Functional analysis of human mismatch repair gene mutations identifies weak alleles and polymorphisms capable of polygenic interactions

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Many of the mutations reported as potentially causing Lynch syndrome are missense mutations in human mismatch repair (MMR) genes. Here, we used a *Saccharomyces cerevisiae*-based system to study polymorphisms and suspected missense mutations in human MMR genes by modeling them at the appropriate *S. cerevisiae* chromosomal locus and determining their effect on mutation rates. We identified a number of weak alleles of MMR genes and MMR gene polymorphisms that are capable of interacting with other weak alleles of MMR genes to produce strong polygenic MMR defects. We also identified a number of alleles of *MSH2* that act as if they inactivate the Msh2-Msh3 mispair recognition complex thus causing weak MMR defects that interact with an *msh6Δ* mutation to result in complete MMR defects. These results indicate that weak MMR gene alleles capable of polygenic interactions with other MMR gene alleles may be relatively common.

hereditary nonpolyposis colorectal cancer | lynch syndrome | *Saccharomyces cerevisiae* | mutator phenotype | genome instability

Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), is an inherited cancer susceptibility syndrome characterized by predisposition to develop colorectal cancer and other cancers (1–3). Inherited DNA mismatch repair (MMR) gene defects underlie many cases of Lynch syndrome (4–7). The two major Lynch syndrome genes are *MSH2* and *MLH1*, encoding MutS and MutL homologs, respectively (4–7). Defects in other MMR genes are found in Lynch syndrome much less frequently but are found in other types of suspected inherited cancer susceptibility such as Turcots syndrome and familial cancers associated with weaker family histories, later age of onset, and potentially a different cancer spectrum (1, 8–14). Large numbers of Lynch syndrome cases have been screened for mutations in MMR genes and consequently large numbers of mutations and genetic variants have been published and reported in public databases.

Many reported mutations in *MSH2* and *MLH1* are missense mutations (1, 4–6); unfortunately, it is difficult to assess the functional and clinical significance of such mutations. Some studies report data demonstrating cosegregation of a missense variant with disease and lack of the variant in normal controls; however, such data do not definitively distinguish a pathogenic mutation from a rare, nonpathogenic variant. Functional studies have been performed to determine if missense variants affect protein function as loss-of-function would provide evidence for a pathogenic mutation. The approaches used in these studies include bioinformatic and structure-based predictions; evaluation of mutations in human cells, mutant mice, and human cell extract-based MMR reactions; biochemical evaluation of mutant proteins; and a number of *Saccharomyces cerevisiae*-based assays (15–27). Although most assays used have limitations, useful functional data for a number of potential mutations have emerged.

Results

Identification of Human MMR Gene Mutations and Polymorphisms that Are Weak Alleles. Here, we evaluated 11 reported polymorphisms in *MSH2*, *MLH1*, *MSH6*, and *PMS2* (*PMS1* in *S. cerevisiae*) and 14 reported missense mutations in these genes by making equivalent mutations at the analogous position in the corresponding chromosomal gene in *S. cerevisiae* and evaluating their effect on MMR using mutator assays (Tables 1 and 2; *S. cerevisiae* allele designations are used in the text of this report). Some of these variants have been characterized in other studies using *S. cerevisiae*-based assays (16–19, 23, 25) but mostly by testing them as plasmid-borne mutations (16–19, 23)(Table 2). The mutator assays used include *CAN1* gene inactivation that detects mutations inactivating the *CAN1* gene and *hom3-10* and *lys2-10A* frameshift reversions that detect mutations that revert +1T and +1A insertions, respectively, present in mononucleotide repeats (28–30). The latter two assays are extremely sensitive. Of the 11 polymorphisms tested, six resulted in a mutation rate indistinguishable from the wild-type rate. Five of the polymorphisms caused weak but significant mutator phenotypes in one of the mutator assays, almost always the sensitive *lys2-10A* assay. Of the 14 suspected pathogenic mutations tested, four caused effectively complete MMR defects, three caused small but significant mutator phenotypes in multiple mutator assays, three caused a small but significant mutator phenotype in a single mutator assay, and four caused no apparent defect. Mutations and polymorphisms causing little or no MMR defects have been reported in other functional studies (17–19, 23). That we detected a large proportion of weak or silent alleles may reflect the fact that the mutations we selected for study were mostly from cases where there was little if any clinical data supporting potential pathogenicity.

