Polymerase Chain Reaction Detection and Restriction Enzyme Typing of Human Papillomavirus in Cervical Carcinoma

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Summary

Specific human papillomavirus (HPV) types have been implicated in the development of cervical carcinoma worldwide. Novel molecular techniques have facilitated the detection and typing of HPV in cervical lesions. DNA preparations from a series of 23 histopathologically confirmed cervical carcinoma patients were analyzed by polymerase chain reaction (PCR) using degenerate primers for the presence of HPV DNA sequences. A total of 22 of 23 cases studied (95.7%) were found positive for HPV DNA sequences. Further studies by DNA hybridization with viral specific probe and restriction enzyme analysis demonstrated the presence of HPV 16 in 73.9% (17/23) and HPV 18 in 65.2% (15/23) of the cases examined. Interestingly, the uncommon HPV 31 and 33 were also found but with a lower percentage (16.9%). It was noted that HPV 16 frequency in the carcinoma increased with age but HPV 18 was evenly present at all ages investigated. We found that HPV was frequently associated with the majority of the cervical carcinomas, and in all but one case, oncogenic high risk HPV genotypes were present. We conclude that HPV infection of the genital tract has an important role in the development of the disease in Malaysia.

Key Words: PCR, Human papillomavirus, HPV, Cervical carcinoma, Hybridization

Introduction

Epidemiological and molecular studies demonstrate that several human papillomavirus (HPV) types are closely associated with the development of human cervical cancer. The HPV are a heterogenous group of viruses with over 70 different genotypes identified and isolated from different lesions of epithelial cell origin[1]. The heterogeneity of the HPV group in general probably reflects an adaptation of these viruses to specifically differentiated epithelial tissue. However, only certain types of HPV, particularly 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52 and 56 are preferentially found in the genital tract and some of these may occasionally be found in oral lesions and laryngeal tissue[2,3,4]. An analysis of various tumour types shows that HPV 6

and 11 are rarely found in high grade premalignant lesions and often associated with the benign disease. Recent studies also demonstrate that HPV 6 and 11 may persist in tissues visibly unaffected by cytopathogenic changes[5,6,7]. On the other hand, HPV 16, 18, 31 and 33 are considered high-risk types and women infected with these genotypes have a greater risk of developing cervical cancer[8,9,10,11].

In Malaysia, little is known of the geographic distribution of oncogenic genotypes of HPV in the cancer of the cervix and other anogenital sites. We have now developed and optimized techniques for the evaluation of tissues for the presence of HPV DNA sequences. Here we report the application of the polymerase chain reaction (PCR) technique for the detection of several HPV genotypes, namely, 6, 11, 16, 18, 31 and 33, in cervical carcinoma. We believe this is the first report showing the prevalence of HPV types in cervical carcinoma from Malaysia.

Materials and Methods

Clinical specimens

The study group comprised of 23 women undergoing surgery at the General Hospital, Kuala Lumpur. The tissues were histopathologically confirmed for carcinoma of the cervix. The tissues were immediately frozen in dry ice at collection and stored in a -70⁰C freezer for later extraction of DNA.

Extraction of DNA from tissues

About 1 gm of tissue was minced into small pieces and placed in a dounce (Bellco, USA) containing 8 ml 1X SSC (150 mM sodium chloride, 15 mM tri-sodium citrate, pH 7.0), 0.5 ml 20% sodium dodecyl sulfate (SDS). The cells were lysed gently with the plunger. Subsequently, 125 μl of 10 mg/ml RNase and 62.5 μl of proteinase K of 20 mg/ml stock was added to the crude DNA and the mixture incubated for 30 minutes at 37⁰C.

The DNA was extracted using the phenol-chloroform mixture as previously described[12]. The DNA was subsequently precipitated in ice-cold absolute ethanol and then spooled out with a sterile pasteur pipette. The DNA was redissolved in sterile deionized water.

Optical density measurements at 260 nm and 280 nm were taken to estimate the nucleic acid concentration and its purity[13]. Samples with 260:280 ratios of 1.8 to 2.0 which indicated fairly pure DNA were used in the study.

DNA extracted from CaSki and HeLa cell lines, which are two human cervical cell lines containing integrated HPV 16 and 18, respectively, served as positive controls. DNA from Raji cells, a human lymphoma cell line, free of HPV served as the negative control.

