In cervical cancer, an important mechanism by which tumor cells escape immune surveillance is loss of HLA class I, enabling tumors to evade recognition and lysis by cytotoxic T lymphocytes. Some tumors, however, escape from immune surveillance without accumulating defects in antigen presentation. We hypothesized that tumors with no or partial loss of HLA class I develop alternative mechanisms to prevent immune surveillance. To investigate this hypothesis, genome-wide expression profiling using Illumina arrays was performed on cervical squamous cell carcinomas showing overall loss of HLA class I, partial and normal HLA class I protein expression. Statistical analyses revealed no significant differences in gene expression between tumors with partial (n = 11) and normal HLA class I expression (n = 10). Comparison of tumors with normal/partial HLA class I expression (n = 21) with those with overall loss of HLA class I expression (n = 11) identified 150 differentially expressed genes. Most of these genes were involved in the defense response (n = 27), and, in particular, inflammatory and acute phase responses. Especially SerpinA1 and SerpinA3 were found to be upregulated in HLA positive tumors (3.6 and 8.2 fold, respectively), and this was confirmed by real-time PCR and immunohistochemistry. In a group of 117 tumors, high SerpinA1 and SerpinA3 expression in association with normal/partial HLA expression correlated significantly with poor overall survival (p = 0.035 and p = 0.05, respectively). This study shows that HLA positive tumors are characterized by a higher expression of genes associated with an inflammatory profile and that expression of the acute phase proteins SerpinA1 and SerpinA3 in HLA positive tumors is associated with poor survival.
Chapter 2

Introduction

The development of cervical cancer is a step-wise process, preceded by dysplastic lesions known as cervical intraepithelial neoplasia (CIN) stage I, II and III. Infection with high-risk human papilloma virus (HPV) plays an essential role in the development of cervical carcinoma [1]. In addition to persistent HPV infection, other factors are necessary and contribute towards the progression of CIN lesions to invasive cancer. The host immune response in particular plays a critical role in determining the progression or regression of HPV-associated CIN lesions [2,3].

Upon infection with HPV, the host initiates a virus-specific cell-mediated immune response, which in most instances leads to regression of the CIN lesions [4]. Cytokines play an important role in regulating the immune response towards either anti-tumor immunity or immune suppression [5]. In addition to the involvement of certain immunostimulatory cytokines, the development of an adequate cell-mediated immune response requires the proper presentation of HPV-associated proteins by human leukocyte antigens (HLA) class I proteins on the cell surface of infected keratinocytes.

Many tumor types have developed mechanisms to escape immune surveillance that involve abnormal HLA class I antigen expression; this may range from loss of a single HLA class I allele to complete loss of HLA class I antigen expression [6]. Loss of HLA class I expression occurs in the majority of cervical cancers via several underlying mechanisms, of which loss of heterozygosity in the HLA region is the most frequent event (50%) [7].

Cervical cancer cells that do express HPV peptides in HLA class I molecules may maintain survival by creating an anergic environment in which the recruited cells from the immune system are incapable of eradicating the tumor cells. Expression of immunosuppressive cytokines, such as TGF-β and IL-10 by tumor cells or suppressor T cells, has been shown to inhibit the generation and function of cytotoxic T cells [8].

Thus far, immune escape mechanisms in cervical carcinoma have been studied primarily individually. Therefore it is unknown whether different immune escape mechanisms occur simultaneously or arise independently. We hypothesize that tumors that retain HLA class I expression employ alternative defense mechanisms to prevent eradication by the immune system.

