A Review

Mechanistic insights into the oncogenic partnership of hADA3 and HPVE6 - paving ways for improved cervical cancer therapy

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High risk Human Papillomavirus (HPV) is considered the primary causative agent of cervical cancer, a devastating malady with significant morbidity. In India, cervical cancer is one of the major reasons of cancer mortality among women. Poor treatment outcomes of this disease is a matter of grave concern and hence demands aggressive research efforts towards discovery of more effective therapies. Understanding the intricacies of HPV oncogenesis at the molecular level can facilitate the discovery of promising anti-viral drugs. Our research aims at catering to the need of the time by revealing some of the key molecular mechanisms that contributes to HPV oncogenesis that can be utilized to discover promising anti-cancer molecules. We delineated the oncogenic connections between hADA3 and HPVE6 and illustrated its critical role in cellular transformation. Our work also shows how HPV oncoproteins exploits the cellular SUMO machinery to downregulate hADA3 to induce malignancy. This intrigued us to identify the hot spots of hADA3-E6 interaction and design therapeutic peptides against HPV induced cervical cancer. Present review is an attempt to outline our research on novel mechanisms of HPV pathogenesis and its implication on development of improved cervical cancer therapies.

Keywords: Cervical cancer, E6, hADA3, HPV, Peptide, SUMOylation

Introduction

Cervical cancer is a significant gynaecological health concern that has evolved into an obliterating plague with a high death toll of 18.7% women deaths in India and 7.7% worldwide for year 2020. Alarmingly, in India, this statistic is predicted to rise with an estimated 47,329 annual deaths by 20401. Prolonged high-risk Human Papilloma Viruses (HPV) infection and lack of comprehensive prognosis testing are the two major factors for increasing mortality rate2. Besides this, smoking, multiple pregnancies, oral contraceptive usage, and poor hygiene conditions are the other common risk factors. Epidemiological investigations identified sexual activity as one of the risk factors for genital HPV infection and cervical cancer in adult young women. Moreover, it acts as a silent killer by being asymptomatic at early stages and symptoms appear only after it has progressed to advanced stages3,4. WHO has taken several initiatives towards the elimination of this disease by speeding up the prevention, screening, and cancer management programs.

Discovery of prophylactic vaccines such as Cervarix, Gardasil-4 and Gardasil-9 has been a major breakthrough for controlling HPV driven cervical cancers. However, the vaccines are restricted to HPV types and prevent the development of cervical lesions only in women who have not been exposed to the vaccine-associated HPV types. At the same time, the mortality rates have failed to reflect the clinical success of such vaccination process because of unavailability of these vaccines. Moreover, exposure to HPV infection prior to vaccination is not beneficial more due to incorporation of the viral DNA into the host genome5. For treatment of cervical cancer at early stages, radiotherapy or surgeries are generally preferred. However, for advanced local tumors there have been no major refinement in the concerned platinum-based chemotherapy drugs currently in use. Such grim clinical scenarios certainly call for more improvement in the targeted strategies and combinational therapies to fight against cervical cancers.

Basic Virology of HPV and Pathogenesis

Papillomaviruses belong to the Papillomaviridae family which encompass 189 papillomaviruses out of which human papillomaviruses are 120. Among these, fifteen are considered high-risk HPV reliant on the
disposition of HPV infected lesion to endure into a malignant form. Most prominently, HPV-16, HPV-18 and HPV-31 are etiologic agents of cervical cancer and anal cancers as they account for over 99% lesions containing viral sequences. While, HPV-6 and HPV-11 are considered mild-risk viruses mostly associated with harmless genital lesions or warts\(^6,7\). HPVs are ubiquitous, non-enveloped viruses, surrounded by icosahedral capsid proteins. The genome of HPV consists of double-stranded circular DNA containing 8kb which is functionally divided into three sections. The first section codes for the early genes E1, E2, E3, E4, E5, E6 and E7, which interact with cellular gene products and facilitates viral DNA replication. The late proteins, major L1 and minor L2 comprising the structural components of viral capsid are involved in the packaging of new virions and are encoded by the second section of the HPV genome. The non-coding section is designated as Long control region which localizes in between ORF L1 and E6. These elements are involved in regulation of DNA replication by controlling the transcription of ORFs\(^7\). Additionally, the available vaccines are designed based on L1 and L2 surface proteins\(^8\).