Human papillomavirus DNA detection

The set of primers used in the PCR were degenerate and therefore capable of general detection of several high risk HPV types including 6, 11, 16, 18, 31 and 33. The primers were obtained commercially (Perkin-Elmer Cetus, USA) and had the following sequence:
Primer 1 (MY09)
5'-CGTCCMARRGGAWACTGATC-3'
and Primer 2 (MY11)
5'-GCMCAGGGWCATAAYAATGG-3' where M = A or C, R = A or G, W = A or T and Y = C or T. The primers resulted in an amplification of a 450 bp fragment DNA from HPV sequences in human genomic DNA. The positive controls used were the DNA from CaSki for HPV 16 and HeLa for HPV 18.

Amplification of a 268 bp product from a cellular gene, ß-globin sequences in human genomic DNA, served as an indicator that the DNA samples were adequate for HPV analysis by PCR.

Polymerase chain reaction (PCR)

Amplification was performed in 50μl reaction mixture containing 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 0.2mM each dCTP, dATP, dGTP and dTTP, 0.001% w/v gelatin, 5 pmole each Primer 1 and 2, 0.1μg template genomic DNA from carcinoma and 2.5U of Taq DNA polymerase (Perkin-Elmer Cetus, USA). The mixture was overlaid with 40μl of silicone oil and amplified using a DNA thermal cycler (Perkin-Elmer Cetus, USA). The conditions for each of the 30 cycles were as follows: denaturation at 95⁰C for 30 seconds, annealing at 55⁰C for 30 seconds and extension at 72⁰C for 60 seconds. The final extension step at the end of the cycles were at 72⁰C for 5

minutes. Since the extreme sensitivity of PCR lends itself to false-positive results due to accidental DNA contamination, we took rigorous precautions to overcome them. Preparation of reaction mixture, PCR amplification and analysis of amplified products were spatially separated. Positive displacement pipettes were used and the pipettes were separated for each reagent. Negative controls in each PCR assay served to monitor potential contamination of the reagents.

Analysis of PCR products

An aliquot of 8μl of the amplified DNA product was subjected to electrophoresis in a 1.5% agarose gel. The gel was stained with ethidium bromide and the DNA bands visualised under ultraviolet light, were photographed.

Restriction enzyme digestion of PCR product

Restriction endonuclease digestion was conducted on PCR amplified products using two enzymes, *Hinf I* and *Pst I*, independently, to determine the HPV types present. The mixture of enzyme and PCR product was incubated at 37°C overnight. The restriction fragments developed were analysed on a 2% agarose gel following electrophoresis. HPV 16 gave one fragment (452 bp) with *Hinf I* and two fragments (224 bp, 228 bp) with *Pst I*. HPV 18 gave one fragment (455 bp) as with *Hinf II* and two fragments (213 bp, 242 bp) with *Pst I*. HPV 31 gave two fragments (215 bp, 237 bp) with *Hinf I* and two fragments (224 bp, 228 bp) with *Pst I*, HPV 33 gave two fragments (215 bp, 234 bp) with *Hinf I* and two fragments (207 bp, 242 bp) with *Pst I*.

Southern blot assay and DNA probes

HPV 16 and HPV 18 DNA probes were labelled with digoxigenin-11-dUTP according to the manufacturer's specifications[14]. Probes for HPV 31 and 33 were not available for hybridization analysis.

The analysis of the HPV type-specific PCR products was carried out by Southern blot hybridization. The gel was denatured in 1.5M NaCl, 0.5M NaOH for 1 hour and neutralised in a solution of 1M Tris.Cl (pH 8.0), 1.5M NaCl for 1 hour. The DNA was then transferred from the gel to a nylon membrane (Hybond, Amersham) by capillary blotting in 10X SSC

overnight[15]. After prehybridization for 1 hour at 68°C, hybridization was carried out in a solution of 5X SSC, 0.1% (w/v) N-lauroylsarcosine.Na-salt, 0.02% blocking reagent containing 100 pg of freshly denatured probe, at 68°C for a minimum of 6 hours. Following hybridization, the membranes were washed twice for 5 min at room temperature with 2X SSC containing 0.1% (w/v) SDS. Membranes were then used directly for non-isotopic detection of the hybridized DNA.

