HPV16-L1-specific Antibody Response Is Associated with Clinical Remission of High-risk HPV-positive Early Dysplastic Lesions

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Abstract. *Background/Aim: The present study was aimed at clarifying if use of a rapid human papillomavirus type 16 L1-specific antibody test could be used to improve clinical management of high-risk HPV-positive low-grade squamous intraepithelial lesion (LSIL)/high-grade squamous intraepithelial lesion (HSIL). Patients and Methods: The study was nested within a prospective study of 801 patients with early dysplastic high-risk HPV-positive lesions to examine the prognostic significance of HPV-L1 protein detection. Serum samples of 87 patients were tested with a rapid HPV16-L1-specific antibody test. The results were correlated with the clinical outcome during 66 months of follow-up. Results: A combined analysis of the 22 antibody-positive women showed that 17 were also L1 protein-positive, and 5 were L1 capsid protein-negative. An HPV-specific immune competence strongly correlates with clinical remission of low-grade squamous intraepithelial lesion (76.6%). For L1 antigen and HPV16-L1 antibody double-positive women, the risk of progression to cervical intraepithelial neoplasia grade 3 was low (5.8%). Conclusion: The rapid anti-HPV16-L1 test could be a promising tool to improve risk assessment and appropriate clinical management of high-risk HPV-positive early dysplastic lesions.*

Despite the increasing use of vaccination, human papilloma virus (HPV) remains a major global health burden. Women in particular are experiencing the consequences of infection with high-risk HPV, with about 500,000 new cases of invasive cervical cancer annually (1).

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The critical importance of an immune response in the control and resolution of HPV infections is well-accepted. On the one hand, HPV vaccine-induced antibodies are very effective in preventing disease, and infiltrating T-lymphocytes as well as macrophages can be observed in spontaneously regressing papilloma. On the other hand, the increased incidence and progression of HPV infection in immunocompromised individuals are obvious (2-4).

Interestingly, even though the HPV lifecycle does not induce keratinocyte death and, especially in high-risk HPV infection, does not result in major proinflammatory signals, a successful immune response to genital HPV infection is established in almost all cases. The time required for clearance of high-risk types, particularly HPV16, averages 12-18 months, which is considerably longer than the 4-9 months needed for clearance of low-risk types (5).

Several studies reported that immunochemical detection of the HPV-L1 capsid protein with Cytoactiv® (Cytoimmun, Pirmasens, Germany) predicts clinical remission and progression of high-risk HPV-associated low- grade squamous intraepithelial lesion/high-grade squamous intraepithelial lesion to cervical intraepithelial neoplasia grade 3+. In the presence of the HPV-L1 capsid protein, spontaneous clinical remission was regularly observed. Only in a minor proportion of cases (20%) did HPV-L1 capsid protein-positive early dysplastic lesions progress to histologically confirmed CIN3. A lack of HPV-specific immunity in 'non immunocompromised' individuals was discussed as a possible explanation for the subsequent development from premalignant to malignant lesions in women with positive HPV-L1 results (6-12).

The aim of the present study was to answer the question if demonstration of immunological activation using a rapid HPV16-L1-specific antibody test could be used as a tool to improve clinical management of high-risk HPV-positive LSIL/HSIL.

Patients and Methods

Patients and design. This study was nested within a larger prospective, international multicenter study of 801 patients with high-risk HPV-positive early dysplastic lesions (LSIL/HSIL) to examine the prognostic significance of HPV-L1 capsid protein detection with Cytoactiv (12). In 2007, randomly selected cases of HIV-negative, non-pregnant, non HPV-L1-vaccinated women diagnosed with LSIL or moderate (HSIL) dysplasia were recruited within the Dysplasia Unit of the Erlangen University Hospital. Two local investigators independently classified the Pap smears as mild (LSIL) or moderate dysplasia (being part of HSIL) and a centralized review of all cases was performed by a reference cytopathologist (Department of Pathology and Cytodiagnostics, Cologne).

