

Unravelling the relationship between Human Papilloma Virus infections with oral squamous cell carcinoma.

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Abstract

Human papillomaviruses (HPVs) are epitheliotropic viruses with an affinity for keratinocytes and are principally found in the anogenital tract, urethra, skin, larynx, tracheobronchial and oral mucosa. On the basis of high, but variable frequency of HPV in oral squamous cell carcinoma (OSCC), malignant potential of HPV infection has been hypothesized but not definitely confirmed. The aim of this review was to highlight the genomic structure and possible mechanism of infection and carcinogenesis by HPV in the oral mucosa and to review the frequency of HPV prevalence in OSCC and oral potentially malignant disorders. A computer database search was performed through the use of PubMed from 1994 to 2014. Search keywords used were: HPV and oral cancer, HPV and oral leukoplakia, HPV and oral lichen planus, HPV and OSCC, HPV and verrucous carcinoma, HPV and proliferative verrucous leukoplakia, HPV and oral papilloma.

Keywords: Human papillomavirus, oral squamous cell carcinoma, oral potentially malignant disorder

INTRODUCTION

Oral squamous cell carcinoma [OSCC] is the sixth most common cancer worldwide. Several risk factors are related to OSCC, with the main being tobacco use, alcohol consumption, and infection by high-risk genotypes of human papillomavirus [HPV].[1] The specific role of HPV in the development of OSCC is still under debate despite its well-established role in the vast majority of squamous cell carcinoma of the cervix.[2] The relationship between HPV and OSCC was first suggested in 1983, but the presence of viral DNA was only confirmed 2 years later, by means of *in situ* hybridization [ISH].[1]

In this article, we are reviewing the genomic structure of HPV, its transmission, life cycle, mechanism of carcinogenesis with review of the studies on prevalence of HPV in OSCC and oral potentially malignant disorders [OPMDs] from 1994 to 2014.

HUMAN PAPILLOMAVIRUS GENOME

Human papillomavirus is a small, nonenveloped, double-stranded, circular DNA virus with a diameter of 52–55 nm. The genome contains a double-stranded DNA molecule that is bound to cellular histones and contained in a protein capsid without envelope.[2,3,4] The HPV-DNA genome encodes approximately eight open reading frames [ORFs]. The ORF is divided into 3 functional parts: The early [E] region comprising 45% of genome, the late [L] region extending for 40% of genome and a long control region [LCR].[2,3,4]

Early ORFs encode for E1, E2, E4, E5, E6, and E7 proteins which are necessary for replication, cellular transformation, and the control of viral transcription. E1 and E2 maintain viral DNA in an episomal form and facilitate the segregation of the viral genome during cell division. During productive infection, E6 and E7 stimulate cell cycle progression. E1, E2, E4, and E5 are required for and expressed during viral DNA amplification which occurs in differentiated cells in upper epithelial layers. Late region encodes the structural proteins or capsid proteins that take part in virion assembly. L1 ORF encodes for major capsid protein and L2 ORF for minor capsid protein. Noncoding upstream regulatory region encompassing the origin of replication, the E6/E7 gene promoter, and the enhancers and silencers, is located between the early and the late regions. LCR is necessary for viral DNA replication and transcription.[2,3,4]

The different HPV types are characterized by genotypic variations in the DNA base sequences of E6 and E7. It is these genotypic differences that permit stratification of the virus oncogenic phenotype into high and low-risk type. High risk includes HPV-16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59 and low risk are HPV 6, 11, 42, 43, 44.[2,3,4]

TRANSMISSION

Prevalence sites of HPVs include the epithelium of the vagina, vulva, penis, anal canal, cervix, perianal region, crypts of the tonsil, and oropharynx. The normal oral mucosa may act as a reservoir for new HPV infections and/or as a source of recurring HPV-associated lesions. The prevalence of HPV in normal oral mucosa range from 0.6% to 81%.[5,6] Multiple pathways for HPV transmission to the oral cavity can exist. These include sexual transmission,

autoinfection, and rarely through perinatal transmission of the neonate during its passage through an infected birth canal of the mother.[5,7] Oral HPV acquisition was found to be more positively associated with number of recent oral sex and open mouth kissing partners than with the number of vaginal sex partners.[5,6]

