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Original Article

Qualitative-based Multiplex PCR: Diagnosis of Papilloma virus Types 16 & 18 in Samples Taken from Patients with Malignant Lesions

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Abstract

Background and Aims: The human papilloma virus was introduced as the major etiological agent in outbreak of cervical cancer in 1970. Since it is very difficult to recognize these viruses and their types using the serological tests and cell culture, molecular methods such as PCR are of great importance. Therefore, in this study, our goal was to use a multiple specific PCR assay on L1 and E6 genes of HPV for molecular detection of this virus and its common type's detection in the society.

Materials and Methods: After collecting the samples from malignant lesions of various patients, the viral DNA was extracted from paraffin blocks of 50 clinical samples and the PCR method was performed on the mentioned samples by specific primers for L1 and E6 genes together with β-globin (as internal control). The PCR product was analyzed on 2% agarose gel and the sensitivity of this test was examined.

Results: from among 50 samples of the patients, 33 cases were HPV positive and 17 ones were negative. The sensitivity of this test was 20 copies from recombinant construct containing target genes for each reaction.

Conclusion: this study confirmed that the designed PCR with specific primers on L1 and E6 genes of HPV proved to be an accurate method for detecting and determining the HPV types.

Keywords: Cancer of cervix, Human Papilloma Virus, PCR

Introduction

Cervical cancer is the sixth prevalent cancer in the world (1). According to the World Health Organization (WHO) 650000 persons are afflicted with this cancer in the world annually. Cervical cancer is highly prevalent in the developing and poor countries and it is said to include 80% of the total cancers women (2). This cancer is known to be the second prevalent cancer among Iranian women (3). The most important types causing cervical cancers are referred to as high-risk types, which include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (3-9).

The types 26, 23, 66 have also been found to be included in high risks types (9, 10). Two

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high-risk types 16 & 18 are the most causative agents of cervical cancer in Iran. High-risk HPV diagnosis in women susceptible to progressive uterine lesions is of great importance (10, 11).

The high prevalent of HPV types causing cervical cancer necessitate, developing a reliable method for detecting different types of HPV for the management and preventing the disease. Many methods have been use for detecting HPV to such as polymerase chain reaction, enzyme analysis probe hybridization, and direct sequencing (12) among which PCR is easier and more sensitive than the other methods (13-18). The reverse hybridization independent-method has recently been used for detecting and typing all HPV types from cervical specimen (15, 19). Using the conserve L gene of virus capsid (20). Since only high risk types of HPV cause cervical cancer, it is necessary to determine the HPV types in clinical samples (21).

Considering the extent of HPV infection, it seems necessary to determine the most prevalent HPV types in each population in order to design an effective screening program, management, and vaccination against the prevalent virus types in the population. Since it is impossible to detect the types of HPV virus using the serological and cell culture tests, molecular methods such as PCR are of great importance for an early detection of this virus (22-23). Therefore, a multiple specific PCR assay was used for detection of HPV types 16 and 18 in cervical cancer lesions.

Methods

Samples collecting. In this study, 50 tissue samples of cervical cancer malignant lesions were gathered from different pathological laboratories in Tehran. Other viral samples containing adenovirus, CMV and HSV were supplied from baghiatallah hospital as negative controls.

Viral DNA extraction. DNA extraction was done from 50 paraffin block samples using the nucleic acid extraction commercial kit (Interon, Korea). The extracted DNA was measured by 260 nm wave length absorbency test and their purity was analyzed by calculating A260/A280 ratio.

primer designing and PCR reaction optimization. in the current study, three pairs of primers were designed in our laboratory and used for the purposes of thermodynamic by CLC main Software were used (CLC Main Workbench 6, USA). These sequences were synthesized by the takapouzist company. The conserved sites of L1 gene (450 bp segment) together with β-globin gene as internal control (268 bp) were used to detect the HPV types. To determine the HPV16 type, the E6 gene upstream regulatory site (217bp) alignment was used and for HPV18, the alignment of E6 gene upstream regulatory site (100bp) together with β-globin (as internal control) were performed and The sequences of primers are shown in Table 1.

The reaction mixture contained specific primers of HPV types 16 & 18 and β-globin (final concentration of 0.4 micromolar), dNTPs (final concentration of 0.2 millimolar per base), MgCl2 (final concentration of 2.5mM), and taq DNA polymerase enzyme (Sinaclone, IR) (final concentration of 0.5 unit) in total volume 25µl. The PCR reaction was carried out, one cycle at 94°C for 5 min followed by

Table 1: The sequences of forward and reverse specific primers.