For conventional Pap smears, the cells were transferred onto glass slides and the remaining cells within the brush were used for HPV-DNA analysis. High-risk HPV association was confirmed with the Hybrid-Capture II test (Qiagen, Hilden, Germany). Serum samples of 87 patients were tested with a rapid HPV16-L1-specific antibody test (HPVix; Cytoimmun-Diagnostics GmbH, Pirmasens, Germany).

The Institutional Review Board of the Erlangen University Hospital (Ethics Committee number 3771) approved the study protocol.

Written informed consent from the patients was not necessary as the samples were received and analyzed anonymously and patients were treated in accordance with the national guidelines in Germany, with cytological follow-up and colposcopy or punch biopsies for histological verification.

Follow-up smears were taken at intervals of 3-6 months or annually after the first negative smear for intraepithelial lesions. Conisation for treatment was performed if clinically indicated, and with the patient's consent.

Owing to the presumed critical importance of immunological reactions, any invasive procedure that may be able to induce an immunological reaction was defined as the clinical endpoint of the study case.

Follow-up ended in June 2012, resulting in a follow-up period of up to 66 months. Women having at least two consecutive smears negative for intraepithelial lesion were considered to be in clinical remission.

Persistence was defined as the state in which mild and moderate dysplasia persisted cytologically over the whole follow-up period or as histologically confirmed CIN1/CIN2. Progression was defined as histologically confirmed CIN3. Histological diagnoses were from conisation specimens or colposcopically guided punch biopsies.

A group of 38 cytologically healthy patients was recruited as a control group. The women visited the Gynecological Outpatient Clinic for routine Pap smears and colposcopy between 2007 and 2012. The patients did not show any evidence of malignancies, pathological Pap smears, genital infections, or pregnancy. Nevertheless we are not able to rule out that the women may have had abnormal Pap smears in the past. All of the women in the control group tested negative for current HPV infection using the Hybrid-Capture II assay. All of the women provided informed consent to the storage of their serum samples and their use for research purposes. Two-sided Fisher's exact test was used for data analysis.

Immunocytochemistry. Routinely processed conventional Pap smears were immunochemically stained with the Cytoactiv Screening Set (Cytoimmun-Diagnostics GmbH), which detects the L1 capsid protein of all known HPV types. Staining was performed according to the manufacturer's protocol, described elsewhere (9). In brief, slides used for the initial morphological diagnosis were subjected to antigen unmasking by microwave treatment after unmounting without prior destaining. Cytoactiv screening-antibody was applied to the slides which were then incubated for 30 min at room temperature, followed by incubation with the detection reagent for 10 min and 3-amino-9-ethylcarbazole (AEC) chromogen for 5 min. After counterstaining with hematoxylin, slides were mounted with Aquatex (Merck, Darmstadt, Germany) and coverslipped. Stained slides were studied by light microscopy independently by two local investigators and a reference cytopathologist, HG. Slides with at least one epithelial cell with distinctly positive nuclear staining were scored as positive.

Rapid HPV16-L1 antibody test. Serum samples of 87 enrolled patients and 38 of the control group were tested with this rapid HPV16-L1-specific antibody test (HPVix; Cytoimmun-Diagnostics GmbH) according to the manufacturer's protocol.

Wold Health Organization (WHO) reference serum and an internal positive control were included within every run.

Results

A total of 150 patients were recruited for the study: 112 women with high-risk HPV-positive early dysplastic lesions served as the study group, and 38 women with 'no intraepithelial lesion detected', as a healthy control group.

The mean age of the 112 women of the study group was 29.4 years (range=18-50 years). The HPV-L1 capsid protein, as relevant antigen for the L1 antibody response, was detected in 70 cases using the conventional Pap smears of these women. The mean age of these Cytoactiv®-positive women was 28.9 years (range=19-44 years). Forty-two cases were HPV-L1 capsid protein-negative. The mean age of these women was 30.2 years (range=18-50 years).

The mean age of the healthy control group (Hybrid-Capture II- and Cytoactiv-negative) was 32.4 years (range=18-59 years).

