—Original—

Prognostic Significance of Ki-67 Antigen Immunostaining (MIB-1 Monoclonal Antibody) in Ovarian Cancer

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Abstract

Objective: To assess the potential usefulness of Ki-67 antigen expression as a predictor of outcome in ovarian cancer through the analysis of MIB-1 monoclonal antibody reactivity.

Methods: Cell proliferation and clinicopathologic variables were assessed in 26 patients with primary epithelial ovarian cancer who had undergone exploratory laparotomy. The expression of primary tumor proliferation related to Ki-67 antigen was immunohistochemically evaluated by MIB-1 monoclonal antibody.

Results: The value of Ki-67 labeling index (LI) ranged between 0 and 92.6% with a mean of 48.9%. Ki-67 LI correlated well with the mitotic index, but not the histological subtype. Ki-67 LI of more than 40% was defined as a higher proliferating tumor by a receiver operating characteristic curve analysis. Higher proliferating tumors were identified in 14 patients (54% of all subjects). The patients with higher proliferating tumors had a statistically significantly worse prognosis compared with those with lower proliferating tumors ($p<0.001$).

Conclusions: The present study demonstrates that the proliferating index detected by Ki-67 antigen immunostaining is a useful factor for predicting the survival of patients with ovarian cancer.

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Key words: ovarian cancer, Ki-67 antigen, MIB-1 monoclonal antibody, prognosis

Introduction

The prognosis of ovarian cancer is poor in gynecological malignant tumors. Several factors are known to influence survival in ovarian cancer, including International Federation of Gynecology and Obstetrics (FIGO) stage at diagnosis, amount of residual disease after surgery, and histological grade[1]. However, these factors failed to account fully for the biological behavior of ovarian cancer and more objective ways to establish the prognosis are needed.

Determination of the proliferative activity has been reported to be of prognostic value, and several methods can be used to estimate the number of proliferating cells. Ki-67 antigen immunostaining is a relatively new technique for estimating the proliferating index of a neoplastic lesion [2,3]. Expression of this antigen occurs preferentially during the late G1, S, G2, and M phases of the cell cycle, while in cells in the G0 phase the antigen

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Table 1 Clinicopathologic characteristics of 26 patients with primary epithelial ovarian cancer

Case no.	Age	FIGO stage	Histological type
1	36	Ia	serous cystadenocarcinoma
2	40	Ic	serous cystadenocarcinoma
3	48	Ic	serous cystadenocarcinoma
4	34	Ic	serous cystadenocarcinoma
5	52	IIa	serous cystadenocarcinoma
6	44	IIIa	serous cystadenocarcinoma
7	56	IIIa	serous cystadenocarcinoma
8	51	IIIa	serous cystadenocarcinoma
9	46	IIIa	serous cystadenocarcinoma
10	55	IIIa	serous cystadenocarcinoma
11	34	Ic	mucinous cystadenocarcinoma
12	57	Ia	mucinous cystadenocarcinoma
13	52	Ic	mucinous cystadenocarcinoma
14	65	IIIa	mucinous cystadenocarcinoma
15	46	Ic	endometrioid adenocarcinoma
16	39	Ic	endometrioid adenocarcinoma
17	46	IIa	endometrioid adenocarcinoma
18	50	IIIa	endometrioid adenocarcinoma
19	48	IIIa	endometrioid adenocarcinoma
20	42	Ia	clear cell adenocarcinoma
21	57	Ia	clear cell adenocarcinoma
22	50	Ic	clear cell adenocarcinoma
23	51	Ic	clear cell adenocarcinoma
24	57	Ic	clear cell adenocarcinoma
25	37	Ic	clear cell adenocarcinoma
26	64	IIIa	clear cell adenocarcinoma

cannot be detected[4,5]. MIB-1 is a murine monoclonal antibody that reacts with the native Ki-67 protein expressed by proliferating human cells[6]. Immunostaining with the monoclonal antibody MIB-1 provides a reliable means of rapidly identifying proliferating normal and neoplastic human cells in histological sections[7]. Previous studies have demonstrated that the proliferative index detected by Ki-67 antigen immunostaining is a useful factor for predicting the survival of patients with tumors of the lymphatic system[8], lung[9], central nervous system[10], and breast[11]. However, relatively little is known about the correlation between Ki-67 antigen immunostaining and the prognosis of ovarian cancer.

The aim of the present study is to investigate the potential usefulness of Ki-67 antigen expression as a predictor of outcome in ovarian cancer, through the analysis of MIB-1 monoclonal antibody reactivity. The correlation between this marker of cell proliferation and other prognostic parameters, e.g. clinical stage, histological findings and mitotic index, is also investigated.

