

ORIGINAL ARTICLE

Detection of the human papillomavirus in cervical carcinoma: a comparison between non-isotopic in-situ hybridisation and polymerase chain reaction as methods for detection in formalin-fixed, paraffin-embedded tissues

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Abstract

Cervical carcinoma, the second most common malignancy in Malaysian females, is aetiologically linked to the human papillomavirus (HPV). A study was conducted at the Department of Pathology, University of Malaya Medical Centre to compare the identification of HPV 6, 11, 16 and 18 in 40 archived formalin-fixed, paraffin-embedded cervical carcinoma by non-isotopic in-situ hybridisation (NISH) and polymerase chain reaction (PCR). HPV L1 ORF consensus PCR was also carried in cases which were negative on HPV type-specific PCR. NISH detected HPV 16 in 13 (32.5%) cases with one case demonstrating a concomitant HPV 18. β -globin DNA PCR was carried out on the same paraffin block as for NISH in 27 cases and on a different paraffin block in 13, with amplification in 9 of the former and 3 of the latter. Thus only 12 cases were subjected to further HPV PCR. HPV was detected in 10 (83.3%) with HPV 16 in 9 cases and HPV L1 ORF in one. When using the same paraffin block for both methods of HPV detection, NISH detected HPV in 6 and PCR in 7. NISH failed to detect HPV in a case detected by PCR. 2 cases were negative for HPV using both methods. Hence, HPV detection results by NISH and PCR were concordant in 88.9%. Interestingly, NISH detected HPV in 2 cases with non-amplifiable β -globin DNA. Using an alternative paraffin block for HPV PCR from NISH, HPV DNA was detected in 3 cases, two of which also showed type-specific positivity on NISH. The third case did not reveal type-specific positivity with NISH or PCR but demonstrated HPV DNA on L1 ORF consensus PCR. It thus appears that PCR and NISH can be successfully used to detect HPV in formalin-fixed, paraffin-embedded tissue and in the presence of intact DNA NISH may be as sensitive as PCR.

Keywords: HPV, cervical carcinoma, formalin-fixed, paraffin-embedded, NISH, PCR

INTRODUCTION

The human papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus, 52-55 nm in diameter which is aetiologically linked to cervical carcinoma.¹ More than 100 types have been identified to date and among these are subsets carrying high, intermediate and low risks for inducing cervical cancers. HPV 16, 18, 31 and 45 are generally considered “high risk”; HPV 33, 35, 39, 51, 52, 56, 58, 59, 68, 73, 82, 83 “intermediate risk” and HPV 6, 11, 26, 40, 42, 53, 54, 55, 57, 66, and 84 low risk.²⁻⁴ Cervical carcinoma is the second most common

malignancy in women on a global scale and is also the second most common cancer among Malaysian females.^{5,6} In view of the aetiological relationship of HPV with cervical carcinoma, we were interested to study the possibility of demonstrating the virus in-situ, in particular on archived, routinely processed, formalin-fixed, paraffin-embedded biopsied tissue sections using probes to the two most common “high risk (16 and 18)” and “low risk (6, 11)” HPV types. An attempt was also made to compare the detection of HPV using the non-isotopic in-situ hybridisation (NISH) technique with the more commonly used polymerase chain reaction (PCR) method.

Although conventional in-situ hybridisation has generally been assumed and shown in some studies to be less sensitive compared with PCR for HPV detection,^{7,8} it has significant advantages of morphological preservation and being technically similar with routine immunohistochemical staining. Thus, NISH as a method of detection for HPV is presumably the most practical method for adoption in the routine histopathology laboratory.

MATERIALS AND METHODS

All cases of invasive cervical carcinoma diagnosed histologically on punch biopsy for the first time at the Department of Pathology, University of Malaya Medical Centre (UMMC) between January 1991 and December 1992 were retrieved from the department files. Cases of microinvasive carcinoma, disease diagnosed outside the period of study which had recurred during the period of study or cases with only stained sections referred from external laboratories for diagnosis were omitted. All cases had been histologically re-confirmed and classified according to the WHO Classification system during a previous study by the authors.⁹ Cases were selected on the basis that (1) the diagnostic punch biopsy had sufficient tissue in the paraffin block for NISH using HPV 6, 11, 16 and 18 probes and (2) usage of the tissue in the paraffin block for this study would not compromise future diagnostic investigations for the patient. Thus taking the latter case selection criterion into consideration although it was preferred that PCR be carried out on the same paraffin block as that for NISH, this was not a pre-requisite and an alternative paraffin block

was selected whenever tissue was considered lacking for PCR amplification. Nonetheless, PCR had at least to be carried out in diagnostic material acquired around the same time as that for NISH and in relation to the same disease episode and carrying the same diagnosis.

