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Assessment of Malignant Potential and Prognostic Correlation in Colorectal Carcinomas Using Monoclonal Antibody Ki-67.

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Summary: The growth fraction in 103 patients with colorectal carcinoma was determined using immunohistochemical staining with the monoclonal antibody Ki-67. The results were correlated with histopathologic findings and clinical outcome. Carcinomas showed a wide range of Ki-67 labeling index (LI), reflecting variation in proliferative activity. Ki-67 LI values paralleled those of the Mitotic Index and DNA polymerase α LI, but were not consistent with the results of DNA flow cytometric analysis, Ki-67 LI was significantly lower in carcinomas confined to the mucosa and submucosa than in those invading the muscularis propria. Poorly differentiated carcinomas demonstrated significantly high LI compared with those that were moderate or well differentiated. Ki-67 LI was particularly higher in carcinomas with lymphatic invasion and/or venous invasion than those without invasion. No correlation of Ki-67 LI with lymph node metastasis or liver metastasis could be found. Four recurrent cases had significantly high LI as compared with eighty-six cases with primary colorectal carcinoma. Carcinomas with high Ki-67 LI (greater than 30%) implicated in poor prognosis, whereas those with low Ki-67 LI demonstrated favorable prognosis.

Immunohistochemical staining of proliferating cells using the Ki-67 monoclonal antibody in not only colorectal carcinomas but also other carcinomas is useful for assessing malignant potential and prognosis.

Introduction

By means of conventional cancer therapy, various forms of adjuvant chemotherapy were performed according to the stage classification based on clinicopathologic findings after performing surgical resection. However, patients with good prognosis and with poor prognosis used to be often experienced, even though these cases were classified as the same stage by clinicopathologic stage classifications. This can be attributed to the proliferation and metastatic activities of individual cancer cells that cannot be evaluated by clinicopathologic evaluation of malignancy. Recently, there has been a trend toward performing treatment taking biological malignancy into consideration, such as flowcytometric DNA analysis, etc., in addition to clinicopathologic malignancy. Consequently, evaluation of proliferation activity of individual cancer cells as one index of biological malignancy appears to be useful.

Although the Mitotic Index[1][2] and labeling index by ^{3}H-thymidine[3][4] have been used in the past as methods for differentiating cancer cells in the proliferation phases, a special facility is needed for ^{3}H-thymidine because an isotope should be employed. Moreover, there are many problems with clinical use of this method. Analysis of the S phase cell percentage using monoclonal antibody for BrdUrd, which is a thymidine analog made by Gratzner, has recently been performed.[5][6] However, there are many difficulties with clinical use of this method due to theoretical problems pertaining to administration of BrdUrd in vivo and problems such as tissue infiltration by BrdUrd in vitro.

Monoclonal antibody Ki-67 is a monoclonal antibody that was first reported by Gerdes[7] in 1983 as an antibody for special nuclear fraction in L428 cells derived from Hodgkin's disease. It is said that during the cell cycle, this Ki-67 specifically recognizes cells in the late G_1, S, and G_2 /M phases of cells in the proliferation phases without reacting with cells in the quiescent phase (G_0 phase). Consequently, it appears that this is one index that shows proliferation activity of individual cancer cells. Moreover, cancer cells in the proliferation phases can be identified in various cancer tissues relatively easily by immunohistochemical staining.

Although there have been several reports in Japan of studies of the proliferating activity of colorectal carcinoma using this Ki-67 monoclonal antibody, there are relatively few subject cases and differences from the results of foreign reports are noted. Therefore, in order to clarify the true meaning of the studies, this study aims at comparing the results with clinicopathologic findings and studying the correlation with prognosis in 103 cases of colorectal carcinoma.

Materials and Methods

The eligible cases in this study were 105 lesions from 103 cases in which suitable samples were obtained from colorectal carcinoma that had been resected at the First Department of Surgery of Nagasaki University and affiliated hospitals from November 1988 to December 1990.

The male:female ratio was 67:36 and the mean age was 66.3 years. The site of the lesion was the cecum in 2 cases, the ascending colon in 13 cases (multiple carcinoma of the sigmoid colon was found in 1 of these cases), the transverse colon in 9 cases, the descending colon in 3 cases, the sigmoid colon in 29 cases, and the rectum in 44 cases (45 lesions; in one case there was multiple carcinoma the Rs and the Rb). Local recurrence was reported in 4 cases.

