

# Immunohistochemical Analysis of Midkine Expression in Preinvasive and Invasive Squamous Cell Neoplasia of the Uterine Cervix

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**Background** : Midkine (MK) is a member of the heparin-binding growth factor family. Overexpression of MK is observed not only in cancerous tissue but also in precancerous lesions of the colon and the prostate. Using immunohistochemical methods, we investigated MK expression in preinvasive and invasive neoplasia of the uterine cervix. **Methods** : We performed immunohistochemical analysis of archived cone biopsy and hysterectomy specimens from 161 squamous cell lesions of the uterine cervix (29 cervical intraepithelial neoplasia 1 (CIN1), 35 CIN2, 49 CIN3, 30 microinvasive squamous cell carcinomas (MIC), and 18 invasive squamous cell carcinoma). In addition, we examined if there is a correlation between MK expression and status of human papilloma virus infection determined by a commercially available DNA chip. **Results** : None of the normal cervical mucosa showed MK immunostaining. The level of MK expression gradually increased according to the histologic grade. Moderate and strong expressions were most frequently observed in cervical tissue with CIN3 and MIC. MK immunostaining was more accentuated in the invasive border of MIC. **Conclusion** : MK may play a functional role in the disease progression of cervical squamous cell neoplasia.

**Key Words** : Cervical intraepithelial neoplasia; Midkine; Immunohistochemistry; Human papillomavirus

Growth factors play fundamental roles in the regulation of differentiation and development.<sup>1</sup> Furthermore, it is well known that overexpression of growth factors and components of their signaling network are implicated in carcinogenesis resulting in promotion of tissue proliferation and induction of malignant transformation.<sup>2,3</sup> Midkine (MK) is one of the developmentally regulated heparin-binding proteins; it was originally discovered during an mRNA analysis of embryonal carcinoma cells treated with retinoid acid.<sup>4</sup> Its role in growth, survival, and differentiation of target cells has also been implicated in development of tumor and tumor progression.<sup>5</sup>

The expression level of MK is enhanced in many human cancers, including carcinomas of gastrointestinal tract,<sup>6</sup> Wilms' tumor,<sup>7</sup> and neuroblastomas.<sup>8</sup> Overexpression of MK has also been reported in carcinomas of lung,<sup>9</sup> breast,<sup>10</sup> and urinary bladder.<sup>11</sup> MK seems to be induced by aberrant splicing of MK mes-

senger RNA resulting in truncated form mRNA,<sup>12-14</sup> which could be used as a molecular marker associated with cellular transformation and promotion of tumor development.<sup>15,16</sup> The plasma or serum level of MK is elevated in various malignant tumors, and it has been proposed as a tumor marker that could be used to monitor the response to therapy and to detect tumor recurrence.<sup>17,18</sup> In addition, MK has been considered as a suitable molecular target for the treatment of cancer.<sup>19,20</sup>

Functional roles of MK in the early stage of carcinogenesis has been demonstrated by a small number of previous studies that have shown enhanced expression of MK in precancerous lesions including colorectal adenoma,<sup>21</sup> high grade vulvar intraepithelial neoplasia (VIN),<sup>22</sup> and prostatic intraepithelial neoplasia (PIN).<sup>23</sup> There have been previous studies showing increased expression level of MK in squamous cell carcinomas of uterine cervix.<sup>24,25</sup> However, there is no systematic report on MK expres-

sion in relation to progression of preinvasive neoplasia of the uterine cervix. Using immunohistochemical methods, we examined MK expression in preinvasive and invasive squamous cell neoplasia of the uterine cervix.

## MATERIALS AND METHODS

### Tissue samples

Archived formalin-fixed, paraffin-embedded cervical tissue sections were collected from 161 patients who underwent cone biopsy or hysterectomy for cervical intraepithelial neoplasia (CIN) or squamous cell carcinoma. The tissue samples included 29 CIN1, 35 CIN2, 49 CIN3, 30 microinvasive squamous cell carcinoma (MIC), and 18 invasive squamous cell carcinoma (ISCC). The mean age of patients who provided the above samples was 40.8, 36.2, 37.3, 45.6, and 51.6 years, respectively. For control

samples, 32 normal cervical tissues were obtained from patients who underwent hysterectomy for leiomyoma. The mean age of the control group was 43 years.

