P16INK4A Immunohistochemistry as a Gold Standard for Cervical Cancer and Precursor Lesions Screening

Mahdieh FARZANEHPOUR¹,², Ahad MUHAMMADNEJAD³, Setareh AKHAVAN⁴, Amir Nader EMAMI RAZAVI³, Somayeh JALILVAND¹, Vahid SALIMI¹, Ebrahim FAGHIHLOO⁵, Ehsan KAKAVANDI¹, Mohammad FARAHMAND¹, Mohammad SHAYESTEHPOUR¹, Farzad BABAKHANI¹, *Talat MOKHTARI AZAD¹

¹. Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
². Department of Microbiology, Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
³. Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran
⁴. Department of Gynecology Oncology, Imam Khomeini Hospital Complex, Valiasr Hospital, Tehran University of Medical Sciences, Tehran, Iran
⁵. Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding Author: Email: Mokhtari@sina.tums.ac.ir

(Received 18 Mar 2019; accepted 11 Jun 2019)

Abstract

Background: High-risk (HR) Human papillomaviruses (HPVs) are known as the main factors implicated in the pathogenesis of cervical preinvasive and invasive lesions. Therefore, the presence or absence of HR-HPV can be followed for the prognosis of low-grade and high-grade squamous intraepithelial lesions. Since the overexpression of p16INK4a protein depends on the presence of transcriptionally-active HPV, and due to its availability and simple interpretation, it may be considered as a proper marker to diagnose cervical cancer.

Methods: An immunohistochemical analysis of p16INK4a was performed in 72 cervical tissue specimens at Imam Khomeini Complex Hospital (Tehran, Iran) from 2016 to 2018. The performance parameters were calculated and compared using receiving operating characteristics curve (ROC) details.

Results: p16INK4a is significantly up-regulated in the cervical cancer samples in comparison with that in normal samples. Moreover, the ROC data showed the potential ability of p16INK4a under determined conditions as a diagnostic marker for CIN 2-3 staging and invasive cervical cancer. The molecular typing disclosed the attendance of HPV DNA in 44.4% of cases (32/72) with a predominance of HPV type 16.

Conclusion: The molecular biomarker p16INK4a can be a good candidate for the early diagnosis and prognosis of cervical cancer in HPV-infected patients. Considering the increase in the expression level of p16INK4a in cancer and precancer tissues, p16INK4a may be used for early detection of cervical cancer.

Keywords: Human papillomavirus; p16INK4A; Immunohistochemistry

Introduction

Human papillomavirus (HPV) has been known by epidemiological and clinical studies as the main pathogen leading to cervical cancer (1). HPV is a non-enveloped, circular double-stranded DNA virus comprising nearly 8,000 base pairs. To date, about 200 subtypes of HPV have been identified based on their L1 capsid protein, sub-categorized into cutaneous or muco-
sal subtypes (2, 3). Another classification into low-risk (LR) and high-risk (HR) types can be performed based on the capability of developing malignancy or cancerous. Since now, 20 HPV genotypes have been identified as high risk which causes uterine cervix, anus, vagina, vulva, penis, and head and neck cancers (4). HR-HPV subtypes, particularly oncogenic types 16 and 18 develop cervical precancerous lesions (5). One of the cost-effective tests to diagnose HR-HPV is following up on the expression of p16INK4a due to its overexpression in the cervical cancerous tissue. Thus, p16INK4a overexpression may be considered as a surrogate biomarker for the presence of high-risk HPV in cervical cancer. Moreover, the correlation between HPV-16, overexpression of p16INK4A, and pRb negativity in oropharyngeal carcinoma have also been reported (6).

