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K-*ras* Codon 12 Mutation Determines the Polypoid Growth of Colorectal Cancer[1]

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ABSTRACT

Colorectal carcinogenesis is widely thought to follow the adenoma-adenocarcinoma sequence. However, there are two morphologically distinct subtypes of colorectal cancer (CRC), polypoid and ulcerative. We conducted a comparative study to clarify whether different combinations of some commonly involved genetic alterations (including mutations in K-*ras*, *p53*, *DCC*, *APC*, and *Rb* genes) may exist between polypoid- and ulcerative-type CRCs, the two morphologically distinct types of CRC. By using PCR-based RFLP, single-strand conformational polymorphism, and loss of heterozygosity analysis, we found that K-*ras* codon 12 mutation was preferentially involved in polypoid tumor ($P < 0.0001$). There were no other significant correlations with *p53* point mutation or loss of heterozygosity in chromosomes 5q, 17p, and 18q and *Rb* gene, which have been suggested to be involved in the progression of CRC of both morphological types. Therefore, different combinations of molecular genetic alterations may be involved in morphologically distinct types of colorectal carcinogenesis, and the K-*ras* codon 12 mutations may play an important role in polypoid growth of CRC. These results shed light on the function of K-*ras* oncogenes involved in colorectal carcinogenesis and may be important in the future design of genetic screening programs, determination of prognosis, and treatment for patients with CRC.

INTRODUCTION

Colorectal tumorigenesis has been widely studied at the molecular genetic level. A number of genetic alterations within oncogenes and tumor suppressor genes have been associated with CRC.[3] Among them, the most common genes involved are K-*ras*, *APC*, *p53*, and *DCC*. Alterations in *APC* and K-*ras* genes are considered to be earlier events, whereas *DCC* and *p53* genes are thought to be involved the late stages of carcinogenesis (1, 2). In terms of gross morphology, however, CRC is not a homogeneous disease entity. There are two morphologically distinct subtypes of CRC, polypoid and ulcerative (Fig. 1, *A* and *B*, respectively). These are distinguished based on differences in gross appearance due to exophytic and endophytic formation. Polypoid tumors always show distinguished exophytic growth with varied degrees of muscular invasion (Fig. 1*A*). They typically have a cauliflower-like appearance. An ulcerative tumor is usually endophytic growth, with the floor of the ulcer below the surface of the surrounding mucosa (Fig. 1*B*). These divergent clinical features raise the possibility that two distinct morphological types of CRC may involve different genetic pathways or different combinations of genetic alteration during tumorigenesis. Histopathologically, however, these two different morphological types of CRC follow the same polyp-cancer sequence as that shown by microscopic findings of adenomatous remnants in carcinoma (3, 4). Little is known about whether or not there are different combinations of genetic alteration(s) between these different types of CRC (5, 6). Therefore, in this study, we tried to clarify whether the presence of different combinations of some commonly identified genetic alterations (including K-*ras*, *p53*, *APC*, *DCC*, and *Rb*) could differentiate between polypoid and ulcerative colorectal carcinoma.

MATERIALS AND METHODS

Sample Collections. We collected 57 fresh tumor specimens of primary CRC from 57 patients who underwent colectomy at Chang Gung Memorial Hospital between June 1995 and June 1996. There were 31 polypoid tumors and 26 ulcerative tumors. The specimens had the histopathological characteristics of adenocarcinoma, with tumor sizes ranging from 2 to 8 cm. The detailed morphological description, histopathological data, and clinical follow-up were recorded for each case. Tumors with exophytic cauliflower-like appearance, with heights exceeding half their diameter, were classified as polypoid. Tumors within depressed ulcers without or with very low elevation edges, showing endophytic growth, were classified as ulcerative. All samples were collected immediately after resection in the operation theater, quickly frozen in liquid nitrogen, and then stored at −80°C freezer until used. Normal mucosa sited ~10 cm from the tumor was also removed as control at the same time.

DNA Extraction. Genomic DNA was isolated from 300 mg of tumor and normal mucosa by standard proteinase K digestion and phenol/chloroform extraction procedure (7). The DNA was precipitated with ethanol, dissolved in 500 μl of H_2O, and used for PCR.

