Human papillomavirus infections: new perspectives for prevention and treatment

Stefano Menzo[1], Katia Marinelli[1], Patrizia Bagnarelli[1], Serena Rolla[2], Massimo Clementi[2,3]

[1]Istituto di Microbiologia e Scienze Biomediche, Università Politecnica delle Marche, Ancona;
[2]Laboratorio di Microbiologia, Diagnostica & Ricerca San Raffaele, Milano;
[3]Laboratorio di Microbiologia, Università Vita-Salute San Raffaele, Milano, Italy

SUMMARY

Human papillomaviruses (HPVs) have been recognized as the main etiologic agent of cervical cancer and other anogenital neoplasms, and a leading cause of death from cancer worldwide. In the last twenty years, extensive research has contributed to document the molecular mechanisms of virus persistence and malignant transformation, confirming a direct role of viral proteins in these processes. A clear understanding of the molecular epidemiology of HPVs and the availability of powerful molecular diagnostic techniques have provided the background for prevention strategies of HPV-related carcinomas. Since these viruses are highly prevalent in the general population, strict screening programs are still necessary. Recently, major breakthroughs have emerged from immunological studies. Indeed, these studies have paved the way for medical treatment of HPV infections and provided the first highly effective preventive vaccines. For these principal reasons, the time has come for a great effort towards the eradication of these important human pathogens. The present review summarizes the main aspects of the virology, molecular epidemiology and molecular biology of HPV infection and highlights the recent perspectives of prevention and treatment of the HPV-related disease.

KEY WORDS: Human papillomaviruses, Cervical cancer, High-risk HPVs, Molecular mechanisms, Diagnosis, Epidemiology, Prevention, Treatment

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INTRODUCTION

The typical papillomavirus (PV)-related lesions, warts, had already been described by the ancient Greeks, but the first hints that PV infection could have a viral origin can be traced to experiments performed at the turn of the nineteenth century (Ciuffo *et al*., 1907) when it was demonstrated that warts were transmissible by filtered extracts from lesions. In 1933 Shope described the first PV as the causative agent of papillomatosis in the rabbit and the first cancer-inducing PV. Since PVs do not replicate in standard tissue culture, viral isolation was delayed until 1975, when the first human papillomavirus (HPV), designated HPV1, was molecularly cloned *ex vivo* using recombinant DNA techniques.

The whole genome's physical map was traced and the open reading frames were extensively studied (Favre *et al*., 1975). Since then, an impressive number of different PVs have been identified in humans and in other vertebrates (including reptiles and fishes), and more recently, papillomavirus type identification boomed after the introduction of polymerase chain reaction (PCR) as a recombinant DNA technique and a powerful diagnostic tool.

Corresponding author
Corresponding author:
Stefano Menzo, M.D.
Servizio di Virologia
Ospedali Riuniti di Ancona
Via Conca 71, 60020 Ancona, Italy
E-mail: menzo@univpm.it

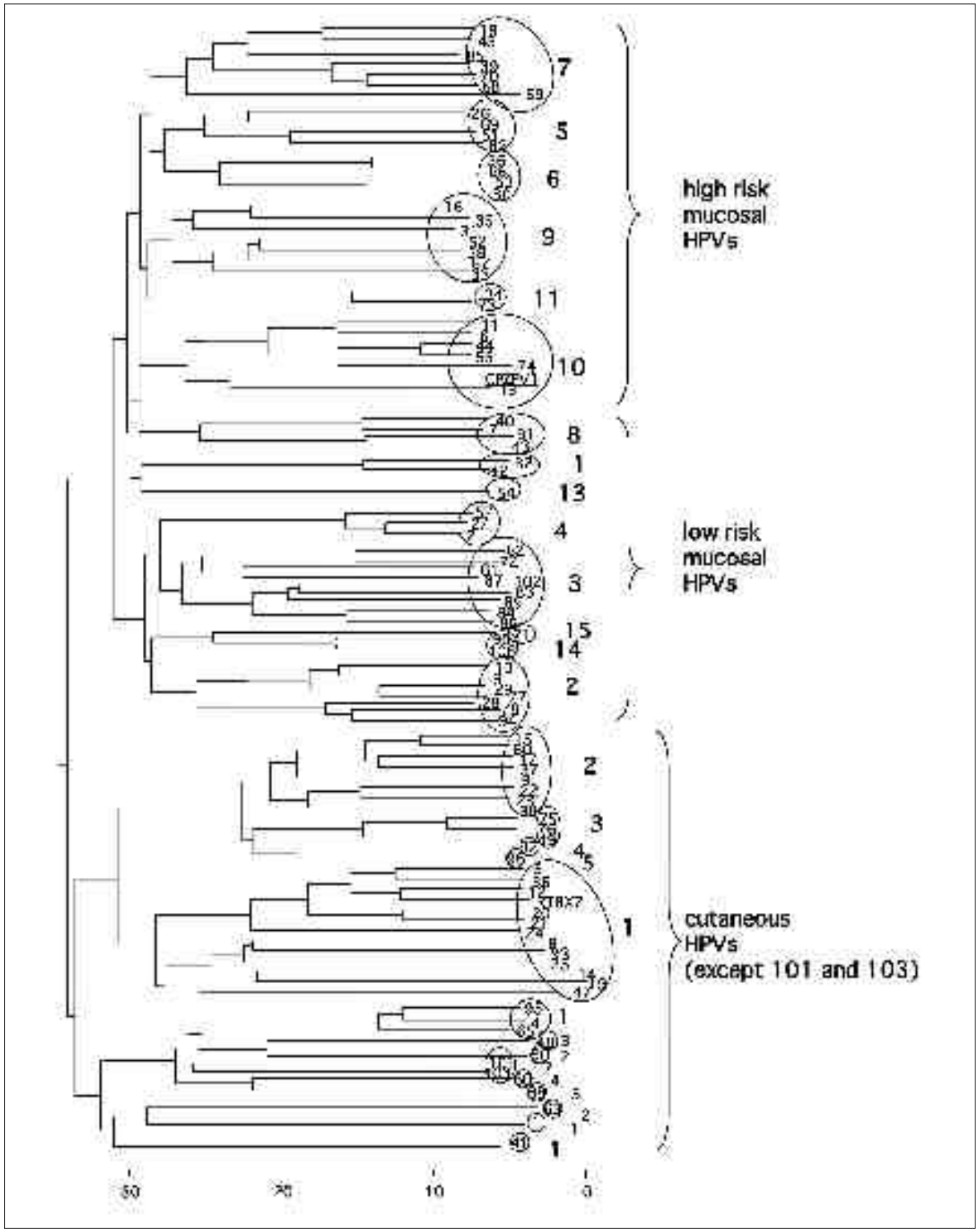

FIGURE 1 - *Schematic representation of phylogenetic relationships among HPV types. Numbers in each circle indicate HPV types included in the same species, numbers on the right of circles indicate species, greek letters at the root of clusters indicate genera.*

These viruses are presently classified as the only genus *papillomavirus* of the family *papillomaviridae*. Members of this family share an unenveloped icosahedral virion structure together with the basic genetic organization and replication strategy of the viral genome, which is a double-stranded, covalently closed circular DNA molecule of approximately 8,000 base pairs.

Most known HPVs share the same number of genes and their functions; phylogenetic studies, based on partial or whole genome sequences have identified them as a fairly homogeneously differentiated group (Figure 1). Viruses which share at least 90% homology are classified as members of the same type. Presently, there are 106 fully characterized HPV genomes (more have been isolated, but need to be fully sequenced and officially typed) and a few animal ones (mostly from primates, rodents and ungulates). Based on phylogenetic analysis, PV types can be divided in species, identified by Greek letters (de Villiers *et al.*, 2004). These species seem to include viruses sharing the same tissue tropism and oncogenic potential, as well as, albeit not rigorously, the natural animal host family. HPVs cluster in four species, genital HPVs cluster in species α, (with the exception of HPV 101 and 103; (Chen *et al.*, 2006)], cutaneous HPVs in species β, γ and μ.

