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# Validity of Flow Cytometric DNA and RNA Analysis in Colon Cancer Cells in Comparison with Histopathological Factors

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The nucleolar DNA and RNA content were analyzed by using flow cytometry in 62 patients with colon cancer. The DNA and RNA index (DI•RI) and ploidy patterns were compared with clinicopathological findings and vascular structures.

The significances of evaluating DNA and RNA contents were clarified in association with biologic characteristics of colon cancer.

Simultaneous analysis in DNA and RNA content was attempted by using AO staining in 43 patients with colon cancer and DNA analysis was performed by using PI staing in 19 patients with colon cancer. Availability of AO staining for DNA analysis is equivalent to that of PI staining.

RNA analysis is of great use to discriminate cancer cells from normal cells. Cell differentiation is well correlated with the frequence of aneuploidy patterns. And also the histologic findings of the disease stage such as the depth, node metastasis, vascular invasion and hepatic metastasis were significantly correlated with DI, RI and ploidy patterns.

According to Dukes classification, high values of DI and RI with ploidy pattern were demonstrated with advances in the disease stage.

In conclusion, evaluation of nuclear DNA in addition to RNA contents is of great benefit in making a precise diagnosis and in applying to an effective therapy.

## Introduction

The development of flow cytometry study enabled biologic behavior of carcinomas to clinically clarify in the field of colorectal surgery.[1-2] There are numerous reports that DNA analysis is essential for assessment of malignant potential of cancer cells.[2-4] It is well recognized that the assessment of biological behavior of malignancy used to be determined by clinicopathological findings and it reflects the prognosis.[4]

It is reasoned that the measurement of nuclear RNA content is mandatory for assessment of biologic features of carcinomas at the proliferative phase.[3,4]

In this study, simultaneous analysis of DNA and RNA contents was attempted and compared with the extension of disease and pathohistological factors.

The purpose of this study is to clarify the significance of the measurement of nuclear DNA and RNA content in colorectal cancer cells and biological feature of colorectal cancer.

## Material and Methods

### *1. Subject*

During the time from June 1984 to February 1986, 62 surgical specimens resected st the First Department of Surgery, Nagasaki University School of Medicine were subjected to this study. 30 were men and 32 were women. The ages ranged from 40 to 86, with an avarege of 62.5 years old. The tumor locations were the colon in 33 and the rectum in 29, respectively. Histological disease stages showed stage I in 14, stage II in 24, stage III in 4, stage IV in 10 and stage V in 10 in accordance with the rule set forth by the Japanese Reseach Society for colorectal cancer.

### *2. Method*

1) Sample treatment

Each one gram tissue was taken from the margin and the center of the tumors in the fresh surgical specimens, cut into pieces, added 1ml of 0.02% collagenase and 3000u/ml of dispase, stirred for 2 hours at room temperature, Thereafter, filtrated with 60$\mu$m nylon filter. The addition of 0.25% trypsin to DNase and treatment at 37℃ for 10 minutes enabled contamination with destroyed cells and debris to minimize.

The samples were fixed with 1:1 of ethanol and aceton and freezed (–80 ℃ ), 10% FCS and 5% dimethyl sulfoxide (DMSO) as a protective material from freezing demage.

2) Simultaneous AO staining of DNA and RNA

The nucleolar DNA and RNA contents were analyzed by means of two step acridine orange staining (AO staining) using FACS IV (Becton Dickinson Co.) according to

Darzynkiewicz method.

The samples were adjusted to 1 to 2 × $10^6$/ml and mixed with 0.4ml of acid detergent in 0.2ml of the samples, kept at room temperature for 5 minutes.

The cells stained with AO were excited by 488nm argon ion laser. The DNA contents were measured with 530nm green fluorescence and the RNA contents were with 640nm red fluorescence.

Histograms were delineated, indicating DNA contents in the ordinate and RNA contents in the abscissa.

3) DNA analysis by propidium iodide (PI)

After defreezing in 37℃ water bath, the samples were fixed with 70% ethanol and treated with 0.5% RNase at 37 ℃ for 30 minutes to remove double helix RNA.

Thereafter, DNA analyzed by PI staining at room temperature.

In this study, the time duration of freezing ranged from 4 to 8 months.