HPV oncoprotein E7 comprises a binding site for retinoblastoma (pRb) that causes inactivation of pRb function. The overexpression of p16INK4a is also occurred in E7 expressing cells, which is probably due to the induction of histone demethylases by HPV E7 (7). Although p16INK4A expresses in individual epithelial cells of the lower genital tract (8), the expression level is higher in cells of high-grade precancerous and cancerous cervical lesions (9, 10). P16INK4a could be considered as the diagnostic tool when the malignant transformation associated with p16INK4a loss in malignant lesions and it also could be a prognostic tool when the malignant transformation accompanies the p16INK4a overexpression as a result of the pRb failure. Therefore, the survey of the p16INK4a expression in human tumors can be of importance to utilize the p16INK4a immunohistochemistry as a diagnostic or prognostic tool. Moreover, there are rare details regarding the subcellular location of p16INK4a, which can help the assessment of p16INK4a overexpression in tumors. Eventually, the information about the p16INK4a expression is required to develop new anticancer drugs which act to restore the p16INK4a functionality as one of the major tumor suppressor (11).

Since incorporating the p16INK4A immunohistochemistry and histopathologic diagnosis examination improves diagnosis of the cervical intraepithelial neoplasia (CIN), p16INK4A immunohistochemistry was assessed as the gold standard for defining the efficiency of cervical cancer screening methods (12). In the present study, the expression of p16INK4a in different samples for the diagnosis of the precancer and invasive cervical cancer in tissue samples was determined. The receiver operating characteristic (ROC) curve analysis was used finding the discriminative value for discriminating the cervical cancer tissues from the precancer and normal tissues.

Materials and Methods

Sample selection and histological analysis
Seventy-two fresh uterine cervix biopsies were fixed in neutral buffered. The whole cervical cancer, precancer, and normal tissue samples were obtained from the cervical tissues of patients with informed consent before operations at Imam Khomeini Complex Hospital (Tehran, Iran) from 2016 to 2018. Patients were also excluded if they had received any neoadjuvant chemotherapy or intraoperative radiation therapy. Slides were reviewed by a single pathologist in a blinded fashion to provide a “study diagnosis” utilized to determine the performance of the different screening tests. All biopsies diagnosed as normal, precancer (CIN1, CIN2, CIN3), or invasive cancer according to international criteria (13). Then, they were reviewed by a second pathologist, and if the second review as opposed to the first, a third pathologist reviewed the case. Considering 2 out of 3 in agreement, a “consensus diagnosis” was obtained.

This study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1395.838).

Immunohistochemistry
Paraffin blocks from 72 biopsies were selected, so that had sufficient diagnostic material remain-
ing for immunohistochemistry. These specimens were included 36 normal, 18 cervical cancer and 18 precancer samples. Five-micron sections were cut and put onto silane-coated slides (Sigma, St. Louis, MO, USA) and processed for immunohistochemistry (14). Anti-human p16INK4A monoclonal antibody (clone E6H4, Dako, Glostrup, Denmark) was used at a 1:50 dilution. Before incubation with the primary antibody, rehydrated sections were microwaved for 15 min in 0.01 citric acid (pH 6.0) and then washed twice with distilled water (15). Endogenous peroxidase activity was terminated by incubation in methanol containing 0.3% hydrogen peroxide for 20 min. Sections were preincubated with 3% normal horse serum in phosphate-buffered saline for 1 h at room temperature (RT), incubated with primary antibody at 4 °C overnight, followed by a 1 h incubation at RT. The avidin-biotinylated-peroxidase complex detection system was used for immunocytochemical localization (Vectastain ABC kit, Vector Laboratory, Burlingame, CA). Immunostaining was imaged using Liquid DAB Pack (BioGenex, CA). For negative controls, slides were incubated with normal rabbit IgG or preimmune serum instead of primary antibody. P16INK4A staining was categorized as either diffuse comprising all layers of the epithelium or basal comprising only the basal and parabasal cell layers and negative. Both diffuse and basal staining could be strong, moderate, or weak.

**Immunohistochemical evaluation**

The microscopic analysis of the slides was separately carried out by two researchers. Digital photographs were recorded with a Nikon Coolpix camera DP12. Quantitative outcomes were stated as the percentage of positive cells per field on total cell count. Only cells within the cervical epithelium were enumerated. The whole section slides were evaluated at 400X magnification and separately assessed by two observers. At least, 200 nuclei were evaluated in each case. The counts were accomplished manually and the percentage of positively stained cells in representative microscopic fields was recorded. The reaction was considered positive for p16INK4A when a dark brown color was seen in the nuclei and/or cytoplasmic compartments.

