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ORIGINAL PAPER

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The hypermethylation and protein expression of *p16^{INK4A}* and DNA repair gene *O^6-methylguanine-DNA methyltransferase* in various uterine cervical lesions

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Abstract *Purpose*: This study is aimed at investigating the significance of gene promoter methylation status and protein expression of p16^{INK4A} and O^6-methylguanine-DNA methyltransferase (MGMT) in the various uterine cervical lesions. *Materials and methods*: Methylation status by using methylation-specific polymerase chain reaction (MS-PCR) and protein expression by using immunohistochemistry for *p16^{INK4A}* and *MGMT* genes were performed in cervical squamous intraepithelial neoplasms (CIN), invasive squamous cell carcinomas (SCC), adenocarcinomas and non-neoplastic cervices. *Results*: None of 20 non-neoplastic cervices showed *p16^{INK4A}* and *MGMT* gene hypermethylation, whereas at least one of these genes was hypermethylated with 50.0% (5/10) of CIN I, 65.0% (13/20) of CIN II–III, 70.2% (33/47) of SCC and 85.0% (17/20) of adenocarcinoma. p16^{INK4A} protein was totally negative in non-neoplastic cervices, but positive with 90.0% of CIN I, 100% of CIN II–III and adenocarcinoma, and 78.7% of SCC. MGMT protein was expressed in 10% of non-neoplastic cervices, but significantly increased in SCC (42.5%) and adenocarcinoma (70.0%). The protein expression of p16^{INK4A} and MGMT was not related to their gene promoter methylation status. *Conclusions*: The hypermethylation of *p16^{INK4A}* and *MGMT* genes in the uterine cervix may indicate the presence of malignant cells, and p16^{INK4A} immunostaining is useful in grading CIN and diagnosing invasive SCC and adenocarcinoma.

Keywords p16^{INK4A} · O^6-methylguanine-DNA methyltransferase · Methylation · Cervical uteri

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Introduction

DNA methylation is an essential mechanism for normal development, X chromosome inactivation, and gene imprinting [8, 44, 46]. However, the aberrant methylation of normally unmethylated CpG islands within the promoter regions of tumor-suppressor or cancer-related genes is a common epigenetic event leading to the silencing of gene expression in human cancers [1, 4, 9, 16, 18, 19, 25, 28, 32, 37].

Uterine cervical cancers are a worldwide common disease, and human papilloma virus (HPV) is a well-known etiologic factor [7, 15]. Cervical cancer development is the result of complex interactions between the host and environmental factors, undergoing stepwise progression from low-grade to high-grade dysplasia to invasive carcinoma. In this pathway, activation or inactivation of various cancer-related genes is involved [3, 17, 31, 39]. Recent studies have demonstrated the promoter hypermethylation of several genes, such as *DAPK, p16^{INK4A}, O6-methylguanine-DNA methyltransferase (MGMT), APC, HIC-1, E-cadherin, RARß, FHIT, GSTP* and *hMLH1*, associated with cervical carcinogenesis [13, 24, 43, 45, 48]. Among these, the

p16^{INK4A} gene controls the cell cycle by inhibiting cyclin-dependent kinase 4 (cdk4)-mediated phosphorylation of several growth regulatory proteins such as retinoblastoma (Rb) [29, 40]. The promoter hypermethylation of *p16*^{INK4A} might result in transcriptional silencing, with a loss of functional activity [6, 28, 33, 47]. However, recent studies reported that p16 protein was strongly expressed in dysplastic and malignant cells of squamous and glandular epithelium of the uterine cervix [33, 38, 42], speculating that high-risk HPV E7 binds Rb protein, and that p16^{INK4A} is overexpressed through a negative feedback [21]. However, the expression of p16^{INK4A} in HPV-negative cancers should be explained by a HPV-independent pathway.

MGMT is a DNA repair protein, which transfers and accepts alkyl groups from the O^6 position of guanine; it is important in protecting the cells from DNA damage [35]. A loss of function is implicated in a variety of human cancers [5, 14]. However, the presence of MGMT can be responsible for chemoresistance and poor prognosis of cancer patients [2, 20, 22, 23, 26]. Several reports have demonstrated the relatively high frequency of hypermethylation in high-grade dysplasia and invasive carcinoma of the cervix, speculating that it plays an important role in cervical carcinogenesis [12, 24, 43, 48].

