The prognostic impact of human leukocyte antigen (HLA) class I antigen abnormalities in salivary gland cancer. A clinicopathological study of 288 cases

Maximilian Müller, Abbas Agaimy, Johannes Zenk,[1] Tobias Ettl,[2] Heinrich Iro,[1] Arndt Hartmann, Barbara Seliger[3] & Stephan Schwarz

Department of Pathology, University of Erlangen, Erlangen, Germany, [1]Department of Otorhinolaryngology-Head and Neck Surgery, University of Erlangen, Erlangen, Germany, [2]Department of Oral and Maxillofacial Surgery, University of Regensburg, Regensburg, Germany, and [3]Department of Medical Immunology, University of Halle-Wittenberg, Halle (Saale), Germany

Aims: To study abnormalities of proteins of the major histocompatibility complex class I in a series of 288 salivary gland carcinomas, and to correlate findings with patients' overall survival (OS).

Methods and results: Protein expression of human leukocyte antigen (HLA)-A, heavy chain (HC)-10, β_2-microglobulin, low molecular weight polypeptides (LMP) 2 and 7, transporters associated with antigen processing (TAP) 1 and 2, calnexin, calreticulin, endoplasmic reticulum (ER) p57 and tapasin was evaluated by immunohistochemistry and semiquantitatively analyzed. As compared with normal salivary gland tissue, HLA-A, LMP7, TAP2 and HLA class I were significantly down-regulated in salivary gland carcinomas, whereas β_2-microglobulin, calnexin, LMP2, and TAP1 were upregulated. Expression of calreticulin, ERp57 and tapasin was unaltered. In univariate Kaplan–Meier analyses, low expression of LMP7 ($P = 0.005$) and high expression of β_2-microglobulin ($P = 0.028$), HLA-A ($P < 0.001$), TAP1 ($P = 0.01$), and tapasin ($P < 0.001$) were significantly associated with shorter OS. In multivariate analysis incorporating tumour stage, nodal/distant metastasis, and grade, HLA-A ($P = 0.014$), LMP7 ($P = 0.033$), and tapasin ($P = 0.024$), as well as distant metastasis ($P = 0.012$) and high tumour grade ($P < 0.001$), remained statistically significant.

Conclusion: The prognostic influence of up-regulated HLA-A and tapasin and down-regulated LMP7 may provide a rationale for targeting these specific components of the antigen processing and presentation pathway in salivary gland carcinomas.

Keywords: HLA class I, immune escape, immunohistochemistry, prognosis, salivary gland cancer

Introduction

Salivary gland carcinoma (SGC) is a complex and heterogeneous disease with an enormous morphological diversity and variable clinical course. Although relatively rare, SGC constitutes the most common adenocarcinoma of the head and neck region. Therapy strategies for patients with SGC are primarily based on surgery with or without postsurgical irradiation. Immunotherapeutic strategies successfully implemented in adenocarcinomas of other origin, e.g. prostate cancer[1] and colorectal cancer,[2,3] are extremely difficult to establish in SGC treatment, owing to the low frequency of the disease and the lack of

Address for correspondence: S Schwarz, MD, Department of Pathology, University of Erlangen, Krankenhausstrasse 8-10, 91054 Erlangen, Germany. e-mail: stephan.schwarz@uk-erlangen.de

comprehensive knowledge of tumour immune escape mechanisms, including alterations of components of the antigen processing machinery (APM) in SGC. To date, only two studies have addressed the expression of proteins of the human leukocyte antigen (HLA) complex in SGC, both focusing on HLA-DR,[4,5] and two other studies have analyzed HLA complex expression in Warthin's tumour[6] and pleomorphic adenoma.[7]

Effective $CD8^+$ cytotoxic T-lymphocyte responses require adequate surface expression of the major histocompatibility complex (MHC) class I antigens. Thus, impaired expression of these antigens might have a negative impact on tumour immunosurveillance, on the course of the disease, and on the outcome of T-cell-based immunotherapies.[8–10] Abnormalities in the MHC class I phenotype of tumours can be caused by distinct molecular defects within the antigen processing pathway.[10,11] These include structural alterations, methylation or dysregulation of genes coding for MHC class I heavy chain (HC) and/or β_2-microglobulin, as well as for other APM components, such as the interferon (IFN)-γ-inducible immunoproteasome subunits, low molecular weight polypeptide (LMP) 2 and LMP7, transporter associated with antigen processing (TAP), and tapasin.

One major prerequisite for the successful implementation of immunotherapy is the appropriate expression of MHC class I molecules on tumour cells. Therefore, the aim of the present study was to analyze the expression of various APM components, such as TAP subunits, calnexin, calreticulin, tapasin, LMP2, LMP7, and HLA class I antigens, in a series of 288 cases of SGC, and to correlate alterations in expression with patient survival.

Materials and methods

PATIENTS AND TUMOUR SAMPLES

Two hundred and eighty-eight carcinomas of major and minor salivary glands diagnosed at the pathology departments of Regensburg University, Erlangen University and Nuremberg Hospital between 1984 and 2008 were reviewed. The medical records were obtained from the clinical tumour registries of Regensburg and Erlangen-Nuremberg, and from the salivary gland carcinoma registry of the Department of Otorhinolaryngology-Head and Neck Surgery, University of Erlangen. The registries and the related translational research activities are covered by ethical regulations of the medical faculties of the Universities of Regensburg and Erlangen-Nuremberg.

CLINICAL CHARACTERISTICS

All patients underwent primary surgery; lymph node dissection was performed for 204 (70.8%) patients. Postsurgical radiotherapy or radiochemotherapy was given in 168 (58.3%) cases with high-grade malignancy, positive resection margins, lymph node metastases, or distant metastasis.

The SGC originated in the parotid (201, 69.8%), submandibular (42, 14.6%), sublingual (1, 0.3%) and minor (44, 15.3%) salivary glands. For 55.2% of the patients, Union for International Cancer Control (UICC) tumour stages at diagnosis were advanced (stages III and IV). Cervical lymph node metastases were obvious in 29.9% of the patients, and distant metastases occurred in 11.1%. In 255 cases (88.5%), tumour-free resection margins (R0) were achieved; 33 (11.5%) patients had microscopic (R1) or macroscopic (R2) residual tumour after surgery.

FOLLOW-UP STUDIES

The patients comprised 138 males and 150 females, with a mean age of 60.7 years (range 11–99 years) at diagnosis. The mean follow-up of all patients was 4.89 years (range 0.1–24.8 years). Recurrence was observed in 68 (23.6%) patients without local or systemic residual tumour after surgery. Death occurred in 109 (37.8%) cases. The 5-year and 10-year overall survival rates of all patients were 52.3% (93 cases censored) and 25.3% (146 cases censored), respectively.

