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Relationship between the Responses to Simultaneous Double Staining for Ki-67 and AgNOR and the Clinicopathological Features of Non-Small Cell Pulmonary Carcinoma

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Samples of stage I to III non-small cell pulmonary carcinoma, resected from 51 patients, were simultaneously doublestained for Ki-67 and AgNOR (argyrophil nucleolar organizer region). The responses to the staining was analyzed in relation to the stage, degree of histological differentiation, extent of pulmonary tumor (factor T), extent of lymph node metastasis (factor N) and DNA ploidy of non-small cell pulmonary carcinoma.

The AgNOR count was significantly higer for Ki-67 positive cells than for Ki-67 negative cells. The Ki-67 positive rate, the AgNOR count in Ki-67 positive cells and the AgNOR count in Ki-67 negative cells differed significantly between stage I and III. The only significant difference observed between well differentiated carcinoma and poorly differentiated carcinoma was the Ki-67 positive rate. When analyzed in more detail, factor T was found to have no correlation with the Ki-67 positive rate. The AgNOR count in Ki-67 positive cells was significantly elevated only in T4 cases which are thought to have a very poor prognosis. When factor N was analyzed, there were significant differences between N1 and N2 cases in terms of the Ki-67 positive rate and the AgNOR count in Ki-67 positive cells. The Ki-67 positive rate and AgNOR count in Ki-67 positive cells were significantly high in N2 and N3 cases. When the AgNOR count was divided into more categories, all cases with less than 18 AgNOR count in Ki-67 positive cells were rated as N0 or N1, while most cases with 20 or more AgNOR count were rated as N2 or N3. These results demonstrate the usefulness of simultaneous double staining for Ki-67 and AgNOR as a means of assessing the biological mailgnancy level of non-small cell pulmonary carcinoma.

Introduction

The number of argyrophil nucleolar organizer region (AgNOR) has been considered to reflect the proliferating activity of cells and to serve as an indicator of the malignancy level of tumor. Comparison of the number of AgNOR between non-proliferating cells and proliferating cells is expected to allow a more detailed assessment of the proliferating activety of cancer cells.

We recently carried out simultaneous double staining of frozen samples of non-small cell pulmonary carcinoma (removed surgically from 51 patients) for Ki-67 (a proliferation-associated antigen) and AgNOR (argyrophil regions of the nucleolus indicating the proliferating activity). The responses of carcinoma to the staining were analyzed in relation to the clinical stage, degree of histological differentiation, extent of pulmonary tumor (factor T), extent of lymph node metastasis (factor N) and DNA ploidy of non-small cell pulmonary carcinoma. Emphasis was placed on analyzing the AgNOR count in Ki-67 positive cells for N2 cases which are known to have a quite poor prognosis.

Materials and Methods

(1) Subjects

The subjects were 51 patients with non-small cell pulmonary carcinoma who were treated surgically at the First Department of Surgery, Nagasaki University Hospital between April 1991 and February 1993. The carcinoma was classified as adenocarcinoma in 31 cases and squamous cell carcinoma in 20 cases. There were 17 cases of well differentiated carcinoma, 25 cases of moderately differentiated carcinoma and 9 cases of poorly differentiated carcinoma. The stage, T and N were assessed, using the conventional method used for assessing the pathologic status. The stage of carcinoma was I in 17 cases, II in 8 cases and III in 26 cases (III a in 16 cases and IIIb in 10 cases). Stage IV cases were excluded from this study (Table 1).

(2) Immunohistochemical staining

Double staining for Ki-67 and AgNOR

Tissues resected surgically were immediately immersed in OCT compound and frozen at −80 ℃. Frozen tissue was then cut into 4 μm slices and fixed on a silane-coated glass slide and dried by air for 20 minutes. The sample was then fixed in 4 % paraformaldehyde (PFA) for 30 minutes and washed in 0.01 M PBS twice (for 5 minutes each time).

