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Genomic Instability Is an Early Event during the Progression Pathway of Ulcerative-Colitis-Related Neoplasia

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Ulcerative colitis (UC) is a chronic inflammatory disease of the colon associated with a high risk of colorectal cancer. This increased cancer risk is thought to result from the cellular damage induced by the inflammatory field. The aim of this study was to determine the pattern and time course of genomic instability occurring in UC-related neoplasia. Sites of cancer, dysplasia, and nondysplasia from 14 UC colectomy cases containing cancer were analyzed for chromosomal alterations by comparative genomic hybridization (CGH) and for microsatellite instability using a series of 10 microsatellite markers. Clonal chromosomal alterations were present in 85% of cancer sites, 86% of dysplasia sites, and 36% of nondysplasia sites. Losses of chromosome 18 or 18q and chromosome 5 or 5q were common in cancer and dysplasia and were occasionally detected in nondysplasia. High-level microsatellite instability was detected in the cancer and dysplasia of two cases. Samples that demonstrated high-level microsatellite instability were unlikely to have chromosomal alterations demonstrable by CGH. These studies suggest that the predominant type of genomic instability in UC-related neoplasia is associated with chromosomal alterations and that this type of genomic instability frequently occurs before the development of histologically defined dysplasia. *(Am J Pathol 1999, 154:1825–1830)*

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon associated with a high risk of colorectal cancer.[1] Colorectal cancer in individuals with UC appears to develop through a multistep process involving genomic instability and the progressive accumulation of genetic alterations, similar to that seen in sporadic colorectal cancer. Unlike sporadic colorectal cancer, which involves the adenoma to carcinoma sequence, UC-related cancers most often arise from flat, dysplastic epithelium that appears grossly identical to adjacent nondysplastic epithelium.

Genomic instability is a fundamental property of neoplastic cells. Different types of genomic instability appear to be involved in the two main neoplastic progression pathways described for sporadic colorectal cancer.[2,3] In the predominant pathway, which accounts for more than 85% of sporadic tumors, genomic instability is evident by the presence of multiple chromosomal alterations. These abnormalities have been detected by cytogenetics, comparative genomic hybridization (CGH), and flow cytometry. This neoplastic progression pathway is frequently associated with alterations in the adenomatous polyposis coli (APC) pathway and with mutations of p53. The other neoplastic progression pathway involves genomic instability that is manifested as errors in DNA replication, usually detected as microsatellite instability (MSI), having an underlying mechanism of deficiency in DNA mismatch repair. Sporadic colon cancers that display replication errors are thought to be the result of somatic mutations in a DNA mismatch repair gene rather than the inheritance of a germline mutation as seen in the hereditary nonpolyposis colon cancer.

It is unclear when genomic instability occurs in the UC-related histological progression pathway from nondysplasia to dysplasia to cancer. Several studies suggest genomic instability occurs early in this histological pathway and may precede the histological development of dysplasia. We recently reported significant chromosomal derangement in nondysplastic epithelium from colectomy specimens containing UC-related cancer.[4]

The aim of this study was to determine the pattern and time course of genomic instability occurring in UC-related neoplasia. We hypothesized that genomic damage occurs before the development of histologically defined dysplasia. This is based on a model in which the sequence of genomic instability, accumulation of genetic abnormalities, clonal expansion, and progression paral-

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lels the simultaneous histological progression from nondysplasia to dysplasia to invasive cancer.

Materials and Methods

Tissue

Fourteen colectomy cases from patients with UC-related colon cancer were identified from the University of California San Francisco (UCSF) Department of Pathology archives. Separate regions, usually on separate blocks, of cancer, dysplasia, and nondysplastic UC-involved epithelium were selected for each case. The histological diagnosis of dysplasia was made using standard criteria.[5] The degree of inflammation was evaluated for each region, and sites were included only if the presence of inflammation did not interfere with histological grading. Dysplasia was high grade in three cases (1, 2, and 9) and low grade in the remainder. Blocks of normal surgical margins from 7 colon tissue samples removed for diverticular disease served as normal controls, as did one block each from 10 colectomy cases from patients with short-term UC (less than 8 years duration, having a low risk for neoplasia). This study was approved by the UCSF Committee on Human Research.

