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

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Promotores:	Prof. Dr. C.J.H. van de Velde Prof. Dr. J.H.J.M. van Krieken (Academisch Ziekenhuis Nijmegen St. Radboud)
Co-promotor:	Dr. R.A.E.M. Tollenaar
Referenten:	Prof. Dr. G.J. Fleuren Prof. Dr. E.M. Noordijk
Overige leden:	Dr. J. Morreau Prof. Dr. T. Wiggers (Academisch Ziekenhuis Groningen)

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Aan mijn ouders
Voor Ivo

CONTENTS

LIST OF ABBREVIATIONS

ACF	Aberrant Crypt Foci
APC	Adenomatous Polyposis Coli
APR	AbdominoPerineal Resection
CDGE	Constant Denaturant Gel Electrophoresis
CIN	Chromosomal INstability
CRAB	Cancer Recurrence And Blood transfusion
CT	ChemoTherapy
DCC	Deleted in Colorectal Cancer
DGGE	Denaturing Gradient Gel Electrophoresis
DPC4	Deleted in Pancreatic Cancer 4
DSS	Disease Specific Survival
EPL	Extended Pelvic Lymphadenectomy
FAP	Familial Adenomatous Polyposis
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IGF	Insulin-like Growth Factor
IKW	Integraal Kankercentrum West
IHC	ImmunoHistoChemistry
IMA	Inferior Mesenteric Artery
LAR	Low Anterior Resection
LND	Lymph Node Dissection
LOH	Loss Of Heterozygosity
LR	Local Recurrence
MCR	Mutational Cluster Region
MMR	MisMatch Repair
MSI	MicroSatellite Instability
OS	Overall Survival
PCR	Polymerase Chain Reaction
Pre/Postop	Pre/Postoperative
Pts	Patients
PTT	Protein Truncation Test
RT	RadioTherapy
SRCT	Swedish Rectal Cancer Trial
SSP	Sphincter Saving Procedure
TGF	Transforming Growth Factor
TME	Total Mesorectal Excision
Vs	Versus
WT	Wild Type

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General introduction and outline of this thesis

PART I: TREATMENT

Colorectal cancer is the most common gastrointestinal cancer in the Western world. In 1995, 8000 new colorectal cancer patients were registered in The Netherlands, of whom about 25% had rectal carcinoma.[1] One of the main problems in the treatment of rectal cancer is the development of local recurrences, of which the reported incidences vary widely.[2,3] Local recurrences cause severe disabling symptoms, are difficult to treat, and usually kill the patient.[4] Recurrences of rectal cancer are often confined to the pelvis without distant metastases, and are considered loco-regional failures.[5,6] Most of them become overt within two years of operation.

Traditional surgical treatment

The surgical principles in the treatment of colorectal cancer were formulated for the first time by Lord Moynihan in 1908.[7] Early in this century the local recurrence rate following surgery for rectal cancer was nearly 100%. Miles described a combined radical abdominal and perineal approach[8] to remove the pelvic mesocolon and the "zone of upward spread" to solve this problem. For a long time Miles operation was the "gold standard" for treatment of rectal cancer, even for tumours above 15 cm from the anal verge.

Since Miles described his abdominoperineal resection (APR) technique, various modifications[9,10] have been proposed to improve patient prognosis. Turnbull[9] described a technique in which lymphovascular isolation and ligation was performed prior to mobilisation of the segment of tumour bearing colon, which was called the "no-touch" isolation technique. In subgroup analysis the concept showed to be of statistically significant benefit only when microscopic vascular invasion was present in the tumour.[11] En bloc resection of lymph nodes at the origin of the inferior mesenteric artery from the aorta, often called "high" ligation, was assumed in the 1960s to give a survival benefit.[10] Two more recent comparative studies have failed however, to show a survival benefit for "high" versus "low" ligation.[12,13]

Improvement of quality of life after surgery was obtained due to the introduction of mechanical stapling devices[14] together with the observation that the safe distal margin is at 2 cm from the primary tumour.[15,16] The combination of these two factors made lower resection with reconstruction possible, guaranteeing an anatomically and functionally intact anal sphincter, instead of the much more mutilating abdominoperineal resection with implicit definite colostomies. Developments of new approaches also included the construction of coloanal anastomoses.[17] This technique has acceptable functional results and complication rates and therefore has become a viable alternative to the APR in the treatment of low rectal cancer. In addition, significant functional improvement, particularly in the first 12 to 24 months after surgery, can be achieved with the use of a colonic J-pouch.[18]

Apart from these surgical technicalities, the availability of blood transfusion and major improvements in anaesthesia, perioperative care management and control of infectious complications, have also enabled surgeons to resect the tumour and reconstruct the continuity of the bowel, rather than to only construct a colostomy and to leave the tumour in situ.[19]

Nevertheless, despite the aforementioned developments, one of the problems in the treatment of rectal cancer surgery has remained the inability of surgeons to optimise local tumour control. The basic conventional procedure involving blunt dissection, often resulted in incomplete removal of mesorectal tissue with high local recurrence rates.[2,20,21] Table 1 shows local recurrence after "curative" surgery" in conventional surgery series; local

recurrence rates from 12% up to 38% have been reported. In addition, damage to the autonomous pelvic nerve plexus with the consequence of a high incidence of sexual[22,23] and bladder dysfunction[24] was very likely in conventional surgery with great impact on quality of life after surgery.

Table 1. Local recurrence after "curative" conventional surgery.

	Patients investigated n	Local recurrence n	Local recurrence %	Remarks
Rao '81[25]	204	44	21.6	
Rich '83[26]	142	43	30.3	
Pahlman '84[27]	197	74	37.6	
Phillips '84[21]	848	124	14.6	
Philipsen '84[28]	382	105	27.5	27% received preop RT
McDermott '85[29]	934	193	20.7	
Pescatori '87[30]	162	19	11.7	
Athlin '88[31]	99	37	37.4	unknown no. of pts received postop RT/CT
Rinnert-Gongora '89[32]	258	53	20.5	
Zirngibl '90[33]	1153	265	23.0	
Akyol '91[34]	294	49	16.7	
Stipa '91[35]	235	42	17.9	
Norstein '93[36]	275	81	29.5	
Adam '94[37]	141	32	22.7	6% received postop RT
Nymann '95[38]	175	37	21.1	
Damhuis '97[39]	902	162	18.0	8% received postop RT
Mollen '97[40]	232	42	18.1	27% received postop RT
Kapiteijn '98[20]	668	150	22.5	36% received postop RT

Variability in outcomes

Inter-institution and inter-surgeon variabilities in colorectal cancer surgery have been shown in several studies with conventional surgery. This applies to immediate results, such as surgical morbidity and mortality,[41-45] as well as long-term results, such as local recurrence and survival.[20,21,41,46-49] An explanation of inter-institution and inter-surgeon variation in outcome remains a delicate matter. The different patient and tumour-related factors have to be considered as well as the surgical technique itself; anaesthesia, pre- and postoperative care (including management of post-surgical complications and further follow-up), additional non-surgical treatment modalities, diagnosis, and management of recurrences.

Table 2 shows an overview of studies which have investigated the influence of hospital- and surgeon-related factors in rectal cancer according to short- and long-term outcomes. This table is a shorter version of the table in a paper by Kapiteijn et al. in which a complete overview of published studies on influence of hospital- and surgeon-related factors in colorectal cancer is given.[50]

With regard to short-term end-points, there are indications that higher volume and specialisation or teaching status are related to better outcomes.[20,21,42-45,48,51-54] Other studies however, found no correlation between hospital- and/or surgeon-related factors and short-term outcomes.[43,55,56]

Several studies have investigated the effect of hospital volume on long-term outcomes.

However, findings in literature are controversial,[20,46-48] with also one report suggesting that e.g. hospital volume predicts clinical outcome for colorectal cancer, but not in the absolute magnitudes in comparison with the variation observed for higher-risk cancer surgeries.[57-59]

The influence of individual surgeon volume and specialisation on long-term outcomes have also been investigated in several studies.[21,41,48,49] Hermanek suggests that, in order to maintain good quality of surgery, the minimum volume per surgeon should be about one or two radical resections per month.[60] In his study[48] however, there was one particular high-volume surgeon with very poor outcome, which makes his conclusion about the role of volume controversial. In the study of Porter et al. it was shown that outcome is improved both with colorectal surgical subspecialty training and a higher frequency of rectal cancer surgery.[49]

When reviewing the data in the literature with respect to volume, it must be considered that the definitions of high volume are different with varying cut-off points. This makes comparison of the studies on hospital- and surgeon-related factors and outcome difficult, also since data sources and statistical methods applied are different. Cut-off points should be defined prospectively to avoid biases inherent in post-hoc analysis (in which cut-off point can be selected to maximise volume-outcome associations).

In conclusion, it is evident from the data published that surgery is less than optimal as reflected by some surgeons or in some hospitals. It is therefore important to give surgeons the opportunity to undergo training and to adopt new and improved techniques. It seems more difficult to find good arguments which support the hypothesis that treatment volume or specialisation in certain centres are important factors. Rather, it could be that the relationship between treatment volume and results is more a consequence of bad organisation or badly trained surgeons than volume or specialisation itself.

Variability in definitions

Apart from variability in hospital and surgeon outcome, important factors responsible for the large range of local failure rates are different definitions used for rectal cancer, curative resection, local recurrence and the methods used for detecting such a local failure.[70] A study of Marsh et al.[71] showed that for the same series of patients local recurrence rate could be calculated as low as 4% or as high as 43% by exclusion or inclusion depending on the used definition. Marsh et al. proposed in their paper that local recurrence after operation for rectal carcinoma should be defined as any detectable local disease at follow-up, occurring either alone or in conjunction with generalised recurrence, in all patients who have undergone resection. Investigators publishing on rectal cancer treatment should clearly state crucial definitions since it is obvious that the diversity in conducting and reporting surgical studies in rectal cancer does little to facilitate interstudy comparison or the evaluation of novel therapies.

Table 2. Studies which have investigated the influence of hospital- and surgeon-related factors in outcome of rectal cancer (including some pivotal studies on combined colon and rectal cancer). NI=not investigated, NR=not reported

Study	Study-period	No. of patients	No. of hospitals	No. of surgeons	Analysed factors	Analysed outcomes	Associations
Short-term outcomes							
Fielding '80[43]	'76-'80	1466 colorectal cancer pts	23	84	-Variation between surgeons -Comparison of teaching and district general hospitals	Leakage	-Variation in leakage 5-30%. The surgeon was probably the most important single factor influencing leakage. -No difference between teaching and district general hospitals.
Hannan et al. '89[44]	'86	10297 colectomies (5 procedures investigated)	250	1997	Hospital and physician volume	In-hospital mortality	Annual hospital thresholds appear to exist at 40 procedures for colectomies
Kessler et al. '93[42]	'84-'86	1115 rectal cancer pts	7	NI	Individual hospitals	Operative/ postop mortality	Institution was an independent factor for operative/postop mortality
Lothian and Borders large bowel cancer project '95[61]	'90-'92	750 colorectal cancer pts, 260 rectal cancer pts	NI	28 consultant surgeons	Variability between surgeons	-Resection type -Leakage	The 5 consultants responsible for half of the rectal cancer pts had similar APR-rates but less leakage in LAR-pts as compared to the others
Long-term outcomes							
Mohner&Slisow '90[62]	'76-'80	15731 rectal cancer pts	334	NI	Degree of centralised treatment in district	Five year survival	Higher 5 year relative survival in districts with a centralisation index of at least 60 (=at least 12 cases/year)
Kapiteijn et al. '98[20]	'88-'92	668 rectal cancer pts	12	NI	Hospital volume	Local recurrence	No correlation between hospital volume and local recurrence
Porter et al. '98[49]	'83-'90	683 rectal cancer pts	5	52	Surgeon -volume -specialisation	-Local recurrence -Disease-specific survival (DSS)	DSS correlated positively with surgeon specialisation and volume
Dahlberg '98[63]	'74-'95	423 rectal cancer pts	1	NI	Concentration of surgery to a colorectal team	Local recurrence	Results of treatment can be improved by concentration of surgery to colorectal team
Blomqvist et al. '99[64]	'73-'92	30811 rectal cancer pts	100	NI	Hospital catchment area	Relative survival	1 yr relative survival was higher in large regional versus small local hospitals

Study	Study-period	No. of patients	No. of hospitals	No. of surgeons	Analysed factors	Analysed outcomes	Associations
Luna Perez '99[65]	'80-'95	82 pts with mid-rectal cancer	NR	NI	Cancer centre versus general hospitals	-Local recurrence (LR) -Overall survival (OS)	Favourable prognostic factor for LR and OS was treatment at a cancer centre
Hermanek et al. '99[66]	'84-'86	1539 colorectal cancer pts; 712 with rectal cancer	3 of 7 departments	43	-Department -Surgeon volume	-Cancer-related 5-year survival	-Surgical volume did not influence prognosis -Department was a significant factor in rectal carcinoma, whereas surgical quality group was only significant when department was excluded from the model Results from earlier publications: -Institution and surgeon are independent prognostic factors for local recurrence and overall survival[48,67] -A minimum of 1 or 2 radical resections/month is necessary to obtain good quality of surgery[60]
Kapiteijn et al. 2001,[68] conventional surgery	'87-'90	269 rectal cancer pts	16	NI	Hospital volume	-Local recurrence -Distant recurrence -Overall survival	Significant association between higher hospital volume and lower distant recurrence risk and higher survival
Kapiteijn et al. 2001,[68] TME-surgery	'96-'99	661 rectal cancer pts	84	NI	Hospital volume and specialisation	-Local recurrence -Distant recurrence -Overall survival	No associations found
Short+long-term outcomes							
Simons et al. '97[47]	'88-'92	2006 rectal cancer pts	125	NI	-Type of hospital -Hospital volume	-Surgical procedure; sphincter saving procedure (SSP) -Survival	-More SSP in teaching and high-volume hospitals -Improved survival in high-volume hospitals
Simunovic et al. 2000[69]	'90	1072 rectal cancer pts	124	NI	Hospital -volume -teaching status	-Treatment measures -Operative mortality -Long-term survival	No effect hospital volume and teaching status on treatment and outcome measures
McArdle and Hole '91[41]	'74-'79	645 colorectal cancer pts	1	13 (specialised consultants)	Variation between surgeons	-Postop compl -Postop mortality -Survival (up to 10 yrs)	Significant variations between surgeons in: curative resections, leakage, postop mortality, local recurrence, survival

(Neo-)adjuvant therapy

In order to improve local control and survival after conventional surgery, additional radiotherapy has been given. The results of studies using radiotherapy for rectal cancer, suggest that preoperative radiotherapy is more effective than postoperative radiotherapy in reducing local recurrence rates.[72-74] Swedish trials showed improved local control and survival with the short-term 5x5 Gy preoperative irradiation scheme.[72,75] So far, chemotherapy alone for rectal cancer has shown little or no effect in combination with conventional surgery on disease-free and overall survival.[76] Combinations of radiotherapy and chemotherapy have also been given with improved outcomes, but sometimes at the expense of severe toxicity.[77-81]

The studies so far published on adjuvant therapy have been carried out without an adequate definition of the surgical procedure and without appropriate quality control. In contrast with radiotherapy and chemotherapy, the quality of surgery has appeared difficult to examine. Nevertheless, standardisation and quality control of surgery are prerequisites to study the effect of (neo)adjuvant therapy reliably, also since the surgeon can be an important factor in the accomplishment of tumour control (Table 2). In some trials, operation reports were reviewed by a surgical board,[79] but otherwise no meaningful quality control on surgery was enhanced. Local recurrence rates in the "surgery alone" control groups of these trials were often high; 20% or higher,[40,75,77,78,82,83] representing non-standardised, conventional surgical techniques.

In addition, in none of the studies were explicit details given of safety margins, excision of mesorectum and lymph node dissection. Optimal quality control of the surgical procedure must also include a standardised examination by pathologists. Quirke et al.[84] described a method of detection of mesorectal spread which required systematical examination of the specimen, by serial sectioning of the whole tumour and the surrounding mesorectum in the transverse plane. This method should be used to monitor differences in operative technique. Furthermore, surgery can be documented photographically due to reproducible gross specimen features.[85]

Lymph node dissection

In order to reduce local recurrence and hence improve survival more radical resections have been devised. Extended lymphadenectomy, involving dissection of pelvic and aorto-iliac lymph nodes without resection of organs other than the rectum, was described as early as 1942.[86] Most studies on extended pelvic lymphadenectomy (EPL)/D3 dissection however, have been retrospective with historical controls as control group. Only one prospective trial has been performed and this could not demonstrate an overall benefit, although in subgroup analysis of mid-rectal Dukes' C cancers this benefit was present.[87] Partly because of the wide variety in lymph node yield and salvage methods, and the differences in definition of lymph node metastasis, there is still wide spread controversy on the extent of lymph node dissection (LND) recommended for primary cancer of the rectum.

Furthermore, extended lymph node dissection carries a higher postoperative morbidity which may be contributed to longer time of operation and increased blood loss. Another strong argument against pelvic LND is the very high rate of bladder and sexual dysfunction[88] as compared to conventional resections. The technique of nerve-sparing LND might decrease these complications,[89] but it requires a meticulous surgical technique and accurate knowledge

of the anatomy of the pelvic autonomic nervous system and prolongs operation time even more significantly.[88]

For the aforementioned reasons, extended lymph node dissection has not become standard surgical practice in the Western world. In Japan however, extended lymph node dissection is the standard surgical procedure since the mid seventies. A possible relevant factor in this is that postoperative morbidity and mortality are minimal in Japanese patients, possibly because of the low prevalence of obesity and atherosclerosis.

TME-surgery

The concept of Total mesorectal excision (TME) was introduced by Heald at the North Hampshire Hospital in Basingstoke in 1979.[90] By using sharp dissection under direct vision a relatively bloodless plane is followed along the lipoma-like outer surface of the mesorectum. The sharp technique used in TME ensures a specimen with intact mesorectum with negative tumour margins in the majority of resectable (i.e. mobile) rectal cancers. Furthermore, the sharp technique allows for preservation of the pelvic autonomic nerves, reducing sexual and urinary dysfunction.

Heald's first series of 112 curative anterior resections showed a cumulative risk of local recurrence at 5 years of 2.7% and an overall corrected survival at 5 years of 87.5% with tumour-free survival of 81.7%. These results were the best reported in rectal cancer treatment up to then.[91] However, many investigators doubted these findings with criticism focused on patient case mix and analytical techniques,[92] unclarified selection process[93] and incorrect use of definitions.[71] In Enker's personal series of 246 curable Dukes' B and C cases only 18 tumours (7.3%) recurred locally, actuarial cancer specific 5-year survival was 74.2%.[94] Aitken published a series of 64 curatively resected TME cases with at least 24 months follow-up: only one patient (1.6%) developed a local recurrence.[95]

The acknowledgement of the important role of circumferential involvement in the occurrence of local recurrences[37,84,96-98] has led to the general introduction of TME-surgery.[63,99,100] In The Netherlands, Sweden and Norway, nation-wide projects have been conducted in which surgeons were trained to perform a proper TME in an attempt to improve their treatment results. Table 3 shows local recurrence rates after TME in several studies, illustrating lower local recurrence rates with TME as compared to conventional surgery (Table 1).

In addition to better results in terms of recurrence, the introduction of TME-surgery has been shown to result in a reduction of abdominoperineal resections.[99,101] However, higher leak rates with TME-surgery as compared to conventional surgery have been reported.[102,103] This increase can be partly explained by the removal of the pain-sensitive peritoneum, which prevents early detection of anastomotic failure.[104] The higher incidence of leakage might also be caused by devascularisation of the anorectal stump during dissection of the distal "tail" of the mesorectum in TME.[103,105] Various other factors such as anastomotic technique,[106,107,108] method of preparation of the bowel,[109] use of a diverting colostomy[104] and the method of pelvic drainage[110] have also been found to be related with leakage.

Since the introduction and application of TME-surgery has resulted in such low local recurrence rates and improved survival, the question has yet to be answered as to whether in combination with TME-surgery, adjuvant therapy is still capable of achieving any further improvement in outcome.

Table 3. Local recurrence after "curative" total mesorectal excision.

