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Research paper

DRH1 – a novel blood-based HPV tumour marker

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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.
Analytical specificity was determined using WHO reference-sera for HPV16/18 and 29 pre- and post-immune sera of Gardasil-9-vaccinees.
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 can 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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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].

Research in context

Evidence before the study

Even with the vaccination era rising Human papillomavirus (HPV)-induced malignancies remain a global health burden, with an estimated seven billion unprotected people at risk and about 400,000 cases of death annually.

Currently used cell-based HPV-related diagnostic procedures and secondary prevention strategies, including the molecular detection of HPV, have proved to be of limited value when the area of cancer origin is hard to access, unknown, or unidentifiable such as very early distant metastasis.

The challenge has been to find an easy to use, blood-based assay as a primary screening tool or as a post-treatment biomarker. However, this has been unattainable without evidence that an antibody is able to discriminate between subclinical HPV-infection and HPV-induced disease.

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. This 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.

Added value

We describe the first blood-based HPV16-specific tumour marker assay by detecting serological response to the DRH1 monoclonal antibody cognate epitope of the viral antigen L1. Human DRH1-equivalent antibodies are related to HPV-induced tumours and predict the course of disease.

In a prospective pilot study, tumour patients tested positive for the DRH1 antibody up to 293 days before their tumour diagnosis had been confirmed. Specificity in the 1064 healthy controls reached 99·4%, with sensitivity up to 95%, and the area under the curve values were calculated with 0·96.

While disease-free survival in HNSCC patients was reflected in decreased antibody levels during follow up, post-treatment increase of DRH1 antibodies was shown to mirror disease recurrence six months earlier than by existing diagnostic methods.

Implications of all available evidence

An independent, blood based tumour marker would facilitate the post-treatment surveillance of HPV-related tumour patients. Early detection of those with recurrent or metastatic disease could enable early intervention using systemic treatment options in the future.

In addition new easy to use secondary preventive approaches may enable to screen for HPV-induced disease in the head and neck as well as the anogenital area.

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

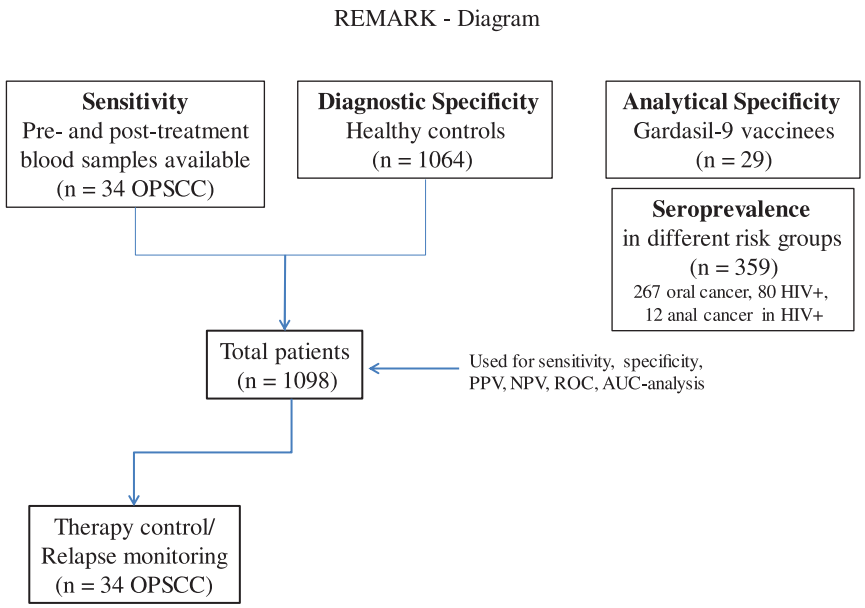

Fig. 1. Remark Diagram of study population and patient subsets used for calculation of sensitivity, specificity, positive predictive value, negative predictive value, ROC- and Area under the curve calculation.

