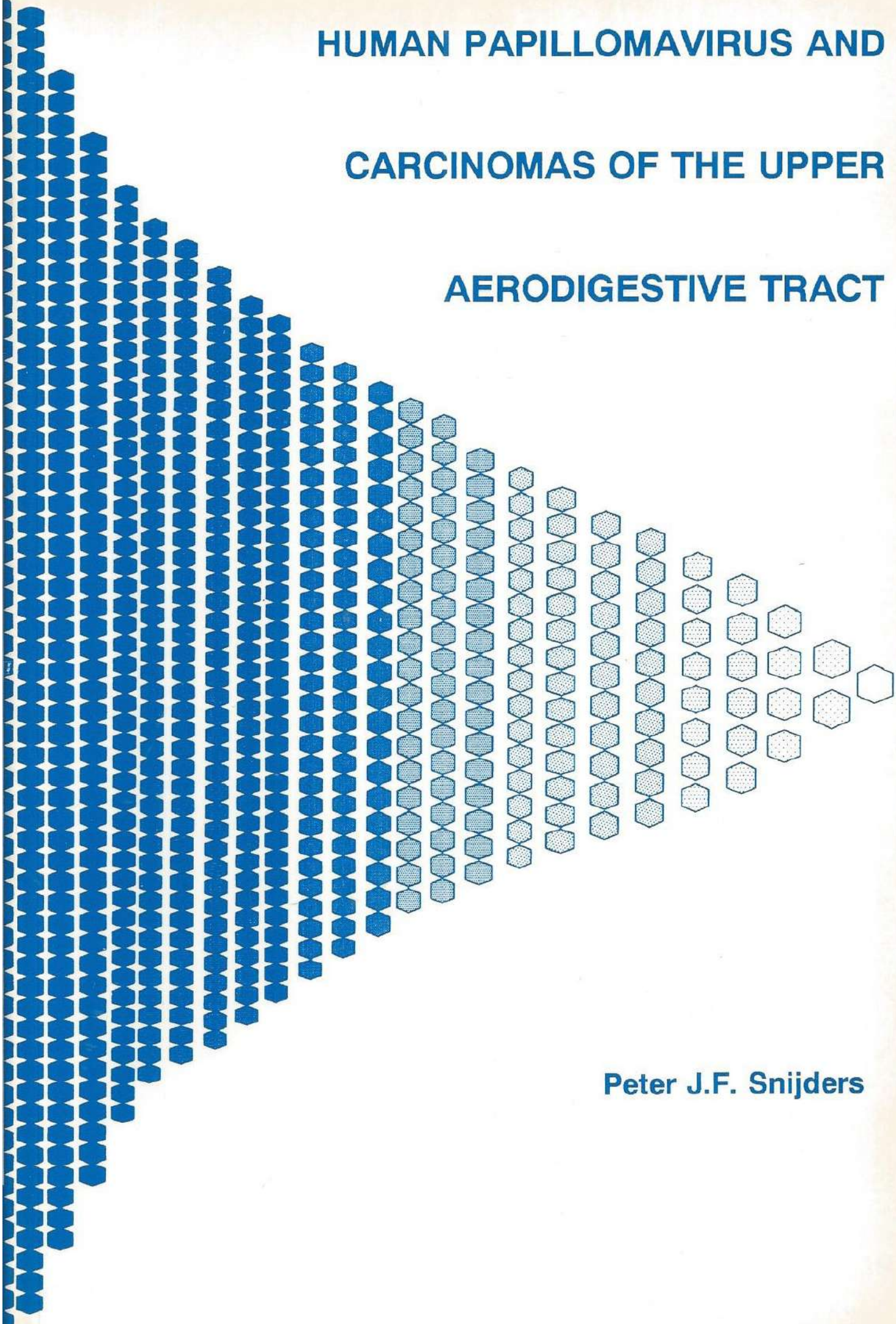
HUMAN PAPILLOMAVIRUS AND CARCINOMAS OF THE UPPER AERODIGESTIVE TRACT



Peter J.F. Snijders

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Het beschreven onderzoek is uitgevoerd in het kader van een samenwerkingsverband tussen de afdelingen Pathologie en KNO van het Academisch Ziekenhuis der Vrije Universiteit te Amsterdam. Het onderzoek werd financieel gesteund door de Nederlandse Kankerbestrijding "het Koningin Wilhelmina Fonds", nummer IKA 89-16. Dit onderzoek maakt deel uit van het VWF onderzoeksprogramma van de vakgroep Pathologie: morfologische, functionele en moleculair biologische aspecten van cellen in chronische ontstekingen en tumoren (GNK 89-07), deelthema II oncologie (zwaartepunt faculteit der geneeskunde VU en VU ziekenhuis). Het proefschrift is bewerkt in het Pathologisch Instituut (hoofd: prof.dr. C.J.L.M. Meijer), sectie Moleculaire Pathologie (hoofd: dr. J.M.M. Walboomers), van het Academisch Ziekenhuis der Vrije Universiteit te Amsterdam.

VRIJE UNIVERSITEIT

HUMAN PAPILLOMAVIRUS AND CARCINOMAS OF THE UPPER AERODIGESTIVE TRACT

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
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in het openbaar te verdedigen
ten overstaan van de promotiecommissie
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door

Petrus Josephus Ferdinandus Snijders

geboren te Nijmegen

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Promotoren: prof.dr. C.J.L.M. Meijer
prof.dr. G.B. Snow
Copromotor: dr. J.M.M. Walboomers
Referenten: prof.dr. L. Gissmann
prof.dr. J. van der Noordaa

Ter nagedachtenis aan mijn vader

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CHAPTER I

Introduction

Introduction

1. Cancer of the upper aerodigestive tract

Nearly every neoplastic disease may have a primary or secondary manifestation in the head and neck. However, squamous cell carcinoma originating from the mucous membranes of the upper aerodigestive tract is by far the most frequent tumor occurring primarily in the head and neck. In the Western world the incidence of head and neck squamous cell carcinoma is generally low, in the order of 5% of the total number of new cancer cases (1), except for a few places where the risk is exceptionally high, such as France, Italy, and Spain. The larynx, oral cavity and pharynx are most commenly involved. In many countries in the developing world, such as India and Pakistan, oral cancer is the commonest form of cancer and this accounts for the fact that oral cancer is one of the ten most frequently occurring cancers in the world. It is of interest that recent epidemiological data suggest that the incidence of head and neck squamous cell carcinoma in the Western world, having fallen gradually earlier in this century, is now rising and that this pattern is present in both men and women (2,3). This appears particularly true for cancers of the oral cavity, the pharynx and the larynx. The disease is more commen in males than in females, generally with a peak incidence in the 7th decade of life. The sex ratio, however, varies considerably for the various sites.

It is very likely that the pathogenesis of head and neck cancer is multifactoral. Both tobacco and alcohol are important risk factors (4). Occupational factors may play a role as well (5). It has not yet been clarified to what extent human papillomaviruses are involved (see I.4). Furthermore, it seems very likely that individual genetic susceptibility to these external carcinogens is important, if only because so many individuals have been and are being exposed, for instance, to tobacco and alcohol, whereas only relatively few actually develop cancer in the upper air and food passages (6). Head and neck squamous cell carcinoma patients are at high risk for developing second primary cancers within the same organ system: the respiratory tract and the upper digestive tract, including the esophagus (11). Many theories have been postulated to explain the phenomenom of multiple primary cancers, the most convincing being "field cancerization" or "condemn mucosa" syndrome (8).

2. Human papillomavirus

2.1. General introduction

Papillomaviruses belong to the family of Papovaviridae and consist of a circular double stranded DNA molecule, approximately 7.8 to 7.9 kilobases (kb) in size, which is packaged into an icosahedral capsid. A characteristic feature of papillomaviruses is that within their genome all major open reading frames (ORFs) are located on the same DNA strand. A functional subdivision of the papillomavirus genome has been made in analogy with other viruses. An "early region" (E-region) is defined, consisting of genes that are supposed to be expressed before onset of viral DNA replication, and a distinct "late region" (L-region) contains genes that encode viral capsid proteins. In addition, an in-between long control region (LCR) or upstream regulatory region (URR) is defined which is built up from sequences involved in the control of viral gene expression and episomal replication. Due to the absence of an *in vitro* HPV culture system and reliable serological tests at this moment, both papillomavirus identification and type differentiation are based solely on nucleic acid criteria. According to these criteria, more than 60 human papillomavirus (HPV) types have been identified to date (9). HPVs are strictly epitheliotropic and are well-known to induce epithelial tumors of the mucosa and skin in man (10,10a). In these lesions HPV production is limited to the upper part of the differentiating epithelium. In general these tumors are benign and ofter regress spontaneously. However, dependent on the HPV type present, some HPV induced lesions may progress to malignancy, a process in which the involvement of co-factors has been suggested (10,11). The majority of HPV types can be grouped together into those associated with cutaneous lesions (e.g. HPV 1, 5, 8, 14) and types associated with mucosal, mainly genital lesions (e.g. HPV 6, 11, 16, 18). However, there exist exceptions to the strict cutaneous versus mucosal classification as reflected by types like HPV 2 and HPV 57, which show an ambivalent tropism (12). In addition, a subdivision can be made between so-called "low risk" and "high risk" HPV types, depending on their association mainly with lesions having a low and high risk for malignant progression, respectively. Amongst the mucosotropic HPVs, the HPV types 6 and 11 are well recognized low risk HPVs and are predominantly associated with genital condylomas and laryngeal papillomas (13,13a,14), which rarely progress to malignancy. Mucosotropic high risk HPV types include HPV 16, HPV 18, HPV 31 and HPV 33, and are implicated in anogenital cancer, in particular cancer of the uterine cervix (15).

2.2. HPV genome organization and encoded functions

Papillomaviruses have a very compact genome organization. Their ORFs are distributed over all three reading frames and there is considerable overlap, particularly between the E-region ORFs. A representative scheme of the HPV genome organization is depicted in Figure 1. The HPVs contain at least six early ORFs (E1 to E7), two late ORFs (L1 and L2) and the LCR, the latter being localized between ORFs L1 and E6. Based on data concerning bovine papillomavirus type 1 (BPV 1) extended with more recent data from some HPVs, the functions of HPV ORFs have been deduced (16). It should be noted, however, that an ORF may not be confounded with a gene. Although some ORFs (e.g. E6 and E7) encode a distinct protein, others (e.g. E4) are non-functional by itself and only provide an exon which should be fused with an exon from a different ORF via RNA splicing to become functional. Therefore, the number of HPV encoded proteins generated following differential RNA splicing may exceed the number of ORFs.

E6 and E7 ORFs. The HPV E6 and E7 ORFs exhibit transforming functions and for the high risk HPVs their products have been assigned as oncoproteins. Both ORFs encode multi-functional proteins with no known enzymatic activities, which are likely to acquire their intrinsic properties via complex formation (17-19).

E1 and E2 ORFs. Although less well studied, data obtained from BPV 1 have suggested that the full-length E1 ORF encodes a viral replication factor (20,20a). Moreover, truncated E1 sequences, fused via RNA splicing with sequences of other ORFs, are involved in the generation of a variety of viral proteins with diverge activities. These include E1-M, E2-C, E1^E4 and E1-M^E2-C fusion proteins (21,22,22a). Of these proteins, E1-M is supposed to act at the level of viral copy number control during replication (23). E2-C, which consists mainly of sequences from the 3' part of the E2 ORF, is likely to be a transcriptional

repressor that acts via binding with E2 responsive sites within the LCR (24). The fusion protein E1-M^E2-C contains both the E1-M replication modulator domain and the E2-C DNA binding domain. Functional analysis has revealed transcriptional repressor activity, triggered by binding to an E2 responsive element. In addition, a role for this protein in controlling episomal DNA replication has been suggested (22a). Also the full-length E2 protein acts by binding to E2 responsive elements within the LCR, thereby triggering positive and negative transcriptional feedback regulation (25-30).

E4 and E5 ORFs. E4 encoding sequences are expressed via an E1^E4 fusion protein that is involved in the collapse of the cytokeratin matrix (31). It has been suggested that by this action a resistant barrier is destroyed which may enhance virus shedding from the epithelial surface. Although the E5 protein of BPV 1 exhibits transforming properties (32), the function of the HPV E5 proteins is still unclear. However, it recently has been found that the E5a protein of HPV 6 contains transforming activity in NIH 3T3 and C127 cells (33).

L1 and L2 ORFs. The HPV L1 and L2 ORFs encode major and minor capsid proteins, respectively.

LCR. The LCR does not contain genes but in analogy with BPV 1 probably contains promoters as well as the replication origin. Several E2 responsive elements are located within the LCR, which mediate transcription feedback regulation by interaction with E2 and E2-C proteins. In addition, an E2 protein-independent enhancer has been identified which exhibits cell-type specificity and is likely to contribute to the strict epitheliotropism of HPVs (26,34). Transcriptional enhancement via this enhancer is mediated by binding and co-operation of several apparently ubiquitous transcription factors including NF1, AP-1, PVF, NFA, oct-1, as well as the hormone stimulated progesterone and glucocorticoid receptors (35,36,37).

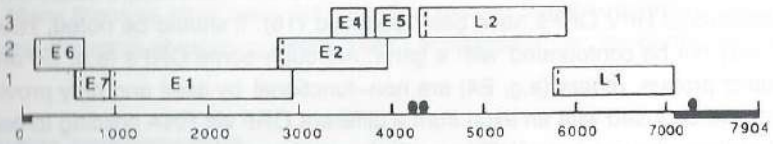

Figure 1: Schematic presentation of the HPV genome organization. The three reading frames are indicated on the left and boxes represent early (E) and late (L) open reading frames. Dotted lines indicate putative initiation codons and filled circles indicate putative poly (A) signals. The location of the LCR is indicated by a bar. The Figure is redrawn from Sousa *et al.* (16).

2.3. HPV detection methods

The lack of a culture system for HPV propagation has resulted in nucleic acid detection assays being widely applied methods of detecting HPV. These assays include hybridization techniques like filter *in situ* hybridization, dot blot hybridization, Southern blot hybridization and nucleic acid *in situ* hybridization, and the polymerase chain reaction (PCR).

2.3.1. Hybridization techniques

These methods have in common that they are based on hybrid formation of denaturated target DNA or RNA strands with a labelled complementary DNA/RNA strand (probe). Probe labelling can be carried out by incorporation or addition of radioactive as well as non-radioactive labels and visualization of the hybrid nucleid acids ultimately can be accomplished by autoradiography and immunohistochemical/immunofluorescence staining procedures, respectively.

Filter *in situ* hybridization. This method is based on the immobilization of crude cell suspensions on a filter support, followed by hybridization (38,39). A major advantage is that the method can be applied rapidly and large numbers of samples can be screened simultaneously. A disadvantage is a limited detection level (10 to 100 HPV copies/cell) and the method is not applicable to solid tissue specimens.

Dot blot method. In this method purified DNA is directly transferred to a filter support and subsequently hybridized to a probe (40,41). The sensitivity is limited to 1 to 10 HPV copies per cell and the method requires about 2 to 5 μg sample DNA.

Southern blot method. In contrast to the dot blot method, the Southern blot technique (42) involves a restriction enzyme digestion step followed by electrophoretical separation of digested DNA fragments on an agarose gel before the DNA is transferred to a filter support. Consequently, application of this method gives rise to a pattern of one or more hybridizing restriction fragments of different sizes, which increases the specificity of the method. The technique usually requires 5 to 10 μg sample DNA and the sensitivity is approximately 0.1 to 1 HPV copies per cell. The Southern blot method is still considered to be the most reliable HPV DNA detection technique and is widely used to confirm results obtained from other HPV detection methods.

***In situ* hybridization method.** In this method (43,44,44a), which usually is applied to tissue sections, hybridization is directly performed within the cells after mild pretreatment to make target DNA/RNA attainable for the probe. This method has a major advantage that it preserves the morphology and allows the identification of cells which harbor HPV nucleic acids. The sensitivity of non-radioactive DNA *in situ* hybridization is approximately 20 HPV copies per cell (45).

2.3.2. Polymerase chain reaction

The recently developed PCR method (46,47) is recognized as the most sensitive DNA detection method. Comparison of different HPV detection methods have revealed that the PCR is superior in sensitivity (48). The technique includes an enzymatic step and is based on the annealing of two short oligonucleotides (primers) to the opposite strands of a certain target DNA molecule, thereby providing free 3'-OH ends for DNA polymerase-mediated chain elongation. Usually, 30 to 40 cycles of amplification are performed, which are composed of a DNA heat denaturation step, a primer annealing step and a chain elongation step. Under optimal conditions a twofold increase in the amount of target DNA, spanned by both primers, can be achieved during each cycle and theoretically the amount of target DNA is amplified more that a millionfold after performance of twenty PCR cycles. Therefore, a sensitivity of 1 HPV copy per sample can be observed by PCR. Ultimately, amplified DNA often can be resolved at the agarose gel level, but additional hybridization by dot- or

Southern blotting is necessary to confirm specificity and enhance sensitivity. The method is not only advantageous because of its high sensitivity, but also by its requirement of relatively low amounts of target DNA (25 to 500 ng), which is important in case of small tissue specimens. Another advantage is that the PCR can be applied to crude cell suspensions (49,50) and crude extracts of formalin-fixed, paraffin-embedded tissue (51). A major drawback of the method is its susceptibility to laboratory contaminations, especially to cloned plasmid DNA and PCR products obtained from previous reactions. Therefore, special precautions should be taken to exclude false positive PCR results (52,53). At present, the PCR is a widely applied HPV DNA detection method.

2.3.3. Problems in HPV detection

Although the methods described above have shown to be successful in the specific detection of a variety of HPV genotypes, problems arise when it is unknown which HPV type might be present in a certain lesion. Owing to the existence of more than 60 different HPV types, it is unfeasible to perform successive type-specific screening assays and more generalized assays are required to screen for the presence of a broad spectrum of, including putatively novel, HPV genotypes. Thus far, several techniques have been used for HPV detection in a rather type-unspecific manner. These include histologic examination, which focuss on cytopathological changes (e.g. koilocytosis) resulting from HPV infections (54), and immunohistochemical detection of group-specific papillomavirus capsid antigens with an antiserum prepared against SDS-disrupted BPV 1 virions (55). The disadvantage of the first method is that agents different from HPV could have caused the histological changes. Moreover, apart from their rather low sensitivity, these methods only can detect productive viral infections, thereby survying a limited field because virus production is known to be abrogated in carcinomas.

Universal HPV DNA detection methods include low stringency Southern blot hybridization (56,57), eventually using an HPV cocktail probe, and reverse blot hybridization (58). The first of these methods makes use of the capability of HPV genotypes to cross-hybridize under these conditions with more or less related HPV genotypes. Inherent in this method is a reduced sensitivity. As shown in Figure 2, the less related HPV types 16 and 18 cross-hybridize under low stringency conditions (Tm-40°C). However, the sensitivity is greatly reduced. Using an HPV 16 probe, HPV 18 DNA can not be detected in the C4-1 (1 to 5 copies per cell) and HeLa (10 to 50 copies per cell) cell lines after a short exposure time (Figure 2A). Although a longer exposure time allowed the resolution of some HPV 18 fragments in the HeLa cell line, a remarkable increase of interfering background signals is evident, making that hybridization signals are difficult to interpret (Figure 2B). After using an HPV 18 probe only HPV 16 DNA in the CaSki cell line (500 to 600 copies per cell) can be detected at reduced sensitivity (Figure 2C).

The reverse blot hybridization method uses cellular DNA as probe for hybridization to a panel of cloned HPV DNAs, immobilized on a filter support. The sensitivity of this method is limited to about 10 HPV genome equivalents per cell (58). Therefore, low copy numbers of a certain type will remain undetectable and it is questionable whether putatively novel, still unidentified HPV types would be detected by this method.

Consequently, this aspect of detecting HPV needs further improvements and the introduction of PCR methods using general/consensus primers offers new perspectives (59,60) and is extensively studied in this thesis.

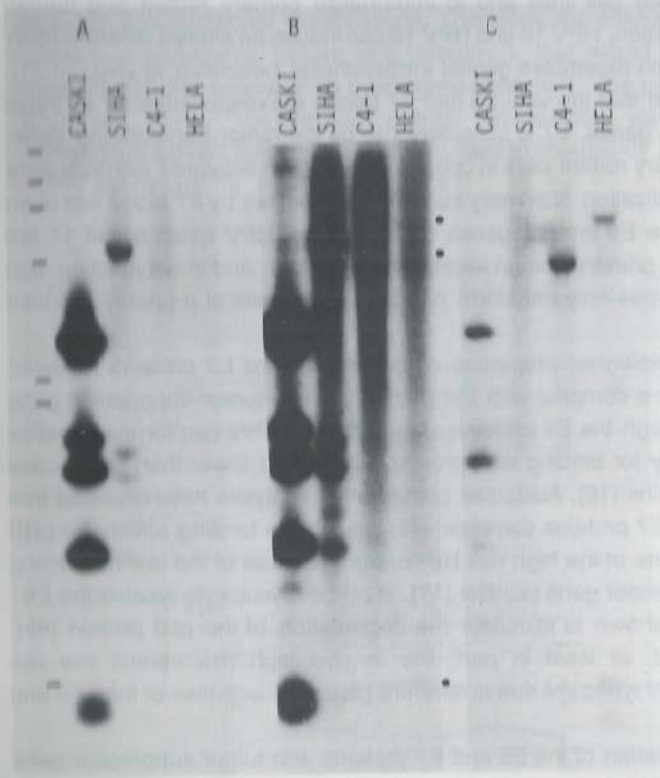

Figure 2: Low stringency Southern blot analysis (Tm-40°C) of 1 μg CaSki DNA (500 to 600 copies of HPV 16 per cell), 10 μg Siha DNA (1 to 10 copies of HPV 16 per cell), 10 μg C4-1 DNA (1 to 5 copies of HPV 18 per cell) and 4 μg HeLa DNA (10 to 50 copies of HPV 18 per cell) digested with *Pst*I. Hybridization was performed with labelled HPV 16 (A and B) or HPV 18 (C) DNA. Autoradiography was performed for one (A and C) and four (B) days. λx *Hin*dIII size markers are indicated on the left. In (B) HPV 18 fragments within HeLa DNA which cross-hybridize with the HPV 16 probe are indicated by filled circles.

3. Human papillomavirus and carcinogenesis: the cervical cancer model

There exists a strong association between certain HPV genotypes and carcinomas of the uterine cervix. Almost all cervical carcinomas have been found to contain HPV DNA (50,52,61), and the most prevalent types in cervical cancer are the HPV types 16 and 18 (62,63). To date, substantial experimental data have been collected which suggest that these specific high risk HPVs are involved in the pathogenesis of cervical cancer (11,64). In this section some aspects will be discussed which are supposed to be essential in carcinogenesis by HPV.

3.1. HPV oncogenesis

Since the majority of cervical carcinomas contain HPV 16 or HPV 18 DNA, studies that have been set up to discover putative oncogenic properties of high risk HPVs mainly have been performed with these types. *In vitro* studies have revealed the abilities of these HPV types to transform established cell lines and to immortalize primary rodent and human keratinocytes (65–69). In addition, HPV 16 and HPV 18 can induce an altered differentiation of human epithelial cells which resembles genital intraepithelial neoplasia *in vivo* (70,71). Further studies have revealed that the viral E6 and E7 genes are responsible for all these properties (72–75). Of these genes, E7 is considered to be the major transforming gene; E7 alone can transform primary rodent cells in cooperation with an activated *ras* oncogene (76,77) and even the immortalization of primary human keratinocytes by E7 alone has been reported (78). In contrast, the E6 and E7 genes of the low risk HPV types 6 and 11 are incompetent in immortalizing primary human keratinocytes (79,80) and in conjunction with *ras*, the E7 genes of these types only transform primary rodent cells at a greatly reduced level (81).

Additional studies on the biological properties of the viral E6 and E7 proteins revealed that the E7 protein can form a complex with the retinoblastoma tumor suppressor gene product, pRB (17a,18). Although the E7 proteins of the low risk HPVs can form a complex with pRB as well, their affinity for binding is approximately tenfold lower than is the case for the high risk HPV E7 proteins (18). Additional comparative analyses have revealed that the transforming capacity of E7 proteins correlate with the relative binding affinity for pRB (19). Moreover, the E6 proteins of the high risk HPVs, but not those of the low risk HPVs, can bind the p53 tumor suppressor gene product (17). In rabbit reticulocyte lysates the E6–p53 interactions have been shown to stimulate the degradation of the p53 protein (82). These findings suggest that, at least in part, the *in vivo* high risk versus low risk characteristics of different HPV types are due to different biological activities of their E6 and E7 proteins.

The idea that complex formation of the E6 and E7 proteins with tumor suppressor gene products is an important event in carcinogenesis, is supported by studies performed on HPV-negative cervical carcinoma cell lines (83,84,85). These studies have revealed that, in contrast to the HPV containing cell lines, the HPV-negative cell lines contain p53 and pRB mutations and abnormal p53 and pRB proteins, suggesting that loss of wild-type p53 and pRB functions is essential for tumor development. The loss of these functions would occur either by mutation within the cellular gene or by expression of viral proteins capable of complexing wild-type cellular proteins.

The importance of the E6 and E7 genes has further been strengthened by their regular transcription into mRNA in cervical carcinomas and cell lines derived from cervical carcinomas (86–89). Also their encoded proteins have been identified in carcinomas and cell lines (88,90–92). Furthermore, interference with E6–E7 expression by antisense RNA constructs has resulted in reduced cell growth of HPV 18 containing C4-1 cervical carcinoma cells (93). In a different study, the continued expression of the HPV 16 E7 protein has been found to be required for maintenance of the transformed phenotype of baby rat kidney cells co-transformed by HPV 16 and an activated *ras* oncogene (94). These data point to a role of E6–E7 genes of high risk HPVs not only in the initiation but also in the

maintenance of the malignant phenotype. Although initially nontumorigenic in nude mice, long-time *in vitro* cultivation of HPV 18 immortalized human keratinocytes have yielded fully malignant cell populations containing chromosomal abnormalities (95). This suggests that high risk HPVs alone can induce malignant growth, if cell proliferation triggered by their E6/E7 functions is allowed to proceed uninterrupted which may lead to spontaneous or virus-induced additional modifications. Therefore, the previously suggested importance of exogenous mutagenic factors (e.g. smoking), that would act synergistically with HPV infections (10,96) seems to be overemphasized (64). The present opinion is that high risk HPVs can trigger uncontrolled cellular proliferation via deregulated expression of endogenous E6–E7 functions, which may lead to aneuploidy and contribute to other cellular events necessary for a full cancer (64,97).

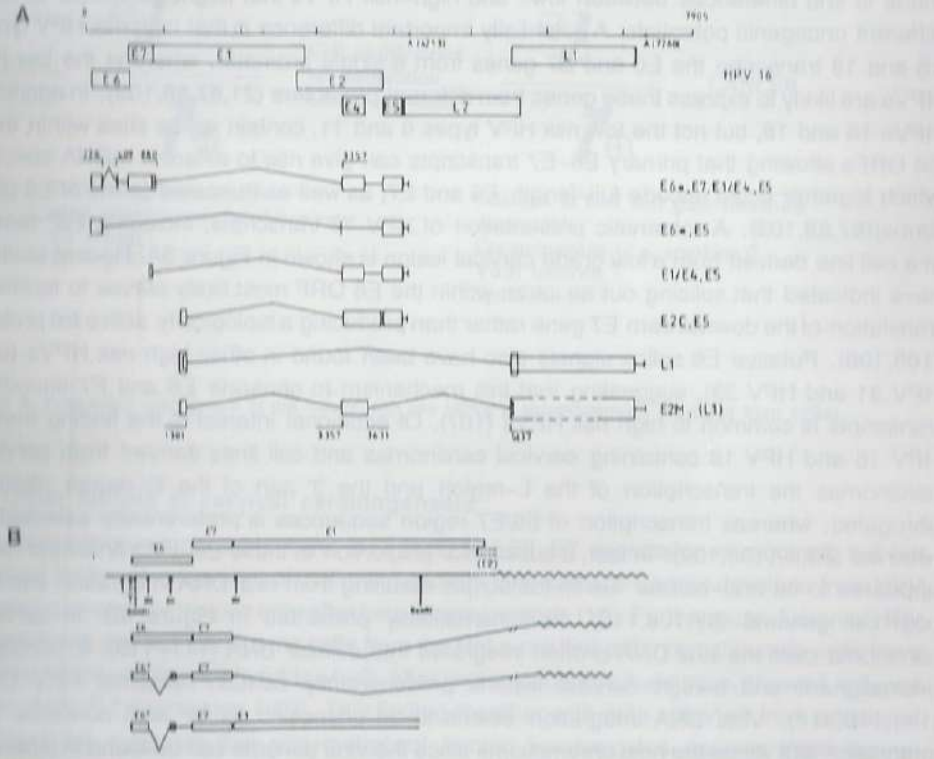

Figure 3: Schematic presentation of HPV 16 transcripts indentified in a cell line derived from a low grade cervical lesion containing multiple HPV 16 copies as stably replicating episomes (A) and of chimeric HPV 18 transcripts in cervical carcinoma cell lines C4-1, HeLa and SW 756 containing integrated HPV 18 DNA (B). Exons are indicated by open boxes and transcripts are depicted in relation to the HPV genome organization. In (B) viral-cellular junction sites are indicated by dotted lines and cellular sequences by zig-zag lines. (A) is redrawn from Doorbar *et al.* (104) and (B) from Schwarz *et al.* (107).

3.2. HPV expression and integration

HPVs can generate a variety of overlapping, mostly polycistronic mRNAs by using alternative promoters and splice sites. Normally, these mRNAs are polyadenylated at one of two sites located at the 3' ends of the E- and L-regions. Studies concerning HPV 1 and HPVs-6 and -11 in plantar warts and genital condylomatous lesions, respectively, have identified several E- and L-region mRNAs containing more or less conserved splice sites (21,22,98-101). *In situ* hybridization of condylomatous lesions with HPV 6 and HPV 11 message specific antisense probes has revealed that both DNA replication and mRNA expression are dependent on cellular differentiation (102). In the basal epithelial layer HPV mRNA is minimally detected but upon differentiation, the copy numbers of mRNAs are increasing, particularly in the upper spinous and granular cell layers. In general, the E1^E4 encoding transcripts predominate throughout in all epithelial layers, while L-region messages are only present in the superficial strata of the epithelium (102).

Especially concerning the expression of the HPV E6 and E7 genes, efforts have been made to find differences between low- and high-risk HPVs that might contribute to their different oncogenic potentials. A potentially important difference is that high risk HPV types 16 and 18 transcribe the E6 and E7 genes from a single promoter, whereas the low risk HPVs are likely to express these genes from different promoters (21,87,88,103). In addition, HPVs 16 and 18, but not the low risk HPV types 6 and 11, contain splice sites within their E6 ORFs allowing that primary E6-E7 transcripts can give rise to different mRNA species which together could encode full-length E6 and E7, as well as truncated forms of E6 (E6* forms)(87,88,103). A schematic presentation of HPV 16 transcripts, including E6* forms, in a cell line derived from a low grade cervical lesion is shown in Figure 3A. Recent studies have indicated that splicing out an intron within the E6 ORF most likely serves to facilitate translation of the downstream E7 gene rather than producing a biologically active E6 protein (105,106). Putative E6 splice signals also have been found in other high risk HPVs (e.g. HPV 31 and HPV 33), suggesting that this mechanism to generate E6 and E7 encoding transcripts is common to high risk HPVs (107). Of additional interest is the finding that in HPV 16 and HPV 18 containing cervical carcinomas and cell lines derived from cervical carcinomas the transcription of the L-region and the 3' part of the E-region often is abrogated, whereas transcription of E6/E7 region sequences is preferentially selected or retained (29,87,108,109). In fact, a substantial proportion of these E6-E7 transcripts have appeared to be viral-cellular fusion transcripts resulting from viral DNA integration into the host cell genome (87,108,110), as schematically presented in Figure 3B. In cervical carcinoma cells the viral DNA is often integrated into cellular DNA (111-116). In contrast, premalignant and benign cervical lesions predominantly contain episomal HPV DNA (111,116,117). Viral DNA integration seems to be unspecific as far as it concerns the integration site within the host chromosome since the viral genome can be found in different locations in different carcinomas and cell lines. However, with respect to the opening and disruption of the HPV genome, integration regularly occurs within the viral E1-E2 region (86,112,118,119), thereby disrupting expression of E2 and additional downstream genes that are disconnected from their natural promoters. Consequently, it has been suggested that viral DNA integration is important in carcinogenesis by triggering the disruption of expression of E2 encoded transcriptional modulator proteins which would result in the uncontrolled expression of the transforming genes E6 and E7. However, additional

suggestions have been made that emphasize a role for HPV DNA integration in carcinogenesis by other means. These include the suggested importance of the utilization of a cellular polyadenylation signal, which may increase the stability of E6/E7 chimeric transcripts, thereby enhancing the expression of E6 and E7 genes (29,110). Furthermore, a role of viral DNA integration at the level of the activation of cellular oncogenes has been proposed. HPV DNA integration near *myc* oncogenes have been reported for some carcinomas and cell lines (120-121) and the *c-myc* gene has been implicated in cervical cancer (122).

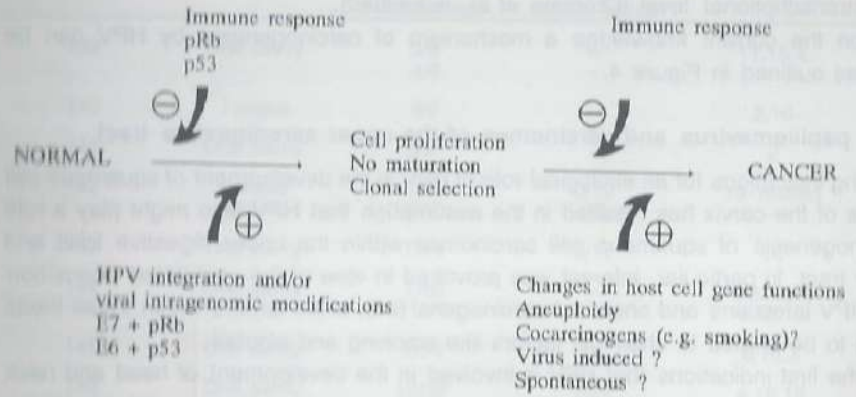

Figure 4: Postulated mechanism of the role of high risk HPVs in carcinogenesis (modified from 129a).

