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ALLELIC LOSSES AT GENOMIC INSTABILITY-ASSOCIATED LOCI IN VILLOUS ADENOMAS AND ADJACENT COLORECTAL CANCERS

Bruce M. Brenner^{a,*}, Daniel L. Stoler^b, Luz Rodriguez^c, Matthew J. Karpenko^d, Helen Swede^e, Nicholas J. Petrelli^f, and Garth R. Anderson^g

^a Division of Surgery, University of Connecticut Health Center, Farmington CT

^b Departments of Head and Neck Surgery and Pathology, Roswell Park Cancer Institute, Buffalo NY

^c Genetics Branch, National Cancer Institute/NNMC, Bethesda MD

^d Department of Internal Medicine, The Ohio State University Medical Center, Columbus OH

^e Neag Cancer Center, University of Connecticut Health Center, Farmington CT

^f The Helen F. Graham Cancer Center, Wilmington, DE

^g Department of Cancer Biology, Roswell Park Cancer Institute, Buffalo NY

Abstract

Allelic imbalances in premalignant villous adenomas were compared with those in adjacent microdissected colorectal carcinoma that had arisen directly from the adenomas. Carcinoma-adenoma pairs were examined from seventeen patients who underwent resections for colorectal cancer. Twenty-eight microsatellite markers were examined, from regions of the genome where individual allelic losses have been associated with overall genomic instability in colorectal carcinomas. Microsatellite instability was also evaluated for each marker in each tissue type. Thirty-five percent of adenomas and sixty-five percent of carcinomas demonstrated loss of heterozygosity (LOH) for multiple markers; the average fractional allelic loss rate was 2.5 times higher in carcinomas than in adenomas. Four of the 17 patients had microsatellite instability for more than 30% of markers in both their adenomas and carcinomas, with no significant differences between the two tissues. Markers with particularly high imbalance rates in adenomas were seen on chromosomes 11, 14 and 15. This study reports further evidence that genomic instability is an ongoing process during carcinogenesis, with a markedly increased frequency of allelic losses seen in carcinomas compared to adjacent adenomas. Markers on chromosomes 11, 14 and 15 may become valuable tools in the identification of patients destined to progress to colorectal carcinomas.

1. Introduction

Colorectal carcinogenesis is the result of a multi-step process involving a series of mutations and/or epigenetic events resulting in the transition from normal mucosa to polyp to carcinoma.

Address correspondence to: Garth R. Anderson, PhD, Department of Cancer Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, Phone: 716-845-4529, Fax: 716-845-8126 e-mail: garth.anderson@roswellpark.org. * Bruce M. Brenner, MD, Division of Surgery, University of Connecticut Health Center, Farmington, CT 06030, Phone: 860-699-2290 e-mail: bbrenner@uchc.edu.

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Together these events allow cells to bypass several of the normal regulatory processes and develop traits that are specific to malignant cells, such as limitless replication, evasion of apoptosis, and tissue invasion (1,2). Since the spontaneous somatic mutation rate is insufficient to account for such a number of mutations, acquisition of a mutator phenotype is generally presumed to be an early event in tumor progression, with additional mutator phenotypes likely entering later during progression (3,4,5). This development is most likely the result of mutations or losses in genes that maintain genetic stability by mediating precise replication, damage repair, or checkpoint activation (6,7).

Genomic instability in colorectal cancer occurs in multiple distinct forms. Microsatellite instability (MSI), the result of defects in mismatch repair, is seen in patients with hereditary nonpolyposis colorectal cancer [HNPCC] and in about 15% of sporadic colorectal carcinomas, but is rare in adenomas (8–12). Chromosomal instabilities appear as changes in chromosomal copy number, chromosomal rearrangements, amplifications and deletions within chromosomes, and in various smaller intrachromosomal events. These are the most abundant types of instability seen in sporadic colorectal adenomas and cancers (4,13). Such instability has been found to occur early in colorectal tumorigenesis, but the mechanisms remain for the most part unclear (5,7,13,14).

Our laboratory and others have quantified genomic instability in sporadic colorectal carcinomas and polyps using several methods that largely detect different forms of genomic instability (5,13,15). The genome sampling technique of inter-simple sequence repeat (inter-SSR) PCR (19,20) reveals a form of instability which is present in a small fraction of aberrant crypt foci, most sporadic colorectal polyps, and most carcinomas; it may reflect an early and ongoing process in carcinogenesis resulting from a mutator phenotype (13,16). Genome-wide allelotyping of 59 sporadic colorectal carcinomas was able to identify seven specific loci where individual allelic loss was correlated with high rates of overall genome-wide fractional allelic loss and/or with high levels of genome-wide intrachromosomal instability measured by inter-SSR PCR (15). The association of overall genomic instability with allelic loss of these specific loci was highly significant statistically with p-values ranging from 0.004 to 0.02; the locus on chromosome 14 had a $p = 0.004$ value (15). Mao et al have recently reported how allelic imbalances appear widespread in colon adenomas, and occur even more frequently in carcinomas (4).

