Correlation between human papillomavirus and p16 overexpression in oropharyngeal tumours: a systematic review

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Background: A significant proportion of squamous cell carcinomas of the oropharynx (OP-SCC) are related to human papillomavirus (HPV) infection and p16 overexpression. This subgroup proves better prognosis and survival but no evidence exists on the correlation between HPV and p16 overexpression based on diagnostic measures and definition of p16 overexpression. We evaluated means of p16 and HPV diagnostics, and quantified overexpression of p16 in HPV-positive and -negative OP-SCCs by mode of immunohistochemical staining of carcinoma cells.

Methods: PubMed, Embase, and the Cochrane Library were searched from 1980 until October 2012. We applied the following inclusion criteria: a minimum of 20 cases of site-specific OP-SCCs, and HPV and p16 results present. Studies were categorised into three groups based on their definition of p16 overexpression: verbal definition, nuclear and cytoplasmatic staining between 5 and 69%, and >70% staining.

Results: We identified 39 studies with available outcome data (n = 3926): 22 studies (n = 1980) used PCR, 6 studies (n = 688) used ISH, and 11 studies (n = 1258) used both PCR and ISH for HPV diagnostics. The methods showed similar HPV-positive results. Overall, 52.5% of the cases (n = 2062) were HPV positive. As to p16 overexpression, 17 studies (n = 1684) used a minimum of 5–69% staining, and 7 studies (n = 764) used >70% staining. Fifteen studies (n = 1478) referred to a verbal definition. Studies showed high heterogeneity in diagnostics of HPV and definition of p16. The correlation between HPV positivity and p16 overexpression proved best numerically in the group applying >70% staining for p16 overexpression. The group with verbal definitions had a significantly lower false-positive rate, but along with the group applying 5–69% staining showed a worse sensitivity compared with >70% staining.

Conclusions: There are substantial differences in how studies diagnose HPV and define p16 overexpression. Numerically, p16 staining is better to predict the presence of HPV (i.e. larger sensitivity), when the cutoff is set at >70% of cytoplasmatic and nuclear staining.

Oral and pharyngeal cancers are the sixth most frequent tumour with over 482 000 new cases and 273 000 deaths worldwide in 2008 (Ferlay et al, 2010). The role of high-risk human papillomavirus (HR-HPV) in the carcinogenesis of the uterine cervix is well recognised (Bosch et al, 1995), and owing to numerous studies in the past 10 years, HR-HPV is now also a well-known risk factor in...
oropharyngeal squamous cell carcinomas (OPSCCs) in addition to established factors such as tobacco and alcohol exposure (Duyzami et al, 2010). Compared with other head and neck squamous cell carcinomas (HNSCCs), HPV-related OPSCCs have different epidemiology, histopathological characteristics, therapeutic response, and clinical outcome (Shah and Patel, 2003; Fakhry and Gillison, 2006; De Vita et al, 2008; Robinson et al, 2010; Westra, 2012).

The small, non-enveloped, DNA virus HPV belongs to the Papillomaviridae family and is known commonly to infect squamous epithelial cells (Doorbar, 2010; Westra, 2012). HPV-positive oropharyngeal cancers are often characterised histologically by a non-keratinising or basaloïd morphologic pattern. Two techniques are generally used to diagnose HPV: polymerase chain reaction (PCR) and in situ hybridisation (ISH). Both have strengths and limitations. Human papillomavirus-specific PCR is not routinely available in most diagnostic laboratories; few HPV PCR tests are approved for clinical use, and the method requires a high level of technical skills and special laboratory facilities to prevent contamination. When applied to extracts made from fresh-frozen biopsy samples, the highest sensitivity is obtained, but the PCR analysis does not distinguish the mere presence of HPV from a clinically relevant HPV infection, where the HPV genome is often integrated into the host genome and actively transcribes HPV oncoproteins. Detection of HPV with ISH provides evidence of viral genomes through mRNA or DNA present in the tumour nuclei and is highly specific, although less sensitive than PCR (Robinson et al, 2010). This method does not differentiate between integrated and non-integrated genomes.

