Chapter 4

Frequent HLA Class I Loss is an Early Event in Cervical Carcinogenesis
Chapter 4

Abstract

Loss at chromosome 6p21.3, the human leukocyte antigen (HLA) region, is the main cause of HLA downregulation, occurring in the majority of invasive cervical carcinomas. To identify the stage of tumour development at which HLA class I aberrations occur, we selected 12 patients with cervical carcinoma and adjacent cervical intraepithelial neoplasia (CIN). We investigated HLA class I and β2-microglobulin expression by immunohistochemistry in tumour and adjacent CIN. Loss of heterozygosity (LOH) was studied using microsatellite markers covering the HLA region. Fluorescence in situ hybridisation (FISH) with HLA class I probes was performed to investigate the mechanism of HLA loss. Immunohistochemistry showed absent or weak HLA class I expression in 11/12 cases. In 10 of these 11 cases downregulation occurred in both tumour and CIN. Only in one case did the concomitant CIN lesion show normal expression. In 9/12 cases LOH was present for at least one marker in both tumour and CIN, one case showed only LOH in the CIN lesion and one case showed retention of heterozygosity (ROH) for all markers in both tumour and CIN. We conclude that HLA class I aberrations occur early and frequently in cervical carcinogenesis. This might allow premalignant CIN lesions to escape immune surveillance and progress to invasive cancer.
Introduction

Cervical carcinoma is the second most common female cancer worldwide and human papillomavirus (HPV) is its most important aetiological factor. Immunological surveillance of HPV-associated lesions is performed by T-cells, which are activated when foreign (antigenic) proteins are presented to the T-cell receptor by human leukocyte antigen (HLA) class I proteins. HLA class I molecules are expressed on the cell surface and consist of a polymorphic heavy α chain, encoded by the HLA class I genes HLA-A, -B and -C on chromosome 6p21.3, in non-covalent association with the light β chain, encoded by the β2-microglobulin (β2m) gene on chromosome 15q21. This association is a prerequisite for the stability of the HLA class I molecule.

Loss of HLA surface expression occurs in various solid tumours and tumour cell lines and might result in escape from cytotoxic T-cell attack. It occurs frequently in cervical carcinoma and is predominantly caused by genetic aberrations at chromosome 6p21.3. Koopman et al. showed that 50% of multiple HLA allele loss is caused by LOH in the HLA region, which is frequently detected in cervical cancer.

Invasive cervical carcinoma is preceded by three stages of cervical intraepithelial neoplasia (CIN). Several studies have shown that the majority of the untreated mild dysplasias regress to normal cytology and only a small proportion of the CIN lesions eventually progress to invasive carcinoma. It is conceivable that the progressive CIN lesions have escaped immune surveillance. Several studies have recently investigated these precursor lesions for losses at 6p21.3, without distinguishing between progressing and regressing CIN lesions.

We selected patients with cervical carcinoma and adjacent CIN lesions to investigate how early and frequently HLA aberrations occur in cervical carcinogenesis. By choosing adjacent CIN, we were able to come as close as possible to selecting only progressive CIN lesions. By including these precursor lesions, we could add to the current knowledge of HLA aberrations during the development of cervical carcinoma.

Material and Methods

Tissue Samples

Since 1989, Surinamese women with cervical carcinoma have come to the Leiden University Medical Centre (LUMC) at Leiden, the Netherlands, to have a Wertheim operation, which is a radical hysterectomy. All the resected tumour tissue is stored in the tissue archive of the Pathology Department of the LUMC. From this group we selected the cases with invasive cervical carcinoma and an adjacent high-grade CIN lesion (CIN III) by reviewing the haematoxylin-eosin-stained slides. We found 15 cases with cervical cancer and an
adjacent CIN lesion, operated between 1989 and 1999, of which 12 had sufficient tissue to investigate. From these 12, tissue blocks containing formalin-fixed paraffin-embedded normal tissue and tumour tissue with adjacent CIN lesion were selected. To study intra-tumour heterogeneity we selected multiple tumour loci per case when possible. In nine cases two tumour sites and in one case three tumour sites were investigated.

All samples were positive for a high-risk HPV genotype, except for S70 which is HPV negative.

