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Comparison of detection of human papillomavirus 16 DNA in cervical carcinoma tissues by Southern blot hybridisation and nested polymerase chain reaction

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Summary. An association between human papillomavirus (HPV) and cervical neoplasia has been widely reported and HPV DNA is commonly detected in cervical carcinoma tissues. However, estimates of the prevalence of HPV infection differs among various detection methods. Seventy cases of cervical carcinoma were screened for HPV 16 infection by Southern blot hybridisation (SBH) and nested polymerase chain reaction (PCR). According to SBH, the prevalences of HPV 16 DNA in stage I ($n = 40$) and stage II ($n = 30$) cervical carcinomas were 52.5 and 63.3 %, respectively, and the overall prevalence was 57.1 % (40 of 70). By nested PCR, the prevalences of HPV 16 infection in stage I and II cervical carcinomas were 87.5 and 93.3 %, respectively, and the overall prevalence was 90.3 %. The prevalence of HPV DNA detected by nested PCR was significantly greater than that detected by SBH. The combined concordance of positive and negative results between SBH and nested PCR was 61.4 %. The discrepancy resulted mainly from 25 cases (35.7 %) that were positive by PCR but negative by SBH. A small copy number of HPV DNA in these 25 cases was documented by a semi-quantitative PCR method. The nested PCR was more sensitive than SBH and detected cases with low amounts of HPV DNA. The detection of HPV infection varied between these two prevailing detection methods and this should be kept in mind in assessing various epidemiological data concerning HPV infection.

Introduction

The association of human papillomavirus (HPV) infection and cervical neoplasia has been widely reported.^{1,2} More than 60 genotypes of HPV have been identified and > 22 genotypes are related to genital infection.^{3,4} Among them, HPV types 6 and 11 are more commonly seen in benign gynaecological lesions such as condylomata accuminata,⁵ whereas HPV types 16 and 18 are more commonly seen in malignancies of the lower genital tract, especially cervical carcinomas.^{6,7} The prevalences of HPV 16 and 18 DNA in cervical carcinoma tissues greatly exceed those in normal cervical tissues.⁸

The prevalence of HPV infection varies between reports. The recorded prevalence rates of HPV 16 or 18 DNA in cervical carcinoma tissues by hybridisation methods in the UK, Germany, the USA and Japan range from 18 to 92%.⁹ The great variation in

prevalence may result from geographic or racial differences.^{6,10} However, other factors such as test design and detection methods may also be responsible for the discrepancy. Although Southern blot hybridisation (SBH) is generally accepted as the ultimate standard method, it is expensive and laborious and a large amount of sample is required for analysis.¹¹ On the other hand, polymerase chain reaction (PCR) is much more sensitive than Southern or other conventional DNA hybridisation methods¹² and a small sample is adequate for PCR analysis.^{13,14} Moreover, the sensitivity and specificity can be increased by means of a two-step PCR with nested primers.¹⁵ Nested PCR has been applied to detect hepatitis C virus (HCV), human immunodeficiency virus (HIV), herpes simplex virus (HSV) and HPV.^{16–19} Exploring the variation in prevalence detected by SBH and PCR is important in assessing epidemiological data of HPV infection. In the present work, HPV 16 DNA, the most prevalent virus in cervical carcinoma tissues, was screened by both SBH and PCR methods and the underlying cause of the discrepant screening results

between these two methods was sought by application of a semi-quantitative PCR analysis.

Materials and methods

Subjects and specimens

Seventy cases of histologically proven cervical carcinoma were studied. The clinical staging of cervical cancer followed the system of the International Federation of Gynecology and Obstetrics (FIGO). There were 40 stage I (57.1%) and 30 stage II (42.9%) cases. The mean ages of cases with stage I and stage II disease were 46.6 and 51.2 years, respectively. The difference in ages between these two groups of patients was not significant ($t = 1.71$, $p = 0.092$). The carcinoma tissues were collected from the surgical specimens immediately after radical hysterectomy and stored in liquid nitrogen until use. The genomic DNA of carcinoma tissues was extracted by a phenol-chloroform-ethanol method and the concentrations of genomic DNA were determined by spectrophotometry.²⁰

Southern blot hybridisation

Southern blot hybridisation was done as described previously.⁸ Briefly, genomic DNA (10 µg) was digested with restriction endonucleases such as *Bam*HI, *Hind*III and *Pst*I, then subjected to agarose gel electrophoresis and subsequently blotted on to nitrocellulose membranes. The HPV probes were prepared by labelling the entire genomic HPV 16 DNA, which had been separated and purified from its vector pBR-322, with [α -³²P]-dCTP. Prehybridisation, hybridisation and washing were performed under highly stringent conditions. Hybridisation was detected by autoradiography. The sensitivity of SBH was c. 1 pg of standard HPV DNA.

