Published in final edited form as:
Expert Opin Med Diagn. 2008 January 1; 2(1): 11–20. doi:10.1517/17530059.2.1.11.

HUMAN PAPILLOMAVIRUS ASSOCIATION WITH HEAD AND NECK CANCERS: UNDERSTANDING VIRUS BIOLOGY AND USING IT IN THE DEVELOPMENT OF CANCER DIAGNOSTICS

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Abstract

The link between human papillomaviruses and human cervical cancers has long been established. However, human papillomaviruses (HPVs) are now being detected in another type of cancer, not previously associated with this virus, head and neck squamous cell carcinoma (HNSCC). This review will focus on experimental data supporting the view that HPVs contribute to the etiology of a subset of HNSCC. We further put forth the argument that HPV-associated HNSCC deserves to be recognized as a distinct disease in the clinic and as such needs to be appropriately diagnosed. We offer an overview of studies that have helped dissect the role of HPVs in HNSCC and that may be helpful in the development of new diagnostic tools for discriminating this type of HNSCC.

Keywords

HPV; Head and Neck Cancer; E6; E7; Biomarkers

Introduction

HPVs were first associated with cervical cancer due to the detection of HPV DNA in tumor biopsies [1]. In these cancers, which frequently harbor HPV genomes integrated into the human genome, selective expression of the viral E6 and E7 genes was detected. Integration events as seen in cervical cancers have since been shown to cause the upregulation of E6 and E7 due in part to the disruption of the E2 open reading frame which encodes a transcriptional repressor of the E6/E7 promoter, and in part due to increased stability of the E6/E7 mRNAs [2,3,4]. Nowadays HPVs are well accepted as the causative agent for the vast majority of cervical cancers occurring worldwide (HPV DNA is detected in >99.7% of cervical cancers), and the predominant HPV type detected is HPV16 [5].

More recent reports have associated high risk HPVs with a subset of HNSCC [6,7,8]. Even though HNSCC is primarily associated with environmental carcinogens such as tobacco and alcohol, emerging evidence shows a clear association between a subset of HNSCC and high risk HPVs. Unlike HPV-negative HNSCC, no overall decline in the number of new cases of this type of cancer has been observed. In these cancers HPV genomes have been detected both in integrated and extrachromosomal forms, with expression of E6 and E7 detected in both cases. HPV-positive HNSCC are primarily found at the oropharynx and account for 20-30% of the total cases of HNSCC. However, at specific sites such as the tonsil there is a particularly high incidence of HPV associated cancers with 50% of HNSCC of tonsilar carcinomas found to harbor HPV DNA. Interestingly, a high frequency of the HPV-positive tonsillar carcinomas harbor the viral genome in the extrachromosomal state [9]. Tumors at other head and neck sites have been reported likewise to be associated with HPV infection such as the base of the tongue

[10] and in some cases the esophagus [11,12]. As in cervical cancer, HPV-16 is the genotype most frequently detected in HNSCC, being found in approximately 90% of HPV-positive HNSCC, with high-risk types 18, 31 and 33 making up the rest of the HPV genotypes detected [7,13,14,15].

Consistent with E6 and E7 functionally contributing to HPV-positive HNSCC, their expression has been correlated to the presence of intact p53 gene [16], as well as decreased levels of pRb, and increased levels of p16 [17,18]. Conversely, in HPV-negative HNSCC, p53 is often mutated, levels of pRb are normal, and levels of p16 are decreased. Other reported differences include the observations that 14-3-3σ and RASSF1A promoters are hypermethylated, and the cyclin D gene is amplified in HPV-negative HNSCC more frequently than in HPV-positive HNSCC [7,19,20]. Furthermore HPV positive HNSCC show transcriptional profiles which are notably different to those of their HPV-negative HNSCC [21,22].