4) DNA and RNA index (DI and RI)

DI and RI were expressed as the following equation

$$\text{DI and RI} = \frac{G_0G_1 \text{ peak in samples}}{G_0G_1 \text{ peak in control}}$$

The control used tumor infiltrating lymphocytes (TIL) as AO staining and lymphocytes in peripheral blood as PI staining.

Diploidy was regarded as a DI of 1.00 and aneuploidy was the others. The stastical analysis was made using t-test for the difference of the mean values of ploidy patterns and using $X^2$ test for the incidence of ploidy patterns.

## Results

### *1. Simultaneous DNA and RNA analysis by AO*

Fig. 1 shows a histogram and dot plot in diploidy and aneuploidy colon cancers. Cancer cells failed to identify from the normal cells on DNA histogram in diploidy patterns. In contrast, it was easy to identify from the normal cells by RNA contents. It was of great use to identify in the case of near diploidy pattern.

### *2. Comparison with PI staining and AO staining*

DNA indices were compared between PI and AO stainings as shown in Fig. 2. It is generally believed that AO staining is inferior to PI staining in association with the amount of RNA and incomplete removal of a binding material with macromolecules except for nucleic acid.

DI values at PI and AO staining were compared in 10 patients with colon cancer. As shown in Fig. 2, the coefficient index was 0.99 and the results by the both stainings correlated well each other.

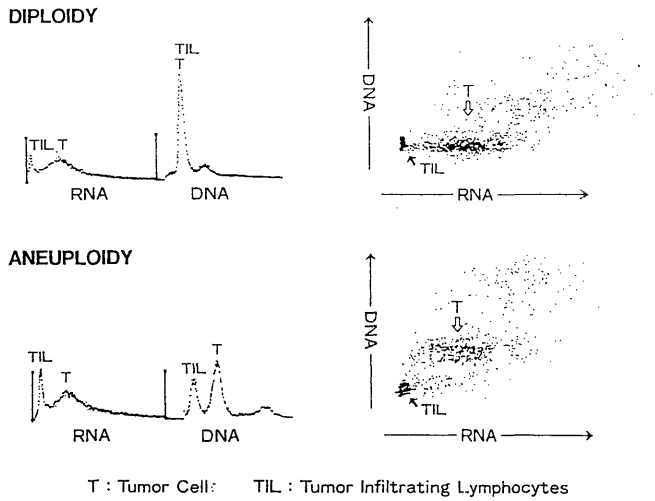


**Fig. 1.** Colon cancer patients with diploidy and aneuploidy patterns.

| Patient No. \ DNA Index | PI | AO |
|---|---|---|
| 1 H. M. | 1 | 1 |
| 2 S. S. | 1 | 1 |
| 3 M. M. | 1.88 | 2.00 |
| 4 Z. M. | 1 | 1 |
| 5 Y. K. | 1.89 | 1.88 |
| 6 T. Y. | 1 | 1 |
| 7 E. N. | 1.16 | 1.13 |
| 8 Y. T. | 1.78 | 1.72 |
| 9 E. M. | 2.02 | 2.07 |
| 10 K. T. | 2.04 | 2.21 |

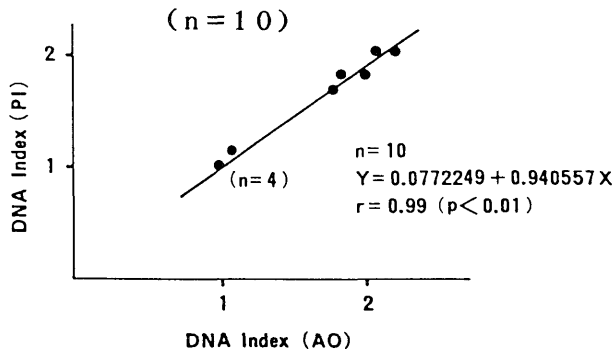


**Fig. 2.** Comparison in DNA index between PI and AO stainings.

### *3. Comparison in DNA and RNA index between the margin and the center of the tumors*

Fig. 3 showed DNA and RNA index in the margin and the center of the tumors. RNA index (4.03 ± 1.50) in the margin of the tumors is usually higher than (3.53 ± 1.43)