We have now investigated if any of the seven instability loci show frequent allelic imbalances in colorectal adenomas, consistent with the possibility that genes responsible for early onset instability are located close to the corresponding markers. One alternative possibility was that these loci are simply associated with furthering genomic instability of carcinomas after they have already arisen. We have utilized a subset of our previously identified instability-associated markers to compare genomic events in a series of villous adenomas specifically presenting simultaneously with associated colorectal carcinomas. Additional markers from regions adjacent to these loci were examined in order to better define sites of recurrent allelic losses associated with genomic instability in adenomas and carcinomas.

2. Materials and Methods

2.1. Patients and Tumors

Tumor samples and normal colonic mucosa were obtained from resected specimens of 17 patients with colorectal cancer that had clearly outgrown from villous adenomas. There were 13 men and 4 women ranging in age from 18 to 78 years (mean 63.9). Patient and tumor features are described further in table 1. All patients underwent surgery at the Roswell Park Cancer Institute between 1999–2001. The tissues were initially quick frozen in liquid nitrogen and subsequently stored at -70°C . The specimens were mounted in cryogel (Instrumedics,

Hackensack, NJ), cut into 30 μ M sections and stained with H & E. The malignant cells were separated from the adenoma cells using microdissection on a light microscope. DNA extraction was performed using a Qiagen DNA isolation kit (Qiagen, Valencia, CA).

2.2. Allelotyping Assays

PCR and allelotyping procedures were as described previously (15). The selection of markers was based on our earlier study allelotyping 59 sporadic colorectal cancers using a screening set of 348 markers spaced approximately 10 cM apart (Research Genetics, Huntsville, AL) (15). Twenty-eight markers that exhibited high rates of allelic loss in association with genomic instability were selected for the present study, along with other markers in regions flanking these loci (Table 2). All primers were obtained from Research Genetics and were end-labeled with 32 P. PCR products were separated on 5 to 8 % denaturing polyacrylamide gels run at 65 watts for 2–3 hours depending on product size. Gels were dried and placed on x-ray films for overnight exposure. Autoradiographs were analyzed visually. Loss of heterozygosity (LOH) was determined by the loss of one allele in the adenoma or carcinoma specimen compared to the normal mucosa. Microsatellite instability was determined by the presence of additional bands in the PCR products of the neoplasms when compared to normal mucosa, as described (15). Examples of loss of heterozygosity and microsatellite instability are illustrated in figure 1.

2.3. Genomic Instability Measurements

The fractional allelic loss (FAL) rate for each marker or neoplasm was determined by dividing the number of LOH events for that marker or lesion by the number of informative PCR reactions. Fractional microsatellite instability (FINST) rate was calculated as the ratio of microsatellite instability events to informative PCR reactions for each lesion. Based on prior analyses in studies of microsatellite instability using numerous markers, an adenoma or carcinoma was considered to be microsatellite unstable if greater than 30% of the informative markers assayed showed instability in that lesion (15,17). Non-parametric Wilcoxon Signed Ranks tests for related samples were employed to assess differences in FAL and FINST values, respectively, between adenomas and malignant tissues from the same patients. SPSS Version 12.0.2 was employed for all analyses (SPSS Inc, Chicago IL).

3. Results

Microsatellite marker PCR was performed on DNA isolated from each of the seventeen adenomas and associated carcinomas, using DNA from normal colonic mucosa from each corresponding patient as a control. Twenty-eight microsatellite markers were examined for allelic losses, including several which had previously been shown by us to be associated with intrachromosomal genomic instability in a separate group of 59 sporadic colorectal cancers (15). Additional markers in adjacent regions were examined in order to better define recurrent LOH sites potentially associated with genome-wide instability. Fractional allelic loss (FAL) and microsatellite instability (FINST) rates were calculated as described in the methods section (Figures 2 and 3).

For the markers examined in this study, the combined fractional allelic loss rate FAL in the adenomas ranged from 0 to 0.192 (mean = 0.052), while in carcinomas it ranged from 0 to 0.636 (mean = 0.135) (Table 3). Twelve of seventeen adenomas (71%) and sixteen of seventeen carcinomas (94%) demonstrated loss of heterozygosity (LOH) for one or more markers, while multiple events were seen in six of seventeen adenomas (35%) and eleven of seventeen carcinomas (65%). Thirteen of the seventeen carcinomas (76%) had increased FAL rates when compared to their adjacent adenomas. In carcinomas, the mean FAL rate of 0.135 was over 2.5 times that seen in adenomas, a statistically significant difference ($Z=-2.59$, Wilcoxon

Signed Rank test, $p=0.010$). This difference remained significant ($Z=-2.39$, $p=0.017$) after a possible outlier (patient 13063) was excluded (see figure 2).

