

Two modes of microsatellite instability in human cancer: differential connection of defective DNA mismatch repair to dinucleotide repeat instability

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Received January 13, 2005; Revised and Accepted February 24, 2005

ABSTRACT

Microsatellite instability (MSI) is associated with defective DNA mismatch repair in various human malignancies. Using a unique fluorescent technique, we have observed two distinct modes of dinucleotide microsatellite alterations in human colorectal cancer. Type A alterations are defined as length changes of ≤ 6 bp. Type B changes are more drastic and involve modifications of ≥ 8 bp. We show here that defective mismatch repair is necessary and sufficient for Type A changes. These changes were observed in cell lines and in tumours from mismatch repair gene-knockout mice. No Type B instability was seen in these cells or tumours. In a panel of human colorectal tumours, both Type A MSI and Type B instability were observed. Both types of MSI were associated with *hMSH2* or *hMLH1* mismatch repair gene alterations. Intriguingly, *p53* mutations, which are generally regarded as uncommon in human tumours of the MSI⁺ phenotype, were frequently associated with Type A instability, whereas none was found in tumours with Type B instability, reflecting the prevailing viewpoint. Inspection of published data reveals that the

microsatellite instability that has been observed in various malignancies, including those associated with Hereditary Non-Polyposis Colorectal Cancer (HNPCC), is predominantly Type B. Our findings indicate that Type B instability is not a simple reflection of a repair defect. We suggest that there are at least two qualitatively distinct modes of dinucleotide MSI in human colorectal cancer, and that different molecular mechanisms may underlie these modes of MSI. The relationship between MSI and defective mismatch repair may be more complex than hitherto suspected.

INTRODUCTION

Microsatellites are repetitive DNA sequences comprising short reiterated motifs dispersed throughout the eukaryotic genome (1). Microsatellite lengths are highly polymorphic in human populations, but appear stable during the life span of the individual. Somatic instability of microsatellite sequences has initially been reported in human colorectal cancer (2,3), and particularly in the familial cancer-prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC) (4,5). In 1993, mutations in one of the genes encoding proteins essential for DNA mismatch repair (MMR) were found in

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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HNPCC individuals (6,7). MMR is an important editing system. It counteracts the base mismatches and strand misalignments that occur during DNA replication and recombination (8). Repetitive sequences such as those comprising microsatellites are particularly prone to polymerase slippage and, consequently, strand misalignment. If these errors remain uncorrected, the mutations are fixed during subsequent replication as addition or deletion of one (or more) repeat units. The phenomenon of unstable microsatellites, i.e. microsatellite instability (MSI), in which tumour cells accumulate this type of repeat length alterations in microsatellites, is considered to reflect MMR deficiency. The MSI⁺ phenotype is frequently associated with various human malignancies (9). As defective MMR is regarded as a risk factor for familial predisposition or second malignancies, analyses of microsatellite instability have been prevalent, particularly in the field of oncology. However, the reported frequency for MSI⁺ tumours in each malignancy differs widely in the literature (9).

Although analysis of MSI is now commonplace, a designation of MSI⁺ may sometimes be a difficult decision. The 1997 National Cancer Institute (NCI) workshop, 'Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition', suggested that the variety of microsatellites used was a major cause of discrepancies among data from various laboratories, and recommended a panel of five microsatellites as 'working reference panel' (10). We believe that, in addition to selection of targets for analysis, methodological problems also account for some of the variability in results. Changes in microsatellite lengths are sometimes minor—as small as loss or gain of a single repeat unit. In addition, cells carrying changes in microsatellite sequences are not always major in a given sample. However, in an assay system using the conventional sequencing gel electrophoresis and autoradiography, it appears difficult to resolve microsatellite PCR products precisely and quantitatively. PCR itself has an intrinsic variability. The most widely used thermostable DNA polymerase (*Taq*) has a terminal deoxynucleotidyl transferase (TDT) activity, which adds one additional base to PCR products in a sequence-dependent manner. TDT activity of *Taq* polymerase is variably expressed, depending on the conditions used. This property, in addition to intrinsic strand misalignment during amplification of microsatellite repeats, increases the complexity of PCR products. In the conventional microsatellite analysis, intrinsic caution and the desire to avoid scoring false-positives may have led to an underestimate of the frequency of minor, more subtle microsatellite changes, such as alterations of limited numbers of repeat units. We have applied our fluorescent technique for microsatellite instability analysis (11) to address these problems. Here, we report that relatively subtle alterations in microsatellites are indeed generally associated with MMR deficiency. In contrast, most HNPCC tumours display much more extensive microsatellite changes. Our findings suggest that there are previously unrecognized aspects of microsatellite instability in human cancer.

MATERIALS AND METHODS

Cells and tissue specimens

Msh2^{-/-} mouse embryonic fibroblast (MEF) cell line, RH95021 (12) and *Mlh1*^{-/-} MEF cell line, MC2, were kindly

provided by Dr Hein te Riele, Amsterdam Cancer Center and Dr Michael Liskay, University of Oregon, respectively. Cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Samples of cancer tissues and the corresponding normal mucosa were obtained from 79 patients with colorectal carcinoma who underwent surgery in the Department of Surgery and Science, Kyushu University Hospital from 1996 to 1999. Written informed consent for studies using the tissues was obtained from each patient. Ethical approval was obtained from the IRB of Kyushu University. Specimens, taken immediately after resection, were placed in liquid nitrogen. High molecular weight DNA was extracted and subjected to microsatellite analyses.

Microsatellite instability

Microsatellite analysis using fluorescence-labelled primers and an automated DNA sequencer has been described in detail (11). Briefly, five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, in genomic DNA from tissue specimens were amplified by PCR. 5' primers were labelled with the fluorescent compound, ROX (6-carboxy-x-rhodamine) or HEX (6-carboxy-2',4',7',4',7,-hexachloro-fluorescein). PCR reactions were done using TaKaRa *Taq* (TaKaRa Co. Ltd., Tokyo, Japan). T4 DNA polymerase was added to the PCR products, followed by incubation at 37°C for 10 min. To compare electrophoretic profiles between two samples, 1.2 µl of ROX-labelled product and 0.3 µl of HEX-labelled product were mixed. Samples were denatured and loaded onto the ABI 373A sequencer (Applied Biosystems, Foster City, CA, USA). The data were processed using the GeneScan software (Applied Biosystems). For mice, three dinucleotide microsatellites, D1Mit62, D6Mit59 and D7Mit91, were analysed.