Information on status of human papilloma virus (HPV) infection of cervix based on an analysis of the cervical swab was retrieved from laboratory data. Status and subtype (high-risk subtype: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 69; low-risk subtype: 6, 11, 34, 40, 42, 43, and 44) of HPV infection was detected using commercially available system (HPV DNA chip<sup>®</sup>, Biomedlab, Seoul, Korea), which is based on polymerase chain reaction and DNA microarray methods.<sup>26</sup>

### Immunohistochemical staining

Paraffin-embedded tissue sections were cut to a 4  $\mu\text{m}$  thickness, attached to silane-coated glass slides, and dried overnight. The sections were deparaffinized in xylene and then rehydrated in graded ethanol. Endogenous peroxidase activity was quenched

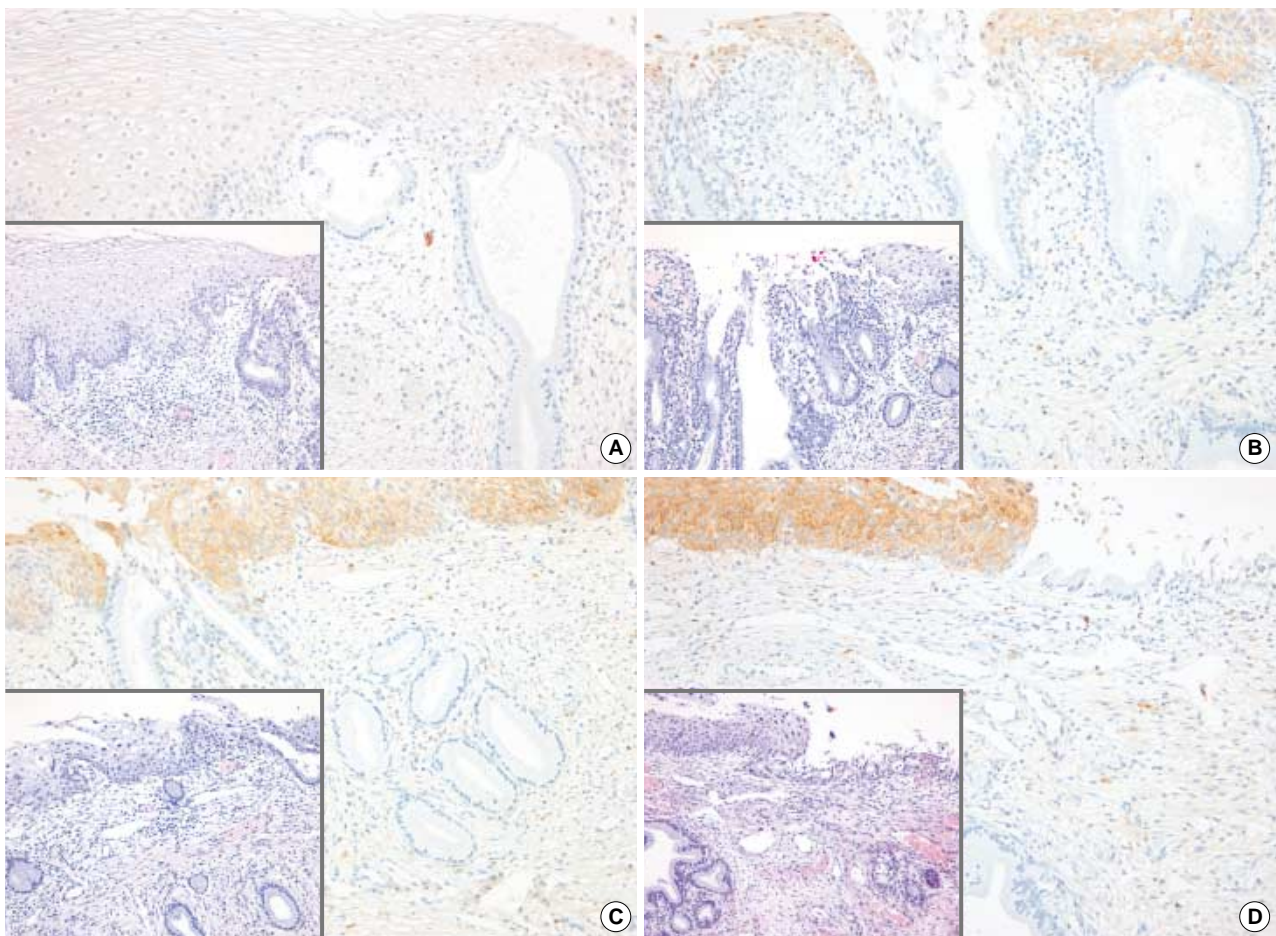


Fig. 1. Intensity of midkine immunostaining (A, no staining in control mucosa; B, weak intensity in CIN1; C, moderate intensity in CIN2; D, strong intensity in CIN3; *Inlets*, hematoxylin and eosin stain).

