GENOMICS OF HUMAN PAPILLOMAVIRUS-INDUCED CERVICAL CANCER

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Abstract

Human papillomavirus (HPV) induced cervical cancer is a serious health issue among most women from the least developed countries of the world due to scarcity of resources. HPVs have evolved a masterly infectious cycle that grabs the advantage of the self-renewal property of stratified cutaneous and mucosal epithelia. Firstly, the viral genome replicates episomally at a low copy number in the epithelial basal layers cells, with minimum viral transcription and translation. After that, when the infected cells are transmitted through the differentiation process maximum viral DNA synthesis and gene expression occur. The two major oncoproteins - E6 and E7, are responsible for inactivating the important tumor suppressor proteins, retinoblastoma (pRb) and p53. Due to the inactivation of these proteins, disruption occurs in the DNA replication, DNA repair mechanisms, oxidative induced damage (8-oxoguanine), aneuploidy, and apoptosis, leading to tumorigenesis. Hence, manipulation of E6 and E7 genes shows a successful result in the treatment of cervical cancer. The diagnosis tests include liquid-based preparations to improve the standard of the Pap smear; computer-based screening methods to improve Pap smear interpretation; and HPV testing methods that may be beneficial in triaging patients with untypical squamous cells of unknown significance or low grade squamous intraepithelial lesions (SILs). The continued studies of the molecular biology of HPVs are necessary to develop advanced screening techniques for testing and prophylactic vaccines for the elimination of HPV infection, also better therapeutic vaccines for the treatment of various HPVs infections.

Keywords: Cervical Cancer, HPVs, Human Papillomavirus, Epithelial Differentiation, Uterine Cervix, Oncogene, Carcinogenesis.

1. INTRODUCTION

Globally, it was estimated that 500,000 new cases of cervical cancer that result in over 250,000 deaths occur every year. Cervical cancer is the fourth major cancer in women and the seventh major common cancer (Schiffman *et al.,* 2007; Bray *et al.,* 2013). HPVs are the causative agents of nearly 99% of cervical cancers cases. Apart from the well-known factor-human papillomavirus infection, poor sanitary conditions, exposure to nonsteroidal estrogen stilbestrol, oral contraceptives (birth control pills) use, smoking, and genetic susceptibility are also supposed to causative factor for cervical cancer. The squamocolumnar junction of cervics is specifically susceptible to transformation by the high-risk HPV and is the region in which over 90 percent of lower genital tract malignancy develop (Mirkovic *et al.,* 2015; Burd, 2016. Women, mostly freshly sexually active and young adult women, may easily acquire HPV via sexual intercourse with an infected male partner (Kong *et al*., 2019). HPV infects the basal layers of epithelial cells through the epithelial injury or corrosion and eventually induces cervical premalignant changes or dysplasia is known as cervical intraepithelial neoplasia (CIN) which generally develops into cervical cancer because of continuous infection of high-risk HPVs (Liu and Baleja, 2008; Small *et al.,* 2017). HPV replication cycle is tightly associated with the differentiation process of the infected cells (Sheila V Graham, 2017). The establishment and consequent development of this form of cancer are dependent on two crucial oncogenes E6 and E7 that are expressed constitutively in infected epithelial cells that lead to tumorigenesis. High copy number of viral genome and formation of a virions assembly occurs in the differentiated sheet of the infected squamous epithelium host cells that should have normally terminated to proliferate. There are various obstacles that HPVs need to be abolished to exhibit a persistent infection such as clearance of infected host cells by programmed cell death, immune responses, and eventually replicative senescence (Serrano *et al.,* 2018).

2. METHODOLOGY

All studies done on HPV-induced cervical cancer were eligible for this review article and the key search terms used for present study were HPVs, Human papillomavirus, HPV vaccination, Cervical Cancer diagnosis, molecular genome etc. through online database such as Scopus, Web of Science, PubMed, ScienceDirect, Elsevier, SpringerLink, PubMed Central (PMC), Google Scholar, PNAS (Proceedings of The National Academy of Sciences of The

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United States of America), Wiley Online Library, Journal of Virology, Nature (Journal), PLOS journals etc.

3. HPVS- CLASSIFICATION AND GENOTYPES:

HPVs belong to the distinct virus family, the Papillomaviridae (Bernard et al., 2010). Papillomaviridae family contains 29 genera which are formed by 189 papillomaviruses (PV) types found from humans- 120, birds- 3, reptiles- 2, and non-human animals 64 types. According to the study, HPVs are members of 5 genera (Alpha papillomavirus, Beta papillomavirus, Gamma papillomavirus, Mupapillomavirus, and Nu-papillomavirus) besides, 2 genera (Eta papillomavirus and Theta papillomavirus) were each comprised of a single bird PV (Bernard *et al.,* 2010)**.** Based on International Agency for Research on Cancer (IARC) data, a sub-group of alpha papillomaviruses-HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59 designates as group 1 (carcinogenic), which are considered as high-risk HPVs type. Also, designates group 2A (probably carcinogenic) and group 2B (possibly carcinogenic) which include several HPVs types namely HPV26, HPV53, HPV66, HPV67, HPV68, HPV70, HPV73, and HPV82. 96 % of the cervical cancers cases are attributed to the most common 13 HPV types which include groups 1 and 2A (Arbyn *et al.*, 2014). Additional alpha papillomaviruses *i.e*., HPV26, HPV30, HPV34, HPV53, HPV66, HPV67, HPV69, HPV70, HPV73, HPV82, HPV85, and HPV97 are related with sporadic incidences of cervical cancer and are designated as group 2B, (Wang *et al.,* 2018). Furthermore, the alpha papillomaviruses also comprise the low-risk human papillomavirus types which have been associated with benign lesions (non-cancerous), such as HPV 6 and HPV11 are responsible for nearly 90% of anogenital warts cases (Hawkins *et al.,* 2013).

4. HPV-GENOME STRUCTURE AND ORGANIZATION

HPV is a non-enveloped double stranded-DNA virus with a circular genome of approximately 8 kbp and up to 10 open reading frames (ORFs**)** (Bernard *et al.,* 2010). The viral genome consists of three functional regions in which two coding regions are found and one is a non-coding region (NCR). First is the Early region(E), the coding region that contains the many open reading frames (ORFs), which encodes for the replication proteins-E1, E2, and E4 and the oncogenic proteins-E5, E6, and E7 that play a role in host's immune evasion for continue growing and also modify the host infected cell to allow the release of progeny virions; (Wang *et*

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al., 2004; DiaMaio and Petti, 2013; Egawa *et al.,* 2015). Second is Late region (L), the coding region that contains the late genes -L1 and L2, which encodes for the major capsid L1 and minor capsid L2 (72 copies) viral proteins. The virions of HPVs are 50- to 60-nm diameter with icosahedral structures consisting of 72 capsomers, and each of the capsomers is composed of 5-L1 molecules (360 copies); (Baker *et al.,* 1991; Trus *et al.,* 1997). The third is NCR- upstream regulatory region (URR) also known as long control region (LCR), located in between E6 and L1 ORFs that consists of DNA regulatory element (Promoter), is involved in the viral gene expression, replication, and transcription (Tommasino Semin, 2014).

