Detection of human papillomavirus in matched cervical smears and biopsy specimens by non-isotopic in situ hybridisation

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Abstract

Aims: To determine the relative diagnostic sensitivity of non-isotopic in situ hybridisation (NISH) for the diagnosis of human papillomavirus (HPV) on matched smears and biopsy specimens; to compare the NISH signal type in the two samples; and to correlate the NISH data with the morphological diagnosis.

Methods: HPV samples were assayed individually by NISH with digoxigenin labelled probes (HPV6, 11, 16, 18, and 33) on routinely collected paraffin wax embedded cervical biopsy specimens and for high risk HPVs with a cocktail of similarly labelled probes (HPV16, 18, 33) on matched smears. These were taken at the same colposcopic examination from 32 patients investigated for an abnormal cervical Papinacolau (PAP) stained smear.

Results: An HPV signal was present in 18 (56%) biopsy specimens and in 14 (44%) smears. There was higher concordance of sets of data in the presence of cytopathic wart virus changes. The superiority of biopsy over smear in detecting HPV was mainly the result of examining the entire cervical biopsy specimen rather than cells scraped from the cervical surface. The NISH signal type in both biopsy specimen and smear was similar; it has been shown that NISH type 1 signal correlates with episomal viral replication and type 2 and 3 signals with viral integration.

Conclusions: These data show that NISH on cervical smears is a worthwhile primary screen for HPV infection. The NISH signal types in cervical smears are similar to those previously described in cervical biopsy specimens.

cultured cells and surgical biopsy specimens was conservatively estimated at 2.5 to 12 copies of HPV per cell,¹⁰ but we have recently shown that one to two copies of HPV16 can be detected in SiHa cells with morphological precision.¹¹ Theoretically, therefore, NISH may be suitable for HPV diagnosis in large scale cervical screening programmes. The relative diagnostic sensitivity of the new NISH methodology^{10,11} has not been investigated on matched smears and biopsy specimens from the same patients.

One of the critical steps in the genesis of cervical cancer may be integration of "high risk" HPVs into the human genome; this hypothesis is based on classic molecular biological approaches.¹² The morphological counterpart of HPV episomal replication or integration may be reflected in the pattern of the HPV NISH signal in cervical dysplasia and cancer.^{13,14} Essentially, HPV gives three signal types on NISH. A type 1 NISH signal for HPV is present diffusely throughout the nucleus. A type 2 signal, represented by discrete dots of nuclear HPV, is associated with sister chromatids in vivo; and a type 3 signal is a combination of types 1 and 2. It has been suggested that a type 1 signal equates with episomal replication; a type 2 signal with viral integration; and a type 3 signal is the morphological correlate of both episomal and integrated HPV.^{13,14} This hypothesis has recently been supported by the demonstration that NISH signal types correlate with episomal and integrated virus in a combined in situ and filter hybridisation study.¹⁵

The aims of this study were three-fold. First, to establish the relative diagnostic sensitivity of HPV detection by NISH on matched cervical smears and biopsy specimens. Second, to determine whether the NISH signal type in smears and biopsy specimens was similar. Third, to correlate these data with the morphological diagnosis of the material studied. These aims were addressed in matched cervical smears and biopsy specimens from the same patients taken at first colposcopic examination.

Methods

Included in the study were 32 patients investigated at the John Radcliffe Hospital colposcopic clinic between January and April 1990. These patients had been referred for colposcopy because a diagnosis of cervical intraepithelial neoplasia (CIN) had been made

Certain HPVs (16, 18, 31, 33, 35, 39, 41, 42, 45, 52) have been implicated in the genesis of cervical cancer.¹ This has led to investigation of the prevalence of HPV in exfoliated cervico-vaginal cells in clinically normal and abnormal populations.²⁻⁹ In these studies, the cells or DNA, obtained by cervical swab or lavage, were tested for HPV by filter hybridisation,^{2,3,7,8} non-isotopic in situ hybridisation (NISH),⁵ or PCR.^{4,6,9} Of these, only in situ hybridisation localises HPV sequences in cells which can be cytopathologically assessed. The sensitivity of HPV detection by NISH in

