Flow Cytometric Detection of Aneuploidy in Colorectal Adenomas

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ABSTRACT

Flow cytometry has been used to study the incidence of aneuploidy in a series of 55 colorectal adenomas (29 tubular adenomas, 22 tubulovillous adenomas, and 4 villous adenomas). For comparison, 5 nonadenomatous polyps, 4 normal mucosa samples from colectomy specimens and 16 colorectal cancers were measured. Fifteen (27%) adenomas were aneuploid, 33 (63%) were diploid, and 7 (11%) were peridiploid. The aneuploidy incidence increased with the size of the adenomas (<1 cm, 0%; 1 to 2 cm, 30%; >2 cm, 50% aneuploid cases, respectively) but was less dependent on the histological type or degree of dysplasia. However, the degree of aneuploidy [mean DNA index of aneuploid stem lines] was significantly higher in tubulovillous adenomas [1.26 ± 0.33 (SD)] than in tubular adenomas [1.09 ± 0.04] and only slightly lower than in carcinomas [1.59 ± 0.26]. The progressive increase in ploidy abnormality with size and histological type strongly supports the evidence for the adenoma-carcinoma sequence in the development of colorectal cancer.

INTRODUCTION

Adenomas of the colon and rectum are generally considered to be the main precursor lesions for colorectal cancer (1). Although the accurate prevalence of colorectal adenomas is difficult to assess, they occur more frequently than do colorectal carcinomas. Therefore, it must be assumed that only some of these lesions progress to cancer within a patient’s lifetime (2, 3). Size, histological type, and degree of atypia are important features in relation to malignant potential and the risk of harboring a carcinoma (3), but these characteristics are insufficient to identify individual high-risk cases.

It is well established now that a great proportion of malignant tumors show numerical and structural chromosome abnormalities (4). For many malignant tumors including colorectal carcinoma, cytogenetic abnormalities have been demonstrated in an indirect way by measuring deviations in total DNA content with cytophotometric techniques (5, 6). On the basis of these studies, aneuploidy may be considered as a specific marker for malignant transformation. From the scanty reports in the literature, it is difficult to assess the actual incidence of aneuploidy in colorectal adenomas since relatively few cases have been studied by karyotype analysis (for review, see Ref. 4, pp. 471–473), whereas the static cytophotometric techniques used in the earlier studies are relatively insensitive to minor ploidy aberrations (6–8).

FCM² is a relatively new technique for DNA ploidy determinations that has found wide acceptance over the past decade because of its high resolution, speed, and reproducibility. The development of procedures for preparing and staining of suspensions of single nuclei from solid-tumor tissue has greatly improved the possibilities for DNA ploidy determinations in oncological studies (5, 9). We have used FCM to study the incidence of aneuploidy in colorectal carcinomas since the higher resolution of this technique might permit the detection of ploidy abnormalities not observable with static cytophotometric techniques (10). By comparing the results with parameters such as size, histological type, and degree of atypia, a better insight may be obtained in the relationship between genotypic and phenotypic changes during the adenoma-carcinoma sequence.

MATERIALS AND METHODS

Selection, Sampling, and Processing of Tissue. The material consisted of an unscreened series of 53 endoscopically removed colorectal polyps including 46 adenomas, 5 nonneoplastic polyps, and 2 adenocarcinomas. The remaining material consisted of 27 colorectal surgical specimens including 9 adenomas, 14 adenocarcinomas of which 12 in polyposus tumors, and 4 samples from normal mucosa. Both polyps and surgical specimens were freshly delivered at the Department of Pathology immediately after removal.

The histological evaluation was made on hematoxylin-eosin-stained sections according to the WHO classification. Histological atypia was graded according to the method of Muto et al. (3). In this grading system, severe atypia includes those features sometimes named focal carcinoma or carcinoma in situ without invasion across the line of the muscularis mucosae. All diagnoses were reviewed by the first author (Table 1).

