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Human papillomaviruses and cervical neoplasia. I. Classification, virology, pathology, and epidemiology

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Introduction

The aetiology of cervical neoplasia has been studied epidemiologically for over 150 years. More recently, experimental approaches have been applied and, with the advent of molecular biology, putative infectious agents can now be studied more easily and with greater precision. There is now considerable evidence that the risk of developing cervical cancer is, in part, sexually transmitted.[1 2] The most likely explanation for this is that an infectious agent is involved but much of the interpretation of epidemiological studies has, of necessity, been performed without knowledge of the identity of this agent. With increasing evidence, both epidemiological and experimental, that human papillomavirus (HPV) is the infectious agent in question, the role of other factors can now be addressed with more confidence. In the first part of this review the molecular evidence implicating HPV in cervical neoplasia will be considered; in the second the evidence for the involvement of other factors will be reviewed and the potential role of HPV testing in clinical practice addressed.

Molecular organisation and classification of human papillomaviruses

Papillomaviruses are non-enveloped, double stranded DNA viruses about 55 nm in diameter and with a circular genome approximately 8 kilobases in length. Traditionally, they have been included in the papovaviridae and this remains the recommendation of the International Committee on Taxonomy of Viruses.[3] However, they are sufficiently dissimilar from the other members of the group, both in terms of virion size and genomic organisation, to be regarded as a separate group.[3] Molecular cloning of viral nucleic acids and more recently polymerase chain reaction (PCR) amplification and sequencing have demonstrated that multiple HPV types exist, with up to 70 types of HPV now described.[3 4] The molecular organisation of all papillomavirus genomes described conforms to the pattern shown in fig 1. The viral genes are divided into two functional groups, the early (E) and late (L) genes. There are seven E genes, each of which serves a different function: the E1 and E2 genes are involved in viral replication and transcriptional control, respectively,[5] and tend to be disrupted by viral integration. The E6 and E7 genes (with some recent evidence implicating E5) are involved in cellular transformation[6 7]; the two L genes encode structural viral proteins and are therefore required for productive viral infection. Between the E and L genes, there is a region of non-coding sequences involved in control of viral gene transcription and known as the upstream regulatory region (URR).

Since 1979, papillomaviruses have been classified according to their degree of homology, assessed by cross hybridisation in solution,[8] and have been numbered in consecutive order of description. Subtypes were defined according to variations in restriction digestion pattern and variants by the presence of a limited, but undefined, number of nucleotide differences. More recently, it has been suggested that a new HPV type be defined as one that has been completely cloned and whose E6 and L1 genes and URR are less than 90% homologous. A subtype would be defined as sharing 90–98% homology in these regions and a variant more than 98%.[3] However, few types have been assigned using this rigorous definition, partly because of the amount of sequencing data required, but analysis of homology in a relatively short segment (384 base pairs) of the E6 gene has permitted the generation of a phylogenetic tree (fig 2), which does not differ substantially from that derived using a larger proportion of the genome.[3] Thus, with further refinements in sequencing technology, particularly automation, and relaxation of the definition to include perhaps only the E6 gene, assignment of HPV type may become relatively straightforward.

With the more widespread application of sequencing to HPV isolates, it has become clear that there is considerable molecular heterogeneity not only among isolates from different ethnic populations[9] but also between individuals from the same population[10] and indeed within the same individual.[11] Thus, HPV infection may be "polyclonal", although the biological significance of this is as yet

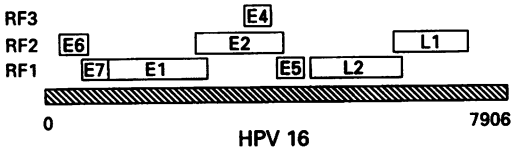

Figure 1 Schematic view of the molecular organisation of the 7906 base pair genome of HPV 16. The boundaries of each gene are those of the open reading frames (ORFs). E and L refer to the early and late genes, respectively. The URR is located between the E and L regions. The three reading frames are denoted by RF1, RF2, and RF3. Derived from data in reference 65.

