RESEARCH COMMUNICATION

Prevalence of Human Papillomavirus Genotypes in Women with Normal and Abnormal Cervical Cytology in Iran

Saeed Reza Ghaffari[1,2,3,4]*, Tayebeh Sabokbar[1], Hamid Mollahajian[1], Jila Dastan[2], Fateme Ramezanzadeh[4], Fereshteh Ensani[5], Fariba Yarandi[6], Alireza Mousavi-Jarrahi[1], Mohammad Ali Mohagheghi[7], Abdolvahab Moradi[8]

Abstract

Introduction: HPV infection has a prime etiologic role in development and progression of cervical cancer, one of the most frequent forms of cancer among women in developing countries. This study was designed to determine the most prevalent HPV genotypes in women with normal and abnormal cervical cytology in Iran. Materials and Methods: Samples from134 patients, including 127 who attended gynecology clinics and 7 with solid cervical tumors were used. All 127 patients underwent routine Pap tests for cytological evaluation and at the same visit a sample of cervical epithelial cells was obtained by scraping the cervix osteum. In each case HPV infection was primarily evaluated by PCR using GP 5/6 primers and then subtyping was performed in proved infected samples with specific primers for HPV 16, 18, 31, 33, 11 and 6. After cytological evaluation, 50 patients with abnormal Pap tests were categorized as the abnormal group and the remaining 77 patients as the normal group. Results: In the normal group, HPV infection was established in 10 cases (13% infection rate), while 30 HPV positive cases were discovered in the abnormal group (60% infected). The most prevalent genotypes among the infected samples were HPV 16 (76%), HPV18 (12.7%) and HPV11/6 (8.5%). Moreover, all 7 tumor samples were positive for HPV general primers of which, 5 samples were infected with HPV 16, two were co-infected with HPV16,18 and HPV16,31 genotypes and one was infected with HPV 18. Conclusions: Infection with HPV 16 was found to be significantly higher in abnormal group in comparison with normal group (42% vs. 11.6%, P value <0.005), likewise HPV18 genotypes were proved to be more prevalent in abnormal group (8% vs. 0%, P value <0.05). No significant relation between other HPV genotypes and pathologic cervical changes was obtained. According to our study high rates of infection with HPV genotypes in sexually active Iranian women makes molecular investigation for HPV16 and 18 very essential in clinical approaches to patients with proven dysplasia in their screening tests and also for those patients with borderline (i.e. ASCUS) or incongruous pathology reports. Larger studies are required to determine the most appropriate vaccine with highest protection in Iranian women.

Key Words: Cervical cancer in Iran - human papillomavirus - HPV infection in Iran

Asian Pacific J Cancer Prev, **7**, 529-532

Introduction

Traditionally the Papanicolaou smear has been used as the screening method for early detection of premalignant lesions in cervix uterine but in view of high number of false negative and positive results, new molecular techniques are being introduced for a more sensitive and reliable approach and also to guide clinical decision making in approaching those patients with border line pathology, like those with ASCUS lesions in their pap test (Lytwyn et al.,2000; Rozendaal et al.,1996). More than 80 types of HPV have been characterized of which some specific types like HPV 16 and 18 have been found to be closely related to pathogenesis of cervical intraepithelial neoplasia (CIN) (Clifford et al., 2003). Epidemiological studies are necessary to pave the way for better understanding and evaluating the risk of developing neoplasia in women who are found to be infected with certain types of HPVs in each population and

*[1]Department of Genetics & Genomics, Cancer Research Center, Tehran University of Medical Sciences, [2]Department of Cancer Research, Gene Institute, [3]Department of Medical Genetics, Tehran Medical School, Tehran University of Medical Science, [4]Valiasr Reproductive Health Research Center, Imam Hospitals Complex, [5]Department of Pathology, Cancer Research Center, Tehran University of Medical Sciences, [6]Department of Oncology, Mirza Koochek-Khan Hospital, [7]Department of Surgical Oncology, Cancer Institute of Iran, University of Medical Sciences, [8]Department of Molecular Virology, Golestan University of Medical Sciences, Gorgan, Iran *Corresponding Author: Fax: 98-21-66933336 E-mail: saeed@ghaffari.org*

also for a precise vaccination program. According to the fact that there may be a localized variation in malignant potential of particular HPV genotypes in different populations (Chan et al., 2002) and in the view of widespread interest in HPV vaccines, we decided to organize a study to characterize pattern of HPV infection in Iranian women in two referral hospitals. During our investigation we also collected seven solid cervical tumors, subsequently proven to be squamous carcinoma of cervix, and compared the HPV genotypes in these end stage lesions with those we had found in women with normal and abnormal cervical cytology.

