High-risk Human Papillomavirus E7 Oncoprotein Detection in Cervical Squamous Cell Carcinoma

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Abstract

Purpose: Persistent infections by high-risk human papillomavirus (HPV) types are the main etiologic factor for cervical cancer. The objective of this study was to evaluate whether high-risk E7 oncoprotein is adequate as a marker for the detection of cervical cancer.

Experimental Design: HPV typing was done in biopsies from 58 cervical carcinoma and 22 normal cervical squamous epithelia. The HPV-16 E7, HPV-18 E7, and HPV-45 E7 oncoprotein levels were monitored by immunohistochemistry and compared with those of p16INK4a and Ki67.

Results: Fifty-five (94.8%) tumors were high-risk HPV-DNA positive (46 HPV-16, 2 HPV-18 and HPV-18, 4 HPV-18, 1 HPV-33, and 2 HPV-45). HPV-DNA could not be detected in three tumors (5.2%). High HPV E7 oncoprotein levels were shown in 57 cervical cancers (98.3%), without correlation between expression levels and tumor stages. Conclusion: This is the first study which systematically analyzes the levels of the major HPV oncoproteins in cervical carcinomas demonstrating that the high-risk HPV E7 proteins are regularly expressed in these cancers. This suggests that high-risk E7 oncoproteins are necessary for cervical cancers and apparently essential as tumor marker.

Human papillomaviruses (HPV) are the main etiologic factors for cervical cancer (1). More than 35 HPV genotypes, which could infect epithelial squamous and glandular cells in the cervical mucosa, have been described. On the basis of epidemiologic and biochemical data, these viruses are classified as high-risk HPV associated with squamous intraepithelial lesions with a high potential for progression to invasive squamous cell carcinoma (SCC) and low-risk HPVs associated with benign hyperplasia. Infections by high-risk genotypes have been detected in virtually all cervical cancers (2), and at least 15 high-risk types of HPV have been associated with this cancer (3). HPV-16 is the most prevalent genotype worldwide with an incidence in SCC of ~55%, followed by HPV-18 (~17%) and HPV-45 (~8%; ref. 3). The persistence of oncogenic HPV is necessary for the development of cervical precancer and cancer (1–3); however, the factors that determine viral persistence and tumorigenic progression are not fully understood. According to the current model, the initial events of cervical carcinogenesis after viral infection are that high-risk HPV types undergo specific changes which overcome the transcriptional control of viral gene expression in the infected keratinocytes (4). Inactivation of these cellular control functions permits deregulated transcription of the early viral genes E6 and E7, and that triggers cell proliferation, inhibition of apoptosis, reprogramming of differentiation, and chromosomal instability (1, 5). These changes could support the integration of episomal HPV genomes into chromosomes of the host cell (6, 7), and contribute to further overexpression of the viral genes E6 and E7 (8, 9), resulting in an increase of the E7 oncoprotein levels during early steps of cervical carcinogenesis (10). The high-risk E7 protein, in cooperation with high-risk E6, can efficiently immortalize human primary keratinocytes (11, 12); and the consistent overexpression of the E6 and E7 oncoproteins is required to induce and maintain the transformed phenotype of cervical cancer cells (13). Immortalization by the E7 oncoprotein involves its ability to bind and thereby functionally inactivate cell cycle–regulatory proteins like the retinoblastoma tumor suppressor protein (10, 14). Further work has shown that E7 acts as a multifunctional protein, deregulating several additional cellular pathways necessary for the oncogenic potential of the virus (15, 16). Thus, overexpression of E7 oncoproteins of carcinogenic HPV types may be a very specific marker for cervical cancer. For these reasons, we conducted a
study to evaluate anti–high-risk HPV E7 antibodies for detection of the HPV-16, HPV-18, and HPV-45 E7 oncoproteins in biopsies (i.e., conisation specimens) from 58 patients with invasive cervical SCC.

