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Development of Vaccines and Gene Therapy Against HPV Infection and Cervical Cancer

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1. Introduction

From the establishment of etiologic relationship between HPV and cervical cancer, researchers have emphasized the importance of prevention by education of people, especially teenagers. This virus is associated with diseases of the cutaneous and mucosal human epithelia, including benign warts and invasive cancer that affect different anatomical regions as skin, cervix, vagina, vulva, anus, penis, head and neck. Controversial evidence suggests a relationship between HPV infection and breast cancer (de Villiers et al., 2005; Khan et al., 2005; Heng et al., 2009) (Table 1).

Clinical manifestation	HPV types often detectable
Verrucae vulgares, verrucae palmares et plantares	1, 2, 4
Verrucae planare	3, 10
Butcher´s warts	7
Squamous cell carcinoma of the finger, Bowen´s disease	16
Epidermodysplasia verruciformis (EV)	3, 5, 8
EV- squamous cell carcinoma	5, 8
Condylomata acuminata	6, 11
High grade-squamous intraepithelial neoplasias and invasive carcinomas of the anogenital tract	16
Bowenoid papulosis, erythroplasia of Queyrat	16
Buschke-Lowenstein tumor	6, 11
Respiratory Papillomatosis	6, 11
Heck´s disease	13, 32
Head and neck cancers (larynx, tonsils, tongue, sinuse, lung)	16, 18, 6, 11
Breast cancer?	16, 18

Table 1. Clinical manifestations and associated HPV types (taken and modified of Handisurya et al., 2009).

Noted that a variety of diseases caused by HPV is largely due to viral tropism, i.e., the preference of a certain type of HPV by a tissue or cell group in particular, depending on factors of virus and host, mainly receptors, transcriptional activators, enhancers, and tissue-specific promoters (Graham, 2010). Based on the tropism, HPV has been classified into two main phylogenetic genera, the α-HPV and the β-HPV, which correspond to the mucosal and cutaneous infective HPV, respectively (de Villiers et al., 2004). The temporal organization of the virus replication cycle is also different between different HPV types perhaps reflecting differences in sites of infection and transmission modes.

Traditionally, cancer and associated lesions have been treated with surgery, radiotherapy and chemotherapy, these treatments produced widely known adverse effects (American Cancer Society, 2007; Instituto Nacional del Cáncer, 2008). However, many efforts have been done in order to find effective preventive and curative options less invasive and with minimal or no side effects. These options are mainly based on molecular biology techniques to develop vaccines and the use of molecules that stimulate the immune and cytotoxic response against HPV infection and cervical cancer. The development of these vaccines and therapeutic procedures is based on *in vitro* culture and knowledge of the life cycle, genome and regulation of viral transcription, which has allowed the identification of potential targets to control genes expression in infectious and / or neoplastic processes (Table 2).

Genome region	Gen/ protein	Expression site	Function
Early	E1	Basal, parabasal and intermediate cells of the host	ATP- dependent DNA helicase; unique enzyme expressed by the virus, which is essential for viral replication
	E2		Helps E1 to locate the origin of replication in LCR, cell cycle and apoptosis regulation
	E4		Cell Cycle arrest, virion assembly, remodels cytokeratin network
	E5		Control of cell growth and differentiation, immune modulation
	E6		Inhibits apoptosis and differentiation
	E7		Cell cycle control, controls centrosome duplication
Late	L1	Superficial cells	Major capsid protein
	L2		Minor capsid protein, recruits L1, virus assembly
Long control region (LCR)			Binds many cellular transcription activators, confers keratinocyte specificity to transcription

Table 2. Role of HPV genome regions

2. Prophylactic vaccines to prevent HPV infection

Prophylactic vaccines currently exist to prevent the spread of HPV infections; these vaccines have the objective to create antigens capable to induce neutralizing antibodies that prevent the entry of virus into host cells. They are based on preventing infection of HPV types most prevalent around the world: types 6 and 11, of low oncogenic risk, associated with the

formation of warts and benign condylomata, and types 16 and 18 of high cancer risk, associated with cancerous and precancerous lesions, which are responsible for approximately 70% of all cervical cancers worldwide (Muñoz et al., 2004).Initially it was suggested to produce a vaccine based on attenuated virus, but its implementation and evaluation in humans was a very high risk due to the presence of oncogenic viral DNA; in addition the growth of virus in *in vitro* culture had been limited until recently when a researchers group managed to establish a reproducible and highly efficient production of HPV type 18 in human keratinocytes, which has a potential value for establishing research models of *ex vivo* viral expression (Castellsagué et al., 2006; Wang et al., 2009).

