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Application of Fluorescence *in situ* Hybridization (FISH) on the Tissue Sections for Detection of Chromosomal Aberrations in the Carcinogenesis in the Colon and Rectum

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To clarify the genetic events and pathway of carcinogenesis in the colorectal neoplasias, fluorescence *in situ* hybridization (FISH), which could detect chromosomal numerical aberrations, is applied to tissue sections of colorectal adenomas and carcinomas using pericentromere specific repetitive DNA probes. When FISH was applied to tissue sections, it would be easy for investigators to distinguish between certain glands and the others in comparison with a hematoxylin-eosin (H-E) staining section in the same specimens. As a fundamental study forward to clinical applications of this new method, then, it was tried to study the feasibility of FISH on paraffin-embedded tissue sections of a spleen, which was surgically resected at operation, driven by necessity. The adequate thickness of the sections was determined as five μm in the study, so the method was applied to the clinical materials, which were collected and fixed in formalin from operative or polypectomy specimens. Biotinylated DNA probes for the centromeric regions of chromosomes 11 and 17 were used. These probes worked well, demonstrating one copy (monosomy) in 26.5 ± 9.7 %, and two copies (disomy) in 66.4 ± 9.9 %, and three copies (trisomy) in 7.1±5.6 % for chromosome 11, and monosomy in 18.4±9.7 %, disomy in 64.3±12.8 %, and trisomy in 17.3± 16.6 % for chromosome 17 in the adenomas. And the probes demonstrated more than three copies of chromosome 17 in 23.6 to 24.6 % in polypoid cancers and the carcinoma component of carcinoma in adenomas. In applying the FISH on tissue section, it should be taken into account that whole nuclei of about ten μm in size could not be encompassed in a five μm thickness section, and that a certain percentage of the cells showed lower copy numbers as a result of truncation. The disadvantage of underestimating the copy numbers due to the nuclear truncation always existed, however, FISH on the tissue section allowed to detect the copy numbers of tumor cells among stromal and inflammatory cells, and to detect intratumor heterogeneity in comparison with the H-E staining sample. The auther emphasized that applying FISH to the paraffin sections enabled to achieve the retrospective study using archival paraffin-embedded specimens.

Introduction

To search for and study the genetic alterations involved in the development in carcinomas of the colon and rectum, numerous data have been accumulated, which suggest that malignant colorectal tumors (carcinomas) arise from preexistng benign tumors (adenomas). The process forward to the formation of the malignancies of the colon and rectum has precisely been investigated. Only recently, however, it has been become possible to identify the molecular events that underlie the initiation and progression of human neoplasia. Vogelstein et al presented the features of the colorectal neoplasia[1,2]. First, colorectal tumors appear to arise from a result of the mutational activation of oncogenes coupled with the mutational inactivation of tumor suppressor genes; the latter changes are predominant. Second, mutations in at least four to five genes are required for the formation of a malignant tumor. Third, although the genetic alterations occur according to a preffed sequnce, the total accumulation of changes, rather than their order with respect to one another, is responsible for determining the tumor's biologic properties. But these data above were mentioned at the DNA level.

The quantitative DNA analysis by flow cytometry (FCM), on the other hand, has provided complementary prognostic informations[3,4,5]. However, the detection of specific chromosome aberrations and minor genomic changes is limited by means of FCM. Although genetic aberrations can be detected more precisely by karyotyping, for solid tumors it is hampered with difficulties such as in low mitotic index, poor banding quality, and a condensed or fuzzy appearance of the chromosomes. Furthermore, the number of cells that can be analyzed is extremely limited[6-10].

FISH using repetitive DNA probes that hybridize to the centromeric associated region of a specific chromosome is a powerful technique to study numerical and, in a limited number of cases, structural chromosome aberrations

within the interphase nuclei of tumor cells[11,12,13]. It has been shown that this method of FISH, also called interphase cytogenetics, allows the analysis of hundreds of (tumor) cell populations, with many different DNA probes[14,15]. As a result, the detection of minor cell populations as well as an imbalance in chromosome copy number within one tumor have become possible.