Materials and Methods

**PCR-based HPV typing.** Formalin-fixed, paraffin-embedded tissues were processed with a QIAamp Tissue kit according to the manufacturer’s instructions (Qiagen). Total cellular DNA was used in the GP5+/GP6+ general primer PCR and the amplicons were used for HPV typing using an enzyme immunoassay (EIA) with different HPV type–specific oligonucleotides as described (17).

**Tissue specimens.** A total of 58 paraffin-embedded conisation specimens with invasive cervical SCC were diagnosed in the Division of Pathology and collected by the National Tumour Registry of the National Health Laboratory, Luxembourg. Twenty-two controls (normal cervical squamous epithelia) were obtained from the Division of Pathology, National Health Laboratory, Luxembourg and the Department of Obstetrics and Gynecology, Medical University of Innsbruck, Austria.

**Indirect immunofluorescence experiments.** Confocal immunofluorescence microscopy was done as described (10, 18). Briefly, cells were fixed with 4% (w/v) PFA/PBS and permeabilized with 0.1% (w/v) sodium citrate/0.3% (v/v) Triton X-100. After blocking with 1% (v/v) bovine serum albumin (BSA)/PBS, cells were incubated for 1 h at room temperature with either affinity-purified goat polyclonal anti–HPV-16 E7 or affinity-purified goat polyclonal anti–HPV-18 E7 or affinity-purified goat polyclonal anti–HPV-45 E7 antibodies (25 µg/mL), which were preincubated with 5 µg/mL of the 20,000 × g supernatant of a NIH3T3 total cell extract in blocking buffer over night at 4°C. Cells were processed for immunofluorescence analysis as described (10) and viewed using confocal microscopy.

**Immunohistochemical detection of HPV E7 proteins, Ki67, and p16INK4a in cervical cancer biopsies.** Immunohistochemistry was done on paraffin-embedded tissue sections derived from invasive cervical SCC conisation specimens or hysterectomy specimens. Two-micrometer sections were mounted on slides, deparaffinized in xylene (2 × 10 min), incubated for 5 min each in 100%, 90%, 80% and 70%, and 50% isopropanol for dehydration and processed for antigen retrieval by treatment for 30 min at 800 W in a pressure cooker in 10 mmol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation in 5% H2O2/methanol for 15 min. Sections were washed in 1× Tris buffer [7.73 g Tris-HCl (pH 7.5), 8.78 g NaCl ad 1 liter H2O] and incubated for 15 min in blocking buffer (10% goat or rabbit serum, dependent on the first antibody) before incubation with the primary antibody overnight at 4°C. Sections were washed in 1× Tris/1% BSA buffer and incubated with anti–HPV E7 antibodies provided by Amynon Biotech GmbH, affinity-purified polyclonal rabbit anti–HPV-16 E7 (50 µg/mL; ref. 10), affinity-purified goat polyclonal anti–HPV-18 E7 (50 µg/mL; ref. 18), and affinity-purified goat polyclonal anti–HPV-45 E7 antibodies (50 µg/mL; ref. 18) for 1 h at room temperature in buffer B (1% BSA/5 µg/mL NIH/3T3 lystate in PBS). The anti-p16INK4a (clone IC 8) and the anti-Ki67 antibodies (both from Neomarkers) were incubated for 1 h at room temperature in 1× Tris/1% BSA. Samples were rinsed in 1× Tris and incubated with secondary IgGs (DAKO) at room temperature. Bound antibodies were visualized with 3,3′-diaminobenzidine solution (Sigma) as substrate chromogen and counterstaining was done with Hemalaun (Merck). The specimens were incubated in increasing concentrations of isopropanol, and mounted using Eukitt (Merck). Bright-field microscopy with photography was done using a Leica DMRB microscope and a Nikon Coolpix 995 camera. We used an arbitrary scoring system to grade the E7 protein staining intensity into three categories. The strongest staining was set at 100% and the staining intensity was rated as follows: 71% to 100% (+++), 41% to 70% (++), and 10% to 40% (+).