3.3. Other factors in cervical carcinogenesis

Several observations support the idea that HPV E6-E7 expression is required, but not sufficient for the development of malignant growth. First, cervical cancer develops from HPV containing precursor lesions only after long latency periods (10). Furthermore, fusion of HPV 18 containing cervical carcinoma cells from the HeLa cell line with normal human cells have resulted in non-malignant hybrids which, after implantation into nude mice, showed reduced levels of E6-E7 expression (123). This finding together with data obtained from additional studies (124) suggests that non-malignant human keratinocytes express trans-acting, negative regulatory factors that reduce transcription of viral genes. A different observation was done by Miyasaka *et al.* (125), who showed that despite the presence of unreduced levels of E6-E7 mRNA, hybrids obtained after fusion of HPV 16 transformed cells of an established rat cell line with primary rat embryo fibroblasts have lost the transformed phenotype. This finding suggests the presence of cellular factors that interfere with HPV 16 E6-E7 functions at the posttranscriptional level. Although the level of action is still speculative, it seems that the loss of one or more genetic functions of the host cell is of

importance in carcinogenesis by HPV. In this context, functions on chromosome 11 have been implicated to play a role since introduction of the normal chromosome 11 into HeLa cells via microcell transfer resulted in the suppression of the tumorigenic phenotype (126). Moreover, human embryonic fibroblasts containing a deletion on one copy of chromosome 11 were susceptible to transformation by HPV 16 E-region DNA whereas normal diploid human embryonic fibroblasts were not (127).

A different, but obviously not less important factor is the immune surveillance of HPV infections. An increased incidence of HPV associated lesions has been found in immunosuppressed and immunodeficient patients (128,129) suggesting that a disturbed immune response can be an additional important factor that contributes to carcinogenesis by HPV. In view of this assumption it is worth noting that recently in 5 out of 10 HPV 16 containing cervical carcinomas, MHC class I expression was found to be downregulated at the posttranscriptional level (Cromme *et al.*, submitted).

Based on the current knowledge a mechanism of carcinogenesis by HPV can be proposed as outlined in Figure 4.

4. Human papillomavirus and carcinomas of the upper aerodigestive tract

The strong indications for an etiological role of HPV in the development of squamous cell carcinomas of the cervix has resulted in the assumption that HPV also might play a role in the pathogenesis of squamous cell carcinomas within the upper digestive tract and respiratory tract. In particular, interest was provoked in view of the postulated interaction between HPV infections and chemical carcinogens (96), since tumors within these tracts are known to be related to chemical factors like smoking and alcohol.

One of the first indications that HPV is involved in the development of head and neck lesions came from clinical observations that juvenile laryngeal papillomas originate from a perinatal infection of mothers with genital condylomatous lesions (130). Indeed, HPV capsid antigens and DNA of HPV types 6 and 11 have been detected in laryngeal papillomas by numerous groups (13a,14,56,131,132) and to date it is widely accepted that the low risk HPV types 6 and 11 are etiologically involved in laryngeal papillomas. In addition, the association of predominantly HPV 13 and HPV 32 with oral focal epithelial hyperplasia (Heck's disease), a benign lesion frequently found among Eskimos and Indians from North and South America, has been documented (133,134). Indications for an association between low risk HPVs and inverted nasal papillomas have been obtained as well (135,136,137).

HPV involvement in carcinomas from the upper aerodigestive tract has been suggested on basis of histological and immunohistochemical studies. Histologic examination of laryngeal squamous cell carcinomas has revealed the presence of condylomatous changes, suggestive for HPV infections in a substantial proportion of cases (54). Moreover, of 40 biopsy specimens of oral squamous cell carcinomas, 16 revealed histological changes suggesting an HPV infection and 8 of them showed positive staining with antiserum raised against the papillomavirus structural antigenes (138). Initial HPV DNA detection studies confirmed the presence of HPV in a proportion of oral carcinomas. Using the Southern blot method with HPV 11 and HPV 16 probes, Löning *et al.* (139) have detected HPV DNA in 3 out of 6 oral carcinomas. One of them contained HPV 11 DNA and one HPV 16 DNA, while typing of the third sample was not possible due to limited amounts of DNA available. Using a cocktail probe, Southern blot analysis of seven tongue carcinomas performed by De Villiers *et al.* (140) has revealed the presence of HPV DNA in 3 cases. DNA from one tumor hybridized with the HPV 2 probe and the remaining two cases showed positivity for HPV 16 DNA. Several additional HPV DNA detection studies using either Southern blot or *in situ* hybridization techniques have been performed on oral carcinomas and the prevalence rates found, ranged from approximately 2.5% to 50%, as summarized in Table 1. These differences may reflect the methods and probes used. Still, HPV 16 is prominent amongst the positive cases, except for verrucous oral carcinomas containing HPV 2.

Table 1. Prevalence of HPV genotypes in carcinomas and normal tissue within the upper aerodigestive tract

References	Site	HPV pos. carcinomas	HPV pos. normal tissue	HPV type(s)	Method
139	Oral cavity	3/6 3/6	- -	11,16,X -	SB IF
140	Tongue	3/7	-	2,16	SB
141	Oral cavity	3/9[a]	-	2	SB
142	Tongue Mouth	2/9 0/10	0/10 0/8	16-related -	SB SB
143	Oral cavity	7/15	5/12	16	SB
144	Oral cavity	1/40 11/40	- -	unknown 6,16,18	ISH PCR
145	Oral cavity	7/74	-	6,16,X	SB,RB
146	Oral cavity	19/39[b]	2/25	4,16,18	PCR
142	Nose Tonsil Pharynx	0/2 2/5 1/7	0/9 0/20 0/13	- 16-related 16-related	SB SB SB
147	Tonsil	6/28	0/30	16	ISH
148	Larynx	1/36	-	16	SB
149	Larynx	6/6[a]	0/3	16-related	SB
57	Larynx	1/42	-	30	SB
142	Larynx	3/57	2/51	11-,16-rel.	SB
150	Larynx	15/116	-	6,11,16	ISH
151	Larynx	26/48 3/3[a]	3/6 3/6	16 16	PCR PCR
152	Larynx	4/10	-	16	PCR
153	Larynx	7/34	-	6,16	PCR
154	Larynx/Pharynx	7/8	9/14	6,11	PCR
155	Larynx/Pharynx	7/16	10/16	6/11,16/33	PCR

[a] Verrucous carcinomas
[b] Also adjacent dysplastic and normal epithelium appeared HPV positive
SB, Southern blot hybridization; IF, immunofluorescence; ISH, *in situ* hybridization; RB, reverse blot hybridization; PCR, polymerase chain reaction

Also laryngeal carcinomas have further been studied for the presence of HPV DNA (Table 1). This led to the establishment of a clear association between HPV and verrucous carcinomas of the larynx, which are characterized by a low incidence (1-2% of all carcinomas of the larynx). By Southern blot analysis at low stringency conditions, HPV 16-related DNA has been detected in all six verrucous carcinomas of the larynx studied by Brandsma *et al.* (149). Squamous cell carcinomas of the larynx have showed HPV occurrence rates ranging from 2.7% to 12.9%, as determined by Southern blot or *in situ* analyses. Brandsma *et al.* (142) have revealed indications for the presence of HPV 11- and HPV 16- related DNA in laryngeal squamous cell carcinomas, which is interesting and may indicate that still unknown, possibly novel HPV types could be specifically associated with carcinomas from this site. Kahn *et al.* (57) have isolated a novel HPV genotype (HPV 30) from a laryngeal carcinoma. However, the prevalence of this type appeared to be rather low since none of 41 additional laryngeal carcinomas tested, were found to contain HPV 30 DNA (57).

Also the newly developed PCR method has been introduced to study HPV prevalence in carcinomas from the oral cavity and larynx (144,146,151-155). In some studies an increased HPV positivity in carcinomas, but also in normal oral or laryngeal epithelium has been found (146,151,155; see Table 1). However, reports of PCR-based HPV association with these carcinomas should be viewed with caution, until they are confirmed by Southern blot analysis, *in situ* analysis or transcript analysis.

Due to the different methods and probes/primers used it is difficult to draw conclusions from all these HPV detection studies performed. However, of interest is the study performed by Brandsma *et al.* (142) using the Southern blot hybridization method to determine the occurrence of HPV DNA in squamous cell carcinomas at different anatomic sites within the head and neck region. Remarkable differences were observed and the highest occurrence rate was found in carcinomas of the tonsil (29%), followed by the tongue (19%), pharynx (13%) and larynx (5%). No HPV DNA was detected in carcinomas of the nose, mouth and esophagus. This finding suggests that different anatomic sites within the upper aerodigestive tract have different susceptibilities to HPV infection or HPV-mediated carcinogenesis. The occurrence of HPV DNA in tonsillar carcinomas also has been reported by others. Niedobitek *et al.* (147) have demonstrated HPV 16 DNA in the neoplastic cells of 6 out of 28 tonsillar carcinomas by *in situ* hybridization. Furthermore, integrated HPV 6 DNA recently has been detected in a tonsillar carcinoma (156).

At last, all data concerning HPV DNA prevalence in carcinomas of the upper aerodigestive tract are summarized in Table 1.

The data obtained thus far suggest an association between HPV, predominantly of type 16 and a proportion of squamous cell carcinomas within the head and neck region. However, this relationship is not as clear as for HPV and cervical carcinomas and there is still insufficient information to support a possible etiological role for HPV in these carcinomas. HPV DNA detection methods need further improvements to minimize the possibility of missing any putatively unknown HPV type and expression studies should be incorporated to resolve the viral behaviour in lesions within this tract.

5. Outline of this thesis

The preceding paragraph indicates that it is still unclear which role HPV plays in the pathogenesis of squamous cell carcinomas within the upper aerodigestive tract. Therefore, the study presented in this thesis was undertaken to answer the question whether HPV can be a candidate that is etiologically involved in carcinomas from this tract. One of the major subjects deals with the detection of HPV DNA. A reliable and sensitive method to detect most if not all members of the heterogeneous HPV group is a prerequisite to make definitive statements about associations.

Due to the relative low sensitivity of conventional HPV detection methods the first question, that was raised is:

Chapter II: Can a PCR method be developed that allows the detection of a broad spectrum of either mucosotropic or cutaneous HPV genotypes?

Two general primer pairs, selected from the conserved L1 region, which are highly homologous to sequences of either mucosotropic or cutaneous HPVs, were tested in the PCR for the detection of a broad spectrum of HPV genotypes.

Despite promising perspectives of the general primer-mediated PCR method described in chapter II, still some HPV types, showing an ambivalent tissue tropism, fail to be detected. This led to the following question:

Chapter III: Can a PCR method be developed utilizing a single primer pair to detect both mucosotropic and cutaneous HPV genotypes?

A single general primer pair that was designed on basis of regions of amino acid conservation was tested in the PCR for its capability to detect both mucosotropic and cutaneous HPV genotypes.

Although the general primer-mediated PCR assays proved to have great value in detecting different HPVs, it was unknown to which extent the method could distinguish between different HPV genotypes and exclude the possibility of detecting sequences from cellular origin. This problem was considered in the next question.

Chapter IV: Can the general primer-mediated PCR method be used both for HPV DNA identification and HPV genotype differentiation?

This chapter describes the sequence analysis and comparison of general primer-mediated PCR products obtained from 22 HPV genotypes in order to determine an HPV consensus sequence and a polymorphic region which together can be used both for confirmation of HPV specificity and for HPV genotype differentiation.

Immunodeficient patients are known to develop HPV containing lesions more frequently. Hairy leukoplakias, which predominantly occur in immunocompromised, HIV-infected patients, were suggested to be associated with HPV. Therefore, hairy leukoplakias could

provide a source of putatively unknown HPV types that specifically infect sites within the aerodigestive tract. Consequently, the following question was raised:

Chapter V: Can HPV DNA be detected in oral hairy leukoplakia?

In this study both the general primer-mediated HPV PCR method and an EBV PCR method were applied to find out whether both viruses are associated with oral hairy leukoplakia.

Preliminary screening of head and neck carcinomas revealed a clear association between HPV DNA and tonsillar carcinomas, which resulted in the next question:

Chapter VI: Is HPV associated with tonsillar carcinomas in a way that support a possible etiological involvement?

Both the presence of HPV DNA and HPV early region transcription was studied in tonsillar carcinomas to get more information about the viral involvement in these carcinomas.

In contrast to cervical carcinomas, HPV 33 appeared to be a major type present in tonsillar carcinomas. The question described in chapter VII deals with the manner in which HPV 33 generates its E7 mRNAs.

Chapter VII: Does HPV 33 generate its E7 mRNA in a manner similar to the high risk types HPV 16 and HPV 18?

HPV 33 early region transcripts in a tonsillar carcinoma were mapped using RNA PCR to discover properties that could be important for the oncogenic character of this virus.

In the cervical cancer model, viral DNA integration is supposed to be essential by triggering an enhanced E6/E7 expression which is required for the maintenance of the malignant phenotype. To find out the role of HPV DNA integration in tonsillar carcinomas the next question was raised:

Chapter VIII: Is expression of E6/E7 mRNA in tonsillar carcinomas always correlated with viral DNA integration?

In this study the viral physical state in HPV 16 and HPV 33 containing tonsillar carcinomas was correlated with the qualitative aspect of E6/E7 mRNA expression.

Results obtained from previous chapters have revealed clear associations between transcriptionally active HPV and the most frequently occurring carcinomas from the oropharynx, namely tonsillar carcinomas. This led to the following question to be answered in the General discussion:

Chapter IX: To which extent can HPV be an etiological factor in squamous cell carcinomas from the upper aerodigestive tract?

In this chapter the technical aspects of HPV detection by PCR and perspectives of detecting novel HPV types specific for the upper aerodigestive tract will be discussed. The clinical aspects of HPV detection in lesions from this tract are also considered.

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CHAPTER II

The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes

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Abstract

A novel polymerase chain reaction (PCR) method was developed that permits the detection of 11 different human papillomavirus (HPV) genotypes using two general primer sets. By computer-assisted sequence analysis, two pairs of general primers were selected from the conserved L1 open reading frame and tested in the PCR on a set of cloned HPV genotypes. Experimental analysis showed that up to three mismatches between primers and target DNA did not influence the efficiency of the assay. The use of these primers in the PCR enabled the detection of HPV genotypes HPV-1a, -6, -8, -11, -13, -16, -18, -30, -31, -32 and -33, and was also succesfully applied to well characterized cervical carcinoma cell lines and clinical samples. For the HPV types tested sub-picogram amounts of cloned DNA could be detected after general primer-mediated PCR and subsequent hybridization. The specificity of the amplification products was confirmed by blot hybridization procedures and *Rsa*I restriction enzyme digestion. The results indicate that this PCR method can be a powerful tool for identifying novel HPV genotypes in dysplasias and squamous cell carcinomas suspected of having an HPV aetiology.

Introduction

During the last few years increasing efforts have been made to determine the role of human papillomavirus (HPV) in malignant transformation of squamous cell epithelium. In particular the finding that almost all carcinomas of the uterine cervix harbour specific HPV genotypes (Dürst *et al.*, 1983; van den Brule *et al.*, 1989), supports a causal relationship between certain HPV types and squamous cell carcinoma of the genital tract and has contributed to a growing field of interest in HPV research. Moreover, a role for HPV in the development of carcinomas of the respiratory tract has been supported as well (Löning *et al.*, 1985; de Villiers at al., 1985; Kahn *et al.*, 1986; Byrne *et al.*, 1987). Because several of these carcinomas were found to contain HPV related DNA (Abramson *et al.*, 1985; Brandsma *et al.*, 1986; Stremlau *et al.*, 1985; Brandsma *et al.*, 1989), it has been speculated that presently unidentified HPV types could be specifically associated with carcinomas of the respiratory tract.

In this study the capability of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) for the detection of a broad spectrum of HPV genotypes was investigated, in order to obtain a powerful tool for the demonstration of unknown HPV types. Based on short regions of homology conserved amongst HPV genotypes whose complete sequences have been determined, two pairs of general primers were designed that match the same sequences in the L1 open reading frame (ORF). A model system of several cloned HPV types (pHPVs) was employed to test these primer pairs in the PCR. With this system experimental conditions could be determined to amplify target DNA of the examined HPV types 1a, 6, 8, 11, 13, 16, 18, 30, 31, 32, and 33. Furthermore the use of one of the primer pairs in the PCR allowed the detection of different HPV genotypes in cervical carcinoma cell lines and clinical specimens that are known to harbour HPV DNA. The results indicate that the primer matched sequences are highly conserved among a broad spectrum of HPV types, suggesting that analogous PCR products can be generated from other, unknown HPV genotypes. This general primer mediated PCR method can therefore be a powerful tool for the detection of presently unidentified HPV types in lesions of the respiratory tract.

Methods

Cell cultures, tissue specimens and HPV clones

The human cervical cancer cell lines CaSki, C4-1 and Siha were obtained from the American Type Culture Collection. HeLa cell line 229 was obtained from Dr. K.H. Thian (Rotterdam, the Netherlands). CaSki/Siha and C4-1/HeLa are known to contain HPV types 16 and 18, respectively (Boshart *et al.*, 1984; Schwarz *et al.*, 1985; Yee *et al.*, 1985). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum. After growth to near confluence cells were harvested by trypsinization, washed with phosphate-buffered saline, spun down and subsequently suspended in 10 mM Tris HCl, 1mM EDTA pH7.5.

Tissue specimens of a laryngeal squamous cell hyperplasia and juvenile papilloma were snap-frozen and stored in liquid nitrogen until use. Further processing and DNA extraction was carried out according to standard procedures (Walboomers *et al.*, 1988). Cervical scrapes were processed as described by van den Brule *et al.* (1989). The presence of HPV DNA in these samples was determined by Southern blot hybridization and PCR with HPV types 6-, 11-, 16-, 18-, and 33-specific primers, as previously described (Melchers *et al.*, 1989; van den Brule *et al.*, 1989).

DNAs of several different HPV types, cloned in pBR322 or pUC19 (pHPVs) were used as targets in a model system for general primer directed amplification. The cloned HPV types 6b, 11, 16, 18, and 30 were kindly provided by Drs. H. zur Hausen and L. Gissmann (Heidelberg, FRG), HPV type 31 by Dr. A. Lorincz (Gaitersburg, Md.), HPV type 33 by Dr. G. Orth (Paris, France) and HPV types 1a, 2a, 8, 13, and 32 by Dr. E.-M. de Villiers (Heidelberg, FRG).

Polymerase chain reaction

A slight modification of the PCR method described by Saiki *et al.* (1988) was used. The PCR was performed on 1 ng DNA of cloned pHPVs or 100 to 500 ng of cellular DNA. In addition dilutions of several pHPV DNAs mixed with 100 ng human placental DNA or diluted Siha DNA were also subjected to PCR, in order to assess the sensitivity of the assay. The reaction mixture of 50 μl also contained 50 mM KCl, 10 mM Tris HCl pH8.3, 0.01 % (w/v) gelatin, 200 μM of each dNTP, $MgCl_2$ at between 1.5 and 10 mM, 1 unit of a thermostable DNA polymerase (*Thermus aquaticus*; Cetus) and 50 pmol of each primer of either the GP5/6 or GP11/12 primer combination (Fig. 1). The mixture was overlaid with several drops of paraffin oil and incubated for 5 min at 94°C for DNA denaturation, followed by 40 cycles of amplification using a PCR processor (Bio-med, FRG). Each cycle included a denaturation step to 94°C for 1 minute, an annealing step to 40°C for 2 minutes and a chain elongation step to 72°C for 1.5 minute. To ensure a complete extension of the amplified DNA, each individual elongation step was increased by 1 s and the final elongation step was prolonged for another 4 min. To avoid contamination by cloned pHPV DNA or PCR products, different steps such as sample preparation and amplification reaction were performed in strictly separated rooms.

Samples containing distilled water were included as negative controls, none of which showed a successful amplification. A total of 10 μl of the PCR mixtures was finally analyzed by agarose gel electrophoresis.

Southern blot analysis of PCR products

Electrophoretically separated DNA fragments were transferred onto nylon membranes (GeneScreen Plus; Du Pont) by diffusion blotting in 0.5 N NaOH, 0.6 M NaCl. GP5/6- or GP11/12-directed, HPV-specific PCR products were used as probes after ^{32}P-labelling by the random priming method. Hybridization was performed at 65°C (Tm-23°C, for high stringency analysis) or 55°C (Tm-33°C, for low stringency analysis) in 0.5 M sodium phosphate pH 7.4, 7% SDS, 1mM EDTA for 16 h. Subsequent washings were carried out at high (Tm) or low (Tm-33°C) stringency in 0.1xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.5% SDS at 65°C or 3xSSC, 0.5% SDS at 56°C, respectively. Autoradiography was performed for 1 day at -70°C with Kodak Royal X-omat film and intensifying screens.

Restriction enzyme analysis

Analysis of PCR products by restriction endonuclease digestion was performed directly on a 10 μl sample of the reaction mixture, without prior purification and resuspendation of the DNA in the recommended restriction buffer (Carman and Kidd, 1989). Two units of *Rsa*I (Boehringer) were added and the digestion was allowed to proceed for 2 h at 37°C. Digestion products were analyzed on composite gels consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% Type 1 agarose (Sigma) to obtain adequate resolution of low M_r DNA fragments.

Dot blot analysis

For dot blotting 1 μg pHPV DNA and pBR322 vector DNA was dotted onto nylon membranes (GeneScreenPlus; Du Pont). HPV specific amplification products of 140 to 150 base pairs (bp) were used as probes. The fragments were electrophoretically separated in low melting point agarose (Bio-Rad), excised from the gel and directly labelled by random primed labelling. Hybridization was performed under high stringency, as described above. Subsequent washings were carried out at high stringency (Tm) down to 0.1xSSC, 0.5% SDS at 65°C. Autoradiography was performed for 1 day at -70°C with intensifying screens.

Computer analysis and primer synthesis

All matrix, homology and restriction site analyses were executed with the Microgenie sequence analysis program (GenBank (Release No. 54), Beckman) developed by Queen and Korn (1984). From the sequence data two sets of general primer sequences were selected that match the same regions within the L1 ORF of all sequenced HPV types (Fig. 1). The primers were synthesized on a DNA synthesizer (Applied Biosystems 380 A) by the methoxy-phosphoramidite method.

Results

Selection of general HPV primer sequences within the conserved L1 open reading frame

Matrix comparison of the sequenced HPV types 1a, 6b, 16 and 18 revealed that the most conserved regions are localized within the E1 and L1 ORFs (Giri and Danos, 1986). As a consequence general oligonucleotide sequences that could be used for the detection of multiple HPV genotypes (general primers), were sought in those ORFs. Although the overall homology between different HPV isolates allows cross-hybridization between the individual HPV genotypes under conditions of low stringency (Tm-40°C), striking heterogeneity is observed on the nucleotide level using the sequence analysis program. This excluded the possibility of selecting linked 20 bp regions that are completely similar in all sequenced HPV types. However, two 20 bp sequences within the L1 ORF were found that are highly conserved but divergent amongst the examined types. Based on these homologies, two pairs of oligomers with a length of 20 nucleotides were designed (names GP5/6 and GP11/12) that are mainly homologous to the corresponding region of the HPV types 6b, 11, 16, 18, 31, and 33 (GP5/6; Fig. 1a) and HPV-1a, -5, and -8 (GP11/12; Fig. 1b), respectively. The oligomer-resembling sequences span a region of approximately 140 to 150 bp in all examined HPV genomes (Table 1).

Application of general primers in the PCR on pHPV DNAs

With both primer pairs the PCR was performed under moderate stringency conditions (3.5 mM Mg++) on 1 ng DNA from several different pHPVs (Fig. 2). In addition to the sequenced HPV types 6b, 11, 16, 18, 31 and 33, the GP5/6 primer set yielded 140 to 150 bp PCR products with the presently unsequenced types 13, 30, and 32. The GP5 and GP6 oligomers did not direct amplification of HPV-1a, -2a, and -8 DNA. In contrast, with primer pair GP11/12 successful amplification resulting in a PCR fragment that was clearly detectable on the gel under UV after ethidium bromide staining, was limited to HPV-8 DNA. Although amplified DNA specific for HPV-1a appeared after hybridization (not shown), lower stringency conditions of annealing were required to yield gel detectable quantities of a GP11/12 directed PCR product of HPV 1a (Fig. 3, lane HPV1a/10 mM Mg++). However, even under lower stringency conditions (10 mM Mg++) none of the primer pairs was found to direct amplification of a 140 to 150 bp DNA fragment specific for HPV-2a (Fig. 2; not shown). Neither GP5/6 nor GP11/12 gave rise to amplification of pBR322 DNA (Fig. 2), indicating that the PCR products were HPV specific and were not the result of cross-reaction with vector sequences. In some instances, co-amplified DNA of lower (40 to 45 bp) and higher M_r was observed (Fig. 2, e.g. lanes HPV-18, -HPV 30 and -HPV 32). The low M_r DNA fragments could reflect the ligation and amplification of the primers, whereas the fragments of higher M_r were presumably the result of primer annealing to additional target sequences within the HPV genome.

To investigate the number of mismatches to be acceptable between primer sequences and target DNA the PCR was performed under different stringency conditions by varying the Mg++ concentration. For that purpose HPV genotypes that

(a) Anti-sense strand 3'-AAACAATGACACCATCTATG-5' GP6 3'-ACTAAATGTCAAATAAAAAG-5' Mismatches
Sense strand GP5 5'-TTTGTTACTGTGGTAGATAC-3' 5'-TGATTTACAGTTTATTTTTC-3' GP5 GP6

HPV1a	...A....A..T.G.....A	HPV1a	....C.TTCT.....AG...	5	7
HPV5	...A.C..A.....T..C.A	HPV5	...AA..TCT..A...C.A.	6	8
HPV6b	.....	HPV6b	.....A.....	0	1
HPV8	.....C.....C.A	HPV8	...AA.TTCCC.C..A..A.	3	10
HPV11	.....	HPV11	.....	0	0
HPV16	.....T..T.....	HPV16	.....	2	0
HPV18	.....	HPV18	.....G.....	0	1
HPV31	.....	HPV31	.....A.....A....	0	2
HPV33	.....	HPV33	....C.....G.....	0	2

(b) Anti-sense strand 3'-AAATAGTGTCACCATCTGTT-5' GP12 3'-ACTTTAAAGAAAATATAATG-5' Mismatches
Sense strand GP11 5'-TTTATCACAGTGGTAGACAA-3' 5'-TGAAATTTCTTTTATATTAC-3' GP11 GP12

HPV1a	.....T.....T.G...T..	HPV1a	...TC.....G.T.	4	4
HPV5	.....T.....	HPV5	.....A.....A..TC...	1	4
HPV6b	...G.T..T.....T.C	HPV6b	...TT.ACAA.....T..T.	5	8
HPV8	...G....T.....	HPV8	.....CC.C.....	2	3
HPV11	...G.T..T.....T.C	HPV11	...TT.ACAG.....T..T.	5	8
HPV16	...G.T..T..T..T..T.C	HPV16	...TT.ACAG.....T..T.	7	8
HPV18	...G.T..T.....T.C	HPV18	...TT.GCAG.....T..T.	5	8
HPV31	...G.T..T.....T.C	HPV31	...TT.ACAA.....T.	5	7
HPV33	...G.T..T.....T.C	HPV33	...TC.ACAG...G.T..T.	5	9

Figure 1. Alignment of sequences from the primers GP5 (A) ,GP11 (B) and the complementary strands of GP6 (A) and GP12 (B) with corresponding sequences of the sense strand within the L1 ORF of the nine sequenced HPV types. Primer sequences are underlined. Dots represent identical bases and mismatched bases are indicated. The number of mismatches is given in the right-hand tables. The primers GP11 and GP12 match the same positions as GP5 and GP6, respectively. The nucleotide position of the first nucleotides of the GP5/GP6 matched sequences are as follows: HPV-1a: 6420/6539; HPV-5: 6940/7074; HPV-6b: 6764/6883; HPV-8: 6871/7005; HPV-11: 6749/6868; HPV-16: 6624/6746; HPV-18: 6600/6725; HPV-31: 6542/6664; HPV-33: 6581/6700. Except for HPV-1a, nucleotide locations are numbered according to the published sequence data (HPV-5: Zachow *et al.*, 1987; HPV-6b: Schwarz *et al.*, 1983; HPV-8: Fuchs *et al.*, 1986; HPV-11: Dartmann *et al.*, 1986; HPV-16: Seedorf *et al.*, 1985; HPV-18: Cole and Danos, 1987; HPV-31: Goldsborough *et al.*, 1989; HPV-33: Cole and Streeck, 1986). The numbering system of the reported HPV-1a nucleotide sequence (Danos *et al.*, 1982) was adapted according to the modified numbering of Danos *et al.* (1983).

show two (HPV-16 and -33), three (HPV 8), four (HPV 1a) or an unknown number (HPV 30) of mismatches with one or both primers were subjected to PCR in the presence of 1.5, 2.5, 3.5, and 10 mM $MgCl_2$ (Fig. 3). The figure shows that two and three mismatches between primer and target DNA, localized more than four bases from the 3' end, are accepted. Under conditions of both high and low stringency GP11/12 and GP5/6 reacted with HPV-8 and HPV- 16/ -33, respectively. With HPV-16 additional faint signals representing a slightly lower M_r can be observed that decrease at higher stringency conditions. It still has to be elucidated whether these fragments result from uncompletely extended or ssDNA from the specific region, or from the co-amplification of additional target sequences within the HPV-16 genome. Furthermore, it was revealed that lower stringency conditions are required to accept four mismatches in the case of HPV-1a and GP11/12. A 139 bp specific DNA fragment became visible on gel only when the Mg++ concentration was raised to 10 mM. Weak signals, slightly increasing in intensity at lower stringency conditions, were also obtained with HPV-30

and the GP5/6 primer pair. Additionally, it appeared that low stringency conditions (10 mM Mg++) of the annealing step resulted in an increase of co-amplified DNA fragments.

Table 1. Sizes of DNA fragments of the sequenced HPV types that are spanned by the GP primer sets

	Total length (bp)	Length of *Rsa*I restriction fragments (bp)
HPV-1a	139	66 73
HPV-5	154	29 125
HPV-6b	139	30 42 67
HPV-8	154	154
HPV-11	139	30 109
HPV-16	142	30 42 70
HPV-18	145	30 38 77
HPV-31	142	30 112
HPV-33	139	30 39 70

The sensitivity of GP-directed PCR was determined by the examination of different concentrations of pHPV and Siha (HPV-16) DNA diluted in human placental DNA. For most pHPVs that show up to three mismatches with one or both primers a detection level, as determined after hybridization of the PCR products with a GP-amplified homologous DNA probe, of 0.1 to 1 fg DNA was found (Fig. 4, HPV-8 and -33). This corresponds to approximately seven to 70 viral genomes. In addition, as little as 10 pg of Siha DNA could be detected after GP5/6 directed amplification (Fig. 4, Siha). Assuming that Siha cells contain 1-10 copies of HPV-16 DNA per genome and that a human diploid cell contains about 5 pg DNA, this implies that 2 to 20 copies of HPV-16 could be detected. A detection level of between 10 fg and 1 pg pHPV DNA,

corresponding to 700 to 70000 viral copies was found with HPV-1a and HPV-30 (Fig. 4, HPV 30).

Analysis of PCR products

The type specificity of the PCR was determined by dot blot analysis. For this purpose 140 to 150 bp PCR products, generated with the GP5/6 or GP11/12 primer set were isolated, labelled and hybridized to a panel of dotted pHPV DNAs (Fig. 5). As can be seen clearly, the PCR products were type specific and did not originate from contaminations with other HPV types. Further analysis by *Rsa*I restriction enzyme digestion confirmed the specificity of the PCR. In Table 1 the sizes of *Rsa*I restriction fragments are given for the predicted GP directed 140 to 150 bp PCR products of the sequenced HPV types. Fig. 6 shows the result of *Rsa*I digestion of the PCR products. In some instances it was obvious that the non-specific 40 to 45 bp PCR products, which can be seen in Fig. 2, interfere with the restriction fragments derived from the 140 to 150 bp products (Fig. 6, lanes HPV-18, HPV-30 and HPV-32). However, despite this interference the calculated sizes of specific restriction fragments from the sequenced HPV types are in agreement with the expected sizes, as estimated from the sequence data. From the PCR products of the unsequenced HPVs, restriction fragments were obtained of approximately 73, 40 and 30 bp (HPV 13), 140 bp (HPV 30) and 110, 30 bp (HPV-32). This indicated that the HPV-13 product contains two *Rsa*I sites, the product of HPV-32 contains one *Rsa*I site, whereas an *Rsa*I site is absent in the HPV-30 fragment.

General primers in the PCR on cellular DNA

Having established the optimal conditions of PCR with general primers, the method was performed with the GP5/6 primer pair on DNAs of cervical cancer cell lines, cervical scrapes and laryngeal lesions that had been well characterized by Southern blot analysis and PCR with HPV type specific primers (van den Brule *et al.*, 1989). In Fig. 7a the PCR products are shown after agarose gel electrophoresis. The presence of additional bands was obvious in most cases. Furthermore, a clear distinction in the detection level between high and low copy numbers of a certain HPV type was demonstrated. From DNA of the CaSki cell line, which contain more than 500 copies of HPV-16 per cell, an HPV-specific PCR product was generated that appeared as a strong band, clearly visible after electrophoresis. In contrast, the HPV product of the low HPV copy number cell line Siha (one to 10 copies of HPV-16 per cell) appeared as a weak signal. In addition, more additional bands were obtained using Siha DNA as target, with a gel band pattern that was hardly distinguishable from that of human placental DNA (not shown) or the cervical scrape (s1) that was negative for a broad spectrum of HPVs. Identical results were obtained with the HPV-18-containing HeLa cell line (10 to 50 copies of HPV-18 per cell) and C4-1 (one to five copies of HPV-18 per cell), and also with cervical scrape s2, laryngeal papilloma p1 and hyperplasia h1, of which the latter two contain low quantities of HPV-6 DNA. HPV-specific PCR products were also generated from DNA of cervical scrapes containing HPV-33 (s3), HPV-11 (s4), HPV-16 (s5) and HPV-18 (s6).

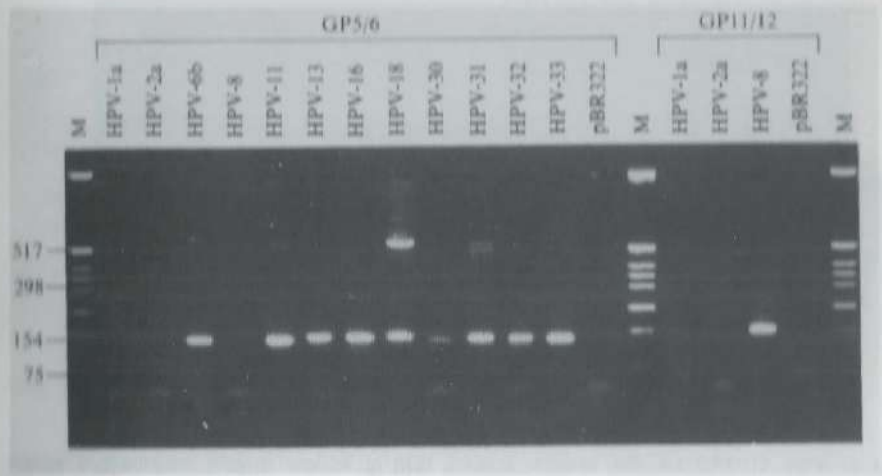

Figure 2. PCR on 1 ng pHPV DNA of several types by using the GP5/6 or GP11/12 primer pair under moderate stringency conditions in the presence of 3.5 mM Mg++. PCR products are shown after electrophoresis, ethidium bromide staining and u.v. irradiation. M, pBR322 fragments (bp) digested with *Hin*fI.

