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Human papillomavirus (HPV) – Pathology, diagnosis and vaccination strategies

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by

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SWORN DECLARATION

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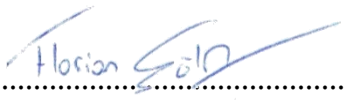

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Wels, January 2017

Abstract

Human papillomaviruses (HPV) are small double stranded DNA viruses and are linked with the occurrence of cervical cancer, one of the most prevalent cancers in women on a global scale, leading to roughly 250.000 deaths per year. It is one of the most transmittable infection by a sexual act. Especially immune suppressed subjects shown a higher susceptibility to HPV and a following diseases outbreak. There are over 100 different types of human papillomavirus known and classified into two major groups. The low-risk classification can cause benign tumors like warts on the skin. The other part are the high-risk human papillomaviruses. An infection with one HPV of this group can results in cancer development of the cervix.
HPV research is tricky because the virus is host and tissue specific. Nevertheless, current research focus on the development of new or approved vaccine strategies as well as the inactivation of the two viral oncogenes E6 and E7. These oncogenes expressed by the high-risk HPV can mutate the cell cycle by disrupting the tumor suppressor p53 and the cell cycle checkpoint pRb is deactivated as well by the HPV oncoproteins. To counteract these viral proteins, the genome editing technique CRISPR/Cas9 highlighted latest promising results in HPV oncogene disruption in vitro. For HPV screening the PAP smears is the most used detection method to detect cell abnormalities, but other nucleic acid based detection methods are in development or are used as a co-detection method.

Based on a global change of the sexual behavior, as well as a lack of information about HPV, the cases of cervical cancer and an oral cancer development is increasing worldwide
Thus, the aim of this paper is to give a comprehensive overview on human papillomavirus and its relevance in different diseases, especially its connections to cancer, vaccines strategies, detection method and to underline the importance of research related to this field.

Table of Content

Introduction

Papillomaviruses belong to the double-stranded DNA-genome viruses. This species of virus is known to cause malign tumors like the cervix carcinoma in humans and the wide spread virus can cause benign skin warts at the human's skin surface as well. This suggested that papillomaviruses have the possibility to regulated and affect the cell division process in the human body. Other types of papillomavirus are found in dogs, sheep, deer, elks, bovines and birds. This kind of virus is strictly epitheliotropic and, thus, the replication is done in terminally differentiated keratinocytes. Due to their host and tissue specify, it is only possible to cultivate the virus *in vitro*. Therefore, to get more information about the biology and the pathogenesis of this virus, a bovine-papillomavirus model is used (Modrow, et al., 2010). Until the possibility of the recombinant DNA technology, scientist imagined that there was only on kind of HPV causing excrescences, which recede spontaneously (Stanley, 2012). Based on the fact mentioned above, most human papillomavirus (HPV) could be detected with an improved nucleic acid isolation technique and a subsequent sequencing process. At present, more than 100 different types of HPV are identified. New types of HPV can be classified, when there is less than a 90% match of the genome sequence, which encode for the E6, E7 and L1 proteins, compared with other currently identified HPV types. New types of HPV, which are characterized by amplification of the genome with polymerase chain reaction (PCR) and sequence analysis are labeled with "cand" (e.g.: candHPV 86). After cloning and approved sequencing analysis, this special term can be removed. Nowadays, there are 16 different genera of papillomaviruses. The virus is classified according to host and tissue specifity, genome organization and sequence characteristics. As highlighted in table 1, HPV are assigned to the genera α-, β-, γ-, and to μ- and ν-papillomavirus. α-papillomavirus infects the mucosal region of the mouth and anogenital area. These viruses could be cause benign tumors, caused by HPV 6, HPV 7, HPV 54, HPV 61 and HPV 71, or malign tumors caused by HPV 26, HPV 34, HPV 53. Lesion of the cutaneous skin are associated with HPV 2 and HPV 10. β- papillomavirus infects immune suppressed people and is responsible for skin cornification, called epidermodysplasia verruciformis. Benign lesions of the skin are caused by γ-Papillomavirus. μ-papillomavirus contributes to benign warts (Modrow, et al., 2010).

Table 1: Classification of papillomaviruses. HPV – human papillomavirus (Modrow, et al., 2013)

Genus	Human virus	Animal virus
Alphapapillomavirus	HPV-2, HPV-6, HPV-7, HPV-10, HPV-16, HPV-18, HPV-26, HPV-32, HPV-34, HPV-53, HPV-54, HPV-61, HPV-71, HPV-90 (wart viruses)[a]	*Maccaca fascicularis* paplillomavirus types 3–11
		Rhesus papillomavirus type 1b
Betapapillomavirus	HPV-5, HPV-9, HPV-49, HPV-92, HPV-96[a]	*Maccaca fascicularis* paplillomavirus types 1 and 2
Gammapapillomavirus	HPV-4, HPV-48, HPV-50, HPV-60, HPV-88, HPV-101, HPV-109, HPV-112, HPV-116, HPV-121[a]	
Deltapapillomavirus		Bovine papillomavirus types 1 and 2[a]
		Ovine papillomavirus type 2[a]
		European elk papillomavirus[a]
		Reindeer papillomavirus[a]
Epsilonpapillomavirus		Bovine papillomavirus type 8
Zetapapillomavirus		*Equus caballus* papillomavirus 1
Etapapillomavirus		*Fringilla coelebs* papillomavirus
Thetapapillomavirus		*Psittacus erithacus timneh* papillomavirus
Iotapapillomavirus		*Mastomys natalensis* papillomavirus
Kappapapillomavirus		Cottontail rabbit papillomavirus (Shope papillomavirus)
		Rabbit oral papillomavirus
Lambdapapillomavirus		Canine oral papillomavirus
		Felis domesticus papillomavirus 1
		Procyon lotor papillomavirus
Mupapillomavirus	HPV-1, HPV-63	
Nupapillomavirus	HPV-41	
Xipapillomavirus		Bovine papillomavirus, types 4, 6 and 9–12[a]
Omikronpapillomavirus		*Phocoena spinipinnis* papillomavirus

(*continued*)

Genus	Human virus	Animal virus
Pipapillomavirus		Hamster oral papillomavirus
		Micromys minutus papillomavirus 1
Rhopapillomavirus		*Trichechus manatus latirostris* papillomavirus 1
Sigmapapillomavirus		*Erethizon dorsatum* papillomavirus 1
Taupapillomavirus		Canine papillomavirus type 2
Upsilonpapillomavirus		*Tursiops truncatus* papillomaviruses 1 and 2
Phipapillomavirus		*Carpa hircus* papillomavirus 1
Chipapillomavirus		Canine papillomavirus types 3 and 4
Psipapillomavirus		*Rousettus aegytiacus* papillomavirus 1
Omegapapillomavirus		*Ursus maritimus* papillomavirus 1
Dyodeltapapillomavirus		*Sus scrofa* papillomavirus 1
Dyoepsilonpapillomavirus		*Francolinus leucoscepus* papillomavirus 1
Dyozetapapillomavirus		*Caretta caretta* papillomavirus 1
Dyoetapapillomavirus		*Erinaceus europaeus* papillomavirus 1
Dyothetapapillomavirus		Feline papillomavirus type 2
Dyoiotapapillomavirus		*Equine caballus* papillomavirus 2

Other genera of papillomavirus like δ-papillomavirus, ε-papillomavirus, ξ-papillomavirus, ζ-papillomavirus, ι-papillomavirus, κ-papillomavirus, π-papillomavirus, λ-papillomavirus and ο-papillomavirus are animal viruses and are not described in this paper.

HPV can be classified into two different groups. The first group are the low-risk HPV, including HPV6 and HPV11, which contribute to genital warts. The high-risk HPVs, for example HPV18 and HPV16 are responsible for cervical cancer. All in all, 15 different HPVs are classified to the high-risk group (Stanley, 2012).

Structure, genome and proteins

The particle of a papillomavirus is a small capsid without a membrane and consists of two proteins. The major structural protein, the L1-protein, builds with 72 pentameric capsomer the particle structure with a diameter of 50 to 60 nm. Twelve to 72 molecules of the minor capsid protein L2, as well as the viral genome, are on the inner side of the particle. In Figure 1 there is a schematic of the particle. Papillomaviruses have a double-stranded DNA with a size of 8000 base pairs, containing eight to nine ORFs. As shown in Figure 2, the circular genome is divided into 2 parts. The first part of the genome is responsible for encoding the proteins E1 to E7. E means early, meaning they are translated first. From the second region, the late proteins L1 and L2 are synthesized. The early region contains more ORFs. Transcriptional regulatory elements like promoter, enhancer and origin of replication (ori) are located in the upstream regulatory region (URR), also known as the long control region (LCR), which is a 100 bp large sequence between the late and early region (Modrow, et al., 2010; Stanley, 2012; Doorbar, et al., 2015).

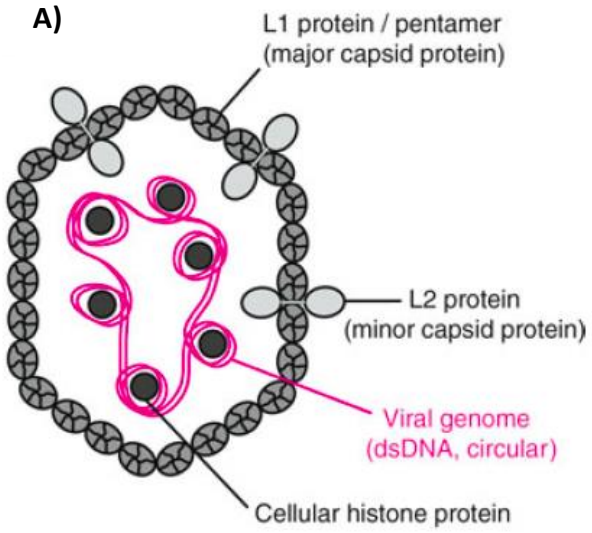

Figure 1: HPV structure

Structure of the viral capsid with the 2 structure proteins L1 and L2 and the circular viral genome associated with histone proteins (Modrow, et al., 2010).

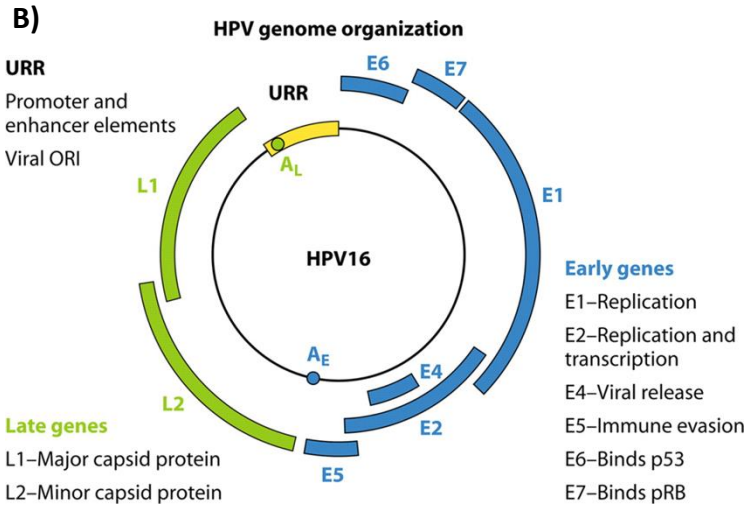

Figure 2: HPV genome organization

Viral genome with the early area encoding for the proteins E1 to E7 and the late region with the 2 late proteins L1 and L2 (Stanley, 2012).

Gene expression is driven by 2 promotors located in the genome of Papillomavirus. The first early promotor, called P97 in HPV16, or P105 in human papillomavirus 18 and P99 in HPV31, as well as P105 in HPV18, P99 in HPV31 and P89 in BPV1 is found upstream of the E6 open reading frame. Some transcription factors, like AP 1, Oct-1, TEF-1, NF-1, TFIID, and KRF-1 regulate the early transcription by binding to the early promotor in the well-studied HPV16. The late promotor is located within the open reading frame of E7 and needs differentiating cells to be active. For promotor activation, the p63 protein is needed in differentiating epithelial cells (Miller & Stack, 2015). The E ("early") proteins are necessary for the regulatory function of the virus, like cell signaling, immune modulation, structural modification and control of apoptosis of the infected cell. The two late proteins L1 and L2 are expressed in the granular layer of the epithelium. In HPV16 infected cervical epithelial cells, four mRNA isoforms of the E6 protein were detected. These isoforms E6*I, FLE6, E6*II and E6*X are evidence, that other virus proteins could have additional important functions. Two other mRNA isoforms have been observed in HPV18 infected cells (Graham, 2010).