DNA sequencing

All the exons and exon-intron junctions of *hMSH2* and *hMLH1* were amplified by PCR using *Taq* polymerase with 3' exonuclease activity, TaKaRa *Ex Taq* (TaKaRa Co. Ltd., Tokyo, Japan). Primer sequences are the same as reported by Kolodner *et al.* (13,14), except that the additional sequence complementary for M13 universal primer was deleted, and that one-step PCR was mainly employed. PCR products were used as a template for cycle sequencing reactions using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Mutations found in one PCR product were verified by reverse sequencing and finally confirmed in two independently amplified PCR products. Sequencing analyses of *p53* gene (exon 5–9) were performed using *p53* primers (Nippon Gene, Tokyo, Japan).

Immunohistochemistry

Tissue specimens were fixed in buffered 10% paraformaldehyde and embedded in paraffin. Prior to the assay, the specimens were sectioned at 4 µm and deparaffinized using xylene. Immunohistochemistry was performed using the streptavidin-biotin-peroxidase complex method (Histofine SAB kit, Nichirei, Tokyo, Japan) using an automated stainer (VENTANA Discovery System, Ventana Medical Systems Inc., Tucson, AZ, USA). At least, two independent antibodies

were used to confirm the status of negative staining. Sections prepared from *Msh2*- and *Mlh1*-knockout mice were also used as negative controls. Antibodies used were as follows: anti-MSH2; NA27 and NA26 (Oncogene Research Products, Cambridge, MA, USA), anti-MLH1; PM-13291A (PharMingen, Hamburg, Germany), NA28 (Oncogene Research Products) and sc-581 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RESULTS

Two modes of dinucleotide microsatellite instability in human cancer

We have established a sensitive fluorescent technique for microsatellite analysis (11). Application of this technique to human cancers revealed a number of previously unrecognized aspects of MSI. In particular, we observed two distinct patterns of alterations at dinucleotide microsatellites in human malignancies (15–17). Examples are shown in Figure 1. In some cases, length changes are relatively small and affect ≤ 6 bp (Type A, Figure 1A–D). In the other, more dramatic changes

involving ≥ 8 bp are observed (Type B) (Figure 1E–H). Because Type B alterations involve large differences in microsatellite length, it can sometimes appear as if a ‘third’ allele is present in addition to the parental alleles (Figure 1E–H). Throughout the analyses using this technique, results were highly reproducible in several independent experiments. Neither additional peaks nor changes in the ratio between peaks were noted.

Microsatellite instability observed in mismatch repair gene-knockout mice

To analyse MSI in a defined genetic background, we used the *Msh2*^{-/-} MEF cell line RH95021 (12). Alterations in the lengths of three dinucleotide microsatellites were analysed in RH95021 subclones. The majority of subclones (14/21) exhibited the same configuration at the D6Mit59 locus (exemplified by clone a, Figure 2A). In clones that deviated from this predominant pattern (Figure 2A, clones b–d), the microsatellite length was altered by ≤ 4 bp. In other words, the microsatellite changes were invariably Type A. A similar pattern of small-scale microsatellite changes was observed at

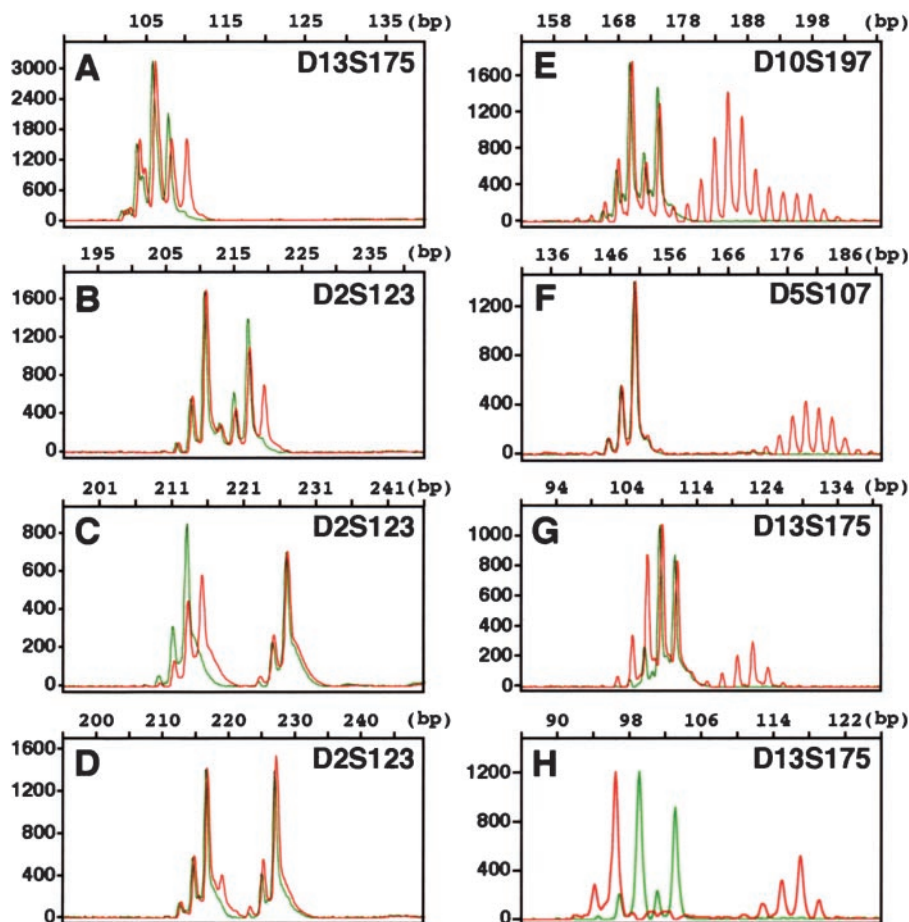


Figure 1. Type A and Type B microsatellite instability observed in human colorectal cancer. Using genomic DNA samples prepared from cancer and the corresponding normal mucosa, microsatellite sequences, indicated at the right top of each panel, were amplified by PCR with primers differentially labelled with fluorescence, then mixed and run on a same lane in an automated DNA sequencer. The amount of each DNA fragment was quantitatively detected and its size was estimated with accuracy of 1 bp, by standardization with size markers run in each lane. Results representative for each mode of microsatellite instability are shown: red lines, cancer; green lines, normal mucosa; Type A, (A) (IC678), (B) (IC810), (C) (IC721) and (D) (IC793); Type B, (E) (IC790), (F) (IC733), (G) (IC690) and (H) (NoTa). Patient codes in the parentheses correspond to those used in Table 1.

