Advances in treatment and new insights in molecular biology of rectal cancer

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Mechanisms of oncogenesis in colon versus rectal cancer

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INTRODUCTION

Several studies have indicated that there are differences in the aetiology, clinical behaviour, pathological features and genetic abnormalities in cancer of the right colon vs. the left colorectum.[1-5] This evidence supports the theory that the oncogenesis of left- and right-sided colorectal cancers may involve, at least partially, different mechanisms.

By far the best chance for cure in patients with colorectal cancer is radical resection at an early stage. The results of traditional rectal cancer surgery, however, are discouraging with high percentages of local recurrence. Two important factors that have been reported to improve outcome are standardised total mesorectal excision (TME)-surgery[6] and short-term preoperative radiotherapy.[7] In contrast, the main problem for colon cancer patients is the development of distant metastasis. Adjuvant chemotherapy has been shown to improve survival in colon cancer patients.[8] An important prognostic factor in colon cancer is TNM staging, whereas in rectal cancer the surgeon[9] and lateral margin involvement,[10] in addition to TNM-stage, are of important prognostic value.

Many studies have been performed in order to find biological parameters that identify a higher degree of aggressiveness, independently of the known prognostic clinicopathological features of colorectal carcinoma. Such knowledge may help to improve treatment strategies. However, few studies have addressed possible biological differences between rectal and colon cancer and if so, they have investigated only one parameter.[11,12] To be able to investigate prognostic markers in rectal carcinoma, standardised surgery is a prerequisite, since treatment-related variation of outcome should be ruled out.

In this study, the aim was to investigate oncogenes and tumour suppressor genes involved in the oncogenesis of colon and rectal cancers. Mutation and expression profiles were investigated and related to tumour site and prognosis. Rectal cancer patients were treated with standardised surgery performed by an experienced rectal cancer surgeon.

METHODS

Study populations

For this project, 35 colon cancer patients were analysed. These patients were operated on at the Department of Surgery, Leiden University Medical Centre by different surgeons between 1990 and 1994. Between November 1994 and February 1995, 42 rectal cancer patients from 24 hospitals throughout the Netherlands were operated on by Y. Moriya (YM) from the National Cancer Hospital, Tokyo, Japan.[13] The surgical technique was focused on nerve preservation and pararectal resection, similarly to the TME technique.[6]

All histopathological slides were reviewed by a senior pathologist (JHJMvK). WHO classification, histological differentiation, growth pattern of the tumour margin (circumscribed, diffuse), degree of the lymphoid reaction that surrounded the tumour (none/few, extensive) and the numbers of eosinophil granulocytes (none/few, moderate, extensive) were evaluated. The presence of lymphangio-invasive growth was also registered.

Mutation analysis of *APC* and *p53*

Fresh frozen tumour samples were investigated from 22 rectal and 8 colon cancer patients. DNA was extracted from tumour tissue by standard procedures of phenol/chloroform extraction and ethanol precipitation.

APC mutation analysis of the mutation cluster region (MCR) in the rectal cancers was

performed using the protein truncation test (PTT) as described by van der Luijt and Meera Khan.[14]

In the rectal cancers, *p53* mutation analysis of exons 5-8 was performed using polymerase chain reaction (PCR) followed by constant denaturant gel electrophoresis (CDGE) as described by Börresen et al.[15] The exon 5-8 regions of the amplified fragment were sequenced to rule out the presence of mutations not detected by screening. The eight colon tumours were also analysed for *p53* mutations by CDGE,[15] but no sequencing was performed.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were cut 4 µm thick and mounted on APES pre-coated slides. After mounting they were kept in an oven at 37°C overnight.

Sections were deparaffinised in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 minutes. Antigen retrieval was performed according to Table 1. Overnight incubation was done with the primary antibody in 1% phosphate buffered saline/bovine serum albumin (1% PBS-BSA).