Materials and Methods

Patients and sample collection

This study was approved by the local university ethical committee. From October 2005 to January 2006, 127 outpatient women were enrolled from two gynecology clinics in Imam Hospital complex and Mirza Koochek-Khan hospital in Tehran. Our aim was to compare prevalence of HPV infection and identify common subtypes in normal women and those with abnormal Pap smear test of high-grade squamous intraepithelial lesions (HSIL), low-grade squamous intraepithelial lesions (LSIL) or abnormal squamous celss of unknown significance (ASCUS) in their medical history within last year.

Each patient was interviewed first and a comprehensive questionnaire containing information about identity, age at sexual activity onset, number of sexual partners, history of genital infection and important medical conditions, utilization of contraception, and history of previous Pap test was filled. We collected 50 patients with abnormal cytology in their previous abnormal Pap smears; the inclusion criterion for inclusion was a cytological report of cervical intraepithelial neoplasia within the last year, those referred for cervical biopsy and or topical treatment being excluded.

We also collected 77 women who never had any cytological report in support of cervical neoplasia nor had HPV cytopathic effects.The third group of samples comprise 7 cervical solid masses from 7 women who had been admitted in surgery ward with proven squamous cell carcinoma of cervix in biopsy. After exposing cervix using sterile speculum, epithelial cells of endocervix were collected by a 360 degree scrape of nylon cytobrush in transitional zone, and then the cells were transferred to sterile vial containing 5 cc of PBS (phosphate buffer saline). The vials containing epithelial suspension were delivered to molecular laboratory for DNA extraction in the same day. After epithelial cell sampling for molecular analysis, each patient underwent a conventional pap test using Ayre's Spatula and a smear of cervical cells was prepared on a sterile blade. Then, after fixation in alcohol 95% the smears were sent to cytology laboratory for evaluation. Those cases with inadequate cells for cytological investigation were excluded or invited for re sampling; therefore each registered case had a sample for molecular evaluation of HPV infection and a cytological file for conventional Pap test.

Precipitation of epithelial cells and DNA extraction:

The cell suspension were centrifuged (10min, 3000xg, 4C) and the lysis buffer (10 m M Tris-HCL; Ph 8, 1m M EDTA, 1% SDS, 1 M NaCl) and the proteinase K (125 Î1⁄4g/ 500 Î1⁄4l) Were added on the pelleted cells and incubated 56 C for 2 hours. The standard phenol-chloroform extraction and the ethanol precipitation were used for DNA purification and the pelleted DNA was resuspended in 50-100 µ1⁄4l of tridistillated sterile water (Husnjak et al., 2000). In order to determine the quality and quantity of the isolated DNA, each DNA was analyzed by electrophoresis on 0.8 % agarose gels stained with ethidium bromide and spectrophotometrically.

Polymerase chain reaction (PCR)

DNA samples were amplified with the GP5+ and GP6+ primers targeting the L1 open reading frame of broad spectrum HPV genomes (Husnjak et al., 2000). In all samples, the constitutively expressed SRY gene was amplified first to confirm the adequacy of the extracted DNA and PCR amplification. DNA from Caski and Hella cells was used as positive control in all preliminary PCR runs. Positive samples were further analyzed with type specific HPV primers to identify high risk (HPV 16 and 18),

Table 1. Primer Sequences for Specific HPV Genotypes, their Targets, Positive Controls and Product Sizes

