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A gene marker panel covering the Wnt and the Ras-Raf-MEK-MAPK signalling pathways allows to detect gene mutations in 80% of early (UICC I) colon cancer stages in humans

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ABSTRACT

Background: Very recently a gene marker panel that allows the mutational analysis of *APC*, *CTNNB1*, *B-RAF* and *K-RAS* was conceived. The aim of the present study was to use the 4-gene marker panel covering the Wnt and Ras-Raf-MEK-MAPK signalling pathways to determine the percentage of sporadic colorectal carcinomas (CRC) carrying at least one of the four above-mentioned genes in a mutated form alone and/or in combination with microsatellite instability (MSI) and to compare the sensitivity of the gene marker panel used in this study with that of gene marker panels previously reported in the scientific literature. *Methods*: *CTNNB1* and *B-RAF* were screened by PCR-single-strand conformation polymorphism analysis and *K-RAS* gene mutations by restriction fragment length polymorphism analysis. For the mutational analysis of the *APC* gene mutation cluster region (codons 1243–1567) direct DNA sequencing was performed. The U.S. National Cancer Institute microsatellite panel (BAT25, BAT26, D2S123, D5S346 and D17S250) was used for MSI analysis. *Results*: It could be shown that about 80% of early stage CRC (UICC stages I and II) and over 90% of CRC in the UICC stage IV carried at least one mutated gene and/or showed MSI. No significant increase in the gene mutation frequencies could be determined when comparing tumours in the UICC stage I with those in UICC stage IV. *Conclusions*: When compared with previously published gene marker panels the 4-gene marker panel used in the present study shows an excellent performance, allowing to detect genetic alterations in 80–90% of human sporadic CRC samples analyzed.

1. Introduction

Colorectal cancer (CRC) is one of the leading cancer diseases in the Western world. In Germany, more than 70,000 patients are diagnosed every year and 29,000 people die from colorectal cancer [1]. This rate is far too high, taking into account that this cancer type grows very slowly and could be cured if it was diagnosed at an early stage. Several publications have described mutational changes in the different types of CRC including hereditary and sporadic forms. Taken together these studies show very clearly that each of these syndromes has a completely different mutation pattern, thereby affecting different signalling pathways.

Hereditary syndromes like hereditary nonpolyposis colorectal carcinoma (HNPCC) and familial adenomatous polyposis (FAP) represent 10–15% of all colorectal carcinomas [2]. Whereas FAP is the result of a germline mutation in the tumour suppressor gene *APC* [3], about 70% of HNPCC cases are caused by mutations in the cellular mismatch repair system, whereby the genes mainly affected are *hMLH1* and *hMSH2* [4,5]. Microsatellite instability (MSI) is found in 75–100% of HNPCC cases and in 15% of sporadic cancers [6]. Ninety percent of cancers with mutations in mismatch repair genes show MSI as well.

In the early 1990s Vogelstein and co-workers [7–9] postulated that sporadic CRC arise as the result of a so-called adenoma–carcinoma sequence, in which an early bi-allelic inactivation of *APC* is followed by an oncogenic mutation of *K-RAS* and culminates in the inactivation of the tumour suppressor gene *p53* at the transition from adenoma to carcinoma. In accordance with this working hypothesis about 60–80% of human sporadic CRC were shown to harbour mutations in the *APC* gene [10,11]. Furthermore,

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Sparks et al. [12] suggested that mutations in *CTNNB1*, which, as *APC*, is involved in the Wnt signalling pathway and encodes the protein β-catenin, could uniquely substitute for *APC* mutations at early stages of CRC development. Later studies reported that *CTNNB1* mutations can be observed in CRC, although at a significantly lower frequency than that originally reported by Sparks et al. [12] and that *CTNNB1* mutations are associated with CRC showing MSI [13–16]. Subsequently Johnson et al. [17] showed that *CTNNB1* mutations are strongly associated with HNPCC.

