Detection of Coding Microsatellite Frameshift Mutations in DNA Mismatch Repair-Deficient Mouse Intestinal Tumors

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Different DNA mismatch repair (MMR)-deficient mouse strains have been developed as models for the inherited cancer predisposing Lynch syndrome. It is completely unresolved, whether coding mononucleotide repeat (cMNR) gene mutations in these mice can contribute to intestinal tumorigenesis and whether MMR-deficient mice are a suitable molecular model of human microsatellite instability (MSI)-associated intestinal tumorigenesis. A proof-of-principle study was performed to identify mouse cMNR-harboring genes affected by insertion/deletion mutations in MSI murine intestinal tumors. Bioinformatic algorithms were developed to establish a database of mouse cMNR-harboring genes. A panel of five mouse noncoding mononucleotide markers was used for MSI classification of intestinal matched normal/tumor tissues from MMR-deficient (*Mlh1*$^{-/-}$, *Msh2*$^{-/-}$, *Msh2*$^{LoxP/LoxP}$) mice. cMNR frameshift mutations of candidate genes were determined by DNA fragment analysis. Murine MSI intestinal tumors but not normal tissues from MMR-deficient mice showed cMNR frameshift mutations in six candidate genes (*Elavl3*, *Tmem107*, *Glis2*, *Sdccag1*, *Senp6*, *Rfc3*). cMNRs of mouse *Rfc3* and *Elavl3* are conserved in type and length in their human orthologs that are known to be mutated in human MSI colorectal, endometrial and gastric cancer. We provide evidence for the utility of a mononucleotide marker panel for detection of MSI in murine tumors, the existence of cMNR instability in MSI murine tumors, the utility of mouse subspecies DNA for identification of polymorphic repeats, and repeat conservation among some orthologous human/mouse genes, two of them showing instability in human and mouse MSI intestinal tumors. MMR-deficient mice hence are a useful molecular model system for analyzing MSI intestinal carcinogenesis.

Key words: coding microsatellite instability; MMR-deficient mice; MSI intestinal carcinogenesis; MSI target genes

INTRODUCTION

The post-replication DNA mismatch repair (MMR) system recognizes and corrects misincorpared bases that occur in newly synthesized DNA strands due to a low but significant intrinsic error rate of replicative DNA polymerase. In mammalian cells, different MMR protein complexes are known to maintain genomic integrity, depending on the type of lesion to be repaired. Heterodimeric complexes of MSH2-MSH6 (MutSα) are thought to be involved primarily in the recognition and repair of base-base mismatches and small insertion/deletion loops. MSH2-MSH3 (MutSß) heterodimers act preferentially on large insertion/deletion loops [1]. Both MutS complexes interact with the MutLα heterodimer consisting of MMR proteins MLH1 and PMS2. The MutLα complex appears to play a key role in coordinating mismatch repair recognition, mutation excision, and resynthesis of corrected bases.

Loss of MMR function is observed in about 15% of colorectal cancers. Inactivation of both alleles of a particular MMR gene causes MMR deficiency either by promoter methylation of the MLH1 gene in sporadic cases or by germline and somatic mutations of MLH1, MSH2, MSH6, PMS2 in the hereditary form of Lynch

Abbreviations: HNPCC, hereditary non-polyposis colorectal cancer; CRC, colorectal cancer; MMR, DNA mismatch repair; MSI, microsatellite instability; MSS, Microsatellite stability; CIN, chromosomal instability; cMNR, coding mononucleotide repeat.

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syndrome [2–6]. MMR-deficient cells accumulate numerous mutations in genomic DNA, most frequently affecting microsatellite sequences leading to the microsatellite instability (MSI) phenotype [7]. If such instability occurs at coding mononucleotide repeats (cMNRs) of expressed genes, the resulting frameshift mutations lead to the synthesis of truncated proteins with partial or complete loss of function. There is strong evidence that mutational inactivation of specific target genes and signaling pathways (TGFBR2, ACVR2, BAX) should provide a growth advantage to MMR-deficient cells [8–10].