In this study, the hypermethylation of *p16*^{INK4A} and *MGMT* genes was studied by methylation-specific PCR (MS-PCR). The expression of *p16*^{INK4A} and MGMT protein was immunohistochemically investigated to define the significance of the promoter hypermethylation and protein expression of these genes in cervical carcinogenesis.

Materials and methods

Materials

One hundred seventeen paraffin-embedded uterine cervical tissues, including 47 invasive squamous-cell carcinomas (nine stage IA, 19 stage IB, 11 stage IIA and eight stage IIB), 20 adenocarcinomas (12 stage IB and eight stage IIA), 30 cervical squamous intraepithelial neoplasia (ten CIN I, three CIN II and 17 CIN III), and 20 non-neoplastic cervices were selected from the Department of Pathology, Anam Hospital of Korea University. The tissues were routinely processed with 10% buffered formalin fixation and paraffin embedding. The hematoxylin-eosin (H-E) stained slides were reviewed, and one appropriate block from each case was selected for this study.

Methods

DNA extraction and methylation-specific PCR

Tissue sections from 117 paraffin-embedded blocks were deparaffinized in xylene before dehydration with 100% ethanol. Then, DNA was extracted by using the 'High Pure PCR Template Preparation Kit' (Roche, Germany). For the relatively specific cancer and CIN specimen selection, H-E slides were reviewed and selected the area of numerous cancer/CIN tumor cells and minor stroma as the target of DNA extraction. The Kai Sterile Dermal Biopsy Punch (Kai Industries, Japan) was used in some difficult cases. One μg of genomic DNA was denatured with 5 M NaOH, and bisulfite treatment was carried out for 16 h at 55°C. DNA samples were then purified using the Wizard DNA Clean-Up System (Promega, Madison, WI), treated again with NaOH, ethanol-precipitated and re-suspended in water, as described by Herman et al. [10].

The modified DNA was used as a template for methylation-specific PCR (MS-PCR). The primer sequences of *p16*^{INK4A} for the unmethylated reaction were sense 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and antisense 5′-CAACCCCAAACCACAACCATAA-3′, which amplify a 151-bp product (annealing temperature: 65°C). The primer sequences of *p16* ^{INK4A} for methylated reaction were sense 5′-TTATTAGAGGGTGGGGC-GGATCGC-3′ and antisense 5′-GACCCCGAACCGC-GACCGTAA-3′, which amplify a 150-bp product (annealing temperature: 60°C). The primer sequences of *MGMT* for the unmethylated reaction were sense 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ and antisense 5′-AACTCCACACTCTTCCAAAAACAAA-ACA-3′, which amplify a 93-bp product (annealing temperature: 66°C). The primer sequences of *MGMT* for methylated reaction were sense 5′-TTTCGACGTTCG-TAGGTTTTCGC-3′ and antisense 5′-GCACTCTTCC-GAAAACGAAACG-3′, which amplify an 81-bp product (annealing temperature: 66°C). [19].

PCR reactions were performed in a 25 μl reaction volume and hot-started at 95°C for 5 min. This was followed by 40 cycles of 95°C for 45 s, appropriate annealing temperature for 45 s and 72°C for 45 s, and concluded at 72°C for 5 min. PCR products were analyzed on 12% polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

Immunohistochemistry for p16^{INK4A} *and MGMT*

For an immunohistochemical study with DAKO LSAB kit (DAKO A/S, Denmark), 4-μm thick tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was eliminated by incubation with 3% H_2O_2 in methanol for 15 min. The antigen was retrieved at 103 kPa for 2 min by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were incubated with the primary monoclonal antibodies for p16^{INK4A} (1:25, mtm Laboratories, Germany) and MGMT (1:50, NeoMarkers, Fremont, CA) for 1 h at room temperature. After incubation at room temperature for 30 min with biotinylated link, the sections were incubated with streptavidin-peroxidase complex at room temperature for 30 min. Immunostaining was visualized by using 3,3′diaminobenzidine. The sections were counterstained

with hematoxylin. As a negative control, 0.1 M Tris buffer (pH 7.6) replaced the primary antibody.

The immunohistochemical results were evaluated as (+) when more than 5% of the epithelial cells were stained.

Statistical analysis

Statistical analysis was performed by using the x^2-test of the SPSS software program. A p value of less than 0.05 was considered statistically significant.