In recent years an alternative concept to the above-mentioned adenoma–carcinoma sequence, the so-called serrated pathway, in which hyperplastic polyps give rise to serrated adenomas, these then leading to the development of serrated carcinomas, has been postulated [18–20]. At the molecular level the serrated carcinomas are characterized by MSI, mutation of *B-RAF*/*K-RAS* (mutually exclusive) as well as extensive DNA methylation [20], whereby *B-RAF*/*K-RAS* mutations represent early steps in tumour development along the serrated pathway [21,22].

In a recent study Jass et al. [23] explored the possibility that the early evolution of CRC is not limited to the adenoma–carcinoma sequence and the serrated adenoma pathway, but often combines components of both pathways in a so-called fusion pathway. Specifically, it was suggested that methylation of the DNA repair gene *O-6-methylguanine DNA methyltransferase*, mutation of *K-RAS* and inactivation of the tumour suppressor gene *p53* provide critical combinations of molecular "cross-over" between the two pathways at the stage of precancerous polyps [23].

In the last two decades research groups worldwide screened thousands of tissue samples from CRC patients with varying panels of molecular gene markers that had been reported to be mutated during CRC development. However, due to the diversity of the CRC types analyzed (in some cases coupled with insensitive screening methods) the sensitivity of the marker panels was rather low or high sensitivities reported in small cohorts were not reproducible if applied to a large number of tissues. Very recently a new panel of primers that allows the analysis of the mutational hotspots within the genes coding for APC, CTNNB1, B-RAF and K-RAS was conceived [24]. The four above-mentioned genes were selected because they are involved in the Wnt and the Ras-Raf-MEK-MAPK signalling cascades (MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase) and therefore play a substantial role in the adenoma–carcinoma and in the serrated adenoma pathways.

The long-term goal is to make use of this new primer panel to develop a non-invasive method to detect colon cancer at a very early stage by stool DNA analysis. In a first step towards this long-term goal the percentage of CRC carrying at least one of the four above-mentioned genes in a mutated form alone and in combination with MSI was determined in 50 sporadic CRC samples by using the new primer panel. Furthermore, the results of this study were compared with those obtained with gene marker combinations previously reported in the scientific literature.

2. Material and methods

2.1. Tissue samples

Fifty colorectal cancer samples from patients who underwent surgery were obtained from the Department of Medicine at the University of Würzburg (Germany). Permission for the study was given by the Ethics Commission of the University of Potsdam (Decision 3/18 taken on July 28, 2004). After being adequately informed all patients gave their written consent to the scientific use of the tissue samples. The number of tumours corresponding to the UICC tumour stages I, II, III and IV was 10, 19, 10 and 11, respectively.

2.2. Reagents and kits

Buffer salts were obtained from AppliChem GmbH (Darmstadt, Germany) and Carl Roth GmbH & Co. KG (Karlsruhe, Germany). PCR reagents were delivered by BIOLINE GmbH (Luckenwalde, Germany), restriction enzymes by Roche Diagnostics GmbH (Mannheim, Germany) and New England Biolabs GmbH (Frankfurt, Germany). Kits for purification of PCR products (QIAquick® PCR purification kit) and gel extraction (QIAquick® gel extraction kit) were purchased from Qiagen GmbH (Hilden, Germany).

2.3. DNA extraction and fragmentation

Genomic DNA was extracted from the tissues using a commercial kit (Stratagene Corp.; Amsterdam, Netherlands) and subsequently submitted to whole genome amplification by using the REPLI-g® Mini kit (Qiagen GmbH) according to the manufacturer's instructions.

2.4. Mutational analysis

Control fragments for use in PCR-single-strand conformation polymorphism (PCR-SSCP) or in restriction fragment length polymorphism (RFLP) analyses were cloned from human carcinoma cell lines with known mutations or were constructed by site directed mutagenesis. The following mutations were used as positive controls: codon 600 (GTG → GAG) in the *B-RAF* gene; codon 33 (TCT → TAT), codon 41 (ACC → GCC and ACC → ATC), codon 45 (TCT → CCT and TCT → TTT) and *del* codon 45 (deletion of the complete codon) in *CTNNB1*; codon 12 (GGT → GAT) and codon 13 (GGC → GAC) in *K-RAS*.

