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Evaluation of Immunostaining for MIB1 and nm23 Products in Uterine Cervical Adenocarcinoma

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Morimura, Y., Yanagida, K., Hashimoto, T., Takano, Y., Watanabe, F., Yamada, H. and Sato, A. *Evaluation of Immunostaining for MIB1 and nm23 Products in Uterine Cervical Adenocarcinoma.* Tohoku J. Exp. Med., 1998, **185** (3), 185-197 —— In this study, we evaluated whether proliferative activity and metastatic potential are prognostic factors in adenocarcinoma of the cervix. Formalin-fixed, paraffin-embedded sections from 34 patients with cervical adenocarcinoma or adenosquamous carcinoma were immunostained with monoclonal antibody MIB1, expressed in proliferating cells, and anti-nm23 antibody, reacts with metastasis suppression gene products. MIB1 positivity ranged from 0.2~54.7% with a mean of 21.4%. The level did not differ significantly between various clinicopathological categories. Although patient survival of high or low MIB1 expressing tumor was not significantly different, the disease-free interval of high ($\geqq$25%) expressing tumor was significantly lower than that of low ($<$25%) expressing tumor. Strong and medium expression for nm23 were detected in 7 (21%) and 3 (9%) of patients. Tumor with strong nm23 expression tended to have a higher relapse rate. Although the survival of strong and weakly nm23 expressing tumor was not significantly different, the disease-free survival of strong nm23 expressing tumor was lower than that of weakly nm23 expressing tumor. MIB1 and nm23 immunostaining might be some of prognostic indicators of recurrence in cervical adenocarcinoma. In cervical adenocarcinoma, the nm23 gene products may not function as metastatic suppression but reflect proliferative activity. —— cervical adenocarcinoma; immunohistochemistry; MIB1; nm23; prognosis

Adenocarcinoma of the uterine cervix is a difficult management problem for gynecological oncologists. Recent reports have indicated an increase in the incidence and frequency of cervical adenocarcinoma (Devesa et al. 1989; Eifel et al. 1990; Miller et al. 1993). There is no general agreement about the factors that predict prognosis. We investigated the tumor proliferative activity with MIB1

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(Immunotech S.A., Marseille, France, anti Ki-67 antigen expressing in the nucleus of cells not in the G0 phase of cell cycle) (Gerdes et al. 1984) and metastatic potential with anti-nm23 antibody (Immunotech S.A., Marseille, France, nm23 isolated as a metastasis suppression gene) (Steeg et al. 1988). In this report, we evaluated the immunostaining and its relationship to relapse and prognosis of patients with uterine cervical adenocarcinomas.

Materials and Methods

Between January 1986 and December 1996, 37 patients with uterine cervical adenocarcinoma or adenosquamous carcinoma were seen at the Obstetrics and Gynecology Hospital, Fukushima Medical University. The patients' records were analyzed for age, Society of Gynecologic Oncology (SGO) stage, treatment, follow up and time to recurrence. Pathologic findings were analyzed for histologic subtype, nuclear atypia, lymph-vascular space invasion, depth of invasion and metastasis of lymph nodes.

The formalin-fixed, paraffin-embedded specimens were immunostained with MIB1 and anti-nm23 monoclonal antibody in a modification of the antigen retrieval technique of Shi et al. (1991). The specimens were deparaffinized, rehydrated and heated with 10 mM sodium citrate buffer (pH 6.0) in a 600 W microwave oven for 15 minutes. The sections were cooled and immunostained using an LSAB kit (Dako, Carpiteria, CA, USA). The primary antibody MIB1 or anti-nm23 was applied overnight at 4°C. After washing in 0.05 M Tris buffer (pH 7.6), the slides were incubated with biotinylated rabbit anti-mouse antibody for 30 minutes at room temperature and then with peroxidase-conjugated streptavidin for 30 minutes. The slides were developed for 10 minutes with 3-amino-9-ethylcarbazole as the chromogen and counterstained with hematoxylin. At least two specimen of a patient were analyzed and the fields were counted in areas of supposed maximal density of positive cells. The MIB1 antigen-positive cells showed red nuclei and all nuclei with red staining irrespective of intensity were counted as positive. The MIB1 positivity was calculated by counting 1500–2000 cells in the subjectively most positive area of adenomatous components and expressed as the percentage of positive cells. Immunostaining for nm23 was revealed diffusely in the cytoplasm. The estimated proportion of intense staining cells were determined and graded as negative (0%), weak (1–50%), medium (51–75%), and strong (76–100%). Quality control of the MIB1 and nm23 assesments was performed by two experienced intraobservers (Y.M. & H.Y.) and one interobserver (A.S.) without knowledge of prognostic significance.