Table 1 Summary of 51cases of non-small cell lung cancers

Sex	♂ : ♀	35 : 16
Age	Mean: 60 age	(48 ~73)
Type of carcinoma	adeno	31 cases
	squamous	20 cases
Histologic grade	Well	17 cases
	Moderately	25 cases
	Poorly	9 cases
Stage	stage I	17 cases
	stage II	8 cases
	stage IIIa	16 cases
	stage IIIb	10 cases
DNA ploidy	diploidy	22 cases
	aneuploidy	29 cases

Immunohistochemical staining was performed by the labeled streptoavidin-biotin (LSAB) method, using a DAKO LSAB kit and alkaline phoshatase. First, the sample was immersed in a blocking solution for 8 minutes. The sample was then incubated at 4 ℃ for 24 hours in the presence of the first antibody, i. e., anti-Ki-67 antibody (DAKO) which had been diluted 1 : 30 with 0.1 M PBS. After the sample was washed in 0.1 M PBS twice (for 5 minutes each time), it was exposed to the second antibody for 15 minutes. The sample was then washed in 0.01 M PBS twice (for 5 minutes each time) and incubated in the presence of streptoavidin for 15 minutes. After the sample was washed in 0.01 M FBS twice (for 5 minutes each time), it was exposed to the Fast Red reagent and observed under a microscope for 4-20 minutes. When the response to this reagent reached a peak, the sample was washed in distilled water three times (for 5 minutes each time). In a dark-room, the sample was immersed for 40 minutes in AgNOR solution, i. e., a 1 : 2 mixture of 2% gelatin-added 1% formic acid solution and 50% silver nitrate solution. The sample was then washed in distilled water twice (for 5 minutes each time) and in running water for 10 minutes. After decoloration using sodium thiosulfate, the sample was observed under a microscope for 1 minutes to 5 minutes. The decolored sample was then immersed in distilled water, followed by aqueous mounting.

(3) Evaluation

Ki-67 labeling index

The slide samples double-stained for Ki-67 AgNOR were observed under a light microscope at a magnification of x200. The chromatic response of 1000 nuclei in the most markedly stained area was assessed to calculate the labeling index (LI).

AgNOR counts in double-stained slide samples

Using the oil immersion technique, the stained sample was carefully observed under a light microscope at a magnification of x1000. The number of AgNOR in 100 Ki-67 positive nuclei and 100 Ki-67 negative nuclei was counted, and the average AgNOR count per nucleus was calculated. M phase cells were excluded from evaluation.

LI and AgNOR counts were analyzed in relation to the clinical stage, degree of histological differentiation, factor T, factor N and DNA ploidy. Differences in data were tested using Wilcoxon test. $P < 0.01$ was regarded significant.

Results

Table 1 shows the details of the 51 patients with non-small cell pulmonary carcinoma. Fig. 1 shows a representative tissue sample double-stained for Ki-67 and AgNOR. In this figure, Ki-67 positive cells have been stained red with Fast Red, and AgNOR is visible as black dots in the nuclei.

Table 2 shows the relationship of Ki-67 LI and the AgNOR counts in Ki-67 positive and negative cells to the stage and degree of histological differentiation of carcinoma. The Ki-67 LI did not differ significantly between any two of stage I through IIIa. It was significantly higher at stage IIIb than at any other stage ($p < 0.005$). Both the AgNOR count in Ki-67 positive cells and the AgNOR count in Ki-67 negative cells differed significantly between stage I and IIIa ($p < 0.05$, $p < 0.01$) and between stage I and IIIb ($p < 0.01$, $p < 0.005$). Thus, AgNOR counts increased significantly as the tumor stage advanced.

