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Alternative genetic pathways in colorectal carcinogenesis

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ABSTRACT **The comparative typing of matched tumor and blood DNAs at dinucleotide repeat (microsatellite) loci has revealed in tumor DNA the presence of alleles that are not observed in normal DNA. The occurrence of these additional alleles is possibly due to replication errors (RERs). Although this observation has led to the recognition of a subtype of colorectal cancer with a high incidence of RERs (caused by a deficiency in DNA mismatch repair), a thorough analysis of the RER frequency in a consecutive series of colorectal cancers had not been reported. It is shown here that the extensive typing of 88 colorectal tumors reveals a bimodal distribution for the frequency of RER at microsatellite loci. Within the major mode (75 tumors, RER− subtype), the probability that a locus exhibited instability did not differ significantly among loci and tumors, being 0.02. The subsequent development of a statistical test for an operational discrimination between the RER− and RER+ subtypes indicated that the probability of misclassification did not exceed 0.001 in this series. The frequency of K-*ras* mutation was found to be equivalent in the two subtypes. However, in the RER+ tumors, the *p53* gene mutation was less frequently detected, the adenomatous polyposis coli (*APC*) mutation was rare, and the biallelic inactivation of either of these genes was not observed. Furthermore, the concomitant occurrence of *APC* and tumor growth factor β receptor type II gene alterations was found only once. These data suggest that the repertoires of genes that are frequently altered in RER+ and RER− tumors may be more different than previously thought.**

Colorectal tumorigenesis has been associated with the progressive acquisition of a variety of genomic alterations in neoplastic cells. Genes of the *ras* family have been found activated by missense mutation in 45% of colorectal cancers (K-*ras* in 40% of cases, N-*ras* in 5%) (1, 2). The tumor suppressor genes *p53* and adenomatous polyposis coli (*APC*) were each found mutated in about two-thirds of the tumors (3, 4). For these latter two genes, frequent association of point mutation in one allele and loss of the other allele has been observed, indicating that their functional inactivation takes place through a two-hit mechanism often involving chromosomal deletions (5–7).

In 1993, comparative analysis at microsatellite loci revealed, in the DNA of some tumors, the frequent presence of alleles that were not observed in the matched normal DNA. These new alleles are possibly generated as the result of errors occurring during replication (replication error, RER) (8). The tumors that exhibit the highest frequency of RER at microsatellite loci, thus termed RER+ (9), were shown to be impaired in the DNA mismatch-repair pathway. Among the genes involved in this pathway, *MSH2* (10, 11), *MLH1* (12, 13), *PMS2* (14), *GTBP/MSH6* (15–17), *MSH3* (17), and perhaps *PMS1* (14) and DNA polymerase δ (18) are the sites of somatic mutations.

RER+ tumors account for 10–15% of all colorectal cancers (19). They arise preferentially from the proximal colon, a site where tumors rarely exhibit losses of 17p, 18q, and 5q (2). Mismatch-repair deficiency may be an early event in tumorigenesis. This deficiency is expected in part to determine the subsequent genetic events associated with tumor progression (20, 21). Indeed, the high mutation rate of the *HPRT* gene in RER+ cell lines grown in the presence of 6-thioguanine (22, 23) is indicative of a genetic instability enabling the rapid accumulation of somatic mutations (24). This high rate may eventually alleviate the requirement of chromosomal loss in the biallelic inactivation of tumor suppressor genes. In support of the latter hypothesis are the observations that RER+ tumors or cell lines infrequently demonstrate allelic losses (25), exhibit normal or quasinormal karyotypes (26), and are the site of biallelic frame-shift mutations in the tumor growth factor β receptor type II (TGF-βRII) gene (27–29) and in the *BAX* gene (30).

With the exception of the TGF-βRII and *BAX* genes, conflicting information is available on the repertoire of genes which, when altered, contribute directly to the oncogenic properties of the RER+ tumors. Reported frequencies of mutations in the three critical genes initially recognized as being recurrently mutated in colorectal cancer, K-*ras*, *p53*, and *APC*, differ markedly among series of RER+ colorectal tumors (8, 31–36). To delineate more accurately the genetic mechanism involved in colorectal cancer we characterized with respect to microsatellite instability tumors that were calibrated for their DNA index and that were also screened for loss of alleles on 17p and 5q, and for point mutations in the *APC*, *p53*, K-*ras*, and TGF-βRII genes.

