

RESEARCH ARTICLE

Comparison Between Two Detection Methods for HPV16, HPV18 and P16Ink4a Biomarkers in Diagnosis of Abnormal Cervical Cytology

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Abstract

Background: Cervical cancer is the second most common cancer among women in many populations. While the Pap smear is a well established screening test it suffers from both false-positive and false-negative results in diagnosis of cancers and precancerous states. In this study, immunocytochemistry of the P16 biomarker and HPV-PCR were compared for their diagnostic potential. **Materials and methods:** In the study, we obtained pairs of specimens from 45 women with cervical dysplasia. One sample was placed in a liquid-based solution, and processed for staining of sections with antibodies to P16. HPV-PCR was performed on the other and the results obtained were analyzed by T-test using SPSS v. 15. **Results:** Using HPV-PCR 71% of the samples were found to be infected with either HPV 16 or HPV 18, and the rate of infection did not have a statistically significant relationship with higher grades of dysplasia ($p= 0.253$). In contrast, with immunocytochemistry evaluation of P16, 64% of the specimens were positive, but the percentage of positive results significantly increased with higher grades of dysplasia ($p= 0.0001$). **Conclusion:** Employment of the P16 marker as an optional test might be preferable over HPV-PCR for cervical dysplasia in our geographical region.

Keywords: Dysplasia- cervix- HPV 16- HPV 18

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Introduction

Cervical cancer is the most common female genital system malignancy in worldwide and the most frequent cause of death by cancers in women specially in western countries (Kónya et al., 1995). It has been reported that accomplishment of cytological screening such as papanicolaou (Pap) smear has been led to significant reduction in prevalence and/or mortality caused by cervical cancer since 1950 (Ryan, 1999; Berek, 2002). In spite of the importance of Pap smear screening, annually more than three million women around the world receive a test result with uncertain cytology (Dehn et al., 2007). This requires further non-invasive tests to diagnose cervical dysplasia as a precancerous lesion (Dehn et al., 2007). Almost all invasive cervical carcinomas pass a stage of intraepithelial growth of abnormal cells (the intraepithelial stage), and the morphological changes of this early stages of cervical cancer are detectable (Johnson et al., 1968). In high grades of dysplasia, there is a several-month to several-year time interval between dysplasia and its transformation into invasive cancer (Berek, 2002; Stoler, 2004). In this regard, cytological detections provide the chance to have an opportunity of appropriate time for diagnosis among the cervical precancerous lesions,

dysplasia, and carcinoma stages. Nevertheless, the problem of false-positive and false-negative results of Pap smear is a worldwide concern, and numerous studies are in progress to reduce the problem resulted from the inter- and intra-observer differences in pathological reports (Berek, 2002; Stoler, 2004; Volkareva et al., 2004).

Epidemiological evidences have been shown that human papilloma virus 16 and 18 (HPV 16 and HPV 18), as the high-risk HPV subtypes, are the most common cause of almost 70% of all cervical cancers (Sargent et al., 2008; Bruni et al., 2010; Piroozmand et al., 2016). Moreover, the prevalence of HPV has been reported to be about 10.4% in women with normal cytology (De Sanjosé et al., 2007; Bruni et al., 2010). In addition, HPV subtype 16 is involved in about 50% of women infected by human papillomavirus (Castle et al., 2005). As the HPV DNA integrates into the host DNA, cervical interepithelial neoplasia (CIN) moves from the polyclonal toward monoclonal proliferation. This monoclonality plays the main role in the low-to-high-grade transformation in cervical neoplasia (Hillemanns and Wang, 2006). The human papillomavirus DNA encodes 8 genes including E1, E2, E4, E5, E6, E7, L1 and L2 that are expressed in several phases of viral differentiation (Doorbar, 2006). If the E2 gene is not integrated into the human genome,

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inactivation of pRb would not occur, and the cells would not turn into neoplasia. Therefore, since HPV DNA test have been reported to be positive in some cases with normal cytology, it is necessary to replace the HPV DNA by an alternative marker, which indicates the integration of viral genome into the host genome; rather than the presence of viral DNA (Zhou et al., 2003; Dueñas-González et al., 2005; Monsonego et al., 2011).