The presence of HR-HPV DNA is insufficient to classify accurately tumours as an HPV infection as it may be biologically inactive and not the cause of malignancy. Along with HPV diagnostics, immunohistochemical detection of p16 (p16-ICH) is often used as a surrogate marker for HPV infection and an activity of viral oncoproteins. P16 is a tumour suppressor gene that inhibits cyclin-dependent kinase 4A. In the presence of transcriptionally active HPV, hypophosphorylated retinoblastoma protein (pRb) bind to the HPV oncoprotein E7, allowing the transcriptional activator E2F to be constitutionally active while effectively stopping the negative feedback of free pRb on p16. Overexpression of p16 ensues. Independent of treatment modality, OPSCC patients with p16 overexpression have better prognosis and clinical outcome (Langendijk and Psyrri, 2010). P16-ICH is generally accessible and its technical costs are estimated to be 2–16 times lower than other HPV-specific tests (Lewis, 2012). Several studies have reported difficulties in HPV and p16 diagnostics, as there is no consensus on defining overexpression of p16 by a clear percentage cutoff level, and definitions vary from >5%, >75% to numerous less specific verbal definitions, for example, ‘diffuse and strong nuclear and cytoplasmatic staining’ (Smeets et al, 2007; Lewis, 2012). This may be problematic because different staining patterns can correlate differently to HPV-positive and -negative tumours, and staining patterns may ultimately distinguish transcriptionally from non-transcriptionally active HPV infections and thereby help determine prognosis and clinical outcomes.

The aim of this systematic review was to define and categorise overexpression of p16 based on immunohistochemical staining and correlate the categories to HPV-positive and -negative OP-SCCs.

METHODS

Search strategy and selection criteria. One author (CGL) undertook electronic literature searches within PubMed (Medline), Embase, and the Cochrane Library. The search strategy was as follows including MESH terms and keywords: ‘HPV’ or ‘papillomavirus’ or ‘papillomaviridae’ and ‘p16’ or ‘cdkn2a’ or ‘cyclin-dependent kinase inhibitor p16’ or ‘p16 genes’ and ‘oropharynx’ or ‘oropharyngeal’ or ‘palatine tonsil’ or ‘tonsil’ or ‘palatine’ or ‘tongue’ or ‘mouth’ or ‘oral’. Two authors (CGL and MG) independently reviewed the relevance of all resulting study titles and abstracts identified through the above search, and full-text copies of potentially eligible articles were assessed. Finally, one author (CGL) reviewed reference lists of the initially included studies. Studies with identical authors were contacted to avoid including the same study population twice.

We included all studies published in English from January 1980 to October 2012 regardless of funding source. The inclusion criteria were restricted to: age above 18 years, a minimum 20 cases of site-specific OP-SCCs (morphologic variants were included), and HPV and p16 results stated.

Data synthesis. Two authors (CGL and MG) independently extracted relevant data from the included studies and entered them into a piloted data extraction form. The following information were recorded: country, year(s) of biopsy collection, demographics, number of cases, tumour site (base of tongue, palatine tonsils, or other), tumour morphology (keratinising, non-keratinising, or mixed), histopathological grade (carcinoma in situ, poor, moderate, or high differentiation), IHC staining probe, definition of p16 overexpression, biopsy preservation (fresh frozen or paraffin embedded), IHC evaluation by pathologists (yes or no), HPV results (negative or HPV-16, HPV-18, HPV-33, HPV-35, and HPV-58 positive), HPV diagnostics (HPV DNA PCR, HPV DNA ISH, and HPV DNA ISH followed by PCR, HPV RNA RT–PCR, and HPV RNA ISH), and the number of p16-positive and negative cases.

Included studies were categorised into three groups by their definition of p16 overexpression: (a) a verbal definition (e.g. ‘Cases were classified in a binary manner as either positive (any cells with nuclear and cytoplasmatic staining) or negative’), (b) 5–69% nuclear and cytoplasmatic staining, and (c) ≥70% staining.

Statistical analysis. Statistics were carried out using IBM SPSS Statistics 19.0 (IBM SPSS, Chicago, IL, USA). Descriptive statistics are presented as actual numbers and percentages, or median and range where appropriate. Cases, tumour site, and HPV results (negative or HPV-16, HPV-18, HPV-33, HPV-35, and HPV-58 positive) were dichotomised for every definition of p16 overexpression.

