in these tumors.

PATIENTS AND METHODS

We retrospectively studied case notes for 90 patients with pharyngeal SCC treated at the Department of Head and Neck Surgery of the University Hospital for Tumors between 1996 and 1999. Data were collected on the TNM stage at the time of diagnosis, treatment, follow-up, and survival. Follow-up for disease-free patients was 5 years.

Tumor specimens were fixed in 10% buffered formalin, embedded in paraffin, cut, and stained with hematoxylin-eosin. Revision of histologic diagnosis and histologic grade was performed. Specific serologic reagents have been developed to assess distribution of CTAs within clinical tumor samples.[23] The 57B monoclonal antibody (mAb) was used in the form of undiluted hybridoma supernatant for the detection of multiple MAGE-A gene products (G. S., University of Basel, Switzerland).[31,32] The B9.8.1.1 monoclonal antibody was used for NY-ESO-1 detection.[33] Immunohistochemical staining was performed by automated DAKO TechMate Horizon immunostainer according to the standardized protocol. Primary antibodies were incubated at +4°C during the night for MAGE-A and for 1 hour for NY-ESO-1.

A semiquantitative scoring method was used to evaluate immunohistochemical staining: negative reaction (-): no staining in tumor cells; weakly positive reaction (+): up to 10% of tumor cells show positive reaction; moderately positive reaction (++): 10% to 50% of tumor cells with positive reaction; strongly positive reaction (+++): more than 50% of tumor cells with positive reaction. The immunohistochemical reaction was considered positive when staining of the cytoplasm occurred.

Only tumors with more than 10% of immunohistochemically positive cells might be considered candidates for immunotherapy. When assessing

the correlation between the expression of analyzed genes and the patient and tumor characteristics, we, therefore, grouped the results of immunohistochemical data as negative and positive. Tumors with negative (-), weakly positive (+), and focally weakly positive (focally +) immunohistochemical reaction were considered negative, and tumors with moderately positive (++), strongly positive (+++), and focally positive (focally ++/+++) immunohistochemical reaction were considered positive.

Patients demographics (age, sex), tumor (localization, TNM stage) data, and treatment (surgery, radiotherapy) data were collected from patient charts. Five-year survival data were collected from the Croatian National Cancer Registry.

Statistical Analysis. Descriptive statistical methods were used to describe the study population. Chi-square test was used to test for the differences between the groups, except in small observed frequencies when Yates corrected chi-square test or Fischer exact test were used. Survival analysis was performed using the Kaplan–Meier method, and differences were assessed with the log-rank test. Statistical significance was set at $p < .05$. Statistica 7.0 was used for all analyses.

RESULTS

Patient Demographics and Clinicopathologic Characteristics of the Tumors. Among 90 patients (5 women and 85 men; mean age 58.8 ± 16.6 years; range age, 39–75 years; median age, 58 years), 46 patients (51.1%) presented with oropharyngeal and 44 patients (48.9%) with hypopharyngeal tumors. We found no differences at baseline between the groups of patients with oropharyngeal and hypopharyngeal tumors according to T classification, N classification, American Joint Committee on Cancer (AJCC) stage, and pathohystologic grade.

The distribution by T classification was 34 (37.8%) small (T1–T2) tumors and 56 (62.2%) locally advanced (T3–T4) tumors. Furthermore, there were 23 (25.6%) N0 patients and 67 patients (74.4%) N+ with metastatic neck lymph nodes. We found no statistically significant difference in the frequency of neck metastasis between early and locally advanced cancers (chi-square = 1.19; df = 1; $p = .275$). In terms of the distribution of AJCC stage (I–IV), we found 1 patient (1.1%) with stage I, 5 patients (5.5%) with stage II, 24 patients (26.7%) with stage III, and 60 patients (66.7%) with stage IV disease. The disease was diagnosed early in 6 patients (6.7%; stage I and II), and at an advanced stage in 84 patients (93.3%; stage III and IV). Regarding histologic grading, there were 17 patients (18.9%) with grade I, 40 patients (44.4%) with grade II, and 33 patients (36.7%) with grade III SCC. Five-year disease-specific survival was 30% for patients with oropharyngeal cancer and 39% for patients with hypopharyngeal cancer. Median survival was 25 months for patients with oropharyngeal and 29.5 months for patients with hypopharyngeal cancer.

