Immunocytochemical Detection of Human Papillomavirus High Risk Type L1 Capsid Proteins in LSIL and HSIL As Compared with Detection of HPV L1 DNA

Peter Melsheimer, M.D., M.I.A.C., Sepp Kaul, Ph.D., Stefanie Dobeck, C.T., Gunther Bastert, M.D.

OBJECTIVE: To investigate the prevalence of HPV L1 capsid proteins in HPV infected HSIL and LSIL. HPV L1 capsid proteins are considered to be a major target of cellular immune response in CIN.

STUDY DESIGN: Cervical smears from 74 women with cytologically and histologically confirmed LSIL (n=32) and HSIL (n=42) were collected prospectively to detect HPV hr (high risk types 16;18;33;39;45;56;58) L1-DNA by standardized L1-consensus primer PCR (MY 09/11) and L1-capsid proteins by immunocytochemistry using the monoclonal antibodies T31 (HPV16) and T16 (HPV hr) in a standardized protocol.

RESULTS: In HSIL and LSIL, L1-DNA was found for HPV hr in 93% and 59% and for HPV16 in 69% and 37% of the specimens, respectively. HPV L1-capsid proteins were detected in HSIL and LSIL for HPV hr in 33% and 44%, and for HPV16 in 29% and 31% of the specimens, respectively. Expression of L1-capsid proteins were significantly reduced by 59.6% for HPV hr L1-DNA positive HSIL (p<0.01) and by 40.4% for HPV16 L1-DNA positive HSIL (p<0.01). In HPV 16 DNA positive and HPV hr DNA positive LSIL, no significant reduction of corresponding L1 capsid protein expression could be demonstrated.

CONCLUSION: These data suggest a disturbed viral cellular interaction in HPV16 and HPV hr infected HSIL with loss of viral L1 capsid antigen. In this context there is a possible role of T31 and T16 as prognostic markers to predict the prognosis of CIN.

Key Words: CIN; Human Papillomavirus; L1 Capsid Proteins; HPV L1 DNA.

From the Department of Obstetrics, Gynecology and Gynecological Oncology, Universitäts Frauenklinik Heidelberg, Germany
Dr. Melsheimer is Consultant Cytopathologist and director, Laboratory of Cytopathology
Dr. Kaul is Director, Laboratory of Oncology
Ms. Dobeck is Cytotechnologist
Dr. Bastert is Professor and Head, Department of Obstetrics, Gynecology and Gynecological Oncology

Financial Disclosure: The authors have no connection to any companies or products mentioned in this article

Corresponding author:
Dr. Peter Melsheimer, M.I.A.C.
Universitäts-Frauenklinik Voßstr. 9
D – 69115 Heidelberg, Germany
E-mail: peter_melsheimer@med.uni-heidelberg.de
Introduction

Nucleic acid amplification techniques regularly identify HPV DNA in 90-95% of cervical cancer specimens. Cohort studies have consistently shown that HPV infections precede the development of cervical cancer by some 10-15 years\(^1\). In addition to HPV DNA detection, markers of neoplastic progression such as HPV type, persistency of the viral detection (as determined by repeated sampling) and viral integration are under investigation. Follow-up studies demonstrated that the presence of HPV DNA is necessary for the development and persistence of cervical neoplasm and that disappearance of the viral DNA anticipates regression of the neoplastic cells\(^2\), even at a stage of high grade squamous intraepithelial lesion (HSIL)\(^3\). Markers to provide information on morphologically identical lesions with opposite prognosis have to be developed. In this connection, viral load, viral integration or markers of viral and cellular genetic interaction are of interest\(^4\).

The aim of our study was to find out whether expression of L1 capsid, which is one of the main targets of cellular immune response, is influenced by HPV type or severity of the CIN lesion.

Materials and Methods

Patients and Design

From December 2000 to December 2001 a prospective study was conducted at the colposcopy clinic of the Universitäts Frauenklinik Heidelberg in 74 consecutive patients with the cytologically and histologically confirmed diagnosis of LSIL or HSIL.

