RNA-based gene targeting therapies for human papillomavirus driven cancers

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Highlights

- RNA-based therapies could be the “clinical key” for treating HPV cancers
- RNAi and CRISPR technology targeting HPV cancers could hold clinical promise
- miRNAs and lncRNAs could serve as future treatment modalities for HPV cancers

Abstract

While platinum-based chemotherapy, radiation therapy and or surgery are effective in reducing human papillomavirus (HPV) driven cancer tumours, they have some significant drawbacks, including low specificity for tumour, toxicity, and severe adverse effects. Though current therapies for HPV-driven cancers are effective, severe late toxicity associated with current treatments contributes to the deterioration of patient quality of life. This warrants the need for novel therapies for HPV derived cancers. In this short review, we examined RNA-based therapies targeting the major HPV oncogenes, including short-interfering RNAs (siRNAs) and clustered regularly interspaced short palindromic repeats (CRISPR) as putative treatment modalities. We also explore other potential RNA-based targeting approaches such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and mRNA vaccines as future treatment modalities for HPV cancers. Some of these technologies have already been approved for clinical use for a range of other human diseases but not for HPV cancers. Here we explore the emerging evidence supporting the effectiveness of some of these gene-based therapies for HPV malignancies. In short, the evidence sheds promising light on the feasibility of translating these technologies into a clinically relevant treatment modality for HPV derived cancers and potentially other virally driven human cancers.

Keywords

siRNA; CRISPR; HPV; miRNA; lncRNA

Introduction

The host immune system is generally efficient at clearing HPV infections. In a situation where it is not, it can persist for years culminating in carcinogenesis [1]. Anogenital, uveal, oropharyngeal, and oesophageal cancers are caused by high-risk HPV types, particularly types 16 and 18 being the two most prevalent HPV-driven cancers, cervical (CC) and oropharyngeal squamous cell carcinoma (OPSCC) cancers [2-6]. Despite preventive vaccination and screening being widely available for cervical cancer, HPV continues to cause gynaecological based cancer deaths in women worldwide with 600,000 cases approximately in 2020, ranking as the ninth most prevalent cancer [7]. Of more significance currently are head and neck cancers (HNCs), which rank as the seventh most prevalent cancer worldwide with an annual incidence rate of approximately 900,000 cases [7]. Around one third of HNCs are now established to be caused by persistent infection with high-risk HPV (hrHPV) type 16 [8, 9]. Indeed, there is a strong association between hrHPV and OPSCC, a subset of HNCs, which has the fastest growing incidence rates among all cancers [10]. It is now the most common HPV-associated cancer, due to persistent infection with hrHPV type 16 [11]. Around 20% of HNCs do not respond to current treatments, have poor prognosis and its relationship to HPV-associated OPSCCs has had a recent dramatic increase in the past few years [9, 12, 13], outnumbering cervical disease in various countries, in particular the USA [14, 15]. Current HPV cancer treatment modalities include surgery or concurrent chemoradiation or both. These treatments have saved many lives, but patient outcomes often remain almost the same with poor prognosis and patient survival [16-18]. Because the process of carcinogenesis takes years, there is a gap for clinical prevention, diagnosis, and treatment that researchers are striving to take advantage of with the development of novel and gene-specific therapies. Here, we aim to review emerging RNA-based gene targeting approaches that have been tested for HPV-driven cancers.

Short-interfering RNA (siRNA)

Sequence-specific short-interfering RNAs (siRNA) are typically 21 nucleotides double stranded RNAs with two base pair 3' overhangs and work by suppressing gene expression via directly degrading target gene mRNA in the cytosol (Figure 1). This leads to the loss of gene target protein expression, with high efficiency and specificity. Three siRNA formulations have been approved by the Food and Drug Administration (FDA) and are now licensed for clinical use: Givlaari (Givosiran) for acute hepatic porphyria (AHP), Onpattro (also known as Patisiran) for hereditary transthyretin-mediated amyloidosis (hATTR) [19], and lumasiran (Oxlumo), for primary hyperoxaluria type 1. Indeed, siRNAs holds the potential as an RNA interference (RNAi)-based therapeutic agent for a range of cancers, including HPV-driven cancers.

Multiple HPV genes have been targeted experimentally for the purposes of therapy, particularly early non-structural genes E1, E2, E4, E5, E6 and E7. HPV frequently integrates into the host genome disrupting gene E2 repressor activity and in every cell cycle, the two notorious oncoproteins, E6 and E7, replicate continuously [20]. E6 and E7 are the most attractive targets as these oncogenes are known to be the main drivers of carcinogenesis. E6 exerts its effect by degrading p53, a tumour suppressor, which makes cells lose control of the mitotic cell cycle in G2/M checkpoint, whereas E7 inactivates phosphorylated retinoblastoma protein (pRb), taking the brakes off the cell cycle and thereby allowing cells to proliferate uncontrollably [21]. Since both genes are expressed from the same promoter, E6 and E7 are found together on the same mRNA transcript, though this differs between HPV types. siRNAs have been shown to reduce E6 and E7 expression, resulting in the subsequent restoration of p53 and Rb function respectively, to induce reduction of tumour growth, and cellular apoptosis or senescence or both (Table 1).