The early and late proteins with their major functions are shown in Table 2. The highly conserved E1 encodes for a virus-specific DNA helicase, needed for replication and amplification of the viral genome (Doorbar, et al., 2015). Depending on the virus type, the ORF of E1 encodes for phosphorylated proteins with a molecular mass between 68 and 85 kDa, which are necessary for replication of the viral genome. The attachment to the ori sequence is mediated and stabilized by the E2 protein. The phosphorylated E1 protein forms hexamers and interacts with other proteins as well as with DNA-polymerase-α and DNA helicase.

Table 2: Expressed proteins by high risk human papillomavirus (Graham, 2010).

Protein	Role in the virus life cycle
E1	Genome replication: ATP-dependent DNA helicase.
E2	Genome replication, transcription, segregation, encapsidation. Regulation of cellular gene expression. Cell cycle and apoptosis regulation.
E4	Remodels cytokeratin network. Cell cycle arrest. Virion assembly.
E5	Control of cell growth and differentiation. Immune modulation
E6	Oncoprotein. Inhibits apoptosis and differentiation. Regulates cell shape, polarity, mobility and signalling.
E7	Cell cycle control. Controls centrosome duplication.
L1	Major capsid protein
L2	Minor capsid protein. Recruits L1. Virus assembly.

The level of phosphorylation of the protein depends on the different phases of the viral infection cycle. In undifferentiated epithelial cells of the skin, E1 controls a limited number of viral genome replications. After initial amplification, there are a constant number of 50 – 400 copies. The replication modulated activity of E1 is responsible for maintenance and transferring of the genome copies during cell proliferation. After development of the undifferentiated cells to fully differentiated epithermal cells, there is a shift of the constant copies to a productive virus enlargement. Thereby a lot of amplified genomes arise. This replication is associated with the production of infectious viruses (Modrow, et al., 2010).

E2 can bind to the viral and cellular genome and is necessary for genome replication, as well as for transcription, segregation and more (Doorbar, et al., 2015). Ensuing from the E2 ORF, different forms of DNA binding proteins are synthesized, which are pretty well characterized in the bovine papillomavirus (BPV). Compared with HPV, there are many conserved amino acids and, therefore, it is assumed that the E2 proteins of HPV and bovine papillomavirus have the same function. The sequence of the E2 binding site is 5'-ACCNNNNNNGGT-3', which is located in different promotor elements at the LCR region close to the ori. The E2 protein with a molecular mass of 48 kDa, consists of two functional domains. At the carboxy-terminal region, there is a DNA binding region, which is also responsible for dimerization. The second domain is a trans-activated acting region at the amino terminal end. Both domains are linked with an amino acid part, whose size and sequence are different among the different papillomaviruses. E2 also controls the transcription of the E6 and E7 gene (Modrow, et al., 2010). Depending on the interaction of E2 with other cellular gene products, it can modify the normal role for the best situation of the virus. To escape from the epithelial surface, the early protein E4 is needed. E5, which is missed in β-papillomavirus, has an important role in genome amplification optimization, due to immune evasion. In α-papillomavirus, the E5 open reading frame is located downstream of E2. The oncoproteins E6 and E7, which could control the cell cycle, are regulated at the transcription level by E2. The proteins allow the amplification of the genome and inhibit the immune response in the mid-layers of the epithelium. In HPV101, HPV103 and HPV108, there is a lack of the E6 protein (Doorbar, et al., 2015). The ORF of E6 contributes to the transformation of the protein, but it could not induce alone. The E6 protein of the high-risk HPV16 has a size of 151 amino acids and builds a complex with two Zn^{2+} ions and, therefore, it could bind to the DNA (Modrow, et al., 2010). In Figure 3, there is a comparison of the E6 protein

expressed by high-risk and low-risk HPV types. The protein consists of two specific zinc fingers, which are necessary for the correct function. Losing the zinc fingers results in inactivity of the protein. In contrast to the conserved central region of the protein, the N-terminus and C-terminus are more different from each other (Miranda, et al., 2002).

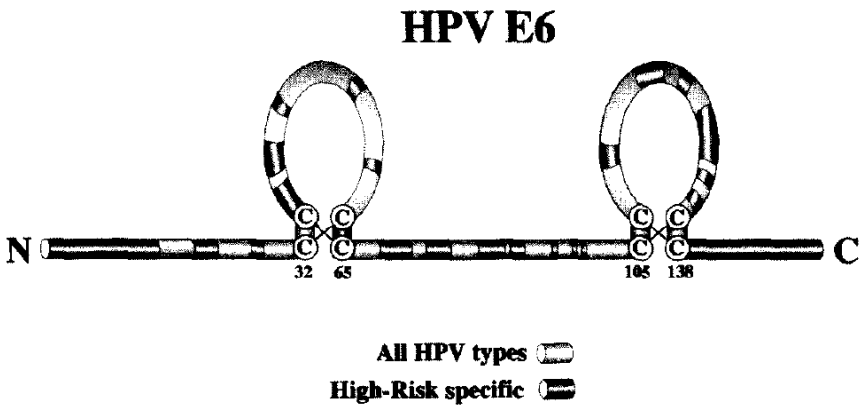

Figure 3: HPV E6 gene

Comparison of high-risk and low-risk HPV E6 protein, where black regions indicate specific sequences of the high-risk types (Miranda, et al., 2002).

The small E7 protein, with a size of 98 amino acids, is the major cause of the transforming potential of certain HPVs. E7 is phosphorylated by the casein-kinase II at the serine residue at position 31 and 32. The correct protein folding and dimerization of the molecules is done by the repetitive amino acid sequence Cys-X-X-Cys. The two late proteins, L1 and L2 are synthesized in fully differentiated keratinocytes. In infectious viruses, there is a 30:1 ratio of both proteins. Without the L2 protein, the virus is not infectious. At the amino-terminus of L2, there is a conserved sequence, which is recognized and cleaved by the protease Furin. The proteolytic cleavage takes place on the cell surface and leads to the infectiousness of the virus. L2 also contributes to the destruction of the endosomal membrane and the transportation of the viral genome into the cell nucleus by receptor-mediated endocytosis (Modrow, et al., 2010).

The 2 major oncoproteins

The two oncoproteins of high-risk HPV, E6 and E7, are associated with the development of cervical carcinogenesis, which is one of the most prevalent cancers in women on a global scale (Yim & Park, 2005), leading to roughly 250 deaths per year (Chen, et al., 2015). 99.7% of all cervical cancers can be linked with high-risk HPV. About 100.000 women will develop precancerous chances in the cervical tissue, whereby a few of these women will develop invasive cancer (Yim & Park, 2005). During the integration of the viral genome to the host DNA, an important viral gene of the replication cycle and for the control of E6 and E7 gene expression, is destructed. Therefore, there is an overexpression of the two oncoproteins, which leads to cell transformation (Hof & Dörries, 2005). In basal cells, both oncogenes are expressed at low levels, but only in fully differentiated cells, transcription is activated (Peralta-Zaragoza, et al., 2006). While the two proteins E1 and E2 of HPV are responsible for the replication, the two oncoproteins E6 and E7 have the purpose to promote cell proliferation. Based on the function to smash the checkpoints of the cell cycle, these two proteins have a key position in causing cervical cancer (Howie, et al., 2009).

Interaction of the E6 oncoproteins and the p53 tumor suppressor

The HPV E6 oncoproteins consist of two CXXC-X_{29}-CXXC domains and have a molecular mass of 18 kDa (Huibregtse & Beaudenon, 1996). The protein, expressed by high-risk types, affects the activation of p53, a tumor-suppressor protein, by inducing a proteasome-depended p53 degradation (Figure 4). The protein labels the p53 protein for degradation, done by a protein complex called the 26s proteasome, due to the transport of ubiquitin peptides from the E6 associated protein (E6AP) to p53. Low-Risk HPV E6 proteins, as cutaneous epithelia-infecting E6 proteins, are unable to target p53 (Yim & Park, 2005) in contrast to HPV16 and

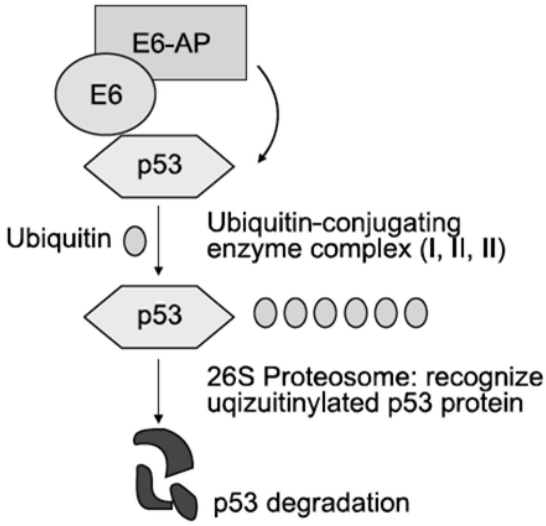

Figure 4: Proteasome pathway of the HPV E6 protein

E6 interacts with the E6 associated protein (E6-AP). The complex binds to p53 and induces its degradation by the 26S proteasome (Yim & Park, 2005).

HPV18, which can exhibit a high transforming potential, which leads to a possible interaction with p53 (Modrow, et al., 2010). Normally the tumor suppressor is encoded in cells with increased mutagenic DNA damage. p53 is responsible for the expression of genes, like a cyclin depending kinase protein $p21^{CIP}$, with the function to stop the cell cycle during G1 phase and defer the transition to the S phase. The lack of p53 leads to an accumulation of mutations, because DNA-repair systems are inoperative. As result of E6AP mediated proteolytic degradation of NFX1-91, the human telomerase reverse transcriptase (hTERT) is activated, leading to an increase in cellular telomerase activity in tumor cells. In consequence to the reproduction of the repeated sequences at the telomeres, done by hTERT, tumor cells are not subject to cellular aging. High-risk E6 proteins have a high affinity to bind to BAK-proteins, which are responsible for apoptosis at the differentiated layer of the skin and, therefore, the normal cellular apoptosis is suppressed (Modrow, et al., 2010), while low-risk HPV6 and HPV11 E6 protein binds very weakly to p53 and, therefore, the protein is not able to degrade the tumor suppressor p53 (Mantovani & Banks, 1999).

Different E6 protein interactions

Various human malignancies, like head and neck cancers, anogenital cancers, squamous cell carcinomas and of course cervix carcinomas are linked with human papillomavirus. E5, E6 and E7 are three oncoproteins expressed by HPV and these proteins could be integrated into the host genome, followed by deletions of the viral genome. Based on the large deletion of the genome, the protein E5 is lost, and E6 and E7 are not altered, but their expression occurred. All in all, there are over 100 human papillomavirus types described, but only a small group, also known as the high-risk group, are associated with human cancer development. The different interactions of the E6 protein are one major reason, why there is a difference in pathology between the HPV types (Miranda, et al., 2002).

Interaction between E6 and cell transformation

In vitro experiments of HPV and human keratinocytes have shown that both oncogenes produced from high-risk HPV contribute to immortalization of the cells. Using E6 and E7 produced by low-risk HPV in rodent cells, these genes are inactive. Another experiment showed a transformation of rodent cells caused by high-risk HPV16 and HPV18. The protein could activate the ras oncoprotein, resulting in

transformation of the rodent cells. E6 proteins from low-risk HPV are not able to transform rodent cells. It is also important to understand that the genes are not able to create a tumor in keratinocytes in an *in vitro* experiment. Therefore, the effect of both genes E6 and E7 was compared in an *in vivo* assay using transgenic mice. A keratin 14 promotor was used to express E6 and E7 in physiological relevant tissues. Expression of E7 alone, leads to development of tumors with higher frequency but these tumors could be classified as benign. In contrast, when E6 is expressed alone, less but more aggressive tumors were produced. Additionally, there was a progression to metastatic cancer. In other studies, in which chemicals were used for cancer development, E7 was indicated as an immortalization promotor, whereas E6 leads to driving cells into full malignancy. Based on these results, chemotherapy against the E6 proteins is a better way to treat cancer caused by human papillomavirus (Miranda, et al., 2002).

Interaction between E6 and bak

Bak is a pro-apoptosis protein and belongs to the Bcl-2 family. The effector protein can induce the apoptotic pathway by help of another pro-apoptotic protein called bax. Both members of the Bcl-2 family act in mammalian cells in mitochondria. They have an important role in programmed cell death. Damage of the mitochondria results in oligomer formation of bax and bak in the outer mitochondrial membrane. This leads to the release of cytochrome c and the formation of an active apoptosome by interactions with Apaf-1 and caspase-9. Activated caspase-9 activates other caspases like caspase-3. As you can see in Figure 5, Bak is targeted by HPV E6. It is a highly-conserved function of the human papillomavirus genome and could be degraded by high-risk, but also by low risk HPV. This happens because the Bak protein is highly expressed in upper epithelial cells, which are also the place for HPV replication (Miranda, et al., 2002; Cooper & Hausmann, 2016).