Numerical and structural aberrations involving several chromosomes, such as chromosomes 1, 5, 7, 11, 17 and 18 have been detected for colorectal carcinomas using metaphase and interphase cytogenetics[16,17]. In particular, allelic losses on 5p,17p, and 18q have been found according to the tumor development in familial adenomatous polyposis colli (FAP) using restriction fragment length polymorphism (RFLP) analysis. And new tumor suppressor genes such as FAP gene, APC (adenomatous polyposis colli) gene were purified and identified in the past decade.

In the present study, FISH was applied to the tissue sections of adenoma, carcinoma in adenoma (CIA) and polypoid cancer in the colon and rectum from archival formalin-fixed, paraffin-embedded specimens using DNA probes for chromosomes 11 and 17 to investigate the specific chromosomal aberrations in the initation and progression of neoplasia in the colon and rectum.

Materials and Methods

(1) Preliminary study using spleen tissue

Before applying the new method of FISH to the clinical materials such as colorectal carcinomas, the feasibility of FISH on tissue sections was investigated with chromosome specific alpha-satellite DNA probes[18]. The purpose of the prelimainary study was the determination for the adequate section in thickness for FISH. The spleen tissue specimen was applied, because almost all of the cells which composed of this organ were homogenously the same size and the same shape. The spleen used in this study was resected in combination with total gastrectomy to ensure the oncological radicality, and it was fixed with non-buffered formalin and embedded in paraffin wax. And it was ascertained that two hybridization signals in the suspended nuclei from the same specimen were detected previously[18]. Then tissue sections cut from 4 to 8 μm in thickness were mounted on the silane-coated glass slides. The method of silane-coating was precisely described by Rentrop et al[19], and his original method was modified. When coating with aminoalkylsilane, it would be allowed to minimize the dettachment of the sample material from the glass surface and the background noise by binding of FITC-avidin to the area instead of target DNA. These sections were dewaxed, rehydrated, and incubated in 0.25% citrate buffer/trysin (pH 7.60) overnight. The next day, after the sections were fixed in 4% paraformaldehyde in PBS, they were digested with 0.5% pepsin (p-7000, Sigma) for the purpose of proteolysis, dipped in 70% acetic acid, incubated in 1% hydroxylamine hydrochloride, and washed in 2 xSSC. Then the target DNA in the material on the glass slide and the probe DNA were denatured at more than 70℃ for 2 and 10 minutes, respectively, and in situ hybridization was performed at 37℃ in humid chamber overnight. The posthybridization washing and detection were precisely described in the chapter of method.

The predicted value of the hybridization signals using alpha-satellite DNA probe was two for autosomes and one fot sex chromosome in normal diploid cells obtained from such as spleen in this preliminary study. Applying the DNA probe for chromosome 17, five μm section in thickness showed 85.2% of two signals, and the value was the most frequent in the preliminary study. As the sections were more thicker, the frequency of three or more hybridized spots were detected because of nuclear overlapping. As for a probe for chromosome Y, on the other hand, one hybridized spot was the predicted value, and 5 μm section showed one signal in more than 91.1% and this was the modal number. It is concluded that the adequate thickness of the tissue section for FISH was 5 μm.

(2) Clinical applications

Ten cases of colonic adenomas, nineteen cases of CIA and fifteen cases of small sized carcinomas (so called polypoid cancers) were analyzed in this study. These were collected from operative or polypectomy specimens in Nagasaki University Hospital and affiliated hospitals from 1985 to 1993. In adenoma, the pathological type in detail were nine tubulovillous adenoma and one villous adenoma. Mean age was 56.1±15.4, sex ratio was 8/2 in male/female, and mean size in diameter was 8.4±2.7 mm. As for polypoid cancers, pathological subtypes were tweleve of mucosal cancer and three of submucosal. And mean age was 63.3 ±8.8 and sex ratio was 9/6 and mean size was 28.3±16.6 mm. In carcinoma in adenoma (CIA), mean age was 63.8 ±8.1 and sex ratio was 10/9 and mean size was 15.0±12.1 mm.