MECHANISM OF INFECTION BY HUMAN PAPILLOMAVIRUS

Human papillomaviruses are characterized by a special tropism for squamous epithelial cells, keratinocytes. The synthesis of viral DNA and the expression of viral genes are linked to the keratinocyte level of differentiation.[8,9]

During the initial phase of infection, the viral genome undergoes episomal replication, and few copies of viral DNA per host cell are present. The episomal form acts as a reservoir of infected cells and is responsible for the latent state of infection.[8]

When the infection becomes productive, the viral genes are expressed sequentially from early genes to late genes, following the epithelial squamous differentiation, starting from basal and parabasal cells, where early portions of the viral genome are more active and proceeding to higher epithelial layers along with the formation of complete virion.[8,10] In HPV-infected basal cells, E1 and E2 proteins are expressed, and they regulate early viral DNA transcription. When expression of E2 is more pronounced, E2 represses viral DNA replication by blocking cellular transcription factors, thus controlling the number of HPV DNA copies in the basal cell by a process analogous to negative feedback.[11,12] In cases of high-risk HPV infection, E6 and E7 may also be expressed in HPV-infected basal cells, and the epithelium may then enter a proliferative phase characterized by an increasing number of HPV-infected basal cells, culminating in intraepithelial or invasive neoplasm.[11,13] As the basal cells divide, E2 mediates distribution of some HPV DNA copies to daughter cells, while some copies remain in the progenitor cells, in both cases, as episomes.[11,14] As the epithelial cells mature, HPV cycle progresses to productive replication.[11,13] In HPV infected epithelium, the matured epithelial cells express HPV E6 and E7 proteins in the suprabasal layers. E6 prevents apoptosis and E7 activates the cellular DNA replication mechanism allowing matured epithelial cells to re-enter the S-phase of the cell cycle, and makes the cellular replication machinery available for viral DNA replication.[11,12,14,15] Specific cellular factors associated with epithelial cell maturation activate late viral promoter located within E7 ORF, which activate late viral gene expression. Eventually, mediated by L1 and L2 proteins, the virus escapes from the shedding

epithelial cells.[11,13]

