

RESEARCH COMMUNICATION

Immunohistochemical Overexpression of p16 Protein Associated with Cervical Cancer in Thailand

Adisorn Jedpiyawongse, Patcharin Homcha-em, Anant Karalak, Petcharin Srivatanakul

Abstract

Cervical cancer is caused by persistent infections through “high risk (HR) types” of human HPVs, particularly HPV 16 and 18. HR-HPV types encode two potent oncogenes, referred to as E6 and E7. Both are required to induce and maintain neoplastic growth of cervical cancer cells. Cyclin dependent kinase inhibitor genes as for example p16INK4A were shown to be negative regulated by active pRb. Inactivation of pRb by E7 thus releases the p16 gene from its negative transcriptional control and results in significant overexpression of p16 encoded protein in HPV transformed cells. It has been demonstrated that p16 protein can be detected in cervical preneoplasia all high grade SIL or invasive cancers, whereas no expression was detected in normal, metaplastic or inflammatory cervical lesions. Moreover, low grade cervical lesions induced by low risk HPV infection but histological indistinguishable from low grade lesions induced by HR-HPV-infections could be clearly differentiated by p16INK4A immunohistochemistry, showing negative staining for p16 protein. The objective of this study is to examine the expression of p16 protein in cervical carcinoma in Thailand. Immunohistochemical analysis of p16INK4A was performed on 53 formalin – fixed and paraffin – embedded samples of various stages of cervical neoplastic lesions. There are squamous cell carcinoma *in situ* 8 cases, squamous cell carcinoma *in situ* with glandular involvement 16 cases, microinvasive squamous cell carcinoma 13 cases and invasive squamous cell carcinoma 16 cases. The specimens were taken from cervical biopsy, cervical conization and hysterectomy in the year 2000 at National Cancer Institute. Strong immunoreactivity for the p16 protein was observed in only the nuclei and cytoplasm of all cervical neoplastic cells. This study supported the idea that immunohistochemical overexpression of the p16 protein may be a useful screening test for cervical cancer. In addition, p16 immunohistochemistry is useful for helping in the interpretation of cervical histology samples, facilitating more rapid diagnosis.

Key Words: Cervical cancer Thailand - p16 protein - immunohistochemistry

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Introduction

The main cause of cervical cancer is a sexually transmitted infection by human papillomaviruses (HPVs) (Munoz and Bosch, 1992; Munoz et al., 1992; IARC, 1995; Walboomer et al., 1999). HPV infection has been detected in almost all preneoplastic and neoplastic lesions of the cervix (Zur Hausen, 1996). HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins, namely E6 and E7. Oncogenes E6 and E7 of HPV have been suggested to play an important role in the differences in oncogenic potential of each HPV subtype in cervical carcinogenesis (Hawley-Nelson et al., 1989; Munger et al., 1989). The oncoproteins encoded by the E6 and E7 genes have the ability to bind host cell regulatory proteins, especially tumor suppressor gene products p53 and hypophosphorylated retinoblastoma (Rb) protein (pRb). These changes may

lead to degradation of p53 by the E6 oncoprotein and to functional inactivation of Rb protein through binding to the E7 gene product (Werness et al., 1990; Dyson et al., 1989). As a result of loss of tumor suppressor function, a decrease in the p21 protein level and liberation of the transcriptional factor E2F-1 from the E2F-Rb complex may occur, allowing activation of cyclin-dependent kinase and transcriptional activation of target promoters respectively (Bagchi et al., 1990; Lechner et al., 1992; Phelps et al., 1988; Amortegui et al., 1995).

