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Genetics

APC mutations in colorectal tumors with mismatch repair deficiency

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ABSTRACT **We have investigated the influence of genetic instability [replication error (RER) phenotype] on *APC* (adenomatous polyposis coli), a gene thought to initiate colorectal tumorigenesis. The prevalence of *APC* mutations was similar in RER and non-RER tumors, indicating that both tumor types share this step in neoplastic transformation. However, in a total of 101 sequenced mutations, we noted a substantial excess of *APC* frameshift mutations in the RER cases (70% in RER tumors versus 47% in non-RER tumors, $P < 0.04$). These frameshifts were characteristic of mutations arising in cells deficient in DNA mismatch repair, with a predilection for mononucleotide repeats in the RER tumors ($P < 0.0002$), particularly $(A)_n$ tracts ($P < 0.00007$). These findings suggest that the genetic instability that is reflected by the RER phenotype precedes, and is responsible for, *APC* mutation in RER large bowel tumors and have important implications for understanding the very earliest stages of neoplasia in patients with tumors deficient in mismatch repair.**

Instability at simple repetitive DNA sequences has been observed in a subset of sporadic colorectal cancers (1, 2) and in essentially all tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) (3–5). Deficiency in binding and repair of DNA mismatches has been demonstrated in tumor cell lines exhibiting such instability (6–10), and studies show an increased spontaneous mutation rate in selectable genes (8, 11–13). In most HNPCC tumors and in many of the sporadic cases, the underlying basis of this mutator or RER (replication error) phenotype is mutational inactivation of a human mismatch repair gene, five of which have so far been described (10, 14–18). With the exception of GTBP/p160 (19), germ-line mutations have been identified in each of these genes in HNPCC families (5, 15–18, 20, 21). However, mismatch repair gene mutations can only be detected in about half of sporadic colorectal tumors that exhibit an RER phenotype (19, 22, 23), suggesting that other genes may also contribute to genetic instability in the nonfamilial cases.

A substantial proportion of RER cell lines have inactivating mutations in the type β transforming growth factor (TGF-β) receptor II (24, 25), but there are little other data on the role of defective mismatch repair (MMR) in the generation of mutations in genes that are specifically implicated in tumorigenesis. In this study, we sought to investigate the influence of genetic instability on the frequency and the spectrum of *APC* (adenomatous polyposis coli) gene mutations in colorectal tumors. Mutational inactivation of the *APC* gene appears to initiate colorectal tumorigenesis, not only in mice and humans with germ-line alterations of *APC*, but also in patient populations with sporadic colorectal neoplasia. Hence it is of considerable interest to determine the nature of processes that may be responsible for the development of such mutations. Here, we show a striking relationship in colorectal tumors between the *APC* mutational spectrum and the presence of genetic instability manifest as the RER phenotype. Our findings provide insight into mechanisms by which defective DNA mismatch repair leads to the development of cancer in humans and demonstrate that such mismatch repair deficiency has important influence from the very earliest stages of tumorigenesis in the colorectal epithelium.

MATERIALS AND METHODS

Collection of Clinical Material and Template Preparation. All patients had undergone resection of histologically verified colorectal adenomas or carcinomas. Familial adenomatous polyposis cases were excluded. The following criteria had to be fulfilled for assignment as an HNPCC case: three first-degree relatives affected by pathologically confirmed colorectal cancer, one affected case arising under 50 years of age, one affected case to be the first-degree relative of the other two, and two or more affected generations (26).

Fresh tumor samples were from patients with colorectal neoplasms undergoing surgery in Edinburgh Royal Infirmary or The Johns Hopkins Hospital. Cell lines were derived from colorectal cancers as described (27) or from American Type Culture Collection and were passaged either in *nude* mice or *in vitro*. DNA and RNA were purified from peripheral blood leukocytes, fresh tumor tissue, adjacent normal colonic mucosa, cell lines, and xenografts by standard protocols. For RER and *APC* mutation analysis of primary tumors, constitutional RNA and DNA were compared with that prepared from fresh tumor tissue taken from cryostat sections (28) or immediately adjacent to regions identified on hematoxylin/eosin staining as containing >60% neoplastic tissue.

