Preface

In the last years a growing number of phenomena of crucial biological importance have been discovered or emphasised, that have no explanation within a static approach to genetics, and are involved in more or less sophisticated dynamic modifications of the genome, both on individual and evolutive scale. Such phenomena can be grouped in a general branch, for which a suitable name is dynamical genetics, and can be often explained by means of a dynamical genetics approach.

As an illustration we quote for example the so-called dynamic genome (McClintock's jumping genes), the generation of the antibody diversity in the immune system, the mechanisms that control the DNA stability, the lateral gene transfer, and the so-called dynamic mutations (instability of micro- and minisatellites) that are the aetiology of some genetic diseases and of some kind of cancer.

Possible mechanisms that act in this framework often exhibit unusual features; for example, peculiar DNA structures that link four DNA strands, the so-called quadruplexes, are very often involved in cases where the genome is dynamic; moreover such mechanisms are usually implemented by enzymatic complexes of many different proteins, where each protein is used in many different complexes for many different reactions.

The chapters of this book address in various ways a number of such phenomena from humans to bacteria, both from an individual and from an evolutionistic viewpoint, both in physiological and in pathological cases (as mental disorders and cancer); they treat also proteins and quadruplexes that together implement the above phenomena, often by smart enzymatic mechanisms managing the DNA; and they consider not only well-known experimental results but also novel experimental techniques and new suggestive hypotheses.

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Microsatellite instability in human cancers: Its virtual and real images

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Abstract

Somatic instability of microsatellite sequences is frequently associated with various human malignancies. Emergence of a familial cancer-prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC) has connected this phenomenon, microsatellite instability (MSI), to a deficiency in an important DNA repair system, DNA mismatch repair (MMR). Efforts have been made to clarify the relationship between MSI and MMR defects in human cancers. However, with the advancement of our knowledge, the image of MSI in cancer has begun to change. MSI was initially regarded as uniformly reflecting defective MMR and characterising a unique tumourigenesis pathway, which is distinct from the classical tumour suppressor pathway. In fact, the MSI$^+$ phenotype observed in various malignancies is not simple and appears to comprise several categories with different molecular

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backgrounds. Indeed, more than two independent molecular mechanisms explain repeat instability in eukaryotic cells. Defective MMR is an attractive hypothesis for the mutator phenotype in cancer. However, there is a paradox. Contribution of defective MMR to repeat instability and tumourigenesis appears more complicated than has been suspected. In this article, changing and current images of MSI in human cancers are discussed.

1. Introduction - Discovery of MSI in human cancers

Microsatellite is one of the most abundant classes of intergenic repeat sequences that contain short repetitive motifs such as one to five base pairs. These sequences, as well as minisatellite sequences, are highly polymorphic in human populations and have been used as a marker for personal identification or pedigree analyses. With the progress of human genome project, numerous microsatellites have been mapped throughout the genome. Instability of microsatellites at the somatic level, *i.e.* changes of the length of microsatellite sequences, has been initially reported in colorectal cancer (1, 2). Mutability of genomic sequences has been determined in diverse organisms. In *E. coli* the rate of spontaneous mutation has been estimated at 1.0×10^{-10}/base pair/replication. This extremely low level of mutability appears to be a result of finely organised cellular systems functioning in maintenance of genetic information. Errors in DNA replication are one of the greatest threats to cells as a major source of spontaneous mutations. Polymerases functioning in DNA replication tend to increase errors on sequences comprised of small reiterative motifs, such as microsatellites, polymerising one more or less repeat unit. This phenomenon is referred to as 'slippage' of polymerases, and each polymerase has this tendency as an intrinsic character. Misalignment formed between the nascent and template strand leads to insertion or deletion mutations after a next round of replication. However, these mutations are suppressed to the above mentioned level, under the existence of normal cellular functions. Somatic instability of microsatellite sequences strongly suggested defects in cellular systems functioning in maintenance of genetic information.