HISTOLOGY AND CLASSIFICATION

All 288 tumours were independently reviewed by two pathologists experienced in salivary gland tumour pathology (S.S. and A.A.) without knowledge of initial histological diagnosis or clinical follow-up, using haematoxylin and eosin (H&E)-stained and periodic acid–Schiff (PAS)-stained slides from at least subtotally embedded tumours. All tumours were classified according to the contemporary World Health Organization (WHO) classification of salivary gland tumours.[12] Tumours were staged according to the present UICC classification,[13] and grading was based on a three-tiered grading system as recently described.[14,15] As shown in Table 1, acinic cell carcinoma, basal cell adenocarcinoma, epithelial myoepithelial carcinoma, cystadenocarcinoma and polymorphous low-grade adenocarcinoma were considered to be low grade (G1), with the exception of dedifferentiated variants, which were classified as high grade (G3). Usual salivary duct carcinoma, adenocarcinoma NOS (not otherwise

Table 1. Distribution of histological subtypes and tumour grades in 288 salivary gland carcinomas

Histology	Grade 1	2	3	Total
Tumours of acinar origin (n = 40)				
Acinic cell carcinoma	35	0	5*	40
Tumours of intercalated duct origin (n = 93)				
Adenoid cystic carcinoma	0	39	11	50
Myoepithelial carcinoma	4	5	9	18
Polymorphous low grade adenocarcinoma	13	0	0	13
Basal cell adenocarcinoma	8	0	0	8
Epithelial myoepithelial carcinoma	4	0	0	4
Tumours of striated duct origin (n = 4)				
Oncocytic carcinoma	0	0	4	4
Tumours of excretory duct origin (n = 137)				
Mucoepidermoid carcinoma	28	4	11	43
Adenocarcinoma NOS	0	0	36	36
Salivary duct carcinoma	0	0	33	33
Squamous cell carcinoma	0	0	25	25
Tumours of other/unclear origin (n = 14)				
Malignant mixed tumour	0	0	5	5
Undifferentiated/large cell carcinoma	0	0	5	5
Small cell carcinoma	0	0	2	2
Cystadenocarcinoma	2	0	0	2
	94	48	146	288

*Dedifferentiated carcinoma.

specified), squamous cell carcinoma, oncocytic carcinoma, malignant mixed tumour, undifferentiated carcinoma, small-cell carcinoma and large-cell carcinoma were classified as high grade (G3). Mucoepidermoid carcinoma was graded according to the criteria proposed in the current WHO classification.[16] Adenoid cystic carcinomas were divided into predominantly tubulo-cribriform (G2) and predominantly solid (G3) tumours. Grading of myoepithelial carcinoma was based on nuclear pleomorphism and mitotic activity, similar to the Elston and Ellis grading of breast cancer with solid growth pattern.[17] The 27 cases of carcinoma ex pleomorphic adenoma were classified and graded according to the malignant component of the tumour. All cases of squamous cell carcinoma were classified as originating from the salivary glands after intensive staging procedures [computed tomography (CT) or magnetic resonance imaging (MRI) of the head and neck, panendoscopy, X-ray, or CT of the chest and ultrasonography of the abdomen] and exclusion of metastasis to the salivary gland.

IMMUNOHISTOCHEMISTRY

A tissue microarray (TMA) with one 2.0-mm-diameter punch core per tumour was constructed from formalin-fixed paraffin-embedded tissue blocks of all patients, as previously described.[18,19] H&E-stained TMA sections were used for reference histology to ensure that the specific tumour type was representatively included in the TMA.

Anti-HLA-A rabbit polyclonal antibody was purchased from Protein Tech Europe (Manchester, UK). The following monoclonal antibodies (mAb) were developed and characterized as described in the related publications: HC-10 (which recognizes β_2-microglobulin-free HLA-A3, HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33, and HLA-B [excluding HLA-B5702, HLA-B5804, and HLA-B73] HC[20,21]; L368 (recognizing β_2-microglobulin)[22]; SY-1 (LMP2)[22]; HB2 (LMP7)[23]; NOB-1 (TAP1)[24]; NOB-2 (TAP2)[24]: TO-5 (calnexin)[25]; TO-11 (calreticulin)[25]; TO-2 (thiol oxidoreductase p57 of the endoplasmic reticulum [ERp57])[25]; and TO-3 (tapasin).[26] All the above-mentioned mAbs are IgG_1, except for HC-10, which is IgG_{2a}. The staining was visualized using the combined mouse/rabbit Envision and Dual Link horseradish peroxidase (HRP) detection system (Dako, Hamburg, Germany).

After staining, all slides were reviewed by one of the authors (M.M.) without knowledge of the clinical data. As previously described,[19,27] an immunoreactivity score was obtained for each case by multiplying a value for staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) by the percentage of positively stained cells; thus, the total could range from 0 to 300. The data for protein expression were then dichotomized into a group with a low expression level (score 0–150) and a group with a high expression level (score 151–300), irrespective of the expression in normal tissue.

Whereas all markers showed cytoplasmic staining, LMP2 and LMP7 could also be detected in the nuclei

of the cells, as previously described by others.[28,29] Therefore, the nuclear and cytoplasmic expression of LMP2 and LMP7 were analyzed separately.

In order to detect alterations of protein expression in tumours as compared with normal tissue, 120 samples of normal salivary glands were used as reference tissue. The tumours were related to the structures of their histogenetic origin, as grouped in Table 1. Thirty samples of each anatomical structure (acinic epithelia, luminal epithelia of intercalated ducts, striated ducts, and excretory ducts) were analyzed.

STATISTICAL ANALYSES

All clinicopathological data were analyzed using SPSS for Windows, version 18.0 (SPSS, Munich, Germany). Student's *t*-test for two independent samples was applied to compare mean values and to confirm statistically significant differences in protein expression between tumour entities and corresponding normal tissue. These analyses were restricted to tumour entities represented by more than seven cases, with the exception of oncocytic carcinoma cases. Up-regulation or down-regulation was noted if the *P*-value was <0.05.

Associations between categorized parameters were analyzed by applying the two-tailed Fisher exact test to contingency tables.

Overall survival (OS), considered to be the primary outcome measure, was calculated as the time from diagnosis to the date of death from any cause or the date when the patient was last known to be alive. Patients lost to follow-up were treated as censored cases on the basis of the date when they were last known to be disease-free or alive, respectively. Survival curves were generated using the Kaplan–Meier method, and log-rank tests were used to compare the distributions between groups.

Clinicopathological parameters were dichotomized as follows: T1/T2 versus T3/T4, N0 versus N1/N2/N3, M0 versus M1, and G1 versus G2/G3.

For multivariate analysis, a Cox proportional hazards model was employed, using a backwards, stepwise elimination approach. At each step, the least significant factor with $P > 0.10$ was eliminated, with reassessment of each factor in the model at each step. A limit for the factors to be included was set at 5%.

Results

EXPRESSION PROFILE OF MHC CLASS I-ASSOCIATED PROTEINS IN SALIVARY GLAND CARCINOMAS

In order to analyze alterations in the expression of MHC class I-associated proteins in SGC with respect to different tumour entities, the tumours were grouped according to their putative histogenetic origin (Table 1). Forty tumours were of acinar origin (acinic cell carcinoma), 93 tumours were of intercalated duct origin (adenoid cystic carcinoma, myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, basal cell adenocarcinoma, and epithelial–myoepithelial carcinoma), four tumours were of striated duct origin (oncocytic carcinoma), and 137 tumours were of excretory duct origin (mucoepidermoid carcinoma, adenocarcinoma NOS, salivary duct carcinoma, and squamous cell carcinoma). Fourteen tumours could not be attributed histogenetically to one of the aforementioned four histogenetic subgroups (malignant mixed tumour, undifferentiated carcinoma, large-cell carcinoma, small-cell carcinoma, and cystadenocarcinoma). Figure 1 illustrates exemplary differences in the expression of LMP7, TAP1, β_2-microglobulin, tapasin and HLA-A in various epithelial types of normal salivary gland tissue and representative tumours. LMP7 (Figure 1E) was moderately expressed in the cytoplasm (mean score 145.0) and nuclei of acinar epithelia (mean score 158.0). Expression was also high in intercalated, striated and excretory ducts (mean cytoplasmic LMP7 expression 232.2, 231.3, and 197.5, respectively; mean nuclear LMP7 expression 209.6, 207.0, and 185.5, respectively). Staining for TAP1 was negative (Figure 1I). β_2-Microglobulin (Figure 1M) was moderately expressed in acini (mean expression 117.7) and showed low-level expression in intercalated, striated and excretory ducts (68.6, 45.2, and 54.0, respectively). Tapasin (Figure 1Q) was highly expressed in excretory, intercalated and striated ducts (mean scores of 157.5, 141.1, and 153.7, respectively), whereas acini were almost negative (mean score of 33.0). HLA-A (Figure 1U) was highly expressed in acinar epithelia (mean score 200.3) as well as in epithelia of intercalated, striated and excretory ducts (mean scores 214.7, 231.0 and 197.0, respectively).

