

Regulation of apoptosis by the papillomavirus E6 oncogene

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Abstract

Infection with human papillomaviruses is strongly associated with the development of multiple cancers including esophageal squamous cell carcinoma. The HPV E6 gene is essential for the oncogenic potential of HPV. The regulation of apoptosis by oncogene has been related to carcinogenesis closely; therefore, the modulation of E6 on cellular apoptosis has become a hot research topic recently. Inactivation of the pro-apoptotic tumor suppressor p53 by E6 is an important mechanism by which E6 promotes cell growth; it is expected that inactivation of p53 by E6 should lead to a reduction in cellular apoptosis, numerous studies showed that E6 could in fact sensitize cells to apoptosis. The molecular basis for apoptosis modulation by E6 is poorly understood. In this article, we will present an overview of observations and current understanding of molecular basis for E6-induced apoptosis.

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Key words: HPV; E6; Apoptosis; Esophageal squamous cell carcinoma

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INTRODUCTION

Papillomaviruses are small DNA viruses that infect various epithelial tissues. Papillomaviruses replicate in the stratified layers of skin and mucosa, and usually give rise to benign lesions such as warts or papillomas. Human papillomaviruses (HPVs) can be classified as either high-risk or low-risk type on the basis of their clinical associations. The high-risk HPV types, of which type 16 (HPV-16) is the most prevalent

type, are commonly associated with lesions that can progress to high-grade intraepithelial neoplasia and ultimately to carcinoma, while the low-risk HPV types, such as HPV-6 and -11, are found associated primarily with benign lesions, which rarely progress to cancer^[1]. A subgroup of low risk HPV types, including HPV-5 and -8, are frequently detected in skin cancers that develop from multiple flat warts when combined with certain physical and chemical carcinogens^[2].

HUMAN PAPILLOMAVIRUS AND THE DEVELOPMENT OF ESOPHAGEAL CANCER

Squamous cell cancer of Esophagus is the pathological type that is most closely associated with HPV infection in gastrointestinal malignancies. Syrjanen group found condyloma-like lesions in the specimens of esophageal cancer in 1982, which links HPV infection to esophageal cancer for the first time^[3]. This finding was soon substantiated by the demonstration of HPV structural proteins in these lesions using immunohistochemistry^[4]. Numerous reports have been published since then. However, there is a wide variation on HPV infection rates among different studies, ranging from 0% to 88%, which make it still hard to consolidate the role of HPV (Table 1). This variation seems

Table 1 Detection of human papillomavirus in esophageal squamous cell carcinomas¹

Area or Country	HPV positive		Detection method	Reference
	%	n		
Germany	0	0/23	PCR	[90]
Japan	0	0/4	IHC	[91]
Italy	4.4	2/45	PCR	[92]
United States	4.5	1/22	PCR	[93]
Belgium	4.8	1/21	PCR	[94]
Linxian, China	6.7	2/32	PCR	[95]
Japan	16	12/75	PCR	[96]
Northern China	16.8	17/101	PCR	[97]
Northern China	16.9	118/700	ISH	[6]
Cixian, China	20.3	26/128	PCR	[98]
Hungary	39	32/82	PCR	[99]
Japan	42	20/48	PCR	[100]
South Africa	46	23/50	PCR	[101]
Italy	47	8/17	PCR	[102]
Anyang, China	63.3	19/30	PCR	[103]
Shanxi and Anyang, China	64	31/48	PCR	[7]
Guangdong, China	65.5	96/176	PCR	[104]
Beijing, China	70	28/40	ISH	[105]
Northern India	74	20/27	PCR	[106]
Beijing, China	83.3	15/18	PCR	[107]
Mexico	88	20/23	PCR	[14]

PCR, polymerase chain reaction, IHC, immunohistochemistry; ISH, *in situ* hybridization. ¹Only publications after 2000 were indexed in this table since there are numerous reports on the HPV infection status in esophageal carcinomas.

to be influenced by methodology of detection, pathological grading, geographic distribution and genetic sensitivity to HPV infection. Even though different opinions do exist, a large portion of them strongly suggest a causal role for HPV in esophageal carcinogenesis, or at least consider HPV as a possible contributor in those HPV prevalent areas such as China and South Africa.