Samples were obtained from the edge of the tumor that had been surgically resected. These were immediately embedded in OCT compound and frozen and stored at − 80 ℃ . With regard to immunohistochemical staining, 5 μ frozen slices were prepared with a cryostat, the slices were dried, they were fixed for 5 minutes with acetone at 4 degrees ℃ , and then one slice was stained by conventional HE staining, while the immunohistochemical staining described below was performed on 2 slices by the avidin-biotin peroxidase complex method (ABC method). After performing a block of nonspecific reactions by first reacting the slices for 7 minutes with PBS to which 3% H_2O_2 had been added and then reacting the slices for 30 minutes with normal saline, they were reacted for 60 minutes with Ki-67 monoclonal antibody made by DAKO Company that had been diluted 50 times with PBS as the primary antibody. The slices were then reacted for 30 minutes with biotin-treated IgG and for 45 minutes with ABC complex as the secondary antibody using an ABC kit (Vectastain, Vector). Furthermore, the slices were washed with PBS after completing each procedure. Tris-buffer solution to which 0.025% 3,3'-diaminobenzidine (DAB) and 0.0075 % H_2O_2 had been added was used for coloration. The coloration time was 3 to 5 minutes. After rinsing with water, light nucleus staining was performed with hematoxylin. The samples were then dehydrated, sealed and microscopically observed.

When HeLa cells of subculture cancer cells in the logarithmic proliferation phases were stained, the entire nucleus or the nucleolus was stained brown, in Ki-67 positive cells the cytoplasm was not stained, and therefore, they could be readily differentiated from negative cells. The following tests were performed on clinical samples by staining and then calculating the ratio of Ki-67 antibody positive cells per 1000 cells and representing this % as the Labeling Index (LI) using HE stained specimens that had been simultaneously prepared as the reference.

The clinicopathologic parameters for colorectal carcinoma were recorded in accordance with General rules for clinical and pathological studies on cancer of colon, rectum and anus,[8] and stage classification was in accordance with the Dukes classification. Moreover, the significant difference between the 2 groups was studied by the Wilcoxon test. The cumulative survival rate was calculated by the Kaplan-Meier method and the significant difference between cumulative survival rates was determined by the Generalized Wilcoxon method.

Results

1) Correlation with cell biological parameters

When the correlation between the Mitotic Index, which is considered to represent the mitotic capability of cancer cells, and the LI by Ki-67 antibody is studied, a correlation is obtained at r=0.70 and therefore, it is concluded that the LI by Ki-67 antibody reflects one aspect of proliferative activity (Fig. 2-1).

Ki-67 antibody appears to react with cells from the late G_1 phase to the G_2/M phase in the cell cycle. However, when the author studied the correlation between the LI by DNA polymerase α , which reacts with all cells in the proliferation phases, and the LI by Ki-67 antibody, although a correlation was seen to a certain extent at r = 0.61, in 15 of 18 cases a tendency was seen for the LI by DNA polymerase α , to be higher than the LI by Ki-67 (Fig. 2-2).