Comparison between MIB1 positivity, nm23 intensity and clinicopathological categories were performed using the Kruskal-Wallis and Mann-Whitney tests. The survival and remission curves were constructed according to the Kaplan-Meier method and difference were established using the Cox-Mantel test and generalized Wilcoxon test. The MIB1 positivity threshold was studied according to the

exploratory method of Kerns et al. (1994). The Spearman method was used to assess the reproducibility of MIB1 positivity and nm23 intensity. The Cox's proportional harzard technique was used to examine the relative prognostic significance of the MIB1 and nm23 positivity in predicting disease free survival.

Results

Table 1 shows the detailed clinicopathological analysis of 34 patients. The mean age was 52.1 years old with a range of 24–87 years old. There were 4 stage 0 (adenocarcinoma in situ), 6 stage Ia, 17 stage Ib, 4 stage IIb and 3 stage IIIb tumors; 29.4% of patients presented with early stage, under stage Ia. Thirty (88%) patients underwent total hysterectomies. A case with stage Ia received conization only for fetility preservation. Three patients with stage IIIb were treated with only cervical biopsy and radiation therapy. A patient with stage Ib diagnosed by biopsy refused any therapy. Lymphadenectomy was performed in 23 cases and 3 patients (12%) showed lymph node metastasis. At the last contact, 30 cases were alive without disease, 4 cases were alive with recurrent disease and 2 cases were dead of disease.

Fig. 1 shows MIB1 immunostaining for cases with high and low antigen expression. MIB1 positivity of 34 patients range from 0.2% to 57% with a mean

Table 1. *Characteristics of 34 patients with cervical adenocarcinoma*

Age	(years)	Histology	(*n*)
Range	24–87	Adenocarcinoma in situ	4
Mean	52.1	Microinvasive adenocarcinoma	6
		Endocervical, well diff.	9
Stage	(*n*)	Endocervical, moderately diff.	8
0	4	Endocervical, poorly diff.	1
Ia	6	Adenosquamous carcinoma	6
Ib	17		
IIb	4		
IIIb	3	Lymphnode metastasis	(*n*)
		Abscent	20
Initial surgery	(*n*)	Present	3
Conizatinon	1		
Simple total hysterectomy	1	Recurrence	(*n*)
Modified radical hysterectomy	7	Free	30
Radical hysterectomy	22	Recurred	4
Others	3		
Adjuvant therapy	(*n*)	Outcome	(*n*)
None	15	Alive	32
Radiation	5	Dead	2
Chemotherapy	14		

