Genetics supersedes epigenetics in colon cancer phenotype

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Summary

A CpG island DNA methylator phenotype has been postulated to explain silencing of the *hMLH1* DNA mismatch repair gene in cancer of the microsatellite mutator phenotype. To evaluate this model, we analyzed methylation in CpG islands from six mutator and suppressor genes, and thirty random genomic sites, in a panel of colorectal cancers. Tumor-specific somatic hypermethylation was a widespread age-dependent process that followed a normal Gaussian distribution. Because there was no discontinuity in methylation rate, our results challenge the methylator phenotype hypothesis and its hypothetical pathological underlying defect. We also show that the mutator phenotype dominates over the gradual accumulation of DNA hypermethylation in determining the genotypic features that govern the phenotypic peculiarities of colon cancer of the mutator pathway.

Introduction

The accumulation of genetic alterations during tumorigenesis substantiates the mutational theory of cancer (Loeb, 1991), but epigenetic alterations are also germane to carcinogenesis (Jones and Laird, 1999). Evolution by natural selection has developed epigenetics as a means to efficiently achieve the regulated fluctuations in gene expression that govern cell differentiation and development. This process has been called the epigenetic code (Breivik and Gaudernack, 1999) or histone code (Jenuwein and Allis, 2001; Turner, 2002). In a highly interrelated and complex process, posttranslational modifications in histones and other chromatin proteins, together with changes in DNA methylation at CpG sequences, lead to changes in gene expression and permanent silencing (Bird, 2001; Baylin and Bestor, 2002).

Somatic hypomethylation and hypermethylation have been associated with tumorigenesis. Global hypomethylation occurs in human tumors (Feinberg and Vogelstein, 1983) and can either suppress (Laird et al., 1995) or induce (Eden et al., 2003) tumors in mice. Hypermethylation has been linked to tumor suppressor gene silencing (Jones and Baylin, 2002). Perhaps the best example is the silencing of *hMLH1* DNA mismatch repair (MMR) gene in colon cancer.

About 13% of unselected colon tumors accumulate hundreds of thousands of somatic mutations in microsatellite sequences (Ionov et al., 1993). MMR deficiency underlies this genome-wide microsatellite instability (MSI) in some hereditary nonpolyposis colorectal cancers (HNPCC) and sporadic gastrointestinal tumors (Kolodner and Marsischky, 1999). Some of these MSI-positive cancers undergo epigenetic silencing of the DNA MMR gene *hMLH1*, a process that is accompanied by hypermethylation of the gene's promoter (Kane et al., 1997). The existence of a CpG island methylator phenotype (CIMP) has been postulated to explain the somatic hypermethylation associated with silencing of the *hMLH1* mutator gene and several tumor suppressor genes (Ahuja et al., 1997; Toyota et al., 1999a).

The CIMP has been proposed to be responsible for the manifestation of the microsatellite mutator phenotype (MMP) characteristic of tumors with MSI (Toyota et al., 1999a; Toyota and Issa, 2000). MSI-positive tumors display a mutator phenotype characterized by an over two orders of magnitude higher mutation rate than normal cells (Ionov et al., 1993; Shibata et al., 1994). The MMP leads to the accumulation of oncogenic mutations in cancer genes (oncogenes and tumor suppressors), ultimately leading to cancer (Perucho, 1996; Kinzler and Vogelstein, 1996).

The MMP is a critical determinant of the fate of the tumor cell and drives tumorigenesis through a defined pathway (Perucho et al., 1994; Perucho, 1996; Olschwang et al., 1997; Breivik et al., 1997). Mutator genes are therefore more fundamental than oncogenes and tumor suppressor genes, as the former cause mutations that trigger the oncogenic potential of the latter. Cancer driven by mutator genes represents a "remote control" mechanism for carcinogenesis, as mutator gene inactivation

SIGNIFICANCE

Silencing of *hMLH1* in tumors of the microsatellite mutator phenotype illustrates the importance of epigenetics in cancer. The bimodal distribution of ubiquitous microsatellite mutations defines the microsatellite mutator phenotype, a critical determinant of tumor cell fate driving tumorigenesis through a specific pathway. The methylator phenotype hypothesis, as begetting the microsatellite mutator phenotype, adds a more fundamental earlier step in carcinogenesis. However, our evidence for the absence of a bimodal distribution for somatic hypermethylation is conclusive since it was obtained by an unbiased approach. The epigenetic origin of the mutator phenotype, and cancer, remains fascinating and mysterious. However, it is not initiated by a punctual event underlying a pathogenic methylator phenotype, but rather by a gradual age-dependent disintegration of the epigenetic code.

does not immediately lead to altered cell growth or survival (Perucho et al., 1994; Perucho, 1996; Cahill et al., 1999).

The sequence of events in the MMP pathway for cancer can be summarized as follows: inactivation of MMR (mutator) genes causes a mutator phenotype, which causes oncogenic mutations, which cause cancer. The implicit sequence of events in the methylator phenotype "ultra remote control" pathway for cancer is as follows: CIMP causes inactivation of MMR, which causes the MMP, which causes oncogenic mutations, which cause cancer (Toyota et al., 1999a). Methylator phenotype is thus even more fundamental than mutator phenotype for cancer pathogenesis, as it generates the MMP in tumors not carrying germline and/or somatic MMR mutator mutations.

Despite its importance, the CIMP concept has not been precisely defined. The current definition rests on the simultaneous tumor-specific hypermethylation of multiple CpG islands. The CIMP conceptually parallels the MMP that was defined by the presence of ubiquitous somatic mutations in mononucleotide repeats (Ionov et al., 1993). But the MMP was defined precisely because the majority of the tumors did not contain these mutations. The bimodal distribution of microsatellite mutations allowed the segregation of colon tumors into tumors with and tumors without these ubiquitous microsatellite mutations (Ionov et al., 1993; Perucho et al., 1994).

The methylator phenotype hypothesis also necessarily implies a clear-cut distinction between tumors with and tumors without an enhanced pathological rate of somatic DNA hypermethylation. This segregation is necessary to postulate that some tumors possess a methylator phenotype, similar to the mutator phenotype possessed by tumors of the MMP. The methylator phenotype concept is important because it implies an underlying defect in the cellular machinery responsible for the generation of hypermethylation events, similar to the MMR deficiency underlying MSI and the MMP (Laird, 2003). We carried out this study to test this hypothesis. We analyzed the hypermethylation alterations occurring in CpG islands in some cancer genes (mutators and suppressors) and random genomic sites, in a panel of colorectal tumors with and without MSI. The results show that tumor-specific somatic hypermethylation is a widespread phenomenon shared by all colon tumors and that the alterations are dispersed into a nearly perfect normal Gaussian distribution when a sufficient number of loci are analyzed. We also show that the MSI phenotype is dominant over the CpG island methylation phenotype.

Results

Hypermethylation of cancer genes and anonymous CpG islands

We examined by methylation-specific PCR (MSP) (Herman et al., 1996) the promoters of six genes in a panel of 207 colorectal cancers and the corresponding paired normal tissue. These included the MMR mutator gene *hMLH1*, the *O^{6}MGMT* DNA repair gene, and the tumor suppressor genes *p16^{INK4A}*, *p14^{ARF}*, *APC*, and *CDH1* (*E-cadherin*) (Figure 1A). All of these genes undergo hypermethylation in colon cancer (Toyota et al., 1999a; Esteller et al., 2001; Shen et al., 2003). A diagram of the tumor arrangement based on the number of methylated loci per tumor formed a gradual pattern rather than a bimodal distribution (Figure 1B). These results are not compatible with the CIMP hypothesis since it is not possible to decide with a sound criterion which of the tumors induce methylation with rates higher than normal cells and which ones do not (Figure 1C). However, because the number of loci analyzed was very small, it was possible that a distinctive group of tumors could contain none or very few methylation alterations (Figures 1B and 1C).

