JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2006, p. 1310–1317 Vol. 44, No. 4
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.4.1310–1317.2006

Presence of E6 and E7 mRNA from Human Papillomavirus Types 16, 18, 31, 33, and 45 in the Majority of Cervical Carcinomas

Irene Kraus,[1]† Tor Molden,[1]†* Ruth Holm,[2] A. Kathrine Lie,[2] Frank Karlsen,[3] Gunnar B. Kristensen,[4] and Hanne Skomedal[3]

Institute of Pathology, National University Hospital, 0027 Oslo, Norway[1]; Department of Pathology, Norwegian Radium Hospital, 0310 Oslo, Norway[2]; NorChip AS, 3490 Klokkarstua, Norway[3]; and Department of Gynaecologic Oncology, Norwegian Radium Hospital, 0310 Oslo, Norway[4]

Received 12 September 2005/Returned for modification 12 December 2005/Accepted 13 January 2006

The oncogenic potential of the human papillomavirus (HPV) early genes E6 and E7 is well established and a source of interest with regard to HPV testing for cervical carcinoma. Here we present a study performed with 204 histologically confirmed invasive cervical squamous cell carcinomas (SCCs) in which we evaluated the HPV E6 and E7 mRNA detection assay PreTect HPV-Proofer for detection of high-risk HPV types 16, 18, 31, 33, and 45. For further evaluation, detection of E6 and E7 mRNA from HPV types 35, 52, and 58 by real-time multiplex nucleic acid sequence-based amplification was also included. For comparison and to assess the overall prevalence of various HPV types, samples were also tested for HPV DNA by both consensus and type-specific PCR, reverse line blotting, sequencing, and in situ hybridization. The overall prevalence of HPV was 97%. HPV E6 and E7 transcripts were detected in 188 of 204 (92%) biopsy specimens, of which 181 contained one of the following HPV types: 16, 18, 31, 33, or 45. Consensus PCR and type-specific PCR detected HPV in 187 of 204 and 188 of 204 (92%) specimens, respectively. In conclusion, this study verifies the presence of HPV E6 and E7 mRNA in SCCs and demonstrates that HPV infections among Norwegian women with SCCs are limited mainly to the five high-risk types, 16, 18, 31, 33, and 45. This, together with the fact that PreTect HPV-Proofer detects the HPV oncogenic transcripts, suggests that the assay is a valuable approach in the field of HPV detection in cervical carcinoma.

The presence of human papillomavirus (HPV) infection in the majority of cases of cervical neoplasia has been considered evidence of an etiological role of HPV in cervical cancer. The association is strong, consistent, and specific to a limited number of viral types (2, 5, 32, 46). On the other hand, the lifelong risk of HPV infection is 80%, and only a small proportion of women infected with HPV will develop high-grade cervical neoplasia (42). Among HPV-positive women, it is therefore of the utmost importance to identify those with an increased risk of developing cervical carcinoma by either methods that reveal persistent HPV infection (38), methods that detect viral oncogene expression (40), or the use of additional human markers for cervical carcinogenesis (45).

The most frequent HPV types found in cervical intraepithelial neoplasia and squamous cell carcinoma (SCC) are HPV type 16 (HPV-16) and HPV-18, which in 1995 were classified as human carcinogens by the International Agency for Research on Cancer. In total, based on their high frequencies in carcinomas, 13 HPV types are now considered carcinogenic types (6). In the largest multinational studies performed so far, HPV types 16, 18, 31, 33, and 45 were shown to be the most prevalent types associated with cervical carcinomas (2, 22, 32), with HPV-16 alone found in more than 50% of the cases. The oncogenic potential of these high-risk HPV types lies in the oncoproteins E6 and E7, which bind to and modulate a number of different gene products, in particular, the tumor suppressors p53 and pRb. These interactions lead to a disturbance of cell cycle control and a deficiency in DNA repair, resulting in genomic instability and an increased risk of malignant transformation (30). However, although the oncogenic potential of E6 and E7 is well established and the expression of E6 and E7 has been found to be necessary for conversion to malignancy (19, 31, 52), few studies have so far been performed to verify the actual presence of these transcripts in clinical cervical carcinoma samples. Such a study would provide valuable information, as the detection of the E6 and E7 transcripts of high-risk HPV types could serve as a better risk evaluation factor than DNA detection for the development of high-grade squamous intraepithelial lesion and the progression to cervical carcinoma (25, 28, 29, 41).

In order to confirm the presence of HPV E6 and E7 mRNA in cervical carcinomas, we performed a study with 204 histologically confirmed invasive SCCs. Additionally, we wanted to investigate which HPV types are the most common among Norwegian women with SCC and performed an evaluation of the HPV mRNA detection assay PreTect HPV-Proofer, which detects HPV types 16, 18, 31, 33, and 45. The PreTect HPV-Proofer assay is based on real-time multiplex nucleic acid sequence-based amplification (NASBA). To assess the overall prevalence of various HPV types, detection of E6 and E7 mRNA from HPV types 35, 52, and 58 by use of a separate multiplex NASBA system was also included, as these are the most prevalent types after the five types included in the assay (5). For comparison and additional evaluation, HPV DNA was detected by consensus and type-specific PCR, PCR-enzyme

* Corresponding author. Mailing address: Institute of Pathology, National University Hospital, Sognsveien 20, 0027 Oslo, Norway. Phone: 47 40403484. Fax: 47 32798801. E-mail: t.e.f.molden@medisin.uio.no.

† Irene Kraus and Tor Molden contributed equally to this work.

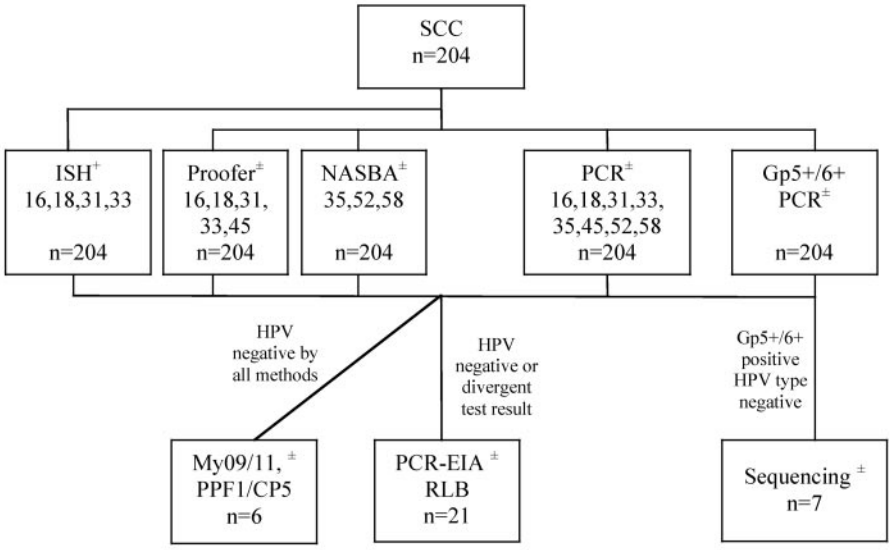

FIG. 1. Flow chart showing the different HPV test methods used with different specimens. Symbols: +, paraffin-embedded tissue; ±, fresh frozen biopsy specimen. Proofer, PreTect HPV-Proofer assay; PCR, type-specific PCR (for HPV types 16, 18, 31, 33, 35, 45, 52, and 58, as indicated); *n*, number of samples tested.

immunoassay (EIA) and reverse line blotting (RLB), and in situ hybridization (ISH).

