CHAPTER 4

ALTERATIONS IN CLASSICAL AND NON-CLASSICAL HLA EXPRESSION IN RECURRENT AND PROGRESSIVE HPV INDUCED VULVAR INTRAEPITHELIAL NEOPLASIA (VIN) AND IMPLICATIONS FOR IMMUNOTHERAPY

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Abstract

Immunotherapy of uVIN is promising however many patients still fail to show clinical responses, which could be explained by an immune escape through alterations in HLA expression. Therefore, we analysed a cohort of patients with a primary (n=43) and subsequent recurrent uVIN lesion (n=20), vaccine treated uVIN patients (n=12), patients with HPV-induced vulvar carcinoma (n=21) and healthy controls (n=26) for the expression of classical HLA-class I/II and non-classical HLA-E/-G and MICA. HLA-class I was downregulated in 70% of uVIN patients, including patients with a clinical response to immunotherapy. Downregulation of HLA-class I is probably reversible, as only 15% of the uVIN cases displayed loss of heterozygosity (LOH) and HLA-class I could be upregulated in uVIN keratinocyte cultures by IFNγ. HLA-class I downregulation is more frequently associated with LOH in vulvar carcinomas (25-55.5%). HLA-class II was found to be focally expressed in 65% of uVIN patients. Of the non-classical molecules, MICA was downregulated in 80% of uVIN whereas HLA-E and -G were expressed in a minority of cases. Their expression was more prominent in vulvar carcinoma. No differences were found between the alterations observed in paired primary and recurrent uVIN. Importantly downregulation of HLA-B/C in primary uVIN lesions was associated with the development of recurrences and progression to cancer. We conclude that downregulation of HLA is frequently observed in premalignant HPV-induced lesions, including clinical responders to immunotherapy, and is associated with worse clinical outcome. However, in the majority of cases downregulation may still be reversible.

Introduction

Usual vulvar intraepithelial neoplasia (uVIN) is a chronic premalignant skin condition, with an increasing incidence mainly in young women, which is caused by a persistent high risk human papilloma virus (HPV) infection in over 90% of cases.[1,2] uVIN causes complaints of severe and long-lasting pruritis, pain and sexual dysfunction and has a malignant potential of 3-4% in treated and of 9% of untreated patients. [1,3] Since conventional treatments for uVIN are characterised by high recurrence rates of 20-40% and psychosexual problems, there is a need for alternative therapies.[4-6] Failure of the immune system to induce a strong and effective immune response to HPV is known to cause viral persistence and development of these premalignant anogenital lesions.[7-9] The microenvironment of uVIN lesions is characterised by high numbers of infiltrating CD4+ T cells as well as regulatory T cells (Tregs) and low numbers of cytotoxic CD8+ T cells (CTLs) compared to controls.[10-12] Immunotherapy aims to overcome the inertia of the immune system and is therefore considered a possible effective treatment option for uVIN.[13-17] Immunotherapy by imiquimod (Aldara®,), photodynamic therapy (PDT) and/or therapeutic vaccination, in both standard and experimental settings, are nowadays widely used as an alternative to conventional treatments, and have shown promising results in uVIN.[13-17] Imiquimod is a topical immune response modulator which activates dendritic cells and induces proinflammatory cytokine expression and T cell activation.[14] PDT leads to tumor directed cell death and induces local inflammation which activates antigen presenting cells and induces effector T cells.[17] Therapeutic vaccines are designed to reinforce HPV specific CD4+ and CD8+ T cell responses, and particularly the protein peptide TA-CIN in combination with imiquimod and the HPV 16 synthetic long peptide (SLP) vaccine have met with clinical success.[13,15] Application of imiquimod, PDT and therapeutic vaccination or a combination of these therapies are all associated with an increase in intralesional CD4+ and CD8+ T cells. Clearance of HPV is associated with a normalization of immune cell infiltration.[12,15,16] Lack of efficacy of immunotherapy was shown to be associated with the presence and increase of Tregs.[11,15,16,18]

The majority of vulvar carcinomas and about 30% of VIN were shown to display down-regulation of human leukocyte antigen (HLA) class I in a single study.[12] This suggests that alterations in the expression of classical, and potentially also non-classical HLA-molecules may result in escape from a specific T cell response, as well as recurrence/progression of the lesions, or unresponsiveness to immunotherapy. Loss of HLA-class I was previously shown to be associated with non-responsiveness to PDT.[12] In analogy, in HPV-induced cervical cancer loss of HLA-A was associated with poor survival.[19] Alterations in HLA-class I expression are caused by a variety of mechanisms and these alterations may be reversible ('soft') or irreversible ('hard'), in case of molecular defects as loss of heterozygosity (LOH) or beta-2 microglobulin (β2M)/HLA-class I mutations.[20-23] Furthermore, downregulation of the non-

classical MHC class I chain related molecule A (MICA), which interacts with the co-stimulatory natural killer cell lectin-like receptor (NKG2)D receptor on natural killer (NK) cells and T cells[24], is observed in cervical cancer where it is associated with a decreased survival when analysed in the context of the CTL/Treg ratio and the expression of classical HLA molecules.[19] Elevated expression of HLA-class II and that of the non-classical molecules HLA-E and HLA-G has also been observed in HPV-induced cervical cancers.[25-29] HLA-E hampers the efficacy of NK and T cells by binding to the inhibitory CD94/NKG2A receptors expressed on tumor infiltrating lymphocytes (TILs). While the expression of HLA-E was associated with poor survival in ovarian cancer it's effect was limited in cervical squamous cell carcinoma and associated with better survival in cervical adenocarcinoma.[27,28] HLA-G inhibits the function of NK cells, T cells and antigen presenting cells (APCs) and induces Tregs and myeloid derived suppressor cells by direct binding to the inhibitory receptors immunoglobulin like transcripts ILT-2 and ILT-4 and the killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4).[30] The expression of HLA-G was preferentially expressed in cervical cancers with high number of TILs and may have caused T cell dysfunction.[29]

Based on these data in HPV-induced cervical cancer, as well as on the fact that HLA downregulation has also been found in vulvar carcinoma, we postulated that similar mechanisms may also play a role in premalignant HPV-induced vulvar neoplasia governing their progression to vulvar carcinoma and non-responsiveness to immunotherapy. Therefore, we analysed the expression of classical and non-classical HLA molecules in the lesions of patients with HPV-induced non-recurrent and recurrent uVIN, and vulvar carcinoma by immunohistochemistry and compared the results to healthy control tissue. Furthermore, the lesions of 12 patients, treated by HPV 16 SLP vaccination with or without clinical success, were analysed to determine the influence of HLA expression alteration on clinical responsiveness. Moreover, we studied whether HLA-class I downregulation could be regarded as 'hard' or 'soft' by LOH analysis and by comparing HLA expression on freshly isolated HPV-infected and non-infected keratinocytes after interferon (IFN)γ stimulation in vitro.

