Mutations in DNA Mismatch Repair Genes Are Not Responsible for Microsatellite Instability in Most Sporadic Endometrial Carcinomas

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Abstract

Endometrial carcinoma is the second most common tumor type in women with hereditary nonpolyposis colorectal carcinoma. Microsatellite instability (MI) has been observed in the inherited (hereditary nonpolyposis colorectal carcinoma-associated) form of endometrial carcinoma as well as in approximately 20% of presumably sporadic cases. Recent studies suggest that MI in many cell lines or xenografts derived from sporadic colorectal carcinomas is not attributable to mutations in four known human DNA mismatch repair (MMR) genes (hMSH2, hMLH1, hPMS1, and hPMS2). Mutational analyses of these four MMR genes in endometrial carcinomas have not been previously reported. We analyzed nine sporadic MI-positive primary endometrial carcinomas for mutations in the above four MMR genes. Mutations were detected in two tumors (in hMSH2), and both of the mutations were acquired somatically. Immunochemical staining revealed a lack of expression of hMSH2 protein in the two tumors containing hMSH2 mutations. Our data suggest that mutations in these four known DNA MMR genes are not responsible for MI in the majority of sporadic endometrial carcinomas displaying this phenotype.

Introduction

Instability of microsatellite DNA sequences was first described in a subset of sporadic colorectal cancers and in cancers associated with the familial cancer syndrome HNPCC (1–3). Endometrial carcinoma is the most common noncolorectal carcinoma occurring in women affected by HNPCC, and MI has been observed in both the inherited form and in approximately 20% of presumably sporadic endometrial carcinomas (4, 5). Studies in microorganisms have shown that mutations in genes involved in DNA MMR lead to instability of simple repetitive DNA sequences (6). These studies provided the initial link between MMR abnormalities and the instability of microsatellite DNA sequences. In addition, when DNA MMR is lacking in microorganisms, the rate at which mutations accumulate is increased (7). The DNA MMR system has been well characterized in microorganisms where it has been shown to detect and repair mispaired bases present in the cellular genome (8). As might be expected, the DNA MMR pathway has been highly conserved throughout evolution. Although the pathway is much less well characterized in mammalian cells, there is substantial evidence suggesting that its functions are very similar to those described in microorganisms. Presently, five human homologues of microbial DNA MMR genes have been identified. Germline mutations in four of the human DNA MMR genes have been found in patients from kindreds that meet the criteria for HNPCC (9–13). Furthermore, most tumors arising in the setting of HNPCC have been found to contain a somatic mutation in the second allele of the same DNA MMR gene. Experimental evidence supports the notion that the inactivation of both alleles of a DNA MMR gene leads to the MI observed in tumors of individuals with HNPCC (14). Recently, a mutational analysis of four DNA MMR genes (hMSH2, hMLH1, hPMS1, and hPMS2) in xenografts and cell lines derived from presumably sporadic MI-positive colorectal carcinomas found that mutations in the four genes are uncommon (14). The identification of a fifth human DNA MMR gene, GTBP, has since been reported and only rare cell lines derived from sporadic colorectal carcinomas have been shown to have mutations in GTBP (15). These findings led to the speculation that MI, in a significant number of sporadic colorectal cancers, must be due to mutations in as yet unidentified human genes. We were interested in determining whether MI in presumably sporadic endometrial carcinomas was caused by mutations, either germline or somatic, in DNA MMR genes. To our knowledge, this study represents the first mutational analysis of primary tissues obtained from MI-positive sporadic human tumors to be reported. We have examined nine MI-positive endometrial carcinomas for mutations in four of the known DNA MMR genes. Our results suggest that mutations in these four human DNA MMR genes are not responsible for MI in most sporadic endometrial carcinomas exhibiting this phenotype. Presumably, as with colorectal cancer, there are additional genes that when mutated give rise to MI in sporadic endometrial carcinoma.

