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Science; May 5, 1995; 268, 5211; Research Library Core
pg. 738

Mismatch Repair Deficiency in Phenotypically Normal Human Cells

Ramon Parsons, Guo-Min Li, Matthew Longley, Paul Modrich, Bo Liu, Theresa Berk, Stanley R. Hamilton, Kenneth W. Kinzler, Bert Vogelstein*

Tumor cells in patients with hereditary nonpolyposis colorectal cancer (HNPCC) are characterized by a genetic hypermutability caused by defects in DNA mismatch repair. A subset of HNPCC patients was found to have widespread mutations not only in their tumors, but also in their non-neoplastic cells. Although these patients had numerous mutations in all tissues examined, they had very few tumors. The hypermutability was associated with a profound defect in mismatch repair at the biochemical level. These results have implications for the relation between mutagenesis and carcinogenesis, and they suggest that mismatch repair deficiency is compatible with normal human development.

Hereditary nonpolyposis colorectal cancer can be caused by germline mutations of the mismatch repair (MMR) genes *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2* (*1*–*4*). The disease is inherited in an autosomal dominant fashion, and non-neoplastic cells of affected patients contain one mutant and one wild-type allele (*3*). These non-neoplastic cells are phenotypically normal and have been assumed to be MMR proficient, as they have shown no evidence of genetic instability or biochemically measurable MMR deficiency in previous studies (*1*, *5*). Indeed, the successful linkage of HNPCC to anonymous markers on chromosome 2p and chromosome 3p was dependent on the stable inheritance of microsatellite DNA (*6*). During tumor development, the wild-type copy of the allele inherited from the unaffected parent is lost or mutated (*3*, *7*). This event is thought to render the neoplastic cells completely MMR deficient, leading to a rapid accumulation of mutations and an accelerated rate of neoplastic progression (*3*, *8*).

We wondered whether the non-neoplastic cells of HNPCC patients might harbor a MMR defect. The stability of short, repeated sequences (microsatellites) provides an excellent indicator of repair proficiency, as these sequences are prone to misalignment during DNA replication (*9*, *10*). We developed a more sensitive strategy for analyzing microsatellite changes, reasoning that if a rare cell in a population harbored microsatellite alterations, the new microsatellite alleles would not be detectable amid the large background of normal alleles. To increase sensitivity, we diluted DNA samples so that the genomes of only one-half to three cells (0.5 to 3 cell equivalents) were used as templates for each of several polymerase chain reactions (PCRs) (*11*). Because any altered microsatellite allele would represent greater than 15% of the alleles in such samples, we could detect alterations present in a small fraction of cells.

We evaluated Epstein-Barr virus–transformed lymphoblasts cultured from HNPCC and control patients, initially examining two microsatellite markers: D2S123, consisting of a $(CA)_n$ repeat, and BAT25, consisting of an $(A)_n$ repeat (*11*). Two control individuals (not from HNPCC families) had no alterations in either microsatellite, nor did patients P1 (*hMSH2* mutation) or P3 (*hPMS1* mutation) (Table 1). However, patients P2 (*hPMS2* mutation) and P4 (*hMLH1* mutation) exhibited a number of alterations in both markers (Fig. 1 and Table 1). More than 20% of the diluted DNA samples from patient P2, for example, contained a novel microsatellite allele. A third, randomly chosen microsatellite marker (BAT40), consisting of an $(A)_n$ repeat, was evaluated, and this marker showed a similar number of alterations (Table 1). Because of their heterogeneity, these alterations were not detectable in undiluted DNA samples (*12*).

The hMLH1 and hPMS2 gene products form a heterodimeric complex (*1*, *13*). Although the microsatellite alterations were observed in lymphoblasts of patients with mutations in *hMLH1* or *hPMS2*, mutations in these genes were not always associated with such instability. For example, patient P5, who had a different mutation of *hMLH1* than did patient P4, did not have microsatellite alterations in lymphoblast DNA samples (Table 1). Nevertheless, the defect appeared to be genetically determined, as P6, a sibling of patient P2 sharing the same germline mutation of *hPMS2*, had a high level of variation (Table 1).

To determine whether uncultured cells from HNPCC patients contained similar alterations, we examined DNA from non-neoplastic colon tissue (*14*). The tissue was microdissected into two fractions, one composed predominantly of epithelial cells (mucosa) and the other of muscular and connective tissue cells (submucosa and muscularis propria). The two normal individuals and patient P1 had few or no microsatellite variants in either fraction, whereas patients P2, P4, and P6 had numerous alterations (Table 1). These alterations were considerably more prevalent in the epithelial fraction than in the nonepithelial fraction (for P2 and P6, probability $P < 0.005$ by χ^2). Numerous alterations were also observed in DNA from epithelial cells of the urinary tract of patient P6 (Table 1).