The colour detection using the digoxigenin - labelled probes was carried out according to the Boehringer method[14]. After equilibration with buffer 1 (100mM Tris-HCl, 150mM NaCl (pH 7.5)) for 1 min, the membrane was incubated with 0.5% (w/v) blocking reagent for 30 min. Thereafter, the membrane was washed again briefly with buffer 1 and incubated with the diluted antibody-conjugate solution (anti-digoxigenin-AP) for 30 min. The membrane was washed again twice for 5 min with buffer 1, equilibrated for 2 min with buffer 3 (100mM Tris-HCl, 10mM NaCl, 50mM $MgCl_2$,pH 9.5) and incubated in the dark in a sealed plastic bag containing freshly prepared solution containing 45μl nitroblue tetrazolium salt and 35μl 5-bromo-4-chloro-3-indoyl-phosphate in 10ml of buffer[3] for development of the colour.

When the desired bands were detected, the reaction was stopped by washing the nylon membrane with buffer[4] (10mM Tris-HCl, 1 mM EDTA, pH 8.0) for 5 min.

Results

Detection of HPV DNA in cervical carcinoma

Genomic DNA was available from 23 cervical tissues that were earlier confirmed on histological examination to be invasive squamous cell carcinoma. The DNA was initially subjected to PCR amplification using primers specific for the common human ß-globin gene. The characteristic 268 bp amplified product was present in all the 23 genomic DNAs indicating that these DNAs were adequate for PCR study and inhibitory factors were absent from them.

Subsequently, PCR amplification for HPV was

performed on the 23 genomic DNAs. The result showed that 20 (86.9%) of the 23 samples were found to contain HPV DNA sequences. A 450 bp viral sequence product was amplified (Fig. 1 and Table I). The three negative DNA samples were subjected to a repeat PCR amplification with a different set of primers but the result again was negative. However, only one of the three negative samples were also negative by hybridization analysis to specific HPV 18 and 16 probes. Thus, only 1 (4.3%) of the 23 samples was negative when both the results were combined (Table I).

**Restriction fragment length analysis of PCR product**

The PCR amplified product (450 bp) was subjected to restriction endonuclease digestion with *Hinf* I and separately with *Pst* I. The product of the digestion was analysed for the HPV genotype following agarose gel separation.

All the PCR amplified product derived from the DNA of cervical carcinoma, were positive for known HPV genotypes, namely, 16, 18, 31 and 33. HPV 16 was the predominant type identified. Nine samples (39.1%) were positive for HPV 16, and seven samples (30.4%)

Table I
Distribution of HPV genotypes in cervical carcinoma in relation to race and age

Patient no.	Race	Age	PCR product (+/- visible/ not visible under uv)	HPV genotype on restriction enzyme analysis	HPV genotype on specific probe hybridization of PCR product
1.	Indian	61	+	18	16, 18*
2.	Malay	35	+	16	16
3.	Chinese	50	+	16	16, 18*
4.	Chinese	40	+	18	16, 18
5.	Chinese	44	+	16, 31	16
6.	Chinese	52	+	18	18
7.	Chinese	47	-	-	16*,18
8.	Chinese	48	+	16, 31	16
9.	Chinese	43	+	16	16, 18
10.	Malay	53	-	-	-
11.	Indian	41	+	16	16, 18*
12.	Chinese	52	+	16, 33	16
13.	Chinese	34	+	16	16, 18
14.	Chinese	55	+	16	16
15.	Chinese	40	+	16	16, 18
16.	Malay	53	+	18	18
17.	Malay	34	-	-	18
18.	Chinese	45	+	16	16, 18
19.	Chinese	33	+	16	16
20.	Indian	52	+	18	18
21.	Chinese	35	+	16, 31	16
22.	Malay	43	+	18	18
23.	Malay	64	+	18	16, 18

