Experimental
&
Clinical Article

Cytologic and Clinical Evaluation of Human Papillomavirus in Women Underwent Routine Gynecologic Examination

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OBJECTIVE: To determine the frequency of Human Papillomavirus infection in routine gynecologic examination and to detect whether there is a relationship between the presence of Human Papilloma Virus and cytologic and gynecological findings.

STUDY DESIGN: Four hundred women applying to Gynecology outpatient clinic for routine gynecologic examination were included. Conventional Pap smears of 400 patients were stained by Papanicolaou technique and examined cytologically. HPV DNA was detected by in-house PCR . Also HPV positive samples were evaluated for HPV 16 and 18 by same method.

RESULTS: Eleven (2.75%) of 400 women were HPV DNA (+). Two of which were type 16 and 18 and one of which was type 18. Nuclear membrane irregularity, hyperchromatism, koilocytotic cell, vacuolisation and post partum period corralated with HPV infection ($p < 0.05$).

CONCLUSION: Pap smear examination is a value of detection of cellular changes belonging Human Papillomavirus and if in this examination it is need to, these patients refer to Human Papillomavirus DNA screening it would be more cost effective.

Key Words: Human Papillomavirus, Polymerase chain reaction (PCR), Pap smear, Koilocytotic cell

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Introduction

Human Papilloma Virus (HPV) is a small, non-enveloped DNA virus which ranks number one among the sexually transmitted virus infections and plays a crucial role in the development of cervical cancers.[1-4] Viral genomes consist of late and early gene regions and the non-coding long control region (LCR) that regulates replication. In the early gene region, there are regions that encode proteins from E1 to E8. These proteins play a role in genome organization, regulation of gene expression, and cellular transport. In the late region, there are L1 and L2 regions. L1 is involved in virus attachment to the host cell.[5] Encoded by the oncogenic HPV types, E6 and E7 oncoproteins are responsible for malignant transformation. These proteins disrupt normal cell growth and proliferation by binding to proteins such as p53 and retinoblastoma protein (pRb), cellular tumor suppressors.[2,6] Causing both benign and malignant lesions, HPV must first infect the divisible basal cell to induce papilloma formation.[2,5,7,8] Viral replication occurs concomitantly with epithelial cell differentiation. Entering the basal cell, HPV replicates simultaneously with epithelial cell differentiation and reaches the keratinized cell [2,4,5,7,8]

The present study investigates the frequency of Human Papillomavirus infection in routine gynecologic examination and relationship between HPV and the all cytologic findings in cervicovaginal smears and evaluates also the HPV-specific cytologic and clinical picture.

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Material and Method

The 400 patients were seen at The Gynecology and Obstetrics Clinics of Hacettepe University for routine gynecologic examination. All women were asked to fill in an questionnaire focusing on detailed history and gynecological symptoms. Clinical evaluations were grouped under three headings: gynecological complaints (discharge, itching, burning, pelvic pain, irregülar menstruation etc.), clinical data (miscarriage, post partum etc.), and pelvic examination findings (hyperemia of cervix, pelvic relaxation etc.). All women underwent a standardized speculum examination. Pregnant women were not included in this study. The study protocol was approved by the ethics review board of the hospital, and all women voluntarily signed informed consent before enrollment. Cervico-vaginal smears were taken with a cytobrush, fixed with absolu alcohol and stained by a routine Papanicolaou technique. All cervico-vaginal smears were evaluated for nuclear (nuclear enlargement, nuclear membrane irregularity, hyperchromatism, karyorrhexis, karyopyknosis, karyolysis), cytoplasmic (koilocytosis, vacuolisation, intracytoplasmic inclusions) and cytologic data. Cervico-vaginal secretion was obtained by swab then collected into tubes which contain physiological saline. It was stored at 4°C, transported to the laboratory and used for HPV DNA extraction. All known precautions to avoid PCR product carry-over and sample-to-sample contamination were taken rigorously. The different steps of the PCR procedure were performed in separate rooms with different positive-displacement pipette tips.