RER Analysis. RER analysis was performed by using four or more microsatellite PCR markers from the following set: D2S123, D2S119, D3S1293, D8S282, D13S160, D18S58, TGF-β receptor II, BAT26, BAT40. Primer sequences and PCR conditions have been reported elsewhere (25, 29, 30). To be categorized as RER, tumors were required to demonstrate variations compared with control tissue in at least half of the markers tested. Following electrophoresis on denaturing gels containing formamide, alleles were visualized either by silver-staining (31), autoradiography of radiolabeled products, or by analysis of fluorescent labeled products using a Pharmacia

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Abbreviations: RER, replication error; MMR, mismatch repair; IVSP, *in vitro* synthesized protein; HNPCC, hereditary nonpolyposis colorectal cancer; TGF-β, type β transforming growth factor.

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automated laser fluorescence (ALF) automated sequencer and the manufacturer's software.

***APC* Mutation Screening Strategy.** Screening for *APC* gene mutations was performed by an *in vitro* synthesized protein assay (IVSP) which allows detection of truncated surrogate APC protein products as described (32–34). In brief, IVSP involved PCR amplification of 100–200 ng of DNA or cDNA by using PCR primers to introduce a 17-bp consensus T7 promoter sequence and a mammalian translation initiation sequence in frame with unique *APC* sequence. PCR primer-pair sequences and PCR conditions are available on request and are similar to those previously described (32). For exons 1–14 (segment 1), cDNA was produced by reverse transcription of tumor RNA followed by PCR amplification (32, 33). Exon 15 was amplified in four overlapping fragments (segments 2–5) from genomic DNA templates. Resultant PCR products were used in a coupled transcription-translation reaction (Promega) incorporating 5–10 mCi (1 Ci = 37 GBq) of ^{35}S-labeled methionine according to the manufacturers instructions. Labeled protein products from the reaction were heat denatured and analyzed by 8%, 10%, and 12% SDS/PAGE gels. Gels were washed in fixative and autoradiography was performed at room temperature overnight (32). All samples showing truncated protein products were independently reamplified and a further IVSP analysis was performed for confirmation.

In 28 cell lines, truncating mutations were identified by using Western blots instead of IVSP assays, performed with monoclonal antibody FE9, as described (35). In 20 of these lines, the presence or absence of truncated *APC* had been reported previously (35).

DNA or cDNA from tumors with reproducible truncations were subjected to independent amplification. PCR fragments were purified and sequenced by using Sequenase 2 (United States Biochemical) with ^{35}S-labeled dATP or SequiTherm polymerase (Epicentre Technologies, Madison, WI) with ^{32}P-labeled primers. Termination of the reactions was followed by electrophoresis in 6% acrylamide under denaturing conditions. Dried sequencing gels were exposed to XAR (Kodak) film overnight.

