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HPV infection, a described cause for human cancer: HPV detection and molecular mechanisms of HPV carcinogenesis (Review).

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Contents

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Abstract

Human papillomavirus (HPV) belongs to the *Papillomaviridae* virus family and it is one of the most common sexual transmission infections. HPV genome is composed of eight genes, including two early genes and six late genes. Among these late genes, E6 and E7 code for proteins that trigger cell-cycle re-entry in infected cells, which can lead to cervical cancer development. The IARC (International Agency for Research Cancer) proposed a guideline based on Hill's criteria to determine whether the relation between HPV infection and cervical cancer is causal or not. Epidemiological studies have demonstrated that HPV infection is a necessary but non-sufficient cause for cervical cancer. Furthermore, HPV infection is considered the first necessary cause described of a human cancer, being HPV16 and 18 carcinogenic to humans and the most studied types. Cervical cancer is the second leading cause of cancer death among women worldwide. Different screening programs are carried out with the aim of preventing cervical cancer; such as cytologies and HPV tests. There are two main methods which are equally usable to detect HPV: the real-time PCR assays and the array assays. Regarding the molecular mechanisms of HPV mediated malignancies, E2, E6 and E7 proteins of HPV16 lead to immune response evasion, inducing IL-10 and TGF-β1 gene expression. Besides, E6 and E7 proteins allow cell-cycle re-entry, phosphorylating RB and ubiquitinating p53 respectively. HPV genome integration in host genome leads to the alteration of host and viral genes expression, including oncogenes and tumor suppressor genes. However, the differences of E6 and E7 oncoproteins in different HPV types is poorly known due to the fact that almost the most studied HPV type has been HPV16.

Laburpena

Giza Papilomaren Birusa (GPB) *Papillomaviridae* birus familiakoa den eta sexu-transmisiozko infekzioak eragiten dituen birus ohikoena da. GPBaren genoma 8 genez osatuta dago, 2 gene goiztiarrez eta 6 gene berantiarrez. Gene berantiarren artean, E6 eta E7 geneek infektatutako zelulen ziklo zelularraren hasiera eragiten duten proteinak kodetzen dituzte; beraz, minbizi zerbikala sorrarazi dezakete. Minbiziaren Ikerketarako Nazioarteko Agentziak (IARC) Hill-en irizpidetan oinarritutako gida proposatu zuen GPB infekzioaren eta minbizi zerbikalaren arteko erlazioa kausazkoa den edo ez aztertzeko. Ikerketa epidemiologikoek adierazi dute GPBaren infekzioa minbizi zerbikala pairatzeko beharrezko kausa dela, baina ez nahikoa. Gainera, GPBaren infekzioa giza minbizi bat gertatzeko beharrezkoa den lehen kausa deskribatua da. GPB16 eta 18 gizakiarentzat kartzinogenikoak eta gehien ikertu diren GPB motak dira. Minbizi zerbikala minbizi ondorioz hilkortasun handiena eragiten duen bigarren kausa da munduko emakumezkoen artean. Badaude hainbat osasun azterketa minbizi zerbikalaren garapena

saihesteko; adibidez zitologiak eta GPB testak. Bi metodo nagusi daude eta biak dira erabilgarriak GPB detektatzeko: denbora errealeko PCR entseguak eta arraietan eginiko entseguak. GPB16 birusaren E2, E6 eta E7 proteinek IL-10 eta TGF-β1 geneen adierazpena induzitzen dute, sistema immuneko erantzuna saihestuz. Bestalde, E6 eta E7 proteinek ziklo zelularraren hasiera eragiten dute. E6 eta E7 proteinek RB fosforilatzen eta p53 ubikitinatzen dituzte hurrenez hurren. GPB genomaren integrazioak zelula-ostalarien genoman birus eta zelula-ostalarien gene-adierazpenak alda ditzake, adibidez onkogene eta tumore-supresoreen adierazpena. Hala ere, gehien ikertu den GPB mota GPB16 denez, GPB desberdinen E6 eta E7 onkoproteinen arteko ezberdintasunak ezezagun diraute gaur egun.

Abbreviations

HPV: Human Papillomavirus
CxCA: Cervical Cancer
CIN: Cervical Intraepithelial Neoplasia
CIN I: Cervical Intraepithelial Neoplasia of Grade 1; nowadays known as LSIL.
LSIL: Low grade Squamous Intraepithelial Lesion
CIN II: Cervical Intraepithelial Neoplasia of Grade 2; nowadays known as HSIL.
CIN III: Cervical Intraepithelial Neoplasia of Grade 3; nowadays known as HSIL.
HSIL: High grade Squamous Intraepithelial Lesion
ASC: Atypical Squamous Cells
CDK4: Cyclin-dependent kinase 4
CDK2: Cyclin-dependent kinase 2
pRB: Retinoblastoma protein
Th1: T helper cell type 1
Th2: T helper cell type 2
TGF-β1: Transforming Growth Factor β1
APOT: Analysis of Papillomavirus Oncogene Transcripts

1. Introduction

Cervical cancer is the second most common cancer among women worldwide (Muñoz N, *et al.* 2003; Köse F, *et al.* 2013; Mammas I, *et al.* 2014), being also the second cause of cancer death after breast cancer among women in developed countries. Every 2 minutes, a woman dies worldwide due to the cervical cancer (Muñoz N, *et al.* 2003; Köse F, *et al.* 2013). Cervical cancers occur mainly in the cervical transformation zone, where the squamous epithelium replaces the glandular epithelium of the cervical canal (Schiffman M, *et al.* 2010). Moreover, cervical cancer has been associated to HPV infection, being the consequence of some mucosatropic HPV type

infections. Thus, the recognition that cervical cancer is a consequence of HPV infection has been an important finding in human cancer and in public health. Although HPV is a necessary, but non-sufficient cause of cervical cancer, its relation with cervical cancer is equally important as cigarette smoking with lung cancer is. HPV infection has been the first necessary cause described for human cancer (Bosch F, *et al.* 2002), when Harald zur Hausen discovered that HPV causes cervical cancer 40 years ago. Moreover, HeLa cancer cell line, which is one of the most used cancer cell lines in cancer biology, is derived from an HPV18 infection (Adams A, *et al.* 2014).

Papillomaviruses are 8000bp DNA viruses than can cause warts in epithelia of many host species (Schiffman M, *et al*. 2011). To date, there are more than 60 animal papillomaviruses (PV) sequenced, together with more than 170 human papillomaviruses (HPV) (Doorbar J, *et al.* 2012; Adams A, *et al.* 2014). HPV are classified in five genera, showing different life-cycles and generating different epithelial diseases, related to their transmission strategies and their interactions with immune system. Nevertheless, among all HPV genera, only Alpha HPV high-risk types can cause cervical neoplasias and cancer. Alpha HPV also include low-risk HPV mucosal types, which can cause benign lesions such as genital warts, but never cause cancer (Doorbar J, *et al.* 2012). Almost all cervical cancer patients present high-risk HPV genomic DNA and oncogenes expression in the cervical transformation zone (Adams A, *et al.* 2014). HPV infection is one of the most common sexual transmission infections, although it can be transmitted vertically from mother to the neonate (Köse F, *et al.* 2013). The risk of HPV infection increases with oral HPV exposure through oral sex and also with the increase of sexual partners (Adams A, *et al.* 2014). The persistent and uncontrolled infection with a high-risk HPV leads to the precursor of the cervical cancer, the Squamous Intraepithelial Lesion (SIL), which can turn into a cervical cancer. The two most prevalent high-risk human papillomaviruses are HPV16 and 18. HPV16 is responsible for 50% of cervical cancer cases, and together with HPV18, both of them cause almost 70% of cervical cancer cases (Torres K, *et al.* 2014; Lu X, *et al.* 2014).

Among young women, under 30 years old, HPV infections are usually temporary. After one year mainly 70% of infections disappear, and in a period of two years, nearly the 90%, thanks to the immune response (Torres K, *et al.* 2014).

As briefly mentioned before, cervical cancer occurs due to the persistent infection of some high-risk HPV types. The persistent infection can be a consequence of immune system evasion or HPV integration in host genome. To date, the study of different HPV types has been based on epidemiological classification depending on the rate of incidence of each HPV type in cervical cancer. However, from a molecular point of view, the differences between low-risk and high-risk HPV types or HPV different genera remain uncertain. The advances in biological molecular technology and sequencing allow a new insight in the study of the relation between HPV and cervical cancer.

