Prognostic relevance of HPV L1 capsid protein detection within mild and moderate dysplastic lesions of the cervix uteri in combination with a second biomarker p16.

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Abstract

Objective: to proof the prognostic relevance of HPV L1 capsid protein detection on colposcopically guided punch biopsies in combination with p16

Study design: Colposcopically guided punch biopsies from 191 consecutive cases with at least 5 years follow up, were stained with HPV L1 capsid protein antibodies (cytoactiv screening antibody) and a monoclonal anti p16 antibody. 50 sections have been derived from a benign group, 91 from CIN I lesions and 50 from CIN II lesions.

Results: 29 of 91 CIN I biopsies had positively stained squamous cell nuclei. 21 out of 29 of the positive lesions went into remission (72,5%) and 8 progressed to histological confirmed high grade lesion (27,5%). 38 out of the 62 L1 negative CIN I lesions have been p16 positive. 24 of these 38 cases progressed to high grade lesions (63,2%), and 14 out 38 cases have shown a remission of the lesion (36,8%). 24 out of the 62 L1 negative CIN I have been p16 negative too. None of these cases progressed.

49 out of 50 CIN II lesions have been L1 negative. All of the cases have shown a progression.

Conclusion: HPV L1 capsid protein detection with cytoactiv screening antibody seems to be a promising new tool to predict the behaviour of HPV associated early dysplastic lesions.

Key words: HPV, L1 capsid protein, cytoactiv, p16, prognostic marker, biomarker, CIN, Pap smear
**Introduction**: The effectiveness of cervical cancer screening programs in detecting preinvasive cervical lesions has brought a decline in invasive cervical carcinomas.\(^1,2\) As a result of this, more cervical cancer precursor lesions are being diagnosed and the costs associated with treatment of low grade cervical lesions have escalated in the US up to $ 6 billion annually in recent years.\(^3\) Even in Germany and Denmark about 725,000 women with abnormal cervical smears have been detected in 2003.\(^4\)

It is known that most of the low grade dysplastic lesions regress spontaneously and only some of them will progress to cervical cancer.\(^5\) But until today no morphological criteria exists to predict the behaviour of a CIN lesion. Therefore it would be most useful to have prognostic markers that will be able to differentiate between patients who will undergo a transition from a precursor state to cancer and those who will not.\(^6\) Thus saving the women's quality of life and health care resources.

Many anogenital cancers, particularly squamous cell carcinoma of the cervix, are induced by the human papillomviruses (HPV), epithelium specific DNA tumor viruses.\(^7\) Many HPV types are known to infect the anogenital tract but only a subset, the so called high risk types, are frequently found in malignant lesions.\(^8\)

In 2003 Melsheimer et al. have shown that most of the HPV high risk associated LSIL are expressing HPV L1 capsid protein, but in most of the HPV high risk associated HSIL the HPV L1 capsid protein is missing. They suggested that this disturbed viral cellular interaction in HPV infected HSIL with loss of viral L1 capsid protein could function as prognostic marker to predict the prognosis of CIN lesions, since L1 capsid
protein, used by most vaccine companies to develop HPV vaccines, is a major target of the immunresponse\textsuperscript{9,10}.

In 2004 Griesser et al have been able to confirm this assumption. He has shown on routinely performed Pap smears that HPV high risk associated low to moderate dysplastic squamous lesions without immunochemically detectable HPV L1 capsid protein are significantly more likely to progress (76.4\%) than are L1 positive cases (23.6\%).\textsuperscript{11}

The aim of our study was to find out wether the prognostic relevance of HPV L1 capsid protein detection found on routinely performed Pap smears could be confirmed on paraffin embedded colposcopically guided punch biopsies and if we could be able to circumvent highly sophisticated DNA methods like PCR or Hybrid capture II by the use of immunochemical staining with p16, as marker to confirm presence of HPV in L1 capsid protein negative cases.

**Material and Methods**

**Histology and immunohistochemistry**

Colposcopically guided punch biopsies were fixed in neutral buffered formalin, embedded in paraffin, sectioned in 6µm slices and than stained with hematoxylin and eosin.