The analysis of variations in the expression pattern of MHC class I APM components was restricted to tumour entities with at least eight cases (with the exception of oncocytic carcinoma). As shown in Figure 2, acinic cell carcinomas were characterized by up-regulation of calnexin, LMP2, TAP1 (see also Figure 1J), TAP2, and tapasin (Figure 1R). Cytoplasmic expression of LMP7 was significantly lower in acinic cell carcinoma (mean score value of 116.8; Figure 1F) than in normal acini (mean score 158.0). In general, tumours of intercalated duct origin showed down-regulation of calreticulin (mean score value of 172.9), HLA-A (mean score of 140.5; Figure 1W),

LMP7 (mean cytoplasmic score of 140.8 and mean nuclear score of 129.9; Figure 1G), TAP2 (mean score value of 159.6), and HLA class I HC (mean score 111.4). TAP1 was up-regulated in myoepithelial carcinoma (*t*-test for independent samples $P = 0.012$) and basal cell carcinoma ($P = 0.268$). Tapasin was down-regulated in adenoid cystic carcinoma ($P < 0.001$; Figure 1S) and polymorphous low-grade adenocarcinoma ($P = 0.03$).

The four oncocytic carcinomas considered to be of striated duct origin showed expression patterns resembling those of tumours of excretory duct origin. With the exception of adenocarcinoma NOS, tumours of excretory duct origin were characterized by overexpression of β_2-microglobulin (mucoepidermoid carcinoma $P < 0.001$, Figure 1P; squamous cell carcinoma $P < 0.001$; salivary duct carcinoma $P < 0.001$) and LMP2 (mucoepidermoid carcinoma $P < 0.01$; squamous cell carcinoma $P < 0.001$; salivary duct carcinoma $P < 0.05$). All tumours originating from excretory ducts showed up-regulated TAP1 ($P < 0.001$; Figure 1L) and down-regulated TAP2 ($P < 0.001$). Oncocytic carcinoma and adenocarcinoma NOS showed slightly reduced nuclear and cytoplasmic expression of LMP7 (oncocytic carcinoma, 185.0 and 152.0, respectively; adenocarcinoma NOS, 167.8 and 187.9, respectively). Salivary duct carcinomas and squamous cell carcinomas showed reduced levels of nuclear LMP7 expression (mean score values of 85.4, and 79.5, respectively).

Regarding the complete series of 288 SGC cases, up-regulation was noted for β_2-microglobulin (*t*-test with $P < 0.001$), calnexin ($P < 0.001$), LMP2 (nuclear and cytoplasmic staining $P < 0.001$), and TAP1 ($P < 0.001$), whereas HLA-A ($P = 0.001$), LMP7 (nuclear and cytoplasmic $P < 0.001$), TAP2 ($P < 0.001$), and HC-10 ($P = 0.018$) were characteristically down-regulated. Expression of calreticulin, ERp57 and tapasin was unchanged.

PROGNOSTIC IMPLICATIONS OF MHC CLASS I-ASSOCIATED PROTEIN EXPRESSION IN SALIVARY GLAND CARCINOMAS

To study the prognostic relevance of the altered expression of MHC class I-associated proteins, the expression data were dichotomized (low expression, score 0–150; high expression, score 151–300). Univariate Kaplan–Meier survival analyzes revealed that high expression of β_2-microglobulin ($P = 0.024$), HLA-A ($P < 0.001$), TAP1 ($P = 0.016$) and tapasin ($P < 0.001$) were significantly associated with shorter overall survival (Figure 3). In contrast, high nuclear expression of LMP7 was a positive prognostic factor ($P = 0.002$). Calnexin, calreticulin, ERp57, LMP2, TAP2 and HLA class I HC did not show any prognostic impact. These factors were not included in further analyses.

INTERDEPENDENCE OF PROGNOSTICALLY RELEVANT MHC CLASS I-ASSOCIATED PROTEIN EXPRESSION AND ASSOCIATIONS WITH CLINICOPATHOLOGICAL PARAMETERS

To study the associative interaction between the five prognostically relevant proteins of the MHC class I complex, contingency tables were drawn up to apply two-sided Fisher exact tests. β_2-Microglobulin was significantly associated with HLA-A, TAP1, and tapasin (all $P < 0.001$). Significant associations were also found between HLA-A and TAP1 ($P = 0.019$) and between HLA-A and tapasin ($P < 0.001$). Also, TAP1 and tapasin were significantly associated ($P < 0.001$). In contrast, LMP7 expression was not associated with the expression of other components, and could be regarded as being independent.

Fisher exact tests were also applied to study the relationship of prognostically significant MHC class I-associated proteins with known tumour-specific clinicopathological parameters used in the UICC classification system (T, N and M stages, and grade). Parameters such as age, sex and tumour localization were not analyzed, irrespective of their clinical prognostic relevance, as a pathogenetic link between them and MHC class I-associated proteins appeared rather unlikely. R-classification was also disregarded, as it depends on the success of surgical therapy, and is therefore not a category of the tumour itself. High expression of HLA-A was significantly associated with high local tumour stage (T3/T4) and positive nodal status ($P = 0.016$ and $P = 0.001$, respectively); high expression of tapasin was associated with positive lymph nodes ($P = 0.01$); and nuclear down-regulation of LMP7 was preferentially found in high-grade tumours (G2/G3, $P = 0.011$).

PROGNOSTIC RELEVANCE OF IMPAIRED MHC CLASS I APM COMPONENT EXPRESSION REGARDING TUMOUR-SPECIFIC CLINICOPATHOLOGICAL PARAMETERS

Multivariate Cox regression analysis was used to assess parameters that turned out to be prognostically relevant in univariate Kaplan–Meier analyses. The multivariate regression model was adjusted by stepwise backwards selection (Table 2). The analysis was

