Defects in the Human Leukocyte Antigen Class I Antigen-Processing Machinery in Head and Neck Squamous Cell Carcinoma: Association with Clinical Outcome

Markus Meissner,[1,3] Torsten E. Reichert,[2] Martin Kunkel,[2] William Gooding,[5] Theresa L. Whiteside,[6] Soldano Ferrone,[7] and Barbara Seliger,[1,4]

Abstract

Purpose: Human leukocyte antigen (HLA) class I antigen defects, which are frequently present in head and neck squamous cell carcinoma (HNSCC) cells may provide the tumor with an escape mechanism from immune surveillance. Scanty information is available about mechanisms underlying HLA class I antigen defects in both lesions and cell lines from HNSCC. In this study, we investigate the role of antigen processing machinery (APM) component abnormalities in the generation of deficient HLA class I surface expression of HNSCC cells.

Experimental Design: Using immunohistochemistry, Western blot, and RT-PCR analyses we correlated the expression of the IFN-γ inducible proteasome subunits and of the peptide transporter TAP with that of HLA class I antigens in biopsies and cell lines from primary, recurrent, and metastatic HNSCC. Furthermore, APM component and HLA class I antigen expression in surgically removed lesions were correlated with the course of the disease in order to assess the clinical significance of deficient expression of these molecules.

Results: A high frequency of LMP2, LMP7, and TAP1 down-regulation or loss was found in tumor lesions and cell lines obtained from HNSCC cancer patients. These defects could be corrected by incubating cells with IFN-γ. Furthermore, LMP2, LMP7, TAP1, TAP2, and HLA class I antigen expression rates in primary HNSCC lesions were found to predict overall survival. Lastly, the level of LMP7 expression was significantly associated with disease recurrence at 2 years.

Conclusions: Our results suggest that the analysis of APM component expression in HNSCC lesions can provide useful prognostic information in patients with HNSCC.

Effective CTL responses require adequate MHC class I antigen surface expression. This mechanism accounts for the negative impact of MHC class I antigen expression abnormalities, which are frequently found in many different types of human tumors, on tumor immunosurveillance, and in the course of the disease as well as on the outcome of T cell–based immunotherapies in malignancies (1–3). Abnormalities in the MHC class I phenotype of tumors are caused by distinct molecular defects, which interfere with several known steps within the antigen processing pathway. These include structural alterations, methylation, or dysregulation of genes coding for the MHC class I heavy chain and/or β_2-microglobulin as well as of those coding for antigen processing machinery (APM) components, such as IFN-γ inducible immunoproteasome subunits LMP2 and LMP7, the peptide transporter TAP, and tapasin (3–5).

Head and neck cancer of the oral cavity, pharynx, and larynx represents only about 6% of newly diagnosed cancers in the United States (6), but is more common in other parts of the world. Despite advances in therapeutic procedures, the benefit of surgery, radiotherapy, and chemotherapy for patients with head and neck squamous cell carcinoma (HNSCC) has not improved in the last three decades (7). The high rate of recurrence, grave functional implications of radical surgery resulting in morbidity and a poor 5-year-survival rate have emphasized the need for alternative therapies for HNSCC patients. T cell–based immunotherapy represents one such alternative, which aims at the induction and/or activation of human leukocyte antigen (HLA) class I antigen–restricted, tumor-associated antigen (TAA)–specific CTL (8, 9). Recent evidence suggests that HNSCC may be a suitable target for T cell–based immunotherapy (10).

One major prerequisite for the successful implementation of such an immunotherapy is the appropriate surface expression of MHC class I molecules on tumor cells. Thus, MHC class I antigen expression was studied in surgically removed HNSCC

Authors' Affiliations: Departments of [1]Internal Medicine and [2]Oral and Maxillofacial Surgery, Johannes Gutenberg-University, Mainz; [3]Department of Dermatology, Johann Wolfgang Goethe-University, Frankfurt; [4]Institute of Medical Immunology, Martin-Luther University, Halle, Germany; [5]Division of Biostatistics and [6]Departments of Pathology and Otolaryngology, School of Medicine and Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania; and [7]Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York

Received 10/20/04; revised 1/5/05; accepted 1/11/05.

Grant support: Deutsche Forschungsgemeinschaft grant SFB432 project A5 (B. Seliger) and project A11 (T. Reichert), Boehringer Ingelheim Fund (B. Seliger) and by PHS grants R01 CA67108 (S. Ferrone), and PO-1DE 12321 (T.L. Whiteside) awarded by the National Cancer Institute. This paper represents a part of the doctoral thesis of M. Meissner.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Barbara Seliger, Institute of Medical Immunology, Martin-Luther University, Magdeburger Str. 2, 06112 Halle, Germany. Phone: 49-345-557-4054; Fax: 49-345-557-4055; E-mail: Barbara.Seliger@medizin.uni-halle.de.

lesions using, in most cases, monoclonal antibodies (mAb), which recognize monomorphic determinants shared by various HLA class I molecules or in a limited number of samples of mAb which recognize allele-specific determinants (11–13). Aberrant HLA class I surface expression accompanied by a significant down-regulation or loss of certain HLA class I alleles (14) as well as by a loss of heterozygosity at chromosome 6p21 (15, 16) has been frequently found in head and neck cancer lesions. The HLA class I allelic loss in HNSCC lesions was significantly correlated with the presence of lymph node metastases, HLA class I defects in the adjacent normal mucosa and subsequent development of new primary cancers (14, 17, 18). In contrast, β_2-microglobulin mutations have not been detected in HNSCC with defective MHC class I antigen surface expression (19).

Altered HLA class I antigen surface expression is likely to play a major role in the escape of HNSCC cells from immune recognition. Furthermore, this mechanism may account for the difficulties in generating HLA class I antigen–restricted, TAA-specific CTL from peripheral blood mononuclear cells of patients with HNSCC (20–25). Therefore, characterization of the molecular defects underlying HLA class I antigen expression abnormalities in HNSCC cells is not only important from the biological viewpoint, but it may also have substantial clinical relevance. Nevertheless, only scanty information is available in the literature in this regard (26). In particular, the role of APM component abnormalities in defective HLA class I antigen surface expression rates by HNSCC cells has been investigated only to a limited extent (27). The lack of information about the mechanisms underlying HLA class I antigen abnormalities has a negative impact on our ability to design strategies to correct these defects in HNSCC lesions. Therefore, in the present study, we have examined a series of surgically removed HNSCC lesions and HNSCC cell lines for both the constitutive and IFN-γ-inducible expression levels of various APM components, such as proteasome subunits, TAP subunits, calnexin, tapasin, calnexin, HLA class I heavy chain, and β_2-microglobulin. In addition, we have correlated the APM component expression rates in HNSCC lesions with their histopathologic characteristics and with patients' survival. Thus, this study provides insights into molecular mechanisms underlying APM deficiencies in HNSCC lesions and, for the first time, assesses their association with patients' survival.