MATERIALS AND METHODS

Cell culture. SiHa (squamous cell carcinoma), CaSki (squamous cell carcinoma), and HeLa (adenocarcinoma) cervical carcinoma cell lines, were obtained from the American Type Culture Collection. The SiHa and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium, and the CaSki cell line was maintained in RPMI medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 25 μg/ml gentamicin. The cells were incubated at 37°C in a 5% CO_2 atmosphere and harvested directly in NucliSens lysis buffer (bioMérieux, Marcy l'Etoile, France) containing 5 M guanidine thiocyanate.

Tissue samples. Tumor samples from 204 patients from Norway who had SCCs and who were admitted to the Norwegian Radium Hospital between 1995 and 1998 were included in the study. Fresh frozen tissue (about 8 mm^3) stored at −70°C was used for the PCR and NASBA analyses. For ISH, formalin-fixed, paraffin-embedded tissue from the same tumors, collected from the files of the Norwegian Radium Hospital, was used. The findings for all samples were histologically confirmed by evaluation of frozen sections before analysis.

Nucleic acid isolation. The material was divided into smaller pieces while it was kept on dry ice (−80°C) and transferred to 1 ml of lysis buffer (bioMérieux), followed by 20 s of homogenization with disposable pestles. One hundred microliters of the sample was further diluted 10-fold in lysis buffer, and 100 μl was then subjected to DNA and RNA extraction with the automated NucliSens extractor (bioMérieux), as described previously (1). The extracted DNA and RNA were eluted with approximately 40 μl of elution buffer (bioMérieux) and stored at −70°C. Five microliters of a fivefold dilution was used in the PCRs and the NASBA reactions. At a later stage, the samples found to be HPV negative by all methods were extracted again by using 100 μl of the undiluted material in lysis buffer. This was done in order to make sure that all HPV-positive samples were detected, including samples containing a low HPV copy number. All cases were retested by NASBA, type-specific PCR, and consensus PCR.

A flow chart illustrating the different test methods performed with the specimens is given in Fig. 1.

HPV RNA analysis by NASBA. (i) HPV types 16, 18, 31, 33, and 45 (PreTect HPV-Proofer). The real-time multiplex NASBA assay PreTect HPV-Proofer (Fig. 2) was performed as suggested by the manufacturer (NorChip AS, Klokkarstua, Norway). Briefly, three premixes were made by reconstitution of reagent sphere, containing nucleotides, dithiothreitol, and $MgCl_2$, in a reagent sphere diluent (Tris-HCl, 45% dimethyl sulfoxide). Then, the primer-molecular beacon mixture U1 small nuclear ribonucleoprotein-specific A protein (U1A)–HPV-16, HPV-33–HPV-45, or HPV-18–HPV-31 was added together with a KCl stock solution. Ten microliters of this premix was distributed to each well in a reaction plate, followed by addition of RNA and 4 min of incubation at 65°C (to destabilize the secondary structures of the RNA) and 4 min of incubation at 41°C. The reaction was started by addition of enzymes (avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase) and was measured in real time by using a Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT) at 41°C for 2 h 30 min. The total reaction volume was 20 μl. A newly developed software package (PreTect analysis software; NorChip AS) was used for analysis of the experimental data. The excitation λ (nm) filters for 6-carboxyfluorescein and Texas Red are 485/20 and 590/20, respectively, and the emission λ (nm) filters are 530/25 and 645/40, respectively.

RNA isolated from CaSki cells was used as a positive control for HPV-16. Artificial and standardized oligonucleotides corresponding to the viral sequence were used as positive controls for HPV types 18, 31, 33, and 45. As a performance control, to avoid false-negative results due to degradation of RNA, we used a primer set and probe directed against mRNA of human U1A. Negative controls consisting of all reagents except RNA were included in each run.

(ii) HPV types 35, 52, and 58. Primer sets and molecular beacon probes were used for amplification and detection of HPV types 35, 52, and 58 (NorChip AS). The real-time multiplex NASBA assay was performed in the same manner as described above for the PreTect HPV-Proofer assay. The reactions were run in two different tubes: HPV-35–U1A and HPV-52–HPV-58. Artificial and standardized oligonucleotides corresponding to the viral sequence were used as positive controls. Negative controls were included in each run.

HPV DNA analysis by PCR. The same nucleic acid extracts and amounts of template used for the RNA analyses were used in the PCRs. PCR was performed with all 204 samples, both by consensus Gp5+/Gp6+ PCR and type-specific PCR, as described below. In addition, consensus My09/My11 PCR and PPF1/CP5 PCR were performed with the samples that tested negative by NASBA, type-specific PCR, and Gp5+/Gp6+ PCR. DNA was amplified in a Primus 96 HPL thermocycler (MWG). Fragments were detected by using the 2100 Bioanalyzer multi-instrument system from Agilent Technologies (Palo Alto, CA). As a DNA control, primers against the human β-globin gene were used (33).

(i) Consensus Gp5+/Gp6+ PCR. The L1 gene-specific consensus Gp5+/Gp6+ PCR (11) was performed with all 204 samples. The PCR amplification was carried out in a volume of 50 μl containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P-40, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 50 pmol of primers Gp5+ and Gp6+, and 1 unit of *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). The first DNA denaturation was performed for 2 min at 94°C; and then 40 cycles of PCR consisting of denaturation for 1 min at 94°C, annealing for 2 min at 40°C, and extension for 1.5 min at 72°C were run, followed by a final extension for 4 min at 72°C.