Analysis of the relationship between Ki-67 LI and the

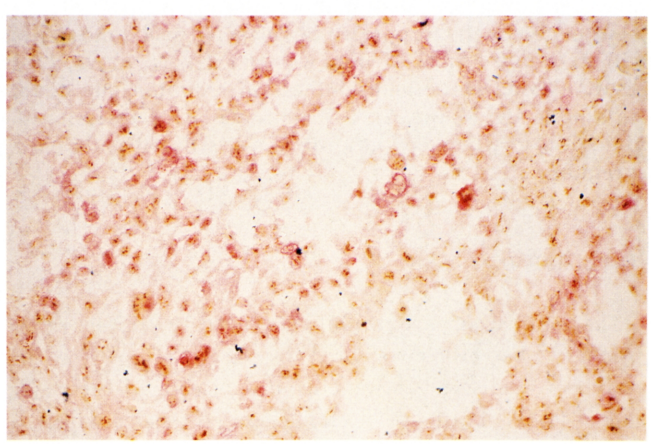

Fig. 1 Tissue section double-stained for Ki-67 and AgNOR. Ki-67 positive cells have been stained red with Fast Red, and AgNOR is visible as black dots in the nuclei.

Table 2 Ki-67 LI and AgNOR Count of Ki-67 positive and negative cells in relation to stage and histological grade.

		KI-67 LI		Ki-67positive AgNORcount		Ki-67negative AgNORcount		
stage	I	15.87 (5.19)		18.01 (2.40)		8.95 (1.48)		
	II	18.56 (3.13)		18.27 (2.42)		9.47 (1.78)		
	III		*2		*1		*1	*2
	IIIa	15.87 (5.19)		19.41 (1.44)		10.16 (1.09)		
	IIIb	23.67 (5.78)		20.51 (2.04)		11.19 (1.68)		
Histologic grade								
Well		15.18 (5.81)		18.21 (2.05)		9.30 (1.38)		
Moderately		20.17 (5.06)	*1	19.42 (2.46)		10.13 (1.82)		
Poorly		22.0 (4.41)		19.21 (1.77)		10.13 (1.44)		
Mean		18.83 (5.78)		18.98 (2.25)		9.85 (1.64)		

*1 $p < 0.01$, *2 $p < 0.005$, *3 $p < 0.001$ Mean (±SD)

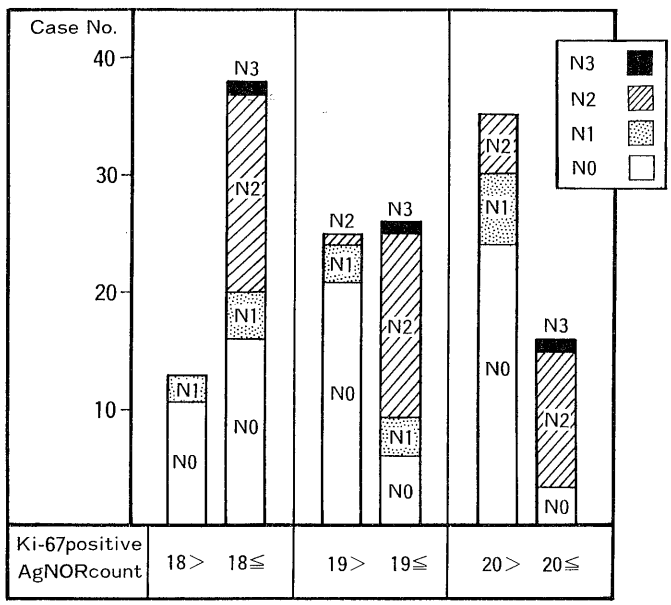

Fig. 2 AgNOR count of Ki-67 positive cells in relation to pN-factor

degree of histological differentiation revealed a significant difference in Ki-67 LI between well-differentiated carcinoma and poorly differentiated carcinoma ($p<0.01$). Thus, Ki-67 LI increased as the degree of differentiation was lower. No particular correlation was noted between the degree of differentiation and the AgNOR count in Ki-67 positive or negative cells.

Following these findings, we analyzed the Ki-67 LI and the AgNOR counts in Ki-67 positive and negative cells in relation to factor T, factor N and DNA ploidy (a cell biological indicator of malignancy level) (Table 3). The Ki-67 LI did not differ significantly between any two categories of T. The AgNOR counts in Ki-67 positive or negative cells showed no significant difference between any two of T1, T2 and T3. The AgNOR count in Ki-67 positive cells was significantly higher in T4 cases than in T3 cases, and the AgNOR count in Ki-67 negative cells was significantly higher in T4 cases than in T1 cases.

