Review

Oncogenic Human Papillomavirus: Application of CRISPR/Cas9 Therapeutic Strategies for Cervical Cancer

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Key Words

Hpv • Cervical cancer • CRISPR/Cas9

Abstract

Oncogenic human papillomaviruses (HPVs) cause different types of cancer especially cervical cancer. HPV-associated carcinogenesis provides a classical model system for clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) based cancer therapies since the viral oncogenes E6 and E7 are exclusively expressed in cancerous cells. Sequence-specific gene knockdown/knockout using CRISPR/Cas9 shows promise as a novel therapeutic approach for the treatment of a variety of diseases that currently lack effective treatments. However, CRISPR/Cas9-based targeting therapy requires further validation of its efficacy *in vitro* and *in vivo* to eliminate the potential off-target effects, necessitates verification of the delivery vehicles and the combinatory use of conventional therapies with CRISPR/Cas9 to ensure the feasibility and safety. In this review we discuss the potential of combining CRISPR/Cas9 with other treatment options as therapies for oncogenic HPVs-associated carcinogenesis. and present our assessment of the promising path to the development of CRISPR/Cas9 therapeutic strategies for clinical settings.

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Introduction

Human papillomaviruses (HPVs) are small DNA viruses with a genome size ~8 kb long (Fig. 1). They infect cutaneous or mucosal epithelial cells, genital tissues, and the upper respiratory tract. To date, over 200 genetically distinct subtypes of HPV have been identified, and approximately 90 genotypes have been fully characterized. Among these types, the high-risk HPVs (HR-HPVs), including HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, are associated with more than 90% of cervical cancers, and to a lesser extent with other anogenital cancers and head and neck cancers [1-4]. Over 50% of HPV-positive cervical

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cancers are with HPV-16, followed by HPV-18 (12%), HPV-45 (8%), and HPV-31 (5%) [5, 6]. Moreover, HPV-16 is associated with a small number of head and neck neoplasia, particularly tonsillar and oro-pharyngeal cancers [7]. Low-risk HPVs (LR-HPVs), including HPV-6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81, have been linked to benign epithelial lesions [8]. In contrast to HR-HPV, infections with low-risk HPVs (LR-HPV) types 6 and 11 are associated with genital warts, essentially laryngeal papilloma, and recurrent respiratory papillomatosis [9].

The HPV genome is composed of an early region (E) that encodes open reading frames involved in the regulation of viral replication and the viral life cycle, and a late region (L) that encodes two ORFs (L1 and L2) that form the viral capsid. During the course of HR-HPV-mediated cancer development, the viral DNAs are frequently integrated into host cell chromosomes, and the proteins encoded by the viral genes play a critical role in carcinogenesis. When infecting host, most HPV are cleared within months, but some HPVs persistently exist and viral oncogenes constantly express to inactivate p53 and Rb, leading to increased genomic instability, accumulation of somatic mutations, and in some cases integration of HPV into the host genome [10, 11].

Fig. 1. Genome of HPV virus.

HR-HPV and Cervical cancer

Cervical cancer is one of the most common types of gynecological malignancies worldwide. According to the World Health Organization, the global prevalence of HPV infections was approximately 630 million cases in 2012, and 190 million cases were clinical infections leading to 528,000 new diagnoses of cervical carcinoma and ~266,000 deaths [12]. Advances in research continue to improve the precautionary methods available in developed countries. Clinical and molecular epidemiological studies have clearly demonstrated that the major cause of cervical cancer is infection with HR-HPVs, such as types 16 and 18. Strength of the association: The strength of association between HR-HPV and cervical cancer is considered one of the strongest for a human cancer. Recent studies have shown that HPV (all types combined) is present in >95% of cervical cancers. Consistency: The presence of HR-HPV in cervical cancer is consistent among a large number of studies, regardless which kind of HPV testing systems being used. There are no published studies with negative observations that challenge the association of HR-HPV and cervical cancer. Specificity: HR-HPVs are related to specific cancers. The type of HPVs is important in the development of specific cancers, such as cervical and head and neck cancers. HR-HPVs are present in the tumor cells. Viral oncogene expression (E6 and E7) occurs in tumor cells, but not in stromal cells. Temporality: HR-HPVs infections precede pre-cancerous cervical lesions and cervical cancer by years to decades. Biological gradient: Unclear, but early studies show that cervical cancer is associated with high viral loads [10-12]. Biological plausibility: HR-HPVs are powerful carcinogens that immortalizes human keratinocytes *in vitro*. HR-HPVs are present in cervical cancer, where they express the oncogenic proteins E6 and E7 to inactivate the host regulatory proteins p53 and RB, respectively. Epidemiological studies support a role for HR-HPV in cervical cancer. Biological coherence: The association does not conflict with what is known about the natural history of cervical cancer development. Experimental evidence: *In vitro* and *in vivo* evidence supports a causal role for HR-HPV in the development of cervical cancer. Analogy: Other DNA viruses can induce cancers in humans, and species-specific papilloma viruses can induce cancers in animals. Although early cervical cancer can be treated with surgery or radiation, metastatic cervical cancer is incurable and new therapeutic approaches are urgently needed. While most HPV infections are cleared within months, some infection persist and

