Evidence That Genetic Instability Occurs at an Early Stage of Colorectal Tumorigenesis

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Abstract

Chromosomal instability is believed to be a common feature of most human tumors, but the stage at which such instability originates has not been defined. At the molecular level, chromosomal instability is characterized by allelic imbalance (AI), representing losses or gains of defined chromosomal regions. We have assessed AI in early colorectal tumors using newly developed methods for assessing AI in complex cell populations. A total of 32 adenomas of average size (2 mm; range, 1–3 mm) were studied. AI of chromosome 5q markers occurred in 55% of tumors analyzed, consistent with a gatekeeping role of the adenomatous polyposis coli (APC) tumor suppressor gene located at chromosomal position 5q21. AI was also detected in each of the other four chromosomes tested. The fractions of adenomas with AI of chromosomes 1p, 8p, 15q, and 18q were 10, 19, 28, and 28%, respectively. Over 90% of the tumors exhibited AI of at least one chromosome, and 67% had allelic imbalance of a chromosome other than 5q. These findings demonstrate that AI is a common event, even in very small tumors, and suggest that chromosomal instability occurs very early during colorectal neoplasia.

Introduction

It is now widely accepted that tumors result from the sequential accumulation of genetic alterations (1). It has also been long recognized that genetic instability may be required to accumulate the large number of genetic alterations that occur during the tumorigenic process (2, 3). It has been proposed recently that two forms of such instability occur in CRCs (13). In ~13% of CRCs, mismatch repair deficiency leads to MIN, characterized by widespread insertions and deletions in microsatellite repeats and associated with numerous nucleotide substitutions (4, 5). In the remaining 87% of CRCs, CIN appears to result in a large number of gross chromosomal changes. Although the biochemical mechanisms underlying MIN are well known, the mechanisms underlying CIN are only beginning to emerge (3, 6).

If genetic instability is essential to human neoplasia, one might expect it to begin early during the tumorigenic process and thereby accelerate the acquisition of growth-promoting mutations. Evidence has been presented that MIN often occurs very early, prior to the occurrence of the APC gene mutations that initiate the abnormal growth phase (7). On the other hand, the timing of CIN is uncertain. Although most CRCs are aneuploid (suggesting CIN), the prevalence of aneuploidy in benign colorectal tumors is less than in cancers and appears to increase as the tumors enlarge in size (8–12). Virtually all adenomas that have been studied through karyotypic or flow cytometric methods have been rather large, containing billions of cells, and there have been few karyotypic or molecular studies focused on very small benign tumors. The choice of tumors studied previously has been dictated by the fact that small benign tumors have relatively few neoplastic cells and a higher fraction of stromal cells, presenting significant experimental obstacles.

To address the question posed above, we have studied a series of colorectal adenomas averaging 2 mm in diameter. AI, reflecting gains or losses of particular chromosomal regions, was used as a marker for CIN. To overcome the obstacles associated with the molecular genetic analysis of these small tumors, we used a recently developed PCR-based approach called digital SNP analysis, in which the alleles within a tumor sample are individually counted, one by one (13, 14). Using this method, we found a remarkably high degree of AI in small tumors, strongly suggesting that CIN occurs early during colorectal tumorigenesis.

Materials and Methods

Tissues and Tumor DNA Samples. Formalin-fixed, paraffin-embedded tissue samples of 32 small adenomas from 27 patients and their associated normal mucosa were retrieved from the surgical pathology files of The Johns Hopkins Hospital. All of the adenomas were from sporadic cases and were obtained either from colonoscopy biopsies or harvested from colorectal resection specimens. Histologically, all adenomas demonstrated low-grade dysplasia without any evidence of high-grade dysplasia or carcinoma. The sizes of the adenomas ranged from 1 to 3 mm, with an average of 2 mm. The adenomatous areas and normal epithelium were microdissected under an inverted microscope and DNA purified using a QiaQuick PCR purification kit (Qiagen, Valencia, CA).

Digital PCR Analysis. SNP markers on chromosomes 1p, 8p, 15q, and 18q were retrieved from the Whitehead human SNP database4 and National Cancer Institute SNP map.5 The SNP markers within the APC gene on chromosome 5q were based on common polymorphisms reported previously (15). Forward and reverse primers were designed for each SNP, allowing the amplification of ~100-bp PCR products (Table 1). Molecular Beacons (16, 17) were designed for each of these 24 polymorphisms. For each case, DNA from normal mucosa was first tested for heterozygosity for SNP markers on all five chromosomes. For each chromosome, one SNP that exhibited heterozygosity in the normal sample was then used to assess AI in DNA from the corresponding tumor.