Materials and methods

Tissue samples

Formalin-fixed, paraffin-embedded squamous cervical carcinomas from 68 patients who underwent radical hysterectomy with bilateral lymphadenectomy between 1991 and 2005 at Leiden University Medical Center, were collected. From these 68 patients, 32 snap-frozen tissues were chosen for subsequent gene expression profiling. The percentage of tumor cells was estimated by visual examination of HE-stained frozen tissue sections (5 μm). Only tissue blocks with areas that contained at least 60% viable tumor cells were selected. The inflammatory infiltrate was microscopically
assessed on HE slides and scored as mild, moderate or extensive. A separate group of 117 formalin-fixed, paraffin-embedded cervical carcinomas embedded in a tissuearray in triplicate, obtained between 1985 and 1999, was used to associate immunohistochemical data with clinicopathological parameters. There was no overlap between the tumors on the tissue array and the tumors used for gene expression profiling. HPV DNA detection was performed by the INNO-LIPA prototype research genotyping assay (Innogenetics, Gent, Belgium) as described previously [9]. Correlation of expression data with clinicopathological parameters or survival was performed with χ² and Kaplan Meier tests. All tissues were retrieved from the archives of the Department of Pathology, Leiden University Medical Center. None of the patients had received radio- or chemotherapy prior to surgery. The local Institutional Review Board approved the use of human tissue material for research.

Immunohistochemistry
Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections as described [10]. For HLA class I staining, antibodies against HLA-A (HCA2); HLA-B and HLA-C heavy chains (HC10, both antibodies were a kind gift from Prof. J. Neefjes, Dutch Cancer Institute); and β2m (A072; DAKO, Copenhagen, Denmark) were used. Normal epithelium, stroma and infiltrating leukocytes served as an internal positive control. Polyclonal antibodies against SerpinA1 (1:12800, A0012, DakoCytomation, Glostrup, Denmark), with proteinase K as antigen retrieval, and SerpinA3 (1:20000, A0022, DakoCytomation) were used for validation of expression microarray data. Immunohistochemical staining was scored based on intensity and percentage of positive tumor cells [11]. The intensity of each marker was scored as 0, 1, 2, or 3, indicating absent, weak, clear or strong expression, respectively. The percentage of positive cells was scored on a scale of 0 (0%), 1 (1-5%), 2 (5-25%), 3 (25-50%), 4 (50-75%), and 5 (75-100%). Samples were categorized in one of three categories of expression based on the sum of the scores: strong expression (total score 6-8), partial loss (3-5) and total loss (0-2). Fisher’s exact tests were computed to determine differences between groups.

Microsatellite analysis
Genomic DNA was isolated from flow-sorted tumor cell subpopulations derived from cervical carcinomas as previously described [12]. Eight fluorescein-labeled primer pairs for microsatellite markers, including MOGC, D6S265, C125, TY2A, TNF, D6S273, D6S294 and D6S1666 (http://www.GDB.org/) were used. PCR, electrophoresis and analysis was performed as described previously [13]. In comparing normal and tumor DNA, a reduction of more than 50% of one allele was assigned as LOH. Tumors were considered to have LOH if all or 2 of the 3 markers spanning the HLA class I region (MOGC, D6S265 and C125) harboured LOH.

RNA extraction, labelling and microarray hybridization
Total RNA was isolated from frozen tissue slides with Trizol (Life Technologies, Carlsbad, CA) and purified as described [14]. The integrity and quantity of the total RNA and the labeled cRNA was assessed by spectrophotometric analysis on the
Agilent 2100 Bioanalyzer conductor with the RNA 6000 Nano Assay Kit (Agilent Technologies, Palo Alto, CA). cDNA was synthesized and transcribed into cRNA using the Illumina Totalprep RNA amplification kit following the manufacturer’s instructions (Ambion, Austin, Texas). Labeled cRNA was hybridized to Illumina Sentrix-human 6 expression bead-chips. These chips have genome-wide coverage with around 47,000 50-mer probes, at approximately 30-fold.

**Microarray analysis**
Gene expression levels were quantified using beadstudio gene expression module 2.1, and data were subsequently normalized using the VSN method [15]. Access to complete microarray datasets are available at GEO, accession number GSE10372. Only the well-annotated genes were selected for further analysis (n = 26091). Differential gene expression analysis was performed first of all by a nested F-analysis using limma which is comparable to ANOVA [16]. After combining group B and C (see Results) T-tests in limma were performed. An FDR threshold of 0.05 was used, according to Benjamini and Hochberg [17], to select differentially expressed genes. The Global test was used [18] to determine differences between the tumor groups in certain Gene Ontology Biological processes. The influence of a different degree of inflammatory infiltrate between tumors on gene expression was tested as confounding factor in the Global test.