Primers		Primer sequences
L1	F	5'-CGTCCACAAGAGGGAATACTGATC-3'
	R	5'-GCACCAGGGATTAACTAATGG-3'
HPV16-E6	F	5’-AAG GCC AAC TAA ATG TCA C-3’
	R	5’-AAG GCC AAC TAA ATG TCA C-3’
HPV18-E6	F	5’- ACCTTAATGAAAAACCACGA -3’
	R	5’- ACCTTAATGAAAAACCACGA -3’
β-Globin	F	5’-GAA GAG CCA AGG ACA GGT AC-3’
	R	5’-CAA CTT CAT CCA CGT TAC ACC-3’

35 cycles of 94°c for 30 sec, 55°C for 30 Sec and 72°C for 30 Sec with a final cycle of 72°C for 1 min. After PCR performing, 10 µl product of each PCR reaction was analysed on 2.5% agarose gel containing 0.005µg ml gel red and the results were documented by BioDoc Analyzer (KIAGEN, IR) as digital file.

Production of the constructs containing of L1, HPV18E6, HPV16E6 genes as positive control and for reaction sensitivity determining. The cloning of 450bp of L1 gene, 217bp of HPV16E6, 100bp of HPV18E6 and 268bp of β-globin genes was performed in pTZ57R vectors using T/A cloning Kit (Fermentas Company). The PCR product extracted from gel was used for cloning. The ligation was performed using the above-mentioned kit. The mixture of ligation reaction was incubated at room temperature for 4 hours. After transformation, the white colonies were selected from the plate containing Ampicilin together with X-Gal substance and IPTG. For the cloning confirmation, 10 of the mentioned white colonies were restreaked on the LB agar plate containing AMP and incubated at 37ºC for 24h. The direct PCR reaction with specific primers was carried out on the designated colonies. Two of these colonies were selected randomly and the plasmid was extracted with the use of Interon plasmid extraction kit (Interon, Koura) which were used as positive control.

Detection limit assessment for PCR reaction. As we mentioned, the fragments were cloned in pTZ57R vector and the recombinant constructs named as; pL1 ، pHPV16 ،pHPV18 and p-β were prepared. For determining the reaction sensitivity, serial dilutions were prepared from each recombinant construct containing 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 copy per reaction and the PCR was done for each dilution by specific primers accordingly. This assay was at repeated three times by three various users in three different days. The highest dilution showing positive results for all the reactions was candidate as the limit of detection.

Multiplex PCR optimization and standardization for HPV16 , HPV18 detection. For synchronous amplification of the L1, HPV16, HPV18 and β-globin genes, 25 µl PCR reaction mixture containing 50 ngr DNA of each plasmid and specific primers of β-globin, dNTPs, MgCl2 and taq DNA polymerase. The PCR reaction was carried out, one cycle at 94°C for 7 min followed by 40

