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Pancolonic Chromosomal Instability Precedes Dysplasia and Cancer in Ulcerative Colitis[1]

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ABSTRACT

Patients with long-standing ulcerative colitis (UC) are at increased risk for colon cancer. These cancers are thought to arise from preexisting dysplasia in a field of abnormal cells that often exhibits aneuploidy and p53 abnormalities. Using dual color fluorescence *in situ* hybridization with centromere probes and locus-specific arm probes for chromosomes 8, 11, 17, and 18, we demonstrate that chromosomal instability (CIN) is present throughout the colon of UC patients with high-grade dysplasia or cancer. In rectal biopsies that were negative for dysplasia, abnormalities in chromosomal arms, especially losses, were most common, whereas centromere gains were most common in dysplasia and cancer. The frequency and type of abnormalities varied between the chromosomes examined; chromosome 8 was the least affected, and 17p loss was found to be an early and frequent event. Chromosomal arm instability showed 100% sensitivity and specificity for distinguishing control biopsies from histologically negative rectal biopsies from these UC patients, raising the possibility that a screen for CIN might detect the subset of UC patients who are at greatest risk for development of dysplasia and cancer. These results suggest that dysplasia and cancer in UC arise from a process of CIN that affects the entire colon; this may provide the mutator phenotype that predisposes to loss of tumor suppressor genes and evolution of cancer.

INTRODUCTION

Extensive UC[3] of more than 8 years in duration confers an increased risk for the development of colorectal cancer (1, 2). The current standard of practice for managing this risk requires lifelong annual colonoscopic surveillance of all such patients to detect dysplasia or early curable cancer (3). We undertook the present study to develop a better understanding of the earliest steps of UC carcinogenesis, in the hope that this knowledge would help identify the subset of patients at highest risk for progression to cancer.

It has been suggested that progression toward cancer in UC proceeds in a stepwise fashion of histological changes from negative for dysplasia → indefinite for dysplasia → dysplasia → cancer (4). Regions of cancer or HGD in UC are often surrounded by mucosa with indefinite or low-grade histology. We and others have shown that these fields of histologically abnormal mucosa are often accompanied by even larger fields of flow cytometrically detectable aneuploidy (5) and/or regions with loss of heterozygosity and mutation of p53 (6). Recently, using comparative genomic hybridization (CGH), we found that approximately 40% of flow cytometrically diploid biopsies near sites of dysplasia or cancer contained clonal cell populations with subtle chromosomal abnormalities.[4] These and other similar findings suggested the possibility that CIN is an early step in neoplastic progression in UC. To pursue this question, we used dual-color FISH with paired green fluorescent centromere probes and red fluorescent arm probes on four different chromosomes. FISH can identify CIN in small subpopulations of interphase cells (7), allowing the detection of infrequent, possibly random changes before they lead to clonal expansion. These experiments demonstrate that CIN is present throughout the colon of patients with dysplasia or cancer in UC.

MATERIALS AND METHODS

Patients. The average age of the 10 non-UC control patients (5 males and 5 females) was 54 years, and the average age of the 17 UC patients (10 males and 7 females) was 48 years. Resections were performed in controls for diverticular disease (4), endometriosis, hernia, rectal prolapse, appendicitis, complications of decubitus ulcer, and tubular adenoma. One biopsy was examined by FISH from each of the non-UC control colons. The known duration of disease in UC patients was 17 ± 12 years (mean $\pm$ SD; range, 2–50 years). Seven of the UC patients had the highest histological grade of cancer, whereas 10 UC patients had dysplasia. Colonoscopy or colectomy biopsies were selected from the 17 UC patients to provide a full spectrum of histological grades for FISH analysis. These consisted of four biopsies with cancer, four with HGD (all from patients in whom this was the highest grade lesion), five with LGD, eight that were IND, seven that were negative for dysplasia (Neg) but near HGD or cancer, and eight rectal biopsies that were negative for dysplasia (NegR). The diagnosis of IND as a histological category between negative and LGD was made according to the consensus criteria (4), with the exception that the indefinite group was not subdivided into three categories. The average distance from the site of highest grade (cancer or HGD) was 10 cm for LGD biopsies, 16 cm for IND biopsies, 10 cm for Neg biopsies, and 49 cm for NegR biopsies. Aneuploidy was examined by flow cytometry as described previously (5) and defined as a distinct nondiploid peak in excess of 5% of cells (8); it was present in three of four cancer biopsies, one of four HGD biopsies, and two of seven Neg biopsies surrounding dysplasia/cancer. All biopsies were frozen at −70°C in MEM with 10% DMSO until use.