HPV infection, both of the epidermis and of mucosal epithelia, seems to be extremely frequent throughout the world. Multiple epidermal infections occur in every individual and are mostly asymptomatic. They persist in limited numbers of cells throughout the body, mainly at the hair roots, rarely they persist in visible lesions such as warts. Cutaneous HPVs are loosely associated with skin cancer: it is still unclear whether they represent a co-factor (the main factor being undoubtedly UV exposure), or merely exploit the favorable proliferative environment of cancer cells, as occurs in other benign proliferative lesions such as scars or psoriasis. Mucosal infections are less common but still very frequent. Indeed longitudinal studies suggest that practically every sexually active individual (depending on the number of sexual partners) becomes infected with one or more mucosal HPV types during a lifetime, most frequently during the first years of sexual activity, when the frequency of new sexual partners is higher, at least in industrialized countries (Giuliano *et al.*, 2002, Molano *et al.*, 2002). Most of these infections are generally cleared after a few weeks or months (although the virus can probably persist in a very limited number of cells). Cross-sectional studies show that HPV DNA is present at detectable levels in the genital tract in 5 to 35% (depending on the country) of sexually active females, slightly less in males. Most of these infections do not lead to major complications. In a small proportion of normal subjects, but in the majority of the immune-suppressed individuals, HPV genital infections can persist for years in clinically relevant lesions. After the first observations that some persistent HPV infections were associated with neoplasms, a few HPV types, termed high-risk types, were identified as the etiologic cause of a variety of cancers, mainly anogenital cancers, but they may also represent a co-factor for some upper-aerodigestive tract cancers. In contrast, other low-risk types have never (or exceptionally) been associated with cancer. HPV-related genital cancers are the second most common cancers in the female population of developing countries (the first in India) and the fourth in industrialized countries (Pisani *et al.*, 2002), with a global prevalence of around 60 per 100,000 population. In the last few decades, the interest in these viruses has grown, and extensive research efforts have identified some of the molecular correlates of malignant transformation as viral and host factors and have traced the pathways for prevention. For these reasons, and given the perspectives of successful intervention, HPV infection deserves the utmost attention from the scientific community and from public health policy-makers.

MOLECULAR BIOLOGY OF THE LIFE-CYCLE OF PVS

PV show a very strict tropism for keratinocytes of multilayered squamous epithelia and transitional epithelia. Only a minority of animal PVs have been shown to infect also fibroblasts in the dermis: among them, the Bovine PV type1 (BPV1). Cell specificity does not seem to be driven by receptor usage: a possible receptor has been identified in the α6 integrin subunit (Evander *et al.*, 1997), but more recently some evidence has pointed to the role of proteoglycans, such as

heparan sulphate (Giroglou *et al.*, 2001). These receptors used by the different PVs abound in most tissues. Restriction of replication seems rather to lie at the level of transcription regulation, as a keratinocyte-specific enhancer (Cripe *et al.*, 1987, Gloss *et al.*, 1987) drives the expression of the viral genes during the stratified epithelium differentiation. The viral particle has a diameter of 52-55 nm, and is composed by two capsid proteins, L1 and L2, possibly in a 60:1 ratio (Trus *et al.*, 1997). Inside the capsid, the DNA genome is coiled around histone complexes, attached to typical Matrix Attachment Regions (MARs) in the viral DNA (Tan *et al.*, 1998). Both viral capsid proteins seem necessary for the interactions with the cell surface (Zhou *et al.*, 1993), the L2 protein being possibly involved in a post-binding interaction with a yet unknown co-receptor (Yang *et al.*, 2003). Upon contact with the cell surface, the viral particles trigger the formation of clathrin-coated pits for endocytosis (Day *et al.*, 2003), but the intracellular route to the nucleus of the viral DNA has not yet been investigated in detail.

The PV genome (in Figure 2 the HPV16 genome) contains a non-coding enhancer region, the Long Control Region (LCR), and a coding portion, organized in a region for early genes and one for late genes.

The virus usually penetrates the squamous epithelia through microscopic lesions, where it infects the basal cells. Here, basal viral early gene expression and low-copy replication of the episomal genome occur in parallel with cell replication. As soon as the infected epithelial cell enters the differentiation pathway, generally in the parabasal layer (Doorbar *et al.*, 1997, Peh *et al.*, 2002), differentiation-specific transcription factors, together with more promiscuous transcription factors such as Sp1, AP1, Yin-Yang-1, octamer binding factor-1, drive the activity of the LCR to express the early and late genes (Carson *et al.*, 2006). Different transcript species can be identified. The splicing pattern is peculiar for each PV, allowing translation from all three possible coding frames in which the genes are organized. Each of these gene products performs one or more functions in the viral life cycle. E6 and E7 proteins act on different components of the cellular cell-cycle regulation machinery (see next chapter) to induce and maintain the S-phase

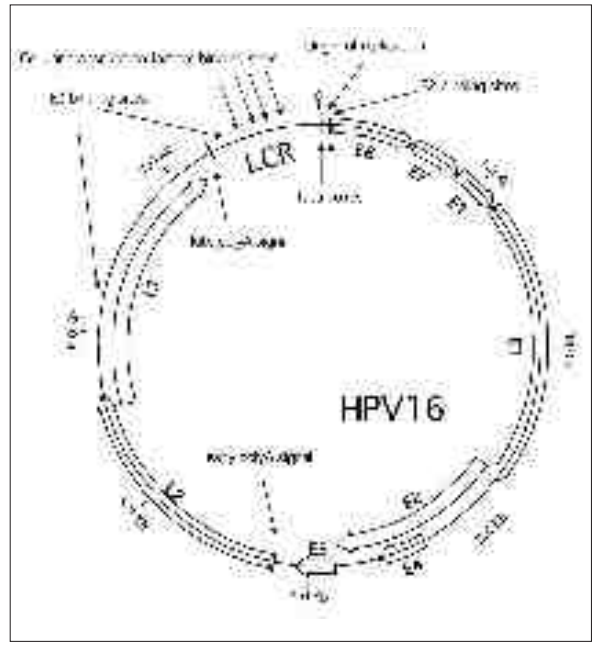

FIGURE 2 - *Graphic representation of HPV16 genome, with genes and regulatory features.*

state, necessary to boost PV genome replication and to protect the cells from senescence or apoptosis. The E1 protein is required for high-efficiency recognition and unwinding (by its helicase activity) of PV origin of replication, located within the LCR. The E2 protein is a DNA-binding factor that recognizes the sequence $ACCN_6GGT$, conserved in all PVs. It performs different functions such as tethering E1 to the viral origin of replication (Frattini *et al.*, 1994) and repressing early and/or activating late gene transcription in a dose-dependent manner (Steger *et al.*, 1997).

Its presence is required to curb E6 and E7 expression and activate transcription of late genes, however its action is also exerted by direct interaction and modulation of the activity of these proteins (Gammoh *et al.*, 2006, Grm *et al.*, 2005). In addition, it may have some effect on the expression of cellular genes (Lee *et al.*, 2002, Steger *et al.*, 2002). The E5 protein might have different functions in different PVs, but the most conserved one is to bind TAP in the Golgi apparatus and alter the cellular processing of MHC Class I and II antigens through the Golgi apparatus to reduce their concentration on the cell membrane (Ashrafi *et al.*, 2000, Zhang *et al.*, 2002). This con-

ceals, as most viruses do, the infected cells from the immune system, in view of the abundant late gene expression. In some genital HPVs the E5 gene is non-functional due to the presence of missense mutations. The E4 protein function is still unclear, it interacts with the cytokeratin network and builds aggregates in the cells (Roberts *et al*., 1993, Roberts *et al*., 1994). It works as a group of different post-translationally cleaved peptides to block the cell at the G2/M passage and seems necessary both for genome amplification and for late gene expression (Nakahara *et al*., 2002, Peh *et al*., 2002).