by 10 min of incubation in 3% hydrogen peroxide in methanol. For antigen retrieval, deparaffinized sections were boiled in 0.01 M citric acid, pH 6.0, for 5 min in pressure cooker. Non-specific binding was blocked by treatment with diluted goat serum for 20 min. The slides were consecutively incubated with avidin and biotin for 15 min to eliminate any endogenous biotin-related background staining. An affinity purified goat polyclonal anti-human midkine antibody (R&D systems, Minneapolis, MN, USA) was used as primary antibody. Tissue slides were incubated with primary antibody (15  $\mu$ g/mL) overnight at 4°C in a moist chamber and staining was then performed using the biotin-streptavidin detection system (Cell and Tissue Staining Kits; R&D Systems). Finally, color was developed in a chromogenic substrate solution of diaminobenzidine-hydrogen peroxide. The sections were then counterstained with hematoxylin, dehydrated through graded alcohol, and cleared in xylene prior to cover-slipping for microscopic examination. Negative control sections were incubated with normal goat serum instead of anti-midkine antibody.

#### Interpretation of immunohistochemical staining

All the slides were evaluated by light microscopy to achieve a semiquantitative estimation of MK expression based on intensity of immunostaining and proportion of stained epithelial cells. The staining intensity was scored with an intensity score (IS) as 0, no staining; 1, weak; 2, moderate; 3, strong (Fig. 1). The number of positive cells was expressed as a percentage of the total number of epithelial cells to define proportional scores (PS), and was subclassified into four categories: 0, 1-4%; 1, 5-25%; 2, 26-50%; 3, >50%. Three independent observers assessed MK immunostaining without having any previous knowledge of the

pathologic features. The sum of IS and PS was determined to define the level of MK expression, which was subclassified to one of the final four categories as follows: negative (0), when  $IS+PS \leq 1$ ; weak expression (1+), when  $IS+PS=2$ ; moderate expression (2+), when  $3 \leq IS+PS \leq 4$ ; strong expression (3+), when  $IS+PS \geq 5$ .

#### Statistical assessment

Statistical analyses were carried out using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). The relation between MK expression and histologic grades was examined using contingency table. The differences in MK expression between the groups were verified using  $\chi^2$  or Fisher's exact test. A linear by linear association test was employed to evaluate the trend for MK expression according to progression of histologic grade. Results were considered significant when a  $p < 0.05$ .

## RESULTS

#### Midkine expression in normal cervical epithelium

We examined 32 normal cervical tissue specimens as controls. Neither surface epithelial cells nor glandular epithelial cells, including those showing mature squamous metaplasia, stained for MK. Normal epithelium adjacent to preinvasive or invasive squamous lesion was also negative for MK staining.

