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A Comparison Between Cytological Method and PCR in the Diagnosis of HPV Infection Among Patients with Cervical Cancer

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Abstract: Present study is aimed at comparison between cytological method and polymerase chain reaction in the diagnosis of HPV infection among patients with cervical cancer. Cervical cancer is one of the common reproductive system cancers in developing countries that involve a much numbers of women annually. It is believed that human papillomavirus protein products including E_6 and E_7 cause transformation. Forty five women out of 166 studied ones were infected by HPV ($p = 0.1$). Among these 45 patients, 24 cases were recognized with HPV 16 ($p = 0.2$), 14 with HPV 18, 3 with both HPV 16 and HPV 18 and 4 with other types of HPV. PCR indicated 31 samples with HPV infection (24.03%) whereas cytology could only find 4 HPV infected patients (3.11%). The difference was statistically significant ($p = 0.003$). We conclude that PCR is more sensitive to diagnose HPV infection and also its type than cytology.

Key words: Cervical cancer, HPV infection, PCR, cytological method

INTRODUCTION

Cervical cancer is the second most common cancer among women next to the breast cancer (Ogunmodede *et al*., 2007). Cervical infection by human papillomavirus is the most important risk factor developing cervical pre-malignant and malignant lesions (Bozzetti *et al*., 2000). A number of secondary factors are thought to influence the likelihood that an HPV infection will persist and progress to cervical cancer such as sexual and reproductive factors (Nabaei and Bahiraei, 2001), sexual intercourse at lower age (Dyson *et al*., 2002), poor socioeconomic conditions, cigarette smoking, long term use of contraceptive pills, nutritional diet and etc (Nabaei and Bahiraei, 2001).

Human papillomaviruses are classified according to their genetic similarities (Swygart, 1997). Up to now, more than 70 types of HPV have been characterized and their DNA sequence were identified (Cubie *et al*., 2001). They tend to special epithelium. The most common HPV are those tend to the epithelium of genital system. However, these viruses are also able to infect other sites such as upper respiratory tracts, connective tissues, paraunguinal tissues and etc; although, the main source of HPV is male and female lining cutaneous and wet mucosal tissue of genital organ (Anderson *et al*., 1997). There are 30 types of HPV that may infect the squamous epithelium of the lower tract of male and female reproductive system (anogenital area). The lesions appear in two forms: cauliflower-like and flat warts. Some other types of these viruses lead to asymptomatic or precancerous diseases (Lytwyn *et al*., 2000). HPV genome is divided into three different regions including early, late and Long Control Region (LCR). Moreover, early region proteins are classified into two groups constitute of E_1, E_2, E_3, E_4 proteins and E_5, E_6, E_7 oncoproteins (Dyson *et al*., 2002). E_5 oncoproteins stimulate the growth of epithelial cells and in many cancers, lead to increased cellular mitosis and consequently, cause papilloma lesions (Prayitno, 2006). E_6 oncoprotein is the most important HPV proteins, responsible for chromosomal abnormalities and progressing cells to neoplasia (Dyson *et al*., 2002). E_6 oncoprotein destroys P_{53} (Pei, 1996). In addition, E_7 oncoprotein bind to Rb gene product and similar proteins, so inactivate them (Kim *et al*., 2001). There are several methods to recognize HPV such as cytological evaluation, colposcopy, biopsy, tissue diagnosis using Schiller's test (Molijn *et al*., 2005) and molecular methods such as

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Southern Blot, Dot Blot Hybridization, Polymerase Chain Reaction and so on (Griffin *et al.*, 1997). According to above mentioned introduction, the purpose of present study is to investigate on sensitivity of PCR versus cytological methods in diagnosis of HPV infection in patients with cervical cancer and to evaluation about their application in screening programs.

MATERIALS AND METHODS

This study was conducted in Cellular and Molecular Biology Laboratory, Khatam University, Tehran, Iran. Samples were prepared from those women who had clinical manifestations or tended to be assured of their health, referred to Shahrara specialized laboratory. In addition, some paraffin embedded tissue blocks were studied and repeated preparations were made.

Cytological method: The swab which had been used to collect endocervical and exocervical sample were placed on a clean glass slide and the specimen was distributed evenly. In order to prevent air drying artifacts, samples were fixed in a fixator. Then the samples were stained with papanicolaou (the slides were placed in ethanol 100°C for 15 min and then washed 0. Moreover they remained in Hematoxylin, ethanol 96°C and EA50 solution for 5, 1-2 and 5 min, respectively. Slides were exposed to ethanol 96°C for 2 or 3 times and then let them dry (Young *et al.*, 1989). Finally, the slides were studied using optical microscope.