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earlier on a routine cervical Papinacolau (PAP) stained smear. At colposcopic examination, two consecutive cervical smears were taken with a spatula from each patient and fixed in ethanol/acetic acid/Carbowax (Cytofix, Cytopath Products, England) in the conventional way. This was immediately followed by colposcopic directed diathermy loop excision of the cervical lesion. One smear was PAP stained and the biopsy specimen processed for routine histopathological diagnosis. NISH was performed on the other smear and serial sections of the matched biopsy specimen. The results obtained on the two NISH samples were compared with each other, and with the cytopathological and histopathological diagnosis in each case. Standard criteria were used for the diagnosis of CIN and wart virus infection on biopsy specimens¹⁶ and smears.¹⁷

NISH ON HISTOPATHOLOGICAL SECTIONS

Sections were tested for HPVs as described previously.¹⁸ Briefly, the sections were mounted on slides coated with 2% aminopropylethoxysilane in acetone, dewaxed, and the target HPV unmasked by digestion with proteinase K (Boehringer Mannheim, Germany) at 500 µg/ml in phosphate buffered saline (PBS = 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 150 mM NaCl, pH 7.4). Sections were hybridised separately with genomic HPV probes 6/11, 16, 18 and 33, and labelled with digoxigenin by nick translation.¹⁹ The hybridisation mix contained 50% (v/v) formamide (Sigma UK), 5% (w/v) dextran sulphate (BDH, UK), 2 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 50 mM TRIS-HCl, pH 7.2, and 5 mM EDTA. Sections and probe in hybridisation mix were covered by glass coverslips and denatured simultaneously at 95°C for 15 minutes and hybridised at 42°C for two hours.¹⁸

NISH ON CERVICAL SMEARS¹¹

Carbowax was removed by washing in freshly prepared methanol/acetic acid (3:1 = v/v) for 10 minutes at 22°C, then smears were fixed in 4% paraformaldehyde (w/v) in PBS, pH 7.4, for 15 minutes at 22°C. The reaction was stopped in PBS containing 0.2% (w/v) glycine, followed by washing in PBS. Endogenous peroxidase activity was blocked in 0.1% (w/v) sodium azide containing 0.3% (v/v) hydrogen peroxide in water for 10 minutes. After washing in PBS, smears were incubated in 1 µg/ml proteinase K in PBS for 15 minutes at 37°C, washed in PBS, postfixed in 4% paraformaldehyde, washed in PBS/glycine, PBS, and air dried at 37°C. A cocktail of HPV16, 18, and 33 probes (2 ng/µl each), labelled with digoxigenin by nick translation,¹⁹ was added to the hybridisation mix; 50 µl of this were added to each smear on a glass slide (25 × 56 mm) and covered with a glass coverslip (22 × 50 mm). Denaturation and hybridisation were carried out as for sections except that the hybridisation mix also contained 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone molecular weight 360 000, 0.2% (w/v) Ficoll

molecular weight 400 000, and 200 ng/µl sheared human DNA.

DETECTION OF NISH SIGNAL ON SMEARS AND SECTIONS

For comparability, the same three-step detection system¹⁰ was used for sections and smears to ensure the same degree of sensitivity on smears and sections. After hybridisation, both samples were washed twice in 4 × SSC and soaked in blocking agent (TBT) containing 50 mM TRIS-HCl (pH 7.2), 100 mM NaCl, 1 mM MgCl₂, 3% (w/v) bovine serum albumin, 0.05% (v/v) Triton × 100. Samples were incubated in antidigoxin monoclonal antibody (Sigma UK) diluted 1 in 10 000 in TBT for 30 minutes, followed by biotinylated rabbit-anti mouse F(ab')₂ fragment (Dako UK) diluted 1 in 200 TBT for 30 minutes. After thorough washing in TBS (50 mM TRIS-HCl, 100 mM NaCl, and 1 mM MgCl₂, pH 7.2), the sections and smears were incubated for 30 minutes in avidin peroxidase conjugate diluted 1 in 75 in TBT containing 5% (w/v) non-fat milk (Marvel, UK). A red NISH signal was developed by incubation in 3-amino-9-ethylcarbazole, H₂O₂ (Zymed, USA) for 10 minutes.¹⁰ The reaction was stopped by washing in water, counterstaining with haematoxylin and mounting in glycerol jelly.¹⁸

Results

Consecutive sections from 32 cervical biopsy specimens were tested by NISH individually for the presence of HPV6/11, 16, 18 and 33 and the results correlated with the histopathological diagnosis (table 1). Signal was detected only in epithelial cell nuclei in 18 (56%) cases. HPV16 was present (figs 1A–3A) in 15 (47%) cases, HPV18 in 2 (6%) cases, and in one case (3%) there was a signal for HPV33. HPV6/11 was not detected in any of these biopsy specimens nor were multiple HPV infections. HPV genomes were detectable in about 60% of high grade dysplasias (CIN II and III) with or without wart virus infection.