In addition to the various molecular differences between HPV-positive and negative cancers, the HPV-associated subset of cancers is epidemiologically distinct from the HPV-negative subset. The patients who develop HPV-positive HNSCC are usually younger and are less likely to be smokers than patients diagnosed with HPV-negative HNSCC [23]. Nevertheless, studies indicate that a history of smoking and high-risk HPV seropositivity together increase the risk for HNSCC suggesting that there is either an additive or synergistic relationship between these two risk factors [24,25]. Interestingly, patients with HPV-positive HNSCC tend to have an improved survival presumably due to enhanced radiation response of the tumors and better overall health of the patients in cases where the patients are non-smokers [9]. Another group of patients that have been shown to be very susceptible to the HPV-positive HNSCC are Fanconi anemia patients [26,27]. These patients have a general predisposition to solid tumors including HPV-associated malignancies. Strikingly in a 2003 study, HPV16 DNA was detected in the cancers 15 of 18 Fanconi anemia patients diagnosed with HNSCC [26]. The underlying cause(s) of the extraordinary susceptibility of this group of patients to HPV-positive HNSCC is currently unknown.

In what ways should we pay attention to this newly established etiology for HNSCC and what are the lessons that we could draw from HPV biology and other HPV-related malignancies? Accumulating evidence discussed in the context of this review suggests that HPV-positive HNSCC should be recognized as a distinct type of HNSCC in terms of mechanism of disease formation, its responsiveness to standard treatments and its prevention. The latter point is of particular note given the recent development of prophylactic vaccines that prevent HPV infection including that of HPV genotypes that contribute to most HPV-positive HNSCC. In order to appreciate the unique characteristics of this type of HNSCC we provide an overview of HPV biology, and the mechanisms by which HPV contributes to the formation of cancers based on *in vivo* studies performed in mice. Emphasis is placed on novel biomarkers that could be used for discriminating HPV-associated HNSCC form HNSCC caused by other etiological factors such as tobacco and alcohol use.

Human Papillomavirus Biology

Human papillomaviruses are DNA viruses that ubiquitously infect humans and have been associated with hyperproliferative lesions [1]. These are small non-enveloped viruses of which over one hundred different genotypes have been described to this date. HPV genotypes are subdivided according to their tissue tropism. A subset of HPVs infects cutaneous epithelia while the group termed mucosotropic HPVs infects anogenital and oral epithelia. Of the mucosotropic HPVs, the so-called “low” risk types, mainly genotypes 6 and 11, are the causative agent of genital warts or condylomas. The “high risk” HPVs have been associated with malignancies, mainly cervical cancer, other anogenital cancers and a subset of head and

neck squamous cell carcinoma (HNSCC). Of the high risk HPVs the most common genotypes are 16, 18, 31 and 45. The most prevalent genotype of HPV detected both in anogenital and in head and neck malignancies is HPV16.

Infection with HPVs is thought to arise in the proliferating basal layer of the epithelium, probably at sites of injury. The viral genome enters the cell nucleus and establishes itself as a low copy number extrachromosomal plasmid. This is termed the non-productive stage of the viral life cycle [28]. The productive stage of the life cycle takes place in the terminally differentiating, suprabasal compartment, where progeny viruses are produced. Because the virus does not express all the necessary factors for its own replication, it is dependent on the host cellular replication machinery. Thus, by altering the replication competence of the suprabasal cells, the virus can complete its life cycle and release progeny virions into the environment through sloughing of dead squames. In the less differentiated layers of the epithelium early genes such as E6 and E7, for which their roles in malignancy will be discussed further, continue to be expressed. One consequence of the expression of these two viral genes is the sustained ability of normally quiescent, differentiated keratinocytes in the suprabasal layers to support DNA synthesis. The roles of the early gene products E1 and E2 lie mainly in supporting and regulating viral DNA replication and transcription from the viral promoters. In the more differentiated keratinocytes the viral protein E1^E4, the most abundantly expressed protein in the life cycle, and E5 are also expressed and are also thought to play a role in the productive stage. Finally, expression of the capsid proteins L1 and L2 allows for virion assembly and viral DNA encapsidation leading to the production of progeny virus that accumulate in the terminally differentiated squames and are released into the environment.