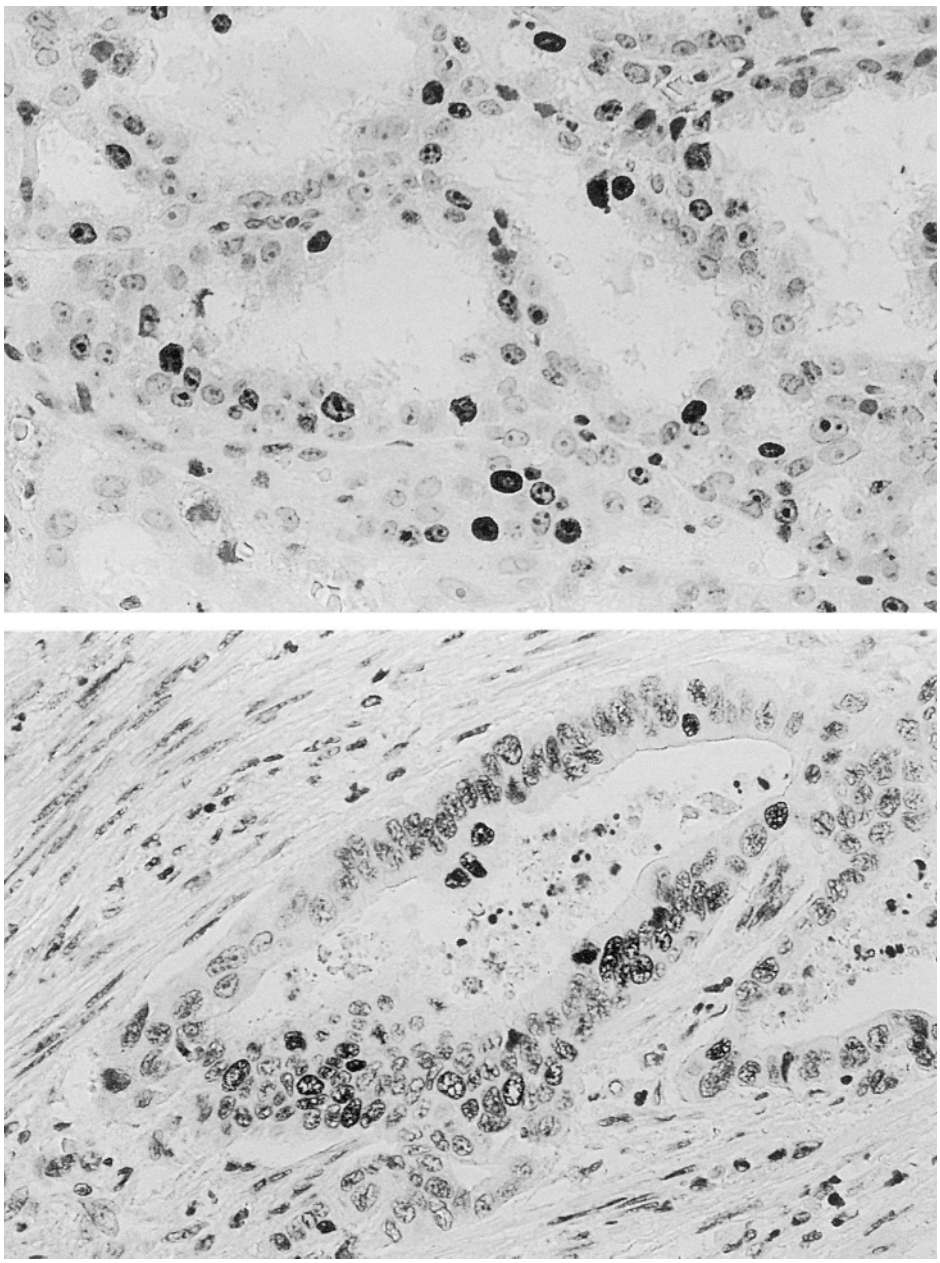

Fig. 1. Immunostainig of uterine cervical adenocarcinoma for MIB1. A: Tumor with high expression (26.0%) of nuclei (upper, ×340). B: Tumor with low (13.4%) expression of nuclei (lower, ×340). (ABC method)

value of 20.8% and standard deviation of 14.4%. Table 2 shows MIB1 positivity with the mean, range and standard deviation for comparison with each clinical and pathological groups. No significant difference was found among groups of varying clinicopathological categories.

The Kaplan-Meier disease free curves for patients whose tumor had greater than 25% MIB1 staining showed an increase chance of recurrence compared to patients whose tumor had less than 25% MIB1 staining ($p<0.05$). (Fig. 2) Although there was tendency of favorable prognosis of patients with low ($<25\%$) MIB1 positivity compared to patients with high ($\geq 25\%$) positivity, no significant all over survival advantage was demonstrated for low proliferative activity

TABLE 2. *Range, mean and S.D. of MIB1 positivity in various clinical categories*

	No.	Range	Mean	S.D.	p-value
Total	34	0.2-54.7	21.4	14.4	—
Stage					
Early (0 and Ia)	10	9.3-30.9	17.5	8.8	0.472
Advanced (over Ib)	24	0.2-54.7	22.0	16.1	
Histology					
Adenocarcinoma in situ	4	15.0-29.0	21.2	7.2	
Microinvasive adenocarcinoma	6	9.3-30.9	20.0	8.2	
Endocervical, well diff.	9	0.2-19.3	17.5	18.8	0.402
Endocervical, modarate diff.	8	22.7-54.7	23.7	15.0	
Endocervical, poorly diff.	1	46.5	46.5	—	
Adenosquamous carcinoma	6	8.5-50.3	25.6	15.4	
Nuclear atypia					
Low	16	0.2-50.4	21.1	15.6	
Mideum	13	3.9-54.7	20.4	13.7	0.741
High	5	5.4-44.5	25.2	14.9	
Lymphovascular infiltration					
Abscent	19	0.2-54.7	18.9	13.9	0.516
Present	12	1.2-44.4	21.6	14.3	
Lymphnode metastasis					
Abscent	20	0.2-54.7	23.2	16.4	0.583
Present	3	11.8-27.8	16.3	10.1	
Reccurence					
Free	30	0.2-54.7	19.5	13.6	0.060
Reccuered	4	5.4-44.5	31.9	9.6	
Outcome					
Alive	32	0.2-54.7	20.4	13.9	0.212
Dead	2	0.8-33.6	30.2	4.9	