Moreover, although the studies on the immunology of HPV have shown antibodies against many different viral products, the best characterized and most type-specific antibodies are those directed against conformational epitopes of the L1 capsid protein. In the 90's it was possible to produce *in vitro*, genetically engineered virus-like particles or VLPs, which consist of L1 or L1 + L2 recombinant protein, obtained by introducing one or both genes, respectively, in cultures of eukaryotic cells (yeast, insect or bacteria). These recombinant proteins have the ability to self-assemble to form three-dimensional structures that are morphological and antigenically identical to the original HPV virions, but not containing the viral genome, so these structures can not replicate or cause infection or cancer (Muñoz et al., 2008).

There have been studies in experimental animal models and humans, where there was a good tolerance to systemic vaccination with L1- VLPs, and induction of serum antibody titers of at least 40 times higher than the titles produced in an natural infection (Lowy & Frazer, 2003). The first large multicenter, double-blind study, with phase III results, was published in 2002, on a monovalent vaccine developed by Merck Laboratories HPV type 16 (Brull & Carrera, 2005). This company created another quadrivalent vaccine called Gardasil, synthesized in the yeast *Saccharomyces cerevisiae*, based on L1- VLPs of oncogenic types most commonly found in cervical dysplasia (HPV types 16 and 18) and non-oncogenic types responsible for approximately 90% of warts genitals and recurrent respiratory papillomatosis (types 6 and 11), which is considered to act on two different hyperproliferative diseases (Schiller & Lowy, 2006). It was approved by the U.S. Food and Drug Administration (FDA) in 2006 and is administered in 3 doses, spread over 6 months (0, 2 and 6 months). Follow-up studies for 3 ½ years after vaccination showed an effectiveness of 94% in persistent infection with HPV types 16, as well as 100% in preventing high-grade intraepithelial lesions associated with types 16 and 18, and prevention of genital lesions related to HPV types 6 and 11 (Mao et al., 2006; Villa et al., 2005). The efficacy against vulvar and vaginal neoplasia grade II and III was 72-100% (Joura et al., 2007). In well designed clinical trials in young women aged 15-25 years who were HPV 16/18 seronegative and DNA negative to 14 HPV high-risk types, high levels of immunogenicity and protection were sustained for follow-up periods of up to 8.4 years (McKeage & Romanowski, 2011).

Each 0.5-mL dose contains 20 μg HPV 6 L1 protein, 40 μg HPV 11 L1 protein, 40 μg HPV 16 L1 protein, and 20 μg HPV 18 L1 protein. VLPs are adsorbed on an aluminum-containing adjuvant. Each 0.5-mL dose contains 225 μg amorphous aluminum hydroxyphosphate sulfate. The formulation also includes sodium chloride, L-histidine, polysorbate 80, sodium borate, and water for injection. The quadrivalent HPV vaccine contains no thimerosal or antibiotics. The vaccine should be stored at 2°C--8°C (36°F--46°F) and not frozen.

GlaxoSmithKline laboratories developed a bivalent vaccine called Cervarix for HPV types 16 and 18, produced in insect cells with baculovirus as expression system, which is also administered in three doses (0, 1 and 6 months). Studies reported 100% efficacy in preventing infections with involved HPV types, with an almost absolute immunogenicity for 4 ½ years after vaccination and the detection of antibody titers from 16 to 26 times higher than detected after natural infection (McKeage & Romanowski, 2011; Bhatla et al., 2010; Muñoz et al., 2008; Harper et al., 2004; Kahn, 2005).

Both vaccines (Gardasil and Cervarix) use aluminum-based adjuvants, which reduces the dose required to induce antibodies peak titer and helps to stabilize the vaccine during storage (Schiller & Lowy, 2006). Overall adverse effects reported in vaccination protocols are limited and include reactions at the injection site (erythema, pain and swelling) and systemic adverse effects (headache, fever and nausea) which were of middle nature, transitory and common for individuals receiving the vaccine or placebo (Paavonen et al., 2007).

Whereas the onset of sexual activity during adolescence, the FDA approved the vaccine Gardasil for girls and women aged between 9 to 26 years, while the Advisory Committee on Immunization Practices of the USA Center for Disease Control and Prevention (ACIP) recommended vaccination in females aged between 11 to 26 years and added males to the population who can benefit from Gardasil vaccination (FDA, 2010).

There has been controversy regarding the vaccination of women alone or the inclusion of men. In this regard, it has been noted that vaccinating girls aged 12 years old, can reduce cases of cervical cancer associated with types 16 and HPV 18 in about 95% and the introduction of vaccination in children would increase this figure three points (Taira et al., 2004). We must also take into account the potential role of vector that man can exert on the transmission of HPV infection, so the inclusion of the male population in vaccination programs could contribute to optimal control of transmission (Tirado-Gómez et al., 2005).