Materials and Methods

Tissue Specimens and DNA/RNA Isolation. Paired tumor and normal samples from 65 patients with endometrial carcinoma were obtained from the Surgical Pathology Tissue Bank of The Johns Hopkins Hospital. All samples were snap frozen in liquid nitrogen and stored at –80°C. The patients ranged in age from 37 to 86 (mean, 62.8) years old, and none of the patients met the Amsterdam criteria for HNPCC (16). Endometrial tissues were classified using the WHO and International Society of Gynecological Pathologists criteria. Genomic DNA was isolated from cryostat sections of the paired normal and tumor tissue samples as described previously (17). The tumor specimens were microdissected such that DNA was prepared only from areas containing greater than 75% neoplastic cells. Cryostat sections from cases with ample tissue were also collected in the same manner for preparation of total cellular RNA using the RNAtarget kit as outlined by the manufacturer (Promega, Madison, WI).

MI Analysis. Instability was assessed for each normal/tumor pair by analyzing eight microsatellite loci. Seven anonymous dinucleotide microsatellite DNA sequences (D2S119, D2S123, D2S147, D10S197, D13S175, D18S58, and D18S99) on chromosomes 2, 10, 13, and 18 were amplified by the PCR using MapPairs (Research Genetics, Huntsville, AL) and the same conditions previously described (4). An additional AT dinucleotide repeat, which lies in...
an intron of the DCC tumor suppressor gene, was amplified and assessed for instability using primers and conditions identical to those previously published (18). As a control, a sample lacking DNA was included for each set of reactions. The radiolabeled products were fractionated on 6% polyacryl-
amide/8 M urea gels and visualized by autoradiography. Tumors were scored as MI positive if they demonstrated alterations in the size of the microsatellite sequences, compared to the product from the matched normal tissue, at a minimum of two of eight loci.

IVSP Assay. First-strand cDNA was synthesized from total cellular RNA using random hexamers and reverse transcriptase as described (19). The coding regions of all four DNA MMR genes were amplified in overlapping fragments by the PCR using primers designed such that the 5′ primer of each set contained the T7 RNA polymerase promoter and a consensus sequence for the initiation of in vitro translation as outlined previously (19). Samples without reverse transcriptase were identically processed as negative controls for reverse transcription-PCR. Three primer sets were used to span the entire open reading frame of the hMSH2 cDNA. The sequences of the primers were: 5′-GGATCC-TTAAATCAGGTCATAAGTTGAGGCT-AT-3′ and 5′-GGCCACGACGCCAAGGGCTCAAG-3′, and 5′-GGATCC-TTAAATCAGGTCATAAGTTGAGGCT-AT-3′ and 5′-GGCCACGACGCCAAGGGCTCAAG-3′. The sequences of the primers used to amplify MI positive if they demonstrated alterations in the size of the microsatellite

Results

MI in Sporadic Endometrial Carcinomas. A total of 65 primary sporadic endometrial carcinomas were analyzed for MI, including 30 cases for which the MI status has been previously reported (4). Six of the 65 tumors included in this study were of the uncommon serous histological subtype while the remaining 59 were of the endometrioid subtype. Eight microsatellite loci were amplified from both the normal and tumor DNA of all 65 cases. MI was observed in 12 (18.5%) of the 65 tumors. Three tumors (E29, E39, and E68) showed shifts at all eight loci, and three additional tumors had shifts at seven of the loci. All MI-positive tumors showed shifts at a minimum of three loci, and nine tumors demonstrated five or more affected loci (Fig. 1). Not a single alteration was detected in the 53 MI-negative tumors at any of the seven anonymous microsatellite loci. However, four tumors showed shifts at the DCC AT locus alone and were not considered to be MI positive. All of the MI-positive tumors were of the more common endometrioid subtype, therefore approximately 20% (12/59) of the endometrioid subtype of endometrial carcinoma demonstrated MI.

IVSP Analysis of hMSH2, hMLH1, hPMS1, and hPMS2 in MI-positive Endometrial Carcinomas. The IVSP assay is capable of detecting mutations in expressed genes that alter the length of the resultant in vitro synthesized polypeptide. These mutations include nonsense mutations, insertions and deletions, and splice site mutations. The assay was originally developed to screen for mutations in the adenomatous polyposis coli (APC) tumor suppressor gene, and it has previously been well described (19). Recently, it was successfully used to identify mutations in four human DNA MMR genes in colorectal carcinoma cell lines and xenografts (10, 11, 14). The results of several studies characterizing inactivating mutations in DNA MMR genes have demonstrated that they often result in the production of a truncated protein. For this reason, we used the IVSP assay to screen MI-positive sporadic endometrial carcinomas for possible mutations in four human DNA MMR genes. Total cellular RNA was isolated from 9 of the 12 MI-positive endometrial cancer cases described above. The entire open reading frame of each gene was amplified from cDNA in overlapping fragments. Each fragment was separately submitted to in vitro transcription and translation, and the products were analyzed for the presence of aberrantly migrating polypeptides. We detected abnormal polypeptides in two tumors (E39 and E68) generated from the middle and 5′ fragment of the hMSH2 gene, respectively (Fig. 2). A product of the expected size was also observed