The incidence of esophageal cancer in Anyang area is one of the highest in China, with a mortality rate of 132×10^5 , significantly higher than the one of 52×10^5 in neighboring area. A 132-case survey in this area showed that the infection rate of HPV-16 is much higher than neighboring area, 1.9 fold by PCR (72% *vs* 37%) and 2.2 fold by immunohistochemistry (49% *vs* 22%), and the infection of HPV is closely related with the degree of dysplasia^[5-26]. Compared to normal adjacent tissues, samples from esophageal carcinoma showed significantly higher infection rate for HPV^[13,20]. The most frequently detected types of HPV in esophageal cancer are HPV-16 and -18^[27].

Some indirect or direct evidences have been shown recently to further substantiate the causal role of HPV. When the genomes of HPV-16 and -18 without E1 and E2 were transfected transiently into esophageal cancer cell, these viral genomes replicated in the absence of E1 and E2, which suggest specific host nuclear factors in esophageal squamous epithelial cells may support HPV replication^[28]. Other researchers have reported that E6 gene can actually associate with the nuclear matrix of esophageal carcinoma cell. Evidence from animal studies showed that persistent papillomatosis and carcinomas in cattle can be experimentally reproduced with bovine papillomavirus 4 (BPV 4) infections in these animals. Up to 96% of the cancer-bearing animals have concomitant papillomas, and the progression from benign papilloma to carcinomas could be clearly identified^[29]. Recently an immortal esophageal cell line was established by transferring HPV 18E6E7 into fetal esophageal epithelium; this cell line showed gradual change from preimmortal, immortal, precancerous to malignantly transformed stages upon prolonged cultivation without any co-carcinogens, which provided valuable direct proof on the role of HPV in carcinogenesis process of esophageal carcinoma^[30,31].

The major role of HPV might be in the early stage of carcinogenesis since it has been shown in several studies that compared to esophagitis, precancerous lesions showed more HPV infection (96% *vs* 26%), while in advanced esophageal cancer specimens, the positive rate leveled off a little (88%)^[25]. The hypothesis might be that HPV play its role in near-normal differentiating cells; this differentiating status is needed for HPV to replicate and when these cells acquire malignant phenotype changes step by step, the differentiation process is reversed. At this stage, HPV will have to face hostile environments to replicate. This may also explain the wide variation on positive rates when detecting HPV in esophageal cancer specimens since the pathological grading may vary greatly. In this aspect, the relation of HPV with esophageal cancer is somehow like the one of HBV with hepatocellular carcinoma. In benign tissue of infected liver such as cirrhosis, HBeAg and HBcAg were easily detected, but after malignant changes happened following virus infection, it became much harder to detect^[32].

Multiple factors besides HPV are considered in carcinogenesis of esophageal cancer, such as some chemicals (nitrosamines, mycotoxins, cigarette smoke, excessive alcohol intake), nutritional deficiencies and physical factors (hot food), thus making it very hard to clearly characterize the significance of HPV in esophageal cancer. More insights will be needed to fully demonstrate the mechanisms involved. Before that it might be hard to draw a final conclusion on the causal role of HPV in esophageal carcinogenesis.

The transforming properties of high-risk HPV's primarily reside in two genes, E6 and E7, which are consistently expressed in HPV-positive cervical cancers and cancer-derived cell lines^[33]. The sustained expression of E6 and E7 is essential to maintain the transformed state of HPV-positive cells^[34]. Independent of E7, E6 exhibits important biological activities. The modulation of E6 in apoptosis will be the focus of this review. However, due to the technical difficulty to establish a normal esophageal keratinocyte cell line, most studies were carried out in keratinocytes from foreskin or skin, or even in unrelated cell types.