Currently the vaccine is not recommended for pregnant women. The long-term effects of the vaccine on fertility are not known, but no effects are anticipated. Although studies on the toxicity to the fetus are inconclusive, FDA has classified the HPV vaccine as a pregnancy Category B medication, meaning that the medication does not appear to cause harm to the fetus in animal studies. Initially, the trials for Gardasil and Cervarix excluded pregnant women. Pregnancy was determined by a sensitive human chorionic gonadotropin (HCG) test on the day of expected vaccination. However, some women became pregnant during the few weeks or months following the receipt of a vaccine or placebo injection. Overall, the proportions of pregnancies with an adverse outcome were comparable in subjects who received Gardasil and subjects who received placebo. However, the clinical trials had a relatively small sample size. Though receiving the HPV vaccine while pregnant is generally considered safe, it is still recommended to wait until after pregnancy to get the vaccine (American Cancer Society, 2008).

Vaccination at early age has led to concerns of parents, researchers and health specialists because of the need to talk with children about sexuality and sexually transmitted diseases, the vaccination charges received by children, the possibility that immunization may lead to the adoption of risky sexual behaviors and concerns about the safety of vaccines (Kahn,

2005). Some researchers have pointed out as risk of vaccination the possible selection of not included HPV types in vaccines or the increase in the prevalence of rare oncogenic HPV types, which can only be known over time, after the mass vaccination had been effective.

Other weaknesses of the prophylactic vaccine against HPV is that it only protects against specific HPV types, leaving out a wide range of viral types, although rare, are also present, and have also been reported conflicting results regarding the existence of cross protection. In addition, the vaccine is preventive (not cure existing infections or injuries) and there is a low percentage of cervical cancer and precursor lesions in which no association has been established with HPV, existing other factors associated with the development of this malignancy, as exposure to mutagens, genetic susceptibility, hormonal status and immune status (Tirado-Gómez et al., 2005; del Amo et al., 2005); unknown the duration of protection provided by the vaccine, it may be necessary to administer a booster dose (Brull & Carrera, 2005; Diestro Tejeda et al., 2007). In this regard, an evaluation showed that the quadrivalent vaccine provides strong and sustained protection against condiloma and vulvovaginal and cervical low grade neoplasia, related to types 6, 11, 16 and 18, for more than four years. The same study suggests that the effectiveness of the vaccine might be less in cases where coinfection with HPV types absent in the vaccine is present (Dillner, 2010).

It is important to take into account the high cost of the vaccines listed, however, this is not comparable to the loss of life or money spending for the treatments of lesions associated with HPV infection. Since vaccines are prophylactic and only provide preventive (no therapeutic) effect, most benefits are gained by vaccinating before infection occurs, ideally before the onset of sexual intercourse. In this sense, the ACIP has recommended the introduction of HPV vaccine in national immunization programs of governments worldwide. It is therefore essential to determine the HPV types circulating in each region and assess the potential impact of preventive vaccination in the respective populations.

Various researchers have emphasized that even with the globalization of preventive vaccination, screening schemes should continue due to multifactorial nature of cervical cancer, only 2 of 15 oncogenic HPV types are included in the vaccine and vaccination programs initially cover women into a limited range of ages, anticipating that at least during 2-3 decades unvaccinated sexually active women remain at risk for the disease (Giuliano, 2007; Stanley, 2008; Hutchinson & Klein, 2008).

The efficacy of L2 vaccination has been proved in pre-clinical and clinical studies. Natural infection does not induce anti-L2 antibodies and many L2 epitopes are not on the virus surface, but during the infection cellular protease furin removes an L2 N-terminal sequence rendering L2 accessible on the capsid surface and displaying the L2-neutralizing epitopes. The L2-neutralizing epitope was inserted on the surfaces of VLPs increasing the titers of neutralizing antibodies approximately 10-fold. A synthetic L2 lipopeptide with concatenated multitype L2 fusion proteins from different papillomavirus types have already been utilized in inducing cross-neutralizing antibodies against several clinically relevant HPV types. This polymeric L2 approach gives rise to antisera, that neutralize at higher titers, not only the types included in the multimeric immunogen but also other types. So, immunization against L2 could be a candidate prophylactic pan-human papillomaviruses vaccine (Alphs et al., 2008; Jagu et al., 2009).

3. Therapeutic vaccines and gene therapy for treatment of cervical cancer

Another line in the study of cervical cancer treatment has pursued in the implementation of therapeutic vaccines and / or gene therapy to cure existing cases, through the transfer of DNA, inserting the normal gene or gene expression regulation.