DNA for CGH and MSI analysis was extracted from thin paraffin sections as previously described.[4] Briefly, regions of interest were microdissected from one to two methyl-green-stained slides and subjected to 3-day proteinase K treatment. DNA was extracted from colonic muscularis propria from each case to define normal allelic ratios.

Comparative Genomic Hybridization

In CGH, total genomic DNA from the colonic sample and human genomic DNA from a normal donor, detected in different colors, are simultaneously hybridized to a normal metaphase spread.[4] The ratio of the colors along the normal chromosomes provides a quantitative map of the relative copy number of DNA sequences in the test sample. Thus, the entire genome can be surveyed in a single step without the need to first select which genetic loci to test.

Amplification of the microdissected DNA was by degenerate oligonucleotide primed (DOP) polymerase chain reaction (PCR) as previously described.[4] DOP PCR is a reliable means of whole genome amplification of DNA. Each PCR run included controls of normal genomic DNA and the MPE-600 cell line. Microdissected DNA (1 to 2 μl) yielded up to 1 μg of PCR product, averaging 400 bp in size (range, 200 bp to 2 kb). Fifty nanograms of fresh DNA (normal and MPE600 cell line) resulted in approximately 2 to 3 μg of amplified DNA.

For normal reference DNA, 20 μl of PCR-amplified DNA was labeled by nick translation with Texas Red-5-dUTP or with Fluorescein-12-dUTP (Dupont, Boston, MA) per 50-μl reaction. Forty microliters of amplified DNA from paraffin sections was used per 50-μl reaction and was labeled with fluorescein-12-dUTP (Dupont) or digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). The optimal size for CGH was 500 to 2000 bp. Nick-translated PCR products were close to the original PCR product size.

Each sample was hybridized as previously described, in duplicate, with different fluorochromes, onto normal male metaphases.[4] Successful hybridizations were judged by the intensity of the tumor and normal signals, by the granularity *versus* smoothness of the signals, by the homogeneity of the signal over the entire metaphase, and by the banding intensity of the DAPI signals used for chromosome identification. Acquisition was performed using our QUIPS analysis system.[6]

High-level amplifications were defined as a peak of the ratio intensity above 2.0, involving less than a whole chromosome arm. Low-level gain or loss was defined as chromosome regions having a ratio above 1.25 or below 0.8. Inverse CGH, using a second hybridization with reversed color labels, allowed greater confidence in making these interpretations. The inverse pair was examined together to allow better discrimination of significant changes. All changes must have been present in both the forward and inverse hybridizations. Interpretation of changes at 1pter, 19, and 22 (and 4 and 13q in the opposite direction) required careful examination of all chromosome profiles, because these loci were likely to show more variability in their ratios.

Microsatellite Instability Analysis

Ten microsatellite loci distributed throughout the genome were examined. Microsatellites of polyA (BATRII, BAT40, and BAT26), dinucleotide (D2S123, D7S507, D18S58, and D16S423), tetranucleotide (D16S539 and D3S2405), and pentanucleotide repeats (D16S476) were used.

The total reaction volume was 25 μl, consisting of 0.1 μl of DNA, 0.125 μl of TaqGold (0.625 U; Perkin Elmer, Norwalk, CT), 2.0 μl of dNTP (200 μmol/L; Boehringer Mannheim), 2.5 μl of 10X PCR buffer (Perkin Elmer), fluorescently labeled primer pairs (Perkin Elmer), $MgCl_2$ (Perkin Elmer), and dH_2O. Primer pairs were sometimes multiplexed in the PCR. Samples were processed at 95°C for 12 minutes, then through 34 cycles at 95°C for 45 seconds, 50°C for 45 seconds, 72°C for 1 minute, and a final extension at 72°C for 7 minutes after the last cycle. The concentration of $MgCl_2$, the annealing temperature, and the time of final extension were optimized for each primer pair.