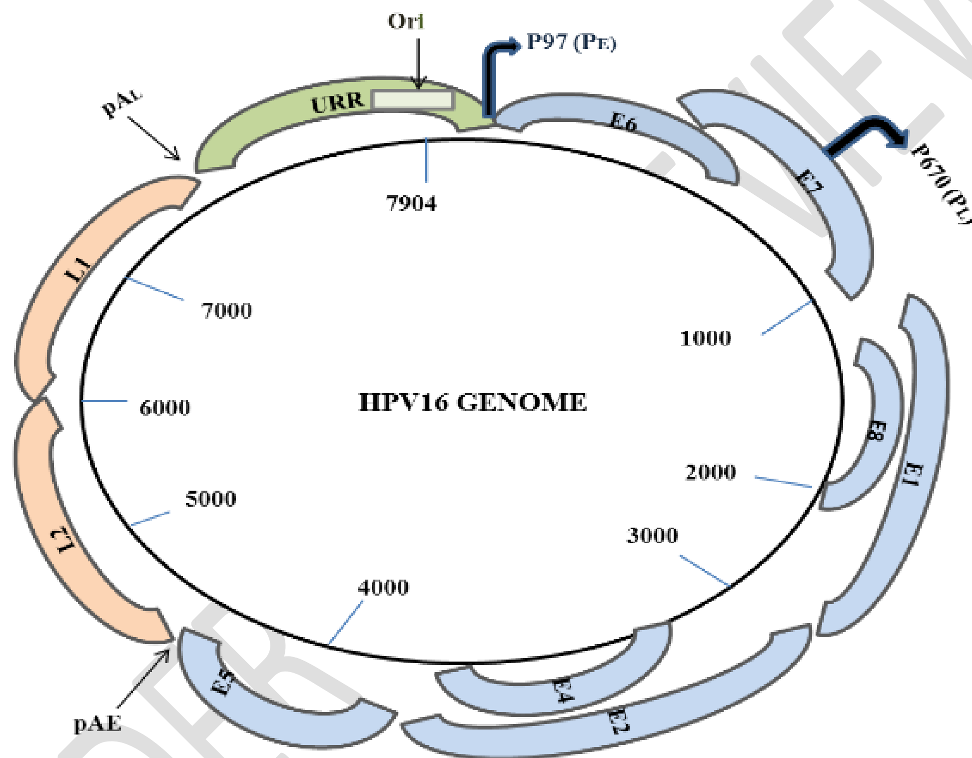

Fig. 1. Human papillomavirus 16 (HPV16) genome structure showing circular dsDNA of HPV16 of approximately 8 kbp. The E1-E8 represents the early genes (shown in blue) responsible for several cellular events that support the complete viral life cycle. Two important structural proteins *i.e.*, late genes (L1 and L2) are shown in orange. Noncoding URR, PE, and PL are the early and late promoters respectively shown in green color. The downward showing arrow indicates the early and late polyadenylation sites, the pAEs, and pALs. The origin of replication (ori) is shown within the region of URR. PE and PL are shown with a darkening arrow that represents the early and late promoters.

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5. HPV LIFE CYCLE

The normal replication cycle consists of an early and a late phase. The early phase involves viral entry and initial genome replication to generate low copy number, stimulation of cell division, and repression of apoptosis in the infected keratinocyte. Late events in the HPV life cycle include vegetative amplification, virion formation, and release into the genital environment from the surface of the keratinocyte. To achieve persistent HPV infection, the lower epithelial cells attached to the basement membrane must get infected (Kaur and Li, 2000). Though given basal epithelial cells are protected by several layers of upper differentiated cells, they are not easily reachable and thus the HPVs must infect those protected cells through wounds that expose lower basal layers (Knipe and Howley, 2013). Later, the infected cells move up through the epithelium as part of the tissue renewal process and acquire characteristics of differentiation till they are slough off from the surface epithelia.

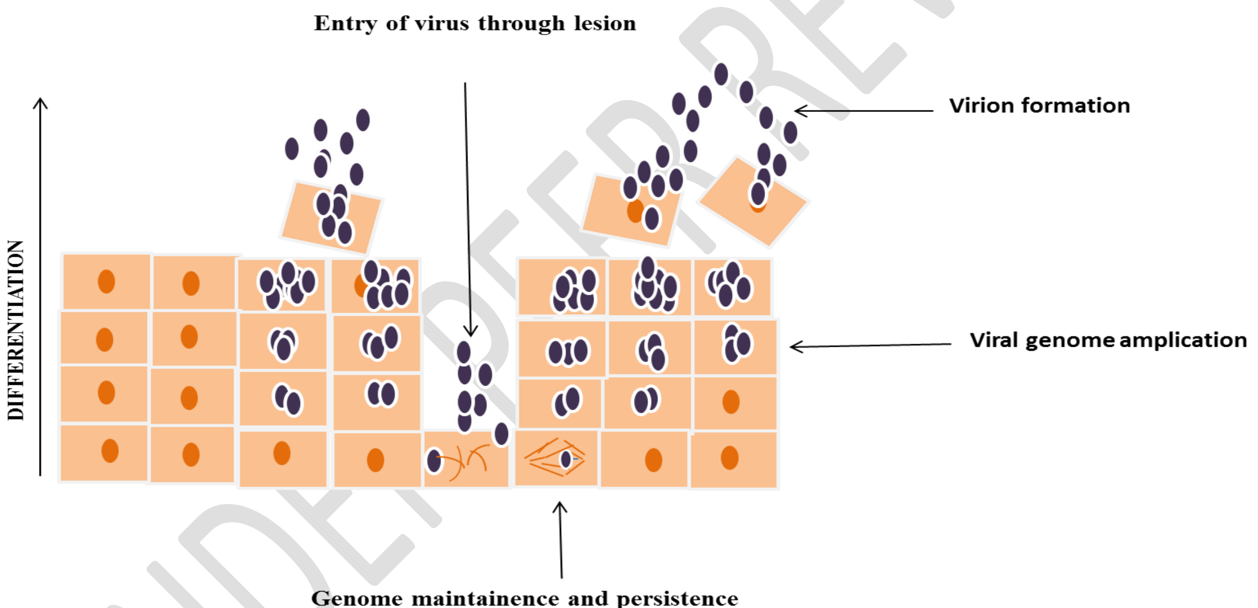

Fig. 2. HPV life cycle coupled with differentiation of host epithelial cells. The virus infects the basal epithelial cells through the lesion and maintains a low copy number. As epithelial cells divide its move up and transform into terminally differentiated cells. HPV is tuned with this process and take advantage by efficiently amplifying high copy number and eventually leads to virions formation.

5.1 Attachment and Entry of HPVs into the Host cell

For non-enveloped (naked) viruses, the proteinaceous coating encircles and protects the viral genetic material and provides the early interaction between viral particles with the host cell.

