4. ROLE OF HUMAN PAPILLOMA VIRUS IN TONGUE CANCERS
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4.1 Background

4.1.1 Historical background

Human Papilloma Virus (HPV) infects a broad spectrum of vertebrates including humans. They are known to cause benign and malignant tumors in humans. The infectious etiology of the warts was proven in the 19th century (Ciuffo 1907). The first reports appeared on HPV as a carcinogen after the finding of cancer development in rabbit infected with cottontail rabbit papilloma extracts (Rous 1934). Harald zur Hausen was the first to demonstrate that genital warts contain human papillomavirus (HPV) genomes (Gissmann 1980; de Villiers 1981) Low-stringency hybridization experiments with HPV sequences isolated from genital warts performed in his laboratory led to the discovery of related HPV sequences in cervical cancer tissues (Durst 1983). It was officially concluded in November 1991 in a workshop convened by the International Agency for Research on Cancer (IARC) and World Health Organization WHO that there was sufficient epidemiological and laboratory evidence linking HPV infection and cervical cancer categorizing HPV as human carcinogens (Bosch 1992)

4.1.2 Taxonomy

HPVs belong to the family Papovaviridae family, which includes subfamilies Papilloma viruses and polyoma viruses (Shah 1990) They are highly species specific and do not infect the species barrier. They are therefore named after the species they infect, and they are numbered according to the order of discovery. A new HPV type is defined by a sequence difference exceeding 10% in the L1 ORF, when compared to that of established genotypes. Approximately 200 different HPVs have now been characterized, of which more than 85 are fully sequenced and new types are regularly added to the list. They have a strong tropism for epithelial cells and they are divided into mucosal and cutaneous HPVs depending on which epithelial surface they infect commonly. Within each of these groups they are further classified onto high, intermediate and low risk based on the propensity for the malignant progression of the lesions they cause (Lorincz 1992). Most HPVs are low risk and produce benign wart that do not undergo malignant transformation.

HPVs 16, 18, 31, 33, 35, 39, 45, 50, 51, 53, 55, 56, 58, 59, 64 and 68 are considered “high risk” types as they are detectable in carcinomas and dysplasias (Lorincz 1992; Clavel
HPVs 31, 33, 35, 51 and 52 are sometimes regarded as “intermediate risks” because they are more common in mild or severe dysplastic lesions than in carcinomas. Among the high-risk strains, HPV 16 and 18 are the most closely associated with cervical and head and neck carcinoma. The HPV16 DNA has been found in more than 50% of squamous cell carcinomas, while the HPV18 DNA has been found in more than 50% of adenocarcinomas in cervical cancers (Milde-Langosch 1993) whereas in head and neck cancers, HPV 16 is the most predominant type.

4.1.3 Virus particle & Genome organization

HPVs are non-enveloped, small circular double stranded DNA viruses with an icosahedral capsid (Syrjänen 1999). The capsid of 72 capsomers is approximately 55nm in diameter (Fig. 4.1). The DNA of HPV is chromatin like and covered by histones (Hausen 1996).

![Figure 4.1: The genome of Human Papilloma Virus (HPV)](image)

The viral genome consists of 7200-8000 base pairs (bp). Only one strand encodes for viral proteins, leading to the transcription that occurs in one direction (Hausen 1996). The genome is divided into three regions: long control region (LCR) ≈1kb and the two open reading frames: early (E) ≈ 4kb and the late (L) region ≈3kb. The upstream regulatory region (URR), also known as the long control region (LCR) is the non-coding region containing a variety of cis elements, which regulate viral replication and gene expression.
The E region codes for regulatory non structural proteins E1, 2, 3, 4-7 and the L region codes for structural capsid proteins L1 and L2. E and L genes are numbered according to size; higher the number, smaller the corresponding open reading frame.

4.1.4 HPV Protein functions

4.1.4.1 L1&L2
The two capsid proteins form the 72 capsomers enclose the viral DNA (Doorbar 1991). L1 is the major capsid protein, contributing to 80-90% of the capsid. The late region units, L1 and L2 encode for viral capsid proteins during the late stages of virion assembly. The protein encoded by L1 is highly conserved among different papilloma virus species (Favre 1975); antibodies against the bovine papilloma virus, therefore, have been used to identify HPV capsid proteins in human tissues. The minor capsid protein encoded by L2 has more sequence variations than that of the L1 protein; hence, antibodies against the L2 protein had been a source of antigen for specific types of HPV antibodies.

4.1.4.2 E1and E2 proteins
They encode proteins that are essential for episomal papillomavirus DNA replication and play a critical role for episomal maintenance. The E1 ORF is after L2, the most conserved region of the papilloma virus genome (Hausen 1996). Though E1 can initiate replication alone, it interacts with E2 and this interaction is needed for maximum efficiency of replication. E2 acts as a transcription factor and regulates viral transcription and therefore playing an important role in the viral life cycle. It encodes two proteins: one, which inhibits transcription of the early region and the other, which increases the transcription of the early region (Ward 1989). This protein represses the expression of E6 & E7, the oncogenes of high-risk types (Thierry 1991). During HPV DNA integration the viral genome usually breaks in the E1/E2 region leading to loss of the E1 and E2 regions. The loss of E2 results in uncontrolled and increased expression of E6 & E7 oncogenic proteins (Schwarz 1985; Cullen 1991; Jeon 1995). HPV viral integration into the host genomic DNA is associated with progression from polyclonal to monoclonal status in CIN and these events play a role in the progression from low-grade to high-grade cervical neoplasia. However the deletion of E2 is a late event in cancer development since most premalignant lesions do not contain disrupted E2. Hence disruption of E2 may not be necessary for HPV induced carcinogenesis (Cullen 1991; Das 1992). Cancer tissues
contain both episomal and integrated HPV DNAs at the same time. Integration appears to occur more frequently in HPV-18 associated cervical cancer than in HPV-16 associated cervical cancer (Crusius 1997).

4.1.4.3 E3 protein

Function is not known

4.1.4.4 E4 protein

The E4 protein originates from an mRNA formed by a splice from E1. It is one of the major transcripts in warts. It associates with the keratin cytoskeleton and induces the collapses of the cytoplasmic cytokeratin network in human keratinocytes, a situation which may assist the release of virions from the infected cell. It is found exclusively in the differentiated layer of the epithelium. It has been speculated that the protein may disrupt normal differentiation in order to establish favorable condition for viral particle formation, thus E4 seems to be important for productive infection.

4.1.4.5 E5 protein

This 83-amino acid membrane protein is highly hydrophobic and found in the Golgi apparatus and the plasma membrane. It has a weak transforming capacity in HPV types like HPV-16 (Pim 1992). The E5 in ORF is often deleted in cervical cancers. It could possibly be important for the initiation of transformation (Schwarz 1985; Haraf 1996), not necessarily essential for maintaining the malignant transformation of the host cell. E5 interacts with various transmembrane proteins like the receptors of epidermal growth factor (EGFR), platelet derived growth factor β, and colony stimulating factor-1 (Hwang 1995). It has been shown that EGFR is necessary for the E5 protein of HPV-16 to transform murine cells (3T3) (Syrjänen 1999). E5 protein of HPV-16 has been shown to reduce degradation of internalized EGFRs (Haraf 1996; Syrjänen 1999). There is experimental evidence that the E5 protein can induce an enhancement of EGFR activation in a ligand-dependent manner (Straight 1995; Crusius 1998).

4.1.5 E6 and E7 oncoproteins

E6 and E7 are the two major oncoproteins. E6 cooperates with E7 in immortalization and transformation of the cells that host the HPV DNA (Bedell 1987.; Phelps 1988). E6 alone can immortalize human mammary epithelial cells (Haraf 1996). The major transforming activities of high-risk HPV E6 and E7 proteins have been linked to inactivation of the p53
and retinoblastoma (pRB) tumor suppressors, respectively (Munger 2004)(Figure 4.2). The ability of the E6 and E7 protein expressed by “high-risk” HPV type 16 to immortalize and transform human keratinocytes was reported1 (Hawley-Nelson 1989).

Figure 4.2: HPV carcinogenesis-E6-p53 and E7-pRb pathways

4.1.5.1 E6 oncoprotein

The HPV E6 protein is a small protein consisting of 150 amino acids. HPV-E6 is a high-risk protein which targets E6-AP, a protein ligase of the ubiquitin pathway to induce ubiquitination and rapid proteasomal degradation of p53 (Huibregtse 1993; Scheffner 1993). E6-AP does not interact with p53 in the absence of E6, and its normal substrates are unknown (Beer-Romero 1997; Talis 1998). The presence of high-risk HPV-E6 and the absence of p53 seem to be associated with an increased risk of development of high-grade cervical disease (Pillai 1996). High-risk HPV E6 protein also has p53-independent transforming activities. The HPV E6 protein contains a carboxyterminal PDZ binding domain.
4.1.5.2 E7 oncprotein

The other major oncprotein, it alone can immortalize human keratinocytes. E7 has the ability to phosphorylate the \( Rb \) proteins, leading to degradation by ubiquitination. This subsequently leads to E2F activation, which produces a family of transcription factors leading to cell proliferation. pRb is the major cellular target protein of the E7 oncprotein of the high risk HPV types (Dyson 1989). Reduced or absent pRb expression was found to be significantly associated with the presence of HPV DNA (Andl 1998) (Wilczynski 1998). The cell cycle components Cyclin D1 and p16INK4a, which are regulated by pRb, were also affected with Cyclin D1 also showing reduced expression and p16INK4a being over-expressed in these tumours. In further support of a causal involvement of HPV, the pRb-defective phenotype of these HPV- positive tumours was also associated with the absence of p53 mutations (Andl 1998). pRb, functionally inactivated releases a transcription factor that causes induction of p16 expression. p16 proceeds to bind all CDK4/CDK6, evicting cyclin D1 from its CDK4/6 association. Cyclin D no longer protected by association with its CDK4/CDK6 partner is degraded resulting in termination of its activity towards the end of G1. The tumor cells express high levels of p16 which binding to CDK4/CDK6, blocks cyclins from complexing with these CDKs resulting in the apparent degradation of the uncomplexed cyclins (Parry 1995). In head and neck squamous cell carcinoma over-expression of cyclin D1 and loss of p16INK4A expression are common genetic events. Cyclin D1 is the regulatory subunit of Cdk-4 and Cdk-6, and overexpression of cyclin D1, with increased formation of Cdk-4 and Cdk-6 complexes, results in hyperphosphorylation and hence functional inactivation of pRb. Over expression has been associated with a more aggressive tumor phenotype and reduced survival.

Braakhuis et al. (Braakhuis 2004) report the striking finding that HNSCCs with active HPV type 16 DNA (i.e., HPV16 DNA that expressed the viral E6 and E7 genes) had substantially lower rates of loss of heterozygosity (LOH) at chromosomal regions 3p, 9p, and 17p than tumors that contained inactive HPV DNA (i.e., HPV DNA that did not express E6 and E7). Because the LOH frequencies in the latter group of chromosomal regions were also low in the E6/E7–negative tumors, it is possible that cells with constitutively expressed E6 and E7 viral proteins may bypass most of the genetic events necessary for malignant transformation in the development of this subset HNSCC. The retention of HPV16 DNA and continued expression of viral E6 and E7 genes are required
to maintain the malignant phenotype of the tumor cells (von Knebel 1992). The finding by Braakhuis et al.—that only HNSCCs that expressed the HPV16 E6 and E7 genes showed distinct patterns of LOH but not the tumors that carried inactive HPV16 DNA (Braakhuis 2004) supports the notion that active HPV16 is required in a subset of HNSCC to maintain the malignant phenotype. The lack of TP53 gene mutations in any of the tumors with active HPV16 in this study, and the observation that tumors with inactive HPV16 DNA had a similar TP53 mutation frequency as tumors that lacked HPV DNA, further underscore the importance of continued activation of HPV16 in maintaining a malignant phenotype. It has been shown that p53 sequence alterations are decreased in the setting of HPV infection, since there is an alternative means of p53 silencing with the production of E6 (Werness 1990; Gillison 2000)

4.1.6 Notch-1

Notch is a single transmembrane receptor that is activated by direct contact with the membrane bound ligands. Delta 1-4 and Serrate/Jagged 1 and 2 (Lindsell 1996). Notch1 has been shown to function as an oncogene in the development of human T-cell leukemia (Ellisen 1991) It can serve as a tumor suppressor or tumor promoter in the same kind of cancer (Yao 2007). Notch1 expression has been found in neoplastic cervical lesions, particularly in well-differentiated squamous cell carcinomas, (Zagouras 1995) it disappears in the late stages or poorly differentiated cervical cancer (Talora 2002). It has been reported that Notch1 expression is reduced or absent in invasive cervical cancers. Conversely, expression of activated Notch1 causes strong growth inhibition of HPV-positive, but not HPV-negative, cervical carcinoma cells, but exerts no such effects on other epithelial tumor cells. Increased Notch1 signaling, but not Notch2, causes a dramatic down-modulation of HPV-driven transcription of the E6/E7 viral genes, through suppression of AP-1 activity by up-regulation of the Fra-1 family member and decreased c-Fos expression. Thus, Notch1 exerts specific protective effects against HPV-induced transformation through suppression of E6/E7 expression, and down-modulation of Notch1 expression is likely to play an important role in late stages of HPV-induced carcinogenesis (Claudio 2002).