Digital SNP was performed as described (13, 14). In brief, DNA samples were diluted and distributed to the wells of a 384-well plate at less than 1 genomic equivalent/well. PCR was performed with a touch-down protocol for all SNPs: 94°C (1 min); 4 cycles of 94°C (15 s), 64°C (15 s), 70°C (15 s); 4 cycles of 94°C (15 s), 61°C (15 s), 70°C (15 s); 4 cycles of 94°C (15 s), 58°C (15 s), 70°C (15 s); and 45 cycles of 94°C for 15 s, 55°C (15 s), 70°C (15 s). After PCR, a pair of Molecular Beacons synthesized by Gene Link (Thorwood, NY) was added to the final PCR product, along with an internal primer that allowed the generation of single-stranded DNA complementary to the Molecular Beacon. Molecular Beacons are single-stranded oligonucleotides containing a fluorescent dye and a quencher on their 5′ and 3′ ends, respec-

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5 Internet address: http://lpg.nci.nih.gov/html-snp/imagemaps.html.
 Results

**Principles of Allele Counting and SPRT Analysis.** The analysis of AI in small tumors is fraught with technical difficulties. Such tumors typically contain a high fraction of nonneoplastic cells, including stromal and inflammatory components (Fig. 1). Microdissection is therefore mandatory to derive a population that is enriched in the neoplastic cell component. But analysis of the microdissected samples with genetic markers is associated with its own set of problems. To overcome such problems, we have used a technique called digital SNP, in which alleles are directly counted, one by one (14). Because SNPs rather than microsatellite markers are used with this method, DNA degradation of the larger microsatellite alleles does not pose a problem (19). However, because only limited amounts of DNA are purified from microdissected lesions, sampling errors can produce artifactual enrichments for one allele when conventional AI assays are used, even with SNPs. For example, if only a few template molecules are successfully amplified in the PCR, one allele may be overrepresented by chance alone. Digital SNP minimizes such sampling errors, because the exact number of alleles evaluated in each assay is determined. Rigorous statistical methods are then used to conclude whether AI is present.

An example of the results obtained with this approach is presented in Fig. 2. DNA from normal and microdissected tumor samples was diluted in a 384-well PCR plate so that there was <1 DNA template/well. PCR was performed using a SNP for which the patient was heterozygous. The resultant PCR products were analyzed using Molecular Beacon probes to determine allelic representation. In the DNA sample from normal colonic mucosa of the patient depicted in Fig. 2A, there were 29 “A” alleles (red; detected with a Hex-labeled Molecular Beacon) and 34 “G” alleles (green; detected with a fluorescein-labeled Molecular Beacon). In the sample from the small adenoma of this patient, there were 33 “A” alleles and 11 “G” alleles (Fig. 2A).

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### Table 1. Primers and probes used for digital SNP analysis

<table>
<thead>
<tr>
<th>SNP</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Internal primer</th>
</tr>
</thead>
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<tr>
<td>Ch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p</td>
<td>CGAP-C-31490</td>
<td>AAAGCTGGGCCCTGATTGG</td>
<td></td>
</tr>
<tr>
<td>15q</td>
<td>CGAP-C-2077</td>
<td>AGATCTGAACTGTCGCTCTT</td>
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<td>CGAP-C-362</td>
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<td></td>
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<tr>
<td></td>
<td>CGAP-C-425</td>
<td>CAGTGCGACGATGACGAGACCGTG</td>
<td></td>
</tr>
<tr>
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<td>CAGTGCGACGATGACGAGACCGTG</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>CGAP-C-1239</td>
<td>CAGTGCGACGATGACGAGACCGTG</td>
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</tr>
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<td>5q</td>
<td>CGAP-C-700</td>
<td>CAGTGCGACGATGACGAGACCGTG</td>
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</tr>
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<td>18q</td>
<td>CGAP-C-331</td>
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<td>CGAP-C-801</td>
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<td>CGAP-C-1468</td>
<td>CAGTGCGACGATGACGAGACCGTG</td>
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**Fig. 1.** A, H&E-stained section of a typical adenomatous polyp used in this study. The adenoma was 2 mm in greatest dimension. B, higher magnification demonstrates dysplastic epithelium overlying the lamina propria, with abundant lymphocytes and stromal cells that are typically observed in these lesions.
distribution.4 resulting from the presence of both alleles. Such results are expected based on a Poisson

cules. Wells that were PCR products derived from only one of the two alleles. Wells with only background

number of alleles counted; the adenoma is above curve 1, and this tumor is interpreted to have AI.

interpreted as being in allelic balance. In contrast, the allelic proportion in the DNA from

dNA from the normal colonic mucosae is below curve 2 and is therefore

interpreted as being in allelic balance. In the example shown in Fig. 2B, the allelic proportion of

DNA from the normal colonic mucosae was below curve 2 and was therefore interpreted as being in allelic balance. In contrast, the allelic proportion in the DNA from the adenoma was above curve 1, and this tumor was interpreted to have AI.