**Results and Discussion**

PCR analysis of biopsies from 58 patients with invasive cervical SCC, using GP5+/GP6+ general primer PCR and HPV typing by EIA, identified infections by various HPV genotypes (Table 1). Fifty-five (95%) of the 58 SCCs were HPV-DNA–positive. Forty-six SCCs were HPV-16 DNA–positive, four were HPV-18 DNA–positive, two were HPV-16 and HPV-18–positive, one was HPV-33 DNA–positive, and two were HPV-45 DNA–positive. In the remaining three cancers, the HPV type was apparently not detectable by the PCR analysis. Moreover, we analyzed normal cervical squamous epithelia taken from 22 hysterectomy specimens with multiple leiomyomas and without cervical lesions derived from women between 37 and 76 years of age. All specimens from this group were HPV-DNA–negative. This is consistent with epidemiologic studies demonstrating that the prevalence of HPV infection strongly declined in women over ~35 years of age and is 4% to 5% when detected with GP5+/GP6+ PCR in fresh cervical smears with normal cytology in women between 34 and 69 years of age (19, 20).

Next, it was analyzed whether high-risk E7 oncoproteins were detectable in these biopsies. To do this, we employed anti–high-risk HPV E7 antibodies, the specificity of which was proven by both Western blotting (see refs. 10, 18) and immunofluorescence experiments using U-2OS cells transiently expressing HPV-16 E7, HPV-18 E7, and HPV-45 E7, respectively (Fig. 1A), and the HPV-positive CaSki (HPV-16), HeLa (HPV-18), and

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<th><strong>Table 1.</strong> High-risk HPV E7 oncoprotein levels in different HPV-DNA–positive cervical SCC</th>
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NOTE: Analysis of paraffin-embedded tissue specimens of 58 SCCs (n = 58 cases) using GP5+/GP6+ general primer PCR and HPV typing by EIA. Paraffin-embedded tissue specimens were HPV-typed and stained with antibodies against HPV-16 E7, HPV-18 E7, and HPV-45 E7 using immunohistochemistry. In 57 specimens (the HPV-33–positive biopsy was not analyzed), E7 expression levels were analyzed by immunohistochemistry and staining intensity rated using a semiquantitative scoring system: (+++) very strong; (+++) strong; (+) moderate staining.
MS751 (HPV-45) cell lines, the HPV-DNA–negative C33A cells served as negative controls (Fig. 1A). The HPV-33 DNA–positive sample was excluded from the analysis because specific antibodies to HPV-33 E7 protein were not available. No E7 staining could be detected in the 22 normal cervical squamous epithelia (Fig. 1B), whereas antibodies against the proliferation marker Ki67 stained the nuclei of the proliferating keratinocytes in the basal and parabasal layers of the normal cervical squamous epithelium.

![Fig. 1. A, detection of HPV-16, HPV-18, and HPV-45 E7 proteins in human cells.](image)

A-HPV-16 E7  A-HPV-18 E7  A-HPV-45 E7
U-2OS/HPV-16 E7  U-2OS/HPV-18 E7  U-2OS/HPV-45 E7
CaSki, HeLa, and MS751 cells were stained with anti–HPV-16, anti–HPV-18, and anti–HPV-45 E7 antibodies as indicated (top). C33A cells were stained with anti–HPV-16, anti–HPV-18, and anti–HPV-45 E7 antibodies as indicated (middle). C33A cells were stained with anti–HPV-16, anti–HPV-18, and anti–HPV-45 E7 antibodies as indicated (bottom). All cells were fixed with 4% PFA/0.1% Triton X-100, blocking and staining with the affinity-purified polyclonal anti–high-risk E7 antibodies was in 1× PBS/1% BSA/5 μg/μL NIH3T3 lysate overnight at 4°C. Cells were then processed for indirect immunofluorescence microscopy by standard methods and viewed using a confocal laser-scanning system. B, protein levels of high-risk HPV E7, p16INK4A, and Ki67 analyzed in normal cervical squamous epithelium. Immunoperoxidase staining of HPV-DNA–negative normal cervical squamous epithelium with anti–high-risk HPV E7 antibodies, anti–p16INK4A antibodies, and anti–Ki67 antibodies stained single cells within the basal and parabasal layers of normal cervical squamous epithelium (middle). No staining was observed with the anti–p16INK4A antibodies (right).
epithelium. The keratinocytes in normal cervical squamous epithelium were also not stained by antibodies against the cyclin-dependent kinase inhibitor p16\textsuperscript{INK4a} (Fig. 1B), in agreement with the observation that p16\textsuperscript{INK4a} expression is confined to cancerous lesions in the cervical epithelium.

