Frameshift Somatic Mutations in Gastrointestinal Cancer of the Microsatellite Mutator Phenotype

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ABSTRACT

An exacerbated genomic instability characterizes hereditary and sporadic gastrointestinal cancer of the microsatellite mutator phenotype (MMP), generating somatic frameshift mutations in genes containing mononucleotide repeats. We have recently shown that approximately 50, 40, and 30% of MMP+ colon tumors harbor frameshift mutations in (G), (A), and (C) tracks within the proapoptotic gene BAX and the hMSH3 and hMSH6 DNA mismatch repair genes, respectively. Here we report a higher incidence of frameshift mutations in these 3 genes in a panel of 25 MMP+ gastric adenocarcinomas: 64% in BAX and hMSH3, and 52% in hMSH6. These results support a multiple mutator gene model for the stepwise unfolding of the MMP and further illustrate the importance of the escape from apoptosis in gastrointestinal cancer. The tumor suppressor role played by BAX is also supported by the finding of other somatic BAX mutations, including recurrent missense mutations, not only in gastrointestinal cancer of the MMP but also in gastrointestinal cancer without the MMP.

INTRODUCTION

A genome-wide instability at simple repeat sequences characterizes gastrointestinal cancer of the MMP (1). By failing to repair the spontaneous errors of replication of these unstable sequences, MMP tumors accumulate hundreds of thousands of insertion and deletion mutations in microsatellites (2). Tumors of the MMP differ from those without enhanced microsatellite genomic instability in many biological, clinical, cytogenetic, and molecular genetic parameters (2–4). These differences support the concept that the MMP underlies a distinct molecular pathway for carcinogenesis (1, 2, 5).

The MMP and the resulting enhanced genomic microsatellite instability are caused by mutator mutations such as those inactivating DNA mismatch repair gene products (6). There are six human gene homologues to the Escherichia coli DNA mismatch repair system: hMSH2, hMSH3, and hMSH6 related to the MutS gene and hMLH1, hPMS1, and hPMS2 related to the MutL gene (7, 8). Germ-line mutations (the vast majority in hMSH2 and hMLH1) have been described in HNPPC families, although about half of the HNPPC tumors do not involve mutations in either of these genes, despite the majority of them exhibiting the MMP (6, 9).

In addition to the multitude of mutations accumulated in neutral microsatellite sequences, the MMP also generates mutations in cancer genes, i.e., those that play an active role in the multistep process of carcinogenesis. Although repeated sequences in the coding region of genes are shorter than the typical microsatellite loci used for gene mapping (10), their propensity for undergoing spontaneous slippage errors of replication is still higher than that of nonrepetitive sequences (11). If the replication fidelity of these unstable sequences is compromised by a defective DNA mismatch repair system, frameshift mutations preferentially accumulate in cancer genes relative to other genes without oncopgenic potential because they are selected for during tumor progression.

The first characterized example of these oncopgenic mutations were the frameshift mutations in a cancer gene with a negative role in cell growth, TGFBRII (12). Mutational targets for the MMP also include other genes involved in cell growth, such as IGFIIIR (13), and genes involved in the presentation of antigens to the immune system, such as the β2 microglobulin gene (14). We have recently shown the relationship between the MMP and the escape from apoptosis by the detection of frameshift mutations in the proapoptotic gene BAX in 50% of MMP+ colorectal tumors (15).

In addition to these cancer genes, MMP tumors contain slippage-related frameshift mutations in some members of the DNA mismatch repair family, such as hMSH3 and hMSH6, which are therefore secondary mutators (16). The frameshift mutations of secondary mutators are presumably induced by primary mutators such as hMLH1 or hMSH2. On the basis of these findings, we have proposed a model by which the unfolding of the MMP occurs in consecutive steps. According to the “mutator that mutates the other mutator” model, secondary mutator mutations enhance the depth and/or width of genomic instability of tumor cells, accelerating the accumulation of oncopgenic mutations in the typical cancer genes of the MMP pathway for carcinogenesis (17).