Of the 14 mutations and polymorphisms tested here that have also been tested in other studies (Table 2), our results differed from those of at least one published study in seven cases (*mlh1-R214C*, *mlh1-R265H*, *msh2-D524Y*, *msh2-E194G*, *mlh1-E129H*, *mlh1-S415N*, and *msh2-G317D*). It seems likely that these differences are because our chromosomal mutation-based assay system in which cells can be grown in rich, nonselective media is more sensitive and accurate for characterizing small differences relative to low wild-type mutation rates than plasmid complementation-based assay systems used in other studies.

Identification of Weak MMR Gene Alleles that Interact with Other Weak MMR Gene Alleles. We previously described a method for screening for enhancer mutations that cause little or no MMR defect alone but significantly enhance the MMR defect caused by a second weakly MMR defective allele (28). To further evaluate the potential significance of polymorphisms and weakly

Author contributions: R.D.K. designed research; S.M. performed research; S.M. and R.D.K. analyzed data; and S.M. and R.D.K. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/1000798107/DCSupplemental.

Table 1. List of mutations tested in this study

Chromosomal alleles

Strain no.	Gene	Human allele	*S. cerevisiae* allele	Source
Mutations				
RDKY7071	*MLH1*	R217C	R214C	a
RDKY7091	*MLH1*	R265H	R265H	a
RDKY7092	*MLH1*	V326A	I326A	a
RDKY7093	*MLH1*	K84E	K81E	a
RDKY7094	*MLH1*	V716M	L731M	a
RDKY7095	*MLH1*	T117M	T114M	a
RDKY7100	*MSH2*	C333R	C345R	a
RDKY7101	*MSH2*	D506Y	D524Y	a
RDKY7102	*MSH2*	E198G	E194G	a; 4, 33
RDKY7103	*MSH2*	I194T	L190T	b
RDKY7104	*MSH2*	E701K	E720K	b
RDKY7107	*MSH6*	S144I	S107I	a
RDKY4493	*MSH6*	V509A	V410A	a
RDKY7110	*PMS1*	E705K	E738K	a
Polymorphisms				
RDKY7096	*MLH1*	D132H	E129H	a
RDKY7097	*MLH1*	H718Y	H733Y	a
RDKY7098	*MLH1*	I219L	V216L	a
RDKY7099	*MLH1*	S406N	S415N	a
RDKY7105	*MSH2*	G322D	G317D	a
RDKY7106	*MSH2*	N127S	N123S	a
RDKY7108	*MSH6*	S503C	S406C	a
RDKY4488	*MSH6*	L396V	L301V	a
RDKY7109	*MSH6*	K1358DfsX1	Asp1239X	a
RDKY7111	*PMS1*	T485K	T505K	a
RDKY7112	*PMS1*	N775S	Q808S	a

Plasmid alleles

Plasmid no.	Gene	Human allele	*S. cerevisiae* allele	Source
Mutations				
pAG207	*MSH2*	C199R	C195R	a; 17
pAG80	*MSH2*	D167H	D163H	a; 17
pAG25	*MSH2*		G122S	c; 17
pAG204	*MSH2*	I145M	I141M	a; 17
pAG203	*MSH2*	N127S	N123S	a; 17
pAG413	*MSH2*	Q61P	Q61P	a; 17
pAG401	*MSH2*	T44M	T44M	a; 17
pAG414	*MSH2*	Y98C	Y104C	a; 17
pRDK1523	*MSH2*	T33P	T33P	a
pRDK1524	*MSH2*	V34E	I34E	a
pRDK1525	*MSH2*	D49V	D49V	a
pRDK1526	*MSH2*	L93P	L99P	a
pRDK1527	*MSH2*	R96H	L102H	a
pRDK1528	*MSH2*	Y103C	Y109C	a
pRDK1529	*MSH2*	V163D	V159D	a
pRDK1530	*MSH2*	L187P	L183P	a

a, inSIGHT database (6) (insight-group.org); b, Available on request; c, *E. coli* mutation (17).

