ORIGINAL ARTICLE

Study of viral integration of HPV-16 in young patients with LSIL

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Aims: To investigate the physical status of human papillomavirus 16 (HPV-16) in low grade squamous intraepithelial lesions (LSILs) as a means of determining the percentage of viral integration.
Methods: Ninety two LSIL/HPV positive Thin Prep® samples were initially tested for the E6 gene by the polymerase chain reaction (PCR) to identify the HPV-16 virus. To avoid false positive results, the specificity of the bands obtained from PCR was confirmed by Southern blot hybridisation with internal oligonucleotide probes. Next, a PCR screen for the E2 gene was performed to identify those samples in which the virus was integrated. Viral integration was detected in just over half of them.
Results: Twenty of the 92 samples were HPV-16 positive, as shown by PCR for the E6 gene. Southern blot analysis confirmed that 13 of these samples were positive for the viral E6 gene. Thus, viral integration was detected in just over a half of the samples positive for HPV-16.
Conclusions: These data show that HPV-16 integration occurs in a subset of LSILs. The measurement of HPV-16 integration would be a helpful complementary tool for cytological evaluation in primary cervical screening to identify those patients at risk of developing high grade squamous intraepithelial lesions and cervical cancer.

Epidemiological and biochemical data support the division of human papilloma viruses (HPVs) into two groups: high risk (for example, HPV-16, HPV-18, HPV-5, and HPV-8) and low risk (for example, HPV-1, HPV-6, and HPV-11) types,[1] and there is considerable evidence showing that cervical dysplasia is induced by persistent infection with high risk HPVs. So far, more than 80 HPV types have been identified and whereas low risk types have been found mostly in benign lesions and low grade squamous intraepithelial lesions (LSILs), HPV-16 and HPV-18 have been categorised as high risk HPV types on the basis of their greater than 50% prevalence in high grade squamous intraepithelial lesions (HSILs) and their 80–90% prevalence in cervical cancers.[2–6] HPV-16 is the most well studied HPV type, and serves as an important model for studying viral carcinogenesis. Therefore, there is increased interest in using the detection of HPV DNA as an adjunct to classic cytological evaluation. In fact, the Papanicolaou test is not perfect and false negative rates of 5–50% have been reported for LSILs.[7 8] For this reason, in the past few years, the use of liquid cytology has made it possible to combine morphological evaluation with the molecular analysis of the cervical cells.

> "There is increased interest in using the detection of human papillomavirus DNA as an adjunct to classic cytological evaluation"

During infection, a subset of several oncoproteins is expressed under a complex regulatory network of cellular and viral transcription factors. Malignant transformation is brought about by the products of the viral E6 and E7 oncogenes, which act by inactivating the tumour suppressor protein p53 and the RB family proteins pRb, p107, and pRb2/p130, respectively.[9–16] In HPV-16, the transcription of both the E6 and E7 genes is under the control of the upstream regulatory region and depends on a single promoter, called p97. The full length E2 gene product, a transcriptional activator protein,[17 16] represses the transcription of the E6 and E7 genes by binding to the p97 promoter region. It has also been shown that the reintroduction of the E2 protein into HPV-16 transformed cervical carcinoma cells upregulates p97 promoter activity and the cells die via apoptosis.[14 18]

We could consider integration as a mutation, with consequences for both the cellular and viral genome. In previous studies on chromosomal locations of the HPV-16 genome in cell lines and carcinomas, integration was found mostly in chromosomes 1, 2, 8, 9, 3, 12, 13, and 20.[19–22] Selected integrations have been described, by other authors, in common fragile sites,[23 24] or in interspersed repetitive sequences of DNA.[25] Nevertheless, it has not been possible to demonstrate a specific and preferred integration site in the human genome. From the viral perspective, the integration produces, first of all, a small deletion of DNA, rarely being more than three kilobases. The E2 open reading frame (ORF) has been identified as the preferential site of integration because it has been found to be disrupted or deleted more frequently than other sites.[26–30] Therefore, the disruption of E2 dependent negative feedback controlling E6 and E7 transcription is considered a selective event in tumour development and progression.[22 28 31 32] Furthermore, several observations have suggested that other E2 functions are necessary to mediate cellular growth arrest. In a recent study,[18] the reintroduction of the E2 protein into an HPV-16 transformed cervical carcinoma cell line resulted in a decrease in growth rate and cell death via apoptosis, by increasing the concentration of free E2F. The same effect was previously described in serum starved cells.[18 33] These and other data suggest the crucial role of inactivation of the E2 gene by integration and explain why E2 damage is associated with poor prognosis and significantly shorter disease free survival for the patient.[34–37]