In support of this hypothesis, we report here that the incidence of somatic frameshift mutations in the hMSH3 and hMSH6 mutator genes and in the BAX gene that promotes apoptosis is even higher in gastric than in colorectal tumors of the MMP.

MATERIALS AND METHODS

Tumor Samples. Gastric tumor samples were obtained as frozen specimens from the Southern Division of the Cooperative Human Tissue Network (University of Alabama, Birmingham, AL), the National Cancer Center Research Institute (Tokyo, Japan), and Sapporo Medical University (Sapporo, Japan). The origin of the colorectal tumor samples has been described previously (15, 16). Genomic DNA was extracted with phenol-chloroform and diluted to a concentration of 20 ng/μl before PCR amplification.

Analysis of Enhanced Microsatellite Instability. Somatic microsatellite alterations were analyzed by PCR as reported previously (2, 18). MMP+ tumors were defined as those with somatic deletion mutations in mononucleotide repeats of (A), in APα3 (2) and/or (A), in intron 5 of hMSH2 (19) and deletions or insertions of more than one repeated unit in dinucleotide microsatellite sequences (D1S158, D5S421, and D8S199) using the corresponding MAPPAIRS primers (Research Genetics). Tumors exhibiting sporadic dinucleotide microsatellite mobility shifts of only one repeated unit were not considered MMP+ (15). These spontaneous microsatellite alterations in the absence of genomic instability are detectable because of their clonality after tumor expansion.

PCR Amplifications. A 94-bp region encompassing the BAX (G) tract was amplified by PCR with primers 5'-ATCCAGGTATGGCAGCCAGGGC-3' and 5'-ATCCGCCTAGCTTTGTTGTT-3'. PCR was carried out with Vent DNA polymerase (New England Biolabs) for 1 cycle of 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in the
presence of 0.2 μCi of [32P]dCTP. PCR products were electrophoresed in a
denaturing 6% polyacrylamide gel (National Diagnostics). The gel was dried
on filter paper and subjected to autoradiography. Mutations in similar repeated
sequences from other genes (see Ref. 15 for details) were analyzed as
described above. The primers used for each are as follows: hMSH3, 5'-AGAT-
GTTAATCCCTAACAAAGC-3' and 5'-ACTCTCCACATGGCCTAAAGA-
AAAT-3'; hMSH6, 5'-GGTGATTGCTAGCTGATGTC-3' and 5'-CGTAGTGC-
GCAAGGATGGCGT-3'; TGFβRII, 5'-AAGCTCCCCATCGACTC-3' and
5'-TGACCTACTACAGCTACAG-3'; IGFIR, 5'-AGGTCCTCGT-
ACTAGAGCT-3' and 5'-CCGCTGAGACTCTCCCGGATGC-3'. We also
analyzed BRCA1 and BRCA2 suppressor genes (GenBank accession numbers
HSU14680 and HSU4376), which both contain (A)8 repeats in their coding
region. The PCR primers were 5'-TGATTAGTCTAGCAGTG-3' and
5'-TCTGTTAGCTAGGAGCT-3' for BRCA1 and 5'-CAGGAT-
CCAGGAGGCC-3' and 5'-CCGCTGAGACTCTCCCGGATGC-3'.

