Human Papillomavirus 16 E6 Variants Are More Prevalent in Invasive Cervical Carcinoma than the Prototype

Ingeborg Zehbe, Erik Wilander, Hajo Delius, and Massimo Tommasino

Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie, D-69120 Heidelberg, Germany; and Pathology Department, University Hospital, S-75185, Uppsala, Sweden [I. Z., E. W.]

ABSTRACT

High-risk human papillomavirus (HPV) is a known risk factor in the etiology of cervical intraepithelial neoplasia (CIN) I–III and invasive cervical carcinoma (ICC). The most severe preinvasive lesion is CIN III, and it is still not entirely understood why some cases progress to invasion, whereas others do not. Our hypothesis that this could be predicted by intratype variation of the immortalizing and transforming early proteins E6 and E7 was tested. Because HPV16 is frequently detected in cervical neoplastic lesions, 25 CIN III and 17 ICC cases from Swedish women, all positive for this genotype, were selected to investigate the E6 and E7 genes for mutations. PCR-amplified products were sequenced by the fluorescent dideoxy termination method. ICC harbored almost exclusively HPV16 E6 variants (94%) and rarely harbored the prototype (6%), whereas CIN III demonstrated a more uniform distribution of variants (56%) and prototype (44%); \( P = 0.013 \). All variants contained variations that were identified in areas likely to be important for protein-protein interaction with p53 or in areas of immunological significance. The most frequent E6 variation was seen at residue 83. This polymorphism was detected alone or in combination with others in 88% of ICC and 44% of CIN III cases. E7 variations were extremely rare and were only detected together with E6 variations in 4% of CIN III and in 6% of ICC cases, suggesting that the HPV16 E7 but not the HPV16 E6 oncprotein is highly conserved in vivo. This indicates that HPV16 E6 variants, specifically those containing the substitution at residue 83, may be more oncogenic than the prototype and thus carry a higher risk for the development of invasive cervical disease. This may be due to subtle differences in the type of transformation produced or to evasion of host immune defenses. These results might have implications for future in vitro studies, diagnostics, treatment, and vaccine design.

INTRODUCTION

The assumption that almost all cervical carcinomas are associated with high-risk HPVs \( ^3 (1) \) has been confirmed by molecular biological studies (2–4) and epidemiological studies (5–7). HPV16 is the most commonly detected genotype not only in ICC (5), but also in its precursor lesions, CIN I–III (8–10). Molecular studies have shown that proteins originating from the early region of HPV16 E6 and E7 are able to immortalize human keratinocytes in vitro and are most probably involved in the malignant transformation of cervical epithelium by interacting with cell cycle-regulating proteins, especially p53 and retinoblastoma protein (2–4). Moreover, a continuous expression of the early E6 and E7 oncoproteins is necessary to retain a malignant phenotype (11). In epidemiological studies, additional risk factors, which may contribute to the development of cervical carcinoma, were identified. These include, \( e.g. \), age at infection, smoking, hormonal factors, genetic predisposition, and immunological response (5–7, 12–14).

Persistent HPV infection, often observed in combination with high-risk rather than low-risk HPVs (15) is a risk factor for developing CIN II and CIN III (16, 17). There is sufficient morphological and epidemiological consensus for the assumption that CIN III is a dynamic disease in which some cases regress spontaneously, whereas others progress to the invasive state (14, 18–21). The progression rates in these reports have varied considerably, with an estimate of approximately 12–69%. Both CIN III and ICC were shown to have similar risk factors, with HPV being the most important, but none was absolutely predictive for progression (14). Instead, additional features other than the presence of high-risk HPV \( \text{per se} \) are likely to be more predictive. Intratype gene variations, especially of HPV16, have been reported, and there seems to be some evidence that these may be involved in developing a persistent infection (22) or a major cervical disease (23–29). Additional, it was demonstrated that naturally occurring variants of HPV16 E6 differ in biological and biochemical properties by having a reduced ability to suppress keratinocyte differentiation responses and to induce p53 degradation. It was reasoned that this might result in differences in pathogenicity (30).

We tested whether progression from CIN III to ICC could be predicted by intratype variations of the immortalizing and transforming early proteins E6 and E7. Because HPV16 is the genotype most often detected in cervical preinvasive and invasive lesions, and because the oncoproteins E6 and E7 play a major role in malignant transformation, the corresponding genes were examined for putative mutations in histologically confirmed cervical biopsies.