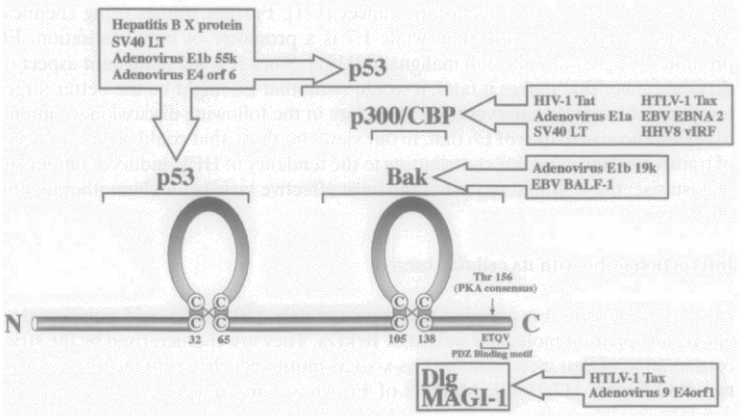

Figure 5: HPV18 E6 protein targeting bak

Further interactions of the E6 protein with cellular targets are shown as well. Other viral proteins are shown in boxes targeting the same cellular components (Miranda, et al., 2002).

Interaction between E6 and the transcription factory p300/CBP

The function of the p300/CBP is to control the correct function of the transcription factory. Therefore, it is very important for the re-modelling of chromatin. As you can see in the Figure above, p300/CBP is the target of some viral genes, like SV40 LT and AD E1A. It is no surprise that there is also an interaction between this important transcription factor and the HPV E6 gene. HPV16 E6 interacts with p300/CBP and reduces its activity. It was also shown that E6 could alter the promotor specify, resulting in viral gene transcription (Miranda, et al., 2002).

Immortalization of infected cells

High-risk HPV infected host cells become immortal by the expression of telomerase activity. The end of the chromosomes, called telomeres, are shortened after each cell cycle leading to senescence of normal cells. In contrary, stem cells require self-renewal property and, therefore, an enzyme called telomerase is active in order to lengthen the 3´- end of the telomeres. In cancer cells, expression of a telomerase subunit (hTERT) occurs, leading to cellular immortalization. Figure 6 shows the process of the proto-oncogene E6 interacting with the proto-oncogene *c-myc* and forming a E6-*c-myc* complex. As a result, hTERT is expressed and leads to cellular immortalization (Fakhry & D'Souza, 2015).

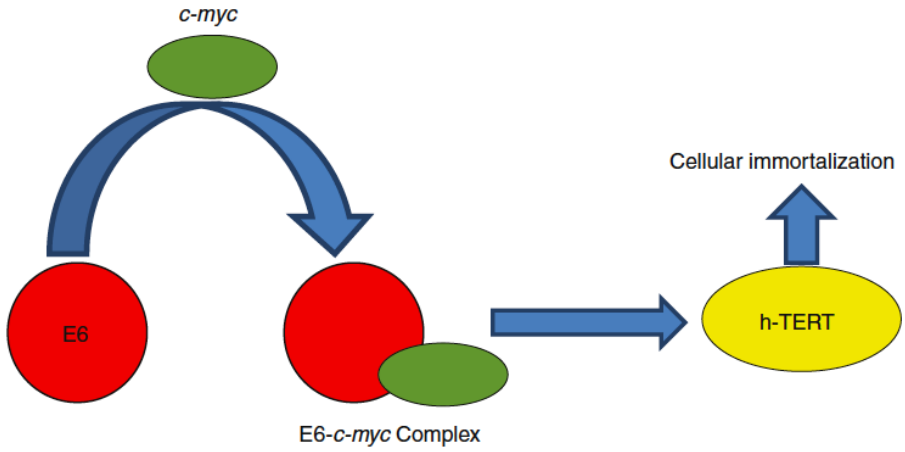

Figure 6: Interaction between E6 and hTERT

Interaction of the HPV E6 oncogene with hTERT results in cellular immortalization by restoring the 3´- end of the telomers (Fakhry & D'Souza, 2015).

E7 interactions

After infection by a HR HPV and insertion of the viral genome into the host DNA, the oncogene E7 binds to the retinoblastoma protein (pRb), a tumor suppressor protein (Figure 7). pRb controls the restriction point at G1 during the cell cycle and prevents the cell entering the S-phase, if DNA is damaged or mutated. Association of pRb and E7 induces ubiquitin mediated degradation of the tumor suppressor, as well as the degradation of p130 and p107. Degradation by the proteasome pathway releases the transcription factor E2F, which helps the cell to enter the S-phase of the cell cycle, leading to uncontrolled cell proliferation and accumulation of mutations. Additionally, cell cycle dependent kinase (cdk) inhibitors, like p21 and p27, are disrupted as well to achieve an uncontrolled cell cycle (Fakhry & D'Souza, 2015).

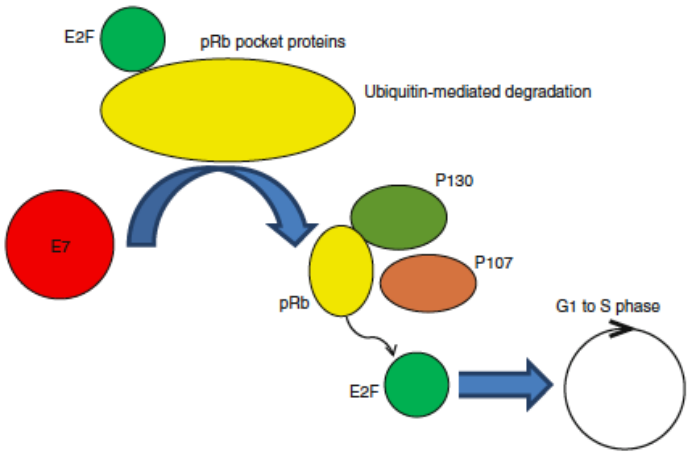

Figure 7: Interaction between the oncoprotein E7 and the retinoblastoma protein (pRb)

Interaction of E7 and pRb causes ubiquitination of the tumor suppressor. Proteasomal digestion of pRb leads to freeing the transcriptional factor E2F. E2F pushes the cell cycle forward, leading to uncontrolled cell proliferation (Fakhry & D'Souza, 2015).

Replication

During the replication process, human papillomavirus manipulates proteins from the host cell, which are responsible for the DNA repair mechanism as well as for the replication factory. Due to the changes of host proteins, HPV ensures the viral survival and the viral propagation in the host cell.

After infection of the basal layer (Figure 8), the HPV genome is replicated in a low copy number. During amplification, happened in differentiated keratinocytes, the copy number increases up to thousands of copies. Late genes are expressed to form a capsid to pack the viral genome. This mature virion is released and infects other hosts after cells are released from the epithelial skin.

There are four major proteins, which are needed for viral replication. The E1 protein consists of a specific sequence, which is cleaved by two caspases, caspase 3 and caspase 7 in order for viral amplification to occur. The same two caspases, as well as caspase 9, are activated in infected cells, and are a signal of an HPV infection. The two oncoproteins E6 and E7 inhibit the checkpoints of the cell cycle and pushes cell proliferation forward. Viral replication needs a specific DNA sequence. This origin of replication is an AT rich region containing an HpaI restriction and a E2 binding site with the palindromic sequence ACC(N_6)GGT. The E1 protein has a high affinity to DNA but it is not very specific. The E2 transcription factor, called E2 transactivation domain, is needed for stable viral replication. It communicates with E1 and recruits a helicase to the origin of replication. After E2 is released, E1 forms a double hexamer with a C-terminal helicase domain and an N-terminal DNA binding domain. Dimerization of the ATPase region of the helicase domain is necessary for its activity. The activated form allows the elongation step. Papillomaviruses are not able to express other enzymes than the needed DNA helicase E1; therefore, HPV depends on other host proteins for replication. E1 contains a nuclear export signal and a nuclear localization signal. A high amount of the E1 protein has cell-toxic properties; therefore, the

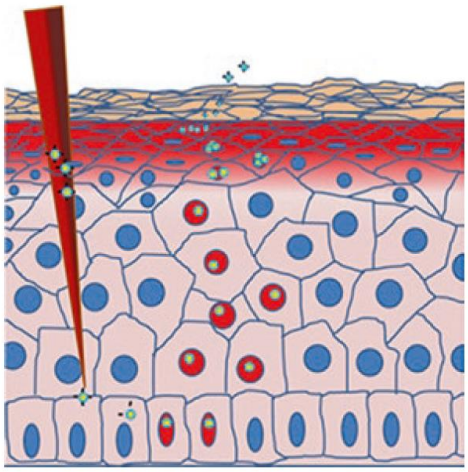

Figure 8: Early infection of the basal layer by the human papillomavirus

The laminin-332 and the heparin sulfate proteoglycan act as a receptor for the virus (small particles) to enter the cells and mutate the cytosol. Viral genomes are located inside the nuclei in a low copy number. In differentiated cells, the copy number increases to a high number (Miller & Stack, 2015).

protein is transported to the cytoplasm during the normal replication process. Phosphorylation of the nuclear export signal during amplification leads to an inactive form, resulting in E1 transport into the nucleus. This nuclear E1 protein innervates the cell DNA damage response, leading the translocation of the cellular machinery to viral DNA.

Transient infection does not require the two oncoprotein E6 and E7, but E6 and its function of disrupting p53 allows a long-term infection. The E6 and E7 complex controls E1 and E2 expression and affects plasmid segregation. For a productive replication, a specific intron of the E6 protein has to be spliced. So, a mutation of the splicing acceptor and or splicing donor could be affected by E1 expression (Miller & Stack, 2015).

Pathology

Human papillomavirus can cause two different kinds of tumors depending on the type of HPV. Low-risk HPV are associated with benign tumors like warts, whereas high-risk HPV are associated with malign tumors, like cervical cancer (Hof & Dörries, 2005). HPV can infect both types of human skin, the mucosal and cutaneous cells. Cutaneous cells are the surface of the skin, for example on legs and arms, whereby mucosal cells are found on the skin surface of the vagina, penis, mouth or anus (Figure 9). The pathogenic virus is transmitted by contact, that means, it is necessary to come in contact with HPV related warts to cause the benign tumor growth on the other subject as well (V. Spencer, 2007).

Benign tumors

Benign genital warts are the best known clinical infection of HPV. Genital warts can occur in the anal and genital zones of women and men, mostly caused by both low-risk HPV 6 and HPV 11. In some cases, a co-infection with one of the oncogenic high-risk types can happen. With a 65% transmission rate, genital warts are extremely infectious. Hence, benign tumors at the genital zone are the most commonly transmitted infection during sexual activity. In the United States, one percent of women and men aged 18 to 49 are infected by low-risk HPV leading to genital warts. However, the highest susceptibility to genital warts is detected by subjects aged 15 to 24. In the last decades, there was a ten-fold increase of genitals warts in the UK. This increase is due to the change of the sexual behavior (Borruto & De Ridder, 2012).

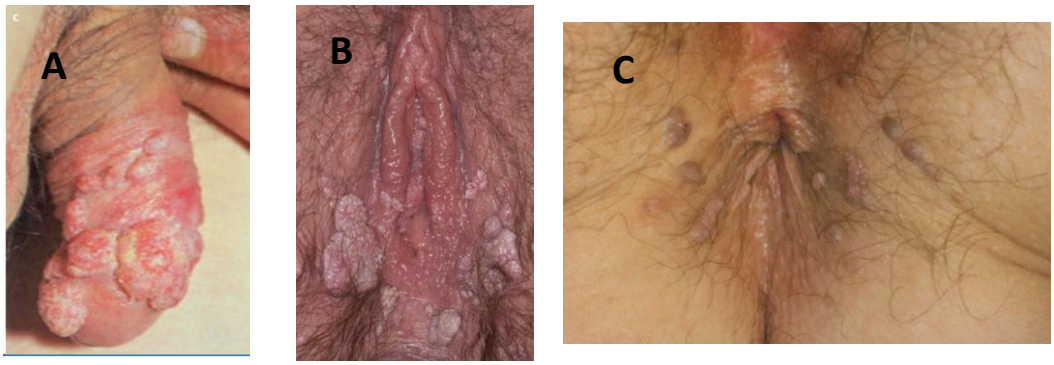

Figure 9: HPV related warts

Benign tumors (warts) at the genital zone (A) of the man, (B) of the woman and (C) at the anal zone (Hof & Dörries, 2005; Borruto & De Ridder, 2012)

Small lesions are sufficient for HPV to enter the cutaneous skin and to infect the epithelial basal layer. HPV specific proteins inhibit tumor suppressor proteins in the host cell and infected cells stuck in the S-phase of the cell cycle produce infectious

virus particles. The uncontrolled proliferation of infected cells results in warts development, linked with cell death of the first cell layer and freeing of the infectious virus particles (Hof & Dörries, 2005).