**Single-Strand Conformational Polymorphism Analysis.** Other BAX so-
matic mutations in gastric and colorectal tumors were searched by single-
strand conformational polymorphism (20) followed by sequencing of bands
with mobility alterations, using the following BAX PCR primers (21) for each
 exon: exon 1, 5'-CGTCAAGGGTTCAAGTTA-3' and 5'-CGAGGCGT-
GAGGAGAT-3'; exons 2-3, 5'-CCCTTAAGAACCGAAGATC-3' and
5'-GGCTGAGAAGCTGCTGTCACAG-3'; exon 4, 5'-TCTCCTGAGAGAGAGAGAT-
TG-3' and 5'-TCCCCAGTCCTCAGATGACTC-3'; exon 5, 5'-CAGGC-
AGTGGGAGCAAGTTGTTGACGGAGGAGAT-3' and 5'-GGCTGAGAAGCTGCTGTCACAG-3'; and exon 6, 5'-CCCTGAGAGGGTCCTGACAG-3' and 5'-AATGCCCAAT
GTCCCTCAATC-3'.

**Sequencing Analysis.** Sequencing was performed as described previously
(15). The PCR products were eluted from the gels, amplified, and subcloned
into pCRTM2.1 (Invitrogen). Recombinant plasmids were sequenced by the
dideoxy chain termination method, using a Sequenase DNA sequencing kit
(United States Biochemical Corp.). DNA was also reamplified with the same
PCR primers, purified using the QIAquick PCR purification kit (Qiagen), and
subjected to direct sequencing using the ABI PRISM™ dye terminator cycle
sequencing kit (Perkin-Elmer Corp.). Mutations were confirmed by the two
sequencing methods.

**RESULTS**

Frameshift Mutations in BAX, hMSH3, and hMSH6 in Gastric
Tumors of the MMP. Twenty-five of 167 (15%) gastric adenocarcino-
mas were MMP+. We compared by PCR amplification the region
comprising the (G)8 tract in the BAX gene and the (A)8 and (C)8 tracks
of the hMSH3 and hMSH6 genes in the 25 MMP+ gastric tumors and
the corresponding normal tissues. Sixty MMP— primary gastric tumors
were also analyzed. Fig. 1 (top two panels) shows the results of the
analysis of 16 of the MMP— (first panel) and MMP+ (second panel)
tumors. BAX frameshift mutations were detected in 64% (16 of 25) of
MMP+ tumors but were not detected in any of the 60 MMP— tumors.
Frameshift mutations in hMSH3 and hMSH6 DNA mismatch repair
genes were detected in 64 (16 of 25) and 52% (13 of 25) of MMP+
and MMP— tumors, respectively, but not in any of the 60 MMP— tumors
(Fig. 1; data not shown). Sequencing analysis confirmed the deletion
or insertion of 1 bp in the mononucleotide repeats from these three
genes (Fig. 2, A and B; data not shown). Mutations in the TGFβRII
(12) and IGFIR (13) genes were also present in 72 (18 of 25) and 8%
(2 of 25) of the MMP+ and MMP— tumors, respectively (Fig. 1; data not shown).
Three tumors (12%) also contained frameshift mutations in the
BRCA1 gene (Table 1).

**Frameshift Mutations in Colorectal Tumors of the MMP.** Fig. 3
shows similar analysis of representative MMP+ colorectal tumors.
BAX mutations at the (G)8 tract were present in 50% (21 of 42) of
MMP+ colorectal tumors (15). The frequency of frameshift mutations
in the hMSH3 and hMSH6 DNA mismatch repair genes (Fig. 3) in the
same MMP+ colorectal tumors was 43 (18 of 41) and 29% (12 of 42; Ref. 16). Frameshift mutations in the TGFβRII and IGFIIIR genes (Fig. 3) were detected in 90 (37 of 41) and 20% (8 of 41) of the tumors, respectively. The summary of the mutational analysis for these genes in gastric and colorectal tumors of the MMP is shown in Fig. 4. The frequency of BAX, hMSH3, and hMSH6 frameshift mutations seemed to be slightly higher in gastric than in colorectal tumors of the MMP. In contrast, TGFβRII and IGFIIIR mutations were less frequent in gastric than in colorectal tumors. Mutations in other repeated sequences of eight nucleotides (see “Materials and Methods”) were absent or significantly less frequent in the same MMP+ tumors.