Cell Preparation. Epithelial cells were isolated from biopsies by the following method: the nonmucosal side of biopsy specimens was affixed to the end of a 2.5-mm-diameter stick with cyanoacrylate glue and soaked for 5 min in Hank's buffer with 20 mM DTT and 5 mM EDTA at 37°C. Samples were transferred to 2 ml of shaking solution (Hank's buffer with 20 mM DTT, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 10% DMSO at 4°C) and vortexed for 5 s, and then the stick with residual stroma was removed. Staining with anticytokeratin antibody showed that the cells in suspension at the end of this procedure were >90% epithelial.

FISH. Cells were washed in 5 mM $CaCl_2$ with 0.1% NP40, spun (10 min at 1000 × g), washed again in 1 ml of 5 mM $CaCl_2$ with 200 μl chilled 3:1 methanol:acetic acid, and spun. Ten μl of nuclear suspension were dropped on plain glass slides and fixed with 3:1 methanol:acetic acid, followed by 1% paraformaldehyde. Replicate slides from each biopsy were hybridized to pairs of FITC-labeled centromere and TRITC-labeled locus-specific probes for chromosomes 8, 11, 17, and 18: (*a*) D8Z2 (centromere), 8q21.3, D18Z1 (centromere), and 18q21.2 (Oncor, Inc., Gaithersburg, MD); and (*b*) p53

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[3] The abbreviations used are: UC, ulcerative colitis; FISH, fluorescence *in situ* hybridization; HGD, high-grade dysplasia; CIN, chromosomal instability; LGD, low-grade dysplasia; IND, indefinite for dysplasia; Neg, negative for dysplasia; ROC, receiver operator curve; NegR, rectal biopsies negative for dysplasia; CGH, comparative genomic hybridization.

[4] Robert F. Willenbucher, Peter Rabinovitch, Daniela E. Aust, Teresa Brentnall, Suzanne J. Zelman, Roger Haggitt, and Frederic M. Waldman. Ulcerative colitis-related dysplasia arises in a genetically abnormal nondysplastic epithelium, submitted for publication.

(17p13.1), 17 α (centromere), cyclin D1 (11q.13), and 11 SG (centromere; Vysis, Inc., Downers Grove, IL). For simplicity, analyses from spectrum orange labeled locus-specific probes on chromosomal arms are referred to as analyses of chromosomal arms. Slides were dehydrated in 70%, 85%, and 100% ethanol at 22°C; denatured in 70% formamide at 72°C; dehydrated in 70%, 85%, and 100% ethanol at −20°C, and incubated overnight with the probe pairs described above. Slides were then washed in 2 × SSC at 72°C and 2 × SSC with 0.03% Tween (pH 5.3). Cells were covered with Anti-fade (Oncor, Inc.) containing 0.25 ng/μl diamindino-2-phenyl indole (Sigma), and slides were examined at ×100 under oil using an epifluorescence microscope equipped with a Hamamatsu C5810 charge-coupled device camera. For each sample and pair of probes, the number of red and green FISH spots was counted for 100 ± 6 nuclei (mean ± SD) by an observer blinded to the sample identity. Counting was repeated blindly on a subset of samples to evaluate reproducibility, which was determined to be within the expected statistical error.

Statistics. Differences in frequencies of abnormalities were estimated and tested for statistical significance using a marginal generalized linear model for cluster correlated data with an independence working correlation matrix and its associated generalized Wald test (9). An overdisperison parameter was included in the marginal model (10). The generalized Wald test was also used to test for differences in the patterns of FISH abnormalities. This test is similar to the commonly used χ^2 test for association between two categorical variables (11), but it also accounts for dependencies between observations from the same person. Abnormality categories with low expected counts were excluded for the last analysis.