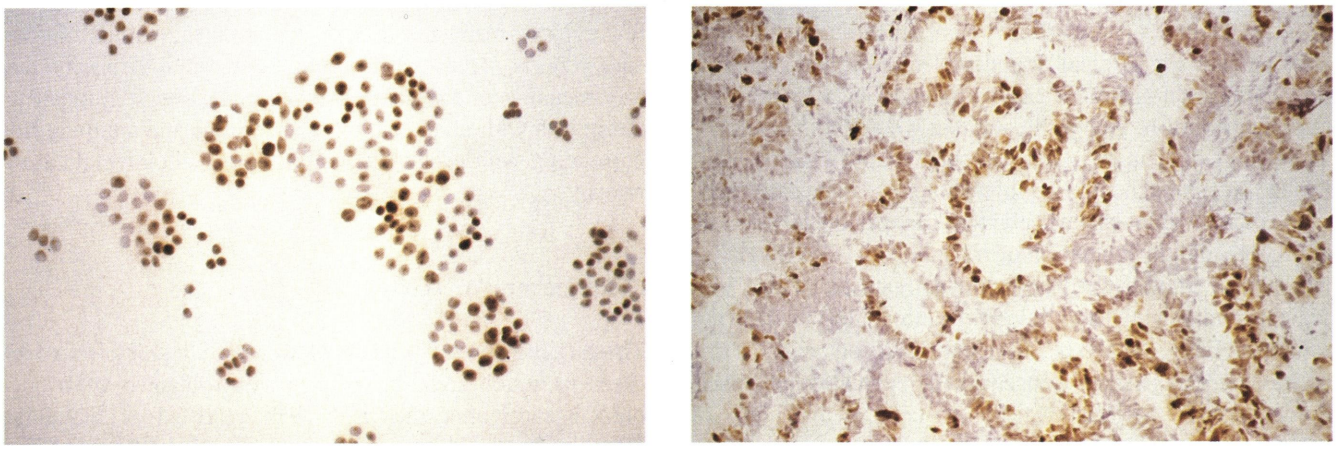

Fig. 1. Photomicrograph of HeLa cell (left) and colon carcinoma (right) stained with monoclonal antibody Ki-67.

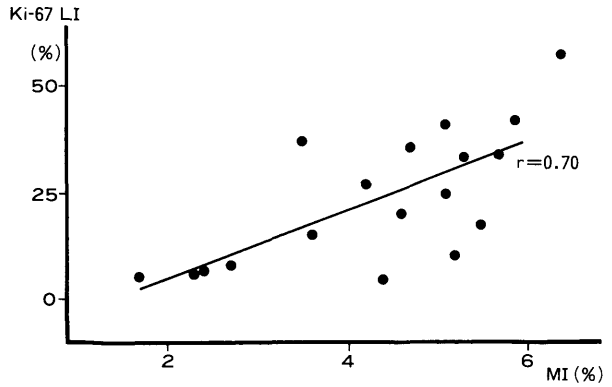

Fig. 2-1. The relation of the Ki-67 Labeling Index with the Mitotic Index (above)

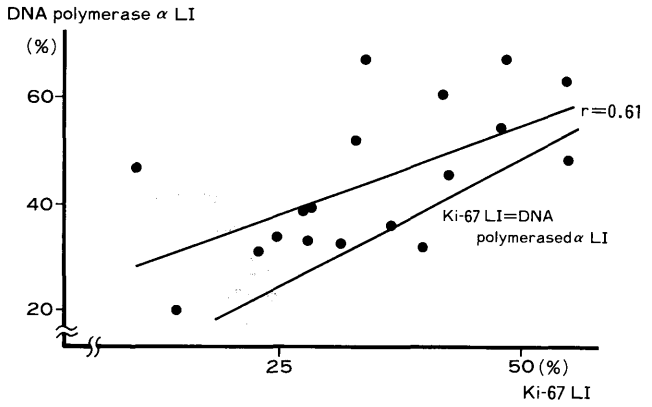

Fig. 2-2. The relation of Labeling Indices between Ki-67 and DNA polymerase α (below)

Moreover, when the total DNA content, which has recently received attention, and LI are compared, in contrast to the fact that LI is 22.7 ± 14.6 in diploid cases, and 33.4 ± 17.7 in aneuploid cases, showing that the LI in aneuploid cases are somewhat higher. However, there is not a significant difference.

2) Correlation with clinicopathologic parameters

With regard to the correlation between macroscopic morphologic classification and the Ki-67 LI, the LI in type 3 and 4, which are invasive type, was 35.2 ± 12.2. when compared to the 22.1 ± 13.0 of type 1 and the 25.5 ± 13.0 of type 2, which are localized forms, the LI of type 3 and 4 was significantly higher (Table 1).

By histologic types, the LI of poorly differentiated adenocarcinoma was 45.3 ± 11.3, the LI of moderately differentiated adenocarcinoma was 26.6 ± 13.9 and the LI of well differentiated adenocarcinoma was 24.1 ± 12.2. Thus, the LI of poorly differentiated adenocarcinoma was significantly higher than that of moderately and well differentiated adenocarcinoma. Moreover, the LI of one case of squamous cell carcinoma and 2 cases of mucinous carcinoma was relatively high when compared to that of adenocarcinoma.

