

Expression and genetic analysis of transporter associated with antigen processing in cervical carcinoma

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Abstract

Objective. Transporter associated with antigen processing (TAP) loss causes human leukocyte antigen (HLA) class I downregulation which is frequently found in cervical carcinomas and their precursors. HLA class I molecules activate T-cells by antigen presentation and are therefore essential for immunological surveillance. To add to the hitherto limited knowledge of molecular mechanisms underlying TAP loss, we investigated TAP expression, loss of heterozygosity (LOH) and possible TAP mutations.

Methods. Twenty-three cervical carcinomas and adjacent precursor lesions were stained with HLA-A-, HLA-B/C-, β_2 -microglobulin-, TAP1- and TAP2- antibodies. In order to separate tumour and non-tumour cells, cervical carcinoma samples were sorted by flow-cytometry and were subsequently analysed for LOH with 3 markers in the TAP region on chromosome 6p21.3. Mutation analysis of the complete TAP1 gene was performed.

Results. Aberrant TAP1 expression was detected in 10/23 cervical carcinoma lesions and in 5/10 adjacent cervical intraepithelial neoplasia (CIN) lesions. All the lesions with low TAP expression also had reduced HLA class I expression. LOH was found in 7 out of 10 lesions with TAP loss. Mutation analysis detected no aberrations, but identified a polymorphism in the 5'-untranslated region (UTR) of the TAP1 gene in two lesions.

Conclusions. This study shows that defective TAP expression in cervical carcinoma is often associated with LOH in the TAP region but not with mutations in the TAP1 gene.

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Introduction

Immunological surveillance of tumour cells and virally infected cells is performed by cytotoxic T-lymphocytes (CTLs), which recognise aberrant peptides presented by human leukocyte antigen (HLA) class I molecules. HLA class I downregulation is caused by different molecular mechanisms and results in escape from CTL attack [1]. Antigen presentation by the HLA molecules to circulating CTLs requires the transporter associated with

antigen processing (TAP). Endogenous proteins (viral or tumour associated products, or waste products from the cell itself) are degraded in the cytosol into smaller peptides. These peptides are subsequently transported by TAP. The latter consists of the subunits TAP1 and TAP2 that form a channel in the endoplasmic reticulum (ER)-membrane [2]. In the ER, the assembly of the HLA class I heavy chain, the β_2 -microglobulin (β_2m) light chain [3] and the peptides [4] is chaperoned by several proteins [5–8]. The newly formed complex is then transported via the Golgi network to the cell surface.

HLA class I downregulation is frequently associated with impaired TAP expression in various tumour types [9–12].

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Limited knowledge is at hand concerning the molecular mechanisms underlying TAP loss. Deletion in the TAP1 gene has been described that leads to rapid degradation of its mRNA in a melanoma cell line [13,14] and recently mutations in the TAP1 and TAP2 genes have been found in colorectal carcinomas [15]. It has been suggested that in renal cell carcinoma TAP defects are caused by regulatory abnormalities [16]. Using real-time PCR it has been shown that in bladder cancer there is a coordinated transcriptional downregulation of the HLA class I antigen processing machinery, including TAP, which causes loss of HLA class I expression [17].

HLA phenotype alterations occur frequently in cervical cancer and its precursor lesions [18–23]. Koopman et al. described that this is caused by extensive loss of heterozygosity (LOH) at chromosome 6p21.3, partly in combination with mutations in the β_2m or HLA class I genes [22]. The TAP expression data from previous studies are inconsistent, with loss of TAP1 being reported in 0–50% of cases [24–27]. Moreover, limited information exists about the genetic mechanisms leading to loss of expression. A recent study of cervical carcinoma lesions has reported possible mutations in the TAP genes, but no sequence analysis was done and the loss of TAP expression was not studied [28].