ROCs were analyzed to determine the sensitivity and specificity of various FISH abnormalities to distinguish individual histologically negative rectal UC biopsies from control biopsies (11). The ROC shows that the trade off between sensitivity and specificity as the threshold for defining a "positive test" is varied. For ROC results based on more than one chromosome, the percentage of FISH abnormalities was calculated by pooling the counts over the chromosomes. The optimal cutoff was determined as that corresponding to the ROC point closest to the upper left corner of the plot. Cutoffs differ, depending on the abnormality.

RESULTS

The probability of finding an abnormal number of either centromere or arm FISH signals (any count other than two centromeres and two arms) was more than 1.9 times greater in histologically negative rectal biopsies from UC patients with dysplasia or cancer elsewhere in the colon (NegR) than in non-UC control biopsies (Fig. 1*a*). This difference was highly significant ($P < 0.0004$). Abnormalities increased further in frequency in HGD and CA. FISH abnormalities were seen less commonly in chromosome 8 than in the other three chromosomes ($P < 10^{-5}$). FISH changes in biopsies that were histologically IND, Neg, or NegR were, with three exceptions, highly variable in nature; no specific category of FISH abnormality (combination of arm and centromere counts of other than two of each) was represented in more than 20% of cells in a biopsy; there were an average of six categories of FISH abnormality per biopsy, with each seen, on average, in 3.2% of the cells; two of the three exceptions were biopsies that were aneuploid by flow cytometry (both Neg) that had more than 50% of cells trisomic for two or more chromosomes. Two HGD biopsies and one cancer biopsy also had more than half of the cells with a single abnormal centromere count (but only 47–58% of these had the same arm count).

Unequal Centromere and Arm Counts. The probability of finding a number of arm FISH signals that was different from the number of centromere FISH signals (Fig. 1*b*) was lower than the probability of finding any FISH abnormality (Fig. 1*a*) due to the fact that a significant number of abnormal FISH counts involved either the loss or gain of both a centromere and an arm (see Fig. 2). Again, the probabilities of unequal centromere and arm counts were significantly lower for chromosome 8 than for other chromosomes ($P < 10^{-5}$).

Abnormal Arm Counts. The pattern of increasing abnormal arm counts with increasing neoplastic progression (Fig. 1*c*) was generally similar to the pattern seen with any FISH abnormality (Fig. 1*a*), although lower in frequency. The difference between abnormal arm counts in NegR biopsies *versus* control biopsies remained highly significant ($P = 10^{-5}$). Chromosome 8 showed significantly fewer abnormal arm counts than the other chromosomes ($P = 0.0002$).

Chromosomal arm losses appeared more likely in early neoplastic progression, whereas gains more commonly occurred later (Fig. 1, *e* and *f*). Chromosomal arm losses were significantly more frequent in NegR ($P < 10^{-4}$) and Neg ($P = 0.0001$) UC biopsies than in non-UC control biopsies. However, losses of chromosomal arms did not increase further in biopsies with dysplasia or cancer. Loss of arms in UC appears to be chromosome specific: chromosome 8 was much less likely to have arm losses than chromosomes 11 or 18 ($P < 10^{-13}$); and chromosome 17 was more likely to have arm losses than chromosomes 11 or 18 ($P < 0.02$).

Gain of chromosomal arms was not as good a discriminator of changes in NegR UC biopsies as was loss of arms (note the difference in confidence intervals between Fig. 1*e* and 1*f*; see Table 1); however, the probabilities of chromosomal arm gains increased further in HGD and cancer (Fig. 1*e*). Chromosome 17 showed fewer arm gains than did the other chromosomes ($P < 0.01$).

Abnormal Centromere Counts. The probability of finding other than two centromeres by FISH (Fig. 1*d*) was less than the probability of finding other than two chromosomal arm markers ($P = 0.04$). Neither gain nor loss of a centromere was effective in discriminating NegR UC biopsies from control biopsies (Fig. 1*g* and *h*; Table 1). Centromere gains were more common in cancer, in dysplasia, and in the surrounding negative mucosa. Chromosome 11 was less likely to have lost a centromere than the other chromosomes ($P < 10^{-10}$), whereas chromosome 8 was less likely than other chromosomes to have gained a centromere ($P < 0.001$).