tumors (Fig. 3).

Immunohistochemical staining for nm23 was observed in the cytoplasm of tumor cells. Fig. 4 shows cases with strongly (4a) and weakly (4b) expression for nm23. Of 34 tumors, seven (21%) stained strongly, three (9%) medium fourteen (41%) weakly and ten (29%) negative. Table 3 shows nm23 intensity for clinicopathological categories. There was no association between intensity of staining and clinical stages, histologic subtype, nuclear grade, lymph-vascular infiltration and lymph node metastasis. Patients with strong and medium nm23 expressing tumors have a tendency of higher relapse rate (29 and 33%) compared to patients with negative and weakly nm23 expressing tumors (0 and 4%) ($p<0.05$). The relapse free survival of patients with strong nm23 expressing tumors was significantly lower than patients with weakly nm23 expressing tumors ($p<$

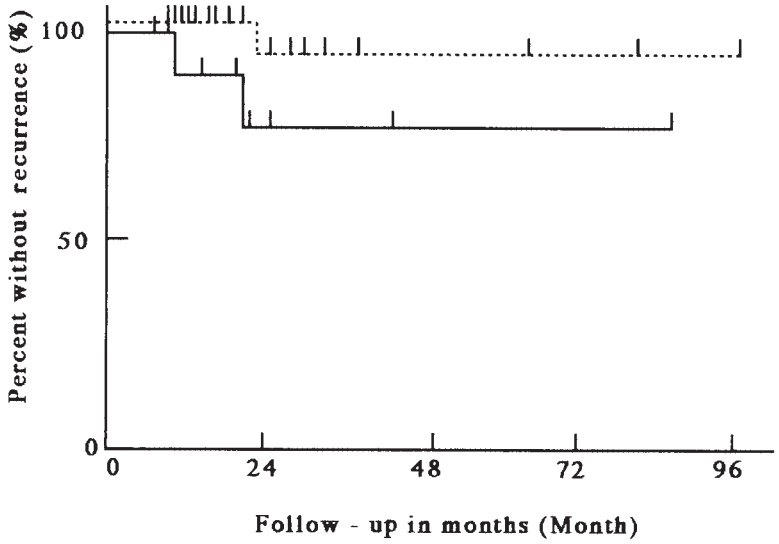

Fig. 2. Disease-free survival of patient expressing high ($\geqq 25\%$; —— [$n=12$]) MIB1 positivity was significantly lower than patient expressing low ($<25\%$, ····· [$n=22$]) MIB1 positivity ($p<0.05$).

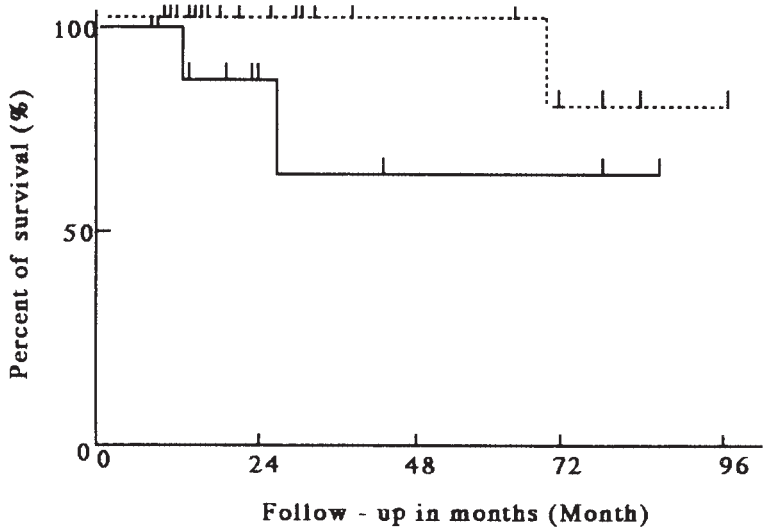

Fig. 3. All over survival of patient between high ($\geqq 25\%$; —— [$n=12$]) and low ($<25\%$; ····· [$n=22$]) MIB1 positivity was not significantly different.