Material and Methods

Patient characteristics and material

All patients treated for uVIN in the LUMC between 1990 and July 2012 of whom archival formalin-fixed, paraffin embedded tissue of the first uVIN lesion was available, were selected and included in this study (N=43, age: mean 47.26 years; range 19-84). Of 20 patients recurrent uVIN lesions were also included. Recurrence free survival was determined as the interval between first therapy and diagnosis of recurrent disease or the last follow up

visit in case of no recurrent disease. Tissues from a cohort of HPV induced micro invasive- (<1mm infiltration) (N=8) and macro invasive vulvar carcinoma (>1mm infiltration) patients (N=13) were included as well to evaluate HLA expression in progressive vulvar neoplasia (age: mean 69.14 years; range 49-95). Samples from elective reductions of the labia minora, which were HPV negative (N=26), served as healthy controls (age: mean 32.96 years; range 16-54). All samples included in this study were typed for HPV by HPV 16 polymerase chain reaction (PCR) with a HPV 16 specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotyping *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV 16 negativity.[31,32] Histologic examination of all controls revealed no dysplasia or other abnormalities. Paraffin embedded uVIN biopsies of patients included in the HPV 16 SLP vaccination trial taken before vaccination were evaluated for tissue availability for HLA-class I immunofluorescent staining (N=12, of which 6 patients with and 6 patients without clinical response measured as lesion reduction).[13] The Leiden University Medical Ethic Committee approved this study on prospective collection of healthy control tissue and for keratinocyte isolation patients were enrolled in the Circle study which investigates cellular immunity against HPV induced neoplasia. Furthermore archival formalin-fixed paraffin embedded patients samples were handled according to the medical ethical guidelines described in the code of conduct for proper secondary use of human tissue of the Dutch federation of Biomedical Scientific Societies.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were stained according to standard protocols as described previously[27] with mouse monoclonal HLA-DR (anti HLA-DR clone TAL.1B5; DAKO (1:400)), mouse monoclonal HLA-DRDQDP (anti HLA-DRDQDPQ clone CD3/43; DAKO (1:2000)) which recognises all HLA class II molecules HLA-DR, -DQ and -DP, rabbit polyclonal MICA (anti MICA LS-B1377; Lifespan BioSciences (1:100)), mouse monoclonal HLA-E (anti HLA-E clone MEM-E/02; Serotec (1:200)) and mouse monoclonal HLA-G (anti HLA-G clone 4H84; Lifespan BioSciences (1:100)). Brown membrane and cytoplasm staining of epithelial cells was indicated positive for HLA expression. In case of no basal membrane staining and only cytoplasm staining this was scored as negative for HLA expression. Stromal and immune cells served as internal positive control and an extra section was stained without primary antibody as a negative control.

Immunofluorescence

Simultaneous detection of HLA-class I was performed by three color fluorescence staining according to standard procedure[27] using mouse monoclonal HCA-2 and HC-10 (anti HLA-A and anti HLA-B/C; kindly provided by Prof. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands (1:400 and 1:2500)) and rabbit polyclonal β2M (anti β2M; clone A-072;

DAKO (1:4000)). HLA-B/C antibody recognises all HLA-B and HLA-C molecules. Staining of lymphocytes was performed by use of rabbit polyclonal CD3 (anti CD3 clone ab828; Abcam (1:100)) and secondary antibodies were all goat anti mouse isotype specific antibodies with Alexa Fluorchromes; Alexa Fluor 488, 546, and 647 (Molecular Probes; 1:200) Five randomly selected representative images were captured using a confocal scanning microscope (LSM510, Zeiss) in a multitrack setting with a 25x/0.80 Plan-NEOFluar objective. Expression of the HLA molecules on the basal membrane was scored and stromal immune cells served as an internal positive control. Two additional sections were stained without primary or secondary antibody as a negative control.

Evaluation of HLA expression and lymphocyte infiltrate

HLA expression patterns were scored according to the scoring system by Ruiter et al.[33] Staining intensity of HLA at the basal membrane was scored as negative, weak, moderate or strong (0 to 3) (Supporting Information Fig.S1) and the percentage of positive cells was scored as: absent (<1%), sporadic (1-5%), local (6-25%), occasional (26-50%), majority (51-75%) or large majority (>75%). Final scores of both the intensity and the percentage were categorized into three groups: 0-1 (negative), 2-6 (weak/moderate) and 7-8 (strong). The slides were scored independently by two researchers (EvE and VB or EJ and MT) without prior knowledge of clinical or histopathological parameters. In case of discrepancies consensus was reached or a third researcher was consulted. Intraepithelial and stromal lymphocyte counts were represented as the number of cells per mm^2 for each slide (average of five 250x image slides) and were manually counted using the LSM 5 Image Examiner software.

LOH

DNA was extracted from micro dissected material (to obtain at least 70% of uVIN DNA) of 14 uVIN, 9 uVIN adjacent to micro invasive carcinoma, 13 HPV positive vulvar carcinoma cases and from the uVIN- and healthy control material from which primary keratinocytes were cultured (see below). DNA was extracted and analysed for LOH on chromosome 6p21, with a minimal input of 10ng DNA/PCR, by PCR amplification using the D6S273 and D6S265 microsatellite markers as previously described.[22] Definitions of thresholds for LOH >1.7, retention of heterozygosity (ROH) 0.76-1.3 and allelic imbalance ("grey area") 0.58-0.75 were used accordingly.[22]

Keratinocyte culture and IFNγ stimulation

Primary keratinocytes were isolated from biopsies or excisions from women undergoing surgery for uVIN, HPV induced vulvar carcinomas or elective reduction of the labia minora and cultured in E-medium in presence of irradiated 3T3J2 mouse fibroblasts and 5-10μM Rho-associated kinase (ROCK) inhibitor (Y-27632). The addition of ROCK inhibitor has proven to be a suitable method to indefinitely extend the life span of primary keratinocytes without

transduction of exogenous viral or cellular genes.[34,35] In case of successful culture the cells were adapted to keratinocyte serum-free medium (K-SFM; Medium 154 supplemented with HKGS kit, Invitrogen, Breda, The Netherlands) for one passage, after which they were stimulated with 0 or 100IU/ml IFNγ for 48 hours. HLA expression was analysed by flow cytometry. Details are given in Supplemental Information Materials and Methods. 3T3J2 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 2mM l-glutamine and 1% penicillin-streptomycin (complete DMEM medium) (Gibco-BRL, Invitrogen).