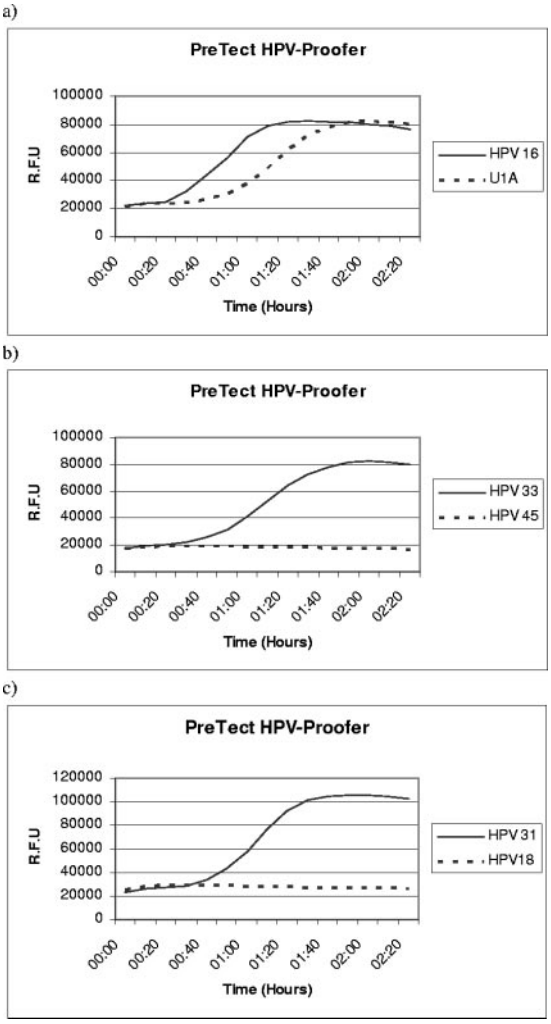

FIG. 2. Amplification of HPV RNA by PreTect HPV-Proofer. The PreTect HPV-Proofer assay detects HPV E6 and E7 mRNA from the high-risk HPV types 16, 18, 31, 33, and 45. The assay is based on real-time multiplex NASBA, detecting in complex HPV-16 and the sample performance control U1A (a), HPV-33 and HPV-45 (b), and HPV-31 and HPV-18 (c). A positive reaction result is revealed as a sigmoid curve.

(ii) Consensus My09/My11 PCR. The L1 gene-specific consensus primers My09 and My11 (26) were used to detect HPV in samples negative for HPV by type-specific or Gp5+/Gp6+ consensus PCR or NASBA. The PCR amplification was carried out in a volume of 50 μl containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P-40, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 30 pmol of primers My09 and My11, and 1 unit of *Taq* DNA polymerase (Sigma-Aldrich). The first DNA denaturation was performed for 2 min at 94°C; and then 40 cycles of PCR consisting of denaturation for 0.5 min at 94°C, annealing for 1 min at 45°C, and extension for 1 min at 72°C were run, followed by a final extension for 10 min at 72°C.

(iii) Consensus PPF1/CP5 PCR. The E1 gene-specific consensus primers PPF1 and CP5 (35) were used to detect HPV in samples negative for HPV by the PCR techniques mentioned above or NASBA. The PCR amplification was carried out in a volume of 50 μl containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of primers PPF1 and CP5, and 1 unit of *Taq* DNA polymerase (Sigma-Aldrich). The first DNA denaturation was performed for 2 min at 94°C; and then 40 cycles of PCR consisting of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 55°C, and extension for 1 min at 72°C were run, followed by a final extension for 10 min at 72°C.

(iv) Type-specific PCR. All samples were tested for HPV types 16, 18, 31, 33, 35, 45, 52, and 58 by type-specific PCR (NorChip AS) (22), without previous selection of positive cases by consensus PCR. The first DNA denaturation was performed for 2 min at 94°C; and then 35 cycles of PCR consisting of denaturation for 0.5 min at 94°C and annealing for 0.5 min at 57°C for HPV types 16, 18, 31, 52, and 58, at 55°C for HPV-35, and at 52°C for HPV-33 were run, followed by a 1-min extension step at 72°C. A final extension step for 4 min at 72°C was included after the 35 cycles.

Sequencing. In order to define the HPV types present in samples negative by the PreTect HPV-Proofer assay, real-time NASBA, or type-specific PCR, we performed sequencing of the PCR products obtained by using the consensus primers Gp5+ and Gp6+. The sequencing was performed at the University of Oslo (Oslo, Norway) by using the MegaBACE sequence analyzer (Amersham Biosciences, Little Chalfont, England), according to the manufacturer's protocol. The sequences were compared to other known sequences in a database search (BLAST; National Center for Biotechnology Information).

HPV DNA analysis by ISH. ISH was carried out with biotinylated probes against HPV types 16 and 18 (gifts from Harald zur Hausen and Ethel-Michele de Villiers, Germany) (3, 12) and HPV types 31 and 33 (Eurodiagnostics BV) and an alkaline phosphatase anti-alkaline phosphatase detection system, as described previously (15). Briefly, sections from formalin-fixed, paraffin-embedded material were treated sequentially with proteinase K (700 μg/ml), 0.2% glycine, and 4% paraformaldehyde. Subsequent to probe and cellular DNA denaturation by heating at 95°C for 10 min, the slides were hybridized for 18 to 22 h at 37°C. Tissue sections were given high-stringency washes before the hybridized DNA was detected by using sequential incubation with mouse antibiotin, rabbit anti-mouse immunoglobulin G, and alkaline phosphatase mouse anti-alkaline phosphatase. The alkaline phosphatase reactions were developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as chromogenes. All series included positive controls. Negative controls were performed by using pBR322 DNA labeled with biotin.

Gp5+/Gp6+ PCR, EIA, and RLB. Samples negative by any of the detection methods described above were extracted again for DNA and RNA and subjected to Gp5+/Gp6+ PCR, followed by EIA for the differentiation of HPV high-risk and low-risk types, with RLB subsequently used for typing (44).