Cervical swabs were obtained with two cytobrushes. From one cytobrush a cytological smear was prepared, fixed in alcohol and Papanicolaou-stained. The top of the cytobrush was transferred into a tube containing PBS buffer, shaken for 30 seconds and stored at 5° C until subsequent use (HPV L1 capsid antigen analysis). The top of the second brush was transferred into a cryotube and immediately quick-frozen in liquid nitrogen until subsequent use (HPV DNA analysis).
HPV L1 Capsid Antigen Analysis

The number of cells in the preserved solution was determined in a Neubauer counting chamber. Approximately 10,000 cells of the homogenous cell suspension were then transferred onto a capillary split slide (Dako, Hamburg, Germany) using a cytocentrifuge device (Shandon Company, 10 minutes, 3000 rpm). The cytospin preparation was air dried overnight.

The cells were fixed first in formaline (3.7% in PBS) for 15 minutes and then in 100% methanol for an additional 5 minutes. They were stored at -20° C in a storage medium (50% glycerol in PBS) until immunocytochemical staining.

For immunocytochemical staining the preparations were boiled for 20 minutes in antigen demasking buffer (Dako, Hamburg, Germany) and then stained in an immune staining machine Techmate 500 (Dako, Hamburg, Germany). The L1 capsid antibodies T16 high risk (HPV hr types 16, 18, 33, 35, 39, 45, 56, 58) and T31 (HPV 16) (now cytoimmun diagnostics, Pirmasens, Germany) were incubated for 25 minutes at room temperature. Detection was conducted with the APAAP Detection System (Dako) and the substrate neufuchsin. Following hematoxilin counter-staining, the preparations were embedded in Apuatex (Merck, Darmstadt, Germany).

All of the preparations were then microscopically evaluated. As previously described\(^5\), heavy nuclear staining in the epithelial cells was the criterion for a positive HPV L1 antigen detection (Fig 1 and 2). The documentation and archiving of the immunohistochemically stained preparations was performed automatically (ACIS, Chromavision).
Fig 1:
Immunocytochemical staining of HPV 16 L1 capsid protein (T31) of cervical smear.
A) Positive staining in a koilocytic cell with dyscariotic nuclei. (*400)
B) Positive staining in groups of dyscariotic cells (HSIL) (*200)

HPV DNA Analysis (L1 Consensus Primer PCR)

DNA from quick-frozen cytobrush samples was isolated using TRIzol (Life Technologies-Gibco BRL, Karlsruhe, Germany) as recommended by the supplier. Degenerated primers MY09 (5'–CGTCCMARRGGAWACTGATT–3') and MY11 (5’–GCMCAGGGWCATAAYAATGG–3’) according to Ting and Manos6 were used to amplify a distinct DNA fragment of approximately 450 bp from the HPV L1 open reading frame of the mucosal HPV types 16, 18, 33, 35, 39, 45, 56, 58. The temperature profile for PCR was as follows: 5 minutes at 94°C for initial denaturation, 40 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, elongation at 72°C for 1 minute, and a final
elongation step at 72°C for 6 minutes. The PCR mixtures were subsequently electrophoresed in 2.0% agarose gels and stained with ethidium bromide. In the event of a positive signal at 450 bp, aliquots of the PCR products were subjected to restriction fragment length polymorphism analysis. Restriction digests were performed with 5 units of the restriction enzymes BamH I, Dde I, Hae III, Hinf I, Pst I and EcoR I (all from Roche Diagnostics, Mannheim, Germany) for 4 h at 37°C in an appropriate buffer. To enhance the specificity of the HPV typing procedure, RFLP fragments were separated by means of agarose gel electrophoresis (3.0 %), stained with ethidium bromide and transferred to nylon membranes (Hybon N+, Amersham Life Science, Buckinghamshire, England). This was followed by hybridization with the generic oligonucleotide probe GOP1 (5'-TTTGTWACYGTKGTRGAYAC-3'), which was deduced from a conserved region within the L1 open reading frame (corresponding to nucleotides 6624-6643 of HPV 16). Labeling and detection of the probe were carried out with the ECL oligolabeling and detection kit (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. HPV typing of the samples was performed as previously described. PCR positive controls consisted of phenol-chloroform-extracted DNA from the cell line SiHa, which contains one to tow copies of HPV16.

