Prevalence and intra-type variation of Human Papillomavirus (HPV) infection in cervical cancers: a nationwide perspective of China

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PREVALENCE AND INTRA-TYPE VARIATION OF HUMAN PAPILLOMAVIRUS (HPV) INFECTION IN CERVICAL CANCERS: A NATIONWIDE PERSPECTIVE OF CHINA

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Cervical cancer is the second most common cancer in women worldwide. Epidemiologic studies have shown that the association of human papillomavirus (HPV) with cervical neoplasia is strong, independent of other risk factors, and consistent in several countries. The etiology of the cervical carcinoma have been linked to several oncogenic HPV types. HPV is the most diverse group of DNA viruses involved in human diseases. To date, 85 HPV types have been identified and more than 120 putative novel types have been partially characterized. The classification of the HPV types is based on the nucleotides sequences of E6 and E7 as well as L1 open reading frames (ORFs). The nucleotide sequences difference between 0 – 2% in the same type are classified as variants. Those differing by 2% to 10% are classified as subtypes.

The International Biological Study on Cervical Cancer (IBSCC) Study Group conducted an international study of HPV on invasive cervical cancer. Specimens from 32 hospitals in 22 countries except China were evaluated. Polymerase chain reaction (PCR) using HPV consensus primer pair MY09/11 was used to screen the 1000 specimens collected. HPV was detected in 93% of the tumors with no significant variation in HPV positivity among countries. Some uncommon HPV types showed geographical variation. HPV 16 was the predominant type in most of the countries. It also predominated in squamous cell carcinomas and HPV 18 predominated in adenocarcinomas and adenosquamous carcinomas.

Cervical cancer is relatively common in the Mainland China. The study of HPV and cervical carcinoma started for more than 13 years. The rate of HPV infection among Shanghai women who had cervical cancer was 86.4% (32/37). In Jiangxi Province, the rate of HPV infection in cervical carcinoma was 100% (40/40). In recent years, some reports have indicated that HPV 52 and 58 are prevalent in cervical cancers among Chinese women.

The aim of this study was to determine the prevalence of HPV in cervical carcinoma over China. It was the first extensive multiregional study in China. More than 800 cervical carcinoma tissues were collected in 5 regions of China; Sichuan, Beijing, Shanghai, Guangdong and Hong Kong. PCRs using HPV

consensus primer pairs MY09/11 and GP5+/6+ were used in the screening of HPV DNA in the samples. DNA sequencing, restriction fragment length polymorphism and HPV type specific PCR were used for HPV typing. Intratype variations of HPV were studied by using the DNA sequencing results.

The overall HPV prevalence in cervical cancer in China was 83.9%, ranged from 79%-88% by regions. The infection rate of HPV 16 among total specimens was 66% whereas the infection rate of HPV 18, HPV 31, HPV 52 and HPV 58 was respectively 6%, 1.4%, 2.1%, and 3.1%. In fact, HPV 16 was the predominated type in all regions and in squamous cell carcinomas as well (68.7% of such specimens). Intratype variations of HPV types were not detected in this study.

The results confirmed the role of etiologic factor of HPV in cervical cancer in China. The uncommon HPV type 52 and 58 were found to be highly prevalent in the southeast part of China. In fact, the information of this study is imperative for the development of HPV vaccines targeted to genital HPVs.

摘要

子宮頸癌是全世界第二常見的婦女癌症。近年，許多流行病學研究指出人類乳頭狀瘤病毒(Human papillomavirus, HPV)感染在宮頸癌發生及發展過程中是重要的危險因子，尤其是 HPV16 型和 18 型。至今已鑑定的 HPV 型類別有 85 種 。HPV 類別的分類是基於 HPV 早期基因 6 區，7 區和晚期基因 1 區不同的基因排列。當同類型 HPV 基因排列有 0 至 2%的變異時，它會被分別爲同型變種類別，而那些有 2%至 10%變異的樣本會被分別爲同型另類類別。

國際子宮頸癌研究小組在全球 22 個國家, 32 間醫院(中國除外)收集超過 1000 個樣本，進行 HPV 基因的檢驗。他們採用 L1 區共用引物 MY09/11 聚合酶鏈反應(Polymerase Chain Reaction, PCR)進行檢測。HPV 的檢出率爲 93%，國與國之間 HPV 的感染率並沒有明顯分別。HPV16 在各國的檢出率比其他類別爲高。在鱗狀細胞癌中以 HPV16 感染爲主，而 HPV18 則在腺癌中最爲常見。

子宮頸癌與 HPV 的研究在中國已有十三年以上的歷史。曾有報告指出 HPV 在上海地區的感染率爲 88%，而在江西省則爲 100%。最近有研究指出 HPV 52 和 HPV 58 在中國婦女子宮頸癌中的感染率較其他國家高。

本研究爲全國首個大型，有系統的宮頸癌與 HPV 的研究。目的是確定 HPV 感染宮頸癌在中國的情況。全國五個地區： 上海，北京，四川，廣東及香港共收集超過 800 個子宮頸癌樣本進行 HPV 基因檢測。在研究中採用了 L1 區共用引物，MY09/11 和 GP5+/6+進行 HPV 感染檢測。HPV 類別鑑定則採用基

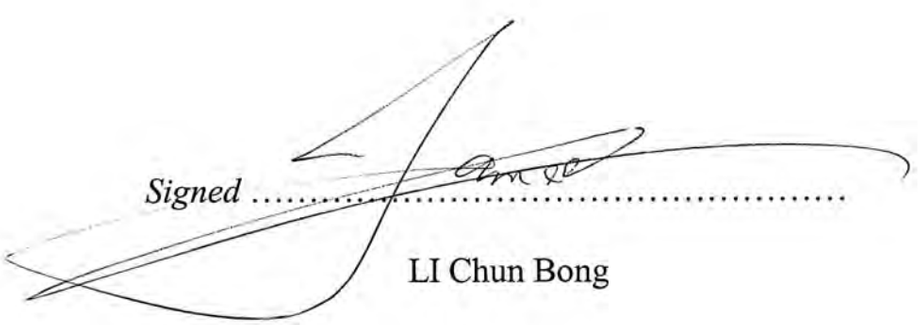
因排序測定，酶切分型及 HPV 類別特定引物 PCR。HPV 基因排序的結果則用來研究 HPV 基因型的變異。

全中國 HPV 在宮頸癌的感染率爲 83.9%，而不同省份中的差別範圍爲 79%至 88%。HPV16 的總感染率爲 66%，而 HPV 18, HPV 31, HPV 52 和 HPV 58 分別爲 6%, 14%,2.1%和 3.1%。HPV16 在鱗狀細胞癌中的感染率爲 68.7%。這些結果與國外其他地區所做的研究相若。

本研究的結果提示 HPV 在流行病學上與宮頸癌有着密切的關係。HPV 52 和 HPV 58 雖然並非最常見的致癌類別，但它們在南中國地區的感染率相對其他中國地區爲高。這些研究結果對發展 HPV 疫苗預防和治療宮頸癌症提供了重要的資料。

DECLARATION

I declare that this thesis represents my own work, except where the due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualification.



Signed .....

LI Chun Bong

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Abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
bp	base pair
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
dGTP	2′-deoxyguanosine 5′-triphosphate
dH_2O	double distilled, deionized water
dNTP	dATP+dTTP+dCTP+dGTP
dTTP	2′-deoxythymidine 5′-triphosphate
E. coli	Esherichia coli
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
GP+	GP5+/6+ primer set
HPV	Human Papillomavirus
IBSCC	International Biological Study on Cervical Cancer
IPTG	isopropylthio-β-D-galactoside
Kb	kilobase pairs
L	liter
MY	MY09/11 primer set
OD	optical denty
ORF	open reading frame
PCR	polymerase chain reaction
rpm	revolutions per minute
sec	second
Tris	tris(hydroxymethyl)aminomethane
TS	HPV type specific primer
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEWS

1.1 Introduction

Cervical cancer is the second most common cancer in women worldwide. Epidemiologic studies have shown that the association of human papillomavirus (HPV) with cervical neoplasia is strong, independent of other risk factor, and consistent in several countries. The etiology of the cervical carcinoma have been linked to several oncogenic HPV types (Bosch *et al.*, 1995). Among all these types, HPV 16 is the most frequently found and a significant risk factor in its aetiology.

To date, 85 HPV types have been identified and more than 120 putative novel types have been partially characterized (de Villiers EM, 1994). Among all these different HPV types, only about 40 types were found in anogenital cancer biopsy specimens. HPV types 16, 18, 31, 33, 39, 45, 52, 58 and 69 are considered to be high-risk types because they are most frequently found in high-grade cervical lesion and in an invasive cancer (Harald zur Hausen, 2000). HPV types 6, 11, 42, 43, 44 are rarely detected in cervical malignant lesions, so they are classified as low-risk types.

The International Biological Study on Cervical Cancer (IBSCC) was organized to study the relationship between cervical carcinoma and HPV. They had collaborated with medical agents of 22 countries to collect the frozen cervical carcinoma biopsies for

the detection of HPV except China. The aim of this study was to determine the prevalence of HPV in cervical carcinoma in a worldwide perspective. Also, the oncogenic relationship between HPV and cervical carcinoma was studied. In fact, there were 93% of the tumors infected with HPV with no significant variation in HPV positivity among countries. HPV 16 was the predominant type in the cervical carcinoma and accounted for 50% of the specimens. HPV 18, HPV 31 and HPV 45 were also found to have high a infection rate from 5% to 14%. There were some rare HPV types detected in the specimens in some countries. HPV 45 was apparent in western Africa and HPV 39 and HPV 59 were detected in Central and South America. These results demonstrated that some of the rare HPV types had geographic variation. (Bosch *et al.*, 1995)

Walboomers *et al* (1999) used the HPV negative specimens from Bosch's study (1995) to perform another experiments for HPV detection and the result was that the detection rate of HPV in cervical carcinoma increased to 99.7% whereas the detection rate of HPV in cervical carcinoma was only 93% in accordance with the study conducted by the IBSCC.

Cervical cancer is relatively common in the Mainland China (Pao *et al.*, 1993). In China, the study of HPV and cervical carcinoma was started for more than 13 years. Most of the studies were conducted in a small scale and were regional-bounded. The rate of HPV infection among Shanghai women who were infected with cervical cancer

was 86.4% (32/37). In Jiangxi Province, the rate of HPV infection was 100% (40/40). All these studies proved that HPV plays an important role in the development of cervical cancer.

In recent years, some reports indicated that HPV 52 and 58 were prevalent in cervical cancers among Chinese women. Another study from Hong Kong showed a significantly alter risk of cervical cancer associated with HPV 58 infection. (Chan et al. 1999) A study in Shanghai showed that HPV 52 and HPV 58 played a more significant role (15 of 35 cases) in the etiology of cervical cancer. (Hunag et. al 1997) Again, according to a study in Jiangxi province, 40 cervical cancer specimens were screened for HPV types and the result showed that HPV 52 and HPV 58 contribute 20% of the total 40 samples. (Lin et al, 1998)

Bosch *et al.* (1995) used a HPV consensus primer pair MY09/11 (MY) that aimed at a 450 base pair (bp) fragment within the HPV L1 open reading frame (ORF) for the detection of HPV DNA in cervical carcinoma. With a view to classifying HPV types in detail, 26 different HPV type specific probes that hybridized on the MY09/11 PCR products were used. In Walboomers's study, another HPV consensus primer pair GP5+/6+ (GP+) that targets the L1 region was also used in screening the HPV negative specimens. The amplified region of GP5+/6+ primer pair was inside the region of MY09/11 primer pair, so a GP+ primer set was actually a nested primer of MY primer set. HPV type specific primers (TS primers) were used to detect the HPV types. These

type specific primers were designed to amplify the oncogenic E7 gene of HPV DNA. In Bosch's study, the infection rate of HPV was 93% but in Walboomers's was 99%. The difference between these two results (6%), indeed, may be either due to the absence of HPV DNA in the specimens or a false-negative HPV result. Integration of HPV DNA in cervical carcinoma may lead to a disruption in PCR primer sequences or a loss of the HPV L1 ORF. The E6 and E7 genes are almost invariable retained, as their expression is necessary for the conversion to and maintenance of a malignant state. (Schearz *et al.,* 1985; Durst *et al.,* 1985; Choo *et al.,* 1987; Wagatsuma *et al.,* 1990; zur Hausen, 1994). These improvements of the detection methods may help to detect the actual HPV DNA in cervical carcinoma.

The aim of this study was to determine the prevalence of HPV in cervical carcinoma over China. This was the first extensive multiregional study in China. It included 5 regions of China: Sichuan (central part of China), Beijing (northern part of China), Shanghai (eastern part of China), Guangdong (southeastern part of China) and Hong Kong (developed city in China). To verify the presence of DNA in the specimens used for PCR, a primer aiming at a 303bp fragments within human β-actin gene was used as an internal control of extracted DNA. All the specimens collected were mounted in a slide stained with H&E, graded, and were classified according to FIGO staging system.

The PCR products of MY primer set were then used as templates of DNA

sequencing or restriction fragment length polymorphism (RFLP) to determine the HPV types. HPV type specific primers targeting E6 and E7 regions of HPV DNA were used as another method to determine the HPV types. When all the above methods were performed with the negative results, GP+ primer set was used as the last experiment to detect the HPV DNA. Intratype variation of the HPV in cervical carcinoma was also determined. This could provide information to see whether the wild types of HPV or its variants infect the cervix.

The results of this study are essential to the development of vaccination strategies to cure cervical cancer. Since of the uncommon HPV type 52 and 58 were found to be comparatively prevalent among the Chinese population, the information gained in this study could help the vaccine developer to have a better understanding of HPV types and its variants in cervical carcinoma.

In the following sections, the background information on the cervical carcinoma, HPV and its detection methods will be introduced.

1.2 Carcinoma of the cervix

The majority of cervical carcinomas develop from an abnormal transformation zone. They are on the ectocervix in the young women and in the endocervical canal in the older woman. The position of the transformation zone determining the position of the tumor. The carcinomas may be exophytic and endophytic. Exophytic can be found in ectocervix and endophytic is in the canal. This causes an expanding tissues and producing the typical barrel-shaped cervix.

Cervical carcinoma can spread locally into cervical stroma, the paracervical and parametrial tissue, the uterus and the vagina. At the later stage of carcinoma, the bladder and rectum can also be affected.

The definitions of squamous carcinoma, adenosquamous carcinoma and adenocarcinoma will be introduced in the following sections.

1.2.1 Squamous carcinoma

Squamous carcinomas constitute more than 70 to 78 percent of cervical malignancies. (Buckley and Fox 1992). They may be well differentiated (large cell tumors showing well-marked keratinization), moderately differentiated (large cell tumors with focal keratinization), or poorly differentiated (large and small cell tumors with minimal evidence of keratinization). The majority of these neoplasms are moderately or poorly differentiated, only about 10 percent being well differentiated. Integrated and episomal HPV types 16 and 18 have been detected in squamous

carcinoma of the cervix.

The well-differentiated carcinomas grow in bands or discrete islands whilst less-well-differentiated neoplasms grow in an infiltrative pattern or form solid sheets. The well-differentiated carcinomas are characterized by intercellular bridges and well-formed epithelial peals.

Moderately differentiated squamous carcinoma shows a greater degree of cellular pleomorphism, higher nucleo-cytoplasmic ratios and more frequent mitoses. Epithelial pearls are rarely seen but individually keratinized, dyskeratotic cells, recognizable by their densely eosinophilic cytoplasm, occur singly or in small groups.

Poorly differentiated squamous carcinomas are often diagnosed because their growth pattern or constituent cells are similar to those of a squamous neoplasm.

1.2.2 Adenosquamous carcinoma

Endocervical-type adenocarcinoma constitutes the largest group of cervical adenocarcinomas. A large number of reports indicate that, overally, adenocarcinomas have no difference in prognosis from squamous tumor. These tumors show a greater tendency to metastasize widely, even when the lesion in the cervix is small, and are disproportionately common in women under the age of 40 years (Buckley et al., 1988).

1.2.3 Adenocarcinoma

Adenocarcinomas of the cervix appear to be increasing in incidence relative to squamous carcinomas of the cervix. Adenocarcinomas now constitute from 10% to 25% of reported cases of cervical carcinoma. This may be due to an absolute increase of the adenocarcinomas or the stability of the adenocarcinomas. The age of adenocarcinoma patients are younger than that of the squamous cell tumors. Even within the adenocarcinomas group, the age is also decreasing.

It is difficult to distinguish adenocarcinoma grossly from squamous carcinoma, having similar morphology and site of origin in the cervix. HPV types 16 and 18 have been identified in different types of adenocarcinomas of the cervix by means of PCR or other hybridization techniques. HPV type 18 is the most common type in adenocarcinomas.

The prognosis for women with adenocarcinoma of the cervix is significantly worse than those with squamous carcinoma. The majority is composed of tissues resembling that of the normal endocervix and may be very well differentiated, so called minimal deviation carcinoma, or more typical endocevical carcinoma. They develop most commonly from the columnar epithelium of an ectopy, or more correctly from an adenocarcinoma *in situ*. Adenocarcinomas also exhibit neuroendocrine differentiation and there may, therefore, be systemic manifestations of their presence. HPV infection has been determined even in tumors of these types.

1.3 Molecular biology of Human papillomavirus

In the age of molecular biology, the technology of cloning and describing the genetics of and organism has been well developed. According to the following studies, the whole organization of the human papillomaviruses has actually been observed.

1.3.1 Genome structure and organization of HPV

Papillomaviruses are small, non-enveloped DNA viruses with a circular, double stranded DNA genome of approximately 7200-8000 base pairs (Figure1.1). All putative protein-coding sequences, called open reading frames (ORF), are restricted to one strand (Pfister *et al.*, 1994; Gross and Krough, 1997) and each ORF segment of DNA is large enough to encode for a protein.

The early (E) and late (L) sequences are classified according to the sequence of expression in the viral life cycle. E genes encode for viral replication and cellular transformation and are detectable in the proliferate areas of HPV-induced lesions. The late (L) genes, L1 and L2 encode for the structural proteins and their expression is restricted to the differentiating part of the epithelium where viral DNA replication also occurs (Fuchs *et al.*, 1994; Turek, 1994; Gross and Krogh, 1997). The promoter and the enhancer DNA sequences are located at the non-coding region (NCR) or long control region (LCR) or upstream regulatory region (URR) which in between E6 and L1 genes. Enhancer and promoter DNA sequences are critical to viral replication.

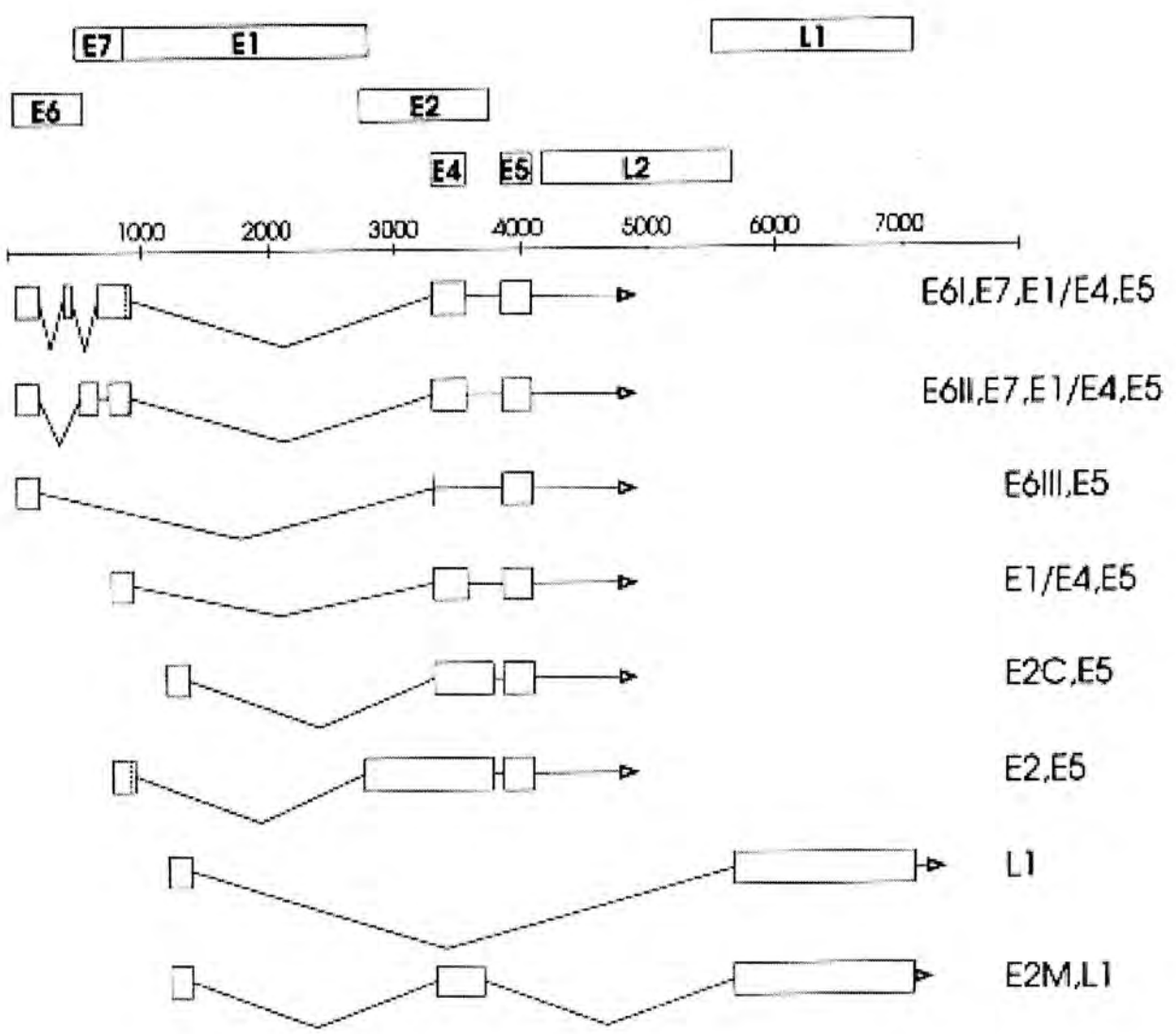


Fig 1.1 Organisation of HPV 16 genome (adapted from Syrjänen, K. and Syrjänen, S. 2000)

1.3.2 Expression of papillomavirus genes

In the following sections, different HPV genes, E1, E2, E4, E5, E6, E7, L1 and L2 will be introduced.

HPV E1 protein

The E1 ORF represents the most conserved structure among different PV types. E1 codes for a polycistronic RNA and the E1 protein contains a highly conserved C-terminal region, with homology to SV 40 large T antigen such as ATP binding, ATPase and helicase domains as well as a divergent N-terminal domain (Clertant *et al.*, 1984; Seo *et al.*, 1993; Yang *et al.*, 1993).

E1 is essential for viral replication (Ustav *et al.*, 1991 a,b) and it also has an important role in retaining the episomal state of the viral molecule, i.e. it remains separate from the host cell chromosome (Sarver *et al.*, 1984, Lusky *et al.*, 1985). Mutational studies have recently shown that a mutation in the E1 ORF resulted in integration of HPV 31 (Frattini *et al.*, 1996).

E1 has a binding site in the origin of replication (*ori*) located in the proximal end of the LCR (Holt *et al.*, 1994). E1 had been shown to interact with cellular DNA polymerase (Park *et al.*, 1994; Bonne-Andrea *et al.*, 1995)

E2 Protein

E2 protein is a DNA-binding protein that regulates viral transcription and replication (Ham *et al.*, 1991; McBride *et al.*, 1991). It also acts as a potent

transcriptional activator and greatly increases viral DNA replication by co-localizing with E1 to the origin of replication. The full-length E2 protein (E2-TA) can function as a trans-activator or a repressor, depending on the location of the E2 binding sites in a responsive promoter.

E4 Protein

The interaction between PVs and the cytoskeleton seem to be mediated by viral E4 proteins derived primarily from the E4 ORF , which represents a region of maximal divergence between different HPV types (Brown *et al.*, 1988; Door bar *et al.*, 1991; Jorreborg *et al.*, 1991). The most abundant viral messages is formed by a single splice between the beginning of the E1 ORF contains the first five amino acids of the E1 ORF fused to the last 85 amino acids of the E4 ORF (Nasseri *et al.*, 1987). Although E4 proteins are expressed in large quantities in benign squamous cell papillomas, their role in the virus life cycle has remained exclusive.

No mutation of HPV 16 E4 sequence results in a mutant protein, which fails to co-localize to the CK intermediate filaments. The C-terminal domain also is not an essential for association with the cytoskeleton, and deletion of the C-terminal sequences (residues 84-92) corresponding to the part of a domain conserved between mucosal E4 proteins, affects the ability of the mutant protein to induce cytoskeletal collapse, despite co-localization with the keratin intermediate filaments (Roberts et al., 1994). These implicate that the E4 proteins include functional domains at the

N-terminus and the C-terminus, with the intervening sequences possible acting as a flexible hinge (Roberts *et al.*, 1997)

E5 Protein

E5 is capable of causing efficient fibroblast transformation in the absence of other viral oncogenes. (Schiller *et al.*, 1989; Horwitz *et al.*, 1991; Auvinen *et al.*, 1997) It is a hydrophobic, 44 amino acid protein with two distinct structural features: (1) a hydrophobic 30 amino acid N-terminal region which functions as a transmembrane domain; and (2) a hydrophilic 14 amino acid C-terminal region which contains two cysteine residues essential for homodimer formateion (Burkhardt *et al.*, 1989 Tada *et al.*, 1989; Sparkowski *et al.*, 1996)

It is less known about the E5 gene in HPV-induced lesions. The E5 ORF is located at the 3' end of the E region. In the nucleotide sequence of HPV 16, a putative E5 ORF was initially localized, but without a translation initiation codon of its own (Seedorf *et al.*, 1985). Subsequently, however, this was found to be due to a sequencing error, and an additional T at nucleotide 3906 was found (Bubb *et al.*, 1988). An initiation codon at nucleotide 3849 similar to the ORF of other PVs was recognized (Bubb *et al.*, 1988; Halbert *et al.*, 1988)

Recently, some degree of homology between the HPV 18 E5 ORF and human or mouse genomic sequences have been found. Kahn *et al.* (1992) described HPV 18 E5-related transcribed mouse sequences, and Q300. Sequence alignments showed a

close similarity as well to Q300 (Kahn et al., 1992) and different viral and human growth factors, allowing it to be fitted into a putative phylogenetic chromosome 7, at 7p13-14, as determined by E5 sequences may constitute an HPV DNA integration target, which is often located near chromosomal breakpoints, oncogens, etc. (Geisen et al., 1995)

E6 Protein

In our study, most of the HPV type specific primers were designed within the E6 and E7 regions. In the next 2 sections, the functions of E6 and E7 proteins will be introduced.

The E6 protein is a major transforming protein of many types of PVs. E6 alone cannot immortalize human keratinocytes (Halbert *et al.*, 1991; Sedman *et al.*, 1991) but is capable of immortalizing mammary epithelial cells in culture (Band *et al.*, 1990). HPV E6 is a basic protein that contains two C-terminal zinc-binding motifs. Mechanistically, the best-characterized E6 proteins are those of the high-risk gential HPVs (e.g. HPV 16 and HPV 18 E6). The schematic structure of HPV 16 E6 protein is given in Figure 1.2 E6 can form complexes in vitro with the tumor suppressor gene product p53 (Werness *et al.*, 1990), sharing similarities in respect with adenovirus E1B protein and the SV40 large T antigen.