*   Hybridization signal was very weak suggesting low copy number in genomic DNA from cervical carcinoma
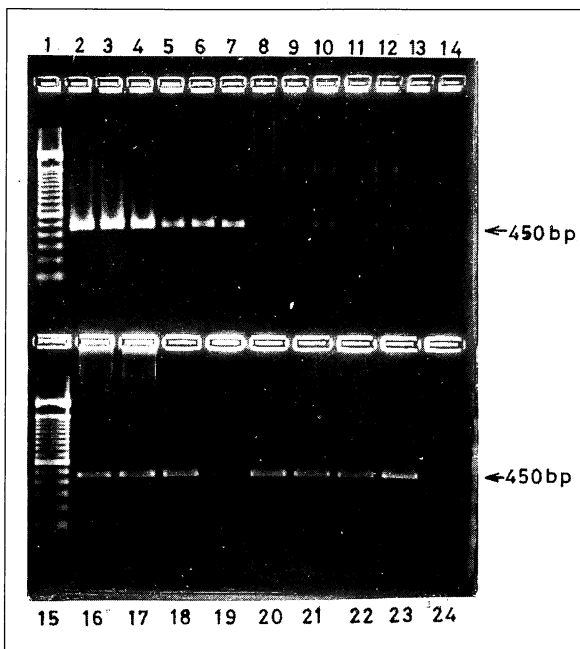
Fig. 1:
The agarose gel electrophoretic pattern of the 450 basepair PCR amplified HPV DNA sequence (arrow) derived from target genomic DNA extracted from cervical carcinoma.

Lane 1 and 15 contain 100 bp molecular weight ladder standard, Lane 2 to 14 and 15 to 22 contain PCR amplified HPV sequence derived from cancer tissue samples. Note absence of 450 bp amplicon in Lane 8, 11 and 19 representing patient 7, 10 and 17 respectively. Lane 23 contains HPV positive controls and Lane 24 HPV negative controls.

were positive for HPV 18. HPV 31 and 33 were encountered relatively less frequently being present in three samples (13.1%) and one sample (4.3%) respectively.

DNA hybridization assay on the amplified products

The amplified products were analysed by DNA hybridization techniques using digoxigenin-labelled probes, specific for HPV 16 and HPV 18.

The presence of HPV 16 and 18 mixed infections were demonstrated in 43% of the samples. On the other hand, 30% of the patients were positive for only HPV 16, and 17% for only HPV 18.

In general, HPV-16 was present in 73% of the samples while HPV-18 infections made up about 65% of the total number of cases studied.

Table II shows the age distribution of the patients in relation to persisting HPV genotypes. In the case of HPV 16 the percentage prevalence increases with age but for HPV 18 there is little difference in incidence with age.

Table II
Proportion of HPV genotypes in cervical carcinoma in relation to ages of patient

HPV genotype	Age in years: No positive/Total studied (percent)			
	25 - 34	35 - 44	45 - 54	55 -
HPV 16	2/3 (66.7)	7/8 (88)	6/9 (66)	3/3 (100)
HPV 18	2/3 (66.7)	5/8 (62.5)	5/9 (55)	2/3 (66.7)
HPV 16 and HPV 18	1/3 (33.3)	5/8 (62.5)	6/9 (66)	2/3 (66.7)

Discussion

In Malaysia, little is known of the geographic distribution of the oncogenic genotypes of HPV. The present limited study shows that several HPV types may be associated with cervical carcinoma. We found that 95.7% of the cervical carcinoma were associated with HPV 16 and 18 genotypes representing 22/23 tumours and another 16.9% (4/23) of tumours were also associated with HPV 31 and 33. Only one tumour was negative using the various tests. Most tumours had multiple HPV infections. It was noted that in 2 occasions the PCR product was not visible under ultraviolet light, following ethidium bromide staining of the gel but the DNA was detectable with Southern hybridization with specific homologous probes. The absence of a clear PCR amplicon in these two samples could be due to low copy numbers of HPV in the original cervical tumour. This raises the importance of performing Southern hybridization with specific probes to confirm the PCR results. Otherwise low levels of PCR product would be missed. Southern hybridization also permitted identification of the HPV genotype which provided more detail on the various persisting HPV genotypes. The results obtained support the currently favoured hypothesis that HPV 16 and 18 play an important role in the development of cervical carcinoma. Infection with HPV 16 occurred in 73.9% of the patients studied compared to 65.2% with HPV 18. Similar results have been reported by Durst et al[9] who noted the presence of HPV 16 DNA in 62% (11/18) of cervical cancer from Germany, 35% (8/23) from Kenya and Brazil. However, a lower proportion (34%) with HPV 16 DNA were found in cervical carcinomas from Japan[16]. On the other hand, HPV 18 was found in 15% (2/13) of those from Germany, 25% (9/36) from Kenya, Uganda and Brazil[17] and 5% (3/56) from Japan[16].

