Chromosomal Instability Correlates with Genome-wide DNA Demethylation in Human Primary Colorectal Cancers

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Abstract

DNA hypomethylation is a common trait of colorectal cancer. Studies in tumor cell lines and animal models indicate that genome-wide demethylation may cause genetic instability and hence facilitate or accelerate tumor progression. Recent studies have shown that DNA hypomethylation precedes genomic damage in human gastrointestinal cancer, but the nature of this damage has not been clearly established. Here, we show a thorough analysis of DNA methylation and genetic alterations in two series of colorectal carcinomas. The extent of DNA demethylation but not of hypermethylation (both analyzed by amplification of intermethylated sites in near 200 independent sequences arbitrarily selected) correlated with the cumulated genomic damage assessed by two different techniques (arbitrarily primed PCR and comparative genomic hybridization). DNA hypomethylation–related instability was mainly of chromosomal nature and could be explained by a genome-wide effect rather than by the concurrence of the most prevalent genetic and epigenetic alterations. Moreover, the association of p53 mutations with genomic instability was secondary to DNA hypomethylation and the correlation between DNA hypomethylation and genomic instability was observed in tumors with and without mutation in the p53 gene. Our data support a direct link between genome-wide demethylation and chromosomal instability in human colorectal carcinogenesis and are consistent with the studies in model systems demonstrating a role of DNA demethylation in inducing chromosomal instability. (Cancer Res 2006; 66(17): 8462-8)

Introduction

Early studies in the 1980s already identified a depletion of the methyl-cytosine content as a landmark in colorectal cancer and other types of tumors (1, 2). DNA hypomethylation has been shown to promote tumorigenesis in murine colon and liver (3) and was included as an early event in the classic Vogelstein’s model for colorectal tumorigenesis (4). Different investigations sustain a causal link between DNA hypomethylation and genetic instability (reviewed in refs. 5, 6), reporting an association between defects in DNA methylation and aneuploidy in human colorectal cancer cell lines (7), increased chromosomal rearrangements in hypomethylated centromeric regions in mitogen-stimulated cells from individuals affected with immunodeficiency, centromere instability and facial anomalies (ICF syndrome; ref. 8), and an increased mutation rate owing to DNMT1 deficiency in murine embryonic stem cells (9) and in murine somatic cells (10, 11). Moreover, it has been shown that DNMT1 deficiency also results in constitutive chromosomal instability in a human colon cancer cell line (12). Fewer studies have investigated the association of DNA hypomethylation with genetic alterations in human primary cancers. Matsuzaki et al. (13) reported that decreased methylation levels in LINE sequences correlate with losses of heterozygosity on discrete chromosomal loci in a series of colorectal carcinomas. A very recent study has shown that DNA hypomethylation precedes genomic damage in gastrointestinal cancer (14). Besides these evidences supporting a close relationship between genome-wide hypomethylation and genomic damage in carcinogenesis, the nature of the genomic damage associated with DNA hypomethylation and its possible interaction with other molecular and clinicopathologic variables is still unclear.

To shed some light into this issue, we have analyzed genome-wide hypomethylation profiles in two series of colorectal carcinomas and studied its association with genomic instability. The extent of DNA hypomethylation was analyzed by amplification of intermethylated sites (AIMS; ref. 15). AIMS profiles represent unique sequences flanked by two methylated CpG sites. Differences in the display of AIMS-amplified bands between paired normal and tumor tissue correspond to changes in the methylation status of specific sequences. AIMS allows the concurrent, but independent, analysis of hypermethylation and hypomethylations in multiple samples; therefore, separate estimates of the two opposite alterations may be obtained and compared (15). Application of AIMS has been instrumental to detect relevant epigenetic changes associated with carcinogenesis (16–19) and aging (20). In a first setting, genomic instability was determined in the form of cumulated genetic alterations by arbitrarily primed PCR (AP-PCR). AP-PCR fingerprints generated from normal and tumor DNA may be easily compared, allowing the detection and characterization of multiple genetic differences (21–23) and the obtainment of unbiased estimates of global genomic disruption (24, 25). Moreover, the application of AP-PCR was instrumental in the discovery of ubiquitous microsatellite instability in a subset of colorectal tumors (26). In a second setting, comparative genomic hybridization (CGH) was used to score genomic damage at chromosomal...
level and to investigate the association of DNA hypomethylation with chromosomal profiles.