0.05) (Fig. 5). All over survival of patients with strong or weakly expressing tumors was not significantly different (Fig. 6).

The relationship for MIB1 and nm23 is shown in Fig. 7. There was a significant correlation between MIB1 positivity and nm23 intensity ($p<0.01$, $r(s)=0.543$). Tumors with strongly nm23 expression have higher MIB1 positivity than tumors with weakly nm23 expression.

Table 4 shows Cox's proportional harzard model using to evaluate the relative importance of putative prognostic factors of cervical adenocarcinoma, including MIB1 and nm23 staining. Among these, MIB1 and nm23 staining did not significantly contribute to the disease free survival. ($p=0.06$ and 0.07)

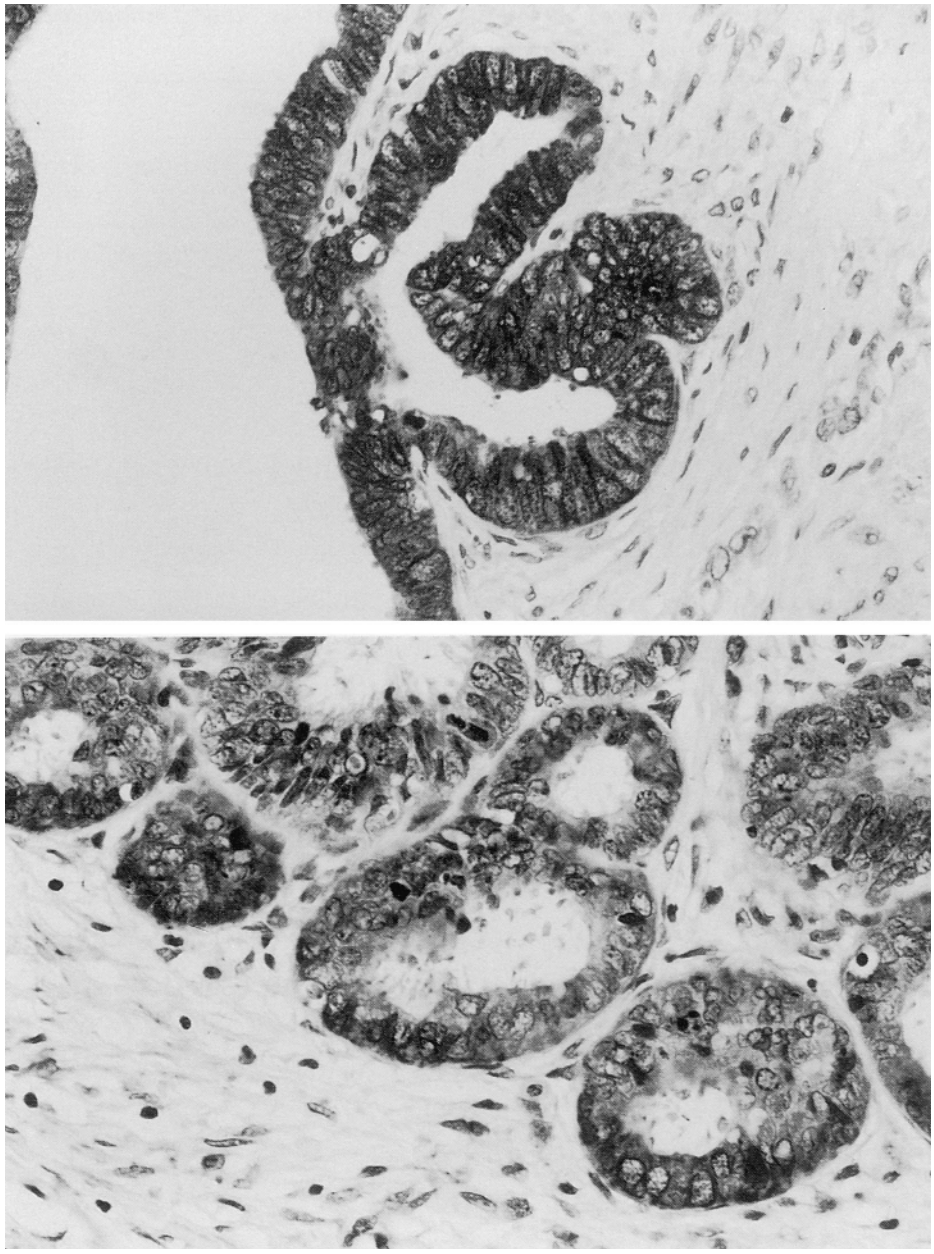

Fig. 4. Immunostainig of uterine cervical adenocarcinoma for anti-nm23. A: Tumor with strongly expression in cytoplasm (upper, ×340). B: Tumor with weakly expression in cytoplasm (lower, ×340). (ABC method)

Discussion

The relative frequency of cervical adenocarcinoma has increased (Devesa et al. 1989; Eifel et al. 1990; Miller et al. 1993). The age of patients with cervical adenocarcinoma was younger than that with squamous carcinoma (Peters et al. 1986; Schwartz and Weiss 1986). In this series, the overall median age of the patients was 50.2 years old and the number of patients younger than 35 years old was small. The stage distribution showed most patients with stage I disease, in accordance with the recent literature (Milson and Friberg 1983; Angel et al. 1992). The incidence of nodal metastasis is similar to previously reported data (Berek et

TABLE 3. *Relationship between clinicopathologic variables and Immunohistochemical expression of nm23 in cervical adenocarcinomas*

Variables	nm23 expression					
	Total (number)	Negative	Weakly (%)	Medium (%)	Strong (%)	p-value
