A Hereditary Nonpolyposis Colorectal Carcinoma Case Associated with Hypermethylation of the MLH1 Gene in Normal Tissue and Loss of Heterozygosity of the Unmethylated Allele in the Resulting Microsatellite Instability-High Tumor

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Abstract

Fourteen suspected hereditary nonpolyposis colorectal carcinoma cases with microsatellite unstable (microsatellite instability-high; MSI-H) tumors but no germ-line MSH2, MSH6, or MLH1 mutations were examined for hypermethylation of CpG sites in the critical promoter region of MLH1. The methylation patterns were determined using methylation-specific PCR and by sequence analysis of sodium bisulfite-treated genomic DNA. In one case, DNA hypermethylation of one allele was detected in DNA isolated from blood. In the MSI-H tumor from this case, the unmethylated MLH1 allele was eliminated by loss of heterozygosity, and the methylated allele was retained. This biallelic inactivation resulted in loss of expression of MLH1 in the tumor as confirmed by immunohistochemistry. These results suggest a novel mode of germ-line inactivation of a cancer susceptibility gene.

Introduction

Approximately 60–90% of CpG dinucleotides are methylated on cytosine in the human genome, although unmethylated GC-rich regions are frequently associated with transcriptionally active genes. Epigenetic alteration of the human genome can affect cytosine methylation and chromatin structure (1–3). A number of studies have demonstrated that DNA methylation can directly prevent the binding of transcription factors, such as E2F, cyclic AMP-responsive element binding protein, and USF (4–6), or act through the binding of methylated CpG-binding proteins to induce chromatin configurations that interfere with the transcription machinery (1, 7). As a consequence, changes in the methylation of CpG-rich regions can result in altered gene expression that is independent of genetic alteration of either coding or regulatory sequences. In the last decade, a number of studies have reported that aberrantly hypermethylated CpG islands are associated with transcriptional silencing of tumor suppressor genes in sporadic cancers (reviewed in Refs. 2, 3). Thus, methylation-associated transcriptional silencing is an alternative to mutational inactivation as a cause of loss of tumor suppressor gene function. Consequently, the analysis of DNA cytosine methylation patterns provides an alternative strategy for the study of tumor suppressor gene inactivation in cases where mutations are not observed. One of many such examples is the MMR3 gene MLH1, where hypermethylation of the MLH1 promoter region is associated with loss of expression and appears to underlie the majority of MMR-defective sporadic cancer cases (8–11).

In a previous study (12, 13), we analyzed 70 HNPPC cases (which met Amsterdam, Modified Amsterdam, HNPPC-like, or Bethesda criteria) for germ-line defects in MSH2, MLH1, and MSH6 genes. For 48 of the cases, a tumor sample was available, and the MSI status of the tumor was determined; 14 cases were MSI-H and showed a germ-line mutation in MSH2 or MLH1, and 14 cases were MSI-H but did not have a germ-line mutation in MSH2, MSH6, or MLH1 (14). The observation of HNPPC cases that have MSI-H tumors but no germ-line mutation in a known MMR gene, as detected by DNA sequencing or other methods for identifying point mutations, has been reported by many studies. Some of these types of case have been attributed to germ-line deletion mutations in MSH2 or MLH1 as well as uncharacterized mutations that cause loss of expression of MLH1 (15, 16). In the present study, the mutation-negative 14 HNPPC cases were examined for hypermethylation of CpG sites in the critical promoter region of MLH1 (−290 to −180 bp relative to the ATG), which shows a strong correlation with the loss of MLH1 expression (17). DNA from blood of one of these 14 cases was found to have one methylated MLH1 allele, and analysis of the MSI-H tumor from this case demonstrated that LOH eliminated the unmethylated MLH1 allele, resulting in loss of expression of MLH1.

Materials and Methods

Tissue and DNA Samples. The HNPPC cases used in the studies described here have been described in detail elsewhere (12, 13). DNA from blood was isolated as described previously and analyzed for the presence of mutations in MSH2, MLH1, and MSH6 by DNA sequencing as reported previously. Tumor DNA from each case was analyzed for MSI using the National Cancer Institute recommended five microsatellite markers as described previously (14). The 14 cases studied here comprised all 14 of the MSI-H cases that did not have mutation in MSH2, MLH1, or MSH6 described previously (14). Control genomic DNA in which MLH1 was not methylated was from blood of the case DF000268 containing the germ-line MSH2 IVS5 + 3A>T splice site mutation (12). Control genomic DNA in which MLH1 was fully methylated was isolated from SW48 cells (American Type Culture Collection CCL-231) with a Puregene DNA isolation kit (Gentra Systems).