These limited and contradictory results prompted us to investigate the loss of TAP in relation to HLA class I expression in invasive cervical carcinoma and adjacent cervical intraepithelial neoplasia (CIN) lesions. Furthermore, we examined possible causative mechanisms of altered TAP expression by performing LOH and gene mutation analysis on flow-sorted pure tumour and normal cell fractions from paraffin embedded cervical cancer tissue.

Material and methods

Tissue samples

Since 1989, Surinamese women with cervical carcinoma have been coming to the Leiden University Medical Centre (LUMC) at Leiden, The Netherlands, to have a radical hysterectomy (Wertheim operation). All the resected tumour tissue is stored in the tissue archive of the Pathology Department of the LUMC. All patients had a FIGO stage Ib or IIa, which qualified them for the radical hysterectomy. Surinamese cervical cancer samples were selected for this study, because of the frequent loss at chromosome 6p that was previously observed [29]. In addition, cervical carcinomas of Surinamese women are usually of substantial size, which provided us with sufficient residual material for the extensive TAP1 gene sequence analysis.

From the group of Surinamese cervical cancer patients that were treated between 1989 and 2004, we selected the cases with invasive cervical carcinoma and an adjacent high-grade cervical intraepithelial neoplasia (CIN III) by reviewing the haematoxylin–eosin-stained slides. Precursor lesions connected to the cervical tumours were required to investigate the timing in addition to occurrence of TAP aberrations. We found 23 cases of cervical cancer, which were operated between 1989 and 2004 that had an adjacent CIN lesion with sufficient tissue to investigate. From these 23, tissue blocks containing formalin-fixed, paraffin-embedded normal tissue and tumour tissue with an adjacent CIN lesion were selected.

Immunohistochemistry

Immunohistochemistry was performed on freshly cut, 3- μ m thick, formalin-fixed, paraffin-embedded sections according to standard procedures [30]. Slides

were incubated overnight with mouse monoclonal antibodies (mAbs) that are suitable for staining paraffin sections, the TAP1-specific mAb NOB-1 (S. Ferrone). This mAb is secreted by a hybridoma derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with splenocytes from a BALB/c mouse immunised with partial length TAP1 recombinant protein (aa 434–735) and a keyhole limpet hemocyanin (KLH)-conjugated TAP1 peptide (aa 717–735). The specificity of the mAb was assessed by its reactivity with the corresponding antigen when tested in Western blotting with a lysate of lymphoid cells which express TAP1 and by the lack of reactivity with a lysate of the T2 cell line, which does not express these molecules [5,11] and anti-TAP2 (clone TAP2.17, Becton Dickinson Biosciences Pharmingen, San Jose, CA, USA). Furthermore, the mouse mAbs HCA2 and HC10 (Dr J. Neefjes, NKI, Amsterdam, The Netherlands) and the primary rabbit polyclonal anti- β_2m (A 072; DAKO, Copenhagen, Denmark) were used. The mAb HCA2 recognises a determinant expressed on β_2m -free HLA-A (excluding HLA-A24), HLA-B7301 and HLA-G heavy chains [31,32]. The mAb HC10 recognises a determinant expressed on all β_2m -free HLA-B and HLA-C heavy chains, as well as on β_2m -free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33 heavy chains [31,33].

Staining was scored semi-quantitatively by the quality control system proposed by Ruiter et al. [34]. The intensity and percentage of positive tumour cells were determined. The intensity of staining was scored as 0, 1, 2 or 3, for 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). In each tumour, stromal cells including lymphocytes served as a positive control for HLA class I and TAP expression.

