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Review

Understanding the HPV integration and its progression to cervical cancer

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ABSTRACT

Cervical cancer is one of the main causes of female cancer death worldwide, and human papilloma virus (HPV) its causal agent. To investigate viral oncogenesis several studies have focused on the effects of HPV oncoproteins E6 and E7 and the mechanisms by which these proteins stimulate the cellular transformation process. However, phenomena such as the physical state of the viral genome (episomal or integrated) and the effects of this integration on cell proliferation contribute new clues to understand how HPV infection causes carcinogenesis. New molecular technologies are currently facilitating these discoveries. This paper reviews the tumor development process initiated by HPV, recent findings on the process of viral integration into the host genome, new methods to detect HPV integration, and derived associated effects.

1. Introduction

Cervical cancer (CC) is the second most common type of cancer worldwide, with 500,000 cases per year (WHO | Human papillomavirus (HPV) and cervical cancer, 2015; Fact Sheets by Cancer, 2014). In Mexico, CC is the second most prevalent neoplasia in women, after breast cancer. In 2012 there were 5571 new cases of severe cervical dysplasia and CC *in situ*, mostly in women between 25 and 44 years of age (WHO | Human papillomavirus (HPV) and cervical cancer, 2015), being responsible for approximately 3880 deaths in women of reproductive age.

This neoplasia is a slow-evolving cellular alteration in the cervix that develops after the human papilloma virus (HPV) infection (Green, 1974; Cervical Cancer Home Page - National Cancer Institute, 2014). A persistent infection with HPV is considered a necessary but not sufficient event for the development of CC (Walboomers et al., 1999). Neoplasia progression has been extensively studied using molecular techniques, but recent research using next-generation sequencing (NGS) technologies have shed light on important aspects not previously described or analyzed. For example, it has been revealed where the host genome and the viral genome break, and it has been proposed how the HPV integration occurs. To understand how these new findings of HPV integration affect the progression of cervical cancer, it is necessary to identify novel and more precise biomarkers and to propose new treatment strategies.

In this review, we focus on describing and analyzing the evidence related to HPV integration, including the forms and integration sites in the host's genome, as well as the implications that such integration has for the host cell.

2. HPV as a causal agent

2.1. Characteristics of the Human Papilloma Virus

HPV is a circular DNA virus with an approximately eight thousand base pairs genome encoding eight genes, classified as early (E) or late (L) according to their expression patterns. There are six E genes (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7*) and two L genes (*L1* and *L2*). HPV infects epithelial

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Table 1
E6 and E7 important targets.

Oncogene	Target	Effect
E6	E6AP	Deregulates the translation signal in cell proliferation
	E6TP1	Inhibits Rap signaling
	E6BP	Inhibits the p53-independent antiapoptotic effect of terminal cellular differentiation
	hDIg	De-regulation of the cell cycle and loss of cellular differentiation
	hScrib	Involved in cellular adhesion and polarity; affects the MAGI-1/2/3-regulated p53-independent antiapoptotic effect
	Mcm7	Stopping point in early G1 phase
	XRCC1	Interferes with DNA repair efficiency
E7	p107, p130, p21, p48	Interruption of cell cycle regulation
	p27	Abrogates TGF-β-related growth and affects interferon-α signaling
	ATPase 4 subunit	Degradation of pRB by the proteasome
	TBP	Interferes with transcriptional initiation and Histone H1 kinase and affects the G2/M transition

cells via lesions in the epithelium, where the viral particles enter and spread to the basal layer. The infected cells start to produce viral particles, which initiate the development of low and high grade lesions or, cervical intra-epithelial neoplasia grade 1, 2 or 3, which can evolve into invasive cancer (Woodman et al., 2007; Munoz et al., 2003). During the infectious process, the virus can be present episomally, integrated into the host cell's genome or combined (episomal/integrated). In its integrated form, the virus can produce changes in cell functions that favor the replication of the viral particles and the cell malignant transformation. There are currently several hypotheses regarding the relationship between viral status and lesion stage. It has been suggested that the virus is completely integrated into the genome in advanced neoplastic lesions. Other studies failed to establish an unambiguous stage of full integration (Shirasawa et al., 1986; Choo et al., 1987a; Wei et al., 2015; Shukla et al., 2014; Ribeiro et al., 2014).

HPV is classified as HR-HPV (High risk Human Papilloma Virus) and LR-HPV (Low risk Human Papilloma Virus) according to its association to carcinogenic potential (Munoz et al., 2003). LR-HPV are found mainly in genital warts, meanwhile HR-HPV have been associated with invasive cervical cancer. These HPV are present in a singular or multiple infection (Del Rio-Ospina et al., 2017; Sohrabi et al., 2017).

A persistent infection with a HR-HPV is the main risk factor for cervical carcinogenesis (Walboomers et al., 1999). In addition, HR-HPV genome integration has been associated to the persistent infection (Manawapat et al., 2012), and this viral persistent infection could lead to a cancer progression. To better understand why HR-HPV are more frequently found to be integrated, it is necessary to understand their carcinogenic potential.