RESULTS

The initial literature search yielded a total of 778 records. From these, we manually selected 160 articles for full-text assessment,
of which 112 articles were later excluded. Accordingly, 48 studies were left eligible for inclusion (Figure 1). Additional three studies were identified through searching reference lists. Studies with identical authors were contacted and resolved in 12 studies excluded; 11 studies were confirmed duplicates by authors; and one study excluded without reply from authors. Thus, a total of 39 studies (n = 3926) were included in the review (Table 1).

In the pooled analysis of all studies with demographic information (n = 3625), the majority of patients were male subjects (n = 2921, 80.6%). Age ranged from 20 to 93 years with a median of 58 years. Thirty-four studies (n = 3420 subjects) were European, Australian, or US based, and five studies (n = 506 subjects) were Asian. Ethnicity was reported in 22 studies (n = 2921, 80.6%) of cases were said to be positive and 59.8% of cases (n = 2092, 53.3%) of unspecified location represent the remaining (n = 770) or based on staining equal to or exceeding 70% (3 centres, n = 562) used a verbal definition. Three centres (n = 194) in Asia used a verbal definition, and two centres (n = 312) defined p16 as positive when staining was between 5 and 69%. No Asian centres defined p16 as positive based on staining equal to or exceeding 70%.

Eleven studies (n = 861) reported data on histopathologic grade (poorly differentiated, moderately differentiated, highly differentiated, or carcinoma in situ), and six studies (n = 634) reported status on tumour morphology (keratinising, non-keratinising, mixed, or unknown). The limited availability of data on tumour morphology did not allow us to examine systematically to what degree the non-keratinising tumours were related to the presence of HPV, as has been observed previously. We found no trends regarding publication year and definition of p16, likely owing to the fact that the included studies were all published in the past 10 years.

Twenty-five studies (n = 2888) provided sufficient information to construct a two-by-two table of both p16-negative/ -positive and HPV-negative/ -positive biopsies. The correlation between HPV and p16 overexpression was numerically greater, when positivity was defined as staining above ≥70% with a sensitivity of 0.927 (95% CI: 0.793–0.974). The verbal group and >5–<70% group had a sensitivity of 0.791 (95% CI: 0.608–0.888) and 0.894 (95% CI: 0.805–0.942), respectively. The false-positive rate of 0.059 (95% CI: 0.031–0.112) for the verbal group was superior to the rate of 0.201 (95% CI: 0.12–0.337) of p16 ≥70% (see Figure 2).