Staining, flow cytometry and sorting

Staining, flow cytometry and sorting of all formalin-fixed, paraffin embedded samples were performed as described previously with minor adjustments [35]. Multiple 0.6 mm punches from representative tumour areas of the paraffin blocks were treated with a combined mechanical/enzymatic method to obtain single cells. Cells were subsequently stained with a mAb mixture directed against keratin and vimentin, containing clones MNF116 (anti-keratin; DAKOCytomation, Glostrup, Denmark), AE1/AE3 (anti-keratin; Chemicon International Inc, Temecula, CA, USA) and V9-2b (anti-vimentin; Department of Pathology, LUMC) [36] or 3B4 (anti-vimentin; DAKOCytomation). Propidium iodide (PI) was used as a DNA stain. Tumour and normal cell subpopulations were flow-sorted based on keratin and vimentine expression, respectively, combined with a gate on DNA content using a FACSVantage flow sorter (BD Biosciences). Flow-sorted cells (yields ranging from 2×10^5 to 1×10^6 cells) were collected in 5.0 ml Falcon™ tubes and centrifuged at $1000 \times g$ for 10 min before DNA extraction [37].

LOH analysis

DNA was extracted from sorted tumour cell subpopulations (keratin positive, vimentin negative) for LOH analysis. The microsatellite markers D6S2444 (UniSTS: 239054); TAP1 (3'-GGACAATATTTTGCTCCTGAGG-5' (F); 3'-GCTTTGATCTCCCCCTC-5' (R)) and M2426 (3'-TTGTGGTTTCAGCTACTCAGG-5' (F); 3'-GTTTCTTTTCTTTTCATTTGGCCTCTACTG-5' (R)) located in the TAP region on chromosome 6p21.3 were used. DNA extracted from the keratin negative, vimentin positive cell fractions was used as a normal DNA reference.

Standard PCR amplifications were performed as described [38]. All reactions were performed at least *in duplo*. The thresholds for retention of heterozygosity (ROH), “grey area” (allelic imbalance) and LOH were applied as previously empirically determined [37]. These were 0.76–1.3 (ROH), 0.58–0.75 or 1.31–1.69 (allelic imbalance) and $0.57 \geq$ or $1.7 \leq$ (LOH).

Sequence analysis of the TAP gene

The 11 TAP1 exons, including the exon–intron boundaries were amplified using the primers shown in Table 1. All primer positions were derived from

Table 1
Primers used for sequence analysis of flow-cytometry sorted, paraffin-embedded tumour samples for mutations in the 11 exons of the TAP1 gene

		Forward primer	Reverse primer
Exon	1-1	ctggtgcaagtggaaaggca	ctggcgaagaagctcagcca
	1-2	gccgctttcgatttcgcttt	gggcctgaagctccgggta
	1-3	ccgccagtagggaggact	ggcgagaagtagcagtagctcc
	1-4	caatggctagcttaggtgt	cacggcccagcggtctca
	1-5	cccggagcttctctcgcat	cagctaattggcttcaaagcag
	1-6	cgctggccgtgctctg	cagtgcagtagcctgggtctatc
	1-7	gttccgagagctgatctcatgg	agcctagaagccgacgcaca
	1-8	ggtcaggcggctctggaa	ttccctgcgttccccttac
Exon	2	tctgactggaactgacctacttag	aactccaactccctcatgtg
Exon	3	aacacacccgtgatccctt	gaacagtagatggctataatg
Exon	4-1	gaacctgtctgattcacctcac	ccagagcatgatcccca
	4-2	gtacctgggtcgaggccctat	gggagatgagggtctgtgtag
Exon	5-1	tgccaaccctgtgatcatct	ttcctaaactctggcctctg
	5-2	gaagcttggccaacgagga	gggaatgggtattcatcttca
Exon	6	ttgtgtctctttatagattcag	caactggggagtgaaggtg
Exon	7-1	ctcactttcactattcttacct	aaggagtgcaacagaccact
	7-2	ttgagtagctggaccgcac	gccagtggaatacagggagtg
Exon	8	gtgtgcttctgtccctcta	caagccacctgctccata
Exon	9	cctgttctatgacttctcatcat	ggctgggtggtgagatga
Exon	10	ggctataccgttctcatcttgg	ccattaaagatgactgcctca
Exon	11-1	cggtctgacggtccgatg	tgcacatggcccagtagca
	11-2	tctggaaggaggcgctatc	gaggagcttggaaaggaggt
	11-3	agctgcctccaggatgagtt	gctgatcatcttccgtaca
	11-4	gggtggtggccagcactctgaa	gtttggtgtgccgaaacat