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Following receptor engagement, the virus particle is internalized through receptor-mediated endocytosis (Bousarghin *et al.,* 2013). This could allow the encapsidated viral genome access to the host cellular transcription and replication machinery. HPVs virion contains two viral late proteins *i.e.*, L1 and L2. L1 is the 55 kDa structural protein that forms the major capsid and the minor capsid protein L2, both capsid genes are crucial for the initial stages of infection, besides genome packing into viral capsids takes place at late stages of viral infection (Wang and Roden, 2013)**.** Subsequently, the initial interaction of the viral particle with the host cell (or the basement membrane), causes a conformational change in the capsid induces exposure of the N-terminus of L2 that is conserved in all known L2 containing species, which allows access of furin to a consensus furin site at the N-terminus (Richards *et al.,* 2006). Furin belongs to the family of calcium-dependent PCs (proprotein convertases), which are eukaryotic subtilisin-like endoproteases (Gensberg *et al.,* 1998). This cleavage plays important role in the entry of the virus, although, it is a reductant for endocytic entry. Furin presents as a functionally active form both on the cell surface and within the compartment of the endomembrane system and, hence, is found in cellular locations where it would interact with incoming virus capsids (Molloy *et al.,* 1999; Thomas, 2002; Mayer *et al.,* 2004). Cleavage of L2 by furin could deliver several functions: First, furin cleavage of L2 could cause the release of the viral L2-genome complex from L1, since L1 doesn't appear to exit from the compartment of the endomembrane system (Day *et al.,* 2004). It crosses the cytoplasm, which is likely due to the interaction between dynein (cytoskeletal motor proteins) and L2, to reach the cell nucleus (Schneider *et al.,* 2011). The Entry of the viral L2-genome complex into the cell nucleus possibly depends on the disassembly of the nuclear envelope (NE), due to the removal of the barrier between the nucleoplasm and the cytosol as infection needs mitotic division so that the HPVs can access the host nucleus for its transcription and replication (Pyeon *et al.,* 2009; Aydin *et al.,* 2014).

Viral uncoating occurs in a vesicular compartment, L2, and that the associated HPV genome localizes adjacent to ND10 through a mechanism independent of the viral DNA sequence. HPVs and host cellular factors are involved in the association of the viral genome with ND10, which results in transcriptional regulation, growth suppression, and apoptosis (Day *et al.,* 2004; Everett, 2006).

5.2 Virus Initial Amplification

In the HPV life cycle, it is normally assumed that there are three different phases of viral replication. The first happens when the viral particle infects the host basal epithelial layers.

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During this phase, the viral genome must undergo a few rounds of unlicensed replication to produce a small number of viral genomes in this newly infected cell. Next, in the second the basal cells divide the viral DNA replicates along with host DNA and are separated into daughter cells. In daughter cells they can either stay in the basal layer and keep on dividing or can move towards the upper region and commence the process of cellular differentiation. Those cells that enter differentiate and can initiate late gene expression (L1 and L2) and the third phase of viral replication *i.e*., Vegetative viral-DNA amplification (Doorbar *et al.,* 2012).

Transcripts encoding the early E1 and E2 replication oncoproteins, that initiated by several cellular factors to assist the first phase of HPV-DNA replication (Ozbun, 2002; McKinney *et al.,* 2016)**.** It was reported that the expression of the HPV E1 and E2 is important for replication of Bovine papillomavirus in mouse C127 cells (Ustav *et al.,* 1991). The 68 kDa E1 protein is a ring-shaped hexameric helicase that belongs to the AAA+ family that catalyzed DNA strand separation and allows for access to replicative machinery (Enemark and Joshua, 2006). On the other hand, 48 kDa E2 functions as a helicase loader that has a dual function, firstly it facilitates binding of E1 protein onto the replication origin (Stenlund, 2003). Secondly, its role in the initiation of viral replication facilitates maintenance and genome partitioning by tethering it to host cell chromatin (Skiadopoulos and McBride, 1998)**.** There is an absolute requirement of a functional E2 binding site as a major cis-Acting replication element that establishes site-specific binding of E2 proteins to the viral replication origin. This origin encompasses an E1 protein binding site, at least one E2 binding site, and an A/T rich region (Mohr *et al.,* 1990; Ustav *et al.,* 1993). Out of three, at least one E2 binding site was needed for origin function and an additional site had a cooperative effect or enhanced this process (Remm *et al.,* 1992). However, HPV E2 proteins do not associate with nuclear chromatin when expressed alone but associate tightly with the E1 protein when expressed together (Sakakibara *et al.,* 2013; Jang *et al.,* 2014).

5.3 Maintenance replication and genome partitioning

Additionally, Minimal replication origin (MO) is an additional element MME (minichromosome maintenance element) in the upstream of the regulatory region of bovine papillomavirus that assures stable replication of the plasmids (containing MO). This element is consisting of multiple binding sites for the E2 however, it is not necessary for stable episomal replication of plasmid MME (Piirsoo *et al.,* 1996). The E2 trans-activator protein is composed of three distinct domains. The amino (N)-terminal has ~200 amino acids that contain the trans-

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activation domain of E2 that interacts with the host mitotic chromosome; and the carboxy (C)-terminal has ~100 amino acids that facilitate DNA binding and dimerization domain (McBride and Myers, 1997). This trans-activation domain is responsible for the DNA replication, segregation, and regulatory functions of the HPV E2 protein (McBride *et al.,* 1989; Bastien and McBride, 2000). Interaction of the viral E2 protein with cellular proteins Bromodomain-containing protein 4 (Brd4), member of the BET (bromodomain and extra terminal domain) family of transcriptional regulators, are playing multiple roles in the HPV lifecycle and showing specific association with the host mitotic chromosome, histone reader that is essential for the regulation of cellular transcription (McBride and Jang, 2013). In several cases Mutation of amino acids R37 and I73 on this surface has been reported , to eliminate alanin and the chromosomal binding ability of E2 (Baxter *et al.,* 2005). The limited replication of the viral genome in undifferentiated cells requires the expression of the conserved E8^E2 (or E8/E2) viral repressor protein, which is a fusion between the E8 gene product and the carboxy-terminal half of the E2 protein. Viral E8/E2 is a sequence-specific DNA binding protein that can inhibit the viral genome replication and viral gene expression (Dreer *et al.,* 2016).

5.4 Genome integration

HPV genome may either get integrated into the host genome or stay as episomal form, with 83 percent of the cervical cancer cases showing evidence of HPV genome integration into the host genome (Burk *et al.,* 2017). In the event of HPV genome integration into the host genome, this frequently leads to the destruction of the E2 gene site. As E2 gene is responsible for repressing E6 and E7 genes, and thus causing activation of E6 and E7 gene by HPV genome integration into the host genome. During infection, this activity is responsible for the amplification of the HPV genome with the help of the host cellular machinery (Neveu *et al.,* 2012).

Previously, Durst et al., in 1987, have reported that the two human HPV 16 flanking sequences derived from the tumor were localized to chromosome region the 20pter----20q13 and 3p25----3qter, that contain the protooncogenes c-src-1 and c-raf-1 respectively, besides, HPV-16 integration site in the SiHa cervical carcinoma-derived cell line is in chromosome region 13q14----13q32. They have also reported about HPV 18 integration site SW756 cervical carcinoma cells is in chromosome 12 but without linking with Ki-ras2 gene and concluded that in two cervical carcinoma cell lines, Hela and C4-I, HPV 18 DNA is integrated into chromosome 8, 5' of the c-myc gene. They have also reported integration sites on chromosomes 3 and 20 in a tumor that

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harbored multiple HPV 16 integration sites and in four cervical carcinomas derived cell lines the integrations sites were reported on chromosomes 8, 12 and 13.