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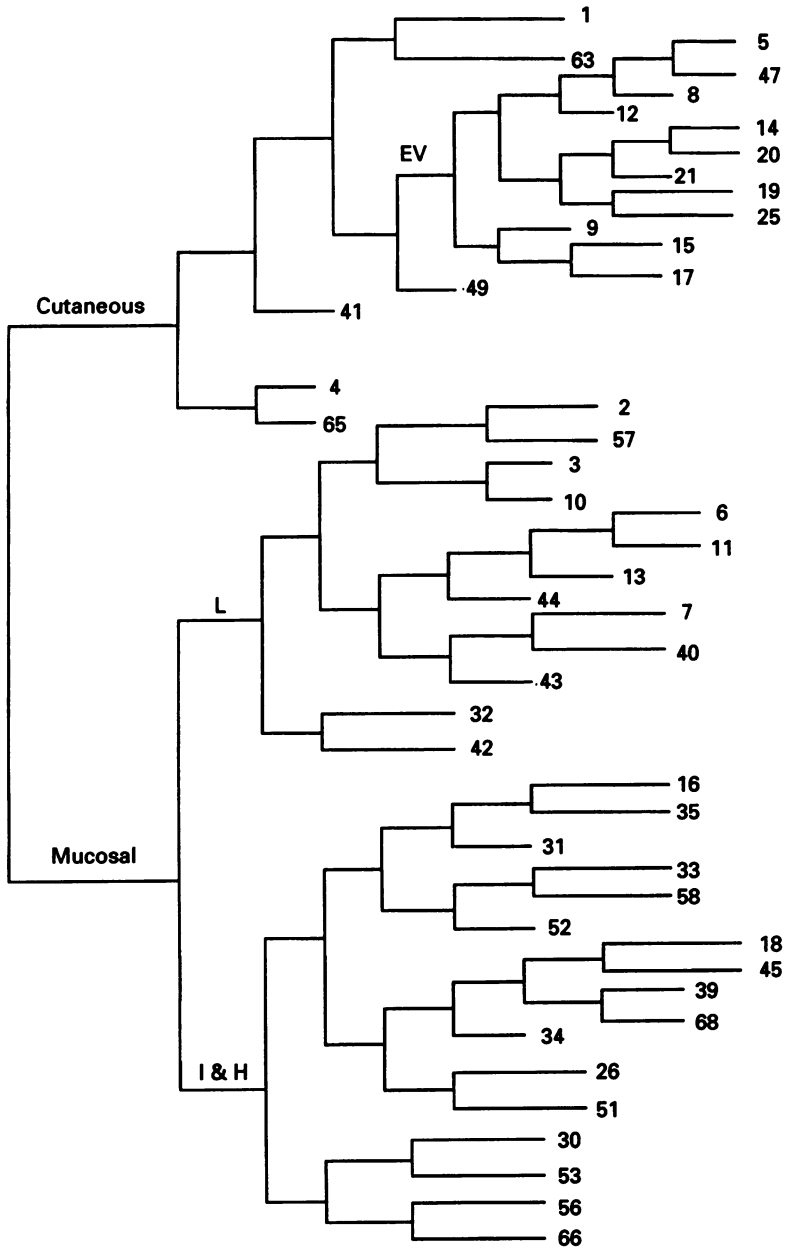

Figure 2 Phylogenetic tree of HPV types based on homology of a 384 base pair segment of the E6 gene. EV = epidermodysplasia verruciformis; L = low risk mucosal types; I & H = intermediate and high risk mucosal types. The numbers refer to HPV type. Drawn from reference 3.

unclear. Similarly, the importance of molecular heterogeneity is not known and, with some studies showing no apparent biological difference between variants,[12] it is likely that many of these variants simply represent polymorphic variation. However, there is a report of a naturally occurring HPV 16 variant with a mutation in the E2 gene which failed to immortalise human keratinocytes.[13] Molecular variation may therefore be of biological importance in some circumstances.