4.1.7 Viral transcription

Viral gene expression is controlled by the LCR, which contains viral promoter and enhancer sequences and the origin of replication. The LCR is also commonly called the upstream regulatory region (URR) or the non-coding region (NCR). The complex regulation of viral
gene expression is controlled by both cellular and viral transcription factors. Examples of cellular transcription factors that bind to the LCR are NF-1, AP-1, Oct-1, TEF-1, TEF-2, SP-1, YY-1 and the glucocorticoid receptor (Haraf 1996). Dysregulation of these transcription factors seemed to be of importance for carcinogenesis in HPV induced lesions. The viral protein E2 acts as a viral transcription factor and has four binding sites in the LCR of HPV-16. E2 represses E6/E7 expression by inhibiting the promoter p97.

4.1.8 Route of infection: HPV life cycle

The life cycle of papilloma virus is linked to the differentiation of the infected epithelial cells. They infect basal epithelial cells, which is the only cell layer that is actively dividing. Though the nature of the HPV receptor remains unclear, integrin $\alpha_4\beta_6$ has been implicated (Evander 1997). The viral DNA persists as nuclear, extra-chromosomal plasmids at moderate copy numbers (e.g., 20 episomes per cell) in the cycling basal and parabasal keratinocytes. Infections generally remain subclinical. Productive infections that can culminate in the release of progeny virions require terminal differentiation of stratified epithelium to enable the activation of differentiation-dependent viral promoters. Reactivation of a latent infection leads to a programmed sequence of high-level expression of the viral genes, vegetative DNA replication and virion morphogenesis in the superficial strata of the epithelium.

![Figure 4.3: HPV Life cycle. Nature Reviews Immunology 2004;4(1)46-54](image)

4.1.9 DNA integration

The HPV DNA is integrated in the host genome in many cancer cells and cancer cell lines. It is usually extra-chromosomal or episomal in benign precursor lesions. Cancer tissues may contain both episomal and integrated HPV DNAs at the same time, although
integration appears to occur more frequently in HPV 18-associated cervical cancer than in HPV 16-associated cervical cancer (Crusius 1997). During HPV DNA integration, the viral genome usually breaks in the E1/E2 region. The break usually leads to the loss of the E1 and E2 regions. The loss of E2, which encodes proteins including one that inhibits the transcription of the E6 and E7 regions, has been known to result in uncontrolled and increased expression of E6 and E7 oncogenic proteins. Increased expression of E6 and E7, meanwhile, has been observed to lead to the malignant transformation of the host cells and to tumor formation (Bosch 1992). HPV viral integration into the host genomic DNA is associated with progression from polyclonal to monoclonal status in CIN, and these events play a fundamental role in the progression from low-grade to high grade cervical neoplasia. HPV may exist in a dormant state and does not necessarily need to produce mRNA continuously to maintain a malignant state.

### 4.1.10 Common detection methods

There are many methods of detection of HPV, each with its own strengths and weaknesses. Some amount of contamination cannot be ruled out even with compulsive isolation techniques. No method is without flaws. Hence the detection method and the clinical significance of the data should be evaluated for appropriate analysis.

**Polymerase chain reaction (PCR)**

It is the most sensitive HPV detection method where it is possible to detect even a single gene copy of HPV. The primers can be either consensus/general primers or type specific. The consensus primers bind to a wide range of HPV types, which is useful in screening approaches. The consensus primers are complementary to sequences (often in L1) in HPV that are highly conserved among many HPV types, while type specific primers bind to a sequence found in a single HPV type (often in E6 or E7) and does not cross to other HPV types. Apart from type specific PCR, sequencing of the PCR products obtained with consensus primers can also distinguish the different types. Negative controls at all steps are of importance to check for false positives. Universal primers to conserved DNA sequences in HPV have been designed to the L1 region (also known as MY09/MY11) the E1 region (also known as CPI and CPII), the E6 region (Maitland 1987) and the E7 region (Evander 1997).

Consensus PCR primers MY09/11 (Bernard 1994), GP5+/6+ (Chaouki 1998) were considered to amplify the 18 HPV types most commonly associated with cervical cancer.
(6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73, 82, and 83; (Clifford 2003) as well as additional high-, intermediate-, and low-risk types. The use of consensus primers vs. type-specific primers would theoretically result in a higher detection rate, since many different types of HPV would be identified. However, one study compared the use of type-specific E6 and E7 primers to L1 consensus primers, and there was no difference in detection rates (Resnick 1990) even though there is a theoretical advantage to using E6 and E7, since these are the known oncogenic proteins with specific molecular downstream effects related to carcinogenesis. This finding is perhaps due to the overwhelming prevalence of HPV-16 and HPV-18, to the exclusion of other types of HPV in the head and neck. A different study in cervical carcinoma samples noted that using several primer sets spanning the different regions would provide a more accurate determination of HPV prevalence (Karlsen 1996).

Quantitative PCR combines the sensitivity of PCR with additional advantage to quantify the number of viral particles per cell.

**In situ hybridization**

Enables not only detection but also the localization of the virus. Tissue sections are put on slides and hybridized with labeled DNA or RNA probes after denaturation. The sensitivity differs between different ISH methods. The sensitivity of this assay was found to be at least 20-50 copies per cell (Syrjanen 1987).

It can also be used to identify whether the virus is integrated or episomal.

Disadvantages:

- ISH depends on the consistency of the complementary sequence present in the sample.
- The presence of HPV DNA in oral cavity samples is inconsistent.
- Storage of samples and degradation of signal over time
- Inter observer variability

**Southern blot hybridization**

It has been one of the gold standard assays for the detection of HPV DNA. It enables the researcher to distinguish between episomal and integrated DNA. It can detect up to 0.1 copy per cell (Syrjanen 1987). It is more specific but less sensitive than PCR (Haraf 1996) There was marked difference in the prevalence of HPV when PCR and Southern
blot were used. Gillison showed that in non-oropharyngeal tumors, Southern blot was rarely positive when compared with PCR (Gillison 2000).

The advantage of the technique is its high specificity, while the major disadvantage is that it is time consuming and requires large amounts of DNA.

**Dot blot hybridization**

In this method, the extracted DNA without enzyme digestion is transferred and bound to a membrane in its single stranded form. HPV specific sequences are identified by hybridization with labeled cloned HPV DNA. The sensitivity is about 1 genome copy per cell, using 300-500ng sample DNA (Syrjanen 1987)

Advantage: relatively fast.

Disadvantage: lower specificity, false positive signals should be controlled for.

Reverse dot blot is another variant for HPV typing, wherein DNA from known HPV types are fixed onto the membrane and labeled genomic DNA or PCR products are used as probes.

**Hybrid capture**

It is the second most sensitive detection method after PCR. The sensitivity is approximately 0.05 HPV DNA copies/cell. It is based on non-radioactive hybridization with HPV RNA as probes, which give the advantage of that the binding is stronger than that of DNA-DNA. The RNA-DNA hybrids are captured in tubes coated with monoclonal antibodies against the hybrid molecule.

4.1.11 Koilocytosis

Koilocytosis was first described by Leopoldo Koss *et al.* in 1956 (Koss 1956). It consists of picnotic nuclei surrounded by extensive clear halos with a volume higher than the cytoplasm. Some view koilocytosis as a pathognomonic sign of HPV infection (Syrjänen 1983; Premoli-de-Percoco 1993). They have the following characteristics:

- Nuclear enlargement (two to three times normal size)
- Irregularity in the nuclear contour (occasionally)
- Hyperchromasia (occasionally)
- Perinuclear clearing
The cost of molecular tests is higher compared to the detection of koilocytosis in H&E slides. It may serve as a screening method in centers where molecular detection methods are not feasible.

Figure 4.4: Low-grade squamous intraepithelial lesion (LG-SIL) exhibiting true koilocytosis

The following should be considered for differential diagnosis:

1. Focal epithelial hyperplasia showing mitosoid cells with altered nuclei.
2. Improper processing of the sample leading to swollen cells, which resembles koilocytes.
3. Glycogen storage disease
4. Any clear cell as a result of cysts, etc which could be eliminated by special stains.

4.1.12 Immune response

The exact role of the immune response against the high risk HPVs is not completely clear. Both cell mediated and antibody immune responses have been demonstrated in humans. But cell mediated immunity plays an important role in controlling HPV infection. The antibodies against HPV reach the maximum levels 6 to 12 months after the beginning of the infection. It is not very clearly known whether antibody formation to any region of the HPV genome is significant, or if there are particular antibodies that herald a worse prognosis. Antibody presence is not necessarily indicative of active infection, latent integration or oncoprotein production that might be a clinically significant contributor to carcinogenesis. In cervical intraepithelial neoplasia and cervical carcinoma, there is an increased prevalence of antibodies against E7 and E4. It is possible that in the future the
measuring of antibodies against E7 will become a marker to assess the response of a specific therapy. The presence of antibodies against E4 is associated with viral replication and is believed to coincide with the first host’s contact with HPV (Dillner 1999).

Antibodies against HPV16 L1 were associated with risk for cancers of the oral cavity (odds ratio=1.5, 95%CI =1.1 to 2.1) and the oropharynx (OR=3.5, CI=2.1 to 5.9). Antibodies against HPV16 E6 or E7 were also associated with the risk of oral cavity cancers (odds ratio=2.9, 95%CI =1.7 to 4.8) and the oropharynx (odds ratio=9.2, 95%CI = 4.8 to 17.7) (Rolando 2003)

4.1.13 HPV in cervical cancers

Literature suggests that HPV can be present in up to 100% of patients with cervical carcinoma (Zur Hausen 2000). The mere presence of oncogenic HPV may increase the relative potential for the development of cervical intraepithelial neoplasm by up to 116 times (Watts 1991; Rozendaal 1996)

4.1.14 HPV in head and neck cancers

The association between HPV and head and neck cancers was observed as early as 1960 in larynx. The association with other subsites was suggested by Luning et al. in 1985 (Luning 1985). During the past two decades, the data supporting HPV as a causative agent in the development and progression of Head and Neck cancers has accumulated. Though the true prevalence of HPV DNA is uncertain, studies have estimated up to 60% positivity in HNSCC. The overall incidence of HPV varies depending on tumor location (McKaig 1998; Schwartz 1998; Gillison 2001). The association with the subsite oropharynx notably tonsil is the strongest (Klussmann 2003; Venuti 2004). In tonsillar cancer, about 45 to 100% tumors are HPV positive (Mellin 2000; Gillison 2001; Mellin 2002; Dahlgren 2003; Mellin 2003). It has also been detected in nasopharyngeal carcinomas. Study conducted by the International Agency for Research into Cancer (IARC) involving 9 countries with 1670 cases (cancers of the oral cavity and oropharynx) and 1732 controls confirmed that HPV is likely to play an etiological role in many cancers of the oropharynx and a small group of oral cavity cancers (Herrero 2003). HPV has been detected in 31% to 74% of oral cancers and is also associated with papillomas, condyloma, verrucous leukoplakia, and carcinoma (Kashima 1990; Chang 1991; Vokes 1993; Franceschi 1996; Steinberg 1996). Results from over 60 studies have revealed that the overall HPV prevalence was 25.9% (Kreimer 2005), which was significantly higher in
oropharyngeal SCCs than oral SCCs or laryngeal SCCs. The presence of HPV was found to be higher in poor tumor grades and oropharyngeal site. Precancerous lesions and metastatic lymph nodes have also been shown to contain DNA of the same HPV type as the primary tumor, supporting the involvement of HPV in the development of squamous cell carcinoma.

