The Genetic of Cervical Cancer and New Therapeutic Approaches

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ABSTRACT

Cervical cancer is known as the second-most common cancer in the world. The high-risk HPV (HR-HPV), as well as environmental, immunological, genetic and epigenetic factors, are the main etiological causes contributing to cervical carcinogenesis; HR-HPV infection influences precursor lesions to form invasive cancer. HPV can immortalize human keratinocytes by the process of DNA transfection and many studies have been demonstrated that the E6 and E7 genes of HPV are transforming genes. The primary diagnostic tools have been cytology and histology. Recently, molecular methods to detect HPV DNA sequences in clinical specimens have been introduced. Alterations to the DNA expression pattern, which have also been described in cervical cancer, contribute to genomic instability, chromosomal rearrangements, and silencing of coding and non-coding genes, such as miRNAs, siRNA and different nanoparticles that conjugated with oligonucleotids.
In this review first we explained cervical cancer genetics and mechanism of action of HPV viruses in carcinogenesis. Then method of detection and early prevention were told, And finally the new therapeutic treatments ways based on genetic described.

INTRODUCTION

Cervical cancer is known as the second-most common cancer in the world. More than 500,000 women worldwide are diagnosed each year suffering this disease and almost half of them will die due to that. Before the invention of the Pap smear procedure in the 1940s, cervical cancer was the major cause of women death due to cancer in the United States [1,2].

After the first identification of HPV DNA in cancer cells it was unclear whether these sequences were of viral origin or not. Although the size of the cloned genomes was characteristic for a papillomavirus genome, it was not clear if these sequences would really stem from an infectious agent that was the cause of cervical cancer [3].

The high-risk HPV (HR-HPV), as well as environmental, immunological, genetic and epigenetic factors, are the main etiological causes contributing to cervical carcinogenesis; HR-HPV infection influences precursor lesions to form invasive cancer. Although HR-HPV induces some changes to the host’s genetic material through unknown mechanisms, it has been proved that integration of the viral DNA into the cellular genome causes genetic (deletions, amplifications and DNA rearrangements) and epigenetic (modifications to the DNA methylation status and aberrant miRNA expression) changes. These can silence the tumor-suppressor genes and result in over expression of oncogenes favoring tumor progression [4,5].

Human Papilloma Virus (HPV)

Rous PJB reported the progression to carcinoma of virus-induced rabbit Papillomas in samples gathered from cottontail rabbits. The association of copulatory and venereal factors with cervical cancer in humans, has been recommended over the past two decades. Cervical cancer has a great incidence among women who have several coital partners, or whose begins their sexual activity at a younger age. But then, nuns show a rare rate of cervical cancer which has been confirmed by many researchers. These findings implicate a venereally transmitted agent in human cervical cancer. In 1970s, Herpes Simplex Virus type 2 (HSV-2) was noted as a potential etiological factor. However, HSV DNA was not found in any cervical cancer, and a prospective epidemiologic study had failed to prove potential of HSV-2 infection in cervical carcinogenesis. The first evidence of association of HPV infection with cervical cancer was recognized by Meisels et al. They had reported that cervical cancer were often an accompanied problem with morphological abnormalities (koilocytosis) which known as the cytopathic effect of HPV. Additional support came from molecular virology which demonstrated the presence of HPV DNA in approximately 70%-80% of cervical carcinomas. According to a recent study using PCR 93% of cervical cancers contained HPV DNA. However, there is a dispute that the high incidence of HPV in cervical cancer may be
due to the high susceptibility of tumor cells to viral infection. This question can be addressed by cohort studies and experimental evidences. Prospective epidemiologic studies have shown that, HPV infection precedes the development of precancerous lesions of the cervix in cytological normal women. For example, in a study of 241 cytological normal women referred to a sexually transmitted disease clinic, the cumulative incidence of high-grade squamous intraepithelial lesions for two years was 28% in HPV-positive women where as the related estimation in HPV-negative women was just about 3%. HPV can immortalize human keratinocytes by the process of DNA transfection and many studies have been demonstrated that the E6 and E7 genes of HPV are transforming genes. Additionally, the E6 and E7 genes in high risk HPV can immortalize primary human keratinocytes, but these same genes in low risk HPV would not be able to immortalize primary human keratinocytes. According to this epidemiological and experimental evidence, it is now obvious that high risk HPV infection is a major risk factor for the subsequent development of cervical cancer, although high risk HPV infection alone is not sufficient [6-12].

**Figure 1:** HPV infection.

**The Structure and Classification of Human Papilloma Virus**

PVs are double-stranded DNA viruses in papovavirus family which are replicated in the nucleus. The PV virion is 55nm in diameter and has an icosahedral capsid composed of 72 capsomers. The outer protein layer consists of a major and a minor capsid protein. The 7900 base pair genome of HPV is classified into three groups: (1) early, (2) late, and (3) control [13]. Moreover, there is a noncoding region referred as the Long Control Region (LCR) which regulates the expression of the ORFs. PVs are categorized by genotype. When a HPV has less than 90% sequence homology in
E6, E7 and L1 ORFs to any of the known HPV types, it will classify as a new type. There are over 90 genotypes at present and new types have been finding every few months. Lorincz AT et al. compared the levels of association between specific HPV types and different disease severities, and categorized the HPV types into four clinical classes, “low risk” (HPV 6, 11, 42, 43, and 44), present in 20.2% of low-grade lesions but absent in all cancers, “intermediate risk” (HPV 31, 33, 35, 51, 52, and 58), detected in 23.8% of high-grade squamous intraepithelial lesions but only 10.5% of cancers, “high risk” (HPV 16) associated with 47.1% of both high-grade intraepithelial lesions and cancers, and “high risk” (HPV 18, 45, 56), found in 26.8% of invasive carcinomas but only 6.5% of high-grade intraepithelial lesions. HPVs are now classified into three categories regarding to their carcinogenic potential, low risk, intermediate risk, and high risk [2, 14-16].