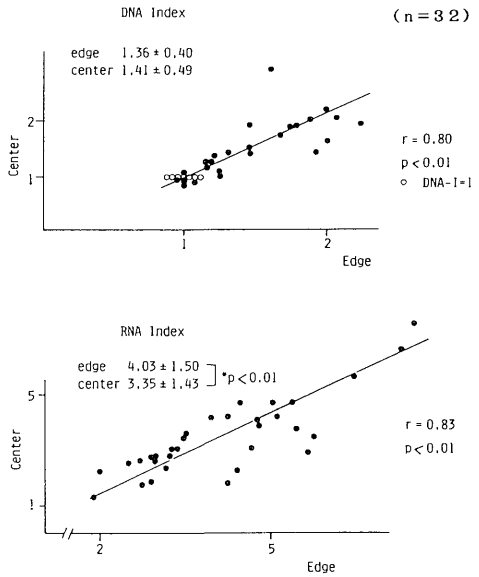


**Fig. 3.** Comparison in DNA•RNA index between the center and the margin of the tumor.

that in the center of the tumors although DI index (1.36 ± 0.40) in the margin is almost the same as that (1.41 ± 0.49) in the center.

*4. Comparison with vascular structure of the tumors and DI•RI*

Table I indicates that RI values in the margin of type A tumors was higher than those of type B tumor in accordance with Shimoyama's classification. Type A; minimal transitional changes in vascular structure in the margin of ulceration. Type B; poorly vascular structure in the margin of ulceration.

*5. Clinicopathologic findings and DIÑRI and DNA ploidy patterns*

(1) Sex and age

There was no close correlation in sexes with clinicopathological findings and patient's ages as well as DI•RI and ploidy patterns as shown in Table 2. In contast, concerning patient's ages, the DI•RI in older patients showed the higher values rather than those in younger in spite of a few older patients as shown in Table 3.

(2) Tumor locations

The DI•RI and ploidy patterns were compared between the colon in 33 and the rectum in 29 in accordance with the tumor location as shown in Table 4.

(3) Correlation with clinicopathological factors

a) Relation to histologic types

The RI showed the higher values according to poorly histological differentiation, and also tended to be an aneuploidy pattern. There was significantly statistic difference in the RI between well and moderate differentiation and in apperance of aneuploidy pattern between poor and well/moderate differentiation as shown in Table 5 and 6.

**Table 1.** Comparison with vascular structure and DNA/RNA index. (Mean ± S. D.)

| | DNA Index | RNA Index |
|---|---|---|
| A type (n = 6) | 1.19 ± 0.35 | 4.35 ± 0.83 |
| B type (n = 6) | 1.62 ± 0.54 | 3.34 ± 1.34 |

**Table 2.** Correlation among sex and DAN/RNA index and incidence of aneuploid. (Mean ± S. D.)

| sex | DNA Index | RNA Index | Aneuploid (%) |
|---|---|---|---|
| Male | 1.35 ± 0.37 (n = 30) | 3.71 ± 1.23 (n = 20) | 63.3 |
| Female | 1.25 ± 0.32 (n = 32) | 4.14 ± 1.53 (n = 23) | 65.6 |

**Table 4.** Correlation with tumor location and DNA/RNA index and incidence of aneuploid. (Mean ± S. D.)

| location | DNA Index | RNA Index | Aneuploid (%) |
|---|---|---|---|
| Colon | 1.30 ± 0.31 (n = 33) | 3.75 ± 1.36 (n = 21) | 69.7 |
| Rectum | 1.30 ± 0.39 (n = 29) | 4.13 ± 1.45 (n = 22) | 58.6 |

**Table 3.** Correlation with ages and DNA/RNA index and incidence of aneuploid (Mean ± S. D.)

| No. | ages | DNA Index | RNA Index | Aneuploid (%) |
|---|---|---|---|---|
| ① | ≧ 40 | 1.33 ± 0.52 (n = 8) | 4.11 ± 2.34 (n = 4) | 62.5 |
| ② | < 50 | 1.10 ± 0.15 (n = 11) | 4.11 ± 1.35 (n = 8) | 45.5 |
| ③ | < 60 | 1.28 ± 0.30 (n = 25) | 4.05 ± 1.60 (n = 17) | 60 |
| ④ | < 70 | 1.33 ± 0.34 (n = 14) | 3.36 ± 0.73 (n = 11) | 78.6 |
| ⑤ | ≧ 80 | 1.79 ± 0.15 (n = 4) | 4.79 ± 0.58 (n = 3) | 100 |
| | | P < 0.05 ④−⑤ | | P < 0.05 ①−③, ①−⑤ |
| | | P < 0.01 ②−⑤, ③−⑤ | | P < 0.01 ①−②, ②−④ |

