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MINIREVIEW

The Role of Human Papilloma Virus in the Molecular Biology of Cervical Carcinogenesis

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Research exploring the E6-p53 and E7-pRb model has resulted in the identity of the viral gene's actions on numerous cellular proteins and processes normally involved in cell growth and proliferation. Specially, several findings have established the various ways by which the HPV-infected cell may escape controls governing cell growth and proliferation, including the fidelity of the host cell's genome and apoptosis.

A large body of knowledge already generated in this area supports the view that high-risk HPV types have the ability to transform cells into a malignant phenotype. Such ability, however, is not sufficient to actually and inevitably produce cervical carcinoma, as indicated by the frequent spontaneous clearance of HPV infection and the long delay between the onset of persistent infection and emergence of the malignancy. Delay in the participation of cofactors has been suggested as explanation in this regard. However, it remains unclear how and when cofactors or factors that are innate in the HPV-infected cells launch the host cells into an irreversible progression to carcinoma.

In November 1991, a workshop convened by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) officially concluded that, based on epidemiological and laboratory data, the association between human papillomavirus infection and cervical cancer is beyond reasonable doubt, and infection with human papilloma virus should be considered as cause to the development of cervical cancer[3]. The publication of the workshop's conclusion marked the beginning of serious efforts by governments and private sectors worldwide to increase global awareness of the grave risks posed by human papillomavirus (HPV) on women's health.

From the perspective of molecular biology, however, awareness of HPV's link to cervical cancer had begun much earlier. Such a role for HPV was suggested as early as 1976[50], 15 years before the 1991 workshop, and the first genital HPV types were identified in 1978[29]. In 1981, Zur Hausen et al. reported the detection of HPV DNA in cervical neoplasia[51]. In 1989, two years before the 1991 IARC-sponsored conference, the ability of the E6 and E7 protein expressed by "high-risk" HPV type 16 to immortalize and transform human keratinocytes was reported[16,27]. HPV is detected by means of polymerase chain reaction (PCR), where L1-PCR was described by Yoshikawa et al[48] and SPF-PCR by Kleter et al[21]. The difference between the two is that, the L1 region is relatively well-conserved and

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several PCR primers from the L1 region were developed. The SPF primers on the other hand, used in the latter type of PCR, only amplify fragment of 65 bp, which is extremely sensitive amplification from a broad array of HPV genotypes. Finally, before the end of the decade, the application of new highly sensitive detection system has shown that nearly 100% of all squamous cell carcinoma of the cervix, and more than 70% of cervical adenocarcinomas, are associated with the presence of HPV DNA[21,44].

Even before the 1991 IARC-WHO workshop, HPV's association with cervical cancer had been so widely observed that by mid-1990s some investigators have begun to regard the virus as the "primary causal agent" in the development of the malignancy and its precursor lesions[30]. In fact, attempt to officially declare such a link was made in an earlier IARC-WHO workshop in 1989, but was ruled out by some participants for lack of well-controlled epidemiologic and laboratory studies to support it. Such evidence were subsequently obtained, under conditions stipulated by the 1989 workshop, and convinced the 1999 meeting to declare the causal role of HPV in cervical cancer.

Today, the study of HPV's carcinogenic role continues to represent the mainstream in the research on the molecular biology of cervical cancer. The status is well deserved as research in this area integrates the major fields of virology, oncology, cellular molecular biology and molecular genetics. Moreover, research in this field possesses practical significance. Cervical cancer is the second most common malignancy among women worldwide and is known to claim more than 400,000 new victims each year[18]. The prophylactic and therapeutic applications of the knowledge from this field, therefore, offers immense benefit to millions of women who are afflicted, or who are at risk of being afflicted, with HPV-induced cervical cancer.

This paper highlights some major recent findings in this field and identifies possible trends in the study of the mechanisms of HPV's participation in cervical carcinogenesis.