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/ −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 $p16^{ink4a}$ (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 C21·0) 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, Abviris, 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 Tables 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, 77·2–99·9%, 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 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 Characteristics				Tumour Characteristics					Serological Status						Surveillance	
Patient ID	Gender	Age at diagnosis	Smoker* /Alcohol**	Localisation	AJCC	HPV DNA	p16	Therapy***	positive/negative	Antibody Conc.	Decrease	Increase	Serum	Follow up	Disease free	Death
	M/W	in years	yes / no							in ng/ml			Samples	in month		
5	W	75	no/no	Tonsil	IV	HPV16	positive	*S*+RCT	positive	28,000	lost	lost	1	0	yes	no
24	M	62	yes/yes	Tonsil	III	HPV16	positive	RCT	positive	22,500	−60%	0	3	16	yes	no
19	M	67	no/no	Tonsil	I	HPV16	positive	*S*+RT	positive	11,100	−90%	0	7	16	yes	no
13	M	79	no/no	Base of Tongue	III	HPV16	positive	RT	positive	9000	−28%	0	6	15	yes	no
28	M	62	yes/yes	Tonsil	II	HPV16	positive	RCT	positive	8400	−63%	0	3	2	yes	no
10	M	57	no/no	Tonsil	II	HPV16	positive	*S*+RCT	positive	5800	−100%	0	8	20	yes	no
34	M	47	no/no	CUP	III	HPV16	positive	*S*+RT	positive	5000	−35%	0	3	1	yes	no
12	M	81	no/no	Base of Tongue	II	HPV16	positive	RIT	positive	4800	−43%	436%	7	19	no	no
15	M	57	no/no	Tonsil	II	HPV16	positive	RCT	positive	4000	−75%	0	7	18	yes	no
27	M	62	no/no	Tonsil	I	HPV16	positive	S	positive	2300	−76%	0	7	16	yes	no
4	W	66	no/no	Tonsil	II	HPV16	positive	*S*+RCT	positive	1900	−74%	0	7	17	yes	no
8	W	65	yes/no	Tonsil	II	HPV16	positive	RCT	positive	1740	−71%	0	7	19	yes	no
16	M	56	no/no	Tonsil	I	negative	positive	RCT	positive	1455	−100%	0	3	2	yes	no
29	W	65	yes/no	Tonsil	III	HPV16	positive	RCT	positive	1425	−60%	0	2	13	yes	no
25	M	70	yes/yes	Tonsil	II	HPV16	positive	RIT	positive	1300	−100%	0	5	16	yes	no
3	M	83	no/no	Tonsil	I	HPV16	positive	S	positive	1285	−53%	0	5	18	yes	no
20	W	77	no/no	Tonsil	I	HPV16	positive	RIT	positive	1250	−100%	0	5	14	yes	no
21	W	58	yes/yes	Base of Tongue	IV C	negative	negative	RCT	positive	1250	−100%	0	3	4	no	yes
11	W	49	yes/yes	Base of Tongue	II	HPV16	positive	*S*+RCT	positive	1184	−54%	0	6	16	yes	no
14	M	64	yes/no	Tonsil	III	HPV16	positive	RCT	positive	1120	0%	148%	6	15	yes	no
1	M	81	no/no	Tonsil	II	HPV16	positive	RT	positive	1070	0%	78%	4	11	yes	no
22	M	52	yes/yes	Base of Tongue	IV A	negative	negative	RCT	positive	1000	−100%	0	3	6	yes	no
7	M	72	no/no	Tonsil	III	negative	positive	RT	negative	965	−100%	0	7	15	yes	no
26	M	51	yes/yes	Soft Palate	IV A	negative	negative	RCT	negative	820	−50%	0	6	10	no	no
17	M	58	no/no	Tonsil	III	negative	negative	RCT	negative	710	−100%	0	6	16	yes	no
31	M	67	no/no	Base of Tongue	III	negative	negative	RCT	negative	700	lost	lost	1	0	lost	lost
32	M	56	yes/yes	CUP	IV A	negative	negative	*S*+RCT	negative	700	stable	stable	2	2	yes	no
9	M	72	yes/no	Base of Tongue	II	HPV33	positive	RCT	negative	630	stable	stable	6	16	yes	no
30	W	68	yes/yes	Base of Tongue	IV A	negative	negative	RCT	negative	600	lost	lost	1	0	yes	no
23	M	59	yes/yes	Tonsil	IV A	negative	negative	*S*+RCT	negative	400	stable	stable	3	6	yes	no
6	M	63	yes/yes	Tonsil	II	HPV33	positive	CT+RCT	negative	56	stable	stable	7	19	yes	no
2	M	55	yes/no	Tonsil	II	HPV16	positive	*S*+RCT	negative	0	stable	stable	7	26	yes	no
18	M	52	lost	lost	lost	lost	lost	lost	negative	0	lost	lost	1	0	lost	lost
33	M	59	yes/no	Base of Tongue	III	negative	negative	*S*+RT	negative	0	stable	stable	6	10	yes	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.