The CDKN2A gene product, p16 protein (p16), is a tumor suppressor protein that inhibits cyclin-dependent kinases (CDK)-4 and -6, which regulate the G1 checkpoint (Serrano et al., 1993; Koh et al., 1995). The CDKs phosphorylate the Rb protein, which results in a conformational change and release of E2F from the Rb protein. Thus, inactivation of either p16 or Rb function allows the cell to enter the S phase after only a pause at

National Cancer Institute, Department of Medical Services, Bangkok, Thailand *For correspondence: adisorn_j2007@hotmail.com

the G1 checkpoint. Rb inactivation was usually reciprocal with p16 expression (Shapiro et al., 1995; Sakaguchi et al., 1996; Kratzke et al., 1996). In several studies of cancer, p16 protein is detectable when the Rb gene is mutated, deleted or inactivated and, conversely, p16 protein is markedly reduced or absent in cell lines and clinical specimens that contain the intact Rb gene. The two most common mechanisms of p16 inactivation are homozygous deletion or hypermethylation of the gene (Herman et al., 1995). In cervical cancer and cervical intraepithelial neoplasia (CIN) lesions, p16 alterations as described earlier may not play a primary role in the development of the tumor. However, it remains to be determined whether the status of p16 and Rb proteins in clinical cervical tumors with relation to HPV is an important event in cervical carcinogenesis.

Cervical cytology is the screening test that has been most widely used. However, sensitivity and specificity of cytology have not been consistently high in a range of settings, especially in those with limited resources (Jacobs et al., 1999). These limitations may reflect the subjectivity of cytological diagnosis. In addition, histological analysis of biopsy samples taken from women with abnormal smears can (as with cytology) be affected by interobserver discrepancies (McCluggage, et al., 1995). The use of specific markers of dysplasia of the cervical epithelium in conjunction with current cytological or histological procedures could greatly improve the accuracy, precision, and sensitivity.

A wide array of immunohistochemical markers have been tested to evaluate their specificity in staining dysplastic cells in either biopsies or cytological smears (Keating et al., 2001). In the previous reports informed p16INK4A has been proposed as a biomarker helpful for the identification of dysplastic cervical cells. Benign cases were negative for p16INK4A, while the high grade lesions showed positive staining. There was a correlation between CIN grade and p16INK4A expression levels with more advanced lesions showing stronger reactivity. Thus p16INK4A expression analysis informed which was consistent with results from the histopathology and was a simple way of emphasizing the presence of premalignant cell reactive atypias. The usefulness of p16INK4A staining can be applied to cytological samples, and might be a complementary prognostic procedure in order to find women at risk for cervical cancer (Norman et al., 2007) and useful for cervical screening (Ekalaksananan et al., 2006). CIN1 cases with diffuse p16INK4A staining had a significantly higher tendency to progress to high-grade lesion than p16INK4A –negative cases. p16INK4A might have the potential to support the interpretation of low-grade lesion of the cervix uteri (Negri et al., 2004). The p16INK4A immunocytochemistry significantly improves specificity and positive predictive value of colposcopy in management of atypical squamous cells undetermined significant cytology (Monsonogo et al., 2007; Nieh et al., 2003). In other reports demonstrated the potential use of p16INK4A as a diagnostic marker for cervical intraepithelial neoplasia, glandular neoplastic, adenocarcinoma and squamous cervical cancer (Murphy et al., 2003; Negri et al., 2003; Mitsuya et al., 2003; Van

de Putte et al., 2003; Sano et al., 1998; Sano et al., 1998). Thus, p16 immunostaining is a useful marker of cervical dysplasia and may help in the interpretation of cervical cytology and histology sample and facilitated more rapid diagnosis and possibly even automated screening of cytologic slides (Trunk et al., 2004; Wentzensen et al., 2005; Klaes et al., 2001; Klaes et al., 2001; Klaes et al., 2002; Horn et al., 2008).

In the present study, we demonstrated the presence of p16INK4A in all stages of the carcinoma of uterine cervix, i.e., squamous cell carcinoma in situ, squamous cell carcinoma in situ with glandular involvement, microinvasive squamous cell carcinoma, invasive squamous cell carcinoma in Thai women by immunohistochemical analysis using an anti-p16INK4A mouse monoclonal antibody.

