Flow Cytometric Nuclear DNA Analysis in Ulcerative Colitis

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The purpose of this study was to assess the usefulness of nuclear DNA analysis in early cancer detection in patients with ulcerative colitis. The results of flow cytometric nuclear DNA analysis of tissue specimens from patients with ulcerative colitis, colonic adenoma, and colorectal carcinoma were compared. DNA aneuploidy was observed in 53.1% of patients with ulcerative colitis and 16.7% of patients with colonic adenoma. The degree of atypism of the tumor cells was related to the incidence of DNA aneuploidy. DNA aneuploidy was noted in 48.6% of the patient with ulcerative colitis and coexistent colorectal carcinoma. The degree of dysplasia of the tumor cells was also correlated with the incidence of DNA aneuploidy. DNA aneuploidy was found in 14.3% of specimens of mucosal tissue that were without cancerous or dysplastic cells. DNA aneuploidy was found in 8.5% of specimens of mucosal tissue collected from patients with ulcerative colitis alone. Our findings showed that abnormalities in DNA content was closely correlated with histological atypism. In addition, our findings suggested that DNA aneuploidy may precede dysplastic changes in some cases of ulcerative colitis. We concluded that DNA analysis using flow cytometry is a useful method for identification of patients with ulcerative colitis at high risk for the development of cancer.

Materials

1. Colorectal carcinoma and colonic adenoma

We studied 32 resected specimens (32 lesions) of colorectal carcinoma. These specimens had been histologically diagnosed as either moderately differentiated or well differentiated adenocarcinomas. There were 115 cases (145 lesions) of colonic adenoma which were treated by endoscopic polypectomy at our hospital between 1985 and 1989. Of these 115 cases, 96 cases (120 lesions) which showed a coefficient of variation (CV) of less than 8% were studied. Carcinoma in adenoma was present in 21 cases. These lesions were trimmed so that the cancerous and adenomatous portions were divided for separate analysis of their DNA content.

2. Case report of a patient with UC and coexistent carcinoma

The patient was a 29-years-old male with total colitis, which was first diagnosed at 18 years of age. The patient received medication for this condition for a short time, after which he discontinued it. Multiple colorectal carcinoma was diagnosed 11 years later, and total colectomy was performed. The resected specimen was sectioned at 5 mm intervals (Fig. 1a) and subjected to histological examination and analysis of nuclear DNA content. The nuclear DNA content of the cancerous portion was measured. The DNA content of the noncancerous portions was measured in each specimen that was divided according to the degree of dysplasia using Riddell's classification.

3. Patients with ulcerative colitis

Sixty-seven patients who were being treated at our hospital underwent 123 endoscopic biopsy examinations...
between 1986 and 1989. Of these 123 specimens, 92 paraffin
embedded blocks from 59 patients with a CV of less than
7% were used to measure DNA content. The specimens
consisted of rectal mucosal tissue, except for 7 specimen
blocks of sigmoid colonic mucosal tissue. Seventy-two
specimen blocks consisted of tissues in active inflamma-
tion whereas 20 consisted of tissues in remission.

The mean age of the patients was 40.4 years (range, 13
to 70 years). The study group consisted of 33 males and 26
females. The mean duration of illness was 6.2 years
(range, 1 year to 26 years). There were 9 patients with
proctitis, 37 of left-sided colitis, and 13 of total colitis.

The past biopsy specimens of those showing DNA
aneuploidy (Aneu) in the present study were reviewed.
Total colonoscopy was performed in the patients with
Aneu, whenever possible, for reexamination of the presence
or absence of cancerous as well as dysplastic changes and
analysis of DNA content.