Early work attempting to silence E6 and E7 *in vitro* using siRNAs were done in HPV positive (+) CC cell lines, CaSki and SiHa [22]. Knockdown of E6 induced accumulation of p53 and reduced

cell growth, while E7 loss drove cells to undergo apoptosis. Since then, numerous studies have examined this strategy to target cancer cell and tumour growth (Table 1). The majority of studies reported effective tumour reduction with siRNA treatment targeting E6 and E7, demonstrating that oncogene targeting siRNAs are highly specific and able to reverse the biological effects of these oncogenes. An effective method for delivering these siRNAs is critical for this therapy to be clinically valid. siRNAs are too large and hydrophilic to travel across the cell membrane. Instead, they must be able to enter target cells to be released into the cytoplasm to knockdown the target mRNA. When administered, siRNAs have to go through numerous host obstacles, including innate immune system recognition, nucleases, non-specific interaction with serum proteins and non-target cells, renal clearance, cell entry, exit from blood vessels to reach target tissues and incorporation into the RNAi system. Over the last two decades, a number of nano-delivery technologies have been developed to surpass these obstacles, which can be improved with chemical modification, nanoparticle complexation and addition of targeting moieties [23-26]. However, this can be overcome with novel nanoparticle formulations, which have been tested for vaginal, intravenous, and intraperitoneal delivery of siRNAs in animal models for HPV cancers [27-31]. siRNA delivery using lentiviruses in HPV cancer animal models have also been done though the feasibility of using this platform in humans remain debatable and its clinical use have been limited to *ex vivo* gene therapy settings [32, 33].

CRISPR gene editing technology

Targeted genome editing technologies using programmable endonucleases is a rapidly evolving field. The most well-known system is the clustered, regularly interspaced, short palindromic repeats (CRISPR) associated endonuclease system. The best characterised CRISPR system uses Cas protein 9 (Cas9) (type II), which induces double stranded breaks (DSB) at specific sites of the

target DNA. Cas9 protein uses dual RNA, or guide RNA (gRNA), for site specific DNA cleavage. After cleavage, the DSBs are repaired by either the error prone non-homologous end joining (NHEJ) pathway, or the high-fidelity homology directed repair (HDR) pathway [34]. More recently, Cas13a (type VI) has also been employed in the CRISPR system to knockdown specific mRNAs [35]. CRISPR technology can be harnessed to generate loss-of-function or gain-of-function mutations in tumour suppressor genes, oncogenes or other modulators of gene expression. CRISPR can also be used to activate genes by inactivating the Cas proteins (dCas) and adding transcriptional activation domains (CRISPRa) [36].

The use of CRISPR mediated therapy for HPV cancers have been explored (Table 2). In the case of CC, tumours rely entirely on the continuous expression of both E6 and E7 proteins to survive [21], which renders these proteins an ideal therapeutic target. In HPV cervical cell lines, targeting E6 and E7 genes with CRISPR restored p53 and pRb cellular levels respectively, resulting in cell death and apoptosis [37, 38]. The same effect was also observed *in vitro*, where CRISPR knockdown of E6 and E7 genes led to inhibition of tumour growth [39]. Recently, another study delivered, nanoparticles with CRISPR targeting E6 or E7 completely ablated tumours in a CC xenograft *in vitro* model [40] further emphasising that these cervical HPV tumours are “oncogene addicted”.

Other strategies knocking out E6 and E7 expression by targeting their common early promoter region, p97, have been attempted using CRISPR [39]. Given that the length of both E6 and E7 genes are short, choosing unique target sequences within these genes can be challenging. Hence, the notion of targeting other regulatory regions might serve as an alternative strategy. In fact, HPV gene expression is largely regulated at the post-transcriptional level, including RNA processing, nuclear export, mRNA stability and translation, in addition to several early and late promoters

controlling the expression at the transcriptional level. Therefore, interrupting these regulatory networks may also be exploited therapeutically. CRISPR-based therapies could also be designed to target the region extending from the end of the L1 open reading frame (ORF) to the late 3' untranslated region (UTR), the region that encodes for key RNA stability regulators that preclude the expression of the HPV capsid proteins. Because these capsid proteins are highly immunogenic, restoring its expression would trigger a robust immune response, thus leading to the clearance of the virus [41]. Beside the early and late promoters, several other promoters have been described in various HPV genomes; such as a late promoter located at the beginning of E4 ORF, which regulates the expression of L1 capsid protein [42], and one located at E5 ORF that encodes for L2 as a first ORF [42]. However, exploiting these regulatory pathways to enhance virus clearance or interrupt gene expression remains hindered by our limited understanding of the molecular interactions involving multiple genomic regions.

