A Pilot Study of DNA Aneuploidy in Colorectal Adenomas and Risk of Adenoma Recurrence

Alan R. Kristal, Mary S. Baker, Michael J. Flaherty, Ziding Feng, Thomas J. Ylvisaker, Andrew D. Feld, and Douglas S. Levine


Abstract
This case-control study examined whether DNA aneuploidy in colorectal adenomas is a risk factor for subsequent adenoma recurrence. Cases (recurrent polyp formers) were defined as persons with diagnoses of new adenomas at 2 colonoscopies following the index (first) adenoma diagnosis; controls were persons with no new adenomas at a follow-up at least 1 year after the index diagnosis. Cases (n = 22) and up to 3 controls (n = 29) were matched for factors known to be associated with polyp recurrence or aneuploidy: (a) age; (b) histology; (c) number of index polyps; and (d) size of largest index polyp. The largest adenoma from the index colonoscopy was removed from the paraffin block and analyzed for DNA content abnormalities by flow cytometry. On the basis of the observed distribution of aneuploidy in case-control sets, the point estimate for the relative odds of recurrence given aneuploidy in the index polyp was infinity (P < 0.035), and the lower bound for the 95% confidence interval was 2.02. We conclude that in this convenience sample, DNA aneuploidy increased the risk of recurrent colorectal adenomas. Larger, preferably prospective studies are needed before DNA content flow cytometric analysis of colorectal adenomas can be recommended as a routine clinical practice, but these results do suggest that polyp ploidy should be assessed in research studies using adenomas as end points or intermediate end point markers.

Introduction
Colorectal cancer is the second leading cause of cancer death in the United States, with an estimated 149,000 new cases in 1994 (1). One approach to the prevention of colorectal cancer is based on primary screening for adenomatous polyps and their removal during colonoscopy (2). Most scientists believe that colorectal cancers originate in adenomas and reason that polypectomy should reduce the subsequent risk of cancer. Questions remain about the efficacy of colonoscopy and polypectomy as a secondary surveillance strategy for preventing colorectal cancer, and the optimal patient management protocols after adenomas are diagnosed and removed are debated. Many clinicians perform repeat colonoscopies within 1 year after a polypectomy and continue prospective colonoscopic surveillance at varying intervals, ranging between 1 and 5 years (3, 4). Several clinical factors may influence recommendations by clinicians about the timing of follow-up colonoscopies, including a previously resected colorectal cancer; a family history of colorectal neoplasms or other solid tissue tumors; an initial diagnosis of multiple synchronous, large, and/or villous colorectal adenomas; and other coexisting medical conditions. However, the optimal time interval for follow-up colonoscopies in individual patients is not known, in part because there are no well established clinical parameters or disease markers that reliably distinguish persons at high or low risk of recurrent adenomas.

Colorectal cancers, like other solid tissue cancers, appear to develop by a multiple step process. As hypothesized by Nowell (5), this involves acquired somatic genetic instability, loss of proliferative controls, and evolution of clones of cells capable of local invasion and metastasis. The manifestations of multistage carcinogenesis of the large intestine are recognized morphologically as a normal → dysplasia (adenoma) → cancer sequence (6), often accompanying progressive proliferative abnormalities (7). Abnormalities reflecting genetic instability, such as point mutations, allelic deletions, and karyotypic abnormalities, often are present in colorectal cancers and precancerous adenomas (5, 8). These progressive somatic genetic changes may become quantifiable in colorectal epithelial cell populations as gross changes in DNA content, or ploidy, as part of a normal diploid → tetraploid → aneuploid → multiple aneuploid genomic sequence suggested for carcinogenesis in the large intestine, in other human solid tissue tumors, and in model systems (9–13). Genetic instability may continue during cancer growth and metastasis, leading to the observed genetic heterogeneity of large neoplasms and of metastatic lesions (12, 13). The mechanisms responsible for generating genetic instability in colorectal cancer, as well as in other solid tissue tumors, are not completely understood.

DNA content flow cytometry is an established method for measuring cellular DNA content in precancerous colorectal epithelium and neoplasms to detect manifestations of genetic instability, including gross abnormalities of DNA content or aneuploidy, as well as proliferative or cell cycle abnormalities (10, 13). The purpose of our study was to evaluate whether the presence of aneuploid cell populations, as measured by DNA content flow cytometry, in adenomas removed at a patient's first colonoscopy, is a risk factor for recurrent colorectal adenomas.
Aneuploidy and Recurrent Colorectal Adenoma Risk

Materials and Methods

This was a case-control study in which demographic and clinical data were abstracted from medical records, and DNA content abnormalities were determined from flow cytometry analyses on paraffin-embedded colorectal adenomas. All data were from GHC of Puget Sound, a large health maintenance organization in western Washington State.