2. HPV Characteristics

HPV belongs to the *Papillomaviridae* family, an 8 kilobase (8000bp), double-stranded and circular DNA heterogeneous virus family (Schiffman M, *et al*. 2011; Köse F,*et al*. 2013). Papillomaviruses, which are specific to species, are able to infect skin and mucosal epithelial surfaces (Köse F, *et al*. 2013). In the case of human papillomaviruses (HPV), the virus invades the basal layer of the epithelial cells, leading to cutaneous and mucosal lesions (Mammas I, *et al*. 2014).

2.1. Phylogeny of HPV

To date, there are more than 170 HPV types described (Adams A, *et al*. 2014), and also more than 60 animal papillomaviruses. There are 5 evolutionary groups among HPV: alpha (α), beta (β), gamma (γ), nu (ν) and mu (μ) human papillomaviruses. All of them can cause benign cutaneous lesions but only α-HPV can cause mucosal lesions. Moreover, α-HPV is the only phylogenetic type associated with cervical cancer (Doorbar J, *et al*. 2012). HPV16, the most studied HPV type, belongs to the α9 phylogenetic group and HPV18, which is also of great interest, belongs to the α7 phylogenetic group (Figure 1).

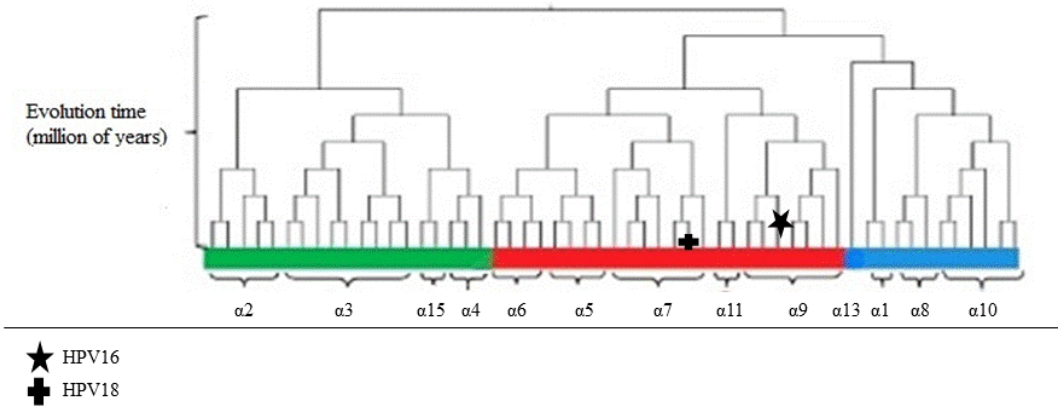

Figure 1. Phylogeny of α-HPV types. HPV types in the green clade cause commensal infections, HPV types in the blue clade cause genital warts. Finally, HPV types in the red clade are related with cervical cancer and CIN III (HSIL). **Adapted from Schiffman M, *et al*. 2011.**

2.2. HPV genome and life-cycle

HPV has a small circular genome that contains 8 genes and a long control region (LCR). These 8 genes, which encode a large number of proteins due to the mRNA splicing, are classified in two different groups: the early genes (E1, E2, E4, E5, E6, E7 and E8) and the late genes (L1 and L2) (Adams A, *et al*. 2014). Besides, as there are two groups of genes, there are also two promoter classes: PE for the early genes and PL for the late genes (Figure 2).

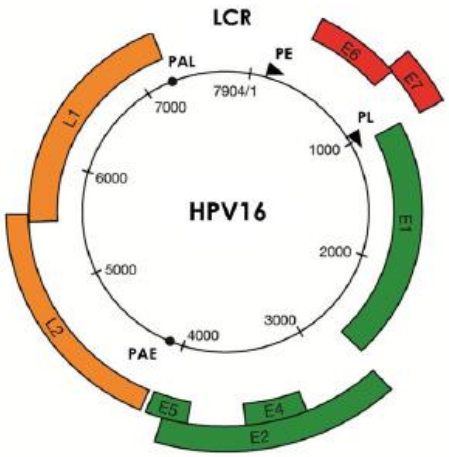

Figure 2. HPV16 genome. LCR (long control region), PE (early promoter), PL (late promoter), PAE (polyadenylation site of early genes), PAL (polyadenylation site of late genes). **Adapted from Doorbar J, *et al*. 2012.**

In a multilayered stratified epithelium, such as the ectocervix, HPV infection is thought to require a microwound which allows the access of the infectious virions to the basal lamina (Doorbar J, *et al*. 2012). Infection is followed by genome amplification and maintenance of the episomes, 200 copies per basal lamina cell. E1 and E2 viral replication proteins are essential in the initial amplification phase; E1 functions as a viral DNA helicase and E2 as a transcriptional activator and repressor (Adams A, *et al*. 2014) regulating the genome partitioning during basal cell division. E2 also regulates E6/E7 abundance by binding to specific sites in the LCR of all HPV types, inhibiting the viral early promoter. E7 viral protein associates with members of the Retinoblastoma (pRB) protein family, which are tumor suppressors, allowing cell cycle re-entry in the basal layer and also in the upper epithelial layers. E6 protein inactivates the tumor suppressor p53 activity and in some HPV types is also able to lead to its ubiquitination and proteasome-dependent degradation. Furthermore, in some HPV types E6 upregulates telomerase activity, leading to the maintenance of telomere integrity during cell division (Doorbar J, *et al*. 2012). E5 protein contributes to genome amplification, activating and stabilizing growth factor receptors, such as EGFR. After amplification, to complete the HPV life cycle, the packaging of viral genomes and virion release must occur. It involves the expression of the late genes (L1 and L2) and also E4. L1 is the major capsid protein and L2 the minor capsid protein. Finally, E4 is thought to be implicated in virion release and infectivity, since it interacts with keratin filaments (Adams A, *et al*. 2014) in the upper epithelial layers.

The expression of the viral products is disrupted in HPV-associated cancers. The disruption of proteins expression is due to the HPV genome integration in host genome.

The integration site of the viral genome is usually within the regulatory E1 and E2 genes. Hence, the loss of E2 activity leads to the loss of E6 and E7 regulation. The overexpression of these

proteins contributes to the accumulation of genetic errors; since both proteins trigger cell-cycle re-entry (Doorbar J, *et al*. 2012).

3. Causal relation between different HPV types and cervical cancer

3.1. Criteria to determine causality between HPV infection and cervical cancer

Epidemiological studies are essential to establish the relation between risk factors and cancer and also to qualify the nature of the association. Epidemiological studies have determined that the key elements for HPV infection are: the number of sexual partners, the age at which sexual intercourse was initiated, and the likelihood that each partner was an HPV carrier. To determine whether the relation between HPV infection and cancer is causal or not, Hill proposed several criteria. These criteria were versioned by the International Agency for Research Cancer (IARC) leading to the following aspects: (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biological gradient, (6) plausibility, (7) coherence, (8) experimental evidence, and (9) analogy. All the 9 aspects are not useful to determine if the association between HPV infection and cancer is causal or not. Nonetheless, a brief explanation of them should be made (Bosch X, *et al*. 2002).

Strength of the association

The strength refers to the magnitude of the ratio of incidence rates. Analysing the odds ratio for the association of human papillomavirus (HPV) DNA and cervical cancer in the IARC multicentre case-control study, it can be inferred that the association between HPV DNA in cervical samples and cervical cancer is one of the strongest ever noted for a human cancer (Thomas DB, *et al.* 2001; Bosch X, *et al.* 2002).

Consistency

It refers to the continuous observation of an association in distinct populations under different circumstances. In the case of HPV, the repeated observation of the association occurs. There are several studies made in different countries using different protocols and HPV DNA testing systems that lead to the same result: there is a causal relation between HPV DNA in cervical specimens and cervical cancer (Bosch X, *et al.* 2002).

Specificity

This criterion implies that a cause should only lead to a single effect. In carcinogenic exposures, the same exposure can induce cancer in different organs. This is why specificity criterion is not usually used when talking about cancer. However, and taking into account that there are more than 40 mucosatropic HPV types, some of them related to cancers of the genital tract, there are some HPV types directly related to cervical cancer. HPV18 and its phylogenetically related family (HPV types 39, 45 and 59) are closely related to adenocarcinomas and adenosquamous cell carcinomas. On the other hand, HPV16 and its phylogenetically related family (HPV types 31, 35

and 52) are closely related to squamous cell carcinomas (Thomas DB, *et al.* 2001; Bosch X, *et al.* 2002).

