Human papillomavirus (HPV) is well known to cause cervical cancer and has now been increasingly implicated as a risk factor for head and neck squamous cell carcinoma (HNSCC). More than 100 types of HPV have been identified but HPV type 16 genome is the one most commonly detected in head and neck cancers, followed by HPV type 18. Two viral oncoproteins E6 and E7 are implicated to play an important role in promoting tumorogenesis by degrading p53 and Rb gene products respectively and thus disrupting the cell cycle. The HPV positive HNSCC patients tend to be younger, non smokers and non drinkers. Oral sex is thought to be the probable mode of transmission for the HPV infection. Patients with HPV positive tumors have at least 50 percent reduced risk of death from HNSCC when compared with HPV-negative patients. Understanding the molecular biology of HPV-positive tumors could lead to the development of diagnostic, therapeutic, preventive and screening measures for HNSCC in the future.

Key Words: Head and neck squamous cell carcinoma (HNSCC), Human papillomavirus (HPV).

Human papillomavirus (HPV) is known to cause tumors in humans. Human papillomaviruses are DNA viruses that are members of the papovavirus family. More than 100 types of humanpapilloma virus (HPV) have been identified and the family is highly ubiquitous in the human population. All the papillomaviruses regardless of the host specificity, are quite similar in physical structure and genome organization. The virion is composed of a double stranded, circular, 8000 base pair DNA genome encased in a naked icosahedral capsid about 55nm in diameter.

Different HPV types are strictly epitheliotropic and this tropism can be further subdivided into mucosal or cutaneous specificity which can also extend to the precise anatomical site susceptible to infection. A number of these papillomaviruses have been implicated in a variety of clinical conditions, ranging in severity from cutaneous warts of the hand (HPV type 1) to squamous cell carcinoma in renal transplant recipients (HPV type 5 and 8) and to anogenital and most importantly cervical cancer (HPV 16, 18, 31, 33 and 45). HPV 16, 18, 31, and 45, account for nearly 80% of the cases of cervical squamous cell carcinoma with HPV 16 alone accounting for about half the cases worldwide. HPV 18 is the most prevalent type in cervical adenocarcinomas (55%), followed by HPV 16 (32%) and HPV 45 (10%).

Oral human papillomavirus (HPV) infection is now recognized to play a role in the pathogenesis of head and neck squamous cell carcinomas (HNSCC). HPV genomic DNA has been detected in head and neck cancers mostly of HPV type 16, 18, 15 but its etiologic role in the development of HNSCC had remained unclear. Recent epidemiologic and molecular data suggest that human papillomavirus (HPV) infection of the upper airway may promote head and neck tumorigenesis. Several studies have shown that tonsillar and oropharyngeal carcinomas contained HPV DNA more commonly than cancers at other head and neck site. This might be because HPV has facilitated access to the basal mucosal cells in the tonsillar crypts.

A clonal association of HPV with cancer cells is strongly supported by a specific localization of HPV in tumor cells at all cancer stages (pre invasive, invasive and lymph node) and its probable integration into the genome of some tumors. In contrast to the HPV negative oropharyngeal cancers these cancers have distinct pathology (more frequently basloid), molecular biology (fewer p53 mutations), risk factors (less associated with alcohol consumption and perhaps smoking), and clinical perspective (good prognosis).

The molecular pathogenesis of HPV in the causation of head and neck cancers that follows would help us have a better insight into the disease process.

Molecular Pathogenesis

Human papillomavirus genome consists of eight to nine open reading frames and non-coding upstream regulatory region containing the origin of replication and enhancer and promoter sequences. The unique factor binding sites likely explain the affinity for different tissues. The viral genes expressed from several promoters via splicing of polycistrionic mRNAs, are termed either early (E) or late (L) depending on when they are expressed during infection. Although the transforming activity of certain HPV types is clinically important, the normal viral life cycle is intended solely to replicate the virus; transformation occurs only as a mistake. During carcinogenic progression the viral genome frequently becomes integrated into the host genome but the integration is non specific with respect to host genome but is specific with respect to viral genome. After integration only
two viral genes E6 and E7 are consistently expressed in HPV associated cancers. Moreover viral transcription/replication factor E2 expression is disrupted which functions as a transcriptional repressor of viral gene expression. As a result, the expression of E6 and E7, which is still driven by viral promoter/enhancer sequences, is dysregulated and is often higher in tumors.

