Research paper

DRH1 – a novel blood-based HPV tumour marker

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ARTICLE INFO

Article History:
Received 15 February 2020
Revised 15 April 2020
Accepted 5 May 2020
Available online xxx

Key Words:
HPV16
Antibodies
Tumour marker
Screening
Blood test
HNSCC

ABSTRACT

Background: To date, no studies have successfully shown that a highly specific, blood-based tumour marker to detect clinically relevant HPV-induced disease could be used for screening, monitoring therapy response or early detection of recurrence.

This study aims to assess the clinical performance of a newly developed HPV16-L1 DRH1 epitope-specific serological assay.

Methods: In a multi-centre study sera of 1486 patients (301 Head and Neck Squamous Cell Carcinoma (HNSCC) patients, 12 HIV+ anal cancer patients, 80 HIV-positive patients, 29 Gardasil-9-vaccinees, 1064 healthy controls) were tested for human HPV16-L1 DRH1 antibodies. Tumour-tissue was immunochemically stained for HPV-L1-capsidprotein-expression.

Findings: The DRH1-competitive-serological-assay showed a sensitivity of 95% (95% CI, 77.2–99.9%) for HPV16-driven HNSCC, and 90% (95% CI, 55.5–99.7%) for HPV16-induced anal cancer in HIV-positives. Overall diagnostic specificity was 99.46% for men and 99.29% for women ≥ 30 years. After vaccination, antibody level increased from average 364 ng/ml to 37,500 ng/ml.

During post-therapy-monitoring, HNSCC patients showing an antibody decrease in the range of 30–100% lived disease free over a period of up to 26 months. The increase of antibodies from 2750 to 12,000 ng/ml mirrored recurrent disease. We also show that the L1-capsidprotein is expressed in HPV16-DNA positive tumour-tissue.

Interpretation: HPV16-L1 DRH1 epitope-specific antibodies are linked to HPV16-induced malignant disease. As post-treatment biomarker, the assay allows independent post-therapy monitoring as well as early diagnosis of tumour recurrence. An AUC of 0.96 indicates high sensitivity and specificity for early detection of HPV16-induced disease.

Funding: The manufacturer provided assays free of charge.

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https://doi.org/10.1016/j.ebiom.2020.102804

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1. Introduction

Human papillomaviruses (HPV) are a large family of epitheliotropic DNA tumour viruses. In the general population, most HPV-infections cause asymptomatic infections, rather than being associated with obvious disease [1,2]. HPV16 is the most carcinogenic of 206 HPV-subtypes identified so far and accounts for up to 90% of HPV-induced cancer deaths [3].

Recent analysis of global cancer registry data showed a constant increase in the incidence of HPV-associated cancers, especially oropharyngeal squamous cell carcinoma (OPSCC) in the Western hemisphere [4–6]. In the US, the number of HPV16-induced OPSCCs has overtaken cervical cancer, highlighting the need for new diagnostic and preventive strategies [7–9]. Current HPV-related secondary prevention strategies in cervical cancer focus on the collection and characterization of suspicious cells, particularly because the area of cancer origin – the squamous columnar junction – is exactly known, limited in size and easily accessible to collect relevant cells by a smear or biopsy.

With oropharyngeal cancer, current approaches of diagnostic specimen collection reach their limits since the tumour can be multifocal, or may hide in the depth of the tonsillar crypts. As a consequence, about 70% of HPV-induced OPSCCs are detected as late-stage tumours after becoming symptomatic [10].

Currently, final diagnosis relies on identifying morphological changes to determine the grade of disease and the detection of HPV nucleic acid. Even though HPV-DNA detection is highly sensitive for viral DNA, it is not necessarily a proof for HPV-driven disease. Therefore HPV-DNA detection is usually combined with more specific assays such as p16, HPV E6/E7-mRNA or miRNA-detection [11,12]. The development of blood-based assays to detect clinically relevant HPV-induced disease like precancerous lesions and tumours has been hampered by various factors: HPV-infection doesn’t necessarily lead to disease and there was no evidence that serum antibodies could discriminate HPV-driven disease from subclinical HPV-infection.

HPV-subtypes are closely related, with DNA sequence homologies of up to 90%. Consequently, all viral proteins, the early (E1–E7) and the late ones (L1, L2), share subtype-specific, as well as functionally highly conserved and broadly cross-reactive adjacent epitopes [1].

Classical serological assays are unable to reliably differentiate between the serological responses to adjacent epitopes [13]. Nevertheless, a high sensitivity and specificity was reported for HPV16 E6 antibody detection for HNSCC and anal cancer, but not for other gynaecological tumours. The clinical significance of such antibodies for early detection is still under debate, since these antibodies are most often detectable several decades before OPSCC diagnosis [14–17]. Subtype-specific conformational epitopes are known to be located on the outer surface of virus-like-particles (VLP) consisting of the HPV-L1-capsidprotein. Prophylactic HPV-vaccines are therefore unable to induce broadly cross-reactive protection against subsequent HPV-infections [18].