Hu *et al.*, 2015, have studied the mechanism of HPV integration in cervical cancer and identified cluster genome hot spots by conducting whole-genome sequence and high-throughput viral integration. They have identified 3,667 HPV integration breakpoint in 26 cervical intraepithelial neoplasias, 104 cervical carcinomas and five cell lines. They have discovered new hot spots *HMGA2* (7.8%), *DLG* (4.9%) and *SEMA3D* (4.9%) besides pre reported *POU5F1B* (9.7%), *FHIT* (8.7%), *KLF12* (7.8%), *KLF5* (6.8%), *LRP1B* (5.8%) and *LEPREL1* (4.9%). They have also reported that protein expression from *FHIT* and *LRP1B* was found downregulated when HPV integrated in their introns and protein expression from *MYC* and *HMGA2* was found elevated when HPV integrated into flanking regions. They found that fusion between viral and human DNA have occurred by microhomology- mediated DNA repair pathway and confirmed that microhomologous sequence between the human and HPV genomes was significantly enriched near integration breakpoints. In high-throughput viral integration detection (HIVID) total of 3,667 HPV integration breakpoint in 103 of 135 samples have been detected including 14 of 26 CINs, 85 of 104 cervical carcinomas and 4 of 5 HPV positive cell lines. It was also reported that HPV may, from the beginning, randomly integrate into the host genome based on genome accessibility but in the case of long-term carcinogenesis integration at recurrent loci gives the advantage to host cells (Wentzensen *et al.*, 2010; Dall *et al.*, 2008; Ferber et al., 2003; Schmitz *et al.*, 2012; Peter *et al.*, 2006). In the study of Hu *et al.*, 2015, it was found that HPV 16 retain intact oncogenes E6 and E7 with the long control region and breakpoint could occur virus to adapt to the changing environment during carcinogenesis. This finding is in disagreement of previous concept of E6 and E7 and warrants further study.

It has been also revealed that HPV integration occurs in initiating stage (ex.- CIN1) for cervical carcinogenesis and increase in both integration rate and number of CIN1 leads to disease progression (Oliveira *et al.*, 2013). It was also reported that integration at *POU5F1B, KLF5-KLF12, FHIT, HMGA2, LRPIB, SEMA3D* are found in both CINs and cancer integration at *POU5F1B* and *KLF5-KLF12* are further being shared by squamous cell carcinoma and adenocarcinomas. The involvement of these hot spot in CINs process proves their role in early progression of cervical carcinogenesis. Besides, it has been also reported that HPV integration exerts different effects on different targets, *viz. – FHIT* and *LRPIB*, results decreased protein expression in neoplastic tissue compared to adjacent normal tissue. When its breakpoint are in

their introns but *MYC* (near *POU5F1B*) and *HMGA2* with HPV integration in their flanking region results increased protein expression in neoplastic tissue compared to adjacent normal tissue. Thus, it become evident that efficiently do the survey of human genome and by activating and inactivating genes that favour positive clonal selection and thus accelerates malignant transformation of host cells (Schmitz *et al*., 2010).

It was found that MH- medicated DNA repair pathways, particularly fork stalling and template switching (FoSTeS). (Lee *et al*., 2007; Zhang, *et al*., 2009) and microhomology medicated break-induced replication (NMBIR) (Zhang, *et al*., 2009; Verdin *et al*., 2013) are mechanism which mediate integration process.

6. DIFFERENTIATION INDUCED VEGETATIVE VIRAL DNA REPLICATION

When the virally infected cells commence the process of cellular differentiation, this ultimately increases the replication activity that results in an induction of late gene (L1 and L2) amplification of the viral genome from approximately 50 episomal copies per cell in basal epithelial to 100-1000 episomal copies of the viral genome in supra-basal epithelial (Laimins 1993). Therefore, the viruses synthesize a large amount of viral DNA in differentiated cells that are in either the G2 phase or have exited the cell cycle. As the viral infected basal keratinocyte divides, progeny cells then start to differentiate, and the late gene functions are induced, which culminate in the formation of viral particles (virions)**.** In several studies, it was found that most HPV transcripts were properly spliced polycistronic RNAs. Most of these RNA transcripts exercise the major splice site donor within the early E1 gene (nucleotide 877) together with the major splice acceptor within E4/E2 region (nucleotide 3295). In undifferentiated cells, the pre-mRNA encoding gene E1 of length 4.2 kb in size (Hummel *et al.,* 1992) arise from the P97, early promoter. Previous studies suggested that differentiation in epithelial cells induces the accumulation of a second E1 mRNA transcript of length 3.7 kb that arises from the P742, differentiation-specific promoter (Ruesch *et al.,* 1998) to a much large extent than E2 (Klumpp and Laimins, 1999). Therefore, late events regulate the expression of late mRNAs, which encode the L1, L2, E1ˆE4, and E5 open reading frames (ORFs) and which originate from the E7 ORF, differentiation-specific promoter (Hummel *et al.,* 1992)**.** By induction of the homologous recombination repair pathways and DNA damage response, the HPV can efficiently replicate daughter genomes in differentiated epithelial cells without interfering with host DNA synthesis. This provides supremacy to viruses to take off and hijack this process. By induction of the DDR

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response in the vicinity of the viral DNA, all elements required for DNA synthesis delivered to the DNA leads to and the DNA damage response foci and becomes virus production factories (Piirso *et al.,* 1996). In the last event of viral replication, a large quantity of daughter viral genomes is synthesized for packaging in viral capsids. This enormous viral replication event intermediates are sensed by the host regulatory system as damaged DNA and hence induces a DNA damage response (Stracker *et al.,* 2002; Lilley *et al.,* 2005). Viral oncoproteins may also persuade a DNA damage response by meddling with host cell cycle regulation that causes replication stress (Bester *et al.,* 2011). The virus takes advantage of host cellular defenses, and, activates an ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) pathway of the DDR responses needed for efficient vegetative DNA replication of the virus (Chaurushiya and Weitzman, 2009: Moody and Laimins, 2009). These virally infected cells originate from an intraepithelial lesion that maintains the viral genome as an episome and during the differentiation process helps it to complete the life cycle (Hummel *et al.,* 1992; Meyers *et al.,* 1992).