Cohort sections were deparaffinized and stained with a monoclonal anti HPV L1 capsid protein screening antibody (cytoactiv screening antibody, cytoimmun diagnostics GmbH, Pirmasens, Germany) and a monoclonal anti p16 antibody (NovaCastra, Newcastle up Tyne, UK) by
immunoperoxidase technique according to the protocol of the manufacturers.
In brief after antigen retrieval for 20 minutes in citric buffer the sections were incubated with the primary antibody for 30 minutes, the detection reagent for 10 minutes and the chromogen for 5 minutes. The sections were intensively washed in washing buffer after each step. After counterstaining with hematoxilin, slides were mounted and coverslipped. Stained slides were studied by light microscopy independent by RH and JH. Evaluation was performed as described elsewhere.\textsuperscript{11,12}

**Results**: Within our study we analysed 191 paraffin embedded colposcopically guided punch biopsies, including 50 from a benign group, 91 CIN I and 50 CIN II/III lesions. (see Table I)
All of the 50 biopsies of the benign group have been negative for HPV L1 capsid protein detection. That means the specificity of HPV L1 capsid protein in our study was 100%.
29 of 91 CIN I lesions have shown positively stained nuclei, called positive for HPV L1 capsid protein detection. Independly of the age of the women 21 out of 29 went into remission (72,5%) and 8 out of 29 progressed to histological confirmed high grade lesion (27,5%).
62 of 91 CIN I lesions have been negative for HPV L1 capsid protein detection. To confirm the association of the lesion with HPV within the group of HPV L1 capsid protein negative CIN I lesions we performed immunochemical staining with an anti p16 antibody.
24 out of the 62 HPV L1 capsid protein negative cases have been negative for p16 too. None of these cases progressed, which confirms the
assumption that L1 and p16 double negative CIN I cases represent lesions that are not associated with HPV and have no potential to progress.

Out of the 38 L1 negative and p16 positive cases 24 have progressed to histological confirmed high grade lesion (63.2%) and 14 have shown a remission of the lesion (36.8%).

49 out 50 CIN II/III lesions have been HPV L1 capsid protein negative and only 1 case has been HPV L1 capsid protein positive. All the CIN II/III have shown a progression of the lesion. (see Table II)

Discussion:
For the first time we have been able to confirm the prognostic relevance of HPV L1 capsid protein detection on paraffin embedded histological sections, initially reported on routinely performed Papanicolaou stained cervical smears and on liquid based cytology (LBC)\textsuperscript{9,11}.

In contrast to these report, the association of the cervical lesions with HPV high risk types in our study was not confirmed by highly sophistic DNA methods like PCR\textsuperscript{9} or Hybrid capture II\textsuperscript{11}, but the use of a second biomarker, p16.

By the use of both biomarkers, L1 and p16 - that can be easily integrated in a histopathology lab - we have been able to confirm the already reported prognostic significance of L1 capsid protein detection for early dysplasitic lesions.

In addition to this we have been able to show that the specificity of HPV L1 capsid protein detection is extremly high. We have also found that all of the 50 biopsies from a benign group have been negative.
The L1 or major capsid protein is a nuclear protein that is expressed by all HPV subtypes. This protein is produced during a productive HPV infection and together with the minor capsid protein (L2) it encapsulates the viral DNA to build infectious viruses. The ratio of the major L1 and the minor L2 capsid protein within the viral particles is 30:1.\textsuperscript{13}

Due to its function the synthesis of HPV L1 capsid protein is linked to the maturation process of basal to superficial epithelial cells and L1 is therefore strongly expressed at the superficial layer of the epithelium after the viral genome replication is completed.\textsuperscript{14, 15}

Within our study about 30% of the CIN I lesions expressed the L1 capsid protein.

Indicative for dysplastic alterations is a disturbed maturation process of the basal cells leading to transcriptional, translational or genetic alterations resulting in a loss of L1 capsid protein within dysplastic lesions.\textsuperscript{16, 17}

In contrast to this an overexpression of p16 could be well documented within the highly dysplastic cells.\textsuperscript{12}

Independently of the age of the women we have found that 72.5% of the L1 positive CIN I lesions have shown a spontaneous remission of the lesion within the follow-up period of at least 5 years.