**Quantitative real-time PCR (qRT-PCR)**
qRT-PCR was performed as described previously [14]. Gene expression was normalized using the Genorm program [19]. Primers for the internal control genes EEF1A1, RPL11 and RPL13 were previously reported [14]. Commercially available primers were used for SerpinA1, SerpinA3 and CCL4 amplification (Superarray, Frederick, USA). Independent T-tests were performed to determine significant differences in expression between the groups. Spearman’s nonparametric correlation coefficients were calculated for the relationship between microarray and real-time expression levels.

**Immunofluorescence**
De-paraffinized tissue sections were incubated with a mix of HC10 (mouse IgG2a) and SerpinA3 (rabbit polyclonal) antibodies. Proteins were visualized using a combination of fluorescent antibody conjugates (goat-anti-rabbit-IgG-Alexa-546 and goat-anti-mouse-IgG2a-Alexa-488). The images were captured with a confocal laser scanning microscope (Zeiss LSM510; Zeiss, Jena, Germany).

**Results**

**Patients**
Of the 68 patients with squamous cell carcinoma, 55 (82%) were diagnosed as FIGO stage Ib, 10 as FIGO Ila (14%) and 2 as FIGO I Ib (3%). Thirty patients (44%) received post-operative radiotherapy due to either tumor positive lymph nodes or a combination of two of the following parameters: depth of infiltration ≥ 15 mm, tumor
size ≥ 40 mm, or the presence of vaso-invasion. Twenty-four patients had lymph node metastases. Twenty-one patients (31%) were HPV16-positive, 18 (27%) were HPV18 positive, 10 (15%) were positive for other HPV types and from 19 patients HPV type was unknown. Prognostic relevance of the findings after microarray analysis was studied with a different group of cervical carcinomas on the tissue array; clinical features from this group are shown in Table 1.

**Table 1.** Clinicopathological features of patients and tumors and association with SerpinA1 and SerpinA3 staining.

<table>
<thead>
<tr>
<th>Patient and tumor characteristics</th>
<th>SerpinA1</th>
<th></th>
<th>SerpinA3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N*</td>
<td>n</td>
<td>p-value</td>
<td>n</td>
</tr>
<tr>
<td>Age</td>
<td>118</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 (mean)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-97 (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA/IB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 40 mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 40 mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Depth of infiltration</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt; 15 mm</td>
<td></td>
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</tr>
<tr>
<td>≥ 15 mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaso-invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV status ‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosquamous**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adeno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N*, number of patients/cervical carcinomas. The total number of reported cases is affected by incidental missing cases. ‡, Association of HPV status with Serpin expression was determined for HPV16 and HPV18. **, Association with histology was performed for adenosquamous/adenocarcinomas versus squamous cell carcinomas. Concerning SerpinA1 and SerpinA3 expression, tumors with less than 2 replicates were excluded from analysis.

**HLA class I protein expression and LOH at 6p21.3 in cervical carcinomas**

Sixty-eight squamous cell carcinomas were immunostained for HLA class I expression to classify tumors into those with normal, partial and overall loss of HLA class I
expression. Eight of the 68 cases (12%) showed normal expression of HLA class I, 43 cases (63%) showed partial downregulation of HLA class I, and 17 cases (25%) displayed total loss or less than 5% of HLA class I expression (β2m, HLA-A and HLA-B/C negative; Table 2). Since LOH is a major mechanism for loss of HLA class I expression and because positive immunohistochemical staining does not reveal allelic loss, we determined LOH at 6p21.3, the locus of the HLA-A, HLA-B and HLA-C genes. Fifty-six percent (33/59) of the tumors showed loss of 6p21.3 at the HLA class I region (Supplementary Figure 1 and Table 2). Thirty-two of the 68 tumors were selected for gene expression profiling based on HLA class I expression and LOH of the respective locus.

**Table 2.** LOH of the HLA class I genomic region at 6p21.3 in squamous cervical carcinoma (n = 59) and expression of HLA class I heavy chains (n = 68).

