THE CARCINOGENIC POTENTIAL OF E6 & E7 GENES OF HIGH-RISK HPV COMPARED WITH E6, E7 GENES OF LOW-RISK HPV IN HUMAN CERVICAL CANCER: A REVIEW

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ABSTRACT: Human papillomavirus is one of the most common causes of sexually transmitted disease in both men and women worldwide. Genital HPV types are divided into high and low-risk types, on the basis of oncogenic gene potential. Molecular & epidemiologic studies have confirmed the interaction between high risk HPV types (especially HPV-16 & 18) and cervical squamous cell low risk strain. In high grade Intra epithelial neoplasias & invasive cancers, generally integration of HPV-DNA into the host genome disrupts or deletes the E2 region, which results in loss of its expression. The E6 and E7 gene deregulate the host cell growth cycle by binding and inactivating two tumor suppressor proteins: the tumor suppressor protein (p53) and retinoblastoma gene (pRb). The HPV E6 gene binds to p53 and targets it for rapid degradation leads to an increased expression of E6 and E7 genes. The inactivations of p53 & pRb proteins can increased proliferation rate & genomic instability. In addition, the potential of activated oncogenes cause chromosome instability may transformed in the host genome such as methylation of viral, cellular DNA, telomerase activation, hormonal & immunogenetic factors. As a result, the host cells accumulate more damage DNA that cannot be repaired, leading to cancerous cells. Low-risk HPV E6 proteins do not bind p53 at detectable levels and have no effect on p53 protein stability in vitro. The E7 protein of low-risk HPV types binds pRb with decreased affinity.

INTRODUCTION:

Human papilloma viruses and cervical cancer: Cervical cancer, the second most common gynecological malignancy worldwide incidence, has been reported to occur in abundance in different populations. In 2010, there were an estimated 12,200 new cases and an associated 4,210 deaths, accounting for around 1% of cancer deaths in women 1.

In 1995 the World Health Organization (WHO) declared HPV as a known carcinogen for causing factor to cervical cancer, because DNA of mucosal high-risk HPV types could be detected in almost all cervical cancers 2.

According to World Health Organization (WHO) 2010, in India approximately 1, 34,420 women are diagnosed with the disease every year, and of them 72,825 die. Cervical cancer is one of the commonest cancers of the female anogenital tract and a leading cause of morbidity and mortality. The association of HPV and cervical cancer was first suggested by Zur Hausen in 1976 3. It is now believed that 94-100% of cervical cancers as well as tumors of the penis, anus, vagina, and vulva is associated with sexually transmitted genital...
infection by the human papilloma virus \(^4, 5\). The link between genital HPV infections and cervical cancer was first demonstrated in the early 1980s by Harold Zur Hausen, a German virologist. In 1996, the World Health Association, along with the European Research Organization on Genital Infection and Neoplasia and the National Institutes of Health Consensus Conference on Cervical Cancer, recognized HPV as an important cause of cervical cancer. Scientists have identified about 30 HPV types that are spread through sexual contact and infect primarily the cervix, vagina, vulva, penis, and anus. Of these, four are most often found within the malignant cells of cervical cancers, with type 16 accounting for about half of the cases in the United States and Europe and types 18, 31, and 45 accounting for an additional 25 to 30% of cases \(^6\).

Cervical cancer is the most common cancer in women in most developing countries and most common cause of cancer deaths \(^7\). To improve the performance of cervical cancer screening mainly in women younger than 30 years, we investigated the use of a quantitative E6, E7 mRNA assay (HPV OncoTect, Incell DX, Menlo Park, California) that determines oncogenes over expression on a cell by cell basis using high throughput flow cytometry \(^8, 9\). In developing countries, cervical cancer is often the most common cancer in women and may constitute up to 25% of all female cancers \(^6\). Cervical cancer is preceded only by breast cancer as the most common cause of death from cancer in women worldwide \(^10\). There are at least 118 fully described forms of the papillomavirus which structurally consists of double-stranded circular DNA surrounded by a viral capsid protein \(^11\). HPVs can infect basal epithelial cells of the skin or inner lining of tissues and are categorized as cutaneous types or mucosal types.

