ORIGINAL ARTICLE

Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma

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Abstract HLA class I loss is a significant mechanism of immune evasion by cervical carcinoma, interfering with the development of immunotherapies and cancer vaccines. We report the systematic investigation of HLA class I and antigen processing machinery component expression and association with clinical outcome. A tissue microarray containing carcinoma lesions from 109 cervical carcinoma patients was stained for HLA class I heavy chains, β_2-microglobulin, LMP2, LMP7, LMP10, TAP1, TAP2, ERAP1, tapasin, calreticulin, calnexin and ERp57. A novel staining evaluation method was used to ensure optimal accuracy and reliability of expression data, which were correlated with known clinicopathological parameters. Partial HLA class I loss was significantly associated with decreased 5-years overall survival (61% vs. 83% for normal expression; $P < 0.05$) and was associated with decreased 5-years disease-free survival (DFS) (65% vs. 82% for normal expression; $P = 0.05$). All APM components except LMP10, calnexin and calreticulin were down-regulated in a substantial number of cases and, except ERAP1, correlated significantly with HLA class I down-regulation. LMP7, TAP1 and ERAP1 loss was significantly associated with decreased overall and (except LMP7) DFS ($P < 0.05$ and 0.005, respectively). ERAP1 down-regulation was an independent predictor for worse overall and DFS in multivariate analysis (HR 3.08; $P < 0.05$ and HR 2.84; $P < 0.05$, respectively). HLA class I and APM component down-regulation occur frequently in cervical carcinoma, while peptide repertoire alterations due to ERAP1 loss are a major contributing factor to tumour progression and mortality.

Keywords Antigen processing machinery · ERAP1 · Cervical carcinoma

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Introduction

Tumour cells utilize several mechanisms to escape immune-mediated recognition and destruction [1]. Loss of surface-expressed human leukocyte antigen (HLA) class I molecules is particularly important, as this enables tumour cells to evade recognition and lysis by cytotoxic T-lymphocytes [2–5]. These molecules consist of a glycoprotein heavy chain (encoded by genes within the HLA regions of chromosome 6p) and a β_2-microglobulin (β_2-m) light chain (encoded on chromosome 15q) [6, 7].

Defects in HLA class I expression are caused by multiple mechanisms, including loss of heterozygosity at chromosome 6p; β_2-m or HLA class I mutations; and defective expression and/or function of components of the antigen processing machinery (APM) [8–10]. The APM is the combination of cellular processes responsible for the presentation of endogenous peptides by HLA class I molecules. These peptides are generated by specialized immunoproteasomes (composed of LMP2, LMP7 and

LMP10 subunits); and transported from the cytoplasm to the endoplasmic reticulum by TAP (transporter associated with antigen presentation) which is composed of TAP1 and TAP2 subunits. Transported peptides undergo length-specific trimming by ERAP1 (endoplasmic reticulum aminopeptidase associated with antigen presentation 1) and are loaded onto empty HLA class I molecules (with involvement of tapasin and the chaperone molecules calnexin, calreticulin and ERp57) [11–14].

Although various in vitro studies have demonstrated partial or total APM component loss in human malignancies, the extent to which this occurs in vivo is largely unknown. Two recent reports describe down-regulation of several APM components in head and neck squamous cell carcinoma and astrocytoma, and demonstrate an association of these defects with prognosis [15, 16]. As HLA class I-restricted processing and presentation of endogenous peptides is crucial to the generation of a virus-specific cytotoxic T-lymphocyte-mediated reaction, APM defects are assumed to contribute significantly to virus-mediated carcinogenesis. Several studies have confirmed interference in various APM processes by viral peptides [17]. However, the extent to which defective expression of APM components contributes to HPV-induced cervical carcinogenesis remains to be established. One study has assessed the expression of various APM components, although only at the mRNA level [18]. Other studies of APM component expression in cervical carcinoma were largely limited to TAP1 and TAP2 and encompassed relatively small sample sizes. Moreover, the expression data yielded by these studies are conflicting, with TAP1 loss being reported in 0–50% of cases [18–20]. Though TAP1 and HLA class I down-regulation has occasionally been shown to be associated with progressive malignant transformation [21–24], a comprehensive study of the association of APM defects with prognostic parameters has yet to be performed.

Since information regarding defective APM component expression in cervical cancer can contribute to the development of T cell-based immunotherapies, we have investigated the expression of all known APM components in a large number of cervical cancer specimens using a tissue microarray (TMA)-based approach [25, 26]. Additionally, we demonstrate for the first time a negative association of APM defects with prognosis and other clinicopathological variables.