Previous studies on cellular DNA replication and repair have elucidated two independent systems which dissolve misalignment formed between the nascent and template DNA strands and, consequently, suppress insertion/deletion mutations derived from polymerase slippage. One is the 'proof-reading' function of DNA polymerases, which is executed by the 3' exonuclease activity of the polymerase complexes, and the other is DNA mismatch repair (MMR). These two systems work on misaligned strands or misincorporated bases, and cooperatively prevent insertion/deletion mutations and base substitutions derived from replication errors. In the summer of 1993, instability of microsatellite sequences was reported in sporadic cases of colorectal cancer and, in addition, in hereditary non-polyposis colorectal cancer (HNPCC) (3). At the end of 1993, mutations in one of the genes functioning in MMR were found in HNPCC kindred (4, 5). In cells defective in MMR, misaligned repetitive sequences, caused by polymerase slippage, are to be left unrepaired. Instability of microsatellite sequences in HNPCC was considered to be an inevitable outcome of defective MMR. Thus, the phenomenon of unstable microsatellites, *i.e.* microsatellite instability (MSI), in which cells accumulate this type of repeat length alterations in microsatellites, is uniformly regarded as reflecting MMR deficiency (Figure 1A). The MSI^+ phenotype is frequently

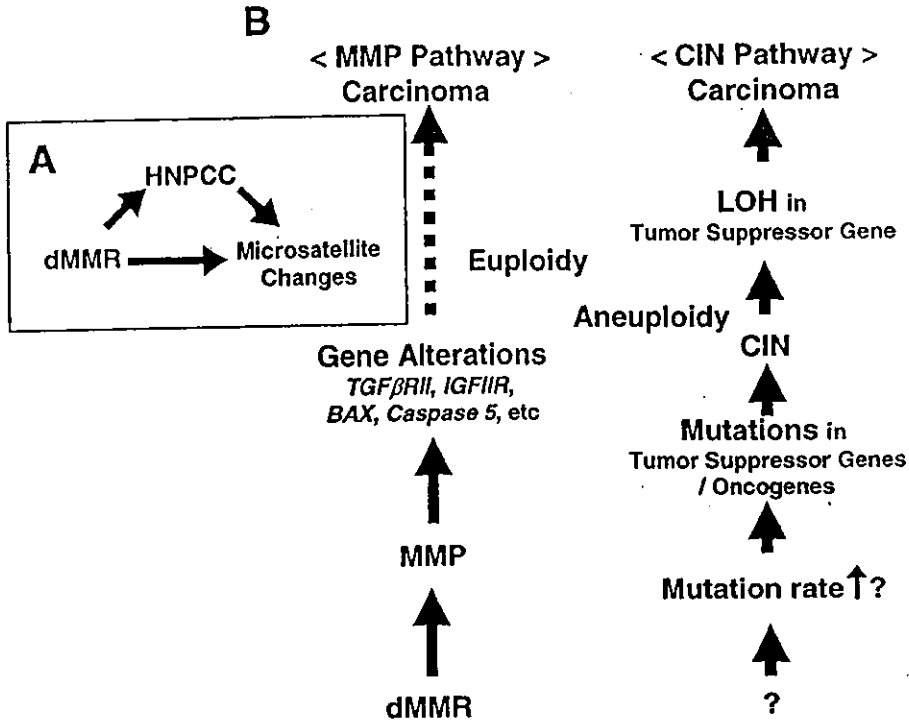

Figure 1. The initially recognised image of MSI and two molecular pathways in colorectal carcinogenesis. A. Microsatellite changes observed in human cancers were initially regarded as uniformly reflecting defective mismatch repair (dMMR). **B.** In colorectal cancer, genetic instability underlying tumourigenesis has been regarded as deriving from two mutually exclusive pathways, 'microsatellite mutator phenotype (MMP)' characterised by microsatellite instability (MSI) derived from dMMR and 'chromosomal instability (CIN)', which is frequently associated with mutations and loss of heterozygosity (LOH) in tumour suppressor gene loci.

associated with various human malignancies. In addition, defective MMR is regarded as one of the important genetic risks for familial predisposition or second malignancies. Analyses of MSI have been prevalent, particularly in the field of oncology. MSI analysis is indeed an efficient approach for detecting defective MMR, since MMR genes have no marked hot spots for mutations. Numerous data have accumulated in the literature. However, the reported frequency for the MSI^+ phenotype in each malignancy differs widely in the literature (6).