The concept of reactivating p53 via CRISPRa, as a way of overcoming E6 mediated inactivation of p53, has yet to be tested but might prove an excellent approach. Tumour suppressor p53 plays an essential role in preventing HPV mediated tumorigenesis. The use of p53 as a gene therapy target for CC has been extensively explored due to the high percentage of p53 inactivation present [43]. As a proof of concept, overcoming p53 inactivation has been shown to work when *in vitro* mediated p53 protein delivery leads to apoptosis, cell cycle arrest and suppression of cell migration and invasion in HeLa cells [44]. As demonstrated in a range of other CC cell lines (HeLa, SiHa and C33A), recombinant adenovirus-p53 treatment inhibited cell growth and enhanced apoptosis by increasing the expression levels of exogenous p53 gene [45, 46]. A biological recombinant human p53 adenovirus gene therapy (Gendicine) was approved in 2003 by the China Food and Drug Administration (CFDA) to treat HNC patients. Gendicine transduces cells to express wild

type p53 protein and is administered either as an intratumorally or intracavity injection or as an intravascular infusion [47]. Although initially approved to treat HNCs, Gemtuzumab has been used to treat various other cancer types, including CC patients in combination with radiotherapy. Though idea of knocking down mRNAs via siRNA was discussed earlier, the CRISPR-Cas13a system possesses a similar ability. This system has been tested in various cancers models, including pancreatic cancer to inhibit mutant KRAS mRNA expression, as well as inhibiting SMAD7 mRNA (mRNA enhanced by oestrogen) [48, 49]. In both cases cellular apoptosis occurred and significant tumour shrinkage was observed. CRISPR-Cas 13a has also been shown to cleave HPV 16/18 E6/E7 mRNAs in SiHa and HeLa cell lines and in SiHa xenograft tumours resulting in cell death and tumour regression [50]. CRISPR gene editing for HPV-driven cancers is a rapidly emerging field, which carries feasible clinical promise as a future therapy.

Future and emerging RNA-based therapies for HPV cancers

Micro RNAs (miRNA)

MiRNAs are single stranded endogenous non-coding small RNA molecules, approximately 19 to 22 nucleotides in length, involved in the regulation of gene expression. They are capable of post-transcriptional changes by binding to the 3' untranslated region of the target mRNA to either affect translation (appoint mRNAs for cleavage and avoid protein synthesis) or reduced mRNAs stability. miRNAs are known to have a role in various cancer related biological processes such as differentiation, apoptosis, proliferation, metabolism, invasion, metastasis, and drug resistance, which makes them function as either tumour suppressors or cancer promoters [51-55]. There are certain miRNAs that may also have an effect in the genes that control some anti-tumour responses making the miRNAs dysregulation somehow related to the origin of certain types of cancer [56].

Genome wide miRNA profiling has shown that there could be different expression patterns when certain miRNAs are present in various human infectious diseases such as Ebola virus, Coronavirus 19 (COVID-19), Human immunodeficiency virus (HIV) and Kaposi sarcoma-associated herpes virus, where latter two are have carcinogenic potential [57-60]. As a result, numerous studies that have used miRNAs as potential biomarkers for cancer diagnosis, prognosis, and cancer staging. miRNA profiles in HPV cancers have also been explored (Table 3). Briefly, miR-10b, miR-15a, miR-20b and miR-16 have been found to be upregulated and miR-139-3p, miR-145, miR-381 and miR-574-3p downregulated in CC and HNC HPV tumours [61]. However, the presence of certain miRNAs was more prevalent in some than other HPV cancer types. In CCs, miR-16, miR-20a, miR-21, miR-34a and miR-182 were more prevalent whereas in HNCs miR-9 is prevalent (Table 3). It was found that miR-16 in non-small cell lung cancer has the ability to induce cell cycle arrest in G1-G0 when Rb is present suggesting that this miRNA may be involved in cell cycle progression in HPV cancer [62]. miR-20a has been shown to be upregulated by HPV16 E6 in CC and promotes cell growth [63]. On the other hand, miR-21 is involved in post-transcriptional downregulation of the tumour suppressor gene, PTEN, to promote cell proliferation and cancer survival [64]. Moreover, it was demonstrated that miR-34a was elevated either by HPV16 E7 or chronic levels of oestrogen, in early cancer stages, but it was downregulated when both factors were present in the late stages [65]. High-risk HPV E7 has been shown to upregulate miR-182 expression through the TGF- β /Smad4 pathway, a pathway critical in cancer progression [66]. It is interesting to note that miR-9 is upregulated only in squamous cell carcinoma caused mainly by HPV16 promoting cell viability and anchorage independence, while in HPV18+ cells it plays a tumour suppressive role [67].