3. Viral oncogenes

E6 and *E7* genes products have shown to be primarily responsible for the cellular transformation process (Takebe et al., 1987; Cripe et al., 1987; Vousden et al., 1988; Hakura et al., 1989). It has been proposed that the integration of HPV into the host genome occurs following a break in the *E2* gene, which has been described as the main repressor of the expression of the *E6* and *E7* oncogenes (Choo et al., 1987b; zur Hausen, 2009). This break results in the loss of repression of these oncogenes, whose proteins interfere with the function of cellular proteins p53 and pRb, respectively. These oncogenes thus, directly and indirectly, influence cellular pathways such as apoptosis, proliferation, growth, and motility and their activation can lead to the onset of tumorigenesis (Rusan et al., 2015).

3.1. E1 and E2 genes

The HPV *E1* gene encodes a helicase protein, essential for the initiation of the viral DNA replication. This gene is expressed at early stages of the viral infection into the host cells. *E2* gene expression also occurs at an early stage of HPV infection, its overexpression reactivates the tumor suppressor pathway and inhibits the expression of the oncogenes *E6* and *E7* (Wu et al., 2000). The E2 protein alters the host gene splicing and it's been reported to affect cell movement and motility pathways in the host genome, this would lead to the loss of motility repression (Gauson et al., 2014). If the expression of E1/E2 is lost, there is no longer inhibition of the expression of E6 and E7 oncoproteins (Bechtold et al., 2003).

3.2. Oncogene E6

The product of the viral oncogene *E6* inhibits p53 and BAK, two key regulators of apoptosis (Boulet et al., 2007). As E6 also promotes cell proliferation by positively affecting telomerase and the SRC kinases family, its activities collectively favor cellular immortalization (zur Hausen, 2002). E6 also affects paxillin, which binds to multiple proteins involved in the regulation of actin cytoskeleton organization and is directly associated with tumor metastasis. The downstream effect is the rupture of the cytoskeleton that affects the cell's interactions with the extracellular matrix (Boulet et al., 2007; zur Hausen, 2002; Scheurer et al., 2005; Turner, 2000). E6 also affects other cellular factors such as IRF-3, thus decreasing the transcription of the *INF-β* gene, and targeting several PDZ proteins, increasing cell proliferation. Additionally, E6 modifies transcription by affecting the expression of *CBP/p300* (Scheurer et al., 2005). Other E6 targets include bax and c-myc, resulting in an anti-apoptotic effect.

3.3. Oncogene E7

Viral protein E7 mainly inhibits pRB, leading to an E2F transcription factor release and, in time, p16 upregulation, a cyclin-dependent kinase inhibitor (CKI) inactivation, that act as tumor suppressors (Boulet et al., 2007). E7 also stimulates cyclins A and E production, and inactivates the CKIs p21 and p27 and promote cell proliferation. This oncoprotein also induces the centrioles amplification, which can lead to aneuploidy (Boulet et al., 2007). All of these mechanisms act together to promote cell immortalization. Table 1 summarizes the most important targets of oncogenes *E6* and *E7* and their effects.

Overexpression of *E6* and *E7* oncogenes explains how the breakage of the *E2* regulator gene can initiate the cellular transformation process. However, this mechanism does not explain the cases of transformation in which no deregulation of viral oncogenes has occurred, i.e., where the viral genome breaks at sites other than *E2* or when the virus is found episomally rather than integrated.

To propose other cancer progression mechanisms, it is necessary to make a deeper study on how the HPV integration affects the normal functions of the host genome.

4. Alternative ways for tumorigenesis

Although several studies have reported increased expression of the viral oncogenes *E6* and *E7* in CC (zur Hausen, 1989), it is now thought that the cancer development can occur independently of these genes

(Olthof et al., 2015; Scarpini et al., 2014; Gray et al., 2010; Hu et al., 2015; Ojesina et al., 2013). Evidence suggests that mechanisms such as viral integration are responsible for genetic alterations that initiate tumorigenesis, proposing it as a driver mutation in the cancer progression (Badaracco et al., 2002; Hopman et al., 2004), without necessarily depending on the *E6* and *E7* oncogenes expression (Hu et al., 2015; Groves and Coleman, 2015). Moreover, the involvement of epigenetic mechanisms affecting the expression of key genes in the tumor transformation process, either by inhibiting the expression of tumor suppressor genes in the host genome or by increasing expression of viral genes for the production of new viral particles, is still unknown (Groves and Coleman, 2015).

There is a huge research area about the alternative ways to produce neoplasia, whether they are E6/E7 independent, or if there are epigenetic or external and genetic factors like viral type, viral load, and the HPV integration sites in the host genome; however, it is needed to explore the later mechanisms used by the HPV to integrate into the host genome. Several researchers have proposed different mechanisms that HPV uses to incorporate its viral genome. Due to the tendency to be found integrated into genes that are constantly being transcript.