Most frameshifts involved the insertion or deletion of only 1 bp, although alterations of 2 bp were also observed in the hMSH3 and TGFβRII genes (for instance, gastric tumor J2 and colon tumor 453 underwent deletions of 2 bp from (A)₈ to (A)₆ in the hMSH3 gene, Figs. 1 and 3). The frequency of deletions versus insertions varied depending on the repeated sequence. Polyadenylic acids were preferentially (TGFβRII) or exclusively (hMSH3) deleted, whereas (G)₈ and (C)₈ repeats also sustained insertions, with variable frequencies depending on the gene. No deletions (or insertions) of 3 bp were detected in any of the genes, including the TGFβRII (A)₁₀ track, despite the detection of (A)₈ to (A)₆ frameshifts in hMSH3. These results support the interpretation that frameshift mutations in cancer

**Fig. 2.** Sequence analysis. A. BAX frameshift mutations in gastric tumors of the MMP. Wild-type allele sequence in normal tissue from tumor A57, a 1-bp deletion in tumor A57, and a 1-bp insertion and deletion in tumor A9 are shown. B. hMSH3 frameshift mutations in gastric tumors of the MMP. Wild-type allele sequence in normal tissue from tumor A57, a 1-bp deletion in tumor A57, and a 1-bp deletion in tumor A9 are shown. C. Somatic missense mutations in codon 169 of BAX in gastrointestinal tumors.
genes containing targets for the MMP are under selective pressure during tumor progression.

**Role of Frameshift Mutations in Gastrointestinal Tumors of the MMP.** The heterozygous or homozygous status of genes with frameshift mutations is difficult to estimate in primary tumors because of the variable amount of contaminating normal tissue. However, by comparing the proportion of wild-type versus mutant alleles in several mononucleotide microsatellite loci, the approximate amount of normal tissue in the tumor samples could be estimated. For instance, gastric tumor A9 seemed to have more normal tissue than tumors A40 or A57, judging by the signal remaining in the wild-type (A)_{26} repeats (Fig. 1, bottom panel). In this manner, we estimate that some gastric (A57 and J2) and colon (73, 442, and 453) tumors harbored more mutant than normal BAX alleles, suggesting that the mutations were homozygous in at least a significant fraction of the tumor cells. Gastric tumors A40, A57, J7, and J40 and colon tumor 405 also exhibited apparent homozygous (or hemizygous) hMSH3 mutations (Fig. 1). Biallelic BAX frameshift mutations (insertion and deletion of one nucleotide) were observed in gastric tumor A9 and in four colorectal tumors.

**Table 1 Frameshift mutations in gastric tumors of the MMP**

In addition, one or no frameshift mutations were found in the genes described in Fig. 4.

<table>
<thead>
<tr>
<th>No.</th>
<th>Case</th>
<th>BAX((G)_8)</th>
<th>hMSH3((A)_8)</th>
<th>hMSH6((C)_8)</th>
<th>TGF(\beta)RII((A)_10)</th>
<th>IGFIIR((G)_8)</th>
<th>BRCA1((A)_8)</th>
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<td>1</td>
<td>A9</td>
<td>+u, d(^a)</td>
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<td>−</td>
<td>+dd(^c)</td>
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<td>−</td>
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<td>+d</td>
<td>+u</td>
<td>+d</td>
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<td>−</td>
</tr>
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<td>+d</td>
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<td>+u</td>
<td>+d</td>
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<td>−</td>
</tr>
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<td>5</td>
<td>J2</td>
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<td>+dd</td>
<td>+u</td>
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<td>−</td>
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<td>+u</td>
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<td>−</td>
<td>−</td>
<td>u +d</td>
<td>−</td>
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<td>−</td>
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<td>+u</td>
<td>+d</td>
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<td>−</td>
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<td>−</td>
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<td>25</td>
<td>J100T</td>
<td>+d</td>
<td>+d</td>
<td>+u, uu(^d)</td>
<td>−</td>
<td>+u</td>
<td>−</td>
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\(^a\) +u, 1-bp insertion; +d, 1-bp deletion.