PCR

Cellular genomic DNA (500 ng, each case) and purified HPV DNA (100 pg, positive control) were used as target DNA. Each sample was amplified in a reaction mixture (100 µl) containing: PCR buffer—50 µM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂ and gelatin 0.01%; primers mix, each 0.2 µM, (outer primer pairs in the first-step PCR and inner primer

pairs in the second-step PCR); dNTP (deoxynucleoside triphosphate, mix: dATP, dTTP, dCTP, dGTP; Sigma, each 50 µM); and *Taq* DNA polymerase (Cetus, USA), 2 units. Salmon sperm DNA (500 ng) was used as the negative control and reaction mixture without target genomic DNA was used as the blank. Amplification was performed with an automated thermal cycler (Perkin-Elmer Cetus, USA) with denaturing conditions of 95°C for 2 min, followed by repeated cycles of 94°C, 55°C and 72°C for 1 min each (35 cycles in total), and a final extension at 72°C for 10 min. After the first-step PCR, a portion (10 µl) of the reaction mixture was used as the source of target DNA in the second-step PCR for further amplification. The PCR reaction was prepared in a hood restricted to this purpose. There was overnight treatment of laminar flow and ultraviolet irradiation in the hood between each run of the PCR. The sequences of nested outer and inner primers used in the two steps of the PCR are shown in table I.

After each run of the PCR, the reaction mixtures were tested for positive signals by gel electrophoresis and dot-blot hybridisation. For gel electrophoresis, 15 µl of reaction mixture was separated by electrophoresis on an agarose 1.6% gel stained with ethidium bromide. Positive bands of corresponding size were recorded (fig. 1). For verification by dot-blot hybridisation, 15 µl of reaction mixtures were dot-blotted on to a nylon membrane. Prehybridisation was followed by hybridisation with non-isotopic, digoxigenin-labelled HPV oligonucleotide probes (10 ng/ml) derived also from PCR incorporating digoxigenin-dUTP.^{21,22} After stringent washing and development with a digoxigenin-anti-digoxigenin-alkaline phosphatase system, according to the manufacturer's instructions (Boehringer Mannheim, Germany), positive signals were recorded for comparison. Only those samples that gave positive bands in electrophoresis and dot signals were interpreted as positive for HPV infection. The sensitivity of nested PCR was tested with serial dilutions of standard HPV DNA. As little as 10⁻⁶ pg of standard HPV DNA was detectable by this method.

Semi-quantitative PCR

To prepare a competitive DNA fragment for semi-quantitative PCR, the originally amplified native HPV 16 DNA fragment (694 bp; nucleotides 7836–625) was

Table I. Sequences of HPV 16 primers used in nested PCR

Primers	5' → 3' sequence	Genomic site	Size (bp)
First-step outer primers			
16 1R	ACTGCACATG GGTGTGTGCA AACC	7836–7859	694
1L	GATCAGTTGT CTCTGGTTGC AAAT	625–602	
Second-step inner primers			
16 2R	ACCGAAACCG GTTAGTATAA AAGC	50–73	552
2L	CTAACATATA TTCATGCAAT GTAG	601–578

Fig. 1. Agarose gel electrophoresis of amplified HPV 16 DNA fragments from genomic DNA of cervical carcinoma tissues following nested PCR. **Upper row** (first-step PCR): **lane 1**, size marker, *Phi-X174/HaeIII* (1 μ g); **2-4**, positive (694 bp) and negative controls and blank, respectively; **5-17**, samples. **Lower row** (second-step PCR): **lane 1**, size marker, *Phi-X174/HaeIII* (1 μ g); **2-4**, positive (552 bp) and negative controls and blank, respectively; **5-17**, samples.

