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Human Papillomavirus 16/18 Expression of Endocervical Glandular Lesions: Relationship with p53 and MIB-1 Expressions

The pathogenesis of endocervical glandular lesions are not clearly understood. The aims of this study are to evaluate the etiologic role of human papillomavirus (HPV) 16/18 and the relationship of HPV 16/18, p53 and MIB-1 expressions in endocervical glandular dysplasia (EGD), adenocarcinoma in situ (AIS) and adenocarcinoma. The materials included 14 endocervical adenocarcinoma and 5 AIS and 18 high grade EGD and 39 low grade EGD. Immunohistochemistry for p53 and MIB-1, and in situ PCR for HPV 16/18 were done. HPV 16/18 positivity was 84.2%, 16.7% and 17.9% in malignant glandular lesion (adenocarcinoma and AIS), high grade EGD and low grade EGD, respectively. P53 protein expression rates of malignant glandular lesions, high grade EGD and low grade EGD were 31.6%, 11.1%, and 0%, respectively. High MIB-1 labelling index was found in 73.7% of malignant glandular lesions, but in only 5.7% and 3.6% of high and low grade EGD, respectively. There were statistically significant differences in HPV 16/18, p53 and MIB-1 expressions between malignant endocervical glandular lesions and EGD, but no significant difference in p53 and MIB-1 expressions in relation to HPV 16/18 expression. In malignant endocervical glandular lesions, HPV 16/18 infection may be a major causative factor, but not be related to p53 and MIB-1 expressions.

Key Words: *Papillomavirus, Human; In Situ Hybridization; Genes, p53; Protein p53; MIB-1; Adenocarcinoma; Cervix Dysplasia*

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INTRODUCTION

Sequential change from precancerous to invasve lesion of squamous neoplasia of the uterine cervix is well known; however, the relationship of endocervical glandular dysplasia (EGD) to the genesis of adenocarcinoma in situ (AIS) and invasive adenocarcinoma remains unclear (1-7). High risk human papillomavirus (HPV) infection is the major causative factor of squamous neoplasia of the cervix, and EGD or AIS foci are occasionally associated with squamous neoplasia, suggesting the possibility of common etiologic factors (8-10).

p53 gene mutation is associated with the development of various human cancers, and MIB-1 is a reliable marker of proliferating activity. The objectives of our study are to evaluate the etiologic role of HPV 16/18 infection by in situ PCR, and to study the relationship of HPV 16/18 expression and p53 protein and MIB-1 expressions in endocervical adenocarcinoma, AIS and EGD.

MATERIALS AND METHODS

Materials

The materials were of two groups. The first group was 14 endocervical adenocarcinomas collected during a period from July 1995 to December 1997. The second group was endocervical glandular lesions recognized on reviewing cervical tissue sections of 195 squamous neoplasia of the uterine cervix, consisting of 63 squamous cell carcinoma in situ, 43 microinvasive squamous cell carcinoma and 89 invasive squamous cell carcinoma, and 120 leiomyoma or adenomyosis. The slides were reviewed by two pathologists (H.K.Y. and M.S.K.), and endocervical glandular lesions were classified into low grade EGD, high grade EGD and AIS according to the classification proposed by Brown et al. (1).

Brown et al. (1) classified endocervical glandular lesion into low grade cervical glandular atypia, high grade

Table 1. Distribution of endocervical glandular lesions

	No. of cases	Presence of endocervical glandular lesions (%)			
		LGD	HGD	AIS	Total
Squamous neoplasia-associated	195	28 (14.3)	13 (6.7)	5 (2.6)	46 (23.4)
Benign lesion*-associated	120	11 (9.2)	5 (4.2)	0	16 (13.3)
Total	315	39 (12.4)	18 (5.7)	5 (1.6)	62 (19.7)

LGD, low grade endocervical glandular dysplasia; HGD, high grade endocervical glandular dysplasia; AIS, adenocarcinoma in situ
* leiomyoma and adenomyosis of uterus

cervical glandular atypia and AIS. The glands exhibiting cervical glandular atypia showed intraluminal tufting and some irregularities of shape. The nuclei were enlarged, hyperchromatic and elongated. The nuclear stratification was limited to the basal two thirds of the epithelium in low grade atypia, and occupied more than two thirds of the height of the epithelium in high grade atypia.