Materials and methods

Clinical characteristics and tissue samples

From 109 patients with cervical carcinoma who underwent radical hysterectomy with bilateral pelvic lymphadenectomy (by the same surgical team) between 1985 and 1999, formalin-fixed, paraffin-embedded tissue blocks were retrieved from the archives of the Department of Pathology, Leiden University Medical Centre, The Netherlands. Twenty-six adenocarcinomas and 83 squamous carcinomas were obtained. These cases were not consecutive, but chosen on the basis of availability of sufficient material. The use of clinical material was approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

All patients were inhabitants of The Netherlands and had not received pre-operative radiotherapy or chemotherapy. Mean age was 48.5 years, the youngest patient being 24 years and the oldest 87 years at the time of surgery. Follow-up of these patients until 2005 provided information concerning recurrence rate and performance state.

Tissue microarray construction

The archival slides for all the cases were reviewed; a slide containing representative tumour was selected, and an area of tumour was encircled on the slide. The corresponding tissue blocks were recovered from the archives and the selected area on the slide was circled on the block for tissue microarray construction. Using a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA), the area of interest in the donor block was cored with a 0.6-mm diameter needle and transferred to a recipient paraffin block. The microarray was constructed with a threefold redundancy (three spots for each patient) to increase accuracy. The finalized arrays were then cut into 3 μm-thick sections and mounted on glass slides using an adhesive tape-transfer system (Instrumedics Inc., Hackensack, NJ, USA) with ultraviolet cross-linking.

Antibodies

The mouse monoclonal antibody (mAb) HCA2 which recognizes a determinant expressed on β_2-m-free HLA-A (excluding HLA-A24), HLA-B7301 and HLA-G heavy chains [27, 28], the mAb HC10 which recognizes a determinant on all β_2-m-free HLA-B and HLA-C heavy chains and on β_2-m-free HLA-A10, -A28, -A29, -A30, -A31, -A32 and -A33 heavy chains [27, 29], the LMP2-specific mAb SY1 [30], the LMP7-specific mAb HB-2 [30], the LMP10-specific mAb TO-7 [30], the TAP1-specific mAb NOB-1 [31], the TAP2-specific mAb NOB-2 [31], the calnexin-specific mAb TO-5 [32], the calreticulin-specific mAb TO-11 [32], the Erp57-specific mAb TO-2 [32] and the tapasin-specific mAb TO-3 [32] were developed and characterized as described.

The rabbit polyclonal anti-β_2-m-antibody A-139 was purchased from DAKO, Copenhagen, Denmark. The rabbit polyclonal anti-ERAP1 antibody has been described previously and was kindly provided by Dr. M. Tsujimoto, RIKEN, Wako, Saitama, Japan [33–35].

Immunohistochemical staining

The slides were dried for 16 h at 56°C before being dewaxed with standard xylene and rehydrated through graded alcohols into water. Standard immunohistochemical staining was performed as described previously [36]. Stromal cells, including infiltrating leukocytes, were used as internal positive controls. Negative control sections of each specimen were processed with omission of the primary antibodies.

Evaluation of TMA immunostaining

Two researchers (A.M. and E.J.) evaluated all patient cores, and scores were averaged to produce a single evaluation with the scorers being blind to the source of the samples. When slides were scored differently, which occurred rarely (<5%), a consensus was reached by simultaneous evaluation. Staining was scored semi-quantitatively by the quality control system proposed by Ruiter et al. [37]. The intensity and percentage of positive cells in the tumour and the stromal cells surrounding the tumour were determined. The intensity of staining was scored as 0, 1, 2, or 3 indicating absent, weak, clear, or strong expression, respectively. The percentage of positive cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The sum of both scores was used to identify three categories of expression: normal expression (total score 7–8), partial loss (3–6) and total loss (0–2). Immunohistochemical staining demonstrated strong positive expression of all examined markers in stromal tissue and tumour-infiltrating inflammatory cells, thereby providing an internal positive control.

Statistical analysis

The Chi-square test or, where appropriate, Fisher's exact test was used for evaluation of associations between expression and clinicopathological parameters. All statistical analyses were performed with the SPSS Version 12 software package. Five-year survival rates were calculated and survival rates were compared according to the Kaplan–Meier method using the log rank test, while multivariate analysis of survival was performed according to Cox proportional hazard models. Overall survival (OS) was defined as survival till death due to cervical carcinoma (the two patients that dyed from other causes were excluded), i.e. disease-specific survival, while disease-free survival (DFS) was defined as time to disease recurrence, metastasis, or disease-specific death. All tests were two-sided and the significance level was set to 5%.

