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A Study of the Correlation Between Cell Proliferation Capacity and Numerical Chromosomal Aberrations in Colon Cancer

Fumitaka AKAMA

The First Department of Surgery, Nagasaki University School of Medicine

We conducted a study of the correlation between numerical chromosomal aberrations and cell proliferation capacity in colon cancer using FISH, PCNA, Ki-67, DNA polymerase α, and DNA ploidy.

The LI of Ki-67 and DNA polymerase a did not show a correlation with any pathological factors. However, the PCNA LI showed a correlation with mitotic index, and a correlation was also observed between venous invasive factors and lymphatic invaseve factors. A high level of the PCNA LI therefore thought to indicate a high degree of melignancy, thus requiring extremely close follow-up.

Among chromosomes No. 7, 11, and 17, a correlation was observed between numerical aberrations in chromosome 17 and the PCNA LI.

Introduction

A variety of studies have been conducted based on the hypothesis that cell proliferation activity in malignant tumors is an indicator of the malignancy of such tumors and is given as one prognostic factor. Substances which show cell proliferation activity include H3-thymidine, Brdu, DNA polymerase α, and Ki-67, and recently, proliferating cell nuclear antigen (PCNA) has also come under study.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of polymerase δ, and it is said that its location in the nucleus changes in correlation with the proliferation status of cells synthesized from the late G_1 to the S phase of the cell cycle[1]. Studies on gastric cancer conducted by S. Jain et al[2] and studies on colon cancer by Ibrahim[3] have shown that the cell proliferation rate determined by PCNA immune staining is an independent prognostic factor. Ki-67 is a monoclonal antibody prepared taking nuclear fractions of culture strains of tumor cells from Hodgkin's disease as antigens. There are also reports that its reactivity with the cells outside the G_2 phase is useful as a histological prognostic factor.[4] DNA polymerase α is considered to occur in the nuclei of all cells in the proliferation cycle, and this substance has been found to be useful in diagnosis of the degree of malignancy in diseases such as lymphoma[5].

Chromosomal aberrations have been clearly shown to play a role in the occurrence of colon cancer[6]. It has been reported that numerical chromosomal aberrations have been observed in cases of colon cancer, and in particular, a correlation between the number 11 chromosome and lymphatic metastasis of colon cancer has been observed[7]. Based on this finding, it would appear that it is possible to carry out reliable evaluation of degree of malignancy by combining cell proliferation activity and numerical chromosomal aberrations. In the present study, we clarified the correlation between cell proliferation activity and pathological factors and investigated the correlation between numerical chromosomal aberrations, cell proliferation activity, and DNA ploidy.

Materials and methods

The subjects of the study were 28 patients with colon cancer who nuderwent surgery-excision at this Departmeit. PC-10 mono-clonal antibody (DAKO Company) was sued as anti-PCNA antibody, PC-K1 monoclonal antibody (DAKO Company) was used as anti-Ki-67 antibody, and CL22-4-42B monoclonal antibody (MBL Company) was used as DNA polymerase α antibody. In FISH, we used the specific repeat sequence DNA probes D7Z1 (p7tetI), D11Z1 (pLC11A), D17Z1 (p17H8) (all provided by Oncor Company), which are specific for No.7, No.11, and No.17 respectively.

(A) Immunohistochemistry

In immune histochemical staining of PCNA, after fixation with 10% formalin, tissue slide sections measuring 4 μm were prepared from paraffin-embedded colon cancer tissue, xylene was used to carry out deparaffinization, and rehydration was carried out on an ethanol column. PC-10 was used as a monoclonal antibody, and the LSAB kit (DAKO) was used for immunohistochemical staining. DAB was used for coloring and hematoxylin was used for counterstaining.

HE staining was also carried out on sections continuous

with sections on which PCNA immunohistochemical staining had been carried out, confirmation of the cancerous portion was carried out, 1,000 cell nuclei or more were observed, and the ratio of cell nuclei undergoing mitosis was evaluated as a mitotic index.

In Ki-67 immunohistochemical staining, 4 μm sections were prepared from fresh frozen specimens frozen immediately after surgical extraction and fixed with 4% paraformaldehyde. After this, using PC-K1 as a monoclonal antibody, immunohistochemical staining was carried out with the LSAB kit in the same manner as PCNA immunohistochemical staining.