viral oncogenes constantly express to inactivate p53 and Rb, leading to increased genomic instability, accumulation of somatic mutations, and in some cases integration of HPV into the host genome [10, 11].

The role of E6 and E7 in carcinogenesis (Fig. 2)

Fig. 2. Oncogenic pathways of E6 and E7.

The HPV E6 protein is a ~150 amino acids-length basic polypeptide containing two zinc-finger motifs [13], each consisting of a CXXC-X29-CXXC sequence and the PDZ-binding epitope at C-terminal [14, 15]. The expression regulation of E6 can be achieved through splice donor sites that give rise to truncated forms of E6, denoted as E6*I~IV, dependent upon the position of the downstream splice acceptors [16-19]. The most abundant splice RNA, E6*I, actually function as an E6 mRNA for efficient E6 translation [20-22]. The HR-HPV E6 protein and the truncated E6*I peptide destabilize several host proteins involved in cell growth and differentiation [19, 23]. Oncogenic HPV infection also deregulates the expression of oncogenic and tumor suppressive miRNAs via E6-TP53 pathways [24, 25].

E6 oncogene is expressed after viral integration. E6 protein facilitates several cellular changes to prolong the cellular lifespan by blocking apoptosis and increasing telomerase activity. The transcriptional activator role of E6 may be coupled with its ability to immortalize and transform cells [26]. E6 binds to cellular proteins, particularly to the HECT domain of ubiquitin ligase E3A [UBE3A, also known as E6-associated protein (E6AP)], and to E6BP (reticulocalbin 2, an EF-hand calcium-binding domain). UBE3A interacts with HPV E6 at a conserved LXXLL motif and forms ternary complexes with TP53, resulting in TP53 degradation through ubiquitin-dependent mechanisms [27-29]. Moreover, E6 can block the translocation of TP53 into the nucleus [30] and thereby inhibit the gene expression regulatory functions of TP53. The promotion of TP53 degradation and block of TP53 transportation into the nucleus by E6 disrupt TP53-mediated cell cycle control, allowing continued cell division despite DNA damage. In previous studies, the results demonstrated that expression of the HPV16 E6* isoform increases oxidative stress and induces oxidative DNA damage in host cells [31, 32]. HR-HPV E6 can also impair apoptosis by accelerating degradation of Bak, c-Myc, FADD, and procaspase-8. Furthermore, E6 binds to E6TP1, hADA3, tuberlin, CBP/p300, and Gps2, interfering with the function of these proteins to finally stimulate cell proliferation. E6 also suppresses the innate immune system through binding to IRF-3, as well as downregulating TLR9 expression [33]. Also oncogenic HPV E6 is capable of regulating the expression of many cellular miRNAs like miR-34a [34].