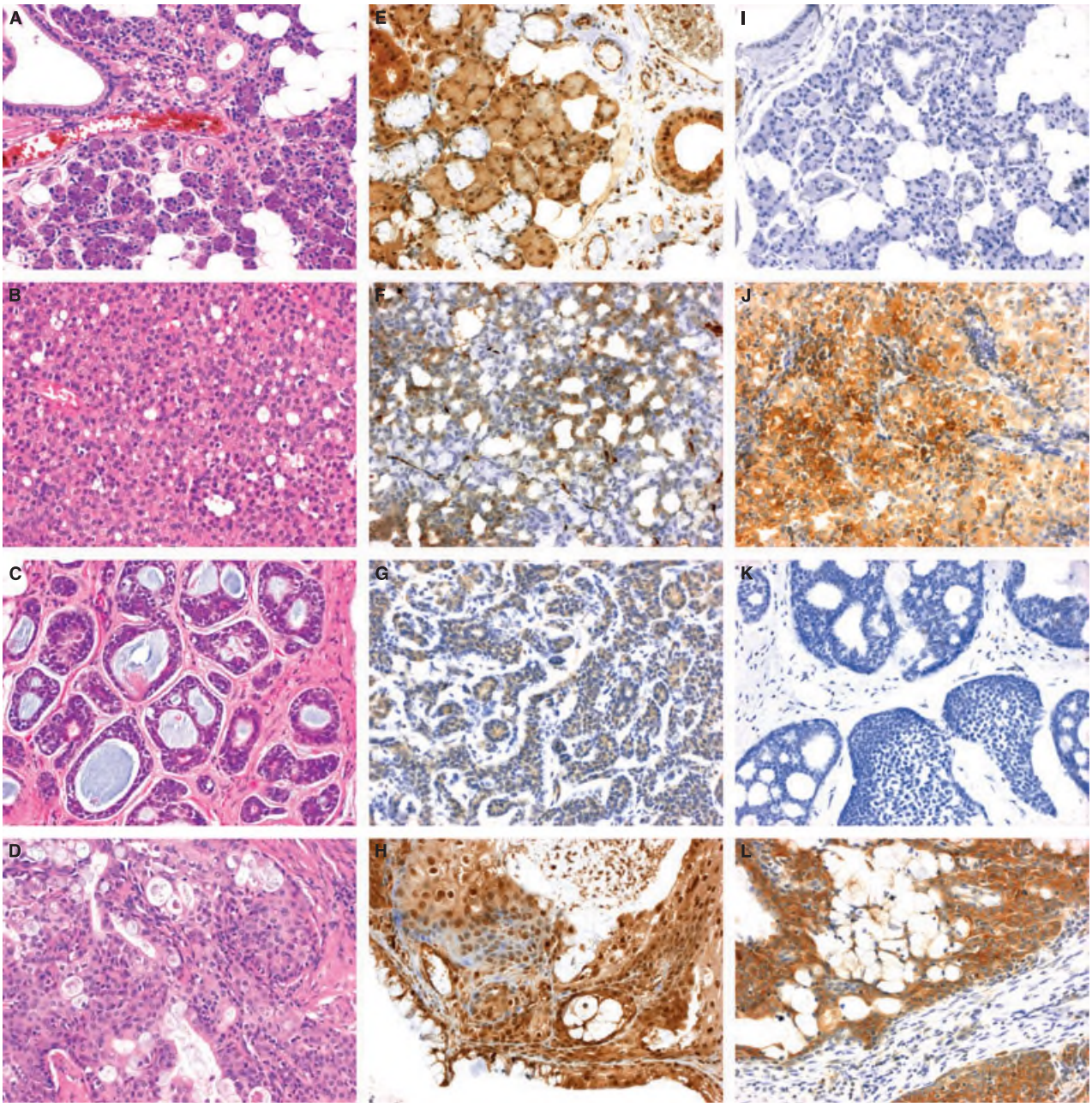

Figure 1. (**A**–**D**), (H&E) staining of normal salivary gland tissue (**A**), acinic cell carcinoma (**B**), adenoid cystic carcinoma (**C**), and mucoepidermoid carcinoma (**D**). (**E**–**L**), Expression of LMP7 and TAP1 in salivary gland normal tissue and carcinomas as detected by immunohistochemistry. (**E**), Moderate cytoplasmic and nuclear expression of LMP7 in acinar epithelia. High cytoplasmic and nuclear expression of LMP7 in ductal epithelia. (**F**), Low expression of LMP7 in acinic cell carcinoma. (**G**), Low expression of LMP7 in adenoid cystic carcinoma. (**H**), High expression of LMP7 in mucoepidermoid carcinoma. (**I**), No expression of TAP1 in normal salivary gland tissue. (**J**), High expression of TAP1 in acinic cell carcinoma. (**K**), No expression of TAP1 in adenoid cystic carcinoma. (**L**), High expression of TAP1 in mucoepidermoid carcinoma. (**M**–**X**), Expression of β_2-microglobulin, tapasin and HLA-A in salivary gland normal tissue and carcinomas as detected by immunohistochemistry. (**M**), Moderate expression of β_2-microglobulin in acini, and low expression of β_2-microglobulin in ductal epithelia. (**N**), High expression of β_2-microglobulin in acinic cell carcinoma. (**O**), Low expression of β_2-microglobulin in adenoid cystic carcinoma. (**P**), High expression of β_2-microglobulin in mucoepidermoid carcinoma. (**Q**), No expression of tapasin in acini. High expression of tapasin in ductal epithelia. (**R**), Moderate expression of tapasin in acinic cell carcinoma. (**S**), No expression of tapasin in adenoid cystic carcinoma. (**T**), High expression of tapasin in mucoepidermoid carcinoma. (**U**), High expression of HLA-A in all epithelia of normal salivary glands. (**V**), High expression of HLA-A in acinic cell carcinoma. (**W**), Reduced expression of HLA-A in adenoid cystic carcinoma. (**X**), High expression of HLA-A in mucoepidermid carcinoma.

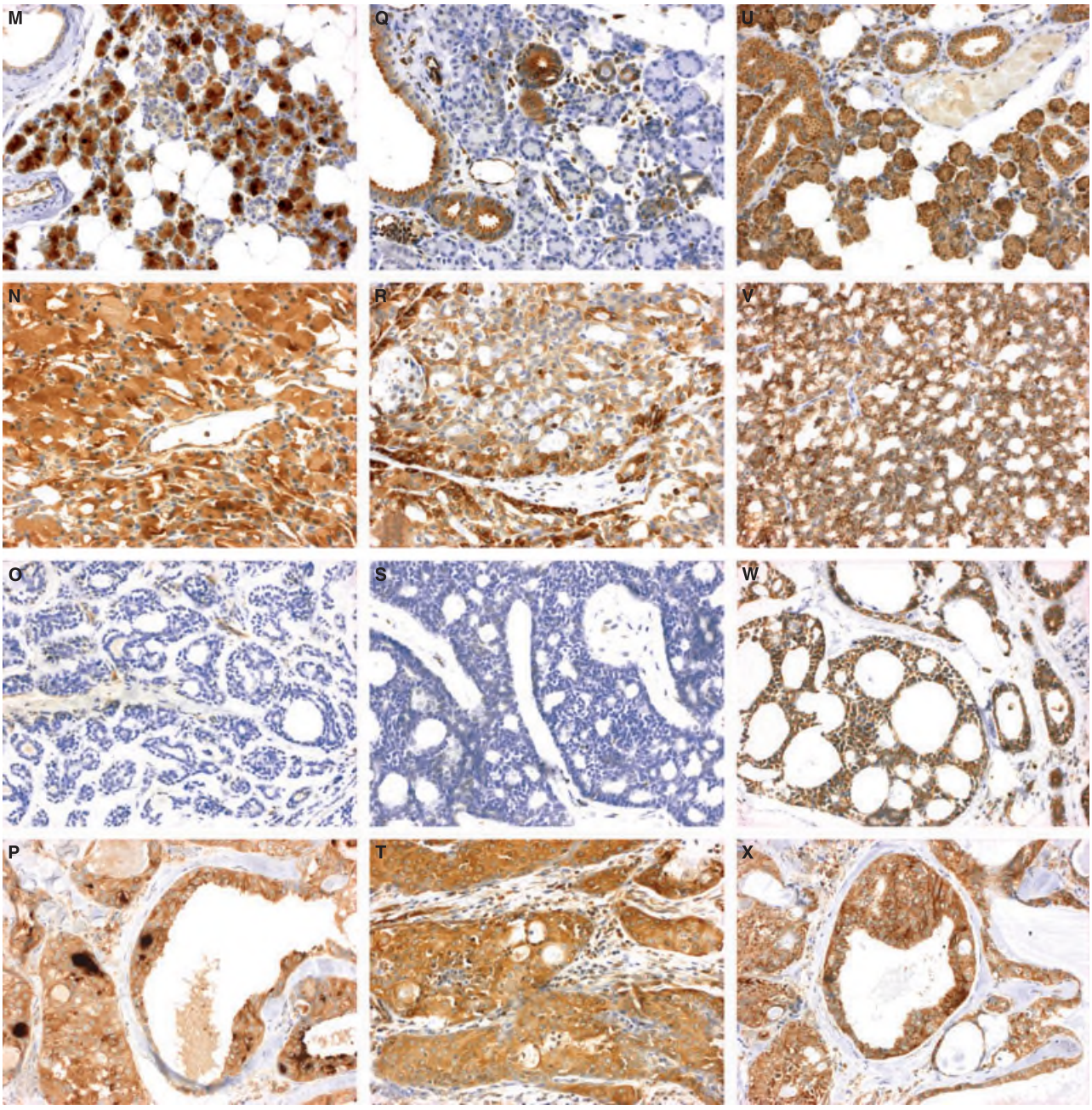

Figure 1. (continued)