As for the relationship with factor N, both the Ki-67 LI and the AgNOR count in Ki-67 positive cells differed significantly between N1 and N2 cases ($p<0.01$). Although only one N3 case was included in the subjects, we noted that both the Ki-67 LI and the AgNOR count in Ki-67 positive cells increased as the extent of lymph node metastasis became higher, and that both parameters differed significantly between the N0+N1 group and the N2+N3 group ($p<0.001$). The AgNOR count in Ki-67 negative cells was not related to factor N.

When the relationship with DNA ploidy was analyzed, neither the Ki-67 LI nor the AgNOR count in Ki-67 positive or negative cells differed between diploid cases and aneuploid cases. When the average of each parameter for all subjects was calculated, Ki-67 LI averaged 18.8, the AgNOR count in Ki-67 positive cells averaged 18.98, and the AgNOR count in Ki-67 negative cells averaged 9.85. AgNOR counts were significantly higher in Ki-67 positive cells than in Ki-67 negative cells.

In patients whose AgNOR count in Ki-67 positive cells was between 17 and 21, the extent of lymph node metastasis was rated as N2 or lower in 13 (100 %) of the 13 cases with an AgNOR count less than 18, and in 24 (96 %) of the 25 cases with an AgNOR count less than 19 (Fig. 2). Because the average Ki-67 LI (18.83) was very close to the average AgNOR count in Ki-67 positive cells, we then analyzed those patients in whom the Ki-67 LI and the AgNOR count in Ki-67 positive cells were 18, 19, 19.5, 20 or

Table 3 Ki-67 and AgNOR count of Ki-67 positive and negative cells in relation to T-factor, pN-factor and DNA ploidy.

		Ki-67 LI		Ki-67positive AgNORcount		Ki-67negative AgNORcount	
T-factor	T1	16.11(6.43)		17.96 (2.68)		8.95 (1.46)	*1
	T2	18.71(4.43)		18.83 (2.41)		9.56 (1.71)	
	T3	16.47(5.66)		18.71 (0.94)	*1	9.92 (0.86)	
	T4	23.67(5.79)		20.51 (2.04)		11.19 (1.68)	
pN-factor	NO	16.2(5.54)		18.11 (2.02)		9.80 (1.55)	
	N1	18.21(3.10)	*1	17.6 (2.44)	*1	8.95 (1.77)	
	N2	22.28(3.34)		20.6 (1.23)		10.9 (1.08)	
	N3	34.8		23.1		12.3	
	N0. N1	16.56(5.20)	*3	18.02 (2.07)	*3	9.24 (1.54)	
	N2. N3	22.98(4.28)		20.73 (1.33)		10.97 (1.10)	
DNAploidy							
diploidy		17.36(6.09)		18.13 (2.42)		9.35 (1.50)	
aneuploidy		19.94(5.37)		19.62 (1.91)		10.23 (1.66)	

*1 $p < 0.01$, *2 $p < 0.005$, *3 $p < 0.001$ Mean (±SD)