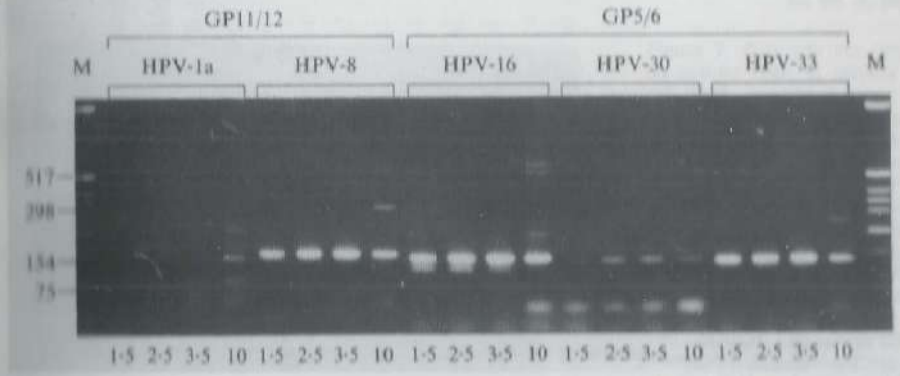

Figure 3. PCR performed under different stringency conditions with DNA of HPV-1a and -8 using the GP11/12 primer pair and DNA of HPV-16, -30, and -33 using the GP5/6 primer pair. Different $MgCl_2$ concentrations used are indicated at the bottom (in mM). M, pBR322 fragments (bp) digested with *Hin*fI.

After blotting and low stringent hybridization with a GP5/6-amplified DNA probe specific for HPV-16, all HPV-specific 140 to 150 bp fragments could be detected

without interference of the additional bands (Fig. 7b). Several samples yielded additional hybridizing DNA fragments of higher M_r. As the HPV-negative sample (s1) did not yield any hybridizing DNA, it is unlikely that these signals appeared from a cross-hybridization to co-amplified cellular DNA.

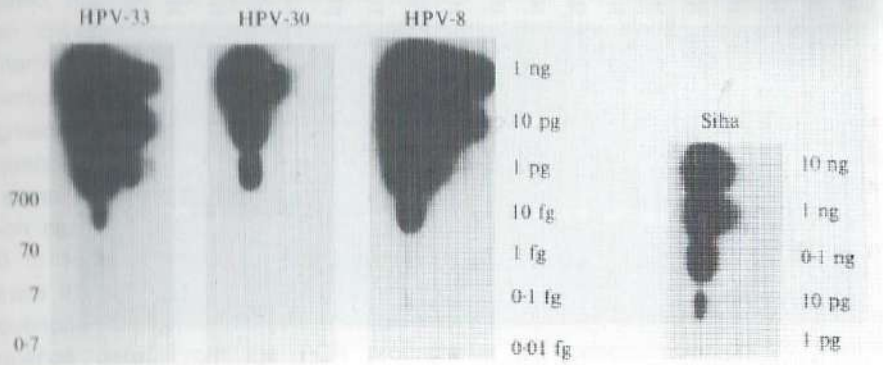

Figure 4. Sensitivity of GP amplification determined with different concentrations of HPV-8, -30 and -33 (1 ng to 0.01 fg) and Siha (10 ng to 1 pg) DNA diluted in 100 ng human placental DNA. PCR was performed with the GP11/12 (HPV-8) or the GP5/6 (HPV-30, -33 and Siha) primer pair. Moderate stringency conditions of annealing (3.5 mM Mg++) were used. Hybridization of Southern blots was performed with GP PCR-amplified probes specific for a certain HPV type (HPV 16 for Siha DNA) under conditions of high stringency. The amounts of target DNA are indicated. In some instances corresponding viral copy numbers are also indicated on the left.

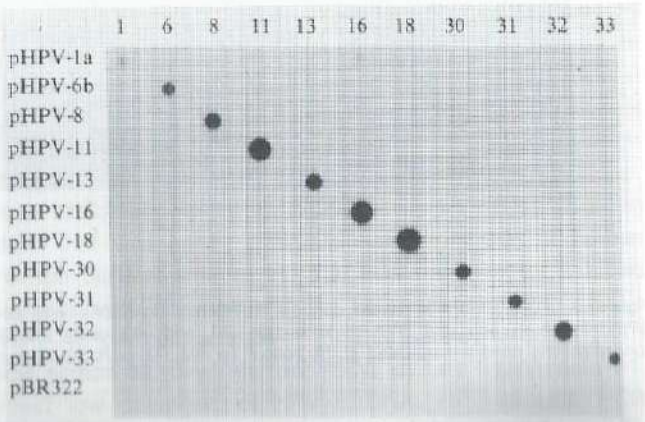

Figure 5. Dot blot analysis of 140 to 150 bp HPV-specific PCR products obtained with the GP11/12 (HPV-1a, and -8) or the GP5/6 (HPV-6b, -11, -13, -16, -18, -30, -31, -32 and -33) primer pair. Hybridization of the specific PCR products (indicated at the top) to dotted pHPV or pBR322 DNA (indicated on the left) was under conditions of high stringency.

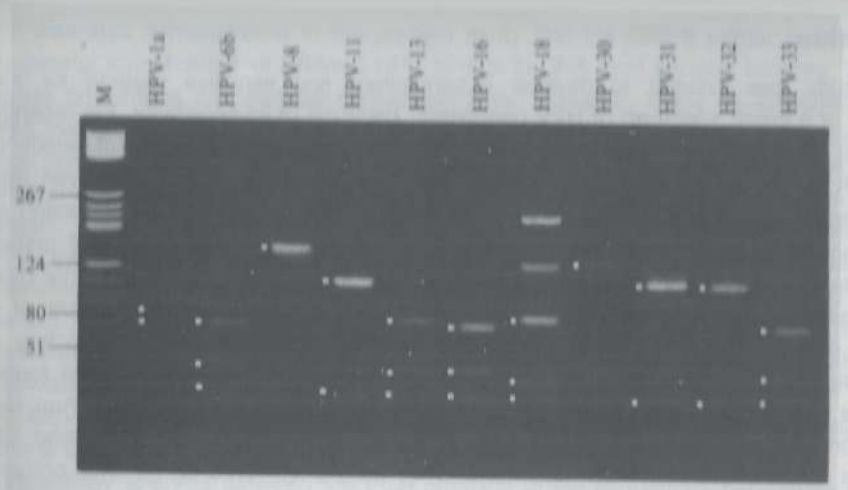

Figure 6. Restriction enzyme analysis of PCR products obtained with the GP11/12 (HPV-1a and -8) or GP5/6 (HPV-6b, -11, -13, -16, -18, -30, -31, -32 and -33) primer pair. PCR products were digested with *Rsa*I and electrophoresed on a composite agarose gel. All DNAs except HPV-1a were amplified under moderate stringency conditions (3.5 mM Mg++). HPV-1a DNA was amplified in the presence of 10mM Mg++. Filled markers indicate the specific restriction fragments derived from the 140-150 bp products, determined after exclusion of non- specific PCR products that can be seen in Fig. 2. In all cases the sums of lengths of the indicated restriction fragments are consistent with the total length of the undigested PCR product. M, pBR322 fragments (bp) digested with *Hae*III.

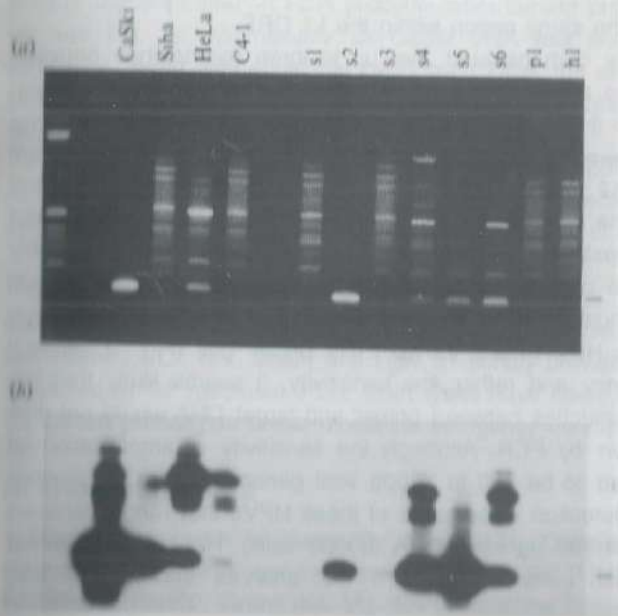

Figure 7. PCR with the GP5/6 pair on cellular DNA of cervical cancer cell lines, cervical scrapes (s1 to s6), a laryngeal squamous cell papilloma (p1) and hyperplasia (h1) under moderate stringency conditions (3.5 mM Mg++). Products are shown after electrophoresis, ethidium bromide and u.v. irradiation (a) or after blotting and hybridization under low stringency conditions with an amplified product specific for HPV-16 (b). The positions of the 140 to 150 bp fragments are marked on the right. The presence of HPV DNA was confirmed by PCR with type specific primers, s1, no HPV; s2, HPV 6; s3, HPV 33; s4, HPV 11; s5, HPV 16; s6, HPV 18; p1, HPV 6; h1, HPV 6; M, pBR322 fragments (bp) digested with *Hin*fI (see Fig. 2).

Discussion

This study was initiated to develop a convenient and sensitive method for the detection of a broad spectrum of HPV genotypes. Because of its sensitivity, the recently developed PCR (Saiki *et al.*, 1988) is rapidly becoming the preferred means of detecting HPV DNA in cervical scrapes and biopsy specimens (Melchers *et al.*, 1989; van den Brule *et al.*, 1989). Consequently, efforts were made to use the PCR for the detection of various unrelated HPV genotypes at one time. This required the selection of primer sequences that are conserved among a broad spectrum of HPV types. As only nine HPV types have been completely sequenced so far, the extent of conservation of a selected primer sequence could only be determined experimentally by subjecting a large group of HPV genotypes to PCR. Two general primer sequences were found within the L1 ORF that match perfectly to HPV 11 and did not differ for more than two bases from the corresponding sequences of the HPV types 6b, 16, 18, 31 and 33. Therefore these oligonucleotides (GP5 and GP6) were considered as general primers for genital HPVs. However, for the skin associated HPV types 1a, 5 and 8, both primers appeared to be more heterogeneous (Fig. 1a), making it unlikely that these HPV genotypes would serve as targets for GP5/6 directed amplification. By changing some bases within the GP5 and GP6 sequences, an additional primer pair was designed (GP11/12) that match, with a maximum of four mispaired bases, to a higher degree to the skin-associated types (Fig. 1b).

In addition to all known HPV DNA sequences, some unsequenced types were found to serve as templates to yield HPV-specific PCR products (Fig. 2), thereby confirming the conserved nature of the selected primer sequences among a broad group of HPV types. This finding suggests that additional HPV types could serve as template to yield analogous PCR products from the same region within the L1 ORF.

Furthermore it was empirically demonstrated that up to three mismatches between primers and target DNA had no effect on the efficiency and sensitivity of the assay. HPV genotypes that posess two (HPV-16 and -33) or three (HPV-8) bases mispairing with one of the primers all showed a sensitive amplification directed by either GP5/6 (HPV-16 and -33) or GP11/12 (HPV 8) pair (Fig. 4). Therefore, both the lower sensitivity of amplification and the faint amplification signals of HPV-30 were supposed to reflect the occurrence of at least three mismatches with one or both primers, possibly in combination with unfavourable positions of the incorrectly matched bases (Newton *et al.*, 1989). Lower stringency conditions were required to ensure the acceptance of four mismatches, as is the case with HPV 1a and the GP11/12 primer pair (Fig. 1). Since it resulted in a decreased efficiency and rather low sensitivity, it seems likely that the presence of more than four mismatches between primer and target DNA would not give rise to a successful amplification by PCR. Although the sensitivity of amplification of HPV-1a and -30 DNA appeared to be 700 to 70000 viral genomes, the GP-directed PCR method still enabled the detection of amounts of these HPVs that range between 0.035 and 3.5 copies per cell in 100 ng input DNA (20000 cells). Hence, this method showed a better sensitivity than genomic Southern blot analysis and is also less laborious.

It was also demonstrated in the present study that the GP5/6 primer pair allowed a successful amplification of different HPV targets in DNA from cervical scrapes, laryngeal biopsy specimens and cervical cancer cell lines. However, many co-amplified cellular DNA fragments were predominantly observed in PCR products of HPV negative or low copy number DNAs. This could be the result of the lack of competition for the primers, since limited or no specific targets were available to withdraw primers from non-specific targets. These co-amplified products made it necessary to confirm the presence of HPV by hybridization and would probably cause problems in the isolation and characterization of an HPV-specific amplification product from a low copy number sample. Therefore, for general primer-mediated PCR, low stringency annealing conditions (3.5 to 10 mM Mg++ at 40°C) are recommended for initial PCRs followed by stepwise increasing of the stringency (by raising the annealing temperature or lowering the Mg++ concentration) to reduce background untill an optimal signal to background ratio is achieved.

Homology analysis between the PCR products of the sequenced HPV types revealed a striking sequence homology ranging from 55.4% (between HPV 1a and HPV 11 products) to 87.8% homology (between HPV-6b and HPV-11 products). This indicates that low stringency conditions of Southern blot analysis should enable the detection of a broad spectrum of HPV specific amplification products, as could be shown by using the HPV 16 specific PCR product as a probe to detect different amplified HPV sequences from cellular DNA (Fig. 7b). Additional hybridizing fragments of higher M_r appeared in all samples containing HPV-18, the CaSki cell line and a cervical scrape containing HPV-11. In part these signals could reflect a cross-hybridization to co-amplified HPV DNA, generated from other regions within the HPV genome. Such co-amplified DNA has already demonstrated in PCR products from certain pHPV DNAs (in particular pHPV-18; see Fig. 2). Alternatively as CaSki DNA was shown to be the only HPV-16-containing target with additional hybridizing DNA, the aberrant fragments could also be generated as a consequence of integration events or rearrangements of the HPV genome.

From the results it can be concluded that the application of the selected primer sequences in the PCR opens new ways of detecting novel HPV genotypes in dysplasias and squamous cell carcinomas suspected to be due to HPV.

Note added in proof: Since this manuscript was submitted for publication two additional HPV types, namely HPV 45 (kindly provided by Dr. E.-M. deVilliers after permission of Dr. K.V. Shah) and HPV 51 (kindly provided by Dr. G. Nuovo) have been subjected to GP mediated PCR. Both types have been found to react successfully with the GP5/6 primer pair under moderate stringency conditions of primer annealing.

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CHAPTER III

Degenerate primers based on highly conserved regions of amino acid sequence in papillomaviruses can be used in a generalized polymerase chain reaction to detect productive human papillomavirus infections

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Abstract

Conserved amino acid sequences within the L1 open reading frame of the human papillomavirus (HPV) genome were used as a basis to design two degenerate primers (GP17 and GP18) and one general probe (GPR22) which direct polymerase chain reaction (PCR) amplification and subsequent detection of a 620 to 660 bp DNA fragment. The conserved nature of the primers and probe was tested experimentally on a panel of 24 cloned HPV DNAs isolated from cutaneous and mucosal lesions, including HPV-2a and -57, which are known to be associated with lesions at both anatomical sites. The sensitivity of this PCR test was at the level of genomic Southern blot analysis, indicating that HPV infections producing high copy numbers can be detected. Positive results were obtained with DNA extracted from clinical samples of genital and cutaneous origin.

During the last decade it has become clear that the human papillomavirus (HPV) group is very heterogeneous at the DNA level. Based on liquid hybridization data, at least 60 different HPV genotypes have been identified and isolated from different lesions of epithelial origin (de Villiers, 1989). The association of certain HPV types with malignancies of the genital tract (Dürst *et al.*, 1983; Boshart *et al.*, 1984), skin (Orth, 1987) and aero-digestive tract (Kahn *et al.*, 1986; Lindeberg *et al.*, 1988) has been documented. Moreover, the involvement of unknown HPV types in squamous cell lesions has been postulated (de Villiers *et al.*, 1985; Kahn *et al.*, 1986).

The present mode of isolating a new HPV type is based on the initial detection of HPV DNA by low stringency Southern blot analysis, probing with an isolated HPV genotype (Gissmann *et al.*, 1982; Kahn *et al.*, 1986). This method, however, is hampered when putative distantly related HPV types lack sufficient homology with the genotype used as a probe. Polymerase chain reaction (PCR) amplification of part of the HPV genome would overcome this problem by generating a type specific DNA fragment that could be used as a probe for high stringency analysis of genomic Southern blots and genomic libraries. Recently, a generalized PCR method for the detection of a broad spectrum of HPV genotypes using general or consensus primers has been introduced (Snijders *et al.*, 1990; Manos *et al.*, 1989; Gregoire *et al.*, 1989). The sequenced HPV genotypes do not show nucleotide sequence identity over a length of more than 12 bases (Gregoire *et al.*, 1990), so the primers or the method used have had to be adapted to ensure the detection of at least a subset of the sequenced genotypes and related HPV DNAs. The difference at the nucleotide level between the sequenced HPV types associated with cutaneous and mucosal lesions has forced us to design two general primer pairs, one specific for "cutaneous" HPV types and one for "mucosotropic" HPV types (Snijders *et al.*, 1990). However, the finding that some types, including the related types HPV-2a and HPV-57, show both a cutaneous and mucosal tropism indicates that there are exceptions to the rule that cutaneous and mucosotropic HPVs form distinct groups (de Villiers *et al.*, 1989; de Villiers, 1989). The existence of additional HPV types showing such ambivalent tropism can not be excluded and "cutaneous-like" HPV types present in mucosal lesions could be missed by general primers selected for mucosotropic HPV types. In this study, a new, generalized HPV PCR assay was developed which makes use of highly degenerate primers and a probe, containing more than one base at a certain position, that were

designed to cover all possible base combinations encoding a certain amino acid (Mack and Sninsky, 1988; Gould *et al.*, 1989) conserved within the L1 open reading frame (ORF) of HPV types, associated with both cutaneous and mucosal lesions.

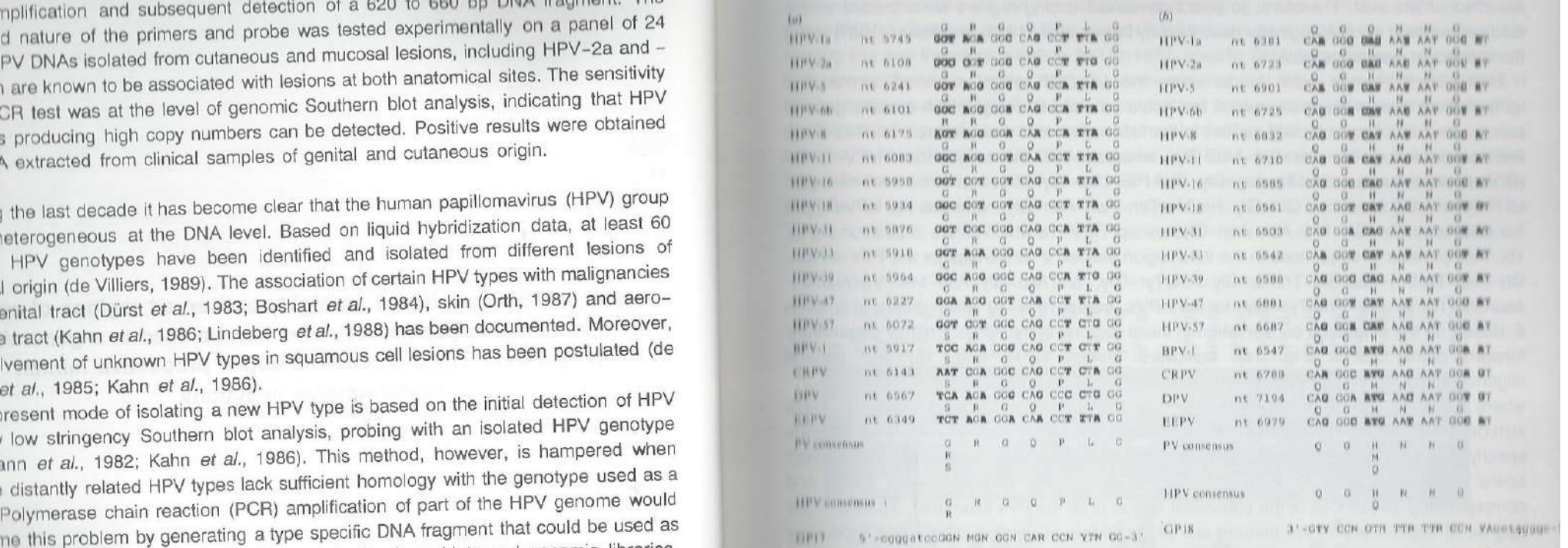

Figure 1. Alignment of PV nucleotide sequences and deduced amino acid sequences in equivalent regions of the L1 ORF which were used for the design of the general HPV primers GP17 (a) and GP18 (b). Only the sequences encoding amino acids which are conserved amongst the HPVs were used to design of GP17 and GP18. Deduced amino acid sequences are shown above the nucleotide sequences. Nucleotides that differ amongst the PV types examined are boxed and in bold. The PV and HPV amino acid consensus sequences are also indicated. Nucleotide changes producing an amino acid change in comparison to the HPV consensus are underlined. Primers GP17 and GP18 were extended at their 5' end with sequences (small characters) which allow *Bam*HI digestion of PCR products for subsequent cloning. Degenerate nucleotide sequences in the oligonucleotides are represented by: Y, C or T; R, A or G; M, A or C; W, A or T; N, A, G, C, or T. Except for HPV-1a, the numbering of the 5' nucleotide is according to the published sequence data. The numbering of HPV-1a (Danos *et al.*, 1982) was adapted according to the modified numbering of Danos *et al.* (1983). The remaining sequence information was from Hirsch-Behnam *et al.* (1990; HPV-2a and HPV-57), Zachow *et al.* (1987; HPV-5), Schwarz *et al.* (1983; HPV-6b), Fuchs *et al.* (1986; HPV-8), Dartmann *et al.* (1986; HPV-11), Seedorf *et al.* (1985; HPV-16), Cole and Danos (1987; HPV-18), Goldsborough *et al.* (1989; HPV-31), Cole and Streeck (1986; HPV-33), Velpers and Streeck (1991; HPV-39), Kiyono *et al.* (1990; HPV-47), Chen *et al.* (1982; BPV-1), Giri *et al.* (1985; CRPV), Groff and Lancaster (1985; DPV), Ahola *et al.* (1986; EEPV).

Extensive comparison of the nucleotide sequences of the L1 ORFs of all sequenced HPV genotypes (HPV-1a, -2a, -5, -6b, -8, -11, -16, -18, -31, -33, -39, -47 and -57),

the animal papillomaviruses (PVs) bovine PV type 1 (BPV-1; Chen *et al.*, 1982), cottontail rabbit PV (CRPV; Giri *et al.*, 1985), deer PV (DPV; Groff and Lancaster, 1985), European elk PV (EEPV; Ahola *et al.*, 1986), and the partially sequenced *Micromys minutus* PV (MmPV; O'Banion *et al.*, 1988) was carried out. The comparison revealed regions in which alterations occurred predominantly at the third base of a codon without affecting the encoded amino acid. Therefore, to select conserved coding regions we extended amino acid comparison studies originally described by Danos *et al.* (1984) and Baker (1987) using the additional sequence data.

Three short regions were found within the L1 ORF which exhibited amino acid conservation over at least six codons and could be used to design HPV-specific general primers and an oligonucleotide probe. In the first region that was selected, all PVs share the sequence Arg-Gly-Gln-Pro-Leu-Gly, whereas, with the exception of HPV-8, the HPVs share the sequence Gly-Arg-Gly-Gln-Pro-Leu-Gly (Fig. 1a). In the second region all PVs have the sequence Gln-Gly-His/Met/Gln-Asn-Asn-Gly, whereas the HPVs share the sequence Gln-Gly-His-Asn-Asn-Gly, except HPV-1a which differs at position 3 (Fig. 1b). Comparison of sequences in the third region yielded a consensus for the PVs of Asp-Gly-Asp-Met-X-Asp/Glu-Thr/Ile-Gly-Phe/Tyr-Gly, and Asp-Gly-Asp-Met-Val/Ala/Ile-Asp/Glu-Thr/Ile-Gly-Phe/Tyr-Gly for the HPVs, except HPV-16 which differs at position 6. Based on the sequence conservation in these regions of the HPVs a highly degenerate forward primer GP17 (Fig. 1a), backward primer, GP18 (Fig. 1b) and general oligonucleotide probe, GPR22 (5'-ARGAYGGNGAYATGRYNGAYAYNGGNTWYGG-3', where Y is C or T, R is A or G, W is A or T, and N is A,G,C or T), were designed; these include most of the nucleotides of the sequenced HPVs and all additional codons specifying amino acids conserved amongst the HPVs. The oligonucleotide probe GPR22 spans nucleotides 6223 to 6253 of the HPV-16 genome (Seedorf *et al.*, 1985) and corresponding sequences of the equivalent region of all the PVs examined. To reduce the degree of degeneracy of the primers and probe, single nucleotide differences producing a codon different to those conserved in the majority of HPV types were not included. Consequently, GP17 shows one mismatch with the HPV-8 sequence (Fig. 1a), GP18 one mismatch with the HPV-1a sequence (Fig. 1b) and GPR22 one mismatch with the HPV-16 sequence. For the same reason, sequences that seemed to be specific for animal PVs (e.g. amino acid position 3 of the GP18 sequence as shown in Fig. 1b) were also not included in primers and probe. As has been determined recently (Sommer and Tautz, 1989; Snijders *et al.*, 1990), sequences with limited mismatches can be used in the PCR, allowing successful amplification of target DNA with only one or a few base differences. To facilitate eventual cloning and to stabilize the primer-template complex (Mack and Sninsky, 1988), primers were extended at their 5' end with sequences containing the *Bam*HI site. The backward primer (GP18) appeared to be from the region used previously by Manos *et al.* (1989) to select the MY11 primer; the forward primer (GP17) is about 624 to 660 nucleotides upstream of this region.

The general properties of the primers and probe were analysed using a panel of representative HPV types isolated from different anatomical sites (de Villiers, 1989). Cloned HPV DNA (1 ng) was subjected to PCR in a total volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl pH8.3, 1.75 mM $MgCl_2$, 0.001% (w/v) gelatine, 200 mM of each dNTP, 1 unit Amplitaq DNA polymerase (Cetus) and 50 pmol of primers GP17 and GP18. Forty

cycles of amplification were performed as described previously (Snijders *et al.*, 1990) except that the annealing temperature was 52°C. The resulting PCR products, visualized after electrophoresis on an 1.5% agarose gel, are shown in Fig. 2a. Except for the HPV-16 and HPV-30 clones, all the HPV clones examined (pHPV-1a, -2a, -5, -6b, -7, -8, -11, -13, -18, -25, -31, -32, -33, -38, -39, -41, -43, -45, -46, -51, -56 and -57) were successfully amplified resulting in a PCR product ranging in size from about 600 to 700 bp. For pHPV-16 (Seedorf *et al.*, 1985) and pHPV-30 (Kahn *et al.*, 1986) the primers flank the cloning site and therefore a 600 to 700 bp HPV-specific DNA fragment was not amplified. This was confirmed by further analysis which revealed that HPV-16 and HPV-30 DNA in clinical specimens and HPV-16 DNA in cervical cell lines were amplified successfully (Fig. 4). The specificity and generality was confirmed by the fact that no amplification signal was obtained with pBR322 vector DNA, whereas with BPV-1, which shows two mismatches with GP17 and three mismatches with GP18, a weak amplification signal was obtained (Fig. 3e).

Figure 2. Analysis of GP17/GP18-directed PCR of cloned HPV DNAs by electrophoresis of 10 µl of the PCR products on a 1.5% agarose gel and staining with ethidium bromide (a), or blotting and hybridization with the GPR22 probe (b). Oligonucleotides were synthesized on a Gene Assembler Plus synthesizer (Pharmacia) by the methoxy-phosphoramidite method. Amplified DNA was transferred to a nylon membrane (Biotrace; German Sciences) by diffusion blotting in 0.5 N NaOH, 0.5 M NaCl. The probe was radiolabelled with (γ-^{32}P) ATP using T4 polynucleotide kinase (Pharmacia) as previously described (van den Brule *et al.*, 1989). Hybridization was performed at 55°C in 0.5 M sodium phosphate pH 7.4, 7% SDS, 1 mM EDTA overnight. Subsequently, the filters were washed three or four times in 3xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.5% SDS at 55°C for 15 min. Autoradiography was for 4 h with Kodak Royal X-Omat film and intensifying screens. For pHPV-7 the subgenomic clone 7/5 was used; the other subclone, 7/4, was negative. For pHPV-43 subclone 2B was positive whereas subclone 2A was negative. Lane M, pBR322 DNA digested with *Hin*fI.

After blotting and hybridization with the 5' end ^{32}P-labelled GPR22 probe, the products of all types except HPV-38 and HPV-41 could be detected easily after a 4 h exposure (Fig. 2b and 3f for BPV-1). After an overnight exposure a clear hybridizing signal also was obtained with HPV-38 and HPV-41 (Fig. 3c and d). Therefore, it appeared that the primers

and probe amplify and detect sequences from HPVs found mainly in skin lesions (HPV-1a, -5, -7, -8, -25, -38, -41 and -46), oral mucosa (HPV-13 and -32), genital mucosa (HPV-6, -11, -16, -18, -30, -31, -33, -39, -43, -45, -51 and -56), and in both mucosal and cutaneous lesions (HPV-2a and -57). These include both so-called oncogenic and non-oncogenic HPV types. The successful application to HPV-38, isolated from a malignant melanoma (Scheurlen *et al.*, 1986), and HPV-41, a distantly related type that shows little homology to the other isolated types (Grimmel *et al.*, 1988), is also striking. Moreover, it is worth noting that despite the presence of mismatches with both primers BPV-1 DNA could be amplified. This finding is of importance if we assume that putative, unknown HPV types could be related more closely to the animal PVs than to other HPV types.

The sensitivity of the method was determined by testing serial dilutions of cloned HPV DNA in 100 ng of human placental DNA. On the average the sensitivity appeared to be about $7x10^5$ (10 pg pHPV DNA), $7x10^4$ (1 pg pHPV DNA), and $7x10^3$ (0.1 pg pHPV DNA) viral copies per sample in agarose gels, and after GPR22 hybridization with a 4 h and an overnight exposure, respectively. However, for HPV-38 and HPV-41 DNA the sensitivity appeared to be slightly lower; approximately $7x10^6$ (0.1 ng pHPV DNA) and $7x10^5$ viral copies per sample could be detected in agarose gels, and $7x10^4$ copies per sample after an overnight exposure for HPV-38 (Fig. 3a and c) and HPV-41 (Fig. 3b and d), respectively. These difference between gel and hybridization detection indicate that the amplification step is critical for HPV-38, whereas the hybridization step is less efficient for HPV-41. It can be concluded that the relatively low sensitivity of the method ($7x10^3$ to $7x10^4$ viral copies in 100 ng DNA, dependent on the type, after an overnight exposure), which is comparable to genomic Southern blot analysis, is probably due to the high degeneracy of the primers, enhancing annealing to cellular DNA and affecting the efficiency of amplification of HPV-specific target DNA.

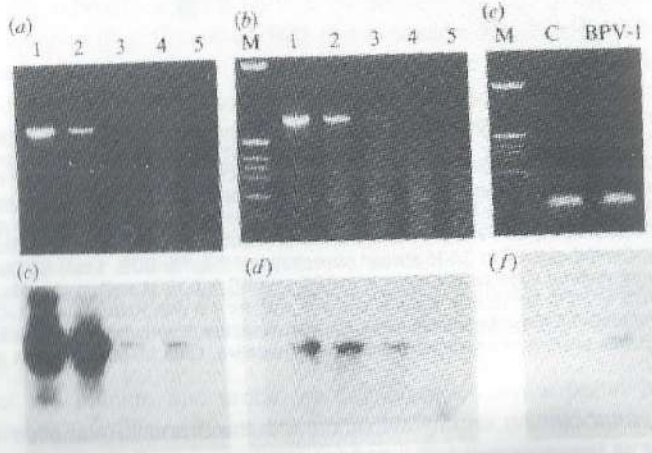

Figure 3. Test of the sensitivity of the GP17/GP18-directed PCR (a to d) and PCR assay of BPV-1 DNA (e and f). Serial dilutions of cloned HPV-38 (a and c) and HPV-41 (b and d) DNA in 100 ng of human placental DNA, and 1 ng BPV-1 DNA (e and f) were subjected to PCR. PCR products were analysed by agarose gel electrophoresis (a, b and e) and Southern blot hybridization with GPR22 (c, d and f) as described for Fig. 2. Autoradiography was overnight (c and d) or for 4 h (f). The amount of cloned HPV DNA was 1.0 ng, 0.1 ng, 10 pg, 1.0 pg and 0.1 pg (lanes 1 to 5; a to d). For BPV-1, the agarose gel signal is so weak that it can hardly be seen (e and f). C, Distilled water; M, pBR322 DNA digested with *Hin*fI.

For further analysis of the application of the generalized PCR, the method was used to test 50 to 250 ng DNA from lesions from different sites and cervical scrapes, some of which had previously been found to contain HPV DNA (Snijders *et al.*, 1990) whereas others were suspected to contain HPV DNA owing to koilocytotic changes observed in histological sections (not shown). DNA from cervical cancer cell lines Siha (HPV-16), CaSki (HPV-16), C4-1 (HPV-18), and HeLa (HPV-18) was also examined. For most samples an HPV-specific fragment of between 600 and 700 bp was amplified and was visible in agarose gels (Fig. 4a). The background smears probably reflect the high degeneracy of the primers allowing a certain degree of cross-annealing to cellular DNA. After a 4 h exposure after hybridization to GPR22, all HPV-specific sequences could be detected clearly, whereas no signal appeared with human placental (Hp) DNA (Fig. 4b). After prolonged exposure of up to 2 days no interfering hybridization signal was obtained with several independent Hp DNA isolates, indicating the high specificity of the combination of these primers and probe. The HPV-positive clinical samples included two skin warts (cu1 and cu5; cu1 was found to contain HPV-7 DNA but cu5 has not been typed although it is negative for the common genital HPV types -6, -11, -16, -18, -31 and -33), a case of Bowen's disease of the hand containing HPV-16 (cu6), two papillomatous laryngeal lesions containing HPV-6 (p1 and h1), and cervical scrapes containing HPV-6 (s2), -11 (s4), -16 (s5), -18 (s6), -30 (s7), -31 (s8 and s9) or -33 (s3) DNA.