5. Mechanisms of viral genome integration

HPV genome integration has been found in two types, as a single genome integrated and as multiple tandem repeats of the viral genome into the cellular genome (McBride and Warburton, 2017). HPV takes advantage of existing DNA repair and replication mechanisms to insert its genome into the host's. Several insertion and breakage mechanisms have been proposed for this process. The “looping” model is among the most accepted and explains the occurrence of the two breakpoints found in the SiHa cell line (Fig. 1) (Akagi et al., 2014). This model states that HPV integration is mediated by DNA replication and recombination, that may result in DNA concatemers (Xu et al., 2015). This could lead to the disruption of genes involved in tumorigenesis, oncogene amplification, inter or intra chromosomal rearrangements and/or genetic instability (Akagi et al., 2014).

Hu et al. have proposed another integration model mediated by micro-homologies (Hu et al., 2015). This is based on findings of microhomology-rich zones between the viral genome and the host genome near the integration sites. The authors highlight two integration mechanisms: FoSTeS (fork stalling and template switching) and MMBIR (microhomology-mediated break-induced replication). The FoSTeS mechanism involves the integration of the viral genome during a halt of the replication fork. Here, HPV “hijacks” this pathway and exchanges the template of the host genome to integrate its own. In MMBIR, however, the replication is induced by a break mediated by microhomologies, where HPV integrates its genome into the host genome during the replication of the host DNA (Fig. 2).

These mechanisms are activated during HPV infection, especially in the presence of certain local genomic elements, such as satellite DNA, Alu and SINE repetitive sequences, as these form the micro-homologies flanking the breakpoint. In this way, HPV “hijacks” the DNA reparation pathways to fuse its genome into the host's nicked chromosomes (Hu et al., 2015).

6. Mechanisms of transformation into the host genome

Rusan et al. proposed a scheme of modifications and alterations generated by the HPV integration into the host genome that can lead to carcinogenesis (Fig. 3). The authors described three main pathways: 1) loss of function of tumor suppressor genes, 2) increase in oncogene expression, and 3) inter- and intra-chromosomal rearrangements (Rusan et al., 2015). These pathways are described below.

6.1. Loss of function of tumor suppressor genes

HPV can provide itself with a selective advantage in the host cell by integrating its genome into a tumor suppressor gene and thereby inactivating it. Integrations in an intron of the *RAD51B* gene have been identified. This gene is involved in the DNA repair pathway, and its loss of function can result in genomic instability. Integrations in this gene have also been reported in other studies (Ojesina et al., 2013; Khoury et al., 2013; Parfenov et al., 2014).

Another human tumor suppressor gene affected by the HPV integration is *ETS2*, a transcription factor that regulates genes involved in metabolic pathways of cellular growth and apoptosis, among others. This integration results in the expression of a truncated protein (Parfenov et al., 2014).

6.2. Increase in oncogene expression

Viral genome integration in the host cell can also lead to key genes deregulation. Ojesina et al. (2013) revealed that the HPV integration upstream the *NR4A2* gene (oncogenic member of the nuclear receptor family) could lead to its overexpression, including different genes that could be involved in its pathway. Other studies have reported oncogenes amplification: *FOXE1* (transcription factor), *PIM1* (Ser/Thr protein kinase), and *SLC47A2* (member of the transporter family); in other cases, the integration occurs near or inside the *MYC* locus (Akagi et al., 2014; Durst et al., 1987; Ferber et al., 2003; Wentzensen et al., 2002; Cid Arregui et al., 2012).

6.3. Inter- and intra-chromosomal rearrangements

Parfenov et al. reported a rearrangement between chromosomes 3 and 13 close to the HPV integration site (Parfenov et al., 2014). The rearrangement occurred in a non-coding region but involved a region of chromosome 3 where the genes tumor regulated protein p63 1 (*TRPG1*) and *TP63*, a member of the p53 transcription factor family, are located. Moreover, the *KLF5* gene in chromosome 13 acts as a cell proliferation suppressor. These rearrangements affect these genes expression, resulting in increased cell proliferation.

7. Integration points

7.1. Genomic breakpoints

The first HPV integration into the human genome was described in 1987, between *KLF5* and *KLF12* in the SiHa cell line (Hu et al., 2015; el Awady et al., 1987). Since then, many reports have described breakage sites in the human genome caused by HPV. Most of them focus on the *E6* and *E7* gene retention for the progression of cancer. These analyses have the disadvantages of limited number of samples, relying on the technology available at the time, and focusing mainly on the *E1* and *E2* gene as the breakpoint in the viral genome.

Recent studies using massive parallel sequencing have shown that *E2* gene breakage is not required an uncontrolled expression of the viral oncogenes *E6* and *E7*, as other breakage sites along the viral genome have been identified, suggesting other mechanisms regulating the expression of *E2* (Hu et al., 2015; Ojesina et al., 2013; Bhattacharjee and Sengupta, 2006). Tumor-initiating mechanisms that cause the inhibition of *E2* expression without relying on the breakage of this gene are currently being researched (Xue et al., 2012).