Results

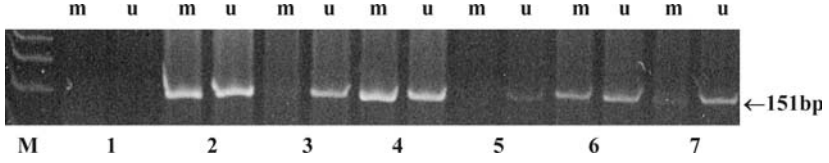
Hypermethylation of *p16 INK4A* and *MGMT* genes

All of the cases showed only unmethylated or methylated DNA band or both. Hypermethylation was detected in 20.0% (2/10) and 30.0% (3/10) of CIN I, 50.0% (10/20) and 35.0% (7/20) of CIN II–III, 53.2% (25/47) and 38.3% (18/47) of SCC, and 60.0% (12/20) and 45.0% (9/20) of adenocarcinoma, respectively. Significantly, at least one of *p16^{INK4A}* and *MGMT* hypermethylation was detected in 50% (5/10) of CIN I, 65.0% (13/20) of CIN II–III, 70.2% (33/47) of SCC, and 85.0% (17/20) of adenocarcinoma. In SCC, stage IIB lesions showed a higher rate of methylation, 87.5% for *p16^{INK4A}* and 50.0% for *MGMT*. The prevalence of methylation in one of the genes was progressively increased according to the stages, without statistical significance. All 20 cases of non-neoplastic cervices showed an unmethylation pattern for both *p16^{INK4A}* and *MGMT*. (Fig. 1 and 2)

Immunohistochemistry for p16^{INK4A} and MGMT protein expression

p16^{INK4A} was specifically stained in the cytoplasm and/or nucleus of the dysplastic and malignant cells, but not in the reactive and normal cervical epithelial and stromal cells. The staining for MGMT was mainly found in the suprabasal layer of non-neoplastic cervices and stromal lymphocytes. p16^{INK4A} protein was expressed in 78.7% (37/47) of SCC, 90.0% (9/10) of CIN I, 100% (20/20) of CIN II–III, and 100% (20/20) of adenocarcinoma, but not expressed in any non-neoplastic cervices. However, MGMT protein showed immunoreactivity in 10% (2/20) of non-neoplastic cervices, whereas its expression was seen in 42.5% (20/47) of SCC and 70.0% (14/20) of adenocarcinoma. The positive signals were stronger in SCC and adenocarcinoma than in the non-neoplastic cervices. There were no significant differences among the clinical stages. (Fig. 3 and 4)

Fig. 1 Methylation-specific polymerase chain reaction for *p16^{INK4A}* methylation. *Lane 1*: negative control; *lanes 2* and *3*: squamous cell carcinoma; *lanes 4–6*: CIN III; *lane 7*: non-neoplastic cervix. (*M* 50 bp size marker, *m* methylation, *u* unmethylation, *CIN* cervical squamous intraepithelial neoplasm)



Relationship between protein expression and hypermethylation of p16^{INK4A} and MGMT

The expression of p16^{INK4A} was higher in methylated groups (91.8%) than in unmethylated groups (60.3%) without statistical significance. The expression of MGMT was similar in methylated (48.6%) and unmethylated (38.8%) groups. The protein expressions of p16^{INK4A} and MGMT were not correlated with the hypermethylation of promoter genes.

Discussion

The hypermethylation of promoter regions is one of the major mechanisms by which cancer-related genes, such as *p14 ARF*, *p15 INK4b*, *p16 INK4a*, *VHL, Rb1, hMLH, HIC, MGMT, RAR-β2,* and *DAPK,* are inactivated [4, 13, 16, 28, 37, 45, 47]. This epigenetic alteration has been described in uterine cervical cancers. Dong et al. [13] investigated promoter hypermethylation of six genes, *p16^{INK4A}, APC, HIC-1, DAPK, MGMT* and *E-cadherin*, in squamous cell carcinoma and adenocarcinoma of the uterine cervix, and detected at least the aberrant methylation of one of these genes in 79% of the cases. Virman et al. [43] also analyzed aberrant methylation of *p16^{INK4A}, RARβ, FHIT, GSTP1, MGMT,* and *hMLH1* in normal and neoplastic cervical tissues and cell lines, reporting that the aberrant methylation occurred at an early stage during multistage pathogenesis and increased with a pathologic change. Cohen et al. [12] described the hypermethylation of *RASSF1A* promoter in uterine cervical adenocarcinoma, but not in squamous cell carcinoma. Recently, Yang et al. [48] detected hypermethylation in one of *DAPK, p16^{INK4A}*, and *MGMT* genes in 75.3% of tumors and 55% of plasma of cervical cancers.

