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Featured Article

Different Genetic Features Associated with Colon and Rectal Carcinogenesis

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ABSTRACT

Purpose: **The issue of whether colon and rectal cancer should be considered as a single entity or two distinct entities is still debated, and there is a need to improve studies addressing the heterogeneity of the pathogenetic pathway leading to sporadic colorectal cancers (SCRCs) as well as to identify biological and/or molecular differences between colon and rectal cancers.**

Experimental Design: **Specimens of SCRCs were analyzed for somatic mutations in *APC*, *K-ras*, and *TP53* genes and loss-of-heterozygosity of chromosome 18.**

Results: **Eleven SCRCs showed microsatellite instability. *APC* mutation frequency was significantly lower in microsatellite instability (MIN+) than in MIN− SCRCs. All MIN− SCRCs showed β-catenin overexpression. A combined analysis of the biomarkers revealed two pathways mainly represented by MIN− SCRCs and differently followed on the basis of tumor location, *APC-K-ras-TP53-Ch18q* and *APC-TP53-Ch18q*.**

Conclusions: **The APC-β-catenin pathway is inactivated in MIN− SCRCs and represents the first hit of SCRC development. Two preferential pathways followed by SCRCs occur, one K-ras dependent, in agreement with the Fearon and Vogelstein model, and the other K-ras independent. Significant differences between colon and rectal tumors occur in our series of MIN− SCRCs. The different pathways observed and their distribution can be summarized as follows: (*a*) *K-ras* mutations were more commonly detected in colon than in rectum; (*b*) the number of mutations detected was significantly higher in colon than in rectal tumors; and (*c*) a mutational pattern restricted to the *APC* gene was more common in rectal than in colon tumors. This molecular characterization can be translated into a clinical setting to improve diagnosis and to direct a rationale pharmacological treatment.**

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INTRODUCTION

Colorectal tumor is the second leading cause of cancer-related death in the Western world and first when smoking-related cancers are excluded (1, 2). The issue of whether colon and rectal cancer should be considered as a single entity or two distinct entities is still debated. The good correlation between cancer incidence rates for both the sites observed in different ethnic populations (1, 2) and the shared similar etiology, type of precancerous lesions as well as mode of spread, all give evidence in favor of the first assumption (2). However, differences exist between colon and rectal carcinomas with respect to age and gender of the patient as well as tumor progression and adjuvant treatments (3–7).

At a molecular level, much progress has been made in the last two decades in the identification and characterization of the genetic changes involved in the malignant colorectal transformation process. The model proposed (8) foresees a colon cancer where the temporary progression from healthy mucosa to carcinoma *in situ* is supported by mutations in *APC*, K-*ras*, *TP53*, and *DCC* genes. The model, originally formulated for sporadic colorectal cancer (SCRC) development, is also valid for familial adenomatous polyposis patients, who carry an *APC* germline mutation (9, 10). A second pathway of colorectal tumorigenesis has been depicted in cases with a normal karyotype but carrying genetic instability at microsatellite loci attributable to alterations in the DNA mismatch repair genes. The latter, when present in germinal cells, are responsible for the familial syndrome hereditary nonpolyposis colorectal carcinoma (9, 11, 12) and when affecting somatic cells may cause microsatellite instability (MIN) in a subset (up to 15%) of sporadic colorectal tumors (9, 13). According to these data, the stepwise progression postulated by the aforesaid model (8) seems to be representative of colorectal tumor development in about 90% of SCRCs and indirectly supported by a number of epidemiological, clinical, histopathological, and genetic studies (1, 14, 15). However, the subsequent discovery of several other genes involved in colorectal tumor development supports the notion that the model of Kinzler and Vogelstein could be more complex than originally proposed (16). In a recent study based on an analysis of *APC*, K-*ras*, and *TP53* genes in the same samples (17), multiple alternative genetic pathways were shown to lead to tumor progression, and more importantly, the originally postulated path-

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way including simultaneously all of the three genetic alterations was found in only 6.6% of the analyzed cases.