Forty six endocervical glandular lesions were recognized from 195 squamous neoplasia of the uterine cervix, and consisted of 28 low grade EGD, 13 high grade EGD and 5 AIS cases. From 120 leiomyoma or adenomyosis, 16 endocervical glandular lesions, composed of 5 high grade EGD and 11 low grade EGD, were found. In total, 62 endocervical glandular lesions were detected, in which 39 were low grade EGD, 18 were high grade EGD and 5 cases were AIS (Table 1).

Immunohistochemistry

From formalin-fixed, paraffin-embedded tissue blocks, 4 μm thick, serial two sections for p53 and MIB-1 were obtained. After deparaffinization and hydration, the sections were incubated in 0.01 M citrate buffer for 10 min in a microwave oven at 800 W, and treated with 3% hydrogen peroxide for 15 min and rinsed. Primary antibodies of p53 (DAKO, Carpinteria, C.A. U.S.A.) and MIB-1 (Immunotech, Marseille, France) were diluted at 1:50 and 1:100, respectively. LSAB (labelled strepavidin biotin) kit (DAKO) was used for detection. The chromogen was AEC (amino-ethyl-carbazole), and counterstaining was performed using Mayer's hematoxylin. For p53 interpretation, more than 5% of tumor cells with positive nuclear reaction was defined as positive. For MIB-1, the reaction was interpreted as negative if less than 1% of nuclei were positive, low if 1-10% of nuclei were positive, and high if more than 10% of nuclei were positive. Appropriate positive and negative control sections were used.

In situ PCR for HPV 16/18

Endocervical glandular lesion-containing areas were selected from 4 μm thick section. After deparaffinization and hydration, the slides were incubated in 0.02 M HCl for 10 min, in 3% H_2O_2 for 10 min, and in 0.01% Triton ×100 solution for 10 sec. After rinsing with PBS, 50 μg/mL proteinase K was treated for 10 min at 30℃, 2 μg/mL glycine/PBS for 5 min and 20% acetic acid for 15 sec to inactivate endogenous alkaline phosphatase activity. After rinsing, the slides were air dried. Following step was performed by TaKaRa PCR HPV detection kit. The PCR mixture was 100 μL, containing 10× PCR buffer 10 μL, 25 mM $MgCl_2$ 20 μL, dNTPs 16 μL, 1 mM biotin-16-dUTP 4 μL, 2 U *Taq* (TaKaRa ex Taq) 2 μL, and forward 5 μL and reverse 5 μL of 20 p/μL primer. The forward and reverse primers were 5'-TGTCAAAAACCGTTGTGTCC-3' and 5'-GAGCTGTCGCTTAATTGTC-3', respectively. The PCR mixture was put on the sections and the slides were sealed using TaKaRa slide seal for in situ PCR. After denaturation for 5 min at 94℃, PCR was done for 35 cycles (5 sec at 94℃, 1 min 30 sec at 55℃, and 1 min at 72℃) and post-extension for 5 to 10 min at 72℃ before cooling down at 4℃. Streptavidin antibody solution (DAKO) was treated, and then AEC was treated for 10 min at 37℃ and counterstained with Mayer's hematoxylin for 10 sec. Nuclear reaction was microscopically examined. Appropriate positive and negative control sections were used.

Statistical anaysis

P53, MIB-1 and HPV 16/18 expressions according to the grade of endocervical glandular lesions, and the correlations between p53 and MIB-1 and HPV 16/18 expression were analyzed using chi-square or Fisher's exact t-test (SAS 6.12). p value less than 0.05 was defined as statistically significant.