Table 2. Mismatch repair defects caused by mutations and polymorphisms tested

Strain no.	Genotype	Mismatch repair defect* Thr$^+$	Lys$^+$	Can^r	Refs†
Controls					
RDKY3590	Wild-type	1	1	1	
RDKY3591‡	*msh2Δ*	1105	7480	55	
RDKY3684§	*msh6Δ*	19	182	18	
Mutations					
RDKY7071	*mlh1-R214C*	NS	NS	NS	18, 23
RDKY7091	*mlh1-R265H*	71	163	10.4	18, 23
RDKY7092	*mlh1-I326A*	4.4	3.9	3.8	18, 19, 23
RDKY7093	*mlh1-K81E*	2389	4835	26	18
RDKY7094	*mlh1-L731M*	NS	NS	NS	18
RDKY7095	*mlh1-T114M*	2952	8730	40	18, 19
RDKY7100	*msh2-C345R*	2623	6625	35	
RDKY7101	*msh2-D524Y*	NS	NS	NS	16
RDKY7102	*msh2-E194G*	20	13.6	NS	17
RDKY7103	*msh2-L190T*	NS	NS	NS	
RDKY7104	*msh2-E720K*	NS	2.4	NS	
RDKY7107	*msh6-S107I*	NS	2.0	NS	
RDKY4493	*msh6-V410A*	NS	2.3	NS	
RDKY7110	*pms1-E738K*	2092	5183	59	25
Polymorphisms					
RDKY7096	*mlh1-E129H*	NS	4.0	NS	18
RDKY7097	*mlh1-H773Y*	NS	NS	NS	18
RDKY7098	*mlh1-V216L*	NS	NS	NS	18
RDKY7099	*mlh1-S415N*	NS	2.3	NS	18
RDKY7105	*msh2-G317D*	NS	NS	NS	16, 23
RDKY7106	*msh2-N123S*	NS	2.6	NS	
RDKY7108	*msh6-S406C*	NS	1.9	NS	
RDKY4488	*msh6-L301V*	NS	NS	4.2	
RDKY7109	*msh6-Asp1239*	NS	NS	NS	
RDKY7111	*pms1-T505K*	NS	NS	NS	
RDKY7112	*pms1-Q808S*	NS	NS	NS	

*Individual experiments were performed in which the mutation rates for one or more mutants and at least one wild-type strain were determined in the same experiment. Mutant and wild-type rates were compared within each experiment and in only those cases where the *P* value for the significance was $P < 0.05$ in a Mann Whitney test the fold-increase in mutation rate relative to wild-type was calculated; in two of the cases where a fold-increase is reported, the P value was 0.025, whereas in the remaining cases the *P* values were < 0.005. Each mutant rate was independently determined from two to eight times and the average fold-increase in mutation rate is reported. In addition, we required that the increase in mutation rate be greater than one standard deviations of the average for 24 independent determinations of the wild-type mutation rate (Thr$^+$ = 1.85 $^{+/-}$ 1.9 x 10^{-9}, Lys$^+$ = 1.38 $^{+/-}$ 0.92 x 10^{-8}, Can^r = 1.08 $^{+/-}$ 0.83 x 10^{-7}). Otherwise, NS (no significant difference) is indicated.

†References to other studies concerning the indicated mutations.

‡The mutation rates for RDKY3591 *msh2Δ* and paired wild-type RDKY3590 were [Thr$^+$ = 4.66 x 10^{-6}, Lys$^+$ = 1.21 x 10^{-4}, Can^r = 5.62 x 10^{-6}] and [Thr$^+$ = 4.21 x 10^{-9}, Lys$^+$ = 1.61 x 10^{-8}, Can^r = 1.02 x 10^{-7}], respectively. The mutation rates for *msh2Δ*, *mlh1Δ*, and *pms1Δ* strains were not significantly different from each other.