PATIENTS AND METHODS

Patients. This study was based on two series of tumors. The first series was collected from 96 patients (mean age, 66 ± 11.3 years) undergoing surgery for colorectal cancer in our institution. Identification of Ki-*ras* mutations and determination of DNA index and allelic losses on chromosomes 17p and 5q have been in part previously reported in a larger series of 109 tumors (2, 37, 38).

A second, more recent, series of 219 colorectal tumors was made from surgical specimens provided to our laboratory by several collaborating institutions. Flow cytometric analysis revealed in this series 70 tumors with near diploid DNA index ($0.95 < n < 1.05$). These tumors were selected for further analysis.

Tumors collected from patients with familial adenomatous polyposis or with a family history of colorectal cancer suggestive of hereditary nonpolyposis colon cancer (HNPCC; Amsterdam criteria) were excluded. In all cases, the freshly

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Abbreviations: APC, adenomatous polyposis coli; RER, replication error; TGF-βRII, tumor growth factor β receptor type II; FUM, fraction unstable microsatellites; LOH, loss of heterozygosity.

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removed tumor and normal mucosae samples were rapidly frozen and stored in liquid nitrogen.

Genotyping of Microsatellite Loci. A total of 110 highly polymorphic microsatellite loci evenly distributed on the autosomes were selected from the Genethon database (list available on request). They were used to determine the RER status of 88 colorectal carcinomas. Each locus was scored for its stability status according to the absence or presence of mobility shifts or additional bands in the amplification product from the tumor as compared with that from normal DNA. A partial characterization of microsatellite instability has previously been reported for 46 tumors of the first series and for 44 tumors of the second series (39).

Identification of TGF-βRII Mutations. Codons 110–134, including 10 repeating adenines of TGF-βRII gene, were amplified using the following primers: sense 5′-CTTTATTCTGGAAGATGCTG-3′ and antisense 5′-GAAGAAAGTCTCACCAGGC-3′. Each case was scored for mutation status according to the size of amplified products.

Identification of *APC* Mutations. Search for somatic *APC* mutations was performed in 85 tumors of the first series by using sequentially two pre-screening methods based on denaturing gradient gel electrophoresis (DGGE) (40, 41). In addition, the PTT procedure was applied to exon 15, using 5 overlapping 1,800-bp fragments (Table 1) (42). All electrophoretic variants were sequenced directly from PCR-amplified products prepared as previously described (41) using Prism ready reaction dye primer cycle sequencing kits (Applied Biosystems) and an Applied Biosystems 373A sequencer. When a mutation was found in a tumor DNA sample, the corresponding normal DNA was systematically checked for the absence of this mutation.

Identification of p53 Mutations. A total of 87 tumors from the first series was screened for somatic mutations in p53 exons 4 to 8 (36).

Allelic Losses. In addition to the previously reported determination of allelic losses on 17p and 5q by using restriction fragment length polymorphism (RFLP) loci and the Southern technique (2, 38), the tumors of patients that were heterozygous for intragenic polymorphisms in the *p53* and *APC* genes or those that carried a somatic mutation in these genes could be directly monitored for the loss of alleles of the corresponding genes by examining the sequence profile of the relevent amplified product.

Statistics. Qualitative variables were compared with respect to one another by using χ^2 analysis with Yates' correction when necessary. The FUM values (see below) and instability rate at each locus in RER− tumors were tested for a Poisson distribution by a Kolmogorov–Smirnov test.

The probability for observing m unstable microsatellite loci among n tested in RER− tumors was calculated from a binomial distribution using p and n as parameters. The p parameter was derived assuming a Poisson distribution for the frequency of unstable microsatellite loci (FUM value) in RER− tumors.

