The Relationship between Numerical Aberrations of Chromosome 17 and Nuclear DNA Content in Colorectal Carcinoma Detected by Fluorescent In Situ Hybridization (FISH) and Cytofluorometry Using Auto-scanning Stage

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Recently, interphase cytogenetics using fluorescent in situ hybridization (FISH) was performed on various kinds of solid tumor, and their inherent karyotypic heterogeneities were revealed. Concerning this heterogeneity, we evaluated both the exhibiting number of chromosome 17 and nuclear DNA content on an identical nucleus by means of computer-controlled auto-scanning stage in order to demonstrate the alteration in number of chromosome 17 among cytofluorometrically distinct subpopulations.

We investigated 8 lesions of surgically resected colorectal carcinomas, which were classified as aneuploid in quantitative DNA analysis and also exhibited an increase of 17-aneusomy nuclei. We used paraffin-embedded archival blocks. First, we prepared isolated cell specimens, and memorized the position of the cells on a glass slide using computer-controlled auto-scanning stage. Next, the specimens were stained with propidium iodide, and the fluorescent intensity was evaluated as nuclear DNA content in the order of cell position data. And lastly, FISH was performed with (peri) centromere-specific DNA probes for chromosome 17, and we enumerated the number of signals in a nucleus also according to cell position data. Then, we compared the distribution of number of chromosome 17 among cytofluorometrically distinct subpopulations.

Three of 8 lesions showed a single GO + G1 peak, and the rest exhibited plural GO + G1 peaks in DNA profile. And 4 of 5 lesions, which showed plural GO + G1 peaks, presented a peak at the DNA value of (near) 2c. We could detect an alteration in the distribution of number of chromosome 17 between diploid peak and aneuploid peaks in 4 of 4 lesions which presented a peak at the DNA value of (near) 2c. However, we could not find a difference in the distribution of number of chromosome 17 between diploid peak and G2 + M peak. These observations indicate that the distribution of number of chromosome 17 reflects an endoreduplication of genome content, yet, it does not alter in accordance with the phase of cell cycle. It is necessary to evaluate nuclear DNA content simultaneously in order to assess an essential cytogenetic change.

Key words: Colorectal cancer, In situ hybridization, Numerical chromosome aberration, Nuclear DNA content, Multiparametric analysis

I. Introduction

The fluorescent in situ hybridization (FISH) technique using (peri) centromeric DNA probe has been applied to various kinds of solid tumor to analyze chromosome abnormalities, and numerical chromosome aberrations have been demonstrated [6, 9–3, 15, 16, 19–23]. In these studies, tumor cell population usually consists of several karyotypically different subpopulations with respect to the exhibiting numbers of a particular chromosome. In some cases, quantitative DNA analysis could reflect such karyotypic heterogeneity, yet, DNA ploidy analysis and
cytogenetic analysis using FISH showed discrepant results in other cases [6, 13, 15]. For example, the tumors showing diploid pattern in DNA profile often exhibited a heterogeneity in the cytogenetic analysis using FISH. Therefore, the relationship between nuclear DNA content and numerical aberration of a particular chromosome has been considered to be complicated.

In colorectal tumors, numerical aberrations of chromosome 7, 8, 12, 17 and 18 were demonstrated in combination with clinicopathological analyses, immunohistochemical analyses, or DNA ploidy analyses [3, 9, 20]. And such numerical aberrations in chromosomes were discussed as related to the grade of tumors, proliferation activity, or DNA aneuploidy. On the other hand, DNA ploidy analyses were more widely applied to various grades of colorectal tumors [4, 5, 8], and the correlation between the DNA aneuploidy and the grade of tumor was also demonstrated. An alteration of nuclear DNA content may involve chromosome numerical aberrations, and both the DNA aneuploidy and numerical chromosome aberrations correlate to the grade of tumor, whereas discrepant results are often observed between them.