Case and Control Selection. Cases and controls were a subset of participants in a larger, previously published study (14). To be eligible for the present study, paraffin-embedded tissue blocks containing colorectal adenomas had to be available for analysis. All cases and controls received their index (first) diagnosis of colorectal adenomas at GHC between 1974 and 1988. All index and follow-up colonoscopies were complete to the cecum, and all polyps were removed and submitted for pathological analysis. Cases were patients with recurrent adenomas, defined as a diagnosis of new adenomas removed at two colonoscopies following the index colonoscopy. This conservative definition of recurrent adenomas was used to reasonably assure that cases had metachronous adenomas and not just missed synchronous adenomas from the index colonoscopy. Controls were patients without new adenomas at a follow-up colonoscopy that followed the index colonoscopy by 1 or more years. Fig. 1 shows the distribution of length of follow-up for controls (to the single follow-up colonoscopy) and cases (to the first and second follow-up colonoscopies). For controls, the median time to follow-up was 24 months (mean, 36.4; range, 9–159); for cases, the median time to first follow-up was 22 months (mean, 33.0; range, 5–138) and to second follow-up was 40 months (mean, 54.3; range, 19–150). Due to an error in record transcription, a single control observation had only a 9-month follow-up after the index colonoscopy.

Demographic and Clinical Parameters. GHC medical charts, including endoscopy and pathology reports, were reviewed for all cases and controls. Demographic information included patient age and gender. Clinical data included time intervals between index and follow-up colonoscopies, estimates of adenoma diameter from endoscopists, descriptions of polyps as pedunculated (stalked) or sessile, anatomic sites of adenomas, methods of adenoma removal (snare electrocautery or multiple biopsies), number of adenomas removed at the index colonoscopy, presence of hyperplastic polyps at the index colonoscopy, and verification of the calendar dates of all colonoscopies, as well as the presence of adenomatous pathology and the architectural type of adenoma (villous versus tubulovillous or tubular) described in the pathology reports.

DNA Content Flow Cytometry. Paraffin tissue blocks containing colorectal adenomas were coded and analyzed as follows. For each tissue block, one 4–5-μm-thick section was obtained for histopathological analysis. Next, three 50–60-μm-thick sections were obtained, deparaffinized in xylene, rehydrated through graded ethanol solutions, digested in pepsin, stained with 4,6-diamidino-2-phenylindole, and processed for DNA content flow cytometry with the use of methods described previously (10, 15). DNA content flow histograms were analyzed without knowledge of demographic, clinical, or histopathological information with the use of methods described previously (10, 13) to generate the following parameters: (a) DNA content; (b) total aneuploid cell fractions; and (c) cell cycle fractions of aneuploid and diploid cell populations (S- and G2-phase) with their coefficients of variation. We defined aneuploidy as the detection of a resolvable peak on the DNA flow histogram separate from the diploid peak. In this sample, the minimum DNA content of an aneuploid peak was 2.3%. The mean coefficient of variation of DNA content from the flow histogram did not differ between cases (5.9 ± 1.3) and controls (6.1 ± 1.3). We did not analyze results related to cell cycle fractions because we and others (16, 17) find these flow cytometry data problematic when based on analyzing paraffin-embedded tissue.

Histopathology. We examined morphological features of all tissue blocks as a quality control procedure to determine whether differences in morphology between case and control tissues could confound DNA content analyses. One 4–5-μm-thick section from each tissue block was stained with hematoxylin and eosin, coded, and examined by light microscopy.

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1 The abbreviation used is: GHC, Group Health Cooperative.
without knowledge of demographic, clinical, or flow cytometric information. The following common morphological features were evaluated: (a) atypism, as fraction of adenomatous epithelium with high-grade, or severe, dysplasia (18, 19); (b) villous architecture, defined as villous if >75% villous, as tubulovillous if 25–75% villous, or as tubular if <25% villous; (c) fractions of adenomatous epithelium versus normal colorectal epithelium; (d) fractions of colorectal epithelium versus nonepithelial stromal elements; (e) presence of acute or chronic inflammation; (f) presence of electrocautery artifact; and (g) the age of each tissue block. There were no differences in the distributions of these factors across case and control tissues, suggesting that the inferences from DNA content analyses were not confounded by technical aspects of flow cytometry.

