Expression of MAGE-A Cancer/Testis Antigens in Esophageal Squamous Cell Carcinomas

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Abstract. *Background: Although the diagnosis and therapy of esophageal cancer have improved over the past decade, the prognosis remains dismal. Since MAGE-A cancer/testis antigens (CTA) are potential targets for immunotherapy, this study was aimed at evaluating their expression in these patients and its prognostic value. Materials and Methods: Using 57B monoclonal antibody, MAGE-A CTA expression was analyzed in paraffin-embedded tumor specimens of 98 patients with esophageal squamous cell carcinoma or adenocarcinomas who had undergone surgical resection. For all patients, a postoperative follow-up of at least 4 years was available. The expression was quantified using a scoring system considering intensity and homogeneity of the immunostaining. The prognostic relevance of MAGE-A expression was analyzed in univariate analyses as well as Cox proportional hazard regression analysis. Results: 57B positivity could be detected in 38 tumors (38.8%). Positive staining was observed in five out of 32 adenocarcinomas (15.2%) and in 33 out of 66 (50%) squamous cell carcinomas. MAGE-A expression did not correlate with the TNM classification, grading or age of the patients. Both univariate (p=0.88) and multivariate analyses (p=0.82) revealed that MAGE-A expression lacked prognostic significance in esophageal carcinomas. Conclusion: MAGE-A was expressed in half of the squamous cell carcinomas of the esophagus, but rarely in adenocarcinomas. Although its immunodetection was insufficient for prognostic evaluation, the high expression rate suggests MAGE-A as a potential target for immunotherapy in the first group with the ability for pretherapeutic testing.*

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During the last decade, a worldwide increase in the incidence of esophageal cancer, mainly adenocarcinomas, has been observed (1). Although progress in the surgical, radiation and chemotherapeutical treatment of these patients has been achieved, the clinical outcome of patients with esophageal carcinoma is not satisfying. High percentages of patients are diagnosed in locally or systemically advanced tumor stages and curative treatment is usually not available for these patients. Therefore, multimodal strategies were developed combining pre- and postoperative therapy with local resection (2, 3). New concepts were introduced to supplement standard therapies and enable individualized therapeutic regimens. For example, immunotherapy is aimed at the activation of biological defense mechanisms against tumor cells (4).

Cancer/testis antigens (CTA), including the MAGE-, BAGE- and GAGE families among others, are a group of proteins characterized by a peculiar expression profile, mostly limited to male germinal cells and cancer cells of diverse histological origin (5). Since the cells that physiologically express these molecules lack the required HLA-molecules at their surface for immunological targeting, CTA appear to be ideal targets for immune therapy (6).

Various 'shared' MAGE-A antigens are frequently coexpressed in tumors, such as in melanomas, lung and bladder cancer (7-10). This coordinated expression of different MAGE-A antigens in tumors suggests a unique activation mechanism of their transcription, and promoter demethylation in tumor cells has been proposed to be involved in this process (11). For example, increasing demethylation in dedifferentiated tumors correlates with enhanced MAGE-A expression. Furthermore, the X-chromosome, where all MAGE-antigens are located, is specifically susceptible to impaired methylation. This genetic localization, however, does not result in gender-specific expression patterns of MAGE antigens in different

tumor types (12-14). Recently, a few studies with small numbers of cases demonstrated that various MAGE antigens are also expressed in esophageal squamous cell and adenocarcinomas (15-20). These expression rates, however, showed high variability in both histological entities. In adenocarcinomas, MAGE antigens were found in 15-37%, whereas squamous cell carcinomas expressed these antigens at higher frequency (50-84%). Only one of these expression studies included an analysis of the correlation between MAGE expression and patient survival, but this study did not find any prognostic relevance (20).

The relatively high specificity of MAGE-A expression in tumor cells qualifies these surface proteins as potential prognostic markers with surrogate function. Therefore, in this study the expression profile of MAGE-A CTA was investigated in a large number of patients with squamous cell carcinomas or adenocarcinomas of the esophagus. Furthermore, the correlation of its expression with tumor characteristics and its potential use as a prognostic marker were analyzed using univariate and multivariate approaches.