Cutaneous types of HPV are epidermotropic and target the skin of the hands and feet. Mucosal types infect the lining of the mouth, throat, respiratory tract, or anogenital epithelium. More than 100 HPV types are known to occur that are divided into three broad categories depending upon their oncogenic potential: high risk types including HPV- 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82; intermediate types including HPV- 26, 53, 66 and low-risk types including HPV- 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 \(^12\).

Eighty-five HPV genotypes are well characterized. An additional 120 isolates are partially characterized potential new genotypes \(^13\). Infection with high-risk HPV types is the critical aetiological factor in the development of cervical cancer. Certain cervical intraepithelial neoplasias (CINs) with persistent HPV infection progress to invasive cervical cancer although a fraction of them regress during the course of time – 60% in case of CIN-1, 40% of CIN-2 and 33% of CIN-3 \(^14\). At least 40% of all CINs persist and only about 1% of CIN-1, 5% of CIN-2 and 15% of CIN-3 advanced to invasive cancers \(^14\).

The use of HPV testing for cervical cancer screening is growing worldwide. HPV-DNA assays determining the presence or absence of high-risk HPV genotypes have been extensively utilized over the past decade; however, newer HPV-RNA based assays are becoming popular because of the hope of increased specificity compared to HPV-DNA assays including genotyping. One of the issues with other HPV-RNA assays such as GenProbe Aptima and BioMerieux NucliSSENS Easy Q HPV is that they genotype HPV from the E6, E7 transcript \(^15\).

In \(^16\) found the interaction between tobacco smoke and HPV-16 E6/E7 oncoproteins for malignant transformation and tumorigenesis of lung epithelial cells. Persistent infection with oncogenic high-risk subtypes of human papillomavirus (HPV) leads to cervical cancer \(^17\) and over 50% of the cases are HPV-16 \(^2\). In cancer development, HPV-16 early proteins E6 and E7 are often believed to act as oncoproteins as both are crucial for immortalization and transformation of cervical keratinocytes \(^18\). The E6 and E7 oncoproteins work synergistically to deregulate cell cycle controls through a variety of mechanisms. The E6 oncoprotein promotes ubiquitination and proteasomal degradation of the tumor suppressor protein p53 and also deregulates the cell cycle \(^19\). The E7 protein binds to and inactivates the function of retinoblastoma protein pRb. It disrupts the complex between pRb and the E2F transcription factor family, which controls the expression of genes involved in cell-cycle progression. Thus, destabilization of p53 and hypophosphorylated pRb by the expression of two viral oncoproteins E6 and E7 promotes chromosomal instability, foreign DNA integration, and other mutagenic events in the cell.
**HPV Oncoproteins:** HPVs encode two major oncoproteins, E6 and E7, which are consistently expressed in cervical carcinomas. E6 and E7 lack intrinsic enzymatic activities and transform cells by stimulating cell growth and inactivating tumor suppressor pathways. Expression of HPV-16 E6 and E7 oncoproteins in primary human epithelial cells causes genomic instability.

**E6 protein:** The E6 protein of HPV is an 18 KDa phosphoprotein, which is localized in the nucleus and in non-nuclear membranes. E6 is a critical factor in tumor formation and acts to destabilize the tumor suppressor p53. The p53 tumor suppressor protein, in turn, regulates the transcription of several genes that keep cell proliferation in check by inducing cell cycle arrest, DNA repair, or apoptosis. The E6 protein forms a complex with p53 and the cellular ubiquitin ligase causing a deregulation of the cell cycle control at the G1/S and G2/M check points, an important step for the replication of HPV, because a productive infection cycle is only possible in cells, which are in the S-phase of the cell cycle. However, this cell cycle manipulation can lead to activation of oncogenes or inactivation of tumor suppressors and consequent DNA damage cannot be repaired. This leads to genetic instability and to malignant transformation of high-risk HPV-infected cells. Another important way how E6 proteins of genital HPV contribute to transformation is the activation of the human telomerase reverse transcriptase promoter, which controls the transcription of the catalytic telomerase subunit. E6 proteins of cutaneous HPV do not interact with p53 and do not degrade (p53).