HPV type	Primer sequence (5'-3')	Size	Positive control	Region	Reference
GP6+/GP5+	Forward: TTTGTTACTGTGGTAGATAC Reverse: GAAAAATAAACTGTAAATCA	142 bp	Hella/caski	L1	(Snijders et al.,1991)
HPV16	Forward: CCCAGCTGTAATCATGCATGGAGA Reverse: GTGTGCCCATTAACAGGTCTTCCA	253bp	Caski cell line	E6/E7	(Soler et al.,1991)
HPV18	Forward: CGACAGGAACGACTCCAACGA Reverse: GCTGGTAAATGTTGATGATTAACT	201bp	Hella cell line	E6/E7	(Soler et al.,1991)
HPV31	Forward: ATGGTGATGTACACAACACC Reverse: GTAGTTGCAGGACAACTGAC	514bp	cervical scrape	L1	(van den Brule et al., 1990)
HPV33	Forward: ATGATAGATGATGTAACGCC Reverse: GCACACTCCATGCGTATCAG	456bp	cervical scrape	E1/E2	(van den Brule et al.,1989)
HPV6/11	Forward: TACACTGCTGGACAACATGC Reverse: GTGCGCAGATGGGACACAC	302 bp	cervical scrape	E6/E7	-(Soler et al.,1991)

intermediate risk (HPV 31 AND 33) and low risk HPV 6/11 (Table1).The results of genotyping were verified by restriction fragment length polymorphism (RFLP) which used restriction enzymes BamHI, DdeI, HaeIII, HinfI, Pst I, RsaI and Sau 3AI (Bernard et al., 1994).CaSki cell line (containing a high copy number of HPV 16), HeLa cell line (containing HPV 18), HPV 31 positive cervical scrape, HPV 33 positive cervical scrape and, HPV 16 and HPV 33 positive cervical scrape were all received from Magdalena Grce.

Statistical analysis

Statistical analysis was performed using SPSS 11 software. Chi square; Student's t-test and Mann-Whitney test were used to investigate intergroup significance.

Results

In this study we included 127 women who attended gynecology clinics in Tehran university hospitals. We also collected 7 solid cervical tumors which were subsequently proved to be squamous carcinoma of cervix. All 127 women underwent routine Pap test for cytological evaluation and at the same visit a sample of cervical epithelial cells was obtained by scraping the cervix osteum. After cytological evaluation and review of medical records, 50 women with abnormal cytology in their Pap smears were categorized in abnormal group and the remaining 77 women with normal Pap smear and no previous abnormality in their records were categorized in normal group. The third group comprised 7 patients with cervical Squamous cell carcinoma.Women with normal cytology and those with abnormal cytology were similar in age (33.0 and 35.3 years, respectively P=0.52), adopting contraception methods (40/50 vs.55 /77, P>0.05) and age at beginning of sexual activity (18.2 vs. 20.2 years respectively). None of the women reported cigarette smoking or having multiple sexual partners. In normal group HPV infection was established in 10 cases (13%), while 30(60%) HPV positive cases were found in patients with abnormal cytology and interestingly all of the 7 cancerous patients (100%) were positive for HPV infection which yields a significant relation between HPV infection and cervical neoplasia (P value <0.001).

Those positive cases for HPV infection were further underwent subtyping using specific HPV primers. The frequency of different genotypes is presented in (Table 3).Overall, the prevalence of HPV 16, 18, 11/6 and 31 in all infected samples was respectively 76%, 12.7%, 8% and 2%. All 7 tumor samples were positive for HPV general primers of which, 5 samples were infected with HPV 16, two samples were co-infected with HPV16,18 and HPV16,31 genotypes and one sample was infected with HPV 18.