To directly address this possibility, we analyzed the global methylation pattern in 32 colorectal cancer specimens by methylation-sensitive amplified fragment length polymorphism (MS-AFLP). The technique permits the unbiased analysis of the methylation status of a random subset of CpG sequences throughout the genome (Yamamoto et al., 2001). In these particular experiments, we scanned the status of the two CpG sites contained in the NotI restriction endonuclease methylation-sensitive recognition site (G**CG**GC**CG**C). In MS-AFLP fingerprinting, tumor-specific somatic hyper- and hypomethylation are recognized as differences of band intensity between PCR products derived from normal and tumor DNA (Figure 2).

About 100 bands appeared in each experiment. Of these, about 75 were universally amplified from normal tissue DNA (Figure 2). Nine of eleven such bands, characterized by cloning and sequencing, matched human sequences derived from the Human Genome Project through BLAST search. Only one of the NotI sites in the nine characterized sequences was not inside a well-defined CpG island according to the currently accepted criterion (Gardiner-Garden and Frommer, 1987).

Hypermethylation and hypomethylation in colon cancer

About 40% of all CpG sequences analyzed in these experiments exhibited methylation alterations. Hyper- and hypomethylation were each observed in about 30 of the 75 bands ($\sim$40%) with a 20% overlapping (six bands showing both hyper- and hypomethylation in different tumors). The average level of hypermethylation in the 32 tumors analyzed was 21.5% (203 hypermethylated bands of 944 total analyzed bands) while the hypomethylation average was 10.2% (108 of 1056 total analyzed bands). The average number of altered bands per tumor was 6.34 for hypermethylation and 3.3 for hypomethylation. There was no correlation between hypermethylation and hypomethylation. Although tumors with levels of hypermethylation higher than average had a higher level of hypomethylation, and vice versa, the differences were not significant (data not shown).

In this report, we focus on hypermethylation and we will not further elaborate on hypomethylation. The methylation status of 30 bands showing tumor-specific hypermethylation is summarized in Figure 3A. Some bands without methylation changes (positions 5 and 17 of Figure 3A top panel and the last two positions in bottom panel) were included in this analysis because they exhibited alterations in a parallel analysis of 20 gastric cancers. Bands with no methylation alterations in colon and gastric cancers were excluded. Some bands exhibited more alterations than others (top panel), but the alterations were dispersed gradually among the colon tumors (bottom panel). The results with the gastric cancers analyzed were similar (data not shown).

Distribution of genetic and epigenetic somatic alterations

Figure 3B shows the effect of increasing the number of loci analyzed in the shape of the distribution of methylation alterations in the tumors. Analysis of only 5 loci was ambiguous, with a gradual diminishing of tumors containing multiple methyl-

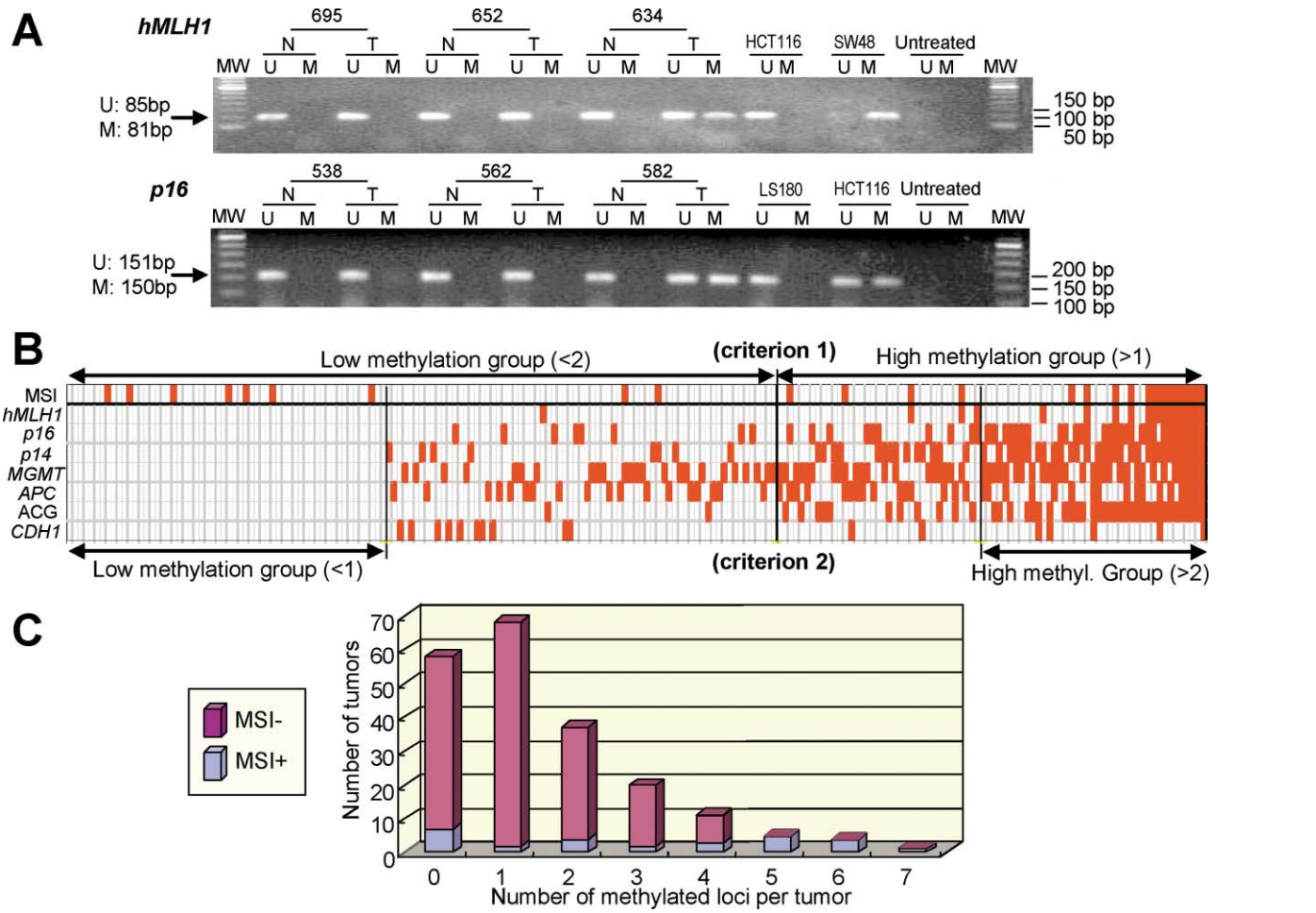

Figure 1. Gradual distribution of CpG island DNA methylation alterations in colon cancer

A: Representative MSP experiments for methylation analysis of *hMLH1* and *p16* genes. PCR products amplified with unmethylated (U) and methylated (M) sequence-specific primers. Untreated DNA is a negative control in which the sodium bisulfite treatment was omitted. MW: DNA fragment ladder used as molecular weight marker.