RESULTS

HPV RNA. All 204 samples were positive for the RNA control, U1A, which was included to avoid false-negative results due to degradation of RNA. The PreTect HPV-Proofer assay, which detects E6 and E7 mRNA from the high-risk HPV types 16, 18, 31, 33, and 45, identified 181 of 204 (89%) biopsy

TABLE 1. Detection of HPV by PreTect HPV-Proofer and PCR

Assays compared and result of first assay	No. of samples with the following result by the second assay:		Total
	Negative	Positive	
Proofer[a] vs PCR type[b]			
Negative	21	2	23
Positive	3	178	181
Total	24	180	204
Proofer vs Gp5+/Gp6+ PCR[c]			
Negative	10	13	23
Positive	7	174	181
Total	17	187	204
PCR type vs Gp5+/Gp6+ PCR[c]			
Negative	12	12	24
Positive	5	175	180
Total	17	187	204

[a] Proofer, PreTect HPV-Proofer.
[b] PCR type, type-specific PCR for HPV types 16, 18, 31, 33, and 45.
[c] Gp5+/Gp6+ PCR, HPV consensus PCR.

specimens as HPV positive (Table 1; Fig. 2). In order to evaluate the overall prevalence of various HPV types, primers and probes against HPV types 35, 52, and 58 were also included. In total, HPV E6 and E7 expression was detected in 188 of 204 (92%) cases: 121 of 188 (64%) samples with HPV-16, 21 of 188 (11%) samples with HPV-18, 10 of 188 (5%) samples with HPV-31, 11 of 188 (6%) samples with HPV-33, 18 of 188 (10%) samples with HPV-45, 3 of 188 (2%) samples with HPV-35, 4 of 188 (2%) samples with HPV-52, and 2 of 188 (1%) samples with HPV-58. Three cases of multiple infections were found: HPV-51 and HPV-58, HPV-16 and HPV-52, and HPV-33 and HPV-58 (see Table 3).

HPV DNA. All samples tested positive for the β-globin DNA control. Type-specific PCR for the high-risk HPV types 16, 18, 31, 33, and 45 identified 180 of 204 (88%) biopsy specimens as HPV positive (Table 1). In total, type-specific PCR detected HPV in 188 of 204 (92%) samples: 122 of 188 (65%) samples with HPV-16, 21 of 188 (11%) samples with HPV-18, 8 of 188 (4%) samples with HPV-31, 12 of 188 (6%) samples with HPV-33, 17 of 188 (9%) samples with HPV-45, 5 of 188 (3%) samples with HPV-35, 5 of 188 (3%) samples with HPV-52, and 2 of 188 (1%) samples with HPV-58. Five cases of multiple infections were found: one sample containing HPV-16 and HPV-52, one sample containing HPV-33 and HPV-58, one sample containing HPV-51 and HPV-58, and two samples containing HPV-31 and HPV-35 (see Table 3).

By using the consensus Gp5+/Gp6+ PCR directed against the L1 gene, which encodes the HPV major capsid protein, DNA was detected in 187 of the 204 biopsy specimens (92%), of which 7 were negative by type-specific PCR (see Tables 1 and 3). Sequencing of these seven negative samples revealed HPV types 6, 26, 66, and 69 and three cases of HPV-73. In addition, one biopsy specimen initially positive by the Gp5+/Gp6+ PCR yet negative by the type-specific PCR was sequenced as HPV-51. This sample was then tested again with more concentrated material and was revealed to be HPV-58 positive by both NASBA and PCR. To further explore whether both HPV types 51 and 58 were present, the sample was also tested for HPV-51 by type-specific PCR and NASBA. HPV-51 was demonstrated by both methods. The same sample was confirmed to be HPV-51 and HPV-58 positive by EIA and RLB. Analysis by EIA and RLB, performed for the samples that were HPV negative by every other method used in the study, did not reveal any further HPV-positive samples. Additional consensus primers (My09/My11, PPF1/CP5) were used to test the HPV-negative biopsy specimens but revealed no further HPV infections.

ISH detected HPV types 16, 18, 31, and 33 in 160 of 204 (78%) samples (Table 2). Of the virus-infected samples, 119 of 160 (74%) contained HPV-16, 26 of 160 (16%) contained HPV-18, 12 of 160 (7%) contained HPV-31, and 12 of 160 (7%) contained HPV-33. One hundred percent of the virus-infected samples had a punctate signal, indicating integrated virus DNA. In addition, a diffuse signal was found in 75 of 160 samples (47%), indicating episomal virus DNA: 66 of 119 (55%) HPV-16-positive samples, 1 of 26 (4%) HPV-18-positive samples, 4 of 12 (33%) HPV-31-positive samples, and 6 of 12 (50%) HPV-33-positive samples. Seven cases of multiple infections were found (Table 3). In 66 of 75 (88%) of the cases with mixed populations, a homogeneous pattern of a punctate-diffuse signal was seen throughout the tissue section. In only 9 (12%) of the 75 cases a punctate signal and a punctate-diffuse signal were found in different parts of the tumor. By examination of serial sections for the cases with multiple infections, two or more HPV types were found within one cell; in three of the cases different HPV types were identified both within the same cell and in different tumor cells (Table 3; Fig. 3).

TABLE 2. Detection of HPV types 16, 18, 31, and 33 by PreTect HPV-Proofer and PCR compared to that by ISH

Assay and result[a]	No. of specimens with the following result by ISH:		
	Negative	Positive	Total
Proofer			
Negative	38	3	41
Positive	6	157	163
Total	44	160	204
PCR type			
Negative	36	5	41
Positive	8	155	163
Total	44	160	204

[a] Proofer, PreTect HPV-Proofer; PCR type, type-specific PCR.

Overall HPV detection. The overall HPV prevalence determined by all methods was 97% (198 of 204 samples). By NASBA and type-specific PCR, HPV (types 16, 18, 31, 33, 45, 35, 52, and 58) was detected in 190 of 204 (93%) biopsy specimens. The discrepancies between the different methods are presented in Table 3. E6 and E7 mRNA from the five HPV types included in the PreTect HPV-Proofer assay (HPV types 16, 18, 31, 33, and 45) was present in 181 of these 190 (95%) HPV-infected biopsy specimens. By consensus PCR, seven additional HPV-positive samples were detected; three represented HPV-73, most commonly described as being a high-risk type, or HPV types presumed to be low-risk HPV types (HPV types 6, 26, 66, and 69). When the results of ISH were also considered, the number of SCCs demonstrated to be positive for high-risk HPV types turned into 195 of 204 (96%) because of the counting of 1 additional HPV-18-positive sample and the finding of HPV-16 in the sample found HPV-69 positive by sequencing. We did not find any relation between the HPV type present and the stage of the tumor.

DISCUSSION

In the present study, 204 histologically confirmed SCCs of the uterine cervix were investigated by both RNA-based and DNA-based detection methods. The E6 and E7 mRNA detection assay PreTect HPV-Proofer, based on real-time multiplex NASBA, as well as a separate real-time multiplex NASBA assay, were used to study E6 and E7 mRNA expression from HPV types 16, 18, 31, 33, 35, 45, 52, and 58, which are the most prevalent high-risk HPV types in Europe and North America (5). The results were compared to those of consensus and type-specific PCRs and ISH.