	Patients investigated n	Local recurrence n	Local recurrence %	Remarks
Heald '86[91]	112	3	2.7	
Colombo '87[111]	89	10	11.2	
Belli '88[112]	72	3	4.2	
Kirwan '89[113]	67	3	4.5	
Karanjia '90[114]	152	4	2.6	
Cawthorn '90[96]	122	9	7.3	
Dixon '91[115]	227	9	4.0	
Moran '92[116]	55	4	7.3	only LAR
Tagliacozzo '92[117]	248	41	16.5	
Jatzko '92[118]	187	25	13.4	
MacFarlane '93[3]	135	7	5.2	
Enker '95[94]	246	18	7.3	70 pts had perioperative RT with or without CT
Aitken '96[95]	64	1	1.6	
Eu '97[119]	278	26	9.4	
Carvalho '97[120]	51	1	1.9	adjuvant therapy was given in 33 pts
Hainsworth '97[121]	45	8	17.8	
Arenas '98[122]	64	4	3.1	42 pts received pre -or postop RT
Maas '00[123]	42	3	7.1	
Martling et al. '00[99]	381	21	5.5	54% of the pts received 5x5 Gy preop RT
Kapiteijn '00[68]	661	57	8.6	
Tocchi '01[124]	53	5	9.4	only LAR

Recent developments in The Netherlands

In The Netherlands, standards of care for rectal cancer surgery have been subject of interest for some years. The results of extended pelvic lymphadenectomy in Japan and the excellent results of TME by Heald and Enker were welcomed with interest but also with scepticism; could these results be repeated in all surgeon's hands? Initially, attention was focused on the Japanese style extended lymphadenectomy. In his thesis, Steup concluded that the value of extended lymphadenectomy should be studied in a randomised controlled trial[125] and a trial was proposed to compare the D3 lymphadenectomy technique with TME. Many Dutch surgeons however, feared a considerable morbidity with the Japanese D3 technique in Dutch patients. A second and third proposal was to compare conventional surgery with TME-surgery or compare in a two by two factorial design yes/no short-term preoperative radiotherapy and conventional vs. TME-surgery. Both designs for trials however, would allocate 25-30% of the patients to the inferior arm of conventional surgery without preoperative radiotherapy. Literature data were so convincing with regard to the superiority of the TME technique,[3,94,95] that a majority of the Dutch surgeons had the opinion that it would be unethical to randomise patients in such a design. Furthermore, there are potential difficulties in a surgeon randomly applying different surgical techniques.[126] Finally, the last proposal was made for the TME-trial: compare TME-surgery with or without preoperative radiotherapy.

Y. Moriya from the National Cancer Centre Hospital in Tokyo visited The Netherlands in

1994-1995 to assess the feasibility of nerve preservation and pararectal resection comparable to the TME-technique in a series of 47 Dutch patients.[127] The nerve-preserving technique yielded good results in terms of morbidity and functional outcome. Of the 42 curatively operated patients, 3 (7.1%) developed a local recurrence. Sixty-seven percent were overall free of recurrence after a median follow-up of 42 months. From these results it was concluded that preservation of the pelvic autonomous nerve system does not compromise radicality in mesorectal excision.[123] This study comprised the pilot study of the TME-trial.

The TME-trial

A large prospective randomised trial (TME-trial) was started in 1996 under the auspices of the Dutch ColoRectal Cancer Group (DCRCG) to document local control when standardised TME is used and to answer the question whether 5x5 Gy preoperative radiotherapy[75] is still beneficial in TME treated patients.[101] Eligibility criteria included histological confirmed resectable primary adenocarcinoma of the rectum without evidence of distant metastases.

An extensive structure of workshops (run together with Heald, Enker and Moriya), symposia and instruction videos helped to accomplish that TME was performed according to strict quality demands. In addition, a monitoring committee of specially trained instructor-surgeons was installed for on-site instructions. In each hospital, the first five TME procedures had to be supervised by an instructor-surgeon.[101] Special training courses were given to pathologists for instruction of the protocol of Quirke et al.[84] The results of histopathological examination of the specimens were reviewed by a panel of supervising pathologists and a quality manager.[128] Eligibility, treatment and follow-up details were checked by the study-coordinators. Fresh frozen and paraffin-embedded tissue samples were collected of each patient for molecular biological research purposes.

The TME-trial was one of the first randomised trials with standardisation and quality control of all participating disciplines. Both of these are prerequisites to study the effect of (neo)adjuvant therapy reliably. In this thesis the set-up and results of the TME-trial are extensively described.

PART II: MOLECULAR BIOLOGY

A genetic model for colorectal cancer

Colorectal cancer is one of the best-characterised cancers from the perspective of understanding the genetic events which underlie the development of malignancy. The majority of colorectal cancers develop from benign preneoplastic lesions, the adenomatous polyps or adenomas. Progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, which is referred to as the adenoma-carcinoma sequence.[129] The aetiology of colorectal cancer is multi-factorial, involving environmental factors, genetic susceptibility and somatic changes during its initiation and progression.[130]

The genetic model for the progression of colorectal neoplasia is the molecular counterpart of the morphological adenoma-carcinoma sequence. This model represents a simplified picture of the complex process of tumour initiation and progression. Alterations in several oncogenes (K-ras) and tumour suppressor genes (APC, DCC/DPC4, p53), as well as epigenetic changes (methylation) are implicated in the stepwise development of colorectal neoplasia (Figure 2). The accumulation of these changes is associated with a gradual increase

in the size, disorganisation and malignancy of colorectal tumours.

APC

Inactivation of the Adenomatous Polyposis Coli gene (APC) on chromosome 5q has been shown to be the underlying defect in familial adenomatous polyposis (FAP), in which germline mutations in *APC* are found. However, inactivation of APC is also one of the earliest events in the development of sporadic colorectal cancers with somatic mutations in the mutation cluster region (MCR).[131,132] In the majority of colorectal neoplasia, the APC gene is either deleted or mutationally inactivated by the introduction of premature termination codons. This inactivation is already observed in the smallest precursor lesions of adenomas, the dysplastic aberrant crypt foci[132,133] and therefore, APC is called the "gatekeeper" of colorectal epithelial cell proliferation, as its inactivation is a rate-limiting event in the initiation of the adenoma-carcinoma sequence.

APC has 3 binding sites which allows for interaction with ß-catenin, a well-known adhesion and signaling molecule which is associated with E-cadherin in the formation of epithelial cell-cell contacts. By the interaction with ß-catenin, APC is a key member of the Wnt signal transduction pathway (Figure 1), which is recognised to function in critical biological processes such as embryonic induction, the generation of cell polarity and the specification of cell fate.[134] In general, secreted Wnt/Wingless glycoproteins interact with receptors of the frizzled gene family, thereby activating the cytoplasmatic phosphoprotein dishevelled (dsh). Dsh inhibits the function of the serine/threonine kinase GSK3β. Inhibition of GSK3β results in the accumulation of β-catenin in the cytoplasm and in its translocation to the nucleus where it forms complexes with the TcF/Lef family of HMG transcription factors.[135] These complexes can activate target genes, of which c-myc[136] and cyclin D1[137] are examples. In the absence of the Wnt signal, GSK3β forms a complex together with conductin, axin, APC and β-catenin promoting the rapid degradation of β-catenin.[138] Hence, loss of APC results in a critical loss over β-catenin control, leading to constitutive signaling to the nucleus and activation of downstream target genes.

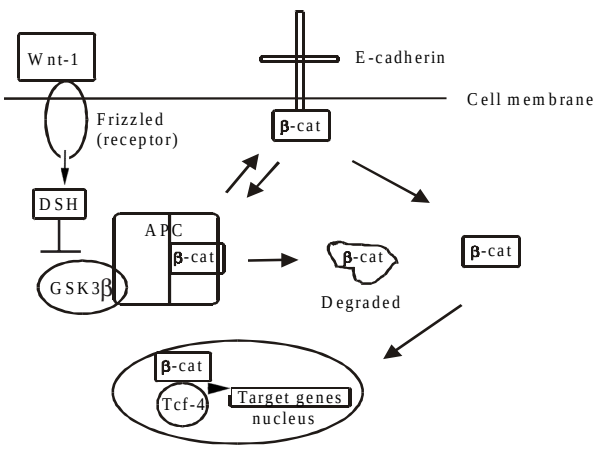


Figure 1. The Wnt signal transduction pathway. APC acts as a negative regulator of β-catenin accumulation and signaling. In the absence of the Wnt signal, GSK-3β forms a multiprotein complex with APC and β-catenin, triggering β-catenin degradation. In the presence of the Wnt signal, Dsh is activated, which inhibits the function of GSK-3β. APC remains unphosphorylated and unable to downregulate intracellular β-catenin, leading to its translocation to the nucleus where it can activate transcription of target genes, including c-myc and cyclin D1.[135]

K-ras

Further clonal expansion of benign tumour cells is driven by mutations at the *K-ras* proto-oncogene on chromosome 12p. *K-ras* mutations are frequent in colorectal cancer and usually involve missense mutations of codon 12 and 13.[139] These mutations result in an increase of the GTPase activity of the ras protein, which is part of a signal transduction pathway. The ras signaling pathway relays signals from the cell surface to the nucleus and has an important role in the control of cell proliferation. Consecutive activation of ras in the pathway has been shown to lead to oncogenesis.

DCC/DPC4

Alterations in the Deleted in Colorectal Cancer (DCC) and/or Deleted in Pancreatic Cancer 4 (DPC4, both on chromosome 18) and p53 (on chromosome 17) tumour suppressor genes occur during the later stages of tumourigenesis, and result in the progression from the benign to the malignant state of colorectal neoplasia. The DCC gene was originally identified due to its high frequency of deletion in colorectal cancer and was mapped to chromosome 18q.[140] Frequent loss of heterosygosity at the DCC locus and loss of DCC expression have been observed in colorectal cancers.[140,141] However, although the DCC gene might play some role in progression of colorectal cancers, the frequency of loss of heterozygosity (LOH) on 18q in some tumours does not correlate simply with the low frequency of mutations on the *DCC* gene.[142] The DCP4/SMAD4 gene, lying in close proximity to the DCC gene at 18q21.1, was recently identified as a candidate suppressor for a predisposing gene for Juvenile Polyposis Syndrome (JPS).[143] This gene functions as a cytoplasmic mediator in the signaling pathway of transforming growth factor (TGF)-β. Inactivation of both alleles of the DPC4/SMAD4 gene was also demonstrated to occur in a substantial proportion of sporadic colorectal cancers.[144] Loss of DCC and DPC4 has been observed to occur independently from each other.[145]

p53

Mutations of the *p53* tumour suppressor gene on chromosome 17p are the most frequently found genetic alterations in human cancer.[146] LOH of the p53 region is observed in more than 75% of colorectal tumours, and usually correlates with point mutation of the remaining allele.[147,148] p53 has been named the "guardian of the genome" because of its capacity to monitor the integrity of the DNA.[149] The function of p53 is to maintain genetic stability of cells by eliminating cells with damaged DNA and by facilitating the repair of such damage.[150] Therefore, it has an important role in several apoptotic pathways. Elimination of p53 tumour-suppressor activity by mutations in the gene will lead to escape of neoplastic cells with DNA alterations from p53 induced growth arrest, that would normally be followed by either DNA repair or apoptosis (programmed cell death).[151]

DNA repair genes

Apart from alterations in the aforementioned genes, inactivation of genes which control the rate of mutations (DNA repair genes) is also observed in colorectal cancer. A link between DNA repair deficiency and colorectal cancer is seen in Hereditary Non-Polyposis Colorectal Cancer (HNPCC). Individuals with HNPCC carry germline mutations in DNA-mismatch repair genes (most frequently in *hMLH1* and *hMSH2*), and exhibit microsatellite instability

(MSI) in their colorectal tumours.[152] A target often hit in these tumours is TGF-β-RII. The TGF-β/SMAD signaling pathway is involved in a variety of biological functions; i.e. cellular differentiation, embryonal morphology and in immunological defense. Finally, TGF-β/SMAD signaling usually inhibits growth in epithelial tissue.[153]

Proliferation/apoptosis

Dividing normal cell populations maintain the balance between cell proliferation and cell loss. This is important for maintaining a constant number of cells within a tissue. If there is increased proliferation, decreased apoptosis or both, uncontrolled growth occurs and this may result in tumour formation.[154] Amongst the cell proliferation markers are Ki-67 and PCNA.[155] An important apoptotic gene other than p53 is Bcl-2. Overexpression of Bcl-2 protects cells against induction of apoptosis by a variety of stimuli, including irradiation and most clinically used chemotherapeutic drugs.[156] Bcl-2 is the founding member of a family of proteins that can either repress (e.g. Bcl-2, Bcl-Xl, Bcl-W) or promote apoptosis (e.g. Bax, Bak, Bcl-Xs).[157]

Cell adhesion

In the progression of colorectal cancers (i.e. invasion and metastasis), microenvironmental interactions are important. Loss of cell adhesion leads to a reorganisation of epithelial cells to make invasion and metastasis possible.[158] E-cadherin is a cellular adhesion molecule, which has an important role regulating cell differentiation and establishing surface-membrane polarity. When E-cadherin is lost, epithelial cells dedifferentiate, cell adhesion and polarity are lost and the cells become invasive.[159,160] In cell-cell adhesion, E-cadherin is associated with the actin cytoskeleton via cytoplasmic proteins, including α-, β-, and γ-catenins, which together form the cadherin/catenin complex.[161] The epithelial cell adhesion molecule (EpCAM) is a homophylic cell adhesion molecule, which is thought to be important for cell segregation. EpCAM has attracted attention as a potential tumour marker, because it is expressed in a vast majority of carcinomas.[162] EpCAM has been shown to affect in vitro expression of the intercellular adhesions mediated by cadherins.[163]

Angiogenesis

Angiogenesis is mediated by multiple molecules that are released by both tumour cells and host cells including endothelial cells, epithelial cells, mesothelial cells and leucocytes. Among these molecules are members of the Fibroblast Growth Factor (FGF) family, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), TGF-β and others.[164,165] Angiogenesis is essential in tissue development, reproduction and wound healing.[166] In addition, angiogenesis has been described as vital for tumour growth and expansion; influx of new blood vessels may facilitate dissemination to distant sites.[167,168]

Other genes

Numerous other genes have been identified to play a role in the tumourigenesis of colorectal tumours. For a full understanding of the process of normal cells becoming malignant tumours, all the genetic pathways and mechanisms need to be identified. A complete genetic pathway would include all the genes involved, whether mutated or functionally altered without mutation, and the order in which they become involved from the first change through to

metastasis in different tissues. In addition, a full description of all modifier genes and the impact of their alleles on progression along the genetic pathways is desirable. These will form the basis of eventually achieving complete understanding of all the functional effects and interactions of these genes to reveal exactly how a tumour evolves.

Genomic instability

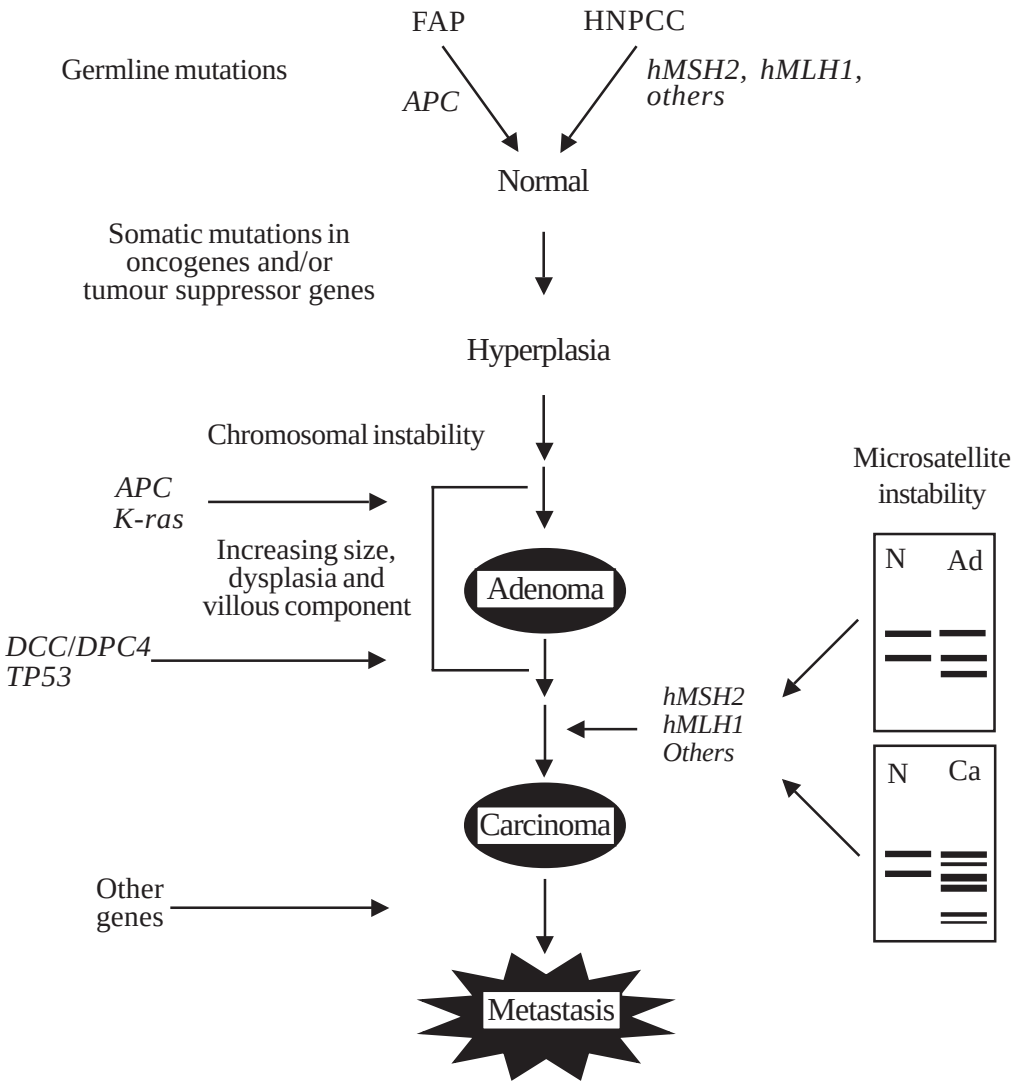
Within the adenoma-carcinoma sequence, two mechanisms of genomic instability have been identified (Figure 2). Genomic instability is considered to play a crucial role in leading to the accumulation of alterations in oncogenes and tumour suppressor genes necessary for malignant transformation.

The first form of genomic instability is known as chromosomal instability (CIN). Due to loss or gain of genetic material at a specific chromosomal region on one or both alleles of the tumour cells, these cells show an altered ratio of both alleles when compared with corresponding normal tissue. CIN is characterised by gross chromosomal segregation abnormalities and is commonly detected as aneuploidy.[169] The majority (85-90%) of sporadic colorectal carcinomas is associated with chromosomal instability.[129] Despite the fact that CIN is a relatively common phenotype, its mechanistic basis has mainly been unclear. Molecular mechanisms thought to be involved are p53-inactivation,[150] changes in mitotic checkpoint genes (*Bub1*),[170] failure of DNA-damage checkpoints (*ATM*)[171] and the JC-virus.[172] Recently it was demonstrated that loss of APC sequences that lie C-terminal to the β-catenin regulatory domain contributes to CIN in colorectal cancer, which was independent of its role in signal transduction. Loss of APC function therefore probably initiates tumourigenesis by constitutively activating Wnt signaling and probably elicits CIN in later stages of malignant progression with the cooperation of other acquired mutations.[173]

Another form of genomic instability in colorectal tumours is Microsatellite Instability (MSI). MSI, characterised by an altered number of repeat units that constitute DNA microsatellite repeats in tumour DNA,[174,175] has been identified in the majority of hereditary non-polyposis colorectal cancers and is caused by germline mutations in human DNA mismatch repair genes.[130] MSI also occurs in 10-15% of sporadic colorectal tumours,[177] mainly by somatic inactivation of hMLH1.[176] MSI reflects the failure to repair replication errors within repeat sequences contained in genes relevant for growth control and differentiation.[178]

MSI-positive colorectal carcinomas have specific clinical and pathological manifestations as compared to MSI-stable cancers, such as right-sided predominance, occurrence in young patients, tumour multiplicity, mucinous histopathological type, prominent lymphoid inflammatory response and diploid DNA content.[175,179-183] Furthermore, MSI-positive cancers appear to show a molecular genetic spectrum that is distinct from CIN-tumours. The genes that are most frequently affected in CIN-tumours are *APC*, *K-ras* and *p53*. Mutations in these genes seem to occur with reduced frequency in MSI tumours,[184,185] but other studies have found *APC*, *K-ras* and *p53* alterations at frequencies similar to MSI-stable cancers.[175,186,187] In MSI-tumours, mutations within small repeated sequences are usually found in genes such as *TGF-β-RII*, *Bax* and *insulin-like growth factor receptor II* (*IGF-RII*).[184,188,189] However, *TGF-β-RII* mutations are also present in 15% of MSI-stable colorectal cancer cell lines, although these are not frameshifts of the (A)10 tract typically mutated in tumours with MSI.[190]

Figure 2. Alterations in several oncogenes and tumour suppressor genes according to two major mechanisms of genomic instability: microsatellite instability (MSI) and chromosomal instability (CIN). N=normal, Ad=adenoma, Ca=carcinoma.