With regard to the correlation between the histologic depth of carcinoma and the Ki-67 LI, the LI of m and sm carcinomas, which are cancers in the early stage, was 13.8 ± 4.8 and when compared to advanced cancer of pm or deeper, the LI was significantly low. Moreover, when compared to the mean LI of all cases, which was 26.4, the LI of cancers in the early stage was low. On the other hand, a difference from the mean LI was not seen in advanced cancers of pm or deeper (Fig. 3).

Table 1. Correlation of Ki-67 LI and clinicopathologic fidings

Gross type		
Type 1	22.1±13.0 (n=13)	*
Type 2	25.5±13.0 (n=76)	**
Type 3, 4	35.2±12.7 (n=10)	*
Type 5	39.3 (n= 1)	
Histologic type		
Well differentiated	24.1±12.2 (n=42)	
Moderate differentiated	26.6±13.9 (n=53)	**
Poorly differentiated	45.3±11.3 (n= 3)	*
others	37.0± 7.1 (n= 3)	
Wall invasion		
m, sm	13.8± 4.8 (n=11)	*
pm	32.3±16.0 (n=18)	*
SS, a_1	25.8±12.9 (n=53)	*
S, Si, a_2, ai	29.9±11.3 (n=19)	
Stage		
Dukes A	18.4± 8.2 (n=17)	**
Dukes B	27.4±12.9 (n=38)	*
Dukes C	26.8±13.6 (n=32)	**
Dukes D	31.1±12.3 (n= 9)	
Lymphatic invasion		
negative	18.3± 9.7 (n=20)	**
positive	28.6±13.6 (n=80)	*
ly1	24.7±13.3 (n=37)	**
ly2	30.9±12.3 (n=34)	*
ly3	35.9±15.6 (n= 9)	**
Vessel invasion		
negative	24.0±12.7 (n=74)	**
positive	33.8±13.3 (n=26)	
Metastasis		
DukesA, B	23.5±12.5 (n=47)	**
Lymph node metastasis positive	27.6±15.1 (n=34)	
Liver metastasis positive	21.2± 9.8 (n= 5)	
Peritoneal dissemination positive	48.9± 9.7 (n= 4)	
DNA flow cytometric analysis		
Diploid	22.7±14.6 (n=17)	
Aneuploid	33.4±17.7 (n= 9)	
Reccurence		
primary lesion	26.4±13.5 (n=86)	**
local reccurent lesion	41.6± 6.9 (n= 4)	

*=P<0.05 **=P<0.01

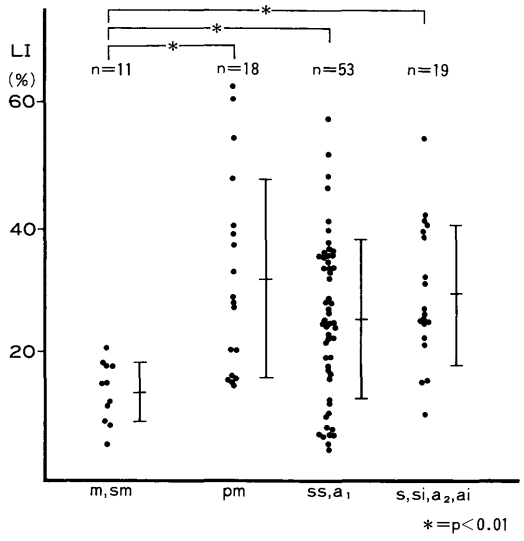

Fig. 3. Correlation of Ki-67 LI and wall invasion

In terms of the presence of lymph node metastasis, while the LI of primary lesions in cases where metastasis was histologically positive was 27.6 ± 15.1, the LI of primary lesions in which metastasis was negative was 23.5 ± 12.5 and a significant difference was not found. As for the extent of lymph node metastasis, the LI was 52.1 ± 12.1 for primary lesions in n_4 cases and the LI was significantly higher, when compared to that of n_0-n_3 cases.