Abbreviations: HPV, human papillomavirus; HSIL, high grade squamous intraepithelial lesion; LSIL, low grade squamous intraepithelial lesion; ORF, open reading frame; PCR, polymerase chain reaction; SCC, saline sodium citrate; SDS, sodium dodecyl sulfate

Although some authors have shown the coexistence of episomal and integrated forms in cervical cancer,[38–40] according to most data in the literature, viral DNA is usually integrated into the cellular genome in lines derived from cervical carcinomas, in addition to HSILs and invasive cervical carcinomas.[6 22 40] Therefore, the phenomenon of integration, leading to progression of dysplasia into carcinoma, is considered to be an important mechanism for tumour progression in the cervix.

In inflammatory states and LSILs, the virus is usually detected as an episomal form.[6 21 41] Nevertheless, we focused on LSIL samples because in these early lesions the frequency of integration is still a matter of conjecture.

Several methods have been used to detect integration, such as Southern blot analysis, two dimensional gel electrophoresis, amplification by the polymerase chain reaction (PCR) of the E2/E1 region of the virus, and the ANCHOR PCR, although some of them are relatively insensitive and the results are not always clear. Furthermore, most of these methods require large amounts of DNA and are still too complicated and time consuming for use in daily clinical practice.

We initially tested the samples for the E6 gene by PCR to identify the HPV-16 type virus. The specificity of the bands, obtained from the first PCR, was confirmed by Southern blot hybridisation with internal oligonucleotide probes. Then, a PCR screen for the E2 gene was performed to identify those samples in which the virus was integrated.

MATERIALS AND METHODS

Clinical samples

Residual material from ThinPrep® samples of 92 LSIL/HPV positive cases was obtained from Thomas Jefferson University Hospital, USA. The diagnosis of LSIL and the presence of HPV were determined after cytological evaluation only, and not confirmed by biopsy because most LSILs are not referred to colposcopy for biopsy. The study population was chosen among young women between 20 and 35 years old with no previous abnormal cytological test. The samples were selected consecutively.

Cytological diagnosis

The samples were prepared for liquid based cytology with the ThinPrep technique (Cytyc Corporation, Marlborough, Massachusetts, USA) and 4 ml of the sample was used to process the slides for cytomorphological evaluation. Samples were classified according to the Bethesda system for reporting cervical/vaginal cytological diagnoses.[42] The cytotechnicians and the pathologists involved in our study were not informed about the results of the HPV testing because this was a retrospective study.

HPV-16 testing

The material remaining from the morphological evaluation was used for HPV molecular analysis.

DNA extraction

The cells were centrifuged at 60 000 ×*g* for 30 minutes, resuspended, and digested in 200 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for one hour at 37°C. The DNA was extracted with phenol/chloroform, precipitated with ethanol, then dissolved in water and used as a template in PCR reactions.

PCR amplification

Ninety two samples were tested for the presence of HPV-16 by PCR amplification of the E6 gene. The primers used were: 5′-AAGGGCGTAACCGAAATCGGT-3′ and 5′-CATATACCTCACG TCGCAG-3′.[43] To detect integration, the DNA from all the 13 samples that had tested positive for the E6 gene at PCR and Southern blot analysis was tested by amplifying the E2 ORF of HPV-16, using specific primers 5′-CTTGGGCACCGAAG AAACAC-3′ and 5′-TTGGTCACGTTGCCATTCAC-3′. The reaction volume was 50 µl and PCR was carried out in the presence of 2.5mM $MgCl_2$ and 0.5 µM of each primer. All the other reagents were used according to the suggestions of the manufacturer. After five minutes at 95°C to denature the DNA, 35 cycles were performed. Each cycle consisted of: denaturation at 95°C for one minute, annealing for one minute, and extension at 72°C for one minute. The annealing steps were performed at temperatures of 58°C for the E2 gene and 60°C for the E6 gene. Samples amplified in the absence of template DNA served as a negative control. SiHa cells were used as a positive control.