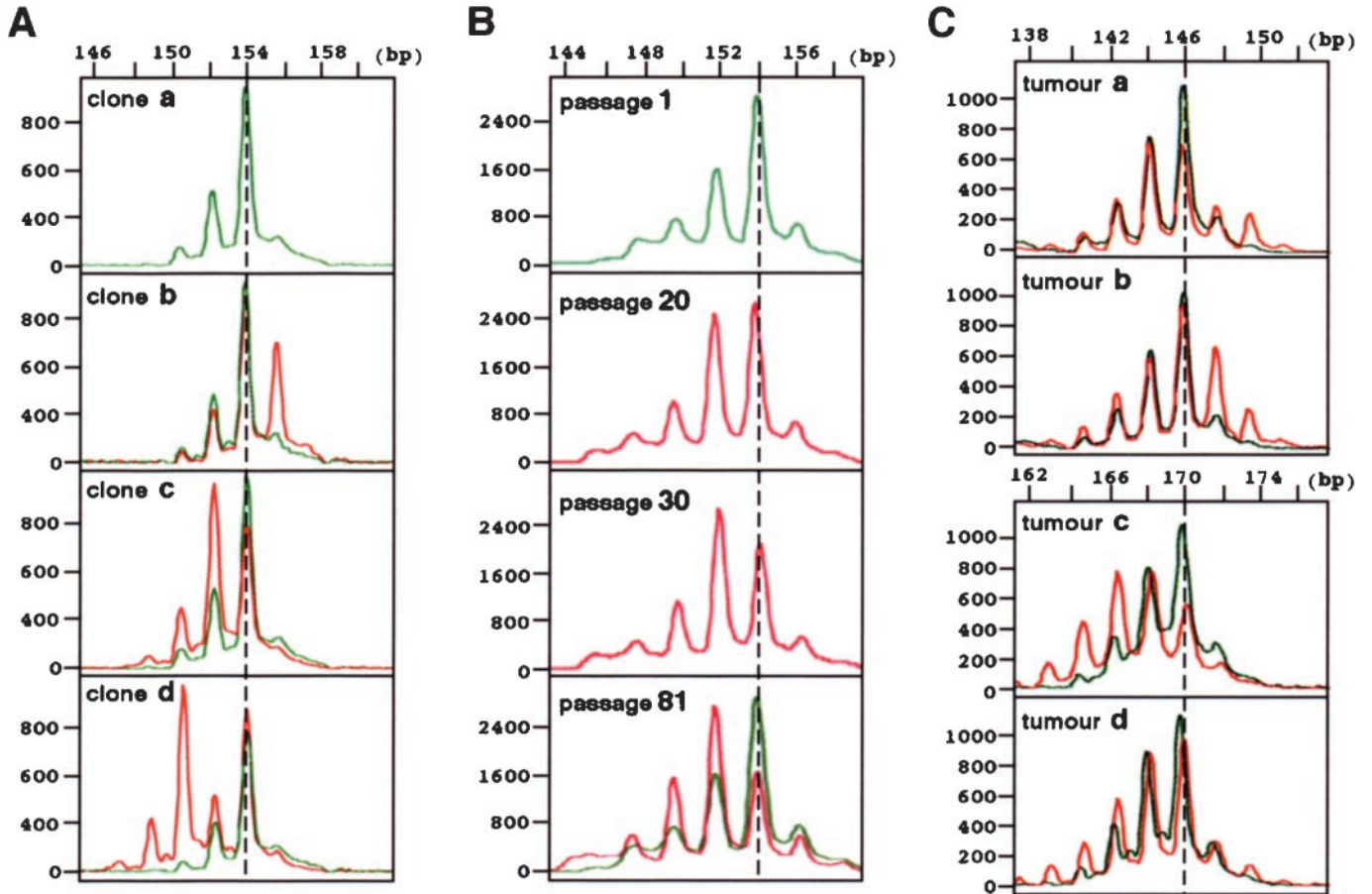


Figure 2. Microsatellite changes in *Msh2*^{-/-} mouse embryonic fibroblast (MEF) cells and in tumours that arose in *Msh2*-knockout mice. (A) More than 20 subclones were isolated from RH95021 (*Msh2*^{-/-}) MEF cells and microsatellite changes were compared among these subclones. The majority exhibited the same configuration (clone a) and a few deviated from this predominant pattern (clone b–d). In each clone, the pattern of clone A has been superimposed with green lines, to facilitate comparison. Results obtained in D6Mit59 microsatellite are shown. (B) RH95021 cells were continuously cultured and sampled at different passages. Results obtained in D6Mit59 microsatellite at passage 1, 20, 30 and 81 are shown. In passage 81, the initial profile at passage 1 has been superimposed with a green line. (C) Tumours that arose *in vivo* in *Msh2*-knockout mice were analysed. Representative results obtained in D7Mit91 (tumours a and b) and D6Mit59 (tumours c and d) microsatellites are shown: red lines, tumour; green lines, the corresponding normal tissue.