HPVs can also be classified on the basis of the site of infection, resulting in two main HPV groups: cutaneous and mucosal (fig 2). The former are found in cutaneous warts and patients with epidermodysplasia verruciformis and the latter predominantly in anogenital lesions. This approach parallels the molecular classification, although some more recently described types cross the boundary, being found in both cutaneous and mucosal lesions.[4]

Virology and pathology of HPV infection

Viral infection involves interaction between virus and host cell and only certain cell types can support vegetative viral production. Cells which do not support infection are termed non-permissive, non-transformable: most cells fall into this category for HPV infection. HPVs are notably epitheliotropic and have been found in squamous epithelium (both original and metaplastic) at almost every site. The means by which the virus gains access to basal epithelial cells is not known and, although abrasion of the epithelium is a postulated explanation, evidence for this hypothesis is lacking.[14] Only a low number of viral genomes is present in the basal and suprabasal layers of the epithelium[14] and, in permissive cells, HPV replication, gene expression, and protein synthesis are closely linked to differentiation and hence keratinisation. Amplification of viral genomes, therefore, only occurs in the strata spinosum and granulosum, as shown by the presence of a high viral copy number in the superficial epithelial cell layers (fig 3).[15] Analysis of viral messenger RNA (mRNA) production using in situ hybridisation has shown that E gene expression is found throughout the epithelium, but L gene expression is only seen in terminally differentiated keratinocytes, where it is accompanied by capsid protein production.[16] This form of infection leads to the cytopathic effect of HPV in squamous epithelial cells, with koilocytosis, nuclear enlargement, dyskeratosis, and multinucleation being the major changes.

Non-permissive transformable infection describes the situation in which viral replication and vegetative viral production do not occur, but viral DNA persists within the cell either as an extra-chromosomal element or by integration into the host genome. This persistence may be associated with cellular transformation as in high grade cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (ISCC) (see Part II). HPV infection of columnar glandular epithelia is being increasingly described,[17] and may also represent this form of infection.

From a clinical point of view, three forms of infection can be defined: latent HPV infection in which HPV DNA is detectable by molecular means but without accompanying clinical, cytological, or histological abnormality; subclinical HPV infection which is not detectable by naked eye examination—that is, it requires colposcopy, or microscopy for identification; and clinical HPV infection which is visible to the unaided eye.[14] The HPV types found in subclinical and clinical anogenital infections are associated more with the histopathology than the site of the lesion. Thus, a condyloma acuminatum is likely to be infected with HPV 6 or 11 whether it is of anal, vulval, penile, or cervical origin. Similarly, the flat condyloma of the cervix and Bowenoid papulosis of the penis are both associated particularly with HPV 16 and 18. Dysplastic lesions from all anogenital sites have been associated with HPV infection by HPV 16, 18, and related viral types analysed by both epidemiological and experimental

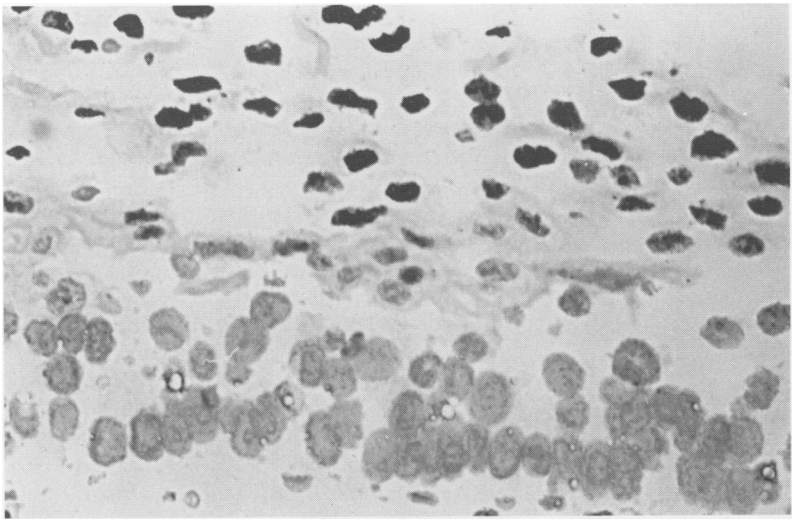

Figure 3 In situ hybridisation of a routine cervical biopsy with a digoxigenin labelled probe for HPV 33. Note the presence of intense staining within superficial epithelial cell nuclei indicating replicative viral infection.