Several mouse models have been developed to study MMR function and how its loss contributes to cancer predisposition. In general, the phenotypes of MMR mutant mice correlate well with observed repair defects in HNPCC patients [11]. For example, the majority of *Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$ mice developed T-cell lymphomas at an early age closely paralleling the findings in rare human patients with homozygous MMR mutations. In addition, these homozygous mutant mice also showed strong penetrant gastrointestinal and skin cancer susceptibility with high levels of MSI in all somatic tissues tested [12–17]. MSI in tumors of these mice affects both mono- and dinucleotide repeats at high frequency similar to *MSH2* or *MLH1*-deficient colorectal tumors in Lynch syndrome patients. Moreover, cell lines derived from HNPCC tumors or MMR-deficient mice displayed increased mutation rates and increased resistance to genotoxic effects of DNA damage-inducing agents. However, *Msh3*$^{-/-}$ *or Exo1*$^{-/-}$ mice only develop tumors at very old ages, and there is no evidence so far for the involvement of these two genes in the pathogenesis of Lynch syndrome tumors. Instability of mononucleotide repeats in tumors of *MSH3*$^{-/-}$ and *Exo1*$^{-/-}$ mice is moderate to high, respectively. *MSH3*$^{-/-}$ mice develop GI tumors, whereas lymphomas prevail in *Exo1*$^{-/-}$ mice [18,19]. *Msh6*$^{-/-}$ mice show a tumor spectrum similar to *Msh2*$^{-/-}$ mice but the onset of tumor development is delayed. These mice are highly predisposed to tumor development including lymphomas, GI and tumors of the skin with lack of mononucleotide repeat instability and low level of dinucleotide instability [20]. In addition, mice carrying a dominant *Msh6*$^{TD/TD}$ missense mutation have been generated which—in contrast to the *Msh6* null mice—showed high level of MSI at mono- and dinucleotide repeats similar to the MSI pattern found in *MLH1* deficient mice [21]. These *Msh6*$^{TD/TD}$ mice had a reduced lifespan and increased cancer susceptibility. The majority of these mice developed B- or T-cell lymphomas, but GI tumors and tumors of the skin were also found. In *Pms2*$^{-/-}$ mice, MSI occurs at both mono- and dinucleotide repeats. These mice also show a higher proportion of frameshift mutations compared with other MMR-knock-out models such as *Mlh1*$^{-/-}$ and *Msh2*$^{-/-}$ mice [22]. Moreover, *Pms2*$^{-/-}$ mice as well as patients with deleterious homozygous *PMS2* mutations show decreased isotype switching [23,24]. *Pms2*$^{-/-}$ mice are predisposed to develop lymphoma and sarcoma but not GI tumors [14,25,26]. The development of hematological malignancy in MMR-deficient individuals, similar to that seen in homozygous knockout mice, suggests that the mouse may be a useful model to study human lymphomagenesis. Recently and in contrast to all the mice decribed above, a conditional MMR-knockout mouse model has been created (*VCMsh2*$^{LoxP/LoxP}$) that enables Villin-Cre mediated specific ablation of *Msh2* function in intestinal epithelial cells [27]. These mice develop tumors in the small intestine but no lymphomas.

Several reasons have been proposed to account for the observed differences between MMR-deficient human and mouse tumors and their MSI phenotypes. First, unlike heterozygous MMR germline mutation carriers in HNPCC families, heterozygous MMR mutant mice do not develop tumors throughout life, most likely due to the shorter lifespan and small size of the mice or possible environmental factors, such as diet and/or genetic factors such as modifier genes. Second, MMR-deficient tumors of Lynch syndrome patients predominantly arise in the colon, whereas MMR-deficient mice develop tumors in the small intestine apart from lymphomas. This difference in organ manifestation has been suggested to be attributable to absence or disruption of cMNR sequences in many of the mouse genes, making their coding regions of the mouse orthologs less vulnerable to mutation. However, even when disrupted, during tumorigenesis such cMNR sequences might still be selective targets of instability as demonstrated by a high frequency of frameshift mutations in the discontinuous murine *Tgfbr2* coding repeat (AAAAA-GAAAA) suggesting that some hematolymphoid malignancies may share pathogenetic mechanisms similar to some solid cancers [28]. Third, some tumors from MMR-knockout mice exhibit lower frequencies of MSI [12,14,29] when compared to the high prevalence of MSI in tumors arising in human MMR mutation carriers which might reflect the lack of sensitivity of the commonly used dinucleotide microsatellites in detecting MSI. In fact, a marker panel consisting of mononucleotides only or a combination of mono- and dinucleotide microsatellites proved to be most sensitive in detecting MSI in murine tumors [30,31] emphasizing the utility of diagnostic mononucleotide repeats for correctly determining the MSI phenotype.

In this proof-of-principle study, we sought to identify mouse genes with extended mononucleotide repeats in their coding regions and assessed their potential as mutation targets in a small set of MMR-deficient mouse intestinal tumors. Our results suggest the utility of five mouse mononucleotide microsatellites for detecting MSI in these murine tumors. We also identified frameshift mutations in

cMNRs of six candidate genes in these MSI intestinal tumors. The cMNRs in two of these mouse candidate genes (*Rfc3, Elavl3*) are conserved in type and length in the orthologous human genes and thus suggest potential overlaps as well as distinctions between mouse and human MSI intestinal tumorigenesis.

MATERIALS AND METHODS

Bioinformatic Analysis

To study cMNRs, we screened all mouse gene sequences in the mouse ensembl database (rel. 75.38). Perl scripts were developed to use the ensembl API and a rigorous redundancy check at the 98% level was applied. All annotation based cMNRs with a minimal repeat length of four mononucleotides were retrieved. Using several filters, repeat tracts within pseudogenes, vector sequences as well as homopolymeric nucleotide stretches at the most 5′ or 3′ ends of sequences were excluded. All candidate sequences were stored in a relational database for further analysis. Automated primer design was performed by a perl script using primer3_core (Primer 3 version 0.1; http://www.broad-institute.org/genome_software/other/primer3.html) in combination with a self-constructed human mispriming repeat library (containing L1, ALU, etc.). Exon-intron boundaries were verified by integrated BLASTN analysis, matching a candidate cDNA with homologous genomic DNA sequences. A randomly selected set of 12 long A/T and 3 shorter G/C cMNR sequences was chosen for frameshift mutation analyses.

