Acta Med. Nagasaki 39:100－105

Study of Nuclear DNA Content and Chromosomal Numerical Aberrations Using Fluorescence *in situ* Hybridization in Colorectal Polyps and Colorectal Adenomas

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In this study, nuclear DNA content were compared with chromosomal numerical aberration by Fluorescence *in situ* Hybridization method in 41 cases of colorectal carcinomas and 12 cases of colorectal adenomas.

In the adenomas, DNA aneuploidy was shown in 8.3%, and moreover, monosomy of the chromosome 11 was shown in 10% and trisomy of the chromosome 7 was shown in 28.6%. However, no abnormality in the chromosome 17 was defined.

In contrast, with regard to the colorectal carcinomas, DNA aneuploidy was shown in 43.9%. Furthermore, it was observed that the rate of appearance of the chromosomal numerical aberrations was high. In the chromosome 17, the rate of appearance of trisomy was significantly higher in DNA aneuploidy cases as compared with DNA diploidy one. However, there was no significant difference in the chromosome 7 and the chromosome 11.

In addition, with regard to the colorectal carcinomas in which the chromosome 17 was disomy, there were significantly a large number of cases with positive v factor, and in trisomy cases, there were a lot of cases with negative v factor.

In conclusion, it is conceivable that detection of abnormality in chromosomes by FISH method provides a new index of the prognosis and the grade of malignancy in the colorectal carcinomas.

Introduction

Recently, advances have been made in the analysis of malignant tumor cells at the level of chromosomes and genes as the cell technology was developed and a lot of information has been obtained. With regard to methods for detection of chromosomal aberrations, Fluorescence *in situ* hybridization (FISH) method was developed and it takes the place of conventional chromosome banding method, and has become utilized for detection. FISH method has the advantage that there is no necessity for culturing of the cells, and information about the nucleus at the interphase is obtained in comparison with the analysis for chromosomes by banding method. Therefore, it is assumed that FISH method is very useful especially in detection of chromosomal aberrations on solid tumors. In addition, the measurement of the nuclear DNA contents by the use of FCM is of application in medical practice including diagnoses and determinations of prognosis of various tumors.

In this study, the relationship between aberrations on DNA ploidy by the use of FCM and chromosomal aberrations by FISH method was investigated in patients with colorectal carcinomas and colorectal adenomas. Moreover, great concern was concentrated on the presence and the characteristics of chromosomal aberrations on DNA diploidy cases which are not able to be detected by FCM. In addition, chromosomal numerical aberrations was compared with various factors in clinical pathology.

Materials and Methods

Materials

Materials for this study were 41 specimens of colorectal carcinomas which included fresh surgical specimens having been obtained as excised ones at our department and facilities concerned from April, 1992 to March, 1993 and specimens obtained from paraffin-embedded tissue fixed with formalin, and 12 fresh specimens of colorectal adenomas excised in colon fiber. (Table 1)

Tablel 1. Materials

Carcinoma :41Cases	Adenoma :12cases
Male : 31 Female : 10 cases	Male : 9 cases Female : 3 cases
Paraffin-embedded tissue : 19 Fresh tissue : 22 cases	Fresh tissue : 12 cases
Dukes A : 8 B : 21 C : 12 cases	DNA Diploidy : 11 DNA Aneuploidy : 1 cases
DNA Diploidy : 23 DNA Aneuploidy : 18 cases	

Methods

Preparetions and the measurement of nuclear DNA content

The tissues, 1×1 cm, obtained from excised specimens of the fresh ones were minced with scalpel, and then, were stirred in 0.05% collagenase, and were filtered through the mesh of 100um, and centrifuged. Then, they were washed with PBS for making the cell suspension. The 4-5 drops of the cell suspension were centrifuged again, and were stained with propidium iodide after the treatment with Triton X, and then the amount of nuclear DNA content was measured by the use of the program consort 30 of FACScan made by Becton & Dickinson Co. The rest of the specimens were fixed with ethanol/acetic acid (3:1), and were conserved at -20℃. 5-6 sections (40um) were cut from paraffin-embedded tissue, then the cell suspension was made by overnight trypsin method after deparaffinized by Xylen and rehydration. Then, the nuclear DNA content was measured by FACScan and the rest of the specimens were fixed with ethanol/acetic acid for conservation at -20℃.