8. Viral integration and its effect on gene expression

Genes localized near the integration sites of viral genomes can experience changes in expression levels, both positive and negative. In 2015, a significant decreased expression of *FHIT* and *LRPB1* genes was reported in neoplastic samples where HPV was found integrated into

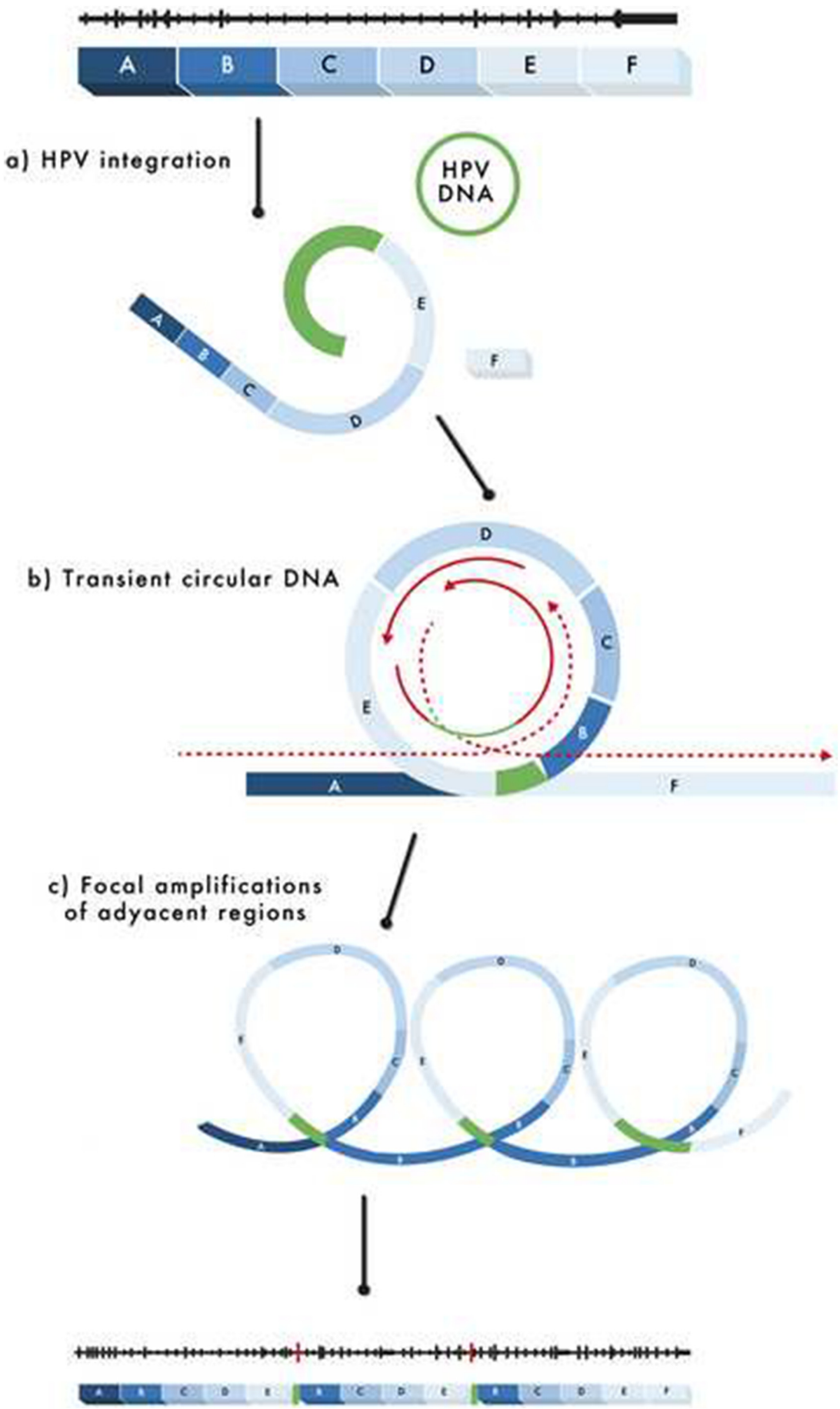

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Fig. 1. Looping model. The model used to explain the integration phenomenon found in the SiHa cell line. Based on Akagi K, et al. it generalizes how HPV integration produces a structural variation in a gene. a) HPV integration occurs between E and F region. b) A transient circular DNA is formed including the viral sequences, meanwhile DNA polymerase starts the DNA replication and forms concatemers. c) Focal amplifications and rearrangements are formed closed to the viral integrations (Akagi et al., 2014).

their introns, while *MYC* was overexpressed if HPV was integrated in its flanking region (Hu et al., 2015). A recent integration sites analysis, reported genes affected by the viral genome integration, with roles in pathways such as angiogenesis, transcription, cellular differentiation, migration, proliferation, and regulation of cell death, among others (Zhang et al., 2015).

Interestingly, in 2014, Akagi et al. reported the complete sequencing of ten cell lines, two of them from cervical cancer samples. No evidence of recurring integration into chromosomal hot spots was found. However, breakpoints tended to concentrate in specific chromosomal regions in the different cell lines (Akagi et al., 2014). This pattern contradicts the breakpoints reported by Hu et al. (Hu et al., 2015), although it is important to consider that cell lines were used in this study, while the latter analyzed patient samples. In addition, this analysis was not exclusive to CC cell lines, as most samples were head and neck cancer cells.