B: Summary of CpG hypermethylation. Upper: Methylation status of six CpG islands from known suppressor/mutator genes as well as an anonymous CpG island (ACG) identified through MS-AFLP (see Figure 2) in 207 colorectal cancers (filled: methylated, blank: unmethylated). Microsatellite instability (MSI) is indicated at the top row (filled, MSI-positive, blank, MSI-negative). The cut off points for the grouping of tumors into two groups with relatively high and low methylation alterations are indicated (see text).

C: The methylated loci per tumor from the above data show a nonbimodal distribution. MSI-positive tumors were also evenly distributed.

ated loci (Figure 3B, top), similar to the curve of the functional loci (Figure 1C). However, when more loci were analyzed, the distribution profile acquired a distinctive Gaussian shape, reflecting a normal distribution of random events (Figure 3B, lower panels). No single tumor remained without methylation alterations after all 35 loci were considered. Therefore, tumor-specific somatic hypermethylation is a widespread and gradual phenomenon. In contrast, the distribution of microsatellite mutations was distinctively bimodal, regardless of the number of loci analyzed (Figure 3B).

Thus, there is a fundamental difference between the tumor-specific accumulation of CpG methylation alterations and microsatellite mutations. The methylation alterations distributed gradually, without a defined boundary (Figure 3A). Mutations in microsatellite sequences exhibited on the other hand a discontinuous distribution of (Figure 3C), with a sharp border splitting the tumors with from the tumors without abundant microsatellite mutations.

Methylator versus mutator genotype-phenotype relationships

We next compared the genotypic features of colon tumors with respect to their mutator and 'methylator' phenotypes. We divided the tumors according to the methylation status of the CpG islands analyzed by MSP (Figure 1B), into a group with two or more methylated CpG islands and another with one or none (criterion 1). This is a division at approximately the median value for methylation (1.5 methylated loci per tumor). The results showed a significant association between methylation and right side location (Figure 4A). Comparison of tumors with no methylation alterations with those with more than 2 alterations (criterion 2), that resembles the comparison of lower and higher quartiles

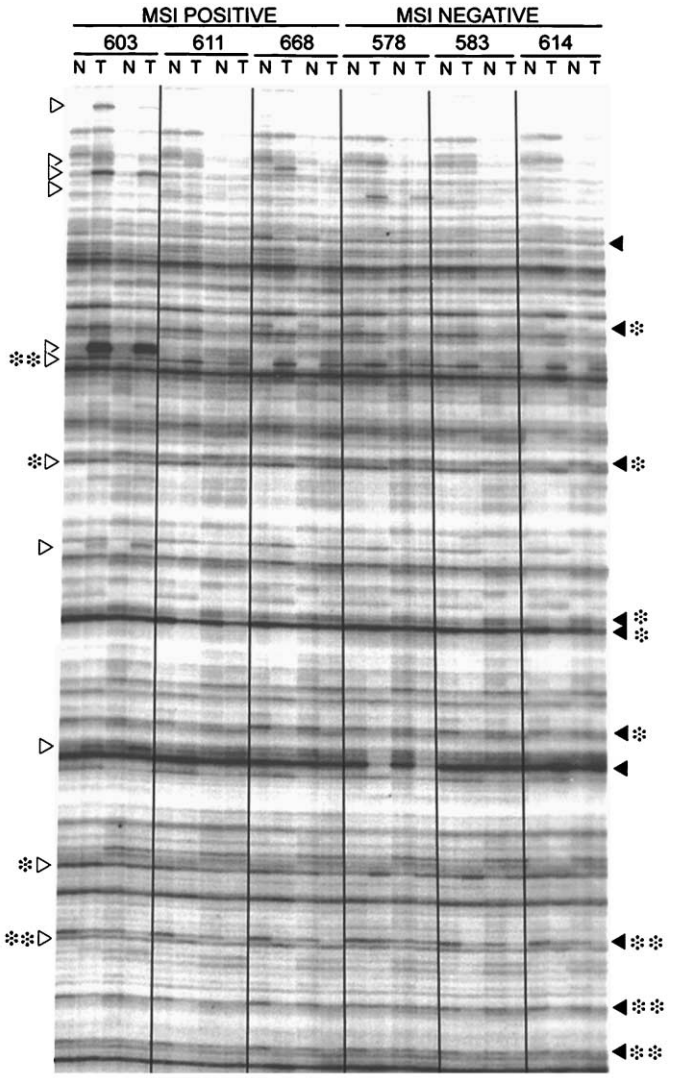

Figure 2. DNA hypermethylation in colon cancer detected by MS-AFLP DNA fingerprinting

Autoradiogram of a MS-AFLP fingerprint of colon tumors with and without MSI. Two different amounts of template DNA (10 and 15 ng) were amplified for each sample. Left empty arrowheads represent hypomethylation, whereas right solid arrowheads indicate hypermethylation. Asterisks denote bands exhibiting recurrent hypo- or hypermethylation in several tumors, with the most common alterations highlighted by double asterisks. N: normal, T: tumor DNA. Additional altered smaller bands were detected in the same experiment by shorter electrophoresis.

(Figure 1B), yielded similar results (data not shown). No significant associations were observed with the rest of the parameters analyzed (gender, progression, differentiation, K-*ras* or *p53* mutations).

On the other hand, MSI positive tumors presented significant differences compared to MSI negative tumors, in accord with our previous findings (Ionov et al., 1993; Yamamoto et al., 2002). MSI positive tumors were predominantly found in the proximal colon, were less advanced in tumor progression, and many exhibited a poorly differentiated phenotype. MSI positive tumors also displayed a low mutational incidence in the cancer genes prototypical in colorectal tumorigenesis, K-*ras* oncogene and *APC* and *p53* tumor suppressors (Figure 4B).

Comparison of the genetic and epigenetic alterations in regard to tumor cell phenotype revealed that the mutator phenotype and the genetic alterations (MSI) were dominant over the "methylator phenotype" and the epigenetic alterations (CpG island methylation). The differences in genotype and phenotype observed between MSI-positive and-negative tumors were independent on CpG island methylation status (Figure 4D). On the other hand, filtering out the MSI-positive cases from the population of tumors diminished the asymmetries between relatively high and low CpG island methylation in stage and grade, and K-*ras* and *p53* mutations (Figure 4C). Therefore, while tumors with and without the MMP exhibited marked differences in genotype and phenotype, tumors with relatively high and low CpG island DNA methylation were essentially indistinguishable.

The preferential location in the proximal colon was shared by DNA hypermethylation and MSI, with the latter showing an even more pronounced tendency (Figure 5). The only significant feature of DNA hypermethylation that was not shared with MSI was its association with older age. The methylation of each CpG island in particular, and the average of all the CpG islands, increased with the patients' age (Figure 6).

Discussion

Since the methylator concept was proposed in colon cancer (Ahuja et al., 1997; Toyota et al., 1999a), this hypothesis has received considerable attention, and the CIMP is currently regarded as the determining event driving a particular carcinogenesis pathway (Toyota et al., 1999a; Toyota and Issa, 2000; Elsaleh et al., 2000; Peltomaki et al., 2000; Baylin and Bestor, 2002; van Rijnsoever et al., 2002; Iacopetta, 2003). The CIMP has also been assigned to various types of tumors other than colorectal cancer. So far, the CIMP has been reported in neoplasms of stomach (Toyota et al., 1999b), pancreas (Ueki et al., 2000), ovary (Strathdee et al., 2001), hepatocellular carcinoma (Shen et al., 2002), and adenoma (Rashid et al., 2001), as well as hyperplastic polyp-polyposis (Chan et al., 2002) of the large intestine. Two groups have recently reported the absence of evidence supporting the CIMP model. One group interpreted their data as not supporting the CIMP hypothesis in esophageal adenocarcinoma (Eads et al., 2001) and another group reported data inconsistent with the CIMP in colorectal cancer, although the results were not explicitly interpreted as contradicting the CIMP hypothesis (Hawkins et al., 2002).