Materials and Methods

Cell lines, cell culture, and cytokine treatment. With the exception of the HNSCC cell line SCC-4, which was purchased from American Type Culture Collection (CRL-1624), all other tumor cell lines studied were established from patients with HNSCC at the University of Pittsburgh and have been described elsewhere (24, 27). The characteristics of the HNSCC cell lines in terms of tumor localization, stage, and grade as well as HLA phenotype determined by DNA typing are summarized in Table 1. The human keratinocyte cell line HaCaT (28) and the renal cell carcinoma cell line MZ1257RC (29) served as controls. All cell lines were maintained in DMEM (Biochrom KG, Berlin, Germany) supplemented with 10% FCS (Greiner, Munich, Germany), 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin (Life Technologies, Karlsruhe, Germany) at 37°C in an atmosphere of 5% CO_2 in air.

Patients and tissue samples. The study group consisted of a sample from all patients between the ages of 37 and 79 who had a histologically confirmed diagnosis of HNSCC at the Department of Oral and Maxillofacial Surgery at the Johannes Gutenberg University, Mainz, between 1999 and 2001. From a total of 75 patients meeting these criteria, a sample of 25 was randomly selected. Twenty of the 25 patients were treated with surgical resection for primary diseases; the remaining 5 underwent surgery for recurrent disease. Each tumor was histopathologically classified according to the WHO classification system. The TNM status, tumor stage, grade, localization, and lymph node involvement of all the studied patients are listed in Table 2.

Surgically removed biopsies were immediately snap-frozen in liquid nitrogen and stored at −70°C for immunohistochemical staining. In addition, paraffin-embedded HNSCC tissues were available from which autologous tumor cell lines had been established. Tonsils were used as control tissues. The study was approved by the Institutional Review Committee at the University of Mainz. Informed consent was obtained from all patients enrolled in this study.

Monoclonal and polyclonal antibodies. The mouse mAb W6/32, which recognizes a framework determinant expressed on β_2-microglobulin associated HLA-A, -B, and -C heavy chains, the mAb HC10 recognizing HLA class I heavy chains, the mouse anti-TAP1 mAb 148.3, the mouse anti-TAP2 mAb 429.4, and the mouse anti-tapasin mAb have been described elsewhere (30–33). The mouse anti-PA28, mouse anti-calnexin, and mouse anti-calreticulin as well as the rabbit anti-LMP2 and anti-LMP7 antibodies were purchased from Affinity (Mamhead, United Kingdom). FITC-conjugated anti-HLA-A, -B, -C mAb, an IgG_{2a}, and an irrelevant FITC-conjugated mAb IgG_{2a} were purchased from Beckman/Coulter, Krefeld, Germany. Alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibodies and peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies were purchased from Dako (Hamburg, Germany). All antibodies were stored at 4°C or at −20°C and were diluted with sterile PBS (pH 7.2) before use.

Human recombinant IFN-γ. IFN-γ (specific activity of 3×10^6 units/mg protein) was purchased from Boehringer Ingelheim, Ingelheim, Germany.

RT-PCR analyses. Total cellular RNA was extracted using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) followed by DNase digestion according to the manufacturer's instructions. The different primer sets for APM components used for PCR and the PCR conditions employed were recently described elsewhere (30, 34).

Western blot analyses. Protein was extracted from 1 to 5×10^6 HNSCC cells which were either left untreated or treated with 20 μg/mL IFN-γ for 24 hours at 37°C. Protein (40 μg per lane) was analyzed by Western blots analyses as recently described (30). Filters were incubated overnight with the anti-human (mAb) specific for TAP1 (mAb 148.3; ref. 31), TAP2 (mAb 429.4; ref. 33), PA28α, calnexin, calreticulin, or the polyclonal rabbit anti-human LMP2 antibody (Affinity). After extensive washing with TBS/0.1% Tween 20, membranes were incubated for 30 to 45 minutes either with a horseradish peroxidase–conjugated rabbit anti-mouse immunoglobulin antibody (Dako) for TAP1, TAP2, PA28α, calnexin, calreticulin, or with a peroxidase-conjugated goat anti-rabbit immunoglobulin antibody as secondary antibody (Dako) for LMP2. After rinsing twice, filters were developed using nitroblue tetrazolium/X-phosphate (Roche Diagnostics, Mannheim, Germany) or the enhanced chemiluminescence system (ECL, Amersham, Braunschweig, Germany) as substrate.

Flow cytometry. Indirect immunofluorescence staining of cells was done as previously described (5). Briefly, 5×10^5 cells were incubated with the appropriate concentration of FITC-conjugated anti-HLA-A, -B, -C mAb for 30 minutes at 4°C. An irrelevant FITC-conjugated mAb IgG_{2a} served as a control. Stained cells were analyzed on a flow cytometer (Epics XL MCL, Beckman/Coulter). The results were expressed as mean specific fluorescence intensity.

Immunohistochemistry. Snap-frozen HNSCC biopsies embedded in the OCT compound (Tissue-Tek, Sakura, Tokyo, Japan) as well as paraffin-embedded HNSCC tissues from which tumor cell lines were available were serially cut into 5 μm sections on a Leica CM1900 cryostat and mounted on electrostatically precharged slides (Superfrost Plus; Menzel-Gläser, Frankfurt, Germany). Consecutive sections from paraffin-embedded tissues were deparaffinized and rehydrated. After

Table 1. Characteristics of HNSCC cell lines and their clinicopathologic parameters

Cell lines	Tumor localization	Type of lesion	TNM	Grading	Patient survival (months)	HLA phenotype	MHC class I expression (mean specific fluorescence intensity)	IFN-γ induction *x*-fold
PCI-1	larynx	recurrence	$T_2N_0M_0$	moderately well differentiated	137	A1, A2, B8, B27, Cw1, Cw7	86	1.6
PCI-4B	larynx	involved lymph node	$T_2N_2M_0$	moderately well differentiated	117	A24, A26, B35, B44, Cw4, C14	71	1.6
PCI-13	RMT	primary	$T_4N_1M_0$	poorly differentiated	107	A2, A3, B7, B35, Cw12, Cw15	25	2.4
PCI-15A	pyriform sinus	primary	$T_2N_1M_0$	poorly differentiated	10	A3, B7, Cw7	40	1.9
PCI-15B	pyriform sinus	involved lymph node	$T_2N_1M_0$	poorly differentiated	10	A3, B7, Cw7	63	1.6
PCI-30	base of tongue	primary	$T_3N_1M_0$	moderately well differentiated	6	A2, A28, B49, B53, Cw4, Cw7	44	1.4
PCI-52	AE fold	primary	$T_2N_0M_0$	moderately well differentiated	49	A23, B58, B82, Cw6,Cw10	17	1.5
SCC-4	tongue	n.a.	n.d.	n.d	n.d.	A2, B7, B44, Cw4, Cw7	25	1.8
SCC-68	tongue	primary	$T_4N_0M_0$	well differentiated	47	A1, A25, B18, B40, Cw10(3), Cw12	72	1.7
SCC-210	tongue	primary	$T_4N_0M_0$	poorly differentiated	4	A31, B40, B51, Cw3,Cw15	58	1.6
HaCaT	keratinocytes	—	—	—	—	n.d	113	1.5
MZ1257RC	renal cell carcinoma	primary	n.a.	moderately differentiated	>100	A2, A3, B7, B44, Cw5, Cw7	136	1.5

NOTE: Ten HNSCC cell lines established from primary, recurrent, and metastatic lesions from patients with HNSCC were analyzed for HLA phenotype, histopathologic characteristics, and expression of MHC class I surface antigens. The ability of IFN-γ to induce expression of MHC class I molecules (as described in Materials and Methods) is also listed. Survival of the patients (in months) from whose tumors the lines were established is listed as well. n.a., not available; n.d., not determined.

endogenous peroxidase quenching (3% H_2O_2 in PBS for 30 minutes), antigens were retrieved by microwave treatment.