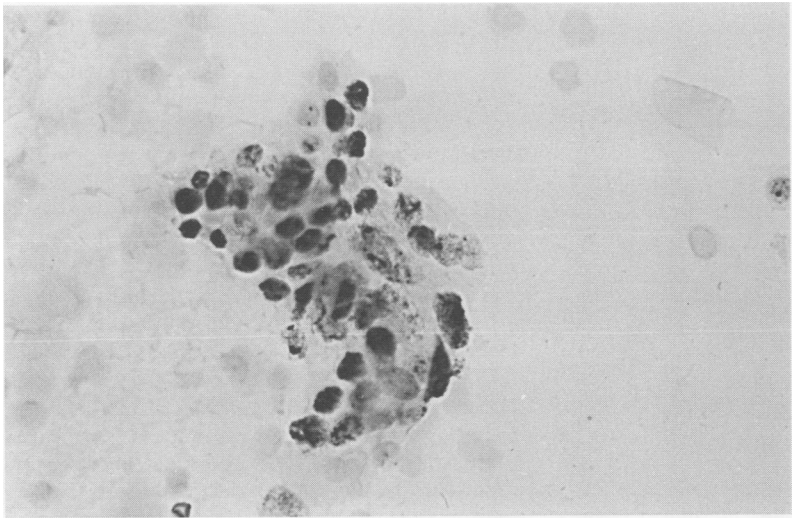

Figure 4 In situ hybridisation of a routine cervical smear with a cocktail of digoxigenin labelled probes for HPV 16, 18, 31, and 33. Note the presence of signal (seen here as black) within the nuclei of a cluster of epithelial cells.

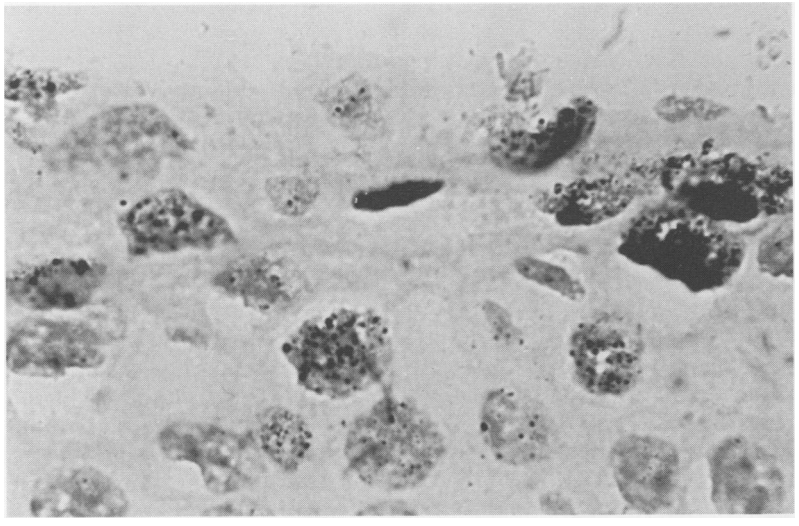

Figure 5 In situ hybridisation of a routine cervical biopsy showing CIN III with a digoxigenin labelled probe for HPV 16. Note the presence of both diffuse and punctate signal within epithelial cell nuclei.

means. These clinical associations have led to the definition of "high risk" (particularly HPV 16 and 18), "intermediate risk" (particularly HPV 31 and 33), and "low risk" (particularly HPV 6 and 11) viral types (fig 2).[4 18] ISCCs of the vulva, penis, anus, and cervix are all associated with the same HPV types, suggesting a common aetiological link. One apparent exception to this is verrucous carcinoma of the cervix, which is associated with HPV 6/11 infection. However, this assertion has recently been questioned with the suggestion that most verrucous carcinomas can be re-classified histopathologically, falling into one of three categories: giant condyloma, condylomatous CIN, and ISCC.[19] HPV 6/11 infection may therefore simply indicate the presence of a giant condyloma. Nevertheless, HPV 6 has been unequivocally identified within squamous carcinoma cells in the absence of other HPV types.[20]

Detection of HPV in clinical material

The detection of nucleic acid in clinical material is the most appropriate way of achieving a diagnosis and of investigating the epidemiology and natural history of HPV infection because (i) clinical features are not diagnostic; (ii) the cytopathic effect is insensitive and non-specific; and (iii) viral protein production is not constitutive. Either DNA or RNA can be analysed but the detection of RNA is technically more difficult and is dependent on viral gene transcription, which is not constitutive. This was highlighted recently by a study of cytologically normal cervical smears using reverse transcriptase PCR in which E6 mRNA transcripts could not be demonstrated despite the presence of HPV DNA.[21] Although the biological relevance of the presence of HPV DNA in the absence of RNA production is unknown, viral DNA is generally analysed. This can be achieved in two basic ways: after extraction from tissue with or without amplification by PCR or directly by in situ hybridisation.