2. Changing images of MSI in human cancers

Microsatellite instability (MSI) has initially been reported in colorectal cancer (1, 7). It is now widely accepted that MSI is most frequently observed in colorectal cancer and cancers occurring in the endometrium, both of which feature HNPCC, as defined in the Amsterdam criteria II (8). However, frequencies for MSI^+ tumours reported in the literature are not uniform even in these malignancies (6). These discrepancies may derive from methodological problems left in assay techniques. Analysis of MSI is now commonplace. However, a precise designation of MSI^+ is sometimes difficult. In the most popular and conventional assay systems using radiolabelled primers and manual sequencing gel electrophoresis, various problems have remained to be overcome (9).

Recently, more caution has been paid for methodological aspects of assay techniques and their effects on results of MSI analyses. Several new assay systems using fluorescence labelling and automated sequencer have been reported (10), and improvement of sensitivity and accuracy in these systems is elucidating previously unrecognised aspects of MSI in cancer. Discrepancies in the data may also derive from a variety of microsatellite markers analysed. In 1997, the National Cancer Institute (NCI) sponsored an workshop titled as "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition", in order to review and unify the confused field (11). In this workshop, it has been concluded that the diversity in data derives mainly from the variety of microsatellite markers used and, therefore, a panel of five microsatellites was recommended as 'working reference panel'. In addition, MSI were recommended being classified into two different grades, MSI-H and MSI-L. The first has been defined as ones showing microsatellite alterations in 'the majority of markers' (*e.g.* ≥ 30-40%) and the second as ones exhibiting changes only in 'a minority of markers' (*e.g.* < 30-40%). This distinction of MSI, *i.e.* MSI-H and MSI-L, has been widely accepted since then. However, contrary to expectation, the variety in the data did not diminish and rather increased (Table 1). In this workshop, MSI-H has been defined as *bona fide* MSI derived from defective mismatch repair (MMR). However, MSI-L has not been well characterised. Recently, controversies on the entities of these two categories of MSI have been raised (12-15). Laiho P *et al.* reported that MSI-L was observed in 80% of colorectal tumours without alterations in a mononucleotide microsatellite, BAT26, which are typical in MSI-H tumours, when 377 markers were analysed. Moreover, no significant difference in clinicopathological and molecular variables was observed between MSI-L tumours and ones without MSI. Halford S *et al.* similarly concluded that MSI-L occurs in most colorectal tumours, and that the difference between MSI-L and the microsatellite-stable phenotype is not qualitative but quantitative (15). On the other hand, some reports have shown significant correlations between MSI-L and mutation in *K-ras* or *p53* that is infrequent in MSI-H tumours, which implies that MSI-L tumours form a unique entity (12, 16, 17). Each microsatellite is located in a different chromosome context and exposed to a different risk for accidents in the processes of DNA metabolism, including polymerase slippage. It appears difficult to discriminate MSI^+ tumours from ones without MSI, merely depending on the frequency of observed changes in a given set of markers.

What is remarkable in MSI data is not only the difference in the frequency of alterations, but also the difference in the form of changes. Oda S and colleagues (9, 18, 19) reported that MSI^+ tumours are classifiable into two distinct subgroups, Type A and Type B, according to the length change observed in dinucleotide microsatellites (Figure 2). Based upon observations in mouse or human cell lines with a known defect in MMR genes ((20) and unpublished data), Type A MSI was defined as length changes of ≤ 6-base pairs, and Type B as more drastic modifications involving ≥ 8-base pairs. In fact, Thibodeau *et al.*, one of those who first reported MSI, noticed these qualitative differences (2). They divided microsatellite changes into two categories, Type I and II mutations; the former was defined a 'significant increase (expansion) or decrease (deletion) in the apparent fragment size' and the latter as a 'single 2-bp change'. This classification may be close to the former distinction. Intriguingly, inspection of published data reveals that MSI thus far reported in tumours occurring in HNPCC