PILLOMAVIRUS E6 PROTEINS

The papillomavirus E6s are relatively small proteins. For example, HPV-16 E6 protein is a small protein of 151 amino acids (Figure 1). E6 proteins from different HPV types or among the animal and human papillomaviruses show moderate amino acid homology. The common feature of most E6 proteins is the presence of four putative Cys-X-X-Cys motifs that are capable of binding zinc^[35-37]. The importance of Cys-X-X-Cys motifs for E6 proteins has been implicated in functions such as transcriptional activation, transformation, immortalization, and association with cellular proteins^[37-42]. There is a PDZ-binding motif in high-risk HPV's E6 that is important for association with PDZ containing proteins^[43,44]. A phosphorylation site for protein kinase A on E6 has also been identified^[45].

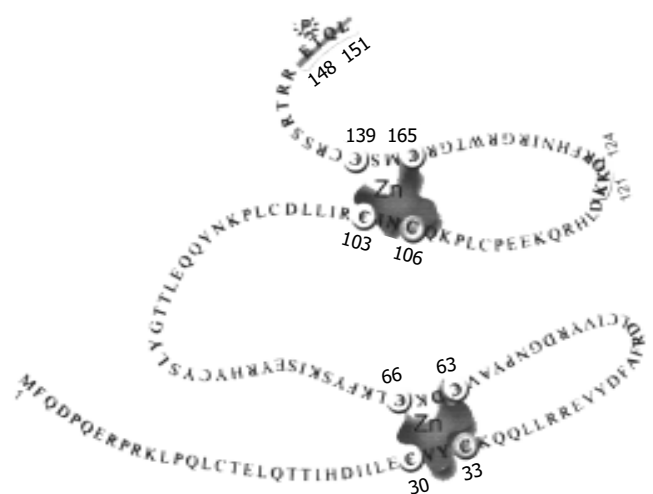


Figure 1 Sequence of HPV-16 E6 protein. Single-letter designations are used to represent the amino acids. The sequence is arranged into a zinc finger configuration. The amino acid residues (121-124)KKQR₁₂₄ essential for nuclear import and the PDZ domain-binding motif (148-151)ETQL₁₅₁ are marked. T₁₄₉ is a putative phosphorylation site for protein kinase A.

Localization of E6 has been controversial and complex, partly due to its very low level in the cells. Nevertheless, E6 proteins have been localized to the nuclear, cytoplasmic, and non-nuclear membrane (including Golgi membrane) fractions in a variety of cells^[46,47]. A recent study showed that HPV-18 E6 localization is an actively controlled process^[47]. Nuclear entry of HPV-16 E6 was shown to occur via several pathways^[48]. Some recent studies also revealed differences in cellular localization between E6 proteins from high-risk and low-risk HPVs^[47].

Non-specific double-stranded DNA-binding by E6 has been observed *in vitro*^[37,49]. Sequence-specific binding to the HPV long control region has also been described for HPV-16 E6^[50]. Recently, specific recognition of Holiday junctions by E6 from high-risk HPVs was demonstrated^[51,52].

The oncogenic activities of E6 have been demonstrated in multiple biological assays. These include immortalization of primary human epithelial cells, transformation of established mouse fibroblasts, transcriptional activation, resistance to terminal differentiation of human keratinocytes, modulation of apoptosis, and tumorigenesis in animals^[53]. Some recent studies showed that E6 played an essential role in HPV life cycle^[54]. Although E6, along with E7, efficiently immortalizes primary human epithelial cells, is not sufficient in induction of human cell transformation; additional alterations are required for the cells to be fully transformed^[55].

Association of E6 with p53 is mediated by the ubiquitin ligase E6AP that leads to the degradation of p53 by the ubiquitination pathway^[56,57]. One of the most important p53-induced gene product is the universal cyclin-dependent kinase (CDK) inhibitor p21^{Waf1/Cip1}^[58]. Notably, posttranscriptional down regulation of p21 by E6 has also been reported in several normal cell types^[59]. Consistent with these observations, differential expression of p53 and p21 in cervical squamous intraepithelial lesions infected with HPV has also been observed^[60]. E6 also has functions independent of inactivating p53 and has been shown to interact with multiple additional cellular proteins^[53]. These include the pro-apoptotic protein Bak, tumor necrosis factor receptor 1, and the DNA repair protein MGMT and XRCC1^[61,62].