With respect to the Ki-67 LI and the Dukes classification, the LI of Dukes A cases was 18.4 ± 8.2, and it was apparently lower as compared with the LI of Dukes B. This is obvious that the LI in early stages was significantly lower. Although it is reported that there is almost no difference in LI values between Dukes B and Dukes C cases, the LI was 32.9 ± 17.0 in 6 cases of the Dukes C in which lymphnode metastasis was positive with the depth of pm. This was high when compared to the LI of the Dukes A cases. Moreover, the mean LI of the Dukes D cases was somewhat higher as compared to the other grades.

The LI in 5 cases with simultaneous liver positive metastasis was 21.1 ± 9.8 and was not significantly different when compared to that with negative. On the other hand, the LI in cases of peritoneal dissemination was 48.9 ± 9.7 and was significantly higher than that with negative.

With regard to the correlation between the presence of vascular invasion and LI, the LI in negative cases of lymphatic invasion was 18.3 ± 9.7. In contrast, the LI in positive cases was 28.6 ± 13.6. Moreover, the LI in negative of venous invasion was 24.0 ± 13.7, meanwhile, the LI in positive cases was 33.8 ± 13.3, showing a significantly higher LI than negative cases. Moreover, there was a tendency for the LI to rise significantly in accordance with the degrees of lymphatic invasion.

As compared the LI between primary lesions and recurring lesions, the LI of primary lesions was 26.4 ± 13.5, the LI of recurring lesions in 4 was 41.6 ± 6.9, with a significantly higher value.

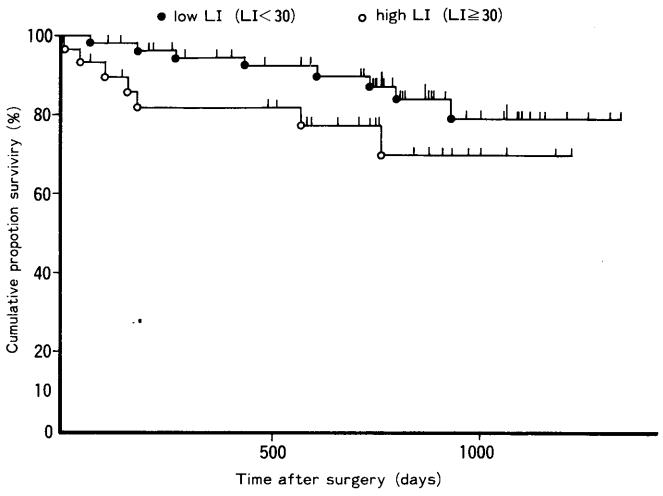

Fig. 4. Overall survival in patients with colo-rectal carcinomas showing high Ki-67 LI and low LI.

The correlation between the Ki-67 LI and the prognosis was evaluated in 67 cases in whom the prognosis for 2 years following surgery was defined, comprised of 55 patients who survived for 2 years and 12 patients who died from cancer recurrence, the LI of the surviving cases was 24.7 ± 12.8, while that of death due to cancer was 30.1 ± 14.5. Although the difference was not significant, there was a tendency for the LI to be higher in the latter cases. Moreover, the survival rates were compared between more than or less than 30 of LI in the 86 patients whose prognosis were clarified. There was no statistically significant difference although showing a favorable prognosis in patients with less than 30 of LI. (Fig. 4).

Discussion

Malignancy of solid tumors had been evaluated by determining clinical stage based on pathological study of the resected sample and taking into consideration pathological findings, such as the degree of differentiation, the presence of vascular invasion, etc. Evaluation of malignancy based on clinicopathology do not coincide with prognosis of each case. Therefore, this research was performed as to whether evaluations of proliferation activity of individual cancer cells can provide more accurate evaluations of malignancy or not.

It is known that monoclonal antibody Ki-67 specifically recognizes cells in the proliferation phases (late G_1 phase to G_2/M phase) without reacting with cells in the quiescent phase (G_0 phase) during the cell cycle. Sasaki et al.[9] clarified an increase in the amount of Ki-67 antigen from the G_1 phase to the S-G_2/M phase in analyses using FCM double staining of Ki-67 antigen and nuclear DNA and the results similar to those were obtained in this series. When immunohistochemical staining is performed using this monoclonal antibody Ki-67, cells in the proliferation phases can be identified relatively easily, as shown in the staining results with Hela cells, and therefore, colorectal carcinoma cells in the proliferation phases were analyzed using this antibody and the clinicopathologic findings and prognosis were compared.