## Results

Clinical features

Of the total group of 109 patients, 52 were diagnosed as FIGO stage IB1, 30 as IB2, 21 as FIGO IIa and 2 as FIGO IIb. For four patients no FIGO staging was available. Fifty-five patients (50%) received post-operative radiotherapy due to narrow tumour-free margins of excision, lymph node metastases, vaso-invasive growth, parametrial infiltration, depth of infiltration exceeding 15 mm and/or tumour size exceeding 4 cm. Twenty-seven patients had lymph node metastases. Fifty-one patients (58%) were HPV16-positive, 21 (24%) were HPV18-positive, while the remaining patients were positive for HPV31, HPV33, HPV45, HPV52 or HPV68. At the end of follow-up, 80 patients were alive, 2 suffered from recurrent disease, 3 had a metastasis, 22 had died of the disease and 2 had died of causes unrelated to the primary disease. The median follow-up time was 45 months.

HLA class I and APM component expression

Representative staining patterns for the HLA class I heavy chains and various APM components are shown in Figs. 1 and 2. Expression of the HLA class I heavy chains and β_2-m is summarized in Table 1. Of the 109 cases, eight exhibited total β_2-m loss; correspondingly, these cases were also negative for HLA heavy chain cell surface expression. As HLA class I expression, and therefore tumour immunogenicity, is not affected by the APM in a β_2-m-negative background these eight cases were excluded from further analysis of APM effects. Furthermore, as HLA class I expression is regulated by the APM in a β_2-m-independent manner, the overall HLA class I expression score was based on expression of the heavy chains. If either or all the heavy chains were expressed normally, overall HLA class I expression was considered to be normal. Total loss of all heavy chains was counted as total HLA class I loss, and all other cases were counted as partial loss.

As shown in Table 2, expression of all APM components except LMP10, calnexin, and calreticulin was lost in some of the cases. This down-regulation was significantly associated with HLA class I down-regulation for all components, except ERAP1. Moreover, defects in overall TAP expression (defined as partial or total loss of either or both of the TAP subunits) were significantly associated with

Fig. 1 Immunohistochemical analysis. Staining patterns of **a–c** HLA-A, **d–f** HLA-B/-C, **g–i** LMP7 and **j–l** TAP1 showing **a, d, g, j** normal expression, **b, e, h, k** partial loss of expression and **c, f, i, l** total loss of expression
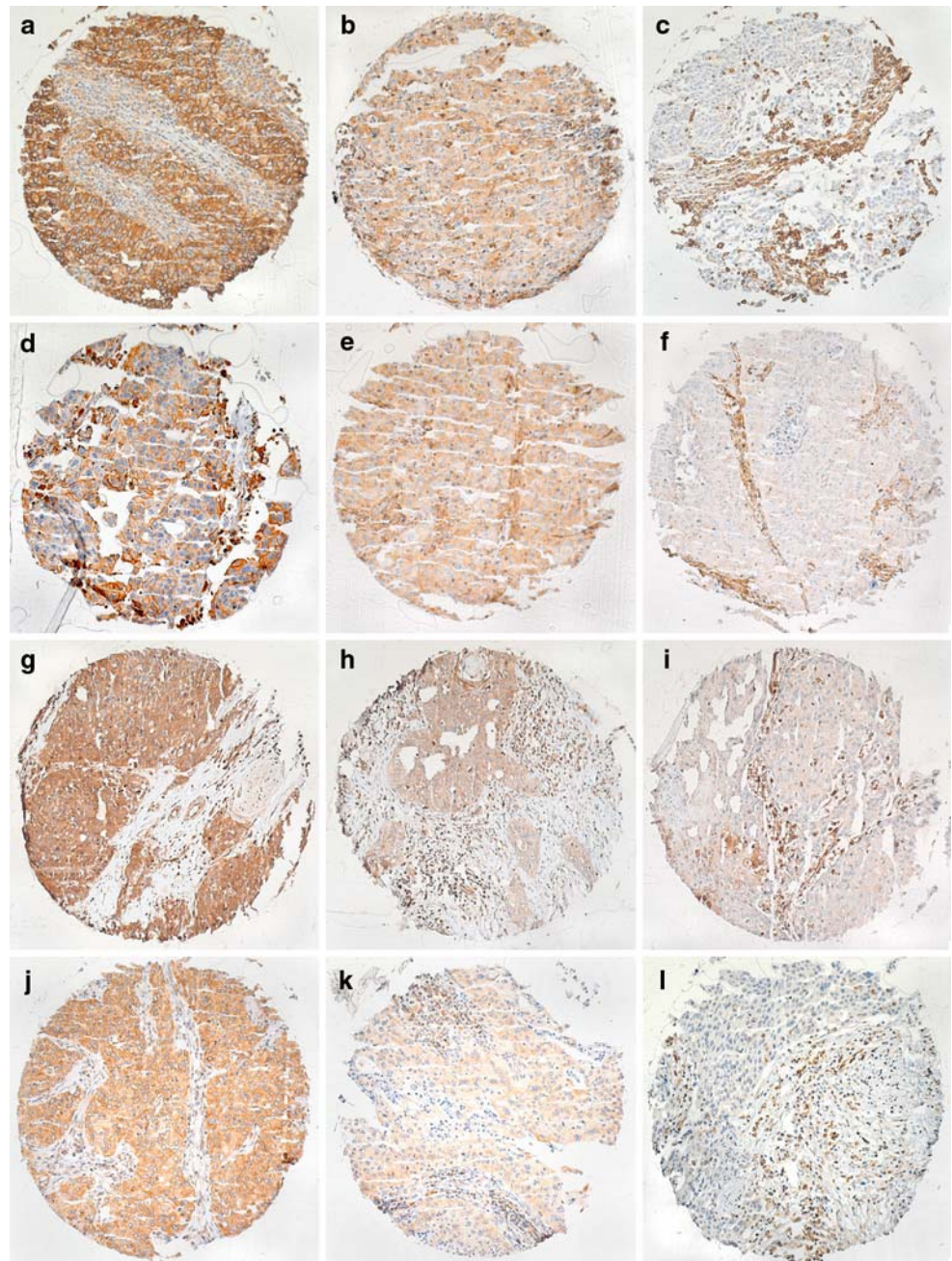
HLA class I down-regulation ($P < 0.001$). Down-regulation of any APM component was significantly associated with HLA class I down-regulation ($P < 0.001$).