Chromosome numerical aberrations have been discussed as a result of chromosome instability, which has been considered as a consequence of DNA injury and the malfunction in the process of DNA repair. Mutation of p53 gene, which locates on the short arm of chromosome 17, is considered to be one of the most representative causes which yield the malfunction in the process of DNA repair, and the relationship between the accumulation of mutant p53 protein and DNA aneuploidy has been proved [2, 14, 18]. By the mutation of p53 gene, chromosome 17 acquires the instability for itself, and moreover, the process of DNA repair becomes malfunctioned. On this point of view, we considered that chromosome 17 was the most suitable for investigating the relationship between numerical chromosome aberration and nuclear DNA content.

Although both nuclear DNA content and number of a particular chromosome should be evaluated simultaneously on an identical nucleus, we usually could not obtain a successful result, because the denaturation process, which is required in FISH procedure, hinders correct evaluation of nuclear DNA content. In the present study concerning colorectal carcinoma, we have collected both of these values sequentially on an identical nucleus by using computer controlled auto-scanning stage [1]. We have paid attention to the heterogeneity in DNA profile, and compared the number of signals in a nucleus (number of S/N) among cytofluorometrically distinct subpopulations, in order to investigate the correlation between nuclear DNA content and numerical aberration of chromosome 17.

II. Materials and Methods

Materials and sample preparation

We investigated 8 lesions from surgically resected colorectal carcinomas. These lesions were classified as aneuploid by quantitative DNA analysis, and also classified in 17-aneusomy by cytogenetic analysis using FISH (Table 1).

Formalin fixed and paraffin-embedded archival tissues were used for this study. First, we made alternative 4 μm- and 100 μm-thick sections, and stained 4 μm-thick sections with hematoxylin and eosin (H & E). Guided by identification on the H & E stained sections under a microscope, we cut out 100 μm-thick sections from each corresponding neoplastic lesion. The small tissue fragments were digested in 75 μg/ml Proteinase K solution for 30 min at 37°C and then homogenized. After filtration of the suspensions, nuclei were cytocentrifuged onto poly-L-lysine coated glass slides and air dried. Next, these specimens were digested in 0.1% pepsin/0.1 N HCl solution for 30 min at 37°C and subsequently incubated in 1 mg/ml RNase A solution for 30 min at 37°C. After a brief wash, the specimens were post-fixed in 4% paraformaldehyde solution, dehydrated in graded series of EtOH and air dried. Then the slides were stored at −20°C until the analyses.

Efficiency of hybridization on PI pre-stained sample

Since propidium iodide (PI) combines with DNA by intercalation between molecular combination of A-T and G-C, PI staining may prevent proper hybridizations of the probes. To examine the efficacy of hybridization on PI pre-stained sample, we used non-neoplastic lymphocytes. The lymphocyte samples were prepared from a suspension of white blood cells separated from human blood obtained from a healthy Japanese male. The suspension of white blood cells was cytocentrifuged onto poly-L-lysine coated glass slides and air dried. These specimens were fixed in 4% paraformaldehyde solution, digested in 0.05% pepsin/0.1 N HCl solution for 10 min at 37°C, and subse-

<table>
<thead>
<tr>
<th>No.</th>
<th>Peak DNA value</th>
<th>Aneusomic subpopulation detected by FISH analysis (% of nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4c</td>
<td>Tri (48.6%) Tetra ≤ (16.4%)</td>
</tr>
<tr>
<td>2</td>
<td>2.6c</td>
<td>Tri (17.9%)</td>
</tr>
<tr>
<td>3</td>
<td>2.8c</td>
<td>Mono (35.1%) Tetra ≤ (19.7%)</td>
</tr>
<tr>
<td>4</td>
<td>4.6c</td>
<td>Tri (34.2%) Tetra ≤ (12.4%)</td>
</tr>
<tr>
<td>5</td>
<td>3.2c</td>
<td>Tri (19.6%)</td>
</tr>
<tr>
<td>6</td>
<td>3.6c</td>
<td>Tri (33.5%)</td>
</tr>
<tr>
<td>7</td>
<td>3c</td>
<td>Tri (28.5%) Tetra ≤ (9.8%)</td>
</tr>
<tr>
<td>8</td>
<td>5.4c</td>
<td>Tetra ≤ (38.8%) Tri (22.7%)</td>
</tr>
</tbody>
</table>

*: Mono, Tri and Tetra are the abbreviations for monosomy, trisomy and tetrasyomy of chromosome 17, respectively.
fluently incubated in 1 mg/ml RNase A solution for 30 min at 37°C. Then, the specimens were post-fixed in 4% paraformaldehyde solution, dehydrated in EtOH series and air dried.