<table>
<thead>
<tr>
<th></th>
<th>β2m</th>
<th>HLA-A</th>
<th>HLA-B/C</th>
<th>Total class I</th>
<th>LOH 6p21.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No expression</td>
<td>13</td>
<td>32</td>
<td>19</td>
<td>17*</td>
<td>8/16</td>
</tr>
<tr>
<td>Moderate expression</td>
<td>31</td>
<td>25</td>
<td>29</td>
<td>43**</td>
<td>22/35</td>
</tr>
<tr>
<td>Strong expression</td>
<td>24</td>
<td>11</td>
<td>20</td>
<td>8***</td>
<td>3/8</td>
</tr>
</tbody>
</table>

Microsatellite analysis and immunohistochemistry was performed as described in Material and Methods. * Tumors without expression of HLA class I are negative for HLA-A and HLA-B/C. ** Weak expression of HLA-A and/or HLA-B/C or strong expression for HLA-A but not for HLA-B/C or vice versa. *** Strong expression for β2m, HLA-A and HLA-B/C.

**Microarray analysis**

The 32 selected tumors were divided in three groups: group A, tumors with less than 5% or no HLA class I positive tumor cells (n = 11); group B, tumors with no or partial loss of HLA class I expression and LOH at 6p21.3 (n = 11); and group C, tumors with normal HLA class I expression and no LOH at 6p21.3 (n = 10). Group C included 5 cases with minimal loss, defined as weak expression of HLA-A or HLA-B/C. Statistical analysis revealed no differentially expressed genes between group B and C. Only 15 genes were significantly differentially expressed between group A and B, and 15 genes between group A and C (Supplementary Figure 2). Since no differences in gene expression were observed between the groups with HLA expression (B and C), we combined the samples of group B and C into one group. Overall, 150 genes were significantly differentially expressed between the HLA negative group (group A, n = 11) and the HLA positive group (group B and C, n = 21).

**Differences in gene expression and gene ontology**

All genes exhibiting statistically significant differences in expression (SSDG) with a defined biological process (n = 105, 68%) were classified by GO terminology. Defense response was the process encompassing most of the SSDGs (Figure 1 and Table 3) and these genes were mainly upregulated in HLA positive tumors (24/27). The program “Global test” was then used to investigate if the defense response as a GO biological process and related subprocesses were significantly different between HLA positive and HLA negative tumors. Indeed, the defense response (which
Figure 1. Categorization of genes with statistically significant differences in gene expression (SSDG) into biological processes (using Gene Ontology terms). The numbers behind the bars depict the total number of annotated genes represented on the array for the biological processes.

included 1032 genes, p = 0.0008) as well as the subprocess ‘immune response’ (925 genes, p = 0.0008) showed significant differences (data not shown). The most prominent subprocesses within the immune response cluster were the “inflammatory response” (256 genes, p = 0.0003, data not shown) and “acute phase response” (33 genes, p = 0.0006, Figure 2). Differences in the amount of inflammatory infiltrate did not influence the differences observed in immune (sub)processes between HLA negative and HLA positive tumors. A correction for the amount of inflammatory infiltrate in the Global test even improved the P-values (data not shown). In the inflammatory response cluster, all SSDGs (11 genes) were upregulated in HLA positive tumors (Table 3). Five of these represented chemokines, such as CCL3 and CCL4. The highest of the SSDGs was SerpinA3, which showed an 8.2 fold change in gene expression (Table 3). In the acute phase response (Figure 2), SerpinA1 and SerpinA3 were both found to be SSDGs.

qRT-PCR verification of microarray data
Because gene expression differences between the HLA negative and HLA positive group were the most prominent in the inflammatory and acute phase responses, we chose gene products from these processes to validate the microarray results using qRT-PCR. The expression of SerpinA1, SerpinA3 and CCL4 was significantly increased in the HLA positive tumors (Figure 3). We detected a strong correlation between gene expression data from the microarray and the qRT-PCR experiments (p < 0.001).
Figure 2. The difference in expression profile of genes involved in the ‘Acute Phase response’ biological process (defined by Gene Ontology) between tumors positive for HLA expression (grey) and tumors without HLA expression (white). The graph was retrieved from the program Globaltest. The bars indicate the magnitude of difference, and the horizontal black lines in the bars depict standard deviation (SD).