Statistical analysis

For data analysis the statistical software package SPSS 20.0 (SPSS Inc., Chicago, IL) was used. Group comparisons of categorical data were performed by χ^2 test, and the Fishers exact test in case of small groups. The non-parametric Mann-Whitney U test was used for continuous variables. The paired McNemars categorical test or the paired Wilcoxon Signed Rank test for continuous variables were used to determine differences in primary and secondary lesions. The Spearman correlation coefficient was used to detect correlation in the non-parametric data and in case of normality Pearson correlation coefficient was used. The Shapiro-Wilk test was used to determine a normal distribution. The Bonferroni correction was applied for multiple testing revealing a *P*-value of <0.004 as significant. Both univariate and multivariate analysis, corrected for multifocality of uVIN, were performed by a Cox proportional hazard model for recurrence-free survival analysis (RFS) and progression free survival (PFS). Two sided *P*-values <0.05 were considered significant. GraphPad Prism 5.04 (Graphpad Software Inc., LA Jolla, CA, USA) was used to illustrate the data by graphs and figures.

Results

Patients

The clinical characteristics of the patient cohort are shown in Table 1. In 79% of uVIN patients HPV type 16 was detected, in the other uVIN samples HPV 33 (12%), HPV 73 (2%) or multiple high risk HPV infections (7%) were detected. First lesions were unifocal in 58.1% of cases and therapy consisted of excision in 51.2% (N=22), laser therapy in 30.2% (N=13), imiquimod in 7% (N=3) and a combination of laser and excision in 11.6% (N=5) of patients. Use of immunosuppressive medication was reported in 7 (16%) of patients and 80% of the patients were smokers.[36] The majority of patients with HPV-induced vulvar carcinomas had a low stage of disease according to the International Federation of Obstetrics and Gynaecologists (FIGO) and only 1 patient was diagnosed with a lymph node metastasis in the groins. None of these patients died in follow up and in 3 patients (14%) a recurrent vulvar carcinoma occurred after a median time of 7 months (range 6-46).

Table 1: Patient Characteristics

Characteristic	VIN patients (n=43)	Carcinoma patients (n=21)	HPV 16 SLP vaccination patients (n=12)	Controls (n=26)
Lesion histology				
High grade uVIN	43 (100%)	-	12 (100%)	-
Microinvasive carcinoma	-	8 (38.1%)	-	-
Macroinvasive carcinoma	-	13 (61.9%)	-	-
No dysplasia	-	-	-	26 (100%)
Age at diagnosis (years)				
Mean	47.3	69.1	38.2	33.0
SD	16.5	14.0	8.7	10.9
Range	19-84	49-95	29-60	16-54
Follow up time (months)				
Mean	85.2	96.5	24.1	n.a.
Median	50.0	8.5	23.7	
SD	85.0	76.5	2.1	
Range	0-307	2-242	20.5-28.1	
Lesion type				
Unifocal	25 (58.1%)	unknown	5 (41.7%)	n.a.
Multifocal	18 (41.9%)		7 (58.3%)	
Recurrent uVIN				
Yes	20 (46.5%)	9 (42.9%)	n.a.	n.a.
No	23 (53.5%)	12 (57.1%)		
Smoking status				
Yes	34 (79.1%)	9 (42.9%)	8 (66.7%)	-
No	6 (14%)	5 (23.8%)	-	-
Unknown	2 (4.7%)	7 (100%)	4 (33.3%)	26 (100%)
HPV type				
16	34 (79.1%)	14 (66.7%)	12 (100%)	-
33	5 (11.6%)	5 (23.8%)	-	-
16 + 33	1 (2.3%)	1 (4.8%)	-	-
Multiple hrHPV (e.g. 33,31,51,44)	2 (4.7%)	-	-	-
73	1 (2.3%)	-	-	-
18	-	1 (4.8%)	-	26 (100%)
Negative	-	-	-	-
Progression to carcinoma				
Yes	8 (18.6%)	n.a.	3 (25%)	n.a.
No	35 (81.4%)		8 (66.7%)	
Unknown	-		1 (8.3%)	
Immunosuppressive medication				
No	36 (83.7%)	20 (95.2%)	12 (100%)	26 (100%)
Yes (e.g. HIV, allograft recipient, autoimmune disease)	7 (16.3%)	1 (4.8%)	-	-

HLA expression in progressive course of disease

Expression of classical and non-classical HLA molecules at different stages of disease are depicted in Fig. 1 and Supporting Information Fig.S2. Partial downregulation of HLA-class I was found in 72% of uVIN lesions, in 88% of uVIN adjacent to micro invasive carcinoma, in 84% of vulvar carcinomas and in 8% of the controls. Total loss of HLA-A was detected in 9.3% of uVIN lesions and in 13% of uVIN lesions adjacent to microinvasive carcinoma. Total loss of HLA-B/C was found in 9% of uVIN lesions and in 38% uVIN lesions adjacent to micro invasive carcinoma whereas total loss of β2M was seen in 7% of uVIN, 13% of uVIN adjacent to micro invasive carcinoma and in 8.3% of vulvar carcinomas. Downregulation of β2M was more prominent in 83% - 100% of vulvar carcinoma compared with 58% of uVIN lesions (p=0.030). HLA-class II expression, determined by either HLA-DR or HLA-DRDQDP positivity, was focal in areas of the basal layer in 65% of uVIN, in 75% of micro invasive carcinomas, in 46% of the vulvar carcinomas and was absent in the controls. In 7% of uVIN lesions HLA-class II expression was limited to the expression of HLA-DQDP. The expression of the non-classical molecules HLA-E (14%) and HLA-G (16%) in uVIN lesions was low but increased with the progressive course of vulvar neoplasia. Both HLA-E and -G were expressed in 25% of micro invasive carcinoma and in 54% (p=0.041) and 46% (p=0.133), respectively of the macro invasive vulvar carcinomas. MICA was downregulated in approximately 80% of uVIN lesions, in a comparable number of the micro- and macro invasive vulvar carcinomas (100% and 92%, respectively) and in 7.7% of control tissues.