Analysis of K-*ras* Mutation. Mutations in K-*ras* exon 1 codon 12 were screened by mismatched primer-mediated PCR amplification followed by RFLP analysis (8, 9). A nested PCR amplification, with the pattern A and B followed by A and C, was performed with the following primers (8): upstream A, 5′-ACTGAATAAACTTGTGGTAGTTGGACCT-3′; downstream B, 5′-TCAAAGAATGGTCCTGGACC-3′; and downstream C, 5′-TAATATGTC-GACTAAAACAAGATTTACCTC-3′. The primers were used in 50 μl of reaction mixture containing 1 unit of Taq polymerase, 1 μM each primer (A and B or A and C) dNTPs (dATP, dCTP, dGTP, and dTTP) at 0.2 mM each, 2.0 mM $MgCl_2$, 60 mM KCl, and 10 mM Tris-HCl (pH 8.8). The reaction mixtures were then subjected to amplification, each cycle comprising 94°C for 1 min, 56°C for 1 min, and 72°C for 30 s. The first PCR comprised 40 cycles, followed by a *Bst*NI digestion for 1 h at 60°C. Five hundred-fold dilution of the first digestion was used as template for the second PCR. The product was then cleaved again by *Bst*NI and electrophoresed on 3% Nusieve agarose gel. PCR products encoding the wild-type and mutant sequences were distinguished as 114- and 143-bp fragments, respectively, by digestion with the restriction enzyme *Bst*NI (New England Biolabs). The second amplification gave rise to a 135-bp product, which cleaves with *Bst*NI, if codon 12 is normal, to a 106-bp fragment (Fig. 2) but fails to cleave if there is a mutation in the first two bases of codon 12.

Analysis of *p53* Point Mutation. By using a PCR-SSCP approach (10, 11), we analyzed all 57 CRCs for mutations in exons 5, 6, 7, and 8 of the *p53* gene. The 10-μl PCR mixture contained 50 ng each of the labeled primers shown in other studies (10, 11), 10 nmol each of the four deoxynucleotides, 0.1 μg of genomic sample DNA, and 0.5 unit of Taq polymerase in 10× PCR buffer specified in the GeneAmp Kit (Perkin-Elmer Cetus). Thirty cycles of the reaction at 95°C for 1 min, 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min were run in a thermocycler (Perkin-Elmer/Cetus). An equal amount (10 μl) of loading dye was added, and electrophoresis using GeneGel Excel 12.5/24 (12.5% T, 2% C) kit (Pharmacia Biotech, Uppsala, Sweden) was performed at 60 W for 80 min with a constant temperature system (Gene Phor Electrophoresis Unit; Pharmacia Biotech). Gels were stained using Plus One Silver Staining Kit according to the manufacturer's guidelines (Fig. 3).

Analysis of 5q, 17p, 18q, and *Rb* LOH. All samples were also examined for genetic alterations at the loci of seven separate microsatellites, including 5q

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[3] The abbreviations used are: CRC, colorectal cancer; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity.