To quantitate the microsatellite instability at the cellular level, we examined individual clones of lymphoblasts (*15*). Twenty-four clones from patient P6 were compared with 18 clones from P7, a patient with familial adenomatous polyposis, a hereditary colorectal cancer syndrome not associated with MMR gene defects (*16*). The DNA of each clone was not diluted, so that only the predominant pattern in each clone was observed. This analysis revealed substantial alterations in the P6 clones for each

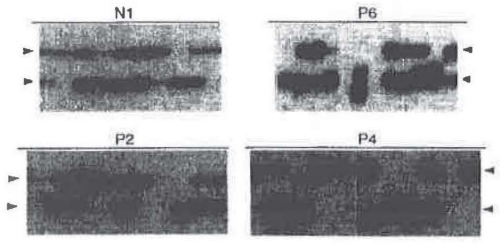

Fig. 1. PCR analysis of lymphoblasts derived from HNPCC patients. PCR reactions, each containing 0.5 to 3 cellular equivalents of DNA, were amplified with primers for the microsatellite marker D2S123. Arrowheads indicate the positions of the major PCR products from undiluted template DNAs. N1 is a non-HNPCC control patient; P2, P4, and P6 are HNPCC patients with germline mutations of either *hPMS2* or *hMLH1* (Table 1). Fragments with abnormal mobilities were present in the P2, P4, and P6 lanes but not in the N1 lanes. As expected from a Poisson distribution, some lanes contained zero or only one allele, giving rise to zero or one band, respectively, instead of the two that were always generated from undiluted DNA templates.

R. Parsons, B. Liu, K. W. Kinzler, B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD 21231, USA.
G.-M. Li, M. Longley, P. Modrich, Department of Biochemistry, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA.
T. Berk, Familial GI Cancer Registry, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.
S. R. Hamilton, Departments of Pathology and Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.
*To whom correspondence should be addressed.

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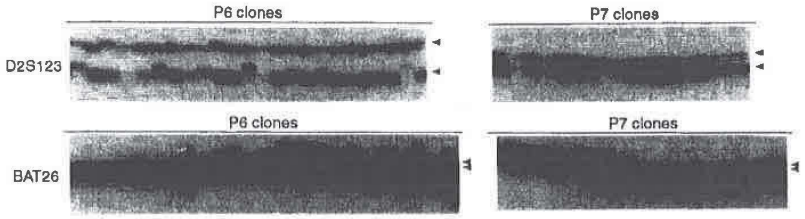

Fig. 2. Microsatellite alterations in individual clones of lymphoblasts. DNA (undiluted) from P6 or P7 clones was amplified with primers specific for microsatellite markers D2S123 and BAT26 (*10*, *14*). Arrowheads indicate the position of alleles derived from DNA of uncloned P6 and P7 cells.

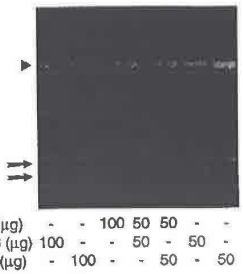

Fig. 3. Nuclear extracts of P6 are deficient in MMR. Nuclear extracts of the cell lines TK6, SO, and P6 were incubated with 24 fmol of a G-T mismatch substrate (arrowhead) containing a single-stranded nick 125 bp 5′ to the mispair (*17*). MMR restores a Hind III endonuclease recognition site. Repair was scored by cleavage with Hind III and Bsp 106. Arrows indicate repair products.

of the five markers tested. Markers BAT25, D2S123, BAT26, D18S58, and APΔ3 revealed alterations in 75, 38, 92, 67, and 54% of P6 clones, respectively (Fig. 2). No alterations were observed in P7 clones with four markers, and only one alteration with the fifth (BAT25). This comparison between clones P6 and P7 was statistically significant ($P < 0.005$ by χ^2).

To determine whether the observed instability was due to a defect in MMR, we measured MMR activity in extracts from the lymphoblasts. Two DNA substrates were used for these assays, one containing a single GT mispair and one containing a CA dinucleotide insertion in one strand (*17*). Extracts from H6 cells, a tumor cell line containing a homozygous mutation of *hMLH1* and no wild-type *hMLH1* gene (*4*, *5*), displayed no measurable MMR activity (Table 2 and Fig. 3). Extracts from SO cells, a colorectal cancer cell line without microsatellite instability (*5*), had MMR activity that was at least 20 times that of H6. Substantial MMR activity was also observed in extracts from three lymphoblastoid lines derived from HNPCC patients P8, P9, and P10, each with an *hMSH2* mutation not resulting in microsatellite alterations in non-neoplastic cells. Extracts from the well-studied non-HNPCC lymphoblastoid cell line TK6 (*18*) also had substantial activity in these assays. However, the three patients (P2, P4, and P6) with microsatellite instability in their non-neoplastic cells had little or no measurable activity in identically prepared extracts (Table 2 and Fig. 3).