Materials and Methods

Patients. Using a retrospective approach, 98 patients with esophageal carcinomas, who had undergone surgical resection at the Department of General Surgery, University Hospital Münster, Germany, between 1993-1998, were evaluated. Demographic data, histological tumor classification (reclassified according to UICC 2002), tumor type and grading and treatment modalities were recorded. None of the patients received neo-adjuvant therapies. In all patients, the tumors were localized in the middle or distal part of the esophagus, but cardia carcinomas were excluded. Furthermore, follow-up data were available for at least 4 years after resection. The follow-up investigations had been performed according to the guidelines of the German Cancer Society.

MAGE-A CTA immunodetection. The 57B mAb was generated using recombinant MAGE-A3 molecules as the immunogen. However, studies performed in different laboratories have revealed that this reagent recognizes a wider range of MAGE-A gene products and can be considered as a multi-MAGE-A specific reagent (21). Formalin-fixed and paraffin-embedded tissue samples were deparaffinized, rehydrated and washed using phosphate-buffered saline (PBS) for 5 min. For antigen recovery, the sections were heated (90˚C) in citrate buffer for 90 sec. After washing (PBS), the sections were incubated with mouse anti-MAGE-A hybridoma (57B-AL, 1:100 dilution) (22, 23) at 4˚C overnight. Subsequently, the biotinylated secondary antibodies were incubated for 30 min. Immunostaining was performed using Vectastain ABC-reagent and DAB as the chromogen (Vector Laboratories Inc., Burlingame, CA, USA). Counterstaining was done with Mayer's Hemalaun. Testis was used as positive control, whereas negative controls were performed with unspecific IgG for primary incubation.

For the evaluation of the immunohistochemical staining, a scoring system was used considering homogeneity (points: 0 - negative; 1 - positive cells <25%; 2 - positive cells 25-50%; 3 - positive cells 50-75%; 4 - positive cells >75%) and intensity (points: 0 - negative; 1 - weak; 2 - intermediate; 3 - strong) of the color reaction. The intensity was determined by comparison with positive (for strong staining) and negative control samples and was evaluated using a graded system. The group assigned 3 - strong - showed equal staining intensity compared to the positive control, the group assigned 1 - weak - had decreased but clearly positive staining intensity and the group designed as 0 had no evidence of staining. Intermediate staining values (2 points) were assumed for intensities between strong and weak color reactions. Score values were obtained as the product of intensity and homogeneity. Maximal values of 12 points were available. All samples were scored by two independent investigators. In seven cases, different scoring values of intensity were obtained and the final evaluation was performed after re-evaluation. For prognostic evaluation, MAGE-A staining was dichotomized into MAGE-A-negative (score=0) and MAGE-A-positive (score >0) cells.

Statistical analysis. The primary statistical analysis tool used was the Cox proportional hazard regression (PHR) procedure (24) of the Statistical Analysis System (SAS, Cary, NC, USA). The Cox PHR procedure was used for univariate and multivariate analyses. The outcome variable was overall survival as measured from the day of surgery. The primary predictor variable (explanatory variable) of interest was the dichotomous MAGE result (positive/negative). Additional covariates available for analysis included: resection (R0 *versus* non-R0), histological type of tumor (adenocarcinoma *versus* squamous cell carcinoma), UICC stage, histological grading (1 to 3), type of treatment (surgery alone *versus* surgery plus adjuvant therapy) and age. The TNM classification was not used in our models as it would have resulted in multicolinearity with the UICC stage. The purpose of PHR modeling was to explore whether the covariates carried prognostic information relative to the outcome (overall survival). One objective of the present investigation was to assess whether MAGE-A carries additional prognostic information beyond that of the other covariates. PHR models of each covariate were estimated separately as a preliminary step (univariate analysis). To assess the risk-adjusted (independent) impact of the primary predictor variable (MAGE positive *versus* negative) on the outcome, the PHR models including all covariates were then computed. The level of statistical significance was 0.05. All tests were two-sided. Estimated distributions of time-to-event data were displayed as Kaplan-Meier plots.