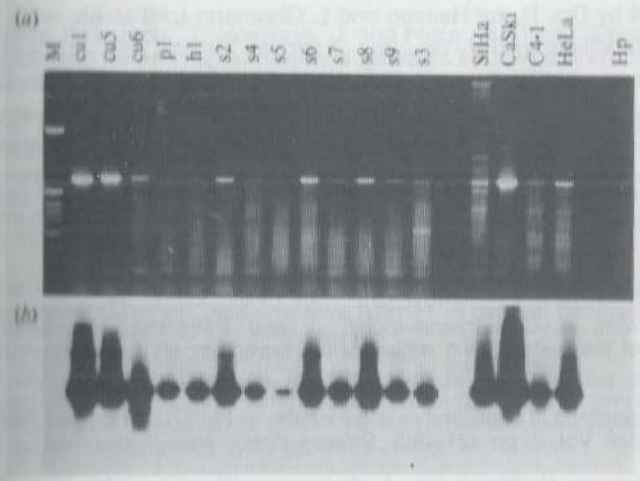

Figure 4. GP17/GP18-directed PCR of clinical samples and cell lines. DNA from the cervical cancer cell lines (CaSki, Siha, HeLa and C4-1) and tissue specimens was extracted as described earlier (Walboomers *et al.*, 1988). Cervical scrapes were processed as described by van den Brule *et al.* (1989). Electrophoretic analysis (a) and GPR22 hybridization analysis (b) are shown. Autoradiography was for 4 h. The clinical samples examined included three cutaneous lesions (cu1, cu5 and cu6), two of which were warts (cu1 and cu5) and one was a case of Bowen's disease of the hand (cu6), two papillomatous laryngeal lesions (p1 and h1) and cervical scrapes (s2 to s9). Lane M, pBR322 DNA digested with *Hin*fI; Hp, human placental DNA.

The sensitivity of this method is comparable with that of genomic Southern blotting, so samples must be selected carefully for the GP17/GP18 primers to be applied reliably. The method can be of value as a first step to the isolation of new, distantly related HPV types

from lesions in which histologically and/or immunohistologically the presence of cytopathological changes (e.g. koilocytosis) and/or late viral capsid antigens can be observed and a productive HPV infection is suspected. This has been substantiated in the skin lesions described (Fig. 4, samples cu1, cu5 and cu6) which fulfil these requirements. Therefore, these "productive" primers may be useful for the investigation of papillomatous benign and pre-malignant lesions of different organs.

In malignancies, however, other problems may arise, e.g. low HPV copy number per cell and deletion or partial disruption of part of the L1 ORF in the primer matched regions caused by integration. In these situations general primers directed against the oncogenic viral regions (i.e. E6 and E7 ORFs) would be more suitable for reliable assesment in this kind of lesions. These regions, however, are very heterogeneous and primers with the optimal general properties could not be selected. More biological data on the oncogenic character of the E6 and E7 ORFs, and sequence alterations not affecting the loss of oncogenic potential are necessary to design general oncogenic HPV-detecting primers.

In conclusion, this report has demonstrated that the use of degenerate primers and a probe produced on the basis of amino acid conservations in the PCR has great value in the detection of distantly related HPV types in productively infected tissue, independent of anatomical site.

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CHAPTER IV

General primer PCR in combination with sequence analysis to identify still unsequenced and potentially novel HPV genotypes in cervical lesions

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Abstract

We recently described the detection of still unsequenced HPV genotypes (HPV X) in cervical smears (J. Clin. Microbiol. 1990, 28: 2739-2743) using general primer mediated polymerase chain reaction (GP-PCR). In this study HPV specificity of HPV X assigned GP-PCR products was determined by sequence analyses. Therefore M13 bacteriophage clones of PCR products derived from cloned unsequenced HPV genotypes 13, 32, 35, 43, 44, 45, 51 and 56 were subjected to dideoxy sequencing. Analyses of the putative amino acid sequences of these HPV types in addition to published HPV sequence data revealed stretches of highly conserved amino acid residues present in all HPV types, resulting in a HPV amino acid consensus sequence.

Subsequently HPV X assigned PCR products found in premalignant cervical lesions (n = 3), carcinoma *in situ* (n = 6) and invasive cancer (n = 6) were analyzed for their nucleotide sequences. Sequence comparison with published HPV nucleotide sequences and data obtained in this study revealed three times HPV 35, twice HPV 45, once HPV 51, twice HPV 56 and 6 different still unidentified HPV X sequences of which 3 types were present in 4 cases of carcinomas (*in situ*). Nucleotide sequences determined appeared to be unique after data bank search. Furthermore all HPV X isolates fulfilled the HPV amino acid consensus sequence, thus confirming HPV specificity.

This study illustrates the powerful use of GP-PCR in combination with sequence analysis to determine HPV specificity and genotyping of PCR products derived from sequenced as well as unsequenced HPVs, including novel, not identified HPV types.

Introduction

At present the heterogeneous family of human papillomaviruses (HPV) consists of more than 60 different epitheliotropic viruses found in cutaneous and mucosal lesions (9). Only 11 of the 27 mucosotropic HPV types reported to date, are thought to possess oncogenic potential. Several HPV genotypes, i.e. HPV 16, 18, 33, 35, 56 and 58 have been isolated from carcinomas of the uterine cervix (1, 3, 10, 19, 20, 22). HPV types 31, 39, 45, 51 and 52 have been associated with cervical cancer (2, 18, 23, 24, 26). Based on their prevalence rates in cervical carcinomas and on *in vitro* transforming capability (25), HPV genotypes have been grouped into high risk (HPV 16, 18), intermediate risk (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58) and low risk (HPV 6, 11) for the development of cervical cancer (12, 18, 20). The remainder of the 27 oral-anogenital HPV genotypes (9) have mainly been found in normal mucosa, benign lesions and low grade intraepithelial neoplasia, suggesting that they belong to low or intermediate risk types. With respect to the broad spectrum of the HPV family and the still ongoing isolation of new types, it is interesting to study whether additional oncogenic HPV types are present in carcinomas of any origin. Squamous cell carcinomas of the cervix uteri can provide a valuable source of these types.

The recently described general primer mediated PCR (GP-PCR; 29, 30) has proven to detect a broad spectrum of HPVs including unsequenced types (HPV X), using a single pair of primers. With the applicance of this approach some unidentified HPV genotypes have already been successfully detected in cervical scrapes (30, 31). To exclude the detection of co-amplified cellular DNA, HPV specificity of GP-PCR products was determined by expected size, additional hybridization (30, 31) and *Rsa*I restriction mapping

(29). In this study nucleotide sequence analysis is used to confirm the specificity and for identification of HPV genotypes. Based on the presence of both a conserved and a polymorphic region, both specificity and genotype of HPV could be determined. Using this approach 3 carcinoma-associated HPV X and 3 HPV X present in cervical dysplasia were detected, which could be novel, still unidentified HPV types.

Materials and methods

HPV clones and clinical specimens

Cloned HPV types 6b, 11, 16, 18 and 30 were kindly provided by Drs H. zur Hausen (Heidelberg, F.R.G.), HPV 31 by Dr A. Lorincz (Gaithersburg, MD, U.S.A.), HPV 5, 33 and 39 by Dr. G. Orth (Paris, France), HPV 45 by Dr K.V. Shah (Baltimore, MD, U.S.A.), HPV 51 by Dr G. Nuovo (New York, NY, U.S.A.) and HPV types 1a, 2a, 8, 13 and 32 by Dr E.-M. de Villiers (Heidelberg, F.R.G.). Cloned HPV types 35, 43, 44 and 56 were derived from American Type Culture Collection (Rockville, U.S.A.).

Cervical scrapes were used for cytological classification and for HPV detection studies (31). For the latter spatulas were placed in 5 ml phosphate-buffered saline (PBS) and vigorously vortexed. In this way Pap IV (carcinoma *in situ*)- and Pap V (invasive cancer) assigned cell suspensions (n = 40) and scrapes with dysplastic cells (n = 3; selected group) have been collected and stored at -40 °C.

Archival paraffin embedded invasive cervical carcinomas (n = 65) were serially sectioned. The first and last sections were heamatoxylin eosin stained and histologically analyzed for the presence of neoplastic cells.

HPV detection

HPV detection of cloned HPV types was performed by PCR on purified DNA. Clinical specimens were pretreated before subjection to PCR. HPV was directly detected in crude cervical cell suspensions by the PCR as previously described (31). Briefly, 10 μl of suspended cells were frozen at -40 °C , thawed, boiled for 10 min, cooled on ice and spinned down before adding the PCR components. In addition, HPV was detected in a single paraffin embedded section, which was deparaffinized by xylene and washed with 96 % ethanol. After the tissue was centrifuged and air dried, the pellet was suspended in 50 μl distilled water and frozen at -80 °C for at least 30 min. After thawing a 50 μl proteinase K mix (10 mM TrisHCl pH 7.5, 1.5 mM MgCl2, 0.45 % Tween 20 and 60 μg/ml proteinase K (Boehringer Mannheim, Mannheim, F.R.G.) was added and the mixture was incubated at 55 °C for 1 hour. Finally samples were treated at 100 °C for 10 min and centrifuged. Twenty μl of the supernatant was used in the PCR reaction.

Samples were subjected to general primer mediated PCR (GP-PCR) followed by type specific PCR (TS-PCR) in a total volume of 50 ul as previously described (30, 31; also for primer sequences). Briefly, general primers GP 5/6 were used in the PCR, which permits the detection of the sequenced mucosotropic HPV types 6, 11, 16, 18, 31 and 33, but also unsequenced HPV types (HPV X) at the subpicogram level (29). After low-stringency Southern blot analyses with probes of HPV specific PCR products, the GP-PCR positive samples were subjected to TS-PCR. A mixture of HPV 6, 16, 33 and HPV 11, 18, 31 specific "anti-contamination" primer sets (31) were used respectively to detect the

sequenced HPV genotypes. Samples which were positive by GP-PCR and negative by TS-PCR were suspected of containing HPV X.

M13 cloning and sequencing

Samples containing oncogenic HPV X types were subjected to GP-PCR in 4-fold with general primers extended at the 5' end with an 8 nucleotide tail including the *Bam*HI restriction site (BGP5: 5'- acggatccTTTGTTACTGTGGTAGATAC -3' and BGP6: 5'- acggatccGAAAAATAAACTGTAAATCA -3'). GP-PCR products were pooled. After addition of 2 volumes ethanol/2M ammoniumacetate at room temperature, the PCR products were centrifuged at 10,000 g to get rid of the primers. The ends of PCR products were filled in using 2 units Klenow DNA polymerase (Pharmacia, Uppsala, Sweden) according to standard procedures (21). After Na-acetate/ethanol precipitation for 30 min at - 80 °C and centrifugation, the PCR products were digested by an excess of 40 units *Bam*HI restriction enzyme (Pharmacia) according to the manufacturer's instructions. Digested GP-PCR products were subsequently ligated into the *Bam*HI cloning site of M13 bacteriophage mp 18. Ligation reaction was performed with the total amount of GP-PCR products, 50 ng M13 DNA and 2 units T4 DNA ligase (Pharmacia) according to standard procedures (21) overnight at 16 °C. Half of the ligation product was presented to $CaCl_2$- competent cells of Escherichia coli strain JM101 (21). HPV containing M13 clones were identified after a plaque lift to a DNA binding nylon support (Biotrace, Gelman sciences, Ann Arbor, MI). Filters were hybridized under conditions of low stringency (Tm -33 °C) with a mixture of HPV 6, 11, 16, 18, 31 and 33 specific GP-PCR products as probe as previously described (30). Subsequent washings were done at low stringency and autoradiography was performed with Kodac Royal X-omat film and intensifying screens overnight at - 80 °C.

Overnight 1 ml cultures of HPV containing M13 clones were pelleted and 800 µl of supernatant was used for single stranded DNA purification (21). DNA was finally dissolved in 10 µl bidest. Five µl of M13-HPV DNA was used for dideoxy sequencing by means of T7 polymerase sequencing kit (Pharmacia) and (α-^{32}P)-dCTP according to manufacturer instructions. Reaction products were analyzed after 8 % polyacrylamide gel electrophoresis and autoradiography.

Results

Sequence analyses of PCR products derived from cloned HPVs

GP-PCR permits the detection of a broad spectrum of HPV types by the amplification of approximately 150 base pairs of the L1 region (29, 30) as shown in Figure 1A for cloned genital HPVs. Additional hybridization under low stringency conditions using a cocktail probe of HPV 6, 11, 16, 18, 31 and 33 specific GP-PCR products confirms HPV specificity (Figure 1B; reference 30). Samples which were positive by GP-PCR and negative by TS-PCR were suspected of containing presently unsequenced HPV types assigned as HPV X in this study. So the group of HPV X can consist of yet identified types, such as HPV 45 and 51, as well as still unidentified, novel HPV types. GP-PCR products derived from plasmid clones of unsequenced HPV 13, 32, 35, 43, 44, 45, 51 and 56 were cloned into bacteriophage M13 and nucleotide sequences were determined (Figure 2). Nucleotide sequences were compared to each other and to data bank information (Micro Genie

release 6.0; Beckman, Berkeley, CA). HPV X sequences were not identical to sequenced HPV genotypes (HPV 1, 2, 5, 6, 8, 11, 16, 18, 31, 33, 39, 47, 57 and 58; references are listed in legend to Figure 3), sequenced animal PV types (bovine PV (BPV) 1, 2 and 4; cotton tail rabbit PV (CRPV), deer PV (DPV), european elk PV (EPV) and feline PV (FPV) 1) nor to other viral and cellular genes known to date.

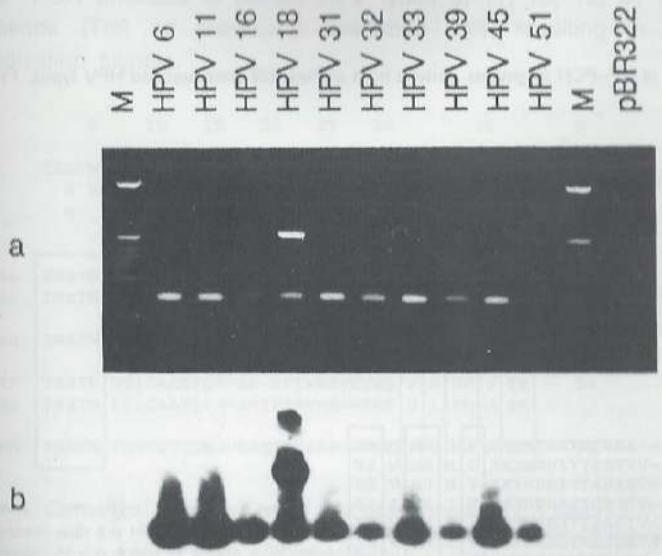

Figure 1. Detection of cloned HPV genotypes by general primer - mediated PCR (GP-PCR). PCR was performed on different amounts of purified DNA. (a) GP-PCR products after electrophoresis on a 1.5 % agarose gel and ethidium bromide staining. (b) Southern blot analysis under low stringent hybridization conditions with labelled GP-PCR products derived from cloned sequenced HPV types 6, 11, 16, 18, 31 and 33. Washes were also performed under conditions of low stringency. M, pBR322 DNA digested with *Hin*fI.

In addition, the short stretches of nucleotide sequences from GP-PCR products flanked by both primers and equivalent regions of HPVs with published sequence data were translated to the putative amino acid sequence, which was numbered according to the position of the L1 start codon found in already sequenced HPV genotypes (7, 12). Alignment of these amino acid sequences of cutanous and mucosotropic HPV types (Figure 3) revealed the presence of strongly conserved amino acid residues at both ends of the GP-PCR products, which allows the determination of a HPV consensus sequence. In contrast, the internal region differing from 8 to 13 amino acid residues in length was found to be polymorphic.

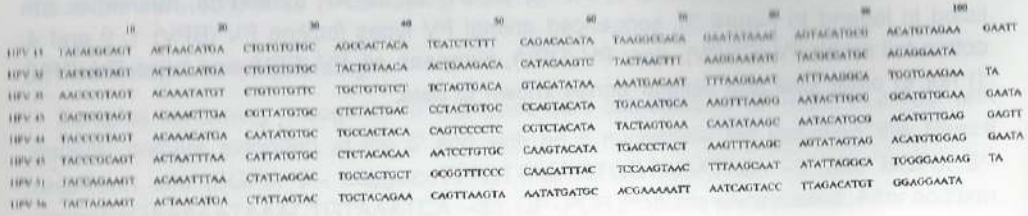

Figure 2. Nucleotide sequence of GP-PCR fragments derived from cloned, still unsequenced HPV types. Primer sequences were excluded.

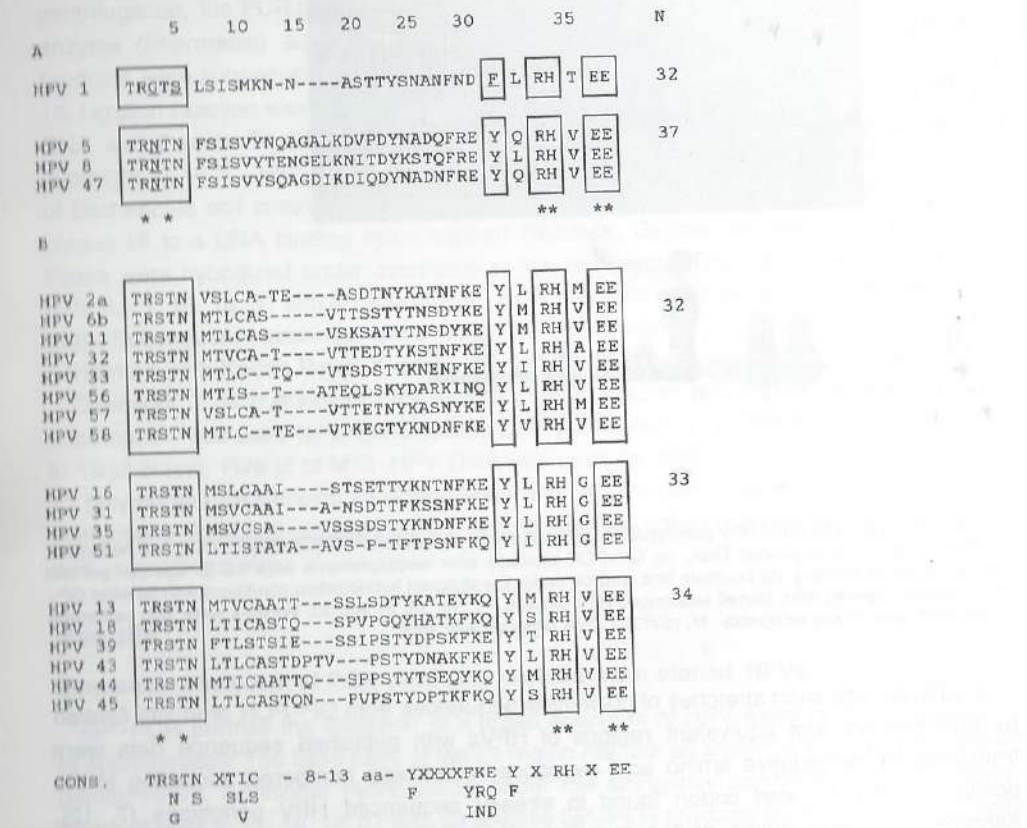

Figure 3. Putative amino acid sequence encoded by internal DNA segments (excluding the primers) of GP-PCR products or equivalent sequences of cutaneous HPV types (A) and mucosotropic HPV types (B), resulting in a HPV amino acid consensus (CONS). HPV 2a and 57 are listed as mucosotropic types according to Hirsch-Behnam *et al.* (8). Boxes indicate conserved regions among mucosotropic HPV types; _ = amino acid residues different from conserved regions of mucosotropic types;the first nuceotide is not underlined since it differs from animal PVs; * = PV specific amino acid conservation, including animal PVs; N = number of amino acid residues. Published nucleotide sequence information used was derived from reference 4 (HPV 18), 5 (HPV 33), 6 (HPV 1a), 8 (HPV 11), 11 (HPV 8), 12 (HPV 31), 13 (HPV 2, 57), 16 (HPV 58), 17 (HPV 47), 27 (HPV 6b), 34 (HPV 39) and 35 (HPV 5).

Sequence analyses of carcinoma-associated HPV X

Carcinoma (*in situ*) specimens (n = 105) were analyzed by GP-PCR followed by TS-PCR (data not shown). This revealed the detection of HPV X genotypes in 12 cases (Pap IV, n = 6; Pap V, n = 1 and paraffin embedded cervical carcinomas, n = 5). Although this study aims to investigate carcinoma (*in situ*)-associated HPV X types, also 3 HPV X assigned PCR products present in premalignant cervical lesions were analyzed. HPV X assigned GP-PCR products were verified in Southern blot analyses with a probe mixture of GP-PCR products of cloned HPV types 6, 11, 16, 18, 31 and 33 under stringent conditions (Tm) as previously described (30), resulting in the disappearance of hybridization signals.

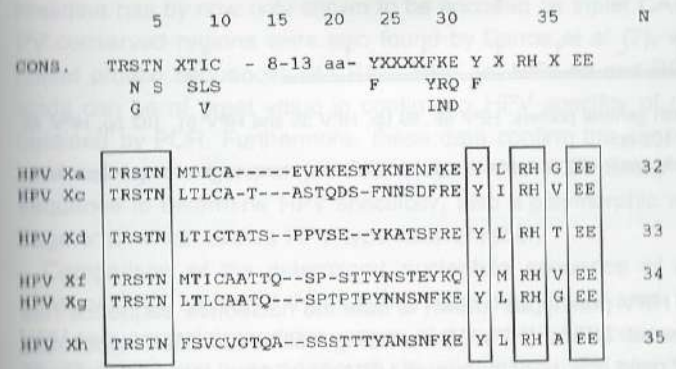

Figure 4. Comparison of putative amino acid sequences of HPV X assigned PCR products (excluding primer sequences) with the HPV amino acid consensus (CONS). Boxes indicate conserved regions among mucosotropic HPV types. N = number of amino acid residues.

M13 clones of these HPV X specific GP-PCR products were sequenced. All separate M13 clones were analyzed for the presence of the HPV specific consensus amino acid sequence (Figure 4). These HPV specific sequences were compared with already known sequence data of animal PV (BPV 1, 2 and 4; CRPV, DPV, EPV and FPV 1) and HPV types (HPV 1, 2, 5, 6, 8, 11, 16, 18, 31, 33, 39, 47, 57 and 58) in addition to sequence information of HPV 13, 32, 35, 43, 44, 45, 51 and 56 obtained in this study. Seven carcinoma (*in situ*)-associated HPV X were typed: HPV 35 three times, HPV 45 twice, HPV 51 once and HPV 56 once. The remaining carcinoma-associated HPV genotypes (HPV Xa, Xc and Xd) and dysplasia-associated types (HPV Xf, Xg and Xh) were unique in their sequence and fulfilled to the HPV amino acid consensus (Figure 4). Table 1 shows the distribution of the different unique HPV genotypes Xa, Xc and Xd in addition to HPV 35, 45, 51 and 56 found in cervical carcinomas (*in situ*), and the length of the generated GP amplification products. Homology comparisons of these unique GP-PCR sequences (alignment function; Micro Genie, Beckman, Berkeley, CA) with all known other PV sequences showed homology of more than 55 % at the nucleotide level with sequenced mucosotropic HPV types. Much lesser homology was observed with cellular and other viral sequences after databank searches. Determined nucleotide sequences of co-amplified GP-PCR products of 80 to 120 base pairs in length that derived from human placenta DNA

showed less homology than 40% with HPV derived sequences and did not fulfill the HPV amino acid consensus.

Table 1. Summary of sequenced HPV-X associated with cervical carcinoma and carcinoma *in situ*

Clinical sample	n	HPV genotypes						
		35	45	51	56	Xa	Xc	Xd
Cervix ca.	5	2	1	1	1	–	–	–
[1]PAP V	1	–	–	–	–	1	–	–
[1]PAP IV	6	1	1	–	1	1	1	1

Lenghts of sequences flanked by both general primers: HPV 56, 99 bp; HPV 35 and HPV 51, 102 bp; HPV 45, 105 bp; Xa and Xc, 99 bp; and Xd, 102 bp.
[1]PAP IV and PAP V were histologically confirmed as carcinoma *in situ* (CIS) and invasive carcinoma, respectively.

Discussion

Of only a fraction of the 60 HPV genotypes known to date the nucleotide sequence has been determined. Unsequenced HPVs (HPV X) including still unidentified types are abundantly present in normal cells (2% HPV X; overall HPV prevalence rate of 3.5%) and dysplastic lesions (20% HPV X; overall HPV prevalence of up to 80%) of the cervical epithelium as shown in a large screening cohort using GP/TS-PCR (32). This study has focussed on the presence of HPV X types in premalignant lesions and cervical carcinomas (*in situ*), an effort to identify all HPV genotypes with possible oncogenic potential. Cervical carcinoma (*in situ*) - associated unsequenced HPV X types and some dysplasia-associated HPV X as detected by GP-PCR were further characterized. HPV specificity of these GP-PCR products is normally confirmed on basis of their expected sizes and by Southern blot analyses using HPV specific probes. However, additional nucleotide sequence analysis is the most reliable approach to rule out the detection of cellular or viral fragments cross-hybridizing with the HPV probe. Direct sequencing of PCR products failed in our hands since this method needs large amounts of homogenous PCR products. Therefore in this study GP-PCR products were cloned in M13 bacteriophage. At least three M13 clones of each HPV X assigned PCR product were sequenced in both directions to exclude reading errors of the *Taq* DNA polymerase (15). Although most M13 clones were identical, variations were sometimes found at the nucleotide level at 2-3 different locations. This may indicate the presence of strain variants of a particular HPV genotype as also recently shown by Ho *et al.* (14).

Comparison of putative amino acid sequences of the GP-PCR products and of published PV sequences resulted in a striking homology as indicated in Figure 3. Eleven mucosotropic HPV genotypes and 4 cutaneous HPV types have been analyzed. All HPV types fullfil to the amino acid consensus sequence as shown in Figure 3. The mucosotropic HPVs contain the TRSTN - amino acid sequence at the 5' part of the GP-PCR product.

The sequenced cutaneous HPV types only differ from this conserved region at amino acid position 3 (HPV 1, 5, 8 and 47) and 5 (HPV 1)(underlined in Figure 3). HPV types 2 and 57 contain the mucosotropic specific TRSTN-sequence, which is in agreement with their recent assignation as mucosotropic types (13). When the consensus is extended, it will be specific for all PVs including animal PV types (not shown). Amino acid residues marked by asterisks in Figure 3 are strongly conserved among all papillomaviruses sequenced thusfar, including the animal types BPV 1, 2 and 4, CRPV, DPV, EPV and FPV 1. Of additional interest is the conserved pentamer RHXEE at the 3' end of the GP-PCR product, since this sequence is also reflected by a conservation at the nucleotide level. Glutamic acid (E) and histidine (H) are encoded by only two different triplets of nucleotides (GAG, GAA and CAT, CAC respectively), which restrict the possible variation at the nucleotide level. Histidine has by now only shown to be encoded by triplet CAT (see also Figure 2). These PV conserved regions were also found by Danos *et al.* (7), who previously compared L1 coded protein sequences of CRPV, HPV 1a, HPV 6b and BPV 1. HPV conserved amino acids can be of great value in confirming HPV specifity of new nucleic acid sequences obtained by PCR. Furthermore, these data confirm the conserved nature of the L1 ORF spanned by the general primers used in this PCR assay (29). Besides a consensus sequence to determine HPV specificity, also a polymorphic region exists in the GP-PCR fragments which allows HPV type differentiation.

Comparison of the determined nucleotide sequence of a small part of the strongly conserved L1 region of the carcinoma (*in situ*)-associated HPV X types with published HPV sequence data and sequences of cloned HPV X obtained in this study, revealed HPV 35, 45, 51, 56 and three additional still unidentified HPV genotypes (HPV Xa,Xc,Xd). These potential oncogenic HPV X as well as all dysplasia-associated HPV X analyzed (HPV Xf-h) were unique in their sequence. Indeed, all these HPV types fulfilled the amino acid consensus indicating HPV specificity (Figure 4). In addition a comparison was made between the determined sequences of HPV Xa, Xc, Xd, Xf-h and HPV 41 (L. Hirt, A. Hirsch-Behnam and E.-M. de Villiers, submitted), and with preliminary HPV sequences which have been determined in a systematic sequencing effort at Heidelberg (HPV 4, 7, 9, 10, 12, 14, 15, 17, 19, 25, 30, 51, 52 and 53; H. Delius, unpublished). The HPV Xa L1 ORF sequence could be identified as HPV 52, leaving HPV types Xc, Xd, Xf, Xg and Xh still unidentified.

Sequences of these new HPV X types showed more than 55 % homology when compared with known sequenced mucosotropic HPVs. HPV type Xa (HPV 52) was highly homologous (71-75%) to HPV 16 and HPVs of the 30's-group. HPV Xc showed approximately 70 % homology with HPV types 16, 32 and 45. The observed homologies of up to 75 % is less than those found when comparing GP-PCR sequences of different, well established HPV types closely related, i.e. HPV 6b and 11 (84 %), HPV 2 and 57 (82 %), HPV 18 and 45 (80 %), and HPV 33 and 58 (86 %). This suggests that the HPV X assigned PCR products found have been derived from different HPV types and do not represent HPV strain variants. HPV typing based on differences found in short stretches of 99 to 114 nucleotides of these GP-PCR products seems therefore to be well possible. This is supported by the use of short sequences of L1 ORF (200 bp) in a phylogenetic study which revealed similar results using larger fragments of L1 and other ORFs (33).

In this study it is shown that HPV Xc and Xd types did not represent HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58, known to be associated with cervical cancer. This means that at least 2 HPV X genotypes, detected by GP-PCR, may represent HPV types, until now not found in carcinomas, i.e. new oncogenic HPV types. Additional confirmation of the possible oncogenic potential of these HPV X types needs to be performed by DNA *in situ* hybridization studies which show their exclusive presence in carcinoma cells and not in the surrounding normal or dysplastic cells. Complete cloning of these putative new oncogenic HPV types may be facilitated by using the specific GP-PCR products as probe to screen the appropriate genomic library.

In conclusion, the GP-PCR is a very useful technique to collect information concerning the great sequence variation within this virus group. This method in combination with sequence analysis is a rapid and efficient approach for HPV typing and detection of novel HPV types in carcinomas of the cervix uteri and other neoplastic tissues suspected for HPV etiology as well.

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CHAPTER V

Detection of human papillomavirus and Epstein-Barr virus DNA sequences in oral mucosa of HIV-infected patients by the polymerase chain reaction

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Abstract

The presence of human papillomavirus (HPV) and Epstein-Barr virus (EBV) was analyzed in 21 oral biopsy specimens of HIV-infected patients using the polymerase chain reaction (PCR) method. Biopsies were catagorized as hairy leukoplakia (HL) (n=12), candidiasis (n=3), oral warts (n=2), and clinically normal epithelium (n=4). For HPV detection a modified general primer mediated PCR method (GP-PCR), which detects a broad spectrum of HPV genotypes at sub-picogram levels, was used. Human papillomavirus DNA was only found in two oral warts and was identified as HPV type 32. Epstein-Barr virus DNA was detected in 16 biopsy specimens, including the 12 HLs, 2 cases of candidiasis, and 2 samples of normal epithelium. Epstein-Barr virus positivity in HL could be confirmed by Southern blot analysis and DNA *in situ* hybridization using biotinylated DNA probes (bio-DISH). Epstein-Barr virus bio-DISH was also positive in one sample of normal epithelium from a patient with HL. The results indicate that HL is strongly associated with EBV and not with any of the common HPV types that react with general HPV primers in the PCR. However the detection of EBV in normal oral epithelium by PCR and bio-DISH suggests that the presence of this virus is not exclusively related to HL.

Introduction

Oral mucosal lesions frequently have been reported in patients infected with human immunodeficiency virus (HIV) (1,2). Some of these lesions have been linked to viral infections. These include oral warts (3) and oral hairy leukoplakia (HL) (4,5). The association of HL with specific viruses has been reported. However the presented data show some discrepancies. The results of initial studies have suggested an association of HL with both a papillomavirus and a herpesvirus (4). The herpesvirus has been identified as Epstein-Barr virus (EBV) (5). It has now been suggested that an accurate diagnosis of HL requires the demonstration of EBV in the epithelial cells of oral white lesions in HIV-infected patients by DNA *in situ* hybridization using biotinylated DNA probes (bio-DISH) (6). Recently Eversole *et al.* (7) described, in addition to EBV DNA, the presence of human papillomavirus (HPV) genus-specific antigen in 15% of the examined cases of HL. However in a single case HPV-16, -18 or -related DNA sequences have been identified by bio-DISH under low stringent conditions. For this reason the possibility of the presence of distantly related HPV genotypes in HL can not be excluded. Other recent investigations have failed to reveal evidence for the presence of HPV in HL (8,9,10). This possibly could be due to the procedures that have been applied for HPV detection. Most HPV detection studies have been performed using DNA hybridization procedures and have consequently been limited to the demonstration of only a small spectrum of HPV genotypes with a rather low sensitivity (11).

As the result of advances in nucleic acid amplification technology (12,13), it is possible to detect even one molecule of HPV DNA in clinical specimens using the polymerase chain reaction (PCR) (14, 15). Apart from its sensitivity, the method is advantageous because it allows the amplification of target DNA in formalin-fixed, paraffin-embedded tissue sections without the need for prior DNA purification (14). Recently a modified PCR method has been introduced that permits the amplification of a broad spectrum of HPV genotypes at once (16,17,18,19) by use of consensus or general primers, homologous to sequences

of the conserved E1 and L1 open reading frame (ORF)(20). The application of this general primer-mediated PCR method (GP-PCR) would enable a more accurate assessment of the presence of both sequenced and yet unsequenced common or possibly novel HPV types in oral lesions of HIV-infected patients.

The presented study aims to assess the presence of HPV by GP-PCR and EBV genotypes in normal and pathological oral mucosa of HIV-infected patients and shows that only EBV is strongly associated but not exclusively related to HL. No association between HL and a broad spectrum of HPV genotypes was found.

