CONCISE COMMUNICATION

Absence of Antibody against Human Papillomavirus Type 16 E6 and E7 in Patients with Cervical Cancer Is Independent of Sequence Variations

Ingo Nindl,1* Klaus Zumbach,1 Michael Pawlita,2 Karin Teller,1 Achim Schneider,1 and Matthias Dürst1

It is not known whether the lack of antibody response against human papillomavirus (HPV) type 16 E6 and E7 among some cervical cancer patients is due to naturally existing sequence variations. In this study, naturally occurring HPV-16 E6 and E7 variants (including the prototype) were cloned, antigens were expressed by in vitro transcription and translation, and the humoral immune response of 34 HPV-16-positive cervical cancer patients was analyzed by radioimmunoprecipitation assay (RIPA). In addition, the RIPA results were compared with those of a sandwich-protein ELISA, to further substantiate antibody status. Sera lacking E6 reactivity by RIPA remained negative by protein ELISA. All E6 antigens (the prototype and the variants 350G[83V], 131G[R10G]/350G[83V], 335T[H78Y]/350G[83V], 345G[Y81C]/350G[83V], and African 2 [A2]) showed cross-reactivity by RIPA. The lack of HPV-16 E6 or E7 antibodies is independent of naturally occurring variants in cervical cancer patients. Thus, testing for HPV-16 E6 or E7 prototype antigens seems to be sufficient in serological assays.

A causal association between cervical cancer and human papillomaviruses (HPVs), of which HPV-16 has the highest prevalence, has been established by molecular, biological, and epidemiological studies [1]. The HPV-16 oncoproteins E6 and E7 are consistently expressed in cervical cancer cell lines, as well as in biopsy specimens from patients with cancer, and they play a key role in HPV-induced carcinogenesis [2]. Antibodies against HPV-16 E6 and E7 are strongly associated with HPV-16-induced cervical cancer [3]. Serological assays, such as radioimmunoprecipitation assays (RIAs) or ELISAs, performed by using the entire E6- or E7-prototype protein under native conditions have a sensitivity and specificity higher than those of assays using denatured proteins or linear peptides [4, 5]. However, 20%–40% of patients with HPV-16 DNA-positive cervical carcinoma lack antibodies against E6 or E7 or both. Previous studies showed that distinct HPV-16 sequence variations exist [6]. HPV-16 E7 is highly conserved, and only a minority of sequence variations lead to an amino acid change [7, 8]. Sequence variations of HPV-16 E6 were detected in 50%–90% of cervical cancer patients [6, 9, 10], and specific variants might serve as additional risk factors for cervical cancer. Variations in amino acid sequence were detected in 19 (56%; E6) and 0 (E7) of 34 HPV-16 DNA-positive cervical cancer patients, who were part of a group of 37 patients described elsewhere [10]. In the present study, we examined the effect of the HPV-16 E6-variant status on seroreactivity.

Patients and Methods

Patients. Sera and biopsy specimens from 34 patients with clinically diagnosed cervical cancer and confirmed HPV-16 infections were collected at the Department of Gynecology, Friedrich-Schiller University, Jena, Germany, during 1995–1998. The median age of the patients was 43 years (range 26–74 years). Squamous cell carcinoma of the uterine cervix (33 patients) and adenocarcinoma (1 patient) were histologically diagnosed, according to the staging categories of the International Federation in Gynecology and Obstetrics (FIGO), as stage I in 21 patients (62%; median age 41 years), stage II in 10 patients (29%; median age 46 years), and stage IV in 3 patients (9%; median age 57 years). Sera were stored at −20°C until use. Sera of 3 patients who were negative for HPV DNA and who had negative cytology were included as controls.