DNA extraction:

The sample was mixed by using vortex for 2 minutes, the swab was removed and the upper aqueous layer was transferred to 1.5 ml microcentrifuge tube. It was centrifuged for 5 minutes at 1000rpm. The upper aqueous layer was removed. 100µl distilled water was added, the sample was homogenized and separated into 2 tubes. 400 µl lyses/binding (Metis Biotechnology) solution was added to 50 µl cell suspension and vortexed. The samples were incubated as following: 65°C for 10 minutes, 4 °C for 2 minutes and then the tube was put in ice. 500 µl precipitation solution (Metis Biotechnology) was added, vortexed for 2 second. The sample was centrifuged for 15 minutes at 13000rpm. The supernatant was separated after centrifugation. 0.5ml washing solution (Metis Biotechnology) was added, vortexed for 2 second and the sample was centrifuged for 5 minutes at 13000 rpm. The supernatant was separated. The sample was left for 10 minutes at room temperature. 20 µl dilution solution (Metis Biotechnology) was added, vortexed for 2 second. the samples were incubated 10 minutes at 65 °C, the sample was centrifuged for 1 minute.

DNA amplification and Detection of PCR Products

DNA was amplified by the PCR using consensus primers; MY09, MY11 and two type specific probes, specific for HPV 16 (MY 9, MY14,) and HPV 18 (MY 9, WD 74) (Metis Biotechnology). MY09 and MY 11 consensus primers amplify approximately 450 base pair of ORF L1. For every reaction, a negative and positive control were prepared. Amplification reaction was performed in total volume of 50µl. The amplification mixture contained 100pmol of each consensus primer 100 µM of each dNTP, 0,25 µl Tag DNA and 5 µl of DNA sample. The mixture was first denaturized at 94 °C for 5 min. Then, 35 cycles of PCR were performed with denaturation at 94 °C for 20s, primer annealing for 45s at 55 °C and primer extension for 7min at 72 °C. At the end of the last cycle, the mixture was incubated at 72 °C for 4 min. Aliquots of the PCR product was analyzed by electrophoresis using 1.5 % agarose gel. The molecular-weight marker was included for the detection of the DNA size of the amplification product. For every reaction, a negative control and also a positive control were prepared Following electrophoresis agarose gel were colored with ethidium bromide and visualized DNA fragments by UV light box and photographed with a Polaroid camera.

Genotyping of HPV positive patients for HPV 16 and HPV 18

HPV positive samples were evaluated for HPV 16 and HPV 18. DNA was amplified by the PCR type specific probes, specific for HPV 16 (MY09, MY 14) and HPV 18 (MY09, WD 74) (Metis Biotechnology) Amplification reaction was performed in total volume of 50µl. The amplification mixture contained 20 pmol of each consensus primer 100µM of each dNTP, 0,3 µl Tag DNA and 5 µl of DNA sample. The mixture was first denaturated at 94 °C for 5 min. Then, 35 cycles of PCR were performed with denaturation at 94 °C for 20s, primer annealing for 45s at 55 °C and primer extension for 1 min at 72 °C. At the end of the last cycle, the mixture was incubated at 72 °C for 5 min. PCR products were analyzed on agarose gel stained with ethidium bromide and transluminating band was detected with UV transluminator encompassing 110 bp for HPV 16 and 140 bp for HPV 18.

All PAP smear and gynecological data were documented in computer. All analyses were performed SPSS programme (SPSS 11, SPSS Inc). The chi-square and Fisher's exact tests were used for the statistical analysis. P values <0.05 were considered to indicate statistical significance.

Results

HPV DNA was detected in 11 (2.75 %) of 400 patients applied for routine control to our clinic. Using two type-specific primers, both HPV DNA 16 and 18 were detected in 2 of 11, HPV 18 was detected in 1 of 11 HPV positive patients. In the light microscopic examination of cervicovaginal smears were first evaluated for infectious agents. In the cytologic examinations of the HPV-positive patients, bacterial vaginosis was de-

tected in one patient infected with one type of the virus other than 16 and 18. Patients have only HPV were the study group (n=10). Patients without infectious agents (n=357) were the control group. The mean ages of the study and control group were 40,5±14.50 (range 22-67) , 42,5±10.12.

The most frequent HPV types detected in HPV positive patients were type 18 (27.2%, 3 cases). No cancer cells were detected in the Pap smears of the patients diagnosed with HPV type 16 and 18; nevertheless, given the possibility that the cells in the smears of such patients might, in time, display atypical changes under viral influence, we concluded that these patients should be regularly followed up with routine cytologic and gynecologic examination.