AI in Small Adenomas. We selected 32 adenomas with an average diameter of 2 mm (range, 1–3 mm) for this study (Fig. 1). Neoplastic cells from these adenomas were carefully microdissected with the contamination from nonneoplastic cells estimated at 30–60% by histological examination. DNA was purified from the microdissected tissue and used as template for digital SNP analysis. Five different chromosomal arms were evaluated for AI. For each chromosomal arm, we developed Molecular Beacon pairs that reliably distinguished paternal from maternal alleles. The sequences of the relevant primers and Molecular Beacons for the 24 different SNPs studied are detailed in Table 1. Using these 24 markers, we were able to find at least one heterozygous SNP for each chromosomal arm in the great majority of patients studied.

All of the normal mucosa samples analyzed were found to be in allelic balance, because their allelic proportions were below curve 2 (data not shown). Scatterplots of the data from the tumors are shown in Fig. 3 and summarized in Fig. 4. Among the informative cases, the percentages of samples exhibiting AI of chromosomes 1p, 5q, 8p, 15q, and 18q were 10, 55, 19, 28, and 28%, respectively. There was no significant correlation between AI and the site of adenomas or the age and sex of patients. Over 90% of the adenomas exhibited AI of at least one chromosomal arm. When chromosome 5q was excluded from analysis, 66% of the adenomas exhibited AI of at least one of the other chromosomal arms. There was no obvious clustering of the AI in the tumors (Fig. 4).

Discussion

The results presented above show that AI is present in the great majority of very small adenomas. This imbalance was seen not only in chromosome 5q, home of the APC gene, but also in several other chromosomes. Our interpretation of these data is that CIN occurs early during colorectal neoplasia, long before the advent of malignancy. These results support the idea that such instability is essential for neoplasia (20).

Although the above represents our preferred interpretation, several alternative interpretations should be pointed out:

(a) We have studied tumors at a single time point. Although our data are consistent with the existence of a true instability (which can only be measured through a time course, not through a single determination), other scenarios are conceivable. For example, it is possible that one abnormal division occurred during the tumorigenic process,

only 50% of the DNA from the microdissected samples originated from neoplastic cells and the other 50% originated from nonneoplastic cells, the observed allelic proportion would be 66.7%, not 100%. SPRT curves were therefore constructed to choose between the hypotheses \( P = 50\% \) and \( P = 66.7\% \), using the conservative assumption that at least 50% of the DNA in the sample was derived from neoplastic cells. The two curves representing these two assumptions are depicted in Fig. 2B. The number of alleles studied in each sample is plotted on the abscissa, and the observed ratio of the two alleles is plotted on the ordinate. The ratio is defined as the number of wells containing the allele that appeared to be in excess to the total number of wells containing either allele. Samples represented by points above curve 1 were interpreted to have AI. It is important to note that the confidence interval of each point above curve 1 excludes allelic proportion values that are expected to occur in normal cells (14, 18). Points below the bottom curve were categorized as being in allelic balance. In the example shown in Fig. 2B, the allelic proportion of DNA from the normal colonic mucosae is below curve 2 and was therefore interpreted as being in allelic balance. In contrast, the allelic proportion in the DNA from the adenoma is above curve 1, and this tumor was interpreted to have AI.

To determine whether there was substantial statistical evidence for AI, we used the SPRT (19). This method allows two probabilistic hypotheses to be compared as data accumulate and facilitates decisions about the presence or absence of AI after study of a minimum number of samples. Hypothesis 1 is that the sample is in AI, i.e., that the alleles have the expected normal allelic proportion of 50%. Hypothesis 2 is that the same one of the two alleles was absent in every tumor cell (14). However, this does not correspond to an allelic proportion of 100% in the tumor samples because isolation of pure tumor cell populations from small adenomatous polyps is not feasible with the microdissection methods we used (Fig. 1). For example, if

Fig. 2. A, examples of digital SNP genotyping data. DNA from microdissected normal (upper panel) and adenoma (bottom panel) samples was distributed to a 384-well PCR plate. After PCR, a pair of allele-specific Molecular Beacons, labeled with fluorescein or Hex, was added to each well to determine allelic status. The fluorescein: Hex fluorescence ratio was then determined. Wells that were green or red corresponded to those containing PCR products derived from only one of the two alleles. Wells with only background fluorescence were uncolored and represent wells that contained no DNA template molecules. Wells that were yellow contained an intermediate green: red fluorescence ratio, resulting from the presence of both alleles. Such results are expected based on a Poisson distribution. B, SPRT analysis of normal and adenoma samples from A. X axis, total number of alleles counted; Y axis, observed proportion of the two alleles. The allelic proportion of DNA from the normal colonic mucosae is below curve 2 and is therefore interpreted as being in allelic balance. In contrast, the allelic proportion in the DNA from the adenoma is above curve 1, and this tumor is interpreted to have AI.
leading to AI at multiple loci, and that there was no persistent chromosomal instability in these lesions. We cannot infer from our static observations any quantitative estimate of the rate of chromosomal loss and can only state that the AI we observed is consistent with a chromosomal instability such as that formally demonstrated in CRC cell lines in vitro (21).