The measurement of the nuclear DNA content by using the fresh specimens was performed at the same time and in the measurement, peripheral blood lymphocytes were added as the internal standard. DNA Index (DI) was calculated with the peak value of the G0/G1 cell in the histogram obtained through the measurement. Moreover, the case, DI=1, was defined as diploidy and the case, DI was not 1, was defined as aneuploidy, in addition the cases of which CV was 8% or less were the subjects for estimation.

Hybridization and detection

Hybridization was performed in the same way as the method of Pinkel et al[1]. The specimens of colorectal carcinomas were expanded on the poly-L-Lysine coating slides and denature was done at 70℃ with 70℃ formamide for two minutes after the treatment with pepsin, moreover denature of the probe was carried out at 70℃ for 10 minutes. Hybridization mixture was dropped on the slide and hybridization was performed overnight in the wet chamber under the coverslip at 37℃. With regard to specimens of colorectal adenomas, samples were stamped on the coating slides and hybridization was performed in the same way as specimens of colorectal carcinomas. After hybridization, the samples of adenomas were washed with 2×SSC, 60% Formamide, and Tween20. Then 1% Bovine serum albumin was added and incubation was carried out for 20 minutes. In addition, the samples were washed with Tween20, and 40ul of FITC-avidin DCS was added. Moreover, incubation was done for 30 minutes. In the samples of colorectal cancer, incubation was carried out for 30 minutes after 40ul of biotinylated anti-avidin DCS was added and FITC-avidin DCS was added again for 20 minutes incubation. Finally, counterstain was performed with 0.2ug/ml of Propidium iodide. Then the number of hybridization spots was counted, and moreover, the number of nuclei in which each of the counted spots was observed, was counted up to approximately 200 pieces as one sample by using an OLYMPUS Co. made fluorescence microscope, Model BHS-RFK. With regard to probes, Oncor Co. made biotinylated repetition DNA probes specific to chromosome 7, 11, and 17 each were used.

In determination of chromosomal numerical aberrations, the case that the rate of the population in which the spots had been increased in number as compared to the normal case (three spots or more) was over 15% of the whole or the rate of the population in which the spots had been decreased in number (single spot) was over 20% of the whole was determined as significantly aberrant, and the number of the spots in the population was considered to be the number of each chromosome. The case that similar aberrations were not observed was determined as normal, that is, disomy. Moreover, we did not count the nuclei in which the spots were not observed. X^2 test was used for examination of the difference between the incidence of various factors in clinical pathology and that of chromosomal numerical aberrations.

Results

DNA aneuploidy was observed in 1/12 (8.3%) of colorectal adenomas and 18/41 (43.9%) of colorectal carcinomas. Percentage of chromosomal aberrations on the adenomas is shown in Fig.1. Monosomy was observed on 10% of chromosome 11, trisomy was observed on 28.6% of chromosome 7, and there was no aberration on chromosome 17.

The signal spots in colorectal carcinomas were able to be observed as yellow green clear spots in the nuclei stained orange color with Propidium Iodide as well as colorectal adenomas (Fig.2 and 3). Percentage of chromosomal numerical aberrations in colorectal carcinomas is shown in Fig.4. The incidence of disomy was only 41.7%, 37%, and 35% on chromosome 7, 11, and 17 each, and monosomy,