Fig. 1. Microsatellite instability in cases E39 and E68. Autoradiographs of microsatellite assays showing paired normal (N) and tumor (T) DNA for cases E39, E40, E68, and E69. Microsatellite D2S123 in case E39 (A) and microsatellite D18S58 in case E68 (B) demonstrate instability in comparison to tumors E40 and E69, which lack instability at the respective loci. Tumors E39 and E68 demonstrate similar instability at all eight tested microsatellite loci.

A

39 40

N T N T

B

69 68

N T N T

5557
in tumor E39 that comigrated with the product generated from the same individual's normal control tissue (Fig. 2A). In contrast, there is only a faint band of normal size in tumor E68 (Fig. 2B). Furthermore, the samples derived from normal tissue lack the smaller products, supporting their authenticity as tumor-specific products.

Sequence Analysis of the Full-Length cDNA of the hMSH2 Gene in MI-positive Endometrial Carcinomas. The entire open reading frame of hMSH2 was sequenced in 8 of the 12 MI-positive tumors. The open reading frame was amplified in seven overlapping fragments, and each fragment was subsequently directly sequenced. Abnormal sequence was detected in one fragment in tumor E39. All other amplified fragments generated only the wild-type sequence in all eight tumors with the exception of a germline G to A substitution in codon 322 in case E6, leading to an amino acid change from glycine to aspartic acid. This change has previously been reported and is thought to represent a polymorphism with an allele frequency of approximately 2% (14). For tumor E68 only the segment of hMSH2 cDNA producing the abnormal IVSP polypeptide was sequenced.

The fragment that gave rise to the mutant sequence in tumor E39 was amplified from primers located in exons 7 and 9 (codons 351–558). Sequence analysis of the precipitated PCR products produced from this primer set in tumor E39 revealed an unreadable sequence ladder. This result was explained by the presence of two different sized fragments when the amplified products were analyzed by agarose gel electrophoresis (data not shown). In addition to the product of the expected size, a fragment of approximately 100 bp smaller was observed. Subsequently, the two bands were separately isolated, purified from agarose, and independently submitted to direct sequencing. The normal sized fragment revealed the wild-type sequence. The smaller product demonstrated deletion of exon 8, with the direct splicing of exon 7 to exon 9 (Table 1). A frameshift mutation caused by the skipping of exon 8 led to the presence of a stop codon (nucleotide 1397) at the fourth codon of exon 9. The early truncation of the hMSH2 protein in tumor E39 would give rise to a polypeptide in the IVSP assay of approximately Mr 23,000. This is the approximate size of the larger of the two aberrantly sized migrating polypeptides (Fig. 2A). The smaller polypeptide may represent either a degradation product or an internal translation initiation. The latter may be more likely since there is an internal methionine at a position that would give rise to a polypeptide of the size of the smaller polypeptide (Fig. 2A). To characterize the genetic alteration responsible for the skipping of exon 8, the intron-exon boundaries of exons 7–9 were amplified from genomic DNA isolated from cryostat sections of tumor E39. A G to A transition was detected in the first nucleotide in the intron of the splice donor at the 3' end of exon 8 (Fig. 3A). Such mutations have been shown to result in the skipping of the corresponding exon, in this case exon 8 (20, 21). Matched normal genomic DNA lacked this change, indicating that the mutation was somatic.