**Statistical Analysis.** Cases and controls were matched for the following characteristics at the index colonoscopy: (a) age (within 5 years); (b) estimates of adenoma diameter (<10 mm, 10–19 mm, 20–29 mm, >30 mm) by endoscopists; and (c) the architectural type of adenoma based on the pathology report (villous versus tubulovillous or tubular). One to three control matches were found per case. One case subject was matched to a control within 6 years of age (42-year-old case subject and 46-year-old control). We calculated the odds ratio and its 95% confidence interval for the risk of polyp recurrence comparing persons with and without aneuploidy in the index polyp. Because of the small sample size, we tested the null hypothesis (that the odds ratio was 1.0) by calculating the exact conditional probability of observing data as or more extreme as we observed if the null hypothesis were true. We calculated the test-based confidence interval by finding the value of the odds ratio for which the probability of observing data as or more extreme than we observed was 0.05. We also used a conditional logistic model to examine whether variables not matched (gender and time from index colonoscopy to follow-up) or not precisely matched (age) were significant predictors of recurrence. This statistical model examines unmatched covariates while incorporating all matching criteria and is described in detail by Breslow and Day (20).

This study was designed originally to have approximately 100 case-control pairs, yielding 75% statistical power to detect a relative risk of 3.0 with a two-sided α error of 5%. We planned a group sequential analysis (21) to save time and costs for the laboratory procedures. We planned for 4 repeated tests for statistical significance, to be completed after each of the 25 sets of case-control data were accumulated, using a nominal significance level at 0.0182. We suspended further data collection after analysis of the first set of case-control pairs reached the 0.01 level for statistical significance. We later found that due to errors when selecting paraffin blocks for analysis, three case subjects could not be matched to at least one control. This left 22 matched case-control sets for the analysis. Results from the final data set did not reach the nominal significance level for early suspension of data collection, and for this reason we report these results as a pilot study.

**Results**

Table 1 gives demographic and clinical data for 22 case subjects and 29 controls. The 22 case:control matches included 17 pairs (1:1), 3 triplets (1:2), and 2 quadruplets (1:3). Mean age of subjects was 62 years; 59% were men and 59% had more than 1 synchronous polyp. On the basis of the largest polyp removed at polypectomy, only 11% were villous adenomas, 15% were >20 cm in diameter, and 25% had DNA aneuploidy.

### Table 1 Clinical and demographic characteristics at index colonoscopy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (recurrent polyps)</th>
<th>Controls (no recurrence)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (± SD)</td>
<td>62.7 ± 8.9</td>
<td>62.7 ± 6.9</td>
<td>62.7 ± 7.8</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>68.2</td>
<td>51.7</td>
<td>58.8</td>
</tr>
<tr>
<td>2+ synchronous adenomas (%)</td>
<td>69.6</td>
<td>52.6</td>
<td>59.0</td>
</tr>
<tr>
<td>Size of largest adenomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>36.4</td>
<td>48.3</td>
<td>43.1</td>
</tr>
<tr>
<td>10–19</td>
<td>45.5</td>
<td>37.9</td>
<td>41.2</td>
</tr>
<tr>
<td>20–29</td>
<td>13.6</td>
<td>10.3</td>
<td>11.8</td>
</tr>
<tr>
<td>3+</td>
<td>4.6</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Architecture of largest adenomas (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villous</td>
<td>13.6</td>
<td>10.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Tubulovillous or tubular</td>
<td>86.4</td>
<td>89.7</td>
<td>88.2</td>
</tr>
<tr>
<td>Aneuploidy in largest adenomas (%)</td>
<td>40.9</td>
<td>10.3</td>
<td>23.5</td>
</tr>
<tr>
<td>n</td>
<td>(22)</td>
<td>(29)</td>
<td>(51)</td>
</tr>
</tbody>
</table>