Patients and Methods

Patients

Twenty-six patients with primary epithelial ovarian cancer served as subjects for this study. The patients underwent exploratory laparotomy at the Department of Obstetrics and Gynecology, Nippon Medical School Tama Nagayama Hospital between 1998 and 2000. All of these subjects provided written informed consent for participation in this study, which was approved by the institutional review board. As shown in **Table 1**, the patients ranged in age from 34 to 65 years (mean 48.3 years). Following the classification of FIGO, 15 patients had stage I disease, 2 had stage II, and 9 had stage III. With regard to histological type, 10

patients had serous cystadenocarcinoma, 7 patients had clear cell adenocarcinoma, 5 patients had endometrioid adenocarcinoma and 4 patients had mucinous cystadenocarcinoma.

Standard operative procedures were total abdominal hysterectomy, bilateral adnexectomy, omentectomy, and lymph node sampling. As a postoperative treatment, chemotherapy was performed in cases higher than stage Ic. Recurrence was defined as a demonstrable disease by both clinical and instrumental examinations such as magnetic resonance imaging and computed tomography. Patients were classified as alive without evidence of disease, alive with recurrence or progression of disease, or dead due to disease.

For each patient, tumor samples were taken at the time of primary surgery and processed at the Institute of pathology of Nippon Medical School Tama Nagayama Hospital as follows: specimens were fixed for 24 hours in neutral-buffered formalin (10%), dehydrated in alcohols, cleared in xylene and embedded in paraffin. One of the authors (S.M.) reviewed all the archival histological slides stained with hematoxylin and eosin, with the aim to control the quality of the histological material and select blocks for immunostaining.

Mitotic figures were identified by morphological features of metaphase, anaphase, or telophase. The mitotic index was calculated from the numbers of mitotic cells in 10 random high-power fields (×400). The sum total of mitotic cells among them was defined as the mitotic index. The indices were classified into one group of 0～30 and another group of 30 or more according to the method previously described[12].

Immunohistochemical Procedures

MIB-1 positive cells were determined with immunohistochemical analysis. MIB-1 rose against recombinant parts of the Ki-67 antigen and worked in formalin-fixed, paraffin-embedded tissue sections. The monoclonal antibody MIB-1 (Immunotech, Marseille, France) sufficiently reliably recognizes the Ki-67 antigen in routine materials[13].

Conventional 3-μm thick histological sections were cut from the sectioned blocks. The tissue sections were dewaxed in xylene, and rehydrated in a series of ethanol graded concentrations according to standards. Rehydrated slides were placed in plastic jars filled with 10 mM citrate buffer (pH 6.0) and heated. They were allowed to cool down to room temperature for 20 minutes and were briefly washed with Tris-buffered saline. Immunolocalization of Ki-67 antigen was performed using the DAKO LSAB kit (DAKO, Carpenteria, CA). Endogenous peroxidases were quenched with 0.3% hydrogen peroxide (H_2O_2) in methanol. The slides were then incubated with primary antibodies. Following 30 minutes of incubation with primary antibodies, they were rinsed gently with buffer solution from a wash and placed in a fresh buffer bath. They were incubated for an additional 10 minutes with a biotinylated antimouse IgG secondary antibody solution and were rinsed. Then they were incubated for 10 minutes with streptavidin-peroxidase conjugate. 0.06% 3, 3'-diaminobenzidine (DAB) with a peroxidase concentration of 0.03% was used as a chromogen. Finally they were counterstained with hematoxylin, and mounted in glycerin jelly.

For the analysis of MIB-1 immunostaining, sections were evaluated by one of the authors (S.M.) using a microscope. Multiple observers independently evaluated the same field on the monitor screen and a consensus on the final result was obtained. The Ki-67 labeling index (Ki-67 LI) in this study was defined as the percentage of MIB-1 positive cells in 1,000 randomly selected tumor cells under the same observation conditions. For judging positivity of immunostaining, only strong nuclear immunostaining was regarded as positive; weak nuclear or cytoplasmic staining was regarded as negative.

Time needed to analyze each case was approximately 40 minutes. Reproducibility was tested by duplicate evaluation in 6 cases, two for each diagnostic category, but no statistically significant differences were found.

Statistical Analysis

The disease-free survival was analyzed according to the methods of life table analysis described by Kaplan and Meier and with the log rank test.