#### Midkine expression in cervical intraepithelial neoplasia 1

MK expression was not observed in majority of the 29 speci-

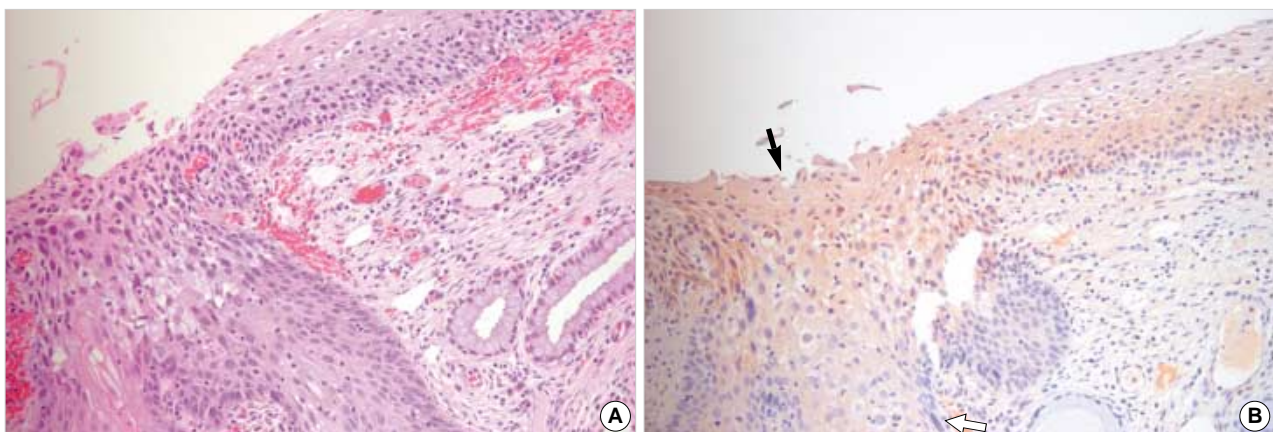


Fig. 2. Midkine immunostaining in CIN1. Weak immunoreactivity in the cytoplasm of dysplastic cells (arrow) contrasts with no staining in the squamous metaplastic area (open arrow). (A, hematoxylin and eosin stain; B, midkine immunostaining).

mens with CIN1 except 2 cases, which showed weak MK expression (6.9%, Fig. 2). However, neither moderate nor strong expression was observed.

### Midkine expression in cervical intraepithelial neoplasia 2

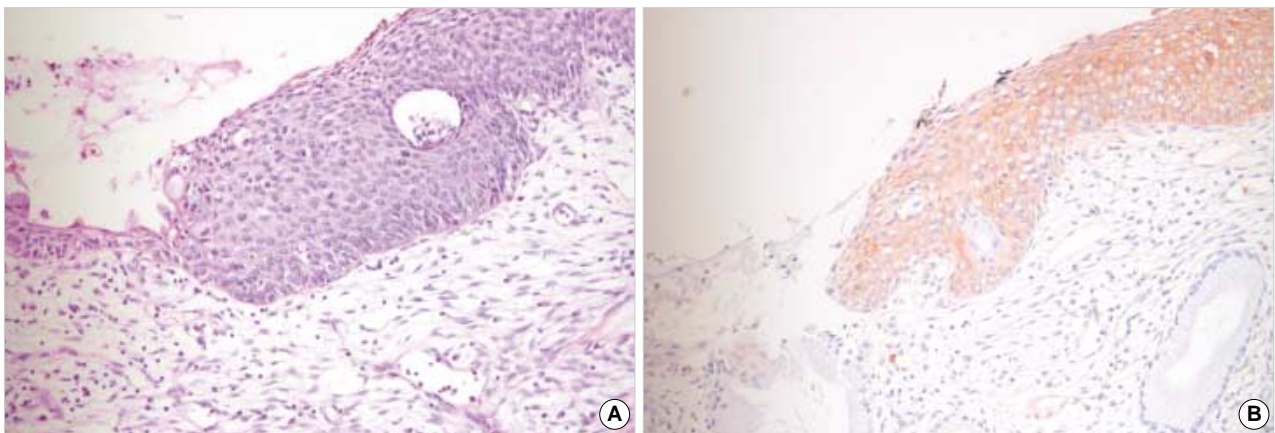
Among 35 specimens with CIN2, MK expression was observed in 12 cases (34.3%). Five cases (14.3%) showed moderate or strong MK expression (Table 1).