**Evaluation of immunostaining results**

For the quantitative evaluation of p16INK4A staining, the percentage of positive cells was measured and then classified according to nuclear and cytoplasmic staining. Immunoreactivity to p16INK4A was classified into three groups according to the percentage of stained cells; weak, variable and strong corresponding to less than 5% of the cells, 5%-50% of the cells (containing weak and strong areas of intensity), and more than 50% of the cells stained for p16INK4A, respectively. Allred score was calculated by measuring the percent of stained cells scored as 0 to 8 and intensity score (weak, intermediate, and strong) (Table 1). The possible values of Allred score are 0 – Allred 0; 1 – Allred 2, 3, 4; 2 – Allred 5, 6; 3 – Allred 7, 8 (Allred score 1 is not possible) (Fig.1).

Table 1: The Allred score

| Proportion score (PS) | Value | Significance |
|-----------------------|-------|-------------|
| 0                     | 0     | None        |
| 1                     | <1%   |            |
| 2                     | 1–10% |            |
| 3                     | 10–33%|            |
| 4                     | 33–66%|            |
| 5                     | >66%  |            |

| Intensity score (IS) | Value | Significance |
|----------------------|-------|-------------|
| 0                    | 0     | None        |
| 1                    | 1     | Weak        |
| 2                    | 2     | Intermediate|
| 3                    | 3     | Strong      |

Available at: [http://ijph.tums.ac.ir](http://ijph.tums.ac.ir)
Nested Polymerase Chain Reaction for HPV detection

DNA from each of the selected specimens was extracted with the High Pure DNA extraction kit (Roche, Germany), according to the manufacturer’s protocol. The concentration of DNA was then quantified by NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The quality of the extracted DNA was further checked by PCR amplification of a fragment of the β-globin gene amplified by PC03/PC04 primers (16). The detection of HPV DNA was conducted by two sets of consensus primers, MY09/MY11 and GP5+/GP6+ (16), which amplify a 450 bp and an internal 150 bp region, respectively, in the highly conserved L1 HPV gene. Afterward, the reaction products were electrophoresed on 2% agarose and visualized by SYBR Safe dye.

Statistical analysis

The Mann-Witney non-parametric test and the one-way ANOVA were carried out to analyze the statistical difference among groups using Graph-Pad Prism (7.0.1) software. A P-value of less than 0.05 was considered remarkable. The receiver operating characteristic (ROC) curves were drawn to find the highest sensibility and specificity point. The area under receiver operating characteristic (ROC) curves were calculated using R software (ver. 3.4.4).

Results

Patient and control data

The mean age of cervical cancer, precancer, and normal groups were 61 (range: 45–81), 47 (range: 27–57), and 36 (range: 23–49), respectively.

The p16INK4A expression profile in the tissue samples

The results showed a higher significant expression of p16INK4A in the tissue of cancerous samples than those in normal samples with a P-value <0.0001. Moreover, the expression of p16INK4A was remarkably increased in the cancer group in comparison with the precancer
group with a $P$-value of 0.0002. The same result was obtained from the comparison between the precancer and normal groups with a $p$-value of 0.0013 (Fig. 2).

![Image]

**Fig. 2:** The relative expression level of p16INK4A

**Receiver operating characteristic (ROC) curve analysis**

The ROC curves were generated and the area under curves (AUC) was analyzed to evaluate the diagnostic value of the p16INK4A expression level in cervical cancer, precancer and normal samples (Table 2).

|                         | Cervical cancer and Normal groups | Cervical cancer and Precancer cervical groups | Precancer cervical and Normal groups |
|-------------------------|----------------------------------|---------------------------------------------|--------------------------------------|
| p16INK4A                | AUC                              | AUC                                         | AUC                                  |
| 95% CI : 1              | 95% CI: 1                        | 95% CI: 0.95                               | 0.89-1                               |
| (1-1)                   | (1-1)                            |                                             |                                      |