The use of miRNAs for targeting HPV cancers has been investigated but not to the degree that siRNAs have been used. Targeting HPV16/18 E6 mRNA with miRNAs in HeLa and SiHa cells resulted in apoptosis but failed to observe any regression in HeLa derived tumour growth *in vitro* [68]. Another study showed that inhibition of miR-93-5p, which regulates B-cell translocation gene 3 (BTG3) expression, blocks the proliferation, invasion and migration and promote apoptosis of HPV+ CC cells, CaSki and HeLa [69]. It was also found that miR-3156-3p expression inhibited cell growth and promoted apoptosis in HPV18 (HeLa) and HPV16 (SiHa and CaSki) + cells [70]. Furthermore, it was also shown that miR-155-5p enhances autophagy of cancer cells by targeting the expression of PDK1 and inhibiting the Akt/mTOR signalling pathway [71]. In addition, overexpression of miR-10b in HeLa and SiHa repressed cell proliferation, migration, invasion, and induced apoptosis [72]. T-cell lymphoma invasion and metastasis 1 (Tiam1) is a direct and functional target of miR-10b which when suppressed by the miRNA acted as a tumour suppressor. In conclusion, there are potential miRNA candidates that could be utilised for HPV cancer therapy but further work to understand the use of miRNAs such setting is warranted. Despite all this, miRNAs have not been widely used to treat HPV infections because their mechanisms of action are not well understood and are still being studied. Only few miRNAs have been demonstrated to be altered in HPV-associated cancers. Therefore, it is likely that miRNAs will be initially used as biomarkers for HPV+ cancers as further work is needed before moving forward with using miRNAs therapeutically.

Long non-coding RNAs (lncRNA)

lncRNAs are transcripts that range in size (100 - 100000 nucleotides) and are diverse in structure. They function either as primary or spliced transcripts, are polyadenylated or non-polyadenylated and can be found in either the nucleus or the cytoplasm. lncRNAs lack evident ORFs and

sometimes can be transcribed by RNA polymerase II and/or III. LncRNAs are also less expressed than protein coding genes, though this depends on the cell type. LncRNAs are still being characterised as their exact function remains elusive. As with many ncRNAs, lncRNAs could serve as potential biomarkers for certain human diseases, including HPV cancers. Indeed, the role of lncRNAs in HPV-driven pathogenesis is well-known [73]. In the context of HNCs, 140 lncRNAs have been identified to be differentially expressed in HPV+ HNC samples compared to HPV- HNC samples [74].

There is also an opportunity to analyse particular roles of certain lncRNAs and determine possible targets for targeted therapy. One group studied the expression and regulation of lncRNA T5CF-7 and miR-155 in HPV 18+ CC cells [75]. Knockdown of T5CF-7 inhibited the cell growth and that miR-155 overexpression reverses this. In another study, lncRNA SNGH8 was shown to induce CC cell proliferation and migration when it binds to enhancer of zeste homolog 2, nuclear regulatory protein (EZH2) and inhibits transcriptionally reversion inducing cysteine rich protein, stops angiogenesis (RECK) expression making SNGH8 a good target to directed therapy for CC [76]. Another study found an interplay between HPV16 E7 oncoprotein and lncRNA HOX antisense intergenic RNA (HOTAIR) [77]. E7 downregulates HOTAIR causing anomalies in the chromatin remodelling complex PRC2 (creating gene silencing H3K27 me3) alongside the cellular processes of cell proliferation and metastasis. Recent work has shown that the damage-induced lncRNA, DINO, that is known to bind and stabilise TP53, is expressed at low levels in a range of HPV+ CC cell lines as a result of HPV E6-mediated TP53 degradation [78]. Indeed, acute ectopic expression of DINO sensitises cells to standard-of-care CC chemotherapeutics, including cisplatin. lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) working alongside miR-124 and growth factor receptor-bound protein 2 (GRB2) was found to promote high-risk HPV

cancers [79]. This suggests that lncRNAs do not work alone, as they can indirectly regulate mRNAs expression by trapping miRNAs acting as sponges for other RNAs to regulate metabolic pathways. However, there remains little evidence to support proposal of using lncRNAs for HPV cancer therapy. Further work is needed to explore this possibility.

mRNA-based HPV vaccines

Current vaccines available for CC include, Cervarix® (HPV 16, 18), Gardasil4® (HPV 16, 18, 6, 11), and Gardasil9® (HPV 6, 11, 16, 18, 31, 33, 45, 52, 58) and have shown to confer good protection [80]. This protection is mediated by neutralising antibodies generated against HPV L1 capsid protein, which are immunogenic [81]. Prophylactic vaccines currently available are not effective either when the infection is prevalent or has established intraepithelial lesions. In the carcinogenic stage, the microenvironment is immunosuppressed by HPV (particularly by E6 and E7) [82] and T-cells are either anergic or exhausted and fail to detect viral antigens. Hence the challenge is to create a therapeutic vaccine that generates cytotoxic T-lymphocytes that recognise HPV antigens.

Synthetic mRNA was first administered into mouse skeletal muscle in 1990 and demonstrated that it can express the target protein in cells [83]. The recent COVID-19 epidemic has led to the advent of the rapid development of mRNA vaccines. mRNA vaccines carry several advantages; it is safe (no virus/protein contamination or genome integration) and activates a strong adaptive immune response without the need for adjuvants. In the case of HPV, the putative targets are E6 and E7. HPV cancer vaccines, that target tumour associated antigens (E6 and E7), have been developed in the form of a lipidic protein with the mutant form of HPV16 E7 (rlipo-E7m) and has shown to inhibit tumour growth in mice [84]. A phase II trial of imiquimod and a HPV therapeutic vaccine

(a fusion HPV16 E6E7L2 protein formulation called TA-CIN) in patients with vulval intraepithelial neoplasia reported complete regression of lesions in 63% of the patients [85].