HPV infection is known to be transmitted during sexual activities via micro-wounds on the genital skin surface or other epithelial lesions that expose the basal layer of squamous epithelia to the viruses. In normal epithelia, the basal cell divides to produce transit amplifying daughter cell which differentiates to give rise to an upper proliferation deficient epithelial layer. However, HPV exposure manipulates this scenario by entering into the undifferentiated host cells through common cell surface receptors such as heparin sulphate proteoglycans. L1 capsid protein facilitates the viral entry via calveolar or clathrin-mediated endocytosis. Following infection, virus undergoes uncoating and its genome enters the nucleus wherein it exploits the host replication machinery by maintaining its extrachromosomal element or episome at a lower level. This extended HPV infection in the basal layers drive the inception of new viromes upon desquamation. Thus, completion of the viral life cycle is achieved without causing cytotoxicity and systemic viraemia or apparent inflammation by evading the local immune responses\(^7,9\).

**Role of HPV E6 and E7 in hijacking host cellular proteins to mediate oncogenesis**

HPV oncoproteins, mainly E6 and E7, cooperatively contribute to human malignancies by interfering with the activities of key cell cycle regulators and tumor suppressors. E6 is a small basic protein of 151 amino acids and approximately 18 kDa in size. It has two characteristic conserved zinc-finger domains formed by two pairs of C-X-X-C motifs. E6 is known to mediate its interaction with host proteins via LXXLL motif. Importantly, the C-terminal region of E6 protein contains PDZ-binding motif that enables E6 to interact with multiple host proteins through its phospho acceptor site. Popular E6-mediated degradation of illustrious p53 tumor suppressor via complex formation with the host E3 Ubiquitin ligase E6-Associated Protein (E6AP). This is considered to be the best trick of HPV’s transformative landscapes where a viral oncoprotein hijacks both the ubiquitin-mediated protein degradation pathway and a powerful tumor suppressor pathway, simultaneously\(^12,13\). Additionally, E6 weakens the p53 regulated transcription by capturing the transcriptional co-activators CBP, p300 and hADA3. The anti-apoptotic function of E6 is majorly driven via p53 degradation, it also achieves the same goal by targeting numerous pro-apoptotic factors like hADA3, Bak, Procaspase 8, FADD and TNFR1. The cellular DNA replication machinery is another hostage of this oncoprotein that provokes genomic instability\(^14\). E6 is also capable of eliciting increased chromosome abnormalities by binding to mini-chromosome maintenance 7 protein, resulting in its subsequent degradation in an E6AP dependent manner. E6 plays a crucial role in hampering the DNA repair by associating with XRCC1 and MGMT. Intriguingly, studies also showed the interaction of E6 with DNA repair factors such as Breast Cancer 1 Gene (BRCA1) and BRCA1-associated ring domain protein 1.
(BARD1). One of the important mechanisms through which E6 stimulates cellular immortalization is by escalating telomerase activity through proteolysis of the NFX1-91 repressor. This is how E6 prevents erosion of telomeres and surpasses replicative senescence\textsuperscript{15}. E6 also promotes invasion by perturbing the epithelial characteristics of cells that include disruption of cell-cell contacts and its polarity. Hence, it is not surprising that E6 targets focal adhesion component paxillin, hScrib, and the MAGI family of proteins to abrogate tight junction integrity. E6 also dysregulates trafficking of proteins by targeting sorting nexin 27 to disrupt cellular homeostasis and proliferation\textsuperscript{16}.

E7 is the other most powerful modulator of HPV with a size of \~100 amino acids and three defined domains that interact with different cellular proteins. E7 promoted cellular transformation occurs by its critical association with different regulators of cell cycle as retinoblastoma tumor suppressor and its family members as p107, p130, E2F1, DREAM complex to halt the cell cycle at different checkpoints. Apart from this, E7 is also capable of eliciting abnormal cell proliferation, inhibition of apoptosis, and overriding checkpoints after DNA damage, causing loss of cell-cell adhesion, development of chromosome abnormalities and aneuploidy\textsuperscript{17,18}. A summarized view of E6 and E7 hijacking of various host cellular pathways has been depicted in (Fig. 1).

Investigations are still on to uncover other host targets of HPV oncoproteins. Extensive research is needed to understand the missing links and mechanisms of HPV pathogenesis for development of improved treatment strategies and discovery of novel drugs. Our research goal aligns with the similar area to decipher prominent pathways hijacked by HPV for cellular transformation. Findings of such study can be exploited to devise better intervention strategies for cancer treatment.