All 87 serum samples available for the study group were tested for the presence of HPV16-L1 antibodies with the rapid test (Table I).

Within the healthy control group, we found two (4%) patients to be HPV16-L1 antibody-positive. The remaining 36 (96.0%) serum samples were negative.

The mean age of the antibody-positive women in the control group was 28.2 years (range=21-41 years) and 33 years (range=18-59 years) for the antibody-negative women. Twenty-two (26%) patients of the study group but only two (4%) women of the control group showed an HPV16-L1-specific antibody response.

This 6.5-fold increase of the frequency of antibody response observed in women with high-risk HPV LSIL/HSIL is indicative of an L1-specific activation of the immune response during a productive HPV infection.

Table I. *Characterisation of the high-risk human papillomaviruses (HPV) HPV-positive early dysplastic lesions in relation to the HPV L1 results.*

	HPV L1 antibody-positive		HPV L1 antibody-negative		Total	
HPV L1 result	n (%)	Mean age (range), years	n (%)	Mean age (range), years	n (%)	Mean age (range), years
Positive	17 (19.5)	26.5 (19-40)	42 (48.3)	30.7 (20-44)	59 (67.8)	29.5 (19-44)
Negative	5 (5.8)	23.2 (18-25)	23 (26.4)	29.5 (18-46)	28 (32.2)	28.4 (18-46)
Total	22 (25.3)	25.8 (18-40)	65 (74.7)	30.3 (18-46)	87	29.1 (18-46)

Clinical outcome of the study group after 66 months of follow-up. Overall, only 4 (8.7%) out of 46 cases with histologically-confirmed CIN were classified as double-positive, meaning that the HPV-L1 antigen as well as HPV16-L1 antibodies were detected (Table I).

Remarkably, only one case showed a progression to histologically-confirmed CIN3 for these 17 double-positive women, three persisted as CIN2 and CIN1 but 13 showed a clinical remission of the lesion (Table II) (p-value=0.012).

This seems to be indicative of the effectiveness of the immune system in clearing the HPV16-associated early dysplastic lesion once the immune system has responded to the HPV-L1 antigen stimulus.

Interestingly four out of five L1 antibody-positive women within the group of L1 capsid protein-negative women showed a clinical remission during the follow-up period, which could be indicative of a false-negative L1 antigen result.

Table II. *Clinical outcome of high-risk human papillomaviruses (HPV) HPV-positive early dysplastic lesions in relation to the result of L1 capsid protein detection and the L1 antibody response.*

	HPV L1 antigen/antibody result			
Lesion type	Double-positive n (%)	Single/double-negative n (%)	Total	p-Value*
CIN 3	1 (5.9)	16 (94.1)	17	
CIN 2	2 (10.0)	18 (90.0)	20	
CIN 1	1 (11.1)	8 (88.9)	9	
Clinical remission	13 (31.7)	28 (68.3)	41	0.012
Total	17	70	87	

*Fisher's exact test.

Discussion

In the present study, we showed that an HPV16-L1-specific antibody response is strongly associated with a clinical remission of high-risk HPV-positive early dysplastic lesions and, therefore, could be a useful tool to improve the clinical management of these women.

Only one (5.9%) HPV16-L1 antibody-positive woman out of 17 with a productive high-risk HPV-positive early dysplastic lesion progressed to histologically-confirmed CIN3 during the follow-up period of 66 months. In addition to that, only 4 (8.7%) out of 46 women with a CIN lesion during follow-up were L1 antigen and L1 antibody double-positive.

It is generally accepted that regression of HPV-associated lesions is due to a cell-mediated immune response to early, but not late HPV proteins, such as L1. Nonetheless, our data are not in contradiction to the well-established model that the cell-mediated immune response is the key driver destroying virus-infected cells.

Measurements of this cell-mediated immune response in clinical practice is not easily performed, but the detection of antibodies in patient serum is routine and the standard-of-care for most infectious agents. As shown here, this approach is indicative of an HPV-specific immune competence in patients and positive test results are associated with clinical remission.