Furthermore E6 proteins of both cutaneous and anogenital HPV are able to target the proapoptotic protein for ubiquitin-dependent degradation by assembling E6-p53, thereby inhibiting apoptosis.

**E7 protein:** E7 is an 11 KDa protein with a zinc finger motif. It acts as an oncogene in genital high risk HPV and is able to immortalize primary foreskin keratinocytes. The major part of the transforming potential of E7 is due to binding and induction of ubiquitin-dependent degradation of the tumor suppressor retinoblastoma protein (pRb). The competitive binding of E7 to pRb and its degradation lead to segregation of the transcription factor E2F. In the G1-phase, E2F is inactivated in a complex with pRb. After segregation, E2F can induce the expression of genes, which are important for DNA synthesis and cell cycle control. Additionally E7 can bind the inhibitors of cyclin dependent kinases p21CIP1 and p27KIP1 and inhibit their functions. Both events direct the cell into the S-phase and enable the viral replication. Since only a certain fraction of HPV-infected CINs progress to invasive cancer after a long latent period and the incidence of tumors are less frequent than the HPV infection, additional events also play crucial role in making HPV infection persistent, leading to oncogenicity. Some high-risk HPV viruses are often found in squamous intraepithelial lesions (SILs) (Table 1). Some authors refer to these HPV types as intermediate risk. The magnitude of the association between HPV and cervical squamous cell carcinoma is higher than that for the association between smoking and lung cancer.

**TABLE 1: HPV TYPE A AND B DISEASE ASSOCIATION**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>HPV type a &amp; b</th>
<th>Risk HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Plantar warts</td>
<td>1, 2, 4, 63</td>
</tr>
<tr>
<td>2.</td>
<td>Common warts</td>
<td>2, 1, 7, 4, 26, 27, 29, 41, 57, 65, 77, 1, 3, 4, 10, 28</td>
</tr>
<tr>
<td>3.</td>
<td>Flat warts</td>
<td>3, 10, 26, 27, 28, 38, 41, 49, 75, 76</td>
</tr>
<tr>
<td>4.</td>
<td>Other cutaneous lesions (e.g., epidermoid cysts, laryngeal carcinoma)</td>
<td>6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73</td>
</tr>
<tr>
<td>5.</td>
<td><em>Epidermodysplasia verruciformis</em></td>
<td>2, 3, 10, 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50</td>
</tr>
<tr>
<td>6.</td>
<td>Recurrent respiratory papillomatosis</td>
<td>6, 11</td>
</tr>
<tr>
<td>7.</td>
<td>Focal epithelial hyperplasia of Heck</td>
<td>13, 32</td>
</tr>
<tr>
<td>8.</td>
<td>Conjunctival papillomas/carcinomas</td>
<td>6, 11, 16</td>
</tr>
<tr>
<td>9.</td>
<td>Condyloma acuminata (genital warts)</td>
<td>6, 11, 30, 42, 43, 45, 51, 54, 55, 70</td>
</tr>
<tr>
<td>11.</td>
<td>Low risk</td>
<td>6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 74</td>
</tr>
<tr>
<td>12.</td>
<td>High risk</td>
<td>16, 18, 6, 11, 31, 34, 33, 35, 39, 42, 44, 45, 51, 52, 56, 58, 66, 68, 70</td>
</tr>
<tr>
<td>13.</td>
<td>Cervical carcinoma</td>
<td>16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58, 66, 68, 70</td>
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</table>
The HPV Genome - key players: The circular HPV DNA is 6800 to 8000 base pairs in length and codes for eight genes - E6, E7, E1, E2, E4, E5, L1 and L2. The first six are "early" viral genes which code for proteins produced during the early phase of infection in the basal cell layer. They are responsible to enhanced proliferation of the infected cells and their lateral expansion. The E5 Protein has been shown to complex with epidermal-growth-factor receptor, platelet-derived-growth factor receptor and the colony-stimulating factor-1 receptor, which promotes growth. E5 also appears to inhibit programmed cell death. Nevertheless the fact that the viral E5 gene is often deleted during the process of viral DNA integration with the host cell genome suggests a dispensable role in oncogenesis. E6 and E7 genes and their proteins appear to have a central role in HPV-induced cervical cancer. They are expressed in cervical cancers and are individually able to immortalise various human cell lines in vitro but when expressed together their efficiency is enhanced. The E6 Protein has significant effects by virtue of its interaction with, and degradation of p53. p53 is also known as the "guardian of the genome" and is crucial in protecting normal cells when exposed to stress (e.g. radiation, UV light or chemicals).