Cloning of HPV-16 or HPV-18 E6 or E7 open-reading frames (ORFs). Nested polymerase chain reaction (PCR) and sequencing...
of the biopsy samples from the 34 patients with confirmed cervical cancer were described elsewhere in a sequence-variation study [10]. Ten picograms of purified, nested PCR fragments, spanning the region of HPV-16, nucleotides (nt) 57–890, was used to amplify the E6 and E7 ORFs with designed primers. To clone prototypes, 1 pg of the plasmid containing HPV-16 or HPV-18 was used to amplify the ORFs. The E6 and E7 sense primers from HPV-16, 5'-CTCTTCTCAGGAATGTGTTCCACACAGG-3' (nt 101–122) and 5'-CTCTTCTCAGATCATGCAATGGAATGACCACCC-3' (nt 559–578), and from HPV-18, 5'-AAAACTGCAGACACA- CCACAATACTATGCG-3' (nt 90–109) and 5'-AAAACTGCAGATAATATTTAAGTGATGCG-3' (nt 578–597), include the recognition sequence for restriction enzyme PstI and for the antisense primers for EcoRI. The sequences of the antisense primers are as follows: for E6 and E7 of HPV-16, 5'-CTCTGAATTCTGATTACAGCTGAGCTTGTCCTTTC-3' (nt 562–543) and 5'-CTCTGAATTTGAGATTATGTTCTGAG-3' (nt 845–864); for E6 and E7 of HPV-18, 5'-CCGGAAATTTCTAATATATTACCTTGTTTGTTTC-3' (nt 587–567) and 5'-CCGGAAATTTCTGTTGCTACTGCTGGGATGC-3' (nt 913–898). The PCR started with a denaturation step of 4 min at 95°C, followed by 40 cycles of a denaturation step at 95°C for 1 min, an annealing step at 55°C for 2 min, and a chain-elongation step at 72°C for 2 min, and ended with a final elongation step at 72°C for 4 min. To confirm the sequence of the plasmid DNA, the ORFs of the HPV-16 and HPV-18 E6 and E7 prototypes and the 5 HPV-16 E6 variants were analyzed from both orientations by primer-cycle sequencing by using the M13 reverse or M13 universal primer.

**In vitro transcription/translation and RIPA.** The plasmids pBK-CMV, containing the HPV-16 or HPV-18 E6 or E7 prototype or natural HPV-16 E6 sequence variations, were linearized by using HindIII. One microgram of linearized DNA was transcribed in vitro according to the instructions of the supplier (Promega, Mannheim, Germany). For in vitro translation, 3–5 μg RNA was used with 0.5 mL nuclear-targeted rabbit reticulocyte lysate (Promega) in the presence of 200 μCi 35S-cysteine, according to the instructions of the supplier. RIPA was performed overnight at 4°C by rocking the mixture containing 2–5 μL protein lysate, 10 μL human serum, 40 μL protein A-Sepharose (Serva, Heidelberg, Germany) washed in PBS and diluted 1:1 in PBS and RIPA buffer (0.1 M NaCl; 1% NP40; 0.1 M Tris/HCl, pH 8.0) in a final volume of 250 μL. The Sepharose beads were washed 5 times in 500 μL RIPA buffer and resuspended in 20 μL twice-concentrated SDS gel-loading buffer (0.1 M Tris/HCl, pH 6.8; 0.2 M dithiothreitol; 4% SDS; 0.2% bromophenol blue; and 20% glycerol) and heated at 95°C for 5 min. The immunoprecipitated proteins were analyzed on 15% SDS polyacrylamide gels. The gels were incubated in 1 M sodium salicylic acid for 30 min, and the antigens were detected by autoradiography, as shown in a representative experiment (figure 1). A strongly reacting rabbit polyclonal anti–HPV-16 E6 antisera (raised against the HPV-16 E6/1 peptide aa 1–23) or the HPV-16 E7–specific monoclonal antibody IV, described by Oltersdorf et al. [11], and 3 control sera of HPV- and cytology-negative women were included in each experiment with a specific HPV-16 E6 or E7 antigen. Each sample was tested twice, and only the sera that scored positive in both assays were considered seropositive.