Southern blot hybridisation

Southern blot hybridisation was performed on all of the 92 samples to confirm the PCR data for the E6 gene. Aliquots (20 µl) of the E6 gene PCR product were electrophoresed on a 2% agarose gel and transferred overnight in 0.5N NaOH to a positively charged nitrocellulose membrane (Hybond N+; Amersham, Arlington, Illinois, USA). Filter immobilised DNA was prehybridised for two hours at 52°C in a solution containing 5× sodium saline phosphate/EDTA (0.15M NaCl, 0.01M sodium phosphate, 0.001M EDTA, pH 7.7), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml of fresh denatured sheared salmon sperm DNA, and hybridised for 12 hours with a ^{32}P end labelled oligonucleotide, corresponding to the region from nucleotide 136 to nucleotide 161 of the HPV genome sequence, using 1×10^6 counts/minute/ml. The oligonucleotide was labelled with T4 polynucleotide kinase (Promega Corporation, Madison, Wisconsin, USA), according to the manufacturer's instructions. After hybridisation, the membrane was washed twice in 2× saline sodium citrate (SSC), 0.1% SDS at room temperature for 10 minutes, washed in 0.5× SSC, 0.1% SDS three times at 52°C for 15 minutes, and exposed to *x* ray.

RESULTS

Ninety two samples cytomorphologically diagnosed as LSIL/HPV positive were analysed. The E6 gene was detected in 20 samples by PCR, which is in accordance with the data concerning the proportion of high risk HPVs found in LSIL.[44] Southern blot hybridisation was performed on the same samples and 13 of 92 were confirmed as positive (fig 1).

We analysed these 13 samples for integration and in seven we could not detect the E2 gene PCR product, suggesting that the virus was integrated into the cellular genome of these HPV-16 positive cases. Figure 2 shows the E2 PCR products of some of the 13 samples that were positive by both PCR and Southern blot for the E6 gene, and hence thought to be HPV-16 positive.

We followed the patients in whom the virus was found to be integrated for two years by cytological evaluation. Two of them had negative cytology, three had persistent HPV infection, and the remaining two showed moderate dysplasia confirmed by histology. The data are summarised in fig 3.

The use of Southern blot analysis has several advantages: it allows confirmation of the type of HPV identified, which was type 16 in those samples in which we found more than one PCR product; it decreases the risk of false negatives because it can detect small amounts of DNA, which are often undetectable by ethidium bromide staining (fig 1; lane 6); and moreover, this method reduces the risk of obtaining false positives because it makes it possible to discriminate small differences in size between two PCR products (fig 1; lane 9). For example, in the primary screening, 20 of the 92 samples were found to be HPV-16 positive, whereas Southern blot analysis allowed us to determine that only 13 of these samples were really positive.

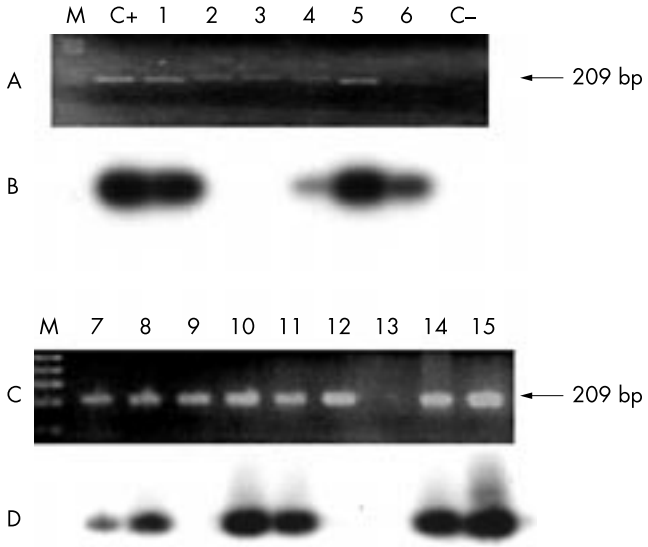

Figure 1 Identification of human papillomavirus 16 (HPV-16) in low grade squamous intraepithelial lesions. Ninety two samples were tested for the presence of HPV-16 by polymerase chain reaction (PCR) amplification of the E6 gene. (A,C) Some of the samples positive for E6 are shown (lanes 1–15). C+ and C– correspond to the positive and negative controls, respectively. Arrows on the right show the expected molecular weight of the E6 gene fragment (209 bp). (B,D) The E6 gene PCR product (20 µl) was electrophoresed on a 2% agarose gel and hybridised using a single strand oligonucleotide corresponding to the region of the HPV genome spanning nucleotides 136 to 161.