Immunohistochemical staining was done at room temperature using the avidin-biotin technique with mAb and rabbit antibodies. Optimal working dilutions of the primary antibodies were determined in titration experiments using human tonsils. Staining was developed with the standard streptavidin-biotin-immunoperoxidase technique (LSAB 2 system-HRP, Dako) and visualized with 3,3′-diaminobenzidine tetrahydrochloride (Dako). The slides were counterstained with hematoxylin (Merck, Darmstadt, Germany) and mounted after dehydration in Eukitt (Kindler, Freiburg, Germany). All immunostained sections were coded and separately examined in a light microscope by two investigators (T.E. Reichert and M. Kunkel). Natural stromal cells and tumor-infiltrating lymphocytes were used as positive controls. Results were scored as positive, heterogeneous, or negative, when the percentage of stained tumor cells in each section was >75%, between 25% and 75%, and <25%, respectively. The staining intensity of nonmalignant oral epithelium adjacent to the carcinoma was used as a reference to define strong and weak staining intensity of HNSCC cells. Negative controls were done by omitting primary antibodies.

Statistical analysis. Overall survival and presence of disease recurrence were analyzed by a series of nonparametric tests for each of the five markers analyzed (LMP2, LMP7, TAP1, TAP2, and HLA class I antigens). The analysis was limited to 19 patients treated for primary disease having adequate follow-up data. Five patients treated with recurrent disease and one patient with unknown survival data were excluded.

(*a*) The expression level scores were dichotomized by combining scores of 0 and 1 or 1 and 2, depending on the score frequencies. A log rank test was applied to the dichotomized data. (*b*) The expression level scores were used as is and tested with an exact log rank test. Monte-Carlo sampling was used to simulate the permutation distribution. (*c*) Patients were classified as either disease-free or with recurrent disease 2 years after surgery. The Cochran-Armitage trend test was then used to identify a trend in the proportion of patients with recurrent disease by expression level score. All tests resulting in *P* values were adjusted by the step-down Bonferroni procedure.

Peptide transport assay. The peptide translocation experiments were done as recently described using the [^{125}I]-labeled model peptides, RYWANATRSI and TNKTRIDGQY (35) which were kindly provided by F. Momburg (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Results

Antigen processing machinery component and human leukocyte antigen class I antigen down-regulation in head and neck squamous cell carcinoma lesions. The histopathologic characteristics of 25 surgically removed tumor lesions obtained from patients with either primary, recurrent, or metastatic HNSCC as

Table 2. Clinicopathologic characteristics of patients with HNSCC and APM component expression in the malignant lesions

No.	Age	Gender	Tumor/site	TNM	Stage	Grade	Clinical status	Survival/death (months)	MHC I	TAP1	TAP2	LMP2	LMP7
1	43	M	floor of the mouth	$T_4pN_1M_0$	4	3	local recurrence	Death (7)	0	0	0	1	1
2	68	M	tongue	$T_1N_0M_0$	4	2	second primary tumor	Alive (29)	1	2	1	1	1
3	64	F	tongue	$T_4N_1M_0$	4	2	distant metastasis	Death (38)	1	2	1	1	2
4	52	M	palate/tongue	$T_4N_{2b}M_0$	4	1	NED	Alive (28)	2	1	1	2	2
5	62	M	tongue (no curative)*	$T_4N_{2b}M_0$	4	3	local progression	Death (n.d.)	2	1	2	1	1
6	55	M	mandible	$T_4N_{2b}M_0$	4	2	NED	Alive (25)	2	2	1	2	2
7	49	M	retromolar trigone	$T_4N_1M_0$	4	2	NED	Alive (31)	2	2	2	1	2
8	74	F	tongue	$T_1N_0M_0$	1	3	local recurrence	Death (19)	2	2	1	1	1
9	37	F	tongue (recurrent)*	$T_1N_1M_0$	3	3	local recurrence	Death (25)	1	0	1	1	1
10	53	F	retromolar trigone	$T_4N_{2b}M_0$	4	2	local recurrence	Death (n.d.)	2	1	1	1	2
11	48	M	tongue	$T_2N_1M_0$	3	2	neck failure	Death (21)	1	0	0	1	1
12	43	M	palate	$T_4N_0M_0$	2	2	NED	Alive (47)	2	2	2	1	2
13	56	M	tongue	$T_4N_{2b}M_0$	4	2	local recurrence	Death (13)	2	2	1	0	1
14	56	F	tongue (recurrent)*	$T_1N_0M_0$	1	2	second primary tumor	Alive (96)	2	1	0	1	1
15	42	F	palate/retromolar trigone	$T_4N_0M_0$	4	2	local recurrence	Death (10)	0	1	0	0	1
16	61	M	mandible	$T_4N_0M_0$	4	1	NED	Alive (55)	1	1	1	1	1
17	72	M	floor of the mouth	$T_4N_0M_0$	4	2	local recurrence	Death (28)	1	0	0	1	0
18	59	M	tongue (no curative)*	$T_3N_{2c}M_0$	4	n.d.	local progression	Death (n.d.)	2	2	1	1	2
19	56	M	tongue	$T_3N_0M_0$	3	2	NED	Alive (38)	2	2	1	1	2
20	66	M	palate	$T_4N_0M_0$	4	2	local recurrence	Death (22)	0	0	0	1	0
21	45	M	retromolar trigone	$T_4N_{2b}M_0$	4	2	NED	Alive (54)	2	2	1	2	2
22	79	F	floor of the mouth	$T_4N_{2b}M_0$	4	2	local recurrence	Death (15)	1	2	1	0	0
24	60	M	tongue (no curative)*	$T_4N_2M_0$	4	2	local progression	Death (7)	1	0	0	1	1
25	70	M	retromolar trigone	$T_1N_2M_0$	4	2	local progression	Death (15)	1	0	0	0	0

NOTE: Twenty-five surgically resected lesions from patients with HNSCC were analyzed for histopathologic characteristics and for the HLA class I antigen and APM component expression as described in Materials and Methods. Patients' survival in months from the date of the surgery is listed in parentheses. n.d.: not determined.
*Excluded from survival analysis.

well as the patients' survival time are summarized in Table 2. Immunohistochemical staining of snap-frozen tumor lesions and autologous normal epithelium of HNSCC patients with APM component–specific antibodies showed a highly variable expression profile for LMP2, LMP7, TAP1, TAP2, and HLA class I antigens (Figs. 1 and 2) ranging from a total loss to heterogeneous but decreased expression levels. Specifically, LMP2 was not detectable in 16% and down-regulated in 72% of the lesions. LMP7 was not detectable in 16% and down-regulated in 44% of the lesions. TAP1 was not detectable in 28% and down-regulated in 24% of the lesions. TAP2 was not detectable in 32% and down-regulated in 56% of the lesions. HLA class I antigens were not detectable in 12% and down-regulated in 36% of the lesions (Table 2). The three tumors with a total HLA class I antigen loss also lacked TAP protein expression, suggesting that in these lesions, deficient peptide translocation may be the molecular mechanism underlying this lack of HLA class I surface expression. A combined deficiency of various APM components was found in 80% of the tumor specimens analyzed. A coordinated down-regulation of the various proteasome subunits, TAP, and HLA class I antigens was the most frequent phenotype of the tumors studied (Table 2).