MATERIALS AND METHODS

Tumor Specimens. The cervical carcinoma prevention program in Sweden requires that women with a series of abnormal Papanicolaou smears must be biopsied to establish the ultimate diagnosis. Formalin-fixed punch biopsies from 90 such women (age range, 22–81 years; mean age, 41 years), diagnosed as having CIN III \( (n = 58) \) or ICC of squamous cell type \( (n = 32) \) were randomly selected for HPV testing at the Pathology Department of the University Hospital in Uppsala, Sweden. Only HPV-positive cases remained eligible for the present E6 and E7 polymorphism study. We also included the HPV16 reference strain, the so-called HPV16 prototype (kindly received from Dr. Ethel de Villiers, Deutsches Krebsforschungszentrum, Heidelberg, Germany), which was isolated from a German cervical carcinoma patient (31).

PCR. HPV detection was performed with the hot-start modification (9) by using consensus primers from the L1 region (32). These primers, denoted GP5+ (5’-TTTGTATCTGGTAGTATACATC-3’) and GP6+ (5’-GAAAAATAACCTGTAATACATTC-3’), define a region of the L1 ORF from nt 6624–6765 for HPV16 and the corresponding regions of the other genital HPVs. HPV typing was performed with single-strand conformational polymorphism (33). According to the above-mentioned selection criteria, 25 HPV16-positive CIN III and 17 HPV16-positive ICC cases along with the HPV16 reference strain were subjected to hot-start PCR with primers amplifying the entire coding region of the HPV16 E6 (nt 83–559) and E7 ORFs (nt 562–858). The E6 ORF primers were 5’-CGAAAATCTGTAAATCCATTC-3’ and 5’-GTATCTCCATGCATGATT-3’, spanning nt 52–575 (23) and the E7 ORF primers were 5’-AAATATAAGGAGTGTTGCGTG-3’ and 5’-GTATTGTTGATGATCATTT-3’, spanning nt 480–985 (34). Forty amplification...
cycles were run in the Peltier thermal cycler (PTC-200; MJ Research Inc., Watertown, MA) with a 94°C denaturation step (1 min), a 55°C annealing step (1 min), and a 72°C extension step (2 min), including an initial denaturation step of 3 min and a final extension step of 7 min. PCR products were checked by ethidium bromide agarose gel electrophoresis. All samples were successfully amplified with the HPV16 E6- and E7-specific primers.

Cycle Sequencing. After post-PCR clean-up with microspin S-300HR columns (Pharmacia Biotech, Uppsala, Sweden), E6 and E7 PCR products were sequenced by the fluorescent dye dideoxy termination method using an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). For the sequencing reaction, the same primers were used as for the PCR reaction. The accuracy of the method was verified by repeating the PCR template production and the sequence determination using a second aliquot of 21 HPV DNAs and determining the sequence of both strands of all PCR products. In parallel, the HPV16 E6/E7 prototype was analyzed and found to be identical with the published sequence (31).

Establishment of E6 and E7 Variants. In 9 of 42 (21%) cases, the E6 and E7 sequences corresponded to the HPV16 prototype (31). The remaining 33 (79%) cases contained 22 E6 and 7 E7 mutations, of which 14 E6 and 2 E7 mutations resulted in amino acid changes (variations). Altogether, 12 variants were identified containing 1 or more amino acid changes. All showed E6 variations, but only two variants also included E7 variations (Fig. 1). No mutations occurred in the E6/E7 promoter sequence or at splice junctions, and there was no evidence of premature stop codons or deletions. All patterns were consistent in independent amplifications from a given specimen.

Fig. 1 lists the E6 and E7 mutations with predicted amino acid changes and their distribution in CIN III and ICC. Three cases (two CIN III cases and one ICC case) with nonsynonymous mutations were assigned to the prototype. Regarding E6, variations at residues 10, 14, 27, 78, and 83 were seen in both CIN III and ICC, of which the substitution of amino acid 83 with a change from leucine to valine (L83V) was the most prevalent. ICC harbored L83V alone or in combination with others in 15 of 17 (88%) cases. The corresponding figures for CIN III were 11 of 25 (44%) cases. Residues 10, 14, and 27 showed several amino acid substitution possibilities. Five E6 variations were exclusively found in CIN III (residues 3, 11, 58, 60, and 61) and two were found in ICC (residues 25 and 55), respectively. Regarding E7, the variations at residues 29 and 51 were detected in CIN III and ICC, respectively.