the genomic sequence of the gene (Ensembl accession number: ENSG00000168394). The only available DNA isolates were obtained from flow-sorted, paraffin-embedded material; we thus used several different primer sets for some exons in order to acquire easily amplifiable products. Sequencing was performed by the LGTC (Leiden Genome Technology Centre, The Netherlands). The obtained sequences were compared with DNA sequences of sorted autologous normal material (vimentin positive, keratin

negative) and with the corresponding allele sequences from the Genome Database (<http://www.gdb.org>).

Results

Loss of HLA class I and TAP expression in cervical carcinoma and adjacent CIN

Tissue sections with both tumour and adjacent CIN tissue were stained for HLA-A (HCA2), HLA-B/C (HC10), β_2m , TAP1 and TAP2 expression. The data are displayed in Fig. 1, grouped based on the TAP1 expression of the samples: absent, weak and positive. Complex combinations of expression patterns for the different molecules studied were observed in the majority of cases. Only one case (S87) showed complete loss of TAP1 expression and resulting HLA-A and HLA-B/C loss of expression. In this case, β_2m was expressed and TAP2 was weakly expressed underlining the importance of TAP1 expression for stability of the HLA/ β_2m /antigenic peptide complex and eventual HLA surface expression. In 9 additional tumour samples, TAP1 expression was weak. In one of these cases (S85) the adjacent CIN lesion was negative, in 3 cases (S84, S31, S04) the CIN followed the expression pattern of the tumour sample, while in 4 other cases (S86, S61, S08, S10) the CIN lesions were positive. In S71, the adjacent CIN showed weak TAP1 expression while the tumour was positive. In 5 of the 9 cervical carcinoma lesions with TAP1 downregulation, TAP2 was also weakly expressed (S85, S84, S31, S04, S86). Total HLA class I expression was downregulated in 8 of the 9 cases with TAP1 defects (only case S04 showed positive expression for HLA-A). In 4 of those 9 cases β_2m expression was altered as well (S85, S84, S86, S38), which points towards