**Figure 2:** The HPV genome and its expression within the epithelium.

**The Genetic Mechanism of Carcinogenesis**

Recent experimental surveys have shown that the E6 and E7 gene products play a critical role in cervical carcinogenesis. The E6 of high-risk HPV intervene in the p53 function and deregulates the cell cycle. The E6 product attaches to p53 to form a stable complex and this complex endures proteolysis [17]. In this process E6AP which combines with E6, acts as an E3 ubiquitin ligase [18]. The E6 of high-risk HPV also down-regulates p53 activity by targeting the transcriptional coactivator CBP/p300, which has a function in the cell cycle and differentiation. However, the roles of these protein-protein interactions with E6 is still unknown [19]. E7 protein represents similarities to the adenovirus E1A, and the SV40 large T-antigen, and the host cellular protein cyclin D1. The conserved regions of the amino acid sequence of these proteins organize inactivating complexes with the retinoblastoma (pRB) antioncprotein by competitive binding to the “retinoblastoma pocket”. This binding releases a transcription factor, E2F which free form of this factor speeds up DNA synthesis and cell-cycle progression [20,21].
HPV DNA is presented in an extrachromosomal form in benign and premalignant lesions. But then, HPV DNA is integrated into the host cell's chromosomes in cervical cancer [4,22,23]. Integration of virus genome to the different locations of host’s chromosome in different cancers implies that this process might be totally random. Occurrence of integration in the E1/E2 region disrupts the E2 viral genome [20]. E2 represses the promoter from which the E6 and E7 genes are transcribed in high-risk HPV [24]. Hence, after HPV DNA integration with disruption of the E2 gene, the expression speed of the E6 and E7 genes are increased, resulting in the accumulation of DNA damage and the development of cancer cells over an extended period of time.

![Figure 3: Mechanism of E6, E7 in Carcinogenesis of Cervical Cancer.](image)

**Cervical Intraepithelial Neoplasia (CIN) and HPV**

Cervical intraepithelial neoplasia (CIN) is a good model for a multistage disease beginning with CIN I, progressing to CIN III, and in some cases, converting to invasive carcinoma. The risks of CIN progression are shown in. Most CIN I cases regress spontaneously, also, not all cases of CIN progress necessarily. An effort has been made to recognize the prognostic factors which rule the regression, persistence and progression. In 1986, Campion MJ et al. prospectively studied 100 women with CIN I, and 2 types of HPV, HPV16 and HPV 6 were. 22 of 39 HPV16-positive CIN I progressed to CIN III, while only 4 of 61 HPV16-negative CIN I showed progression [25]. Many studies have been supported the fact that persistent high-risk HPV infection is a major risk factor for CIN progression [26-28].
Common and Molecular Methods Diagnosis of Cervical Cancer

Most mild and moderate dysplasias are more likely have tendency to regress than progress [29]. The risk of converting a stage of dysplasia to a higher grade depends on the stage where tissue is at; for example, the risk of progression of mild dysplasia to severe dysplasia was only 1% per year, while the risk of progression of moderate dysplasia to severe dysplasia was 16% within 2 years and 25% within 5 years. Nevertheless, it is agreed that progression to cancer can be prevented through early detection and subsequent early treatment of HPV in precancerous lesions [30]. As mentioned above, HPV cannot be cultured in the laboratory from clinical specimens and immunologic approaches are not sufficient for detection of HPV infections. The primary diagnostic tools have been cytology and histology. Recently, molecular methods to detect HPV DNA sequences in clinical specimens have been introduced.

Conventional Cytology

The preliminary method for detection of high-risk HPV is still the Papanicolaou-stained (Pap) smear. George Papanicolaou was a pathologist who introduced this method in 1949 before the cause of cervical cancer was known [31]. Since its introduction, the Pap smear has helped reduce cervical cancer incidence and mortality dramatically, rates by roughly half to two-thirds [32]. The Pap smear is a screening tool that looks for alterations in cells of the transformation zone of the cervix. HPV is often responsible for development of these changes. The Pap smear approach has some limitations. Received specimens contain only about 8% of required sample which is
Insufficient. False-negative rates as high as 20 to 30% have been reported. False-negative results can occur from clumping of cells when the cells are not spread equally and uniformly on the microscope slide [30].

Figure 5: The Normal and Malignant Cervical Cancer Cells in Papanicolaou-stained.

Monolayer Cytology

New methods of collection and preparation of specimens for Pap smears have recently been invented to help lowering the number of false-negative results. In these approaches, the specimens are collected in a preservative solution instead of being spread directly on the microscope slide by hand. Immediate fixation of cells resulted in better preservation of cellular structure. Moreover, specimen is collected with a cervical brush, which provides almost twice as many epithelial cells as do other collection devices [33].

HPV DNA or RNA Detection

The genetic material of HPV can be exhibited in biopsy tissues by in situ hybridization with probes labeled with either radioisotopes or chemically reactive ligands which are detected by autoradiography, fluorescence, or a detection of color reaction. In situ hybridization can localize HPV nucleic acid sequences inside individual cells while maintaining cell and tissue morphology to allow simultaneous evaluation of the morphological changes related to the lesion. In situ methods can perform in varying forms such as non amplified, target amplification by PCR, or
signal amplified. Non isotopic probes are suitable for HPV detection, and enzymatic methods are preferred over fluorescence methods for facilitate of interpretation. Features of the signal (confluent versus punctate) may reflect either the episomal or integrated form of the viral target DNA [34].