Immunohistochemical analysis of HPV-16, HPV-18, and HPV-45 E7 protein expression was done in 54 HPV-DNA-positive (HPV subtypes 16, 18, and 45) and three HPV-DNA-negative SCCs and the results in relation to HPV subtype are summarized in Table 1. To determine whether the HPV-16 E7 oncoprotein

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**Fig. 3.** Comparative expression analysis of different high-risk HPV E7 oncoproteins, p16\textsuperscript{INK4a}, and Ki67 in high-risk HPV-DNA-positive cervical SCCs. Consecutive sections of 30 paraffin-embedded cervical cancer specimens (n = 24, HPV 16; n = 4, HPV 18; n = 2, HPV 45) were stained with the anti – high-risk HPV E7 antibodies (top), anti-Ki67 antibodies (middle), and anti-p16\textsuperscript{INK4a} antibodies (bottom) using immunohistochemistry. The anti – high-risk HPV E7 antibodies stained the same tumor cell pattern as the anti-Ki67 and anti-p16\textsuperscript{INK4a} antibodies in all cervical SCC specimens analyzed.
can be detected in all HPV-16 and HPV-16/-18 DNA–positive cervical carcinoma biopsies, immunohistochemistry experiments with the affinity-purified polyclonal anti–HPV-16 E7 antibodies were done on paraffin sections of the 46 HPV-16 and 2 HPV-16/-18 DNA–positive invasive cervical cancers (Fig. 2A). In these biopsies, the anti–HPV-16 E7 antibodies recognized almost all epithelial cells within the tumor islets but did not stain any cells in the adjacent connective tissues (Fig. 2A and E, left). Dysplastic tumor cells in precancerous squamous epithelium (classified histologically as CIN II and CIN III) adjacent to the cancerous tumor islets in the same section were also stained positive (Fig. 2E, middle). Keratinocytes in areas of nonepithelial squamous epithelium in these cancer biopsies were not stained (Fig. 2E, right). In keeping with a previous study (10), these findings suggest that E7 can be considered as a marker for premalignant cervical intraepithelial lesions. Whereas all HPV-16 DNA–positive carcinomas stained positive for E7, a more detailed analysis revealed strong variations in E7 oncoprotein expression levels across the individual biopsies. Using an arbitrary scoring system, we judged the E7 expression levels by semiquantitative analysis in three categories: 8 carcinomas were classified as +3 or higher, 30 as +2, and 8 as +1 (Fig. 2A; Table 1). The anti–HPV-16 E7 antibodies also stained the three SCCs which were analyzed as HPV-DNA–negative in the PCR analysis (Fig. 2B), suggesting that these biopsies are still HPV-positive cervical cancers infected either by HPV-16 or by a related high-risk HPV type. These findings prove the high specificity of the affinity-purified anti–HPV-16 E7 antibodies.