E7 ORF encodes an acidic phosphoprotein with zinc-binding motifs consisting of CXXC-X29-CXXC [35] that is essential for proper protein folding and stability. The E7 protein is primarily localized in the nucleus, capable of inducing cellular proliferation, immortalization, and transformation [36]. HR-HPV E7 confers transforming activities and therefore immortalizes human keratinocytes via interacting with factors involved in the regulation of cell growth [37]. Most E7 proteins contain a strictly conserved LXCXE-binding motif that interacts with members of the RB family of tumor suppressors, resulting in ubiquitin-mediated degradation of the RB family members [38]. Binding of E7 to hyper-phosphorylated RB (pRB) results in the release of the E2F transcription factor, activating gene transcription [39]. E7 from both HR and LR-HPVs possesses the ability to bind to pRB, although the interaction between LR-HPVs E7 and pRB is much weaker [40]. Furthermore, HR-HPV E7 interacts with and upregulate expression of cyclins A and E, inactivates the cyclin-dependent

kinase (CDK) inhibitors CDKN1A (p21CIP1) and CDKN1B (p27KIP1), finally promotes cell cycle progression [41]. HPV-16 E7 protein can modulate the cytoplasmic localization of CDKN1B (p27KIP1) and in turn regulate tumor metastasis/aggressiveness through the PI3K/AKT pathway [42].

Therapeutics against HPVs

In 2006, the US Food and Drug Administration (FDA) approved Gardasil to be used in girls and women for the prevention of cervical, vaginal, and anal cancers and genital warts. In 2009, FDA approved it to be used in boys and men to prevent anal cancers and genital warts. In 2014, a new 9-valent HPV vaccine was approved. However, current vaccines are not evidently effective for the therapy of cervical cancer patients [43, 44].

For cervical cancer therapy, E6 or the E6/UBE3A complex deserve special attention as specific targets. Several strategies targeting E6 or the E6/E6-AP complex have been developed, exemplified by cytotoxic drugs, a zinc-ejecting inhibitor of the viral E6 oncoprotein, an E6-AP mimetic epitope peptide (mimotope), an anti-E6 ribozyme, peptide aptamers targeting the viral E6 oncoprotein, siRNAs of the viral E6 oncogene, and combinations of all these therapies [45-53]. A new strategy is to induce viral E6 and E7 instability by using HSP90 and GRP78 inhibitors for the treatment of cervical cancer [54]. An E7 antagonist peptide reactivating pRB *in vitro* and *in vivo* shows antitumor effects [55]. GS-9191, a nucleotide analog prodrug, shows the anti-proliferative effect *in vitro*, and its topical application reduces the size of papilloma in the cottontail rabbit papillomavirus model [56]. Chitosan hydrogel containing granulocyte-macrophage colony-stimulating factor (GM-CSF) in combination with anticancer drugs, cyclophosphamide in particular, results in antitumor effects through CD8+ T cell immunity [57]. Heparin-like glycosaminoglycans inhibit tumor growth by downregulating HPV18 long control region activity in transgenic mice [58]. Finally, 5-aza-2'-deoxycytidine, a demethylating agent, and 5, 6-dimethyl xanthenone-4-acetic acid, a vascular disrupting agent, have each been combined with therapeutic HPV DNA vaccines [59, 60], showing significant antitumor therapeutic effects *in vivo*.

Additionally, antiviral RNAi therapies have been developed and tested in clinical trials with short interfering RNAs (siRNAs) [61]. siRNAs have been demonstrated to be capable of selective silencing of endogenous genes in mammalian cells [62, 63], and of selectively silencing viral genes in virus-induced diseases [64-66]. Remarkably, it has been reported that siRNA targeting E7 or E6/E7 promotes the accumulation of TP53 and/or pRB, eventually leading to the induction of apoptosis and/or senescence in HPV16-positive cervical cancer cell lines [67, 68], as well as in HPV18-positive human cervical cancer cells [69, 70]. It has been reported earlier that, silencing of both E6 and E7 produces greater anticancer activity than silencing E6 alone [71-73].

Recently, CRISPR/Cas9 has been developed as a novel therapeutic strategy and has entered into clinical trials. The difference between RNAi and CRISPR/Cas9 is shown in Table 1. The therapeutic mechanism of CRISPR/Cas9-mediated silencing of both E6 and E7 mainly depends on the reactivation of TP53 and pRB to induce apoptosis and cellular senescence.