An E8 protein is present in some cutaneous HPVs, where it downregulates gap junction intracellular communication, but its function is currently unknown. After the early genes have conditioned the cell to host PV replication, and as this proceeds further in the differentiation process to the upper strata of the epithelium (spinosum through corneum), the accumulation of E2, E4 and episomic viral DNA, together with cellular differentiation factors, shifts the expression from early to late genes, L1and L2. Expression of E4 and E5 is maintained at a high level throughout the late viral life cycle.

A late promoter is sometimes located downstream from the LCR (e.g. at position 2443 in the BPV genome or at similar positions in other cutaneous PVs), in the early gene coding region. In most PVs, however, the promoter for late genes seems to be located in the LCR. An additional mechanism to explain the transcriptional restriction of late genes has recently been proposed (Collier *et al*., 2002).

In this model, based on the finding of transcriptional terminators at the 5' end of the L1 transcripts, there would be no need for a differentiation-specific late promoter. The terminator sequences would specifically inhibit expression of the late transcripts, without affecting early ones, until an unknown cellular factor, induced by the differentiation process, could allow read-through transcription. Differentially spliced late transcripts use a specific polyadenylation site, located in the LCR. Late transcription leads to abundant synthesis of capsid proteins, which translocate into the nucleus and colocalize in the ND10 (Day *et al*., 1998) subnuclear structures. Here the assembly of the viral particles around the viral genome occurs. Both naked and histone-complexed genomes can be incorporated, although the latter display a greater infection efficiency (Shäfer, personal communication). In the outer strata of the infected epithelia the viral particles are finally released, when the cells loose their structural integrity and are shed.

MOLECULAR MECHANISMS OF CELL TRANSFORMATION BY HPVS

It has been known for many years that the transforming properties of some PVs are associated to the presence of the E6 and the E7 proteins (Matlashewski *et al*., 1987). The first to be extensively studied, the E7 protein of HPV16, which had been identified as the HPV type most frequently associated with human cancer, shares structural homology with the E1A protein of adenovirus and the SV40 Large T antigen: proteins displaying oncogenic properties. Its molecular mechanisms of action and transforming properties have therefore been thoroughly studied (Phelps *et al*., 1988).

Current understanding of the oncogenic potential of this crucial protein indicates that its activity resides in the interaction with components of the cell-cycle system that regulates the onset of the S-phase. A key component of this system is the E2F transcription factor, which is responsible for the activation of the genes involved in the S phase. In quiescent cells (G0/G1 phases), the activity of E2F transcription factor is normally inhibited, because it is bound to the hypophosphorylated form of the retinoblastoma protein (pRB) and other similar proteins of the same family (p107 and p130). These proteins are termed "pocket proteins" after their binding pocket for the viral oncoproteins mentioned. When the S phase is due, pRB becomes phosphorylated by cyclin kinases 4 and 6 and releases E2F, which is thus free to exert its function. E7 binds to the pocket proteins (functional sequestration) (Dyson *et al*., 1989) and leads them to degradation through the ubiquitin-proteasome pathway (Boyer *et al*., 1996), thus freeing E2F function independently of cell cycle regulation. Similarly, E7 can bind to and abrogate the function of p27kip1 (Zerfass-Thome *et al*., 1996) and, maybe more importantly, of p21cip (Funk *et al*., 1998,

Helt *et al.*, 2002), which in keratinocytes are both inhibitors of cyclin dependent kinases required for cycling. HPV6 E7 protein binds to pRB with lower affinity (Gage *et al.*, 1990) and this might partially explain its lower transforming activity than that of HPV16/18 (Barbosa *et al.*, 1990). In some experiments, the transforming potential of HPV E7 correlated directly with its ability to bind pRB (Heck *et al.*, 1992). Similarly, such a difference was observed between HPV38 E7, an oncogenic cutaneous PV, and that of HPV10 and 20, non-oncogenic viruses (Caldeira *et al.*, 2003). In the case of HPV1, E7 is unable to lead pRB to degradation (Giarre *et al.*, 2001, Gonzalez *et al.*, 2001).

The transforming role of HPV16 E6 protein first became apparent when it was shown to cooperate with E7 for transforming human primary cell lines (Hawley-Nelson *et al.*, 1989, Watanabe, 1989). Like the E7 protein, it contains two double cysXXcys motifs, which constitute zinc-finger domains. High-risk HPV E6 shares a fundamental function with proteins of other DNA tumor viruses (adenovirus E1B and SV40 large T antigen): it abrogates the activity of the tumor suppressor protein p53, a protein acting as a trascriptional activator for a number of growth-suppressing proteins, the most important of which is believed to be the p21cip1 formerly mentioned as a target of E7. Other targets of p53 are the genes involved in growth arrest and apoptosis, (see, for review, Vogelstein *et al.*, 2000, Giono *et al.*, 2006). Unlike adenovirus E1B or SV40 large T antigen, E6 achieves p53 inhibition by redirecting specifically to it the activity of the E6AP or other ubiquitin ligases (Scheffner *et al.*, 1993).

The interaction between E6 and E6AP is mediated by an amino terminal binding domain (Huibregtse *et al.*, 1993), capable of binding several other α helix partners (Nguyen *et al.*, 2002). This interaction forms a ternary complex and ends with the specific ubiquitination of p53 and its subsequent degradation through the proteasome pathway (Varshavsky *et al.*, 2000). In addition, E6 is capable of inhibiting *de novo* p53 dependent expression by binding to the CBP/p300 transcriptinal coactivator (Zimmermann *et al.*, 1999). A direct consequence of p53 activity abrogation in HPV-infected cells would be the overriding of the growth control mechanisms that keep in check the normal growth processes in the cells and drive cells with metabolic or genotoxic damage to apoptosis. A few studies have focussed on the role of a p53 polymorphism at codon 72 in E6 binding efficiency. Most human populations bear either an arginine (R) or a proline (P) residue in that position, the allele distribution being slightly variable in different human populations.

After the first report that the homozygous R at position 72 was associated with increased p53 degradation and cancer risk (Storey *et al.*, 1998), other groups have studied the role of this allele, but most of them have failed to confirm the finding**.** In addition to p53, E6 has been demonstrated to bind other proteins and to lead them to degradation (see, for review, Munger *et al.*, 2002). Among these are the MCM7 subunit of replication licensing factor (Kuhne *et al.*, 1998), B1k (Oda *et al.*, 1999), ADA3 (Zeng *et al.*, 2002), MAGI1 (Thomas *et al.*, 2002), E6TP1 (Gao *et al.*, 2002) and paxillin (Tong *et al.*, 1997). E6 of high risk HPVs (but not of low-risk ones) contains a binding motif for the PDZ domain, present in a group of different proteins, such the human homologue of the *Drosophila melanogaster* Disks Large (hDlg) and Scribble (hScrib) tumor suppression proteins, or MAGI family members.

Most of these proteins perform their function in cell cycle regulation, and E6 probably evolved in the simplest way, by exploiting these common motifs, to act on different targets for more efficient cell-cycle deregulation. In addition, among the recently described targets of E6-E6AP interaction, two proteins required for DNA repair processes have been reported: XRCC1 (Iftner *et al.*, 2002) and MGMT (Srivenugopal *et al.*, 2002). These interactions might explain the genomic instability (see, for review, Duensing *et al.*, 2002) and high mutation rates described in cells expressing the E6 proteins, two factors that might contribute to malignant cell transformation. The effects mentioned above seem to be peculiar to high-risk HPV E6 proteins. However all PV E6 are capable of performing different functions, including transcriptional activation or repression of a number of genes (Sedman *et al.*, 1991, Brehm *et al.*, 1999), by modulating the activity of transcription factors. Among these, some are responsible for enhanced cell growth (Malanchi *et al.*, 2002); in one case E6, together with E7, seems

to be implicated in the inhibition of transcription of Il-8, a key factor for the initiation of the local immune response (Huang *et al.*, 2002). Recently two novel HPV types have been identified, HPV101 and HPV103, which apparently lack the E6 gene (Chen *et al.*, 2006).