The definition of the CIMP has been in constant evolution since its inception (Toyota et al., 1999a, 1999b, 2000; Ueki et al., 2000; Shen et al., 2002, Rashid et al., 2001; Chan et al., 2002). Initially the concept was defined by the "frequent concordant methylation of the type C clones examined" (Toyota et al., 1999a). The CIMP-positive group was classified as having a high level of type C methylation (three or more loci) (Toyota et al., 1999a). Subsequently, the CIMP has been diversely characterized from a methylation tendency (Peltomaki et al., 2000) to a variable pattern of hypermethylation of CpG islands in tumor suppressor genes (Shen et al., 2002). The current common definition of simultaneous methylation of multiple CpG islands is not very precise. The issue is further complicated because tumors were classified into two distinct categories (CIMP+ and CIMP−) only in the first report of colorectal tumors (Toyota et

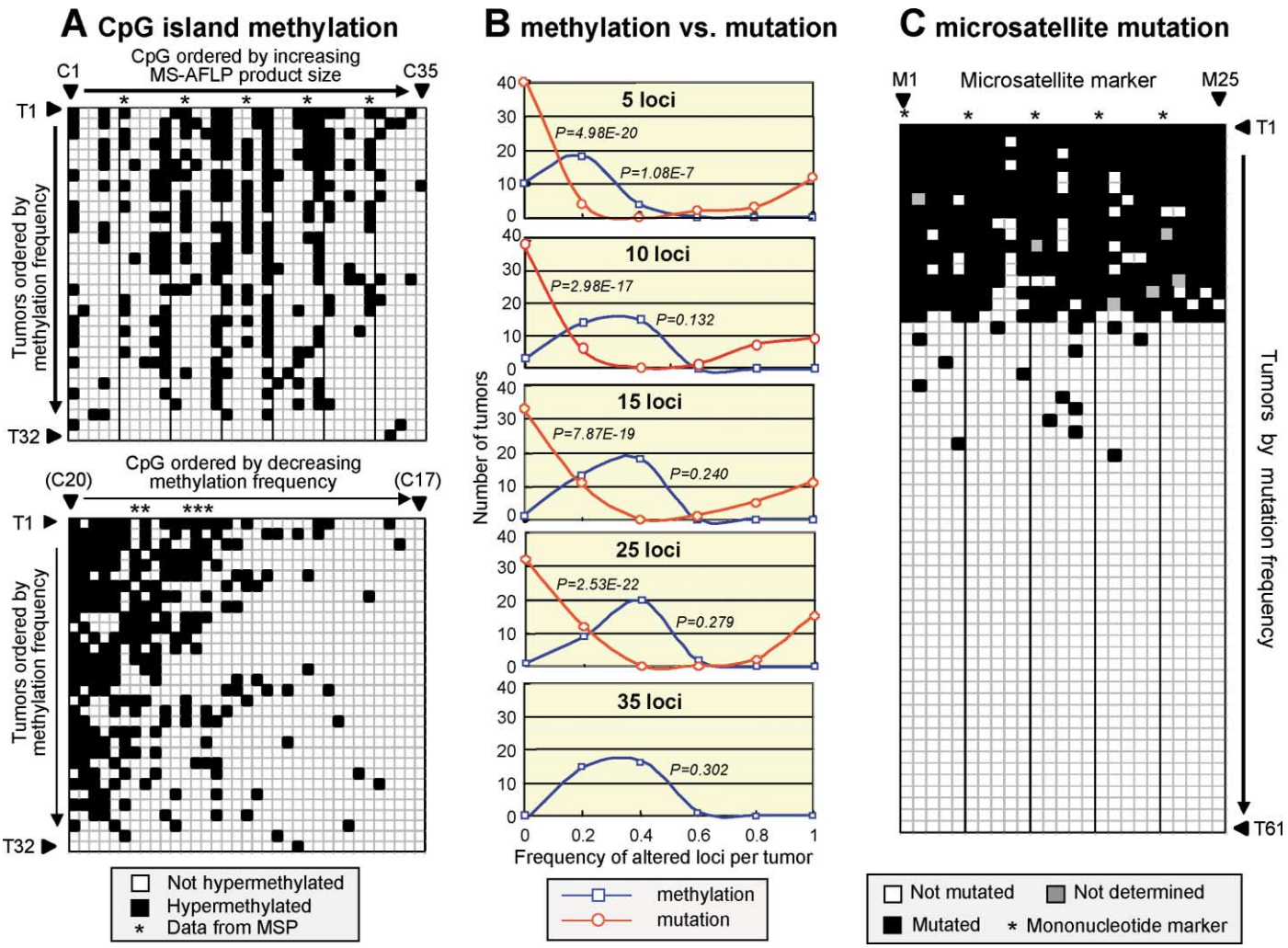

Figure 3. DNA hypermethylation follows a normal distribution in contrast with the bimodal distribution of MSI

A: DNA hypermethylation is gradual in colon cancers. The methylation status of 30 NotI CpG sites analyzed by MS-AFLP is shown. The five loci analyzed by MSP are also included. In the top panel, the 35 CpGs (each lane) are ordered in an unbiased manner according to the size of the MS-AFLP fingerprint band. The five MSP loci are evenly distributed after every five loci. In the bottom panel, the 35 CpG loci and the 32 colorectal cancers are sorted out by decreasing methylation frequency.

B: The distribution of CpG epigenetic alterations is dependent on the number of loci analyzed, in contrast with the independency of MSI-genetic alterations. The tumors are distributed by percentage of methylated and mutated loci analyzing 5 (top) to 35 (bottom) CpGs. The microsatellite markers are also distributed in groups of five. The DNA methylation data is derived from the top panel of Figure 3A and the mutation data from Figure 3C. The first five loci in this panel correspond to the first 5 CpGs and the first 5 microsatellite markers at the left in panel A top and panel C, respectively. Scale on the X axis: 0 = tumors with no hypermethylation or mutation (0%); 0.2 = tumors with one or more loci altered up to 20%; 0.4 = tumors with more than 20% up to 40% loci altered, and so on. P values were obtained from the statistical comparison of observed values with those predicted for a normal distribution.

C: MSI is discontinuous in colon cancer. The extent of MSI was analyzed using 25 microsatellite markers (5 mononucleotide and 20 dinucleotide markers) in 61 colorectal cancer specimens, which are sorted out by mutation frequency.

al., 1999a). In almost all subsequent publications (Toyota et al., 1999b; Ueki et al., 2000; Shen et al., 2002; Rashid et al., 2001; Chan et al., 2002), additional groups such as CIMP-I (intermediate) or CIMP-L (low) were used to embrace tumors with intermediate levels of methylation. Despite these difficulties, the classification of CIMP-positive tumors is usually done by selecting tumors with 2–3 altered genes of the commonly used 3–6 loci. This is arbitrary, as recognized by the same authors of these reports (Shen et al., 2002; van Rijnsoever et al., 2002). Nevertheless, the CIMP model remains generally supported even when the experimental data appears inconsistent with the concept (Ueki et al., 2000; Rashid et al., 2001; Shen et al., 2002, 2003; van Rijnsoever et al., 2002; Chan et al., 2002).