Patterns of FISH Abnormalities. The comparisons above address differences in the frequencies of classes of FISH abnormalities (*e.g.*, any abnormality, abnormal arm counts, and abnormal centromere counts). We also addressed the question of whether the distribution of specific abnormal FISH counts, as shown in Fig. 2, differed between chromosome probes. (It is possible, for example, for the frequencies of any abnormality to be the same for two chromosomes while the distributions [patterns] over the specific abnormalities differ). We used the Wald test (see "Materials and Methods") to examine differences in the pattern of abnormalities between chromosomes within the NegR category, and to examine differences in the patterns of abnormalities in NegR *versus* non-UC samples. Within NegR biopsies, the distributions of abnormalities for chromosomes 17 *versus* 11, 17 *versus* 18, and 11 *versus* 18 were all different from each other ($P = 0.002$, $P < 10^{-8}$, and $P = 0.0002$, respectively). The distribution of abnormalities was different in NegR *versus* non-UC controls within chromosomes 11 ($P < 10^{-10}$) and 17 ($P < 10^{-8}$).

Fig. 2 illustrates that the most significant abnormality in NegR biopsies was arm losses (1 arm-2 centromeres and 0 arm-2 centromeres), especially in chromosome 17, where 9% of NegR cells had loss of one arm of 17p, and 5% of cells had loss of both arms of 17p. As suggested by Fig. 1*f*, these arm losses did not increase further with increasing histological grade. Consistent with Fig. 1*g*, Fig. 2 also shows a pattern of increasing frequencies of centromere gains (0a3c, 2a3c, and 2a4c) in HGD and CA.

Sensitivity and Specificity of FISH Findings. The sensitivity and specificity of FISH abnormalities for distinguishing individual NegR UC biopsies from control biopsies were determined from ROCs see ("Materials and Methods"). Sensitivity in this context refers to the probability of a positive test result in an individual UC biopsy.