Additional analyses assessed the clinical significance of APM component down-regulation patterns in HNSCC lesions. For this study, only 19 lesions were utilized, since 5 tumor lesions were from surgically resected recurrent disease, and no information was available about the survival of one HNSCC patient. Clinical, pathologic, and survival data obtained from the 19 HNSCC patients were examined for association with the expression of LMP2, LMP7, TAP1, TAP2 and HLA class I antigens using three nonparametric tests for each of these five markers. The expression of all five markers was associated with patients' overall survival (Fig. 3); the association was stronger for LMP2, LMP7, and TAP2 expression with adjusted *P* values according to the two-group log rank test of 0.005, 0.045, and 0.0044, respectively than for TAP1 and HLA class I antigen expression with adjusted *P* values of 0.0775 and 0.0580, respectively (Table 3). In summary, the high

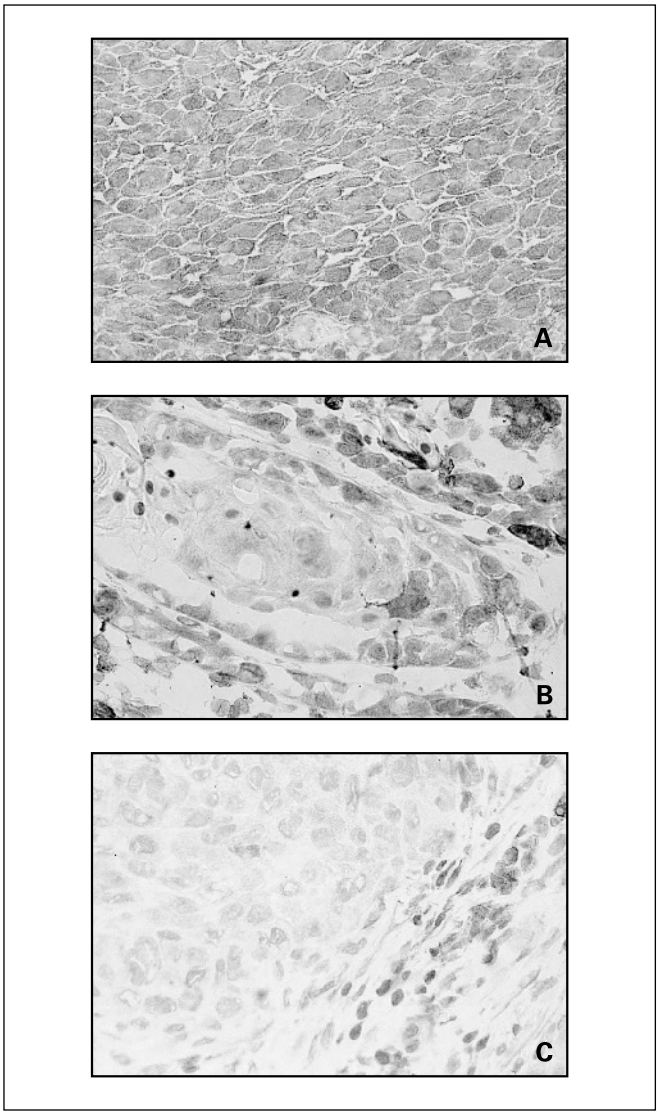

Fig. 1. Immunohistochemical analysis of HNSCC lesions with HLA class I antigen – specific mAb. Representative immunoperoxidase staining patterns of frozen sections of three distinct HNSCC lesions with HLA class I antigen – specific mAb W6/32. *A*, strong expression of HLA class I antigens in almost all tumor cells. *B*, heterogeneous expression of HLA class I antigens in HNSCC cells. *C*, low HLA class I antigen expression in HNSCC cells. The tumor-infiltrating lymphocytes in (*C*) show normal expression of HLA class I molecules. Original magnification (×400).