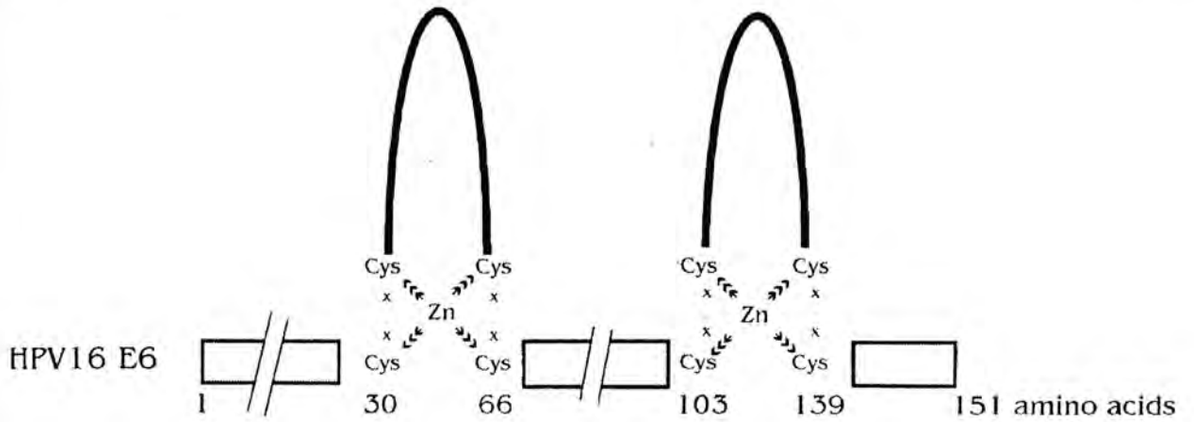


Figure 1.2 Structure of HPV 16 E6 protein (adapted from Syrjänen, K. and Syrjänen, S. 2000)

In contrast to E1B and large T, which inactivate p53 through their binding, E6-p53 complex formation requires an additional cellular protein, E6-AP (Huibregtse *et al.*, 1991), leading to complete degradation of p53. E6 binds directly to E6-AP protein, which functions as a specific ubuqutin-protein ligase, E3 targeting the E6-E6-AP complex to p53 (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993). The degradation of p53 proceeds through the ubiquitin-dependent pathway, which involve the activation of ubiquitin-conjugating enzyme E2 by ubiqitin-activating enzyme E1, and transfer of uniquitin (E3) to a lysine reside of the target protein. E6, E6-AP and p53 is an absolute requirement for degradation. E6 mutants are capable of binding with p53 but have lost their ability of mediate degradation. (Crook *et al.*, 1991, Pim *et al.*, 1994; Dalal *et al.*, 1996). E6 can form a complex with p53 in the absence of E6-AP, although this interaction is much weaker than with E6-AP. (Lechner *et al.*, 1994).

The liner binding site for E6 within the p53 is still unknown. Different regions of

p53 are shown to be involved, and the conformation of p53 is important to form this complex (Scheffiner *et al.*, 1992; Marston *et al.*, 1994; Mansur *et al.*, 1995; Kubbutat *et al.*, 1996). Pim et al., (1997) recently reported that HPV 18 E6* protein will interact both with the full-length E6 proteins from HPV 16 and HPV 18, and also with E6-AP, and subsequently blocks the association of the full-length E6 protein with p53. They also showed that, as a result of this block, E6* can inhibit E6-mediated degradation of p53, both in vivo and in vitro. The biological consequences of this are: (1) an increased transcriptional activity on p53-responsive promoters; and (2) an inhibition of cell growth in cells transfected with E6* (Pim *et al.*, 1997). This may represent a means by which HPV can modulate the activity of the full-length E6 protein with respect to p53 during viral infection.

E7 Protein

E7 is a small acidic nuclear phosphoprotein with a zinc-binding motif located in its C-terminal half that is important for its stability and dimerization. The N-terminal region contains two conserved regions, CR1 and CR2, which have sequence similarity to E1A protein of adenovirus and large T antigen of SV40. Both regions are important for transformation *in vitro*. Mutations in these regions destroy transforming activity. The LXCXE motif, located within CR2 (residues 22-26), includes the binding site for the tumor suppressor gene product, retinoblastoma protein p105 RB and the related p107 and 130 proteins. (Dyson *et al.*, 1989, 1992; Firzlaff *et al.*, 1989; Vousden, 1993;

Kubbutat *et al.*, 1996). These phosphorylation sites are needed for efficient transformation but not for pRB binding. Binding of E7 to the hypophosphorylated form of RB releases the transcription factor E2F. E2F motif has been found in the promoters of several cellular genes involved in cell proliferation. The structure of HPV 16 E7 protein is illustrated in Figure 1.3

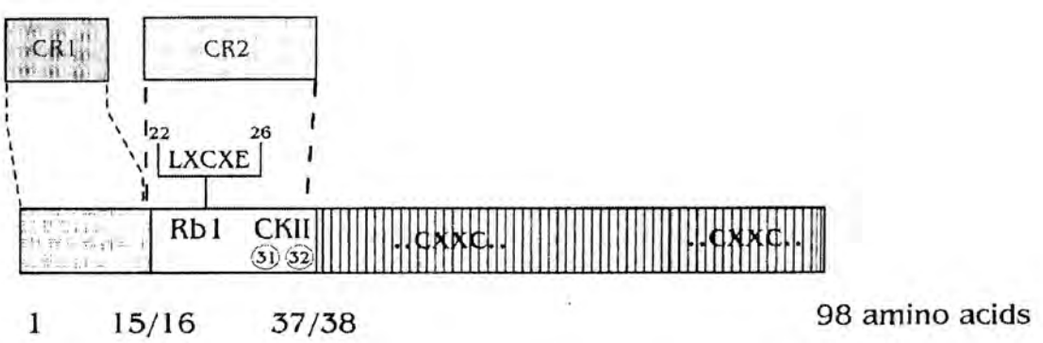


Figure 1.3 Structure of HPV 16 E7 protein (adapted from Syrjänen, K. and Syrjänen, S. 2000)

Although efficient keratinocyte immortalization requires cooperation between the E6 and E7 genes, HPV E7 (but not E6) can immortalize human keratinocytes alone when expressed at high levels (Halbert et al., 1991; Sedman et al., 1991). E7 protein of the low risk HPV types also binds to pRB but with lower affinity, indicating its significance in transformation. However, it should be noted that E7 of the low-risk HPV 1 can bind to pRB with the same affinity as E7 of HPV 16, indicating that mechanisms other than pRB binding are of importance for E7-associated malignant transformation (Ciccolini et al., 1994)

These studies suggest that both E6 and E7 maybe important proteins for both cell transformation and regulating the cell's mitotic cycle, allowing continuous proliferation of host cells. Human cancers are characterized by failure of cell cycle checkpoints, resulting in genetic instability. Clearly, E6 and E7 oncoproteins from high-risk HPV types are able to disrupt the integrity of these checkpoints.

L1 Protein

The 2 consensus primers used in our study were designed to amplify the L1 (55kDa) region because it was conserved among different HPVs. It is a major capsid protein. Cryo-electron microscopic image reconstructions of HPV capsids show that L1 proteins from pentameric capsomers with a similar structure to those of polyomavirus VP1 protein, including a central hole in each capsomer similar to that of polyomavirus (Baker *et al.*, 1991).

The mechanisms that control HPV L gene expression are still not fully elucidated. Studies on the interaction of HPV with the host cells and the control of HPV L gene expression are hampered by the lack of an *in vitro* cell culture system for efficient propagation of the virus. The onset of the L genes coding for L1 and the production of the virus are strictly linked to the differentiation stage of the infected cells, and L1 proteins are detected primarily in terminally differentiated cells in the upper layers of the epithelium, the intimate association between HPV late gene expression and epithelial cell differentiation is likely to be regulated by *cis*-acting sequences on the

viral gene of viral mRNAs and by *trans*-acting viral or cellular factors produced in infected cells.

L2 Protein

L2 protein's (50kDa) function is unclear evidence suggests that although it is not require for receptor binding, L2 is required for infectivity (Mose-Larsen *et al.*, 1987; Zhou *et al.*, 1993; Roden *et al.*, 1994a) L2 quantity is approximately 1/10 that of L1 (Doorbar *et al.*, 1987). Recombinant L1 protein self-assembles into particles resembling virus capsids, but assembly is enhanced by the presence of L2 protein, which may be required for the assembly of infectious virions as well as for DNA-binding (Hagensee *et al.*, 1993; Zhou *et al.*, 1991, 1993).

While studying the capsidation of HPV DNA, Zhou *et al.* (1994) showed that L2 protein, but not L1 protein, could bind the HPV DNA. Only the N-terminal of L2 was required for DNA binding.

1.3.3 Taxonomy of HPV

Papillomaviruses have been classified as a subfamily (Shah & Howley, 1992) together with polyomaviruses in the family Papovaviridae. There have been several reviews of the classification of papillomaviruses, both by phenotypic aspects and sequence similarities (Chan *et al.*, 1992; van Ranst *et al.*, 1992; Bernard *et al.*, 1994; Myers *et al.*, 1994; de Villiers, 1994; Chan *et al.*, 1995) The characterization of a new HPV type was based on the viral DNA sequence from ORFs E6, E7 and L1 and currently only from L1. The nucleotides sequences of the E6, E7 and L1 genes differ by more than 10% from those of any previously described HPV types (de Villiers, 1994) will be classified as a novel type of HPV. The difference of the DNA sequences of these 3 genes is less then 2% is classified as variants. The difference between 2% to 10% is classified as subtypes. (Gissmann *et al.*, 1983; Deau *et al.*, 1993; Ho *et al.*, 1993; Ong *et al.*, 1993)

The similarities of E6, E7 and L1 genes were used to identify and grouping of related HPVs into 5 subgroups. In the 5 subgroups of papillomaviruses, three contain HPVs (supergroups A, B, E). (Chan *et al.*,1995) The largest supergroup, A, includes 44 HPV types and untyped genomes (Chan *et al.*, 1995), can be subdivide into eleven subgroups. One of these groups contains HPV 16, HPV 31, HPV 33 and HPV 35 and another contains HPV 18 and HPV 45, the HPV types most frequently reported in association with malignant genital lesions. (Figure 1.4)

Until now, 85 HPV types have been identified and fully sequenced; more than 120 putative novel types have been partially characterized. (zur Hausen, 2000). All identified types infected epithelial cells either of the skin or of the anogenital and oropharyngeal mucosa. No evidence for HPV infections has yet been found in the gastric, ileojejunal, or colon mucosa.

The taxonomic status and the origin of the cloned genome from HPV 1 to HPV 70 were summarized in Figure 1.5.

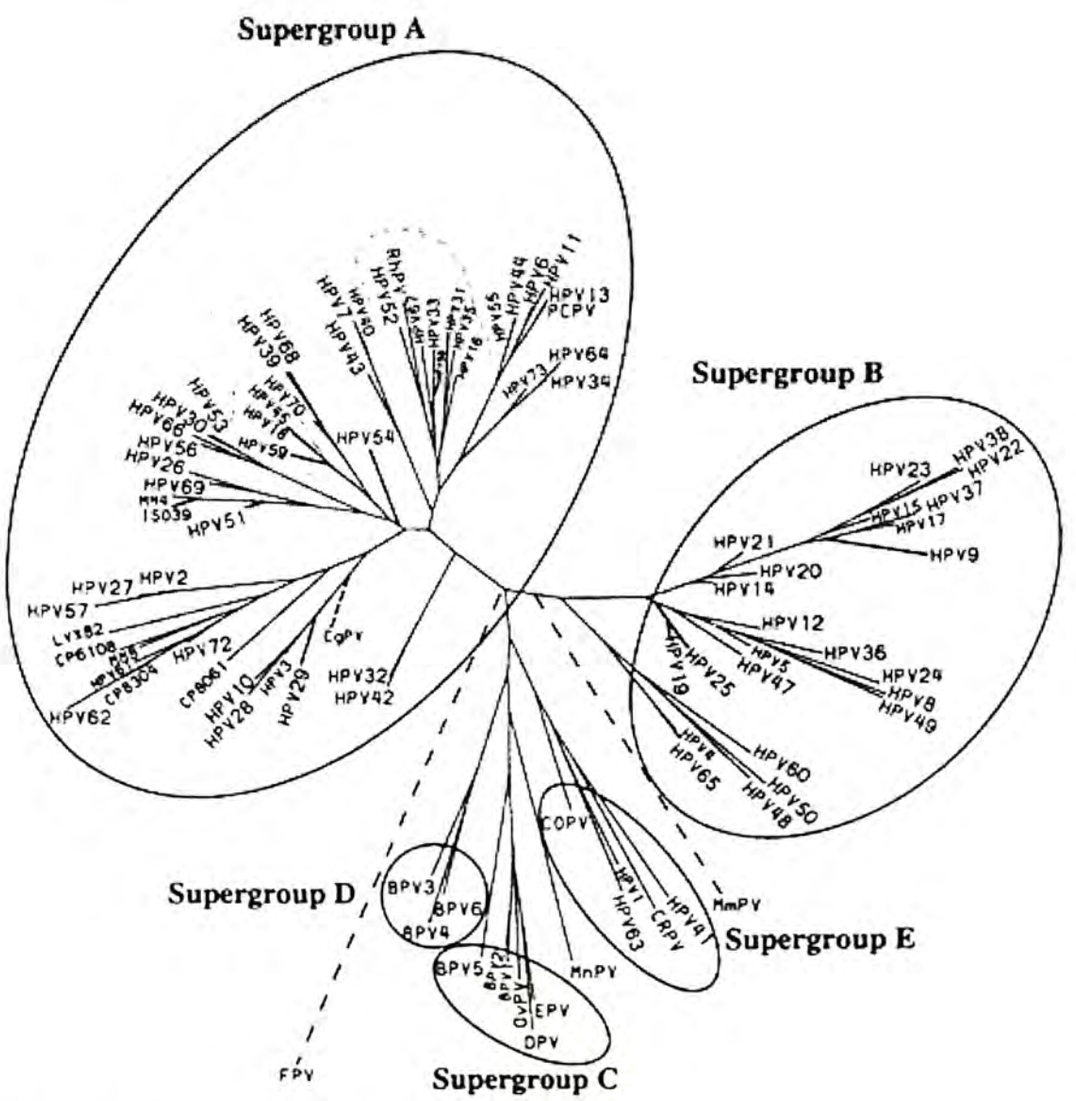


Figure. 1.4 Relationship among 95 human and animal papillomaviruses, in the form of a phylogenetic tree (adapted from Chan *et al.*, 1995)

HPV type	Taxonomic status (supergroup)[a]	Origin of cloned genome[b,c]
HPV-1	E	Verruca plantaris
HPV-2	A	Verruca vulgaris
HPV-3	A	Verruca plana
HPV-4	B	Verruca vulgaris
HPV-5	B	EV lesion
HPV-6	A	Condyloma acuminatum
HPV-7	A	Butcher's wart
HPV-8	B	EV lesion
HPV-9	B	EV lesion
HPV-10	A	Verruca plana
HPV-11	A	Laryngeal papilloma
HPV-12	B	EV lesion
HPV-13	A	Focal epithelial hyperplasia
HPV-14	B	EV lesion
HPV-15	B	EV lesion
HPV-16	A	Cervical carcinoma
HPV-17	B	EV lesion
HPV-18	A	Cervical carcinoma
HPV-19	B	EV lesion
HPV-20	B	EV lesion
HPV-21	B	EV lesion
HPV-22	B	EV lesion
HPV-23	B	EV lesion
HPV-24	B	EV lesion
HPV-25	B	EV lesion
HPV-26	A	Verruca vulgaris
HPV-27	A	Verruca vulgaris
HPV-28	A	Verruca plana
HPV-29	A	Verruca vulgaris
HPV-30	A	Laryngeal carcinoma
HPV-31	A	CIN
HPV-32	A	Focal epithelial hyperplasia
HPV-33	A	Cervical carcinoma
HPV-34	A	Bowen's disease
HPV-35	A	Cervical carcinoma
HPV-36	B	Actinic keratosis
HPV-37	B	Keratoacanthoma
HPV-38	B	Malignant melanoma
HPV-39	A	PIN
HPV-40	A	PIN
HPV-41	E	Disseminated warts
HPV-42	A	Vulvar papilloma
HPV-43	A	Vulvar hyperplasia
HPV-44	A	Vulvar condyloma
HPV-45	A	CIN
HPV-46 (= HPV-20b) (not recognized as separate type)		
HPV-47	B	EV lesion
HPV-48	B	Cutaneous squamous-cell carcinoma
HPV-49	B	Verruca plana
HPV-50	B	EV lesion
HPV-51	A	CIN
HPV-52	A	CIN
HPV-53	A	Normal cervical mucosa
HPV-54	A	Condyloma acuminatum
HPV-55	A	Bowenoid papulosis
HPV-56	A	CIN, cervical carcinoma
HPV-57	A	Inverted papilloma of the maxillary sinus
HPV-58	A	CIN
HPV-59	A	VIN
HPV-60	B	Epidermoid cyst
HPV-61	A	VaIN
HPV-62	A	VaIN
HPV-63	E	Myrmecia
HPV-64	A	VaIN
HPV-65	B	Pigmented wart
HPV-66	A	Cervical carcinoma
HPV-67	A	VaIN
HPV-68	A	Genital lesion
HPV-69	A	CIN
HPV-70	A	Vulvar papilloma

Figure 1.5 Taxonomic association and origin of HPV types 1 to 70

(adapted from IARC monographs volume 64)

1.4 Diagnostic techniques in HPV detection

There are no suitable serological tests available for diagnosis of HPV infections, and the virus cannot be isolated from clinical specimens by cell culture techniques. Rapid progress made in the field of recombinant DNA technology, with the availability of specific probe, has enabled the detection of HPV genomic sequences in different clinical HPV lesions. Cytology and histology are usually sufficient for the proper diagnosis of clinical HPV infections, whereas DNA tests are necessary for reliable detection of subclinical and latent HPV infections. HPV DNA detection, however, increase the accuracy of HPV diagnostics.

1.4.1 Southern blot analysis

The extracted DNA from tissue, smear or cell cultures are digested with restriction enzyme or undigested. It can be electrophoresis according to their size to give different DNA fragments. The DNA fragments migrate to the cathode at different speed according to their size. Appropriate DNA size standard are run simultaneously. The DNA can be visualized on the agarose gel that stained with ethidium bromide under ultraviolet light illumination. The DNA will blotted on a membrane. The image of the electrophosis will exactly transfer to the membrane. After transfer to the membrane, a HPV type specific probe can bind on the membrane if the HPV DNA is stack on it. Nowadays, the probe can be radioactive or non-radioactive. By varying the hybridization conditions, heterologous DNA fragments can also be detected. After 48

hours of hybridization, the membrane is washed and put into the cassette for autoradiography for several days to weeks. The hybridized labeled probe yields distinct bands, corresponding to the complementary sequences in the cellular DNA. (Figure 1.6)

Southern blot technique can be determining the physical status of HPV in the samples detected. By using this technique, it can distinguish between episomal and integrated viral DNA. The physical status of the HPV in the host might provide an important information for the study of the transition of pre-malignant lesions to cervical cancer. Episomal DNA is mainly detected in benign lesions while integrated HPV DNA is mainly detected in cervical cancer. Moreover, sometimes rearrangements in the genome can also be detected.

Southern blot is considered to be the "golden standard" for HPV detection and typing. The sensitivity of this method is about 1pg HPV DNA in 10μg genomic DNA can be detected. This method is time consuming and labor intensive process. It requires large quantities of sample DNA.

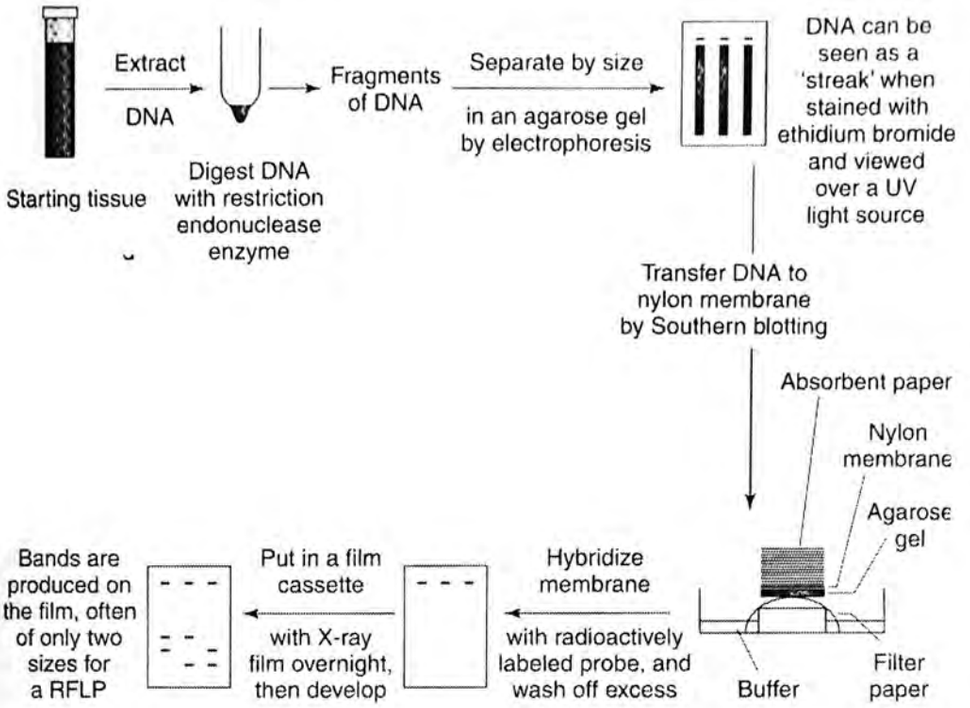


Figure 1.6 Schematic representation of the process of Southern Blot

1.4.2 Dot blot analysis

The dot blot procedure is a more rapid method for DNA hybridization. Using this technique, the extracted total cellular DNA is not digested with restriction enzyme. The DNA is not electrophoretically separated and fixed onto the membrane. The amount of DNA used in this technique is fewer than southern blot. Only 300-500ng of DNA per membrane is required. The sensitivity of dot blot hybridization is approximately one copy per genome equivalent when 500ng of cellular DNA is applied to the filter. The other reason why dot blot is not as accurate as southern blot is the band size is not determined. The signals of the dot blot produced may due to non-specific hybridization. This can be avoided by using high-stringency condition of each probe. Also if high copy number of HPV present in the sample, other type of HPV probes can hybridize to

the membrane. In this case, false positive result produced and this sample should not be interpreted as true positive. In addition, it has been shown that all the samples, whether HPV positive and negative, will hybridize with the HPV-probe under low-stringency conditions, because all DNA is focused on a single spot. (Melki *et al.*, 1988; Roman *et al.*, 1989). High quality of DNA, especially its purity, is mandatory for the specificity of the hybridization result.

Dot blot analysis had been used to develop commercial HPV detection kits, called ViraPap and ViraType. They are developed by Digene, Silver Spring, Maryland, USA and have been widely used in several large scale epidemiological studies. (Munoz *et al*, 1988, 1992a, c; Kjaer *et al.*, 1989, 1990).

1.4.3 In Situ Hybridization

The strength of the *in situ* hybridization (ISH) technique is the preservation of morphology of the specimen, which permits localization of HPV within tissue and the infected cell (Brigati *et al.* 1983). Histological sections are cut on specially treated slides (Rentrop *et al.*, 1989) to avoid the detachment of the sections during the hybridization procedure. The probe can be permeable to the cell by the proteinase digestion so the nucleic acid can be access to the sample. Denaturation of the target DNA and the labeled probe is done simultaneously, using high temperature. The probe will hybridize on the slide and the excess probe will be washed away. The detection of

target-probe duplexes is done either by autoradiography (for ^{35}S- or ^{3}H-labelled probes) or by enzymatic detection methods (i.e. colour precipitation of labeled hybrids as a result of catalysis by enzymes, such as alkaline phosphatase or horseradish peroxidase).

It is very difficult to detect the sensitivity of this method because the viral copy of each sample is different. Base on the cultured cell line analysis, known copy number of HPV is used. The sensitivity of the ^{35}S-probes vary between 20 and 100 copies per cell (Ostrow *et al.*, 1987a; Collins *et al.*, 1988). The sensitivity of biotinylated DNA probes varies from as low as 800 copies per cell (Collins *et al.*, 1988) to the levels comparable with or even higher than that of ^{35}S-labelled probes (Burns *et al.*, 1987; Syrjanen *et al.*, 1988; Heiles *et al.*, 1988; Mullink *et al.*, 1989). This wide variation in the sensitivity of biotinylated probes is due to the different detection system, rather than biotin itself. Sense and anti-sense RNA probes are both use in radiolabelled or biotinylated. Sense probe is RNA that is identical to mRNA and this will detect the DNA. Anit-sense RNA probes hybridized to non-denatured tissue sections will detect mRNA. It has been shown that RNA probes are more sensitive and specific than the comparable DNA probes.

The benefit of *in situ* hybridization is that localize of the infected cell can be achieved. Only the HPV infected cells can be labeled, so the pathologists can be easily distinguished. The other advantage is the rate of false positive signals will be less. Using two consecutive sections stain together can exclude the false positive signals.

For paraffin-embedded samples that is poorly suited for dot blot or southern blot, ISH can be give a reliable result in detecting HPV.

1.4.4 Hybrid Capture System

Diagene Diagnostics has been one of the most active companies working with the development of HPV tests of clinical routine. The first two diagnostic tests provided by Digene Diagnostics were ViraPap (for HPV screening) and ViraType (for HPV typing), which were widely adopted in HPV research and routine clinical diagnosis as well. After a few years of use, however, it was realized that both these tests shared several disadvantages, inherent to dot blot.

The most updated FDA approved HPV test is hybrid capture test. This is based on the solution hybridization principal. Hybird capture is a simple, non-radioactive immunoassay that employs RNA probes to detect single-stranded target DNA (Lorincz, 1992; Ferenczy, 1995a). Hybrids are captured onto the surface of a plastic tube by immobilized antibodies specific for RNA-DNA hybrids. Detection of the hybrids is done with an alkaline phosphatase-conjugated RNA-DNA antibody, followed by incubation in a chemiluminescent substrate. A luminometer records the results, yielding quantitative results within 4 hours (Sun *et al.*, 1995; Ferenczy *et al.*, 1996; Reid *et al.* 1996).

Hybrid capture test can detect up to 16 different HPV types in a single reaction.

Those 16 HPV types are commonly found in lower genital tract. The sensitivity of this method is comparable to the traditional southern blot technique. Evaluation of hybrid capture test with highly sensitive primer-specific (GP+ primers) PCR had been done. Compare the hybrid capture system and consensus primer PCR for high-risk HPVs and reported a 86% agreement in a series of unselected consecutive patients (Farthing *et al.*, 1994). In screening cohort analyses of 967 smears, Schneider *et al.* (1996) found that hybrid capture detected only 50% of the HSILs.