An overall HPV detection rate of 97% was achieved, which is comparable to those achieved in other studies (5, 32, 46), confirming the importance of HPV in cervical carcinogenesis. No HPV was detected in 3% of the SCCs. In these cases HPV

TABLE 3. Discrepancies in HPV detection between the different methods[a]

Sample no.	β-Globin/U1A[b]	Gp5+/Gp6+[b]	HPV type detected				
			Seq.[b]	PCR type[b]	NASBA[b]	ISH[c,j]	Gp5+/Gp6+ RLB[d]
Single infections							
3307	+	+	16	16[e]	−	−	X (HR)
3393	+	+	6	−	−	−	6
3472	+	+	66	−	−	−	66
3697	+	+	69	−	−	16P	69
3808	+	+	26	−	−	−	26
3843	+	+	73	−	−	−	73
3931	+	+	31	52	31	31P	31
3959	+	+	33	33[e]	−	−	33
3980	+	+	73	−	−	−	73
4023	+	+		45	45	31P	
4111	+	+	73	−	−	−	
3311	+	−		52	52	−	52
3443	+	−		16	16	16P	−
3477	+	−		18	18	18P	−
3573	+	−		31	31	31P	−[f]
3647	+	−		45	45	−	−
3711	+	−		31	31	31P/D	31
3830	+	−		−	45	−	−[f]
3909	+	−		52	52	−	52
3988	+	−		−	−	18P	18
4011	+	−		52	52	−	52
4104	+	−		−	31	31P	31
Multiple infections							
3712	+	+	51	58[g]	58[g]	−	51/58
4075	+	+		31, 35	31	16P/D, 31P/D, 33P/D[h]	
3943	+	+		16, 52	16, 52	16P/D	
3606	+	+	31	31, 35	31	31P/D	
3874	+	+		33, 58	33, 58	33P	
3388	+	+		16	16	16P/D, 18P, 31P[h]	
3535	+	+		16	16	16P, 18P[i]	
3745	+	+		16	16	16P/D, 18P[h]	
3881	+	+		16	16	16P, 18P[i]	
4109	+	+		16	16	16P, 18P[i]	
4113	+	+		16	16	16P/D, 31P[i]	

[a] PCR type, type-specific PCR. The NASBA was for HPV types 16, 18, 31, 33, 45, 35, 52, and 58. The ISH was for HPV types 16, 18, 31, and 33. Symbols: +, positive result; −, negative result; blank cell, not analyzed.
[b] PCR and NASBA were performed with DNA/RNA extracted from fresh frozen tissue. Sequencing (Seq.) was performed with the Gp5+/Gp6+ PCR product.
[c] ISH was performed with paraffin-embedded tissue; P, punctate signal (indicates integration); D, diffuse signal (which indicates an episomal state).
[d] RLB was performed with newly extracted DNA/RNA.
[e] PCR was performed with more concentrated material in order to get a positive result.
[f] NASBA performed with the same newly extracted DNA/RNA used for RLB gave a negative result.
[g] Confirmed to also contain HPV-51 by PCR and NASBA.
[h] HPV types in the same tumor cells as well as in different tumor cells.
[i] HPV types in the same tumor cells.
[j] In addition, six samples showing a positive HPV result by type-specific PCR and NASBA were negative by ISH.

types that are not detected by the methods used may have been present; alternatively, carcinoma development may be caused by other mechanisms, such as p53 mutations, which are more frequently found in HPV-negative carcinomas (14).

E6 and E7 transcripts from the high-risk HPV types 16, 18, 31, 33, 35, 45, 52, and 58 were detected in 92% of the biopsy specimens, while 89% contained transcripts from at least one of the following HPV types: 16, 18, 31, 33, or 45. Type-specific PCR primers, which identified the same eight HPV types, also detected HPV DNA in 92% of the samples, although not exactly the same samples in which HPV was detected by the RNA assays. When the same HPV types are considered, previous studies show a somewhat lower HPV prevalence (4, 5). This may be explained by the use of L1 gene-specific consensus PCR prior to typing, which possibly misses the cases in which loss of the L1 gene has occurred during the process of integration (43). Our findings show that 10 and 8 of the 17 Gp5+/Gp6+ PCR-negative cases were detected by NASBA and type-specific PCR, respectively, suggesting that for 4 to 5% of the HPV-positive samples, loss of the L1 gene has occurred.

Nine samples positive by consensus PCR were found to be negative by the PreTect HPV-Proofer assay. Seven of these were identified by sequencing as HPV type 6, 26, 66, 69, or 73. HPV-73 was detected in three cases. Similar results have been reported in other European studies (5, 47), and in certain European countries, HPV-73 may have the same importance as HPV types 35 and 52 in the development of SCC. Further studies of the nature of these transcripts will add valuable information to the importance of the respective HPV types. The two remaining consensus DNA-positive, RNA-negative

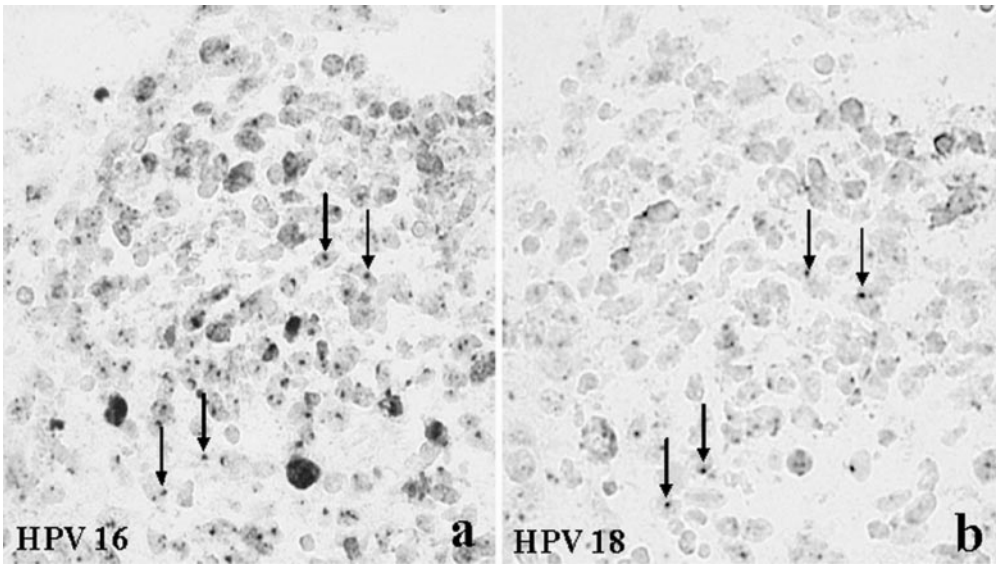

FIG. 3. Examination of serial sections by ISH. (a) Punctuate and diffuse ISH signal, indicating the presence of both integrated and episomal HPV-16; (b) punctuate ISH signal, indicating integrated HPV-18. Some tumor cells were infected with both HPV-16 and HPV-18 (arrows).

samples were found to be positive for HPV type 16 or 33 by type-specific PCR. A negative result by the PreTect HPV Proofer assay could be explained by low transcriptional activity or the occurrence of mutations in the regions covered by the primers or probes. These two samples were also negative by ISH, and more concentrated material had to be tested for the samples to become positive by PCR, indicating a low HPV copy number.