HLA expression in recurrent uVIN and recurrence free survival

We analysed 20 pairs of a primary uVIN lesions and the corresponding lesion that recurred after primary treatment. Analysis of the alterations in classical and non-classical HLA molecules revealed no overt differences between primary and subsequent recurrent lesions (Fig. 2). There was a clear correlation between the downregulation of HLA-B/C and the recurrences of uVIN lesions (p=0.029) as well as progression to carcinoma (p=0.016). Furthermore, our data suggest that the combination of HLA-class I and MICA downregulation was also associated with lesion recurrence (p=0.023) albeit that there was no difference in the risk of progression to cancer (Supporting Information Table S1). Expression of the other classical and non-classical HLA molecules was not predictive for recurrent uVIN lesions or progressive course of disease. Considering the time until recurrence or progression, the alterations in HLA expression had no influence on RFS neither on PFS (data not shown).

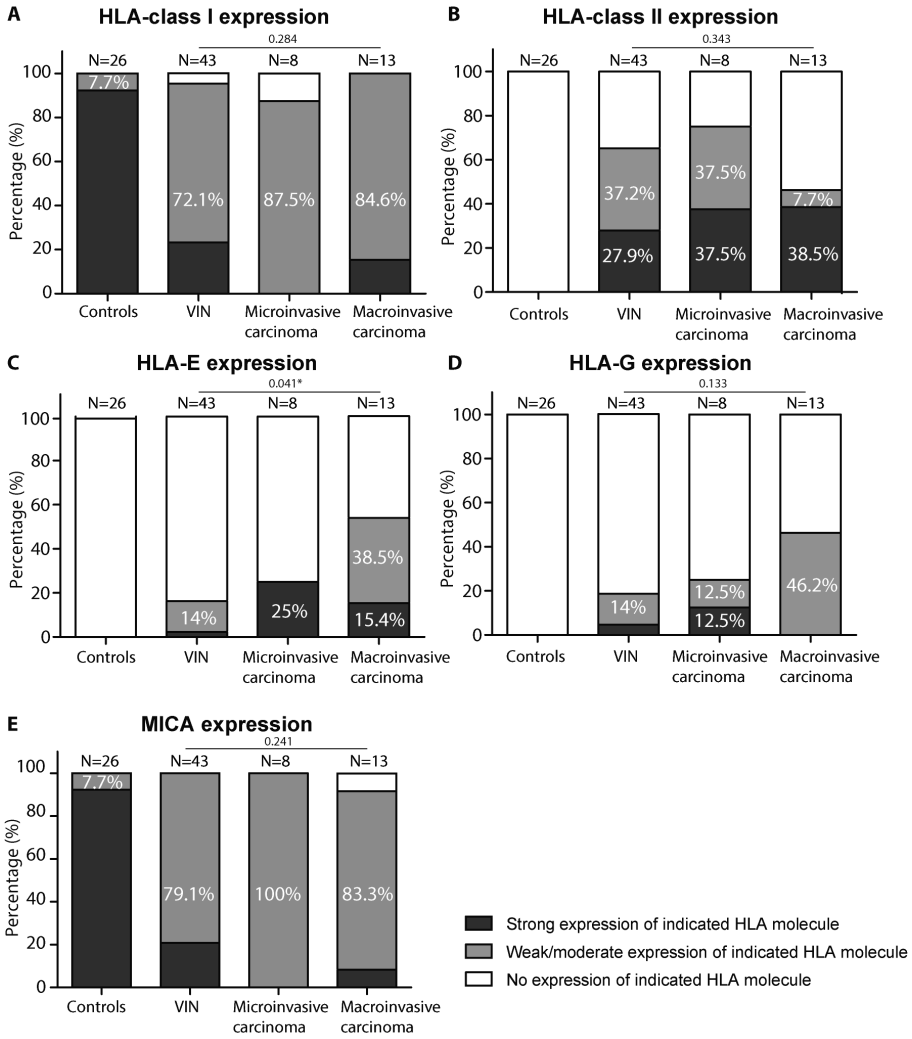

Figure 1: Classical HLA-class I, HLA-class II, HLA-E, -G and MICA expression in the progressive course of HPV induced vulvar neoplasia

Paraffin embedded tissues of healthy controls (n=26), high grade uVIN lesions (n=43), microinvasive- (n=8) and macroinvasive vulvar carcinomas (n=13) were stained by immunohistochemistry for the indicated HLA molecules. A: membranous expression of HLA class I was evaluated by use of antibodies to β2M, HLA-A (HCA-2 antibody) and HLA-B/C (HC-10 antibody). Weak HLA-class I expression was indicated in case of weak to moderate HLA-A and/or HLA-B/C membrane expression compared to stromal expression. No expression was indicated in case of total loss of β2M, HLA-A and HLA-B/C on the membrane of basal epithelial layer. B: expression of membranous HLA class II was evaluated by use of weak, moderate or strong membrane expression compared to stromal expression of HLA-DR or HLA-DRDQDP and healthy controls. Membranous expression of HLA-E (C), HLA-G (D), and MICA (E) was considered positive in case of moderate or strong expression. The expression of these molecules was considered downregulated in case of none or weak expression.
HLA expression is determined according to the scoring system of Ruiter et al.[33] (see example in supplemental figure 2)

