

Molecular Targeting of Human Papillomavirus oncogene expression using RNA interference Technology

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Cervical cancer is a killer disease that claims innumerable lives of women all over the world. Human Papilloma Virus (HPV) serotypes 16 and 18 have been implicated as the causal organism of the disease. Initiation of sexual intercourse at an early age, multiple sex partners, poor hygiene etc are regarded as risk factors that are also associated with progression of HPV infection. Two genes in HPV encode the oncoproteins E6 and E7. These proteins have been found to be in high content in cervical cancer positive cell lines. Attempts have been made to selectively inhibit the expression of these two oncogenes by molecular biological techniques. Of these RNA interference (RNAi) deserves special mention. RNAi occurs naturally in mammalian cells through which expression of a particular gene can be knocked down with high precision. Different authors from time to time have targeted to knock out these E6 and E7 proteins as a preventive measure of cervical cancer. The present review portrays the state of the art evaluation of retrospective as well as recent literature on these challenging techniques.

Keywords: cervical cancer; RNAi; antisense RNA; ribozyme; siRNA; shRNA

1. Introduction

Cervical cancer is the second most common cancer in women throughout the world. Human Papilloma Virus (HPV) infection is the most important risk factor for cervical intraepithelial neoplasia and invasive cervical cancer [1,2, 3]. Cervical cancer occurs early during the productive period of a woman's life. HPV serotypes 16 and 18 account for nearly 76.7% cervical cancer. Other factors like high parity, early initiation of sexual intercourse, multiple sex partners, low socioeconomic status, poor hygiene etc. have been found to be associated with progression from HPV infection to pathological consequences of cancer. [4].

Physical ablation of HPV induced lesions like cryo therapy, photo therapy, laser cone biopsy and localized radiotherapy are effective to some extent in treating lesions. Removal of afflicted

tissue is accomplished by removal of keratinocytes harboring HPV. These are the conventional approaches for treatment of cervical cancer. Prophylactic vaccines are available and should be administered prior to viral exposure. Initiatives for vaccination have been undertaken by the governments and also non-governmental agencies. Attempts are being made to develop therapeutic vaccines that would treat prevailing HPV infection. Use of immunomodulators like interferon and imiquimod induce cytokine production to increase host immune response against HPV infection [5]. Many antioxidants and poly herbal formulations are also in use. Molecular targeting of HPV gene expression using RNA interference technology includes use of antisense oligonucleotides, ribozymes, short interfering RNAs (siRNA), short hairpin RNA (shRNA) etc. The present review is a cri-

tique of RNAi approaches used for prevention of cervical cancer.

RNA interference (RNAi) occurs naturally in mammalian cells, through which expression of a particular gene can be knocked down with high specificity and selectivity [6]. Pre-clinical studies confirm that RNAi techniques can be used to silence cancer related targets [7]. *In vivo* studies have also shown favorable outcomes by RNAi targeting of components critical for tumor cell growth, metastasis, angiogenesis and chemo resistance.

2. Anti sense RNA therapy for cervical cancer

HPV has been implicated as the causal agent in cervical cancer. Two genes in HPV encode the oncoproteins E6 and E7. E6 can form complexes and also can degrade p53. E7 protein binds to the Rb protein preventing it from binding to its normal substrate E2F-1 protein. Attempts have been made to selectively inhibit the expression of these two oncogenes by antisense RNA. Steele observed [8] that a plasmid expressing antisense RNA of HPV18 decreased the growth rate of human cervical cancer cell line HeLa. Antisense E6 and E7 oligonucleotides have been shown to inhibit the growth of HPV positive cancer cells [9]. However when the oligonucleotides were withdrawn after 3 days the remaining cells in the culture recovered and grew as before. To explore the potential of an adenoviral antisense RNA transcript for gene therapy of cervical cancer, Hamada et al. [10] introduced the antisense RNA transcript of E6 and E7 genes of HPV 16 into cervical cancer cells via a recombinant adenoviral vector. They observed that the growth of infected cells was greatly suppressed as evidenced by decrease in cell counts. In an *ex vivo* study in nude mice tumorigenicity was completely inhibited. It was suggested by the authors that transfection of cervical cancer cells with HPV16 E6/E7 antisense RNA is a potential novel approach for treatment of cervical cancer. Inhibition of E6 and E7 by complementary antisense transcripts led to reduced growth rates, loss of transformed phenotype of cervical and oral carcinoma cell lines and inhibition of tumor formation exhibited in an animal model.