The location of the hMSH2 mutation found in tumor E39 correlated with what would have been predicted from the results of the IVSP assay. Therefore, we chose to sequence only the region of hMSH2 in tumor E68 that gave rise to the abnormal polypeptide. A fragment was amplified using primers located in exons 3 and 8 (codons 189–433), and the PCR product was submitted to direct sequencing. The sequence suggested the possibility of a single base pair deletion at the junction of exons 5 and 6. To verify the sequence, the PCR product was cloned into the pCR II vector, and independent clones were sequenced. Three of six clones showed a deletion of a single nucleotide (guanine) in codon 315 (Fig. 3B). This deletion leads to a frameshift that creates a stop codon at codon 330 (Table 1). Again, as in tumor E39, this mutation explains the production of the abnormal polypeptide generated in the IVSP analysis of tumor E68 (Fig. 2B). To determine the nature of the mutation at the genomic level, a portion of the introns and the entirety of exons 5 and 6 were amplified, cloned, and sequenced. Tumor E68 revealed a single base pair substitution at the intron-exon boundary of the splice acceptor at the 5' end of exon 6. The substitution was a G to an A transition at the first nucleotide in the intron that would change the splice recognition signal such that a single G at the junction of exons 5 and 6 would be deleted from the spliced product, as was detected in the cDNA of tumor E68 (data not shown).

Immunohistochemical Analysis of hMSH2 Expression in MI-positive Endometrial Carcinomas. To determine whether the expression of hMSH2 in MI-positive endometrial carcinomas correlated with the presence of a mutation in the gene, we stained 11 of the 12 MI-positive tumors with a monoclonal carboxyl-terminal hMSH2 antibody. All of the tumors were positive with the exception of tumors E39 and E68. The positive tumors showed strong nuclear staining of the neoplastic cells, whereas the neoplastic cells in tumors E39 and

![Fig. 2. Mutational analysis of hMSH2 by IVSP assay in cases E39 and E68. In A, in case E39, translation of codons 256–668 from cDNA of tumor tissue (Lane T) produces two smaller polypeptides not present in cDNA extracted from matched normal tissue (Lane N). The dominant polypeptide seen in Lane N is of the size (approximately Mr 46,000) expected for wild-type hMSH2. Arrow, truncated polypeptide due to the introduction of a stop codon by a mutation in hMSH2 in tumor E39. In addition, the normal size polypeptide is present in tumor E39. In B, translation of codons 1–434 from tumor cDNA of case E68 (Lane T) generates a faster migrating polypeptide (arrow) that is not seen in cDNA derived from normal tissue (Lane N).](image-url)

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Fig. 3. Sequence analysis of genomic DNA of case E39 and cDNA of case E68. In A, sequence analysis of the intron-exon boundary of the splice donor of exon 8 reveals a G to A transition (arrow) in DNA from tumor E39 (T) that is not present in the patient’s normal DNA (N). It is detected as a C to T transition due to the use of an antisense primer in the sequencing reaction. In B, cDNA sequence from tumor E68 reveals a deletion of a single G (arrow) at the junction of exons 5 and 6 when compared to the normal sequence (N).

E68 completely lacked staining (Fig. 4). The nuclei of non-neoplastic stromal cells demonstrated expression of hMSH2 in both the positive tumors and in tumors E39 and E68.

Discussion

Previous studies have conclusively shown that MI in tumors arising in patients with HNPCC is due to the presence of mutations in one of four known (hMSH2, hMLH1, hPMS1, and hPMS2) DNA MMR genes (9–11). The association of endometrial carcinoma with HNPCC hints that such mutations may also account for the MI observed in the subset of presumably sporadic endometrial carcinomas demonstrating this phenotype. Our results are the first to suggest that instability of simple repetitive DNA sequences in most sporadic endometrial carcinomas displaying MI is not caused by mutations in the four MMR genes above.

