Reduced Expression of the Antigen Processing Machinery Components TAP2, LMP2, and LMP7 in Tonsillar and Base of Tongue Cancer and Implications for Clinical Outcome[1]

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Abstract

OBJECTIVES: Patients with human papillomavirus (HPV)–positive tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC) have a better clinical outcome than those with corresponding HPV-negative tumors. Moreover, there is a strong positive correlation between absent/low as opposed to strong HLA class I expression and favorable clinical outcome for HPV-positive tumors, while the reverse applies to HPV-negative tumors. The expression of the antigen processing machinery (APM) components TAP1, TAP2, LMP2, and LMP7 in these tumors in relation to HPV status, HLA class I expression, each other, and clinical outcome was therefore investigated. *MATERIAL AND METHODS*: Formalin-fixed paraffin-embedded TSCC and BOTSCC, derived from 151 patients and previously analyzed for HPV DNA, HLA class I, and LMP10 expression were stained by immunohistochemistry for TAP1, TAP2, LMP2, and LMP7. *RESULTS*: Absent/low TAP2, LMP2, and LMP7 expression, similar to HLA class I and LMP10, was common in TSCC and BOTSCC, irrespective of HPV status. Expression of TAP1 and TAP2 was correlated, as was LMP2 to LMP7. LMP2 and LMP7 expression was also associated to HLA class I expression. Moreover, absence of LMP7 was linked to increased disease-free survival in both HPV-positive and HPV-negative cases. *CONCLUSION*: Reduced expression of TAP2, LMP2, and LMP7 was frequent in TSCC and BOTSCC and their expression as well as that of TAP1 was often interrelated. Furthermore, low LMP7 expression correlated to better clinical outcome and may, together with HPV status, potentially be used for prediction of treatment response.

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Introduction

Recently, an increased incidence of oropharyngeal squamous cell carcinoma (OPSCC) was noted throughout the Western world, in particular for tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC), the most common OPSCC types [1–7]. This increase was attributed to an increased prevalence of human papillomavirus (HPV) in OPSCC, especially in TSCC and BOTSCC, rather than smoking and alcohol, the two other main risk factors for these tumors [8]. Currently, a substantial part of TSCC and BOTSCC are HPV positive and have a better survival than the corresponding negative tumors (with roughly 80% to 40% 5-year survival) [2,5,7,9–12]. The reason for the increased survival for patients with HPV-positive tumors has not been established. However, a positive correlation between the number of tumor-infiltrating CD8^{+} lymphocytes and clinical outcome has been

demonstrated, indicating an influence of the immune system [13,14]. It was therefore surprising that in HPV-positive tumors, absent or low

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HLA class I expression (in 44% of the tumors) correlated to a favorable clinical outcome, while the opposite applied for HPV-negative TSCC and BOTSCC, where 31% of the tumors had absent or low expression [15,16]. Low or absent HLA class I expression, frequently found both in head and neck squamous cell carcinoma (HNSCC) and in other tumors, should abrogate an immune response and is, in general, correlated to a poor clinical outcome [17–22]. In this context, further analysis on the expression of other components of the antigen processing machinery (APM), such as LMP2, LMP7, LMP10, TAP1, and TAP2, affecting the formation of peptide presenting HLA-b2m complexes, seemed of importance [23,24]. LMP2, LMP7, and LMP10 are subunits of the immunoproteasome, responsible for the processing of proteins to peptides, while TAP1 and TAP2 transport peptides from the cytoplasm to the endoplasmic reticulum [25,26]. This was emphasized by our recent finding that a low nuclear LMP10 expression was correlated to a favorable clinical outcome in HPV-positive tumors, whereas for HPV-negative tumors a moderate/high cytoplasmic LMP10 expression correlated to a good clinical outcome [27]. Thus, the HPV status of the tumor is critical for the evaluation of both HLA class I and LMP10 in relation to clinical outcome.

In contrast to the many reports on HLA class I expression and cancer, including HNSCC, fewer studies have been performed on other APM components besides HLA. In cervical carcinoma, where virtually all tumors are HPV positive, defects in APM components are commonly found [26]. Furthermore, expression of APM components LMP2, LMP7, TAP1, TAP2, and HLA class I correlated to clinical outcome in HNSCC [21]. However, the relation in expression between different APM components, besides their association with prognosis, is rarely evaluated nor, to our knowledge, have HPV status been taken into account in previous studies of APM components and HNSCC.

In this study, we have analyzed the expression of APM components TAP1, TAP2, LMP2, and LMP7, their potential interrelationships, as well as possible association to HLA class I and LMP10 expression, using previously obtained data on HLA class I and LMP10 expression in TSCC and BOTSCC [15,16,27]. Furthermore, we specifically investigated if HLA class I expression was correlated to that of any other APM component and whether the expression of any additional APM component was correlated to clinical outcome.