RESULTS

Immunohistochemical stains and in situ PCR were done in 28 low grade EGD except 11 cases showing no reproducible EGD lesions on deeper levels, 18 high grade EGD, 5 AIS and 14 adenocarcinomas. Immunohistochemical expression rate for p53 protein of malignant

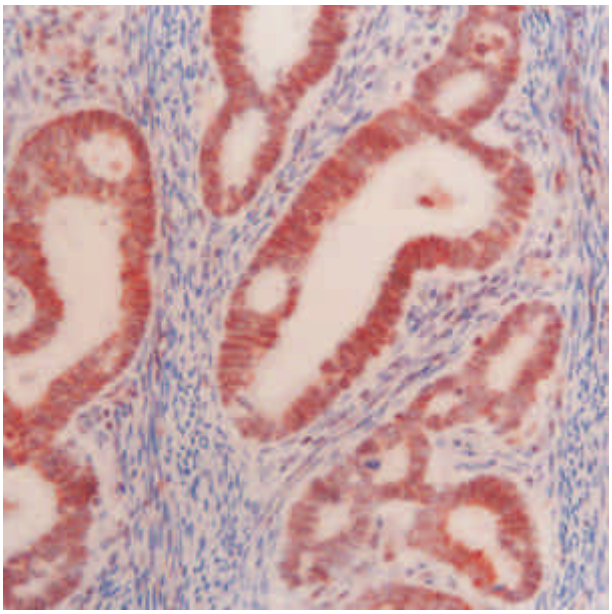

Fig. 1. Positive reaction for p53 in adenocarcinoma (LSAB method, ×200).

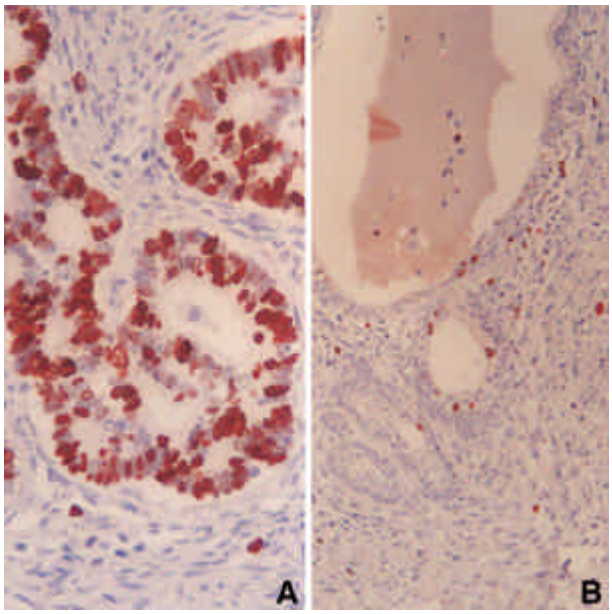

Fig. 2. High MIB-1 labelling index in adenocarcinoma (A), and a few positive cells for MIB-1 in benign glandular epithelium (B) (LSAB method, A: ×200, B: ×100).

Table 2. p53, MIB-1 and HPV 16/18 expressions in endocervical glandular lesions

Group	No. of cases	p53†	MIB-1†	HPV 16/18†
		Positive (%)	High* LI (%)	Positive (%)
LGD	28	0 (0.0)	1 (3.6)	5 (17.9)
HGD	18	2 (11.1)	1 (5.7)	3 (16.7)
AIS+ACA	19	6 (31.6)	14 (73.7)	16 (84.2)

ACA, endocervical adenocarcinoma; LI, labelling index
† $p<0.05$; ‡ $p<0.001$
* more than 10%

glandular lesion (14 adenocarcinoma and 5 AIS) was 31.6% (Fig. 1), and that of high grade EGD was 11.1%. In low grade EGD, no positive cases were seen (Table 2). There were statistically significant differences of p53 protein expression among the endocervical glandular lesions ($p<0.05$).

High MIB-1 labelling index was noted in 14 (73.7%) of 19 malignant endocervical glandular lesions (Fig. 2A), in 1 (5.7%) of 18 high grade EGD and in 1 (3.6%) of 28 low grade EGD, and benign endocervical lining cells revealed small number of positive nuclei (Fig. 2B). The incidence of high MIB-1 labelling index was significantly higher in the cases of malignant endocervical glandular lesions than in the cases of EGD (Table 2) ($p<0.001$).