Table 1. PCR conditions for protein truncation test

Fragment	Primer	Annealing temp; °C
I	5′-(a)GGAACTTTGTGGAATCTCTC	60
	5′-TTCGGTTTTACTGCTTTGTCC	
II	5′-(a)GTTTCTCCATACAGGTCACG	60
	5′-TGTAGGAATGGTATCTCGTTT	
III	5′-(a)GCAGTAAATGCTGCAGTTCAGAGG	50
	5′-CTTTTTTGGCATTGCGGAGCTT	
IV	5′-(a)GATGATGTTGACCTTTCCAGGG	58
	5′-GTTGACTGGCGTACTAATACAG	
V	5′-(a)GCAAACATGCCTTCAATCTCTCG	58
	5′-CCCTCTAACAAGAATCAAACCT	

(a) indicates GGATCCTAATACGACTCACTATAGGGAGACCACCATG: T7 RNA polymerase promoter and translation initiation consensus sequences.

RESULTS

Classification of RER+ and RER− Tumors. To investigate microsatellite stability in a series of colorectal tumors, an initial set of 110 microsatellite loci scattered over the entire genome was chosen. Using this set of markers, we characterized a group of 46 tumors by comparing the typing data of matched normal and tumor DNA samples (Fig. 1*a*). These abnormal bands correspond to the somatic generation of new alleles. Occasionally, multiple additional bands were observed, resulting in a ladder. Most tumors demonstrated little or no microsatellite instability in spite of the fact that more than 80 loci were informative for each tumor. In contrast, a small group of tumors demonstrated many instabilities.

A quantitative estimate of the frequency of unstable microsatellite loci in each tumor was derived by computing the ratio of the number of unstable microsatellite loci to the total number of typed microsatellites. By analogy with the FAL parameter, which measures the fractional allelic losses (43), this ratio was termed FUM, for fractional unstable microsatellites. Analysis of the FUM values revealed a bimodal distribution (Fig. 2). The first subgroup, which included 37 tumors, demonstrated FUM below 0.06 (minimum 0; mean 0.02). These tumors were classified as RER−. In this subgroup, the numbers of microsatellite instabilities per tumor and per locus are compatible with a Poisson distribution (Kolmogorov–Smirnov test, $p = 0.35$ and $p = 0.41$, respectively), thus failing to reveal heterogeneity in the FUM values of the tumors or in the frequency with which microsatellite loci would demonstrate instabilities. The second subgroup included 9 tumors that had FUM values larger than 0.53 (maximum 0.86; mean 0.70). They were termed RER+. This group was too small to test for heterogeneity. No tumor had a FUM value between 0.06 and 0.53 (Fig. 2).

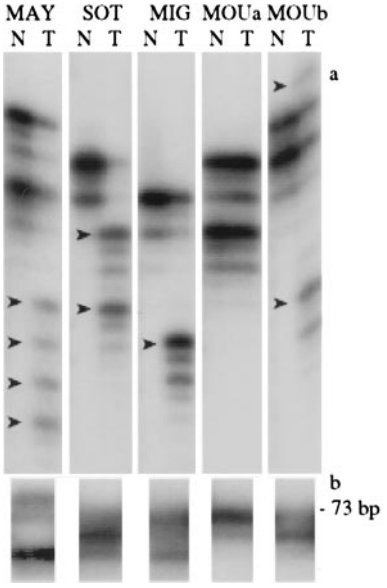

FIG. 1. Detection of instability at microsatellite loci. (*a*) The PCR products generated at *D9S178* from normal (N) and matched tumor (T) DNAs are compared for five cases. No difference was observed for sample MOUa. Arrows indicate the presence of additional alleles (RER) in the four other cases. (*b*) The PCR product generated by primers spanning the coding A_{10} tract of the TGF-βRII gene from the tumor DNA is shown for the same five cases. The product generated from normal DNA is 73 bp long. This is the only product observed for case MOUa. In the four other cases, additional product(s) demonstrate(s) the presence of frameshift mutation(s).

