Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic in situ hybridisation

K Cooper, C S Herrington, J E Stickland, M F Evans, J O'D McGee

Abstract

It was postulated that non-isotopic in situ hybridisation (NISH) signal types 1–3 for human papillomavirus in cervical biopsy specimens represent episomal or integrated virus. The aim of this study was to validate this hypothesis by independent molecular techniques. Fresh cervical intraepithelial neoplasia (CIN) and squamous cell cancer (SCC) tissue were examined for NISH signal pattern by hybridising with digoxigenin labelled HPV 16. DNA was extracted from the same samples and analysed by restriction endonuclease digestion and Southern blotting to determine the physical state of the viral genome. Six CIN biopsy specimens showed a type 1 NISH signal for HPV 16. On Southern analysis these biopsy specimens contained only episomal HPV 16. Three SCC with a type 2 NISH signal contained integrated HPV 16 by Southern analysis. Two specimens, a CIN 3 and an SCC with a type 3 NISH signal for HPV 16, showed the presence of both episomal and integrated HPV 16 with conventional Southern analysis and two dimensional gel electrophoreses.

These results show that episomal HPV can be reliably determined by NISH type 1 signal, integrated HPV by type 2, and a combination of both episomal and integrated HPV, by a type 3 signal in archival paraffin wax embedded cervical biopsy specimens. This will add another variable to the epidemiological studies of HPV infection. In particular, it will now allow retrospective studies to be done to define the role of episomal and integrated HPV in the evolution of cervical intraepithelial neoplasia and other cervical disease associated with this virus.

Certain human papillomavirus (HPV) types, particularly HPV 16 and 18, and to a lesser extent 31, 33, 35, 45, 51, and 52, have been associated with cervical intraepithelial neoplasia and invasive cancer. Although epidemiological studies have produced conflicting results, there is experimental evidence to support a pathogenetic role for HPV in cervical neoplasia and cancer. The part played by integration of HPV DNA into the human genome has attracted increasing interest. HPV DNA is integrated into the host genome in immortalised human keratinocytes, cell lines derived from cervical cancers, cervical intraepithelial neoplasia (CIN), and squamous cell cancers (SCC). Integration disrupts the E1/E2 open reading frames with consequent derepression of viral gene expression and subsequent overexpression of the E6 and E7 ORFs. Clinical studies that correlate E6 and E7 overexpression with poor prognosis in CIN lend support to the potential importance of detection of HPV integration in cervical neoplasia.

Viral integration can be shown in several ways in clinical material. All require destruction of tissue morphology, however, and are technically time consuming; restriction digestion and Southern blot analysis provide indirect evidence, while the cloning of virus-host cell junction fragments is definitive. Direct morphological evidence of viral integration is provided by in situ hybridisation on metaphase chromosomes derived from cervical carcinoma cells but this requires cell culture. None of these methods is routinely applicable to small clinical biopsy specimens. Recently it was shown that HPV DNA is present in three morphologically distinct forms in the nuclei of cervical preneoplasia and neoplasia by non-isotopic in situ hybridisation (NISH). These forms are referred to as NISH signals types 1, 2, and 3. A type 1 signal is diffuse and present throughout the nucleus; a type 2 signal is punctate; and type 3 is a combination of type 1 and 2. It was postulated that a type 1 signal represents episomal virus, type 2 integrated virus, and type 3 a combination of both. To examine this hypothesis further, we have now examined fresh CIN and SCC tissue for an NISH signal pattern, extracted DNA from the same biopsy specimens and analysed them by Southern blotting to determine the physical state of the viral genome.

Methods

Cervical biopsy specimens came from patients with HPV infection, CIN, or SCC, as defined by conventional morphological criteria. Samples were snap frozen in liquid nitrogen and stored at −70°C until used. The CaSki, HeLa, and SiHa cell lines (ATCC, USA) derived from cervical carcinoma were grown and prepared as previously described.

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Frozen sections (5 μm) were cut from biopsy specimens and treated as for cultured cell lines by fixation in methanol-acetic acid (3:1 v/v), 4% (w/v) paraformaldehyde, and washed in phosphate buffered saline (PBS) (10 mM phosphate, 150 mM NaCl, pH 7.2) containing 0.2% glycine. Slides were washed in PBS, air dried, and stored at −70°C. One section was stained with haematoxylin and eosin, and the remainder were analysed by NISH.