Using a 16/18/33 HPV probe cocktail on the 32 corresponding smears, NISH detected viral genomes (Figs 1B–3B) in 14 (44%) cases; the cytological diagnosis differed slightly from the biopsy diagnosis in seven cases (tables 2 and 3). The HPV positivity on smears was 71% for CIN II + wart virus infection, and 38% for CIN III with or without wart virus infection.

RELATIVE DIAGNOSTIC SENSITIVITY

The relative sensitivity of HPV diagnosis by NISH on biopsy specimens and smears in

Table 1 HPV genotyping and biopsy specimen diagnosis

Biopsy diagnostic group	n =	HPV type			
		6/11	16	18	33
CIN1	1	0	0	0	0
CIN2 + wart virus infection	5	0	2	0	1
CIN3	8	0	4	1	0
CIN3 + wart virus infection	18	0	9	1	0

Figure 1(A) HPV 16 in CIN II + wart virus infection (case 2). The cells in the superficial epithelial layers show a diffuse nuclear staining (type 1 NISH signal) (haematoxylin counterstain). **(B)** NISH positive superficial squamous cervical cells in the matched smear from the same biopsy specimen. The cell nuclei show the same NISH signal (haematoxylin counterstain).

individual patients was assessed from data in table 3. Overall, 18 biopsy specimens and 13 (72%) corresponding smears were HPV positive; one smear was positive and the biopsy specimen negative. Compared by biopsy diagnostic category, three biopsy specimens (60%) and four (80%) smears of five cases of CIN II + wart virus infection were HPV positive; there were no cases of CIN II without wart virus infection. Ten (56%) biopsy specimens and seven (39%) corresponding smears from 18 patients with histopathologically confirmed CIN III + wart virus infection were NISH positive. Of eight cases of CIN III without wart virus infection, five (63%) biopsy specimens and three (38%) smears gave a positive NISH signal. The single case of CIN I was HPV negative on smear and biopsy specimen.

Analysis of the same data by cytological diagnosis—that is, moving cases 10, 16, 19, 20, 24, 26, 29 into the appropriate cytological category—shows that in CIN II + wart virus infection, 71% of smears and 57% of biopsy specimens were positive; in CIN III + wart virus infection, 42% of smears and 50% of biopsy specimens were HPV positive; and in CIN III 33% of smears and 67% of biopsy specimens were positive for HPV.

Of the 18 patients with an HPV positive biopsy specimen, only 10 showed HPV DNA in the first block containing CIN examined. On this basis, therefore, cytological testing for HPV is as, or more, sensitive than biopsy typing. However, screening of two or more CIN blocks increased this number to 18. In case 4 only two of eight blocks from the entire cervical biopsy specimen were HPV positive. In those biopsy specimens scored as negative

for HPV, all blocks were analysed by NISH.

Several controls were used in all NISH experiments. Biopsy specimens known to be positive with each probe were included with each set of specimens studied here. As controls for cervical smears, CaSki cell smears (which contain integrated HPV16²⁰) and cytologically normal cervical smears (from a separate cohort) were hybridised with HPV16 and total human DNA,²¹ respectively. All of these controls gave the appropriate signal.

NISH SIGNAL TYPE

Three morphologically different types of HPV NISH signal have been described in cervical biopsy specimens of CIN¹³ and invasive cancer.¹⁴ The matched biopsy specimens and smears were analysed for these three signal types and correlated with the morphological diagnosis (table 3). A type 1 signal was present only in NISH positive biopsy specimens that also showed morphological evidence of wart virus infection (figs 1A, B). Interestingly, three of the four cases which showed this signal type had CIN II + wart virus infection. A type 2 signal was present in five of eight cases with CIN 3 without evidence of wart virus infection. Case 24 with CIN III + wart virus infection also contained a type 2 signal, but the NISH signal in this case was confined to the undifferentiated/dysplastic cells of the lesion and was not detectable in cells showing cytopathic viral change. A type 3 signal occurred only in CIN III + wart virus infection (eight cases); the signal was present either in or adjacent to cells showing morphological wart virus infection.