Kapiteijn *et al.* (18) showed that nuclear expression of β-catenin, a critical mediator of the *wnt* transcriptional response negatively regulated by adenomatous polyposis coli (APC; Ref. 19), and TP53 overexpression are significantly more common in rectal than in colon cancers and that TP53 overexpression is related to a worse disease-free survival in rectal but not in colon tumors.

In the light of the aforementioned considerations, there is a pressing need to improve studies addressing the heterogeneity of the pathogenetic pathway leading to SCRCs as well as to identify possible biological and/or molecular differences between colon and rectal cancers, in a possible attempt to translate the molecular knowledge into a clinical setting to improve the diagnosis and to direct a rationale pharmacological treatment. Thus, we investigated the occurrence of somatic mutations in *APC*, K-*ras*, and *TP53* genes and loss-of-heterozygosity (LOH) of nine microsatellite loci spanning the long arm of chromosome 18, where the *DCC*, *SMAD4/DPC4*, and *SMAD2* tumor suppressor genes are located, in 99 specimens of SCRCs. To our knowledge, the present analysis is the most comprehensive genetic characterization of colorectal cancer, the results of which emphasize the genetic heterogeneity of colorectal carcinogenesis as well as the differences between colon and rectal cancers.

MATERIALS AND METHODS

Patient Specimens. Tissue specimens from 99 SCRCs were obtained with informed consent from previously untreated patients who underwent surgical resection at the Istituto Nazionale per lo Studio e la Cura dei Tumori of Milan between 1998 and 2000. Tumor specimen and its surrounding normal mucosa were selected by an experienced pathologist from formalin-fixed, paraffin-embedded material, as well as from cryopreserved tissue.

The patient group included 42 women and 57 men. Ages ranged from 41 to 89 years (median, 65 years). All tumors were adenocarcinomas and were graded according to the WHO criteria, and the anatomical distribution was described as follows: rectum; left colon (including sigma and splenic flexure); right colon (caecum, right flexure, transverse colon). Overall, 72 tumors were localized in the colon and 27 in the rectum. The distribution according to the modified Dukes' classification was as follows: 8 A; 32 B; 25 C; and 34 D cases.

Mutational Status of *APC*, K-*ras*, and *TP53*. Genomic DNA was extracted from stored tissues using the QIAamp Tissue Extraction kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Samples identified mutated by screening for *APC*, *K-ras*, and *TP53* mutations were subjected to automated sequencing by ABI Prism 377 (Applied Biosystems, Foster City, CA) and analyzed with Sequencing Analysis and Sequence Navigator software programs by ABI Prism.

APC. Codons 686-1693 were analyzed for mutations using the *in vitro* synthesized protein assay as described previously (20). After verifying the presence of amplified products for each fragment on a 1% agarose gel, PCR reactions (3 μl) were used directly without purification as template in 10 μl of T7-Coupled *in vitro* Transcription/Translation System (Promega, Madison, WI), and [^{35}S]methionine was incorporated into the translation products according to the manufacturer's instructions.

K-ras. Mutations were searched throughout for the entire sequence of the gene. Screening of mutations in codons 12 and 13 was accomplished through mismatched primer-mediated PCR amplifications followed by restriction-fragment length polymorphism analysis using restriction endonucleases as already reported (21, 22). Mutated samples were then sequenced. Exon 2 of the *K-ra*s gene was examined as follows: 200 ng of genomic DNA was amplified in 50 μl of final volume reaction with 1× PCR Buffer II; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; and 0.5 μM appropriate primer and 2 units of AmpliTaq. PCR amplifications were performed as follows: initial denaturation step of 96°C for 3 min; 45 cycles of 96°C for 30 s; 50°C for 1 min; 72°C for 1 min; and a final elongation step of 72°C for 4 min. The samples were run on 4% polyacrylamide gels and then sequenced.