Tumor Specimens

A pair of matched normal (tail) and intestinal tumor tissues each were collected from nine mice of three different genotypes: $Mlh1^{-/-}$ (n = 4); $Msh2^{-/-}$ (n = 1); $Msh2^{LoxP/LoxP}$ (n = 4). The $Mlh1^{-/-}$ and $Msh2^{-/-}$ mice exhibit constitutional MMR-deficiency, whereas the $Msh2^{LoxP/LoxP}$ mice show intestine-specific ablation of MMR function. Due to limited tissue amounts, not all cMNRs could be analyzed in all tissues. The Qiamp Tissue Kit (Qiagen, Hilden, Germany) was used for extraction of genomic DNA from total tissues without microdissection according to the manufacturer's instructions.

Microsatellite Analysis

Microsatellite instability was determined using a panel of five microsatellites consisting exclusively of long mononucleotide repeats. These microsatellites were chosen from two previous studies [30,31] and are indicated in Table 1. DNA fragment analysis of diagnostic markers was performed as described for cMNR frameshift mutation analysis. A marker was defined as unstable if novel peaks occurred in tumor compared to normal tissue or if the ratio of peak areas of corresponding peaks in tumor and normal tissue revealed values ≤ 0.5 or ≥ 2. Tumors were scored as MSI if 2/5 markers showed instability.

cMNR Frameshift Mutation Analysis

Primers were designed with the software primer3 and selected to generate short amplicons in order to assure robust PCR amplification (Table 1). One primer of each primer set carried a fluorescent (FITC) label at the 5′-end. PCR was performed in a total volume of 5 μl containing 0.5 μl 10 × reaction buffer (Invitrogen, Karlsruhe, Germany), 1.5 mM MgCl2, 200 μM dNTPs, 0.3 μM of each primer, and 0.1 U Taq DNA polymerase (Invitrogen), and 10 ng of genomic DNA, using the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and primer extension at 72°C for 30 s. The final extension step was carried out at 72°C for 6 min. PCR fragments were separated on an ABI3130xl genetic analyzer (Applied Biosystems, Darmstadt, Germany). Raw data were analyzed with Genescan analysis software (Applied Biosystems). Instability was scored, if novel peaks were obtained in tumor compared to normal tissue, or if the ratio of peak areas of corresponding peaks in tumor and normal tissue revealed values ≤ 0.5 or ≥ 2 [32].

Table 1. Mononucleotide Markers Used for Classification of Mouse MSI Intestinal Tumors

Name	Repeat	Gene	Chromosome	Product Size [bp]	Primer [5′ > 3′]
AA003063	A23		16	87	for: ACGTCAAAAATCAATGTTAGG rev: CAGCAAGGGTCCCTGTCTTA
U12235	A24	uPAR Plasminogen Activ.	7	86	for: GCTCATCTTCGTTCCCTGTC rev: CATTCGGTGGAAAGCTCTGA
L24372	A27	Scgb1a1 Secretoglobin	19	90	for: GGGAAGACTGCTTAGGGAAGA rev: ATTTGGCTTTCAAGCATCCATA
AC096777	T27		17	138	for: TCCCTGTATAACCCTGGCTGACT rev: GCAACCAGTTGTCCTGGCGTGGA
AC096777	A33		17	153	for: TACAGAGGATTGTCCTCTTGGAG rev: GCTGCTTCACTTGGACATTGGCT

RESULTS

Coding Microsatellites in the Mouse Genome

Since mismatch repair deficient tumors of HNPCC patients and MSI tumors of MMR knockout mice show partially overlapping tumor phenotypes, it is reasonable to assume that functional inactivation of the same pathways might occur in MSI tumors of both species, though the individual cMNR containing genes affected by frameshift mutations might be different. Therefore, we initiated a search of cMNRs (≥ 4 repeat units) in the mouse genome using the same bioinformatic strategy originally developed for the identification of human cMNRs [32]. Similar to the distribution of human cMNRs (Figure 1A), very short coding repeats (length < 7 bp) showed the highest prevalence in the mouse genome whereas their abundance decreased with increasing repeat length (Figure 1B). At increased repeat length (≥ 10), C and/or G cMNR occurrence showed a different distribution among the mouse and human genome. Initial sequence comparison of some human cMNR containing genes like *TGFBR2, BAX*, and *TCF-4* with their mouse orthologs also revealed that these repeats are interrupted (discontinuous) in many mouse genes. However, we observed repeat identity in some human-mouse orthologs like the RNA-binding protein ELAVL3/HuC (G_9), the tyrosine phosphatase PTPN21(A_8), and the replication factor RFC3 (A_{10}) suggesting that there is evolutionary conservation of such cMNRs across species (Table 2).