**E6 Protein**

E6 protein from HPV 16 and 18 are able to bind to the p53 product and this binding promotes the degradation of p53 by the ubiquitin pathway.\(^3,28\) P53 links cell damage with DNA repair, cell cycle arrest, and apoptosis. Following DNA damage, there is rapid increase in p53 levels. At the same time, kinases such as DNA-dependent protein kinase and ATM (ataxia-telangiectasia mutated) are activated and phosphorylate p53 which becomes an active transcription factor and increases the transcription of the Cyclin dependent kinase (CDK) inhibitor p21. p21 then haults the cell at G1/S check point giving the cell enough time to repair the DNA damage. This is done by GADD45 (growth arrest and DNA damage) the transcription of which is also increased by phosphorylated p53. If the DNA is repaired successfully p53 activates MDM2 by feedback mechanism which degrades p53 itself relieving the cell cycle block. If there is a failure of DNA repair p53 increases the transcription of pro-apoptotic gene BAX resulting in cell apoptosis.

E6 mediated degradation of p53 is dependent upon a cellular protein, E6 associated protein (E6-AP) also known as UBE3A.\(^29,31\) E6-AP does not require E6 in order to function as a ubiquitin protein ligase since it has been shown that E6-AP alone is capable of ubiquitinating itself and a number of other cellular proteins\(^32\) and E6-AP, in fact, belongs to the family of closely related ubiquitin ligase. E6 protein has been shown to stimulate the degradation of both c-Myc and Bak through the interaction with E6-AP\(^33,34\) but in these cases it appears to be an enhancement of a normally occurring interaction. In the degradation of p53, E6 appears to have diverted E6-AP to an unnatural target. It has been assumed that degradation of p53 contributes to the onco-genic potential of high risk HPV. E6 protein from both high and low risk HPV were also shown to be capable of binding to p53 without inducing this degradation.\(^35,36\) This interaction prevents the p53 mediated transcriptional repression of TATA containing promoters\(^37\) and also represses p53’s transactivation of promoters containing p53 response elements.\(^38\) P53 degradation emerges as one of the prime causes for chromosomal instability of high risk human papillomavirus containing cells,\(^39,40\) with resulting mutational consequences for HPV-positive cells. The presence of E6 also enhances the integration of foreign DNA into host cell genome.\(^41\) Recent observation suggests that E6-AP mediates ubiquitination and degradation of the src family tyrosine kinase Blk but it is conceivable that the presence of E6 partially blocks this degradation and thereby stabilizes the respective kinase and stimulates mitotic activity.\(^43\) This could explain, in part, growth-stimulatory functions of the E6 protein of high risk HPVs.\(^44\)

In addition, however E6 reveals a remarkable pleiotropy in binding further host-cell proteins:

It interacts with calcium-binding protein ERC 55 homologue, E6BP.\(^35\) This appears to be a vitamin D receptor (VDR)-associated factor\(^46\) and E6’s interaction may perhaps prevent the mediation of vitamin D3’s growth suppressive effect.

E6 also interacts with paxillin.\(^47,48\) Paxillin is involved in signal transduction between the plasma membrane and focal adhesions and the cytoskeleton and it is activated in response to a number of mitogenic stimuli. It is possible that E6 induced disruption of the cytoskeleton and consequent interference with signal transduction can release the cell from certain cell cycle controls.\(^47\)

E6 also interacts with mammalian homologue of the Drosophila discs large protein (DLG).\(^39\) Since DLG has been implicated in the control of cell-cell contact and cell polarity\(^50,51\) it is reasonable to speculate that this activity of E6 might be relevant for tumor progression.

E6 targeted protein 1 (E6TP1) is a negative regulator of Rap1 GTPase and could therefore be involved in the negative regulation of mitogenic signaling. E6 induced degradation of E6TP1 might restore this signaling and thence contribute to cellular immortalization.\(^52,53\)

E6 also binds to the interferon regulatory factor3 and inhibits the induction of interferon beta messenger RNA (mRNA) following Sendai virus infection.\(^54\) This inhibition is not mediated by ubiquitination or degradation.

E6 has also been shown to overcome p53 independent apoptosis, this is at least in part, the result of E6-induced ubiquitin-mediated degradation of c-Myc and Bak proteins, both of which are associated with induction of apoptosis.\(^33,34,54\)

Human papillomavirus has been shown to specifically upregulate telomerase activity during immortalization.\(^55-58\) Mutational analysis of E6 reveals that the up regulation of telomerase, rather than the degradation of p53 is important for its immortalizing activity.