Cytologic findings of only HPV positive patients were documented in Table 1. The most commonly detected nuclear changes were nuclear membrane irregularity and karyorrhexis, karyopyknosis, karyolysis in HPV positive patients. These were followed by hyperchromatism (3 case) and nuclear enlargement (2 case). The most commonly observed cytoplasmic change was koilocytosis (7 case) in HPV positive patients (Figure 1) which was followed by vacuolization (3 case) (Figure 2) and intracytoplasmic inclusions (1 case). These vacuoles amply found in the cell cytoplasm gave the cytoplasm a foamy apperance. The difference between of HPV and koilocytotic cell, vacualisation, nuclear membrane irregularity, hyperchromatism was statistically significant ($p < 0.05$).

Clinical evaluations of only HPV positive patients were documented in Table 2. The most commonly reported gynecologic findings were discharge, pelvic pain and hyperemia of cervix (3 case). 2 case of our patients with HPV is in postpartum period. Statistical evaluations revealed that there is a significant difference between HPV presence and postpartum period ($p < 0.05$).

Table 1: Cytologic findings of only HPV positive patients

Cytologic Findings	HPV + (n= 10)	
	Present	Absent
Nuclear enlargement	2(10.5%)	8(2.2%)
Nuclear membrane irregularity †	4(50%)	6(1.6%)
Hyperchromatism †	3(37.5%)	7(1.9%)
Karyorrhexis, karyolysis, karyopyknosis	4(6%)	6(1.9%)
Koilocytotic cell †	7(24.1%)	3(0.88%)
Vacuolisation †	3(27.2%)	7(1.9%)
Intracytoplasmic inclusions	1(7.1%)	9(2.5%)
Metaplasia	3(4.05%)	7(2.3%)
PMNL	9(3.5%)	1(0.88%)
Doderlein bacilli	4(2.4%)	6(2.9%)
Erytrocytes	2(1.49%)	8(3.4%)
Macrophage	1(2.1%)	9(2.8%)
Cocci	1(1.75%)	9(2.9%)
Superficial cell	8(2.6%)	2(2.9%)

Footnote: HPV; Human Papillomavirus. † ($p < 0.05$).

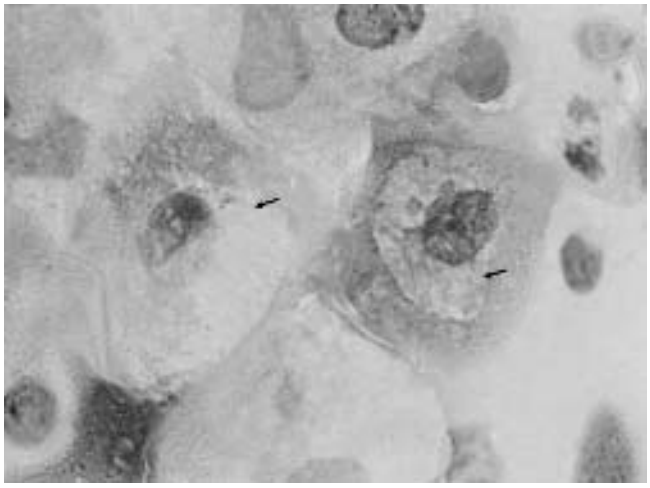

Figure 1: Koilocytotic cells were seen at arrows. The nucleuses are surrounded by a large clear area. The peripheral cytoplasm is quite dense. (Papanicolaou stain x1250)

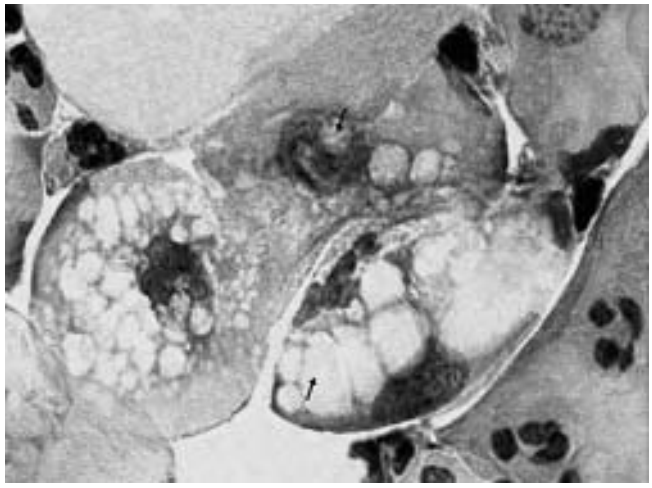

Figure 2: Epithelial cells showing with cytoplasm and nucleus filled with vacuoles (arrow). Nuclear and cytoplasmic vacuolization leading to a honeycomb-like picture. (Papanicolaou stain x1250)