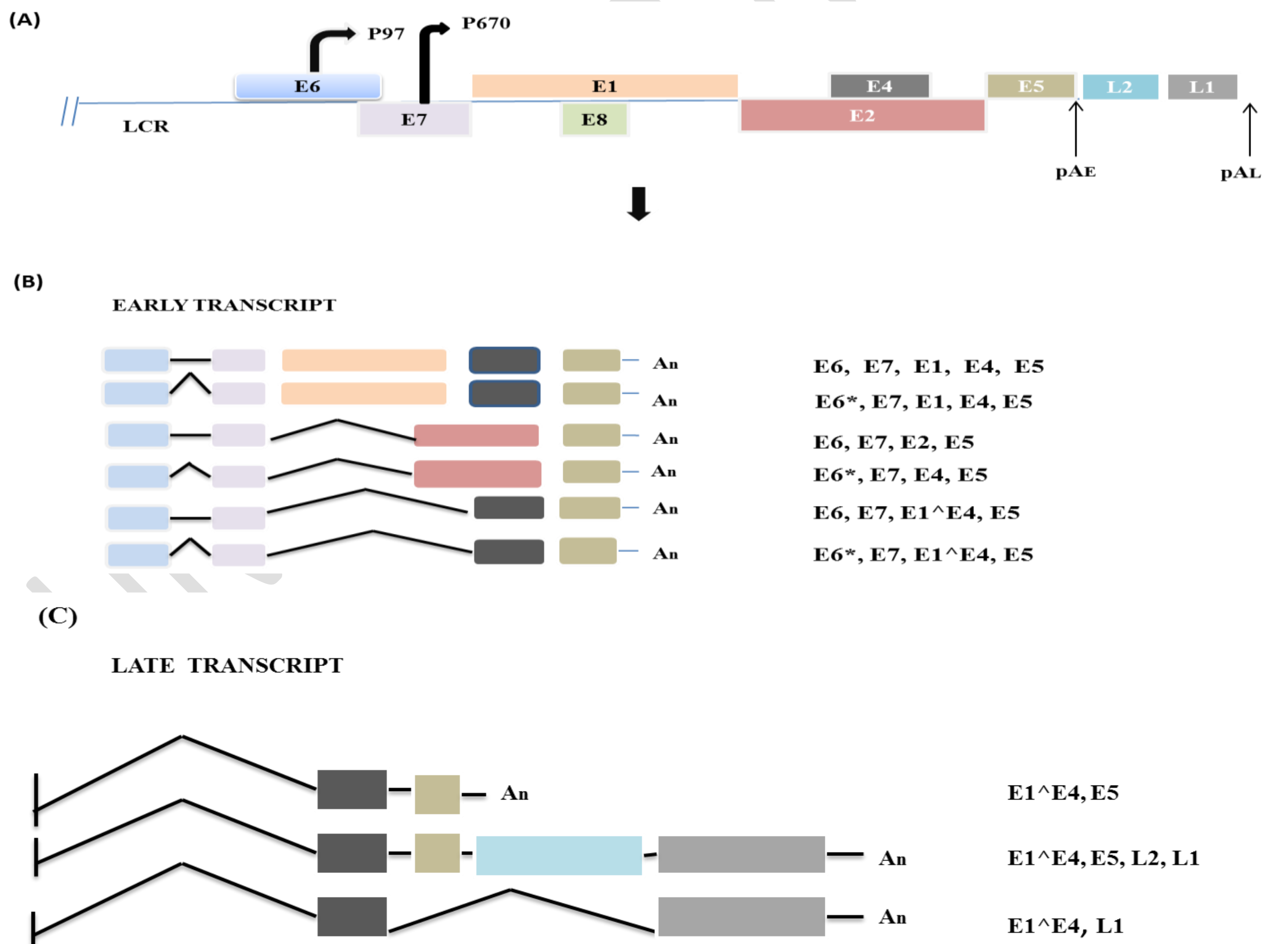

Fig. 3. The diagram showing splicing events for the generation of HPV 16 early and late mRNAs transcript. (A) HPV16 genome. Colored boxes represent viral open reading frames (ORFs). Arrows indicated the promoter (P) region. (B) the diagram represents the main splicing events for the generation of HPV early mRNAs. Again, colored boxes indicate ORFs. Uphill lines indicate introns that are spliced out. (C) Late transcript with its splicing events.

7. E6 AND E7 GENE - THE ONCOPLAYERS

High-risk HPVs are encoded with two early oncogenic proteins, E6 and E7, and each individually extend the life of primary host cells and when they co-expressed, facilitate their immortalization, undergo malignancy when exposed to a further oncogenic stimulant (Hawley-Nelson *et al.,* 1989; Munger *et al.,* 1989; Halbert *et al.,* 1992). In the course of replicating the HPV genome, each can induce all the hallmarks of a cancerous cell, i.e., uncontrolled cellular growth, invasion, angiogenesis, unrestricted telomerase activity, and metastasis along with the bypassing of growth suppressors and apoptosis activity by interfering with various cellular pathways. Such one pathway is the retinoblastoma E2F pathway (Rb-E2F) leads to DNA damage, replication stress, and malignant transformation (Bester *et al.,* 2011). Retinoblastoma (Rb) is a key player in the regulation of the cell cycle, limiting cell proliferation by inhibiting E2F transcription factors for the cells to proceed through G1/S phase transition (Heuvel and Dyson, 2008). Deregulation of the Rb-E2F pathway by the E7 plays a role in the activation of the ATM (Ataxia Telangiectasia Mutated Protein) response both directly (Moody and Laimins, 2009) and indirectly by causing cell replication stress due to a deficiency in nucleotides (Bester *et al.,* 2011), In infected cells, E7 targets pRb for ubiquitination, leading to the release of E2F transcription factors, which transcribe cyclin A, cyclin E, and p16 (also known as p16-INK-4A, an inhibitor of CDK4/6), drives the cells entry into the premature S-phase (Boyer *et al.,* 1996). p16 (tumor suppressor protein) is a key target of E7 to regulate the cell cycle. E7 triggers the gene expression of p16 not only through pRb disintegration but also by epigenetic de-repression through the KDM6B family (H3-K27 specifically demethylase 6B) (Drubin *et al.,* 2013)**.** Abnormal activation of this pathway either through HPV E6/E7 or cyclin E oncogenes ultimately decreases the level of cellular nucleotide in the transformed cells (Frame *et al.,* 2006). This leads to programmed cell death or senescence, providing a barrier to tumor progression. To overcome this barrier, HPV16 E6 oncogenes causes proteasomal degradation of p53 (also known as TP53). E6 mediated degradation of p53 allows various cellular changes to

become a cell cancerous, one of such brings uncontrolled cell proliferation by evading the cell cycle checkpoints. The 53 kDa molecular weight protein, p53 like the Rb, is a tumor suppressor protein and is often called the "Guardian of the Genome" because it determines the fate of a cell in stressed conditions. When the cell undergoes stress conditions due to oxidative damage or any other cause, it activates and acts as a transcription factor to transcribe the critical genes for either cell cycle arrest or apoptosis (Pflaum *et al.,* 2014). On the other side, Mouse double-minute 2 homolog (MDM2), functions as an E3 ubiquitin ligase and helps to keep p53 at a basal level in a normal cell. Thus, p53 inactivation by E6 is critical to ensure continuous cell proliferation. Therefore, both E6 and E7 enable the proliferative property of viral-infected cells (Duensing and Münger, 2004).