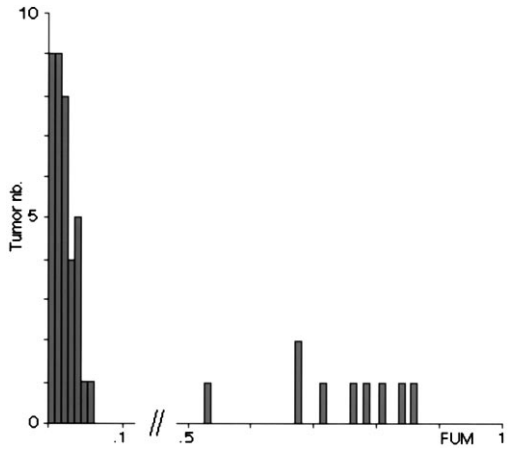

FIG. 2. FUM distribution in colorectal cancers. A total of 46 tumors that had been typed on 110 dinucleotide repeat loci are ordered according to their FUM values. The tumors that have a FUM value less than or equal to 0.06 were classified in the RER− subtype. Although about 4,000 elementary genotypic data on microsatellite loci were available for this subgroup, statistical analysis revealed heterogeneity neither for FUM values nor for the occurrence of RER at specific loci.

Taking into account the presence of two clearly distinct subtypes, the number of microsatellite loci to be typed was decreased to 20 for the classification of an additional group of 42 tumors. Again none of the tumors had a FUM value between 0.06 and 0.5. This second group was found to contain 4 RER+ and 38 RER− tumors. Thus the merged subgroups of this first series contained a total of 75 RER− and 13 RER+ tumors. All RER+ tumors were found to have a near-diploid DNA content ($n < 1.3$, Table 2).

Frequency of *p53*, *APC*, K-*ras*, and TGF-βRII Mutations in RER+ and RER− Tumors. The screening for *p53* and *APC* mutations that was applied to this series of tumors was performed for exons 4 to 8 of the *p53* gene and for all coding exons of the *APC* gene with the exception of the newly identified small alternative exon 10b, in which no mutation has been described in the literature (44, 45). For both genes, the intron/exon boundaries were also investigated. The details of the *p53* mutations have been reported elsewhere (6). Of the 13 tumors that were classified as RER+, 4 (31%) demonstrated a *p53* mutation (IVS3–1G → A, CCC152CC, AGG249TGG, and CGT273TGT). *APC* mutations were found in 59 different tumor samples. Fourteen tumors were found to carry two mutations (Table 3). A single RER+ tumor (11%) demonstrated an *APC* mutation (CGA1450TGA) (Table 2).

Table 2. Relation of RER phenotype with single genetic alterations in the first series of tumors

		Tumors, *n*		
Measurement	Criterion	RER+	RER−	χ^2 *P*
DNA index	$n < 1.3$	13	29	$<10^{-4}$
	$n \geq 1.3$	0	46	
LOH				
17p	Yes	0	50	$<10^{-4}$
	No	13	21	
5q	Yes	0	44	2×10^{-3}
	No	9	24	
Mutation				
p53	Yes	4	41	NS
	No	9	33	
K-*ras*	Yes	2	24	NS
	No	7	39	
APC	Yes	1	53	2×10^{-4}
	No	8	15	
TGF-βRII	Yes	13	1	$<10^{-4}$
	No	0	74	

LOH, loss of heterozygosity; NS, not significant.

Mutation of K-*ras* was searched for in codons 12 and 13 by using oligonucleotide-specific hybridization. Mutations were found in 2 of 9 RER+ tumors (22%) and in 24 of 63 RER− tumors (38%).

Mutations for TGF-βRII were revealed by a deletion or insertion of 1 to 2 bp in the A_{10} tract (Fig. 1*b*). Mutations were found in all 13 RER+ tumors and in 1 of the 75 RER− tumors (Table 2). Because the presence of contaminating normal DNA may lead to erroneous conclusions when LOH at microsatellite loci is monitored by PCR, it was not possible in most cases to decide reliably whether one or both alleles were altered.