Immunohistochemical Analysis. Examples of MAGE-A and NY-ESO-1 specific stainings are shown in Figures 1 and 2. We detected MAGE-A gene products in 63 patients (70.0%) with pharyngeal tumors and NY-ESO-1 in 30 tumors (33.3%; Table 1).

The distribution of MAGE-A expression was similar in all analyzed subgroups in terms of the size of the tumor (chi-square = 0.01; df = 1; $p = .924$), the presence of neck metastases (chi-square = 0.71; df = 1; $p = .399$), the AJCC stage of the disease at presentation (chi-square = 0.42; df = 1; $p = .519$), and the histologic grade (chi-square = 1.25; df = 2; $p = .536$). Similarly, no differences were seen in the distribution of NY-ESO-1 reactivity according to the size of the tumor (chi-square = 1.16; df = 1; $p = .282$) and

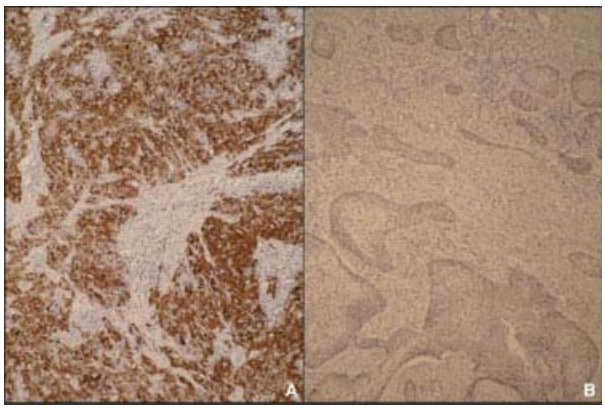

FIGURE 1. MAGE-A strongly positive immunohistochemical reaction (**A**) and a negative reaction (**B**) (original magnification ×100). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

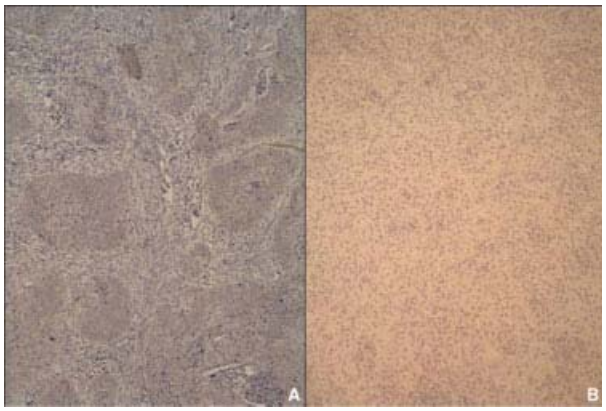

FIGURE 2. NY-ESO-1 weakly positive immunohistochemical reaction (**A**) and a negative reaction (**B**) (original magnification ×100). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the presence of neck metastases (chi-square = 0.47; df = 1; $p = .494$). However, a significantly smaller proportion of poorly differentiated tumors showed NY-ESO-1 expression ($p = .003$).

Our analysis of antigen expression showed that 22 tumors (24.4%) were negative for both antigens, 65 (72.2%) were positive for one antigen of which 60 (66.7%) for MAGE-A and 5 (5.6%) for NY-ESO-1, and 25 tumors (27.8%) were positive for both antigens.

Clinical Correlations. Survival analysis showed a trend toward a shorter 5-year disease-free survival (log-rank test, $p = .122$) in the group of patients with MAGE-A–positive tumors (Figure 3). In contrast, a trend toward improved 5-year disease-free survival (log-rank test, $p = .219$) was observed in the group of patients bearing NY-ESO-1 positive tumors (Figure 4).

Table 1. MAGE-A and NY-ESO-1 expression according to tumor characteristics.