MODULATION OF APOPTOSIS BY E6

Apoptosis is a genetically programmed process of cellular destruction that is indispensable for the normal development and homeostasis of multi-cellular organisms^[63]. Apoptosis is characterized by plasma membrane blebbing, condensation, and fragmentation of cells and nuclei, degradation of chromosomal DNA into nucleosomal units^[64]. Apoptosis serves to eliminate cells that are no longer required or potentially dangerous, such as radiation-damaged, aberrantly growing due to oncogene activation, and virally infected cells. Regulation of apoptosis is very important in terms of pathogenesis of diseases. Inappropriate occurrence of apoptosis results in neurodegenerative diseases and AIDS, while the failure of appropriate apoptosis contributes to autoimmune diseases and cancer. Many viral proteins have been found to modulate apoptosis^[65]. Both pro- and anti-apoptotic activities for papillomavirus E6 have been described. While the anti-apoptotic function of E6 can be

attributed in part to its ability to degrade p53, little is known regarding how E6 sensitizes cells to apoptosis.

INDUCTION AND SIGNAL TRANSDUCTION OF APOPTOTIC PATHWAYS

The apoptotic signal may originate endogenously, for example, from DNA damage, uncoordinated induction of cell cycle, or disruption of the cellular metabolism. This pathway involves the mitochondria and more specifically cytochrome c, the protein localized in the inner mitochondrial membrane and the inter-membrane space^[66]. During apoptosis, cytochrome c is released in the cytosol and together with Apaf-1, activates procaspase 9. Activated caspase 9 then cleaves and activates the executioner caspase 3, an event that leads to the cleavage of other death substrates, cellular and nuclear morphological changes, and ultimately to cell death^[67]. Apoptotic signals can also be triggered externally once the suitable surface death receptors are ligated. For example, the Fas (CD95/APO-1) receptor transduces apoptotic signals upon cross-linking with the Fas ligand (FasL). FasL binding triggers trimerization of the Fas receptor and recruitment on the cytoplasmic death domain DD of death-inducing signaling complex (DISC), which includes the adaptor FADD and pro-caspase 8 as crucial physiological death effectors. Coupling of pro-caspase 8 to Fas results in proteolytic activation of caspase 8. Two pathways have been shown for the signal transduction downstream of caspase 8, which are used in different cell types (types I and II)^[68]. In type I cells, caspase 8 directly activates procaspase 3; in type II cells, caspase 8 cleaves Bid, a proapoptotic member of the Bcl-2 family^[69,70]. The cleaved Bid translocates to the mitochondria and stimulates the release of cytochrome c (Figure 2).

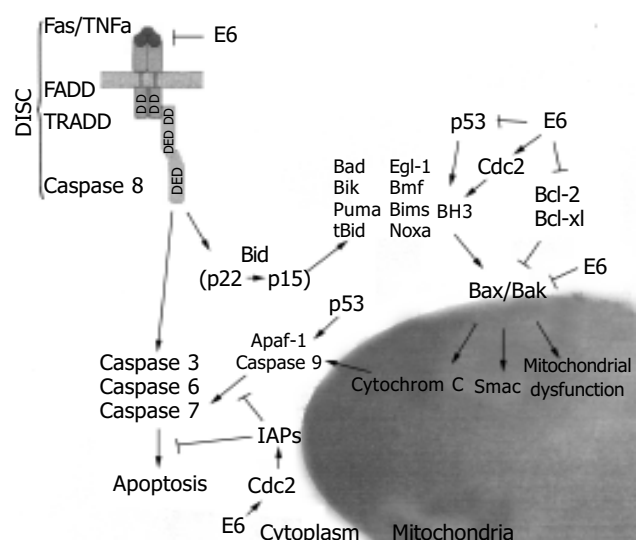


Figure 2 Modulation of apoptosis by E6.