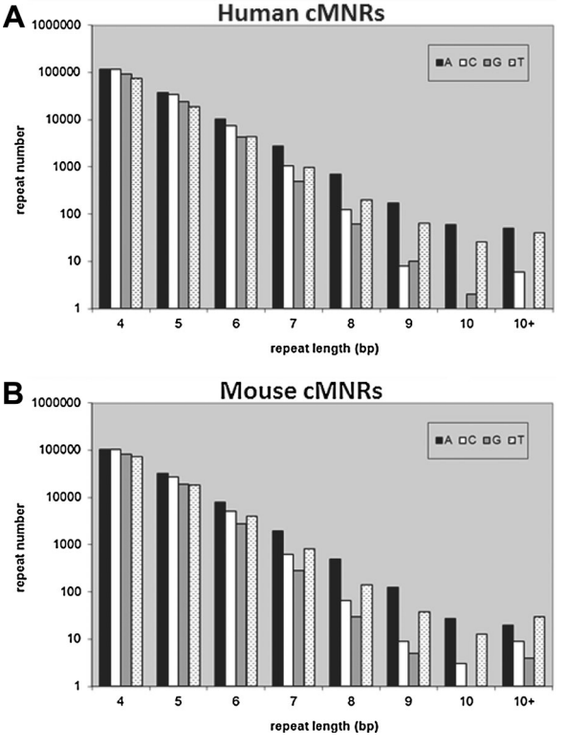

Figure 1. Distribution of coding mononucleotide microsatellites in the (A) mouse (ensembl release Mus_musculus_75_38) and (B) human (ensembl release Homo_sapiens_75_37) genomes. Mononucleotide repeat occurrence is depicted according to repeat length and nucleotide type.

Microsatellite Instability Classification of MMR-Deficient Murine Intestinal Tumors Using Mononucleotide Microsatellites

It is also largely unknown whether murine cMNRs are in fact targets of insertion/deletion mutations. In a first attempt to address these questions, we examined a small set of nine matched normal tail/intestinal tumor pairs for our initial studies. Since the MSI status of these tumors was unknown, we used a set of five long mononucleotide microsatellites (≥ 23 repetitive units) as a diagnostic marker panel (Table 1). Several reasons account for the selection of this marker panel: First, mononucleotide microsatellites show the highest sensitivity in detecting MSI particularly when using a panel consisting exclusively of such MNRs. This strategy is different to previous studies which used a combination of mono- and dinucleotide markers and often required semi-nested PCR strategies on single genome equivalent template DNA to detect MSI in murine tumors. Second, instability of diagnostic mononucleotide microsatellites is considered a key factor for detecting instability of coding region mononucleotides which are usually of shorter length. Using this marker panel all nine intestinal tumors from different MMR-deficient mice (*Mlh1*$^{-/-}$, *Msh2*$^{-/-}$, *Msh2*$^{LoxP/LoxP}$) proved to be MSI when compared to non-tumorous tissue. Notably, each tumor showed instability in all five markers (Figure 2) indicating a high level of microsatellite instability. Overall, these data demonstrate the utility of this marker set for MSI typing of murine tumors.

Murine MSI Intestinal Tumors Show Coding Microsatellite Instability

Subsequently, we analyzed the instability of 10 candidate cMNRs in MSI intestinal mouse tumors and their corresponding non-tumorous tissue. These cMNRs were selected randomly from a set of 113 candidate cMNRs in our mouse database that possess 10 or 11 repetitive A/T units and thus are expected to be highly susceptible to instability in MMR-deficient cells. We also included two of the above mentioned cMNRs which were found to be conserved in type and length among the human and mouse orthologous genes (*Ptpn21, Rfc3*) and which are known to be mutated in human MSI tumors (Table 3). 6/12 candidate genes (*NM025833, NM178420, Phactr4, Senp6, Sdccag1, Rfc3*) showed instability in murine MSI intestinal tumors. The observed shifts of fragment length were exclusively 1 bp deletion mutations and only affected one allele (Figure 3).

Based on our previous findings that some cMNRs in the human genome turned out not to be located in

Table 2. Conserved cMNR Gene Sequences in the Human and Mouse Genome

Gene (Repeat)	Species		[a]cMNR Sequence (5′ > 3′)	
PTPN21 (A8)	Human	2506	CTAGGGGGAATGAAAAAGACTCGAGTAGATGC**AAAAAAAA**TTGGTCCTCTTAAACTGGCT	2565
			++++++++ ++++++++ +++++++ ++++++++++++++++++++ ++ ++ +++++	
	Mouse	2512	CTAGGGGGGATGAAAAAAACTCGAGCAGATGC**AAAAAAAA**TTGGTCCCCTCAAGCTGGCA	2571
			[b]Human Tumor Mutation Frequency: 12.9% in MSI Colorectal Cancer	
			17.6% in MSI Gastric Cancer	
RFC3 (A10)	Human	220	ATCACAACTCCATCT**AAAAAAAAAA**TTGAAATTAGCACCATTGCAAGTAACTACCACCTT	279
			++++++++ ++ ++++++++++++++++++ ++++++++++++++ ++++++++++++	
	Mouse	220	ATCACAACCCCGTCT**AAAAAAAAAA**TTGAAATCAGCACCATTGCAAGCAACTACCACCTT	279
			[b]Human Tumor Mutation Frequency: 25% in MSI Colorectal Cancer	
			24% in MSI Gastric Cancer	
			5.6% in MSI Endometrial Cancer	
ELAVL3 (G9)	Human	10	CAGATACTGGGGGCCATGGAGTCTCAGGT **GGGGGGGGG**CCCGGCCGGCCCGGCCCTGCC	69
			++++++++++++++++++++++++++ +++++++++ ++++++++++++++++++++	
	Mouse	10	CAGATACTGGGGGCCATGGAGTCTCAGGTT**GGGGGGGGG** CCGGCCGGCCCGGCCCTGCC	69
			[b]Human Tumor Mutation Frequency: 35% in MSI Colorectal Cancer	
			6% in MSI Gastric Cancer	
			33% in MSI Endometrial Cancer	