**The Detection of HPV mRNA**

In the Cell (Invirion, Frankfurt, Mich.) viral load test has been developed for detection of E6 and E7 transforming genes of HPV-mRNA has made researchers need less to detection of different HPV serotypes. In this case, the assay actually determines whether the HPV genes that cause malignant changes are present and active. The test can be automated on any analytic tools that detect fluorescence. Flow cytometry instruments are readily appropriate for this assay by utilizing liquid-based cytology specimens. The experiment can also be performed directly on Pap smear slides and visualized using a fluorescence microscope. The sensitivity and specificity of this assay is reported as 100% and 70% respectively in comparison with Pap smear. Apparent false-positive results are responsible for the lowered specificity; however, these false-positives may in fact not be real false but may be caused by early upregulation of the E6 and E7 genes [35-37].

**Cervical Cancer Therapies Based on Genetics Methods**

Most HPV-induced cervical cell changes are temporary, and 90% regress spontaneously within 12 to 36 months as the immune system eliminates the virus [38-41]. Cell-mediated response is the primary immune response to HPV infection which induced at local lymph nodes. Local levels of HPV-specific immunoglobulin G (IgG) and IgA in tissue after developing of humoral immune response do not correlate with clearance of virus [42]. Systemic levels of HPV-specific IgA were correlated with virus clearance. In case of HPV-specific IgG, Systemic levels of antibody were detected more frequently in patients with persistent HPV infection. The propensity toward regression of HPV infection correlates with the severity of cervical disease inversely. Invasive cancer develops in a small proportion of mild and moderate cervical diseases, but severe cervical cellular abnormalities, may convert to invasive carcinoma with a rate of at least 12% [41]. Genetic predisposition, frequency of reinfection, intratypic genetic variation within HPV type, coinfection with more than one HPV type, hormone levels, and immune response are examples of factors may influence the ability to clear an HPV infection.

Determination of choosing proper treatment procedure depends on several factors such as stage, size, and histologic characteristics of the tumor, lymph node involvement, risk factors for complications from surgery or radiation, and patient. Generally, noninvasive intraepithelial lesions identified only microscopically can be treated using superficial ablative procedures such as cryotherapy or laser therapy. These are outpatient office procedures, and fertility is maintained. Cryotherapy destroy abnormal tissue and the surrounding 5 mm by freezing with a super cooled probe. A single freeze usually cannot induce necrosis, so the area is allowed to thaw and is frozen again. Ablation of tissue with a carbon dioxide laser beam is as effective as cryotherapy. Faster
healing time and less distortion are advantages of laser therapy, but this procedure is more expensive than cryotherapy. The treatment of choice for noninvasive squamous lesions is a group of procedures based on loop electrosurgical excision. In these procedures, the transformation zone and distal endocervical canal excise using electrically charged wire which is less expensive than laser therapy and preserves the excised tissue for histologic examination of margin status. Regardless to the kind of techniques used for treatment of noninvasive intraepithelial neoplasia, there is always a potential risk of leaving dysplastic cells behind. Recurrence rates as high as 31% with a recurrence mean time of 11.9 months have been reported following loop diathermy procedures in immunologically normal patients [43]. Having positive margins in patients showed higher recurrence rate (47%) compared with those whom their margins were clear (26%). Human immunodeficiency virus-infected women have a significantly higher recurrence rate (87%) than uninfected women (18%), showing the importance of an intact immune system in resolution of HPV-associated disease [44]. Although, progression to invasive disease is rare (<2% in most series), these data emphasize the importance of follow-up surveillance in treated patients. Preliminary evidence suggests that tracing of HPV DNA using molecular approaches may be able to help detect residual lesions following treatment [45]. Detection of high-risk HPV DNA at 6 months after treatment was more sensitive than abnormal cytology findings in patients with moderate or severe cervical disease before treatment. The negative predictive value of absence of high-risk HPV DNA and normal cytology test results in these patients was 99%. An evaluation of the efficiency of HPV DNA testing for residual disease following treatment of lower grades of dysplasia should be implemented. Excisional cone biopsy should perform to excise micro invasive cancers less than 3 mm in size conservatively. Radical hysterectomy or external-beam high-energy (to 18 MV) radiotherapy and implants loaded with $^{192}$Ir are major approaches for managing early invasive cancers. Destroying malignant cells in the cervix, paracervical tissues, and regional lymph nodes is the main goal of this therapy. Selected patients also benefit from concurrent chemotherapy. Radiotherapy is the approach which manages locally advanced cancers by affecting primary tumor and potential sites of regional spread.

Except surgical and cytodestructive procedures, several antiviral and immunomodulatory agents have been studied as treatment for HPV-associated cervical lesions. Cidofovir is an acyclic nucleoside phosphonate derivative which has wide range of activity against DNA viruses and is using for clinical treatment of CMV infections. Exposure of human carcinoma cell lines containing HPV-16 or HPV-18 and human cervical keratinocytes immortalized by HPV-33 to Cidofovir led to inhibition of cell proliferation. The in vitro antiproliferative activity was shown to be selective for the highly proliferating HPV-infected cells when normal primary human cervical keratinocytes were treated similarly. 15 women received 1% Cidofovir gel topically without side effects every other day for 1 month to treat with severe CIN [46]. Complete or partial response was seen in 80% of patients as evaluated by histology and detection of HPV DNA by PCR [47].

Podophyllin, a cytotoxic agent that stops mitosis in metaphase (also used to treat genital warts), in combination with vidarabine which is a DNA polymerase inhibitor, suppressed
HPV gene expression and cell growth in cervical cancer cell lines [48]. The expression of HPV-16 E6 and E7 gene products in normal cervical keratinocytes in vitro in the presence of either Podophyllin or vidarabine made these cells susceptible to apoptosis. Combined topical therapy with Podophyllin and vidarabine ointments in 28 patients with mild to moderate CIN resulted in regression of lesions and complete elimination of HPV-16 or HPV-18 DNA in 81% of patients.