Table 2. Gynecologic findings of only HPV positive patients

Gynecologic Findings	HPV + (n= 10)	
	Present	Absent
Discharge	3(3.1%)	7(2.5%)
Itching	2(9.5%)	8(2.3%)
Burning	1(5%)	9(2.5%)
Pelvic pain	3(7.3%)	7(2.1%)
Irregular menstruation	2(3.6%)	8(2.5%)
Pelvic relaxation	1(5.5%)	9(2.5%)
Miscarriage	1(5.8%)	9(2.5%)
Postpartum period †	2(20%)	8(2.2%)
Hyperemia of cervix	3(4.6%)	7(2.3%)

Footnote: HPV; Human Papillomavirus. † ($p < 0.05$).

Discussion

Human Papillomaviruses are the most common sexually

transmitted viral infective agents in the world found both among men and women.[1,2,4] It has been reported that the virus has a prevalence of 5-46% among healthy and sexually active women and the infection increases in the age range of 16-25 years, while it decreases with advanced age.[9] In a study conducted in Turkey, an evaluation of cervico-vaginal smears from 3230 patients revealed changes in favor of HPV infection in 0.3% of the patients.[10] The frequency rate of HPV infection among low-risk women for cervical cancer was 6.1% in other study.[11] Ozturk et al. reported the frequency of HPV DNA positivity was found 2.1% in women with normal cervical cytology and 42.9% in women with epithelial cell abnormalities.[12] In our study, HPV infection was detected in 2.75%. The HPV rate as 2.75% in a Turkish population seems to be in accordance with the previous report from our country.[10,11,12] But this rate is lower than that reported from other countries. [13,14] A majority of the epidemiological studies on HPV worldwide has been conducted on patients with squamous and adenocarcinoma and they reported a much higher prevalence of HPV. Among non-pregnant women with normal Pap smears, the prevalence of HPV is 4-43%. Geographical and cultural diversities also affect the prevalence of the virus.[9,15] The low prevalence of HPV infection observed in our study has been attributed to the facts that our patients applied to our clinic for routine gynecological examinations, that they had high social and economical income level and that they regularly attended their follow-up visits. And also since monogamy is common in our country may be affect the HPV infection prevalance. Recently obtained data have revealed that different HPV types and multiple infections in the same person have a higher incidence than predicted.[3,16] In our study, the genotyping of the samples from HPV-positive patients revealed concomitant presence of HPV type 16 and 18 in two of the 11 (18%) patients.

A common feature of Human papillomavırus infection is the appearance of koilocytes in the differentiated layers of squamous epithelium. The greatest change caused by HPV in the epithelial cell cytoplasm is the hollows called koilos. These koilocytes are epithelial cells that contain a hyperchromatic, acentric, moderately enlarged nucleus. The genesis of koilocytes is somewhat puzzling because most HPV proteins are localizes in the nucleus.[17] In the literature, there are some views on the formation mechanism of koilocytotic cells.[18,19,20] According to Rapp and Chen, the first holds that the E6 protein synthesized from the early gene region of the virus and the E6-associated protein E6-AP are effective in this formation. The second view on koilos formation pertains to the E6 protein and paxillin, a cellular protein which E6 also interacts with. By interacting with paxillin, the E6 protein of the virus disrupts the actin filament formation and the regulation of cytoskeleton. In the light of this information, the disruption of actin filament formation and thus, cytoskeleton disruption are believed to affect the formation of koilos around the nucleus.[18] The third view relates to E1^E4 protein. Recent studies have introduced a new protein of HPV type 16 that could disrupt the epithelial cytoskeleton and named it E1^E4 protein. It has been reported that this protein degrades the cytoskeleton by interacting with epithelial cell proteins.[19,20] According to Krawczyk et al. E5 from high- risk and low-risk HPV types cooperates with high and low-risk E6 to induce koilocytes in vitro.[17] Drawing upon this information, it has been suggested that koilos formation might be linked to cytoskeleton degradation. Furthermore, another striking finding of the present study is that the koilos were mostly detected in surface cells. The already known fact that the matured virus is finally released into cornified cells supports this finding.[5] Another cytoplasmic change was vacuoles observed in both the nucleus and the cytoplasm.[2,21] The role of cytoplasmic vakuolization in viral life cycle is unclear. This change was attributed to virus activation. Although the cause of the changes such as nuclear membrane irregularity, karyorrhexis, karyolysis, karyopyknosis, nuclear enlargement, and hyperchromatism is unclear, these changes might have been caused by the nuclear membrane damage that occurs as the proteins synthesized in the cytoplasm are transported into the nucleus passing through the cytoplasm during epithelial cell replication.[5] The other cytoplasmic changes is intracytoplasmic inclusion Ustacelebi emphasis these inclusions are viral nucleic acids and proteins accumulated in nucleus and cytoplasm.[22]