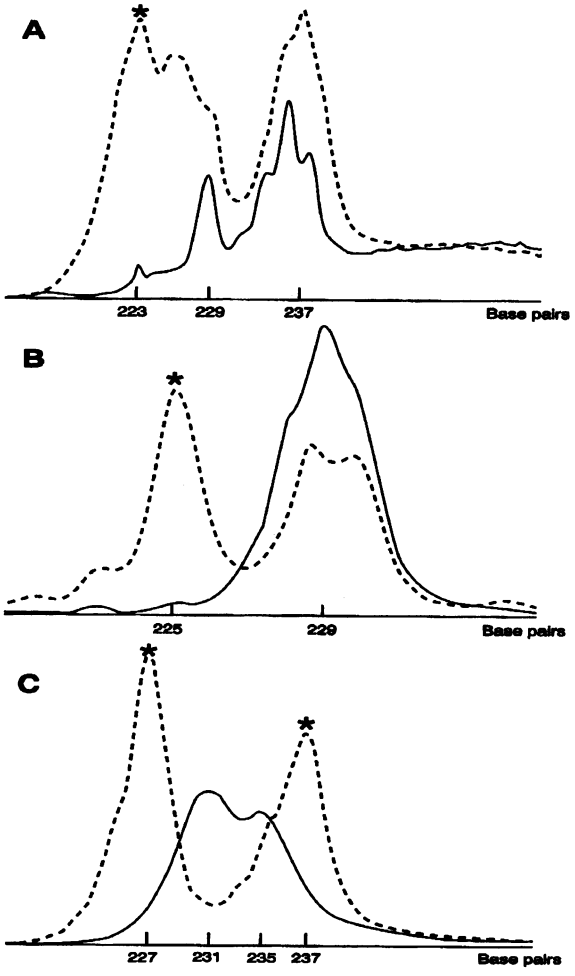

FIG. 1. Microsatellite instability. Representative PCR amplified products from the Pharmacia ALF automated sequencer using fluorescent-labeled primers for D13S160 in three separate tumors with normal DNA from the same patient for comparison. Allele sizes are given along the *x* axis. Tumor marker patterns are represented by broken lines and normal, constitutional PCR products from the same patient by continuous lines. Each of these tumors exhibits microsatellite instability and shows novel, tumor-specific alleles (indicated by *). Contractions of one allele are shown in tumors from patients A and B, while in the tumor from patient C there has been both expansion and contraction at the D13S160 locus.

RESULTS

Prevalence of Truncating *APC* Mutations in RER Tumors. To allow meaningful assessment of the prevalence of *APC* mutations in RER tumors, we analyzed a sufficient number of primary tumors and cell lines to identify a relatively large number of RER cases. In all, 52 RER tumors were identified for mutation analysis of the *APC* gene by combining 20 tumors from a new prospective cohort with 32 tumors identified in our previous studies of genetic instability (3, 5, 6, 22, 25). Nineteen of these 52 tumors were derived from patients who were members of different HNPCC kindreds, and the remainder were not associated with any known family history of colorectal cancer. Sixty-three non-RER tumors were selected randomly from the 412 sporadic tumors analyzed to acquire the 52 RER cases. The fraction of RER tumors among the total sporadic cases examined (15.8%) was similar to that reported in previous studies (1–3, 36, 37). Examples of the microsatellite assays employed for determination of RER status are shown in Fig. 1.

Screening for truncating *APC* mutations was performed on the 115 tumors in the combined RER groups by using either IVSP assays (32–34) or Western blots (35) as described. Both assays are highly reliable indicators of *APC* mutation status, as virtually all pathogenic *APC* mutations result in truncated proteins (38). Examples of positive IVSP assays are shown in Fig. 2. The prevalence of truncated *APC* gene product detectable by our assays was somewhat lower in the RER tumors (29

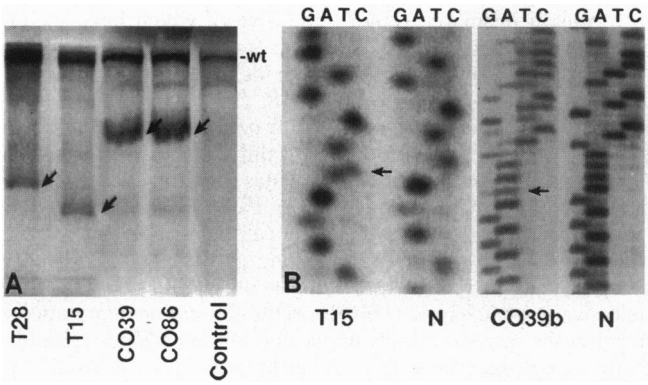

FIG. 2. *APC* mutation analysis. IVSP data for *APC* gene segment encompassing codons 1099–1693 are shown in *A* for non-RER tumors T28 and T15 and for RER tumors CO39 and CO86 with a control reaction using lymphocyte DNA from a healthy donor as template. Arrows show truncated protein products, and the sequence alterations responsible for each of these are listed in Tables 1 and 2. Example sequence analysis (*B*) of purified PCR products amplified from tumors T15 and CO39 with constitutional genomic DNA from the same patient as control in each case.