The IFNs and intravaginal 5-fluorouracil have shown variability in response in clinical and in vitro studies. IFN-α is approved for administration in genital warts. The effects of IFN-α, IFN-β, and IFN-γ in several human carcinoma cell lines containing HPV-16 or HPV-18 have been evaluated [49]. Response was observed in some cell lines but not others. All IFNs suppressed the levels of HPV E6 and E7 gene transcripts in HPV-18 HeLa cells. IFNs had not any effect in HPV-18 C-411 cells. In HPV-16 CaSki and HPK1A cells, only IFN-γ was effective. It is possible that, since IFN-responsive elements appear to be down-regulated by at least some oncogenic HPV types, the usefulness of IFN therapy in cervical disease will be restricted [50].

The HPV Vaccines

Young sexually active women are the common candidates for Genital HPV, and in the majority of these women the infection is self-restricted. The role of the immune system in viral clearance is not revealed yet. HPV infection and HPV-related lesions are more common in immunosuppressed hosts such as those infected with HIV [51,52]; this fact suggests that cell-mediated immunity plays a critical role. The success of prototypic vaccines in animal models of PV infection raises the hope that prophylactic vaccines could be developed for clinical use [53,54]. Culture cells have not been capable to produce a large amount of PV virions. PV virions contain oncogenic genome, so they are possibly not suitable for vaccines. Therefore, majority of studies have been carried out using a recombinant viral protein. However, recombinant subunit vaccines based on the major capsid protein L1 were found to have limited effectiveness in animal models. The solution was made by Kirnbauer et al. [55] who found that L1 during its high level expression in cultured insect cells self-assembles into virus-like particles (VLPs) which induced the production of neutralizing antibodies to conformational epitopes. Additionally, vaccination with VLPs has been shown protective effects against experimental infection in animal models [56,57]. These magnificent results in animal models have persuaded several commercial and public institutions to set about clinical trials of VLP-based vaccines. Schiller JT et al. [58] represented the primary results of the phase I trials conducted at Johns Hopkins University. Seventy-two men and women who had four or fewer sex partners were enrolled. They were randomized to take 10µg or 50µg VLPs and a placebo with or without adding adjuvant. The clinical grade VLPs consume from purifying the HPV16 L1 recombinant Baculovirus-infected Sf-9 insect cells. All vaccinated individuals receiving VLP were seroconverted with in a month, as measured in a VLP-based IgG ELISA, while none of the placebo-vaccinated subjects was seroconverted during the course of the study. The problem is that it is unknown if serum IgG antibodies alone are sufficient for protection or not.
Using DNA vaccines is another approach. Newly, it was proved that DNA uptake in the muscle cells and expression of the protein encoded by the DNA can be promoted by an intramuscular (i.m.) injection of DNA expression vectors in mice [59]. Ulmer JB et al. [60] reported that an injection of plasmid DNA encoding influenza-A nucleoprotein led to the generation of nucleoprotein-specific cytotoxic T cells (CTLs) which consume a protection from subsequent challenge with a heterologous strain of influenza-A virus. Naked DNA vaccines produced by conflating one or more of the HPV surface protein genes with plasmid DNA are under progress.

HPV E7 noted as a tumor rejection antigen. Chen L et al. [61] demonstrated that protection against transplanted cells from an HPV-16 E7-positive syngeneic tumor can be emerged from immunizing mice with syngeneic non-tumorigenic fibroblast-like cells containing the HPV-16 E7 gene. The E6 and E7 oncoproteins of HPV are constitutively expressed in cervical cancer to maintain the cells in a transformed state. Hence, E6 and E7 oncoproteins became ideal targets for the immune response and are candidates for active immunotherapy. One of the most important defense mechanisms against viral infection and tumors is CTL responses. CD8+ CTL detects peptides derived from HPV-related proteins presented on the MHC class I molecule at the cell surface. These peptides usually have 8 to 11 amino acids in length. Ressing ME et al. [62] evaluated the immunogenicity of 9 HLA-A0201 binding peptides encoded by HPV16 E6 and E7, and detected three highly immunogenic peptides for CTL induction in the peripheral blood mononuclear cells (PBMC) of HLA-A0201 healthy donors. Human CTL clones specific for these three peptides were able to lyse the HPV16 E7-containing HLA-A0201 cervical carcinoma cell line CaSki. A HPV-specific CTL response was also identified in patients with CINIII [63], and CD4+ tumor infiltrating lymphocytes (TIL) in cervical cancer recognize HLA-DR-restricted peptides prepared by human HPV-E7 [64].