Table 2. Frequency of HPV Infection, Determined with the GP5+/GP6+ Primers

Study Groups	Total	Negative	Positive
Women with abnormal cytology	50	20 (40%)	30 (60%)
Women with cervical carcinoma	7	0 (0%)	7 (100%)
Women with normal cytology	77	67 (87%)	10 (13%)

Table 3. Comparison of HPV Genotypes in Different Pathological Grades

Pathology	Total	HPV infection					
		(GP05/06)	16	18	31	33	6/11
Normal	77	10 (13)*	9 (12)	0	0	0	1 (1)
ASCUS*	31	18 (58)	11 (35)	3 (9)	0	0	2 (6)
LSIL	12	7 (58)	5 (41)	1 (8)	0	0	1 (8)
HSIL	7	5 (71)	5 (71)	0	0	0	0
SCC**	7	7 (100)	6 (85)	2 (28)	1 (14)	0	0
Total	134	47	36	6	1	0	4

*Number (%). ASCUS=atypical squamous cells of undetermined significance; LSIL=Low grade squamous intraepithelial lesion HSIL= High grade squamous intraepithelial lesion; SCC=Squamous cell carcinoma *: Two samples could not be sub-typed with available specific primers. **: Two samples were co-infected with. HPV16, 18 and HPV16, 31.

Discussion

Cervical cancer is the most common malignancy among females in developing countries, with highest prevalence in Latin America, Asia and Africa (Parkin et al., 1999). Likewise, in Iran cervical cancer has been the most common cancer amongst women. The high rate of cervical cancer fell from 19.4% to 11.4% of all reported malignancies10 (Mortazavi et al., 2000) mainly because of adopting screening methods, literally Pap test, and early detection of precancerous lesions. HPV plays a central role in pathogenesis of cervical cancer and this viral infection is considered to be a necessary although not always a sufficient cause. HPV DNA has been shown to be present in over 99% of cervical cancers (Walboomers et al., 1999) and it is now clear that persistent infection with high risk HPV increases the risk of developing cervical carcinoma (HO GY et al., 1998). Recently, prophylactic (Harro et al., 2001; Koutsky et al., 2002) and therapeutic vaccination (IM and Monk, 2002) have shown promising results for preventing HPV infection as well as cervical neoplasia. Noticeably there are evidences that vaccinating the subjects who were infected with HPV (PCR positive) can reduce the incidence of CIN 1-3 in affected women (Olson, 2006).Development of effective vaccines would require a comprehensive study on HPV genotypes in different regions of the world.

Worldwide, HPV 16 is the most common type followed by HPV18 and geographical variations in non-HPV16 distribution have been noted (Clifford et al., 2003). To the best of our knowledge, there are no previously published studies from Iran that have investigated the prevalence of different HPV genotypes among women with normal and abnormal cervical cytology. In one study on different HPV genotypes in cervical cancer among Iranian women (Mortezavi et al., 2002), HPV16 (73.9%) and HPV18 (11.6%) were the most common detected viruses in cervical tumors, followed by HPV33 .In our study, prevalence of HPV16 in women with proved cervical SCC, abnormal cytology (ASCUS, LSIL AND HSIL) and those with normal

Pap test were 85%, 42% and 11.6% respectively. Moreover, HPV16 made up 76% of all HPV infections in all women in different groups, which makes it as the main culprit for cervical neoplasia in Iranian women. In addition, there was a meaningful relation (P value=0.013) between HPV18 infection and pathologic cervical lesions as 2 samples of the 7 cervical tumors (28%) and four women with an abnormal Pap test (8%) were HPV18 positive while no HPV18 infection was detected in women with normal cytology.

Among the 134 women who were enrolled in this study we did not find any habitual cigarette smoker or any case with a history of multiple sexual partners. Therefore, theses risk factors are not likely to be influential among the majority of Iranian women at the moment. There was neither significant difference in other risk factors including age of sexual activity onset, parities and using contraception between different groups of the study.

According to our study high rates of infection with HPV genotypes in sexually active Iranian women make molecular investigation for HPV16 and 18 essential in clinical approach to patients with proved dysplasia in their screening tests and also to those patients with borderline or incongruous pathology reports. Since the vaccines have already been tried in humans and found to be very effective in preventing cervical neoplasia, their utilization will be soon justified in developing countries like Iran. However, it is essential to choose those which are compatible with prevalent genotypes. Further studies with larger scales of samples and investigation of wider spectrum of HPV genotypes are required to choose the best between available monovalent prototype HPV16 vaccine and quadrivalent (HPV 6,11,16,18) vaccines.