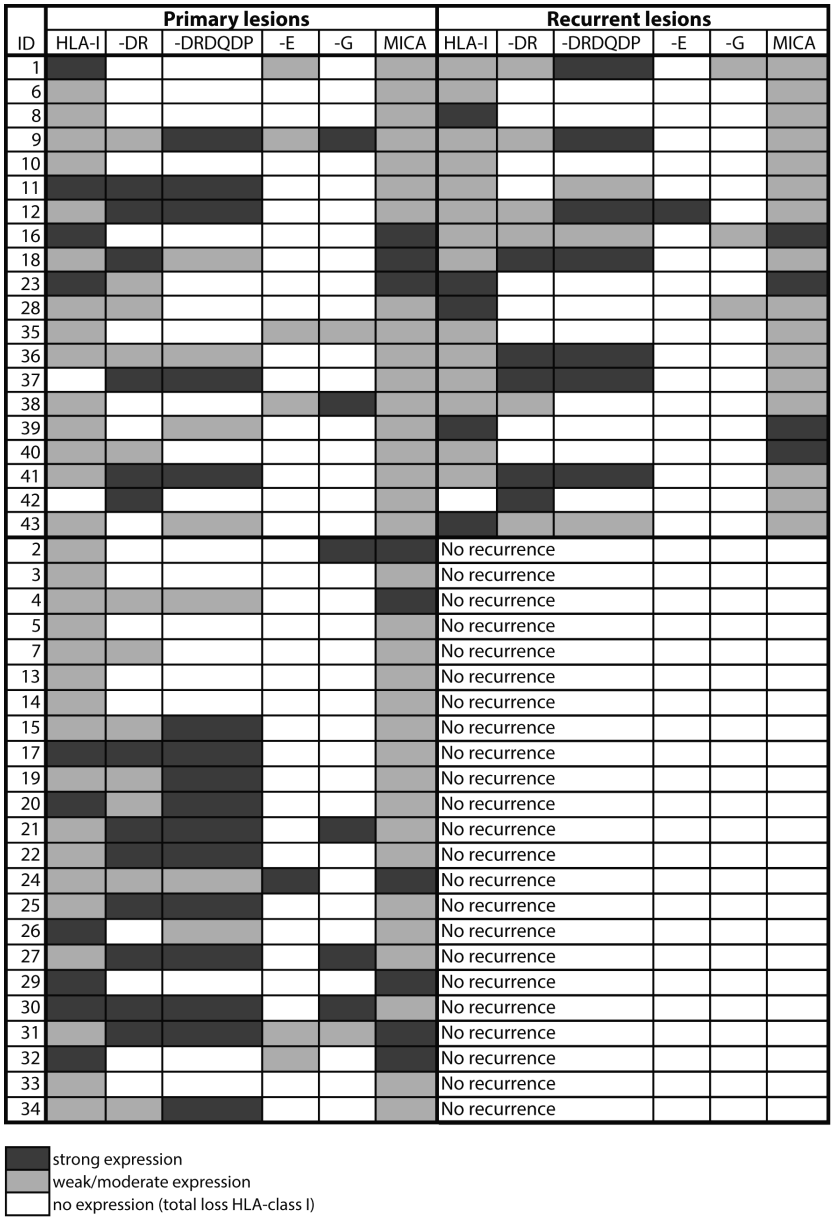

Figure 2: Classical and non-classical HLA expression in primary and recurrent uVIN lesions for each individual patient.

Primary lesions of high grade uVIN lesions (n=43) and concomitant secondary recurrent uVIN lesions (n=20) were analysed by immunohistochemistry for HLA-class I expression as described in figure 1. Depicted are the expression levels of all indicated molecules for each patient in the primary and the concomitant recurrent uVIN lesion showing that there are no distinct differences in alterations of classical and non-classical HLA molecules between the primary and recurrent lesions.

Hard and soft wired downregulation of HLA-class I in uVIN and vulvar carcinoma

In order to determine whether the observed downregulation of HLA by immunohistochemistry was 'hard'- or 'soft'-wired we selected 14 uVIN lesions based on tissue availability, 9 uVIN lesions adjacent to HPV positive micro invasive carcinomas and 12 HPV-positive macro invasive carcinomas for LOH evaluation (Supplemental Table 2). For 13 of the 14 uVIN lesions and for all carcinomas selected for LOH analysis, reliable results were obtained. Immunohistochemistry revealed downregulation of HLA-class I in 85% of these uVIN patients, in 100% of the micro invasive carcinomas and in 92% of the macro invasive carcinomas. The frequency of LOH was low (2 of 13; 15%) in the uVIN patients. In uVIN adjacent to micro invasive carcinoma LOH was found in 5 out of 9 cases (55.5%) and in 3 out of 12 (25%) of the macro invasive carcinomas LOH could be detected. In most cases the results of immunohistochemistry and LOH were concurrent. However, in 1 uVIN case LOH was found while immunohistochemistry revealed a strong expression of HLA-A, -B/C and β2M. The high percentage of partial HLA downregulation in uVIN lesions as detected by immunohistochemistry without concurrent detection of LOH suggests that the observed HLA-class I downregulation in uVIN is 'soft' wired and therefore, may be restored upon exposure to proinflammatory cytokines. To test this, we isolated keratinocytes from 3 healthy controls, 4 uVIN lesions and 2 vulvar carcinomas and analysed the expression of HLA-class I directly or after stimulation with IFNγ. The cultured keratinocytes of vulvar neoplasia patients displayed a lower expression of HLA-class I compared to controls (p=0.018; mean fluorescence 31.6 ± 19.1 vs. 66.1 ± 14.4) but stimulation with IFNγ resulted in an upregulation of HLA-class I expression in all samples (p=0.842; mean fluorescence index 143.7 ± 85.8 vs. 131.8 ± 25.8) (Fig. 3a). The expression level detected by in vitro analysis corresponded to the expression of HLA-class I obtained by immunohistochemistry on uVIN lesions of these patients (Fig. 4). In 2 cases, the cultured keratinocytes showed LOH and IFNγstimulation revealed upregulation of HLA-class I expression in both cases. This is probably explained by the upregulation of HLA-class I expression based on the one allele that was still present (Fig. 3a). As a positive control the IFNγ-mediated upregulation of HLA-DRDQDP expression was analysed. The expression of HLA-class II was upregulated in all cases and the expression levels did not differ (p=0.382; mean fluorescence 7.0 ± 4.4vs. 10.4 ± 4.7) (Fig. 3b).