NISH

After unmasking with proteinase K (Boehringer-Mannheim, Germany) at concentrations ranging from 0.1 to 1 mg/ml (depending on the batch of enzyme and the sample) for 15 minutes at 37°C, NISH was carried out as previously described using nick-translated digoxigenin labelled HPV 16 probes and a three step antibody-avidin detection method with aminoethylcarbazole as the final substrate. An HPV 16 positive control biopsy specimen was included in each experiment and six other digoxigenin labelled HPV probes (HPV 6, 11, 18, 31, 33, 35) were used on each biopsy specimen as negative controls.

DNA EXTRACTION

From a set of 53 biopsy specimens, 11 containing HPV 16 detected by NISH were selected. DNA was extracted and analysed by restriction digestion and Southern blotting. HeLa cell DNA (which contains integrated HPV 18) and breast tumour DNA were used as negative controls. CaSkI and SiHa cells contain integrated HPV 16 in 300–500 and one to two copies, respectively, and were used as positive controls.

DNA was extracted by incubation in 10 mmol/l TRIS-HCl, pH 7.8, 150 mmol/l NaCl, 10 mmol/l EDTA, 0.6% (w/v) sodium dodecyl sulphate (SDS), and 100 μg/ml proteinase K (Boehringer-Mannheim, Germany) at 37°C overnight; it was then extracted once with phenol, twice with phenol-chloroform, and twice with chloroform-isooamy alcohol (24:1). DNA was precipitated with ethanol and resuspended in TRIS-EDTA buffer (10 mmol/l TRIS-HCl, pH 8, 1 mmol/l EDTA). The yield of DNA ranged from 60 to 500 μg per biopsy specimen.

SOUTHERN BLOT ANALYSIS

This was performed according to standard techniques. Genomic DNA (10 μg) was digested with three restriction endonucleases: BamHI, which cuts wild-type HPV 16 once at position 6150 in the conserved late region, and HindIII and Xhol, which cut tumour DNA but not HPV 16. After electrophoresis on 0.8% (w/v) agarose gels the digested DNA was transferred to Hybond N (Amersharn) nylon membrane and cross linked with ultraviolet light using a Stratalinker (Stratagene).

HPV 16 genomic DNA was excised from the vector pAT 153 and isolated on DEAE NA-45 paper. The HPV 16 insert was labelled with 32P-dCTP by random primer directed synthesis according to the manufacturer’s instructions (Boehringer-Mannheim, Germany) and residual unincorporated nucleotides removed on a Sephadex G-50 column. A specific activity of 1.5 x 106 dpm/μg DNA was typically obtained.

Southern blots were prehybridised for two hours at 42°C in prehybridisation solution containing 50% deionised formamide, 1 x Denhardt’s solution (100 x Denhardt’s solution = 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone), 5 x SSPE (20 x SSPE = 3.6 M NaCl, 0.2 M sodium phosphate, 0.2 M EDTA, pH 7.5), 1% (w/v) sodium dodecyl sulphate (SDS) and 0.2 mg/ml of denatured sheared herring sperm DNA. Filters were hybridised overnight at 42°C in prehybridised solution to which 5% (w/v) dextran sulphate and denatured labelled probe (1–2 x 106 cpml/ml) were added. After hybridisation, washing was carried out in two changes of 2 x SSPE containing 0.2% SDS for five minutes at room temperature followed by one of 0.2 x SSPE/0.2% SDS at 65°C for 20 minutes and signal detected by autoradiography for three to 12 days at −70°C using Fuji RX film. These conditions represent calculated hybridisation and washing stringencies of Tm-20°C and Tm-10°C, respectively. HindIII digested lambda phage was used as a molecular marker in all electrophoretic agarose gels.

Two cases in which complex restriction patterns were obtained were reanalysed by two-dimensional gel electrophoresis to clarify the physical states of the HPV DNA. HindIII digested DNA (10 μg) was run on a 0.4% agarose gel at 25 V for 14 hours. The lane was then excised and recast with a 0.8% electrophoretic agarose gel and run at 90° to the original direction for five to six hours at 60 V.

Results

From 53 fresh frozen biopsy specimens, 11 were selected for further study. The criteria for inclusion were that on frozen sections a type 1, 2, or 3 NISH signal was evident and that there was sufficient tissue for DNA extraction and characterisation by restriction enzyme digestion and Southern blotting.