TP53. All samples were screened for the presence of *TP53* mutations in the most frequently affected exons (5 through 8) of the gene, by double gradient-denaturing gradient gel electrophoresis and, in the initial phase of the study, by single-strand conformation polymorphism, as described previously (23, 24).

Immunohistochemistry. β-Catenin expression was analyzed in formalin-fixed, paraffin-embedded samples as already reported (25) with minor modifications. Antigen-retrieval was performed using 5 mM citrate buffer (pH 6) in a steamer at 95°C for 6 min. As a primary antibody, a mouse monoclonal anti-β-catenin antibody (Transduction Laboratories, Lexington, KY) was diluted at 1:2000 in a blocking solution containing 0.05 M PBS, 1% BSA, and 0.1% sodium azide. Incubation in the absence of the primary antibody was used as a negative control, and β-catenin protein was evaluated for the presence of nuclear, cytoplasmic, and membranous accumulation in tumor and normal surrounding tissues.

LOH Analysis. Nine sets of polymorphic microsatellite sequences (*D18S478*, *D18S1102*, *D18S474*, *D18S64*, *D18S68*, *D18S61*, *D18S1161*, *D18S462*, and *D18S70*), spanning the entire long arm of chromosome 18, were obtained from Applied Biosystems. The two loci *D18S474* and *D18S64* flank the region where three tumor suppressor genes (*DCC*, *SMAD4/DPC4*, and *SMAD2*) are located. PCR reactions were carried out according to the manufacturer's instruction. The PCR products were run on 5% polyacrylamide gel containing 6 M urea and 1× Tris-borate-EDTA buffer in an ABI PRISM 377 sequencer (Applied Biosystems) according to the manufacturer's instructions, and the collected data were analyzed by Gene Scan 3.1 software program (Applied Biosystems). LOH was defined when at least 40% signal reduction intensity of one allele was observed in neoplastic tissue compared with the matched allele in the healthy mucosa specimens in >30% of microsatellite loci tested. MIN was defined when an additional band was detected in the tumor tissue specimen compared with the healthy mucosa specimen.

Statistical Analysis. Wilcoxon's rank-sum test, χ^2 test and Fisher's exact test were used where appropriate. All *P*s were two-sided.

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Table 1 Gene mutations and clinicopathological features in sporadic colorectal adenocarcinomas without microsatellite instability

	No. of cases	Frequency (%) of alterations[a] in the following:			
		APC	K-*ras*	*DCC*	*TP53*
Overall series	88	75.0	50.0	60.2	56.8
Age					
≤60 yr	25	76.0	52.0	60.0	60.0
>60 yr	63	74.6	49.2	60.3	55.5
Gender					
Female	36	77.7	61.1	50.0	58.3
Male	52	73.0	42.3	67.3	55.7
Location					
Proximal colon	31	77.4	61.3[b]	64.5	58.0
Distal colon	30	73.3	53.3	60.0	53.3
Rectum	27	74.0	33.3	55.5	59.2
Tumor size (cm)					
≤3	14	78.5	64.2	50.0	50.0
3.1–6	56	75.0	48.2	57.1	58.9
>6	14	71.4	42.8	78.5	64.2
Grade					
Well/moderate	78	76.9	48.7	61.5	60.2
Poor	8	62.5	50.0	37.5	25.0
Stage[c]					
A	8	75.0	50.0	50.0	50.0
B	22	72.7	50.0	72.7	68.1
C	24	70.8	29.1	58.3	54.1
D	34	79.4	64.7	55.8	52.9

[a] *APC* (exon 15); K-*ras* (exons 1, 2); *DCC* (loss of heterozygosity in *D18S478*, *D18S1102*, *D18S474*, *D18S64*, *D18S68*, *D18S61*, *D18S1161*, *D18S462*, *D18S70*); *TP53* (exons 5–8).

[b] $P = 0.09$; proximal colon *versus* rectum, $P = 0.033$; distal colon *versus* rectum, $P = 0.12$; colon *versus* rectum, $P = 0.037$.

[c] Stage, modified Dukes' classification.