**Protein-ELISA.** To detect antibodies against E6 and E7 proteins of HPV-16 and HPV-18, we used a protein ELISA as described elsewhere [5], but with minor modifications. Complete, highly purified and refolded E6 (900 ng per well) and E7 (200 ng per well) proteins were used as antigens. Cutoff values (0.044 for HPV-16 E6, 0.029 for HPV-16 E7, 0.048 for HPV-18 E6, and 0.033 for HPV-18 E7) were calculated from a control group as mean + 3 SD of net optical densities, excluding all positive outliers. For each sample from a cervical cancer patient, 2 control samples from age-matched healthy women were used (n = 68), selected from a larger serum collection (n = 1644) representative of the general population of German women [12, p. 8].

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**Figure 1.** Radioimmunoprecipitation assay (RIPA) with the prototype protein and 5 variant antigens using HPV-16 DNA–positive sera from patients with cervical cancer. In all experiments, the same 6 human sera were used to detect antibodies against various HPV-16 E6 proteins. Prototype (PT) and the following 5 variant antigens were used for RIPA: 350, 350G (L83V); 131/350, 131G (R10G)/350G (L83V); 335/350, 335T (H78Y)/350G (L83V); 345/350, 345G (Y81C)/350G (L83V); and Af2, 132T (R10I), 134G/145T (Q14D)/286A/289G/335T (H78Y)/403G (table 1). All human sera were from cervical cancer patients infected with the prototype or with 1 of the HPV variants. Sera of cervical cancer patients infected with the following HPV-16 E6 variants were used for RIPA: lane 1, control serum (HPV negative); lane 2, 353T (H78Y)/350G (L83V); lane 3, 131G (R10G)/350G (L83V); lane 4, prototype; lane 5, 350G (L83V); and lane 6, prototype. In all experiments, lanes 2, 4, and 6 were scored positive. **Arrows** indicate radiolabeled E6 proteins after immunoprecipitation.
Results

Detecting antibodies against the prototype E6 and E7 proteins by ELISA versus RIPA. Thirty-four sera from HPV-16 DNA-positive cervical cancer patients, who were previously analyzed in an HPV-16 E6 and E7 sequence-variation study [10], were examined. Prototype nucleotide sequences of the E6 and E7 ORFs were found in 44% and 85% of patients, respectively, and variants were found in 56% and 15%, respectively. Antibodies against the complete HPV-16 E6 and E7 prototype proteins were assayed by ELISA and RIPA. When tested with all the HPV prototype proteins analyzed, only 1 of 68 control sera reacted with HPV-16 E7 by ELISA. Overall reactivity against E6 and E7 was found in 19 (56%) and 12 (35%), respectively, of the 34 samples by ELISA versus 27 (79%) and 11 (32%) by RIPA. No significant difference was found for the distribution of the 34 samples by ELISA versus 27 (79%) and 11 (32%) by RIPA. In total, 7 sera showed no reactivity with E6 or E7 or both types of antigens when tested by ELISA versus 11 sera by RIPA. In total, 7 sera showed no reactivity with E6 or E7 or both types of antigens when tested by using ELISA or RIPA or both.

To estimate the analytical sensitivity of each method, we performed dilution experiments, using a serum sample with low reactivity and another with high reactivity. The analytical sensitivity of the ELISA (final volume 100 µL) versus RIPA (final volume 250 µL) was 1 : 1600 versus 1 : 250 in the serum with low reactivity and 1 : 25,600 versus 1 : 2500 in the serum with high reactivity. The HPV type specificity of both methods was determined by analyzing the reactivity of the HPV-16 DNA-positive patients’ sera against HPV-18 E6 and E7 antigens. Only 1 serum showed a reaction with HPV-18 E7 (OD, 0.249), and no sera reacted with HPV-18 E6 by ELISA. Antibodies against HPV-18 E6 or E7 were detected in 3 and 0 sera, respectively, by RIPA.