A phase I-II clinical trial was carried out involving vaccination with the HPV16 E7 peptides of patients (HLA-A0201) whom suffering from HPV16-positive cervical carcinoma which was resistant to common treatments. The vaccine composed of two HPV E7 peptides and one helper peptide emulsified in adjuvant. Fortunately, no unfavorable side-effects were reported. Of 19 patients listed, 2 were stable for one year after the vaccination, 15 showed progressive cancer, and 2 showed tumor-regression after chemotherapy following the vaccination [65]. Another phase I trial of peptide vaccine was performed by Munderspach L, et al. [66] Eighteen HPV16 and HLA-A2 positive women with high stage cervical and vulvar intraepithelial neoplasia, were treated with a vaccine composed of a 9-amino acid peptide from amino acids 12-20 encoded by the E7 gene emulsified with incomplete Freund’s adjuvant. Only 3 patients of 18 cleared their dysplasia, but all 6 patients whom undergo the test showed increased dendritic cell infiltration. Adams M et al. represented that the intradermal administration of live vaccinia virus HPV16 and 18 E6/E7 construct can induce a clinical response in 1/3 advanced cervical cancer and 3/12 CIN III. These results were preliminary but promising [67].
Dendritic cells (DC) are believed to be essential for the induction of CTL responses. Numerous studies have been administrated using peptide-, tumor lysate-pulsed, and genetically engineered DC for the induction of antitumor immunity \[68,69\]. Tuting et al. \[70\] genetically modified DC using particle-mediated transfer of the HPV16 E7 gene. The I.V injection of these genetically modified DC induced antigen-specific CD8\(^+\) CTL in vivo and raising the rejection rate of a subsequent, normally vital challenge with an HPV16-transformed tumor cell line. Although, the DC-based immunotherapy was successful in animal models \[71\], only a few primary studies have been represented in humans. Schoell WMJ et al. \[72\] described that co-culture of PBMC and HPV16 E711-20 peptide-pulsed DC can be induced by CTL activity in vitro. Also, CTL activity could be induced by co-culture with DC transfected with the HPV16 E7 gene by an adeno-associated virus (AAV) vector \[73\]. Santin AD et al. \[74\] has represented a case with multiple lung metastasis secondary to recurrent HPV18-associated cervical adenocarcinoma. Administration of DC pulsed with HPV18 E7 oncoprotein subcutaneously followed by 14 vaccination episodes with low-dose interleukin-2, and CT scans caused that no evidence of tumor progression during 13 months of therapy be shown.

The experiments on DC-based immunotherapy in humans are preliminary and unsatisfactory; however, future optimizations of this strategy to boost antigen-specific immunity should be explored.

**Methylation of DNA in Cervical Cancer**

Regulation of gene expression is a crucial process that specifies the profile of proteins required to insure the proper occurrence of processes including development, cellular differentiation, organogenesis, cellular stress response and programmed cell death \[75\]. In intact tissues, epigenetic events such as DNA methylation, histone acetylation and expression of miRNAs, and other small RNAs regulate the expression of genes taking part in the activation of the processes differentiation as well as cellular functions that contribute to cellular homeostasis. Methylation of CpG islands which are generally found in the promoter regions of protein-coding genes, make the process of expression silenced. Non-coding genes, such as miRNAs, are also subject to regulation by methylation \[76-78\].

DNA modifications such as global DNA hypomethylation in repetitive regions and hypermethylation in CpG island regions of tumor-suppressor gene promoters are commonly found early during cancer development \[76,79\]. Alterations to the DNA methylation profile, which have also been explained in cervical cancer, contribute to genomic instability, chromosomal rearrangements, and silencing of coding and non-coding genes, such as miRNAs \[80-82\]. DNA hyper methylation causes silencing of tumor-suppressor genes which is linked to the development of different types of cancers, including cervical cancer, and is substantially associated with poor clinical results. As respects, silencing of tumor suppressor miRNAs through hyper methylation of CpG islands in their promoter regions has also been involved in carcinogenesis \[82,83\].
DNA methylation in HPV Genome

It is proved that HR-HPV can provoke alterations in DNA methylation and histone acetylation and also cause aberrant miRNA expression. Some researchers have demonstrated that upon HPV 16 infection, cellular DNA endures epigenetic alterations induced by the E6 and E7 oncoproteins [84-86]. It has been suggested that methylation has created as a defense mechanism by the host cell to silence viral DNA [87-89]. Expression and activity of DNMT1 can be increased by E6 and E7 oncoproteins of HR-HPV [86]. E6 does so by degrading p53.

The function of DNMT1 in cervical carcinogenesis has been reported by Jin-Tao et al. [90]. They used both in vitro and in vivo studies and found that low amounts of serum folate and high expression of DNMT1 protein or mRNA were considerably associated with cervical carcinogenesis (p=0.001). The DNA of HPV 16 and HPV 18 is methylated After its integration into the human genome [81,91]. After all, controversial results regarding the participation of HPV in the aberrant DNA methylation that has been observed in cervical cancer still exist. In addition, Leonard et al. [35] studied the whole-genome methylation profile after transfection with the episomal forms of HPV 16 and HPV 18 and found a considerable increase in the methylation status of 5,607 and 2,387 genes, respectively. They also found a reduction in the methylation status of 3,568 and 4,160 genes, respectively. 2,295 and 1,023 genes showed non-overlapping increases and decreases in methylation, respectively [92]. Altered miRNA expression observed in cervical cancer might be related to the aberrant methylation of miRNA promoters. Hence, HR-HPV could indirectly induce aberrant miRNA methylation [80,93].

Deregulation of miRNA and Their Function in Cervical Cancer

miRNAs are small, non-coding RNA molecules and include about 22-25 nucleotides (nt) in size. They are usually phylogenetically conserved and have a tissue- and time-specific expression pattern [94,95]. miRNAs have been categorized as epigenetic regulators, which control gene expression without changing the DNA sequence. The expression pattern of miRNAs in cell lines and cervical cancer tissues represents that aberrant miRNA expression involves in the development of cervical cancer and HR-HPV-induced precursor lesions [96-98]. Defects in miRNA expression have been related to: i) genetic alterations, such as deletions, amplifications and point mutations and ii) epigenetic alterations, like histone modifications and improper DNA methylation [78,93,99].

Currently, it is known that miRNA biogenesis is regulated at several levels: i) at a transcriptional level, which in, RNA polymerase II and III transcribe pri-miRNA; ii) at a post-transcriptional level, including miRNA maturation, which involves the processing of pri-miRNA to pre-miRNA, export into the cytoplasm, and incorporation into the RISC complex and iii) at a level of miRNA locating within the genome [93,100].