We prepared 2 specimens, one for conventional FISH procedure, and the other for FISH on PI pre-stained sample. We acquired cell position datas from both of these specimens using computer controlled auto-scanning stage. One of these specimens was stained with PI [1], observed under excitation light at 530 nm, and washed briefly. Then we performed FISH on both of these specimens with the same method as described below. The number of S/N was counted at a 480 nm excitation wavelength. We compared the distribution of number of S/N statistically between a plain sample and a PI pre-stained sample.

Combined analysis of nuclear DNA content and numerical aberration of chromosome 17 using autostage cytofluorometry

The combined analysis was carried out with following 4 steps.

Acquisition of cell position data
A slide was put onto a Nikon P1 fluorescent microscope (NIKON, Tokyo, Japan), into which a personal computer-controlled auto-scanning stage (Autostage) was installed. We observed 500-600 nuclei under the phase contrast view, and saved the position data of each nucleus.

DNA cytofluorometry
After positioning data were stored in the computer, the same slide was processed for nuclear DNA staining with PI solution (2.5 μg/ml), and then was put onto the Autostage again. According to the position data of the nuclei, the slide was automatically scanned, and the DNA content of each nucleus was measured under the excitation light at 530 nm. The DNA content data of each nucleus was saved along with the nuclear position data. Nuclear fluorescence intensities of lymphocytes on the same specimen were used as an internal standard of diploid DNA content (2c).

FISH for chromosome 17
After DNA cytofluorometry, the coverslip was removed and FISH was performed on the same slide. In situ hybridization was carried out mainly according to the method of Pinkel et al [17]. Briefly, the specimens were denatured in 70% formamide (FA)/2xSSC (Standard Saline Citrate) for 2 min at 70°C, and quickly chilled in cold 70% EtOH. Then the specimens were dehydrated in EtOH series and air dried.

We used digoxigenin labeled DNA probes specific for (peri) centromeric regions of chromosome 17 (D17Z1) which was purchased commercially (Oncor, Gaithersburg, MD, USA). The DNA probe was prepared in 50% FA, 10% dextran sulfate, 0.1% tween 20, and 10 μg/ml salmon sperm DNA / 2xSSC, at a probe concentration of 1 μg/ml hybridization mixture. The hybridization mixture was denatured for 5 min at 75°C, immediately chilled on ice, and applied onto each slide. Hybridization was carried out for 16-24 hr at 37°C. The hybridization probes were immunohistochemically detected by subsequent 3 incubations of the slides with sheep anti-digoxigenin antibody (Boehringer, Heidelberg, Germany; dilution 1: 100), biotinylated anti-sheep IgG antibody (Vector, Burligame, CA, USA; dilution 1: 100), and fluorescein isothiocyanate (FITC) conjugated streptavidin (Vector; dilution 1: 100) each for 2 hr at 37°C. Then the slides were soaked quickly in 0.5 μg/ml PI solution for counterstaining.

Scoring of FISH results
The specimen was put onto the Autostage, and automatically scanned again. Number of S/N was enumerated at a 480 nm excitation wavelength. Broken nuclei and nuclei with no signal were omitted from the analysis. The number of the S/N data was also saved with the corresponding nuclear position data.

Comparison of FISH results with nuclear DNA content
The data of DNA content and number of S/N were combined, and the histograms of FISH results were drawn independently for the cytofluorometrically distinct subpopulations. We compared the histograms by means of Chi square test, and the significance was defined with p-value <0.05. The subpopulations containing a small number of nuclei were excluded from the analysis.

III. Results
Fig. 1 shows the representative views of combined analysis of nuclear DNA content and number of chromosome 17.