Temporality

Temporality means that the cause precedes the effect in time. It is a *sine qua non* condition for establishing causality. Epidemiological data shows that HPV infection precedes cervical precancerous lesions and cervical cancer by a large amount of years (Bosch X, *et al.* 2002).

Biological gradient

The biological gradient refers to the presence of a dose-response curve between the cause and the effect, which means that the magnitude of the exposure is related to the risk of disease. In the case of HPV infection and cervical cancer, women with high viral loads of HPV16 had a 30 fold higher risk of developing cervical cancer than HPV negative women (Bosch X, *et al.* 2002).

Biological plausibility and coherence

The epidemiological data shows that the association of HPV DNA in cervical specimens and cervical cancer is plausible and coherent. This means that the cause and effect interpretation for the association (HPV infection and cervical cancer), does not removes what is known of the biology of the disease (cervical cancer). In other words, the causal relation between HPV infection and cervical cancer does not exclude what is known about the biology of cervical cancer (Bosch X, *et al.* 2002).

Experimental evidence

This criterion is rarely found in human populations. Nevertheless, the results obtained in animal models for the study of HPV related lesions, including cancer, suggest that papillomaviruses induce papilloma and cancer in susceptible hosts. For example, canine oral papillomavirus (COPV) causes oral papilloma, although it does not lead to malignant transformation (Bosch X, *et al.* 2002).

Analogy

The analogy criterion defends that if one virus can cause cancer, other viruses could too. Nowadays, the analogy criterion is not viewed as an essential criterion for causality determination. Although analogy criterion is not used, the HPV and cervical cancer model is analogous to other animal examples of PV (papillomavirus) that induced papilloma and carcinomas (Thomas DB, *et al.* 2001; Bosch X, *et al*. 2002).

3.2. Other risk factors for the development of cervical cancer

The sexual behaviour, including the number of sexual partners and the age at first exposure, were thought to be directly related to HPV infection and cancer development. Moreover, there are other risk factors that have been studied; such as, cigarette smoking, the use of oral contraceptives, and parity (Santos C, *et al.* 2001).

Cigarette smoking

Smoking has been related to cervical cancer for more than 30 years. A review published by the IARC in 1998 evidenced the carcinogenic effect of cigarette smoking in women with HPV infection. The review concluded that "ever smoking" supposed a twofold increased risk of developing cervical cancer (Santos C, *et al.* 2001; Thomas DB, *et al.* 2001; Bosch X, *et al*. 2002).

Oral contraceptives

A study made by the IARC, concluded that the usage of oral contraceptives for five or more years, supposed a fourfold increased risk of developing cervical cancer among women which are infected by the HPV. However, the effect of different oral contraceptives depends on the intensity, duration or the chemical composition of the exposure (Thomas DB, *et al.* 2001; Bosch X, *et al*. 2002).

Parity

In an IARC multicentre study, the results showed that HPV-positive women who reported seven or more pregnancies, had a twofold increased risk of developing cervical cancer compared with HPV-positive women who reported one or two pregnancies. If women with multiple pregnancies were compared with nulliparous HPV-positive women, a fourfold increased risk was observed.

Although the risk factors mentioned above could increase the risk of developing cervical cancer, HPV infection is the most important risk factor. Hence, HPV infection is considered the first necessary cause described of a human cancer. Furthermore, HPV is a necessary, but non-sufficient cause of cervical cancer. There are other endogenous factors (disruption of cell-cycle) that together with HPV lead to cervical cancer (Thomas DB, *et al.* 2001; Bosch X, *et al*. 2002). The review done by the IARC concluded that HPV types 16 and 18 were carcinogenic to humans.

3.3. Classification of carcinogenic HPV types

The epidemiologic classification of HPV differentiates high- and low-risk HPV types. The number of high-risk HPV types varies from 13 to 19, and their classification is made according to the rate of incidence of the HPV type in different cervical cancer patients. The classification can also be made by phylogenetic clustering, based on an informative region on the L1 gene. The phylogenetic classification enhances the reliability of the standard classification including HPV types in high- or low-risk groups depending on their L1 segment (Muñoz N, *et al*. 2003).

According to a pooled data from 11 studies, including 9 countries with 1918 women with histologically confirmed cervical cancer and 1928 control women, 15 HPV types were considered as high-risk types, carcinogenic to humans: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. 4 HPV types were considered as probably high-risk types (should be also considered carcinogenic): types 26, 53, 66 and 85. Finally, 17 were classified as low-risk types: types 6, 11, 40, 42, 43, 44, 54, 61, 62, 70, 71, 72, 81, 83, 84, 89 and CP6108 (Table 1).

Table 1. Phylogenetic and Epidemiologic classification of different HPY types. There are two types of HPV according to their incidence in cervical cancer samples: high-risk and low-risk HPV types. **Adapted from Muñoz N, *et al*. 2003 and Dunne, *et al*. 2007.**

	Epidemiologic Classification	
Phylogenetic Classification	**Low-risk**	**High-risk**
Low-risk	6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, 89, CP1608	73
High-risk	70	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 82, 85

The epidemiologic classification of HPV patients in the Basque Country, agrees with the IARC classification (Figure 3).

HPV16 is present in 59% of HSIL (CIN II, III) or cervical cancer Basque patients, being among the HPV high-risk types the most dangerous (Figure 3).

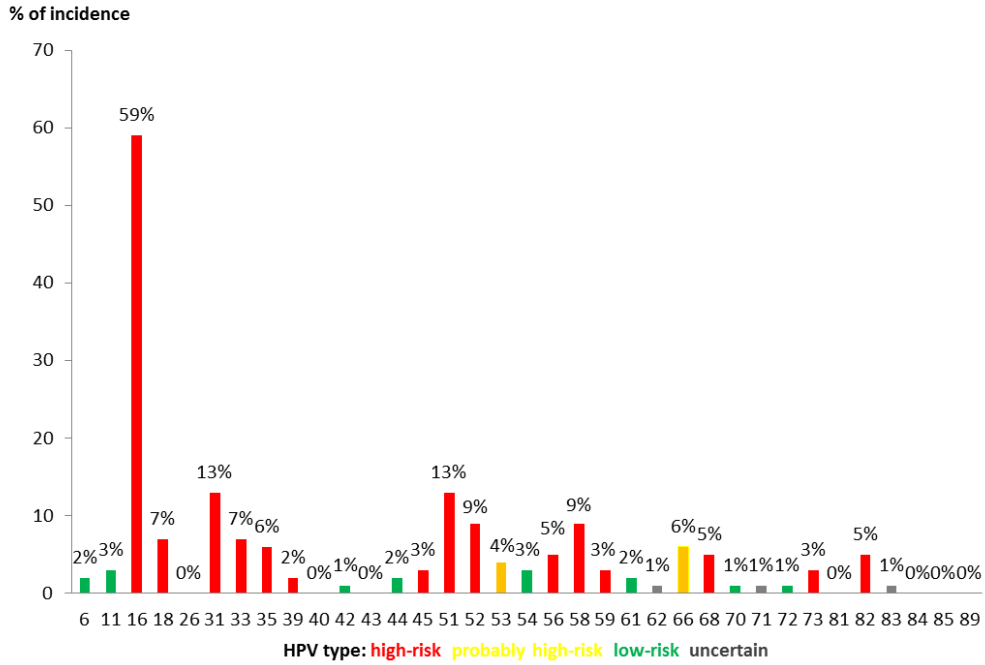

Figure 3. HPV epidemiological classification according to the frequency of the virus in HSIL (CIN II, III) or/and Cervical Cancer samples of Basque patients from 2006 to 2012. Adapted from Huarte M, *et al.* 2013.

The Cervical Intraepithelial Neoplasia (CIN) is an abnormal growth of cells that occurs in the cervix, which can form a mass of cells, a tumor. Regarding other HPV high-types, it should be outlined that in Basque patients HPV18 is not the second most common type, although HPV18 is usually the second type that most appears worldwide. HPV types 31 and 51 are present in 13% of

Basque patients (Figure 3), being after HPV16 the types that present more prevalence among CIN and cervical cancer Basque patients (Huarte M, *et al.* 2013).