Materials and Methods

Tissue specimens

A total of 53 cervical biopsy samples were studied from Pathological Division of National Cancer Institute, Bangkok, Thailand. The specimens were taken from cervical biopsy, cervical conization and hysterectomy. All samples were fixed in formalin and embedded in paraffin wax by conventional techniques. H&E stained slides of all samples were reviewed and classified by a certified pathologist. The samples were 8 squamous cell carcinoma in situ, 16 squamous cell carcinoma in situ with glandular involvement, 13 microinvasive squamous cell carcinoma, invasive squamous cell carcinoma (14 squamous cell carcinoma large cell non keratinizing type, 1 squamous cell carcinoma small cell type, 1 squamous cell carcinoma large cell keratinizing type)

Hematoxylin and eosin (H&E) staining procedure

Paraffin embedded sections were cut in 3 micrometers. The slides were deparaffinized and hydrated to running water 5 minutes, before staining. Sections were stained in Mayer's hematoxylin solution for 15 – 30 minutes then washed in running tap water for 15 – 30 minutes and counterstained in eosin – phloxine solution for 2 minutes. The sections were dehydrated and cleared through 2 changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene, 2 minutes each. The sections were mounted with toluene solution.

Immunohistochemistry for p16

Sections were cut from formalin fixed, paraffin wax embedded biopsy samples and mounted on glass slides. Sections were dewaxed by passage through xylene and then rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubating in 0.5% H₂O₂ / methanol 30 minutes, rinsed in running water 1 minute and dipped in distilled water 2-3 dips. Antigen retrieval was performed by boiling in 10mM citrate buffer pH6.0, 4 minutes 95-100 °C in micro-wave, and then left to cool for 25 minutes, rinsed in running water 1 minute and dipped in distilled water 2-3 dips, washed in phosphate buffer saline (PBS) pH7.2, washed in PBS. Non-specific antibody binding was reduced by incubating in 3% non-

immune horse serum (NHS) for 20 minutes, washed in phosphate buffer saline pH7.2. The sections were incubated overnight in p16INK4A specific monoclonal MTM-E6H4 antibody (MTM LABORATORIES AG, Heidelberg, Germany) at a 1/200 dilution with 3% NHS in PBS at 40°C. After washing thoroughly with PBS, the slides were incubated in secondary antibody Rabbit anti-mouse IgG/Biotin (Dako A/S, Glostrup, Denmark) dilution 1/500 with 3% NHS for 30 minutes, washed in PBS, then incubated in third antibody, Peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) dilution 1/1000 with 3% NHS for 60 minutes, after that washed in PBS. The sections were developed with 0.01% 3,3'-diaminobenzaminidine tetrahydrochloride containing 0.003% H₂O₂ in 10 mmol/L imidazole/PBS for 20 minutes, washed in PBS, washed in deionized water and counterstained lightly with hematoxylin. The sections were dehydrated and cleared through 2 changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene, 2 minutes each. The sections were mounted with toluene solution. All slides were qualitatively graded intensive staining in nucleus, cytoplasm, nuclear-cytoplasm ratio in term of score and percentage base on intensity grading of ER, PGR of DAKO protocol (ER/PR pharmDxTM/ Interpretation Manual Dako).

Results

From H&E staining showed 8 cases of squamous cell carcinoma in situ (Figure 1a), 16 cases of squamous cell carcinoma in situ with glandular involvement (Figure 2a), 13 cases of microinvasive squamous cell carcinoma (Figure 3a), 14 squamous cell carcinoma large cell non keratinizing type (Figure 4a), 1 squamous cell carcinoma small cell type (Figure 5a), 1 squamous cell carcinoma large cell keratinizing type (Figure 6a).