of 52; 56%) compared with non-RER tumors (47 of 63; 75%), but this difference was not statistically significant ($0.10 > P > 0.05$). There was no difference in the *APC* mutation prevalence between primary RER tumors (19 of 34; 56%) and RER cell lines (10 of 18; 56%). In addition, *APC* mutation prevalence was similar whether the RER tumor was derived from HNPCC patients (10 of 19; 53%) or from patients without a strong family history of colon or rectal cancer (19 of 33; 58%).

Comparison of *APC* Mutations in RER and non-RER Tumors. For comparison of the *APC* mutation spectrum in RER tumors with that in non-RER cases, *APC* sequence data were collated from two sources: from sequence analysis of tumors shown to have *APC* truncations in the IVSP or Western blot assays described above, and from sequence data obtained from tumors studied previously in our laboratories (39–42), for which we determined the RER status as part of the present study. These two sources provided a total of 101 *APC* mutations, 37 from RER tumors and 64 from non-RER lesions, sufficient to determine whether the RER phenotype had any influence on the *APC* mutational spectrum in colorectal tumors. The sequence alterations and target sequences for each of these 101 mutations are shown in Table 1 for RER tumors and in Table 2 for non-RER tumors. Mutations that have previously been reported are indicated in each table.

Of the whole set of 101 *APC* mutations, 51 were newly characterized from tumors shown to have an *APC* truncation in the assays described above. Sequencing was performed on PCR products amplified from genomic DNA or cDNA templates, and representative results are shown in Fig. 2, along with corresponding protein truncation data. In all, there were 27 mutations from 22 RER tumors and 24 mutations from 23 non-RER tumors. All 29 RER tumors with truncations were analyzed in detail but precise determination of the underlying mutation was not possible in 7 cases because of insufficient high quality nucleic acids or because the mutation could not be identified despite extensive sequencing. We selected 25 non-RER cases with *APC* truncations, largely on the basis of nucleic acid availability, and determined the sequence alteration responsible for the truncation noted on IVSP assay or on Western blot in all but 2 of these cases. These *APC* sequence data from the two RER tumor groups were combined with a total of 50 mutations identified in 36 tumors that have previously been reported from our laboratories (39–42). The RER status of these tumors had not been determined and so these were systematically assessed using microsatellite markers as described in *Materials and Methods* and on exactly the same basis as the assignment of the RER status to the tumor set assessed in the protein truncation assays. Ten *APC* mutations