Four biopsy specimens showing morphological features of CIN 2 or 3 with coincident wart virus infection and two with wart virus infection changes only gave a type 1 diffuse NISH signal pattern (table) (fig 1A). Three SCC gave a punctuate/dot type 2 NISH
signal in most of the tumour cells, with about one to three signals/cell⁹ (fig 1B). A type 3 signal was present in a CIN 3 lesion showing coincident morphological evidence of wart virus infection (fig 1C) and in an SCC where most of the tumour cells had a type 2 signal and isolated scattered cells a type 1 signal¹⁹ ¹⁰(fig 1D).

A type 2 NISH signal was obtained by NISH using HPV 16 in CaSki cells (three to 20 dots per cell)²² and SiHa cells (one to two dots per cell)²⁹ Both these cell lines contain integrated HPV 16²³-²⁶ and were used as positive controls for Southern blot analysis. HeLa cells contain integrated HPV 18³³ and gave no signal with the HPV 16 probe. None of the biopsy specimens studied here reacted with HPV 6, 11, 18, 31, 33 or 35 on NISH.

NISH TYPE 1 SIGNAL AND EPISOMAL HPV
The methods used for determining the physical state of HPV DNA in fresh frozen cervical material were adapted from those described by Durst.³⁸ The detection of episomal viral DNA is based on the fact that the HPV 16 genome is a double stranded, circular DNA molecule 7-9 kilobases long. This form of the virus (Form 1) is supercoiled and has the greatest electrophoretic mobility in agarose gels with an apparent molecular weight of about 4-4 kilobases. After DNA extraction a nick may be introduced into one of the supercoiled strands. This produces the nicked/relaxed form (Form 2; "open" circular), which has a lower electrophoretic mobility than Form 1. Digestion with a restriction endonuclease that cleaves the viral genome once—for example BamHI, generates the linearised form of the viral DNA (Form 3); this produces a band at a position of 7-9 kilobases on agarose gels with an electrophoretic mobility intermediate between forms 1 and 2.²⁸

Using these principles, the episomal state of HPV DNA was clearly shown in cases 1, 2, 5, 8, 10 and 13 (table) all of which showed type 1 NISH signal (fig 1A). The episomal bands noted on the HindIII and XhoI digested DNA lanes are identical with those seen with the uncut genomic DNA because these enzymes do not cut the HPV 16 genome. After digestion with BamHI (which cleaves HPV 16 only once), both forms 1 and 2 of the episomal viral DNA are linearised to 7-9 kilobases; the absence of additional off-sized bands in this lane excludes the possibility of integrated viral genome in these biopsy specimens (fig 2).

The high molecular weight bands in cases 5 and 13 most likely represent oligomeric circular DNA arranged as catenates. The distinct linearisation of this form to 7-9 kilobases with BamHI digestion in all six cases in this study, as well as the absence of noticeably different band sizes after HindIII and XhoI digestion, confirms that these biopsy specimens with a type 1 NISH signal contain only episomal HPV 16. The 7-9 kilobase bands in the uncut, HindIII and XhoI digested lanes is probably as result of linearisation of circular HPV 16 following DNA extraction.

NISH TYPE 2 SIGNAL AND INTEGRATED HPV
This was shown by the changed migration, when compared with the undigested sample, of extracted DNA after digestion with two different restriction endonucleases which have no cleavage sites in the HPV 16 genome. HPV
enzymes have episomal viral particles. (single and type labelled HPV 16 DNA. Both cases showed histological features of integration by exposure* (see results).

HindIII of Harbour only integrated virus; enzymes) undigested (U) DNA 23.1-9.4- 9.4-6.6 6.6-23.1- '*, 9.4- of autoradiographic NISH lane bands in each) digested with XhoI and NISH junction fragments. the X and H lanes also HPV 16 exposure) CASE 135 of HPV 16 genome). Southern specific signal is present for days 1-2 7 9 and human DNA extracted from a breast cancer, did not hybridise with the HPV 16 probe (data not shown).

NISH TYPE 3 SIGNAL AND EPISOMAL AND INTEGRATED HPV Cases 12 and 121 both showed a type 3 NISH signal (figs 1C and D). On Southern analysis of case 12 (fig 5), episomal HPV 16 bands were present in all three lanes of HindIII, XhoI, and digested DNA. BamHI linearised most of the HPV DNA to the 7-9 kilobase band, suggesting the presence of episomal viral DNA. An off-sized fragment of about 23-1 kilobases was also present, suggesting the presence of virus-host cell junction fragments and implying HPV integration as well. A similar situation existed in case 121 (fig 5) with episomal bands in the HindIII, XhoI, and uncut lanes in addition to bands at and smaller than 4-4 kilobases after BamHI digestion. This suggested HPV integration as well as the presence of episomal viral DNA. In this case, however, the bulk of HPV DNA did not linearise to 7-9 kilobases. This may be due to the presence of HPV multimers containing deletions or duplications with resultant unpredictable off-sized fragments or an HPV variant in which the episomal form lacks the BamHI restriction site. Alternatively, they may represent integrated sequences.