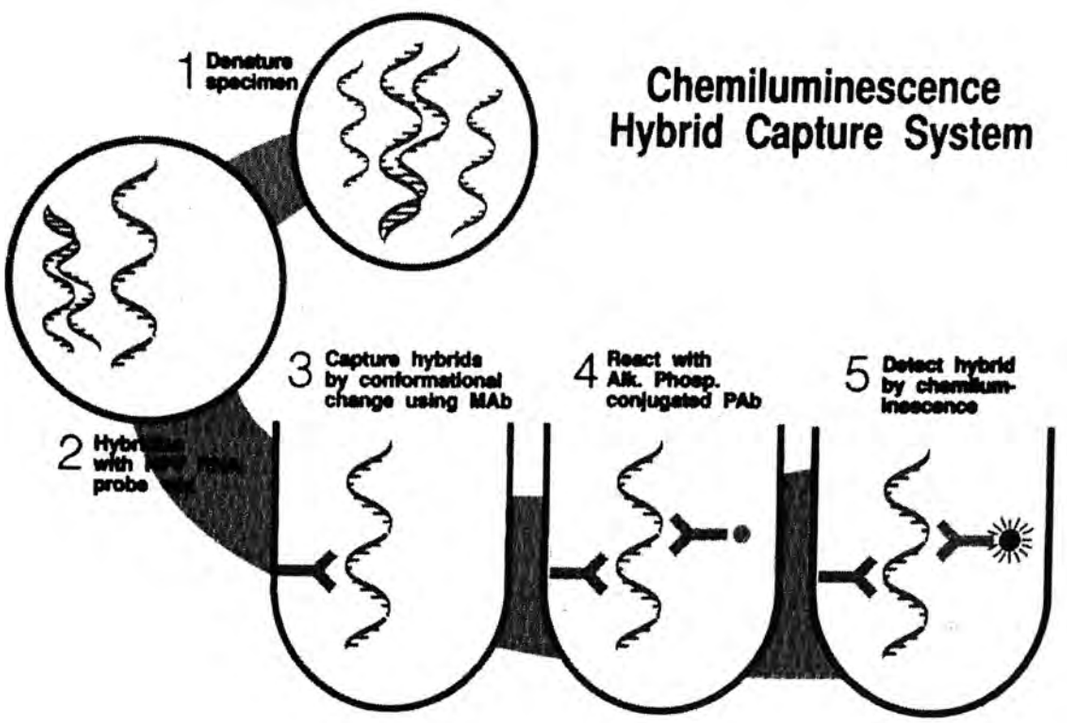


Figure 1.7 Schematic representation of the process of Hybird Capture System

1.4.5 Polymerase Chain Reaction

PCR is an *in vitro* method for primer-directed enzymatic amplification of specific target DNA sequences over a million times in a few hours. (Saiki *et al.*, 1985; Mullis *et al.*, 1987; Morris *et al*, 1988) PCR amplification of DNA is achieved by using oligonucleotide primers, also known as amplimers. These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on single-stranded denatured DNA (template) by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. The results in the synthesis of new DNA stands are complementary to the template strands. These strands exits at this stage as double stranded DNA molecules. Strand synthesis can be repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. Each new DNA stand synthesis comprises a cycle of amplification. Each new DNA stand synthesized becomes a template for any further cycle of amplification and so the amplified target DNA sequence is selectively amplified cycle after cycle. (Fig 1.8) The first extension products result from DNA synthesis on the original template and these do not have a distinct length as the DNA polymerase will continue to synthesize new DNA until either stops or is interrupted by the start of the next cycle. The second cycle extension products are also of indeterminate length; however, at the third cycle,

fragments of 'target' sequence are synthesized which are of defined length corresponding to the positions of the primers on the original template. From the fourth cycle onwards the target sequence is amplified exponentially.

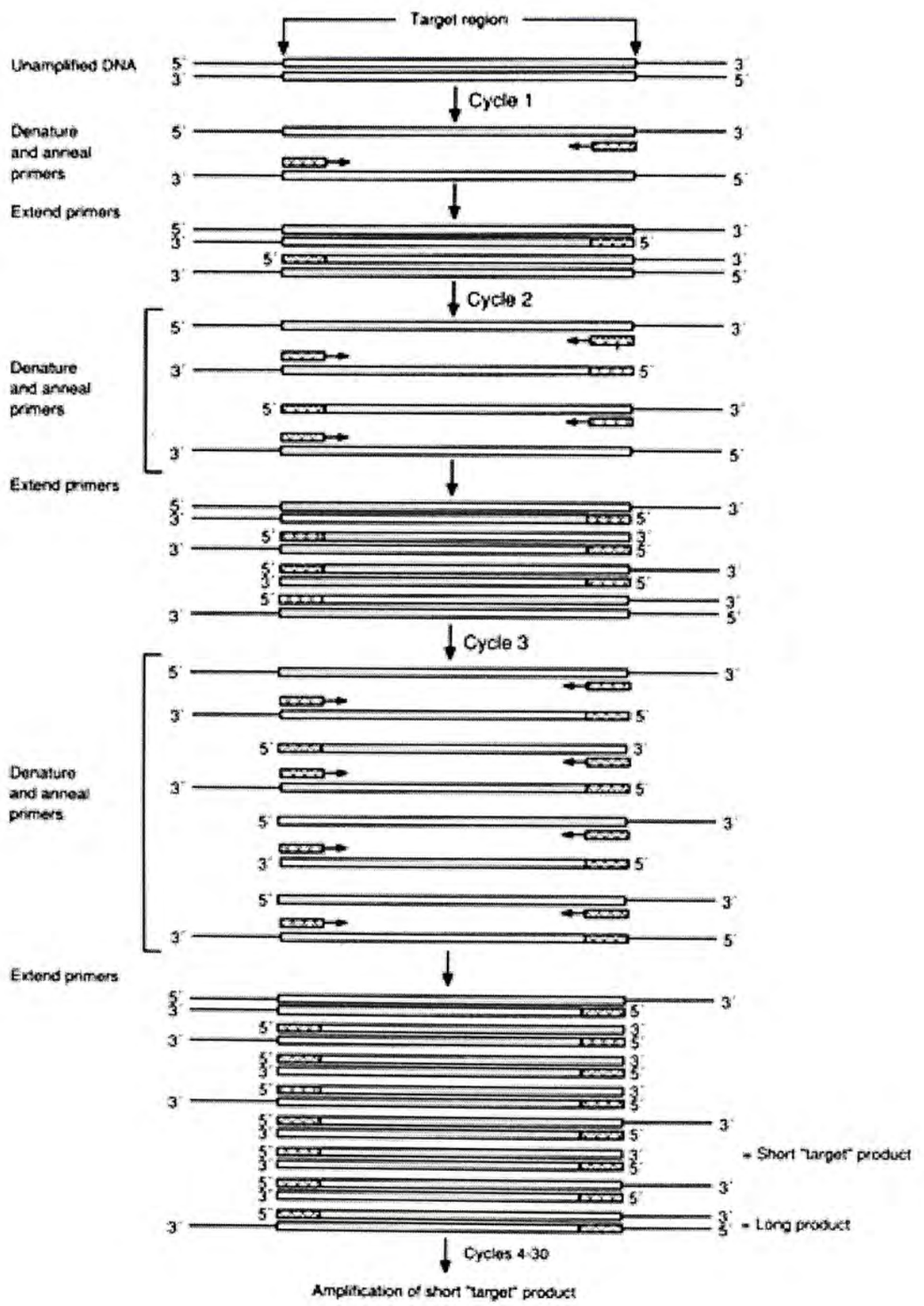


Figure 1.8 Schematic representation of the process of polymerase chain reaction

If enough amplified DNA is available, diagnosis can be read directly on the gel after electrophoresis and ethidium bromide staining. However, all gels have to be hybridized with specific oligonucleotide probes covering part of the amplified DNA to confirm the specificity of the PCR product. Hybridization will usually result in additional positive samples not visible in the gel, because it sensitizes the method further. In additional to hybridization, the specificity of PCR results can be confirmed by restriction enzyme digestion or direct sequencing of the PCR product. The sensitivity of PCR depends on several factor, including the concentrations of target DNA, polymerase enzyme, Mg-ions, primers etc.

1.5 Human papillomavirus in cervical carcinoma

1.5.1 Prevalence

From the epidemiological point of view, it is of critical importance to establish the prevalence of genital HPV infection at the population level, as a measure of the disease burden in the general population (Bosch *et al.*, 1995; IARC, 1995). This can be measured in different populations with a suspected different prevalence of HPV infections, including virgins and nuns, healthy women with normal PAP smear (a low prevalence group), those attending STD clinics (high prevalence), those suffering from immunosuppression (due to AIDS or transplantation), as well as women with a detected cervical cancer (Fisher *et al.*, 1991; Griffiths, 1991; Chang *et al.*, 1992; Lorincz *et al.*, 1992; Meekin *et al.*, 1992; Agorastos *et al.*, 1995; Bosch *et al.*, 1995, Persson *et al.*, 1996). The detection rate of HPV is critically depends of the sensitivity of the detection techniques, and only the most sensitive PCR methods can provide useful information on the prevalence of HPV in heavy mucosa (IARC, 1995).

As the development of PCR technique, there are many studies on the prevalence of HPV infection had been published. The studies used PCR amplification for the detection of HPV, the variation reported (0-47.9%) in the prevalence of HPV DNA in these PAP smear-negative women is remarkably high. One of the sources of inconsistency is the technique of PCR amplification. It is because PCR can detect even 1 copy of HPV DNA. This will give the false positive result. However, some of the

studies showed the HPV DNA prevalence is age-dependent, with the highest prevalence figures found in younger women. (Purola et al., 1977; Syrjanen, 1979a,b). Some of the studies showed that HPV DNA seems to be virtually absent in women with no reported sexual activity (Fairley et al., 1992; Rylander et al., 1994; Critchlow et al., 1995a) According to the data; there is no geographical variation in the prevalence and the type distribution of HPV infection. These data are collected in the PAP smear-negative women. However, IARC had done a research on worldwide prevalence of HPV infection. In this finding, there are significant geographic and ethnic differences in the distribution of certain HPV subtypes and HPV type variants.

Prevalence of HPV in cervical cancer in China

The study of HPV in cervical cancer has been started for 13 years and still ongoing now. Most of the studies were in small scale and regional bounded. There were several studies on HPV in cervical cancer conducted in Sichuan, for instance, Tang *et al* (1991) conducted a study on the prevalence of HPV in the general population of high incidence regions and low incidence regions of cervical carcinoma of Sichuan. Meanwhile, the risk factors were also studied in his study. In accordance with Tang's study, HPV infection rate in high incidence area was 18% and that in low incidence area was 3.4% in general population. This showed that the HPV infection rate in high cervical carcinoma incidence area was 5 times higher than that in the low incidence

area. It also suggested that the number of sexual partners and the nutrients absorption could be the risk factors of HPV infection in Sichuan.

Another study was mainly on the relationship between HPV and cervical carcinoma not in the general population. The result of this study showed that only 35% (35/101) of cervical cancer cases were HPV-infected. The positive specimens were infected with HPV 16 and none from HPV 33. A study performed by Stephen Al *et al.* (2000) showed that the infection rate of HPV in Sichuan was 88% (28/34) and this rate was much higher than that in Peng's study in 1991. The infection rate of HPV 16 and HPV 18 was 61% and 9% respectively. HPV types 33, 45, 58 and 59 were also identified in the samples.

Apart from Sichuan, the situation of HPV and cervical cancer also studied at Shanghai. At 2 hospitals of Shanghai, the HPV 16 infection rates were 27.8% and 15.8% respectively whereas in Jiangxi the infection rate was 70.9%. In the fresh tissue specimens from Shanghai and Jiangxi, HPV 52 were detected but none could be found in the paraffin-embedded tissue but in Shanghai frozen tissue, the percentage of infection was 16.7% and there was only 2.5% in Jiangxi. When comparing the prevalence of HPV 58 in Shanghai and Jiangxi, two different regions had no significant difference, varying from 15.8% to 22.2%.

In Beijing, Zhang *et al.* had conducted a research using 3H-dCTP-labelled HPV-16 probe. The results showed that the HPV-16 DNA positive rate was 74.4%

(29/39). Another research in Shanxi Province using dot blot hybridization to detect the HPV DNA showed 40% of the invasive cervical carcinomas were infected with HPV 16. Using L1 consensus primer for detection of HPV in cervical cancer tissues was another method used in Shanxi Province (Zhao *et al.*,1994). The HPV infection rate of total 40 samples was 85%. For adenocarcinoma, HPV 18 were detected in 9 specimens (45%) and only 5 specimens were detected with the infection of HPV 16 (15%). HPV 16 were detected in 80% of the squamous cell carcinoma samples where HPV 18 only counted for 5%. The results indicated that HPV type 16 was detected more often in squamous cell carcinoma than in adenocarcinomas while HPV type 18 was detected significantly more often in adenocarcinoma tissues.

There were several studies conducted by the University of Hong Kong and the Chinese University of Hong Kong in HPV and cervical cancer. In 1994, Ngan *et al.* used HPV 16 and HPV 18 E6 type specific primers to screen the 64 cervical carcinomas and it was discovered that 90% of the samples show positive in the HPV DNA screening and 67.2% had HPV 16 infection. Chan *et al.* conducted a research on HPV and abnormal PAP smear in 1998. Among 18 cervical carcinoma cases, 14 were infected by HPV. There were 8 specimens which were found to be infected by HPV 16 (44.4%), 6 by HPV 58 (33.3%), and 1 by HPV 18 and HPV 31 respectively. High prevalence of HPV 58 in cervical carcinoma and also in other grades of cervical lesions was detected. These observations might indicate that the worldwide uncommon

genotype, HPV 58 played an important role in the development of cervical cancer in Chinese.

In Taiwan, the combined presence of HPV 52 and HPV 58 was detected separately or together in over 25% of HPV positive subjects compared with the fact that only 5% of HPV positive subjects were detected in a large international study conducted by IBSCC in 1995 as well as by Liaw *et al* (1995).

From the studies conducted, HPV 16 is the dominant HPV type in cervical carcinoma and HPV 52 and HPV 58 have a significant high infection rate in China than that of other countries.

1.5.2 Transmission

Most of the genital warts are transmitted sexually. This kind of transmission is well accepted and regard as the most important route of infection in sexually active adults (Oriel, 1971b, 1981, 1988). Data confirming this concept are derived from different epidemiology approaches. In the study of virgin and nuns, there are no HPV found. (Fairley et al., 1992; Gutman et al., 1994; Rylander et al., 1994, Critchlow et al., 1995a). With increasing sexual experience, the risk of infecting with HPV seems to be increased. Critchlow et al. (1995a) suggested that this risk increase in parallel with the number of sexual partner. At present, evidence implicating the number of sexual partners as one of the most significant risk factors for genital infections is compelling

(IARC,1995). When studying the transmission of HPV, the virus reservoirs have to found. Most of the HPV found in the epithelium of the penis, urethra and prostate. (Hillman et al., 1993a, Kyo et al., 1994; Rotola et al., 1994; Baken et al., 1995) This is confirmed using highly sensitive PCR-based HPV detection techniques. The other transmission is maternal-infant transmission. Roman et al. (1989) suggested this assumption because he found HPV 6 and HPV 16 DNA in the normal foreskin of newborn by dot blot hybridization.

In non-sexual transmission of HPV, autoinoculation, infection from moist dwellings, contaiminated instruments and underwear and vertical transmission from an infected mother to her newborn baby are some of the routes. The vertical transmission from an infected mother to her newborn baby is of particular clinical relevance. There is HPV 16 and 18 DNA sequences have been found in nasopharyngeal aspirates from neonates, laryngeal tissue from infants, and cytological samples from virgins. The conclusion seems inescapable that some but not all cervical HPV infection is acquired by means other than sexual intercourse.

1.5.3 Risk Factors

Before the link of HPV and cervical cancer was established, compelling epidemiological evidence suggested that cervical cancer bears close similarities with STDs. One of the characters of STD is a multiple sexual partner. Some of the studies had shown that women with multiple sexual partners, promiscuous sexual partners, and early onset of sexual activity are high risk group having cervical cancer. (Munoz et al., 1989; Bosch et al., 1994, 1995; Ponten et al., 1995; Schiffman, 1995a, b; Morris et al., 1996; Franco et al., 1997). In the late 70's, strong evidence from the different approach indicated that HPV, certain oncogenic types of HPV, may play an important role in pathogenesis of cervical cancer. In addition to these infection agents, other factors have been suspected or shown to increase the risk for cervical cancer: low socioeconomic status, reproductive history, smoking habits, oral and barrier contraceptive use, dietary factors, immunosuppression, frequency of PAP smears, and characteristics of the male sexual partners. (Munoz et al., 1989; Bosch et al., 1994, 1995; Ponten et al., 1995; Schiffman, 1995a,b; Morris et al., 1996; Franco et al., 1997). Many of these factors are the same as those predisposing women to genital HPV infections and CIN. All the above factor are closely related and it is difficult to assess their independent roles as risk factors. But most recent studies emphasizing the role of HPV as an independent risk factor (Morris et al., 1996; Franco et al., 1997)

Sexual Characteristics

One of the sexual characteristics linked with an increased risk of cervical cancer was number of male sexual partners. (Bosch et al., 1989, 1993, 1994; Brinton, 1992). As the number of male sexual partners increased, the risk of cancer precursors and invasive cervical cancer increase parallel. So this is a strong risk factor of cervical cancer. (Herrero et al., 1990; Bosch et al., 1992; Koutsky et al., 1992; Munoz et al., 1993a, b) Indeed the number of sexual partners was a significant risk factor for genital HPV, as demonstrated in the early case-control study of Syrjanen et al. (1984b).

Another sexual risk factor is the first age of having sexual intercourse. (Herrero et al., 1990; Bosch et al., 1992; Koutsky et al., 1992; Munoz et al., 1993; Schiffman et al., 1993) There are several studies show that an early onset of sexual activity on the risk of cervical cancer after controlling for potential confounders, other studies have failed to show any such independent effect (Jones et al., 1990).

Reproductive Factors

There are some reproductive characteristics have been analyzed as risk factors for cervical cancer. (Chang, 1989; Hildesheim et al., 1990; Brinton, 1991; Guo et al., 1994; Key, 1995) These include: age at menarche, age at menopause, parity, number of spontaneous or induced abortions, age at first pregnancy, age at first live birth, age at last birth, number of vaginal deliveries, and number of Caesarean sections. (Herrero et al., 1990; Bosch et al., 1992; Kjaer et al., 1992; Munoz et al., 1993; Becker et al., 1994)

Smoking Habits

Cigarette smoking is classified as one of the most important risk factor of cervical cancer. These studies have been work over than 20 years. Until 1990, Winkelstein (1990) and several have provided evidence supporting an association between cigarette smoking and invasive cervical cancer or CIN. The smokers have shown at least two-fold increased risk of getting cervical cancer. After the adjustment of other factors such as sexual behavior, the risk a slight decreased but still significant high and independent. (Greenberg et al., 1985; Brock et al., 1989; Daling et al., 1992a, 1996) The reason of cigarette smoking cause cancer is due to the biochemical element. In a cigarette, some of the carcinogens such as nicotine, cotinine and other mutagenic substances has been found in the cervical mucus of women actively or passively exposed to tobacco smoke. (Hellberg et al., 1988; Prokopczyk et al., 1997) These carcinogens may cause DNA damage due to direct carcinogenic effect, co-carcinogenic effect or local immunosuppressive effect. The DNA damages levels in cervical tissue cells and exfoliated cells of female smokers was significant higher then non-smokers. But now most of the recent studies have not supported smoking as an independent risk factor for cervical cancer. The detection rate of HPV DNA was identical in smokers and in women who had stopped smoking, suggesting that smoking does not increase risk of invasive cancer by prolonging the persistence of HPV DNA (Fairley et al., 1995).

Oral Contraceptive

When the oral contraceptive starts to use, many studies start to blame contraceptives as a risk factors for many diseases including CIN and cervical cancer. But during the research and interpretation of the epidemiological data, the scientists found many problems. Even the recent studies controlling the HPV status are equally conflicting with the previous. (Brinton et al., 1990b; Bosch et al., 1992l Koutsky et al., 1992; Becker et al, 1994) We cannot conclude that oral contraceptive use is an independent risk of cervical cancer.

Dietary Factors

It is difficult to determine the relationship independently of certain nutrients and th human malignancies. Some studies suggest 60% of cancers in women and 40% of cancers in men could be ascribed to nutritional factors. The action of nutrients usually show protective again neoplasm rather that positive risk association. So there are some nutrients have protective effect against cancer, such as vitamin A, carotenoids, vitamin C and vitamin E and folic acid. (Schneider et al., 1989; Cuzick et al., 1990b; Ziegler et al., 1990; Amburgey et al., 1993; Liu et al., 1993; Potischman et al. 1996) Epidemiological studies on the association of vitamin A, carotenoids, vitamin C and folic acid with cervical cancer are hampered by difficulties in measuring nutrients levels (Potischman et al., 1996). Predictably this source of bias has led to conflicting results and difficulties in comparing findings. (Schneider et al., 1989; Cuzick et al.,

1990b; Ziegler et al. 1990) The study of nutrients effect should be focusing on the interaction with other risk factors such as smoking and HPV. Also the role of different nutrients intake among distinct ethnic and racial groups should be examined in more details.

Human Papillomavirus

When studying the relationship between HPV and cervical cancer, the method is the identical to those described before for CIN. When first report on the morphological evidence of HPV involvement in an invasive cervical cancer (Syrjanen, 1979a), many other studies of cervical cancer were detecting the presence of HPV DNA using different techniques. As the detection techniques and the sensitivity improved as time, the detection rate are keep on increasing. In the 70's and 80's, southern blot or other hybridization techniques were used as a gold standard in detecting HPV DNA in the cervical cancer tissue. When the PCR techniques developed, most of the research are relies on this. The HPV detection rates vary between than 50% to 100% (IARC, 1995). Comparing the research using PCR techniques rather then other hybridization techniques, the detection rate of PCR-based research is around 75% (57%-100%) which was higher than the average of hybridization techniques. From these results, it can conclude that the correlation between HPV and cervical cancer is strong. A large number of studies on extensive series of cervical carcinoma s almost unanimously confirm the detection of HPV DNA in the vast majority of these lesions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The chemicals and reagents listed below were used in this study.

2.1.1 Chemicals and regents

Chemicals	Companies / Manufacturers
1. [γ^{32}P] ATP (10mCi/ml)	(Amersham, Buckinghamshire, UK)
2. 10X Taq DNA polymerase buffer	(GibcoBRL, Grand Island, USA)
3. 6% GENE-PAGE PLUS, EZ SQUEEZE™	(Amersco, Ohio, USA)
4. Absolute ethanol (analytical grade)	(Sigma, St.Louis, USA)
5. Agaroses 3:1 (High Resolution Blend)	(Amersco, Ohio, USA)
6. Boric acid (Ultra pure)	(GibcoBRL, Grand Island, USA)
7. Bovine serum albumn (Fraction V)	(Sigma, St.Louis, USA)
8. Bromophenol blue	(Sigma, St.Louis, USA)
9. Chloroform	(Sigma, St.Louis, USA)
10. dNTP (2'-deoxynucleoside 5'-triphosphate)	(GibcoBRL, Grand Island, USA)
11. Ethidium bromide	(Sigma, St.Louis, USA)
12. Ethylenediaminetetraacetic acid (EDTA)	(Sigma, St.Louis, USA)
13. Ficoll (Type 400)	(Pharmacia, Buckinghamshire,UK)

14. Formamide (Amersco, Ohio, USA)
15. Isopropylthio-β-D-galactoside (IPTG) (Promega, USA)
16. Phenol/chloroform/isoamy alcohol (25:24:1) (GibcoBRL, Grand Island, USA)
17. Polyvinylpyrrolidone (Sigma, St.Louis, USA)
18. Proteinase K (Sigma, St.Louis, USA)
19. Restriction enzyme Rsa I and Dde I (10U/ μ l)(Roche, GmbH, Germany)
20. Salmon sperm DNA, denatured, fragmented (Sigma, St.Louis, USA)
21. Sodium acetate (GibcoBRL, Grand Island, USA)
22. Sodium chloride (Amersco, Ohio, USA)
23. Sodium citrate (Amersco, Ohio, USA)
24. Sodium dodecyle sulfate (SDS) (GibcoBRL, Grand Island, USA)
25. T4 polynucleotide kinase with forward buffer(GibcoBRL, Grand Island, USA)
26. *Taq* DNA polymerase (5U/ μ l) (GibcoBRL, Grand Island, USA)
27. 5-Brono-4-chloro-3-indoyl-β-D-galactoside (Promega, USA)
    (X-Gal)
28. Xylene cyanol FF (Sigma, St.Louis, USA)
29. Xylene (Sigma, St.Louis, USA)
30. ΦX-174 RF DNA (GibcoBRL, Grand Island, USA)

Reagents

LB Medium (Luria-Bertani Medium) per liter

To 950ml of deionized water. add:

Bacto-trypton	10g
Bacto-yeast extract	5g
NaCl	10g

Adjust the pH to 7.0 with 5N NaOH (~0.2ml). Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 minutes at 15 lb/sq in. on liquid cycle.

SOC Medium

To 950ml of deionized water. add:

Bacto-trypton	20g
Bacto-yeast extract	5g
NaCl	0.5g
1M glucose	20ml

Adjust the pH to 7.0 with 5N NaOH (~0.2ml). Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 minutes at 15 lb/sq in. on liquid cycle.

TE Buffer

Tris Cl (pH 7.4)	10mM
EDTA (pH 8)	1mM

Sterilize by autoclaving for 20 minutes at 15 lb/sq in. on liquid cycle.

PNK Buffer

Tris Base	10mM
Ethylenediaminetetraacetic acid (EDTA)	10mM

Loading dye

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%

Ficoll (Type 400) in water

50X Denhardt's reagent

Ficoll (Type 400, Pharmacia)	5g
Polyvinylpyrrolidone	5g
Bovine serum albumn (Fraction V, Sigma)	5g

Adjust the volume of the solution to 500ml with deionized water and sterilize by filtration through a 0.22-micron filter.

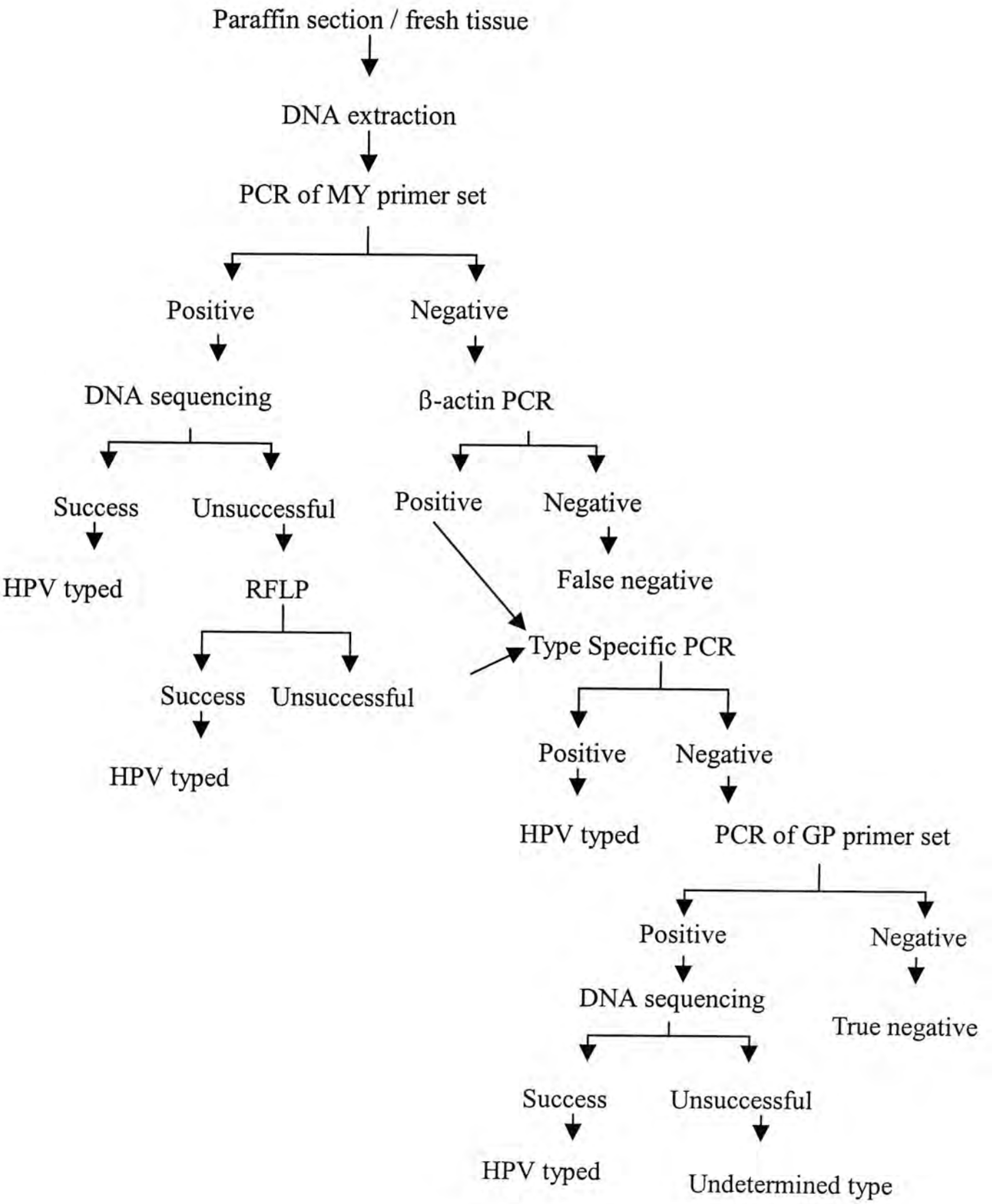
2.1.2 Specimens collection

One hundred and ninety-five cervical cancer tissues were collected at Prince of Wales Hospital, Hong Kong Special Administrative Region, China from 1992 to 1998. They were snap frozen in liquid nitrogen and embedded in OCT compound, stored in -80℃ before DNA extraction. Six hundred and sixty-eight paraffin-embedded cervical carcinoma tissues were collected from Shanghai (eastern of China), Sichuan (central of China), Beijing (northern of China) and Guangdong (Southern of China) of China for this study.