\(^b\) −, wild type.

\(^c\) +dd, 2-bp deletion.

\(^d\) +uu, 2-bp insertion.

Fig. 3. Frameshift mutations in colorectal tumors of the MMP. The PCR experiment shows a representative set of tumors positive for BAX frameshift mutations. Symbols are the same as those described in the Fig. 1 legend. Bottom panel, absence of hMSH6 frameshift mutations in MMP− colorectal tumors.
Fig. 4. Incidence of frameshift mutations in gastrointestinal tumors of the MMP. The graph represents the percentage of somatic frameshifts in repetitive sequences in the genes indicated at the bottom (the coding region for BAX, IGFIIR, HMGH6, DRP, NSEP, HMGH3, BRCA1, BRCA2, ICE, HPS2, and Polyja and the noncoding region for the other genes (3' end noncoding region of IGFIIR and NGFIIR, the promoter region of GS, and introns of the RB gene and the FNIII gene)). For details on these genes, see Refs. 15 and 16 and "Materials and Methods." The number of positive cases is shown above the bars. *nd*, not determined. Statistics (Fisher's exact test) for the association of these mutations with the MMP were as follows: BAX, *P < 10^-2*; BAX versus IGFIIR, GS, and NGFIIR, *P < 10^-2*; DRP, *P < 10^-2*; hMSH6 versus DRP, *P < 10^-2*; hMSH3 versus ICE, hPMS2, Polya, RB, and FNIII were not significant. Statistics for mutations in different genes were as follows for gastric tumors: BAX versus IGFIIR, *P < 10^-2*; BAX versus IGFIIR, GS, and NGFIIR, *P < 10^-2*; hMSH6 versus DRP, *P < 10^-2*; hMSH3 versus NSEP, *P < 10^-2*; hMSH3 versus BRCA1, *P < 10^-2*; hMSH3 versus BRCA2, *P < 10^-2*; hMSH6 versus DRP, *P < 10^-2*; hMSH6 versus NSEP, *P < 10^-2*; hMSH3 versus BRCA1, *P < 10^-2*; hMSH3 versus BRCA2, *P < 10^-2*; hMSH6 versus ICE, hPMS2, Polyja, RB, and FNIII, *P < 10^-2*.

(15). Other tumors seemed heterozygous for frameshift mutations in these loci.

Other Somatic BAX Mutations in Gastrointestinal Tumors of the MMP. The role in tumorigenesis of heterozygous mutations in these genes is not clear, especially in recessive mutator genes. However, tumors with frameshift mutations in one allele could have other different mutations in the other allele. We tested this hypothesis by searching for somatic BAX mutations in MMP+ tumors in addition to the frameshifts in the (G)8 track. The BAX gene is more amenable to this type of analysis than the DNA mismatch repair genes because of its smaller size. Somatic BAX gene mutations were found in both gastric and colorectal tumors (Table 2). These included frameshift insertions (gastric tumor 42S and colon tumor 437) and a G to A transition generating a stop codon in gastric tumor J18. We also found several other missense mutations, with a hot spot of transitions at codon 169. The threonine at this position was replaced by an alanine (ACG—*ATG) in colon tumor 446 (Fig. 2C). This tumor was analyzed with additional microsatellite loci (3 mononucleotide and 1 3 dinucleotide repeats), but no evidence of microsatellite alterations was found (data not shown). No frameshift mutations in any of the genes analyzed in this work were detected in this colon tumor. We conclude from these results that somatic mutations at the proapoptotic gene BAX also occur in tumors without the MMP.