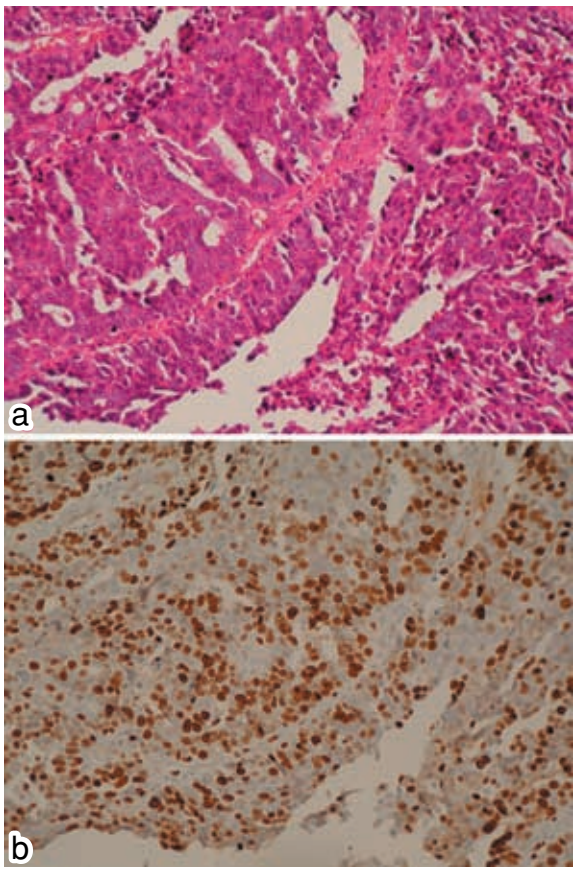

Fig. 1 H-E staining (a) and MIB-1 immunostaining (b) of serous ovarian carcinoma: (a) × 200; (b) × 200. MIB-1 immunostaining was confined to the nucleus and ranged from granular to diffuse. The immunostaining pattern was usually heterologous throughout the tumor; the evaluation was therefore done in the most positively stained areas.

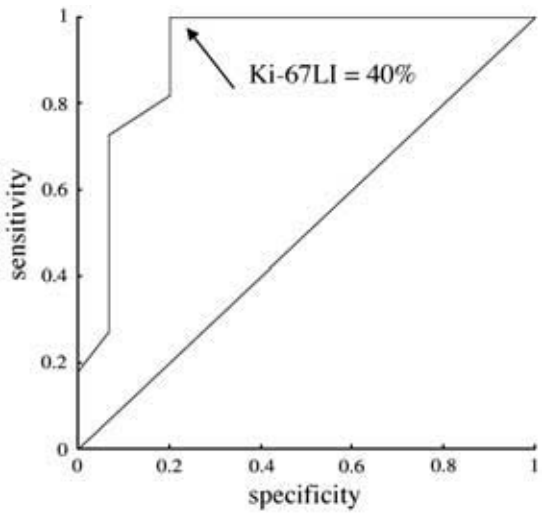

Fig. 2 The receiver operating characteristic (ROC) curve of Ki-67 LI that is relative to the probability of a disease free survival with primary epithelial ovarian cancer.

Regression analysis was used to evaluate relationships between Ki-67 LI and the mitotic index. The selection of an appropriate cut-off value for Ki-67 LI was determined using a receiver operating characteristic curve analysis. Evaluation of a possible statistically significant association between proliferative activity and other clinical and histopathological data was performed by chi-square test. Differences with a P value of less than 0.05 were considered to be statistically significant.

Results

In all the cases examined, MIB-1 immunostaining was confined to the nucleus and ranged from granular to diffuse, faint to intense; cytoplasmic positivity was observed only during mitosis (**Fig. 1**). The immunostaining pattern was usually heterogeneous throughout the tumor; the evaluation was therefore done in the most positively stained areas.

The value of Ki-67 LI ranged between 0 and 92.6% with a mean of 48.9%. A receiver operating characteristic curve analysis identified the optimal cut-off point for Ki-67 LI that is reactive to the probability of a disease-free survival (**Fig. 2**). Ki-67 LI of 40% was identified as the optimal cut-off value. Ki-67 LI of ≧40% was significantly associated with FIGO stage ($P = 0.04$) and mitotic index ($P < 0.0001$), whereas no significant association was found with histological type (**Table 2**).

As indicated in **Table 3**, the group of patients who had recurrence was comparable in terms of FIGO stage, histological type, Ki-67 LI and mitotic index, with the group who had no recurrence. The group of patients who had recurrence was significantly correlated with FIGO stage ($P = 0.02$), Ki-67 LI ($P = 0.0003$) and mitotic index ($P < 0.0001$), whereas there was no significant correlation with histological type.

The relationship between Ki-67 LI and mitotic index is listed in **Fig. 3**. There was a significant correlation between Ki-67 LI and mitotic index[13].