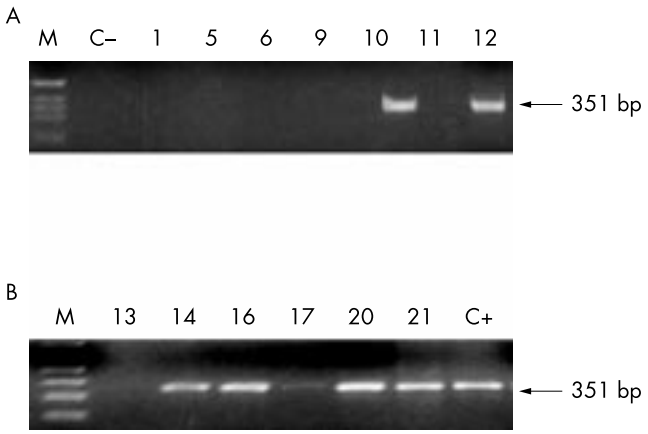

Figure 2 Study of viral integration. For the detection of integration, the E2 open reading frame of HPV-16 was amplified. Examples of viral genomic integration (lanes 1, 5, 6, 9, 11, and 13) and of viral episomal forms (lanes 10, 12, 14, 16, 17, 20, and 21) are shown. Arrows on the right show the expected molecular weight of the E2 gene fragment (351 bp).

Phase 1: Identification of HPV type 16

Method: PCR for HPV16 E6 gene			
Presence of E6 gene PCR product	No.	Total no.	%
	20	92	21.7

Phase 2: Control of the E6 gene PCR product specificity

Method: Southern blot hybridisation for E6 gene PCR product			
Presence of hybridisation	No.	Total no.	%
	13	92	14.1

Phase 3: Assessment of viral integration

Method: PCR for HPV16 E6 gene			
Absence of E2 gene PCR product: integration	No.	Total no.	%
	7	13	53.8

Phase 4: 2 years' follow up of the 7 patients in which the virus was found to be integrated

No.	Clinical outcome	Method of diagnosis
2	Negative	Cytology
3	Persisting HPV	Cytology
2	Moderate dysplasia	Cytology confirmed by histology
Total no. 7		

Figure 3 Diagnostic method of identification of human papillomavirus 16 (HPV-16) and the detection of viral integration. The figure shows, in a temporal sequence, the steps followed in HPV-16 identification and in the detection of viral integration, ending with the follow up. Results of each phase of the study (numbered from 1 to 4) are shown below. In phase 1, HPV-16 was detected by polymerase chain reaction (PCR) for the E6 gene and 20 of the 92 cases were positive. In phase 2, Southern blot hybridisation was performed on the E6 gene PCR product as a control. Thirteen of the 92 cases were confirmed to be HPV-16 positive. Phase 3 was the study of viral integration among the HPV-16 positive cases. The absence of E2 gene PCR product implies viral integration and this was found in seven of the 13. In the last phase, the clinical outcome is summarised (after two years of follow up) for those patients in whom the virus had been integrated. In five of these seven patients, HPV infection or moderate dysplasia persisted during the two following years.

DISCUSSION

It is now accepted that the integration of high risk HPVs into the host cell genome is one of the major contributing factors to genital malignant transformation. To provide a better understanding of this complex phenomenon, it would be interesting to establish temporal relations in HPV induced carcinogenesis. Most authors agree with the hypothesis that the integration of the HPV genome takes place very early in the development of cancer. The question is: how early? Considering viral integration as the key point in cervical carcinogenesis, we wanted to investigate the physical status of HPV-16 in LSILs.