[a]Gene-specific coding mononucleotide repeat (cMNR) sequences (*bold underlined*) and their flanking regions; nucleotide identity among both species is marked (*plus*); nucleotide numbers refer to the first nucleotide of the ATG start codon.
[b]taken from [33,34].

gene coding regions due to allele size variations in normal tissues, we also examined the allele size pattern of our set of 12 murine candidate cMNRs in a series of genomic DNAs from normal tissues of 12 wild-derived and 12 laboratory inbred mouse strains (inter- and intra-subspecies variation). Variations in repeat allele sizes in the constitutional DNA of these control mice were observed in 7/12 murine cMNR-harboring genes also including those three that exhibited instability in murine intestinal MSI tumors.

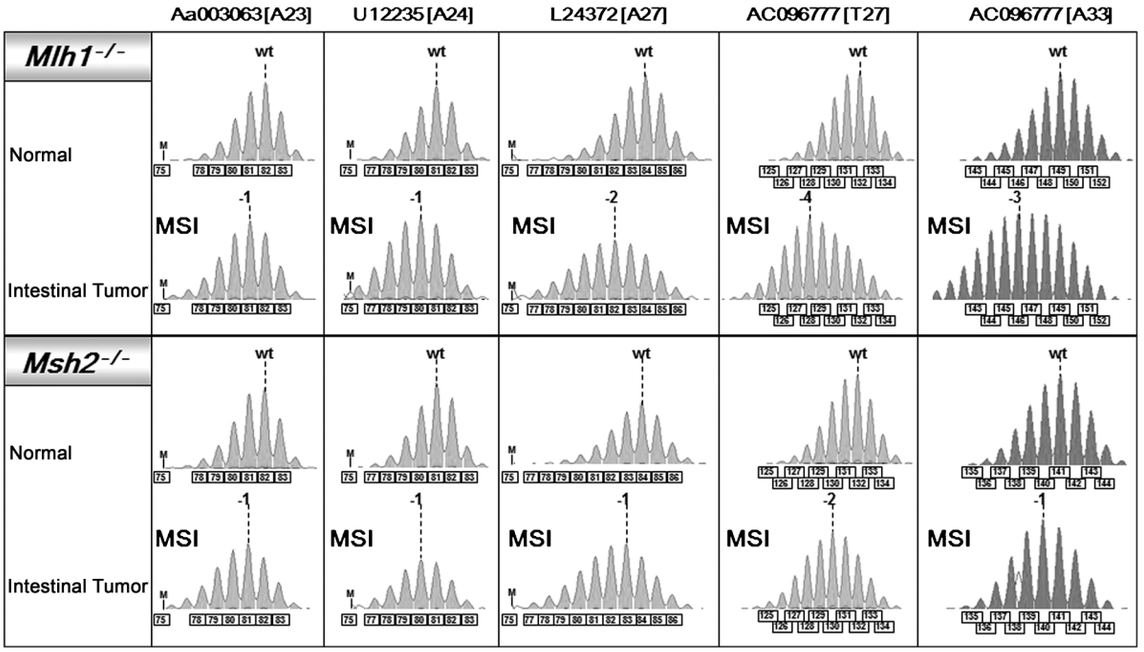

Figure 2. Representative microsatellite instability (MSI) pattern of intestinal tumors from MMR-deficient mice (*Mlh1*$^{-/-}$, *Msh2*$^{-/-}$) using five diagnostic mononucleotide markers. The highest peak in normal/tumor tissue is marked by a vertical line and corresponds to the major allele. Tumor-specific MSI manifests as allele length variation usually occurring as deletions (*negative numbers*). MSI was defined and determined at the marker and tissue level as described in Materials and Methods. Length of alleles and size markers (M) are indicated (*boxed numbers*).

Table 3. Candidate Mouse Genes Used for cMNR Mutation Analyses in Mouse MSI Intestinal Tumors