Table 1. *hMSH2* and *hMLH1* alterations found in tumours exhibiting Type A and Type B MSI

PATIENT	MSI A/B	NUCLEAR EXPRESSION		SEQUENCE																			SUMMARY																	
		<i>hMSH2</i>	<i>hMLH1</i>	<i>hMSH2</i>									<i>hMLH1</i>																											
				exon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
IC669	A	P	N																																					cytoplasmic hMLH1
IC678	A	P	P																																					L521I (CTC to ATC)
IC692	A	P	P																																				G132G (GGC to GGA)	
IC721	A	P	P																																				L390F (CTT to TTT)	
IC724	A	P	P																																				G178R (GGA to AGA)	
IC759	A	P	P																																					
IC793	A	P	N																																				<i>hMLH1</i> silencing	
IC807	A	P	P																																				I219V (ATC to GTC)	
IC810	A	P	P																																			<i>hMSH2</i> ; L390F / <i>hMLH1</i> ; I219V		
IC815	A	P	P																																					
IC824	A	P	P																																					
IC860	A	P	P																																					
IC622	B	P	P																																					
IC653	B	P	P																																					
IC676	B	P	P																																					
IC690	B	P	N																																				R226Q (CGA to CAA) / cytoplasmic hMLH1, HNPCC	
IC698	B	P	P																																				I219V (ATC to GTC)	
IC733	B	P	P																																					
IC790	B	P	P																																					
IC853	B	P	P																																				G132G (GGC to GGA)	
IC873	B	P	P																																				Q604Q (CAA to CAG)	
NoTa	B	P*	N*																																			<i>hMLH1</i> silencing, HNPCC		

MSI, microsatellite instability; P, positive nuclear staining in immunohistochemistry; N, negative; *, determined by immunoblotting; Closed circle, base substitution with amino acid change; open triangle, possible polymorphism; Open rectangle, base substitution without amino acid change.

two other dinucleotide microsatellites, D1Mit62 and D7Mit91 (data not shown). To investigate whether Type B variations might simply reflect the accumulation of numerous smaller alterations over many generations, RH95021 cells were continuously cultured, and sampled periodically at different passages. As shown in Figure 2B, even after undergoing 81 population doublings (2^{81} corresponds to 10^{24}), there was no detectable appearance of shorter or longer D6Mit59 alleles. Similar patterns were observed at the two other microsatellite loci (data not shown). Thus, there was no evidence of the accumulation of changes consistent with Type B instability at any of the three microsatellites in these MMR-defective MEFs. Similar data were obtained using a second MMR-deficient MEF cell line, MC2, which derived from an *Mlh1*^{-/-} mouse (data not shown).

We also examined microsatellite instability in lymphomas and adenocarcinomas that arose in various organs of *Msh2*^{-/-} mice. Among 16 tumours that were analysed at the three microsatellite loci, each contained alterations at one or more locus. In all cases, changes were limited to ≤ 6 bp (examples are shown in Figure 2C) and no Type B alteration was observed. Intriguingly, an examination of published microsatellite changes in cells of MMR gene-knockout mice clearly indicates that most changes are of Type A (12,18,19).

Analysis of microsatellites in MEFs and tumours from MMR-defective animals therefore indicates that Type A MSI is a direct consequence of defective MMR. The absence of more extensive microsatellite length changes may indicate further that an *Msh2* or *Mlh1* defect is insufficient for the development of Type B instability.

Mismatch repair gene inactivation is associated with both Type A and Type B MSI in human colorectal cancer

HNPCC patients inherit mutations in MMR genes (6,7). More than 90% of HNPCC tumours are MSI⁺ (20). The MSI⁺ phenotype is also frequent among sporadic colorectal carcinomas (2–4,6,21). Inspection of published data derived from the conventional microsatellite analysis reveals that microsatellite changes thus far reported in various tumours, including those in HNPCC individuals, are largely Type B (2–4,20–22). We considered the possibility that the more subtle Type A MSI might have remained undetected in some cases. Using our fluorescent assay with a panel of five dinucleotide repeat microsatellites, we found that the frequencies of Type A and Type B MSI among 79 colorectal carcinomas were 30% and 17%, respectively. In agreement with previous observations that Type B instability is common in HNPCC colon tumours, the IC690 tumour and the colorectal carcinoma cell line, NoTa (Table 1), both of which were derived from patients who fulfilled the Amsterdam Criteria II for HNPCC (23), exhibited Type B instability (Figure 1G and H). Our finding that 17% of colorectal tumours display Type B MSI is consistent with the generally reported figure of around 20% for MSI among colorectal carcinomas (20,24,25). The observation that a further 30% of tumours displayed Type A MSI suggests that the frequency of MSI, at least in colorectal carcinomas, has previously been underestimated.

The relationship between MSI and defective MMR in our set of colorectal tumours was investigated further. *hMSH2* and