It is the fact that the deregulation of several genes plays the important role in human cancers. Among them, P16INK4a gene which encodes a tumor suppressor protein with the normal function of preventing cell division is mutated and/or inactivated by mutation in development of cancer (Rocco and Sidransky, 2001; Ellenson et al., 2010). P16INK4a as an inhibitor of cyclin dependent kinases (CDKs) suppresses G1-S cell cycle progression by formation of inhibitory cdk4-6/P16 complex which led to degradation of free cyclin D and maintenance the dephosphorylated form of retinoblastoma protein (pRB). It has been suggested that production of viral oncoproteins E6 and E7 is associated with increased expression level of P16 in cervical cancer (Sano et al., 1998). E6 and E7 as the two transcriptional units of HPV 16 and 18 encodes proteins involved in binding to host regulatory proteins particularly tumor suppressors. For instance, deregulation of P53 by interaction with E6 and inactivation of pRB through binding to E7 led to unchecked cell cycle progression (Dyson et al., 1989; Amortegui et al., 1995). Oncoprotein E7 negatively enhances the expression of P16, owing to the existence of a negative feedback loop between pRb and P16 (Xiong et al., 1996). Furthermore, in most cervical cancers, inactivation of pRb protein leads to progression of cell proliferation (Amortegui et al., 1995; Ryan, 1999; Ellenson et al., 2010). Therefore, overexpression of P16 in neoplasia could be an alternative marker for HPV E7-mediated pRb downregulation, which is a mechanism to control cell proliferation and indicates the existence of an infection with high risk for turning into neoplasia (von Knebel Doeberitz, 2002; Smeets et al., 2011; Gajanan et al., 2015).

In an investigation using HPV-PCR, the expression levels of high-risk HPV were reported to be 70% and 80% in the high-grade squamous epithelial lesion (HSIL) and squamous cell carcinoma (SCC), respectively (Chen et al., 2009). Meanwhile, 100% of the cases were positive for the expression of P16INK4a marker, which suggests the contribution of some factors other than high-risk HPVs in the progression of cervical cancer (Nieh et al., 2003; CHEAH et al., 2016). Now, the question is whether the percentage of the cervical dysplasia in the studied geographical region is associated with high-risk HPV infections. Are all individuals who express P16INK4a marker associated with high-risk HPV infections? Therefore, the aim of the current study is to evaluate the association between HPV 16 and HPV 18 on one hand, and P16INK4a on the other hand, in patients with cervical dysplasia using immunocytochemistry and polymerase chain reaction (PCR).

Materials and Methods

Tissue specimens and cytological preparation

The study was carried out on patients with the Pap smear report of squamous dysplasia, or carcinoma in 2005-2006 at Kermanshah. After obtaining the consent from patients, another sample was obtained. Specimens divided in to groups, one group was exposed to cytology and the other was prepared for PCR. For preparation of specimens for cytology, the liquid-based technique was used to avoid false positive results due to staining of normal cells and bacteria (Sahebali et al., 2006). For this aim, patient specimens were placed into a liquid-based solution, and were kept at 2-8 °C to conserve the normal structure of cells. Then, cellular samples of the patients were mounted on slides for immunocytochemistry (ICC) staining and conventional slides for Papanicolaou (Pap) staining. The results of slide staining with Pap stain were evaluated by a pathologist.

Immunocytochemistry (ICC)

ICC staining was performed using incubation of slides in Epitope Retrieval Solution (DAKO.K5339) and heating in a steamer at 90 °C for 45 min in the antigen retrieval step. The samples were then incubated with the primary antibody of Mouse Anti-Human P16INK4a Protein (DAKO.k5340) at the room temperature in the dark and humidity for 45 min. We used monoclonal antibody of E6H4 in the study. The incubation was followed by addition of secondary antibody with Visualization Reagent (DAKO.K534) at the room temperature, in the dark and humidity for 30 min. The samples were then covered by Substrate-Chromogen Solution (DAB) for at least 10 min, and then counterstained by hematoxylin (Fig.1). In evaluation of the results of ICC staining of P16INK4a, the sample was considered to be positive for P16 marker if at least 10 dysplastic cells were stained, cytoplasmic or nuclear (Bibbo et al., 2002).