For p53 and Bcl-2, rabbit-anti-mouse (RAM) was applied as secondary antibody and swine-anti-rabbit (SWAR) as tertiary antibody. For hMLH1, hMSH2, E-cadherin and ß-catenin, the streptavidin-biotin complex (sABC) staining method was applied, after incubation with biotinylated rabbit anti-mouse (RAM). Staining was performed with AEC (3-amino-9-ethylcarbazol in dimethylformamide). Finally, the sections were counterstained with haematoxylin. Incubation with PBS instead of the primary antibody served as a negative control.

Table 1. Antibodies with their dilution and pre-treatment.

Antibody	Dilution	Pre-treatment
MLH1 (Oncogene research products)	1:50	boiling EDTA
MSH2 (Oncogene research products)	1:100	boiling EDTA
Bcl-2 (mAb clone 124, Boehringer Mannheim)	1:50	boiling citrate buffer
p53 (mAb NCL-p53-DO-7, Novocastra Laboratories Ltd.)	1:1000	boiling citrate buffer
E-cadherin (Anti-E-Cadherin clone HECD-1, Zymed Laboratories Inc.)	1:1000	boiling citrate buffer
β-catenin (Transduction laboratories)	1:24000	boiling citrate buffer

Analysis of staining patterns

The slides were assessed independently by three observers (EK, LCL and GJL). All sections were reviewed by a pathologist experienced in the assessment of immunohistochemical staining (JHJMvK) and discussed until agreement was reached.

For Bcl-2 staining, infiltrating lymphocytes were used as an internal positive control.[16] Bcl-2 was scored as positive if Bcl-2 expression was seen in the cytoplasm of tumour cells, regardless of the number of cells stained.

Nuclear p53 staining was scored in four categories; 0-25%, 26-50%, 51-75% and 76-100%. Results were compared with *p53* mutation analysis to define the immunohistochemical cut-off point for *p53* mutation.

Expression of membranous E-cadherin and ß-catenin was scored as loss/negative (0-75% of the tumour cells positive) or no loss/positive (76-100% of the tumour cells positive).

Normal colorectal tissue served as positive internal control. Apical E-cadherin[17] and nuclear ß-catenin[18] were also scored. Any degree of apical E-cadherin or nuclear ß-catenin staining was accepted as positive.

In order to check staining variability and intra-observer variation, 37 of the 77 ß-catenin slides were stained and analysed a second time. Of 37 slides, only two (5%) were scored differently from the initial scoring, implying good reproducibility of the staining technique and assessment of staining patterns.

Statistics

Data were analysed using SPSS statistical software (version 9.0 for Windows, SPSS, Chicago). Some clinicopathological variables were categorised in fewer categories to avoid statistics with small numbers. Chi-square tests were applied to assess differences in the distribution of parameters among groups. Mann-Whitney tests were used for comparison of continuous variables. Univariate survival analyses were carried out by the Kaplan-Meier method and differences between groups were compared with the log-rank test. For overall survival, all deaths, irrespective of cause, were considered as events. For disease-free survival, events were defined as recurrence of disease or death. Cases with macroscopically incompletely resected tumour or metastases at operation were given a disease-free survival of 0 months. The Cox proportional hazards model was used for multivariate analysis. A P-value of 0.05 or less was considered statistically significant.

RESULTS

The rectal series consisted of 30 males and 12 females (Table 2). Median age of the 42 rectal cancer patients was 66.0 years (range 30-85 years). Median follow-up was 41 months (range 32 to 48 months). Thirty-nine patients (93%) underwent a macroscopically curative resection. Of these, nine (24%) developed distant recurrences in the follow-up and four (10%) developed local recurrences without distant metastases. Disease-free and overall survival were 50% and 66% at four years.

The colon series consisted of 15 males and 20 females. Median age of these patients was 70.0 years (range 39-89 years). Median follow-up was 55 months (range 45 to 91 months). Twenty-seven patients (77%) underwent a curative resection. Of these, seven (26%) developed distant recurrences in the follow-up while no local recurrences were reported. Disease-free and overall survival were 49% and 49%, respectively, at four years.