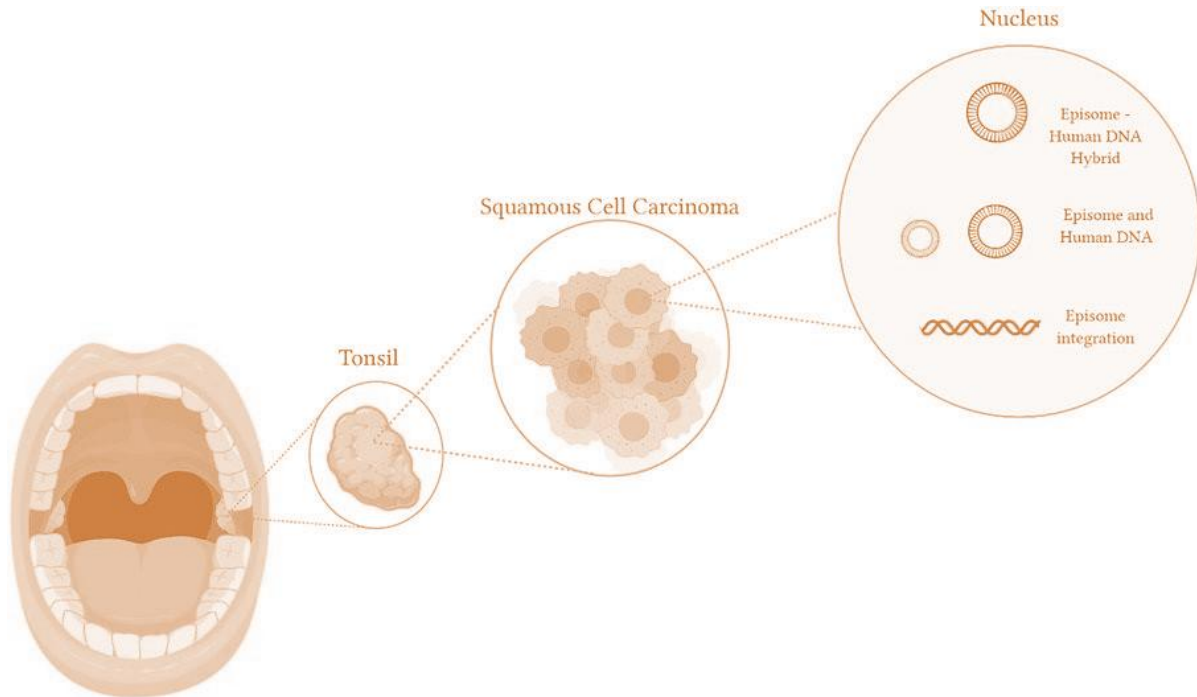


Figure 1 - Human Papilloma Virus transmission into the host nucleus can result in (A) Viral DNA integration, (B) a hybrid of conjoined viral-DNA and host DNA, (C) independent viral-DNA, not integrated or linked with the host DNA.

MECHANISM OF HUMAN PAPILLOMAVIRUS INDUCED CARCINOGENESIS

The possibility of evolving into direction of malignancy depends on the type of virus, the synergic action with different physical, chemical, and biological agents, the genetic constitution, and the immune defense mechanisms of the host, all of which are able to modify the course of HPV infection. In the case of high-risk HPV infection and under favorable conditions, the viral genome is integrated into the host genome, which is the necessary event for the keratinocytes immortality.[3]

During this process of integration, the circular form of viral genome breaks at the level of the E1 and E2 regions. The loss of E2 during this process of integration produces the loss of E6 and E7 control.[2,3,4] Therefore, the sequences E6 and E7 are directly involved in the cellular cycle by inhibiting the normal functions of p53 and pRb, respectively.

The most manifest function of the E6 protein is to promote the degradation of p53 through its interaction with a cellular protein, E6 associated protein [E6AP]. The p53 tumor suppressor

gene itself regulates growth arrest and apoptosis after DNA damage. In addition, E6 interferes with other pro-apoptotic proteins, Bak, and procaspase 8, to comprehensively prevent apoptosis.[16,17,18] Recently, the product of the notch1 gene has been identified as a novel target of p53.[16,19,20]

Over the past dozen years or so, an increasing number of other proteins have also been revealed to be target proteins of E6 that might contribute to cellular transformation, with telomerase as one probable important example.[16]

E7 is known to bind to the retinoblastoma tumor suppressor gene product, pRb, and its family members, p107 and p130. In the hypophosphorylated state, pRb family proteins can bind to transcription factors such as E2F family members and repress the transcription of particular genes involved in DNA synthesis and cell cycle progression.[16,21] Because E7 is able to bind to unphosphorylated pRb, it may prematurely induce cells to enter the S phase by disrupting pRb–E2F complexes. The E7 protein function enables HPV replication in the upper layers of the epithelium where uninfected daughter cells normally differentiate and completely exit the cell cycle. P16INK4a, which prevents the phosphorylation of pRb family members, is overexpressed when pRb is inactivated by HPV E7. Thus, overexpression of p16INK4a is suggested to be a useful biomarker for evaluating HPV pathogenic activity.[16,22]

E6 and E7 can cooperate with cellular oncoproteins such as ras and myc, which enables the virus to act at the level of growth factors and cellular and nuclear metabolism producing oncogenic cells.[16]