Table 1. Frequencies of MSI reported in the literature: a case of gastric cancer

Author	Year	MSI	MSI-H	MSI-L	Note	Reference
Shinmura K	1995	24%				81
Keller G	1995	23.90%				82
Ottini L	1997	48.20%				83
Shinmura K	1997	36.30%				84
Wirtz HC	1998	44.50%				85
Keller G	1998		11%	28%		86
Nakachi A	1999	20%				87
Sepulveda AR	1999		50% 7% 15%	9% 5% 20%	Korea USA Colombia	88
Artunedo Pe P	2000		37.8%			89
Schneider BG	2000		18.2%	10.6%		90
Philp AJ	2000		16%			91
Leung WK	2000		26.7%	50%		92
Miyoshi E	2001		3%	8%		93
Ogata S	2001		24.2%	6.1%		94
Lee HS	2001	9.40%				95
Palli D	2001	34.10%				96
Flocca R	2001	14.60%				97
Tamura G	2001	0%				98
Simpson AJ	2001	15% 16% 27% 16% 21% 23% 50%			Japan Korea Taiwan USA UK Portugal Italy	99
Theur CP	2002	39% 20%	0% 15%		Japan USA	100
Lee HS	2002	9.50%				101
Lawes DA	2003		5% 2~15%	16~39% 24~84%	Japan Western countries	102

individuals is predominantly Type B/Type I mutation. In addition, in colorectal cancer, Type B MSI tends to occur in the majority of markers analysed, while Type A/Type II mutations have a tendency to be noted in a limited number of markers. Therefore, in colorectal cancer, Type A/B MSI, or Type II/I mutations, may correspond to MSI-L/H, respectively (Table 2). The Bethesda classification, *i.e.* MSI-H and –L, is based on these observations. Type A MSI/Type II mutations are observed in approximately 25% of sporadic colorectal tumours (18, 19). Intriguingly, MSI observed in mouse and human cell lines with a known defect in MMR genes is uniformly Type A ((20) and Oda S *et al.* in preparation). In addition, there is a series of findings to suggest that tumours exhibiting Type A MSI and Type B tumours arise from different molecular backgrounds. It is well known that MSI-H/Type B tumours occur more frequently in the proximal colon. On the other hand, it has been recently reported that all the microsatellite changes observed in tumours occurring in the rectum are Type A (18). Moreover, while it is widely accepted that mutations in oncogenes or tumour suppressor genes, such as *K-ras*, *p53* etc, are infrequent in MSI-H/Type B tumours, Type A MSI appears to be strongly correlated with mutations in these genes (Oda S *et al.*, in preparation). This observation

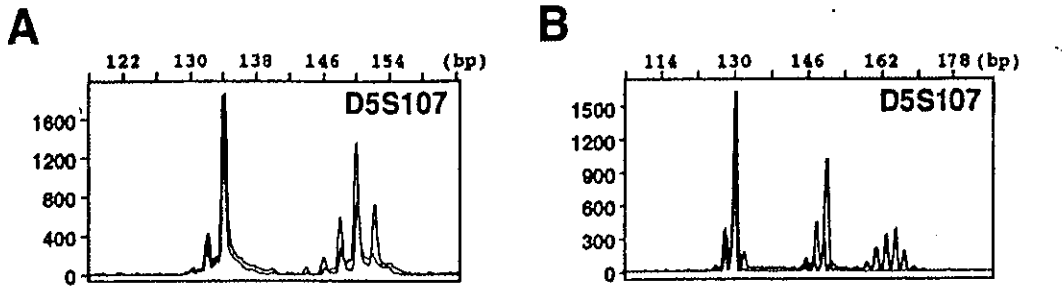

Figure 2. Type A and Type B microsatellite instability observed in human cancers. Dinucleotide microsatellite sequences, indicated at the right top of each panel, were amplified by PCR with primers differentially labelled with fluorescence, then mixed and run on a same lane in an automated sequencer. The x axis corresponds to the fragment length standardised using size markers run in the same lane, and the y axis indicates the quantitatively detected amount of each DNA fragment. Results typical for each subtype of microsatellite instability are shown: red lines, cancer; green lines, normal mucosa. **A.** Type A alteration defined as length changes of ≤ 6-base pairs. **B.** Type B change involving more drastic modifications involving ≥ 8-base pairs with 'jump'-like expansions.

Table 2. Relationships between Type A/B MSI and MSI-L/H in colorectal cancer

	Type A	Type B	Total
MSI-H	2	12	14
MSI-L	28	0	28
Total	30	12	42

MSI: microsatellite instability
(modified after reference 18)

may be compatible with several recent reports that have shown correlations between MSI-L and mutations in *K-ras* or *p53* (12, 16, 17), since MSI-L corresponds to Type A in colorectal cancer (Table 2). Thus, MSI^+ tumours appear to include, at least, two distinct entities with different molecular backgrounds, *i.e.* tumours with MSI-H/Type B MSI and ones with Type A MSI, in colorectal cancer. This may be rather possible since more than two distinct molecular mechanisms may contribute to microsatellite changes occurring in eukaryotic cells, as discussed later.