A recent multi-center study revealed the prevalence of HPV DNA in 18.3% of oropharynx cancers and 3.9% of oral cavity cancers (Herrero 2003). The majority of HPV positive cases had HPV 16, the most common type in genital cancers. Another observation of the study was an increased prevalence of HPV in patients without any carcinogen exposure.

There are studies from India demonstrating the prevalence of HPV ranging from 31% to 39% in head and neck cancers (Pillai 1999; Das 2002; Kumar 2003; Koppikar 2004). Pillai et al have shown 64% of the oral cancer samples (39/61) positive for HPV 16/18. In a study by Balaram et al (Balaram 1995) with 91 Indian oral cancer patients, predominantly betel quid chewers, it was found that HPV DNA was detected in 74% of these lesions, of which 41% had multiple HPV infections. HPV-16 was detected in 15% of oral tumors, 34% of potentially malignant lesions and 31% of the corresponding normal mucosa in the patients with oral lesions (D'Costa 1998). In a study conducted in rural India, it was found that 27.7% of oral cancers were positive for HPV16 (Gheit 2009).

Oral cancers especially occurring in the young has long been thought as etiologically distinct group that is increasing in evidence. However the relationship between HPV and age is controversial. Some reported an association with older age (Lindel 2001) and others have found no association (Klussmann 2001; Herrero 2003). There appears to be a trend for HPV to be detected in younger age groups (Ringstrom 2002). There are reports saying that the median age of the HPV positive group is less compared to the HPV negative group (Gillison 2000). These studies definitely implicate the virus in the progression of the disease and further studies involving different populations will hence help in understanding the role of HPV as a causal agent of cancers of the head and neck.

As detection of HPV DNA in tumor biopsies is alone not a sufficient evidence of causation, molecular biology studies have helped to identify a subset of these cancers that may be the consequence of HPV infection (Gillison 2000; Herrero 2003).
4.1.15 HPV in oral tongue cancers

There are only few studies reporting the association between HPV status and tongue cancers, the frequency ranging between 0-81% (Kantola 2000; Koskinen 2003; Liselotte 2004). The table 4.1 gives the percentage positivity among tongue cancers in different studies and the methods employed (Hönig 1992). And a study by Balaram et al in India among betel quid chewers, the lesions of the tongue had the highest rate 81%(9 of 11) of HPV infection (Balaram 1995). In this study with 91oral cancer patients, 42% and 47% of the oral cancer cases were positive for HPV16 & 18 respectively.

Researchers from UB and Roswell Park Cancer Institute published the first study showing an association between long-standing periodontitis and risk of tongue cancers (Tezal 2007). Studies suggest that periodontal pockets act as reservoirs for human papilloma virus (Hormia 2005).

4.1.16 HPV in premalignant lesions

The prevalence of HPV in premalignant lesions varies between 0% to as high as 85% (Table 4.2) (Fouret 1995; Bouda 2000).

4.1.17 HPV in normal individuals

There is considerable literature to suggest the presence of HPV in normal subjects. This reveals that all infections do not necessarily lead to carcinogenesis. Hence identification of factors leading to carcinogenesis is important. Geographic, exposure related and other behavioral influences play role in individuals with normal mucosa. The prevalence rate varied between 0% and 81% (Jalal 1992; Lawton 1992; Lambropoulos 1997; Smith 1998; Terai 1999). The table 4.3 gives the percentage positivity among normal individuals in different studies and the methods employed. The sensitivity of detection varied depending upon the sample used (buccal scrapings, biopsy or mouthwash) and the technique followed. The finding of high risk HPV in normal mucosa implies that the infection is dormant and could contribute to the development of oral cancer in the future.
Table 4.1: Prevalence of HPV in oral tongue cancers

<table>
<thead>
<tr>
<th>Study</th>
<th>Mode of detection</th>
<th>HPV % (n)</th>
<th>HPV16 % (n)</th>
<th>HPV18 % (n)</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koskinen et al, 2003</td>
<td>Broad spectrum SPF10 PCR and a microtiterplate based probe hybridization assay</td>
<td>73 (11/15)</td>
<td>47 (7/15)</td>
<td>-</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Ribeiro da Silva et al, 2007</td>
<td>PCR</td>
<td>74 (37/50)</td>
<td>-</td>
<td>-</td>
<td>Tongue SCC</td>
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<tr>
<td>E.-M. De Villiers et al, 2006</td>
<td>Southern blot analysis</td>
<td>43 (3/7)</td>
<td>29% (2/7)</td>
<td>-</td>
<td>Tongue cancers</td>
</tr>
<tr>
<td>Masanobu Shindoh et al, 1991</td>
<td>PCR and the dot-blot hybridization technique</td>
<td>33(8/24)</td>
<td>33(8/24)</td>
<td>4(1/24)</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Honig et al, 1992</td>
<td>Non-radioactive ISH</td>
<td>60 (7/12)</td>
<td>-</td>
<td>-</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Balaram et al, 1995</td>
<td>PCR amplification and direct DNA sequencing</td>
<td>81 (9 of 11)</td>
<td>*</td>
<td>*</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Xin-Hua Liang et al, 2008</td>
<td>Consensus primer L1&amp; type specific</td>
<td>1.96 (1/51)</td>
<td>1.96 (1/51)</td>
<td>-</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Im Il Na, et al, 2007</td>
<td>Genotyped using an Easy HPV DNA CHIP</td>
<td>(0/70)</td>
<td>-</td>
<td>-</td>
<td>Tongue SCC</td>
</tr>
<tr>
<td>Liselotte Dahlgreen et al, 2004</td>
<td>Consensus primer L1&amp; type specific</td>
<td>10.9 (12/110)</td>
<td>8 (9/110)</td>
<td>-</td>
<td>Mobile and base tongue cancers</td>
</tr>
</tbody>
</table>

*-See text on HPV in oral tongue cancers
<table>
<thead>
<tr>
<th>Study</th>
<th>Mode of detection</th>
<th>HPV % (no:</th>
<th>HPV16 % (no:</th>
<th>HPV18 % (no:</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holladay et al., 1993</td>
<td>E1 PCR + Slot blot hybridization</td>
<td>29 (13/45)</td>
<td>29 (13/45)</td>
<td>-</td>
<td>CIS, dysplasia, inflammation, hyperplasia</td>
</tr>
<tr>
<td>Nielsen et al., 1996</td>
<td>ISH/HPV16PCR</td>
<td>40.8 (20/49)</td>
<td>20/49</td>
<td>40.8</td>
<td>Dysplasia, leukoplakia</td>
</tr>
<tr>
<td>Bouda et al., 2000</td>
<td>Nested consensus PCR</td>
<td>85 (29/34)</td>
<td>-</td>
<td>-</td>
<td>Hyperplasia, dysplasia</td>
</tr>
<tr>
<td>Sand et al., 2000</td>
<td>Consensus primer L1 &amp; type specific</td>
<td>27.6 (8/29)</td>
<td>-</td>
<td>-</td>
<td>Lichen planus, leukoplakia</td>
</tr>
<tr>
<td>Jenice De Costa et al, 1998</td>
<td>Southern hybridization analysis of the PCR products</td>
<td>-</td>
<td>34 (27/80)</td>
<td>-</td>
<td>Premalignant lesions</td>
</tr>
<tr>
<td>Zeuss et al, 1991</td>
<td>In situ hybridization</td>
<td>0 (0/15)</td>
<td>-</td>
<td>-</td>
<td>Epithelial dysplasia</td>
</tr>
</tbody>
</table>
Table 4.3: Prevalence of HPV in normal controls

<table>
<thead>
<tr>
<th>Study</th>
<th>Mode of detection</th>
<th>HPV % (n)</th>
<th>HPV16 % (n)</th>
<th>HPV18 % (n)</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jalal et al., 1992</td>
<td>HPV-16 specific primers</td>
<td>-</td>
<td>43.8(21/48)</td>
<td>-</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Holladay et al., 1993</td>
<td>E1 PCR + Slot blot hybridization</td>
<td>16.7(1/6)</td>
<td>16.7(1/6)</td>
<td>16.7(1/6)</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Eike et al., 1994</td>
<td>Consensus primer PCR</td>
<td>0 (0/61)</td>
<td>-</td>
<td>-</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Ostwald et al., 1994</td>
<td>Consensus primer-PCR</td>
<td>1 (1/97)</td>
<td>-</td>
<td>-</td>
<td>Normal buccal mucosa</td>
</tr>
<tr>
<td>Cruz et al., 1996</td>
<td>Consensus and type specific primer PCR</td>
<td>0(0/12)</td>
<td>-</td>
<td>-</td>
<td>Normal gingival mucosa</td>
</tr>
<tr>
<td>Nielsen H et al., 1996</td>
<td>DNA-DNA ISH, PCR analysed by Southern blot-HPV16 Probe</td>
<td>(0/20)</td>
<td>-</td>
<td>-</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Lamropoulos et al., 1997</td>
<td>Detection by PCR, typing by Southern blot hybridization</td>
<td>9.5(16/169)</td>
<td>2.4(4/169)</td>
<td>0(0/169)</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Smith et al., 1998</td>
<td>32P-labelled generic probes &amp; sequencing</td>
<td>5 (10/205)</td>
<td>-</td>
<td>-</td>
<td>Mouth rinse collection of cells in the oral cavity</td>
</tr>
<tr>
<td>Terai et al., 1999</td>
<td>PCR(L1) based sequencing</td>
<td>81(30/37)</td>
<td>6.7(2/30)</td>
<td>86.7(26/30)</td>
<td>Scrapings from normal oral mucosa</td>
</tr>
<tr>
<td>Bouda et al., 2000</td>
<td>Nested consensus PCR</td>
<td>0 (0/16)</td>
<td>-</td>
<td>-</td>
<td>Normal oral mucosa</td>
</tr>
<tr>
<td>Study</td>
<td>Methodology</td>
<td>HPV DNA Status</td>
<td>Risk Group</td>
<td>Normal Tissue</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>Sand <em>et al.</em>, 2000</td>
<td>Consensus primer L1 &amp; type specific</td>
<td>0(0/12)</td>
<td>-</td>
<td>Normal oral mucosa</td>
<td></td>
</tr>
<tr>
<td>Nagpal <em>et al.</em>, 2002</td>
<td>Consensus primer</td>
<td>26.9(7/27)</td>
<td>-</td>
<td>Normal oral mucosa</td>
<td></td>
</tr>
<tr>
<td>Priya Koppikar <em>et al.</em>, 2005</td>
<td>sequencing</td>
<td>5(5/102)</td>
<td>-</td>
<td>Exfoliated buccal cells</td>
<td></td>
</tr>
</tbody>
</table>

### 4.1.18 Role with established risk factors

HPV positive tumors are not found exclusively in patients with no risk habits but also in patients with risk habits of tobacco and alcohol consumption. Numerous studies have reported the additive or synergistic effect of tobacco/- alcohol consumption and HPV. In a study by Schwartz *et al* it was reported that the HPV-VLP (Virus like particles) – sero-positive smokers had a higher risk (OR=8.5, 95%CI=5.1-14.4) for HNSCC development compared to the sero-negative smokers(Schwartz 1998). Also it was reported that the effect of smoking and alcohol with HPV VLP serology was multiplicative. Another study by Smith *et al* (Smith 2004)also reported a synergistic effect of heavy tobacco and alcohol use with a positive high risk HPV DNA status (synergy index=6.0, 95%CI=1.1-32.1)

Severe alcohol and tobacco use and poor oral hygiene lead to ulcerations and other tissue damage in the oral cavity and these are significant additional risk factors for oral carcinogenesis (Field 1992). In a healthy individual, HPV infection is a rare event.