Materials and Methods

Patients and tumor samples. Two series of fresh-frozen (–80°C) colorectal carcinomas, including the paired normal tissue, were used in this study. Series HSP included 83 patients diagnosed with colorectal cancer at the Hospital de la Santa Creu i Sant Pau (Barcelona, Catalonia, Spain). Series HUB included 50 patients diagnosed of Dukes B or Dukes C colorectal cancer at the Hospital Universitari de Bellvitge (L’Hospitalet, Catalonia, Spain). These cases were part of two larger series of patients preoperatively diagnosed with colorectal cancer, and prospectively included in a study designed to evaluate the prognostic value of specific genetic and epigenetic alterations. Sample collection, patient selection criteria, and main clinicopathologic characteristics of the patients are described in Supplementary Data. Transformed cell content was >75% in most tumor specimens as assessed by histologic examination. DNA amenable for genetic and epigenetic analysis was obtained by using standard procedures.

Quantification of the degree of hypermethylation and hypomethylation. Comparative fingerprints representing the methylhyme of the tumor and the normal tissue were obtained by AIMS (15). AIMS bands correspond to selected genomic sequences flanked by two methylated SmaI sites (CCCCGGG). Lack of methylation at either site prevents amplification of the band. Three independent experiments were done, resulting in the reproducible display of 208 bands that were initially considered for analysis. Assay conditions and technical validation of the approach have been described before (15, 17). Bands with age-related display in the normal tissue were excluded in normal-tumor comparisons. A total of 193 sequences were scored for differential methylation between the normal and the tumor tissue. The index of hypomethylation was calculated as the number of hypomethylated sequences (bands with a decreased intensity in the tumor compared with the normal tissue) divided by the total number of bands analyzed. Simultaneously, an index of hypermethylation (bands with an increased intensity in the tumor compared with the normal tissue) was calculated in the same way. For each AIMS-tagged band, a hypomethylation rate was calculated as the fraction of tumors showing hypomethylation with respect to the total number of informative cases.

Estimation of the genomic damage fraction. Genomic alterations at chromosomal and subchromosomal level were assessed using the DNA fingerprinting technique AP-PCR. AP-PCR is based on the amplification by PCR of genomic DNA using primers of arbitrarily chosen sequence and initial cycles of low stringency. Because the primer anneals to multiple sites, many PCR products are generated and result in a reproducible fingerprint when analyzed by gel electrophoresis. Three independent experiments were done, generating information on a total of 181 independent loci distributed genome wide (although not all of them were informative in all the samples). An index of cumulated alterations [genomic damage fraction (GDF)] was calculated as the number of bands with differential display in the tumor with regard to the paired normal tissue divided by the total number of bands visualized. Assay conditions and main associations of the GDF with genetic and clinical features of the tumors have been described in detail elsewhere (24).

Analysis of chromosomal alterations by CGH. Normal human genomic DNA (control) and tumor DNA (test) were labeled with Spectrum Green-dUTP and Spectrum Red-dUTP, respectively, by nick translation (Vysis, Downers Grove, IL). Equal amounts of control and test-labeled probes were coprecipitated and dissolved in 12 μL hybridization buffer, denatured, and hybridized in a moist chamber for 48 to 72 hours on normal metaphase spreads (Vysis). Slides were washed according to the protocol of the manufacturer. Chromosomes were counterstained with 4,6-diamine-2-phenylindole (Sigma, St. Louis, MO) and analyzed using Cytovision Ultra workstation (Applied Imaging, Sunderland, United Kingdom). Ratio values obtained from at least 10 metaphases were averaged. Ratio values >1.25 and <0.75 were considered to represent chromosomal gains and losses, respectively.

Competitive hybridization of AIMS products to metaphase chromosomes. Unrestrained AIMS were done as described (15) in 10 normal tumor pairs. Products were purified using Concert Rapid Purification System (Life Technologies, Carlsbad, CA) and labeled with SpectrumRed dUTP or SpectrumGreen dUTP (Vysis) using a nick translation kit (Vysis). Both normal and tumor probes from the same patient were cohybridized to metaphase chromosomes. Procedures and image analysis were done as described for conventional CGH (27).

High-performance capillary electrophoresis. 5-Methylcytosine content was determined by high-performance capillary in 16 tumors exhibiting a wide range of hypomethylation index electrophoresis and their paired normal tissue, as previously described (28). Two cases were excluded due to technical failure; therefore, 14 cases remained available for analysis. Briefly, genomic DNA samples were boiled and treated with nuclease P1 (Sigma) for 16 hours at 37°C and with alkaline phosphatase (Sigma) for an additional 2 hours at 37°C. After hydrolysis, total cytosine and 5-methylcytosine content was measured by capillary electrophoresis using a P/ACE MDQ system (Beckman-Coulter, Fullerton, CA) with UV detection. Relative 5-methylcytosine content was expressed as a percentage with respect to the total cytosine content (methylated and nonmethylated).