Materials and Methods

Patients and Tumor Biopsies

Two patient cohorts, one diagnosed with TSCC and one with BOTSCC, with a total of 151 patients, all treated at Karolinska University Hospital (Stockholm, Sweden) were included in the study (for details, see Table 1). The first sample set consisted of 78 TSCC samples (ICD-10 C09.0-9), derived from patients diagnosed from 2000 to 2006, treated with the intention to cure and with available pretreatment paraffin-embedded tumor biopsies. This set included 48 HPV DNA–positive and 30 HPV-negative TSCC samples, derived from a set of 83 TSCC samples included in the analysis of HLA class I and tumor-infiltrating CD8$^+$ and FoxP3$^+$ but with five tumors excluded due to lack of material (for details, see [13,15]). The second set consisted of all 73 BOTSCC samples (ICD-10, C01.9), 53 HPV DNA positive and 20 HPV negative, from patients diagnosed from 2000 to 2007, with available pretreatment biopsies, and 66 of these were treated with curative intent, while the rest received palliative

Table 1. Characteristics of Patients with TSCC and BOTSCC and Their Tumors

Patient Characteristics	HPV-Positive Tumors		HPV-Negative Tumors		All Patients/Tumors		P Value
	101		50		151		
	n	Percentage	n	Percentage	n	Percentage	
Age (years)							
Mean	61		63		62		.227
Median	61		62		61		
Diagnosis							
TSCC	48	47.5%	30	60.0%	78	51.7%	.149
BOTSCC	53	52.5%	20	40.0%	73	48.3%	
Sex							
Male	71	70.3%	39	78.0%	110	72.8%	.317
Female	30	29.7%	11	22.0%	41	27.2%	
Tumor size							
T1	26	25.7%	10	20.0%	36	23.8%	.024
T2	38	37.6%	9	18.0%	47	31.1%	
T3	18	17.8%	16	32.0%	34	22.5%	
T4	19	18.8%	15	30.0%	34	22.5%	
Nodal disease							
N0	15	14.9%	23	46.0%	38	25.2%	<.001
N1	28	27.7%	4	8.0%	32	21.2%	
N2a	15	14.9%	3	6.0%	18	11.9%	
N2b	29	28.7%	10	20.0%	39	25.8%	
N2c	11	10.9%	6	12.0%	17	11.3%	
N3	3	3.0%	4	8.0%	7	4.6%	
NX	0	0.0%	0	0.0%	0	0.0%	
Distant metastasis							
M0	97	96.0%	50	100.0%	147	97.4%	.362
M1	3	3.0%	0	0.0%	3	2.0%	
MX	1	1.0%	0	0.0%	1	0.7%	
Stage							
I	1	1.0%	8	16.0%	9	6.0%	.003
II	6	5.9%	4	8.0%	10	6.6%	
III	29	28.7%	11	22.0%	40	26.5%	
IV	65	64.4%	27	54.0%	92	60.9%	
Treatment							
Curative	98	97.0%	46	92.0%	144	95.4%	.167
Palliative	3	3.0%	4	8.0%	7	4.6%	

n denotes number of patients/tumors.
P value for comparison of HPV-positive versus HPV-negative tumors/patients.

treatment. Data on HPV DNA status and p16^{INK4a} (p16) status (by immunohistochemistry) were obtained from previous studies [1,6,11,28]. As described in these studies, detection of HPV DNA was performed by polymerase chain reaction using the general primer pairs GP5 +/6 + and CPI/IIG, HPV16-specific primers, and in some cases, sequencing. HPV DNA–positive and p16-negative samples were excluded from the sample sets to minimize the risk of including samples with inactive HPV DNA. Thus, HPV status in the presented study was defined as positive for tumors with both presence of HPV DNA and expression of p16 or negative for tumors lacking HPV DNA, regardless of the expression of p16. Of the 101 HPV DNA–positive tumors, 91 were HPV16, 6 HPV33, 2 HPV35, and 1 each with HPV56 and HPV58. Treatment of the 144 patients treated with intention to cure consisted of accelerated radiotherapy (RT) (1.1 + 2.0 Gy/day for 4.5 weeks, total dose: 68 Gy) or conventional RT (2.0 Gy/day, for 6.5-7 weeks, total dose: 68 Gy) in 111 cases and induction chemotherapy followed by concomitant RT in 33 cases. After therapy, patients were followed up by clinical examination every 3 months during the first 2 years and every 6 months starting from the third year. The study was approved by the Regional Ethical Committee at Karolinska Institutet (Stockholm, Sweden) according to ethical permissions 2005/431-31/4, 2005/1330-32, and 2009/1278-31/4.

Antibodies

For staining the following antibodies and dilutions were used: for TAP1, rabbit polyclonal H-300 (1:100); for TAP2, rabbit polyclonal H210 (1:100), both from Santa Cruz Biotechnology, Inc (Dallas, TX, USA); for LMP2, rabbit polyclonal antibody ab3328 (1:1000); for LMP7, ab3329 (1:500), both from Abcam (Cambridge, United Kingdom). As secondary antibodies, BA-1000 anti-rabbit (1:200) and BA-2000 anti-mouse (1:200) both from Vector Laboratories (Burlingame, CA, USA) were used. The tumors included in the present study were formerly evaluated for LMP10 with ab C-2 (Santa Cruz Biotechnology, Inc) [27], and for HLA class I with mouse monoclonal antibodies HCA-2 and HC-10 [15,16]. Data from these studies have been included in the present study for comparison to other APM components.