DNA Probes and In Situ Hybridization

Biotinylated repetitive DNA probes (Oncor Inc., Gaithersburg, MD,USA) for specific for the centromeric regions of chromosomes 11, 17(D11Z1, D17Z1) were used. The highly repetitive sequences of the DNA were confirmed near the centromeric regions.

First, the 5 μm slides in thickness on which the target materials were mounted were deparaffinized in xylen twice for 20 minute, rehydrated in gradient ethanol series (100, 95, 70%) for 10 minutes each and rehydrated in distiled water (DW3) for 10 minute. Then the slide were incubated in trypsin/citrate buffer solution (pH 7.60) overnight to

remove interstitial materials roughly. This trypsin/citrate buffer solution contained 3 mM trisodium citrate, 0.1N nonidet P-40, 1.5mM spermine, 0.5mM hydroxymethyl amino methane, and 0.25 % trysin. The next day, the slides were washed with phosphate buffer saline (PBS) in koplinger, fixed with 4 % paraformaldehyde/PBS for 10 minutes at 4℃, and washed with PBS for 5 min two times. After digestion with 0.5 % pepsin in 0.2N HCL for 15 to 25 minutes at 37.0℃, there were dipped in 70.0 % acetic acid for 90 seconds to remove dirt, and incubated in 1.0 % hydroxylamine hydrochloride (H-2391, Sigma, USA) > Acetylation was done with acetic anhydrate in Tris-HCl for blocking the non-specific binding between DNA probe and free base of target DNA. Then the slides were rinsed with 2 xSSC (standard saline citrate).

The target DNA on the slides were denatured in 70 % formamide in 2 xSSC at 70℃ for two minutes, immersed in 70 % ethanol for quickly cooling and dehydrated in gradient ethanol series and the slides were allowed to dry with cool air. The hybridization mixture containing probe DNA, on the other hand, was denatured for 10 minutes at 70℃. Then the probe mixture was applied to the target DNA on the slides, under a coverslip and sealed with paper bond (Kokuyo, Japan). Then in situ hybridization was performed in humid chamber at 37℃ for 10 to 16 hours.

After in situ hybridization, the slides were washed two times for ten minutes in 2 xSSC at 42℃. They were then washed under stringency condition (60 % formamide in 2 xSSC) for fifteen minutes at 42℃ to remove and washout non-specific hybrids on the target DNA, followed by successive washes in 2 xSSC and 0.05 % Tween 20 in PBS for 5 minutes each at 42℃. At the next step, incubation with 1.0 % bovine serum albumin/0.05 % Tween 20 in PBS was performed for blocking. The hybridized probe was detected by fluorescein-avidin DCS (Vector Labboratories, Burlingame, CA) at a final concentration of 5 μg/ml in TBS buffer (TBS was contained of 1.0 % bovine serum albumin, 4 xSSC, Tween 20). They were rinsed in 4 xSSC in 0.05 % Tween 20 in PBS for five minutes each at 42 ℃. Amplification was carried out by using biotinylated goat anti-avidin D (Vector Labboratories) at a concentration of 5 μg/ml in TBS buffer, followed by another layer of fluorescein-avidin. All the immunohistochemical steps were preformed for 30 minutes at 37 ℃. Nuclei were counterstained with an antifade solution, containing propidium iodide (1.0 μg/ml), and the FISH signals were counted and photographed using an Olympus BH-2 fluorescent microscope with epifluoresnce using Fuji film (Fujichrome ASA 100, Tokyo, Japan).