In the future, the development of an mRNA vaccine for HPV cancers can utilise other oncoproteins (other than E6 and E7) or specific components of the virus making the vaccine safer, cheaper, and not only prophylactic but possibly therapeutic in cancer later stages.

Conclusions

Currently, the promise of genetic based treatments for HPV remains in preclinical stages. Promising results are clear and await support for clinical translation. mRNA vaccines for COVID-19 are now a reality and can potentially be applied for HPV cancers. Given that the use of siRNAs as therapeutic agents have already reached the clinic for a number of diseases [19], this could potentially be a reality for HPV cancers. Using CRISPR as a potential therapeutic for HPV cancers may become a reality with the recent successful Phase I/II CRISPR-based therapy tested in patients with transthyretin amyloidosis (ATTR), a rare disease caused by the progressive accumulation of misfolded TTR protein in tissues [86]. Indeed, CRISPR gene editing of the TTR gene resulted in demonstrable loss of serum TTR protein in patients. Given that CC is “oncogenically addicted” to E6 and/or E7 [40], the use of CRISPR therapy to edit one of these oncogenes, could prove to be therapeutically feasible clinically. CRISPR could become a concomitant therapy to the standard treatment modality (surgery, radiation, and chemotherapy) for HPV cancers.

miRNAs and lncRNAs have been studied in a myriad of settings on HPV derived cancers.

Initially proposed to be used solely as cancer biomarkers, it is becoming increasingly evident that miRNAs and lncRNAs could be utilised as therapeutic targets. Given that multiple miRNAs and lncRNAs play a role in HPV cancers, it seems clinically unfeasible to target multiple miRNAs and lncRNAs though it could possibly work when combined with standard treatment protocols

for HPV cancers. In conclusion, nanoparticle/viral vector-based co-delivery of chemotherapy drugs or radiotherapy with RNA-based gene targeting therapies offers a new alternative to start eliminating HPV derived cancers at present. The future of RNA-based gene targeting therapies for HPV cancers is exciting and much anticipation is expected with the advent of RNA-based therapies being deployed for COVID-19 in recent times.

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Tables

Table 1 siRNA - mediated targeting of HPV E6 and E7 oncogenes

Target	Delivery vehicle	Experimental model	Findings	Reference
HPV16 E6	(DSPC), (DMG-PEG), and (DSPE-PEG-Ome) lipid nanoparticle with targeted antihuman EGFR mAbs and siRNA - (tLNP-siRNA)	Cell lines (FaDu, 2A3, SCC104, and SCC90) Xenograft model athymic Foxn1 nude female mice (SCC104)	>49% tumour growth inhibition with siRNA E6 in tLNP-siE6	[87]
HPV18 E7	siRNA+paclitaxel (PTX) in poly lactic-co-glycolic acid (PLGA) nanoparticles surrounded by camouflaging HeLa cell membranes (Si/PNPs@HeLa)	Cell line (HeLa) Xenograft model BALB/c nude mice (HeLa)	>80% tumour growth inhibition with siRNA E7 in Si/PNPs@HeLa	[88]
HPV16 E6	Oligofectamine (Invitrogen)	Cell line (CaSki)	>42% loss of cell viability due to apoptosis with siRNA	[89]

HPV16 E6/E7	Oligofectamine (Invitrogen)	Cell lines (SCC2, SCC104, SCC90 and 147T) Xenograft model BALB/c nude mice (SCC104)	16E6/E7 siRNA resulted in >35% growth tumour inhibition	[90]
HPV18 E6/E7 HPV16 E6/E7 MCL-1 (antiapoptotic protein)	Oligofectamine (Invitrogen)	Cell lines (CaSki, SiHa, UPSCI-SCC154, C4II, HaCaT)	siRNA MCL1 plus siRNA E7 resulted in apoptosis of CaSki (10%), SiHa (26%), UPSCI-SCC154 (9%), C4II (6%)	[91]
HPV18 E6/E7 HPV16 E6/E7	DharmaFECT transfection reagent (Thermo Scientific, USA)	Cell lines (HeLa CaSki) Xenograft model BALB/c nude mice (HeLa)	Simultaneous use of 18E6/E7 siRNA with cisplatin, and paclitaxel resulted in >60% growth tumour inhibition	[92]
HPV18 E6/E7 HPV16 E6/E7	Core-shell type polyion complex micelles with biocompatible outer shell of modified polyethylene glycol (PIC micelles) Lipofectamine 2000 (Invitrogen)	Cell lines (HeLa CaSki, SiHa, C33A) Xenograft model BALB/c nude mice (HeLa and SiHa)	SiHa and HeLa cell tumours were reduced by 60% post-treatment with PIC micelles loaded with si16E6/E7 and si18E6/E7	[93]
HPV18 E7	PEGylated liposomes encapsulating siRNA (PLEsiRNA)	Cell line (HeLa)	PLEsiRNA with siRNA 18E7 diminished mRNA expression in HeLa cells	[94]
HPV16 E7	Lipofectamine 2000 (Invitrogen)	Cell line (CaSki)	16E7 siRNA diminished HPV16 E7 protein expression	[95]
HPV16 E6/E7	RNAiFect Transfection Reagent (Qiagen, USA) for cells 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) neutral nanoliposomes (NP) for mice	Cell line TC-1 (murine C57BL/6 lung epithelial cells transformed with HPV16 E6, E7 and h-ras oncogenes) Xenograft model C57BL/6	65% tumour reduction after treatment with siRNAE7-DOPC-NP	[96]
HPV18 E6/E7 HPV16 E6/E7	DharmaFect (Dharmacon, Lafayette, CO, USA)	Cell lines (HeLa, Hela-Luc, SiHa) Xenograft model BALB/c nude mice (HeLa)	>50% growth tumour inhibition with radiation and HPV18 or HPV16 E6/E7-specific siRNAs	[97]
HPV16 E6/E7	Cationic liposome-encapsulated siRNAs (ClesRNA)	Cell line (TC-1) Xenograft model C57BL/6 (TC-1_	60% tumour inhibition post-administration of liposome encapsulated with bicistronic E6/E7 siRNA	[98]