PCR method: Sampling swab was put in a microtube containing 1 mL of PBS solution and then rotated. In order to extract DNA, 1 mL of lysing buffer was added [including 10 mM Tris (pH = 8), 1 mM EDTA, SDS 1%, 200 μg mL^{-1} Proteinkinase K and 1 μL of 2ME (mercaptoethanol)] into microtube and put it in Vortex for 3 min. These tubes were placed for 5 min in heating block with a temperature of about 65°C. Then 130 μL of KCl was added to the contents of tubes and remained in a temperature of about -20°C for 5 min. Samples were centrifuged in 13000 rpm for 10 min, let the proteins deposit. In order to concentrate DNA, 500 μL Isopropanol and 60 μL sodium acetate 3M were added to the samples, then they were left for 10 min in -20°C. Sodium acetate caused DNA to be ionized and decreased its solubility in water (Shibata *et al.*, 1988). Also, adding highly purified ethanol led to decreased water contents of the samples that per se contributed to the appearance of DNA in the form of non-soluble opacities. From samples that were sent to the laboratory in paraffin embedded blocks, we performed sections and omitting paraffin and also the hydration before DNA extraction.

PCR phases: We concerned a 0.5 mL microtube for each of purified DNA samples. Then, 7.2 mL PCR mix (contained PCR buffer including 10 mM Tris HCl in pH = 9, 1 mM $MgCl_2$, 50 mM KCl and 0.2 mM of each dNTP) was added. Additionally, 50 pm of each primer including MY_{09} (5'GCTCC[C/A]A[G/A][G/A]GGA [T/A]ACTGAT3') and MY_{11} (5'GC[C/A]CAGGG[T/A] CATAA[T/C]AATGG3') was added (Marybeth *et al.*, 1996). At the next step, 2.5 μL purified DNA with respect to the numbering following 0.1 μL (0.5 U) Taq Polymerase enzyme were added. Preventing materials vaporization, 2 drops of paraffin were added finally and caps of microtubes were fastened. The microtubes were placed in Termocycler. First PCR product was used as the target DNA for second PCR. In this phase, each patient two microtubes were concerned (for HPV 16 and HPV 18). All of the mentioned process phases were repeated again, except adding specific primers for HPV 16 and HPV 18 including (Kampion, 1992):

HPV 16
forward primer: 5′-GAACAGCAATACAAVCAAA-3′
HPV 16
reverses primer: 5′-CCATGCATGATTACAGCTGG-3′
HPV 18
forward primer: 5′-TGCCAGAAACCGTTGAATCC-3′
HPV 18
reverses primer: 5′-CAATGTCTTGCAATGTTGCC-3′

Termocycler program constituted of 35 repetitive cycles. First of all, the samples were left in 94°C temperature for 3 min and in 94°C temperature for 30 sec (to separate double strands); then in 50°C temperature for 1 min (to bind the primers to the strands) and in 72°C temperature for 30 sec (to elongate the concerned strand). They were left in 72°C temperature for additional 3 min for assurance. The second PCR phase was similar to the first one. In order to deposit and stain DNA, PCR products that were 11 μL were combined by 2 μL loading buffer and then microfuged (Pernoll and Benson, 1987). Agarose gel was used for electrophoresis of the samples. Samples were studied to observe the specific bands under UV transilluminator.

RESULTS AND DISCUSSION

The traditional way of classifying tumors is by histopathology; the staining and analysis of tissue samples. Now, the ability to analyze change in the levels of the transcripts and/or protein products for literally thousands of genes promises interesting possibilities as a research tool-for understanding the underlying molecular mechanisms, but also for automated tissue

Table 1: Result of studying HPV infection by PCR

PCR results	No.	Frequency (%)
Common HPV	4	2.41
HPV16	24	14.46
HPV18	14	8.43
HPV16 and HPV18	3	1.81
Normal	121	72.89

Table 2: Result of studying HPV infection by cytology

PCR results	No.	Frequency (%)
Normal	61	47.2
Begin cellular change	61	47.2
Ascus	4	3.1
CIN1	3	2.4

diagnosis (Drain *et al*., 2002; Shin *et al*., 2003). The diagnosis of cancer relies primarily on invasive tissue biopsy, as non invasive diagnostic test are generally insufficient to define a disease process of cancer. Molecular medicine has led to the discovery and application of molecular tumor markers, which make histology more accurate and additionally help to prognosticate cancer. The diagnosis of cancer involves the analysis of tissue and cytology specimens obtained through several procedures, including surgical biopsy, endoscopic biopsy, Polymerase Chain Reaction etc. Target-amplified HPV assays, such as PCR, produce highly concentrated samples of a specific DNA genetic sequence. The DNA samples are then probed to identify which specific HPV genotypes are present. PCR is the most common target amplified technique; its inherent strength lies in its capacity to detect have programmatic implications. While HPV is an objective test with rapid turnaround, the test results are not immediate. In addition, quality control mechanisms for HPV testing need further evaluation. The referring women ranged between 19 and 65 years of age and their mean age was 38.3 years. Forty five women out of 166 studied ones were infected by HPV ($p = 0.1$). Table 1 shows result of studying HPV Infection by PCR.