Efficacy of hybridization after PI staining
Fig. 2 shows the result of counting the number of S/N for each sample with D17Z1 probe. Since these 2 samples showed concordant distribution of number of S/N, we concluded that the prestaining with PI does not affect the efficacy of hybridization.

Numerical aberration of chromosome 17 in cytofluorometrically distinct subpopulations
In 3 of 8 lesions, we could detect an obvious primary peak at the DNA value of more than 2c, and a secondary peak at about twice the DNA value of a primary peak (Table 2; Case 4-6). Other 5 lesions presented more than 2 peaks in DNA profiles (Table 2; Case 1-3, 7, and 8). Of these 5 lesions, 4 lesions showed a peak at the DNA value of 2c or near 2c, and 2 lesions presented a minor peak at about the DNA value of 4c other than aneuploid peaks.

Fig. 3 shows a DNA profile of Case 4, and also illustrates the distributions of number of S/N in the sub-
Fig. 1. Representative figures of combined analysis of FISH and nuclear DNA content. After the process of DNA cytofluorometry with PI staining (left), the samples were denatured and hybridized (right). Panel A) shows a nucleus of Case 1, the DNA value and number of S/N were 5.2c and 1. Panel B) also shows a nucleus of Case 1, the DNA value and number of S/N were 6c and 3, respectively. ×400 (original magnification).

Fig. 2. Efficiency of hybridization on PI pre-stained sample. Plain sample and PI pre-stained sample showed similar distribution of number of S/N. The concordance between them was proved statistically (p<0.01; Concordance test).

population of a primary peak and a secondary peak, respectively. We could not detect a statistically significant difference in the distribution of number of S/N between a primary peak and a secondary peak. Fig. 4 shows a DNA profile of Case 1 and the distribution of number of S/N in each peak. We could detect significant differences between the cytofluorometric subpopulations of 1.4c–2.2c and 3c–3.6c, 1.4c–2.2c and 4.4c–4.8c, and 1.4c–2.2c and 5.2c–7.2c, respectively, while we could not find a remarkable difference among the aneuploid subpopulations. As shown in Fig. 5, a similar observation could be found in Case 8. In this case, the cytofluorometric subpopulation exhibiting the DNA value of 1.6c–2.6c showed an increase of 17-monosomy nuclei, whereas the aneuploid subpopulations showed an increase of 17-trisomy and more than 17-trisomy nuclei.

Overall, we could detect a significant difference in the distribution of number of S/N between diploid and aneuploid subpopulations in 4 of 4 lesions (Cases 1, 3, 7 and 8), although we could not find a difference among aneuploid and tetraploid subpopulations in 7 of 8 lesions (Table 2). Fig. 6 shows a DNA profile of Case 6 and the distribution of number of S/N in each peak. In this case,
Table 2. Summary of results

