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Genetic instability and methylation tolerance in colon cancer

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Summary. - Microsatellite instability was first identified in colon cancer and later shown to be due to mutations in genes responsible for correction of DNA mismatches. Several human mismatch correction genes that are homologous to those of yeast and bacteria have been identified and are mutated in families affected by the hereditary non-polyposis colorectal carcinoma (HNPCC) syndrome. Similar alterations have been also found in some sporadic colorectal cancers. The mismatch repair pathway corrects DNA replication errors and repair-defective colorectal carcinoma cell lines exhibit a generalized mutator phenotype. An additional consequence of mismatch repair defects is cellular resistance, or tolerance, to certain DNA damaging agents.

Key words: *hMSH2, hMLH1, GTBP, PMS1, PMS2*, HNPCC, mismatch repair, methylation tolerance.

Riassunto (*Instabilità genetica e tolleranza alla metilazione nel cancro del colon*). - Nel cancro del colon-retto è stata osservata l'instabilità genetica di una classe di sequenze ripetute del DNA, i microsatelliti; questo fenotipo è la conseguenza di mutazioni nei geni responsabili della correzione dei "mismatch" del DNA. E' stato dimostrato che il genoma umano contiene una serie di geni, omologhi a quelli dei batteri e del lievito, che hanno il compito di correggere i "mismatch" che si formano sul DNA. Mutazioni in questi geni sono state osservate nelle famiglie affette dalla sindrome ereditaria associata con una alta insorgenza di carcinomi del colon-retto di tipo non poliposico (HNPCC). Alterazioni genetiche negli stessi geni sono state identificate in alcuni carcinomi sporadici del colon-retto. La via di riparazione dei "mismatch" corregge gli errori che avvengono durante la replicazione del DNA e linee cellulari difettive in questa funzione sono caratterizzate da un fenotipo mutatore esteso a tutto il genoma. Le cellule difettive in questa via riparativa sono inoltre caratterizzate dalla resistenza, o tolleranza, agli effetti citotossici di alcune classi di agenti chimici che danneggiano il DNA.

Parole chiave: *hMSH2, hMLH1, GTBP, PMS1, PMS2*, riparazione dei mismatches, tolleranza alla metilazione.

Introduction

It has long been suggested that spontaneous errors in DNA replication play an important role in neoplastic transformation. Replication errors have also been invoked to explain the multiple genetic alterations seen in cancer cells [1]. An increase in mutation rate would lead to a higher probability of oncogene activation or of loss of tumor suppressor gene expression, thus conferring the selective growth advantage necessary for tumor progression. A defect in the control of DNA replication fidelity has long been hypothesized but, until recently, its identification has proven to be elusive. There is no evidence that increased mutation rates are generally associated with neoplastic transformation. Comparison of spontaneous mutation frequencies in normal primary cultures of rodent cells and in their chemically-transformed counterparts did not show significant differences [2]. Similarly the fidelity of DNA replication by extracts of a normal fibroblast strain was found to be similar to its Ha-*ras* transformed derivative [3]. The first evidence that a defect in DNA replication might be one of the causative factors in human neoplasia came from the observations that subtle alterations of the genome were present in repeated DNA sequences in tumors of the colon and rectum and not in DNA from normal cells of the same patient [4]. These alterations were then characterized as somatic deletions of up to 4 bases in monotonic runs of dA [5]. Similarly, differences between tumor and normal DNA were detected in 28% of colorectal carcinomas [6], showing deletions or insertions in a variety of simple repeated sequences such as di-, tri- and tetranucleotides (microsatellite DNA) [5, 7]. This microsatellite instability was significantly correlated with some of the characteristic of hereditary non polyposis colorectal carcinoma (HNPCC), such as the location of the tumor in the right side of the colon and the near-diploid karyotype of tumor cells. These findings suggested that a heritable defect that allowed the accumulation of replication errors at microsatellite sequences was associated with HNPCC tumors. By analogy to bacteria

