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Abnormal DNA Content as a Biomarker of Large Bowel Cancer Risk and Prognosis

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Abstract Aneuploid cell populations can be defined as those that contain an abnormal number of chromosomes or an abnormal amount of DNA. Aneuploidy can be reliably detected by flow cytometric analysis of DNA content. This technique not only identifies aneuploid cell populations but can also quantify the percent of cells in various phases of the cell cycle, thus giving an indication of the proliferative activity of a tissue. Aneuploidy occurs in approximately 60% of established colorectal cancers, and many studies have demonstrated that patients with aneuploid tumors have a poorer prognosis than patients with diploid colon cancers. Some studies have suggested that the proliferative rate of tumors, as assessed by the percent of cells in S phase, also has prognostic significance. Until recently, aneuploidy was thought to occur only in malignant tissues, but it has been clearly shown that aneuploid cell populations can be identified in benign adenomatous polyps as well as in non-neoplastic-appearing mucosa of patients with chronic ulcerative colitis and Barrett's esophagus. In chronic ulcerative colitis, aneuploidy occurs more frequently in patients with dysplasia or cancer than in those with no evidence of neoplasia. Similarly, dysplastic and malignant biopsies are more commonly aneuploid than non-neoplastic biopsies. Patients who have undergone colectomy for cancer or dysplasia in the setting of chronic ulcerative colitis frequently have multiple areas of aneuploidy throughout the remainder of their colon. Whether aneuploidy can be useful as a marker of cancer risk in patients with chronic ulcerative colitis deserves further investigation.

Key Words: aneuploidy, chemoprevention, chronic ulcerative colitis (CUC), colonic DNA content, flow cytometry, intermediate biomarker

The most commonly used feature of flow cytometry is its ability to measure the intensity of fluorescence in an individual cell or nucleus. Isolated cells or nuclei from the tissue of interest are prepared and labeled with a fluorescent probe. The fluorescent label may be a fluorescent-tagged antibody directed against a cellular constituent or, in the case of DNA analysis, a fluorescent dye that binds quantitatively to DNA such as propidium iodide. The fluorescent-labeled cells or nuclei are dispersed into single droplets of saline and passed singly through a laser beam which excites the fluorescence; a fluorescence detector is used to measure the intensity of fluorescence in each individual cell or nucleus. The flow cytometric (FCM) data can then be displayed in a histogram (Figure 1), plotting the number of cells versus the intensity of fluorescence. Computer analysis can be used to calculate the proportion of cells with any given intensity of fluorescence.

Generally, an internal standard is run with each sample so that the amount of DNA in each peak can be quantitated. Most cells in normal tissue contain two copies of each chromosome (2N DNA) and are represented by the major peak in the G0 and G1 phase of the cell cycle. A small peak of cells has twice as much intensity as the 2N peak. These latter cells contain four copies of each chromosome (4N DNA) and are the cells in the G2 and M phases of the cell cycle. The cells synthesizing DNA (those in S phase) are seen as a plateau of cells between the 2N and 4N peaks.

Aneuploidy is defined as a population of cells containing an abnormal amount of DNA; an

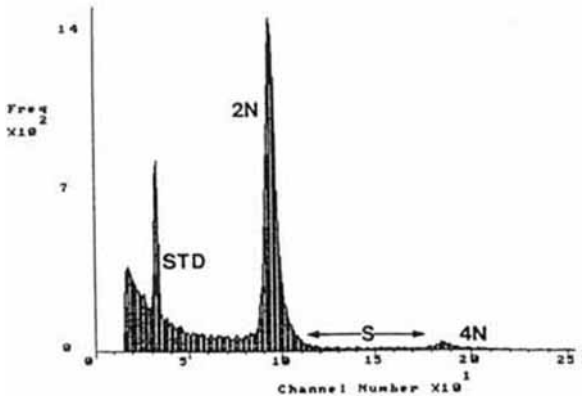

Fig. 1. FCM analysis of DNA content. In this FCM histogram the intensity of fluorescence is plotted on the horizontal axis and the number of cells is plotted on the vertical axis. An internal standard (STD) is run with each sample to allow quantitation of each of the fluorescent peaks. The major population of cells contains two copies of each chromosome (2N DNA). A smaller peak of cells with twice as much fluorescence contains four copies of each chromosome (4N DNA) and represents the cells in G2 and M phases of the cell cycle. The cells and assays of the cycle are represented by the cells between the 2N and 4N peaks.

aneuploid population appears by flow cytometry as a peak of cells with DNA content that is discrete from the G0/G1 peak or the G2/M peak. Such an aneuploid population is illustrated in Figure 2. This peak is distinctly separate from the G0/G1 peak and has a DNA index which contains about 1.2 times the normal amount of DNA in diploid cells.

