

Human Papillomavirus Type Specific DNA and RNA Persistence—Implications for Cervical Disease Progression and Monitoring

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In 2000, we monitored the course and persistence of human papillomavirus (HPV) infection in 54 women who were HPV positive and free of any cytological disease using HPV-DNA genotyping with a linear array assay (baseline). The impact of HPV infection on development of cervical cytological abnormality (dyskaryosis) was monitored by repeat HPV genotyping and cytological assessment 2 years later. Detection of mRNA transcripts of known HPV oncogenes E6 and E7 using NASBA methodology and specific molecular beacons for five common HPV types was also performed at both time points. A total of 11/54 (20%) women developed dyskaryosis after 2 years with 31/54 and 23/54 women exhibiting transient and persistent infections respectively, as monitored by DNA genotyping. Women who maintained type-specific persistent HPV infection were significantly more likely to develop dyskaryosis compared to those who exhibited a transient infection ($P=0.001$). The presence of HPV mRNA E6/E7 transcripts was less sensitive but more specific for the detection of disease at follow up. Moreover, women who were DNA positive and also positive for mRNA transcripts at baseline were significantly more likely to harbour persistent infection compared to those in whom DNA only was detected at baseline ($P=0.013$). This study highlights the importance of detecting persistent type specific HPV infection to identify those women more at risk of developing cervical abnormalities, either by repeated DNA genotyping, or potentially by RNA based techniques that may be more predictive of persistent infection if performed at a single time point. **J. Med. Virol. 73:65–70, 2004.** © 2004 Wiley-Liss, Inc.

KEY WORDS: HPV; genotyping; E6/E7 transcript detection; dyskaryosis

INTRODUCTION

Infection with human papillomavirus (HPV) is considered to be a pre-requisite for the development of

cervical cancer and associated neoplastic precursor lesions [Zur Hausen, 2002]. However, even in “low-risk” populations HPV prevalence within a community can be high and the significant majority of infected women will successfully clear HPV with no deleterious pathological effects [Nobbenhuis et al., 2001]. Thus there is still debate as to where, in cervical screening programmes, HPV DNA testing would prove most effective.

The majority of existing or proposed HPV screening protocols have involved the application of HPV DNA based tests for structural genes, most commonly the *L1* capsid gene utilising the PGM1 or GP5+/6+ primers. However, as it is viral persistence that is considered to be the true pre-cursor of neoplastic progression [Kjaer et al., 2002], DNA genotyping approaches designed to identify type specific persistence, could be more appropriate for identification of those individuals at greater risk of disease progression compared with a presence/absence test. Furthermore, detection of RNA transcripts of genes known to be involved in oncogenesis may be more useful than DNA tests for detection of active and potentially persistent infections [Ylitalo et al., 2000]. Appropriate target oncogene candidates are the early genes *E6* and *E7*, considered fundamental for progression and maintenance of the neoplastic state [Watanabe et al., 1989]. The E6 protein causes degradation of the tumour suppressor protein p53 [Scheffner et al., 1993] whereas E7 affects the retinoblastoma gene product by releasing it from E2F-type transcriptional control leading to unchecked cell cycle progression [Dyson et al., 1989]. Moreover, the relative expression levels of E6 and E7 have been found to increase in tandem increasing severity of cervical disease [Wang-Johanning et al., 2002].

Grant sponsor: Chief Scientist Office of the Scottish Executive.

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Accepted 8 December 2003

DOI 10.1002/jmv.20062

Published online in Wiley InterScience
(www.interscience.wiley.com)

We performed a pilot study to track the natural course of HPV infection over time by application of both E6/E7 RNA and L1 DNA genotyping techniques, whilst assessing concurrent cytological evidence of disease. We analysed and compared the impact of HPV DNA and RNA type-specific persistence on disease progression. We also assessed whether individuals who were HPV DNA positive and equivalent HPV RNA transcript positive (HPV RNA) were more likely to exhibit persistent infection, compared to those who were HPV DNA positive alone at baseline. Finally, cross-sectional analysis was carried out on follow-up samples to analyse the specificity/sensitivity of the HPV DNA and RNA testing methodologies for the detection of disease at a single time point.