In conclusion, at least two different molecular pathways are involved in the development of colorectal cancer: the APC/β-catenin (Wnt) mutational pathway, usually associated with CIN, and DNA mismatch repair (MMR) pathway, associated with MSI with often inactivation of TGF-β-RII.[191] These routes are not totally independent, but show crosstalk with mutations in certain genes (*APC*,[186] *TGF-β-RII*[190] and *axin*[192]) in both pathways. The APC and MMR pathways show mutations in different parts of cell regulation mechanisms, but these may both result in growth advantage for tumour cells.

Colon vs. rectal tumours

First, it is important to mention that colon vs. rectal cancers can also be defined as right-sided vs. left-sided or proximal vs. distal colorectum. The rectum is regarded as the left-sided or distal colorectum.

Tumours located in the distal colorectum 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[193] and 18q[194] allelic loss, p53 accumulation,[195] c-myc expression[196] and aneuploidy.[197] Right-sided tumours are more often mucinous,[198] diploid[197] and of the MSI-phenotype.[179] Furthermore, clinical behaviour has appeared different in that in rectal cancer local recurrence has been the major problem and in colon cancer distant metastasis. However, through the recent introduction of a better surgical technique (TME) for rectal cancer, this difference in clinical behaviour may disappear. Nevertheless, it is still reasonable to suggest that the molecular basis differs between the colon and rectum.

Cellular responses to ionising radiation damage

Ionising radiation, as an effective physical agent for cancer therapy, targets primarily DNA molecules and produces an array of lesions that include single-strand breaks, base alterations and double-strand breaks. These lesions are repaired by distinct DNA repair mechanisms, each covering a specific spectrum of damage. In addition to repair pathways, DNA lesions are also recognised by components of the DNA damage cell cycle checkpoint pathways.

The function of p53 in normal cells is to respond to DNA damage by ionising radiation by either causing cell cycle arrest or by forcing damaged cells to go into apoptosis. The stability of the p53 protein is regulated by binding to MDM2, a protein that degrades p53 and consequently inactivates the transcriptional function of p53.[199,200,201] Mutations in *p53* prevent degradation by MDM2, allowing stabilisation and detection of the protein by immunohistochemistry.

The induction of the CDK inhibitor $p21^{waf1}$ after ionising radiation leads to a G1 growth arrest, thus allowing the cell to repair the damage.[202] Apart from induction by wild type p53, activation of the $p21^{waf1}$ gene can also occur through mechanisms independent of p53.[203] TGF-β, the BRCA1 gene products and Nerve Growth Factor (NGF) are examples of factors that promote $p21^{waf1}$ transcription by p53-independent mechanisms.[204-206] In addition to a role in the repair process, $p21^{waf1}$ has an important function during differentiation of cells.[207]

Role of molecular investigations in rectal cancer treatment

Investigations of the genetic pathways involved in rectal cancer give insight in the mechanisms of development of these tumours and may provide new therapeutic targets. In addition, biological parameters that identify a higher degree of aggressiveness, independent of known clinicopathological features of colorectal carcinoma may help to improve treatment strategies. However, to be able to investigate genetic pathways and prognostic markers in rectal carcinoma, standardised treatment is a prerequisite, since treatment-related variation of outcome should be ruled out.

OUTLINE OF THIS THESIS

In this thesis, we have studied clinical and molecular aspects of rectal carcinoma. Most results reported in this thesis are based on the prospective randomised TME-trial, a large trial investigating the role of short-term preoperative radiotherapy in combination with standardised TME-surgery. The first part of this thesis focuses on advances in the treatment of rectal cancer, while the second part involves new insights in molecular biology of rectal carcinomas. We investigated both aspects of rectal cancer since investigation of molecular parameters can provide a better understanding of clinical outcome.

PART I: ADVANCES IN TREATMENT

The basic conventional procedure involving blunt dissection, often resulted in incomplete removal of mesorectal tissue with high local recurrence rates. **Chapter 2** describes a retrospective analysis of local recurrence rate in a regional cancer centre in the west Netherlands of rectal cancer patients diagnosed between 1988 and 1992. In this study, we evaluated patients who were treated with conventional surgery.

In Europe, TME has become the preferred standard of operative management for rectal cancers. Current clinical trials examining the role of adjuvant therapy in patients who are undergoing standardised operations are now setting the standard of care in several European countries. **Chapter 3** provides an overview of present European trials in which TME-surgery is intentionally performed.

The TME-trial was set up to document local control when standardised TME is used and to answer the question whether short-term preoperative radiotherapy is still beneficial in TME treated patients. However, when investigating (neo)adjuvant therapies, side effects must be weighed against potential benefits with regard to recurrence and survival. In **Chapter 4**, short-term results of the combination of preoperative radiotherapy and TME-surgery are presented.

Before the start of the TME-trial there were doubts whether the excellent results of specialised surgeons performing TME-surgery could be repeated in a large multicentre trial. **Chapter 5** compares outcomes of rectal cancer patients in a former randomised trial, the Cancer Recurrence And Blood transfusion (CRAB)-trial in which conventional surgery was applied, with the TME-trial, in which standardised TME-surgery was introduced under extensive quality-control. Furthermore, the influence of hospital volume and specialisation was investigated

In **Chapter 6**, the main objective of the TME-trial, the role of preoperative radiotherapy in combination with TME-surgery, is analysed. Both short-term preoperative radiotherapy and TME have independently demonstrated to improve local control in rectal cancer, but the combination of these treatment modalities was never investigated.

PART II: NEW INSIGHTS IN MOLECULAR BIOLOGY

Several studies have suggested that the development of colon and rectal cancers may involve different mechanisms. In **Chapter 7** we investigated different genes involved in oncogenesis of colon and rectal cancers, and analysed their prognostic value. Cases were obtained from consecutive series of colon carcinomas and standardised treated rectal tumours from the pilot study of the TME-trial. Mutation analysis was performed for *p53* and *APC*. hMLH1, hMSH2, Bcl-2, p53, E-cadherin and β-catenin were investigated immunohistochemically.

Two pathways of genomic instability have been identified in colorectal carcinoma; chromosomal instability (CIN) and microsatellite instability (MSI). These pathways are characterised by their own genetic alterations and clinicopathological characteristics, although mutations in certain genes can be present in both pathways. **Chapter 8** describes the investigation of genomic instability patterns in rectal carcinomas with microsatellite markers, immunohistochemical, *p53* mutational and gene expression array analyses.

In the TME-trial, half of the patients received 5x5 Gy irradiation before the operation. This trial design provided an excellent opportunity to investigate the influence of irradiation on expression of genes involved in the cell cyclus and DNA-repair of cells. In **Chapter 9**, the influence of radiotherapy on the expression of p53 and $p21^{waf1}$ was investigated in normal mucosa and tumour tissue in vivo.

Since radiotherapy, surgery and pathology were standardised in the TME-trial, optimal conditions were provided for studying prognostic markers. In **Chapter 10**, we examined immunohistochemical expression of E-cadherin, α-, β-, γ-catenin, EpCAM and CD31, to analyse the influence of irradiation on the expression of cell adhesion molecules and microvessel count. Furthermore, the prognostic value of these factors was investigated.

A summary of this thesis is given in **Chapter 11** in English and in **Chapter 12** in Dutch.

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2

Local recurrence in patients with rectal cancer, diagnosed between 1988 and 1992: a population-based study in the west Netherlands

E. Kapiteijn[1], C.A.M. Marijnen[2], A.C. Colenbrander[6], E. Klein Kranenbarg[1], W.H. Steup[5], J.H.J.M. van Krieken[3], J.C. van Houwelingen[4], J.W.H. Leer[2], C.J.H. van de Velde[1]

Departments of Surgery[1], Radiotherapy[2], Pathology[3] and Medical Statistics[4], Leiden University Medical Centre, Leiden; Department of Surgery, Leyenburg Hospital[5], Comprehensive Cancer Centre West (CCCW), Leiden[6], The Netherlands

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INTRODUCTION

Colorectal cancer is the most common gastrointestinal cancer in the Western world. In 1992, 7700 new colorectal cancer patients were registered in The Netherlands, of whom about 35% had rectal carcinoma.[1] One of the major problems in the treatment of rectal cancer is the appearance of local recurrences. These cause severely disabling symptoms, are difficult to treat and usually have a fatal outcome.[2] Most of them become overt within 2 years of surgery.

Surgery is still the primary therapy for rectal cancer. In the literature, the reported local recurrence (LR) incidence after curative resection varies widely, between 5% and 45%.[3,4] The inter- and intra-institutional local recurrence rates vary to the same extent.[5] This emphasizes the importance of varying levels of surgical skill in the genesis of local failures.

Conventional non-standardised surgery of rectal cancer consists of a partial blunt dissection, directed "cone-wise" towards the rectal wall through the mesorectum. A high incidence of local recurrence is associated with this procedure. Surgeons specialised in rectal surgery have results of improved local control (5-8%) and survival in their series with standardised surgery. In the Western world the concept of circumferential or total mesorectal excision (TME) is advocated.[6,7] In Japan the extended lateral pelvic lymph node dissection is routinely conducted in a standardised procedure.[8]

Important factors of local recurrence and survival in rectal cancer are Dukes' Astler-Coller stage[9-11] and lateral margin involvement.[12,13] Other important factors include tumour grade,[14] fixation,[15,16] level of the tumour in the rectum,[17,18] blood[19] and lymphatic[20] vessel invasion and inadvertent perforation of the tumour during resection.[9,21,22] Sex[23] and age[24-26] are patient-related prognostic features. Prognostic factors can be used to assign patients with unfavourable prognosis to the most suitable adjuvant protocol to improve outcome.

In different studies, the benefit of radiotherapy (RT) in the treatment of rectal cancer has been demonstrated. Both pre- and postoperative radiotherapy have been shown to improve local control and disease-free survival.[27-29] In a large, prospective Swedish trial it was shown that preoperative hypofractioned radiotherapy results in better local control than postoperative radiotherapy.[30] Recently, results of the Swedish Rectal Cancer Trial showed reduced rates of local recurrence and also improved survival with the preoperative short-term 5x5 Gy regimen.[31] Shorter overall treatment time and better treatment compliance are probably the most important reasons for better results of short-term preoperative radiotherapy compared to postoperative radiotherapy.

A population-based retrospective study in patients with primary rectal cancer was set up by the Tumour Study Group Gastroenterology of the Comprehensive Cancer Centre West (CCCW). The objectives were to compile an inventory of overall local recurrence rate after non-standardised conventional surgery for rectal cancer and inter-institutional recurrence rate variability, and to investigate correlations between patient- and tumour-related factors and recurrence rate. In this study radiotherapy was given postoperatively according to CCCW guidelines. We also investigated compliance to these guidelines and whether violation of the protocol had consequences for local recurrence rate. Patients in this study were treated during the period 1988-1992 in 12 hospitals.

METHODS

The medical records of 1105 patients with rectal cancer diagnosed between January 1988 and December 1992 were reviewed. Basic data were obtained from the population-based registry of the CCCW, which derives its data from clinical records in hospitals upon notification of pathology laboratories and medical record administration. The minimum follow-up date was set on 1 February, 1995, the follow-up period being 3-8 years. Information on stage, type of surgery, local recurrence and survival was extracted from the clinical records. Additional information on dates of recurrence or death was provided by general practitioners.

Data from rectal endoscopy were used to determine the exact location of the tumour: ≤6 cm, 6.1-12 cm and 12.1-18 cm. The surgical procedure was considered to be curative when the surgeon stated in his surgical report that local tumour resection had been macroscopically radical without intraoperative detection of metastases. Extent of the tumour was recorded according to the modified Astler and Coller classification.[32] Tumours diagnosed in 1988 were classified according to the extent of disease (EOD) system[33] in the CCCW registry. As a consequence this left us with B1/B2 and C1/C2 tumours which we could not specify further. Tumour spill was defined as rupture of the tumour during resection. Surgical margins were considered tumour-free when proximal and distal margins were microscopically negative. Residual tumour (R1) was defined as either tumour spill or positive surgical margins.

Local recurrence was defined as tumour growth in the pelvis. This definition also included anastomotic recurrences and perineal wound recurrences in abdominoperineal resection (APR) patients. Distant metastases were defined as recurrence of tumour growth outside the pelvis. This definition also included "recurrence" at the side of the stoma or in para-aortic lymph nodes.

Indications for postoperative radiotherapy were Dukes' Astler-Coller B2 and C tumours, positive surgical margins, or tumour spill, according to the guidelines of the CCCW. Patients with an indication for radiotherapy received a dose between 50 and 60 Gy in fractions of 1.8-2.0 Gy, five fractions per week, depending on the institute. Treatment was ideally started within 6 weeks.

Hospitals were analysed separately, but also by volume category. The average number of patients per hospital was 56. The categories of low- and high-volume were determined by dividing the 12 hospitals into two volume-groups: hospitals with a number of patients lower than or equal to the average number of patients per hospital,≤56, were classified as low-volume hospitals, and hospitals with a number of patients higher than the average number of patients,>56, were classified as high-volume hospitals.

Statistical methods

Univariate analysis of local recurrence rates was performed using Chi-square tests. Odds ratios and 95% confidence intervals (CI) represent the relative risk compared to a reference category.

Logistic regression analysis was used for multivariate evaluation of prognostic factors including age (groups of <65, 65-75, >75 years), gender, location of the tumour (cm), type of surgery, intraoperative tumour spill, surgical margins, residual tumour, Dukes' Astler-Coller stage, adjuvant radiotherapy, adjuvant chemotherapy, hospital, and the low- and high-volume hospital categories. The categories of variables were represented by indicator

variables and their predictive value was assessed using the P-value of the log-likelihood. Only variables which significantly improved the fit of the model (P<0.10) were included in the final model.

Survival rates were calculated using the Kaplan-Meier method and differences between groups were assessed with the log-rank test.

RESULTS

Of 1105 cases, 437 were ineligible. 205 (19%) cases were excluded because of missing medical records, no carcinoma, or incorrect registration; 107 (10%) because of no laparotomy; 75 (7%) because of a non-curative resection; and 50 (5%) were lost to follow-up. The median age of the 1105 patients was 68.6 years (range 24.4-98.5). The baseline characteristics of these patients are shown in Table 1. For our final analysis, 668 curative resection cases were left. Median follow-up of these patients was 4.6 years.

Table 1. Baseline characteristics of the 1105 registered patients.

	Total
Eligible	668
Ineligible	437
-missing medical record/no carcinoma/incorrect registration	205
-no laparatomy	107
-non-curative resection	75
-lost to follow-up	50
Sex	
-male	592
-female	513
Age (years)	
-mean	68.6
-range	24.4-98.5

Local recurrence rate

The overall local recurrence rate was 22.5% (150/668). Sixty-six percent (99/150) of the local recurrences were diagnosed within 2 years and 88% (132/150) within 3 years.

In the univariate analysis, tumour location (P=0.001), intraoperative tumour spill (P=0.002), positive surgical margins (P=0.001), residual tumour (P<0.001), Dukes' Astler-Coller stage (P<0.001), and adjuvant radiotherapy (P=0.03) were significant prognostic factors for the risk of local recurrence. These results are shown in Table 2. The local recurrence rate was highest (29%) for patients with a tumour in the most distal part of the rectum (0-6 cm). Patients with intraoperative tumour spill had a higher local recurrence rate than patients without tumour spill, 47% vs. 21%, although tumour spill was reported in only 34 (5%) of the patients. Local recurrence rate was significantly higher in patients with tumours with positive surgical margins than for patients with negative surgical margins, 56% vs. 22%, although only 16 (2%) patients had tumours with positive margins. Obviously, residual tumour (R1), defined as either tumour spill or positive surgical margins, was also a significant factor. The local recurrence rate was highest for patients with a Dukes' C tumour. It is surprising however, that patients with C1 tumours had a higher recurrence rate than patients with C2 tumours, 40% vs. 32%. Patients who had received postoperative

radiotherapy had a significantly higher local recurrence rate than patients who had not received radiotherapy, 27% vs. 20%.

In the multivariate analysis tumour location (P<0.001), residual tumour (P=0.001) and Dukes' Astler-Coller stage (P<0.001) were significantly related to local recurrence. Figures 1 and 2 show local recurrence risks for the R0 and R1 groups by Dukes' Astler-Coller stage and tumour location as obtained from the logistic regression model. The risks shown in these figures are not the fractions by subgroup. The risks probably correspond with these fractions, but in the logistic regression model risks can also be calculated in almost empty subgroups. Patients with C1 tumours, tumours at 0-6 cm from the anal verge and R1 status had the highest risk for local recurrence.

Local recurrence rates varied from 9% to 36% between the 12 hospitals (Table 2). However, these differences were not statistically significant on univariate or multivariate analysis. There was also no significant difference in local recurrence rate between the low- and high-volume hospital groups; low-volume hospital LR rate, 22%, vs. high-volume hospital LR rate, 23%. Figure 3 shows the 95% confidence intervals of the local recurrence rates of the 12 hospitals in the univariate analysis, ranged according to volume. The lowest and highest recurrence rates are observed in hospitals with low numbers of patients. High-volume hospitals have average recurrence rates.

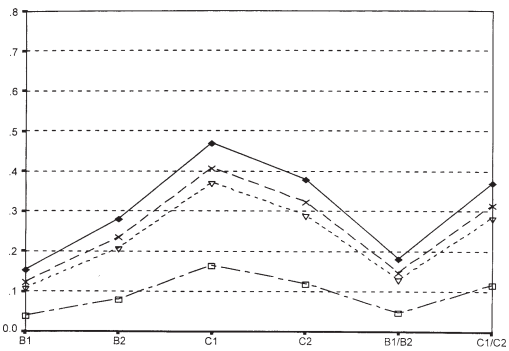

Figure 1. Risk of local recurrence by Dukes' Astler-Coller stage and tumour location for the R0-group as obtained from the logistic regression model. Distance from anal verge: ——, 0-6 cm; · · ·, 6.1-12 cm; - · -, 12.1-18 cm, - - -, unknown.

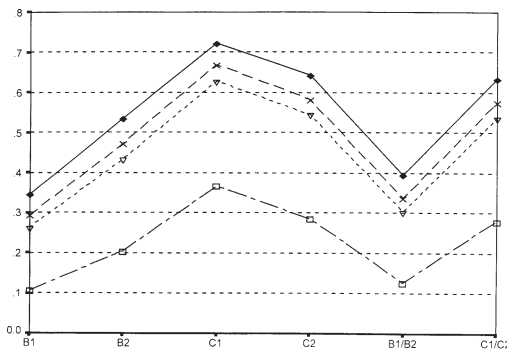

Figure 2. Risk of local recurrence by Dukes' Astler-Coller stage and tumour location for the R1-group as obtained from the logistic regression model. Legend as for figure 1.

Recurrence-free and overall survival

In studying the time to local recurrence one should note that patients who die before local recurrence will not experience a recurrence. Therefore, using the Kaplan-Meier method with death as a censoring variable is not appropriate because such an analysis assumes that patients can still experience a recurrence after death! A correct analysis will set the time to local recurrence at a very high value (e.g. 4000 days) for patients who die before recurrence. The resulting Kaplan-Meier is shown in figure 4 and estimates the chance of ever having experienced recurrence at about 24%. This is very close to the observed overall local recurrence rate we found (22.5%). Figure 4 also shows that there is hardly any censoring due to short follow-up before the Kaplan-Meier curve reaches its plateau.

Table 2. Local recurrence rate (LR) for rectal cancer patients (668), univariate analysis.

	n (%)	LR	% LR	P	Odds ratio (95% CI)
Age (years)				0.47	
-<65	230 (34)	57	25		
-65-75	241 (36)	54	22		
->75	197 (30)	39	20		
Gender				0.71	
-male	374 (56)	86	23		
-female	294 (44)	64	22		
Tumour location (cm)				0.001	
-12.1-18	99 (17)	8	8		1.0
-6.1-12	257 (43)	56	22		3.2 (1.5-6.9)
-0-6	236 (40)	68	29		4.6 (2.1-10.0)
-unknown	76	18	24		3.5 (1.4-8.6)
Surgery				0.18	
-APR*	238 (36)	62	26		
-LAR**	400 (60)	80	20		
-Hartmann procedure	30 (4)	8	27		
Tumour spill				0.002	
-no	634 (95)	134	21		1.0
-yes	34 (5)	16	47		3.3 (1.6-6.7)
Surgical margins				0.001	
-negative	652 (98)	141	22		1.0
-positive	16 (2)	9	56		4.7 (1.7-12.7)
Residual tumour				<0.001	
-R0	622 (93)	128	21		1.0
-R1	46 (7)	22	48		3.5 (1.9-6.5)
Dukes' Astler-Coller stage				<0.001	
-B1	173 (27)	22	13		1.0
-B2	230 (35)	53	23		2.1 (1.2-3.5)
-C1	30 (5)	12	40		4.6 (1.9-10.8)
-C2	145 (22)	47	32		3.3 (1.9-5.8)
-B1/B2	60 (9)	9	15		1.2 (0.5-2.8)
-C1/C2	14 (2)	5	36		3.8 (1.2-12.4)
-unknown	16	2	13		1.0 (0.2-4.6)
Adjuvant radiotherapy				0.03	
-no	429 (64)	85	20		1.0
-yes	239 (36)	65	27		1.5 (1.0-2.2)
Adjuvant chemotherapy				0.55	
-no	645 (97)	146	23		
-yes	23 (3)	4	17		
Hospital				0.30	
-1	94 (14)	19	20		
-2	32 (5)	3	9		
-3	21 (3)	3	14		
-4	63 (9)	13	21		
-5	44 (7)	10	23		
-6	64 (10)	12	19		
-7	65 (10)	13	20		
-8	94 (14)	19	20		
-9	48 (7)	14	29		
-10	50 (7)	14	28		
-11	65 (10)	20	31		
-12	28 (4)	10	36		
Hospital volume					
-low-volume (6 hospitals)	237 (35)	52	22		
-high-volume (6 hospitals)	431 (65)	98	23	0.81	

* abdominoperineal resection.