The study of Nicholls *et al*., who provided a complete chronological picture of wart regression, supports our serological data (13). They showed for canine oral papillomavirus that a clinical remission of the lesions were due to a cell-mediated immune response that was closely-followed by seroconversion and production of antibodies to the major coat protein, L1 (14).

On the other hand, our findings regarding the clinical outcome of the L1 antibody-positive women with abnormal smears (LSIL/HSIL) are in clear contrast to the results reported by deGruijl *et al*. and Ochi *et al*. Even with a similar setting as 'prospective, non-intervention, cohort study' deGruijl *et al*. reported that seropositivity for HPV16-L1/L2 capsids was strongly-associated with an increased cervical cancer risk, but not clinical remission of early dysplastic lesions (15). Using a HPV16 neutralisation assay Ochi *et al*. reported similar findings (16). The reasons for these completely different outcomes are difficult to explain.

Our and de Gruijl *et al*.'s study protocol were nearly identical, looking at the numbers of enrolled patients (133 *vs*. 112), the cytology-based classification as mild-to-moderate dysplasia, and the follow-up times (56 months *vs*.

66 months). The study protocol of Ochi *et al*. differed slightly, recruiting 242 patients with punch biopsy-confirmed CIN1 and CIN2. This invasive procedure was avoided in both cytology-based studies because of the potential negative impact on the natural outcome of HPV infection due to wound-healing processes and 'non natural' antigen release. Follow-up time of this study was a little bit longer, with up to 84 months. In all three studies, the age of the patients enrolled ranged from 18-56 years (18-50, 19-56, 19-52 years, respectively). The mean age of our patients was 29.4 years, whereas the women were a little bit older in the other two studies, at 35.1 and 35.7 years (15, 16).

In contrast to de Gruijl *et al*. and Ochi *et al*., we used Hybrid-Capture II test for high-risk determination but not a HPV16 type-specific Polymerase Chain Reaction. This means that we are not able to rule out that non-HPV16 infections were also followed-up.

However, assuming a similar HPV-type distribution of the 87 high-risk HPV-positive dysplasias in our study as described by Delere *et al*. for a similar German collective, we would expect that about 50% (42/85) of our cases would most likely have had HPV16 infection (17). Overall, 22 cases in our study showed an HPV16-VLP antibody response, which would be 52.4% of the expected HPV16-positive cases. This positivity rate is similar to that reported by deGruijl *et al*. (55.1%), Ho *et al*. (56.7%), Carter *et al*. (59.5%) as well Ochi *et al*. (61.8%) (15-19).

Interestingly Choi *et al*. reported that 50% of HPV16-L1 capsid protein-positive dysplasias regressed within one year (10).

In addition to that, and even more conclusive due to the use of the known type-restricted HPV16-VLP serological assay, we can be quite sure that HPV16 infection was present to induce the antibodies measured in our study. This means that the different HPV-DNA detection systems used in the different studies most likely do not explain the discrepant clinical outcomes observed.

One additional difference in the studies is the characterisation of the lesions enrolled. Ochi *et al*. used biopsies confirmed of CIN1 and CIN2 lesions, whereas de Gruijl *et al*. used only cytological criteria to classify the lesions as mild to moderate dysplasia. Both were unable to differentiate the early dysplastic lesions in productive and non-productive infections. We, for the first time, added HPV-L1 capsid protein detection with the L1 biomarker, Cytoactiv. As reported previously, this allows for identification of lesions with different risk profiles for remission and progression within the heterogenic group of early dysplastic lesions.

Therefore, in contrast to de Grujil *et al*. and Ochi *et al*., we are able to link the L1 antibody response to the expression of the L1 capsid protein, as Nicholls *et al*. also did (13, 15, 16). Twenty-two (26%) patients of the study group but only two (4%) women of the control group showed an HPV16-L1-specific antibody response. This observed 6.5-fold increase of the antibody response for women with high-risk HPV-positive early dysplastic lesion is indicative of an L1-specific activation of the immune response during a productive HPV infection.

