Review: The Discovery of the Widespread Microsatellite Instability Phenotype and Distinction of the Mutator and Suppressor Pathways for Gastrointestinal Cancer

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ABSTRACT

This short review summarizes the molecular genetics of gastrointestinal cancer. The first part describes the serendipitous discovery of the widespread microsatellite instability (MSI) phenotype in 1993 during the analysis of genetic alterations in colorectal cancer progression by unbiased arbitrarily primed PCR (AP-PCR) DNA fingerprinting. The concept of ubiquitous mutations in simple repeated sequences is explained in this context. We also describe criteria for the distinction between true genomic instability versus sporadic alterations in microsatellite sequences due to the high background mutation rate of these unstable sequences, which become detectable in tumors because of their clonality. In the second part, we describe the molecular features that distinguish two pathways for gastrointestinal cancer, the suppressor and the mutator pathways. The model of the mutator that mutates other mutators is also discussed. As an illustrative example of the distinctive features in the genotype of tumors from the two pathways, the results of analysis of targeted mutations in the pro-apoptotic gene *Bax* are described in some detail.

Key words : Microsatellite instability, DNA fingerprinting, DNA repair, Frameshift mutation, Apoptosis

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INTRODUCTION

1. The discovery of the widespread microsatellite instability phenotype by AP–PCR

Tumors with the widespread microsatellite instability (MSI) phenotype accumulate hundreds of thousands of somatic mutations in simple repeated sequences or microsatellites[1]. These staggering numbers are simple extrapolations made possible by the unbiased nature of the DNA fingerprinting approach, which was instrumental to their detection[2]. The unbiased search of the genome achieved by AP–PCR is derived from the arbitrary nucleotide sequences of the PCR primers (Fig.1).

ARBITRARILY PRIMED PCR (AP-PCR)

John Welsh & Michael McClelland[2]

CYCLE 1: **Low** annealing temperature (about 45°C) and *thermostable polymerase*.
The primer makes imperfect but sufficiently good match in many sites of genomic DNA.

CYCLE 2: Heat to 95°C then **low** annealing temperature (normally about 45°C).
The primer makes imperfect but sufficiently good matches in a few products from CYCLE 1.

CYCLE 3: Heat to 95°C then **high** annealing temperature (about 65°C).
Perfect matches for all successful priming events from CYCLES 1 and 2.
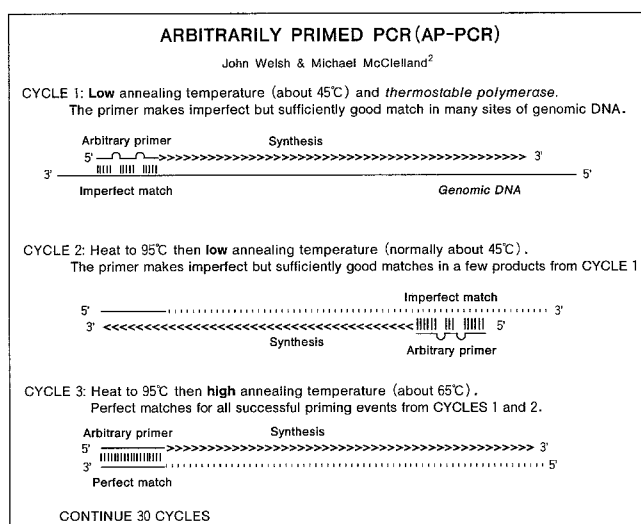
CONTINUE 30 CYCLES

Fig. 1 The principle of the Arbitrarily Primed PCR unbiased DNA fingerprinting

Each primer generates a different fingerprint composed of many bands that originate from random sites in the genome. This knowledge steemed from the observation that most of the fingerprint bands represented different single copy sequences[3]. Cloning and sequencing of fingerprint bands led to the revelation that they were localized in different chromosomes[3,4]. DNA sequences corresponding to chromosomal regions that have undergone gains or losses in tumors can be easily identified, isolated and characterized by AP–PCR (Fig.2, left).