As cases 12 and 121 gave an episomal and integrated pattern by Southern blot analysis, 2-dimensional gel electrophoresis (2-D gel) was used to separate episomal and integrated sequences further. The integrated HPV 16 fragments co-migrate with the curved pathway of cellular DNA, which was marked out on the gel with ultraviolet illumination before Southern blotting (data not shown). The circular episomal molecules in both cases 12 and 121 remain closer to the lane of the first dimension due to their slower electrophoretic mobility.
Discussion

HPV DNA is found in human epithelia in two forms, either individually or in combination: episomal (extrachromosomal) particles or integrated into the human genome. The integration of HPV 16 DNA has been shown directly on metaphase chromosomes from cervical carcinoma derived cell lines by in situ hybridisation and by cloning viral-cellular junction fragments from clinical cervical cancer. Southern analysis has led to the same conclusions in some cases of CIN, SCC, and cell lines derived from cervical cancers. This study explores the hypothesis that the NISH signal type 1 is indicative of the physical state of the HPV genome in clinical samples. Using cryostat sections of frozen cervical tissue, NISH signals types 1 (diffuse throughout the nucleus), type 2 (punctuate/dot), and type 3 (combined 1 and 2) were shown in CIN (types 1 and 3) and SCC (types 2 and 3). The same cervical biopsy specimens were analysed by Southern blotting to investigate the physical state of the virus and to determine, by using another parameter, whether a type 1 signal represents episomal virus, type 2 signal represents integrated virus, and a type 3 signal represents the coexistence of both physical forms in the same lesion.

The presence of episomal HPV 16 was shown in six cases by the presence of forms 1–3 which linearise to 7-9 kilobases with BamHI. The absence of additional off-sized bands in these biopsy specimens confirms that no integrated viral sequences was present in these lesions. Although small amounts of integrated viral DNA could, theoretically, be masked by the large excess of episomal sequences, this would only occur if the bands generated from the integrated sequences co-migrated with linear HPV 16. This is unlikely to occur other than by chance. The presence of high molecular weight bands in all six cases containing episomal virus probably represent oligomeric circular DNA arranged as catenates. This is not an unusual feature and has been noted in 72% of cases in a previous study. Therefore, an episomal pattern on Southern analysis correlates with NISH type 1 signal.

Integrated HPV was demonstrated in two ways: firstly, an altered migration of HPV 16 signal after digestion with two restriction enzymes that cut human DNA but not HPV 16 DNA, and secondly the presence of off-sized bands in BamHI digested DNA. As BamHI cuts HPV 16 DNA once it indicates the presence of integrated HPV DNA. This integrated DNA, bands of size other than 7-9 kilobases are produced by digestion with this enzyme if integration has occurred at a site other than the BamHI restriction site. As viral breaks occur consistently in the E1/E2 open reading frames which do not contain the BamHI site, integration will almost invariably lead to the production of off-sized virus-cell junction fragments. The presence of a 7-9 kilobases band after BamHI digestion can be explained by the presence of integrated HPV 16 in the form of head-tail tandem repeats which has been demonstrated in CaSki cells. This pattern correlates with a NISH type 2

compared with integrated DNA when the gel concentration is increased. The linear (7-9 kilobases) HPV 16 DNA which co-migrated with the cellular DNA is either integrated HPV DNA or more likely episomal viral particles linearised during the process of DNA extraction. This confirmed the presence of both episomal and integrated virus in cases 12 and 121 (fig 6) which showed type 1 and 2 NISH signals. The clinical diagnosis, NISH signal, and Southern blot patterns for each case studied are summarised in the table.

Figure 4 Southern blot analysis of DNA from two cell lines derived from cervical cancer containing integrated HPV 16. These restriction patterns served as positive controls and are similar to those described in fig 3.