§The mutation rates for RDKY3684 *msh6Δ* and paired wild-type RDKY3590 are reported in Table 3 and Table S1.

defective mutations in MMR genes, we selected two weak alleles (*msh2-E194G*, *mlh1-I326A*) and three polymorphisms (*msh2-G317D*, *msh6 -L301V*, *mlh1-E129H*) and screened 6,000–12,000 mutagenized survivors for enhancer mutations. In the case of the *msh2-E194G* mutation, 12 enhancer mutations (*msh6-T312I*, *msh6-E624K*, *msh6-G477D*, *pms1-P343L*, *msh6-G1064E*, *msh6-T474I*, *msh6-E395K*, *msh6-E442K*, *pms1-R610K*, *mlh1-G144D*, *mlh3-D556N, msh6-G1066R*) were found. Reconstruction experiments demonstrated that although each single mutant had little if any MMR defect, the double mutants could be almost completely MMR defective (Table 3). Enhancer screens with the *mlh1-I326A* mutation and the *mlh1-E129H* polymorphism had a lower yield of three and two enhancer mutations each, respectively (*mlh1-I326A - msh2-T743I*, *msh2-P640S*, *pms1-G173E*; *mlh1-E129H - msh6-G1064E*, *pms1-E191K*), whereas enhancer screens with the *msh2-G317D* and *msh6-L301V* polymorphisms did not yield enhancer mutations (of note, one dominant *msh6* mutation was identified in the *msh2-G317D* screen; Table S1) (31). These

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Table 3. Genetic interactions with the *msh2-E194G* mutation

Strain no.	Genotype	Mutation Rate		
		Thr$^+$	Lys$^+$	Can^r
RDKY3590	Wild-type	2.0×10^{-9} (1)	1.1×10^{-8} (1)	9.0×10^{-8} (1)
RDKY7102	*msh2-E194G*	2.3×10^{-8} (11.5)	1.54×10^{-7} (14)	1.35×10^{-7} (1.5)
RDKY7114	*msh6-E624K*	6.32×10^{-9} (3.2)	1.22×10^{-7} (11.1)	2.7×10^{-7} (3)
RDKY7115	*msh6-E624K msh2-E194G*	1.44×10^{-6} (720)	2.19×10^{-5} (1991)	1.53×10^{-6} (17)
RDKY7116	*msh6-G477D*	2.07×10^{-9} (1)	2.08×10^{-8} (1.9)	1.32×10^{-7} (1.5)
RDKY7117	*msh6-G477D msh2-E194G*	6.55×10^{-7} (327.5)	3.11×10^{-6} (283)	6.81×10^{-7} (7.6)
RDKY7118	*pms1-P343L*	1.37×10^{-7} (68.5)	1.12×10^{-5} (1018)	2.84×10^{-7} (3.2)
RDKY7119	*pms1-P343L msh2-E194G*	7.6×10^{-7} (380)	1.26×10^{-5} (1145)	5.14×10^{-7} (5.7)
RDKY7120	*msh6-T312I*	3.73×10^{-9} (1.9)	5.87×10^{-8} (5.3)	3.41×10^{-7} (3.8)
RDKY7121	*msh6-T312I msh2-E194G*	1.67×10^{-6} (835)	2.9×10^{-5} (2636)	1.82×10^{-6} (20.2)
RDKY3684	*msh6Δ*	3.8×10^{-8} (19)	2.0×10^{-6} (182)	1.62×10^{-6} (18)
RDKY7122	*msh2-E194G msh6Δ*	2.4×10^{-6} (1200)	6.49×10^{-5} (5900)	3.20×10^{-6} (35.5)

The number in parentheses is the fold-increase in mutation rate relative to the wild-type strain RDKY3590. For comparison, mutation rates for the RDKY3591 *msh2Δ* strain were [Thr$^+$ = 4.66×10^{-6}, Lys$^+$ = 1.21×10^{-4}, Can^r = 5.62×10^{-6}], which are representative of a complete loss of MMR.

screens are not saturated given they would be expected to yield allele-specific, likely rare mutations; however, it seems likely that the differences in yields obtained reflect differences in the diversity of enhancer mutations each allele might be capable of interacting with, given the similar number of mutations tested in each screen.