Table 1. *APC* mutations and target sequence in RER tumors

Tumor ID	Family history	*APC* change, nt	Codon	Target sequence
		Frameshift mutations		
Mutation target in mononucleotide repeat				
Ca6#	S	1 bp del	758	AAC**a**AAAAGCC
Ca18*#	S	2 bp del	773	GAAAC**tt**TTGAC
Ca8#	S	1 bp del	801	TATG**t**TTTTGAC
Cx7*	H	1 bp ins	847	TCTG**(A)**AAAAAGAT
K10	H	1 bp del	907	TCT**g**GGTCT
CO86*	H	1 bp ins	941	TCGG**(A)**AAAATTCA
CO39*	NK	1 bp del	975	GGT**a**AAAGAGGT
Ca18*	S	1 bp ins	1307	GCAG**a**AAATA
T19	NK	2 bp del	1354	GAA**tt**TTCTT
S175	S	1 bp del	1429	GGAC**a**AACC
C081, TSh40	S,S	1 bp del	1437	AGT**a**AAACACC
K40	H	1 bp ins	1454	CCT**(A)**AAAAATAAA
Ca10*#	S	1 bp del	1454	CCT**a**AAAATAAA
18*, Ca10*#	S,S	2 bp del	1461	GCTG**aa**AGAG
22	H	1 bp ins	1554	GCAG**(A)**AAAAAACTAT
18*	S	1 bp del	1554	GCAG**a**AAAAACTAT
CO88	S	1 bp del	1818	TTTG**a**AAAACG
Mutation target in dinucleotide repeat				
CO39*, CO86*	NK,H	2 bp del	1462	A**ag**AGAGAGAGTGG
Mutation target in nonrepeat sequence				
K39	H	2 bp del	799	GAT**ta**TGTT
Ca20#	S	1 bp del	1405	TCC**g**TTCAG
CO10*	NK	1 bp ins	1431	CAAACC**(A)**ATGCC
T8	S	1 bp ins	1500	GGA**(A)**TTTTCT
Cx10	H	1 bp ins	1935	TTTCCCC**(A)**AGTCA
		Transition mutations		
Ca12*#	S	C → T	232	CpG
Ca1#, Ca12*#	S,S	C → T	564	CpG
T27	H	C → T	876	CpG
CO10*	NK	C → T	1114	CpG
T533	H	C → T	1338	CpG
Mx14	S	C → T	1367	CpA
CO20	S	C → T	1444	CpA
K1, Cx7*	H, H	C → T	1450	CpG
		Transversion mutations		
CO30	S	G → T	1499	GpG

APC mutation data for RER tumors including sequence alteration and mutational target. Insertion (ins) mutations are underlined and the inserted sequence is shown in uppercase lettering in parentheses. The sequence removed by deletion (del) mutations is underlined. Tumors marked with * indicate those cases in which more than one mutation was identified. Mutations identified by our laboratories that have previously been reported in the literature (39–41) are indicated by #. A family history indicated by H or S denotes HNPCC or sporadic cases, respectively, as defined in the text, and NK was entered where the family history was incomplete.

proved to be from RER tumors and the remaining 40 from non-RER cases.

A comparison of the nature and location of the whole set of 101 *APC* mutations in RER and non-RER tumors is presented in Table 3. There were a number of striking differences in the mutation spectrum between the two RER tumor categories. A substantial excess of frameshift mutations in the RER tumors was observed: 70% of the mutations in RER tumors were insertions or deletions, while only 47% of the mutations in the non-RER tumors were of this type ($P < 0.04$, $\chi^2 = 4.3$; Table 3). The excess of frameshift mutations was observed in both HNPCC (8 of 12; 75%) and sporadic tumors (14 of 20; 70%) exhibiting the RER phenotype.

Most of the insertions or deletions in the RER tumors were within simple repeated sequences (Table 1 and 3). Twenty-one of 26 (81%) frameshift mutations in RER tumors occurred within such microsatellites, compared with only 11 of 30 (37%) in non-RER tumors ($P < 0.003$, $\chi^2 = 9.33$). Mutations within $(A)_n$ tracts were especially prominent in the RER tumors, accounting for 49% of all *APC* mutations detected in RER tumors, while only