DNA extraction and Polymerase chain reaction

The sample was obtained by a brush and placed in 15 cc falcon tubes containing 3 cc of sterile PBS buffer. The DNA of the cells was extracted according to the salting out method. In brief, 6 µM NaCl was added to tissues and centrifuged at 3500 rpm for 30 min. Then, cold isopropanol was added to precipitate DNA. The amount of DNA extracted was determined by spectrophotometer. To evaluate the presence of HPV 16 and HPV 18 DNA, lyophilized kits of Human Papilloma Virus (code: KP085, Bioscience Dynamic) with positive and negative controls were used. The DNA of HPV 16 and HPV 18 were amplified by the fragments of 512- and 415-bp length, respectively, by primers for HPV16 and HPV18. Then, amplified DNAs were visualized by electrophoresis. We used positive control sample and also 100-bp DNA fragment (Fermentas.SMO323) to evaluation of DNA fragments.

Statistical evaluation

The results obtained from the ICC staining for P16 marker and HPV-PCR was analyzed using T-test in SPSS

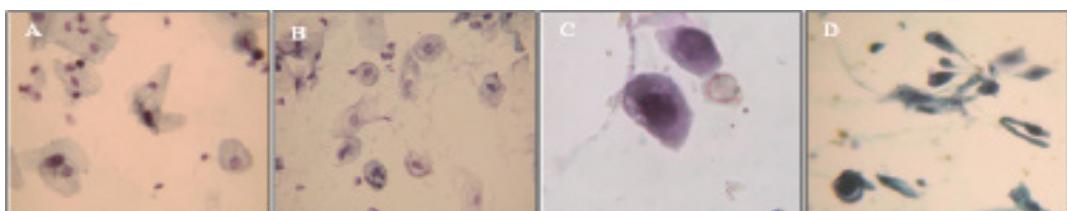


Figure 1. Cytological Features Using Papanicolaou (Pap) Staining of Cervical Squamous Cells of Patients. The cases were diagnosed as (A) atypical squamous cells of undetermined significance (ASCUS), (B) low-grade squamous intraepithelial lesion (LSIL), (C) high-grade squamous intraepithelial lesion (HSIL), and (D) squamous cell carcinoma (SCC), [IHCx400]

Table 1. Results of PCR and P16 ICC Staining in Specimens with Abnormal Cytology

Cytology diagnosis	Test result	Test used	
		P16 n = 45	HPV (16 and 18) n = 45
Ascus (n = 13)	+	(3/13)(23%)	(8/13)(61.5%)
	-	(10/13)(77%)	(7/13)(38.5%)
LSIL (n = 18)	+	(12/18)(67%)	(12/18)(67%)
	-	(6/18)(33%)	(6/18)(33%)
HSIL(n = 11)	+	(11/11)(100%)	(10/11)(91%)
	-	(0/11)(0%)	(1/11)(9%)
S.C.C (n = 3)	+	(3/3)(100%)	(2/3)(67%)
	-	(0/3)(0%)	(1/3)(33%)
Total (n = 45)	+	(29/45)(64%)	(32/45)(71%)
	-	(16/45)(36%)	(13/45)(29%)

(v.15). The type of dysplasia and levels of P16, P18 and HPV16 were summarized in a one-dimensional frequency distribution table. Distribution of HPV and P16 were evaluated by two-dimensional tables according to the type of dysplasia. In addition, several tables were provided to distinguish the concordance between HPV and P16. In this line, χ^2 statistical test was used to compare the results of HPV and P16.