MATERIALS AND METHODS

Study Group and Sample Collection

Liquid based cytology (LBC) samples were obtained in 2,000 from more than 3,000 women as part of an ongoing study designed to assess HPV persistence (baseline). LBC involves rinsing a cervical specimen into a vial containing a cellular preservative solution, rather than depositing it directly on a slide as is performed during the conventional Papanicolaou smear. Primary care personnel carried out specimen collection, flat layer slides were created by the ThinPrep[®] procedure and cytological grading was performed according to British Society for Clinical Cytology guidelines. A cohort of 54 women were selected on the basis of having a cytologically normal result at baseline but who were also HPV DNA positive for at least one of the following "high-risk" HPV types: 16,18,31,33 and 45, considered the most commonly found high-risk types in Europe and implicated in >90% of cancers [Muñoz et al., 2003]. It should be noted that an individual could have been infected with other HPV genotypes, in addition to of these five. Women were recalled for a follow up LBC smear 2 years after baseline when cytological assessment and both HPV DNA and RNA testing was performed.

Sample Processing and Nucleic Acid Extraction

After cytology, residual cells in the LBC sample were centrifuged at 3,500 rpm for 10 min and stored as split cellular pellets at -70°C prior to nucleic acid extraction and HPV detection. Local Research Ethics Committee approval was granted for HPV testing and informed consent was obtained. Automated DNA extraction was performed using a BioRobot 9604[®] (QIAGEN Ltd., Crawley, UK) according to a protocol described previously, [Cuschieri et al., 2003] using the reagents supplied with the QIAamp[®] 96 DNA Swab BioRobot[™] Kit whereas RNA extraction was performed by application of RNeasy columns (QIAGEN Ltd.) following the protocol for isolation from animal cells, according to the manufacturer's instructions. Nucleic acid was stored at -70°C prior to HPV detection.

HPV Detection

HPV DNA genotyping was undertaken by linear array hybridisation assay (LA) which involved the hybridisation of a 450 nt PCR amplicon generated by the PGMY primer set to a nylon strip containing immobilised probes [Gravitt et al., 2000; Coutlee et al., 2002]. The strip contained two levels of β -globin control probes, 18 high-risk HPV (HR-HPV) probes; 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, 83 and 9 low-risk HPV (LR-HPV) probes: 6, 11, 40, 42, 53, 54, 57, 66, 84. PCR reagents, probe strips and developing reagents were supplied by Roche Molecular Systems, Inc. (Alameda). Any sample that tested β -globin negative would be excluded from analysis. RNA amplification was achieved via an isothermal NASBA amplification [Kievits et al., 1991] and type specific detection was performed using molecular beacon (MB) probes directed against full-length E6/E7 mRNA for HPV types HPV 16, 18, 31, 33, 45. All reagents required for NASBA amplification and HPV detection were supplied as part of the PreTect[®] HPV-Proofer Kit, (NorChip, Klokkearstua, Norway). Fluorescent detection of accumulated mRNA product was performed in real time using a NucliSens EasyQ Analyzer and fluorescent profiles analysed using the PreTect analysis software (PAS, NorChip).

As a performance control, to avoid false negatives due to degradation of nucleic acid, we used probes directed against the human U1 small nuclear ribonucleoprotein specific A protein (U1A mRNA). Standardised oligonucleotides corresponding to the viral E6/E7 mRNA sequence were used as positive controls for all HPV types.

RESULTS

Cytological Evidence of Dyskaryosis

Of the 54 women (mean age 28.9) that were normal cytologically at baseline but who contained detectable HPV, 11 exhibited some level of cytological abnormality/dyskaryosis in their follow-up LBC sample (20%). Seven of these were classified as low-grade cytological disease (indicative of either borderline or mild dyskaryotic changes) whereas four were categorised as high-grade cytological disease (indicative of either moderate or severe dyskaryotic changes). The remaining 43 women exhibited concurrent cytology that was classified as within normal limits. No LBC smears were considered unsatisfactory for cytological assessment.