Considering that miRNA genes are expressed in a tissue- and time-specific way and their promoters possess specifications such as CpG islands, TATA boxes, TFIIB recognition elements, and initiators that are similar to the promoters of protein-coding genes, epigenetic mechanisms, such
as nucleosome remodeling and DNA methylation can regulate miRNA expression [93,100,101]. An important factor for the regulation or deregulation of certain miRNAs is their location within the genome, given that several miRNAs have been mapped to or fragile sites, minimal regions of loss of heterozygosity, amplification, common breakpoint regions and transcriptionally active regions that have been related to cancer in humans [98,100]. miRNA expression profiling has revealed that their expression in cancer tissues is either increased or decreased in comparison with healthy tissue. They are differentially expressed in various types of tumor, cell lineages and tumor stages [102,103].

**The miRNA promoter Methylation and This Role in Cervical Cancer**

It is probable that the aberrant methylations of miRNA promoters are responsible for the modified expression of some miRNA genes with tumor-suppressor or oncogenic functions in cancer. Few studies exist regarding DNA methylation in the deregulation of miRNA expression in cancer, but deregulation of miRNA expression in cervical cancer has been proposed to be able to explicate alterations to the methylation status of miRNA genes [80,104]. Involvement of the HR-HPV genotypes in the methylation process of miRNAs in cervical cancer can be possible. However, in vitro findings suggest that the methylation events occur after cellular immortalization and are not directly linked to the presence of HR-HPV [80]. It is likely that detection the methylation status of miRNAs could be suitable for the prognosis of precursor lesions and cervical cancer [80].

The finding that alterations in miRNA expression and methylation of key genes involved in cell cycle regulation are abundant events in the process of carcinogenesis indicates a challenge and a motivation for this field of research [105]. Comprehensive study has been implemented to identifying variations in miRNA expression and the expression of miRNA targets in cervical cancer and its precursor lesions [106]. However, knowledge considering the role of miRNAs in the carcinogenic process is still in primary stages. Evidence represents that in cervical cancer hyper methylation of miRNA promoters contributes to the lowered expression of miRNAs with tumor-suppressor gene functions and supports overexpression of miRNAs with oncogenic functions [80]. Methylation is one of the most important mechanisms in the HPV viral cycle. Some factors such as HPV infection, the viral genotype, the physical state of the viral DNA, and oncogenic risk are involving in alterations to the methylation status of cellular DNA. The E6 and E7 oncoproteins of HPV 16 induce the overexpression of DNA methyl-transferase enzymes, which can catalyze the aberrant methylation of protein-coding and miRNA genes that are capable to regulation by methylation. Additionally, HPV implements E6 and E7 oncoproteins to deregulate the expression of miRNAs with loci located at fragile sites. p53 is one of targets of these proteins include transcription factors of miRNAs [106,107].

**The Effect of Reduced Expression miR-143 in Cervical Cancer**

The completely different frequency level of miR-143 between normal cervical samples and cervical cancer cell lines led researchers to investigate its association with cervical cancer
development. The expression of miR-143 is significantly lower in most of the tumors as compared with their normal counterparts, which is in agreement with the cloning data. Similarly, significantly lowered levels of mature miR-143 in different tumor types, e.g., colorectal tumors, sarcomas, breast, prostate, and lymphoid cancer cell lines [108,109], represents that miR-143 might have suppressor roles in a wide range of tumor cells. It is good to note that ERK5 (also known as MAPK7) which is the only experimentally verified target for miR-143 is known to increase cell growth and proliferation in response to tyrosine kinase signaling [96]. In addition, abnormal levels of ERK5 expression have been observed in some cancer types [110,111], representing its remarkable role in tumor development. More investigations are required to reveal further information about aERK5 role in cervical cancer cells and its regulation by miR-143.

**The Effect of Increased Expression miR-21 in Cervical Cancer**

miR-21 was the most frequently recovered miRNA species in the entire cervical cancer cell lines ever examined, showing significantly lower abundance in the normal cervical samples analyzed. Compatibly, the elevated expression of miR-21 was also found in a preponderance of tumors. Frequent miR-21 may be a general, albeit not universal, feature of tumor cells. Moreover several hybridization-based profiles of different tumor types (e.g., glioblastomas, breast cancer) and cancer cell lines have been shown strong miR-21 signals (e.g., HCT-116, colorectal carcinoma; HeLa, cervical adenocarcinoma, and glioblastoma cell lines [112,113]. Chan et al. [114] recently reported that activation of caspases can trigger suppression of miR-21 in glioblastoma cells and lead to increased apoptotic cell death. Conversely, Cheng et al. reported that knockdown of miR-21 can increase cell growth in HeLa cells [115]. On the whole, these findings suggest that miR-21 may influence different biological processes in different cellular contexts. Interestingly, miR-21 is located in the fragile site FRA17B region, which is one of the HPV16 integration loci at 17q23.2 [116,117]. It is revealed that integration of the HPV into the host cell genome can cause some genetic alterations (such as deletions, amplifications, or complex rearrangements) and epigenetic alteration, therefore, intriguing to speculate that the expression of cellular miRNA genes at or near HPV integration sites may contribute to the tumor phenotype.

**Treatment Cervical Cancer with siRNA**

siRNAs are able to silencing nucleic acid sequence-based gene, and have been used as a powerful tool in functional genomics. Drug targeting potentially can be applicable for genes involved in the pathogenesis of human diseases using this technology. For instance, it has been reported that siRNAs against Fas and TNF can control fulminant hepatitis and septic shock in mice, respectively, while others have been shown successful inhibition of replication of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1 [118,119]. Using of a siRNA for cancer therapy appears to be more complicated; recent molecular analyses have revealed that there are no or only subtle sequences changes such as point mutations and translocation in most oncogenes involved in carcinogenesis. Therefore, the first problem to overcome is the ability to design specific siRNA for targeting gene without disturbing the expression of the normal allele.
Recent studies contrary to initial reports which showing a high specificity for siRNA-directed RNAi, have revealed that siRNAs induce off-target effects by various mechanisms [120,121]. siRNA can tolerate some mismatches with its target mRNA when enduring directed target cleavage, though increases in the number of mismatches cause decreases in enzyme efficiency [120]. In fact, the study showed that a single nucleotide mismatch in the central portion of an siRNA targeting mRNA made a fivefold decrease in the value of IC\textsubscript{50}. siDirect software selects siRNA sequences which have at least three base mismatches with non-redundant sequence sets of human genes. Thus, some off-target effects applied through RISC cleavage may be attenuated by reducing the siRNA concentration.