Immunohistochemistry

Staining was performed essentially as described in Näsman et al. [15]. Briefly, formalin-fixed paraffin-embedded tumor biopsy slides (4 mm) were deparaffinized in xylene and rehydrated in ethanol of decreasing concentrations. Heat-mediated antigen retrieval took place in citrate buffer (pH 6.0). Horse serum (1.5%) diluted in phosphate-buffered saline was used for blocking of unspecific sites, followed by overnight incubation (+8°C) with primary antibodies in a moist chamber. Secondary antibodies were applied and the Avidin-Biotin-Peroxidase Complex (ABC) Kit (Vectastain; Vector Laboratories, Burlingame, CA, USA) was used for antigen detection. Chromogen-39-diaminobenzidine (DAB) was used for visualization, and hematoxylin was used for counterstaining. Staining of tissue sections with secondary antibody alone served as negative controls.

Evaluation of Immunostaining

Staining of tumor samples, including negative and positive controls, was evaluated by two researchers blinded for all other information about the samples. For cases where the evaluation differed, a consensus was reached. The fraction of malignant cells stained for each of the markers was evaluated separately for cytoplasmic and nuclear compartments. The percentage of stained tumor cells was scored as follows: 0—0%, 1—1% to 25%, 2—26% to 50%, 3—51% to 75%, or 4—76% to 100%. Staining intensity was scored separately as absent, weak, moderate, and strong. All examined APM components showed strong expression in stromal tissue as well as in tumor-infiltrating immune cells, thus serving as internal positive controls. In cases where tumor cell staining was uneven, the intensity of the majority of the cells was used in the analysis. Cases were the staining was not possible to evaluate adequately were excluded.

Statistical Evaluation

Student's t test was used for comparison of mean values, and Fisher exact test was used for categorical data. Spearman rank correlation test was used for the comparison of the expression of the different APM components together as well as with the expression of HLA class I (HCA-2 and HC-10 antibodies). Clinical outcome and survival of patients were measured in years from the date of diagnosis until the occurrence of an event or until 3 years after diagnosis, where patients were censored. Events were defined as death due to any cause (overall survival, OS), death with TSCC or BOTSCC present (disease-specific survival, DSS) or recurrence in disease [disease-free survival (DFS)]. Patients who died without a documented TSCC or BOTSCC present were considered as a censored observation in DSS and patients who died without a prior recurrence were censored at day 0 in DFS. The Kaplan-Meier estimator was used for the estimation of DFS, DSS and OS, and differences in survival were tested using the log-rank test. For the P values obtained and presented in Figure 2, all three groups were compared. All analyses were performed using IBM Corp SPSS Statistics version 21.0 except from Fischer exact test that was performed in R statistical software version 2.15.3 [29].

Results

Patient and Tumor Characteristics

The main characteristics of the patients and their tumors are presented in Table 1. As noted above, the study cohort consisted of 151 tumor samples, 78 TSCC and 73 BOTSCC samples. In total, 67% of the tumors were HPV DNA positive, 48% and 52% for TSCC and BOTSCC, respectively. As noted in the Material and Methods section, no HPV DNA–positive and p16-negative samples were included in this study. As has been stated elsewhere, HPV DNA–positive tumors were, on average, associated with a higher nodal staging score [16].

Expression of APM Components in TSCC and BOTSCC

For each of the included APM components, TAP1, TAP2, LMP2, and LMP7, between 127 and 147 of the 151 included TSCC and BOTSCC samples were stained and evaluated for nuclear and cytoplasmic expression. Representative staining patterns of both nuclear and cytoplasmic expression of the different APM components are presented in Figure 1.

The expression of the APM components, evaluated by intensity of cytoplasmic or nuclear staining, for TSCC and BOTSCC separately and together, and according to HPV status (as defined in the Material and Methods section), is shown in Tables 2 and 3, respectively. For comparison, data on LMP10 expression in these tumors, derived from the study by Tertipis et al. [27], are also included in both tables.

As presented in Table 2, cytoplasmic expression of TAP2, LMP2, and LMP7 was absent or weak in a substantial fraction (33-59%) of the tumors, while LMP10 was reduced in even more (87%) of the tumors, whereas TAP1 only had a reduced expression in 9%. Similarly, nuclear TAP1, TAP2, LMP2, LMP7, and LMP10 expression was absent or weak in 18% to 58% of the tumors, with the least for TAP1 and the most for LMP2 (Table 3).

### Expression of APM Components in Relation to Tumor Site

The expression of the APM components was generally similar in TSCC and BOTSCC both with regard to cytoplasmic and nuclear expression. However, there was a slightly lower cytoplasmic expression of LMP7 and possibly LMP2, as well as lower nuclear expression of LMP10 and a higher expression of TAP1 in TSCC as compared to BOTSCC (Tables 2 and 3). The LMP10 data are from Tertipis et al. [27], but there they were not presented per subsite.

Expression of APM Components in Relation to Tumor HPV Status

The expression of APM components was also evaluated in relation to the HPV status of the tumors (Tables 2 and 3). There was no difference in TAP1, TAP2, LMP2, and LMP7 expression in HPV-positive and HPV-negative tumors, while, as presented in Tertipis et al., there was a minor difference with regard to cytoplasmic LMP10 expression [27].