Based on the findings of this study, as well as those of other studies performed with carcinoma material, discussions related to the question of the classification of low-risk versus high-risk HPV types may evolve. In February 2005, the International Agency for Research on Cancer concluded that the 13 HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 can be classified as carcinogenic (6). In addition, types 68, 73, and 82 are considered to be associated with cervical carcinoma. To further stratify the different HPV types according to their importance for cervical carcinogenesis, the types have been sorted as follows by their decreasing prevalence based on a meta-analysis of 10,058 carcinoma samples worldwide (5): HPV types 16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 6, 51, 68, 39, 82, 73, 66, and 70. In the present study, HPV types 16, 18, 45, 33, and 31, listed by decreasing prevalence, were detected in 91% of all cases and represented 93% of all cases positive for HPV. Supplementation with HPV types 35, 52, and 58 increased the detection rate only marginally; seven additional samples were detected, giving a detection rate of 94%. Other Norwegian studies have shown the same low prevalence of HPV types 52 and 58 in cervical intraepithelial neoplasia 3 (24, 25). On the other hand, prevalence alone does not give an accurate picture of the carcinogenic potential of HPV, exemplified by the frequency of the low-risk HPV type 6 as compared to less-frequent "high-risk" HPV types. Conversely, most viral infections that occur in the uterine cervix, including high-risk HPV types, produce transient low-grade lesions and not high-grade cervical neoplasia. Furthermore, HPV types like types 39, 51, 56, and 66 are found more frequently in precancerous samples than in carcinomas (10) and are therefore suggested to be cleared out during carcinogenesis, possibly not being able to uphold the carcinogenic phenotype on their own. HPV types 26 and 66 were recently classified as probable high-risk types, whereas HPV-69 was not listed in any group (32). The biopsy specimen that was found positive for HPV-69 by sequencing was by ISH found to be positive for HPV-16. This suggests that HPV-69 may be only a passenger and is not involved in the process of carcinogenesis.

A relatively low number of multiple HPV infections was detected, which is in agreement with previous findings (32, 36, 39), as the frequency of multiple HPV infections in cervical carcinomas is generally lower than that in precancerous lesions (8, 17, 34). In this regard, RNA data may add to the basis for evaluating the role of different HPV types in cervical carcinogenesis, since continuous expression of E6 and E7 RNA is necessary for the maintenance of the malignant phenotype (51). In the present study, 11 cases of multiple infections were detected, of which 6 cases were found only by ISH. This may be explained by the targets and nature of the different methods used and the fact that different parts of tumor tissue were used for ISH.

In two of the five samples infected with multiple HPV types, as determined by PCR, RNA from only one of the types was detected. For the two cases positive for both HPV type 31 and HPV type 35 DNA, a detectable level of E6 and E7 RNA was found only for HPV-31, verifying the higher relative importance of HPV-31 over HPV-35 in cervical carcinogenesis. In biopsy specimens infected with HPV-18 and/or HPV-31 in conjunction with HPV-16 (as determined by ISH), only HPV-16 E6 and E7 RNA was detected. As shown previously, this may reflect a higher probability of persistent infection of HPV-16 (9) and implies that HPV infections with no detectable E6 and E7 mRNA may not contribute to carcinoma development. However,

the role of multiple infections in cancer progression still needs clarification.

Initially, one sample showed a different HPV type result by PCR versus NASBA: sequencing of the Gp5+/Gp6+ consensus PCR product reported the presence of HPV-51, whereas type-specific PCR and NASBA gave positive results for HPV-58. To explore if this could be due to a multiple infection, this sample was also tested for HPV-51 by type-specific PCR and NASBA. HPV-51 was demonstrated by both methods, and the HPV-51-positive, HPV-58-negative result by Gp5+/Gp6+ PCR may be explained by a higher viral load of HPV-51 than of HPV-58. Additionally, the consensus Gp5+/Gp6+ PCR has been reported to amplify HPV-58 rather poorly compared to the level of amplification obtained with the My09/My11 primer pair (37). The presence of both HPV types was confirmed by EIA and RLB, which verifies the importance of a hybridization step subsequent to Gp5+/Gp6+ PCR (44), since by sequencing a bias toward the detection of single infections is often created, and thus, the sensitivity of detection of other HPV types present is decreased.

A feature that has been demonstrated to be an important event in carcinogenesis is the integration of the virus into the host genome (7, 21, 49, 50), which leads to an increase in E6 and E7 expression (51) and a possible improved stability of the E6 and E7 transcripts (20). In this study, the physical state of HPV was investigated by ISH. However, it is important to keep in mind the use of different kinds of material for the RNA and PCR analyses compared to that used for ISH, and therefore, we cannot draw definite lines between the data. Still, the relatively small size of the tumors strengthens the probability that the sample material used for the different methods represents the same conditions. Additionally, the majority of the samples showed a homogeneous ISH pattern throughout the tumor; we saw heterogeneous patterns in only nine of the samples, denoting a greater possibility that the condition of the material used for ISH and for PCR and NASBA is not consistent. For the samples showing a homogeneous pattern, we find it reasonable to believe that the conditions were the same throughout the tissue. All samples found to be positive for HPV types 16, 18, 31, and 33 showed a punctate ISH signal, demonstrating integrated virus DNA (16). However, 47% of the samples also had an additional diffuse signal, indicative of episomal virus DNA. This is in accordance with previous findings, demonstrating that most SCCs and the cell lines derived from them contain the integrated form of HPV, either alone or together with the episomal form (7, 13, 23). However, the prevalence of episomal HPV diverged between the different HPV types. For HPV types 16 and 18, additional episomal HPV was found in 55% and 4% of the samples, respectively, which is in accordance with previous findings (7, 18, 21, 48). For HPV types 31 and 33, data on HPV integration are limited. We found that 33% of the HPV-31-positive samples and 50% of the HPV-33-positive samples contained episomal HPV, in addition to integrated HPV, suggesting similarity to HPV-16. Nevertheless, the implication of the diverging frequency of episomal HPV for different HPV types remains to be investigated.