Therapeutic vaccines are composed of peptides homologous to the viral proteins, as indicated in the treatment of dysplasia and invasive cervical cancer or as adjuvant therapy for locally recurrent or metastatic (Diestro Tejada et al., 2007). DNA vaccines have also been developed, which are considered as stable, safe, can be prepared in large quantities and repeatedly administered without significant adverse effects. In addition, the DNA tends to be preserved in the receptor cells, ensuring long-term expression of the encoded antigen and reinforcing the maintenance of immunological memory.

Generally, the immune response generated from DNA alone is weak, so it has been tested the adjuvant effect of several molecules, and the combination of specific genes (Kim et al., 2004). However, vaccines have been developed based on naked DNA, viral or bacterial vector, tumor cells and dendritic modified cells.

Other molecules in use for gene therapy of cervical cancer are cytokines which has immunoregulatory effect that promotes maturation, activation and migration of effector cells of the immune response to the tumor site. Of particular interest are interferons, interleukin 2 or IL-2 (which activates T cells, NK cells, macrophages and the release of other cytokines) and the aforementioned IL-12 (whose anti-tumor effect individually or in combination with *E6* and *E7* is dependent on the activation of CD8 + cytotoxic T lymphocytes and NK cells at the site of immunization). Intratumoral administration of these molecules significantly reduced the progression of HPV-associated tumors and inhibited recurrent tumor formation after being removed by surgery (Frechtel, 2005; Bubenik et al., 2003).

In 1999, a group of researchers developed and tested the vaccine TA-GW based on the L2 and E7 fusion proteins of HPV type 6, with Alhydrogel as adjuvant, for the treatment of condilomas, reporting immunogenicity and cure in approximately 62% of lesions 8 weeks after vaccination, without considerables adverse effects (Lacey et al., 1999).

In 2002, Kaufmann et al. performed one of the first multicenter and multinational studies with a recombinant vaccine, TA-HPV, consisting of attenuated vaccinia virus genetically engineered to express the E6 and E7 proteins of HPV types 16 and 18. The protocol included two doses of the vaccine in patients with early-stage cervical cancer, and the induction of humoral and cell-mediated response with low side and/or toxic effects was observed.

In 2004, Gutierrez et al. evaluated the effect of the recombinant vaccine MVAE2, consisting of attenuated vaccinia virus and the E2 gene of HPV, in squamous intraepithelial lesions of high and low grade. Treatment consisted in the administration of six doses, one every week, injected directly into the cervix. During the observation of treatment results, the reduction of injuries was monitored by colposcopy and histological analysis. The immune response was determined by measuring of antibodies againts MVAE2 and analysis of cytotoxic lymphocyte activity against cancer cells with oncogenic human papillomavirus. The presence of viral DNA and viral load were determined using the Hybrid Capture method (Digene).

After treatment it was possible the elimination of pre-cancerous lesions and even cancer (cancer *in situ*), with an efficiency of 95% in the first and 40% in the latter. All patients developed antibodies to the vaccine and a specific cytotoxic response against HPV-transformed cells. These results were compared with those obtained in a similar group of patients treated with cryosurgery, a technique that is able to eliminate low-grade lesions in all patients; but there was not observed cytotoxic activity against cancer cells.

As for of the virus detection, there was no evidence in 50% of the treated sample after treatment and in the remaining 50% was only detected 10% of the original viral load. Assessments of MVAE2 vaccine in women with cervical cancer are in Phase III, consisting of a multicenter study in 250 patients with cancer *in situ* in the Juarez Hospital of the Ministry of Health of Mexico.

This vaccine was also tested in men for the treatment of urethral condilomas, showing the stimulation of the immune response against HPV and regression of lesions in 93% of cases, 4 weeks after therapy. These assessments are in phase II. The results are promising and show that local therapeutic vaccination with MVAE2 is an effective tool for stimulating the immune response to HPV infection and the presence of virus-transformed cells, as well as regression of high and low grade cancer lesions (Albarran y Carvajal et al., 2007).

On the other hand, researchers have highlighted the potential utility of gene gun used in the administration of DNA-based vaccines as part of the antigenic systems strategy for the control of cancer and infectious diseases, projecting itself as an important tool in antigen-specific immunotherapy (Kim et al., 2008a).