Analysis of the NISH signal in the 13 HPV positive cases on both biopsy specimen and

Figure 2(A) HPV 16 in CIN III (case 7). There are discrete nuclear dots (type 2 NISH signal) in the middle two thirds of the epithelium (haematoxylin counterstain). **(B)** HPV positive squamous cervical cells in the matched smear. A type 2 NISH signal is present (haematoxylin counterstain).

Figure 3(A) HPV 16 signal in CIN III + wart virus infection (case 22). The nuclei show both a diffuse (type 1) and punctate signal (type 2) in the same or adjacent cells (a type 3 NISH signal) (haematoxylin counterstain). (B) NISH positive squamous cervical cells in the corresponding smear. A type 3 NISH signal is present (haematoxylin counterstain).

smear showed absolute concordance of the signal type in nine cases. Of the four other cases, two (cases 9 and 10) had a type 3 signal on smear, while the corresponding biopsy specimen contained a type 2 signal, suggesting that the “episomal + integrated HPV” containing cells in the smear had been removed by the spatula before biopsy. A type 1 signal was present in the smear from case 18 and a type 3 signal on biopsy. This suggests again that “episomal HPV containing” cells had been removed by smear taking. The fourth case (20) showed a type 2 signal on smear and a type 3 on biopsy specimen. In this patient there were foci of cells in the biopsy specimen with a type 2 signal only, and in morphologically separate areas a type 3 signal was present. The smear showed a type 2 signal, indicating that in this case only these former cells were sampled. Given these assumptions, there was absolute concordance of NISH signal in biopsy specimen and smear. It should be noted that a type 3 signal is a combination of type 1 and 2.

Discussion

To assess the clinical importance of the relation between HPV and cervical dysplasia and cancer, data on the prevalence of the virus in normal and pathological populations, together with the natural history of the associated lesions on follow up, are required. Consequently, exfoliated cervico-vaginal cells have been extensively tested for the presence of HPV, using filter hybridisation methods^{2 3 7 8} and more recently by the polymerase chain reaction (PCR).^{4 6 9} The results are conflicting, probably reflecting differences in study design and in sensitivity of the two techniques. Filter in situ hybridisation detects HPV derived from exfoliated cells and cervico-vaginal fluid where they may exist outside cells. The PCR measures HPV in the same sites but also any

contaminant introduced by smear taking or in the laboratory performing the analysis. HPV genomes can only be unequivocally attributed to epithelial cells by in situ hybridisation.

In the present study NISH was performed on routine cervical smears and the results compared with in situ detection of HPV on the matched biopsy specimen. This approach offers the advantage of comparing a highly sensitive technique (2.5 to 12 copies of HPV/cell¹⁰), performed on the individual cells with the tissue from which they were collected. Apart from different fixation and pretreatment of the smears and biopsy specimen hybridisation was carried out under the same stringency conditions and HPV was visualised by the same detection system.

A high rate of concordance was observed when wart virus infection changes were associated with cervical dysplasia, HPV being detected in 57% of the biopsy specimens and 53% of the smears. The only significant difference in the results on the two sample types was in the case of CIN III, without wart virus infection, where 63% of biopsy specimens contained a signal compared with 33% on smears. These data underline the different rate of HPV detection in dysplastic smears with and without wart virus infection associated changes and concur with data reported by isotopic in situ hybridisation.²² Notably, in three biopsy specimens NISH signal was limited to scattered dysplastic cells in the intermediate and lower epithelial cell layers (cases 8 and 16) or to the atypical metaplastic epithelium in endocervical glands (case 11). The matched cervical smear was negative in all these cases. Cytological samples are not representative of the deeper epithelial layers, and smears from these cases might reasonably be expected to be HPV negative. In cases 15 and 16 there was a lack of concordance between biopsy specimen and smear, presumably due to sampling. However, in only 10 cases was the first block containing CIN examined HPV positive compared with 14 HPV positive smears. Therefore, although HPV typing is more sensitive on biopsy than on smear, care has to be taken to examine at least two and even more blocks from a cone excision to achieve this. In routine practice, therefore, it could be argued that HPV typing of cervical smears is more cost effective, less invasive than biopsy, and almost as sensitive. It is justifiable, therefore, to recommend cervical screening by NISH as a preliminary screen for HPV.

Table 2 HPV genotyping by NISH on cervical smears

Cytological diagnostic group	n =	NISH*	
		Positive	Negative
CIN1	1	0	1
CIN2 + wart virus infection	7	5	2
CIN3	12	4	8
CIN3 + wart virus infection	12	5	7

*Smears were hybridised with a cocktail of digoxigenin labelled HPV16, 18, 33 probes.