restricted to 265 cases with a complete dataset of five MHC class I APM components and the main four tumour-specific clinicopathological parameters (local tumour, nodal metastasis, distant metastasis, and grade). In the first step of the Cox regression model with the inclusion method, distant metastasis, high tumour grade, high expression of HLA-A and tapasin and low nuclear expression of LMP7 were considered to be significant negative prognostic factors. After adjustment of the model in step four, these five parameters remained statistically significant.

Discussion

As effective MHC class I antigen processing and presentation is required for proper recognition by T lymphocytes, alterations in the MHC class I APM play an

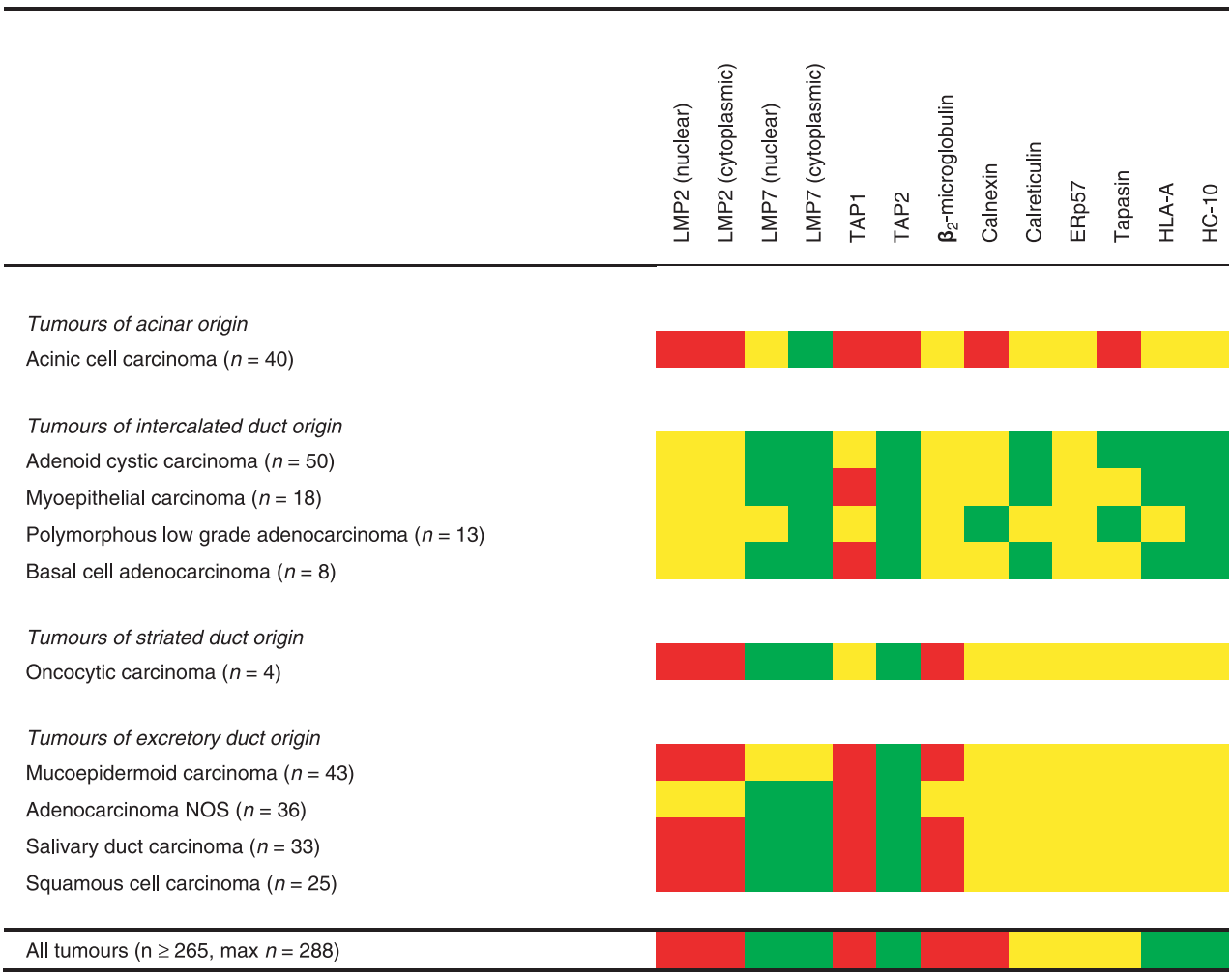

Figure 2. Expression profile of MHC-I associated proteins in salivary gland carcinomas in comparison to normal structures of histogenetic origin. Results of *t*-tests comparing mean immunohistochemical expression (score) values of independent groups (red: significantly higher expression, green: significantly lower expression, yellow: no difference).

important role in the immune escape of tumour cells, and have been described in a variety of malignancies.[10,11,30,31] As antibodies detecting proteins involved in the MHC class I pathway are available, several immunohistochemical studies of their clinical relevance have been performed in different tumour entities, such as breast cancer,[32] Hodgkin lymphoma,[33] malignant melanoma,[34] prostate cancer,[35] and squamous cell carcinoma of the head and neck,[36] and linked to clinical parameters. As most of the studies only analyzed paraffin-embedded tissues, MHC class I surface expression could not be determined by staining with the mAb W6/32 recognizing the MHC class I HC–β_2-microglobulin–peptide complex, which can only be performed on fresh frozen tissues. As no study has to date extensively focused on SGC, the aim of the present investigation was to identify alterations in the expression of components involved in the following four different steps of antigen processing and presentation[11]: (i) peptide generation/trimming—LMP2 and LMP7, representing interferon-γ-inducible subunits of the proteasome, are involved in the degradation of endogenously synthesized and ubiquitinated proteins, which are then trimmed by cytosolic peptidases; (ii) peptide transport—the heterodimeric transporters associated with antigen processing (TAP1 and TAP2) are responsible for the transport of the resulting peptides from the cytosol into the ER; (iii) MHC class I assembly—the chaperones calnexin, calreticulin and ERp57 assist the association and stabilization of MHC class I HC and β_2-microglobulin, and tapasin, a chaperone that is linked to ERp57 within the peptide loading complex,[37] is required for stabilization of TAP and