Methods

1. Histological evaluation

   a) Colonic adenoma: The grade of atypism was classi-
      fied according to the criteria of Morson8) (mild atypism,
      moderate atypism, and severe atypism [designated as
      intramucosal carcinoma in Japan], submucosal infiltrat-
      ing carcinoma [sm]).

   b) Ulcerative colitis: The grade of dysplasia was deter-
      mined according to the standardized classification of
      Riddell et al9) as negative for dysplasia [NEG], indefinite
      for dysplasia [IND], low grade dysplasia [LGD], high
      grade dysplasia [HGD], and carcinoma [CA]. The highest
      grade of atypism observed within a single specimen was
      regarded as the grade of atypism for that particular tissue
      specimen.

2. Analysis of nuclear DNA content

   a) Materials: Three or four 50 µm sections were cut
      from each formalin fixed and embedded in paraffin (Table
      1). In a patient with UC and coexistent colorectal carci-
      noma, seven blocks each from specimens classified as NEG,
      IND, LGD, HGD, and CA were subjected to trimming to
      remove the muscularis mucosae. Only the lamina propria
      was used for measurement of the DNA content.

   b) Preparation of the specimen: The specimens were
      prepared according to the method of Schutte et al10.

      The specimens were deparaffinized in xylene for an hour.
      This procedure was repeated twice. The specimens were
      then rehydrated for 30 minutes in each descending con-
      centration of ethanol (100%, 96%, 70%, and 50%).
      Subsequently, the specimens were rehydrated for 20 min-
      utes in tertiary distilled water. The specimens were then
      incubated in 0.25% trypsin citrate buffer (3 mM tri-
      sodium citrate, 2H2O, 0.1% Nonidet P-40, 1.5 mM Tris,
      pH 7.6) while being shaken at a rate of 100/min for 24
      hours at 37°C. Naked single cells were obtained in this

Table 1. Analysis of nuclear DNA content by flow cytometry

| 1) 3 or 4 fragments of 50 µm section |
| 2) trimming                          |
| 3) xylene (60 minutes for 2 times)   |
| 4) 100%, 96%, 70%, 50% ethanol (30 minutes each) |
| 5) trypsin sodium citrate buffer (37°C, 24 hours) |
| 6) 50 µm nylon mesh filter           |
| 7) centrifugation (1000 rpm for 5 minutes) |
| 8) sodium citrate buffer (200 µl)    |
| 9) solution A (1800 µl)              |
| 10) solution B (1800 µl)             |
| 11) solution C (1800 µl)             |
| 12) FACScan                          |
manner.
c) DNA staining: Nuclear DNA was stained according to the methods of Vinderov et al. Single cells were suspended in 200 µl of citric acid buffer (sucrose 250 mM, tri-sodium citrate, 2H2O 40 mM, dimethylsulfoxide 50 ml, distilled water 1000 ml, pH 7.6). Next, 1800 µl of solution A (trypsin 15 mg, stock solution [tri-sodium citrate, 2H2O 3.4 mM, 0.1 % Nonidet p-40, spermine tetrachloride 1.5 mM, Tris 0.5 mM, distilled water 2000 ml, pH 7.6] 500 ml) was added to the specimens and left to stand at room temperature for 10 minutes. This was followed by the addition of 1500 µl of solution B (250 mg of trypsin inhibitor, 50 mg of RNase A, 500 ml of stock solution, pH 7.6) to the specimens and left to stand for another 10 minutes. Staining was performed by the addition of 1500 µl of solution C (208 mg of propidium iodide, 580 mg of spermine tetrachloride, 500 ml of stock solution, pH 7.6) to the specimens and allowing the mixture stand for 10 minutes at room temperature.

Flow cytometry: FACScan (Becton-Dickinson Co.) The cells were passed individually through an argon laser beam having a wavelength of 488 nm. A DNA histogram was plotted using a long pass filter of 580 nm. Nuclear DNA content was assessed using the DNA index. A smaller GO/G1 peak was regarded as due to normal cells. Cells showing a single GO/G1 peak on the histogram were considered as having a diploidy pattern (Di). Cells showing 2 or more GO/G1 peaks and having a DNA index of greater than 1.1 were considered as having an Aneu pattern.