With regard to Ki-67 monoclonal antibody and other indices of proliferation activity, Sasaki et al.[10] and Nishizaki et al.[11] noted that there was a close correlation between the LI by Ki-67 antibody and the LI by BrdUrd. Isola et al.[12] reported that there was a correlation between the Ki-67 LI and Mitotic Index. In the studies, there was a good correlation of Ki-67 LI with the Mitotic Index and there was a correlation with the LI by DNA polymerase α, which is said to react with all cells in the proliferation phases. Consequently, it appears that LI by Ki-67 is an accurate index showing one aspect of proliferation activity of cancer cells. Moreover, with regard to the correlation with the nuclear DNA that attention has been recently paid,

Nishizaki et al.[11] reported that there was a tendency for the Ki-67 LI to be somewhat higher in aneuploid cases when compared to diploid cases. The course of the disease in 3 of 13 cases in whom malignant meningioma were clinicopathologically diagnosed was fair. Although these three cases were aneuploid cases, the Ki-67 LI and BrdUrd labeling rate were not reported to be high. On the other hand, Isola et al.[12] reported that the LI of aneuploid cases was significantly higher than the LI of diploid cases. Therefore, the results varied with the report. In this series, there was no significant difference between diploid and aneuploid in DNA analysis in spite of somewhat higher LI in aneuploid cases. Consequently, it appears that the LI by Ki-67 antibody is a valid index of biological malignancy that stands apart from nuclear DNA.

There have been several reports of the results of studies using this antibody for tumors in other sites: Burger et al.[13] and Giangaspero et al.[14] reported that in analysis in cases of brain tumor, this antibody had been useful in confirming cells in the proliferation phases. Soyer et al.[15] noted that Ki-67 LI reflected well proliferative activity in cases of melanocytic skin tumor and was useful in diagnosis and evaluation of prognosis. Moreover, Hoang et al.[16] and Johnston et al.[17] reported that when the Ki-67 LI was compared between colorectal carcinomas and adenomas, the LI was significantly higher in carcinomas. Lelle et al.[18] compared the LI between benign tumors of the breast and breast cancer and noted that the LI in breast cancer was approximately 3 times higher than that in benign tumors. Moreover, Gerdes et al.[19] reported that the LI of benign tumors of the breast was 3 %, that of breast cancer was 16.6 %. The author also compared the LI of normal tissue and of cancer tissue in colorectal carcinomas and lung cancers. The LI in normal tissue was 10% or less and was significantly lower when compared to the LI of cancer tissue. Moreover, since it was found that reliable staining results were obtained from biopsied tissues, the LI was usefull for differentiating cancer from severe dysplasia in preoperatively biopsied specimens.

Furthermore, there are many reports pertaining to a comparison with clinicopathologic parameters. Gerdes et al.[19] clarified a close correlation with the grading of breast cancer, but they emphasized the fact that a low LI was presented even when histologically classified as Grade 3. Moreover, Walker et al.[20] reported the same findings. Isola et al.[12] noted that although LI showed a correlation with grading of breast cancer, it did not correlate with the presence of lymph node metastasis. Yonemura et al.[21] clarified the higher possibility of lymph node metastasis in cases of the LI of 22% or higher in carcinomas of the stomach, indicating more significant lymphatic invasion or more severe serous invasion rather than in cases of the LI below 22%.

In comparison of the Ki-67 LI with clinicopathological findings in colorectal carcinoma, Shepherd et al.[22] stressed in 108 patients with colorectal carcinoma that there was no correlation between the Ki-67 derived proliferative score and known prognostic parameters, such as the Dukes' classification, New Prognostic Classification grade, lymph node involvement, tumor cell differentiation, venous spreading, invasive margin, lymphocytic infiltrate around the tumor, and curative versus palliative surgery. Porschen et al.[23] reported that there was no correlation between Ki-67 staining and various clinicopathological features, including age, sex, the tumor size, tumor cell differentiation, the location and the tumor stage, and the Ki-67 LI increased in recurrent carcinomas. Moreover, Hoang et al.[16] concluded that there was no difference in the 21 patients with colorectal carcinoma between Ki-67 LI and the depth of cancer infiltration or the degree of cell differentiation. Johnstone et al.[17] reported that the degree of cell differentiation and Dukes' stage in the 28 patients with colorectal carcinoma did not correlate with Ki-67 LI.