The relative extent of genomic damage among different tumors can be estimated by comparative analysis of the number of changes in their AP–PCR fingerprints. These arbitrary values reflect the degree of tumor cell aneu-
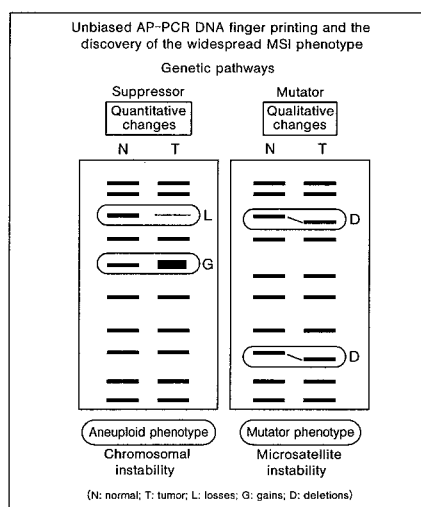
Fig. 2   AP-PCR and the discovery of the MSI phenotype and its application for the diagnosis
of two molecular pathways for gastrointestinal cancer

ploidy and have applications for cancer prognosis[5,6]. The chromosomal loca-
lization of the amplified fragments can be determined by AP-PCR of somatic
rodent/human monochromosome cell hybrids[4]. Thus, consistent gains and
losses of sequences from known chromosomal origins can be readily identified
in particular types of tumors[7]. Therefore, DNA fingerprinting by AP-PCR
provides a molecular approach for cancer cytogenetics[3,5,7].

The fingerprints of a minority of colon cancers revealed one or a few
bands with shifts in mobility relative to normal tissue (Fig.2, right). These
shifts were confirmed to represent somatic mutations by cloning and sequenc-
ing of the fingerprint bands. The number of mutations accumulated in these
tumors was estimated to be hundreds of thousands by simply dividing the
size of the human genome (3×10^9) by the accumulative average size of dif-
ferent genomic sequences amplified in each fingerprint (calculated by adding
the size of bands, about 30 Kb). Thus, $3 \times 10^9 / 3 \times 10^4 = 10^5$. The detection
of these ubiquitous somatic mutations left no doubt that these tumors followed
the cancer as a mutator phenotype hypothesis[8]. Spontaneous errors of re-
plication due to slippage by strand misalignment[9] are fixed as mutations and
accumulate because of defects in replication fidelity of these unstable se-
quences, such as a defective DNA mismatch repair machinery[10]. Tumors of
the hereditary non-polyposis colorectal cancer (HNPCC) syndrome and some
sporadic tumors of the gastrointestinal and urogenital tracts belong to the
widespread MSI phenotype[11]. The MSI phenotype accounts for the mutational

activation and inactivation of cancer genes (those with positive and negative roles in cell growth or survival), which drive multistep carcinogenesis[12,13].

2. "Microsatellite instability" and the concept of ubiquitous somatic mutations

Microsatellite instability is frequently used to describe the genomic instability underlying the pathogenesis of HNPCC and other MSI tumors. However, microsatellite instability is a misnomer because these sequences are intrinsically unstable. Detection in tumors of sporadic alterations in microsatellite sequences is not necessarily diagnostic of genomic instability, because they could be spontaneous errors of replication of these unstable sequences in the absence of any defects in the cellular replication machinery. These mutations are detected in the tumors because their clonal expansions, which are invisible in the polyclonal normal tissue, unveil them. Microsatellite mutations are thus useful as markers of clonality or of mitotic activity, but they may be completely unrelated to the widespread MSI phenotype.

The distinction between true instability and clonality is diagnostic of two distinct molecular genetic pathways for gastrointestinal cancer. Microsatellite clonality in the absence of instability is diagnostic of the classical tumor suppressor pathway for aneuploid cancer[13]. True microsatellite instability is, on the other hand, diagnostic of the MSI pathway for (pseudo)diploid gastrointestinal cancer[10,14].

Tumors in the suppressor pathway may achieve derailment of the homeostatic control of gene expression possibly required for tumor development, by altering the chromosomal balance. This not only unmasks recessive tumor suppressor genes but also increases the amounts of other cancer gene products with positive roles in cell growth or survival[7]. In contrast, tumors with the widespread MSI may achieve the same alteration of the overall patterns of gene expression by the sheer numbers of frameshift and other (point) mutations occurring not only in coding but also in regulatory gene regions[15].

The diagnostic detection of MSI in gastrointestinal tumors is also of practical value in the clinical arena, because it may enable detection of hereditary cases[16]. In addition, it has prognostic value since tumors in the mutator pathway are less aggressive than those in the suppressor pathway.