**Table 5.** Pathologic factor and index. (Mean ± S. D.)

| Pathologic factors | DNA Index | RNA Index |
|---|---|---|
| Defferentiation | | |
| well | 1.25 ± 0.34 (n = 25) | 3.41 ± 1.11 (n = 18) ** |
| moderate | 1.38 ± 0.37 (n = 28) | 4.18 ± 1.40 (n = 18) ** |
| poor | 1.13 ± 0.17 (n = 4) | 5.51 ± 2.39 (n = 3) |
| Depth of cancer infiltration | | |
| m, sm | 1.02 ± 0.03 (n = 3) * | 2.95 ± 0.29 (n = 3) |
| pm | 1.31 ± 0.35 (n = 59) * | 4.02 ± 1.42 (n = 40) |
| m, sm, pm | 1.21 ± 0.30 (n = 13) | 3.17 ± 0.52 (n = 9) * |
| ss, $s_1$ | 1.34 ± 0.36 (n = 49) | 4.12 ± 1.49 (n = 34) * |
| Node Meta | | |
| n (–) | 1.27 ± 0.36 (n = 43) | 3.83 ± 1.43 (n = 32) |
| n (+) | 1.39 ± 0.32 (n = 17) | 4.26 ± 1.34 (n = 11) |
| lymphatic invasion | | |
| Ly (–) | 1.26 ± 0.35 (n = 23) | 3.35 ± 0.94 (n = 17) * |
| Ly (+) | 1.32 ± 0.35 (n = 39) | 4.33 ± 1.53 (n = 26) * |
| Venous invasion | | |
| V (–) | 1.30 ± 0.36 (n = 32) | 3.94 ± 1.30 (n = 23) |
| V (+) | 1.38 ± 0.37 (n = 20) | 3.94 ± 1.59 (n = 17) |
| Hepatic metastasis | | |
| H (–) | 1.30 ± 0.35 (n = 52) | 3.86 ± 1.29 (n = 38) |
| H (+) | 1.35 ± 0.33 (n = 8) | 4.13 ± 2.11 (n = 3) |

*P < 0.01, **P < 0.05

**Table 6.** Correlation with pathologic factor and ploidy pattern. (Mean ± S. D.)

| Pathologic factors | Diploid/Aneuploid | the incidence of Aneuploid (%) |
|---|---|---|
| Defferentiation | | |
| well | 11/14 | 56 |
| moderate | 9/19 | 67.9 * |
| poor | 0/ 4 | 100 * |
| Depth of cancer infiltration | | |
| m, sm | 2/ 1 | 33.3 |
| pm | 20/39 | 66.1 |
| m, sm, pm | 6/ 7 | 53.8 |
| ss, $s_1$ | 16/33 | 67.3 |
| Node Meta | | |
| n (–) | 18/25 | 58.1 * |
| n (+) | 4/15 | 78.9 * |
| lymphatic invasion | | |
| Ly (–) | 11/12 | 52.2 |
| Ly (+) | 11/28 | 71.8 |
| Venous invasion | | |
| V (–) | 11/21 | 65.6 * |
| V (+) | 4/16 | 80 * |
| Hepatic metastasis | | |
| H (–) | 21/31 | 59.6 * |
| H (+) | 1/ 7 | 87.5 * |

*P < 0.01

b) Relation to the depth of cancer infiltration

With the depth of cancer infiltration, the DI values in pm and deeper invasion patients were significantly higher than those in m and sm patients as well as a high RI and the higher incidence of aneuploidy patterns. The RI increased higher with high incidence of aneuploidy pattern when cancer infiltration had been extending from the submucosal layer to the muscular tunics as shown in Table 5 and 6.

c) Relation to nodal involvement

The DI and RI increased with nodal involvement and also the aneuploidy pattern revealed a high incidence as shown in Table 5 and 6.

d) Relation to vascular invasion (Table 5, 6)

The RI in patients with positive lymphatic invasion was remarkably high when compared with those with negative lympatic invasion as well as the DI and the incidence of the aneuploidy pattern.

