Journal of Cytology & Histology

ISSN: 2157-7099

Sivars, et al., J Cytol Histol 2014, S4:2
DOI: 10.4172/2157-709.S4-014

Research Article Open Access

A Simple Approach for Determining Presence of HPV DNA from Slides Previously Stained for P16ink4a

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Rec date: Oct 06, 2014, **Acc date:** Nov 04, 2014, **Pub date:** Nov 06, 2014

Abstract

Aim: To test for human papillomavirus (HPV) DNA in material obtained from p16INK4a (p16) immunohistochemistry (IHC) stained slides of formalin-fixed paraffin-embedded (FFPE) tumor biopsies.

Background: HPV is a favorable prognostic factor in oropharyngeal squamous cell carcinoma (OSCC), where tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC) dominate. Hence. HPV DNA testing as a predictive marker is desirable even when FFPE material is unavailable. Glass slides with p16 staining, often used as a surrogate marker for HPV, are however generally available. Furthermore, the combination of HPV DNA and p16 positivity is established as being better than testing for HPV DNA, or p16 alone, and almost equivalent as testing for E6 and E7 mRNA for determining HPV driven OSCC. Presence of HPV DNA was therefore examined in cells from glass slides previously stained for p16.

Materials and methods: DNA extracted from cells scraped off glass slides from 23 TSCC; 24 BOTSCC and 50 cancers of unknown primary (CUP) of the head and neck, with known HPV DNA and p16 status was tested for presence of HPV by a bead-based multiplex assay.

Results: DNA was obtained from 95.9% of the TSCC/BOTSCC/CUP slides and exhibited 93.5% (87/93) concordance with the HPV DNA status of the original FFPEs, with 38/42 (90.5%) of the originally HPV DNA positive/p16 positive remaining HPV DNA positive and all HPV DNA negative samples staying negative.

Conclusion: A 93.5% concordance was obtained between the HPV DNA status of the original FFPEs and DNA obtained from p16 stained glass slides. Of the HPV DNA positive IHC samples 38/38 100% were derived from HPV DNA positive/p16 positive FFPE biopsies, while all HPV DNA negative samples stayed HPV-negative. This approach allows for safer identification of HPV-positive tumors, and useful for following patients with HPV-positive tumors in retrospective studies and for future decision-making.

Keywords: HPV-typing; Formalin-fixed paraffin embedded tissue; Oropharyngeal cancer; Tonsillar cancer; Base of tongue cancer; Cancer of unknown primary of the head and neck

Introduction

Human papillomavirus (HPV), particularly HPV16, was classified as a risk factor for oropharyngeal squamous cell carcinoma (OSCC), in addition to smoking and alcohol, by the International Agency for Research on Cancer (IARC) in 2007 [1]. HPV is mainly found in tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC respectively), together covering 80% of all OSCC, with HPV-positive cases having a better clinical outcome than the equivalent HPV-negative cases (80% vs. 40% 5-year disease free survival) [2-6]. HPV is also sometimes present in lymph node metastasis on the neck, where the primary tumor is not found despite a thorough examination - so called cancers of unknown primary (CUP) [7]. If the metastasis is HPV-positive it has been shown that the patients with CUP have a similar beneficial clinical outcome as patients with HPV-positive OSCC, suggesting that the primary tumor in these cases may be an HPV-positive OSCC [7].

The past decade, others and we have shown an epidemic increase in the incidence of OSCC, especially for TSCC and BOTSCC, mainly attributed to an increase in HPV-positive tumors in many Western countries [8-18]. The group of patients with HPV-positive tumors is thus increasing [14,18,19,21,22]. Of late, due to the poor prognosis of head and neck cancer in general, treatment has been intensified with induction chemotherapy and hyperfractionated radiotherapy and in some cases treatment with Erbitux leading to increased side effects [20-22]. More recently, it has been suggested that patients with HPV-positive OSCC, TSCC or BOTSCC may benefit from individualized treatment based on HPV status rather than routinely receiving the most aggressive treatment [21,22]. Nonetheless, before initiating clinical trials with less intensified treatment it is important to analyze clinical responses in relation to HPV status of the tumors. In some cases, especially in retrospective studies, neither fresh frozen nor formalin-fixed paraffin-embedded (FFPE) samples are available. In these cases, performing further diagnosis of HPV status on glass slides may be of use.