Results

The study series contained specimens from 32 women with LSIL and 42 women with HSIL. In all women diagnosis was confirmed by cytology (Papanicolaou stained smears) and histology (colposcopically guided punch biopsies). Mean age of all patients was 36.5 years, standard deviation (sd.) 11.3 years and range (r.) 17-82 years. Mean age of the LSIL patients was 36.7 years (sd. 11.3; r. 17-73); mean age of the HSIL patients was 36.3 years (sd.11.4; r. 19-82). Differences between the groups were not significant (Wilcoxon test for unpaired samples).

HPV typing by HPV DNA analysis (L1 consensus primer PCR) detected HPV 16 DNA in 37.5% (12/32) and 69.0% (29/42) of LSIL and HSIL specimens respectively. HPV high risk types 16, 18, 33, 35, 39, 45, 56, 58 (HPV hr) were detected in 59.4% (19/32) and 92.9% (39/42) of LSIL and HSIL specimens
respectively. The prevalence was significantly higher (p<0.01; Fisher’s exact test) in HSIL than in LSIL with an Odds Ratios (OR) of 3.7 for HPV 16 DNA and an OR of 8.9 for HPV hr DNA infection (table 1).

T31 antibodies (HPV16) antibodies showed positive immunocytical staining in 31.3% (10/32) of LSIL and 28.6% (12/42) of HSIL. T16 antibodies (HPV types 16, 18, 33, 35, 39, 45, 56, 58) showed positive immunocytochemical staining in 43.7% (14/32) and 33.3% (14/42) of the LSIL and HSIL specimens respectively. (table 2).

In HSIL the difference between proportions of positive detection of L1 capsid proteins and positive detection of L1 DNA was 40.48% for HPV16 infection and 59.52% for HPVhr infection (table 3). The reduction of L1 capsid protein expression in HPV 16 DNA positive and HPV hr DNA positive HSIL was statistically significant (p<0.01; McNeamar test for matched pairs). In HPV 16 DNA positive and HPV hr DNA positive LSIL, no significant reduction of corresponding L1 capsid protein expression could be demonstrated. Differences between proportions were 6.25% (95%CI: -17.77%; 23.50%) for HPV 16 and 15.63% (95% CI: -7.51%; 30.01%) for HPV hr. (table 3).

Table 1
Detection of HPV 16 and HPV high risk types** DNA by L1 consensus primer PCR (MY 09/11) in cervical smears from 32 women with LSIL and 42 women with HSIL.

<table>
<thead>
<tr>
<th></th>
<th>LSIL (n=32)</th>
<th>HSIL (n=42)</th>
<th>OR</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16 DNA</td>
<td>12</td>
<td>29</td>
<td>3.72*</td>
<td>1.41; 9.81</td>
</tr>
<tr>
<td>HPV hr** DNA</td>
<td>19</td>
<td>39</td>
<td>8.9*</td>
<td>2.26; 35.00</td>
</tr>
</tbody>
</table>

* Significant OR
** High risk types (hr) 16, 18, 33, 35, 39, 45, 56, 58

Table 2
Detection of L1 capsid antibodies T31 (HPV 16) and T16 for (HPV hr*) in cervical smears from 32 women with LSIL and 42 women with HSIL.

<table>
<thead>
<tr>
<th></th>
<th>LSIL (n=32)</th>
<th>HSIL (n=42)</th>
<th>OR</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>T31 (HPV 16)</td>
<td>10</td>
<td>12</td>
<td>0.88</td>
<td>0.32; 2.40</td>
</tr>
<tr>
<td>T16 (HPV hr*)</td>
<td>14</td>
<td>14</td>
<td>0.64</td>
<td>0.25; 1.66</td>
</tr>
</tbody>
</table>

* High risk types (hr) 16, 18, 33, 35, 39, 45, 56, 58
Table 3
Numbers and proportions of positive L1 DNA detection and positive L1 capsid protein expression for HPV16 and HPVhr in cervical smears of 42 HSIL and 32 LSIL lesions and the corresponding differences of positive proportions.