A collateral target for siRNA-mediated translation inhibition is a mRNA that has a 3′ untranslated region containing a sequence complementary to nucleotide 2–7 of the 5′ end of the guide strand, analogous to the seed region of miRNA [122-124]. Those studies also represented that all siRNAs potentially have miRNA-like activities. To avoid such off-target effects, analysis of complementarity between the guide strand and human genes should be implemented carefully. Chemical modifications of siRNAs, such as a 2′-O-methyl ribose modification, have been reported to lessen the miRNA-like off-target effect without compromising RNAi activity [125].

siRNAs can also apply unintended effects through producing of IFN-I and inflammatory cytokines. Double-stranded RNA-dependent protein kinase (PKR) and toll-like receptors (TLRs), such as TLR3, TLR7 and TLR8 are Receptors of siRNAs for immune-stimulation. siRNAs with sequences of 5′-GUCCUUCAA-3′, 5′-UGUGU-3′ and 5′-UGUCU-3′ have been shown to be mouse TLR7 and, most likely, human TLR8 stimulators [118,119]. Among the E6 and E7 siRNAs selected in this study, siRNA 497 was the only siRNA that contained the 5′-UGUCU-3′ sequence. Delivery of a siRNA in a cholesterol-conjugated or atelocollagen-complexed form may bypasses activation of TRLs as well as following chemical modification of the RNA backbone [126].

The exhibition mechanism of off-target effects on HPV16− cells by present siRNAs was not revealed. However, nonspecific growth suppression of three of the siRNAs (497, 573, 752) was dramatically improved without compromising their powerful growth suppression of HPV16+ cells by reducing the siRNA concentration to as low as 1 nM. To further improve the off-target effect, researchers are now working on backbone modification of these siRNAs, which has been reported to attenuate miRNA-like activity and cytokine response [127,128].

Establishing a proper delivery system for siRNA convince us to believe that HPV-caused cervical cancer can be treated by an E6-targeting siRNA. E6 and E7 viral proteins, the responsible agents in cancer development, are necessary for inactivating cellular wild-type p53 and Rb and subsequently maintaining cancer phenotypes. Hence, process of senescence, growth arrest, or apoptosis in tumor cells should be initiated by knockdown of the viral proteins. From what we found, the superior therapeutic strategy to silence both E6 and E7 might be the selective E6 silencing, since expression of E7 alone might activate genes involved in the induction of growth.
suppression and apoptosis. Further experiments are being undertaken in our laboratories to elucidate this hypothesis. Before clinical application of such, it is also necessary to specify the E6 target sequences of siRNA that show the strongest RNAi activity toward cancer cells [129,130].

**Drug Delivery in Cancer with Therapeutic Nanoparticles**

Cancer nanotherapeutics are progressively improving and are being used to solve several restrictions of conventional drug delivery systems such as nonspecific biodistribution and targeting, insufficient oral bioavailability, lack of water solubility, and low therapeutic indices. Optimal nanoparticles have been designed to refine the biodistribution of cancer drugs, by optimization of their size and surface characteristics to increase their circulation time in the bloodstream. Some unique pathophysiology characteristics of tumors such as their enhanced permeability and retention effect and the tumor microenvironment make nanoparticles capable to carry their loaded active drugs to cancer cells. In addition to this passive targeting mechanism, there are active targeting strategies using ligands or antibodies against selected tumor targets which amplify the specificity of these therapeutic nanoparticles [131-134].

![Figure 6: The mode of action of targeted multifunctional nanoparticle (NP).](image-url)
Different Nanoparticles Used as Drug Delivery

Nanoparticles used in Drug delivery systems are submicron-sized particles (3-200 nm), which applied as devices or systems that can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), viruses (viral nanoparticles), and even organometallic compounds.

The Polymeric nanoparticles

Polymers such as albumin, chitosan, and heparin occur naturally and have been an ideal choice for the delivery of oligonucleotides, DNA, and protein, as well as drugs. Newly, a nanoparticle formulation of paclitaxel has been invented, in which serum albumin is applied as a carrier [nanometer-sized albumin-bound paclitaxel (Abraxane); it has been applied for the treatment of metastatic breast cancer clinically [135]. Moreover, Abraxane has also been evaluated in clinical trials involving many other cancers than breast cancer including non–small-cell lung cancer (phase II trial) and advanced nonhematologic malignancies (phase I and pharmacokinetics trials) [136,137].

A novel TPGS-b-(PCL-ran-PGA) nanoparticle modified for the first time which polyethyleneimine was applied to be a vector of TRAIL and endostatin for cervical cancer gene therapy. According to resulted data, the nanoparticles could deliver plasmids into HeLa cells efficiently and approaches such as RT-PCR and Western blot analysis applied to verify the expression of TRAIL and endostatin. The TRAIL/endostatin-loaded nanoparticles represent considerable potential as a desired candidate for in vivo cancer gene delivery [138,139].

Figure 7: Polymeric Nanoparticles.
**The Polymeric micelles**

The functional characteristics of micelles are based on amphiphilic block copolymers, which gather to nanosized core/shell structure in aqueous media. In this case, the hydrophobic core region acts as a reservoir for hydrophobic drugs, while the hydrophilic shell region stabilizes the hydrophobic core by surrounding it and renders the polymers water-soluble, making the particle an appropriate candidate [140]. Polymeric micelles can load drug in two ways: physical encapsulation or chemical covalent attachment [141].