Materials and methods

Patients, biopsy specimens and viral DNA clones

Between October 1986 and August 1989, oral biopsies were obtained from HIV-infected patients, attending the Departments of Internal Medicine and Oral & Maxillofacial Surgery and Oral Pathology. Two patients were at high-risk for HIV infection but did not grant permission to be tested for HIV antibodies. Lesions were diagnosed according to criteria described by Schulten *et al.* (2). Of 19 biopsies taken from this patient group, 12 showed histologic features consistent with HL, 3 biopsies were taken from white lesions that showed histologically candidiasis. These lesions initially disappeared after antifungal treatment. Furthermore two biopsies were oral warts, while two were clinically normal gingival specimens. The latter two gingival specimens were obtained during tooth extractions. Furthermore, two biopsies were taken during necropsy from an additional patient, one from the gingiva and one from the tongue. Biopsies of oral lesions obtained from patients who did not belong to a high-risk group of HIV infection were studied as control specimens (including white sponge nevus and leukoplakia). Biopsies were divided and one part was fixed in 4% buffered formalin and embedded in paraffin. From 17 specimens, the other part was snap-frozen and stored in liquid nitrogen until use.

DNAs of several cloned HPV types (pHPVs) were tested in the PCR using general HPV primers. The pHPVs 6b, 11, 16, 18, and 30 were provided by Drs. H. zur Hausen and L. Gissmann (Heidelberg, FRG), pHPV 31 by Dr. A. Lorincz (Gaithersburg, MD), pHPV 33 by Dr. G. Orth (Paris, France) and pHPVs 1a, 8, 13 and 32 by Dr. E.-M. de Villiers (Heidelberg, FRG). The cloned subgenomic *Bam*HI W fragment of the large internal repeat region (IR1) of the EBV genome was purified from vector sequences by agarose gel electrophoresis and subsequently used as probe.

Primer synthesis and sequences

Primers were synthesized on a DNA synthesizer (Pharmacia/LKB Gene Assembler plus, Pharmacia, Uppsala, Sweden) by the methoxy-phosphoramidite method. General HPV primers specific for a wide range of mucosotropic (GP5/6) and cutaneous (GP11/12) HPV types were selected and have been described in detail elsewhere (17). GP5 and GP6 match to the same sequences in the conserved L1 ORF as GP11 and GP12 respectively. For EBV detection a single pair of primers (EBV primer A and primer B) selected from the *Bam*HI W fragment of the B 95-8 strain was used (Jiwa *et al.*, submitted for publication). These primers are complementary to the sense (EBV primer A) and anti-sense (EBV primer B) DNA strand and consist of nucleotides 781-800 (EBV primer A) and 1001-1020

(EBV primer B). Primer sequences were: HPV GP5: 5'-TTTGTTACTGTGGTAGATAC-3'; HPV GP6: 5'-GAAAAATAAACTGTAAATCA-3'; HPV GP11: 5'-TTTATCACAGTGGTAGACAA-3'; HPV GP12: 5'-GTAATATAAAAGAAATTTCA-3'; EBV primer A: 5'-CTCTGGTAGTGATTTGGCCC-3'; and EBV primer B: 5'-GTGAAGTCACAAACAAGCCC-3'.

Polymerase chain reaction

The PCR was performed on purified DNA of 17 snap-frozen tissue specimens and four paraffin-embedded tissue sections. DNA was extracted from frozen tissue specimens as described by Walboomers *et al.* (11). Paraffin-embedded tissue sections were prepared as described by Shibata *et al.* (14). A combination of two general primer (GP) pairs for HPV detection (GP5/6 and GP11/12) (17) was used. For EBV detection, a specific primer set that recognizes at least 100 different EBV isolates (Jiwa *et al.*, submitted) was used. The HPV-specific primer pairs direct for the amplification of a DNA fragment of 140 to 150 basepairs (bp), whereas the EBV-specific primer pair allows the amplification of a 240 bp fragment. The reaction mixture contained 1 ng of cloned HPV DNA, 10 to 50 ng purified DNA of clinical samples or the product of a deparaffinized and washed tissue section, 50 mM KCl, 10 mM Tris HCl, pH 8.3, 0.01% (w/v) gelatin, 200 μM of each dNTP, 3.5 mM $MgCl_2$, 50 pmol of each primer of either the GPs or the EBV-specific primer pair, and 1 U of *Taq* polymerase (*Thermus aquaticus*; Amplitaq; Cetus Corp., Emeryville, CA). The mixture was overlaid with 25 μl of paraffin oil to prevent evaporation and incubated for 5 minutes at 94°C for DNA denaturation. Subsequently 32 or 40 cycles of amplification were performed with purified DNAs and paraffin-embedded tissue sections, respectively, using a PCR processor (Biomed, Theres, FRG). Each cycle included a denaturation step to 94°C for 1 minute, an annealing step to 40°C (for the HPV GPs) or 55°C (for the EBV specific primer pair) for 2 minutes, and a chain elongation step to 72°C for 1.5 minute. The final elongation step was prolonged for another 4 minutes to ensure a complete primer extension of the amplified DNA fragments.

A total of 10 μl of the reaction products was analyzed on 2% agarose (Sigma Chemical Co., St. Louis, MO) gels or composite gels consisting of 3% NuSieve agarose (FMC Bioproducts, Rockland, ME) and 1% Type 1 agarose (Sigma). Subsequently the DNA was transferred by diffusion blotting onto nylon membranes (Biotrace, Gelman Sciences, Ann Arbor, MI) in 0.5 N NaOH, 0.6 M NaCl. A mixture of GP-directed amplification products of HPV types 1, 6, 8, 11, 13, 16, 18, 30, 31, 32, and 33 was ^{32}P-labelled by the random priming method and used as cocktail probe for HPV detection by GP-mediated PCR. For the specific detection of amplified EBV sequences, the purified subgenomic *Bam*HI W fragment of EBV was used as probe. Hybridization was performed at 56°C (low stringency for HPV detection) or 65°C (high stringency for EBV detection) in 0.5 M sodium phosphate, pH 7.4, 7% SDS, 1 mM EDTA for 16 hours. The filters were washed three times (for HPV detection) or once (for EBV detection) in 3xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate)/0.5% SDS at 56°C and 65°C, respectively, for 15 minutes, and (for EBV detection) subsequently once in 0.5xSSC/0.5% SDS at 65°C for 15 minutes, and twice in 0.1xSSC/0.5% SDS at 65°C for 15 minutes. Autoradiography was performed for 1 day at -80°C with Kodak Royal X-omat film (Kodak, Rochester, NY) and intensifying screens. Subsequently for HPV detection additional washings at higher stringency down to 0.5xSSC/0.5% SDS at 65°C were performed.

Dot blot analysis of PCR products

Human papillomavirus positive amplification products were further analyzed by dot blotting. One μg of cloned HPV DNA and pBR322 vector DNA was dotted onto nylon membranes (Biotrace). The amplified fragments of 140 to 150 bp were electrophoretically separated in low melting point agarose (Bio-Rad, Richmond, CA), excised from the gel, and ^{32}P- labelled by the random priming method. Hybridization was performed at 65°C and washings were carried out down to 0.5xSSC/0.5% SDS at 65°C. Autoradiography was performed as described above.

Southern blot analysis of restriction endonuclease digested cellular DNA

Seventeen tissue specimens were analyzed for the presence of EBV DNA by the Southern blot method. Cellular DNA (100 ng to 1 μg) was digested with *Bam*HI (Pharmacia) according to recommendations of the manufacturer and subsequently electrophoresed on an 1 % agarose gel. Dilutions of the *Bam*HI W fragment of the EBV genome in 1 μg human placental DNA corresponding to about 20 and 200 EBV genome equivalents per cell were included. In addition DNA from a leukoplakia of the tongue from a subject not belonging to a high-risk group for HIV infection and a white sponge nevus were included. Blotting and hybridization conditions were as described above using the EBV *Bam*HI W probe for hybridization. Autoradiography was performed for 3 days at -80°C.

DNA *in situ* hybridization

The *Bam*HI W fragment of the EBV genome was biotinylated by incorporation of biotin-11-dUTP (BRL, Gaithersburg, MD) in a nick translation assay and purified by Sephadex G50 (Pharmacia) gel filtration. Biotin-11-dUTP-labelled probes, with a mean size of 300 bp, were used throughout the experiments.

Dewaxed paraffin-embedded sections were treated with 0.5% pepsin in 0.2 N HCl for 20 minutes. After rinsing in PBS, sections were dehydrated in graded alcohols and air dried.

Prehybridization was carried out by dripping 10 to 20 μl hybridization mixture without DNA probe on the dried section, which then was covered with a coverslip and incubated for 1 hour in an humidified atmosphere at 37°C. The hybridization mixture consisted of 50% formamide, 10x Denhardt's (0.2% ficoll, 0.2% BSA, 0.2% polyvinylpyrrolidone), 2x SSC, 10% dextran sulphate, and 250 μg/ml sheared salmon sperm DNA. After washing in distilled water, the sections were dehydrated and air dried again. Depending on the size of the section, 10 to 20 μl hybridization mixture, containing 2 ng/μl biotinylated DNA probe was added and mounted with a glass coverslip. Both DNA probe and target DNA in the tissue section were denaturated by heating at 95° to 100°C for 4 minutes. Then hybridization was carried out for 18 hours at 37°C in a humidified container. After hybridization, tissue sections were subsequently washed in 2xSSC (to loosen the coverslips) and 3 x 20 minutes in 50% formamide with 0.1x SSC at 37°C.

For detection of the biotinylated DNA hybrids a streptavidin-biotinylated polyalkaline phosphatase kit (BRL) was used; subsequently sections were incubated with (1) PBS + 3% BSA (30 minutes), (2) 0.4 μg/ml streptavidin in PBS + 3% BSA for 1 hour, and (3) 0.2 μg/ml biotinylated polyalkaline phosphatase in PBS for 1 hour. After steps 2 and 3, sections were thoroughly washed with PBS. Alkaline phosphatase staining was performed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (0.33 and 0.17 mg/ml 0.1 M Tris-HCl with 0.1 M NaCl and 50 mM $MgCl_2$ at pH 9.5) for 20 to 45 minutes.

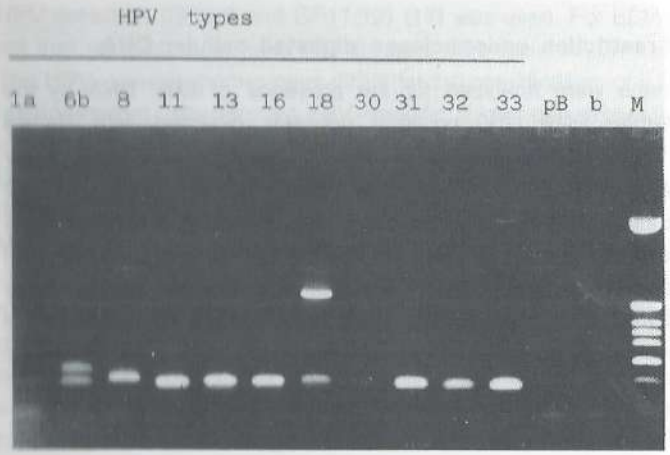

Figure 1. HPV general primer-mediated PCR on 1 ng of cloned HPV or pBR322 DNA. PCR products are shown after agarose gel electrophoreses, ethidium bromide staining and ultraviolet illumination. pB, pBR322 DNA; b, distilled water; M, pBR322 DNA digested with *Hin*fI.

Results

HPV/EBV detection by PCR

To show the detection of a broad spectrum of HPV genotypes by GP-PCR a mixture of the primer pairs GP5/6 and GP11/12 was used in the PCR on a set of cloned HPV genotypes (Figure 1). Cutaneous, genital, and oral HPV types yielded specific PCR products of the expected size (approximately 140 to 150 bp). The sensitivity of this method appeared to be at the subpicogram level, as shown in reconstruction experiments and as published previously (17).

In Figures 2a and b, the results of GP-PCR on clinical samples are shown. The demonstration of HPV sequences was limited to two oral warts of one HIV-infected patient (Figure 2, lanes 23 and 24). Human papillomavirus positivity of these specimens was clear after hybridization with the HPV cocktail probe (Figure 2b).

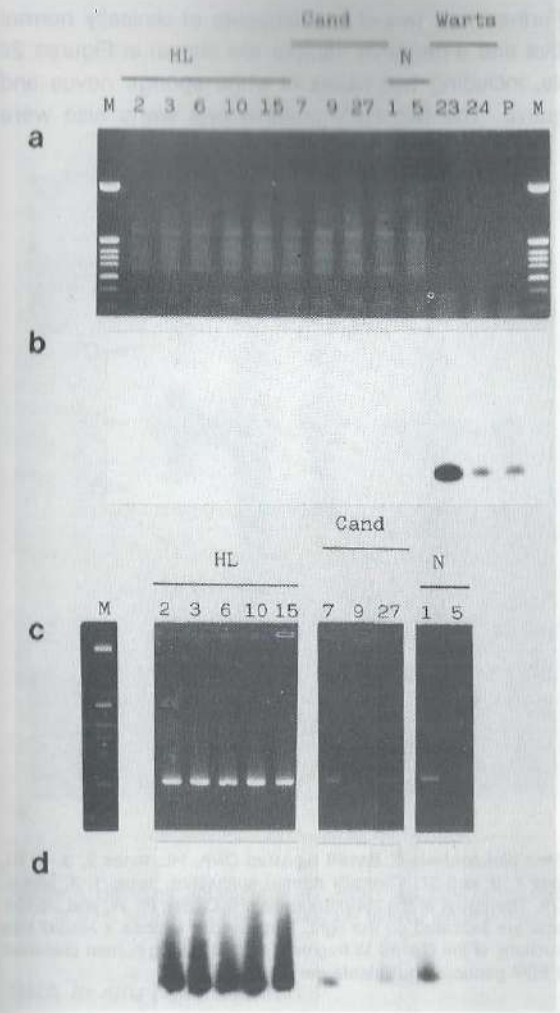

Figure 2. HPV- (a,b) and EBV- (c,d) PCR on clinical samples. PCR products are shown after agarose gel electrophoreses, ethidium bromide staining, and ultraviolet illumination (a,c), as well as after subsequent blotting and hybridization (b,d) with a cocktail probe of HPV-specific amplification products (b) and the EBV *Bam*HI W fragment (d), respectively. a: The open circle in lane 23 indicates the HPV-specific amplification product. p, papillomatous lesion on the tongue of a patient without sign of HIV infection; M, pBR322 DNA digested with *Hin*fI; HL, hairy leukoplakia; Cand, candidiasis; N, clinically normal epithelium.

Further characterization by dot blotting demonstrated that both warts contained HPV type 32 DNA (not shown). A control specimen of a papillomatous lesion on the tongue also scored HPV positive by GP-PCR (Figure 2, lane p) and the HPV type was also identified as HPV 32 by dot blot analysis. All 12 HL specimens (Figures 2a and b, lanes 2,3,6,10 and 15), three cases of candidiasis (Figures 2a and b, lanes 7,9 and 27) and clinically normal epithelium (Figures 2a and b, lanes 1 and 5) were negative.

The EBV-PCR (Figures 2c and d) demonstrated a high prevalence of EBV DNA in oral biopsies from HIV-infected patients. Of the 21 biopsies examined, a total of 16 specimens (76%) were found to contain EBV sequences. Of the 15 clinically white lesions, 14 (93%)

were found to contain EBV DNA, including all 12 lesions showing histologic features consistent with HL (Figures 2c and d, lanes 2,3,6,10 and 15) and two cases of candidiasis (Figures 2c and d, lanes 7 and 27). Furthermore two of four biopsies of clinically normal epithelium also were positive. A positive and a negative sample are shown in Figures 2c and d (lanes 1 and 5). Seven controls, including two cases of white sponge nevus and three cases of leukoplakia were negative. The two HPV positive oral warts also were negative (data not shown).

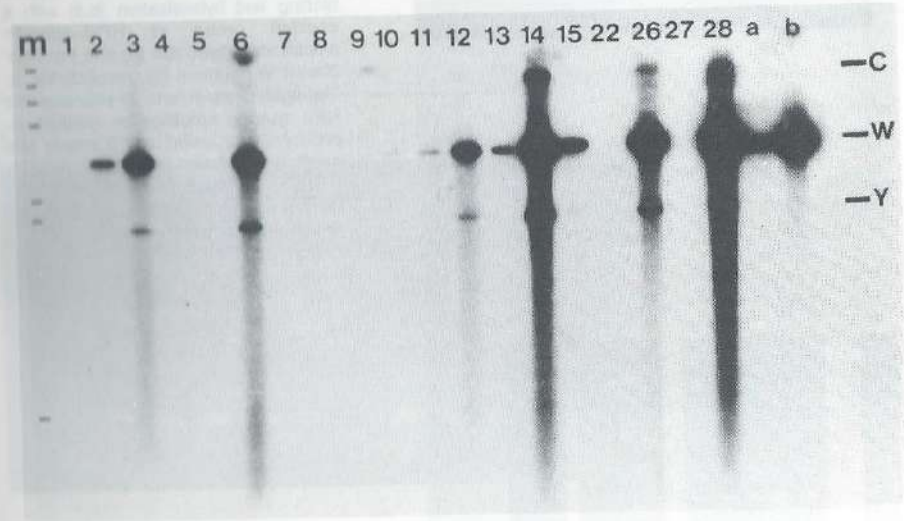

Figure 3. EBV detection in oral tissue by Southern blot analysis of *Bam*HI digested DNA. HL, lanes 2, 3, 6, 10, 11, 12, 13, 14, 15, 26, and 28. Candidiasis, lanes 7, 9, and 27. Clinically normal epithelium, lanes 1, 4, and 5. Leukoplakia, lane 8. White sponge nevus, lane 22. The levels of the hybridizing *Bam*HI C, *Bam*HI W, and *Bam*HI Y fragments of the IR1 region of the EBV genome are indicated on the right. Furthermore lambda x *Hin*dIII size markers (m) are indicated, in addition to reconstructions of the *Bam*HI W fragment diluted in 1 μg human placental DNA corresponding to about 20 (a) and 200 (b) EBV genome equivalents per cell.

Southern blot analysis

For Southern blot analysis the *Bam*HI W fragment of the EBV genome was used as probe. This fragment allows the detection of tandem repeated *Bam*HI W fragments of about 3.1 kilobases (kb) and in addition two flanking *Bam*HI fragments C and Y (Figure 3). All 11 HL specimens examined, were found positive for EBV DNA (Figure 3, lanes 2,3,6,10,11,-12,13,14,15,26 and 28). The other oral lesions (candidiasis; Figure 3, lanes 7,9 and 27), clinically normal epithelium (Figure 3, lanes 1,4 and 5) and two controls, a white sponge nevus and leukoplakia (Figure 3, lanes 8 and 22) were negative. Comparison of the hybridizing signals with those of the reconstructions (Figure 3, lanes a and b) revealed that most HLs contain a copy number of EBV DNA corresponding to approximately 50 (lanes 6, 10, and 11) to 200 (lanes 14, 26, and 28) EBV-DNA equivalents per cell. This value was

calculated after correction for low amounts of DNA input (Figure 3, lanes 2,3,10, and 11) and the determination of the epithelium/stroma ratio of the sample.

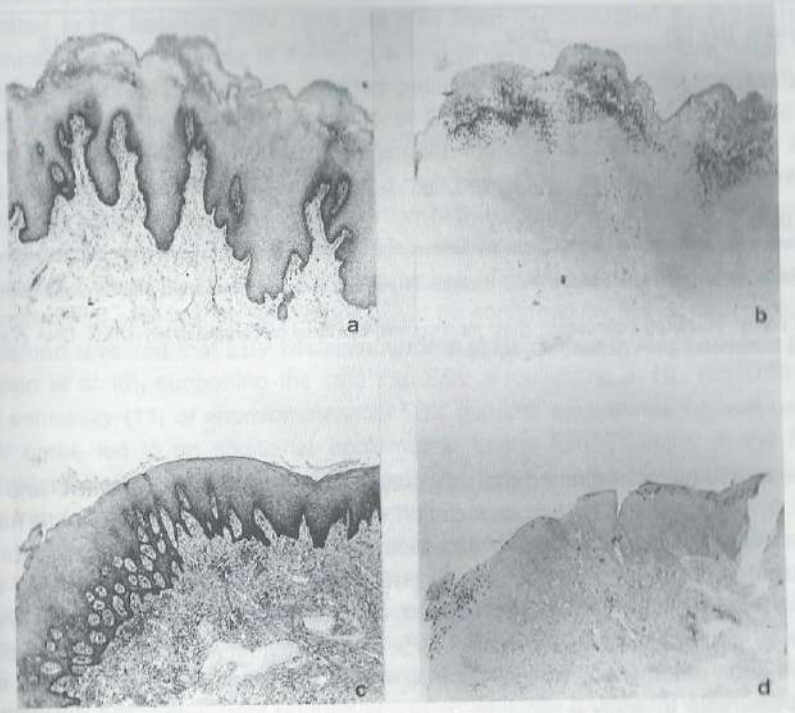

Figure 4. Histologic appearance (a,c; H&E) and EBV demonstration by bio-DISH with the EBV *Bam*HI W probe (b,d) of a HL biopsy (a,b) and a clinically normal gingival biopsy (c,d) (original magnification x 50).

DNA *in situ* hybridization

All biopsies were subjected to bio-DISH using the EBV *Bam*HI W probe. All 12 HL specimens scored positive for EBV DNA, which is consistent with results of the PCR and Southern blot procedures. The location of EBV DNA in HL was limited to the upper most spinous cell layers of the epithelium (Figures 4a and b). In addition the clinically normal gingival epithelium with an EBV positivity by PCR, but not by Southern blot analysis, was found to be EBV positive by bio-DISH (Figures 4c and d).

All the clinical findings and the results of different DNA detection techniques are summarized in Table 1.

Table 1. HPV and EBV DNA detection in oral mucosa of HIV-infected patients

	HPV	EBV		
Variable	GP-PCR	PCR*	SB	bio-DISH
Hairy leukoplakia (N=12)	0/12	12/12	11/11	12/12
Candidiasis (N=3)	0/3	2/3	0/3	0/3
Warts (N=2)	2/2	0/2	ND	0/2
Normal gingiva (N=3)	0/3	1/3	0/2	1/3
Normal tongue (N=1)	0/1	1/1	0/1	0/1

* PCR was performed on paraffin-embedded tissue sections of one HL, two oral warts, and one clinically normal epithelium.
GP-PCR, general primer-mediated polymerase chain reaction; SB, Southern blot analysis; bio-DISH, DNA *in situ* hybridization using biotinylated DNA probes; ND, not done.

Discussion

Initial studies on HL have suggested the association with both a papillomavirus and a herpesvirus (4, 5). However the regular association of HL with an HPV genotype still has not been demonstrated. A problem for HPV detection is the existence of a broad spectrum of HPV types, of which 60 different types already have been isolated and characterized to date (21). Until recently, the HPV-PCR required accurate sequence information and consequently was applicable only to the nine HPV genotypes, of which the nucleotide sequences have been determined. However, for a reliable assessment of the presence of HPV in HL, it is desirable to apply a sensitive detection method that permits the detection of a broad spectrum of HPVs. To ensure the detection of multiple HPV types, a modified PCR method was applied, which is based on general primers that anneal under moderate stringency conditions to the highly conserved L1 ORF of many, including yet unsequenced HPV genotypes (17,19). Initial studies with the two general primer pairs GP5/6 (selected for mucosotropic HPVs) and GP11/12 (selected for cutaneous HPVs) revealed that this method enables the detection of a broad spectrum of HPVs, including HPV types 1, 6, 8, 11, 13, 16, 18, 30, 31, 32, 33, 45, and 51 (17). In addition to the common HPV types 6, 11, 16, 18, 31, and 33, less common and yet unsequenced HPV types have been detected in cervical scrapes by GP-PCR (19). Therefore the lack of GP-PCR positivity in the HL specimens suggests that HL is not regularly associated with a broad spectrum of HPV types. However, that other HPV types that do not react with the GP pairs are preferentially associated with HL cannot be excluded.

The amplified DNA sequences in the two oral warts could be typed as HPV 32 specific. HPV 32 originally was isolated from an oral focal epithelial hyperplasia (22) and has also been found in oral warts of HIV-infected patients (3). The absence of common HPV types in the other oral lesions examined is remarkable and unexpected. Maitland *et al.* (23) found that HPV sequences, predominantly of HPV type 16, occurred in a high proportion (80%) of biopsies taken from areas of keratosis and lichen planus and even in 41% of unaffected

oral mucosal biopsies from the general population. However, in the present study, special precautions such as physical separation of the PCR actions and a strong laboratory discipline were used to prevent false positivity due to contamination (17, 19, 25).

The association of HL with EBV has been well documented (5,7,8). However it has been reported also that the presence of EBV in oral tissue of HIV-infected patients is not strictly limited to HL because EBV DNA has also been demonstrated by *in situ* hybridization in clinically normal mucosa of the tongue and gingiva (8). Furthermore it has been reported that a high proportion of HIV-infected patients are shedding EBV from the oropharyngeal mucosa (6). Therefore the presence of EBV demonstrated by PCR could indicate both shedding of EBV from the oropharyngeal mucosa or EBV infection of the oral tissue studied. Although the PCR revealed a high prevalence rate of EBV in oral tissues of HIV-infected patients, a significant proportion of these samples was scored negative (Table 1).

In addition to PCR, the Southern blot method and bio-DISH methods were applied for a reliable assessment of the EBV prevalence in oral tissue. It appeared that the EBV PCR positivity of only the HL specimens could be confirmed by the Southern blot method. The method revealed that EBV DNA is abundant in HL. These findings confirm data of Greenspan *et al.* (5), supporting the idea that EBV is replicating in HL. Bio-DISH analysis with a sensitivity (11) of approximately 25 EBV genome equivalents per cell using the *Bam*HI W probe led to an additional confirmation of the EBV positivity in the HL specimens. Epstein-Barr virus DNA was localized mainly in a large amount of cells in the upper spinous layer of the HL epithelium, which is consistent with data of previous studies (6,8). In one additional clinically normal gingival specimen of a patient with HL, focal EBV signals could be detected by bio-DISH. The relation between this site of EBV infection and the coexisting HL is still unknown. Because it concerned a focal signal, the total EBV copy number might have been too small to allow detection by the Southern blot method. The Southern blot method, which has a greater sensitivity than the bio-DISH in detecting uniformly infected tissue (11), requires approximately 20,000 EBV genomes in 1 μg cellular DNA (corresponding to an average of 0.1 viral genomes per cell) for a minimal detection.

The significance of the presence of low copy numbers of EBV DNA in the remaining PCR positive biopsies is still unknown. Using the PCR, Sixbey and coworkers (24) could make a clear distinction in the demonstration of two EBV strains (types A and B) in throat washings. Although in throat washings of HIV-infected patients both EBV types could be demonstrated, there were indications that HL consistently contains EBV type B (24). Therefore the significance of oropharyngeal or oral EBV infection for the development of HL could depend on the type of EBV.

We have shown definitively that in our patients HL was not associated with common HPV genotypes. However a strong association was found between EBV and HL, but this association was not exclusive.

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CHAPTER VI

Prevalence and expression of human papillomavirus in tonsillar carcinomas indicates a possible viral etiology

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Abstract

The presence of human papillomavirus (HPV) DNA was assessed in biopsies of tonsillar carcinomas (n=10) and cases of tonsillitis (n=7), serving as controls, by general primer-mediated PCR (GP-PCR). All carcinomas appeared HPV positive whereas all cases of tonsillitis were HPV negative. Additional type-specific PCR for HPV-6, -11, -16, -18, -31 and -33 revealed that four carcinomas contained HPV 16 DNA, four carcinomas contained HPV 33 DNA and one carcinoma contained an HPV 16/33 double infection. False positivity was excluded by additional Southern blot analysis of type-specific PCR positive samples (n=4). Further characterization of GP-PCR products by sequence analysis revealed that two carcinomas contained still unknown HPV genotypes, one carcinoma which also contained HPV 33 DNA and one carcinoma that was negative by type-specific PCR. Application of RNA PCR revealed expression of HPV 16 or HPV 33 E7 encoding spliced E6*I transcripts in all tonsillar carcinomas (n=4) examined. Additional nonradioactive RNA *in situ* hybridization performed on three biopsies revealed the presence of HPV 16 or HPV 33 E7 transcripts exclusively localized within the carcinoma cells whereas stroma stained negative. These findings strongly support a role for certain HPV types in the pathogenesis of tonsillar carcinomas.

Introduction

To date increasing experimental data have emerged which support the concept that certain human papillomavirus (HPV) types play an etiological role in human anogenital cancer, particularly carcinomas of the cervix (zur Hausen and Schneider, 1987). The postulated interaction between HPV infections and chemical factors associated with cigarette smoking and alcohol consumption (zur Hausen, 1986) have suggested a role for HPV in the development of carcinomas within the upper aerodigestive tract as well (zur Hausen, 1989). HPV DNA has been detected in carcinomas of the oral cavity (Löning *et al.*, 1985; de Villiers *et al.*, 1985), tonsil (Brandsma and Abramson, 1989; Niedobitek *et al.*, 1990) and larynx (Brandsma *et al.*, 1986; Kahn *et al.*, 1986). However, there is still insufficient evidence to support strongly the hypothesis that there exists a causal relationship between HPV infections and carcinomas from this tract. This is partly due to reported HPV prevalence rates being variable and generally lower than those found in genital carcinomas. Moreover, studies thus far have focussed primarily upon HPV genotype detection, thereby survying a limited field and leaving the involvement of viral properties like expression, especially of the transforming genes E6 and E7, an open question.

HPV DNA detection studies have been hampered by the existence of more than 60 different HPV types (de Villiers, 1989), while practical limitations of conventional DNA detection methods only allowed a small subset of them to be screened for with an optimal sensitivity. Consequently, HPV types that do not react with specific hybridization probes or primers could have been missed in previous studies. Recently, we have developed a general primer-mediated PCR method (GP-PCR) for the detection of a broad spectrum of HPV genotypes (Snijders *et al.*, 1990), which appeared succesful in detecting both known and still unidentified HPV genotypes in cervical scrapes (van den Brule *et al.*, 1990).

Since tonsillar carcinoma is the most dominant oropharyngeal malignancy which account for about 75% of carcinomas of this region (G.B. Snow, personal communication), we first studied the HPV prevalence in this type of carcinomas. The HPV GP-PCR method and HPV type-specific PCR in combination with genomic Southern blot analysis were employed for HPV detection, typing and confirmation, respectively. Furthermore, transcription of the E7 open reading frame (ORF), encoding a major transforming gene (Vousden *et al.*, 1988), was examined using HPV RNA PCR and RNA *in situ* hybridization. The results strongly support an active role for certain HPV types in the pathogenesis of tonsillar carcinomas.

Materials and methods

Clinical specimens and viral DNA clones

Of biopsy specimens of tonsillar carcinomas taken from ten patients, one part was snap-frozen and stored in liquid nitrogen until use. A second part of the biopsies was fixed in 4% buffered formalin and embedded in paraffin for histologic examination and eventually RNA *in situ* hybridization. Six patients were male and four patients were female. The age of patients ranged from 46 to 79 years and the mean age was 64 years. Five carcinomas were poorly differentiated, one was moderately differentiated, three were well differentiated and one was an undifferentiated squamous cell carcinoma. In addition seven tonsils surgically removed for tonsillitis were snap-frozen and served as control specimens.

DNA of HPV 16 cloned into the *Bam*HI site of pBR322 (Dürst *et al.*, 1983) and of HPV 33 cloned into the Bgl II site of pBR322 (Beaudenon *et al.*, 1986) was purified from vector sequences by agarose gel electrophoresis and subsequently used as probe.

Isolation of DNA and cytoplasmic RNA

Snap-frozen samples were serially sectioned (10-15 sections) on a cryostate. The first and last sections (5 μm) were used for hematoxylin eosin (HE) staining to assess the percentage of carcinoma cells present in these samples. The intermediate sections were used for DNA and RNA isolation. DNA was extracted as described by Walboomers *et al.* (1988) and cytoplasmic RNA was isolated according to Gough (1988) as previously described (van den Brule *et al.*, 1991a). The integrity of RNA samples was determined by agarose gel electrophoresis and only the samples that clearly showed the 28S and 18S ribosomal RNA fragments without any sign of degradation were taken into consideration for RNA PCR analysis.

DNA polymerase chain reaction

The GP-PCR was performed as described previously with the GP5 (5'-TTTGTTACTGTGGTAGATAC-3')/GP6 (5'-GAAAAATAAACTGTAAATCA-3') primer combination (Snijders *et al.*, 1990). To allow subsequent cloning of PCR products for sequence analysis, sequences containing the *Bam*HI recognition site were added to the 5'-ends of GP5 and GP6. Type-specific PCR was carried out with cloning site flanking primers as described by van den Brule *et al.* (1990) except that separated PCR assays instead of multiplex PCR were performed. Additional type-specific PCR was performed with an E6/E7 region primer combination specific for HPV 16 (E6: nt 141 5'-CACAGTTATGCACAGAGCTG-3' and E7: nt 625 5'-TAATTGCTCATAACAGTAGAG-3') and HPV 33 (E6: nt 144 5'-GCATGATTTTGTGCCAAGCAT-3' and E7: nt 636 5'-

TAATTGCTCATAGCAGTATAG-3'). PCR reaction mixtures contained 50 to 100 ng purified DNA of clinical samples, 50 mM KCl, 10 mM Tris HCl, pH 8.3, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 50 pmol of each primer of either the GPs or type-specific primer pairs, and 1 U of thermostabile DNA polymerase (Amplitaq; Cetus). Mixtures were incubated for 5 minutes at 94°C for DNA denaturation. Subsequently, 40 cycles of amplification were performed using a PCR processor (Biomed). Each cycle included a denaturation step to 94°C for 1 minute, an annealing step to 40°C (for the HPV GPs) or 55°C (for the type-specific primer pairs) for 2 minutes and a chain elongation step to 72°C for 1.5 minute. The final elongation step was prolonged for another 4 minutes. To avoid contamination by cloned pHPV DNA or PCR products, different steps like sample preparation and amplification reaction were performed in physically separated rooms and samples containing distilled water were included as negative controls. Ten μl of the PCR mixtures was finally analyzed by agarose gel electrophoresis. Subsequently, DNA fragments were transferred onto nylon membranes (GeneScreenPlus; Du Pont) by diffusion blotting in 0.5 N NaOH, 0.6 M NaCl. A mixture of GP5/6 directed PCR products derived from cloned HPVs HPV-6, -11, -16, -18, -31 and -33 was used as probe for analysis of GP-PCR products. For type-specific analysis specific oligonucleotide probes were used as described elsewhere (van den Brule *et al.*, 1990). Hybridizations were performed at 55°C in hybridization mixture containing 0.5 M sodium phosphate, pH 7.4, 7% SDS, 1 mM EDTA for 16 hours. Subsequent washings were carried out in 3xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.5% SDS at 55°C. Autoradiography was performed overnight at -80°C with Kodak Royal X-omat film and intensifying screens.