Malign tumors

Malign tumors can occur by an infection with the high-risk HPV type in the genital tract. In contrast to benign tumors, where the viral genome remains episomal, the viral genome is integrated into the host DNA, resulting in an overexpression of viral proteins and inactivation of tumor suppressors of the infected cell. A long-term transformation of the target cells leads to cervical cancer development over years (Hof & Dörries, 2005).

Cervical Cancer

With about 530.000 new infections and over 275.000 deaths by the year 2008, cervical cancer is placed third worldwide of cancer in women. Cervical cancer occurs as a consequence of sexual activity after an infection by one of the 40 types of high risk human papillomaviruses (Borruto & De Ridder, 2012). The cervix is the lower end of the uterus and is the connector to the vagina. Cervical cancer develops very slowly over years and is induced by uncontrolled cell growth at the cervix. Figure 10 shows the infected zones by HPV with a corresponding CIN lesion. The lack of cell contact inhibition of cancer cells leads to tumor formation with the ability to invade other parts of the body. Regular screening can help for an early detection of

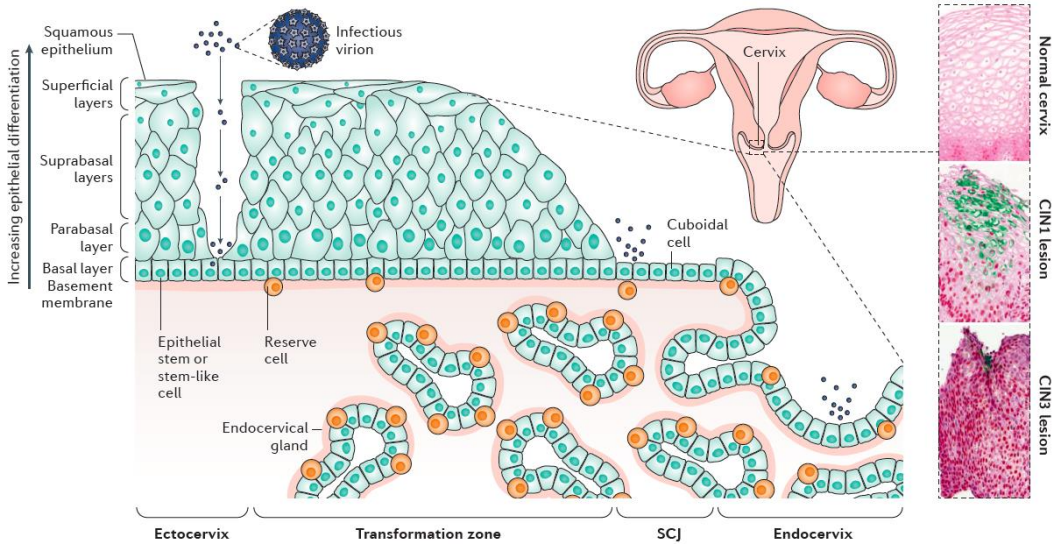

Figure 10: Viral infection zones of cervical cancer

Transformation zone of HPV infection, including immunohistochemistry images of a normal cervix with a CIN-1 and CIN-3 clinical lesion. CIN stands for Cervical intraepithelial neoplasia and is an abnormal growth of the squamous cells of the cervix (Schiffman, et al., 2016).

abnormal cells in a precancerous state. This precancerous state, called dysplasia, can easily be detected by the Pap smear and infected cells or tissues can be removed before the development of cervical cancer. Regular screening is recommended because there are no typical symptoms regarding cervical cancer. Possible symptoms of a cervix carcinoma could be bleeding between menstrual cycles, pelvic pain or a pain during sexual activity, but these do not implicitly indicate cervical cancer. Some factors like smoking, lack of nutrients, immune deficiency or inherited predisposition of cervical cancer in family history can increase cervical cancer development, but does not induce cancer formation. An infection with a high-risk HPV is necessary to induce cancer of the cervix. Both cell types of the cervix, the squamous epithelial cells (SEC) and the columnar epithelial cells, can be infected by HPV, but many cervical cancers arise from the squamous epithelial cells. The transformation zone in the cervix is the main target of HPV infection. The transformation zone is a location where the squamous cells meet up with columnar epithelial cells. Samples for the Pap smear are collected from this region (V. Spencer, 2007).

As shown in Figure 11 of the cervical cancer ranking, considering women of all ages, about 85% of the cases occur in developing regions, which corresponds to 13% of cancer in women (Borruto & De Ridder, 2012).

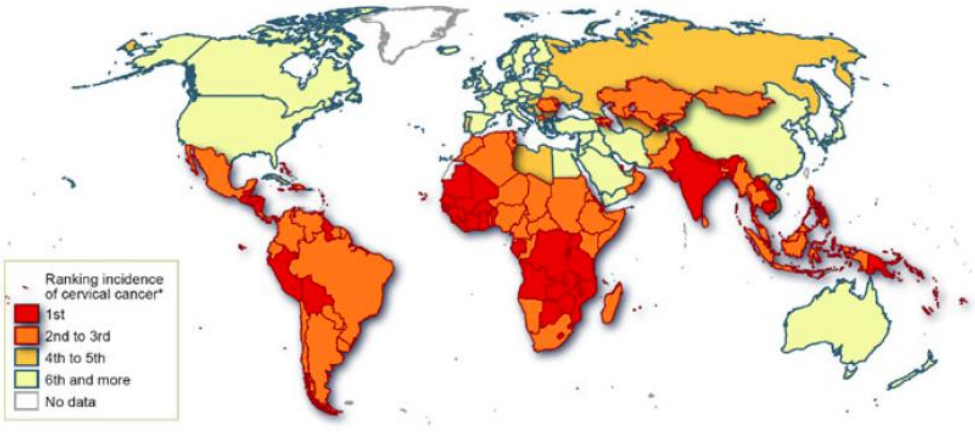

Figure 11: Cervical cancer ranking

Overview of the cervical cancer ranking including women of all ages. Developing countries in Africa and South Amerika indicate a high level of cervical cancer development, whereas a cervix carcinoma is placed on the 6th place of the cancer ranking in the US, Europe, Australia and China (Borruto & De Ridder, 2012).

Oral infections

Normally, a tumor initiator like tobacco and tumor promotors such as alcohol are the common risk factors for head and neck cancer, which make up to 75% of all cases worldwide and 50% of the cases in the United States. All risk factors, which can lead to oral cancer development, are not completely understood. Whereas a 43% abundance rate of HPV related cervical cancer exists in the states, oral HPV occurrence is less than 10%. However, immune-depressed people, smokers, older people and persons with a high change of sex partners have a higher risk of getting oral cancer. This means, there is a 3-fold increase of cancer development by tobacco users compared to nonsmokers. Nevertheless, HR-HPV16, one of the major causes of oropharyngeal cancer, has shown traits to create malign tumors at the oropharynx. Smoking, which is generally known as an immune system suppressor, can increase the rate of a viral infection and boost a possible cancer development of the cervix, as well as an oral HPV infection. HIV positive subjects also show a higher prevalence to get an oral infection by the human papillomavirus. Therefore, it´s likely that a down regulation of the immune system increases the susceptibility to HPV. Oropharyngeal cancer caused by the high-risk subset of human papillomavirus differs from the HPV negative cancers in epidemiological, molecular and clinical characteristics. These HPV related tumors emerge from the tongue or the tonsils as seen in Figure 12. Compared with a total number of 85.000 cases of oropharyngeal cancer in the year 2008, 22.000 cases are the result of a HPV infection.

Based on the reduction of tobacco consumption, an overall reduction of head and neck cancer could be noted, but oropharyngeal cancer is increasing in the USA. This increase of HPV related infection is likely based on a change of sexual activity of the population in the last decades. A study highlighted that young persons between 18 and 24 years have a low rate of oral cancer development with 3%. In contrast, people aged 55 to 74 years are hit by up to 6%. Genital infections are caused by HPV transmission due a sexual act, but oral infection remains unclear.

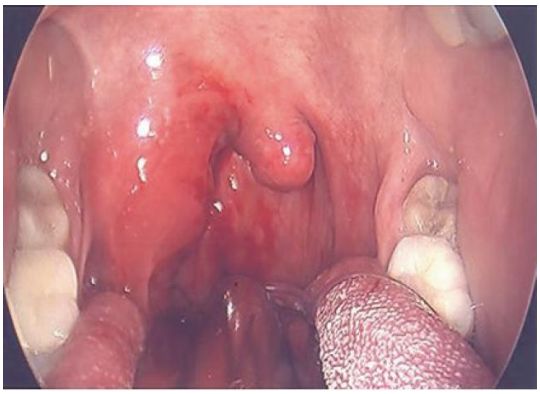

Figure 12: Oral HPV infection

Picture of the oropharynx with a tonsil tumor on the right side (Miller & Stack, 2015).

A study shows that patients with genital HPV infection have a higher prevalence to get an oral infection, potentially by another high-risk HPV type. Up to 71% of the tested women with an oropharynx infection also have a genital infection but only 10% of a cervix infection show an oral infection by HPV (Fakhry & D'Souza, 2015).

Human papillomavirus detection

Genital warts caused by the low-risk group of human papillomavirus do not require any additional screening methods. Internal infection mediated by HPV requires ancillary screening methods, because eye-based detection is not sufficient (Bonnez, 2009).

By the year 1943, G. Papanicolaou had revolutionized HPV screening by the introduction of the Pap smear. Cervical cancer as well as precancerous lesions could be detected with this screening of cervix samples, leading to a 75% decrease of cervical cancer, and leading to the implementation of Pap smear as the standard method for HPV screening (Smith, 2013).

More than 30 different types of oncogenic HPV strains are known, which lead to approximately 70% of all cervical cancers. After HPV infection, viral DNA is inserted into the host DNA, to express both oncogenes E6 and E7, leading to malignant transformation and dysplasia of the infected cells. About 50% of the women show signs of disease four years after sexual act, of which 15% show a possible dysplasia. This means, that HPV infection is preconditioned but not enough for malignant transformation. Therefore, HPV DNA testing becomes a significant topic. In Table 3, you can see the recommended screening schedule for HPV related cervical cancer in women between the age of 21 to 65 (Smith, 2013).

Cancer development slows over the years; therefore, screening for cervical cancer is not necessary for women younger than 21. It will not influence mortality of infected subjects. Women between 21 to 65 years old should attend HPV screening with a Pap smear cytology every 3 years. A co-testing (HPV testing) is recommended for women between 30 to 65 years (Smith, 2013).

Table 3: Screening recommendation for cervical cancer by the US Preventive Service Task Force (UPSTF) (Smith, 2013)

Population	Women ages 21 to 65	Women ages 30 to 65	Women younger than age 21	Women older than age 65 who have had adequate prior screening and are not high risk	Women after hysterectomy with removal of the cervix and with no history of high-grade precancer or cervical cancer	Women younger than age 30
Recommendation	Screen with cytology (Pap smear) every 3 years. Grade: A	Screen with cytology every 3 years or co-testing (cytology/HPV testing) every 5 years Grade: A	Do not screen. Grade: D	Do not screen. Grade: D	Do not screen. Grade: D	Do not screen with HPV testing (alone or with cytology) Grade: D

Cytology is the most widely used method for detecting HPV infections. During cytology, cells of a potential infected tissue are evaluated under a microscope, but this screening shows lower sensitivity than newly introduced HPV testing. Therefore, it requires a shorter schedule of screenings. HPV mRNA testing is more sensitive, but is not robustly proven at this time (Schiffman, et al., 2016).

Colposcopy

Colposcopy is a method for a visual triage (Schiffman, et al., 2016). This screening method uses magnifying optics of the cervix, for the determination of abnormal differentiations. The use of acetic acid (3-5%) causes whitening of the appropriate infected tissue. The more abnormalities there are, the stronger are the colorimetric changes, guiding the physician to sample biopsies. To determine the grade of cervical intraepithelial neoplasia (CIN, Figure 13), an additional screening method called “Schiller-negative” could be used. In this method, Lugol´s solution is used to stain cells.