FCM ANALYSIS OF DNA CONTENT IN COLON CANCERS

Aneuploidy occurs in a fraction of cancers of many types, and in breast cancer and lymphomas aneuploidy is a poor prognostic sign. About 60% of colon cancers are aneuploid and in general patients with aneuploid tumors have a higher recurrence rate and poorer survival than those with diploid tumors (Table 1).

It is not yet certain whether aneuploidy is an independent prognostic variable after correcting for stage and histologic differentiation, but several studies suggest that it is [1,3,5,7,10].

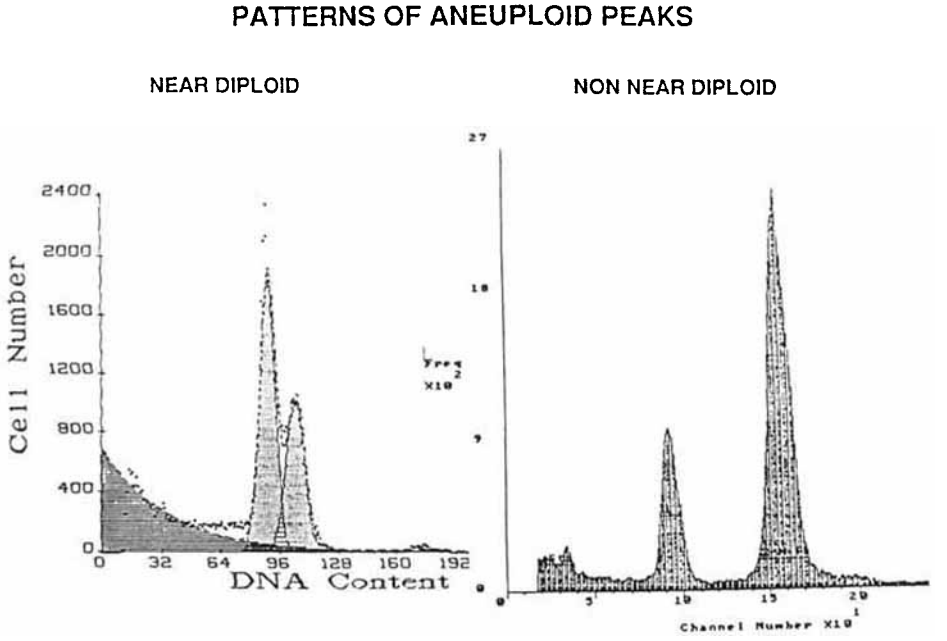

Fig. 2. Patterns of "aneuploid" peaks. These two histograms demonstrate different patterns that could be interpreted as aneuploidy. On the left, a population of cells with a DNA index of 1.2 is plotted. There is a discrete peak of cells with the intensity of fluorescence approximately 1.2 times the major G0/G1 peak. On the right, a large aneuploid population is seen with a DNA content approximately 1.7 times the normal diploid peak.

TABLE I. Prognostic Significance of Ploidy Status in Colon Cancer

					5 Year Survival	
Ref	Pts	Tissue	Location	% Aneuploid	Dipl vs. Aneu	p Value
1	279	Archival	Colon/Rectum	58	80 70(4)	<0.05
2	125	Archival	Rectal	54	57 35(5)	0.02
3	121	Archival	Rectosigmoid	46	65 42(5)	0.01
4	279	Archival	Colon/Rectum	62	75 65(4)	0.07
5	264	Archival	Colon/Rectum	52	64 50(5)	0.17
6	166	Fresh	Rectosigmoid	75	80 58(5)	>0.05
7	37	Fresh	Colon/Rectum	62	69 40(3)	0.007
8	100	Fresh	Colon/Rectum	63	64 49(5)	>0.05
9	123	Fresh	Colon/Rectum	67	64 34(3)	0.007
10	694	Archival	Colon/Rectum	49	65 53(5)	<0.001

Two other FCM parameters (an increased proliferative rate and a high DNA index) have been found to have prognostic value in recent studies [10,11] and deserve to be evaluated further.