The high detection rate of HPV DNA in our study was attributed to the application of PCR and Southern hybridization to the DNA samples. The analysis of distribution of HPV infection in relation to age revealed that despite the slight drop in age group 45-54, the frequency of HPV 16 infection increased with age. Our results are in agreement with those of Meanwell et al[18] who reported that the frequency of HPV 16 infection increased with age. The prevalence

of HPV 16 contrasts with that of HPV 18 in the cervical carcinoma. We found as did Yoshikawa et al[16], that HPV 18 frequency in cervical carcinoma does not change with age[16]. However, because of the large proportion of females with HPV 16 and 18 in our samples the trend of HPV with age was weak in our case. HPV 16 is the predominant HPV type worldwide and is associated with more than 50% of the malignant lesions while other HPV types specifically 18, 31 and 33 differ in their abundance geographically[19]. HPV 16 and 18 have been consistently detected in invasive tumours at a high frequency and therefore are regarded as "high risk" viruses with an oncogenic potential for malignant conversion of normal epithelial cells[9,20]. While HPV 18 appears to be the second most frequent type in cervical carcinoma it is more often found in adenocarcinomas of the cervix than HPV 16[21]. A strong correlation between the presence of HPV 16 and 18 in the cervix and the greater probability of progression from low grade to advanced carcinoma *in situ* has been documented[22,23]. These observations suggest that the pathogenesis of and the possible progression to cervical cancer may depend on the genotype of HPV infection.

Experimental evidence exists for the direct role of HPV in oncogenic transformation of infected epithelial cells. Analysis of tumour tissue from cervical carcinoma shows that the HPV genome is integrated into the host DNA at random locations. The papillomavirus normally exists as a circular entity but in the process of integration into the host DNA the virus is linearised by a cut and splice mechanism that leaves 2 viral genes, namely E6 and E7, expressed from a strong viral promoter. These two viral genes are oncogenic and these viral transcripts need to cooperate for the initiation of proliferative changes in the transfected host cells. In general HPV 16, 18, 31 or 33 DNA sequences but not HPV 6 or 11 have the ability to immortalise primary epithelial cells[2].

The viral encoded oncoprotein E6 and E7 both form complexes with host cell protein product of two tumour suppressor gene products, namely p53 and Rb, which function to negatively regulate the replication of the cells. Thus, the E6 protein binds to p53 protein and E7 to the Rb gene product. The viral host protein

interaction results in the rapid degradation of the host tumour suppressor proteins and this causes a loss of the negative cell proliferative control exerted by p53 and Rb[24]. Moreover, the continuous interaction of the E6 and E7 proteins with the host cell cycle regulatory proteins, causes chromosomal instability and accumulation of mutations[25]. Thus, the infection of an epithelial cell with an oncogenic HPV is by itself sufficient for malignant conversion of the cell provided that sufficient time elapses following primary infection with HPV. However, epidemiological studies indicate that quite often cofactors are important and facilitate the process of transformation of the infected cell. Some cofactors recognised to play an important role in the progression of HPV infected cells to malignancy include concurrent infection with herpes simplex virus[26], smoking[27] and oral contraceptives and hormones[28,29] and certain microorganisms[30,31].

A small number of cancers of the cervix have been reported without persisting HPV DNA[32]. In our study, one tumour was found to be HPV-negative after PCR and Southern hybridization. The tumour was in a 53-year old Malay patient. One possible explanation for the non-detection could be that the particular cervical cancer was associated with HPV types which were unrelated to the HPVs used in the study and therefore undetectable under our experimental conditions. While it is important to establish whether such tumours are truly negative present methodology does not allow detection of all including as yet unidentified papillomavirus types. Moreover, specific HPV may have been present in the initial stages of the tumour development but were lost in the course of the disease. Nevertheless, there is a possibility that the development of the carcinoma in this Malay patient was indeed HPV-independent and that other factors might be involved in the initiation of the tumour[32]. It has been shown recently for HPV-negative cervical cancers, that the wild tumour suppressor activity of the p53 proteins are inhibited by point mutations resulting in a dysregulated p53 genes in these carcinomas[33].

Acknowledgement

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