	No. (%) by MAGE-A expression		No. (%) by NY-ESO-1 expression	
Tumor characteristics	Negative	Positive	Negative	Positive
T classification				
T1-T2	10 (29.4)	24 (70.6)	25 (73.5)	9 (26.5)
T3-T4	17 (30.4)	39 (69.6)	35 (62.5)	21 (37.5)
N classification				
N0	9 (39.1)	14 (60.9)	14 (60.9)	9 (39.1)
N+	18 (26.9)	49 (73.1)	46 (68.7)	21 (31.3)
AJCC stage				
I-II	3 (50.0)	3 (50.0)	4 (66.7)	2 (33.3)
III-IV	24 (28.6)	60 (71.4)	56 (66.7)	28 (33.3)
Histologic grade				
I	7 (41.2)	10 (58.8)	9 (52.9)	8 (47.1)
II	11 (27.5)	29 (72.5)	24 (60.0)	16 (40.0)
III	9 (27.3)	24 (72.7)	27 (81.8)	6 (18.2)
Total	27 (30.0)	63 (70.0)	60 (66.7)	30 (33.3)

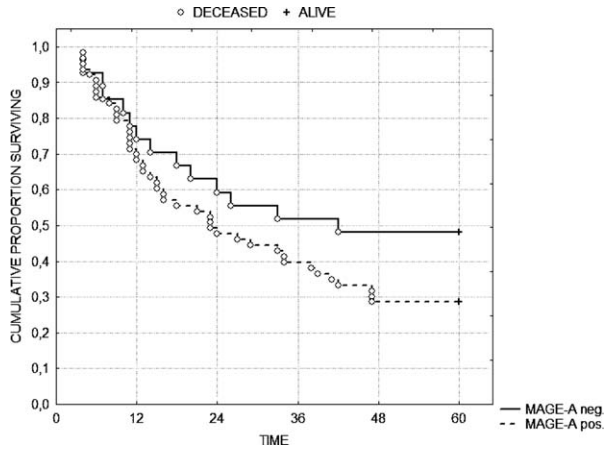

FIGURE 3. Five-year disease-free survival according to MAGE-A immunohistochemistry reaction (Kaplan–Meier method).

DISCUSSION

The prognosis for patients with HNSCC is generally poor, and treatment planning is based on the accepted prognostic factors. The tumor's aggressiveness often makes multimodal treatment mandatory. Insufficient progress in head and neck oncology brings out the importance of molecular genetic studies.[34] Redefining of the prognostic factors and treatment protocols based on detection of gene changes in tumor cells could guide contemporary treatment advancements.

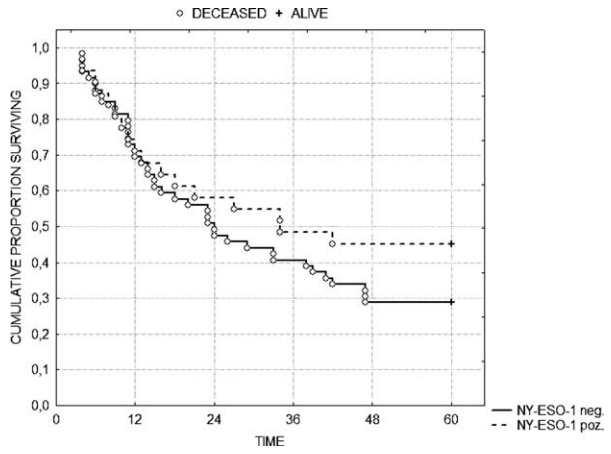

FIGURE 4. Five-year disease-free survival according to NY-ESO-1 immunohistochemistry reaction (Kaplan–Meier method).