The *msh2-E194G* mutation screen yielded large numbers of enhancer mutations. This mutation alters an amino acid located at the boundary of Msh2 domains I and II and would be predicted to interfere with the correct positioning of domain I. Domain I is required for Msh2 to function in the Msh2-Msh3 complex but not the Msh2-Msh6 complex (32) and consistent with this, the *msh2-E194G msh6Δ* double mutant had a synergistic increase in mutation rate in the frameshift reversion assays compared to the respective single mutants (Table 3). This result and the observation that it is possible to isolate many enhancer mutations that cause much weaker MMR defects than a *msh6Δ* mutation indicates that the *msh2-E194G* mutation sensitizes the Msh6-dependent pathway to inactivation in addition to causing a defect in the Msh3 pathway. The human mutation, *msh2-E198G*, was initially found in a Lynch syndrome family with microsatellite stable tumors consistent with a weak MMR defect (4, 33). Four of the affected mutation carriers also inherited a missense variant in *MLH3* (34). However, our studies suggest that the *mlh3* missense mutation might not be an enhancing mutation as the human *msh2-E198G* mutation (*Sc msh2-E194G*) would be predicted to already cause a defect in the Msh3 pathway where Mlh3 functions (35).

Identification of Weak Lynch Syndrome-Associated *MSH2* Alleles that Inactivate the Msh2-Msh3 Complex. Interestingly, numerous Lynch syndrome-associated *msh2* missense mutations affecting Msh2 domains I and II have been reported, and many of those tested in functional assays cause no MMR defect (6, 17). These mutations potentially inactivate the function of the Msh2-Msh3 complex but not the Msh2-Msh6 complex, and such mutations would be expected to cause synergistic increases in the rate of accumulating frameshift mutations when combined with an *msh6Δ* mutation. To investigate this possibility, we first tested 16 such mutations for MMR defects by determining their ability to complement an *msh2Δ* mutation as plasmid borne alleles using patch tests. Of these, eight had been previously tested in functional assays (17) and eight had not been previously studied. We confirmed that five of the previously studied mutant alleles (*T44M*, *Q63P*, *Y104C*, *N123S*, *I141M*) were fully complementing and had no effect on MMR, and one was a loss of function mutation (*C195R*). Two of these were not fully complementing (*G122S, D164H*) and caused weak MMR defects (Table 4). Of the newly studied mutations, we found that three mutations (*L102H*, *Y109C*, *V159D*) had no effect on MMR, three mutations (*I34E*, *D49V*, *L183P*) were loss of function mutations, and

Table 4. Interaction between plasmid *MSH2* alleles and an *msh6Δ* mutation

Plasmid no.	*MSH2* Allele	RDKY2706 *msh2Δ*		RDKY4136 *msh2Δmsh6Δ*	
		Mutation rate		Mutation rate	
		Thr$^+$	Can^r	Thr$^+$	Can^r
pMSH2	*MSH2*	21.27×10^{-8} (1)	2.81×10^{-7} (1)	4.54×10^{-8} (1)	4.29×10^{-6} (1)
pRS413	Vector	1.45×10^{-5} (1142)	6.49×10^{-6} (23)	1.19×10^{-5} (262)	9.37×10^{-6} (2.2)
pAG80	*msh2-D164H*	1.24×10^{-7} (9.8)	7.92×10^{-7} (2.8)	7.87×10^{-7} (13)	3.48×10^{-6} (0.8)
pAG25	*msh2-G122S*	1.11×10^{-7} (8.7)	4.65×10^{-7} (1.7)	2.49×10^{-6} (55)	7.37×10^{-6} (1.7)
pRDK1523	*msh2-T33P*	4.25×10^{-7} (33)	4.01×10^{-6} (14.3)	2.86×10^{-5} (630)	2.21×10^{-5} (5.2)
pRDK1526	*msh2-L99P*	6.43×10^{-7} (51)	9.97×10^{-7} (3.5)	1.70×10^{-5} (374)	1.13×10^{-5} (2.6)
pRDK1527	*msh2-L102H*	1.44×10^{-8} (1.1)	3.04×10^{-7} (1.1)	1.15×10^{-5} (253)	1.09×10^{-5} (2.5)
pRDK1528	*msh2-Y109C*	5.70×10^{-8} (4.5)	4.04×10^{-7} (1.44)	2.75×10^{-5} (606)	2.02×10^{-5} (4.7)