2.2 Methods

In this study, several HPV detection methods were used. Firstly, the DNA was extracted from the paraffin sections and fresh tissues. It would undergo MY09/11 PCR screening. The positive PCR products were used in DNA sequencing and RFLP for HPV typing. Those samples that negative with MY09/11 screening were screened using β-actin PCR. The negative specimens were excluded in this study. The second part of screening was used the HPV type specific primers. The samples that shown negative in DNA sequencing or RFLP and those positive with β-actin PCR were screened by using HPV type specific primers. Finally the GP5+/6+ consensus primers were used to screen the negative samples in the HPV type specific primers PCR screening. The summarized methodology was showed at section 2.2.1.

2.2.1 Summary of methodology



2.2.2 DNA extraction from fresh and paraffin embedded tissues

A snap frozen cervical carcinoma tissue was grounded in a 15ml Falcon tube. The grounded tissue was then re-suspended in Proteinase K buffer (50mM Tris (pH8.0), 1mM EDTA, 0.5% Tween 20). 1/10 volume of the Proteinase K was added to the tissue and incubated at 37°C water bath overnight. The tissue was then extracted with equal volume of phenol/$CHCl_3$/isoamy alcohol phenol twice and once with equal volume of chloroform.

The supernatant was transferred to the eppendorf tubes, 3M NaOAc (pH5.2) and absolute ethanol was added to the samples and left at 4°C overnight (16 hours) to precipitate the DNA. Next day, the tubes were centrifuged at 13,000 rpm for 15 minutes; the pellet in each tube was washed with 70% ethanol. The DNA pellet then was dried in SpeedVac concentractor, dissolved DNA with TE buffer. The samples were finally stored at -20°C for future use.

A single 10 μm formalin fixed, paraffin-embedded cervical carcinomas tissue section was put in the autoclaved 1.5ml eppendorf tube. It was deparaffinized by extraction twice with 1ml xylene for 20 minutes each at room temperature with constant shaking. Absolute ethanol (1ml) was then added to the tube for 5 minutes. The sample digested with proteinase K solution overnight followed by phenol-choloroform extraction twice and ethanol precipation as described above. The DNA was precipitated by adding 40µl 3M NaOAc (pH 5.2) and 1 ml absolute ethanol, and left overnight at

-20°C.The DNA samples were lastly prepared for later use: recovering the DNA by spinning it at 13,000rpm for 20 minutes at room temperature, washing it with 70% ethanol, drying the DNA pellet in SpeedVac concentrator, dissolving in 10μl TE buffer, and storing at -20℃ until use.

Microdissection of cancer cells was performed when the cancer cells were less than 50% from the whole tissue section. Three consecutive 5-micron sections from a formalin fixed paraffin embedded block were cut and placed onto slides. Another section stained with H&E was used as a guilded section. A sterilize new #11 blade and needles were used to collect the dissected areas. The collected cells were placed carefully into the 0.5 ml tube with the TE buffer and extracted as above.

The following 16 steps were adopted in DNA extraction from paraffin sections

1. Extract 10μM section with 1000μl xylene incubate at Room Temp. for 20 minutes.

2. Spin for 5 minutes at 13000rpm in microfuge

3. Decant xylene

4. Repeat steps 1-3

5. Extract with 1000μl ETOH, incubate at Room temp. for 5 mins.

6. Spin for 5 minutes at 13000rpm in microfuge

7. Decant ETOH

8. Repeat steps 5-7

9. Dry samples in Speed-Vac for 20 mins

10. Resuspend in 300μl PNK buffer with 3μl prot.K. Incubate at 37°C for 8-18hrs

11. Boil sample for 5 minutes at 95°C

12. Extract 1X with phenol/CHCL3/isoamy alcohol

    → add 300μl of the mixture to the sample

    → mix the mixture in voltex → cent for 10min at 13000rpm

    → transfer the upper layer to the new tube

    → repeat step (12)

13. Extract 1X with $CHCl_3$

    → add 200μl chloroform

    → mix the mixture in voltex → cent for 10min at 13000rpm

→ transfer the upper layer to the new tube

14. Add 1/10 volume 3M NaOAc, Abs ETOH ppt.

→ add 20µl 3M NaOAc

→ add 400µl ETOH

→ cent for 15 mins at 13000rpm

→ stand overnight at 4°C

15. Wash with 70% ETOH

→ add 500µl 70% ethanol

→ voltex → cent 15 min

→ remove the supernatant

16. Dry; resuspend in 10µl TE

→ Vaccum Dry for 20min

→ add 10µl TE

→ store at -20°C

*PNK buffer: 50mM Tris (pH8.0), 1mM EDTA, 0.5% Tween 20.

2.2.3 Polymerase Chain Reaction using HPV Consensus Primer MY09/11

HPV consensus primer pair MY09/11 (MY09, 5'-CGT CCM ARR GGA WAC TGA TC-3'; MY11, 5'- GCM CAG GGW CAT AAY AAT GG –3') targeted at L1 region of the HPV DNA to amplify about 450bp DNA sequences. (Manos *et al.,* 1989)

2.2.3.1 Template for PCR

Six hundred and fourteen paraffin-embedded cervical carcinoma tissues from Shanghai, Sichuan, Beijing and Guangdong, and one hundred and ninety-five fresh cervical carcinoma tissues collected at Prince of Wales Hospital, Hong Kong, China were screened with MY09/11 primer set.

2.2.3.2 PCR Amplification

A typical 50 μl reaction mixture contained 1.5mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer, 2 units of *Taq* polymerase (GIBCO BRL, Life Technologies) and 1 μl of cervical cancer sample DNA. The PCR was performed with a modification of the original method using GeneAmp® PCR System 9600 or GeneAmp® PCR System 9700 (Applied Biosystems, USA) (Manos et al., 1989). The thermal cycling condition started at 94℃ for 3 minutes for denaturation. Thirty-five cycles of amplification were carried as following: denaturation at 94℃ for

30 seconds, primer annealing at 55℃ for 30 seconds and primer extension at 72℃ for 1 minute. After the 35 amplification cycles, 10 minutes at 72℃ was for the complete extension of the primers.

Several precautions were taken to prevent the false positive result and cross contamination. All PCR reagents were taken in aliquots from uncontaminated stock reagents. Different sets of pipettes and disposable sterile pipette tips were used for the PCR reagents and the preparation of the amplification were worked in strictly separated rooms. Each set of PCR included a distilled water samples as a control experiment of the reagents. HPV-negative human placenta DNA PCR was performed with each amplification reactions as a negative control for monitor sample-to-sample carried over. None of these control samples was positive for any of the assays.

2.2.3.3 PCR product analysis

The products of the reaction were electrophoresed with 2% agarose gel and stained with ethidium bromide running at 100V for 1 hour in 1%TBE buffer. The PCR products were visualized with an ultraviolet light source and analysed by imaging system GelDoc 1000 by BioRad Laboratories, Inc., USA.

2.2.4 DNA sequencing

Automatic sequencers did DNA sequencing, both ALF*express* DNA automatic sequencer AM 3.0 from Amershan Pharmacia Biotech, and ABI 377 DNA sequencer from Applied Biosystems were using dye primer cycle sequencing.

2.2.4.1 DNA sequencing reaction for ALF*express* DNA automatic sequencer

ALF*express* DNA automatic sequencer was designed for automatic detection of fluorescently labeled DNA molecules separated by electophoresis. The basic difference from the tranditional Sanger dideoxy sequencing method was the use of a fluorescently labeled primer or fluorescently labeled dNTP instead of radioactive deoxynucleotides.

SequiTherm EXCEL™ II Long-Read ™ DNA Sequencing Kits-LC from Epicentre Technologies, UK was used for ALF*express* DNA automatic sequencing. Either Cy5™ labeled MY09 primer or Cy5™ labeled MY11 primer were used in sequencing reactions.

The templates for the sequencing reaction were the PCR products of MY09/11 primer set. Microspin Column S-300 (Amerasm Pharmacia, USA) were used to purify 50μl of PCR products; extracting the DNA from agarose gel was the other method to purify the templates. The PCR products were electrophoresed with 2% agarose gel and stained with ethidium bromide and visualized under UV light. The desired band was cut

by sterilize razor blade and put into the eppendorf tube. The same sample's PCR products were pooled in the same tube and extracted using QiAquick Gel Extraction Kit by Qiagen.

In order to clean the glass plates and spacers of the ALFexpress sequencer, they were rinsed under room temperature running tap water with great care, as all the components were very fragile. Then each glass and spacer was polished by Kimwipe sheets, and was rinsed with 70% ethanol. This washing process was repeated for three times. All items were examined for any remaining gel residues and lastly Kimwipe sheets dried them.

The cassette was assembled following the ALF*express* manual. The comb was inserted and checked that if the pressure was correct and the comb slid in too easy; if the pressure was uneven, changed the clamps on the loose side until an even pressure was reached.

5% EZ Squeeze ™ Gene-PAGE PLUS ™ 6M Urea sequencing gel from Amresco,, Ohio, USA was used for ALF*express* autosequencing. The gel solution was warmed at room temperature for 10 minutes and was mixed by gentle inversion for 2 minutes with the ammonium persulfate TEMED polymerization catalyst and initiator disks. The mixed solution was poured into prepared glass plate assembly. Then let the gel solution polymerize at room temperature for at least 2 hours.

After the gel was polymerized in the cassette, mounted the cassette into ALFexpress, connected the water circulation and aligned the laser. Afterwards, carefully examined the laser beam for dust particles and for reflections on backside of the glass plates. The dust must be avoided by adjusting the height adjustment wheels. 2 litres of 0.6X TBE buffer was prepared and added to the upper and lower buffer tank, allowing the gel to reach working temperature. Then removed the comb and rinsed the wells with buffer using a syringe, loaded 6 μl of each denatured samples into the wells starting from left in the following order: A, C, G and T followed by re-aligned the laser and started the run for 420 minutes. The result sequences were read and analysis using the evaluation programme.

2.2.4.2 ABI comparative PCR sequencing

ABI Prism 377 DNA Sequencer from Perkin Elmer was a fast and reliable sequencing method for clones screening. It runs based on the multiple fluorescent dyes labeled DNA cycle sequencing with AmpliTaq Polymerase, FS. The steps involved purifying DNA template, amplifying DNA by PCR, preparing of sequencing temple, performing fluorescent sequencing reaction, electrophoresizing dye-labeled samples, analyzing and comparing of data in gene bank.

Purified PCR product of MY09/11 primer was used as the temple for auto-sequencing, 1 μg of template DNA was added to 3.2 pmole sequencing primer in a

total volume of 12 μl with ddH_2O, then 8 μl of terminator ready reaction mix from ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA) was mixed together with the template and primer. Afterwards, underwent cycle sequencing on the thermal cycler and repeated the following condition sequence 25 times: 96°C for 20 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Then rapid thermal ramp to 4°C, spun down the contents of the tubes in a microcentrifuge. Excess dye terminators were removed by ethanol precipitation. For 20 μl PCR reaction, 2 μl of 3M sodium acetate (pH 4.6) and 50 μl of 95% ethanol were added. Vortex the tubes and placed on ice for 3 hours to precipitate the extension products. Then spun the tubes in a microcentrifuge at 13,000 rpm for 20 minutes at 4°C, carefully aspirated the supernatant with a pipette and discard, rinsed the pellets with 250 μl of 70% ethanol. Spun for another 5 minutes, carefully discards the supernatant, and dried the pellet in a vacuum centrifuge for 15 minutes.

In order to store the samples, each sample was resuspended in 12 μl of Template Suppression reagent (TSR). Before the reaction started, the samples in TSR were vortex and spun. The DNA were denatured by heating at 95°C for 2 minutes, it then chilled on ice until ready for use.

2.2.4.3 DNA sequence analysis

GCG package (version 8.0) from Genetics Computer Group Inc. was currently installed in the network system in The Chinese University of Hong Kong. The DNA sequences obtained from the sequencing of screening clones were directly input into the GCG program. Data searching of the entire HPV gene was performed by the FASTA command. They were then retrieved into the computer for further manipulation. Any updated sequences was download directly from the internet website of the Genebank (http://www.ncbi.nlm.nih.gov).

2.2.5 Restriction Fragment Length Polymorphism

RFLP is a molecular biology technique using the restriction enzymes to cut the DNA sequences into short fragments. Restriction enzymes have the ability to cut the nucleotides according to unique DNA sequence. Each type of HPV have its own DNA sequences so using specific restriction enzymes can produced unique patterns.

Double infection of HPV could detect using RFLP technique. If the specimen infected by 2 types of HPV, MY09/11 primer pair could amplify both types of HPV DNA. In this case, both type's digestion pattern could detected.

2.2.5.1 Template preparation

The templates for Restriction Fragment Length Polymorphism (RFLP) were the PCR product of MY09/11 primer set. Five micro littlers of the crude PCR products were used in the RFLP.

2.2.5.2 Restriction enzyme digestion

Aliquots of the PCR products were subjected to digest with restriction enzyme Rsa I and Dde I (SuRE/Cut Buffer Set; Boehringer-Mannheim). Five units of enzymes were mixed with its specific buffer in total volume 10 μl. Reactions were incubated in a 37°C water bath for 1 hour.

2.2.5.3 Agarose gel electrophoresis analysis

The products of the reaction were electrophoresed with 3% agarose gel stained with ethidium bromide run at 50V for 1 hour in 1% TBE buffer. The digestion products were visualized with an ultraviolet light source and analysis by imaging system GelDoc 1000 by BioRad Laboratories Inc. The sizes of the restriction enzyme digestion products were confirmed with reference to the DNA standard size marker.

2.2.6 HPV Type Specific PCR

Since the PCR carried out on crude samples was easy to perform, HPV type specific PCRs developed and applied to a variety of clinical specimens. HPV type specific primers targeted in several parts of the HPV genomes and could be synthesized in large amounts.

2.2.6.1 Preparation of positive control DNA

There were total six types of HPV type specific primers, HPV 16, HPV 18, HPV 31, HPV 33, HPV 52 and HPV 58. (Table 2.1) The positive control DNA of HPV 16, HPV 31 and HPV 33 PCR were cervical carcinoma tissues collected from Hong Kong. They were screened by MY09/11 and typed with RFLP and DNA sequencing to confirm the presence of HPV 16, HPV 31 and HPV 33 DNA. HeLa cell was used as positive control of HPV 18 PCR.

2.2.6.2 Preparation of HPV 52 and HPV 58 type specific PCR

Plasmids of HPV 52 and HPV 58 were kind gifts of Prof. Wayne Lancaste and Prof. Toshihiko Matsukura respectively. HPV 52 plasmids were directly transformed with JM 109 High Efficiency Competent Cells (Promega, USA). At first thawed the vials of JM109 Competent ncells on ice. Then, 2 μl of HPV 52 plasmid reacted into the competent cells and stirred gently with pipette tip to mix. In order to heat shock the cells, the vials containing the cells were incubated on ice for 30 minutes and then

placed in a 42°C water bath for exactly 30 seconds. Next immediately placed the vials on ice for 2 minutes and added 450 μl of SOC medium to each vial, shaked the vials at 37°C in a shaker at 220 rpm for 1 hour. The transformed cells were then plated 50 μl, 100μl and 200 μl on an LB plate containing 50 μg/ml ampicillin, 20 μl X-Gal and 50 μl IPTG. Incubated the plates at 37°C overnight, and shifted the plates to 4°C for colour development. Plasmid DNA was isolated from 3 white transformants and analyzed for the presence and HPV 52 DNA by RFLP.

For HPV 58 sequence, additional of A-tail sequence to the genome was performed. 2μl of the HPV 58 genome were added to the 1μl of Taq DNA polymerase 10X reaction buffer with $MgCl_2$. Total 0.2mM dATP and 5U of Taq DNA Polymerase were added to the reaction mixture.

pGEM-T Vector System from Promega was used for the cloning of HPV 58 sequence from the original HPV 58 genome. Ligation reaction was set up by adding 1 μl of the fresh genome (~10 ng) into 1 μl pGEM-T vector (50 ng/μl), 5 μl 2 X rapid ligation buffer and 1 μl T4 DNA ligase in a volume of 10 μl with ddH_2O, incubated at 14 °C overnight for the ligation reaction. In the following day, transformation was done as the same as HPV 52 plasmid preparation.

Small-scale preparation of plasmid DNA, better known as minipreps was done by Wizard™ minipreps DNA purification system (Promega, USA). Each miniprep started from a 3ml LB medium containing 50 μg / ml ampicillin overnight culture in 15

ml culture tubes, which were the selected white recombinants colonies containing the pGEM-T vector with the genome as inserted. Allowed the culture to grow at 37°C with shaking at 250 rpm overnight.

The cultured cells were collected by centrifugtion for 10 minutes at 2000 rpm at 4°C. Resuspended the cell pellet in 200 µl cell resuspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A). Then transferred the resuspended cells to an 1.5ml eppendorf tube. Added 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) and mixed by gently inverting the eppendorf for several times until it turned to clear.

Afterwards, added 200 µl of neutralization solution and mixed by inverting the eppendorf several times. Spun at 13,000 rpm for 5 minutes at room termperature, then decanted the clear supernatant to a new centrifuge tube.

Added 1 ml of the Wizard™ minipreps DNA purification resin to the clear supernatant and mixed by inverting the tube. For each miniprep, prepared one new Wizard™ minicolumn.

The resin / DNA mix were pipetted into the minicolumn, spun at 13,000 rpm for 1 minute. In order to wash the minicolumn, 2 ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA in 55% ethanol) were used. Finally transferred the minicolumn to a new centrifuge tube, applied 50 µl of water to the minicloumn and waited for 1 minutes. To elute the DNA, spun at 13,000 rpm for 1

minute at room temperature, then removed and discarded the minicolumn, stored the plasmid DNA in eppendorf at -20°C for future used.

2.2.6.3 PCR primer design

The primer sequence for HPV 16, HPV 18, HPV 31, HPV 33 were adopted from Baay 1996. (Table 2.1)

The sequence of the HPV 52 and HPV 58 specific oligonucledtide primers were newly designed used to amplify a unique region of the boundary of the E_7 and E_1 proteins and was based on the published sequences of the HPV genome. The sequences were chosen with the aid of computer programmes and the comparison with existing sequences in the gene bank. Primers sequences for HPV 52 (5'-GCATTCATAGCACTGCCAC – 3'; 5' – GCCTCTACTTCAAACCAGCC – 3') corresponded to position of the sense strand 761 – 779 and antisense strands 909 – 928. This primer pairs produced an amplified product of 168 bases.

HPV 58 type specific primers sequences corresponded to position 833 – 851 of sense strand (5' - CATGTACCATTGTGTGCCC – 3') and position 932 – 950 of antisense strand (5' – ACCGCTTCTACCTCAAACC – 3'). This pair of primers gave 118 bases of the amplified product. The sequences of the probes for confirming the amplicons in Southern Hybridization were designed within the amplified sequence. The oligonucleotide sequences were: HPV 52: 5'- ACACAACTTGTAATGTGCCC – 3'; HPV 58: 5'- TGCACAGCAATAAACACATC – 3'.

Table 2.1 Different HPV type specific primers sequences

Primer pair	Sequence	Amplimer length (bp)
HPV 16 sense *	5'- GGT CGG TGG ACC GGT CGA TG – 3'	96
HPV 16 antisense *	5'- GCA ATG TAG GTG TAT CTC CA – 3'	
HPV 18 sense *	5' – CCT TGG ACG TAA ATT TTT GG – 3'	115
HPV 18 antisense *	5' – CAC GCA CAC GCT TGG CAG GT – 3'	
HPV 31 sense *	5' – GGG ATT GTT AGA AAA GCT ACC – 3'	110
HPV 31 antisense *	5' – CGC TTA GTA GAC GTC GTC GC – 3'	
HPV 33 sense *	5' – CCA CCA CTG CTT CTT ACC TC – 3'	114
HPV 33 antisense *	5' – ACC ATT TTC ATC AAA TGG GA – 3'	
HPV 52 sense	5'- GCA TTC ATA GCA CTG CCA C – 3'	168
HPV 52 antisense	5' – GCC TCT ACT TCA AAC CAG CC – 3'	
HPV 58 sense	5' – CAT GTA CCA TTG TGT GCC C – 3'	118
HPV 58 antisense	5' – ACC GCT TCT ACC TCA AAC C – 3'	

*Journal of Clinical Microbiology, Mar. 1996, p.745-74

2.2.6.4 PCR Amplification

PCR amplification conditions of HPV 16, 18, 31 and 33 were similar to MY09/11 PCR amplification. A typical 50 μl reaction mixture contained 1.5mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer, 2 units of *Taq* polymerase (GIBCO BRL, Life Technologies) and 1 μl of cervical cancer sample DNA. The thermal cycling condition was started at 94℃ for 3 minutes for denaturation. Thirty-five cycles of amplification were carried as follows: denaturation at 94℃ for 30 seconds, primer annealing at 55℃ for 30 seconds and primer extension at 72℃ for 1 minute. After the 35 cycles amplification, 10 minutes at 72℃ was for the complete extension of the primers.

For PCR amplification of HPV 52 and HPV 58, a typical 50 μl reaction mixture contained 1.5mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer, 2 units of Taq polymerase (GIBCO BRL, Life Technologies) and 1 μl of cervical cancer sample DNA. The PCR condition was performed with a modification of the original method (Saiki et al., 1988). The thermal cycling condition was started at 94 ℃ for 3 minutes for denaturation. Thirty-five cycles of amplification were carried as follows: denaturation at 94℃ for 30 seconds, primer annealing at 59℃ for HPV 52 and 62℃ for HPV 58 for 30 seconds and primer extension at 72℃ for 1 minute. After the 35 cycles amplification, 10 minutes at 72℃ was for the complete extension of the primers. The products of the reaction were electrophoresed on 2% agarose gel and

stained with ethidium bromide in TBE buffer running at 100V for 1 hour. The PCR products were visualized with an ultraviolet light source.

Southern hybridization analysis was used to confirm the HPV 52 and HPV 58 type specific PCR. An aliquot (25 μl) of the PCR products was electrophoresed on 2% agarose gel and was then used for Southern blot analysis. The gel was shaken twice with 0.4 M NaOH, 1M NaCl for 15 minutes and 20 minutes respectively, and then rinsed with ddH_2O. A piece of nylon membrane (Hybond-N, Amersham) was cut and put on top of the gel. At the bottom of the gel, a paper bridge with 3 MM Whatman paper was set up and 0.4 M NaOH, 1M NaCl was used for overnight transfer. The following day, the membrane was baked for 2 hours in 80℃ oven for fixing the DNA on the membrane. Membrane was prehybridised in 10ml of prehybridization solution containing 5X Denhardt's reagent, 6X SSC (1X SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulphate (SDS) and 100 μg/ml of denatured salmon sperm DNA for 2 hours at 50℃, and hybridized in the same buffer containing 0.5 pmol/ml of a purified [γ-^{32}P] ATP 5' end-labeled oligonucleotide probe (2 x 10^6 cpm/ml) overnight at Tm -10℃. Membrane was rinsed briefly in 2X SSC, 0.1% SDS at room temperature and then twice for 10 minutes at 40℃; 1X SSC, 0.1% SDS four times at room temperature for 5 minutes each and 0.1X SSC, 0.1% SDS twice at room temperature for 10 minutes. The membranes were exposed to X-ray film (Kodak Biomax MS) in a cassette with intensifying screen for 6 hours at -80℃.

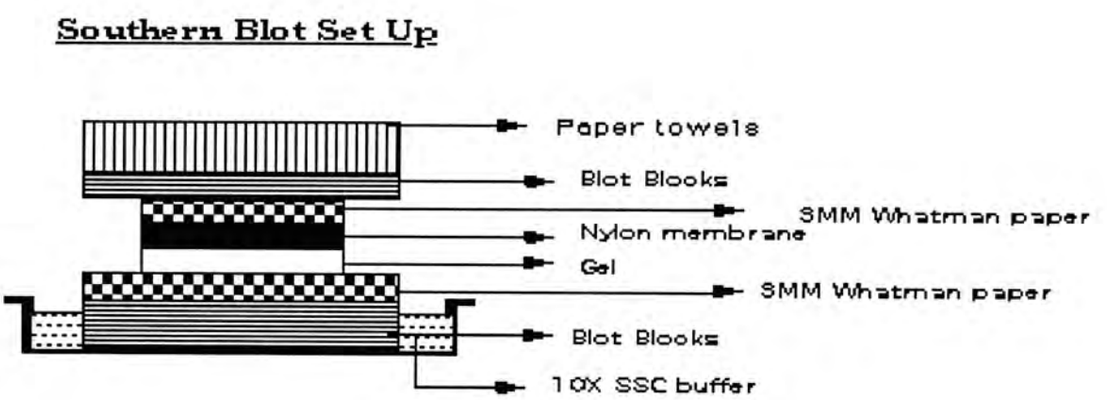


Fig 2.1. Set up of Southern Hybridization

2.2.7 Polymerase Chain Reaction using HPV Consensus Primer GP5+/6+

HPV consensus primer GP5+/6+ (GP5+, 5'-TTT GTT ACT GTG GTA GAT ACT AC- 3'; GP6+, 5'- GAA AAA TAA ACT GTA AAT CAT ATT C-3') targets at L1 region of the HPV DNA to amplify about 150bp DNA sequences. This primer set consists of a fixed nucleotide sequence for each primer and detects a wide range of HPV types. (de Roda *et al.,* 1995)

2.2.7.1 Template for PCR

One hundred and forty four paraffin-embedded cervical carcinoma tissue blocks from Shanghai, Sichuan, Beijing and Guangdong were screened with GP5+/6+ primer set.

2.2.7.2 PCR Amplification

A typical 50 μl reaction mixture contained 1.5mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer, 2 units of *Taq* polymerase (GIBCO BRL, Life Technologies) and 1 μl of cervical cancer sample DNA. The thermal cycling condition was started at 94℃ for 3 minutes for denaturation. Thirty-five cycles of amplification were carried as follows: denaturation at 95℃ for 1 minute, primer annealing at 42℃ for 1 minute and primer extension at 72℃ for 1

minute. After the 40 cycles amplification, 10 minutes at 72℃ was for the complete extension of the primers.

2.2.7.3 PCR product analysis

The products of the reaction were electrophoresed with 2% agarose gel and stained with ethidium bromide in TBE buffer run at 50V for 2 hour. The PCR products were visualized with an ultraviolet light source and analysis by imaging system GelDoc 1000 by BioRad.

2.2.8 Statistical analysis

Statistical analysis of the comparison in terms of HPV type and histology was performed using the JUMP IN for Windows (SAS Institute). Chi-square test and Fisher's Exact test were done to assess statistical significance for the difference in the prevalence of HPV types by the histologic type of cervical cancer and the viral type and geographic region.

CHAPTER 3

RESULTS

3.1 Histology review of tumor specimens

A total 863 tumor specimens, including paraffin-embedded and snap frozen tissue blocks, were sectioned and stained with H&E. Dr. May Chan and Prof. Y.F. Wong of the Chinese University of Hong Kong reviewed the histologic type and grade of differentiation of the specimens (Table 3.1 and 3.2). Among those specimens, thirty-three specimens from Shanghai, twenty specimens from Sichuan and one from Beijing were excluded from the study as no cancer cells were found in the corresponding slides.