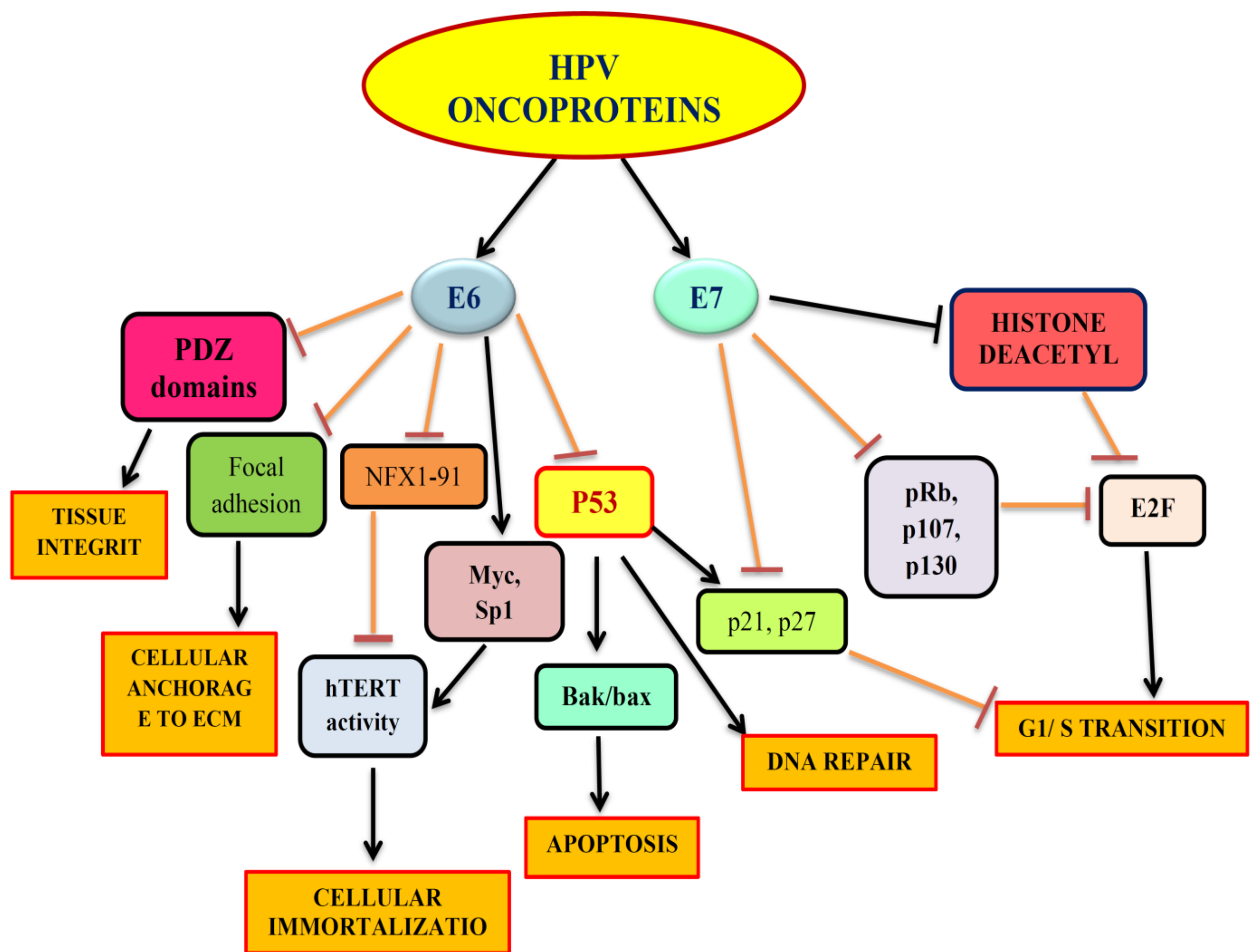

Fig. 4. Showing molecular mechanisms of HPV Oncoproteins induced cell proliferation and genomic instability, a hallmark of cancerous cells.

7.1 Resisting Programmed Cell Death

E6 causes degradation of p53 through ubiquitination process along with the help of E6-AP (E6-associated protein) also called UBE3A (Scheffner *et al.,* 1990). HPV E6 oncoproteins have been found to bind to the LxxLL-consensus sequence to form a heterotrimeric complex - E6/E6AP/p53, which ultimately leads to the degradation of p53. This drives the cells through uncontrolled cell proliferation, evading the cell cycle checkpoints (Nguyen *et al.,* 2002). Thus, escaping cell-cycle arrest or programmed cell death. Several studies showed that E6 was found to block programmed cell death through both p53-dependent and p53-independent manners. HPVs E6 can directly interact with BAK (members of the Bcl-2 family) which leads to the degradation of BAK through the interaction of p53-E6AP (Garnett and Duerksen-Hughes, 2006). In p53 independent manner, E6 has been found to hinder Tumor necrosis factor (TNF) mediated extrinsic mode of programmed cell death through the PDZ-domain of HPV E6, which can bind to TNFR1 and protect TRADD from interacting with it (Filippova *et al.,* 2002).

7.2 Continuous Proliferative Signaling

A healthy cell progresses through cell-cycle and divisional stages in response to the various growth-promoting signals, which are formed and regulated in a controlled fashion. These signals are recognized by the receptor kinases present either on the cell surface or inside the cells and transmitted to the nucleus through several branched pathways. In the case of a cancerous cell, these signaling pathways have been de-regulated which leads to the sustenance of uncontrolled cell proliferation. One such signaling pathway that induces cell survival and proliferation is mediated by the oncogenic RAS, which drives the infected cells to tumor progression through downstream effectors of these pathways, like Phosphoinositide-3-kinase (PI3K) Protein kinase B (PKB)/Akt, and MAP kinase (MAPK) pathways. E6 has been found to activate the MAPK pathway (Chakrabarti *et al.,* 2004). HPV-E6 and E7 both have been found to have a deep involvement in the mTOR (mechanistic target of rapamycin) pathway to regulate cellular proliferation too. Studies also showed that HPV E7 expressing infected cells were found to undergo autophagy (or auto phagocytosis) even in nutrient-rich conditions (Zhou *et al.,* 2009). Whenever nutrients were in limited amount, E7 expressing infected cells unlike the healthy cells could continue to proliferate and eventually lead to CICD (caspase-independent cell death) a process called a “trophic sentinel” response (Eichten *et al.,* 2004). Although, in the presence of HPV E6, this will be avoided, as E6 had been found to activate mTORC1 signaling to enhance protein formation even in the absence of cell growth-factors through the upstream kinases PDK1

(key regulator, transmitting PI3K dependent signaling pathway) and mTORC2 (Zhou and Spangle, 2009; Spangle and Münger, 2010). This would lead to rising in mTORC1-dependent cell growth and proliferation even in the lack of cellular growth factors and help in the tumor progression.

7.3 Permit Replicative Immortality

In every new round of cell replication, the telomeres shorten in length with cellular aging. Hence, in a cancer cell, cells need to protect the telomeres shortening to continue the tumor progression. Telomerase is the ribonucleoprotein enzyme, which plays a role in the chromosomal end replication, and hence it is overexpressed in the cancer cell and becomes inactive in a healthy cell. The telomerase complex consists of two core subunits, a template RNA subunit (ERC) and the catalytic unit - human telomerase reverse transcriptase (hTERT) (Shay *et al.,* 2002). In HPV infected cells E6 and E7, the oncoproteins manage the expression of hTERT constitutively to sustained replicative immortality. HPV E6 has been found to activate the promoter of hTERT with the help of c-myc, Sp1, and NFX1 along with ubiquitin ligase E6AP (Liu *et al.,* 2009). NFX1 is a negative transcriptional repressor of the hTERT gene and thus gets disrupted by E6/E6AP oncoproteins that lead to activation of the hTERT promoter. E6- mediated hTERT expression is also get established through the epigenetic mechanism, histone demethylases and methylases are manipulated accordingly to decrease the level of repressive H3K9Me2 chromatin mark and increase the activating H3K4Me3 chromatin mark (Gewin *et al.,* 2004; Zhang *et al.,* 2017). While the other two, proteins *i.e*., Sp1 and c myc function as a positive transcriptional regulator and hence get activated by HPV E6 (Liu *et al.,* 2009)**.**