In such cells it causes cell cycle arrest preventing a cell with damaged DNA from multiplying, and allowing the cellular repair systems to fix any damaged DNA. If repair is not feasible then p53 induces apoptosis (programmed cell death).

Since all cancers arise on a background of DNA mutations, p53 has a key role in preventing carcinogenesis and unsurprisingly 50-60% of all cancers have p53 mutations. Other effects of the E6 protein include degradation of the pro-apoptotic BAK protein which is involved in the intrinsic (mitochondrial) death pathway. BAK has a physiological role in the cellular response to stress, in that it can promote opening of the mitochondrial permeability pores releasing intra-mitochondrial cytochrome-c which induces apoptosis. E6 also activates telomerase and stabilises active Src-family kinases involved in enhanced cell survival, proliferation, and motility. The E7 Protein binds to and degrades the Retinoblastoma (pRb) protein.

The pRb gene, initially identified as the gene responsible for childhood eye tumors, was one of the first tumors suppressor genes to be discovered and led to Knudson's famous "two-hit" hypothesis of cancer development. The pRb protein normally inhibits proliferation by binding to the E2F transcription factor – a key player controlling the G1/S phase checkpoint of the cell cycle. Loss of pRb by HPV E7 protein can therefore result in uncontrolled cell division.

A normal cell would react to excessive E2F-mediated growth signals by p53-dependent apoptosis; however the presence of E6 protein counteracts this by p53 and BAK degradation which prevents apoptosis. The end result of their combined action is host cell DNA which is prone to accumulate chance errors unchecked by physiological repair or programmed cell death. The functions of oncogenic genes of HPV are presented in Table 2 briefly.

TABLE 2: HUMAN PAPILLOMA VIRUS EARLY AND LATE GENES WITH POSTULATED FUNCTIONS

<table>
<thead>
<tr>
<th>Gene Category</th>
<th>Gene Function</th>
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<tbody>
<tr>
<td>E1</td>
<td>E1 Viral replication</td>
</tr>
<tr>
<td>E2</td>
<td>E2 Modulation of transcription and replication</td>
</tr>
<tr>
<td>E3</td>
<td>E3 Unknown</td>
</tr>
<tr>
<td>E4</td>
<td>E4 Productive viral infections</td>
</tr>
<tr>
<td>E5</td>
<td>E5 Transforming properties</td>
</tr>
<tr>
<td>E6</td>
<td>E6 Oncoprotein; interaction with p53 protein</td>
</tr>
<tr>
<td>E7</td>
<td>E7 Oncoprotein; interaction with pRb protein Early genes</td>
</tr>
<tr>
<td>E8</td>
<td>E8 Unknown</td>
</tr>
<tr>
<td>Late gene L1</td>
<td>Late genes L1 Major capsid protein</td>
</tr>
<tr>
<td>Late gene L2</td>
<td>Late genes L2 Minor capsid protein</td>
</tr>
</tbody>
</table>