Correlations between the Different APM Components

To investigate whether there was any association in expression between different APM components, the Spearman rank correlation
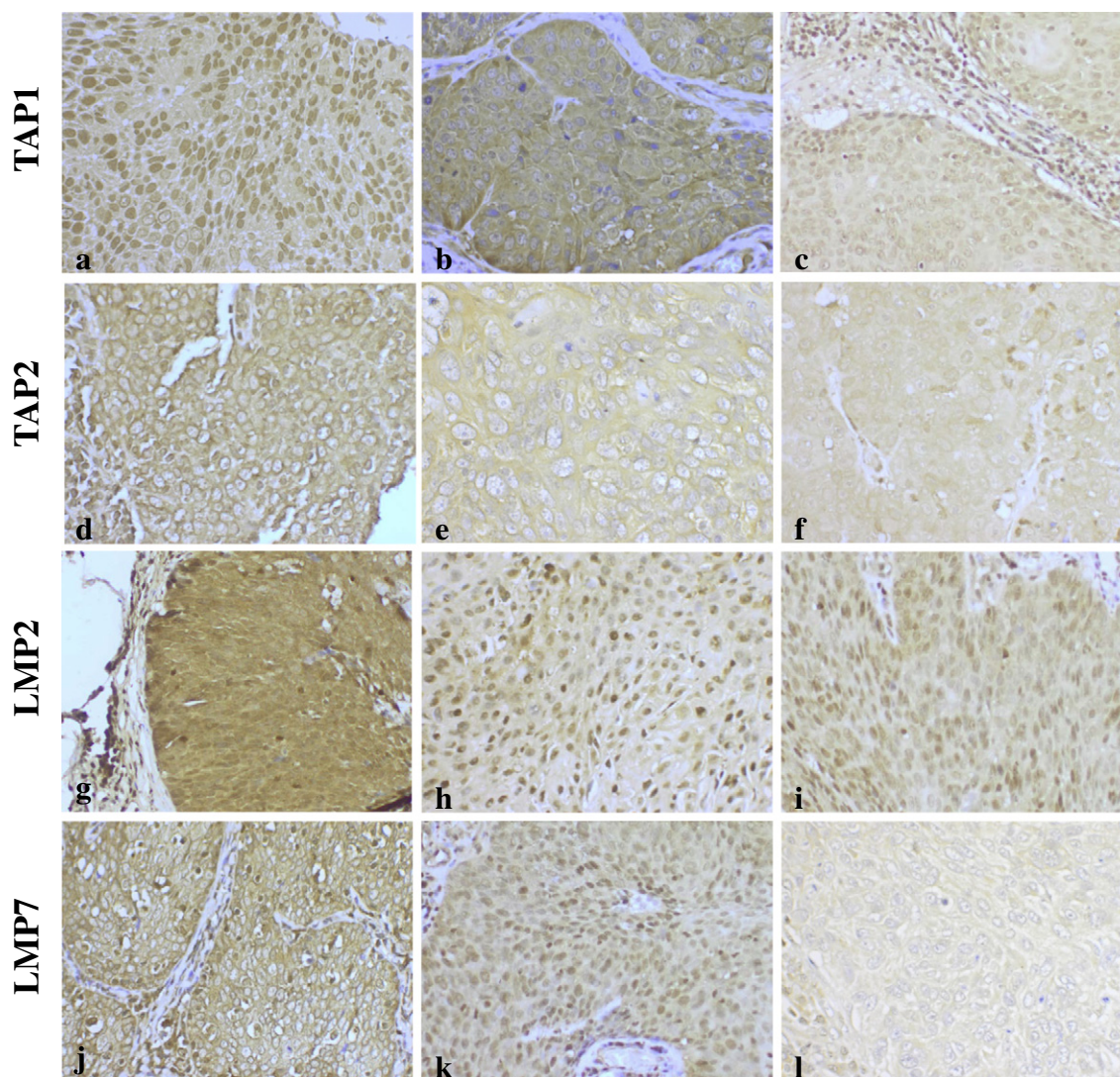
Figure 1. Examples of some of the expression patterns observed for APM components in TSCC and BOTSCC. (A–C) TAP1: (A) strong nuclear and moderate cytoplasmic; (B) absent nuclear and strong cytoplasmic; (C) moderate nuclear and cytoplasmic. (D–F) TAP2: (D) moderate nuclear and cytoplasmic; (E) absent nuclear and weak cytoplasmic; (F) weak nuclear and cytoplasmic. (G–I) LMP2: (G) strong nuclear and cytoplasmic; (H) strong nuclear and weak cytoplasmic; (I) moderate nuclear and cytoplasmic; (J–L) LMP7: (J) moderate nuclear and strong cytoplasmic; (K) moderate nuclear and weak cytoplasmic; (L) absent nuclear and cytoplasmic. Original magnification, x400.

coefficient was calculated, separately for cytoplasmic and nuclear expression, as well as for intensity and fraction of positive cells, and presented in cross-tables. The results of these calculations for the intensity of the cytoplasmic and nuclear staining are presented in Tables 4 and 5, respectively, while the results for the fraction of positive cells are not presented. All calculations were performed separately for HPV-positive and HPV-negative tumors. In this evaluation, earlier data obtained for LMP10 [27] and for HLA class I expression (evaluated by both HC-10 and HCA-2 antibodies) [15,16] were included in the analysis. As presented in both Tables 4 and 5, several significant correlations were found.