For DNA polymerase α, 4 μm sections were prepared from fresh-frozen specimens and fixed with 4% paraformaldehyde, and munohistochemical staining was carried out using the PAP kit (MBL).

Concerning the evaluation method, several visual fields which showed favorable staining under a low-magnification optical microscope were selected as desired, and the cell nuclei which showed even a little DAB coloration were evaluated as positive. Observations were conducted at a magnification of 400X on 1,000 cells or more, and evaluation was conducted by means of the positive cell ratio (labeling index: LI).

(B) DNA ploidy

DNA ploidy was measured by the method of Ishikawa[8] from part of FISH-treated specimen following trypsin treatment. In conducting this measurement, the FACScan (Becton Dickinson) was used, and analysis was conducted using the Cellfit (Becton Dickinson).

(C) FISH

We used a method independently developed by us for detection of numerical chromosomal aberrations[9]. Specifically, after first carrying out 10% formalin fixing, 40 μm sections were prepared from paraffin-embedded colon cancer tissue. After deparaffinization was carried out with xylene, rehydration was carried out on an ethanol column, and after trypsin treatment, fixing was carried out in an ethanol-acetic acid solution, and development was carried out on slides.After thorough drying, dehydration was carried out on the ethanol column, deproteinization was carried out using pepsin, and immersion was carried out in 70% acetic acid. After immersion in 1% hydroxylamine for 15 min., dehydration was again carried out on the ethanol column and acetylation was carried out for 10 min. using 0.25% anhydrous acetic acid. After washing with 2 × SSC, heat-denaturation of the chromosomes of the sample was carried out using 70% formamide /2 × SSC heated to 70℃. After 2-3 min., fixation was quickly carried out for 5 min. with 70% ethanol at -20℃, and the probe DNA was heated to 70-80℃ during this period to carry out heat-denaturation. After the slide specimens were dehydrated on an ethanol column, the probe was titrated onto the slide and subjected to overnight hybridization at 37℃. After rinsing was carried out under high-stringency conditions, detection was carried out by a FITC-avidin reaction for 30 min. at 37℃. After rinsing with 0.05% Tween 20/PBS, signal enhancement was carried out with biotinylated anti-avidin antibody-FITC-avidin, and a counterstain was conducted using propidium iodide. Using a fluorescence microscope, 100-200 nuclei showing a favorable state were selected, and the number of signals per nuclei was evaluated[10) 11) 12)].

There have been various reports on methods of evaluating the number of chromosomes, but in the present study, we used the method of Waldman et al[13]. Specifically, in cases where an aneusomic subpopulation accounted for 50% or more, this subpopulation was used. However, when aneusomic subpopulations was not observed and aneusomic population exceeded 20% of the whole, the subpopulation thereof was used. In cases where, populations exceeding 20% were not observed, this was evaluated as disomy. Moreover, in carrying out FISH, normal human peripheral lympho-cytes were used as a control, two specific target spots were detected, and hybridization specificity was confirmed.

Statistical analysis was carried out using Fisher's exact test.

Results

Fig.1 shows a photograph of FISH results. Hybridization spots labelled yellow by FITC were observed in nuclei dyed red by PI.

The results are shown in Table 1. The average value for the PCNA LI was 51.4±9.6, and a correlation between the stage and degree of penetration and PCNA LI was not observed, but the PCNA LI of the venous invasive factor

Fig.1 FISH spots: It shows two yellowish with FITC-avidin on a red nuclei which is couterstained with propidium iodide.