Has the molecular background of MSI-H/Type B MSI been well understood? In more than 90% of tumours occurring in HNPCC individuals, this type of MSI is observed (21). As mutations in major MMR genes were found in HNPCC kindred (4, 5), this phenotype of MSI has been directly connected to defective MMR (Figure 1.A). However, as mentioned above, microsatellite changes observed in MMR gene-knock out mice, including ones in tumours occurring in the mouse bodies, was uniformly Type A (Oda S *et al.* in preparation), which strongly suggests that Type A MSI is a direct consequence of defective MMR, and that deficiency in MMR itself is not sufficient for Type B MSI. Previously unrecognised molecular abnormalities, in addition to defective MMR, may underlie the development of Type B MSI. This hypothesis may be compatible with the findings that a generally reported figure of mutation frequency in

major MMR genes in HNPCC kindred is sometimes lower than 50% (22-27). Needless to say, the relationship between molecular abnormalities causing these genomic changes and pathogenesis in HNPCC remains unclear. However, such drastic and multicentric changes in the genome may influence the structure of chromatin domains and, consequently, expression of the genes within. In tumours exhibiting typical MSI, *i.e.* MSI-H or Type B, base substitution mutations in representative tumourigenic genes, such as *APC*, *K-ras* or *p53*, are rare and, instead, insertion/deletion mutations are found in mononucleotide runs within the genes of a different variety, such as *TGFβRII* (28), *IGF2R* (29), *BAX* (30), *Caspase 5* (31) etc (32, 33). Alterations in these genes functioning in growth control and apoptosis have been highlighted as a cause of tumourigenesis. Insertion/deletion mutations in mononucleotide runs in the coding region may cause a shift of the reading frame, and finally lead to a change in protein structure and, possibly, in gene function. Because a T cell clonality reactive to these altered proteins is indeed found in patients with MSI-H tumours (34), proteins with these structural alterations appear to be expressed in MSI-H tumour cells. Intriguingly, in mice, such long mononucleotide runs are not found in the counterpart genes (35). Pathogenic significance of these gene alterations remains unclear.

Clinical and pathological features of MSI-H tumours appear more established. It is now widely accepted that MSI-H tumours occur more frequently in the proximal colon, and possess a reduced metastatic potential and an increased sensitivity to chemotherapy (2, 36-41). Histopathologically, they frequently exhibit poor differentiation, mucinous component and lymphocyte infiltration (37, 39, 41-43). However, in fact, MSI^+ colorectal tumours appear to arise via two distinct pathways in tumourigenesis; one is MSI-H tumour occurring in HNPCC individuals in which deleterious germ line mutations are found in major MMR genes, and the other is sporadic MSI-H colorectal cancer. It was originally expected that all MSI-H tumours harbour an inherited defect, *i.e.* mutation in MMR genes. However, most MSI-H tumours are sporadic and germ line mutations are not found. In population-based studies, it is suggested that germ line mutation in major MMR genes accounts for 2 – 5% of colorectal cancer (44, 45). At present, generally accepted frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, are beneath 30% in all of sporadic MSI-H colorectal tumours. Instead, in sporadic MSI-H tumours, a role of epigenetic silencing of *hMLH1* is assumed to be more important. It has been reported that colorectal tumours which do not express hMLH1 comprise approximately 70% of all MSI^+ tumours (46-50). In MSI-H tumours, epigenetic events may play a critical role in tumorigenesis, particularly in the sporadic setting. In this context, distinction of MSI-H tumours comprised of two distinct categories, *i.e.* familial MSI-H and non-familial MSI-H colorectal cancer, has been emphasised by Jass JR and colleagues (43, 49, 51). They found differences in clinicopathologic features between these two categories. Indeed, familial MSI-H and non-familial MSI-H colorectal cancer show a line of significant histopathological differences, including mucinous component, lymphocyte infiltration and concomitant serrated adenoma, which is recently suggested to be a precursor lesion for sporadic MSI-H colorectal cancer (52, 53). MSI-H tumours occurring in HNPCC individuals and sporadic MSI-H tumours may be more different than have been suspected.