There was a significant correlation in cytoplasmic intensity between TAP1 and TAP2, and also between LMP2 and LMP7, irrespective of HPV status (Table 4). Notably, for the fraction of cells positive in the cytoplasm, both LMP2 and LMP7 were significantly correlated to HLA class I (HC-10) expression in HPV-positive tumors (data not shown). In addition, several weaker and more inconsistent correlations were

noted. As expected, given the large overlap in their targets, there was a strong correlation in the staining with the two HLA class I antibodies HC-10 and HCA-2 (Table 4).

Nuclear intensity staining was also significantly correlated between TAP1 and TAP2 as well as between LMP2 and LMP7, irrespective of HPV status (Table 5). Furthermore, both LMP2 and LMP7 nuclear intensity staining was correlated to HLA class I (HC-10) expression in HPV-positive tumors (Table 5). Moreover, LMP7 expression was correlated to TAP2, irrespective of HPV status, and to TAP1 in HPV-negative tumors (Table 5). LMP10 was, in general, not correlated to any of the other examined APM components.

Correlation of Expression of APM Components with Clinical Outcome

DFS, disease-specific survival (DSS), and overall survival (OS) in relation to cytoplasmic and nuclear expression of TAP1, TAP2,
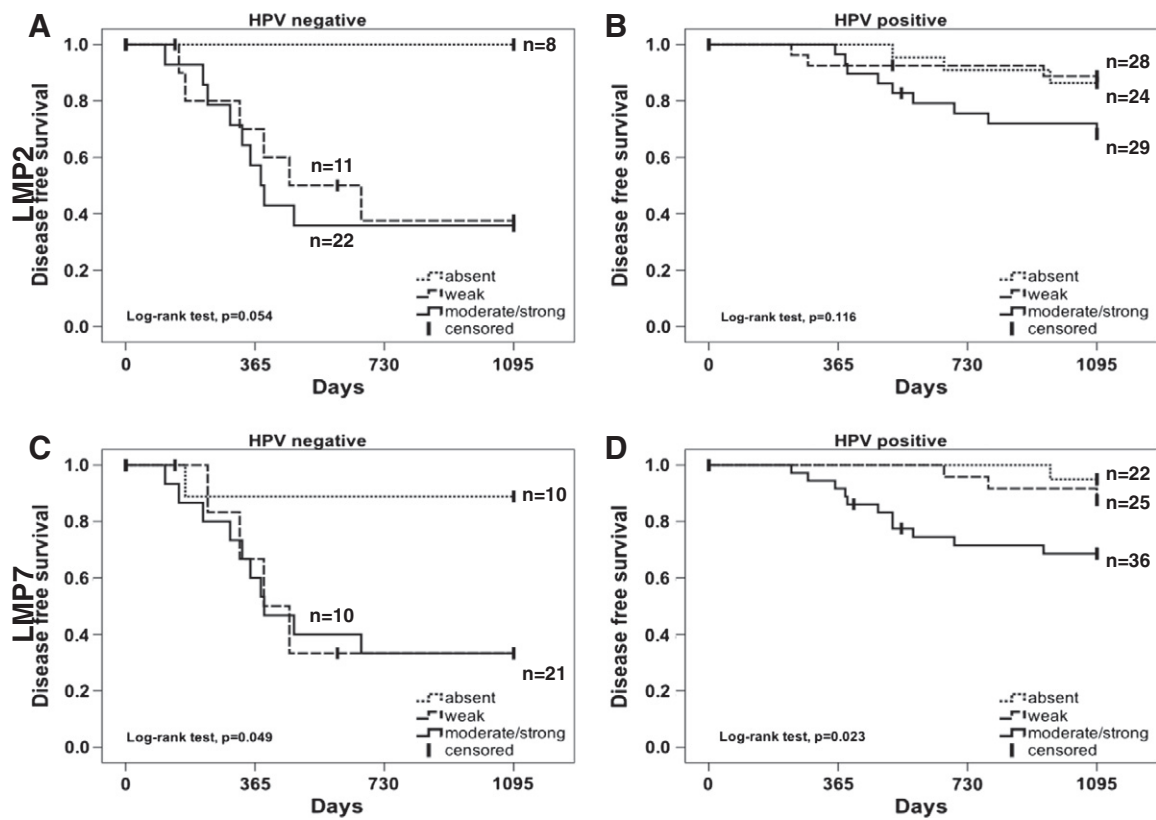
Figure 2. Kaplan-Meier curves for DFS of patients with TSCC and BOTSCC treated with intention to cure stratified by the intensity of nuclear staining for LMP2 for HPV-positive (A) and HPV-negative (B) cases and for LMP7 for HPV-positive (C) and HPV-negative (D) cases; *n* denotes the number of patients in each group.

LMP2, and LMP7 were analyzed only for patients treated with curative intent, and separately for patients with HPV-positive and HPV-negative tumors (Figure 2), since the former usually have a better clinical outcome [7].