**Table 1.** Midkine (MK) expression in normal, preinvasive, and invasive lesions

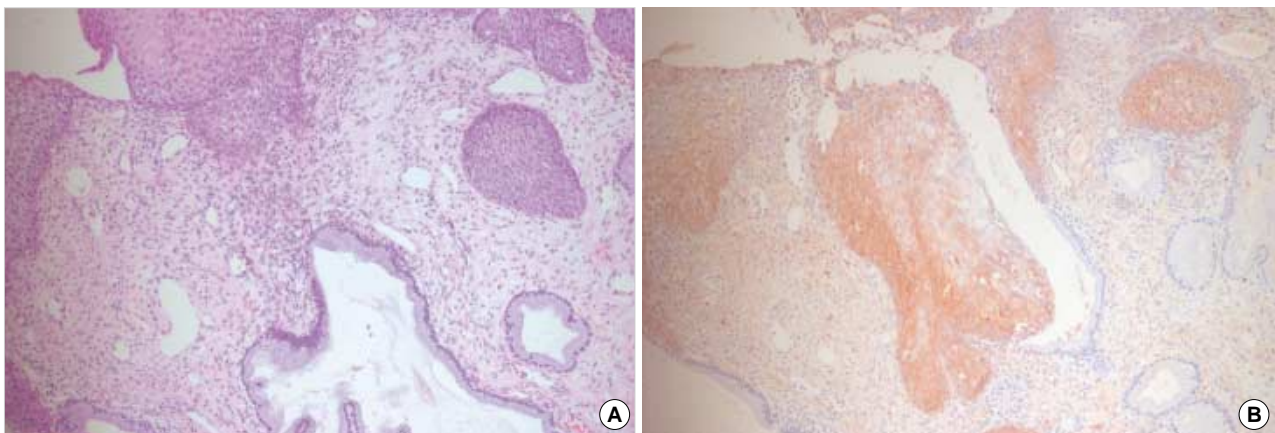
Histologic diagnosis (n)	MK expression, n				MK expression $\geq 1+$ , n (%)	MK expression $\geq 2+$ , n (%)	(MK expression $\geq 2+$ )/ (MK expression $\geq 1+$ ), n (%)
	0	1+	2+	3+			
<sup>a</sup> Normal (32)	32	0	0	0	0 (0)	0 (0)	
<sup>b</sup> CIN1 (29)	27	2	0	0	2 (6.9)	0 (0)	
<sup>c</sup> CIN2 (35)	23	7	3	2	12 (34.3)	5 (14.3)	5/12 (41.7)
<sup>d</sup> CIN3 (49)	12	11	10	16	37 (75.5)	26 (53.1)	26/37 (70.3)
<sup>e</sup> MIC (30)	6	5	10	9	24 (80)	19 (63.3)	19/24 (79.2)
<sup>f</sup> ISCC (18)	11	1	3	3	7 (38.9)	6 (33.3)	6/7 (85.7)

$\chi^2$  test, Fisher exact test, and  $\chi^2$  test for trend were employed. <sup>a,b</sup> $p=0.475$ , <sup>b,c</sup> $p=0.008$ , <sup>c,d</sup> $p<0.0001$ , <sup>d,e</sup> $p=0.797$ , <sup>e,f</sup> $p=0.004$ .

1+, weak expression; 2+, moderate expression; 3+, strong expression. CIN, cervical intraepithelial neoplasia; MIC, microinvasive carcinoma; ISCC, invasive squamous cell carcinoma.



**Fig. 3.** Midkine immunostaining in CIN3. Midkine immunoreactivity is observed in the whole layer of the dysplastic epithelium (A, hematoxylin and eosin stain; B, midkine immunostaining).



**Fig. 4.** Midkine immunostaining in CIN3 lesion with glandular involvement shows characteristic accentuation of MK immunostaining in the basal and parabasal layer (A, hematoxylin and eosin stain; B, midkine immunostaining).

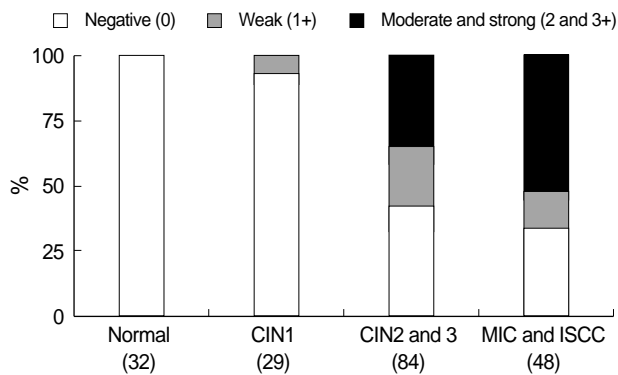


Fig. 5. Trend of midkine expression according to histologic grade. The gradual increase of MK expression was evidenced when it was subcategorized trichotomously in triad [i.e. negative (0), weak (1+), and moderate and strong (2 and 3+)] and the cervical tissue specimens were grouped in tetrad (i.e. normal, CIN1, CIN2/3, MIC/ISCC).  $p < 0.001$ , Test for trend by linear by linear association. CIN, cervical intraepithelial neoplasia; MIC, microinvasive carcinoma; ISCC, invasive squamous cell carcinoma.