HPV16 E6/E7	Lipofectamine RNAiMAX (Invitrogen)	Cell line (UM-SCC47) Xenograft model BALB/c nude mice (UM-SCC47)	UM-SCC47-derived tumours reduced by 60% with the combination of siRNA 16E6 and 16E7	[99]
HPV18-E6/E7	Lipofectamine 2000 (Invitrogen)	Cell lines (VUP-HPV18+, HeLa, OCM1, OM431, ARPE-19, SP6.5)	VUP-HPV18+ cells transfected with HPV 18 E6/E7-specific siRNA resulted in 40% cell growth inhibition	[100]
P97 HPV16 HPV16 E6	Lentiviral vectors (LV-shRNA)	Cell lines (CaSki, SiHa, HeLa and C33A) Xenograft model BALB/c nude mice (SiHa, CaSki)	Inhibition of SiHa tumour growth >80% and CaSki tumour >94% when using P97 and E6 siRNA	[101]
HPV16 E6/E7	Lipofectamine 2000 (Invitrogen)	Cell lines (SiHa) Xenograft model BALB/c nude mice (SiHa)	>81% tumour reduction with siRNA E6/E7 and co expression of p53	
HPV16 E7	Chitosan/siRNA nanoparticles (CsiN)	Cell line (CaSki)	>30% CaSki cells became apoptotic after treatment with CsiN complexed 16E7 siRNA	[102]
P97 HPV16 HPV16 E7	Lipofectamine™ RNAi MAX (Invitrogen)	Cell line (SiHa) Xenograft model BALB/c nude mice (SiHa)	Inhibition of tumour growth of 89.7% when using in vitro P97 HPV16 siRNA and 64% with HPV16 siRNA	[103]
HPV18 E6 HPV18 E6/E7	Oligofectamine (Invitrogen)	Cell lines (HeLa, SiHa, CaSki) Xenograft model BALB/c nude mice (HeLa, SiHa, CaSki)	Simultaneous use of 18 E6/E7 siRNA and cisplatin decreased tumour growth by 80%	[104]
HPV16 E6/E7	Hydration-of-freeze-dried-matrix (HFDM)-PEGylated liposomes	Cell line (TC-1) Xenograft model C57B/6 mice (HeLa) Murine lung epithelial cancer cells (MLECC)	HPV16 E6/E7 siRNA decreased tumour growth by 50% (P<0.01)	[31]
HPV16 E6/E7	PEGylated liposomes	Cell line (TC-1) Xenograft model C57B/6 mice (TC-1)	HPV16 E6/E7 siRNA decreased tumour growth by 45%	[28]