One could argue that the antibody response measured by de Gruijl *et al*. and Ochi *et al*. has to be due to the expression of the L1 capsid-protein, even the expression was not confirmed (15, 16).

Nevertheless, the detection of the serological antibody response is not indicative of when the antibodies were generated, *i.e*. during a past infection or the present ongoing infection. Ochi *et al*. for example reported that 12.5% of HPV16-DNA-negative early dysplastic lesions were positive in the HPV16 neutralisation assay, which could be indicative of HPV16 antibodies produced in the past. However, even this would only be able to explain some, but not all, of the discrepant clinical outcomes (16).

Interestingly in our study, 4 out of 5 L1 antibody-positive women within the group of L1 capsid protein-negative women showed clinical remission during the follow-up period, which might mean that even antibodies produced in the past could be indicative of clinical outcome for early dysplastic lesions.

Most likely, the different findings in the clinical outcome can be ascribed to the different serological assays used in the studies. de Gruijl *et al*. and Ochi *et al*. measured L1/L2 VLP antibody responses, whereas we used an epitope-specific, competitive HPV16-L1 VLP assay (15, 16). This means that deGruijl *et al*. and Ochi *et al*. measured antibodies against L1 and L2, whereas we in contrast measured only the L1-specific antibody response of a specific epitope.

Nevertheless, it is still not clear to us why the HPV-specific immune response measured by deGruijl *et al*. and Ochi *et al*. was not supportive in controlling the infection, but was accompanied by disease progression in almost all cases. Unfortunately they did not discuss this issue (15, 16).

We are convinced that our data is well in accord with Stanley's model (5). Papilloma viruses have developed several mechanisms to escape immune recognition. There is no viremia in natural infections, and free virus particles are shed from the surfaces of squamous epithelia, with poor access to vascular and lymphatic channels and thus to lymph nodes, where immune responses would be initiated. Nevertheless, these free viral particles, consisting of 360 L1 capsid proteins, are the only fully-accessible antigen source in the early stage of virally induced SIL. These particles are released from the apical layers of keratinocytes during the productive phase at the end of the natural viral lifecycle of the HPV infection.

To generate an effective virus-specific immune response the virus particles have to be detected by antigen-presenting

cells of squamous epithelium. We imagine that this L1 stimulus might function like an adjuvant, attracting additional immune cells to this area, which could lead to a T-cell-specific immune response against the early HPV proteins, subsequently or in parallel. Microlesions in the epithelial transformation zone could promote this process, also leading to an activation of B-lymphocytes as antibody-producing cells. A block at any stage of the activation cascade, such as no or diminished antigen release, reduced numbers of antigen-presenting cells or Major.

Histocompatibility Complex (MHC) incompatibility or deficiency, may result in an ineffective immune response, manifest as the lack of L1 antibody response, and failure to clear HPV, propagating progression of the intraepithelial lesion. In addition to the established model of a cell-mediated immune response to early proteins, such as E1 and E6, it remains to be elucidated if an L1-specific cell-mediated immune response is also of importance in the clinical remission of early dysplastic lesions. Additional studies are necessary to answer this question.

Taken together, our data emphasize the importance of the immune response in the control of HPV infections and HPV-related pre-malignant lesions. HPV-specific immune competence as evidenced by the detection of HPV16-L1-specific antibodies strongly correlates with clinical remission of early dysplastic lesions. The risk of progression to CIN3 for L1 antigen and HPV16-L1 antibody double-positive women is extremely low (about 6%) and very rarely (in 8.7%) associated with CIN during follow-up. Therefore, the rapid HPV16-L1 antibody test could be a promising tool to improve the risk assessment and appropriate clinical management of high-risk HPV-positive early dysplastic lesions when combined with the Cytoactiv® test.

Conflicts of Interest

Ralf Hilfrich works as an employee for Cytoimmun Diagnostics GmbH. The remaining authors declare no conflict of interest.

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