![Figure 8: Polymeric micelles.](image)

**Dendrimers**

Dendrimers are synthetic polymeric macromolecules with nanometer dimensions. They composed of multiple highly branched monomers which emerge radially from the central core. Characteristics associated with these dendrimers such as their monodisperse size, modifiable surface functionality, multivalency, water solubility, and available internal cavity make them susceptible for drug delivery [142]. In comparison with other tested formulations, DF3 containing siRNA against E6 and E7 was found to knock down the target genes considerably [143].
Liposomes

Liposomes are self-assembling closed colloidal structures consist of lipid players and have a spherical shape in which a central aqueous space is surrounded by an outer lipid bilayer. Currently, several types of cancer drugs have been applied to this lipid-based system using a variety of preparation procedures. Among them, liposomal formulations of the anthracyclines doxorubicin (Doxil, Myocet) and daunorubicin (DaunoXome) are approved for using in the treatment of metastatic breast cancer and AIDS-related Kaposi's sarcoma [144-146]. In addition to these approved agents, various liposomal chemotherapeutics are currently being evaluated in clinical trials [147]. Immunoliposomes may be the next generation of liposomal drugs, which deliver the drug to the desired sites of action in a more selective manner [147].

Liposomes are used in vaginal instillation of small-interfering RNA (siRNA) to silence endogenous genes in the genital tract and protect it against challenge from infectious disease. Although siRNA lipoplexes are easily constructed, several of the most effective commercial transfection agents may be toxic to the mucosal epithelia and none are able to provide controlled or constant release. Moreover, nanoparticles penetrated deep into the epithelial tissue which is the first report demonstrating that biodegradable polymer nanoparticles are efficient delivery vehicles for siRNA to the vaginal mucosa [148].
The main aim of this study was to evaluate the uptake of E6 mRNA antisense into cervical cancer cells, induced by human papilloma virus (HPV). In this study, the carrier of the antisense was tri-calcium phosphate nanoparticles (TCP NPs) conjugated with dioleoylphosphatidylethanolamine (DOPE) and/or anti-E6 antibody. At first, TCP NPs were synthesized, coated with carboxy-polyethylene glycol, and then conjugated with anti-E6 antibody and/or DOPE by carbodiimide cross-linker. Then, a single stranded DNA, which was complementary (antisense) of E6 mRNA, was attached to each one. Finally, the uptake of conjugated and unconjugated TCP NPs into HeLaS3 cells was separately evaluated by Fourier transform infrared spectroscopy, optical microscopy, and fluorescent microscopy. Also, the cytotoxicity of these carriers was measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Overall, 4 types of TCP NPs were used in this study, including 1) TCP NPs conjugated with DOPE (TCP NPs/DOPE), 2) TCP NPs conjugated with DOPE and antibody (TCP NPs/DOPE/Anti-E6 Ab), 3) TCP NPs conjugated with antibody (TCP NPs/Anti-E6 Ab), and 4) TCP NPs which not conjugated with DOPE and antibody (unconjugated TCP NPs). Uptake tests showed that although all types of TCP NPs could transfer antisense of E6 mRNA into HeLaS3 cells, TCP NPs/DOPE and TCP NPs/DOPE/Anti-E6 Ab had more uptake than TCP NPs/Anti-E6 Ab and unconjugated TCP NPs. Moreover, MTT assay showed that TCP NPs/DOPE was more toxic than TCP NPs/DOPE/Anti-E6 Ab, TCP NPs/Anti-E6 Ab, and unconjugated TCP NPs. It can be concluded that TCP NPs/DOPE/Anti-E6 Ab is a good choice for oligonucleotide delivery, because of higher uptake and less toxicity, compared with other formulations [152].

Figure 10: Liposome.
Figure 11: Viral nanoparticles.

Figure 12: The image of the cervical cancer cells, obtained by optical microscopy, exposed to nanoparticle 1 (a), 2 (b), 3 (c), and 4 (d). The image of the cervical cancer cells, obtained by fluorescent microscopy, treated with nanoparticle 1 (e), 2 (f), 3 (g), and 4 (h).
Viral Nanoparticles for Therapeutic Purposes

A wide range of viruses including cowpea mosaic virus, cowpea chlorotic mottle virus, canine parvovirus, and bacteriophages have been developed for biomedical and nanotechnology applications that include tissue targeting and drug delivery. Some targeting molecules and peptides can be represented in a biologically functional form on their capsid surface using chemical or genetic means. Hence, several ligands or antibodies including transferrin, folic acid, and single-chain antibodies have been associated with viruses to target specific tumor \textit{in vivo} [149]. Also, a subset of viruses, such as canine parvovirus, has innate affinity for receptors like transferrin receptors that are up-regulated on a variety of tumor cells [150]. A dual-function protein cage with specific targeting and doxorubicin encapsulation has been developed using target of heat shock protein [151].

CONCLUSION

Cervical cancer is a common disease with a high mortality risk in women. The most common diagnosing cervical cancer is the Pap test, But in this method we have up to 20\% false negative result which is not desirable for a diagnostic test. Common treatment methods include radiotherapy, chemotherapy and radical hysterectomy, Not only this methods do not have affect definitive improvement ,but also many side effects on the patient and their fertility. Therefore, the use of less invasive and powerful methods to diagnose and treatment is required. Considering that more than 90\% of cervical cancer caused by HPV infection and two oncogenes are known well, Cervical cancer is a good choice for diagnosis and treatment with genetic methods. Use of antisense oligonucleotide, miRNA,and siRNA, can destroy copies of the gene expression or stop translation phase. As a result progress in genetic therapeutics, can hope to an effective and safe treatment for cervical cancer.

References