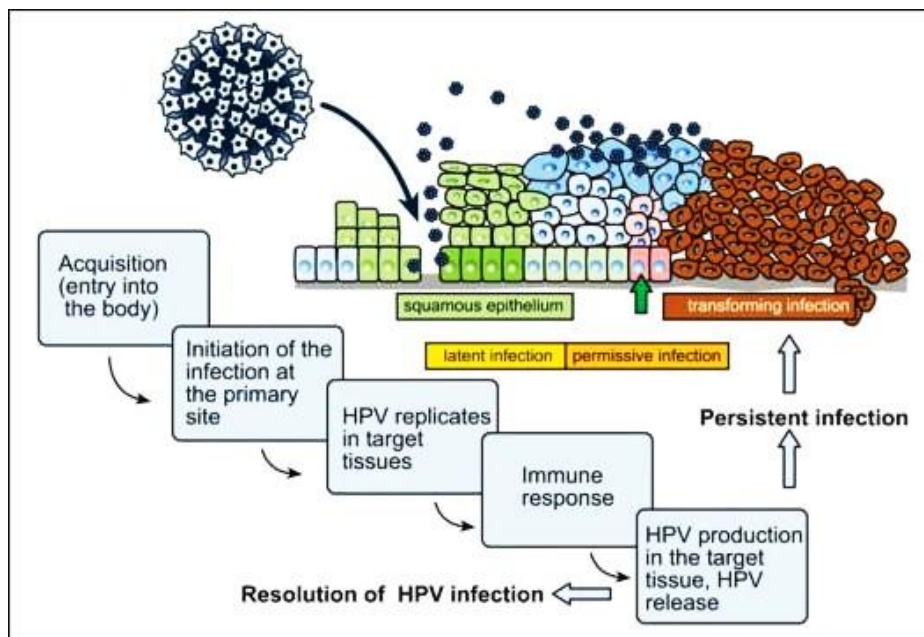
Considerations about viral detection

The screening of a limited number of genotypes is another important factor that may lead to false negative results, and consequently, to an underestimate of HPV role in OSCC. A remarkable example is the study by Kojima *et al.*[23], in which ISH-tyramide exposed an elevated prevalence of HPV-38 [35/53 [66%]], a genotype rarely investigated in other studies. Meanwhile, Pannone *et al.*[37] detected genotypes 44, 53, and 70, by DNA sequencing following PCR, but were not successful with the ISH method, as the available commercial kits do not include the corresponding probes: they are designed to detect high-risk genotypes associated with anogenital lesions.

The amount of viral copies may vary depending on the differentiation stage of the host cell,

reaching the highest levels in differentiated keratinocytes of the most superficial layers, where viruses are in their vegetative phase. About the sensitivity of the employed techniques, ISH is only able to detect HPV when the rate is higher than 10 copies of viral DNA per cell, being considered of low sensitivity. The methods of moderate sensitivity, like Southern blot, dot blot, and reverse blot hybridization, can detect it from a rate of one viral copy per cell. PCR demonstrates an expressive ratio, able to detect HPV at a rate lower than a copy of viral DNA per cell, being considered of high sensitivity. Immunofluorescence and immunoperoxidase assays are considered of low sensitivity[32].

Some authors affirm that ISH sensitivity could be improved by means of a biotinyl-tyramide-based detection system, amplifying the signal and, thus, allowing the detection even of a single viral copy per infected cell[9 , 28]. According to Syrjänen *et al.*[50], ISH is perhaps more sensitive than PCR, especially when few cells in the specimen contain viral copies that may not be detected by PCR, depending on the tissue portion used for DNA obtainment, what demonstrates the importance of studies using *in situ* approaches.



Human papillomavirus infections in the oral mucosa

It is interesting to note that technical specificity may also influence HPV detection, and consequently, prevalence rates. Still according to the meta-analysis by Miller and White[32], studies involving PCR using primers to detect early HPV genes yielded an average prevalence of 42.7% [88 out of 206], whereas those using primers designed for HPV late regions presented prevalence of 22.5% [48 out of 213]. The discrepancy is enlarged as one considers only studies

that detected HPV-16/18, with prevalence rates of 46.7% [84 out of 180] for the early region, and 15.9% [33 out of 208] for the late region. Probably, such differences are due to the disruption of the viral genome, most frequently in sites located in the late region, during integration to the host genome. As a result, false negative results may occur when primers are used in the late regions. However, disruption may eventually occur in early regions, with a possible underestimate of prevalence when using primers for this region of viral genome[16].