Statistical analysis. Results are expressed as a mean ± SD, except where indicated. Statistical differences between variables were analyzed with unpaired/paired t or nonparametric tests as appropriate. Correlation
Results and Discussion

Cumulated genomic damage correlates with genome-wide demethylation. In a first setting, we analyzed abnormalities at DNA methylation and genomic level using two DNA fingerprinting methods. DNA hypermethylation and hypomethylation profiles were analyzed by AIMS in 83 colorectal carcinomas and their paired normal tissues (referred here as series HSP). The extent of genomic alterations was assessed by AP-PCR (24). Illustrative examples of the AIMS and AP-PCR fingerprints are shown in Fig. 1. HSP series did not include any case with microsatellite instability to avoid the contribution of microsatellite-related instability in the calculation of the genomic damage index.

DNA methylation profiles generated by AIMS rendered an average of 185 informative bands per case. In AP-PCR experiments, an average of 141 ± 27 informative loci were scored per case. Indices of hypomethylation and hypermethylation and the index of genomic alterations (GDF) were calculated as the fraction of bands with differential display between paired normal and tumor according to AIMS (DNA methylation changes) and AP-PCR fingerprints (GDF). DNA hypomethylation indices and GDF are shown in Table 1. Tumors with p53 mutations exhibited higher levels of GDF but not of DNA hypomethylation (Table 1). No differences in the GDF and hypomethylation indices were observed when tumors were classified according to clinicopathologic features (Supplementary Table S1), except for sex: Men showed higher hypomethylation levels than women as previously reported (17).

A correlation between GDF and the hypomethylation index ($r = 0.250, P = 0.022$; Fig. 2A), but not the hypermethylation index ($r = 0.020, P = 0.856$; Supplementary Fig. 1A), was observed. Therefore, it can be concluded that demethylation, but not de novo DNA methylation, correlates with the number of genetic alterations in colorectal cancer. In a recent study in gastrointestinal cancer, Suzuki et al. (14) have reached similar conclusions demonstrating the dominant role of hypomethylation over hypermethylation on genomic damage.

Classification of the cases according to clinicopathologic variables revealed that the correlation was strengthened in younger patients (≤67 years) and men (Supplementary Table S2). Because age-related hypermethylation and hypermethylation have been reported in normal mucosa (14, 29), it can be speculated that the epigenetic “noise” associated with aging is lower in younger patients, bearing an undisturbed and enhanced correlation between the epigenetic and genetic damage.

DNA hypomethylation–related genomic instability is of chromosomal nature. The genetic alterations revealed by AP-PCR may be of multiple and different nature (structural and numerical chromosomal alterations, point mutations, short insertions, and deletions; reviewed in ref. 30). In the first study, we excluded tumors exhibiting microsatellite instability. Microsatellite instability is also detectable in AP-PCR fingerprints (21, 26) and the mechanisms underlying this type of instability are well known. Therefore, we knew that the genomic damage detected by AP-PCR did not include microsatellite instability. However, we wondered if the genomic instability associated with hypomethylation was the reflection of multiple types of genomic damage or rather of a specific type of alteration. Hence, we analyzed DNA methylation profiles by AIMS and genomic alterations by CGH in a second series of 50 colorectal carcinomas (series HUB). The indices of DNA methylation and genomic instability are shown in Table 1 and Supplementary Table S1. Noteworthy, the correlation between the hypomethylation index and the number of chromosomal alterations was highly significant ($r = 0.514, P < 0.001$; Fig. 2B). We also investigated if the type of chromosomal alteration (structural, numerical, gains, and losses) was differentially associated with the hypomethylation index. In all cases, the correlation was highly significant, as it was among the measures of each category of chromosomal alteration (Supplementary Table S3), suggesting that chromosomal instability is manifested in different types of alterations concurrently and that DNA hypomethylation does not associate preferentially with one specific kind of chromosomal damage.