**DISCUSSION**

This is the first systematic review exploring the correlation between HPV infection and p16 overexpression in OPSCCs. This review shows that p16 overexpression correlates numerically better to HPV results if staining of tumour cells exceeds 70% rather than lower percentages or positivity based on a verbal definition. The issue of determining a specific cutoff value for p16 positivity has earlier been addressed in smaller samples supporting staining above 75% or staining above 50% combined with >25% confluent areas to define p16 positivity (Begum and Westra, 2008). We found
| Author (year) | Country | Year | Study years | n   | Age (years) | M/F | HPV | Analysis | P16 cutoff (%) | P16 antibody | Sensitivity of p16 (%) | Specificity of p16 (%) |
|--------------|---------|------|-------------|-----|-------------|-----|-----|----------|----------------|--------------|----------------------|-----------------------|
| Al-Swahb et al (2010) | Taiwan | 2010 | 1992–2008 | 220 | 51 | 206/14 | PCR | 36 | ≥5% and < 70 | Clone unknown (Neomarkers, Fremont, CA, USA) | 86 | 99 |
| Ang et al (2010b) | USA | 2010 | 2002–2005 | 323 | Unknown | 271/52 | PCR | 214 | ≥70 | E6H4 (MTM Laboratories AG, Heidelberg, Germany) | 90 | 93 |
| Charfi et al (2008) | France | 2007 | 1987–2005 | 52 | 61 | 36/16 | PCR | 25 | ≥5 and < 70 | E6H4 (MTM Laboratories AG, Heidelberg, Germany) | 84 | 59 |
| Chenevert and Chiosea (2012) | USA | 2012 | 1956–1969 and 2007–2009 | 97 | 58 | 80/17 | ISH | 57 | ≥70 | G175-405 (BD Pharmingen, San Diego, CA, USA) | 69 (second period: 86%) | 80 (second period: 90%) |
| El-Mofty and Patil (2006) | USA | 2006 | Unknown | 20 | Unknown | 12 | ISH | 11 | Verbal definition | Clone unknown (Novacstra Labs Ltd, Newcastle, UK) | 100 | 89 |
| El-Mofty et al (2008) | USA | 2008 | Unknown | 32 | Unknown | 22 | PC and ISH | 26 | ≥70 | JCB (Lab Vision, Fisher Scientific, Pittsburgh, PA, USA) | 85 | 100 |
| Evans et al (2011) | USA | 2011 | 2000–2007 | 30 | 55 | 25/5 | PCR | 19 | ≥70 | Clone unknown (Neomarkers, Fremont, CA, USA) | NA | NA |
| Farshadpour et al (2011) | The Netherlands | 2011 | 1980–2004 | 32 | Unknown | 14 | ISH | 14 | ≥70 | Clone unknown (Santa Cruz Biotechnology, Santa Cruz, CA, USA) | 93 | 100 |
| Friedland et al (2012) | Australia | 2011 | 1996–2008 | 20 | Unknown | 19 | PCR | 19 | Verbal definition | Clone unknown (Santa Cruz Biotechnology, Santa Cruz, CA, USA) | 100 | 100 |
| Gao et al (2013) | USA | 2012 | 1997–2006 | 150 | 56 | 136/14 | PCR | 131 | ≥5 and < 70 | E6H4 (MTM Laboratories AG, Heidelberg, Germany) | 93 | 100 |
| Hafkamp et al (2008) | The Netherlands | 2008 | 1992–2001 | 81 | 58 | 59/22 | PCR | 33 | ≥5 and < 70 | E6H4 (Dako, Glostrup, Denmark) | 86 | 98 |
| Hoffmann et al (2012) | Germany | 2012 | 2004–2009 | 20 | Unknown | 16/4 | ISH | 14 | ≥5 and < 70 | CiNtecO (MTM Laboratories AG, Heidelberg, Germany) | 79 | 83 |
| Holzinger et al (2012) | Germany | 2012 | 1990–2008 | 196 | 57 | 146/50 | PCR | 97 | ≥5 and < 70 | E6H4 (MTM Laboratories AG, Heidelberg, Germany) | 78 | 59 |
| Hong et al (2010) | Australia | 2010 | 1987–2006 | 195 | 59 | 159/36 | PCR | 83 | ≥5 and < 70 | JC2 (Neomarkers, Fremont, CA, USA) | 95 | 84 |
| Junor et al (2012) | Scotland | 2012 | 1999–2001 and 2003–2005 | 254 | 60 | 182/72 | PCR | 133 | ≥5 and < 70 | E6H4 (MTM Laboratories AG, Heidelberg, Germany) | 95 | 46 |
| Kim et al (2007) | South Korea | 2007 | 1995–2005 | 52 | Unknown | 38 | PCR | 37 | Verbal definition | Unknown | NA | NA |
| Klussmann et al (2009) | Germany | 2009 | 1997–2005 | 60 | 60 | 47/13 | PCR | 29 | ≥5 and < 70 | 16p04 (Neomarkers, Fremont, CA, USA) | NA | NA |
| Kuo et al (2008) | Taiwan | 2008 | 1997–2005 | 92 | 51 | 79/13 | PCR and ISH | 69 | ≥5 and < 70 | JC2 (Neomarkers, Fremont, CA, USA) | NA | NA |
| Laco et al (2011) | Czech Republic | 2010 | 2000–2009 | 22 | 60 | 13/9 | PCR | 18 | ≥5 and < 70 | CiNtec (MTM Laboratories AG, Heidelberg, Germany) | NA | NA |
| Lewis et al (2010) | USA | 2010 | 1997–2008 | 239 | 55 | 211/28 | ISH | 144 | ≥5 and < 70 | Clone unknown (MTM Laboratories AG, Heidelberg, Germany) | 74 | 90 |
| Li et al (2007) | China | 2007 | 1985–2004 | 49 | 58 | 37/12 | PCR and ISH | 9 | ≥5 and < 70 | 16p04 (Neomarkers, Fremont, CA, USA) | 89 | 95 |
Table 1. (Continued)