The combined action of E6 and E7 of high-risk HPVs readily achieves transformation of rodent cell lines *in vitro,* but it is not capable *per se* of inducing neoplasia *in vivo*. Additional factors seem to be necessary for the onset of truly malignant cell growth in humans. Longitudinal studies have shown that the time of persistence is a key factor for the onset of cancer. In the case of HPV16, infections progress to high-grade dysplasia after a median of five years of persistence. This time lag seems to be necessary for a number of molecular events to occur in the infected cells. The most important is certainly viral genome integration in the cellular chromosomes. First observed in cell lines such as HeLa (HPV18) and CaSki (HPV16), derived from invasive cancers, it is now apparent that the event occurs in the majority of truly neoplastic genital HPV-related lesions. Integration occurs by chance and in random chromosomal locations and is not necessary in the viral life cycle, in fact it might ultimately lead to the shut-off of viral replication.

Most integrational events that come to our attention (those that become fixed in neoplastic clones), interrupt the E2 open reading frame, suggesting that abrogation of E2 expression is necessary to confer growth advantage on the cell. Indeed, expression of E2 protein in cell lines derived from cervical cancer is capable of partially reversing the malignant phenotype (Goodwin *et al.*, 1998) or of leading cells to apoptosis (Demeret *et al.*, 1997). Furthermore the acquisition of the transformed phenotype in primary cultures from genital lesions has been demonstrated to coincide with viral integration (Yokoyama *et al.*, 1995, Johansson, personal communication). The transformed phenotype depends on overexpression of E6 and E7, due to the absence of E2.

In addition to viral recombination, cellular factors are important for the development of HPV-related tumors. Somatic mutations are found to activate oncogenes such as those in the *ras* and *myc* families. In any case hypermutation in the infected cells might be favored by HPV infection, and could be either the consequence of faster cycling, or, more likely, the effect of the viral oncogenes on centromeric stability and on the DNA repair processes. P53-inactivating mutations, a common finding in many tumors, seem to be under-represented in HPV-related neoplasms, possibly because they are irrelevant in the context of E6 inactivation of p53.

The third viral protein that is believed to contribute to malignant transformation is E5. It interacts with the cellular protein 16k ductin/subunit C (Goldstein *et al.*, 1991), which is a component of the gap junction structures (ductin) and with the V0 sector of the vacuolar proton pump (subunit c).

This interaction seems responsible for the down-regulation of gap junction intercellular communication and consequent disruption of cell homeostasis, and for the inhibition of acidification of endosomes and Golgi apparatus (Straight *et al.*, 1995), with consequent sustained activation of growth factor receptors including the epidermal growth factor receptor.

Finally, in addition to viral and host factors, a few external factors have been suspected as enhancing the risk of developing genital cancer. Among the most suspected (parity, use of oral contraceptives, HSV2 infection, cigarette smoking), only smoking has survived scrutiny and is considered a true independent risk factor (Castle *et al.*, 2002). Parity and extended use of oral contraceptives are associated with enhanced cancer risk only as a consequence of enhanced HPV DNA persistence, and their effect is probably mediated by the action of progesterone, a hormone that was shown to stimulate the viral enhancer and therefore may favor viral persistence.

NATURAL HISTORY OF HPV INFECTIONS AND RELATED DISEASES

In HPV-infected cells, the E6 and E7 proteins interfere with the cell-cycle regulation machinery, driving the infected cells towards pathologic growth. Such molecular interactions, which have been extensively demonstrated *in vitro* for many HPVs, lead *in vivo* to the generation of hyper-dysplastic lesions: warts in the skin and papillomas in the upper respiratory tract or eye

conjunctiva. In the genital mucosa, they appear as condylomas (flat or exophytic) or other dysplastic lesions, histologically grouped as Cervical Intraepithelial Neoplasia, grade I to II (CIN, in other districts Anal, Vaginal, Vulvar or Penile, AIN, VAIN, VIN or PIN). These lesions are reversible as the infection is cleared. High-grade dysplasias (grade III) or frankly malignant lesions arise only when overt infection with specific HPV types, persistent for many years, has lead to viral genome integration into the cell chromosomes and/or to the accumulation of somatic mutations in the cellular genes and are therefore irreversible. Microscopically, the infected cells acquire the typical koilocytotic aspect, with high cytoplasm to nucleus ratio and vacuolated appearance. The different pathologies related to HPV infections and the type of viruses that have been associated with each are summarized in Table 1.

In the genital tract, the great majority of benign lesions, which appear between 2 weeks to 8 months after infection, are usually short-lived, and spontaneous regression usually occurs in immunocompetent individuals, although the time needed for the lesions to regress varies in different individuals and for different viruses. In general, high-risk mucosal HPVs tend to persist longer than low-risk ones. In particular, oncogenic types average a longer median time of persistence (9.8 vs 4.3 months compared to low-risk ones) (Giuliano *et al.*, 2002).

Important factors for persistence reside in the peculiar strategies evolved by PVs to evade the immune response. The most important is their ability to infect cells that never cross the basal membrane and that start producing viral antigens where immune system cells are quite scarce, namely in the outer layers of the stratified squamous epithelia. Viral particles themselves are not released until the infected cells are shed in the environment. There is reduced opportunity for extensive antigen uptake by the Langerhans cells (LC), the main antigen presenting cells in the squamous epithelia. Even when a response has

TABLE 1 - *HPV-related diseases and types implicated. HPV types are listed progressively according to type number, not frequency of incidence.*

Cutaneous Warts	1, 2, 3, 4, 7, 10, 26, 27, 28, 29, 38, 41, 49, 57, 63, 65, 75, 76, 77, 80, 92, 95, 96	Many EV-related viruses have also been found in warts in immunocompromised subjects, although with low frequency
Epidermodysplasia verruciformis (EV)	2, 3, 5, 8, 9, 10, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 38, 46, 47, 50	
Recurrent respiratory papillomatosis	6, 11	
Focal epithelial hyperplasia (Heck's disease)	13, 32	
Conjunctival papillomas	6, 11, 16	
Condylomata acuminata	6, 11, 30, 42, 43, 45, 51, 53, 54, 55, 56, 62, 70, 84, 87, 90, 91	
Cervical (vaginal, vulvar, anal or penile) intraepithelial neoplasia	2, 6, 7, 11, 16, 18, 26, 27, 30, 31, 33, 34, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 57, 58, 59, 60, 61, 62, 64, 66, 67, 68, 69, 70, 71, 73, 74, 82, 83, 84, 85, 86, 87, 89, 91, 94, 97, 101, 102, 103, 106	Many genital PVs have been found associated to benign and malignant lesions of the aerodigestive mucosa. HPV2, 7 27 and 57 are also capable of infecting the epidermis outside the genital area.
Cervical carcinoma (high risk types, data from (Munoz, N. *et al.*, 2003))	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, 82	The listed types accounted for 93.5% of cancers. A few additional known HPVs, phylogenetically related to these high risk types, were not looked for in the study: 30, 53, 67 and 69.