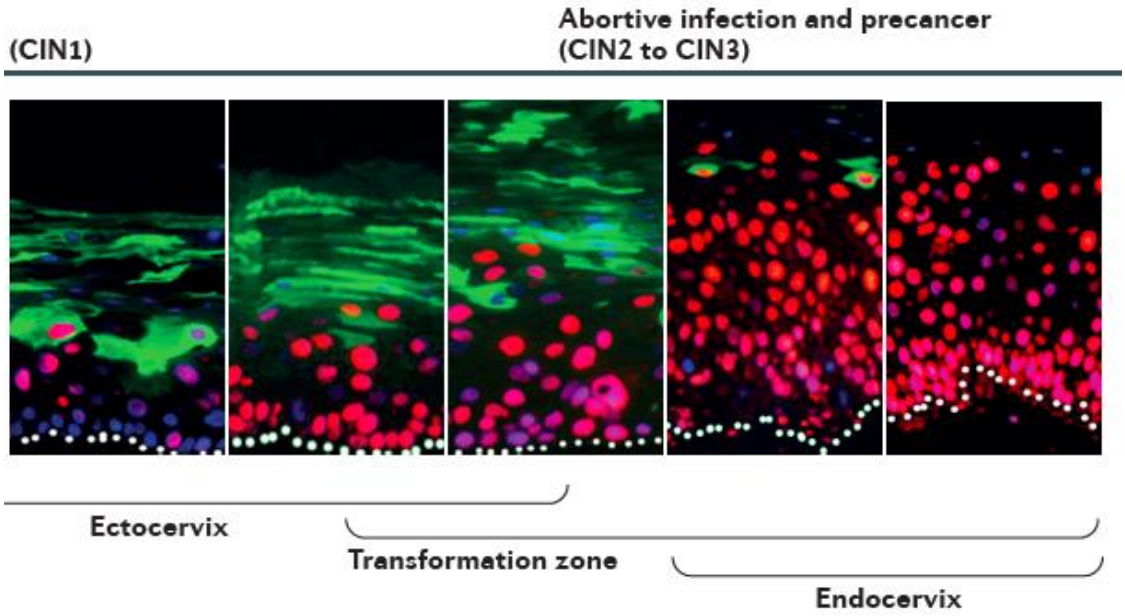

Figure 13: Immunofluorescence imaging of a cervix HPV Infection

Green fluorescent signals the E4 protein and the two late viral proteins L1 and L2, whereas the two oncogenes E6 and E7 are stained red during immunofluorescence. During cervical intraepithelial neoplasia grade 1 (CIN1), there is a base level expression of E6 and E7, required for genome amplification. During CIN2 or CIN3 there is an overexpression of both oncogenes, indicating precancerous state (Schiffman, et al., 2016).

Normal cells show a brown color, whereas infected carcinomatous cells are “Schiller-negative” (Figure 14). Cervical intraepithelial neoplasia can be classified into 3 grades according the color changes shown in table 3 (Bonnez, 2009). With colposcopy, major abnormalities of the cells can be detected but to distinguish between a precancerous state and minor changes is still difficult (Schiffman, et al., 2016).

Table 4: Scoring system of CIN according "Schiller-negative" test method. The highest grade indicates the highest presumption of neoplasia (Bonnez, 2009).

	Score			
	0	I	2	3
Color	Low intensity, semitransparent, insdistinct	Shiny, gray-white, intermediate	Dull, oyster-white, gray	Yellow, necrotic, friable
Margins	Microcondylomatous, micropapillary, indistinct, feathered, flocculated, angular, jagged	Regular, symmetrical, smooth	Rolled, peeling edges, internal borders	Exophytic, nodular, ulcerated
Mosaicism, punctation	Fine	None	Coarse	

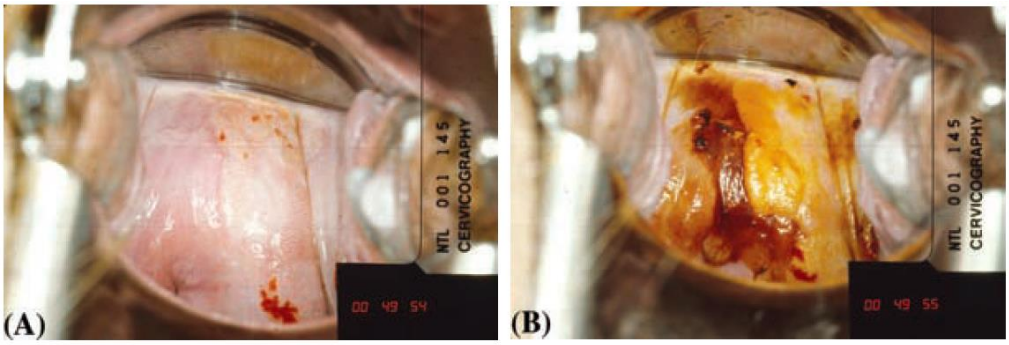

Figure 14: Schiller negative test

Screening of a vagina after 3% acetic acid treatment (A) and after Schiller´s negative test (B) (Bonnez, 2009).

According the US Food and Drug Administration (FDA), there are currently five approved HR HPV detection assays on the market (Smith, 2013). A short summary of the screening methods is shown in Table 5.

Table 5: Several high-risk HPV detection methods with sensitivity and specificity of cervical intraepithelial neoplasia grade II (CIN2), costs and whether both major oncogenic HPV types HPV16 or HPV18 could be detected (Smith, 2013)

	HC2 (SZAREWSKI ET AL, 2012)	Cervista (EINSTEIN ET AL, 2010)	Cobas (SZAREWSKI ET AL, 2012)	Aptima (STOLER ET AL, 2013)	CareHPV (QIAO ET AL, 2008)
Technique	Nucleic acid hybridization	Invader technology	PCR amplification	Target amplification assay for mRNA	Signal-amplification
Sensitivity ≥ for CIN2	96.3% (93.8-98.0)	92.8% (84.1-96.9)	95.2% (92.5-97.2)	93.3% (84.1–97.4%)	84.3% (75.8-92.8)
Specificity ≥ for CIN2	19.5% (16.7-22.6)	44.2% (41.5-46.9)	24.0 (20.9-27.2)	61.5% (58.3-64.7%)	87.5% (86.1-88.8)
Approximate Cost	$100	$50	$50	$75	$4
HPV 16/18 testing available	No	Yes	Yes	No	No

Hybrid Capture 2 (HC2)

The co-screening assay with cytology is the Hybrid Capture 2 from QUIAGEN. With this test, an infection of one of 13 high-risk human papillomaviruses (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68) is possible, but it is not possible to distinguish between the different HPV strains. The measurement is based on nucleic acid hybridization. The DNA is hybridized with the specific human papillomavirus RNA probe mentioned above. Hybrids are incubated on a microplate and incubated with antibodies containing alkaline phosphatase and a substrate. Evaluation is done with a luminometer. A relative light unit greater than 1.0 means 1 pg HPV DNA ml^{-1} and is classified as high-risk HPV positive. To compare the results of the luminometer assay you can use a PreservCyt solution containing the Pap smear. This solution can be stored up to 3 months, which allows testing of one sample with a cytological assay and the co-screening assay (Smith, 2013).

Cervista HPV HR test kit.

This detection kit by HOLOGIC can be used to analyze an infection by 14 different HR HPV types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, HPV68). Using amplification of specific nucleic acid sequences, positive results lead to fluorescence signals, which can be detected. This requires less laboratory equipment, like a PCR based detection assay. This detection kit also contains internal controls as well as a software for a better analysis of the results. As mentioned before, this kit can detect both high-risk HPV types HPV16 and HPV18, which are the major cause of cervical cancer. With a sensitivity of 68.8% and a specificity of 69.3%, this kit is not recommended for primary screening (Smith, 2013).

APTIMA HPV

This assay produced by GenProbe is used to detect the mRNA from both major oncogenes E6 and E7. This assay can be done with the PreservCyt solution. This method is based on an isothermal transcription mediated amplification, of the same 14 HPV types that the Vercista HPV HR test kit uses (Smith, 2013).

Cobas 4800 test

This HPV detection kit is currently the most used kit for HR HPV diagnostics, manufactured by Roche Molecular Diagnostics. After DNA extraction of the test sample, a real-time PCR is performed to detected HPV infections. Human β-globin is used as a reference control during the qPCR amplification reaction. A big advantage of this system is to analyze multiple HPV types and whether HPV 16 or HPV18 is present. Compared with HC2, there was an approximately 94% agreement of both test methods, but there is a decrease of the Cobas´ 4800 sensitivity after a coinfection with different HPV types (Smith, 2013).

careHPV

A new HPV detection method was designed, but at the time, it is not used in the United States. This system developed by QIAGEN is a low resource system and can detect up to 14 different high-risk HPV types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, HPV68) in only 2.5 hours. It requires no running water or electricity and a technician without special training can use the detection technology. The testing method of this cheap system can be compared with HC2, because it uses relative light units (RLU) to determine positive signals, where an RLU of 0.5 is considered a positive result (Smith, 2013).

Vaccines

Most cutaneous warts are benign; therefore, a specific therapy is not necessary. Silver nitrate or a cryotherapy application can be used to remove benign cutaneous warts for cosmetic reasons. After a treatment with salicylic acid, warts disappear after 5 days. Furthermore, a surgical intervention could be done to remove plantar warts. The only way to remove diseases around the genital area is the surgical intervention. An early detection of HPV related diseases in the nether regions is crucial to prevent metastases. Nowadays vaccination strategies are available for non-infected persons. In the year 2006, the first quadrivalent vaccine was approved in the EU. This vaccine counteracts a HPV6, HPV11, HPV16 and HPV18 infection. Another vaccine, which protects against the two high-risk HPV16 and HPV18 was approved in the year 2007. Both vaccines are based on the L1 capsid protein with specific HPV type dependent proteins and induce the production of antibodies. The vaccination should be administered before adolescence, recommended to girls between 12 and 17 years of age. After the vaccination, it is not ruled out that an infection with a high-risk HPV could happened. Therefore, a cancer check-up should be done regularly (Modrow, et al., 2010).

The bivalent (HPV16 and HPV18) and the quadrivalent vaccines (HPV6, HPV11, HPV16 and HPV18) are effective to prevent the development of a precursor of cervical cancer mediated by the high-risk strains HPV16 and HPV18. Additionally, the quadrivalent vaccine also prevents genital warts caused by HPV6 and HPV11. The vaccines had shown that they are able to prevent precursors of anal cancer, vulvar cancer and vaginal cancer. Therefore, there is a shift of a female-only vaccine to a prevention of HVP related diseases for males, too. Most vaccination programs target young persons between 9 to 12 years of age. Vaccination before sexual activity had shown the most effective strategy for HPV mediated diseases.

A long-term protection of both vaccines could be estimated up to 10 years. The quadrivalent HPV vaccine shows no breakthrough disease. The bivalent vaccines have shown a full protection (100%) up to 9.4 years.

Studies of the HPV vaccine protection against non-cervical diseases had shown, that the quadrivalent vaccine protects up to 99% of genital warts by young women between 16 to 26 years of age, whereas a protection up to 90% for young men was observed. The efficiency was achieved in non-infected situations. The effectiveness of the vaccines decreased to 62% when used in infected persons. The quadrivalent

version of the HPV vaccine highlighted a 59% to 95% prevention of an infection with anal disease correlated to HPV strains. In a Costa Rica trial, there was a 62% to 84% reduction of anal HPV16 and HPV18 infection with the bivalent vaccine. Both vaccines have no therapeutic properties, but it was shown that male and female persons have fewer recurrent diseases by HPV. The bivalent and the quadrivalent version of HPV vaccine are prophylactic and they are most effective when administered before adolescence. The European Medicines Agency (EMA) approved both vaccines for a 2-dose application. The suggested interval should be between 6 to 12 months. Studies have shown that the interval of vaccination is the most important thing. In a three-dose schedule, where the third dose was forgotten, led to an inferior immune response. The next goal was to cover more oncogene disease mediated HPV strains after the successful application of the quadrivalent vaccine. Therefore, the food and drug administration (FDA) approved a nine-valent version of HPV vaccine. This vaccine covers the four strains of the quadrivalent version and covers additionally the strains HPV31, HPV33, HPV45, HPV52 and HPV58. These five strains are responsible for 90% of a precursor of cervical cancer and for invasive cervical cancers. An application of the new vaccine to quadrivalent vaccinated patients is safe and no decreasing immune response was observed.
Originally, HPV vaccines were developed for protection against cervical cancer for young women, but in recent years HPV vaccination became common for an extended range of protection. Nowadays, the vaccines on the market are used by both sexes. A two-dose schedule with an early vaccination before sexual activity shows the best results to prevent HPV infections. Instead of a female-only concept for HPV prevention, boys and men should have also the right for personal protection, including men, which have sex with other men. A gender-neutral vaccination would improve the protection for HPV mediated diseases. Therefore, Austria, the United States of America and Australia have introduced this gender-neutral vaccination program (Pils & Joura, 2015).