Immunohistochemistry

Immunohistochemistry was performed on freshly cut, 3-μm thick buffered, paraffin-embedded tissue sections according to standard procedures\(^2\). Slides were incubated overnight with mouse monoclonal antibodies (mAbs) suitable for paraffin sections: HCA2 and HC10 (Dr J. Neefjes, NKI, Amsterdam, the Netherlands) and the primary rabbit polyclonal anti-β\(_2\)m (A 072; DAKO, Copenhagen, Denmark). HCA2 recognises a determinant expressed on β\(_2\)m-free HLA-A (excluding HLA-A24), HLA-B7, and HLA-G heavy chains\(^2\). The mAb HC10 recognises a determinant on all β\(_2\)m-free HLA-B and HLA-C heavy chains, as well as on β\(_2\)m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and HLA-A33 heavy chains\(^2\). Immunodetection was performed as previously described\(^1\).

In each tumour, stromal cells including lymphocytes served as a positive control for HLA class I expression. Tumour cells were only scored negative if no staining was present compared to a strong staining of internal control cells. If some staining was present but reactive cells stained much stronger, tumour cells were scored as weakly positive.

Microdissection, DNA Extraction and Quantification

DNA was extracted as previously described, with minor adjustments\(^2\). Paraffin-embedded (normal and tumour/CIN) tissues of the 12 cases were cut in 10 μm sections and stained with haematoxylin. Before the normal dehydration steps, the staining procedure was interrupted to use the slides for microdissection. CIN lesions were microdissected using a needle under direct light-microscopic visualisation. Areas containing over 70% tumour cells, as well as normal control tissue were obtained using the same procedure and all tissue was transferred to sterile microcentrifuge tubes and incubated for 12 hours in 186 μl of PK1 buffer (10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM MgCl\(_2\), 0.45% NP40, 0.45% Tween 20, 0.01% gelatine), 5% Chelex (Chelex 100; Bio-Rad Laboratories, Hercules, CA) and 10 μl of a 10 μg/μl solution of proteinase K at 56°C. This was followed by incubation at 100°C for 5 minutes to inactivate the proteinase K. After 5 minutes of centrifugation at full speed (16,000 x g) the supernatant was transferred to new sterile microcentrifuge tubes. The DNA content was quantified using Picogreen double-stranded DNA (dsDNA) quantification reagent (Molecular Probes Europe BV, Leiden, the Netherlands), an ultrasensitive fluorescent nucleic acid stain for quantifying dsDNA in solution, according to the
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manufacturer’s instructions. Instead of the prescribed 10 mM Tris (pH 7.6), 0.1 mM EDTA dilution buffer, PK1 buffer was used, in accordance with the DNA isolation method.

LOH Analysis

DNA from microdissected normal, CIN and tumour material from all 12 cervical carcinoma cases was analysed for LOH on chromosome 6 by polymerase chain reaction (PCR) amplification using seven highly informative di-, tri- and tetrancleotide microsatellite markers. These are listed in Table 1.

Standard PCR amplifications were performed according to a protocol previously described, with some adjustments. To circumvent PCR artefacts, 10 ng/μl purified template DNA was used in a 12 μl reaction volume containing 6 pmol of each primer, 2 mM dNTPs, 0.1 mg/ml BSA, Taq polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.01% (w/v) gelatine, 0.1% Triton) and 1.0 unit AmpliTaq Gold polymerase (Perkin Elmer, Applied Biosystems Inc., Foster City, CA, USA). Either the forward or reverse PCR primer was fluorescently labelled with FAM or TET, respectively. Samples were denatured for 5 minutes at 96°C and amplified for 33 cycles consisting of 1 minute of denaturation at 94°C, 2 minutes of primer annealing at 55°C and 1 minute of elongation at 72°C, followed by a final extension step of 6 minutes at 72°C. For each primer set, PCR products of tumour DNA and normal DNA were mixed 1:1 and red-coloured GENE-SCAN-500 ROX (Perkin Elmer Cetus, Norwalk, CT, USA) was added as an internal DNA size marker. After denaturation and electrophoresis the PCR products were visualised as peaks and analysed.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Map Position</th>
<th>Forward and Reverse Primer Sequences Forward</th>
</tr>
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<tbody>
<tr>
<td>D6S89</td>
<td>6p22.3</td>
<td>CAAGGGAATAGTGAAGATTGCCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATGAGAAGGGCCAGCTTGC</td>
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<tr>
<td>D6S105</td>
<td>6p22.1-6p21.3</td>
<td>GCCCTATAAAATCCTAATTAC</td>
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<tr>
<td></td>
<td></td>
<td>GAAGGAGATGTGAAATCC</td>
</tr>
<tr>
<td>MOGc</td>
<td>6p21.3</td>
<td>GAATGTAGATTAAGAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATAAAAGGGAATCTACATA</td>
</tr>
<tr>
<td>D6S265</td>
<td>6p21.3</td>
<td>ACGTTCGTACCCATTAACCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATCGAGGTAACAGACGAAA</td>
</tr>
<tr>
<td>C143</td>
<td>6p21.3</td>
<td>AGCCTGGGTGACAGAACAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGATTAACCTGGAGACTCCT</td>
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<td>TNFa</td>
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</tr>
<tr>
<td></td>
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<td>CCTCTCTCCCCCTGCAACACACAC</td>
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<td>6p21.3</td>
<td>CTGAGTTTGCGACAGACATTTG</td>
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<tr>
<td></td>
<td></td>
<td>ACCCAGCATTTCCTGAGTGT</td>
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on an ABI 310 automatic sequencer. GeneScan Analysis Software (Perkin Elmer, Applied Biosystems Inc., Foster City, CA, USA) was used to quantify each peak in terms of size (base pairs), peak area and height. Each PCR was performed at least twice.