Previous attempts to develop a clinically relevant subtype-specific serological assay by utilizing these VLPs as antigen failed because a specificity of up to 95% was not sufficiently high enough for clinical use [19].

In addition, it has been reported that HPV-L1-related antibody levels are mostly stable over time, correlate with the number of sexual partners, but not with disease, with no observable change in antibody levels, even after treatment [20,21].

It has been suggested that HPV-L1-seropositivity reflects lifetime exposure rather than acute disease [13]. Therefore, L1 serum antibodies measured with traditional assays have been classified as a marker of infection, unsuitable for serological tumour diagnostics.

This study reports the performance and results using an HPV16 subtype-specific competitive serological assay, based on the HPV16-L1-specific monoclonal antibody clone DRH1.

2. Material and methods

In a multi-centre study, blood sera of collectively 1486 patients were gathered and analysed to assess the serological HPV16-L1-specific antibody status in different patient groups using a competitive DRH1 epitope-specific rapid test (PrevoCheck, Abviris, Germany). Seven institutions in Austria and Germany provided serum samples of different cancer patients, HIV positive patients as well as healthy controls to evaluate the presence of HPV16-L1-specific antibodies.
either in a qualitative or in a quantitative approach. Fig. 1 shows a REMARK-diagram. All serum samples were collected using standardised collection devices and stored at −20 °C to 80 °C. This was carried out in a prospective (pre- and post-treatment analysis) or retrospective manner further elucidated as follows.

2.1. Longitudinal DRH1 serum levels in HNSCC patients in Graz

34 patients with histologically confirmed and previously untreated head and neck cancer (ICD-10-Codes C01.9, C02.4, C05.1, C09 and C10, C80) treated at the Department of Otorhinolaryngology - Head and Neck Surgery in Graz were included in the study. 31 patients suffered from OPSCC, in two patients CUP-syndrome was diagnosed. One patient originally suspected to suffer from CUP-syndrome had to be excluded because histology revealed malignant melanoma. Serum samples were collected prior to treatment and in intervals of 3–6 months between September 2016 and November 2018 during clinical follow up, which was delivered in accordance with national guidelines, including visual inspection, palpation, ultrasound and imaging (MRT/CT) if indicated.

Furthermore, HPV-DNA analysis of tumour specimen was carried out using the 3.5 LCD-Array Kit (Chipron, Berlin, Germany) according to manufacturer’s protocol and immunohistochemistry for p16ink4a (Ventana Roche Diagnostics, Basel, Swiss) was performed.

2.2. Cross sectional serum analysis in patients with tumour of the oral cavity in Halle and Mainz

176 patients from Halle and 91 patients from Mainz treated because of cancer of the oral cavity (ICD C02-C06 and C14) at the local Department for Oral and Maxillofacial Surgery were included in the study. Serum samples were collected prior to treatment. Again, HPV-DNA analysis and immunohistochemistry for p16 were carried out.

2.3. Pre- and post-treatment serum analysis of HIV positive anal cancer patients in Bochum

Pre- and post-treatment serum samples chosen for 12 male HIV-positive anal cancer patients (ICD-10-CM C210) in Bochum were analysed, which belong to the bio- and data-bank of the HIV patient cohort of the German Competence Network for HIV/AIDS (KompNet HIV/AIDS) [22–24].

2.4. Serum analysis of a high-risk population: randomly selected HIV positive patients in Munich

From the HIV outpatient clinic of the Department of Dermatology and Allergy in Munich, serum samples of 80 randomly selected HIV-positive patients with no history of HPV induced tumours were analysed.

2.5. The vaccine-study in Berlin to assess analytical specificity

The vaccine study with Gardasil-9 was carried out in the Clinic for Gynaecology, Gynaecological Tumour Immunology, Charité in Berlin. Sera of 29 female patients were collected immediately before the first immunization with Gardasil-9 (MSD, Kenilworth, USA) was applied. Post-vaccination serum samples were collected 3–6 weeks after the third immunization.

2.6. Diagnostic specificity in a healthy control-group in Ingelheim

The control group, consisting of 1064 randomly selected serum samples of healthy patients with C-Reactive-Protein (CRP) negative test results, were kindly provided by Bioscientia Laboratories (Ingelheim, Germany).

2.7. Competitive serological detection of human antibodies to HPV16-L1 DRH1 epitope

Serological detection of HPV16-L1-specific antibodies was carried out using a competitive DRH1 epitope-specific rapid test (Prevo-Check, Abirirs, Germany), according to the manufacturer’s instruction. In Graz, Bochum, Berlin and Ingelheim, 5 μl serum was used for antibody quantification. After pre-incubation of the serum with an HPV16-L1-specific reagent for 5 min the mixture was transferred onto a lateral flow test cassette. 15 min later the test result was measured using an EseQuant-reader (QIAGEN, Germany). 25 μl of serum
respectively 40 µl of whole blood were used for qualitative analysis (Mainz, Halle, Munich). The purified mouse monoclonal antibody DRH1, World Health Organization (WHO) reference sera for HPV16 and HPV18 served as standards.