Table 1 Results

CASE	DEPTH	V	LY	STAGE	LABELING INDEX PCNA	KI-67	POLY	MITOTIC INDEX	FISH SPOTS C7	C11	C17	DNA PLOIDY
1	pm	0	0	1	40.7	30.0	53.0	2.27	3	1	1	*
2	ss	0	1	4	40.6	23.2	38.7	2.90	2	3	3	A
3	ss	0	1	4	30.1	31.5	54.1	1.54		3	2	D
4	pm	0	2	4	65.9	59.0	34.6	4.69		2	3	D
5	ss	1	3	5	64.9	55.5	40.8		2	3	3	A
6	a2	0	3	2	61.3	45.4	25.9	4.97	2	1	1	D
7	pm	0	2	1	66.8			3.11	3	2	3	A
8	ss	0	1	2	56.3	44.9	47.0	3.68	2	1	1	D
9	ss	0	1	2	63.0	51.1	53.5	4.10	3		3	D
10	ss	0	0	5	40.1	58.9	43.5		2	1	1	*
11	ss	0	1	3	50.9		54.5	3.95			3	*
12	ss	0	2	2	50.6	28.2	56.7	2.08	3	1	3	A
13	sm	0	0	1	53.3	48.3	34.9	2.30	3	1	3	A
14	ss	0	1	2	47.4	40.9	51.0	2.49		3	3	D
15	a1	0	2	2	51.6	47.9	60.7	2.77	2	1	3	A
16	a2	2	3	2	67.6	52.9	47.1	3.98	3	3	3	D
17	ss	0	1	2	38.5	51.2	42.7	2.43			2	A
18	ss	0	3	4	46.0	24.2		2.45	3	3	2	A
19	a2	2	1	2	47.2	44.5		1.99	2	2	2	D
20	a1	0	0	1	41.5		21.0	2.70	3		2	D
21	a2	2	1	3	52.6	31.5	45.3	4.06	3	3	3	A
22	a2	2	1	3	57.6	50.7	61.3	4.49			3	A
23	a2	1	1	5	49.1	62.1	34.7		2	2	2	D
24	a2	2	3	4	55.5	32.7		4.31		2	3	A
25	a1	1	3	5	54.7	62.5	50.9		3	1	1	D
26	s	0	2	3	40.3	39.0	40.8	2.37			3	*
27	ss	1	2	2	51.1	58.3		3.75		3	3	*
28	sm	1	1	1	53.6	41.1	44.0	3.70	2	2	2	*

*:UNCLASSIFIED

(V factor) positive group was 55.9±6.42, significantly high compared to the PCNA LI of the V factor negative group of 48.4±10.3 ($p = 0.030$). Moreover, the PCNA LI of groups having 2 or more lymphatic duct invasive factors (LY factors) was 56.4±8.9, a significantly higher value than the PCNA LI of the groups with fewer than 2 LY factors of 47.7±8.6 ($p = 0.029$). On the other hand, the average value for Ki-67 LI was 44.7±12.0, and the average value for the DNA polymerase α LI was 45.1±10.4, but the Ki-67 LI and the DNA polymerase α LI did not show correlations with any histological factor.

The average value for the mitotic index used to evaluate cell division capacity was 3.21±0.97, showing a correlation with PCNA LI with a level of significance of $p<0.01$ and a correlation with Ki-67 LI with a level of significance of $p<0.05$, but no correlation with the DNA polymerase α LI was observed (Fig.2). Because of this, PCNA LI and the mitotic index was found to show a more favorable correlation than the other two.

Fig.3 shows the results of a comparison of hybridization spots and the LI of various proliferation-related factors. The average value of the PCNA LI was 51.6±8.48 in the