Determination of HPV by non-isotopic in-situ hybridisation (NISH)

Procedures were adapted from Morris et al.¹⁰ and according to Technical Aid Report (TMS-5).¹¹ Four consecutive 5 um sections were cut from the paraffin blocks on to silanised slides. The sections were dewaxed and rehydrated before immersion in 0.02N hydrochloric acid. This was followed by treatment with 0.01% Triton X-100 (Sigma) for 1.5 min and incubation with 250 ug/ml proteinase K (Sigma) at 37°C at pH 7.6 for 10 min. After washing with glycine/PBS (2 mg/ml), 20% acetic acid was added for 15 s and the reaction kept at 4°C. The sections were post-fixed in 4% paraformaldehyde and dehydrated in varying gradients of alcohol. 50 µl of hybridisation mixture, consisting of 140 ng/ml of respective digoxigenin-labelled HPV probe (subcloned from whole genomes of HPV 6, 11, 16 and 18) in hybridisation buffer made up of 2 X SSC, 5% dextran sulphate, 10% deionised formamide and 0.2% skimmed milk was layered on the tissue sections and covered with Gelbond (FMC Bioproducts). Denaturation of the double stranded HPV DNA probe and cellular DNA was carried out at 90°C for 10 min before hybridisation at 42°C for 16 h in a humidified chamber. This was followed by stringency washes with 2 X SSC at 60°C for 20 min, 0.2 X SSC at room temperature for 10 min,

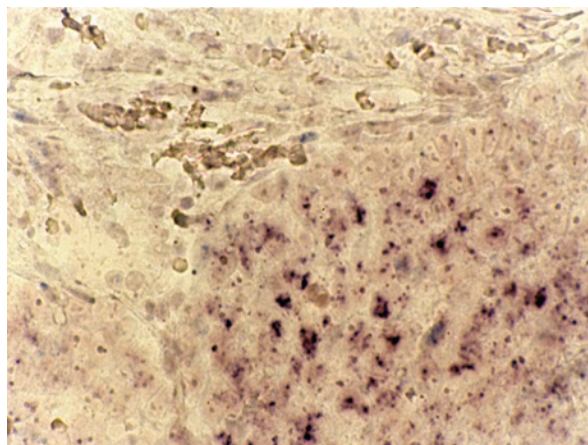


FIG. 1: Intranuclear HPV 16 signals noted in a cervical squamous cell carcinoma

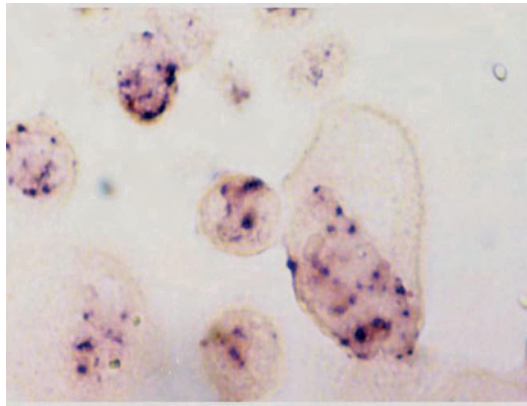


FIG. 2: Intranuclear signals of HPV 16 in CaSki cells

0.2 X SSC at 42 °C for 20 min and 0.1 X SSC at room temperature for 10 min. The hybridised probe was detected as dark blue intranuclear signals (Figure 1) using a digoxigenin detection kit (Boehringer Mannheim: DIG DNA labelling and detection kit). Positive controls for HPV 6 and 11 consisted of formalin-fixed, paraffin-embedded biopsied tissues of known HPV 6 and 11 positive laryngeal polyps respectively. Formalin-fixed, paraffin-embedded cell blocks of CaSki (American Type Culture Collection: CRL 1550) and HeLa cells (American Type Culture Collection: CCL 2) formed positive controls for HPV 16 (Figure 2) and 18 respectively. Substitution of ultrafiltered water for probe in the respective positive control sections were used as negative controls. Relevant positive and negative controls were run with each batch of cases.