Basic Virology: Papillomaviruses are members of the Papovaviridae family, which also includes polyomavirus and simian vacuolating virus. HPV is a relatively small, nonenveloped virus, 55 nm in diameter. It has an icosahedral capsid composed of 72 capsomers, which contain at least two capsid proteins, L1 and L2. Each capsomer is a pentamer of the major capsid protein, L1. Each virion capsid contains several copies (about 12 per virion).
of the minor capsid protein, L2. The virus is said to somewhat resemble a golf ball when viewed by electron microscopy. The HPV genome consists of a single molecule of double-stranded, circular DNA containing approximately 7,900 bp associated with histones. All open reading frame (ORF) protein-coding sequences are restricted to one strand.

The genome is functionally divided into three regions (Fig. 1):

(i) The first is a non-coding upstream regulatory region of 400 to 1,000 bp, which has been referred to as the non-coding region, the long control region (LCR), or the upper regulatory region. This region contains the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. This region also contains the highest degree of variation in the viral genome.

(ii) The second is an early region, consisting of ORFs E1, E2, E4, E5, E6, and E7, which are involved in viral replication and oncogenesis.

(iii) The third is a late region, which encodes the L1 and L2 structural proteins for the viral capsid. By definition, the nucleotide sequences of the E6, E7, and L1 ORFs of a new HPV type should be no more than 90% homologous to the corresponding sequences of known HPV types. HPVs have further been classified into subtypes, when they have 90 to 98% sequence similarity to the corresponding type and variants when they show no more than 98% sequence homology to the prototype. Some naturally occurring variants have different biological and biochemical properties important in cancer.

HPV life cycle and pathogenesis: HPV are non-lytic, non-enveloped, icosahedral-shaped viruses that replicate their genomes in the nuclei of host cells using host cellular machinery. Their 7.9-kb circular, double-stranded DNA genome has a simple organization and can be separated into two coding regions and a non-coding regulatory region. The coding regions are denoted E and L for “early” proteins and “late” proteins, and encode on average 8 major open reading frames that are expressed from polycistronic mRNAs transcribed from a single DNA strand. The six E proteins are responsible for viral DNA replication, transcription and cellular transformation. They fulfill regulatory functions vital for the production of viral progeny while the two L proteins are the capsid proteins responsible for virion assembly and DNA packaging. The functions of HPV genes have been discussed in detail elsewhere. Although HPV virions have been shown to bind heparin, a ubiquitous polysaccharide that may provide the initial attachment and heparin sulfate has been shown to be required on the cell surface for HPV infection, the receptor for HPV entry into basal cells has not been functionally identified.

Thus, the precise steps of the entry process are yet to be elucidated and additional work is required to understand the mechanism by which HPV virions infect host basal epithelial cells. HPV infection of basal cells leads to the activation of a cascade of viral gene expression that resulted in the replication of the viral genome.

However, the expression of viral genes is largely suppressed and there is limited expression of specific early viral genes. The first viral genes to be expressed are the replication factors E1 and E2 which form a complex that bind to the origin of
replication and act to recruit cellular polymerases and accessory proteins that mediate DNA replication 46,47.

Three HPV proteins (E5, E6 and E7) possess proliferation-stimulating activity. E5 stimulates cell growth by forming complexes with the epidermal-growth-factor receptor (EGFR), the platelet derived growth-factor-β receptor and the colony-stimulating factor-1 receptor 27. It has also been shown to prevent apoptosis following DNA damage 28. E6 and E7 are independently able to immortalize human cell types in tissue culture, but the efficiency is increased when they are expressed simultaneously 29,48. Together, expression of these viral proteins results in blockage of exit from the cell cycle and enhanced proliferation of infected cells containing hundreds of copies of the HPV genome per cell. As infected cells divide, viral genomes are partitioned into the daughter cells.