Search for Evidence of Biallelic Inactivation of *p53* and *APC*. This series of tumors had previously been screened for allelic losses on 17p and 5q by typing a set of restriction fragment length polymorphisms located at these two chromosomal segments. During the screening for point mutations in *APC* and *p53* genes, a number of patients were identified as being heterozygous for intragenic polymorphic DNA variations that could be used to further document allelic losses. A final method to monitor LOH that was also used took advantage of the detection of somatic mutations. Sequencing of the DNA fragment containing the mutation, obtained by PCR amplification, from the tumor DNA sample permitted documentation of loss or conservation of the nonmutated allele. All three methods provided consistent data when used on the same tumor. Allelic losses on 17p and 5q were observed in 50 and 44, cases respectively. All tumors that demonstrated LOH in these chromosome segments belonged to the RER− group of tumors (Table 2). Tumors were classified according to the number of alterations (point mutation and LOH, counting each for one alteration) that had been detected at a given locus. None of the 42 tumors with two *APC* alterations, and only 1 of 22 tumors bearing a single *APC* alteration, was found to exhibit a RER+ phenotype. In contrast, 8 of 13 tumors (62%) in which no APC alteration was evidenced demonstrated a RER+ phenotype. This difference was highly significant (χ^2 test, 2 degrees of freedom, $P < 10^{-4}$). Similarly, 36 tumors inactivating both *p53* alleles and 19 of 23 tumors bearing a single *p53* alteration were of RER− phenotype; 9 of 26 tumors in which no *p53* alteration was detected exhibited a RER+ phenotype. This difference was also found statistically significant (χ^2 test, 2 degrees of freedom, $P = 6 \times 10^{-4}$). Biallelic inactivation of *APC* and/or *p53* gene(s) was not observed in tumors with a TGF-βRII gene mutation (Table 4).

Search for *APC* Mutation in Tumors with TGF-βRII Gene Mutation. A systematic screening for TGF-βRII gene was established in the laboratory and applied to a new set of 70 consecutive sporadic colorectal carcinomas with near diploid DNA content. An out-frame mutation in the A_{10} coding sequence was identified in 23 tumors. On 11 occasions, the absence of the normal PCR product derived from the A_{10} sequence indicated that both alleles were altered. For the remaining 12 tumors its persistence precluded any conclusion on the inactivation of the second allele because of the possible presence of nonneoplastic cells contaminating the tumor specimen. Of the 34 mutated TGF-βRII alleles that could be evaluated the A_{10} sequence was reduced to A_8 in 11 cases, to A_9 in 19 cases, and increased to A_{11} in 4 cases. Typing of 16 poly(CA) polymorphisms revealed for all 23 tumors more than 8 RERs. Thus these tumors met the criteria that had been defined for being classified as RER+. Search for *APC* mutation in this subgroup of tumors using the *in vitro* transcription–translation test failed to reveal any truncated APC protein. This observation contrasts with the detection, in 48 of 68 RER− tumors, of a mutation in exon 15 leading to a truncated protein (Fig. 3).