Determination of HPV by polymerase chain reaction (PCR)

HPV status was determined by PCR using type specific (HPV 6, 11, 16 and 18) and L1 ORF consensus primers (MY09/MY11). DNA template was prepared by digestion of two 10 µm sections of the formalin-fixed, paraffin-embedded tissue with 0.06 mg/ml proteinase K in 10 mM Tris, 0.1 mM EDTA (pH 8.0) at 48°C for 5 days.^{12,13} This was followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and subsequent precipitation in 0.5 volume 7.5M ammonium acetate and 2.5 volume ethanol. DNA template was only considered suitable for HPV PCR on amplification of a 268 bp human β-globin segment using primers GH20/PCO4. If tissue from the selected paraffin block for the case was not β-globin amplifiable, no attempt was made to select another block for study. For HPV PCR, 30 cycles of amplification were

carried out, with DNA denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min, using the respective type specific or L1 ORF consensus primers, in an automated thermal cycler (Perkin Elmer: DNA Thermal Cycler 480). Single bands of expected amplicon lengths identified on 2% agarose gel electrophoresis were considered positive whereby cases of HPV type specific PCR positivity were further confirmed by dot blot in-situ hybridisation using digoxigenin-labeled oligoprobes. Cloned plasmids with full HPV genomes (HPV 6, 11, 16 and 18) served as positive controls for the relevant specific HPV types while any of the cloned plasmids served as the positive control for L1ORF consensus PCR and were included in each amplification run. Heart muscle, acquired at post-mortem examination from a patient who succumbed from multiple injuries sustained in a motor vehicular accident, which was β-globin segment amplifiable and known not to contain any HPV DNA, was used as negative control with each batch of HPV PCR analysis.

RESULTS

Forty invasive cervical carcinoma histologically diagnosed for the first time between 1991 and 1992 by punch biopsy were entered into the study. Of these, 39 were squamous and 1 adenocarcinoma. Table 1 illustrates the type of HPV detected by NISH according to histological type. NISH detected type specific HPV in 13 (32.5%) cases. Of these, single HPV 16 infection was detected in 12 (11 squamous and 1 adenocarcinoma) while 1 squamous carcinoma showed a mixed HPV 16 and 18 infection.

Table 2 shows β-globin and HPV PCR amplification results of the cases. β-globin DNA

TABLE 1: Detection of HPV by NISH according to histological type of invasive cervical carcinoma (n=40)

Histological type	HPV type			
	6	11	16	18
<i>Squamous carcinoma (n=39)</i>	0	0	12	1
<i>Adenocarcinoma (n=1)</i>	0	0	1	0
Total	0	0	13	1

was not amplified in 28 squamous carcinomas of the 40 cases. Thus only 12 cases (11 squamous and 1 adenocarcinoma) could be subjected to further HPV PCR. HPV 16 was identified in 8 squamous and the only adenocarcinoma. A squamous carcinoma, negative on type specific PCR, revealed HPV DNA on L1 ORF consensus PCR. Thus HPV DNA was detected in 10 (83.3%). HPV 6, 11 and 18 were not detected in any of the cases. The squamous carcinoma with mixed HPV 16 and 18 infection detected on NISH had non-amplifiable DNA template and was not subjected to HPV PCR.

Table 3 compares HPV detection by NISH against PCR with reference to β -globin DNA amplification, and according to the paraffinised tissue blocks used for NISH and PCR purposes. PCR was carried out on the same paraffin block as for NISH in 27 cases and on a different paraffin block in 13. β -globin DNA was amplifiable in 9 of the former (8 squamous and 1 adenocarcinoma) and 3 of the latter (all squamous carcinomas). When using the same paraffin block for both

methods of HPV detection, NISH detected HPV 16 in 6 (5 squamous and 1 adenocarcinoma) and PCR in 7 (6 squamous and 1 adenocarcinoma). NISH failed to detect HPV 16 in a case of squamous carcinoma that was detected by PCR. 2 cases were negative for HPV using both methods. Hence, HPV detection results by NISH and PCR were concordant in 88.9%. Interestingly, NISH detected HPV 16 in 2 paraffin blocks (both squamous carcinomas) with non-amplifiable β -globin DNA.

Using an alternative paraffin block for HPV PCR from NISH, HPV DNA was detected in 3 squamous carcinoma, 2 of which also showed similar HPV 16 positivity on NISH. The third case did not reveal HPV 6, 11, 16 or 18 with NISH or PCR but HPV DNA was detected on L1 ORF consensus PCR. 3 other squamous carcinomas showed HPV signals on NISH although the alternative selected paraffinised tissues DNA was unsuitable for HPV PCR amplification. Single HPV 16 infection was identified in 2 of the above while 1 showed HPV 16 and 18.