There were more male patients in the rectal group than the colon group (P=0.01), but no other differences could be found between the series with regard to clinicopathological characteristics. Median follow-up of the 77 colorectal cancer cases was 45 months. Disease-free and overall survival were 48% and 56%, respectively, at four years.

Rectal cancer cases

Analysis of the MCR of *APC* by the PTT revealed 18 truncating mutations in 22 rectal cancers (82%). No correlation could be found between nuclear ß-catenin expression and *APC* mutation analysis (P=0.42, Table 3); there were nine nuclear ß-catenin negative tumours, of which eight showed an *APC* truncating mutation and 12 nuclear ß-catenin positive tumours, of which nine showed an *APC* mutation.

p53 mutation analysis showed mutations in 15 of 22 rectal cancers (68%); of these, 14

Table 2. Clinical and histopathological data for the 77 colorectal cancer patients, n (%).*

	Total (n=77)	Colon (n=35)	Rectum (n=42)	Colon vs. Rectum
Gender				P=0.01
male	45 (58)	15 (43)	30 (71)	
female	32 (42)	20 (57)	12 (29)	
Age (yrs)				P=0.13
median	67.0	70.0	66.0	
range	30-89	39-89	30-85	
Tumour site				NA
caecum	19 (25)	19 (54)		
ascending colon	3 (4)	3 (9)		
transverse colon	6 (8)	6 (17)		
descending colon	7 (9)	7 (20)		
rectum	42 (54)		42 (100)	
WHO classification				P=0.35
adenocarcinoma n.o.s.	64 (87)	27 (79)	37 (93)	
mucoid carcinoma	7 (9)	4 (12)	3 (7)	
adenosquamous carcinoma	2 (3)	2 (6)	-	
undifferentiated carcinoma	1 (1)	1 (3)	-	
unknown	3	1	2	
Differentiation grade				P=0.14
well/moderately	19 (26)	10 (29)	9 (23)	
poorly/undifferentiated	54 (74)	24 (71)	30 (77)	
unknown	4	1	3	
Tumour infiltration				P=0.14
circumscribed	45 (64)	20 (59)	25 (69)	
diffuse	25 (36)	14 (41)	11 (31)	
unknown	7	1	6	
Lymphoid reaction				P=0.12
none/few	59 (84)	27 (79)	32 (89)	
extensive	11 (16)	7 (21)	4 (11)	
unknown	7	1	6	
Eosinophil infiltration				P=0.71
none/few	53 (73)	26 (76)	27 (69)	
moderate/extensive	20 (27)	8 (24)	12 (31)	
unknown	4	1	3	
Lymph-angio invasive growth				P=0.20
no	47 (67)	22 (65)	25 (69)	
yes	23 (33)	12 (35)	11 (31)	
unknown	7	1	6	
TNM stage				P=0.86
I/II	47 (61)	21 (62)	26 (62)	
III/IV	30 (39)	14 (38)	16 (38)	
Curative resection				P=0.06
curative	66 (86)	27 (77)	39 (93)	
non-curative	11 (14)	8 (23)	3 (7)	

* Unknown: in some slides it was not possible to determine all the histological characteristics.
NA=not applicable.

(93%), showed more than 25% p53 overexpression (one tumour with a *p53* mutation was not analysed for p53 immunohistochemistry). Of six tumours without a mutation, only one showed more than 25% overexpression; the cut-off point of 25% was thus shown to be both sensitive and specific for *p53* mutation (P<0.001, Table 3).

No association was found between *APC* and *p53* mutation rate (P=0.75); of the 18 tumours with an *APC* mutation, 12 tumours (67%) showed a *p53* mutation; of the four without an *APC* mutation, three (75%) showed a *p53* mutation. *APC* mutation was not associated with p53 immunohistochemistry either (P=0.69).

In the univariate analysis, no significant correlations were found between marker expression and clinicopathological parameters. Our survival analysis, however, showed a significant correlation between p53 overexpression and worse disease-free survival (P=0.008, Figure 1). Analysis of local recurrence-free survival and distant recurrence-free survival showed that p53 was prognostic for local (P=0.02), but not for distant recurrence (P=0.13). Besides p53, advanced TNM stage was correlated with worse disease-free survival (P=0.03). The Cox regression model showed that p53 expression (P=0.03) was an independent predictor of disease-free survival, but not TNM stage (P=0.15).