With regard to patient population, 729 (90.1%) patients were diagnosed as squamous cell carcinoma; 64 (7.9%) as adenocarcinoma; 10 (1.2%) as adenosquamous carcinoma and 6 (0.8%) with other diagnosis. The results of grading were as follows: grade 1, 218 (26.9%); grade 2, 416 (51.4%) and grade 3, 175 (21.7%).

Table 3.1 Histologic types of cervical carcinoma tissues from different regions of China

Regions / HistologicType	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong
Squamous	146	146	150	125	162
Adenocarcinoma	3	11	0	21	29
Adenosquamous	1	3	0	3	3
Small-cell Undifferentiated	0	2	0	2	1
Clear Cell Adenocarcinoma	0	1	0	0	0
Total	150	163	150	151	195

Table 3.2 Histologic grades of cervical carcinoma tissues from different regions of China

Regions / Differentiation*	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong
Grade 1	39	27	30	75	47
Grade 2	80	75	104	54	103
Grade 3	31	61	16	22	45
Total	150	163	150	151	195

* Grade 1: well differentation

Grade 2: moderate differentation

Grade 3: poorly differentation

3.2 Polymerase chain reaction using HPV consensus primer MY09/11

PCR using HPV consensus primer MY09/11 was used for screening of HPV genome in the cervical carcinoma tissues. HPV amplification products of the expected size (~450bp) were observed in ethidium bromide stained gel (Fig.3.1). A total 809 paraffin-embedded and fresh cervical carcinoma tissues were subjected to this screening. A total 451 specimens were shown positive in this PCR including 88 out of 150 (58.67%) specimens from Shanghai; 54 out of 163 (32.1%) specimens from Sichuan; 52 out of 150 (34.7%) specimens from Beijing; 104 out of 151 (68.8%) specimens from Guangdong and 153 out of 195 (78.4%) specimens from Hong Kong showed positive results in this PCR. From the above, it was obvious that the efficiency of MY detection in Sichuan was lower than Hong Kong ($p=0.00$)

Serial dilution test was carried out to determine the sensitivity of HPV detection using primer pair MY09/11. HeLa cells, which contained 10-50 copies of HPV 18 genome in the cell, were used as the template of PCR. The DNA extracted from Hela cells were used in the dilution test in which 100,000 Hela cells were used for this test. Then, 10,000 Hela cells were used for the test. After that 5,000 Hela cells followed by 1,000 Hela cells, 500 Hela cells, 100 Hela cells, 50 Hela cells, 10 Hela cells, 5 Hela cells until 1 Hela cell were used for the test. In fact, the lowest limit of this PCR was 1 copy of Hela cell, the lowest limit of this PCR was 1 copy of HeLa cell (Fig. 3.2).

PCR using beta actin primer

The β-actin PCR was performed to access the integrity of DNA. A total 358 specimens that were negative after the test of MY PCR would be used to amplify a DNA fragment at 303bp within the β-actin region and they were found to be visible in ethidium bromide stained gels. (Fig. 3.3)

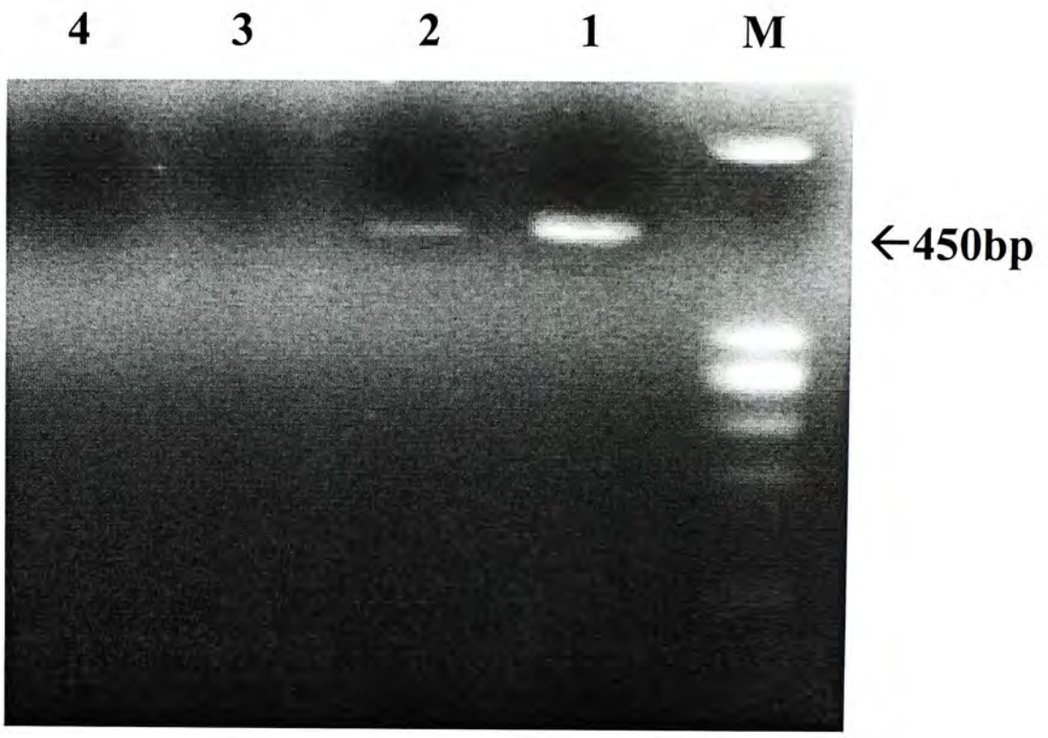


Fig. 3.1 **Electrophoresis of PCR products from L1 region of HPV genome in cervical carcinoma tissues in agarose gel stained with ethidium bromide. Lane 1: HPV DNA positive sample; Lane 2: positive control using HeLa cell DNA; Lane 3: negative control of DNA isolated from human placenta; Lane 4: negative control of distilled water; M: size marker, Φx-174/HaeIII Markers.**

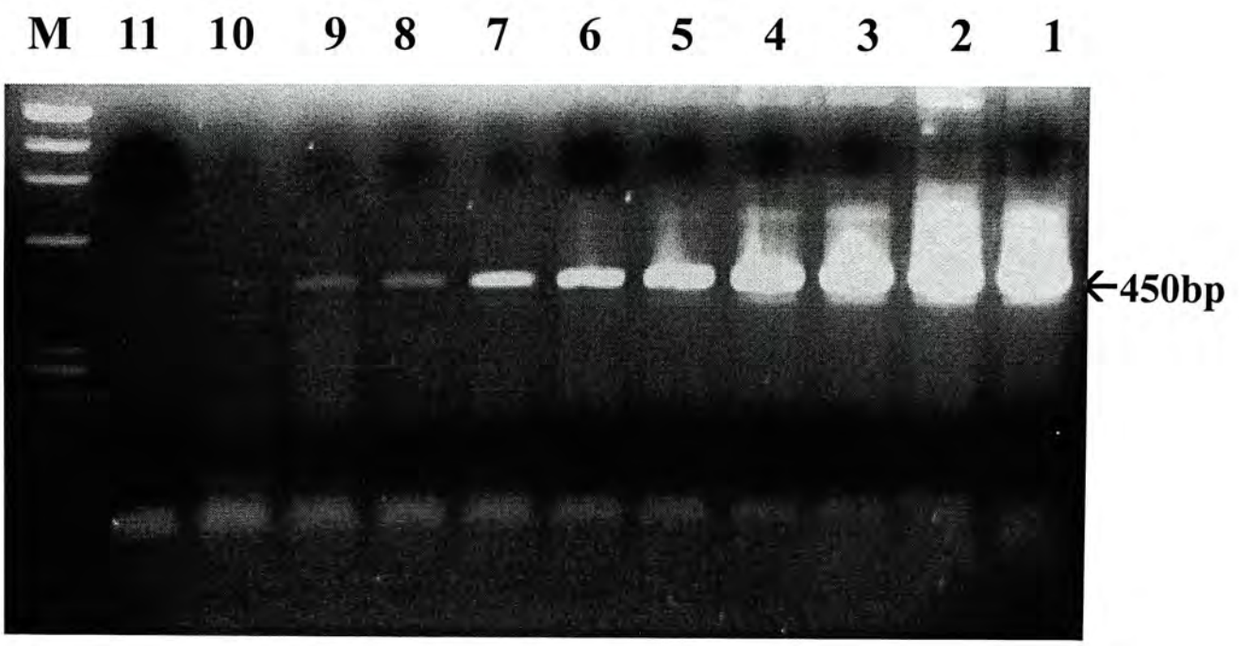


Fig 3.2 HPV PCR products of HeLa cells using MY09/11 primer pair in 2% ethidium bromide stained agarose gel. Lane 1, 100,000 HeLa cells; Lane 2, 10,000 HeLa cells; Lane 3, 5,000 HeLa cells; Lane 4, 1,000 HeLa cells; Lane 5, 500 HeLa cells; Lane 6, 100 HeLa cells; Lane 7, 50 Hela cells, Lane 8, 10 HeLa cells; Lane 9, 5 HeLa cells; Lane 10, 1 HeLa cell; Lane 11, Human plasma cells as negative control of this PCR, M, size marker Φx-174/HaeIII Markers.

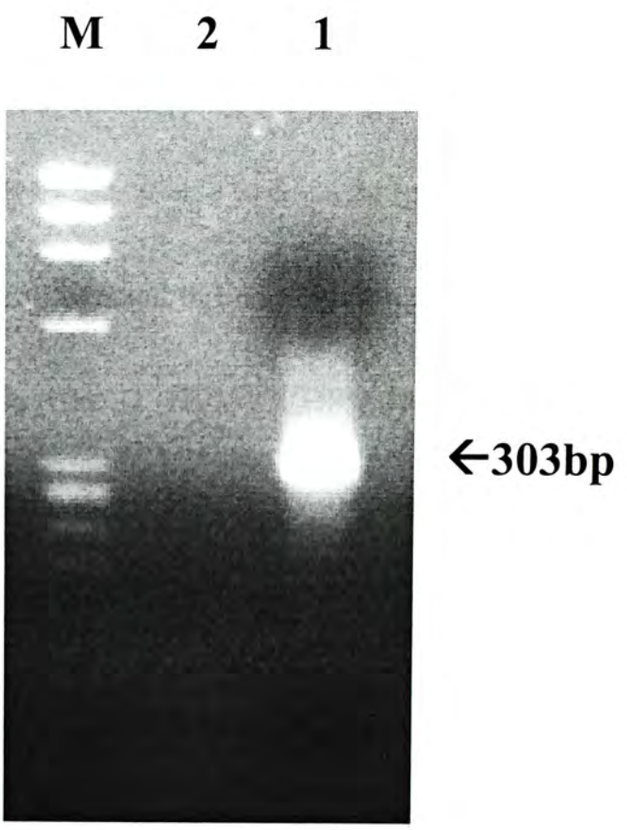


Fig. 3.3 **Electrophoresis of PCR products from β actin region of human genome in 2% agarose gel stained with ethidium bromide. Lane 1: Human placenta DNA as positive sample; Lane 2: negative control of distilled water; M: size marker Φx-174/HaeIII Markers.**

3.3 DNA sequencing reaction

There were 451 cervical carcinoma specimens, which were positive with MY09/11 PCR undergoing DNA sequencing reaction. HPV typing was obtained in 283 specimens including 23 (26.1%) samples from Shanghai, 30 (55.6%) samples from Sichuan, 20 (38.5%) from Beijing, 71 (68.3%) from Guangdong and 138 (90.2%) from Hong Kong. The efficiency of DNA sequencing in Guangdong and Hong Kong was statistically higher than that in Shanghai and Sichuan (p=0.00 by Fisher's exact test)

Ten types of HPV were detected in the cervical carcinoma tissues. These included HPV 11, HPV 16, HPV 18, HPV 31, HPV 33. HPV 45, HPV 52, HPV 58 and HPV 70. Besides HPV 11 which was low risk HPV type, the rest were high or intermediate risk HPV types. The detection of HPV 16 in DNA sequencing successful specimens were found in 21 of 23 (91.3%) in Shanghai; 26 of 30 (86.7%) in Sichuan; 17 of 20 (85%) in Beijing; 52 of 72 (72.2%) in Guangdong and 85 of 153 (55.6%) in Hong Kong. HPV 16 detection rate was predominated in most of the regions except Hong Kong (p=0.1653 by Fisher's exact test) The infection of HPV 18 were found in 2 (8.6%) specimens in Shanghai; 3 (10%) in Sichuan; 1 (5%) in Beijing; 5 (7%) in Guangdong and 20 (14.4%) in Hong Kong. (Table 3.3)

The sequencing results obtained from 150 to 410 nucleotides were compared to known HPV sequences available in the genebank. The aligned sequences of HPV 16 (sample from Sichuan), HPV 58 (sample from Guangdong) and HPV 18 (sample from Shanghai) were shown in Fig. 3.4 – Fig 3.6, respectively.

Table 3.3 HPV typing results using DNA sequencing with MY09/11 primer pair

HPV type \ Regions	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong
11	0	0	0	1	2
16	21	26	17	52	85
18	2	3	1	5	20
31	0	0	0	2	1
33	0	0	0	0	9
45	0	0	0	2	0
52	0	0	0	4	8
53	0	0	0	0	1
58	0	1	2	6	11
70	0	0	0	0	1
Total typed samples	23	30	20	72	138
Total samples	88	54	52	104	153

```
97 6521 :1    gttactgttgttgatactacacgcagtacaaatatgtccttatgtgctgccatatctact 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16: 991  gttactgttgttgatactacacgcagtacaaatatgtccttatgtgctgccatatctact 1050

97 6521:61   tcagaaactacatataaaaatactaactttaaggagtacctacgacatggggaggaatat 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16:1051 tcagaaactacatataaaaatactaactttaaggagtacctacgacatggggaggaatat 1110

97 6521: 121  gatttacagtttatttttcaactgtgcaaaataaccttaactgcagacgttatgacatac 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16: 1111 gatttacagtttatttttcaactgtgcaaaataaccttaactgcagacgttatgacatac 1170

97 6521: 181  atacattctatgaattccactattttggaggactggaattttggtctacaacctccccca 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16: 1171 atacattctatgaattccactattttggaggactggaattttggtctacaacctccccca 1230

97 6521: 241  ggaggcacactagaagatacttataggtttgtaacatcccaggcaattgcttgtcaaaaa 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16: 1231 ggaggcacactagaagatacttataggtttgtaacatcccaggcaattgcttgtcaaaaa 1290

97 6521: 301  catacacctccagcacctaaagaagatccccttaaaaaatacacttttgggaagtaaat 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV 16: 1291 catacacctccagcacctaaagaagatccccttaaaaaatacacttttgggaagtaaat 1350

97 6521: 361  ttaaaggaaaagttttctgcagacctagatcagtttcc 398
              ||||||||||||||||||||||||||||||||||||
HPV 16: 1351 ttaaaggaaaagttttctgcagacctagatcagtttcc 1388

Identity  : 398/398 (100%)
```

Fig 3.4 Alignment of positive strand of HPV sequence from the genebank compared to the 398bp HPV PCR (Sichuan sample, #97 6521). This sample's sequences were found to be 100% identical to the HPV type 16 L1 region sequences of the genebank (HPV type 16 Sen 32 late major capsid protein, L1 gene).

```
113   : 1    accgtggatgataccactcgtagcactaatatgacattatgcactgaagtaaataaggaa 60
              |||||||*|||||||||||||||||||||||||||||||||||||||*|||||||
HPV58: 6636 accgtggttgataccactcgtagcactaatatgacattatgcactgaagtaactaaggaa 6695

113   :   61   ggtacatataaaaatgataattttaaggaatatgtacgtcatgttgaagaatatgactta 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV58: 6696 ggtacatataaaaatgataattttaaggaatatgtacgtcatgttgaagaatatgactta 6755

113   : 121  cagtttgtttttcagctttgcaaaattacactaactgcagagataatgacatatatacat 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV58: 6756 cagtttgtttttcagctttgcaaaattacactaactgcagagataatgacatatatacat 6815

113   : 181  actatggattccaatattttggaggactggcaatttggtttaacacctcctccgtctgcc 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV58: 6816 actatggattccaatattttggaggactggcaatttggtttaacacctcctccgtctgcc 6875

113   : 241  agtttacaggacacatatagatttgttacctcccaggctattacttgccaaaaaacagca 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV58: 6876 agtttacaggacacatatagatttgttacctcccaggctattacttgccaaaaaacagca 6935

113   : 301  ccccctaaagaaaaggaagatccattaaataaatatacttttgggaggttaacttaaag 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV58: 6936 ccccctaaagaaaaggaagatccattaaataaatatacttttgggaggttaacttaaag 6995

113   : 361  gaaaggttttctgcagatctagatcagtatcccctgggacg 401
              ||||*|||||||||||||||||||||*|||**|||||||
HPV58: 6996 gaaaagttttctgcagatctagatcagtttcctttgggacg 7036
```

Identities = 395/401 (98%)

Fig 3.5 Alignment of positive strand of HPV sequence from the genebank compared to the 401bp HPV PCR (Guangdong sample, #113). This sample's sequences were found to be 98% identical to the HPV type 58 L1 region sequences of the genebank (Human papillomavirus type 58, complete genome). The next homologies were to HPV 33 (84%) and HPV 52 (81%). Alterations from the original HPV 58 sequence are as follows: (marked by {*}, A-T transversion; nt 6643, nt 7024; A-C, nt 6688; G-A, nt 7000; C-T, nt 7028 and 7029.

```
4314 : 66  tgtgcttctacacagtctcctgtacctgggcaatatgatgctaccaaatttaagcagtat 125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV18: 88  tgtgcttctacacagtctcctgtacctgggcaatatgatgctaccaaatttaagcagtat 147

4314 : 126 agcagacatgttgaggaatatgatttgcagtttatttttcagttgtgtactattacttta 185
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV18: 148 agcagacatgttgaggaatatgatttgcagtttatttttcagttgtgtactattacttta 207

4314 : 186 actgcagatgttatgtcctatattcatagtatgaatagcagtattttagaggattggaac 245
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV18: 208 actgcagatgttatgtcctatattcatagtatgaatagcagtattttagaggattggaac 267

4314 : 246 tttggtgttcccccccccgccaactactagtttggtggatacatatcgttttgtacaatct 305
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV18: 268 tttggtgttcccccccccgccaactactagtttggtggatacatatcgttttgtacaatct 327

4314  : 306 gttgctattacctgtcaaaaggatgctgcaccggctgaaaataaggatccctatgataag 365
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV18: 328 gttgctattacctgtcaaaaggatgctgcaccggctgaaaataaggatccctatgataag 387

4314 : 366 ttaaagttttggaatgtgga-ttaaaggaaaagttttctttagactta 412
           ||||||||||||||||||^||||||||||||||||||||||||||
HPV18: 388 ttaaagttttggaatgtggatttaaaggaaaagttttctttagactta 435
```

Identities = 347/348 (99%), Gaps = 1/348 (0%)

Fig 3.6 Alignment of positive stand of HPV sequence from the genebank compared to the 348bp HPV PCR (Shanghai sample, #4314). This sample's sequences were found to be 99% identical to the HPV type 18 L1 region sequences of the genebank (Human papillomavirus type 18, isolate IS002, major capsid protein L1 (L1) gene). The next homologies were to HPV 45 (85%) and HPV 59 (80%). Alterations from the HPV 18 sequence are as follows: (marked by {^} as a gap in the sequence), nt 408

3.4 Restriction fragment length polymorphism

Total 169 paraffin-embedded and frozen cervical carcinoma tissues were re-amplified with MY09/11 and typed using RFLP. 145 (85.7%) samples gave recognizable digestion patterns of HPV DNA (Fig 3.7 – Fig 3.8) and 24 samples were still untyped. These 24 samples were then analyzed again by using HPV type specific primer PCRs.

Five types of HPV were detected in the cervical carcinoma specimens using RFLP. These included HPV 16, HPV 18, HPV 33, HPV 52 and HPV 58. The detection of HPV 16 in the cervical carcinoma tissues were 91% (34/37) in Shanghai; 100% (5/5) in Sichuan; 92.3% (72/78) in Beijing; 100% (6/6) in Guangdong and 75% (15/20) in Hong Kong. There was no statistically significant difference in different regions ($p=0.1325$ by Fisher's exact test). (Table 3.4)

Double infection of HPV 16 and HPV 58 was detected in one sample from Shanghai.

Table 3.4 Results of HPV typing using RFLP

Regions / HPV types	**Shanghai**	**Sichuan**	**Beijing**	**Guangdong**	**Hong Kong**
16	34	5	72	6	15
18	0	0	3	0	4
33	0	0	0	0	1
52	0	0	2	0	0
58	3*	0	1	0	0
Total	37*	5	78	6	20

* Double infection of HPV 16 + HPV 58 was counted

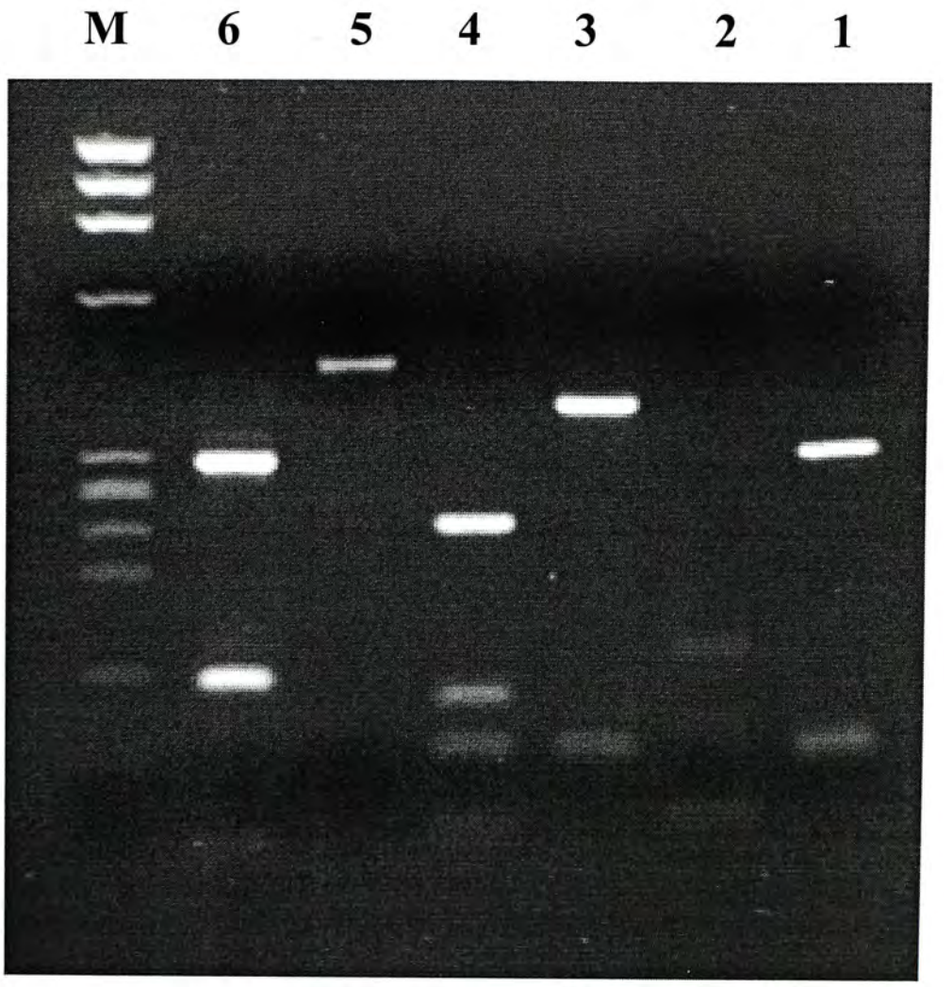


Figure 3.7 Restriction fragment length polymorphism patterns of MY09/11 L1 PCR products used to identify genital HPV types. Electrophoresis of restriction enzyme Rsa I digestion of different PCR products in 2% agarose gel stained with ethidium bromide. Lane 1: HPV 16 (310bp, 72bp, 70bp) ; Lane 2: HPV 18 (135bp, 125bp, 85bp, 72bp, 38bp); Lane 3: 31 (380bp, 72bp) ; Lane 4: HPV 33 (236bp, 102bp, 72bp, 39bp); Lane 5: HPV 52 (449bp) ; Lane 6: HPV 58 (306bp, 111bp, 32bp); Lane 7: size marker Φx-174/HaeIII Markers.

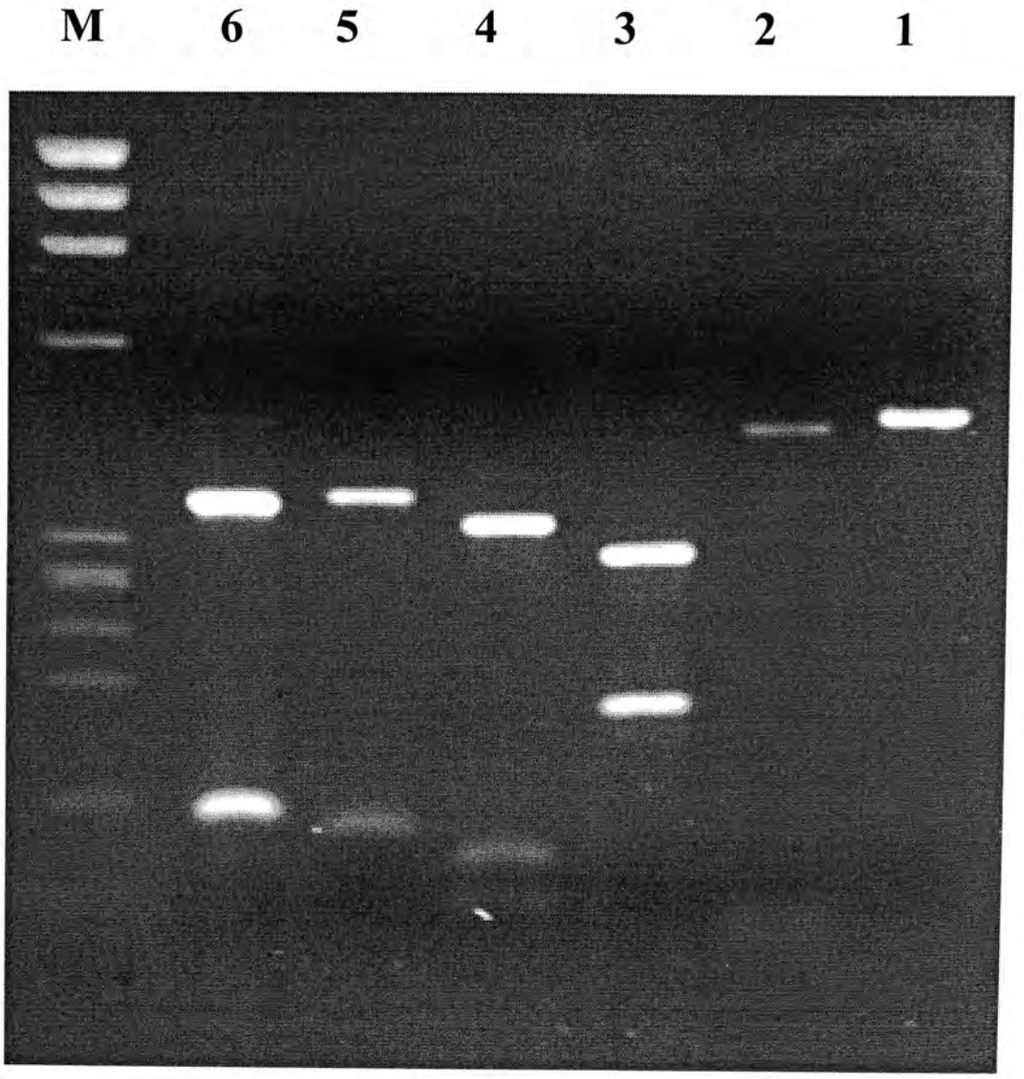


Figure 3.8 Restriction fragment length polymorphism patterns of MY09/11 L1 PCR products used to identify genital HPV types. Electrophoresis of restriction enzyme Dde I digestion of different PCR products in 2% agarose gel stained with ethidium bromide. Lane 1: HPV 16 (452bp); Lane 2: HPV 18 (432bp, 23bp); Lane 3: HPV 31 (283bp, 167bp, 2bp); Lane 4: HPV 33 (320bp, 77bp, 52bp); Lane 5: HPV 52 (357bp, 92bp); Lane 6: HPV 58 (348bp, 101bp); Lane 7: size marker Φx-174/HaeIII Markers.