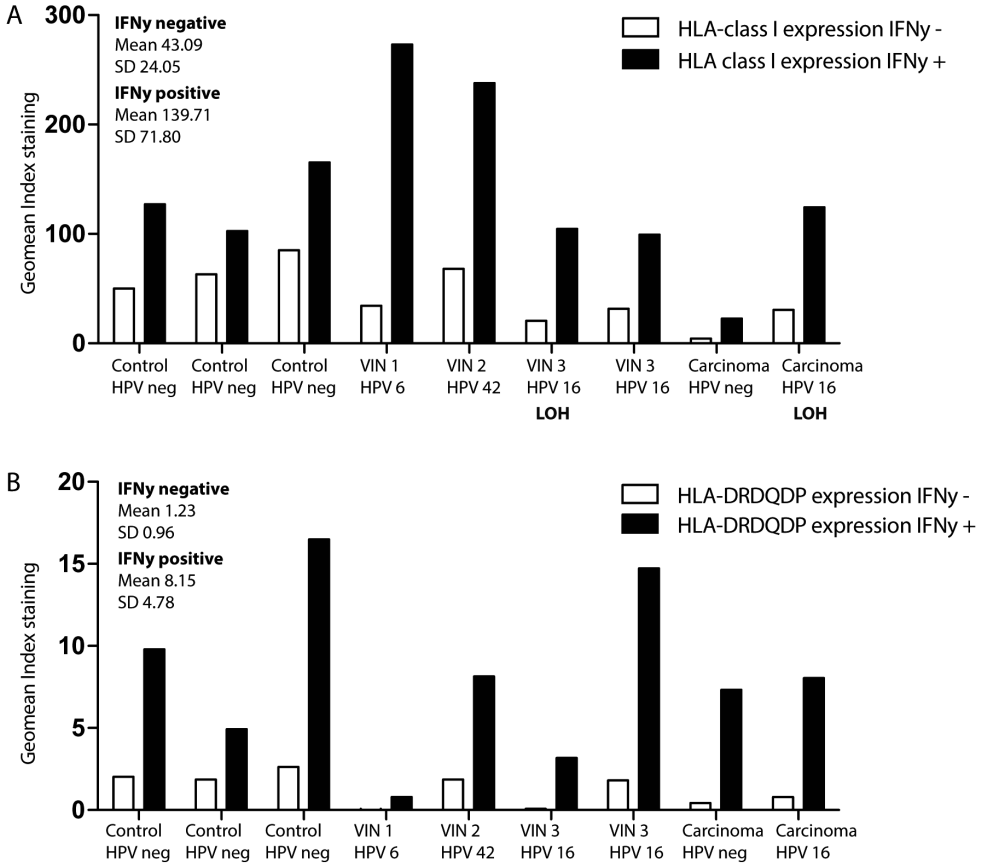

Figure 3: HLA-class I (A) and HLA-DRDQDP (B) expression on cultured patient-derived keratinocytes before and after 48h of IFNy stimulation.

Primary keratinocytes were isolated from biopsies or excisions of the vulva from women undergoing surgery for labia reduction, uVIN or HPV induced vulvar carcinomas. Keratinocytes were cultured in E-medium in presence of irradiated 3T3J2 mouse fibroblasts and 5-10µM Rho-associated kinase (ROCK) inhibitor (Y-27632). When cells grew out they were adapted to keratinocyte serum-free medium for one passage, after which they were stimulated with 0 or 100IU/ml IFNy for 48 hours. HLA expression was analysed by flow cytometry and the Geomean Index was used to depict the intensity of HLA-class I or - II staining. The cultured keratinocytes of vulvar neoplasia patients display lower expression of HLA-class I compared to controls whereas stimulation with IFNy results in an upregulation of HLA-class I expression in all samples. As a positive control the levels and IFNy mediated upregulation of HLA-DRDQDP expression was analysed.

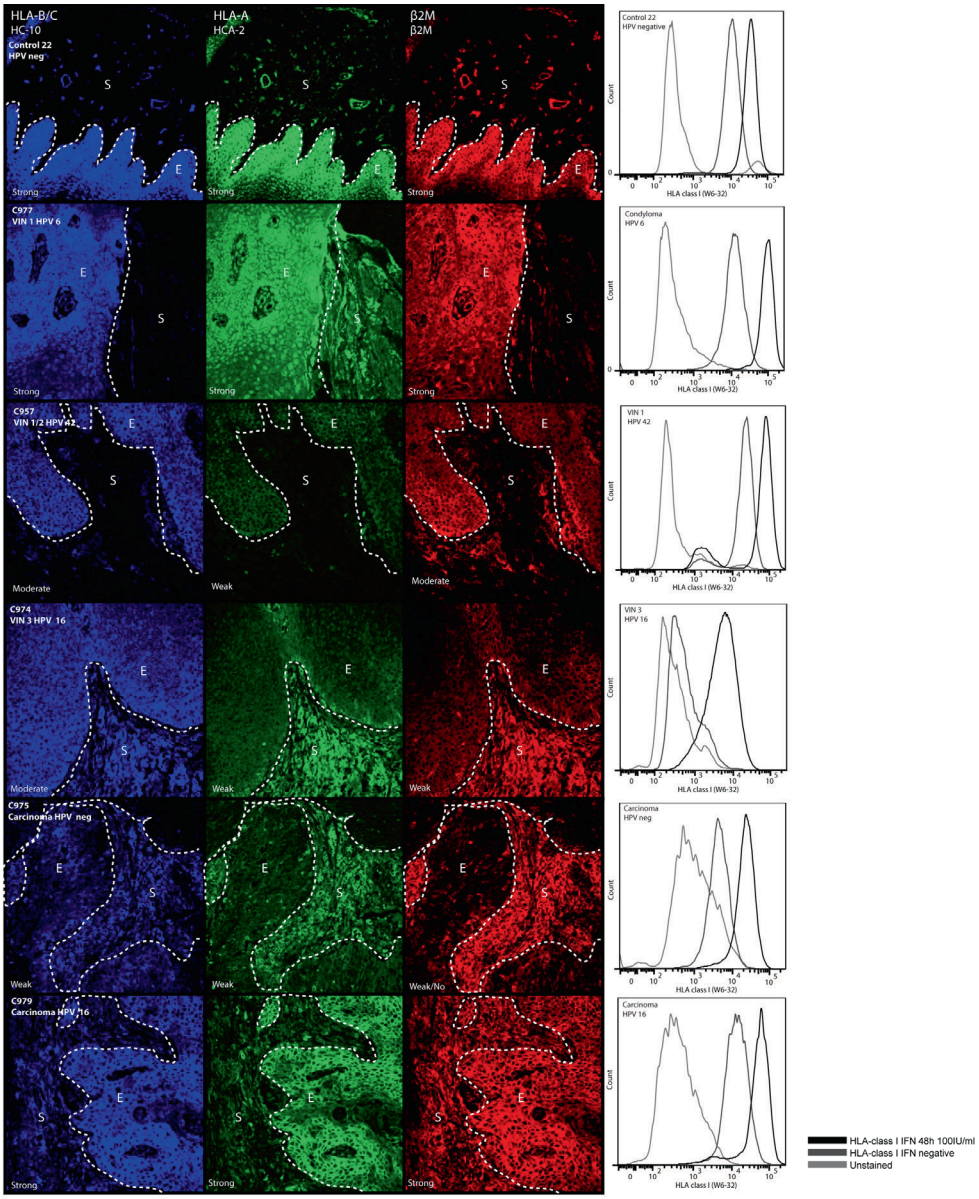

Figure 4: Immunofluorescent staining of HLA-class I in paraffin-embedded tissue and the effect of IFNy stimulation on HLA expression on corresponding isolated keratinocytes