Human Papilloma Virus

HPV Types

More than 80 types of human papilloma viruses (HPVs) are known today, and they are generally classified according to their potential to induce malignant transformation. HPVs 16, 18, 31, 33, 35, 39, 45, 50, 51,53, 55, 56, 58, 59, 64 and 68 are considered "high risk" types because they are detectable in carcinomas and dysplasias[8,23]. HPVs 31, 33, 35, 51 and 52 are sometimes regarded as "intermediate risks" because they are more common in mild or severe dysplastic lesions than in carcinomas. Among the high-risk strains, HPV 16 and 18 are the most closely associated with cervical carcinoma. The HPV16 DNA has been found in more than 50% of squamous cell carcinomas, while the HPV18 DNA has been found in more than 50% of adenocarcinomas[25].

HPV Genome

HPVs are DNA tumor viruses whose genome is organized in three regions: the early gene (E1 to E7), the late gene (L1 and L2) regions and the upper regulatory region (URR). The early and late gene regions are both protein-encoding, but the URR is non-encoding[30]. The URR possesses numerous binding sites for many repressors and activators of transcription, suggesting that it may play a part in determining the range of hosts for specific HPV types[41].

E1 and E2, meanwhile, encode proteins that are vital for extrachromosomal DNA replication and the completion of the viral life cycle. The E2 also encodes two proteins: one, which inhibits transcription of the early region; and the other, which increases the

transcription of the early region[45]. A hallmark of HPV-associated cervical carcinoma is loss of the expression of the viral E2 protein[39].

The E4 protein is expressed in the later stages of infection when complete virions are being assembled, and is not known to have transforming properties; however it is considered to play an important role for the maturation and replication of the virus[4]. The E4 protein also induces the collapse of the cytoplasmic cytokeratin network in human keratinocytes, a situation which may assist the release of virions from the infected cell[13].

The E5 in open reading frame (ORF), meanwhile, is often deleted in cervical carcinoma cells, indicating that it might not be essential in maintaining the malignant transformation of the host cell. When present, E5 interacts with various transmembrane proteins like the receptors of the epidermal growth factor, platelet-derived growth factor β, and colony stimulating factor-1[18]. A study using HPV 16-infected cells found the E5 protein to possess weak transforming activity[9].

In the protein-encoding regions, the E6 and E7 ORF are considered to play the most major roles. These two units encode for oncoproteins that allow replication of the virus and the immortalization and transformation of the cell that hosts the HPV DNA[2,32,36].

The late region units, L1 and L2 encode for viral capsid proteins during the late stages of virion assembly[30]. The protein encoded by L1 is highly conserved among different papilloma virus species; antibodies against the bovine papilloma virus, therefore, have been used to identify HPV capsid proteins in human tissues. The minor capsid protein encoded by L2 has more sequence variations than that of the L1 protein; hence, antibodies against the L2 protein had been a source of antigen for specific types of HPV antibodies.

DNA Integration

The HPV DNA is usually extrachromosomal or episomal in benign cervical precursor lesions. However, in many cervical cancer cells as well as in cervical cancer cell lines and HPV-transformed human keratinocytes in vitro, the HPV DNA is integrated in the host genome[30]. Cancer tissues may contain both episomal and integrated HPV DNAs at the same time, although integration appears to occur more frequently in HPV 18-associated cervical cancer than in HPV 16-associated cervical cancer[9].

During HPV DNA integration, the viral genome usually breaks in the E1/E2 region. The break usually leads to the loss of the E1 and E2 regions. The loss of E2, which encodes proteins including one that inhibits the transcription of the E6 and E7 regions, has been known to result in uncontrolled and increased expression of E6 and E7 oncogenic proteins. Increased expression of E6 and E7, meanwhile, has been observed to lead to the malignant transformation of the host cells and to tumor formation[3,13,32]. HPV viral integration into the host genomic DNA is associated with progression from polyclonal to monoclonal status in CIN, and these events play a fundamental role in the progression from low-grade to high-grade cervical neoplasia[42].