been primed, infected cells could be difficult for the immune system to detect, given the down-regulation of MHC antigens on the cell surface by the E5 protein. Other mechanisms interfere with MHC antigen expression in the PV infected cells. The E7 protein of oncogenic HPVs represses the promoters of MHC class I heavy chain (Georgopoulos *et al.*, 2000) and of TAP-1 (the protein responsible for transporting antigenic peptides in the Endoplasmic Reticulum (ER) for assembly with MHC class 1 proteins), while HPV6/11E7 seems to bind and interfere directly with the activity of TAP-1 (Vambutas *et al.*, 2001). Molecular mechanisms inside the infected cell, induced by the activity of the E6 and E7 proteins, might make the cell resistant to the action of interferons (IFNs). HPV16 E7 inhibits the induction of IFNα-inducible genes inactivating the ISGF-3 complex (Barnard *et al.*, 1999), in a similar way to that of adenovirus E1A protein. E7 has also been shown to have a physical interaction with IFN regulatory factor (IRF)-1 which inhibits activation of the IFN-β (Park *et al.*, 2000). Both of these functions of E7 are mediated by the portion of E7 that binds the cellular protein Rb, by which E7 exerts its oncogenic effect, demonstrating the multi-functionality of this region of the protein. Multiple IFN-responsive genes, including IFN-α, IFN-β and STAT-1 are downregulated by HPV-16 E6 (Nees *et al.*, 2001). In addition, E6 can bind a second IFN response protein, IRF-3, which delays the expression of IFN-β (Ronco *et al.*, 1998), and can physically associate with Tyk2 and impair Jak-STAT activation of the IFN pathway by IFN-α (Li *et al.*, 1999).

In general, HPV infection seems to preferentially elicit a Th2-type response: patients with recurrent respiratory papillomatosis express Th2 cytokine mRNAs in response to autologous papilloma tissue (Bonagura *et al.*, 1999), and a Th2 profile is associated with progression to cervical cancer (Clerici *et al.*, 1997). It has been observed that expression of E6 results in down-regulation of IL-18 expression in HaCat cells (transformed keratinocytes) and in several cervical tumour lines. This effect is independent of p53 and is not caused by the direct binding of E6 to IL-18 (Cho *et al.*, 2001). Since IL-18 is essential for building a Th1 response and for NK activation, down-regulation of IL-18 might interfere with both an effective CTL response and the NK response, which could otherwise target the low MHC-expressing infected cells.

Despite all these means to evade the immune response, cell-mediated immunity is readily detected in HPV-infected individuals (de Gruijl *et al.*, 1996) and is certainly the factor determining lesion regression (Coleman *et al.*, 1994). However, in some cases, especially in immune suppressed subjects, this response is insufficient to clear the infection promptly. This immunity can be easily and effectively enhanced: a commonly referred phenomenon is self-vaccination, which occurs when cutaneous warts are subjected to physical treatment (e.g. electrocoagulation diathermy or laser), and spontaneous regression of other lesions, that may be present in different locations, is subsequently observed. Physical treatment destroys tissue integrity, releases antigens and stimulates the convergence on the infected cells of large quantities of different inflammatory cells, with massive release of soluble immune mediators, sufficient to amplify the previously weak response. Indeed, a wealth of data from the use of immune modulators and therapeutic vaccinations in man and in animal models is confirming that the weak natural response to HPV can be successfully boosted by therapeutic interventions.

The humoral response to genital HPV infections has been extensively studied (Mann *et al.*, 1990, Strickler *et al.*, 1994, Van Doornum *et al.*, 1994, Wikstrom *et al.*, 1995, de Gruijl *et al.*, 1996), and current knowledge shows that antibodies are generally produced against both early and late viral antigens, and correlate with the presence of HPV DNA in population based and longitudinal studies. Both serum IgG and secreted IgA (Dillner *et al.*, 1989) have been documented. However, compared with other viral diseases, antibody titers are somehow unpredictable in the single individual, sometimes they rise slowly and persist from former cleared infections; cross-reactivity among different HPV types is normally observed. These aspects make serologic assays unreliable diagnostic markers. Furthermore, anti-HPV antibodies, while effective in blocking new infections, as is becoming apparent in vaccination studies, are ineffective in clearing ongoing ones, especially those of high-risk HPVs. Strong antibody responses to E6 and E7 are consistently

present in the case of invasive lesions which have extended past the basal membrane of the epithelium (Lehtinen *et al.*, 1992). Again this kind of response seems helpless against tissue invasion, but it could be exploited for diagnostic purposes as a marker of invasion. Cross-sectional studies show that seroprevalence, measured as reactivity to *in vitro*-generated virus-like particles tends to rise with age in the general population, with a peak at 50 years, reflecting cumulative risk and multiple exposures during the sexually active lifetime, and is more than double in women, suggesting that in men the infections are generally superficial and more rapidly cleared (Stone *et al.*, 2002).

Genetic host factors are also important in determining the efficacy of the immune response to HPVs: among HLA class II alleles three sets of alleles/haplotypes have been most extensively studied (for a comprehensive review, see Hildesheim *et al.*, 2002): (I) DQB1*03 alleles (DQB1*0301, *0302, and *0303); (II) DRB1*1501 and DQB1*0602 alleles (in linkage disequilibrium: they occur in the same haplotype); and (III) DRB1*13 (consisting of DRB1*1301-5 alleles) and DQB1*0603 (in linkage disequilibrium with DRB1*1301). While in some of the studies the first two sets of alleles, particularly the first one, appeared to be associated (although inconsistently) with increased disease risk, in virtually all studies the last set was associated with decreased risk of infection or developement of cancer. In addition, a more recent study associated the DRB1*0301 allele with recurrent respiratory papillomatosis, a disease caused by HPV6/11 (Gelder *et al.*, 2003). Other studies have proposed a relationship between HPV infection and MHC class I C*0202 (negative association) (Wang *et al.*, 2002) or A2 and B7 (positive association) (Davidson *et al.*, 2003), but the evidence is still weak and needs confirmation. Although yet to be demonstrated, these associations should be related to the ability of the immune response to control persistent infection.

A specific, though unknown mechanism leads to HPV persistence in the skin, followed by malignant transformation, in a rare genetic disease named Epidermodysplasia Verruciformis (EV, see Table 1 for EV-related viruses). Patients with this rare autosomal recessive disease are affected during their lifetime by large numbers of cutaneous wart-like lesions of viral origin. Occasionally some of these warts degenerate to true neoplastic lesions, posing a threat to the lives of the patients. In the normal population and in immunosuppressed subjects, their DNA is usually found at the root of plucked eyebrows (Boxman *et al.*, 1997), and, rarely, in warts, where other types may also be found. Sometimes they are associated with other hyperplastic disease of the skin such as psoriasis or actinic keratosis or with physiological skin regeneration in the healing process of burns (Bouwes Bavinck *et al.*, 2001), probably as opportunistic infections exploiting the favourable environmental conditions. Association of these viruses with cancer, in non-EV patients, is still controversial. The genes responsible for EV have been identified and denominated EVER1 and EVER2 (Ramoz *et al.*, 2002), members of a novel Transmembrane Channel-like (TMC) family of genes. Their products are localized in the ER, where they might function as ion-channel transporters; their impact on HPV replication is currently unknown.

MOLECULAR EPIDEMIOLOGY AND RISK OF MALIGNANT TRANSFORMATION

The risk of progression to cancer, the main clinical issue associated with HPV infection, is not equal for infections with different HPV types. The distinction between high- and low-risk types is not clear-cut, because if low-risk types are virtually never associated with the development of cancer, high-risk ones are quite different in their oncogenic potential. For genital tract HPVs, a recent international study conducted on populations in different parts of the world, including Africa, Asia, America and Europe (Munoz *et al.*, 2003), has shed some light on this particular issue. In this study, 1,739 HPV-positive cervical cancer patients and 259 matched cancer-free subjects were evaluated to calculate the relative risk of cancer development specific to each HPV type (33 types were evaluated; unfortunately, for the other 26 known genital HPVs, not included in the study, only anecdotal data are available). This study drew up a reliable classification of high- and low-risk types, together with the relative risk (Odds Ratio, absence of HPV infection =1) of progression to cancer (Figure 3, panel A).