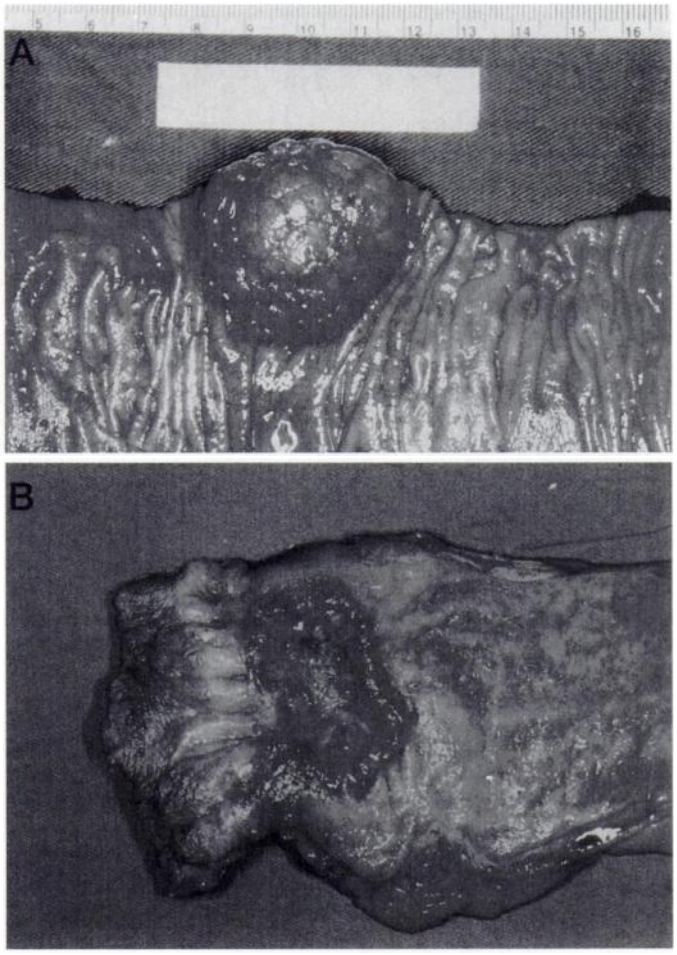

Fig. 1. *A*, polypoid tumor. *B*, ulcerative tumor.

[*D5S136* (*APC*); Ref. 37], 17p (*TP53* and *D17S513*; Refs. 38 and 39), 18q (*D18S34*, *D18S35*, and *DCC*; Refs. 40 and 41), and *Rb* (42). All these microsatellites are dinucleotide repeats. PCR analyses of these microsatellite DNA markers were amplified in a thermocycler (Perkin-Elmer/Cetus). The PCR was performed in a total volume of 25 μl containing 50 ng of genomic DNA template; 0.4 μM unlabeled primer; 0.4 μM of end-labeled primer; 200 μM each dATP, dCTP, dGTP, and dTTP; 1× PCR buffer [10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin]; and 0.6 unit of Taq DNA polymerase. Samples were processed through 40 temperature cycles, the parameters of which depended upon each specific probe. Amplified PCR products were analyzed on 6% polyacrylamide DNA sequencing gel. Gels were dried and subjected to autoradiography for 10–16 h. The data were analyzed by Digital Imaging & Analysis Systems (Alpha Innotech Corporation, CA). As shown in Fig. 4, LOH was considered present in a tumor when the PCR assay for the normal tissue showed heterozygosity of the microsatellite marker and the relative intensity of the two alleles in the tumor DNA differed from the relative intensity in the normal DNA by a factor of at least 1.5.

Statistical Analysis. To compare the incidence of each genetic alteration between the polypoid and ulcerative groups, the Student's *t* test was used. $P < 0.05$ was considered to be significant.

RESULTS

The basic characteristics of clinicopathological factors between polypoid and ulcerative subgroups are shown in Table 1. There were no significant differences found between these two subgroups in terms of clinical parameters, including age, sex, tumor size, tumor stage, and tumor differentiation, except that the right colon had a higher incidence of polypoid tumor than the left colon. The frequencies of the K-*ras* and the *p53* point mutation, as well as of LOH on 5q, 17p, 18q, and *Rb* gene overall and in subgroups, are shown in Table 2. The overall frequencies of these genetic alterations in our study were: point mutation in K-*ras*, 46%; point mutation in *p53*, 40%; LOH in 5q, 20%; LOH in 17p, 32%; LOH in 18q, 44%; and LOH in *Rb*, 13%. These findings are similar to previous reports (13, 18). However, if we compare the genetic alterations between these two subgroups, there was a significant difference in K-*ras* codon 12 mutation frequency (*i.e.*, polypoid, 75% *versus* ulcerative, 12%; $P < 0.0001$) that was found to correlate with growth appearance of CRC. The other genetic mutations (*p53*, *APC*, *DCC*, and *Rb*) showed similar mutation rates (Table 2). Microsatellite instability was also found with higher frequency in polypoid tumor, but this difference was marginally significant ($P = 0.06$).

Interestingly, among 57 cases, 2 had concomitant polypoid and ulcerative tumors. K-*ras* codon 12 mutation was only found in the polypoid tumor, not in the ulcerative tumor, although the two types of tumor were synchronously present, whereas *p53* point mutation and LOH in 18q, 17p, *APC*, and *Rb* were randomly present in both types of tumor (Table 3).