Table 3. Results of APC mutation analysis and β-catenin immunhistochemistry, p53 mutation and immunohistochemistry analysis in 22 rectal cancer patients.

	APC mutation analysis	Nuclear β-catenin IHC	p53 mutation analysis			p53 IHC
			Codon	From Base	To Base	
1	trunc	-	WT			-
2	trunc	+		insertion		+
3	trunc	+	272	GTG	TTG	+
4	WT	-		exon 5		+
5	WT	+	273	CGT	TGT	+
6	trunc	-	WT			+
7	trunc	-	248	CGG	TGG	+
8	trunc	-	175	CGC	CAC	-
9	trunc	+	WT			-
10	trunc	-	282	CGG	TGG	+
11	trunc	-	WT			-
12	trunc	+	151	CCC	ACC	+
13	trunc	ND	194	CTT	CGT	+
14	WT	+	WT			-
15	trunc	+	151	CCC	TCC	+
16	trunc	+		exon 5		ND
17	trunc	-	WT			-
18	trunc	+	282	CGG	TGG	+
19	trunc	+	216	GTG	ATG	+
20	trunc	+	WT			-
21	trunc	-	248	CGG	TGG	+
22	WT	+	193	CAT	TAT	+

ND= not determined.

Colon cancer cases

In the colon tumours, no discrepancy was found between *p53* mutation analysis and p53 immunohistochemistry. In four cases, a *p53* mutation was found together with more than 25% p53 expression. The four cases without a mutation did not show more than 25% p53 expression.

In the univariate analysis, no significant correlations were found between marker expression and clinicopathological parameters, nor did survival analysis show significant correlations between p53 (Figure 2) or other marker expression and disease-free or overall survival. Advanced TNM stage (P=0.0004) and male gender (P=0.03) were significantly associated with worse disease-free survival.

Colorectal cases, colon vs. rectal tumours

In Table 4, the results of positive marker expression are shown. There are missing cases, since some staining was not successful. All evaluated colon and rectal cases were positive for hMLH1 and hMSH2. Positive membranous ß-catenin expression was found in 61% of the cases. Rectal cancers showed nuclear ß-catenin expression significantly more often than colon cancers (65% vs. 40%, P=0.04). In total, 22% of the tumours were positive for Bcl-2. Rectal cancers showed positive p53 expression significantly more often than colon cancers (64% vs. 29%, P=0.003). Fifty-eight percent of the tumours showed positive membranous E-cadherin expression and 34% apical E-cadherin expression. No differences were found in Bcl-2, E-cadherin and membranous ß-catenin expression between colon and rectal cancers.

In the univariate analysis, no significant associations were found between marker expression and clinicopathological parameters. Our survival analysis, however, showed a significant correlation between p53 overexpression and worse disease-free survival (P=0.03, Figure 3). Advanced TNM stage (P=0.0004) and age >65 years (P=0.03) were correlated with worse disease-free survival. The Cox regression model showed that age (P=0.007) was an independent predictor for disease-free survival, but not TNM stage (P=0.12) or p53 expression (P=0.09).

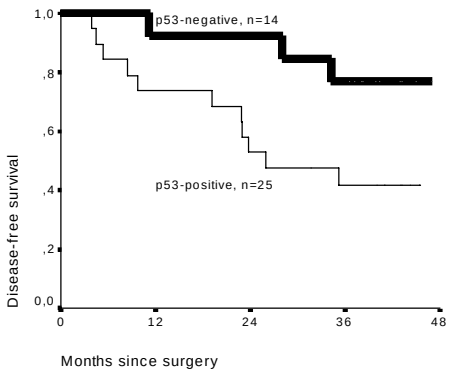
Table 4. Results of positive immunohistochemical marker expression for 77 colorectal cancer patients.*