3.4. Different methods to prevent HPV infection and cervical cancer

The integration of vaccination programs against HPV is the first way to prevent HPV infection, and the screening programs are the second way. There are two vaccines: Gradasil® and Cervarix®, which are based on the L1 region of the HPV. Cervarix® is a bivalent vaccine which prevents patients from HPV types 16 and 18. On the other site, Gradasil® is tetravalent vaccine that prevents patients from HPV types 16, 18, 6 and 11. In addition, this last vaccine also provides partial protection against HPV types 31 and 45, which are phylogenetically related to HPV types 16 and 18, respectively (Schiffman M, *et al.* 2011; Cortés J, *et al*. 2011). There are 45 countries that have integrated vaccination programs and other 16 have programmed to integrate them between 2013 and 2016 (González H, 2013). Although screening programs do not prevent directly the HPV infection as vaccination does, they are more used due to the fact that they do not cause side effects.

4. Inclusion of HPV tests in the screening programs to prevent the development of cervical cancer

4.1. Different screening programs

There are different screening programs depending on the tests used to detect HPV infection or cervical lesions. Nevertheless, the aim of all of them is to detect the viral infection or/and the lesion, before cancer develops (Schiffman M, *et al*. 2011).

Screening using Cervical Cytology

The Papanicolaou cytological test is the screening method most used worldwide. It is based on the smears morphology of the exfoliated cervical cells. And, depending on the morphology of the cells, they are classified in 4 different groups: CIN I, CIN II, CIN III and ASC (atypical squamous cells). CIN III is the previous stage for developing cervical cancer but women who present this grade of lesion can be treated before cancer appears.

Nonetheless, screening using only the Papanicolaou test requires a screening period of few years, since a lesion classified as grade one (CIN I), can rapidly develop into a second or third grade lesion, increasing the risk of developing cervical cancer (Schiffman M, *et al*. 2011).

Screening using HPV Tests

Screening programs using tests for the detection of carcinogenic HPV DNA are more sensitive than cytological screening programs for detecting CIN III. Besides, a negative HPV test provides a long-term insurance of not developing CIN III and a stronger insurance of not developing invasive cancer than a negative cytology (Schiffman M, *et al*. 2011).

Screening using Co-testing with Cervical Cytology and HPV tests

The usage of both screening programs allows the extension of screening intervals in case of both tests being negative. If there were discordant results, the management of the patient would be according to the type of discordance. For example, the most common discordant result is finding a HPV-positive and cytology-negative woman; whose risk of CIN III (HSIL) is greater than a HPV-negative woman, but lower than a HPV and cytology-positive woman (Schiffman M, *et al*. 2011).

The best option is to include both HPV tests and cervical cytologies in the screening programs, due to the fact that the reliability of the screening program increases if more tests are done. Apart from co-testing, there is another screening program that sums up both tests, the triage. Triage is similar to co-testing except that in triage, the second test is done only if the first one gives an equivocal result (Schiffman M, *et al*. 2011).

4.2. The management of the obtained results

As said before, the co-testing allows longer screening intervals than cytology or HPV tests (Köse F, *et al*. 2013). When both cytology and HPV tests are negative, the screening will be repeated in 3 years. If the HPV test is positive and the cytology is not clearly negative, a colposcopy will be practiced. A colposcopy is an examination of the cervix using light and a microscope after the application of a 3-5% acid acetic solution, which aids to detect abnormal areas in the cervix that might be previous lesions to neoplasia (Massad L, *et al*. 2013). The colposcopy makes the selection of the biopsy area easier. Depending on the results of the colposcopy, if a cervical intraepithelial lesion is detected, the management of the patient will be according to the ASCCP Guideline (Figure 4).

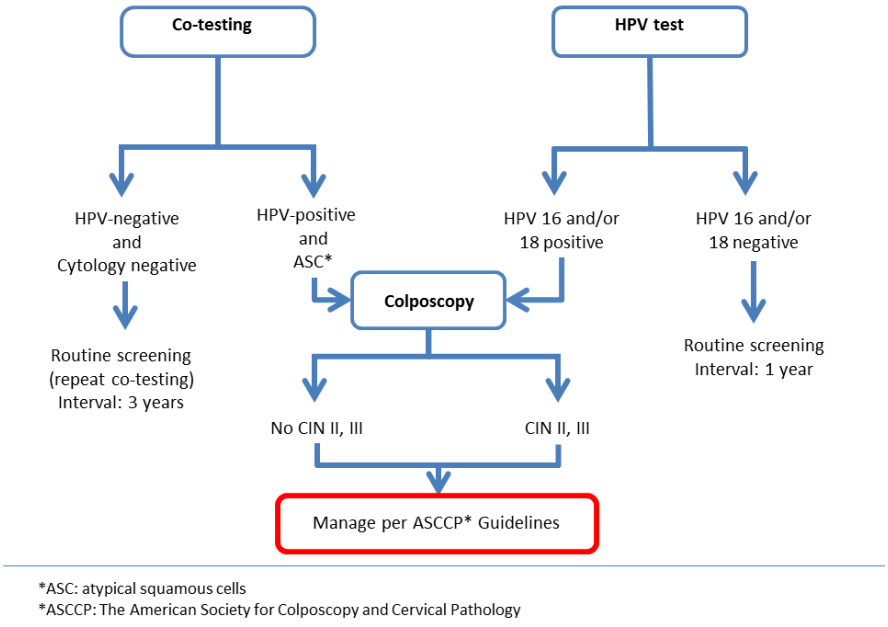

Figure 4. Management of HPV-positive cases with negative cytology. Adapted from Köse F, *et al.* 2013.

On the other hand, the co-testing also allows determining the risk of developing a CIN III lesion (HSIL) (Schiffman M, *et al*. 2011). When both cytology and HPV tests are negative, the risk of developing a CIN III lesion (HSIL) is very low; however, the risk increases when HPV infection and abnormal squamous cells (ASC) are detected (Figure 5).

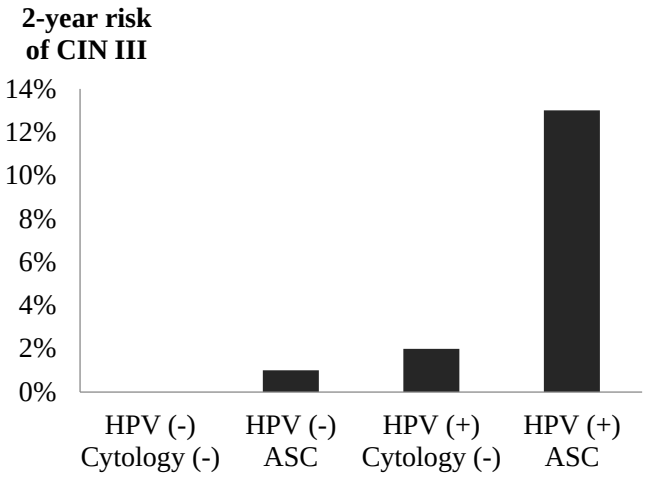

Figure 5. The percent risk of developing CIN III lesions (HSIL) depending on the tests results. Adapted from Schiffman M, *et al*. 2011.

With regard to the HPV detection, there are different methods to detect HPV DNA in cervical cells: the real-time (multiplex or not) PCR assays and the array assays (Roberts C, *et al.* 2011).

5. The effectiveness of different methods to detect HPV

As mentioned before, there are two main methods which are used for HPV DNA detection in human epidemiological studies: the real-time (multiplex or not) HPV PCR assays and the array (linear or microarray) HPV genotyping PCR assay. Both of them are based on PCR assays and present similar sensitivity and accuracy to detect different HPV types. The region of the HPV genome detected by the two methods is the same, the L1 region (Roberts C, *et al.* 2011). In the case of the first method, other regions can be detected, such as the E6 and E7 regions.

5.1. Real-Time (Multiplex or not) HPV PCR Assays

There are different real-time PCR assays; but all of them have a common characteristic: the PCR products can be analysed *in situ.* It involves the detection of intercalated fluorescent molecules in double-stranded DNA; or the detection of a marked probe that is added to the oligonucleotide pool. In the first method, the DNA-intercalating dye gives off a fluorescent signal that is detected by the real-time PCR machine after each cycle (Figure 6). Nonetheless, the fluorescent molecule binds to unspecific double-stranded DNA, disallowing the multiple HPV detection.