3. Criteria for classification of gastrointestinal cancer with MSI

The criteria of MSI for colorectal cancers proposed by the National Cancer Institute (NCI) workshop[16,17] were. Analysis of a panel of 381 unselected colorectal tumors according to these criteria yielded 46 high- frequency MSI

(MSI-H) (12%), 36 low-frequency MSI (MSI-L) (9.4%), and 299 micro-satellite stable (MSS) (78.5%) tumors. The results are summarized in Fig. 3. Analysis of the frequency of mutations in target genes for the MSI (see sections 4 and 5) revealed that they were absent in MSI-L tumors (Fig.4). Similar results were also obtained in gastric tumors[18]. Conclusions from
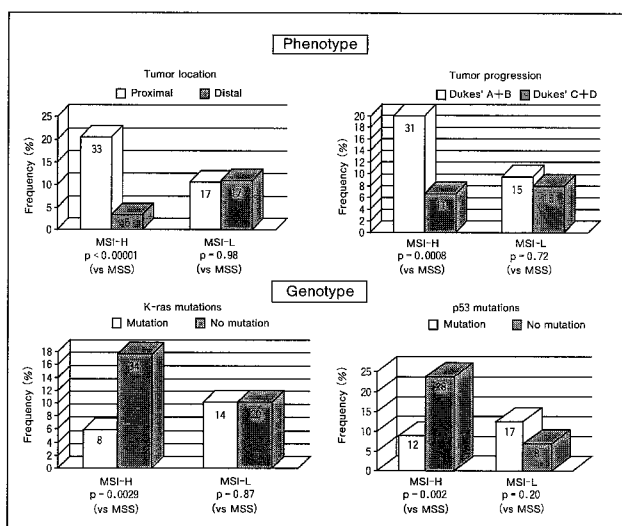

Fig. 3 Comparative features of colorectal tumors according to their MSI status. The percent values shown on the MSI-H vs MSI-L series reflect the proportion of represented cases vs. the rest of the cases. The probability values have been calculated by Chi square test with Yates correction or Fisher exact test.
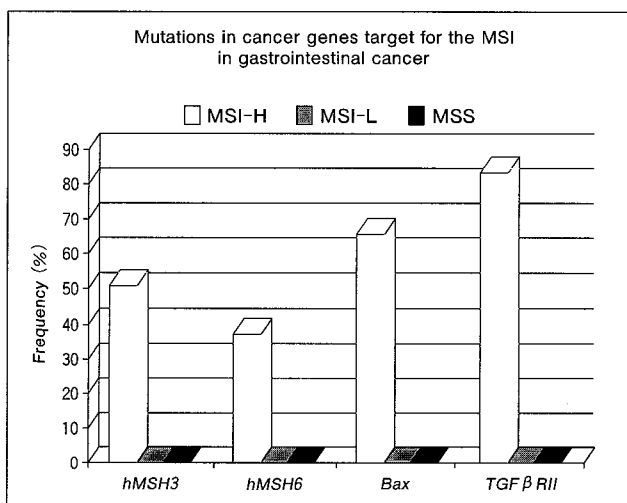

Fig. 4 MSI-L tumors do not accumulate mutations in MSI target cancer genes

these findings are: MSI–H tumors differ from the other gastrointestinal tumors in most clinical, biological and molecular parameters. Therefore, microsatellite alterations in MSI–H tumors represent true genomic instability, underlying a mutator pathway for cancer. MSI–L tumors are indistinguishable from those without microsatellite alterations in every parameter analyzed. Therefore, these isolated microsatellite alterations, although useful markers of clonality or mitotic activity, may simply represent spontaneous errors of replication in the absence of genomic instability. For all practical purposes related to genomic instability and the corresponding diagnostic applications for cancer and cancer susceptibility, MSI–L tumors can be considered as tumors without detectable microsatellite alterations.

4. Gastrointestinal cancer pathways

Two apparently mutually exclusive genomic instabilities define two distinct pathways for gastrointestinal cancer[10,14]. Chromosomal instability is associated with the suppressor pathway for aneuploid cancer, and MSI–H underlies the mutator pathway for (pseudo)diploid cancer. Tumors of the mutator and suppressor pathways follow Knudson's "two hit" model. In hereditary cancers, one mutation is present in the germline and the other is somatic, while both mutations are somatic in sporadic cases. The difference distinguishing the suppressor from the mutator pathway is that suppressor mutation leads to growth or territorial expansion advantage, while mutator mutation does not (Fig.5).
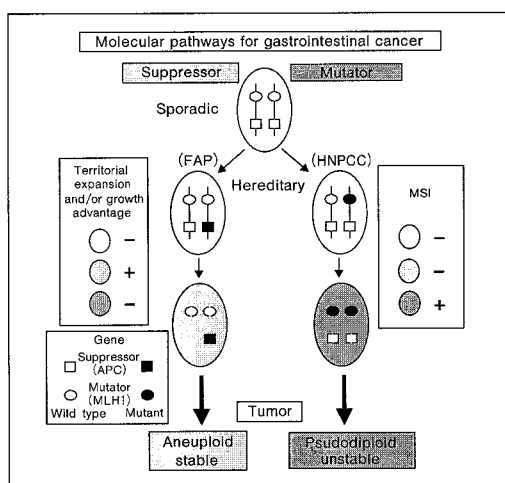