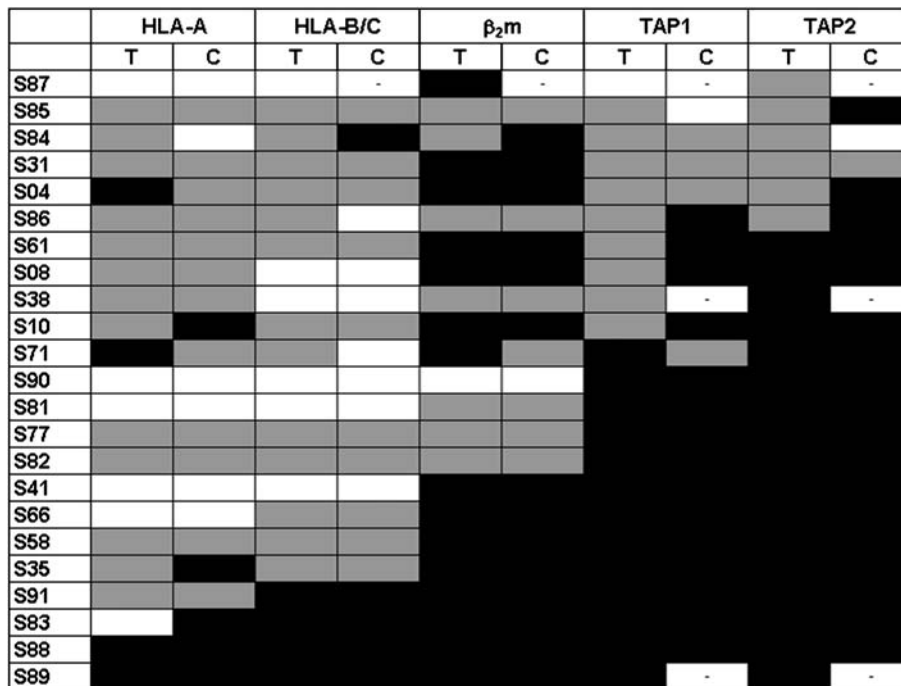


Fig. 1. Expression patterns of HLA-A (HCA2), HLA-B/C (HC10), β_2m , TAP1 and TAP2, grouped with regard to TAP expression. (T) Tumour; (C) CIN lesion. White squares represent loss of expression; grey squares represent low expression; black squares represent normal expression. (-) No data.

cumulative negative effects of alterations in TAP and β_2m on HLA expression. In 12 (of the 23) cases, TAP expression was not affected. In only 1 case (S90), total HLA class I loss could be explained by total β_2m absence. Two tumours (S77, S82) showed weak total HLA expression in concordance with the weak β_2m expression observed, while in sample S81 weak β_2m expression was probably accompanied by a second hit at the HLA class I region on chromosome 6p21, as this case was negative for HLA expression. In cases S35 and S91, low expression of both HLA-A and HLA-B/C, and solely HLA-A, respectively, was found, indicative of possible genetic aberrations in these genes such as mutations or LOH. Tumour S83 was solely negative for HCA2 implying a specific mutation in the HLA-A gene. Two samples (S88 and S89) were positive for expression of all molecules studied. Expression of all studied molecules was not always concordant between tumour and adjacent CIN. In the majority of cases, the tumour sample showed weaker or absent expression as compared to the tumour-associated CIN (i.e. S86, S61, S08, S10 for TAP1 expression). In Fig. 2, a representative case is shown stained with the 5 antibodies used.

LOH on flow-sorted tumour cells

The 23 tumour samples were all flow-sorted to obtain pure tumour cell fractions (keratin positive), assuring the precision and reliability of the LOH results (Fig. 3A). Stromal cells and infiltrating lymphocytes are responsible for masking true LOH. These cells (vimentin positive) were also sorted and used as a normal control in all experiments. We performed LOH analysis only on the tumour cell fractions and not on the adjacent CIN cells as we were not able to obtain sufficient amounts of flow-sorted CIN material. Three markers that cover the TAP region on chromosome 6p21.3 were applied. A representative example of LOH for the TAP1 marker is shown in Fig. 3B. In 4 cases none of the markers was informative and other 4 showed retention of heterozygosity for all markers (Fig. 3C). LOH for at least 1 marker was found in 9 of the cases. The TAP1 marker, which is located in the TAP1 gene, was lost in 6 cases (S87, S04, S86, S61, S41, S91). The TAP2 gene is located in between the TAP1 marker and the D6S2444 marker but the latter is situated at approximately 100 kb from the TAP2 gene. This marker showed LOH in 5 cases (S04, S86, S61, S08, S10) but