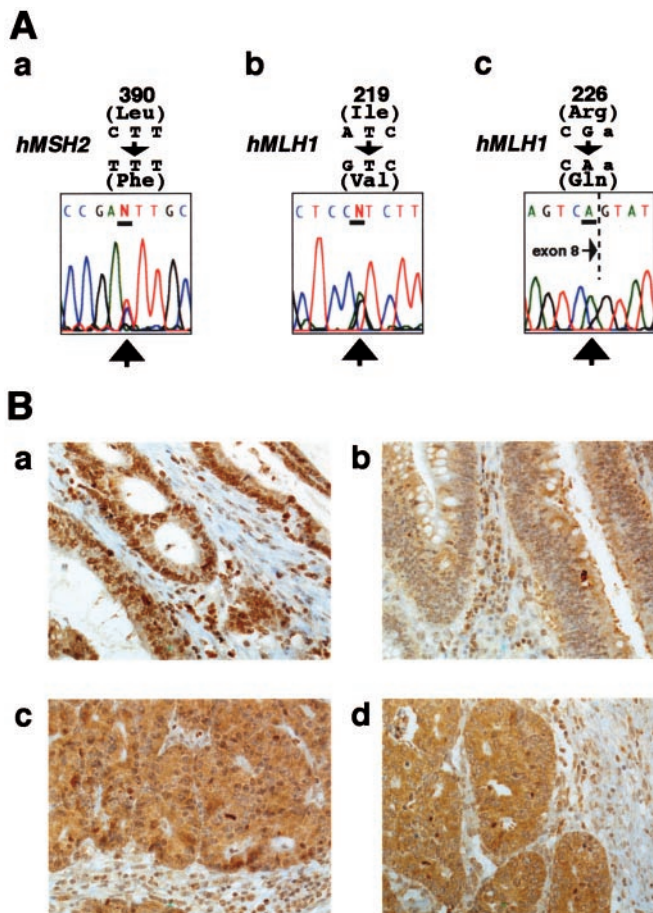


Figure 3. *hMSH2* and *hMLH1* alterations in tumours exhibiting Type A or Type B microsatellite instability. (A) Sequences for all the exons including exon–intron boundaries of *hMSH2* and *hMLH1* were determined using an automated sequencer. Sequence alterations found at (a) codon 390 of *hMSH2* (patient IC810), (b) codon 219 of *hMLH1* (patient IC810) and (c) codon 226 of *hMLH1* (patient IC690) were shown. (B) Abnormal expression of hMLH1 proteins observed in a panel of human colorectal carcinomas. (Panel a) A typical result with a strong nuclear staining implying normal hMLH1 expression (IC853). (Panel b) Complete loss of hMLH1 expression in tumour cells, which suggest a possible epigenetic silencing (IC793). (Panels c and d) Results without evident nuclear staining, but with an accumulation of hMLH1 antigens in the tumour cytoplasm, which may suggest an abnormal intracellular distribution of this protein (IC690 and IC669).

hMLH1 MMR genes of 12 tumours with Type A and 9 with Type B MSI were sequenced. The same genes in the NoTa cell line were also sequenced (Figure 3A and Table 1). Sequence alterations causing amino acid substitutions were identified in 5 of the 21 tumours. Four of these (80%) were associated with Type A MSI. In addition, one patient with Type A MSI (IC793) in whom no mutation was identified was negative for immunohistochemical staining of hMLH1 (Figure 3B, panel b). This is consistent with a possible epigenetic *hMLH1* gene silencing (26–28). In one other Type A case (IC669) and one Type B case (IC690), there was an abnormal intracellular distribution of hMLH1 which remained predominantly cytoplasmic (Figure 3B, panels c and d). Among the 10 tumours displaying Type B MSI, there was an example of base substitutions causing amino acid change in *hMLH1*. In this case, IC690, the failure of hMLH1 to localize to the nucleus was associated with the codon 226 mutation in exon 8 (Figure 3A,

Table 2. *p53* mutations found in 79 colorectal carcinomas

No.	Patient	EX05	EX06	EX07	EX08	EX09	Codon change	Base substitution	(type)	AA change	MSI
1	IC628				273		CGT → CAT	G:C → A:T	TS	Arg → His	N
2	IC630		196				CGA → TGA	G:C → A:T	TS	Arg → stop	A
3	IC634				306		CGA → TGA	G:C → A:T	TS	Arg → stop	N
4	IC668		193				CAT → CGT	A:T → G:C	TS	His → Arg	N
5	IC669	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
6	IC673	176					TGC → AGC	A:T → T:A	TV	Cys → Ser	N
7	IC674				285		GAG → AAG	G:C → A:T	TS	Glu → Lys	N
8	IC680		ND	255			ATC → ACC	A:T → G:C	TS	Ile → Thr	A
9	IC693	179					CAT → CTT	A:T → T:A	TV	His → Leu	A
10	IC694				273		CGT → CAT	G:C → A:T	TS	Arg → His	A
11	IC711			239			AAC → GAC	A:T → G:C	TS	Asn → Asp	N
12	IC721	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
13	IC748		190			ND	CCT → CTT	G:C → A:T	TS	Pro → Leu	A
14	IC754		196				CGA → CCA	G:C → C:G	TV	Arg → Pro	N
15	IC763	151					CCC → CAC	G:C → T:A	TV	Pro → His	N
16	IC772	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
17	IC778	175	ND				CGC → CAC	G:C → A:T	TS	Arg → His	N
18	IC784		214		ND	ND	CAT → CGT	A:T → G:C	TS	His → Arg	A
19	IC808		205				TAT → GAT	A:T → C:G	TV	Tyr → Asp	A
20	IC812		190				CCT → CTT	G:C → A:T	TS	Pro → Leu	N
21	IC816				273		CGT → CAT	G:C → A:T	TS	Arg → His	A
22	IC819			248			CGG → CAG	G:C → A:T	TS	Arg → Gln	N
23	IC860				273		CGT → CAT	G:C → A:T	TS	Arg → His	A

MSI, microsatellite instability; N, negative; A, Type A MSI; TS, transition; TV, transversion; ND, not determined. Bold codon numbers indicate the acknowledged hot-spots for mutation.

panel c). These findings suggest that Type A instability, as well as Type B, is indeed associated with MMR defects.

***p53* mutation is strongly associated with Type A MSI in human colorectal cancer**

One view of the involvement of MMR defects in cancer development is that the 'microsatellite mutator phenotype (MMP)' (29,30) in mismatch repair-defective cells offers an alternative to chromosomal instability as a mechanism for genetic instability in cancer (31). On this model, MSI and chromosomal instability represent mutually exclusive pathways of tumour development. This reasoning is based partly in the observation that *p53* mutations, commonly associated with chromosomal instability, are infrequent among MSI⁺ tumours (2,32–34). To examine the relationship between Type A/B instability and *p53* mutation, we sequenced the *p53* gene in our panel of 79 colorectal tumours. *p53* mutations resulting in an amino acid substitution were detected in 23 tumours (29.1%). The mutations were predominantly transitions in acknowledged hot spots; codons 175, 248 and 273 (Table 2). Of the *p53* mutations that were found in MSI⁺ tumours, all were associated with Type A MSI (Tables 2 and 3). No *p53* mutations were detected among the 14 Type B tumours. Among Type A tumours, the frequency of *p53* mutation approached 50% (12/25). These findings confirm that *p53* mutations are rare in tumours with Type B MSI. More importantly, they suggest that, in contrast to prevailing opinion, defective MMR is significantly associated with *p53* mutation, at least in human colorectal cancer.

