Colorectal Cancer With and Without Microsatellite Instability Involves Different Genes

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There is evidence supporting a multistep genetic model for colorectal tumorigenesis. In familial adenomatosis polyposis (FAP), the inherited defect is a mutation in the *APC* gene. The vast majority of all sporadic colorectal cancers also show mutations in the *APC* gene, and the tumorigenesis in sporadic colorectal cancer and FAP is assumed to involve the same genes. Hereditary nonpolyposis colorectal cancer (HNPCC) is associated with germline mutations in DNA mismatch repair genes and, as a result of defective mismatch repair, microsatellite instability (MSI) is frequently seen. Tumorigenesis in HNPCC was first thought to involve mutations in the same genes as in FAP and sporadic colorectal cancer. Recently, however, an alternative pathway to development of colorectal cancer has been suggested in colorectal tumors with MSI, compared to those tumors without the MSI phenotype. We used a consecutive series of 191 sporadic colorectal cancers to find out if there were any differences between the two groups of tumors regarding the prevalence of mutations in the *APC*, *KRAS*, *TP53*, and *TGFβR2* genes. As expected, 86% (19/22) of MSI-positive tumors showed a mutation in *TGFβR2*, while only one of 164 (0.6%) MSI-negative tumors did. A highly statistically significant negative association was found between MSI and alterations in *APC* and *TP53*. The MSI-positive tumors were screened for mutations in exon 3 of β-catenin, which has been suggested to substitute for the *APC* mutation in the genesis of colorectal cancer, without finding mutations in any of the 22 MSI-positive tumors. The number of mutations found in *KRAS* was lower in MSI-positive than in MSI-negative tumors but the difference was not statistically significant. Our results strongly support the idea that carcinogenesis in MSI-positive and MSI-negative colorectal cancer develops through different pathways. *Genes Chromosomes Cancer 26:247–252, 1999.*

INTRODUCTION

It appears that multiple mutations lead to progression from normal epithelium to metastatic colorectal carcinoma (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). Two common autosomal dominant syndromes with a high risk of colorectal cancer are known (Kinzler and Vogelstein, 1996). In familial adenomatosis polyposis (FAP), the inherited defect is a mutation in the adenomatous polyposis coli (*APC*) gene, which is suggested to work as a gate keeper. The vast majority of all sporadic colorectal cancers also show mutations in the *APC* or β-catenin genes, and the tumorigenesis in sporadic colorectal cancer and FAP has been assumed to involve the same genes (Kinzler and Vogelstein, 1996). The other heritable syndrome, hereditary nonpolyposis colorectal cancer (HNPCC), comprising about 1%–5% of all colorectal carcinomas (Lynch, 1986; Mecklin, 1987), is associated with germline mutations in DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*, *MSH6*) (Kinzler and Vogelstein, 1996; Akiyama et al., 1997; Miyaki et al., 1997). Colorectal cancer in HNPCC was first suggested to occur as a result of mutations in the same genes as in FAP and unselected colorectal cancer (Aaltonen et al., 1993). It appeared that in many tumors the deficiency in mismatch repair occurred before the *APC* mutation, which often seemed to be a direct result of the defective mismatch repair genes in HNPCC (Huang et al., 1996). However, this observation could not be confirmed in a series of unselected colorectal cancers (Homfray et al., 1998).

The presence of mobility shifts or additional bands in the PCR product using microsatellite markers was first observed in unselected colorectal cancer (Ionov et al., 1993; Thibodeau et al., 1993). Microsatellite instability (MSI) is a characteristic phenotype of HNPCC tumors and can be detected in the majority of these tumors (Aaltonen et al., 1993; Lindblom et al., 1993). Huang et al. (1996) showed that also in sporadic MSI-positive colorec-

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tal cancer, the *APC* gene was frequently mutated, often as a result of defective mismatch repair. Thus, the same genes seemed to be involved in all colorectal cancers; only the mechanisms for how mutations occur vary. Besides the *APC* gene, the *KRAS* gene is frequently mutated in sporadic and HNPCC-associated colorectal cancer (Forrester et al., 1987; Aaltonen et al., 1993). The *TP53* gene is mutated in almost all types of cancer (Hollstein et al., 1991), including sporadic and HNPCC-associated colorectal cancer (Baker et al., 1989; Aaltonen et al., 1993).