DNA can be extracted from peripheral blood, cellular suspensions, frozen tissue, and fixed, processed embedded material. It is therefore possible to analyse the DNA content of any clinical sample encountered in everyday practice using conventional molecular techniques such as restriction mapping, and Southern and dot blotting.[22] The basic PCR can be used to amplify a specific DNA region by as much as 10^8-fold and can be performed on the smallest of clinical samples with simple equipment.[23] This combination of sensitivity and flexibility has prompted its widespread use for the detection of HPV sequences and, if product identity is established by restriction digestion, it is routinely applicable. However, if Southern blotting or nucleotide sequencing are used, the time and expertise required are correspondingly increased. Several PCR based systems are available for HPV detection[24 25] but those most commonly used involve a generic amplification step which

amplifies a very wide range of HPV types (some of which are uncharacterised) using degenerate primer pairs, coupled with either more specific amplification[24] or typing by dot blot hybridisation.[25] Both approaches allow high sensitivity and broad-spectrum HPV typing. In situ hybridisation allows cellular localisation of signal in both cytological and histological material, thus increasing specificity for cellular infection but with lower absolute sensitivity[26] and can distinguish integrated from episomal viral sequences on morphological grounds (figs 3–5).[27]

Detection of HPV in clinical material is therefore most commonly achieved by DNA analysis, particularly by Southern or dot blotting and PCR, or by in situ hybridisation. Recently, the hybrid capture technique, in which hybrids formed between HPV DNA and a RNA probe are detected by antibody binding, has been described.[28] This technique is of similar sensitivity to PCR but the biological importance of small amounts of HPV DNA, which may not be derived from within cells and are not always associated with RNA expression,[21] has yet to be defined.

Molecular epidemiology

Early molecular studies suggested the association of HPV 6/11 with condylomata acuminata and CIN I and of HPV 16 and 18 with CIN II and III, and ISCC of the cervix. In view of this observation it was postulated that HPV 16 and related viral types were causally related to the development of CIN and ISCC. The prevalence of HPV 16/18 increases with the severity of the lesion, viral DNA being integrated in the majority of CIN III lesions and ISCC.[29] Progression from HPV infection alone to CIN to ISCC has been documented histopathologically[30] and an almost 16-fold increased risk for the development of carcinoma in situ has been reported in patients with cytological evidence of wart virus infection.[31] The natural history of HPV infection appears to be similar to that of CIN, but CIN associated with HPV infection occurs in women about 10 years younger than CIN alone. In a large follow-up study a high proportion of infections regressed but progression of lesions was highly associated with the presence of HPV 16.[32] These data suggest that HPV infection, particularly with certain HPV types, is a precursor lesion to the development of CIN. This has been confirmed by a recent cohort study in which the relative risk of development of CIN II or III in the presence of HPV 16 or 18 DNA, as determined by Southern blotting, was 11·0.[33] Analysis of eight cross-sectional studies involving 2627 patients confirmed the logic of separating HPV types into "low", "intermediate", and "high risk" types, showing a greater association of HPV 18 with invasive carcinoma then with CIN II or III. HPV 16 was equally associated with invasive carcinoma and CIN II or III, and "intermediate risk" HPV types were more associated with CIN II or III than with invasive carcinoma. Overall, the presence of oncogenic types was associated with an increased risk ranging from 65·1 to 235·7 for CIN II or III and from 31·1 to 296·1 for an invasive lesion.[18]