PCR-SSCP mutational analysis of *B-RAF* and *CTNNB1* was performed as follows: 20 ng genomic DNA were amplified by conventional PCR with sequence-specific primers flanking codons 582–620 and 6–76 [24], respectively. The amplicons were purified from a 2% agarose gel. Electrophoresis of 4 μl denatured product was performed by using a 16 cm × 20 cm non-denaturing polyacrylamide gel in 1 × TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA; pH 8.0) with 20 mmol/l *N*-(2-hydroxyethyl)piperazine-1-*N*′-(2-ethanesulfonic acid). In order to detect the *B-RAF* mutation, a 10% polyacrylamide gel was used at 18 °C with 300 V for 4.5 h. In the case of the *CTNNB1* mutation analysis a 13% gel was run at 26 °C with 250 V for 5 h and an 8% gel at 21 °C with 200 V for 5 h. Gels were silver stained and shifted bands were cut out, reamplified, purified and sequenced.

K-RAS gene mutations were screened by RFLP analysis according to the method described by Schimanski et al. [25]. Briefly, 2 μl of the whole genome amplification product obtained by using the REPLI-g Mini kit were PCR-amplified with the primer pair Ras A and Ras B (for primer sequences see Schimanski et al. [25]), generating a 166 bp amplicon. The mismatch primer Ras A hybridizing with codons 2–11 of the *K-RAS* gene exon 1 introduces a restriction site for *Bst*XI or *Xcm*I into the amplicon. In case that a template is mutated in codon 12 or 13, the restriction site is lost. The subsequent enzymatic restriction leads to an enrichment of mutated DNA strands. Gel bands having the size of the mutated control fragments were cut out and sequenced.

For the mutational analysis of the *APC* gene mutation cluster region (codons 1243–1567) direct DNA sequencing was used. Sequencing was performed at GATC Biotech AG (Konstanz, Germany).

2.5. MSI analysis

The U.S. National Cancer Institute microsatellite panel (BAT25, BAT26, D2S123, D5S346 and D17S250) was used for MSI analysis

Table 1
Primer sequences for MSI analysis.

Marker	Sequence	Chromosomal localization
D2S123	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	2p16.3
D5S346	ACTCACTCTAGTGATAAATCGGG AGCAGATAAGACAGTATTACTAGTT	5q22.2
BAT25	TCGCCTCCAAGAATGTAAGT TCTGCATTTTAACTATGGCTC	4q12
BAT26	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACCC	2p16
D17S250	GGAAGAATCAAATAGACAAT GCTGGCCATATATATATTTAAACC	17q11.2–17q12

[26]. The corresponding primer sequences are shown in Table 1. For PCR one of the primers of each primer pair is labeled with a fluorescent dye (Cy 5 and Cy 5.5; TIB MolBiol, Berlin, Germany) that enables detection of the resulting PCR product. PCR was performed in a 25 μl reaction mix containing 50 nM primers, 200 μM dNTPs, 1.5 mM $MgCl_2$ in 1 × PCR buffer, 50 ng template DNA, and 0.4 U *Taq* Polymerase. Initial denaturation for 3 min at 96 °C was followed by 33 cycles at 96 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s. Electrophoretic separation of the amplified PCR products was performed using a CEQ8000 capillary electrophoresis system (Beckman-Coulter, Krefeld, Germany). An external size standard allows size determination of the PCR products. Carcinomas were scored as high MSI (MSI-H) when ≥2 markers showed instability and as low MSI (MSI-L) when 1 marker displayed instability.

2.6. Statistical analysis

The Fischer's exact test was applied for comparison of the categorical variables. All tests were two-sided. Probability (*P*) values of less than 0.05 were considered to be statistically significant. All data were analyzed with the statistical software SPSS version 11.0 (SPSS Inc., Chicago, USA). In order to perform the Fischer's exact test, the data for the distal colon and the rectum were grouped together.