### 4.1.19 Clinical implications of HPV as an etiological agent of head and neck cancers

There is no cure for HPV infection although the infection usually resolves on its own. Natural immunity eliminates 70% of the infection within 1 year, 90% within 2 years. It is thought that only 10% of infections involve cancer-causing strains. It is possible that the virus remains in dormant state and could be reactivated years later. Vaccines do not eliminate or reduce pre existing infection. Prophylactic vaccines (Gardasil and Cervarix) against HPV 16 & 18 induce the generation of neutralising antibody to the virus coat.
protein and have shown promising results but will be effective before exposure to the virus. Therapeutic vaccines are aimed at eliminating existing infection with the focus on the main HPV oncogenes, E6 and E7. It is hoped that immune responses against the two oncogenes might eradicate established tumors.

HPV detection may have future implications for the diagnosis, prognosis, therapeutics and prevention of head and neck squamous cell cancers. The biological behavior of HPV positive cancer is significantly different from that of HPV negative cancer. The prognosis is better for HPV positive tonsillar patients than with HPV negative patients (Mellin 2000; Li 2003; LiW 2003; Ritchie 2003).

Prophylactic vaccines based on viral capsids of HPV 16 and 18, and types 6 and 11, responsible for the major types in cervical cancers and genital warts respectively have shown great promise in advanced clinical trials (Street 1999; Devaraj 2003; Tomson 2004; Brinkman 2005) and are expected to become commercially available shortly. A prophylactic vaccine composed of the HPV16 viral capsid protein has recently been shown to prevent persistent HPV16 infection and the development of cervical dysplasia in phase three randomized controlled trials (Koutsky 2002; Harper 2004).

The majority of oral cancers (approximately 90%) caused by HPV are identified as HPV 16 positive. Therefore, HPV-associated oral cancers could be prevented by a prophylactic vaccine if the vaccine was demonstrated to be capable of preventing oral HPV 16 infection. These findings have created new potential opportunities for the primary prevention of oral cancers.

In a recent prospective clinical trial, the association of tumor HPV status with therapeutic response and survival was evaluated among 96 patients with stage III or IV HNSCC of the oropharynx or larynx who participated in an Eastern Cooperative Oncology Group (ECOG) phase II trial. The HPV positive cancer is more sensitive to therapy with higher response rates to chemotherapy, chemoradiation and better overall survival (Fakhry 2008). The authors insist on stratifying the head and neck cancer patients based on tumor HPV status for future clinical trials. Though currently a diagnosis of HPV-positive malignancy is clinically relevant for prognostication, it may have future diagnosis and therapeutic implications, as well as implications for prevention and screening.
4.1.20 Variants

Any change in the sequences of the genes E6 & E7 may lead to altered biological function of the proteins encoded by the genes, which in turn influence the natural history of the infection. If amino acid changes in the E6 protein are located in regions critical for immune recognition, vaccines developed for a particular variant virus type may have a reduced efficiency against other variants. Local population immunogenetics adds an additional layer of complexity to this challenge. Identification of HPV variants is important for the rational design of newer diagnostic and therapeutic interventions in cervical cancer as well as for vaccine development strategies.

4.2.1 Primary objective

To determine the prevalence of HPV in tongue cancer patients with and without risk habits.

4.2.2 Secondary objectives

1. To identify whether HPV infection is episomal or integrated.
2. To correlate the expression of cell cycle regulatory proteins (p53, cyclin D1, p16) and determine the cell cycle pathway (either E6 - p53 or E7 - pRb) involved in HPV carcinogenesis.
3. To correlate the HPV infection with the histological change (koilocytosis)

4.2.3 Methodology Adopted

HPV DNA analysis

DNA

<table>
<thead>
<tr>
<th>PCR consensus primer</th>
<th>MY11/9, GP5+/6+, CPI/II</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR type specific primer HPV 16</td>
<td>HPV 16 (L1), E6</td>
</tr>
<tr>
<td>Viral integration PCR E2 primer</td>
<td>Confirmation by ISH, SEQUENCING</td>
</tr>
</tbody>
</table>
HPV protein analysis

<table>
<thead>
<tr>
<th>IHC studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6, p53, E7, pRb, p16, cyclin D1, Notch-1, EGFR</td>
</tr>
</tbody>
</table>

Koilocytosis

| Routine H & E |

Briefly, DNA was isolated from the tissues and PCR was done with the Consensus primer MY11/09. The samples negative for the above said primer were repeated with two other primers-GP5+/6+ to exclude false negatives. Then PCR was done with type specific primer (HPVL1& E6). After purifying the DNA from the gel, few representative PCR samples were subjected to DNA sequencing by dye dideoxy termination method and compared with a standard sequence (GenBank K02718/HPV16R) to confirm the specificity of the amplified product. Mere presence of HPV 16 DNA does not imply its role in carcinogenesis. To assess the integrity of HPV in the cell, PCR was done with E2 primers. Also the samples positive for HPV16 were subjected to catalyzed signal amplified colorimetric in situ hybridization to confirm the presence of HPV16 DNA in the tumor cell.

There are two major pathways involved in HPV carcinogenesis- E6-p53 and E7-pRb. Hence immunohistochemical analysis of the proteins was done in HPV positive patients to detect the predominant pathway involved in HPV mediated carcinogenesis.

Since the presence of koilocyte is pathognomonic of HPV infection, it was evaluated in hematoxylin-eosin slides.

4.3 Materials and Methods

4.3.1 Study Subjects

A case control study was designed to evaluate the presence of HPV in oral tongue cancer (Fig 4.5). Sixty consecutive patients with histologically confirmed squamous cell carcinoma of the oral tongue accrued from December 2004 to August 2007, with the tumor tissue archived in the Head and Neck biorepository, formed the study group. Archiving of specimens was undertaken after obtaining institutional review board approval. The tumor samples were collected after obtaining informed consent. The
specimens were obtained during surgery, snap frozen in liquid nitrogen and stored at –80°C. Thirty of these patients were with risk habits and thirty without risk habits. The control groups (n=46) were subjects enrolled from the Dental College, AIMS, who underwent routine dental extractions. The subjects were age and sex matched. Of which 25 subjects were with risk habits and 21 were with out risk habits. Samples were processed as per standard procedure and Hematoxylin and eosin staining was carried out on 1 or 2 sections to confirm that the epithelium was normal.

![Figure 4.5: Case control study – schematic representation](image)

Demographic details, risk habits, tumor characteristics (stage, pathology) and disease status were obtained from the patient records. Presence of risk habits were defined as regular use of tobacco, pan chewing, or consumption of alcohol at least five days per week for a minimum period of two years.

Survival and recurrence were measured in months from the date of diagnosis until death or until the patient was last known to be alive. Recurrence in patients was measured in years from the date of diagnosis until recurrence. Dates of death or dates last known to be alive were available from the medical records, the department follow-up register.

4.3.2 Statistical analysis

Chi-square test was employed to test the association of different variables between males and females and between HPV positive and negative patients. Independent sample t test
was employed to compare the mean age of cases and controls and HPV positive and negative cases.

The overall survival analysis was done. Survival curves were estimated by the Kaplan–Meier method and the difference between curves was tested by the log-rank test. All survival curves were generated in SPSS version 11. Statistical analyses were performed using Systat12.

4.3.3 HPV detection and confirmation

The following molecular methods were performed for HPV detection and confirmation.

1) DNA Isolation
2) Polymerase chain Reaction
3) Immunohistochemistry
4) In Situ-hybridization.
5) Sequencing

4.3.3.1 DNA Isolation from tissue

Reagents
1. Proteinase K (pH-8.0)  
   20mg/ml in sterile 50mM Tris and 1.5mM Calcium acetate.
2. 1X TE Buffer (pH-8.0)  
   100mM Tris (0.060g) and 10mM EDTA (0.018g)
3. 20% Sodium Dodecyl Sulphate
4. 3M Sodium Acetate (pH-5.2)
5. Chloroform-isoamyl alcohol (24:1)
6. RNAase (20mg/ml)
7. Tris Saturated Phenol (pH-8.0)
8. 95% and 70% Ethanol
9. 1X TNE buffer (pH-9.0)  
   0.05M Tris Cl, 0.15M NaCl and 5mM EDTA
Method

Tissue was homogenized with 500µl of 1X TNE buffer and to the homogenate 50µl of 20% SDS and 12.5µl of Proteinase K were added and incubated at 55°C for 3 hours. To remove RNA contamination, 0.5µl of RNase was added and again incubated 55°C for 15 min. Phenol and Chloroform-Isoamyl alcohol was added in 1:1 ratio (250µl of each) and incubation was further continued for 30 min at room temperature with continuous shaking. The sample was centrifuged at 14000 rpm for 15 minutes. The upper aqueous phase was transferred to another micro centrifuge tube and 500µl of Chloroform-Isoamyl alcohol was added and centrifuged at 14000rpm for 10 minutes. To precipitate the DNA, 25µl of 3M sodium acetate and 1ml of absolute ethanol were added to the aqueous layer and mixed well and centrifuged at 14000 rpm for 10 min. The precipitated DNA was pelleted down by centrifugation and washed with 1ml of 70% ethanol and centrifuged at 10000 rpm. The dried DNA pellet was dissolved in 50µl of 1X TE buffer and stored overnight at -20°C.

DNA Quantification

The isolated DNA samples were quantified using DNA/RNA quantifier (Gene Quant PRO, AP Biotech USA) at wavelengths 230nm, 260nm, 280nm and 320nm (DNA program). The 260nm value indicated the quantity of DNA samples and the 260nm/280nm ratio provided the quality.

4.3.3.2 HPV Detection by Polymerase chain Reaction (PCR)

Reagents

1. PCR grade MilliQ water
2. Taq DNA polymerase (5U/µl)
3. 10X PCR Buffer
4. dNTP mix (2.5mM)
5. Forward primer and Reverse primer (5 pmol/µl each)
Table 4.4: List of primers for HPV PCR analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>5* → 3* Nucleotide Sequence</th>
<th>Target</th>
<th>Strand</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-F²</td>
<td>TCACCAGGGCTGCTTTTAACTC</td>
<td>GAPDH</td>
<td>+</td>
<td>150</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>ATGACAAGCTTCCCGTTCTCAG</td>
<td>GAPDH</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MY 11¹</td>
<td>GCMCAGGGWCATAAYAATGG</td>
<td>HPV L1</td>
<td>+</td>
<td>450</td>
</tr>
<tr>
<td>MY09</td>
<td>CGTCCMARRGGAWACTGATC</td>
<td>HPV L1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>GP5⁺¹</td>
<td>5’ TTTGTTACTGTGAGATACTAC 3’</td>
<td>HPV L1</td>
<td>+</td>
<td>150</td>
</tr>
<tr>
<td>GP6⁺</td>
<td>5’ GAAAAATAAAACTGAAATCATT 3’</td>
<td>HPV L1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CP I¹</td>
<td>5’ TTATCWATATGCCCAYTGTACC 3’</td>
<td>HPV E1</td>
<td>+</td>
<td>188</td>
</tr>
<tr>
<td>CP II</td>
<td>5’ATGTTAATWSAGCCWCCAAAATT 3’</td>
<td>HPV E1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HPV16 F²</td>
<td>TGCTAGTGCTATGACGCAAAATT</td>
<td>HPV16 L1</td>
<td>+</td>
<td>152</td>
</tr>
<tr>
<td>HPV16 R</td>
<td>ATTTACTGCAACATTTGACT</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E6 F³</td>
<td>AAGGCGTAACCCAGAATTCGTT</td>
<td>E6</td>
<td>+</td>
<td>209</td>
</tr>
<tr>
<td>E6 R</td>
<td>TTGTCAGCTGCAATTCA</td>
<td>E6</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E2 F³</td>
<td>CTTGGGCACCAAGAAAC</td>
<td>E2</td>
<td>+</td>
<td>351</td>
</tr>
<tr>
<td>E2 R</td>
<td>TTGGTCACGGCCATTCA</td>
<td>E2</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>