TABLE 2: HPV PCR detection following human β -globin amplification according to histological type of invasive cervical carcinoma (n=40)

Histological type	β -globin positive					PCR		β -globin negative
	HPV positive				HPV negative			
	6	11	16	18	L1 ORF			
<i>Squamous carcinoma (n=39)</i>	0	0	8	0	1	2	28	
<i>Adenocarcinoma (n=1)</i>	0	0	1	0	0	0	0	
Total	0	0	9	0	1	2	28	

TABLE 3: HPV (includes 6, 11, 16 and 18) detection by NISH compared with PCR for HPV (includes 6, 11, 16, 18 and L1 ORF using consensus primers) after human β -globin gene amplification in invasive cervical carcinoma (n=40) and according to paraffin block used for NISH and PCR analyses

		PCR analysis						Total
		HPV+/ β globin +		HPV-/ β globin +		β globin -		
		Same block	Different block	Same block	Different block	Same block	Different block	
NISH	HPV +	6	2	0	0	2	3	13
	HPV -	1	1 (L1 ORF)*	2	0	16	7	27
Total		7	3	2	0	18	10	40

Note: * This case was negative by PCR for HPV 6, 11, 16 and 18 but showed positivity with consensus primers to L1 ORF

DISCUSSION

HPV was detected by NISH in 13 (32.5%) of 40 cases of invasive cervical carcinoma on the archival formalin-fixed, paraffin-embedded tissues. In comparison, HPV was detected by PCR in 10 (83.3%) of 12 of the above cases in which DNA templates could be used for amplification. Although immediately appearing less sensitive than PCR for HPV detection, it is important to assess NISH in the light of the study design.

In the 9 cases where both NISH and PCR HPV (types 6,11,16 and 18) detection were carried out on the same paraffin block, NISH detected HPV in 6 and PCR in 7 with 2 cases consistently negative using both methods. Thus, concordance of 88.9% was reached between the 2 methods and is similar to that achieved in other studies.¹⁴ This perhaps presents a fairer comparison of the two methods in detection of HPV. The observation of a low detection rate of 32.5% by NISH compared to 83.3% by PCR is probably due to the unsolicited subject of all cervical carcinoma cases for NISH testing without taking into account the quality of the target DNA. In contrast, PCR was carried out only when the ubiquitous 268 bp β -globin DNA was amplifiable by GH20/PCO4 primers. Based on the large number of cases (70.0%) in this study which were β -globin DNA non-amplifiable, it can be surmised that DNA of a large majority were fragmented and chemically altered, a well-acknowledged effect of formalin-fixation and paraffin-embedding.¹⁵ This probably affected NISH detection and led to the high

rate of false negativity in this study. The observation that NISH detected HPV in 66.7% (8/12) β -globin DNA amplifiable and 17.9% (5/28) non-amplifiable cases also lends support to the above notion. That NISH was able to detect HPV in 5 cases deemed unsuitable for PCR amplification is interesting. While NISH may lag slightly in sensitivity, its seemingly less fastidious requirement for DNA integrity may be an advantage when working on formalin-fixed, paraffin-embedded tissue. It is also obvious from these observations that although β -globin DNA amplification may provide surrogate indication of DNA suitability for NISH, it is not always reflective. It may still be worthwhile considering in-situ probing of an endogenous target such as human placental DNA¹⁶ to empirically optimise protein digestion of samples prior to NISH to improve sensitivity.

HPV L1 ORF was detected by PCR in a squamous carcinoma negative for HPV 6, 11, 16 or 18 confirming the general observation that while HPV 16 and 18 account for more than 70% of cervical carcinoma,^{17,18} other types are also involved in cervical carcinogenesis. Single infection by HPV 16 was the most prominent pattern seen. Only one case of squamous non-keratinising carcinoma exhibited concurrent HPV 16 and 18 infection. Mixed infections, with varying frequencies and involving different combinations of HPV types, have also been reported.^{19,20}

In conclusion, it appears that (1) HPV infection is prevalent in 83% or possibly more of invasive cervical carcinoma in Malaysians; (2) both PCR and NISH can be successfully

used to detect HPV in formalin-fixed, paraffin-embedded tissue and (3) when DNA integrity is more assured, NISH appears to be almost as sensitive as PCR in detection of HPV.

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