Results

Tumor stage and survival rates. The mean age of the patients was 61.5±9.4 years (31-78 years). Twenty-two females (22.4%) and 76 males (77.6%) were included. In 32 cases, adenocarcinomas were found (32.6%), whereas 66 patients had squamous cell carcinomas (67.3%).

More than 70% of the patients were diagnosed in locally advanced tumor stages (T3/4) with lymph node involvement. In the earlier years of the study period, sufficient palliative treatment was not available and, therefore, 23 patients were resected as palliative treatment who had distant metastases at the time of resection. Differences in these characteristics were not found between adenocarcinomas and squamous cell

Table I. *Histopathological tumor parameters in patients with esophageal carcinomas.*

	Adenocarcinoma (n=32)	Squamous cell carcinoma (n=66)
Local invasion		
pT1	1 (3%)	1 (1,5%)
pT2	8 (25%)	14 (21%)
pT3	22 (69%)	48 (73%)
pT4	1 (3%)	3 (4.5%)
Lymph nodes		
pN0	7 (22%)	20 (30%)
pN1	25 (78%)	46 (70%)
Metastasis		
pM0	23 (72%)	52 (79%)
pM1	9 (28%)	14 (21%)

Table II. *Risk factors affecting overall survival rate in univariate analysis.*

	P-value	Hazard ratio	95% CI
Age (decades)	0.20	1.02	[0.99, 1.04]
Histological type (squamous *vs.* adeno)	0.74	0.92	[0.57, 1.50]
UICC stage (I-IV)	**0.0004**	**1.71**	**[1.27, 2.31]**
Grading (I, II, III)	0.77	1.07	[0.68, 1.69]
MAGE-A (pos. *vs.* neg.)	0.88	0.96	[0.59, 1.56]
Therapy (surgery plus adjuvant treatment *vs.* surgery alone)	**0.04**	**0.58**	**[0.35, 0.97]**

95% CI: 95% confidence interval.

Table III. *Estimates from the proportional hazard regression model of overall survival.*

	P-value	Hazard ratio (95% CI)
Age (decades)	0.04	1.03 [1.00, 1.06]
Histological type (squamous *vs.* adeno)	0.03	0.50 [0.28, 0.92]
UICC stage (I-IV)	<0.001	2.10 [1.49, 2.96]
Grading (I, II, III)	0.48	0.84 [0.51, 1.38]
MAGE-A (pos. *vs.* neg.)	0.82	1.07 [0.62, 1.84]
Therapy (surgery plus adjuvant treatment *vs.* surgery alone)	0.02	0.48 [0.26, 0.88]

95% CI: 95% confidence interval.

carcinomas (Table I). The histological differentiation was also comparable in both entities and more than 50% of the tumors showed poorly-differentiated patterns (G3/4). The median overall survival of all patients was 362 days (range: 38-1661 days) with 1-, 2- and 5-year survival rates of 51.1%, 29.7% and 6.1%, respectively. Patients with squamous cell carcinomas had significantly better survival rates (1-year: 51.1%; 2-year: 29.7%; 3-year: 21.6%) compared with adenocarcinoma patients (1-year: 41.1%; 2-year: 11.7%; 3-year: 11.7%) (Table II and III).

Expression of MAGE-A CTA. Using the 57B-AL hybridoma antibody, the expression of MAGE-A was observed with variance in intensity and homogeneity within the tumors and between different patients (Figure 1). In 38.8% of all 98 patients, MAGE-A expression was found. More than half (n=20; 52.6%) of these positive samples showed positive staining in 50-75% of the tumor and 21.1% (n=8) had >75% positive tumor cells. The non-homogeneous expression was not related to different cellular elements within the tumor environment. Combining this homogeneity with intensity staining, scores were obtained as shown in Figure 2. Approximately 40% of all positive tumors, but only two of the adenocarcinomas, reached score values of more than 6 points corresponding to high expression profiles. The staining intensity and homogeneity, as well as the resulting score values, did not show any correlation with other tumor characteristics (Figure 3c+d). Therefore, for further statistical analysis, 57B-AL positivity was dichotomized as MAGE-A-positive (any positive staining) or MAGE-A-negative (score value = 0).