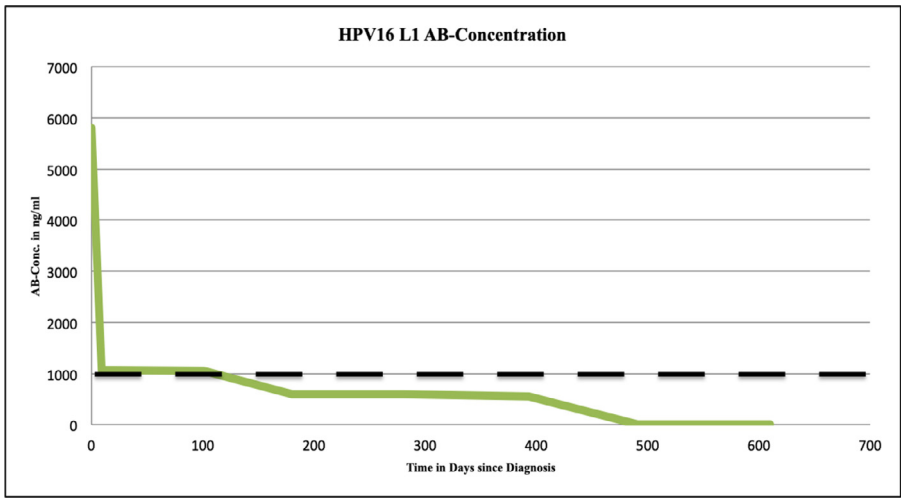

Fig. 2. a–d. Characteristic antibody graphs during follow up.
Fig. 2a: Classical antibody decrease during follow up, indicating a successful treatment understood as successful removal of tumour cells, which is associated with disease free overall surveillance.
The black spotted bar at 1000 ng/ml represents the cut off antibody concentration discriminating positive and negative DRH1 test results.
Patient characteristics of patient 10 as described in Table 1: Male, 58 year old, carcinoma of the tonsils, HPV16 DNA positive, p16 positive
Therapy: Surgery + adjuvant Radiotherapy
Decrease of antibody concentration by 90% within 6 months, and 100% within 18 months.

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 1–92 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 heterogeneously 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

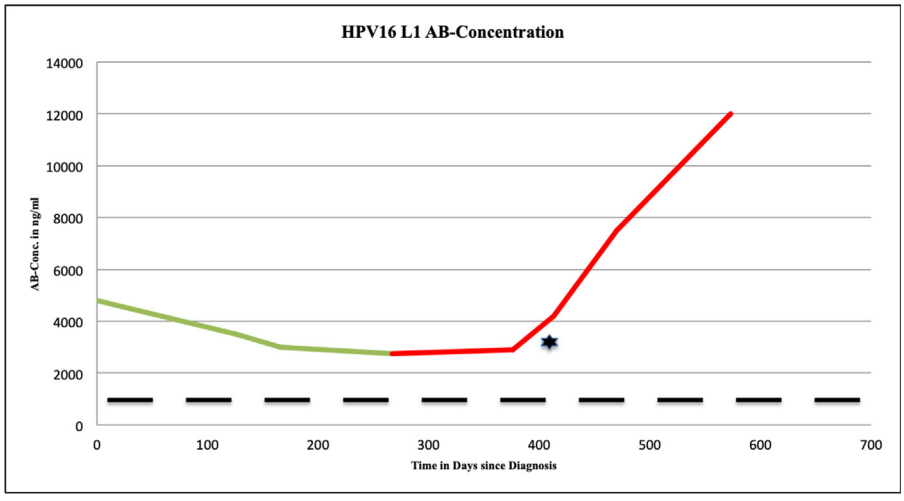

Fig. 2. a–d. Characteristic antibody graphs during follow up.
Fig. 2b: Antibody concentration during follow up of patient No 12.
Initial antibody decrease during follow up by 43% after 9 months (in green), indicating a successful treatment, was followed by a sudden increase of antibody concentration (in red) 3 months later– soon after tumour recurrence in the lungs was diagnosed.
The black spotted bar at 1000 ng/ml represents the cut off antibody concentration discriminating positive and negative DRH1 test results.
Patient characteristics of patient 12 as described in Table 1: Male, 81 year old, base of tongue carcinoma, HPV16 DNA positive, p16 positive
Therapy: Radioimmunotherapie with Cetuximab
*Time of diagnosis of tumour recurrence