Fig. 2. Relative intensity of amplified HPV DNA bands on electrophoresis following semi-quantitative PCR. **Lane 1**, size marker, *Phi-X174/HaeIII*; **2-4**, 0.1, 0.01 and 0.001 pg, respectively, of native HPV DNA in addition to 0.1 pg of modified HPV 16 DNA in the reaction mixtures; **5-7**, 0.1, 0.01 and 0.001 pg, respectively, of native HPV DNA in addition to 0.01 pg of modified HPV 16 DNA; **8-10**, 0.1, 0.01 and 0.001 pg, respectively, of native HPV DNA in addition to 0.001 pg of modified HPV 16 DNA. The iso-dense bands of the amplified native (694 bp) and the modified competitive (494 bp) HPV DNA appeared when the relative amounts of these two DNA templates in the samples were similar, e.g., lanes 2 and 6.

cut with *HinfI* at the sites (GATC) of nucleotides 274 and 474 into three fragments—343 (7836–274 ntd), 200 (274–474) and 151 (274–675) bp.²³ The flanking sequences of the 343- and 151-bp fragments were purified by electrophoresis and electro-elution, then ligated with a DNA ligase to form a novel DNA of 494 bp. This 494-bp DNA fragment was used as a competitive target DNA for amplification because it possessed the same flanking sequences as that of native DNA (694 bp). The novel competitive short (494-bp) DNA fragment could be amplified by PCR and quantified for further use. Moreover, the amplified native 694-bp and competitive 494-bp DNA fragments were clearly distinguished by their different sizes on gel electrophoresis. In the semi-quantitative PCR, the competitive DNA at a known concentration was added to the reaction mixture. The predominance of DNA bands (competitive or native DNA) on electro-

phoresis after PCR depended on the relative amounts of these two target DNAs in the samples before amplification. The appearance of two iso-dense bands or the transition from one dominant band to another, either native or competitive DNA fragments, on electrophoresis indicated that the amount of the native DNA in this sample was identical to or near the known amount of competitive DNA fragment multiplied by a factor 16 (7904 bp/494 bp). In this design of semi-quantitative PCR, the concentrations of HPV DNA in samples could be estimated at less than pg levels (fig. 2).

Results

Prevalence by Southern blot hybridisation and nested PCR

The prevalences of HPV 16 DNA in cervical carcinoma tissues by SBH in stage I and stage II patients were 63.3% (19 of 30) and 52.5% (21 of 40), respectively. The overall prevalence rate was 57.1% (40 of 70) (table II). Conversely, the prevalences of HPV 16 DNA in cervical carcinoma tissues by nested PCR in stage I and stage II patients were 87.5 (35 of 40) and 93.3% (28 of 30), respectively. The overall prevalence rate was 90.0% (63 of 70). The difference in

Table II. Stage-specific prevalence of HPV 16 DNA in cervical carcinoma tissues by SBH and nested PCR

Method	Status	Number (%) of samples		
		Stage I (n = 40)	Stage II (n = 30)	Total (n = 70)
SBH	+ve	21 (52.5)	19 (63.3)	40 (57.1)
SBH	–ve	19 (47.5)	11 (36.7)	30 (42.9)
PCR	+ve	35 (87.5)	28 (93.3)	63 (90.0)
PCR	–ve	5 (12.5)	2 (6.7)	7 (10.0)

Table III. Comparison of the prevalence of HPV 16 DNA in cervical carcinoma tissues detected by SBH and nested PCR

Method	Status	Number (%) of samples		
		SBH +ve	SBH -ve	Concordance
Stage I (n = 40)				
Nested PCR	+ve	19 (47.5)	16 (40.0)	55 % (22/40)
Nested PCR	-ve	2 (0.5)	3 (7.5)	
Stage II (n = 30)				
Nested PCR	+ve	19 (63.3)	9 (30.0)	70 % (21/30)
Nested PCR	-ve	0 (0.0)	2 (6.6)	
Stage I & II (n = 70)				
Nested PCR	+ve	38 (54.3)	25 (35.7)	61 % (43/70)
Nested PCR	-ve	2 (2.9)	5 (7.1)	

prevalence between stage I and stage II patients was not significant either by SBH ($\chi^2 = 0.439$, $p = 0.508$) or by nested PCR ($\chi^2 = 0.162$, $p = 0.687$). However, the overall prevalence rate by nested PCR (90.0%) was much greater than that by SBH (57.1%) ($\chi^2 = 17.78$, $p < 0.001$).