Epithelial cells were scraped from the entire surface of the polyps with the edge of a glass slide. This resulted in only limited tissue damage that did not interfere with the histological evaluation. For technical reasons, only polyps with a diameter of at least 5 mm were sampled. The scraped material was suspended in 3 ml 40 mM citrate buffer, pH 7.6, containing 250 mM sucrose and 5% (v/v) dimethyl sulfoxide (9). This buffer permits storage of the cell samples at −70°C until further processing for FCM.

Flow Cytometry. Suspensions of single nuclei were prepared from stored or fresh samples with the detergent-trypsin procedure of Vindelov et al. (9) and stained with propidium iodide (Sigma Chemical Co., St. Louis, MO). In contrast to the procedure of Vindelov, the cells were pelleted before treatment with the detergent-trypsin procedure by centrifugation at 1100 x g for 5 min. TRBC were added to the sample at the beginning of the preparation and staining procedure to serve as an internal DNA ploidy standard (9). The DNA content of these cells is about 80% of that of human diploid cells. The amount of TRBC to be added arrived at equal proportions of TRBC and epithelial cells was estimated on the basis of the pellet size of the scraped cells. This procedure was found to be more practical than was determination of the cell concentration in the scraped material with a hemocytometer because of the presence of cell sheets and aggregates.

Stained samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA). Filtered demineralized water was used as sheath fluid. For excitation of propidium iodide fluorescence, LP 515 and SP 560 filters were used in combination with a 560 nm chromatic beam splitter. Emission was measured using an LP 590 barrier filter.

A lesion was considered to be diploid when its DNA profile showed only one G0.1 population with a G0.1/TRBC ratio (modal channel number

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²The abbreviations used are: FCM, flow cytometry; TRBC, rainbow trout red blood cells; DI, DNA index.

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G₀,i, population/modal channel number TRBC population) ranging between 1.19 and 1.31. This range is equal to the 99.7% confidence interval for a mean G₀,i/TRBC ratio of 1.25 with a standard deviation of 0.02 found for diploid cells in a previous study (11). Lesions were classified as aneuploid when the DNA profile showed at least 2 distinct G₀,i populations or when the G₀,i/TRBC ratio of the single population was <1.19 or >1.31. The degree of aneuploidy was expressed as the DI:

\[
\text{DI} = \frac{\text{Modal channel number of aneuploid G₀,i fraction}}{\text{Modal channel number diploid G₀,i population}}
\]

For a diploid cell population, DI = 1.00.

Statistical Analysis. Differences between means were analyzed with Student's t test, whereas differences in aneuploidy incidence were evaluated with 2 x 2 and 2 x 3 contingency tables.

RESULTS

Aneuploidy Incidence, Relationship with Age and Sex of the Patients. Aneuploid G₀,i populations in addition to a diploid G₀,i population were detected in 15 (27%) of 55 colorectal adenomas and in 9 (62%) of 16 colorectal carcinomas (Table 1). Aneuploidy (DI = 1.13) was also found in an inflammatory polyp from a patient with Crohn's colitis. All other polyps (hyperplastic, hamartomatous, and juvenile) were diploid. Typical examples from DNA profiles are shown in Chart 1. Two distinct aneuploid populations were found in a tubulovillous adenoma with severe histological atypia but without histological signs of invasive carcinoma, (Fig. 1; Chart 2). Multiple aneuploid populations were also found in a tubulovillous adenoma harboring an adenocarcinoma (Chart 2) and in an adenocarcinoma from a second patient. The incidence of multiploid stem lines in aneuploid adenomas (1 of 15) was lower than that in aneuploid carcinomas (2 of 9), but the difference was not statistically significant.

Six adenomas the DNA profile of which showed only one G₀,i population had borderline or low abnormal G₀,i/TRBC ratios just within the range shown by aneuploid G₀,i populations (Chart 3). Since we were not sure whether these elevated ratios had to be attributed to experimental variation or represented minor ploidy abnormalities not resolvable as a distinct peak, these were classified as "peridiploid." Also, for the same reasons one carcinoma was classified as peridiploid.