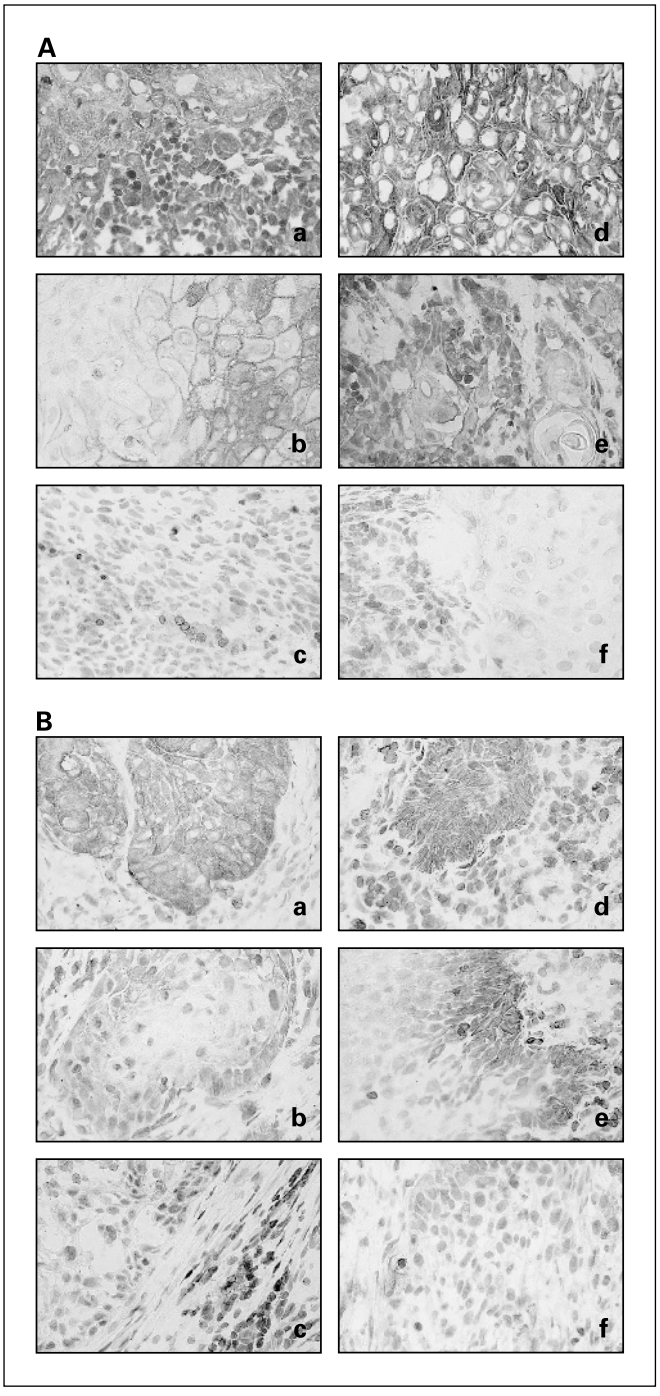

Fig. 2. Immunohistochemical staining of HNSCC lesions with LMP- and TAP-specific antibodies. *A*, representative immunoperoxidase staining patterns of oral HNSCC lesions with TAP1- (*a-c*) and TAP2- (*d-f*) specific mAb. TAP1 and TAP2 expression is high and homogeneous in (*a*) and (*d*), heterogeneous in (*b*) and (*e*), and not detectable in (*c*) and (*f*), respectively. Original magnification (×400). *B*, representative immunoperoxidase staining patterns of oral HNSCC lesions with LMP2- (*a-c*) and LMP7- (*d-f*) specific mAbs. LMP2 and LMP7 expression is high and homogeneous in (*a*) and (*d*), heterogeneous in (*b*) and (*e*), and not detectable in (*c*) and (*f*). Original magnification (×400).

expression levels of the five markers analyzed were consistent with improved patients' survival (Fig. 3). The association of APM component expression pattern with disease recurrence was less clear except for a statistically significant association of LMP7 down-regulation (*P* value, 0.0395) with disease recurrence at 2 years (Table 3).

Impaired expression and function of antigen processing machinery components in cell lines derived from head and neck squamous cell carcinoma lesions. In order to assess functional consequences of impaired APM gene expression rates observed in tumor biopsies, we analyzed 10 HNSCC cell lines for APM component mRNA and protein expression levels. The keratinocyte cell line HaCaT and the renal cell carcinoma cell line MZ1257RC, known to have a normal HLA class I phenotype, served as controls. When compared with HaCaT cells, all HNSCC cell lines displayed reduced levels of total HLA class I surface antigens, which was accompanied by heterogeneous mRNA and protein expression levels of the various APM components analyzed (Fig. 4). In the case of PC-15A/B cells,

which exhibit a low HLA class I surface expression, it could not be determined whether the patient is HLA homozygous or whether there exists a HLA haplotype loss in the tumor cell, since normal cells from this patient were not available for such an analysis. Furthermore, highly variable TAP1 protein levels were often correlated with impaired TAP function as measured by the peptide translocation assay (Fig. 5). PCI-13 and PCI-15A cells displayed peptide transport rates ranging from 2.1% to 5.8%. The peptide transport rate in both HNSCC cell lines was dependent on the model peptides employed, since peptide #63 was more efficiently transported into the endoplasmic reticulum than peptide #600.

Antigen processing machinery component and human leukocyte antigen class I antigen down-regulation in both head and neck squamous cell carcinoma lesions and autologous cell lines. To determine whether the levels of the APM components and of the HLA class I antigen surface expression in HNSCC cell lines correlated with those in the lesions from which the cell lines had been originated, we compared LMP2, LMP7, TAP1, TAP2, and HLA class I heavy chain expression levels in three formalin-fixed, paraffin-embedded HNSCC lesions to that of the autologous HNSCC cell lines PCI-13, PCI-30, and PCI-52 (Table 4). With the exception of the HLA class I heavy chain expression in PCI-13 cells, the expression levels of APM components and HLA class I heavy chains in the three HNSCC lesions were similar to those found in the corresponding cell lines as determined by RT-PCR and Western blot analyses (Fig. 4).

IFN-γ-mediated restoration of antigen processing machinery component expression in head and neck squamous cell carcinoma cell lines. It is generally accepted that IFN-γ transcriptionally modulates the expression of most APM components, including the proteasome subunits LMP2, LMP7, and LMP10/MECL-1 as well as their activators PA28α and β, next to TAP, tapasin, and HLA class I antigens (3, 5, 36). To provide insights into the underlying mechanism(s) of APM component abnormalities in HNSCC cells, such as structural alterations or deregulation, APM component expression levels were analyzed by flow cytometry, RT-PCR, and/or Western blot in the 10 HNSCC cell lines incubated in the absence or presence of IFN-γ (200 units/mL for 24 hours at 37°C). As shown in Fig. 4, PA28α, LMP2, TAP, and tapasin expression was induced by IFN-γ, whereas calnexin and calreticulin transcription and/or translation were not up-regulated by this treatment. The IFN-γ-mediated increased expression of PA28α, LMP, TAP, and tapasin was directly associated with an enhanced HLA class I antigen expression (Table 1).

Discussion

Abnormalities in MHC class I antigen surface expression and down-regulation of different APM components have been identified in a substantial number of human tumors of distinct