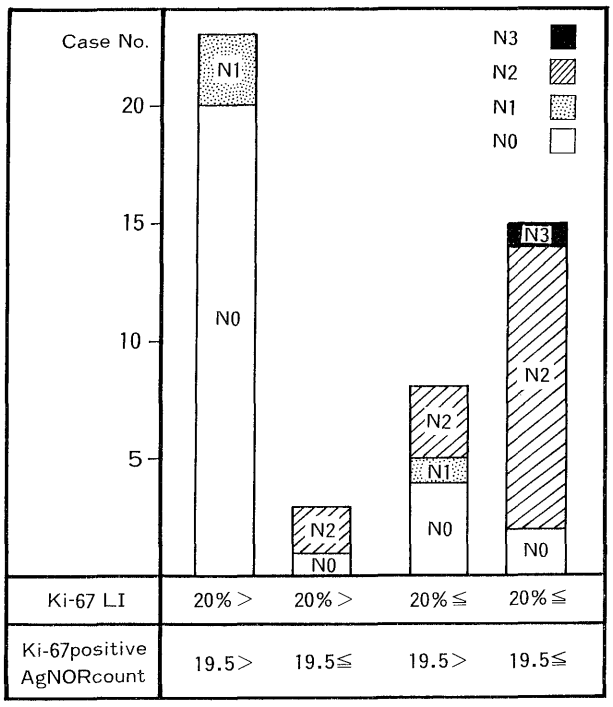

Fig. 3 Ki-67 LI and AgNOR count of Ki-67 positive cells in relation to pN-factor.

21. When patients were divided into two groups on the basis of Ki-67 LI (less than or over 20 %) or the AgNOR count in Ki-67 positive cells (less than or over 19.5), differences in the extent of lymph node metastasis (N1 or lower vs. N2 or higher) tended to be more clearly reflected in Ki-67 LI and AgNOR count. Of the 15 patients in whom Ki-67 LI was over 20 % and the AgNOR count in Ki-67 cells was over 19.5, 13 patients (87 %) were rated as N2 or higher. The extent of lymph node metastasis was N1 or lower in all patients in whom Ki-67 was less than 20 % and the AgNOR count in Ki-67 positive cells was less than 19.5 (Fig. 3).

Discussion

Non-small cell pulmonary carcinoma is one of the cancers which are likely to recur soon after surgery and which have a poor prognosis[1–4]. The prognosis is particular poor in cases where extent of lymph node metastasis is N2 or higher. The relationship between factor N and the biological malignancy level of this carcinoma has been studied by a number of investigators, but no widely accepted relationship has been identified[5–7]. To explore a highly reliable indicator of the biological malignancy level of this carcinoma, we double-stained samples of non-small cell pulmonary carcinoma for Ki-67 and AgNOR simultaneously[8], and analyzed the relationship between the responses to these stains and factor N which has been clinicopathologically established as a reliable indicator of the malignancy level of this carcinoma.

AgNOR stain was developed in 1980 by Howell and modified in 1986 by Ploton et al.[9]. Since its usefulness as an indicator of the malignancy level of malignant lymphoma was reported in 1987 by Crocker et al.[10], this stain has been used for the assessment of various malignant tumors and has been established as a useful indicator of

the malignancy level as viewed from the proliferative activity of cells. AgNOR represents the specifically stained areas where r-RNA related to the formation of nucleoli is present. Human solid cancer cells consist of non-proliferating cells and proliferating cells. AgNOR counts may differ between these two types of cancer cells. Also in the present study, the AgNOR count in Ki-67 positive cells was significantly higher than its count in Ki-67 negative cells. It is also possible that the AgNOR count in proliferating cells differs depending on the phase of the cell cycle. A correlation between cell cycle and AgNOR was confirmed in 1984 by Fields et al.[11]. Subsequent studies also revealed that AgNOR correlated with the S phase fraction when examined using flow cytometry[12,13]. When examined using conventional immunohistochemical staining, most cells constituting clinical samples of cancer are in the G0 phase (resting stage), and proliferating cells are estimated to account for about 20-30 %. When AgNOR staining alone is used, both the resting cells and the proliferating cells are tested together. To assess the proliferating potential of cancer cells using AgNOR staining, it is necessary to count AgNOR in the nuclei of cells at the proliferating stage.

In the present study we used double-staining for AgNOR and Ki-67 to distinguish non-proliferating cells from proliferating cells. Anti-Ki-67 antibody is a monoclonal antibody first prepared by Gerdes et al.[14], using the nuclear fraction of cultured Hodgkin's disease cells. Ki-67 is reported to appear in late G1 through M phases of the cell cycle[15]. It can appear in all but G0 cells. Ki-67 has been used as a sensitive marker of cell proliferation in cases of various tumors such as malignant lymphoma and breast cancer. Although some investigators reported the usefulness of Ki-67 as a prognostic marker, there is no widely accepted view concerning the usefulness of Ki-67[8,16-19]. Ki-67 considered to correlate with the degree of histological differentiation of cancer. In the present study of pulmonary carcinoma, Ki-67 LI was higher in cases of poorly differentiated carcinoma than in well differentiated carcinoma, but no correlation was noted between the degree of differentiation and the AgNOR count in Ki-67 positive cells.