No significant differences were found for the mean age of patients in the different ploidy classes (Table 2). The aneuploidy incidence in adenomas from female patients was significantly higher than in adenomas from male patients (Table 2). However, if the aneuploid cases were pooled with the peridiploid cases, the difference was no longer statistically significant.

Relationship with Histological Type, Size, and Degree of Atypia. There were no statistically significant differences between the percentages of diploid, peridiploid, and aneuploid cases for the different types of adenomas, although tubulovillous adenomas tended to be slightly more often peridiploid or aneuploid than tubular adenomas (Table 1). Aneuploid tubular adenomas showed only minor ploidy abnormalities as indicated by the low mean DI (Table 3). The mean DI of the aneuploid stem lines in tubulovillous adenomas was significantly higher. An even higher value was found for carcinomas, although the latter difference was not statistically significant.

The mean size of aneuploid adenomas [21.3 ± 14.2 (SD)] was significantly larger than that of the diploid adenomas [14.7 ± 9.0]. An intermediate value [18.0 ± 11.1] was found for peridiploid adenomas.

Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Total</th>
<th>Diploid</th>
<th>Peridiploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal carcinoma</td>
<td>16</td>
<td>6 (38)</td>
<td>1 (6)</td>
<td>9 (56)</td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>29</td>
<td>19 (66)</td>
<td>3 (10)</td>
<td>7 (24)</td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>22</td>
<td>12 (55)</td>
<td>3 (14)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>4</td>
<td>3 (75)</td>
<td></td>
<td>1 (25)</td>
</tr>
<tr>
<td>Hyperplastic polyp</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamartomatous polyp</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory polyp</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Juvenile polyp</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>4</td>
<td>4 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses, percentage.
b Multiple aneuploid stem lines present in 2 cases.
c Multiple aneuploid stem lines present in one case.

Fig. 1. Detail of a hematoxylin-eosin-stained histological section from a tubulovillous adenoma with atypia without signs of invasion. The DNA profile from this lesion (Chart 2) showed 2 distinct aneuploid stem lines. × 50.
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Chart 1. Typical FCM DNA profiles from adenomas. Top, DNA profile from a tubulovillous adenoma showing a diploid G₀-I population, a hyperdiploid population (DI = 1.17), and a hypotetraploid population (DI = 1.84). Middle, DNA profile from a tubular adenoma showing a diploid G₀-I population, a hyperdiploid population (DI = 1.06), and a hypotetraploid population (DI = 1.73). Bottom, DNA profile from a tubulovillous adenoma showing a diploid population (DI = 1.00) and a hypotetraploid population (DI = 1.73).

Chart 2. DNA profiles from lesions with 2 different aneuploid stem lines. Top, DNA profile from a tubulovillous adenoma showing a diploid G₀-I population, a hyperdiploid population (DI = 1.17), and a hypotetraploid population (DI = 1.84). Bottom, DNA profile from an adenocarcinoma in a tubulovillous adenoma also showing a diploid G₀-I population, a hyperdiploid population (DI = 1.12), and a hypotetraploid population (DI = 1.93) + aneuploid G₂-M population.

Chart 3. Histogram of the distribution of the G₀-I/TRBC ratios measured in samples from benign and malignant colorectal tissue specimens. Solid line, G₀-I/TRBC ratios from distinct aneuploid peaks. With one exception, these fall outside the confidence interval (1.19 to 1.31) for diploid populations. From the cases with only one G₀-I population (C), 6 fall within the range of the aneuploid peaks. These are classified as peridiploid.

Adenomas. No aneuploidy was found in adenomas smaller than 1 cm which were predominantly of the tubular type (Table 4). The aneuploidy incidence for adenomas in the 1- to 2-cm size class was 30%, and for lesions over 2 cm it was even 50%. The latter group, which also showed the highest percentage of peridiploid lesions, consisted predominantly of tubulovillous and villous adenomas. For the 1- to 2-cm size class, no marked differences in aneuploidy incidence exist between tubular and tubulovillous adenomas.