There are many ways of determining the presence of HPV DNA in FFPE samples [23-28]. However, the presence of HPV DNA does not necessarily prove that HPV is functionally active, and therefore today

the golden standard for determining HPV status is considered to be the analysis of the presence of E6 and E7 mRNA expression by RT-PCR [29]. This analysis is optimally performed on fresh frozen material, which unfortunately is frequently unavailable [29]. Notably, it has recently been reported that the presence of HPV DNA (analyzed by PCR) on FFPE material together with overexpression of p16INK4a (p16) analyzed by immuno-histochemistry (IHC) was almost as sensitive and specific as defining HPV status by the golden standard [29]. The use of this combination has thus been very useful in many retrospective studies [21,22,29]. However, in some cases FFPE samples may be unavailable and overexpression of p16 by IHC of the cells has instead been considered as a surrogate marker for positive HPV status, and using different degrees of p16 positivity was also suggested [30-35]. Nevertheless, even when defining p16 overexpression as >70% of the cells expressing nuclear or cytoplasmatic p16 staining, still roughly 8-18.9% of HPV-negative OSCC may overexpress p16, clearly incorporating the risk of categorizing HPV-negative tumors as being HPV-positive [35-36].

To potentially increase the accuracy to determine HPV status, e.g. in retrospective studies where FFPE biopsies of OSCC are unavailable, we here used tumor material from glass slides previously stained for p16 by IHC for complementary testing for the presence of HPV DNA.

Materials and Methods

Patient and tumor material:

Totally, 23 patients with TSCC (ICD-10 code C09.0-9), 24 with BOTSCC (ICD-10 code C01.9) and 50 with CUP of the head and neck region (ICD-10 code C77.0) treated 2000-2009 at Karolinska University Hospital, Stockholm, Sweden and described previously were included in the study [6,7,14,16]. All patients had FFPE pre-treatment tumor biopsies, or FFPE lymph node metastases, earlier tested for presence of HPV DNA using the assay described below or other PCR based methods, as well as available histology slides earlier stained for p16 as described previously [6,7,14,16]. Glass slides randomly selected from 47 TSCC and BOTSCC FFPEs on the basis that the slides included ample tumor material were used for the initial analysis [6,14,16]. Furthermore, no TSCC and BOTSCC FFPE samples were selected that were HPV positive and p16 negative, since in such cases, HPV is not regarded as being functionally active [29]. Thereafter for validation all slides from 50 patients with CUP included in a previously described study, irrespective of metastasis section size on the slides were examined [7].

As shown in Table 1, among patients with TSCC and BOTSCC, originally, 24 pre-treatment tumor FFPE biopsies were HPV DNA positive/p16 positive; 20 HPV DNA negative/p16 negative; and 3 HPV DNA negative/p16 positive. In Table 2 the patients with CUP are depicted, with 18 FFPE metastasis being HPV DNA positive/p16 positive; 27 HPV DNA negative/p16 negative; 2 HPV DNA positive/p16 negative, and 3 HPV DNA negative/p16 positive [7].

Immunohistochemistry of p16

Briefly, tumor sections (4-5 µm) were de-paraffinized and rehydrated, followed by antigen retrieval in citrate buffer (pH 6). Blocking of unspecific binding sites was done with 1 % horse serum in PBS before the sections were stained with the primary antibody mAb p16INKA4a (clone: JC8, dilution 1:100, Santa Cruz Biotech, Santa Cruz, CA, USA) at +8°C overnight. The slides were then incubated for 45 min. with the secondary biotinylated anti-mouse antibody (dilution 1:200, Vector Laboratories, Burlingame, CA, USA). For antigen detection, the avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) was used according to manufacturer's instructions. Slides were developed in chromogen 3'-diaminobenzydine (DAB) (Vector Laboratories, Burlingame, CA, USA) and haematoxylin was used as a counter stain. The slides were then washed and dehydrated and cover slips were mounted using the VectaMount permanent mounting media (Vector Laboratories, Burlingame, CA, USA). P16 staining was regarded as positive if >70% of the tumor cells were strongly p16 positive [31,34].