Table 3. Somatic *APC* mutations

Patient	Location	Nucleotide change	Consequence	LOH	Patient	Location	Nucleotide change	Consequence	LOH
DEM	EX5	C607T	Q203X	−	SCH	EX15	3155insCGAC	Frameshift	
VER	EX5	C637T	R213X	+		EX15	3926del5	Frameshift	−
GIM	EX6	C646T	R213X	+	GAL	EX15	3191insT	Frameshift	
RUB	EX6	C646T	R213X	−		EX15	T4565A	L1522X	−
MARb	EX6	C667T	Q223X	−	BON	EX15	C3340T	R1114X	−
DUT	EX6	C694T	R232X	+	LEB	EX15	G3466T	E1156X	+
MARc	EX6	C694T	R232X		ELO	EX15	G3625T	E1209X	+
	EX15	C3581A	S1194X	−	BOG	EX15	C3682T	Q1228X	+
THE	EX8	C847T	R283X	+	MOUa	EX15	C2626T	R876X	
BOU	EX8	C904T	R302X			EX15	C3682T	Q1228X	−
	EX9	983del7	Frameshift	−	DEP	EX15	G3856T	E1286X	+
LAS	EX8	A931T	K311X	+	KAT	EX15	3859del	Frameshift	+
FLE	IVS8a	IVS8a-2A → T	Splice mutation	−	SAU	EX15	3872insA	Frameshift	+
SER	EX9	1253del	Frameshift		COU	EX15	G3883T	E1295X	+
	EX15	3926del5	Frameshift	−	LAN	EX15	G3916T	E1306X	+
NOV	IVS9	IVS9+2insTAT	Splice mutation		LIS	EX15	3926del5	Frameshift	+
	EX15	C3638A	S1213X	−	PIS	EX15	3926del5	Frameshift	+
LET	EX14	1944insA	Frameshift		ROB	EX15	G3934T	G1312X	+
	EX15	C4348T	R1450X	−	NOE	EX15	3950insG	Frameshift	+
HAM	EX15	G1972T	E658X	−	BOR	EX15	C4031A	S1344X	+
VAS	EX15	G2572T	E858X	−	MIG	EX15	4060insT	Frameshift	−
FRE	EX15	C2626T	R876X	+	GAU	EX15	C4099T	Q1367X	+
LOU	EX15	C2626T	R876X	+	AVE	EX15	4146del	Frameshift	+
ARG	EX15	C2626T	R876X		DESb	EX15	4147delAT	Frameshift	+
	EX15	4661insAA	Frameshift	−	JEA	EX15	4185insT	Frameshift	−
LEC	EX15	C2626T	R876X		GOD	EX15	G4189T	E1397X	+
	EX15	4242del17	Frameshift	−	DAU	EX15	C4199A	S1400X	−
DESa	EX15	C2626T	R876X		DER	EX15	C4202A	S1401X	+
	EX15	4243del	Frameshift	−	POU	EX15	G4222T	E1408X	−
PET	EX15	C2626T	R876X		SOUa	EX15	4233del	Frameshift	+
	EX15	C4348T	R1450X	−	LEF	EX15	C4448T	R1450X	+
JAN	EX15	2857del	Frameshift		FAL	EX15	C4448T	R1450X	−
	EX15	G4138T	E1380X	−	HUB	EX15	G4381T	E1461X	+
LEO	EX15	G2950T	E984X		SOUb	EX15	4462del	Frameshift	−
	EX15	C4285T	Q1429X	−	BRI	EX15	4462del	Frameshift	+
MARa	EX15	A2977T	E993X	+	LEG	EX15	4489del	Frameshift	−
					WIT	EX15	4589del	Frameshift	+

Descriptions are according to the update on nomenclature for human gene mutations (49). EX, exon; IVS, intervening sequence; del, deletion; ins, insertion.

DISCUSSION

The typing of at least 20 (more than 100 for half of the cases) dinucleotide repeat polymorphisms for each of 88 colorectal carcinomas has revealed, on the basis of their FUM values, two distinct groups of tumors, which were qualified RER+ and RER−. In each group the average FUM values were 0.7 and 0.02, respectively. Given the size of each group, it was not possible to distinguish within each of them subgroups for the FUM values.

Simulation studies were performed to derive, from the set of data collected on RER− tumors, the probability of finding RERs as a function of the number of tested loci (Table 5). The minimum FUM value that is to be observed for a tumor to exclude it from the RER− subtype (i.e., to classify it as RER+) was derived from these empirical probabilities. The resulting values are in close accordance with those derived assuming that RERs in RER− tumors were the result of stochastic events, following a Poisson distribution, which would occur with an equal probability of 0.02 on the different

Table 4. Classification of the first series of tumors according to the number of altered *APC* or *p53* alleles for the RER tumor subtype or the presence of TGF-βRII mutation

Gene	Mutated/deleted alleles, *n*	Tumors, *n*			No. with mutation in TGF-βRII		
		RER+	RER−	χ^2 *P*	Yes	No	χ^2 *P*
APC	2	0	42		0	45	
	1	1	21	$<10^{-4}$	2	22	$<10^{-4}$
	0	8	5		8	8	
p53	2	0	36		0	39	
	1	4	19	6×10^{-4}	5	20	2×10^{-3}
	0	9	17		9	22	