Localization of E6 and E7 Variations. Fig. 2 demonstrates the positions of E6 and E7 variations with respect to protein structure. Ten E6 variations were found in the amino-terminal half, in regions before and within the first predicted zinc finger, and two were seen in the region linking the two zinc fingers. One E7 variation was seen in the amino-terminal, in coding region 2, and the other one was seen in the carboxyl-terminal half, in coding region 3, just before the predicted zinc finger.

Prevalence of Variants in CIN III and ICC. In CIN III, 14 of 25 cases (56%) contained variants and 11 of 25 cases (44%) contained the prototype. ICC harbored variants in 16 of 17 cases (94%) and contained the prototype in just 1 case (6%). The correlation of disease status with prototype and variant was statistically significant (P = 0.013; Fig. 3).

Regarding E7, the variations at residues 29 and 51 were detected in CIN III cases and one ICC case) with nonsynonymous mutations were assigned to the prototype. Regarding E6, variations at residues 10, 14, 27, 78, and 83 were seen in both CIN III and ICC, of which the substitution of amino acid 83 with a change from leucine to valine (L83V) was the most prevalent. ICC harbored L83V alone or in combination with others in 15 of 17 (88%) cases. The corresponding figures for CIN III were 11 of 25 (44%) cases. Residues 10, 14, and 27 showed several amino acid substitution possibilities. Five E6 variations were exclusively found in CIN III (residues 3, 11, 58, 60, and 61) and two were found in ICC (residues 25 and 55), respectively. Regarding E7, the variations at residues 29 and 51 were detected in CIN III and ICC, respectively.

Fig. 1 lists the E6 and E7 mutations with predicted amino acid changes and their distribution in CIN III and ICC. Three cases (two CIN III cases and one ICC case) with nonsynonymous mutations were assigned to the prototype. Regarding E6, variations at residues 10, 14, 27, 78, and 83 were seen in both CIN III and ICC, of which the substitution of amino acid 83 with a change from leucine to valine (L83V) was the most prevalent. ICC harbored L83V alone or in combination with others in 15 of 17 (88%) cases. The corresponding figures for CIN III were 11 of 25 (44%) cases. Residues 10, 14, and 27 showed several amino acid substitution possibilities. Five E6 variations were exclusively found in CIN III (residues 3, 11, 58, 60, and 61) and two were found in ICC (residues 25 and 55), respectively. Regarding E7, the variations at residues 29 and 51 were detected in CIN III and ICC, respectively.

Localization of E6 and E7 Variations. Fig. 2 demonstrates the positions of E6 and E7 variations with respect to protein structure. Ten E6 variations were found in the amino-terminal half, in regions before and within the first predicted zinc finger, and two were seen in the region linking the two zinc fingers. One E7 variation was seen in the amino-terminal, in coding region 2, and the other one was seen in the carboxyl-terminal half, in coding region 3, just before the predicted zinc finger.

Prevalence of Variants in CIN III and ICC. In CIN III, 14 of 25 cases (56%) contained variants and 11 of 25 cases (44%) contained the prototype. ICC harbored variants in 16 of 17 cases (94%) and contained the prototype in just 1 case (6%). The correlation of disease status with prototype and variant was statistically significant (P = 0.013; Fig. 3).
Fig. 2. The position of the E6 and E7 variations. Often, E6 variations were identified in regions known to be important for the transforming activity of the protein (residues 3, 10, 11, and 78; Ref. 35) and likely to be significant for host immune recognition (residues 3, 11, 14, 25, 27, 55, 58, 60, 78, and 83; Ref. 38). E7 variations were not located within domains known to be important for the transforming activity of this protein, i.e., the retinoblastoma protein-binding motif LXCE (residues 22–26) and the casein kinase II phosphorylation site (residues 31 and 32; Ref. 39). E7 variations were not located within domains known to be important for the transforming activity of this protein, i.e., the retinoblastoma protein-binding motif LXCE (residues 22–26) and the casein kinase II phosphorylation site (residues 31 and 32; Ref. 39). E7 variations were not located within domains known to be important for the transforming activity of this protein, i.e., the retinoblastoma protein-binding motif LXCE (residues 22–26) and the casein kinase II phosphorylation site (residues 31 and 32; Ref. 39).