Analysis of MLH1 Methylation Using MS-PCR. One μg of total genomic DNA was modified by sodium bisulfite treatment using a CpGenome Modification Kit (Intergen) according to the manufacturer’s instructions and suspended in 10 μl of 1× TE buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA]. The methylation status of the MLH1 gene was then determined by MS-PCR.

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3 The abbreviations used are: MMR, mismatch repair; HNPPC, hereditary nonpolyposis colorectal carcinoma; MSI, microsatellite instability; MSI-H, MSI-high; LOH, loss of heterozygosity; MS-PCR, methylation-specific PCR.
A HNPCC CASE WITH HYPERMETHYLATION OF MLH1

In a previous study, we identified 14 suspected HNPCC cases (which met Amsterdam, Modified Amsterdam, HNPCC-like, or Bethesda criteria) that had MSI-H tumors but did not have a germ-line mutation in MSH2, MSH6, or MLH1. To understand the genetic basis for the MMR defect in these 14 HNPCC cases, we screened them for the MMR defect in these 14 HNPCC cases, we screened them for

hypermethylation of the MLH1 gene. The MLH1 DNA methylation pattern was determined by sequencing PCR products amplified from bisulfite-modified DNA isolated from blood and tissues. The region from bisulfite-modified DNA and sequenced the resulting PCR products.

To analyze the presence of a polymorphism in the 5'-untranslated region of the MLH1 gene, genomic DNA from tumor and blood samples was used as template in PCR reactions using primers specific for the sequences that result after bisulfite modification of methylated and unmethylated DNA generate two fragments of different lengths containing the same region, 91 and 103 bp long, respectively. The size of the resulting fragments allows discrimination between methylated and unmethylated alleles by acrylamide gel electrophoresis. The region of MLH1 selected for analysis, −290 to −180 bp relative to the ATG, contains nine CpG sites close to the CCAAT box and different transcription factors binding sites, and hypermethylation of this region is associated with loss of expression of MLH1.

Using MS-PCR, one case (which met Bethesda criteria 4 alone; diagnosis at 25 years of age) was found to have both methylated and unmethylated MLH1 alleles when blood DNA was analyzed, and only methylated alleles when tumor DNA was analyzed (Fig. 1). Only unmethylated alleles were observed when unmethylated control DNA was analyzed, and conversely, only methylated alleles were observed when fully methylated DNA was analyzed from the SW48 cell line, which is MMR defective and does not express MLH1. To further analyze the hypermethylation status of this region, we PCR amplified the region from bisulfite-modified DNA and sequenced the resulting PCR products (Fig. 2). The chromatograms showed that the MLH1 promoter region was partially methylated (a mixture of C and T is seen at critical Cs in the region analyzed) in blood DNA and totally methylated (only C is seen at the critical Cs in the region analyzed) in tumor DNA; analysis of an area containing two CpG sites (8 and 15 bp downstream from the CAAT box located at position −282) is

Fig. 1. Analysis of the methylation status of the MLH1 gene in an HNPCC case using MS-PCR. M and U, the 91- and 103-bp PCR products amplified using primers specific for methylated and unmethylated alleles, respectively. B, blood DNA; T, tumor DNA. Me+, DNA from the SW48 cell line in which MLH1 is fully methylated; Me−, DNA sample with a genetic alteration in MSH2 gene, in which MLH1 is unmethylated.

Fig. 2. Sequence analysis of bisulfite-modified DNA. Control, unmethylated DNA from a MSH2 mutant HNPCC case showing all Ts at positions −268, −264, and −261 attributable to complete modification of the DNA; Blood, DNA isolated from blood from the case showing a mixture of C and T at positions −268, −264, and −261 attributable to partial modification of the DNA because of partial methylation of the DNA; Tumor, DNA isolated from tumor from the case showing an increase in the level of C at positions −268, −264, and −261 attributable to reduced modification of the DNA because of increased methylation of the DNA; SW48, DNA from the SW48 cell line in which MLH1 is completely methylated showing a high level C at positions −268, −264, and −261 attributable to reduced modification because of complete methylation of the DNA.
shown in Fig. 2. Sequencing of unmethylated and fully methylated control DNAs detected only unmethylated and methylated alleles, respectively (Fig. 2). Sequencing 20 cloned PCR products from each sample (data not shown) revealed a mixture of methylated and unmethylated clones from blood DNA and an increased proportion of methylated clones from tumor DNA, consistent with the results obtained by direct sequencing of PCR products shown in Fig. 2. The observation that ~50% of the DNA from blood from the mutation-negative HNPCC case described above showed MLH1 methylation by direct bisulfite sequencing suggests that the observed MLH1 methylation is unlikely to result from shedding of tumor cells into the blood (18), because this would likely yield lower levels on MLH1 methylation than observed here.