Fluorescently labeled PCR products were analyzed using the Applied Biosystems (Foster City, CA) 377 sequencer. A positive result was defined as a shift of any magnitude in PCR product length compared with the patient's normal muscle DNA run on the same gel. Samples that demonstrated MSI were categorized as either high-level MSI (MSI-H, having four or more positive markers) or low-level MSI (MSI-L, having one to three positive markers) based on recently established guidelines.[7] Samples with no positive markers were termed microsatellite stable (MSS).

Table 1. Clinical Information

Case	Sex	Age (years)	Duration (years)	Extent of disease	Stage	Grade	Sampling sites		
							Nondysplasia	Dysplasia	Carcinoma
1	Male	38	20	Hepatic flexure	T1N1M0	G1*	Midtransverse colon	Rectum	Rectum
2	Male	36	17	Pancolitis	T3N2M0	G3	Proximal margin (36 cm from CA)	Right colon	Right colon
3	Male	60	11	Pancolitis	T2N0M0	G1	Proximal margin (23 cm from CA)	Right colon	Right colon
4	Male	26	9	Pancolitis	T2N0M0	G2	Transverse colon (26 cm from CA)	Rectum	Rectum
5	Female	60	23	Pancolitis	T3N0M0	G2	Right colon (126 cm from CA)	Right colon (116 cm from CA)	Rectum
6	Female	27	13	Left sided	T1N1M0	G2	Distal margin (8 cm from CA)	Rectum	Rectum
7	Male	70	30	Pancolitis	T1N1M0	G2	Descending colon (45 cm from CA)	Ascending colon	Ascending colon
8	Male	67	20	Pancolitis	T3N2M0	G3	Sigmoid	Sigmoid	Sigmoid
9	Male	65	12	Pancolitis	T3N0M0	G2	Ascending colon	Ascending colon (8 cm distal CA)	Ascending colon
10	Male	30	12	Pancolitis	T3N0M0	G3	Ascending colon	Ascending colon	Ascending colon
11	Male	63	25	Left sided	T2N0M0	G2	Sigmoid (2 cm from CA)	Sigmoid (2 cm from CA)	Sigmoid
12	Female	26	Unknown	Pancolitis	T2N0M0	G1	Proximal margin (50 cm from CA)	Proximal margin (50 cm from CA)	Descending colon
13	Male	37	10	Pancolitis	T3N3M1	G1	Right colon	Right colon	Right colon
14	Male	42	25	Pancolitis	T3N1M0	G3	Rectum	Rectum	Rectum

Staging was according to TNM-Atlas 4th edition 1997 (Ref. 31). CA, carcinoma.
*G1, highly differentiated; G2, moderately differentiated; G3, poorly differentiated.

Results

Fourteen colectomy specimens from patients with UC-related cancer were analyzed for chromosomal alterations by CGH and for MSI. The clinical information is summarized in Table 1. The average age at the time of surgery was 46.2 years, and the average duration of disease was 17.5 years.

Chromosomal Alterations by Comparative Genomic Hybridization

Relative chromosomal losses and gains were identified by CGH analysis of microdissected sites of cancer and dysplastic and nondysplastic UC-involved epithelium from each of the 14 UC-related colectomy specimens. Chromosomal abnormalities detected by CGH are summarized in Table 2. CGH results of cases 1 to 5 have been reported previously.[4] CGH of epithelium from 10 low-risk colitis patients (disease duration less than 8 years) and 7 normal diverticulitis samples did not reveal any chromosomal aberrations by CGH.

CGH-detectable chromosomal alterations were present in 85% of cancer sites, 86% of dysplasia sites, and 36% of nondysplasia sites. Only two sites of cancer (cases 9 and 12) and two sites of dysplasia (cases 5 and 12) did not have CGH-detectable chromosomal alterations. The mean number of chromosomal alterations per site was 8.0 for cancer and 5.3 for dysplasia. In the five nondysplastic sites with CGH-detectable alterations, the mean number of alterations was 3.6 per site. One site of cancer (case 4) yielded an insufficient amount of DNA, resulting in a small PCR product that was inadequate for CGH.