Aneuploidy was originally thought to occur only in malignancies; however, it is now clear that it is seen in some histologically benign-appearing neoplasms such as adenomatous polyps [12,13]. Interestingly, clones of aneuploid but histologically non-neoplastic-appearing colonocytes have been identified in the colonic mucosa of some patients with ulcerative colitis [14]. It is not yet known if the presence of aneuploid clones in these clinical conditions is predictive of an increased risk of the subsequent development of dysplasia or carcinoma, but their presence can be viewed as an example of clonal expansion of a genetically abnormal (initiated?) cell.

FCM ANALYSIS OF DNA CONTENT IN CHRONIC ULCERATIVE COLITIS

The initial work of Hammarberg *et al.* [15,16] stimulated interest in FCM analysis of DNA content in chronic ulcerative colitis (CUC). These authors found a high (90%) frequency of aneuploidy in colon cancers in the setting of ulcerative colitis and a low (<5%) frequency of aneuploidy in normal tissue. Intermediate levels of aneuploidy were identified in dysplastic mucosa and inflamed, atrophic or hyperplastic mucosa in CUC (Figure 3). Two subsequent studies from Great Britain, however, came to divergent conclusions [17,18]. The results of the analysis of the individual biopsies in these two studies are plotted in Figure 4. The study from Leeds [17] showed no significant difference in the frequency of aneuploidy from biopsies showing ulcerative colitis only, dysplasia, or cancer, with a frequency between 18% and 24% in all groups. In contrast, a gradient of increase in frequency of aneuploidy in these three biopsy classifications was seen by the St. Marks group [18]. These observations were particularly disturbing because the studies were done in a very similar manner using archival specimens (fixed and paraffin-embedded) and they both used similar FCM techniques. It was initially difficult to envision a systematic difference in preparation or interpretation that might account for the differences because the Leeds group found both a higher frequency of aneuploidy in the non-neoplastic biopsies and a substantially lower frequency of aneuploidy in the dysplastic and cancer biopsies than the St. Marks group. The Leeds group subsequently reported that the frequency of aneuploidy in cancer in their series had increased to about 46% so that the remaining disparity was in the biopsies graded negative for dysplasia or cancer.

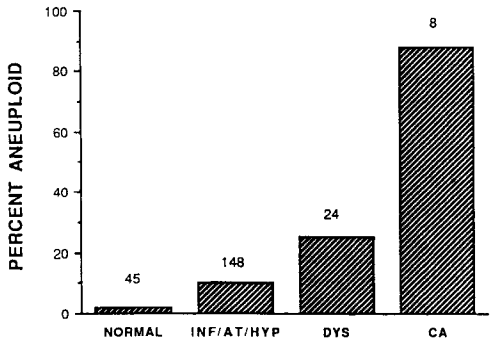

Fig. 3. The frequency of aneuploidy in CUC. The percent of biopsies that are aneuploid is plotted for groups of biopsies that are interpreted as normal, inflamed, atrophic, or hyperplastic (INF/AT/HYP), dysplastic (DYS), or cancerous (CA). The number above each column represents the number of biopsies analyzed in each group. Redrawn from Hammarberg *et al.* [15].

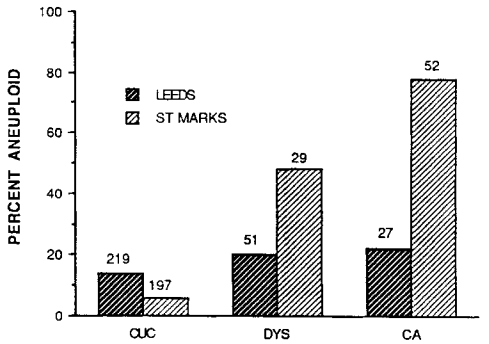

Fig. 4. The frequency of aneuploidy in CUC biopsies from two studies from Great Britain. The data from the Leeds group is shown in the dark cross-hatch bars and that from the St. Marks group in the light cross-hatch bars. The percent of biopsies that are aneuploid is plotted for each of three groups of biopsies interpreted as showing only ulcerative colitis without dysplasia (CUC), dysplasia (DYS), and cancer (CA). The numbers at the top of each column are the numbers of biopsies analyzed in each group. Redrawn from Fozard *et al.* and Melville *et al.* [17,18].