MLECC				
HPV18 E6/E7 HPV16 E6/E7	Lipofectamine 2000 (Invitrogen) in cells	Cell lines (HeLa, CaSki, C33A) Xenograft model BALB/c AnN- Foxn1nu/CrlNarl nude mice (HeLa)	HPV-18E6 siRNA decreased tumour growth >50% at day 30	[105]
P97 (Promoter of HPV16 E6/E7)	CodeBreaker siRNA Transfection Reagent (Promega)	Cell lines (SiHa, C33A)	siRNA P97 induced 4.4 ± 0.8% apoptosis and 57.1 ± 4.4% cell death	[106]
HPV16 E6/E7	Dharmacon RNA Technologies' SiQuest transfection reagent	Cell line (CaSki) Xenograft model female nu/nu athymic mice (CaSki)	>75% tumour reduction with siRNA E6 and a 60% tumour reduction with siRNA E7	[107]
HPV16 E6/E7	Lipofectamine 2000 (Invitrogen)	Cell lines (CaSki, SiHa, HeLa, SK- OV-3) Xenograft model NOD/SCID mice (SiHa)	>35% reduction in tumour volume with siRNA targeting E6/E7	[108]
HPV16 E6/E7	Lipofectamine 2000 (Invitrogen)	Cell line (SiHa)	siRNA E6 + E7 induced 63.7% of cell apoptosis	[109]
HPV16 E7	Lipofectamine 2000 (Invitrogen)	Cell lines (SiHa, CaSki, C33A)	pSIREN-16E7 transfected SiHa cells underwent apoptosis (>50%)	[110]
HPV18 E6 HPV18 E7	Oligofectamine (Invitrogen)	Cell lines (HeLa, C4I, H1299)	Majority of E6 and E7- siRNA treated HeLa cells died	[111]
HPV16 E6 HPV18 E6	HiPerFect (Qiagen)	Cell lines (HeLa, CaSki, SiHa)	80% reduction in cell proliferation when treated with a combination of siRNA16 E6, 4C6 (antibody against HPV16 E6), and K15C (allows formation of antibody-lipid complex)	[112]
HPV18 E6/E7	sureFECTOR (B-Bridge International)	Cell lines (HeLa, SKG-II, SKG-IIIa) Xenograft model athymic nude mice (SKG-II)	>65% of tumour growth reduction when treated with HPV18 E6/E7 siRNAs and atelocollagen	[113]
HPV16 E6	TransMessenger™, Qiagen, Valencia, CA	Cell line (CaSki)	CaSki cells derived tumours reduced by	[114]

		Xenograft model mice (CaSki)	>75% after treatment with HPV16 E6 siRNA	
HPV16 E6/E7 HPV18 E6/E7	Oligofectamine (Invitrogen)	Cell lines (CaSki, SiHa, HeLa)	siRNAs targeting E6 reduced HeLa cell viability by 60% to 80%	[115]
HPV18 E6	Lentiviral vectors (LV-shRNA) 18-E61/18E6-2	Cell line (HeLa) Xenograft model Rag-/- mice (HeLa)	High dose (4x) of LV-shRNA 18E6-1 caused complete tumour ablation	[32]
HPV18 E6/E7	Oligofectamine (Invitrogen)	Cell line (HeLa)	18E6 siRNA decreased the number of surviving HeLa CMV cell clones by ~20%	[116]
HPV16 E6 HPV18 E6	Oligofectamine (Invitrogen) RNAi lentivirus	Cell lines (SiHa, CaSki, HeLa, C33A)	CaSki cells after transfection with siRNA E6 reduced cell viability >60% HeLa cells infected with lentivirus delivering a shRNA plasmid against HPV18 E6 in HeLa with cisplatin reduced cell viability >60%	[33]
HPV16 E7	Agarose/liposome/E7 siRNA formulation (gel)	Cell line (SiHa)	>19% of SiHa cells became apoptotic after gel delivery containing E7 siRNA	[117]
HPV16 E6	Lipofectamine 2000 (Invitrogen)	Cell lines (SiHa, COS-1) Xenograft model NOD/SCID mice	>40% reduction in SiHa cell-derived tumour volume post-treatment with HPV16 E6 targeting siRNAs	[118]
HPV16 E6 HPV18 E6	Oligofectamine (Invitrogen)	Cell lines (HeLa, SiHa, MCF-7, MeWo, H1299)	siRNAs targeting HPV16 E6 or HPV18 E6 resulted in increased p53 expression and cell apoptosis	[119]
HPV18 E7	Oligofectamine (Invitrogen)	Cell lines (HeLa, C33A)	E7 siRNA reduced E7 and E6 protein expression and induces senescence >80% in HeLa cells	[120]
HPV16 E6/E7	Oligofectamine (Life Technologies)	Cell lines (CaSki, SiHa)	>20% cells became apoptotic after E7 siRNA treatment in SiHa	[22]