Results

Criteria for the Evaluation of FISH Signals

To detect and count the FISH signals, more than 200 nuclei in the tumors were counted in each case. Pericentromeric probes for chromosome 11 and 17 demonstrated disomy in approximately 60-80 % of nuclei examined from normal mucosal cells in the same specimens. The threshold of aneusomy should be set in avoiding the underestimation of monosomy. Then three cases of carcinomas and four cases of adenoma or CIA were randomly picked up and signals were counted on the area of normal appearance for setting the threshold. The mean value was 23.5±7.5 % of monosomy, 62.9±19.6 % of disomy, 7.2± 5.5 % of trisomy for chromosome 11, and 14.3±3.6 %, 76.1±13.6 %, and 16.9±0.7% for chromosome 17, respectively. The threshold was set as mean value plus one standard deviation (mean+1SD), then the loss of signal for a certain probe was determined that the population was over 30 %. On the contrary, chromosomal gain was determined as polysomy that the population was over 18 % (Table 1B). Major fluorescence of normal mucosal cells and tumor cells were counted on interphase nuclei. Results were interpreted as significant for polysomy when greater than 18 % of the nuclei had more signals than diploid for a specific chromosomal DNA probe, while determination of hypoploidy was based on finding that more than 30 % of the cells had fewer signals than expected for a diploid cell. The range of monosomy with the probes in normal colonic mucosa was 3.8-36.0 %, with an average monosomic frequency of 19.1 %. Because thin section as applied in this study did not encompass the whole nucleus, some artificial loss of the disomic signals is to be expected in all tissues.

Table 1. Signal distrbution in normal colonic mucosa

Case	Probe	Depth of invasion	Monosomy	Disomy	Trisomy (%)
910621	17	m	14.3	65.8	16.9
910469	17	m	16.7	68	15.3
930472	17	m	21.2	74.6	3.7
930476	17	m	18.3	76.1	5.5
582	17	s	18.7	72.4	8.9
582	17	s	26.3	68.3	5.4
944	17	pm	27.9	67.1	5
944	17	pm	36	60.7	3.3
988	17	s	32.5	66.8	0.7

The range of trisomy in normal epithelium was 3.3-13.6 % with the probes, for an average trisomic frequency of 7.2 %.

Criteria for the evaluation of FISH signals were the following. (a) Overlapping interphase nuclei were not counted. (b) Signals within one nucleus should have more or less the same size or intensity. (c) Splitting shaped or adjacent spots were counted as one signal. (d) Unusual or non-specific spots such as minor binding sites were not counted, If no or only one FISH signal was seen in the abundance of tumor cells, the hybridization procedure was regarded as suboptimal and should be repeated[22]. (e) Small fluorescent signals were counted more than two hundred nuclei per one sample, and in histologically heterogenous sample as carcinoma in adenoma, FISH signals should be counted in both regions, respectively. (f) Nuclei which signals were different from the others in signal intensity or size were excluded because such nuclei were not representative.

Statistical comparisons were evaluated by chi-square test.

Comparison between FISH to the isolated nuclei and FISH on the section in the same specimens

The validity and specificity of the detection of FISH signals were determined on interphase nuclei obtained from isolated cells and paraffin sections from the samples. The tumors which were applied to the test were consisted of one mucosal, one submucosal, one proper mucosal, one serosal and 4 subserosal cancers (Table 2). As for chromosome 11, FISH on the tissue section was performed to 8 cases and the suspension FISH was done to 6 cases. For chromosome 11 the former was seven and the latterr was eight cases. The accordance ratio with section FISH and suspension FISH was 3 to 6 (50.0 %) for chromosome 11 and 5 to 7 (71.4 %) for shromosome 17. There was no correlation between signal numbers and the depth of invasion and no relationship between signal numbers and DNA index (DI).

Table 3. Hybridization results of adenoma

	Chromosome 11		Chromosome 17	
	Number	%	Number	%
Monosomy	6	60.0	3	30.0
Disomy	4	40.0	4	40.0
Trisomy	0	0	3	30.0

Table 4A. Hybridization results of adenoma component in CIA

	Chromosome 11		Chromosome 17	
	Number	%	Number	%
Monosomy	5	29.4	3	17.7
Disomy	8	47.1	12	70.6
Trisomy	4	23.5	2	11.7

Clinical application to colorectal neoplasias

In application of FISH on the paraffin sections to the clinical materials as adenomas, CIA and polypoid cancers, numerical aberrations could be detected in almost all cases in this study.

In ten cases of adenoma, mean size of them was 8.39 ± 2.69 mm in diameter and the locations were four in sigmoid colon and rectum, two in descending colon, and pathological features were nine cases of tubulo-villous adenoma with moderate to severe atypia and the rest was villous adenoma. Monosomy 11 was detected in 6 cases (6/10, 60.0 %, one case was undetectable because of poor probe penetration), and disomy 11 was in 4 (40.0 %). As to chromosome 17, monosomy was in 3 cases (30.0 %), disomy in 4 (40.0 %), and trisomy in 3 (30.0 %) (Table 3).