3.5 HPV type specific polymerase chain reaction

The band size of HPV 16, 18, 31, 33, 52 and 58 positive samples was 96bp, 115bp, 110bp, 114bp, 168bp and 118bp respectively. HPV DNA was identified in 227 out of 371 (61%) specimens. (Fig. 3.9)

There were 128 specimens from Sichuan and after screening, 105 (82%) out of them showed positive. No evidence of any HPV DNA presented in the specimens from Hong Kong. In Shanghai, Beijing and Guangdong, the positive rates were 61%, 41% and 61% respectively. This showed that the HPV type specific primers PCR was efficient in detecting the HPV types in Sichuan specimens and least effective in Hong Kong specimens from Hong Kong.

HPV 16 infection was the most common type found in this screening and 202 specimens were found to be infected. The difference was not statistically significant ($p=0.364$ by Fisher's exact test) in different regions of HPV 16 infection. There were 14 specimens infected by HPV 18, 4 specimens by HPV 52 and 2 specimens by HPV 58.

Among the specimens with HPV infection there were 2 specimens having more than one type of HPV infection including one case with HPV 18/52 in Sichuan, and another case with HPV 18/31 in Guangdong. (Table 3.5)

Table 3.5 Results of HPV typing using HPV type specific primers PCR

Regions / HPV types	**Shanghai**	**Sichuan**	**Beijing**	**Guangdong**	**Hong Kong**
16	50	91	21	40	0
18	2	9*	0	3*	0
31	2	0	0	1*	0
33	1	1	0	0	0
52	1	2*	0	1	0
58	0	2	0	0	0
Total No. of typed sample	56	104	21	44	0
Total screened	91	128	51	73	28

* Double infections of HPV 52 + HPV 18 and HPV 18 + HPV 31 were counted

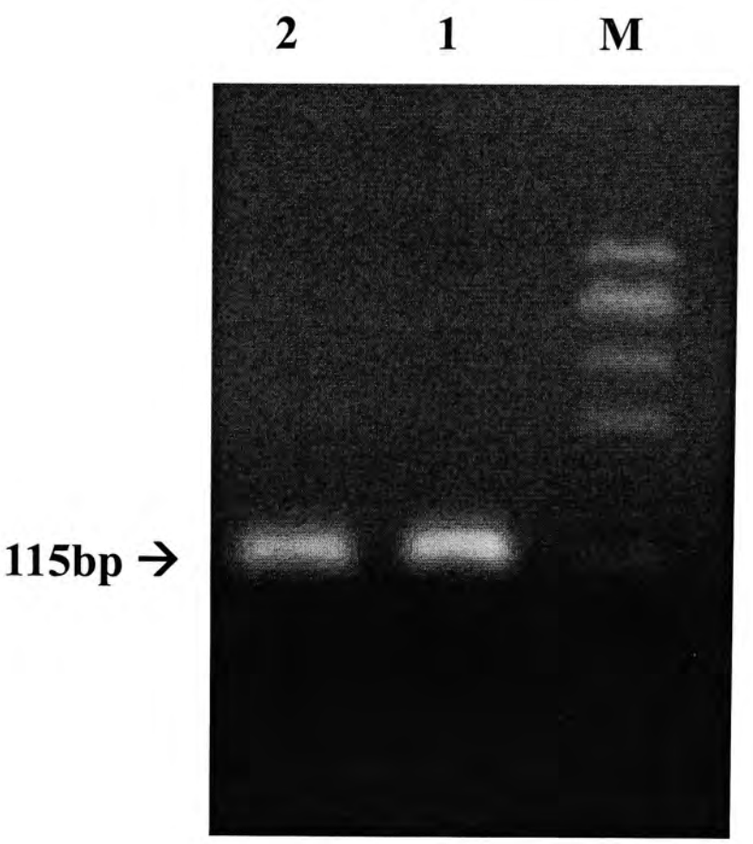


Figure 3.9 Electrophoresis of HPV type 18 specific PCR in 2 % agarose gel stained with ethidium bromide. Lane 1: HeLa cell DNA; Lane 2 HPV 18 specimen from Sichuan; M: size marker Φx-174/HaeIII Markers

HPV 52 and 58 type specific polymerase chain reaction

Primers for HPV 52 and 58 type-specific polymerase chain reactions were newly designed for this study. Prof. Wayne Lancaster and Prof. Toshihiko Matsukura kindly provided the HPV 52 plasmid and HPV 58 genome respectively.

Transformation of HPV 52 and 58 plasmids were done to produce enough HPV 52 and HPV 58 DNA for the development of HPV type-specific primers. The transformation efficiency of HPV 52 plasmids was 100% and it was 70% for HPV 58.

HPV 52 plasmid DNA was extracted and digested with EcoR I. The 8kb-size DNA was the genome of HPV 52 while the 3kb-size DNA was the cloning vector. NcoI and Not I were used to digest the HPV 58 plasmid into the 8kb-size DNA (HPV 58 genome) and 3kb-size DNA (pGEM-T vector) (Figure 3.10)

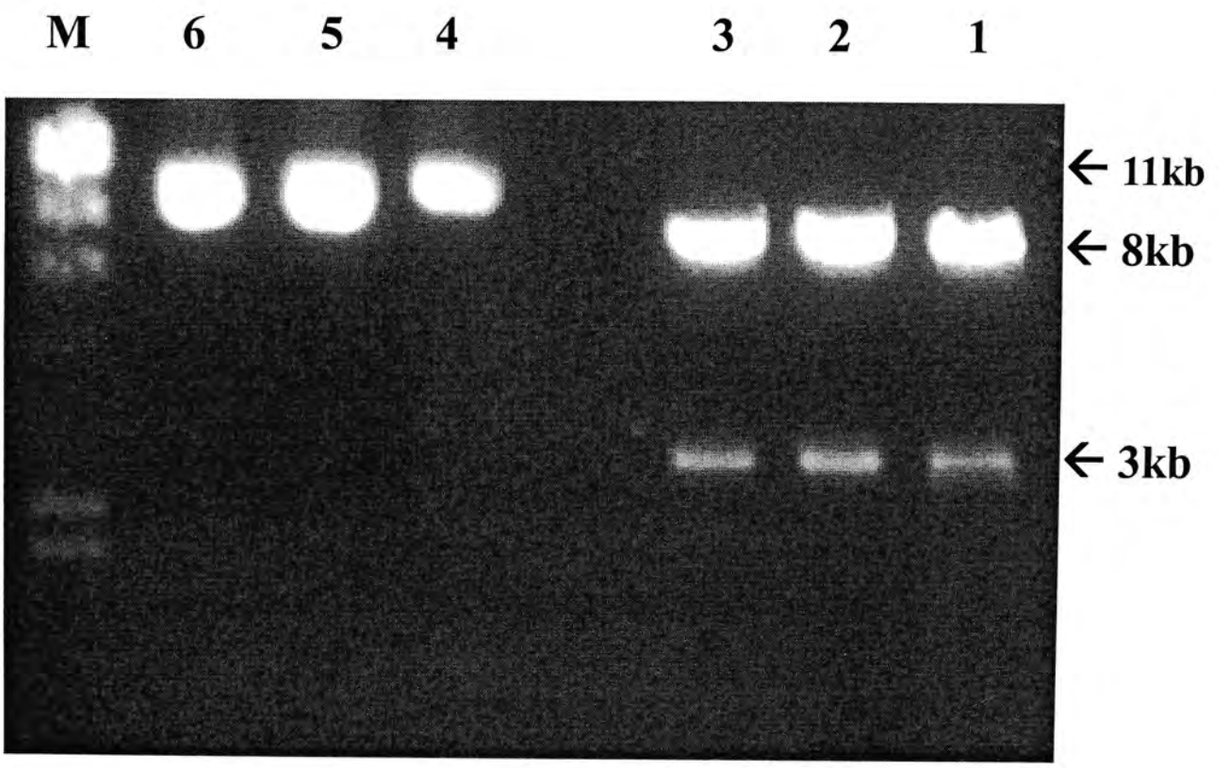


Fig 3.10 DNA extracted from HPV 52 and HPV 58 plasmid. Lane 1: HPV 58 plasmid digested with Nco I and Not I; Lane 2 and 3: HPV 52 plasmid digested with EcoR I; Lane 4: Uncut HPV 58 plasmid; Lane 5 and 6: Uncut HPV 52 plasmid, M: size marker Lambda DNA/Hind III Markers

Specificity of the reaction

Five different types of HPV DNA (HPV 16, HPV 31, HPV 33, HPV 52, HPV 58) and HeLa cells, which were used as a positive control of HPV 18, were used in different PCR to determine the specificity of the primers. HPV 52 yielded a band of 168bp and there was no this band appearing in all the rest of the samples. A band of 118bp was only observed in the PCR with HPV 58 DNA but could not be observed in other types of HPV DNA. (Fig. 3.11)

Southern hybridization was performed with 5'-end labeling method. After gel electrophoresis, PCR products were transferred onto the nylon filter membranes by Southern-blot. Then, the membranes were hybridized with a HPV 52 and HPV 58 ^{32}P-labelled oligonucleotide probes respectively. (Fig 3.12)

Sensitivity of the methods

The sensitivity of the PCR was determined by HPV DNA serial dilution test. The PCRs were started with 1 ng of HPV 52 and HPV 58 plasmid DNA with serial ten-fold dilutions. The lowest limit of HPV DNA detection in PCRs was 10fg of HPV DNA. (Fig. 3.13-3.16)

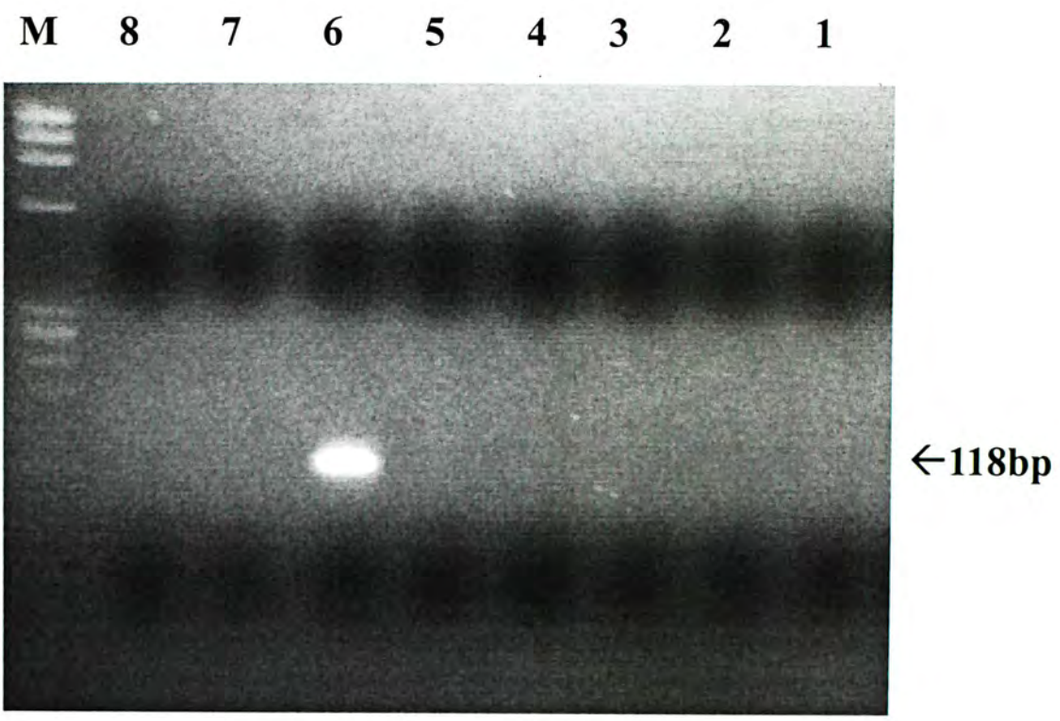


Fig 3.11 Detection of HPV 58 DNA using HPV 58 type specific PCR with other types of HPV infected cervical cancer specimens. PCR products were shown after electrophoresis on 2% agarose gel and ethidium bromide staining. Lane 1-6; HPV 16, HPV 18, HPV 31, HPV 33, HPV 52 and HPV 58 DNA respectively. Lane 7: negative control of DNA isolated from human placenta; Lane 8: negative control of distilled water; M: size marker, Φx-174/HaeIII Markers.

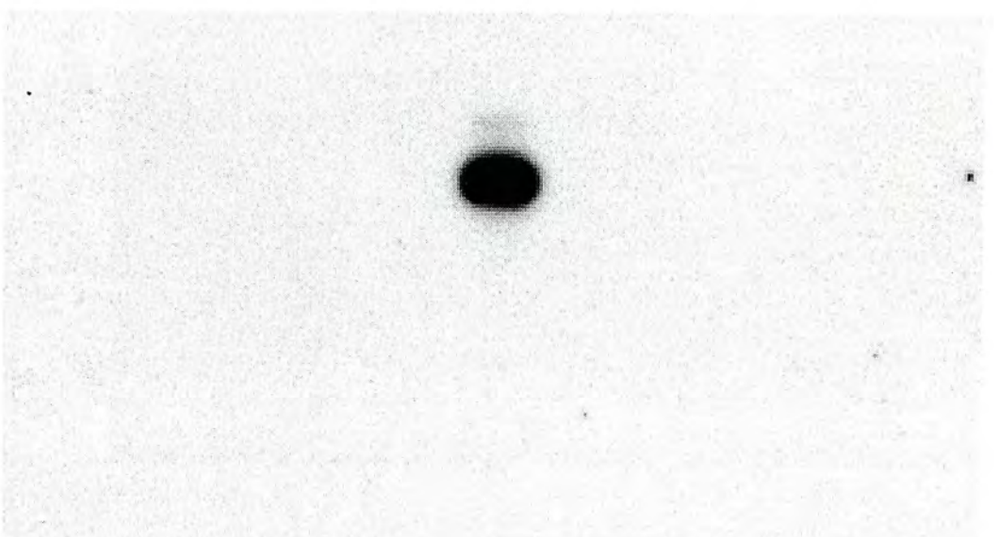


Fig 3.12 Southern hybridization of HPV 58 DNA transferred from the agarose gel in Fig. 3.11 using a [γ-^{32}P] labeled HPV 58 type specific probe. The arrow indicated the HPV 58 positive case.

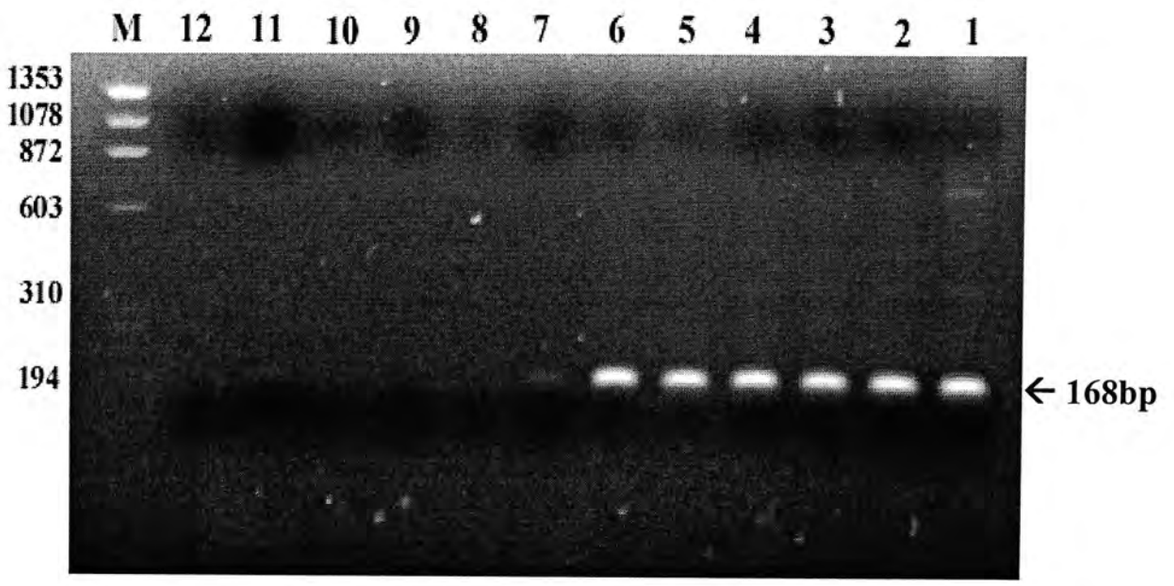


Fig 3.13 **Sensitivity of the HPV 52 type specific PCR. Lane 1-8: amplification products of several 10-fold dilution of HPV 52 plasmid DNA, from 10ng, lane 1 to 1 fg, lane 8 were analysed on 2% agarose gel stained with ethidium bromide. Lane 9: negative control of DNA isolated from human placenta; Lane 10: negative control of distilled water; M: size marker, Φx-174/HaeIII Markers.**

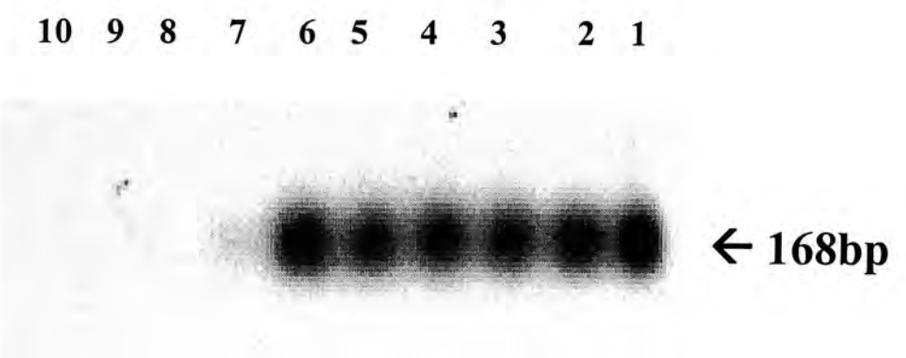


Fig 3.14 Southern hybridization of HPV 52 DNA transferred from the agarose gel in Fig. 3.13 using a [γ-^{32}P] labeled HPV 52 type specific probe. The arrow indicates the HPV 58 positive case. The lane arrangement was the same in Fig. 3.13

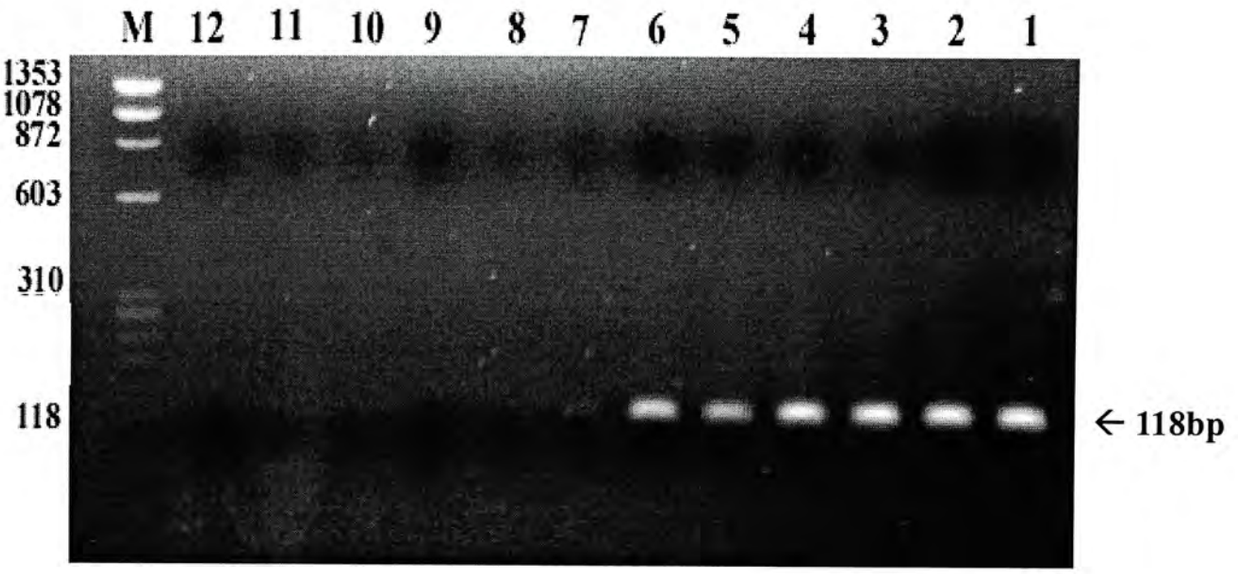


Fig 3.15 **Sensitivity of the HPV 58 type specific PCR. Lane 1-8: amplification products of several 10-fold dilution of HPV 58 plasmid DNA, from 10ng, lane 1 to 1 fg, lane 8 were analysed on 2% agarose gel stained with ethidium bromide. Lane 9: negative control of DNA isolated from human placenta; Lane 10: negative control of distilled water; M: size marker, Φx-174/HaeIII Markers.**

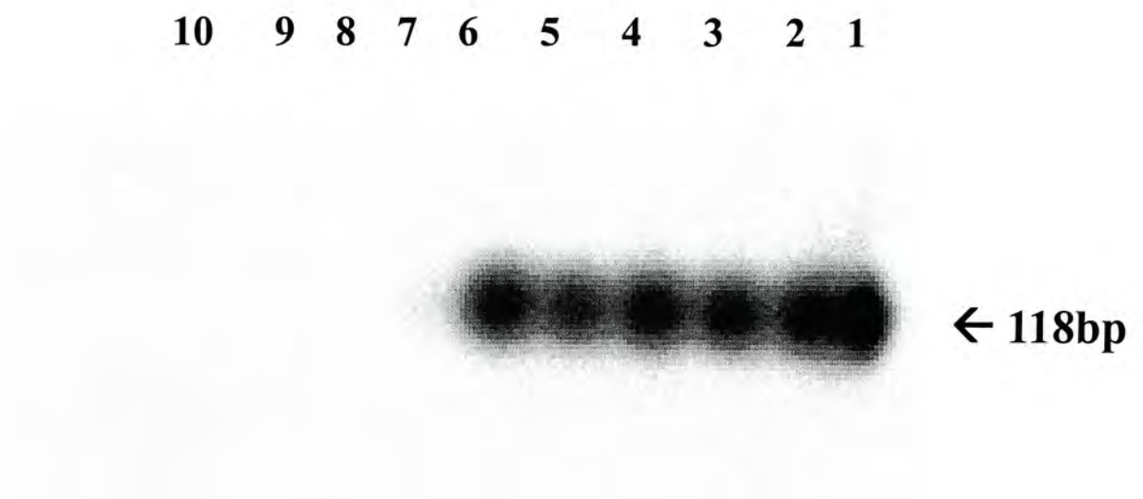


Fig 3.16 Southern hybridization of HPV 58 DNA transferred from the agarose gel in Fig. 3.15 using a [γ-^{32}P] labeled HPV 58 type specific probe. The arrow indicated the HPV 58 positive case. The lane arrangement was the same in Fig. 3.15.

Validation on snap-frozen tissues and paraffin-embedded clinical specimens

The HPV 52 and HPV 58 type specific PCR were tested on paraffin-embedded and fresh tissues. There were 5 fresh samples and 3 out of 4 paraffin-embedded tissues shown positive with HPV 52 type specific PCR whereas there were 6 fresh samples and 4 paraffin-embedded tissues showing positive for HPV 58 type specific PCR. The results showed that the efficiency of these 2 types specific PCR were high. (Fig 3.17 – 3.20)

Comparison of HPV consensus primers and type specific primers

There were 15 and 10 specimens of HPV 58 and HPV 52 respectively. showed positive in this study. 3 out of 15 (20%) HPV 58 positive specimens and 6 out of 10 (60%) HPV 52 positive specimens were MY09/11 negative. The sensitivity of the MY09/11 PCR was 10 copies of HPV DNA. All the specimens after the use of MY09/11 and GP5+/6+ primers were negative but positive to HPV 52 and 58 type-specific primers were paraffin-embedded tissues. This result showed that the HPV 52 and HPV 58 type-specific primers were more sensitive in paraffin-embedded specimens than MY and GP+ primer sets. (Fig. 3.21 – 3.22)

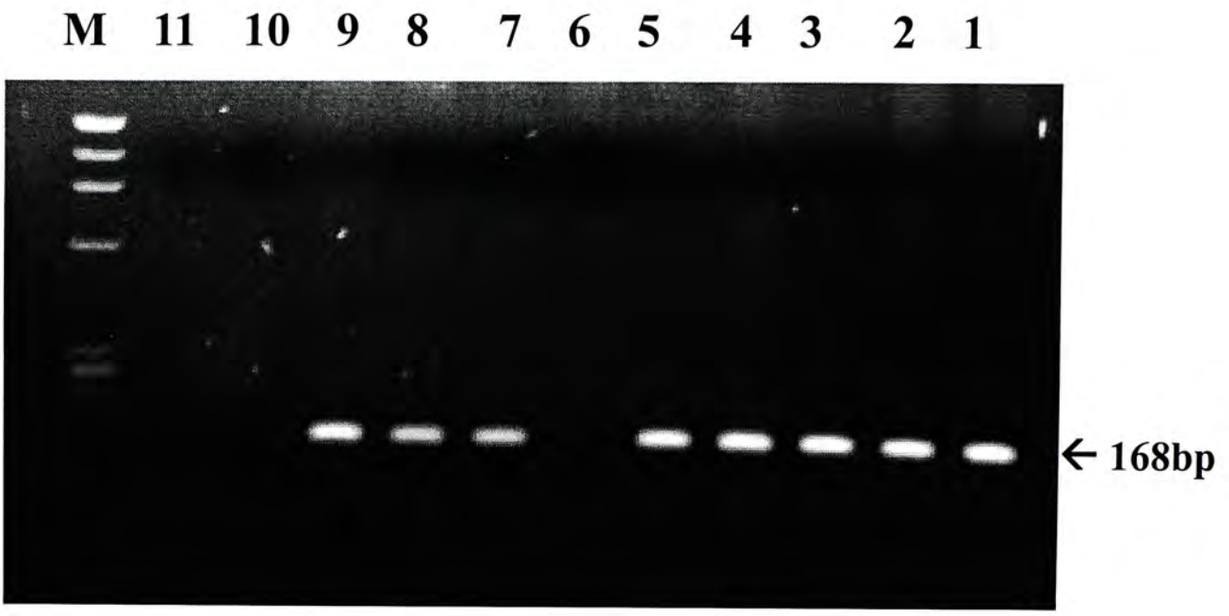


Fig. 3.17 HPV 52 type specific PCR. Lane 1-5: analysis of fresh prepared cervical cancer DNA from 5 different patients; Lane 6-8: analysis of 4 paraffin-embedded cervical cancer tissue biopsies; Sample 6 was negative for HPV 52; Lane 9: positive control of HPV 52 plasmid; Lane 10: negative control of Hela cell DNA. Lane 11: negative control of distilled water.