The ROC curves showed that the AUC values in cervical cancer and normal groups were 1 (95% CI: 1-1), in cervical cancer and precancer groups were 1 (95% CI: 1-1), and in the precancer and normal groups were 0.95 (95% CI: 0.89-1) (Fig. 3). Therefore, the highest AUC value was obtained from comparing cervical cancer and the normal groups and also cervical cancer and precancer groups. P16INK4A has a strong potential diagnosis value for diagnosing cervical cancer from precancer and normal groups (Table 3).
Fig. 3: Receiver-operating characteristics (ROC) curve analysis using p16INK4A for discerning different groups in tissue samples. Cervical cancer and normal groups (a), Precancer samples and normal groups (b), cervical cancer and precancer groups (c)
Table 3: The sensitivity and specificity estimation of p16INK4A according to the ROC curves results in tissue samples

| Variable                  | Sensitivity and Specificity | Cervical cancer and Normal groups | Cervical cancer and Precancer cervical groups | Precancer cervical and Normal groups |
|---------------------------|-----------------------------|----------------------------------|----------------------------------------------|-------------------------------------|
| p16INK4A                  |                             | 100                              | 100                                          | 100                                 |
|                           | Sensitivity                 | 100                              | 89.47                                        | 78.95                               |

**HPV typing**

The molecular typing revealed the presence of HPV DNA in 44.4% of the cases (32/72), with a predominance of HPV type 16 (Table 4). Stratification of the pathological status showed that HPV 16 was found in all samples (100%) in the cancer group; HPV 16 and 53 were detected at 50% and 5.5%, respectively, in the precancer group; and HPV 16, 66 and 68 were identified at 5.5%, 2.7%, and 2.7%, respectively, in the normal group.

Table 4: The distributions of the HPV genotypes

| Groups       | Virus genotype | Percentage |
|--------------|----------------|------------|
| Cancer (18)  | HPV-16         | 18 (100)   |
| Precancer (18)| HPV-16         | 9 (50)     |
|              | HPV-53         | 1 (5)      |
| Normal (36)  | HPV-16         | 2 (5.5)    |
|              | HPV-66         | 1 (2.7)    |
|              | HPV-68         | 1 (2.7)    |

**Discussion**

In the present study, the overexpression of p16INK4a in the cervical cancer samples determining by the percentage of positive squamous cells per category was reported. Moreover, to test the predictive power of p16INK4a as a diagnostic marker for precancer and invasive cervical cancer, ROC curves were applied. ROC analysis and calculation of AUC specified the variance of sensitivity and specificity (17). A cut-off value of 54.43% was established and relatively high sensitivity (100%), specificity (100%) were obtained. Accordingly, p16INK4a could be an applicable surrogate marker to discern CIN from other similar tumors and assess the risk of CIN 2-3. These findings are agreement with previous reports regarding the marker potential of p16INK4a for prediction of CINs (18-21). A dramatic increase in the p16Ink4a expression has been reported in the transformation from normal tissue to preneoplastic lesions, and also from preneoplastic lesions to carcinoma in several types of cancer (22-26). The p16INK4A overexpression has been reported at the invasive front of endometrial, colorectal and basal cell carcinoma (27-30).