and yeast, the mechanism responsible for this genomic instability was hypothesized to be a defect in the repair pathway responsible for the correction of DNA replication errors. Mutations in the *E. coli* mismatch recognition genes *mutS* and *mutL* lead not only to large increases in both classes of transitions (GC to AT and AT to GC) [8], but also to high frequencies of ±2 frameshifts in sequences comprising tandemly repeated CA or GT dinucleotides [9]. *Saccharomyces cerevisiae* mutants in the mismatch correction genes (*mlh1*, *pms1*, *msh2*) show destabilization of tracts of dinucleotide repeats of this type [10]. These results indicated that replication intermediates containing 1 or 2 extrahelical bases could be recognized and corrected *via* the same mismatch repair pathway that corrected mispaired bases. Cloning of the genes responsible for HNPCC (*hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*) demonstrated that they were indeed human homologs of the mismatch correction genes of *Escherichia coli* and yeast [11-15]. Mutations in these genes were found in tumors of patients with HNPCC as well as, in the heterozygous state, in HNPCC kindreds [11-23]. A list of the mutations in mismatch correction genes found in human tumors is shown in Table 1.

Parallel to the discovery of mutations in the mismatch correction genes of HNPCC colorectal cancers, cell lines established from some colorectal carcinomas were also shown to be unable to perform mismatch correction in an *in vitro* assay. This repair assay uses mismatch-containing restriction sites that are resistant to cleavage by restriction enzymes. When a mismatch is corrected to a complementary base pair by the cell extracts, subsequent cleavage with the restriction enzyme produces fragments which are diagnostic for repair [24-26]. This approach demonstrated that defective mismatch correction was not restricted to colorectal carcinoma cell lines [27] but also responsible for the microsatellite instability present in some endometrial cancer cell lines [17, 28].

The inability to correct DNA mismatches may lead not only to instability of microsatellite sequences [29] but also to a more generalized mutator phenotype. Human cell lines with defects in mismatch correction have greatly enhanced spontaneous mutation rates. Mutation rates at hypoxanthine guanine phosphoribosyl-transferase (*HPRT*) are 10 to 1000-fold higher than *HPRT* mutation rates in normal human fibroblasts or mismatch repair-competent colorectal carcinoma cell lines [30-32]. Some human tumors have a mutator phenotype in expressed genes. Accumulation of multiple mutations (up to 6 per gene) were found in the *APC* and *p53*, in an HNPCC patient with an inactivated *hMSH2* gene [33]. Furthermore frequent inactivation of the TGF β receptor by frameshift mutations was observed in colorectal carcinoma cells with microsatellite instability [34]. Inactivation of mismatch repair genes is thus a source of progressive accumulation of mutations which may lead to changes in critical transforming genes.

Biochemical analysis of mismatch correction proteins

Restoration of mismatch correction to defective human cell extracts by fractions of wild-type cell extracts allowed the purification of mismatch recognition proteins. Recognition of mismatches involves a heterodimer formed by hMSH2 and a protein of 160 kDa [35, 36]. The 160 kDa protein, the product of the *GTBP* gene, has been identified as another MutS homolog [36]. Two cell lines with mutations in the *GTBP* gene (MT1 and DLD-1) accumulate alterations in mononucleotides tracts while they are apparently stable at microsatellites formed by dinucleotides repeats [29, 30, 37]. A tumor xenograft mutated in the *hMSH2* gene shows instabilility at both mono and dinucleotides [37]. The ovarian carcinoma cell line 2774 mutated in *hMSH2* also displays dinucleotide repeat instability [38]. It seems likely therefore that the mammalian mismatch repair pathway is branched at the recognition step. The mismatch recognition complex of each subdivision recognizes a different set of mismatches. The GTBP protein, complexed with hMSH2, is selectively responsible for the recognition of one extrahelical base and of single base mismatches (Fig. 1). hMSH2, or a second complex also containing hMSH2, might recognize extrahelical dinucleotides (Fig. 1) [39, 40]. It is interesting that a third *MutS* homolog has been identified in mammalian cells, the *MRP1* gene, which is located in a head-to-head configuration with the *DHFR* gene and directed by a promoter with bidirectional activity [41]. *MRP1* is the human homolog of the yeast *MSH3* gene and yeast mutants in this gene display dinucleotide repeat instability with no concomitant general increase in mutation rates [40]. The product of the *MRP1* gene might then be the missing link in the first step of mismatch recognition. These data all seem to indicate that, in contrast to bacteria, multiple mismatch recognition proteins maintain the accuracy of human DNA replication.