	No of samples/ group	p16 status	HPV DNA status from FFPE material	HPV DNA+ status after using material from glass slides
TSCC	13	+	+	13/13
TSCC	9	-	-	0/9
TSCC	1	+	-	0/1
BOTSCC	11	+	+	9/11
BOTSCC	11	-	-	0/11
BOTSCC	2	+	-	0/2
Total	24	+	+	22/24
Total	20	-	-	0/20
Total	3	+	-	0/3

Table 1: HPV status and p16 overexpression in pre-treatment biopsies and p16 stained glass slides derived from 47 patients with tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC).

	No of samples/ group	p16 status	HPV DNA status from FFPE material	HPV DNA+ status using material from glass slides
CUP	18	+	+	16/18
CUP	27	-	-	0/23 *
CUP	2	-	+	0/2
CUP	3	+	-	0/3
* 4 samples lacked b-globin				

Table 2: HPV status and p16 overexpression in tumor metastasis and p16 stained glass slides derived from 50 patients with cancer of unknown primary (CUP) in the head and neck region.

DNA extraction from p16 stained slides

The slides were put in xylene for 2 days or until cover slips were easy to remove. The tumor sections were thereafter scraped off into Eppendorf tubes using sterile surgical blades. After each scraping a removal of the gloves was appropriate to exclude cross contamination, while for every fifth sample, a blank control sample was added and treated the same way for the same purpose.

Xylene (300 µl) and 150 µl of ethanol (99.5%) were added to the samples followed by vortexing and centrifugation for 2 min. at 13.200 rpm. The supernatant was removed and the pellet resuspended in 250 µl ethanol (99.5%) prior to centrifugation for 2 min. at 13.200 rpm, the supernatant then removed and the pellet dried for 5 min. in a centrifugal evaporator.

DNA extraction was thereafter performed using the High-Pure RNA paraffin kit according to the instructions of the manufacturer with the exclusion of the DNase treatment (Roche Diagnostics Scandinavia).

Determination of HPV status by a multiplex bead based assay

Using 10 µl of the DNA extracted sample from cells obtained from glass slides stained for p16, DNA of 27 different mucosal HPV types (HPV6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73 and 82), HPV16E6, HPV33E6 and b-globin (as a control for DNA quality) was analyzed. This was done by PCR, followed by a bead-based multiplex assay on a MagPix instrument (Luminex Corporation, Austin, TX, USA) as described previously [28,37]. A positive signal for having sufficient amplifiable DNA was regarded as having a positive median fluorescent intensity (MFI) for b-globin after subtracting the result with 1.5 x background - 15, while a positive signal for HPV was also regarded as having a positive MFI after using the above formula. The sensitivity of the assay allows for the detection of 5 copies of HPV16 genomic DNA when obtained from cells [37].

For the corresponding TSCC and BOTSCC biopsies as well as CUP metastases, DNA had previously been extracted from 30µm FFPE tissue sections using the same kit and tested for presence of HPV DNA status as described before [7,28,37].

Statistical analysis

Concordance between the results obtained from the scraped samples previously stained for p16 and the DNA obtained from the FFPE blocks for the same samples was estimated by determining the Kappa coefficient (95% CI) using the Kappa test of concordance. The Kappa coefficient interpretation was done according to Landis and Koch [38]. Statistical analysis was performed using IBM Corp., SPSS Statistics, Version 21.0.

Results

Presence of HPV DNA in material from p16 IHC slides from TSCC and BOTSCC: Amplifiable DNA was retrieved from all 47 samples with ample material as exhibited by the presence of a positive signal for ß-globin in the multiplex assay. In total, 45/47 (95.7%) of the TSCC and BOTSCC slides showed complete concordance with the HPV DNA status of the biopsies irrespective of p16 status (Table 1). More specifically, 22/24 (91.7% sensitivity) of the slides from FFPEs originally HPV DNA positive/p16 positive were still HPV DNA positive; and 23/23 (100% specificity) of the slides, from FFPEs originally HPV DNA negative, irrespective of p16 status remained HPV DNA negative (Table 1). A kappa test of concordance, on the total number of tested samples, revealed an almost perfect agreement between the results obtained from the scraped samples and the FFPEs ($k=0.915$, $p<0.001$).