**Method**
The PCR reaction mix was prepared with 50-100ng/µl of the DNA sample, 5pmol each of forward and reverse primers (Table 4.4), 1X PCR buffer, 200µmol of dNTP and 1 unit of Taq polymerase. The cycling conditions are given in the following table.
Table 4.5: Cycling conditions for HPV PCR analysis

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Initial Denaturation</th>
<th>Denaturation</th>
<th>annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY11/09</td>
<td></td>
<td>40cycles</td>
<td>57.8°C-1min</td>
<td>72°C –1min</td>
<td></td>
</tr>
<tr>
<td>GP5+/6+</td>
<td>94°C-5min</td>
<td></td>
<td>57.8°C-1min</td>
<td>72°C –30sec</td>
<td></td>
</tr>
<tr>
<td>CPI/IIG</td>
<td>94°C-1min</td>
<td>61.7°C-1min</td>
<td>72°C –30sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16L1</td>
<td></td>
<td>55°C-1min</td>
<td>72°C –1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>94°C-1min</td>
<td>57.8°C-1min</td>
<td>72°C –1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td>57.8°C-1min</td>
<td>72°C –1min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Among the DNA samples isolated from clinical specimens and controls, only those successfully amplified GAPDH (house keeping gene) fragment were considered for HPV analysis. The presence of HPV DNA was confirmed with the general primers for the conserved L1 or E1 viral regions namely MY11/09, GP5+/6+, CPI/IIG (which detect multiple HPV types 6, 11, 13, 16, 18, 26, 31–35, 39, 40, 42–45, 51–59, 61, 62, 64, 66–74, 81–85). In all PCR reactions HPV viral DNA was used as positive control and absence of template DNA was used as negative control. Samples negative in the MY11/09 PCR were confirmed with GP5+/6+ and CPI/CPII primers to exclude false negatives.

Samples positive for HPV infection with the consensus primers were further confirmed for HPV16 type with HPV 16L1 & E6 specific primers.
**Agarose Gel Electrophoresis**

**Reagents**

1. 0.5X TBE (Tris-borate-EDTA) stock buffer

   A concentrated (5X) stock solution of TBE was prepared by weighing 54 g Tris base (FW = 121.14) and 27.5 g boric acid (FW = 61.83) and dissolving both in approximately 900 mL deionized water. And 20 mL of 0.5 M EDTA (pH 8.0) was added to adjust the solution to a final volume of 1 L. This solution was stored at room temperature.

2. 1.5% Agarose

3. Ethidium bromide (10 mg/ml)

4. Gel loading dye
   - 40% Sucrose
   - 0.25% Xylene cyanol.
   - 0.25% Bromophenol blue

   Agarose gel (1.5%) was prepared by dissolving 1.5 g of agarose in 100 ml of 0.5X TBE buffer in a conical flask by heating. To the melted agarose gel 2 µl of Ethidium bromide (10 mg/ml) was added and the gel was poured into the gel platform without any air bubbles. After solidification, 6 µl of sample along with 1 µl of loading dye was loaded into the wells. The gel was electrophoresed at a constant voltage of 50V for a period of 1 hour.

**4.3.3.3 Immunohistochemistry**

Slides from all blocks were reviewed by a pathologist to select the most representative areas of the tumor for further proceeding to IHC, ISH, Koilocytosis. Only blocks with estimates of atleast 70% tumor cells were included. Sections were cut using a clean pair of gloves for each block. Sections from different blocks were each cut using a new microtome blade to prevent cross contamination from the blade.

**Reagents**

1. Xylene

2. 100% alcohol
3. 75% alcohol
4. 50% alcohol
5. Deionised water
6. PBS 1L (1X) (pH 7.4)
   a. Sodium chloride - 8g
   b. Potassium chloride - 0.2g
   c. Di Sodium Hydrogen phosphate (Na$_2$HPO$_4$) - 1.44g
   d. Potassium di hydrogen phosphate (KH$_2$PO$_4$) - 0.24g
   All the above were mixed in 800ml of D.H$_2$O and made up to 1000ml.
7. 3% H$_2$O$_2$ – 3ml H$_2$O$_2$ diluted in 97ml of methanol
8. Citrate unmasking solution (pH 6.0)
   Trisodium citrate – 2.94 g to 1 litre of d. H$_2$O
9. Citrate wash buffer (pH 7.4)
   1X PBS + 0.1% Tween 20
10. Blocking agent:
    The universal blocking reagent for IHC, ISH, EIA and Blots is 3% BSA in citrate wash buffer or Power block™ which contains casein and Sodium azide (Biogenex – 25ml).

**IHC in Paraffin embedded blocks**

The 3-5µ sections were prepared from paraffin embedded blocks as follows

1. Deparaffinisation
   The sections were cut, floated on demineralised water and placed on the pretreated slides. The overnight incubation was carried out at 37° C. After incubation the sections were baked for 30-45 min at 65°C.
2. Dewaxing in xylene
   The sections were treated twice with fresh xylene for 5 min for deparaffinisation
3. Rehydration

The sections were rehydrated with absolute ethanol for 5 min and then thrice with 75% ethanol for 5 min. This was followed by treatment with 3% \( \text{H}_2\text{O}_2 \) in methanol for 10 min then thrice in 50% ethanol for 5 min. Finally the slides were washed in running water. The slides were kept in citrate antigen unmasking solution (pH-6.0) in the microwave, allowed it to boil for 5 minutes, cooled for one minute and the process is repeated once. After unmasking the antigen the slides were incubated in the buffer at room temperature for 20 minutes. This was followed by incubation twice in deionised water for 5 min. The slides were then washed in citrate wash buffer for 5 minutes.

4. Staining

The sections were treated with blocking agent for 30 minutes at RT. After wiping the slide, the primary antibody was added and incubated overnight at 4°C. At the end of incubation the primary antibody was drained and the slides were washed thrice in PBS buffer for 5 min. The secondary antibody (Dako kit –anti-mouse and anti-rabbit) was added and after incubation for 30 minutes it was drained. The slides were washed thrice in PBS buffer for 5 min. The bound antibodies were visualized with DAB (3.3′-Di Amino Benzidine) provided in the kit (1:50 diln) after a 10 min treatment. The sections were counterstained with hematoxylin for 1 minute. The slides were air dried and dipped in xylene before mounting with DPX mountant. The details of the dilution of the antibodies and their working condition are given in Table 4.6.

Immunohistochemistry evaluation

Immunostaining for all antibodies was quantified by counting the cells exhibiting positive staining with a given antibody in 10 randomly selected high-power fields (X40) and the results were expressed as percentages of all epithelial cells in those areas (minimum of 2,000 cells). For E6 and E7 expression, only the presence or absence of immunoreactivity was considered. For other proteins, it was considered significant when characteristic nuclear/cellular immunoreactivity was seen in at least 10% of the tumor cells. In addition to this, an expression index was also created. This was done by classifying the protein expression into four categories. Thus, grade 1 included those samples with less than 10% positive cells (0=insignificant); grade 2 included samples showing 11–30% positive cells (1+mild expression), grade 3 included samples showing 31–60% positive cells (2+moderate expression), and grade 4, samples showing more than 61% positive cells.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>dilution</th>
<th>Primary antibody incubation</th>
<th>Staining pattern</th>
<th>Positive control</th>
<th>Manufacturer</th>
<th>Antigen retrieval with trisodium citrate (pH 6.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>MMAb</td>
<td>1:25</td>
<td>Overnight at 4°C</td>
<td>Nuclear and cytoplasmic</td>
<td>Cervical cancer</td>
<td>Santa Cruz</td>
<td>Heating in a microwave</td>
</tr>
<tr>
<td>E7</td>
<td>MMAb</td>
<td>1:100</td>
<td>1 hour at 37°C</td>
<td>Nuclear and cytoplasmic</td>
<td>Cervical cancer</td>
<td>Santa Cruz</td>
<td>Heating in a hot chamber at 95°C</td>
</tr>
<tr>
<td>p53</td>
<td>MMAb</td>
<td>1:250</td>
<td>1 hour at 37°C</td>
<td>Nuclear</td>
<td>Breast cancer</td>
<td>Neomarkers</td>
<td>Heating in a hot chamber at 95°C</td>
</tr>
<tr>
<td>pRb</td>
<td>MMAb</td>
<td>1:25</td>
<td>Overnight at 37°C</td>
<td>Nuclear</td>
<td>Breast cancer</td>
<td>Novo Castro</td>
<td>Heating in a microwave</td>
</tr>
<tr>
<td>p16</td>
<td>MMAb</td>
<td>1:50</td>
<td>Overnight at 4°C</td>
<td>Nuclear and cytoplasmic</td>
<td>Cervical cancer</td>
<td>Santa Cruz</td>
<td>Heating in a microwave</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>Rabbit IgG</td>
<td>1:50</td>
<td>1 hour at 37°C</td>
<td>Nuclear</td>
<td>Cervical cancer</td>
<td>Neomarkers</td>
<td>Heating in a hot chamber at 95°C</td>
</tr>
<tr>
<td>Notch-1</td>
<td>MMAb</td>
<td>1:50</td>
<td>1 hour at 37°C</td>
<td>Nuclear, cytoplasmic, membranous</td>
<td>Breast cancer</td>
<td>Neomarkers</td>
<td>Heating in a hot chamber at 95°C</td>
</tr>
<tr>
<td>EGFR</td>
<td>Rabbit/Goat IgG</td>
<td>1:50</td>
<td>Overnight at 4°C</td>
<td>Membranous</td>
<td>HNSCC</td>
<td>Santa Cruz</td>
<td>Heating in a microwave</td>
</tr>
</tbody>
</table>
Also the intensity of staining was graded as mild (1+), moderate (2+) and intense (3+).

4.3.3.4 In Situ-hybridization

All tissue sections positive for HPV 16 DNA by PCR were subjected to In-situ hybridization. Catalyzed signal amplification colorimetric-in situ hybridization (CSAC-ISH) was performed as described below. A tissue block from a confirmed HPV-positive cervix carcinoma was used as positive control. For the negative control, the probe was omitted. The whole slide was scanned and the presence or absence of signal was evaluated. Dark blue punctate or diffuse nuclear staining was regarded as being positive for HPV. HPV present as episomes leads to diffuse staining, while integration of viral DNA into host cell DNA gives punctate signals.

Table 4.7: Details of the probe used in ISH

<table>
<thead>
<tr>
<th>Name</th>
<th>5* → 3* nucleotide sequence</th>
<th>Target</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16 oligo probe*</td>
<td>GCAAACCACCTATAGGGGAACACTGGGGCA</td>
<td>L1</td>
<td>30mer</td>
</tr>
</tbody>
</table>

Source*-(May, Lau et al. 1996)

Reagents

1. Xylene
2. 100% alcohol
3. 90% alcohol
4. 75% alcohol
5. Deionised water
6. PBS 1L (1X) (pH 7.4)
   a. Sodium chloride - 8 g
   b. Potassium chloride – 0.2 g
   c. Di Sodium Hydrogen phosphate (Na₂HPO₄) – 1.44 g
d. Potassium di hydrogen phosphate (KH$_2$PO$_4$) – 0.24 g

All the above were mixed in 800ml of D.H$_2$O and made up to 1000ml.

7. PBS with 0.3% Triton X

8. Proteinase K in PBS (125 µg/ml).

9. 0.4% formaldehyde

10. H$_2$O$_2$

11. Hybridization buffer

   4X SSC + 50% deionised formamide

12. Probe cocktail (7 ml)

   a. 40% deionised formamide - 2800 µl
   b. 0.25% BLOTTO-17.5 mg
   c. 20X SSC - 1400 µl
   d. 1 mg/ml sheared salmon sperm DNA -7 mg
   e. 10 mM DTT 10.78 mg
   f. 10% dextran sulfate - 0.7 g,

The biotin labeled probe (20ng/µl) was added to the probe cocktail.