RESULTS

Microsatellite Analysis in SCRCs. In our patient group, we found 11 SCRCs showing a high degree of MIN (>40% of analyzed loci) at the chromosome 18q level. These samples were subsequently investigated through the National Cancer Institute panel, and the analysis confirmed these tumors as highly unstable (and therefore named MSI+ colorectal cancer; data not shown).

Mutational Status of *APC*, *K-ras*, and *TP53* in SCRCs. The analysis of exon 15 of the *APC* gene showed mutations in 66 of 88 (75%) MIN− CRCs (Table 1). In particular, 53 cases presented a single mutation, and most were non-sense mutations (30 cases; Table 2). A double mutation implying a complete inactivation of the *APC* gene was found in 13 cases where the MCR alteration was always present. The *APC* mutational pattern of the present series of MIN− SCRCs was compared with that observed in the series of 11 MIN+ SCRCs (Table 2). It is noteworthy that the nature of the *APC* mutation was different between MIN− SCRCs and MIN+ SCRCs. All *APC* mutations in MIN+ SCRCs were insertion or deletion mutations (5 of 5 = 100%), whereas only 32 of 82 (39%) mutations were insertions or deletions in MIN− SCRCs, in which non-sense alterations predominantly occur (50 of 82 = 61%; Table 2). Moreover, the frequency of *APC* mutations was significantly lower in MIN+ SCRCs (4 of 11 = 36%) than in MIN− SCRCs (75%; $P = 0.0079$).

K-ras mutations were present in 50% of MIN− SCRCs (Table 1), and bp transversions were the predominant alteration. Nucleotide substitutions were observed in codon 12, 13, 61, and 63 (34, 7, 2, and 1 case, respectively).

The *TP53* gene DNA-binding domain was screened, and 50 of 88 (56.8%) MIN− SCRCs showed an alteration (Table 1), which was subsequently characterized through automated sequencing. Moreover, four tumors showed two distinct mutations, two of which had mutations in two exons.

LOH Analysis. Nine microsatellite loci spanning the long arm of chromosome 18 were amplified to analyze the status of three tumor suppressor genes (*DCC*, *SMAD4/DPC4*, and *SMAD2*) located in the 18q21-2 region, and chromosome 18q losses were found in 53 of 88 (60.2%) of the MIN− SCRCs analyzed (Table 2). When the analysis was restricted to the two loci bordering the region 18q21-2 (*D18S474* and *D18S64*), no differences were found compared with the analysis of the entire loci panel.

Gene Alterations and Clinicopathological Features. *K-ras* mutations were significantly more prevalent in tumors of the colon than in those of the rectum ($P < 0.037$) and were predominant in the proximal colon (Table 1). There was no significant association between *APC*, *TP53*, or *LOH* alterations, or any clinico-pathological feature such as age and gender of patient, location and size of tumor, histological grading and Dukes' stage (Table 1).

APC–β-Catenin Pathway Status. To overcome the problem that the analysis of *APC* mutations was restricted to exon 15 and to elucidate the APC-β-catenin pathway status in *APC*-negative specimens, immunohistochemical evaluation of β-catenin expression was performed. Fig. 1*A* shows an example of an *in vitro* synthesized protein experiment in the *top*, where two samples (T2 and T3) showed the presence of an abnormally truncated APC protein. The subsequent automated sequence characterization of the T2 sample, reported in the *bottom* of Fig. 1*A*, showed that the APC protein was abnormally truncated for the presence of a frame-shift alteration in exon 15 of the *APC*-coding sequence.