Detectable HPV DNA Genotypes at Baseline and Follow-Up

All baseline and follow-up DNA extracts tested positive by β -globin PCR. Of the 54 samples that were HPV DNA positive at baseline, 27 (50%) were mono-infections (HPV 16 $n = 16$, HPV 31 $n = 4$, HPV 33 $n = 2$, HPV 45 $n = 4$ and HPV 18 $n = 1$) and 27 were mixed infections that contained at least one of the above. The full results of DNA genotyping are displayed in Table I. A total of 31 HPV DNA transient infections were observed (HPV DNA

TABLE I. Longitudinal Detection of HPV by DNA Genotyping and RNA Transcript Detection in a Cohort of Individuals Tested in 2000 (Baseline) and 2002 (Follow-Up)

Case no.	HPV DNA genotype 2000	HPV RNA transcript 2000	HPV DNA genotype 2002	HPV RNA transcript 2002	Cytology result 2002	DNA persistent	RNA persistent
1	18, 83	18	18	—	Normal	+	—
2	16, 31	31	31	—	Normal	+	—
3	16	16	16	—	Normal	+	—
4	16	16	16	16	Normal	+	+
5	33	33	33, 84	16, 33	Normal	+	+
6	16, 18, 52	18	18, 52	—	Normal	+	—
7	16, 18, 84	16, 18	16, 33, 54	16	Normal	+	+
8	16, 56, 42, 53, 66	16	16, 51, 56, 42, 53, 66	—	Normal	+	—
9	16, 59	—	59	—	Normal	+	—
10	16, 51, 42	—	16, 52, 42	16	Normal	+	—
11	45	—	45	—	Normal	+	—
12	16	—	16	—	Normal	+	—
13	16	—	16	16	Normal	+	—
14	16	16	—	—	Normal	—	—
15	16	16	51	—	Normal	—	—
16	16	16	—	—	Normal	—	—
17	16	16, 45	—	—	Normal	—	—
18	45	45	—	—	Normal	—	—
19	16, 51	16	31, 52	16	Normal	—	+
20	45, 52	45	73	—	Normal	—	—
21	16	16	84	—	Normal	—	—
22	16	16	18, 66	—	Normal	—	—
23	31, 33	31	51	—	Normal	—	—
24	18, 52	18	—	18	Normal	—	+
25	16, 73	16	—	—	Normal	—	—
26	31, 51	—	—	—	Normal	—	—
27	45	—	—	—	Normal	—	—
28	16, 18, 52, 73, 6, 53, 66	—	—	—	Normal	—	—
29	16, 31, 55	—	—	—	Normal	—	—
30	31, 56, 54, 66	—	—	—	Normal	—	—
31	45, 56, 68	—	—	—	Normal	—	—
32	16, 73, 56	—	42	—	Normal	—	—
33	45, 6	—	—	—	Normal	—	—
34	31	—	—	—	Normal	—	—
35	45	—	—	—	Normal	—	—
36	16	—	—	—	Normal	—	—
37	33, 42, 53	—	35, 51	—	Normal	—	—
38	31	—	—	—	Normal	—	—
39	31	—	42	—	Normal	—	—
40	18, 42	—	16, 53	16	Normal	—	—
41	33	—	—	—	Normal	—	—
42	18	—	39, 84	—	Normal	—	—
43	52, 45	—	—	—	Normal	—	—
44	16	—	16	16	Severe	+	—
45	16	16	16	16	Severe	+	+
46	16, 18	16, 18	18	18	Moderate	+	+
47	16, 58	16	16, 58	16	Mild	+	+
48	16	16	16	16	Moderate	+	+
49	31	—	—	—	Mild	—	—
50	33, 58	33	33, 82	—	Mild	+	—
51	16, 52, 82, 6, 42	16	16, 53	16	Mild	+	+
52	16	16	16, 33	16, 31	Mild	+	+
53	16, 58	—	16, 58	16	Mild	+	—
54	16	16	16	—	Borderline	+	—

A borderline or a mild cytology result is suggestive of low-grade abnormalities whereas a moderate or a severe cytology result is suggestive of high-grade abnormalities.

transient being defined as the absence of an HPV DNA type in the follow-up sample, that was previously detected in the baseline sample). Thus an individual could have remained HPV DNA positive and still be classed as having a transient infection, for example case 19, who tested positive for HPV 16 and HPV 51 at baseline but was positive for HPV 31 and HPV 52 at

follow up. Only one case of transient infection progressed to cytological abnormality (case 49).