In the particular case of uterine cancer, Kim et al. (2008b), in a mouse model, used a Helio gun to dispense gold microparticles coated with E6 DNA of HPV type 16 attached to an expression regulator of major histocompatibility complex class I molecules (human calreticulin). They observed an increased cellular and humoral immune response and antitumor effect, from the increased processing and presentation of antigens to T cells, together with the regression of tumors, enhanced antigen-specific memory and prolonged survival of vaccinated mice. The authors highlight the potential clinical benefits of this therapeutic strategy in humans, which may include co-administration of molecules with other properties, e.g. DNA encoding anti-apoptotic or angiogenic proteins (Kim et al., 2004; Kim et al., 2008b).

This vaccine was combined with E7 and L2 proteins of HPV, also observed significant therapeutic effects against E6/E7 expressing tumor cells, and generate a potent L2-antigen specific response, thereby protecting against pseudovirion infection. These results highlight the potential clinical benefits of this vaccine (Kim et al., 2008b).

Ahn et al. (2004) performed in mice, direct intratumoral injection of an adenoviral vaccine carrier *E7* sequence of HPV type 16 and interleukin 12 (IL-12) as adjuvant, which induces cellular immune responses for protection against tumor formation. They observed partial or complete regression of the tumors and long-term immunity against recurrence of the malignancy, and this effect was much greater with the vaccine formed with the all components, compared with the injection of any of the separate components. The IL-12 is one of the most widely used cytokine on gene therapy against cervical cancer, due to its effect in inhibiting tumor growth and experimental metastasis, dependent on the activation of NK cells.

However, Sin (2009) reported that IL-12 and *E7* HPV type 16 cDNA-based vaccine lost its antitumor and immunoprotective effect when it was combined with nitric oxide (used and

known for its adjuvant effect in routine protocols for vaccination), which demonstrates an immunosuppressive effect of the compound (nitric oxide) in the system used.

Peng et al. (2010) in a preclinical model about recurrent respiratory papillomatosis (RRP), generated a DNA vaccine that encodes the HPV-11 *E6* and *E7* genes in a pcDNA3 backbone plasmid. Vaccinated mice generated strong CD8+ T cell response against the $E6_{aa44-51}$ peptide, which is presented by the major histocompatibility complex class I molecule. Results revealed that the $E6_{aa44-51}$ peptide contains the most immunogenic region for HPV-11 viral type, making it a candidate for the development and evaluation of novel vaccine strategies targeting the RRP patient population.

On the other hand, it has been established that an L1 molecule of various HPV types contains several cysteine residues at markedly similar relative positions, strongly suggesting that these cysteine residues play important roles in the structure and function of the HPV capsids, especially in the viral capsid assembly. Ishii et al. (2007) , in an *in vitro* model (HeLa cells), observed that HPV type 16-pseudovirions lost their infectivity after incubation with thiol-reactive reagents that bound to the free thiol of pseudovirions major capsid protein L1, due to conformational changes that result in the inhibition of the entry and trafficking of this molecules. Therefore, the authors suggest that these reagents might function as practical inhibitors of HPV infection. These reagents could be used in drug design or in combination with preventive and/or therapeutic strategies. It would be necessary further evaluation on this topic.

3.1 Antisense molecules and RNA interference in cervical cancer treatment

Another line of investigation for the treatment of cervical cancer by gene therapy has been successful in testing antisense molecules as the ribozyme R434 (which, through its catalytic activity, specifically destroys the HPV types 16 *E6* and *E7* mRNA and prevents the growth of immortalized cells in the presence of virus), antisense oligonucleotides (AS-ODN) that hybridize with viral messenger blocking viral translation, and interference RNA (RNAi) (Álvarez Salas, 2006; Hamada et al., 1996; Hall & Alexander, 2003) (table 3).

Both technologies, antisense and RNAi, consist of gene silencing (interruption or suppression of the expression of a gene at transcriptional or translational levels).

Agent	Mechanism	Result
Most drugs	Bind to target protein	Protein inhibition
RNase H-independent ODNs	Hybridize to target mRNA	Inhibition of translation of the target protein
RNase H-dependent ODNs	Hybridize to target mRNA	Degradation of the mRNA by RNase H
Ribozymes and DNA enzymes	Catalyze cleavage of target mRNA	Degradation of the mRNA
siRNA	Hybridize to target mRNA by its antisense strand and guide it into endoribonuclease enzyme complex RISC	Degradation of the mRNA

Table 3. Comparison of different gene silencing strategies.

The second, based on double-stranded RNA has proven to be more powerful than the first, based on single-stranded RNA (Mao et al., 2007).

The antisense oligonucleotides have shown effectiveness in inhibiting the expression of *E6* and *E7* oncogenes, and also produced the release and / or activation of molecules involved in defense mechanisms (such as cytochrome c and procaspases 3 and 9), the induction of apoptosis and inhibition of telomerase activity. However, it has been reported that these molecules are unstable and design and management are very expensive (Choo et al., 2000).