Table 2 **CRISPR - mediated targeting of HPV E6 and E7 oncogenes**

Target	Delivery vehicle/Transfection process	In vitro/In vivo model ¹	Findings	Reference
HPV18 E6/E7	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) liposomes	Cell lines (HeLa, HEK293, RD, A549, HEPG2, 4T1, CaSki) Xenograft model Female BALB/c nude mice (HeLa)	>95% HeLa cells tumour growth inhibition with 18E6/E6 sgRNA CRISPR/Cas9 plus docetaxel inside liposome DOTAP	[121]
HPV16 E6 HPV18 E6	High-capacity adenoviral vectors (HCAdVs)	Cell lines (HeLa, SiHa, CaSk, A549)	>85% SiHa significant cell reduction with HPV16-E6-specific CRISPR-HCAdV, the other cells were not significant	[122]
HPV18 E6/E7	Lipofectamine 3000 (Invitrogen)	Cell lines (SiHa, HeLa) Xenograft model BALB/c nude mice (HeLa)	>59% SiHa cells tumour growth inhibition with 18E6/E6 sgRNA CRISPR/Cas9	[123]
HPV16 E6/E7 HPV18 E6/E7	X-tremeGene HP with nucleofection (Roche)	Cell lines (SiHa, HeLa, C33-A) Xenograft model Female BALB/c nude mice (SiHa, HeLa)	>70% SiHa cells tumour growth inhibition with 16E6 crRNA CRISPR/Cas13a	[50]
HPV16 E7 HPV18 E7	Poly(amide-amine)-poly(β -amino ester) hyperbranched copolymer (hPPC)/PBAE-GFP polyplex nanoparticles	Cell lines (SiHa, HeLa) Xenograft model Female nude (nu/nu) mice (SiHa)	90% tumour growth inhibition (SiHa cells) with hPPC1-16E7 CRISPR/Cas9	[124]
HPV16 E7 HPV18 E7	PEGylated liposomes	Cell lines (HeLa, CasKi) Xenograft model Rag 1 mice (HeLa, CasKi)	100% HeLa cells tumour clearance with 18E7 and CasKi cells tumour with 16E7 gRNA CRISPR/Cas9	[40]
HPV18 E7	Self-assembled micelle composed of quaternary ammonium-terminated poly (propylene oxide) (PPO-NMe ₃) and amphiphilic Pluronic F127 (F127/ PPO-NMe ₃ /pCas9-GFP)	Cell line (HeLa) Xenograft model Female nude (nu/nu) mice (HeLa)	>38% tumour growth inhibition with (F127/ PPO-NMe ₃ /pCas9-GFP/gRNA) micelles with E7 gRNA	[125]

HPV18 E6	Adeno-associated virus (AAV) vector	Cell lines (HeLa, HCS-2, SKG-1, HEK293) Xenograft model BALB/c nude mice (SKG-1)	>85% tumour growth inhibition with E6 gRNA CRISPR/Cas9	[126]
HPV16 E6/E7 Promoter of HPV16 E6/E7	Lipofectamine 2000 (Invitrogen)	Cell lines (SiHa, C33-A) Xenograft model BALB/c nude mice (SiHa)	>90% tumour growth inhibition with promoter+E6+E7 gRNA CRISPR/Cas9	[39]
HPV16 E7	X-tremeGENE HP DNA transfection reagent (Roche) jetPEI polymer-based DNA transfection reagent (Polyplus)	Cell lines (SiHa, CaSki, HEK293, C33A)	56% apoptosis in SiHa cells and 48% apoptosis in CaSki cells with E7 gRNA CRISPR/Cas9	[38]
HPV18 E6/E7 HPV16 E6/E7	FuGENE® 6 (Promega)	Cell lines (HeLa, CaSki)	>80% with E6 sgRNA and >56% with E7 sgRNA apoptosis in HeLa cells	[37]

Table 3 miRNAs in HPV derived cancers

Expression status	miRNAs		References
	Cervical cancers	Head and neck cancers	
Upregulated	miR-9, miR-10b*, miR-15a*, miR-16 (4), miR-20a (2), miR-20b*, miR-21 (5), miR-25, miR-27a, miR-34a (4), miR-92a, miR-106b, miR-124, miR-135b, miR-141, miR-146a, miR-155, miR-182 (2), miR-183, miR-196a, miR-210, miR-221, miR-223, miR-301b, miR-378, miR-449a	miR-9 (3), miR-10b*, miR-15a*, miR-16, miR-20b*, miR-134, miR-210, miR-455, miR-196b, miR-338, miR-376c, miR-378a, miR-380, miR-493	CC [61, 63-66, 127-133] HNC [61, 67, 134-136]
Downregulated	miR-22, miR-27a, miR-29a, and miR-100, miR-125a, miR-126, miR-195, miR-218, let-7a, miR-139-3p*, miR-145*, miR-381* and miR-574-3p*	miR-29, miR-139-3p*, miR-145*, miR-381* and miR-574-3p*	CC [61, 63, 131, 132, 137, 138] HNC [61, 136]

*both upregulated/downregulated in CC and HNC

Figures

Figure 1 Mechanism of action of therapeutic RNAi targeting HPV E7 delivered using lentiviruses or lipid nanoparticles. RNAi targeting HPV E7 mRNA can either be delivered using lipid nanoparticles or lentiviral vectors. Lentiviral vectors carrying short hairpin RNA (shRNA) is taken up by cells through receptor-mediated endocytosis. Viral material containing targeting shRNA is then released into the cytosol, reverse transcribed before integrating into the host cell genome. The shRNA is then expressed and released into the cytosol. On the other hand, naked shRNA and siRNAs can also be delivered into the cells using lipid nanoparticles (LNPs), which will release the RNAi cargo into the cytosol. shRNA is cut by an RNase III endonuclease, DICER,

and forms a protein complex called RNA induced silencing complex (RISC), whereas exogenous siRNA directly forms a complex with RISC. Here the siRNA-RISC complex cuts the complementary E7 mRNA resulting in endonucleolytic mRNA cleavage and subsequent loss of E7 expression. Figure was created with BioRender.com.