The most common alterations seen were deletions involving chromosomes 18 and 5. Loss of the entire chromosome 18 or just 18q was present in 69% of cancers and 43% of the dysplasias, whereas loss of chromosome 5 or 5q was present in 54% of cancers and 36% of the dysplasias. Of the five nondysplastic sites having at least one chromosomal abnormality, two had loss of chromosome 18 or 18q and two had 5q loss (case 2 had both 5q and 18q losses, in addition to 19p gain). Gain of chromosome 8 or 8q was the next most frequent, present in 38% of cancers and 36% of dysplasias. Chromosome 20 or 20q gain was seen in 38% of the cancers and 21% of dysplasias.

In some cases, similarities were seen between the changes detected in the cancer and the dysplasia. For example, cases 6, 10, 13, and 14 showed high concordance between the chromosomal alterations in the cancer and the dysplasia in the same case. In case 2, loss of both 5q and 18q were seen in all three tissue sites. A statistical model[8] to define nonrandom similarities (data not shown) suggests that changes in cancer and dysplasia within case pairs show significantly greater similarity than do non-case pairs.

Microsatellite Instability

Analysis for MSI was performed from the same DNA used for CGH analysis. Ten microsatellite loci of different repeat lengths were used. The results of MSI analysis are shown in Table 2. Only two cases (cases 9 and 12) demonstrated MSI-H (as defined by more than four positive markers). In both of these cases, MSI-H was present in both the dysplasia and cancer. One case (11) demonstrated MSI-L (as defined by one to three positive markers). In this case, only the dysplasia had a single positive marker (BAT40). The microsatellite analysis for the three cases demonstrating MSI-H or MSI-L are summarized in Table 3. There were no positive markers in the nondysplasia of any of the cases.

Table 2. Genetic Alterations in UC-Related Neoplasia

Case	Nondysplasia		Dysplasia		Carcinoma	
	CGH	MSI	CGH	MSI	CGH	MSI
1	No changes	MSS	−11, 18q12-qter−	MSS	2pter-p21++, 5q13-qter−, 8p−, 12p12-q13.1+, −17, 18q−, −Y	failed
2	5q−, 18q−, 19p+	MSS	5p+, 5q−, 8q23-24.1+, 18q−	MSS	5q−, 8p12-pter−, 13q+, 18q−, +20	MSS
3	+19	MSS	−18, +X	MSS	−18, +X	MSS
4	No changes	MSS	5q−, 8q21.1-qter+, 12q13-qter−, −17	MSS	Inadequate	MSS
5	No changes	MSS	No changes	MSS	18q−	MSS
6	No changes	MSS	1q31-32.3−, 4pter-q28−, 5q31.1-qter, 14q−, −16, −17, 22q−	MSS	1q31-32.3−, −3, −4, 5q31.1-qter−, 10p+, 11p+, 13q+, −16, 17q−, 18q−, 22q−	MSS
7	No changes	MSS	+7, 8p21.3-p11+, 8q+, +9, 12pter-q22+, 13q+, +19	MSS	+6, +7, 8q+, 13q+, 17p−, −18, +20, +X	MSS
8	No changes	MSS	+7, 9p21-pter−, 13q+, 14q+, −17, +20, +X	MSS	−3, 4p−, 5q−, 6q−, +7, 8pter-q22.3−, 8q23-qter+, 9p21-pter−, 10pter-q24.3−, 10q25.1-qter+, 11q−, 13q+, 14q+, +19, Xp−	MSS
9	No changes	MSS	6q16.1-22.2−, 12p12.3-pter−	MSI-H	No changes	MSI-H
10	−4, 5q−, +9, 11q−	MSS	1q+, 3q22-qter+, 4q−, 5q−, +7, 8q21.1-qter+, 9p+, −10, 11q14.1-22.3+, 13q+, 14q11.2-24.3+, −17, 18q−, 20q+, 21q−, 22q−, −Y	MSS	3q22-qter+, 4q−, 5q−, +7, 8q21.1-qter+, 9p+, 11q14.1-22.3+, 13q+, 14q11.2-24.3+, −17, 18q−, 20q+, 21q−, 22q−, −Y	MSS
11	9p+, −12, 13q+, 15q−, −17, −18, +X, +Y	MSS	17q−	MSI-L	−1, 3q−, 4q21.1-qter−, −5, 7p+, 8p21.1-pter−, −8p12qter+, 9p−, 11p+, −17, −18, 20q+, 21q11.2-21−, −Y	MSS
12	No changes	MSS	No changes	MSI-H	No changes	MSI-H
13	No changes	MSS	+8, 18q−	MSS	+8, 18q−	MSS
14	+7, +8	MSS	−4, 5p+, 5q−, 7p+, 8p−, +9, 15q−, −18, +20	MSS	−4, 5p+, 5q−, 8q+, 15q26-qter+, +20, +Y	MSS