3. Results

Clinicopathological data regarding the patients and the 50 tumours analyzed are summarized in Table 2. Patients were on average 67.8 years old; 27 were male and 23 female. The mean age of the patients with a CRC in the UICC stage I was somewhat higher than that of patients with CRC in the UICC stages II–IV. However, a detailed analysis of the data revealed that most of the patients with CRC in the UICC stages I–IV were 55–75 years old (UICC I: 7/10; UICC II: 16/19; UICC III: 7/10; UICC IV: 8/11), only one or two patients in each group being younger than 55. Furthermore, no significant association between the age of the patients and the frequency of a certain gene mutation/MSI was found.

Table 2
Patient and tumour characteristics.

	UICC I	UICC II	UICC III	UICC IV
Gender, *n*				
Male/female	4/6	9/10	8/2	6/5
Mean age, years (range)	71.1 (53–89)	66 (30–78)	68.9 (54–90)	65 (44–83)
Tumour location				
Proximal colon	4	9	4	4
Distal colon	1	2	1	2
Rectum	5	8	5	5
Differentiation grade, *n*				
1	1	0	0	0
2	8	16	8	6
3	1	3	2	5

Twenty-one tumours were located in the proximal colon including caecum, ascending colon, hepatic flexure, transverse colon and splenic flexure, 6 tumours were located in the distal colon, consisting of the descending colon and sigmoid colon, and 23 were located in the rectal part composed of the rectosigmoid junction and rectum. Differentiation of tumours decreased with progression of the disease, the number of dedifferentiated tumours (grade 3) in UICC stages I, II, III and IV being 1, 3, 2 and 5, respectively. During the 69 months following surgery 4 patients showed disease recurrence (1 stage II patient and 3 stage III patients) and 14 patients died from cancer (4 out of 10 stage III patients and 10 out of 11 stage IV patients).

Mutations verified by DNA sequencing as well as MSI status are shown in Table 3. Overall, 43 mutations and 10 cases of MSI were detected. *APC* was the most frequently mutated gene among the examined targets (22 mutations) followed by *K-RAS* (12 mutations), *B-RAF* (7 mutations) and *CTNNB1* (2 mutations). The same was true for the mutations within every single tumour stage. However, no significant increase in the amount of mutations or in the number of tumours with MSI was observed between the different tumour stages.

All the mutations detected in *K-RAS*, *B-RAF* and *CTNNB1* were single base substitutions, whereas in the case of *APC* insertions and deletions were more frequent than single base substitutions (Table 3). The mutations detected in the *K-RAS* gene affected eleven times codon 12 and once codon 13. All seven mutations detected in the *B-RAF* gene affected codon 600, while two *CTNNB1* mutations, affecting once codon 41 and once codon 45, were identified. Whereas each of the single base substitutions in *K-RAS*, *B-RAF* and *CTNNB1* leads to the exchange of an amino acid in the corresponding gene product, the three single base substitutions in the *APC* gene results in a stop codon leading to protein truncation. Deletions in *APC* were detected in 11 cases, insertions in 8 cases. Codon 1309 (deletion of 5 bp), codon 1400 (deletion of a single C) and codon 1556 (insertion of a single A) of the *APC* gene were found to be modified in two tumour samples each. All determined deletions or insertions cause frameshifts. Concomitant mutations of *K-RAS* and *APC* within the same sample were found in 6 out of 50 tumours and mutations of *CTNNB1* and *APC* within the same sample in 1 out of 50 tumours.

By using the selected primer panel mutations were detected in 8 out of 10 stage I carcinomas, whereby 2 carcinomas were mutated in the *K-RAS* as well as in the *APC* gene. The percentage of mutated tumours in stages II–IV was 74% (18 mutations in 19 stage II carcinomas, including three carcinomas with mutations in *K-RAS* as well as in the *APC* gene and one carcinoma with a mutation in the *APC* as well as in the *CTNNB1* gene), 50% (5 mutations in 10 stage III carcinomas) and 82% (10 mutations in 11 stage IV carcinomas including one tumour with a mutated *APC* as well as a mutated *K-RAS* gene), respectively (Table 4). Taken together, 36 out of 50 carcinomas (72%) carried at least one mutation in the *APC*, *K-RAS*, *B-RAF* or *CTNNB1* gene.