In colorectal cancer, genetic instability underlying tumourigenesis has been regarded as deriving from two mutually exclusive pathways, 'chromosomal instability (CIN)'

frequently associated with mutations in various oncogenes or tumour suppressor genes such as *APC*, *K-ras*, *p53* etc – and 'microsatellite mutator phenotype (MMP) (54, 55)', which is characterised by MSI (56, 57) (Figure 1B). However, recent studies on genetic instability in colorectal cancer suggest that this distinction may be an oversimplification, and that these two pathways are not always independent and overlap in some tumours. Heterogeneity in MSI-H tumours, *i.e.* familial MSI-H and non-familial MSI-H colorectal cancer, and entity of MSI-L or Type A MSI have already been discussed. In addition, tumours in which MSI and LOH are coincident (58) and biologically distinct diploid tumours without MSI (59) have been recently reported. In the former report, more than twenty percent of MSI-H tumours exhibited LOH events at acknowledged tumour suppressor loci. It is widely accepted that tumours with MSI-H are largely diploid and that aneuploidy is frequently observed in tumours without MSI, *i.e.* CIN tumours. Diploid tumours with stable microsatellites also appear to exist. They represent a biologically distinct phenotype, *i.e.* a significantly high metastatic potential at an early clinical stage. This complexity in the relationship between the CIN and MMP pathways may derive from an oversimplification of the both phenotypes (Figure 3). CIN may be comprised of numerical and structural instability in chromosomes. The former is characterised by aneuploidy, and the latter by diverse chromosome aberrations including LOH. Different molecular abnormalities may underlie the two CIN phenotypes. Detailed characterisation of the CIN and MMP pathways is necessary for a better understanding of tumourigenesis in the colorectum, and, possibly, other organs.

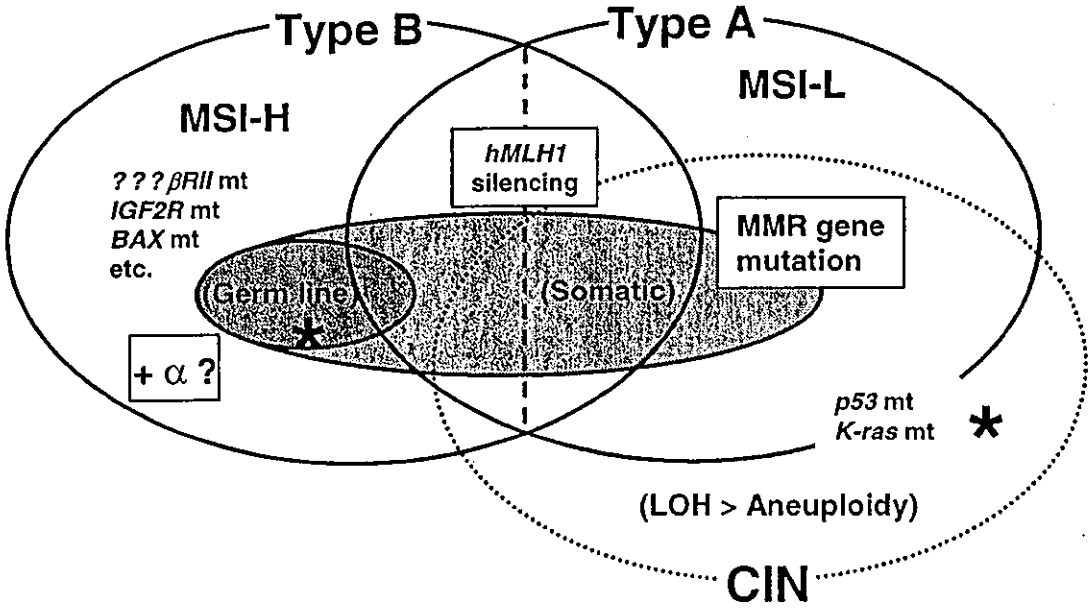

Figure 3. Complicated realities of MSI$^+$ phenotypes in human colorectal cancer. Molecular mechanisms underlying each category are shown in rectangles. The closed and open asterisks indicate the classical MMP and CIN pathways, respectively. MSI, microsatellite instability; dMMR; defective mismatch repair; CIN, chromosomal instability; LOH, loss of heterozygosity; mt, mutation.