Although few studies have been conducted on the relationship between AgNOR and malignancy level of non-small cell pulmonary carcinoma, Yoshida et al.[7] reported that survival rates were high in cases with low AgNOR counts. No more detailed study of the relationship between survival rate and AgNOR count has been reported. In the present study, the AgNOR count in Ki-67 positive and negative cells had no correlation with the degree of histological differentiation or DNA ploidy. However, both the AgNOR count in Ki-67 positive cells and the AgNOR count in Ki-67 negative cells tended to increase as the tumor stage advanced. Following this finding, we analyzed two determinants of stage, i. e., factors T and N, in relation to AgNOR counts. This analysis revealed significantly higher AgNOR counts in Ki-67 positive and negative cells for T4 carcinoma than for carcinoma of any other T level. This finding suggests the high clinical malignancy of T 4 cancer well. No such finding has been obtained by the single Ki-67 staining. Regarding factor N, it has been reported that the prognosis is quite poor in cases rated as N2 or higher and relatively good in cases rated as N1 or lower. In the present study, the Ki-67 LI and the AgNOR count in Ki-67 positive cells were significantly higher in cases rated as N2 or higher. It is noteworthy that the difference in this parameter between N1 and N2 cases was statistically significant ($p < 0.01$).

These results indicate that a marked increase in pulmonary cancer cells with a proliferating potential, i. e., cells in the proliferating stage, stimulates the activity of nucleoli, resulting in metastasis to the mediastinal lymph nodes and to remote tissue or organs[20].

If the number of proliferating cells and the activity of nucleoli can be assessed simultaneously, it will contribute to determining the malignancy level and prognosis of cancer. Simultaneous double-staining for Ki-67 and AgNOR is very useful in assessing the activity level of proliferating cells.

Therefore, when AgNOR (an indicator of the proliferating activity of cancer) is to be used for assessing clinical samples of non-small cell pulmonary carcinoma, it is advisable to combine AgNOR staining with Ki-67 staining so that AgNOR can be assessed separately for non-proliferating cells and proliferating cells, because solid cancer is known to be composed of a mixture of proliferating and non-proliferating cells. In the present study, the poor prognosis of non-small cell pulmonary carcinoma with N2 or severer lymph node metastasis was clearly supported by the double-staining. The double staining for AgNOR and Ki-67 can be therefore regarded to provide a highly reliable means of assessing the prognosis and biological malignancy level of non small cell lung carcinoma. Although the final outcome of individual cases has not yet been analyzed, we have noted that most of the patients with 20 or more AgNOR in Ki-67 positive cells were rated as N2 or higher (i. e., types with a poor prognosis), and three N0 cases with 20 or more AgNOR in Ki-67 positive cells have developed recurrence within 2 years. It seems therefore necessary for us to pay special attention to N0 and N1 cases with this group when we follow the patients examined in the present study.