Table 3. Significant differentially expressed genes involved in defense response in HLA-positive compared to HLA-negative tumors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Fold change</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>chemokine (C-C motif) ligand 3</td>
<td>CCL3</td>
<td>3.0</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-C motif) ligand 3</td>
<td>CCL3L1</td>
<td>2.7</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-C motif) ligand 4</td>
<td>CCL4</td>
<td>2.7</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-C motif) ligand 7</td>
<td>CCL7</td>
<td>1.5</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-X-C motif) receptor-like 2</td>
<td>CCR3L2</td>
<td>1.9</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>CXCL2</td>
<td>3.5</td>
<td>up</td>
</tr>
<tr>
<td>chemokine (C-X-C motif) ligand 3</td>
<td>CXCL3</td>
<td>2.7</td>
<td>up</td>
</tr>
<tr>
<td>defensin, beta 100A</td>
<td>DEFB106</td>
<td>1.8</td>
<td>down</td>
</tr>
<tr>
<td>FYB binding protein</td>
<td>FYB</td>
<td>1.8</td>
<td>up</td>
</tr>
<tr>
<td>ISG15 ubiquitin-like modifier</td>
<td>G1P2</td>
<td>3.4</td>
<td>up</td>
</tr>
<tr>
<td>complement factor 1</td>
<td>IF</td>
<td>4.3</td>
<td>up</td>
</tr>
<tr>
<td>interleukin 18 receptor accessory protein</td>
<td>IL18RAP</td>
<td>2.0</td>
<td>up</td>
</tr>
<tr>
<td>interleukin 21</td>
<td>IL21</td>
<td>1.5</td>
<td>up</td>
</tr>
<tr>
<td>interleukin 29 receptor, alpha</td>
<td>I2L29A</td>
<td>2.2</td>
<td>down</td>
</tr>
<tr>
<td>integrin, beta 2</td>
<td>ITGA2</td>
<td>1.9</td>
<td>up</td>
</tr>
<tr>
<td>small conductance calcium-activated channel, subfamily N, member 4</td>
<td>KCNN4</td>
<td>3.1</td>
<td>up</td>
</tr>
<tr>
<td>leukocyte immunoglobulin-like receptor, subfamily A, member 2</td>
<td>LILRA2</td>
<td>1.5</td>
<td>up</td>
</tr>
<tr>
<td>N-myc (and STAT1) interactor</td>
<td>NMI</td>
<td>1.8</td>
<td>up</td>
</tr>
<tr>
<td>nuclear receptor subfamily 3, group C, member 1</td>
<td>NR3C1</td>
<td>1.4</td>
<td>up</td>
</tr>
<tr>
<td>2',5'-oligoadenylate synthetase 1</td>
<td>OAS1</td>
<td>2.3</td>
<td>up</td>
</tr>
<tr>
<td>2',5'-oligoadenylate synthetase 2</td>
<td>OAS2</td>
<td>2.3</td>
<td>up</td>
</tr>
<tr>
<td>serpin peptidase inhibitor, clade A, member 1</td>
<td>SERPINA1</td>
<td>3.6</td>
<td>up</td>
</tr>
<tr>
<td>serpin peptidase inhibitor, clade A, member 3</td>
<td>SERPINA3</td>
<td>8.2</td>
<td>up</td>
</tr>
<tr>
<td>toll-like receptor 8</td>
<td>TLR8</td>
<td>1.4</td>
<td>down</td>
</tr>
<tr>
<td>G4N2-related adaptor protein 2</td>
<td>GRAP2</td>
<td>1.5</td>
<td>up</td>
</tr>
</tbody>
</table>

Up: upregulated in HLA positive tumors; down: downregulated in HLA positive tumors. The genes shown in bold are genes involved in the inflammatory process (defined by Gene Ontology).
Figure 3. Differences in gene expression between HLA negative and HLA positive tumors. Box plots show differences in gene expression as measured by qRT-PCR of SerpinA1, SerpinA3 and CCL4. P-values were calculated with a Mann-Whitney test.