Association with histopathological parameters

Depth of invasion exceeding 15 mm and vaso-invasive growth are unfavourable prognostic indicators. Of the cases examined, 51 (45%) demonstrated depth of invasion greater than 15 mm and 38 (33%) demonstrated vaso-invasive growth. Total loss of overall HLA class I expression and of any APM component was found to be

significantly associated with depth of invasion exceeding 15 mm ($P = 0.034$ and 0.045, respectively), while no significant association was found for defective expression of any marker and vaso-invasion.

None of the markers was found to be significantly associated with FIGO stage, corresponding to the lack of a significant association with histopathological parameters. No association of any of the markers with specific HPV status of the patients was detected. Interestingly, partial LMP2 and/or LMP7 loss was significantly associated with an absence of detectable lymph node metastases ($P = 0.023$ and 0.021, respectively).

Fig. 2 Immunohistochemical staining patterns of ERAP1. **a** Normal expression. **b** Partial loss of expression
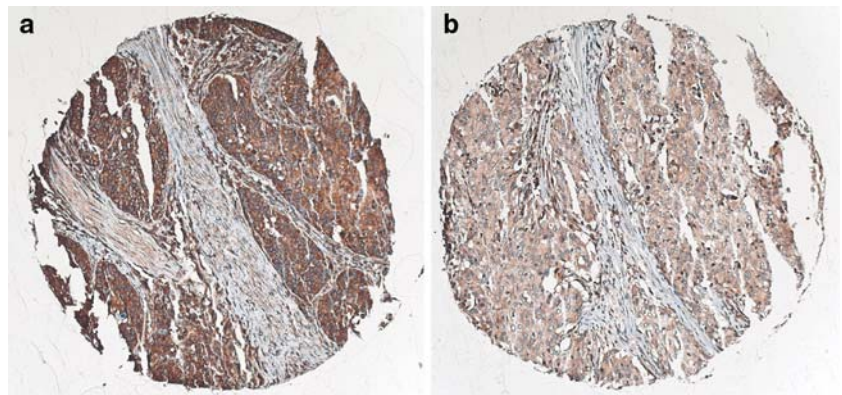

Table 1 Expression of HLA class I heavy chains, β_2-microglobulin and overall HLA class I