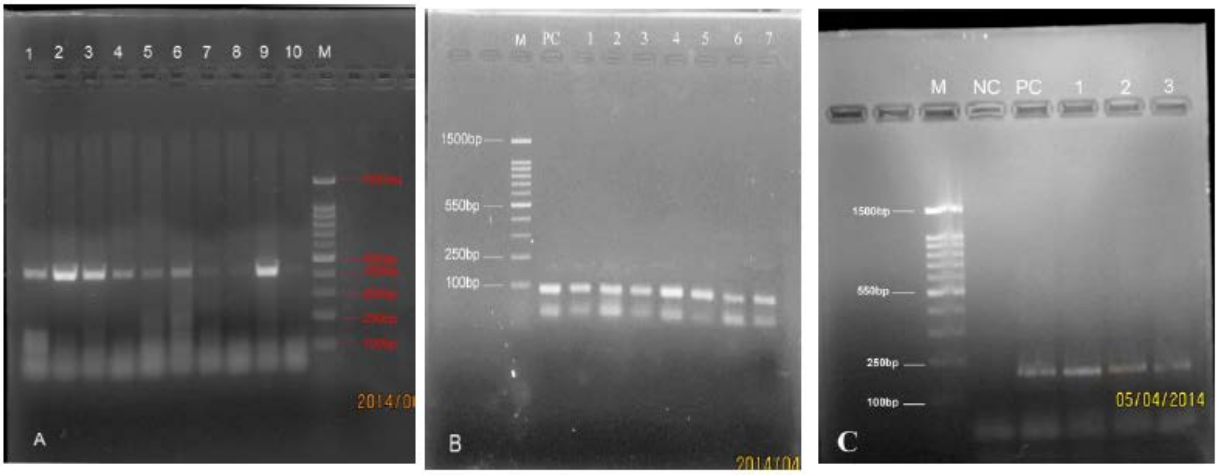

Fig. 1. Prevalence of tissue samples taken from the cervical lesions: A)column 1-10: the result of target region of L1 replication and observing 450 bp bond. M: 100 bp MW marker (Cina gene, Iran#PR901644). B) the result of target region gene HPV18 replication. Column 1-7: 100 bp products related to HPV 18 E6 amplification. PC: Positive control C) the result of HPV16 target gene region amplification. NC: negative control, PC: Positive control column 1-3: 217 bp product related to HPV16 amplification.

cycles of 94°C for 45 sec, 55°C for 30 sec and 72°C for 30 sec with a final cycle of 72°C for 1 min.

Results

Prevalence of HPV in tissue samples taken from the cervical malignant lesions. In our studied population (50 participants), 33 ones were HPV positive (figure1/a) and 17 ones were reported HPV negative. In other words, the rate of HPV infection was high in this population (around 66%) among which 18 cases were HPV positive (figure 1/b) and 15 ones were HPV16 positive (figure 1/c).

The evaluation of PCR characteristic (specificity). The result of PCR specificity test using L1 gene specific primers for papilloma samples was positive, while none of the other virus samples (containing adenovirus, CMV and HSV) was diagnose by this PCR. In fact, the results demonstrated in figure 2 confirm the test specificity.

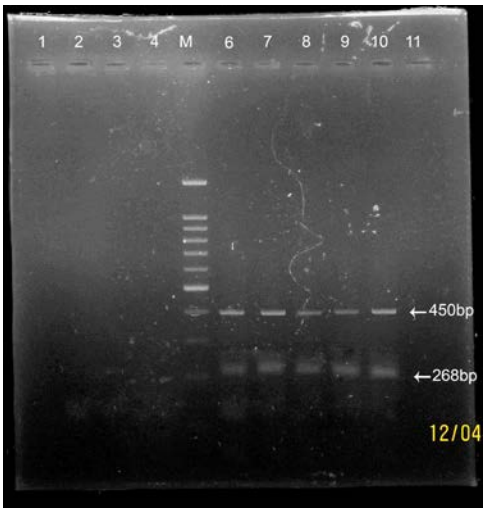

Fig. 2. The results of PCR characteristic surveying: columns 1 and 11: empty. Columns 2 to 4: the analysis result of DNA adenovirus, HSV and CMV respectively with the specific primer L1, lacking the target region. Columns 6 to 10: the 450 bp segment of DNA amplification in papilloma samples together with β-globin (268 bp) as the internal control, M: 100 bp weight marker (Cina gene, Iran # PR901644).

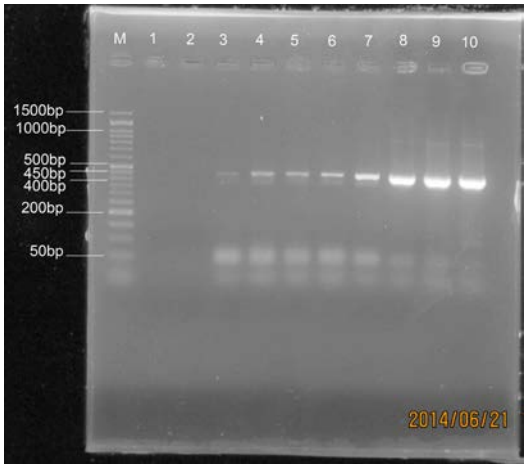

Fig. 3. The results of PCR sensitivity analysis test. M well: 50 bp Mw marker (Cina gene, Iran #PR901633). Wells 1 to 10: the analysis results of pL1 recombinant construct serial dilutions respectively contain 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 copy per assessing reaction. As demonstrated the well number 3 expresses the detection limit of this method which equals 20 copies of recombinant construct containing L1 in each assessing reaction.