Hypermethylation of *p16^{INK4A}* in uterine cervical cancers was detected in 28.2% in a study by Yang et al. [48], and 53% by Lea et al. [24]. Virmani et al. [43] reported a progressive increase of hypermethylation during cervical carcinogenesis; 3% of non-dysplasia/low-grade CIN, 24% of high-grade CIN and 42% of invasive cancers. Dong et al. [13] and Lea et al. [24]

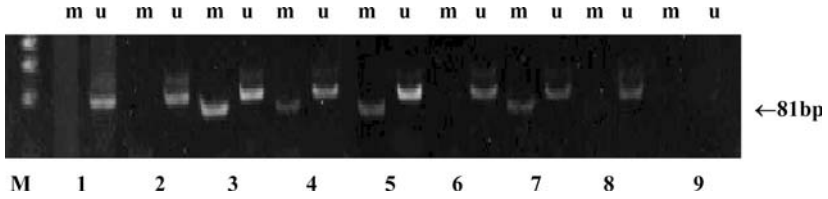


Fig. 2 Methylation-specific polymerase chain reaction for *MGMT* methylation. *Lanes* 1 and *2*: non-neoplastic cervices; *lanes 3* and *4*: squamous cell carcinoma; *lanes 5* and *6*: cervical adenocarcinoma; *lanes 7–9*: CIN III. (*M* 50 bp size marker, *m* methylation, *u* unmethylation, *CIN* cervical squamous intraepithelial neoplasm)

reported more frequent hypermethylation of $p16^{INK4A}$ in SCC than in adenocarcinoma (39% vs 18%, and 61% vs 37%), but this was not found in the study of Yang et al. [48]. In our study, the prevalence of $p16^{INK4A}$ hypermethylation was 53.2% of SCC and 60.0% of adenocarcinoma.

The reported frequencies of *MGMT* promoter hypermethylation in uterine cervical cancers were 10% of SCC and 5% of adenocarcinoma in the study by Dong et al. [13] and 3%, 29% and 26% of non-dysplasia/low-grade CIN, high-grade CIN and invasive cancers in the study by Virmani et al. [43]. Yang et al. [48] reported 18.8% of *MGMT* hypermethylation in cervical cancers (11% of SCC and 5% of adenocarcinoma). In our study, *MGMT* hypermethylation was seen in 30% of CIN I, 35.0% of CIN II–III, 38.3% of SCC and 45.0% of adenocarcinoma. However, all 20 cases of non-neoplastic cervices showed an unmethylation pattern. *MGMT* methylation indicates cellular changes of malignant possibility.

Hypermethylation of $p16^{INK4A}$ and *MGMT* was more frequent in our study than in the previous ones. It may be due to the scanty contamination of normal cells by selecting the tumor cells by dissection.

Hypermethylation in at least one of $p16^{INK4A}$ and *MGMT* genes showed statistically higher frequency in CIN, SCC and adenocarcinoma than in the non-neoplastic cervices; however, it did not show any correlation with histologic types and clinical stages in this study. The combined detection of $p16^{INK4A}$ and *MGMT* methylation status might be useful in detecting a possible presence of cervical dysplasia and malignancy.

The results of $p16^{INK4A}$ immunohistochemistry showed that all cases of non-neoplastic cervices were negative. The positive rate increased significantly in CIN I (90.0%), CIN II–III (100%), SCC (78.7%) and adenocarcinoma (100%). The positive signals were specifically located in the cytoplasm and nucleus of the dysplastic or malignant cells, but not in the reactive and normal cervical epithelium and stromal components. The data were consistent with other previous studies [21, 38, 42], strongly indicating that $p16^{INK4A}$ immunostaining is useful in grading CIN, and evaluating the glandular lesions. Depending on the antibodies used in immunohistochemistry, some of the normal or reactive squamous and