	HLA-A			HLA-B/-C			β_2-microglobulin			HLA class I		
	Total loss	Partial loss	Normal expression	Total loss	Partial loss	Normal expression	Total loss	Partial loss	Normal expression	Total loss	Partial loss	Normal expression
Overall ($n = 109$)	36 (33)	22 (20)	51 (47)	24 (22)	29 (27)	56 (51)	8 (7)	28 (26)	73 (67)	21 (19)	24 (22)	63 (59)
Tumour type												
AC ($n = 26$)	6 (23)	5 (19)	15 (58)	4 (15)	8 (31)	14 (54)	1 (4)	7 (27)	18 (69)	4 (15)	5 (19)	17 (66)
SCC ($n = 83$)	30 (36)	17 (21)	36 (43)	20 (24)	21 (25)	42 (51)	7 (8)	21 (25)	55 (67)	17 (21)	19 (23)	46 (56)

Data shown as n (%)

Table 2 Overall and histological type-specific expression of APM components

	Overall ($n = 101$)				AC ($n = 25$)				SCC ($n = 76$)			
	Total loss	Partial loss	Normal expression	P	Total loss	Partial loss	Normal expression	P	Total loss	Partial loss	Normal expression	P
LMP2	14 (14)	30 (30)	57 (56)	0.014	5 (20)	11 (44)	9 (36)	0.772	9 (12)	19 (25)	48 (63)	0.001
LMP7	3 (3)	16 (16)	82 (81)	<0.001	2 (8)	9 (36)	14 (56)	0.100	1 (1)	7 (9)	68 (90)	<0.001
LMP10	0	0	101 (100)									
TAP1	4 (4)	19 (19)	78 (77)	<0.001	0 (0)	4 (16)	21 (84)	0.230	4 (5)	15 (20)	57 (75)	0.001
TAP2	11 (11)	26 (26)	64 (63)	<0.001	4 (16)	6 (24)	15 (60)	0.005	7 (9)	20 (26)	49 (65)	<0.001
ERAP1	0	14 (15)	80 (85)	0.314	0 (0)	2 (9)	20 (91)	0.582	0	12 (16)	60 (84)	0.307
Tapasin	1 (1)	24 (24)	76 (75)	<0.001	0 (0)	10 (40)	15 (60)	0.284	1 (1)	14 (18)	61 (81)	<0.001
Calnexin	0	0	101 (100)									
ERp57	12 (12)	40 (40)	49 (48)	0.017	1 (4)	6 (24)	18 (72)	0.615	11 (15)	34 (45)	31 (40)	0.002
Calreticulin	0	0	101 (100)									

Data shown as n (%). P values are shown for association with overall HLA class I down-regulation

Association with overall and disease-free survival

As shown in Table 3 and Figs. 3 and 4, partial loss of overall HLA class I expression was significantly associated with decreased OS, and exhibited a trend towards a significant association with DFS, whereas total loss was not significantly associated with survival. Moreover, partial HLA-A loss (evaluated by HCA2 reactivity) was significantly associated with decreased OS and DFS, whereas total loss was not significantly associated with survival. Partial, but not total HLA-B/-C loss (assessed by HC10 reactivity) showed a trend towards significant association with decreased survival.

Decreased OS and DFS was significantly associated with total TAP1 loss, but not with normal expression and partial loss. A similar pattern was observed for partial LMP7 loss, though this was not significantly associated with DFS. ERAP1 loss was significantly associated with

Table 3 HLA class I and APM component expression and 5-years survival rates

	Overall survival			Disease-free survival		
	Normal expression	Partial loss	Total loss	Normal expression	Partial loss	Total loss
HLA-A	42 (83)	12 (55)	29 (81)	42 (83)	13 (60)	28 (77)
HLA-B/-C	45 (81)	19 (64)	18 (75)	46 (82)	19 (67)	18 (73)
HLA class I	52 (83)	15 (61)	16 (77)	52 (82)	16 (65)	15 (75)
LMP7	24 (74)	17 (87)	1 (33)	62 (76)	13 (80)	2 (50)
TAP1	61 (78)	8 (70)	1 (33)	63 (81)	13 (67)	1 (25)
ERAP1	66 (82)	5 (38)	–	65 (81)	7 (50)	–

Data shown as n (%)

decreased OS and DFS as compared to normal ERAP1 expression.

Multivariate analysis

As expected, well known prognostic parameters such as depth of infiltration exceeding 15 mm, presence of vaso-invasive growth, and presence of lymph node metastases were significantly associated with a shorter survival, while FIGO-stages of IB2 or more showed a significant association with shorter DFS (Table 4). HPV type was not significantly associated with survival (data not shown). Subsequently, multivariate analysis was performed on overall HLA class I, TAP1, LMP7 (for OS only) and ERAP1 expression, combined with the aforementioned prognostic factors. ERAP1 down-regulation was demonstrated to be an independent prognostic parameter for shorter OS (hazard ratio (HR) 3.08; 95% CI 1.07–8.90; $P = 0.037$) and shorter DFS (HR 2.84; 95% CI 1.01–8.03; $P = 0.049$), as was presence of lymph node metastases (HR 7.02; 95% CI 2.55–19.32; $P < 0.001$ for OS and HR 6.64; 95% CI 2.53–17.44; $P < 0.001$ for DFS). Total TAP1 loss was also an independent predictor for shorter DFS (HR 6.72; 95% CI 1.29–35.06; $P = 0.024$).