Immunohistochemical analysis of protein expression
The elevated expression of SerpinA1 and SerpinA3 was then verified by immunohistochemistry (Figure 4). Samples with absent or low Serpin expression were combined and compared with the samples that showed strong expression. For both SerpinA1 and SerpinA3, the sum score of protein expression correlated with mRNA expression (p = 0.04 and p = 0.008, respectively). Similar to the mRNA expression data, a significantly higher expression of SerpinA1 (Fisher’s exact, p = 0.05) and SerpinA3 (Fisher’s exact, p = 0.02) protein was observed in the tumors with HLA expression (data not shown). Figure 5 shows immunofluorescence of a tumor that coexpressed HLA class I and SerpinA3 (A) as well as one negative for both HLA class I and SerpinA3 (B).

Association of SerpinA1 and SerpinA3 with clinical parameters and survival
To evaluate the association of Serpin expression with prognostic factors, we immunostained a tissue array that represented 117 tumors. As with the individual slides, the final score for expression of SerpinA1 or SerpinA3 comprised 2 groups: samples with absent/moderate expression, and samples with strong expression. In the tissue array, SerpinA1 expression significantly correlated with HLA expression (p = 0.03), whereas SerpinA3 did not show a significant association with HLA expression (p = 0.14). Tumor size (≥40 mm) and HPV status significantly correlated with strong SerpinA3 expression (p = 0.013 and p = 0.008 respectively, Table 1). No significant correlation was observed between HLA expression and HPV type or tumor histotype. HPV type significantly correlated with histotype (p = 0.02) but not with tumor size (p = 0.06). In addition, a trend for correlation of both SerpinA1 and SerpinA3 with overall survival (p = 0.068 and p = 0.11, respectively) and disease-free survival (p = 0.052 and p = 0.12, respectively) was found (data not shown).

Since expression of Serpins may facilitate the immune escape of HLA positive tumors, we next analysed the effect of Serpin expression on survival in cases with normal/partial HLA expression. Strong SerpinA1 expression correlated significantly with poor overall and disease-free survival (p = 0.035 and p = 0.014, respectively,
Chapter 2

**Figure 4.** Immunohistochemical analysis of SerpinA1 and SerpinA3 protein expression in cervical carcinomas. Arrows indicate tumor areas. A) Strong staining of SerpinA1, B) weak staining of SerpinA1, C) lack of SerpinA1 staining, D) strong staining of SerpinA3, E) mostly weak staining of SerpinA3 and F) lack of SerpinA3 staining.

**Figure 5.** Immunofluorescent staining for SerpinA3 and HLA class B/C. Tumors are depicted with A) positive staining for HLA class I (red, membranous) and SerpinA3 (green, cytoplasmic) or B) no positive staining for HLA class I and SerpinA3, whereas infiltrate cells show positivity.

Figure 6A), and strong SerpinA3 expression showed a correlation with poor overall survival (p = 0.05, Figure 6B).
Figure 6. Association between Serpin expression and survival. Kaplan-Meier curves and log rank test for overall survival. Patients with normal/partial HLA expression (+/-) in association with absent/moderate (-/+) or strong (+) expression of A) SerpinA1 and B) SerpinA3.

Discussion

This study shows that loss of HLA class I, as determined by analysis at the genetic and protein level occurs frequently in squamous cell carcinoma. Eighty-eight percent of the tumors showed a downregulation of HLA class I expression, and 56% of the tumors showed LOH of 6p21.3 at the HLA class I region; this data corresponds to previous results [7].

Gene expression analysis between tumors without or less than 5% of HLA class I positive tumor cells (HLA-) and tumors with normal/partial HLA class I expression (HLA+) showed that “defense response” is the process encompassing most of the SSDGs. No significantly different expression of immunosuppressive cytokines was observed between HLA- and HLA+ tumor groups. In contrast, many genes contributing to inflammatory and acute phase responses, such as CCL3, CCL4, CCL7, CXCL2 and CXCL3, were upregulated in HLA+ squamous cell carcinomas. CCL3 and CCL4 are IFNγ-induced chemokines that initiate the infiltration of macrophages, NK cells and T cells, all of which are cell types that have been associated with anti- and pro-tumor activities. These chemokines can be excreted by stromal cells, immune cells and/or tumor cells.

When we corrected our findings for differences in the extent of inflammatory infiltrate (estimated by visual examination of HE-stained slides) between the groups using the program global test, the immune response (sub)processes remained a significant distinction between HLA+ and HLA- tumors. Thus, the high gene expression profile of inflammatory-related genes in HLA+ tumors is not likely due to a higher number of immune cells.