DISCUSSION

Here, we performed a comparative analysis of genetic changes in the polypoid and ulcerative subgroups of CRC. Our analysis attempted to determine whether identifiable genetic events underlie the process of colorectal carcinogenesis and, thereby, lead to the determination of the morphological differences. The most striking finding was the different incidence of K-*ras* mutations in these two morphologically distinct types of CRC, which both follow the adenoma-adenocarcinoma sequence in the pathogenesis. Morphologically, the gross appearance of advanced sporadic CRC is quite variable. In general, the gross morphology of endoscopically detected CRC can be divided into polypoid and ulcerative types, although intermediate or mixed types can be found (46). Other less frequently encountered types are plateau or flat tumors or the rare pipe-like shape (linitis plastica) tumors. It is usually difficult to categorize those intermediate tumors showing mixed appearance into either the polypoid or ulcerative group. Therefore, we started this study with these two pure groups, pure polypoid and pure ulcerative tumors (Fig. 1, *A* and *B*, respectively). Although a proposed molecular mechanism of colorectal carcinogenesis based on the adenoma-carcinoma sequence (3, 4)

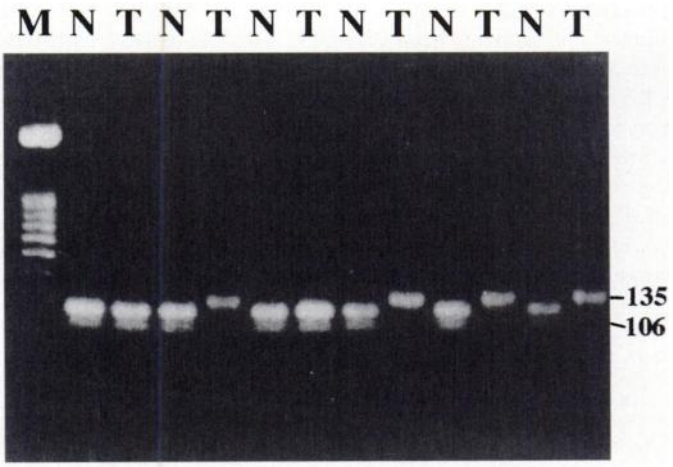

Fig. 2. A nested PCR-RFLP analysis of K-*ras* codon 12 mutations in colorectal tumors. By this method, the wild-type (*Lanes N*) and mutant (*Lanes T*) fragments were detected at positions of 106 and 135 bp, respectively. *Lane M*, molecular size marker (ϕx 174 DNA).

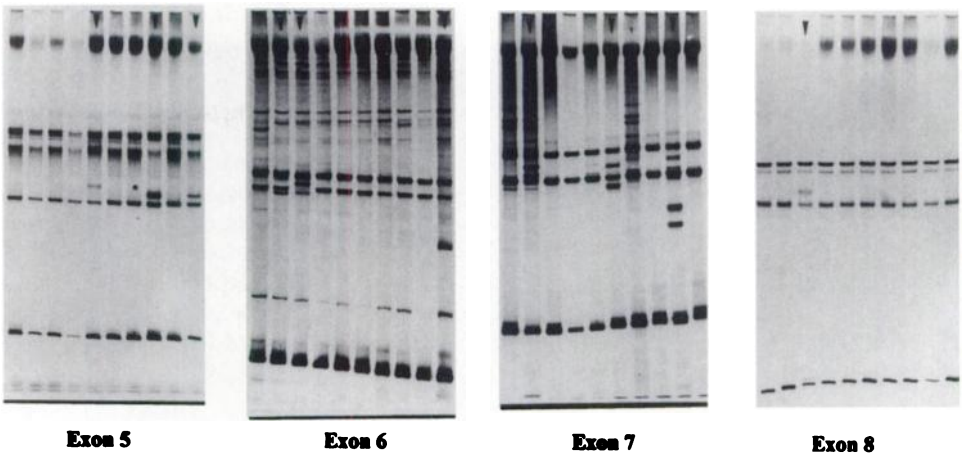

Fig. 3. SSCP analysis of *p53* mutations in colorectal tumors. PCR-amplified fragments run on a GeneGel Excel 12.5/24 at 15°C. Representative sample are shown for exons 5–8. Samples were scored positive for mutations (*arrowheads*) when bands that were different from the normal control were detectable.