Discussion

Despite its origin in a chronic inflammatory field, UC-related neoplasia exhibits the same pattern of genomic instability that predominates in sporadic colorectal neoplasia[2]: gross chromosomal alterations. Of the 14 cases analyzed, only two tumors and one dysplasia did not demonstrate clonal chromosomal alterations by CGH. This similarity with sporadic tumors suggests that neoplastic progression in UC-related and sporadic neoplasia may involve similar genetic events. In fact, alterations in p53,[9–11] K-*ras*,[12–14] and APC[11,13,15] have all been reported in UC-related neoplasia. Also, like sporadic cancer, UC-related neoplasia appears to progress histologically through a model of clonal expansion. This is supported by our finding of genetic similarities between the cancer and dysplasia from the same case, suggesting the existence of a common precursor.

Where UC-related neoplasia appears to differ from sporadic colorectal neoplasia is in the timing of the development of genomic instability in relation to histological progression. In sporadic colon cancer, the earliest histologically definable lesions are aberrant crypt foci (ACF), which appear to be precursor lesions to adenomas.[16] Clonal genetic alterations in K-ras and APC can be demonstrated in ACF but are extremely rare in histologically normal mucosa from colectomy specimens containing sporadic cancer.[17,18] However, genetic alterations appear to be common in nondysplastic epithelium from UC colectomy specimens containing cancer. We previously reported chromosomal analysis of a series of five UC colectomy cases containing cancer.[4] In that study, nondysplastic epithelium was abnormal in all five cases when analyzed by fluorescence *in situ* hybridization analysis for copy number using a panel of five chromosomal probes.

Table 3. Microsatellite Instability

Case	Histology	Poly-A			Dinucleotide				Tetra		Penta, D16S476
		BATRII	BAT40	BAT26	D2S123	D18S58	D7S507	D16S423	D16S539	D3S2405	
9	Nondysplasia	−	−	−	−	−	−	−	−	−	−
	Dysplasia	−	+	+	−	+	+	+	+	+	−
	Carcinoma	−	+	+	−	−	+	+	−	+	−
11	Nondysplasia	−	−	−	−	−	−	−	−	−	−
	Dysplasia	−	+	−	−	−	−	−	−	−	−
	Carcinoma	−	−	−	−	−	−	−	−	−	−
12	Nondysplasia	−	−	−	−	−	−	−	−	−	−
	Dysplasia	−	+	+	+	+	+	−	+	+	+
	Carcinoma	+	+	+	−	+	+	−	−	−	−

In addition, two of the five cases demonstrated CGH changes in nondysplastic epithelium. This current report of a larger series of cases confirms our previous findings. CGH detected clonal chromosomal alterations in nondysplasia in 5 of 14 (36%) cases (3 of 9 additional cases studied).

Our findings of genetic alterations in nondysplastic UC-involved epithelium is consistent with previous studies. Aneuploidy was occasionally found in nondysplastic epithelium from colectomy specimens containing cancer.[10,19,20] There have also been two reports of aneuploidy preceding the development of dysplasia in patients followed prospectively in cancer surveillance programs.[19,20] In addition, there have been reports of p53[21] and K-*ras*[22] mutations in nondysplastic UC-involved epithelium. These studies together with our findings support the hypothesis that genomic instability and clonal alterations occur before the development of histologically defined dysplasia in the progression pathway from nondysplasia to dysplasia to cancer in UC-related neoplasia. The high incidence of genomic instability in nondysplastic epithelium from UC colectomy cases containing cancer as opposed to the extremely low incidence seen in colectomy specimens containing sporadic cancer may be due to the chronic inflammatory field leading to differences in carcinogenesis. Alternatively, histology may be an insensitive measure to detect early neoplastic changes in the setting of chronic inflammation.