2.8. Immunohistochemistry for detection of L1-capsidprotein in tumour cells

For immunohistochemistry, sections were deparaffinized and stained with a monoclonal anti-HPV L1 antibody (cytoactiv, Cytoimmun Diagnostics, Pirmasens, Germany) using immunoperoxidase techniques according to manufacturer’s protocol. For HPV-L1 detection, a modified staining protocol was used. In brief, after antigen retrieval for 20 min in citric buffer, the sections were incubated with the primary antibody, the detection reagent and the chromogen for 1 h each, changing the chromogen every 15 min. The sections were thoroughly washed after each step, counterstained with haematoxylin and cover-slipped.

2.9. Statistical analysis

Sensitivity, specificity, positive and negative predictive -values (PPV, NPV), receiver–operating-characteristic (ROC) -curve-analysis and area-under-the-curve (AUC), as well as confidence intervals, Liu-, Youden–Index and ‘closest-to-(0, 1) criterion’ were calculated externally (p-wert, Jena, Germany).

2.10. Ethic statement

The study was approved by the IRBs responsible for the participating institutes. Written informed consent was obtained from all participants prior to enrolment.

2.11. Data availability

Raw experimental data associated with the figures presented in the manuscript are available from the corresponding author upon reasonable request.

3. Results

3.1. Prospective study design in Graz

34 tumour patients mainly suffering from OPSCC were recruited for this prospective non-interventional study at the Department of Otorhinolaryngology - Head and Neck Surgery in Graz, two of them with CUP-syndrome (one HPV-DNA negative, the other one HPV-DNA positive). In one patient originally suspected to be a CUP-syndrome histology revealed malignant melanoma. He therefore was excluded.

The mean age of all 34 patients was 63.7 years (range 47–83y). The 26 men with a mean age of 63.2 years (range 47–83 years) were slightly younger than the 8 women (65 years (range 49–77 years). The detailed characteristics of all the patients are shown in Table 1.

20 out of 34 patients were HPV16-DNA- and p16-positive. 19 out of these 20 patients showed an antibody level above the cut-off-value of 1000 ng/ml (sensitivity 95%, 95% CI, 772–999%, PPV 45.6%, NPV 99.9%). The antibody level was not associated with tumour localisation or AJCC-classification. An antibody decrease during follow up in the range of 30–100% was associated with disease-free survival (Fig. 2a). An antibody increase during follow up was observed for three patients in the range of 76–436%, which was associated in one case with recurrent disease in the form of distant lung metastasis (Fig. 2b). Stable low antibody levels were observed in two HPV33-positive and two HPV-negative tumour patients, which indicate the HPV16-subtype specificity of the assay (Fig. 2c). Fig. 2d gives an overview of all 34 graphs over the follow up period of 26 month according to Table 1.

3.2. Pre-treatment serum analysis in Halle and Mainz

To identify a subset of HPV16-driven tumours, a cohort of 267 oral cancer patients was tested in Halle and Mainz. The average age of Halle patients was 61.3 (range 30–90 years). The 55 women [mean age 67.4 (range 46–87 years)] were significantly older than the 121 men [mean age 58.5 (range 30–90 years)]. In Mainz, the average age was 67.2 (range 39–93 years). The 60 men with a mean age of 65.9 (range 39–92 years) were slightly younger than the 31 women (mean age 69.7 [range 47–93 years]).

In total, 12 positive results (seven men, five women) were obtained. The DRH1 positivity rate was 4.5% (Halle) respectively 4.4% (Mainz). All these cases could be histologically confirmed as HPV16 and p16 positive tumours indicating the high clinical specificity of DRH1-equivalent testing as indicator for HPV16-driven tumours (Table 3).

3.3. Pre- and Post-treatment serum analysis in Bochum

12 male HIV-positive anal cancer patients had an average age of 45 (range 27–63 years) at the time of diagnosis. The mean time of HIV-infection was 10.2 (range 5 – 19 years).

Within the year prior to tumour diagnosis, 9 out of 10 pre-treatment sera of anal cancer patients showed positive antibody levels of 1000 to 3000 ng/ml (sensitivity 90%, 95% CI, 55.5–99.7%). The earliest detected positive result was received 293 days ahead of tumour diagnosis. The remaining two pre-treatment sera collected 516 and 578 days before tumour diagnosis were antibody negative, indicating a correlation between antibody detection and active tumour development.

During follow up, a decrease of antibody levels ranged from 25 to 60% was observed within 89 days after tumour diagnosis. In one case, post-treatment antibody levels increasing by 30% were associated with recurrence of disease.