Subsequently, the expression of MAGE-A antigens was compared with conventional tumor characteristics. The most obvious difference was found between the two different tumor types. Eighty-seven percent of all MAGE-A-positive samples were squamous cell carcinomas, representing 50% of these tumors. In contrast, only five out of 32 adenocarcinomas (15.2%) demonstrated positive staining (Figure 3a). Comparable differences, however, were not found for tumor staging, grading or UICC classification (Figure 2).

Results of univariate analyses and Cox proportional regression analyses. The results from PHR models of each separate covariate (univariate analysis) on overall survival are provided in Table II. Only the covariates UICC stage ($p=0.0004$) and type of therapy (surgery alone *vs.* surgery plus adjuvant treatment, $p=0.04$) were significant predictors of overall survival in this group of models.

In the PHR models with all covariates (multivariate analyses, Table III), age ($p=0.04$), UICC stage ($p<0.001$), histological type (squamous *vs.* adeno-carcinoma, $p=0.03$) and type of therapy (surgery alone *vs.* surgery plus adjuvant treatment, $p=0.02$) were significant independent predictors of overall survival. The Kaplan-Meier plots in Figure 3b show the overall survival according to the MAGE-A status (positive *versus* negative) and demonstrate a tendency for better survival of MAGE-negative patients. This difference, however, was not significant.