Acknowledgments

This work was supported jointly by the Cancer Research Center and the Gene Institute, Tehran, Iran. The authors would like to thank Dr. Magdalena Grce for providing Hella and Caski cell lines and other positive control samples. We also appreciate the sincere collaboration of Ms. Rezayof.

References

Bernard HU, Chan SY, Manos MM et al (1994). Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphism, nucleotide sequence, and phylogenic algorithms. *JID*, **170**, 1077-85.

Chan PK, Lam CW, Cheung TH et al (2002). Association of human papillomavirus type 58 variant with the risk of cervical cancer. *J Natl Cancer Inst*, **94**, 1249-53.

Clifford GM, Smith JM, Aguado T, Franceschi S (2003). Comparison of HPV type distribution in high cervical lesions and cervical cancer: a meta analysis. *Br J Cancer*, **89**,101-5.

Ho GY, Bierman R, Beardsley L, Chang JC, Burk RD (1998). Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med*, **338**, 423-8.

Husnjak K, Grce M, Magdic L,Kresimir P (2000). Comparison of five different polymerase chain reaction methods for detection of human papillomavirus in cervical specimen's. *Virological Methods*, **88**, 125-34.

Harro CD, Pang YY, Roden RB, et al (2001). Safety and immunogenecity trial in adults volunteers of a human paillomavirus 16 L1 virus like particle vaccine. *J Natl Cancer Inst*, **93**, 284-92

Im SS, Monk BJ (2002). New development in treatment of invasive cervical cancer. *Obstet Gynecol Clin North Am*, **29**, 659-72.

Koutsky LA, Ault KA, Wheeler CM, et al (2002). A controlled trial of a human paillomavirus type 16 vaccine. *New Engl J Med*, **347**, 1654-51.

Lytween A, Sellors JW, Mahony JB (2000).Comparison of human papillomavirus DNA testing and repeat Papanicolauou test in women with low grade cervical abnormalities: a randomized trial. *Can Med Assoc J*, **163**, 701-7.

Mortazavi SH, Zali MR, Raoufi M, et al (2002). The prevalence of human papillomavirus in cervical cancer in Iran. *Asian Pac J Cancer Prev*, **3**, 69-72.

Munoz N, Bosch FX, de Sanchose, et al (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *New Engl J Med*, **384**, 518-27.

Olsson SE (2006). Post infection prophylaxis of a quadrivalent HPV (types 6, 11, 16, 18) L1 virus like particle (VLP) Vaccine. *Eur J Obst Gynecol Reprod Biol*,(in press).

Parkin DM, Pisani P, Ferlay J (1990). Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer*, **80**, 827-41.

Parkin DM, Pisani P, Ferlay J (1993). Estimates of worldwide frequency of eighteen major cancers in 1985. *Int J Cancer*, **54**, 594-606.

Rozendaal L, Walboomers JM, Van der Linden, et al (2000). PCR based high risk HPV test in cervical cancer screenings gives objective assessment of women with cytomorphologically normal cervical smears. *Int J Cancer*, **68**, 766-9.

Snijders P, Van den Brule A, van den Brule et al. (1990). the use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. *J Gen Virol*, **71**, 173-81.

Soler C, Allibert, P., Chardonnet Y, Cros P et al (1991). Detection of human papillomavirus types 6, 11, 16 and 18 in mucosal and cutaneous lesions by

Van den Brule A, Class E, du Maine M et al (1989). Use of anticontamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. *J Med Virol*, **29**, 20-7.

Van den Brule A, Meijer C, Bakels V, et al (1990). Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type-specific polymerase chainreaction. *J Clin Microbiol*, **28**, 2739-43.

Walboomers JM, Jacob MV, Manos MM, et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*, **189**, 12-9.

Yoshikawa H, Kawana T, Kitagawa K, et al (1990). Amplification and typing of multiple cervical cancer-associated human papillomavirus DNAs using a single pair of primers. *Int J Cancer*, **45**, 990-2.

Zur Hausen H (1991). Human papillomavirus in the pathogenesis of anogenital cancer. *Virology*, **184**, 9-13.