Results

The mean age of participants of the study was 42 ± 12 , with the age range of 16-70. Among the participants, 41 women (91%) were married and 4 (9%) were divorced. Results showed that 13 (29%), 18 (40%), 11 (24%), and 3 (7%) of the cases were diagnosed as atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC), respectively.

Among the cases, none were diagnosed as atypical

glandular cells of undetermined significance (AGUS) or adenocarcinoma (Figure 2). As indicated in table 1, out of the patients, 29 cases (64%) were positive for P16, and all the three cases of SCC were diffusely positive in the P16 staining. Moreover, all the 11 cases of HSIL were positive in ICC staining. However, 12 out of 18 cases (67%) of LSIL and 3 out of 13 cases (23%) of ASCUS were positive in the staining (Table 1). In higher grades of dysplasia in the cytological specimens, the percentage of positive cases for P16 marker was higher, and the finding was statistically significant ($p < 0.0001$).

The PCR results demonstrated that 32 participants (45%) were positive for HPV 16, while 24 cases (53%) were positive for HPV 18. Being positive for at least one of the two viruses; HPV 16 and HPV 18, was a significant finding in the study. The results are provided in Table 1 in more details. Being positive for HPV did not have statistically significant relationship with the dysplasia grade ($p = 0.253$).

Discussion

In the current study, the expression of P16 biomarker in cytology samples (Pap smear) was evaluated using the liquid-based method. The results showed that the probability of being positive for P16 marker is elevated at higher grades of dysplasia, and the finding was statistically significant ($p = 0.0001$). Sano and colleagues reported this method to be as valuable as the histological evaluation of the specimens for P16 marker. Moreover, they considered the cleanliness of the slide background and lack of requiring invasive procedures as the advantages of this approach (Sano et al., 1998; Nieh et al., 2003). According to the studies of Bibbo et al., (2002) and Galina Volgareva et al.,(2004) the E6H4 monoclonal antibody is one of the most appropriate markers for evaluation of P16, due to having the highest affinity to the Ag of interest, and the lowest non-specific affinity. In the study performed by Galina Volgareva et al., although the percentage of being positive for P16 was low, the increase in the percentage of

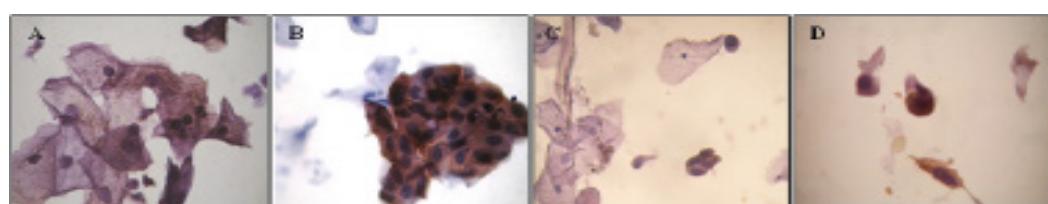


Figure 2. Immunocytochemistry (ICC) Staining of P16INK4a in Patients' Tissues Diagnosed as (A) ASCUS, (B) LSIL, (C) HSIL and (D) SCC, [IHCx400].

cases positive for P16 marker in higher grades of dysplasia was obvious (LSIL, 37%; HSIL, 67%; and SCC, 96%). They considered paraffin blocks stored for some years and the high cut-off point value (positivity of more than 25% of the cells) which was accounted as the reasons for their low percentage of positive cases (Volgareva et al., 2004). Diffuse and strong staining for this marker in high-grade dysplasia and carcinoma is noteworthy (Sano et al., 1998; Nieh et al., 2003; Yildiz et al., 2007; Aslani et al., 2013). This is also the case for all the three SCC samples and most HSIL samples in the current study.