Epidemiological classification usually parallels phylogenetic classification (with 2 exceptions: HPV73, a phylogenetically low-risk and epidemiologically high-risk type, and HPV70, the opposite) and identified 4 definite high-risk HPV species: A9, A7, A5 and A6. The putative risk for the HPV types not included in that study can thus reasonably be inferred from their phylogenetic clustering. HPV16, 59 and 33 are the highest-risk types, while types 35, 56, 51, and 68 are those at lowest risk among the high-risk ones. As HPV26, 39, 53 and 66 were detected only in cancers (albeit in a few cases), their relative risk could not be assessed. In particular, HPV39 was detected in 9 cancer cases and in none of the 259 controls. In our laboratory, HPV39 was detected in 3 out of 1,256 consecutive infections (unpublished data): altogether, these data suggest that HPV39, though rare, could be associated with a quite high risk of malignant transformation. On the other hand HPV66 is one of the most prevalent in our routine samples, suggesting low oncogenic potential. The global prevalence of HPV types in cervical SCCs is listed in Figure 3, panel B.

Additional information on risk comes from the study of viral variants. HPVs can be divided into subtypes (within types, when L1 divergence is between 2% and 10%) and variants (when L1 divergence is lower than 2%). Variants are phylogenetically distinct throughout the viral genome, suggesting independent evolution in different human populations in prehistoric times (Ong *et al.*, 1993, Ho *et al.*, 1993). In the case of HPV16, the different variants found worldwide have been assigned to five lineages according to geographical distribution: European (2 variants with a T or a G at nucleotide position 350 in the

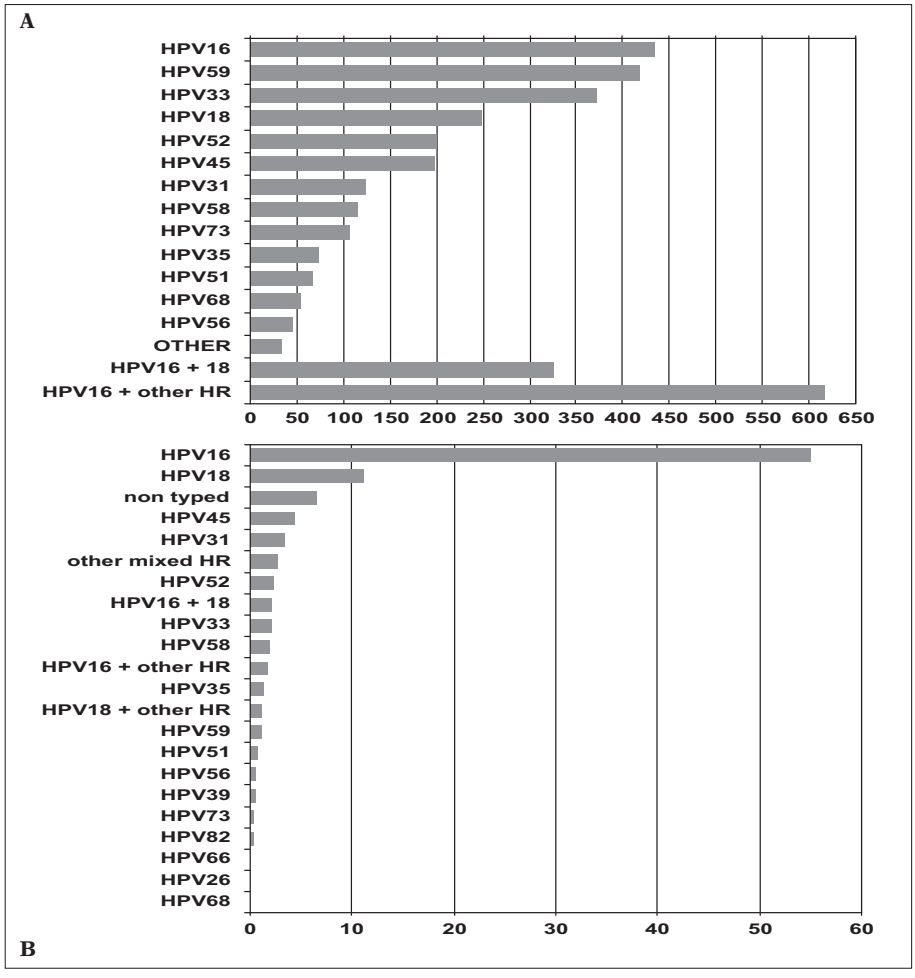

FIGURE 3 - *A) Relative risk of cancer development associated with HR HPV genital infections, compared to being HPN DNA negative (=1).*
B) Prevalence of HPV types in 1739 HPV-related cervical carcinomas: percentage of total cases (modified from Munoz N. et al., 2003).

E6 gene), Asian, Asian-American, African-1 and African-2 (Yamada *et al*., 1997). Recent studies have shown that oncogenic potential may differ among variants of the same HPV type, and that this might depend on the few polymorphic residues in the E6 open reading frames. Some evidence indicates that non-European variants may correlate with longer persistence and greater risk of progression to cancer (Villa *et al*., 2000). A study conducted on Indian patients found that non-European 350G E6 variants bearing a T at position 145 correlated with more aggressive tumors, while those with a G at position 419 were found only in cancer-free subjects (Radhakrishna Pillai *et al*., 2002).

The development of malignant genital tract lesions is much less common in males than in females and also shows different characteristics. Apart from condylomas, which are mostly caused by low-risk HPVs, are generally short-lived and leave no sequelae, Penile Intraepithelial Neoplasia (PIN) is the histopathologic feature associated with persistence of high-risk HPV infection. In a minority of cases, and more often in immunosuppressed subjects, infections persist but are visible only by peniscopy and acetic acid staining (Franceschi *et al*., 2002). They can evolve as three distinct paraneoplastic lesions: Bowenoid Papulosis, Bowen Disease and Erythroplasia of Queirat. While the first two are the true equivalents of the more distal feminine genital lesions (Vaginal or Vulvar Intaepithelial Neoplasia, VAIN or VIN), which are entirely due to mucosal HPVs (Dianzani *et al.*, 2004), the latter is caused by a mixed HPV infection: HPV8 plus a high-risk genital HPV (Wieland *et al*., 2000). PINs, which are virtually always associated with HPV DNA, mostly type 16 (Cupp *et al*., 1995), represent precursor lesions for the development of invasive carcinoma, although a substantial proportion of invasive cancers of the penis test negative for HPV DNA, similarly to vulvar carcinomas. Studies analyzing formalin-fixed, paraffin-embedded material found low percentages of HPV positivity (20-40%), while in those analyzing fresh samples HPV DNA prevalence ranged from 50 to 80%. This suggests that, in contrast to cervical cancer, HPV DNA load is quite scant: the role of HPV in these neoplasms might be quite different from that in cervical cancer.

Mucosal HPVs have also been associated with aerodigestive tract infections and breast tissue infections. Apart from a few typical infections such as Recurrent Respiratory Papillomatosis (RRP) caused by HPV6 or 11 or Focal Epithelial Hyperplasia (FEH) caused by HPV13 or 32, the DNA of the most common genital HPVs has been found in the aerodigestive tract, seldom associated with visible lesions.