<table>
<thead>
<tr>
<th></th>
<th>L1 DNA</th>
<th>L1 protein</th>
<th>Difference</th>
<th>(95% CI)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSIL (n=42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV 16</td>
<td>29</td>
<td>12</td>
<td>40.48%</td>
<td>(40.48%; 40.48%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>HPV hr</td>
<td>39</td>
<td>14</td>
<td>59.52%</td>
<td>(59.52%; 59.52%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>LSIL (n=32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV 16</td>
<td>12</td>
<td>10</td>
<td>6.25%</td>
<td>(-17.77%; 23.50%)</td>
<td>0.7518</td>
</tr>
<tr>
<td>HPV hr</td>
<td>19</td>
<td>14</td>
<td>15.63%</td>
<td>(-7.51%; 30.01%)</td>
<td>0.2278</td>
</tr>
</tbody>
</table>

* McNeamar Test for matched pairs
** High risk types (hr) 16, 18, 33, 35, 39, 45, 56, 58

Discussion
Patients’ mean age was 36.5 years. It has been demonstrated that the prevalence of HPV infection varies with age and geographical region, reaching highest rates below 35 years of age. Comparisons of HPV detection rates with data in the published literature therefore have to be made carefully. In our study, the mean age of the participating women was remarkably high. The detection rate of HPV DNA certainly depends on the kind of HPV test that is used. High sensitivities, specificities and good interlaboratory agreement are reported for protocols employing L1 DNA Consensus Primer PCR (MY09/11).

In our study prevalence of HPV 16 and HPV hr DNA in HSIL (diagnosed by cytology and histology) was 69% and 93% respectively, using HPV L1 DNA Consensus Primer (MY09/11) PCR as the standard test to detect HPV infection. The prevalence of HPV 16 and HPV hr DNA was significantly higher in HSIL than in LSIL. These data are in accordance with very well documented published data.

Our study demonstrates that the expression of L1 capsid proteins is significantly reduced in HPV 16 DNA positive HSIL and HPV hr DNA positive HSIL. A possible explanation may be found in a disturbed viral cellular interaction. Integration may cause a loss of L1 expression by segregating the promoter from the L1 gene or by destroying the L1 gene in the process of integration itself. Because of cis-regulatory effects and an increased stability of transcriptional factors, the integration of viral genomes contributes to deregulated expression...
of viral oncoproteins\textsuperscript{11}. It was demonstrated by published data of the authors and others, that integration of HPV 16 oncogenes can be found in up to 15\% of HPV16 DNA positive HSIL\textsuperscript{12}. Concerning the data, presented in this paper, integrated HPV16 oncogenes might be found in up to 5/29 HPV16 DNA positive HSIL. 17/29 HPV16 DNA positive HSIL had negative L1 capside protein expression. According to these calculations, HPV oncogene integration might not be the only explanation for reduced HPV L1 capside protein expression in HSIL.

The development of viral capsid antigen L1 depends upon transcriptional factors which only can be expressed during maturation process from basal epithelial cell to superficial epithelial cell\textsuperscript{13}. In HSIL the natural structure as well as maturation of the epithelium are disturbed, leaving dysplastic basal epithelial cells as the predominant cell type of the tissue with reduced L1 capsid protein expression.

Semiquantitative counts of slides from the authors as well as from others (personal communication) revealed, that about 20\% of both koilocytes and dyskeratocytes were observed to show positive staining by T31 and T16 antibodies in HPV 16 and HPV hr DNA positive HSIL. It remains to be speculated and further investigated, to which extend HPV 16 and HPV hr L1 DNA positive and L1 capsid antigene negative cells really underwent malignant transformation.

Viral L1 capsid antigen may be considered to be a major target of cellular immune response. A reduction or loss of capsid antigen production therefore might result in a reduction of cellular immune response\textsuperscript{14}. This might explain the role of HPV 16 as the most oncogenic HPV type, causing higher rates of progression and persistence of CIN than other HPV types\textsuperscript{15}.

In this context there is a possible role of T31 and T16 as prognostic markers to predict the prognosis of CIN. This question should be further evaluated by larger prospective studies. Women, who suffer of HPV positive HSIL, might be followed up after cone biopsy in order to find out a possible association between loss of HPV hr L1 capsid protein expression and development of recurrences.
Acknowledgments

The authors express their thanks to Ralf Hilfrich, Ph.D., chief executive officer, and Michael Kohlhoff, Ph.D., manager product development, present address cytoimmun diagnostics, Pirmasens, Germany, for providing T31 and T16 antibodies on cytologic specimen.

12. Melshheimer P, Klaes R, von Knebel-Doeberitz M, Bastert G. Prospective clinical study comparing DNA flow cytometry and HPV typing as predictive tests for persistence and progression of CIN I/II. Cytometry 2001; 46(3):166-71