RNA interference (RNAi) is a process of RNA-based gene silencing, which relies on nucleotide sequence complementarity and is involved in the mobilization of transposable genetic elements, in defense mechanisms and in different cellular events (such as differentiation, metabolism , stress response, propagation and apoptosis). This natural RNA-dependent gene silencing process is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-RNA molecules in a cell´s cytoplasm, where they interact with the catalytic RISC component, protein argonauta, and the enzyme Dicer (Humayun et al., 2008) (figure 1).

This process occurs through effector molecules identified in many eukaryotes, called microRNAs (miRNAs), highly conserved in orthologous species, indicating their importance in basic cellular processes. miRNAs are endogenous short RNA molecules with space-time independents expression patterns that determine inhibition of translation or degradation of target mRNAs when complementarity is incomplete or perfect, respectively (Raia & Calin, 2011). Hence, this methodology has a potential therapeutic for various diseases, additionally it can be used in the evaluation of molecular and metabolic pathways. MiRNAs originate from populations of non-coding small RNAs (they are one of several small non-coding RNAs, including ribosomal RNA, transfer RNA and small nuclear RNA) resulting from transcription of DNA sequences by RNA polymerase II and form secondary structures hairpin loop type. Several investigators have found alterations of these molecules (particularly single nucleotide polymorphism or SNP) in all cancers studied to date and have indicated that miRNAs are expressed abnormally in these patologies and are involved in predisposition, development and progression of cancer, so they can be used for diagnostic and prognostic purposes. In this regard, miRNAs have been detected in body fluids, which favors its use as biomarkers, because their assessment would be less invasive compared with other conventional markers, such as Pap smears and biopsies. Also, they can become as tumor suppressor, inhibiting cancer development, and as oncogenes, stimulate their development, depending on its expression pattern (Patel & Sauter 2011, Vitale et al. 2011).

In the biogenesis of mature miRNAs, act two type III Rnases, Drosha and Dicer, which cut precursor RNAs in double-stranded RNA (dsRNA) molecules with a length of 21 to 25 nucleotides, which will separated to generate single strand molecules (Ketting et al., 2001). In addition to miRNAs, in the RNAi mechanism have also identified other endogenous small RNAs called short interfering RNAs (siRNA) which, like miRNAs, originate from endogenous complementary dsRNA transcripts, but have an exact length of 21 nucleotides and most interesting is that they can derive from mRNA-coding sequences, transposons and heterochromatin (Ghildiyal et al., 2008).