** low anterior resection.

The P-values given are the P-values of the chi-square test in the 2xk table. Odds ratios are given for the prognostic factors showing a significant effect. The most favourable group is chosen as baseline.

Therefore, we can safely assume that the patients censored after 1000 days will only rarely have experienced recurrence. Moreover, it can be concluded that actual experienced local recurrence can be safely taken as our outcome variable (as there are only a few patients with early censoring that might have had a recurrence later on).

The outcome "observed local recurrence" was analysed by logistic regression. To investigate the influence of local recurrence on overall survival we applied Cox regression with the factors from the univariate and multivariate analysis and occurrence of LR as time-dependent covariate. Age, Dukes' Astler-Coller stage, type of surgery and sex were determinants for overall survival. The appearance of a local recurrence influenced overall survival after diagnosis of the recurrence and made the chance of dying 10 times greater than no appearance of local recurrence (data not shown).

The median overall survival in patients with local recurrence was 265 days after diagnosis of the recurrence. Overall survival was 40% for the whole group of patients at the end of the follow-up period (1 February 1995). Cancer-free survival was 60% for the whole group (data not shown).

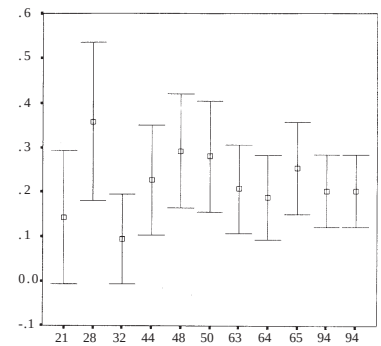

Figure 3. 95% CI intervals of the local recurrence rates of the 12 hospitals, ranged according to volume (univariate analysis).

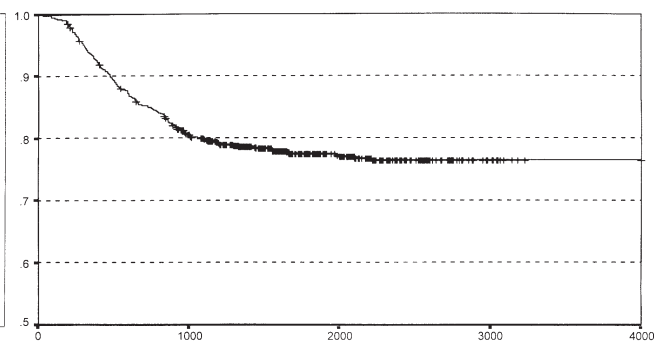

Figure 4. Recurrence-free survival. Kaplan-Meier curve in which the time to local recurrence is set at 4000 days for the patients who have died before recurrence. The possibility of experiencing a recurrence is estimated at about 24%.

Compliance to guidelines for postoperative radiotherapy

Adjuvant radiotherapy was given to 239 patients (36%, Table 2). Twenty-three patients (3%) received adjuvant chemotherapy. Of these patients, 12 also received radiotherapy while 11 did not (data not shown). The chemotherapy was administered within a clinical trial similar to that published by Moertel et al.[34]

In Table 3 the compliance to the guidelines for radiotherapy is shown. According to the guidelines, 432 patients should have received radiotherapy. However, only 214 patients (50%) actually received radiotherapy. Eight (5%) of 163 patients who should *not* have received radiotherapy, did so. In the patient group with an indication for radiotherapy, there was no significant difference in local recurrence rate between patients treated according to the guidelines and those not treated according to the guidelines: 29% vs. 27%. These results are shown in Table 4.

Table 3. Compliance to guidelines for postoperative radiotherapy.

	RT given (222)	RT not given (373)
guideline: RT (432)	214 (50%)	218 (50%)
guideline: no RT (163)	8 (5%)	155 (95%)
unknown: 73*		

* unknown: guidelines were unknown in 73 cases because of unknown Dukes' Astler-Coller stage and Dukes' B1/B2 tumours.

Table 4. Local recurrence rates by guideline and radiotherapy group.

	RT given	RT not given
guideline: RT	63/214 (29%)	58/218 (27%)
guideline: no RT	0/0 (0%)	18/155 (12%)
unknown: 73		

DISCUSSION

This population-based study showed an overall local recurrence rate of 22.5% in patients with primary rectal cancer after non-standardised surgical treatment with or without adjuvant treatment. Independent prognostic factors for the risk of local recurrence were Dukes' Astler-Coller stage, distance from the anal verge and residual tumour. Although there was great variability (9-36%) between local recurrence rates between hospitals, no significant differences in recurrence rates between the separate hospitals or between low- and high-volume hospitals were found. Postoperative radiotherapy guidelines were available in this study. The compliance to these guidelines was only 50%. However, no significant difference in recurrence rate was found between patients treated according to the guidelines and those not treated according to the guidelines.

We performed this population-based study with data collected retrospectively from rectal cancer patients diagnosed between 1988 and 1992 from 12 hospitals in the west Netherlands. The greatest advantage of a population-based study is that analysis is based on an unselected group of patients from oncological centres and peripheral hospitals. This is in contrast with most trials in which, due to the use of inclusion and exclusion criteria, selection or centralisation of treatment inevitably takes place. The minimum follow-up was 3 years. This period seems long enough since most recurrences are diagnosed within 2-3 years. In our study, 88% of the local recurrences were diagnosed within 3 years.

The overall local recurrence rate of 22.5% is not at variance with other studies in which non-standardised conventional surgery was performed.[26,35] However, standardised TME surgery has been shown to result in much lower recurrence rates and improved survival.[6,7] In The Netherlands, TME surgery was introduced a few years ago to improve the results of treatment of primary rectal cancer.

Prognostic factors can be used in selecting patients with tumours with an unfavourable prognosis to be assigned to the most suitable adjuvant protocol to improve outcome. We found a higher local recurrence rate for patients with Dukes' C tumours as compared to Dukes' B tumours. Other authors[9-11] have found that patients with Dukes' B and C tumours have a higher rate of recurrence and a poorer outlook than those with Dukes' A lesions. In our study it was notable that patients with C1 tumours showed a higher recurrence rate

than patients with C2 tumours. This finding can most probably be explained by the fact that the number of C1 tumours was very small. Another explanation is that the C1 group might have been mixed with C2 tumours which were staged as C1 tumours.

We found the highest recurrence rate in patients with tumours located between 0 and 6 cm from the anal verge. The prognostic significance of the tumour level in the rectum seems to be generally accepted; tumours arising at distances of under 6 cm from the anal verge have also been found to be associated with an increased local recurrence rate in other studies.[17,18]

Furthermore, residual tumour was an independent prognostic factor for the risk of local recurrence, while tumour spill and positive surgical margins appeared to be significant in the univariate analysis only. Ranbarger et al.,[36] Slanetz et al.[37] and Zirngibl et al.,[21] also showed in their studies that intraoperative spillage of tumour cells significantly increases the local recurrence rate. Tumour involvement of bowel mucosa at resection margins can also predict a possible anastomotic recurrence. However, most recurrences in the operating field are extraluminal and relate to lateral tumour spread. These local recurrences can only be predicted in less than half of the cases by pathological examination in current use in which only proximal and distal margins are investigated. Quirke et al. clearly demonstrated that a different method of pathological preparation and examination, which can reveal lateral margin involvement, is able to predict a local recurrence in 85% of the cases.[12,13] In our study circumferential margins were not routinely investigated.

Hospital was not a significant prognostic factor in our analysis. No significant differences in local recurrence rate were found between the separate hospitals or between low- and high-volume hospitals. However, the variability of local recurrence rate was large between the hospitals: 9% to 36%. The explanation for the non-significance of this variance might be that the lowest and highest recurrence rates were seen in hospitals with low numbers of patients.

Inter-institution and inter-surgeon variabilities have been shown in other studies. This applies to immediate results, such as surgical mortality and morbidity,[5,38-40] as well as long-term results, such as local recurrence[5,9,41] and survival.[5,41] Philips et al.[9] found that local recurrence rate varied from 5% to 20% between the 20 individual surgeons participating in the Large Bowel Cancer Project. Similarly, McArdle et al.[42] and McArdle and Hole[5] noted recurrence rates ranging from 0% to 21% between the individual surgeons participating in their study. Controversy exists about case volume in itself being important for achieving good results in cancer surgery. It is becoming increasingly clear that specialist interest is also important.[43] The specialist will be familiar with the relevant anatomy and techniques so that good results will be achieved, even though relatively small numbers of patients may be treated. It seems that a certain volume is necessary for good long-term results, but above this level inter-surgeon variability in long-term outcome cannot be correlated with surgeon volume.[44] However, in a recent study of Porter et al.,[45] it was shown that outcome is improved both with colorectal surgical subspecialty training and a higher frequency of rectal cancer surgery.

Radiotherapy was a significant prognostic factor in the univariate analysis for the risk of local recurrence. Patients who received radiotherapy did worse than patients who did not receive radiotherapy. A likely explanation for this finding might be that the patients who received radiotherapy were those whose tumours had the worst behaviour. Apparently,

clinicians were able to select those patients with the highest risk of local recurrence.

The compliance to the guidelines for postoperative radiotherapy was only 50%. Surprisingly, no difference in local recurrence rate was found between patients treated according to the guidelines and those not treated according to the guidelines in the group which should have received radiotherapy. However, it cannot be concluded from this finding that postoperative radiotherapy does not play a role in the prevention of local recurrence. The patients who did not receive radiotherapy, despite the fact that they were entitled to it according to the guidelines, might have done better if they had received radiotherapy, and the local recurrence rate in the group of patients who received radiotherapy might have been higher if these patients had not received any further treatment.

In the Uppsala trial it was shown that a short course of preoperative radiotherapy resulted in better local control compared to postoperative radiotherapy.[46] One explanation for the success of the preoperative radiotherapy is that a radiobiological high dose is given in a short overall time followed by immediate surgery. In our study and in most postoperative radiotherapy studies, treatment ideally starts within 6 weeks and lasts for 5-6 weeks. This long overall treatment time might lead to regrowth of residual tumour. Another explanation for the better results of short-term preoperative radiotherapy is better compliance for preoperative radiotherapy compared to that of postoperative radiotherapy. In the Uppsala trial, 16% of the patients who should have been referred to postoperative irradiation did not receive radiotherapy, usually due to problems with postoperative recovery. The non-compliance for the preoperative short-term radiotherapy scheme was only 0.5%.[46] Within our study, we will investigate the reasons for violations to the postoperative radiotherapy protocol in an additional medical record check.

The objective of this study was to complete an inventory of local recurrence rate after curative non-standardised surgery for rectal cancer. This study was set up prior to the initiation of a nation-wide trial. Since January 1996, surgeons, radiotherapists, and pathologists have been involved in a prospective randomised trial "Total mesorectal exision (TME) with or without preoperative radiotherapy in the treatment of primary rectal cancer".[47] In this trial, surgery, radiotherapy, and pathology are standardised. At present, 1278 patients have been randomised from a large number of hospitals in The Netherlands and several hospitals in other countries. This trial will hopefully result in a low local recurrence rate and provide an answer to the question of whether preoperative short-term radiotherapy is still beneficial in the treatment of primary rectal cancer when standardised TME-surgery and pathology are applied.

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3

European trials with Total Mesorectal Excision

E. Kapiteijn[1], C.J.H. van de Velde[1]

Department of Surgery, Leiden University Medical Centre, Leiden, The Netherlands[1]

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INTRODUCTION

Until a few years ago, results after curative surgery for rectal cancer were not optimal, reflected in high local failure rates.[1-3] The basic conventional procedure involving blunt dissection, has been shown to result in an incomplete removal of mesorectal tissue, with a high risk for local failure and damage to the autonomous pelvic nerve plexus, resulting in a high incidence of sexual[4,5] and bladder dysfunction.[6] Lack of improvement in the surgical results of rectal cancer has prompted many investigators to seek different adjuvant therapy approaches in conjunction with surgery.

In several studies, the benefit of radiotherapy (RT) in the treatment of rectal cancer has been suggested. In a large prospective Swedish trial it was shown that preoperative hypofractioned radiotherapy results in better local control than postoperative radiotherapy.[13] The role of chemoradiotherapy or chemotherapy (CT) as sole treatment still has to be determined. The Netherlands Adjuvant Colorectal Cancer Project (NACCP) did not show an effect of chemotherapy in rectal cancer.[14] In the United States the opinion is that all patients with a Dukes' B or C lesion should have postoperative chemoradiotherapy.[15] Chemo(radio)therapy is not routine in Europe and is still considered investigational therapy in rectal cancer.

Improvement in survival with radiotherapy alone was not demonstrated until the Swedish Rectal Cancer Trial (SRCT).[7] This was the first randomised study to demonstrate a significant survival benefit in patients receiving preoperative radiation (compared with surgery alone). In recent years, local control and survival have been further improved by the introduction of total mesorectal excision (TME) surgery, as first described by Heald.[8-10] With this technique, less morbidity[4,11,12] and a reduction in abdominoperineal resections have also been reported.[10]

A major problem of published studies on adjuvant therapy in the treatment of primary rectal cancer is that surgery has not been standardised in these studies. Moreover, the surgeon remains an important factor in the accomplishment of tumour control and reduction of morbidity.[16-18] Therefore, the effect of adjuvant therapy can only be studied when strict standardised and quality-controlled surgery is performed.

Optimal quality control of the surgical procedure must also include a standardised examination by pathologists.[19-20] Detection of mesorectal spread requires systematic examination of the specimen, by serial sectioning of the whole tumour and the surrounding mesorectum in the transverse plane. This method can be used to monitor differences in operative technique. Furthermore, standardised surgery can be documented photographically, due to reproducible gross specimen features.[21]

In Europe, TME has become the preferred standard of operative management for rectal cancers.[22] Current clinical trials examining the role of adjuvant therapy in patients who are undergoing standardised operations are now setting the standard of surgical care in several European countries. We studied European trials in which TME-surgery is intentionally performed. Trials were classified in neoadjuvant and adjuvant trials. Furthermore a subdivision was made according to short- and long-term radiotherapy. Most of these trials are still in progress and have short follow-ups, so definitive results cannot be presented yet, apart from feasibility and interim analyses.

NEOADJUVANT TRIALS, SHORT-TERM PREOPERATIVE RADIOTHERAPY

TME-trial, Dutch ColoRectal Cancer Group (DCRCG)

In this trial, patients with resectable rectal cancer underwent standardised TME surgery alone, or patients received preoperative radiotherapy (5x5 Gy) followed within 10 days after the start of radiotherapy by TME.[23] Table 1 shows the characteristics of this trial and the other trials described.

TME was performed according to strict and controllable quality demands. An extensive structure of workshops, symposia, and instruction videos helped to accomplish this goal. In addition, a monitoring committee of specially trained instructor-surgeons was formed for on-site instructions to optimise quality. R.J. Heald, W.E. Enker and Y. Moriya were involved as operating surgeons in different hospitals in The Netherlands and as instructors at several workshops about the trial. Pathological examination was done according to the protocol of Quirke.[19,20] Special training courses were given to pathologists. A pathology review panel and a trained quality manager guaranteed quality control.

Taking into account an ineligibility rate and an R1-resection percentage of 25%, it was calculated that 1400 Dutch patients had to be randomised in order to detect a difference of 5% in local recurrence rate (LR) between the R0-patients in the TME alone group (LR 10%) and R0-patients in the RT-group (LR 5%).

From January 1996 until January 2000, 1861 patients were randomised; 1530 patients from 84 Dutch hospitals and 331 patients from 24 hospitals in other countries (mainly Sweden). The European Organisation for Research and Treatment of Cancer (EORTC) participated in the TME-trial under trialnumber 40971, in order to assure quality control of surgery for the EORTC-GastroIntestinal (GI) group.

Of the patients randomised to receive preoperative radiotherapy, 87% received it at the correct dose and were operated within 10 days after its inception. In total, 37% of the operations were attended by instructor-surgeons; in the first quarter, most operations (89%) were supervised by instructor-surgeons. Later on, this percentage decreased to 19% in the last quarter of the trial. Only perioperative blood loss was significantly higher in irradiated patients, and the perineal wound dehiscention rate was higher in irradiated abdominoperineal resection (APR)-patients.[24] The clinical leak rate was 12% in the low anterior resection (LAR)-group, with no difference between the randomisation groups. In the LAR-group, temporary stomas were constructed in 57% of the patients. The percentages for side-end anastomosis and pouch construction were 60% and 28%, respectively. From this trial it can be concluded that performing a large, multicentre trial with quality control of surgery is feasible. The accrual of the trial has been very good, and short-term preoperative radiotherapy was also demonstrated to be safe in combination with TME-surgery.

In a recent study by Nagtegaal et al.,[25] it was shown that pathology data need to be based on pathology reports and controlled by trained quality managers. A retrospective comparison of pathology data case record forms with hospital pathology reports was performed using the data from 300 patients from the TME-trial. Successive rounds of quality control appeared to be required for accuracy and completeness of pathology data.

The overall local recurrence rate up to 1 July 2000 (median follow-up 20 months, range 0.3-48.5 months) was low: 7% in the operated-upon with curative intent group (R0+R1) and 5% in the R0-group. The role of preoperative radiotherapy in combination with standardised TME-surgery is not known yet, because follow-up has not been completed.

Medical Research Council (MRC) CR07 trial

The aim of this trial is to address the following question in operable rectal cancer: Are local recurrence-free rates and quality of life optimised by giving all patients short-course preoperative radiotherapy, or is it preferable to give postoperative chemoradiotherapy only to those at high risk of recurrence (i.e. with involved margins following surgery)?

Randomisation is done for preoperative radiotherapy of 5x5 Gy and selective postoperative radiotherapy of 25x1.8 Gy; if randomised for this arm patients with involved circumferential margins receive chemoradiotherapy. Adjuvant chemotherapy can be given as per local policy to these patients, but also to other randomised patients.

Although the aim of the operation is to achieve complete local excision of the tumour, the performance of a formal total mesorectal excision is left to the surgeon's discretion. All specimens are assessed by the local pathologist using the procedure of Quirke and Dixon[19] and Quirke et al.[20] Pathologists had to attend a training day, and a network of regional pathologists (including P. Quirke) has been established to handle any queries, and to provide further pathology training if required. A system of quality assurance was implemented to ensure uniformity of pathological reporting.

The trial is designed to show that there is less than a 5% difference in local recurrence rates at 2 years. This will require the randomisation of approximately 1800 patients.

The trial started in March 1998, and up to July 2000, 375 patients have been randomised from 46 centres in the UK, including South Africa and New Zealand. From an interim-analysis, it appeared that anastomotic leak rate is 10% in the LAR-group. Of the patients randomised to receive preoperative radiotherapy, 88% received it at the correct dose. Over 80% of patients in the postoperative arm with involvement of the circumferential margin received postoperative radiotherapy, 75% at the dose stated in the protocol.(newsletter MRC CR07, Spring 2000)

Stockholm IV trial

The aim of this study is to compare preoperative radiotherapy treatment with "conventional" fractionation (25x2 Gy) with treatment of 5x5 Gy, and to study a possible effect of different time intervals between the end of radiotherapy and surgery in operable rectal cancer. The main questions to be addressed are: A) Is preoperative radiotherapy given during 5 weeks with a conventional fractionation (25x2 Gy) followed by surgery after 4-8 weeks (arm 1) preferable to treatment with 5x5 Gy during one week followed by surgery within a week (arm 2) or after 4-8 weeks (arm 3)? B) Are there any clinically significant differences in the rate of local recurrence, survival time, postoperative morbidity and mortality, or late morbidity? C) Is the need of a permanent stoma less if the surgery is delayed?

Some centres and some patients will likely not accept the first randomisation arm, due to long treatment time. In these situations patients may be randomised only between the second and third arms.

Specimen-oriented surgery with total or partial mesorectal excision according to Heald is performed in the trial. In Sweden, introduction of TME was done on a general basis several years ago.[10,26] In addition, the protocol of Quirke was introduced and taught to pathologists.[10] In the Stockholm IV trial, the surgical specimen is judged according to this protocol.