Tubular and tubulovillous adenomas with moderate or severe atypia showed a higher aneuploidy incidence (40-50%) than tubular adenomas (10-20%). Adenocarcinoma in tubulovillous adenoma also showed a higher aneuploidy incidence (50-60%) than the adenomas.

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Table 2

<table>
<thead>
<tr>
<th>Type</th>
<th>Age (yr)</th>
<th>No. of males</th>
<th>No. of females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>63.9 ± 11.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 (72)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16 (53)</td>
</tr>
<tr>
<td>Peridiploid</td>
<td>66 ± 12.0</td>
<td>4 (16)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>64.1 ± 13.1</td>
<td>3 (12)</td>
<td>12 (40)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>63.8 ± 12.5</td>
<td>25 (100)</td>
<td>30 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.  
<sup>b</sup> Numbers in parentheses, percentage.  
<sup>c</sup> Statistically significant difference with aneuploidy incidence in male patients (P < 0.05).

Table 3

<table>
<thead>
<tr>
<th>Type</th>
<th>N&lt;sup&gt;2&lt;/sup&gt;</th>
<th>DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular adenoma</td>
<td>7</td>
<td>1.09 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>8</td>
<td>1.26 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>1</td>
<td>(1.33)</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>10</td>
<td>1.59 ± 0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of aneuploid stem lines.  
<sup>b</sup> Mean ± SD.  
<sup>c</sup> The difference for tubular adenomas is statistically significant (P < 0.025), the difference for carcinomas is not significant (P < 0.20).

Table 4

<table>
<thead>
<tr>
<th>Histology</th>
<th>Size (cm)</th>
<th>Diploid</th>
<th>Peridiploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular adenoma</td>
<td>&lt;1</td>
<td>5 (83)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (17)</td>
<td>7 (32)</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>13 (59)</td>
<td>2 (9)</td>
<td>7 (32)</td>
</tr>
<tr>
<td></td>
<td>&gt;2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>&lt;1</td>
<td>9 (69)</td>
<td>1 (8)</td>
<td>3 (23)</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>3 (33)</td>
<td>2 (22)</td>
<td>4 (44)</td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers in parentheses, percentage.

Table 5

<table>
<thead>
<tr>
<th>Histology</th>
<th>Dysplasia</th>
<th>Diploid</th>
<th>Peridiploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular adenoma</td>
<td>Mild</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>7</td>
<td>2</td>
<td>3 NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>Mild</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>6</td>
<td>2</td>
<td>3 NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>Mild</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td></td>
<td></td>
<td>1 NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> NS, difference in aneuploidy incidence between the atypia classes is not statistically significant.

Table 6

<table>
<thead>
<tr>
<th>Size class (cm)</th>
<th>This study (55 cases)</th>
<th>Muto et al. (2489 cases)</th>
<th>Aneuploidy incidence (%)</th>
<th>Cancer incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>15</td>
<td>59.4</td>
<td>0</td>
<td>1.3</td>
</tr>
<tr>
<td>1-2</td>
<td>67</td>
<td>23.3</td>
<td>30</td>
<td>9.5</td>
</tr>
<tr>
<td>&gt;2</td>
<td>18</td>
<td>17.3</td>
<td>50</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data taken from Muto et al. (3).