Several studies aiming to discover viral integration sites in the genome of host cells have demonstrated frequent integrations in the *MYC*, *TMEM49,* and *FANCC* genes (Zhang et al., 2015). In another report, *POU5F1B, FHIT, KLF12, KLF5, HMGA2, LRP1B, LEPREL1, DLG2,* and *SEMA3D* were found to be among the most recurrent sites for integrations, with up to five independent reports each (Table 2), while *AGTR2, DMD, CDH7, DCC, HS3ST4, CPNE8, C9orf85, MSX2,* and *CADM2* were slightly less common integration sites, with no more than four reports (Hu et al., 2015). Most of these genes are related to cellular repair pathways, tumor suppression, growth, and cell proliferation and, in some cases, code for transcription factors, as summarized in Table 2. Interestingly, the *LEPREL1* gene has been also associated with breast cancer development, while *HS3ST4* has been associated with the pathogenesis of the herpes simplex virus (HSV-1), corroborating the involvement of this gene in the pathogenesis of viral infections (Antoine et al., 2014). Ojesina et al. found viral integrations in the *RAD51B* gene in three separate samples (Ojesina et al., 2013). This gene is required for DNA repair by homologous recombination and has been associated with other types of cancer, including breast, ovary, prostate and colorectal (Scarbrough et al., 2015; Thomas et al., 2009).

9. Viral integration and its effect on epigenetic patterns

In late CC stages methylation patterns are increased, and it has been associated to the viral state (Fernandez et al., 2009). The altered methylation patterns were in the viral genome, E2 binding sites in URR region, L1, and L2 increased methylation are correlated as the cervical lesion increases. As an effect of these alterations, there is a deregulation in E6/E7 expressions.

Methylation patterns are correlated to the number of viral copies integrated in the host genome (Chaiwongkot et al., 2013). In previous studies, HPV 16 positive samples, with one or two copies integrated per cell, they found that URR region didn't show methylation, compared to the high percentage of the majority of lesions with multiple integrated HPV genome copies.

Also, there are significant differences between the methylation levels in samples where the HPV is only in the episomal form, and whether single or multiple copies are integrated into host genome. These findings apply similarly to HPV 18, in which its integration is also associated to methylation increase (Chaiwongkot et al., 2013).

Further studies are still necessary to understand the role between other epigenetic events, like the chromatin structure changes, and their effects on gene expression; and to establish its role to mediate HPV integration into the host genome.

A recent study described the relationship between chromatine structure changes and the viral state in HPV positive in head and neck squamous cell carcinoma (HNSCC) samples. Such as the H3K27ac histone mark, that distinguished tumor samples with and without HPV integration, and this H3K27ac histone mark enrichment colocalized with the different number of HPV integration sites. Additionally, these H3K27ac histone mark differential enrichments were found in upstream genes implicated in HNSCC tumorigenesis, such as *TP63, FOXE1, NOTCH1,* and *EGFL7,* where their expression is enhanced (Kelley et al., 2017).

All this evidence supports the different gene expression patterns between episomal and integrated HPV samples (Zhang et al., 2016). This gene expression altered patterns could not only emerge from histone modifications, but also from acetylation, methylation, mutations, copy number variations, among others, and should be considered as influencers.

10. New proposals of oncogenic mechanisms

This recent evidence shows that HPV integration often affects preferably those genes that are being constantly expressed during DNA transcription and repair (transcription factors and transcription

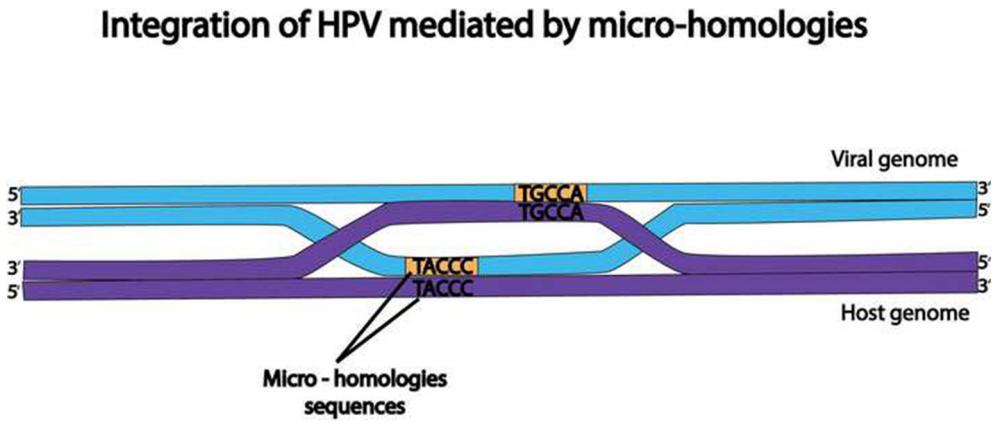

Fig. 2. Illustration of the mechanism where HPV integrates into the host genome, mediated by microhomologies in the sequences. The integration of HPV mediated by micro-homologies occurs during DNA repair, mainly via FoSTeS and MMBIR.