Genomic Southern blot analysis

Restriction enzyme digestions were carried out according to recommendations of the manufacturer (Pharmacia). DNAs were separated on 0.7% agarose gels and transferred onto nylon membranes as described above. Hybridization was performed with HPV 16 or HPV 33 DNA at 65°C for 16 hours. The filters were washed two times in 3xSSC, 0.5% SDS for 15 minutes at 65°C, once in 0.5xSSC, 0.5% SDS at 65°C for 15 minutes and twice in 0.1xSSC, 0.5% SDS at 68°C for 15 minutes. Autoradiography was performed for 5 days at -80°C with Kodak Royal X-omat film and intensifying screens.

DNA sequencing

Sequencing of the GP-PCR products was carried out after cloning fragments into the *Bam*HI site of M13mp18/mp19 vector DNA. At least three independent clones were analysed in order to reduce false sequence information that may be the result of incorporation errors by *Taq* DNA polymerase during PCR. Sequencing was performed using the dideoxy chain termination reaction with a T7 polymerase sequencing kit (Pharmacia) according to instructions of the manufacturer.

RNA polymerase chain reaction

RNA PCR was performed as described previously (van den Brule *et al.*, 1991a; Chapter VII) using the HPV 16 or HPV 33 specific E6/E7 primers, the E7 primers serving as antisense primer used for first strand cDNA synthesis. Briefly, one strand of cDNA was

synthesized in a final reaction volume of 50 μl containing 200 ng cytoplasmic RNA, 25 pmol antisense primer, 50 mM Tris.HCl pH 8.3, 60 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM of each dNTP, 40 units RNasin (Promega) and 7 units AMV reverse transcriptase (RT; Promega). The mixture was incubated at 42°C for 45 minutes. In addition reactions were performed without RT added to allow distinction between final RNA PCR products and products derived from eventually copurified DNA. Ultimately, 5 μl of the cDNA mixture was used for PCR. PCR products were analysed on 1.5% agarose gels and specificity was determined by hybridization with cloned HPV 16 or HPV 33 DNA under stringent conditions as described above.

Nonradioactive RNA *in situ* hybridization

RNA *in situ* hybridization (RISH) was performed as described by van den Brule *et al.* (1991a) for detection of HPV 16 specific E7 mRNA, using β-actin RISH as quality control. Sense and antisense RNAs generated from a *Sau*3A-*Pst*I fragment from the HPV 16-E7 ORF (nt 622-879; kindly provided by Dr. H. Smits, Dept of Virology, University of Amsterdam) and a 156 bp *Msp*I-*Bam*HI fragment from the 5' part of the human β-actin cDNA (Clontech Lab.) cloned into Gemini 4 (Promega) were used as probe. In addition, for detection of HPV 33 specific E6/E7 region mRNA a probe was generated from the amplified 236 bp HPV 33 E6*I cDNA fragment (see Figure 4) which was cloned into the *Bam*HI site of the pBluescript II SK+ vector (Stratagene). This E6/E7 fragment spans a splice junction between nts 231 and 509 within the E6 ORF (see Chapter VII). Biotinylated sense and antisense RNA probes were generated according to Melton *et al.* (1984) with biotin-11-UTP (ENZO Biochemical) using T3 and T7 RNA polymerase (Promega), respectively.

After successive steps of pre-treatment of the formalin fixed tissue section, hybridization and staining (van den Brule *et al.*, 1991a), images were ultimately visualized by confocal laser scan microscopy with reflex contrast using a type II laser scan microscope with synchronously scanned pinhole in the detector beam path, a servo-controlled galvanometer scanner and a dual laser system (Ar, 488 nm; HeNe, 622 nm; Zeiss).

Results

Detection of HPV DNA

The strategy followed in this study was to examine tonsillar biopsy specimens first for the presence of HPV DNA by applying the GP-PCR method with the GP5/GP6 primer pair and subsequently to identify certain sequenced mucosotropic HPV genotypes by successive performance of PCR with primers specific for HPV-6,-11,-16,-18,-31 and -33. The HPV PCR typing results are included in Table 1. All ten carcinoma biopsies examined revealed a hybridizing HPV specific GP-PCR fragment, as illustrated for seven specimens in Figure 1A. The seven cases of tonsillitis, serving as non-malignant controls, appeared GP-PCR negative. Amplification of β-globin sequences with primer pair PCO3/PCO4 (Saiki *et al.*, 1988) was accomplished with all samples, indicating that the failure to react with GP5/GP6 is not owing to inpurities or degradation of target DNA. Type-specific PCR using E6/E7 region primers revealed that four carcinomas contained HPV 16 DNA (Figure

1B, lanes T2, T5 and T7), four carcinomas contained HPV 33 DNA (Figure 1C, lanes T6 and T8) and one carcinoma showed positivity both for HPV 16 and HPV 33 (Figure 1B and C, lane T4). These typing results were confirmed by PCR with cloning site flanking primers localized within the L1 (for HPV 16) or E1/E2 (for HPV 33) ORFs (not shown). In case of two carcinoma specimens, results of GP-PCR and type-specific PCR were not in agreement, suggesting the presence of HPV types different from the common genital HPVs. This concerns a carcinoma that was negative by type-specific PCR (Figure 1, lane T3) and a carcinoma showing a rather weak HPV 33 specific amplification signal in comparison with the GP-PCR signal, suggesting that this sample contains a double infection (Figure 1, lane T8). All samples were negative for the remaining mucosotropic HPV types 6, 11, 18 and 31.

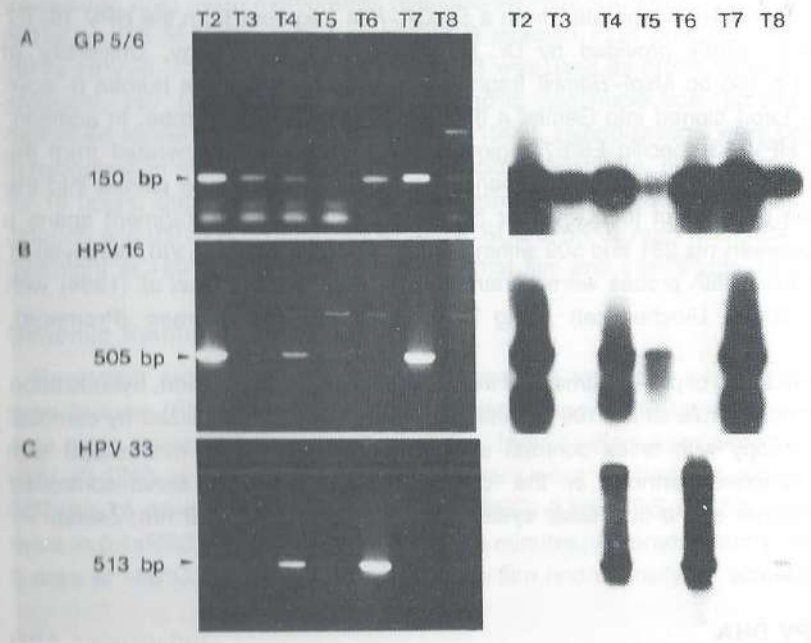

Figure 1. HPV PCR results of seven tonsillar carcinoma biopsies. A) GP-PCR with the GP5/GP6 primer pair. B) HPV 16 specific PCR with E6/E7 primers. C) HPV 33 specific PCR with E6/E7 primers. Agarose gel patterns are shown on the left and results after Southern blot hybridization are shown on the right. Arrowheads point to the level of HPV specific amplification products and sizes are indicated.

Furthermore, genomic Southern blot analysis was applied to confirm type-specific PCR results. By this method HPV DNA of the corresponding type was detected in two out of three HPV 16 PCR positive cases and one out of two cases positive for HPV 33 DNA PCR, as shown in Figure 2. Histologic examination revealed that the two carcinoma specimens which could not be confirmed by genomic Southern blot analysis contained a rather small proportion (less than 10%) of carcinoma cells. In addition, Southern blot

analysis allowed the detection of HPV 33 DNA but not HPV 16 DNA in the HPV 16/33 double infected case, as determined by PCR (Table 1).

Table 1. HPV DNA and RNA detection in tonsillar carcinomas by different techniques

HPV genotype	PCR positive[1]	Southern blot positive[2]	RNA PCR positive[3]	RISH positive[4]
HPV 16	4/10	2/3[5]	1/1	1/1
HPV 33	3/10	1/2[5]	1/1	1/1
HPV 16/33	1/10	1/1[6]	1/1[6]	1/1[6]
HPV X	1/10	nd	nd	nd
HPV X/33	1/10	nd	1/1[6]	nd

[1]PCR data were based on results of GP- and type-specific PCR
[2]Samples containing sufficient DNA were subjected to Southern blot analysis
[3]Samples containing undegraded RNA were subjected to RNA PCR
[4]Samples positive for β-actin RISH were subjected to HPV RISH
[5]Southern blot negative biopsies contained less than 10% carcinoma cells per sample
[6]Positive for HPV 33
nd, not done

The presence of still unknown HPVs in the two samples T3 and T8 was confirmed by cloning and DNA sequence analysis of their GP-PCR products. As shown in Figure 3, deduced amino acid sequences of these GP-PCR fragments were found to fulfill the HPV consensus sequence, recently determined on basis of sequence information of the L1 region flanked by the GP5 and GP6 primers from 22 HPV genotypes (see Chapter IV). Homology comparison revealed that the amplified sequences do not represent HPV-1a,-2a,-5,-6,-8,-11,-13,-16,-18,-31,-32,-33,-35,-39,-43,-44,-45,-47,-51,-56,-57,-58 or six still unidentified HPVs detected in cervical scrapes, indicating that they might represent novel HPV types.

Detection of HPV 16 and HPV 33 E7 encoding transcripts in tonsillar carcinoma biopsies by RNA PCR

Both for HPV 16 (Smotkin *et al.*, 1989) and HPV 33 (Chapter VII) the putative E7 mRNAs are bi- or poly-cistronic and contain an intron within the E6 ORF (E6* mRNAs). For HPV 16 two forms (designated as E6* I and E6*II) have been identified which differ in the splice acceptor site utilized, whereas for HPV 33 only one form (E6*I) has been discovered with E7 coding potential. Recently, it has been shown that these mRNAs easily can be detected by RNA PCR with HPV 16- (Cornelissen *et al.*, 1990; van den Brule *et al.*, 1991a) and HPV 33- (Chapter VII) specific E6/E7 primers flanking the E6 splice sites.

Four carcinomas from which undegraded RNA could be purified as determined by agarose gel analysis were analysed by E6/E7 RNA PCR (Table 1). These include one HPV 16 positive specimen, the HPV 16/33 double infected sample and two specimens containing HPV 33 DNA. The HPV 16 positive sample revealed two amplified cDNA fragments, 505 bp and 323 bp in size (not shown), representing full length E6/E7 mRNA and spliced E6*I mRNA (van den Brule *et al.*, 1991a), respectively. No HPV 16 specific cDNA was amplified from the HPV 16/33 double infected sample. However, in addition to the two HPV 33 positive specimens, this sample appeared to contain HPV 33 E6*I mRNA, as reflected by the production of a 236 bp cDNA fragment (see Chapter VII), shown for two biopsies in Figure 4 (lanes T4+ and T6+). In one HPV 33 positive case also the full length 513 bp fragment was amplified (Figure 4, lane T6+). Since the reaction without reverse transcriptase added also yielded a 513 bp PCR product (Figure 4, lane T6-) it is unclear to which extent this fragment is derived from full length mRNA or from copurified DNA.

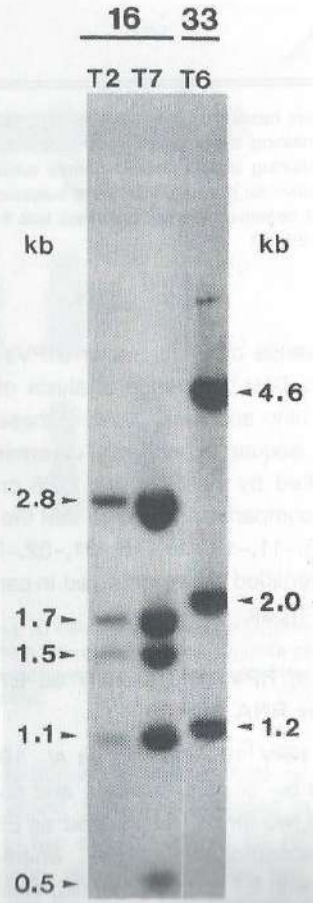

Figure 2. Genomic Southern blot analysis with *Pst*I of two HPV 16 containing carcinomas (T2 and T7) and one HPV 33 containing carcinoma (T6). Sizes of HPV 16 and HPV 33 *Pst*I fragments are indicated on the left and right, respectively.


```
T3 : T R S T N L T L C AATQSPTPTP Y D N S K F K Q Y L R H G E E
     : : : : :   : : :                    :         : : : :   : :
HPV: T R S T N X T I C 8 to 13 aa Y X X X X Y K E Y X R H X E E
       N   S   S L S                  F       F R Q F
       D       V                              I N D
     : : : : :   : : :                    :         : : : :   : :
T8 : T R S T N L S V C ASTTASIPNV Y T P T S F K E Y A R H V E E
```

Figure 3. Alignment of deduced amino acid sequences of GP5/GP6 flanked L1 sequences amplified from carcinoma samples T3 and T8 with the HPV consensus sequence (shown in the middle) determined on basis of information from 22 HPV genotypes (see Chapter IV). Matched amino acids are indicated by dots. The middle part of the sequence, consisting of 8 to 13 amino acids is polymorphic and was not included in the alignment. aa= amino acids.

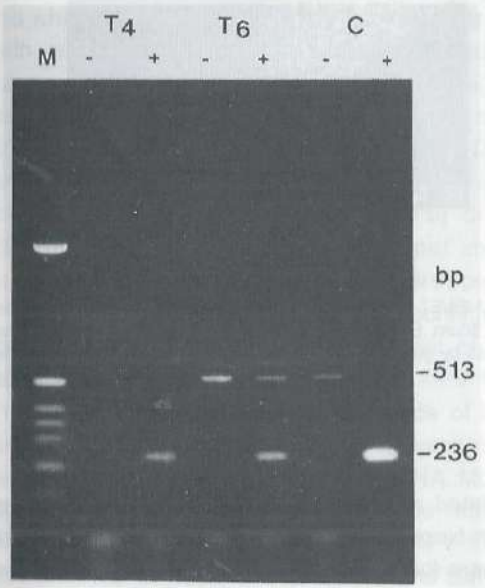

Figure 4. RNA PCR analysis of samples T4, T6 and a HPV 33 containing cervical carcinoma (C) serving as positive control with the HPV 33 E6/E7 primer pair. Reactions were performed with (+) and without (-) reverse transcriptase added during the first strand cDNA synthesis step. Levels of full length fragments (513 bp) and fragments derived from E6*I mRNA, spliced between nucleotides 231 and 509 within the E6 ORF (236 bp), are indicated on the right. M: pBR322 x *Hin*fI size markers.

Nonradioactive RNA *in situ* hybridization

In order to investigate RNA quality for RNA *in situ* hybridization (RISH) analysis, samples were prescreened by β-actin RISH. β-actin mRNA detection showed positivity for one HPV 16 containing case, one HPV 33 containing case and the HPV 16/33 double infected case. Therefore, for a spatial resolution of E7 transcripts these biopsies were subjected to HPV RISH using sense and antisense RNA probes specific for HPV 16 or HPV 33. No signals were obtained with the sense probes used (not shown). As shown in Table 1, the HPV 16 antisense probe yielded specific signals in the HPV 16 containing case, but not in the HPV 16/33 double infected case, similar to the RNA PCR results. However, with the HPV 33 antisense probe signals were obtained in the HPV 16/33 double infected case and the HPV

33 positive case. Signals were exclusively localized in the carcinoma cells whereas stroma stained negative, as shown for the latter case by comparison of the HE stained section (Figure 5A) with the convocal laser scan image of the hybridized section (Figure 5B).

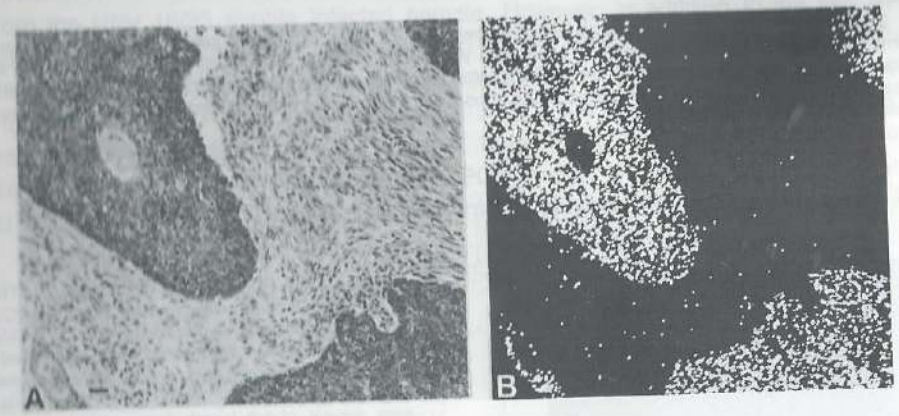

Figure 5. Nonradioactive RNA *in situ* analysis of HPV 33 E6/E7 transcripts in biopsy specimen T6. A) Section after hematoxylin-eosin staining and light microscopy. Bar = 50μm. B) Serial section following RNA *in situ* hybridization with the HPV 33 E6*I antisense probe. Visualization was by gold/silver enhancement staining and confocal laser scan microscopy.

Discussion

A combination of the general primer-mediated and type-specific PCR methods allowed the detection and typing of HPV DNA in ten biopsies of tonsillar carcinomas. In contrast to seven cases of tonsillitis all carcinomas were found to contain HPV DNA as determined by PCR. Type-specific analysis revealed that 5 cases contained HPV 16 DNA and 5 cases contained HPV 33 DNA, including one HPV 16/33 double infection. In order to minimize false positive results due to contamination with PCR products or cloned HPV DNA both E6/E7 and cloning site flanking primers specific for HPV 16 or HPV 33 were employed, which gave identical results. The tonsillar carcinoma biopsies included two cases containing a still unidentified HPV genotype. This was concluded from the GP5/GP6 directed amplification of thus far unique sequences that hybridized with the probe and fulfilled the HPV amino acid consensus sequence. Since GP5/GP6 directed sequences amplified from cellular DNA have failed to show any homology with the HPV consensus (Chapter IV) it is justified to designate these products as HPV specific. As shown in Chapter IV, homologies of GP5/GP6 flanked L1 sequences between highly related HPV types HPV 6 vs HPV 11 and HPV 18 vs HPV 45 were 84% and 80%, respectively. The highest observed homologies of the T3 and T8 products were 71% and 60% with HPV 45 and HPV-18, -31, -39, -45, respectively, suggesting that these products represent different types rather than subtypes of any of the HPVs that were included in the

comparison. Molecular cloning of these unknown HPV types is currently in progress and will offer the possibility of future type-specific screening in lesions of the upper aerodigestive tract.

Recently, HPV 16 genotypes have been demonstrated in oral specimens that did not react with the HPV 16 cloning site flanking primers as a result of an alteration in the L2-L1 region (Yeudall and Campo, 1991). Our results indicate that the HPV 16 and HPV 33 genotypes present in the tonsillar carcinomas studied do not contain major alterations affecting L1 sequences, which is supported by genomic Southern blot analysis showing the typical HPV 16 and HPV 33 prototype *Pst*I fragments (Figure 2).

Preliminary studies on carcinomas from other sites within the aerodigestive tract (i.e. oral cavity and larynx) revealed HPV prevalence rates which do not exceed 30% (see Chapter IX). This indicates that this high HPV prevalence rate is not common to carcinomas from the whole head and neck region but thusfar is specific for tonsillar carcinomas. Compared with cervical carcinomas a different distribution of HPV genotypes amongst tonsillar carcinomas was evident, especially concerning HPV 33. In a similar study that recently has been performed on 50 cervical carcinomas collected from the same geographic region, 68% of cases were found to contain HPV 16 DNA, 10% showed positivity for HPV 18 DNA and 16% of cases contained an HPV 16/18 double infection, whereas only a minority of cases appeared to contain HPV 31 (4%) or HPV 33 (2%) DNA (van den Brule *et al.*, 1991b). Thus, HPV 33 can be of greater importance in carcinogenesis than has been suggested on basis of its association with cervical cancer.

Using the genomic Southern blot technique Brandsma and Abramson (1989) have detected HPV 16-related DNA in 2 out of 7 (29%) tonsillar carcinomas whereas all samples were negative for HPV 11 and HPV 18 DNA. In a different study Niedobitek *et al.* (1991) have demonstrated the presence of HPV 16 DNA by *in situ* hybridization in the tumour cells of 6 out of 28 (21%) biopsies of tonsillar carcinomas whereas all samples were negative for HPV 6 and HPV 11 DNA. Moreover, both groups have not revealed HPV positivity in control tonsil samples. The results obtained in this study are similar with respect to the failure to detect HPV 6, HPV 11 or HPV 18 DNA in the carcinomas and the documented absence of HPV DNA in the control samples. The different prevalence rates found, may reflect the detection methods used, the hybridization methods generally being not as sensitive as the PCR and having less universal properties than the GP-PCR method. Since both groups have not specifically probed with HPV 33, a substantial amount of HPV positivity could have been missed.

Based upon *in vitro* studies of HPV 16 and HPV 18 the E6 and E7 genes are considered the transforming genes (Barbosa *et al.*, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989) and their consistent expression has been demonstrated in cervical carcinomas and cell lines derived from cervical carcinomas (Baker *et al.*, 1987; Schneider-Gädicke and Schwarz, 1986; Smotkin and Wettstein, 1986). We focussed on the detection of putative E7 transcripts which, due to intron sequences spliced out, easily can be detected by RNA PCR without interference of eventually copurified DNA. HPV specific E6*I cDNA was detected in all carcinomas examined. In the HPV 16/33 double infected biopsy only HPV 33 E6*I mRNA could be demonstrated both with RNA PCR and RISH. Thusfar it is unclear whether the failure to detect HPV 16 E6* mRNA in this sample reflects the absence or presence of minor quantities of such transcripts. It is worth noting that HPV 33 was found

to be the major type present in this specimen detectable by genomic Southern blot analysis. RISH analysis revealed that transcripts containing E7 sequences are exclusively localized within the carcinoma cells. Similar data have been documented for cervical carcinomas, showing the presence of HPV 16 E7 transcripts also limited to the carcinoma cells (van den Brule *et al.*, 1991a).

In this study we collected data that strongly support a role for HPV in the pathogenesis of tonsillar squamous cell carcinomas: i) all carcinomas were found to contain HPV DNA including high risk HPV types that also have been associated with cervical cancer. ii) control tonsil samples were HPV negative. iii) HPV E7 mRNA was demonstrated in all carcinomas examined. iiii) expression of E6/E7 sequences was found to be limited to the carcinoma cells.

These findings open a way to identify novel HPV types of the aerodigestive tract and will shed new light upon the role of HPV in the development of carcinomas within this region.

Acknowledgments

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CHAPTER VII

Human papillomavirus type 33 in a tonsillar carcinoma generates its putative E7 mRNA via two E6* transcript species which are terminated at different early region poly (A) sites

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Abstract

HPV 33 specific early region transcripts in a tonsillar carcinoma were analysed using the RNA PCR method. A total of five cDNA species could be identified including species with E6*I, E6*II and E6*III coding potential. As determined by 3' cDNA end mapping one E6*I cDNA species was found to utilize a novel early region poly (A) site and was polyadenylated at or near the putative initiation codon of the E1 ORF. Compared with the HPV16 and HPV18 E6* mRNAs, the HPV33 E6*I and E6*II species utilize different splice acceptor sites, the latter being localized within the E7 ORF. Furthermore, HPV 33 E6* mRNAs were found to contain a short overlapping ORF resulting in alternative coding potentials if translation would start at an internal AUG codon within the E6 region. These results indicate that in addition to HPV 16 and HPV 18 also HPV 33 generates E6* mRNAs which may serve as efficient mRNAs for E7. However, HPV 33 has the ability to generate its putative E7 mRNAs by the utilization of two early region poly (A) sites which offers the possibility to express E7 in different ways.

At least 60 different human papillomavirus (HPV) types have been identified, a subset of which is associated with genital-mucosal lesions (7). Of these types, HPV 6 and HPV 11 are mainly found in benign condylomas and low grade dysplasias and are considered low risk HPV types whereas the high risk types HPV 16 and HPV 18 are the major types increasingly associated with high grade dysplasias and carcinomas of the uterine cervix (43, 46). Additional high risk HPVs like HPV 31, HPV 33 and HPV 35 have been found in only a minority of cervical carcinomas. In contrast, we have detected HPV 33 in a substantial proportion (5 out of 10 cases) of tonsillar carcinomas (see Chapter VI). Moreover, the presence of HPV 33 specific transcripts has been demonstrated in the neoplastic cells by RNA *in situ* hybridization (see Chapter VI). These findings indicate that HPV 33 can be of greater importance in carcinogenesis than previously has been suggested on the basis of its association with cervical cancer.

Transformation and immortalization functions of HPV 16 and HPV 18 (9, 21, 26, 27, 41) have been mapped to the E6 and E7 open reading frames (ORFs) (2, 16, 22), regions which are consistently transcribed in cervical carcinomas and carcinoma derived cell lines (1, 32, 33, 39). The importance of these genes has further been strengthened by the abilities of their products to bind the p53 and retinoblastoma tumor suppressor gene products, respectively (10, 23, 45). Comparative studies have revealed differences in biological activities between the E6 and E7 genes of the low risk type HPV 6 and of the high risk types HPV 16 and 18 (3, 13, 24) which may account for certain *in vitro* immortalization and transformation properties being limited to the high risk HPVs (26, 40). The *in vivo* oncogenic potential may also depend on the mechanism by which the E6 and E7 encoding transcripts are generated and the expression of these genes is regulated (3, 38). Putative E7 mRNAs of HPV 16 and HPV 18 are bi- or poly-cistronic and contain an intron within the E6 ORF (E6* mRNAs) (32, 39). Such spliced E6/E7 transcripts, two of which have been identified for HPV 16 (E6*I and E6*II), failed to be present in HPV 6 and HPV 11 containing condylomas (4, 38). Moreover, HPV 16 and HPV 18 can generate E6/E7 encoding viral-cellular fusion transcripts (17, 29, 32, 37), a phenomenom which is uncommon to the low risk HPVs. Knowledge about the mechanism by which E6/E7 mRNAs

of additional high risk HPVs like HPV 33 are generated is necessary to find out which viral features are most critical in determining the *in vivo* oncogenic potential. In this study we applied the RNA polymerase chain reaction (PCR) to analyse HPV 33 early region transcripts present in a tonsillar carcinoma containing both extrachromosomal and integrated HPV 33 DNA (not shown).

Primer selection and analysis of amplified cDNAs

Three sense primers (E6, E7a and E1a) and three antisense primers (E7b, E1b and E2/E4) were chosen in such a way to flank putative splice sites, as deduced from transcriptional mapping data of HPV 11 (31) and HPV 16 (8, 39). Primer locations in relation to the HPV 33 early region genome structure are depicted in Figure 1A and primer sequences are shown in Table 1. Although we did not investigate the 5' mRNA ends, studies of HPV 16 that have established a major cap site (P97) for E6/E7 transcripts at or near nucleotide (nt) position 97 (29, 37, 39), together with the high sequence homology between HPV 16 (36) and HPV 33 (5) around this region (including TATA box sequences) suggest the existence of an equivalent major cap site for HPV 33. Indeed, all transcripts detectable with the E6 sense primer also could be detected with a sense primer (nt 95, 5'-GGTACTGCACGACTATGTTT-3') specific for this putative cap region (not shown). Although the presence of cap sites further upstream of this region can not be excluded, this finding justifies the inclusion of the putative E6 initiation codon which maps to nt 109 when determining the coding potentials of these transcripts.

Following the identification of HPV 16 and HPV 18 E6/E7 mRNAs, containing an intron within the E6 ORF (32, 39), sequence comparison data have revealed the presence of putative splice signals at equivalent positions within the E6 ORF of the remaining sequenced high risk mucosotropic HPVs (34). For HPV 33 the predicted splice donor and acceptor sites of a putative E6* mRNA have been mapped to nt 231 and nt 414, respectively. The use of primers E6 and E7a which flank these sites and span a region of 513 bp at the DNA level, revealed a single amplified cDNA fragment of 236 bp whereas DNA PCR yielded the expected 513 bp fragment (Figure 2A). DNA sequence analysis of the cDNA product allowed the identification of a splice junction connecting nt 231 to nt 509 (Figure 3A) indicating that for this splice the donor site is identical but the acceptor site is different from the predicted site. Also the acceptor site is not equivalent to the HPV 16 E6*II acceptor site (38), thus it concerns a novel splice junction. RNA PCR of an additional tonsillar carcinoma (not shown) and a cervical carcinoma (Figure 2A) yielded the same 236 bp fragment suggesting that these sites are natural splice sites common to HPV 33. Since we failed to detect a full-length 513 bp RNA PCR product it is assumed that unspliced E6/E7 mRNA, if present, must exist at very low levels within the cytoplasm.

Four cDNA species could be identified using the different HPV 33 specific primer combinations. Primer pair E6-E2/E4 allowed the detection of three cDNA species as reflected by the production of PCR fragments of 714 bp, 438 bp and 328 bp (Figure 2B). The 714 bp fragment clearly presented as the major amplified product (not shown) indicating that the mRNA species represented by this fragment is abundant when compared with the 438 bp and 328 bp species. Moreover, 498 bp and 430 bp amplified cDNA fragments were obtained with primer combinations E7a-E2/E4 and E1a-E2/E4, respectively (Figure 2B). DNA sequence determination revealed that the 714 bp E6-E2/E4

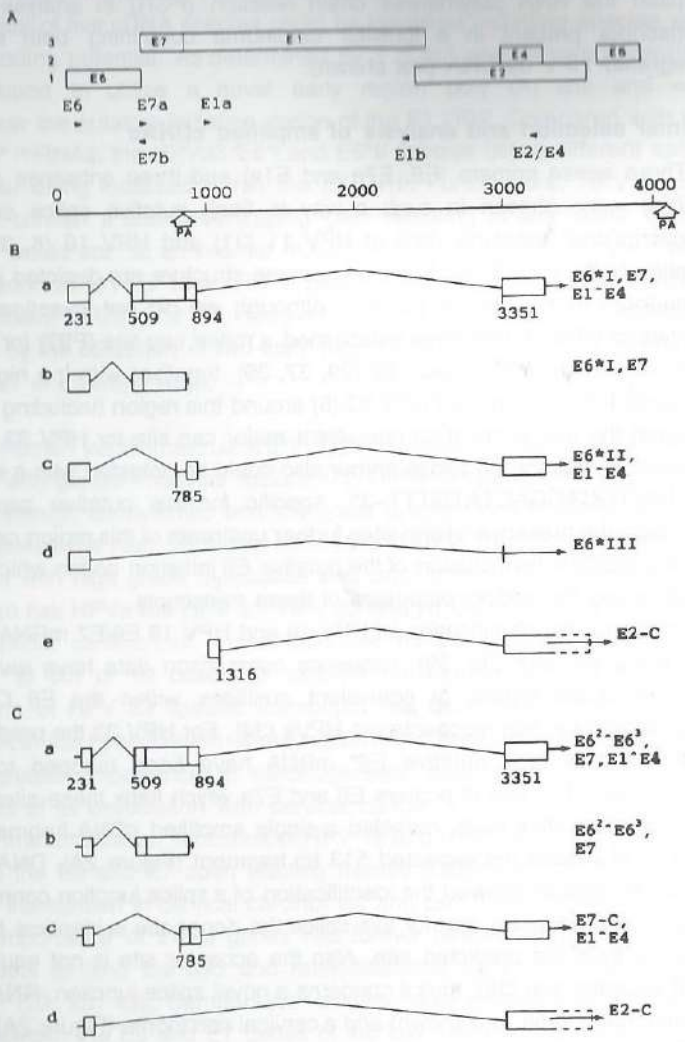

Figure 1. Structures and coding potentials of HPV 33 specific cDNA species identified in the tonsillar carcinoma. (A) Primer positions in relation to the HPV 33 early region genome structure (5) are indicated by arrowheads. Arrowheads point to the 5' to 3' direction. Putative poly (A) sites are indicated by open arrows. Reading frames are numbered according to Cole and Streeck (5). (B) Structures and probable coding potentials of the five cDNA species (a to e) identified, depicted in relation to the genome structure shown in (A). Numbers indicate the splice donor and acceptor sites. Open boxes represent potential coding regions. Coding potentials of the cDNA species are shown on the right. For species a to d the potential to encode E6* proteins depends on the utilization of the E6 AUG codon at nt position 109. (C) Alternative coding potentials of cDNA species a to d depending on the utilization of the second out of frame AUG codon within the E6 region mapping to nt 146. ($E6^2{}^*E6^3$) indicates a putative fusion protein resulting from the in frame connection of E6 sequences of reading frame 2 with E6 sequences of reading frame 3 (see A). (E7-C) indicates a putative protein consisting of a C-terminus encoded by the E7 ORF, joined via splicing to N-terminal sequences encoded by E6 reading frame 2.