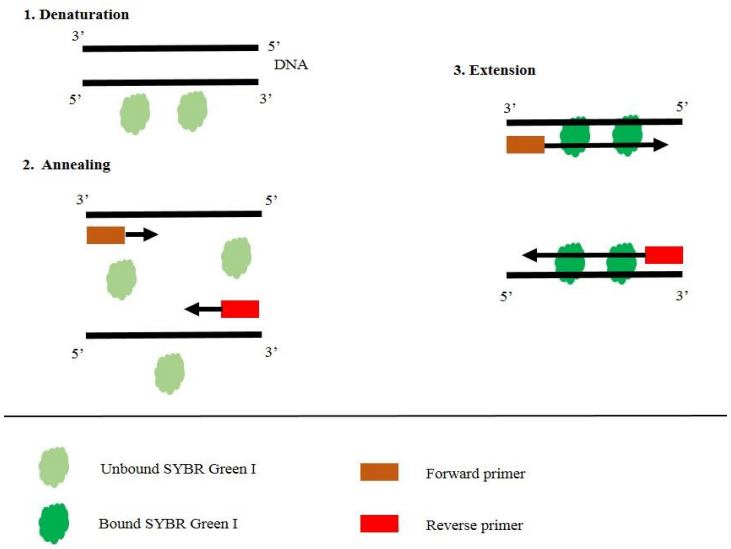

Figure 6. SYBR® Green I molecules (DNA-intercalating dye) intercalation in the double-stranded DNA molecules during the PCR process. The fluorescence of SYBR® Green I increases significantly when it is intercalated in the double-stranded DNA molecules. **Adapted from An Introduction to Molecular Biotechnology, 2011.**

In the second one, the marked probe carries both a fluorophore (reporter) at 5' and a quencher in its 3´-end. The quencher is removed owing to the Taq polymerase exonuclease activity (5´→3´) during the extension process, allowing the real-time PCR to pick it up (Figure 7). Besides, the probe is sequence specific, allowing the detection of different HPV types in each PCR cycle (An Introduction to Molecular Biotechnology, 2011).

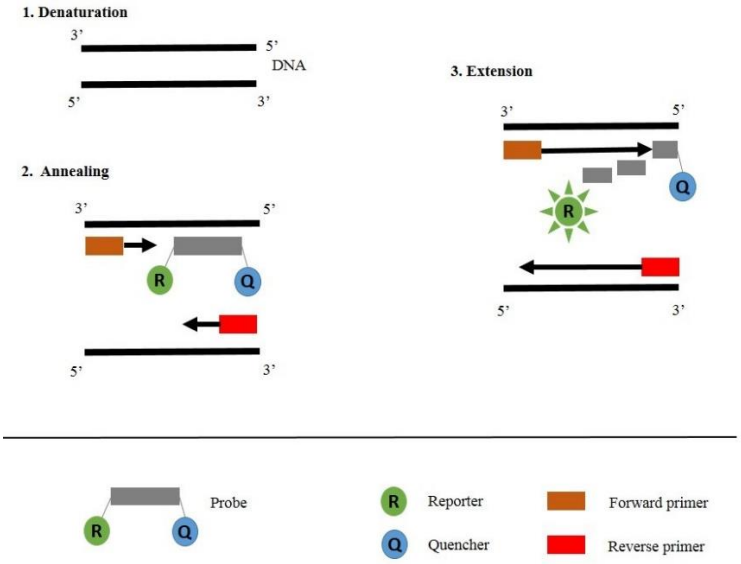

Figure 7. TaqMan® mediated qPCR assay. The fluorescent signal of the reporter is hijacked by the quencher when both of them are bound to the oligonucleotide probe. During the extension process, the reporter is released and the fluorescent signal can be detected. **Adapted from An Introduction to Molecular Biotechnology, 2011.**

5.2. Array HPV Genotyping PCR Assays

As said before, the detected region is a 450bp L1 fragment, which is a highly conserved sequence. Furthermore, the sequence presents enough variations to differ among 35 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85 and 89.

The detection system of the arrays is based on the precipitation of an insoluble product in the microarray area where the hybridization of the specific targets occurs with the specific probes.

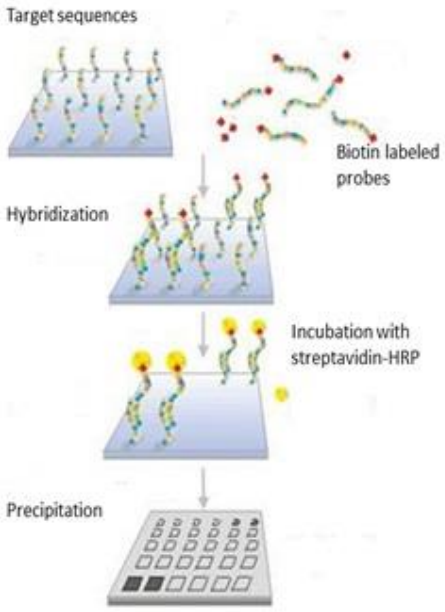

Figure 8. A scheme of the array HPV genotyping PCR assay. Adapted from Clart® guide 2013.

During the PCR, the amplified products are labelled with biotin and after the amplification these products will hybridize with their complementary specific targets, which are located in known microarray areas. After the hybridization, the biotin marked amplifications and their complementary sequences of the microarray are incubated with a streptavidin-HRP (peroxidase) mix. The streptavidin of the mix binds to the biotin of the amplified products and due to the peroxidase activity, an insoluble product appears in the presence of the o-dianisidine substratum (which is in the mix). The insoluble product precipitates in the microarray area where the hybridization has occurred (Figure 8).

Unlike in the real-time PCR assays, in the array tests, the detection of the HPV is made after the PCR amplification, not during it. This means that the product that is detected is not the HPV sequence *per se*, but an amplification of it. Hence, owing to the fact that the detection depends on the amplification products, false negatives may appear. The reason of the false negatives could be the bad quality of the DNA samples or/and the presence of DNA polymerases inhibitors in the samples (Clart® guide, 2013). To determine if the results are valid or not, the amplification control

and the genomic control must be studied. If the result of both tests is negative, the PCR has been inhibited. On the other hand, if the amplification control is positive and the genomic control is negative, the DNA has been lost. Besides, to verify that the sample is HPV positive, the precipitate of a HPV type must appear in the array in three different places (Figure 9).

R	70	71	72	73	81	82	83	84	85	6 ; 11	**R**
51	52	53	54	56	58	59	61	62	66	68	
R	18	26	31	33	35	39	40	42	43	44	45
	81	82	83	84	6 ; 11	89	**Cl**	**DNA**	6	11	16
54	56	58	59	61	62	66	68	70	71	72	73
31	33	35	39	40	42	43	44	45	51	52	53
82	83	84	85	89	**Cl**	**DNA**	6	11	16	18	26
56	58	59	61	62	66	68	70	71	72	73	81
	39	40	42	6 ; 11	44	45	51	52	53	54	
R	**Cl**	**DNA**	6	11	16	18	26	31	33	35	**R**

Figure 9. Spatial position for the different controls and the different HPV types probes in the Clart®2 microarray. R: metallic indicator; Cl: amplification control; DNA: genomic control. There are three probes for each HPV type, which are indicated with the HPV type number. **Adapted from Clart® guide 2013.**

5.3. Comparison of the accuracy and sensitivity of both methods

According to a pooled analysis of 12 studies in 25 different countries, the 15 most common HPV types present in 95% of cervical cancer subjects in descendent order of frequency, were: HPV type 16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 39, 51, 73, 68 and 66 (Muñoz N, *et al*. 2003; Roberts C, *et al*. 2011).

The comparison of the HPV detection by the real-time multiplex HPV PCR assay and by the Linear Array assay was made for 14 HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. The agreement rate (0 to 1) for any of the 14 HPV types evaluated was 0.881, with rates of 0.909 positive agreement and 0.830 negative agreement. Statistically significant differences in the HPV detection were noticed for HPV31, 33, 52 and 56 (Roberts C, *et al*. 2011). Although multiplex real-time HPV PCR assay is able to detect more positive specimens than Linear Array assay, both of them could be used for screening programs; due to the fact that there are no differences in the detection of the two predominant HPV types: HPV16 and HPV18.

In addition, the method used in the Oncologic Foundation of Donostia is the microarray assay, which is able to distinguish 35 HPV different types.