Nuclear expression of LMP7 was significantly correlated to DFS, both for HPV-positive and HPV-negative tumors (P = .023 and P = .049, respectively; Figure 2). Absent nuclear expression of LMP7 was correlated to increased survival, whereas strong LMP7 nuclear expression was

Table 2. Intensity of Cytoplasmic Staining for APM Components in TSCC and BOTSCC in Relation to HPV Status

		Tumor Site		Tumor HPV Status		All Tumors	P Value	P Value[†]
		TSCC	BOTSCC	Positive	Negative			
TAP1	Absent	0.0%	1.4%	1.0%	0.0%	0.7%	.200	.785
	Weak	5.2%	12.9%	10.2%	6.1%	8.8%		
	Moderate/strong	94.8%	85.7%	88.8%	93.9%	90.5%		
TAP2	Absent	0.0%	1.5%	1.0%	0.0%	0.7%	.542	.386
	Weak	35.9%	27.9%	36.1%	24.5%	32.2%		
	Moderate/strong	64.1%	70.6%	62.9%	75.5%	67.1%		
LMP2	Absent	14.1%	10.5%	13.1%	11.4%	12.5%	.063	.724
	Weak	54.9%	35.1%	46.4%	45.5%	46.1%		
	Moderate/strong	31.0%	54.4%	40.5%	43.2%	41.4%		
LMP7	Absent	8.7%	0.0%	3.5%	6.5%	4.5%	.028	.559
	Weak	44.9%	41.3%	44.2%	41.3%	43.2%		
	Moderate/strong	46.4%	58.7%	52.3%	52.2%	52.3%		
LMP10	Absent	29.5%	31.5%	22.8%	46.0%	30.5%	.293	.014
	Weak	52.6%	60.3%	60.4%	48.0%	56.3%		
	Moderate/strong	17.9%	8.2%	16.8%	6.0%	13.2%		

Fisher exact test on TSCC *versus* BOTSCC.
[†] Fisher exact test on HPV-positive *versus* HPV-negative tumors.

Table 3. Intensity of Nuclear Staining for APM Components in TSCC and BOTSCC in Relation to HPV Status

		Tumor Site		Tumor HPV Status		All Tumors	P Value	P Value[†]
		TSCC	BOTSCC	Positive	Negative			
TAP1	Absent	1.3%	10.0%	7.1%	2.0%	5.4%	.049	.242
	Weak	9.1%	15.7%	14.3%	8.2%	12.2%		
	Moderate/strong	89.6%	74.3%	78.6%	89.8%	82.3%		
TAP2	Absent	12.8%	20.6%	16.5%	16.3%	16.4%	.248	.165
	Weak	32.1%	26.5%	35.1%	18.4%	29.5%		
	Moderate/strong	55.1%	52.9%	48.5%	65.3%	54.1%		
LMP2	Absent	29.6%	21.1%	28.6%	20.5%	25.8%	.589	.364
	Weak	32.4%	31.6%	34.5%	27.3%	32.0%		
	Moderate/strong	38.0%	47.4%	36.9%	52.3%	42.2%		
LMP7	Absent	27.5%	23.8%	26.7%	23.9%	25.8%	.507	.650
	Weak	23.2%	30.2%	29.1%	21.7%	26.5%		
	Moderate/strong	49.3%	46.0%	44.2%	54.3%	47.7%		
LMP10	Absent	26.9%	6.8%	16.8%	18.0%	17.2%	.006	.864
	Weak	20.5%	26.0%	21.8%	26.0%	23.2%		
	Moderate/strong	52.6%	67.1%	61.4%	56.0%	59.6%		

Fisher exact test on TSCC *versus* BOTSCC.
[†] Fisher exact test on HPV-positive *versus* HPV-negative tumors.

Table 4. Spearman Rank Correlation of Cytoplasmic Expression of APM Components.

		TAP1	TAP2	LMP2	LMP7	LMP10	HCA2	HC10	
Cytoplasmic Intensity									
TAP1	Rho[a]	1.000	.384**	.040	.137	.003	.054	.030	
	p-value		.000	.721	.210	.980	.608	.772	
TAP2	Rho	.563**	1.000	.174	.308**	.064	.129	.057	
	p-value	.000		.113	.004	.532	.219	.587	
LMP2	Rho	-.008	.072	1.000	.724**	.013	.132	.190	
	p-value	.957	.641		.000	.907	.243	.092	HPV positive
LMP7	Rho	.135	.136	.508**	1.000	-.115	.150	.164	
	p-value	.378	.374	.000		.293	.179	.140	
LMP10	Rho	-.046	-.079	-.062	-.294*	1.000	-.128	-.136	
	p-value	.753	.589	.690	.048		.210	.183	
HCA2	Rho	-.150	-.185	.149	-.125	.249	1.000	.846**	
	p-value	.324	.224	.351	.435	.100		.000	
HC10	Rho	-.229	-.277	.047	-.221	.175	.678**	1.000	
	p-value	.131	.066	.771	.166	.249	.000		
HPV negative									

[a]Spearman correlation coefficient
* Correlation significance at the 0.05 level — Positive correlation
** Correlation significance at the 0.01 level — Negative correlation

Table 5. Spearman Rank Correlation of Nuclear Expression of APM Components.