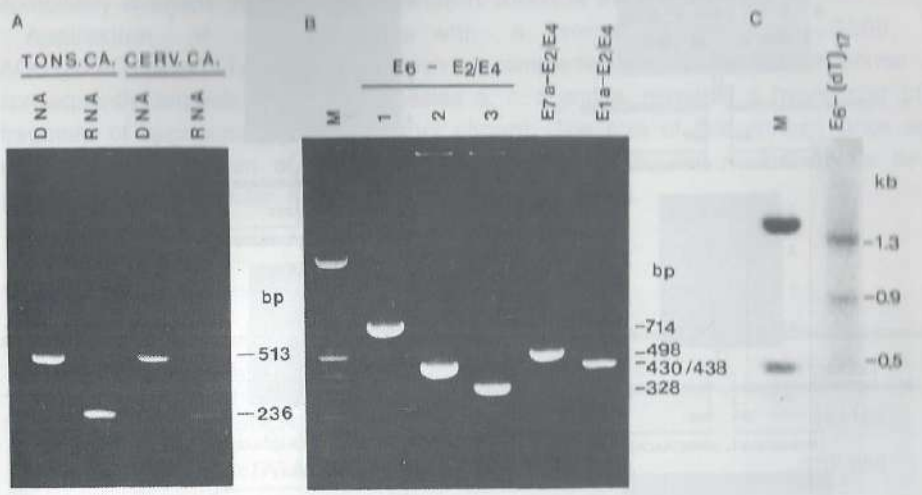

Figure 2. HPV 33 RNA PCR analysis of a primary poorly differentiated tonsillar squamous cell carcinoma. Cytoplasmic RNA was isolated according to Gough (14). One strand of cDNA was synthesized in a final reaction volume of 50 µl containing 200 ng cytoplasmic RNA, 25 pmol antisense primer, 50 mM Tris.HCl pH 8.3, 60 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM of each dNTP, 40 units RNasin (Promega) and 7 units AMV reverse transcriptase (RT; Promega). The mixture was incubated at 42°C for 45 minutes. In addition reactions were performed without RT added to allow distinction between final RNA PCR products and products derived from eventually co-purified DNA. Five µl of the cDNA mixture was used for PCR. Both PCR on cDNA and genomic DNA was performed under identical conditions as prevously described for type specific PCR analysis (42). To determine 3' cDNA ends the method described by Frohman *et al.* (11) was applied. PCR products were analysed on 1.5% agarose gels. Southern blot analysis was carried out (44) using the cloned HPV 33 DNA as probe. (A) Comparison of E6-E7b directed PCR products derived from DNA and RNA of the tonsillar carcinoma and a cervical carcinoma containing HPV 33. The agarose gel pattern is shown after ethidium bromide staining. (B) Detection of RNA PCR products of the tonsillar carcinoma obtained with the E2/E4 antisense primer. Three PCR fragments originally obtained in a single PCR assay with the E6-E2/E4 primer pair were electrophoretically separated and isolated fractions were reamplified to facilitate subsequent sequencing (lanes 1 to 3). M: pBR322 DNA digested with *Hin*fI. (C) Southern blot analysis of the 3' end RNA PCR product generated by the E6-oligo (dT)-adaptor primer combination. M: pBR322 DNA digested with *Hin*fI.

fragment and most likely also the 498 bp E7a-E2/E4 fragment represent cDNA species a. The remaining 438 bp (E6-E2/E4), 328 bp (E6-E2/E4) and 430 bp (E1a-E2/E4) fragments were found to represent cDNA species c, d and e, respectively. The splice patterns and resulting cDNA structures are shown in Figures 1B and C. Species a and c were found to contain at least three exons, the first two of which are joined via nt 231 to nt 509 (species a; Figure 3A) and via nt 231 to nt 785 (species c; Figure 4B). In both species the second and third exon are joined by the same splice via nt 894 to nt 3351 (Figure 3D). Species d and e consist of at least two exons joined via nt 231 to nt 3351 (species d; Figure 3C) or via nt 1316 to nt 3351 (species e; Figure 3E). Except for the first splice junctions of species a and c, equivalent splice junctions have been mapped for HPV 16 (8). cDNA species e only could be detected with the E1a sense primer and failed to be amplified using the E6 or E7a sense primers. This finding together with transcriptional mapping data of HPV 11 and HPV 16 (8, 31) suggests the presence of a cap site for this transcript which maps to the 5' portion of the E1 ORF.

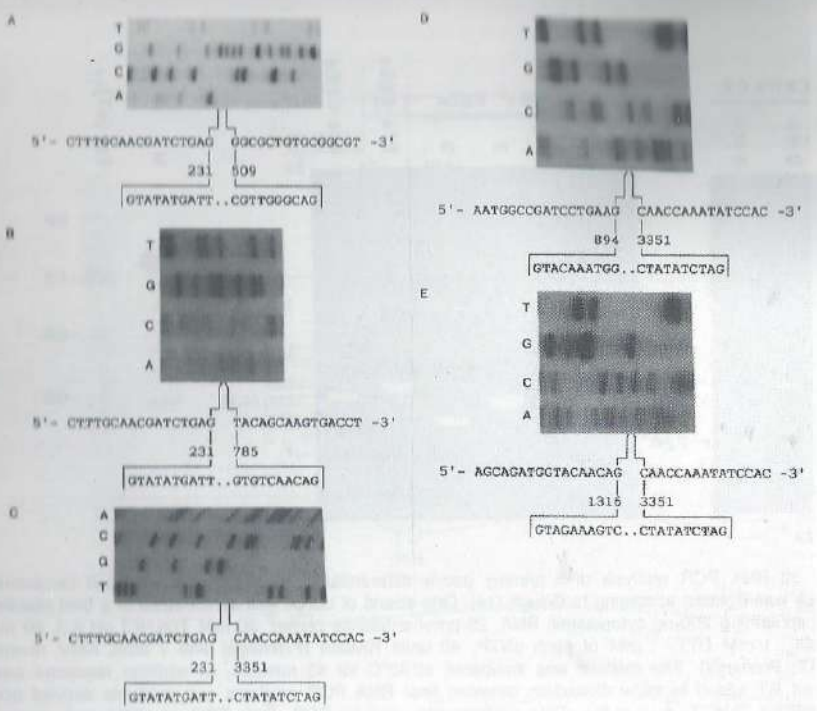

Figure 3. cDNA sequences which span the splice junctions. Sequencing of the amplified cDNA fragments was carried out either directly after a-symmetric PCR (15) or after cloning the product into the *Eco*RI or *Eco*RI/*Bam*HI site of M13mp18/mp19 vector DNA. Sequencing was performed using the dideoxy chain termination reaction with a T7 polymerase sequencing kit (Pharmacia) according to instructions of the manufacturer. Nucleotide positions of splice donor and acceptor sites are indicated. Intron sequences around the splice junctions are shown beneath and between sequences of both exons. Panel A: E6*I splice junction (species a and b), panel B: E6*II splice junction (species c), panel C: E6*III splice junction (species d), panel D: E1/E4 splice junction (species a and c), panel E: E2-C splice junction (species e).

Identification of a novel poly (A) site by 3' cDNA end mapping

3' cDNA ends were analysed by the method described by Frohman *et al.* (11), combining one of the HPV 33 specific sense primers with an oligo (dT)-adaptor primer set. The E6-adaptor primer combination revealed three hybridizing cDNA fragments, approximately 1300 bp, 900 bp and 500 bp in size (Figure 2C). Restriction enzyme analysis using Rsa I and Sau 3A (not shown) suggests that the 1300 bp and 900 bp fragments are likely to represent cDNA species a and d, respectively, uninterrupted downstream of the E4 ORF and utilizing the early region poly (A) motif which maps to nt 4176. Similar full-length cDNA species have been mapped for HPV 16 (8, 29). The 500 bp fragment, however, is aberrant in size and could not be related to any of the transcripts identified thus far. DNA sequence analysis of this product revealed that it concerned a new transcript species (species b; see Figures 1B and C) consisting of two exons, joined via the same splice that was mapped for the first exons of species a and terminated at a cleavage/poly (A) addition site 14 or 17

nts downstream of the poly (A) signal at nt position 862 (Figure 5). This poly (A) signal completely overlaps the putative termination codon of the E7 ORF (5).

Application of the method with a sense primer (nt 3569, 5'-ACCTATAGTGCATTTAAAAG-3') which is complementary to the E2/E4 primer and consequently anneals with cDNA species a, c, d and e, revealed a hybridizing cDNA fragment of approximately 650 bp (not shown). The size of this product once again suggests the utilization of the poly (A) signal at nt 4176, and no indications for the presence of viral-cellular fusion transcripts were obtained.

Table 1. Specification of primers used for RNA PCR

Primer	Sequence[a]	Position
E6	5'-ggaattcGCATGATTTTGTGCCAAGCAT-3'	144-164
E7a	5'-ggaattcTATACTGCTATGAGCAATTA-3'	636-656
E7b	5'-ggaattcTAATTGCTCATAGCAGTATAG-3'	656-636
E1a	5'-ggaattcTGCACTAAAACGAAAGTTTGC-3'	1127-1147
E1b	5'-ggaattcATTTGCACCACGTCCTTGAGA-3'	2715-1695
E2/E4	5'-cgggatccCTTTTAAATGCACTATAGG-3'	3588-3569
$(dT)_{17}$[b]	5'-GACTCGAGGATCCTGCAGCCTTTTTTTTTTTTTTT-3'	
Adaptor[b]	5'-GACTCGAGGATCCTGCAGC-3'	

[a] Sequences written in small characters indicate sequences containing *Eco*RI or *Bam*HI recognition sites.
[b] Primers described by Frohman *et al.* (11).

Coding potentials of cDNAs

The probable coding potentials of the identified cDNA species are shown in Figure 1B. Species a and b may encode an E6*I protein (39). If translation would start from the E6 initiation codon at nt 109 localized in reading frame 1 (5), the first splice would shift translation to reading frame 2 of the E6 region resulting in the termination at the UAA codon which maps to nt 563. This codon lies 7 nts downstream of the termination codon of the full-length E6 ORF and 8 nts upstream of the putative initiation codon of the E7 ORF. The second splice junction of species a and c is equivalent to splice junctions previously mapped for HPV 1 (25), HPV 11 (31), HPV 16 (8) and HPV 18 (30) and allows the ability to encode an E1^E4 fusion protein. However, the presence of an additional E1^E4 encoding message which, similar to that of other HPVs (8, 25, 31) contain a cap site within the E1 ORF can not be excluded. Additional 5' cDNA end mapping is necessary to resolve the presence of this putative mRNA. Species c differs from species a by the utilization of a different splice acceptor site to join the first and second exon and has the potential to encode a novel E6*II protein. Starting from the E6 AUG codon at nt 109 the

splice would shift translation to out of frame sequences of the E7 ORF, resulting in the addition of three amino acids to the E6 C-terminus before reaching a termination codon at nt 794. cDNA species d is equivalent to an HPV 16 message (8) and has coding potential for an E6*III protein. cDNA species e contains AUG codons at nt positions 1276 and 1305, both of which are in frame for initiation of an E2-C protein (carboxy terminal domain of E2) consisting of N-terminal sequences derived from the E1 region. Therefore, if this species would proceed without any interruption beyond the E2 ORF it could encode an E2-C protein.

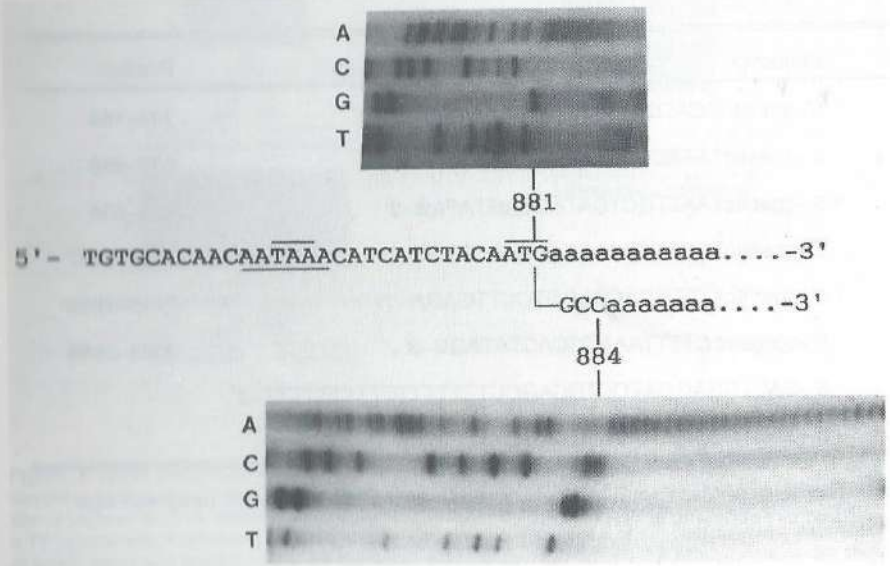

Figure 4. 3' end analysis of cDNA species b. Two cleavage/poly (A) addition sites were found as shown for two M13 clones. The putative E7 termination codon (TAA), the putative E1 initiation codon (ATG) and the poly (A) signal (AATAAA) are indicated. Sequences of the poly (A) tail are shown by small characters.

Within the E6 region of the HPV 33 genome a second short overlapping ORF is present in reading frame 2 which proceeds past the E6 splice donor site (5). Consequently, removal of the first intron of species a to d generates new ORFs in addition to E6* resulting in alternative coding potentials if translation would start at the second AUG codon (nt 146) within the E6 region (Figure 1C). For species a and b this offers the ability to encode a protein containing N-terminal sequences encoded by E6 region reading frame 2 and a C-terminus consisting of ten amino acids encoded by E6 region reading frame 3 ($E6^2{}^\wedge E6^3$). Similarly, species c has additional coding potential for a protein containing a C-terminus of 27 amino acids encoded by the E7 ORF (E7-C). Homology comparison with HPV 16 sequences revealed that this putative protein does not contain sequences that are supposed to be involved in transformation and in the interaction with the retinoblastoma

gene product (24). However, this putative protein contains a CXXH motif at the N-terminus and a CXXC motif at the C-terminus and consequently has the potential to form a hybrid zinc loop drawing the polypeptide ends together. Species d could have additional coding potential for an alternative E2-C protein if not interrupted within the 3' part of the E2 ORF. This putative protein would presumably initiate from the second out of frame AUG codon of the E6 region, at nt position 146 and therefore, its N-terminus consists of E6 instead of E1 sequences.

If no further splicing or interruption would occur downstream of the E4 ORF the utilization of the putative early region poly (A) site at nt 4176 would provide species a, c, d and/or e with additional coding potential for an E5 protein.

Albeit HPV 33 is only associated with a minority of cervical carcinomas, this type belongs to the group of high risk HPV types and *in vitro* transforming activities similar to that of HPV 16 and HPV 18 have been demonstrated (40). Three HPV 33 specific E6* transcripts could be demonstrated in the tonsillar carcinoma studied. Because the E6*II and E6*III acceptor sites map within the E7 ORF and E2/E4 ORFs, respectively, the E6*I species a and b are thus far the only candidates to encode an HPV 33 E7 protein. Recent studies have indicated that splicing out an intron within the E6 ORF most likely serves to facilitate translation of the downstream E7 ORF rather than producing a biologically active E6* protein (28, 35). According to the scanning model for translation proposed by Kozak (18, 20) it has been suggested that splicing within the E6 ORF would provide a more efficient reinitiation of translation at the E7 AUG codon by placing an E6* termination codon more upstream of the termination codon of the unspliced E6 ORF resulting in a larger spacing between both cistrons (38). Studies using different bicistronic mRNAs have revealed that the efficiency of translational reinitiation at the second cistron improves as the distance between the end of the first and the beginning of the second cistron increases (19). However, in contrast to the HPV 16 and HPV 18 E6* mRNAs the HPV 33 E6*I termination codon is placed downstream of that of the unspliced E6 ORF, in close vicinity of the putative E7 AUG codon. This could mean that the HPV 33 specific E6*I mRNAs are less efficient in expressing E7 proteins than the HPV 16 and HPV 18 counterparts, unless a different first cistron would be used. Indeed, a short overlapping ORF exists in these mRNAs, with coding potential for an $E6^2{}^\wedge E6^3$ protein, that has a termination codon positioned at nt 540, that is 23 nts upstream of the E6*I termination codon. Still it is uncertain whether this ORF is expressed since its internal AUG codon lies in an unfavourable sequence context to support translational initiation by the "leaky scanning" mechanism (18). However, also the first AUG codon in the E6 ORF has less than an ideal sequence context for efficient translation, according to the proposed scanning model. Therefore, additional data are required to find out in which way the expression of the HPV 33 E7 ORF is regulated and if the alternative ORFs would be expressed. If expressed, for species a, b and c this most likely would serve to affect the translation of downstream ORFs rather than producing functional proteins since there exist no indications that other HPVs can encode similar proteins. In this context only cDNA species d may encode a novel functional E2-C protein. cDNA species c contains a splice that places the termination codon for the E6*II ORF immediately beyond the splice acceptor site and in this way creates an optimal target for the production of an E1^E4 fusion protein since the putative E1 AUG codon lies in a favourable sequence context for translational reinitiation (18).

Striking is the identification of an E6*I/E7 transcript that utilizes a novel poly (A) site at the 3' end of the E7 ORF. Sequence analysis indicated that HPV 6b, HPV 11, HPV 16, HPV 18 and HPV 31 do not contain putative poly (A) signals in the equivalent region suggesting that the presence of a second early region poly (A) site is not a general feature. Consequently, HPV 33 can express E7 via at least two early region mRNAs.

In general in HPV 16 and HPV 18 containing carcinomas and cell lines derived from carcinomas, transcripts encoding a full-length E6 protein have been demonstrated (17, 33, 37, 39). However, quantities of such transcripts have been found to be relatively low (37, 39). Low amounts of HPV 33 specific unspliced E6 mRNAs could have been missed because the method certainly favours the amplification of the shorter E6* cDNAs. Moreover, in cervical carcinomas and carcinoma derived cell lines containing HPV 16 or HPV 18, the viral genome is often integrated into the host genome, usually interrupting E1/E2 sequences and leaving the E6 and E7 ORFs undisrupted and transcriptionally active by means of the generation of viral-cellular fusion transcripts (17, 29, 32, 37). The carcinoma we investigated was found to contain both integrated and episomal HPV 33 DNA, which is not unique since also a proportion of HPV 16 containing cervical carcinomas have been found to contain both viral forms (6, 12). Since we have no detailed information with respect to integration site and possible head to tail arrangement of integrated HPV 33 DNA, it is still unknown if the identified HPV 33 transcripts are derived from integrated or episomal DNA. Although the method allowed the amplification of fragments more than 2 kb in size, we failed to detect viral-cellular fusion transcripts. However, this does not necessarily mean that these transcripts are not present. Putative chimeric transcripts could have been too large and/or exist at too low levels to allow a successful amplification in the presence of the other transcripts. In a human keratinocyte cell line immortalized by HPV 16 a chimeric transcript has been identified by cDNA cloning which contained a stretch of cellular RNA of approximately 2.8 kb (29), indicating that chimeric HPV 33 transcripts may exist that are too large for efficient PCR amplification. Alternatively, in an integrated state HPV 33 may generate its E6/E7 mRNA different from HPV 16 and HPV 18. If single copy HPV integration occurs, typically within the E1/E2 region, this results in the disconnection of the E6/E7 region from the putative normal early region poly (A) site which is generally localized downstream of the E5 ORF. For HPV 16 and HPV 18 this consequently means that the generation of a chimeric mRNA and the utilization of a cellular poly (A) site is required to ensure the continued expression of the E6/E7 ORFs via a polyadenylated mRNA. For HPV 33 the continued expression of the E6/E7 ORFs would not necessarily depend on a cellular poly (A) site since the E6/E7 region provides its own poly (A) site.

In this study we demonstrated that in addition to HPV types 16 and 18 also the HPV 33 E7 ORF is encoded by spliced E6* messages. At least one E6* splice junction was also found in an additional tonsillar carcinoma and a cervical carcinoma suggesting that this mechanism to generate E7 mRNA is common to HPV 33 independent on the site of infection. The property to generate E7 mRNAs by the utilization of two early region poly(A) sites thus far seems specific for HPV 33 and may offer the possibility to express E7 in different ways.

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CHAPTER VIII

Human papillomavirus type 16 and 33 E6/E7 region transcripts in tonsillar carcinomas can originate both from integrated and episomal HPV DNA

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Abstract

This study was undertaken to find out whether HPV E6/E7 transcription in tonsillar carcinomas is correlated with viral DNA integration. Therefore, tonsillar carcinomas containing HPV 16 (n=2) and HPV 33 (n=2) DNA were analysed for the viral physical state and transcription of the E6/E7 region. Southern blot analysis, DNA PCR and, eventually, two-dimensional gel electrophoresis revealed indications for the presence of only episomal DNA in the HPV 16 containing biopsies and only integrated DNA in one HPV 33 containing biopsy. The second HPV 33 containing carcinoma, of which one biopsy and two resected tumor specimens were analyzed, showed a rather complex physical state profile. The biopsy of this tumor only contained episomal DNA, one resected tumor part only contained integrated DNA and the remaining tumor part contained both integrated and episomal HPV 33 DNA. Independent of the viral physical state, all biopsies and resected tumor parts tested, showed the presence of E6/E7 transcripts, as determined by RNA PCR. The results indicate that E6/E7 transcripts in tonsillar carcinomas can originate from integrated as well as episomal HPV DNA.

Introduction

A distinct subset of human papillomavirus (HPV) types, predominantly HPV 16 and HPV 18, have been implicated in human anogenital cancer and are considered high risk HPV types (zur Hausen and Schneider, 1987). Moreover, we recently have documented a strong association between predominantly HPV 16 and HPV 33 genotypes and tonsillar carcinomas (see Chapter VI).

Based on accumulating experimental data the E6 and E7 open reading frames (ORFs) are supposed to be crucial for transforming properties of high risk HPVs (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989). Of these ORFs, the E7 ORF encodes a major transforming gene (Storey *et al.*, 1988; Phelps *et al.*, 1988; Halbert *et al.*, 1991). Interference with E6/E7 expression has shown to affect the tumorigenicity of cervical carcinoma cells and transformation of rodent cells (von Knebel Doeberitz *et al.*, 1988; Crook *et al.*, 1989). Consequently, it has been suggested that HPV E6-E7 functions are essential both for initiation and maintenance of the malignant phenotype (zur Hausen, 1991). Indeed, the E6/E7 region has been found to be consistently transcribed in cervical carcinomas and cell lines derived from cervical carcinomas (Baker *et al.*, 1987; Schwarz *et al.*, 1985; Smotkin and Wettstein, 1986; van den Brule *et al.*, 1991.), and also tonsillar carcinomas previously tested have shown to contain E6-E7 region mRNA (Chapter VI).

In the proposed model of anogenital carcinogenesis by HPV, viral DNA integration has been suggested to play an essential role, most likely by triggering a dysregulated expression of E6-E7 genes (zur Hausen, 1991). Integration of viral DNA often has been observed in HPV 16 and HPV 18 containing cervical carcinomas (Dürst *et al.*, 1985; Cullen *et al.*, 1991) and cell lines derived from cervical carcinomas (Schwarz *et al.*, 1985; Awady *et al.*, 1987; Yee *et al.*, 1985; Shirasawa *et al.*, 1987). In contrast, premalignant and benign cervical lesions predominantly contain episomal HPV DNA (Dürst *et al.*, 1985; Lehn *et al.*, 1988; Cullen *et al.*, 1991). HPV DNA integration regularly occurs within the viral E1/E2 region (Schwarz *et al.*, 1985; Baker *et al.*, 1987; Kong-Bung Choo *et al.*, 1987; Shirasawa *et al.*, 1989), thereby disrupting E2 expression. Since the HPV E2 ORF encodes transcriptional modulator proteins (Phelps and Howley, 1987; Cripe *et al.*, 1987; Bernard *et al.*, 1989; Romanczuk *et al.*, 1991) it has been suggested that integration -mediated disruption of E2 expression would trigger an uncontrolled expression of the transforming genes E6 and E7. Another event often resulting from HPV 16 or HPV 18 DNA integration is E6/E7 expression via viral-cellular fusion transcripts (Inagaki *et al.*, 1988; Schneider-Gädicke and Schwarz, 1986; Rohlfs *et al.*, 1991; Smits *et al.*, 1991).

This study was undertaken to find out whether transcription of E6/E7 sequences in tonsillar carcinomas is also correlated with HPV DNA integration. The results indicate that E6/E7 region transcripts in HPV containing tonsillar carcinomas can originate both from integrated and episomal viral DNA templates.

Materials and methods

Clinical specimens and viral DNA clones

Biopsy specimens of four tonsillar carcinomas containing HPV 16 (n=2) or HPV 33 (n=2) were snap-frozen and stored in liquid nitrogen until use. From one carcinoma the biopsy was taken one month prior to surgical treatment and two matched specimens taken from separated parts of the resected tumor also were snap-frozen and available for HPV analysis.

DNA of HPV 16 cloned into the *Bam*HI site of pBR322 and of HPV 33 cloned into the *Bgl*II site of pBR322 was purified from vector sequences by agarose gel electrophoresis and subsequently used as probe.

Isolation of DNA and cytoplasmic RNA

Snap-frozen samples were serially sectioned (10-15 sections) on a cryostate. The first and last sections (5 μm) were used for hematoxylin eosin (HE) staining to assess the percentage of carcinoma cells present in these samples. The intermediate sections were used for DNA and eventually RNA isolation. DNA was extracted as described by Walboomers *et al.* (1988). Cytoplasmic RNA was isolated according to Gough (1988). Briefly, tissue sections were suspended in 200 μl cold lysis buffer containing 10 mM Tris.HCl pH 7.5, 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40 and vortexed vigorously. Nuclei and remaining cell debris were removed by centrifugation at 800 g for 5 minutes. The supernatant (cytoplasmic lysate) was transferred to a new tube containing 200 μl 7 M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris.HCl pH 7.5, extracted with phenol/chloroform and precipitated with 96% ethanol. The integrity of RNA samples was determined by agarose gel electrophoresis.

DNA polymerase chain reaction

DNA PCR was performed with combinations of HPV 16 or HPV 33 specific primers located within different parts of the HPV genome. Combinations of the following primers, of which sequences are numbered according to the published sequence data (Seedorf *et al.*, 1985; Cole and Streeck, 1986), were used in the PCR: For HPV 16: E6: nt 141-

160; E7: nt 645–625; E1a: nt 1114–1134; E1b: nt 2720–2700; E2/E4: nt 3633–3614; L1a: nt 6028–6047; L1b: nt 6179–6160. For HPV 33: E6: nt 144–163; E7: nt 656–636; E1a: nt 1127–1147; E1b: nt 2415–2434; E1c: nt 2715–2695; E1d: nt 2695–2715; E1e: nt 2870–2871; E2/E4: nt 3589–3570; L1a: nt 5896–5917; L1b: nt 6561–6542. PCR was performed as described by van den Brule *et al.* (1990) for type-specific analysis. PCR products were analysed by agarose gel electrophoresis and after blotting by hybridization with the cloned HPV 16 or HPV 33 probe.

Southern blot hybridization of one- and two-dimensional agarose gels

One-dimensional Southern blot analysis was carried out using 0.7% agarose gels. Two-dimensional gel electrophoresis was performed as described by Wettstein and Stevens (1982) using no-cut enzymes or undigested DNA. Agarose gels of 0.4% and 1.0% were used for the first and second dimension, respectively. This assay allows separation of circular episomal DNA from linear DNA as the mobility of circular DNA decreases more rapidly than that of linear DNA at increasing gel concentrations. Separated DNAs were transferred onto nylon membranes (GeneScreen Plus, NEN-DuPont) as described above. Cloned HPV 16 or HPV 33 DNA was used as probe after ^{32}P- labelling using an oligolabelling kit (Pharmacia). Hybridization was performed at 65°C in 0.5 M sodium phosphate pH 7.4, 7% SDS and 1 mM EDTA four 16 hours. The filters were washed two times in 3xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.5% SDS for 15 minutes at 65°C, once in 0.5xSSC, 0.5% SDS at 65°C for 15 minutes and twice in 0.1xSSC, 0.5% SDS at 68°C for 15 minutes. Autoradiography was performed for 5 days at -80°C with Kodak Royal X-omat film and intensifying screens.

RNA polymerase chain reaction

RNA PCR was performed as described previously (van den Brule *et al.*, 1991). One strand of cDNA was synthesized in a final reaction volume of 50 μl containing 200 ng cytoplasmic RNA, 25 pmol antisense primer, 50 mM Tris.HCl pH 8.3, 60 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM of each dNTP, 40 units RNasin (Promega) and 7 units AMV reverse transcriptase (RT; Promega). The mixture was incubated at 42°C for 45 minutes and subsequently at 95°C for 5 minutes to inactivate the RT. In addition, reactions were performed without RT added to allow distinction between final RNA PCR products and products derived from eventually co-purified DNA. Five μl of the cDNA mixture was used for PCR in a volume of 50 μl containing a final concentration of 50 mM KCl, 10 mM Tris.HCl pH 8.3, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 50 pmol of both sense and antisense primer and 1 unit thermal stable DNA polymerase (Amplitaq; Cetus). Forty cycles of amplification were performed as previously described for type-specific PCR (van den Brule *et al.*, 1990). HPV-specific primers used were as shown above.

To determine 3' cDNA ends the method described by Frohman *et al.* (1988) was applied using a 35-base $(dT)_{17}$-adaptor primer (5'-GACTCGAGGATCCTGCAGCC-TTTTTTTTTTTTTTTTT-3') for first strand cDNA synthesis. In the subsequent PCR with

single-sided specificity this primer was replaced by a 19 base adaptor primer lacking the (dT) stretch and combined with an HPV-specific primer.

PCR products were analysed on 1.5% agarose gels. Southern blot analysis was carried out as described above using cloned HPV 16 or HPV 33 DNA as probe.

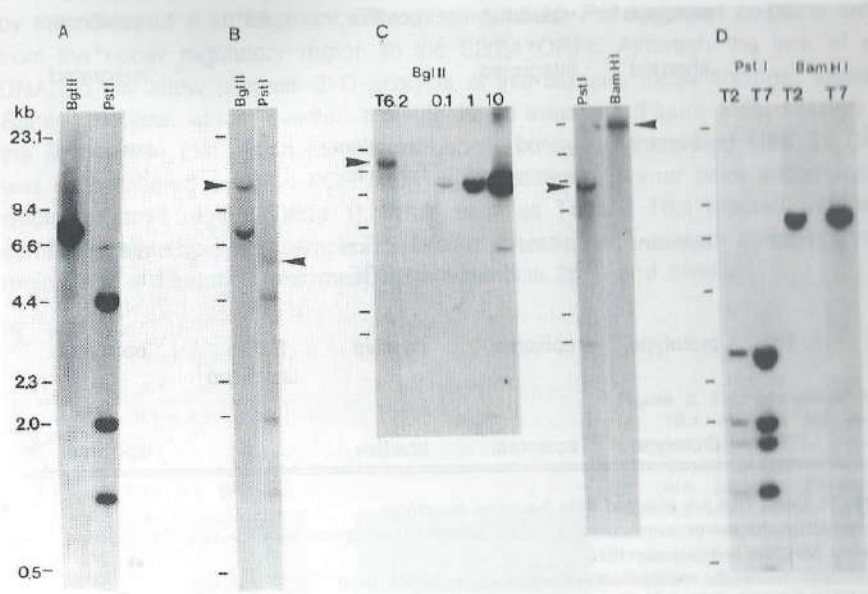

Figure 1. Southern blot analysis of tonsillar carcinoma samples. From one HPV 33 containing carcinoma biopsy sample T6 (A) and the resected tumor samples T6.1 (B) and T6.2 (C) were examined by hybridization with labelled HPV 33 DNA. Panel (C) also includes a reconstruction of human placental DNA mixed with cloned HPV 33 DNA at concentrations corresponding with 0.1, 1 and 10 genome equivalents per cell. Since sample T6.2 contained approximately 50% carcinoma cells the estimated HPV 33 copy number was 1 genome equivalent per carcinoma cell. In each panel λ x *Hin*dIII size markers are indicated on the left. Off-sized restriction fragments are indicated by arrowheads. Panel (D): Analysis of HPV 16 containing biopsies T2 and T7 hybridized with HPV 16 DNA.

Results

Comparative analysis of HPV 33 physical state and E6/E7 transcription in a biopsy specimen and matched resected tumor specimens from the same tonsillar carcinoma

From one HPV 33 containing tonsillar carcinoma both a biopsy specimen (designated as T6) and two resected tumor specimens (T6.1 and T6.2) were available for combined HPV DNA and RNA analysis. Histologic examination revealed that all three specimens were morphological identical, consistent with poorly differentiated squamous cell carcinoma. Furthermore, all these samples consisted for approximately 50% of carcinoma cells. Results of HPV DNA and RNA studies are summarized in Table 1.

Table 1. DNA and RNA analysis on tonsillar carcinoma specimens

Specimen	HPV type	Southern blot[2]	2-D analysis	DNA PCR[3]	RNA PCR[4]	Physical state
T6[1]	33	prototype	episomal	positive	E6*I	episomal
T6.1[1]	33	prototype + aberrant	episomal + integrated	positive	E6*I	episomal + integrated
T6.2[1]	33	aberrant	nd	disruption E2 ORF	E6*I	integrated[5]
T4	33	aberrant	nd	disruption E1 ORF	E6*I	integrated[5]
T2	16	prototype	episomal	positive	E6*I + unspliced	episomal
T7	16	prototype	episomal	positive	nd	episomal

[1]Samples T6, T6.1 and T6.2 are obtained from the same carcinoma
[2]Prototype restriction fragments are according to the published sequence data for HPV 16 (Seedorf *et al.*, 1985) and HPV 33 (Cole and Streeck, 1985)
[3]Positivity indicates successful reaction with all primer combinations used
[4]Concerns RNA PCR detection of transcripts with E7 coding potential
[5]Suggested on basis of Southern blot analysis and DNA PCR only
nd=not done

Standard Southern blot analysis of biopsy sample T6 using the single cut enzyme *Bgl*II and the multi-cut enzyme *Pst*I yielded the prototype HPV 33 fragments, an 8 kb *Bgl*II fragment and *Pst*I fragments of 4.6 kb, 2.0 kb and 1.2 kb (Figure 1A). Two-dimensional analysis with *Bam*HI, a no-cut enzyme for HPV 33, revealed the presence of circular HPV DNA in addition to linear fragments of approximately 8 kb and 16 kb in size (Figure 2A). The linear fragments also appeared after 2-D analysis of undigested DNA (not shown) indicating that they represent linearized monomeric and dimeric episomal DNA rather than integrated HPV DNA. In contrast to the biopsy, resected tumor specimen T6.1 yielded an off-sized 15 kb *Bgl*II fragment and an off-sized 6 kb *Pst*I fragment in addition to the prototype HPV 33 fragments (Figure 1 C). Two-dimensional analysis of sample T6.1 showed the presence of linear HPV 33 DNA of 22-23 kb in addition to the spots of circular DNA (Figure 2B). This finding points to the presence of both integrated and episomal HPV 33 DNA in this sample, the integrated DNA most likely giving rise to the off-sized restriction fragments. Apart from these, the specimens T6 and T6.1 also showed differences in HPV 33 copy number: sample T6

contained approximately 20 copies of HPV 33 DNA per carcinoma cell and sample T6.1 0.5 and 1 genome equivalents per carcinoma cell of integrated and episomal HPV 33 DNA, respectively. The second resected tumor specimen T6.2 showed a restriction enzyme pattern consistent with the presence of integrated HPV 33 DNA exclusively and the estimated copy number was 1 genome equivalent per carcinoma cell (Figure 1 C). Analysis with *Bgl*II revealed the off-sized 15 kb fragment only, whereas the prototype 8 kb fragment was missing. Moreover, the prototype 4.6 kb *Pst*I fragment was replaced by the off-sized 6 kb fragment. The missing 4.6 kb *Pst*I fragment contains sequences from the upper regulatory region to the E2/E4 ORFs. Although the lack of sufficient DNA did not allow ultimate 2-D analysis of this sample, these findings, together with *Bam*HI analysis, which revealed a single 22 kb fragment (Figure 1 C), strongly support the assumption that this specimen exclusively contained integrated HPV 33 DNA. This was substantiated by DNA PCR analysis with different primer pairs encompassing the whole genomic region (Table 1). While samples T6 and T6.1 reacted with all primer combinations employed, sample T6.2 failed to react with primers spanning the E2/E4 region and a disruption was mapped between nts 2870 and 3588.