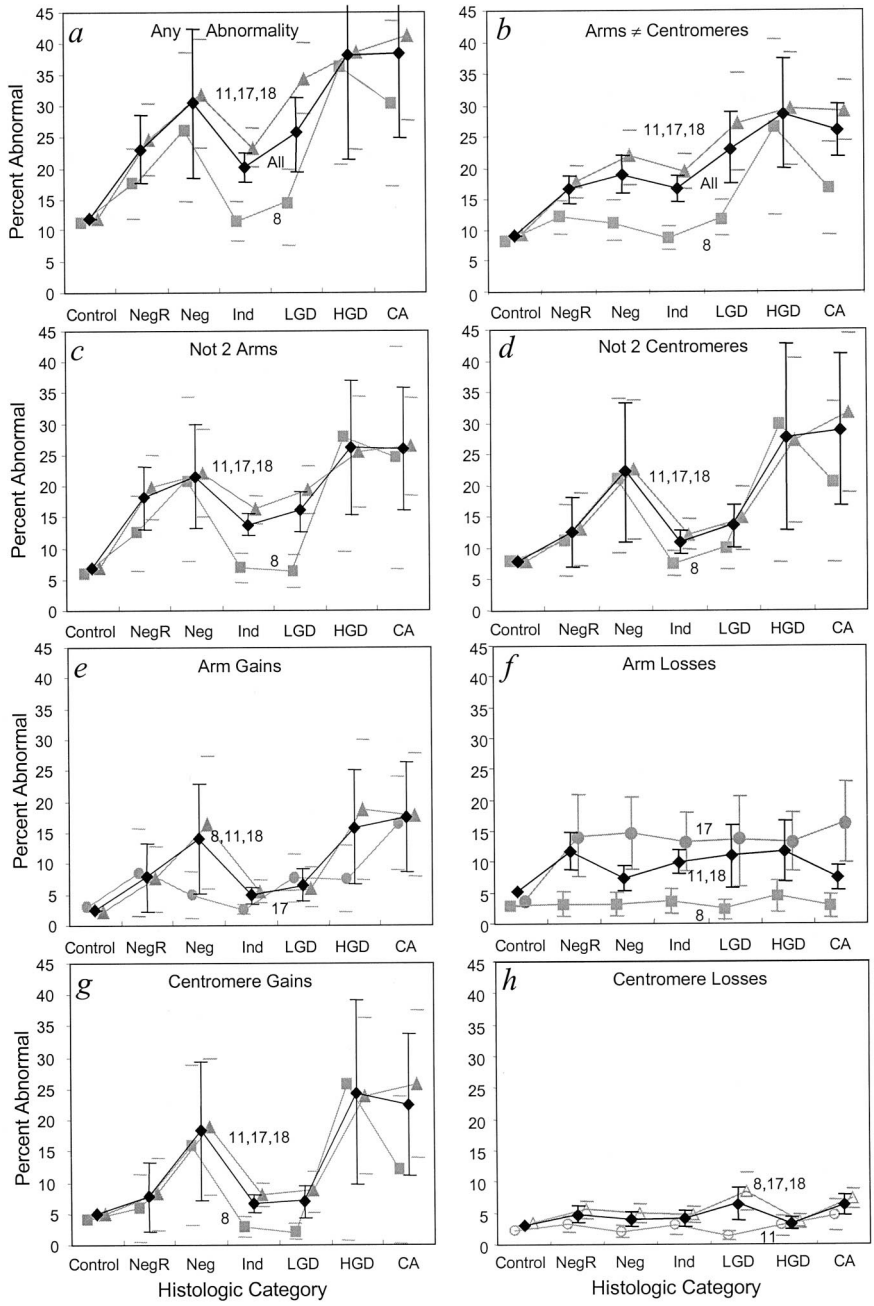

Fig. 1. The average percentage of cells with FISH abnormalities as a function of histology. Data are shown for categories of nuclei with other than two arm and two centromere FISH signals (any FISH abnormality, *a*); unequal numbers of centromere and arm FISH signals (*b*); other than two arm signals, ignoring centromeres (not two arms, *c*); other than two centromere signals, ignoring arms (not two centromeres, *d*); greater than two arm signals (arm gains, *f*); less than two arm signals (arm losses, *g*); greater than two centromere signals (centromere gains, *h*); and less than two centromere signals (centromere losses, *g*). The *error bars* show 95% confidence intervals for differences from non-UC controls. In addition to the average percentage of abnormal counts for all chromosomes taken together (◆), when the frequencies of FISH abnormalities in different chromosomes were statistically different from each other, those curves are shown separately, as indicated. For clarity, the category of all chromosomes is not shown in *f*.

Specificity refers to the probability that the test is negative in a control biopsy. Table 1 shows the optimum combinations of sensitivity and specificity derived from these curves. When the category of any FISH abnormality was considered, optimum sensitivity and specificity for separating individual NegR UC biopsies from control biopsies was derived from the analysis of chromosomes 11, 17, and 18, consistent with the results shown in Fig. 1. The ROC analysis also showed that centromere counts are not as sensitive or specific as arm counts for discriminating NegR UC from non-UC controls. In fact, arm counts from the combination of chromosomes 11, 17, and 18 fully separated individual NegR UC biopsies from control biopsies (sensitivity and specificity = 1.0): all NegR biopsies had more than 11% abnormal counts for pooled chromosome 11, 17, and 18 arm markers; and all controls had less than this. Arm counts from chromosome 17 alone were almost as good (sensitivity and specificity = 0.9; Table 1). Consistent with Fig. 1, arm losses were much better discriminators than gains.