<table>
<thead>
<tr>
<th>No.</th>
<th>Nuclear DNA value</th>
<th>Number of nuclei</th>
<th>Aneusomic subpopulation detected by FISH analysis (% of nuclei)*</th>
<th>Chi-square test b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4–2.2c</td>
<td>87</td>
<td>Tri (20.7%)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>3c–3.6c</td>
<td>229</td>
<td>Tri (55.0%), Tetra ≤ (21.0%)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>4.4c–4.8c</td>
<td>23</td>
<td>Tri (52.2%), Tetra ≤ (8.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.2c–7.2c</td>
<td>47</td>
<td>Tri (61.7%), Tetra ≤ (21.3%)</td>
<td>**</td>
</tr>
<tr>
<td>2</td>
<td>2.2–2.8c</td>
<td>231</td>
<td>Tri (15.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.8c–4.4c</td>
<td>48</td>
<td>Tri (25.0%)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4.6c–5.8c</td>
<td>59</td>
<td>Tri (220.9%), Tetra ≤ (8.5%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.4c–2c</td>
<td>32</td>
<td>Mono (46.9%)</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>2.6c–3c</td>
<td>129</td>
<td>Mono (52.2%), Tri (17.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.8c–5.6c</td>
<td>70</td>
<td>Mono (30.0%), Tri (24.3%)</td>
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</tr>
<tr>
<td>4</td>
<td>4.2–5c</td>
<td>168</td>
<td>Tri (36.9%), Tetra ≤ (10.1%)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>8c–11.4c</td>
<td>36</td>
<td>Tri (38.9%), Tetra ≤ (13.9%)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.8c–3.6c</td>
<td>333</td>
<td>Tri (18.6%), Tetra ≤ (8.7%)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4.8c–7c</td>
<td>40</td>
<td>Tri (32.5%), Tetra ≤ (5.0%)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.4–3c</td>
<td>270</td>
<td>Tri (35.6%)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>4.6c–5.4c</td>
<td>74</td>
<td>Tri (31.1%), Tetra ≤ (20.3%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.4c–2.2c</td>
<td>81</td>
<td>Tri (16.1%), Tetra ≤ (7.4%)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2.6c–3.2c</td>
<td>114</td>
<td>Tri (34.2%), Tetra ≤ (5.3%)</td>
<td>*</td>
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<tr>
<td></td>
<td>5c–6.6c</td>
<td>47</td>
<td>Tri (34.0%), Tetra ≤ (14.9%)</td>
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<tr>
<td>8</td>
<td>1.6c–2.6c</td>
<td>22</td>
<td>Mono (27.3%), Tri (13.6%)</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>5.2c–6.2c</td>
<td>270</td>
<td>Tetra ≤ (38.5%), Tri (25.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.8c–1.2c</td>
<td>23</td>
<td>Tetra ≤ (43.5%), Tri (17.4%)</td>
<td></td>
</tr>
</tbody>
</table>

*: Mono, Tri and Tetra are the abbreviations for monosomy, trisomy and tetrasomy of chromosome 17 respectively.

b: *indicates p<0.05, **indicates p<0.01 and N.S. indicates "not significant"

Fig. 3. Comparison of distributions of number of S/N between cytofluorometrically distinct populations in Case 4. These 2 populations showed similar distribution of number of S/N despite the difference in nuclear DNA content.
Fig. 4. Comparison of distributions of number of S/N among cytofluorometrically distinct populations in Case 1. Significant differences were detected between diploid peak and aneuploid peaks.

Fig. 5. Comparison of distribution of number of S/N among cytofluorometrically distinct populations in Case 8. Significant differences were also detected between a diploid peak and aneuploid peaks. In this case, it was notable that the nuclei exhibiting more than 2 signals increased in aneuploid peaks although the nuclei with 17-monosomy increased in a diploid peak.

we could detect a significant difference in the distribution of number of S/N. The frequency of nuclei exhibiting more than 4 signals increased in the population of a secondary peak. In this lesion, it was notable that a minor population arising at twice the DNA value of the secondary peak could be found. This observation indicated that the population of a secondary peak included the nuclei harboring higher endoreduplication.

IV. Discussion

A quantification of nuclear DNA content could not be made accurately on the specimen upon which FISH had already been proceeded, because the actual amount of nuclear DNA decreased, and the bonding of fluorescent staining agent and genome DNA was weakened after the denaturation process. Therefore, an evaluation of
Chromosome Aberration and DNA Aneuploid

Fig. 6. Comparison of distribution of number of S/N between cytofluorometrically distinct populations in Case 6. In the population showing 4.6c–5.4c of nuclear DNA content, the nuclei exhibiting 4 or more signals in a nucleus increased.

cytogenetic analysis using FISH and quantitative DNA analysis could not be made simultaneously even by a fluorocytometric method using a photo multiplier. The relationship between cytogenetic analysis using FISH and quantitative DNA analysis was studied in the case of bladder carcinoma, hepatocellular carcinoma and colonic carcinoma [13, 15, 20]. In the present study, we have evaluated each parameter sequentially by means of computer controlled auto-scanning stage. We have evaluated nuclear DNA content before the denaturation process of FISH, and then, scanned out the identical nuclei in the order of the nuclear position data after FISH process. This method enables us to acquire both nuclear DNA content and number of FISH signals on each identical nucleus. Thus, we can appreciate the correlation between nuclear DNA content and number of FISH signals.