HPV E6 protein has also been implicated in processes resulting in perturbation of chromosomal structure and the control of normal cellular DNA replication, HPV 18 E6 had been shown to induce the ubiquitin mediated degradation of Mcm7, via E6-AP.\(^59\) The Mcm7 protein is a part of the replication licensing factor whose binding to cellular DNA replication origins (ori) ensures that each ori fires only once in each round of DNA replication.\(^60,61\) From the above discussion it is clear that many of the effects seen in cells upon over expression of E6 are not related solely to the degradation of p53.

**E7 Protein**

The HPV E7 protein is able to bind and interact with Rb gene product\(^62\) and retinoblastoma protein (Rb)-related pocket proteins\(^63\), however the binding affinity of the low-risk
E7 protein is much lower than the high-risk E7 protein.\textsuperscript{64} Rb gene product is a nucleo-phosphoprotein that plays a key role in regulating cell cycle. Rb exists in active hypophosphorylated state in quiescent cells and inactive hyperphosphorylated state in the G1/S cell cycle transition.\textsuperscript{65} Hypophosphorylated Rb binds to a protein complex that contains E2F and a subunit called DP1. The E2F/DP1/Rb complex binds to promoters of E2F-responsive genes. These E2F targets include prominent cell cycle regulatory genes, such as cdc2, thymidine kinase, myb, dihydrofolate reductase, and the E2F1 gene itself.\textsuperscript{66} Bound to the E2F/DP1/Rb complex, such genes are silent because Rb recruits histone deacetylase, an enzyme that causes compaction of chromatin and inhibition of transcription. When the quiescent cells are stimulated by growth factor the concentrations of cyclin D and E go up, resulting in the activation of cyclin D-CDK4 and cyclin E-CDK2 at the G1/S restriction point and causing phosphorylation of Rb. Hyperphosphorylated Rb dissociates from complex activating transcription of E2F target gene that is essential for progression through S phase.\textsuperscript{67} The binding of E7 to Rb protein and Rb-related pocket proteins results in phosphorylation of these protein and their enhanced degradation by ubiquitination,\textsuperscript{68} and the release of transcription factors of the E2F family, activating transcription of genes regulating cell proliferation.\textsuperscript{69,70} E7 protein of high risk HPV are found in cyclin E\textsuperscript{71} and in cyclin A\textsuperscript{72} complexes. These complexes exhibit kinase activity. The activation of cyclin E followed by activation of cyclin A is mediated by E7 sequences required for transformation.\textsuperscript{73} The E7 induced S phase entry is not accompanied by cyclin D activation, probably because of the formation of cyclin D/p16\textsuperscript{INK4} complexes in high risk human papillomavirus infected cells.\textsuperscript{74} p16/INK4 binds to CDK4 and promotes the inhibitory effect of Rb. E7 protein also causes the inactivation of the cyclin-dependent kinase inhibitor p21\textsuperscript{CIP1-75,76} and p27\textsuperscript{KIP1-77}. This interaction uncouples the CDK activity from CDK inhibitors and is a major factor in growth stimulation of HPV infected cells. Similar to E6, E7 protein also inhibits the interferon signaling pathways by binding to the interferon regulatory protein p48.\textsuperscript{78} High risk human papillomavirus E7 expression enhances the integration of foreign DNA into the host cell DNA\textsuperscript{79} results in increase mutagenesis\textsuperscript{74,80} and enhances mutagenicity of chemical carcinogens.\textsuperscript{81}

Although E6 and E7 proteins may immortalize various types of human cells independently, their cooperative interaction leads to substantially enhanced immortalization efficiency.

**EPIDEMIOLOGY & HPV TYPES IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC):** Kreimer et al\textsuperscript{82} did PCR-based assays for HPV DNA in greater than 5,000 HNSCC biopsy specimens from 60 studies and found that one-fifth to one-fourth of SCCs from the oral cavity and larynx were HPV positive, but the prevalence in oropharyngeal SCCs was significantly greater amounting to more than one-third of the cases. This is consistent with the findings of other studies showing that HPV-positive tumors arise largely from the lingual and palatine tonsils in the oropharynx compared with other anatomic sites of the head and neck.\textsuperscript{8,9,15,83,85} Furthermore according to this study,\textsuperscript{82} HPV16, the most common HPV type detected in biopsies from women with cervical SCC was also the most common type detected in biopsies from HNSCCs. In the oropharynx, HPV16 accounted for the overwhelming majority of HPV-positive cases (86.7%), whereas the predominance of HPV16 was less striking in other head and neck sites. HPV18 the second most common type detected in HPV-positive cancers in this study, was found much less frequently in HPV-positive was found much less frequently in HPV-positive oropharyngeal SCCs (2.9%) compared with HPV-positive oral SCCs (34.5%) or HPV-positive laryngeal SCCs (17.2%). It therefore seems that the type distribution of HPVs in HNSCCs may also vary by head and neck site. Aside from HPV16 and HPV18, other oncogenic HPV types commonly detected in invasive cervical cancer biopsies (e.g., HPV31, 33, 35, 45, 56, 58, and 59) were rarely or never detected in HNSCC biopsies. Conversely, HPV 6, which has been designated as “low-risk” or “non-oncogenic” to the cervix\textsuperscript{86} and is the cause of benign tumors in the aerodigestive and genital tract,\textsuperscript{18} was present in a greater number of HNSCCs than any of the oncogenic types other than HPV16 and HPV18. The fact that HPV16 and HPV18 account for almost all oncogenic HPV types detected in HNSCC biopsies suggests that newly developed prophylactic vaccines for cervical cancer\textsuperscript{87} should also be relevant for HNSCCs.