In our study, the patients were evaluated in terms of their clinical information. Boden et al state that discharge, itching, inflammation, and fissures might be experienced in the presence of this virus, while another study has reported that HPV infection may account for acute viral symptoms such as fever and joint pain, and dysuria.[8,23] On the other hand, Mao et al. suggest that acute and chronic HPV infections are not related to discharge, itching, and inflammation.[24] In our study, no relationship was found between these gynecological complaints and HPV (Table 2). 2 of our patients with HPV is in postpartum period. Statistical evaluations revealed that there is a significant difference in terms of HPV presence between those in postpartum period and those who are not in postpartum period ($p<0.05$). Sex hormones can influence genital system infections. It has been shown in humans and animal models that received estrogen and progesteron affect susceptibility of organisms to sexually transmitted infections. These hormones act exactly as a co-factor in HPV 16 infections, increasing HPV E2 and E7 proteins, affect levels of apoptosis that occurs due to these proteins, and thus, play a part in cervical cancer development, even indirectly.[25,26] Pregnancy may foster the development of infections, in particular HPV infections.[27] Given the hormonal change in pregnancy, this finding suggests that, as some researchers argue, HPV might be activated by the hormonal change, progesteron and immunological change in pregnancy.[19,28] A clear explanation of the relationship between

HPV- post partum period requires the screening and typification of the virus in larger populations with post partum.

Consequently, in case that the cellular changes are observed in cervicovaginal smears, suspicion of the presence of this virus will require further investigation and typification of the viral presence through a molecular method. And also early detection of HPV type may identify women who are at risk for cervical cancer. The present study is significant in that it provides HPV prevalence in routine gynecological examinations by a specific and sensitive assay like PCR and reveals the relationship between HPV and cytologic and gynecologic findings.

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Human Papilloma Virüsün Rutin Jinekolojik Kontrole Gelen Kadınlarda Sitolojik ve Klinik Olarak Değerlendirilmesi

AMAÇ: Rutin jinekolojik incelemede Human Papilloma Virus (HPV) sıklığını belirlemek ve bu virüs ile hücresel değişiklikler, sitolojik ve jinekolojik bulgular arasında ilişki olup olmadığını değerlendirmek.

GEREÇ VE YÖNTEM: Rutin jinekolojik muayeneleri için Jinekoloji kliniğine başvuran dörtyüz hasta çalışma kapsamına alınmıştır. Bu hastalara ait konvansiyonel serviko-vajinal yaymalar Papanicolaou yöntemine göre boyanmış ve sitolojik olarak incelenmiştir. HPV DNA Polimeraz Zincir Reaksiyonu (PCR) ile değerlendirilmiştir. HPV pozitif örnekler ise yine aynı yöntemle HPV tip 16 ve 18 açısından incelenmiştir.

BULGULAR: HPV DNA 400 hastanın 11 (%2.75)'inde saptanmıştır. HPV pozitif hastalar HPV 16 ve 18 açısından PCR ile değerlendirildiğinde ise 2 hastada HPV 16 ve 18 birlikte bulunurken 1 hastada sadece HPV 18 saptanmıştır. HPV ile çekirdek zar düzensizliği, hiperkromatizm, koilositotik hücre, vakuolizasyon ve post partum dönemde bulunma arasında anlamlı ilişki saptanmıştır ($p<0.05$).

SONUÇLAR: Pap simir incelemesinin HPV'a ait hücresel değişikliklerin tesbiti için değer taşıdığı ve eğer simir taramasında şüphelenilirse hastanın HPV DNA taramasına yönlendirilmesinin maliyet açısından da yararlı olacağı düşüncesindeyiz.

Anahtar Kelimeler: Human papilloma virüs, Polimeraz zincir reaksiyonu (PCR), Pap simir, Koilostotik hücre

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