The thresholds for retention of heterozygosity (ROH), “grey area” (allelic imbalance) and LOH were applied as previously empirically determined\(^\text{31}\). These were 0.76-1.3 (ROH), 0.58-0.75 & 1.31-1.69 (allelic imbalance) and 1.7< (LOH).

**Interphase FISH analysis on Isolated Nuclei**

To study the mechanism of HLA aberrations we performed interphase fluorescence in situ hybridisation (FISH) analysis with HLA-A and -B/C probes. In seven cases, nuclei were isolated from formalin-fixed, paraffin-embedded material as previously described\(^\text{32}\). The suspension was applied to microscope slides as described for interphase FISH on frozen material\(^\text{33}\). The slides were air-dried and used for hybridisation. All probes used were obtained and labelled as previously described 10. The \(\alpha\)-satellite centromeric 6-probe (D6Z1, Oncor, Gaithersburg, MD) was biotin-16-dUTP-labelled by nick translation (Roche, Basel, Switzerland). Cosmid c109K2118, derived from the ICRF flow-sorted chromosome 6 library, was obtained from the Resource Centre/Primary Database of the German Human Genome Project (Berlin, Germany). PAC238M10 was isolated from the RCPI-1 Human PAC Library of the Roswell Park Cancer Institute (obtained by Dr J. den Dunnen, Genome Technology Centre, LUMC, Leiden, the Netherlands) using an HLA-C probe. Cosmid and PAC probes were digoxigenin-12-dUTP-labelled by nick translation. Hybridisation was performed as previously described 10,33. Hybridisation mixture (5 \(\mu\)l) that contained 3 ng/\(\mu\)l of the centromere 6 probe combined with 3 ng/\(\mu\)l of the cosmid or PAC probe, 1.5 \(\mu\)g human Cot-1 DNA and 3 \(\mu\)l hybridisation mix (50% formamide, 10% dextran sulphate, 50 mM sodium phosphate (pH 7.0), 2x sodium chloride/sodium citrate [SSC]) was applied to the slides. After denaturation for 8 minutes at 80°C, nuclei were hybridised overnight at 37°C in a moist chamber. Immunodetection was performed as previously described 33. Slides were analysed with a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany). Tonsils of healthy individuals were used as controls. The cut-off level for homozygous and hemizygous deletions was set as described previously\(^\text{32}\).