All of the indicated *MSH2* alleles were made by site-directed mutagenesis in the pRS413 *HIS3 MSH2* vector pMSH2; two of the plasmid mutants (pAG80 and pAG25) were obtained from Dr. Alison Gammie (Princeton University, Princeton, NJ). The number in () is the fold-increase in mutation rate relative to the value obtained with the pMSH2 wild-type plasmid.

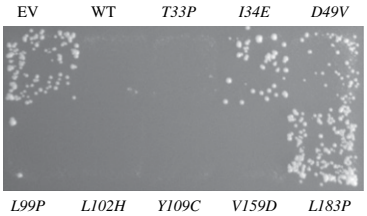

Fig. 1. Complementation of a chromosomal *msh2Δ* mutation by plasmid *MSH2* alleles assessed by patch tests. An *msh2Δ* mutant strain RDKY2706 containing the indicated plasmid MSH2 alleles was patched onto His drop out media and then replicated onto Thr^- His^- CSM media to assess the frequency of accumulation of *hom3-10* (Thr^+) revertants. EV indicates Empty Vector.

two mutations (*T33P*, *L99P*) were not fully complementing and caused weak MMR defects (Fig. 1). Each of the alleles that caused either no or weak MMR defects were then tested as plasmid borne alleles in an *msh2Δ msh6Δ* double mutant in patch tests to determine if they caused synergistic increases in the rate of accumulating frameshift mutations when combined with an *msh6Δ* mutation, and the interacting mutations were then characterized by performing quantitative mutation rate assays. Six mutations did not cause an MMR defect and did not cause synergistic increases in the *hom3-10* frameshift reversion assay in combination with an *msh6Δ* mutation in patch tests (*T44M*, *Q63P*, *Y104C*, *N123S*, *I141M*, *V159D*) and were not studied further. Of the mutations studied further, five caused synergistic increases in mutation rates in combination with an *msh6Δ* mutation in the *hom3-10* frameshift reversion assay (*T33P*, domain I; *L99P*, domain I; *L102H*, domain I; *Y109C*, domain I; *G122S*, domain I) and hence behaved as if they inactivated the Msh2-Msh3 complex, whereas one mutation (*D164H*, domain II) caused at most a very small synergistic increase in mutation rate in combination with an *msh6Δ* mutation and was likely a weakly MMR defective mutation (Table 4).

Discussion

Our studies have used a *S. cerevisiae*-based system to explore the genetics of polymorphisms and suspected mutations in human MMR genes. The limitations of the system are that only mutations affecting conserved amino acids can be analyzed, and it is