The p16INK4A upregulation was associated with the expression of other molecules such as the γ2 chain of laminin 5 and β-catenin related to invasive status (27, 28, 31). Furthermore, in vitro studies have demonstrated that p16INK4A is involved in the regulation of matrix-dependent cell migration (32), in glioma invasion (33). Moreover, p16INK4a expression has been reported in many cases of endometrial adenocarcinomas (34-37). The concurrent evaluation of HPV status and p16INK4a expression in en-
dometrial carcinomas have been evaluated in only a few cases (34, 35, 37). P16INK4a is known as a common immunohistochemical marker in gynecologic pathology. The nuclear and diffuse cytoplasmic expression of p16INK4a in squamous cell carcinomas of the female genital tract are extremely accompanied by high-risk HPV infection and neoplasms of cervical origin (38). Similar nuclear staining for p16INK4a leads to a change in the cytoplasmic intensity corresponding to the CIN grade. This finding suggests that the hyper-synthesis of p16INK4a in higher grade lesions is a reflection of the overexpression of p16INK4a in the cytoplasm (21). Consistent with other previous studies, a continuous staining pattern from the basement membrane was found that expanded upward in proportion to the lesion grade (39). The staining pattern may be considered as a reliable variable to interpret the p16INK4a rather than other signal intensity determination. Moreover, p16INK4a expression was introduced as an applicable marker to the diagnosis of CIN2 (40). However, these reports only showed the sensitivity and specificity of p16INK4a. In order to establish the best cut-off point for p16INK4a, the ROC curves were established. On the other hand, p16INK4a upregulates as a result of HR-HPV E7 expression in proliferating cells (7, 41-47). The increase in intracellular expression of p16INK4A is observed upon the binding of HR-HPV derived E7 oncoproteins to the retinoblastoma gene product. Considering the association between HR-HPV and cervical cancers, the over-expression of p16INK4A in HPV-induced neoplasia is expected (19, 48-52). Therefore, based on the immunohistochemical analysis in neoplastic cervical lesions, diffuse p16INK4a positivity can be considered as an indicative marker for the presence of HR-HPVs (50).

**Conclusion**

The expression level of p16INK4A changes in cervical cancer cells. The identification of p16INK4A is beneficial to cancer prognosis and early treatment. The up-regulation of p16INK4A in the tissue is a noteworthy biomarker for the diagnosis of HPV-associated cervical cancer. Molecular typing manifested the dominant presence of HPV 16 DNA in cervical cancerous cells. Therefore, our results can help to identify the possible biomarker for HPV-induced cancers.

**Ethical considerations**

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

**Acknowledgments**

This study has been funded and supported by Tehran University of Medical Sciences (TUMS), Grant no. 31386. It has also been part of a Ph.D. thesis supported by Tehran University of Medical Sciences; Grant no. 31386.

**Conflict of interests**

The authors declare that there is no conflict of interest.

**References**

1. Bosch FX, Lorincz A, Munoz N, et al (2002). The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol*, 55(4):244-65.
2. Swick AD, Chatterjee A, De Costa A-MA, et al (2015). Modulation of therapeutic sensitivity by human papillomavirus. *Radiother Oncol*, 116(3):342-5.
3. Suzich JA, Ghim SJ, Palmer-Hill FJ, et al (1995). Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proc Natl Acad Sci U S A*, 92(25):11553-7.
4. Lyon (2007). Human papillomaviruses. IARC monographs on the evaluation of carcinogenic risks to humans. 90:1-636.
5. Walboomers JM, Jacobs MV, Manos MM, et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol, 189(1):12-9.

6. van Bogaert LJ (2012). P16INK4a immunocytochemistry/immunohistochemistry: need for scoring uniformization to be clinically useful in gynecological pathology. Ann Diag Pathol, 16(5):422-6.

7. McLaughlin-Drubin ME, Crum CP, Munger K (2011). Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. Proc Natl Acad Sci USA, 108(5):2130-5.

8. Bibbo M, DeCecco J, Kovatch AJ (2003). P16INK4A as an adjunct test in liquid-based cytology. Am J Clin Pathol, 120(3):358-64.

9. Cuschiiri K, Wentzensen N (2008). Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev, 17(10):2536-45.

10. Tsoumpou I, Arbyn M, Kyrgiou M, et al (2009). p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer Treat Rev, 35(3):210-20.

11. Romagosa C, Simonetti S, Lopez-Vicente I, et al (2011). p16(INK4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene, 30(18):2087-97.

12. Crum CP (1998). Detecting every genital papilloma virus infection: what does it mean? Am J Pathol, 153(6):1667-71.

13. Doutre S, Omar T, Goumbri-Lombo O, et al (2018). Cervical intraepithelial neoplasia (CIN) in African women living with HIV: role and effect of rigorous histopathological review by a panel of pathologists in the HARP study endpoint determination. J Clin Pathol, 71(1):40-45.

14. Nicot AF, Nuovo GJ, Salomao-Estevez A, et al (2008). Immune factors involved in the cervical immune response in the HIV/HPV co-infection. J Clin Pathol, 61(1):84-8.