The data described here document a profound MMR defect in the phenotypically normal cells of a subset of HNPCC patients. Our results suggest that normal human development is compatible with greatly reduced levels of MMR. Although we have not assessed embryonic cells directly, we assume that the same defect present in the adult cells was also present during embryogenesis.

One explanation for the MMR deficiency in these patients is that the wild-type allele co-inherited with the mutant MMR gene was lost or mutated somatically, as was observed in HNPCC tumors. However, this seems unlikely because no evidence of mutation or loss of the unaffected allele could be detected by sequencing the relevant complementary DNA or searching for truncated proteins (*12*). Alternatively, these patients might have inherited mutations of other genes that participate in MMR, with multiple germline mutations leading to a reduction of MMR activity. Yet no mutations were detected in the other known MMR genes with the same methods. Another explanation for the observed deficien-

Table 1. Microsatellite alterations in phenotypically normal tissues. For lymphoblastoid cells the differences between patients P2, P4, and P6 and the other individuals were significant (χ^2 test, $P < 0.005$, 0.005, and 0.025, respectively). Their colonic epithelial fraction was also significantly different from the epithelial fraction of the other tested individuals ($P < 0.01$ by χ^2). Epith., epithelium; Q, Gln; R, Arg; ter, termination codon; ND, not done.

Patient	Mutated gene	Mutation*	Tissue fraction	% of PCR reactions revealing an alteration in microsatellite†		
				BAT25	D2S123	BAT40
N1	None	—	Lymphoblastoid	0	0	0
			Colon epith.	0	ND	0
N2	None	—	Lymphoblastoid	0	0	0
			Colon epith.	4	ND	0
P1	*hMSH2*	Codons 265 to 314 deleted	Lymphoblastoid	0	0	ND
			Colon epith.	0	ND	0
			Colon nonepith.	0	ND	ND
P2	*hPMS2*	R134 ter	Lymphoblastoid	22	29	28
			Colon epith.	25	ND	50
			Colon nonepith.	6	ND	10
P3	*hPMS1*	Q233 ter	Lymphoblastoid	0	0	ND
P4	*hMLH1*	Codon 618 deleted	Lymphoblastoid	7	10	12
			Colon epith.	4	ND	22
			Colon nonepith.	0	ND	8
P5	*hMLH1*	Frame shift at codon 347	Lymphoblastoid	0	0	ND
P6	*hPMS2*	R134 ter	Lymphoblastoid	14	33	33
			Colon epith.	20	ND	53
			Colon nonepith.	6	ND	3
			Urinary tract epith.‡	20	ND	33

*Described in (*4*, *23*). †Because 0.5 to 3 cellular equivalents of DNA were used for each PCR, the fraction of cells with altered alleles can be estimated by dividing the values by a factor of 0.5 to 3. ‡Cells derived from urine sediment.

Table 2. Mismatch repair activity of nuclear extracts. The extracts were tested for MMR activity with 24 fmol of mismatched substrate (*18*).

Cell line	Repaired substrate (fmol)	
	3′ CA	5′ G-T
	HNPCC lymphoblastoid	
P8	4.9	8.6
P9	3.5	2.0
P10	2.3	3.5
P4	<0.3	<0.3
P2	<0.3	<0.3
P6	<0.3	<0.3
	Control lymphoblastoid	
TK6	7.6	8.6
	HNPCC colorectal cancer	
H6	<0.2	<0.2
	Sporadic colorectal cancer	
SO	11	5.8

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cy would entail a dominant negative effect of specific *hPMS2* and *hMLH1* mutations. The mutated proteins resulting from such mutations might be able to interact with hMSH2 bound to mismatched DNA but not be able to recruit the enzymes necessary for proper excision and repair. Although inhibition of repair was not seen in mixing experiments (Fig. 3), it is possible that the mutant proteins might have been sequestered in a preexisting protein complex.

Given their elevated rates of mutation, it was surprising that these patients did not have more colorectal cancers (CRCs). Patient P2 had no CRC at age 14, P6 had one CRC at age 12, and P4 had two CRCs at age 31. An exponential increase in CRC formation would be expected from standard models of tumorigenesis which assume that multiple rate-limiting mutations drive the process [reviewed in (*19*)]. One interpretation of these observations is that mutations per se may not be sufficient for a high rate of tumorigenesis. It has been argued that exogenous mutagens are carcinogenic not only because they induce mutations but also because they induce them in cells actively regenerating as a result of the extensive cellular damage associated with mutagenic compounds (*20*). Although cells in normal environments undergo apoptosis when damaged (*21*), cells in regenerating tissues may have their apoptotic pathways repressed, allowing cells with mutations in growth-controlling genes to expand clonally. The patients described here had a continuously high mutation rate, but perhaps their cells did not receive the tissue regenerative stimulus afforded by exposure to high concentrations of mutagens, and this may explain why they did not develop larger numbers of tumors.