A similar *in vitro* complementation approach allowed the identification of another protein complex which is required for human mismatch repair, the heterodimer hMutLα formed by hMLH1 and hPMS2 [42] (Fig. 1). This heterodimer appears to be the functional analog of the *E. coli* MutL protein [43] or the yeast MLH1: PMS1 complex [44] and is recruited at the site of the mismatch. Tumor cells containing

Table 1. - Mutations in mismatch correction genes in human tumors with microsatellite instability and HNPCC kindreds

Sample		Exon	Map site	Sequence	DNA change	mRNA change	Predicted protein change	Alleles affected	Reference
hMSH2									
CX2	T *(a)*	1-6	NI *(b)*	NI	deletion	no transcript	no protein	both	[21]
955 *(c)*	T	2	intron 2: +29bp	acttatttttttaagag *(d)*	-1 T	NI	NI	one	[61]
LoVo	T	4-8	NI	NI	deletion	no transcript	no protein	both	[21]
family 1	K	5	838-39	A CTC TTA TCA G	+T; new stop 7 bp downstream	loss of the exon with stop codon	exon 5 del.	one	[33]
NL-10	K	5	930	TT GGA CAG TTT	C to T		Gln to stop	one	[22]
MF, C, RB	K	5	intron 5: + 3bp	CAG gtaaaaaa	A to T	exon 5 del.		one	[16]
NL-21	K	6	1084-6	GGA CAA AGA CT	-AA		stop at site 1094	one	[22]
NL-7	K	7	1205-7	AA GAT TTA CTT	-T		stop at site 1300	one	[22]
8	K	7	1284	GT TAC CGA CTC	C to T		Arg to stop	one	[16]
NL-23	K	8	1353	AA CAC CAG AAA	C to T		Gln to stop	one	[22]
3106	K	8	1441	A ACT TTA GA	T to G		Leu to stop	one	[16]
1286 *(c)*		9	1486	A ACA TTA ATA	T to G		Leu to stop	one	[61]
	T	13	2240-41	A ATC ATA GAT	-TA				
HEC59	T	9	1510-13	AA TTA AGA GA	4 bp duplication		stop at site 1526	one	[17]
I-219	K	9	1511-2	AA TTA AGA GA	+A		stop at site1526	one	[22]
2774	K	9	1571	AC TTT CGT GTA ACC	G to C		Arg to Pro	one (e)	[38]
NL-13	K	10	1662	AA AAA GTC CTT	+G		stop at site1676	one	[22]
family 1	K	12	1869	TTA GCT CAG CTA	C to T		Gln to stop	one	[18]
J	K	12	1933	A CGA CCA GCC A	C to T		Pro to Leu	one	[16]
JV	K	12	1983	CC AGG CAT GCT	C to T	new splice site	stop at site 2090	one	[16]
JV/ Cx10	T	12	2055	AA CAG ATG TTC	A to TG		stop at site 2090	both	[12]
1314 *(c)*	T	12	2020	TG GGA GGT AAA	G to A		Gly to Ser	one	[61]
family 2	K	12	2053-4	AAA CAG ATG TT	-AG		stop at site 2090	one	[18]
A *(e)*	K	13	intron 12: -6 bp	ttttgtagGC CCC AA	T to C	NI	NI	one	[11]
	T								[62]
TM	K	13	NI	NI	NI	exon 13 del.	stop at site 2302	one	[16]
NL-203	K	14	2413-5	GCA ACC CAT TTT	-C		stop at site 2500	one	[22]
1186 *(c)*	T	14	2466	GTC TGT GAT CAA	T to A		Cys to stop	one	[61]
JG	K	15	intron 15: +1	GA GAG gtttg	G to A	exon 15 del.	stop at site 2708	one	[16]
DH	K	8-15	NI	NI	NI	exons 8-15 del.	stop at site 2708	one	[16]
hMLH1									
family 2	K	2	152	A AAA TCC ACA A	C to T		Ser to Phe	one	[13]
BR	T	2	205	CAG ATC CAA GAC	C to T		Gln to stop	both	[21]
H6 (HCT116)	T	9	775	C TAC TCA GTG A	C to A		Ser to stop	both	[14]
SNUH-H7	K	9	intron 9: +2	TCAACCgtaagtta	T to A	abnormal splicing	NI	one	[20]
SNUH-H4	K	11	1027-9	C CTG GGC TCC AA	-1 bp			one	[20]
RA	K	12-13	1120		- 371 bp		deletion	one	[14]
HNPCC family	K	13	1575	GGACATGAGGgtac	+T			one	[19]

Table 1 (continued)