Research Trends

E6-p53 and E7-pRb Model

Because of their oncogenic properties, E6 and E7 proteins have been the focus of most studies in the cervical carcinogenesis during the past 20 years. A vital portion of the mechanism by which these two oncoproteins induce their effects was elucidated between the late 1980s and early 1990s. During that period, it was established, among others, that E6 binds to and inactivates the tumor suppressor p53, while E7 binds to and degrades the tumor suppressor pRb[37,46]. The establishment of this link created what may be called the E6-p53 and E7-pRb model, the exploration of which may represent the HPV's role in cervical

Table I. Binding of cellular proteins by the high-risk HPV oncoproteins E6 and E7

HPV oncoprotein	Cellular-binding protein	Investigators (year)
E6	* p53	Werness et al. (1990)
		Nagpal et al. (2002)
		DeFilippis. (2003)
	*E6-associated protein	Scheffner et al. (1993)
	*ERC55	Cehn et at. (1995)
	*hDLG	Kiyono et al. (1997)
	*Paxillin	Tong and Howley. (1997)
		Vande Pol et al. (1998)
	*Interferon regulator factor 3	Rnonco et al. (1998)
	*Bak	Thomas and Banks. (1999)
	*E6TPI	Gao et al. (1999)
	*P16 (INK4a)	Masumoto et al. (2003)
E7	*Retinoblastoma protein (pRb)	Dyson et al. (1989)
		Salcedo M et al. (2002)
		DeFilippis. (2003)
	*Rb-related pocket proteins	Dyson et al. (1992)
	*E2F/cyclin A complex	Arroyo et al. (1993)
	*Histone H1 kinase	Davies et al. (1993)
	*TATA box-binding protein	Massimi et al. (1996)
	*Cyclin E	McIntyre et al. (1996)
	*Subunit 4 (S4) adenosine Triphosphatase	Berezutskaya et al. (1997)
	*c-jun	Nead et al. (1998)
	*hTid-1	Schilling et al. (1999)
	*Mi2-pyruvate kinase	Zwerschke et al. (1999)
	*p48	Barnard et al. (1999)

carcinogenesis. The integration of high-risk HPV is likely to be a critical event in cervical carcinogenesis, and this even precedes the development of chromosomal abnormalities[31]. Recent studies by Masumoto et al revealed that inactivation of Rb protein by HPV 18 E7 protein may be associated by carcinogenesis of small cell carcinoma the in same manner as inactivation of Rb protein by HPV 16 E7 protein is associated with carcinogenesis of squamous cell carcinoma[24].

The discovery of the link between these oncoproteins and tumor suppressors might not have been entirely surprising; the degradation of tumor suppressors p53 and pRb by other oncogenic viruses had been known previously[5,52]. The demonstration of these tumor suppressors' inactivation by the E6 and E7 HPV oncoproteins, however, provided a basic explanation on how high-risk HPV types induce their oncogenic effects on cervical cells[17].

When a cell suffers DNA injury or damage, p53 protein activates the transcription of genes like p21 (CIP1/WAF1) or GADD45, effecting a delay in the cell's entry into the S phase until DNA repair is accomplished. Alternatively, p53 may induce apoptosis by activating genes such as bax 1 and the fas receptor gene. The inactivation of p53 by the E6 oncoprotein, therefore, results in the deregulation of the cell cycle and allows cellular mutations to occur[36]. The relationship between p53 codon 72 polymorphism and

Table II. Identified functions of the high-risk HPV oncoproteins E6 and E7

HPV oncoprotein	Identified function	Investigators (year)
E6	*Cell immortalization	Band et al. (1990)
	*Binding of E6-associated protein results in degradation of specific host cell proteins(p53)	Werness et at. (1990) Scheffner et al. (1993) Tommasino et al (2003)
	*Antiapoptic effect	Werness et al. (1990) Thomas and Banks. (1998)
	*Chromosomal destabilization	White et al. (1994)
	*Enhancement of foreign DNA integration and mutagenicity	Kessis et al. (1996) Havre et al. (1995)
	*Activation of telomerase	Klingelhutz et al. (1996)
	*Blockade of interferon	Ronco et al. (1998)
	*E6 I/E6 II gene expression	Moodley et al. (2003)
	*E2F-regulated mitotic genes	Thierry et al. (2004)
E7	*Cell immortalization	Munger and Phelps. (1993)
	*Activation of cyclins E and A	Arroyo et al. (1993) Zerfass et al. (1995)
	*Inactivation of retinoblastoma protein-related pocket proteins	Dyson et al. (1989, 1992)
	*Induction of apoptosis	Puthenveettil et al. (1996)
	*Inhibition of cyclin-dependent kinase inhibitors	Jones et al. (1997) Funk et al. (1997)
	*Enhancement of foreign DNA integration and mutagenicity	Kessis et al. (1996) Reznikoff et al. (1996)
	*Degradation of tyrosine kinase	Oda et al. (1999)
	*Numerical and structural chromosomal abnormalities	Pett et al. (2004)
	*E2F-regulated mitotic genes	Thierry et al. (2004)

susceptibility to development of HPV-associated cervical cancer has been reported[28]. Majority of the cervical cancer cells have a wild-type p53 gene[40].