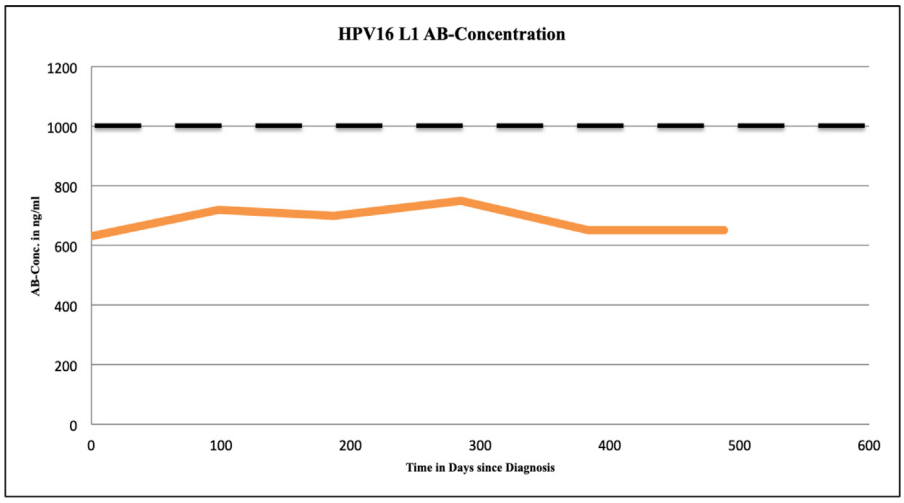

Fig. 2. a–d. Characteristic antibody graphs during follow up.
Fig. 2c: Characteristical graph in a patient with an HPV33 associated OPSCC showing HPV16 L1 antibody concentration at a constant low level indicating the type-specificity of the assay.
The black spotted bar at 1000 ng/ml represents the cut off antibody concentration discriminating positive and negative DRH1 test results.
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

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.