Sample		Exon	Map site	Sequence	DNA change	mRNA change	Predicted protein change	Alleles affected	Reference
hMLH1									
SNUH-H2	K	14	1646	G GCA C**A**G CAT CA	A to T		Gln to Leu	one	[20]
SNUH-H1	K	15	1741	T GTT C**T**C AGG TT	T to C		Leu to Pro	one	[20]
595	T	15	1752	TTA TC**G**gtaagttt	G to A	exon 15 del	deletion	both	[21]
F2, F3, F6, F8, F10, F11,F59,	K	16	1752-1917	NI	NI	exon 16 del	in-frame deletion	one	[14]
JPN-1	K	16	1765	CA CCG **C**TC TTT G	C to G		Leu to Val	one	[20]
SNUH-H5	K	16	1777-79	C CTT **GCC** ATG CT	+1 bp			one	[20]
KRH-H1	K	16	1777-79	C CTT **GCC** ATG CT	+1 bp			one	[20]
family 14	K	16	1873-5	G AAG **AAG** GCT G	-AAG		- Lys	one	[23]
US-6	K	16	1874	G AAG A**A**G GCT G	A to C		Lys to Thr	one	[20]
L7	K	19	2199-2202	TC**A CAC** ATT C	-ACAC			one	[14]
L2516	K	19	2289	AGGTGT TAA	+TTGT		extension of COOH-terminus	one	[14]
SW48	T		NI	NI	NI	no trascript	no protein	both	[21]
VACO5	T		NI	NI	NI	no trascript	no protein	both	[21]
VACO6	T		NI	NI	NI	no trascript	no protein	both	[21]
hPMS1									
C.W.	K		777	GAA TCT **C**AG ATT	C to T	loss of the exon containing a stop codon	exon deletion (codons 195-233)	one	[15]
hPMS2									
family 12	K		424	GGA ACT **C**GA CTG	C to T		Arg to stop	one	[23]
HEC-1-A	T		2428	CTT TCC **C**GA GTC	C to T		Arg to stop	both	[28]
G.C.	K		826-2056		-1230 bp		in-frame deletion del. codons 301-381	one	[15]
G.C.	T		NI					both	
Polymerase δ									
587X *(f)*	T		1557	GGG ACC **G**AC CAG	G to A		Asp to Asn	one	[63]
DLD-1	T		1570	CC CGC C**G**C CGC CT	G to A		Arg to His	one	[63]
HCT15									
GTBP									
DLD-1	T		709	GA AGG **C**TG AAC	-C		Leu to stop	both	[37]
			3381-90	**-TTGATAGAGT**	+TTTGT		stop 9bp downstream		
543X	T		1916-21	**AAA AAA** TGC CT	+A			both	[37]
MT1 *(g)*	T		3511	T GTG G**A**T GAA T	A to T		Asp to Val	both	[37]
			3651	AA AAT **G**TT GCT	G to A		Val to Ile		

(a) T: tumor; K: kindred; *(b)* NI: not identified; *(c)* somatic mutations found in colorectal carcinomas; *(d)* the mutation was heterozygous in the normal tissue of the patient; in the tumor (an ovarian serous cystadenocarinoma) the normal allele was lost; *(e)* polymorphism of the general population, possibly related to cancer proneness in colon carcinoma and ulcerative colitis patients; *(f)* mutation found both in tumor and in normal tissue; *(g)* two different mutations in the two alleles