Although the Southern blot requires a significant amount of DNA for its conduction, it allows defining whether HPV is in the episomal or integrated form. Moreover, it is not prone to contamination errors, an event that may occur with PCR and is difficult to control, because PCR is very sensitive, besides offering simply a binary finding. Thus, techniques as the Southern blot and quantitative PCR [qPCR] provide means for quantification to differentiate low-level positivity from contamination[16].

Controversy about the role of HPV in the oral carcinoma

Although the presence of HPV in a percentage of oral carcinomas has been confirmed, as previously exposed, viral detection only does not prove the causal relationship between this agent and the aforementioned neoplasia. These results, from studies of diverse designs, allow inferring the possibility of the cited virus participation in oral carcinogenesis, but direct experimental evidence establishing cause and effect is required for confirmation. For Feller *et al.*[10], it is necessary that a significant number of individuals harboring HPV in the oral mucosa develop carcinoma in a certain period, as can be seen in cases of squamous carcinoma of the uterine cervix. The presence of high-risk genotypes with confirmed expression of E6 and E7 oncoproteins in the malignant cells, both in the primary site and in metastases, and viral monoclonality are also decisive.

The existence of a substantial number of viral DNA copies [viral load] in the neoplastic tissue also provokes an involvement of the microbial agent. However, evidence warns that this finding is not a reliable prediction of HPV-induced neoplastic progression, as the determination of viral load does not distinguish between an infection of many cells containing few copies of viral DNA per cell and an infection of few cells with a large number of viral copies per cell, or even between long-standing infection and recent infection[10].

Although the mucosae of the oral cavity and uterine cervix share histologic similarities, significant differences are observed as to neoplastic development. The oral mucosa, for

example, is more exposed to a series of carcinogens such as alcohol, tobacco and betel, which play an important role in the etiology of oral neoplasms. In the uterine cervix, HPV seems to be the main triggering factor, with more than 90% of association. Regarding HPV in squamous oral carcinomas, other differences have been observed, among which, the infrequent viral integration and isolation of genotypes 31, 33, and 35, commonly observed in cervical carcinomas, as well as the relatively lower overall prevalence in oral carcinomas. These examples illustrate the differences in mechanisms of oncogenesis between both sites; it is possible, then, that HPV presents a distinct behavior in the oral mucosa, and still plays a role in the neoplastic process [10 , 32].

Concerning viral integration into the host genome, although it is a strong indicator of the viral oncogenic role, the presence of high viral load followed by the high-risk E6 and E7 mRNA active expression may occur, for instance, in malignant cells of squamous carcinoma of the oropharynx in the episomal form[56].

Other aspects that hinder define the role of HPV in oral carcinogenesis include its identification in samples of normal mucosa, benign leukoplakia and intraepithelial neoplasia, with significant prevalence rates of 13.5%, 14.8%, and 27%, respectively, according to a meta-analysis carried out by Miller and White[32]. Even more intriguing is the detection of high-risk genotypes in the mentioned samples, besides the scarcity of cases of oral carcinoma for which HPV is the only known risk factor, that is, without including tobacco use and/or alcohol consumption, what would provide a more accurate analysis of the isolated viral action. In general, this group of patients, when observed, represents a small part, around 7.3% of the studied samples[32]. The limited number of studies assessing the expression of viral oncoproteins and their effects on oral lesions also makes it difficult to understand viral behavior *in situ*.

Castro and Bussoloti Filho[6] highlight also the fact that the virus is detected just in a subset of tumor cells when cell localization techniques are used, usually with low viral load. Both findings, added to the presence of HPV in samples of normal oral mucosa and pre-neoplastic lesions, may suggest that this virus is merely a passenger in the oral cavity. On the other hand, we cannot exclude the possibility of a progressive loss of viral genome copies after the establishment of neoplasia, a mechanism named "hit and run". Thus, HPV would play an important role in tumor development that could be underestimated due to the loss of viral copies during the process, with the resulting failure of the detection techniques[10 , 32].