Another noteworthy finding was positivity of all the HSIL and SCC samples for P16. In other studies, it was reported that as the malignancy grade was higher, the positivity rate increased, and the percentage was very high (Sano et al., 1998; Bibbo et al., 2002; Saqi et al., 2002; Dehn et al., 2007; Lesnikova et al., 2009). The percentage of positivity for P16 in cervical carcinoma has not been reported to be less than 90% in other studies (Bibbo et al., 2002; Saqi et al., 2002). Even in the study by Galina Volgareva et al., despite the low overall percentage of positivity for P16 marker, only 3% negative result was obtained for cervical carcinoma. This indicates the worth of P16 in detection of high-grade dysplasia (Volgareva et al., 2004). With regard to the positive ASCUS cases for P16 (3 out of 13, 23%), it seems that these are the ASCUS cases with high probability for progression to dysplasia of higher grades. This is explained in the Bethesda system of standard report also, defined as ASC-H (Solomon and Nayar, 2004). This also becomes authentic for the negative cases of LSIL which is assumed as the reversible cases of LSIL (Ryan, 1999; Zhou et al., 2003). However, it is required follow up the patients in another study to confirm the results. According to PCR results, elevated probability of being positive (percentage of positive cases) does not have a statistically significant relationship with higher grades of dysplasia ($p= 0.253$). This was more prominent in the study of Sano et al., as they could not find high-risk HPV in 17 out of 53 cases using PCR and in situ hybridization (ISH) (Sano et al., 1998; Yıldız et al., 2007). Galina Volgareva et al. also reported some cases of high-grade dysplasia, which were negative for HPV by using Western Blot as a standard method along with the PCR technique (Volgareva et al., 2004). It is well-known that PCR is an acceptable method with reliable results due to high sensitivity and specificity and applicability of the test even with minute amounts of the sample are the advantages of PCR.

Sano and colleagues (1998) considered some other causes for the HPV-negative cases with high-grade dysplasia and carcinoma, including Loss of subgenomic regions on the DNA of HPV, low copy number that it is not detectable by PCR, and existence of some high-risk HPVs other than HPV 16 and HPV 18. A question which was proposed since the early studies in this field was that if there is any correlation between the positivity for P16 marker and positivity for DNA from HPV 16 and HPV 18. The current study does not show a statistically significant correlation between these two items (kappa coefficient= 13.9%, which is not desirable). This discrepancy was also reported by Galina Volgareva et al., (2004). It was stated

that HPV is not useful in grading of SIL (Nieh et al., 2003; Yıldız et al., 2007). However, the studies carried out by Saqi et al., (2002); Bibbo et al., (2002) and Sano et al., (1998) found statistically significant correlation between the presence of high-risk HPVs and being positive for P16 in the cytological or histological samples of cervix. Furthermore, it has been reported that HPV-negative cell line C-33A and HPV-negative adenocarcinomas were positive for P16. This indicates that there is a pathway for P16 expression independent of HPV (Milde-Langosch et al., 2003; Murphy et al., 2003). On the other hand, some HPV-positive cases have been reported in samples with normal cytology (Bruni et al., 2010). Various studies have shown that test for P16 marker possesses high values of sensitivity and specificity (probability of negative result in normal people (Nieh et al., 2003; Holladay et al., 2006; Sahebali et al., 2006). Thus, P16 is the most reliable marker for cervical dysplasia and its expression has not been observed in normal cervix tissue (Murphy et al., 2005). However, as the malignancy grade increases, the frequency of positive cases of P16 will increase, which indicates that P16 is a diagnostic tool more effective and helpful than the high-risk HPV viral load for detection of cervical dysplasia (Nieh et al., 2003; Murphy et al., 2005).

In the current study, we observed an inconsistency between HPV results and cases with cervical dysplasia. Moreover, most cases of dysplasia were positive for P16 biomarker. Considering these data and the low infection rate of HPV in the geographical region studied and lack of multiple sexual partners in women living in this region, carrying out ICC test for P16 is preferred over HPV-PCR as a complementary tool in detection of dysplasia (Bruni et al., 2010). Nevertheless, it should be mentioned that this biomarker is not sufficient for diagnosis of the cancer, and further clinical information is required to achieve definite diagnosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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