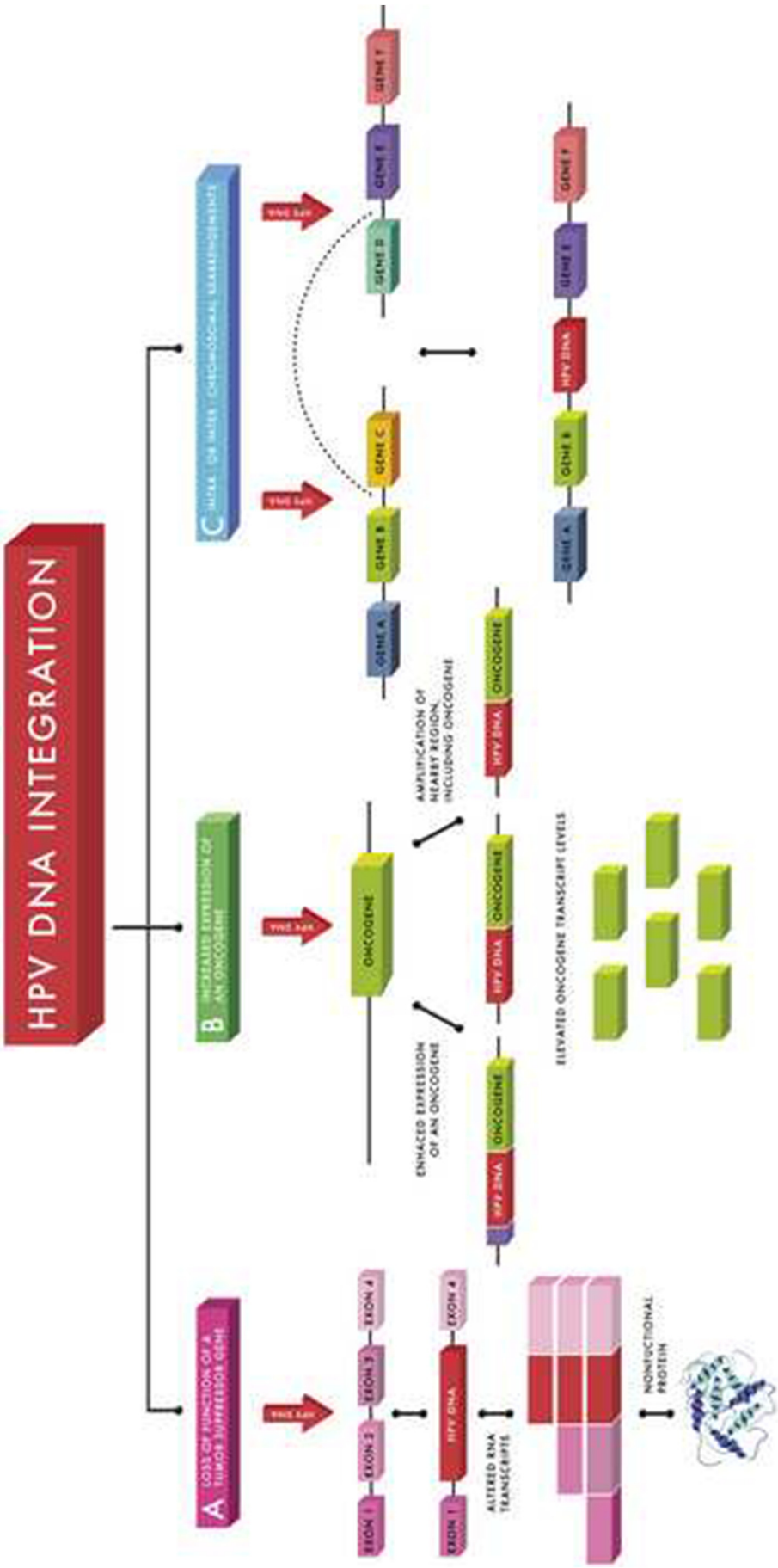

Fig. 3. Alterations produced by viral integration in the host genome. These integration mechanisms can lead to alterations in key genes. A. Loss of function of a tumor suppressor gene, with transcripts producing truncated or non-functional proteins. B. Increased expression of an oncogene can occur when the virus integrates upstream of an oncogene in the host cell or when the site of integration stimulates the promoters of viral oncogenes. C. Intra- or inter-chromosomal rearrangements can result in altered expression of multiple genes in the regions affected by the viral integration site. This schematic is based on the figure from Rusan et al. (2015).

Table 2
Major genes affected by viral integration and their cellular functions.