MSI was detected in 10 out of 50 tumours. Four out of these 10 MSI-positive tumours showed a low level (MSI-L) and the other 6 MSI-positive tumours a high level (MSI-H) of MSI (Table 3). The MSI frequencies were 3 out of 10 for stage I tumours, 4 out of 19 for stage II tumours and 3 out of 11 for stage IV tumours. No MSI was found in stage III tumours.

About half (19/40) of the microsatellite stable (MSS) tumours contained mutations in the *APC* gene, 11/40 had mutations in *K-RAS*, 3/40 in *B-RAF* and 1/40 in *CTNNB1*. In the case of the

Table 3
Mutations and MSI status of CRC tissues (UICC stages I–IV).

Tumour stage (UICC)	Patient no.	Tumour location[a]	MSI status[b]	Mutations			
				K-ras	B-raf	CTNNB1	APC
I	1	1	MSS	Cd 12 GGT → GTT			Cd 1509 Ins[c] A
I	3	11	MSS				Cd 1361 Del C
I	6	10	MSS				
I	8	1	MSI-L	Cd 12 GGT → GAT			Cd 1427 Del CC
I	9	2	MSI-H			Cd 45 TCT → GCT	
I	33	7	MSS				Cd 1465 Del AG
I	34	9	MSS				Cd 1505 Ins T
I	37	1	MSI-H		Cd 600 GTG → GAG		
I	39	12	MSS				Cd 1438 Del A
I	49	8	MSS				
II	7	6	MSS	Cd 12 GGT → GAT			
II	10	2	MSI-L		Cd 600 GTG → GAG		
II	17	8	MSS				Cd 1295 Del A
II	19	2	MSS	Cd 12 GGT → TGT			Cd 1556 Ins A
II	22	9	MSS	Cd 12 GGT → GTT			Cd 1322 GAA → TAA STOP
II	24	10	MSI-H				Cd 1556 Ins A
II	25	2	MSI-H				
II	27	5	MSS		Cd 600 GTG → GAG		
II	30	2	MSS		Cd 600 GTG → GAG		
II	31	8	MSS				
II	32	4	MSS	Cd 12 GGT → TGT			Cd 1450 CGA → TGA STOP
II	35	7	MSS				
II	36	1	MSS	Cd 13 GGC → GAC			
II	41	11	MSS			Cd 41 ACC → ATC	Cd 1309 Del 5 bp
II	42	11	MSS				
II	44	10	MSS				
II	46	4	MSS	Cd 12 GGT → TGT			
II	47	10	MSS				Cd 1294 Del CA
II	48	2	MSI-H		Cd 600 GTG → GAG		
III	4	9	MSS				
III	5	12	MSS				
III	15	1	MSS				Cd 1400 Del C
III	16	4	MSS				Cd 1394 Ins CAGTTCACTT
III	20	8	MSS	Cd 12 GGT → GAT			
III	21	2	MSS		Cd 600 GTG → GAG		
III	23	3	MSS				
III	38	7	MSS				
III	40	10	MSS				
III	50	11	MSS				Cd 1472 Ins 98 bp
IV	2	9	MSS				Cd 1371 Ins A
IV	11	2	MSI-L				Cd 1400 Del C
IV	12	10	MSS				Cd 1309–1311 Del AAAGA
IV	13	10	MSS				Cd 1411 Del T
IV	14	1	MSI-L				
IV	18	1	MSS				Cd 1414 Ins T
IV	26	7	MSS				
IV	28	5	MSI-H		Cd 600 GTG → GAG		
IV	29	11	MSS	Cd 12 GGT → TGT			
IV	43	10	MSS	Cd 12 GGT → GAT			
IV	45	7	MSS	Cd 12 GGT → GAT			Cd 1553 GAA → TAA STOP