The pronounced inflammatory gene expression profile of HLA+ tumors could be due to an interaction between immune cells and tumor cells that present viral and/or tumor derived peptides by HLA, leading to activation of the tumor infiltrate and
subsequent release of inflammatory cytokines. Although an inflammatory response is required for the development of an effective T-cell response against tumor cells, it can also contribute to tumor growth and invasion by providing growth factors and releasing proteases [20].

The highest differential expression between the HLA- and HLA+ tumor groups involved the SerpinA1 and SerpinA3 acute phase response genes which are upregulated during inflammatory processes. Our study showed that SerpinA1 and SerpinA3 expression at both the mRNA and protein level correlated with the presence of HLA. Both SerpinA1 and SerpinA3 expression showed a trend towards poor prognosis. Previously, high expression of SerpinA1 in tumors was correlated with poor prognosis in different types of adenocarcinomas [21,22].

The mechanisms by which SerpinA1 and SerpinA3 promote tumorigenesis and tumor growth remain unclear. In colorectal and lung adenocarcinomas, SerpinA1 expression correlated with the invasive and metastatic capacity of tumor cells [23,24]. Also, an immunosuppressive role for SerpinA1 as strong inhibitor of NK cytotoxicity in vitro has been reported [25]. Consistent with this, SerpinA1 was shown to be one of the most potent in vitro inhibitors of granzyme B [26], a trypsin-like serine protease by which cytotoxic T cells (CTL) and NK cells mediate apoptotic death of target cells. SerpinA3 expression has been detected in various tumors, including gastric and hepatocellular carcinomas [27,28]. Chymotrypsin, a target of SerpinA3, can induce apoptosis in leukaemia and hepatoma cells in vitro, and SerpinA3 inhibits apoptosis of hepatoma cells [29,30]. Thus, although a precise role for SerpinA1 and SerpinA3 in cancer remains to be elucidated, these proteins could be involved in tumor invasion or inhibition of apoptosis.

We recently used the tissue array described in the present study to show that partial loss of HLA, compared to complete loss or normal HLA expression, correlated with disease progression [31]. Low expression of HLA class I could assist the tumor cell in escaping from both CTL and NK cell-mediated killing [32]. Since SerpinA1 and SerpinA3 expression did not show a significant correlation with survival, the influence of these proteins on carcinogenesis is likely dependent on other factors. As SerpinA1 and SerpinA3 expression was most prominent in HLA+ tumors, we investigated whether Serpin expression may contribute to disease progression in HLA+ tumors. We observed that strong expression of both SerpinA1 and SerpinA3 in association with normal/partial HLA expression correlated with poor prognosis. Thus, immune evasion in HLA+ tumors could be attributed in part to factors such as SerpinA1 and SerpinA3 that possibly mediate an invasive and/or anti-apoptotic effect, thereby contributing to a more aggressive behaviour of the tumor.

In conclusion, here we show that the gene expression profile of HLA+ tumors differs from that of HLA- tumors with respect to expression of genes involved mainly in inflammatory and acute phase immune responses. Elevated expression of SerpinA1 and SerpinA3 in HLA+ tumors was confirmed by qRT-PCR and immunohistochemistry. Furthermore, strong expression of either SerpinA1 or SerpinA3 associated with HLA expression is detrimental for survival.
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References

Supplementary Figure 1. Microsatellite analysis at 6p of 59 squamous cell carcinomas. As described in Materials and methods, microsatellite analysis was performed on flow-sorted tumor cell subpopulations and frequently approached 100% signal reduction in one of the alleles. A.I.: allelic imbalance.
Supplementary Figure 2. Number of significant differences in gene expression between tumors with overall loss of HLA class I (A), tumors with partial loss of HLA class I (B) and tumors with no or minimal loss of HLA class I expression (C). Statistical analysis was performed by a nested F-analysis as described in Materials and Methods. The Venn diagram demonstrates 15 genes to be differentially expressed between group A and B and 15 genes between group A and C of which 6 genes are shared. No differences in gene expression were observed between group B and C.
SerpinA1 and SerpinA3 expression in HLA-positive cervical carcinoma