Presence of HPV DNA in material from p16 IHC slides from CUP of the head and neck region: Amplifiable DNA was retrieved in 46/50 (92.0%) of the samples, exhibited as presence of a positive signal for ß-globin in the multiplex assay. In total, 42/46 (91.3%) of the CUP slides with sufficient DNA demonstrated complete concordance with the HPV DNA status of the biopsies irrespective of p16 status and the details are shown in Table 2. More specifically, 16/18 (88.9% sensitivity) of the slides from FFPEs originally HPV DNA positive/p16 positive remained HPV DNA positive and 26/26 (100% specificity) of the slides, originally HPV DNA negative irrespective of p16 status were still HPV DNA negative (Table 2). None of the slides that were HPV DNA positive/p16 negative 0/2 (0%) remained HPV DNA positive. A kappa test of concordance for the whole set of samples revealed an almost perfect concordance ($k=0.819$, $p<0.001$). More specifically, of the 20 samples that were HPV positive in the FFPEs, 16 remained positive giving a sensitivity of 80%, while for the HPV negative samples, all remained negative (26/26) giving a specificity of 100%. If excluding the HPV DNA positive/p16 negative samples, then a kappa test of concordance resulted in $k=0.904$, $p<0.001$, i.e. almost identical to that of the TSCC and BOTSCC group, where such samples were not included.

Presence of HPV DNA in material from p16 IHC slides from TSCC, BOTSCC and CUP of the head and neck region: Taken together, 93/97 (95.9%) of all TSCC/BOTSCC/CUP slides had amplifiable DNA for further HPV analysis, resulting in complete concordance in HPV status in 87/93 (93.5%) of the cases with the HPV DNA status of original FFPEs. More specifically, using the proposed algorithm (HPV DNA positive/p16 positive) by Smeets et al., [29] as the definition of HPV positive status, we show by this method a high sensitivity 90.5% (38/42) and a high specificity 100% (38/38) for HPV detection (Tables 1 and 2). Furthermore, 49/49 (100%) of the slides, originally HPV DNA negative irrespective of p16 status were still HPV DNA negative (Tables 1 and 2). Presence of HPV DNA could not be demonstrated in two CUP FFPEs originally HPV DNA positive/p16 negative (Table 2).

Discussion

p16 IHC stained tumor or metastasis biopsies on glass slides from 23 TSCC, 24 BOTSCC and 50 CUP yielded DNA in 95.9% of the cases for HPV DNA analysis by a multiplex bead based assay, and resulted in a 93.5% HPV status concordance to that obtained when testing the original FFPE biopsies [6,7,14,16]. Notably, 90.5% of the originally HPV DNA positive/p16 positive FFPE samples remained HPV DNA positive and 100% of the originally HPV DNA negative FFPE samples stayed HPV DNA negative. HPV DNA was not found in the two previously HPV DNA positive/p16 negative FFPE CUP samples [7]. Taken together, PCR amplifiable DNA was readily obtained from glass slides, and HPV DNA positive status was obtained only from slides where the original FFPEs had been HPV DNA positive, while no slides from originally HPV DNA negative FFPEs gave HPV DNA positive results.

That PCR amplifiable DNA was obtained from most glass slides was not only due to that some samples with ample material were selected, since 92% of the unselected CUP material yielded amplifiable DNA as well. However, no glass slides contained material older than 15 years, and it is possible that using older material may be less optimal [39]. Furthermore, using a sensitive method for HPV DNA detection, when extracting DNA this way was also most likely of importance, since the material obtained was limited [28,37].

Noticeably, all 38 cases with HPV DNA positive status were derived from tumors or metastasis both presenting HPV DNA positive status and p16 overexpression in the original FFPE biopsies and could with very high probability be regarded as HPV positive [29, 31]. Furthermore, HPV DNA positive status was obtained to a similar extent from material derived from tumor slides (91.7%) and metastasis slides (88.9%), when the original FFPEs tested HPV DNA positive/p16 positive. In four HPV positive/p16 positive cases, however HPV DNA was no longer detected. Two of these samples, both CUP, had very weak HPV16 MFI signals already in the original FFPEs and were negative for HPV16E6 (data not shown) [7]. Thus although most (38/42, 90.5%) HPV DNA positive cases among originally HPV DNA positive/p16 positive cases were identified using material from slides, four cases tested HPV-negative, suggesting that when using DNA from slides one may lose somewhat in HPV sensitivity. Moreover, HPV DNA positivity was not obtained from slides of the two HPV DNA positive/p16 negative FFPE metastasis, also implying that present approach is less sensitive for HPV DNA detection, apart from also arguing for not regarding HPV positive/p16 negative tumors as being truly HPV positive [29,31]. Nevertheless, using the combination of HPV DNA and p16 positivity as an alternative to the golden standard, these two latter cases would not have been categorized as HPV positive to start with [29]. To summarize, using material from slides is not surprisingly somewhat less sensitive than using FFPE material directly with regard to testing for the presence of HPV DNA.