DISCUSSION

The fluorescent technique we used here allows the unequivocal designation of Type A and Type B MSI, and has revealed

Table 3. *p53* mutation highly correlates with Type A MSI

		MSI			Subtotal
		Type A	Type B	Negative	
<i>p53</i>	Wild type	13	14	29	56
	Mutant	12	0	11	23
	Subtotal	25	14	40	79

$p = 0.006$. MSI: Microsatellite instability.

a previously unrecognized complexity in the relationship between dinucleotide MSI and defective DNA MMR in human cancer. Type A MSI (changes ≤ 6 bp) is clearly linked with MMR inactivation in both mice and humans, which implies that Type A MSI is a direct consequence of defective MMR. Since we found no evidence of Type B instability (changes ≥ 8 bp) in MMR-defective animals, it is possible that changes in addition to repair deficiency contribute to, or are responsible for, Type B MSI. One important finding of this study is that Type A instability is frequent among human tumours. Type A MSI predominated in our large panel of colorectal carcinomas. We suggest that, because the changes associated with Type A instability are more subtle, the frequency of MSI among colorectal tumours may have been considerably underestimated. Our findings also reveal a hitherto unrecognized association between defective MMR and *p53* mutation. Significantly, Type A MSI was strongly associated with *p53* mutation in human colorectal tumours. Since Type A instability is unequivocally associated with MMR deficiency, this novel finding implies that, in contrast to prevailing opinion, *p53* mutations are common in MMR-defective tumours, at least in human colorectal cancer.

Established guidelines for classification of MSI utilize the frequency of changes in a defined set of microsatellite

markers, i.e. MSI-H and -L (10). However, qualitative differences in microsatellite changes are not widely discussed. In one of the earliest report of the MSI phenomenon, however, Thibodeau *et al.* divided microsatellite changes into two categories; Type I and II mutations (3). 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 distinction has received little attention since then. Our data indicate that Type A MSI, which appears similar to their Type II mutation, is more frequent than hitherto suspected, and suggest that it represents the bona fide MMR-deficient phenotype. On the other hand, Type I mutations may correspond to our Type B instability. The problem is that mutations in MMR genes have been reported in tumours displaying this type of instability. More than 90% of HNPCC tumours are MSI⁺ (20), and this type of MSI can be categorized as Type B/Type I (2–4,20–22). However, the frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, in HNPCC kindred are not high in some reports (35–40). Among the panel of tumours displaying Type B MSI, we found a base substitution mutation in *hMLH1* and one case with a possible *hMLH1* silencing. This incidence of MMR gene inactivation in the Type B group is not unduly low, compared with the reported frequencies in the literature (25,36,41–43). The relationship between Type B MSI and defective MMR is probably more complex than hitherto suspected.

Type B MSI may involve molecular abnormalities in addition to defective MMR. We suggest that whereas Type A MSI probably reflects the uncorrected DNA polymerase slippage events that accumulate in MMR-defective cells, inappropriate processing of damage by recombinational DNA repair may contribute to Type B MSI. This hypothesis may be supported partly by our observation that there was no evidence of the emergence of Type B instability in MMR gene-knockout animals. It is known that microsatellite alterations occur via several independent mechanisms, including recombination (44–46), and MMR counteracts incorrect strand alignment during homologous recombination (47). The drastic microsatellite changes in Type B MSI may be more consistent with dynamic events, such as recombination, than with replication slippage. In this context, defective MMR might be a promoting, and consequently highly coincidental, but insufficient factor for Type B changes. Connection between MSI and the recombinational repair pathway in tumours, particularly in HNPCC, may warrant attention.

Type A MSI is also strongly associated with *p53* mutation in human colorectal tumours. This observation may be compatible with several recent reports (36,48,49) that have shown a connection between *p53* mutation and the MSI-L phenotype, since in colorectal cancer Type A MSI tends to be observed in a limited number of markers and, consequently, categorized as MSI-L. This finding may also provide an insight to the mechanism of genetic instability in tumours. Genetic instability in tumours 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 *p53*—and 'microsatellite mutator phenotype (MMP)' (29,30), in which *p53* mutations are rare and, instead, mutations are found in mononucleotide repeats within genes of a different variety. Several recent reports suggest that there might be an oversimplification

in this distinction (50–53). From our observations, dinucleotide MSI in tumours can be divided into two modes, Type A and Type B, and Type A instability is the direct consequence of defective MMR. A close association of Type A MSI with *p53* mutation may suggest a hitherto unrecognized causal relationship between *p53* mutation and defective MMR. *p53* mutations may derive from a state with an elevated mutation rate, i.e. MMR-deficient phenotype, as initially suspected. Thus, our observations suggest added complexities to the relationship between MMR defects and MSI, and also shed light on previously unrecognized fundamental processes in the molecular mechanisms of genetic instability underlying tumour development.

ACKNOWLEDGEMENTS

We are extremely grateful to Drs H. te Riele and M Liskay for providing the RH95021 and MC2 cell lines, respectively. The expert assistance in DNA sequencing by Y. Baba, K. Miyamoto, Y. Ikematsu and K. Funatsu is also gratefully acknowledged. This study was supported by a Grant-in-aid for Cancer Research from the Ministry of Health, Labour and Welfare, and grants from the Ministry of Education, Science, Sports and Culture of Japan. The Open Access publication of this article was also supported in part by these grants.

Conflict of interest statement. None declared.