[a] *Tumour location*: 1: caecum; 2: ascending colon; 3: hepatic flexure; 4: colon transversum; 5: splenic flexure; 6: descending colon; 7: sigmoid; 8: rectosigmoid; 9: rectum <4 cm; 10: rectum <8 cm; 11: rectum <12 cm; 12: rectum >12 cm.
[b] MSS: microsatellite stable; MSI-L: low microsatellite instability; MSI-H: high microsatellite instability.
[c] Del: deleted; Ins: inserted.

microsatellite unstable (MSI-L and MSI-H) tumours ($n = 10$) three samples carried a mutated *APC*, one sample a mutated *K-RAS*, one sample a mutated *CTNNB1* and four samples a mutated *B-RAF* (Table 5). The correlation between *B-RAF* mutation and MSI was statistically significant ($P = 0.023$).

Overall, 38/50 tumours were at least positive for one of the examined tumour markers (i.e. microsatellite instability or a mutation in *APC*, *K-RAS*, *B-RAF* or *CTNNB1*). Thus, MSI analysis increased the percentage of tumours being positive for at least one of the examined tumour markers to 79% in the case of stage II carcinomas and to 91% in the case of stage IV carcinomas (Table 4). MSI did not enhance the percentage of tumours being positive for at least one of the examined tumour markers in the case of stage I and stage III tumours.

Table 4
Sensitivity of the gene marker panel with and without MSI.

Sensitivity of CRC detection (%)	UICC I	UICC II	UICC III	UICC IV	Total
Mutation markers	80	74	50	82	72
Mutation markers + MSI	80	79	50	91	76

Table 5
Number of MSS, MSI-L and MSI-H tumours with a specific gene mutation.

Gene	MSS	MSI-L	MSI-H	MSI-L + MSI-H
APC	19	2	1	3
CTNNB1	1	0	1	1
K-RAS	11	1	0	1
B-RAF	3	1	3	4

Table 6
Number of tumours with a specific gene mutation in the proximal colon, in the distal colon and in the rectum.

Localization	*APC*	*CTNNB1*	*K-RAS*	*B-RAF*
Proximal colon	8/22	1/2	6/12	7/7
Distal colon	2/22	0/2	2/12	0/7
rectum	12/22	1/2	4/12	0/7

Table 7
Number of MSS, MSI-L and MSI-H tumours in the proximal colon, in the distal colon and in the rectum.

Localization	MSS	MSI-L	MSI-H	MSI-L + MSI-H
Proximal colon	12/21	4/21	5/21	9/21
Distal colon	6/6	0/6	0/6	0/6
Rectum	22/23	0/23	1/23	1/23

In the case of *APC* 19 out of 22 gene mutations were detected in MSS, 2 out of 22 in MSI-L and 1 out of 22 in MSI-H carcinomas (Table 5). Eleven out of 12 *K-RAS* mutations were detected in MSS and 1 out of 12 in MSI-L carcinomas. One *CTNNB1* mutation was identified in a MSS and one in a MSI-H tumour. Three out of seven *B-RAF* mutations were detected in MSS, one out of seven in MSI-L and three out of seven in MSI-H carcinomas.

In a last step the correlation between tumour location and gene mutations/MSI status of tumours was evaluated. The number of proximal colon, distal colon and rectum carcinomas carrying gene mutations was 18/21, 3/6 and 15/23, respectively (Table 6). Mutations affecting the Wnt pathway (*APC* and *CTNNB1*) were significantly more frequent in the colorectum ($P = 0.018$), whereas mutations in *B-RAF* and *K-RAS* genes tended to be more frequent in proximal colon tumours. In the case of *B-RAF* all mutations were found in proximal colon carcinomas, so that this correlation showed a high statistical significance ($P = 0.001$). Nearly all (9/10) tumours showing MSI were present in the proximal colon (Table 7), where they accounted for 43% of the tumours. The association of MSI with tumours developing in the proximal colon was highly significant as well ($P = 0.001$). In contrast, in the rectum only 1 out of 23 tumours showed MSI. There were no tumours with MSI in the distal colon.