Many types of tumors, including colorectal cancer, have lost the ability to respond to growth inhibition by the transforming growth factor-beta through TGF beta receptors (Massague, 1996). The small repeat sequences in the type II TGF beta receptor (*TGFβR2*) make it a favorable target for MSI-associated mutator mechanisms and mutations in the *TGFβR2* are prevalent in cancers with mismatch repair deficiency (Markowitz et al., 1995; Parsons et al., 1995). The recent report that germline mutations in *TGFβR2* are associated with an increased risk for colorectal cancer in HNPCC families emphasizes the importance of *TGFβR2* in colorectal cancer tumorigenesis (Lu et al., 1998).

Several studies have suggested that the *APC*, *KRAS*, and *TP53* genes are infrequently mutated in HNPCC and sporadic MSI-positive colorectal cancer (Ionov et al., 1993; Heinen et al., 1995; Cottu et al., 1996; Kahlenberg et al., 1996; Konishi et al., 1996; Breivik et al., 1997; Losi et al., 1997; Olschwang et al., 1997), where instead *TGFβR2* is frequently involved. These observations indicate that there are alternative pathways to carcinogenesis in colorectal cancer. Some of these studies were, however, performed on a small number of colorectal cancer cell lines only (Heinen et al., 1995; Cottu et al., 1996) or HNPCC tumors only (Losi et al., 1997). Others have used a relatively small number of tumors and/or studied only the mutation frequency of one particular gene.

Based on the partly contradictory results from previous studies as well as the limitations of some of the studies performed, there was a rationale for an additional study. In this study, a significant number of consecutive unselected colorectal cancer cases, divided into MSI-positive and MSI-negative, have been used to compare the frequency of mutations in all genes studied in the various prior studies. We used a consecutive series of 191 unselected colorectal tumors with a defined subgroup of 22 MSI-positive tumors to find out if there were any differences between the two groups of tumors regarding the prevalence of mutations in the *APC*, *KRAS*, *TP53*, and *TGFβR2* genes.

MATERIALS AND METHODS

Tumor Samples and Family History of Cancer

This study was based on 191 consecutively collected tumors from unrelated patients with colorectal cancer, treated at the surgical departments in Uppsala and Falun, Sweden, between 1988 and 1992.

DNA Extraction

The samples were frozen and stored at -70°C prior to DNA extraction. DNA was prepared by proteinase-K digestion and phenol-chloroform extraction as described previously (Tannergård et al., 1997).

Microsatellite Instability

MSI was established using three mono- and six dinucleotide repeats markers (*D22S272*, *D22S428*, *PDGF*, *TGFβR2*, BAT-26, and BAT-25). High microsatellite instability (MSI-H) was defined as an alteration in two or more of five informative markers. Tumors showing one alteration but not fulfilling the criteria above were called MSI low (MSI-L), and tumors with no alteration in five informative markers were called microsatellite stable (MSS) (Boland et al., 1998; Percucho et al., 1999). In samples where constitutional DNA was not available, dinucleotide markers could not be used, and MSI was defined as an alteration in at least two loci of three tested mononucleotide markers. The PCR conditions have been previously described (Salahshor et al., 1999).