In DNA profile, the population of a primary peak consists of the cells situated on G0+G1 phase, and the population of a secondary peak, which usually arises at twice the DNA value of a primary peak, mainly consists of the cells situated on G2+M phase. Regarding the tumors growing in vivo, we can often find minor subpopulations showing different degrees of endoreduplication as small peaks apart from these major peaks. Devilee P. et al. demonstrated that the distribution of number of S/N of a particular chromosome was not affected by the fraction of S+G2+M phase by comparing it between non-neoplastic lymphocytes and Con-A stimulated lymphocytes [6]. We could demonstrate the similar finding that the distribution of number of S/N did not alter between the cells situated on G0+G1 phase and the cells situated on G2+M phase in aneuploid colorectal carcinomas. Although the genome content actually increases in the nuclei situated on G2+M phase, the analysis of chromosome numerical aberration using FISH technique precisely interprets the intrinsic karyotype.

In estimating the number of S/N using (peri)centromere specific DNA probe for a particular chromosome, we usually suffered from not a few errors, such as insufficient hybridization, non-specific signal expression and/or counting error. Therefore, we usually did not pay attention to the karyotypic heterogeneity in the whole population of tumor cells. In this study, we could demonstrate the alteration in the distribution of number of S/N according to the endoreduplication of nuclear DNA. And, in some cases, the heterogeneity detected by FISH analysis reflected the cytofluorometrical heterogeneity. On this point, a minor subpopulation identified by FISH analysis could indicate the subpopulation which acquired different property in the manner of proliferation. However, we also found the heterogeneity of number of S/N within cytofluorometrically identical subpopulation. This heterogeneity may arise from an inherent change as well as estimation errors. It may be difficult to detect the significance of this heterogeneity because we cannot distinguish an intrinsic change from estimation errors. Gobhart E. et al. demonstrated the existence of microclones which harbored single aberrant karyotype among the interphase cell population by analyzing numerical aberration for multiple chromosomes in the case of aneuploid leukemia [7]. We also consider that an essential karyotypic heterogeneity exists within cytofluorometrically identical cell population. Although tumor cell population shows an apparent homogeneity in quantitative DNA analysis, yet it may consist of plural subpopulations which harbor slightly different karyotype. Such subpopulation, which acquires high proliferating activity, metastasizing activity or invading activity, may arise among these slightly different karyotypic subpopulations, and, later on, may dominate the characteristics of tumor.
This speculation should be proved by comparing the manner of progression or the proliferating activity among these slightly different karyotypic subpopulations.

Numerical aberration of a particular chromosome in diploid tumors was demonstrated in the case of bladder carcinoma, breast carcinoma and colorectal carcinoma [6, 9, 15]. These numerical aberrations were considered as a specific change for each tumor, and as an early state of aneuploid [6]. We could not distinguish these numerical aberrations from the numerical aberrations accompanied by aneuploidization with a single analysis of FISH. Similarly, we could not distinguish the nuclei, which exhibited disomy of a particular chromosome despite showing aneuploid in quantitative DNA analysis, from intact nuclei with a single analysis of FISH. Therefore, we can conclude that cytogenetic analysis using FISH reflected aneuploidization of nuclear DNA, although, in some cases, cytogenetic analysis using FISH indicated the discrepant result from quantitative DNA analysis.

In conclusion, we could examine both nuclear DNA content and number of chromosome 17 on each identical nucleus by means of computer controlled auto-scanning stage. We could demonstrate that the number of S/N did not alter between the nuclei situated on G0+G1 phase and those on G2+M phase, and that cytogenetic analysis using FISH could reflect aneuploidization of nuclear DNA. What is more, we could detect a cytogenetic heterogeneity in the cytofluorometrically identical subpopulation. Cytogenetic analysis using FISH and quantitative DNA analysis often showed discrepant results, because these 2 analyses reflected different aspects of alteration in genome content. Therefore, in analyzing cytogenetics using FISH, we should perform quantitative DNA analysis simultaneously in order to assess an essential cytogenetic change.

V. References