The evaluation of PCR test detection limit (the minimum diagnosis or sensitivity). The serial dilutions of recombinant constructs pL1, pHPV16, pHPV18 and p- β containing 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 copy in each PCR reaction. The test sensitivity was 20 copies of recombinant construct per PCR reaction. This level was the highest dilution, all of the reactions performed on which were positive or we can say it was the highest dilution in which reaction positive results was 100 % repeatable and can be an estimated standard of viruses numbers containing the mentioned gene. The results of the sensitivity test on these serial dilution of the above recombinant construct are shown in figure 3.

The multiple polymerase chain reaction. Sample analysis with four different primers shows 4 bonds with the sizes 450bp(L1), 217bp (HPV16), 100 bp (HPV18) and 268 bp (β-globin)in which L1 was used as positive HPV segment and β-globin as the internal control and depending on the HPV type, 217

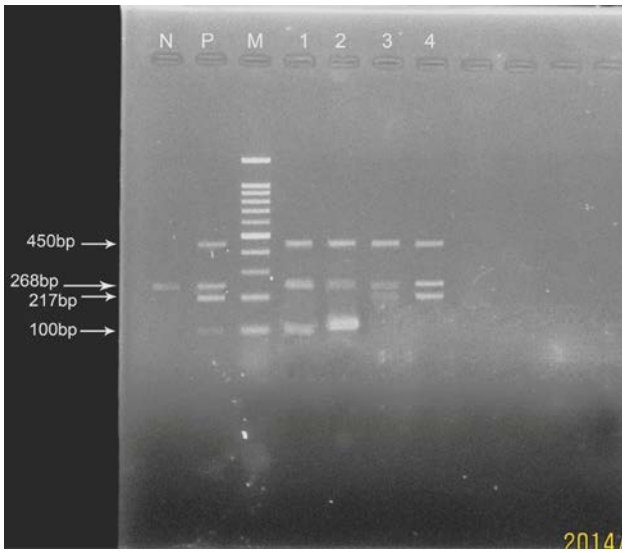

Fig. 4. The results of multiple PCR product of L1(45bp), HPV16 E6(217bp) HPV18 E6(100bp) and β-globin(internal control) (268bp). N: the negative control showing the 268bp and lacking the 450bp segment. P: the positive control containing the recombinant plasmids pL1, pHPV16, pHPV18 and p-β, observing 4 bonds containing the indicated fragments of the above genes alignment. M: 100bp weight marker (Cina gene, Iran # PR901644). Columns 1 and 2: HPV18 positive sample, containing L1, β-globin and HPV18-E6 genes (respectively 450, 268 and 100bp) PCR products. Columns 3 and 4: HPV16 positive sample containing L1 , β-globin, HPV16 E6 genes (respectively 450, 268 and 217 bp).

bp segment (HPV16) or 100bp (HPV18) were demonstrated (Figure 4).

Discussion

According to the previous studies, some HPV types known as high risk types play important roles in causing cervical cancer and these types have been detected in more than 99% of cervical cancers worldwide(24-26). Unfortunately there is not any comprehensive statistical data on the prevalence of oncogenic types of this virus related to cervical cancer in Iran. Many molecular diagnostic methods have been developed for detecting HPV infection related to viral carcinogenesis. Pop smear test has long been one of the prevalent methods specially in developing middle Asian countries (27). However this method is not acceptable by all the specialists (27) and since HPV culture is not practical, developing molecular methods is of great important. Various molecular methods have been developed for diagnosing and assaying HPV infection such as PCR, DNA probes, hybrid capture2 (HC2), dot blot, DNA methylation (20, 28-30) which can detect HPV in cervical biopsy samples in a short time (31). Among these methods, the ones relying on PCR are of great acceptance in developing countries. Formulating the diagnosis approach, PCR related methods can screen HPV samples using a simple and economical procedure (1). In present time, HPV recognition using the conserved regions of viral genome makes it possible to determine different HPV viral genotypes (17). Making a distinction between high risk (HR1) and low risk (LR2) types of HPV is of great important for predicting the outcome and progression of the disease (19). We have made an effort to run a conventional method relying on PCR with high sensitivity for detecting and recognition of HPV and its prevalent high risk types through amplifying the target regions of L1 and E6 genes. The significance of this study is in HPV diagnosis and determining the various types of HPV16 and HPV18 together with the β-globin gene target region as an internal control to confirm the results of polymerase chain reaction procedure. The conserved region of L1 gene in all HPV types is an appropriate choice for designing primers olygonucleotide in order to detect HPV based on the common region of E6 gene between HPV16 and HPV18. Since the β-globin gene is one of the house keeping genes in the cell, it was used as the internal control to confirm the PCR reaction process. Other validating methods such as repeatability, specificity and sensitivity proved this method to be a reliable as compared to histological approaches. During some studies in japan, Sasagawa and his colleagues invented the same method relying on Nested PCR on LCR-E7 gene and analyzing the nucleotide sequences of low risk types, declared that sensitivity of this method was 100 viral particles (20). In the