Total	34	10(29%)	14(41%)	3(9%)	7(21%)	—
Stage						
Early (0 and Ia)	10	0(0%)	7(50%)	0(0%)	3(43%)	0.125
Advanced (over Ib)	24	10(100%)	7(50%)	3(100%)	4(57%)	
Histology						
Adenocarcinoma in situ	4	0(0%)	3(21%)	0(0%)	1(14%)	
Microinvasive adenocarcinoma	6	0(0%)	5(36%)	0(0%)	1(14%)	
Endocervical, well diff.	9	4(40%)	2(14%)	2(67%)	1(14%)	
Endocervical, modarate diff.	8	1(10%)	4(29%)	0(0%)	3(43%)	0.222
Endocervical, poorly diff.	1	1(10%)	0(0%)	0(0%)	0(0%)	
Adenosquamous carcinoma	6	4(83%)	0(0%)	1(33%)	1(14%)	
Nuclear atypia						
Low	16	5(50%)	8(57%)	1(33%)	2(29%)	
Medium	13	3(30%)	6(43%)	1(33%)	3(43%)	0.176
High	5	2(40%)	0(0%)	1(33%)	2(29%)	
Lymphovascular infiltration						
Abscent	19	4(40%)	10(71%)	2(100%)	3(43%)	0.209
Presnt	12	6(60%)	4(29%)	0(0%)	2(29%)	
Lymphnode metastasis						
Abscent	20	5(63%)	9(100%)	2(100%)	4(100%)	0.040
Present	3	3(37%)	0(0%)	0(0%)	0(0%)	
Recurrence						
Free	30	10(100%)	13(93%)	2(67%)	5(71%)	0.045
Recurred	4	0(0%)	1(7%)	1(33%)	2(29%)	
Outcome						
Alive	32	10(100%)	14(100%)	2(67%)	6(86%)	0.089
Dead	2	0(0%)	0(0%)	1(33%)	1(14%)	

al. 1985). The prognostic factors for cervical adenocarcinoma have been controversial. Several authors reported lymph node metastasis as the most commonly accepted factor predicting recurrence (Berek et al. 1985; Greer et al. 1989). Several authors suggested tumor volume as the most important prognostic factor (Berek et al. 1985; Eifel et al. 1990). Matthews et al. (1993) suggested patients with stage I cervical adenocarcinoma, whose tumor shows lymph-vascular space invasion have a high pelvic relapse rate. The histologic subtype was not related to patient survival (Korhonen 1984; Hopkins et al. 1988). In this series, the factors including clinical stage, histologic subtype, nuclear grade, vascular