6. Biological mechanisms of HPV carcinogenesis

6.1. Molecular mechanisms to prevent cancer: RB and p53 tumor suppressor genes

RB and p53 are both well-known tumor suppressor genes. The pRB family interacts physically with many proteins. One of those proteins is the E2F protein family, which is a transcription factor protein family. E2F (protein family) regulates the expression of genes necessary for DNA replication, as well as those genes which code for enzymes that synthesize and metabolize DNA. Furthermore, E2F also regulates E and A cyclins, which are necessary to complete the cell cycle (Sherr C, *et al.* 2002). Finally, E2F also regulates the CDKN2A gene, which is another tumor suppressor gene. When RB is bound to E2F, it inactivates the transcription factor function of E2F. Mitogen stimulation induces D cyclin expression and its assembly with CDK4 and p27, leading to the activation of E-CDK2. Active E-CDK2 is able to phosphorylate p27 leading to its ubiquitination and degradation. Both active cyclin-dependent kinases (4 and 2) phosphorylate pRB inactivating it and releasing E2F transcription factor. E2F will lead to the S phase entry through the regulation of genes necessary for DNA replication (Figure 10).

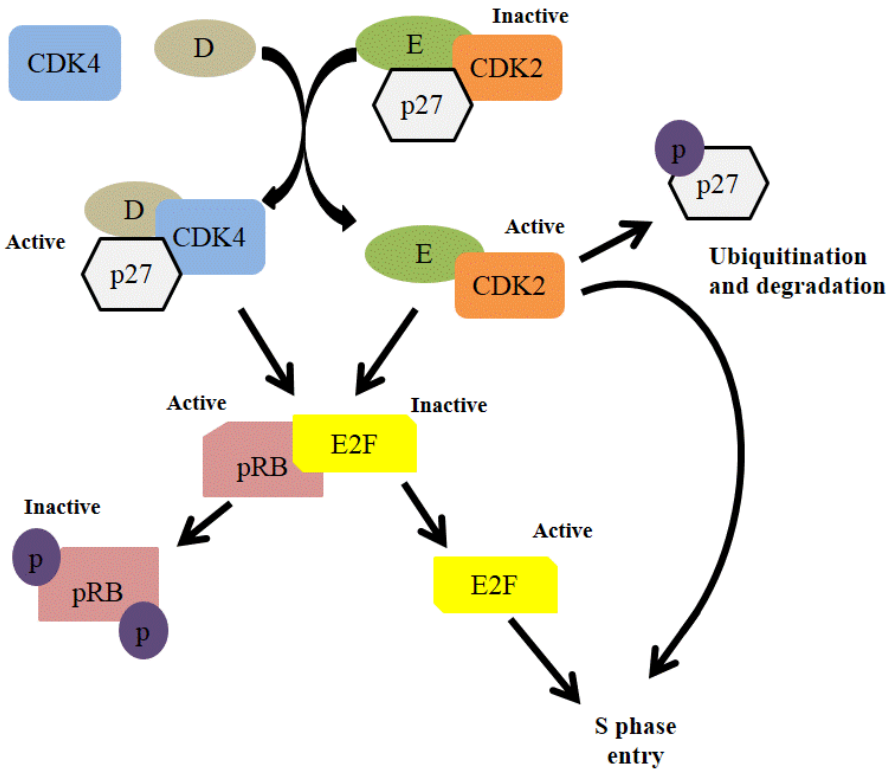

Figure 10. Transition from G1 to S phase through pRB (retinoblastoma) phosphorylation by cyclin (D and E)-dependent kinases (CDK4 and CDK2). Adapted from Sherr C, *et al.* 2002.

CDKN2A gene (*INK4a* locus) codes for the $p16^{INK4a}$ protein. $p16^{INK4a}$ protein inhibits the activity of cyclin D-dependent kinases, preventing the phosphorylation of pRB and avoiding the transition

from G1 to S phase (Figure 11). This regulatory mechanism acts as tumor suppressor and a mutation in $p16^{INK4a}$, CDK4, or pRB could lead to the development of cancer (Hanahan D, *et al.* 2000).

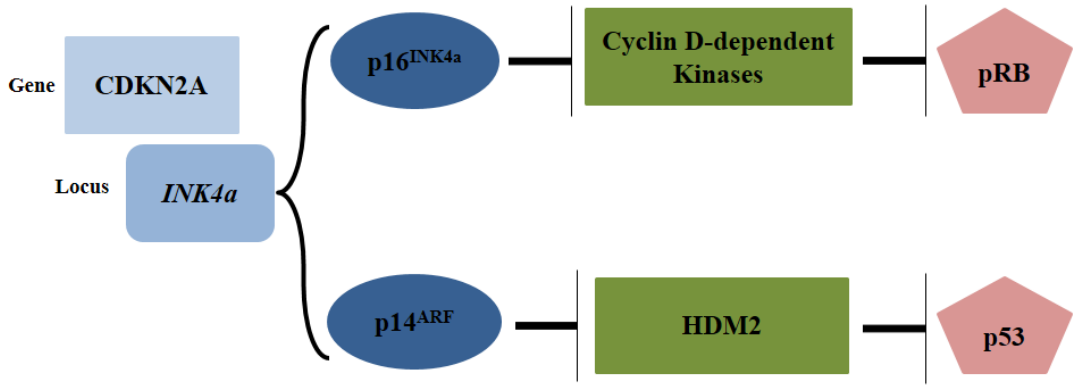

Figure 11. CDKN2A gene products and their functions as tumor suppressors. Adapted from Sherr C, *et al.* 2002.

On the other hand, the p53 transcription factor, which is induced in response to hypoxia, DNA damage and oncogene activation after mutations in the RB pathway, also controls the step from G1 to S phase. p53 is the transcription factor, among others, of p21, which is an inhibitor of cyclin D-CDK4 and cyclin E-CDK2, avoiding the phosphorylation of pRB. Furthermore, if DNA damage is irreversible, p53 triggers apoptosis (Speidel D, *et al.* 2015). The *INK4a* locus (CDKN2A gene) mentioned before encodes another product apart from the $p16^{INK4a}$: the $p14^{ARF}$ protein. $p14^{ARF}$ inhibits HDM2, avoiding its binding to p53 (Figure 11). When HDM2 binds to p53, the transcription function of the last one is inhibited. Moreover, HDM2 also catalyzes p53 ubiquitination, targeting its degradation. Hence, $p14^{ARF}$ acts as a tumor suppressor enhancing p53 stability and avoiding cell proliferation (Sherr C, *et al.* 2002).

In more than 50% of human cancers, the p53 gene is mutated. Tumors that maintain the p53 gene without mutations, may have mutations in other genes encoding for p53 regulators, such as HDM2 or CDKN2A (Sherr C, *et al.* 2002).

6.2. HPV molecular carcinogenesis

As mentioned before, HPV genome has some segments related with genome replication and with the cell cycle re-entry (Bosch X, *et al.* 2002). Among these genome segments, E6 and E7 early genes should be mentioned. One key function of E7 is binding to pRB, releasing E2F transcription factors, which are necessary to promote DNA synthesis and to transcribe genes needed in the S phase (Bosch X, *et al.* 2002; Adams A, *et al.* 2014). On the other hand, E6 binds to p53 inactivating its tumor suppressor activity. Besides, in high-risk HPV types, for example HPV16, E6 leads to p53 ubiquitination and triggers p53 proteasome-dependent degradation. Last but not least, in some high-risk HPV types, E6 is also able to activate telomerase reverse transcriptase

activity (TERT), contributing to keratinocyte immortalization (Bosch X, *et al.* 2002; Doorbar J, *et al.* 2012; Adams A, *et al.* 2014). In low-risk HPV types, E6 products have not been detected (Doorbar J, *et al.* 2012).

E7 binds to pRB releasing E2F, which will transcribe some genes needed for S phase entry but also other genes related with cell cycle regulation. Among these late set of genes, CDKN2A should be outlined. CDKN2A leads to the production of two proteins: $p14^{ARF}$ and $p16^{INK4a}$. The first one enhances p53 level and stability. Nonetheless, E6 activity will degrade p53. With regard to the $p16^{INK4a}$, it binds to CDK4 avoiding the phosphorylation of pRB. However, it does not recognize E7, so E7 will continue binding to pRB and releasing E2F (Figure 12).