Gene	Integrations reported	Cellular function
C9orf156	15	Nucleosomes assembly.
MYC	13	Cell cycle, apoptosis, cellular transformation
POU5F1B	10	Transcription factor
FHIT	9	Purine metabolism; associated with translocations in cancer
KLF12	8	Transcription factor
HMGA2	8	Transcriptional regulation factor
KLF5	7	Post-translational modifications; suppressor/promoter of cell proliferation
DIAPH2	7	Development and normal function of the ovaries. Associated with premature ovarian insufficiency
TP63	6	Transcription factor. Skin development, maintenance, and premature aging.
LRP1B	6	Normal cell development.
NFIB	6	Transcription factor. Regulates cellular and viral gene transcription.
MACROD2	6	Modifies proteins involved with gene transcription and regulates cell signaling.
PVT1	5	c-Myc activator. Binding site of many transcription factors.
LEPREL1	5	Assembly, stability and chain crosslinking; decreased in breast cancer.
DLG2	5	Cellular signaling.
SEMAD	5	Receptor activity.
TMEM49	5	Cellular adhesion, cell death.
FANCC	4	DNA repair.
MSX2	4	Transcriptional receptor; Balance between survival and apoptosis
CPNE8	4	Regulator of molecular events in the interphase of the cell membrane and cytoplasm
HS3ST4	4	Enzyme expressed during HPV-1 pathogenesis
DCC	4	Tumor suppressor
CDH7	4	Cadherin; ERK pathway
AGTR2	4	Angiotensin receptor; mediator of cell death
RAD51B	3	DNA repair by homologous recombination; associated with other types of cancer, such as breast, ovary, prostate and colorectal

*Based on findings by Hu et al. (2015), Ojesina et al. (2013), Akagi et al. (2014), Zhang et al. (2015), Park et al. (1999a).

regulation factors) to generate carcinogenesis (Vemula et al., 2015; Guo et al., 2017; Rosenthal et al., 2013). Also, the progression to cancer could be explained by the viral DNA integration into tumor suppressor genes, this integration into host DNA inactivates those genes and it would lead to an uncontrolled growth (Zhao et al., 2016).

Evidence suggests that HPV has specific integration points, in or close to fragile sites (Liu et al., 2016), or “hot spots”, where it could alter expression patterns and these altered genes would have to be participating in specific pathways that would lead to important cellular changes, and lead to cancer. HPV could be involved in transcription mechanisms or hyperactive epigenetic spots, which could facilitate its integration in those active genes; and we observed repetitive viral integrations due to a possible clonal selectivity. Therefore, HPV integration is proposed as a driver mutation for this cancer progression and it is not E6/E7 overexpression dependent only.

11. Determination of the viral state

11.1. Pre-genomic methods for the detection of the viral state

The first techniques used for the detection of HPV in cells were immunohistochemistry and Fluorescent *in situ* hybridization (FISH), using monoclonal antibodies targeting cell proteins such as p16, which is produced in the presence of HPV infection, primarily when *E7* oncogene is expressed (Park et al., 1999a; Sano et al., 1998; Rufforny et al., 2005), and probes for HPV sequences allowing the fluorescent detection of small segments of the viral genome in the chromosomes of the host cell (Lizard et al., 1994; Siadat-Pajouh et al., 1994; Adler et al., 1997). Later, HPV detection was performed by polymerase chain reaction (PCR) (Carmody et al., 1996), and it was possible to detect the presence of the *E2*, *E6,* and *E7* genes, allowing researchers to determine the viral state in the infected cell (episomal/integrated) by measuring the amplification of these sequences (Donaldson et al., 1993). These integration-detecting methods improved with real-time PCR, with the ability to observe with higher precision the *E2/E6* or *E2/E7* ratio in real time (Zheng et al., 2006; Fujii et al., 2005).

PCR methods to identify integration sites in the host were based on Alu repetitive or intercalated sequences (IRS interspersed repetitive sequence) and involve specific primers for HPV and Alu sequences. Amplification of the viral-host DNA occurs when the binding sites for the HPV primer and the repetitive sequence primer are separated by < 3 to 4 kb (Carmody et al., 1996). The products generated are sequenced using Sanger methods (Park et al., 1999b) to determine the union points between the two genomes and reveal the viral integration by aligning the sequences with the human genome. All these methods are based in HPV E1/E2 breakage to incorporate its genome into host's genome, which was known before more sensitive techniques, as NGS, arrived.

11.2. Post-genomic methods for the detection of the viral state

Since NGS arrival, there have been several studies using it to determine HPV integration points through a whole genome or exome sequencing (Akagi et al., 2014; Chung et al., 2015; Liang et al., 2014). However, the disadvantage is the number of reads in each sequencing run. It would need a deeper sequencing to find rare HPV-human reads.

More recently, Zhen Hu et al. reported a new method for integration and breakage sites detection in the viral genome using massively parallel NGS. The researchers compared their method, HIVID (High throughput viral integration detection) (Li et al., 2013), against whole genome sequencing (WGS) and determine if HIVID identifies a higher number of breakpoints than the WGS. In this new method, HPV probes first hybridize with the samples and then are enriched prior to sequencing, with a higher number of reads per base. The authors reported that this method yielded coverage of the relevant sequences 600 times higher than WGS. The method was validated in 135 samples, identifying 3666 breakpoints. This study identified several sites with higher viral integration frequency, in some cases more than five events, that often affected candidate genes.