13. 2X SSC, pH 7.2-7.4

14. 0.2X SSC, pH 7.2-7.4

15. 0.1X SSC, pH 7.2-7.4

16. TNB Blocking Buffer

   a. 0.1 M Tris-HCl, pH 7.5
   b. 0.15 M NaCl
   c. 0.5% Blocking Reagent (supplied in kit)

17. TNT wash buffer

   a. 0.1 M Tris-HCl, (pH 7.5)
   b. 0.15 M NaCl
Other wash buffers such as PBS may be used. Substitution of 0.3% Triton X-100 for the 0.05% Tween-20 is also possible.

18. DNP amplification reagent

19. Anti-DNP-AP

20. BCIP/NBT

a. 200 µl of NBT/BCIP was mixed in 10ml of diluent and stored at 4°C. The diluent was prepared with 0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl₂

Method

The 5µ sections (at least 3 in number) for each block were prepared.

1. Deparaffinisation

The sections were cut, floated on demineralised water and placed on the pretreated slides. The sections were baked for 45 min at 60°C.

2. Dewaxing in xylene

The sections were treated twice with fresh xylene for 15 min for deparaffinisation

3. Rehydration

The sections were rehydrated thrice with absolute ethanol for 5 min then again thrice with 75% ethanol for 5 min. This was followed by treatment with 3% H₂O₂ (in methanol) for 30min followed by rehydration thrice in 50% ethanol for 5 min. Finally the slides were rinsed in deionized water for 5 min. The slides were then washed in PBS with 0.3% Triton X-100 for 5 min.

4. Proteinase K treatment

An aliquoted frozen stock solution of Proteinase K (10 mg/ml, in H₂O) was thawed and diluted to the working concentration of 0.125 mg/ml in PBS. Each section was treated with 20–30 µl of the working solution of Proteinase K for 20 min at 37°C. During the proteolytic digestion the sections were covered with cover slips and placed in a humid chamber. After the treatment with Proteinase K, the cover slip was removed and the slides were washed twice with PBS for 5 minutes. The sections were then fixed in 0.4% formaldehyde for 5 min at 4°C and washed twice with PBS for 5 minutes.
5. Prehybridization

The sections were then incubated in pre-hybridization buffer (4X SSC and 50% deionised formamide) for 30 min at 37°C.

6. Hybridization

The pre-hybridization solution was drained from the slides and the sections were overlaid with 5-10 µl of the probe cocktail. The negative control was prepared by covering one section from each block with a “blind” probe cocktail, i.e. a probe cocktail containing all the ingredients, except the labeled HPV DNA. The cover slips were placed over each section and the DNA was denatured by placing the slides in the hybridization chamber at 95°C for 10 min continuously. The slides were incubated at 42°C overnight in a humid chamber.

7. Washes

After removing the cover slips the slides were treated with increasing stringencies of SSC i.e, in 2X SSC at 37°C for 10 min, 0.2X SSC at RT for 10 min and 0.1X SSC at RT for 10 min.

8. Detection of Hybrids

1. Blocking Step

The sections on the slides blocked in TNB buffer at RT for 30 min.

2. Incorporation of HRP

The slides were incubated with appropriate HRP reagent SA-HRP (1:1000 in TNB buffer) for 30 min. @ RT. Then the slides washed thrice in TNT buffer for 5 min at RT with agitation

3. TSA Plus Amplification

The TSA plus amplification was performed with TSA™ DNP (AP) System NEL746A001KT (Perkin Elmer, USA) using DNP amplification reagent kit which consists of Anti–DNP-AP (150µl), Blocking Reagent (3gm), 1X Plus Amplification Diluent (15ml). This kit is sufficient for 50-150 slides.

The slides were incubated in DNP Amplification Reagent working solution (1:50diln) for 10 min at RT then washed thrice in TNT buffer for 5 min at RT for 5 min with agitation.
4. Chromogenic Visualization

i) The slides were incubated in anti-DNP-AP (1: 100 in TNB buffer) for 30 min at RT in a humidified chamber and washed thrice in TNT buffer for 5 min at RT for 5 min with agitation. The BCIP/NBT solution was added and incubated for 10-30 minutes in the dark. The slides were washed 3 times with PBS for 5 min. Finally the slides were counter stained with nuclear fast red, washed with water, dehydrated and mounted for microscopy. The slides should not be dipped in xylene in this case.

4.3.3.5 Koilocytosis

The presence of koilocytes was evaluated in the hematoxylin-eosin stained slides with optical microscopy.

4.3.3.6 Sequencing

The PCR products amplified with HPV consensus primers (MY 09/11, CP I/II), E6, E2, and L1 were purified by gel extraction and subjected to sequencing.

DNA gel extraction

The PCR products were run by agarose gel electrophoresis. The prominent band confirming the amplicon was cut out and transferred to a 1.5 ml microcentrifuge tube. The gel was dissolved with 5 volumes of buffer GB (approximately gel slice of 0.1 g has a volume of 100 µl) and incubated at 50°C. The pH was neutralized with the addition of 5 µl of 3 M sodium acetate (pH 5.2) and mixed thoroughly. The sample color changing from pink to yellow indicated the neutralization. The sample was transferred into a column (max 1ml) assembled in a clean 2ml collection tube and centrifuged at 10,000 x g for 1 min. The flow through was discarded. The remaining sample was added into the column by repeating the above step. The column was washed with the addition of 750 µl of Wash buffer, incubated at room temperature for 2 minutes to remove any residual agarose. The column was centrifuged at 10,000 x g for 1 minute. The flow through was discarded. The column was centrifuged at 10,000 x g for 1 minute to remove residual ethanol. The column was placed into a clean 1.5 ml microcentrifuge tube, 50µl of Elution buffer (10 Mm Tris-HCl, pH 8.5) was directly added on the column membrane and incubated at room temperature for 2 min. The DNA was eluted by centrifuging the column at 10,000 x g for 1 minute. The eluted DNA was quantified as mentioned before and stored at –20°C.
Sequencing by chain termination method

The sequencing of the PCR products using forward and reverse primers was performed by chain termination method (Sanger 1977) using Bigdye Terminator kit V3.1 (Applied Biosystems, USA). The sequencing reaction performed was as follows: To each well in the 96-well reaction plate 1 µl of Bigdye Terminator, 2 µl of sequencing buffer, 1 µl of primer (1 pmol/µl), 1µl of PCR product (50 ng), 1 µl of betaine (5 M) and 4 µl of PCR grade water were added. The plate was kept in the thermal cycler and cycle sequencing was performed with the following thermal conditions: 95°C for 5 min, 30 cycles of 95°C for 1 min, 50 °C for 30 sec, 62°C for 1 min and final extension at 72°C for 5 min (seq cycle is different from the usual PCR cycle). The extension products were purified by adding 2.5 µl of EDTA (125 mM) and 30 µl of absolute ethanol, incubation at room temperature for 15 min and centrifugation at 2500 x g at 4°C for 30 min. The supernatant was removed and the plate was subjected to reverse spin to remove any residual ethanol. The purified extension products were washed by adding 30 µl of 70% ethanol and centrifugation at 1650 x g at 4°C for 15 min. The final pellet was dissolved in 10 µl of HiDi formamide and the plate was placed in the sequencer. The sequencer detector V3.0 performed the sequencing and the chromatogram files generated were stored in a specific location in the sequencing server. The data analysis was performed with Sequence Analysis V5.0 program and the chromatograms that were passed the analysis (<50% QV ratio and with nonoverlapping peaks) were certified. The sequences obtained were further confirmed by BLAST analysis, comparing the sequences with the existing HPV sequences from the database. It was confirmed that the sequences were matching with the respective genes of HPV.

4.4 Results

The results will be discussed under the following headings:

1. Details of study groups
2. Prevalence of HPV
3. HPV Integration
4. Detection of HPV by Koilocytosis
5. Pathways involved in carcinogenesis
4.4.1 Details of study groups

This case control study consisted of 60 cases and 46 controls. Among the cases, 68% were males and 32% were females. The patients’ age ranged from 28 to 83 years (mean = 55 years, SD=13.3). Among the cases, 30 (50%) had at least one of the established risk habits. Among the controls, males were 76% and females were 24%, the age ranged from 27 to 80 years (mean = 52 years, SD=13.8). Twenty-four (25%) of the controls had at least one of the risk factors. The cases and controls are statistically comparable with respect to gender, mean age and the prevalence of risk habits (Tables 4.8, 4.9 and 4.10).

Table 4.8: Comparison of cases and controls by gender

<table>
<thead>
<tr>
<th></th>
<th>CASES n (%)</th>
<th>CONTROLS n (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MALE</td>
<td>41 (68.3)</td>
<td>35 (76.1)</td>
<td>0.508</td>
</tr>
<tr>
<td>FEMALE</td>
<td>19 (31.7)</td>
<td>11 (23.9)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9: Comparison of mean age of cases & controls

<table>
<thead>
<tr>
<th></th>
<th>MALES</th>
<th>FEMALES</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>n</td>
<td>41</td>
<td>35</td>
<td>19</td>
</tr>
<tr>
<td>Mean</td>
<td>55.49</td>
<td>54.71</td>
<td>54.11</td>
</tr>
<tr>
<td>SD</td>
<td>11.9</td>
<td>13.6</td>
<td>16.1</td>
</tr>
</tbody>
</table>

p = 0.792     p = 0.114     p = 0.313

Table 4.10: Presence of risk habits in cases and controls-gender wise

<table>
<thead>
<tr>
<th></th>
<th>MALES</th>
<th>FEMALES</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk factors</td>
<td>Cases (n = 41)</td>
<td>Controls (n = 35)</td>
<td>Cases (n = 19)</td>
</tr>
<tr>
<td>n (%)</td>
<td>27 (65.9)</td>
<td>24 (68.6)</td>
<td>3 (15.8)</td>
</tr>
</tbody>
</table>

p = 0.995     p = 1.000     p = 0.804
The characteristics of the cancer cases (n = 60) are presented in table 4.11. Tumor staging was assessed according to the American Joint Committee on Cancer staging criteria. Thirty two percent presented at stage I, 18% at stage II, 22% at stage III and 28% at stage IVA. Histological grading was performed following the WHO criteria for oral squamous cell carcinomas. There was no significant difference between males and females with respect to staging and grading among the cancer cases.

**Table 4.11: Characteristics of cases**

<table>
<thead>
<tr>
<th></th>
<th>Males n = 41</th>
<th>Females n = 19</th>
<th>P</th>
<th>Total n = 60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Grading of cancers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wdscc*</td>
<td>23 (56.1)</td>
<td>11 (57.9)</td>
<td></td>
<td>34 (56.7)</td>
</tr>
<tr>
<td>mdscc#</td>
<td>14 (34.1)</td>
<td>8 (42.1)</td>
<td>0.329</td>
<td>22 (36.7)</td>
</tr>
<tr>
<td>pdscc@</td>
<td>4 (9.8)</td>
<td>0 (0)</td>
<td></td>
<td>4 (6.6)</td>
</tr>
<tr>
<td><strong>Staging of cancers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14 (34.1)</td>
<td>5 (26.3)</td>
<td></td>
<td>19 (31.7)</td>
</tr>
<tr>
<td>II</td>
<td>5 (12.2)</td>
<td>6 (31.5)</td>
<td>0.353</td>
<td>11 (18.3)</td>
</tr>
<tr>
<td>III</td>
<td>9 (22.0)</td>
<td>4 (21.1)</td>
<td></td>
<td>13 (21.7)</td>
</tr>
<tr>
<td>IV</td>
<td>13 (31.7)</td>
<td>4 (21.1)</td>
<td></td>
<td>17 (28.3)</td>
</tr>
</tbody>
</table>

* - wdscc-well differentiated squamous cell carcinoma
# - mdscc-moderately differentiated squamous cell carcinoma
@ - pdscc- poorly differentiated squamous cell carcinoma

Survival analysis was performed as detailed in materials and methods section 4.3.2. The overall survival rate was 80%. Twelve patients (20%) had recurrence, 5 had in the primary
site, 4 had nodal recurrence and 3 skeletal metastasis. The overall mean survival was 43 months (CI: 38.12-46.48). Log rank test was performed to assess the relationship between different variables and survival (Table 4.12). The variables included in the analyses are i) age (≤45, >45), ii) sex (male, female), iii) risk factors (absent, present), iv) stage of tumor (1-stage I & II, 2-stage III& IV), v) grade of tumor (1-wdsc, 2-mdsc+pdscc). There was no statistically significant difference in the survival experience with respect to different groups among the test variables.