Antibody response against variant HPV-16 E6 by RIPA. All E6-variant antigens reacted during RIPA with the rabbit polyclonal anti–HPV-16 E6 antiserum raised against the HPV-E6/1 peptide aa 1–23. Sera negative for antibody to HPV-16 E6 or E7 or both, as determined by both detection methods, were from patients infected with HPV-16 E6 prototype in 2 patients, variant 350G (L83V) in 3 patients, and variant 131G (R10G)/350G (L83V) in 2 patients. To investigate the possible lack of reactivity or cross-reactivity of sera with each variant protein, we have reanalyzed all sera with these 2 variant antigens and, in addition, with 3 further variants: 335T (H78Y)/350G (L83V), 345G (Y81C)/350G (L83V), and 132T (R10I)/143G/145T (Q14D)/286A/289G/335T (H78Y)/403G (Af2) (table 1). We observed no difference in reactivity in sera of cervical cancer patients with the prototype or any of the variant antigens, as shown in a representative experiment (figure 1). To test the quality of these nonreacting sera, they were analyzed for the presence of IgG antibodies against Epstein-Barr virus and cytomegalovirus antigens. All were positive for antibodies of ≥1 of the 2 viruses.

Discussion

Our results revealed sequence variations leading to an amino acid change in the HPV-16 E6 protein, but not in the E7 protein, in 56% of the patients with cervical cancer. Examination of the antibody response against HPV-16 E6 variants showed cross-reactivity among 6 natural HPV-16 E6 proteins (including the prototype).

High specificity of the protein ELISA for E6 and E7 of HPV types 16 and 18 was reported [5]. We observed cross-reactivity of HPV-16 and HPV-18 in only 1 serum by ELISA and in 3 sera by RIPA in HPV-16 DNA-positive cervical cancer patients, confirming earlier reports.

In contrast to the numerous genotypic analyses, only 2 serological studies using different HPV-16 L1-variant antigens had been done, and no different serotypes were found [14, 15]. Cheng et al. [14] investigated 2 HPV-16 L1-variant antigens with 1 (northern Europe variant) and 8 (central African variant) amino acid changes. Both variant strains were serologically cross-reactive. Touze and coworkers [15] reported serological cross-reactivity of 3 variants of HPV-16 L1, with 1 (Philippine strain) cross-reactive.}

| Table 1. Alignment of E6 amino acid sequences corresponding to naturally occurring human papillomavirus (HPV) type 16 E6 variants used as antigens to detect human IgG antibodies in 34 patients with cervical cancer. |
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| HPV-16 E6 strain | 1 | 10 | 14 | 27 | 81 | 83 | 151 |
| Prototype | Met/M | Arg/R | Gin/Q | His/H | Tzy/Y | Leu/L | Leu/L |
| V-350 | — | — | — | — | — | Val/V | — |
| V-131/350 | — | Gly/G | — | — | — | Val/V | — |
| V-335/350 | — | — | — | Tyr/Y | — | Cys/C | Val/V |
| V-Af2 | — | His/I | Asp/D | Tyr/Y | — | — | — |

*NOTE.* Each amino acid is represented by the 3- and 1-letter code. The amino acid positions are compared with those of the prototype protein [13]. Dashes indicate an amino acid identical to that of the prototype. V-350, variant 350G(L83V); V-131/350, variant 131G(R10G)/350G(L83V); V-335/350, variant 335T(H78Y)/350G(L83V); V-345/350, variant 345G(Y81C)/350G(L83V); and Af2, 132T (R10I), 143G/145T (Q14D)/286A/289G/335T (H78Y)/403G (Af2) [6].
4 (Algeria type) and 8 (Senegal type) amino acid changes, indicating that HPV-16 L1 variants belong to the same serotype. We analyzed the prototype and 5 variants of HPV-16 E6 antigens with 1, 2, and 3 amino acid changes in serum samples from patients with cervical cancer (table 1). The same seroreactivity was observed for all antigens used (figure 1). Thus, the cross-reactivity of variant antigens in all sera from cervical cancer patients indicates that these 5 HPV-16 E6 variants do not define different serotypes. These findings are in accordance with the observations made with HPV-16 L1 variants.

In this study, 21% of sera from HPV-16 DNA-positive cervical cancer patients lacked antibodies against 5 HPV-16 E6 variants and the prototype. No sera showed reactivity, even when the corresponding antigen was used, indicating that some cervical cancer patients lack antibodies against E6. Thus, HPV-16 E6 variants need not be used in serological testing for cervical cancer.

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References