Table 2. *APC* mutations and target sequence in non-RER tumors

Tumor ID	*APC* change, nt	Codon	Target sequence
	Frameshift mutations		
Mutation target in mononucleotide repeat			
Ca16#	1 bp del	869	GaAAAT
S82#	1 bp del	1490	TTATTACAtTTTGCCA
UC5*#	1 bp del	1534	AATgGGAAT
Ad1*#, UC4*#, Ad4*#	1 bp ins	1554	GCAG(A)AAAAAACTAT
H-Ad4	2 bp del	1309	GaaAAG
H65	2 bp del	1420	AGccCCAGT
Ca14*#	2 bp del	1461	GCTGaaAAGAGA
Mutation target in dinucleotide repeat			
UC5*#	1 bp del	1464	AAGAGAGaGAGTGG
S80#	2 bp del	1328	TGTcaCAGCA
Ad3#	4 bp ins	1465	GAGAGAGAG(AGAG)TGG
Mutation target in nonrepeat sequence			
Ad2#	1 bp del	403	CGTgAAATC
H-Ad9	1 bp del	1317	GCTgAAG
H-Ad3	1 bp del	1407	CAGAGtGAA
H-Ad1	1 bp del	1416	AGTgGCATT
T10	1 bp del	1416	AGTGGcATTATA
Ca4*#	1 bp del	1443	CCTCCtCAA
T775	1 bp del	1489	TTAtTACATTT
T1	1 bp ins	1022	CTAG(T)ATA
AM13	1 bp ins	1318	GAA(A)GAT
Ad10#	1 bp ins	1373	GTCCA(C)CCTGA
R5#	1 bp ins	1376	CACT(A)ATGTT
S79#	1 bp ins	1578	GAAGAA(T)TGTATT
Ad9#	1 bp ins	1941	GACATA(A)CCAGAC
H-Ad2	2 bp del	1489	TTATtaCATTT
UC9#	4 bp del	1156	GAAGAAGaagaGAG
T34#	5 bp ins	289	GCC(CAGCC)AGT
UC11#	8 bp del	1489	TTATtacattttGCCA
Ca19#	17 bp del	1354–9	TGAattttcttcaggagcgaAATCT
	Transition mutations		
Ad4*#	G → A (splice)	141–177	GpG
Ca2#	A → G (splice)	278–311	ApT
T27#	C → T	332	CpG
Ad4*#	C → T	405	CpG
T9	C → T	805	CpG
S78, Ca4*#, Ad1*#	C → T	876	CpG
T12	C → T	1114	CpG
T201, T607	C → T	1338	CpA
Ad7#, T15, AM31	C → T	1367	CpA
H-Ad13	C → T	1378	CpA
T24	C → T	1406	CpA
Ad12#, UC8#, T7, Ca22*#, TSh16, Ca14*#, UC4*#	C → T	1450	CpG
5	C → T	1469	CpG
	Transversion mutations		
Ca21#	A → T	670	ApA
T32	A → T	1359	ApA
Ca22*#	C → A	906	TpC
Ad11#	C → A	1281	CpA
S83#	C → A	1344	CpA
Ca25#	C → A	1346	CpA
S77#	G → T	1020	GpA
S84#	G → T	1312	GpG
T28	G → T	1353	GpA
Ad15#	G → T	1408	GpA

APC mutation data for non-RER tumors with deleted (del) or inserted (ins) sequence shown in the same format as in Table 1. Tumors with more than one sequenced mutation are identified by an * and mutations previously reported from our laboratories (35, 39–41) by #. All but one (T10) of these non-RER tumors were from patients with no family history suggestive of HNPCC.

11% of the mutations in non-RER cases were of this type ($P < 0.00007$, $\chi^2 = 15.93$). The complete lack of deletion or insertion of more than 2 bp in RER tumors was also notable.

Point mutations were accordingly less frequent in the RER cases (30%) than in the non-RER cases (53%). In contrast to the frameshifts noted above, the targets for point mutation were largely similar in the two RER groups. Although there was a tendency for transitions rather than transversions in the RER tumors, this difference was not statistically significant (Table 3). All point mutations identified in RER tumors occurred at C:G base pairs and all but one resulted in C → T changes (Table1 and 3). In both the RER and non-RER cases, CpG dinucleotides seemed to be the favored mutation sites, being affected in 8 of 11 (73%) and 15 of 34 (44%) of the point mutations in RER and non-RER tumors, respectively (P = not significant) (Tables 1 and 2).