Our data with colon (Figures 1 and 3) and gastric (not shown) cancer, as well as the data from others (Eads et al., 2001; Hawkins et al., 2002; Shen et al., 2002, 2003), show that it is impossible to draw a precise borderline between CIMP-positive and -negative tumor groups due to the gradual distribution pattern of CpG island somatic hypermethylation, which is far from the bimodal distribution reported in the original paper (Toyota et al., 1999a). The reason for this discrepancy might partly rest on the criterion that originated the CIMP in the initial report.

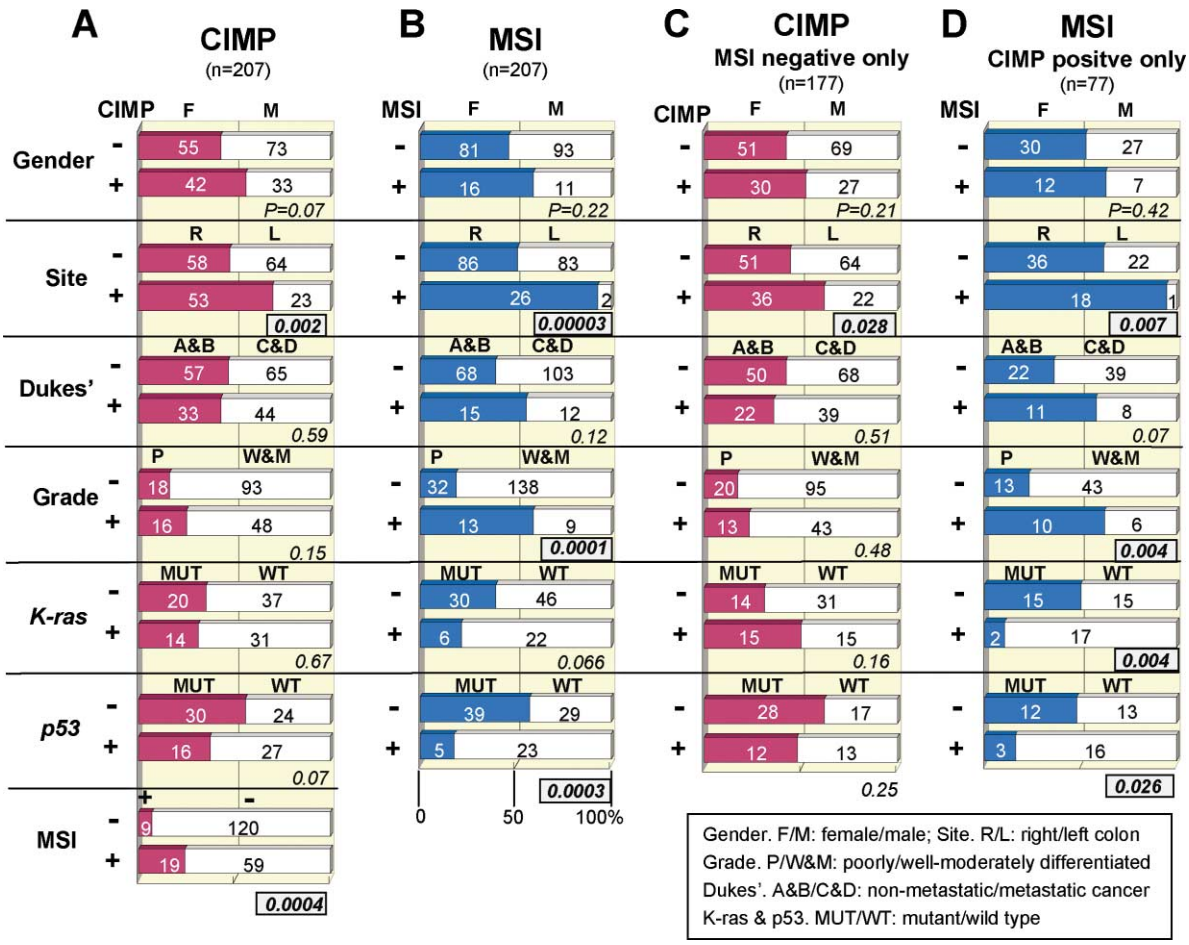

Figure 4. Genetics, but not epigenetics, underlies colon cancer clinicopathological features

A: CpG island methylation associates to proximal location but not to any of the other phenotype parameters. Tumors with weak methylation (CIMP−) were compared with those with intense methylation (CIMP+) using the MSP data from the 207 cases. Numbers on bars represent the actual number of cases, and p values less than 0.05 are highlighted. The borderline between CIMP− and CIMP+ was derived by criterion 1 elaborated in Figure 1. Criterion 2 also yielded similar results, with only the site showing significant right side predominance ($p = 0.00001$). The MSI/CIMP relationship also remained significant ($p = 0.004$).

B: Mutator phenotype exhibits strong associations with many parameters, including right side preference. The panel demonstrates the results from the 207 cases used for the MSP analysis. The distribution of MSI-positive/negative tumors regarding tumor progression was 3/11 (21%); 12/57 (17%); 11/63 (15%); 1/35 (2.7%); and 0/5 (0%) for Dukes' A:B:C:D: liver metastases, respectively ($p = 0.024$ χ^2 test for trends). Results obtained from a larger tumor sample for which the MSI status has been previously determined (Malkhosyan et al., 2000) showed that the significant associations of MSI-positive tumors extended to gender (female predominance, $p = 0.0017$); to Dukes' stage (nonmetastatic predominance, $p = 0.003$); and K-*ras* (wild-type predominance, $p = 0.0001$). *APC* suppressor gene mutations were also analyzed in a subset of tumors with a significantly lower mutational incidence in MSI-positive cancers (14 *APC* mutant and 36 wild-type in MSI-positive tumors versus 36 *APC* mutant and 37 wild-type in MSI-negative tumors, $p = 0.017$).

C: CIMP does not differentiate among colon cancers. MSI-positive cases were eliminated from the analysis.

D: MSI is dominant over CIMP. MSI-positive and MSI-negative tumors were compared using only cases with intense methylation (CIMP+) according to criterion 1 (Figure 1).

Analysis of individual CpG islands (not shown) showed that methylation of *hMLH1* correlated with right side ($p = 0.0004$) and female predominance ($p = 0.06$), old age ($p = 0.003$), poorly differentiated histological phenotype ($p = 0.0002$), and low mutational incidence at K-*ras* and *P53*. Similarly, *p16* methylation correlated with right side, poorly differentiated phenotype, and methylated *p14* and *MGMT* exhibited right side predominance. These characteristics overlapped with the features of MSI-positive colon cancer. Except right side location, these differences lost their statistical significance in multiple regression analysis and univariate analysis after MSI-positive tumors were eliminated (not shown).

Mutation analysis for K-*ras* and *p53* was performed as previously described (Ionov et al., 1993; Yamamoto et al., 1997, 1999). Somatic *APC* mutations (from codons 865 to 1590, comprising the mutation cluster region in exon 15) were identified and characterized by SSCP and sequencing of normal-tumor DNA as described (Yamamoto et al., 1997, 1999). p values were calculated by Fisher's exact test or Chi square with Yates correction.