3.4. Serum analysis of randomly selected HIV positive patients in Munich

12 (15%) out of 80 HIV-positive patients from the LMU outpatient clinic were tested positive. This was 30 times higher than in the regular German population (Table 3). The mean age was 51.9 (range 23–79 years) with 48.7 (range 28–66 years) for seven women and 52.1 (range 23–79 years) for 73 men.

3.5. The vaccine-study in Berlin

To assess analytical specificity, pre- and post-vaccination sera of 29 women with an average age of 27.7 (range 20–41 years) were recruited. The pre-vaccination sera were collected immediately before the first immunization. Post-vaccination sera were collected 3–6 weeks after the third (Table 4).

All 29 women showed higher antibody concentrations after the third immunization. The average pre-immune antibody level of 364 ng/ml (range 0–2900 ng/ml) increased due to the vaccination by more than 100-fold to 37,500 ng/ml (range 3000–237,500 ng/ml). The lowest increase ranged from 575 to 3500 ng/ml to the highest from 0 to 237,500 ng/ml.

3.6. The control-group in ingelheim

1064 healthy control group samples were split into three age groups (Table 2). Within the 559 men (mean age 48.1 [range 1–93
Table 1
Baseline characteristics of 34 patients with head and neck cancer (Graz).

| Patient ID | Gender | Age at diagnosis in years | Smoker* / Alcohol** | Localisation | AJCC | HPV DNA | p16 | Therapy*** | Serological Status | Antibody Conc. Decrease | Increase | Serum | Follow up in month | Surveillance Disease free | Death |
|------------|--------|---------------------------|---------------------|--------------|------|---------|------|-----------|-------------------|------------------------|----------|-------|-------------------|------------------------|-------|
| 5          | W      | 75                        | no / no             | Tonsil IV    | HPV16 positive | S+RCT | positive | 28,000 | lost       | lost              | 1         | 0     | yes no             | 16 yes no               | no    |
| 24         | M      | 62                        | yes / yes           | Tonsil III   | HPV16 positive | RCT   | positive | 22,500 | 0         | -60%              | 0         | 3     | 16 yes no          | 16 yes no               | no    |
| 19         | M      | 67                        | no / no             | Tonsil I     | HPV16 positive | S+RT  | positive | 11,100 | 0         | -90%              | 0         | 7     | 16 yes no          | 16 yes no               | no    |
| 28         | M      | 79                        | no / no             | Base of Tongue III | HPV16 positive | RT    | positive | 9000   | 0         | -28%              | 0         | 6     | 15 yes no          | 15 yes no               | no    |
| 10         | M      | 57                        | no / no             | Tonsil II    | HPV16 positive | S+RCT | positive | 8400   | 0         | -63%              | 0         | 3     | 2 yes no           | 2 yes no                | no    |
| 34         | M      | 47                        | no / no             | CUP III      | HPV16 positive | S+RCT | positive | 5800   | 0         | -100%             | 0         | 8     | 20 yes no          | 20 yes no               | no    |
| 12         | M      | 81                        | no / no             | Base of Tongue II | HPV16 positive | RCT   | positive | 5000   | 0         | -35%              | 0         | 3     | 1 yes no           | 1 yes no                | no    |
| 15         | M      | 57                        | no / no             | Tonsil II    | HPV16 positive | RCT   | positive | 4800   | 0         | -43%              | 436%       | 7     | 19 no no           | no no no                | no    |
| 27         | M      | 62                        | no / no             | Tonsil I     | HPV16 positive | S    | positive | 2300   | 0         | -76%              | 0         | 7     | 16 yes no          | 16 yes no               | no    |
| 4          | W      | 66                        | no / no             | Tonsil II    | HPV16 positive | S+RCT | positive | 1900   | 0         | -74%              | 0         | 7     | 17 yes no          | 17 yes no               | no    |
| 8          | W      | 65                        | yes / no            | Tonsil II    | HPV16 positive | RCT   | positive | 1740   | 0         | -71%              | 0         | 7     | 19 yes no          | 19 yes no               | no    |
| 16         | M      | 56                        | no / no             | Tonsil III   | HPV16 positive | RCT   | positive | 1455   | 0         | -100%             | 0         | 3     | 2 yes no           | 2 yes no                | no    |
| 29         | M      | 65                        | yes / no            | Tonsil III   | HPV16 positive | RCT   | positive | 1425   | 0         | -60%              | 0         | 2     | 13 yes no          | 13 yes no               | no    |
| 25         | M      | 70                        | yes / yes           | Tonsil II    | HPV16 positive | RT    | positive | 1300   | 0         | -100%             | 0         | 5     | 16 yes no          | 16 yes no               | no    |
| 3          | M      | 83                        | no / no             | Tonsil I     | HPV16 positive | S    | positive | 1285   | 0         | -53%              | 0         | 5     | 18 yes no          | 18 yes no               | no    |