The suprabasal, differentiating layers of the host epithelium are the sites for synthesis of progeny virus DNA. Since the virus does not encode its own DNA polymerase the fact that the replication occurs in the suprabasal layers, where DNA polymerase is limiting is paradoxical; but the virus has developed strategies to reprogram suprabasal cells so they can support DNA synthesis. The ability of the virus to do so is thought to be largely dependent on the E6 and E7 proteins. Both these proteins have been shown to be able to bind and modulate important cellular proteins that are responsible for allowing cell cycle re-entry. The best characterized interactions of E6 and E7 are those with the cellular tumor suppressor proteins p53 and pRb, respectively. High risk E6 binds p53, which is involved in mediating cell stress responses, in a complex with the ubiquitin ligase E6AP and targets p53 for degradation [29, 30,31]. High risk E7 binds the pRb protein, which is involved in cell cycle regulation, and lead to its degradation [32].

Roles of E6 and E7 in Carcinogenesis

As indicated above, the E6 and E7 viral gene products play very important roles in the life cycle of the virus. In the case of the papillomavirus originally used as a model for papillomavirus-associated oncogenicity, bovine papillomavirus type 1 (BPV1), the main transforming oncogene in tissue culture was shown to be E5 [33]. However, for the high-risk human papillomaviruses, E6 and E7 have been characterized as the main oncogenes. Consistent with this concept, in human cervical cancers, HPV genomes are frequently found to be integrated into the host genome, and this integration results in a selective increase in the expression of E6 and E7 [2,3,4]. Similarly, expression of E6 and E7 has been detected in HPV-positive HNSCC both from integrated and extrachromosomal genomes. Continued expression of E6 and E7 is required for the continued growth of cell lines derived from cervical cancers [34,35]. E6 and E7 have demonstrated transforming properties in tissue culture in combination with other oncogenes and therefore are considered to be the papillomaviral oncogenes responsible, at least in part, for the onset as well as persistence of cervical cancer [36,37,38].

Since reports have implicated high-risk HPVs in head and neck cancers the role of E6 and E7 in the neoplastic transformation of oral keratinocytes has begun to be investigated. Transformation studies using oral keratinocytes argue for a synergy between the viral oncogenes and tobacco carcinogens. Even though HPV16 E6 and E7 are sufficient to immortalize human oral keratinocytes, and organotypic raft cultures generated using the immortalized cells have a dysplastic phenotype, exposure to tobacco carcinogens is required for these cells to become tumorigenic in nude mice [39,40,41]. Also, E6 and E7 have been shown to lead to transformation of normal oral epithelial cells in combination with Erb2 overexpression and these transformed cells form tumors in athymic nude mice [42]. In oral keratinocytes, as in the cervical keratinocytes, HPV cannot lead to transformation independently but does so in collaboration with other oncogenes, consistent with the long latency between infection and presentation of neoplastic disease. It has not yet been demonstrated whether cell lines derived from HPV-positive HNSCC are dependent on the continued expression of E6 and E7; however, it is very likely that this will be the case given the growth-dependence of HPV-positive cervical cancer-derived cell lines on continued expression of E6 and E7 [34,35].