High-risk types seem to be the most prevalent, and they are suspected of acting as co-factors for the development of Head and Neck Squamous Cell Carcinomas (HNSCC), especially tonsillar carcinomas (Snijders *et al*., 1992, De Petrini *et al*., 2006). HPV DNA is readily detected in a substantial proportion of these carcinomas, but it can also be found, with comparable prevalence, in swabs from normal subjects (Nagpal *et al*., 2002). More specific evidence is E6 mRNA expression in a subset of carcinomas (van Houten *et al*., 2001), or the higher prevalence of HNSCC in anti-HPV16 positive subjects (Mork *et al*., 2001). Epidemiological evidence includes the higher frequency of HNSCC in subjects with a history of anogenital cancer (Gillison *et al*., 2001). HPV16 accounts for nearly 90% of the infections (Klussmann *et al*., 2001). Despite this evidence, the role of HPV in HNSCC is not as clear as in cancers of the genital tract, and it might concern only a subset of tumors (possibly around 10-20%), in the context of other strong and consistent risk factors such as tobacco smoking or chewing (betel quid chewing in India and other Asian countries), and consumption of alcohol or hot tea.

Even greater uncertainty regards SCC of the skin (in non EV patients). Cutaneous HPV DNA is detected in a very high percentage of these lesions, often as multiple infections (Euvrard *et al*., 1993, Shamanin *et al*., 1996).

However, a high prevalence of HPV DNA is also found in other pathologic or physiologic hyperplastic lesions of the skin, in both immunosuppressed and immunocompetent individuals (Meyer *et al*., 2003). Malignant lesions sometimes seem to arise on preceding warts in immunosuppressed subjects (Noel *et al*., 1994), but exposure to UV radiation is a more consistent factor. The HPV types involved in SCCs are often the EV-related ones, but benign types such as HPV1 or 2 have also frequently been found.

Current knowledge suggests that HPV proliferation in benign lesions may enhance the oncogenic potential of the most important risk factor for the development of SCC, UV exposure, and concur to select for malignant cell clones. The higher load of proliferative HPV infections in immunosuppressed individuals makes these subjects also at risk for the development of SCC of the skin.

LABORATORY DIAGNOSIS AND SCREENING

Early diagnosis of papillomavirus infection is a key issue for the prevention of HPV-related cancers. The incidence of these neoplasms in some developing countries is approximately tenfold that in industrialized Countries (Parkin *et al.*, 2001). In India and other South-Eastern Asian Countries, in most of sub-Saharan Africa, and in Central and South America, the incidence of cervical cancer reaches 93 in 100,000 per year and represents the most prevalent neoplastic pathology. In addition, the mortality rate is also high in these countries because, in the absence of controls, the disease becomes apparent at a very late stage. Of course, different factors influencing sexual habits, such as religion or socioeconomic status, may play a role in generating this huge difference, but most of it is explained by the success of preventive screening programs implemented for many years in industrialized countries.

Screening programs have been, and are, mainly based on the examination of cytologic smears from the cervical canal stained by Papanicolau staining technique (Pap-test). The currently most used reporting method for the results of Pap smears has recently been updated within the Bethesda system (Broder 1992; Solomon *et al.*, 2002). Basically three levels are recognized for squamous cell abnormalities: Atypical Squamous Cells of Undetermined Significance (ASC-US), Low-grade Squamous epithelial Lesion (LSIL) and High-grade Squamous epithelial Lesion (HSIL). Each abnormality should suggest a different diagnostic triage.

Since antibody response is unreliably detected and does not necessarily correlate with current viral presence, the only diagnostic virologic assays for HPV infection are based on HPV DNA detection and typing. Direct hybridization assays are still widespread (Hybrid Capture II), but the cost, the limited sensitivity and the vast number of HPV types not detectable by these methods are gradually leading to the predominance of Polymerase Chain Reaction (PCR) based tests.

Different PCR assays, mostly using general primers for the conserved L1 or E1 regions, have been developed: first generation L1 PCR (Bosch *et al.*, 1995, Snijders *et al.*, 1990) and subsequent methods which optimize primer use to obtain greater sensitivity and a broader range of detectable HPV types (Kleter *et al.*, 1998, Menzo *et al.*, 2001). In some cases, nested protocols using combinations of the mentioned set of primers can be used. Typing is achieved by sequencing, restriction endonuclease digestion or probe hybridization, all performed on the amplified product.

None of these methods can be considered a golden standard. Sequencing cannot resolve multiple infections, restriction endonuclease analysis fails to resolve a few types without further digestions or sequencing, and current commercial linear array hybridization assays presently lack the number of probes for adequate typing (Crapolicchio *et al.*, 2006) and/or suffer from low specificity, thus overestimating multiple infections. Microarray based assays are currently being developed to improve specificity and type range. The World Health Organization has set up an international collaborative group for quality control and standardization of HPV DNA assays (Quint *et al.*, 2006). In the last few years HPV DNA (Schlecht *et al.*, 2003, Flores *et al.*, 2006) or RNA (Andersson *et al.*, 2006, de Boer *et al.*, 2007) quantitation, as well as integration status of HPV genomes in the cellular DNA (Arias-Pulido *et al.*, 2006, De Marco *et al.*, 2007) have been shown to correlate with histologic markers of progression, but the clinical utility of these assays as prognostic markers is still unclear.

No official consensus has been reached on this issue, but a reasonable screening/diagnostic pathway could be a Pap smear every two/three years for all women from age 25 to 75 followed by HPV testing and typing in cases of ASC-US and LSIL. Colposcopy and further analysis could

be limited to infections with high-risk HPV types or whenever HSIL is found. In this context, HPV testing could be extremely useful to detect high-risk type infections at their very early stages (ASC-US), allowing colposcopy to be restricted to high-risk type infections or in case of LSIL or HSIL. The presence of a new histologic marker, p16INK4a (a cyclin dependent kinase inhibitor over-expressed in cells infected with HR HPVs) can be sought in cytologic or bioptic specimens. This could allow detection of HR infections without DNA-based assays (Sano *et al*., 2002). Alternatively, total substitution of cytologic screening with HPV DNA testing would be cost-effective only for tests costing less than 5 US $ (Mandelblatt *et al*., 2002) in industrialized countries. This threshold has already been reached in some settings, and it could be helpful to start planning long-term follow-up studies with this strategy.

Cytologic screening is quite expensive for the health service, and applying it to reduce the incidence of cervical cancer in the countries where it is more prevalent is an appalling challenge. On the other hand, the morbidity of HPV-induced cancer is also costly, and the rise in prevention costs could be offset by a decrease in invasive cancer treatment costs. Some studies performed in South Africa have shown that even sporadic cytologic testing can achieve a 30% reduction in lifetime risk of cancer (Goldie *et al*., 2001), with very limited costs. However the cytologic screening in the developing countries has some important drawbacks that limit its implementation. HPV DNA testing, performed using molecular assays, might be a real alternative strategy.

Unlike cytologic screening, a swab drawn from anywhere in the vagina or from the vaginal fornices, is adequate for obtaining a reliable result, since shed infected cells and virions, with their high load of viral DNA, spread throughout the genital tract (similarly, in non-circumcised males, in the absence of visible lesions, the balano-preputial groove is the best place to obtain a reliable swab, because most shed debris, including that from the urethra, accumulates there). HPV DNA testing performed on self-collected swabs would sensibly reduce sampling costs and make the screening more acceptable to the population.

MEDICAL TREATMENT

Physical and surgical treatments of malignant and benign HPV-related lesions are still the most successful treatments, but a new role is emerging for some recent pharmacological treatment options. In the past, IFN-α and -β, alone or associated with antiblastic agents, have been the most widely used medical treatments, but because of the high costs and the inconsistent success (except possibly as adjuvant in surgical interventions for RRP), they are unsuitable as treatment alternatives, and other compounds have been tested.

Podophyllin, a cytotoxic agent that arrests mytosis at the metaphase, has been successfully used in combination with vidarabine, a DNA polymerase inhibitor, to treat CIN in 28 patients, achieving a regression rate of 81% (Okamoto *et al*., 1999). Although podophyllin is currently used for the treatment of skin warts, its use for genital tract dysplasias is limited.