In Japan Yamaguchi et al.[24] reported that the LI was significantly high in 32 carcinomas with the findings of invasive types, poorly differentiated adenocarcinoma, positive venous invasion and lymph node metastasis, and that there was a close correlation between the LI and the depth of the tumor. Moreover, Terabe[25] reported that the LI was significantly higher in patients with submucosal invasion rather than those with limited mucosal invasion in analysis of the 56 patients. Moreover, the LI of patients with positive lymphatic invasion was significantly higher than those with negative cases, but it has no correlation with other clinicopathologic factors, including the serum CEA level.

Thus, there are some differences between the results reported in Japan and in other countries. The reason for this appears to be based on the fact that few cases were used as subjects and therefore, more accumulated data is required.

In this series, the LI was significantly high in poorly differentiated adenocarcinoma when compared to moderately and well differentiated adenocarcinomas. Moreover, the levels of the LI were more significantly elevated in advanced cancers than those in the early stage. In addition, when compared to localized type, the LI in invasive cancers was significantly higher. However, a difference in LI based on the presence of lymph node metastasis was not found. This appears to indicate that lymph node metastasis is caused not only by the malignant behavior of the cancer cells but also by other factors. For instance, it is sometimes experienced that the tumors with biologically malignant behaviors dose not show any lymph node metastasis. This is also confirmed by the fact that although there is no correlation between the LI and the presence of lymph node metastasis, there is a good correlation between the LI and the presence and extent of vascular invasion. Agrez et al.[26], Minsky et al.[27], Hatta[28] and Shiromizu et al.[29] reported that a close correlation was seen between the extent of lymphatic invasion and prognosis, and their results emphasized

to be usefulness of Ki-67 LI for the evaluation of prognosis. Although, there was no correlation with the LI of primary lesions and the presence of liver metastasis, it was very interesting that there was a good correlation with the LI of primary lesions and the presence of peritoneal disseminated metastasis, and showing high LI values in localized recurrent lesions.

Retrospective studies fail to be performed on the prognosis for the reasons of not applicable method of immunostaining with antibody. However, Suzuki et al.[30] studied a correlation with their prognosis in 56 patients with colorectal cancer and reported the results of comparative study in the survival times between high LI group of 21 patients and low one of 35 patients. The survival times were not significantly different between the two groups. In the series, the LI of patients who died of recurrent cancer within less than 2 years following surgery showed a tendency to be higher when compared with the LI of patients who survived for 2 years or more. Based on the cumulative survival rate, there was a tendency for prognosis to be poor in cases of a high LI when compared to cases of a low LI. This result is different from the report of Suzuki et al.[30]. Long-term prognosis should be investigated in the future by follow-up study. Moreover, it is dubious at the present time as to what is a reasonable cut off value of the LI. In this study the cut off values of 30 % was used as a reference.

The results in this series indicate that when cancer invasion reaches and extends the submucosa, rapid proliferatic activity must be enhanced by some types of proliferation promoting factors. Moreover, when cancer cells acquire a rapid proliferatic activity, they involve the surrounding tissue so that the tumor grow rapidly and become an invasive type macroscopically, demonstrating a histologic pattern of venous and lymph vessel invasion.

In conclusion the immunohistochemical staining method with monoclonal antibody of Ki-67 makes it possible to evaluate the aggressive grade of biologic behavior of malignant cells and to know the prognosis more accurately. Therefore it benefits from preventing recurrence by help of the use of potent anticancer agents. In addition, the LI is more sensitive to implantable cells such as peritoneal dissemination so that the LI response to nodal involvement is different from that to peritoneal dissemination. The LI of Ki-67 is specific for implantable cells. Therefore, this method is of great use in estimating cell origins of tumor progression to differentiate direct spreading cells from implantable or disseminated cells.

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