Persistent HPV DNA infection was defined as the detection of an HPV DNA type in the follow-up sample that had previously been detected in the appropriate baseline sample. Persistent infection with at least one genotype was observed in 23 women (Table II) and of

TABLE II. Proportion of Detectable Persistent HPV Infections in Individuals With and Without Concurrent Evidence of Dyskaryosis on Follow-Up as Detected by DNA Genotyping and HPV RNA Transcript Detection

Cytological assessment on follow-up	No. of cases	No. of persistent infections (DNA)	No. of persistent infections (RNA)	No. of persistent infections (DNA or RNA)
Abnormal	11	10 (90.1)	6 (54.5)	10 (90.1)
Normal	43	13 (30.2)	5 (11.6)	15 (34.8)

these 10 (43%) developed cervical abnormalities (1 borderline, 5 mild, 2 moderate and 2 severe dyskaryosis). A comparison of proportions revealed that a cytological abnormality was significantly more likely to develop if an individual was infected persistently with HR-HPV ($P = 0.001\%$) when compared to those who harboured a transient infection.

The acquisition of new HR-HPV types was noted in 17 women, with normal and abnormal cytology at follow-up apparent in 14 and 3 women respectively. In 12 of the 17, the acquisition included HR-HPV types while in the remaining five, new types were all low risk and the cytology was normal (cases 7, 21, 32, 39, 42).

Detectable HPV mRNA Transcripts at Baseline and Follow-Up

RNA transcripts were detected in 20/54 individuals at baseline with a single type-specific transcript detected in the majority and two types of transcripts detected in a further three (cases 7, 17 and 46).

Of the 43 cases with normal cytology at follow-up, only 8 had evidence of HPV RNA transcripts: 6 exhibited HPV 16 alone (cases 4, 7, 10, 13, 19, 40) one exhibited HPV 18 (case 24) and one HPV 16 and HPV 33 (case 5). In this last case, HPV 16 DNA was not detected at either baseline or follow up.

A total of 11 women showed type-specific persistent RNA infections, of which 6 (55%) developed dyskaryosis and of the 43 cases where persistent RNA infection was not detected, only 5 (12%) progressed to dyskaryosis (Table II). Again, a comparison of proportions indicated that the difference was highly significant ($P = 0.006$) suggesting that an individual who is persistently infected with HPV (as detected by HPV RNA) is more likely to develop abnormality. A breakdown of HPV persistence as monitored by DNA and RNA detection methodologies is detailed in Table II.

Baseline Detection of E6/E7 Transcripts to Predict Persistence

Of 54 HPV DNA positive samples at baseline, 28 subjects were also found to be RNA transcript positive and of these 18 (64%) went on to be infected persistently. In contrast, of the 26 transcript negative samples, only 7 (27%) progressed to persistent infection, as demonstrated by type specific RNA, DNA or both. The difference in percentage between persistent infections in the transcript positive and negative populations at baseline was found to be significant ($P = 0.05$) indicating that an HPV DNA infection was significantly more

likely to persist if its equivalent transcript was also detected.

Comparison of HPV DNA and RNA Detection With Cytological Evidence of Dyskaryosis at a Single Time Point

Table III demonstrates the sensitivity and specificity of DNA and RNA testing for the detection of disease at a single time point (i.e. the 2 year follow up samples). Cross-sectional analysis revealed that all four cases of high grade disease were detected by DNA genotyping and RNA transcript detection. Of the seven low grade disease cases, 4/7 were RNA transcript positive with 6/7 testing positive via DNA genotyping whereas 1/7 tested negative for both HPV DNA and RNA.