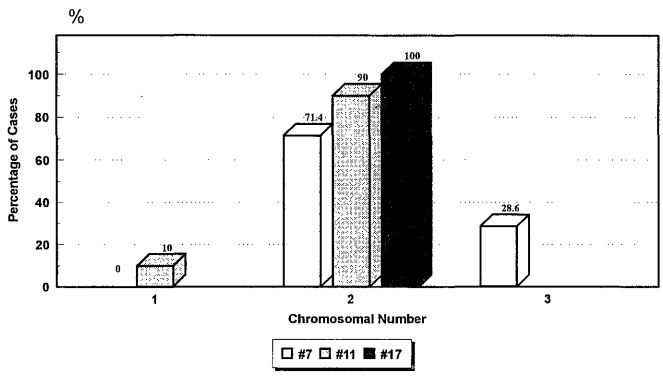

Fig. 1. Percentage of chromosomal numerical aberrations on colorectal polyps

trisomy, and tetrasomy were observed. However, monosomy was not observed on chromosome 7. When percentage of chromosomal aberrations was examined from the standpoint of DNA ploidy types (Fig.5), the rate of numerical aberrations on DNA diploidy was also high and there was not a significant difference in percentage of chromosomal aberrations between diploidy and aneuploidy on chromosome 7 and 11. In contrast, the rate of trisomy was significantly high in aneuploidy on chromosome 17 (Fig.6). Percentage of chromosomal numerical aberrations was examined from the standpoint of age (less than

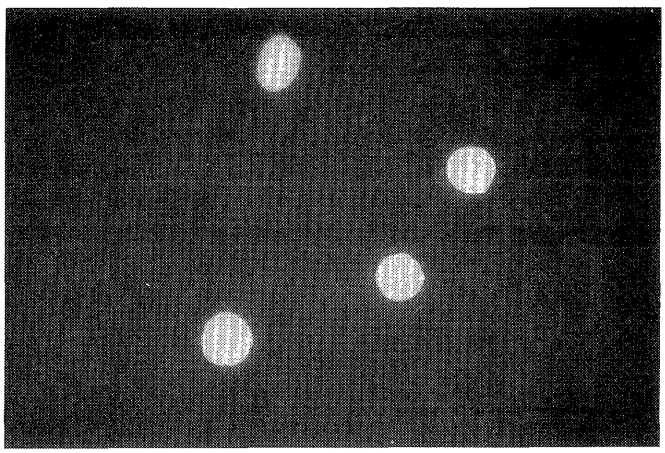

FIg. 2. Sample from fresh tissue, Stage II, ss, Dukes B DNA aneuploidy (DI=1.64), chromosome 11 showing 4 hybridization spots/nucleus

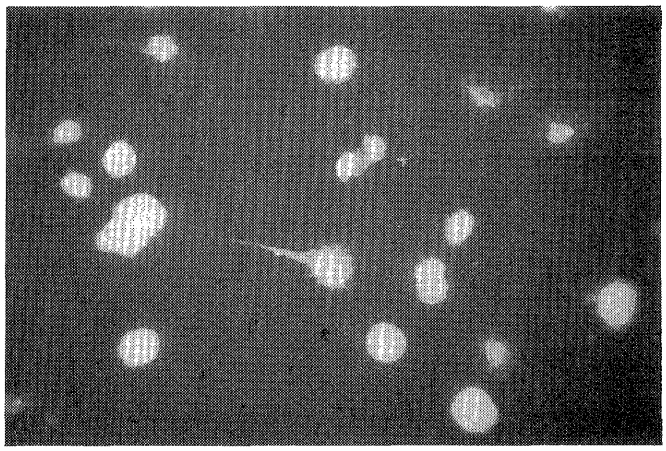

FIg. 3. Sample from paraffin embedded tissue Stage IV, ss, Dukes C DNA aneuploidy (DI=2.05), chromosome 17 showing 1-4 hybridization spots/nucleus