Sixteen (84.2%) of 19 malignant endocervical glandular lesions showed positive reaction for HPV 16/18 using in situ PCR (Fig. 3); however, 3 (16.7%) of 18 high grade EGD and 5 (17.9%) of 28 low grade EGD

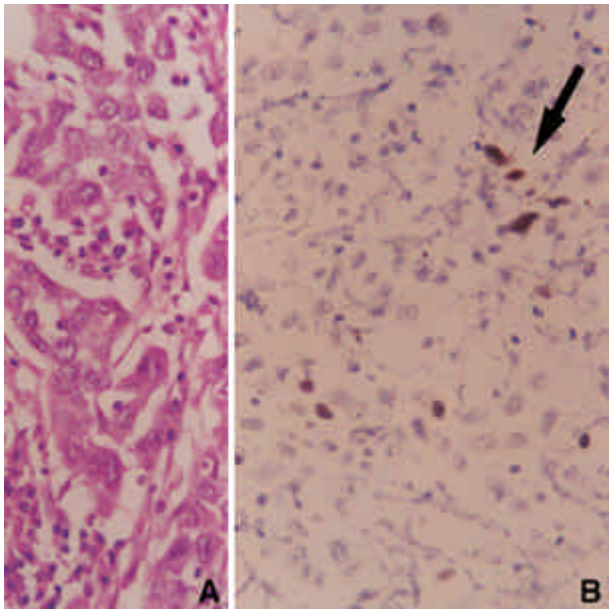

Fig. 3. Poorly differentiated endocervical adenocarcinoma (A) and scattered positive nuclei (arrow) on in situ PCR for HPV 16/18 (B) (×200).

displayed positive reactions. There was a significant difference of HPV 16/18 expression rate between malignant endocervical glandular lesions and EGD (Table 2) ($p<0.001$).

In malignant endocervical glandular lesions, 6 (37.5%) of 16 cases showing HPV 16/18 positive reaction were p53 positive but all 3 cases showing HPV 16/18 negative

Table 3. Relationship of p53, MIB-1 and HPV 16/18 expressions in malignant endocervical glandular lesions

		p53†		MIB-1†	
		Negative	Positive (%)	Negative	High* LI (%)
HPV 16/18	Negative	3	0 (0.0)	1	2 (66.7)
	Positive	10	6 (37.5)	4	12 (75.0)

† p=0.200; ‡ p=0.764
* more than 10%

reaction were negative for p53. The incidences of high MIB-1 labelling index in the cases with HPV 16/18 positive and negative reaction were 75.0% and 66.7%, respectively. No significant differences of p53 protein expression and high MIB-1 labelling index according to HPV 16/18 expression were noted (Table 3).

DISCUSSION

The term endocervical glandular dysplasia or atypia has been used in cases showing less marked cytologic and architectural atypia compared with adenocarcinoma. However, histologic criteria for endocervical glandular dysplasia or atypia are still unclear (11). EGD was regarded as a precancerous lesion that may develop to adenocarcinoma (1, 2). Cervical intraepithelial glandular neoplasia (CIGN) was suggested by Gloor and Hurlimann (12) and subdivided into grade I, II and III. Continuity between AIS and adenocarcinoma was suggested because cytologic and histologic findings of AIS and adenocarcinoma were very similar (3) and because AIS lesions were coexistent in 9.2% of adenocarcinoma (4).

However, Yeh et al. (13) reported that the term CIGN was not appropriate because of no proven clinicopathologic relationship and no evidence of progression from CIGN I to II to III. According to Goldstein et al. (5), low grade EGD and CIGN I were regarded as reactive lesions because EGD was not easily found in AIS cases and showed diffuse distribution. And CIGN II was defined as a borderline lesion between EGD and AIS, and CIGN III was comparable to AIS; therefore strict histologic criteria for endocervical glandular lesions should be needed.