| Author (year) | Country | Year | Study years | n  | Age (years) | M/F | HPV + | Analysis | P16 cuto (%) | P16 antibody | Sensitivity of p16 (%) | Specificity of p16 (%) |
|---------------|---------|------|-------------|----|-------------|-----|-------|----------|--------------|--------------|------------------------|------------------------|
| Licitra et al (2006) | Italy | 2006 | 1990–1999 | 90 | 58 | 69/21 | 17 | PCR | 32 | Verbal definition | Clone unknown (Neomarkers, Fremont, CA, USA) | NA | NA |
| Lindquist et al (2012) | Sweden | 2012 | 1970–2002 | 73 | 59 | 59/14 | 36 | ISH | 40 | ≥5 and <70 | Clone unknown (Pharmingen, San Diego, CA, USA) | 73 | 60 |
| Mellin Dahlstrand et al (2005) | Sweden | 2005 | 1983–1999 | 51 | 63 | 39/12 | 25 | PCR | 27 | ≥5 and <70 | E6/4 (DakoCytomation A/S, Carpinteria, CA, USA) | 74 | 79 |
| Mills et al (2012) | USA | 2012 | Unknown | 62 | Unknown | Unknown | 33 | PCR and ISH | 37 | ≥5 and <70 | E6/4, predilute, Tris (pH 9.0) (MTM Laboratories Inc., Westborough, MA, USA) | NA | NA |
| Nichols et al (2009) | USA | 2008 | Unknown | 44 | Unknown | Unknown | 35/9 | PCR | 27 | Verbal definition | Clone unknown (MTM Laboratories, Heidelberg, Germany) | 100 | 100 |
| Ukpo et al (2011) | USA | 2011 | Unknown | 211 | 56 | 188/23 | 153 | ISH | 148 | Verbal definition | E6/4 (MTM, Laboratories Inc., Westborough, MA, USA) | 98 | 90 |
| Park et al (2012) | Korea | 2011 | 2002–2007 | 93 | 62 | 80/13 | 53 | PCR | 46 | Verbal definition | P2D11F11 (Novocast Labs Ltd, Newcastle, UK) | 100 | 85 |
| Preuss et al (2008) | Germany | 2008 | 1998–2005 | 106 | 57 | 77/29 | 30 | PCR | 61 | Verbal definition | E6/4 (Novocast Labs Ltd, Newcastle, UK) | 100 | 85 |
| Quon et al (2013) | USA | 2011 | Unknown | 48 | Unknown | Unknown | 36 | PCR and ISH | 35 | Verbal definition | E6/4 (MTM Laboratories Inc., Westborough, MA, USA) | 91 | 69 |
| Reimers et al (2007) | Germany | 2007 | 1997–2002 | 106 | 59 | 83/23 | 30 | PCR | 29 | ≥5 and <70 | E6/4 (Neomarkers, Fremont, CA, USA) | 86 | 86 |
| Schache et al (2011b) | UK | 2011 | 1988–2009 | 108 | 58 | 83/25 | 36 | PCR and ISH | 42 | ≥70 | CINtec (MTM Laboratories AG, Heidelberg, Germany) | NA | NA |
| Semrau et al (2012) | Germany | 2012 | 2000–2008 | 52 | 56 | 42/10 | 15 | PCR and ISH | 17 | ≥5 and <70 | Clone unknown (Roche MTM Laboratories, Westborough, MA) | NA | NA |
| Shi et al (2009) | Canada | 2009 | 2003–2006 | 111 | 57 | 82/29 | 73 | PCR and ISH | 72 | Verbal definition | CINtec (MTM Laboratories, Westborough, MA, USA) | NA | NA |
| Thavaraj et al (2011) | UK | 2011 | Unknown | 142 | 58 | 108/34 | 100 | PCR and ISH | 90 | ≥70 | CINtec (MTM Laboratories Westborough, MA, USA) | 97 | 75 |
| Ukpo et al (2012) | USA | 2012 | 1996–2007 | 154 | 56 | 133/21 | 89 | ISH | 104 | Verbal definition | E6/4 (MTM Laboratories Inc., Westborough, MA, USA) | NA | NA |
| Weinberger et al (2010) | USA | 2010 | 1980–1999 | 140 | 60 | 106/34 | 58 | ISH | 25 | Verbal definition | JC8 (Abcam Corporation, Cambridge MA, USA) | 100 | 57 |
| Weiss et al (2012) | Germany | 2012 | Unknown | 61 | Unknown | Unknown | 36 | PCR | 30 | ≥5 and <70 | Unknown | NA | NA |
| Zhao et al (2012) | USA | 2012 | 2002–2006 | 38 | Unknown | Unknown | 14 | ISH | 22 | Verbal definition | MAB4133 (Chemicon International Company/Millipore Corporation, Temecula, CA, USA) | NA | NA |