Table 1. Comparisons between RNAi, CRISPR centric technologies

	RNAi	CRISPR/Cas9
Loss-of-function mechanism	Post-transcriptional RNA degradation	Frame shift DNA mutation
Result	Reversible knockdown	Permanent knockout
Transgenes	si/shRNA	Cas9 nuclease
Guiding sequence	si/shRNA	gRNA
Required sequence information	Transcriptome	gRNA
Off-target space	Transcriptome	Transcriptome
Transcript variants	All variants via conserved region	Genome, Cuts as monomer
		All variants via conserved region

Yu et al. [74] have reported a CRISPR/Cas9 sequence targeting the E6 mRNA reduces the level of full-length E6 mRNA and increases the level of TP53 protein. Hu et al. [75] also suggested that CRISPR/Cas9 inhibition of E7 as a potential therapeutic intervention for the treatment of cervical cancer.

Our study [76] has recently reported that CRISPR/Cas9 targeting the promoter and ORF of E6/E7 transcripts reduces E6 and E7 mRNA level, increases TP53 protein levels, decreases RB protein level, promotes apoptosis and inhibits growth of SiHa cells. Similarly, CRISPR/Cas9 transfected cells exhibits mitigated growth *in vivo*. Kennedy et al. [77] have designed CRISPR/Cas9 that specifically targeted E6/E7 mRNA of HPV16 or HPV18. Its intratumoral administration has resulted in the inhibition of tumor growth and induction of apoptosis *in vivo*, promising CRISPR/Cas9 as a potential adjuvant therapy for cervical cancer.

CRISPR/Cas9 technology: molecular tool for gene therapy

Clustered regularly interspaced short palindromic repeats (CRISPR) and their associated proteins (cas proteins) form an adaptive immune system in most bacteria and archaea to target and inactivate invading phages, plasmids and other genetic elements [78-80]. There are three types of CRISPR systems (I, II, III), among which the type II CRISPR system from *Streptococcus pyogenes* has received the most attention and been summarized below.

In the CRISPR/Cas9 system, sgRNA directs Cas9 DNA endonuclease to the target DNA sequence next to the protospacer adjacent motif (PAM) for site-specific cleavage and produces sequence-specific double-strand breaks (DSBs) [81] (Fig. 3). Consequently, various mutations such as substitutions, deletions and insertions in the target genome are introduced by the host DNA repair machinery [82] including nonhomologous end joining (NHEJ) at the binding site [83] or homologous-dependent repair (HDR) [84]. Designs of sgRNAs for intended genes are flexible to cleave virtually any DNA sequence by simply designing a single RNA sequence that matches the targeted DNA [85]. In addition, because the sgRNA component is physically separate from Cas9 expression, the sgRNA is easily “programmed”, with the possibility of many sgRNAs targeting multiple DNA sites to be expressed simultaneously with the same Cas9 [86]. Therefore, CRISPR/Cas9 has shown great promise in realizing potent and multiplex genome editing and regulation of gene expression without host dependence (Table 1). CRISPR/Cas9-based tools have been successfully applied in diverse organisms and in a broad range of research fields, including high throughput genetic screens [87-89], generation of gene knockouts in several species [90], and targeting of pathogens to eradicate infections such as HPV, HIV and HBV [76, 91, 92]. In our previous study, Cas9 has been used with cognate gRNAs to

disrupt the HPV E6 and E7 coding sequences in SiHa cervical cancer cells that retains integrated HPV-16, a subtype of HPV that has been associated with greater than 50% of cervical tumors. Forty-eight hours after transfection of plasmids encoding Cas9 and gRNAs, viral transcript levels

Fig. 3. The mechanism of CRISPR/Cas9 system.

were reduced over 90% relative to cells transfected with control vectors. The reduction in E6 and E7 transcript levels correlated with an increase in host p53 and p21 (a cyclin-dependent kinase inhibitor used as a readout for Rb) production, as well as a significant reduction in cell viability. Our another study has also demonstrated that both HBV cccDNA is susceptible to Cas9-mediated cleavage in cell culture as well as *in vivo*. Ye et al. [92] have recently demonstrated that deletion of the 32 bp region of the ccr5 gene with Cas9:grNA complexes confers resistance to CCR5-tropic HIV-1 in monocytes and macrophages differentiated from induced pluripotent stem cells.