3. The origins of ambiguity

Frequency or qualitative changes

Tomlinson I and colleagues (15) have concluded in their recent report that MSI-L occurs in most colorectal tumours, and that the difference between MSI-L and the microsatellite-stable phenotype is not qualitative but quantitative. In this report, no difference in clinicopathological features and molecular backgrounds was found between MSI-L and microsatellite-stable tumours. On the other hand, some reports have shown significant correlations between MSI-L and mutation in *K-ras* or *p53* (12, 16, 17), which implies that MSI-L tumours form a distinct entity. Although MSI-H tumours appear to form unique clinicopathological and molecular entities, there seems to be a limit in discussing MSI merely from the frequency of changes in a given set of markers. As mentioned above, it may be also informative to note qualitative aspects of MSI. More than two distinct molecular mechanisms may contribute to microsatellite changes occurring in eukaryotic cells, as discussed below. These molecular mechanisms should work differently on repeat sequences with different unit lengths, and may lead to different modes of length changes in repeat sequences. Qualitative differences in dinucleotide MSI have been pointed out by Thibodeau SN *et al.* (2). More caution is recently being paid for methodological aspects of MSI assay. Fragment analysis using fluorescent PCR primers and an automated sequencer has greatly improved sensitivity and quantitativity, and enabled us to observe microsatellite changes in detail (10). This approach is now widely used and elucidating qualitative differences of MSI (9, 19).

The second 'marker problem': Mono-, di- or tri-nucleotide?

The above mentioned NCI workshop concluded that the diversity in MSI data in the literature derives mainly from the variety of microsatellite markers used, and recommended a 'working reference panel' of markers. Recently, selection of targets for analysis has become controversial once again. Jass JR *et al.* pointed out that instability in dinucleotide microsatellites is not identical to mononucleotide MSI, and that use of dinucleotide markers for detecting MSI-H is problematic (43). Mononucleotide markers, such as BAT25, BAT26 etc, exhibit typical 'jump'-like band shifts in MSI-H tumours. On the other hand, instability in dinucleotide microsatellites is observed mainly in MSI-L. The 'working reference panel' recommended by NCI includes two dinucleotide markers, which appear to be employed because of their sensitivity to MSI-L. In fact, molecular mechanism causing repeat instability are not single. In addition to polymerase slippage and defective mismatch repair (MMR), erroneous proofreading by polymerase complexes and misalignment in the processes of recombinational repair are possible. An important finding is that mismatch recognition complexes such as MutSα work mainly on loop-outs comprised of one or two base and much less on larger loop-outs (60-62). Instability in trinucleotide repeats is known in some neurodegenerative disorders (63), but MMR is not regarded as a mechanism of this category of repeat instability. Instead, recombinational models are now proposed (64). Proofreading, *i.e.* 3' exonuclease activity, of polymerase complexes may work mainly on one base loop-outs. Thus, in each of genetic backgrounds defective in these cellular functions, instability of repeat sequences comprising different unit lengths should manifest itself differently (Table 3).

Table 3. Possible molecular mechanisms underlying different modes of repeat instability in eukaryotic cells.

	Repeat Instability		
	mono-	di-	tri-
Proof-reading (-)	(+)(A)	(-)	(-)
MMR (-)	(+)(A)	(+)(A)	(±)(A)
? (+ MMR (-))	(+)(B)	(+)(B)	(+)(B)

MMR: DNA mismatch repair, (A): Type A mode, (B): Type B mode.

Moreover, there are two modes of dinucleotide MSI (9, 19), *i.e.* Type A MSI/Type II mutations and Type B MSI/Type I mutations with 'jump'-like expansion. In the latter, misalignment in the processes of recombinational repair may be involved. Indeed, MMR counteracts incorrect strand alignment during homologous recombination (65). Such drastic and 'jump'-like changes in Type B MSI may be more consistent with dynamic events such as recombination, than with polymerase slippage. In this hypothesis, defective MMR is a promoting, and consequently highly coincidental, but insufficient molecular abnormality for Type B MSI. This hypothesis appears to provide a relatively better explanation for the genetic data in tumours exhibiting Type B MSI/MSI-H, including HNPCC, and MMR gene-knock out mice. Clarifying relationships between these molecular mechanisms and each category of repeat instability is of urgent necessity.