Discussion

In the present study we have investigated HLA class I and APM component expression in cervical carcinoma patients and have evaluated the relationship of these factors with survival and other clinicopathological parameters.

Our results show that HLA class I down-regulation occurs frequently (~40% of cases) in cervical carcinoma, corresponding to previous reports [19, 21]. Synchronous partial or total loss of various APM components was found in a substantial number of cases and was demonstrated to be significantly associated with HLA class I down-regulation. We observed partial HLA class I loss to be significantly associated with decreased OS. Of the APM

components, TAP1, LMP7 and ERAP1 down-regulation exhibited significant associations with decreased survival. ERAP1 down-regulation was shown to be an independent, significant predictor of shorter survival.

Though defective APM component expression is hypothesized to be an important mechanism of HLA class I down-regulation and therefore immune evasion by tumour cells, information regarding APM down-regulation in carcinomas is limited. This is partly due to the scarcity of reliable antibodies for most APM components other than the TAP subunits. Furthermore, most previous studies of APM expression in cervical cancer were limited by small sample sizes. Lastly, the use of various scoring methods for evaluation of immunohistochemical staining has rendered proper interpretation of findings difficult, leading to conflicting data, e.g. regarding the frequency of TAP1 loss [9, 18–20].

In this study, a panel of recently reported monoclonal antibodies was used to investigate expression of all known APM components in a large set of cervical carcinoma lesions using a TMA-based approach [30–33]. Staining was evaluated according to a previously reported quality control system [37], which is more stringent in defining total and partial expression loss than other evaluation methods, which consider ≤25% positive cells as total loss. The system used here defines total loss as complete or near-complete negativity and is therefore more reliable. Moreover, by excluding β_2-m-negative cases from the analysis, all outcome parameters can be reliably correlated to expression levels of the various APM components, as any influence of defective APM expression will only be detectable in a β_2-m-positive setting.

As an APM component, ERAP1 has been shown to be an important determinant of the repertoire of HLA class I-presented peptides [14, 34, 38–41]. However, to date no systematic investigation of its potential role in HLA class I down-regulation in tumours has been performed. To our knowledge, this is the first description of ERAP1 down-regulation in a human carcinoma. Additionally, we have identified ERAP1 loss as an independent predictor of