15. Shi SR, Key ME, Kalra KL (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem, 39(6):741-8.

16. Svec A, Mikyskova I, Hes O, et al (2003). Human papillomavirus infection of the epididymis and ductus deferens: an evaluation by nested polymerase chain reaction. Arch Pathol Lab Med, 127(1):1471-4.

17. Eng J (2005). Receiver operating characteristic analysis: a primer. Acad Radiol, 12(7):909-16.

18. Branca M, Ciotti M, Santini D, et al (2004). p16INK4A expression is related to grade of cin and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. Int J Gynecol Pathol, 23(4):354-65.

19. Tringler B, Gup CJ, Singh M, et al (2004). Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. Hum Pathol, 35(6):689-96.

20. Wang SS, Trunk M, Schiffman M, et al (2004). Validation of p16INK4a as a marker of oncogenic human papillomavirus infection in cervical biopsies from a population-based cohort in Costa Rica. Cancer Epidemiol Biomarkers Prev, 13(8):1555-60.

21. Queiroz C, Silva TC, Alves VA, et al (2006). P16(INK4a) expression as a potential prognostic marker in cervical pre-neoplastic and neoplastic lesions. Pathol Res Pract, 202(2):77-83.

22. Dai CY, Furth EE, Mick R, et al (2000). Overexpression of p16INK4a begins early in human colon neoplasia and correlates inversely with markers of cell proliferation. Gastroenterology, 119(4):929-42.

23. Mikle-Langosch K, Bamberger AM, Rieck G, et al (2001). Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. Breast Cancer Res Treat, 67(1):61-70.

24. Di Vinci A, Perdelli L, Banelli B, et al (2005). p16(INK4a) promoter methylation and protein expression in breast fibroadenoma and carcinoma. Int J Cancer, 114(3):414-21.

25. Zhao P, Mao X, Talbot IC (2006). Aberrant cytological localization of p16 and CDK4 in colorectal epithelia in the normal adenoma carcinoma sequence. World J Gastroenterol, 12(39):6391-6.

26. Hilliard NJ, Krail D, Sellheyer K (2009). p16 expression differentiates between
27. Jung A, Schrauder M, Oswald U, et al (2001). The invasion front of human colorectal adenocarcinomas shows co-localization of nuclear beta-catenin, cyclin D1, and p16INK4A and is a region of low proliferation. *Am J Pathol*, 159(5):1613-7.

28. Natarajan E, Saeb M, Crum CP, et al (2003). Co-expression of p16(INK4A) and laminin 5 gamma2 by microinvasive and superficial squamous cell carcinomas in vivo and by migrating wound and senescent keratinocytes in culture. *Am J Pathol*, 163(2):477-91.

29. Svensson S, Nilsson K, Ringberg A, et al (2003). Invade or proliferate? Two contrasting events in malignant behavior governed by p16(INK4a) and an intact Rb pathway illustrated by a model system of basal cell carcinoma. *Cancer Res*, 63(8):1737-42.

30. Horree N, van Diest PJ, Sie-Go DM, et al (2007). The invasive front in endometrial carcinoma: higher proliferation and associated derailment of cell cycle regulators. *Hum Pathol*, 38(8):1232-8.

31. Palmqvist R, Rutegard JN, Bozoky B, et al (2000). Human colorectal cancers with an intact p16/cyclin D1/pRb pathway have up-regulated p16 expression and decreased proliferation in small invasive tumor clusters. *Am J Pathol*, 157(6):1947-53.

32. Fahraeus R, Lane DP (1999). The p16(INK4a) tumour suppressor protein inhibits alphavbeta3 integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of alphavbeta3 to focal contacts. *EMBO J*, 18(8):2106-18.

33. Chintala SK, Fueyo J, Gomez-Manzano C, et al (1997). Adenovirus-mediated p16/CDKN2 gene transfer suppresses glioma invasion in vitro. *Oncogene*, 15(17):2049-57.