![Figure 4.6: Overall survival of the patients-curves based on Kaplan-Meier method.](image)
The plus marks on curves indicate censored observations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean survival time in months (CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>41.32 (36.58-46.08)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>42.15 (37.74-46.56)</td>
</tr>
<tr>
<td>Age</td>
<td>≤45</td>
<td>43.15 (39.37-49.63)</td>
</tr>
<tr>
<td></td>
<td>&gt;45</td>
<td>40.93 (39.53-45.92)</td>
</tr>
<tr>
<td>Risk factors</td>
<td>Absent</td>
<td>50.27(43.76-56.78)</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>44.98(42.58-47.37)</td>
</tr>
<tr>
<td>Stage of cancers*</td>
<td>1</td>
<td>43.59(39.71-47.48)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>39.48(33.26-45.71)</td>
</tr>
<tr>
<td>Grading of cancers*</td>
<td>1</td>
<td>42.84(38.72-46.96)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>39.48(33.26-45.71)</td>
</tr>
</tbody>
</table>

* - see text for grouping
4.4.2 Prevalence of HPV

PCR was employed as the gold standard method to determine the prevalence of HPV in the study groups. Briefly, DNA was isolated from the tissues and PCR was done with the Consensus primers followed by typing with HPV 16 specific primers. The amplicons were confirmed by sequencing. All samples negative with HPV primers were checked with GAPDH primers to ensure that the DNA is amplifiable (Fig. 4.7).

Fifty percent of cases (30/60) and sixty seven percent of controls (31/46) were positive for HPV infection in general (Fig. 4.7). When the samples were tested for oncogenic HPV 16 positivity by PCR, it was found that 48.3% of cases were positive for HPV16 (Fig 4.8), while none of the controls were positive.

Sequencing was performed for representative amplicons for each primer pair as per the procedure in materials and methods section and the sequences obtained were further confirmed by Basic Local Alignment Search Tool (BLAST, NCBI) analysis with the existing sequences in the database. The sequences corresponded to that of the respective genes of HPV (Fig 4.9).

4.4.3 HPV Integration

The integration of the HPV DNA into the host cell was detected by PCR with E2-specific primer (Fig 4.8). If the virus is integrated, E2 will be disrupted; hence the presence of HPV infection with loss of E2 was considered indicative of integration. Eighty three percent (24/29) of the HPV cases showed integration by E2 PCR.

Further confirmation of integration of the HPV DNA in the tumor DNA was performed by Catalyzed Signal Amplification Colorimetric/in situ hybridization (CSAC-ISH). It was found that 67% of the PCR positive cases showed integration as revealed by punctate signals in the tumor nuclei (Fig 4.10).
Figure 4.7: Polymerase chain amplification of HPV with GAPDH and consensus primers in tumor and control DNA samples

Legend: The genomic DNA isolated from the patients and controls samples were amplified with GAPDH and HPV consensus primers as described under materials and methods (section 4.3)

Lane 1: 100 bp DNA ladder, Lane 2: HPV positive control,
Lanes 3-5: Patient samples, Lanes 6-8: Control samples
Figure 4.8: Polymerase chain amplification of HPV E6, E2 and L1 in genomic DNA positive for HPV consensus primers

Legend: The genomic DNA that were positive for HPV consensus primers were amplified with HPV L1, E6 and E2 primers as described under materials and methods (section 4.3)

L1
Lane 1: HPV positive control; Lane 2-16: HPV consensus primer positive DNA

E2 & E6
Lane 1: HPV positive control, Lane 2-8: HPV consensus primer positive DNA
Figure 4.9: Confirmation of HPV by sequencing in tumor and control DNA samples

Legend: The genomic DNA isolated from the patients and controls samples were sequenced with HPV specific primers as described under materials and methods (section 4.3). The sequences were confirmed with BLAST analysis.

A: HPV16 consensus sequence, B: HPV E2 gene sequence, C: HPV E6 gene sequence, D: HPV L1 gene sequence
Figure 4.10: Analysis of HPV DNA in tumor samples by in situ hybridization

Legend: The tumor sections were analyzed for HPV infections by in situ hybridization using oligo probe labeled with biotin as described under materials and methods (section 4.3).

a: Negative control
b: Tumor sample positive for HPV infection showing punctate signals
Table 4.13: Prevalence of HPV in cases and controls-gender wise

<table>
<thead>
<tr>
<th></th>
<th>MALES</th>
<th>FEMALES</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n = 41)</td>
<td>Controls (n = 35)</td>
<td>Cases (n = 19)</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>HPV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 (53.7)</td>
<td>0 (0)</td>
<td>7 (36.8)</td>
</tr>
</tbody>
</table>

p<0.001         p=0.02           p=0.001

The prevalence of HPV16 in cases was statistically significant when compared to controls (Table.4.13.). The clinical and histopathologic characteristics of the HPV positive and negative patients were compared in order to document any major differences between them. There was no significant difference between the groups with respect to gender, age group, presence of risk factors, staging, and survival except the tumor grading (Table.4.14 and 4.15). HPV positivity is more common in well-differentiated cancers (p=0.041). Survival analysis by Kaplan Meier method showed that there was no significant difference between the HPV positive and negative patients (Fig 4.11)(Table.4.16.).

Table 4.14: Comparison of age between HPV positive and negative cases

<table>
<thead>
<tr>
<th>AGE</th>
<th>HPV+</th>
<th>HPV-</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>29</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>56.7</td>
<td>53.5</td>
<td>.349</td>
</tr>
<tr>
<td>SD</td>
<td>14.3</td>
<td>12.2</td>
<td></td>
</tr>
</tbody>
</table>
Table. 4.15: Association of HPV positivity with different variables

<table>
<thead>
<tr>
<th></th>
<th>HPV +ve (n = 29)</th>
<th>HPV-ve (n = 31)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (75.9)</td>
<td>19 (61.3)</td>
<td>0.349</td>
</tr>
<tr>
<td>Female</td>
<td>7 (24.1)</td>
<td>12 (38.7)</td>
<td></td>
</tr>
<tr>
<td><strong>AGE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤45 years</td>
<td>8 (27.6)</td>
<td>8 (25.8)</td>
<td>0.891</td>
</tr>
<tr>
<td>&gt;45 years</td>
<td>21 (72.4)</td>
<td>23 (74.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>17 (58.6)</td>
<td>13 (41.9)</td>
<td>0.301</td>
</tr>
<tr>
<td>Absent</td>
<td>12 (41.4)</td>
<td>18 (58.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Recurrence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2 (0)</td>
<td>10 (9.7)</td>
<td>0.014</td>
</tr>
<tr>
<td>Absent</td>
<td>27 (100)</td>
<td>21 (90.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Grading of cancers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wdscc</td>
<td>21 (72.4)</td>
<td>13 (41.9)</td>
<td>0.041</td>
</tr>
<tr>
<td>mdsc c</td>
<td>6 (20.7)</td>
<td>16 (51.6)</td>
<td></td>
</tr>
<tr>
<td>pdsc c</td>
<td>2 (6.9)</td>
<td>2 (6.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Staging of cancers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10 (34.5)</td>
<td>9 (29.0)</td>
<td>0.975</td>
</tr>
<tr>
<td>II</td>
<td>5 (17.2)</td>
<td>6 (19.4)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>6 (20.7)</td>
<td>7 (22.6)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>8 (27.6)</td>
<td>9 (29.0)</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.11: Overall survival by HPV status.** The green line is for HPV negative and the pink line is for HPV positive patients respectively.

**Table 4.16: Comparison of survival between HPV positive and negative patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean survival time in months (CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>43.71 (39.48-47.92)</td>
<td>0.714</td>
</tr>
<tr>
<td>Negative</td>
<td>38.3 (33.19-44.41)</td>
<td></td>
</tr>
</tbody>
</table>

**4.4.4 Detection of HPV by Koilocytosis**

Since the presence of koilocyte is pathognomonic of HPV infection, it was evaluated in hematoxylin-eosin slides. Koilocytes were detected in 45% of the cases (27/60) (Fig 4.12). Forty one percent of the HPV positive cases showed koilocytes with a sensitivity of 41% compared to PCR, the gold standard. It is evident from this study that atleast around 41% of the HPV positive cases could be detected by routine careful H&E examination.
Figure 4.12: Koilocytes in routine H & E evaluation

Legend: Koilocytes showing picnotic nuclei with clear halos surrounded by a rim of cytoplasm
4.4.5 Pathways involved in carcinogenesis

The two major pathways involved in HPV carcinogenesis are E6 mediated degradation of p53 and E7 mediated degradation of pRb (Fig 4.2). In the HPV positive tumors, the concomitant expression of E6 protein and the absence of p53 in cancer indicate the complex formation of E6 with wild type p53 and subsequent degradation of p53; implicating the E6-p53 pathway in HPV mediated carcinogenesis. The pRb tumor suppressor is altered by interaction with viral oncoprotein E7, leading to its degradation, p16 over-expression and cyclinD1 degradation. Down regulation of Notch-1 is also known to play an important role in HPV mediated carcinogenesis. EGFR over-expression is a constant characteristic seen in HPV positive tumors.

IHC was hence performed with these proteins in an effort to identify the pathway involved in the HPV positive cases. Hence immunohistochemical analysis of the E6, E7 oncoproteins and the downstream regulatory proteins-p53, pRb, p16, CyclinD1 was carried out in HPV positive cases to detect the predominant pathway involved. Additionally, analysis of the Notch-1, EGFR proteins was also performed.

Out of 29 positive cases, blocks were available for 27 cases and immunohistochemical analysis of the above-mentioned proteins was performed as mentioned in materials and methods section 5.2.4. As mentioned previously the presence or absence of immunoreactivity was considered for E6 & E7 proteins while for other proteins, it was considered significant when characteristic nuclear/cellular immunoreactivity was seen in at least 10% of the tumor cells. The proteins, p53, pRb and cyclin D1 showed distinct nuclear localization, E6, E7, p16 showed nuclear and/or cytoplasmic immunoreactivity. Notch-1 showed cytoplasmic immunoreactivity while EGFR was distinctly positive in membranes (Fig. 4.13 and 4.14).

Analysis of the E6 and E7 expression revealed 63% and 78% positivity in cases respectively with only 11% (3/27) of the cases negative for E6/E7. Among those cases positive for E6 and E7 expression, 41% and 33% respectively had both nuclear and cytoplasmic staining.

Among the p16 positive cases (37% of the cases), 30% had both nuclear and cytoplasmic staining with majority of the positive sections having grade 2 staining (11-30% cells) and moderate intensity. 63% of the samples were negative for p16 with <10% positive cells.
Figure 4.13: Immunohistochemistry analysis of E6, E7, p53 and p16 proteins in HPV positive tumor samples

Legend: The HPV positive tumor sections were analyzed by immunohistochemistry for E6, E7, p53 and p16 proteins using monoclonal antibody as described under materials and methods (section 4.3).

a: E6 negative, b: E6 positive, c: E7 negative, d: E7 positive
e: p53 negative, f: p53 positive, g: p16 negative, h: p16 positive
Figure 4.14: Immunohistochemistry analysis of pRb, CyclinD1, EGFR and notch1 proteins in HPV positive tumor samples

Legend: The HPV positive tumor sections were analyzed by immunohistochemistry for pRb, CyclinD1, EGFR and notch1 proteins using monoclonal antibody as described under materials and methods (section 4.3)

a: pRb negative, b: pRb positive, c: CyclinD1 negative, d: CyclinD1 positive
e: EGFR negative, f: EGFR positive, g: notch1 negative, h: notch1 positive
Wild type p53, pRb and cyclin D1 expression were seen in 56%, 11% and 33% of cases. Notch-1 expression as cytoplasmic staining was observed in 26% of cases. EGFR membrane staining was observed in 89% of cases.