**Summary odds ratio:** 95% confidence interval (2.02, ∞).

**Controls, no polyp recurrence.**

**Cases, recurrent polyps.**

**+,” aneuploidy.**

**“-,” no aneuploidy.**

**Cases**

### Table 2 Matched analysis of aneuploidy in index polyp and risk of polyp recurrence

<table>
<thead>
<tr>
<th>Matched pairs controls</th>
<th>Cases</th>
<th>Controls</th>
<th>Total</th>
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<tr>
<td>+</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Matched triplets controls**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Matched quadruplets controls**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2 gives the matched analyses of the 22 case:control sets. All three aneuploid controls were matched to aneuploid cases. There were no matched sets with aneuploid and diploid cases; thus, the point estimate of the odds ratio is infinity. The probability for the two-sided exact test that the summary odds ratio from these three tables is not different from 1.0 is 0.035. The lower bound of the 95% confidence interval is 2.02 (the upper bound is infinity). We also completed an additional analysis to examine whether three additional covariates, gender, months from index colonoscopy to last follow-up (both of which were not matching variables), and age (which was matched imprecisely) were associated with risk of recurrence. The odds ratio associated with being male was 1.46 (95% confidence interval, 0.36–7.02), with time to last follow-up was 1.01 (95% confidence interval, 0.99–1.03), and with age was 1.04 (95% confidence interval, 0.78–1.39). We conclude that risk of polyp recurrence was significantly higher in persons with DNA aneuploidy in the largest polyp removed at the index colonoscopy, and this increased risk was independent of age at diagnosis of index polyp, number and size of index polyps, and whether an index polyp was characterized as a villous adenoma.
We completed several exploratory analyses to examine whether there were differences in matching criteria between the concordant and discordant matched sets. We compared the sets in which both cases and controls were positive for aneuploidy (n = 3); both cases and controls were negative for aneuploidy (n = 13), and cases were positive and controls were negative for aneuploidy (n = 6). The only statistically significant difference was in the percentage of matched sets in which the polyps were villous adenomas (66.7, 7.7, and 0%, respectively; P < 0.05). Although not significantly different statistically, the mean ages of these groups were 70.7, 64.5, and 60.0 years, respectively. The distributions of number and size and index polyps were similar across these groups.

Discussion

We found a significant increase in the risk of recurrent colorectal adenomas in patients with aneuploid cell populations detected in an index adenoma. Due to the small sample size and distribution of case-control matches, we could not calculate a point estimate for the increase in risk, although the lower bound of the 95% confidence interval was a 2-fold increased risk.

Several aspects of our study design should be considered when interpreting these results. Most important, the results are based on 22 case-control sets, and only 6 sets were discordant for ploidy. The decision to suspend further data collection was justified by reaching the nominal significance level for sequential testing, but we lost three case-control sets due to technical errors. Additional data would have provided greater precision in our risk estimates; we must therefore consider the results presented here as preliminary. Another potential weakness of this study is in the definition of cases and controls. We defined cases as patients with new adenomas diagnosed at the two colonoscopies following the index colonoscopy in order to reduce the chance that new adenomas were synchronous adenomas missed at the index colonoscopy. We defined controls as patients without new adenomas at colonoscopies performed at least 1 year after the index colonoscopy. Thus, while there are clear differences between cases and controls, it is possible that some controls could be misclassified because they could have developed recurrent adenomas during more extended follow-up. These definitions for cases and controls reflect the colorectal cancer prevention surveillance practices in place at GHC during the 1980s. Generally, when a colorectal adenoma was removed, a second follow-up colonoscopy was scheduled approximately 1 year later. If additional adenomas were removed, a third follow-up colonoscopy was scheduled 1 year after the second. Only when a colonoscopic examination failed to identify new adenomas would the interval between surveillance colonoscopies be increased to between 3 and 5 years. On the basis of these practices, it would not have been possible to select controls that were matched precisely to cases on intensity of colonic follow-up. Most follow-up colonoscopies were much later than proscribed by the surveillance protocol of GHC, but the distributions of time to follow-up for controls and time to first follow-up for cases were similar. This suggests that there were no biases associated with access to or use of medical care between the two groups. Whether the longer time to second follow-up for cases introduces an important bias is difficult to assess, and only a prospective study with uniform time to follow-up colonoscopies will be capable of eliminating this problem entirely.

There were several strengths to this study: (a) the matched case-control design allowed control for important known, as well as theoretical confounding variables. Our observed association of aneuploidy with recurrent colorectal adenomas was independent of patient age, adenoma size, and high grade epithelial dysplasia; (b) a variety of parameters that may have affected the technical quality or accuracy of the DNA content analyses were well controlled. False aneuploid peaks may result from tissue autolysis (22) but are reported to be rare in DNA flow histograms generated from paraffin-embedded tissue (23). The ability to detect aneuploid cell populations in adenomas could have been diminished by dilutional effects of increased inflammatory cells or nonepithelial stromal elements. However, the analyses of the histopathological data indicate that potentially confounding factors that might have affected the results of DNA content analyses did not differ significantly in cases versus controls; and (c) the matched design also was efficient, yielding statistically meaningful results with relatively few observations.