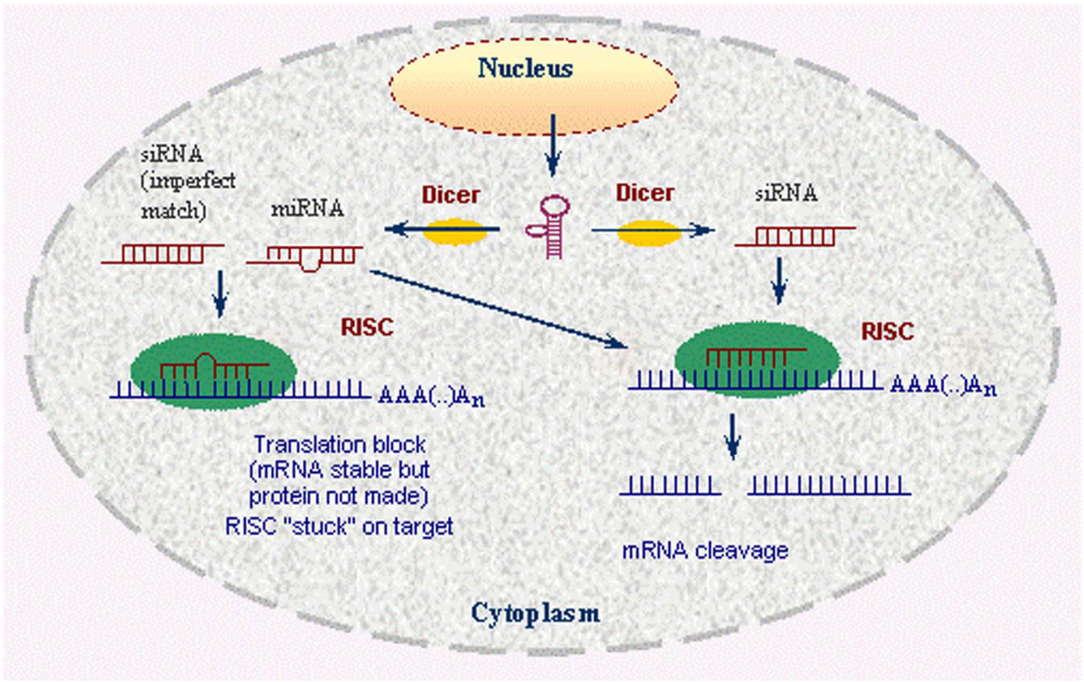

Fig. 1. A simplified model for the RNAi pathway.
The model has two steps, each involving ribonuclease enzyme. In the first step, the trigger RNA (either dsRNA or miRNA primary transcript) is processed into an short, interfering RNA (siRNA) by the RNase II enzymes Dicer and Drosha. In the second step, siRNAs are loaded into the effector complex RNA-induced silencing complex (RISC). The siRNA is unwound during RISC assembly and the single-stranded RNA hybridizes with mRNA target. Gene silencing is a result of nucleolytic degradation of the targeted mRNA by the RNase H enzyme Argonaute (Slicer). If the siRNA/mRNA duplex contains mismatches the mRNA is not cleaved. Moreover, gene silencing is a result of translational inhibition.
Source: National Center for Biotechnology Information
(http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechRnai.shtml)

siRNAs can be designed and artificially synthesized by chemical methods or by molecular cloning vectors and have been used to induce gene silencing *in vitro* and *in vivo* models, showing that perform the same biological functions as the natural miRNAs. The synthetic siRNAs can be transfected into mammalian cells by cationic lipofection, where they bind to liposomes as transport vehicle. In general, although it has achieved the efficient silencing of target genes, this strategy has a high cost and initially required the administration of multiple dose in mice, because a considerable percentage of the siRNAs is degraded by the action of endogenous endonucleases. Recently, the application of liposomes contained in Biogels, has overcome this limitation. The main disadvantage is that synthetic siRNAs have a short half-life after application (Sioud & Sorensen, 2003; Jiang et al., 2004).

It has been suggested that the efficiency of silencing by RNAi not only depends on the activity of RISC by itself, but also other factors such as accessibility of RISC to the target sequence (which is affected by RNA secondary structure and interaction of target mRNA with other molecules) and cleavage and release of cleaved RNA. Moreover, recent studies have shown that siRNA has nonspecific effects, ie in addition to its complementary target

sequence (Schubert et al., 2005); studies of cervical cancer reported the silencing of the gene of interest and the production of inflammatory cytokines and interferon, simultaneously. This was particularly evident when using high concentrations of siRNA. That is the reason because the researchers recommend dose-response studies on a given system in order to select the lowest concentration of siRNA to observe the desired result, eliminating or reducing non-specific responses (Yamato et al., 2008). These nonspecific responses may also be caused by mismatches present in a siRNA in a tolerable level with its mRNA target (Haley et al., 2004). All these elements must be taken into account to design siRNA sequences and protocols in order to obtain the greatest effectiveness and specificity. Currently there is software that facilitates the design of these molecules.

The DNA sequence to silence can also be introduced into a vector that allows the transcription of siRNA. These vectors include siRNA expression plasmids, in which the DNA insert is placed under the control of the promoter of RNA Pol III (H1 gene or U6 gene) and form hairpin secondary structures that are processed by RISC and hybridize to target mRNA for its degradation (Brummelkamp et al., 2002).

Another type of widely used vectors are viruses, because they have a wide capacity of cell infection, especially retroviruses and adenoviruses. The former have the ability to integrate into the genome of replicating cells and therefore are useful for stable transfection of cancer cells. However, it has been reported that the transfection rate is low (de Felipe & Izquierdo, 2000). Adenoviruses have the ability to infect quiescent cells and dividing cells and produce a higher transfection rate than the retroviruses, but are more difficult to handle because of the size of its genome (Carette et al., 2004).

Noted that the use of this technology in the treatment of cervical cancer is possible because while *E6* and *E7* sequester the activities of p53 and pRb proteins in HPV-induced malignancies, copies of *p53* and *pRb* wild genes are usually conserved, which confers a reversible character of the malignancy, whereas reducing the expression of *E6* and *E7* in HPV-transformed cells could restore the activity of endogenous tumor suppressor and thus prevent the proliferation of these cells (Webster et al., 2000).

It has also been shown that RNAi technology against *E6* and *E7* genes induces senescence, apoptosis or inhibition of cancer cell growth in cell models (Webster et al., 2000; Butz et al., 2003; Niu et al., 2006) and destroys or suppresses the growth of tumors in mice injected directly with short molecules of RNAi (siRNA) against E6/E7 (Niu et al., 2006; Fujii et al., 2006). The first work on gene silencing by RNAi in uterine cancer was conducted by Jiang and Millner (2002). The researchers, in an *in vitro* model, administered synthetic siRNAs against *E6* and *E7* oncogenes of HPV 16. They observed the degradation of the *E6* and *E7* mRNA, with consequent expression of *p53*, decreased cell proliferation and induction of cell death by apoptosis. In particular, the induction of apoptosis or senescence in tumor cells has been effective since the introduction of the transcriptional regulator *E2* and the reduction or inhibition of *E6* and *E7* genes expression (Butz et al., 2003).