Gene	Repeat	Description	Product size [bp]	*Primer [5′ > 3′]
C79407	A10	EST ?	105	for: AAGACCAGACAGGAAACAGCA
				rev: ATACGTGATGCTGGCCTTTT
Dock4	A10	Dedicator of cytokinesis protein 4	237	for: ATAGCCAAGTGCCACAACAA
				rev: AGGGGACTTTCAGGATAGCA
Grb14	A10	Growth factor receptor-bound protein 14	147	for: GCATTTGCAGCTTTTCAGTG
				rev: GGCTGAGGCTTGCTTTACTC
Kcnma1	A10	Ca++-activated potassium channel subfamily M, alpha subunit 1	123	for: CACAAGCTGCCTGTATTTGC
				rev: TGCTTACCTCATCAGCTTCG
Phactr4	A10	Phosphatase and actin regulator 4	133	for: ATCTTCAAGCCGTGGAAATG
				rev: CCAGGGCTTTGCTCAAACTA
Sdccag1	A11	Serologically defined colon cancer antigen 1	124	for: GCCAGCATTGAGAACAGTGA
				rev. CTGACAGGCTGAGATCCACA
Senp6	A11	SUMO/sentrin-specific protease 6	118	for: GTGAGCCTTGTTACCGGAGA
				rev: CAAATGTCCTGGCACGTAGA
NM_025833	T11/G13	Receptor kinase ?	146	for: TCCAATCTCAACACCCCATAA
				rev: GCAAAGGGGTTTTCTCCACT
NM_178420	C11	EST ?	145	for: CCAACGTCCTAACACCCAGT
				rev: GTCACCGAAGTGTTTCCTG
NM_199470	A11	Cadherin-like 24/EY-cadherin/ cadherin 14-like	115	for: CCAAGATTTCATTCCCAGCA
				rev: TAAAAGCATTGGCAGTGGTG
Ptpn21	A8	Protein tyrosine phosphatase N 21	176	for: TTTGAAAGGTGTCTGGGTACG
				rev: CGCTCTGGTGGACACTTCTT
Rfc3	A10	Replication factor C, subunit 3	165	for: TTGGCAGTAGTGATTTGGTGA
				rev: AGGAGCAGTTTACCTGGGATT
Elavl3	G9	Embryonic lethal, abnormal vision-like 3; Hu C	119	for: CGGGTACGACCTGTTTTCTC
				rev: GTCAGTGGCTCCATTTGTCC
Glis2	C9	GLIS family zinc finger 2	131	for: TTCCAGCCACTTCGCTATTT
				rev: GGTGAGAGGCACTTGTCCTT
Tmem107	G9	Transmembrane protein 107	127	for: GAGCGATGGGAATGTACCAC
				rev: TCTAGGTGGGGGAAACCTTC

*for, forward; rev, reverse.

Thus, these repeats most likely do not comprise coding and translated sequences and observed allelic shifts in MSI intestinal tumors are expected to remain without functional consequences. However, cMNRs in the remaining 5/12 mouse genes (*Kcnma1*, *Sdccag1*, *Senp6*, *Ptpn21*, *Rfc3*) displayed invariant repeat length in constitutional DNA from all 24 wild-derived and laboratory inbred mouse strains (Table 4). Based on our DNA fragment analyses, the cMNR 1 bp deletions in 3/12 candidate genes (*Sdccag1, Senp6, Rfc3*) observed in MSI intestinal tumors are expected to represent inactivating frameshift mutations. Affected genes code for a serologically defined colon cancer antigen (Sdccag1/NY-CO-1) implicated in colon and lung cancer suppression, a SUMO-1 protease (*Senp6*) and the clamp loader replication factor 3 (Rfc3).

In addition to these repeats, which are expected to be highly susceptible towards mutation in MMR-deficient cells, we also examined three G/C repeats with a repeat length shorter than 10 repeat units (Table 3). Also for these repeats located in the coding region of the genes *Elavl3 (G9)*, *Glis2 (C9)*, and *Tmem107 (G9)*, microsatellite mutations were detected in mouse MSI intestinal tumors (Figure 3). These results underline that MMR deficiency in murine tumors has broad consequences, inducing