Fig. 3 Kaplan–Meier curves for overall survival. Patients with normal expression, partial loss, and total loss of **a** HLA-A, **b** HLA-B/-C, **c** overall HLA class I, **d** LMP7, **e** TAP1 and **f** ERAP1 expression. *P* values are shown for comparison with normal expression
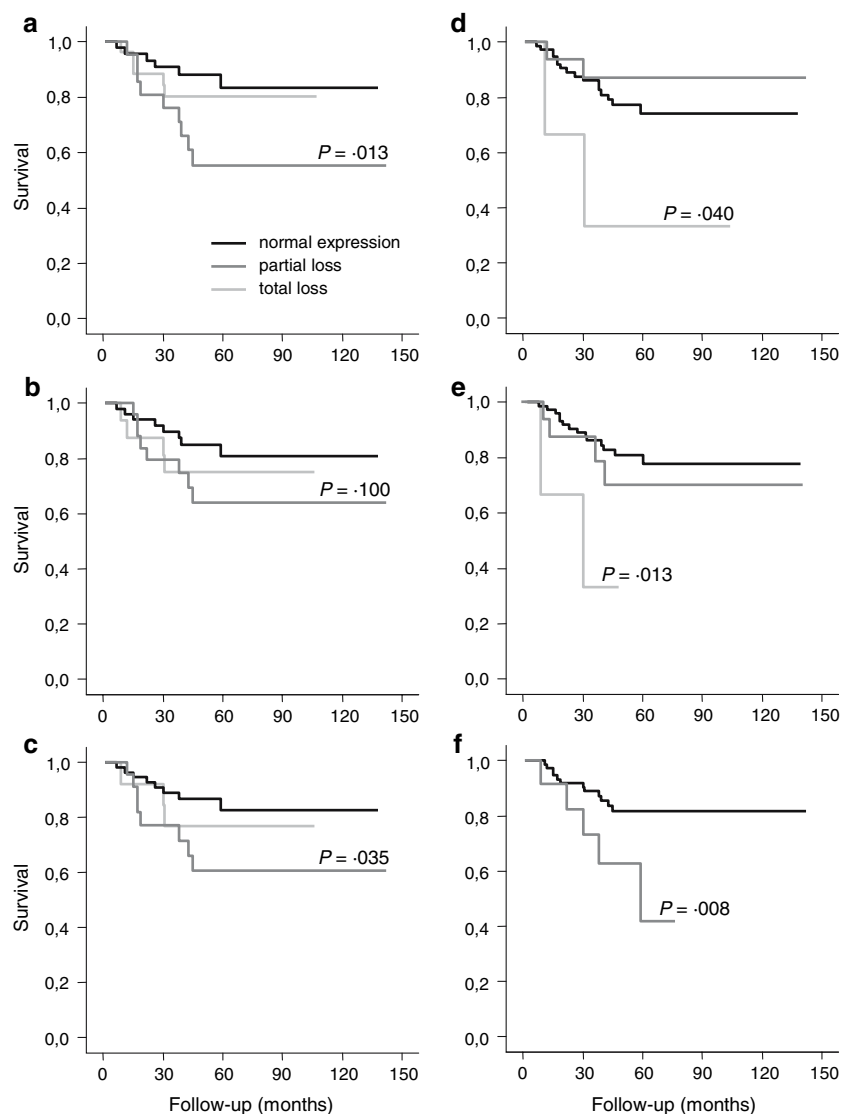
shorter survival, in the absence of associated HLA class I down-regulation. A possible explanation for this observation is the role of ERAP1 in shaping the antigen repertoire: ERAP1 down-regulation may lead to the preferential loading and presentation of non-tumour-associated peptides, thereby yielding a less immunogenic phenotype and facilitating tumour growth and progression. This hypothesis is supported by previous reports on the disrupted nature of presented peptide repertoire in ERAP1-deficient mice [41, 42]. Also, the effect of down-regulation of several APM components on the presentation of specific HPV 16 E6-derived epitopes has been firmly established [43–45].

Although we found that total TAP1 loss was an independent predictor for shorter DFS, this finding should be interpreted with caution as only four patients exhibited this loss. The decreased significance of ERAP1 as an independent predictor for shorter DFS can be explained by the

interdependency of ERAP1-mediated peptide trimming and TAP-mediated peptide transport.

An interesting finding in this study is the observed association of LMP2 and LMP7 down-regulation with an absence of lymph node metastasis; this finding is seemingly corroborated by reports that certain immunogenic peptides in melanoma and renal-cell carcinoma are not generated by immunoproteasomes and that presentation of these peptides is reduced upon up-regulation of the immunoproteasomal subunits [46]. Though altered proteasomal cleavage of endogenous proteins can lead to an alternative, less immunogenic antigen repertoire, an adequate mechanism for the association of LMP down-regulation with an absence of lymph node metastases remains to be postulated.

Interestingly, we have recently observed that genetic variation in various APM components is significantly