At the molecular level, it was observed that *E6* silencing induced accumulation of p53 cellular protein and transactivation of *p21* cell cycle control gene (Jiang & Milner, 2005). On the other hand, has been demonstrated the specificity of the technique since it was found that *E6* RNAi of HPV type 16 was less efficient in silencing *E6* gene in cells infected with other HPV types (Niu et al., 2006). These findings support the usefulness of this technique as

a tool for investigating the mechanisms involved in the establishment and development of malignancy, as for the creation of therapies for treatment and healing.

It is noteworthy that there are studies that report the simultaneous silencing of *E6* and *E7*, while in others there was particular silencing of only one of these oncogenes, when using the mechanism of siRNA. It was explained that this is due to the existence of the bicistronic E6/E7, and simultaneous or individual silencing will depend largely on the particulars sequences of siRNA used and their positions of complementarity with the target mRNA (they may hybridize at a point where affect the expression of both genes or only one). In this sense, it has been suggested that alternative splicing events of *E6* and *E7* oncogenes of HPV preced events of silencing by siRNA (Lea et al., 2007).

Another target in the treatment of cervical cancer with RNAi is the telomerase *hTERT* gene, which has been cloned in several siRNA expression plasmids. This enzyme helps in maintaining the genomic stability by synthesizing the telomeres of eukaryotic chromosomes to protect them from degradation events, fusion and recombination. Overexpression of this enzyme void aging and cell death, as in most somatic cells telomerase activity is very low or absent, whereas in undifferentiated or immortal cells is considerable. Using siRNA plasmids for *hTERT in vivo* and *in vitro* models, it has been observed the target gene silencing, with the consequent decrease of telomerase activity, inhibition of cell proliferation, increased activity of caspase 3 and death of tumor cells by apoptosis (Wang et al., 2007).

It has also been observed that the application of siRNA technology increases the sensitivity of malignant cells to chemotherapy and radiotherapy. This has been demonstrated with cisplatin (study where *E6* and *E7* oncogenes were silenced), which can then be used in lower concentrations with the consequent reduction of its negative effects (Putral et al., 2005). The sensitivity of HeLa cells to radiotherapy increased with the *hTERT* gene silencing by siRNA, which allowed the establishment of a relationship between sensitivity to radiotherapy and telomerase activity in this type of cancer (Wang et al., 2007). This observation can be taken into account when designing treatment protocols for a given patient. Thus, the combination of siRNA with chemotherapy or radiotherapy may be synergistic in reducing cancer resistance to conventional therapies, which may promote recovery and / or survival with these therapies.

4. Conclusions

While it has been estimated the impact that preventive vaccination may have on the transmission of HPV infection and the development of cervical cancer and precursor lesions, is important to note the existence of other factors that may affect or influence the development of this pathology as well as existing cases prior to vaccination, so the effect of a preventive vaccine in the prevalence of cervical cancer may involve several decades. Moreover, prophylactic vaccines do not protect against infection (or malignancy) caused by other HPV types not contained in them, so cases of disease will still arise and require treatment.

Faced with these limitations of preventive vaccines, therapeutic vaccines based primarily on molecular resources and gene therapy are currently being evaluated and could become as an effective tool for the treatment of cervical cancer and low or high grade lesions, contributing together to preventive vaccines for a better control of this disease.

In this regard, studies carried out until to date about the effect of gene therapy on cervical cancer, project this technology as an useful and specific tool for the activation of the short

and long term immune response, reducing metastasis and regression and even producing the elimination of tumors when applied alone or in combination with routine therapies (chemotherapy and radiotherapy) and surgery.

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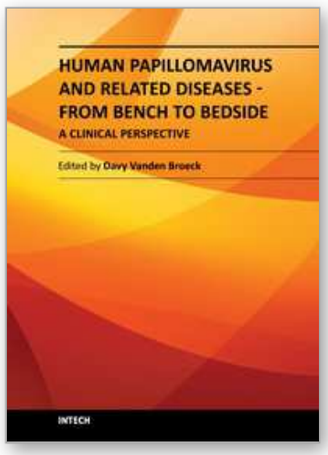

Human Papillomavirus and Related Diseases - From Bench to Bedside - A Clinical Perspective

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

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