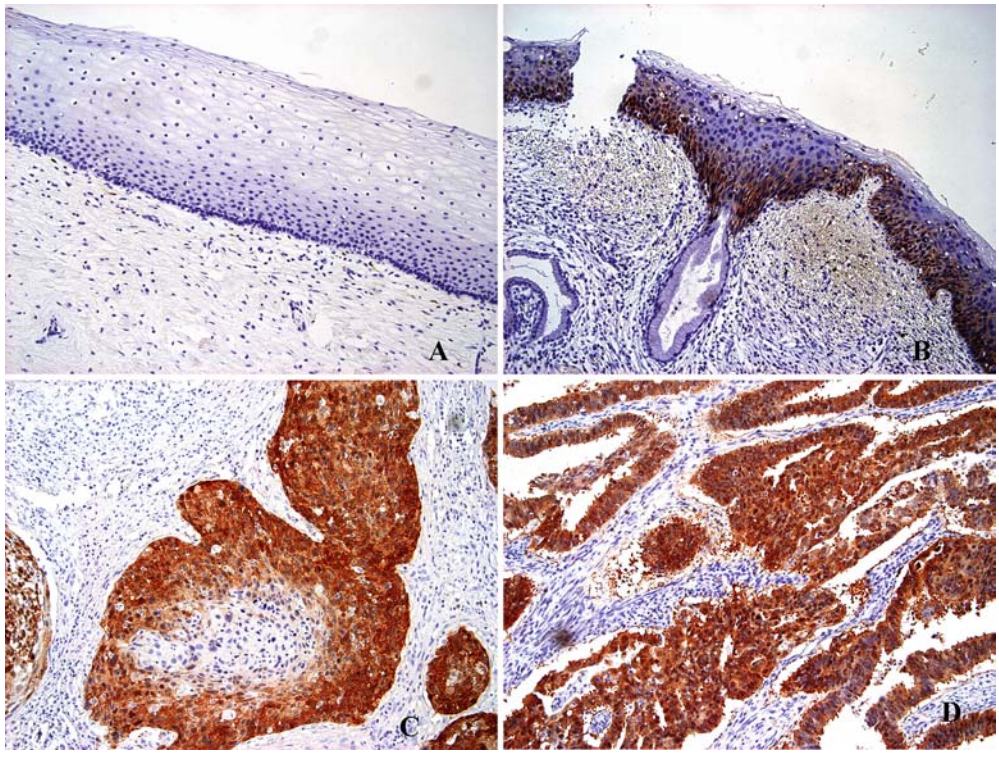


Fig. 3 Immunostaining for $p16^{INK4A}$ in the uterine cervical lesions. (LSAB kit, ×200). The staining for $p16^{INK4A}$ is negative in the normal cervix (**A**). The dysplastic squamous epithelium of CIN II is positive for $p16^{INK4A}$ (**B**). Squamous cell carcinoma (**C**) and adenocarcinoma (**D**) are strongly positive for $p16^{INK4A}$ (*CIN* cervical squamous intraepithelial neoplasm)