The pathways of cell proliferation and apoptosis are tightly coupled. Inappropriate proliferation of somatic cells may trigger apoptosis. Activation of p53 by DNA damage induces either cell cycle arrest or apoptosis^[71]. The cytostatic

effect of p53 is largely mediated by transcriptional activation of p21, whereas the apoptotic effect is mediated by transcriptional activation of pro-apoptotic genes including BAX and PUMA^[72,73]. Compared to many normal tissues, cancer cells are highly sensitized to apoptotic signals, and survive only because they have acquired lesions such as loss of p53 that prevent or impede cell death^[74]. Much effort has gone into determining the effects of p53 inactivation on the response of cancer cells to the therapeutic agents. The results have been conflicting, with some studies indicating enhanced sensitivity and others indicating increased resistance^[74]. For example, one study showed that the p53-deficient cells were sensitized to the effects of DNA-damaging agents as a result of the failure to induce expression of p21, while resistant to the effects of the antimetabolite 5-fluorouracil; p21 was shown to inhibit Cdc2-associated apoptosis^[75]. Inappropriate activation of Cdc2 has been implicated or shown to be required for apoptotic cell death^[76,77]. In some other systems, however, inactivation of Cdc2 increased the level of apoptosis^[78]. The discrepancy regarding Cdc2's contribution to cell death or survival probably depends on phosphorylation of its downstream targets including BAD and survivin^[79-81].

So far, numerous studies addressing the role of E6 in apoptosis have been reported (Figure 2). Since different systems have been used, conflicting and sometimes confusing results have been obtained. As cell types could affect experimental results, we will first focus on apoptosis modulation by E6 in its natural host cells, the keratinocytes or keratinocytes-derived cancer cells. Some interesting observation made in other cell types will be discussed in the third section. For additional information, please see other related reviews^[82].

E6 MODULATION OF APOPTOSIS IN HPV NATURAL HOST CELLS

In primary human foreskin keratinocytes, expression of HPV 16 E6 slightly increased spontaneous apoptosis^[83,84]. After induction with chemotherapeutic agents such as cisplatin, etoposide, and mitomycin C, enhanced sensitivity in E6 expressing cells was observed^[85]. In contrast, E6 inhibited apoptosis during serum- and calcium-induced differentiation of human foreskin keratinocytes^[86]. E6 expression correlated with prolonged expression of Bcl-2, reduced elevation of Bax, and loss of p53^[86]. While the role of Bcl-2 and Bax in this process remains to be determined, p53 inactivation or E6BP binding do not appear to be essential^[87]. Furthermore, co-expression of E6 abrogated E7-mediated apoptosis by TNF^[84].

In human keratinocytes immortalized by E6, low levels of apoptosis as compared to the non-immortalized control cells were observed after CD95 (Fas) agonist treatment^[88]. Interestingly, in addition to p53 and p21, protein levels of anti-apoptotic proteins Bcl-2 and Flip were reduced. Proteasomal inhibition increased the susceptibility of E6 expressing cells to CD95-mediated apoptosis. But it remains to be determined whether this sensitization is due to increased protein levels of E6, p53, or some other molecules. In another study, E6 reduced UVC-, mitomycin C, and serum starvation-induced apoptosis in the immortalized human

keratinocytes (HaCaT) bearing mutated alleles of p53^[89,90].

Expression of HPV-16 E6 in HeLa cervical carcinoma cells where the endogenous HPV-18 E6 and E7 transcription were repressed, slightly increased the number of apoptotic cells after prolonged incubation^[91]. However, expression of E6 to allow E7 to induce apoptosis is implicated in this study. Similarly, intracellular targeting of HPV-16 E6 by E6-binding polypeptide resulted in apoptosis of HPV-16-positive cervical cancer cells^[92]. In contrast, HPV-16 E6 expression in cervical carcinoma C33A cells leads to atractyloside-induced apoptosis^[93]. C33A cells do not contain HPV but express mutant p53.

In summary, expression of E6 in primary human keratinocytes or keratinocyte-derived cells consistently induces low level of spontaneous apoptosis. Depending on the agents used, E6 could either sensitize or inhibit keratinocytes to apoptosis after treatment with chemotherapeutic agents.