In the past, vaccines were only available in high income countries and after decades vaccines were introduced on the market in the low and middle-income countries. Based on the support of the World Health Organization (WHO) and Gavi, the Vaccine Alliance, as well as the United Nations Children´s Fund, new vaccines are launched faster in low- and middle-income countries. By the year 2012, the HPV vaccine was only used in Europe, North America and Australia, but the WHO recommended a

vaccination for adolescent girls in countries where cervical cancer prevention is necessary. By the year 2016, the human papillomavirus vaccine is available in 67 countries all over the world (Figure 15), designated for adolescent girls, whereby, according to Gavi, HPV pilot demonstration projects were conducted in 23 countries. HPV vaccinations are introduced into the national immunization schedule, based on the worldwide recognition of immunization as an element of human right and health. Nevertheless, HPV vaccination is only used in 35% of the world (Loharikar, et al., 2016).

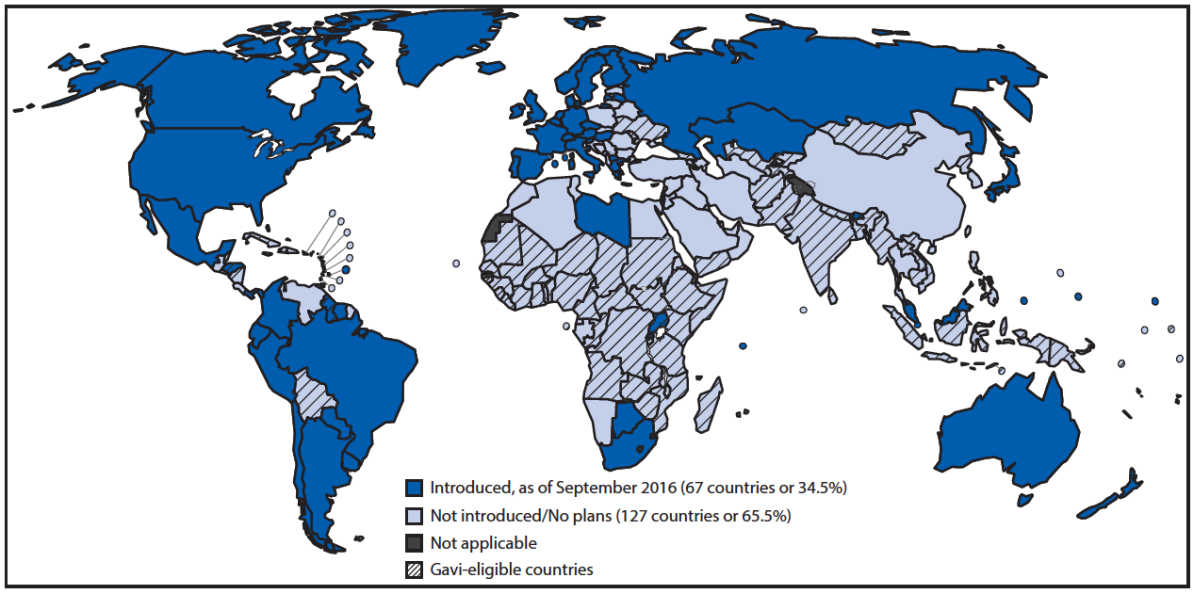

Figure 15: Map of the HPV vaccination program

Overview of countries with a current or planned HPV vaccination program. Gavi = Gavi, the Vaccine Alliance (Loharikar, et al., 2016).

Quadrivalent HPV vaccine

This vaccine targets the four HPV strains, which are responsible for approximately 90% of genital warts (low-risk HPV6 and HPV11) and about 70% of cervical cancers (High-risk HPV16 and HPV18). About 17.000 young women attended the clinical trial to approve the vaccine and the efficiency against both high-risk HPV was 97% to 100%. In the year 2006, the vaccine was approved by the FDA. The pharmaceutical company Merck launched the vaccine on the market under the name Gardasil (Pils & Joura, 2015).

Figure 16: Gardasil vaccine

A quadrivalent HPV vaccine targeting HPV6, HPV11, HPV16 and HPV18 (Greenmedinfo)

Gardasil was created using a recombinant non-infectious virus like particle (VLP). The major capsid protein L1 of the four HPV strains is produced by a separate fermentation process using a recombinant *S. cerevisiae* strain. The VLP is released from the strain by cell disruption and the protein is purified using several physical and chemical methods. To adsorb the purified proteins, a preformed aluminum containing adjuvant was used. Each sterile quadrivalent HPV vaccine contains the purified and adsorbed L1 VLPs of each HPV type with additional compounds like aluminum, sodium chloride, sodium borate and the amino acid L-histidine.

Gardasil is designed for young female persons, as well as for boys and men between 9 and 26 years of age. The vaccine should prevent the females from getting cervical, vulvar, vaginal and anal cancer caused by the two high-risk HPVs and from contracting genital warts caused by the two low-risk HPV. Gardasil also provides a protection to precancerous or dysplastic lesions like cervical intraepithelial neoplasia grade 2/3 (CIN), cervical adenocarcinoma *in situ* (AIS), cervical intraepithelial neoplasia grade 1, vulvar intraepithelial neoplasia grades 1 to 3 (VIN) and anal intraepithelial neoplasia grades 1, 2 and 3 (AIN). For the males, a vaccination with Gardasil prevents anal cancer (HPV16 and HPV18), genital warts (all four types of HPV) and anal intraepithelial neoplasia grades 1, 2 and 3 (all four types of HPV). A successful vaccination requires a three-dose application with 0.5 ml after 0, 2 and 6 months (Merck & Co.).

Bivalent HPV vaccine

The bivalent HPV vaccine is used to prevent the high-risk HPV16 and HPV18 mediated cervical cancers. In Europe, the vaccine was introduced on the market by the year 2007. Since 2009, the bivalent vaccination is also available in the United States of America. Over 19.000 females between 15 and 25 years of age joined the clinical studies to test the effectiveness of the vaccine. The study was carried out as a twice double-blinded and randomized controlled clinical study (GlaxoSmithKline).

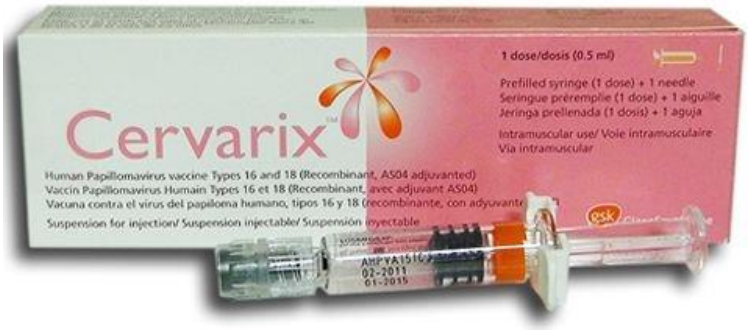

Figure 17: Cervarix

A bivalent HPV vaccine against the two high-risk strains HPV16 and HPV18 (Yibada).

Compared to the quadrivalent vaccine, a higher number of produced antibodies against the two high-risk types were observed by Cervarix (Pils & Joura, 2015). Cervarix is recommended for use in young female persons between 9 and 26 years of age, preferably to 9 year old girls. The key fact is, that girls receive the vaccine before the first sexual contact. Once the person is infected with one of the human papillomaviruses, Cervarix may not work (Anon.). The vaccine should prevent the females from contracting cervical cancer caused by the two high-risk HPV16 and HPV18. It also provides a protection to cervical intraepithelial neoplasia grade 2 (CIN), cervical adenocarcinoma in situ (AIS) and cervical intraepithelial neoplasia grade 1. A successful vaccination requires a three-dose application with 0.5 ml after 0, 1 and 6 months.

Possible side effects of Cervarix could be pain, redness and swelling at the injection site. Other unlikely effects could be headache, feeling tired and muscles aches. The bivalent vaccine contains recombinant VLPs from both HPV types and also contains additional ingredients like sodium chloride, aluminum hydroxide, sodium dihydrogen phosphate dehydrate and 3-O-desacyl-4´-monophosphoryl lipid A (GlaxoSmithKline).

Nine-valent HPV vaccine

The next milestone in HPV protection was the development of a nine-valent vaccination. This vaccine protects against the two high-risk HPV16 and HPV18, as well as the two low-risk HPV6 and HPV11, which are the same as covered by the quadrivalent vaccine.

Figure 18: Gardasil 9

A nine-valent vaccine against HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58. A vaccination protects you for diseases like cervical cancer, anal cancer and genital warts caused by the HPV (Helfand, 2015).

Additionally, a vaccination with the nine-valent vaccine also prevents diseases mediated by five other HPV strains. HPV31, HPV33, HPV45, HPV52 and HPV58 are oncogenic types, which contribute to 90% of a precursor of cervical cancer. To approve this vaccine, a clinical study was performed with 14.200 young females between 16 and 26 years of age. Based on an already available effective vaccine on the market, no placebo control group was necessary for the trial. The achieved efficiency against infection and diseases caused by the 5 additional human papillomavirus strains was >96%. Compared to the enrolled subjects aged 16 to 26 years of age, a bridge study was performed with 9 to 15 year old boys and girls. This bridge study revealed a higher antibody production in the young population. In the year 2009, the vaccine was licensed by the FDA. The pharmaceutical company Merck launched the vaccine on the market under the name Gardasil 9 (Pils & Joura, 2015). Gardasil 9 is designed for girls and women aged 9 to 26 and prevents HPV mediated diseases like, cervical, vulvar, vaginal and anal cancer (HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, HPV58), genital warts (HPV6 and HPV11), as well as precancerous or dysplastic lesions like cervical intraepithelial neoplasia grade 2/3 (CIN), cervical adenocarcinoma *in situ* (AIS), cervical intraepithelial neoplasia grade 1, vulvar intraepithelial neoplasia grades 2 to 3 (VIN), vaginal intraepithelial neoplasia (grade 2 and 3) and anal intraepithelial neoplasia grades 1, 2 and 3 (AIN). For males, a vaccination with Gardasil 9 prevents anal cancer (HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, HPV58), genital warts (HPV6 and HPV11) and anal intraepithelial neoplasia grades 1, 2 and 3. A successful vaccination requires a two-dose

application with 0.5 ml in a 0 and 6 to 12 months schedule for 9 to 14 year old recipients. If the second dose is given before 5 months after the firth dose, a third dose is required at least 4 months after the second dose. A 3-dose regimen is also possible with 0, 2 and 6 month intervals. For men and women aged 15 to 26, a three-dose treatment after 0, 2 and 6 months is scheduled (Merck & Co.).

HPV and CRISPR/Cas

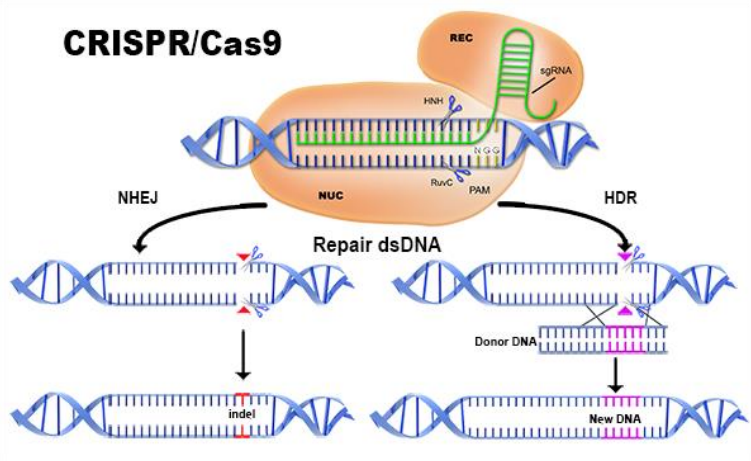

Figure 19: CRISPR/Cas9

Genome engineering by the CRISPR/Cas system using a sgRNA and the two DNA repair mechanism (AATI-US, 2015).

In the last few years, a gene editing method was developed for effective genetic modification in mammalian cells called CRISPR/Cas system (Cooper & Hausmann, 2016). This CRIPSR (clustered, regularly interspaced, short palindromic repeat) and Cas9 (CRISPR associated protein 9) system is used by prokaryotes as an adaptive immune response mechanism, against foreign nucleic acid elements, such as plasmids and viruses (Ceasar, et al., 2016; Cooper & Hausmann, 2016). The function of creating breaks at a specific DNA site, has been adapted for genome editing in mammalian cells (Ceasar, et al., 2016). This genome engineering system is classified into the type I, II and III type, but only class II requires Cas9 for DNA degradation, matching a sgRNA (single guide RNA). The nuclease Cas9 contains two nucleic acid binding grooves. The first one is called REC (recognition) and the second groove is known as NUC (nuclease), both grooves are connected by a helix bridge. Normally, Cas9 is inactive until it is activated by combining with an sgRNA, located in the REC groove (Song, et al., 2016). The CRISPR RNA recognizes a specific target sequence, followed by cleavage of the DNA by the Cas9 nuclease. The gRNA (guide RNA) directs Cas9 to the target gene (Figure 19). The nuclease could be used in two different variants. The non-homologous end joining (NHEJ) and the homology-directed repair (HDR). At NHEJ, Cas9 contains two nuclease cleavage sites and, therefore, the double stranded DNA is broken. Non-homologous recombination results in small deletions or insertions in the target gene. HDR uses one cleavage site and homologous recombination like DNA repair mechanisms, resulting in an introduction of a specific mutation in the gene (Cooper & Hausmann, 2016). A big advantage of CRISPR/Cas9 is that the system does not require a protein engineering step. This is well applicable to test multiple gRNA for the target gene, leading to a cheap molecular genome editing system usable in any biotechnology laboratory (Bortesi & Fischer , 2015).