Results

1. Colorectal carcinoma and colonic adenoma

Of 32 patients with colorectal carcinoma, 17 (53.1 %) showed DNA aneuploidy. In colonic adenoma, there were 45 lesions showing mild atypism, 39 showing moderate atypism, 25 showing severe atypism, and 11 with sm carcinoma. The incidence of DNA aneuploidy in each grade of atypism was investigated. None of the 45 lesions with mild atypism showed DNA aneuploidy. Eight of 39 lesion (20.5 %) with moderate atypism showed DNA aneuploidy. Six of 25 lesions (24%) with severe atypism, and 6 of 11 (54.5 %) with sm carcinoma showed DNA aneuploidy. The incidence of DNA aneuploidy increased in parallel with the grade of atypism. Analysis by the chi-square test showed that the differences in the incidence of DNA aneuploidy were significant among the four groups classified according to their grade of atypism (Table 2). The normal colonic mucosal tissue specimens which served as controls all showed a Di pattern.

Specimens diagnosed as carcinoma in adenoma were divided into their cancerous and noncancerous portions by trimming. The nuclear DNA content was separately measured in each of these divided portions. Of 21 lesions of carcinoma in adenoma, 9 (42.9%) showed DNA aneuploidy in the cancerous portion of these tumors. DNA aneuploidy was noted in the noncancerous portion in 7 of these 21 lesions (33.3 %). The incidence of DNA aneuploidy in the cancerous and noncancerous portions was similar. Histological examination of the cancerous portion of these lesions revealed well differentiated adenocarcinoma whereas that of the noncancerous portion showed moderate atypism.

2. Cancerous complications in a patient with ulcerative colitis

a) Histological assessment

i) Cancerous complications: As shown in Fig. 1-a, cancers were observed in the rectum, sigmoid colon, and descending colon. These tumors appeared slightly depressed. Histological findings were similar in all cancers. The cancers were composed mainly of well differentiated adenocarcinomas. Poorly differentiated cancer cells including those derived from signet ring cell carcinomas were occasionally noted. All cancers had invaded the pm. The cancers in the rectum had penetrated among the ss. In addition, minute early cancers were observed dispersed among the mucosal specimens (Fig. 1-b). These early cancers mostly consisted of poorly differentiated adenocarcinomas. In total, we observed 4 advanced cancers and 30 or more early cancers in this patient.

ii) Mucosa of the noncancerous portions of the lesions: The noncancerous portions of the lesions included sites of active inflammation as well as those without histological evidence of active inflammation. Various degrees of dysplasia, as determined according to the criteria proposed by Riddell et al. (Fig. 1-b), were observed in the mucosal specimens. HGD were mainly noted in the left side colon.
The DNA indices with regard to the DNA aneuploidy patterns at the various sites with different degrees of dysplasia were 1.2, 1.3, 1.4, 1.5, 1.6, and 1.8 respectively. The chi-square test showed that the differences in the incidence of the Aneu pattern were significant among these 5 groups of specimens.

The relationship between the incidence of Aneu and the degree of dysplasia determined according to the criteria proposed by Riddell et al. was investigated. The Aneu pattern was found in 1 of 7 blocks (14.3%) at the NEG sites, whereas 17 blocks showed the Aneu pattern (48.6%). The DNA aneuploidy pattern was detected in multiple biopsy examinations in 3 patients. The DNA index of the Aneu pattern was relatively low at LGD and less dysplastic sites. In contrast, the same incidence was high at HGD sites and at the cancerous portions of the lesions. Analysis by the chi-square test showed that the differences in the incidence of the Aneu pattern were significant among these 5 groups of specimens.

The DNA indices with regard to the DNA aneuploidy patterns at the various sites with different degrees of dysplasia were 1.2, 1.3, 1.4, 1.5, 1.6, and 1.8 respectively.