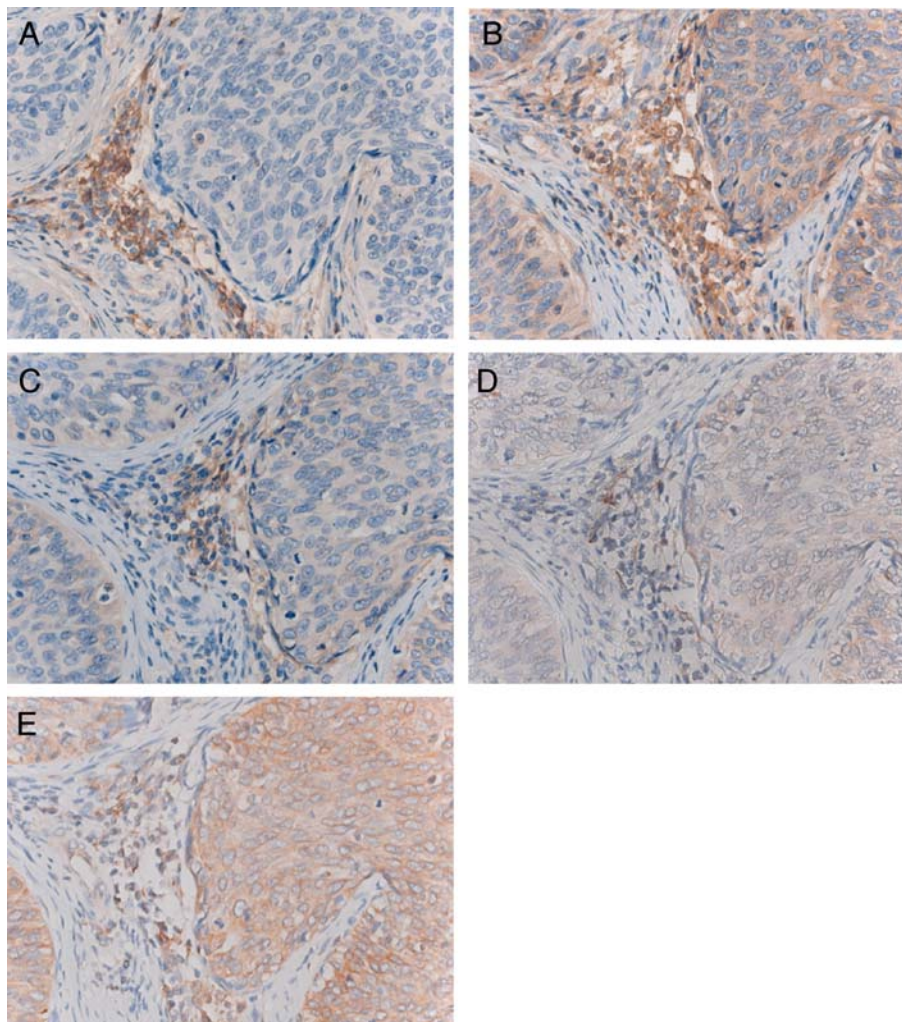


Fig. 2. Immunohistochemical staining of a cervical carcinoma lesion (sample S87). Detail (400 \times magnification) of the same group of tumour cells, stained with TAP1 (negative) (A); TAP2 (positive) (B); HLA-A (C) and HLA-B/C (D) (weak cytoplasm, negative membrane); β_2M (positive cytoplasm) (E).