E6 and E7 inactivation in Cervical Carcinoma cells

Recent research highlighted that the oncogenes E6 and E7 expressed by high-risk human papillomavirus could be inactivated by using the bacterial CRISPR/Cas endonuclease.

Kennedy, et al., 2014, showed in their experiments a possible inactivation of the HPV oncogenes E6 or E7 in cervical carcinoma cells resulting in their subsequent death. Both human papillomavirus genes are targeted by the Cas9 nuclease from a *S. pyrogenes* strain using a self-designed sgRNA. Figure 20 shows the designed fusion protein, including an HIV1-Rev tag at the amino terminal end acting as an epitope tag and an in-frame HPV18 target sequence such as an enhanced green fluorescent protein (eGFP) open reading frame. Two single guided RNAs were designed for targeting the N-terminus of HPV18 E6 or E7 proteins. These proteins were used for a reporter assay. Cells were analyzed by Western blotting to detect the Rev expression. eGFP positive cells were detected using flow cytometry after 3 days of transfection.

Both designed sgRNAs with a specifity to the E6 or E7 oncogenes led to a reduction of eGFP-positive cells. The transfected cells were analyzed by flow cytometry after 72 hours and the number of eGFP expressing cells and the mean fluorescence intensity (MFI) are demonstrated in Figure 21.

Using the designed sgRNAs, the expression of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21 with its chief function as a regulator of the cell cycle was induced, as shown in Figure 22.

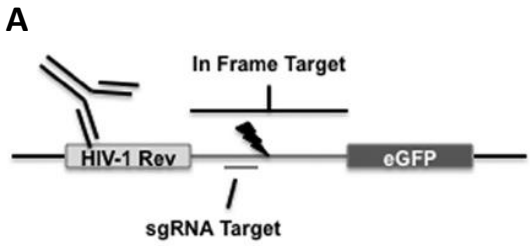

Figure 20: Fusion protein

Schematic illustration of the fusion protein, containing an N-terminal HIV1 Rev fragment, an HPV18 derived target sequence and a C-terminal eGFP open reading frame (Kennedy, et al., 2014).

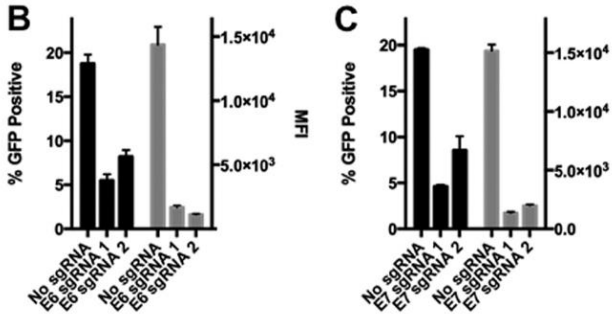

Figure 21: eGFP expression of transfected cells

Analyzing of eGFP expression of transfected cells with an sgRNA specificity to HPV18 E6 gene, a control construct and the indicator plasmid. Analysis was done by Flow cytometry (Kennedy, et al., 2014).

In addition, induction of Rb expression was achieved by the cleavage of the E7 protein by the E7 sgRNA1.

To be sure that re-expression of p53 by the sgRNA1 targeting E6 occurred exclusively by the cleavage of the HPV oncogene E6, a mutant of the gene was designed to block sgRNA mediated cleavage but did not alter the amino acid sequence of the E6 gene. As shown in Figure 23, you can see the mutant version compared to the wild type E6 oncogene. You can also see p53 expression based on the cleavage by E6 sgRNA1, whereby there is no significant tumor expression induced by the mutant version of E6 sgRNA1. This paper shows that the inactivation of the oncogenes E6 and E7 in HPV18 or HPV16 transformed cells in culture is possible by cleavage applying the CRISPR/Cas9 method. As the group expected, the inactivation of E6 leads to expression p53 and p21, while inactivation of E7 results in Rb expression. This induces cell cycle arrest followed by apoptosis of the infected cells (Kennedy, et al., 2014).

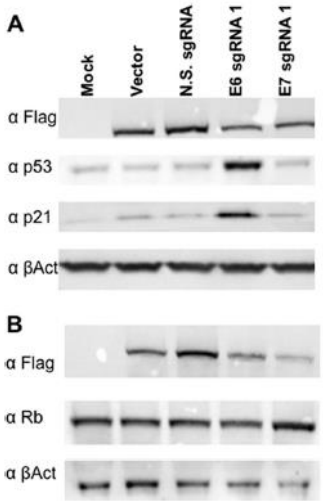

Figure 22: Western blot analysis of tumor suppressor expression

Induced tumor suppressor based on cleavage of the oncogenes by sgRNA1, analyzed with Western blotting. Lysate was probed for p21 and p53 expression. β-actin was used as a loading control. Detection of the Cas9 protein using an antibody specific for the Flag epitope. In segment B of the graph, the lysate was probed for Rb. Mock transfection HeLa cells was used as a control (Kennedy, et al., 2014).

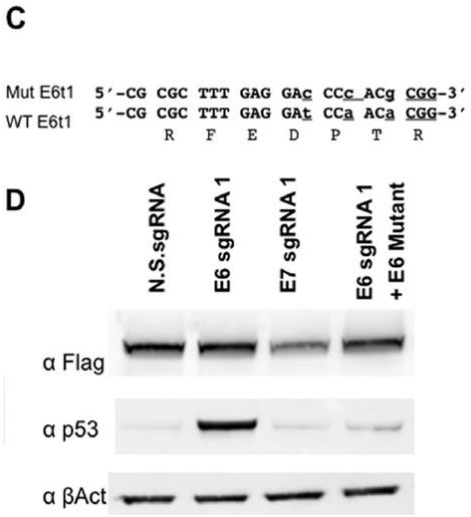

Figure 23: Western blot analysis using a mutant version of E6

A mutant version of the E6 gene was designed to show the effect of re-expression of the tumor suppressor by cleavage of the sgRNA. The shown sequence of the mutant is resistant to cleavage by the Cas9 protein. The protospacer adjacent motif (PAM) is underlined and the mutations of the sequence are in lowercase type., N.S. → non-specific (Kennedy, et al., 2014).

Growth suppression of HPV16 positive cervical cancer cells

A second research group demonstrated stable suppression of both high-risk HPV16 oncogenes E6 and E7 expression *in vitro*. Additionally, an experiment using nude mice was performed to show *in vivo* inhibition of cervical tumor growth using the CRISPR/Cas9 system.

For the *in vitro* assay, a single copy of HPV16 was integrated in the chromosome of the human cervical carcinoma cell line SiHa to express E6 and E7. A second cervical carcinoma cell line C33-A was used as a negative control.

Table 6 contains the different sets of oligonucleotides, which were designed to target the two oncogenes of HPV16. gRNA expression plasmids were created, by preparing a 100 bp double stranded DNA (dsDNA) insert fragment with a 20 bp targeting sequence and a PAM sequence. The BbsI restriction site of the gRNA cloning vector was used to insert dsDNA.

To detect expression of p53 and p21, a protein extraction was prepared and analyzed by Western blotting using mouse monoclonal anti-p53 and goat polyclonal anti-p21 primary antibodies. As an internal control the GADPH (Glycerinaldehyd-3-phosphat-Dehydrogenase) was used. After 2 days of infection, an increased expression of p53 and p21 in CRISPR/Cas9 infected SiHa cells could be detected. As shown in Figure 24 re-expression of p53 and p21 proteins occurred in the cervical carcinoma infected SiHa cells transduced by cells infected with Promotor 1 sequence (HPV1) and the HPV4 oligo-set, compared to the control. Up-regulation of p21 also occurred by using

Table 6: Sequence of the designed oligonucleotides of the gRNA to target the expressed E6 and E7 gene of the high-risk HPV16. The number of HPV in the column “Name” does not correspond to the eponymic HPV type. All sequences were designed for HPV16. The first sets of sequences (HPV1-2) are two promotor sequences, whereas the sets of HPV3-5 targets the E6 oncogene. The last two sets HPV6 and HPV7 was designed for E7 (Zhen, et al., 2014).

The sequences and location of gRNA targeting HPV16 E6 and E7.

Name	Genomic target	Target location
HPV1	CACCGACTAAGGGCGTAACCGAAAT AAACATTTCGGTTA CGCCCTTAGT	23
HPV2	CACCGGTTTCGGTTCAACCGATTT AAACAAATCGGTTG AACCGAAACC	36
HPV3	CACCGACTTTCTGGGTCGCTCCTGT AAACACAGGAGCGA CCCAGAAAGT	E6(115)
HPV4	CACCGCAACAGTTACTGCGACGTG AAACCACGTCGCAG TAACTGTTGC	E6(205)
HPV5	CACCGCTAATTAACAAATCACACAA AAACTTGTGTGATT TGTTAATTAG	E6(386)
HPV6	CACCGTCCGGTTCTGCTTGTCCAGC AAACGCTGGACAAG CAGAACCGGA	E7(682)
HPV7	CACCGACACGTAGACATTCGTACTT AAACAAGTACGAAT GTCTACGTGT	E7(777−)

Sequence design of HPV-16–CRISPR/Cas9.

CRISPR+Cas9+promotor1 and CRISPR+Cas9+E7-6.

Cell viability was tested performing a MTT colorimetric assay. Figure 25 represents the *in vitro* growth inhibition assay. *In vitro* cell viability experiments showed significant results for specific growth inhibition using the CASPR/Cas9 system. HPV16 infected SiHa cells and HPV16 negative control cells C33-A were transfected with the Promotor 1 sequence as wells with E6-4 and E7-6. An *in vivo* assay was performed by transfecting SiHa cells with CRISPR+Cas9+promoter-1 and CRISPR+Cas9+E6-4, CRISPR+Cas9+E7-6 and CRISPR+Cas9+ (promoter-1+E6-4+E7-6). As a control sample, transfected SiHa cells with CRISPR+Cas9+gRNA were used. After a 2-day cultivation, cells were inoculated into nude mice. As shown in Figure 26, there was a slower tumor formation in promoter-1+E6-4+E7-6 treated cells, compared to the control.

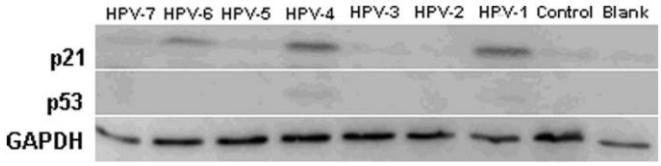

Figure 24: Western Blotting of induced p21 and p53 expression in SiHa cells

CRISPR/Cas9 method was used to target the E6 and E7 protein in HPV positive SiHa cells. Expressed proteins were labeled using the primary antibodies mouse monoclonal anti-p53 and goat polyclonal anti-p21. GAPDH was used as a control sample for protein expression. Up-regulation of p21 and p53 was determined by target the promotor1 and E6-4, as E7-6 sequence (Zhen, et al., 2014).

After the mice were sacrificed, tumors were weighed and a significant lower weight was measured in cells transfected with the promotor + E6 + E7, compared to control samples.

Results showed, that there is a stable and specific suppression of the target E6 and E7 oncogenes, as well as an enhanced re-expression of p53 and p21. Furthermore, growth inhibition of infected cervical carcinoma cells as wells as growth inhibition of tumors could be observed. These findings could provide an important application of the CRISPR/Cas9 system to target high risk human papillomavirus as a possible treatment strategy in cancer therapy (Zhen, et al., 2014).

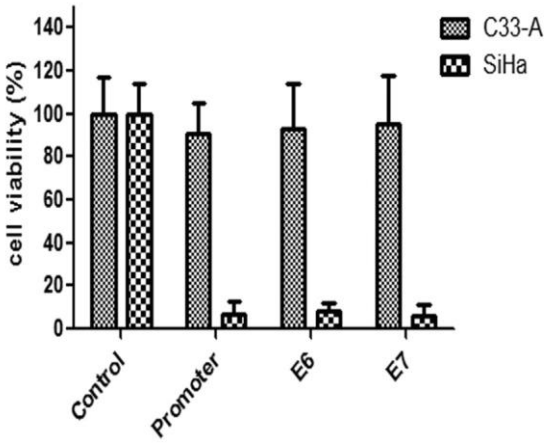

Figure 25: Determination of cellular proliferation using a MTT colorimetric assay

SiHa (HPV16 positive) and C33-A cells (HPV16 negative) were transfected with HPV16 promotor, E6 and E7. Control sample was transfected with gRNA empty and hCas9 plasmid (Zhen, et al., 2014).