Adenoma component of 19 cases of CIA, the group was constituted of 5 cases of monosomy (29.4 %), 8 of disomy (47.1 %), and 4 of trisomy (23.5 %) for chromosome 11 accordining to the criteria above (Table 4A). And as for chromosome 17, on the contrary, the other group was constituted of 3 cases of monosomy (17.7 %), 12 of disomy (70.6 %), and 2 of trisomy (11.7 %). The frequency of monosomy 11 in adenoma component in CIA was lower

Table 2. Comparison between FISH to the isolated nuclei and FISH on the section in the same specimens

Case	Depth of inuasion	Chromosome 11		Chromosome 17		DNA index
		Section FISH	Suspension FISH	Section FISH	Suspension FISH	
902	m	1	ND	3	2	1.00
968	sm	1	2	3	2	1.00
944	pm	3	ND	3	3	1.94
828	ss	3	4	3	3	1.71
882	ss	1	1	ND	3	1.72
947	ss	1	1	1	1	1.00
978	ss	1	2	3	3	1.32
903	s	1	3	3	3	1.20

ND;not done

Table 4B. Hybridization results of carcinoma component in CIA

	Chromosome 11		Chromosome 17	
	Number	%	Number	%
Monosomy	9	56.3	1	5.6
Disomy	4	25.0	5	27.8
Trisomy	3	18.7	12	66.6
	※		※p = 0.0027	

Table 5. Hybridization results of polypoid carcinoma

	Chromosome 11		Chromosome 17	
	Number	%	Number	%
Monosomy	5	41.7	1	6.7
Disomy	3	25.0	2	13.3
Trisomy	4	33.3	12	80.0
	※		※p = 0.0366	

than that of simple adenoma (5/17, 29.4 %). Then the frequency of chromosomal aberrations between simple adenoma and adenoma component of CIA was not significantly different. Mean signal distribution for chromosome 11 was 26.5 % of monosomy and 66.4 % of disomy and 7.1 % of trisomy. Following the criteria of signal evalution, four cases (50.0 %) were dominant in monosomy and the rest four (50.0 %) in disomy for chromosome 11. On the other hand, for chromosome 17 (Fig. 1A, 1B), mean distribution was 18.47±9.7 % of monosomy and 64.3±12.8 % of disomy and 17.3±16.6 % of trisomy. Three cases (42.9 %) were dominant in monosomy, one (14.2 %) in disomy, and three (42.9 %) in trisomy.

As for cacinoma component of 19 cases of CIA, the following aberrations were found; 9 cases of monosomy (56.3 %), 4 of disomy (25.0 %), and 3 of trisomy (18.7%) for chromosome 11, and one of monosomy (5.6%), two of disomy (27.8 %), tweleve of trisomy (66.6 %) for chromosome 17, respectively. The frequencies of monosomy 11 and trisomy 17 were significantly high (p=0.0027). (Fig.2A, 2B).

Fifteen cases of polypoid cancers consisted of 5 cases of monosomy (41.7 %), 3 of disomy (25.0 %), 4 of trisomy (33.3%) for chromosome 11 and one of monosomy (6.7 %), two of disomy (13.3 %), and 12 of trisomy (80.0 %) for chromosome 17 (Table 5). The monosomy for chromosome 11 and the trisomy for chromosome 17 were significantly dominant in polypoid cancers (p=0.0366).

When examined these cases by fluorescence microscopy, the HE staining slides of the same paraffin blocks were always compared with the slides of FISH (Fig.1A, 1B, 2A, 2B).