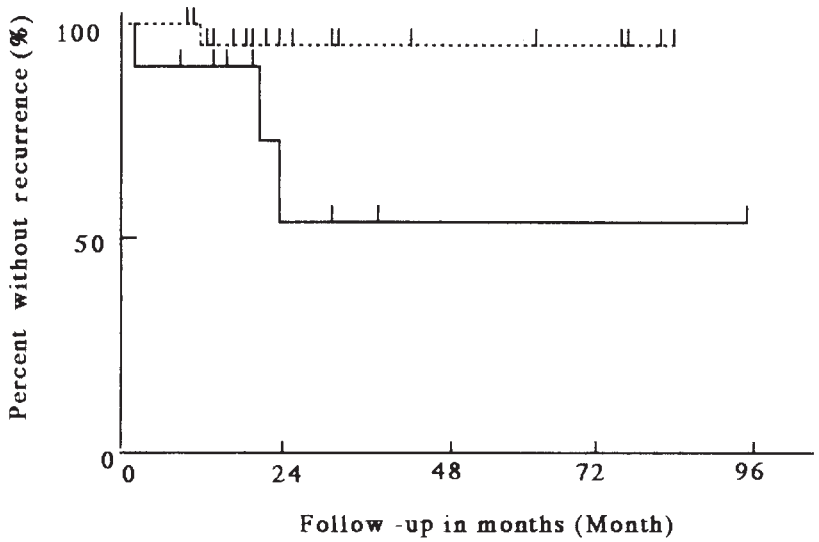

Fig. 5. Disease-free survival of patient with high (medium and strongly; —— [$n=10$]) nm23 expressing tumors was significantly lower than patient with low (weakly or negative; ····· [$n=24$]) nm23 expressing tumors ($p<0.05$).

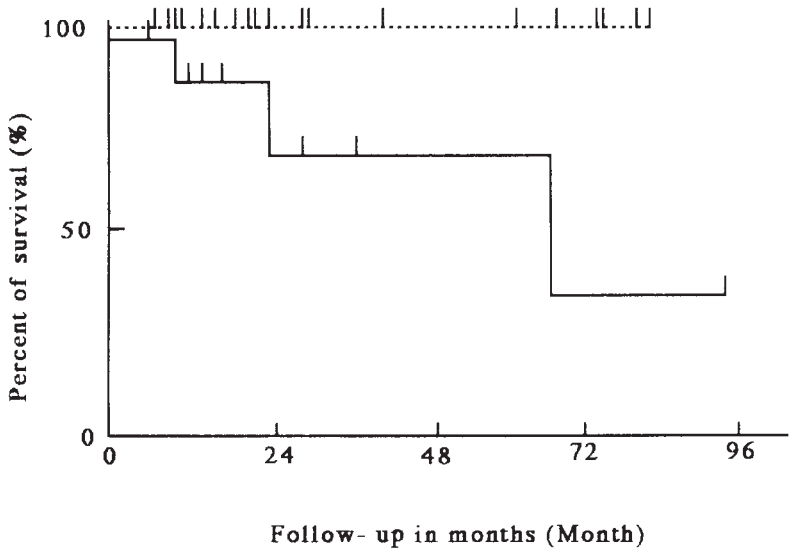

Fig. 6. All over survival of patient between low (negative or weakly; ····· [$n=24$]) and high (medium or strongly; —— [$n=10$]) nm23 expressing tumors was not significantly different.

involvement and nodal status did not predict prognosis of patients.

Tumor proliferation activity has been prognostic value in various tumors such as malignant lymphoma, colorectal cancer, breast cancer and pulmonary cancer (Shepherd et al. 1988; Walker and Camplejohn 1988; Schwart et al. 1989; Soomro and Whimster 1990). The Ki-67 antigen is expressed in the nucleus of cells except in GO phase (Gerdes et al. 1984). MIB1, monoclonal antibody to the Ki-67 antigen is a marker of cell proliferation (Cattoretti et al. 1992). Several authors reported the value of MIB1 in the assessment of gynecological malignancies. Kerns et al. (1994) reported that high MIB1 positivity had worse