Table 2. Somatic BAX mutations in gastrointestinal tumors of the MMP

<table>
<thead>
<tr>
<th>Tumors*</th>
<th>BAX(G)8h</th>
<th>Exon</th>
<th>Nucleotide</th>
<th>Codon</th>
<th>Domain</th>
<th>Alteration</th>
<th>bp alteration</th>
<th>Amino acid alteration</th>
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<td>42S</td>
<td>—</td>
<td>2</td>
<td>84-86</td>
<td>29</td>
<td>BH3</td>
<td>Insertion</td>
<td>GGC to GGGG</td>
<td>Frameshift</td>
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<tr>
<td>68</td>
<td>—</td>
<td>3</td>
<td>174</td>
<td>58</td>
<td>BH3</td>
<td>Insertion</td>
<td>AA to AAC</td>
<td>Lys to Asn</td>
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<td>—</td>
<td>3</td>
<td>203</td>
<td>68</td>
<td>BH3</td>
<td>Transversion</td>
<td>GAC to GTC</td>
<td>Asp to Val</td>
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<td>437</td>
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<td>4</td>
<td>266-267</td>
<td>89</td>
<td>Insertion</td>
<td>CGA to CGTA</td>
<td>Frameshift</td>
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<td>4</td>
<td>276</td>
<td>92</td>
<td>BH2</td>
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<td>TGT to TGA</td>
<td>Phe to Leu</td>
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<td>J18</td>
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<td>5</td>
<td>453</td>
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<td>6</td>
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<td>Carb end</td>
<td>Transition</td>
<td>ACG to ATG</td>
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</table>

a Tumors 42S, J18, J13, and J40 (gastric) and 68, 504, 437, 149, 91, and 446 (colorectal).
b Presence (+) or absence (−) of frameshift mutations in BAX (Table 1).

* Colon tumor without the MMP.
APOTOTIC AND MUTATOR MUTATIONS IN GASTROINTESTINAL CANCER

DISCUSSION

We have analyzed the presence of frameshift mutations in genes containing mononucleotide repeats in stomach and colon cancers of the MMP. We describe here the presence of these mutations in some genes previously not reported, such as BRCA1 and BRCA2, although with low frequency. These mutations could have contributory roles in cancer progression either as inactivating their tumor suppressor functions or as secondary mutator mutations (see below), because the products of these genes may exhibit DNA repair activity (22). Another question that we have studied in this work is the extent of involvement of BAX mutational inactivation in gastric and colorectal cancer of the MMP. The BAX gene is a key player in the homeostatic equilibrium controlling programmed cell death (23). The importance that inactivation of the proapoptotic activity of the BAX gene product plays in cancer is illustrated by the common presence of BAX frameshift mutations in colorectal tumors of the MMP (15). Other tumors from the gastrointestinal tract, especially gastric cancer, also develop through the MMP pathway (24). It was of interest to extend the BAX gene mutational analysis to gastric cancer. We show here that the incidence of BAX frameshift mutations is higher in gastric tumors than in colorectal tumors, suggesting that these mutations are common mechanisms for escaping from apoptosis in the MMP pathway for gastrointestinal tumors.

BAX frameshift mutations were present in 64% (16 of 25) of MMP+ gastric tumors but absent in 60 MMP− gastric tumors, indicating that these mutations are specifically associated with the MMP. Moreover, in the same tumors, similar frameshift mutations were also absent or were much less frequent in other genes with tumor suppressor function or within genes whose inactivation would not presumably lead to a selectable advantage by the corresponding tumor cells (Fig. 4). Thus, BAX frameshift mutations are under strong positive selective pressure in MMP+ gastric tumors.