APC Mutation Screening

Codon 686–1706, a 3-kb fragment of the *APC* exon 15, was amplified using two pairs of primers. This fragment was divided into two overlapping fragments, A and B. Fragment A was amplified using the T7-modified sense primer apc AF: (5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGG/ATGCATGTGGAACTTTGTGG-3′) in combination with the antisense primer apc AR: (5′-GAGGATCCATTAGATGAAGGTGTGGACG-3′). Fragment B was amplified using the T7-modified sense primer apc15eT7: (5′-GGATCCTAATACGACTCACTATAGGAACAGACCACCATGCTTAAATATTCAGATGAGCAGTTGAA-3′) in combination with the antisense primer apc 15j3: (5′-GAGCCTCATCTGTACTTCTGC-

3′) (Powell et al., 1993; Van Der Luut et al., 1994). Approximately 200 ng genomic DNA and 350 ng of each PCR primer were dispensed in 50-µl PCR reaction buffer (200-µM dNTP, 1.5-mM $MgCl_2$, and 2 units Taq polymerase). PCR conditions were 96°C for 1.30 min, followed by 35 cycles (96°C for 30 sec, 60°C for 1.30 min, and 70°C for 1.30 min), and a final elongation at 70°C for 5 min. A protein truncation test (PTT) was performed according to the manufacturer's instructions with some modifications. Two µg of the PCR-amplified product were used in a TnT T7-coupled reticulocyte lysate system (Promega). Incorporation of [^{35}H]methionine (Amersham) was used to detect the translation products after electrophoretic separation on 5% SDS-polyacrylamide gel. The gel was then dried on a vacuum gel dryer and exposed to film overnight at -70°C.

KRAS Mutation Screening

All samples were screened for *KRAS* mutation in codons 12 and 13 using temporal temperature gradient gel electrophoresis (TTGE) (Kressner et al., 1998a).

TGF Beta Type II Receptor Mutation Screening

A 173-bp region of the *TGF*β*R2* (nucleotides 263–35) was amplified and PCR products were separated in a 6% polyacrylamide gel (Tannergård et al., 1997).

TP53 Mutation Screening

Immunohistochemistry (IHC) was used for evaluation of TP53 protein overexpression (Kressner et al., 1996) and cDNA sequencing was used for mutation screening of the entire coding region of the *TP53* gene (Kressner et al., 1998b).

β-Catenin Mutation Screening

Exon 3 of β-catenin was amplified by PCR using primers as described previously (Kitaeva et al., 1997). Fifty ng of DNA was amplified in each reaction with the following reaction conditions: 1 cycles of 96°C for 2 min, 35 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 1 min, and 1 cycle of 72°C for 10 min. The PCR products were purified using Microcon (Amicon). Fifty ng of the PCR products was sequenced using internal primers (Kitaeva et al., 1997) by ThermoSequenase (Amersham) and ^{33}P-labeled ddNTPs (Amersham) according to the manufacturer's instructions.

TABLE 1. Somatic Mutation Frequency in Colorectal Tumors

	Number of mutations (total number of tumors studied)		
Gene	MSI-H	MSI-L	MSS
TGFβR2	19 (22)	1 (7)	0 (158)
APC	5 (22)	1 (6)	90 (158)
KRAS	4 (22)	1 (7)	56 (161)
TP53 (IHC)[a]	1 (22)	2 (7)	84 (154)
TP53 (mut)[a]	1 (22)	3 (6)	92 (154)
β-catenin[b]	0 (22)		

[a]IHC, immunohistochemical detection, which indicates overexpression of *TP53*; mut, mutations identified by direct sequencing.
[b]Only MSI-H tumors were tested for mutations in β-catenin gene.

RESULTS

The frequency of microsatellite instability (MSI-H) in our unselected material was 12% (22/191 tumors). Seven tumors showed allelic instability in only one of the tested markers (MSI-L). One of those seven MSI-L tumors displayed a mutation of *TGF*β*R2*, while the other six all demonstrated a mutated BAT-26.

It has been shown that in sporadic colorectal cancer, microsatellite instability is mostly found within the proximal (right) colon in individuals with a deficient mismatch repair system (Ionov et al., 1993; Thibodeau et al., 1993). In our material 86% (19/22) of MSI-H tumors were located in the proximal colon.