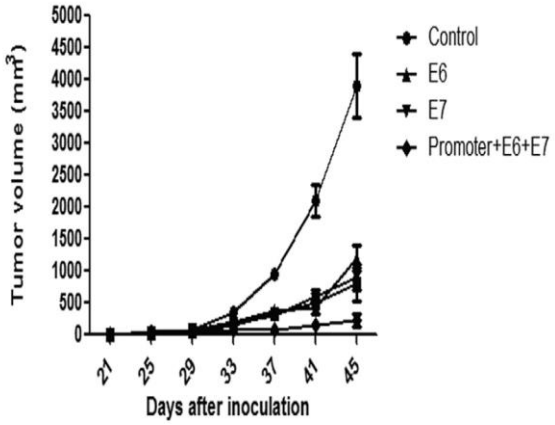

Figure 26: *In vivo* assay to suppress tumor volume in infected nude mice

SiHa cells were treated with CRISPR+Cas9+promoter-1, CRISPR+Cas9+E6-4, CRISPR+Cas9+E7-6 and CRISPR+Cas9+ (promoter-1+E6-4+E7-6) and infected to nude mice (Zhen, et al., 2014).

Disruption of HPV16 E6 gene by CRISPR/Cas9

Another study shows a potential strategy for cervical cancer treatment using CRISPR/Cas9. In Figure 27, you can see several gRNA (guidance RNAs) constructs targeting the E6 oncogene, which leads to a double stranded break of the DNA by the Cas9 enzyme. Disruption of the gene occurs by the non-homologous end joining repair pathway of the DNA break. In Table 7, the gRNAs and PAM sequences are represented. After inactivation of E6, cellular apoptosis and growth inhibition is induced.

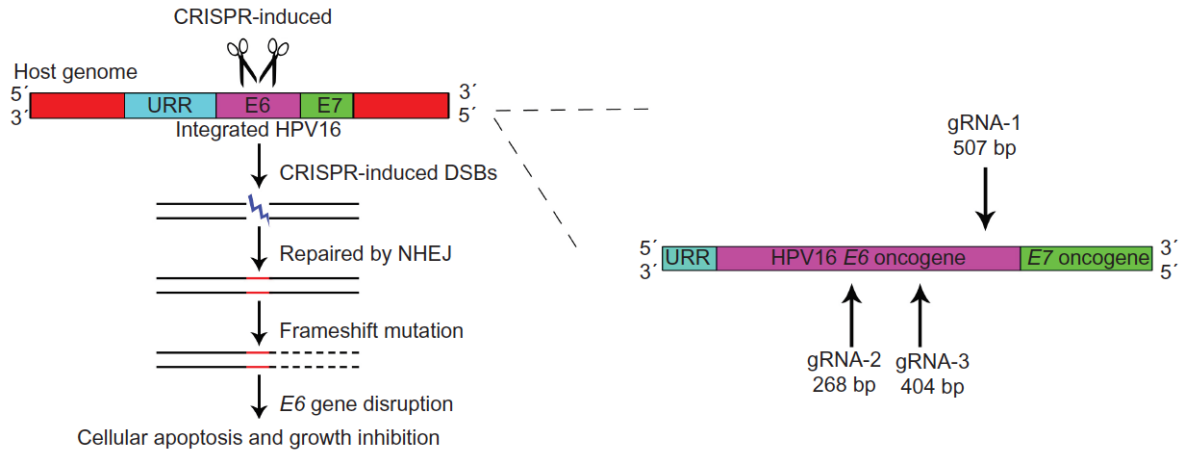

Figure 27: Visualization of CRISPR- induced DSB and E6 gene disruption

Designed gRNA targets and disrupts the HPV16 E6 gene by NHEJ. The coordinates of the three gRNAs are referred to the double-strand break (Yu, et al., 2014).

To test the effectiveness of the gRNAs in different assays, 4 cell lines were used. The HEK293 cell line was used for a single-strand annealing luciferase reporter assay and SiHa and CaSki cell lines were used for the T7 endonuclease I assay. The single-strand annealing luciferase reporter assay showed, that 2 of the created gRNAs show an activity, which means that the gRNA could target the E6 gene and induced a double-strand break (Figure 28). To detect the double strand breaks, a luciferase gene was transfected into the cells, and the active form of the luciferase gene was created during homologous recombination. The third designed gRNA shows no significant activity. A control group was also prepared with Renilla luciferase to measure the cytotoxicity of the

Table 7: Sequences of the three designed gRNAs with the corresponding PAM sequence (Yu, et al., 2014).

Name	gRNA sequence (5′-3′)	PAM sequence (5′-3′)
gRNA-1	gtcgatgtatgtcttgttgc	CCG
gRNA-2	ttatgcatagtatatagaga	TGG
gRNA-3	cgttgtgtgatttgttaatt	AGG

gRNAs, but no significant cytotoxicity was measured. A confirmation of the gRNAs´ activity was shown performing a T7 endonuclease I assay. SiHa cells and CaSki cells were transfected with each gRNA/Cas9 plasmid and after performing a PCR, cell lines were treated with T7E1. Figure 28 (C and D) illustrate that gRNA2 and gRNA3 are able to cleave the plasmid, where gRNA1 remains still uncleaved. For analysis of a possible apoptosis resulting from the designed gRNAs, cells were stained with propidium iodide and fluorescein isothiocyanate conjugated annexin V (FITC) for a flow cytometry analysis. Induced apoptosis is shown in Figure 29 (A – D). Used cervical cancer cell lines showed high apoptosis rates after treatment with gRNA2 and gRNA3. Corresponding apoptosis rates are shown in Figure 29 (E and F) (Yu, et al., 2014).

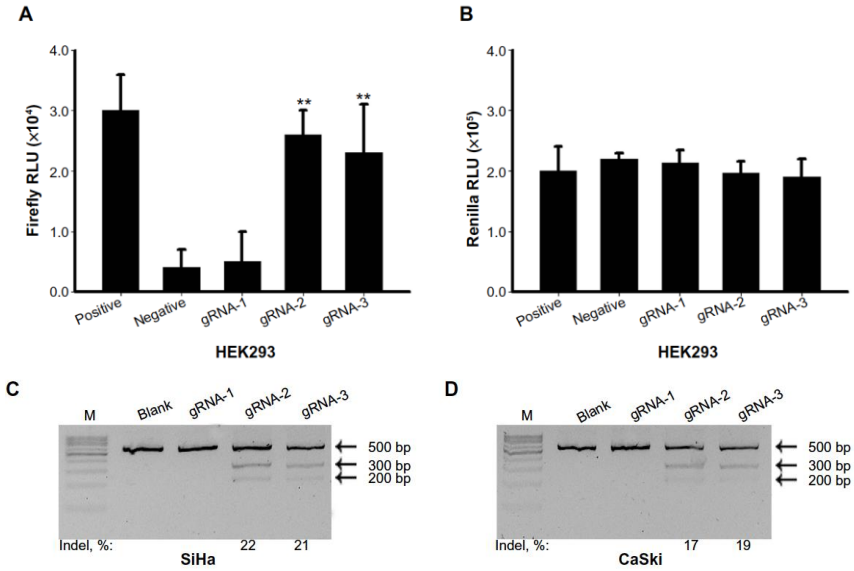

Figure 28: Results of effectiveness of the constructed gRNAs in HEK293 cervical carcinoma cell line

(A): Cleavage of the double-strand DNA leads to form an active luciferase gene by homologous repair. Using a luminometer, the activity of the luciferase was measured. gRNA2 and gRNA3 showed a high effectivity, whereas gRNA had no activity compared to a negative control (only Cas9 plasmid). (B): Renilla luciferase was tested too as a control group for cytotoxicity of the gRNA. gRNAs showed no relevant cytotoxicity. For the positive control, cells were transfected with the luciferase gene and two GATA zinc fingers. (C and D): Western blot analysis of the E6 gene cleavage mediated by CRISPR gRNA/Cas9 in SiHa cells (C) and CaSki cell line(D). gRNA2 and gRNA3 demonstrated a 200bp and 300 bp cleavage products, whereby no cleavage product by gRNA1 was visible. A 100 bp DNA marker (M) was used to compare the size of the products. Cells treated with only Cas9 were used as blank (Yu, et al., 2014).

The results of these experiments show again, that the CRISPR/Cas9 system, could be a possible treatment option for infected patients with high risk human papillomavirus possibly causing cervical cancer. With this technique, it is possible to target the E6 oncogene, induce a double-strand break of the DNA and disrupt the oncogene. This leads to re-expression of the tumor suppressor p53 resulting in cell apoptosis.

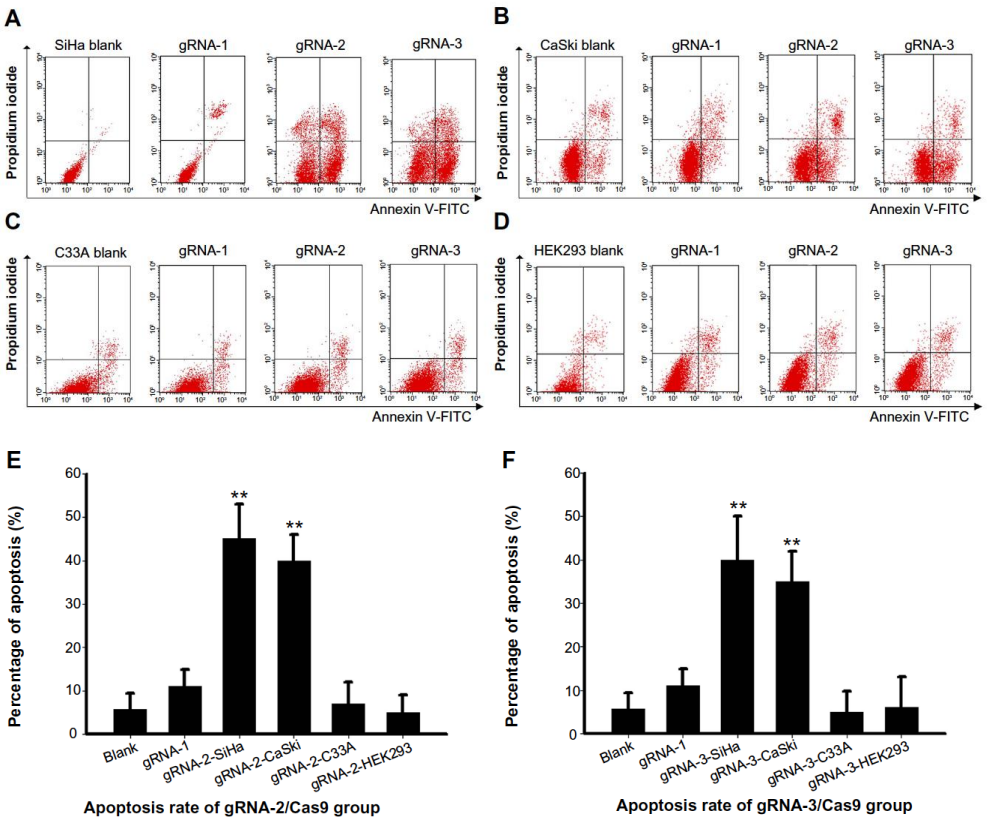

Figure 29: Analysis of gRNA mediated apoptosis

(A-D): Flow cytometry analysis of SiHa (A), CaSki (B), C33a (C) and HEK293 (D) cells stained with annexin V – FITC and propidium iodide. Increased apoptosis of transfected CaSki and SiHa cells with gRNA2 and gRNA3 was measured. No significant induced apoptosis was observed in HEK293 and C33a cells. Treated cells with only Cas were used as blank. No significant induced apoptosis was measured by gRNA1 (Yu, et al., 2014).

Closing words

Human papillomaviruses are one of the most transmittable infection after sexual act. The high-risk type of HPV can cause cervical cancer and every year 250.000 subjects die in consequence of an infection by HPV. At the time, there are 3 different vaccines on the market, which prevent you for the most common causer of a cervix carcinoma or genital warts. Young girls and boys should get the vaccine before their first sexual act, because the vaccine can only protect you for an infection by HPV, but it shows no therapeutical properties after an infection by the human papillomavirus. With the Pap smear method, cervical cancer decreased up to 75%, nevertheless current research focus on nucleic acid based detection methods and on new HPV treatments. The latest genetic modification method CRISPR/Cas9 shows promising opportunity of a new and effective treatment of HPV infected cells.

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