REFERENCES

- Weissenbach,J., Gyapay,G., Dib,C., Vignal,A., Morissette,J., Millasseau,P., Vaysseix,G. and Lathrop,M. (1992) A second-generation linkage map of the human genome. *Nature*, **359**, 794–801.
- Ionov,Y., Peinado,M.A., Malkhosyan,S., Shibata,D. and Perucho,M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558–561.
- Thibodeau,S.N., Bren,G. and Schaid,D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816–819.
- Aaltonen,L.A., Peltomaki,P., Leach,F.S., Sistonen,P., Pylkkanen,L., Mecklin,J.P., Jarvinen,H., Powell,S.M., Jen,J., Hamilton,S.R. *et al.* (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812–816.
- Peltomaki,P., Lothe,R.A., Aaltonen,L.A., Pylkkanen,L., Nystrom-Lahti,M., Seruca,R., David,L., Holm,R., Ryberg,D., Haugen,A. *et al.* (1993) Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res.*, **53**, 5853–5855.
- Fishel,R., Lescoe,M.K., Rao,M.R., Copeland,N.G., Jenkins,N.A., Garber,J., Kane,M. and Kolodner,R. (1993) The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell*, **75**, 1027–1038.
- Leach,F.S., Nicolaides,N.C., Papadopoulos,N., Liu,B., Jen,J., Parsons,R., Peltomaki,P., Sistonen,P., Aaltonen,L.A., Nystrom-Lahti,M. *et al.* (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215–1225.
- Modrich,P. and Lahue,R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Ann. Rev. Biochem.*, **65**, 101–133.
- Arzimanoglou,I.I., Gilbert,F. and Barber,H.R. (1998) Microsatellite instability in human solid tumors. *Cancer*, **82**, 1808–1820.
- Boland,C.R., Thibodeau,S.N., Hamilton,S.R., Sidransky,D., Eshleman,J.R., Burt,R.W., Meltzer,S.J., Rodriguez-Bigas,M.A., Fodde,R., Ranzani,G.N. *et al.* (1998) A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.*, **58**, 5248–5257.

11. Oda,S., Oki,E., Maehara,Y. and Sugimachi,K. (1997) Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. *Nucleic Acids Res.*, **25**, 3415–3420.
12. de Wind,N., Dekker,M., Berns,A., Radman,M. and te Riele,H. (1995) Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell*, **82**, 321–330.
13. Kolodner,R.D., Hall,N.R., Lipford,J., Kane,M.F., Rao,M.R., Morrison,P., Wirth,L., Finan,P.J., Burn,J. and Chapman,P. (1994) Structure of the human MSH2 locus and analysis of two Muir-Torre kindreds for msh2 mutations. *Genomics*, **24**, 516–526.
14. Kolodner,R.D., Hall,N.R., Lipford,J., Kane,M.F., Morrison,P.T., Finan,P.J., Burn,J., Chapman,P., Earabino,C. and Merchant,E. (1995) Structure of the human MLH1 locus and analysis of a large hereditary nonpolyposis colorectal carcinoma kindred for mlh1 mutations. *Cancer Res.*, **55**, 242–248.
15. Maehara,Y., Oda,S. and Sugimachi,K. (2001) The instability within: problems in current analyses of microsatellite instability. *Mutat. Res.*, **461**, 249–263.
16. Tokunaga,E., Oki,E., Oda,S., Kataoka,A., Kitamura,K., Ohno,S., Maehara,Y. and Sugimachi,K. (2000) Frequency of microsatellite instability in Breast cancer determined by high-resolution fluorescent microsatellite analysis. *Oncology*, **59**, 44–49.
17. Ikeda,Y., Oda,S., Abe,T., Ohno,S., Maehara,Y. and Sugimachi,K. (2001) Features of microsatellite instability in colorectal cancer: comparison between colon and rectum. *Oncology*, **61**, 168–174.
18. Edelmann,W., Cohen,P.E., Kane,M., Lau,K., Morrow,B., Bennett,S., Umar,A., Kunkel,T., Cattoretti,G., Chaganti,R. *et al.* (1996) Meiotic pachytene arrest in MLH1-deficient mice. *Cell*, **85**, 1125–1134.
19. Baker,S.M., Bronner,C.E., Zhang,L., Plug,A.W., Robatzek,M., Warren,G., Elliott,E.A., Yu,J., Ashley,T., Arnheim,N. *et al.* (1995) Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell*, **82**, 309–319.
20. Liu,B., Parsons,R., Papadopoulos,N., Nicolaides,N.C., Lynch,H.T., Watson,P., Jass,J.R., Dunlop,M., Wyllie,A., Peltomaki,P. *et al.* (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Med.*, **2**, 169–174.
21. Gryfe,R., Kim,H., Hsieh,E.T., Aronson,M.D., Holowaty,E.J., Bull,S.B., Redston,M. and Gallinger,S. (2000) Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *New Engl. J. Med.*, **342**, 69–77.
22. Aaltonen,L.A., Peltomaki,P., Mecklin,J.P., Jarvinen,H., Jass,J.R., Green,J.S., Lynch,H.T., Watson,P., Tallqvist,G., Juhola,M. *et al.* (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res.*, **54**, 1645–1648.
23. Vasen,H.F., Watson,P., Mecklin,J.P. and Lynch,H.T. (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology*, **116**, 1453–1456.
24. Lothe,R.A., Peltomaki,P., Meling,G.I., Aaltonen,L.A., Nystrom-Lahti,M., Pylkkanen,L., Heimdal,K., Andersen,T.I., Moller,P., Rognum,T.O. *et al.* (1993) Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res.*, **53**, 5849–5852.
25. Liu,B., Nicolaides,N.C., Markowitz,S., Willson,J.K., Parsons,R.E., Jen,J., Papadopoulos,N., Peltomaki,P., de la Chapelle,A., Hamilton,S.R. *et al.* (1995) Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genet.*, **9**, 48–55.
26. Thibodeau,S.N., French,A.J., Roche,P.C., Cunningham,J.M., Tester,D.J., Lindor,N.M., Moslein,G., Baker,S.M., Liskay,R.M., Burgart,L.J. *et al.* (1996) Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes. *Cancer Res.*, **56**, 4836–4840.
27. Herman,J.G., Umar,A., Polyak,K., Graff,J.R., Ahuja,N., Issa,J.P., Markowitz,S., Willson,J.K., Hamilton,S.R., Kinzler,K.W. *et al.* (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl Acad. Sci. USA*, **95**, 6870–6875.