A high frequency of MSI-H tumors (19/22, 86%) showed a mutation in the exonic poly-A tract in the *TGF*β*R2*, whereas none of 158 MSS (microsatellite stable) tumors did (Table 1). There was a highly significant association between MSI-positive tumors and mutations in *TGF*β*R2*, regardless of whether we used MSI-H only or included also the MSI-L among the MSI-positive tumors (Table 2).

Mutations in the *APC* gene were detected in 96 tumors, in 5 of the 22 MSI-H, in 1 MSI-L, and in 90 of the 158 MSS tumors (Table 1). A statistically significant negative association was found between MSI and *APC* gene mutations, regardless of whether MSI-positive tumors included those considered only MSI-H or both MSI-H and MSI-L tumors (Table 2).

It has been suggested that β-catenin mutations might occur more frequently in MSI-positive tumors lacking *APC* mutations, than in MSI-negative tumors with an *APC* abnormality (Kitaeva et al., 1997; Muller et al., 1998; Sparks et al., 1998). We tested the 22 MSI-positive tumors in our material for activating mutations in exon 3 of β-catenin. We screened only this part of the gene, since exon 3

TABLE 2. Somatic Mutation in MSI-Positive versus MSI-Negative Tumors

Gene	Number of samples[a]	Mutation frequency Total (%)	MSI-positive (%)	MSI-negative (%)	χ^2 P
MSI-positive tumors (MSI-H), versus MSI-negative (including MSS and MSI-L)					
TGFβR2	187	20 (11)	19 (86)	1 (0.6)	<0.001
APC	186	96 (51)	5 (23)	91 (55)	<0.01
K-*ras*	190	61 (32)	4 (18)	57 (34)	<0.5
TP53 (IHC)	183	87 (48)	1 (5)	86 (53)	<0.001
TP53 (mut)	182	96 (53)	1 (5)	95 (59)	<0.001
MSI-positive tumors (including MSI-H and MSI-L) versus MSS					
TGFβR2	187	20 (11)	20 (69)	0 (0)	<0.001
APC	186	96 (51)	6 (21)	90 (57)	<0.001
K-*ras*	190	61 (32)	5 (17)	56 (35)	<0.1
TP53 (IHC)	183	87 (48)	4 (14)	83 (54)	<0.001
TP53 (mut)	182	96 (53)	4 (14)	92 (59)	<0.001

[a]Number of informative samples. IHC, immunohistochemical detection, which indicates overexpression of *TP53;* mut, mutations identified by direct sequencing.

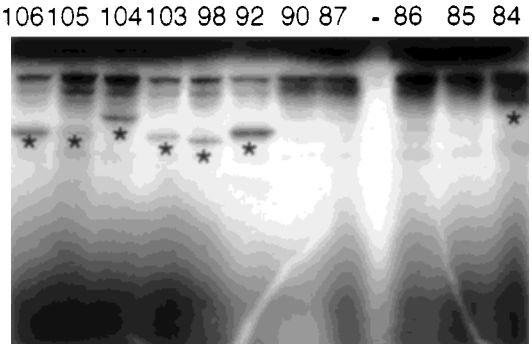

Figure 1.Detection of truncating mutations in exon 15, fragment B (codons 1024–1706) of the *APC* gene by PTT test. Asterisk indicates truncated APC protein.

encodes the NH_2 terminal regulatory domain of β-catenin, previously found to contain activating mutations (Morin et al., 1997; Rubinfeld et al., 1997). None of *22* MSI-positive tumors was mutated in this region. Thus, the low frequency of *APC* mutations in this subset of tumors did not seem to be substituted for by a mutation in the β-catenin gene (Table 1).

KRAS displayed mutations in 18% (4/22) of MSI-H tumors, in 14% (1/7) of MSI-L tumors, and in 35% (56/161) of MSI-negative tumors (Table 1). These differences were not statistically significant regardless of whether the MSI-positive included both MSI-H and MSI-L or not (Table 2).