The CIMP concept was reached after selecting particular types of CpG islands showing methylation, those denominated class C or cancer specific. Class A, or age-specific, CpG islands were excluded from the analysis (Toyota et al., 1999a). However, the distinction of class A from class C CpG islands depended on whether methylation could be detected in normal tissues, while the semiquantitative method utilized for detection was not very sensitive (Toyota et al., 1999a; Shen et al., 2002, 2003). A CpG island showing a faint band in the MSP experiments from the normal tissue DNA was classified as belonging to class A,

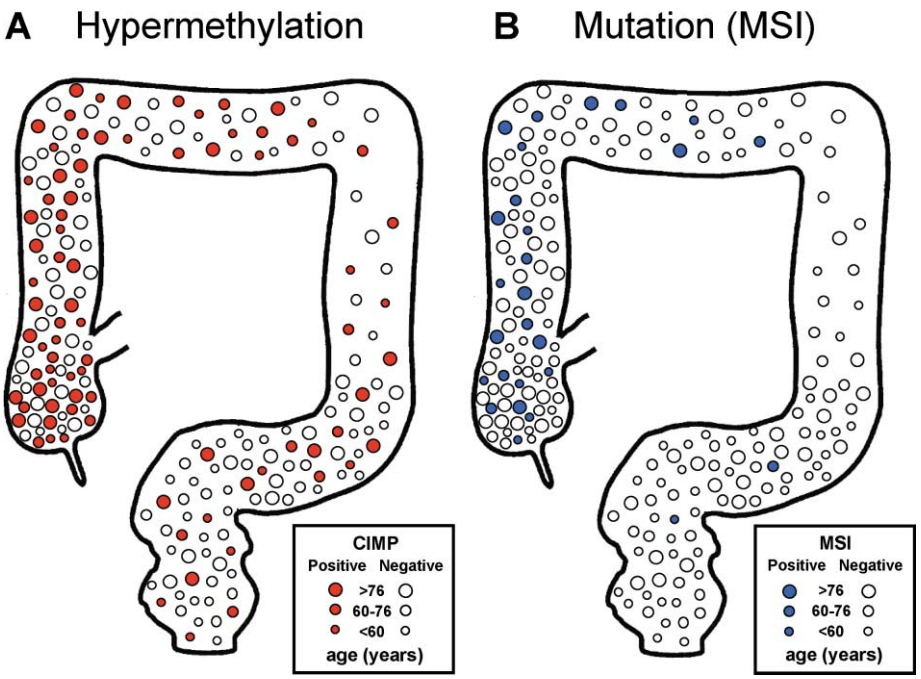

Figure 5. Gradient of CpG island DNA methylation and MSI in colon cancers in the large intestine

The tumors are classified for CIMP according to criterion 1 (Figure 1).

but another CpG island failing to reveal a band was classified as class C. However, the CpG islands initially classified as type C have been subsequently shown to be methylated in normal tissues when the sensitivity of the detection assays was increased (Kuismanen et al., 1999; Shen et al., 2003). Moreover, these class C loci also have been found to have a tight connection with aging, which was a peculiarity of the class A CpG islands (Kuismanen et al., 1999; Malkhosyan et al., 2000; Shen et al., 2003; this work). In other words, a class C CpG island is no more than a disguised class A CpG island.

There is no clear boundary between class A and class C CpG islands, and they follow a gradual distribution regarding their presence and detectability in normal tissues. Furthermore, a CpG island that is classified as class C in one type of tumor is classified as class A in another type of tumor (Eads et al., 2001; Ueki et al., 2000). But if there is hypermethylation of a particular CpG island in *any* normal tissue, this means that it is not cancer specific. The restriction of some class C CpG islands to a particular tumor type would lead to the enigmatic concept of a tissue-specific and tumor-specific methylator phenotype. Therefore, the CIMP has become a convoluted, ambiguous, and untenable concept. The contradictory nature of what CIMP has

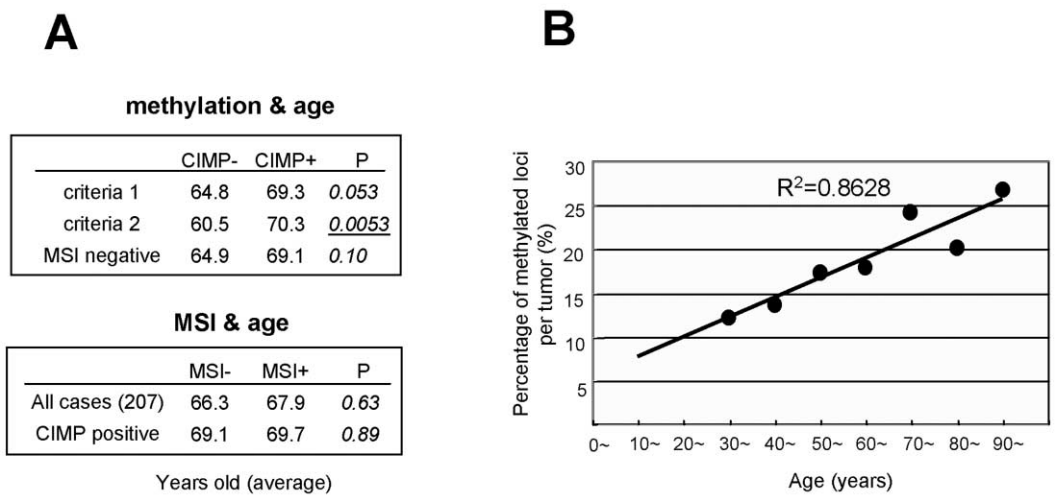

Figure 6. Age is the only parameter where DNA hypermethylation is independent of MSI status

A: Average ages of each patient group of the 207 tumor cohort (Figure 4) were compared by Student's t test.
B: The 207 patients were divided into seven groups according to age with a 10 year interval. The average of methylated loci per tumor (percentage of the seven loci) was plotted for each age group.

become can be exposed by the statement that the cancer-specific hypermethylator phenotype can also occur in normal tissue (Peltomaki et al., 2000).

The data from Figure 3 imply that up to 40% of all genomic CpG islands undergo hypermethylation in some colon cancers. This extrapolation is based on the unbiased nature of the NotI sites analyzed by MS-AFLP DNA fingerprinting (Yamamoto et al., 2001) and the calculation that the vast majority (~80%) of NotI sites are located in CpG islands (Kutsenko et al., 2002). The last premise for such an extrapolation is that there are no structural or functional differences between CpG islands with and without NotI sites. In this line, other different NotI sites amplified with different primer/adaptor combinations also have similar levels of hypermethylation (unpublished observations). Therefore, if hypermethylation were due to defects in transacting factors required for the protection of CpG islands from encroaching methylation (Laird, 2003), individual "universal" transacting factors would be required to cover a significant fraction of all the genomic CpG islands. Therefore, it is more likely that the methylation changes (both hyper- and hypomethylation) represent frequent and accumulative stochastic fluctuations (Laird, 2003) occurring prior to and/or during tumorigenesis.

Many of these individual random CpG sequences were hypermethylated in a majority of the colon and gastric tumors (six different MS-AFLP bands were each hypermethylated in over 50% of the tumors, Figure 3A and data not shown). The results also imply that widespread CpG island hypermethylation often affects the same genomic loci in tumorigenesis. Therefore, if different defects were to lead to hypermethylation of distinct groups of structurally similar CpG islands (Laird 2003), this would require the concomitant existence in each tumor of several defective methylator genes. The results obtained by unsupervised clustering that show groups of tumors with high and low levels of concordant methylation (Yan et al., 2002) can be better explained by arbitrary ordering of the clustered tumors and arbitrary selection of subclusters of CpG islands.