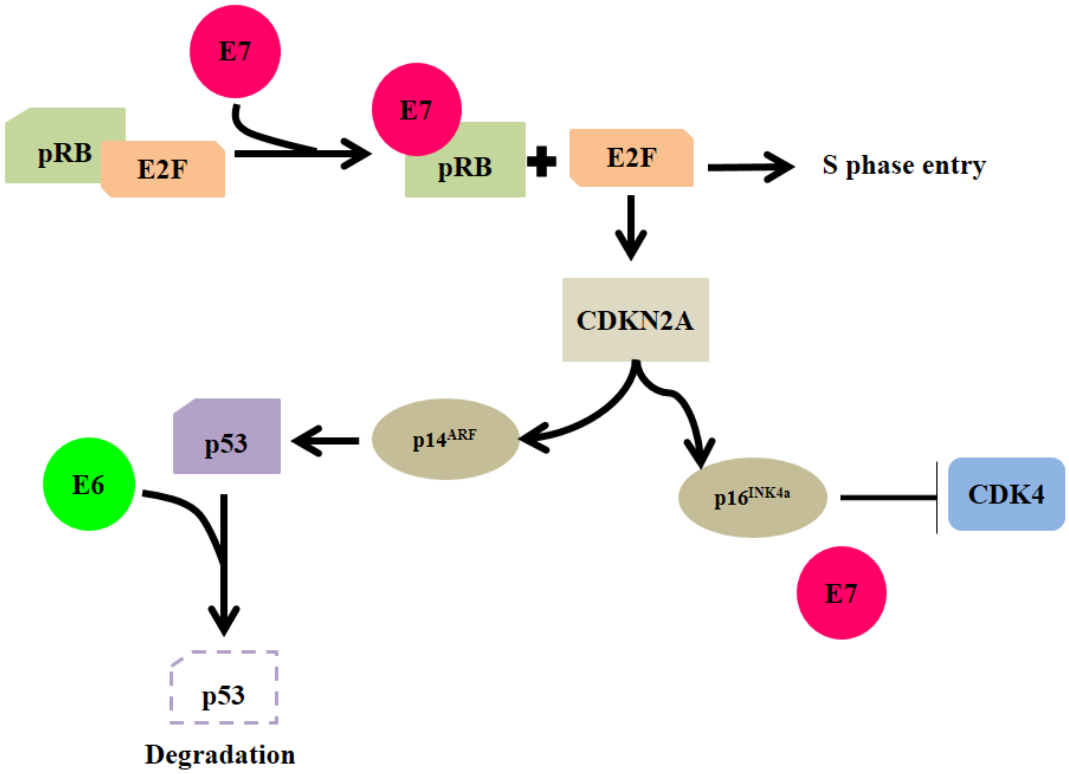

Figure 12. A part of the cell cycle of a high-risk HPV infected cell. Functions of E6 and E7 HPV proteins in the cell cycle and their effect in its regulation. Adapted from Doorbar J, *et al.* 2012.

Cervical carcinomas usually present HPV E7 proteins that bind to pRB and pRB family members. Moreover, in cervical carcinomas that do not present HPV E7, the inactivation of pRB occurs through somatic mutation (Sherr C, *et al.* 2002). Cells infected with high-risk HPV types are not able to prevent the accumulation of mutations, since E6 triggers p53 destruction. Hence, HPV E6 and E7 proteins are key factors for the development of cancer, due to the fact that they direct the cell into the S phase although it has DNA damage. In HPV patients with a high grade CIN or cervical cancer, great amounts of E6 and E7 have been detected (Bosch X, *et al.* 2002).

With regard to the HPV carcinogenesis, the relation between HPV products and host immune system should be mentioned. Th1 cytokines activate the antiviral immune response in cervical cancer and Th2 cytokines inhibit it (Torres K, *et al.* 2014). It has been noticed that women with CIN III lesions (HSIL) or cervical cancer present a low Th1 cytokine mediated immune response to HPV E6 and E7 proteins, compared with HPV infected women who do not present cervical lesions. The lack of Th1 cytokine mediated immune response to HPV infection could lead to the prevalence of HPV and the progression of cervical cancer (Bosch X, *et al.* 2002). Moreover, the activity of IL-10 and TGF-β1 has been related to the HPV prevalence and its evasion to the immune response. IL-10 is a Th2 anti-inflammatory cytokine that regulates the immune response inhibiting T cell proliferation and inflammation and also reducing the synthesis and differentiation of Th1 cytokines. Human gene IL-10 regulatory regions are activated through Sp1 transcription factor (Roitt's Essential Immunology, 2011; Torres K, *et al.* 2014). Regarding TGF-β1, which is also regulated by the Sp1 transcription factor, it is a multifunctional cytokine that decreases immune response inhibiting T cell proliferation and differentiation (Torres K, *et al.* 2014).

HPV E2 protein binds to the IL-10 gene promoter, inducing IL-10 expression. IL-10 induces TGF-β1 expression and vice-versa. With regard to the HPV16 type, E6 and E7 proteins also interact with immune system elements. Both proteins bind to the previously mentioned Sp1 transcription factor. The E6-Sp1 and E7-Sp1 complexes bind to the IL-10 and TGF-β1 regulatory region (GGGGCGG) activating the transcription of IL-10 and TGF-β1. Furthermore, IL-10 induces the transcription of the E6 and E7 early promoters leading to the expression of more E6 and E7, generating an endless cycle (Figure 13). High amounts of IL-10 and TGF-β1 lead to the decrease of Th1 cytokine mediated immune response, allowing the evasion of HPV from immune system and the progression of cervical lesions.

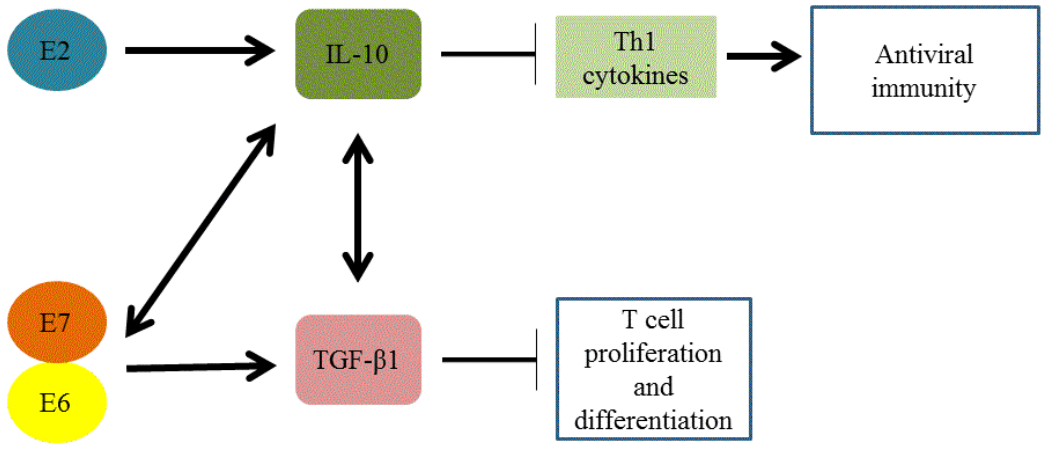

Figure 13. Interactions between HPV16 products and Th2 immune system elements. Adapted from Torres K, *et al.* 2014.

6.3. A possible explanation of why HPV could lead to cervical cancer: The integration of HPV genome in host genome and altered function of genes are associated with the development of cervical cancer.

The integration of HPV genome is known to be a key event in cervical carcinogenesis, since it can lead to the disruption of the E2 viral gene in host cells. As previously mentioned, E2 regulates E6 and E7 expression binding to specific sites located in the LCR of the HPV genome. The disruption of E2 removes its inhibition on early promoters and E6 and E7 will be overexpressed, leading to the cell-cycle re-entry and immune response evasion (Adams A, *et al.* 2014; Lu X, *et al.* 2014). Besides, the integration of HPV genome in host genome would change the function and expression of both viral and host genes; for example, activating oncogenes or inactivating tumor suppressor genes. To date, only transcription patterns of HPV16 and 18 in cervical cancer tissues have been reported; which included episomal HPV early gene transcripts and also integrated HPV transcripts (Lu X, *et al.* 2014).

Regarding HPV16 genome integration in host genome, a recent study has been carried out seeking HPV16 oncogene transcripts to infer the integration sites of HPV in host genome. After an APOT assay -which is based on a 3' rapid amplification of cDNA ends PCR assay that achieves amplification and cloning of the region between a cDNA sequence and its unknown 3'-end (An Introduction to Molecular Biotechnology, 2011) - the cDNA segments were sequenced, and finally they were analysed using the BLAST program. As mentioned before, the aim of the study was to infer the integration pattern and host genome sequences that were placed on the 3'-end of the cDNA sequences; since the known 5'-end sequences contained viral oncogenes, such as E7. The study shows that there are different transcription patterns and integration sites (Lu X, *et al.* 2014).