DISCUSSION

It is postulated that UC cancer is derived from a field of precursor cells, based on the presence of dysplastic mucosa that surrounds and abuts the cancer. Abnormal fields of an even larger extent have been demonstrated based on flow cytometric aneuploidy, CGH, and mutated p53 (5, 6, 12). However, each of these methods can only detect abnormalities when clonal expansion produces an appreciable subpopulation of cells with a uniform change. FISH can detect nonclonal chromosome abnormalities, and a previous report suggests that these indeed may be found in UC mucosa that does not show abnormalities by ploidy or CGH (12). These authors also found that centromeric FISH abnormalities were present in nondysplastic UC epithelium, a result that we confirm and extend. In this report, the application of interphase FISH demonstrates that the process of chromosomal instability is seen throughout the entire colon, including tissue in the most distal and nondysplastic rectum: the abnormal field is essentially the

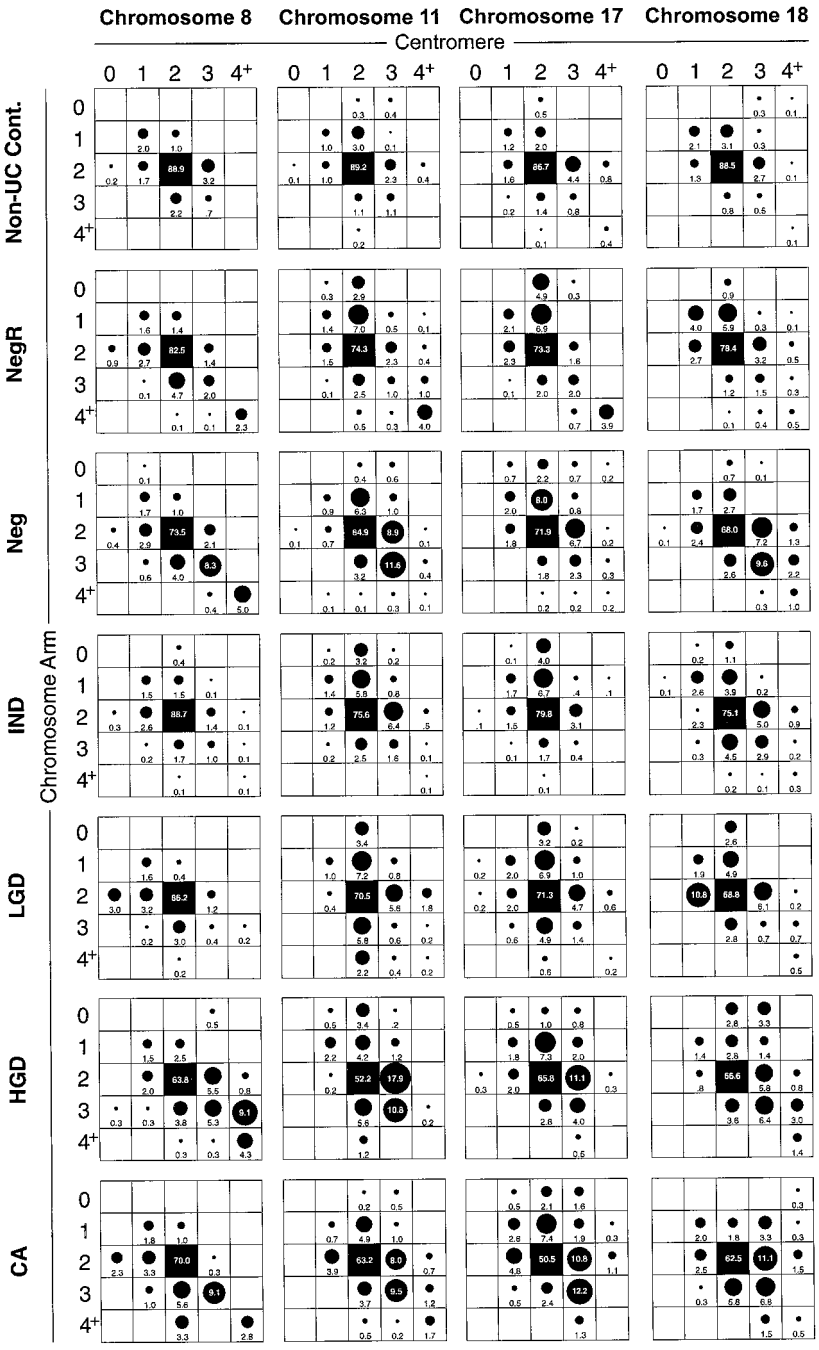

Fig. 2. Summary of FISH counts according to histological category (*rows*) and chromosome examined (*columns*). In each position, a five-by-five matrix shows the percentage of nuclei with each combination of chromosomal arm FISH counts (vertical dimension) and centromere counts (horizontal). The area of each circle is proportional to the percentage of cells seen with that count; the percentage is shown in small numbers. The percentage of cells with normal (*2a2c*) counts is shown within the ■. For example, in the *top left matrix*, for chromosome 8, non-UC control cells had on average 88.9% 2a2c counts and 3.2% 2a3c counts. In the Neg category, almost all of the 3a3c counts, almost all of the chromosome 8 4a4c counts, and approximately half of the 2a3c counts were due to two cases in which the majority of cells had the same aneusomy, as described in "Results."

entire organ. The CIN involves diverse changes in multiple chromosomes (Fig. 2), with loss of chromosomal arms being the most frequent early event (Fig. 1*f*). In lower histological grades, most individual biopsies showed diverse FISH abnormalities, suggesting that CIN was either random or involved clonal outgrowths that comprised only a small fraction of the biopsy. Based on these findings, we hypothesize that neoplastic progression in UC begins with CIN and random chromosomal arm losses over a very large field of cells; some of these will result in the loss of tumor suppressor loci, with subsequent clonal expansion and neoplastic evolution, as suggested by Nowell (13). The initial CIN would be consistent with the hypothesis initially formulated by Loeb (14) that a mutator phenotype contributes to the accumulation of genetic alterations in cancer.