Total accrual will be 840 patients. When 300 patients have been included and followed

for at least 2 years in the two-armed comparison, an interim analysis regarding cumulative local recurrence will be undertaken.

This study started in 1999 and up to 1 July 2000, 50 patients have been randomised.

NEOADJUVANT TRIALS, LONG-TERM PREOPERATIVE RADIOTHERAPY

Chirurgische Arbeitsgemeinschaft fur Onkologie (CAO)/Arbeitsgemeinschaft Radiologische Onkologie (ARO)/Arbeitsgemeinschaft Internistische Onkologie (AIO), Rectal Cancer Study

In this trial, patients with advanced rectal cancer (uT3/4 or uN+ or Mason III/IV) are randomly assigned to pre- or postoperative radiochemotherapy: 50.4 Gy are applied to the pelvis. 5-fluorouracil (FU) is administered concomitantly as 120h-continuous infusion. Four cycles of 5-FU maintenance chemotherapy are applied. Radiochemotherapy is identical in both arms except for a small-volume boost of 5.40 Gy in the postoperative setting. The time interval between radiochemotherapy and surgery is 4-6 weeks in both arms.

Techniques of surgery are standardised and total mesorectal excision is mandatory for lesions in the lower and middle parts of the rectum. The pathology examination is done according to standardised procedures that were established by P. Hermanek.

A decrease in local recurrence rate is expected from 15% to 5-10% in the arm with preoperative radiochemotherapy vs. the arm with postoperative therapy. Furthermore a survival advantage of 5-10% is expected, with no difference in, or even lower toxicity. In total, 680 patients must be randomised in order to detect these differences.

Regular study meetings are held twice a year with review of patients charts including surgery and pathology reports and data of radiochemotherapy, to ensure quality control.

This trial started in the summer of 1994, and up to 1 July 2000, 597 patients have been recruited from 26 participating institutions. Accrual of the trial will probably close in December 2000, when more than 800 patients will have been randomised. From an interim-analysis it was concluded that accrual of the trial is going well and that neoadjuvant radiochemotherapy is tolerated excellently and bears no higher risk for peri- and postoperative morbidity.[27]

EORTC 22921

This trial is conducted by the EORTC Radiotherapy Group and evaluates in a four-arm randomised study the effects of combining 5-FU/leucovorin (LV) with preoperative irradiation (45 Gy) vs. radiotherapy alone (45 Gy), and of postoperative 5-FU/LV vs. no adjuvant therapy in patients with resectable T3/4 adenocarcinoma of the rectum.

The surgical procedure should be performed as soon as possible 3-10 weeks after preoperative treatment in both arms. It is advised to perform a total dissection of the mesorectal fat in any case. In the trial pathological examination is not strictly performed according to Quirke. However, on the pathology form information is asked about the status and quality of the circumferential margin.

In a previous study of the EORTC-GI group using preoperative irradiation, the 5-year survival was 52% in clinically selected patients. The minimal clinically significant survival difference of interest is 10%. If it is assumed that 25% of the randomised patients will become potentially ineligible, a total of 992 patients have to be entered.

Quality control procedures take place by means of audits of the Radiation Physics Quality Assurance Committee of the cooperative group of Radiotherapy[28] and a Datamanagement

Study Group.

This trial started in April 1993 and up to 1 July 2000, the accrual was 759 patients.

ADJUVANT TRIALS

Study 92/157-004

Randomised phase III studies for a direct comparison of 5-FU/LV + monoclonal antibody (mAb) 17-1A vs. 5-FU/LV in colon cancer are completed, but not yet analysed for outcome. A comparable study for rectal cancer has not been conducted yet. Therefore, the 92/157-004 study was set up by the Austrian cooperative group. The objective of this study is to assess the efficacy of preoperative radiotherapy in combination with postoperative chemotherapy or postoperative immuno-chemotherapy.

At first all patients were treated with 10x2.5 Gy (5 days of 2x2.5 Gy) preoperative radiotherapy followed by surgery. The rationale for this scheme was the fear of side effects with the larger single dose of 5 Gy. After surgery, Dukes' B or C tumour patients were randomised between 5FU/LV vs. 5-FU/LV/mAb 17-1A. This trial has in the meantime been amended. Investigators are allowed to choose prolonged fractionation in order to permit inclusion in the study of patients whose tumours at presentation are felt to be unresectable (and those who have a bulky, but resectable tumours). Operable tumours at presentation may receive either short or prolonged fractionation radiotherapy according to individual practise.

In carcinoma of the middle or lower third of the rectum, the mesorectum should be removed completely, laterally as well as caudally, up to the visceral pelvic fascia of the pelvic floor. Pathological examination is done according to the protocol of Quirke.

The study is designed to detect a difference in 5 year survival from 70% for chemotherapy alone, as compared to 80% for chemotherapy plus mAb 17-1A. Total accrual is calculated to be 700 patients, with 350 patients per arm.

This trial started in July 1997 and accrual up to 1 July 2000 was 278 patients.

PROCTOR-trial, Dutch ColoRectal Cancer Group (DCRCG)

The successor to the TME-trial is the PROCTOR-trial (Preoperative Radiotherapy and/Or adjuvant Chemotherapy combined with Tme-surgery in Operable Rectal cancer). In this trial, the role of adjuvant chemotherapy (5FU/LV) is investigated in combination with standardised TME-surgery and pathology. Randomisation for preoperative short-term radiotherapy is continued in the PROCTOR-trial, but hospitals can also choose for an own policy of yes/no preoperative radiotherapy, until the outcome of the TME-trial is known. After surgery and pathological examination, TNM-stage II or III, R0 patients are randomised for chemotherapy with 5FU/LV or observation. The setup for this trial is the same as that for the TME-trial.

The overall survival in the arm treated without chemotherapy is expected to be approximately 60%. Assuming an improvement in overall survival from 60% to 70% in the arm with chemotherapy, 500 R0, TNM-stage II or III patients are needed per arm.

The PROCTOR-trial has been accruing patients since April 2000; up to 1 July 2000, eight patients have been randomised.

Table 1. Randomised phase III treatment trials in rectal cancer.

Trial	Year started	Year closed	Design	Inclusion-criteria	Stratification	No. of partici-pating centres	Ac-crual	Target accrual	Main outcome parameter	Difference to be detected
Neoadjuvant, short-term radiotherapy										
TME-trial, DCRCG [23,24]	1996	2000	-TME -5x5 Gy RT + TME	Resectable adenocarcinoma within 15 cm of anal verge, without evidence of distant metastases, no upper age limit	Hospital, type of resection	108	1861	1400 (Dutch patients)	Local recurrence	5%
MRC CR07 trial	1998	Still open	-5x5 Gy + surgery -Surgery + selective 45 Gy	Resectable adenocarcinoma within 15 cm of anal verge, without evidence of distant metastases, upper age limit 75	A number of factors including surgeon	46	375	1800	Local recurrence	5% at 2 years of FUP
Stockholm IV	1999	Still open	-50 Gy + surgery -5x5 Gy + surgery within one week -5x5 Gy + surgery after 4-8 weeks	Operable adenocarcinoma, without evidence of distant metastases	Hospital	unknown	50	840	Postop morbidity and mortality, rate of sphincter preserving surgery, late morbidity, local recurrence, survival	When 300 patients have been included and followed for at least 2 years in the 2-arm comparison (2nd and 3rd arm), an interim-analysis regarding LR will be undertaken.
Neoadjuvant, long-term radiotherapy										
CAO/ARO/ AIO[27]	1994	Still open	-Surgery + 50.4 Gy+5.4 Gy boost/5-FU -50.4 Gy/5-FU + surgery	Advanced rectal cancer (uT3/T4 or uN+ or Mason CS III/IV) within 16 cm of anal verge, without evidence of distant metastases, upper age limit 75	Surgeon	26	597	680	Overall survival	10%
EORTC 22921	1993	Still open	-45 Gy + surgery -45 Gy/5FU-LV + surgery -45 Gy + surgery + 5FU-LV -45 Gy/5FU-LV + surgery + 5FU/LV	Resectable T3/T4 adenocarcinoma, within 15 cm of anal verge, without evidence of distant metastases, upper age limit 75	Institution, sex, tumour location (0-5, 5-10, 10-15 cm), stage (T3/T4)	40	759	992	Disease-free and overall survival	10% overall survival
Adjuvant										
Study 92/157-004	1997	Still open	-5x5 Gy or 10x2.5 Gy or 40-45 Gy + surgery + 5FU/LV -5x5 Gy or 10x2.5 Gy or 40-45 Gy + surgery + 5FU/LV/mAb17-1A	Dukes' B or C rectal tumour, within 16 cm of anal verge, R0 resection, at least 8 histologically investigated lymph nodes, upper age limit 80	Centre, preop RT-regimen, T-stage, N-stage	50	278	700	Overall survival	10%
PROCTOR -trial, DCRCG	2000	Still open	-no RT or 5x5 Gy + surgery -no RT or 5x5 Gy + surgery + 5FU/LV	TNM-stage II or III tumours, within 15 cm of anal verge, R0 resection	Centre, preoperative RT	6	8	1000	Overall survival	10%

DISCUSSION

The outcome after surgery for rectal cancer differs markedly between patients series, regarding both local recurrence rates and survival. A high incidence of local recurrence is associated with conventional, nonstandardised procedures.[1-3] To improve the results of surgery, various additional treatments, such as radiotherapy, chemotherapy, and immunotherapy, have been tested.

The Swedish Rectal Cancer Trial was the first trial to show that better local control achieved with preoperative radiotherapy resulted in improved survival.[7] The results found in this trial confirmed the studies from P. Hermanek, which showed a clear correlation between survival and local recurrence rate.[18] This can be explained by the fact that the disease recurs locally first and then disseminates to other organs in 20-30% of all patients with a recurrent rectal cancer.

In recent years local control and survival have been further improved by the introduction of the TME-technique, as first described by Heald.[8-10] TME is accomplished by precise sharp dissection within the true pelvis around the integral mesentery under direct vision, enveloping the entire mid-rectum, with preservation of the hypogastric plexus.

The studies published so far on adjuvant therapy have been carried out without any adequate definition of the surgical procedure and without appropriate quality control. In none of the cases were explicit details given of the procedure applied, together with the criteria to be met with respect to safety margins, excision of mesorectum and lymph node dissection. Local recurrence rates in the “surgery alone” control groups of former trials were often high; 20% or more.[7,29-33] Therefore, it is necessary to determine whether a further improvement can be obtained by adjuvant therapy, given the currently achievable global local recurrence rates of less than 10% by standardised TME-surgery alone. If optimal TME-surgery can be widely implemented, outcome improvement could, by the calculations of Hermanek, be four times as great as that achievable by adjuvant therapy, and at a fraction of the cost.

In this work an overview is given on European trials in which TME-surgery is intentionally performed. Most of these trials are still in progress and have too short a follow-up, so definitive results, apart from feasibility and interim-analyses, cannot be presented yet. In all trials described, TME-surgery is indicated, or at least advised, in low or middle rectal cancers. However, the extent of surgical quality control differs between the trials. In Sweden and The Netherlands, nation-wide projects have been conducted in which surgeons were trained to perform a proper TME in an attempt to improve their treatment results. The study of Martling et al.[10] showed that local recurrence rate decreased by more than 50% as a result of a surgical teaching initiative in the county of Stockholm. The effect of the introduction of TME-surgery with quality control on outcome of rectal cancer was also investigated in The Netherlands. We compared results from Dutch patients in the TME-trial with results from a former randomised trial (CRAB-trial), in which conventional surgery was performed without quality control. It was found that the introduction of TME-surgery with quality control led to a substantial decrease in local recurrence rate and cancer death in The Netherlands.(paper submitted)

Besides better local control and survival, sharp TME-dissection has been associated with a higher incidence of sphincter preservation and pelvic autonomic and plexus preservation, avoiding both colostomy and impotence, as well as blood transfusions.[4,11,12]

The proportion of abdominoperineal resections has come down from 35% or more[1,34,35] to 30% or less in recent years.[10,23] However, one adverse effect of the higher rate of sphincter-preserving procedures may be a higher rate of anastomotic leakage.[36] Higher leak rates with TME-surgery as compared to conventional surgery, have been reported.[9,37] Several conventional surgery studies report overall anastomotic leak rates between 0% and 17.4%.[35,38,39] The clinical leak rate of 12% in the Dutch TME-trial and 10% in the MRC CR07 trial are quite high, but within this range. Perhaps the construction of a high number of temporary colostomies and side to end anastomoses or pouches, have prevented increased clinical leak rates.[40-42]

Due to the extreme disability caused by local failure, a gain in local pelvic control per-se is generally accepted as a main objective of any adjuvant treatment.[15] In terms of tumour biology, preoperative radiotherapy is preferred to postoperative radiotherapy because the tumour cells before the operation have a higher oxygen saturation and are therefore more sensitive to irradiation. Furthermore, preoperative radiotherapy devitalises tumour cells that may be dispersed in the course of the operation. Preoperative radiotherapy over several weeks results in "down-staging" in many cases, i.e. the size of the primary tumour is reduced, the possibility of lymph node metastases is reduced, and in isolated cases complete remission occurs.[29,43] The timing dose and fractionation of preoperative radiotherapy in combination with TME-surgery are currently under investigation in the Stockholm IV trial.

One advantage of postoperative radiotherapy is that it allows the exclusion of patients with tumours in stages Dukes' A or D. To be effective, postoperative radiotherapy should begin within 4-6 weeks of the operation to prevent tumour cell proliferation in the postoperative fibrous, hypoxic tissues. However, at this point many patients have not yet recovered from the operation and therefore, there is often a delay before they receive the adjuvant radiotherapy.[13,44] In the MRC CR07 trial, the compliance of patients in the postoperative radiotherapy arm was lower than in the preoperative short-term radiotherapy arm. Therefore, higher compliance rates of the short-term preoperative radiotherapy regimen (compared to the postoperative radiotherapy plan), as shown in the Dutch TME and MRC CR07 trials, could explain the better results.

The probability of resistant cell lines may be smaller when irradiation is combined with chemotherapy and when these are given earlier in the disease, i.e. sooner in the treatment plan. Concurrent 5FU may have a sensitising effect on the radiotherapy. However, the role of chemoradiotherapy in the treatment of primary rectal cancer still has to be determined. In the United States the opinion is that all patients with a Dukes' B or C lesion should have postoperative chemoradiotherapy.[15] Chemoradiotherapy is not routine in Europe and is still considered investigational therapy in rectal cancer. The CAO/ARO/AIO and EORTC 22921 trials are ongoing to determine the role of chemoradiotherapy in combination with TME-surgery.

In the Dutch NACCP-study, chemotherapy as sole treatment showed no effect in combination with conventional surgery in the prevention of distant recurrences and improvement of survival.[14] This may in part be explained by the high rate of local recurrence, which might have masked the beneficial effect of chemotherapy. The role of chemotherapy has never been tested in combination with standardised TME-surgery, but is now under investigation in the PROCTOR-trial. The efficacy of preoperative radiotherapy in combination with postoperative chemotherapy or postoperative immuno-chemotherapy is currently being

investigated in the Austrian 92/157-004 study.

For optimal quality control of the surgical procedure standardised examination by pathologists is important.[19,20] In all trials, the circumferential margin was included for investigation. However, the protocol of Quirke was not used in all trials. In the Dutch, MRC CR07 and Swedish trials, this protocol has been used in combination with extensive pathological quality control.

The Dutch TME trial is one of the first trials in which the effect of adjuvant therapy in combination with TME surgery was evaluated. Standardisation of surgery, radiotherapy, and pathology were achieved. The accrual progressed swiftly and the trial was shown to be quite feasible. The design of the TME-trial has already resulted in a large reduction of the number of local recurrences. The first results on the role of preoperative radiotherapy in combination with standardised TME-surgery are presented at a large congress in April 2001 in The Netherlands (www.colorectal2001.com). Results of the other European TME-trials will take longer.

An effort will be made to achieve further standardisation and quality assurance of diagnostics in the PROCTOR-trial. Based on the data of the TME-trial, 20% of the cases with primary rectal cancer underwent an irradical resection either defined as an R1 (microscopic tumour left behind) or R2 (macroscopic tumour left behind) resection. Better selection of patients for preoperative radiotherapy in case of a locally advanced tumour or palliative procedures in patients with disseminated disease can be achieved by preoperative staging of rectal cancer with the routine use of a spiral computed tomography scan.

CONCLUSIONS

During the past decade, it has been clearly demonstrated that adjuvant treatment has the potential of improving not only prognosis in terms of local recurrence, but also in terms of overall survival. However, the question has yet to be answered as to whether in combination with TME-surgery, adjuvant therapy is capable of achieving any further improvement in outcome. In Europe, mesorectal excision, with its persistent decline in local recurrence rates, has become the new standard of operative management for rectal cancers, replacing conventional resection technique.[22] Current clinical trials examining the role of adjuvant therapy in patients who are undergoing standardised operations, are now setting the standard of care in several European countries.

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4

Acute side effects and complications after short-term preoperative radiotherapy combined with total mesorectal excision in primary rectal cancer

Report of a multicentre randomised trial

C.A.M. Marijnen[1,2], E. Kapiteijn[2], C.J.H. van de Velde[2], H. Martijn[3], W.H. Steup[4], T. Wiggers[5], E. Klein Kranenbarg[2], J.W.H. Leer[6] and the cooperative investigators of the Dutch ColoRectal Cancer Group

Departments of Clinical Oncology[1] and Surgery[2], Leiden University Medical Centre, Leiden; Department of Radiotherapy, Catharina Hospital, Eindhoven[3]; Department of Surgery, Leyenburg Hospital, The Hague[4]; Department of Surgery, Groningen University Hospital, Groningen[5]; Department of Radiotherapy, University Medical Centre St. Radboud, Nijmegen[6], The Netherlands

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INTRODUCTION

In the treatment of rectal cancer local recurrences are a major problem and the rate varies between 15% and 45%.[1-4] Local recurrences cause severe disabling symptoms and are difficult to treat. In order to reduce local recurrence rates after curative surgery, additional radiotherapy has been given either preoperatively[5-14] or postoperatively.[4,15-18] In a large Swedish trial short term, preoperative radiotherapy resulted in better local control than postoperative radiotherapy (13% vs. 22% local recurrences).[5] All trials with short-term preoperative radiotherapy show lower local recurrence rates in the radiotherapy arm.[7,12,13,19] Results of the Swedish Rectal Cancer Trial (SRCT) even showed an improved overall survival with the short term 5x5 Gy regimen compared to surgery alone, with 58% 5-years survival in the irradiated group versus 48% in the non-irradiated group.[14] However, this beneficial effect of preoperative radiotherapy was observed in combination with conventional surgery. This conventional procedure implies partially blunt dissection of the rectum along the presacral fascia, resulting in incomplete removal of mesorectal tissue. This possible residue of tumour cells was a logical rationale behind application of radiotherapy. The acknowledgement of the important role of circumferential margin involvement in the appearance of local recurrences in rectal cancer has led to the general introduction of total mesorectal excision (TME) surgery, as advocated by Heald[20] and Enker.[21] The main principle of this technique is to achieve a radical resection by sharp dissection within the true pelvis around the intact mesorectum under direct vision, thus enveloping the entire midrectum with the tumour. This technique has shown to reduce the number of local recurrences significantly in retrospective series.[22] A second beneficial effect of TME surgery is the possibility to preserve the autonomic pelvic nerve plexus, resulting in less bladder dysfunction and less sexual morbidity.[23,24]

To answer the question whether preoperative radiotherapy is still beneficial in TME treated patients a randomised, prospective international multicentre trial was conducted under the auspices of the Dutch ColoRectal Cancer Group (DCRCG) to compare the effect of preoperative, hypofractionated radiotherapy combined with TME surgery with TME surgery alone.[25] Any benefit regarding a reduced local recurrence rate and possible improved survival must be weighed against potential adverse effects in both the short- and the long-term. Several trials with preoperative, short-term radiotherapy have shown that preoperative 5x5 Gy followed by surgery within one week is a safe procedure.[12,26-28] In these studies however, the preoperative radiotherapy was combined with conventional surgery.

The present study was undertaken to assess the side effects of short-term, preoperative radiotherapy in rectal cancer patients operated with the TME surgical technique and to study the influence of 5x5 Gy on surgical parameters, postoperative morbidity and mortality in patients randomised in the TME trial.

METHODS

Study population

From January 1996 until December 1999, 1861 patients were randomised to preoperative radiotherapy followed by standardised TME surgery or to TME surgery only in a large international multicentre trial.