One daughter cell migrates to the suprabasal layers and undergoes differentiation, while the other continues to divide and is the reservoir for continuous viral replication. In uninfected cells, differentiation would lead to exit from the cell cycle, but infected cells remain in active cell cycle due to the action of E7 49 and continue to replicate the viral genome. The functions of E4 and E5 in the replication process are not well understood, though they both have been proposed to be involved in the regulation of late viral functions 20. Early proteins are predominantly expressed in the basal and suprabasal layers of the epidermis. E5, E6 and E7 are oncogenic proteins (Fig. 2). E5 expression enhances oncogenic potential 50,51.

Following entry into the suprabasal layers, “late” viral gene expression is initiated and the structural proteins (L1 and L2) are expressed. The L1 and L2 proteins spontaneously self-assemble into icosahedral capsids 52,53 that contain the viral genome and mature virus particles are released from the uppermost layers of the epithelium when the differentiated cells are sloughed off at the end of their lifespan.

During the initial phase of infection, HPV is present as an episome, but in the majority of cancers, HPV has integrated into the host genome 54. Integration results in the inactivation of the E2 open reading frame and a loss of its repressor function for E6 and E7 transcription, which allows for an accumulation of genetic changes 38.

While the molecular pathogenesis of cancer caused by the high-risk HPV types is not fully understood and although they are self-sufficient to induce carcinogenesis, the infection itself is not able to induce the malignant transformation of infected cells. The development of cervical cancer and the invasive phenotype requires more changes that are reviewed in detail elsewhere 43.

**Prevention of Apoptosis:** Apoptosis, or programmed cell death, is initiated by specific biological signals and two main apoptotic routes have been identified 55,56. In the extrinsic death receptor pathway, receptors are activated specifically by their cognate ligands, such as Fas-Fas ligand (FasL) interaction. The intrinsic mitochondrial pathway is used in response to many nonspecific stimuli, e.g. DNA damage, radiation, and osmotic stress 57, resulting in cytochrome c release from the mitochondrial inter-membrane space. Both pathways converge at the level of caspase-3 activation. Inhibition of apoptosis of HPV-infected cells could be a mechanism to promote survival of the virus.

Furthermore, if the expression of death receptor ligands were up-regulated on the surface of infected cells, they may induce apoptosis of activated T-cells that mount an immune response. The E6 protein of HPV-16 and other HPV binds cellular p53 and the binding correlates with the in vivo clinical behavior and the in vitro transforming activity of these different papilloma viruses 30. E6 binding to p53 stimulates the degradation of p53 in an ATP-dependent manner that involves the ubiquitin dependent protease system. Thus, E6 prevents intrinsic, p53-dependent apoptosis of infected cells. E5 suppresses FasL mediated-apoptosis and this is associated with a two-fold reduction in Fas expression. Although E5 also suppresses tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated-apoptosis, no such down-regulation was observed 58.

This was further investigated using raft cultures because it allows analysis of apoptosis under more tissue-like conditions by mimicking the stratified organization of a normal surface epithelium and similar results were obtained 58. Thus, inhibition of
apoptosis may be a primary function of the HPV-16, E5 protein needed to prevent apoptosis at early stages of viral infection and promote its survival. Whether FasL expression on infected cells is up-regulated by any of the HPV proteins has yet to be determined.

**FIG. 2:** INTEGRATION OF ONCOGENIC GENES WITH THE HOST CELL UNDER MECHANISM OF PATHOGENESIS OF HPV

**CONCLUSIONS:** The incidence of cervical cancer and its concerned mortality have declined in recent years, largely due to the widely use methods of testing for detection of abnormal cervical cells. Molecular and epidemiologic studies have solidified the association between high-risk strains of HPV and cervical squamous cell carcinoma. Tests have been developed to detect high-risk HPV-DNA with high sensitivity and specificity in cervical samples. Early identification and intervention will probably have a significant impact on the reduction of cervical cancer morbidity and mortality.

In addition to changes in screening strategies, effective therapeutic and preventive vaccines may be developed that have the potential to contribute significantly to the control and prevention of cervical cancer.

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