### Table 3. Incidence of the aneuploidy pattern according to the degree of dysplasia in the patient with ulcerative colitis complicated by colorectal carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Diploidy</th>
<th>Aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>6 (86%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>IND</td>
<td>5 (71%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>LGD</td>
<td>5 (71%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>HGD</td>
<td>2 (29%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>7 (100%)</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>18 (51%)</td>
<td>17 (49%)</td>
</tr>
</tbody>
</table>

**chi-square test (significant difference among the 5 groups [P < 0.01]**

NEG: negative for dysplasia  
IND: indefinite for dysplasia  
LGD: low grade dysplasia  
HGD: high grade dysplasia  

### Figure 2  DNA aneuploidy in the cancerous and high grade dysplastic portions of cancer complicated by ulcerative colitis

#### b) Analysis of nuclear DNA content

Of a total of 35 specimen blocks, 18 (51.4%) showed a Di pattern, whereas 17 blocks showed the Aneu pattern (48.6%). The Aneu pattern was mainly observed in the left side colon.

The relationship between the incidence of Aneu and the degree of dysplasia determined according to the criteria proposed by Riddell et al. was investigated. The Aneu pattern was found in 1 of 7 blocks (14.3%) at the NEG sites. It was noted in 2 of 7 (28.6%) at the IND sites and LGD sites. The Aneu pattern was observed in 5 of 7 blocks (71.4%) at the HGD sites and at all sites within the cancerous portions of the lesions (Table 3, Fig. 2). The incidence of the Aneu pattern was relatively low at LGD and less dysplastic sites. In contrast, the same incidence was high at HGD sites and at the cancerous portions of the lesions. Analysis by the chi-square test showed that the differences in the incidence of the Aneu pattern were significant among these 5 groups of specimens.

The DNA indices with regard to the DNA aneuploidy patterns at the various sites with different degrees of dysplasia were 1.2, 1.3, 1.4, 1.5, 1.6, and 1.8 respectively.

### 3. Patients with ulcerative colitis

Fifty-nine biopsy specimens obtained from patients with ulcerative colitis between 1986 and 1989 were examined. Of these 59 specimens, 5 showed the Aneu pattern. Histological examination of these 5 specimens revealed that ulcerative colitis took the form of total colitis in 2 patients and left-sided colitis in 3. The duration of illness ranged from 3 to 16 years. The clinical course of our patients was characterized by intermittent attacks, with remissions between attacks. No histological evidence of dysplasia or cancer was noted in the 59 specimens examined. The nuclear DNA content of paraffin-embedded biopsy specimens from the 5 patients who showed an Aneu pattern was measured. The biopsy specimens were those obtained from the 5 patients who had undergone multiple biopsies in the past 10 years. Patient 1 had undergone 5 biopsy examinations during this time, of which 2 showed an Aneu pattern. Of the 7 biopsy examinations performed in patient 2, the results in 2 showed an Aneu pattern. An Aneu pattern was observed in 2 of 3 biopsy examinations in patient 3 (Fig. 3). The DNA aneuploidy pattern was detected in multiple biopsy examinations in 3 patients. The DNA index
Figure 3  Clinical course of patients showing DNA aneuploidy

Figure 4  Histogram of a patient showing DNA aneuploidy (case 1)
Table 4. Characteristics of patients with ulcerative colitis showing DNA aneuploidy

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Extent of disease</th>
<th>Duration of illness</th>
<th>Type of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>70</td>
<td>total colitis</td>
<td>16yrs</td>
<td>intermittent attacks</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>50</td>
<td>total colitis</td>
<td>3yrs</td>
<td>intermittent attacks</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>63</td>
<td>left side colitis</td>
<td>14yrs</td>
<td>intermittent attacks</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>22</td>
<td>left side colitis</td>
<td>3yrs</td>
<td>intermittent attacks</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>26</td>
<td>left side colitis</td>
<td>5yrs</td>
<td>intermittent attacks</td>
</tr>
</tbody>
</table>