The enhancement in the correlation between DNA hypomethylation and cumulative genomic damage determined by CGH (compared with that obtained from AP-PCR data) suggests that the main type of genomic instability resulting directly from DNA hypomethylation is most probably of chromosomal nature. By using resampling techniques, we calculated that the correlation hypomethylation index/number of chromosome alterations is

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<th>Table 1. Genomic damage and hypomethylation index in colorectal cancer</th>
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<td>All tumors</td>
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<td>p53 status</td>
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<td>Wild-type</td>
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NOTE: Values are expressed as mean ± SD. Numbers in parentheses indicate range. $P$ values are estimated from two-tailed $t$ test.
greater than the correlation hypomethylation index/GDF with a 95% confidence. The method used to calculate this value was based only on the observed data and took into account that different samples of patients were used to analyze each measure of genomic damage. Even if we exclude three cases of the HSP series that show very high values of GDF and low DNA hypomethylation index, the probability that the correlation is greater for the number of chromosome alterations is 80%. The positive correlation was maintained when tumors were classified by clinicopathologic features, although some differences could be observed according to sex and tumor location (Supplementary Table S2). Similar to what we observed in series HSP, the hypermethylation index showed no correlation with cumulated genomic alterations (Supplementary Fig. S1B).

**Genomic extent of hypomethylation related instability.** To determine which chromosomes were more affected by DNA hypomethylation, we performed an analysis of variance (ANOVA) on the genomic extent of hypomethylation (GEXH) for each chromosome. The results showed that chromosomes 1, 3, 8, 10, 12, 17, and 20 had a higher GEXH compared to the rest of the chromosomes (Supplementary Table S3). These findings are consistent with previous studies that have shown a higher incidence of chromosome breaks in these chromosomes.

**Figure 2.** Scatterplot of the distribution of the hypomethylation index and the cumulated genomic damage determined by two different techniques in colorectal carcinomas. **Box A**, GDF detected by AP-PCR in HSP series (n = 83). **Box B**, number of chromosomal alterations detected by CGH in the HUB series (n = 50). **Boxes C and D**, multiple regression analysis after categorization of tumors by the p53 mutational status. p53 mutation showed an additive effect in the number of genetic alterations (GDF, box C; number of chromosomal alterations, box D) to the hypomethylation index. Top regression line, p53 mutated tumors (crosses); bottom regression line, wild-type p53 tumors (open circles).
demethylation, we compared, for each chromosome alteration, the hypomethylation index between tumors with and without any given alteration. For most of the chromosomes, the hypomethylation index was higher in tumors exhibiting the specific alteration than tumors without (Fig. 3). Representation of the individual data illustrated that alterations for any given chromosome tended to occur more frequently in tumors with high hypomethylation levels (Fig. 3). This result suggests that DNA hypomethylation is likely to affect the stability of all the chromosomes, and that the differences in the frequency of alterations among chromosomes are rather a consequence of differential selective pressure (31).

Finally, to exclude a possible effect of chromosomal alterations on the determination of differential methylation indexes, we analyzed gross DNA methylation changes at chromosomal level by competitive hybridization of AIMS products to metaphase chromosomes in 10 tumors. This approach allows the detection of hypomethylations and hypermethylations at a genomic scale (16, 20). The differential methylation profiles were compared with the chromosomal profiles of losses and gains determined by CGH and no consistent parallelism between genetic and epigenetic alterations (gains with hypermethylation and losses with hypomethylation) was observed (Supplementary Figs. S2 and S3), indicating that AIMS changes are unaffected by chromosomal copy number alterations.

Demethylation variables that correlate with chromosomal instability. Hypomethylations detected by AIMS are broadly distributed throughout the genome (15, 17) and its computation represents a natural measure of demethylating events occurring in the tumor cells compared with its paired normal tissue. This measure, when transformed in an index, represents an estimation of genome-wide hypomethylations. Because AIMS screening through the genome is uneven, with a more intense representation of euchromatin and gene-rich regions (15, 17), the hypomethylation index calculated from AIMS fingerprints cannot be considered a representation of global hypomethylation, which also affects heterochromatin (32, 33). To get insights into the DNA methylation variables that are more likely to play a role in chromosomal instability, we did global and specific analyses. At global level, we determined 5-methylcytosine content in normal and tumor tissue in a short subset of cases. 5-Methylcytosine content was lower in tumors (normal 3.3 ± 1.0; tumor 2.7 ± 0.4; Wilcoxon test P = 0.026), but neither the relative loss of 5-methylcytosine (ratio of 5-methylcytosine content in the tumor with respect to the paired normal tissue) nor the tumor 5-methylcytosine showed statistically significant correlation with the hypomethylation index determined by AIMS (Supplementary Fig. S4).