In addition to some tumors with biallelic mutations, other tumors seemed to harbor more mutant than normal alleles, implying the existence of homozygous BAX frameshift mutations. This interpretation is consistent with a recent report showing that 8% (4 of 48) of gastric tumors contained no BAX-immunopositive cells and that 17% (8 of 48) of tumors consisted of over one-half BAX-immunonegative tumor cells (25). On the other hand, some tumors seemed heterozygous or heterogeneous for BAX frameshift mutations. Heterogeneous frameshift mutations in the TGFβR1 gene have been reported in intratumor regions of MMP+ gastric tumors (26). Nevertheless, the presence of a frameshift mutation in only one of the BAX alleles does not necessarily means that the gene is functionally heterozygous, because the other allele could have another type of mutation. This hypothesis was confirmed by the detection of other frameshift and nonsense BAX somatic mutations in gastric tumors, some of which contained frameshift mutations (Table 2). Therefore, these tumors, apparently heterozygous at first, contained homozygous inactivating mutations at the BAX locus.

The existence of other BAX somatic mutations added further support to the concept that BAX plays a suppressor role in the MMP pathway for gastrointestinal cancer. The functional significance in cancer development of the missense mutations detected in the BAX gene remains to be determined. However, several of these base substitutions lead to amino acid changes in protein domains with characterized functions in protein-protein interactions (27). In addition, the somatic mutation at codon 68 that we found in a colon tumor has been previously described to lead to the inactivation of BAX dimerization ability (28). The BAX proapoptotic activity is dependent on its relative intracellular abundance that favors its dimerization (23).

The finding of a mutational hot spot at codon 169 also supports the relevance of this mutation and suggests that this residue may be critical for BAX function. It is remarkable that independent mutations occurred in this codon, although replacing the wild-type threonine by two different amino acids. This suggests that replacement of the threonine 169 may inactivate the proapoptotic BAX function, possibly by preventing protein phosphorylation.

In contrast with slippage-induced frameshift mutations, missense mutations are not exclusively associated with the MMP. This raised the possibility that missense BAX mutations could also be present in tumors without enhanced microsatellite instability. This prediction was confirmed by finding a codon 169 mutation in a colon tumor negative for microsatellite alterations despite an exhaustive search. This observation is consistent with the proposal that BAX plays a tumor suppressor role in cancer development, regardless of its molecular pathway. In tumors of the MMP, BAX frameshift mutations at the (G)8 track may generally occur before mutations in other genes of the apoptotic cascade, such as p53, which do not have clear targets for the mutagenic action of the MMP. Inactivation of p53, by loss of heterozygosity and mutation, has been reported in about 60% of gastric tumors (29). Given our results and the transcriptional activation of BAX by wild-type p53 (30), we postulate that MMP+ gastric tumors, like MMP+ colorectal tumors (2), have a negative correlation with mutant p53. The relative mutation frequencies in these two genes are thus reversed in gastrointestinal tumors with and without the MMP. This hypothesis may provide a mechanistic explanation for the molecular genetic differences between these two cancer pathways by highlighting the role that the presence or absence of simple repeats in critical cancer genes plays in the etiology of mutations driving carcinogenesis.

The second issue addressed in the present study was the incidence of frameshift mutator mutations in MMP+ gastric tumors. We have recently reported hot spots for frameshift mutations in DNA mismatch repair genes hMSH3 (40%) and hMSH6 (28%) in MMP+ colorectal tumors (16). These observations led to the postulation of the “mutator that mutates the other mutator” model to describe the stepwise nature of the unfolding of the MMP (17). We examined the presence of frameshift mutator mutations in our panel of 25 MMP+ gastric tumors. We found that 64 and 52% of these tumors contained frameshift mutations in hMSH3 and hMSH6, respectively. Therefore, the common occurrence of slippage frameshift mutations in hMSH3 and hMSH6 adds further support to the multiple mutator model for the development of enhanced microsatellite genomic instability during the MMP pathway for gastrointestinal cancer.