To determine whether the HPV-18 E7 and HPV-45 E7 oncoproteins could be detected in the HPV-18 and HPV-45 DNA–positive SCCs, immunohistochemistry experiments with the affinity-purified polyclonal goat antibodies against HPV-18 E7 and HPV-45 E7 were done on paraffin sections of the four HPV-18 DNA–positive cervical cancer biopsies, the two mixed HPV-16/-18 DNA–positive cervical cancer biopsies, and the two HPV-45 DNA–positive cervical cancer biopsies (see Fig. 2C and D; Table 1). Similar to the anti–HPV-16 E7 antibodies, the anti–HPV-18 and -45 E7 antibodies stained almost all cells within the squamous cell tumor islets of the eight respective SCCs but did not stain cells in the adjacent connective tissue. Again, the E7 expression levels of these eight HPV-18 and HPV-45 DNA–positive SCCs were judged by semiquantitative analysis (Table 1; Fig. 2C and D); one carcinoma was classified as +3 and seven as +2. In conclusion, the tumor cells in all cervical carcinoma analyzed in this study were stained positive by the anti–HPV-16, anti–HPV-18, and anti–HPV-45 E7 antibodies. This finding reveals that E7 oncoproteins are highly expressed in most (if not in all) cervical cancers, at least for HPV subtypes 16, 18, and 45, which together make up ~80% of all cases worldwide (3).

It has been shown that the cyclin-dependent kinase inhibitor p16\textsuperscript{N\textsubscript{K4A}} is frequently up-regulated in HPV-positive cervical carcinomas, suggesting that p16\textsuperscript{N\textsubscript{K4A}} expression could play a role as a surrogate marker for cervical cancers (21, 22). We stained consecutive sections from 30 out of the 58 SCCs (24 HPV-16–positive specimens, 4 HPV-18–positive specimens, and 2 HPV-45–positive specimens) with anti-p16\textsuperscript{N\textsubscript{K4A}} antibodies (an example is shown in Fig. 3, bottom). We found the same tumor cell staining pattern as for the anti–HPV-16, anti–HPV-18, or anti–HPV-45 E7 antibodies, respectively (Fig. 3, top). This indicates that the anti-p16\textsuperscript{N\textsubscript{K4A}} antibodies can detect invasive cervical cancer cells positive for different high-risk HPV types. Finally, we employed an antibody against Ki67 to stain the proliferating cells in the cervical cancers (Fig. 3, middle). The anti-Ki67 antibodies stained the same tumor cell pattern as the anti–high-risk HPV E7 and the anti-p16\textsuperscript{N\textsubscript{K4A}} antibodies in all cervical cancer specimens analyzed. These findings underline that high-risk HPV E7 oncoproteins are highly expressed in cervical cancers and that p16\textsuperscript{N\textsubscript{K4A}}, as far as analyzed in this study, is suitable as a surrogate marker for the detection of high-risk HPV-positive cervical carcinoma cells. Because the anti-Ki67 antibodies also stained the normal proliferating cells in the basal layers of the squamous epithelium, Ki67, known as biomarker for proliferating cells (23), was not suitable as a specific marker for cervical cancer cells.

Tumor size, depth of invasion, and grading of the invasive tumor front are actually the main prognostic factors in early cervical SCC (24). In the present study, we compared the E7 oncoprotein levels in 57 cervical SCC with the histopathologic staging and grading of these lesions according to the criteria defined by the tumor-node-metastasis system of the Union Internationale Contre le Cancer (25). However, whereas the detection of the E7 oncoprotein always coincided with the presence of an invasive cervical carcinoma, there was no correlation between the E7 protein levels and the pathologic prognostic variables (tumor size, lymph node status, metastases at distance, or histologic grading).

There is strong evidence that expression of the E7 oncogene is necessary for the induction and maintenance of the transformed phenotype (1); however, the role of the E7 oncoprotein in cervical carcinogenesis has remained elusive, due to the lack of antibodies allowing its detection in clinical samples. In this study, we analyzed for the first time, the high-risk E7 oncoprotein levels in HPV-DNA–positive invasive cervical cancer biopsies from a defined set of 58 patients. Except for one HPV-33 DNA–positive carcinoma, which could not be analyzed for technical reasons, we show high-risk HPV oncoprotein levels in all of these cervical cancer biopsies. These findings indicate that the high-risk E7 oncoproteins of HPV-16, HPV-18, and HPV-45 are expressed continuously in invasive cervical carcinoma. The prevalence of these three HPV types in cervical cancers is ~80% worldwide (3), suggesting that the anti-E7 antibodies may have diagnostic potential as markers for the detection of cancerous and precancerous cervical lesions.

References
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