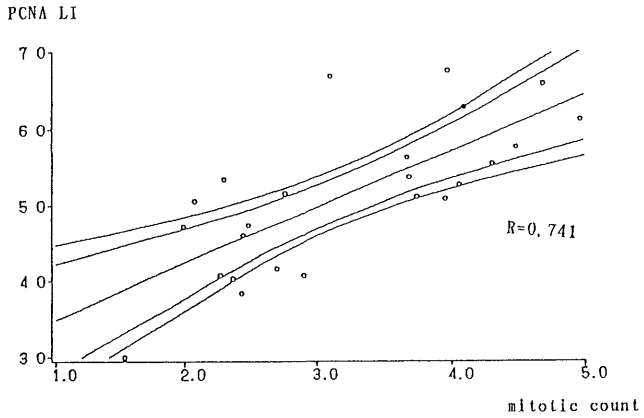

Fig.2a Relationship between mitotic count and PCNA LI

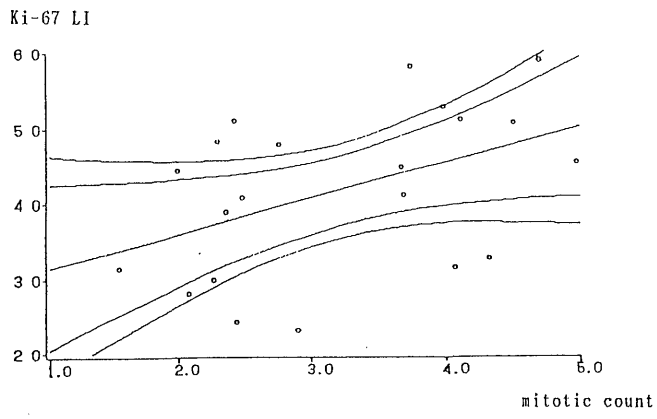

Fig.2b Relationship between mitotic count and Ki-67 LI

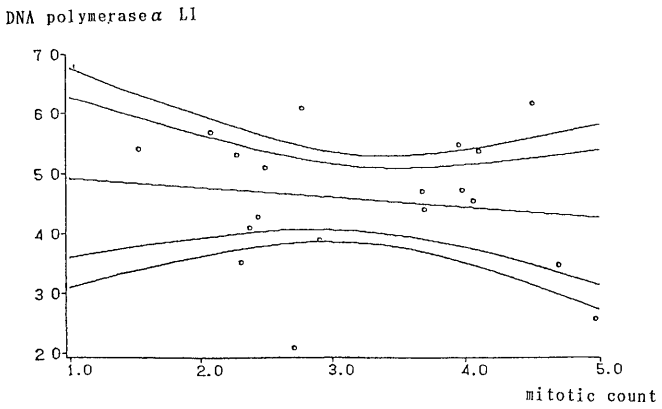

Fig.2c Relationship between mitotic count and DNA polymorase α LI

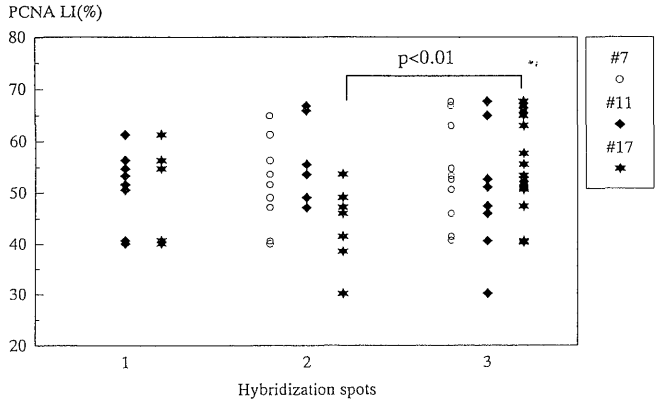

Fig.3a Relationship between PCNA LI and hybridization spots for chromosome 7, 11 and 17

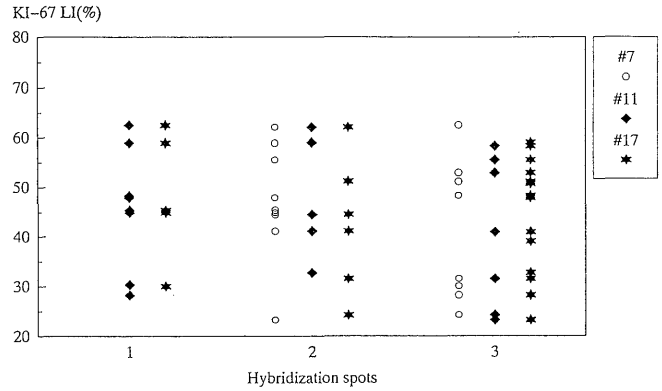

Fig.3b Relationship between KI-67 LI and hybridization spots for chromosome 7, 11 and 17

case of 2 spots of #7 and 53.7±9.66 for 3 spots. In the case of 11, 1, 2, and 3 spots showed values of 51.1±7.35, 56.4 ±8.31, and 47.9±11.5 respectively. In the case of 17, the respective values were 50.6±9.64, 43.7±7.76, and 55.0± 8.71. Comparing the PCNA LI of 2 spots and 3 spots of #17, 3 spots showed a significantly higher value ($p<0.01$). No clear correlation with the LI of other proliferation-related factors was observed.