4. Discussion

By combining the use of a novel primer panel [24] with a MSI analysis it could be shown that about 80% of early stage CRC (UICC stages I and II) and over 90% of CRC in the UICC stage IV carried at least one mutated gene and/or showed MSI. The fact that only one half of the CRC in the UICC stage III were "positive" for at least one of the tumour markers studied remains presently unexplained. Nevertheless, the mutation frequencies of all four genes analyzed in the present report are within the range of those of previous studies [16,27–29], in which some of the genes screened in the present study were analyzed together with other gene markers. Interestingly, no significant increase in the mutation frequencies of all four genes studied could be determined when comparing tumours in the UICC stage I with those in UICC stage IV. It is assumed that the marker genes analyzed represent early and initiating mutations in CRC development, so that one would not expect to see an increase in the mutation frequencies of these genes but rather mutations in further target genes as tumours progress from benign to malignant stages, as previously pointed out for *APC* mutations [10].

In a next step the ability of the primer panel used in the present study to detect mutated genes in CRC samples was compared with that of previously published and often cited gene marker combinations (Table 8). Table 8 shows that the percentage of CRC carrying certain gene mutations in previous publications varies between 55% and 87% [16,29–33]. If one takes into account that three out of six gene marker combinations [16,30,32] detect mutations in $\leq$60% of the tumours analyzed and that in one study [31] the *CTNNB1* status was analyzed by immunohistochemistry, the primer panel [24] used in the present study shows an excellent performance. In particular, it should be pointed out that with the primer combination used gene mutations were detected in 80% of the analyzed sporadic CRC in UICC stage I, a percentage not achieved with any of the previously published gene marker panels. The results obtained in previous studies as well as those in the present report show that in about 10–20% of CRC up to now not further characterized genetic alterations might occur and that colorectal cancer might even develop along a pathway other than the adenoma–carcinoma sequence [7–9], the serrated adenoma pathway [18–20] or the fusion pathway [23].

The assumption that the gene panel analyzed in the present study as well as MSI play a decisive role in colon cancer development is strongly supported by the data of a very recent oncogenetic tree analysis performed on 971 colon tumours by Sweeney et al. [34]. An oncogenetic tree is a model intended to describe the pathways and sequence of somatic alterations in carcinogenesis without assuming that tumours will fall in mutually exclusive categories. The oncogenetic tree analysis resulted in a reproducible tree with three branches: (1) the first branch was initiated by the methylation of "methylation in tumour" (MINT) sites, predisposing to MSI, methylation of *MLH1* and *TP16*, and *B-RAF* mutation; (2) the second branch was initiated by an *APC* mutation and followed by a *p53* mutation; (3) the third

Table 8
Comparison of the mutational analysis results obtained in the present study with those obtained by using different gene marker combinations previously published in the literature.

Reference	*n*[a]	*APC*[b]	*KRAS*[b]	*p53*[b]	*BRAF*[b]	*CTNNB1*[b]	*Axin2*[b]	MSI[c]	MLH1[d] hypermethylation	%[e]
Deuter and Müller [30]	23	22	22	43						56
Zhang et al. [31]	74		42			27[f]				78
Domingo et al. [32]	40	25	20	22.5	30		20	100[g]	27.5	60
Fransén et al. [33]	130	29	40	42	11.5	0.8		9		78
Conlin et al. [29]	107	56	27	61						87
Lüchtenborg et al. [16]	656	37	36			1				55
Present study	50	44	24		14	4		18		80–91

[a] Number of carcinoma samples analyzed.
[b] Percentage of carcinoma samples carrying the corresponding gene in a mutated form.
[c] Percentage of carcinoma samples showing MSI.
[d] Percentage of carcinoma samples showing *MLH1* hypermethylation.
[e] Percentage of carcinoma samples carrying at least one of the markers used in each individual study.
[f] Activated β-catenin, determined by immunohistochemistry.
[g] Only tumours showing MSI were analyzed.

branch was due to a *K-RAS* mutation and was not followed by any other genetic alteration [34].