In our material, only 1 of *22* (5%) MSI-H tumors showed a *TP53* mutation and only 1 (5%) showed TP53 protein overexpression using immunohistochemistry. Two of the seven MSI-L tumors showed TP53 overexpression on immunohistochemistry, and three of six tumors in the same group had a mutation. In 92 of the 154 (59%) MSS tumors mutations in the *TP53* gene were found and 84 tumors showed protein overexpression (Table 1). The difference between MSI-positive and MSI-negative tumors remained statistically significant even if the MSI-L group was included among MSI-positive tumors (Table 2).

DISCUSSION

The results of the present study show that in unselected colorectal MSI-positive tumors there is a lower incidence of *TP53* and *APC* mutations, and a tendency to a lower incidence also of *KRAS* mutations compared to MSI-negative tumors. The *TGFβR2* mutations were found almost exclusively in the MSI-positive tumors. These results are in agreement with previous data presented by Olschwang et al. (1997).

In contrast to Olschwang et al. (1997), we found convincing evidence for a negative correlation between *TP53* mutations and MSI-positive tumors. The reason for this discrepancy between the two studies is most likely due to the large number of tumors in the present study and to the fact that we studied the whole gene. Our results on *TP53* are supported by a study by Kahlenberg et al. (1996), who used 58 sporadic tumors, including 10 MSI-positive, and the study by Breivik et al. (1997), who used an extended number of unselected colorectal tumors and found a negative correlation between MSI-positive tumors and *TP53* mutation. In two studies done by Konishi et al. (1996) and Losi et al. (1997), a lower (<20%) incidence of *TP53* mutations in HNPCC with microsatellite instability was found. In summary, several studies demonstrate a low *TP53* involvement in MSI-positive unselected and HNPCC tumors, in contrast to the early studies on HNPCC (Aaltonen et al., 1993).

In accordance with the findings by Olschwang et al. (1997), but in contrast to the early studies (Aaltonen et al., 1993; Huang et al., 1996), a lower mutation frequency in the *APC* gene was found in MSI-positive tumors compared to MSS tumors. We screened the *APC* gene from codons number 686–1706, which include the mutation cluster region for mutations (Fig. 1). Huang et al. (1996) reported that the mutations in MSI-positive and MSI-negative tumors did not reveal a different distribution, only a different spectrum of mutations indicative of the mechanism behind the etiology of the mutation. Thus, since the mutation cluster region is supposed

to reveal the vast majority of *APC* mutations in colorectal cancer, our design does not explain the difference between MSI-positive and MSI-negative tumors in this respect.

Our results demonstrate that β-catenin mutations occur less frequently than previously suggested in MSI-positive tumors and it seems that activation of the β-catenin/*Tcf* pathway is not always critical for tumor initiation. However, activation through other mechanisms than mutations in the coding sequences or alterations in other components of this pathway may be present in these tumors (Willert et al., 1998).

A lower mutation frequency in *KRAS* was found in MSI-positive tumors. Since the first study by Aaltonen et al. (1993), who found the *KRAS* mutations to be of similar frequency in HNPCC-associated and sporadic carcinomas, several studies, including ours, have demonstrated a lower mutation frequency in MSI-positive tumors. Only one study, by Konishi et al. (1996), has shown a statistically significant difference between MSI-positive and MSI-negative tumors, but in that study the number of MSI-H tumors was very low.

The *TGFβR2* gene has previously been reported to be inactivated by mutation in 90% of human MSI-positive colon tumors (Markowitz et al., 1995), which is in line with the present findings. The high frequency of mutations is not unexpected since the mechanism for this particular mutation is the MSI phenotype itself, and indicates that the *TGFβR2* mutation may be one of the most important in the genesis of MSI-positive colorectal tumors.

In summary, our results support the idea that carcinogenesis in MSI-positive and MSI-negative unselected colorectal cancer develops through mutations in different genetic pathways. The low incidence of mutations in *TP53* and *APC* in MSI-positive tumors shows that mutations in these genes are not necessary for tumor initiation or progression in colorectal cancer.

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