DISCUSSION

Two major conclusions can be drawn from this study. First, *APC* mutations occur in the majority of RER and of non-RER tumors, suggesting that both tumor types proceed along a similar pathogenetic pathway. Second, the *APC* mutational spectrum is substantially different in RER tumors from that in non-RER tumors. This observation provides additional evidence that genetic instability is not simply an epiphenomenon associated with tumorigenesis, but has a direct, causal role in colorectal neoplasia.

Many previous observations indicate that mutational inactivation of *APC* plays a central role in colorectal tumorigenesis. Gene carriers from familial adenomatous polyposis families and mice with heritable mutations of *APC* develop numerous colorectal and small intestinal tumors (38, 43–45). Truncation or complete absence of *APC* protein can be demonstrated in the great majority of colorectal tumors (38, 40, 42, 46–48). Such mutations can be detected in the smallest adenomas studied (40, 46–48) as well as in dysplastic aberrant crypt foci, which appear to be the very earliest histological manifestation of neoplasia (41, 49). In light of the differences in genetic instability and other features characteristic of RER compared with non-RER tumors, it was possible that the molecular pathogenesis of these two tumor types differed considerably. Indeed, TGF-β receptor II mutations have been identified exclusively in RER cases (24, 25). However, the data presented here demonstrate that a critical and probably initiating step in colorectal neoplasia, namely *APC* mutation, is common to both tumor types. Data from other studies indicate that *ras* and *p53* mutations also occur in both tumor types (1, 3, 50), although larger studies will be needed to better quantify the relative prevalence and spectra of these alterations. The clinical and biological differences that exist between RER and non-RER tumors may result from genetic alterations that occur exclusively in RER cases, such as those demonstrated in the TGF-β receptor (24, 25), although host factors are also likely to play a part.

Table 3. Summary of *APC* mutations in RER and non-RER tumors

	RER tumors	Non-RER tumors
Total no. of mutations	37	64
Point mutations	11 (30%)	34 (53%)
Transitions		
C:G → T:A	10	22
A:T → G:C	0	2
Transversions		
C:G → A:T	0	4
A:T → T:A	0	2
G:C → T:A	1	4
Frameshift mutations	26 (70%)	30 (47%)*
Deletion		
1 bp	11	11
2 bp	7	5
4 bp	0	1
8 bp	0	1
17 bp	0	1
Insertion		
1 bp	8	9
4 bp	0	1
5 bp	0	1
Frameshifts at each target repeat		
Mono-	19 (51%)	9 (14%)†
(A)n	18 (49%)	7 (11%)‡
Mono- or di-	21 (57%)	12 (19%)§
Tri-	0	1
Nonrepeat	5 (14%)	18 (28%)

Summary data on the 101 APC gene mutations characterized by sequencing, comparing the mutational spectrum and target sequences in RER tumors with those in non-RER tumors.
*$P < 0.04$; †$P < 0.0002$; ‡$P < 0.00007$; §$P < 0.0003$.