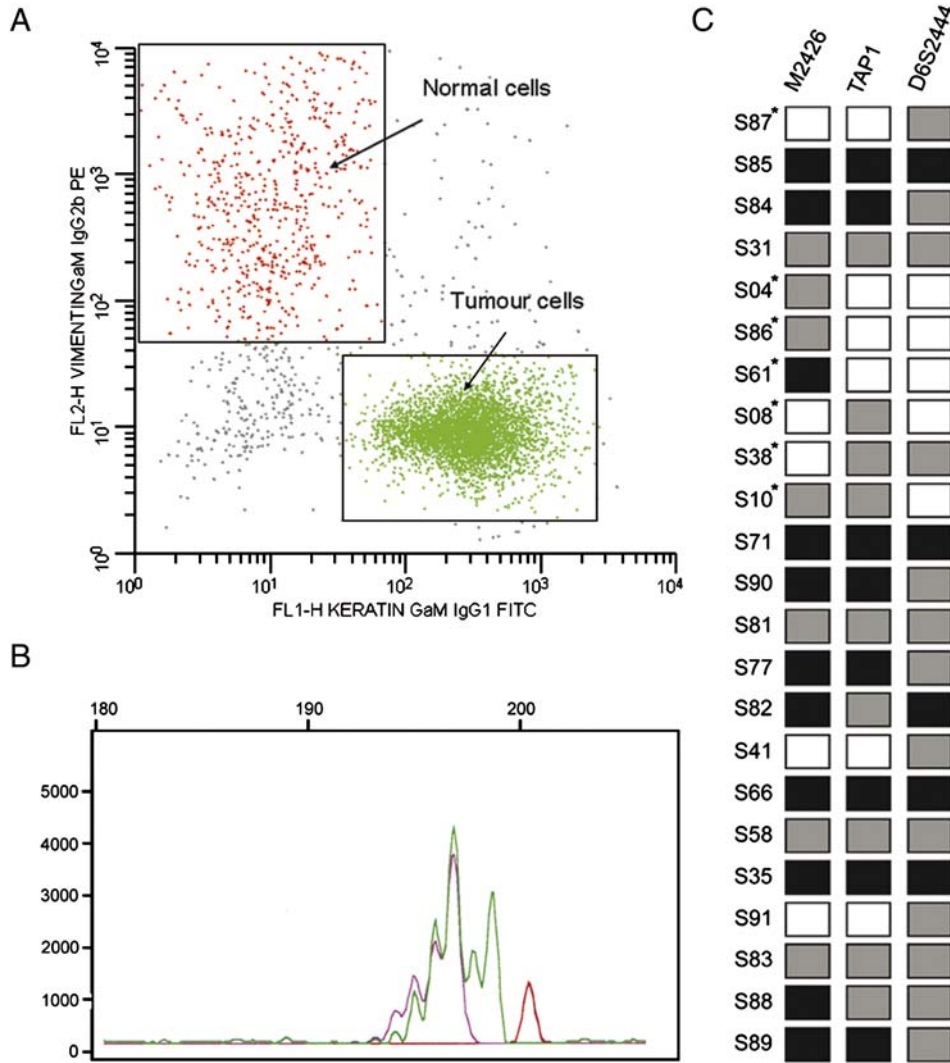


Fig. 3. LOH analysis results. (A) A representative example of flow-sorting data of a cervical cancer sample (S41). The keratin positive (tumour, FITC-labelled) cells and the vimentin positive (normal, PE-labelled) cells were flow-sorted and used in further analyses [35]. (B) LOH results (S87) at marker TAP1 for tumour (pink, one peak) and normal (green, two peaks) sorted cell fractions (S87). A size marker is depicted in red. (C) The complete LOH data of the three microsatellite markers used per tumour sample, represented as ROH (black squares); LOH (white squares) and not informative (grey squares). The same order of samples is used as in Fig. 1.

was also often non-informative (13 cases). When linking TAP protein expression to LOH, 2 cases with LOH had retained TAP expression. However, in 7 cases with LOH at at least 1 marker in the TAP region, TAP loss or weak expression was observed (S87, S04, S86, S61, S08, S38, S10) (Fig. 3C).

Mutation analysis

The 7 cases with loss of TAP expression and LOH at at least 1 marker located in the TAP region were selected for mutation analysis of the complete TAP1 gene (11 exons). To assure that no mutations could be missed as a result of contaminating normal cells present in the samples, sequencing was performed on the flow-sorted pure tumour cell fractions. Two cervical carcinomas carried a polymorphism located in the 5'-untranslated region (UTR) preceding exon 1 (Ensemble SNP annotation: rs3216794). A 1-nucleotide "C" deletion was observed in both the normal and tumour sorted cell fractions

(data not shown). No mutations or other polymorphisms were found.

Discussion

In the present study, we determined the association between loss of TAP expression and loss of HLA class I in cervical carcinogenesis and examined possible causative molecular mechanisms to add to the hitherto limited knowledge of TAP aberrations in cervical cancer.