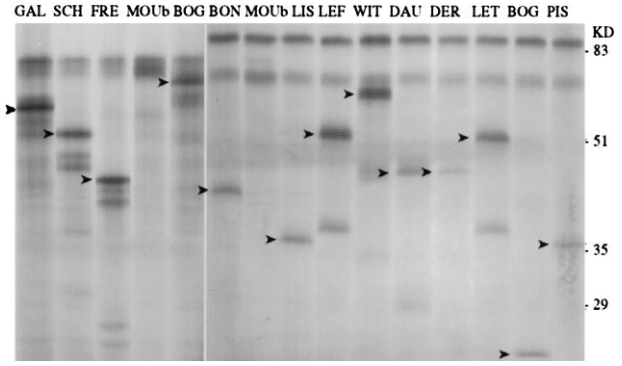

FIG. 3. Detection of truncating mutation in exon 15 of the *APC* gene. *Left* and *Right* show polypeptides derived from the *in vitro* transcription–translation test performed on fragments I and II of *APC* exon 15, respectively. Fragment I is expected to give rise to a normal 72-kDa polypeptide that is observed in all lanes due to contamination of tumor fragments with nonneoplastic cells. Observation of shorter polypeptides (arrowheads) is indicative of the presence of an *APC* mutation that was in all cases confirmed by sequencing. Fragment II encodes a normal 85-kDa polypeptide. Note that in sample BOG shorter polypeptides are generated from both fragments I and II. This observation was suggestive of a localization of the truncating mutation on the DNA portion overlapping I and II, a hypothesis that was confirmed by sequencing.

microsatellite loci tested and in the different tumors studied (Table 5).

None of the RER+ tumors demonstrated either allelic loss on 17p or loss on 5q, an observation that is compatible with previous cytogenetic (26) or allelic loss studies (25). These observations suggest that, if biallelic inactivation of p53 and APC were to occur in the RER+ tumors, an increased frequency of point mutations would be expected. However, the observed *p53* and *APC* mutations were markedly less frequent in the RER+ tumors as compared with the RER− tumors. This unexpected observation may be due to a lower efficiency of our mutation screening methods in RER+ tumors as compared with RER− tumors or to a high incidence of mutations in RER+ tumors in regions that were not screened in this work. Alternatively, it may reflect a true low incidence of *p53* and *APC* mutation in RER+ tumors. The recent observation of the presence of functional p53 mRNA in 4 of 4 RER+ cell lines derived from colorectal cancer (36) supports the proposal that development of RER+ tumors is frequently compatible with the continued presence of an intact *p53* gene. No functional test is yet available for the *APC* gene. However, by far the most frequent mode of first-hit inactivation of the *APC* gene occurs through mutations causing protein truncation. The observation that full-length APC protein is present in RER+ cell lines indicates that this gene may also be frequently functional in these tumors (33).

The present study may help to resolve apparently conflicting conclusions recently reached by independent groups of investigators concerning the frequency of microsatellite instabilities in colorectal cancer and its relationship to *APC* mutation.

Konishi *et al.* (35) distinguish severe and mild RER+ phenotypes. The criterion for the severe RER+ phenotype (i.e., 3 to 5 instabilities of 5 tested loci) is, according to our statistical analysis, discriminative with a probability of misclassification lower than 10^{-4} (Table 5). In this subgroup the *APC* mutation prevalence is small (1 mutation in 10 cases) and compatible with that observed in our group of RER+ tumors. The criterion for their mild RER+ phenotype is the observation of 1 or 2 unstable loci among 5 typed dinucleotide repeats. In their series 11% of the tumors meet this criterion, a percentage that is comparable to the expected proportion of RER− tumors (9.6%) that would yield this observation (Table 5). It follows that most tumors classified by Konishi *et al.* in the mild RER+ subtype may correspond to tumors that we would have categorized RER−. In accord with this hypothesis, the mild RER+ tumors of Konishi *et al.* demonstrate a high frequency of *APC* mutation (35).

Huang *et al.* (34) suggest that *APC* mutation rates do not differ markedly in RER+ and RER− subtypes. In their report, the RER status determination was based on the typing of 4 or more microsatellite loci per tumor and tumors were entered in the RER+ subtype when at least half of the tested loci demonstrated RER. The RER+ subgroup thus defined may contain a minute proportion of tumors that would have been classified RER− in our study. This proportion, however, is not sufficient to account for the high *APC* mutation rate observed in this series of RER+ tumors. Although we cannot

Table 5. Analysis of the criteria for the classification of colorectal tumors in the RER− subtypes