Nevertheless, even though no CIMP underlies the common somatic methylation alterations observed in many tumors, there is an unequal distribution of methylation alterations following an increasing gradient from the distal to proximal colon (Figure 5). Another critical variable is the age. Methylation of all CpG islands analyzed in colorectal cancer demonstrated an inclination toward older age (Figure 6). Together with other reports (Kuismanen et al., 1999; Wiencke et al., 1999; Malkhosyan et al., 2000; van Rijnsoever et al., 2002; Shen et al., 2002), these results show that hypermethylation of most CpG islands is a process strongly age dependent in gastrointestinal tumorigenesis. Thus, proximal colon cancers in old patients have more methylation than distal cancers in young patients (Figure 5).

The reason underlying this distortion remains mysterious, but it implies a disintegration of the epigenetic code during aging in tissues with high cell turnover. However, to assign a methylator phenotype to the increased gradient of methylation in the proximal colon of old cancer patients (from whatever unknown reasons) would be equally inadequate as to assign a mutator phenotype to a concentration gradient of carcinogens causing mutations at p53 in the distal colon (Breivik and Gaudernack, 1999).

DNA hypermethylation at multiple loci correlates with MSI-positive colon cancer (Figure 4A). This is because many tumors with MSI, especially sporadic cases, owe the mutator phenotype to *hMLH1* silencing and this is linked to its hypermethylation. But the determinant genotypic feature that propels the phenotypic differences of colorectal cancer is MSI and mutator phenotype and not CpG island hypermethylation and methylator phenotype (Figure 4). Our results show that differences in genotype and phenotype (K-*ras* and p53 mutations, differentiation and progression) are independent of the status of CpG island methylation (Figure 4D). On the other hand, if MSI-positive cancers are eliminated, the remaining tumors with higher DNA methylation were indistinguishable from the group of tumors with lower methylation, regardless of the criteria for their segregation (Figure 4C).

The distinctive features that MMP tumors display compared with tumors without MSI is due to the particular spectrum of mutated cancer genes in MSI-positive tumors (Markowitz et al., 1995; Rampino et al., 1997; Lindblom, 2001). MMP tumors display a low incidence of mutations in the *APC* and *P53* tumor suppressor genes and K-*ras* oncogene, prototypical for colon cancer (Ionov et al., 1993; Perucho et al., 1994; Kim et al., 1994; Olschwang et al., 1997; Breivik et al., 1997). Instead, MSI-positive colon tumors carry a plethora of different mutated genes, such as *TGFßRII* and *Bax*, which are rarely found in MSI-negative tumors (Markowitz et al., 1995; Rampino et al., 1997; Woerner et al., 2003). This is because in a MMR deficiency background, mutations occur preferentially in genes with simple repeats in their coding or regulatory sequences (Perucho, 1996; Suzuki et al., 2002). The differences in genotype can explain the differences in phenotype displayed by MMP tumors, such as poorly differentiated histological features, a less advanced stage of tumor progression, and a better survival (Ionov et al., 1993; Thibodeau et al., 1993; Boland et al., 1998; Elsaleh et al., 2000).

The anatomical distribution of MSI tumors overlaps the preferential localization of hypermethylation in the proximal colon (Figures 4 and 6). The reason for the exacerbated asymmetry in anatomical location for both mutator and methylator phenotypes is intriguing. HNPCC tumors also show a preferential localization in the proximal region of the large bowel, although the disparity (about 70%) (Kuismanen et al., 2000) is not so remarkable as in sporadic tumors (about 90%) (Kuismanen et al., 2000; Figure 4).

The preferential proximal colon location of both MSI-positive tumors and CpG island methylation tumors could be due to an intrinsic asymmetry in some critical cellular processes between proximal and distal colon, such as stem cell renewal and mitotic activity (Lipkin et al., 1962; Potten et al., 1992). A higher mitotic activity may be sufficient to increase the probability of occurrence of both mutator mutations (somatic structural alterations inactivating MMR genes) and epigenetic silencing of *hMLH1*, and more importantly, the necessary additional cell replications *before* neoplastic transformation can eventually occur (Ionov et al., 1993; Perucho et al., 1994; Perucho, 1996; Tsao et al., 2000). The age dependence of MSI-positive tumors with *hMLH1* methylation is also consistent with this hypothesis. The difference in incidence of MSI-positive proximal tumors between hereditary and sporadic cases could be explained by the lower dependence of the hereditary cancers on mitogenesis, as these cancers have a shortened pre-neoplastic period due to the germline mutations.

This hypothesis predicts that MSI-positive tumors with low methylation will be also preferentially found in the proximal colon regardless of their hereditary or nonhereditary origins. Our data

are consistent with this hypothesis although corroboration with a larger sample is needed: 7 MSI-positive/51 MSI-negative proximal versus 1 MSI-positive/64 MSI-negative distal ($p = 0.020$) in the CIMP-negative group and 18/36 versus 1/22 in the CIMP-positive group ($p = 0.007$; Figure 4D).

The reason for the age-dependent silencing and hypermethylation of *hMLH1* remains to be elucidated. However, the apparent lack of association of MSI with aging is explained by the heterogeneous mixture of familial and sporadic tumors in unselected colorectal cancer series. Familial cancers occur in younger patients while silencing and methylation in older tumor patients, and the two extremes neutralize each other (Malkhosyan et al., 2000).

While age dependence and anatomical localization are MMP independent, the genotype and phenotype features of colon cancers with MSI supersede those displayed by the high hypermethylation tumor group. There is no clear explanation for the association of MSI and CpG methylation with gender (Breivik et al., 1997; Malkhosyan et al., 2000; Elsaleh et al., 2000), and environmental and genomic factors may play roles. However, anatomical location, gender, and age preempt cancer development and fall beyond cancer cell genotype-phenotype relationships.

In conclusion, after its manifestation, the microsatellite mutator phenotype appears to determine the fate of the tumor cell and to be dominant over the age-dependent epigenetic alterations that may originate the mutator phenotype itself. Thus, there are two distinct groups of colon cancers defined by the presence or absence of MSI and the underlying MMP. Whether the MSI-negative tumors can be subdivided into two classes depending upon their degree of epigenetic alterations remains to be demonstrated. Nevertheless, the original seminal observation of the association of hypermethylation with aging (Issa et al., 1994) is thus reinforced, as it represented the first description of a phenomenon that seems to reach vast and deep ramifications. The task ahead is to find the mechanistic links between epigenetics, aging, and cancer unveiled by these recent studies. Notwithstanding the importance of the disruption of the epigenetic code for cancer development, our studies show that, regarding the neoplastic phenotype, genetics transcends epigenetics.