Concerning DNA ploidy, there was correlation with

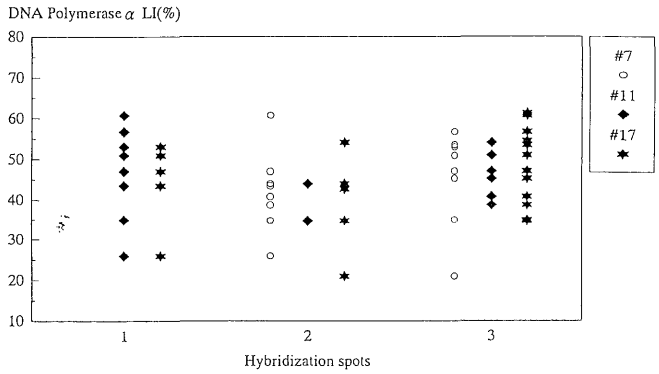

Fig.3c Relationship between DNA polymerase α LI and hybridization spots for chromosome 7, 11 and 17

neither the numerical aberrations in the 3 chromosomes nor the 3 proliferation-related factors LI examined in the present study.

Discussion

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of polymerase δ which is used to evaluate the proliferation capacity of tumor cells synthesized from the late G1 to the S phase of the cell cycle. Ibrahim[3] et al reported that it is able to predict long-term survival in colon cancer using the PCNA proliferation rate, and Gabriella et al[14] reported a correlation between immune activity of PCNA and vascular invasion, and these results correlate with those of the present study. According to experiments conducted by the authors, PCNA LI shows the strongest correlation with the mitotic index[15] which is said to correlate with histological degree of malignancy. This might be due to the evaluation with serial samples. Based on the above, in evaluating cell proliferation capacity, PCNA LI was found to show the most outstanding results. Moreover, from a technical standpoint as well, in immunohistochemical staining using anti-PCNA antibodies, detection evaluation from conventional formalin-stained paraffin-embedded sections is possible, and while immunohistochemical staining using anti-Ki-67 antibodies and anti-DNA poly-merase α antibodies requires fresh-frozen specimens, it is an extremely simple procedure, and it can be retrospectively studied, making it very useful.

Numerical chromosomal aberrations are observed in many types of malignant tumors such as colon cancer, and numerical aberrations in the No.11 chromosome in particular have already been reported to show a correlation with lymph node metastasis of colon cancer[7]. However, concerning research on the correlation between numerical chromosomal aberrations and proliferation-related factors, studies have been conducted by Waldman[12] and Van Dekken[16], but both of these studies were conducted on bladder cancer, and no reports are available on colon

cancer.

Considering methods for detecting numerical chromosomal aberrations, these is able the chromosomal banding method, but while FISH is also capable of detecting chromosomal numerical aberrations in the nuclear division stage and therefore does not require cell culturing, it is far simpler than the chromosomal banding method. Concerning the accuracy of detection of aberrations as well, the same results have been indicated[17] Moreover, as FISH can be used not only on fresh specimens but also with conventional formalin-fixed and paraffin-stained tissue specimens for detection and evaluation, this point also makes it very useful.

There have been reports that the PCNA gene is located in the vicinity of chromosome 20p12-13[18)19)], but all of these studies were conducted on a small number of subjects, so doubts remain as to whether or not it is really located at this site. Moreover, it must be borne in mind that FISH, which uses a DNA probe to confirm the vicinity of the centromeres used in the present study, only detects numerical chromosomal aberrations and dose not detect changes in chromosome structure. Moreover, in the present study, a significant correlation was observed only between the No.17 chromosome and the PCNA LI. On the No.17 chromosome, the c-erb-B2 gene[20)], which has already been clinically applied as a prognostic factor in breast cancer, and the p53 gene, which is thought to play a role in the occurrence and spreading of various types of cancer such as colon cancer, are present. Experimental results have indicated that when anti-bodies to p53 are infused into normal cells, the shift from the Go to the S phase is disturbed[21)], and it is thus thought to be indispensable in proliferation. This p53 gene is said to be present in 17p13, and in many cases of colon cancer, the No.17 chromosome is found to show a short-arm defect[22)]. In the present study, on the contrary, the number of chromosomes was increased in many cases, and the PCNA LI showed a higher tendency than in cases of trisomy than disomy. This is an extremely interesting finding, and it is though that the study of both will be extremely useful in the future in evaluating the degree of malignancy of colon cancer taking cell proliferation capacity as an index.

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