Fig. 4 Kaplan–Meier curves
for disease-free survival.
Patients with normal expression,
partial loss, and total loss of
a HLA-A, **b** HLA-B/-C,
c overall HLA class I, **d** LMP7,
e TAP1 and **f** ERAP1
expression. *P* values are shown
for comparison with normal
expression
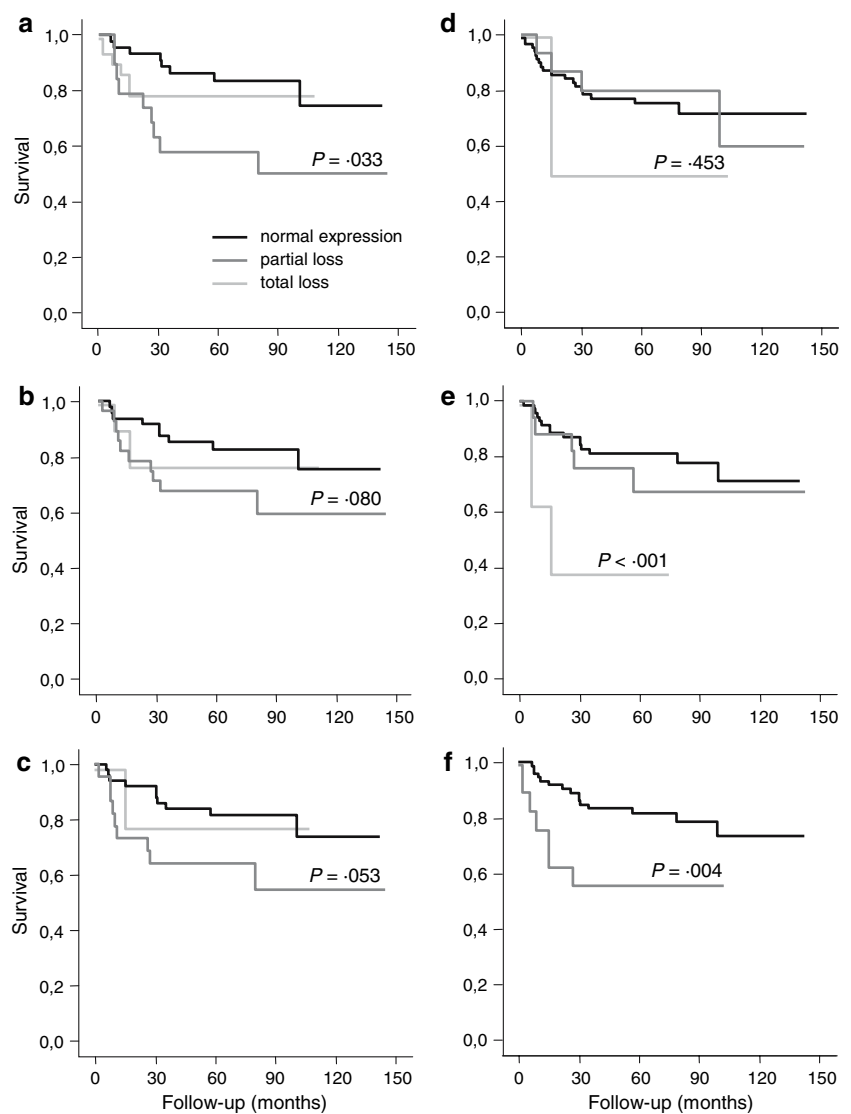
associated with increased cervical carcinoma risk.
Although the underlying mechanisms of APM component
loss are largely unknown, the occurrence of single nucle-
otide polymorphisms in the various APM genes could form
a genetic basis predisposing to either mutations, or regu-
latory down-regulation [47, 48].

Partial HLA class I loss was observed to be significantly
associated with decreased OS; correspondingly, reports in
colon cancer have shown partial, but not total HLA class I
loss to be associated with worse OS [49]. This finding can
be explained by the ability of low levels of HLA class I
molecules to provide tumour cells with an escape not only
from cytotoxic T lymphocyte-mediated, but also from
natural killer (NK) cell-mediated cytotoxicity [2]. This
hypothesis is further supported by the association of
decreased survival with total TAP1 loss, which has been
shown previously to lead to partial HLA class I loss
[2, 50–52]. In the present study, an association of loss or

down-regulation of expression of the various APM mole-
cules with aberrant HLA expression was observed. More-
over, the partial HLA loss observed is most likely an
underestimation of the extent of HLA alterations present as
immunohistochemical staining on paraffin sections does
not reveal loss of specific HLA alleles [8]. However, there
are also other aberrations known to lead to HLA class I
alterations and it is plausible to hypothesize that all these
mechanisms can coexist in the same tumour sample.

In conclusion, we have shown here that defective
expression of HLA class I and of various APM components
occurs frequently in cervical carcinoma. Partial HLA class
I loss and total TAP1 loss are associated with decreased
survival, while down-regulation of immunoproteasomal
subunits is associated with decreased risk of lymphogenic
metastasis. Moreover, we report the first description of
ERAP1 down-regulation in a human carcinoma, and have
found that this is an independent prognostic parameter for

Table 4 Histopathological parameters and 5-years survival rates

	Overall survival		Disease-free survival	
	%	P	%	P
Depth of infiltration				
<15 mm (n = 59)	88		90	
≥15 mm (n = 51)	60	<0.001	57	<0.001
Vaso-invasion				
Absent (n = 68)	81		85	
Present (n = 38)	59	0.031	54	0.002
Lymph nodes				
Negative (n = 80)	86		86	
Positive (n = 27)	43	<0.001	39	<0.001
Histopathological class				
SCC (n = 84)	75		76	
AC (n = 26)	77	0.924	74	0.662
FIGO stage				
≤IB1 (n = 52)	82		91	
≥IB2 (n = 53)	69	0.060	57	0.002

decreased survival. A complete understanding of the mechanisms and relevance of HLA class I and APM component down-regulation and immune evasion may contribute to the rational design of tumour vaccines and T-cell-based immunotherapies.

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