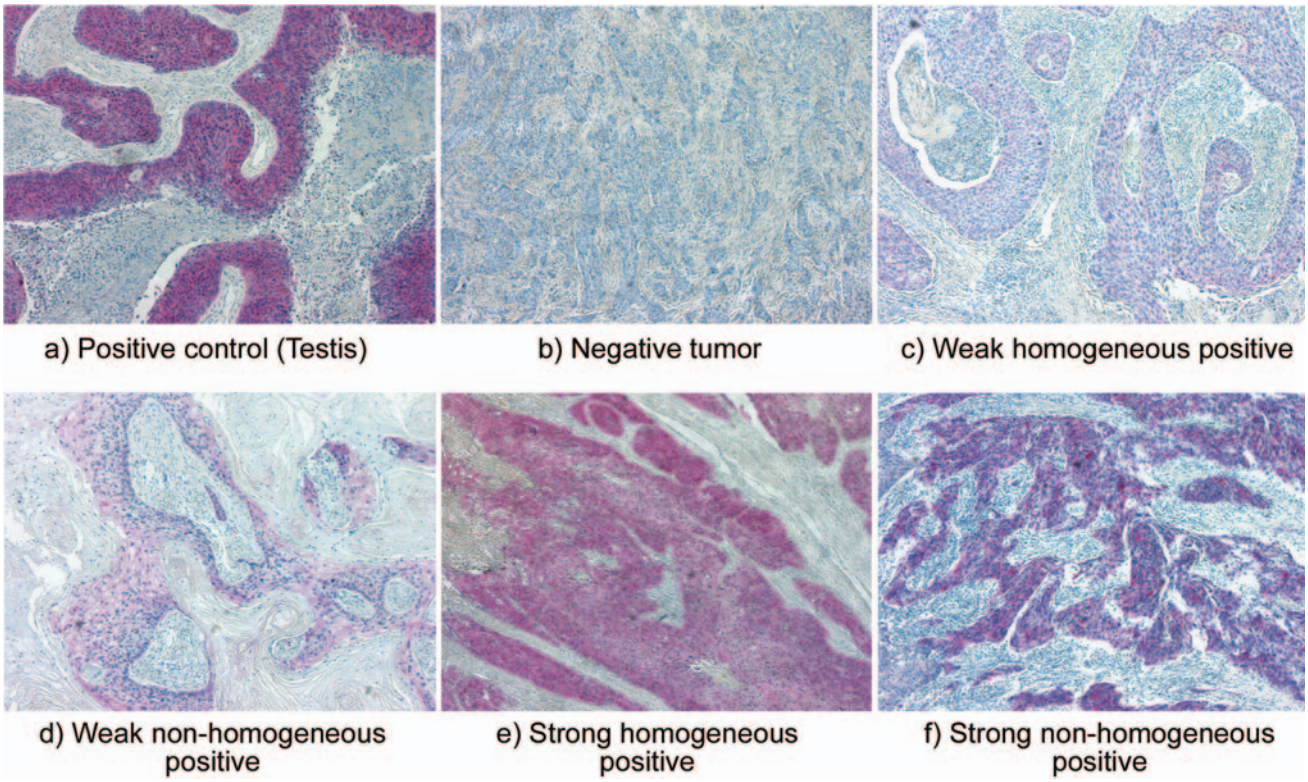

Figure 1. *Expression of MAGE-A with different intensity und homogeneity. Representative examples of negative as well as for weak and strong expressions for both homogeneous (4 points) and non-homogeneous (1-3 points) expression patterns are shown. Testis was used as positive control.*

Discussion

This retrospective study investigated the role of MAGE-A antigens as a potential therapeutic target and prognostic parameter for patients with esophageal carcinomas. More than half of the patients had been diagnosed in advanced tumor stages, but 80% had been resected with curative intention. During the first years of inclusion, sufficient palliative treatment had been limited and therefore some patients had received palliative resections. Although multimodal treatment strategies were used for these patients, their overall prognosis remained poor, with a median survival of approximately 21 months. The theoretical aspects of the molecular characteristics of the MAGE-A antigen family and previous results in other tumor entities, such as bladder cancer, suggested that these proteins may be useful as predictive markers and/or novel targets for immune therapy in those patients (25-27). Established prognostic factors, such as tumor stage and UICC classification, can predict potential outcome as confirmed in our analysis, but appear to be insufficient for individual evidence-based therapeutic decisions.

Previous studies revealed high variations of MAGE antigens in esophageal carcinomas (MAGE-A1 18-62%; MAGE-A3 2-57%), but the numbers of investigated patients were small (9, 18, 19). In our study, positive immunostaining for MAGE-A CTA was found in 38.8% of all cases, with a high variability in intensity and homogeneity within the tumors. Positive staining of MAGE-A CTA was almost exclusively found in squamous cell carcinomas (50%) and only rarely in adenocarcinomas of the esophagus (15%). The non-homogenous MAGE-A expression, as well as different detection techniques, may explain the differences of expression rates, especially in squamous cell carcinomas (9, 15, 19). For example, the 57B mAb was initially used for the detection of MAGE-A3, but was later recognized to cross-react with other members of the MAGE-A family. Other investigations were performed at the mRNA level (15). Although the expression of MAGE-A antigens has been proposed to be associated with dedifferentiation in various tumor entities (28), we and others did not find correlations between MAGE-A expression scores and the tumor differentiation, TNM- or UICC classifications (29). Similarly, other CTA expression (GAGE, NY-ESO-1, and SSX) was also not correlated with prognosis in esophageal cancer patients (29). Furthermore, 57B immunostaining was not a significant predictor of overall survival in uni- or multivariate analyses. Since MAGE-A is an X-chromosome-related