On the other hand, the DI showed the higher values in patients with positive vascular invasion rather than negative one.

e) Relation to hepatic metastasis (Table 5 and 6)

The DI and RI in patients with hepatic metastasis were higher than those in non-hepatic metastasis and the incidence of aneuploidy pattern was significantly higher in patients with hepatic metastasis.

(4) Relation to the progression of the disease stage

The DI in stage III was higher than that in stage I. And also the RI in stage II was higher than that in stage I as shown in Table 7, Fig. 4 and 5. The incidences of the aneuploidy pattern in stage III, IV and V were significantly higher in those in stage I and II (Table 8). According to Duke's classification,[6] the DI, RI and the incidence of the aneuploidy pattern were higher with the progression of the disease stage.

There was a significant difference in DI between Duke's A and C and in both RI and aneuploidy patterns among Dukes A, B and C (Table 7, 8 and Fig. 4, 5).

**Table 7.** Correlation with stage and index. (Mean ± S. D.)

| Disease stage | DNA Index | RNA Index |
|---|---|---|
| stage classification | | |
| I | 1.19 ± 0.29 (n = 14) | 3.17 ± 0.52 (n = 9) * |
| II | 1.28 ± 0.36 (n = 24) ** | 4.27 ± 1.67 (n = 19) * |
| III | 1.62 ± 0.34 (n = 4) | 3.87 ± 0.95 (n = 3) |
| IV | 1.37 ± 0.37 (n = 10) | 4.09 ± 1.31 (n = 7) |
| V | 1.28 ± 0.32 (n = 10) | 3.93 ± 1.60 (n = 5) |
| Dukes classification | | |
| A | 1.18 ± 0.30 (n = 13) | 3.18 ± 0.55 (n = 8) ** |
| B | 1.28 ± 0.36 (n = 30) ** | 3.99 ± 1.59 (n = 24) ** * |
| C | 1.41 ± 0.34 (n = 19) | 4.39 ± 1.25 (n = 11) * |

*P < 0.01, **P < 0.05

**Table 8.** Correlation of disease stages and ploidy pattern. (Mean ± S. D.)

| Disease stage | Diploid /Aneuploid | the incidence of Aneuploid (%) | |
|---|---|---|---|
| stage classification | | | |
| I | 7/ 7 | 50 | |
| II | 9/15 | 62.5 | * |
| III | 0/ 4 | 100 | |
| IV | 3/ 7 | 70 | |
| V | 3/ 7 | 70 | |
| Dukes classification | | | |
| A | 7/ 6 | 46.3 | |
| B | 11/19 | 63.3 | * |
| C | 4/15 | 78.9 | |

*P < 0.01, **P < 0.05

(5) The disease stage and RNA index in patients with diploidy pattern of colon cancer

The RI increased with advances in the disease stage (Table 9). The nuclear RNA contents tended to correlate well with the disease stage in accordance with progression of the diseases by Duke's classification even in patients with a similar nuclear DNA content (Table 9).

**Table 9.** Correlation with stages and RNA index in diploidy patient. (Mean ± S. D.)

| Disease stage | RNA Index |
|---|---|
| stage classification | |
| I | 3.31 ± 0.89 (n = 3) |
| II | 4.59 ± 1.31 (n = 7) |
| III | (n = 0) |
| IV | 3.38 ± 1.34 (n = 2) |
| V | 4.17 ± 1.65 (n = 2) |
| Dukes classification | |
| A | 3.31 ± 0.89 (n = 3) |
| B | 4.32 ± 1.44 (n = 8) |
| C | 4.22 ± 1.17 (n = 3) |

(6) Relation between progression of the disease and DNA index in consideration of RNA index

The biological meaning of DNA and RNA index were individually different each other. However, comparative study was made between RI and DI × 3 which was based on the fact that the mean RI was 3 times as great as the mean DI.

There was a significant difference in the disease stage between stage I and II, I and III and in Duke's classification between A and B, A and C, respectively (Table 10).