In this study we show that, when it comes to cancerous samples, there are only minor differences between the mRNA and the DNA detection rates. However, a main problem with HPV DNA testing is the high prevalence of HPV among women with a cytological normal Pap smear, atypical squamous cells of undetermined significance, or low-grade squamous intraepithelial lesion compared to the number of women who actually develop severe dysplasia. Here, HPV E6 and E7 mRNA detection may have a diagnostic and prognostic advantage over DNA detection, with this advantage related to the mRNA expression pattern observed in relation to dysplastic development. For latent and abortive infections, the E6 and E7 proteins have been detected only in the basal cell layers. In contrast, a transforming infection is related to the higher expression of oncogenic E6 and E7 proteins, and in severe dysplasias these proteins have been identified throughout the entire cell layer (27). Therefore, detection of E6 and E7 mRNA may reflect a lesion more likely to persist or progress.

In summary, this study verifies the presence of HPV E6 and E7 mRNA expression in SCCs and demonstrates that HPV infections among Norwegian women with SCC are mainly limited to the five high-risk types 16, 18, 31, 33, and 45. This, together with the finding of a high agreement to other detection methods, demonstrates that the PreTect HPV-Proofer assay is a valuable approach for cervical carcinoma detection. To further establish the potential of HPV E6 and E7 mRNA detection in finding women at risk for the development of cervical carcinoma, extensive follow-up studies are ongoing.

ACKNOWLEDGMENTS

This work was supported by grant 145316/213 from the Research Council of Norway. The consumables were provided by NorChip AS.

We thank Petter Grønn, Inger Marie Falang, Marit Øder Øye, Vivi Bassøe, and Mai Thi Phuong Nguyen for technical assistance and Peter Snijders' group for performing the Gp5+/Gp6+ EIA and RLB assay.

Frank Karlsen is a stock holder in NorChip AS.

REFERENCES

1. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Bosch, F. X., M. M. Manos, N. Munoz, M. Sherman, A. M. Jansen, J. Peto, M. H. Schiffman, V. Moreno, R. Kurman, K. V. Shah, et al.** 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Natl. Cancer Inst. **87:**796–802.
3. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3:**1151–1157.
4. **Clifford, G. M., J. S. Smith, T. Aguado, and S. Franceschi.** 2003. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br. J. Cancer **89:**101–105.
5. **Clifford, G. M., J. S. Smith, M. Plummer, N. Munoz, and S. Franceschi.** 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br. J. Cancer. **88:**63–73.
6. **Cogliano, V., R. Baan, K. Straif, Y. Grosse, B. Secretan, and F. El Ghissassi.** 2005. Carcinogenicity of human papillomaviruses. Lancet Oncol. **6:**204.
7. **Cullen, A. P., R. Reid, M. Campion, and A. T. Lorincz.** 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J. Virol. **65:**606–612.
8. **Cuschieri, K. S., H. A. Cubie, M. W. Whitley, A. L. Seagar, M. J. Arends, C. Moore, G. Gilkisson, and E. McGoogan.** 2004. Multiple high risk HPV infections are common in cervical neoplasia and young women in a cervical screening population. J. Clin. Pathol. **57:**68–72.
9. **Cuschieri, K. S., M. J. Whitley, and H. A. Cubie.** 2004. Human papillomavirus type specific DNA and RNA persistence—implications for cervical disease progression and monitoring. J. Med. Virol. **73:**65–70.
10. **Depuydt, C. E., A. J. Vereecken, G. M. Salembier, A. S. Vanbrabant, L. A. Boels, E. van Herck, M. Arbyn, K. Segers, and J. J. Bogers.** 2003. Thin-layer liquid-based cervical cytology and PCR for detecting and typing human papillomavirus DNA in Flemish women. Br. J. Cancer **88:**560–566.
11. **de Roda Husman, A. M., J. M. Walboomers, A. J. van den Brule, C. J. Meijer, and P. J. Snijders.** 1995. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences im-