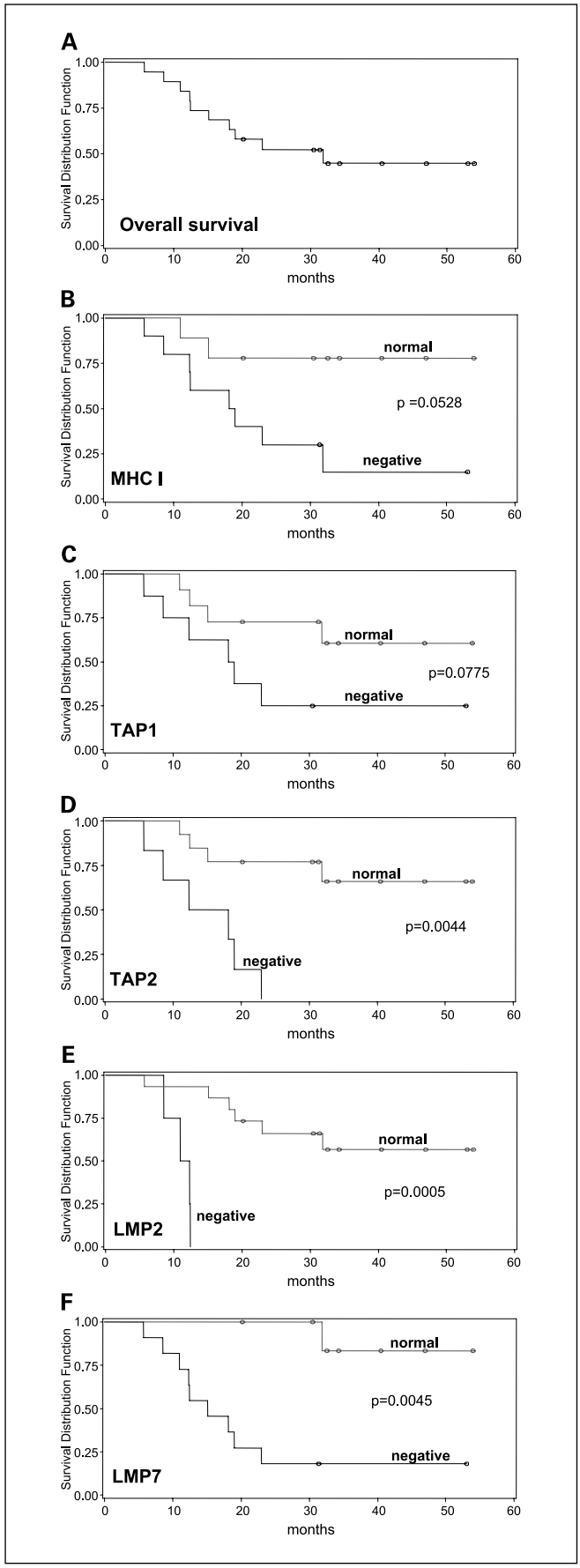

Fig. 3. Association of APM component expression patterns in primary HNSCC lesions with patients' survival. Overall survival of the 19 patients examined (*A*). Survival of 19 patients according to HLA class I antigen (*B*), TAP1 (*C*), TAP2 (*D*), LMP2 (*E*), and LMP7 (*F*) expression. Adjusted *P* values are shown for the log rank test to compare the two groups.

Table 3. Statistical analyses of APM component expression levels in comparison with survival and disease recurrence

	Adjusted *P* value		
Biomarkers	**Two-group log rank test**	**Three-group exact Monte Carlo log rank test**	**Cochran-Armitage trend test for recurrence**
MHC I*	0.0580	0.0426	0.0576
TAP1*	0.0775	0.0633	0.1836
TAP2†	0.0044	0.0208	0.0576
LMP2†	0.0005	0.0208	0.1836
LMP7*	0.0045	0.0105	0.0395

Note: *P* values < 0.05 after adjustment are shown in boldface.
*For two-group analysis those who have expression scores 0/1 have been compared with those who have high expression levels (2).
†For two-group analysis those who have no expression (score 0) have been compared with those who have heterogeneous or high levels of expression (score 1 and 2).

histology (3) and may be related to the metastatic potential of some tumor types (37, 38). In HNSCC patients, MHC class I alterations were found in up to 50% of the tumor lesions (11, 14, 15, 18). Although the underlying molecular mechanisms of deficient HLA class I antigen expression rates in HNSCC cells have not yet been determined in detail, microsatellite instability analyses showed loss of heterozygosity of chromosome 6p21 in HNSCC lesions (16, 39). Here, we extended these findings to other HLA class I molecules and APM components showing a high frequency of down-regulation or even loss of LMP2, LMP7, TAP1, and HLA class I antigens in both tumor biopsies as well as cell lines of HNSCC patients when compared with normal control cells (Fig. 2; Tables 1 and 2). A similar coordinated down-regulation of APM components in tumor cell lines has been described by Johnson et al. (40). In addition, down-regulation of one or more APM components is often associated with deficient HLA class I antigen surface expression (41).

It is noteworthy that APM abnormalities in HNSCC seem to be mainly attributable to deregulation rather than to structural gene abnormalities, since normalization of APM component expression could be induced by IFN-γ treatment (Table 1). This is further underlined by the fact that structural alterations of APM components, such as TAP, seem to be a rather rare event

Table 4. Summary of APM component deficiencies detected in the HNSCC tumor biopsies and in HNSCC cell lines

(A) APM component deficiencies detected in the HNSCC tumor biopsies and in HNSCC cell lines*

	Deficiencies/tumor biopsies	Cell lines/deficiencies	
APM component	**Protein level**	**RNA level**	**Protein level**
TAP1	13/25	3/10	3/10
TAP2	22/25	0/10	0/7
Tapasin	n.d.	3/10	n.d.
LMP2	22/25	6/10	6/10
LMP7	15/25	10/10	n.d.
LMP10	n.d.	2/10	n.d.
Calnexin	n.d.	0/10	1/10

(B) Comparison of APM components expression in HNSCC cell lines and corresponding tumor lesions †

		HLA I	TAP1	LMP2
PCI-13	cell line	1	1	2
	tumor	2	2	1
PCI-30	cell line	1	1	1
	tumor	0	1	0
PCI-52	cell line	1	1	1
	tumor	1	1	1

Abbreviation: n.d., not determined.
*The results are shown as numbers of tumor biopsies/cell lines with APM deficiencies versus numbers of samples tested.
†The results are expressed as level of protein expression: 0, <25% stained cells; 1, 25% to 75% stained cells; 2, >75% stained cells.

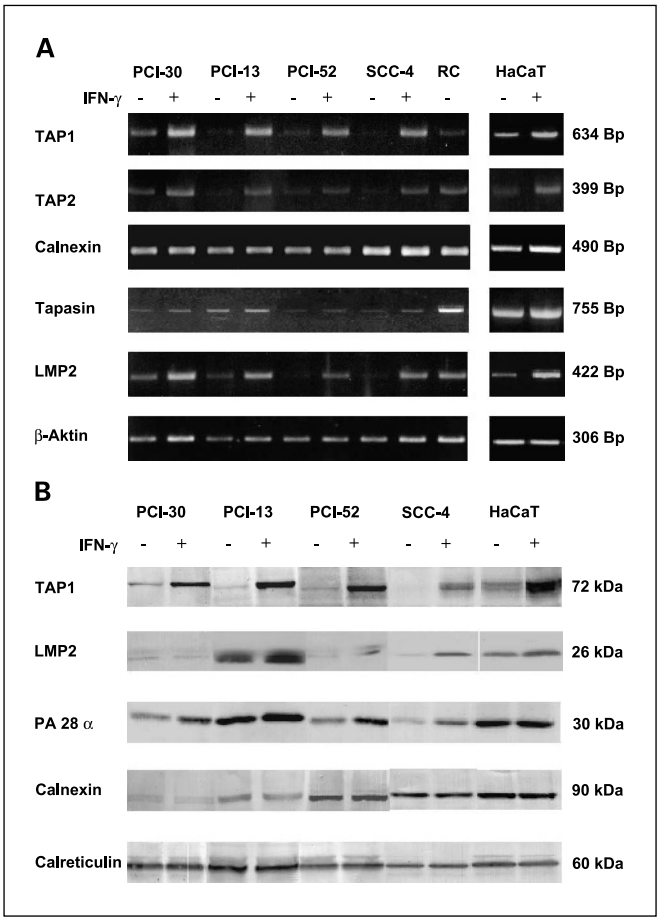

Fig. 4. Constitutive and IFN-γ-mediated mRNA and protein expression patterns of APM components in HNSCC cell lines. *A*, total cellular RNA from untreated and IFN-γ-treated HNSCC cell lines and HaCaT, MZ1257RC control cells, respectively, was used for RT-PCR analysis of APM component mRNA using a panel of APM component – specific primer sets as described in Materials and Methods. *B*, lysates of HNSCC cell lines (40 μg of total protein per lane) were electrophoresed and then tested in Western blotting with APM component – specific mAbs.