3. Ribozyme mediated therapy in cervical cancer

Ribozymes have several advantages over antisense RNA. They are capable of catalytic activity and do not require the presence of an auxiliary enzyme like RNase H. More over one molecule of ribozyme can bind and cleave many molecules of mRNA [5]. Chen et al. [11] showed that ribozymes can be used for effective cleavage of HPV transcripts and can also inhibit E6/E7 mediated immortalization [12]. Zheng et al [13] demonstrated that a hammerhead ribozyme Rz 170 can specifically target HPV 16 E6/E7 transcripts and was effective in inhibiting cell growth and promoting apoptosis. Rao et al [14] demonstrated that anti HPV 16 E6 ribozyme when transfected into CaSki cell increased the sensitivity to cisplatin, the chemotherapeutic agent used to treat cervical cancer. The increase of sensitivity to cisplatin in these cells may be associated with increased expression of p53, Bax protein and decreasing expression of Cmyc, Bcl-2 proteins.

4. shRNA mediated treatment for cervical cancer therapy:

HPV is known to play an important role in the pathological manifestation of the disease. Its early genes E6 and E7 can activate several intracellular signal pathways for carcinogenesis. *In vitro* knock down of E6 and E7 can reduce the survival ability of cervical cancer cell lines. Chen et al [11] studied the effect of lentiviral shRNA against E6/E7 on the tumor growth of xenografted cervical cancer model in mice. The growth of HeLa cells transduced with lentiviral shRNA against E6/E7 was compared to controls, *in vitro*. *In vivo* the RAG^{-/-} mice, which were deficient in T and B lymphocytes were used to establish the HeLa xenografted model. HeLa cells transduced with lentiviral shRNA against E6/E7 grew slower than those with control vector. Lentiviral shRNA significantly reduced the size of xenografted tumors compared with controls. According to these authors RNAi could be used for the treatment of cervical cancer by intra tumor injection.

Gu et al. [15] reported inhibition of cervical cancer cell growth *in vitro* and *in vivo* with

lentiviral vector delivered short hairpin RNA targeting HPV E6 and E7 oncogenes.

RNAi holds great promise for the treatment of cervical cancer but delivery remains a key issue. Lentiviral vectors are widely used for stable transfer of short hairpin RNA (shRNA) into cells and are expected to deliver a stable and durable interference. Gu et al. have shown that lentiviral delivered shRNAs directed against HPV E6/E7 oncogenes are effective for less than three weeks. This short lived RNAi was not due to the loss of the vector in the host cells but was more likely to be related to shRNA expression or RNAi machinery itself. Using this vector to carry two copies of the same shRNA or two shRNAs targeting at different genes (HPV E6 and VEGF) was more effective at silencing the gene targets and inhibiting cell or tumor growth than their single shRNA counterparts. These results indicate that a multi shRNA strategy is a more attractive approach than single shRNA for developing RNAi treatment for cervical cancer.

5. siRNA mediated approaches for cervical cancer therapy

A major advancement in RNA interference methods in recent years have been specifically aimed at HPV oncogenes E6 and E7. It has been established that siRNA can induce selective silencing of exogenous viral genes in mammalian cells. Moreover, the silencing process does not interfere with the recovery of cellular regulatory systems previously inhibited by viral gene expression. It is conjectured that since E6 and E7 genes are absent in normal cells, RNAi based therapies would not affect them.

Yoshinouchi et al [16] targeted a gene specific therapy for HPV related cancer. They studied the effects of E6 siRNA on the expression of E6 and E7 oncogenes and on the cell growth of HPV16 related cervical cancer cells. Using SiHa cell line these authors showed that E6 siRNA decreased the levels of mRNA encoding E6 as well as E7 proteins and induced nuclear accumulation of p53 which is the most important target of E6. It was found that E6 siRNA suppressed monolayer and anchorage-independent growth of SiHa cells. This cervical cancer cell line treated with E6 siRNA formed tumors in

NOD/SCID mice that were significantly smaller than in those treated with control siRNA. It was suggested that HPV E6 siRNA could be a candidate for gene specific therapy for HPV related cervical cancer.

Koivusalo et al [17] observed that HPV18 positive HeLa cervical cancer cells when transfected with short interfering RNA (siRNA) molecules targeting HPV18 E6 mRNA before treatment with chemotherapy compounds showed nuclear accumulation of p53. But the effect was transient despite continuously suppressed HPV mRNA levels. According to these authors activating p53 by degrading E6mRNA may either increase or decrease the chemosensitivity of cervical cancer cells depending upon the drug used for chemotherapy.