We focused on type 16 HPV because its behaviour seems to differ from that of other high risk papilloma viruses. As shown in a recent study,[40] cervical carcinoma reveals differences in the integration profile depending on the virus type. For HPV-18, HPV-31, and HPV-35, the viral genome is always present in the integrated form, whereas for HPV-16 the episomal and integrated forms coexist even in cancer,[22 38–41 45] and the concordance between integration and carcinogenesis is less evident. Moreover, we were interested in LSIL because the correlation between cytological and histological findings is often poor in the diagnosis of LSIL. The literature reports a rate of cytological overdiagnosis of 15% and underdiagnosis of between 28% and 62%.[46–49] In addition, there is currently no consensus as to the appropriate management of women with this kind of lesion; opinions include immediate colposcopy and directed biopsy, as with cytological HSILs, follow up with repeat cytology every four to six months and colposcopy (indicated only if an abnormality persists), or triage using DNA testing for cancer associated HPV types.[50 51] Therefore, we chose to investigate the frequency of integration of a high risk HPV (HPV-16) in LSIL.

We found that HPV-16 was integrated in more than half of the cases that were HPV-16 positive. These results contrast with most of the studies in the literature[41 45] that have looked

Take home messages

- Viral integration was detected in just over half of the samples positive for human papillomavirus type 16 (HPV-16)
- The measurement of HPV-16 integration would be a helpful complementary tool for cytological evaluation in primary cervical screening to identify those patients at risk of developing high grade squamous intraepithelial lesions and cervical cancer

at inflammatory states and LSIL, in which the virus is usually detected in its episomal form, as a 8 kb circular molecule. We agree that viral integration is a very early event, as already postulated by most authors, but our results show that it occurs earlier than the onset of morphological changes, which could indicate a high grade lesion. In attempts to explain our data, we suggest that molecular events precede morphological features leading to malignancy, and that integration does not always temporally coincide with a high grade lesion. It is also possible that viral integration is not necessarily always followed by immediate viral oncoprotein expression. Nevertheless, our data concerning the persistence of HPV or LSIL after two years of follow up in five of seven patients carrying the integrated virus lead us to suppose that integration represents a point of "no return" in the natural history of the lesion.

> "We agree that viral integration is a very early event, as already postulated by most authors, but our results show that it occurs earlier than the onset of morphological changes, which could indicate a high grade lesion"

Several techniques have been described to detect the physical status of HPV-16, such as Southern blot analysis, two dimensional gel electrophoresis, amplification by PCR of the E2/E1 region of the virus, and ANCHOR PCR. However, the interpretation of the results remains difficult. In the procedure we describe, we initially tested the samples for the E6 gene by PCR to identify the HPV-16 type virus. The specificity of the bands obtained from the first PCR was confirmed by Southern blot hybridisation with internal oligonucleotide probes on all of the samples. Then a PCR screen for the E2 gene was performed to identify those samples that were HPV-16 positive in which the virus is integrated. This method has several advantages: it allows the detection of integration using a small amount of DNA (0.3–1.0 mg); the interpretation of the results is easier than with other techniques because it is based on the presence or absence of PCR products; furthermore, the results obtained by the PCR technique are confirmed by Southern blot hybridisation analysis.

These days, cytomorphological evaluation alone is not thought to be sufficient for grading cervical dysplasia, and these results need to be supported by molecular HPV testing. In this regard, liquid based cytology has the double advantage of having a sensitivity significantly higher than that of conventional cytology (87.8% *v* 68.1%; $p < 0.05$)[52–55]; moreover, it allows us to combine the morphological examination with molecular HPV testing.

Very recently, most authors have focused their attention on the issue of whether HPV testing might be of value in primary screening or in the assessment of defined patient groups with borderline changes and mild dyskaryosis.[46 56] For example, the combination of liquid based cytology and HPV testing enhances sensitivity to ensure that all patients with dyskaryotic lesions are identified. Nevertheless, sensitivity and specificity are generally inversely related to one another, and the higher sensitivity could lead to an increase in the detection of HPV DNA even in patients without detectable disease. Therefore, if HPV testing only provides us with data about the presence or absence of the virus, it is not as useful as a test that also provides information about the physical status (integration) of the virus in the host cell.

Our strategy appears to be a helpful complementary tool for cytological evaluation because it can reduce unnecessary colposcopy guided biopsies in women with a cytological diagnosis of LSIL and might help identify those patients who are at risk for developing HSIL and cervical cancer. Moreover, we suggest the use of this technique in clinical practice.

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