### ADA3, a prominent host regulator

Co-activator Alteration/deficiency in activation-3 (Ada3) was first identified by means of genetic strategy employed in yeast by Berger \textit{et al.} in 1992\textsuperscript{19}. yADA3 was shown to play a pivotal role in imparting the cytotoxicity in the yeast cells by enabling the constitutive expression of GAL4-VP16\textsuperscript{20}. Mammalian ADA3 was identified as an interactor of multi-protein histone acetyl transferase (HAT) complex containing p300/CBP Associated Factor (PCAF)\textsuperscript{21}. The ADA complex constitutes of ADA3, ADA2 and a HAT general control nonrepressed 5 (GCN5)\textsuperscript{22}. Loss of hADA3 has been associated with embryonic lethality which reflects its crucial role in cell survival and cell proliferation\textsuperscript{23}. It associates with various HAT complexes like PCAF, STAGA, p300 and GCN5 to aid in the process of histone acetylation leading to chromatin remodelling\textsuperscript{24}. One of the primary functions of hADA3 is to enhance the transcriptional activity of p53 via its acetylation upon DNA damage leading to its stabilization\textsuperscript{25,26}. Various studies have established that hADA3 is a target of high risk HPV16E6 oncoprotein\textsuperscript{27}. 

![Fig. 1 — A plethora of host proteins and pathways reported to be hijacked by HPV oncoproteins E6 and E7 contributing towards malignant transformation. Integration of HPV DNA into the host genome results in deregulated overexpression of E6 & E7 which targets different proteins involved in several pathways which eventuates into host cell immune evasion, inhibition of apoptosis, dysregulation in cell polarity & cell-cell contacts, DNA damage and defective repair. The cumulative effect of these changes in cellular programming causes tumorigenesis. FADD, Fas-associated protein with death domain; BAK, Bcl-2 homologous antagonist/killer; TNFR1, tumour necrosis factor receptor 1; CASP8, caspase-8; ADA3, alteration/deficiency in activation 3; hTERT, human telomerase reverse transcriptase; SNX27, sorting nexin 27; NHERF1, Na+ /H+ exchange regulatory factor 1; DLG1, discs large homologue 1; MAGI, Membrane-Associated Guanylate Kinase; Paxillin; MCM-7, mini-chromosome maintenance 7 protein; BRCA1, Breast Cancer 1 Gene; IFNα, interferon alpha; IRF, IFN regul atory factor; pRb, retinoblastoma protein; DREAM, dimerization partner, pRb-like, E2F and multi-vulval class B; CDK2, cyclin-dependent kinase; pCAF; p300/CBP-associated factor; DNMT1, DNA methyltransferase 1; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3- related; IGFBP-3, insulin-like growth factor binding protein 3, GSTP-1, glutathione S-transferase P1](image-url)
Mechanism of hADA3 degradation by HPV E6

Though it has been established that hADA3 is degraded by high-risk HPV E6 protein\textsuperscript{28,29}, the machinery and the motive of this degradation in HPV induced carcinogenesis was still a mystery. Though the existing literature corroborated that the function of hADA3 is disrupted by HPV16E6\textsuperscript{27}, but the insights into its molecular architecture has not been explored thoroughly. We happen to observe that the levels of hADA3 were lower in HPV positive cells in comparison to HPV negative cells\textsuperscript{24}. This captivated our interest to closely look into the degradation mechanism of hADA3 by high risk HPV16E6. Accumulation of hADA3 by the proteasomal inhibitor, MG132 further fired up our idea about the involvement of ubiquitin proteasome system in this process. Since ubiquitin mediated degradation is an essential part of regulating protein homeostasis in cells, the next obvious thing to check was the ubiquitination status of hADA3. Involvement of ubiquitination was investigated \textit{in vivo}, which revealed accumulated levels of ubiquitinated hADA3 upon E6 overexpression. This was further confirmed by half-life assay of hADA3 by overexpressing E6. E6AP is an established ubiquitin E3 ligase, known to facilitate ubiquitination of numerous HPV16E6 targets\textsuperscript{30,31}. Our experiments further provided evidence for the participation of E6 in the destruction of hADA3.