The expression rate for p53 protein was reported as 70% in adenocarcinoma and 20% in AIS but negative in benign glandular lesion, and these findings suggested that p53 gene mutation might be the late event in the endocervical carcinogenesis (14). Cina et al. (15) reported that p53 protein expression was negative in AIS but positive in case showing florid microglandular hyperplasia, and p53 protein expression did not mean malignant nature. In our study, p53 protein expression rate of malignant endocervical glandular lesions (adenocarcinoma and AIS) was 31.6%, which was lower than other reports (14, 15), and 11.1% in high grade EGD, and no positive case in low grade EGD.

MIB-1 expression rates of AIS and adenocarcinoma were 47.5% and 60.0%, respectively (16), and 30 to 80% of positive rate in adenocarcinoma was reported (17, 18). Cina et al. (15) reported no MIB-1 reactivity in EGD, but Kim et al. (18) and Kim et al. (19) reported 26.6% and 18% of MIB-1 labelling index in cases with EGD, respectively. According to Leteutre et al. (20), endocervical glandular atypia showed focal expression with 40% of labelling index, but endocervical glandular dysplasia displayed diffuse expression with more than 50% of labelling index.

Malignant endocervical glandular lesion showed more than 60% of MIB-1 labelling index in contrast to less than 10% in benign endocervical glandular lesion (19), so MIB-1 labelling index could be a useful marker for the differentiation of endocervical glandular lesions. In our study, the incidence of high (more than 10%) MIB-1 labelling index was much higher in adenocarcinoma and AIS than high and low grade EGD. Our results were consistent with previous report (19), and high MIB-1 labelling index could be a suggestive finding for malignancy.

It was reported that HPV expression rate of AIS was 70%, and EGD adjacent to AIS also showed positive reaction for HPV; therefore HPV might be the causative agent in the development of EGD and AIS (9). Higgins et al. (10) reported that HPV expression rate of CIGN was 95.2% and HPV might be involved in the early stage of carcinogenesis because higher grade lesions showed more strong expression. In other report (21), HPV was expressed in AIS lesion, but negative in endocervical glandular atypia and normal endocervix. Tase et al. (22) regarded EGD as a reactive lesion because HPV expression rate of EGD was only 6% in contrast to 64% of coexisting AIS, microinvasive adenocarcinoma and cervical intraepithelial neoplasia (CIN) lesion.

AIS lesion was occasionally associated with CIN and both lesions expressed HPV 16/18, which suggested that HPV played a role in the development of both squamous and glandular lesions (23). Duggan et al. (24) studied HPV 16/18 expression in AIS associated with CIN, and

HPV expression rate was 66% and HPV 18 might be more important in the development of AIS. According to Anciaux et al. (25), adenocarcinoma showed similar HPV expression rate regardless of coexistent squamous neoplasia; however, HPV expression rate of EGD associated with squamous neoplasia was 64.2% and that of EGD without squamous neoplasia was 16.7%. Their results indicated that squamous and glandular neoplasia of the cervix might have different pathways in carcinogenesis. In other report (26), HPV might not be important in the development of endocervical adenocarcinoma because HPV expression rate of adenocarcinoma using in situ hybridization technique was much lower than using PCR. In our study using in situ hybridization, high expression rate of HPV 16/18 in malignant endocervical glandular lesions indicates that HPV has been involved in the development of endocervical adenocarcinoma, but much lower expression rate of HPV 16/18 in EGD suggests that EGD might not be related to HPV infection.

HPV-negative, p53-positive adenocarcinoma was regarded as a separate entity with poor prognosis due to inverse correlation between p53 expression and HPV expression (27). However, there were no significant differences of p53 expression and MIB-1 labelling index according to HPV 16/18 expression in our study.

In conclusion, HPV 16/18 might be a major causative factor in malignant endocervical glandular lesions, but p53 gene mutation and HPV infection might be involved separately in the development of endocervical adenocarcinoma and HPV 16/18 infection might not influence the proliferating activity of the tumor cells. EGD revealed significantly low p53 positivity, MIB-1 labelling index, and HPV 16/18 expression rate compared to malignant endocervical glandular lesion, however, it is not definable that EGD is a reactive process or a premalignant lesion.

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