Analysis of β-catenin expression in *APC* double-mutated MIN− SCRCs revealed an immunohistochemical staining limited to the plasma membrane in healthy mucosa, whereas β-catenin immunoreactivity was found in the plasma membrane as well as in the cytoplasm and nucleus in tumor tissue specimens (Fig. 1*B, top*). A similar β-catenin expression pattern was detected in all *APC*-negative MIN− SCRCs, as depicted in the *bottom* of Fig. 1*B*. In contrast, in *APC*-negative MIN+ SCRCs, β-catenin immunoreactivity was limited to the plasma membrane (Fig. 1*C, top*). A similar β-catenin expression pattern was observed in 3 of 3 MIN+ SCRCs with a single *APC* mutational event in tumor and in healthy mucosa specimens (Fig. 1*C, bottom*). However, the only MIN+ SCRC sample with a double *APC* exon 15 mutation showed an immunohistochemical β-catenin overexpression similar to that observed in MIN− SCRCs specimens.

Genetic Pathways in MIN− SCRCs. A combined analysis for the presence of the observed single mutations was performed considering the first three molecular markers involved in the original model (*APC*, *K-ras*, and *TP53* genes; Ref. 9). Only 6% of tumors did not show any alteration in these

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Table 2 *APC* gene mutations in MIN− SCRCs and MIN+ SCRCs[a]

	No. of cases	Hotspot codon mutated (no. of cases)
MIN− SCRCs	88	
wt	22 (25%)[b]	
Single *APC* mutation	53	
Nonsense	30	1450 (7), 1286 (2), 1294 (2), 876 (1), 1114 (1)
Deletions	13	1312 (3), 1467 (3), 1414 (2), 1471 (2), 1472 (1), 1506 (1)
Insertions	10	1558 (4), 1385 (2)
Double *APC* mutation	13	
Nonsense	20	876 (5), 1450 (3), 1114 (2)
Deletions	4	859 (1), 1413 (1), 1472 (1), 1506 (1)
Insertions	2	1558 (2)
MIN+ SCRC	11	
wt	7 (64%)[b]	
Single *APC* mutation	3	2 deletions and 1 insertion
Double *APC* mutation	1	1 deletion and 1 insertion

[a] MIN, microsatellite instability; SCRC, sporadic colorectal cancer.
[b] MIN− SCRCs *versus* MIN+ SCRCs, $P = 0.0079$.

genes, although β-catenin overexpression was always detected, and 27% of tumors were mutated in only one gene [*e.g., APC* (14 cases, 16%), *K-ras* (2 cases, 2%), or *TP53* (8 cases, 9%)]. Moreover, the most common mutated pathways were *APC-K-ras-TP53* (23%), *APC-K-ras* (21%), and *APC-TP53* (18%).

Extension of the analysis to chromosome 18q increased the patterns of mutational events, and the altered APC-β-catenin pathway in the present series of MIN− SCRCs evidenced the following two dominant combinations of genetic alterations: *APC-K-Ras-TP53-Ch18q* (17 of 88 cases, 19.5%) and *APC-TP53-Ch18q* (17 of 88 cases, 19.5%; Fig. 2). The altered *APC*-β-catenin pathway alone was observed in only 9% of cases (8 of 88).

Genetic Pathways and Primary Location in MIN− SCRCs. The combinations of genetic alterations were found to be different throughout tumor site (Fig. 3). Altogether, the number of mutational events were higher in the colon than in the rectum ($P = 0.017$). In particular, the two most frequently