Chang et al. [18] developed nine siRNAs against either the E6 or E7 genes of HPV-16 or HPV-18 in several combinations, yielding siRNAs targeting 16E6, 16E7, 18E6 and 18E7. HPV siRNAs significantly reduced cell growth, colony formation and apoptosis in HPV positive CaSki (HPV-16) or HeLa(HPV-18) cell lines, and had no effect in HPV negative C33A cells. These authors also found that intratumoral injection of the siRNAs reduced tumor growth in BALB/c nude mice. According to these authors siRNA treatment has potential as adjuvant therapy for cervical cancer.

Palanichamy et al [19] observed silencing of integrated HPV-16 oncogenes by siRNA mediated heterochromatinization. These authors have screened multiple siRNAs homologous to one of the NF-1 binding sites in the HPV-16 enhancer and identified one siRNA which causes specific transcriptional gene silencing of the HPV-16 oncogenes E6 and E7 when transfected into HPV-16 positive cell lines SiHa and CaSki. This phenomenon was found to be specific to the HPV-16 enhancer and showed no effect on HPV-18 enhancer. Gene silencing was found to be associated with heterochromatinization of the target region of the enhancer. However no DNA methylation was reported by these authors during the time period studied. These authors proposed that this siRNA causes simultaneous silencing of E6 as well as E7 oncogenes by an epigenetic mechanism for HPV-16 positive cervical and other epithelial cancers.

Dutta et al. [20] proposed a dendrosome based delivery of siRNA against E6 and E7 oncogenes in vitro in cervical cancer. Since siRNAs are large molecules and polyanionic in nature, they do not freely cross the cell membrane. Different types of synthetic vectors have been investigated for gene silencing. Cationic lipids, liposomes, cationic polymers, cationic dendrimers and cationic cell penetrating peptides have been used by different authors for delivery of siRNA from time to time. Rapid enzymatic degradation, limited permeability across the cell membrane and substantial liver and renal clearance restricted the therapeutic applications of siRNA in vivo. According to Dutta et al. [20] the dendrosomes hold potential for the delivery of siRNA in vivo.

Eaton et al. [21] studied the efficacy of TRAIL (Tumor Necrosis Factor related apoptosis inducing ligand) treatment against HPV16 injected SiHa cells undergoing senescence following siRNA knock down of E6/E7 genes. They observed that E6/E7 siRNA induces senescence rather than apoptosis in SiHa cells. These findings are significant for combinatorial strategies for cancer therapy because the induction of senescence can preclude apoptosis rendering cells to be recalcitrant to TRAIL treatment.

Yang et al. [22] evaluated the efficiency of chitosan based HPV 16 E7 siRNA delivery and also studied the chitosan /HPV 16 E7 siRNA complex to induce apoptosis in HPV 16 positive CaSki cells. Chitosan is derived from the biopolymer chitin and is biologically safe, nontoxic, biodegradable and biocompatible. Chitosan/HPV16 E7 siRNA nanoparticles were delivered to CaSki cells and induced apoptosis. According to these authors, the delivery of nanoparticles *in vivo* may serve as a promising therapy for cervical cancer.

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor constitutively active and aberrantly expressed in cervical cancer. A study by Shukla et al. [23] performed on HPV-16 positive cervical cancer cell lines (SiHa and CaSki) and primary tumor tissues revealed a positive correlation of STAT3 with expression of HPV16 E6 and E7 oncoproteins and a negative association

with levels of p53 and pRB. STAT3 specific siRNA targeting showed accumulation of p53 and pRb in cervical cancer cell lines. These authors have found a positive correlation of active STAT3 with E6 and E7 and an inverse relation with p53 and pRB pools. Specific targeting of STAT3 expression in cervical cancer cell lines have been performed earlier using recombinant adenoviral dominant negative STAT3 or STAT3 specific siRNA. Specific silencing of E6/E7 using specific siRNA also results in similar growth inhibition of cervical cancer cells, loss of transformed phenotype, induced apoptosis and replicative senescence and inhibited tumor formation in animal models. Delivery of E6/E7 siRNA into nude mice has shown significant reduction in the number of tumor nodules and retarded tumor growth of HPV16 positive cells in NOD/SCID mice. According to these authors the loss of expression of oncoproteins is not only the direct and sole cause of p53 and pRB accumulation in cervical cancer cells. However, leads obtained from this study provide a strong rationale for developing novel STAT3 based approaches for therapeutic interventions against HPV infections to control cervical cancer.

6. Conclusion

Molecular targeting of HPV oncogene expression using RNAi technology has tremendous potential for therapeutic use. The challenge might be overcome by application of different delivery systems of siRNA to specifically target the E6 and E7 oncogenes.

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Conflicts of Interest

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE “uniform requirements” for biomedical papers.

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