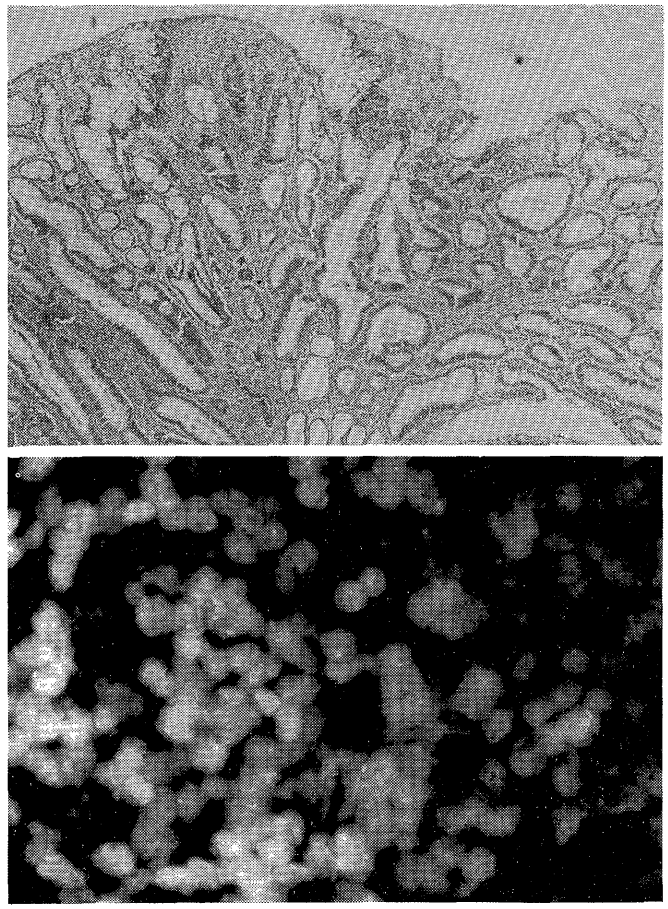

Fig. 1A ; It shows a case of carcinoma in adenoma, HE staining of adenoma coponent.
1B ; Hybridization signals in adenoma component, which shows mainly monosomy with DNA probe for chromosome 17.

Discussion

The flow cytometric (FCM) technique quantitated the total nuclear DNA content of a tumor cell population, and FCM provided rapid information about the ploidy of the tumor[3,4,5]. But it had limitations in the detection of minor quantitative DNA changes, namely, it gave no information about specific chromosome aberrations. Furthermore, its changes of less than 4% in the DNA content were not detectable by FCM[20].

Chromosome analysis of cancer cells by karyotyping (metaphase cytogenetics) faciliated that identification of small deviations in chromosome content and chromosome structure. And the principal reason for the delay of solid tumor cytogenetics establishment was difficulty in bringing the neoplastic cells of many solid tumor types to divide in vitro and the banding quality of metaphase spreads was also generally poor, and furthermore, such analyses were often hampered by the small number of recognizable metaphase, the lack of chromosome spreading, poor banding quality, and a condensed or fuzzy appearance of