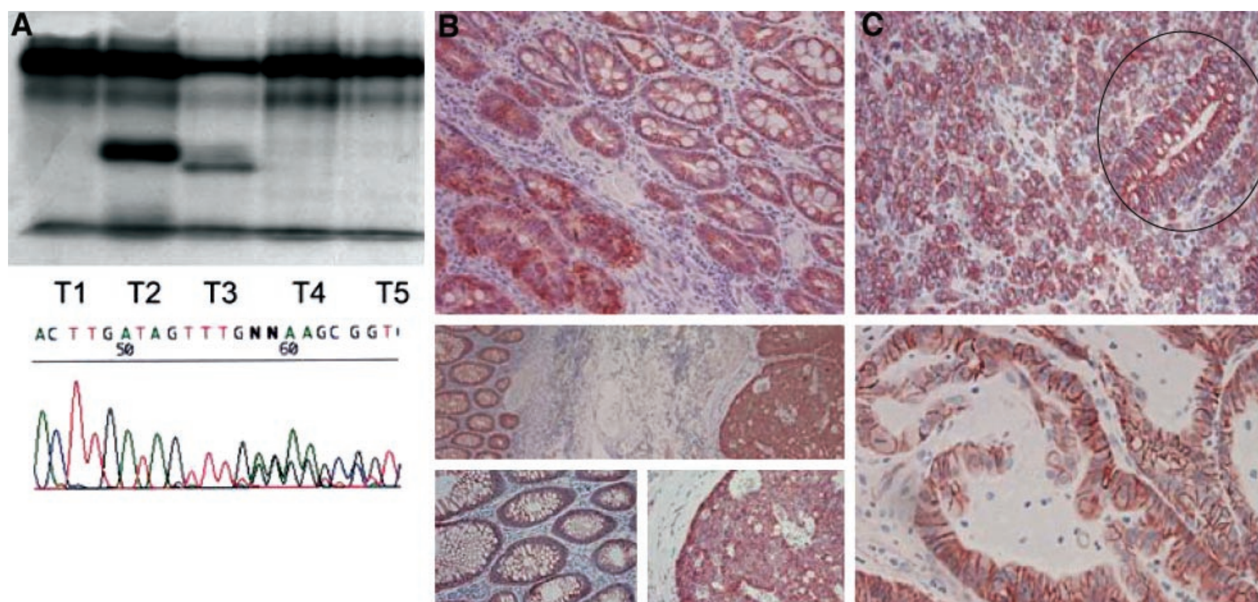

Fig. 1 APC-β-catenin pathway analysis. *A, top,* example of *in vitro* synthesized protein experiment representing an initial screening for the detection of *APC* exon 15 mutation. T2 and T3 samples show an APC protein abnormally truncated. *A, bottom,* characterization through automated sequencing with internal primers of the samples showing an *APC* alteration after *in vitro* synthesized protein assay. The molecular characterization of a T2 sample carrying a frame-shift alteration is shown. *B, top,* immunohistochemical staining of β-catenin protein in a MIN− SCRC sample carrying two distinct *APC* mutations. *On the right,* the healthy mucosa and *on the left* the tumoral portion are represented, respectively. *B, bottom,* immunohistochemical staining of β-catenin in MIN− SCRC samples lacking *APC* mutation. Healthy mucosa is *on the left* and tumoral tissue *on the right*. *On the bottom,* the two representative portions are depicted in *a wide window*. *C, top,* immunohistochemical staining of β-catenin in a MIN+ SCRC lacking *APC* mutation. The expression is similar in the tumor (*on the left*) as in an entrapped colon crypt (*in the black circle on the right*). *C, bottom,* immunohistochemical staining of β-catenin in a MIN+ SCRC carrying two distinct *APC* mutations. Only the tumor portion is depicted. *SCRC*, sporadic colorectal cancer; *MIN*, microsatellite instability; *APC*, adenomatous polyposis coli.

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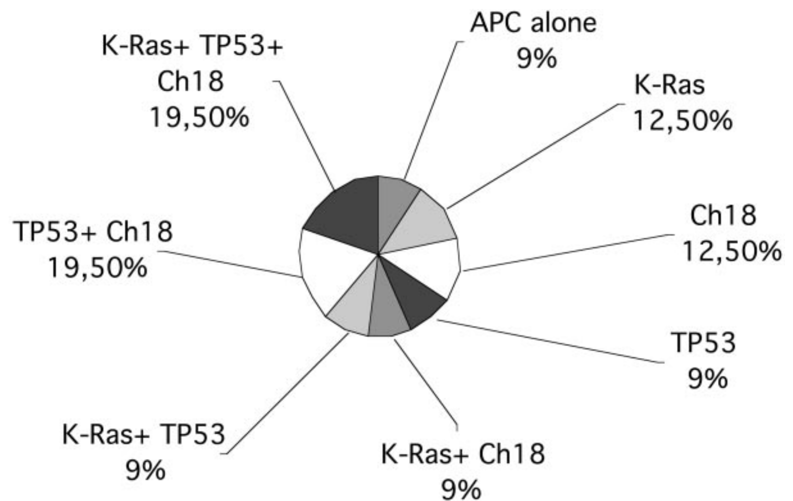
Fig. 2 Different genetic pathways of tumorigenesis in MIN− SCRCs according to the frequencies found in our patient group. The APC-β-catenin pathway is altered in all cases. *SCRC*, sporadic colorectal cancer; *MIN*, microsatellite instability; *APC*, adenomatous polyposis coli.