The usefulness of Ki-67 LI as a predictor of clinical outcome was also examined. The Kaplan-Meier

Table 2 The relationship between the clinicopathologic characteristics and Ki-67 LI

	Ki-67 LI ≧ 40 (n = 14)	Ki-67 LI < 40 (n = 12)	p value *
FIGO stage			
Ⅰ	5	10	
Ⅱ, Ⅲ	9	2	0.04
Histological type			
serous	6	4	
mucinous	3	1	
endometrioid	4	1	
clear cell	1	6	0.083
Mitotic index			
< 30	1	12	
≧ 30	13	0	< 0.0001

* For statistical evaluation, chi-square test was used.

Table 3 Clinicopathologic characteristics of patients who did not recur versus patients who recurred

	Patients who did not recur (n = 15)	Patients who recurred (n = 11)	P value
FIGO stage			
Ⅰ～Ⅱ	12	3	
Ⅲ	3	8	0.02
Histologic type			
Serous	5	5	
Mucinous	2	2	
Endometrioid	2	3	
Clear cell	6	1	0.357
Ki-67 LI			
≧ 40	12	0	
< 40	3	11	0.0003
Mitotic index			
≧ 30	13	0	
< 30	2	11	< 0.0001

disease free survival curves were generated for 14 patients (54% of all subjects) with Ki-67 LI≧40% and 12 patients with Ki-67 LI<40%. The Kaplan-Meier curves showed that patients with Ki-67 LI≧40% had significantly worse disease free survival than those with Ki-67 LI<40% (P<0.001) by log-rank analysis (**Fig. 4**).

Discussion

The main findings of this investigation demonstrate that patients with higher proliferating tumors had a statistically significant worse prognosis than patients with lower proliferating tumors. The results suggest the usefulness of Ki-67 antigen expression as a predictor of outcome in ovarian cancer. Because Ki-67 LI correlated well with the mitotic index, but not with histological subtype, the proliferating index is apparently more significant than the other known prognostic indicators and is probably independent of those markers. To the best of our knowledge, the current study is the first to define independently an optimal Ki-67 LI value that is relative to the probability of aggressiveness in ovarian cancer, thereby establishing what should be considered a higher proliferating tumor. A Ki-67 LI

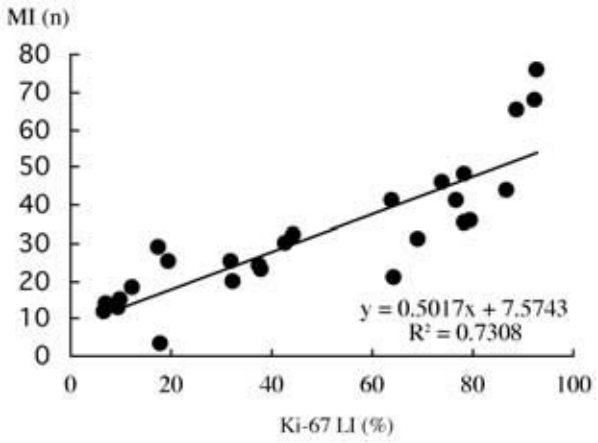

Fig. 3 The relationship between Ki-67 LI and mitotic index (MI). There was a significant correlation between Ki-67 LI and mitotic index (MI).

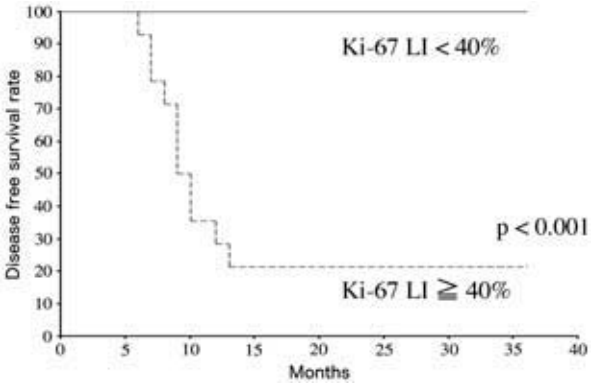

Fig. 4 Kaplan-Meier disease free survival curves with Ki-67 LI. Kaplan-Meier disease free curves showed that patients with Ki-67 LI of ≧ 40% had significantly worse disease free survival than those with Ki-67 LI value＜40% (P＜0.001) by log-rank analysis.

value of 40% was the optimal point defined by the receiver operating characteristic curve analysis.