This is also the case if one analyzes the data with respect to the patients, rather than the individual biopsies, as shown in Figure 5. Thus, it seems that the major difference between these two studies is the finding of a higher frequency of aneuploidy in the non-neoplastic biopsies in patients by the group at Leeds (40%) than that at St. Marks (10%). It is possible that a difference in the criteria used for the classification of aneuploidy between the two studies could account for such a difference.

Several other groups have also studied the frequency of aneuploidy in CUC. Two studies reporting the correlation between aneuploidy and histology in individual biopsies are shown in Figure 6. In studies from Bergen, Norway [19], and from Umea, Sweden [20], a substantially higher frequency of aneuploidy was seen in biopsies showing dysplasia than in those that were negative for dysplasia. Interestingly, the absolute frequency of aneuploidy in the non-dysplastic biopsies differed by almost 2-fold between the two reports. The results of two other studies of the relationship between aneuploidy and histology as analyzed by patient groups are shown in Figure 7. The study from Stockholm, Sweden [21], and the study from Seattle [22] both show a gradient of increasing frequency of aneuploidy in patients who have biopsies that are only negative for dysplasia, those that have one or more biopsies graded as

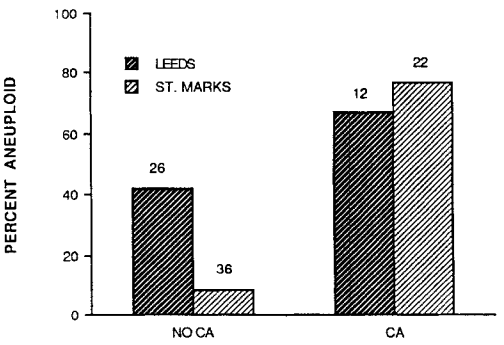

Fig. 5. The frequency of aneuploidy in any group from patients with and without carcinoma from two studies from Great Britain. The study from Leeds is shown by the dark cross-hatch bars while those from St. Marks are shown on the light cross-hatch bars. The frequency of aneuploidy found in any biopsy is plotted for two groups of patients, those that have no evidence of cancer in any biopsy (no CA) and those that have cancer in one or more biopsies (CA). The number at the top of each bar represents the number of patients in each group. Redrawn from Fozard *et al.* and Melville *et al.* [17,18].

indefinite for dysplasia, and those that have one or more biopsies graded as definite dysplasia.

The bulk of the evidence in the literature about the relationship between aneuploidy and dysplasia in CUC does suggest that biopsies that are dysplastic and patients that have dysplasia have a higher frequency of aneuploidy

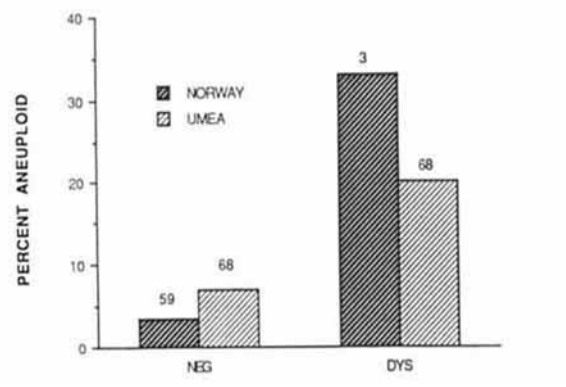

Fig. 6. The frequency of aneuploidy in biopsies from patients with CUC from two studies is presented. The study from Bergen, Norway is shown in the dark cross-hatch bars and the study from Umea, Sweden is shown in the light cross-hatch bars. The percentage of biopsies that are aneuploid is plotted for those that were graded as non-dysplastic (NEG) and those interpreted as dysplastic (DYS). The number of biopsies in each group is shown above the bars. Redrawn from Borkje *et al.* and Rutegard *et al.* [19,20].