observed combinations, *APC-K-ras-TP53-Ch18q* and *APC-TP53-Ch18q*, were represented in 23% and 15%, respectively, of the colon cancers, in contrast to 11% and 30%, respectively, of the rectal cancers. Moreover, *APC-K-ras-Ch18q* combined alterations were detected in colon (13%) but not in rectal cancers ($P = 0.048$), and alterations restricted to the APC-β-catenin pathway were more present in rectal (15%) than in colon (7%) cancers.

DISCUSSION

A number of molecular studies have shown that colon carcinogenesis results from an accumulation of epigenetic and genetic alterations, including activating mutations of the *K-ras* proto-oncogene and inactivating mutations of *APC* and *TP53* tumor suppressor genes (as originally proposed in 1990 by Fearon and Vogelstein; Ref. 8) or of DNA repair genes (15). However, this stepwise model of colorectal tumorigenesis has been mainly validated conceptually, and there is mounting evidence that alternative genetic events may occur during colorectal carcinogenesis, sometimes preferentially, sometimes randomly, and sometimes with an overlap (16, 26). A recent analysis of the three key genes, *APC*, *K-ras*, and *TP53*, demonstrated that the most common combination (27% of cases) of mutations involved *APC-TP53*, whereas the pattern *K-ras-TP53* was infrequent, and only 6.6% of SCRCs showed mutations in all of the three molecular markers (17). On the whole, these data suggest that the model originally proposed (8) will predict a pattern of combined genetic events leading to tumor development in an extremely low number of SCRCs.

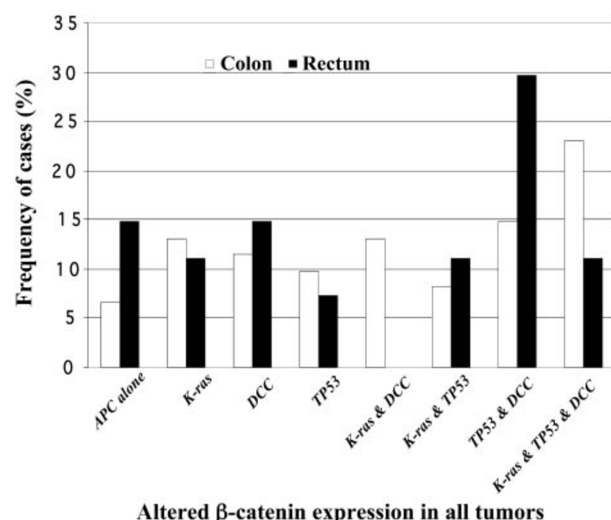


Fig. 3 Comparison of genetic pathway frequencies on the basis of tumor location (colon and rectum) in MIN− SCRCs. The APC-β-catenin pathway is altered in all cases. *SCRC*, sporadic colorectal cancer; *MIN*, microsatellite instability; *APC*, adenomatous polyposis coli.

Our findings indicated that in MIN− SCRCs the APC-β-catenin pathway is always inactivated and represents the first hit leading to the sporadic development of colorectal neoplasia. In contrast, the MIN+ SCRC specimens with a high-MIN (sustained by the National Cancer Institute panel and 18q analysis) showed a low *APC* mutational frequency. These data were confirmed by immunohistochemical staining that demonstrated a cytoplasmic and nuclear β-catenin overexpression in all MIN− SCRCs in contrast to a β-catenin expression confined to the plasma membrane in MIN+ SCRCs (with the exception of the *APC* double-mutated case). The data strongly support that MIN+ SCRCs follow an APC-independent pathway of tumor development, different from that of SCRC without MIN.