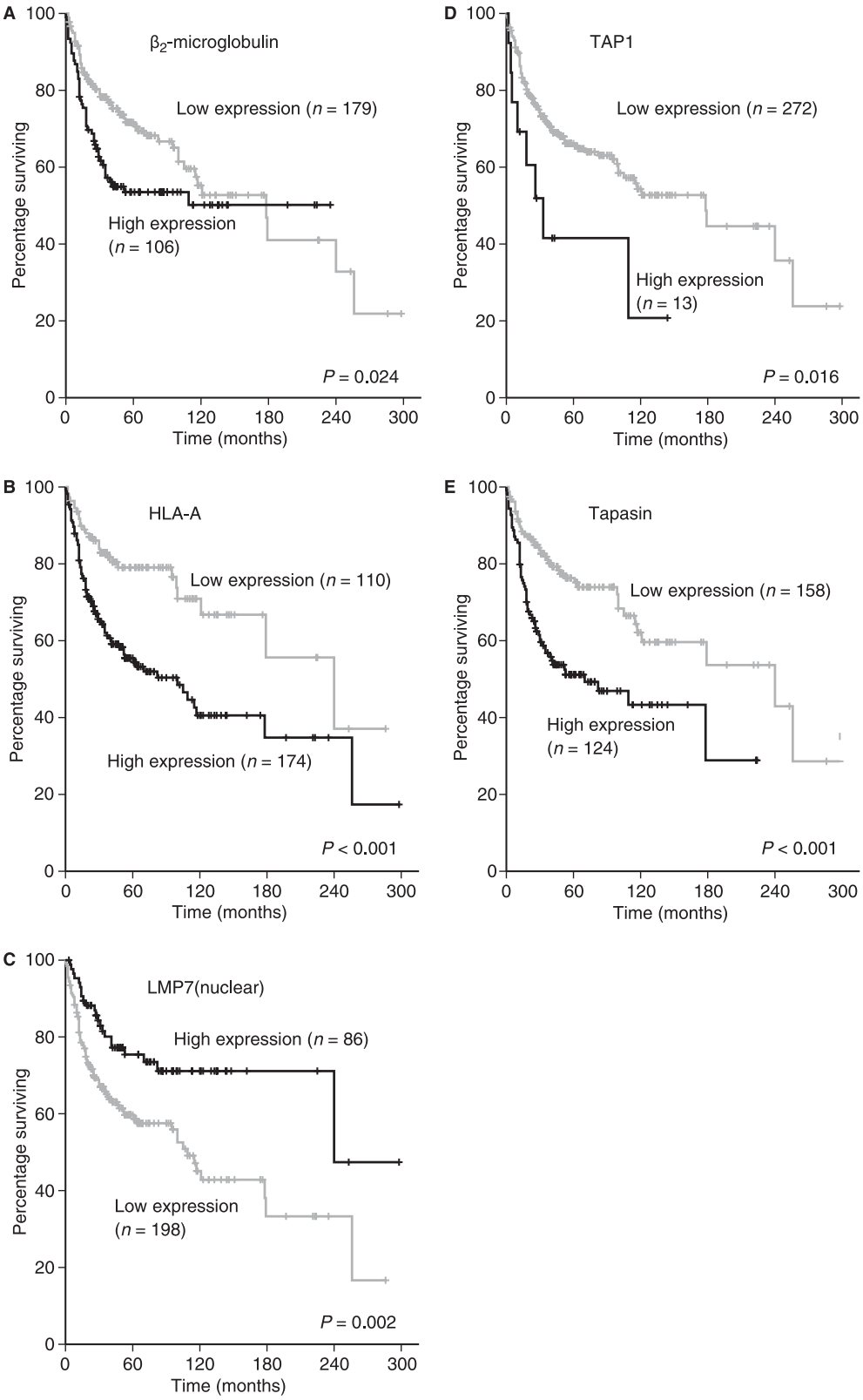

Figure 3. Univariate Kaplan–Meier analysis of the prognostic impact of MHC-I associated proteins in salivary gland carcinomas. **A**, β_2-Microglobulin. **B**, HLA-A. **C**, Nuclear expression of LMP7. **D**, TAP1. **E**, Tapasin. Log-rank statistics.

Table 2. Univariate analysis (Kaplan–Meier survival and Log rank statistics) and multivariate analysis (Cox regression with stepwise backwards selection): encountered *P* values*

Variable	Coding†	Univariate (*n* = 265) Log rank	Multivariate (*n* = 265) Step 1	Step 4	HR (95%CI)
Clinico-pathological parameters					
Age	>60 years (*n* = 142) versus ≤60 years (*n* = 123)	<0.001	–	–	–
Sex	Male (*n* = 129) versus female (*n* = 136)	0.002	–	–	–
Localisation	Major glands (*n* = 227) versus minor glands (*n* = 38)	0.02	–	–	–
Residual tumour	R1/R2 (*n* = 25) versus R0 (*n* = 240)	<0.001	–	–	–
Tumour specific parameters					
Local tumour	T3/T4 (*n* = 100) versus T1/T2 (*n* = 165)	0.001	0.088	0.062	1.459 (0.946–2.250)
Nodal metastasis	N1/N2/N3 (*n* = 77) versus N0 (*n* = 188)	<0.001	0.295	–	1.269 (0.813–1.981)
Distant metastasis	M1 (*n* = 24) versus M0 (*n* = 241)	<0.001	0.015	0.012	1.948 (1.140–3.330)
Grade	G2/G3 (*n* = 182) versus G1 (*n* = 83)	<0.001	<0.001	<0.001	4.107 (1.938–8.704)
Expression of MHC class I associated proteins					
LMP7 (nuclear)	High (*n* = 80) versus low expression (*n* = 185)	0.005	0.038	0.033	0.582 (0.349–0.970)
TAP1	High (*n* = 13) versus low expression (*n* = 252)	0.01	0.308	–	1.510 (0.684–3.331)
β_2-microglobulin	High (*n* = 99) versus low expression (*n* = 166)	0.028	0.825	–	0.947 (0.585–1.533)
Tapasin	High (*n* = 118) versus low expression (*n* = 147)	<0.001	0.045	0.024	1.700 (1.011–2.857)
HLA-A	High (*n* = 164) versus low expression (*n* = 101)	<0.001	0.028	0.014	1.947 (1.076–3.523)

*Abbreviations: *n* = Number of patients; HR = Hazard ratio; CI = Confidence interval.
†Negative prognostic parameter versus positive prognostic parameter except for variables with HR<1 (i.e. LMP7).

facilitates peptide loading; and (iv) antigen presentation—the trimeric complex comprising HLA class I HC, β_2-microglobulin and peptide is transported through the Golgi complex to the cell surface, and then presented to $CD8^+$ cytotoxic T cells.[10]

MHC class I antigen down-regulation or loss represents one important strategy that allows tumours to escape from immunosurveillance. Altered expression of APM components has been found in renal cell carcinoma,[38] bladder cancer,[39] squamous cell carcinoma of the head and neck,[36,40] breast cancer,[32] cervical cancer,[28] endometrial cancer,[41] and malignant melanoma,[34,42] and was often associated with disease progression, early disease relapse, or a high tumour grade.

Conflicting results were obtained in studies focusing on colorectal cancer. Whereas some authors detected down-regulation of HLA-A and other APM components in the majority of invasive tumours and precancerous lesions, such as high-grade intraepithelial neoplasia,[43] others found marked staining variability of HLA-A, HLA-B and HLA-C in tumours and normal colonic tissues, with up-regulation and down-regulation. The majority of tumour cells mimicked their normal counterparts; deviations from the expression pattern in the normal paired mucosa (both increases and decreases) correlated with poor survival.[44] In contrast to Atkins *et al.* [43], who found APM abnormalities to be associated with K-RAS mutation, a known negative prognostic factor in colorectal cancer, Menon *et al.*[45] demonstrated a correlation of down-regulated HLA-A expression with a better prognosis in colorectal cancer patients, and an inverse correlation of intraepithelial $CD8^+$ T-cell infiltration with HLA-A ($P = 0.04$) and HLA-B/C ($P = 0.04$) expression. Furthermore, a link between high levels of $CD57^+$ natural killer (NK) cell infiltration and down-regulated HLA-B/C expression ($P = 0.04$) exists. As the presence of $CD57^+$ NK cells was a positive prognostic factor, the association of HLA class I down-regulation with better prognosis might be related to the elimination of HLA class I-negative tumour cells by NK cells, thereby leading to attenuated tumour aggressiveness.[45] Immunohistochemical analysis of oesophageal cancer cases associated down-regulation of HLA class I HC with an unfavourable prognosis,[46] whereas up-regulation of TAP1 and β_2-microglobulin was found in the majority of cancer tissues. Interestingly, up-regulation of TAP1 appeared to be an independent negative prognostic factor ($P = 0.015$).