Tested loci, *n*	Probability of observation of RER at dinucleotide repeat loci in RER− tumors					Minimum FUM requested for classification of a tumor as not belonging to RER− subtype*		
						Risk of misclassification =		
	None	1	2	1 or 2	≥3	$P < 0.05$	$P < 0.01$	$P < 0.001$
2	0.960	0.039	0.000	0.040	—	0.50	—	—
	0.967	*0.033*	*0.000*	*0.033*		*0.50*		
3	0.941	0.058	0.001	0.059	0.000	0.66	0.66	—
	0.943	*0.056*	*0.000*	*0.057*	*0.000*	*0.66*	*0.66*	*0.66*
4	0.922	0.075	0.002	0.078	0.000	0.50	0.50	0.75
	0.933	*0.065*	*0.002*	*0.067*	*0.000*	*0.50*	*0.05*	*0.75*
8	0.851	0.139	0.010	0.149	0.000	0.25	0.25	0.38
	0.87	*0.122*	*0.009*	*0.131*	*0.000*	*0.25*	*0.25*	*0.38*
12	0.785	0.192	0.022	0.214	0.002	0.17	0.25	0.33
	0.806	*0.175*	*0.017*	*0.192*	*0.001*	*0.17*	*0.25*	*0.33*
16	0.724	0.236	0.036	0.273	0.004	0.13	0.19	0.25
	0.758	*0.208*	*0.030*	*0.238*	*0.003*	*0.13*	*0.19*	*0.25*
20	0.668	0.272	0.053	0.325	0.007	0.15	0.15	0.20
	0.700	*0.247*	*0.048*	*0.295*	*0.005*	*0.10*	*0.15*	*0.20*

The data were obtained either from a binomial distribution, assuming that the probability for any locus to exhibit replication error in a RER− tumor is equal to 0.02 (numbers in roman type) or, to derive probabilities that are independent of the assumption of Poisson distribution, from simulations that were performed by choosing randomly tumors and loci within the set of data obtained on the RER− tumors. Each line corresponds to 5,000 trials (numbers in italic type).

*Because the FUM is the ratio of two integers, occasionally, the minimum FUM to avoid misclassification does not decrease regularly as the number of tested loci increases.

provide a specific explanation for this discrepancy with our present observation, it should be noted that the published data of Huang *et al.* (34) do suggest a significant difference in the rate of *APC* mutation in RER+ and RER− tumors (47 mutations in 63 RER− tumors versus 29 mutations in 52 RER+ tumors, χ^2 test, 1 degree of freedom, $P = 0.03$).

It is suggested that to avoid future confusion, the classification of a colorectal tumor in the RER+ subgroup should rely on stringent criteria. Those advocated here may be provisionally adopted. We may not exclude, however, that subsequent larger series of data would reveal within each subtype or among microsatellite loci, smaller groups that would exhibit subtle differences in stability. Mononucleotide repeats such as Bat-26 have been shown to be systematically unstable in RER+ tumors (39). This locus also has demonstrated instability in the HCT 15 cell line, which has a low rate of alteration at dinucleotide repeats and an inactivation of the *MSH6* gene.

When cell lines derived from RER+ tumors are grown under selective conditions, the genes that are placed under selective pressure exhibit a high rate of mutation (22, 23). It is therefore likely that the low incidence of mutation in *p53* and *APC* in RER+ tumors reflects the lack of a strong requirement for mutation in these genes during tumor initiation and progression. It has been observed that genes that carry mononucleotide or dinucleotide repeats may be more readily mutated in RER+ tumors as compared with RER− tumors as exemplified by mutations of the A_{10} coding sequence of the TGF-βRII gene (27–29), the G_8 coding sequence of the *BAX* and *IGFIIR* genes (30, 46), and the β_2-microglobulin gene (47). Our data and those published by Konishi *et al.* indicate that mutation in the A_{10} tract of TGF-βRII and mutation in *APC* occur as alternative events in colorectal tumors. Interestingly, search for mutation in the entire coding sequence of the TGF-βRII gene for 30 colorectal tumors has revealed a mutation in a region distinct from the A_{10} tract. This single mutation had occurred in a tumor that displayed microsatellite instability (48). Taken together, these observations strongly suggest that the somatic mutagenesis associated with tumor initiation and progression of RER+ and RER− involves groups of target genes that are markedly more different than previously suspected. It prompts the search in RER+ tumors for additional genes that when mutated may contribute to the tumor phenotype.

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