CTAs have been shown to induce specific immune responses of potential clinical relevance.[10–12] Indeed, so far, clinical immunotherapy trials, mostly based on the administration of synthetic peptides in the presence or absence of GM-CSF or loaded on dendritic cells, have only met limited clinical efficacy,[35] possibly due to unfavorable tumor microenvironment and to the generation of regulatory T cells upon vaccination.[36] However, promising ongoing research focuses on the use of CTA vaccines in combination with mAbs blocking inhibitory circuits[37] or novel adjuvants, and as adjuvant treatment for minimal residual disease.[35] In addition, most interestingly, infusion of NY-ESO-1-specific autologous CD4+ T cells has recently been reported to result in durable remission in a patient with metastatic melanoma.[38]

Notably, CTA expression is considered to be an unfavorable prognostic marker, among others, in bladder cancers and SCC of the lung.[25,26] A number of studies have focused on CTA gene expression in HNSCC.[27–30] However, data on expression of specific gene products are scarce. Immunohistochemical analysis allows for a precise evaluation of the expression. This information could be of particular relevance for the planning of immunotherapeutic treatments in HNSCC. CTAs have shown promising characteristics for the development of immunotherapy, as target antigens for specific cytotoxic lymphocytes, which are induced by peptide or protein vaccines.[10,20,39]

Our study, to the best of our knowledge, is the largest to date to investigate MAGE-A and NY-ESO-1 expression in HNSCC. In previous studies, MAGE-A expression occurred in 43.4% to 72% of HNSCC.[27–30] In our study, MAGE-A was expressed in 70.0% of pharyngeal cancers. The majority of previous studies found no correlation between MAGE-A expression and clinical characteristics of the disease, histologic characteristics of the tumor, or survival,[27,29,30] and our study supports these findings. Different distribution, depending on the localization of tumors and histologic degree of differentiation, was described only by Eura et al.[28] MAGE-A tumor positivity has been shown to negatively correlate with the histologic grade in melanoma and breast cancer;[21,22,24] the expression is higher in advanced tumors and associated with a poor prognosis. However, no correlation with the histologic grade or stage of the disease has been established for pharyngeal cancer.

We found that MAGE-A expression in pharyngeal cancer was predictive of poor prognosis, although the correlation did not reach statistical significance. MAGE-A expression does not seem to be a powerful prognostic marker and does not seem to hold the potential for modifying treatment plans for patients with pharyngeal cancer. The product of MAGE-A gene belongs to tumor antigens, and the efficiency of immunotherapy in patients with MAGE-A–positive melanoma is currently being investigated. In this study, MAGE-A shows an overall high positivity and indicates the need to study the use of immunotherapy in MAGE-A–positive pharyngeal cancer.

In our study, NY-ESO-1 expression was present in 33.3% of analyzed tumors, which is markedly higher than previously reported (5.9% and 6.6%).[27,30] It occurred less frequently in poorly differentiated cancers and was predictive of a more favorable prognosis. Similar results have previously been shown for esophageal SCC.[40] The link between NY-ESO-1 expression and favorable prognosis is probably due to the patient's immunologic response. This result opens many questions and warrants further investigation.

Metastatic neck disease is common in pharyngeal tumors and is the most important prognostic factor.[6–8] It would have been interesting to investigate expression of the analyzed genes in the metastatic lymph nodes in our study; however, we were unable to perform such analyses. A comparison of the level of mRNA and protein for MAGE-A and NY-ESO-1 would have also been of interest; however, we have no access to fresh frozen materials and paraffin-embedded tissues in our hands do not represent a reliable source of total cellular RNA for RT-PCR.

CONCLUSIONS

In our sample of patients with pharyngeal SCC, MAGE-A protein expression was present in 70.0% of tumors and NY-ESO-1 was present in 33.3%. No correlation was established between MAGE-A or NY-ESO-1 expression and TNM staging at presentation. We found no correlation between MAGE-A protein expression and histologic grade, but NY-ESO-1 expression was less frequent in poorly differentiated tumors. No correlation was found between MAGE-A or NY-ESO-1 expression and metastatic potential and

biologic behavior of the tumor. We found a tendency toward a poorer prognosis in pharyngeal cancer patients with MAGE-A–positive tumors, compared with MAGE-A–negative tumors, and toward a better prognosis in patients with NY-ESO-1–positive tumors, compared with NY-ESO-1–negative tumors, although the results did not reach statistical significance. Immunotherapeutic potential of NY-ESO-1 expression is limited due to its overall low expression in HNSCC. However, a high proportion of MAGE-A antigen expression in HNSCC holds promise for including immunotherapy in future treatment protocols.

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