	Total positive cases / n (%)	Colon positive cases / n (%)	Rectum positive cases / n (%)	Colon vs. Rectum
MLH1	66/66 (100)	31/31 (100)	35/35 (100)	P=NA
MSH2	67/67 (100)	31/31 (100)	36/36 (100)	P=NA
β-catenin membranous	41/67 (61)	18/30 (60)	23/37 (62)	P=0.86
β-catenin nuclear	36/67 (54)	12/30 (40)	24/37 (65)	P=0.04
Bcl-2	16/73 (22)	6/33 (18)	10/40 (25)	P=0.48
p53	35/73 (48)	10/34 (29)	25/39 (64)	P=0.003
E-cadherin membranous	43/74 (58)	19/35 (54)	24/39 (62)	P=0.53
E-cadherin apical	25/74 (34)	10/35 (29)	15/39 (38)	P=0.37

* Missing cases have been excluded.
NA=not applicable.

Colorectal cases, right- vs. left-sided tumours

In an additional analysis, descending colon tumours were analysed together with the rectal tumour group, in order to investigate expression profiles in right- vs. left-sided tumours. We found the same differences as in our analysis of colon vs. rectal cases. Right-sided tumours showed significantly less nuclear ß-catenin (36% vs. 64%, P=0.025) and p53 overexpression (26% vs. 61%, P=0.004) than left-sided tumours. Furthermore, our survival analysis showed a significant correlation between p53 expression and disease-free survival (P=0.008) in the left-sided tumour group, but not in the right-sided group.



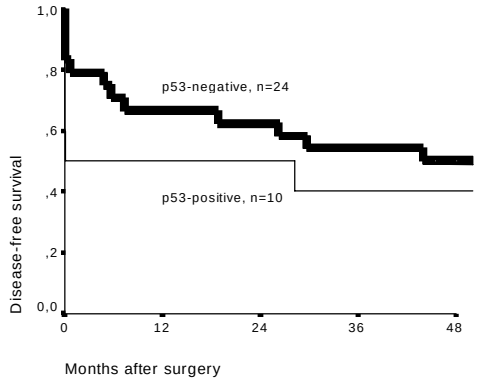


Figure 1. Kaplan Meier curve of 39 (out of 42) rectal cancer patients with regard to negative and positive p53 immunohistochemical expression (P=0.008).

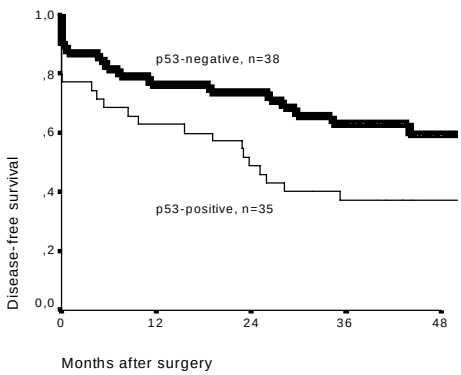


Figure 2. Kaplan Meier curve of 34 (out of 35) colon cancer patients with regard to negative and positive p53 immunohistochemical expression (P=0.37).

Figure 3. Kaplan Meier curve of 73 (out of 77) colorectal cancer patients with regard to negative and positive p53 immunohistochemical expression (P=0.03).

DISCUSSION

Tumours located in the distal colon have been proposed to arise and progress by pathways distinct from those originating in the proximal colon. Distal tumours display a higher frequency of 17p[19] and 18q[20] allelic loss, p53 accumulation,[11] c-myc expression[21] and aneuploidy.[22] Right-sided tumours are more often mucinous,[23] diploid[22] and of the microsatellite instability (MSI) phenotype.[5] Furthermore, clinical behaviour is different, in that in rectal cancer local recurrence is the major problem, whereas in colon cancer it is distant metastasis. It is therefore reasonable to suggest that the aetiological factors and the molecular basis may differ between the colon and rectal cancer.