P16INK4A immunostaining showed normal cells, inflammatory cells, connective tissue, superficial, intermediate and parabasal cells, from normal ectocervical stratified squamous epithelium in the normal area near the lesions exhibited negative staining with the p16INK4A that presented resemble to H&E staining results. The cervical neoplasia, 8 cases of squamous cell carcinoma in situ (Figure 1b), 16 cases of squamous cell carcinoma in situ with glandular involvement (Figure 2b), 13 cases of microinvasive squamous cell carcinoma (Figure 3b) showed clear and distinct positive staining for p16 throughout the nuclei and cytoplasm of the tumor cells when compared to the surrounding normal, hyperplastic, metaplastic epithelial and mesenchymal cells corresponded to the histological slides. Although weak immunoreactivity for p16 was seen in the nuclei and cytoplasm of proliferating fibroblasts, endothelial cells and some inflammatory cells adjacent to the cancer tissue. But the weak staining was judged negative for p16 immunoreactivity. The neoplastic cells from invasive cancer, 14 squamous cell carcinoma large cell non keratinizing type (Figure 4b), 1 squamous cell carcinoma small cell type (Figure 5b), 1 squamous cell carcinoma large cell keratinizing type (Figure 6b) also showed intense and diffuse p16 reactivity in both nuclei and cytoplasm.

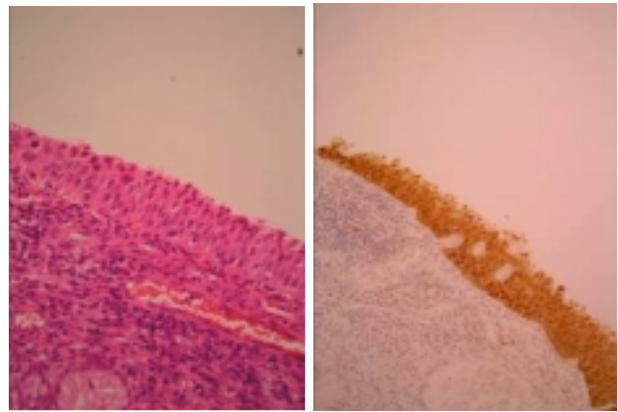


Figure 1. a) H&E staining and b) Immunohistochemistry for p16 are in the Same Lesion

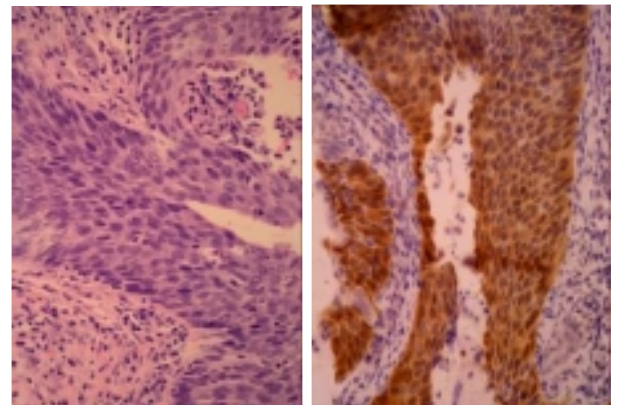


Figure 2. a) Squamous Cell Carcinoma in situ with Glandular Involvement and b) Intense p16 reactivity in both Nuclei and Cytoplasm

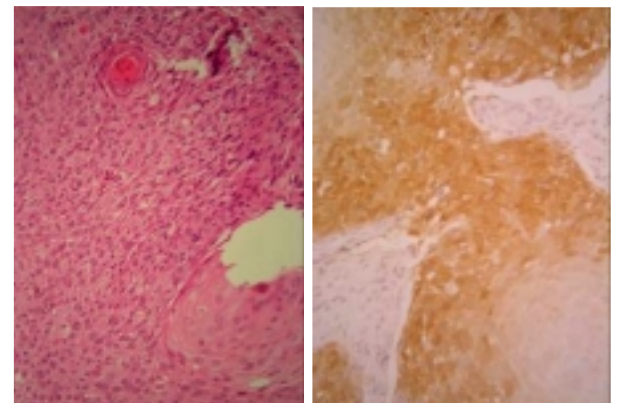


Figure 3. a) Squamous Cell Carcinoma, Keratinizing Type and b) Strong and Diffuse Immunostaining for p16 in Cervical Cancer, but not Keratinized Pearls

All patterns of staining showed the strong nuclear and cytoplasmic staining. (All slides were qualitatively graded intensive staining in nucleus, cytoplasm, nuclear - cytoplasm ratio in term of score and percentage).