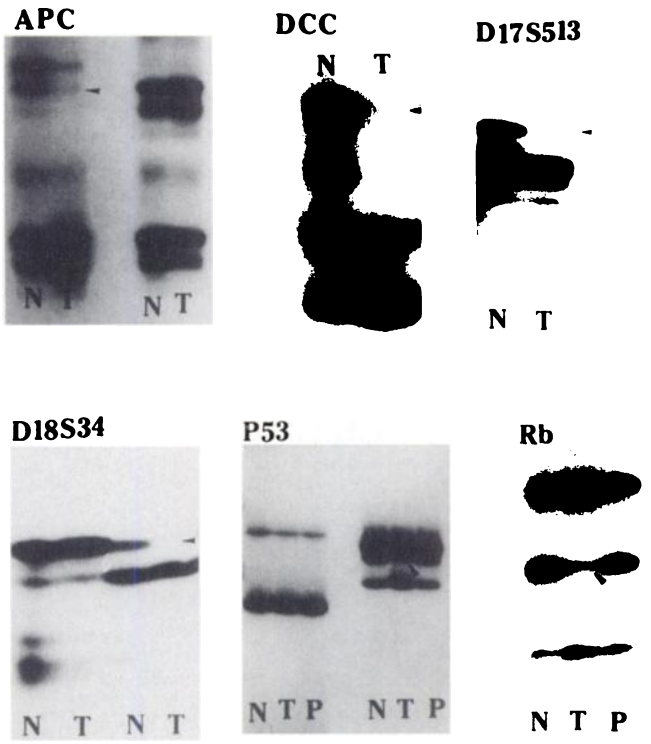

Fig. 4. Examples of the LOH revealed by each of the polymorphisms used in this study. *Arrowhead*, position of the missing allele. *Lanes N*, fragments produced from normal tissue; *Lanes T*, fragments produced from tumor; *Lanes P*, fragments produced from polyp.

appears to be reasonable (1, 12), actually, the accumulation of genetic mutations should not be viewed as an orderly progression of mutations in different genes (2). Rather, it appears that genes are affected in a multistep chain of genetic mutation that occurs in a random combination. Therefore, the diversity of gross appearance in colorectal tumor may be one reflecting the different combinations of underlying genetic alterations (5, 6).

The reported incidence of K-*ras* mutation in CRC has varied from 17 to 53% in different series of sporadic or hereditary CRC (1, 13–18). It is interesting to note that these varied data perhaps reflect the different polypoid:ulcerative adenocarcinoma ratios included in these studies. Our data might also explain the higher incidence of K-*ras* mutation in large polyps than in carcinoma in the model of Vogelstein *et al.* (1) because ulcerative CRCs usually have a higher incidence than polypoid CRCs. Besides, varied prevalence of K-*ras* mutation has already been observed in CRC of different etiologies. For example, a lower prevalence of K-*ras* mutation (8–24%) was found in ulcerative colitis-associated adenocarcinoma, which occurs in a dysplasia-carcinoma sequence (19–21). Interestingly, these tumors almost showed ulcerative appearance. Some other studies have also reported a lower prevalence of K-*ras* mutation among flat-type colorectal adenomas (22, 23). When our findings are considered in the context of previous reports, it becomes clear that K-*ras* codon 12 mutation is associated with polypoid, protruding, or exophytic growth-type adenocarcinoma, whereas low frequency of K-*ras* mutation is found in superficial, ulcerative, or endophytic growth types of colorectal tumor. Furthermore, our study includes K-*ras*, *p53*, *APC*, *DCC*, and *Rb* genes to show that the different mutation patterns in polypoid and ulcerative CRC may manifest in a different route of malignant transformation and may indicate a different histogenesis. This finding suggests that two different molecular genetic pathways underlying the adenoma-adenocarcinoma sequence might be present, one of which involves the K-*ras* codon 12 mutation and another that does not.

The high incidence (90%) of K-*ras* mutation in polypoid CRC can be partially attributed to different environmental factors or carcinogens. Some experimental studies in carcinogenesis of CRC have already demonstrated that K-*ras* mutations can be preferentially induced by different carcinogens in different types of tumor. For example, a high incidence of K-ras mutation (90%) has been reported for

Table 1 *Basic characteristics between polypoid and ulcerative CRC*

Patient characteristic	Tumor type	
	Polypoid (n = 31)	Ulcerative (n = 26)
Age, yr (mean)	29–81 (62)	42–83 (61)
Sex, no. of patients		
Male	17	16
Female	14	10
Tumor location, no. of patients		
Right colon	5	1
Left colon	11	9
Rectum	15	16
Tumor stage (Dukes'), no. of patients		
A	4	0
B	16	14
C	9	9
D	2	3
Tumor size, no. of patients		
>5 cm	11	7
≤5 cm	20	19
Tumor differentiation, no. of patients		
Well	10	6
Moderately	19	20
Poorly	2	0