MODULATION OF PROGRAMED CELL DEATH BY E6 IN OTHER SYSTEMS

Numerous studies have been conducted to explore the function of E6 or p53 using cells unrelated to keratinocytes. Different cell types, reagents, and assays were employed. The results are quite inconsistent and sometimes confusing. It is impossible to discuss every report in this review. For this reason, only some representative studies considered to be of special interest will be discussed.

Some early studies showed that E6 inhibited E7-induced apoptosis through p53-independent mechanism in the developing lens of transgenic mice^[94,95]. Similarly, E6 could functionally substitute the insulin-like growth factor 1 receptor in inhibiting staurosporine-induced apoptosis in mouse fibroblast, including p53-null cells^[96]. Expressing of E6 in human foreskin fibroblasts also inhibited caspase 3 activation after treatment with thiol-containing antioxidant penicillamine^[97]. HPV-18 E6 protected cancer cells from Bak-induced apoptosis^[98]. E6 of both cutaneous and genital HPVs promoted proteolytic degradation of Bak^[61,98]. The role of Bak in UV-induced apoptosis in skin cancer has also been implicated^[61].

Several studies have examined the sensitivity of cells expressing E6 to TNF. HPV-16 E6 was shown to bind TNF receptor 1 (TNF R1) and protect cells from TNF-induced apoptosis in mouse fibroblasts and human histiocyte/monocyte and osteosarcoma cells^[62,99]. E6 binding to TNF R1 probably interfered with formation of the death-inducing signaling complex and thus with transduction of apoptotic signals. However, E6 did not appear to have much effect on TNF susceptibility in human keratinocytes^[84,88]. In contrast, in human ovarian and colon cancer cells, HPV-16 E6 enhanced susceptibility to TNF-induced apoptosis^[100]. This effect of E6 appeared to be p53-independent but may involve down-regulation of NF-kappa B. Notably, the BPV-1 E6 oncoprotein sensitized cells to TNF-induced apoptosis^[101]. This BPV-1 E6-induced sensitization to apoptosis is distinct from its transforming activity^[102]. Interestingly, expression of HPV-16 E6 sensitized murine fibrosarcoma L929 cells to TNF-induced necrosis instead of apoptosis^[103]. The E6-enhanced cytolysis correlated with an increase in reactive oxygen species level and was independent of p53 and caspases^[103].

In human diploid fibroblasts, expression of HPV-16 E6 resulted in an inhibition of oxidant-induced apoptosis as compared to vector control within 24 h but a sensitization after prolonged incubation^[104], indicating that time point at which cell death is measured also contributes to the outcomes. Dying E6 cells exhibited a G2/M phase distribution with elevated cyclin B/Cdc2 levels and activity. The death of E6 cells has some features of oncosis. It remains to be determined to what extent the elevated cyclin B/Cdc2 activity contributes to the cell death in E6-expressing cells. Notably, Normal human fibroblasts expressing HPV-16 E6 showed increased cytotoxicity to taxol^[105]. Mutational analysis indicated that reduced levels of p53 correlated with increased G2/M phase arrest and taxol-induced apoptosis^[105]. Adriamycin and cisplatin-treated human foreskin fibroblasts expressing E6 also were arrested at G2 with increased cyclin B/Cdc2 kinase activity but no apoptosis^[106]. Apparently, activation of cyclin B/Cdc2 kinase in G2/M arrested cells is not by itself sufficient to trigger cell death. E6 expressing cells could also die at other cell cycle stages. For example, when treated with cisplatin, normal human foreskin fibroblasts expressing HPV-16 E6 showed increased cytotoxicity associated with delayed progression through S phase^[107].

CONCLUSIONS

Progress has been made on observations of E6 regulation of apoptosis. However, the precise mechanism by which E6 modulates apoptosis remains to be explored. In particular, we know little about how E6 sensitizes cells to apoptosis independently of p53. Few studies have addressed the functions of low-risk HPV E6s on cell proliferation and apoptosis. Future studies should also establish the role of more than twenty E6-interacting proteins identified during the past decade. Understanding the mechanism by which E6 regulates apoptosis will certainly help us fully demonstrate the significance of HPV in the etiology of esophageal cancer and possibly have some therapeutic significance.

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