DISCUSSION

The results presented indicate that aneuploidy occurs frequently in adenomas of all 3 histological types, although the incidence is less than the 60 to 80% aneuploidy found in colorectal adenocarcinomas and other types of solid tumors (6, 12–17). The aneuploidy incidence in adenomas is similar to that found in dysplastic mucosa and polyps from patients with ulcerative colitis, a well-known high-risk group for colorectal cancer (18). With the present FCM technique, the detection of a distinct aneuploid stem line in heterogeneous cell populations, e.g.,
consisting of epithelial and stromal cell types, requires a minimum DNA difference of about 4 to 5% (10). Smaller differences in DNA content cannot be resolved in 2 distinct peaks but may give slightly shifted G0/M/TRBC ratios. It is not unlikely, therefore, that some of the cases classified as periploid actually had minor ploidy abnormalities.

The incidence of aneuploidy may also have been underestimated due to sampling errors, since the scraping technique is a compromise between optimal sampling for FCM and optimal tissue preservation for histodiagnostic evaluation.

The correlation between aneuploidy incidence and size of the adenomas is similar to the relationship between size and cancer incidence in adenomas found by other investigators (Refs. 3 and 19; Table 6). The discrepancy for the 1- to 2-cm size class may be attributed to differences in size and composition of the 2 series of patients, e.g., to the overrepresentation of adenomas in the 1- to 2-cm size class in our material.

An alternative explanation is that only part of the aneuploid lesions progress to invasive cancer although studies on cervical neoplasia and bladder tumors indicate that aneuploid lesions are unlikely to regress and have a relatively high potential for progression to invasive cancer (17, 20, 21).

It is generally known that the cancer incidence in villous adenomas is much higher than in pure tubular adenomas (3, 19). Size and a villous growth pattern often are closely linked, however, and therefore the increased malignant potential is considered to be primarily an effect of size (3, 19). This opinion is supported by our results showing a stronger effect of size than of histological type on the aneuploidy incidence.

Although histological atypia, particularly in tubular adenomas, is also associated with an increased cancer incidence (3), the relationship with the aneuploidy incidence failed to reach the level of statistical significance. This may be caused by the small number of cases in our atypia classes or by a different use of the grading criteria.

Alternatively, the phenotypic changes such as atypia and the appearance of a villous component may occur relatively late in the adenoma-carcinoma sequence, depending on the progression of initial genetic changes by a clonal evolution process (22). Evidence for this is presented by the progressive increase in the degree of aneuploidy (mean DI) and chromosome abnormalities with the appearance of a villous component (Ref. 4, pp. 471–473; Refs. 23 and 24), whereas the finding of aneuploidy, chromosome abnormalities, and the elevated expression of the ras oncogene family in tubular adenomas without marked atypia indicates that the genetic events, associated with or perhaps causal to the process of malignant transformation, indeed are more early phenomena (25, 26).

The underrepresentation of small adenomas (<1 cm) in our series and the resolution limits of FCM make it still impossible to identify more accurately the stage (or size class) in the adenoma-carcinoma sequence where the first cytogenetic changes have occurred.

Since the incidence of colorectal cancer in females is not higher than in males, the higher aneuploidy incidence found in female patients is probably accidental and may be associated with the overrepresentation of tubulovillous adenomas in this group.

The finding of an aneuploid inflammatory polyp in a patient with an 8-year history of Crohn’s disease may be connected with the estimated 20-fold higher incidence of colorectal carcinoma in these patients compared with the general population (27).

In conclusion, our results give supportive evidence for the adenoma-carcinoma sequence on the basis of the following arguments: (a) aneuploidy is found in all 3 types of adenomas; (b) ploidy abnormalities become more severe in lesions with a villous component which is considered to be a phenotypic marker for progression towards malignancy; and (c) the aneuploidy incidence increases with size, which is a well-known risk factor.

There is now growing evidence that aneuploidy may be associated with a more aggressive clinical course of several types of cancers including colorectal carcinoma (15, 16, 28, 29). This may form a reason for a closer follow-up of patients with aneuploid lesions, even when no histological signs of invasion have been found. FCM could possibly contribute to the identification of high-risk subgroups which could lead to better guidelines for the long-term follow-up of polypectomy patients which forms an important clinical and economical problem (30).

ACKNOWLEDGMENTS

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REFERENCES


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