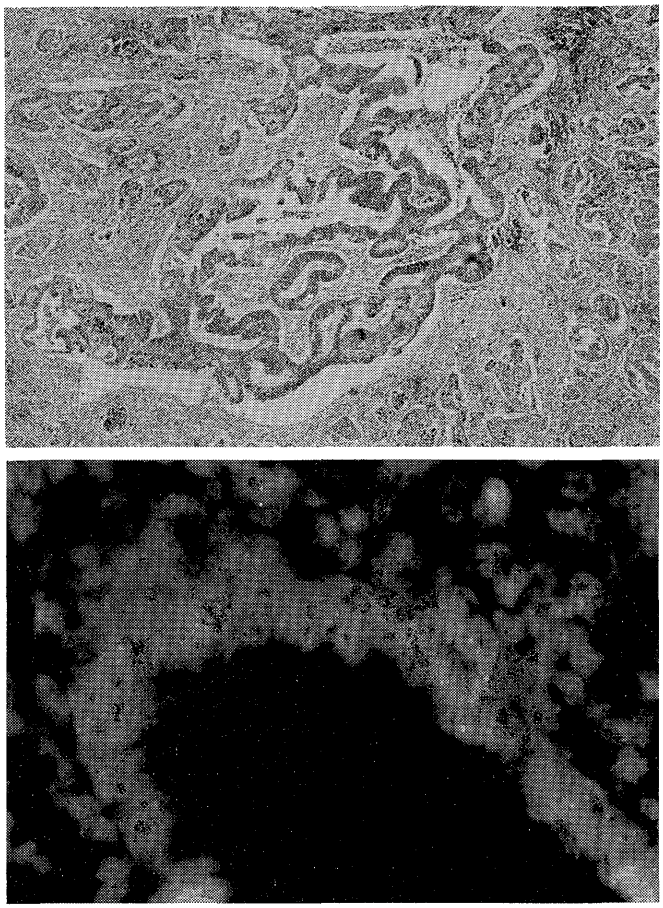

Fig. 2A ; It shows the carcinoma component of the same case, HE staining.
2B ; Hybridization signals in carcinoma component, which shows mainly disomy.

chromosomes[21].

Multiple molecular techniques, as DNA sequencing, Southern and Nothern blotting, RFLP analysis, and PCR (polymerase chain reaction), made it possible to study genes, their copy number, structure, and the regulation of their expressions. These techniques have identified different genes involved in cancers, such as proto-oncogenes and tumor suppressor genes. Although the sensitivity of these molecular techniques was high, partially as a result of the large amount of starting material, no information was obtained on the single-cell level, and heterogeneity within a population of cells was often difficult to detect and recognize.

Recently, some studies were published, comparing cytogenetic procedure using non-radioactive ISH with conventional cytogenetic analyses of cell lines derived from solid tumors, in neoplastic cells from bone marrow and peripheral blood cells. But chromosomal analysis which was applied to solid tumors without tissue culture were very rare. Then, as a fundamental study before inducing the clinical materials, the feasibility of FISH was investigated using chromosome specific repetitive DNA probes for chromosomes 17 and Y on formalin fixed paraffin embbedded tissue sections of spleen tissue specimens, first of all, the adequate thickness of FISH on the tissue sections was determined as five μm.

In the present study, interphase cytogenetics was performed using adenoma and carcioma in adenoma (CIA) and polypoid cancer in the colon and rectum. Each tumor nuclei were considered more larger than splenic nuclei and the sizes and the shapes of the tumors were not homogenous. Then eight cases of colorectal neoplasias were randomly picked up, and 5 μm tissue section and isolated nuclei were obtained from the same paraffin blocks and FISH was done for the materials.

The reasons we selected the two probes for chromosomes 11 and 17 in this study was that the following genes played an important role in tumor progression. First, the short arm of chromosome 17 (17p) contained the tumor suppressor gene p53, and loss of heterozeigosity in 17p was strongly related to the progression of colorectal tumorigenesis.[1),2)] And recently chromosome deletions and/or allelic losses from 11q are frequently observed in tumors of the large intesitine[1,2,23,24)], indicating the possible location in this chromosomal arm of an as yet unidentified tumor suppressor gene, whose inactivation may play an important role in colorectal tumorigenesis. Konstantinova et al performed cytogenitic and restriction fragment length polymorphism (RFLP) analyses in a large series of patients, and they found 11q 22-23 deletions and/or allelic losses in 23 of 39 (59 %) informative colorectal carcinomas[24)]. The role of chromosome 11 in tumor suppression was confirmed by suppression of the tumorigenic phenotype on the introduction of the chromosome into the tumorigenic Hela cell-derived hybrid cell lines by Misra et al[25)].

In the previous study by our co-workers, numerical aberrations for chromosome 11 were investigated with fresh and paraffin blocks. Monosomy for chromosome 11 was mainly recognized in early cancer and in a colorectal cancer with no metastasizing lymphnode. In comparison with the results for isolated nuclei and that for tissue section samples, the same results were obtained in two cases (cases 882 and 947, depth of invasion was ss in both), in which main copy number was monosomy (Table 2). As for the remaining four cases, the copy numbers of FISH for sections were lower than that for suspended nuclei. For chromosome 17, on the contrary, the copy number for the centromere specific DNA probe tended to increase according to the depth of invasion. The concordant ratio between the results for isolated nuclei and that for tissue section was 71.4%(5/7). It was noteworthy that the copy numbers of tissue section for chromosome 17 was superior to that of isolated nuclei in two cases, in which the depth of invasion were m and sm. Then, hybridization results of suspension as cases 902 and 968 was not representative for tumor properties, and section FISH was more reliable than