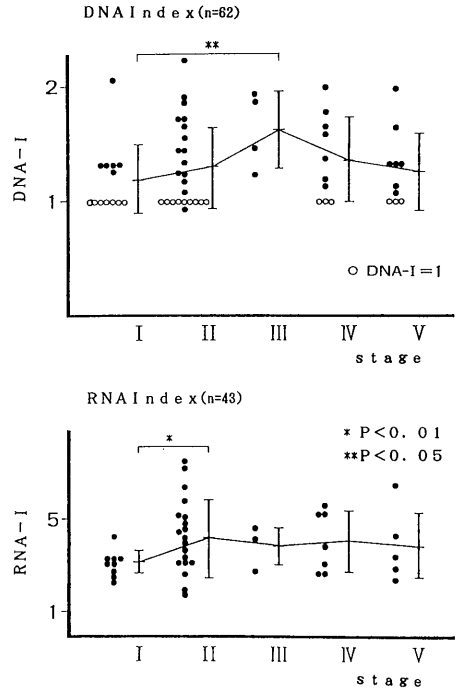


**Fig. 4.** Comparison with stage classification and DNA. RNA index.

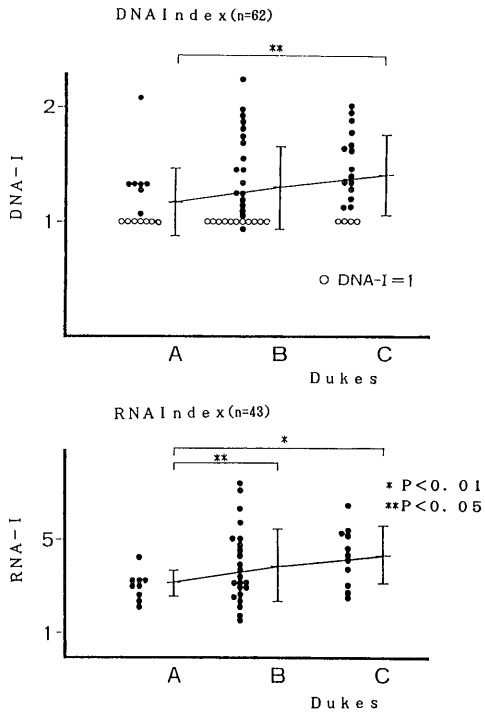


**Fig. 5.** Comparison with Dukes classification and DNA. RNA index.

**Table 10.** Correlation of disease stage and DNA in addition to RNA index. (Mean ± S. D.)

| Disease stage | Index (DI × 3.00 + RI) | |
|---|---|---|
| stage classification | | |
| I | 6.96 ± 1.06 (n = 9) | |
| II | 8.17 ± 1.64 (n = 20) | ** |
| III | 8.47 ± 1.80 (n = 3) | |
| IV | 8.25 ± 2.12 (n = 7) | |
| V | 8.02 ± 2.05 (n = 4) | |
| Dukes classification | | |
| A | 6.96 ± 1.06 (n = 9) | |
| B | 8.03 ± 1.68 (n = 23) | ** |
| C | 8.54 ± 1.82 (n = 11) | |

**P < 0.05

## Discussion

The incidences of colon cancer is increasing with changes in dietary life improvement of diagnostic techniques. Detection of early colon cancer is enhanced in recent year. The improvement of surgical outcome should be mandatory for refinement of surgical technique and potent adjuvant chemotherapy for advanced cancer patients with non-curative operation and/or recurrence.

The DNA and RNA analysis should be applied for clinical use of improving surgical outcome except for identifying biological behaviors on the basis of the result of pathohistogical findings as usual.

Recent studies have defined biological malignancy based on chromosomal aberration and the measurement of nucleaolar DNA content.[8,9]

On the other hand, FCM has enabled nuclear DNA content to measure quickly, objectively and accurately.[10] It is generally accepted that the process of cell metabolism is constant without any effect which is regarded as DNA constancy theory as reported by Boivin.[11] And also it is well known that RNA in cells is an active material and showed markedly quantitative changes from one minute to the next.

In this study, it is evaluated as to whether the measurement of RNA in cancer cells is beneficial for judging the grades of cell proliferation.

At present, the method of two step acridine orange has been worked out with respect to AO concentration, AO temperature and the time of staining.