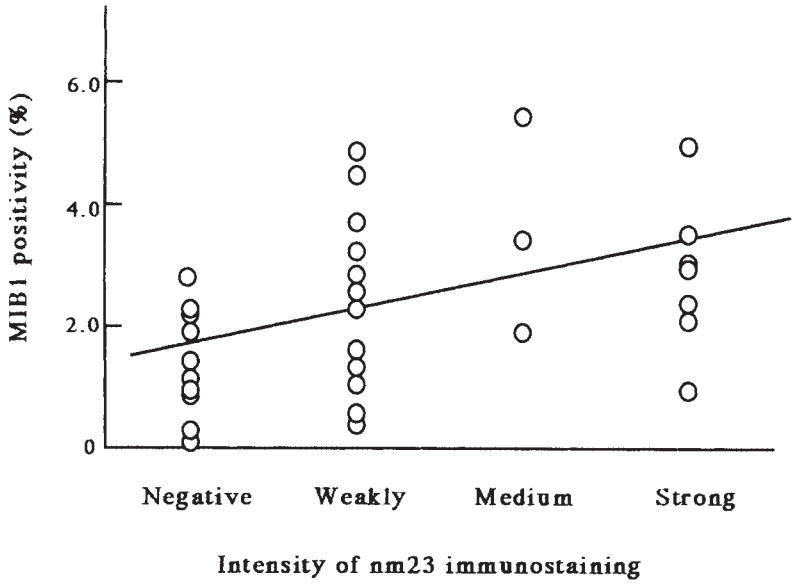

Fig. 7. MIB1 positivity correlated with nm23 intensity. ($p<0.01$, $r(s)=0.534$, $n=34$)

TABLE 4. *Cox's proportional hazard model by MIB1 and nm23 immunostaining on disease free survival of crevical adenocarcinoma*

	NED*	Relapsed	RR	95%GI	p-value
High ($\geq$25%) MIB1 positivity	9	3	9.163	.686～122.386	.0939
Medium or strongly nm23 expression	7	3	9.384	.721～122.107	.0872

* NED, no evidence of disease.

prognosis than low MIB1 positivity in stage III and IV advanced ovarian cancer. We reported that ovarian clear cell adenocarcinoma with high MIB1 positivity had poor prognosis despite early stage and complete cytoreduction (Morimura et al. 1996). Geiser et al. (1996) showed a high relapse rate of endometrial cancer with over 39% of MIB1 positivity. In this study, MIB1 immunostaining was found to be related to disease free survival. We thought that the proliferation index by MIB1 immunostaining might be a predictor of recurrence.

The nm23 gene was first isolated as metastasis suppression gene in a murine melanoma cell line (Steeg et al. 1988). Transfection of nm23 into highly metastatic murine melanoma cells reduced their metastatic potential. Decrease of nm23 correlated with high metastatic potential and poor prognosis in breast cancer, hepatocellular carcinoma and melanoma (Barnes et al. 1991; Hirayama et al. 1991; Florens et al. 1992; Yamaguchi et al. 1994). In contrast, tumors with high nm23 expression had poorer survival in pulmonary, thyroid, pancreatic and ovarian cancers (Hailat et al. 1991; Engel et al. 1993; Nakanishi et al. 1993; Zou et al. 1993; Srivatsa et al. 1996). Although Kristensen et al. (1996) found no prognostic value of nm23 immunostaining in cervical adenocarcinoma, Mandai et al. (1955) showed that tumors with reduced nm23 expression had a high incidence

of lymph node metastasis and poor prognosis. We found a high relapse rate and shorter desease-free survival in cervical adenocarcinoma with the nm23 strongly positivity.

The nm23 products play a major role in the regulation of microtubles in the mitotic spindle (Liotta and Steeg 1988). Igawa et al. (1994) demonstrated high expression of nm23-H1 mRNA in late G1, early S and G2∼M phase in prostatic carcinoma cell lines. These reports suggest that nm23 might be related to tumor proliferating activity. We consider nm23 to reflect proliferating activity in cervical adenocarcinoma. The result of correlation between intensity of nm23 and MIB1 positivity in our study supports this hypothesis. Further molecular biological investigation should be performed to clarify the role of nm23.

Although MIB1 and nm23 immunostaining were not significantly prediction of disease free survival in multivariate analysis, there was a tendency of high MIB1 and/or nm23 expression to be related to higher relapse rate. We think that the number of cases was small in this study and studies of large number of cases were necessary. However, we consider the MIB1 and nm23 immunostaining might be some of predictor in recurrence of uterine cervical adenocarcinomas.

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