Experimental procedures

DNA preparation and MSI screening

Surgically removed frozen tissues of colorectal cancers and paired adjacent noncancerous tissue were obtained from the Cooperative Human Tissue Network. The tumors analyzed represent a random subgroup of 207 tumors from a consecutive collection of over 700 unselected colorectal cancers. Genomic DNA was prepared by standard phenol-chloroform extraction and ethanol precipitation. Radioactive PCR using ^{32}P-αdCTP (NEN-life Science Products, Boston, Massachusetts) was carried out to analyze MSI as described previously (Yamamoto et al., 1999). Microsatellite status was determined using two mononucleotide markers (*BAT-26* and *APΔ3*) and one dinucleotide marker (*D1S158*). Tumors with deletion of multiple repeat units in any of the two mononucleotide loci were defined as MMP positive, although the vast majority of MMP-positive cancers exhibited mutation in both the two mononucleotide markers as well as the dinucleotide marker (see Figure 3C). This analysis classified the tumors into 28 (14%) MMP-positive, and 154 (75%) MMP-negative. MMP-positive tumors in our classification (Rampino et al., 1997; Perucho, 1999) correspond to MSI-H tumors in the Bethesda classification (Boland et al., 1998). In addition, 22 (11%) displayed sporadic alterations (deletion or insertion of one or two repeat units) only in the dinucleotide repeat. A subset of the cancers was analyzed with additional microsatellite repeats, including mono- and dinucleotide repeats (the list of the loci and PCR primer information available upon request). Up to 30% of the tumors shifted from MMP negative to the category of MSI-L (as defined by the Bethesda Criteria of tumors with at least one and no more than 40% mutated microsatellite markers) after analysis of 75 dinucleotide markers. We extrapolated that all tumors would fall into the MSI-L category after analysis of 200–250 dinucleotide repeats. Therefore, MSI-L cancers were grouped together with the MMP-negative.

Methylation-specific PCR (MSP)

MSP was carried out based on the original method developed by Herman et al. (1996) with minor modification. Five hundred nanograms to one microgram of genomic DNA were subjected to sodium bisulfite treatment, then purified using Wizard DNA Clean-Up system (Promega) and stored at −20°C. After bisulfite treatment and subsequent purification, DNA was amplified separately using specific primers for unmethylated and methylated genomic sequences. We used the published MSP primers and identical annealing temperatures for *hMLH1* (Yamamoto et al., 2001), *p16* (Herman et al., 1996), *p14* (Shen et al., 2003), *MGMT* (Esteller et al., 1999), promoter 1A of *APC* (Tsuchiya et al., 2000), and *CDH1* (Graff et al., 1997, Island 3). Primers for an anonymous CpG island (ACG) identified by MS-AFLP were designed based on the BLAST search data (GenBank AC008425). The sequences for unmethylated-specific primers were 5′-GGGTTTGGGTAAATTTGTTGTTT-3′ (nt 7733–7755) and 5′-AATCAAACACATCTCACA-3′ (nt 7879–7862), which amplify a 147 bp PCR product. For the methylated-specific reaction, the primer sequences were 5′-TGGGTAAATTCGTCGTTC-3′ (nt 7738–7735) and 5′-ATCAAACGCATCTCGCGA-3′ (nt 7878–7861), which amplify a 141 bp product. The annealing temperature for the ACG primers was set at 58°C. A total of 10 μl PCR mixture consisted of 1 μl bisulfite-treated DNA, 1× PCR buffer (16.6 mM of ammonium sulfate, 67.0 mM of Tris-HCl, 6.7 mM of magnesium chloride, and 10 mM of 2-mercaptoethanol), 0.4 mM dNTP, 0.5 μM each primer, and 0.25 unit of Platinum Taq polymerase (Gibco BRL, Rockville, Maryland). The amplification was started at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, various annealing temperature (58°C –65°C) for 30 s, and 72°C for 30 s, and finished with 8 min of final extension at 72°C. The PCR products were loaded on a 2.0% agarose gel and visualized under ultraviolet illumination with ethidium bromide.

Methylation-sensitive amplified fragment length polymorphism (MS-AFLP)

MS-AFLP was performed as described previously (Yamamoto et al., 2001) with slight modifications. Briefly, 1 μg of genomic DNA was digested overnight with 5 units of methylation-sensitive restriction endonuclease NotI (Roche, Indianapolis, Indiana) and 2 units of methylation-insensitive MseI (NE Biolabs, Beverly, Massachusetts) at 37°C. Two pairs of oligonucleotides were annealed overnight at 37°C to generate NotI (5′-CTCGTAGACTGCG TAGG-3′ and 5′-GGCCCCTACGCAGTCTAC-3′) and MseI (5′-GACGAT GAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) specific adaptors. The digested DNA was ligated to 1.25 μl each of 5 pmol/μl NotI and 50 pmol/μl MseI adaptor using 1 unit of T4 DNA ligase (Roche) overnight at 16°C. A primer complementary to the NotI adaptor (NotI primer, 5′-GACTGCG TAGGGGCCGCG-3′) was labeled at the 5′ end using ^{32}P-γATP (NEN) and T4 polynucleotide kinase (Promega, Madison, Wisconsin). The adaptor-ligated template DNA was amplified by PCR using the ^{32}P-labeled NotI primer and not-labeled MseI primer (5′-GATGAGTCCTGAGTAAC-3′). A total of 20 μl PCR mixture contained 6 ng of ^{32}P-labeled NotI primer, 30 ng of MseI primer, 0.4 mM dNTP, and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, California) and 5 to 15 ng of template DNA. The PCR started at 72°C for 30 s, 94°C for 30 s, then followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min. The final extension was performed for 10 min at 72°C. Each PCR sample was electrophoresed on a denaturing gel (Sequagel-6, National Diagnostics, Atlanta, Georgia) after heat denaturing. The gel was dried on a gel drier and exposed to an X-ray film.

Scoring of methylation alteration in tumors by MS-AFLP

The sensitivity of MS-AFLP is highly dependent on the degree of normal tissue contamination in the tumor specimens. A considerable amount of normal cells in the tumor sample may obscure the intensity differences. We were able to approximately estimate the contamination level in MSI-positive samples from the autoradiographs of BAT-26 and other mononucleotide

repeats as described previously (Suzuki et al., 2002). To evaluate the contamination level in MSI-negative cases, LOH analysis using 5–7 polymorphic dinucleotide markers was conducted prior to MS-AFLP. Samples showing a LOH pattern with the band corresponding to the lost allele with an intensity less than 25% of the band corresponding to the retained allele at any marker were regarded as adequate for MS-AFLP analysis.

Only major bands in the fingerprints were considered for the assessment of methylation alterations. Scoring of quantitative changes between normal and tumor DNA were made by visual inspection as described previously (Yamamoto et al., 2001). Scoring of signal intensity changes was done by two observers independently. Only commonly detected alterations by the two independent investigators were considered for the analysis. Furthermore, only changes commonly detected in at least two independent fingerprints with duplicates of DNA from normal and tumor tissue were included.

DNA sequencing

Bands showing intensity alterations in MS-AFLP fingerprints were excised from the gels and subjected to a second round PCR using the same primer pair. After column-purification (QIAquick PCR purification kit, Qiagen, Valencia, California), the PCR products were directly sequenced using ABI Prism BigDye Terminator Cycle Sequence Ready Reaction Kit and a DNA sequencer (Perkin-Elmer). The sequences were subjected to a BLAST search to identify their origins in the human genome.

Comparison of MS-AFLP with MS-PCR

To contrast the sensitivity and specificity of the two methods, one of the MS-AFLP bands showing methylation alterations was isolated, and the characterized CpG island containing the NotI site (ACG) was further analyzed by MS-PCR in parallel to the six loci examined in the panel of 206 tumors. The results showed an 85% concordance between MS-AFLP and MS-PCR. The same tumors that exhibited tumor-specific hypermethylation of the ACG sequence by MS-AFLP also showed hypermethylation in tumor tissue by MS-PCR.

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