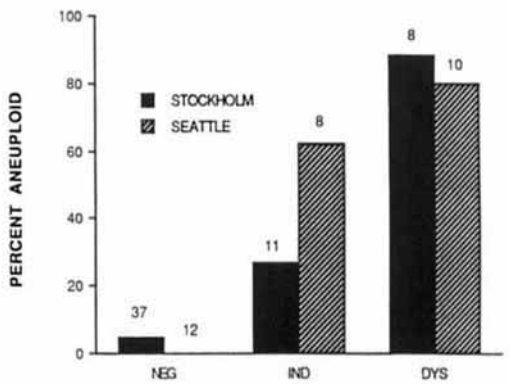

Fig. 7. The frequency of aneuploidy in patients with CUC from two studies as presented. A study in Stockholm, Sweden, is shown in the closed bars and a study from Seattle, Washington, in the cross-hatch bars. The frequency of aneuploidy in three patient groups is plotted. Those whose biopsies were all graded as negative for dysplasia (NEG), those with one or more biopsies graded as indefinite for dysplasia (IND), and those with one or more biopsies graded as dysplastic (DYS). The number above each bar represents the number of patients in each group. Redrawn from Lofberg *et al.* and Levine *et al.* [21,22].

than those with no evidence of dysplasia. Nonetheless, there is a significant discrepancy concerning the frequency of aneuploidy in non-dysplastic mucosa. We have had the opportunity to perform a large number of FCM studies in patients with CUC. Patients evaluated in our studies include patients entered from the Denver Dysplasia in Ulcerative Colitis Study as well as patients enrolled in the Cleveland Clinic Chronic Ulcerative Colitis Registry. We have analyzed our data to not only look at the relationship between dysplasia, cancer, and ploidy status, but also to see whether it might explain the variance in the literature concerning the frequency of aneuploidy in the non-dysplastic mucosa.

We have performed FCM analysis of DNA content in colonic biopsies from three groups. The first group is controls that have been found by colonoscopy to have no colonic neoplasia and have no personal history of CUC, adenomas, or colon cancer. The other two groups are patients with CUC who are either enrolled in our surveillance program or have come to colectomy. The surveillance group consists of patients who have long standing (>8 years), extensive (proximal to the splenic flexure) ulcerative colitis. These patients are enrolled in surveillance studies in which yearly colonoscopy is advised and multiple biopsies are obtained throughout the colon. The routine procedure in these patients is to obtain two biopsies every 10 cm throughout the colon as well as additional biopsies of any lesions seen. One biopsy at each site is prepared for histologic evaluation for dysplasia; the second is prepared for FCM analysis of DNA content. We have thus far evaluated over 1000 biopsies from 100 patients in the surveillance group. The colectomy group consists of patients with ulcerative colitis who have come to colectomy for intractable disease, dysplasia, or cancer. In this group, two side-by-side, four quadrant biopsies are taken every centimeter through the entire resected specimen. As in the surveillance group, one biopsy at each site is prepared for flow cytometry, the other for histology. Thus far, we have had the opportunity to evaluate over 800 biopsies from 15 patients in this group.

The criteria we used to define aneuploidy is the presence of a discrete peak of cells distinct from the G0/G1 and G2/M peaks that contained at least 5% of the total cell population. Using these criteria alone, a high frequency of aneuploidy (about 15% of the biopsies, 40% of the patients) have one or more biopsies that were aneuploid. We have noted that a large percent-

age of these putative aneuploid peaks are near diploid peaks, that is, they are discrete peaks, but they have DNA content less than 1.2 times the diploid peak. In the surveillance group, over 75% of biopsies that were interpreted as aneuploid fell into the near diploid group. The frequency of aneuploidy in the non-dysplastic tissue is thus influenced greatly by whether one interprets the near diploid peaks as aneuploidy. Analysis of our data suggests to us that variability in interpretation of the near diploid peaks may account for some of the differences reported in the literature about the relationship between aneuploidy, dysplasia, and cancer in CUC. Inclusion of the near diploid populations in our data markedly increases the overall frequency of aneuploidy, particularly in the non-dysplastic group. Interobserver variation in the interpretation of near diploid peaks has been noted in other tissues [23]. We believe that this difference may well account for much of the variability in the literature cited above. As in the other studies and literature, we found a highly significant increase in the frequency of aneuploidy, both in dysplastic biopsies as well as in patients who have dysplasia or cancer in our studies.