From a number of patients with uVIN lesion we obtained fresh tissue for keratinocyte culture and paraffin embedded tissue of the same lesion used for histology. (left) Immunohistochemistry of HLA-B/C (blue), HLA-A (green) and β2M (red) staining of the paraffin embedded biopsy is shown. (right) Flow cytometry histograms representing HLA-class I expression (using the W6-32 antibody) of the keratinocytes from corresponding lesions with or without stimulation with 1000 IU/ml IFNy for 48 hours. Unstained is the background fluorescence of keratinocytes. The expression level (low, high) detected by in vitro analysis of keratinocyte cultures corresponded to the expression of HLA-class I determined by immunohistochemistry. E: Epithelium, S: Stroma

Lymphocyte infiltrates

Higher numbers of T cells, identified by the expression of CD3, were found in the stroma (p=<0.01) but not in the epithelium of uVIN and vulvar carcinoma lesions when compared to control tissues (Fig. 5). No correlations were found between CD3+ T cell infiltrates in stroma or epithelium and recurrent or progressive uVIN lesions (Supporting Information Table S1) neither was there an association with recurrence- or progression free survival (data not shown). Moreover the number of CD3+ cells did not correlate to the upregulation of HLA-class II, HLA-E or HLA-G or to the downregulation of HLA-class I, HLA-A, HLA-B/C or MICA.

HLA expression in uVIN biopsies of patients treated by HPV 16 SLP vaccination.

Evaluation of HLA expression in 12 uVIN biopsies taken before inclusion in a HPV 16 SLP vaccination trial[13] revealed that both clinical responders (n=6; ID 206, 207, 210, 216, 227, 229) and non-responders (n=6; ID 201, 202, 203, 212, 222, 228) showed partial downregulation of HLA-class I. Overall 75% of uVIN biopsies revealed partial downregulation. In 3 of the 6 non-responders a strong expression of HLA-class I could be observed whereas all 6 tested clinical responders revealed partial downregulation of HLA-class I. Of the clinical responders four patients displayed partial downregulation of HLA-A and –B/C whereas 2 patients displayed partial downregulation of HLA-A alone.

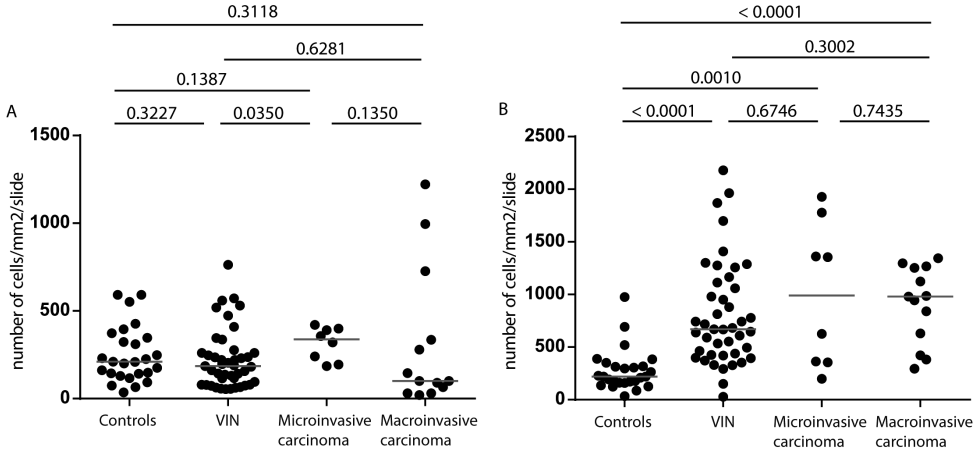

Figure 5: CD3 infiltrates in progressive course of HPV induced vulvar neoplasia

Paraffin embedded tissues of healthy controls (n=26), high grade uVIN lesions (n=43), microinvasive vulvar carcinomas (n=8) and macroinvasive vulvar carcinomas (n=13) were analysed for lymphocyte infiltration as identified by the expression of CD3 in both the epithelium (A) and stroma (B). An increased number of CD3+ T cells were found in the stroma (p=<0.01) but not in the epithelium of uVIN and vulvar carcinoma lesions when compared to healthy control tissues.

A: epithelial CD3+ infiltrates B: stromal CD3+ infiltrates

Discussion

This study shows that alterations in classical and non-classical HLA molecules is not limited to HPV-induced cancers but can already be observed at the premalignant stage of HPV-induced vulvar neoplasia. HLA-class I is partially downregulated in over 70% of uVIN lesions and in 80% of HPV-induced vulvar carcinomas. Loss of either one allele of HLA-A or HLA-B/C was found in only 9% of uVIN lesions and total HLA loss of both alleles in only 2 (5%) of uVIN lesions. These results do not completely correspond to the previously reported total loss in 19%, and partial downregulation of HLA-class I (allelic loss 9%) in uVIN lesions, probably due to the inclusion of HPV negative uVIN lesions in the previous study.[12] Importantly, our study revealed that both HLA-class I and MICA downregulation is an early event in the immune escape mechanism of HPV-induced premalignant uVIN lesions. HLA-B/C downregulation and possibly also the combined downregulation of HLA-class I and MICA are associated with the recurrence of uVIN after treatment. These data once again show the impact of the immune system in HPV-induced diseases as downregulation of HLA-class I and MICA are a well-documented events in both HPV-induced cervical intraepithelial neoplasia (CIN) and cervical cancer and both weak HLA-A and MICA expression are an independent prognostic factor for a decreased 5-year survival rate.[19,22,26,37] Alterations in the other HLA molecules (-class II, -E and -G) have no influence on recurrent or progressive course of uVIN lesions, and neither is the expression of both classical and non-classical HLA molecules altered from primary to recurrent uVIN lesions.