current study the sensitivity was per 20 particles which showed 5 fold sensitivity increase compared to the previous studies (20). The existing restrictions in histological tests (pop smear) for prognosing cervical carcinoma led to development of more sensitive method for HPV detection such as PCR tests and related procedure. Considering the deficiencies of histological tests and inefficiency of diagnosis test in cancer development phases, the molecular diagnosis of this oncogenic virus appears to be a necessity (8, 32). Although more sensitive molecular methods such as Real-Time PCR and probe assaying are more accurate and sensitive compared to the PCR test, the latter appears to be more cost effective which can be available in developing countries (32). Because of high prevalence of this virus in cervical cancer tissues, this method can be used as a suitable candidate for screening and detecting the high risk types in Iranian population.

Conclusion

Since there is a high prevalence of HPV infection among young women suffering from cervical cancer because of the long pre cancer period in patients infected with high risk HPV types methods such as cytology and molecular tests for detecting the viruses are highly recommended. Considering these facts, it is quite logical to implement a method for early screening and viral detection which can prevent primary cell transformation progression toward cervical dysplasia. Since there is no cell culture system for HPV, the best way to study the existence of HPV infection is molecular method such as PCR which can detect HPV in cervical biopsy samples very shortly(31). This study proved that PCR with specific primers on L1 and E6 genes, a suitable, accurate, sensitive and specific for detecting and type determination of HPV. So making use of this method beside pop smear screening in the sample with dysplasia diagnosis is highly suggested.

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References

1. Chaudhary A, Pandya S, Singh M, Mehrotra R. Identification of high-risk human papillomavirus-16 and-18 infections by multiplex PCR and their expression in oral submucous fibrosis and oral squamous cell carcinoma. Head Neck Oncol. 2013;5:4.
2. Romero-Pastrana F. Detection and Typing of Human Papilloma Virus by Multiplex PCR with Type-Specific Primers. International Scholarly Research Notices. 2012;2012.
3. Keyhani E, Kohannia N, Izadimood N, Keyhkhaee M, Najmabadi H. The prevalence of human papilloma virus (HPV) in malignant cervical lesion, using multiplex PCR. Tehran University Medical Journal. 2006;64(3):95-101.
4. Bosch F, Lorincz A, Munoz N, Meijer C, Shah K. The causal relation between human papillomavirus and cervical cancer. Journal of clinical pathology. 2002;55(4):244-65.
5. Chichareon S, Herrero R, Muñoz N, Bosch FX, Jacobs MV, Deacon J, et al. Risk factors for cervical cancer in Thailand: a case-control study. Journal of the National Cancer Institute. 1998;90(1):50-7.
6. Lehtinen M, Luukkaala T, Wallin K-L, Paavonen J, Thoresen S, Dillner J, et al. Human papillomavirus infection, risk for subsequent development of cervical neoplasia and associated population attributable fraction. Journal of clinical virology. 2001;22(1):117-24.
7. Schiffman MH, Bauer HM, Hoover RN, Glass AG, Cadell DM, Rush BB, et al. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. Journal of the National Cancer Institute. 1993;85(12):958-64.
8. Schiffman MH, Castle P. Epidemiologic studies of a necessary causal risk factor: human papillomavirus infection and cervical