Discussion

Immunohistochemical staining of p16INK4A antibody in normal tissues were negative. All patterns of cervical neoplasia staining showed positive immunostaining with p16INK4A monoclonal antibody like in the previous reports which were observed

p16INK4A protein localization is thought to be nuclear. The presence of p16INK4A in the cytoplasm may result from a type of post transcriptional modification or more simply, over production of protein may force its transfer into the cytoplasm. (Sano et al. 1998; Sano et al., 1998; Tam et al., 1994). Strong and diffuse immunoreactivity for p16 in both the nuclei and cytoplasm of all HSILs and invasive cancer lesions as well as those of many LSILs in marked contrast with non-neoplastic tissues. (Sano et al., 1998; Sano et al., 1998; Horn et al., 2008; Murphy et al., 2003)

The Papanicolaou (Pap) testing allows the identification of asymptomatic woman who have preneoplastic lesion or early cancer of the uterine cervix. But Pap smear test is limited with respect to sensitivity and specificity. False negative rates for cervical premalignant lesions and cervical cancer lie between 15% and 50% and false positive rates of approximately 30% have been reported (Jacobs et al. 1999). In addition, in the other study reported that p16INK4A marks dysplastic squamous and glandular cells of the cervix with a sensitivity of 99.9% and a specificity of 100% (Murphy et al. 2003) so that p16INK4A is a reliable marker for dysplastic squamous and glandular cervical cells in tissue sections and in cervical Thin-Preps. The use of p16INK4A immunohistochemical analysis as a complement to conventional screening programmes and would help reduce false positive and false negative results which could reduce patient anxiety and the overall cost of cervical cancer screening programmes (Murphy et al. 2003).

Another report revealed the overexpression of p16INK4A has been proposed as a biomarker helpful for the identification of dysplastic cervical epithelial cells on histological slides as well as cervical smears. The application of conventional cytomorphologic criteria to identify dysplastic cells turned out to be helpful since evaluation of the nuclear structure is easy in p16INK4A immunostained and haematoxylin-counterstained slides. And the other report suggested immunohistochemical study was the possible usefulness of p16 protein as a marker to differentiate high and intermediate-risk-HPV-related neoplastic lesions from low-risk-HPV-related lesion in the cervix. It was particularly noteworthy that p16 staining appears to be most useful in LSIL cases, which may contain low-or high-risk HPV, because the lesions would progress to HSIL or cancer if they were caused by high-risk HPV (Lorincz et al. 1992; Matsukura et al. 1995; Yoshikawa et al. 1991). The diffuse and strong p16 immunoreactivity was so highly specific and sensitive among the high-and intermediate-risk-HPV-related lesions that its overexpression in cervical precursor lesions, especially in LSIL, may provide valuable information regarding the HPV subtype infecting the lesions without the need for molecular techniques such as polymerase chain reaction (PCR), southern blotting or in situ hybridization (Sano et al., 1998). The other study suggested that the combination of p16 and HPV detection may be useful in cervical cancer screening to identify cervical cells with minor abnormalities and a high risk of progressing to cervical neoplasia, and so defining cases requiring early management, or at least close surveillance.

Further follow up studies of such cases are required (Ekalaksananan et al. 2006). Nevertheless, histological analysis of biopsy sample taken from women with abnormal smears can be affected by interobserver discrepancies. The need for the identification of specific biomarkers for abnormal tissue of cervical epithelium in conjunction with histological procedures could greatly improve the accuracy, precision and sensitively (Klaes et al.,2002).

P16INK4A immunohistochemistry may help to conventional H&E-stained specimen to avoid ambiguities in the interpretation and more reproducible diagnosis of cervical intraepithelial neoplasia and facilitate more rapid diagnosis and may be a valuable aid for the interpretation of cervical histology of cervical cancer identification. Our study showed that the lesions of cervical cancer of Thai women exhibited positive staining of p16INK4A. There is possible the usefulness of p16 protein as a marker to differentiate normal and cervical lesions in Thai women. It was particularly noteworthy that p16 staining appears to be most useful for aid in the interpretation of cervical histology.

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