EGFR showed high grade (3 & 4) membrane staining with moderate/intense intensity in maximum number of samples, with the overall positivity being 89%. This is in accordance with the over-expression of the gene observed in HNSCC. The patterns of staining/intensity obtained with each antibody is mentioned in the table (Table 4.17).

It was observed that 33% of the cases (n = 9/27) had active E6–p53 pathway with E6 expression and p53 degradation. Similarly 67% (18/27) of the cases had active E7-pRb pathway with E7 expression and pRb degradation. Nineteen percent (5/27) had both the pathways active.

**Table 4.17 Immunohistochemistry results**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>E6</th>
<th>E7</th>
<th>p53</th>
<th>pRb</th>
<th>p16</th>
<th>Cyclin D1</th>
<th>Notch-1</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Slides showing positive expression. n (%)</td>
<td>17 (63)</td>
<td>21 (78)</td>
<td>15 (56)</td>
<td>3 (11)</td>
<td>10 (37)</td>
<td>9 (33)</td>
<td>7 (26)</td>
<td>24 (89)</td>
</tr>
<tr>
<td>Extent of staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1 (-)</td>
<td>9</td>
<td>6</td>
<td>17</td>
<td>24</td>
<td>17</td>
<td>18</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Grade 2 (1+)</td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Grade 3 (2+)</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Grade 4 (3+)</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Intensity of staining</td>
<td>mild (1+)</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>moderate (2+)</td>
<td>8</td>
<td>14</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>severe (3+)</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
4.5 Discussion

The prevalence of HPV infection in head and neck cancers was studied extensively in different parts of the world suggesting a strong link of HPV primarily to oropharyngeal cancers, especially tonsil. There are very few studies to link HPV infection with tongue cancers. As observed in our epidemiology study (chapter 3) despite reduction of overall incidence of head and neck cancer, there is a significant increase in incidence of tongue cancer, which may not be attributed to the existing risk factors like tobacco and alcohol. An alternate causative factor should exist to explain this increase in incidence of tongue cancer. HPV as a potential aetiologic factor has been suggested by other authors (Davis 1987; Schantz 2002). HPV may act as an independent aetiologic factor or may have synergistic effect with other prevailing risk factors. The biologic effect may be apparent only in those who are susceptible to these risk habits (Fig 4.15)

![Diagram](image)

**Figure 4.15: Malignant transformation of the normal oral mucosa-effects of risk factors**

Systematic review of 60 studies involving 5026 head and neck cancer by Kreimer et al, (Kreimer 2005) has shown 25.9% HPV positivity. Oropharynx subsite had the most significant correlation with HPV infection (35.6%). There is paucity of data in the literature investigating correlation of HPV infection and tongue cancer.

The frequency of detection of HPV in tongue cancers ranged from 0-81%, the highest rate detected by Balaram et al in his study on the prevalence of HPV infection among betel quid chewers (Balaram 1995; Kantola 2000). In this study, a total of only 11 tongue
cancers were analyzed and it was observed that 9 out of 11 subjects were positive for HPV infection in general. And the prevalence of HPV 16 was 42% out of 91 oral cancer cases. The detection rate depends on the sample size and the method employed for detection. The sample size used in previous studies on tongue cancers ranged from 7 to 50, majority of the studies employing either PCR or hybridization as the detection method. Studies on the prevalence of HPV in tongue cancers exclusively are very few, in most of the studies, tongue cancers formed a subset of head and neck cancers which were analyzed. In a study which included base tongue and oral tongue cancers with the sample size of 110, it was observed that 11% and 9% of the tongue cancer cases were positive for HPV infection in general and HPV 16 respectively (Liselotte 2004).

In this project we have carried out a systematic study of HPV infection in oral tongue cancer. This included identifying prevalence of oncogenic HPV 16 in tongue cancer, its integration and correlating the positivity with different proteins implicated in HPV mediated carcinogenesis.

In our present study, a combination of multiple PCR assays was done in order to avoid false negatives. It was observed that 48% of the tongue cancers were positive for HPV16 infection.

The mere presence of HPV DNA does not indicate its role in carcinogenesis. Therefore, a demonstration of HPV integration in genomic DNA is essential for establishing its role in the pathogenesis of a tumor. Molecular biology studies help to identify a subset of the cancers that may be the consequence of HPV infection. Eighty three percent and 67% of HPV 16 positive cases showed integration by PCR and ISH respectively suggesting the role of HPV in carcinogenesis in this subset. The sensitivity of ISH was found to be atleast 20-50 copies per cell, hence the difference in the positivity rate. Many studies have shown similar decreased positivity with ISH detection when compared to PCR. In a study with 51 uterine cervical cancers, it was observed that HPV16 positivity by PCR was 57% whereas it was only 10% by ISH (Kim 1997). In another study with 59 cases of OSCC, it was found that 12% of the cases were positive for HPV by PCR, but none of the cases were positive by ISH (Sonja 2006).

There is a strong notion that HPV plays a role in carcinogenesis in subjects without established risk factors (Haraf 1996; Gillison 2000; Lindel 2001; Klussmann 2003; Ritchie 2003). HPV cannot infect the normal epithelium; it enters through a breach in the
epithelium. Habitual use of alcohol and tobacco, poor oral hygiene generally leads to ulcerations through which HPV enters the oral mucosa. In our study HPV infection was not statistically associated with the presence of risk habits. It is evident from this study that HPV positive tumors are not found exclusively in patients with no risk habits but also in patients with risk habits of tobacco and alcohol consumption. Numerous studies have reported the additive or synergistic effect of tobacco/- alcohol consumption and HPV. Hence HPV may act as an independent risk factor or act with other established risk factors like tobacco and or alcohol to induce cancer.

HPV positive tumors may have different clinical and biologic behavior, with improved overall survival and favorable prognosis (Licitra 2006). HPV positive tumors showed better response to chemotherapy and radiotherapy. Previous studies reveal that HPV positive cancers were more common in the younger age group (Mellin 2000; Strome 2002), non smokers (Gillison 2004), associated with advanced TNM stage and poorly differentiated tumors (Haraf 1996; Wilczynski 1998; El-Mofty 2003). In our study it was found that HPV positive cases were well differentiated and had better disease free survival than their negative counterpart implicating that HPV positive cancer might have good prognosis. But there was no statistically significant difference between HPV positive and HPV negative cases with respect to age, staging and survival. This may be due to the smaller sample size or due to different modality of treatment employed in this patient group. As the standard of care for oral tongue cancer is surgery followed by adjuvant treatment, this was employed in this subset of patients. In the report by Fakhry et al (Fakhry 2008), where a significant difference in treatment outcome observed in correlation with HPV status, chemo-radiotherapy was employed to treat stage 3 or 4 laryngo-pharynx cancers.

The two main pathways involved in HPV carcinogenesis are E6–p53 and E7-pRb leading to aberrations in p53 expression and pRb degradation. HPV positive tumors tend to have wild type p53, because p53 is functionally inactivated by viral E6 oncoprotein (Gillison 2000; Wiest 2002; Balz 2003). Pillai et al has shown that the aberrant expression of high risk HPV16/18 E6 protein is a critical event in HPV carcinogenesis (Pillai 1999). However mutations are also common in tongue cancers resulting in the production of functionally defective p53 protein. Sometimes as a result of excess DNA damage, there might be increased synthesis of p53 in cancers. Eighteen percent (5/27) of the cases showed p53 expression with E6 expression. Similarly the concomitant expression of E7
and the absence of pRb indicate the complex formation of E7 with pRb followed by pRb degradation; implicating E7-pRb pathway in HPV mediated carcinogenesis. In our study 33% and 67% of the HPV positive cases showed that E6-p53 and E7-pRb pathways were active respectively and hence might be associated with carcinogenesis. Both the pathways were active in nineteen percent of the subjects. As a result of reduced or absent pRb expression, over-expression of downstream p16 and down regulation of cyclinD1 occur. In our study it was observed that p16 over-expression and cyclinD1 loss was seen in 37% and 67% of cases respectively. As is evident from these results, the E7-pRb pathway seems to be more predominant among the HPV infected cases.

p16 over-expression is highly correlated with HPV infection especially in cervical cancers speculating p16 over-expression may be used as an alternative to HPV DNA detection by routine methods (Ruediger 2001). Also studies have shown improved prognosis of HPV positive oropharyngeal cancers with high levels of p16 expression. Maintenance of senescence in tumor cells after treatment such as radiation might be dependent on p16 levels and that would explain the difference in outcome between p16 expressors and non-expressors. But our study in tongue cancer patients showed only 37% of cases with p16 over-expression indicating that its association with the head and neck cancers might vary from that of cervical cancer. Cyclin D1 expression was associated with unfavorable prognosis (Bae 2001). Cyclin D1 has been reported to be under-expressed in cervical cancers as a result of pRb degradation. Similar results were obtained in our study with 67% of the case showing down regulation.

Previous studies have shown that Notch-1 gene expression is markedly reduced in cervical carcinoma cells. Activated Notch-1 causes growth inhibition of HPV positive, but not HPV negative carcinoma cells suggesting that Notch-1 exerts specific protective effects against HPV-induced transformation through suppression of E6/E7 expression. Hence it has been indicated that down-modulation of Notch1 expression might play an important role in late stages of HPV-induced carcinogenesis (Claudio 2002). Our present study showed a high percentage of HPV positive patients with down regulation of Notch-1 (74%) indicating a causative effect in this subset of patients.

There are two different schools of thought with regards to EGFR expression in HPV positive cancers. One thought is that the E5 protein interacts with EGFR and alters the cell response to signals for growth and differentiation (Pim 1992). It is proposed that the E5 protein expression destabilizes the cells at an early stage rendering them susceptible to
the action of E6 & E7 oncoproteins (Syrjanen 1987). In other studies, it has been reported that the increase of EGFR is due to HPV E6 & E7 rather than a consequence of E5 mediated post translational mechanism (Conrad 1994; Hwang 1995). In our study it was observed that 89% of the HPV positive cases showed EGFR expression. Further investigations should be carried out to better understand the molecular mechanisms involved. The present study implicates that both the pathways are involved in HPV mediated carcinogenesis with E7-pRb preponderance.

Our study has its own limitations. The lack of correlation of HPV infection and clinical and pathological behavior could have been due to the small sample size. Though PCR is the most sensitive method to detect HPV positive cases, quantification of the viral load would further help in assessing the degree of infection. The best way to undertake the same would be microdissecting the tumor cells from blocks and performing Quantitative Real Time PCR. Immunoprecipitation of E6 and E7 proteins will prove beyond doubt their interactions with p53 and pRb proteins and their causative role in carcinogenesis.

4.6 Conclusion and future directions/prospects

The present study shows the definite implication of the role of HPV infection in tongue cancers. Since multiple PCR assays were used for HPV detection it is unlikely that we missed any HPV positive case in our study. And the presence of HPV DNA in tumors was demonstrated clearly in our study, we could conclude that tongue SCCs is significantly associated with high risk HPV 16. Since the sensitivity of detection of HPV by routine HE–Koilocytic change is only 41% we could not propose that as a standard screening method, but the detection of a major subset of the patients by routine HPE indicated that this technique should not be completely ignored. In centers where sophisticated molecular techniques cannot be done, careful assessment of the HE slide could result in detecting atleast a significant proportion of the HPV positive cases. It could be recommended to perform PCR to confirm HPV infection in those patients with koilocytosis. So far clinical trials have not evaluated the effect of HPV vaccine in oral cancers. Given this high rate of HPV infection in oral cancers, the vaccine does have the potential on the incidence of oral cancer if specifically targeted to HPV16.