neoplasia. Journal of the National Cancer Institute. 2003;95(6):E2-E.
9. Wright Jr TC, Cox JT, Massad LS, Twiggs LB, Wilkinson EJ. 2001 consensus guidelines for the management of women with cervical cytological abnormalities. Jama. 2002;287(16):2120-9.
10. Burger M, Hollema H, Pieters W, Quint W. Predictive value of human papillomavirus type for histological diagnosis of women with cervical cytological abnormalities. BMJ. 1995;310(6972):94-5.
11. Rozendaal L, Walboomers J, Van Der Linden J, Voorhorst F, Kenemans P, Helmerhosrt TJ, et al. PCR-based high-risk HPV test in cervical cancer screening gives objective risk assessment of women with cytomorphologically normal cervical smears. International Journal of Cancer. 1996;68(6):766-9.
12. Nishiwaki M, Yamamoto T, Tone S, Murai T, Ohkawara T, Matsunami T, et al. Genotyping of human papillomaviruses by a novel one-step typing method with multiplex PCR and clinical applications. Journal of clinical microbiology. 2008;46(4):1161-8.
13. Baay M, Quint W, Koudstaal J, Hollema H, Duk JM, Burger M, et al. Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. Journal of clinical microbiology. 1996;34(3):745-7.
14. de Roda Husman A-M, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. Journal of general virology. 1995;76(4):1057-62.
15. Kleter B, Van Doorn L-J, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. Journal of clinical microbiology. 1999;37(8):2508-17.
16. Manos M, Ting Y, Wright D, Lewis A, Broker T, Wolinsky S. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer cells. 1989;7(17):209-14.
17. Sotlar K, Diemer D, Dethleffs A, Hack Y, Stubner A, Vollmer N, et al. Detection and typing of human papillomavirus by E6 nested multiplex PCR. Journal of clinical microbiology. 2004;42(7):3176-84.
18. van Doorn L-J, Quint W, Kleter B, Molijn A, Colau B, Martin M-T, et al. Genotyping of human papillomavirus in liquid cytology cervical specimens by the PGMY line blot assay and the SPF10 line probe assay. Journal of clinical microbiology. 2002;40(3):979-83.
19. Gravitt P, Peyton C, Alessi T, Wheeler C, Coutlee F, Hildesheim A, et al. Improved amplification of genital human papillomaviruses. Journal of clinical microbiology. 2000;38(1):357-61.
20. Sasagawa T, Minemoto Y, Basha W, Yamazaki H, Nakamura M, Yoshimoto H, et al. A new PCR-based assay amplifies the E6–E7 genes of most mucosal human papillomaviruses (HPV). Virus research. 2000;67(2):127-39.
21. Rajaram S, Gupta G, Agarwal S, Goel N, Singh K. High-risk human papillomavirus, tumor suppressor protein p53 and mitomycin-C in invasive squamous cell carcinoma cervix. Indian journal of cancer. 2006;43(4):156.
22. Lazo P. The molecular genetics of cervical carcinoma. British journal of cancer. 1999;80(12):2008.
23. Zanotti KM, Kennedy AW. Screening for gynecologic cancer. Medical Clinics of North America. 1999;83(6):1467-87.
24. Franco EL, Duarte-Franco E, Ferenczy A. Cervical cancer: epidemiology, prevention and the role of human papillomavirus infection. Canadian Medical Association Journal. 2001;164(7):1017-25.
25. LORINCZ AT, REID R, JENSON BA, GREENBERG MD, LANCASTER W, KURMAN RJ. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstetrics & Gynecology. 1992;79(3):328-37.
26. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al.

Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. The Journal of pathology. 1999;189(1):12-9.
27. zur Hausen H. Papillomaviruses in the causation of human cancers—a brief historical account. Virology. 2009;384(2):260-5.
28. Contorni M, Leoncini P. Typing of human papillomavirus DNAs by restriction endonuclease mapping of the PCR products. Journal of virological methods. 1993;41(1):29-36.
29. Jacobs MV, Snijders P, Van Den Brule A, Helmerhorst T, Meijer C, Walboomers J. A general primer GP5+/GP6 (+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. Journal of clinical microbiology. 1997;35(3):791-5.
30. Resnick RM, Cornelissen MT, Wright DK, Eichinger GH, Fox HS, ter Schegget J, et al. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. Journal of the National Cancer Institute. 1990;82(18):1477-84.
31. Eslami G, Golshani M, Rakhshon M, Fallah F, Goudarzi H. The study on relation of Human Papillomavirus with bladder transitional cell carcinoma. Cancer Therapy. 2008;6:355-60.
32. Dorostkar R, Hashemzadeh MS, Tat M, Shafaati MR, Najarasl M, Zahiri Yeganeh S. Molecular Recognition of Human Papilloma Virus (HPV) Using Proprietary PCR Method Based on L1 Gene and the Evaluation of its Frequency in Tissue Samples from Patients with Cervical Cancer. Arak University of Medical Sciences Journal. 2015;18(3):28-36.