The high frequency of HLA-class I downregulation in uVIN may compromise the efficacy of immunotherapy because HPV peptides are insufficiently presented to T cells. However, in contrast to the vulvar carcinomas tested in this study, the majority of uVIN lesions display downregulation of HLA-class I expression that is not associated with LOH suggesting that HLA-class I downregulation is soft-wired and that expression can be restored upon changes in the microenvironment. Indeed, we were able to show that the lower expression of HLA-class I by HPV-infected keratinocytes freshly isolated from uVIN lesions could be increased by stimulation with IFNγ. Note that we only assessed general HLA-class I expression (by the W6-32 antibody) which may obscure subtle allele specific alterations. The reversible nature of the HLA-class I downregulation, however, may form an explanation for the lack of clinical impact of partial downregulation of HLA-class I in patients responding to HPV 16 SLP vaccination since for instance vaccine-induced HPV 16-specific type helper T cells infiltrating the lesion can provide IFNγ in the microenvironment.[13,18] The lack of overt differences in alterations of the expression of HLA between primary uVIN and the corresponding recurrent uVIN lesions suggests that the same possibility exists to restore HLA expression in recurrent uVIN lesions, however, maybe the recurrent lesions more often express LOH as we detected LOH in 55% of the uVIN adjacent to micro invasive carcinoma. The latter is in the same range as LOH in

CIN adjacent to carcinoma (75%) and in cervical cancer (50%).[22,23] We did not assess if there was a structural loss of β2M expression as total loss of HLA-class I expression was found in only 2 uVIN lesions. It will be important to test this as hard-wired HLA downregulation may directly affect the ability of lesions to regress. For instance, in melanoma it was the type of HLA-class I downregulation that determined the difference between regressing (soft-wired) and progressing (hard-wired) metastatic melanoma lesions.[38] Moreover in cervical cancer patients complete loss of HLA-class I was associated with a failure to induce systemic HPV-specific T cell responses as measured by CD4+ T cell responses.[25] This may indirectly cause a failure of HPV-specific T cell response induction since cancer cells with normal HLA-class I expression will be more efficiently destroyed by CTLs, resulting in an enhanced antigen uptake and presentation by HLA-class II on APCs that can stimulate the CD4+ T cells required for the maintenance and functions of CTLs.

The upregulation of HLA-class II is an early event in the carcinogenesis of vulvar neoplasia. HLA-class II is not expressed on non-dysplastic epithelium whereas in 65% of uVIN lesions and in 46-75% of vulvar carcinomas the expression is upregulated, corresponding to previous data on cervical cancer and 28% upregulation in uVIN (in a single study of only 7 cases).[25,26,39] The expression in uVIN is mainly focal (43.5% of cases <10% upregulation) and corresponds to high stromal infiltrates adjacent to the epithelium suggesting that upregulation is potentially related to cytokines in the local inflammatory environment. The effect of HLA-class II upregulation is unclear and may on one hand elicit activation of CD4+ T cells and enhance recruitment of CTLs but on the other hand it may as well hamper the immune response by induction or activation of Tregs.[24,40]

The expression of the non-classical molecules HLA-E and-G increases with the progressive course of HPV-induced vulvar neoplasia. These data are corresponding to cervical neoplasia where HLA-E expression gradually increases from CIN towards cancer and in cervical cancer was correlated to a large tumor size and lymph node metastasis.[27,28,41] HLA-E, expressed in 56-83% of cervical cancers and in 69% of cervical adenocarcinomas, is able to induce tumor tolerance by binding to its ligand CD94/NKG2A expressed on both NK and CTLs.[27,28,42] CD94/NKG2A expression can be induced by the cytokines interleukin (IL)-15 and tumor growth factor (TGF)-β present in the microenvironment of tumors.[43,44] Apart from its immune inhibitory function HLA-E can also bind to the immune stimulatory molecule NKG2C, which is expressed on the majority of NK cells and on some CTLs.[27] However the number of infiltrating NK cells in the microenvironment of both uVIN lesions and cervical cancer is low.[27]

HLA-G may induce immune evasion by inhibition of T cells, NK cells and APCs as well as by stimulating the production of Th2 cytokines.[45,46] Upregulation of HLA-G in tumor cells may be induced by IFNγ in the anti-tumor elimination phase, by epigenetic alterations of tumor cells, by hypoxia or immunosuppressive factors in the tumor environment such as IL-

10.[47] HLA-G expression was previously shown to be associated with progression of cervical lesions[29,41,48,49] and other types of cancer.[47] In addition, lack of HLA-G expression was an independent prognostic indicator of prolonged survival in patients with colorectal cancer.[50] These studies indicate that the increased expression of HLA-E and HLA-G in progressing lesions can contribute to escape from the immune system as well as form a barrier to successful immunotherapeutic approaches, although their rare expression in uVIN suggests that they are not able to mediate these effects during immunotherapy of uVIN.
In summary, alterations in classical HLA-class I and -II and non-classical MICA expression are already present in premalignant uVIN lesions and therefore of potential influence on immunotherapy in these lesions whereas expression of the non-classical HLA-E and -G are limited to the progressive course of disease. Remarkably, all evaluated uVIN patients who showed good clinical responses upon HPV SLP vaccination had partial HLA-class I downregulation indicating the potential reversibility of downregulation in premalignant uVIN upon proinflammatory cytokine response. These premalignant uVIN lesions may indeed be regarded as 'soft' lesions, considering the low percentage of LOH in uVIN and the reversible HLA-class I downregulation upon IFNγ stimulation in HPV infected keratinocytes.

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Supporting Information

Supplemental data available on: http://onlinelibrary.wiley.com.ezproxy.leidenuniv.nl:2048/doi/10.1002/ijc.28713/suppinfo

Supplemental Table 1: HLA expression and CD3 infiltrates associated with recurrence or progression of uVIN

Supplemental Table 2: LOH percentage in the progressive course of HPV induced vulvar neoplasia

Supplemental Figure 1: HLA expression intensity in immunohistochemistry staining according to Ruiter et al.[33]

Supplemental Figure 2: Expression of β2M, HLA-A, HLA-B/C and HLA-DR or total HLA class II (DRDQDP) in the progressive course of HPV induced vulvar neoplasia