We investigated markers that have a function in the oncogenesis of colorectal cancer. In rectal cancer, the surgeon is an important factor in outcome[9] and the role of prognostic factors can only be studied when standardised surgery is performed. In our study, rectal cancer patients were treated with standardised surgery performed by one experienced rectal

cancer surgeon. There was a significant difference only in gender; more male patients were present in the rectal than the colon group, which agrees with a previous report.[2]

Expression of mismatch repair genes did not differ between colon and rectal cancers and was positive in all cases. This implies that in our series, no HNPCC patients with *hMLH1* or *hMSH2* mutations were present. HNPCC patients show MSI in 95% of their colorectal tumours, but MSI has also been reported in 15-20% of sporadic colorectal tumours, with a difference between colon (30% MSI) and rectal tumours (4% MSI).[24] In another series of 79 rectal tumours, we found MSI in only one tumour (1%), which also indicates that MSI does not play a major role in the development of rectal cancers.(paper submitted)

Almost all of the mutations of *APC*, both germline and somatic, result in truncation of the gene product.[25] The somatic mutations exhibit a definite accumulation in an area termed the MCR,[25] so the protein truncation test of the MCR is an ideal procedure for *APC* mutation analysis in sporadic colorectal tumours. In our rectal cancer series, we found truncating *APC* mutations of the MCR in 18 of 22 (82%) cases. This mutation rate is comparable to the *APC* mutation rates of sporadic colorectal cancers described in literature.[25,26]

Mutations of *APC* have been shown to result in the stabilisation or nuclear localisation of ß-catenin, whilst *ß-catenin* mutations can also contribute to high/nuclear ß-catenin levels.[27] Significantly more nuclear ß-catenin expression was found in rectal cancers than in colon cancers (65% vs. 40%, P=0.04), but this was not associated with the presence of an *APC* mutation. This could be due to other factors being capable of destabilisation or nuclear localisation of ß-catenin. No association was found between *APC* and *p53* mutation rate or p53 immunohistochemistry. This seems to contradict the findings of Narayan and Jaiswal, who support a model featuring a direct link between p53 and APC in response to a DNA alkylating agent and suggest a novel role for p53 in a stress-response pathway involving APC.[28] However, in our study, no DNA-alkylating agents were given.

p53 mutations have been mainly found in the best conserved regions of the gene, exons 5-8, which harbour 95% of all mutations.[29] We found *p53* mutations in 15 of 22 (68%) rectal and four of eight (50%) colon cases. The mutational spectrum of these mutations was comparable with that described in literature.[29,30] *p53* mutation analysis and p53 immunohistochemistry corresponded very well, so our p53 immunohistochemistry results are reliable. We found more p53 overexpression in rectal than colon tumours, indicating a higher rate of *p53* mutations in rectal cancer. This agrees with a previous report of Scott et al.,[4] but Zeng et al.[31] and Yamaguchi et al.[32] did not confirm our observation.

Several studies have shown that p53 overexpression, either in the nucleus or in the cytoplasm, is related to unfavourable survival in patients with colorectal cancer,[31,32] but others have not found this relationship.[4,16] We did not find a prognostic value for p53 expression in the colon cancer group, but a significant relationship was found between positive p53 expression and shorter disease-free survival in the rectal cancer group and total colorectal group. In the Cox regression model, p53 expression was found to be an independent predictor for disease-free survival in the rectal cancer group, but not in the total colorectal group. It is difficult to explain the higher rate of *p53* mutations in rectal cancer than in colon cancer. The different bacterial flora and longer transit time in the rectum might change the contact between potential carcinogens or promoters in the faecal stream, which might lead to more (exogenous) mutations of *p53*.

In conclusion, we investigated oncogenes and tumour suppressor genes in colon and

rectal cancers. Rectal cancer patients were treated with standardised surgery to provide optimal conditions for studying prognostic markers. Our results indicate that rectal cancer may involve more nuclear ß-catenin in the APC/ß-catenin pathway than colon cancer, and/or nuclear ß-catenin may have another role in rectal cancer independently of APC. The p53 pathway also seems to be more important in rectal cancer, in which p53 expression also has independent prognostic value. This study shows that when prognostic markers are investigated in larger series, differences in biological behaviour between colon and rectal cancer should be considered.

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