proves human papillomavirus detection by PCR. J. Gen. Virol. **76:**1057–1062.
12. **Durst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80:**3812–3815.
13. **Durst, M., A. Kleinheinz, M. Hotz, and L. Gissman.** 1985. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. J. Gen. Virol. **66**(Pt 7)**:**1515–1522.
14. **Helland, A., F. Karlsen, E. U. Due, R. Holm, G. Kristensen, and A. Borresen-Dale.** 1998. Mutations in the TP53 gene and protein expression of p53, MDM 2 and p21/WAF-1 in primary cervical carcinomas with no or low human papillomavirus load. Br. J. Cancer **78:**69–72.
15. **Holm, R., F. Karlsen, and J. M. Nesland.** 1992. In situ hybridization with nonisotopic probes using different detection systems. Mod. Pathol. **5:**315–319.
16. **Hopman, A. H., M. A. Kamps, F. Smedts, E. J. Speel, C. S. Herrington, and F. C. Ramaekers.** 2005. HPV in situ hybridization: impact of different protocols on the detection of integrated HPV. Int. J. Cancer **115:**419–428.
17. **Huang, L. W., S. L. Chao, P. H. Chen, and H. P. Chou.** 2004. Multiple HPV genotypes in cervical carcinomas: improved DNA detection and typing in archival tissues. J. Clin. Virol. **29:**271–276.
18. **Hudelist, G., M. Manavi, K. I. Pischinger, T. Watkins-Riedel, C. F. Singer, E. Kubista, and K. F. Czerwenka.** 2004. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. Gynecol. Oncol. **92:**873–880.
19. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laiminis.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol. **64:**519–526.
20. **Jeon, S., and P. F. Lambert.** 1995. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc. Natl. Acad. Sci. USA **92:**1654–1658.
21. **Kalantari, M., E. Blennow, B. Hagmar, and B. Johansson.** 2001. Physical state of HPV16 and chromosomal mapping of the integrated form in cervical carcinomas. Diagn. Mol. Pathol. **10:**46–54.
22. **Karlsen, F., M. Kalantari, A. Jenkins, E. Pettersen, G. Kristensen, R. Holm, B. Johansson, and B. Hagmar.** 1996. Use of multiple PCR primer sets for optimal detection of human papillomavirus. J. Clin. Microbiol. **34:**2095–2100.
23. **Klaes, R., S. M. Woerner, R. Ridder, N. Wentzensen, M. Duerst, A. Schneider, B. Lotz, P. Melsheimer, and M. von Knebel Doeberitz.** 1999. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. Cancer Res. **59:**6132–6136.
24. **Kraus, I., T. Molden, L. E. Erno, H. Skomedal, F. Karlsen, and B. Hagmar.** 2004. Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. Br. J. Cancer **90:**1407–1413.
25. **Lie, A. K., B. Risberg, B. Borge, B. Sandstad, J. Delabie, R. Rimala, M. Onsrud, and S. Thoresen.** 2005. DNA- versus RNA-based methods for human papillomavirus detection in cervical neoplasia. Gynecol. Oncol. **97:**908–915.
26. **Manos, M. M., Y. Ting, D. K. Wright, A. J. Lewis, T. R. Broker, and S. M. Wolinsky.** 1989. Use of polymerase chain reaction amplification for detection of genital human papillomavirus. Cancer Cells 209–214.
27. **Middleton, K., W. Peh, S. Southern, H. Griffin, K. Sotlar, T. Nakahara, A. El Sherif, L. Morris, R. Seth, M. Hibma, D. Jenkins, P. Lambert, N. Coleman, and J. Doorbar.** 2003. Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. J. Virol. **77:**10186–10201.
28. **Molden, T., I. Kraus, F. Karlsen, H. Skomedal, J. F. Nygard, and B. Hagmar.** 2005. Comparison of human papillomavirus messenger RNA and DNA detection: a cross-sectional study of 4,136 women >30 years of age with a 2-year follow-up of high-grade squamous intraepithelial lesion. Cancer Epidemiol. Biomarkers Prev. **14:**367–372.
29. **Molden, T., J. F. Nygard, I. Kraus, F. Karlsen, M. Nygard, G. B. Skare, H. Skomedal, S. O. Thoresen, and B. Hagmar.** 2005. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-Proofer and consensus PCR: a 2-year follow-up of women with ASCUS or LSIL Pap smear. Int. J. Cancer **114:**973–976.
30. **Munger, K., and P. M. Howley.** 2002. Human papillomavirus immortalization and transformation functions. Virus Res. **89:**213–228.
31. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
32. **Munoz, N., F. X. Bosch, S. de Sanjose, R. Herrero, X. Castellsague, K. V. Shah, P. J. Snijders, and C. J. Meijer.** 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. **348:**518–527.
33. **Mwenda, J. M., T. Shotake, T. Yamamoto, R. Uchihi, C. S. Bambra, and Y. Katsumata.** 1999. DNA typing of primate major histocompatibility complex (Mhc)-DQA1 locus by PCR and dot blot hybridization. Cell Mol. Biol. (Noisy-le-Grand) **45:**1249–1256.
34. **Park, T. C., C. J. Kim, Y. M. Koh, K. H. Lee, J. H. Yoon, J. H. Kim, S. E. Namkoong, and J. S. Park.** 2004. Human papillomavirus genotyping by the DNA chip in the cervical neoplasia. DNA Cell Biol. **23:**119–125.
35. **Petry, K. U., S. Menton, M. Menton, F. Loenen-Frosch, G. H. de Carvalho, B. Holz, B. Schopp, S. Garbrecht-Buettner, P. Davies, G. Boehmer, A. E. van den, and T. Iftner.** 2003. Inclusion of HPV testing in routine cervical cancer screening for women above 29 years in Germany: results for 8466 patients. Br. J. Cancer **88:**1570–1577.
36. **Pirog, E. C., B. Kleter, S. Olgac, P. Bobkiewicz, J. Lindeman, W. G. Quint, R. M. Richart, and C. Isacson.** 2000. Prevalence of human papillomavirus DNA in different histological subtypes of cervical adenocarcinoma. Am. J. Pathol. **157:**1055–1062.
37. **Qu, W., G. Jiang, Y. Cruz, C. J. Chang, G. Y. Ho, R. S. Klein, and R. D. Burk.** 1997. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. J. Clin. Microbiol. **35:**1304–1310.
38. **Remmink, A. J., J. M. Walboomers, T. J. Helmerhorst, F. J. Voorhorst, L. Rozendaal, E. K. Risse, C. J. Meijer, and P. Kenemans.** 1995. The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. Int. J. Cancer **61:**306–311.
39. **Schellekens, M. C., A. Dijkman, M. F. Aziz, B. Siregar, S. Cornain, S. Kolkman-Uljee, L. A. Peters, and G. J. Fleuren.** 2004. Prevalence of single and multiple HPV types in cervical carcinomas in Jakarta, Indonesia. Gynecol. Oncol. **93:**49–53.
40. **Sotlar, K., H. C. Selinka, M. Menton, R. Kandolf, and B. Bultmann.** 1998. Detection of human papillomavirus type 16 E6/E7 oncogene transcripts in dysplastic and nondysplastic cervical scrapes by nested RT-PCR. Gynecol. Oncol. **69:**114–121.
41. **Sotlar, K., A. Stubner, D. Diemer, S. Menton, M. Menton, K. Dietz, D. Wallwiener, R. Kandolf, and B. Bultmann.** 2004. Detection of high-risk human papillomavirus E6 and E7 oncogene transcripts in cervical scrapes by nested RT-polymerase chain reaction. J. Med. Virol. **74:**107–116.
42. **Syrjanen, K., and S. Syrjanen.** 2000. Papillomavirus infections in human pathology. John Wiley & Sons, Ltd., Chicester, United Kingdom.
43. **Tate, J. E., Y. C. Yang, J. Shen, C. M. McLachlin, E. E. Sheets, and C. P. Crum.** 1996. A comparison of early (E7) and late (L1) primer-mediated amplification of papillomaviral DNA in cervical neoplasia. Mol. Cell Probes **10:**347–351.
44. **van den Brule, A. J., R. Pol, N. Fransen-Daalmeijer, L. M. Schouls, C. J. Meijer, and P. J. Snijders.** 2002. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J. Clin. Microbiol. **40:**779–787.
45. **von Knebel Doeberitz, M.** 2002. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. Eur. J. Cancer **38:**2229–2242.
46. **Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. **189:**12–19.
47. **Wallin, K. L., F. Wiklund, T. Angstrom, F. Bergman, U. Stendahl, G. Wadell, G. Hallmans, and J. Dillner.** 1999. Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. N. Engl. J. Med. **341:**1633–1638.
48. **Watts, K. J., C. H. Thompson, Y. E. Cossart, and B. R. Rose.** 2002. Sequence variation and physical state of human papillomavirus type 16 cervical cancer isolates from Australia and New Caledonia. Int. J. Cancer **97:**868–874.
49. **Wentzensen, N., S. Vinokurova, and M. von Knebel Doeberitz.** 2004. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. Cancer Res. **64:**3878–3884.
50. **Ziegert, C., N. Wentzensen, S. Vinokurova, F. Kisseljov, J. Einenkel, M. Hoeckel, and M. von Knebel Doeberitz.** 2003. A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques. Oncogene **22:**3977–3984.
51. **zur Hausen, H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer **2:**342–350.
52. **zur Hausen, H., and E. M. de Villiers.** 1994. Human papillomaviruses. Annu. Rev. Microbiol. **48:**427–447.