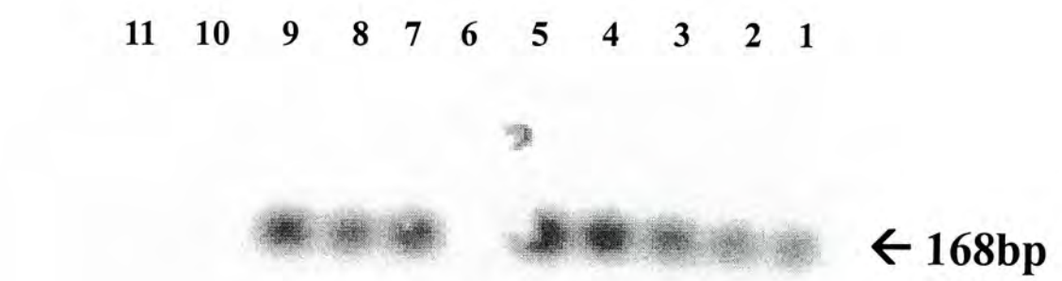


Fig 3.18 Southern hybridization of HPV 52 DNA transferred from the agarose gel in Fig. 3.17 using a [γ-^{32}P] labeled HPV 52 type specific probe. The arrow indicated the HPV 52 positive case. The lane arrangement was the same in Fig. 3.17

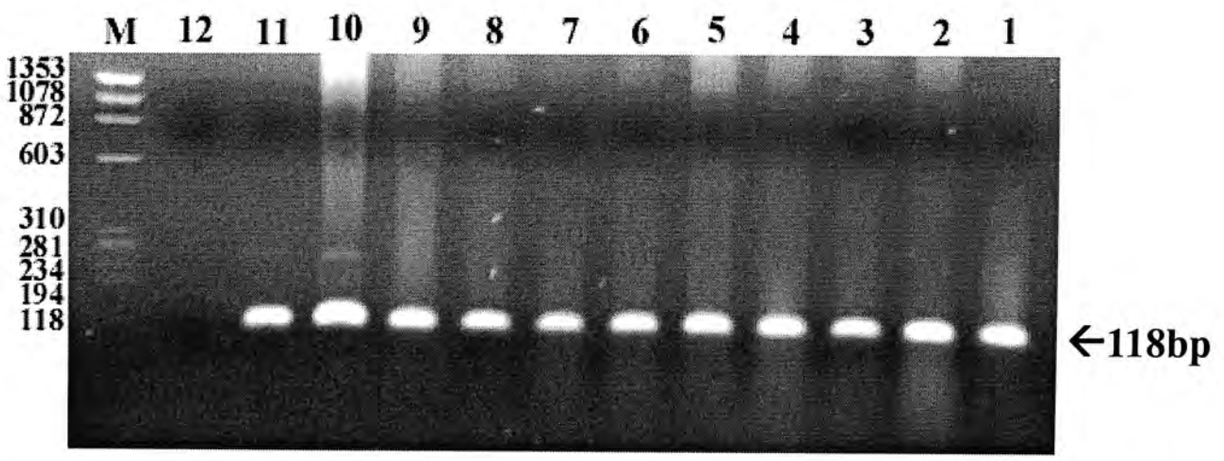


Fig. 3.19 HPV 58 type specific PCR. Lane 1-6: analysis of fresh prepared cervical cancer DNA from 6 different patients; Lane 7-10: analysis of 4 paraffin-embedded cervical cancer tissue biopsies; Lane 11: positive control of HPV 58 plasmid; Lane 12: negative control of distilled water

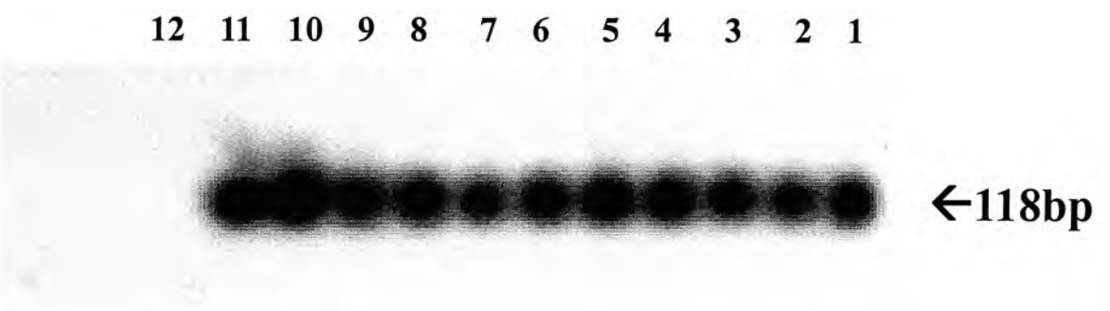


Fig 3.20 Southern hybridization of HPV 58 DNA transferred from the agarose gel in Fig. 3.19 using a [γ-^{32}P] labeled HPV 58 type specific probe. The arrow indicated the HPV 58 positive case. The lane arrangement was the same in Fig. 3.19

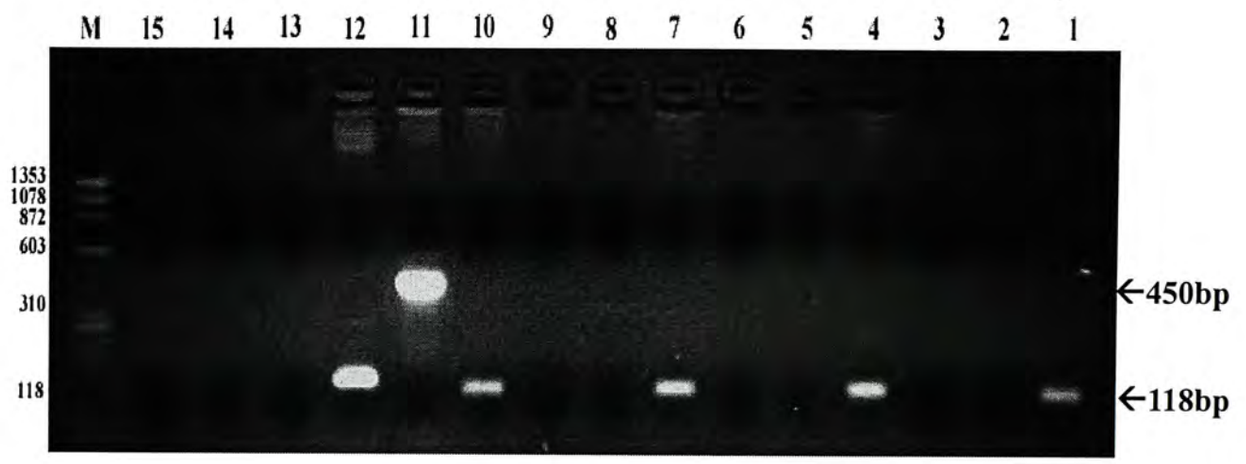


Fig 3.21 Comparison of PCR using HPV 58 type specific primer, MY09/11 and Gp5+/6+ in paraffin-embedded HPV 58 samples. Lane 1, 4, 7: HPV 58 type specific primer; Lane 2, 5, 8: MY09/11 primer; Lane 3, 6, 9: GP 5+/6+ primer; Lane 10: HPV 58 type specific primer with HPV 58 plasmid; Lane 11: MY09/11 primer with HPV 58 plasmid (450bp); Lane 12: GP5+/6+ primer with HPV 58 plasmid; Lane 13-15: negative control of distilled water for each set of PCR.

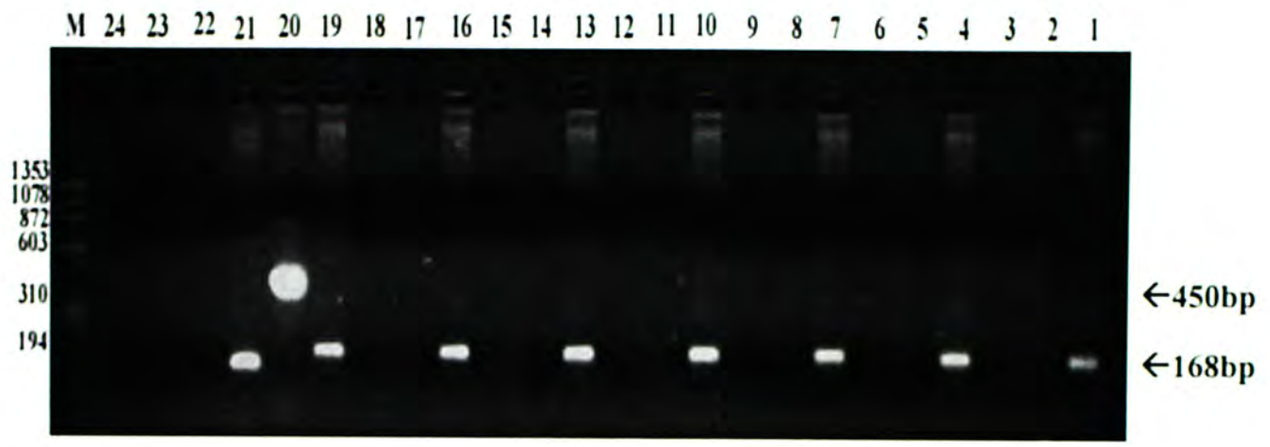


Fig 3.22 Comparison of PCR using HPV 52 type specific primer, MY09/11 and Gp5+/6+ in paraffin-embedded HPV 52 samples. Lane 1, 4, 7,10, 13, 16: HPV 58 type specific primer. Lane 2, 5, 8, 11, 14, 17: MY09/11 primer. Lane 3, 6, 9,12,15,18: GP 5+/6+ primer. Lane 19: HPV 52 type specific primer with HPV 52 plasmid. Lane 20: MY09/11 primer with HPV 52 plasmid (450bp). Lane 21: GP5+/6+ primer with HPV 52 plasmid. Lane 22-24: negative control of distilled water for each set of PCR.

3.6 Polymerase Chain reaction using HPV consensus primer GP5+/6+

Total 144 cervical carcinoma specimens that were negative with MY primer DNA sequencing, RFLP and HPV type specific primers PCR were screened with HPV consensus primer pair GP5+/6+. 17 specimens were detected with HPV DNA among which 3 (8.5%) from Shanghai, 3 (13%) from Sichuan, 4 (13%) from Beijing, 3 (10.7%) from Guangdong and 4 (14.2%) from Hong Kong (Fig 3.23).

Five types of HPV were detected in the 17 cervical carcinoma tissues using DNA sequencing with GP5+/6+ primer pair. The sequencing results obtained from 90 to 120 nucleotides were compared to known HPV sequences available in the genebank. These included HPV-16, HPV 35. HPV 45, HPV 59 and HPV 67. Except HPV 16 which was high risk HPV type, the rest were intermediate risk HPV types. HPV 16 were detected in 1 specimen from Sichuan, Beijing and Guangdong respectively. (Table 3.6)

Table 3.6 Results of GP5+/6+ PCR screening and HPV typing by DNA sequencing using GP5+/6+ primer pair

Regions / HPV types	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong
16	0	1	1	1	0
35	0	0	0	0	1
45	0	0	1	0	0
59	1	2	0	2	2
67	0	0	0	0	1
Undetermined	2	0	2	0	0
Total HPV typed samples	3	3	4	3	4
Total screened	35	23	30	28	28

The successful rate of this reaction was statistically no different within 5 regions.

$\chi 2 = 0.65$, $p = 0.957$ by Fisher's exact test

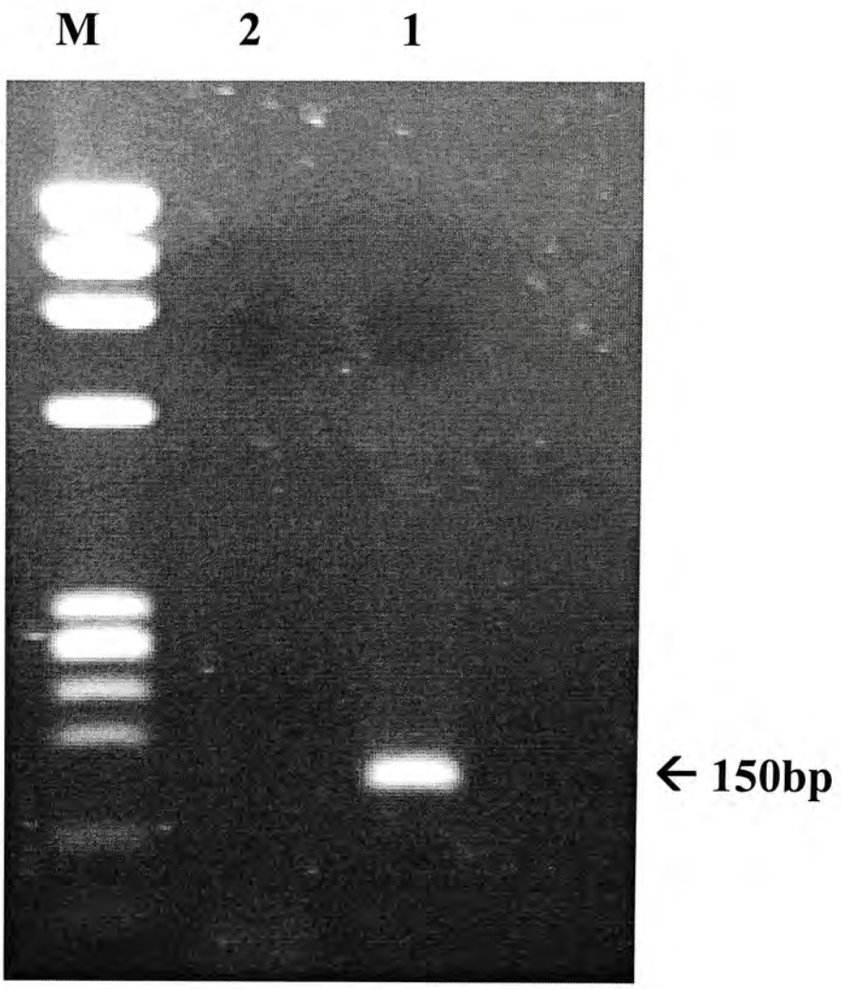


Figure 3.23 Electrophoresis of GP5+/6+ PCR products from L1 region of HPV genome in cervical carcinoma tissues in agarose gel stained with ethidium bromide. Lane 1: HPV DNA positive samples; Lane 2: negative control of distilled water; M: size marker, Φx-174/HaeIII Markers.

3.7 Correlations of HPV prevalence, geographical variation, histology and age of the cervical cancer patients

The overall HPV prevalence in cervical carcinoma in China was 83.9% and ranged from 79% - 88% by region. Thirteen types of viruses were detected through different methods. HPV 16 accounted for 79% of the total HPV positive specimens and did not have geographical variation ($p = 0.00$ by Fisher's exact test). The HPV 16 phylogenetic group, including HPV 16, 31, 33, 35, 52 and 58, represented 87.3% of the viruses found. HPV 18 related group including HPV 18, 45 and 59, represented 8.6%. HPV 35, 53, 67, 70 were each found once in the specimens. The prevalence of HPV 52 and 58 in Hong Kong and Guangdong were statistically higher than other regions of China ($p = 0.00092$ for HPV 52 and $p = 0.011$ for HPV 58 by Fisher's exact test). Three cases of double infection were detected in Shanghai, Sichuan and Guangdong. This showed that some rare HPV types varied geographically. (Table 3.7)

The relationship between HPV type and histology was shown in Table 3.8. HPV 16 or related virus accounted for 76.5% of the viral types found in squamous cell carcinoma ($p = 0.00$ by Fisher's exact test). HPV 18 and related viruses accounted for 22.6% of the viral types in adenocarcinoma and 5.8% of the viral types in squamous cell carcinoma as well. HPV 18 was more prevalent in adenocarcinoma than squamous cell carcinoma. ($p = 0.00$ by Fisher's exact test).The prevalence of HPV 16 in squamous cell carcinoma was higher than that in adenocarcinoma. ($p = 0.004$ by

Fisher's exact test). No relationship was observed between HPV 52 or HPV 58 infections and histology of the specimens ($p = 0.843$ and $p = 0.541$ by Fisher's exact test respectively). (Table 3.8)

The correlation of the age of the Hong Kong patient and the HPV type were studied. The mean age of the specimens was 55.2. HPV 16 accounted for 60% (95/156) of Hong Kong patients. There was no significant difference in the incidence of HPV 16 among the 3 age groups. However, HPV 52 and HPV 58 showed more prevalent in elder patients then younger patients ($p = 0.008$ for HPV 52 and $p = 0.356$ for HPV 58 by Fisher's exact test). (Table 3.9)

Different detection methods were applied to determine the HPV types in cervical carcinoma. The efficiency of each method varied in different regions and the results were summarized in Table 3.10.

Table 3.7 Prevalence of HPV in cervical cancer by different regions of China.

Regions / HPV types	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong	Total
11	0	0	0	1	2	3
16	105	123	111	99	100	538
18	3	11	4	7	24	49
31	2	0	0	2	1	5
33	1	1	0	0	10	12
35	0	0	0	0	1	1
45	0	0	1	2	0	3
52	1	1	2	5	8	17
53	0	0	0	0	1	1
58	2	3	3	6	11	25
59	1	2	0	2	2	7
67	0	0	0	0	1	1
70	0	0	0	0	1	1
16,58	1	0	0	0	0	1
18,31	0	0	0	1	0	1
18,52	0	1	0	0	0	1
Unknown	2	1	3	7	0	13
Negative	32	20	26	19	33	130
Total	150	163	150	151	195	809

Table 3.8 Prevalence of HPV type by histologic tumor characteristics

	Histologic type, No. (% of total specimens)		Histologic differentiation degree, No. (% of total specimens)		
Type	**Squamous**	**Adenocarcinoma**	**Poor**	**Moderate**	**Well**
HPV 16	500 (68.7)	28 (45.2)	96 (60.9)	281 (67)	165 (72.6)
HPV 16 related*	17 (2.4)	0 (0)	3 (2.2)	11 (2.2)	4 (1.9)
HPV 18	33 (4.6)	13 (21)	11 (7)	25 (6)	13 (5.7)
HPV 18 related	9 (1.2)	1 (1.6)	3 (2.2)	3 (0.7)	2 (1.2)
HPV 52	16 (2.2)	2 (3.2)	2 (1.7)	13 (3.1)	0 (0)
HPV 58	25 (3.4)	1 (1.6)	7 (4.8)	8 (1.9)	10 (4.4)
Other	15 (2.1)	0 (0)	4 (2.5)	6 (1.6)	4 (1.9)
HPV negative	114 (15.4)	17 (27.4)	28 (17.2)	70 (17.1)	29 (12.3)
Total No. of specimen	729 (100)	62 (100)	157 (100)	417 (100)	227 (100)
	$\chi 2 = 39.67$, p=0.00		$\chi 2 = 23.47$, p=0.052		

* HPV 52 and HPV 58 were excluded

Table 3.9 Prevalence of HPV in cervical cancer by age in Hong Kong.

HPV type \ Age group	<40	40-60	>60	Total
11	0	1	0	1
16	18	42	35	95
18	7	15	2	24
31	0	0	1	1
33	2	3	5	10
35	0	0	1	1
52	1	2	5	8
53	0	1	0	1
58	0	2	9	11
59	0	1	1	2
67	0	0	1	1
70	1	0	0	1
Total	29	67	60	156

Table 3.10 Efficiency of different detection methods in different regions of China

	Regions, No. (% of total specimens)				
Method	Shanghai	Sichuan	Beijing	Guangdong	Hong Kong
MY-DNA squencing	22 (18)	30 (21)	20 (16)	72 (57)	138 (85)
RFLP	36 (31.5)	5 (3)	78 (64)	6 (5.7)	20 (12.6)
TS-primer	57 (48)	104 (74)	21 (17)	44 (35)	0 (0)
GP-DNA Sequencing	3 (2.5)	3 (2)	3 (2)	3 (2.3)	4 (2.4)
Total	119	143	121	126	162

CHAPTER 4

DISCUSSION

4.1 Prevalence of HPV infection in cervical cancer in China

The International Biological Study on Cervical Cancer conducted a research on the prevalence of HPV in cervical cancer in 22 countries except China (Bosch *et al.,* 1995). The HPV prevalence ranged from 75% to 100% in different countries. It was found that HPV16 was the predominant type in cervical carcinoma in most countries and some of the intermediate risk HPV types were only found in certain countries. It was found that HPV 45 was predominant in western Africa, whereas HPV 39 and HPV 59 were predominant in Central and South America.

The studies of the relationship of HPV and cervical cancer in China were started in 1987 (Zhang *et al.,* 1987; Shu *et al.,* 1987). After that several studies was conducted and continue until now. Among those studies, most of them were small-scaled and regional bounded. The detection methods used in the prevalence studies could influence the actual detection rate of HPV. In order to obtain a more accurate prevalence of HPV in cervical cancer, the detection methods were standardized. These include DNA sequencing technique, RFLP, HPV type specific primer PCRs and 2 sets of HPV consensus primer pairs. Nearly all the HPV types could be classified.

Sichuan

The HPV detection rates in early 90s were lower than that research conducted in recent years. This was because the detection methods they choose. When using one or two HPV type specific primers and probes, only few HPV types could be detected. As the detection methods improved, using several HPV consensus primers and DNA sequencing could detect all the HPV types and this increased the detection rate of HPV in recent studies. The report of Peng in 1991 showed that only 35% (35/101) of cervical cancer cases were due to the infection of HPV. The positive specimens were the result of the infection of HPV 16. Both studies performed by Stephen AL *et al* (2000) and ours showed that the infection rate of HPV in Sichuan was 88% which were found to be higher than Peng's in 1991. The infection rate of HPV 16 and HPV 18 in Stephen's study was 61% and 9% respectively. HPV types 33, 45, 58 and 59 were also identified in the samples. Among our samples, the HPV infection rate was 88% (143/163) and the infection rate of HPV 16 was 86% (123/143). HPV 18 was the second most infected type that accounts for 8.4% of the positive cases. HPV 33, HPV 52, HPV 58 and HPV 59 were also detected in the specimens. A novel finding in Sichuan was that the rate of HPV 58 and 52 infections came third and forth in the study. This showed that HPV 58 and 52 were the high-risk HPV types in the development of cervical cancer in Sichuan.

In our study, the relationship of histology types and HPV infection was also studied. Most of the HPV 16 infected samples were squamous cell carcinoma. In the

case of the infection of HPV 18, 33.3% were adenocarcinoma. This finding was coincidence with other research (Bosch *et al.,* 1995).

In order to study the risk factors of HPV in cervical cancer, Tang *et al* conducted a study on the prevalence of HPV in regions with high incidence and low incidence of cervical carcinoma respectively within Sichuan in 1992. The samples collected by PAP smear of 317 cases from high incidence area and 500 from low incidence area were used for the HPV 16 and HPV 33 type specific primers screening. The HPV infection rate in high incidence area was 18% whereas it was 3.4% in low incidence area. This revealed that the HPV infection rate in high incidence area of cervical carcinoma was 5 times higher than that in the low incidence area. For the risk factors study, women were recruited from 2 different villages to complete a survey about personal hygiene situation, cigarette-smoking habit and sexual behavior with partners. It was concluded that in low incidence area, the marital status was stable and 95% of women had only one sexual partner. Besides, the harvest of the western part of Sichuan was much better than that in any other part of Sichuan, the vitamin absorption was more adequate than that in high incidence area. These suggested why the cervical carcinoma incidence was higher in high incidence area.

Shanghai

In Shanghai, several studies on the relationship between HPV and cervical cancers had been reported. Hunag *et al* has conducted a survey of HPV prevalence in cervical cancer in Shanghai in 1997. In his study, specimens from 40 patients with uterine cervix carcinoma were collected from Shanghai Tumor Hospital and were examined for HPV DNA by PCR. The researchers used consensus primers (MY09/11) located in the L1 region, as well as 10 different HPV type specific probes located at the same L1 regions to detect the HPV DNA. During the HPV screening, positive results were shown in 35 samples, in which HPV 16 counted for 7.5% while HPV 18 counted for 10% of the total. Moreover, HPV 52 and 58 demonstrated a high prevalence in this study, each of them counted for 15% of the HPV positive cases.

Another research was conducted by Lin *et al* (1998) included 37 cases collected from 3 different hospitals in 2 different regions in eastern China: Shanghai and Jiangxi. Eighteen samples of frozen tissue were obtained from the Shanghai Tumor Hospital; while in the Shanghai Hospital of Obstetrics and Gynecology, 19 samples of formalin-fixed, paraffin-embedded tissues were found. Besides, 40 cases of frozen cervical cancer tissue were obtained from the Jiangxi Provincial Maternity Institute. 72 cases were diagnosed as squamous cell carcinoma of the cervix and 5 cases as cervical adenocarcinoma. In this study, two sets of HPV consensus primer pairs including MY09/11 and GP5+/6+ were used for HPV DNA screening. A prevalence rate of HPV

infection in frozen tissues and paraffin-embedded tissues were 83.3% and 89.5% respectively. In Jiangxi, the infection rate of HPV in cervical carcinoma was 100%. Among the 3 hospitals, different HPV types had different prevalence. The prevalence of HPV 16 detected in cervical carcinoma was different in Shanghai and Jiangxi Province. At 2 hospitals in Shanghai, the HPV 16 infection rates were 27.8% and 15.8% respectively, whereas the rate in Jiangxi was 70.9%. However, HPV 52 was absent in the paraffin-embedded tissue when it was detected in the fresh tissue specimens from Shanghai and Jiangxi. In the frozen tissue from Shanghai, the percentage of infection was 16.7%, but it was only 2.5% in Jiangxi. When comparing with the prevalence of HPV 58, there was no significant difference in the infection rate of the two places, they are 15.8% to 22.2%.

In our study, 150 paraffin-embedded cervical carcinoma tissues were collected for HPV DNA screening. HPV DNA was detected in 80% of the samples, while HPV 16 accounted for 90% of them. Moreover, HPV 18, HPV 31, HPV 33, HPV 52, HPV 58 and HPV 59 were also detected. My finding about HPV 16 infection rate was similar to the pervious one and was dominate in the cervical carcinoma (Lin *et al.,* 1998). HPV 18 counted for 2.5% of total. In Lin's study, HPV 18 was not detected in any specimens from Shanghai. This demonstrated that the infection rate of HPV 18 in Shanghai was relatively lower than the others. The International Biological Study on Cervical Cancer also gave a report on the prevalence of HPV in cervical cancer in a worldwide

perspective by stating that 13.7% of HPV infected cervical carcinoma were infected by HPV 18.

Besides HPV 16, HPV 58 was the second most commonly found in our study, counting for 2.5% of the HPV infected cervical carcinoma. The positive rate of this study was lower than the previous one done by Huang *et al* and Lin *et a*l. The difference between the result of my study and those of the pervious ones might be attributed to the sampling from different regions. Lin *et al* suggested that the risk factors of different regions might cause the difference in the prevalence of HPV types.

HPV 52 was another type of HPV, which was uncommon in other countries. From the pervious studies, the infection rate of HPV 52 was higher than other HPV types (Lin *et al.*, 1998). In my study, only 1 case of HPV 52 was detected. However, this observation might indicate a variation of HPV epidemiology among Shanghai living in different geographic areas.

Beijing

In 1990, Zhang *et al.* conducted a research on the relationship between HPV and cervical cancer. Thirty-eight cervical carcinomas and 1 cervical condyloma were examined for the presence of HPV type 16 DNA by using 3H-dCTP-labelled HPV-16 probe and the results showed that the HPV-16 DNA positive rate was 74.4% (29/39).

In 1991, Zhang *et al.* did another research on HPV and cervical carcinoma. A large-scale survey was conducted to investigate the association between cervical

carcinoma and human papillomavirus infection in a high-risk area, Xiangyuan Country, Shanxi Province. There were 6710 women examined using Dot blot hybridization with HPV 6B/11, 16 and 18 DNA probes. Fourteen cases were diagnosed as invasive cervical carcinoma and 40% of them were infected by HPV 16.

Zhang *et al.* also conducted the third research on HPV and cervical carcinoma. This time, he used HPV 16 E6 type specific primer for screening and the result was that 30 specimens were shown to be infected by HPV 16.

In our 150 specimens, all were classified as squamous cell carcinomas in which HPV 16 was also dominant (111/124, 90%). This finding was similar with the pervious studies.

Besides Zhang, Zhao *et al.* did a research by using L1 consensus primer for the detection of HPV DNA in cervical cancer tissues in Shanxi Province. In his study, the PCR amplification was carried out. After that, the PCR products for HPV typing were used for carrying out restriction enzyme digestion. By using this method, HPV 16 and HPV 18 could be classified. In fact, 20 cervical adenocarcinoma and 20 squamous cell carcinoma specimens were collected for this study and the result was that the HPV infection rate of total 40 samples was 85%. For adenocarcinoma, HPV 18 were detected in 9 specimens (45%) and only 5 specimens were detected with the infection of HPV 16 (15%). For squamous cell carcinoma, 80% of the samples were detected with the infection of HPV 16 while 5% were detected with the infection of HPV 18.