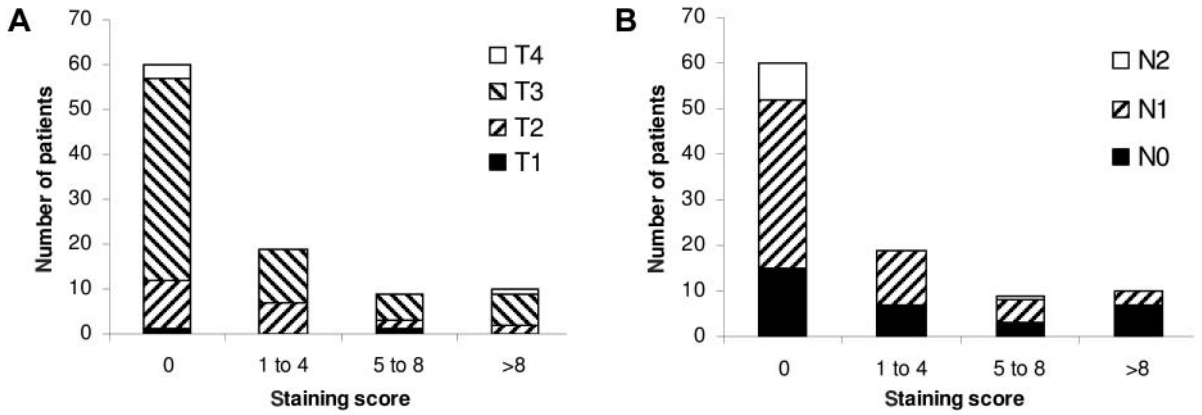

Figure 2. *Staining scores of MAGE-A expression. Score values were summarized into four groups according to the staining points (0 - negative tumors; 1-4 low; 5-8 intermediate; >8 strong expression). The distribution of A) tumor stage and B) nodal involvement for these score values is shown.*