| 20         | W      | 77                        | no / no             | Tonsil I     | HPV16 positive | RIT   | positive | 1250   | 0         | -100%             | 0         | 5     | 14 yes no          | 14 yes no               | no    |
| 21         | W      | 58                        | yes / yes           | Base of Tongue IV C | negative negative | RCT   | positive | 1250   | 0         | -100%             | 0         | 3     | 4 no yes           | yes yes yes              | yes   |
| 11         | W      | 49                        | yes / yes           | Base of Tongue II | HPV16 positive | S+RCT | positive | 1184   | 0         | -54%              | 0         | 6     | 16 yes no          | 16 yes no               | no    |
| 14         | M      | 64                        | yes / no            | Tonsil III   | HPV16 positive | RCT   | positive | 1120   | 0%        | 148%              | 6         | 15    | yes no             | yes yes yes              | yes   |
| 1          | M      | 81                        | no / no             | Tonsil II    | HPV16 positive | RCT   | positive | 1070   | 0%        | 78%               | 4         | 11    | yes no             | yes yes yes              | no    |
| 22         | M      | 52                        | yes / yes           | Base of Tongue IV A | negative negative | RCT   | positive | 1000   | 0         | -100%             | 0         | 3     | 6 yes no           | yes yes yes              | no    |
| 7          | M      | 72                        | no / no             | Tonsil III   | negative positive | RT   | negative | 965    | -100%    | 0                  | 7         | 15    | yes no             | yes no yes               | no    |
| 26         | M      | 51                        | yes / yes           | Soft Palate IV A | negative negative | RCT   | negative | 820    | -50%    | 0                  | 6         | 10    | 0 no no            | no no no                | no    |
| 17         | M      | 58                        | no / no             | Tonsil III   | negative negative | RCT   | negative | 710    | -100%    | 0                  | 6         | 16    | yes no             | yes no yes               | no    |
| 31         | M      | 67                        | no / no             | Base of Tongue III | negative negative | RCT   | negative | 700    | lost     | lost               | 1         | 0     | lost lost          | lost lost               | yes   |
| 32         | M      | 56                        | yes / yes           | CUP IV A     | negative negative | S+RCT | negative | 700    | stable   | stable             | 2         | 2     | yes yes            | yes yes yes              | no    |
| 9          | M      | 72                        | yes / no            | Base of Tongue II | HPV33 positive | RCT   | negative | 630    | stable   | stable             | 6         | 16    | yes no             | yes yes yes              | no    |
| 30         | W      | 68                        | yes / yes           | Base of Tongue IV A | negative negative | RCT   | negative | 600    | lost     | lost               | 1         | 0     | yes no             | yes no yes               | yes   |
| 23         | M      | 59                        | yes / yes           | Tonsil IV A  | negative negative | S+RCT | negative | 400    | stable   | stable             | 3         | 6     | yes no             | yes no yes               | no    |
| 6          | M      | 63                        | yes / yes           | Tonsil II    | HPV23 positive | CT+RCT | negative | 56     | stable   | stable             | 7         | 19    | yes no             | yes no yes               | no    |
| 2          | M      | 55                        | yes / no            | Tonsil II    | HPV16 positive | S+RCT | negative | 0      | stable   | stable             | 7         | 26    | yes no             | yes no yes               | no    |
| 18         | M      | 52                        | lost                | lost lost    | lost lost     | lost lost | negative | 0      | lost     | lost               | 1         | 0     | lost lost          | lost lost               | yes   |
| 33         | M      | 59                        | yes / no            | Base of Tongue III | negative negative | S+RT  | negative | 0      | stable   | stable             | 6         | 10    | yes no             | yes yes no              | no    |

Description of 34 tumour patients, 31 of them with OPSCC, 2 with CUP syndrome and one drop out, showing baseline characteristics, tumour description containing HPV DNA and p16 status from the tumour specimen and serological HPV16 L1 antibody status.

* ≥ 20 pack years tobacco use.

** regular alcohol consumption.

*** S = Surgery, RT = Radiotherapy, CT = Chemotherapy, RCT = Radiochemotherapy, RIT = Radioimmunotherapy.
years], three positive results (0.54%) were obtained with an antibody level above 1000 ng/ml, resulting in an overall specificity of 99.46%.

Within the 505 women (mean age 49.4 [range 19–92 years]), 22 positive results were obtained. Three positive results were obtained from the 424 women aged 30 and older. This resulted in a specificity of 99.29%. Receiver-Operating-Characteristic (ROC) curve analysis, with an area under the curve of 0.96 (95% CI, 0.91–1), was calculated for 20 HPV16 driven OPSCCs and 1064 controls (Fig. 3).