Many of the problems encountered in the analysis of epidemiological data regarding the prevalence of HPV infection have been because of variations in methodology. This is particularly true of PCR, which has suggested that up to 84% of women with normal cervical smears carry HPV 16 DNA. However, more recent data have shown that a high rate of false positive results is obtained with PCR if measures are not taken to eliminate or control for sample contamination.[23] With the appropriate precautions, however, PCR can be used fruitfully and more recent studies have found much lower carriage rates for high risk HPV types in the community, estimates ranging from 3·5 to 30%,[24 34 35] and show that the prevalence of HPV is age dependent, being consistently only 1–2% over the age of 35 years.[36] Clearly, the figures obtained partly depend on the population studied but there does appear to be a difference between patients with normal and dyskaryotic smears. A recent PCR based case-control study suggests that most grades of CIN can be attributed to HPV infection.[37]

The use of semiquantitative PCR techniques has revealed a correlation between higher amounts of "intermediate" and "high risk" HPV DNA and CIN II or III, a finding which is consistent with results obtained using in situ hybridisation.[39–42] This may simply represent the molecular equivalent of distinguishing between latent and subclinical/clinical HPV infection by these types, an interpretation which is supported by the observation that, in all cases in which apparently normal cervical smears were positive on in situ hybridisation, the morphology of the positive cells appeared abnormal.[26] Nevertheless, this distinction may be of clinical relevance (see Part II).

Molecular pathology of cervical lesions and cell lines

There are four main events in the interaction between HPVs and the host cell: infection, integration, transcription, and translation. Most data are available for squamous lesions, which will be considered first.

SQUAMOUS CERVICAL LESIONS

HPV sequences have been localised to squamous epithelial cell nuclei by in situ hybridisation.[43] Invasive cervical carcinoma and CIN III lesions often contain areas of lower grade CIN adjacent to the main lesion. Analysis of this situation by in situ hybridisation has shown that the same HPV type is present in areas of all grades of CIN including the main lesion, suggesting that these areas represent sequential steps in the same process.[44] This observation also suggests that HPV infection occurs early in the process of cervical carcino-

genesis. "Intermediate" and "high risk" HPV types are associated more with CIN II and III than with HPV infection alone or CIN I both on in situ hybridisation[27] and PCR,[45] an observation which is consistent with the epidemiological data. ISCCs consistently contain HPV sequences, particularly "high risk" types, with most studies reporting over 85% of tumours positive and some series 100% positivity on PCR.[35]

The HPV genome is integrated in all HPV containing cell lines studied to date either arising spontaneously[46] or induced in vitro.[47] Viral integration is also associated with CIN III and ISCC, being demonstrable by both conventional molecular techniques and in situ hybridisation (fig 5).[27 29] However, integrated sequences often co-exist with episomal sequences within the same lesion[27 29 48] and not all invasive carcinomas contain integrated sequences. Viral integration into the host genome appears to occur at random at fragile sites[46 47] but the site of breakage of the viral genome is more consistent, occurring in the E1/E2 region. The E2 gene is involved in the control of viral transcription,[8] suggesting that a break at this point may lead to enhanced transforming gene transcription. The finding that mutation of the E1/E2 genes of HPV 16 enhances viral immortalising capacity[49] is consistent with this possibility in view of the role of the E2 gene in the repression of E6 and E7 gene expression.[5]

The pattern of viral transcription in condylomata and low grade CIN mirrors that of permissive viral infection, with all E genes expressed and L gene mRNA only present in terminally differentiated keratinocytes.[50] CIN II and III lesions show a different pattern, with loss of L gene expression but increased E gene expression,[16 50] with de-repression particularly of E6/E7. This pattern is consistent with viral integration in these lesions, with disruption of the E2 gene and hence up-regulation of E6/E7 gene transcription.

Viral mRNA is produced by HPV containing cervical carcinoma derived cell lines,[51] the major transcripts being derived from the E6 and E7 genes and the continued requirement of E7 transcription for maintenance of the transformed phenotype has been demonstrated.[52] This is supported by the observation that inhibition of E6/E7 gene activity using anti-sense oligonucleotides led to reduced growth and phenotypic alteration in HPV 18 containing cell lines.[53]

The viral proteins found in cervical carcinoma derived cell lines are the products of the E6 and E7 genes. This correlates with the major transcripts found in these cells. These proteins bind to cellular proteins involved in the regulation of growth control, thus suggesting a mechanism for the interaction between HPV infection and integration and aberrant cell growth (see Part II). However, one study using in situ hybridisation failed to show the presence of transcripts capable of encoding full-length E6 or E7 proteins in intraepithelial or invasive lesions,[54] thus questioning the universal in vivo role of E6 and E7 proteins.