Patients entering the trial were required to have biopsy confirmation of a rectal adenocarcinoma, resectable tumours as judged by clinical examination, tumours with the

inferior margin within 15 cm of the anal verge and no hereditary colorectal cancer syndrome. Distant metastases had to be excluded by chest X-ray and ultrasound or CT scan of the liver. Patients in whom previously a malignancy was diagnosed were not included in the study. The World Health Organisation (WHO) performance score had to be less than or equal to two. The patient had to give written or oral informed consent, depending on local hospital regulations.

Stratification took place for institute of surgery and expected type of resection, i.e. Abdomino Perineal Resection (APR) or Low Anterior Resection (LAR). Balanced randomisation lists with a block size of six were used for central randomisation at the Datacentre in Leiden.

The majority of the included patients (1530) were from the Netherlands; the other 331 patients were included by Swedish, other European and Canadian co-investigators. For the final analysis of the trial, all patients will be analysed. Since the Dutch follow-up has been extremely thorough, data about the Dutch patients considering treatment characteristics, toxicity, complications and mortality are very complete and checked by the study coordinators. We therefore only included the Dutch patients in the current analysis.

Preoperative radiotherapy

Patients assigned to preoperative radiotherapy received a total dose of 25 Gy in 5 fractions over 5-7 days. The prescribed dose was specified according to ICRU 50 guidelines.[29] The clinical target volume included the primary tumour and the mesentery with vascular supply containing the perirectal, presacral and the internal iliac nodes (up to the S1/S2 junction).

The recommended upper border was at the level of the promontory. The perineum was included if an APR was planned, whereas the lower border was 3 cm above the anal verge if the planned operation was a LAR. The treatment was delivered with three portals or with a four-portal "box" technique, depending on the institutes' preference.

Shielding of the lordotic area at the dorsum of the sacrum was recommended. The protocol recommended a treatment time from Monday till Friday, with surgery on the following Monday, Tuesday or Wednesday. In case treatment started on other days and was interrupted during the weekend, the time between the first radiotherapy fraction and the day of surgery was not to exceed 10 days.

In case of resection margins smaller than 1 mm or tumour spill during operation, postoperative radiotherapy was mandatory for the TME only patients.

Treatment details were reported on a radiotherapy form and checked by a radiation oncologist for inconsistencies.

Surgery

All patients underwent surgery according to the Total Mesorectal Excision principle, as advocated by Heald.[20] An extensive structure of workshops, symposia and instruction videos ensured the instruction of this novel technique. In addition, a committee of instructor surgeons was formed to optimise quality. In each participating hospital the first five TME procedures had to be supervised by an instructor surgeon.

A surgery- as well as a post-surgery form, on which all operation characteristics, operative and postoperative complications were recorded, was completed by the operating surgeon. These forms were compared with the operation report and discharge letter by the surgical

trial coordinator and checked for inconsistencies. Additional information was requested when data were not clear or incomplete.

Pathology procedures

Standardised routine pathology examination was performed as described by Quirke et al.[30] Pathologic information on the resected tumour was recorded by pathologists from the referral hospital on a pathology case record form for all patients. A pathology quality manager and a pathology review committee were installed to ensure constant quality of all pathology data and procedures.[31] Tumour staging was performed using the TNM classification.[32]

Side effects and complications

Radiation oncologists were asked to score acute side effects within 3 months from the start of radiotherapy according to the Radiation Therapy Oncology Group (RTOG) scoring system.[33] –In general, grade 0 represents no complaints, whereas grade 5 is any toxicity leading to death. The RTOG system has no scoring system for acute neurological symptoms. Since acute plexopathy was observed in the SRCT,[34] we introduced a scoring system for neurological complaints, with the following categories for painful buttocks or legs: 0: no complaints, 1: mild or intermittent pain not requiring intervention, 2: moderate constant pain requiring narcotics or adjustment of the treatment, 3: intractable severe pain or treatment interruption. This scoring system was introduced in 1997, a year after the start of the trial, explaining the missing data for patients randomised in 1996.

For the postoperative complications, all complications during the first admission were taken into account and the following definitions were used.

Anastomotic leaks included those clinically apparent or after suspicion determined on a contrast-enema. An abscess around the anastomosis was recorded as leakage. Since it is very difficult to discriminate between perineal dehiscence or perineal wound infection these complications were recorded as *perineal wound complication*. Rare complications were classified as *other*. Two categories were used: *moderate* consisting of complications that needed non-invasive treatment or *serious* defined as complications that required reintervention or caused a prolonged hospital stay.

Hospital death was defined as any death occurring during first admission, whereas *postoperative mortality* was defined as any death occurring during the first 30 days after the operation.

A *reintervention* was defined as any surgical procedure that took place in the operating room after the initial operation during the first admission. Only the first reintervention was taken into analysis. Elective procedures like removal of gauzes left behind during the initial operation for bleeding or opening/closure of stoma were not considered as a reintervention. Re-resections for positive margins were not considered as reinterventions.

Data collection and statistics

All case record forms were sent to the central data office in Leiden. After several checking rounds, the data were entered in a database and analysed with SPSS statistical software (version 9.0 for Windows, SPSS, Chicago).

Mann-Whitney tests were used to compare quantitative and ordered variables and Student's t-tests were used to analyse differences in normally distributed data between the two groups.

Chi-square tests were used to compare proportions. A P-value of 0.05 or less was considered statistically significant.

RESULTS

Patients

Of the 1530 Dutch patients included in the trial, 116 turned out to be ineligible. Reasons for ineligibility are recorded in Table 1. In some institutes, a CT-scan for treatment planning of the radiotherapy was performed, leading to detection of metastasis or irresectability. Consequently, more TME only patients turned out to be irresectable or metastasized during the operation. Thus, 1414 patients remained evaluable: 695 in the radiotherapy group and 719 in the surgery alone group. Table 2 shows well balanced clinical and tumour characteristics over both treatment arms. There was also no difference in the distribution in TNM stages or in the percentage of patients with a positive circumferential margin.

Table 1. Patients excluded from analysis.

Randomised	RT+TME n=761	TME n=769	Total n=1530
Ineligible at randomisation	22	27	49
no adenocarcinoma	4	3	7
other/previous malignancy	10	15	25
double tumour	1	5	6
other	7	4	11
Ineligible after randomisation	44	23	67
withdrawn informed consent	11	2	13
sigmoid carcinoma	2	-	2
unresectable on CT-scan	5	-	5
M1 on CT-scan	4	-	4
RT not possible	4	-	4
other	5	1	6
no resection	13	20	33

Radiotherapy

Delivery

In the radiotherapy group, the following minor protocol violations occurred. Treatment was not completed in 14 patients. The interval between the first day of radiotherapy and the day of surgery exceeded 10 days in 11% of the patients (range 11-60). In 85 patients (12%) the upper border of the treatment field was at the level of S1/S2 and in 6 patients the upper border was at the level of L4 or L5 instead of the promontory. In 40 patients undergoing an APR, the perineum was not included in the treatment field. All patients with minor protocol violations were included in the analyses.

Radiotherapy was given with 3 portal fields in 75% of the patients and with four portal fields in 25% of the patients. Fifty-three percent of the patients were treated in supine position. Of the 322 patients treated in prone position, 92 (29%) were treated on a belly board. The dorsal sacrum and lordotic curve was shielded in 90% of all patients.

The median interval between randomisation and surgery was 21 days in the radiotherapy group and 14 days in the surgery group, indicating that postponement of surgery did not occur more often in the radiotherapy group, since it was anticipated that radiotherapy increased the treatment time by a maximum of 10 days.

Table 2. Clinical and pathological characteristics.

	RT+TME n=695		TME n=719		Total n=1414
	n	%	n	%	n
Age (mean, range)	64.1	26-88	64.1	23-92	64.1
Sex					
male	455	65	455	63	910
female	240	35	264	37	504
Tumour level inferior margin					
0-5 cm	202	30	225	32	427
5.1-10 cm	290	42	281	40	571
10.1-15 cm	193	28	204	28	397
missing	10		9		19
Operation type					
APR	214	31	220	30	434
LAR	439	63	465	65	904
Hartmann	42	6	34	5	76
TNM-stage					
0	10	1	15	2	25
I	218	31	203	28	421
II	191	28	190	26	381
III	235	34	272	38	507
IV	41	6	39	6	80
Circumferential margin					
> 1 mm	572	82	578	80	1150
≤ 1 mm	122	18	141	20	263
missing	1				1

Toxicity

During radiotherapy, any kind of side effect was reported in 26% of all irradiated patients (Table 3). Nineteen percent was grade 1 toxicity, representing only minor complaints. In 7% of the patients there was a grade 2 or 3 complication.

Acute transient neurological complaints were recorded in 53 patients, of which 35 had grade 1, not requiring any intervention. In 2 patients the shielding was adjusted and the upper border was lowered in 3 patients. In 13 patients treatment was interrupted due to serious pain in the gluteal region or legs. Remarkably, of these 13 patients, 6 patients were treated in one radiation institute. No relation with number of portals, upper border, treatment position or shielding could be found. Due to the fact that the neurotoxicity score was introduced in 1997, data about neurotoxicity are missing in 178 patients.

In four (<1%) patients other grade 3 toxicity was reported, leading to postponement of the operation in two patients with thrombo-embolic complications. One patient required a catheter due to urinary retention after the radiotherapy. The last patient had anal blood loss 2 months after radiotherapy and proctoscopy confirmed a proctitis.

Table 3. Number of patients with radiotherapy toxicity.

	RTOG grading			
	0	1	2	3
Skin	685	8	2	0
Gastrointestinal	605	75	14	1
Genitourinary	676	16	2	1
Neurological	464	35	5	13
Other	655	31	7	2

Table 4. Surgery characteristics.

	RT+TME n=695		TME n=719		
	n	%	n	%	P
Operation characteristics					
time (median, range)	180	65-390	180	70-380	ns
blood loss (median, range)	1100	50-20000	1000	20-15000	<0.001
LAR	1025		800		<0.001
APR	1200		1300		ns
hospital stay (median, range)	15.0	3-179	14.0	0-169	ns
Operation type when LAR planned					
LAR	408	85	435	89	ns
APR	45	9	35	7	
Hartmann	30	6	21	4	
Stoma in LAR patients					
no stoma	176	36	216	43	0.05
stoma	263	64	249	57	
Anastomosis in LAR patients					
side-end	261	60	278	60	ns
end-end	54	12	50	11	
pouch	122	28	132	29	
missing	2		5		

Operation time in minutes, blood loss in ml and hospital stay in days. ns=not significant

Surgery

Surgical characteristics

To evaluate whether preoperative radiotherapy influences operation procedures, surgery characteristics are compared in Table 4. There was no significant difference in median operation time or median hospital stay between both treatment arms. Total blood loss was slightly increased (100 ml) in the irradiated (RT+) group (P<0.001). Subset analysis revealed that the difference in median blood loss was mainly present in the LAR patients: 1025 ml in the RT+ group vs. 800 ml in the non-irradiated (RT-) group (P<0.001), whereas median blood loss in the APR patients was not significantly different over the treatment arms.

Of the patients planned to undergo a LAR operation, 9% in the RT+ group and 7% of the patients in the RT- group underwent an APR. In APR patients, conversion to a sphincter saving procedure took place in 20% of the irradiated patients and in 19% of the TME alone group. A pouch reconstruction was done in 28% of the irradiated patients undergoing a

LAR vs. 29% of the non-irradiated patients.

More RT+ patients received a temporary diverting stoma at the time of TME surgery than RT- patients did (64% vs. 57%, P=0.05). Postoperatively, slightly more RT- patients required a stoma due to complications, resulting in a not significantly different overall number of temporary stomas in both groups (68% vs. 63%, P=0.2), as is shown in Figure 1.

Complications

There was no difference in the percentage of patients with complications during the operation. Bleeding during operation occurred in 13% of the patients in both groups. In 8% of the irradiated patients and in 7% of the non-irradiated patients, an unintended organ injury occurred.

All reported postoperative complications are listed in Table 5. For most complications there was no difference between the two treatment arms. The overall postoperative complication rate was 48% in the irradiated group vs. 41% in the non-irradiated group (P=0.008). This difference was mainly attributable to the difference in perineal wound healing.

In APR patients, perineal wound complications were significantly increased in the irradiated patients (29% vs. 18%, P=0.008), whereas there was no difference in the abdominal wound complications. Application of an omentoplasty did not lead to a reduction in perineal complications. In 40 irradiated APR patients the perineum was not included in the treatment field. Seven of these patients (18%) had perineal problems, vs. 54 (31%) of the 174 patients in which the perineum was included in the treatment field.

The percentage of LAR patients showing clinical leakage postoperatively was 11% (n=105) and was not statistically different for irradiated and non-irradiated patients (11% vs. 12%). Leakage was less common in patients with a diverting stoma (8% vs. 16%, P=0.001). In patients with an end-end anastomosis leakage occurred in 16% of the LAR patients, whereas only 9% of the patients with a pouch reconstruction experienced anastomotic failure. In patients with a side-end anastomosis this percentage was 12%. There was no influence of the distance of the tumour from the anal verge or age on the occurrence of leakage. Twenty percent of the patients with leakage were treated conservatively, whereas 80% required a surgical reintervention.

In total, 201 patients (14%) underwent one or more reinterventions with 103 patients in the RT+ group and 98 in the RT- group. Indications for reinterventions are listed in Table 6. No difference between the number of reinterventions in the LAR or APR patients was observed.

Twenty-eight patients (4%) died in hospital in the RT+ group vs. 24 (3.3%) in the RT- group (P=0.49). Postoperative mortality (<30 days) was 3.5% in the RT+ group vs. 2.6% in the RT- group (P=0.38). There was a strong correlation between age and hospital death (P<0.001, Figure 2). Causes of hospital death are given in Table 7. In the RT+ group 10 patients died of cardiac problems versus 3 patients in the RT- group (P=0.04). Anastomotic leakage contributed to postoperative mortality in 12 patients (23% of all in-hospital mortalities).

Table 5. Postoperative complications.**

	RT+TME n=695		TME n=719	
	n	%	n	%
Infectious				
wound infection	43	6	45	6
abscess	31	5	20	3
haematoma	7	1	2	<1
sepsis/fever	63	9	50	7
other	2	<1	2	<1
Any infectious complication	120	17	105	15
General				
cardiac	36	5	22	3 #
multi-organ failure	11	2	10	1
pulmonary	53	8	57	8
thrombo-embolism	11	2	12	2
line-sepsis	9	1	9	1
neurological	10	1	12	2
psychological disorders	28	4	10	1 *
renal	4	1	6	1
other	25	4	23	3
Any general complication	161	23	30	18 #
Surgical				
leakage (LAR)	49	11	56	12
perforation	8	1	7	1
intestinal necrosis	6	1	7	1
fistula	8	1	14	2
stoma complications	14	2	12	2
bleeding	23	3	29	4
abdominal dehiscence	16	2	25	4
perineal complications (APR)	61	29	39	18
diarrhoea	11	2	2	<1 #
ileus	37	5	48	7
other	22	3	10	1 #
Any surgical complication	209	30	191	27
Any complication	336	48	297	41 *

# P<0.05

* P<0.01

** The numbers and percentages of the separate complications do not summate "any complication" since some patients had more than one complication. They were registered for each separate complication, but for "any complication" they were counted as one.

Table 6. Indications reintervention.

	RT+TME	TME	Total
Anastomotic leakage	23	31	54
Abscess	27	13	40
Bleeding	11	16	27
Abdominal dehiscence	8	13	21
Perineal complications	4	2	6
Complications stoma	3	4	7
Other complications surgery	6	6	12
Peritonitis or sepsis	7	2	9
Ileus	11	9	20
Other	3	2	5
Total	103	98	201

Table 7. Causes of hospital mortality.

	RT+TME	TME	Total
Abscess	1	1	2
Anastomotic leakage	4	8	12
Bleeding	1	1	2
Perforation bowel	3	1	4
Complications mechanic ileus	-	3	3
Necrosis bowel	2	2	4
Sepsis	1	2	3
ARDS	1	-	1
Cardiac	10	3	13
Pulmonary embolism	2	2	4
Pneumonia	3	1	4
Total	28	24	52

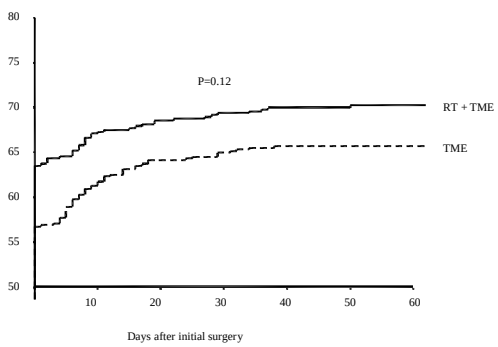

Figure 1. Percentage of LAR patients with a diverting stoma per randomisation group (P=0.12).

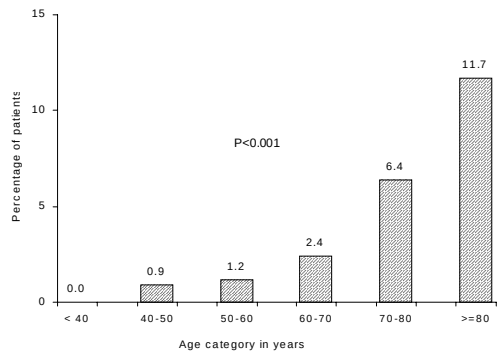

Figure 2. Percentage of hospital deaths per age category (P=0.001).

DISCUSSION

The results of this study indicate that short-term, preoperative radiotherapy does not complicate TME surgery, although there is a slight increase in complications in the preoperatively irradiated patients.

Acute side effects of preoperative, hypofractionated radiotherapy include nausea, diarrhoea and skin erythema. These side effects develop to some degree in most patients, but usually resolve within a few weeks. In this trial, few early side effects for radiotherapy were reported. This may be attributed to the fact that most patients were operated in the week after radiotherapy and not seen by the radiation oncologist until several weeks after the operation. By this time, most side effects will have resided.

Lumbosacral plexopathy was a major cause of concern in the Swedish Rectal Cancer Trial (SRCT) since six patients developed long-standing pain and/or neurological symptoms at the level of the lower lumbar plexus.[34] These six patients all complained about pain during the radiotherapy. An extensive study on dose distribution showed that these patients might have received a higher dose (112%) at the level of the lumbar vertebrae, when the dorsal shields were inappropriately placed.

In our study, 53 patients had pain or a feeling of discomfort in the legs or in the gluteal region, of which 18 needed medication or treatment interruption. In these patients, a careful evaluation of the treatment fields and the dorsal shielding was done and adjustments were made in 5 patients. As precaution, treatment was interrupted in 14 patients. So far, with a median follow up of 25.4 months, there are no reports of longstanding pain or neurological symptoms. This might be attributed to the fact that the upper border of the radiation field was defined as L5/S1, as opposed to mid L4 in the Swedish trials. This prevents the irradiation of the lower dorsal lumbar roots.

Although there was initial concern that irradiation would hamper the operation, this was not reflected in the parameters of the surgical procedure. There was no increase in the duration of the operation and although the difference in blood loss was significant, an increase of 100 ml is not a serious clinical problem. Irradiation did not influence the choice of the surgeon to perform a LAR or an APR procedure.

The relatively high incidence of postoperative complications in our trial (45%) might be explained by the great effort taken to meticulously register all possible complications. Apart from data from the case record forms as recorded by the surgeon, data from operation notes and discharge letters were taken into account as well. Similar complication rates were reported in a prospective comparison of conventional and TME surgery.[35]

The mortality rate in the Stockholm I trial with 5x5 Gy was 2% in the RT- group vs. 8% in the RT+ group.[27] In the Imperial Cancer Research Fund (ICRF) trial where patients were treated with 3x5 Gy, these percentages were 7% vs. 12%, respectively.[7] The difference could mainly be contributed to an increase in cardiovascular deaths, particularly in patients aged over 75 years. Therefore, patients elder than 80 years were excluded from the Stockholm II trial and the SRCT. The explanation for the increased mortality rates in the Stockholm I trial and the ICRF trial is possibly the suboptimal treatment technique. In these trials, the treatment was given by two opposed fields, which increases the volume treated with 25 Gy considerably. Later trials therefore requested a three or four portal technique in order to reduce the treated volume. In the SRCT 48 patients were treated with a two-portal technique and those patients showed a higher mortality rate than the patients treated with three or four

portals.[26] In the Stockholm II trial there was no longer a difference in mortality within 30 days between the two treatment arms: 2% in the irradiated group vs. 1% in the non-irradiated group. In-hospital mortality rates in the SRCT were 4% in the RT+ vs. 3% in the RT- group. The in-hospital mortality rate in our trial showed no difference between the treatment arms and was 4% in the RT+ group vs. 3.3% in the RT- group. This can be considered as a satisfying result, taking into account that patients above the age of 80 were included in our trial. Our results demonstrate that the introduction of TME surgery after preoperative radiotherapy does not lead to an increase in the postoperative mortality rate, as long as at least three portals are used for the radiotherapy.