34. Ansari-Lari MA, Staebler A, Zaino RJ, et al (2004). Distinction of endocervical and endometrial adenocarcinomas: immunohistochemical p16 expression correlated with human papillomavirus (HPV) DNA detection. *Am J Surg Pathol*, 28(2):160-7.

35. Horn LC, Richter CE, Einenkel J, et al (2006). p16, p14, p53, cyclin D1, and steroid hormone receptor expression and human papillomaviruses analysis in primary squamous cell carcinoma of the endometrium. *Am J Diag Pathol*, 10(4):193-6.

36. McCluggage WG, Jenkins D (2003). p16 immunoreactivity may assist in the distinction between endometrial and endocervical adenocarcinoma. *Int J Gynecol Pathol*, 22(3):231-5.

37. Melgoza F, Brewster WR, Wilczynski S, et al (2006). p16-Positive small cell neuroendocrine carcinoma of the endometrium. *Int J Gynecol Pathol*, 25(3):252-6.

38. Cioffi-Lavina M, Chapman-Fredricks J, Gomez-Fernandez C, et al (2010). P16 expression in squamous cell carcinomas of cervix and bladder. *Appl Immunohistochem Mol Morphol*, 18(4):344-7.

39. Galgano MT, Castle PE, Atkins KA, et al (2010). Using biomarkers as objective standards in the diagnosis of cervical biopsies. *Am J Surg Pathol*, 34(8):1077-87.

40. Guedes AC, Brenna SM, Coelho SA, et al (2007). p16(INK4a) Expression does not predict the outcome of cervical intraepithelial neoplasia grade 2. *Int J Gynecol Cancer*, 17(5):1099-103.

41. Khleif SN, DeGregori J, Yee CL, et al (1996). Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. *Proc Natl Acad Sci U S A*, 93(9):4350-4.

42. Carozzi F, Confortini M, Dalla Palma P, et al (2008). Use of p16-INK4A overexpression to increase the specificity of human papillomavirus testing: a nested sub-study of the NTCC randomised controlled trial. *Lancet Oncol*, 9(10):937-45.

43. Carozzi F, Gillio-Tos A, Confortini M, et al (2013). Risk of high-grade cervical intraepithelial neoplasia during follow-up in HPV-positive women according to baseline p16-INK4A results: a prospective analysis of a nested sub-study of the NTCC randomised controlled trial. *Lancet Oncol*, 14(2):168-76.

44. Denton KJ, Bergeron C, Klement P, et al (2010). The sensitivity and specificity of p16(INK4a) cytology vs HPV testing for detecting high-grade cervical disease in the triage of ASC-US and LSIL pap cytology results. *Am J Clin Pathol*, 134(1):12-21.

45. Guo M, Hu L, Baliga M, He Z, et al (2004). The predictive value of p16(INK4a) and hybrid capture 2 human papillomavirus testing for
high-grade cervical intraepithelial neoplasia. *Am J Clin Pathol*, 122(6):894-901.

46. Nieh S, Chen SF, Chu TY, et al (2005). Is p16(INK4A) expression more useful than human papillomavirus test to determine the outcome of atypical squamous cells of undetermined significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies. *Gynecol Oncol*, 97(1):35-40.

47. Yoshida T, Fukuda T, Sano T, et al (2004). Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: a comparative study using simultaneously sampled histology materials. *Cancer*, 102(2):100-8.

48. Bosch FX, de Sanjose S (2003). Human papillomavirus and cervical cancer--burden and assessment of causality. *J Natl Cancer Inst Monogr*, (31):3-13.

49. Klaes R, Benner A, Friedrich T, et al (2002). p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol*, 26(11):1389-99.

50. Klaes R, Friedrich T, Spitkovsky D, et al (2001). Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic cervical cells of the cervix uteri. *Int J Cancer*, 92(2):276-84.

51. Negri G, Egarter-Vigl E, Kasal A, et al (2003). p16INK4a is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors: an immunohistochemical study with immunocytochemical correlations. *Am J Surg Pathol*, 27(2):187-93.

52. Sano T, Oyama T, Kashiwabara K, et al (1998). Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *Am J Pathol*, 153(6):1741-8.