Even though loss of TAP expression has previously been reported and found to be associated with HLA class I downregulation in cervical carcinoma lesions, the reported results are contradictory with 0–50% of the cases being negative [24–27]. Discrepancies are probably due to the frequently divergent scoring methods. In the majority of studies, cases containing less than 25% positive tumour cells are scored as negative [11,12,15,16]. Here, we applied the semi

quantitative scoring system as proposed by Ruiter et al. [34], which in our opinion gives a more accurate estimation of expression. Using this method downregulation of TAP1 expression was observed in 10/23 cases. In 6 of these 10 cases TAP2 expression was also low. None of the samples had loss of TAP expression throughout the whole tumour area with scattered nests of positive tumour cells. Although the group of patients described here is quite small, we were interested in determining TAP expression in cervical tumours with adjacent CIN lesions and these are scarce. Only some of the adjacent CIN lesions displayed low TAP expression, indicating that the moment of occurrence of these aberrations varies between cervical tumours. In several cases the altered TAP expression was more extensive in CIN than in the invasive tumour tissue. This could imply that the CIN and the tumour have a different clonal origin. In addition, probably only TAP negative CIN lesions could survive T-cell attack activated by the tumour's presence.

In cervical cancer, loss of HLA class I cell surface expression is predominantly caused by extensive LOH at chromosome 6p21.3, where the HLA class I genes encoding the heavy chains of the HLA molecules are located [22,23]. Additional mutations in these genes have also been described [22]. The genes encoding the TAP1 and TAP2 molecules are located in the same region of chromosome 6p21 [39]. Several studies have investigated the presence of mutations as well as regulatory and transcriptional abnormalities affecting the TAP genes in various tumour types [13–17,40]. A recent study on alterations of the TAP genes in cervical carcinoma was performed using single strand polymorphism PCR on blood and tumour samples [28]. The authors proposed that the major cause of TAP loss is the presence of TAP gene mutations. However, neither direct sequencing analysis nor expression analysis was performed to determine whether the loss of specific TAP alleles in fact leads to TAP protein loss.

To unravel the molecular mechanisms leading to loss of TAP expression in cervical cancer, we performed LOH analysis on flow-sorted tumour keratinocytes (keratin positive) isolated from 23 paraffin-embedded cervical carcinoma samples using concurrently sorted vimentin positive normal cells as an autologous control. In cervical cancer, some of the tumour cells might express both keratin and vimentin, which would lead to a biased selection for the solely keratin positive population using the flow-sorting method. Although such double positive cells were shown to have higher proliferation rates and invasive potential in breast cancer [41], this does not seem to be the case in cervical tumours (manuscript in preparation). In addition, in the present group of patients the keratin positive/vimentin negative tumour cells represented the major subpopulation in the samples.

Seven of the tumours with downregulation of TAP expression were shown to have LOH using markers specific for the TAP genes. It is plausible to suggest that when one allele of either one or both of the TAP genes is absent as a result of LOH, this will lead to aberrant TAP protein expression.

In addition, thorough sequence analysis of the TAP1 gene was also performed on flow-sorted pure tumour cell fractions

from the 7 cervical carcinoma samples with altered TAP expression and LOH. Although the studied cases had heterogeneous low TAP expression, which might obscure the detection of mutations, previously colon tumours with similar TAP staining patterns were shown to carry mutations [15]. However, in the present study, no mutations were detected in any of the cases.

In summary, we detected altered TAP expression in a substantial number of cervical carcinoma to be associated with LOH of 6p21.3 where the TAP genes are located and not with mutations in TAP1. The applied flow-sorting procedure allowed us to perform precise molecular analysis of the tumours, without admixture of stromal cells, infiltrating lymphocytes and other normal cells, which are known to affect LOH analysis and to obscure mutation detection [42,43]. Currently, we are studying the presence of TAP polymorphisms in cervical carcinoma to determine whether LOH is associated with the retention of specific TAP alleles.

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