### Midkine expression in cervical intraepithelial neoplasia 3

The immunohistochemical staining pattern of MK was analyzed in 49 CIN3 lesions. MK expression was observed in 37 cases. Moderate or strong MK expression was observed in 26 cases (Table 1). In the most CIN3 lesions that showed MK expression, immunoreactivity was demonstrated in the whole layer of the dysplastic epithelium (Fig. 3). Discernible differences in the immunostaining pattern were observed between CIN3 lesion and adjacent low-grade squamous intraepithelial lesion in the CIN3. Accentuation of MK immunostaining in the basal and parabasal layer was frequently observed in CIN3 lesion with glandular involvement (Fig. 4).

### Midkine expression in microinvasive squamous cell carcinoma

Among 30 MIC lesions, MK expression was observed in 24 MIC specimens (80%). Moderate or strong MK expression was observed in 19 cases (63.8%, Table 1). Interestingly, there was a case initially interpreted as CIN 3 with extensive glandular involvement, which on the step section for the present study demonstrated a focus of early stromal invasion with accentuated MK immunoreactivity.

### Midkine expression in invasive squamous cell carcinoma

We examined 18 specimens of ISCC, in which MK expression was noted in 7 cases (38.9%). There were 6 cases (33.3%)

Table 2. Midkine (MK) expression and status of human papillomavirus (HPV) infection

Histologic diagnosis	HPV status <sup>a</sup>	MK expression		*p value
		≤ 1+, n	≥ 2+, n	
Normal	-	7	0	
	+	2	0	
CIN1	-	10	0	
	+	8	0	
CIN2	-	8	0	0.052
	+	10	6	
CIN3	-	1	10	0.206
	+	9	23	
MIC	-	0	8	0.120
	+	3	8	
ISCC	-	1	2	0.386
	+	7	4	
Total		66	61	

<sup>a</sup>Of 193 cases, data on HPV DNA Chip test were available in 127 cases. \*, Fisher's exact test; ≤ 1+, negative and weak expression; ≥ 2, moderate and strong expression.

showing moderate or strong MK expression (Table 1), in which 5 cases were large cell keratinizing type.

### Comparison of midkine expression according to the histologic subgroups

When the tissue specimens were grouped in four subgroups, i.e. control, low-grade CIN (CIN1), high grade CIN (CIN2 and 3), and invasive lesion (MIC and ISCC), there was a gradual increase of MK expression according to increase of the histologic grade ( $p < 0.001$ ,  $\chi^2$  test for trend by linear by linear association, Fig. 5). When data were analyzed after dichotomization of MK expression (negative and weak versus moderate and strong), moderate and strong MK expression was most frequently observed in MIC specimens (19/30; 63.3%) with significant differences from lesions with CIN or ISCC (Table 1). MIC lesions showed more frequent MK expression compared to that of ISCC lesions ( $p = 0.004$  by  $\chi^2$  test). However, there was no significant difference between CIN3 lesion and MIC lesions ( $p = 0.797$  by  $\chi^2$  test).

### Comparison of midkine expression according to the status of human papillomavirus infection

Of 193 cases in the present study, data on the status and subtypes of the HPV DNA were available in 127 cases (65.8%). About two thirds of the specimens retrieved for this study were positive on HPV DNA. However, MK expression was not associated with status of HPV infection ( $r = 0.043$ ,  $p = 0.603$  by Spear-

mann's rho correlation test, Table 2). Furthermore, subtypes (high risk group versus low risk group) of HPV did not affect MK expression.

## DISCUSSION

MK is a member of heparin-binding growth/differentiation factor. Its involvement in carcinogenesis is evidenced by its biologic roles associated with mitosis, angiogenesis and transformation of cells.<sup>27,28</sup> Enhanced expression of MK in cervical carcinoma has been reported in a couple of previous studies,<sup>24,25</sup> in which MK expression was correlated with clinical stage. To the best of our knowledge, there are no studies on expression of MK in precancerous lesions of the uterine cervix, which led us to immunohistochemical analyses for MK expression on archived cervical tissue specimens with CIN.