In the present series of MIN− SCRCs, when we limited the analysis to mutational spectra of *APC, K-Ras*, and *TP53* genes, in agreement with Smith *et al.* (17), we found that one of the most common combinations was not only *APC-TP53* but also *APC-K-ras-TP53* and *APC-K-ras* mutational patterns were highly represented. This has led to a tentative conclusion that the model of Fearon and Vogelstein, although it remains the most common pathway in colorectal tumorigenesis, is representative for only a subset of MIN− CRCs (about 25%). The greater number of mutations detected in our series could be justified by the lower frequency of *APC* and *K-ras* mutations reported by

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Smith *et al.* (17), most likely because of the use of less sensitive techniques.

We subsequently extended the analysis to nine microsatellite loci of *Ch18q*. It is noteworthy that, to our knowledge, this is the first investigation where the four molecular markers are characterized together in terms of gene mutations or losses in a large series of SCRCs. The expanded analysis showed a wide variability of combinations of mutations with a prevalence of two combinations that cumulatively encompassed about 40% of the MIN− SCRCs (*e.g.,* the *APC-K-ras-TP53-Ch18q* and the *APC-TP53-Ch18q*). These results strongly support the existence of two preferential pathways followed by sporadic colorectal tumorigenesis [*e.g.,* one *K-ras* dependent, in agreement with the model of Fearon and Vogelstein (8), and the other *K-ras* independent].

Despite clinical evidence of differences between colon and rectum cancers in terms of metastatic sites, treatment modalities, and outcome, few studies have addressed to molecular and/or biological differences between the two diseases and generally have focused on a single marker (27, 28). In a recent contribution, Kapitejin *et al.* (18) through the analysis of several molecular markers, showed a significantly different β-catenin and TP53 expression in a series of colon and rectal cancers and concluded that these two diseases could follow different mechanisms of oncogenesis. Expanding the spectrum of molecular alterations, we confirmed the presence of significant differences between colon and rectal tumors in our series of MIN− SCRCs. The most striking difference was seen in the K-ras-dependent pathway, which is preferentially followed by colon cancer, in agreement with the model of Fearon and Vogelstein. In contrast, the K-ras-independent pathway was predominant in rectal cancer, because in this tumor the alterations in the four molecular markers were rarely detected, and the combined alterations *APC-K-ras-Ch18q*, the second dominant pathway in colon cancer, were absent. Overall, the different pathways observed and their distribution can be summarized as follow: (*a*) *K-ras* mutations were more commonly detected in colon than in rectal samples; (*b*) irrespectively of the genes involved, the number of mutations detected was significantly higher in colon than in rectal tumors; and (*c*) a mutational pattern restricted to the *APC* gene was more common in rectal than in colon tumors.

In conclusion, by applying a comprehensive molecular approach, we were able to identify at least one genetic lesion in 97% of the analyzed colorectal tumors. It is therefore reasonable to predict that the molecular diagnosis can be translated into a clinical setting to improve the diagnosis and to direct a rationale pharmacological treatment. However, it should be remembered that although the feasibility of a multi-target assay based on a panel of molecular markers has been proposed for the analysis of body fluids (29), *APC* and *TP53* display a large mutational spectrum, and their analyses are expensive and time-consuming. Furthermore, the analysis of *K-ras* mutations, which we found to be more specifically detectable in colon than in rectal carcinoma, is not always informative, because *K-ras* alterations cannot be easily detected in stools of patients with right-sided colon cancer (30, 31). To take full clinical advantage of these molecular advances, we therefore need to develop new sensitive and easy-to-handle methodological approaches to the already known genetic markers and to extend the repertoire of possible additional genetic alterations involved in colorectal carcinogenesis.

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