Yamaoka[12] reported that the concomitant measurement of DNA and RNA contents is of great use to identify biological features of lung cancer. In contrast, there are few reports concerning simultaneous analysis of DNA and RNA contents in colon cancer except for DNA analysis alone as reported by Petersen[13] and Wolley.[3]

In this study, the troubles about mingling debris and destroyed cells were solved with the use of 0.25% trypsin and DNase. Yamaoka[12] emphasized that density gradient centrifugation by Ficoll Conray is of great benefit to remove contamination of normal cells in the analysis of lung cancer cells.

Ensley[14] reported that there was no definite alternation in the analysis of DNA histogram by PI staining from frozen samples preserved during one year period as reported by Vindelov.[15]

In the method of AO staining, CV of 5 or more did not impaired the measurement steps of nuclear DNA contents.

It is easy to judge cancer cells with the diploidy pattern to be a carcinoma by measuring RI of more than 3 in the region of the diploidy pattern.

In this study, comparative study in heterogeneity was made between the center and the periphery of the tumors. There was a significant difference in RI although no remarkable difference in DI between the center and the periphery of the tumors.

This indicates that RI is one of the excellent indicators for judging the grades of cell proliferation. With respect to the grades of development of tumor vascularity, high RI values were seen in the region of rich blood supply with hypervascularity on micro-angiography. It is assumed that with advancing patient's age, a high RI was recognized in relation to a weak immune response. On the other hand, it is reported that the cell nucleus enlarged with ages. Kaji[16] reported that the ploypoidy pattern increased with ages.

In contrast, a high DI was recognized in male and a high RI was seen in female without a statistic difference. With respect to tumor locations, a high RI was seen in the rectum although there was no significant difference in DI between the colon and the rectum.

It is believed that an aneuploidy pattern implies biological behaviors of malignancy in a range of 60 to 80% incidences. The poorer cell differentiation, the higher the RI was seen and the more the incidence of the aneuploidy pattern increased with a significantly statistic difference. Araki[17] also reported that poorly differentiated carcinomas showed higher incidence of the aneuploidy pattern rather than well or moderately differentiated carcinomas. With respect to the depth of cancer infiltration which is said to be correlated well with the prognosis, a high RI and a high incidence of the aneuploidy pattern are not infrequently seen when cancer extension spreads across the tunica muscularis.

The incidence of the aneuploidy pattern in patients with hepatic metastasis was markedly higher than that without metastasis.

Shida[18] pointed out that there was a close correlation with vascular invasion in the submucosal layer and hepatic metastasis.

On the other hand, Tribukait[2] reported that hepatic metastasis tended to metastasize elsewhere in the liver.

As for the disease stage, DI was high in stage III although RI kept a high level in stage II with a statistic

difference. Also it is reported that high incidence of the aneuploidy pattern was shown in stage III inclusive of positive nodal involvement.

As reported by Tribukait,[2] the results of this study also revealed that DI was high in accordance with advancing the disease stage. The incidence of the aneuploidy pattern increased with advancing the disease stage according to Araki's and/or Dukes classification with a statistic difference.

The validity of measuring RI is in stage III in patients with the diploidy pattern according to Duke's classification and the disease stage.

In conclusion, the measurement of DI and RI is of great use to judge biological bihaviors of the tumor cells. It is defined that the number of chromosoms is proportional to nuclear DNA contents as reported by Atkin. Therefore, the cell kinetics of the stem cell should be taken into consideration by measuring nuclear DNA content. In contrast, it is suggestive of alteration of stem cell lines by the investigation between primary and metastatic foci and between adenomas and carcinomas. Based on the result of this investigations, the DNA and RNA contents increased in variation of cell milieu with heterogeneous cell groups. A peak of DNA implies that the stem line cells accommodate the environment of cells and grow as a dominant cell group.

Until recently, proliferation of cancer cells has been investigated by uptake of $^{2}$H-thymidin and the use of anti-BrdU antibody. Yamaoka[12] emphasized that the RNA/DNA ratio is of great value to judge the progression of the disease. In particular, the measurementof RNA contents is useful for knowing the degree of cell proliferation in relation to blood supply.

Trends in future research focus on simple concomitant DNA and RNA measurement to exactly assess biological behaviors of malignancy in cancer cells on the basis of a result of this study.

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