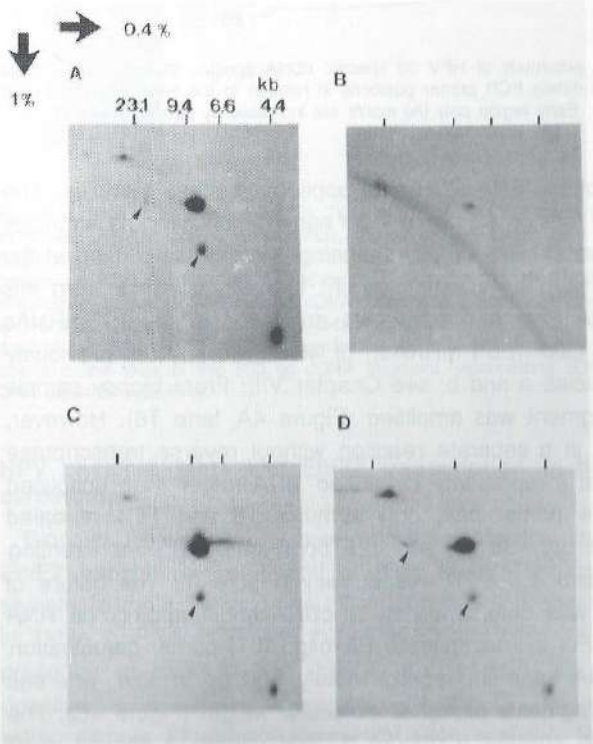

Figure 2. 2-D analysis of samples T6 (A), T6.1 (B), T2 (C) and T7 (D). Samples T6 and T6.1 were digested with *Bam*HI and hybridized with HPV 33 DNA. Samples T2 and T7 were digested with *Hin*dIII and hybridized with HPV 16 DNA. λ x *Hin*dIII size markers are indicated in addition to directions of electrophoresis in the first (0.4%) and second (1%) dimension. Linear restriction fragments of 8 kb and 16 kb are indicated by arrowheads.

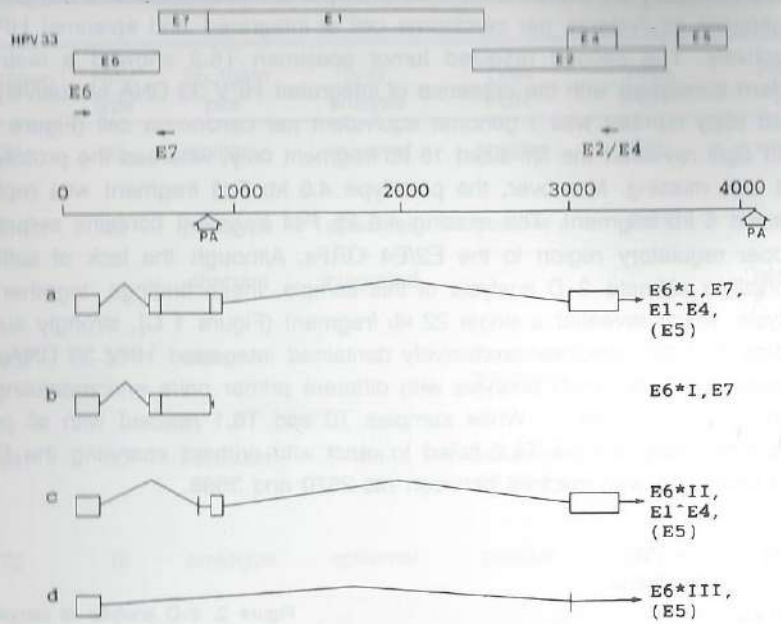

Figure 3. Structures and probable coding potentials of HPV 33 specific cDNA species that previously have been identified (see Chapter VII). Arrows indicate PCR primer positions in relation to the HPV 33 early region genome structure (Cole and Streeck, 1986). Early region poly (A) motifs are indicated by open arrows.

To study E6/E7 region transcription, RNA PCR was applied on these samples. The location of PCR primers utilized in relation to the HPV 33 early region genome structure is depicted in Figure 3. Primer combination E6/E7, spanning a region of 513 bp at the DNA level, revealed an amplified 236 bp cDNA fragment for all specimens from this carcinoma (Figure 4A). This cDNA fragment represents putative HPV 33 E7 mRNAs containing an intron within the E6 ORF (E6*I mRNAs), of which two species previously have been mapped (Figure 3, species a and b; see Chapter VII). From biopsy sample T6 also the full length 513 bp fragment was amplified (Figure 4A, lane T6). However, this fragment also was generated in a separate reaction without reverse transcriptase added (not shown) suggesting that it represents copurified DNA rather than unspliced E6/E7 mRNA. With the E6-E2/E4 primer pair, only samples T6 and T6.1 revealed amplified cDNA fragments of 714 bp, 438 bp and 328 bp (Figure 4B), representing cDNA species a (E6*I), c (E6*II) and d (E6*III), respectively (Figure 3). The nature of the E7 mRNA from sample T6.2 was determined by 3' cDNA end mapping via RNA PCR with single-sided specificity. For this sample the E6-oligo (dT) primer combination revealed a single hybridizing cDNA fragment, approximately 500 bp in size, whereas sample T6.1 in addition revealed fragments of higher molecular weigth (Figure 4C). The 500 bp fragment represents cDNA species b (Figure 3) that utilizes a second early region poly (A) signal at nt 862 and has a coding potential limited to E6*I and E7 proteins (Chapter VII).

Figure 4. RNA PCR on samples T6, T6.1 and T6.2 from the same HPV 33 containing tonsillar carcinoma. (A) PCR with E6-E7 primer combination. Levels of full length 513 bp fragments and spliced E6*I 236 bp fragments are indicated by arrowheads. (B) PCR with HPV 33 E6-E2/E4 primer combination. Agarose gel pattern of PCR products and hybridization analysis with an HPV 33 probe are indicated on the left and right, respectively. Levels of hybridizing cDNA fragments corresponding with cDNA species a (714 bp), c (438 bp) and d (328 bp) (see Figure 3) are indicated by arrowheads. (C) Agarose gel pattern (left) and hybridization analysis of RNA PCR products generated by the E6-oligo-(dT)-adaptor primer combination. Arrowhead points to the level of the 500 bp cDNA fragment representing cDNA species b (see Figure 3). M, pBR322 DNA digested with *Hin*fI.

HPV physical state and E6/E7 transcription in biopsies from different tonsillar carcinomas

Biopsies obtained from three additional tonsillar carcinomas also were examined for E6/E7 transcription and viral physical state. This concerned one HPV 33 containing sample (sample T4) and two HPV 16 containing cases (samples T2 and T7). As shown in Table 1, RNA PCR with the HPV 33 and HPV 16 E6-E7 primer pairs revealed the presence of HPV 33 E6*I mRNA and of HPV 16 E6*I (Smotkin *et al.*, 1989) and unspliced E6/E7 mRNA in samples T4 and T2, respectively, while degradation of RNA within sample T7 did not allow HPV RNA analysis. By standard Southern blot analysis sample T4 revealed an off-sized 11 kb *Bgl*II fragment. DNA PCR analysis of this sample with different HPV 33 specific primer combinations revealed a disruption within the E1 ORF which maps between nts 1136 and 2396 (Table 1). Although this is

suggestive for integration, the amount of DNA available did not suffice for further analysis so that no supporting evidence could be obtained. The HPV 16 containing biopsies both revealed the prototype HPV 16 *Bam*HI fragment of 8 kb and the prototype HPV 16 *Pst*I pattern (Figure 1D, lanes T2 and T7). No off-sized restriction fragments could be detected in these samples. Two-dimensional analysis with *Hin*dIII, a no-cut enzyme for HPV 16, revealed the presence of circular HPV 16 DNA in both samples (Figure 2, panels C and D). Furthermore, these samples also showed linear DNA fragments of 8 kb (samples T2 and T7) and 16 kb (sample T7) consistent with linearized monomeric and dimeric episomal DNA. Histologic examination revealed that samples T2 and T7 contained approximately 70% and 50% carcinoma cells, indicating that the failure to detect clonally integrated DNA is not owing to the presence of low percentages of neoplastic cells. Two-dimensional analysis using a model system containing 5 μg DNA of the Siha cell line (which contains one copy of integrated HPV 16 DNA per cell) mixed with 5 μg human placental DNA and cloned pHPV 16 DNA at a copy number of 50 genome equivalents per cell allowed the detection of the integrated HPV 16 DNA (not shown).

Discussion

Recently, a strong association between certain HPV genotypes and tonsillar carcinomas has been documented (Chapter VI). HPV 16 and HPV 33 containing biopsies of tonsillar carcinomas have been found to express putative E7 mRNAs which contain an intron within the E6 ORF. In addition, RNA *in situ* hybridization has demonstrated the presence of E7 transcripts exclusively localized within the carcinoma cells, suggesting an etiological role for HPV in tonsillar carcinoma. This study was undertaken to find out whether these E6/E7 mRNAs are expressed from integrated or episomal HPV DNA. The viral physical state was studied by genomic Southern blot analysis, DNA PCR and, if sufficient DNA was available, by additional 2-D analysis.

The HPV 33 containing carcinoma from which a biopsy and two resected tumor parts were obtained, showed a rather complex physical state profile reflected by subpopulations of tumor cells containing episomal DNA and different subpopulations containing integrated HPV 33 DNA. Biopsy specimen T6 from this tumor showed a profile consistent with the presence of episomal DNA. No evidence for the presence of clonally integrated HPV 33 was obtained. Resected tumor specimen T6.1 contained episomal as well as integrated DNA, resected tumor specimen T6.2 contained integrated HPV 33 DNA exclusively. The off-sized restriction fragments of specimens T6.1 and T6.2 were equal in size suggesting that they represent the same integrated viral DNA status. PCR data from sample T6.2 suggest that integration resulted in the interruption of the E2/E4 region. The reason for the heterogeneity observed in this carcinoma is still unknown. One explanation could be that viral DNA integration, coupled with the loss of episomes, had taken place at a late stage of tumor development so that only a subpopulation of carcinoma cells contained integrated HPV 33 DNA at the time of surgical treatment. Alternatively, viral DNA integration might have been an early event followed by the loss of integrated DNA in some tumor cells.

Independent of the viral physical state, expression of E7 encoding HPV 33 E6*I mRNA was evident for all tumor cell populations represented by the three different specimens obtained from this carcinoma. No evidence for the presence of unspliced HPV 33 E6/E7 mRNA with E6 coding potential could be obtained in this carcinoma. This indicates that HPV 33 E6 mRNA, if present, exists at very low levels. Since HPV 33 integration within this carcinoma probably resulted in the disconnection of upstream E6/E7 sequences from the putative early region polyadenylation signal which maps to nt 4176 we supposed that the integration event would affect the transcriptional pattern. Indeed, further comparison of early region transcripts between samples T6.1 and T6.2 revealed that mRNAs encompassing most of the early region (Figure 3, species a,c and d) were not generated from integrated HPV 33 DNA. In fact, the shorter E6*I species b, which utilizes a polyadenylation signal at the 3' end of the E7 ORF, was the only mRNA originating from integrated HPV 33 DNA that could be detected. This finding is consistent with data obtained from cervical carcinomas and cell lines containing HPV 16 and HPV 18 DNA, showing that transcription of E6/E7 region sequences is preferentially selected or retained after viral DNA integration (Schneider-Gädicke *et al.*, 1986; Inagaki *et al.*, 1988; Shirasawa *et al.*, 1988; Broker *et al.*, 1989). No evidence could be obtained for the involvement of a cellular polyadenylation signal in the transcription of E6/E7 region sequences. It is likely that integrated HPV 33 DNA can generate E7 transcripts independent of cellular poly (A) signals due to the presence of a second early region poly (A) motif. In contrast, HPV types 16 and 18 do not contain an equivalent second early region poly(A) signal and in a similar integrated state these types would require a cellular poly (A) signal to provide their E7 expression via polyadenylated mRNA.

It should be noted, however, that this heterogeneous distribution of integrated and episomal HPV DNA could reflect an uncommon rather than a general event. Still this finding suggests that tonsillar carcinoma cells can acquire E6/E7 transcript both from integrated and episomal DNA. To test this assumption, biopsies of three additional tonsillar carcinomas were analysed. Of these samples, only one HPV 33 containing biopsy (T4) showed an aberrant Southern blot pattern. In conjunction with the abnormal DNA PCR profile this is suggestive for the presence of integrated HPV DNA in this sample. However, although a disruption was mapped within the E1 ORF, it still can not be excluded that this sample contained altered episomal HPV 33 forms rather than integrated HPV 33 DNA. Analysis of sample T4 by DNA PCR with single-sided specificity (Riley *et al.*, 1990) to detect putative viral-cellular junctions is presently in progress to find out whether the HPV DNA is integrated.

The two HPV 16 containing tonsillar carcinoma biopsies revealed hybridization and PCR results consistent with the presence of only episomal HPV 16 DNA in a clonal arrangement. Since in the model system of Siha and human placental DNA half a copy per cell of integrated HPV 16 DNA could be demonstrated in the presence of excess (50 copies per cell) HPV 16 DNA, a clonal distribution of single copy integrated HPV 16 DNA would have been detected in these biopsies.

The results obtained from this study imply that transcription of HPV 16 and HPV 33 E6/E7 mRNA in tonsillar carcinomas is not necessarily dependent on viral DNA integration. Consequently, the maintenance of the malignant phenotype of tonsillar carcinomas does not seem to require integrated HPV DNA.

Recently, similar data have emerged with respect to cervical carcinomas. Also a proportion of HPV 16 containing cervical carcinomas have been found to contain episomal HPV DNA only (Fuchs *et al.*, 1989; Matsukura *et al.*, 1989; Cullen *et al.*, 1991). Furthermore, Nasseri *et al.* (1991) have found that the presence of HPV 16 E2 encoding transcripts does not affect the expression levels of the E7 protein in immortalized cervical keratinocytes suggesting that E2 interruption triggered by integration is not causually related to increased E7 expression. Still HPV DNA integration could be important in carcinogenesis. However, it is likely that also viral intragenomic modifications or changes in host cell gene expression affecting viral E6/E7 expression may take place as alternative events to gain an equivalent effect from viral episomes.

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CHAPTER IX

General discussion

General discussion

1. HPV DNA detection using general primers in the PCR

In this thesis two general primer-mediated PCR methods were described. Of these methods, the GP5/GP6 mediated PCR shows the highest sensitivity and is therefore the most appropriate assay for a first screening of clinical samples. For HPV typing, GP5/GP6 PCR positive samples have to be subjected to type-specific PCR with primers directed against the sequenced HPV genotypes. If a given sample is negative by type-specific PCR, the original GP5/GP6 PCR product has to be sequenced to find out whether the HPV type detected is a novel type.

Due to its greater universal properties, the GP17/GP18 PCR method has to be applied to GP5/GP6 negative samples to screen for the presence of putative distantly related HPV types. This technique can be of additional value since it generates a larger amplified fragment that can be used either to select type-specific primers more easily or as a suitable probe for additional type-specific hybridization.

However, it has to be kept in mind that to explore an etiological role of HPV additional techniques have to be applied. In particular HPV RNA-DNA *in situ* analysis is necessary to localize HPV in the neoplastic cells.

2. Clinical aspects and future perspectives

The main question to be answered in this thesis is whether HPV is a candidate to be etiologically involved in carcinogenesis in the upper aerodigestive tract. The question can be divided in two sections, i.e. the role of HPV in the pathogenesis of tonsillar carcinomas and HPV in relation to other carcinomas of the upper digestive tract and the respiratory tract.

2.1. Tonsillar carcinomas

All tonsillar carcinomas tested, were found to contain HPV DNA. In addition, transcription of the E6-E7 region was demonstrated and E6-E7 mRNAs were exclusively localized within the neoplastic cells. These findings indicate an association which resembles the association between HPV and cervical carcinomas. Consequently, HPV is likely to be involved in the pathogenesis of tonsillar carcinomas as well. To determine the HPV prevalence rate in nonmalignant tonsils, large numbers of samples from matched individuals, including clinically normal tonsils, have to be investigated.

2.2. Carcinomas from other sites within the upper digestive and respiratory tracts

The question whether HPV is involved in the genesis of squamous cell carcinoma of the other sites in the air and upper food passages is more complicated. HPV DNA appeared to be present only in about 20% of these carcinomas (Table 1). However, the finding that HPV 16 DNA, present in a carcinoma of the floor of the mouth, is transcriptionally active, as determined by HPV RNA-PCR and *in situ* analysis (Table 1), argues for a role of HPV in the pathogenesis of these carcinomas as well. The finding that HPV DNA is also found in 1 out of 4 laryngeal hyperplasias with atypia, which are the precursor lesions of these carcinomas (Table 1), supports this hypothesis. Moreover, it recently has been found that

both human oral and tracheal epithelial cells can be immortalized by HPV 16 and HPV 18 (Park *et al.*, 1991; Conrad *et al.*, 1991; Sexton *et al.*, 1991), indicating the oncogenic potential of these viruses in epithelium of both the upper digestive tract and the respiratory tract.

Table 1. HPV GP5/GP6 PCR and HPV typing on biopsy specimens from sites within the upper digestive and respiratory tracts[1]

Specimens	HPV GP-PCR positive	HPV type
SCC[2] tongue (n=9)	2 (22%)	1x HPV 33 1x HPV X[3]
SCC buccal mucosa (n=5)	1 (20%)	HPV 16
SCC floor of mouth (n=6)	1 (17%)	HPV 16[4]
SCC nose (n=3)	0	-
SCC tonsil (n=10)	10 (100%)	4x HPV 16 3x HPV 33 1x HPV 16/33 1x HPV X[3] 1x HPV X[6]/33
Tonsillitis (n=7)	0	-
Papillomas larynx (n=2)	2[5] (100%)	2x HPV 6
Hyperplasia larynx (n=4)[7]	1 (25%)	HPV 16
SCC larynx (n=12)	2 (17%)	1x HPV 11 1x HPV X[3]
SCC lung (n=15)	3 (20%)	1x HPV 6 2x HPV X[3]

[1]Application of GP17/GP18 PCR and PCR with the E1 region primer pair GP1/GP2 (van den Brule *et al.*, 1990) did not result in the detection of additional HPV positive cases
[2]SSC = Squamous cell carcinoma
[3]HPV X = Negative by PCR specific for HPV 6, 11, 16, 18, 31 and 33
[4]HPV 16 positive case also showed E6/E7 expression which was limited to the neoplastic cells as determined by RNA PCR and RNA *in situ* hybridization
[5]Confirmed by genomic Southern blot analysis
[6]Determined by cloning and sequence analysis of the GP-PCR product
[7]Hyperplasias with atypia

There are several explanations to be given for the relative low HPV prevalence rate in these squamous cell carcinomas. It is unlikely that these results reflect the presence of HPV types containing alterations in the primer matched regions of the L1 ORF because also the E1 region general primer pair GP1/GP2 and type-specific primers located

elsewhere in the HPV genome did not reveal additional HPV positivity (Table 1; Snijders, P.J.F., unpublished results). The HPV positive squamous cell carcinomas of the tongue, larynx and lung include cases containing thus far unknown HPV sequences. Therefore, characterization of the GP5/GP6 PCR products of these samples followed by molecular cloning of these genotypes and further type-specific screening by PCR has to be carried out and will answer the question whether novel HPVs are preferentially associated with these carcinomas.

Alternatively, screening of these carcinomas for the presence of mutations in the tumor suppressor genes p53 and pRb could give interesting information in view of recent findings concerning HPV-positive and HPV-negative cervical carcinoma cell lines. In particular modification of p53 functions, either by mutations or by interactions with viral encoded oncogene products, has shown to be a common genetic change in several human cancers (Levine *et al.*, 1991). Therefore, carcinomas lacking mutations of these tumor suppressor genes could be more likely to be associated with HPV. Hence, the significance of HPV in carcinogenesis within the upper digestive and respiratory tracts has to be further investigated by comparative analysis of HPV positive and HPV negative carcinomas for the presence of p53 and pRb mutations.

At last, it appears of interest to find out whether HPV infection in head and neck cancer patients has any prognostic value for the development of second primary neoplasms within the same organ system. Also the significance of HPV present in premalignant lesions, e.g. laryngeal hyperplasias, has to be further defined and HPV studies on these samples have to be extended.

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CHAPTER X

Summary / Samenvatting

Summary

This thesis describes studies which have been performed to elucidate the relationship between human papillomaviruses (HPVs) and squamous cell carcinomas of the head and neck region.

Previous studies on cervical cancer have revealed a strong association between so-called high risk HPV types and cervical carcinomas. Additional investigation of these HPV types has resulted in the discovery of oncogenic properties, suggesting that these HPVs are involved in the pathogenesis of cancer of the uterine cervix.

Chapters II and II describe the development of methods which permit the detection of a broad spectrum of HPV genotypes. The HPV group comprise more than 60 different types and it was not predictable which of them could be associated with head and neck carcinomas. Furthermore, it could not be excluded that unknown, still unidentified HPV types are specifically associated with these carcinomas. Consequently, attention was focussed on the development of a sensitive and universal method of detecting HPV. For this purpose the recently developed polymerase chain reaction (PCR) was used. This method is able to amplify specific viral DNA fragments in such a manner, that theoretically one HPV DNA molecule can be detected. The use of the PCR as a universal HPV detection method requires the selection of primers which anneal to multiple HPV genotypes (general primers). As only a limited number of HPV types have been sequenced, the general properties of general primers selected, have had to be determined experimentally by subjecting a large group of HPV genotypes to PCR. In the study described in chapter II, two general primer pairs appeared successful in detecting a broad spectrum of either mucosotropic (GP5/6) or cutaneous (GP11/12) HPVs. Chapter III describes the use of a single general primer pair (GP17/18) in the PCR, which was designed on basis of conserved amino acid sequence in papillomaviruses. These degenerate primers reacted with all HPV genotypes that have been examined, including those associated with both mucosal and cutaneous lesions. However, the use of this primer pair in the PCR showed a reduced sensitivity; so, samples must be selected carefully before applying this method. The study described in chapter IV deals with the analysis of the GP5/6-PCR method which is described in chapter II. Comparative sequence analysis of GP5/6-PCR products allowed the determination of an HPV consensus sequence. In addition, a polymorphic region could be found within the GP5/6-amplified viral region. Together, these regions can be used both for confirmation of HPV specificity and for HPV genotype differentiation.

In chapter V the PCR was used to analyse the presence of HPV- and EBV-DNA in oral biopsies, including biopsies of hairy leukoplakias, of HIV-infected patients. The results indicate that hairy leukoplakia is strongly associated with EBV and not with any of the HPV types that react with general HPV primers in the PCR.

Chapter VI describes the use of the GP-PCR method to assess the presence of HPV DNA in biopsies of tonsillar carcinomas. All carcinomas (n=10) appeared HPV positive by GP-PCR. Additional type-specific PCR revealed that four carcinomas contained HPV 16 DNA, three carcinomas contained HPV 33 DNA and one carcinoma contained an HPV 16/33 double infection. Furthermore, two carcinomas contained still unknown HPV genotypes (HPV X), one carcinoma which also contained HPV 33 DNA and one carcinoma which was negative by type-specific PCR. False-positivity was excluded by additional Southern blot analysis of HPV 16 and HPV 33 PCR positive samples. Homology comparison of amplified sequences derived from the HPV X genotypes revealed that these sequences do not represent any of 28 HPV types included in the comparison. Application of RNA PCR revealed expression of HPV 16 or HPV 33 E7 encoding transcripts in all carcinomas examined and RNA *in situ* analysis showed that these transcripts were exclusively localized within the carcinoma cells.

Also HPV 33 specific early region transcripts were mapped in a tonsillar carcinoma (chapter VII). By RNA PCR a total of five transcript species were identified which include spliced E6* species. As determined by 3' cDNA end mapping, one E6* species was found to utilize a second early region poly (A) signal.

The study described in chapter VIII deals with the question whether HPV E6/E7 transcription in tonsillar carcinomas is correlated with viral DNA integration. Tonsillar carcinomas containing HPV 16 and HPV 33 were analysed for the viral physical state and transcription of the E6/E7 region. Independent of the viral physical state all samples tested, showed E6/E7 transcription indicating that E6/E7 transcripts in tonsillar carcinomas can originate from integrated as well as episomal HPV DNA.

The data collected in these studies suggest a role of HPV in the development of tonsillar carcinomas.

Samenvatting

In dit proefschrift worden studies beschreven die tot doel hebben de relatie tussen humane papillomavirussen (HPV's) en plaveiselcelcarcinomen uit het hoofd-halsgebied op te helderen.

Uit eerder onderzoek naar baarmoederhalskanker is gebleken dat bepaalde, zogenaamde kwaadaardige HPV typen zeer frequent voorkomen in baarmoederhalscarcinomen. Verdere studies naar deze virussen hebben geleid tot de ontdekking van oncogene eigenschappen, waardoor het aannemelijk is dat deze virussen een rol spelen bij het ontstaan van baarmoederhalskanker.

De hoofdstukken II en III beschrijven de ontwikkeling van methodieken die het mogelijk maken een breed spectrum aan verschillende HPV typen aan te kunnen tonen. De HPV groep bestaat immers uit meer dan 60 typen en het was op voorhand niet te traceren welke van deze typen konden voorkomen in hoofd-halscarcinomen. Tevens kon de mogelijkheid dat onbekende, nog niet geïdentificeerde HPV typen voorkomen in deze carcinomen niet worden uitgesloten. Derhalve werd ruime aandacht besteed aan de ontwikkeling van gevoelige, universele HPV detektiemethoden. Hiervoor werd de recent ontwikkelde polymerase kettingreaktie (PCR) gebruikt. Deze methode is in staat om heel specifiek kleine fragmenten van het virale erfelijke materiaal, het DNA, te vermeerderen. De mate waarin deze vermeerdering plaatsvindt is dusdanig, dat theoretisch één HPV DNA molecuul kan worden aangetoond. De moeilijkheid voor het gebruik van de PCR methode als universele HPV detektiemethode lag op het niveau van de selektie van geschikte algemene primers. Primers zijn korte, synthetische DNA moleculen die als "starters" fungeren voor de vermeerderingsreaktie en bepalen welke DNA moleculen kunnen worden vermeerderd. De basenvolgorde van een primer moet geheel of grotendeels overeenkomen met die van het te vermeerderen DNA molecuul. Aangezien van de meeste HPV typen geen basenvolgorde bekend is, moesten algemene HPV primers worden geselekteerd aan de hand van de basenvolgorde van een beperkt aantal HPV's. Het universele karakter van deze primers werd vervolgens proefondervindelijk vastgesteld door een breed panel aan HPV typen te onderwerpen aan de PCR. In hoofdstuk II wordt het succesvolle gebruik van twee algemene primercombinaties (GP5/6 en GP11/12) beschreven, die het mogelijk maakten om HPV typen voorkomend in respectievelijk afwijkingen van de slijmvliezen en huidafwijkingen aan te tonen. Hoofdstuk III beschrijft het gebruik van één primercombinatie (GP17/18) die reageerde met alle HPV typen die zijn onderzocht. Inherent aan het gebruik van deze primercombinatie is echter een gereduceerde gevoeligheid zodat klinisch materiaal moet worden voorgeselekteerd alvorens een PCR met deze primers toe te passen. Hoofdstuk IV behandelt de verdere uitwerking van de GP5/6-PCR methode zoals deze is beschreven in hoofdstuk II. Door de basenvolgorden van GP5/6-PCR produkten, afkomstig van verschillende HPV typen, te vergelijken, kon een HPV specifieke regio (consensus) alsmede een heterogene regio worden vastgesteld. Tesamen maken deze regio's het mogelijk om zowel de virale herkomst van een GP-PCR produkt te bevestigen als onderscheid te maken tussen verschillende HPV typen.

In het onderzoek beschreven in hoofdstuk V is de PCR methode gebruikt om het voorkomen van HPV- en EBV-DNA te bestuderen in orale weefsels, waaronder biopten van harige leukoplakie, van HIV-seropositieve patienten. Geen samenhang tussen harige

leukoplakie en de aanwezigheid van HPV DNA kon worden aangetoond. Wel werd een sterke associatie van harige leukoplakie met EBV DNA aangetoond.

In hoofdstuk VI is het gebruik van de GP-PCR methode beschreven om het voorkomen van HPV te bestuderen in plaveiselcelcarcinomen van de tonsil (amandel). Alle tonsilcarcinomen die zijn onderzocht (n=10), vertoonden positiviteit voor HPV DNA. Typering met behulp van HPV type-specifieke PCR analyse gaf aan dat het in 4 gevallen HPV 16 DNA betrof, terwijl 3 carcinomen HPV 33 DNA bevatten. Daarnaast werd een HPV 16/HPV 33 dubbelinfectie aangetoond alsmede een geval met een HPV X/HPV 33 dubbelinfectie en een geval met HPV X DNA, waarbij X een nog onbekend HPV type aanduidt. De positiviteit voor HPV 16 en HPV 33, typen die ook voorkomen in baarmoederhals-carcinomen, kon in een aantal gevallen worden bevestigd met behulp van genomische Southern blot hybridisatie. Nadere analyse van de PCR produkten afkomstig van de HPV X-en leerde dat deze typen verschillen van 28 HPV typen die eerder konden worden aangetoond met de GP-PCR methode en reeds waren gekarakteriseerd. Verdere analyse van HPV 16 en HPV 33 bevattende carcinomen gaf aan dat deze typen RNA, coderend voor de virale onco-eiwitten, tot expressie brengen en RNA *in situ* analyse heeft aangetoond dat deze expressie beperkt is tot de carcinoomcellen.

Hoofdstuk VII beschrijft de analyse van transcripten uit het vroege gebied van HPV 33 in een tonsilcarcinoom. Dit leidde tot de identificatie van zogenaamde E6* mRNA's en tevens werd een tweede polyadenyleringssignaal in het vroege gebied van HPV 33 ontdekt.

In hoofdstuk VIII werd bekeken of HPV 16 en HPV 33 transcripten in tonsilcarcinomen, die coderen voor het E6-E7 gebied, afkomstig zijn van extrachromosomaal viraal DNA of van viraal DNA dat is geïntegreerd in het gastheer DNA. E6-E7 RNA kon worden aangetoond in zowel gevallen met geïntegreerd DNA als gevallen met extrachromosomaal DNA.

De resultaten verkregen uit bovengenoemde studies maken het aannemelijk dat HPV typen een rol spelen bij het ontstaan van tonsilcarcinoom.

Publications / Curriculum vitae

Publications

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Curriculum vitae

De schrijver van dit proefschrift werd op 5 augustus 1961 geboren te Nijmegen. In 1979 behaalde hij het VWO diploma aan de scholengemeenschap Canisiuscollege-Mater Dei te Nijmegen. In juli van datzelfde jaar trad hij vervroegd in dienst bij de luchtmacht ter vervulling van de militaire dienstplicht. In 1980 begon hij zijn studie Biologie aan de Katholieke Universiteit te Nijmegen. In maart 1984 behaalde hij het kandidaatsexamen Biologie (B1g). In het kader van zijn doctoraalstudie volgde hij bijvakken op de afdelingen Chemische Cytologie (Dr. F. Wanka) en Farmacologie (Dr. J.F. Rodrigues de Miranda). Hij volgde zijn hoofdvak op de afdeling Moleculaire Biologie (Prof. Dr. J.G.G. Schoenmakers en Prof. Dr. R.N.H. Konings) en het doctoraalexamen werd afgelegd op 26 mei 1987.

Vanaf mei 1987 is hij werkzaam op het Pathologisch Instituut van het Academisch Ziekenhuis der Vrije Universiteit te Amsterdam, alwaar hij tijdelijk onderzoek verrichtte naar erfelijke darmtumoren. Vanaf 1 april 1988 werd het onderzoek verricht waarvan het resultaat is beschreven in dit proefschrift.

Stellingen
behorende bij het proefschrift

Human papillomavirus and carcinomas of the upper aerodigestive tract

1. Het voorkomen van transcriptioneel actieve HPV typen in de neoplastische cellen van tonsilcarcinomen maakt een causale relatie aannemelijk *(dit proefschrift)*.

2. Papillomavirussen zijn niet geassocieerd met harige leukoplakie *(dit proefschrift)*.

3. De aanwezigheid van een splice-acceptor in het E6 open leesframe van HPV 33 die verschilt van de voorspelde acceptor, geeft aan dat voor de vaststelling van feitelijke splice-plaatsen niet enkel de aanwezigheid van geconserveerde splice consensus sequenties in aanmerking genomen mag worden, maar dat hiervoor analyse van RNA of cDNA vereist is *(dit proefschrift)*.

4. De polymerase kettingreactie is niet alleen van waarde voor het aantonen van reeds gekarakteriseerde HPV typen, maar kan ook waardevol zijn bij de ontdekking van nog onbekende HPV typen *(dit proefschrift)*.

5. Het gevaar voor vals-positiviteit bij ondeskundig gebruik van de polymerase kettingreactie zou extra moeten worden benadrukt door als alternatief PCR te lezen als "**P**revent **C**ontamination **R**eaction".

6. De konklusie dat hybridiserende restrictiefragmenten van afwijkende lengte een nieuw lid van een genfamilie vertegenwoordigen, mag niet worden gebaseerd op het resultaat verkregen met slechts één restrictie-enzym *(Meakin et al. (1985). Mol. Cell. Biol. 5, 1408-1414)*.

7. Karakterisering van genen die geassocieerd zijn met translocaties gevonden in maligniteiten kunnen leiden tot de isolatie van nieuwe cellulaire oncogenen *(Fisher et al. (1988). Br. J. Haematol. 71, 31-36)*.

8. Het frusterende maar tevens boeiende aspekt aan wetenschapsbeoefening is dat de uitkomst van een experiment meer vragen kan opwekken dan beantwoorden.

9. De kreet "Steeds meer weten over steeds minder leidt tot alles weten over niets" geeft aan dat een specialisatie niet te ver mag voeren daar anders het contact met de realiteit verloren gaat.

10. De toenemende populariteit van de telefax kan ertoe leiden dat het huis van de toekomst niet meer in het bezit is van een brievenbus.

11. De bevinding dat de werkplek op vier kilometer afstand van het huis sneller met de fiets dan met de auto wordt bereikt, bevestigt het gegeven dat het Nederlandse wegennet overvol is *(eigen ervaring)*.

Peter Snijders, 28 februari 1992