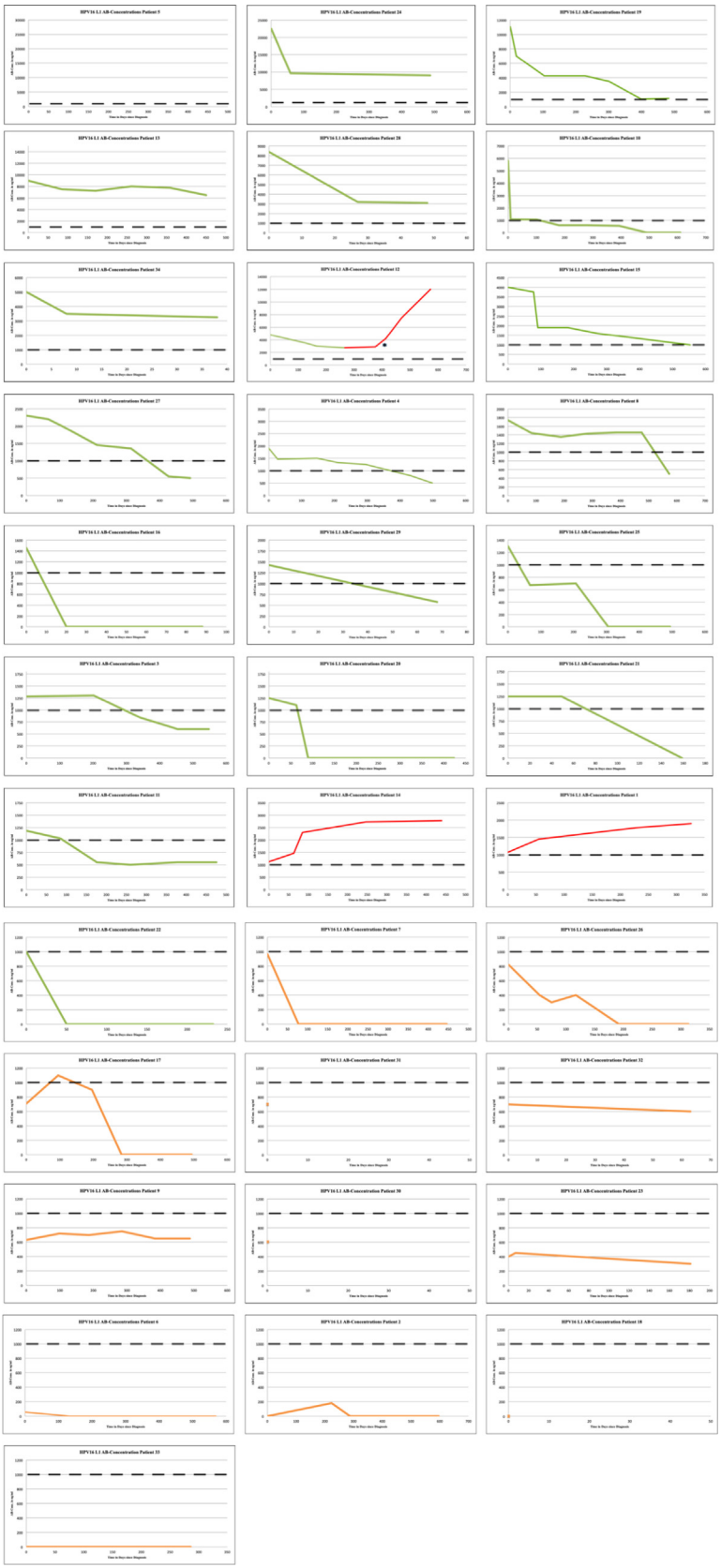

Fig. 2. a–d. Characteristic antibody graphs during follow up.

Fig. 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.

Table 2
Diagnostic specificity within healthy ‘CRP negative’ blood donors.

female	DRH1 negative		DRH1 positive		in total
	0 ng/ml	1 – 999 ng/ml	≥ 1000 ng/ml	Spec. in%	
0–19 years	19	8	10		37
(in%)	(51·4)	(21·6)	(27·0)	(73·0)	(100)
mean in ng/ml	0	306	7345		2051
20–29 years	25	10	9		44
(in%)	(56·8)	(22·7)	(20·5)	(79·5)	(100)
mean in ng/ml	0	515	3892		913
30 y and older	348	73	3		424
(in%)	(82·1)	(17·2)	(0·71)	(99·3)	(100)
mean in ng/ml	0	220	1510		49
in total	392	91	22		505
(in%)	(77·6)	(18·0)	(4·4)	(95·6)	(100)
mean in ng/ml	0	260	5136		270

male	DRH1 negative		DRH1 positive		in total
	0 ng/ml	1 – 999 ng/ml	≥ 1000 ng/ml	Spec. in%	
0–19 years	31	3	0		34
(in%)	(91·2)	(8·8)	0	(100)	(100)
mean in ng/ml	0	217	–		19
20–29 years	45	9	0		54
(in%)	(83·3)	(16·7)	0	(100)	(100)
mean in ng/ml	0	128	–		21
30 y and older	405	63	3		471
(in%)	(86·0)	(13·4)	(0·6)	(99·4)	(100)
mean in ng/ml	0	168	6566		64
in total	481	75	3		559
(in%)	(86·1)	(13·4)	(0·5)	(99·5)	(100)
mean in ng/ml	0	165	6566		57

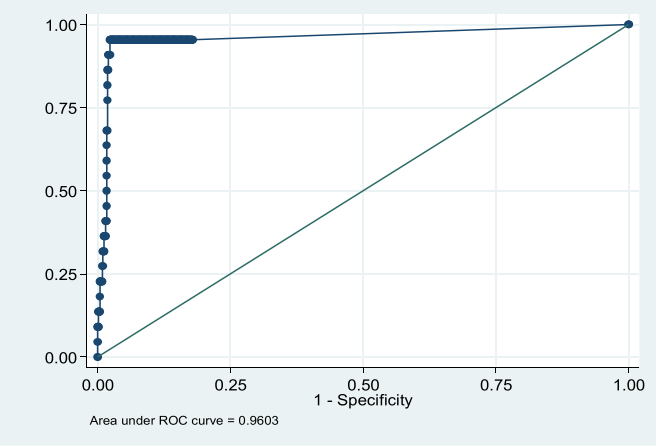

Fig. 3. Receiver-Operating-Characteristic (ROC)–curve analysis, with an ‘area under the curve’ of 0·96 (95% confidence interval 0.91–1), was calculated for 20 HPV16 driven OPSCCs and 1064 controls.