In order to better characterize the *in vivo* contributions of E6 and E7 to carcinogenesis, our lab has previously generated *K14E6* and *K14E7* transgenic mice that express the individual HPV16 oncogenes, E6 and E7, respectively [43,44]. In these mice a human keratin 14 construct is used to drive expression of the E6 or E7 open reading frames (ORF) to the basal layer of stratified squamous epithelia that lines the epidermis, the anogenital tract, the oral cavity, esophagus and forestomach of mice. In these *K14E6* and *K14E7* mice both the E6 and E7 ORFs are present as there are splicing signals in both ORFs that are thought to contribute to efficient gene expression. In the *K14E6* mice expressing only E6, a translation termination linker is placed in the E7 ORF that introduces stop codons in all three open reading frames preventing expression of the E7 protein. The expression of E6 is prevented in an analogous way in *K14E7* mice. The oncogenic phenotypes of the *K14E6* and *K14E7* mice have been well characterized in the cutaneous and cervical epithelia [43,45,46]. In order to develop a model for HPV-associated HNSCC, bitransgenic animals were generated by crossing *K14E6* and *K14E7* animals, as these were shown previously to have more severe phenotypes in other tissues [46]. Even though E6 and E7 can induce suprabasal DNA synthesis in the oral cavity, the mice do not spontaneously develop HNSCC. Likewise, spontaneous head and neck tumors have not been observed in these animals, although homozygous *K14E7* neonates display severe runting and increased mortality due to esophageal hyperplasia, which obstructs perinatal feeding [43].

In order to examine the roles of E6 and E7 in the context of HNSCC, mice were treated with the oral carcinogen 4-nitroquinoline-n-oxide (4-NQO) in their drinking water as a co-carcinogen [47]. 4-NQO is a synthetic carcinogen known to induce DNA damage similar to that observed with tobacco associated carcinogens, and shown to cause oral cancers in rodents [48,49,50,51,52]. The 4-NQO treated HPV16 E6/E7 bitransgenic animals were dramatically more susceptible to carcinogenesis and developed tumors almost fully penetrantly as compared to the low tumor incidence in the like-treated non-transgenic control group. Histopathological analysis revealed that the tumors in the 4-NQO-treated HPV transgenic mice were of a higher grade compared to that of the like-treated nontransgenic mice, similar to that described for human patients with HPV-positive HNSCC. Furthermore, molecular differences such as the differential expression of p16 paralleled those reported in literature for human HNSCC. MCM7, previously identified as a useful biomarker for HPV-positive cervical cancers both in mice and in humans was identified as useful in distinguishing between E6/E7 positive and negative head and neck lesions in the mouse and is a candidate for future investigation, as a useful biomarker in human cancer samples [47].

Both E6 and E7 are likely to contribute to tumorigenesis through their ubiquitously characterized interactions with the tumors suppressors, p53 and pRb, respectively. However, both proteins are multifunctional and have been reported to interact with numerous cellular factors. It is unclear which of these numerous interactions contribute to the viral proteins' roles in the viral cycle, carcinogenesis, or both. The interaction of the E6 oncoprotein with p53 was shown to mediate formation of a trimeric complex between E6, p53 and the ubiquitin ligase E6AP a member of the HECT ubiquitin ligase family [29]. The p53 protein is not a natural target of E6AP in the absence of E6 [53,54]. The E6-mediated p53 degradation results in decreased transactivation of p53 target promoters, which transcriptionally regulate a number of genes involved in the DNA damage response [55]. The interaction of E6 with the α-helix binding partners as well as that with its PDZ binding partners has been shown to contribute to tumorigenesis in tissues other than the head and neck *in vivo* [56,57], and thus are likely to contribute in these tissues as well.

The E7 oncoprotein is likewise multifunctional but its most heavily studied interactions are those with the *RB* family of proteins, particularly the one with pRb, which regulates entry to S-phase and cell cycle progression [32]. E7 can interact with these proteins through an *LxCxE* motif and target them for degradation and these interactions have been shown to be important for the viral life cycle [58]. The ability of E7 to target the *RB* family proteins has been linked to its ability to activate E2F-regulated transcription and has also been shown to be important for the *in vitro* transforming abilities of E7 [59,60]. Because the other *Rb* family members, p107 and p130, have not been shown to be human tumor suppressors, the abilities of E7 to destabilize pRb and activate E2F transcription have long been postulated to be the main way in which E7 contributes to tumorigenesis.