MSI was detected in 10 out of 50 tumour samples, whereby 4 were classified as MSI-L and 6 as MSI-H tumours. By including the MSI analysis as a further "tumour marker" the sensitivity of the 4-gene marker panel to detect tumours in the UICC stages II and IV increased by 5% and 9%, respectively, while it did not result in an increase of sensitivity in the case of tumours in the UICC stages I and III. Overall, a modest increase of about 4% was recorded, which is in accordance with the fact that MSI is observed much more frequently in hereditary syndromes such as HNPCC and FAP than in sporadic CRC [6].

APC and *K-RAS* were by far more frequently mutated in MSS tumours than in MSI tumours. In the case of *APC* similar results have been reported by Thorstensen et al. [35], whereas in the case of *K-RAS* Konishi et al. [36] and Jass et al. [37] have shown that it is more frequently mutated in MSI-L than in MSS tumours. The *CTNNB1* gene was only found to be mutated in 2 out of 50 tumours, which is in agreement with previously published data reporting that *CTNNB1* mutations were present in up to 3% of the sporadic CRC analyzed [13–16] and that they are mainly associated with HNPCC [17]. *B-RAF* mutations were found to be present in four MSI-L/H and in three MSS tumours, the mutation frequency being in the range of that reported by Fransén et al. [33].

In the present study all tumours carrying a mutated *B-RAF* gene and 9 out of 10 tumours showing MSI arose in the proximal colon. The results presented herein are in accordance with the observation by Domingo et al. [32] that *B-RAF* mutations are strongly associated with proximal DNA mismatch repair-deficient CRC. All in all 9 out of 21 proximal tumours showed MSI, a percentage that is similar to that previously reported by Thibodeau et al. [38].

In contrast, *APC* gene mutations were more frequently detected in the rectum than in the proximal and distal colon. This observation is in line with the suggestion that toxic compounds in food (e.g. heterocyclic aromatic amines, HCA) may remain quite a long time in direct contact with the epithelial cells in the distal colon and rectum mucosa prior to evacuation, thus facilitating the uptake of these compounds in this particular part of the gastrointestinal tract. Furthermore, it has been shown that male rats fed 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant HCA in cooked meat, develop colon tumours, in which specific-1G mutations within 5′-GGGA-3′ sequences of the *APC* gene were detected [39,40]. Moreover, the same authors suggested that these specific mutations could be useful for mutational fingerprinting of human cancers. None of the colon cancer tissues analyzed in the present study carried such mutations. This could be due to the fact that the above-mentioned PhIP-induced *APC* mutations are only observed in rats, that the patients did not take up significant amounts of PhIP or that the PhIP-induced gene mutation pattern in humans is different from that in rats.

In the present study the mean age of the patients with CRC in the UICC stage I was slightly higher than that of patients with CRC in the UICC stages II–IV. Regarding the association of gene mutations with tumour location one could argue that age possibly is a confounder. Although no obvious association between the age of the patients and the frequency of a certain gene mutation/MSI was observed, this point cannot be ultimately cleared due to the low sample size.

Taken together the results presented in this study show that: (1) the combined use of a 4-gene marker panel [24] together with an MSI analysis allows to detect genetic alterations in 80–90% of human CRC samples (UICC stages I–IV); (2) the primer combination alone allows to detect gene mutations in 80% of CRC in UICC stage I. At the present time the same primer combination is being used to analyze genetic alterations in hyperplastic polyps and adenomas removed from human colon.

Conflict of interest statement

Regarding the primer combination used in this study the University of Potsdam (Potsdam, Germany) has applied for an international patent and Bettina Scholtka and Pablo Steinberg are named as inventors in this application.

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