Interestingly, the number of chromosomal alterations, although showing a good correlation with the hypomethylation index, showed no correlation with any of the 5-methylcytosine variable considered: global loss of 5-methylcytosine (T/N ratio), normal 5-methylcytosine content, and tumor 5-methylcytosine content (Supplementary Fig. S5). We think that these results are not in contradiction with previous studies done in model systems defective in DNMT1 and demonstrating a relationship between global hypomethylation and genomic instability (3, 10–12). The content of 5-methylcytosine in the tumor is consequence of the initial 5-methylcytosine content (that varies in a wide range from...
individual to individual, from 2.5 to 6.3%) and two opposite and independent processes: demethylation and de novo methylation. Therefore, the lack of correlation in our assessment was predictable.

Because AIMS bands showed a wide distribution in the rate of hypomethylation (0% to 78%), we wondered if the effect of hypomethylation on chromosomal instability could be attributed to a fraction of the sequences represented in the AIMS fingerprints, or, alternatively, it was a cooperative effect. We compared the number of chromosomal alterations in tumors with hypomethylation versus no hypomethylation for each one of the AIMS bands showing recurrent hypomethylation (in six or more tumors, 12%). No trends were observed between the hypomethylation rate (fraction of tumors showing hypomethylation for any given band) and the number of chromosome alterations (Supplementary Fig. S6). This implies that the association of hypomethylation with chromosomal instability results from the cumulated effect of multiple independent loci distributed genome wide (Supplementary Fig. S7). Therefore, it can be concluded that the observed correlations are explained by a genome-wide cumulative effect rather than by the concurrence of the most prevalent genetic and epigenetic alterations.

It should be noted that previous studies have attributed the global DNA hypomethylation observed in cancer cells to a demethylation of repetitive elements and heterochromatin (32). Nevertheless, most of the hypomethylated sequences isolated from AIMS fingerprints are of heterogeneous nature and map in gene-rich regions (15, 17),4 supporting the notion that DNA hypomethylation also involves unique sequences and euchromatin.

DNA hypomethylation and p53 mutations define the degree of chromosomal instability. It is well known that inactivation of the tumor suppressor gene p53 also plays a pivotal role in genetic instability (34). In agreement with previous studies, we observed increased rates of genomic alterations in tumors displaying mutations in the p53 gene (Table 1) in both series of cases. On the other hand, p53 mutations were not associated with a higher hypomethylation index. To fully dissect the possible interaction, we did regression analysis of genomic damage and hypomethylation index. To disentangle the interactions and the cause-consequence order of the different factors involved.

Compared with tumors with wild-type p53. In both cases, the correlation between DNA hypomethylation and genomic instability was maintained (Fig. 2C-D; Supplementary Table S2). The P value for the differences in the linear relationship according to p53 was 0.86 for GDF and 0.69 for CGH.

Whereas we do not show a causal relationship between DNA hypomethylation and genomic instability, an issue that has already been addressed in cell lines and murine models (see above), our results support the hypothesis that chromosomal instability is a direct outcome of DNA hypomethylation. Hence, p53 inactivation does not seem as the primary cause of genomic instability (35), but would contribute by releasing instability repression, which is manifested as an increase in the number of genetic alterations. This scenario is compatible with the early appearance of DNA demethylation in tumor progression (17, 33, 36).

Implications. This is a comprehensive study assessing the effect of genome-wide epigenetic deregulation on genomic instability in human colorectal carcinogenesis. Our results are consistent with a critical role of genome-wide hypomethylation in carcinogenesis. Its early contribution to genomic instabilization is supported by previous studies in model systems and hereditary cancer. DNA hypomethylation-induced chromosomal instability boosts the rate of losses of heterozygosity at the APC locus (the second hit in Knudson’s model) in APC(Min+/-) DNMT1 hypomorphic mice (with reduced genomic methylation), which results in an increased number of microadenomas (3). Moreover, a recent study suggests that chromosomal instability caused by another early alteration, APC mutation, is likely to be subtle and that chromosomal instability may even precede adenoma onset in polyposis patients (37).

Cumulated data have outlined tumor progression as alternative genetic pathways in colorectal cancer (24, 38, 39). Each pathway displays particular molecular profiles and confers differentiated biological and clinical behavior. Based on our results, we hypothesize that DNA hypomethylation is likely to induce a cascade effect with direct implications in the determination of the progression pathway, and hence the patient’s outcome. Future studies should dissect the interactions and the cause-consequence order of the different factors involved.

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References


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