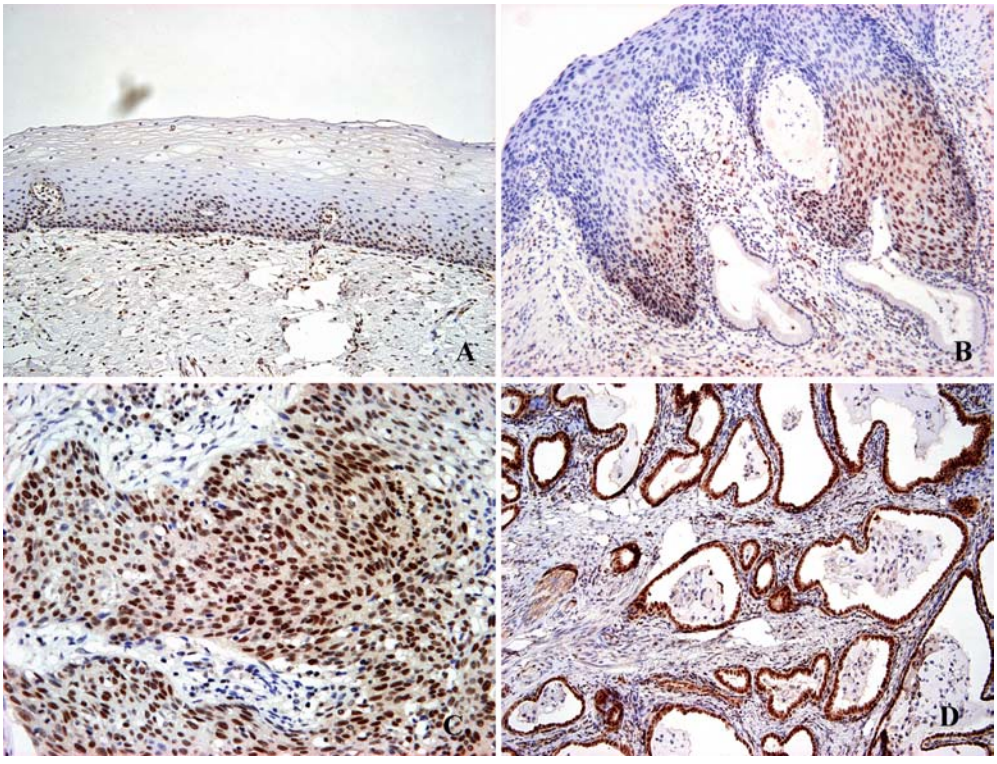


Fig. 4 Immunostaining for MGMT in uterine cervical lesions. (LSAB kit, ×200). The cells of basal and suprabasal layers of the squamous epithelium are positive for MGMT. The lymphocytes in stroma are also positive (**A**). The dysplastic epithelium in CIN II is strongly positive for MGMT, in contrast to the negative staining in the normal gland (**B**). Squamous cell carcinoma (**C**) and adenocarcinoma (**D**) are strongly positive for MGMT (*CIN* cervical squamous intraepithelial neoplasm)

glandular epithelial cells were positive for $p16^{INK4A}$ [30]. The antibody used in our study was consistently negative in non-neoplastic cells. The expression of p16 in high-grade CIN and invasive squamous cell carcinoma has been considered the result of high-risk HPV E7-mediated degradation of protein Rb (pRb). However, the fact that adenocarcinoma, less commonly associated with high-risk HPV than squamous lesions, showed a strong expression of p16 in almost all cases, suggesting that other mechanisms should be defined.

Nuovo et al. [34] nicely detected hypermethylation of the $p16^{INK4A}$ gene by methylation-specific PCR in situ hybridization (MSP-ISH) and demonstrated a loss of protein in hypermethylated cervical cancer cells. Considering the fact that the high proportion of the cervical squamous lesions is associated with HPV and shows positive staining for $p16^{INK4A}$, hypermethylation of $p16^{INK4A}$ does not seem related with the loss of protein. In our study, the expression of $p16^{INK4A}$ was strong and high in both squamous lesions and adenocarcinoma, without regard to gene hypermethylation status. According to Dong et al. [13], there was no correlation between the promoter hypermethylation of $p16^{INK4A}$ and the presence of HPV.

According to the cell type, the amount of MGMT protein differs and decreases in some tumors in comparison with their normal counterparts [10, 11]. Several studies have demonstrated that the inactivation of a repair gene by promoter hypermethylation resulted in the impaired expression of MGMT protein [2, 9, 14, 18, 22]. The loss of protein was uniform in lymphoma, but tumors of the colon, lung, and brain showed cellular heterogeneity or stronger expression than adjacent normal tissues [14, 23, 27, 36, 41, 49]. According to Kokkinakis et al. [23], MGMT protein was absent in the hyperplastic and normal components of pancreatic tissues, but invariably present in dysplastic foci and especially strong in the invasive component of pancreatic cancer. They suggested that MGMT activity is up-regulated in dysplastic epithelium and that its expression increases during a tumor progression. In our study, only two of 20 non-neoplastic cervices showed MGMT expression in the suprabasal layers of squamous epithelium, whereas dysplastic and malignant lesions showed a significantly increased expression (40% and 42.5%, respectively, of CIN II–III and SCC, and 70.0% of adenocarcinoma). However, the protein expression of MGMT was not correlated with promoter hypermethylation. This result was concordant with the previous reports [36]. Since MGMT is a DNA repair protein, the intracellular localization should be nuclear. However, our study showed that the immunohistochemical staining for MGMT was found both in the cytoplasm and nucleus. Belanich et al. [5] demonstrated the clear cytoplasmic and nuclear localization of MGMT in brain tumors, but only nuclear localization in the breast cancer. The significance of cytoplasmic localization of MGMT should be defined.

The protein expression and methylation status of both $p16^{INK4A}$ and *MGMT* genes were not different in the various stages of cervical cancers, suggesting that $p16^{INK4A}$ and *MGMT* gene alterations might play a role in early carcinogenesis. Furthermore, the lack of difference of $p16^{INK4A}$ and MGMT expression between methylated and unmethylated lesions raises the possibility of other

mechanisms participating in controlling p16 ^{INK4A} and MGMT protein expression.

In conclusion, *p16^{INK4A}* and *MGMT* hypermethylation may play an important role in the progression of uterine cervical cancer. Consequently, p16^{INK4A} immunostaining can be used as a diagnostic marker of intraepithelial and invasive neoplasm of squamous and glandular epithelium.

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