**Results**

**Loss of HLA class I expression detected in paraffin cervical tumour sections**

Tissue sections with both tumour and CIN tissue were stained for \(\beta_2\)m, HLA-A and HLA-B/C expression (**FIGURE 1**). Using the available antibodies for use on paraffin sections, only the loss of both of the A- or B/C-alleles will result in a negative score. In S66 no expression of HLA-A, in S8 and S38 no expression of HLA-B/C and in S41 no expression...
Analysed LOH data together with immunohistochemistry results. LOH results for seven markers on chromosome 6p for CIN (C) and tumour (T) are presented on the left side. O Retention of heterozygosity (ROH); ⊘ Non-informative; ● Loss of heterozygosity (LOH). Immunohistochemistry results are presented on the right for the HLA proteins and for the $\beta_2m$ protein (encoded on chromosome 15). □ Normal expression; ◼ Weak expression; □ Absent expression; □ Heterogeneous: weak and normal expression; ◼ Heterogeneous: absent and normal expression. The cases are grouped with regard to the results obtained for the tumour samples studied. (A) No expression of one or both HLA class I molecules in combination with LOH on 6p21.3. (B) Downregulation of $\beta_2m$ combined with ROH at 6p21.3. (C) Weak expression of HLA-A and/or HLA-B/C. (D) Lack of HLA class I expression combined with ROH and normal $\beta_2m$ expression. (E) Normal HLA class I expression and LOH.
of both HLA molecules was seen in the invasive tumour tissue and adjacent CIN. Heterogeneous loss of expression with the two HLA class I antibodies was seen in three cases (S38, S77, S61), of which one (S38) had similar results in tumour and CIN. In four other cases homogenous or heterogeneous loss of expression was seen in the CIN lesions with one or both HLA class I mAbs, but weak expression was seen in the adjacent tumour tissue. Absent or very low $\beta_2$m expression provided an explanation for the absent or low expression of HLA-A and HLA-B/C in both tumour and CIN in two cases (S38, S77) and in the CIN lesion alone in one case (S71).

**LOH Analysis in Tumour and Adjacent CIN Cases**

We performed LOH analysis on 12 invasive cervical carcinomas with adjacent CIN III lesions using seven markers on chromosome 6p. Multiple tumour loci of the invasive tumour were studied, but in all patients except for one (S38) the results were similar. Only for this patient are the data from both tumour loci shown. An overview of the results is depicted in **FIGURE 1**.

In 9 of 12 cases at least one of the markers showed LOH in the invasive tumour and CIN lesion. In five of these nine cases, all markers showed LOH in both tumour and CIN lesion. In four of the nine cases, discrepancies between the results obtained for CIN lesion and tumour were seen (**FIGURE 1**). In S38, the LOH pattern of the accompanying CIN lesion differed only for D6S1666, a marker that showed ROH in the tumour. In S61, the tumour showed ROH for all markers, whereas the CIN lesion showed LOH for markers D6S89, D6S105, MOGc and D6S265. In S70, marker D6S265 showed ROH in the tumour and LOH in the CIN lesion. In S77, most markers showed ROH in the tumour and CIN, but at TNF-a LOH was detected only for CIN.

In S10, all markers showed LOH in both the tumour and CIN lesion, except for C143, located in the HLA-E – HLA-C region. This marker showed homozygosity in the normal sample, but two alleles of different molecular weights in both the tumour and CIN lesions. This phenomenon is termed elevated microsatellite instability at selected tetranucleotide repeats (EMAST) and has been described in other human cancers as a novel form of microsatellite instability (MSI)$^{34-36}$.

**Interphase FISH Results**

Interphase FISH analysis was performed on nuclei isolated from paraffin-embedded material of different tumour and CIN localisations from eight cases: S10, S16, S38, S41, S61 (C+T), S70, S71 and S77. HLA class I-specific clones (HLA-A and HLA-B/C) in combination with a probe for centromere 6 were applied. No homozygous or heterozygous deletions were found. Most cases showed nuclei with three and nuclei with four copies of chromosome 6. In all cases, nuclei with three copies were detected. The highest percentages were found in S10 (28%), S16 (31%) and S41 (59%). In all but two cases (S16 and S71), nuclei
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with four copies of chromosome 6 were seen, with the highest percentage in S38 (28%). In one case (S77) FISH revealed two centromeric signals and three signals for the whole HLA class I region in 16% of the nuclei, suggesting a possible duplication and translocation (FIGURE 2).

Combined Allelic Imbalance and Immunohistochemistry Results

The analysed immunohistochemistry results together with the LOH data are presented in FIGURE 1. The cases are grouped with regard to the results obtained for the tumour samples studied. Because the majority of the results obtained by the different techniques used were in concordance, only the results whose interpretation is more complicated are reported below.

In S38, where CIN and tumour site 1 (C1+T1) showed ROH but site 2 showed LOH, the immunohistochemistry data also differed somewhat: in site 1 the expression was heterogeneous with both alleles absent from some of the cells, whereas site 2 had weakly expressed HLA-A in both the tumour and CIN. β2m was weakly expressed in both tumour sites. In group D, lack of HLA class I expression in combination with ROH and normal β2m expression was found in S61 (T) and in S66 (C+T). Remarkably, S61, with ROH in the tumour and LOH in the CIN, displayed (heterogeneous) negative expression of the HLA molecules in the tumour and weak expression in the CIN. Only in S70 was normal HLA class I expression seen despite LOH (group E).