28. Cunningham,J.M., Christensen,E.R., Tester,D.J., Kim,C.Y., Roche,P.C., Burgart,L.J. and Thibodeau,S.N. (1998) Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.*, **58**, 3455–3460.
29. Perucho,M. (1996) Cancer of the microsatellite mutator phenotype. *Biol. Chem.*, **377**, 675–684.
30. Perucho,M. (1996) Microsatellite instability: the mutator that mutates the other mutator. *Nature Med.*, **2**, 630–631.
31. Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1998) Genetic instabilities in human cancers. *Nature*, **396**, 643–649.
32. Cottu,P.H., Muzeau,F., Estreicher,A., Flejou,J.F., Iggo,R., Thomas,G. and Hamelin,R. (1996) Inverse correlation between RER+ status and p53 mutation in colorectal cancer cell lines. *Oncogene*, **13**, 2727–2730.
33. Simms,L.A., Radford-Smith,G., Biden,K.G., Buttenshaw,R., Cummings,M., Jass,J.R., Young,J., Meltzer,S.J. and Leggett,B.A. (1998) Reciprocal relationship between the tumor suppressors p53 and BAX in primary colorectal cancers. *Oncogene*, **17**, 2003–2008.
34. Salahshor,S., Kressner,U., Pahlman,L., Glimelius,B., Lindmark,G. and Lindblom,A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosome Cancer*, **26**, 247–252.
35. Froggatt,N.J., Brassett,C., Koch,D.J., Evans,D.G., Hodgson,S.V., Ponder,B.A. and Maher,E.R. (1996) Mutation screening of MSH2 and MLH1 mRNA in hereditary non-polyposis colon cancer syndrome. *J. Med. Genet.*, **33**, 726–730.
36. Konishi,M., Kikuchi-Yanoshita,R., Tanaka,K., Muraoka,M., Onda,A., Okumura,Y., Kishi,N., Iwama,T., Mori,T., Koike,M. *et al.* (1996) Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology*, **111**, 307–317.
37. Weber,T., Conlon,W., Petrelli,N., Rodriguez-Bigas,M., Keitz,B., Pazik,J., Farrell,C., O'Malley,L., Oshalim,M., Abdo,M. *et al.* (1997) Genomic DNA-based hMSH2 and hMLH1 mutation screening in 32 Eastern United States hereditary nonpolyposis colorectal cancer pedigrees. *Cancer Res.*, **57**, 3798–3803.
38. Scartozzi,M., Bianchi,F., Rosati,S., Galizia,E., Antolini,A., Loretelli,C., Piga,A., Bearzi,I., Cellerino,R. and Porfiri,E. (2002) Mutations of hMLH1 and hMSH2 in patients with suspected hereditary nonpolyposis colorectal cancer: correlation with microsatellite instability and abnormalities of mismatch repair protein expression. *J. Clin. Oncol.*, **20**, 1203–1208.
39. Wahlberg,S.S., Schmeits,J., Thomas,G., Loda,M., Garber,J., Syngal,S., Kolodner,R.D. and Fox,E. (2002) Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. *Cancer Res.*, **62**, 3485–3492.
40. Pucciarelli,S., Agostini,M., Viel,A., Bertorelle,R., Russo,V., Toppan,P. and Lise,M. (2003) Early-age-at-onset colorectal cancer and microsatellite instability as markers of hereditary nonpolyposis colorectal cancer. *Dis. Colon Rectum*, **46**, 305–312.
41. Kuismannen,S.A., Holmberg,M.T., Salovaara,R., de la Chapelle,A. and Peltomaki,P. (2000) Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. *Am. J. Pathol.*, **156**, 1773–1779.
42. Percepe,A., Borghi,F., Menigatti,M., Losi,L., Foroni,M., Di Gregorio,C., Rossi,G., Pedroni,M., Sala,E., Vaccina,F. *et al.* (2001) Molecular screening for hereditary nonpolyposis colorectal cancer: a prospective, population-based study. *J. Clin. Oncol.*, **19**, 3944–3950.
43. Potocnik,U., Glavac,D., Golouh,R. and Ravnik-Glavac,M. (2001) Causes of microsatellite instability in colorectal tumors: implications for hereditary non-polyposis colorectal cancer screening. *Cancer Genet. Cytogenet.*, **126**, 85–96.
44. Sia,E.A., Jinks-Robertson,S. and Petes,T.D. (1997) Genetic control of microsatellite stability. *Mutat. Res.*, **383**, 61–70.
45. Richard,G.F. and Paques,F. (2000) Mini- and microsatellite expansions: the recombination connection. *EMBO Rep.*, **1**, 122–126.
46. Lenzmeier,B.A. and Freudenreich,C.H. (2003) Trinucleotide repeat instability: a hairpin curve at the crossroads of replication, recombination, and repair. *Cytogenet. Genome Res.*, **100**, 7–24.
47. Evans,E. and Alani,E. (2000) Roles for mismatch repair factors in regulating genetic recombination. *Mol. Cell. Biol.*, **20**, 7839–7844.
48. Jass,J.R., Biden,K.G., Cummings,M.C., Simms,L.A., Walsh,M., Schoch,E., Meltzer,S.J., Wright,C., Searle,J., Young,J. *et al.* (1999) Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. *J. Clin. Pathol.*, **52**, 455–460.
49. Kambara,T., Matsubara,N., Nakagawa,H., Notohara,K., Nagasaka,T., Yoshino,T., Isozaki,H., Sharp,G.B., Shimizu,K., Jass,J. *et al.* (2001)

- High frequency of low-level microsatellite instability in early colorectal cancer. *Cancer Res.*, **61**, 7743–7746.
50. Hawkins,N.J., Tomlinson,I., Meagher,A. and Ward,R.L. (2001) Microsatellite-stable diploid carcinoma: a biologically distinct and aggressive subset of sporadic colorectal cancer. *Br. J. Cancer*, **84**, 232–236.
 51. Young,J., Simms,L.A., Biden,K.G., Wynter,C., Whitehall,V., Karamatic,R., George,J., Goldblatt,J., Walpole,I., Robin,S.A. *et al.* (2001) Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. *Am. J. Pathol.*, **159**, 2107–2116.
 52. Jass,J.R., Walsh,M.D., Barker,M., Simms,L.A., Young,J. and Leggett,B.A. (2002) Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. *Eur. J. Cancer*, **38**, 858–866.
 53. Goel,A., Arnold,C.N., Niedzwiecki,D., Chang,D.K., Ricciardiello,L., Carethers,J.M., Dowell,J.M., Wasserman,L., Compton,C., Mayer,R.J. *et al.* (2003) Characterization of sporadic colon cancer by patterns of genomic instability. *Cancer Res.*, **63**, 1608–1614.