This report confirms our preliminary finding of a high incidence of loss of chromosome 18 or 18q in UC-related neoplasia.[4] Loss of chromosome 18 or 18q was present in 69% of cancers, 43% of dysplasias, and in two of five nondysplastic sites demonstrating CGH changes. 18q21 is the location of three putative tumor suppressor genes, *DCC*,[23] *SMAD4*,[24] and *SMAD2*,[25] suggesting that one or more of these genes may play an important role in UC-related carcinogenesis. In sporadic colorectal cancer, allelic loss of 18q is thought be a later event and is associated with metastatic disease.[26] In UC, the frequent loss of 18q in dysplasia and occasionally in nondysplasia suggests this may be an important early event in UC-related neoplasia. We also found frequent loss of chromosome 5 or 5q in cancers (54%) and dysplasias (36%). The role of APC in UC-related neoplasia has not been clearly defined, but our findings are consistent with other reports of allelic loss at APC.[11,15]

Two of the fourteen cases demonstrated a high level of MSI (MSI-H) as defined by 4 or more of the 10 markers assayed being positive. This recently proposed definition of high-level MSI[7] is a more restrictive definition of MSI than has been used in many previous studies involving colorectal neoplasia. These two cases (9 and 12) most likely developed through a pathway of deficient DNA mismatch repair. Interestingly, the dysplasia of case 9 demonstrated only two partial chromosomal losses, and the remainder of the sites in both MSI-H cases were normal by CGH. This is consistent with previous reports demonstrating a low incidence of chromosomal alterations in tumors associated with disordered DNA mismatch repair.[27,28] Although we did not assess DNA mismatch repair genes directly, MSI-H has been shown to be highly correlated with loss of mismatch repair gene (*hMSH2* or *hMLH1*) expression whereas MSI-L (one to three markers positive) was highly correlated with normal mismatch gene expression.[7] Other investigators have reported a higher frequency of MSI in UC-related neoplasia than we have found,[11,29] although many of the tumors and dysplasias analyzed in these reports were positive in only one marker. In one study of 68 tumor samples, only five tumors demonstrated MSI in more than one marker.[29] MSI-L in these studies might reflect a stochastic allelic alteration that is locked in by tumor clonality[30] rather than disordered DNA mismatch repair. It is possible that our microsatellite analysis may have failed to detect MSI in some samples (ie, false negatives), but using a panel of 10 microsatellite markers minimized the probability of this.

CGH and microsatellite analysis allow one to address the role of biological selection in neoplastic progression by comparing the cancer to the histological precursor (dysplasia). In this study, the clonal relationship of the cancer and the dysplasia is not entirely clear. A statistical model showed that changes in cancer and dysplasia within case pairs showed greater similarity than non-case pairs. In addition, there was considerable overlap of abnormal microsatellite loci in cancer and dysplasia in the two cases that demonstrated MSI-H. This suggests that the cancer and dysplasia within a case may have evolved from a common precursor. However, it is possible that in many cases the cancer and dysplasia are the result of independent, clonally unrelated areas of neoplastic progression in a high-risk epithelium.

The pattern of genomic instability that predominates in UC-related neoplasia is associated with multiple chromosomal alterations. The type of genomic instability associated with high-level MSI appears to be present in a small minority of UC-related dysplasias and cancers. These represent new findings over our previous work. In many cases of UC-related neoplasia, genomic instability appears to precede the development of histologically defined dysplasia. This confirms our preliminary report demonstrating chromosomal alterations in nondysplastic epithelium.[4] This may represent a distinct difference from neoplastic development in sporadic colorectal neoplasia. The presence of clonal genetic abnormalities in nondysplastic epithelium in patients with UC-related neoplasia may be especially relevant to development of cancer surveillance tests and chemoprevention trials in this high-risk patient group.

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