FIGURE 2
Examples of the FISH results. (A) In case S77, FISH showed two centromeric signals and three signals of the whole HLA class I region in 16% of the nuclei, suggesting a possible duplication and translocation. (B) Example of aneusomy 6, found in most of the cases.
Chapter 4

Discussion

To investigate the timing, frequency and mechanism of HLA class I downregulation in cervical carcinogenesis, we performed immunohistochemistry, LOH analysis and FISH on cervical carcinoma specimens and adjacent CIN lesions. Including the precursor lesions in our study permitted us to add to the current knowledge of HLA aberrations in invasive cervical carcinoma\textsuperscript{11}. The present study demonstrates that HLA class I downregulation occurs frequently and early in cervical carcinogenesis.

In cervical cancer LOH at chromosome 6p21.3, which occurs at high frequencies\textsuperscript{12-16}, represents an important and common mechanism by which HLA genes and their products are abolished\textsuperscript{11}. This remains unclear in cervical precursor lesions. By choosing CIN III lesions that are adjacent to invasive cervical carcinoma, we were able to come as close as possible to selecting only CIN lesions that were progressing to invasive carcinoma. Nonetheless, we cannot exclude the possibility that multiple CIN lesions are present in the same patient. Thus, the CIN lesion adjacent to the invasive carcinoma could originate from a clonal process different from that of the invasive carcinoma.

The absence of HLA class I expression was explained by β\textsubscript{2}m loss in two samples. This did not apply, however, to all HLA class I downregulation observed. Complex genetic changes involving various loci could be an alternative explanation. The weak but not absent expression of HLA class I that was observed in several samples suggests a genetic aberration in only one HLA allele, at 6p21.3. In most patients, CIN and invasive tumour samples provided similar results, supporting the hypothesis that both are from the same clonal process and demonstrating that HLA loss is an early event in cervical carcinogenesis.

The specificity of antibodies available for immunohistochemistry on paraffin-embedded tissue is limited\textsuperscript{23-25,37} and this might have led to an understatement of HLA downregulation in the present study.

Bontkes et al. reported loss of HLA class I expression in CIN lesions progressing from low to high grade, supporting the results in our study\textsuperscript{38}. Several studies performed on solitary CIN lesions whose connection with invasive carcinoma was unknown did not find any HLA class I downregulation\textsuperscript{39,40}. Other studies detected LOH at chromosome 6p21.3 in 25-75% of low and high grade CIN lesions\textsuperscript{20,21}. We found even more LOH in our group, which could reflect the fact that it consisted of progressing CIN lesions.

Tumour heterogeneity occurs in cervical carcinoma\textsuperscript{11} and can obviously be represented in different stages of carcinogenesis, explaining the rare differences observed between the results from tumour and adjacent CIN in the present study. When feasible, we selected multiple tumour loci per case (results not shown) to account for possible allelic imbalance variation caused by different tumour fractions, that is, diploid and aneuploid fractions. In one case, different tumour loci from the same cervical carcinoma had different LOH results and another case showed ROH for all markers in the tumour, but LOH in the CIN
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lesion. This could implicate different clonal origins. In addition, in three of our cases some markers showed LOH in the CIN lesion but not in the invasive cancer. This could be due to the fact that the tumour tissue is frequently surrounded and infiltrated by lymphocytes that can contaminate the sample, whereas the CIN lesions are better separated from the stroma. Another possibility is that only HLA negative CIN lesions survived T-cell attack activated by the tumour’s presence.

Several explanations exist for the multiple HLA aberrations we found in most of the tumour samples and the adjacent CIN lesions. The failure to express HLA class I could result from LOH at 6p21.3 in combination with a locus-restricted event in this area. Such an event might be a point mutation, small deletion, methylation, chromosome loss or large deletion. We found no homozygous or heterozygous deletions with FISH using cosmid and BAC probes covering the HLA-A and HLA-B/C region. We did find aneusomy 6 in most cases, which is in concordance with the study by Koopman et al. In one case we found a possible duplication and translocation of the HLA class I region (FIGURE 2A). Such a triplication should lead to LOH, but ROH was detected even in sorted cells and was probably caused by tumour heterogeneity.

We conclude that HLA class I aberrations occur not only frequently, but also early in cervical carcinogenesis. This phenomenon might allow the premalignant CIN lesion to escape immune surveillance and progress to invasive cancer.
References