The functional significance of these frameshift mutator mutations and the mechanisms by which these secondary mutators contribute to the acquisition of enhanced genomic instability remain to be established. The hot spot for frameshift mutations in hMSH3 resides at the beginning of the coding region (16), presumably leading to a complete loss of protein function, similar to the BAX frameshift mutations (15). In contrast, the frameshift hot spot in hMSH6 is localized in the carboxyl-terminal region. The carboxyl end of hMSH6 shares a high degree of homology with the other MutS genes, hMSH2 and hMSH3. This region also includes a highly conserved Walker type A nucleotide binding motif (31). Moreover, germ-line mutations very close to the carboxyl end of hMSH2 have been described in several HNPCC families (19). Frameshift mutations in the (C)8 tract of hMSH6 would thus result in the abolishment of this conserved and functionally important region of the gene product. Therefore, these mutations may have an impact in the genomic instability of MMP+ tumors that would explain why they are under a positive selective pressure during cancer development. Whether these mutations may exert their effect in a homozygous or heterozygous state remains to be elucidated.

The heterozygous or homozygous nature of these frameshift mutator mutations is relevant because of the recessive nature of the MMP (32). Some primary gastric and colorectal MMP+ tumors harbored apparently homozygous (or hemizygous) frameshift mutations in
hMSH3. We have also found homozygous hMSH3 frameshift mutations in MMP+ colon tumor cell lines.4 In contrast, frameshift mutations in hMSH6 seemed heterozygous in most of the primary MMP+ gastrointestinal tumors. Analysis of somatic mutations in the entire regions of hMSH3 and hMSH6, like the analysis reported here for hBAX and hMSH3 frameshift mutator mutations in MMP+ sporadic and hereditary gastrointestinal tumors. Preliminary evidence suggests that hMSH6 mutations, similar to BAX mutations, may be homozygous, because tumors with frameshift mutations at the (C)8 tract also contain other mutations in the other allele.4

Recent in vitro studies of yeast and human MSH homologues have shown the redundancy of MSH3 and MSH6 in MSH2-dependent mismatch repair (31, 33, 34). In this regard, it is remarkable that 19 (8 of 41) and 40% (10 of 25) of MMP+ colon and gastric tumors, respectively, harbored double hMSH3/hMSH6 frameshift mutations. In addition, we have found several cases of primary colon and stomach MMP+ tumors and cell lines that harbor concomitant inactivating mutations in hMSH2 and hMSH3 or hMLH1 and hMSH3 and/or hMSH6 mutator genes (16). Therefore, we propose that the sequence of primary-secondary mutator events preferentially but not exclusively involves the MMP-independent mutational inactivation of hMLH1 followed by the MMP-dependent mutational inactivation of the members of the MutS family, for instance, by frameshift mutations in hMSH3 and hMSH6.

The molecular mechanisms governing the genomic instability in sporadic and hereditary MMP+ gastrointestinal tumors and the relative hierarchies and timing of occurrence of these mutator mutations are still incompletely defined. Nevertheless, the presence or occurrence of secondary mutator mutations should be independent of the hereditary or sporadic nature of the tumors in the MMP pathway for carcinogenesis. In this context, we do not find differences in the mutational frequencies for BAX, hMSH3, and hMSH6 genes between MMP+ HNPCC tumors (by the Amsterdam criteria) and MMP+ tumors without documented familial history.5

In conclusion, our results suggest that MMP-induced frameshift mutations in genes controlling apoptosis and DNA mismatch repair play an important role in the MMP+ pathway for gastrointestinal cancer. Identification of other inactivating somatic mutations in BAX, not only in MMP+ but also in MMP− tumors, adds strong support to the tumor suppressor role of BAX in gastrointestinal cancer development. At the same time, these mutations should provide informative clues to understand the function of BAX in regulating apoptosis. The analysis of frameshift and other mutations in hMSH3 and hMSH6 secondary mutators should also provide clues to the molecular mechanisms underlying the profound genomic instability accompanying tumor progression in the MMP pathway for gastrointestinal cancer.

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Frameshift Somatic Mutations in Gastrointestinal Cancer of the Microsatellite Mutator Phenotype

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