Whereas down-regulation of major APM components was also frequently detected in prostate cancer, disease progression and/or metastasis was associated with weak expression of calnexin in one study,[35] but with overexpression of β_2-microglobulin in another study.[47] These examples of prostate and colorectal cancer indicate that tumour progression and aggressiveness are not necessarily based on the general concept of APM breakdown, T-cell-based immune escape, and tumour growth.[10] Other immunological and non-immunological factors may also influence the course of the disease.

The present study is the first to analyze the major MHC class I APM components in a variety of SGC subtypes that are prone to low or high clinical aggressiveness. Regarding the expression of APM components, the different SGC entities clustered according to their histogenetic origin (Figure 2). Some markers, such as β_2-microglobulin and LMP2, were up-regulated in tumours of striated and excretory ducts, whereas loss of HLA-A, HLA class I HC and calreticulin was predominantly observed in tumours of the intercalated ducts. LMP7 and TAP2 were down-regulated more or less independently of tumour type. ERp57 expression was not significantly altered. Acinic cell carcinomas resembled tumours of the intercalated ducts in some respects (β_2-microglobulin), but tumours of the excretory ducts in others (LMP2 and TAP1). However, they seem to have their own signature as well: overexpression of calnexin, TAP2, and tapasin, which was not observed in other entities. This heterogeneous expression pattern might be attributable to the histogenetic heterogeneity of acinic cell carcinoma, with acinar, ductular and ductulo-acinar subtypes.[48]

With respect to the clinical outcome of the SGC patients, we demonstrated that down-regulation of nuclear LMP7 was associated with shorter survival (Table 2). In contrast, low expression of β_2-microglobulin, HLA-A, TAP1 and tapasin turned out to be positive prognostic factors—for HLA-A and tapasin this could also be demonstrated in multivariate analysis including tumour-specific parameters such as T, N and M stage, and tumour grade (Table 2).

HLA-A, β_2-microglobulin, TAP1 and tapasin were significantly associated with each other, forming a group of interdependent parameters in the APM. LMP7 regulation seemed to be independent of these parameters. As already mentioned, LMP7 is involved in peptide generation and trimming, whereas the other proteins are responsible for peptide transport, MHC class I assembly, and antigen presentation. The data indicate that peptide generation and trimming might either be independent of or inversely associated with later steps of the APM. They also illustrate that

SGC lesions do not fit into the model of APM breakdown, immune escape and tumour progression that has been well established in renal cell carcinoma and malignant melanoma,[34,38,42] but behave more like colorectal carcinomas, which are also known to constitute a heterogeneous group of tumours with microsatellite stable and unstable types, and mucinous and medullary types.[43,45]

The negative prognostic impact of nuclear down-regulation of LMP7 was recently reported in ovarian cancer: high nuclear expression was associated with better disease-specific survival.[29] Differences in the compartmental localization of LMP7 have been described previously, and are mainly attributable to the fact that LMP2 and LMP7 subunits are synthesized as precursor proteins of 24 kDa and 30 kDa, respectively, and that only the processed 21-kDa and 23-kDa subunits form part of the 20S proteasome complex.[49] The presence of LMP2 and LMP7 in 26S proteasomes (but to a lesser extent than in 20S proteasomes) is consistent with a role for 26S proteasomes and, possibly, ubiquitin-dependent proteolysis in antigen processing.[50] It has been demonstrated that 26S proteasomes are relatively more abundant in the nuclei of mammalian cells, and that they are distributed throughout the nucleus.[51] Therefore differences in the nuclear expression of LMP2 and LMP7 might be more significant than variations in the cytoplasmic expression.

To summarize, the present study demonstrates that the histogenetic and morphological heterogeneity of SGC goes along with heterogeneous patterns of alterations of the APM machinery. Regardless of the different entities, down-regulation of LMP7 and up-regulation of HLA-A and tapasin were independent and statistically significant markers of poor prognosis in multivariate survival analyzes of the present cohort of 288 salivary gland carcinomas. These findings might provide a rationale for targeting the specific components of the antigen processing and presentation pathway in the management of this disease.

Acknowledgements

The authors thank Mr Rudolf Jung for excellent technical assistance. They also thank Dr Sabrina Petsch, Tumour Center Erlangen-Nuremberg, and Dr Monika Klinkhammer-Schalke, Tumour Center Regensburg, for providing the clinical follow-up data of the patients. This work was sponsored by the DFG-581-9-1 (B. Seliger).

References

1. Risk M, Corman JM. The role of immunotherapy in prostate cancer: an overview of current approaches in development. *Rev. Urol.* 2009; **11**; 16–27.
2. Rao B, Han M, Wang L *et al.* Clinical outcomes of active specific immunotherapy in advanced colorectal cancer and suspected minimal residual colorectal cancer: a meta-analysis and system review. *J. Transl. Med.* 2011; **9**; 17.
3. Speetjens FM, Zeestraten EC, Kuppen PJ, Melief CJ, van der Burg SH. Colorectal cancer vaccines in clinical trials. *Expert Rev. Vaccines* 2011; **10**; 899–921.
4. Genetzakis M, Gomatos IP, Georgiou AN *et al.* BCL-2, p53 and HLA-DR antigen expression in surgically treated parotid cancer patients. *Eur. Arch. Otorhinolaryngol.* 2009; **266**; 417–424.
5. Hamano H, Haneji N, Yanagi K, Ishimaru N, Hayashi Y. Expression of HLA-DR and cytokine genes on interferon-gamma-stimulated human salivary gland cell line. *Pathobiology* 1996; **64**; 255–261.
6. Ogawa Y, Hong SS, Toyosawa S, Chang CK, Yagi T. Expression of major histocompatibility complex class II antigens and interleukin-1 by epithelial cells of Warthin's tumor. *Cancer* 1990; **66**; 2111–2117.
7. Thrane PS, Sollid LM, Brandtzaeg P. Abundant dendritic cells express HLA-DR in pleomorphic adenomas. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* 1990; **59**; 195–203.
8. Garrido F, Ruiz-Cabello F, Cabrera T *et al.* Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* 1997; **18**; 89–95.
9. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.* 2000; **74**; 181–273.
10. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today* 2000; **21**; 455–464.
11. Seliger B. Different regulation of MHC class I antigen processing components in human tumors. *J. Immunotoxicol.* 2008; **5**; 361–367.
12. Barnes L, Eveson JW, Reichart P, Sidransky D eds. *Pathology and Genetics of Head and Neck Tumours. World Health Organization Classification of Tumours*. Lyon: IARC Press, 2005; 430.
13. Sobin LH, Gospodarowicz MK, Wittekind C, International Union against Cancer. *TNM Classification of Malignant Tumours*, 7th ed. Chichester: Wiley-Blackwell, 2010.
14. Jouzdani E, Yachouh J, Costes V *et al.* Prognostic value of a three-grade classification in primary epithelial parotid carcinoma: result of a histological review from a 20-year experience of total parotidectomy with neck dissection in a single institution. *Eur. J. Cancer* 2010; **46**; 323–331.
15. Therkildsen MH, Christensen M, Andersen LJ, Schiodt T, Hansen HS. Salivary gland carcinomas—prognostic factors. *Acta Oncol.* 1998; **37**; 701–713.
16. Goode RK, El-Naggar AK. Mucoepidermoid carcinoma. In Barnes L, Eveson JW, Reichart P, Sidransky D eds. *Pathology and Genetics of Head and Neck Tumours. World Health Organization Classification of Tumours*. Lyon: IARC Press, 2005; 219–220.
17. Elston EW, Ellis IO. Method for grading breast cancer. *J. Clin. Pathol.* 1993; **46**; 189–190.
18. Milanes-Yearsley M, Hammond ME, Pajak TF *et al.* Tissue micro-array: a cost and time-effective method for correlative studies by regional and national cancer study groups. *Mod. Pathol.* 2002; **15**; 1366–1373.