3.7. Immunohistochemical detection of the HPV-L1-capsidprotein in tumour tissue

As shown in Fig. 4, the L1-capsidprotein seems to be heterogeneous expressed within different HPV16-DNA-positive and p16-positive tumours. Fig. 4 shows a tumour where about half of the cells, equally distributed throughout the whole tumour, are stained. Fig. 4 shows a tumour with an inner border-like structure where one half...
of the tumour is homogenously stained, whereas the other shows no staining at all.

4. Discussion

For the first time, we can report that an HPV-related antibody is indicative of the course of disease in patients with HPV16-induced oropharyngeal cancer, raising the test’s potential as an independent, post-treatment biomarker. Showing excellent results concerning sensitivity and specificity in a broad range of different study populations, the test may also serve as a reliable screening tool in a secondary preventive approach. Furthermore, using a modified IHC-method, we have been able to show for the first time that tumour cells are also capable of producing L1-capsidprotein, although at levels much lower than known from precancerous lesions. HPV16-induced oropharyngeal cancer, especially in the USA, has already surpassed the incidence of traditional anogenital cancers like cervical, anal, vaginal or vulvar cancer [7].

If this virus-related cancer epidemic is to be addressed, novel prevention strategies are needed to overcome the limitations of currently used cell-based screening systems. Our focus has been on the HPV-L1-capsidprotein and the related antibody response in clinical as well as preventative settings. Until now, the HPV-L1-capsidprotein and especially its related antibody response have not been considered a suitable target for the early detection of HPV-related tumours. The idea that the L1-capsidprotein itself could be a new target for oncology was hampered by two main contradictions: the view that the expression of the L1-capsidprotein is restricted to terminally differentiated cells and cannot take place in tumour cells and that the L1-related antibody response reflects life time exposure to HPV rather than acute disease. This paper challenges that belief. Several studies have shown that traditional HPV-L1-based ELISAs are sensitive but not specific enough.

Assays using bacterially expressed Glutathion-S-Transferase (GST)–L1 fusion proteins hide the issue that the antigen presents cross-reactive and subtype-specific epitopes next to each other. Therefore, this approach lacks the capacity to discriminate the different HPV-subtypes reliably. The specificity of such assays is typically in the range of 70% [13]. Assays using virus-like particles (VLP) as antigen show with up to 95% a much higher specificity, because most cross-reactive epitopes are hidden inside the VLP, and are not accessible to the related cross-reactive antibodies [25,26]. These traditional HPV-L1 ELISAs are useful in assessing cumulative lifetime exposure to HPV, but they cannot indicate acute HPV-induced disease [27].

Within our study, the newly developed HPV16-L1 assay showed a sensitivity of 95% for HPV16-driven oropharyngeal and 90% for HPV16-induced anal cancer within HIV-positive patients. Overall diagnostic specificity, using the cut off level of 1000 ng/ml, was 99.46% for men and 99.29% for women age 30 and over. With the Youden-, Liu-Index and 'closest-to-(0,1) criterion' three different calculations determined the cut-off point. The area under the curve was calculated with 0.96 (95% confidence interval 0.91–1).

We noted a remarkable decrease in serum antibodies after removal of the antigen expressing tumour cells. Levels were patient specific and not linked to special TNM-characteristics. Although the numbers of cases studied were limited, all patients showing an antibody decrease were alive and disease-free over a period of up to 26 months. Even more interesting were patients showing an increase in antibody levels after apparently successful treatment. The rare observation of an immediate antibody increase after treatment did trigger clinical alarm, indicating that an on-going release of antigen by hidden tumour cells was taking place, with continued stimulation of antibody production and thus rising antibody levels. Three cases with such an increase in antibody levels were observed. The more than four-fold increase in the antibody level from 2750 to 12,000 ng/ml within ten months was accompanied by recurrent disease, a distant lung metastasis. Even after one year, recurrent disease had not been diagnosed clinically in the two other patients. However, a 78%–rise from 1070 to 1900 ng/ml and a 148%-rise from 1120 to 2775 ng/ml (see Table 1: Patient 1 and 14) respectively may indicate slowly progressing recurrent disease and these patients are under careful observation.

Fig. 2. a–d. Characteristic antibody graphs during follow up.

Fig. 2c: Characteristic graph in a patient with an HPV33 associated OPSCC showing HPV16 L1 antibody concentration at a constant low level indicating the type-specificity of the assay.

Patient characteristics of patient 9 as described in Table 1:

Male, 72 years old, base of tongue carcinoma, HPV33 DNA positive, p16 positive

Therapy: Radiochemotherapy

![Graph showing HPV16 L1 AB-Concentration](image-url)
Fig. 2d: Overview of antibody concentrations of all HNSCC patients from Graz during follow up, baseline characteristics can be seen in Table 1. Green curves: HPV16-L1 immunoassay positive, Orange curves: HPV16-L1 immunoassay negative, Red curves: Increasing antibody titers in three HPV16-L1 immunoassay positive patients.
In our study, the assay had the potential to indicate disease recurrence much earlier than current clinical practice. In turn, this might justify earlier treatment in the future before tumour recurrence is revealed macroscopically.