DISCUSSION

This investigation has shown that ICC almost exclusively harbored HPV16 E6 variants rather than the prototype, whereas in CIN III, prototype (44%) versus variant (56%) was more equal. This supports our hypothesis that progression from CIN III to ICC could be predicted by intratype variations, at least regarding HPV16 E6. Moreover, our results are within the range of previously estimated progression rates of between 12 and 69% (14, 18–21). It was a surprising observation that the HPV16 prototype, which was isolated from a German cervical carcinoma patient (31), had a detection frequency of only 6% in the same disease in a Swedish population. In a recent study conducted by Yamada et al. (26), the prevalence of the prototype, referred to as E-P-350T, amounted to 34% of the investigated cervical carcinomas in three European countries (Germany, Poland, and Spain). This prevalence is considerably higher than that in our study, but both sets of results indicate that the reference HPV16 E6 is relatively uncommon in European women with ICC. Analogous results have recently been obtained in a North American prospective study including female university students and women attending a sexually transmitted disease clinic, suggesting that the risk of developing CIN II and III is not the same with all variants of HPV16 (29).

In the present study, just 1 of 17 cases of ICC was found to be infected with the prototype, and 15 cases infected with variants invariably included the same E6 L83V variation, either alone or in combination with other variations. Interestingly, two cervical carcinoma-derived cell lines, CaSki and SiHa, also contained this polymorphism (data not shown). Moreover, in Central and South American women with ICC, E6 L83V was significantly increased compared to the prototype (26). In a British follow-up study of CIN, a striking association of persistent cervical lesion with E6 L83V was observed (28). It was shown that 10 of 12 women infected with this HPV16 E6 variant developed CIN III, regardless of the initial morphological status, compared to only 1 of 16 women infected with the HPV16 E6 prototype. Although a study of CIN, three women who had ICC at their initial visit were also included, and all carried the E6 L83V variation. These and our results indicate strongly that E6 L83V alone or in combination with other variations seems to be indicative for progression not only from CIN I to CIN III but also from CIN III to ICC.

It is rather unlikely that the identified mutations in our material would have evolved during the viral life cycle in the host cell or as a result of uncontrolled proliferation during tumor development. HPVs, in general, mutate extremely slowly. A majority of the biopsies examined were shown to contain typical mutations described in other investigations (Refs. 24–26, 28, and 29; Fig. 1), and most of our isolates could be assigned to European lineages, confirming the geographically related distribution of intratype variation within HPV types (26). Often, identical HPV16 E6 variants were found both in women with ICC and in women with CIN III (Fig. 2). This is well in accordance with other studies (24, 28). Finally, the accumulation of mutations not leading to amino acid changes was somewhat higher in CIN III than in ICC in the present study (Fig. 1).

Several HPV16 E6 amino acid changes were identified in positions crucial for p53 interaction, as anticipated. Additionally, amino acid changes were even found in positions likely to be important for host immune surveillance. Certain mutants of the HPV16 E6 protein, in regions to which our identified variations 3, 10, 11, and 78 localize have been generated by site-directed mutagenesis and were found to impair the biological and biochemical properties of E6 in vitro (35–37). Thus far, no mutational analysis is available for the other identified HPV16 E6 variations of our cases, i.e., residues 14, 25, 27, 55, 58, 60, 61, and 83. It remains to be seen how specific amino acid substitutions and their combinations in a given specimen correlate with the biological properties of the protein. For this reason, functional in vitro studies will have to be performed to characterize these naturally isolated amino acid substitutions. Moreover, the aforemen-
tioned variations, except the ones at residues 10 and 61, were all found to be positioned within E6 epitopes that can bind to various HLA class I peptides (38). Interestingly, 11 of 12 variants included one or more such variations. This is important because CTL responses are considered to be necessary for the elimination of established viral infections (39). In due course, these data might also provide a basis for CTL-based prophylactic and therapeutic vaccines.

HPV16 E7 mutations, especially those resulting in amino acid changes, were exceedingly rare in the tested biopsies. Similar data were obtained in several other studies (34, 40, 41). This suggests that the HPV16 E7 but not the HPV16 E6 protein is highly conserved in vivo, and that the tertiary structure of HPV16 E7 does not tolerate such variations. This is important because CTL responses are considered to be positioned within E6 epitopes that can bind to various HLA class I molecules.

ACKNOWLEDGMENTS

We thank Dr. Ethel de Villiers and Professor Harald zur Hausen for stimulating discussions and kind support, Dr. Lutz Edler for the statistical analysis, Dr. Lutz Gissmann and Dr. Valerie Bosch for revising and critically reading the manuscript, and Ulike Hebling for technical assistance.

REFERENCES


Human Papillomavirus 16 E6 Variants Are More Prevalent in Invasive Cervical Carcinoma than the Prototype

Ingeborg Zehbe, Erik Wilander, Hajo Delius, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/4/829

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.