The breakdown of the obtained 55 HPV DNA negative cases has already partially been discussed above. In a randomized clinical trial with de-escalated therapy, none of the six cases originally HPV DNA positive with HPV DNA negative scrapes would be included. The remaining 49 HPV DNA negative cases were all derived from originally HPV DNA negative cases, of which 43 were p16 negative and six p16 positive samples. If p16 had been used as a surrogate marker for HPV positivity these six HPV DNA negative samples would have been classified as HPV-positive.

Obviously, there are several limitations with this study. First of all it is a retrospective study. Furthermore, p16 glass slides from 47 TSCC and BOTSCC with ample material were selected primarily in order to first test if it would be possible to obtain PCR amplifiable DNA, which did prove to be the case, though the numbers of HPV DNA positive samples slightly declined. However, subsequently a complete cohort of 50 metastasis of CUP from the head and neck region was also included [7]. In this cohort, only 92.0% of the slides had amplifiable DNA. Moreover, 88.9% of the CUP compared to 91.6% of the TSCC/BOTSCC disclosed HPV DNA positivity in previously HPV DNA positive/p16 positive FFPE samples, indicating that there were no major differences determining presence of HPV DNA using this approach, between selected and none selected materials.

A third limitation of this approach is the potential loss of sensitivity for detecting HPV DNA positive samples, despite the very sensitive method used for detection of HPV DNA, allowing for the detection of 5 copies of HPV16 genomic DNA from cells [37]. Whether this is due to that less DNA is available in general when obtained from glass slides, or that the quality is not as high as when preparing DNA from FFPEs directly has to be examined further. Nevertheless, no HPV DNA positive cases were detected among previously HPV DNA negative FFPE biopsies, suggesting that all slides overexpressing p16 and testing HPV DNA positive, can be regarded being HPV positive.

Finally, using this approach two HPV DNA negative/p16 negative samples were derived from two HPV DNA positive/p16 negative FFPEs. This should however not create a problem in determining the HPV status of these samples, if requesting presence of HPV DNA together with p16 overexpression for HPV positivity, nor would such patients likely be included in a group where therapy would be tapered.

Despite the above limitations, our approach of determining HPV DNA status in tumor/metastasis material from p16 stained glass slides increases the reliability of the assay for the determination of truly HPV-positive tumors compared to using p16 overexpression alone.

In summary, extracting DNA from IHC slides for HPV DNA analysis is especially useful for identifying HPV-positive tumors, defined as HPV DNA positive/p16 positive tumors, and allows for better follow up of such patients both in retrospective and prospective studies when FFPE material is unavailable. The method also identifies patients with HPV DNA negative, p16 negative tumors, but with slightly less sensitivity, since a very small proportion of those tumors may still be HPV DNA positive. Finally, with this assay, samples that present as being HPV DNA negative and p16 positive, should for safety reasons not be classified as being either HPV-positive or HPV-negative. Larger studies with more samples could be helpful to prove the value of this method and to calculate the specificity and the sensitivity of the method.

To conclude, supplementing p16 IHC with HPV DNA analysis is desirable and the method described here allows for this being both sensitive and specific, but the obtained data, more specifically those presenting as HPV DNA negative/p16 positive need to be treated with caution.

Acknowledgements

This study was supported by grants from The Swedish Cancer Society, The Swedish Medical Research Council, The Stockholm Cancer Society, Henning and Ida Perssons Research Foundation, Karolinska Institutet, The Stockholm Country Council, The Swedish Cancer and Allergy Foundation, and the Stockholm County Council ALF.

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This article was originally published in a special issue, entitled: **"Histology and Histopathology",** Edited by Borislav A. Alexiev, University of Maryland Medical Center, USA