Among these 45 patients, 24 cases were recognized with HPV 16 ($p = 0.2$), 14 with HPV 18, 3 with both HPV 16 and HPV 18 and 4 with other types of HPV. Table 2 shows result of studying HPV infection by cytology.

Patients with HPV 16 and HPV 18 might be infected with other types. Frequencies of HPV infection revealed by cytology and PCR are shown by Fig. 1.

HPVs frequently infect humans. They are classified into categories of low-risk types responsible for the most common sexually-transmitted viral infections and high-risk types which are crucial etiological factors for cervical cancer development (McFadden and Schmann, 2001). It is extremely important to detect and genotype HPVs at an early stage of the infections as to direct clinical treatment and reduce the incidence HPV-related carcinomas, especially cervical cancer (Bosch and de Sanjose, 2002). The traditional method for HPV detection, are morphological and immunological methods. Currently, the methods for HPV detection are molecular biological methods, including nucleic acid hybridization-based and PCR-based methods. Since cervical cancer has a long

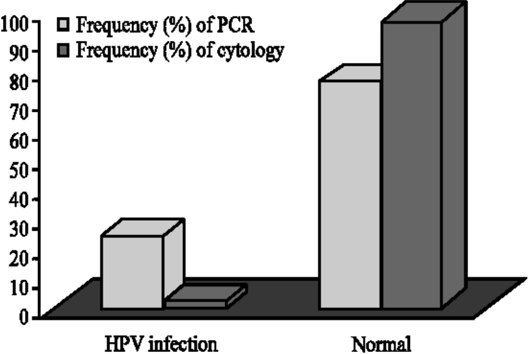

Fig. 1: Frequencies of HPV infection revealed by cytology and PCR

preinvasive period, it is clear that early diagnosis and preventing the onset of invasive form and also its treatment is very important. Previously, cervical cytopathology was used for diagnosis. This method could only confirm HPV infection without the determination of its type. High rate of false negatives is one of the cytology method defects. There are remarkable differences in the results of this test and it has low reproducibility. Its reliability depends on two main factors including expert sampling and interpretation in addition to some problems such as inadequate cell sampling, unsuitable fixation or obscuring blood or inflammation which my lead to cytopathologist misinterpretation (Koss, 1992; Valente *et al*., 1996).

In this study, PCR indicated 31 samples with HPV infection (24.03%) whereas cytology could only find 4 HPV infected patients (3.11%). The difference was statistically significant ($p = 0.003$). This result is in accordance with previous research. For example, Naucler *et al*. (2004) using PCR method found that HPV-16 and-18 are the most frequent HPV infections associated with cervical cancer in Mozambique and PCR is more sensitive than cytological method in diagnosis of HPV infections. Guerrero *et al*. (1992) by comparison of Viral Pap, Southern Hybridization and PCR for HPV identification, suggest that PCR-based HPV identification is the method of choice for future epidemiological investigation. We believe that low quality Pap smear especially with inadequate cell sample is etiologic. On the other hand, cytology disability for diagnosis of unclear HPV infection may result in false negative results. Unlike to the cytology, PCR is able to recognize symptomatic and

asymptomatic HPV infection and also its type. There are two major restrictions that may obstruct the use of PCR in cervical cancer screening programs: (1) the methods and instrumentation required to process cervical specimens and (2) the technical equipment requirements for interpreting test results. Regarding the first restriction, it is possible that instrumentation and processing of samples may be simplified by developments in isothermal amplification of the target HPV DNA. As implied by its name, isothermal amplification does not require the constant change in temperature generally needed to separate, hybridize and amplify target DNA. Instead, enzymes catalyze the formation of daughter strands identical to the targeted section of DNA. These enzymes are effective in all three phases of amplification, which can proceed at room temperature. This technology is still in development. The second restriction ultimately may be addressed through adaptations of current approvals and/or development of simple, rapid, endpoint read-out systems using a lateral flow (immunochromatographic) technology.

In conclusion, PCR sensitivity for diagnosis of HPV infection is 4 fold higher than cytology, immunohistochemistry, histopathology and colposcopy. Among different molecular methods, PCR is the most sensitive method diagnosing HPV infection. It is recommended to apply polymerase chain reaction with specific pair primers for detection of HPV infection and related types of it, rather than conventional methods. However there is some limitation such as need to expensive laboratory equipments and reagents in polymerase chain reaction to detection of HPV in cervical cancer. Use of other complementary method of nucleic acid amplification such as isothermal amplification may be result in more convince in cervical cancer screening programs.

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