		TAP1	TAP2	LMP2	LMP7	LMP10	HCA2	HC10	
Nuclear Intensity									
TAP1	Rho[a]	1.000	.456**	.156	.168	-.090	-.009	.053	
	p-value		.000	.159	.124	.380	.928	.609	
TAP2	Rho	.700**	1.000	.204	.304**	.182	.081	.045	
	p-value	.000		.062	.005	.074	.442	.672	
LMP2	Rho	.220	.449**	1.000	.721**	.177	.188	.252*	
	p-value	.152	.002		.000	.107	.095	.024	HPV positive
LMP7	Rho	.446**	.566**	.753**	1.000	.117	.266*	.273*	
	p-value	.002	.000	.000		.285	.016	.013	
LMP10	Rho	.160	.133	-.133	.054	1.000	.008	.090	
	p-value	.273	.363	.391	.719		.939	.382	
HCA2	Rho	-.278	-.345*	-.039	-.127	.073	1.000	.846**	
	p-value	.065	.020	.808	.430	.636		.000	
HC10	Rho	-.271	-.341*	-.043	-.180	.057	.678**	1.000	
	p-value	.072	.022	.791	.260	.710	.000		
HPV negative									

[a]Spearman correlation coefficient
*Correlation significance at the 0.05 level — Positive correlation
**Correlation significance at the 0.01 level — Negative correlation

correlated to poor survival, irrespective of HPV status. Notably, weak LMP7 expression fell into a category between those with absent and those with a moderate/strong expression in HPV-positive tumors, while in HPV-negative tumors it was correlated to poor survival. In addition, absence of LMP7 nuclear expression in HPV-negative tumors was also significantly correlated to both increased OS and DSS (P = .016 and P = .042, data not shown). LMP2 nuclear expression in relation to DFS showed a very similar pattern to that of LMP7, although this result was not statistically significant (Figure 2). Cytoplasmic expression of LMP2 and LMP7 were, however, not associated to survival (data not shown). Similarly, no significant correlation was found for TAP1 and TAP2 in relation to clinical outcome (data not shown).

Discussion

In this study, the expression of TAP1, TAP2, LMP2, and LMP7 in TSCC and BOTSCC was found, with the exception of TAP1, to be frequently reduced, irrespective of HPV status. Possible correlations between the above APM components and LMP10 and HLA class I expression from previous data [15,16,27] were also analyzed. There were significant correlations in the expression of TAP1 and TAP2, as well as between LMP2 and LMP7, irrespective of tumor HPV status. In addition, nuclear LMP2 and LMP7 expression was correlated to HLA class I expression in HPV-positive tumors. Finally, of note, absent/low LMP7 nuclear expression was correlated to better DFS in patients with HPV-positive tumors.

Reduced expression of APM components in tumors is common and is often shown for HLA class I antigens and regarded as a way to evade the immune defence ([18,21,22,30–33]. We have also reported that HLA class I expression is frequently absent or low in both HPV-positive and HPV-negative TSCC and BOTSCC. Notably, in HPV-positive tumors, this was correlated to a favorable clinical outcome, while the reverse was true for HPV-negative cancer [15,16]. Reduced expression of other APM components besides HLA class I has also been demonstrated, e.g., in HNSCC, laryngeal squamous cell carcinoma, cervical and urothelial cancers, and malignant melanoma [21,22,26,30,31]. Thus, in HNSCC and cervical carcinoma, TAP1, TAP2, LMP2, and LMP7 expression was frequently reduced, although LMP7 reduction was not as pronounced in the latter [21,26]. The frequencies of absent or reduced TAP1, TAP2, LMP2,

and LMP7 expression in the HNSCC study by Meissner et al. were also somewhat higher than those presented here, but in line with our data, TAP1 was less frequently reduced than TAP2 [21].

In the current study, all APM components were evaluated for both cytoplasmic and nuclear expression. It was noted that, for some tumors, the expression of specific APM components was more pronounced in the cytoplasm, while others were mainly present in the nucleus. The cytoplasm is assumed to be the active compartment for these proteins, but the significance of nuclear staining for peptide presentation by HLA class I antigens especially in tumors as well as their effects on the immune response has not been investigated thoroughly. Variations in cytoplasmic and nuclear localization of APM components have been observed previously. In one report, the thymus presented pronounced LMP2 and LMP7 nuclear localization, while in liver cells their distribution of LMP2 and LMP7 between the cytoplasm and nucleus was more even [34]. In the present study, both strong cytoplasmic and nuclear LMP2 and LMP7 staining of the stroma and tumor-infiltrating lymphocytes were observed. Furthermore, notably, in an earlier study, we have shown that low nuclear LMP10 expression was found to be significantly correlated to a favorable clinical outcome, demonstrating the validity of evaluating nuclear staining of APM components in relation to clinical outcome [27].