Since the initial discovery of MI in sporadic and inherited colorectal cancer, a large number of studies have reported the finding of MI in a wide variety of tumors. It has recently been noted that tumors showing MI can be separated into three distinct classes: (a) the sporadic colorectal cancers that show instability at numerous microsatellite loci; (b) tumors associated with HNPCC that also show alterations in many tested microsatellite loci; and (c) other types of sporadic tumors (e.g., pancreas, lung, breast, and prostate) that show fewer and less dramatic microsatellite alterations than those observed in the first two classes (14). Most recently, a fourth class of tumors has been reported that primarily demonstrate instability of mononucleotide repeats (15). Notably, the instability present in the sporadic endometrial cancers analyzed herein is similar in both quantity and quality to the first two classes of MI-positive tumors. Therefore, we believe they should be included with tumors that fall into the first class. This finding provides yet another reason, in addition to their common occurrence in HNPCC, to believe that endometrioid endometrial and colorectal cancers of both the sporadic and inherited forms share similar pathogenetic mechanisms. This notion is further supported by the lack of mutations in the four DNA MMR genes in sporadic MI-positive endometrial carcinoma that we have reported here. This is the first report we are aware of that has analyzed, in primary tumors, the mutational status of MMR genes in noncolorectal cancers. It will be of interest to know the mutational status of these genes in the third class of tumors with MI. It seems quite likely that the genetic mechanisms underlying the instability in those tumors will be different than those in the first two classes.

It is possible that our molecular studies have underestimated the number of cases with mutations in the MMR genes. Our study was performed on tissues harvested directly from hysterectomy specimens removed for endometrial carcinoma. Analysis of primary tumors is advantageous in that it eliminates the possibility of detecting alterations that may arise in cell culture or in the expansion of tumor cell populations in animals during the production of xenografts. On the other hand, analyses of primary tumors are complicated by the contamination of tumor by normal cells. It has been reported that several colorectal cancer cell lines and xenografts have mutations in hMSH2 and hMLH1 that lead to the lack of a detectable transcript (14). This type of mutation may not be detected by the molecular genetic analyses used in our present study due to the contribution of tran-

Fig. 4. Immunohistochemical staining for hMSH2. A, tumor E39, a MI-positive tumor with a mutation in hMSH2, completely lacks detectable staining for hMSH2 protein in the neoplastic cells. Both tumors demonstrate positive staining in non-neoplastic stromal cells. B, a MI-positive tumor without a detectable mutation in hMSH2 (tumor E30) showing strong nuclear reactivity in tumor cells. Arrows, neoplastic cells; arrowheads, non-neoplastic stromal cells.
scripts from contaminating normal cells. In addition, missense mutations and some deletions (both intragenic and subchromosomal) will not be detected with the IVSP assay. However, the IVSP assay has been shown to identify 77% of the mutations in hMSH2, hMLH1, hPMS1, and hPMS2.5

In an attempt to determine the sensitivity of our molecular genetic methods, we examined all of the MI-positive endometrial carcinomas for the expression of hMSH2 by immunohistochemistry. To date, the majority of inactivating mutations of hMSH2 lead to a lack of expression or the expression of a truncated protein not detectable by the antibody used in these studies.6 The results of the immunohistochemical staining have two implications. First, they suggest that mutations in hMSH2 are not common in MI-positive sporadic endometrial carcinomas. Second, they indicate that our analyses were both sensitive and specific, since mutations were detected only in tumors that lacked hMSH2 expression. It is possible that we have missed mutations in the other three genes for which antibodies are not available. However, given the above results, and the reported findings in sporadic colorectal cancer, it is unlikely that mutations in the three genes account for MI in the remaining MI-positive endometrial carcinomas we have analyzed. Recently, a fifth human DNA MMR gene, GTBP, was identified and cloned (15, 22, 23). Mutations in GTBP have been found in several xenografts derived from sporadic colorectal cancers and one primary colorectal cancer. However, the tumors in which the mutations were found demonstrate instability in mononucleotide tracts with only rare instability at dinucleotide loci (15). The tumors we have analyzed show marked dinucleotide tract instability. Therefore, it is unlikely that mutations in GTBP underlie the instability phenotype demonstrated by this subset of sporadic endometrial carcinomas.

It has been proposed that in mammalian cells, instability of microsatellite DNA sequences may simply serve as an indicator of the mutator phenotype, and that the neoplastic process is driven by an increase in the rate of mutations in oncogenes and tumor suppressor genes. This increase in mutation rate is thought to be caused by the same underlying defect that gives rise to the MI. While the phenotype of MI can be due to mutations in the known MMR genes, it now seems likely that there are additional genes that cause an identical phenotype in both sporadic colorectal and endometrial cancer. In the future, it will be of interest to determine the nature of the additional genetic alterations that lead to MI and whether they, too, are shared by colorectal and endometrial carcinoma.

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References


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