19. Ettl T, Stiegler C, Zeitler K, *et al.* EGFR, HER2, survivin, and loss of pSTAT3 characterize high-grade malignancy in salivary gland cancer with impact on prognosis. *Hum. Pathol.* 2012; **43**; 921–931.
20. Stam NJ, Spits H, Ploegh HL. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 1986; **137**; 2299–2306.
21. Perosa F, Luccarelli G, Prete M, Favoino E, Ferrone S, Dammacco F. Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J. Immunol.* 2003; **171**; 1918–1926.
22. Lampson LA, Fisher CA, Whelan JP. Striking paucity of HLA-A, B, C and beta 2-microglobulin on human neuroblastoma cell lines. *J Immunol* 1983; **130**; 2471–2478.
23. Bandoh N, Ogino T, Cho HS *et al.* Development and characterization of human constitutive proteasome and immunoproteasome subunit-specific monoclonal antibodies. *Tissue Antigens* 2005; **66**; 185–194.
24. Wang X, Campoli M, Cho HS *et al.* A method to generate antigen-specific mAb capable of staining formalin-fixed, paraffin-embedded tissue sections. *J. Immunol. Methods* 2005; **299**; 139–151.
25. Ogino T, Wang X, Kato S, Miyokawa N, Harabuchi Y, Ferrone S. Endoplasmic reticulum chaperone-specific monoclonal antibodies for flow cytometry and immunohistochemical staining. *Tissue Antigens* 2003; **62**; 385–393.
26. Temponi M, Kageshita T, Perosa F, Ono R, Okada H, Ferrone S. Purification of murine IgG monoclonal antibodies by precipitation with caprylic acid: comparison with other methods of purification. *Hybridoma* 1989; **8**; 85–95.
27. Ettl T, Baader K, Stiegler C, *et al.* Loss of PTEN is associated with elevated EGFR and HER2 expression and worse prognosis in salivary gland cancer. *Br. J. Cancer* 2012; **106**; 719–726.
28. Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren GJ. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol. Immunother.* 2008; **57**; 197–206.
29. Leffers N, Gooden MJ, Mokhova AA *et al.* Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. *Gynecol. Oncol.* 2009; **113**; 256–263.
30. van Endert PM. Genes regulating MHC class I processing of antigen. *Curr. Opin. Immunol.* 1999; **11**; 82–88.
31. Chapman DC, Williams DB. ER quality control in the biogenesis of MHC class I molecules. *Semin. Cell Dev. Biol.* 2010; **21**; 512–519.
32. Vitale M, Rezzani R, Rodella L *et al.* HLA class I antigen and transporter associated with antigen processing (TAP1 and TAP2) down-regulation in high-grade primary breast carcinoma lesions. *Cancer Res.* 1998; **58**; 737–742.
33. Murray PG, Constandinou CM, Crocker J, Young LS, Ambinder RF. Analysis of major histocompatibility complex class I, TAP expression, and LMP2 epitope sequence in Epstein–Barr virus-positive Hodgkin's disease. *Blood* 1998; **92**; 2477–2483.
34. Kageshita T, Hirai S, Ono T, Hicklin DJ, Ferrone S. Down-regulation of HLA class I antigen-processing molecules in malignant melanoma: association with disease progression. *Am. J. Pathol.* 1999; **154**; 745–754.
35. Seliger B, Stoehr R, Handke D *et al.* Association of HLA class I antigen abnormalities with disease progression and early recurrence in prostate cancer. *Cancer Immunol. Immunother.* 2009; **59**; 529–540.
36. Meissner M, Reichert TE, Kunkel M *et al.* Defects in the human leukocyte antigen class I antigen processing machinery in head and neck squamous cell carcinoma: association with clinical outcome. *Clin. Cancer Res.* 2005; **11**; 2552–2560.
37. Vigneron N, Peaper DR, Leonhardt RM, Cresswell P. Functional significance of tapasin membrane association and disulfide linkage to ERp57 in MHC class I presentation. *Eur. J. Immunol.* 2009; **39**; 2371–2376.
38. Atkins D, Ferrone S, Schmahl GE, Storkel S, Seliger B. Down-regulation of HLA class I antigen processing molecules: an immune escape mechanism of renal cell carcinoma? *J. Urol.* 2004; **171**; 885–889.
39. Cathro HP, Smolkin ME, Theodorescu D, Jo VY, Ferrone S, Frierson HF Jr. Relationship between HLA class I antigen processing machinery component expression and the clinicopathologic characteristics of bladder carcinomas. *Cancer Immunol. Immunother.* 2010; **59**; 465–472.
40. Ogino T, Bandoh N, Hayashi T, Miyokawa N, Harabuchi Y, Ferrone S. Association of tapasin and HLA class I antigen down-regulation in primary maxillary sinus squamous cell carcinoma lesions with reduced survival of patients. *Clin. Cancer Res.* 2003; **9**; 4043–4051.
41. Bijen CB, Bantema-Joppe EJ, de Jong RA *et al.* The prognostic role of classical and nonclassical MHC class I expression in endometrial cancer. *Int. J. Cancer* 2010; **126**; 1417–1427.
42. Seliger B, Ritz U, Abele R *et al.* Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. *Cancer Res.* 2001; **61**; 8647–8650.
43. Atkins D, Breuckmann A, Schmahl GE *et al.* MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. *Int. J. Cancer* 2004; **109**; 265–273.
44. Benevolo M, Mottolese M, Piperno G *et al.* HLA-A, -B, -C expression in colon carcinoma mimics that of the normal colonic mucosa and is prognostically relevant. *Am. J. Surg. Pathol.* 2007; **31**; 76–84.
45. Menon AG, Morreau H, Tollenaar RA *et al.* Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab. Invest.* 2002; **82**; 1725–1733.
46. Tanaka K, Tsuchikawa T, Miyamoto M *et al.* Down-regulation of human leukocyte antigen class I heavy chain in tumors cancer patients. *Int. J. Oncol.* 2011; **40**; 965–974.
47. Huang WC, Wu D, Xie Z *et al.* Beta2-microglobulin is a signaling and growth-promoting factor for human prostate cancer bone metastasis. *Cancer Res.* 2006; **66**; 9108–9116.
48. Schwarz S, Zenk J, Muller M *et al.* The many faces of acinic cell carcinomas of the salivary glands: a study of 40 cases relating histological and immunohistological subtypes to clinical parameters and prognosis. *Histopathology* 2012; **61**; 395–408.
49. Frentzel S, Kuhn-Hartmann I, Gernold M, Gott P, Seelig A, Kloetzel PM. The major-histocompatibility-complex-encoded beta-type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. *Eur. J. Biochem.* 1993; **216**; 119–126.
50. Rock KL, Goldberg AL. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* 1999; **17**; 739–779.
51. Brooks P, Fuertes G, Murray RZ *et al.* Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem. J.* 2000; **346**(Pt 1); 155–161.