The correlation between the expression of different APM components, e.g., between TAP1 and TAP2, between LMP2 and LMP7, and between TAP2 and LMP7, was not unexpected. The genes for TAP1, TAP2, LMP2, and LMP7 are all located within a narrow region of the class II cluster of the major histocompatibility complex on chromosome 6 [35], with TAP1/LMP2 regulated by a bidirectional promoter, and e.g., in Ad 12–transformed mouse cells, all four are downregulated, indicating a common regulation [36,37]. In contrast, the expression of LMP10 was not correlated to any of the other investigated APM components, possibly due to its gene location on chromosome 16 [38]. However, despite LMP10 not being located in the vicinity of LMP2 and LMP7, the expression of all three genes is regulated by interferon-γ [39].

To investigate whether the reduced HLA class I expression in TSCC and BOTSCC was dependent or independent of the other analyzed APM components, possible correlations were examined. For HPV-positive tumors, both LMP2 and LMP7 expression were correlated to HLA class I expression, and when HLA class I expression

was reduced, expression of LMP2 and LMP7 was often also reduced. This is in line with a study in feline mammary carcinoma by Favole et al., where the expression of LMP2 and LMP7 were both correlated to that of HLA class I [40]. Similarly, in a study on laryngeal squamous cell carcinoma, a correlation between the expression of LMP2 and HLA class I was noted [22].

Earlier studies have shown that the HLA class I heavy chain promoter can be repressed by the HPV16 and 18 E7 proteins [41]. Besides, the bidirectional promoter of TAP1 and LMP2 is repressed by HPV18 E7 but not by HPV16 E7 [41]. It is therefore conceivable that other APM components can be repressed by E7. In this study, with absolute dominance of HPV16, LMP2 reduction should consequently not be dependent on E7 repression. Moreover, since no major differences were observed between HPV-positive and HPV-negative tumors in the decreased expression of TAP2, LMP2, LMP7, and LMP10, the decline was likely not due to HPV. Several other different mechanisms for down-regulation of the APM components have been described, such as mutations, promoter methylation, and transcriptional or post-transcriptional regulation [33].

Notably, low nuclear expression of LMP7 was significantly correlated to longer DFS, both for HPV-positive and HPV-negative tumors, and with OS and DSS only for HPV-negative tumors. LMP2 showed a similar tendency, although not statistically significant. This finding is analogous to the earlier demonstration that low HLA class I and low nuclear LMP10 expression both are positive factors in HPV-positive TSCC and BOTSCC [15,16,27]. However, in the present study, the absence of LMP7 expression was a positive factor for survival also in patients with HPV-negative tumors and did not parallel the worse survival for patients with HPV-negative low HLA class I expressing TSCC or BOTSCC or low LMP7 expressing HNSCC [15,21]. Correlations between APM component expression and clinical outcome are generally interpreted to be associated to effects on the ability of the immune system to elicit an immune response to the tumor. However, LMP2, LMP7, and LMP10 also play a role with regard to cell survival and proliferation and protect cells against oxidative damage [25]. A high nuclear expression of LMP2 and LMP7 would thus enhance cell survival and proliferation, this way resulting in a poorer clinical outcome.

Notably, a high expression of LMP2 and LMP7 is not always associated with an increased antigen presentation. Thus, in a study on Epstein-Barr virus-transformed B cells as well as in other cell types, the presence of the immunoproteasome instead of the standard proteasome inhibited the presentation of specific peptides [42]. In addition, in a report by Mehta et al., it was noted that down-regulation of LMP2 and LMP7 was associated with an absence of lymph node metastasis, possibly due to a similar mechanism [26]. It is possible that the results obtained in the present study, at least partially, may be caused by a decreased recognition of specific peptides, e.g., HPV-derived peptides, by immune cells due to a difference in peptides produced by the immunoproteasome compared to those produced by the standard proteasome.

It is important to note that the expression of APM components was here analyzed in pretreatment tumors, in a situation where the tumor is still growing and the immune defence has so far failed to reject the tumor. We do not know whether expression of these components is affected by treatment and whether the immune defence plays a role during successful, relapse-free, treatments. The results in the present study indicate that LMP7, and possibly also LMP2, can potentially be useful together with other biomarkers, including HPV status, for predicting clinical outcome [13,16,27,43].

There are some limitations in the present study. Although 151 tumors were included, when separated for HPV-positive and HPV-negative status and analyzed for correlations between different APM components, random correlations may still be obtained. For this reason, only the stronger associations, found for both HPV-positive and HPV-negative tumors, were noted, although some weaker correlations may also be valid. Furthermore, not all APM components were evaluated. An analysis of the expression of additional components, e.g., β_2-microglobulin, calnexin, tapasin, and ERp57, may have contributed by giving a fuller view of the combined expression of different APM components in these tumors. For the staining of TAP1, TAP2, LMP2, and LMP7, we have used commercially available antibodies and we have trusted the manufacturers' descriptions of their specificity. However, we cannot fully exclude the possibility of cross-reactions with other proteins that we are not aware of.

In conclusion, TAP2, LMP2, and LPM7 expression was, similar to HLA class I and LMP10, frequently absent or reduced in many TSCC and BOTSCC, and correlations between the analyzed APM components were noted. In addition, for HPV-positive tumors, LMP2, LMP7, and HLA class I expression were correlated. For HPV-positive TSCC and BOTSCC, absent/low nuclear LMP7 expression was correlated to better clinical outcome in line with earlier data on absent/low HLA class I and/or nuclear LMP10 expression [15,16,27].

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