The study also reported a higher number of viral integrations in squamous cell carcinoma samples than in adenocarcinoma. There is also a significant difference in the number of viral integrations in lesion samples compared with cancer samples, suggesting that this event can be used as a predictive marker for the risk of evolving into invasive cancer (Hu et al., 2015).

There are also several reports where HPV integration is observed at

the latest stages of the cervical lesions and at cervical cancer, and the pure episomal HPV forms are observed at early stages of cervical lesions such as low grade lesions (Shukla et al., 2014; Cricca et al., 2007).

12. Viral state and pathology

As mentioned previously, the prevalence of HPV states (episomal and integrated) varies between the different stages of this disease. Although the virus is generally mostly integrated at advanced stages, viral integration has also been detected in some early lesions (CIN) (Wei et al., 2015), indicating that viral state can serve as a progression biomarker for this neoplasia (Shukla et al., 2014; Kahla et al., 2014a). Nevertheless, the implications of the different viral state proportions are still unknown, as the studies performed to date are limited, and it has not been clearly determined whether identifying the virus in a combined and integrated state at early stages has clinical significance as a progression biomarker (Wei et al., 2015; Li et al., 2011, 2012). However, all these reports have focused on the most prevalent HPVs, such as HPV 16 and HPV 18, and there isn't enough data about other HR-HPV integration status (Vinokurova et al., 2008). There is a need to research other HPV viral state in early lesions and cancer. Such as how the HPV multiple infections participate and evolve along the different stages of this neoplasia. Further research is still required to understand which of them integrate, which would stay in an episomal state, how the different HPV interact with each other in multiple infections, and if its integration is necessary to produce cervical cancer.

As a result of these new NGS-facilitated discoveries, the methods to determine the viral state (episomal/integrated/combined) in HPV-positive samples must be modified to yield a more realistic landscape of the integration process. The conventional methods developed before massive parallel sequencing was available are based on the *E6/E2* ratio, which assumes that viral *E2* gene breaks when the virus integrates into the host's genome (Choo et al., 1987b; Kahla et al., 2014a; Cricca et al., 2009; Tornesello et al., 2013). But, recent studies have shown the break occurs in the *E1* gene (Tsakogiannis et al., 2015) or in one of the viral oncogenes (*E6* or *E7*) in most samples. In prior studies that assessed the viral state as a possible progression biomarker, there was a discrepancy regarding whether the integrated or combined state is associated with progression: some reports established a higher probability of progression in those patients where the virus existed in a combined state versus those where the virus was completely integrated in the genome (Kiseleva et al., 2013, 2014; Kahla et al., 2014b; Ho et al., 2011).

Furthermore, the results of other studies where the expression of the *E6* and *E7* oncogenes was measured as a marker of latent infection should be re-analyzed because the viral genome can also break at these loci, thereby affecting their expression. It will be necessary to identify those reports where the viral oncogenes expression was not detected. These new results may explain some discrepancies between different research groups reports (Li et al., 2008, 2011; Andersson et al., 2005; Khouadri et al., 2007; Lillsunde Larsson et al., 2014; Zhang et al., 2014; Shin et al., 2014; Liu et al., 2010; Briolat et al., 2007; Arias-Pulido et al., 2006), where the relevance of *E6* and *E7* expression and their use as biomarkers for latent infection are discussed. It is important to consider that the expression detected in the analyzed samples could derive from viral particles found episomally in the sample and not from the viral oncogenes integrated in the host's genome. The new NGS methods will allow us to determine viral breakage sites across samples before measure the viral oncogenes expression, and considering the viral state (episomal/integrated/combined).

13. Conclusions

In this review, the advances in the detection of viral genome integration are described. Relevant research has revealed previously unrecognized breakpoints in the viral genome, with the disruption of the *E1* gene being the most common. These findings indicate tumorigenesis not only depends on viral integration and inhibition of *E2* expression for the deregulation of *E6* and *E7*; rather, there are other genomic mechanisms that affect the development of this neoplasia.

This review also describes preferential points of viral integration across the host genome. The gene with the highest known integration frequency is *C9orf156* a factor in nucleosomes assembly, followed by *MYC*, key in cell cycle, and previously associated with cancer. However, it is important to note that HPV integrates throughout the entire human genome, in many preferred “hot spots” as well as fragile sites and regions of highly active genes (Christiansen et al., 2015). Some of these genes affected by the viral genome integration have been associated with tumorigenesis, cell proliferation, cell migration, apoptosis, and kinase-controlled metabolic pathways. A better understanding of viral integration loci should help to clarify the basis for the persistence and evolution of lesions to cancer.

The probable mechanisms of viral integration into the host genome are also described, focusing on how HPV “hijacks” DNA repair and transcription pathways, how the virus “chooses” integration sites via microhomologies between the genomes, and the mechanisms the virus integrates its DNA into the human genome.

More evidence is still needed to determine whether the physical state of the viral genome can be used as a biomarker to predict the evolution of low and high grade lesions to invasive cancer. The resolution of this issue should emerge from new technologies capable of detecting the breakpoints in the viral genome as well as the insertion points in the host genome.

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Conflicts of interest

The authors have no conflicts of interest.

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