Figure 5 Southern blot analysis of DNA from case 12 (CIN3/HPV) and case 121 (squamous cell cancer), both of which contained an NISH signal type 3. Each lane comprised 10 μg DNA: undigested (U), HindIII (H), Xho1 (X) (both these enzymes do not cut HPV 16) and BamHI (B) (cuts HPV 16 once). Most of the bands in the uncut lane of case 12 linearise to 7-9 kilobases in the BamHI lane indicating predominantly episomal virus in this biopsy specimen. An off-sized band in this lane indicates the presence of integrated virus in addition, Case 121 is more complex in that two off-sized bands indicative of viral-cell junction fragments are present in addition to the 7-9 kilobase band after BamHI digestion. The smear in the uncut lane is also characteristic of integrated HPV DNA, which migrates with the bulk of the undigested genomic DNA.
signal both in biopsy specimens and positive control cell lines. In the latter, integration has been established by both molecular means and in situ hybridisation.\(^6\)\(^7\)\(^17\)

The presence of both integrated and episomal virus would, theoretically, result in a mixture of the two Southern blot patterns referred to above. Distinction by Southern analysis is complicated, however, by the relative amounts of episomal and integrated HPV in a given lesion, with one pattern tending to predominate. This difficulty was overcome by two-dimensional gel electrophoresis which showed the presence of both episomal and integrated virus in two cases in which a type 3 NISH signal was obtained. A dominant type 2 signal was obtained by NISH in case 121, with only occasional nuclei containing a diffuse signal. This correlates with the predominance of the integrated pattern over episomal bands by Southern analysis. In this case, however, the complex bands did not linearise to 7-9 kilobases. The presence of off-sized fragments with BamH1 digestion and co-migration with human DNA on the two-dimensional gel confirms the presence of integrated virus. Episomal virus is similarly visible on this gel. The converse was true of case 12, with a dominant diffuse signal by NISH and only occasional cells showing a punctuate signal. Again, this correlated with the signal intensity on both conventional and two-dimensional gels. The NISH type 3 signal therefore correlates with the presence of both episomal and integrated viral sequences. Southern analysis, however, cannot determine whether these two physical states of HPV coexist within the same cell or adjacent cells.\(^9\) This distinction is clearly made by NISH analysis (figs 1C and D).

These results are further evidence in favour of the hypothesis that a punctuate NISH signal represents viral integration and permits further interpretation of previous data.\(^18\) In these studies all CIN2 that contained HPV DNA showed a diffuse type 1 signal. On the other hand, half the archival lesions defined morphologically as CIN3 with morphological evidence of HPV from patients from Britain and South Africa contained either a type 2 or type 3 NISH signal.\(^19\) This indicates that these cervical biopsy specimens contained integrated virus without or with episomal forms, respectively. Similar conclusions have been arrived at by others using different methodologies. HPV DNA integration has been observed in CIN lesions\(^6\)\(^12\) and has even been shown in mild cervical dysplasia.\(^30\) In one study 86% of HPV positive CIN lesions and 100% of HPV positive carcinoma in situ (CIS) and SCC contained HPV 16 in an integrated state.\(^36\) The co-existence of integrated and episomal forms of HPV has also been previously shown in CIN and SCC\(^13\) and in cell lines derived from cervical cancers.\(^6\)\(^7\) Type 3 NISH signal pattern shows that these two physical states of HPV DNA may coexist in the same cell or in adjacent cells in the same lesion.

In another study we have shown a type 2 or 3 signal in 100% of HPV DNA positive SCC, indicating the integration of HPV in these tumours.\(^19\) A type 2 or 3 signal was also obtained with HPV 16, 18, and 33 in lesions from two different geographical regions, South Africa and Britain, implying integration of these three HPV types in SCC.

There is therefore a pattern regarding the physical state of the HPV DNA in CIN and SCC; that of episomes predominating in the early stages of CIN, with integrated virus being detected more frequently in HPV positive high grade CIN and SCC. The latter may or may not contain episomes as well. This idea is not new. It has been proposed that the physical state of HPV DNA may reflect a progression towards the malignant phenotype\(^32\) and that higher grade CIN lesions may originate from a single cell clone in which viral integration had occurred.\(^9\)\(^36\) Therefore apart from the fact that lesion grade and HPV type—that is, 16 and 18—are established risk factors in clinical progression\(^37\)\(^38\) the physical state of HPV DNA may also be relevant. The ability to determine the presence of integration in archival biopsy specimen by NISH will allow the importance of the physical state of HPV DNA and its role in progression or regression to be investigated further. This question could be addressed with retrospective or prospective studies where it has been shown that 56% of woman with HPV infection regressed spontaneously, 30% persisted, and a substantial proportion (14%) progressed (some to CIS).\(^39\)

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