CRISPR/Cas9-based combination therapeutics against HPV

Several CRISPR/Cas9 studies have been used in human cancer cell lines to identify modulators of drug sensitivity [93-98]. These studies address whether combination therapy with CRISPR/Cas9 can significantly enhance the chemo/radio sensitivity of cancer cells or prevent chemo/radio resistance resulting from long-term low-dose chemo/radio therapy. From a clinical perspective, if the combined effect is equal to the sum of the effects of the individual modalities, the effect of the multiple modalities is additive. If the combined effect is greater than the predicted effect of the multiple individual modalities, the interaction is synergistic. The multiple modalities may also interact in an antagonistic fashion. Statistical analysis using the Chou-Talalay method must be applied to determine the synergistic, additive, or antagonistic effects of CRISPR/Cas9-based combination therapies.

In our study, we have validated the role of CRISPR/Cas9 targeting the HPV E6 and E7 oncogenes as a potential sensitizer to CDDP [99] and radiation therapy (unpublished data) for the treatment of cervical carcinoma. Thus, the combination of CRISPR/Cas9 targeting E6 or E6/E7 with cytotoxic agents may have synergistic effects on the restoration of TP53 and/or pRB function, and may be a more effective therapeutic modality for the treatment of cervical cancer. Further studies are required to overcome all the key points mentioned above and to develop clinically applicable combination therapies based on CRISPR/Cas9. Most importantly, to establish proof-of-concept for CRISPR/Cas9-based combination therapeutics, the mechanism underlying the synergy between the treatments should be elucidated. The results of *in vivo* experiments have demonstrated that combination therapy is significantly superior to either modality. Moreover, assessments to verify the absence of off-target effects and minimal induction of interferon are a prerequisite for the clinical application of CRISPR/Cas9-based combination therapeutics.

Conclusion and future perspective

In spite of recent progress and various treatment modalities that have been proved beneficial to some extent, no effective treatment is currently available for HPV-associated carcinogenesis. Even though the precise molecular targets have been characterized and several approaches for their inhibition have been demonstrated, not currently possessing an effective treatment approach constitutes a problem as important as finding novel methods for the known targets and mechanisms. Nevertheless, the use of combinatorial treatment approaches with CRISPR/Cas9 appears to be best suited for clinical protocols. Since its discovery, CRISPR/Cas9 has enabled interrogation of the role of individual genes in complex cellular processes. Advancements in CRISPR/Cas9-based screening technologies have fueled the anticipation of new discoveries. However, CRISPR/Cas9-based therapeutics has encountered many barriers in clinical trials, including the potential off-target effect, optimization of target sites, *in vivo* safety and limited choice of delivery systems for treatment. For example, methods need to be optimized for efficient and safe delivery of the CRISPR/Cas9 components into the desired tissues. In the past 25 years, many clinical trials for gene therapy with conventional ectopic overexpression vectors have been conducted [100, 101].

These studies provide promising results for gene delivery using both viral and non-viral vectors, which could potentially be adapted to deliver CRISPR/Cas9. Adeno-associated virus shuttle vectors seem particularly promising because of their high efficiency in transducing a broad range of cell types and their low cytotoxicity and immunogenicity. Another hurdle for applying CRISPR/Cas9 in patients is safety concerns resulting from the potential off-target effects [102-107]. Two complementary strategies have been used in order to detect potential off-target sites: (1) biased methods that rely on bioinformatics algorithms to predict potential off-target sites and (2) unbiased methods that identify genome-wide DSBs generated by the SNs. Biased methods are, at the moment, unable to detect a large number of off-target sites and have become therefore a method to complement unbiased methods. Indeed, several unbiased technologies have been described (WGS, IDLV capture, GUIDE-seq, and LAM-HTGTS) that have shed light on the real specificity of several SNs, confirming some off-target sites predicted by bioinformatics algorithms and uncovering many more. All of these obstacles must be overcome if CRISPR/Cas9-based treatments for cancer are to be successful. CRISPR/Cas9 has the potential to become a reliable and facile genome editing tool after addressing the aforementioned issues. Benefiting from the simplicity and adaptability of CRISPR/Cas9, it opens the door for revealing gene function in biology and correcting gene defects seen in diseases. For instance, recent genome-wide deep sequencing results will be helpful for selecting suitable target sites and designing highly specific gRNA. In addition, associations and synergies between CRISPR/Cas9 and other chemo/radio therapeutic agents may open new avenues for treatment and improve the clinical outcome of patients with cervical cancer.

Disclosure Statement

The authors declare no conflicts of interest.

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