Another intriguing finding is the relatively favorable prognosis of HPV-associated oral carcinomas. In this context, the metaanalysis conducted by O'Rorke *et al.*[36] concluded that not only OSCC cases, but head and neck carcinomas in general exhibited better survival in relation to HPV-negative cases. It is admitted that HPV-positive tumors commonly present the wild-type *TP53* gene, whereas cases not related to HPV harbor mutations in the referred gene which, in their turn, are associated with worse prognosis. That would help explain the prognostic profiles verified among HPV-positive and HPV-negative cases[26].

Evidence confirming the role of HPV

The findings that confirm HPV participation in the development of oral squamous carcinomas include the predominant detection of high-risk genotypes [HPV-16/18], the increased prevalence of HPV in dysplasia and squamous carcinomas compared with the normal mucosa, mainly as to high-risk genotypes, indicating HPV as an independent risk factor for the mentioned neoplastic type[6 , 21 , 50]. Correspondence around 76% has been found between genotypes identified in tumor sites and metastatic lymph nodes[32]. Lind *et al.*[27] reported that seven of 13 cases of HPV-positive leukoplakias progressed to oral carcinoma within a 10-year period, but genotypes were not reported. Nonetheless, studies show that oral human keratinocytes expressing E6 and E7 genes from HPV-16 become immortal, as previously demonstrated in other keratinocyte cell lines[34].

We must remember there is no certainty as to the necessity of viral integration, in the oral cavity, for the activation of viral oncogenesis[16]. HPV isolation, however, especially high-risk, in non-tumoral oral mucosa may be related to the latency period of the virus in the tissue. Subclinical infections of the oral and oropharyngeal mucosae by HPV are common. It is possible that these sites function as reservoirs where viruses remain quiescent, and later, when activated, may play a role in the development of local carcinomas[10]. Besides, viral presence does not necessarily indicate active viral expression, as demonstrated in studies with cases of HPV-positive head and neck squamous carcinoma, in which less than half of the cases [9 out of 20] showed E6 mRNA expression[54].

Miller and White[32], based on the body of evidence observed until then, ascertain that HPV involvement in the neoplastic process is clear, and warn that possibly some genotypes still have not been identified in oral lesions, what would explain the relatively low prevalence. If HPV

were transient, at least one of the following characteristics should be verified: 1. similar prevalence of HPV among samples of tumor, normal mucosa and pre-neoplastic lesions, including non-tumoral specimens obtained from sites far from the lesions in patients affected by squamous carcinoma; 2. viral prevalence differences in biopsy samples and in oral squamous carcinoma cell lines, due to the potential selectivity of HPV-negative cells during culture; 3. indifferent prevalence of high- and low-risk genotypes in oral cancer samples. Such characteristics were not verified in retrospective analyses, though.

As stated by Syrjänen *et al.*[50] when considering the modified Koch's postulates, at least three conditions necessary to formally confirm the role of HPV as an etiological agent of OSCC have already been satisfied: 1. presence of viral genome in tumor lesions or tumor cells; 2. ability of the virus or viral protein to transform cells *in vitro*; 3. ability of the virus or viral protein to promote tumor formation in animals. As to the other criteria: 4. viral infection precedes cancer development - in spite of the few prospective studies, we must not forget the work by Lind *et al.*[27], in which some cases of infected leukoplakia progressed to carcinoma within a 10-year period; 5. epidemiologic association between presence of the virus and development of cancer - although the association has not been proved, several works demonstrate that the prevalence of HPV is higher in pre-neoplastic lesions and carcinomas when compared with the normal mucosa[21 , 50]; and finally, 6. prophylactic HPV vaccination would eliminate OSCC - this effect will only be correctly assessed years after implementation of a vaccine program.