Two other aspects of these FCM studies relate to the potential value of ploidy status as a marker of cancer risk in CUC. First, patients who come to colectomy because of dysplasia or cancer are commonly found to have markedly abnormal FCM analysis, frequently with multiple areas of aneuploid populations of different DNA index [14]. For example, five of six patients with high grade dysplasia or cancer studied by Levine *et al.* had multiple aneuploid cell populations in regions remote from the areas of cancer or high grade dysplasia (aneuploidy was also present in the area of dysplasia). Two patients had 14 or more different aneuploid cell populations. We have also observed the presence of multiple aneuploid populations in our patients with dysplasia and cancer in CUC. These observations suggest that aneuploidy is a prominent feature of the colonic mucosa in the late stages of carcinogenesis in the setting of CUC.

The colectomy data also suggest that aneuploid populations can appear over wide expanses of the colonic mucosa [14]. This kind of observation can be viewed as an example of clonal expansion in this mucosa. It is possible that the ulceration and denudation of the mucosa that occurs in ulcerative colitis allows the opportunity for a clone of cells that has growth advantage to repopulate large areas of the colonic mucosa. In the absence of such denudation, perhaps this abnormal clone would be limited to a single crypt, as the cells would normally be sloughed into the lumen when they reached the top of the crypt. In the context of multi-stage carcinogenesis, one can view the appearance of aneuploidy in multiple areas of the colon as an example of clonal expansion. If this aneuploid population were to represent a clone of cells with one or more of the genetic events responsible for a step(s) in carcinogenesis, its expansion over a large patch of the colon would mean that a large number of cells would be available for subsequent carcinogenic steps. Thus, progression to cancer would be more likely to occur. The most commonly held current view is that aneuploidy does not itself represent a specific genetic event necessary for carcinogenesis but is a reflection of the genomic instability that characterizes the premalignant mucosa.

We have been encouraged also by the frequent finding that not only do dysplastic biopsies frequently show aneuploidy, but the same aneuploid population can often be seen in the non-dysplastic mucosa adjacent to dysplasia. For example, one of our patients had two biopsies at the hepatic flexure which showed dysplasia and which were aneuploid with a DNA index of about 1.7. In addition, four adjacent non-dysplastic biopsies showed the same aneuploid population. This type of observation suggests the possibility that dysplasia may arise within a pre-existing aneuploid population and that aneuploidy may occur more diffusely than dysplasia. If this is so, it could decrease the problem of sampling error when looking for dysplasia. These observations have encouraged us to continue to evaluate aneuploidy as a promising biomarker of cancer risk in CUC.

Taken together, the current data suggest that aneuploidy occurs commonly in CUC and that it correlates with the established markers of cancer risk in this setting (dysplasia, established cancer). Aneuploidy is found commonly in patients with dysplasia and/or cancer, but not all patients with neoplasia have detectable aneuploidy. Aneuploidy is more commonly

found also in dysplastic and cancerous biopsies than in non-dysplastic mucosa, but not all neoplastic biopsies are aneuploid. Aneuploidy clearly can occur in non-neoplastic mucosa. The clinical meaning of the presence of aneuploidy in non-dysplastic mucosa is not yet known, and we do not currently recommend altering clinical decision-making on the basis of a single aneuploid population in a patient with ulcerative colitis. Nonetheless, in our opinion, aneuploidy is the most promising of the new markers for the detection of cancer risk in ulcerative colitis and deserves further evaluation.

SUMMARY

Aneuploidy is found in about 60% of sporadic colon cancers. Aneuploidy is a poor prognostic feature in colon cancer but it does not occur in the non-neoplastic mucosa of sporadic colon cancer patients. In CUC, however, aneuploid populations occur in non-neoplastic mucosa. Studies in CUC have shown a significant relationship between aneuploidy and the current best markers of cancer risk in this population. Aneuploidy certainly deserves rigorous testing to determine if its presence will be of clinical value as an intermediate biomarker in assessing cancer risk in patients with CUC.

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