Role of SUMOylation in accelerating the degradation of hADA3

After analyzing the ubiquitination status of hADA3, it intrigued us to investigate the possibility of SUMOylation as well. This is because SUMOylation is a similar post-translational modification (PTM) which maintains protein homeostasis in cells. Also, it can affect the protein stability and localization in cellular compartments. Ours was the first study that demonstrated covalent modification of hADA3 by SUMO1, SUMO2 and SUMO3. We could also predict the potential SUMOylation sites using SUMOplot software. Since Ubiquitination and SUMOylation both utilize lysine residues for conjugation, we were curious to find out the effect of SUMOylation on hADA3 ubiquitination and stability. Interestingly, turnover of hADA3 was seen to be drastically reduced by SUMOylation. Covalent addition of SUMO to hADA3 might bring about conformational changes leading to exposure of hidden ubiquitination sites making hADA3 more prone to ubiquitin mediated destruction.

E6, the master regulator of SUMOylation

Next interesting task was to elucidate how high-risk HPV E6 influences hADA3’s SUMOylation status. This aspect was investigated by utilizing SUMOylation assay, where overexpression of E6 was seen to augment the basal levels of SUMOylated hADA3. We also utilized some of the important enzymes of SUMOylation machinery like Ubc9 and PIAS, to provide additional supporting evidence for E6 mediated SUMOylation of hADA3. Dominant negative mutants of Ubc9 as well as E6AP were used to validate the results. All these data further solidifies the idea that E6AP mediated ubiquitination of SUMOylated hADA3 leads to its degradation \textit{via} HPV16E6.

Further validation of hADA3 destruction \textit{via} E6 leading to tumorigenesis was analyzed in cervical cancer cell lines expressing HPV. Depletion of hADA3 in SiHa cells escalated its malignant behavior, thereby proving the point that E6 requires hADA3 to exert its oncogenic transformation function. Other biological assays were also performed to assess cancerous growth of cervical cancer cells in hADA3 depleted cells. Outcome of all these experiments repeatedly indicated that loss of hADA3 is an important facet leading to emergence of cancerous phenotype in SiHa cells. Importantly, restoration in the levels of hADA3 led to suppression of the tumorigenic phenotype of SiHa cells. The mechanism by which E6 regulates hADA3 and its functions is portrayed in (Fig. 2). Based on the above findings, it was obvious that the oncogenic interaction of hADA3-E6 plays a crucial role in cellular malignancy. Therefore, disruption of such interaction can be utilized as an excellent approach for the development of targeted therapy against cervical cancer.

Peptide based strategy as reliable therapy against cancer

Rational design of drugs based on structure has been a pioneering technology in the design of various therapies available commercially for numerous diseases\textsuperscript{32,33}. Though this technique has tremendous advantage over traditional method of drug discovery\textsuperscript{34}, it requires a reliable 3D- structure of the protein and its targets. Due to unavailability of fully resolved structures of hADA3 and E6 proteins, this necessitates the modelling of hADA3-E6 complex to identify the competing molecules. In the past, anticancer effects were shown utilizing peptides\textsuperscript{35-37}, which generally provide some advantage over small molecules-based drugs\textsuperscript{38}. There have been only few
attempts at the design of peptides derived from the E6 protein sequence. Another set of peptides were tested possessing alpha helical component that resembled E6AP motif responsible for binding to E6. Though these inhibitors were potent in disruption of E6/E6AP interaction, they were selected from peptide libraries and not tailored specifically for E6 binding resulting in offsite targeting and side effects. In our investigation, we focused on structure-based rational design of peptides, which would result in enhanced specific interaction leading to little side effect. Figure 3 illustrates the overall devised strategy to restore the normal levels of hADA3 as well its functions.

**Modelling the 3D-structure of hADA3**

To initiate this investigation, first of all we needed the 3D-structure of hADA3 that was not available. This intrigued us to generate this model ab initio. Five putative models were generated based on a set of...
stereo chemical and experimental parameters. These models were shortlisted based on secondary structure, conformation, solvent accessibility, NR-Boxes, solvent accessibility, SUMOylation site availability, Ramachandran plot statistics and various geometrical considerations. After determining the structure, we wanted to analyze the stability and conformation of the model. For this, MD simulation coupled with Ramachandran statistics for favored and allowed regions were utilized to select the best model. Putative models were validated by checking their interaction in silico utilizing known binding partners from the literature such as RARα ligand binding domain (RARLBD), p53 and p300. Next, the hADA3 model was refined by exploiting the data of solvent accessibility required by the lysine residues for successful SUMOylation. This is because they are generally expected to be displayed on the exterior surface of the protein. The finally selected model was found to display K85, K178 and K319 sufficiently exposed as well as having high solvent accessibility thus allowing for successful post translational modification.