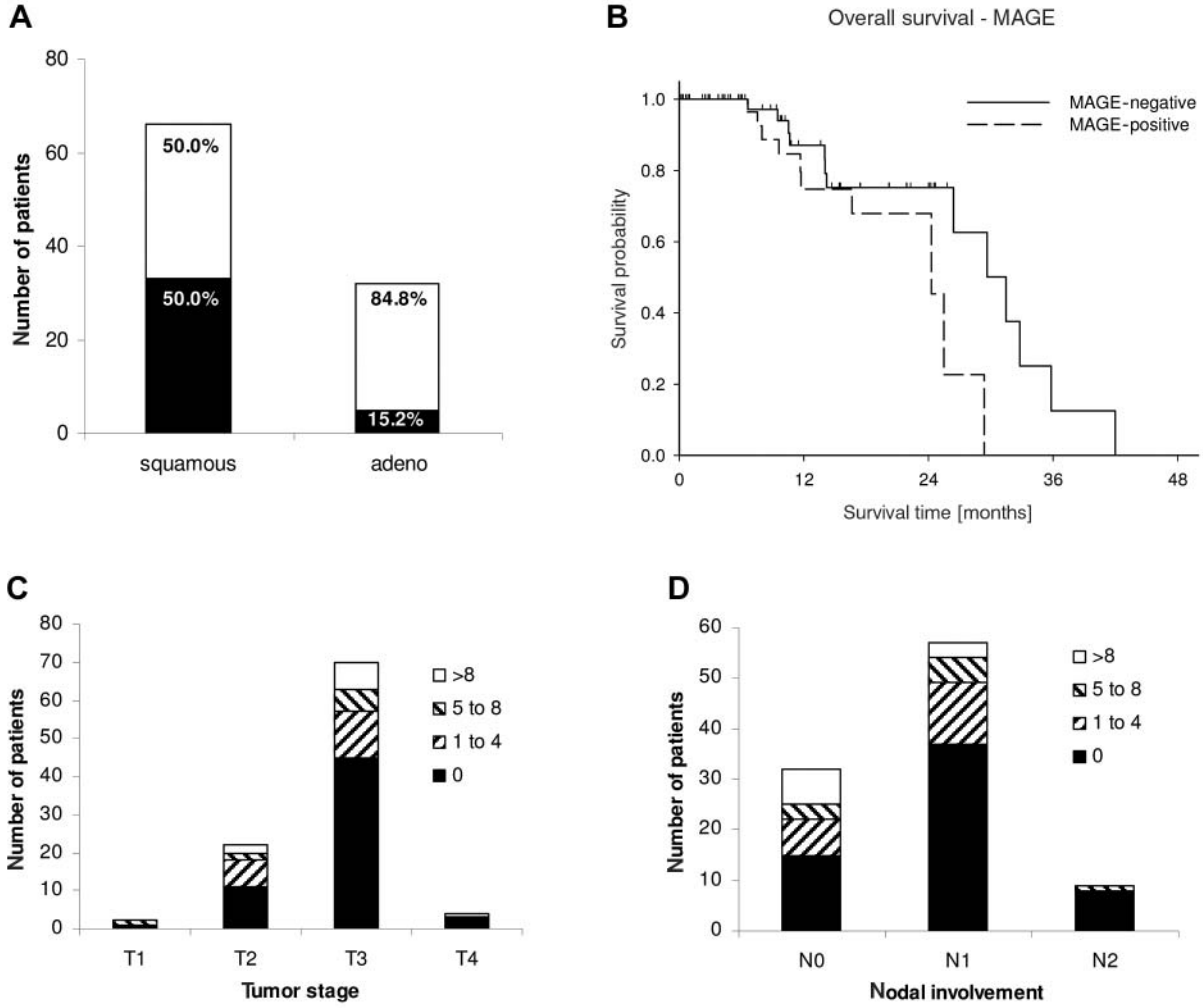

Figure 3. *MAGE-A expression and tumor characteristics. (A) The expression of MAGE-A antigens was significantly more frequent in squamous cell carcinomas as compared with adenocarcinomas ($p<0.0001$). (B) The expression of MAGE-A was not a significant predictor of overall survival for patients with esophageal carcinomas. This expression did not correlate with tumor stage (TNM), grading or UICC classification; distribution of MAGE-A staining scores depending on (C) local invasion (T-classification) and (D) nodal involvement (N-classification) (reclassified UICC2002).*

antigen, we also compared its expression in tumors from females and males. Although most patients in our study were male, thus limiting the possibility to compare both groups, MAGE-A-positive tumors were found at similar rates in both genders (30-32).

High expression of MAGE-A is probably not useful as a surrogate marker in esophageal cancer patients or for other tumor entities (20, 30-32); in approximately half of the squamous cell carcinomas, these antigens may represent potential immunotherapy targets recognizable by specific cytotoxic T-lymphocytes (CTL) (33, 34). There are several reports of the successful induction of HLA class I-restricted antitumor CTLs using MAGE peptides, and some clinical trials with these immunogenic peptides were reported as effective for some patients with malignant melanoma. The results of initial clinical studies also suggested that dendritic cell vaccination with MAGE-3 peptide appears to be a safe and promising approach in the treatment of gastrointestinal carcinomas (35). Thus, our data support the application of tumor vaccines directed against these antigens in esophageal cancers. In addition, tumor samples can be used for the direct detection of this target, offering an option for pretherapeutic testing.

This study included the largest number of investigated patients with esophageal carcinomas regarding MAGE-A expression. However, the retrospective analysis of follow-up and the relatively long period of recruitment are limitations of our investigation. Furthermore, different adjuvant treatment modalities had been used in our patients, but the number of patients was insufficient for the inclusion of these different subgroups in the statistical evaluation. For further evaluation, a prospective analysis that considers different adjuvant treatment – possibly in a multicenter design – appears to be required.

In summary, our study revealed that MAGE-A CTA are frequently expressed in squamous cell carcinomas of the esophagus, but rarely in adenocarcinomas. Their up-regulated expression appears to be independent of local tumor invasion, tumor progression and differentiation. Moreover, MAGE-A expression lacks a prognostic value for evaluating overall patient survival. However, the high expression rate in squamous cell carcinomas suggests a potential usefulness as a novel target for immunotherapy in these patients, as proposed for other tumor entities (36, 37). Although, to our knowledge, CTA-based immune therapy has not been investigated in esophageal patients yet, our study and others suggest that patients positive for MAGE-A should be included in further clinical studies.

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