Table 5. Analysis of DNA content by flow cytometry
(data obtained from the literature in Western countries)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Incidence of DNA aneuploidy</th>
<th>correlation with dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986 Fozard surgical</td>
<td>dys (++) 21%</td>
<td>dys (-) 15% negative</td>
</tr>
<tr>
<td>1987 Lofberg biopsy</td>
<td>5/53 (9%) including</td>
<td>4/3 dys positive</td>
</tr>
<tr>
<td>1988 Rutegard surgical</td>
<td>6/73 (8%)</td>
<td>dys (-) 5% negative</td>
</tr>
<tr>
<td>1988 Melville biopsy</td>
<td>dys (++) 39%</td>
<td>dys (-) 5% positive</td>
</tr>
</tbody>
</table>

dys: dysplasia

remained constant in each of these patients at all examinations.

Except for patient 5 who moved to another prefecture and therefore was lost to follow up, all patients underwent repeat total colonoscopy and serial biopsy examinations. The results of these examinations showed no evidence of cancer development or dysplastic changes. Only the rectal tissue specimen from patient 1 showed an Aneu pattern. All other specimens showed a Di pattern.

Discussion

The DNA ploidy pattern in various malignant neoplasma have been investigated in recent years using FCM). The incidence of the Aneu pattern has been reported to be high in patients with malignant neoplasmas. The Aneu pattern has also been associated with the progression and prognosis of cancers. These observations have lead some investigators to speculate that the DNA ploidy pattern in an indicator for the grade of malignancy. The Aneu pattern occurs in 40% to 75% of patients with colorectal carcinoma. It has also been reported that the Aneu pattern is correlated with the stage of colorectal carcinoma (determined according to Dukes classification) and the prognosis of this disease). The degree of dysplastic changes in colonic adenoma has been reported to be correlated with the incidence of the Aneu pattern). The Aneu pattern was observed in 17 of 32 patients (53.1%) with colonic adenoma. In patients with colonic adenoma, the Aneu pattern was noted in 20 of 120 lesions (16.7%). The relation between the incidence of the Aneu pattern and the grade of atypism was investigated. This analysis revealed that all lesions with mild atypism showed the Di pattern. Lesions with moderate atypism showed the Aneu pattern in 20.5%, those with severe atypism showed the same pattern in 24%. More than half (54.5%) of the sm cancers showed the Aneu pattern. The incidence of the Aneu pattern increased along with the grade of atypism. This indicated that the grade of atypism was associated with nuclear DNA content. It is widely accepted that colonic adenomas have a great potential for malignant transformation. Thus, most investigators agree that the condition should be regarded as a premalignant condition). Therefore, the adenoma-carcinoma sequence can also be speculated from the analysis of nuclear DNA content.

The Aneu pattern becomes detectable by the analysis of nuclear DNA content only if chromosomal changes are present in the cells). Thus, the presence of the Aneu pattern in colonic adenomas suggests that cancer development is manifested at the chromosomal level before it becomes morphologically apparent. We investigated whether the Aneu pattern can be used to detect the development of colorectal cancer in patients with ulcerative colitis.

The development of colorectal cancer in patients with ulcerative colitis have been reported by several investigators). The incidence of such a complication has been lower in Japan than that in Western countries. However,
Incidence of polyploid cells in patients with UC and analyzed the nuclear DNA content in patients with UC development of cancer (Table 5). In Japan, Suzuki et al." noted close association between these 2 parameters and the degree of dysplasia and the incidence of the Aneu pattern. However, their findings showed that both the Aneu pattern and dysplasia are related. Studies of patients with UC complicated by colorectal cancer have shown that dysplasia is present in the mucosal tissues of the noncancerous portion of the lesions. Of the 36 patients with UC and associated colorectal cancer that we collected from the literature, 28 showed coexistent dysplastic changes. These observations suggest a close relationship between dysplastic changes and the development of carcinoma. Morson and Pang reported that endoscopic biopsy examination for the presence of dysplastic changes is useful in detecting early cancers.