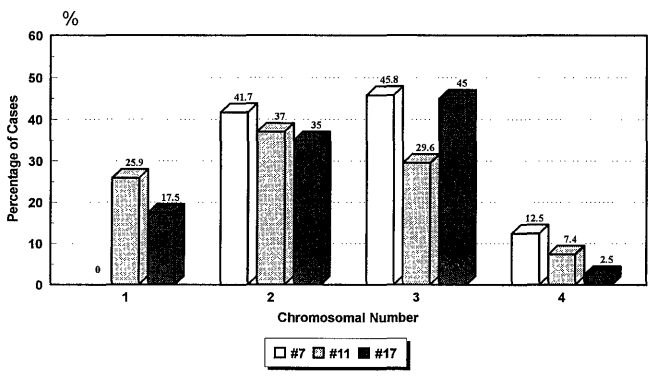

Fig. 4. Percentage of chromosomal numerical aberrations on colorectal carcinomas

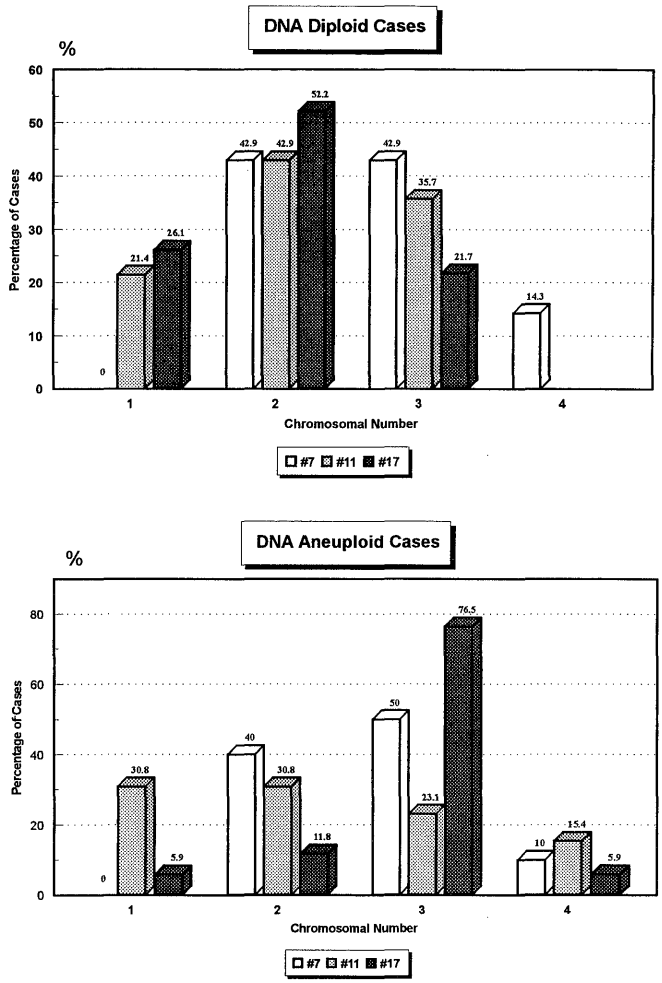

Fig. 5. Percentage of chromosomal numerical aberrations on DNA diploid and aneuploid colorectal carcinomas

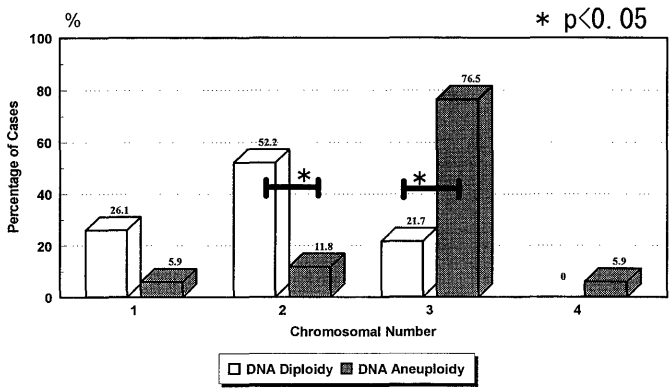

Fig. 6. Percentage of chromosomal numerical aberrations of chromosome 17 on colorectal carcinomas

70 years old, and 70 years old or more), sex, Dukes stage, the existence of metastasis in lymph node, and the existence of invasion in lymph vessels, we did not observe a significant difference in percentage of chromosomal numerical aberrations. There was no distant metastasis in this study cases. Therefore, a close relationship between chromosomal numerical aberrations and distant metastasis was not difiend. However, it was observed that there were significantly a lot of cases of positive for vein invasion in disomy of chromosome 17, and a lot of cases of negative for vein invasion in trisomy of chromosome 17 (Fig.7). Moreover, in the relationship between vein invasion and chromosome 17 limited to DNA diploidy cases, though there was not a significant difference, a lot of disomy cases tended to be positive for invasion and a lot of trisomy cases tended to be negative with invasion (Fig.8).