The above analysis documented an HNPCC case in which normal tissue contained a mixture of methylated and unmethylated MLH1 alleles, whereas tumor tissue contained only methylated MLH1 alleles. There are two possible explanations for the observation that the blood DNA contained one methylated and one unmethylated MLH1 allele while the tumor DNA only contained methylated alleles; the unmethylated allele could have been deleted or methylated in the tumor. Sequencing of the promoter region from unmodified DNA from this case revealed that the blood DNA was heterozygous for a G→A polymorphism at position -93 bp, whereas DNA from the tumor only had a G at this position.

Discussion

A growing body of evidence has suggested that the majority of HNPCC families showing MSI can be accounted for by mutations in MSH2 and MLH1 (see Refs. 12, 13, 14, 16). Initial studies documented missense, nonsense, frameshift, splice site, and specific deletion mutations in MSH2 and MLH1, and more recent studies have documented large MSH2 and MLH1 deletion mutations and uncharacterized mutations that eliminate expression of these genes (15, 16). In the present study, we have identified an HNPCC case in which one allele of MLH1 was methylated in DNA isolated from normal tissue (blood) and somatic LOH of the unmethylated allele subsequently occurred, resulting in a tumor that did not express MLH1 and was MMR defective. These results raise the possibility that methylation and associated silencing of MLH1 could represent a germ-line alteration that underlies some HNPCC cases; however, samples from the parents were not available. Therefore, it was not possible to determine whether the methylated allele was actually inherited. We have detected one such case in our sample set of 14 mutation-negative, MSI-H HNPCC cases, indicating that germ-line silencing of MLH1 could underlie a small but significant proportion of HNPCC cases. Larger scale studies will be required to definitively identify other HNPCC cases similar to the one described here and to determine how frequently this unique mechanism of tumorigenesis occurs.

Fig. 3. Detection of LOH by sequence analysis of the 5’ untranslated region of the MLH1 gene. DNA isolated from blood was heterozygous for a G→A polymorphism at position -93 bp, whereas DNA from the tumor only had a G at this position.

Fig. 4. Immunohistochemistry analysis of MLH1 expression. MLH1 staining is in brown. N, normal tissue; T, tumor tissue.
The region of MLH1 found to be methylated in our studies contains CpG sites close to the CCAAT box at position −282, and methylation of this region is associated with inactivation of the MLH1 gene. The CCAAT box is recognized by CBF, and binding of this factor effects MLH1 promoter activity. Functional studies have shown that methylation of this region interferes with binding of CBF, resulting in decreased transcription of MLH1 (22). In addition to decreasing the binding of critical transcription factors, methylation of this region of MLH1 likely increases the affinity of methyl-binding proteins and histone deacetylases, leading to the assembly of inactive chromatin configurations. Consistent with this view, full reactivation in tumor cell lines requires treatment with both inhibitors of DNA methylation and histone deacetylation (23). These observations support the view that the methylation of the region of MLH1 observed here is directly associated with or underlies the silencing of the gene observed in the HNPCC case reported here.

A number of examples of loss of function of different tumor suppressor genes attributable to somatic silencing of the genes associated with hypermethylation have been reported. The types of examples reported in sporadic cancer include biallelic methylation, somatic methylation associated with LOH, and mutation associated with methylation (reviewed in Ref. 2). These observations have led to the hypothesis that hypermethylation and associated silencing of tumor suppressor genes represents an alternate pathway of the two-hit tumor hypothesis that hypermethylation and associated silencing of tumor suppressor genes attributable to somatic silencing of the genes associated with hypermethylation have been reported. The types of examples reported in sporadic cancer include biallelic methylation, somatic methylation associated with LOH, and mutation associated with methylation (reviewed in Ref. 2). These observations have led to the hypothesis that hypermethylation and associated silencing of tumor suppressor genes represents an alternate pathway of the two-hit tumor hypothesis that hypermethylation and associated silencing of tumor suppressor genes attributable to somatic silencing of the genes asso-

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