The two major causes of postoperative mortality in our trial were cardiovascular problems and complications due to anastomotic failure in LAR patients. Anastomotic leakage is a major clinical problem in rectal or anal anastomoses. The reported clinical leakage rate after anterior resection varies from 3% to 11%.[36-39] Karanjia et al. showed that a diverting colostomy is an important measure in reducing the complications of anastomotic leakage.[37] After TME surgery, an increase in serious anastomotic leakage has been reported as compared to conventional surgery.[35,40] This increase can be partly explained by the removal of the pain-sensitive peritoneum, which prevents early detection of anastomotic failure.[37] In our study, the number of patients with clinical anastomotic leakage was 105 (11%). This is consistent with other reports in which TME surgery was applied. It is particularly reassuring since this trial was a large multicentre study, whereas most other reports concern single institution experience. No difference in clinical leakage rate between the RT+ and RT- patients was observed, which is in agreement with previous reports about preoperative radiotherapy.[11,12,27,28] Since patients with a diverting colostomy developed fewer leaks, we recommend a diversion in case there is any doubt about the quality of the anastomosis.

Increase in perineal dehiscence after preoperative RT has been observed by several authors, both after short-term as well as after long-term preoperative radiotherapy. Although results are difficult to compare, due to various definitions of perineal dehiscence, a twofold increase is generally reported after RT.[11,12,26-28] In our study, 100 patients suffered from perineal complications with 18% in the RT- group vs. 29% in the RT+ patients. When the perineum was not included in the target volume, there was no increase of perineal complications as compared to the non-irradiated patients. However, avoidance of irradiation of the perineum is not desirable in APR patients since this might lead to an increase in local recurrences.

In conclusion, our results show that although application of short term, preoperative radiotherapy in combination with TME surgery leads to an increase in overall postoperative complication rate when compared to TME surgery alone, the number of complications leading to reintervention or even mortality are similar in both treatment arms. Although follow-up is too short to comment on the occurrence of late side effects, long term results from the SRCT give no reasons for concern so far. Therefore, preoperative hypofractionated RT is to be considered a safe procedure also in patients treated with TME surgery, despite a slight increase in complications when compared to TME surgery only.

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5

Impact of surgical training on recurrence and survival in rectal cancer

Analysis of rectal cancer patients from two prospective, randomised trials in The Netherlands

E. Kapiteijn[1], H. Putter[2], C.J.H. van de Velde[1] and cooperative investigators of the Dutch ColoRectal Cancer Group (DCRCG)

Departments of Surgery[1] and Medical Statistics[2], Leiden University Medical Centre, Leiden, The Netherlands

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INTRODUCTION

In The Netherlands, 8000 new colorectal cancer patients were registered in 1995, of whom about 25% had rectal carcinoma.[1] A major problem in the treatment of rectal carcinoma is local recurrence. In literature, the reported local recurrence incidence after curative resection varies widely with high incidences of local recurrence with conventional, often bluntly dissectioning, non-standardised procedures.[2-4] In recent years local control and survival have improved by the introduction of total mesorectal excision (TME) surgery.[2,5-8] TME is accomplished by precise sharp dissection within the true pelvis around the integral mesentery under direct vision, enveloping the entire mid-rectum, with preservation of the hypogastric plexus. With the TME-technique, also a reduction in abdominoperineal resections has been achieved.[7]

Inter-institution and inter-surgeon variabilities in colorectal cancer surgery have been shown in several studies. This applies to immediate results, such as surgical morbidity and mortality,[9-13] as well as long-term results, such as local recurrence and survival.[4,9,14-18] Obviously, surgical technique is a critical factor for immediate outcome, as well as good postoperative care. Disease-free and overall survival can be influenced by tumour-related factors (stage, lateral margin involvement), treatment related factors (surgical technique, adjuvant therapy) and patient-related factors (gender, age). The treating institution as well as the individual surgeon can be further prognostic factors.

The Dutch ColoRectal Cancer Group (DCRCG) was installed to conduct trials in order to improve outcome of colorectal cancer treatment in The Netherlands. In the Cancer Recurrence And Blood transfusion (CRAB)-trial, colorectal cancer patients were randomised between transfusion of leukocyte-depleted or buffy-coat-depleted blood between 1987 and 1990.[19] In this trial, conventional surgery was performed without quality control. The randomised TME-trial investigated the role of preoperative short-term radiotherapy in combination with standardised TME-surgery in rectal cancer patients and was conducted between 1996 and 1999. In the trial, TME-surgery was introduced on a nation-wide basis and performed according to strict and controllable quality demands.

The aim of this paper was to assess the effect of the introduction of TME-surgery on outcome of rectal cancer in The Netherlands. Therefore, we compared results from the TME-trial with outcomes from the CRAB-trial. Furthermore the influence of hospital volume was investigated in both trials. The role of specialisation was analysed by comparing outcomes of hospitals with specialised instructor-surgeons vs. hospitals without instructor-surgeons in the TME-trial.

METHODS

The CRAB-trial

Between June 1987 and December 1990, colorectal cancer patients were randomised between transfusion of leukocyte-depleted or buffy-coat-depleted blood and received blood transfusion upon indication in the CRAB-trial. Eligibility criteria were: histologically or radiologically proven, primary adenocarcinoma of the colorectum without clinical evidence of distant metastases. In total, 1108 colorectal cancer patients were randomised from 16 Dutch hospitals. Colorectal cancer recurrence rates were not influenced by blood transfusion; however, blood transfusion resulted in more infections and affected overall survival by an association with non-cancer death.[19]

Rectal cancer affected 331 patients. A rectal tumour was defined as a tumour with an inferior margin within 15 cm of the anal verge (as measured preoperatively during withdrawal of a flexible endoscope). The CRAB rectal cancer patients had conventional, non-standardised surgery without quality control. The procedure was considered to be curative when a macroscopically local radical resection was performed in the absence of intraoperative metastases. Of the 331 rectal cancer patients, 281 patients were eligible and curatively operated. The pathology of the carcinomas was classified according to the TNM system.[20]

Patients received preoperative radiotherapy of 30 Gy in 10 days in case of large tumours (T3-T4) in a few hospitals. Indications for postoperative radiotherapy of 45-60 Gy were tumour spill during operation or Dukes' Astler-Coller B2 and C tumours and positive margins. Median follow-up of living patients in the CRAB-trial was 78 months (range 34-114).

The TME-trial

A phase III trial "Total mesorectal excision with or without short-term preoperative radiotherapy in the treatment of primary rectal cancer" was conducted from January 1996 until December 1999. This trial evaluated the effect of 5x5 Gy preoperative radiotherapy in combination with standardised TME-surgery and pathology.[21] Patients were randomised by Dutch, Swedish, other European and Canadian participants; however, this paper only concerns Dutch patients. Clinically operable patients with a histologically proven, primary adenocarcinoma of the rectum without evidence of distant metastases were eligible. In total, 1530 patients were randomised from 84 Dutch Hospitals. The trial was shown to be feasible; the only significant differences between irradiated and non-irradiated patients concerned more blood loss and a higher perineal wound complication rate in abdominoperineal resection (APR) patients in the radiotherapy group.[21,22]

The height of the tumour was defined preoperatively by its inferior margin as measured at withdrawal of a flexible scope. An extensive structure of workshops, symposia, and instruction videos helped to accomplish that TME was performed according to strict quality demands. In addition, a monitoring committee of specially trained instructor surgeons was formed for on-site instructions. TME was taught to surgeons who generally deal with rectal cancer (1-3 surgeons per hospital surgical unit). In each participating hospital the first five TME 's had to be supervised by an instructor surgeon. A curative resection was defined as a macroscopically radical local tumour resection without intraoperative detection of metastases. Of the 1530 patients, 1352 patients were eligible and curatively operated.

Pathological examination was performed according to the protocol of Quirke[23] with special attention for circumferential margin involvement, and the carcinomas were classified according to the TNM system.[20] Patients randomised for preoperative radiotherapy received 5x5 Gy. In patients having TME alone, postoperative irradiation of 50-60 Gy was mandatory in case of tumour spill during surgery or tumour infiltration within 1 mm from the circumferential resection margin. Median follow-up of living patients in the TME-trial was 28 months (range 3-56).

CRAB- vs. TME-trial

We compared patients who were eligible and curatively operated by abdominal surgery to enable homogeneity between the trials. Furthermore patients who had preoperative radiotherapy were excluded from the analysis because the outcome of the TME-trial (=the role of preoperative radiotherapy in combination with standardised TME-surgery) was not known at the time of this analysis.

Of the 281 eligible rectal cancer patients curatively operated in the CRAB-trial, 9 patients were excluded who received preoperative radiotherapy and 3 patients who underwent polypectomy. In the TME-trial, 691 preoperatively irradiated patients were excluded. The analysed numbers of eligible, curatively operated and preoperatively non-irradiated patients were 269 patients in the CRAB-trial and 661 patients in the TME-trial.

Analysed short-term outcomes in both trials were operation time, blood loss during operation, hospital stay, leakage, wound infection and 30-day mortality. The breakdown of anastomotic integrity was defined on clinical grounds. Wound infection was defined as either abdominal or perineal wound infection. Deaths within 30 days after surgery were those which occurred postoperatively either in- or outside the hospital.

Long-term outcomes included local and distant recurrence and overall survival. Local recurrence was defined as the presence of any anastomotic, pelvic, or perineal tumour, as proven by histology or radiology. Distant recurrence involved evidence of tumour in any other area than the pelvis. Overall survival concerned deaths of any cause, with and without tumour, as event. To ensure valid comparisons, we analysed only events occurring within 2 years of surgery in both trials.

Hospital volume was determined per trial and based on the number of included eligible, curatively operated, preoperatively non-irradiated patients treated in a certain hospital. Furthermore the role of specialisation was analysed by comparing hospitals with instructor-surgeons vs. hospitals without instructor-surgeons only in the TME-trial.

Statistics

Chi-square tests were used to compare proportions. Mann-Whitney tests were used to compare quantitative and ordinal variables. Multivariate analyses of determinants of short-term outcomes, including type of surgery (conventional (CRAB-trial) vs. TME (TME-trial)), were done with the linear and logistic regression models. For long-term outcomes, multivariate analyses were performed with the Cox proportional hazards model. Interaction-terms of factors with type of surgery were included in the regression models to correct for differences in the effect of factors between the trials. The effect of e.g. postoperative radiotherapy (RT) in each trial might have been different since the indications for RT were different between the trials.

Hospital volume was analysed as a continuous variable. For analysis of hospital volume and specialisation all individual risks for an hospital with regard to short-term outcomes were summated; the deviation of the expectation was calculated by subtracting the expected complication rate (based on clinicopathological characteristics) from the observed rate. The deviation was divided by the number of individuals for the hospital to obtain the crude hospital effect. For long-term outcomes crude estimations of the hospital effects were obtained by calculating the ratio of residual (observed minus expected=O-E) number of deaths (or recurrence of disease) to expected (=E, based on clinicopathological

characteristics) number of deaths (or recurrence of disease). An (O-E)/E ratio greater than (less than) zero was an indication that an hospital was experiencing more (fewer) events than would have been expected after adjustment for the burden of illness in that hospital patient population. Subsequently, the crude estimates and standard errors of both short- and long-term outcomes were used to analyse the effects of hospital volume and specialisation by means of linear regression. By application of the empirical Bayes method,[24] a more realistic view on the results of especially, hospitals with low numbers of patients, was obtained.

RESULTS

Clinicopathological characteristics (Table 1)

In the CRAB-trial significantly more female patients (P=0.002) and older patients (P=0.03) were treated. More patients had postoperative radiotherapy (P<0.001) in the CRAB-trial, while postoperative chemotherapy was more often applied in the TME-trial (P<0.001). Tumour location, type of resection and TNM-stage did not differ between the trial-groups. The variables in this table and their interactions with type of surgery were used in the multivariate regression models to compensate for differences in case-mix.

Table 1. Eligible, curatively operated, preoperatively non-irradiated patients; clinicopathological characteristics, univariate analysis, n (%).†

	CRAB (n=269)	TME (n=661)	P
Gender			0.002
-male	140 (52)	415 (63)	
-female	129 (48)	246 (37)	
Age (yrs)			0.03
-median	67.0	66.0	
-range	36-89	23-92	
Tumour location			0.31
- 0-5 cm	69 (33)	215 (33)	
- 5.1-10 cm	93 (44)	260 (40)	
- 10.1-15 cm	48 (23)	183 (28)	
- unknown*	59	3	
Type of resection			0.70
-LAR	173 (64)	432 (65)	
-APR	89 (33)	206 (31)	
-Hartmann	7 (3)	23 (3)	
TNM-stage			0.09
-I	92 (34)	203 (31)	
-II	87 (32)	186 (28)	
-III	90 (33)	272 (41)	
Adjuvant radiotherapy (RT)			<0.001
-none	168 (62)	587 (89)	
-RT	101 (38)	74 (11)	
Adjuvant chemotherapy (CT)			<0.001
-none	269 (100)	626 (95)	
-CT	-	35 (5)	

* The inferior margin of the tumour was unknown in 59 patients in the CRAB-trial and in 3 patients in the TME-trial.

† Because of rounding, percentages may not total be 100.

Short-term outcomes (Table 2)

Operation time, blood loss during operation and hospital stay did not differ significantly between the two trials, although the difference in blood loss was of borderline-significance (median 900 vs. 1000 ml, P=0.06). Clinical leakage was reported significantly more often in the TME-trial (P=0.046), despite a higher number of temporary stomas in LAR-patients in this trial (CRAB: 25% vs. TME: 55%, P<0.001). The rates for wound infection were 8% and 9% and for 30-day mortality 4% and 2%, respectively in the CRAB vs. TME-trial; these rates did not differ significantly between the trials. In the multivariate analysis, type of surgery was not an independent predictor for leakage when corrected for case-mix.

Table 2. Short-term outcomes; univariate analysis, n (%).*

	CRAB (n =269)	TME (n=661)	P
Operation time (min)			0.50
-median	180	180	
-range	65-420	70-380	
Blood loss during operation (ml)			0.06
-median	900	1000	
-range	0-9445	20-15000	
Hospital stay (days)**			0.11
-median	15.0	14.0	
-range	1-120	0-169	
LAR-group, clinical leakage	n=173	n=432	0.046
-yes	11 (6)	51 (12)	
Wound infection (abdominal or perineal)			0.49
-yes	21 (8)	61 (9)	
Mortality < 30 days			0.10
-yes	12 (4)	16 (2)	

* Type of surgery was not an independent predictor for any of the short-term outcomes, including leakage.

** Postoperative deaths were included for analysis of hospital stay.

Long-term outcomes (Table 3 and 4)

Local recurrence (P=0.002) and overall survival (P=0.002) at 2 years of surgery differed significantly between the CRAB vs. TME-trial. No significant difference was found in distant recurrence risk between the trials (P=0.86, Table 3). In the multivariate analysis (Table 4), type of surgery was an independent predictor for local recurrence (hazard ratio (HR) TME 0.017, 95% confidence interval (CI) 0.001-0.22, P=0.002, Figure 1), in addition to TNM-stage (P=0.006). For distant recurrence (Figure 2), TNM-stage was the only independent predictor (P<0.001). Type of surgery was also an independent predictor for overall survival (HR TME 0.21, 95% CI 0.057-0.78, P=0.019, Figure 3), in addition to TNM-stage and age. Interactions between factors and type of surgery were corrected for, but not mentioned in Table 4 when a significant independent predictive value was found.

In order to check whether there was a bias in the databases (data of the CRAB-trial were more mature than those of the TME-trial due to longer follow-up), we repeated the multivariate analysis for long-term outcomes separately for events occurring in the first year vs. those in the first and second year. No major differences in size or direction of the Cox regression coefficients were found between these analyses.

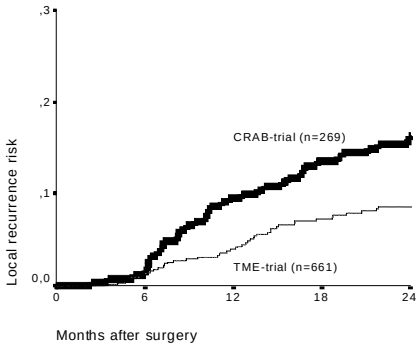
Table 3. Long-term outcomes; univariate analysis, %.

	CRAB (n=269)	TME (n=661)	P
Local recurrence risk after 24 months	16.3%	8.6%	0.002
Distant recurrence risk after 24 months	17.4%	17.1%	0.86
Overall survival after 24 months	77.0%	85.5%	0.002

Table 4. Multivariate Cox regression model using the baseline characteristics of Table 1 as input variables, including type of surgery, and long-term outcomes as outcome variables; only significant input variables listed.*

Outcome variables	Input variables	Hazard ratio	95% Confidence interval	
Local recurrence risk	-Type of surgery			P=0.002
	conventional	1.00		
	TME	0.017	0.001-0.22	
	-TNM-stage			P=0.006
	I	1.00		
	II	2.37	0.85-6.64	
	III	5.18	1.86-14.4	
Distant recurrence risk	-TNM-stage			P<0.001
	I	1.00		
	II	2.90	0.75-11.2	
	III	9.87	2.67-36.5	
Overall survival	-TNM-stage			P=0.004
	I	1.00		
	II	1.80	0.77-4.19	
	III	3.95	1.70-9.20	
	-Type of surgery			P=0.019
	conventional	1.00		
	TME	0.21	0.057-0.78	
	-Age	1.80	1.01-3.29	P=0.05

* Age was analysed as a binary variable; cut-off point was the median (66 yrs).



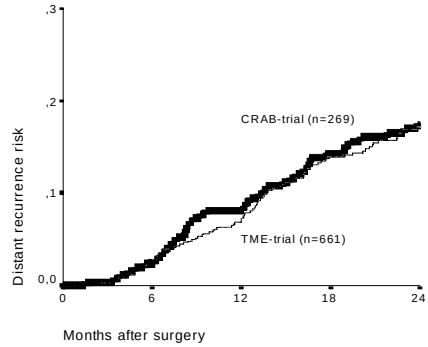


Figure 1. Kaplan Meier plot using local recurrence risk as outcome variable, influence of type of surgery (Cox model: HR TME 0.017, 95% CI 0.001-0.22, P=0.002).

Figure 2. Kaplan Meier plot using distant recurrence risk as outcome variable, influence of type of surgery (Cox model: HR TME 0.32, 95% CI 0.06-1.59, P=0.16).

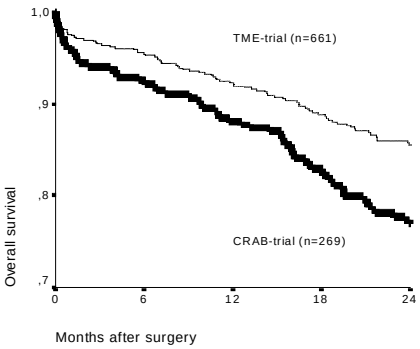


Figure 3. Kaplan Meier plot using overall survival as outcome variable, influence of type of surgery (Cox model: HR TME 0.21, 95% CI 0.057-0.78, P=0.019).

Investigation of hospital volume and specialisation (Table 5)

Hospital volume did not have an effect on short-term outcomes in the CRAB- and TME-trials. No effect of hospital specialisation was found either on short-term outcomes in the TME-trial. For long-term outcomes however, higher hospital volume was significantly associated with lower distant recurrence (P=0.006) and higher overall survival (P=0.011) within 2 years in the CRAB-trial. The effect of hospital volume on local recurrence was of borderline-significance (P=0.07) in the CRAB-trial. In the TME-trial, hospital volume did not have an effect on local recurrence (P=0.57), distant recurrence (P=0.88) and overall survival (P=0.65). Hospital specialisation was also not of significant value for long-term outcomes in the TME-trial.

Table 5. Multivariate Cox regression and empirical Bayes models; associations of hospital volume and specialisation with long-term outcomes in the CRAB and TME-trials.*,**

	Local recurrence risk			Distant recurrence risk			Overall survival		
	ß	SE	P	ß	SE	P	ß	SE	P
CRAB-trial									
Hospital volume	-0.046	0.022	0.07	-0.46	0.017	0.006	-0.037	0.014	0.011
TME-trial									
Hospital volume	-0.014	0.024	0.57	-0.0013	0.009	0.88	0.008	0.017	0.65
Hospital specialisation	-0.49	0.35	0.17	-0.064	0.25	0.80	-0.093	0.26	0.72

* ß=regression coefficient, SE=standard error.

** A negative ß means that lower volume or non-specialised hospitals did worse than higher volume or specialised hospitals.

DISCUSSION

The major problem in the treatment of rectal cancer is local recurrence. A high incidence of local recurrence is associated with conventional, non-standardised surgical procedures.[3,4] In recent years local control and survival have improved by the introduction of TME-surgery.[2,5-8] The aim of this study was to assess the effect of the introduction and training of TME-surgery on outcome of rectal cancer by analysing data from two large, prospective randomised trials performed in The Netherlands. In the CRAB-trial[19] conventional surgery was applied and in the TME-trial[21] TME-surgery was introduced, quality-controlled by specially trained instructor-surgeons.