High rates of CIN have previously been found in aneuploid colon cancer cell lines and been termed the CIN phenotype (7). The frequency of FISH abnormalities that we observe in UC cancer and HGD are similar to those reported previously in CIN cell lines. Diploid colon cancer cell lines are not reported to show this phenotype but are typically deficient in DNA mismatch repair, leading to the microsatellite instability phenotype (7, 15). We observe more FISH abnormalities in diploid UC biopsies that are Neg and IND than have been reported for diploid (microsatellite instability) cancer cell lines, suggesting that the preneoplastic cells in UC exhibit the CIN phenotype well before aneuploidy develops. Flow cytometric aneuploidy was present in two of seven Neg, one of four HGD, and three of four cancer biopsies. When aneuploid biopsies were removed from the analysis, no differences in overall trends were observed, except that the probability of FISH abnormalities was lower in the one diploid cancer biopsy (data not shown). Notably, the finding of increasing probabilities of FISH abnormalities in higher grades of dysplasia and

Table 1 *Sensitivity and specificity of FISH for discriminating negative rectal UC from non-UC control biopsies*

FISH result	Sensitivity	Specificity
Any abnormality		
All chromosomes	1.0	0.9
Chromosomes 11, 17, and 18	1.0	0.9
Chromosome 17 only	0.8	0.9
Chromosome 18 only	0.9	0.7
Chromosome 11 only	0.9	0.9
Abnormal arm count		
All chromosomes	0.9	1.0
Chromosomes 11, 17, and 18	1.0	1.0
Chromosome 17 only	0.9	0.9
Chromosome 18 only	0.8	0.9
Chromosome 11 only	0.6	1.0
Arm loss		
All chromosomes	0.8	1.0
Chromosomes 11, 17, and 18	0.9	1.0
Chromosome 17 only	0.8	1.0
Chromosome 18 only	0.6	0.9
Chromosome 11 only	0.6	0.8
Arm gain		
All chromosomes	0.4	0.8
Chromosomes 11, 17, and 18	0.3	0.8
Chromosome 17 only	0.3	0.9
Chromosome 11 only	0.4	0.9
Chromosome 18 only	0.5	0.7
Abnormal centromere count		
All chromosomes	0.4	0.8
Chromosomes 11, 17, and 18	0.4	0.8
Chromosome 17 only	0.4	0.8
Chromosome 18 only	0.6	0.8
Chromosome 11 only	0.4	0.9
Centromere loss		
All chromosomes	0.8	0.8
Chromosomes 11, 17, and 18	0.6	0.8
Chromosome 17 only	0.6	0.7
Chromosome 18 only	0.8	0.9
Chromosome 11 only	0.5	0.8
Centromere gain		
All chromosomes	0.1	1.0
Chromosomes 11, 17, and 18	0.5	0.2
Chromosome 17 only	0.3	0.3
Chromosome 18 only	0.5	0.7
Chromosome 11 only	0.4	0.3

more frequent centromere and arm gains, rather than losses, in dysplastic as compared to negative biopsies (Fig. 1) persisted in diploid samples. We suggest that this progression is a feature of the developing CIN phenotype in UC and that aneuploidy is a secondary event.