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 (4·5)	168 (95·5)
men	121	58·5	30–90	4 (3·3)	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	
Pre-immun	n (%)	n (%)	n (%)	n (%)	95% CI
20–29 years	8 (40)	9 (45)	3 (15)	20 (100)	
mean (ng/ml)	0	382	2300	517	127–906
≥ 30 years	6 (66·6)	3 (33·3)	0	9 (100)	
mean (ng/ml)	0	75	0	25	0–201
in total	14 (48·3)	12 (41·4)	3 (10·3)	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	15.544 – 69.396
≥ 30 years	0	0	9 (100)	9 (100)	
mean (ng/ml)	0	0	26.456	26.456	7.296 – 45.615
in total	0	0	29 (100)	29 (100)	
mean (ng/ml)	0	0	37.500	37.500	

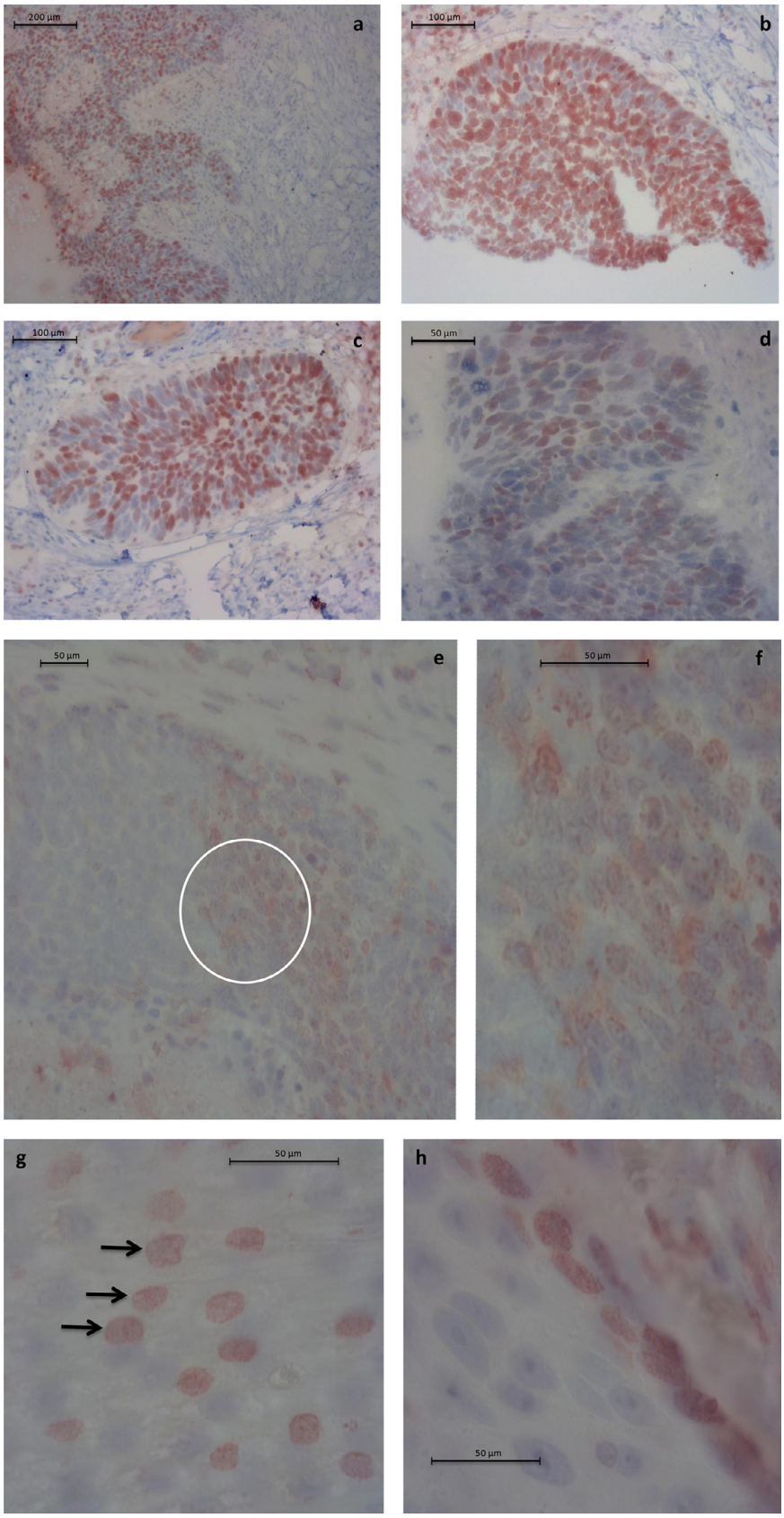

Fig. 4. : HPV16-L1 capsid protein expression in tumour cells

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 ínner 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 couldnt 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.

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