There is only a single previously published investigation of whether aneuploidy in colorectal adenomas is a risk factor for the development of metachronous adenomas (16). This study, based on a cohort of 50 persons, found no association between polyp ploidy at the index colonoscopy and adenoma recurrence by the 10-year follow-up. There were many aspects of the design of this study that make evaluation of this result difficult. The analyses were based on the number of adenomas (n = 61) and not on the number of individuals; thus, the estimate of risk associated with an index aneuploid polyp is not interpretable. The analyses were not controlled for known risk factors for recurrence or aneuploidy such as age, polyp size, polyp histology, or number of synchronous polyps; thus, confounding was likely. Further, of the 27 patients classified without recurrent polyps, 4 (15%) had a previous adenoma diagnosis at least 1 year before the “index” diagnosis, suggesting that these patients were misclassified. Finally, any adenomas found before 12 months were classified as synchronous, but not all patients received follow-up colonoscopies within 12 months. Thus, many adenomas classified as metachronous could well have been synchronous.

There is also a single study (24) of the association of aneuploidy in a polyp and subsequent risk of colorectal cancer. In this study, 13% of a group of patients with colorectal cancer had aneuploid cell populations in colorectal adenomas removed at least 6 months before colectomy. Several aspects of this study also limited the conclusions of the author regarding the lack of usefulness of aneuploidy in adenomas as a predictor of subsequent colorectal neoplasia. In particular, there was no control group, the high coefficient of variation (8.2%) reported for DNA flow histograms may have led to an inability to detect near-diploid aneuploid peaks, and the prevalence of aneuploidy in adenomas was considerably lower than the 22–35% prevalence figures (30–38% prevalence for adenomas >1 cm) reported by others (16, 25–32).

Detection of DNA content abnormalities predicts risk of progression to high grade dysplasia and adenocarcinoma in other premalignant disorders of the alimentary tract, including Barrett’s esophagus (33) and ulcerative colitis (34). The development of aneuploidy in these disorders and in colorectal adenomas may reflect constitutional genetic abnormalities that predispose to neoplastic progression in intestinal epithelium, intraluminal factors, or interaction between these environmental influences and the somatic genome present in epithelial cells. The importance of genetic susceptibility in the pathogenesis of colorectal neoplasia is suggested by an investigation showing that the prevalence of aneuploidy in adenomas of patients with a family history of colorectal cancer (78%) was significantly greater than that in adenomas of patients without such a family history (20%;
Ref. 35). Other investigators also have provided data to support the hypothesis that colorectal adenomas and cancers develop as a result of an inherited genetic susceptibility or predisposition (36). Candidate genes for cancer susceptibility include cell division cycle checkpoint genes that maintain genetic stability and a diploid DNA content in normal proliferating cells, which must accurately replicate and then segregate all chromosomes between daughter cells during cell division. It has been suggested that defects in such checkpoint genes may lead to the development of aneuploidy and an increased risk of progression to cancer (37).

The results of our study are potentially relevant for clinical care of patients at risk for colorectal cancer. DNA content flow cytometry is available in most hospital pathology laboratories, and it is used routinely to assess the severity of disease for breast, colorectal, and other tumors. Therefore, evaluation of adenomas for aneuploidy also could become a routine clinical practice. If prospective studies prove that aneuploidy in adenomas is a sensitive and specific marker for increased risk of recurrent colorectal adenomas, it may become appropriate to individualize time intervals for follow-up surveillance colonoscopies based on assays of DNA content in index adenomas.

The results of our study also are relevant for research investigations on colorectal cancer and its prevention. Recurrence of colorectal adenomas is currently being used as an intermediate end point marker of cancer in intervention trials. Detection of aneuploidy in colorectal adenomas may be useful to distinguish adenomas with greater neoplastic potential, as well as to identify patients at higher risk of recurrent adenomas. Therefore, measurement of DNA content in adenomas could be expected to improve the efficiency of clinical trials using adenomas as an intermediate marker. For example, a trial to prevent recurrent colorectal adenomas that is restricted to patients with aneuploid index adenomas would yield higher recurrence rates and allow for reduced study sample sizes. However, patients with aneuploid colorectal adenomas also could possibly have an irreversibly elevated risk of recurrent adenomas, and the intervention under investigation might be judged inappropriately to be ineffective.

Aneuploidy reflects genetic instability of sufficient severity to produce abnormalities in DNA content that are relatively gross and thus are quantifiable by flow cytometry (10, 13). Therefore, significant genetic abnormalities in colorectal adenomas that do not lead to a change in ploidy will not be detectable by this assay technique. Identification of such genetic changes that are shown to be more sensitive and specific in predicting the risk of colorectal neoplasia may prove ultimately to be most valuable in patient management and research study design.

Acknowledgments

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References


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