Resected colonic specimens were examined for determination of the different degrees of dysplasia (NEG, IND, LGD, and HGD) and the presence of carcinoma. The specimens were then sectioned according to the degree of dysplasia. Cancerous portions of the specimens were also separated for examination. The DNA content was measured in each of these sectioned specimens. The Aneu pattern was found in 14.3% of the sectioned specimens with NEG, in 28.6% each of the sectioned specimens with IND and LGD, and 71.4% of the sectioned specimens with HGD. The Aneu pattern was present in all the cancerous tissues. The incidence of the Aneu pattern was high in specimens showing a high grade of atypism. The Aneu pattern was observed in 23.8% of noncancerous mucosal specimens. The Aneu pattern was noted in 14.3% of mucosal tissue specimens without cancer or dysplasia (NEG).

The analysis of nuclear DNA content using FCM has already been reported in Western countries. Hammarberg et al., and Loftberg et al. reported that the incidence of the Aneu pattern and the degree of dysplasia was related. They found a few patients in whom the Aneu pattern was observed before the appearance of cancer or HGD lesions. Melville et al. also noted a high incidence (39%) of the Aneu pattern in dysplastic mucosa. On the other hand, Fozard et al. and Rutegard et al. found no correlation between the incidence of the Aneu pattern and the degree of dysplasia. However, their findings showed that both the degree of dysplasia and the incidence of the Aneu pattern were increased in long standing cases of UC, suggesting a close association between these 2 parameters and the development of cancer (Table 5). In Japan, Suzuki et al. analyzed the nuclear DNA content in patients with UC using microspectrophotometry. They reported a high incidence of polyploid cells in patients with UC and associated cancer or dysplasia. These findings show that dysplastic changes frequently occur in UC patients with associated malignancy. These patients also showed a high incidence of the Aneu pattern.

The Aneu pattern was observed in 14.3% of the noncancerous and nondysplastic tissues obtained from our patient with UC complicated by cancer. The Aneu pattern was found in 5 of 59 patients (8.5%) with UC which is no development of cancer or dysplastic changes. The Aneu pattern was detected in several times it was examined in 3 patients. The DNA index also remained constant in each of these patients. Sixteen lesions were obtained from the 5 patients for examination of the Aneu pattern. Of 10 lesions with histological evidence of active inflammation, 6 showed the Aneu pattern. Of 6 lesions with their mucosal tissue in remission, 3 showed the Aneu pattern. Thus, it was found that the severity of inflammation was not related to the incidence of the Aneu pattern.

Several investigators have speculated on the significance of the presence of the Aneu pattern at sites with no evidence of cancer or dysplasia. Fujita proposed the "cross linkage hypothesis" to explain the occurrence of aberrations in DNA content. DNA injury predisposes the chromosomes to become unstable. Division of these cells results in the appearance of polyploid and aneuploid cells which are the earliest changes observed in the process of malignant degeneration. He speculated that atypism in the cells and tissues are still not manifest during this stage. Sasak hypothesized that colonic adenomas showing the Aneu pattern are already in the process of malignant degeneration, though their grade of morphological atypism may not indicate so. The presence of the Aneu pattern in nondysplastic mucosa probably represents the initial stage of malignant degeneration. DNA injury due to recurrent or persistent inflammation is common in patients who have had UC for extended periods of time. Since DNA injury predisposes the patients to cancer development, it seems reasonable that patients with long-standing UC have a higher risk of colorectal carcinoma than the general population.

At present, we are following up our patients with long-standing UC at regular intervals because we believe that they are at high risk for the development of cancer. One study reported the importance of surveillance colonoscopy for detection of dysplastic changes. Nuclear DNA content is closely associated with the development of cancer or dysplastic changes. Studies have suggested that aberrations in DNA content precedes histological changes, such as malignant degeneration or dysplasia. The analysis of nuclear DNA content along with the detection of dysplastic changes are useful in the identification of patients with ulcerative colitis at high risk of developing colon cancer.
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References