Table 3 HPV genotyping on matched biopsy specimens and smears

Case No	Biopsy/smear diagnosis	Biopsy†				Smear† 16/18/33	NISH signal type‡	
		6/11	16	18	33		Biopsy specimen	Smear
1	CIN1	0	0	0	0	0	0	0
2		0	+	0	0	+	1	1
3		0	0	0	+	+	1	1
4	CIN2 + WVI	0	+	0	0	+	1	1
5		0	0	0	0	+	0	1
6		0	0	0	0	0	0	0
7		0	+	0	0	+	2	2
8	CIN3	0	+	0	0	0	2	0
9	CIN3	0	+	0	0	+	2	3
10	CIN3/CIN2 + WVI	0	0	+	0	+	2	3
11	CIN3	0	+	0	0	0	2	0
12	CIN3	0	0	0	0	0	0	0
13	CIN3	0	0	0	0	0	0	0
14	CIN3	0	0	0	0	0	0	0
15	CIN3 + WVI	0	+	0	0	0	3	0
16	CIN3 + WVI/CIN3	0	+	0	0	0	3	0
17	CIN3 + WVI	0	+	0	0	+	3	3
18	CIN3 + WVI	0	+	0	0	+	3	1
19	CIN3 + WVI/CIN3	0	+	0	0	0	1	0
20	CIN3 + WVI/CIN3	0	+	0	0	+	3	2
21	CIN3 + WVI	0	+	0	0	+	3	3
22	CIN3 + WVI	0	+	0	0	+	3	3
23	CIN3 + WVI	0	+	0	0	+	3	3
24	CIN3 + WVI/CIN3	0	0	+	0	+	2	2
25	CIN3 + WVI	0	0	0	0	0	0	0
26	CIN3 + WVI/CIN3	0	0	0	0	0	0	0
27	CIN3 + WVI	0	0	0	0	0	0	0
28	CIN3 + WVI	0	0	0	0	0	0	0
29	CIN3 + WVI/CIN2 + WVI	0	0	0	0	0	0	0
30	CIN3 + WVI	0	0	0	0	0	0	0
31	CIN3 + WVI	0	0	0	0	0	0	0
32	CIN3 + WVI	0	0	0	0	0	0	0

*The biopsy diagnosis is indicated first and the cytological diagnosis only when it differed—for example, case 10, CIN3/CIN2 + wart virus infection, showed the former on biopsy and the latter on histology.

†Biopsy specimens were typed by NISH for the individual HPV types shown. Smears were typed with a cocktail of 16/18/33 HPV probes only because no biopsy specimen was 6/11 positive.

‡Signal types categorised as described in text.^{4,5}

The three types of NISH signal in those CIN biopsy specimens confirm previous findings.¹³ The distribution of a type 1 NISH signal is similar to immunocytochemical detection of HPV capsid protein,²³ and may represent viral particles in an episomal physical state within cells showing squamous differentiation. A punctate/dot (type 2) NISH signal was confined to undifferentiated/dysplastic cells in CIN III lesions. Furthermore, this punctate/dot signal (associated with type 1 signal) was also present in CIN III lesions with wart virus infection. A type 2 signal has also been described in CaSki cells and in invasive cancer of the cervix.¹⁴ This correlation of type 2 signal with integrated virus has also been recently confirmed using combined in situ and Southern blot analysis.¹⁵ This evidence, as well as the presence of a type 2 signal only in high grade dysplastic lesions^{13,14} confirmed in this study, supports the hypothesis that signal type 2 represents integrated virus, irrespective of HPV type. The implication of this type of analysis is that the discrimination of integrated virus could have potential in determining which CIN lesions are likely to be more aggressive. The type of NISH signal present in smears was, in most, if not in all cases, concordant with that in the biopsy specimens. This is the first time that these signal types have been reported in cervical smears.

In conclusion, we have shown that non-isotopic in situ hybridisation on cytological smears is useful for detecting HPV infection, being of almost comparable diagnostic sensitivity with NISH performed on cervical

biopsy specimens in the presence of morphological wart virus infection. The population of women studied in this report consisted of patients seen in a colposcopic clinic, selected by smear abnormalities. It will now be necessary to evaluate the rate of HPV detection on cytological smears in a large unselected population and correlate this with progression/regression of CIN. As the present NISH methodology is being automated, it should be possible to mount a large scale screening programme to answer these questions.

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