(41–43). However, detailed sequence analyses of TAP as well as other APM components are needed to better understand the molecular basis of escape mechanisms utilized by human malignancies, including HNSCC.

Impaired APM component expression might influence both the induction and the effector phase of TAA-specific immune responses. Indeed, abnormalities of MHC class I surface expression attributable to APM deficiencies enable tumors to evade immunosurveillance and thereby developing a more aggressive phenotype (3). Such an immune escape mechanism has also been suggested for HNSCC cells and might be reflected by a low T cell infiltration of tumors or by inefficient activation and generation of HNSCC-associated antigen–specific CTL as described by several investigators (20, 22, 25). Restoration of antigen presentation by cytokine treatment and/or gene transfer of deficient APM component consequently renders cells sensitive to CTL-mediated lysis. These strategies should be considered for the development of novel T cell–based immunotherapies for HNSCC patients. In this context, it is noteworthy that other mechanisms besides APM down-regulation may be involved in selective HLA losses. Indeed, Maleno et al. (44) described multiple mechanisms of HLA class I down-regulation pattern in laryngeal carcinomas. These include a high frequency of haplotype loss associated with loss of heterozygosity as well as total HLA class I loss, HLA-A and -B locus–specific down-regulation and HLA class I allelic loss. Such structural alterations cannot be restored by cytokine treatment.

It has been suggested that the survival of cancer patients might be influenced by the functional state of their immune system. Clearly, the ability of the host immune system to control tumor growth is likely to be impaired in the presence of HLA antigen expression abnormalities. To the best of our knowledge, only a few studies have examined the correlation between loss of APM component expression and clinical outcome (26, 37, 38, 43). In melanoma, breast, lung, and cervical carcinoma, the lack of HLA class I antigen surface expression and/or TAP expression was reported to be associated with poor tumor differentiation, increased frequency of aneuploidy as well as reduced survival rate (37, 38, 45, 46). Conflicting information is available about the association between HLA class I antigen down-regulation and patients' survival in HNSCC patients (25, 26, 47). Our results are in accordance with those reported by one of the authors (26) showing a significant correlation between the survival time and a loss or down-regulation of APM components in primary maxillary sinus squamous cell carcinoma lesions. Although preliminary, our results suggest that the APM component levels might serve as valuable prognostic markers in this disease (Fig. 3). However, our study has limitations including the small number of HNSCC patients analyzed thus far and the fact that preoperative radiation was used in several of our patients, which might affect the pathologic stages as well as clinical responses and patient survival. In addition, the node stage, which is considered to be the best predictor of survival or recurrence in HNSCC patients, could not be correlated with survival or recurrence in this cohort of patients (48). To further confirm the intriguing possibility that the APM components could serve as independent predictors of survival in HNSCC patients, it will be necessary to perform a larger retrospective or prospective study

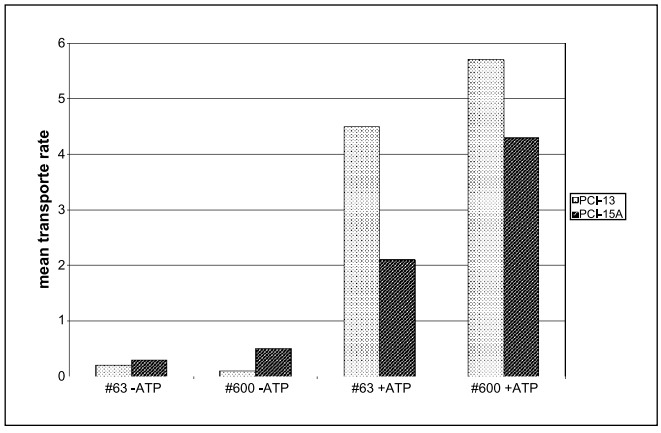

Fig. 5. Distinct peptide translocation in HNSCC cell lines. Peptide translocation assays were done with SLO-permeabilized HNSCC cell lines using the reporter peptides #63 (RYWANATRSI) and #600 (TNKTRIDGQY) in the presence and absence of ATP. Results are expressed as the percentage of translocated peptides.

specifically designed to measure survival. Nevertheless, the observed correlation between deficiencies in APM components and survival represents a novel finding suggesting that the host's immune system/tumor cell interactions determine the disease outcome and that long-term prognosis depends on these interactions.

Acknowledgments

We thank Dr. Robert Tampé (University of Frankfurt, Frankfurt, Germany) for his generous gift of antibodies, M. Post (German Cancer Research Center, Heidelberg, Germany) for technical assistance, and I. Schmidt and Liis Pellenen for help in the preparation of this manuscript.