Similar results were found within the HIV-positive anal cancer patients. A positive DRH1 result was measured 293 days before clinical tumour diagnosis. In addition, at 99.4% the specificity of the assay in 895 apparently healthy individuals 30 years and older was extremely high, further

| Table 2 |
| --- |
| Diagnostic specificity within healthy CRP negative blood donors. |
| **female** | DRH1 negative 0 ng/ml | DRH1 positive 1 – 999 ng/ml | DRH1 positive ≥ 1000 ng/ml | Spec. in % |
| 0 – 19 years (in %) | 19 (51.4) | 8 (21.6) | 10 (27.0) | 37 (73.0) | 100 |
| mean in ng/ml | 0 | 306 | 7345 | 2051 |
| 20 – 29 years (in %) | 25 (56.8) | 10 (22.7) | 9 (20.5) | 44 (79.5) | 100 |
| mean in ng/ml | 0 | 515 | 3892 | 913 |
| 30 y and older (in %) | 348 (82.1) | 73 (17.2) | 3 (0.71) | 424 (99.3) | 100 |
| mean in ng/ml in total | 0 | 220 | 1510 | 49 |
| (in %) | 392 (81.6) | 91 (18.4) | 22 (4.4) | 505 (95.6) | 100 |
| mean in ng/ml | 0 | 260 | 5136 | 270 |

| **male** | DRH1 negative 0 ng/ml | DRH1 positive 1 – 999 ng/ml | DRH1 positive ≥ 1000 ng/ml | Spec. in % |
| 0 – 19 years (in %) | 31 (91.2) | 3 (8.8) | 0 (0) | 34 (100) |
| mean in ng/ml | 0 | 217 | – | 19 |
| 20 – 29 years (in %) | 45 (83.3) | 9 (16.7) | 0 (0) | 54 (100) |
| mean in ng/ml | 0 | 128 | – | 21 |
| 30 y and older (in %) | 405 (86.0) | 63 (13.4) | 0 (0) | 471 (100) |
| mean in ng/ml in total | 0 | 168 | 6566 | 64 |
| (in %) | 481 (87.6) | 75 (14.0) | 3 (0.5) | 559 (100) |
| mean in ng/ml | 0 | 165 | 6566 | 57 |

In our study, the assay had the potential to indicate disease recurrence much earlier than current clinical practice. In turn, this might justify earlier treatment in the future before tumour recurrence is revealed macroscopically.

Similar results were found within the HIV-positive anal cancer patients. A positive DRH1 result was measured 293 days before clinical tumour diagnosis.

In addition, at 99.4% the specificity of the assay in 895 apparently healthy individuals 30 years and older was extremely high, further

| Table 3 |
| --- |
| DRH1 test results in different risk groups. |
| **Total** | Mean age in years | Range in years | DRH1 Positive (in %) | DRH1 Negative (in %) |
| **Munich: HIV patients** | | | | |
| Total | 80 | 51.9 | 23 – 79 | 12 (15.0) | 68 (85.0) |
| men | 73 | 52.1 | 23 – 79 | 12 (16.4) | 61 (83.6) |
| women | 7 | 48.7 | 28 – 66 | 0 (0) | 7 (100) |
| **Halle: Oral cancer patients** | | | | |
| Total | 176 | 61.3 | 30 – 90 | 8 (45) | 168 (95.5) |
| men | 121 | 58.5 | 30 – 90 | 4 (33) | 117 (96.7) |
| women | 55 | 67.4 | 46 – 87 | 4 (7.3) | 51 (92.7) |
| **Mainz: Oral cancer patients** | | | | |
| Total | 91 | 67.2 | 39 – 93 | 4 (4.4) | 87 (95.6) |
| men | 60 | 65.9 | 39 – 92 | 3 (5) | 57 (95) |
| women | 31 | 69.7 | 47 – 93 | 1 (3.2) | 30 (96.8) |

| Table 4 |
| --- |
| DRH1 pre- and post-immun test results of Gardasil 9 vaccinees. |
| **Seronegative 0 ng/ml** | **Seropositive 1 – 999 ng/ml** | **Seropositive ≥ 1000 ng/ml** | **in total** |
| **Seronegative 0 ng/ml** | **Seropositive 1 – 999 ng/ml** | **Seropositive ≥ 1000 ng/ml** | **in total** |
| **Pre-immun** | n (%) | n (%) | n (%) | n (%) |
| 20 – 29 years | 8 (40) | 9 (45) | 3 (15) | 20 (100) |
| mean (ng/ml) | 0 | 382 | 2300 | 364 |
| ≥ 30 years | 6 (666) | 3 (333) | 0 | 127 – 906 |
| mean (ng/ml) | 0 | 75 | 0 | 25 – 0 – 201 |
| in total | 14 (483) | 12 (414) | 3 (103) | 29 (100) |
| mean (ng/ml) | 0 | 305 | 2300 | 364 |
| **Post-immun** | n (%) | n (%) | n (%) | n (%) |
| 20 – 29 years | 0 | 0 | 20 (100) | 20 (100) |
| mean (ng/ml) | 0 | 0 | 42.470 | 42.470 |
| ≥ 30 years | 0 | 0 | 9 (100) | 9 (100) |
| mean (ng/ml) | 0 | 0 | 26.456 | 26.456 |
| in total | 0 | 0 | 29 (100) | 29 (100) |
| mean (ng/ml) | 0 | 0 | 37.500 | 37.500 |
Fig. 4: HPV16-L1 capsid protein expression in tumour cells