Among all these studies, it was obvious that HPV 16 was found to be dominated in cervical carcinoma and in squamous cell carcinoma whereas HPV type 18 was found to be more prevanlent in adenocarcinoma tissues. However, since either HPV 16 or HPV 18 type specific probes or primers was the only tools used for detecting HPV 16 or HPV 18, except the one by Zhao *et al.*, it could not reflect the real situation of HPV infection in cervical carcinoma.

In order to determine the real situation of HPV in cervical cancer, a large scaled and standardlized methods were used in this study. One hundred and fifty paraffin-embedded cervical carcinoma tissues were collected in Beijing and screened by using HPV consensus primer pairs MY09/11 and GP5+/6+. DNA sequencing, restriction fragment length polymorphism and HPV type specific primers were used to determine the HPV types. The infection rate of HPV in cervical carcinoma was 82.6% and HPV 16 counted for 90% of the HPV infected samples. There were many other types of HPV detected, HPV 18, HPV 45, HPV 52 and HPV 58. HPV 18 was all the second most common infection in this study. Since in these 150 specimens, all were classified as squamous cell carcinomas in which HPV 16 was dominant, this explained why the infection rate of HPV 18 was not as higher as other studies.

HPV 58 and HPV 52 were also shown to have a high prevalence than other types were compared with Shanghai (eastern part of China) and Sichuan (western part of China), the prevalence of HPV 52 and HPV 58 was higher than that of other

intermediate risk HPV types in other countries.

Guangdong Province

Guangdong Province is located in the southwestern part of China. In our study, Guangdong Province was chosen to compare the difference of HPV prevalence in cervical carcinomas with that of the other parts of China. The cervical carcinomas tissues collected from Beijing, Sichuan and Shanghai representing different situation in different parts of China.

Besides, in this study, the specimens were collected at Zhongshan city in Guangdong Province and no study about cervical cancer and HPV conducted at this city. In our research, there were 151 paraffin-embedded cervical carcinoma tissues collected during the period 1998-2000 and among them, one hundred and thirty-two specimens (87.4%) contained HPV DNA, in which 75% were found to be infected by HPV 16, 8 specimens were infected by HPV 18, 6 were infected by HPV 58 and 5 specimens were infected with HPV 52. Meanwhile, HPV 31, HPV 45 and HPV 59 were also detected. However, 7 specimens were detected with the infection of HPV DNA but they could not be typed.

Hong Kong

Since 1990's, the scientists of the University of Hong Kong and the Chinese University of Hong Kong has conducted studies on the HPV and cervical carcinoma. Yiu *et al.* had conducted a research, in which 15 cervical carcinoma tissues classified as

squamous cell carcinoma undergoing Southern blot hybridization were used to test the presence of HPV DNA. The result was that 13 specimens (86.7%) were showed to have HPV DNA in which 9 of them were infected with HPV16, 2 of them were infected with HPV 18 and 2 were infected with HPV 16 related types.

In 1994, Ngan *et al.* used HPV 16 and HPV 18 E6 type specific primers to screen 64 cervical carcinoma tissues. The result was that 90% (58/64) of the samples showed positive in the HPV DNA screening and 67.2% had a HPV 16 infection.

In 1996, Chan *et al.* collected 488 Hong Kong Chinese women's cervical scrapes obtained from the colposcopy, general, and antenatal clinics and the result was that nine out of 217 patients seen in colposcopy clinic were diagnosed as squamous cell carcinoma and adenocarcinoma. 88% of cervical carcinoma specimens were detected with HPV DNA infection.

From these studies, HPV was predominant in cervical carcinoma. In our study, the HPV DNA was found in 162 cases (83%) and HPV 16 was still the predominant type of HPV high risk type in cervical cancer. This result was similar with the pervious studies. (Yiu *et al.,* 1991; Ngan *et al.,* 1994; Chan *et al.,* 1996)

In 1999, Chan *et al.* conducted a research on 322 Hong Kong women with abnormal Papanicolaou smears using PCR and RFLP analysis. Only 18 specimens were invasive cervical carcinoma. Among the cervical carcinoma, 14 were infected with HPV. There were 8 specimens infected by HPV 16 (44.4%), 6 by HPV 58 (33.3%), and

HPV 18 and HPV 31 were detected only once respectively. In his study, the high prevalence of HPV58 in cervical carcinoma and also in other grades of cervical lesions were detected.

Another study conducted by Liaw's also shown high prevalence of HPV 52 and HPV 58. They were detected among 25% of HPV positive subjects compared with a large international study conducted by IBSCC in 1995 that only 5% of HPV positive subjects were used for the detection of HPV 52 and HPV 58.

The incident of HPV 58 in this study was high (6.79%, 11/195). This observation was similar to others (Liaw *et al.,* 1995; Huang *et al.* 1997; Chan *et al.,* 1999) that HPV 58 showed high prevalence in cervical cancer among Chinese population.

These observation indicated the presence of the worldwide uncommon genotype; and among them, HPV 58 plays an important role in the development of cervical cancer among Chinese.

In this study, 195 fresh cervical carcinoma tissues from Hong Kong were collected at the Prince of Wales Hospital, HKSAR, China from 1991 to 1998. Although the tissue preparation of Hong Kong was different from other sets of specimens that were paraffin-embedded, the screening methods were the same. The HPV DNA was found in 162 cases (83%). These included HPV 16 in 100 (61%) cases, HPV 18 in 24 (14.8%) cases, HPV 31 in 1, HPV 33 in 10 (6.17%), HPV 35 in 1, HPV 52 in 8 (4.93%) cases, HPV 58 in 11 (6.79%) cases, HPV 59 and HPV 11 in 2 respectively and HPV 67 and

HPV 70 in 1 respectively. The variety of HPV types in Hong Kong was great. Twelve different HPV types were detected. HPV 16 was still the predominant type of HPV high risk type in cervical cancer. Eleven (45%) cases of HPV 18 infected were classified as adenocarcinoma.

Regional comparison of HPV prevalence

In the studies of Hong Kong and Guangdong, the high prevalence of HPV 58 and HPV 52 were detected. In Beijing, Sichuan and Shanghai, the prevalence of HPV 52 and HPV 58 were comparatively lower than that of Hong Kong and Guangdong. However, these uncommon HPV types were shown more prevalent in China than other countries in the world. Comparing the prevalence of different HPV types in different countries, Japanese studies showed a lower prevalence of HPV 16 and HPV 18, but higher prevalence of HPV 52 and HPV 58. (Yajima H *et al.,* 1988, Matsukura T *et al.,* 1990) In the United States, studies showed a higher prevalence of HPV 16 and HPV 18 but lower prevalence of HPV 52 (Shimoda *et al.,* 1988). The prevalence of different HPV types in different geographical locations might indicate different etiologies of cervical cancer. In this study, the prevalence of HPV 52 and HPV 58 in the southeastern part were higher than that of other parts of China. Although the overall prevalence of HPV among Chinese women was similar to that described in Western countries, the distinct high proportion of HPV 52 and HPV 58 infection drawn an attention. During designing the HPV detection methods and clinical treatment methods in cervical

carcinomas of Chinese, 2 HPV 16 related types, HPV 52 and HPV 58 should be carefully considered.

The overall prevalence of HPV in cervical cancer of 5 regions of China was 83.93% and there was no significant difference in geographical distribution. According to Walboomer's study, the HPV prevalence in cervical cancer was 99.7%. The reason for this high prevalence may be the improper sampling; disruption of HPV by integration events; the existence of still unidentified HPVs; sensitivity of the method; and the mechanism of transformation. The advantages and disadvantages of different detection methods will be discussed in next section.

In this study, the prevalence of HPV 16 was still predominated in cervical cancer. This results were consistent with many reports on HPV in cervical cancer internationally (zur Hausen, 2000; Walboomers *et al.,* 1999; Bosch *et al.* , 1995). The overall HPV prevalence did not vary between histologic tumor types, suggesting an etiologic consistency among squamous cells tumors, adenocarcinomas and adenosquamous carcinomas.

4.2 DNA extraction and HPV detection methods

Due to the DNA extraction methods and HPV detection methods could influence the detection rates of HPV, the discussion on these subjects will be make in this section.

The paraffin embedded tissues were collected from 4 different regions in China. Paraffin wax embedded tissues are valuable source of DNA for analysis. The use of paraffin-embedded for tissue storage is easy and it has been used for years. In fact, there were many factors affecting the DNA quality of the paraffin embedded tissues, they were the use of fixative, the fixation time, the storage time and condition and the extraction method.

In modern science, PCR is a routine method to detect the presence and amplify the DNA. The effect of fixative in paraffin embedded tissues should be studied in order to avoid the negative effect in PCR. Buffered neutral formalin (BNF) was used in preparing the paraffin-embedded tissue. In two studies, the sizes of DNA fragments from the tissues were fixed in 10% buffered neutral formalin ranged from 100 to 1,500bp (Goelz *et al.,* 1985) to from 4,000 to 9,000bp (Arnheim *et al.,* 1990). The extracted DNA from paraffin embedded tissues was the template for the PCR, therefore, the researcher should know the preparation condition since it would be an advantage in the analysis of the result. Greer *et al.* conducted a study on the effect of fixative and the fixation time of paraffin-embedded tissues in PCR amplification. In his study, 11 fixatives and 3 fixation time slots were tested. 10% buffered neutral formalin were

found to be the best for the subsequent analysis by PCR. Extraction method of paraffin-embedded tissues was another factor affecting the efficiency of PCR. Chan *et al* (2001). conducted a research by using different DNA extraction methods including tradition phenol/chloroform, microwave based (Banerjee *et al.,* 1995) and commercial available spin column based DNA extraction kit. The traditional method of purifying DNA from archival tissues using xylene dewaxing followed by phenol/chloroform purification is laborious and requires multiple steps that are prone to cross contamination. Although the performance of phenol/cholorform in extracting paraffin-embedded DNA is not as good as other methods, it is cheap and readily available in most laboratories. In Chan's research, primers targeted at a 509bp fragment and 355bp fragment in β globin gene were chosen for DNA testing. The positive rates of phenol/chloroform, microwave based, and Qiagen spin column methods were, 50%, 60% and 50% for the 509 bp β globin PCR respectively; for 355bp β globin PCR, the figures were 90%, 95% and 95% respectively. Extracted DNA were also subjected to two sets of HPV consensus PCR, MY09/11 and GP5+/6+. The positive rates of phenol/chloroform, microwave based, and Qiagen spin column methods were 25%, 50% and 65% for MY09/11 respectively; for GP5+/6+ PCR, the figures were 60%, 60% and 75% respectively (Chan *et al,* 2001).

In our study, phenol/chloroform was used to extract the DNA from paraffin-embedded tissues and the extracted DNA were subjected to a primer pair

targeting at a 303bp fragment of the human β actin gene (GeneBank Accession No. X00351) for the assessment of integrity of DNA extraction. There were 358 paraffin-embedded tissues subjected to β actin PCR and all gave a positive results.

According to the study of Chan *et al*, DNA extraction by using phenol/chloroform method might be underestimated for detecting the HPV DNA. In our study, the phenol/chloroform extracted DNA were first screened with MY09/11 PCR. This screening ensured the picking up of HPV in phenol/chloroform extracted DNA. The MY09/11 negative samples were then screened using β actin primer and TS primer. These methods eliminated the underestimation of the HPV samples.

In detecting the HPV DNA in the cervical carcinomas tissues, HPV DNA could be integrated into the host DNA or in episomal circular form. This explained that even the β actin gene extracted, the HPV DNA could still not be extracted and vice versa. In fact, even the copy numbers of β actin gene were lower than HPV DNA, the β actin PCR negative result samples could also contain the HPV DNA. Therefore the copy numbers of HPV DNA in the cervical carcinoma tissues might affect the efficiency of extraction of DNA. In a way that the higher the copy numbers of HPV DNA was, the greater the chance of HPV DNA extracted.

Polymerase chain reaction

Polymerase chain reaction is a very useful and common molecular method to amplify a fragment of DNA sequence. There were many HPV consensus primer pairs developed to detect different HPV types by using single PCR. There were 2 most commonly used PCR primer sets, MY09/11 and GP5+/6+ and they were used for the detection of HPV DNA in clinical cervical carcinoma tissues. The MY09/11 primer set was synthesized with several degenerate nucleotides in each primer and was thus a mixture of 25 primers. The GP5+/6+ primer set consisted of a fixed nucleotides sequence for each primer and thus it detected a wide range of HPV types by using lowered annealing temperature during PCR. Both the MY and GP+ primer sets targeted at the highly conserved L1 ORF of the HPV genome. The GP+ primer set was complementary to the part of the L1 region located inside the sequence recognized by the MY primer set. Therefore, they can be used either as a single primer or in the nested PCR after the amplification with the MY primer set (Evander *et al.,* 1992) MY09/11 primer set yielded about 450bp of DNA fragment in PCR and GP5+/6+ primer yielded 150bp DNA fragment.

In fact, MY09/11 primer set first was used first to screen HPV DNA in paraffin embedded and fresh prepared cervical carcinoma tissues and the result was that from 37.7% to 78.8% for paraffin embedded and 94.4% for fresh prepared tissue. The overall positive rate using MY09/11 was 60%. The specimens from Sichuan and Beijing

showed relatively low positive rate in using MY primer set. The reason for that difference may be due to the storage time of the paraffin-embedded tissues. The paraffin-embedded tissues from these 2 regions were stored for about 5 years and the samples from Shanghai and Guangdong were stored less than 2 years. Although Shibata *et al.* succeeded in amplifying a 40-year-old paraffin-embedded tissues but Goelz *et al.* reported that the longer the storage time was the shorter the DNA fragments produced when comparing that of a fresh prepared paraffin-embedded tissues. The use of formalin in the preparation of the paraffin-embedded tissues may cause the disruption of L1 regions of HPV DNA to produce shorter fragments in DNA extraction. Several authors have reported difficulties in reproducing PCR results with formaldehyde-fixed tissues (Ben-Ezra *et al.,* 1991; Goodrow *et al.,* 1992; Thompson *et al.,* 1991). Karlsen *et al* had described the antagonistic effect of formaldehyde fixation resulting from DNA modification, which led to the inhibition of PCR amplification of longer than 200bp amplimers. Since MY09/11 amplified a long L1 region (450bp) in HPV, the chance of DNA disruption within this region was high. In this case, the PCR showed a negative result. This would produce an underestimated result of HPV infection. As a result, the quality of extracted DNA from Sichuan and Beijing might not suitable for the MY PCR due to the longer storage time and tissue preparation.

Different HPV types have different disruption patterns. In Walboomers (1999) study, PCR assay targeting at the L1 fragments only had 50 percent positive rate of the

E7 PCR positive cases and this suggested the presence of interruptions or deletions in the HPV DNA at the level of the L1 ORF. HPV 18 was more often disrupted in the L1 region than other HPV types, this would imply that PCR assays targeting at other ORFs were required to determine the true prevalence in adenocarcinomas because it usually infected by HPV 18 (Walboomers *et al.,* 1999).

In order to solve the motioned difficulties, different HPV type specific primer PCRs that could amplify other ORFs, E6 and E7 were used. Before the discussion of the use of HPV type specific primer PCRs, 2 HPV typing methods, Restriction Fragment Length Polymorphism and DNA sequencing will be introduced.

Restriction fragment length polymorphism

The restriction enzymes were used to digest the DNA to give distinct fragments in Restriction Fragment Length Polymorphism. Each type of HPV DNA genome had their unique DNA sequence. When digesting with different kinds of restriction enzymes, it would possess a unique DNA fragments. By interpreting the fragments, HPV types can be identified. The templates for RFLP were the PCR products of MY09/11 primer pair. Since MY09/11 primer pairs were commonly used in the routine HPV detection, by using the products of the PCR, the typing procedure could be simple and easy to prepare. Since each PCR produced 50 μ l amplified DNA, and each RFLP just used 5 μ l of the amplified DNA, so even just 1 reaction tube could produce enough template for

typing. This could be valuable if the extracted DNA was in a limited amount. Apart from material saving, the reaction time was also short. The required reaction time for RFLP was about 1 hour, so the HPV type could be confirmed within 4 hours from the MY PCR to RFLP. These advantages make RFLP method become a powerful, quick, cheap and reliable HPV typing tool.

In order to avoid potential errors in the visual scanning of the DNA bands, a high concentration of agarose gel was used in the study since high resolution of DNA fragments could be achieved by high concentration of agarose gel. Also the computerized image taking system could help to produce higher resolution of the gel image. In our study, Gel Doc 1000 system by BioRad laboratories was used to produce a high-resolution gel image.

All the above advantages were the reasons to use RFLP for HPV typing in this study. In our study, Rsa I and Dde I were chosen to perform RFLP. These 2 restriction enzymes produced distinct DNA fragments for high-risk types of HPV, so it was easily to be distinguished from type to type. All the MY09/11 positive specimens and unsuccessful in DNA sequencing were undergone RELP analysis. For example, in Beijing, 63% of the HPV specimens could be typed by using RFLP analysis. In Shanghai and Hong Kong, about 30% and 12% of HPV types were typed by RFLP respectively. In Sichuan, only 3.4% of specimens were successfully typed by using RFLP for HPV typing and it was because the qualities of PCR products amplified by

MY09/11 were not suitable for both DNA sequencing and RFLP. Using HPV type specific primers could type most of the specimens from Sichuan.

DNA sequencing

DNA sequencing is a powerful and accurate tool in determining the nucleotides sequence of HPV DNA. The advantages of DNA sequencing are that all HPV types can be confirmed by comparing the Genebank sequence data and it is easy to confirm a new type or variants of HPV.

The quality of PCR products played a very important role in preparing the template for sequencing. In this study, the templates of the sequencing reaction were the PCR products of MY and GP+ primer sets. They were purified by Microspin coloumn provided by Pharamica, USA. Another method to purify the template was DNA extraction from agarose gel. After the agarose gel electrophoresis of the PCR products, the target band was excised under UV light and extracted using QiAqucik gel extraction kit. After the DNA extracted, they stored in 50 μl TE buffer or autoclaved water. As the DNA should be concentrated for DNA sequencing, the 50 μl DNA were dried under vacuum for 15 minutes to 6 μl before performing DNA sequencing.

It should be reminded that the time of exposure of UV light to the agarose gel should be minimized since UV could cause cross-link of DNA and would degrade it. Also a study indicated that when such direct sequencing is used, it would be necessary

to have reamplification and cloning to resolve some sequence results that are difficult to interpret.

The drawback of the direct sequencing methods was the low quality of the sequencing reaction on the template of PCR fragments, characterized by the frequent "strong stops". This resulted in the fact that the bands in all four lanes (A, G, C, T) could not be distinguished secondary structure related impediments instead of the desired termination only at the places of dideoxymucleotide incorporation. Indeed, the existence of the latter result was due to an incorrect DNA chain termination. Cycle sequencing is a method to reduce the secondaty strcutre-related sequencing ambiguities.

HPV Type Specific PCR

The HPV type specific PCR was another method for HPV typing. All the HPV type specific primers targeted at the HPV E6 or E7 ORFs regions. To use the E6 and E7 ORFs for typing specific regions could avoid the disadvantages of using L1 primers that discussed in the pervious secrions. HPV E6 and E7 were the oncogenes causing cervical carcinomas and they had to be present in the tissue. The primers targeted at these regions could easily amplify the target HPV DNA if the specimens were infected by HPV.

The detection rate of using MY09/11 primer set screening in the whole study was 60%. After HPV type specific PCRs, the detection rate increased to 83.3%. This result

showing that using MY09/11 L1 consensus primer was not enough to detect the HPV DNA in the samples, there was a need to combine both consensus structural L1 gene derived primers and type-specific viral oncogene E6 and E7 derived primers in order to detect HPV DNA in cervical cancer specimens more accurately.

After the screening of MY09/11 and type specific primers, another set L1 consensus primer pair, GP5+/6+ was used to screen the HPV DNA. The reason for performing this screening was to detect the uncommon HPV types that could not be detected by pervious methods. The specimens showed positive results of GP5+/6+ all undergone DNA sequencing. HPV 35, 45, 59 and 67 could also be detected from these specimens.

In this study, using RFLP and DNA sequencing could detect most of the HPV types even the novel HPV types. However, the limitation of these 2 methods was that they all needed MY09/11 PCR products as the templates. If the samples were negative with MY09/11 PCR, HPV could not be typed. As a result, using HPV type specific primer PCRs targeted the E6 or E7 regions to screen the specimens could help to detect the HPV DNA when HPV L1 regions was disrupted. So, as we can see, the whole methodology established in this study could help detecting most of the HPV types in the paraffin-embedded and fresh cervical carcinoma specimens including those rare ones.

4.3 Intratype variation of HPV

Several researchers had done the studies of intratype variation in HPV. Most of these studies targeted of the genome with high intratype variation, such as the URR, the E6 and the L2 ORFs. The reason to choose the L1 region for detection of intratype variation was that the variation in this region allowed the distinction and assessment of known and novel HPV types and for HPV diagnostic purposed as well.

Yamada suggested that nucleotides changes in the L1 region were linked to different regions of HPV genome (Yamada *et al,* 1995). Linkage between intratype variant patterns in different parts of the genome should not be taken as an evidence for a causal connection, since mutation in different parts of the genome might be independent events.

The definition of new HPV type is more than 10% difference in its nucleotide sequence compared with other known HPV types in the E6, E7 and L1 open reading frames combined (de Villiers *et al.,* 1989; van Ranst *et al.,* 1993). Isolates within the same type differing by 0 to 2% in their nucleotide sequences compared with the reference sequence are regarded to as variants, and those differing by 2 to 10% are regarded to as subtypes. Stewart *et al.* had done the study on the L1 region by using MY09/11 primer set. In his study, the PCR products were cloned and about 410 nucleotides were sequenced. Within a single HPV type, nucleotide diversity varied

between 0.2% and 2.9%

In some previous studies, the intratype variation for genital HPV types varied from 1% - 5% within ORF; in URR, the variation was approximately 5%.

PCR amplification using *Taq* polymrase could produce amplification error of the sequences. The transitions of T → C or A → G and G → A or C → T were common errors using *Taq* polymerase.

In my study, using ALFexpress from Amersham Pharamacia Biotech. and ABI 377 DNA Sequencer from Applied Biosystem sequenced 451 specimens of MY09/11 PCR products. The sequencing reaction resulted in a range from 150 nucleotides to 404 nucleotides and the diversity was varied between 0 to 2%. This showed that there was no intratype variation in the specimens.

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

Extensive, accurate and systemic methods were used to determine the prevalence of HPV in cervical cancer in China. PCR with HPV consensus primer pairs MY09/11 and GP5+/6+ were used in the screening for HPV DNA in the samples. DNA sequencing, restriction fragment length polymorphism and HPV type specific PCR were used for HPV typing.

The results showed that the mean prevalence of HPV infection in cervical cancer in five regions of China was 83.93% (range: 80% - 88%). There was no statistically significant geographic difference in HPV infection in cervical cancer.

The findings from this study indicated that HPV 16 was the predominant type of HPV infection in cervical carcinoma in nation-wide China. The result was consistent with other reports (zur Hausen, 2000; Walboomers *et al.,* 1999; Bosch *et al.,* 1995). The infection rates of HPV 18, HPV 31, HPV 52 and HPV 58 were 6%, 1.4%, 2.1%, and 3.1% of the total specimens respectively. The infection rates of HPV 52 and 58 in the southeast part were higher than that in other regions of China. Specific HPV types might pose different relative risks for cervical cancer in different geographic regions.

The histological distribution of the tumors was as follows: squamous cell

carcinoma - 729 (90%); adenocarcinoma - 62 (7.6%); and other types - 18 (2.4%).

The overall HPV prevalence did not vary among different histologic tumor types, and this suggested an etiologic consistency among squamous cells tumors, adenocarcinomas and adenosquamous carcinomas.

The PCR products of MY09/11 and GP5+/6+ were sequenced and compared with the known HPV sequences in the genebank. The variance of HPV type sequences in this study was 1 - 2%, indicated that there was no intratype variation of HPV in these specimens.

Future development in HPV DNA detection

The detection methods for HPV DNA have been updated recently. A new set of HPV general consensus primer, SP10, was developed. It is used to amplify a fragment of 65bp from the L1 region. (van Doorn *et al,* 2000) L1 region is still the target region in the development of new consensus primers. The trend of development of new sets of primers are aiming at producing short PCR products that the MY primer set is 450bp whereas GP5+/6+ is shortened to 150bp and now the new SP10 is just 65bp. In fact, the shorter size of PCR products gives an advantage in detecting clinical samples in. In the sense that most of the false negative results were due to the misamplification of the HPV DNA as discussed before. So, The

shorter the target PCR product can enhance the efficiency even the L1 region is easy to be disrupted, the chance of breakage at the 65bp region is low.

Apart from the new consensus primer, which provides an effective, rapid and simple method in detecting the HPV DNA, quantitative PCR is another method to detect the HPV DNA in the cervical carcinomas. By using the fluorescent 5' exonuclease assay the number of oncogenic HPV can be detected and quantitated. In fact, quantitation over a range of 10 to 10,000,000 initial HPV copies was possible by using real-time detection of the accumulation of fluorescence with cycle numbers. By using this technique, not only the HPV DNA can be detected, the copy numbers of HPV can also be determined.

Future perspective in HPV and cervical cancer research

The molecular pathogenesis of cancer caused by high-risk HPV infections is not fully understood now. In the HPV 2000 International Papillmavirus Conference, some studies on the biology of papillomavirus had been done. Some novel function of viral protein, transcription control mechanisms, and intratype variants had been published. The availability of pseduovirions and the use of raft cultures continued to be useful for unraveling the life cycle of PVs, which will allow new approaches for the therapeutic interventions.

The development of PVs vaccine is an important issue in clinical field. Studies

of the immune response of HPV provide valuable information in developing the vaccine. The experiments performed to measure the immune response to the PVs antigens in human are useful in the sense that they help finding out the natural history of PV infections. The role of specific PVs infection in diseases is not yet clarified. (Bosch *et al.,* 2001) . VLP based vaccines are still the most promising agents in preventing PV infection including cervical cancer. In the near future, the study of the immunotherapy for PV infection will help to the development of powerful and antigen specific vaccine to control the PV associated cancer.

Another aspect related to HPV is the cervical cancer screening program. For years, cervical cancer screening program is a major prevention program for women to prevent cervical cancer by simple and fast methods - PAP smear. As there is an improvement in the HPV detection methods, more accurate detection of HPV types in the specimens can have a prognostic significance to the cervical cancer. So in the course of development of detection methods, HPV type specific detection come to the first priority. Since knowing the prevalence of HPV types in cervical cancer in China can help the development of more effective treatment.

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RELEVANT PUBLICATIONS

1. Paper

Keith W.K. Lo, T.H. Cheung, Tong K.H. Chung, V.W. Wang, J.S. Poon, **J. C. B. Li**, Peggo Lam, Y.F. Wong. (2000). Clinical and prognostic significance of human papillomavirus in a Chinese population of cervical cancers
Gynecol Obstet Invest 2001; ***51***:202-207

2. Poster in 18th International Papillomavirus Conference

T.K.H. Chung, T.H. Cheung, M. K. M. Chan, **J.C.B. Li**, C. S. Poon, Y. F. Wong (2000). Intratype variation in human papillomavirus 16, 18 and 58 in cervical cancer: A regional perspective in Chinese Women

3. Poster in 18th International Papillomavirus Conference

Keith W.K. Lo, T.H. Cheung, Tony K.H. Chung, V.W. Wang, J.S. Poon, **J.C.B. Li**, Peggo Lam, Y.F. Wong (2000) Clinical and Prognostic Significance of HPV Subtypes in Chinese Population of Cervical Cancer