These results also have potential clinical implications. Drugs designed to be lethal to MMR-deficient tumor cells would likely be toxic to the non-neoplastic cells of the MMR-deficient patients described here. Careful evaluation of the germline defects in HNPCC patients would therefore seem critical once such therapeutic agents are developed.

Finally, the results suggest a strategy for making any cell MMR-deficient by the transfer of genes encoding the mutant proteins found in patients P2 and P4. The expression of the altered gene products could be driven by cell type–specific promoters to create highly mutable cell types in transgenic organisms. This strategy might be useful for a variety of experimental purposes.

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11. DNA was diluted to 0.5 to 3 cellular equivalents per reaction and used in multiple parallel PCR reactions of 40 cycles. PCR was performed in 96-well plates with a ^{32}P-labeled primer (>10^8 cpm/μg) as described [J. Jen *et al.*, *N. Engl. J. Med.* **331**, 213 (1994)]. Samples were analyzed by electrophoresis in urea-formamide polyacrylamide gels, fixed in methanol-acetic acid (5% each) for 20 min, dried, and exposed to film. PCR markers included D2S123 (primers 5′-AAACAGGATGCCTGCCTTTA-3′ and 5′-GGACTTTCCACCTATGGGAC-3′) (*22*), D18S58 (*22*), APΔ3 (*9*), BAT25 (5′-TCGCCTCCAAGAATGTAAGT-3′ and 5′-TCTGCATTTTAACTATGGCTC-3′) (*12*), BAT26 (5′-TGACTACTTTTGACTTCAGCC-3′ and 5′-AACCATTCAACATTTTTAACCC-3′), and BAT40 (5′-ATTAACTTCCTACACCACAAC-3′ and 5′-GTAGAGCAAGACCACCTTG-3′). D2S123 and D18S58 contained dinucleotide repeats, and the other markers contained mononucleotide repeats.
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14. Formalin-fixed, paraffin-embedded sections were prepared and stained with hematoxolin and eosin. The epithelial and nonepithelial components were separated with the aid of a dissecting microscope. Pools of 10 sections of the epithelial fraction (mucosa) or the submucosal-muscularis propria fraction were incubated at 58°C in proteinase K (0.5 mg/ml), 1% SDS, 500 mM tris-HCl (pH 8.9), 20 mM EDTA, and 10 mM NaCl. Samples were then boiled for 10 min, extracted with phenol-chloroform, ethanol precipitated, and resuspended in 50 μl of 3 mM tris-HCl (pH 7.5) and 0.2 mM EDTA. Samples typically contained 1000 to 10,000 genome equivalents per microliter. Serial 10-fold dilutions of DNA were used to determine the DNA concentration that would allow amplification of one to six alleles per reaction.
15. The P6 and P7 clonal cells were cultured in 96-well plates in the presence of GM1899B feeder cells (5 × 10^4 per well) that had been irradiated previously with 40 Gy (absorbed dose of ionizing radiation). Clones were expanded in the absence of feeder cells before DNA was purified.
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17. Repair assays were performed with 50 μg of nuclear extract and 24 fmol of mispaired DNA at 37°C for 15 min. The 3′ CA substrate was a CA dinucleotide insertion heteroduplex containing a nick 181 base pairs (bp) 3′ to the mismatch (*5*). The 5′ G-T mispair contained a G T mismatch and a nick 125 bp 5′ to the mispair [S.-S. Su, R. S. Lahue, K. G. Au, P. Modrich, *J. Biol. Chem.* **263**, 6829 (1988)]. The repair of the substrate restored a restriction endonuclease site. The repair efficiency was measured by digestion of the reaction products with the appropriate restriction enzymes and resolving them on an agarose gel. None of the defective extracts inhibited a repair reaction containing a wild-type nuclear extract.
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24. We thank A. Knudson and A. de la Chapelle for helpful discussions and T. Gwiazda for preparation of the manuscript. Supported by the Clayton Fund, translational funds from the Duke Comprehensive Cancer Center, NIH grants GM45190, CA35494, CA62924, and CA09320, and American Cancer Society (ACS) grant PF-3940. B.V. is an ACS Research Professor.

3 February 1995; accepted 17 March 1995

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