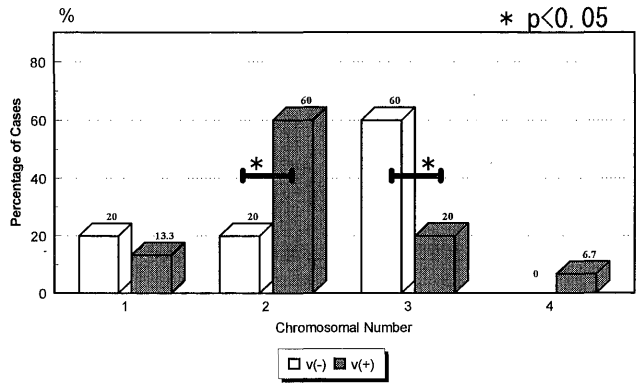

Fig. 7. Percentage of chromosomal numerical aberrations of chromosome 17 and v factor on colorectal carcinomas

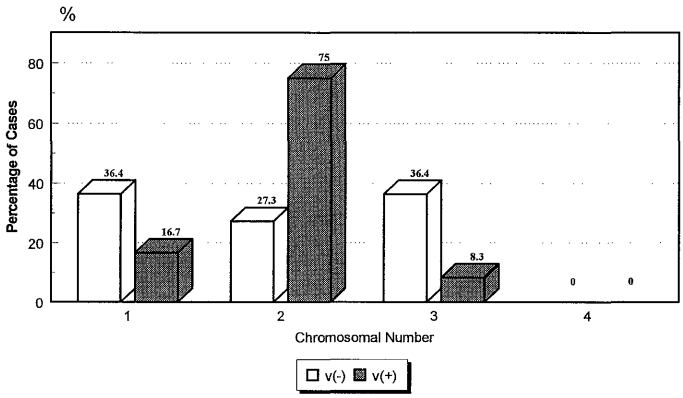

Fig. 8. Percentage of chromosomal numerical aberrations of chromosome 17 and v factor on DNA diploid colorectal carcinomas

Discussion

In this study, fresh specimens and the specimens obtained from paraffin-embedded tissues were used and it has been reported that FISH method by using the specimens from paraffin-embedded tissue was equally comparable to the method with fresh specimens[2], It is well known that FISH method is applicable to paraffin-embedded tissues preserved routinely. This fact shows that FISH method is able to be applied in retrospective study and it is thought that the application of this method can be expanded further. Moreover, it is reported that sensitivity of FISH method is equal to or better than the banding method. In addition, reliability of the data is thought to be sufficient for studies.

It is reported that percentage of DNA aneuploidy on colorectal adenomas is approximately 4.4-30% and the incidence is high in the cases with large lesions and with high atypia[3][4]. In this detection, aneuploidy was observed in one case out of 12 cases (8.3%). The 12 cases were adenomas of 1 cm or less in diameter excluding one case, and the one of 2 cm in diameter was diploidy. Moreover, the degree of atypia of all cases ranged from low to medium, and there was only one aneuploidy case. Therefore, the incidence of aneuploidy concerning the size and the degree of atypia failed to be examined. Detection of LOH by the unilization of DNA marker showing RFLP as well as DNA ploidy is extensively carried out. However, it is difficult to determine aberrations on the adenoma in which no aberrations are present in the cell population. In this study, chromosomal numerical aberrations on colorectal adenomas were lower in frequency as compared with carcinomas. With regard to FISH method, information about each of the cells is obtained. Therefore, it is possible that chromosomal aberrations on individual cells provide a good information for the lesion such as colorectal adenoma which seems to be precancer. Ikeuchi et al. reported that trisomy on chromosome 7 was observed in 35% of colorectal adenomas which originated in patients with FAP[5]. In this study, trisomy on chromosome 7 was observed in 28.6% of the adenomas. This is the most frequently observable chromosomal aberrations on adenomas. Definitive concluision failed to be drawn that trisomy on chromosome 7 indicated an early stage of adenoma-carcinoma sequence. Moreover, aberrations on chromosome 17 were not observed in adenomas and as is shown in the multiple process progression theory of Vogelstein about chromosomal aberrations on progression of carcinomas[6], there is some doubt that aberrations on chromosome 17 participate in the transition from adenomas to carcinomas rather than simple alteration of normal mucosa to adenomas in the light of chromosomal numerical aberrations only.