Table 5. *S. cerevisiae* strains used in this study

Strain no.	Relevant genotype	Source
RDKY3590	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2-10A*	28
RDKY3023	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2ΔBgl, his3Δ200, ade2Δ1::hisG, ade8*	39
RDKY2706	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2ΔBgl, his3Δ200, ade2Δ1::hisG, ade8, msh2Δ::hisG*	This study
RDKY4136	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2ΔBgl, his3Δ200, ade2Δ1::hisG, ade8, mshΔ2::hisG, msh6Δ::hisG*	This study
RDKY3591	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2-10A, msh2Δ::hisGURA3hisG*	This study
RDKY3684	*MATa, ura3-52, leu2Δ1, trp1Δ63, hom3-10, lys2-10A msh6Δ::hisG*	This study
RDKY7071	RDKY3590 *mlh1-R214C*	This study
RDKY7091	RDKY3590 *mlh1-R265H*	This study
RDKY7092	RDKY3590 *mlh1-I326A*	This study
RDKY7093	RDKY3590 *mlh1-K81E*	This study
RDKY7094	RDKY3590 *mlh1-L731M*	This study
RDKY7095	RDKY3590 *mlh1-T114M*	This study
RDKY7096	RDKY3590 *mlh1-E129H*	This study
RDKY7097	RDKY3590 *mlh1-H733Y*	This study
RDKY7098	RDKY3590 *mlh1-V216L*	This study
RDKY7099	RDKY3590 *mlh1-S415N*	This study
RDKY7100	RDKY3590 *msh2-C345R*	This study
RDKY7101	RDKY3590 *msh2-D524Y*	This study
RDKY7102	RDKY3590 *msh2-E194G*	This study
RDKY7103	RDKY3590 *msh2-L190T*	This study
RDKY7104	RDKY3590 *msh2-E720K*	This study
RDKY7105	RDKY3590 *msh2-G317D*	This study
RDKY7106	RDKY3590 *msh2-N123S*	This study
RDKY7107	RDKY3590 *msh6-S107I*	This study
RDKY7108	RDKY3590 *msh6-S406C*	This study
RDKY7109	RDKY3590 *msh6-Asp1239X*	This study
RDKY7110	RDKY3590 *pms1-E738K*	This study
RDKY7111	RDKY3590 *pms1-T505K*	This study
RDKY7112	RDKY3590 *pms1-Q808S*	This study
RDKY4488	RDKY3590 *msh6-L301V*	This study
RDKY4493	RDKY3590 *msh6-V410A*	This study
RDKY7113	RDKY3590 *msh6-R1024C*	This study
RDKY7114	RDKY3590 *msh6-E624K*	This study
RDKY7115	RDKY3590 *msh2-E194G, msh6-E624K*	This study
RDKY7116	RDKY3590 *msh6-G477D*	This study
RDKY7117	RDKY3590 *msh2-E194G, msh6-G477D*	This study
RDKY7118	RDKY3590 *pms1-P343L*	This study
RDKY7119	RDKY3590 *msh2-E194G, pms1-P343L*	This study
RDKY7120	RDKY3590 *msh6-T312I*	This study
RDKY7121	RDKY3590 *msh2-E194G, msh6-T312I*	This study
RDKY7122	RDKY3590 *msh2-E194G, msh6Δ::hisG*	This study

tedious to make mutations at the chromosomal MMR gene of interest. The strength of the system is that sophisticated genetic analysis can be used. We found that most polymorphisms are likely silent but that at least five polymorphisms, including one previously associated with increased cancer susceptibility (*mlh1-E129H*) (36, 37), cause very weak MMR defects. Our results also indicate that although many reported missense mutations in MMR genes are likely to be loss-of-function mutations, numerous reported mutations have either no affect on MMR or cause very weak MMR defects underscoring the need for functional testing before concluding missense mutations are pathogenic. Finally, we identified a number of weak alleles that are capable of interacting with other weak alleles in a number of different MMR genes or that interact with an *msh6Δ* mutation to produce strong polygenic MMR defects, indicating such alleles may be relatively common. These latter results reveal a level of genetic complexity that is relevant to the genetics of cancer susceptibility not previously demonstrated for human MMR genes.

How might weak alleles result in increased development of cancer? It is possible that such defects simply lead to low rates of accumulating mutations resulting in the need for increased numbers of cell generations before sufficient numbers of mutations occur in critical tumor suppressor genes and proto-oncogenes, compared to complete loss of MMR. Alternatively, such mutations might be separation-of-function mutations that primarily cause DNA damage response defects rather than MMR defects (27, 38). These types of defects would have lower penetrance than complete MMR defective mutations. Our results demonstrate two other potential mechanisms. One is that inherited weak alleles might participate in polygenic interactions with other inherited weak alleles. However, unless the interacting alleles were common, the cancers due to inheritance of two such alleles would likely be seen as isolated cancer cases rather than showing the dominant, high penetrance segregation patterns characteristic of Lynch syndrome families. Furthermore, becasue two "second hits" would be required to inactivate the wild-type alleles in the resulting double heterozygotes, it is likely that cancer in these cases would show a later age of onset than seen in Lynch syndrome. The other mechanism is that in patients with an inherited weak allele, somatic second hits of the type seen here could occur in a different MMR gene resulting in strong MMR defects; this type of genetic interaction could be identified by sequencing appropriate target MMR genes in tumors from Lynch syndrome cases where an inherited weak allele has been implicated. In the case of mutations affecting Msh2 domains I and II, the *MSH6* gene would be the most likely target for enhancing mutations.