The binding of the E7 oncoprotein on pRb provides a complementary function. The binding releases transcription factor E2F that activates the expression of genes that stimulate DNA synthesis in the cell. If earlier E6 action had freed the same cell from p53 control, that cell survives into the S phase with a damaged DNA and, through E7 action, is able to replicate the HPV DNA[5]. It was reported that the heterogenous pRb immunostaining in the different stages of cervical carcinogenesis could be a common feature implicated in the pathogenesis of uterine cervical cancer[33].

The oncogenic properties of E6 and E7, as well as their effects on p53 and pRb, have provided the general basis for further investigations of the role of HPV in carcinogenesis in the HPV-infected cervix. Major findings in these areas are summarized in Tables I and II[53]. Briefly, research in the actions of the two oncoproteins have shown how they subvert key cell cycle and regulatory processes such as cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDIs) to transform and immortalize the host cell[35].

However, the oncogenic effects of HPVs can be suppressed or reversed. For instance, dexamethasone can reverse E6/E7 expression in cancer cell lines and prevent their

development into malignant phenotype[43]. Moroever, viral oncogene antisense constructs, specific ribozymes or antisense oligonucleotides can inhibit the growth of cervical cancer cells containing HPV oncoproteins. Subsequent to somatic hybridization, HPV-immortalized cells may, intracellularly, also complement each other, albeit in a still undefined manner, and undergo senescence despite their continuing transcription of HPV E6/E7 mRNA[7]. Inside HPV-immortalized cells, some cytokines, as well as transforming growth factor-ß (TGF-ß), may also suppress HPV gene transcription. Specifically how the oncogenic effects of the HPV oncoproteins are suppressed or reversed in such conditions, and when such conditions occur physiologically, remains to be understood. DeFilippis et al recommended that strategies that inhibit the expression or activity of either E6 or E7 protein are likely to inhibit the growth of HPV-associated cancers[11].

HPV-Induced Carcinogenesis Theory

Continuing research on these viral genes and their actions has produced a wealth of data during the past decade. Such volume of information, related but generally unconnected, has begun to encourage attempts at integrating them into a unifying explanation or theory for HPV-induced carcinogenesis.

Tewari and associates recently proposed such a theory, one which highlights the disruption by E7 of the functional link between pRb and histone deacytelase-1 (HDAC1) and the subsequent aberrant expression of Notch-1 proteins which, in turn, leads to squamous cell carcinoma of the cervix[38].

HDAC-1 tightens the association between DNA and nucleosomes, preventing the access of transcription factors to DNA recognition elements. This barrier, according to the theory, is removed when E7 degradation of pRb prevents the binding of HDAC1. As a result, there is an aberrant expression of genes that participate in cellular proliferation as well as of Notch and other genes that determine cell fate.

Notch-1 encodes a large transmembrane protein and participates in determining the progression of immature cells to a more differentiated state[47]. The presence of Notch-1 in the cervical transformation zone has been established, particularly in the reserve cells beneath the differentiated columnar epithelium and in the metaplastic areas[49]. Moreover, Notch-1 levels in cervical cells have been shown to increase from mild dysplasia to severe dysplasia, and are highest in invasive carcinoma cells[10]. This pattern was also observed to be parallel to the rise in the level of E6 and E7 transcripts in lesions during the progression from mild dysplasia to invasive cancer.

The theory proposed by Tewari and associates suggests, therefore, that as a result of E7-pRb disruption in HDAC1 binding, the normal cell fate-determination function of Notch-1 is disrupted, and Notch-1 aberrantly functions to assymetrically favor the precursor cells differentiation to squamous epithelia instead of columnar epithelia. The action of HPV viral genes in the squamous cells prevents apoptosis, leading to hyperplasia. The theory additionally proposes that persistent presence of E6 and E7, plus the action of oncogenic co-factors, subsequently results in squamous cell carcinoma.