Cumulative FAL rates were calculated for each individual marker tested (Figure 4). FAL rates in adenomas ranged from 0 to 0.267, while in carcinomas it ranged from 0 to 0.29. Fifteen of twenty-eight markers (54%) showed LOH events in one or more adenomas, while twenty-six of twenty-eight markers (93%) demonstrated LOH events in carcinomas. Multiple LOH events were observed for five markers (18%) in adenomas and fifteen (54%) in carcinomas. The five markers with frequent LOH in adenomas were located on chromosomes 11p15, 11q23, 14q24, 14q32, and 15q26. Twenty-two of the twenty-eight markers (79%) had higher FAL rates in carcinomas than in adenomas. These differences ranged from 0.005 to 0.23, with a mean of 0.104. Of the remaining six markers, five were increased in adenomas, by 0.008 to 0.084 (mean 0.04).

Fractional microsatellite instability for the markers examined (FINST) in adenomas ranged from 0 to 76.9%, while that in carcinomas varied from 0 to 70.4% (Table 3). Using instability in 30% of informative markers as a cutoff to define microsatellite unstable (MSI) lesions, four of the seventeen (23%) patients demonstrated microsatellite instability in both adenomas and carcinomas. These four patients had microsatellite instability in 37, 52, 62, and 77% of informative markers in their adenomas and 33, 48, 64, and 70% of informative markers in their carcinomas. One additional adenoma showed instability in 50% of markers, but only 19% in the carcinoma; this patient, had a small number of informative PCR reactions in the adenoma and microsatellite instability therefore may be overestimated for this particular sample.

For the microsatellite stable tumors, the mean FINST rate was 0.063 for the adenomas and 0.057 for the carcinomas, which was not a significant difference ($Z=-1.162$, Wilcoxon Signed Rank test, $p=0.25$). For the four tumors with frank microsatellite instability, the mean FINST value for the adenomas (0.57) was nearly identical to the mean FINST rate for the corresponding carcinomas (0.54), suggesting for this small sample that additional microsatellite alterations were not widespread as adenomas progressed to carcinomas.

Discussion

This study examined the progression of genomic instability in a series of colorectal carcinomas arising from villous adenomas; our results support the model wherein mutations are facilitated by genomic instability, with ongoing accumulation and selection of genetic events. We have found that the previously identified loci (15) associated with genomic instability in colorectal carcinomas have already developed allelic imbalances by the adenoma stage; this particularly applies to loci on chromosomes 11, 14, and 18.

Allelic losses were seen in multiple adenomas in five chromosomal regions in this study: 11p, 11q, 14q (2 markers), and 15q. Loss of 11p has been reported by others in colorectal cancers and a variety of other solid malignancies (18,19); 11p is the site of putative tumor suppressor genes including WT1 (20) and p53 (21). LOH at 11q23-24 was reported in colorectal (22) and ovarian (23) cancers. LOH at 14q has been reported in colorectal cancers and has been associated with early onset and advanced stage (24,25). 14q32 has been identified as the site of a potential tumor suppressor gene and demonstrated high rates of LOH in sporadic colorectal cancers, particularly in nonsmokers (15,26,27,28). A locus in the corresponding region of the mouse genome has also been associated with colorectal cancer incidence (29). Allelic losses were seen at 15q in a family with inherited colorectal adenomas and carcinomas; the THBS1 gene in this region was targeted as a candidate gene involved in carcinogenesis (30).

Our study results further confirm the recent study by Mao et al, where allelic loss measurements used in a genome-wide survey showed a generalized increase in allelic losses during the

progression of colorectal adenomas to carcinomas (4). Other genomic alterations also increase during the progression of colorectal adenomas to carcinomas, as seen with BAC-microarray comparative genomic hybridization (CGH), which measures relatively large-scale genomic events at least equal in size to the 150 kilobase size of the average BAC clone insert (5).

Our finding of a significant elevation in the level of allelic losses at specific instability-associated loci in carcinomas, as compared to their adjacent villous adenomas, may reflect that additional forms of instability come into play as progression advances. A large majority of the patients in our study had an increased number of mutations observed in their carcinomas; FAL was seen both in a larger number of markers and at higher rates for most markers in the carcinomas. In contrast, microsatellite instability was not significantly different between the adenomas and carcinomas. The rate of microsatellite instability we observed in villous adenomas was slightly higher than that seen in previous studies of sporadic adenomas; this difference is likely due either to our selection of markers, or that the adenomas we selected for study all contained adjoining regions that had progressed to malignancies (12,15). Instability as quantified by inter-(simple sequence repeat) PCR present in sporadic polyps previously has been shown to also be similar to the level detected in synchronous malignancies (13). This methodology generally detects smaller events, distinct from those seen with microsatellite instability (13,15,31).