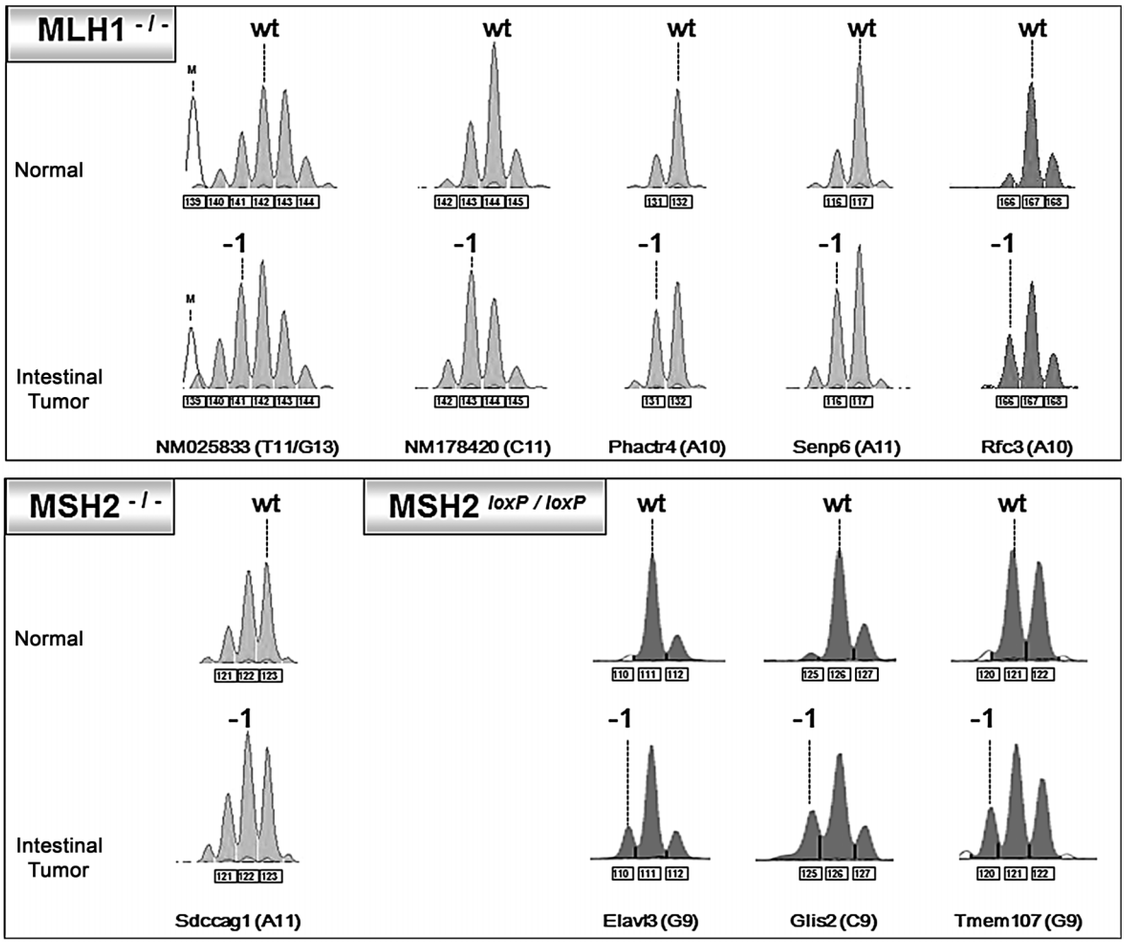

Figure 3. Coding repeat mutations of candidate target genes in MSI intestinal tumors of MMR-deficient mice ($Mlh1^{-/-}$, $Msh2^{-/-}$, $Msh2^{LoxP/LoxP}$). Wildtype (wt) and mutant cMNR alleles (−1) in normal and MMR-deficient intestinal tumors are indicated by vertical arrows, respectively. Length of alleles and size markers (M) are indicated (*boxed numbers*).

mutations of A/T as well as G/C repeats, including those with a short length and even affecting cMNRs (*Rfc3, Elavl3*) that are conserved among humans and mice and known to be mutated in human MSI colorectal, endometrial and gastric tumors.

DISCUSSION

This proof-of-principle study provides evidence for (i) the utility of a mononucleotide marker panel for detection of MSI in murine tumors; (ii) the existence of cMNR instability in MSI murine tumors; (iii) the utility of a set of mouse subspecies DNA for identification of polymorphic repeats; and (iv) repeat conservation among some orthologous human/mouse genes with two of them showing instability in both human and mouse MSI intestinal tumors. Hence, this latter observation challenges the general assumption that human and mouse MSI molecular tumorigenesis differ by cMNR mutations in different target genes.

In human MSI tumor diagnostics, the recommended consensus marker panel includes two mono- and three dinucleotide microsatellite markers [35]. However, subsequent analyses revealed that mononucleotide repeats proved to be more sensitive than dinucleotide repeats in MSI tumor classification [36]. Moreover, the quasimonomorphic nature of such homopolymeric repeats overcomes the requirement of non-tumorous tissue as a reference. For MSI analyses in mouse tumor tissues, no consensus marker panel is currently available. This is mainly due to the fact that tumors of MMR-deficient mice exhibit a less pronounced MSI phenotype, that is, the proportion of mutant alleles in a given tumor is small. In the present study, we used a marker panel consisting of five mononucleotide repeats retrieved and combined from two previous studies [30,31]. This panel proved to be useful in detecting MSI. Although our results are obtained from a very small sample set, they suggest that the observed shifts in microsatellite

Table 4. Mono-/Polymorphic cMNRs of Murine Candidate Genes Occurring in Laboratory and Wild-Derived Inbred Mouse Strains

	Gene (cMNR)											
Strain	C79407 (A10)	Dock4 (A10)	Grb14 (A10)	*Kcnma1 (A10)	Phactr4 (A10)	*Sdccag1 (A11)	*Senp6 (A11)	025833 (T11/G13)	78420 (A11)	199470 (A11)	*Ptpn21 (A8)	*Rfc3 (A10)
Laboratory												
129X1/SvJ	†+	+	+	+	+	+	+	−	−	+	+	+
A/J	+	+	+	+	+	+	+	+	−	+	+	+
AKR/J	+	+	+	+	+	+	+	−	−	+	+	+
BALB/cByJ	+	+	+	+	+	+	+	+	−	+	+	+
C3H/HeJ	+	−	+	+	+	+	+	+	−	+	+	+
C57BL/6J	+	+	+	+	+	+	+	−	−	−	+	+
C58/J	+	+	+	+	+	+	+	−	−	−	+	+
DBA/2J	+	+	+	+	+	+	+	+	−	+	+	+
FVB/NJ	+	+	+	+	+	+	+	+	−	+	+	+
NOD/LtJ	+	−	+	+	+	+	+	+	−	+	+	+
NZB/BINJ	+	−	+	+	−	+	+	+	−	+	+	+
SJL/J	−	−	+	+	+	+	+	−	−	+	+	+
Wild-Derived												
LEWES/EiJ	+	+	+	+	+	+	+	−	−	+	+	+
MOR/Rk	+	−	+	+	+	+	+	+	−	−	+	+
PERA/EiJ	−	+	+	+	+	+	+	+	−	+	+	+
PERC/EiJ	+	+	+	+	+	+	+	−	−	+	+	+
SF/CamEIJ	+	−	+	+	+	+	+	−	−	+	+	+
WSB/Ei	+	+	+	+	+	+	+	+	−	+	+	+
ZALENDE/Ei	+	+	+	+	+	+	+	−	−	+	+	+
CZECHII/EiJ	+	−	+	+	−	+	+	−	−	−	+	+
SKIVE/EI	+	−	−	+	−	+	+	−	−	−	+	+
MOLF/EiJ	+	−	−	+	+	+	+	+	−	−	+	+
CAST/EiJ	+	−	−	+	−	+	+	+	−	+	+	+
SPET/Ei	+	+	−	+	−	+	+	−	−	+	+	+

*Genes harboring monomorphic cMNRs.
†Constitutional normal (+) and altered (−) cMNR alleles.

length are often subtle and obviously require highly sensitive mononucleotide markers for MSI assessment.