Genome-wide mutations at $(A)_n$ tracts are common in colorectal cancers exhibiting a mutator phenotype (1), and the data presented here demonstrate a direct link between such genetic instability and cancer gene mutations. The reason why $(A)_n$ tract mutations were more common than dinucleotide repeat alterations likely involves the relative frequency of mononucleotide and dinucleotide repeats within *APC*. For example, runs of three or more (CA), (AT), (AG), or (TG) dinucleotide repeats occur 3, 2, 8, and 1 times, respectively, within the entire *APC* coding sequence compared with 278 runs of $(A)_{\geq 3}$, 103 runs of $(A)_{\geq 4}$ and 33 runs of $(A)_{\geq 5}$. The relatively high frequency of $(A)_n$ tracts within the *APC* gene may in part explain the tumor spectrum arising in HNPCC patients. We hypothesize that genes involved in the initiation of tumors that are common in HNPCC, such as colorectal and uterine cancers, may be prone to mutation because of a higher frequency of microsatellites within their coding sequence. Thus, the initiating role of inactivating *APC* mutations in colorectal tumorigenesis and a corresponding relative abundance of microsatellite sequences in the *APC* coding sequence may be analogous to the situation in uterine cancer, for which the initiating gene remains, as yet, uncharacterized. In contrast, such microsatellites may be rare in genes involved in the initiation of lung cancer, which is not part of the HNPCC tumor spectrum. A lower frequency of $(A)_n$ tracts in the murine *APC* sequence may also partly explain the absence of colon tumors in transgenic mice with homozygous defects in mismatch repair genes (51, 52). Runs of 3, 4, and 5 $(A)_n$ repeats are 25%, 43%, and 32% more common, respectively, in the human than in the murine *APC* sequence. Interestingly, in TGF-β receptor II, the only other gene identified so far as a specific target for mutation in RER tumors, the microsatellite content differs considerably between the human and mouse homologs. The $(A)_{10}$ tract in the human sequence that is mutated in 90% of RER colorectal tumors (24, 25) is completely absent from the mouse homolog.

The predominance of −1 or +1 frameshifts in RER tumors and the complete absence of deletions or insertions involving more than 1 bp in tumors with mismatch repair gene mutations indicates either that larger replication slippages do not occur in the *APC* gene or that they are efficiently repaired by other mechanisms. Indeed the only frameshift mutations of more than 2 bp noted in this study were in non-RER tumors.

An excess of A:T transversions or −2 deletions has been noted previously in the *aprt* gene in a clone of CHO cells with a mutator phenotype and defective mismatch binding (8, 11), but this was not apparent in RER tumors in this study. However, observations in a variety of cell lines indicate that the mutator phenotype is a heterogeneous phenomenon with substantial variation between cell lines in both mutation rate and in the type of mutation that arises (12, 13, 19, 53). Hence, detailed analysis of a large series of primary tumors, such as reported here, allows independent determination of the effect of genetic instability on mutations in genes known to be involved in tumorigenesis and is a robust method of assessing its relevance in an *in vivo* human context.

Mismatch repair capacity is intact in normal tissues of most HNPCC patients because of the presence of the relevant wild-

type mismatch repair allele inherited from the unaffected parent. At some stage during tumorigenesis, the wild-type allele is inactivated, leading to mismatch repair deficiency in the tumor. In sporadic RER cases, both alleles of a MMR gene are inactivated somatically. It has not been clear when these mismatch repair deficiencies appear. Genetic instability occurring in the late stages of tumorigenesis, accelerating the rate of tumor progression, was a reasonable assumption. However, the substantial differences in mutational spectra in RER and non-RER tumors observed in this study now strongly suggest that MMR deficiencies *precede APC* mutations in the majority of cases. *APC* gene alterations appear to be required for neoplastic transformation and so our findings indicate that, in patients with RER tumors, genetic instability leads to tumorigenesis via *APC* inactivation and hence is indirectly responsible for both tumor initiation and later progression. This hypothesis is compatible with some, but not all, previous data. It is consistent with the observation that most adenomas from HNPCC patients are MMR deficient (4), and that microsatellite instability is demonstrable at an early stage in some sporadic adenomas (53). However, the observation that adenomas are not much more frequent in HNPCC cases than in the general population (54, 55) would not necessarily have been predicted on the basis of this hypothesis. Perhaps adenomas progress so rapidly to carcinomas in HNPCC patients that adenomas only *appear* to occur at lower frequency than expected (55). Alternatively, a significant proportion of early adenomas with MMR deficiency regress due to the host responses alluded to above, leaving only those tumors capable of escaping such responses. The latter adenomas would then rapidly progress to malignancy due to a reduced capacity for DNA repair, manifest as an elevated mutation rate. Further studies correlating incidence of early colorectal lesions and genetic instability in HNPCC patients may clarify this issue.

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