Both E6 and E7 have been detected in HNSCC but their *in vivo* contribution to head and neck carcinogenesis had not been investigated until recently in these tissues. From work done in transgenic mice in our lab, E7 was found to be the major transforming oncogene at the head and neck sites with a likely role for E6 at the later stages of carcinogenesis[61]. Contrary to what was expected, loss of *RB* in these tissues did not recapitulate the effects of E7, which suggests that the involvement of E7 in oncogenesis is more complex than merely the inactivation of pRb.

Even though E6 and E7 are the main focus of research in HPV-associated cancers, another HPV protein, E5 is also worth consideration. Recent studies from our lab in mice transgenic for a codon-optimized HPV16 E5, showed that E5 is an oncogene in its own right and can contribute to tumorigenesis *in vivo* [62]. E5 is likely to be expressed in HPV-positive HNSCC that harbor the virus extrachromosomally [9], and therefore maintain an intact E5 open-reading frame. The E5 oncoprotein is thought to activate EGFR signaling [63,64] and thus could provide a point of similarilty between the HPV-positive and negative cancers which often overexpress the EGFR [65]. The EGFR pathway has also been explored as a target for therapeutics, and could also be useful in targeting E5-expressing HPV-positive cancers [66].

Mechanistic studies that shed light on the mechanism of HPV-associated HNSCC could eventually be extrapolated in the clinic. The overexpression of MCM7 in HPV-associated cancers first described in mouse models [47], should be examined for its possible use in the clinic as a surrogate marker along with the prersence of HPV DNA in a tumor. MCM7 detection would corroborate the active involvement of the virus in the cancers where it is detected and particularly the involvement of the E7 oncogene.

Mouse models could also be used to understand the underlying mechanisms of increased radiosensitivity of HPV-associated head and neck tumors [9,67]. Furthermore they could be used as a means of pre-clinical testing of therapies specifically targeted to patients with HPV-

positive tumors, particularly treatments aimed at the E7 oncoprotein, which seems to be the driving force at least in early stage carcinogenesis [61].

Expert Opinion

The molecular characteristics of HPV-positive HNSCC and epidemiological profiles of these patients define these patients as a distinct patient group from the patients with HPV-negative cancers. However, both patient groups are treated under the same criteria in the clinic, even though, as previously mentioned, patients with HPV+ cancers have improved survival [9,67]. The reasons underlying the improved survival are not clearly understood. They may include epidemiological reasons, such as reduced exposure to tobacco and alcohol, which implies improved overall health for the group of patients where HPV is a co-factor for carcinogenesis. Others have suggested that the lack of p53 mutations seen in cancers that express HPV E6 may be a reason for improved response to radiation therapy, as E6 is thought to only partially inactivate p53. Another possible explanation could involve the less differentiated/ more proliferative characteristics of HPV-positive cancers that could make them more susceptible to radiation therapy. The reasons for this improved response to radiation of HPV-positive disease are an important focus for future research. It is also important to recognize that the implications for patient treatment may be significant, in that patients with HPV-positive disease may be treated less aggressively than those with HPV-positive disease. It is not common practice for HNSCC patients in the clinic to be screened for HPV. However, it is reasonable to consider different treatment strategies aimed at reducing the aggressiveness in treatment of HPV-positive patients since they will frequently have a more positive outcome. Thus reducing the aggressiveness of treatment and thereby reducing the undesired side-effects of such aggressive treatment in these patients may be appropriate to consider.

Along with customized guidelines for the treatment of these different diseases at the same site it would be important to develop guidelines for diagnostics that will be the most predictive of virus involvement in the cancer. HPV-positivity in HNSCC patients can be tested by means of serology (not entirely predictive) or by the use of PCR-based tests that detect the viral DNA in tumor biopsies. Since only high-risk HPVs have been shown to contribute in the formation of cancer, it would be reasonable and expedient to only test for those types which are most frequently implicated (16, 18 etc.). As previously discussed the E6 and E7 viral genes are the driving force leading to cancer associated with HPVs. Therefore, diagnostics indicative of E6 and E7 function would help verify a causative rather than a bystander role for the virus in the cancer. Such tests could include direct detection of E6 and E7 mRNA or protein in tumor biopsies. Verification that the viral oncogenes are actively being transcribed would support the assumption that they are actively participating in the process of carcinogenesis. However, the detection of several surrogate markers can also serve to confirm the action of E6 or E7, which themselves are difficult to detect.