GLANDULAR CERVICAL LESIONS

There has been considerable debate regarding the role of HPV infection in glandular lesions of the cervix.[55] Although PCR based studies have generally identified a high proportion of cases containing HPV sequences, this is not universally so.[56] Moreover, derivation of the HPV sequences from adjacent squamous epithelium cannot be excluded without microdissection techniques. However, in situ hybridisation studies have localised HPV sequences to glandular epithelial cell nuclei and have demonstrated viral integration.[57] Glandular lesions of the cervix are associated more commonly with HPV 18 than with HPV 16 or other types in most studies both from Europe and the United States, suggesting that HPV 18 may be more capable of infecting glandular epithelial cells. Thus, although there is some variation between studies, there appears to be an association between HPV infection and glandular cervical lesions, with the association being stronger for HPV 18. More data are required, however, before this association is firmly established.

Molecular experimental pathology

There are three approaches to the experimental study of the effect of HPV sequences on cells in vitro: transfection studies; raft culture techniques; and the use of transgenic mice. These will be considered in turn.

IN VITRO TRANSFORMATION

The transforming ability of HPV 16 was initially demonstrated by direct transfection of NIH-3T3 fibroblasts with HPV 16 DNA, while the E6/E7 region of HPV 18 was sufficient for transformation of these cells.[58] However, as NIH-3T3 cells cannot be considered normal, the ability of HPV sequences to transform them does not imply that the transformed phenotype can be produced in normal cells by the same mechanism.

The approach to the study of cultured normal cells involves the use of the cooperation assay which has defined two categories of oncogenes: establishment genes and transforming genes.[59] In general, one or more genes from each of these groups are required for cellular transformation, and both human fibroblasts and keratinocytes can be immortalised, but not transformed, by HPV 16 DNA. Transformation, therefore, requires other genes and is discussed in Part II.

RAFT CULTURES

In vitro collagen raft culture systems, where keratinocytes are permitted to differentiate morphologically, enable the effect of HPV DNA on "epithelial" morphology to be studied.[60] HPV 16 DNA induced morphological changes in foreskin keratinocytes were indistinguishable from CIN in this system and were associated with viral integration, aneuploidy, and chromosome aberrations.[60] E6/E7 mRNA was also present in the abnormal cells. However, morphological CIN was produced after only a few hundred generations and

therefore this model does not accurately reflect the time-course of cervical neoplasia, suggesting that viral integration may be accelerated in this system. Fetal ectocervical cells transfected with HPV 16 showed abnormal patterns of differentiation similar to CIN in a similar system[61] and the culture of cell lines containing episomal viral sequences has now been achieved.[62] These studies show that HPV sequences can induce lesions with the morphology of CIN and that productive viral infections can be cultivated in vitro. Therefore, these systems will be of importance in the analysis both of productive viral infection itself and of events involved in the genesis and progression of CIN.

TRANSGENIC MICE

Transgenic mice have been used to examine the effects of the E6/E7 genes of HPV 16 in vivo. When coupled to a β-actin promoter, these genes led to the formation of neuroepithelial tumours in the transgenic clones.[63] However, E6/E7 RNA was only expressed in the tumours and not in surrounding tissues, suggesting a localised effect. Linkage of the E6/E7 genes to the α-crystallin promoter led to microphthalmia and cataracts, and the formation of lens tumours in a large proportion of transgenic animals, showing that localised gene expression can lead to tumour development at that site.[64]

These studies provide evidence that the E6/E7 genes of HPV 16 have transforming function in vivo as well as in vitro and further study of transgenic models will allow analysis of the other factors involved in tumour progression.

Conclusions

There is clear evidence that different HPV types are associated with different lesions allowing the distinction of "high", "intermediate", and "low risk" HPV types. Molecular epidemiological and pathological studies confirm this stratification and support a role for HPV in the process of cervical carcinogenesis. However, HPV infection often regresses and, where it is associated with neoplasia, is an early event. Therefore, other factors must be involved and these are discussed in Part II.

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