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Reference

1) Martini N, Flehinger BJ, Zaman MB, et al : Results of resection in non-small cell carcinoma of the lung with mediastinal lymphnode metastases. Ann. Surg., 198 : 386-397, 1983.
2) Martini N, Flehinger BJ : The Role of Surgery in N2 Lung Cancer. Surgical Clinics of North America, 67 : 1037-1049, 1987.
3) Toyohiro Isimatu, Takashi Yoshimatu, Kazuhiro Dobashi : Evaluation of Surgical Resection in pN2 Non-Small Cell Lung Cancer. Lung Cancer, 33 : 471-480, 1993.
4) Masao Ichiki, Masanori Sakurai, Izumi Hayashi : Prognosis of P-N2 Non-small cell Lung Cancer with Special Emphasis on the Significance of a Chest CT Diagnosis of Metastasis to Mediastinal Lymp Nodes. Lung Cancer, 33 ; 1031-1036, 1993.
5) Hidemi Ouwada, Masahiko Ishibasi, Kennzi Hiroshima : Differences in Number of AgNORs and Rate of PC-NA immunoreactiveity of Central and Peripheral Zone Cancer Cells within Primary Tumors of Squamous Cell and Adenocarcinouma of the Lung-Lung Cancer, 33 : 907-916, 1993.
6) Kyouko Hara : Immunohistochemical Approach to Evaluation of Growth Potential of Lung Cancer Using Monoclonal Antibody Ki-67. Lung Cancer, 33 : 177-186, 1993.
7) Yasuhiro Yoshida, Kouki Inai, Shuji Yonehara : Argyrophil Nucleoar Organizer Regions in Small-sized Adenocarcinomas of Peripheral Lung, with Reference to Prognosis. Lung Cancer, 32 : 213-221 1992.
8) Murray, P. G., Boldy, D. A. R., Crocker, J. andAyres, J. G. : Sequential demonstration of antigen and AgNORs in frozen and paraffin sections. J. Pathol., 159 : 169-172, 1989.
9) Ploton. D, Menager. M, Jeannesson. P : Improvement in the staining and in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer regions at the optical level. Histochem. J., 18: 5-14, 1986.
10) J. Crocker, Paramjet. Nar, : Nucleolar organizer regions in lymphomas. J Pathol, 151 : 111-118, 1987.
11) Diane H. Field, Peter H. Fitzgerald, Frank Y. T. Sin : Nucleolar silver-staining patterns related to cell cycle phase and cell generation of PHA-stimulated fuman lymphocytes. Cytobios, 41 : 23-33, 1984.
12) Riaz M. Jan-Mohamed, Susan J. Armstrong, John Crocker : The Relastionship between number of interphase NORs and NOR-bearing chromosomes in non-Hodgkin's Lymphoma. J. Pathol., 158 : 3-7, 1989
13) Crocker J. Macartney JC, Smith PJ : Correlation between DNA flow cytometric and nucleolar organizer region date in non-Hodgkin's lymphomas. J. Pathol., 154 : 151-151, 1988
14) Gerdes, J., Lemke, H., Baisch, H. et : Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J. Immunol., 133 : 1710-1715, 1984
15) Hall, P. A., Richards, M. A., Gregory, W. M., d'Ardenne, A. J., Lister, T. A., and Stansfeld, A. G. : The prognostic value of Ki-67 immunostaining in non-Hodgkin's lymphoma. J. Pathol., 154 : 223-235, 1988
16) E Leonardi, S Girlando, G Serio, : PCNA and Ki-67 expression in breast carcinoma : Correlations with clinical and biological variables. J. Clin, Pathol., 45 : 416-419, 1992
17) K. Sasaki, K Matsumura. T Murakami : Measurement of Bromodeoxyuridine Labeling index, Ki-67 Score and Ag-NORcount in Breast Carcinomas. Oncology, 49 : 147-153, 1992
18) A. Pich, G. Valente, L. Azzoni : Argyrophilic Nucleolar Organizer Region Counts and Ki-67 Scores in Human Renal Cell Carcinoma. Path. Res. Pract., 187 : 482-486, 1991
19) Y Okabe, S Nakamura, H Okumura : The Relation of Argyrophilic Proteins of Nucleolar Organizer Regions (AgNORs) to the Proportions of Ki-67 or DNA Polymerase α -Reacting Cells in non-Hodgkin's Lymphomas. Anticancer Research, 11 : 2031-2036, 1991
20) Satosi Matsuo : A Study of the Pattern of Recurrence Using Flow Cytometric DNA Analysis and PCNA/AgN OR Sequential Staining in Non-Small Cell Lung Cancer. Acta Med. Nagasaki., 38 : 257-264, 1993