DISCUSSION

This study supports the theory that type-specific persistent infection with HPV is a significant risk-factor for the development of cervical abnormalities. Our study differs in part from other investigations on the natural history of HPV infection in that it monitored longitudinal type specificity rather than generic HPV detection in which a presence/absence screening test could suggest persistent HPV infection, yet actually reflect sequential transient infections with different types. Persistent infection could be further refined by a sequencing approach to assess and account for the presence of molecular variants within a single type such as HPV 16. If the increasing evidence base continues to show that persistent infection is a necessary cause of neoplasia, it will be relevant clinically to look for consistent activity of specific types. While generic HPV tests have a clear role in screening, second stage testing should incorporate HPV genotyping. Such assays are being developed and their diagnostic potential for cervical disease assessed. They include techniques for detecting and monitoring early oncogene expression. Soltar et al. [1998] developed a sensitive, RT-PCR for spliced oncogene transcripts of E6 and E7 and more recently, Wang-Johanning et al. [2002] and Lamarq

TABLE III. Comparison of DNA Genotyping and RNA Transcript Detection for the Detection of 11 Cases of Dyskaryosis

Method of detection	Sensitivity		Specificity	
DNA	10/11	90.9%	19/43	44.2%
RNA	8/11	72.7%	35/43	81.4%

et al. [2002] developed a real-time quantitative RT-PCR to evaluate levels of E6/E7 expression and E7 expression in clinical samples respectively. These and related studies have concentrated on cross-sectional analysis of a set of samples that are either free of or possess histologically confirmed disease and conclude that a significantly higher percentage of abnormal samples harbour oncogenic transcripts with titres increasing coordinately with increasing severity of disease. Cross-sectional analysis of our data revealed a higher specificity for the detection of dyskaryosis when using RNA based detection. Sensitivity appeared slightly lower than for DNA based detection, as two cases of low grade dyskaryosis were missed. However, such cases have a high chance of resolving [Zielinski et al., 2001] and since the number tested was small, a much larger study would be required to determine if this was a clinically significant reduction in sensitivity. If a DNA based approach was adopted to check persistence, genotyping would have to be performed repeated at intervals of approximately 1 year. The cost implications, clinical effectiveness of repeated tests and delay in treatment may make this less suitable than single time point RNA expression analysis.

In our study, detection of mRNA E6/E7 transcripts identified which infections were more likely to persist, in addition to picking up 8/11 cases of dyskaryosis, suggesting it has two significant benefits: high specificity for detecting high-grade cases and identification of infections which are more likely to persist and induce abnormalities in the future. At present only five types are detected by the RNA methodology described here and this may be considered a disadvantage in comparison with available DNA genotyping strategies such as the LA assay which can detect a broad range of 27 HPV types. Nevertheless, the significant majority of cancers will contain at least one of these five core types [Muñoz et al., 2003] and indeed all the HPV positive cytologically abnormal cases in our study contained at least one of the five. However, the incidence of different HPV types in cervical cancers varies with geographical populations [Bosch et al., 1995] and it may be advisable to assess the molecular diversity of HPV types associated with cervical disease in a given population so that an appropriate probe set can be selected.

Another interesting finding of this study is that there were four samples that exhibited detectable E6/E7 transcripts of more than one HPV genotype. The role of multiple infection as opposed to single HPV infection in cervical disease progression has still to be fully elucidated. It could be argued that pathogenic HPV associated effects are conferred by one dominant type within a multiple infection, with the rest representing a commensal population. This notion was supported by Stoler and Baber [2002] in an in situ study where detection of E6/E7 mRNA in cervical cancer biopsies, known to contain multiple HPV DNA types, revealed that only one type was transcribed. In our study 3/4 "multiple mRNA" positive samples were evident in individuals with normal cytology and only one in an

individual with mild dyskaryosis. Using the method described above, it would be of interest to investigate whether histologically confirmed cancers exhibited multiple mRNA transcripts. Of the four cases of high-grade disease, all showed one predominant transcript. It may be that selection of the "aggressive" genotype/integrated clone is based on level of transcript produced at an earlier stage of the dysplastic sequence, this being the type that goes on to pre-dispose to higher levels of malignancy. Quantitative assays could address this hypothesis.

In summary, we have shown that detection of type specific persistent HPV infection as measured by both DNA and RNA techniques selects individuals with a significant risk of developing cervical disease. Moreover, the detection of oncogenic transcripts has the potential to identify which high-risk infections may persist without having to perform repeat testing.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Frank Karlsen and colleagues NorChip AS and Dr. Janet Kornegay and colleagues at Roche Molecular Systems for providing reagents and technical support. We also wish to thank Iris Krell for useful comments and Cat Graham at the Western Clinical Trust Research Facility for the statistical analysis.

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