HPV prevalence in oral SCCs from Asia was reported to be considerably higher compared with the other geographic locations. Similarly, HPV prevalence was significantly higher in oropharyngeal SCCs from North America and Asia compared with Europe.\textsuperscript{82} However, in the IARC study of HPV and oral and oropharyngeal cancers HPV prevalence did not differ significantly among Europe, North and South America, Asia, and Africa.\textsuperscript{88} Important gaps remain on the topic of HPV prevalence in HNSCC in many parts of the world. In particular, almost 84% of the information on oropharyngeal SCCs derived from studies conducted in Europe and North America.

**Risk factors & Clinical Implications**

The etiology of head and neck squamous cell cancer is multifactorial with alcohol and tobacco consumption considered to be the main risk factors.\textsuperscript{89-92} Average and poor oral hygiene and inadequate dental status\textsuperscript{93} marijuana use\textsuperscript{94} and genetic factors\textsuperscript{95} are also implicated in increasing the risk for head and neck squamous cell carcinoma. The association between high-risk human papillomavirus infection and the risk of head and neck squamous cancer development dates to 1985, when HPV 16 was detected in oral squamous cell tumours.\textsuperscript{96} Since then, HPV DNA has repeatedly been found in a variable proportion of head and neck cancers\textsuperscript{9,14,83,97} \textsuperscript{and...}
it has been proposed that HPV DNA positive tumors form an etiologically distinct group of oral cavity/oropharyngeal tumors. In this group of tumors, distinct clinical and epidemiological characteristics can be found. The risk factors for HPV-positive head and neck squamous cell carcinoma are discussed briefly in the following paragraphs.

The HPV-positive patient appears to be distinct from the HPV-negative patient with regard to alcohol and tobacco exposure history. HPV-positive HNSCC is more likely than HPV-negative HNSCC to occur in the nonsmoker and nondrinker. Non smokers are approximately 15-fold more likely to develop HPV-positive HNSCC than smokers. An inverse association between HPV status and alcohol use has also been reported. Although evidence suggests that HPV is associated with cancers in nonsmokers and nondrinkers, the degree to which oral HPV infection may combine with tobacco and/or alcohol use to increase risk of cancer is currently unclear, with some studies suggesting a synergistic effect with tobacco or alcohol, whereas others have found no such synergy.

How oral HPV infection occurs was not firmly established but sexual transmission was thought to be the likely mode since numerous case control studies of cervical cancer patients have indicated that HPV infection is predominantly sexually transmitted. Recently oral HPV infection has also been associated with sexual behavior, in particular with number of oral sex partners. Other factors associated with elevated risk of oral HPV infection include increasing age, male sex, history of sexually transmitted disease HIV infection and severity of immunosuppression. In three case-control studies, patients with oral cancer had had more sexual partners than controls, although the number of patients and controls who ever had oral-genital contact were not significantly different. One of these studies found that the associations with a higher life-time number of sexual partners and with a total of more than four partners with whom the subject engaged in oral sex was stronger for patients with tumors positive for HPV-16 DNA than for those whose tumors did not contain HPV-16 DNA. Other studies also support the finding that the risk of HNSCC is elevated among women with a higher number of sexual partners. Risk factors among men include young age at first intercourse, number of sexual partners and a history of genital warts. However, no significant sex-related difference in the risk of cancer associated with the presence of anti-HPV antibodies has been found. Patients with HPV-positive HNSCC tend to be younger by approximately 5 years, on average, when compared with HPV-negative HNSCC.