In this study, MK expression was not observed in normal cervical tissue in contrast to a previous immunohistochemical study of MK expression that has shown MK expression in 10.5% of normal cervical tissue.<sup>24</sup> Only focal and weak MK immunostaining was detected in a few CIN1 specimens (6.9%:2/29) in the present study. We observed MK expression in 34.3% (12/35) of CIN 2 specimens in which 41.7% (5/12) cases demonstrated moderate or strong expression. The frequency of moderate and strong MK expression gradually increased according to the histologic severity from preinvasive lesion to invasive cervical lesion. However, MK expression was most frequently observed in cervical tissue with MIC and CIN3 without significant difference between MIC and CIN3. One interesting point was that MK expression was less frequently observed in ISCC compared to CIN3 and MIC. This finding suggests potential roles of MK expression in the relatively early phase of cervical carcinogenesis.

In the previous immunohistochemical study of MK expression in vulvar squamous cell neoplasia, MK expression was seen in 3 of 5 VIN3 specimens but not in VIN1 and VIN2 specimens, suggesting MK expression might be involved in malignant transformation of vulvar squamous cell.<sup>22</sup> However, in this study there was no statistically significant correlation between MK expression, clinical stage, lymph node metastasis and status of HPV infection in vulvar squamous cell carcinoma, which is in contrast to another immunohistochemical study in squamous cell carcinoma of the uterine cervix.<sup>24</sup>

It is well established that development of cervical carcinoma is a result of a sequential transformation of normal epithelium to a precancerous lesion to a final carcinoma, and that high-risk

HPV infection is one of the etiological agents for the development of cervical carcinoma. In several of the clinical samples, we analyzed the relationship between MK expression and the presence of different subtypes of HPV. We failed to demonstrate any significant relationship between MK expression and HPV infection, suggesting that MK expression may be controlled by HPV-independent cellular mechanisms.

There are a small number of previous studies on MK expression in precancerous epithelial lesions.<sup>21-23</sup> In one of the studies, Ye and colleagues<sup>21</sup> have demonstrated elevated MK expression at both the mRNA and protein level in precancerous lesions of human colorectal cancer. These investigators observed MK immunorexpression in 18 out of 29 (62.1%) adenomas with moderate dysplasia, which is in contrast to that found in adenoma with severe dysplasia (100%; 5/5) and adenocarcinoma (100%; 22/22). A study by Konishi *et al.*<sup>23</sup> showed MK expression in 75% of prostatic intraepithelial neoplasia, and suggested MK overexpression might play roles in an early event in the tumorigenesis of prostatic carcinoma. In our study, gradual increase of MK expression was evident when MK expression was subcategorized trichotomously in triad, *i.e.* negative (0), weak (1+), moderate and strong (2 and 3+), and the cervical tissue specimens were grouped in tetrad, *i.e.* normal, CIN1, CIN2/3, and MIC/ISCC. These results suggest MK is a protein that has a role in the early stages of carcinogenesis, and is particularly involved in progression of preinvasive lesion to invasive lesion.

Overexpression of MK protein and messenger RNA has also been shown in papillary thyroid carcinoma,<sup>29</sup> in which the immunoreactivity of MK was fortified at the invading border area of the tumors rather than in the central area. Similar features were seen in cases of cervical tissue specimens with MIC in the current study. In the present study, level of MK expression in ISCC was lower as compared to that of MIC or CIN3. We speculate that increased MK expression might determine early stage of stromal invasion, which is very critical step for development of cervical carcinoma.

With regard to biologic functions of MK in carcinogenesis, several cancer-related activities including mitosis, antiapoptotic, angiogenic, transforming, fibrinolytic and chemotactic events have been proposed,<sup>5</sup> indicating importance of MK in both tumor development and promotion of tumor progression. A functional role of MK as a mediator of angiogenesis has been implicated in tumor growth and progression.<sup>30</sup> In one study (Moon *et al.*<sup>24</sup>), however, no correlation between MK expression and microvessel density measured by CD34 immunostaining in the cervical cancer tissues was seen, which suggested that the biologic role

of MK in cervical carcinogenesis might not be related with angiogenic activity.

In conclusion, we demonstrated MK expression in CIN lesions with gradual increase of its expression associated with tumor progression and discernible accentuation in the early invasive foci. These findings suggest that MK plays a functional role in early step of stromal invasion.

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