(b) A second proviso concerns the term "early." The selected adenomas were the smallest tumors generally noted during pathological examination of surgically excised tissues and were also the smallest from which we could reproducibly obtain a sufficient number of alleles for analysis with several probes. However, these ~2-mm adenomas contain ~1 x 10^6 neoplastic cells, and the term "early" is a relative one. On the basis of clinical studies, it would likely take 10–30 years for these tiny adenomas to progress to malignancy (22), and it therefore is legitimate to label them "early."

How do our results compare with previous molecular studies of AI? Although allelic imbalances of chromosome 5q were expected (15, 23, 24), the other AIs were surprising. For example, loss of heterozygosity of chromosomes 1p, 8p, 15q, and 18q have previously been observed rarely in adenomas, even in large ones (23, 25–28). There are two factors that might reconcile these observations: (a) the digital SNP method we used is more sensitive than previous methods to detect allelic loss. Some of the AIs we observed might have been present in only 50% of the neoplastic cells, for example, and standard techniques would not have detected them. Digital SNP reliably discerns AI but cannot distinguish (for example) whether this AI is attributable to allelic loss in 100% of the neoplastic cells in a specimen composed of 50% nonneoplastic cells versus allelic loss in 60% of the neoplastic cells in a specimen composed of 80% nonneoplastic cells. We therefore might be observing losses in major subpopulations of neoplastic cells that only become completely clonal at later stages of tumorigenesis. Such progression has been observed previously in colon and other tumors (24, 29, 30). Additionally, our analysis does not distinguish between chromosome gains and chromosome losses as the cause of the AI. Previous studies that concentrated on loss of heterozygosity might not have detected chromosome gains. From a CIN (rather than functional) point of view, chromosome gains and losses are equally important and indicative of instability (3). It is also noteworthy that the chromosomes we analyzed harbor tumor suppressor genes, and losses of such chromosomes may result in a selective growth advantage. Whether such losses confer a selective advantage, or are simply random, does not affect the data or conclusions of this study.

Previous cytogenetic and flow cytometric studies of colorectal neoplasms are in general consistence with our observations, although there have been very few examinations of colorectal tumors as small as those studied here. There have been several reports of aneuploidy
in larger adenomas, with the suggestion that aneuploidy increases with tumor progression (8–12). Accordingly, our study showed that AI in adenomas (Fig. 4) is much less common than that in cancers studied previously. Indeed, it is not uncommon to observe AI in over one-third of the total chromosomes in primary tumors (30), and chromosomes 1p, 8p, and 18q exhibit allelic losses in a much higher proportion of cancers (14, 23, 31, 32) than in small adenomas (Fig. 4). If CIN began early, one would expect to see increasing degrees of aneuploidy as tumors enlarged, because of repeated rounds of clonal selection.

Our observations and conclusions are also consistent with those made on the early stages of other tumor types. For example, AI has been found in metaphasic lesions associated with gastric carcinoma (33), in intraepithelial neoplasia of the vulva (34), in dysplastic esophageal epithelium in Barrett’s esophagus (35), and in fibrocystic changes of breast (36). Although the temporal relationship between such lesions and cancer is less well defined than in colorectal tumorigenesis, such studies support the idea that CIN is an early component of human neoplasia in general.

It is interesting to speculate whether CIN precedes the advent of APC gene mutations. Studies in mice, as well as in humans, have shown that APC inactivation occurs very early during the neoplastic process (reviewed in Ref. 37). In many cases, this inactivation involves mutation of one allele and loss of the other. Such inactivations could occur in two ways. A mutation in one APC allele could occur first, followed by a loss of the second allele, or vice versa. In either case, the allelic loss event could be precipitated by CIN, although occasional allelic losses also occur in normal cells that are chromosomally stable (38–40). As a working hypothesis, we propose that CIN drives the allelic loss of chromosome 5 in some cases and that CIN may already be present in a proportion of the tiny neoplastic lesions containing APC mutations. This hypothesis can only be convincingly refuted or confirmed once the mechanism(s) underlying CIN is identified.

Acknowledgments

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References

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