Cofactors

The ability of the high-risk HPV types to transform and immortalize infected cells has been widely established. However, it is also quite known that many cases of HPV infection clears up spontaneously. Moreover, even in cases where HPV infection persists, cervical cancer most often does not appear until many years or more than a decade later. This long delay has, therefore, supported the view that HPV infection may initiate but not necessarily progress to cervical carcinoma, and that oncogenic influences by other factors, or cofactors, are necessary to fully establish an HPV-induced malignancy. Because it has long been

understood that cancer is a multi-etiological disease[12], the time lag between HPV infection and the diagnosis of cancer also indicates that multiple steps as well as multiple factors may be necessary in the development of cervical cancer.

Previous studies have associated environmental factors and specific aspects of lifestyle with cervical cancer. It is only recently, however, that study has been reported on the relationship between these cofactors and cervical intraepithelial neoplasia (CIN 2-3) among women with history of HPV infection. In that study, Kjellberg and associates observed that smoking was the most significant environmental risk factor for the disease[20]. Pregnancy also showed some degree of influence as a cofactor, but diet, number of sexual partners and prolonged use of oral contraceptives exhibited no significant relationship with HPV-associated CIN[6]. The specific manner by which smoking influences CIN, however, remains to be elucidated.

Infection with Chlamydia trachomatis has also been suggested as a possible contributor to the oncogenic effect of HPV[34]. As in HPV-infected cells, chlamydia has also been observed to inhibit apoptosis in host cells[14]. However, two studies, which statistically adjusted for the concomitant history of HPV exposure of its sample population, showed that chlamydia, particularly serotype G, is by itself a significant risk factor in the development of squamous cell carcinoma of the cervix[1,22]. Despite evidence that the chlamydial infection can be an independent risk factor, the studies' authors nevertheless would not rule out the possibility that the bacteria and HPV can exert a confounding effect on each other in the host cells and lead to cervical cancer.

Another possible cofactor to the oncogenic effect of HPVs is estrogen. The high levels of circulating estrogen during puberty is considered a major influence in the metaplastic changes in the cervical transformation zone during that period. In tissues from the normal adult cervix, which contained the transformation zone and were implanted in severe combined immunodeficiency mice (SCID), estrogen also induced squamous metaplasia as well as hyperplastic change[38]. In the same experiment, implants which were exposed to anti-estrogenic tamoxifen exhibited columnar differentiation exclusively. In vitro studies have indicated that this steroid hormone could assist in the HIV-induced neoplastic transformation in the cervix, as estrogen enhanced the transcription of HPV 16 E6 and E7 oncoproteins in ectocervical cells immortalized by HPV 16. In transgenic mice expressing HPV16, chronic treatment with 17β-estradiol for 3 months resulted in high-grade epithelial dysplasia in the cervix and vagina. After 6 months, 6/8 mice (75%) developed microinvasive or frankly invasive squamous cancer. In contrast, nontransgenic mice given the same treatment showed normal epithelial differentiation pattern[19]. While estrogen may be a potential cofactor in the carcinogenesis in cervix and vagina, the exact nature and mechanism of its participation in HPV-induced malignancy still remains to be clarified. On the other hand, from a molecular standpoint, the role of injectable progesterone does not confirm its role in cervical carcinogenesis[26].

CONCLUSION

Cervical cancer, which continuously plagues women all over the world, still remains to be one of the major focuses of researchers. Infection with HPV has been implicated as an important etiological factor in the causation of uterine cervical cancer. The role of HPV, in association with other factors, has been extensively studied in relation to cervical cancer. High-risk HPV types, specifically types 16 and 18 have been shown to participate in uterine cervical carcinogenesis through the expression of proteins, such as E6 and E7, interaction with different steroid hormones, and genetic susceptibility to develop cervical cancer. Even

with the active research investigations being done, cervical cancer still ranks as one of the leading female cancers. Prevention is still advocated by gynecological oncologists since therapeutic modalities available are still not that 100% effective in curing this disease.

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