The proliferative activity of a tumor has long been considered to bear a relationship to its clinical course, and recent reports[14–16] indicate that measurement of tumor cell proliferation yields useful prognostic information. There are several methods to measure cell proliferation. These include counting mitotic figures, S-phase fraction assessed by DNA flow cytometry, or immunostaining for proliferating cell nuclear antigen or MIB-1/Ki-67 index. MIB-1 monoclonal antibody is a reliable means of assessing the growth fraction of normal tissues and has been used to study the cell proliferation in various cancers[17–20].

Several studies in patients with ovarian tumor demonstrate the potential usefulness of Ki-67 antigen expression, through the analysis of MIB-1 monoclonal antibody reactivity. Isola et al.[21] assessed Ki-67 antigen reactivity and DNA index in 29 patients with different histological type of ovarian cancer; there was a significant relationship between high Ki-67 and high S-phase levels and advanced stage disease and shorter patient survival. Huettner et al.[22] also indicated that malignant neoplasms had a higher median percentage of Ki-67 staining than borderline and benign tumors. Recent studies[2,14] which examine the relationship between Ki-67 antigen expression and long-term survival have reported that the proliferating index is a good predictor of patient outcome in serous epithelial ovarian cancer. The authors[2,14] also demonstrate that there is no relationship between Ki-67 antigen expression and other clinicopathological features such as histological grade and FIGO stage, suggesting a prognostic significance of Ki-67 antigen expression probably independent of the other known prognostic indicators. In agreement with those results, our results on the analysis of disease-free survival demonstrated that patients with higher proliferating tumors have a statistically significant worse prognosis than patients with lower proliferating tumors, although we examined different histological subtypes, e.g. serous cystadenocarcinoma, clear cell adenocarcinoma, mucinous cystadenocarcinoma , and endometrioid adenocarcinoma. In addition, our results on the relationship between Ki-67 antigen expression and the other prognostic indicators, similarly to the previous data obtained in serous epithelial ovarian cancer, suggest that the proliferative index of Ki-67 may represent an additional useful prognostic factor independent of, or if not independent, then more specific than, the histological subtype.

In contrast to this, Hartmann et al.[23] determining the proliferative index in 92 untreated advanced epithelial ovarian cancers by means of proliferating cell nuclear antigen/cyclin immunostaining, obtained apparently conflicting results. In this retrospective

study, there was a significant inverse relationship between the tumor proliferating index and estimated 5-year survival, with significantly greater likelihood of survival in patients with more rapidly proliferating tumors. In clear cell adenocarcinoma, a recent study[24] also indicates that the survival rate for patients with high Ki-67 antigen expression was significantly greater than for those with low Ki-67 antigen expression and suggests that low proliferation activity may contribute to chemoresistance. These conflicting results in the prognostic value reflect the strict relationship between higher tumor proliferating rate and greater sensitivity to chemotherapy; however, it seems important to define if prognosis is more conditioned to drug sensitivity or to tumor biological characteristics because in this series different chemotherapic schedules have been used. The responsiveness to antiblastic drugs is related to improvement of quality of life and short-term survival rather than to overall survival increase. If a higher proliferative rate may render some tumors more sensitive to cytostatic agent with an increase in clinical response, the long-term prognosis for patients with ovarian cancer is only related to individual tumor characteristics, such as the tumor proliferation index, rather than to the treatment administered.

Another possible explanation for the conflicting results is the different choice of the cut-off point to discriminate between low and high proliferative index. In the present study, we used a receiver operating characteristic curve analysis to identify the optimal cutoff point for the Ki-67 LI value. The results differ from the findings of previous reports[2,3,14,24–26]. To our knowledge, however, there are no previous studies which evaluated the cut-off points by appropriate statistical methods because in these reports the cut-off points depend on the arbitrary selection of a decision threshold such as median or mean values. It is important that the reliability of these selections be carefully evaluated before they gain universal clinical acceptance. The receiver operating characteristic curve is shown to be a simple yet complete empirical description of this decision threshold effect, indicating all possible combinations of the relative frequencies of the various kinds of correct and incorrect decisions[27]. Thus, we believe that our results provide the optimal cut-off point of Ki-67 LI value that is relative to the probability of aggressiveness in ovarian cancer.

We acknowledge that our series was probably too limited to draw a conclusion because of the relatively small number of patients with ovarian cancer. There is a need for further study to confirm that the proliferative activity is an independent prognostic variable in several types of ovarian cancer. However, we believe that our results provide new insight into the prognosis of ovarian cancer and that the immunohistochemical testing for Ki-67 antigen should be incorporated into routine diagnosis. It may become possible to define a risk group, in which a more intensive follow-up control would be necessary for early relapse detection and for tumors of favorable degrees of malignancy.

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