Comparison of prevalences detected by Southern blot hybridisation and nested PCR

Comparisons between SBH and nested PCR for detecting HPV 16 DNA in cervical carcinoma tissues are shown in table III. In total, 38 (54.3%) patients had concordant positive and five (7.1%) cases had concordant negative results by SBH and PCR methods. The combined concordance of positive and negative results between these two methods was 61.4% (43 of 70). The discrepancy resulted mainly from 25 (35.7%) cases, whose samples were positive by nested PCR but negative by SBH. The amounts of HPV 16 DNA determined by the semi-quantitative PCR in the samples from these 25 cases were in the range 10^{-2} – 10^{-5} pg. On the other hand, samples from two (2.9%) further cases were positive for HPV 16 DNA by SBH but negative by nested PCR. The concordance of SBH and PCR results in those with stage II cervical carcinomas (70%) was greater than in those with stage I disease (55%) but the difference was not significant ($\chi^2 = 1.506$, $p = 0.304$).

Discussion

HPV 16 is the predominant HPV type found in cervical carcinomas.^{24,25} The prevalence of HPV 16 DNA in cervical carcinoma tissues by SBH in the present work was 57.1%, similar to the 53% reported by Choo *et al.* in Chinese women from the same geographic area of northern Taiwan.²⁶ Both prevalence rates were in the range 35–90% found in patients studied by SBH but in different geographic regions such as Japan (35%), East Anglia (UK) (46%), Norway (56%), UK (66%), Australia (71%) and India (90%).^{24,25,27–30}

As previously reported, the prevalence of HPV infection was not related to the clinical stage of the cervical cancers.^{28,31} The difference in prevalence between stage I and stage II patients, either by SBH or PCR, was not significant in this study. However, the prevalence of HPV infection detected by nested PCR was much greater than that by SBH.

PCR is generally many times more sensitive than Southern blot or other hybridisation methods. Melchers and colleagues reported that the prevalence of HPV DNA in cervical scrapes detected by PCR (70%) was much larger than that by SBH or modified filter *in situ* hybridisation (FISH) (46%).¹² Moreover, the prevalence of HPV infection according to PCR in those with abnormal Pap smear (67%) exceeded that by the ViraPap method in Burmer's series (47%);³² a similar result was reported by Bauer *et al.*³³ In contrast, Nuovo and colleagues reported a lower detection rate of HPV in cervical lesions that lack koilocyte atypia with PCR than with SBH.³⁴ The lower detection rate of HPV with PCR might have been related to the relatively high proportion of "novel" HPV types in such lesions. In the present work, the prevalence of HPV 16 DNA in cervical carcinoma tissues detected by nested PCR was 90%, much larger than that by SBH (57.1%) and also larger than the prevalence by one-step PCR method in cervical carcinoma specimens from women in central China (72%).³⁵ Generally, the sensitivity of two-step PCR is greater than that of one-step PCR and the specificity is also increased by applying nested primers.^{15,19} Moreover, hybridisation of the PCR product with radioactively or non-radioactively labelled oligonucleotide probes increased the sensitivity of HPV detection by 100-fold.³⁶

The combined concordance of positive and negative results of HPV DNA between dot-blot hybridisation and PCR was 69% in Morris's series,³⁷ whereas the concordance between SBH and PCR was > 80% in Guerrero's series.³⁸ In this work, the concordance between SBH and nested PCR was 61.4%. Most discrepancies resulted from the 25 (35.7%) cases that gave positive PCR but negative SBH results. Similar results (27.5% of cancer biopsies were positive by PCR but negative by SBH) have been reported by Kristiansen and colleagues.³⁹ These findings might be explained by the relatively small copy number of HPV DNA in carcinoma tissues from these cases. In semi-quantitative PCR analysis, the amounts of HPV 16 DNA in the samples of these 25 cases with discrepant PCR and SBH results were in the range 10^{-2} – 10^{-5} pg, which was less than the detection limit (1 pg) of SBH. On the other hand, results in two cases (2.9%) were positive for HPV DNA by SBH but negative by nested PCR. This discrepancy might result from non-specific binding by SBH to other HPV DNA or the presence of novel HPV types incapable of amplification by PCR with type-specific primers.³⁴

In conclusion, the variation in prevalence of HPV DNA in cervical carcinoma tissues detected by SBH

and nested PCR was evident and the discrepancy resulted mainly from cases having positive PCR but negative SBH results. Based on semi-quantitative PCR analysis, we believe that more cases with relatively small copy numbers of HPV DNA could be detected by nested PCR than by SBH. The influence of different

screening methods on the prevalence rate of HPV infection is important; this factor should be kept in mind in assessing epidemiological data concerning HPV infection.

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