A possible explanation for this could be that high-risk sexual behaviors were found to be more prevalent among HNSCC patients younger than 55 years when compared with those older than 55 years.

Histologically, HPV-positive tumors tend to have a poorly differentiated and frequently basaloid histology but patients with HPV-positive tumors have improved prognosis when compared with patients with HPV-negative tumors in the majority of studies. The reason for the improved survival is unclear; however, improved radiation responsiveness, immune surveillance to viral antigens, and the absence of field cancerization in these patients who tend to be nonsmokers, have been postulated. In addition, E6-related degradation of p53 in HPV-positive cancers may be functionally inequivalent to HPV-negative p53 mutations, and therefore, HPV-positive tumors may have an intact apoptotic response to radiation and chemotherapy.

Molecular Detection OF HPV

HPV can be detected by many methods but each has its own merits and demerits. The problem of contamination by miniscule amounts of RNA or DNA underlies all of the sensitive molecular assays.

The detection of antibodies to E6 and E7 has been demonstrated in cervical cancer patients by several studies. However, in a small study of HNSCC patients only 12% had antibodies to E6 or E7 and none of the patients with oral tumors demonstrated seropositivity. It is not known if antibody development to any region of the HPV genome is significant, or if there are particular antibodies that herald a worse prognosis. Furthermore antibody presence is not necessarily indicative of active infection, latent integration, or oncprotein production that might be a clinically significant contributor to carcinogenesis. In addition, seropositivity may be a confounding factor associated with other risk factors for oral cancer, including tobacco and alcohol exposure.

HPV DNA and E6 mRNA assays have also been performed. van Houten et al demonstrated by PCR that not all the HPV DNA positive HNSCC samples were positive for E6 mRNA, indicating that the presence of DNA does not necessarily indicate active viral replication. Since it is not clear that integration must occur for HPV to play a role in carcinogenesis and HPV is thought to exist in an episomal form in the oral cavity; it is possible that HPV can be a transient infection that may or may not participate in the foundation of malignancy.

In situ hybridization (ISH) involves the use of type-specific radioactively labeled DNA probes complementary to HPV sequences for detection. The sensitivity of this assay was found to be at least on the order of 20-50 copies per cell. It is the only clinically useful test to confirm the diagnosis of HPV. However, p16 immunohistochemistry may serve as a surrogate marker for high-risk HPV because strong correlations have been found between diffuse nuclear and cytoplasmic p16 staining, HPV DNA by ISH (140) and real-time PCR.

PCR is known to be a very sensitive assay for the detection of HPV DNA in any given sample. Universal primers to conserved DNA sequences in HPV have been designed to the L1 region [also known as MY09/MY11] and the E1 region [also known as CPI and CPII].
and the E7 region. Furthermore, there is a host of other primers utilized that can be type-specific. The majority of studies have settled on the use of MY09/11 primers for detection, which yields a product size of ~ 450 base pairs.

Southern blot has long been one of the gold standard assays for the detection of HPV DNA. It offers the ability to distinguish between episomal and integrated DNA, and it can detect up to 0.1 copy per cell. While Southern blot may boast a theoretically higher specificity, it is clearly less sensitive than PCR.

Quantitative PCR utilizes a fluorescent probe that is cleaved upon each round of amplification by the DNA polymerase, and the degree of fluorescence in the reaction mixture is then measured. Real-time PCR can be performed on microdissected tumor DNA normalized to a single-copy human gene to demonstrate one or more viral copies per tumor cell. Capone et al. reported the detection of HPV DNA in the sera of patients with HPV-associated HNSCC and proposed that quantitative real-time PCR is a more sensitive and specific assay to monitor the serum cell free HPV DNA. Thus this technique combines the sensitivity of PCR reaction with the specificity of a southern blot.

Every method has its drawbacks so critical evaluation of data based on the types of detection methods used as well as determination of what the data means in a clinical context is necessary for appropriate analysis.

Conclusion
There is increasing evidence that human papillomavirus might be one of the causative factors in a unique subset of HNSCC. Molecular mechanisms that reveal the ability of the high risk (type 16 and 18) HPV to disrupt key events in the regulation of cell cycle and apoptosis have now been identified and oral sex is considered to be the most likely route of transmission. The current data is however insufficient to quantify exactly the magnitude of increased risk for HNSCC in individuals infected with HPV. Because of the lack of this information there are currently no screening programmes and the clinical relevance of HPV-positive tumors lie in their good prognostic value. Further research could lead to the development of diagnostic, therapeutic, preventive and screening implications for the HPV-positive HNSCC.

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