7.4 Tumor Angiogenesis

Tumor progression and metastasis also depend on angiogenesis; it is the development of new blood vessels to the transforming cells from the existing vessels triggered by chemical signals generated by tumor cells in a period of rapid growth (Folkman, 1971). This action would maintain equilibrium between angiogenesis-inhibitors and angiogenesis-inducers. HPV E6/E7 helps the infected cells to derive oxygen and nutrition from the surrounding cells through angiogenesis by regulating the gene expressions and activities of the inhibitors and inducers. The key change in the expression of the pRb and p53 (tumor suppressor) by E7and E6, respectively, would connect to the angiogenic modulators. one of the analyses showed that p53 regulated the three major genes to change its expression in infected cells transformed with HPV E6 and E7, including VEGF (vascular endothelial growth factor- angiogenesis inducer), thrombospondin-1,

and maspin (angiogenesis inhibitors) (Toussaint-Smith, 2004). The vascular endothelial growth factor is a potent angiogenic inducer that stimulates endothelial cells to interrupt the extracellular matrix, indorse proliferation, relocation, and form tubes, and also acts as an endothelial cellular surviving influencer. This is negatively regulated by tumor suppressor p53, through the HIF-1α (Hypoxia-Inducible Factor), and in the absence of p53, it would become activated and contribute to angiogenesis. VEGF promoter contains an AP1-binding region, which could be activated by HPV E7. Through several reports, it was also found that IL-8 is known to be a critical angiogenic inducer, found to increase due to the expression of E6 and E7 oncoproteins (Tischer *et al.,* 1991; Pal and Kundu, 2020). Thrombospondin-1 and maspin both act as angiogenic inhibitors and are positively controlled by p53, though p53 is degraded by HPV E6, the angiogenic inhibitors become nonfunctional.

7.5 Invasions and Metastasis

HPV E6 and E7 oncogenic proteins have been reported responsible for the induction of the epithelial-to-mesenchymal transition (EMT), a well-characterized process required for the tumor cells to intravasate into the blood vessel and metastatic progression at a new place in the body. The study showed that ectopically expression of HPV E6/E7 can induce the formation of typical cobblestone-shaped epithelial cells from unique spindle-shaped mesenchymal cells (Kim *et al.,* 2013). Furthermore, it has been also reported that HPV E6/E7 can activate the EMT-activating transcriptional factors (EMT-ATFs) including Snail family of zinc-finger transcription factors –Slug; the basic helix-loop-helix factors-Twist, zinc-finger transcription factors of d-crystallin/E2 box factor (dEF1) family proteins-ZEB1, and Smad interacting protein-ZEB2 followed by an increase in the invasive and migratory potentials of the cancer cells (Perez-Moreno *et al.,* 2001). EMT is characterized by, downregulation of epithelial cell marker E-cadherin, followed by overexpression of mesenchymal cell markers N-cadherin and cytoskeletal alterations (vimentin, and fibronectin) (D'Costa *et al.,* 2012) in response to expression of HPV E6 and E7 (Hellner *et al.,* 2009). Additionally, E6 /E7, E5 have also been found to upregulate the expression of VEGF through (epidermal growth factor receptor) EGFR, MEK/ERK1, and 2 along with PI3K/Akt signaling pathway, which contributes to cell invasion and metastasis (Kim *et al.,* 2006).

8. SCREENING AND DIAGNOSTIC TESTS TO DETECT CERVICAL CANCER

Screening and diagnosis for cervical cancer is crucial process due to delayed symptoms. Today different screening methods, based on traditional and newer advanced technologies, are available to screen the patients for cervical pre-cancers lesion and cancers. No screening test is best to give 100% accurate result and hence the selection of screening test will decide on the situation where is to be applied. Presently there are three different types of effective diagnostic tests for cervical cancer screening- Papanikolaou (Pap) test, visual inspection with acetic acid, HPV typing test.

8.1 Cytology-based screening- Pap test

Cytology-based screening can be performed efficiently only if the framework and laboratory quality assurance demands are constantly met. The different cytology-based screening is as follows: (1) Conventional cytology-based Pap test: This method is broadly used for screening in most well-developed countries, which involves the collection of cells that are scraped from the endocervix and ectocervix, either with a brush or spatula and making their smears and confirmed by microscopic examination. This test is highly particular but sometimes gives false-negative results which have been an area of concern in cytology-based screening, wherein malignant or premalignant epithelial cells have been misidentified as normal cells (Karnon *et al.,* 2004). (2) liquid-based cytology (LBC) using Pap test: In this, the cells are collected the same as conventional Pap using a brush. The tip of the brush is forcefully shaken into a little pot of liquid containing preservative solution, and then the sample is filtered to eliminate debris and excess blood. In the laboratory, the collection of cells is transferred on the slide in a mono-layer. In a Metadata analysis comparing conventional Pap with LBC, no major difference was established in the relative sensitivity (Mishra *et al.,* 2011). (3) (i) Automated Pap testing – In this method computer-based screening to assess Pap smear slides to reduce the chances of errors is being carried out (ii) AutoCyte Screen with AutoPap- This device presents various cell images displaying to a human reviewer, who then defines whether a manual review is required or not. After the reviewer choose its opinion, then the device reveals its decision based on a ranking system as to whether the manual review is warranted. Manual review is needed for any case if designate by either the computer-based ranking or cytologist (Nuovo *et al.,* 2001).

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8.2 Visual examination

Different methods of visual screening have been used in various countries. First, Visual inspection following application of 3-5% acetic acid (VIA) - causes reversible precipitation or coagulation of the cellular nuclear proteins in the region containing dysplasia cervical intraepithelial neoplasia (CIN) or invasive cancer (Belinson *et al.,* 2001). Second**,** Visual inspection following application of 3-5% acetic acid and observed under magnification (VIAM) devices, called a gyroscope, or VIAM shows a similar result when compared with VIA with no extra benefit as noted in Mumbai cervical cancer trial (Shastri *et al.,* 2005). Third, Schiller's test Visual inspection after application of Lugols' iodine (VILI) - Precancer lesions and invasive cancer cells don't take up iodine and appear thick, mustard, or saffron yellow due to lack of glycogen. Although normal squamous epithelium contains glycogen, so iodine reacts with glycogen and gives a mahogany brown color appearance.

8.3 Human papillomavirus (HPV) DNA test

Many studies revealed that women infected with HPV 16 or 18 have a very higher frequency of progression of cervical squamous intraepithelial lesions that leads to cancer. There are several varieties of laboratory-based approaches for the detection of HPV in cervical cancer samples, the Hybrid Capture II kit was approved by the United States Food and Drug Administration (USFDA) is being used frequently. This sample is collected with a cervix swab of the transformation zone and transferred into a transport medium. The test may also be performed from a residual sample collected in a liquid-based medium (LBC) for monolayer preparation. In the laboratory, host cellular DNA is denatured and mixed with a ribonucleic acid (RNA) probe that only binds to HPV DNA. The DNA "hybrid" is then attacked by an antibodies-coated tube. Later, a chemical is added, which causes a chemo-luminescent reaction. The amount of light illuminate determines the presence of HPV and the virus loads (Spitzer, 1998).