Fig. 5 Genetic pathways for gastrointestinal cancer

   The tumor suppressor pathway usually involves mutations in the tumor suppressor genes *APC* and *p53* and the oncogene *K-ras*[13]. The mutator phenotype pathway[14] unfolds after a mutation occurs in a mutator gene (i.e., DNA mismatch repair family). Tumors with MSI-H are distributed unequally along the gastrointestinal tract (Fig.6), although the reasons for this asym-
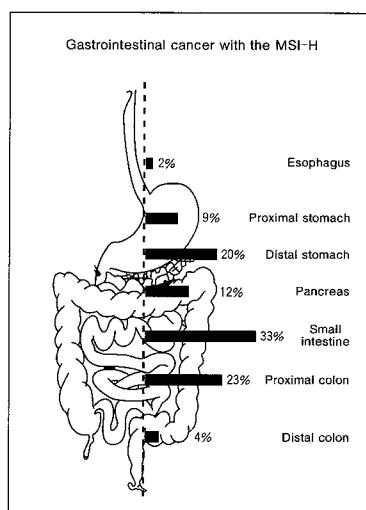

Fig. 6   Incidence of the MSI-H in the gastrointestinal tract

metry are not understood. The MSI-H represents a distinct molecular genetic pathway for gastrointestinal cancer because the cancer genes mutated in MSI-H tumors are generally different from the cancer genes mutated in tumors in the suppressor pathway[10,17,19,20]. This hypothesis originated from an observation that colon tumors with MSI-H displayed paradoxically low mutation frequencies for the two prototypical examples of cancer genes, the *c-K-ras* oncogene and the *p53* tumor suppressor gene[1]. Therefore, while the "distal" molecular genetic cause (the mutator mutations) for cancer with MSI-H[1] was soon confirmed[11], the "proximal" cause for the development of cancer with MSI-H remained unclear until recently.

   An interesting twist to the "two hit" model for cancer is provided by the recent finding that in the mutator pathway for gastrointestinal cancer, inactivation of one mutator allele may also be achieved not by a mutation but by an epimutation. Thus, the inactivation of the *hMLH1* mutator gene is often accomplished by an epigenetic alteration, the hypermethylation of its promoter[21,22].

5. Target cancer genes for MSI-H

The cancer genes mutated in cancer with MSI-H are beginning to be characterized. The TGFβ receptor type II gene (*TGF β RII*) and the pro-apoptotic gene *Bax* are frequently inactivated by slippage-induced frameshift mutations in mononucleotide tracts present in their gene coding regions[23,24]. These findings have provided proof for the causal link between MSI-H and mutations in cancer genes and were also persuasive examples of the differences between the mutator and suppressor pathways for cancer. In contrast with the high incidence of *TGFβRII* and *Bax* frameshift mutations in MSI-H tumors, these mutations are absent in tumors in the suppressor pathway. The *Bax* gene has in its amino terminus a run of 8 Gs, which is a target for the MSI-H, generating insertions and deletions of one bp inactivating the gene product (Fig.7). These frameshift mutations are frequent in colon,
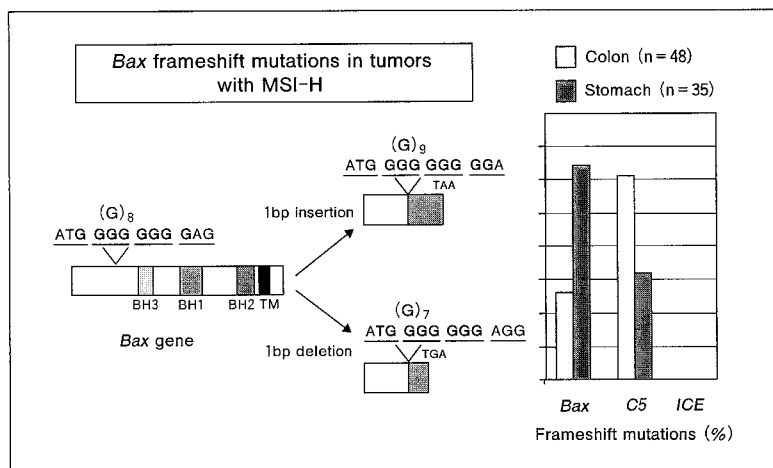