As shown in pictures a - d, about half of the tumour cells show a nuclear staining in red colour for the L1 capsid protein. L1 expression was confirmed by Western Blot analysis. Magnification: 100x (a), 200x (b, c), 400x (d)

Pictures e and f show an ‘inner border like’ staining. This means different clusters of tumour cells next to each other being L1 capsid protein positive (on the right) and L1 negative (on the left). Picture f shows a higher magnification of the area marked by a white circle. Magnification: 400x (e) and 1000x (f).

The dot like staining of the nuclei (black arrows) was always associated with a sporadic (g) or an ‘inner border like’ (h) staining. Like shown here, the inner border like staining showed a sharp border between the L1 positive and the L1 negative cells. Magnification: 1000x each.
emphasizing the correlation of the human-DRH1-antibody equivalents to acute HPV16-induced disease rather than HPV16-infection.

That L1-related antibodies would be suitable as a tumour marker for HPV16-induced disease, especially for oropharyngeal and anal cancer, was very surprising. The expression of HPV-specific oncoproteins E6 and E7 has been shown to be a prerequisite for the development of tumour cells. In accordance, a high sensitivity and specificity was reported for HPV16 driven HNSCC as well as for anal cancer by the detection of anti E6-antibodies. Still under debate is the clinical utility of these antibodies for screening purposes since they are most often detectable several decades before OPSCC diagnosis [14–17].

However, the L1-capsidprotein was believed to be produced only at the end of the viral life cycle, supporting the encapsulation of the replicated viral DNA and the assembly of new, infectious virus particles [2]. In that context, it was shown for precancerous lesions of the cervix uteri that L1-expression is restricted to fully differentiated superficial cells, whereas latent respectively subclinical HPV-infections do not express L1-capsidprotein [28–31]. Since the ability to differentiate from an intermediate into a superficial cell gets lost with increasing severity of the precancerous lesion, L1-capsidprotein expression rate is high in low-grade (CIN1) and declines to a small subset of about 15% of the high-grade lesions (CIN3) [31]. Concordantly the L1-capsidprotein could not be detected in tumour cells, using the traditional immunohistochemical (IHC) or Western-blot staining protocols. Looking back, data supporting an active involvement of the L1-capsidprotein during tumour genesis are rare but striking. In 2009, Bellone [32] and Schmitt [33] reported surprisingly that L1-mRNA-expression in 29 HPV16-positive tumour cases was the rule rather than the exception. They demonstrated a higher L1-mRNA-expression-level than for E6. They concluded that a translational control mechanism may exist in tumour cells to block L1-capsidprotein-expression. That tumour cells are not only capable of producing the L1-mRNA but the L1-protein itself was shown since HPV16-L1-mRNA positive C3 tumour cells could be eliminated by L1-specific CD8+ cytotoxic T-cells [34].

Overall we provide promising findings, especially when compared to the most effective cancer screening test so far, the Pap-smear, which has significantly reduced the incidence of HPV-induced cervical cancer, even with less impressive key data of about 50% sensitivity and 98% specificity.

Our data show that the epitope-specific assay, compared with the traditional ELISAs, differs in results and clinical usability. This new competitive, epitope-specific rapid test detects HPV16-L1 DRH1-related antibodies, which are linked to acute disease, and therefore the assay is a promising tool for the clinical oncologist as post-treatment biomarker as well as for secondary prevention purposes.

Declaration of Competing Interest

All participating authors hereby disclose any financial and personal relationships with other people or organisations that could have inappropriately influenced the current study.

Acknowledgements

I would like to thank Dr. Ralf Hilfrich, Co-Founder and technical Head of the Alviris Company for providing assays free of charge and thereby supporting the realization of the study. Furthermore I would like to thank Prof. Thurnher for his support and all the participating co-authors, especially from the institutions abroad for sharing their data and knowledge.

Funding sources

The manufacturer provided assays free of charge. The Competence Network for HIV/AIDS was supported by the Federal Ministry of Education and Research (grant no. BMBF-01 KI 0501). No additional funding was provided. The funders did not have any role in study design, data collection/analysis, interpretation or writing.

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