Our recent findings have highlighted the importance of hADA3-E6 collaboration for causing HPV stimulated cervical cancer. Therefore, to gain insights into this at a molecular level and thus develop peptides utilizing this information for disruption of this interaction, in silico analysis of this binary complex was investigated. For this, site specific and blind docking was carried out. For site specific docking we defined the docking site residues while in the blind docking, no binding site was demarcated thus allowing both the proteins to interact freely. N terminal of hADA3 was found to interconnect with E6. This was further validated by generating several truncated mutants hADA3 and performing co-immunoprecipitation with E6. Our data revealed that crucial contact points lie between the amino acid residues 255-288 of hADA3 required for E6 binding. Similarly, we mapped the interacting domains for hADA3 and E6AP and demonstrated that hADA3 C terminal region was essential for E6AP association.

**Structure based rational design of peptides**

Knowledge regarding the important residues involved in interaction of hADA3 and E6 provided us the framework for designing the competitive peptides that can dislocate the interaction between hADA3 and HPVE6. From our previous findings we were convinced that hADA3 N terminal region can be utilized for the design of blocking peptides. From top 10 docked models of hADA3-E6, peptides showing strong interaction with G130 of E6 were selected. Next, we were interested in shortlisting the peptides that can enter the cancer cells efficiently. Based on the predictions of Cell PPD and CPPPred, the peptides were scored and the top hits were taken further. The functional activity of these peptides was determined based on their ability to disrupt hADA3-E6 interaction. We performed immunoprecipitation studies by overexpressing hADA3 and E6 in presence and absence of these peptides. Interestingly, one of the peptides showed a significant drop in disrupting hADA3-E6 interaction. We also demonstrated the potency of this peptide in blocking E6 and hADA3 association thus preventing the degradation of the latter. This rescued the endogenous level of hADA3 in HPV positive cancer cells. Following this, we examined the effect of these peptides in curbing the prominent hallmarks of cancer. One of the promising peptides was found to hamper the hyper proliferative and metastatic traits of cervical cancer cells. Taking together, this work helped us to propose the first 3D-structure of hADA3 and discover novel inhibitory peptides with a potential to combat HPV infection and cervical cancer.

**Conclusion**

The secrets behind the tumorigenic properties of HPV are still unclear. Many of its cryptic oncogenic traits are getting unfolded as fresh connections are being built with the new discovery of its interacting partners. Human ADA3 is one such interesting E6 ally that is manipulated to activate cellular malignancy. Our previous investigation underscored the role of hADA3-E6 axis in HPV driven neoplasm and shown the significance of its disruption in designing new therapies. Going forward, elucidation of the high-resolution structure of hADA3 and its complex with HPV16E6 needs immediate attention for devising improved therapeutic peptides or small molecule inhibitors for Cervical cancer therapy. Innovation of highly specific inhibitors, be it peptides or small molecule compounds is a daunting mission and it may take a while to make it a reality. Nevertheless, improvements are possible by tweaking the chemistry of the potential hits. The chemical structure of the peptides can be modified by applying medicinal chemistry knowledge to achieve enhanced bioactivity, improve solubility, stability, and selectivity. Further, targeted delivery of these peptides
can be attained by encapsulating the therapeutics in liposomes and nanoparticles. Delivery vehicles can serve as an excellent tool to enhance the stability and preferred effects of the therapeutic peptides in cancer cells and cause minimum toxicity in normal cells. Needless to mention, studies with appropriate xenograft mice model will help in testing the efficacy, bioavailability and safety profiles of the therapeutic peptides. The detailed role of hADA3 remains a much less explored aspect in the field of cancer biology. It will be worthwhile to enumerate its physiological partners. State of the art omics-based approaches in combination with computational methods can be applied to delineate the transformed pathways when E6 targets hADA3. Evaluating the variations in the proteome complexes of hADA3 in presence of E6 will provide valuable insights into hijacking mechanism of HPV and its intervention.

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Conflict of interest
All authors declare no conflict of interest.

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