This rate is slightly higher than the values reported by Griesser et al. (68%) found on routinely performed Pap smears showing a mild to moderate dysplasia (Pap IIID Munich nomenclature II).

The reasons for this difference seem obvious. In contrast to Griesser we analysed mild dysplasias (CIN I) and moderate dysplasias (CIN II) having different rates of L1 positivity in two different subgroups.
Why L1 capsid protein detection correlates with the remission of the lesion is not totally understood. But different authors have shown that the highly immunogenic HPV L1 capsid protein is able to stimulate L1 specific cytotoxic T-lymphocytes\textsuperscript{18, 19, 20} the most probable way to limit a viral infection.

Griesser has shown that a remission of the HPV L1 capsid protein positive mild to moderate dysplasias is correlated with the elimination of the HPV infection, resulting in Hybrid capture II negative women showing non suspicious cervical smears. (personal communication)

In some cases (27.5\%) we have observed a progression of the CIN I lesions, which is probably indicative for the inability of the immune system of these women to clear the dysplasia even in the presence of the L1 protein.

It seems that this is indicative not for a general but most probably a decreased HPV specific or lack of HPV specific immunocompetence within these women.

Looking at the L1 negative CIN lesions we have only been able to confirm the prognostic relevance within the group of the CIN II/III lesions, proven to be all p16 positive. All of the cases not expressing L1 capsid protein showed a progression of the lesion.

Within the CIN I lesions it was necessary to integrate a second biomarker, p16, to confirm the association of the CIN lesions with HPV. We preferred using p16 instead of other, highly sophisticated methods like PCR or Hybrid capture II because immunostaining is much easier to integrate in a routine histopathology lab.
Of the 38 L1 negative and p16 positive CIN I lesions 63.2% showed a progression of the lesion and only 36.8% regressed spontaneously. This is showing in contrast to Griesser et al. that independent of the age of the women, 76% of the mild to moderate dysplastic lesions progressed to high grade lesions within their study.

The reasons for this difference could be multiple. Cervical smears and histological sections are difficult to compare and in contrast to Griesser we analysed only CIN I lesions where we could expect that the probability to progress is lower.

None of the L1-negative and p16-negative CIN1 lesions progressed to a higher degree. The negative predictive value of the combination of L1 and p16 staining is thus 100%.

In addition to this it is known that p16 overexpression is not absolute specific of HPV high risk infections. Therefore we are not absolutely sure if there aren’t any mild dysplasias associated with HPV low risk infections with no potential to progress to cervical cancer.

Putting the 38 CIN I lesions and the 49 CIN II/(III) lesions being L1 negative and p16 positive together in one group - like Griesser has done according to the Munich nomenclature II for cervical smears - we would find that 83.9% of this lesions progressed and only 15.1% showed a remission of the lesion.

This shows that immunostaining with p16 as additional tool to confirm the association with HPV seems to be equivalent to highly sophisticated methods like PCR or HC II.
Our data shows that HPV L1 capsid protein detection with cytoactiv screening antibody seems to be a promising new tool to predict the behaviour of early dysplastic lesions.

Thus offering the possibility to save the women’s quality of life and health care resources.
References


7. zur Hausen H, Papillomaviruses and cancer : from basic studies to clinical application, Nat Rev Cancer 2002, 2, 342-350


Table I:

<table>
<thead>
<tr>
<th>Histology</th>
<th>L1 positive</th>
<th>L1 negative</th>
<th>p16 positive</th>
<th>p16 negative</th>
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<td>Benign</td>
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<td>CIN I</td>
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<tr>
<td>CIN II</td>
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Table II:

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<th>No. of cases</th>
<th>Remission</th>
<th>Progression</th>
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<tr>
<td>L1 +</td>
<td>29</td>
<td>72,5 %</td>
<td>27,5 %</td>
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<td>L1-, p16+</td>
<td>38</td>
<td>36,8 %</td>
<td>63,2 %</td>
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