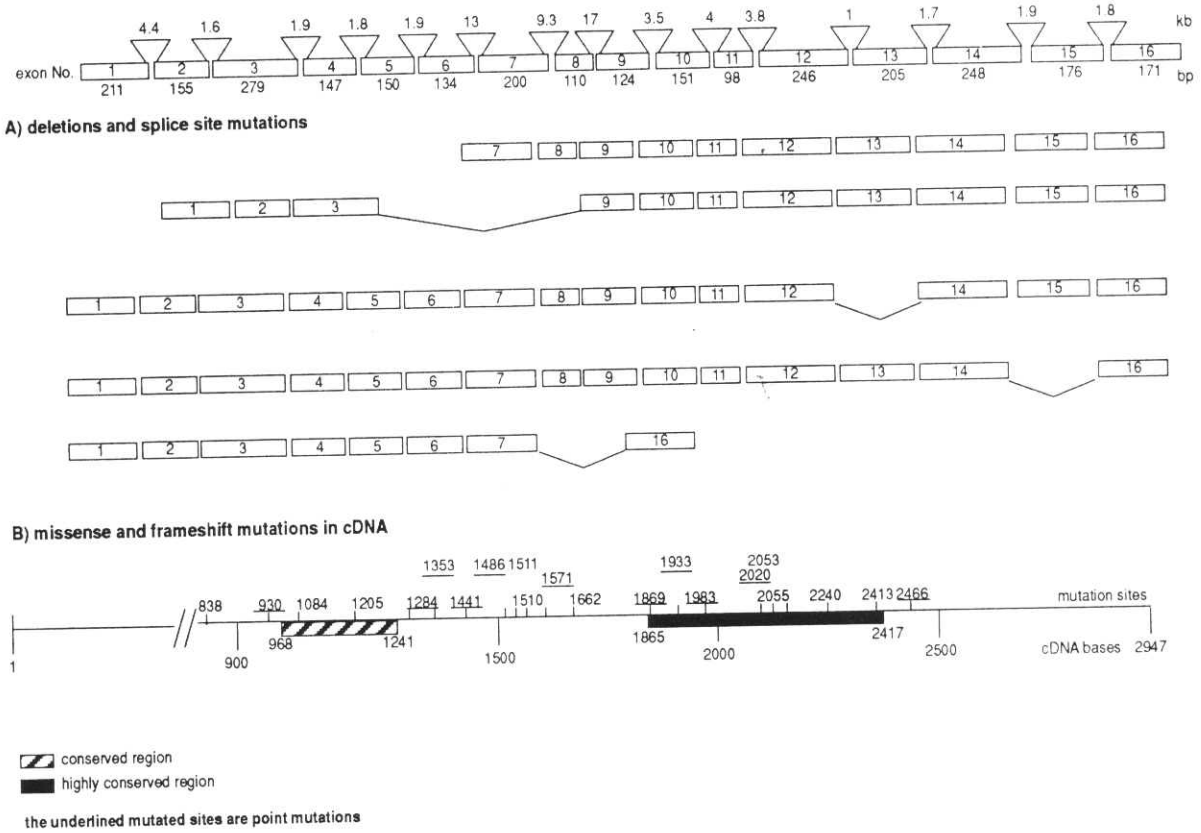

Fig. 1. - Distribution of mutations along the *hMSH2* gene.

mutations in either component of the hMutLa heterodimer show instability of both types of microsatellite repeats as well as high mutation rates at expressed genes. *hPMS1*, another yeast *pms*1 homolog, which is mutated in an HNPCC kindred, has yet to be assigned a biochemical function. The proteins involved in strand discrimination, incision and removal of the mismatched DNA, synthesis and ligation of the repaired DNA tract (Fig. 1) have still to be identified.

All these mismatch correction proteins are strongly conserved from bacteria to man. In Fig. 2 is shown the genomic structure of the *hMSH2* gene and the positions where mutations in human tumors or HNPCC kindreds occur. Since the mismatch correction proteins act in a multi-protein complex, analysis of the mutational spectrum might be informative on which domains of the protein are important in the interaction with the other components of the complex. It is clear from this type of analysis that: a) no mutations were found in the first 4 exons of the gene; b) mutations seem to be randomly scattered along the whole *hMSH2* sequence from exon 4 to exon 15; c) no clusterering of mutations is observed in the conserved domains of the protein. These data seem to suggest that all parts of the human protein are of importance to exert its cellular function. However, this information is derived from mutation analysis by the truncated protein assay. This assay is based on the *in vitro* translation of the mRNA of the putative mutant gene followed by analysis of the protein products by gel eletrophoresis. This method identifies truncated proteins derived from deletions, frameshift mutations or base substitutions producing stop codons but not missense mutations which result in a full length mutant protein. In contrast, analysis of mutations by amplification of single exons using intron-based primers [18] can identify every possible type of base substitution. Inferences of possible exposures to chemical carcinogens responsible for the inactivation of the mismatch correction genes as well as the identification of important regions of the protein await the compilation of a data base of mutations by this method.