9. PROPHYLACTIC AND THERAPEUTIC HPV VACCINES

The level of antibodies produced by epithelial cells in natural infections by HPVs is usually insufficient to prevent consequent reinfection. The infected cervical region lacks secondary lymphoid tissues which contain a large number of memory B cells, later producing antibodies and neutralizing the virus antigens before uptake. Besides, to establish a protective

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immune response in the course of the sexual life, high and persistent levels of nAbs (neutralizing antibodies) induced through vaccination are required. Thus, the ultimate vaccine is needed to protect against high-risk HPV types. Currently, three licensed prophylactic vaccines for prevention of HPV infection that was approved in 2006-07 are available at present- a bivalent vaccine (2vHPV) against HPV16, HPV18 (Cervarix), a quadrivalent vaccine (4vHPV) against HPV 6, 11, 16, 18 (Gardasil), and a non-violent vaccine (9vHPV) against HPV6, 11, 18, 31, 33, 45, 52, 58 (Gardasil 9). The US Food and Drug Administration in 2018 approved the non-violent HPVs vaccine in the USA for 9 to 45 years old. The quadrivalent vaccine is used for the prevention of, precancerous lesions, genital warts, and cervical cancer under the age of 9–26 years. Based on recombinant DNA technology, these HPVs vaccines are prepared from the L1 protein that is self-assemble to form the HPV-specific empty shells (VLPsvirus-like particles) (Stanley *et al.,* 2012). These vaccines are effective in protecting 90% of HPVs infection vaccines by inducing humoral immunity against the target late proteins L1 or L2 which leads to antibody induction but shows limited benefits in eliminating the pre-existing infections (Buck *et al.,* 2013; Wang *et al.,* 2020). Hence, there is a need for progression in the advancement of therapeutic vaccines. Therapeutic vaccines are differing from prophylactic vaccines as they aim to generate a cell-mediated immune response and kill the HPVs infected cells in place of neutralizing antibodies to eliminate the persistent infection and pre-cancerous lesions made by HPVs. Different therapeutic HPV vaccines that target E6, E7, and other viral oncoproteins have been broadly studied, including live vector-based vaccines, nucleic acid, peptide-based or protein vaccines, and cell-based vaccines.

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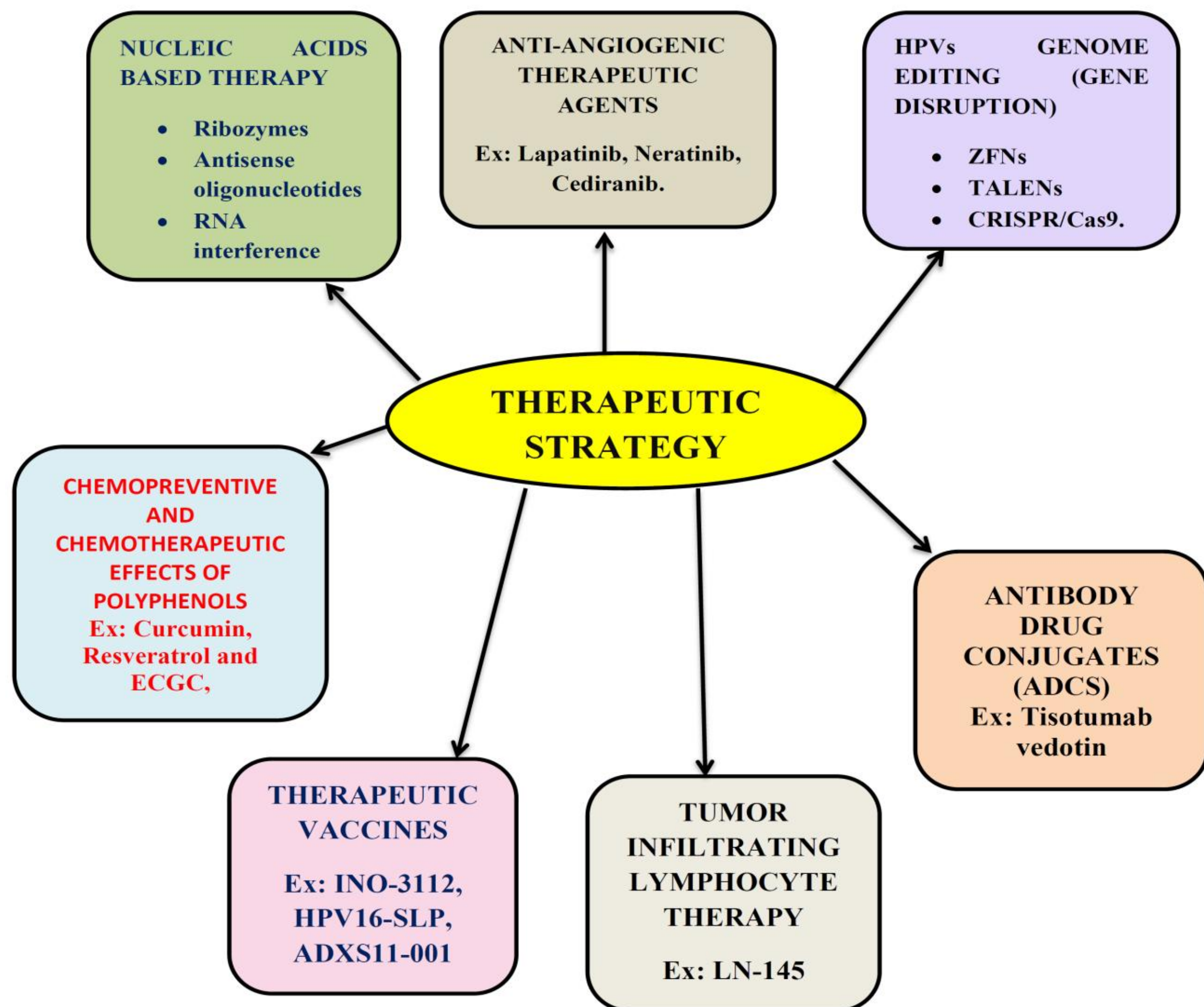

Fig. 5. Showing different therapeutic strategies used to target E6/E7 expression and its activity to prevent beginning cervical cancer.

CONCLUSION

Papillomavirus infection targets squamous epithelial and can cause cervical cancer. Progression of the viral lifecycle requires cellular differentiation of the host and tight regulation between host differentiation events and viral genome replication. Understanding the major molecular mechanisms that become disrupted in the HPV-infected cells and transformation from cervical intraepithelial neoplasia (CIN) to invasive cancer gives an insight into the multiple pathways involved. The overexpression of HPVE6/E7 proteins is the prime factor that affects the activity of tumor suppressor genes (p53 and pRB) that regulate the cell cycle of the host cell, which disturbs many downstream signaling pathways leading to cancer development. Hence, the

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present review concludes that manipulation of two oncogenes E6 and E7 may give significant results in cervical cancer therapy. There is need of further intense study about E6 and E7 oncogenes to develop much more effective mechanism to control cervical cancer.

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