suspension FISH. Considering these results, certain criteria and thresholds should be established in the evaluation of FISH signals on tissue sections. As for normal colonic mucosa, three cases of carcinoma in adenoma (CIA) and three of carcinoma was examined for evaluation for the copy numbers of adjacent normal mucosa (Table 1), the frequency of monosomy was 23.5 ± 7.5 %, 63.0 % for disomy, and 7.2 ± 5.5 % for trisomy. The threshold for monosomy was decided by means of calculation of mean value plus one standard deviation (23.5+7.5 %), so the underestimation of hybridization results could be avoided. Then, when the frequency of one copy number was more than 30.0 %, the final result of the material was monosomy. And aneusomy for a certain chromosome was determined as trisomy when the frequncy of three signals was more than 18.0 %. By setting these criteria for evaluation of FISH signals on five μm tissue sections, which could not encompass a whole nucleus obviously, the data obtained from FISH on tissue sections became similar and responsible to that from isolated nuclei.

For the retrospective study using archival paraffin blocks, the proteolytic digestion step with pepsin was very important for guarantee of a good penetration of the DNA probes and reporter molecules, for preservation of a good morphology in cells, while discrete ISH signals with a high fluorescence intensity were obtained Hopman et al mentioned that they got better results with sodium isothiocyanate before pepsin digestion. Bur more better ISH signals and good morphology have been obtained by our collegue without sodium isothiocyanate. Namely, after incubation in citrate butter trypsin, we treated glass slides dipping into 4 % paraformaldehyde at 4 ℃ for prefixation[22]. Poddighe et al pointed out that ISH, especially double-target ISH could be detected some numerical chromosomal aberrations which the classical cytogenetic analysis could not be confirmed. This could be explained by the fact that the difficulties in the classical karyotyping techniques were often a consequence of the lack of mitoses and/or poor banding quality as a result of condensed or fuzzy appearance of the chromosomes[14]. The nature of the probes used for the ISH in their study was such that they did not allow detection of these aberrations as structural ones. The dicrepancy of the ISH results is due to the fact that the sample used for karyotyping was analyzed 10 years before the ISH data were obtained. This finding could probably be explained by a preparation artifact, since other differnt chromosomes were also missing in these three individual cells. This result illustrated an advantage of the ISH procedure, in which more cells could be analyzed, as compared to karyotyping.

In recent years, gene amplification, which was a characteristic feature of malignant cells needed for acquisition and maintenance of the malignant phenotype could be detected by FISH[28,29]. Kallioniemi et al showed that FISH allowed assessment of the levels of ERBB2 amplification as well as the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas. The major advantage of FISH in comparison with other methods for quantitating ERBB2 genes amplification was that it permitted measurement level of amplification in a tumor as well as the actual number and distribution of ERBB2 genes was in individual, morphologically defined cells. The method was rapid, nonradioactive, and required small amount of tumor material. Their results indicated the average level of ERBB2 amplification determined by FISH was closely correlated with Southern and slot blot data. In tumors with amplification, however, FISH revealed an extensive cell to cell variation in gene copy number. This was expected on the basis of the tumor heterogeneity found in the evaluation of other tumor properites as histologic differentiation, DNA content, or estrogen receptor expression. A unique property of FISH was that it allowed evaluation of the pattern of amplification on the basis of spatial distribution of amplified gene copies in interphase nuclei and metaphase chromosome. They finally suggested that extrachromosomal amplification was an early step, whereas the integration of amplified genes into chromosomes occured later during tumor progression.

Finally, the application of the FISH technique on the paraffin sections of malignancies was a powerful tool for investigation for the retrospective study and a trigger to detect key chromosomes in association with progression in the certain malignant tumors[30,31]. FISH would allow to distinguish the malignant region and the non-malignant region in comparing with the H-E stain section in view of numerical chromosomal aberrations. In addition, it was emphasized that the intratumor heterogeneity could be elucidated at the level of chromosomes.

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