In addition to this instability of long (> 23 repeat units) non-coding mononucleotide microsatellites, we also detected fragment length shifts of shorter mononucleotide stretches (< 12 repeat units) in some mouse genes (*Sdccag1, Senp6, Rfc3, Elavl3, Tmem107, Glis2*) that — if expressed in the intestinal epithelium — are expected to result in translational frameshifts and abrogation of normal protein function. It is generally assumed that cMNR mutations in some but not all affected genes provide a growth advantage to MMR-deficient cells which has been demonstrated for *TGFBR2* and *BAX2* mutations in human MMR-deficient tumor cells. Since the present study for the first time demonstrates the existence of such cMNRs mutations also in MMR-deficient mouse intestinal tumors, it is reasonable to assume that similar molecular alterations might be associated with murine MSI tumorigenesis. So far, however, our observations are descriptive and therefore need to be interpreted with caution until confirmed by functional studies. In terms of mutant allele status, neither the DNA fragment analyses nor the sequencing data provide any evidence for biallelic cMNR mutations in this small set of tumors. However, template DNA was isolated from total tumors without microdissection, and thus our analysis might be confounded by non-malignant epithelial, stromal and inflammatory cells. It is also conceivable that haploinsufficiency might account for the lack of biallelic mutations, and thereby conferring some growth advantage to affected cells as has been demonstrated for human genes such as *PTEN* [37].

Proteins encoded by these six mutated mouse genes are involved in different cellular processes. As a common theme, *Elavl3*, *Tmem107* and *Glis2* appear to be involved in neurogenesis and *Tmem107* and *Glis2* also contibute to the biogenesis

of cilia. However, *Tmem107* and the Krüppel-like zinc-finger protein Glis2 are also expressed in the colonic epithelium. Glis2 has been implicated in the control of epithelial mesenchymal transition and is known to act as a negative regulator of ß-catenin thereby inhibiting TCF/LEF-mediated activation of cyclinD1 [38]. Not much is known about the function of Sdccag1 which has been originally identified by serological analysis of colon cancer patients [39]. It might play a role in nuclear export, and it also has been reported that inhibition of Sdccag1 in normal human bronchial epithelial (NHBE) cells increased invasiveness as determined by invasion chamber assay [40]. In addition, Sdccag1 induction has been linked to growth arrest in lung cancer cells [41]. In human cells, loss of the SUMO-1 protease SENP6 causes a substantial decrease in long-term cell viability [42] and errors in mitotic progression [43]. It also appears that MSH2-MSH3 has a synthetic lethal/sick relationship with Senp6 which means loss of Senp6 reduces viability of MSH2 deficient tumor cells (W. Edelmann and E. Tosti, unpublished results). Another candidate gene affected by cMNR mutation is the replication factor 3 (Rfc3) that serves as a clamp loader of proliferating cell nuclear antigen (PCNA) onto DNA thereby increasing the processivity of DNA polymerases. An integrative genomics study identified human *RFC3* as an amplified candidate oncogene in esophageal adenocarcinoma. These authors showed that RFC3 gain is prevalent in several types of human cancer, that knockdown of RFC3 had an antiproliferative effect, and that RFC3 expression was associated with poor prognosis in multiple patient cohorts [44].

In this context, it is important to note that frameshift mutations in the A10 cMNR and/or loss of RFC3 expression occur in human gastric and colorectal cancers [32,45]. One of the most interesting findings in the present study was the observation that the *Rfc3* A10 and the *Elavl3* G9 coding repeat tracts are conserved in the human and mouse orthologous genes. Although this does not apply to the majority of human/mouse orthologs, it clearly provides proof-of-principle that cMNR identity in some genes is maintained across species. Since *Rfc3* and *Elavl3* cMNR frameshift mutations are found in both mouse and human MSI tumors, common mechanisms in mouse and human MSI tumorigenesis are likely to be involved. Therefore, it will be important to determine such conservation and differences by a systematic comparison of all cMNRs in both species. Similarly, a much larger number of tumors need to be analyzed to confirm our preliminary findings. Interestingly, cMNR frameshift mutations not only occurred in intestinal tumors of $Mlh1^{-/-}$ and $Msh2^{-/-}$ mice exhibiting constitutional MMR-deficiency but also arose in MSI tumors of a recently described *Msh2loxP* mouse line with specific ablation of Msh2 function and subsequent adenoma and carcinoma development in the intestinal epithelium [27] which is currently the best preclinical model for HNPCC/Lynch syndrome. Overall, comprehensive cMNR instability analyses of murine MSI tumors will thus provide important mechanistic insights into the etiology and progression of mouse MMR-deficient tumors.

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