Discrepancies between MSI and MMR gene mutation

In HNPCC individuals, deleterious mutations in major MMR genes are found, not in all but in more than a half. On the other hand, in sporadic MSI-H colorectal tumours, generally accepted frequencies for mutations in the major MMR genes, *hMSH2* and *hMLH1*, are beneath 30%, and missense mutations with unknown pathogenic significance predominate. Instead, a role of epigenetic silencing of hMLH1 is now highlighted. It has been reported that colorectal tumours which do not express hMLH1 comprise approximately 70% of all MSI^+ tumours (46-50). However, it is not always of ease to designate a loss of expression by immunohistochemistry, considering quality of fixed tissue specimens, reactivity of antibodies used and technical variables. In fact, methylation of the proximal region of *hMLH1* promoter and its extent well correlate with loss of hMLH1 expression (66-68). However, results in immunohistochemistry are not always parallel with ones in methylation analyses (69). MSI-H tumours may arise via two different pathways in which defective MMR is differentially involved. Indeed, Jass JR and colleagues propose that MSI-H tumours in these two settings form clinicopathologically different entities (43, 49, 51), which may suggest that additional and previously unrecognised molecular abnormalities underlie the differential tumourigenesis. Nevertheless, MSI-H tumours exhibit a unique and uniform mode of instability, *i.e.*, Type B MSI, either in the hereditary or the sporadic settings. Relationship between defective MMR and this form of MSI appear more complex than has been suspected.

Where does mutation come from? – Defective mismatch repair as a source of mutation

It remains controversial whether a state with an increase in the mutation rate plays an important role in tumourigenesis. Loeb and colleagues (70, 71) propose a state with an elevated mutation rate in tumourigenesis, and this state is now referred to as 'mutator phenotype'. On the other hand, Bodmer and colleagues (72, 73) pointed out that tumour cells which harbour mutations in tumour suppressor genes or oncogenes can be selected merely by phenotypical advantage, without an increased mutation rate. Spontaneous mutation rate on the genome is invariably controlled. Previous studies using *E. coli* mutators suggest that there are several cellular systems, the failure of which will lead to a significant increase in the mutation rate. MMR is also categorised into these systems. In an *E. coli* mutator, in which *mutS*, one of MMR genes, is inactivated, the spontaneous mutation rate is 100-fold higher than the wild type level. Mutations found in this mutator are mainly base substitutions and one base-insertions/deletions (74). Nevertheless, base substitutions in acknowledged oncogenes or tumour suppressor genes are not found in MSI-H tumours that are regarded as MMR-deficient. Instead, only insertion/deletion mutations are observed in mononucleotide runs within the reading frame in several genes of a different variety, such as *TGFβRII*, *IGF2R*, *BAX*, *Caspase 5* etc. It is a remarkable question why only insertion/deletion mutations are found in MSI-H tumour cells, while both of base substitutions and insertions/deletions are increased in cells defective in MMR, either in *E. coli* or in mammals (74-77).

It is widely accepted that chromosomal instability (CIN) and MSI characterise two mutually exclusive pathways of tumour development, and that in CIN tumours point mutations in representative oncogenes or tumour suppressor genes are frequent. Some recent reports have pointed out a connection between MSI-L and mutations in *p53* or *K-ras* genes (12, 16, 17). These observations are consistent with our finding that Type A MSI correlates with *p53* mutation (Oda S *et al.*, in preparation), considering that Type A overlaps a part of MSI-L (Table 2). MSI observed in MMR gene-knock out mice was Type A. *Bona fide* mutator phenotype derived from defective MMR, in which the rate for point mutation, *i.e.* both of base substitution and insertion/deletion, is elevated, may rather underlie tumourigenesis in some of Type A or MSI-L tumours.

Where do mutations found in cancer come from? In MSI^+ colorectal cancer, defective MMR is the most likely candidate for the source of mutation. However, there is a paradox, as discussed above. In tumours with stable microsatellites, such as squamous cell carcinoma in the oesophagus (78), non-small cell lung cancer (79) and breast cancer (80), the source of mutation is unknown. From the previous studies using *E. coli* mutators, it is known that disruption of several cellular components, particularly DNA repair enzymes counteracting oxidative DNA damage and replication polymerises, leads to a marked increase in the spontaneous mutation rate. Abnormalities of these cellular functions in cancer are of particular interest. It appears essential to test these possibilities in various cancers for understanding more of the mutator phenotype underlying tumourigenesis.

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