Table 2 *Genetic alterations in CRC*

Genetic alteration	Type of cancer: All cancer (n = 57), no. (%)	Polypoid cancer (n = 31), no. (%)	Ulcerative cancer (n = 26), no. (%)	P
K-*ras* codon 12 point mutation	26 (46%)	23 (75%)	3 (12%)	<0.00001
p53 point mutation	23 (40%)	10 (32%)	13 (50%)	NS[a]
LOH[b]				
5q (*APC*)	10 (20%)	5/29 (17%)	5/22 (23%)	NS
17p (*TP53, D17S513*)	12 (32%)	4/16 (25%)	8/21 (38%)	NS
18q (*D18S34, D18S35, DCC*)	25 (44%)	12/31 (40%)	13/26 (50%)	NS
Rb	7 (13%)	3/30 (10%)	4/23 (17%)	NS
Microsatellite instability[c]	5 (9%)	5/31 (16%)	0/26 (0%)	0.06

[a] NS, not significant.
[b] LOH was considered present in a tumor only when the PCR assay for the normal tissue showed heterozygotes of the microsatellite marker.
[c] Microsatellite instability was considered present in a tumor when at least two or more markers showed extra bands.

various chemically induced tumors (24–26), whereas a low percentage of mutation in the *ras* gene (0–20%) was detected in others (27–29). The nature of these variations was mainly dependent on the types of cell and the inducing agents or carcinogens.

K-*ras* mutations have been found in the stool in some patients with colon cancer, which suggests that mutational diagnosis might be possible (30, 31). However, our data highlight that future designs for genetic screening of CRC should take into consideration the incidence of specific genetic mutation in subgroups of CRC, in other words, a single genetic marker (*e.g.*, K-*ras* in stool example) may not be sufficient to cover all types of CRC, whereas a combination of K-ras and another marker is probably required for genetic screening study. These results also highlight the importance of careful interpretation of genetic mutation as a prognostic factor, as has been suggested in some reports (18, 32, 33). Different interpretations should be given to results when a genetic marker that never plays a role in the tumorigenesis is chosen and when one that plays an important role but may be absent is chosen.

The mechanism by which K-ras confers a growth pattern is still unclear. However, previous studies provide clues to this mechanism. First, the growth of a tumor larger than a few millimeters without necrosis is only achieved under conditions of adequate supply of oxygen and nutrients. Thus, adequate neovascularization or angiogenesis is required to sustain primary tumor enlargement. The previously reported correlation between K-*ras* mutation and up-regulation of vascular endothelium growth factor suggest that the activated K-ras expression may play an additional role by facilitating angiogenesis through the modulation of vascular endothelium growth factor (34, 35); this, in turn, contributes to polypoid growth. However, if this factor is down-regulated from the beginning of carcinogenesis, it may leads to ulcerative appearance. Second, activation of K-*ras* gene mutations may also result in a loss of the capacity for anchorage-independent growth and altered cell differentiation, cell growth, and cell morphology (36, 43). Signaling pathways can be intimately coupled to physical cell adhesion events making the molecular basis of morphogenesis. For example, a biochemical mechanism for the activation of the Ras/mitogen-activated protein kinase pathway by integrins has been described (44, 45).

Table 3 *Genetic alterations in two cases of synchronous polypoid and ulcerative tumor*[a]

Case no. 47/57	Point mutation: K-*ras*	Point mutation: *p53*	LOH: 5q	LOH: 17p	LOH: 18q
Ulcerative	−/−	−/+	+/−	H/−	+/+
Polypoid	+/+	+/+	−/−	H/−	+/−

[a] +, mutation in K-*ras* or *p53* or LOH; −, no mutation in K-*ras* or *p53* or no LOH; H, noninformative homozygous alleles.

In summary, this comparative study showed that different combinations of commonly involved genetic alterations may be present in different morphological subgroups of CRC and that the K-ras mutation dependent pathway plays an important role in the polypoid growth of CRC. These data strongly suggest that different types of genetic mutation confer CRC with distinct growth appearances. Although tumorigenesis is a multistep process and a single point mutational event is not easily reconciled with the observed complexity of the process, our observations provide new information on the relationships between tumor character and genetic alteration in carcinogenesis.

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