With regard to the transcription patterns, 3 different types were detected: types A, B and C. Moreover, among all the samples containing HPV16 viral transcripts, some of them were directly linked to a poly-A region in their 3'-end. Type A HPV16 viral transcript has E7-E1 sequences which are directly fused with host genome sequences through two different E1 integration sites, nt880 and nt1107. In Type B HPV16 viral transcripts, the E7-E1 region is spliced with the E2 region, which binds to the host genome sequences in the nt2870 integration site. Finally, Type C is similar to Type B; E1-E7 region is spliced with the E4 region, which binds to the host genome sequences in the nt3691 (Figure 14).

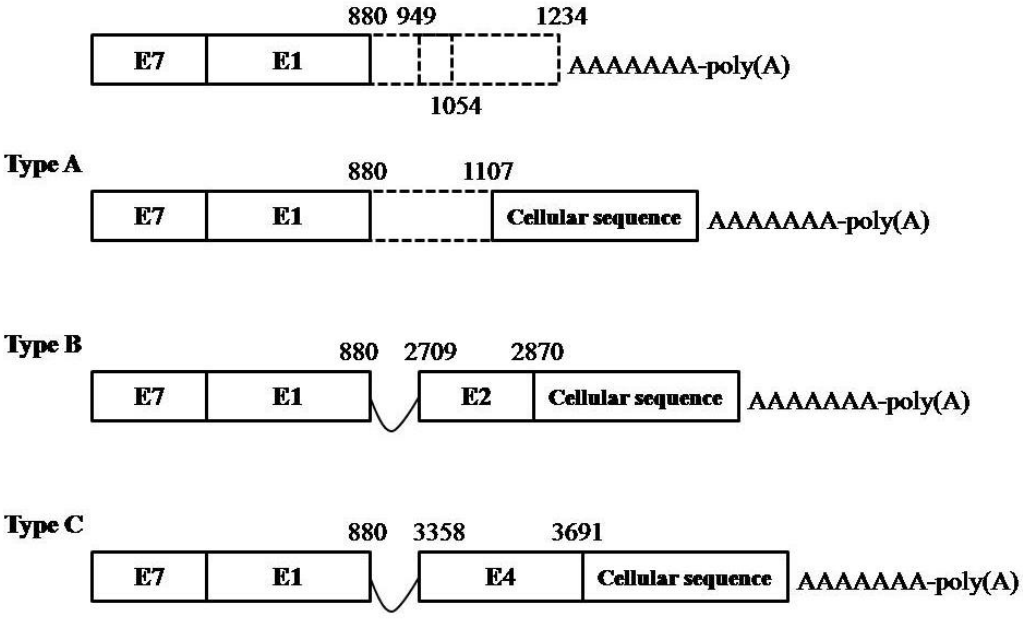

Figure 14. Different transcription patterns of HPV16. The first viral transcript is directly linked to a poly-A region in the 3'-end; the numbers indicate the different E1 integration sites. Type A fuses directly with cellular sequences in two possible E1 integration sites, nt880 andnt1107. Type B's E1 is linked to E2, and E2 is spliced to a cellular sequence in the nt2870. Type C's E1 is linked to E4, and E4 is spliced to a cellular sequence in the nt3691. **Adapted from Lu X, *et al.* 2014.**

Nonetheless, the prevalence of the different transcription patterns varies depending on the grade of the intraepithelial lesion. In LSIL and HSIL samples, only Type A and B transcripts have been detected. Type C is only present in CxCa (cervical cancer) samples. Furthermore, the presence of different fusion transcripts -viral transcripts fused with host genome- is higher in HSIL and CxCa samples than in LSIL samples (Table 2). These results support the idea of HPV genome integration being a key factor in cervical cancer development; since the presence of fusion viral transcripts is higher in lesions that lead to the development of cancer.

Table 2. Transcript number of the three transcript patterns in LSIL, HSIL and CxCa samples. 53 transcripts detected from 40 HPV16 positive cervical samples, 8 LSIL, 24 HSIL and 8 CxCa. Type A is present in all samples with a 100% prevalence in HSIL and CxCa samples; and a 37.5% prevalence in LSIL samples. Type B shows a 100% prevalence in CxCa samples; a 12.5% prevalence in HSIL; and a %12.5 prevalence in LSIL. Type C is only found in CxCa samples, with a prevalence of 75%. **Adapted from Lu X, *et al.* 2014.**

	Patient samples		
Transcription pattern	**LSIL**	**HSIL**	**CxCa**
Type A	3	24	8

Table 2. Continuation

	Patient samples		
Transcription pattern	**LSIL**	**HSIL**	**CxCa**
Type B	1	3	8
Type C	-	-	6

On the other hand, regarding the integration sites where viral sequences bind to, the study results showed that HPV integration in host genome has not any preferential integration site. Integration events have been documented in every human chromosome except for chromosomes 21 and X. In addition, most of the transcripts fused with host genome were located in or near to a fragile site. Fragile sites are known as genome regions where replication mistakes frequently occur. These mistakes may be a result of a low condensed chromatin due to the hypomethylation of the DNA. As briefly mentioned before, the integration of HPV sequences in host genome changes the transcription and function of HPV genes. Besides, this integration event also leads to alterations of host genes expression and function. Many of host genes located in integration sites are tumor suppressor genes or oncogenes, which are directly related with cancer development; such as MSH2 (mutS homolog 2). MSH2 gene is located in the 2p21 position, in the second chromosome. MSH2 gene encodes the MSH2 protein, which repairs DNA mismatches; thus, MSH2 protein is a tumor suppressor related with DNA repair pathway. The integration of HPV genome close to MSH2 gene, leads to the reduction of gene expression, reducing the tumor suppressor activity of MSH2 and increasing the risk of developing cervical cancer (Lu X, *et al.* 2014).

7. Conclusions

To sum up, as we have seen, in order to progress from a CIN III lesion (HSIL) to cervical cancer, HPV infection is a necessary but non-sufficient cause. The evasion of the immune system would explain the persistent high-risk HPV infection. Moreover, the completion of the transformation of cervical cells initiated by E6 and E7 high-risk HPV oncoproteins could be explained by the alteration of host and viral genes through high-risk HPV genome integration in host genomes (Adams A, *et al.* 2014; Lu X, *et al.* 2014).

Nowadays, we know that high-risk HPV types express E6 and E7 oncoproteins in infected basal cells and in differentiating keratinocytes of the upper layers. The expression of these E6 and E7 oncoproteins in differentiating keratinocytes (upper layers) is the natural mechanism to amplify HPV genome and to package it into infectious new virions; since, as we have already seen, E6 and E7 oncoproteins prevent apoptotic mechanisms allowing cell-cycle re-entry (Doorbar J, *et al.* 2012). However, the expression of these oncoproteins in the basal layers only occurs in the high-

risk HPV types, which are those who can cause cervical cancer. E6 and E7 are key proteins to understand HPV interactions with host cellular-cycle pathways. Further research on differences of E6 and E7 oncoproteins in high-risk and low-risk HPV types should be done, in order to obtain more information about which are the differences that make high-risk E6 and E7 oncoproteins more effective or to better know why they are expressed in basal layer cells.

Moreover, to date most research has been focused on two high-risk HPV types: HPV16 and 18, mainly on HPV16. We know how HPV16 E6 and E7 oncoproteins direct infected cells to cell-cycle re-entry, as well as we know how it evades host immune response thanks to E2, E6 and E7 proteins. Furthermore, we know that HPV16 genome integration in host genome leads to the disruption of E2, increasing E6 and E7 levels, which could lead into cervical cancer progression. However, it would be necessary to describe the differences of E2 among different high-risk HPV types to see whether it is directly linked to cervical cancer or not.

Last but not least, in the last decade, HPV has been related with many anogenital cancers; such as vulvar, anal and penile cancers (Adams A, *et al.* 2014). It would be necessary more research focused on different HPV associated diseases. Besides, a new insight studying HPV oncoproteins and host genome tumor suppressor genes expression in different HPV associated cancers may help in better understanding the differences among HPV carcinogenic mechanisms.

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