Abbreviations: HPV = human papillomavirus; ISH = in situ hybridisation; NA = not applicable; PCR = polymerase chain reaction.
no statistically significant difference between groups of p16 definition correlated to HPV, which may be because of the great heterogeneity among studies, including different p16 antibodies. In addition, ISH and PCR methods vary from centre to centre, leading to a loss of statistical power to detect differences. The explanation might also be that all p16 groups are equally correlated to HPV status; thus, the level of p16 staining is less important and the status of positivity or negativity is evident for a given staining, that is, most p16-positive tumours are above 70% when positive. Histopathologic grade and morphology was insufficiently reported and an agreement on a grading scheme applicable to OPSCC and consensus on reporting data is important for future research. As to p16 antibodies, an FDA-approved recommendation might be profitable to uniform research methods. It is widely assumed that HPV-related oropharyngeal tumours are poorly differentiated based on the immature appearance of the tumour cells, but in fact they are commonly highly differentiated as they emulate the specialised epithelium of the tonsillar crypts (Westra, 2009). Further data for analysis on this matter might question the challenge of interpreting p16-IHC in mixed and keratinising SCCs. In addition, it should be considered if carcinoma in situ should be included in future similar studies.

In future studies applying p16-IHC and HPV diagnostics, the real value of IHC must be questioned once the site of the tumour is known (oropharynx) and the morphology is recognised (non-keratinising); the chance of a non-keratinising OPSCC being HPV positive is still not known.

Previous data report a prevalence of HPV in OPSCC of 51%, which is similar to our results (O’Rorke et al, 2012). Regardless if studies used PCR, ISH, or both, similar results were achieved.

Oropharyngeal squamous cell carcinomas are characterised by a heterogeneous clinical and molecular profile (Huang et al, 2002; Shah and Patel, 2003; Bosch et al, 2004; De Vita et al, 2008) and have interestingly proven to have a better prognostic outcome in cases with p16 overexpression (Lewis et al, 2010; Ang et al, 2010a). P16-IHC is, however, a diagnostic method causing much debate, and concerns have been raised: p16 overexpression might be associated with functional pRb disturbances irrelevant for the HPV infection (Marur et al, 2010). High-risk human papillomavirus-infected OPSCCs have not necessarily lost the 9p21 allele encoding p16 (Braakhuis et al, 2004), and p16-IHC has been reported 100% sensitive but 79% specific as to carcinomas with HPV infection (Smeets et al, 2007). P16-IHC is performed on just one slide of tumour tissue and staining might vary allowing false-negative results explaining a lower specificity. Lately, cutoff values above 70 or 75% have proven to be of wider use (Ang et al, 2010a; Evans et al, 2011; Schache et al, 2011a) as compared with, e.g., values >10% as a ‘validated’ definition of p16 overexpression. In a retrospective study based on material from The Danish Society for Head and Neck Oncology (DAHANCA), the cutoff value was changed in a Letter to the Editor after publication from >10 to >70% (Lassen and Overgaard, 2012).

In conclusion, substantial differences exist in the definition of p16 overexpression and means of HPV diagnostics between studies. To achieve the highest correlation between p16-IHC and HPV results, we advise clinicians and researchers to define p16 overexpression as >70% staining of tumour cells. Future research in this field should report on p16 and HPV results, allowing a better understanding of the association between the two.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHORS CONTRIBUTION**

CB concepted the idea of the study. CGL and MG searched the scientific literature, extracted data, and led the writing. LK, CGL,
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