Consequences of loss of mismatch correction

A characteristic of mismatch correction defective cells is tolerance to methylation damage. Exposure to methylating carcinogens introduces the potent mutagenic and cytotoxic base O^6-methylguanine (O^6-megua) into DNA. This methylated base, if not repaired by the

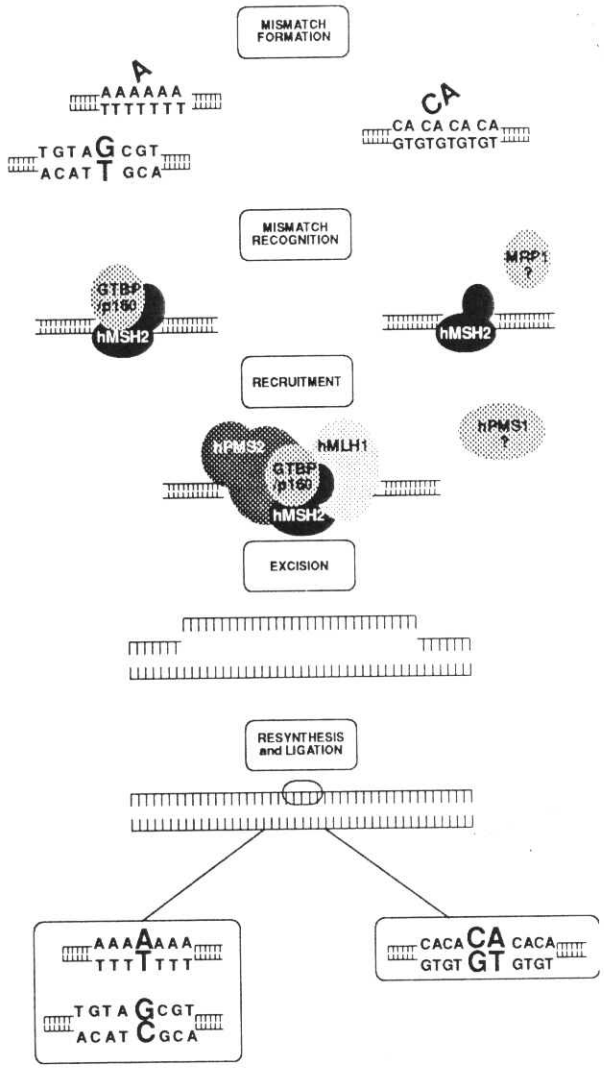

Fig. 2. - Mismatch repair pathway in human cells.

specific enzyme O^6-megua-DNA methyltransferase (MGMT), is able to pair with thymine during DNA replication and this property is responsible for the mutations (almost exclusively GC-> AT transitions) induced by methylating agents. However, O^6-megua either paired with C or T has thermodynamic properties similar to a DNA mismatch [45]. O^6-megua containing base pairs and DNA mismatches can disrupt DNA: protein interactions such as SV40 T antigen binding and unwinding of the viral replication origin [46, 47] or restriction endonuclease recognition and cleavage of specific sequences [48]. The hypothesis has been made that the proteins of the mismatch correction pathway may recognize and attempt to correct O^6-megua base pairs [49]. Attempts of correction might occur at the strand containing the cytosine or the thymine and repeated cycles of excision and resynthesis are produced because no perfect match is available for O^6-megua. These DNA strand interruptions may lead then to cell death [50] (Fig. 3). According to this hypothesis, variants defective in mismatch correction may display resistance to the lethal effects of DNA O^6-megua. This hypothesis has been validated and some mammalian cell lines resistant to the methylating agents N-methyl-N-nitroso-guanidine (MNNG) or N-methylnitrosurea (MNU) are defective in mismatch recognition and/or repair [51-53]. Clone B, a methylation tolerant cell line derived from CHO cells, has a moderate mutator phenotype, unstable dinucleotide repeats and is unable to bind to DNA mismatches or extrahelical dinucleotides [54]. The mutational spectrum at the *APRT* gene indicates that these cells accumulate -2 frameshifts at dinucleotide repeat sequences [55]. A second tolerant cell line, F12 derived from human Raji cells, also displays a 3-fold increase in mutation rate at the *HPRT* gene, is unable to bind a G:T mismatch and to correct a single base mismatch in an *in vitro* assay [53]. The F12 cell line has a wild-type *hMSH2* gene and display instability only at microsatellites formed by mono- nucleotides repeats (R. Hampson, G.A., M.B. and P. Karran, manuscript in preparation). Clone B and F12 might be representative of the two different modes of mismatch recognition (see Fig. 1). The tolerant phenotype is also present in colorectal carcinoma cell lines with defined defects in mismatch correction. SW48, a colorectal carcinoma cell line with a deletion in the *hMLH1* gene is highly resistant to MNU although devoid of MGMT activity [32]. Other examples of MNNG-tolerant cell lines with defective mismatch correction possibly include the *hMLH1*· HCT116 colorectal carcinoma [56] and an endometrial cell line with a mutation in the *PMS2* gene, [28]. Furthermore mice ES cells in which both copies of the *hMSH2* gene have been inactivated display the methylation tolerant phenotype [57].