By using PCR allelotyping to evaluate losses of heterozygosity, we have confirmed the progressive accumulation of mutations resulting from genomic instability. Five potential genomic instability loci have now been found to be frequently lost in villous adenomas, consistent with the possibility they represent potential sites for genes involved in the generation of genomic instability. Finer mapping and identification of these early changes may allow us to further clarify the mechanisms producing genomic instability leading to the development of colorectal cancers.

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Examples of LOH and Microsatellite Instability

Figure 1.

Examples of autoradiographs of polyacrylamide gels of PCR amplification products using microsatellite markers and demonstrating loss of heterozygosity (LOH) and microsatellite instability (MSI) in villous adenomas. Left lane in each example represents normal mucosa with two normal alleles and right lane represents adenoma. Arrow for LOH shows loss of allele in adenoma, while arrow for MSI marks extra band representative of microsatellite instability.

Figure 2.

Comparison of fractional allelic loss rate in adenomas and carcinomas for all informative markers each of the 17 patients. X-axis represents FAL rate calculated as described in the methods section. Y-axis lists patient number. Black bars represent carcinomas, while striped bars represent adenomas.

Figure 3.

Comparison of fractional microsatellite instability rate in adenomas and carcinomas for all informative markers in each of the 17 patients. X-axis represents FINST rate calculated as described in the methods section. Y-axis lists patient number. Black bars represent carcinomas, while striped bars represent adenomas.

Figure 4.

Comparison of fractional allelic loss rate in adenomas and carcinomas for each of the 28 microsatellite markers in all 17 patients. X-axis represents FAL rate calculated as described in the methods section. Y-axis lists marker number. Black bars represent carcinomas, while striped bars represent adenomas.

Table 1Clinicopathologic features of tumors used in these studies^a

Patient	Age	Gender	Cancer Site^b	TNM Stage (AJCC)
14003	58	M	Rt. Colon	T3N0M0 (II)
13078	61	M	Lt.Colon	T3N0M0 (II)
13075	71	M	Rectum	T3N0M0 (II)
13071	65	M	Transv. Colon	T4N1M0 (III)
13067	72	M	Rectum	T3N0M0 (II)
13063	79	F	Rt. Colon	T3N0M0 (II)
13058	78	M	Rectum	T3N1M0 (III)
13055	18	M	Lt.Colon	T3N0M0 (II)
13051	55	M	Rectum	T3N1M0 (III)
13040	43	M	Rectum	T3N0M0 (II)
13038	65	M	Dist.Transv.	T3N1M1 (IV)
13009	75	F	Rt.Colon	T3N0M0 (II)
13005	69	M	Sigmoid	T3N0M0 (II)
12093	75	M	Rt.Colon	T3N2M1 (IV)
12079	62	F	Lt.Colon	T3N2M0 (III)
12075	66	F	Rectum	T3N0M0 (II)
12061	74	M	Rt.Colon	T3N0M0 (II)

^a All patients presented with carcinomas and simultaneous villous adenomas. Stage information listed is provided for the carcinomas.

^b Rt colon, right colon; Lt colon, left colon; Transv colon, transverse colon; Dist Transv, distal transverse colon.

Table 2
Microsatellite Markers Characterized for Loss of Heterozygosity

Chromosome	Marker ^a
2	D2s1399
5	D5s2501
	D5s408
8	D8s1477
	D8s1119
11	D11s1984
	D11s2362
	D11s4464
	D11s934
	D11s2359
13	D13s325
14	D14s742
	D14s306
	D14s588
	D14s53
	D14s606
	D14s1434
	D14s987
	D14s979
	D14s1426
15	D15s652
17	D17s1293
18	D18s481
	D18s535
	D18s483
	D18s878
	D18s477
	D18s466

^aThe 28 microsatellite markers used in these studies are listed by chromosome and distance from the telomere.

Table 3Mean Fractional Allelic Loss And Microsatellite Instability For Adenomas and Carcinomas^a

	ADENOMAS	CARCINOMAS	P VALUE ^b
Mean FAL	0.052	0.135	0.010 ^c
Mean FINST (ALL)	0.215	0.181	n.s.
Mean FINST (MSI-)	0.063	0.057	n.s.

^a Mean fractional allelic loss (FAL) and fractional microsatellite instability (FINST) for adenomas and carcinomas. Mean FINST values are provided for the entire set of tumors (microsatellite unstable and microsatellite stable: ALL) and for the set of microsatellite stable tumors alone (MSI-).

^b Wilcoxon Signed Rank test for related samples. n.s. = not significant (> .05)

^c p=0.017 when FAL data of patient 13063 was excluded from analysis due to appearance as outlier.