The most promising surrogate marker arising from various epidemiological studies is the overexpression of the cdk inhibitor p16 detectable by immunohistochemistry on tumor biopsies. In fact it has been shown in several studies to correlate with HPV positivity in head and neck and also cervical premalignant and malignant lesions [17,18,68]. Mechanistically this overexpression of p16 in lesions harboring HPV can be attributed to the function of the E7 oncoprotein, which perturbs the function of pRb and related proteins. The frequent epigenetic silencing of the *CDKN2A* locus in HPV-negative cancers leads to minimal detection of p16 in those cancers. Other possible biomarkers to consider could also be products of the deregulation of the pRb pathway, since microarray studies have indicated that most of the transcriptional differences between HPV-positive and negative disease are indicative of differences in that pRb/E2F pathway.

Some good insights for potential biomarkers could be gained from these microarray studies that compare the transcriptional profiles of HPV+ and HPV-cancers. Interestingly different sets of cell cycle regulated genes are upregulated in the cancers in the presence of the virus, another piece of evidence that supports a causative role for the virus in these cancers. Several of these cell cycle regulated genes could be considered, in addition to p16, as possible biomarkers. Most compelling perhaps are the MCMs, which are components of the DNA replication machinery. Several of the MCMs appear to be selectively upregulated in HPV+ head and neck cancers as well as cervical cancers, and MCM7 has been shown to be selectively upregulated at the protein level in a mouse model for HPV HNSCC [21,22,47,69,70]. Perhaps most importantly, MCMs have been a focus as adjunct biomarkers for cervical cancer screening and recently been shown to be more effective than Pap-screening alone in diagnosing cervical cancers [70]. The use of MCMs in cancer diagnostics is currently being explored for clinical use. Other cell cycle regulated genes that are good candidates for biomarkers include cyclins E [21,71,72] and B, which are selectively upregulated in HPV-positive cancers and cyclin D, which is selectively upregulated in HPV-negative cancers [20,21].

Another group of candidate biomarkers, which emerges from the microarray study by Pyeon *et al*, are testis specific antigens, which were found to be selectively upregulated in HPV-positive cancers [21]. These antigens would be quite useful as biomarkers because they are normally expressed only in germ line cells and not detected in normal tissue. Their expression was confirmed to be upregulated in keratinocytes as a result of E6 and E7 expression, a result which supports the hypothesis that expression of these genes is driven by the virus. Of particular interest is the antigen TCAM-1. TCAM-1 is a transmembrane protein, therefore lends itself as a target for diagnostics and therapeutics due to its accessibility. The selective upregulation of TCAM-1 in HPV-positive HNSCC was also observed by Slebos *et al* [22].

The advent of successful prophylactic vaccination for high-risk HPVs may eventually have an impact on the number of cases of HPV-positive HNSCC. However, such an outcome will likely not be evident for years as vaccination was not shown to be effective in already infected individuals. Furthermore, the effect will be dependent on the extent to which individuals actually receive the vaccine, something which at least for males will not initially be widespread. Until then, it is important to acknowledge that HNSCC can have variable etiology, and that its association with HPV can lead to more informed decisions in the clinic, and treatment that is more tailored to the patient.

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Table 1

Summary of Candidate Biomarkers for Distinguishing between HPV-Positive and Negative Cancers

Biomarker	Differences described in:
P16	[17,18,21,22,68]
MCMs	[21,22,70,73]
Cyclin E	[21,71,72]
Cyclin D	[20,21]
Testis specific antigens eg. TCAM1	[21,22]