HUMAN PAPILLOMAVIRUS AND ITS ASSOCIATION WITH ORAL SQUAMOUS CELL CARCINOMA AND ORAL POTENTIALLY MALIGNANT DISORDERS WITH REVIEW OF LITERATURE

Low-risk HPV mainly HPV-6 and 11 appears to be closely associated with a range of oral benign papillomatous lesions including oral squamous papilloma, oral verruca vulgaris, oral condyloma accuminatum and focal epithelial hyperplasia. High-risk HPV, that is, HPV-16, 18 are in turn associated with OPMDs and OSCC.[3] HPV-16 and 18 has been found to be associated with OSCC and HPV-16 with oral leukoplakia [OL] including proliferative verrucous leukoplakia [PVL]. The reported rates of HPV DNA detection in OPMDs and OSCC

range from 0% to 100%. This extreme variation is owing to difference in ethnicity, geographic locations to variations in methods used for detection of HPV.[8]

We carried out PubMed search for prevalence of HPV in oral lesions including Oral Lichen Planus, lichen planus, OSCC, oral papilloma, verrucous carcinoma [VC], and PVL during period of 1994–2014.

A total of 50 studies are included in this review including frequency of HPV in OSCC, OL, oral lichen planus, VC, PVL, and benign and malignant papillary lesions. Except for 8 studies which utilized *In situ* hybridization [ISH] as the assay for detection, most of the authors quantified HPV-DNA using polymerase chain reaction [PCR]. The frequency of HPV in OSCC varied from 0% to 80%. The HPV type most commonly detected in OSCC and OPMDs was HPV-16, 18 with HPV-6, 11 found in only a few studies. Whereas, HPV type found in oral benign lesions and papilloma was HPV-6 and 11.

Miller and Johnston in a meta-analysis of OSCC observed that HPV may be a significant and independent risk factor. The prevalence of HPV in OSCC varies depending on several parameters such as geographic differences in population, type of specimen, selection of preparation method, and use of HPV detection method.[71] A brief review of the association of various parameters and OSCC based on these studies is presented as follows:

Molecular factors

Human papillomavirus-positive OSCCs seem to have a different molecular profile compared with that of HPV-negative cancer. In addition, HPV-positive cancers share some similarities with cervical carcinoma. By immunohistochemistry [IHC], most HPV-positive tumors show p16 overexpression. The expression of p53 and bcl-2 is not associated with HPV-positive OSCC and mutations in p53 are rarely seen in HPV-positive tumors compared with HPV-negative tumors.[72] Genetic signatures of HPV-positive OSCC have been shown to be different from those of HPV-negative OSCC.[72]

Influence of method used for analysis

There is a wide array of assays used for detection of HPV in sample including PCR, ISH, IHC, and Western blot analysis with PCR being the most widely used to estimate the HPV-DNA in samples. Besides the method used, different results are obtained when using fresh frozen or formalin-fixed paraffin-embedded material.[73] HPV in saliva and oral exfoliated cells has

been detected in some recent studies, but the sensitivity and specificity are too low, and the role of HPV detection in saliva is still uncertain.[74]

Other risk factors and human papillomavirus

No correlation between HPV-positive OSCC and tobacco or alcohol consumption has been found. A strong association has been found between sexual behavior and risk of HPV infection.[75] In India, HPV-DNA was detected less frequently in tumor specimens from tobacco chewers than in those from nonchewers.

Patient factors

Patients with HPV-positive OSCC usually are younger and more often present at a higher stage and with large metastatic lymph nodes.

Prognostic factors

Many studies have now confirmed that HPV-positive tumors in head and neck area have a better prognosis compared with those that are HPV-negative. The better prognosis is independent of the treatment given. The positive prognosis is also more pronounced in HPV-positive patients who are also p16 positive.

CONCLUSION

Risk factors mainly responsible for OSCC include tobacco, alcohol, ultraviolet rays but many cases have none of these identifiable risk factors. On the basis of high frequency of HPV in some types of OSCC and OPMDs, an oral malignant potential of HPV infection in oropharyngeal carcinoma is likely. Of particular significance is the association of high frequency of HPV in oral cancers involving base of the tongue, in those occurring in younger patients and without the prior history of exposure to the usual risk factors. Still further research is needed in order to standardize a particular protocol for screening of patients with OSCC and OMPDs for HPV as well as to determine a specific and universal method/assay for analysis.

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