Fig. 7 Frameshift mutations are frequent in the proapoptotic gene *Bax* in MSI-H tumors

stomach, and endometrial cancers with MSI-H[24-26]. The high incidence of *Bax* frameshift mutations in MSI-H tumors and their absence in MSI-L or MSS tumors suggest that these mutations are under a selective pressure during tumor progression. This hypothesis was also supported by the absence of frameshift mutations in identical repeated sequences present in other genes[24-26]. Several other *Bax* missense mutations, with a "hotspot" of transitions at codon 169, have been reported in gastrointestinal tumors with MSI-H[25]. The threonine at this position was replaced by an alanine or by a methionine. Both missense mutations at codon 169 of *Bax* have been shown

to be functional because they inhibit its apoptotic activity[27].

6. The mutator that mutates other mutators

Due to the still limited knowledge of the human genome and the strong mutator phenotype of these tumors, it is likely that there are many target genes mutated in cancer with MSI-H[28]. The targets for MSI-H are not only cancer genes, such as *TGFβRII* or *Bax*, but other mutator genes as well. These secondary mutators are inactivated by the mutagenic effect of primary mutators. The model of the "mutator that mutates another mutator"[29] was proposed because of the detection of frequent frameshift mutations in mononucleotide tracts present in the coding region of *hMSH3* and *hMSH6* DNA mismatch repair genes[25,30]. The secondary mutator mutations may increase the depth or width of the tumor cell genomic instability, accelerating tumor progression.

7. Sporadic and hereditary tumors with MSI-H are indistinguishable in phenotype and genotype

While the clear differences in phenotype and genotype of tumors with or without MSI-H provided the rationale for distinguishing these two pathways for carcinogenesis[1,24], tumors with MSI-H, either hereditary or sporadic, are indistinguishable in all molecular genetic parameters analyzed (Fig.8).
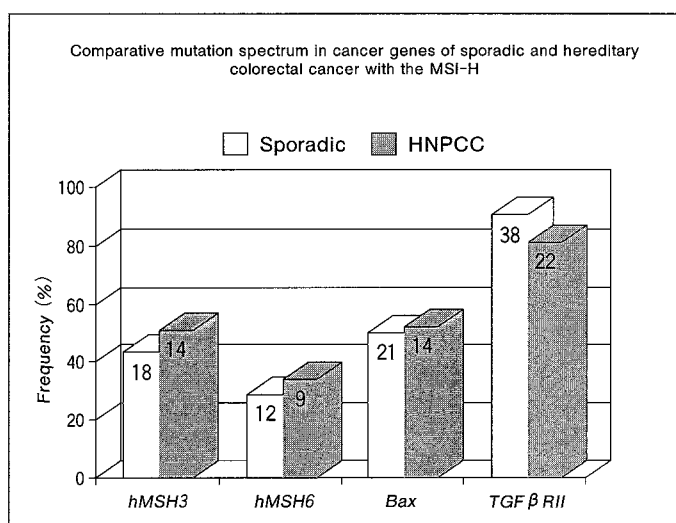

Fig. 8 Hereditary and sporadic tumors with the MSI-H are identical in genotype

This includes somatic inactivation of the primary (*hMSH2* and *hMLH1*) and secondary (*hMSH3* and *hMSH6*) mutators, as well as the cancer genes targets for the MSI (*TGFβRII* and *Bax*)[31]. If these tumors have the same phenotype (hundreds of thousands of somatic clonal microsatellite mutations), it would be surprising if they were to have any significant difference in genotype.

8. Mutations in target genes for MSI-H and the molecular genetic basis for the existence of distinct molecular pathways for cancer

The identification of *Bax* mutations also helped to explain the paradoxical low *p53* mutation frequency in the MSI pathway for cancer. p53 is a transcription activator, and *Bax* is one of its targets. This mediates the apoptotic signaling by p53. However, in the presence of frameshift *Bax* mutations, its transcriptional activation by p53 in response to DNA damage would be futile[24,25]. Once the MSI unfolds, the mutational events leading to cancer are stochastic but predictable because mutations in the *Bax* slippage hotspot usually occur sooner than in the *p53* gene, which lack such repeats.

In conclusion, the presence of simple repeated sequences in subsets of cancer genes, in concert with defective machinery to correct their spontaneous slippage-induced mutations, is the ultimate reason for the existence of these two pathways for cancer and for the biological and clinical differences in corresponding tumors. Once the mutator phenotype is manifested, the mutations in cancer genes with slippage targets occur sooner than in cancer genes without targets, which are involved in tumors of the suppressor pathway with no preference for mutational hot spots for MSI.

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