Cidofovir is an acyclic nucleoside phosphonate with broad-spectrum activity against different DNA viruses. Its action in HPV infected cells seems to trigger apoptosis and to lead cells to death (Andrei *et al*., 2000). Its clinical use as a topical cream or an injected solution, intralesionally or systemically, has been tested in different HPV-related pathologies, including RRP, anal condylomas, skin warts and CIN with success (Snoeck *et al*., 1998, Martinelli *et al*., 2001, Snoeck *et al*., 2000). In particular, it seems to act conveniently as a coadjuvant to physical or surgical treatment (Orlando *et al*., 2002).

Other potentially useful molecules for the medical treatment of HPV infections are the synthetic immunomodulating agents such as imiquimod or inosiplex. Imiquimod's action is exerted by inducing the release of proinflammatory Th-1 class cytokines, principally INF-α, tumour necrosis factor-α and IL -12 by macrophages and lymphocytes (Stanley 2002), thus boosting cell-mediated local immunity. It has been administered to treat other viral infections such as herpes simplex and molluscum contagiosum (poxvirus infection) and non-melanoma skin cancer. In cases of HPV infection, it has been used with some success for HPV-related pathologies such genital lesions (Edwards *et al*., 1998; Tyring, S.K. *et al*., 1998; Kaspari *et al*., 2002;

Sauder *et al.*, 2003), cutaneous warts (Grussendorf-Conen *et al.*, 2002) and in a case of carcinoma *in situ* (Orengo *et al.*, 2002). Inosiplex (inosine pranobex, methisoprinol), is a compound formed from the p-acetamido benzoate salt of N,N-dimethylamino-2-propanol and inosine in a 3:1 molar ratio. Its action is still unclear but it may function by interacting with the intracellular interferon pathways. It can be administered either locally or orally, and some success has been reported after its use (Georgala *et al.*, 2006).

PREVENTION

Although the primary prevention of HPV infection by behavioral change is theoretically feasible, the extent to which possible interventions on behavioral risk factors could have some success in practice is negligible. Of course some (limited) reduction of the risk of cervical cancer could be achieved by reducing the number of sexual partners, the number of pregnancies, the use of oral contraceptives or by eliminating cigarette smoking, but this remains speculation. Physical or chemical contraceptives such as condom, diaphragm or spermicide, have shown limited efficacy (Celentano *et al.*, 1987, Parazzini *et al.*, 1989, Kjaer *et al.*, 1997, Franceschi *et al.*, 2002); besides, increased use of such means in developing countries, where they would be most needed, is unlikely.

The true breakthrough for the prevention of HPV infection has been the recent approval and marketing of two prophylactic vaccines for HPV16 and -18 (Cervarix by Glaxo Smith Klein and Gardasil by Merck Sharp & Dome, which also covers HPV6 and -11). Preventive vaccines have been designed to induce effective production of neutralizing antibodies. Different antigen formulations have been tested for this purpose, the most effective being Viral-Like Particles (VLPs) obtained by self-assembly of L1 proteins produced in bacteria, yeast, insect cells or even plants, with or without the addition of other fused HPV sequences or L2 proteins. Different candidate vaccines have been successful in animal models (Jochmus *et al.*, 1999) in eliciting high titers of neutralizing antibodies. Some induced mucosal immunity without parenteral inoculation (Dupuy *et al.*, 1999, Nardelli-Haefliger *et al.*, 1999, Reuter *et al.*, 2002), opening the way for the development of cheap oral vaccines (Rose *et al.*, 1999, Bermudez-Humaran *et al.*, 2002, Franconi *et al.*, 2002, Lin *et al.*, 2002) which are possibly the future of mucosal vaccination. The most encouraging results are those obtained with the use of the recently approved VLP vaccines (data from clinical trials) which show >90% efficacy (Koutsky *et al.*, 2002, Koutsky *et al.*, 2006), but other phase II/III clinical trials with other VLP vaccines are in progress. Although reliable results on the reduction of cancer incidence could take years to emerge, those on the prevalence of HPV persistence should become available quite soon. In the meantime, epidemiological models of HPV16/18 infections and their related pathologies in different population-wide vaccination programs have been proposed (Taira *et al.*, 2004, Elbasha *et al.*, 2007).

Basically the introduction of such vaccines (efficacy >90%, average immunity around 10 years, cost 320 $, <12 years old female only vaccination) would be clearly cost-effective in adding quality-adjusted years of life to the population in United States. The outcome of such models depends on parameters that may significantly vary in other countries, such as the penetration of current screening programs, the global incidence and prevalence of HPV infection and of cervical cancer, hence cost-benefit models should be calculated specifically for each country. Italy is the first country to implement a mass vaccination program for 12 year old girls, despite being (curiously enough) the country where Gardasil (the vaccine adopted) is sold at the highest price (188 euros/dose compared to 155 in Germany, 146 in France, 120 in the Netherlands and 120$ in the United States; three doses are necessary for full vaccination).

However, most countries with limited resources, where the burden of HPV-related pathologies is heavier, cannot afford such high vaccine costs. The same countries, of course, cannot currently afford to implement screening programs, but while screening costs are not bound to decrease significantly any sooner, cheaper vaccines (and vaccines targeting more high-risk HPVs) will hopefully be available in the near future. The next important step should be oral preventive vaccines that could reach a penetration sufficient to erad-

icate these dangerous infections worldwide. Many research groups are already studying models for effective oral vaccination, and they deserve the maximum support from the institutions.

In contrast to prophylaxis, therapeutic vaccines have been designed to enhance cell-mediated immunity against E6 and E7 in order to achieve regression of HPV induced dysplasias and neoplasias.

Different animal models have shown that this approach can be very effective (Sundaram *et al*., 1998, Chen *et al*., 1999, Han *et al*., 2000, Han *et al*., 2000), and that the best solutions seem to need a prime/boost approach based on E6 and E7 toxoid. DNA prime/protein boost (possibly with the addition of various immunomodulating genes such as IL-2, IL-12, Heat Shock Proteins or others response modifiers such as ubiquitin (Tan *et al*., 1999, Hung *et al*., 2001, Smahel *et al*., 2001, Velders *et al*., 2001), protein prime/protein boost or protein prime/recombinant vaccinia boost strategies have been adopted. A few small clinical trials are currently in progress for this kind of therapeutic vaccines (Garcia-Hernandez *et al*., 2006, Fiander *et al*., 2006): the results are promising and confirm those achieved with animal models. More work is still needed in this field to identify the most effective therapeutic vaccination strategies, which, if implemented, could revolutionize the treatment of HPV infections and related precancerous or neoplastic lesions in a relatively short time.

CONCLUDING REMARKS

In the recent past, new insights have emerged regarding the mechanism of transformation that leads from infection with HPVs to cervical cancer. The molecular epidemiology of the most dangerous among these infections has also been sufficiently clarified.

Together with the availability of more sensitive diagnostic techniques, this knowledge has made it possible to aim second-line diagnostic procedures and treatments at the subset of persisting, high-risk HPV infections. This approach allows early conservative treatment only where needed and obviates it in other situations. Sensitive molecular assays also ensure a more reliable follow-up. Up to 90% reduction in the risk of progression to cancer can be achieved by the correct implementation of the screen-diagnose-treat strategy. However, this strategy has probably reached its limits of efficacy, is scarcely cost-effective and cannot be applied in settings with limited resources. Improving the prevention of cervical and other HPV-related cancers is possible, both in industrialized and in developing countries. Current data from the use of the first commercially available vaccines indicate that a dramatic reduction of high-risk HPV infection through vaccination is achievable. Extensive research efforts should therefore be aimed at developing and testing new candidate vaccines, cheap and active against the whole range of potentially dangerous HPVs. Rational public health planning should be implemented in a concerted action worldwide, as was successfully achieved in the past for smallpox and polio: eradication of these insidious pathogens is now feasible.

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