It is interesting to note that cells tolerant to the lethality of methylation damage arise after multiple exposures to methylating carcinogens [58]. Among these variants some display the mutator phenotype characteristic of mismatch correction variants. It is possible that methylating agents exert a selective pressure such that mismatch correction variants are isolated due to their accompanying resistance to the cytotoxicity of these chemicals [59].

The existence of the methylation tolerant phenotype in mismatch correction defective tumors has important clinical implications. Several methylating agents, such as procarbazine, dacarbazine and triazines, are used in cancer chemotherapy. Their toxicity is based on the formation of O^6-megua in DNA and they will therefore be ineffective in the treatment of this class of tumors. More importantly there is increasing evidence that cells with a tolerant phenotype or with mismatch correction defects are sensitive to the chemotherapeutic

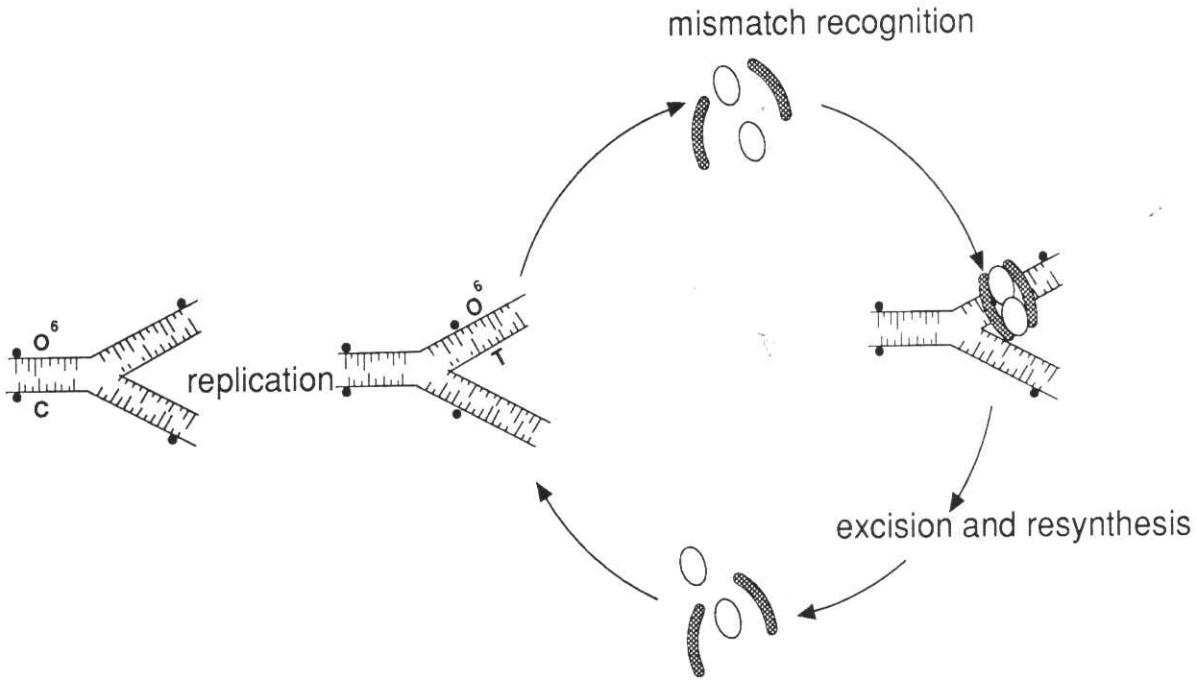

Fig. 3. - Model for the contribution of mismatch correction to the cytotoxicity of DNA methylation damage. Futile T insertion/removal cycle creates long-lived strand interruptions that are postulated to lead to cell death.

chloroethylnitrosoureas ([60] and G. Aquilina and M. Bignami, unpublished data). This indicates that a subset of colon cancer may be particularly responsive to this type of chemotherapy and screening for microsatellite instability may thus identify potentially curable tumors.

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