References

1. Garrido F, Ruiz-Cabello F, Cabrera T, Perz-Villar JJ, Lopez-Botet M, Duggan-Keen M. Implications for immunosurveillance of altered HLA class I phenotypes in human tumors. Immunol Today 1997;18:89–95.
2. 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.
3. Seliger B, Maeurer MJ, Ferrone S. Antigen processing machinery breakdown and tumor growth. Immunol Today 2000;21:455–64.
4. Restifo N, Esquivel F, Kawakami Y, et al. Identification of human cancers deficient in antigen processing. J Exp Med 1993;177:265–72.
5. Seliger B, Schreiber K, Delp K, et al. Downregulation of the constitutive tapasin expression in human tumor cells of distinct origin and its transcriptional upregulation by cytokines. Tissue Antigens 2000;57:39–45.
6. Hoffman HT, Karnell LH, Funk GF, Robinson RA, Menck HR. The national cancer database report on cancer of the head and neck. Arch Otolaryngol Head Neck Surg 1998;124:951–62.
7. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J Clin 1999;49:8–31.
8. Whiteside TL, Letessier E, Hirabayashi H, et al. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. Cancer Res 1993;53:5654–62.
9. Chikamatsu K, Reichert TE, Kashii Y, et al. Immunotherapy with effector cells and IL2 of lymph node metastases of human squamous cell carcinoma of the head and neck established in nude mice. Int J Cancer 1999;82:532–7.
10. Chang AE, Li Q, Jiang G, Teknos TN, Chepeha DB, Bradford CR. Generation of vaccine-primed lymphocytes for the treatment of head and neck cancer. Head Neck 2003;25:198–209.
11. Esteban F, Concha A, Huelin C, Perez-Ayala M, Pedrinaci S, Ruiz-Cabello F. Histocompatibility antigens in primary and metastatic squamous cell carcinoma of the larynx. Otolaryngol Head Neck Surg 1989;103:14–24.
12. Houck JR, Sexton M, Zajdel G. HLA class I and class II antigen expression on squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 1990;116:1181–5.
13. Mattussen V, DeMulder PH, Schalwuk L, Manni JJ, Hof-Grootenboer BV, Ruiter DJ. HLA antigen expression in routinely processed head and neck squamous cell carcinoma. Br J Cancer 1991;6:95–100.
14. Vora AR, Ridgers S, Parker AJ, Start R, Rees RC, Murray AK. An immunohistochemical study of altered immunomodulatory molecule expression in head and neck squamous carcinoma. Br J Cancer 1997;76:836–44.
15. Feenstra M, Verdaasdonk M, Wil van der Zwan A, De Weger R, Slootweg P, Tilanus M. Microsatellite analysis of microdissected tumor cells and 6p high density microsatellite analysis in head and neck squamous cell carcinoma with downregulated human leukocyte antigen class I expression. Lab Invest 2000;80:405–14.
16. Feenstra M, Veltkamp M, van Kuik J, et al. HLA class I expression and chromosomal deletions at 6p and 15q in head and neck squamous carcinomas. Tissue Antigens 1999;64:235–45.
17. Cabrera T, Salinero J, Angustias Fernandez M, Garrido A, Esquivias J, Garrido F. High frequency of altered HLA class I phenotypes in laryngeal carcinomas. Hum Immunol 2000;61:499–506.
18. Rubin-Grandis J, Falkner DM, Melhem MF, Gooding WE, Drenning SD, Morel PA. Human leukocyte antigen class I allelic and haplotype loss in squamous cell carcinoma of the head and neck: Clinical and immunogenic consequences. Clin Cancer Res 2000;6:2794–802.
19. Feenstra M, Rozemuller E, Duran K, et al. Mutations in β-2m is not a frequent event in head and neck squamous cell carcinomas. Hum Immunol 1999;60:697–706.
20. Taitz A, Petruzelli G, Schmidt Pak A, et al. Immune parameters of mice bearing human head and neck cancer. Cancer Immunol Immunother 1999;40:283–91.
21. Lang S, Whiteside T, Lebeau A, Zeidler R, Mack B, Wollenberg B. Impairment of T cell activation in head and neck cancer *in situ* and *in vitro*. Arch Otolaryngol Head Neck Surg 1999;125:82–8.
22. Cruz I, Meijer CJ, Walboomers JM, Snijders PJ, Van der Waal I. Lack of MHC class I surface expression on neoplastic cells and poor activation of the secretory pathway of cytotoxic cells in oral squamous cell carcinomas. Br J Cancer 1999;81:881–9.
23. Okada K, Yasumura S, Muller-Fleckenstein I, et al. Interactions between autologous $CD4^+$ and $CD8^+$ T lymphocytes and human squamous cell carcinoma of the head and neck. Cell Immunol 1997;177:35–48.
24. Yasumura S, Hirabayashi H, Schwartz DR, et al. Human cytotoxic T-cell lines with restricted specificity for squamous cell carcinoma of the head and neck. Cancer Res 1993;53:1461–8.
25. Esteban F, Redondo M, Delgado M, Garrido F, Ruiz-Cabello F. MHC class I antigen and tumor infiltrating leukocytes in laryngeal cancer: long-term follow-up. Br J Cancer 1996;74:1801–4.
26. Ogino T, Nobuyuki B, 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;9:4043–51.
27. Matsui M, Ikeda M, Akatsuka T. High expression of HLA-A2 on an oral squamous cell carcinoma with downregulated transporter for antigen presentation. Biochem Biophys Res Commun 2001;280:1008–14.
28. Boukamp P, Petrussevska RT, Breitkreuz D, Hornung J, Markham A, Fusenig NE. Normal keratinisation in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761–71.
29. Seliger B, Höhne A, Knuth A, et al. IFN-γ mediated transcriptional regulation of the human TAP1 and LMP2 genes in human renal cell carcinoma. Cancer Res 1996;56:1756–60.
30. Delp K, Momburg F, Hilmes C, Huber C, Seliger B. Functional deficiencies of components of the MHC class I antigen pathway in human tumors of epithelial origin. Bone Marrow Transplant 2000;25:88–95.
31. Meyer T, van Endert P, Uebel S, Ehring B, Tampé R. Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. FEBS Lett 1994;351:443–7.
32. Brodsky FM, Parham P, Barn CJ, Bodmer WF. Monoclonal antibodies for analysis of the HLA system. Immunol Rev 1979;47:342–67.
33. Van Endert PM, Tampé R, Meyer TH, Tisch R, Bach JF, McDevitt HO. A sequential model for peptide binding and transport by the transporters associated with antigen processing. Immunity 1994;1:491–500.
34. Neumann E, Engelsberg A, Decker J, et al. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: Candidates for T-cell-based immunotherapies? Cancer Res 1998;58:4090–5.
35. Neefjes JJ, Momburg F, Hämmerling GJ. Selective ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 1993;261:769–71.
36. Eppersson DE, Arnold D, Spiess T, Cresswell P, Pober JS, Johnsson DR. Cytokines increase transporter in antigen processing-1 expression more rapidly than HLA class I expression in endothelial cells. J Immunol 1993;149:3297–301.
37. Cromme FV, van Bommel PFL, Walboomers JMM, Galle TJM, Stern PL, Kenemans P. Differences in MHC and TAP-1 expression in cervical cancer lymph node metastases as compared with the primary tumors. Br J Cancer 1994;69:1176–81.
38. Vitale M, Rezzani R, Rodella L, et al. HLA class I antigen and TAP downregulation in high grade primary breast carcinoma lesions. Cancer Res 1998;58:737–42.
39. Jimenez P, Cantón J, Concha A, et al. Microsatellite instability analysis in tumors with different mechanisms for total loss of HLA expression. Cancer Immunol Immunother 2000;48:684–90.
40. Johnson A, France JM, Sy MS, Harding CV. Downregulation of the transporter for antigen presentation, proteasome subunits, and class I major histocompatibility complex in tumor cell lines. Cancer Res 1998;58:3660–7.
41. Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F. Analysis of HLA expression in human tumor tissues. Cancer Immunol Immunother 2003;52:1–9.
42. Chen HL, Gabrilovich D, Tampé R, Girigs KR, Nadaf S, Carbone DP. A functionally detective allele of TAP 1 results in loss of MHC class I antigen presentation in a human lung cancer. Nat Genet 1996;13:139–40.
43. 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;61:8647–50.
44. Maleno I, Lopez-Nevot MA, Cabrera T, Salinero J, Garrido F. Multiple mechanisms generate HLA class I altered phenotypes in laryngeal carcinomas: high frequency of HLA haplotype loss associated with loss of heterozygosity in chromosome region 6p21. Cancer Immunol Immunother 2002;51:389–96.
45. Kageshita T, Hirai S, Ono T, Hicklin D, Ferrone S. Downregulation of HLA class I antigen-processing molecules in malignant melanoma: association with disease progression. Am J Pathol 1999;154:745–54.
46. Redondo M, Concha A, Ruiz-Cabello F, et al. Class I major histocompatibility complex antigens and tumor ploidy in breast and bronchogenic carcinomas. Cancer Detect Prev 1997;21:22–8.
47. Concha A, Esteban F, Cabrera T, Ruiz-Cabello F, Garrido F. Tumor aggressiveness and MHC class I and II antigens in laryngeal and breast cancer. Cancer Biol Ther 1991;2:47–54.
48. Heo DS, Snyderman C, Gollin SM, et al. Biology, cytogenetics, and sensitivity to immunological effector cells of new head and neck squamous cell carcinoma cell lines. Cancer Res 1989;49:5167–75.