Although the FISH methodology is subject to some counting errors (for example, overlapping FISH spots counted as one), these are expected to be similar in control and UC samples. On average, 11% more cells in NegR UC biopsies and 19% more cells from Neg UC biopsies near dysplasia or cancer showed FISH abnormalities than controls (Fig. 1*a*, ◆). Loss of one or more copies of 17p was the single most common abnormality in NegR UC biopsies; it was present in 10.5% more cells than in controls (Figs. 1*a* and 2). Almost half of this excess (4.7%) involved loss of both short arms of chromosome 17 (Fig. 2); loss of both copies of p53 would, by itself, be considered a contribution to cancer risk. If the four chromosomes measured in this report were typical, each cell in the rectal UC biopsies would have, on average, almost three chromosomal aberrations. Because these may not be clonal, they might not necessarily involve the crypt stem cells, in which case, chromosomal aberrations would have to be accumulated at high frequency as the cells move up from the crypt base to surface, where they are sloughed. Measuring chromosomal abnormalities as a function of location in the crypt would require FISH on histological sections; this method, however, yields frequent artifacts due to cut and overlapping nuclei (16).

The high rates of CIN in histologically negative UC epithelium suggest that UC colonocytes are subject to high rates of genetic damage, chromosomal breakage, mitotic infidelity, or all three. UC has been associated with elevated levels of reactive oxygen species and reduced oxidative defenses, both of which might contribute to genetic damage (17–20). Telomeres have been reported to be shortened in histologically negative UC biopsies (21), presumably because of both higher proliferative rates and increased oxidative damage in UC.

Because one function of telomeres is to reduce the fusigenic potential of chromosome ends, shortened telomeres can contribute to CIN via end-to-end chromosome fusion and consequent nondisjunction or bridge-breakage-fusion (22). In fact, 17p has been reported to have the shortest telomeres of any human chromosome (23); although speculative, this might be consistent with our observation that the highest rates of chromosomal arm loss were on 17p. Abnormalities in the fidelity of the mitotic apparatus have been suggested to be important in sporadic colon cancer, including damage to mitotic checkpoints (24). Whereas the high frequency of observed CIN would require improbably high mutation rates in mitotic regulatory or structural genes, it is possible that epigenetic changes, such as methylation, could adversely affect the genes responsible for mitotic fidelity. Overexpression of pericentrin, for example, is frequent in cancers and can result in chromosome missegregation and breakage (25).

The ability of FISH to distinguish individual NegR UC biopsies (obtained from patients with dysplasia or cancer elsewhere in their colon) from controls with 100% sensitivity and specificity using three probes (11q, 17p, and 18q; Table 1) suggests that risk assessment of individuals may be possible by FISH analysis of rectal biopsies. In a preliminary study of eight UC patients who have not developed dysplasia over an average 15-year duration of disease, FISH counts for chromosomes 17 and 11 were no different than those seen in non-UC controls ($P = 0.36$ and 0.73, respectively) and were very different from NegR biopsies in patients with HGD or cancer ($P < 0.0001$).[5] Because we studied UC patients in this report only at the time of diagnosis of dysplasia or cancer, it remains to be proven that CIN can be similarly detected preceding dysplasia and cancer in UC; however, this would be most consistent with both the pancolonic distribution of CIN that we observed and the hypothesis that the process of CIN underlies progression to cancer. If FISH examination of rectal biopsies can indeed identify the subset of UC patients at risk for dysplasia or cancer, this could have considerable impact on the medical management of these patients. It would also give strong impetus to the development of therapeutic strategies to reduce CIN before it progresses to dysplasia or cancer.

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