Materials and Methods

S. cerevisiae were grown on standard media, either yeast extract/peptone/dextrose (YPD), or complete supplement mixture (CSM) medium (US Biological) lacking specific amino acids to select for plasmid markers and/or Lys^+ and Thr^+ revertants; 60 mg/L canavanine was added to CSM–arg medium to select for canavanine resistance. All *S. cerevisiae* strains are isogenic derivatives of S288C. The wild-type strain RDKY 3590 (28) has the genotype *MATa ura3-52 leu2Δ1 trp1Δ63 hom3-10 lys2::InsE-A10* and was the parent for all strains containing the chromosomal mutations studied. The wild-type strain RDKY 3023 (39) has the genotype *MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 ade2Δ1 ade8 hom3-10 lys2-BgI* and was derived from the same parental strain background as RDKY 3590. The strains RDKY 2706 and RDKY 4136, which were used for all plasmid complementation tests, were derived from RDKY 3023 have an *msh2Δ::hisG* mutation or both *msh2Δ::hisG and msh6Δ::hisG* mutations, respectively. The plasmid the *ARS CEN HIS3 MSH2* plasmid pMSH2 and derivatives containing the *S. cerevisiae MSH2* alleles *T44M*, *Q63P*, *Y104C*, *N123S*, *G122S*, *I141M*, *D164H*, and *C195R* were kindly provided by Dr. Alison Gammie (Princeton University, Princeton, NJ); the matched vector, pRS413, was from our laboratory collection. pMSH2 derivatives containing the *MSH2* alleles *T33P*, *I34E*, *D49V*, *L99P*, *L102H*, *Y109C*, *V159D*, and *L183P* were made by site directed mutagenesis. The plasmids are listed in Table 1 and the strains are described in detail in Table 5. All of the methods used in the described studies including those for site directed mutagenesis, verification by DNA sequencing, transferring mutations to the relevant chromosomal locus, construction of strains by gene disruption or intercrossing, and evaluation of mutator phenotypes by patch tests or fluctuation rate analysis were standard methods or were exactly as previously described (28, 29, 40, 41).

Isolation of enhancer mutations was performed exactly as previously described (28). Briefly, a derivative of RDKY 3590 containing one of the indicated mutations or polymorphisms (*msh2-E194G*, *mlh1-I326A*, *msh2-G317D*, *msh6 -L301V*, *mlh1-E129H*) at the relevant chromosomal locus was mutagenized with Ethyl Ethane Sulphonate and 6,000–12,000 survivors were plated onto YPD plates. The resulting colonies were then replica plated onto CSM plates that either lacked lysine or threonine, or lacked arginine and also contained canavanine to identify mutator mutants. The initial mutants were subjected to two cycles of retesting and then each mutator mutant identified was transformed with an *ARS CEN* plasmid containing the wild-type gene that would complement the initial mutation present in the starting strain to identify those mutants whose mutator phenotype was fully complemented by this plasmid. For all complementable mutants, the starting mutant gene was sequenced to identify those mutants where the starting mutant gene only contained the starting mutation and had not acquired a loss of function mutation. Then, as relevant, the *MSH2*, *MSH3*, *MSH6*, *MLH1*, *PMS1*, *MLH3*, *EXO1* and *POL30* genes from each mutant were sequenced; this resulted in the identification of a second mutation in one of these genes in each starting mutant. Finally, each mutant was transformed with an *ARS CEN* plasmid containing the gene predicted to complement the second mutation to verify that the mutator phenotype of each mutant required both the starting mutation and the second mutation.

ACKNOWLEDGMENTS. The authors thank Drs. Jill M. Dowen, Richard Fishel, and Christopher D. Putnam for helpful discussions. Supported by National Institutes of Health Grant GM50006.

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