Van Dekken et al. reported that the DNA histogram by flow cytometry (FCM) well accorded with the result of FISH method in solid carcinomas and noted that the specificity in the measurement of nuclear DNA content by FCM was considerably lower as compared to FISH method[7]. It has been reported that percentage of chromosomal numerical aberrations was high on colorectal carcinomas with DNA diploidy[8], and the aberrations

especially on chromosome 7 and 11 were observed in DNA diploidy cases as well as in aneuploidy cases. Some of the chromosomal aberrations in carcinomas with aberrations which are not able to be detected by FCM are detected by FISH method. The reasons are in that sensitivity of FCM is not sufficient for detection in a case of one or two chromosomal aberration. Moreover, the carcinomatous cells change variously. Consequently, an increase and decrease in the chromosome are sometimes observed concurrently in the same specimen. In that case, a significant aberration of the nuclear DNA content is sometimes not shown in one nucleus so that simultaneous detection fails to define a presence of aberrations on some chromosomes in one nucleus by multicolor FISH method[9]. In addition, structural aberrations on chromosomes is detected by using chromosomal painting method[10)11)]. Thus, FISH method is of great value to detect a fine change in chromosomes.

Reports on detection of chromosomal aberrations on malignant tumors by FISH method have been gradually increasing in number.[12)13)] Hopman et al. showed by FISH method that chromosomal deletion of chromosome 9 in bladder carcinomas was observed frequently in the primary DNA diploidy carcinomas and the finding was important for canceration and progression of carcinomas as a primary event[14]. In addition, Frederic et al. reported that Grade of carcinomas correlated with copy number on chromosome 7 in bladder carcinomas[15]. There was not a significant difference in percentage of numerical aberrations according to Dukes stage, but percentage of trisomy on chromosome 17 was high in aneuploidy cases of colorectal carcinomas and there is a possibility that this chromosomal aberration participates in the transition from carcinoatous cells to aneuploidy.

Generally, it is thought that appearance of chromosomal aberrations means increase in proliferative activity and malignancy. In practice, Akama et al reported that PCNA LI in trisomy cases was inclined to be higher than disomy cases on chromosome 17[16]. Also in this study, significantly a lot of DNA aneuploidy were observed in the cases in which chromosome 17 was trisomy. Nevertheless, the result that there were significantly more negative cases with vein invasion than positive cases in trisomy cases of chromosome 17 is suggestive of the possibility that vein invasion is selectively inhibited with trisomy of chromosome 17. There is no report that the numerical aberration on a specific chromosome has selectively influenced such pathological factors. Therefore, it is difficult to define the significance. Some kinds of genes including p53 as tumor suppresser gene and C-erb-B2 related to carcinomas are present in chromosome 17 and we should consider the relationship to the results obtained from this study. However, the mechanism of the appearance of these genes when chromosomal aberrations including monosomy and trisomy are present has not been elucidated at this stage.

After the measurement of nuclear DNA content obtained from paraffin-embedded tissues by Hedley et al. was reported[17)18)19)], the nuclear DNA content has been measured in a lot of malignant tumors and it was reported that the prognosis of aneuploidy carcinomas were poor in most of carcinomas. Moreover, there are some patients whose prognosis is poor in DNA diploidy carsinomas. In this study, it was defined that percentage of chromosomal numerical aberrations was high in DNA diploidy cases. In conclusion, a study of chromosomal numerical aberrations in DNA diploidy is useful for judging the prognosis more precisely.

Acknowledegment

My special thanks to Professor Masao Tomita for his helpful and instructive deliberations, and to Dr. Yutaka Tagawa for practical and editorial guidance and the other doctors belonging to the First Department of Surgery of Nagasaki University School of Medicine for their kind support.

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