This paper showed that introduction of TME has led to a substantial lower local recurrence rate when analysing events within 2 years; 16.3% in the CRAB-trial vs. 8.6% in the TME-trial. Type of surgery was an independent predictor for local recurrence in the multivariate analysis. Before the start of the TME-trial there were doubts whether the excellent results of specialised surgeons[2,5,6] could be repeated in a large multicentre trial. With the low local recurrence rate in the TME-trial, we conclude that good results can also be achieved in all surgeons' hands with thorough surgical instruction. It is remarkable that this result has been achieved in a relatively short time (4 years) with a great number of surgeons participating in the trial (n=213), especially since some surgeons performed only a restricted number of TME-procedures. Our results are in concordance with the report of Martling et al.[7] They compared the Stockholm I and II randomised trials in which conventional surgery with or without preoperative radiotherapy was performed, with the TME-project introducing the concept of TME to surgeons in Stockholm, and found that 2-year local recurrence rates had decreased from 14-15% to 6%.

Type of surgery also appeared to be an independent predictor for overall survival, with a higher survival rate in the TME-trial. However, we have to be careful with this last conclusion since follow-up of the TME-trial is not as mature as in the CRAB-trial. Type of surgery was not associated with distant recurrence, which is in concordance with results of Kockerling et al.[25] Their and our data suggest that distant recurrence rate is independent of the quality of surgery. This can most likely be explained by assuming that so-called metachronous metastases were already present in the form of systemic minimal residual disease at the time of primary surgery.

In addition to better results in terms of recurrence, the introduction of TME-surgery has been reported to result in a reduction of abdominoperineal resections.[7] However, we did not find this reduction in our comparison of the 2 trials. In the univariate analysis, we found a higher clinical leak rate in LAR-patients in the TME-trial, but this association was not significant anymore when corrected for case-mix. Higher leak rates with TME-surgery as compared to conventional surgery have been reported before.[26,27] An additional analysis showed that clinical leakage was related to postoperative mortality in both trials (P=0.024 and P=0.001 respectively), but no difference in 30-day mortality rate was found. This can be explained by the great number of temporary stomas in the TME-trial (55%) which might have prevented a higher mortality from leakage.[28]

The influence of hospital volume and specialisation was also investigated. Hospital volume was analysed as a continuous variable; this approach has as advantage that when effects are linear, differences come out more clearly than when choosing a cut-off point. In addition, cut-off points are sometimes arbitrary; they should be defined prospectively to avoid biases

inherent in post-hoc analysis (in which cut-off point can be selected to maximise volume-outcome associations). Inter-institution and inter-surgeon variabilities in short-term outcomes in colorectal cancer surgery have been shown in several studies.[9-11,13] A study by Hannan et al.[12] showed that hospitals with volumes of 40 or fewer procedures for colectomies had significantly higher standardised in-hospital mortality rates as compared to hospitals with volumes higher than 40. No effect of hospital volume was found on short-term outcomes in the CRAB- and TME-trials, nor was there any significant effect of hospital specialisation in the TME-trial.

Several studies have investigated the effect of hospital volume on long-term outcomes. However, findings in literature are controversial,[4,14-16] with also one report suggesting that hospital volume predicts clinical outcome for colorectal cancer, but not in the absolute magnitudes in comparison with the variation observed for higher-risk cancer surgeries.[29-31] In the CRAB-trial, hospital volume appeared to have a significant effect on distant recurrence and overall survival. In the TME-trial hospital volume did not have an effect on any long-term outcome. An explanation for the different findings in the CRAB- and TME-trials might be that standardised TME-surgery with quality control was not performed in the CRAB-trial, by which differences between hospitals came out to be significant. It is remarkable however, that hospital volume in the CRAB-trial had mainly influence on distant recurrence and not on local recurrence, although the association with local recurrence was of borderline-significance. Perhaps differences in surgical factors related to distant metastasis (e.g. tumour spill during surgery) might have influenced the association between hospital volume and distant recurrence.

Several studies have investigated the influence of individual surgeon volume and specialisation on outcomes.[9,16-18] We could not analyse individual surgeon volume and specialisation. In the CRAB-trial, no information was available on individual surgeons, while in the TME-trial the effect of surgeon volume was not analysed since most operations were performed by 2 surgeons, so individual surgeon analyses are difficult interpretable.

An advantage of analysing data of prospective trials, is that stringent follow-up, particularly when chronic outcome is an end-point, is often present, whereas in retrospective analysis these data are often absent. Nevertheless, some biases have to be ruled out concerning our analyses. The CRAB- and TME-trials were performed during different time-periods with different numbers of patients and follow-up periods. However, inclusion and classification criteria were the same, data on outcome were collected in an equal uniform way, only events within 2 years were analysed and we corrected for differences in clinicopathological characteristics by means of multivariate analyses. Although we must admit that the data of the CRAB-trial are more mature than those from the TME-trial, we consider minimal follow-up time of 2 years sufficient since 55-80% of local recurrences present during the first 2 years with a peak at 6-12 months.[17,32] In addition, no major differences in size or direction of the Cox regression coefficients were found between the multivariate analysis for events occurring in the first year vs. those in the first and second year. Besides surgical technique, pathology data on circumferential margin involvement may also not be comparable between the trials because pathology in the TME-trial specifically focused on this issue. Staging of tumours however, was performed according to the same classification.[20] Lastly, the question can be raised whether our analysis represents developments in all parts of The Netherlands. We think this is the case, since patients were randomised from hospitals throughout the

whole country in both trials.

In general, it is thought that high volume and specialist care produces superior results to low volume and non-specialist care, especially for those less frequent forms of cancer. Centralisation in fewer hands seems also of importance in technically difficult operations, like those for rectal cancer. The results of the CRAB-study support the idea of volume being important in outcome in rectal cancer. However, limiting the performance of rectal cancer surgery to those who work in specialised colorectal surgery centres or to only those general surgeons who perform more than a certain volume threshold is impractical in view of the prevalence of rectal cancer. The concentration process for patients with rectal carcinomas can also be achieved within the individual clinic. This has been demonstrated in the TME-trial, in which it is shown that training in TME-surgery to surgeons who diagnose and treat rectal cancer (1-3 per hospital surgical unit), leads to improved outcome without volume- or specialisation-related differences.

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6

Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer

E. Kapiteijn[1], C.A.M. Marijnen[1,2], I.D. Nagtegaal[3], H. Putter[4], W.H. Steup[5], T. Wiggers[6], H.J.T. Rutten[7], L. Pahlman[8], B. Glimelius[9,] J.H.J.M. van Krieken[10], J.W.H. Leer[11], C.J.H. van de Velde[1], for the Dutch ColoRectal Cancer Group

Departments of Surgery[1], Clinical Oncology[2], Pathology[3] and Medical Statistics[4], Leiden University Medical Centre, Leiden; Department of Surgery, Leyenburg Hospital, The Hague[5]; Department of Surgery, University Hospital Groningen, Groningen[6]; Department of Surgery, Catharina Hospital, Eindhoven[7]; Departments of Radiotherapy[11] and Pathology[10], University Medical Centre St. Radboud, Nijmegen, The Netherlands; Departments of Surgery[8] and Oncology[9], Akademiska Sjukhuset, Uppsala, Sweden

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INTRODUCTION

Local recurrence is a serious problem in the treatment of rectal cancer, since it causes disabling symptoms and is difficult to treat.[1,2] There is a high incidence of local recurrence (15 to 45%) after conventional surgery, in which blunt dissection of the rectal fascia often fails to remove all the tissue that may bear tumour.[3-5]

In an attempt to improve local control and survival after conventional surgery, radiotherapy has been given. The only randomised trial that compared preoperative and postoperative radiotherapy showed the superiority of preoperative radiotherapy for local control.[6] The Swedish Rectal Cancer Trial (SRCT) found that preoperative radiotherapy also improved the rate of survival at five years.[7] A recent meta-analysis[8] concluded that the combination of preoperative radiotherapy and surgery, as compared with surgery alone, significantly improved overall survival and cancer-specific survival.

The recognition that involvement of the circumferential margin by tumour cells is important in local recurrences has led to the general use of total mesorectal excision,[9-13] in which the entire mesorectum is enveloped and resected by precise, sharp dissection. Improvements in local control with this technique have been shown, mainly in retrospective series.[9-12,14]

In previous studies of radiotherapy for rectal cancer, surgery was not standardised. Since surgical technique is a key factor in the success of tumour control,[15-17] standardisation and quality control with respect to surgery are indispensable for evaluating the effects of adjuvant therapy. Optimal quality must also include the use of standardised methods of pathological examination.[18] A prospective, randomised trial was organised by the Dutch ColoRectal Cancer Group to investigate the efficacy of preoperative radiotherapy in combination with standardised total mesorectal excision in patients with rectal cancer.[19] In this article, we present the results of the trial after a median follow-up of two years.

METHODS

Eligibility, randomisation and sample size

Patients were enrolled between January 1996 and December 1999. To be eligible, patients had to have histologically confirmed adenocarcinoma of the rectum, without evidence of distant metastases, and the inferior margin of the tumour had to be located not farther than 15 cm from the anal verge and below the level of S1–2. Patients with fixed tumours or tumours that were treated by local (transanal) resection were excluded. Patients with previous or coexisting cancer and those who had previously undergone large-bowel surgery, chemotherapy or radiotherapy of the pelvis, were also excluded.

After informed consent had been obtained, we randomly assigned the patients to treatment with preoperative radiation (5 Gy on each of five days) followed by total mesorectal excision or to total mesorectal excision alone. Randomisation was performed at the central trial office and was based on permuted blocks of six, with stratification according to centre and the expected type of operation (low anterior resection or abdominoperineal resection). The trial was approved by the medical ethics committees of all the participating hospitals. The trial design and the calculation of the sample size have been described in detail elsewhere.[19]

Follow-up

Clinical evaluation every three months during the first year after surgery and yearly thereafter for at least two more years was mandatory and included yearly liver imaging and endoscopy.

Local recurrence was defined as evidence of a tumour within the lesser pelvis or the perineal wound. Distant recurrence was defined as evidence of a tumour in any other area. Recurrence at the colostomy site or in the inguinal region was also classified as distant recurrence.

Quality control

In The Netherlands, participating surgeons attended workshops and symposiums, saw instructional videotapes, and were monitored by specially trained instructor surgeons. At each hospital, the first five total mesorectal excisions were supervised by an instructor surgeon.[19] Pathologists were trained to identify lateral spread of tumour according to the protocol of Quirke et al.[18] The results of histopathological examination of the specimens were reviewed by a panel of supervising pathologists and a quality manager.[20] Patients' eligibility and treatment and the details of follow-up were checked by study coordinators. Local and distant recurrences were confirmed radiologically or histologically and checked by a radiation oncologist.

In Sweden, the technique of total mesorectal excision was introduced on a national basis several years ago,[12,13] as was the protocol of Quirke et al.[18] The European Organisation for Research and Treatment of Cancer participated in this trial under protocol 40971. Visits to other participating hospitals and specialists were made before the start of the trial to ensure the quality of treatment at those sites. For logistic reasons, no quality control with respect to radiotherapy, surgery, or pathological examination was performed outside The Netherlands during the trial.

Statistical analysis

Case-report forms were sent to the central trial office, where information on the forms was entered into a data base and analysed with SPSS statistical software (version 9.0 for Windows, SPSS, Chicago). Chi-square tests were used to compare proportions. Mann-Whitney tests were used to compare quantitative and ordinal variables. Univariate analyses of survival were carried out by the Kaplan-Meier method, and the evaluation of differences between the two groups was performed with the log-rank test. The Cox proportional hazards model was used to calculate hazard ratios and 95% confidence intervals in the univariate and multivariate analyses. A two-sided P value of 0.05 or less was considered to indicate statistical significance.

The starting point for the analyses of survival and recurrence was the day of surgery. Data on patients who were alive or free of recurrence were censored at the time of the last follow-up. The analysis of overall survival was performed on an intention-to-treat basis and thus included all the eligible patients. The rate of local recurrence was calculated on the basis of the number of eligible patients who underwent a macroscopically complete local resection. The rate of distant recurrence was calculated on the basis of the number of eligible patients who did not have distant metastasis at the time of surgery. The overall rate of recurrence was calculated on the basis of the number of eligible patients who had macroscopically complete local resection without distant metastasis. Analyses of postoperative morbidity and mortality were based on the total number of eligible patients who underwent resection.

RESULTS

Patients

A total of 1861 patients were randomly assigned to one of the two treatment groups. There were 1530 patients from 84 Dutch hospitals, 228 from 13 Swedish hospitals, and 103 from 11 other European and Canadian centres. Of these 1861 patients, a total of 56 were found to be ineligible before randomisation, including 4 patients for whom there was no information on eligibility. Our analysis therefore included 1805 eligible patients. Of these, 1653 patients had a curative resection. Of the remaining 152 patients, 57 did not undergo a macroscopically complete local resection, and 95 were found to have distant metastasis at surgery (Table 1). The characteristics of the 1805 patients who were eligible for the study and the features of their tumours were similar in the two treatment groups (Table 2). In 28 patients (2%), no tumour was found in the resected specimen, despite a preoperative biopsy that showed an adenocarcinoma.

Table 1. Characteristics of the eligible and ineligible patients and rates of macroscopically complete local resection, according to treatment group.*

	All patients	Treatment group	
Variable	No. (%)	Radiotherapy plus surgery	Surgery alone
Randomly assigned to treatment	1861 (100)	924 (100)	937 (100)
Ineligible for participation	56	27	29
No adenocarcinoma	8	5	3
Fixed tumour	2	-	2
Tumour treated by transanal resection	2	2	-
Tumour location > 15 cm from anal verge	5	4	1
Previous cancer	21	8	13
Coexisting cancer	11	4	7
Previous large-bowel surgery, pelvic radiotherapy, or chemotherapy	3	2	1
No information on eligibility	4	2	2
Eligible for participation	1805 (97)	897 (97)	908 (97)
Incomplete local resection			
Without distant metastases	31	10	21
With distant metastases	26	14	12
Complete local resection	1748 (94)	873 (94)	875 (93)
With distant metastases	95	47	48
Without distant metastases (curative)	1653 (89)	826 (89)	827 (88)

* Percentages are based on the total numbers of patients randomly assigned to one of the two treatment groups.

Protocol violations

Patients with major or minor protocol violations, or both, were included in all the analyses.

Major violations: Of the 897 eligible patients assigned to undergo radiotherapy before total mesorectal excision, 29 did not receive preoperative radiotherapy for the following reasons: known metastases (8 patients), carcinoma in situ (1), sigmoid carcinoma (3), a second cancer (1), withdrawal of informed consent (11), and physical limitations that made radiotherapy impossible (5). Long-term preoperative radiotherapy was given to seven patients for locally advanced tumours. One patient was unable to tolerate surgery and was treated

with long-term radiotherapy alone. Preoperative radiotherapy was discontinued in 14 patients, mainly because of neurotoxicity.

Of the 908 eligible patients assigned to total mesorectal excision alone, 3 patients withdrew their informed consent and requested radiotherapy (5 Gy on each of five days), and 8 patients had advanced local tumours for which long-term preoperative radiotherapy was given.

Postoperative adjuvant therapy was not allowed in patients who had microscopically tumour-free margins without spillage of tumour cells during the operation. Of 1759 eligible patients with available information on margins and tumour spillage, 1351 (77%) had tumour-free margins without tumour spillage. Eighty-five of these patients (38 in the group assigned to radiotherapy and surgery and 47 in the group assigned to surgery alone) received adjuvant therapy (chemotherapy, radiotherapy, or chemoradiotherapy), which was a major protocol violation.

Minor violations: Of the 846 eligible patients randomly assigned to preoperative radiotherapy who received the total dose of 25 Gy, the interval between the first day of radiotherapy and the day of surgery exceeded 10 days in 110 patients (13%). In 127 of the patients (15%), the upper border of the treatment field was at the level of S1–2 instead of at the promontory, and in 161 of the patients undergoing an abdominoperineal resection (19%), the perineum was not included in the treated volume.

Table 2. Characteristics of the 1805 eligible patients.*

	Radiotherapy plus surgery		Surgery alone		
Characteristic	(n=897)	(%)	(n=908)	(%)	
Age (yr)					P=0.79
Median	65.0		66.0		
Range	26-88		23-92		
Sex					P=0.92
Male	573	(64)	578	(64)	
Female	324	(36)	330	(36)	
Distance of tumour from anal verge					P=0.48
10.1-15 cm	267	(30)	280	(31)	
5.1-10 cm	384	(43)	364	(40)	
≤5 cm	244	(27)	263	(29)	
Unknown	2		1		
Type of resection					P=0.12
None	16	(2)	29	(3)	
Low anterior	579	(65)	604	(67)	
Abdominoperineal	251	(28)	234	(26)	
Hartmann†	50	(6)	40	(4)	
Unknown	1		1		
TNM-stage					P=0.53
0	11	(1)	17	(2)	
I	265	(30)	244	(27)	
II	252	(28)	245	(27)	
III	300	(34)	324	(36)	
IV	61	(7)	61	(7)	
Unknown or no resection	8		17		

***** Characteristics were unknown in some cases because not all case-report forms were received. Because of rounding, not all percentages total 100. TNM denotes tumour-node-metastasis.

† A Hartmann resection is a low anterior resection without the construction of an anastomosis.

Postoperative morbidity and mortality

The median interval between randomisation and surgery was 21 days in the group assigned to radiotherapy and surgery and 14 days in the group assigned to surgery alone. The patients assigned to radiotherapy and surgery lost slightly more blood during the operation than those assigned to surgery alone (median loss, 1000 vs. 900 ml, P<0.001), and of the patients who had an abdominoperineal resection, those assigned to radiotherapy had more perineal complications than those assigned to surgery alone (26% vs. 18%, P=0.05). No other significant differences with respect to postoperative morbidity and mortality were found between the two groups.

Follow-up

As of February 2001, surviving eligible patients without local recurrence had been followed for a median of 24.9 months (range, 1.1 to 56.0). Of these patients, 87% were followed for at least one year, 54% for at least two years, 24% for at least three years, and 5% for at least four years. Rates of survival and recurrence are presented here at a follow-up of two years. A reanalysis as of June 1, 2001, produced essentially the same results for all the major end points of the study.

Events

As of February 2001, 365 (20%) of the 1805 eligible patients had died. Of the 365 deaths, 61 occurred postoperatively, 231 were related to rectal cancer (growth of the primary tumour (in cases of macroscopically incomplete resection) or recurrence), and 70 were not related to rectal cancer. In three patients, the cause of death was unknown.

Local recurrence occurred in 87 patients. Of these 87 patients, 45 (52%) had local recurrence alone, 28 (32%) had both local and distant recurrences, and 14 (16%) had local recurrence after distant metastasis was found at surgery (in 9 patients) or during follow-up (in 5). A total of 227 patients were found to have only distant recurrence.

Overall survival

The rate of overall survival at two years was 82.0% in the group assigned to radiotherapy before surgery and 81.8% in the group assigned to surgery alone (P=0.84, Figure 1). The hazard ratio for death in the group assigned to surgery alone as compared with the group assigned to preoperative radiotherapy was 1.02 (95% confidence interval (CI), 0.83 to 1.25).

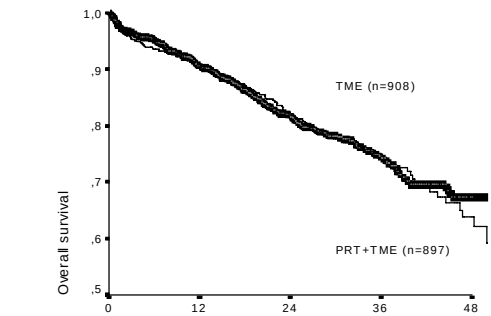
Local recurrence

The rate of local recurrence at two years was 5.3% in the population of 1748 patients who underwent a macroscopically complete local resection. The rates of local recurrence at two years were 2.4% in the group assigned to radiotherapy before surgery and 8.2% in the group assigned to surgery alone (P<0.001, Figure 2). According to a univariate analysis, the hazard ratio for local recurrence in the group assigned to surgery alone as compared with the group assigned to preoperative radiotherapy plus surgery was 3.42 (95% CI, 2.05 to 5.71).

In the univariate analyses, treatment-group assignment (P<0.001), the location of the

tumour (distance of the tumour from the anal verge, P=0.003), and the tumour-node-metastasis (TNM) stage (P<0.001) were significant predictors of the risk of local recurrence. In the multivariate Cox regression analysis (Table 3), the treatment-group assignment (P<0.001), the tumour location (P=0.03), and the TNM stage (P<0.001) were independent predictors of the risk of local recurrence, whereas the type of resection (P=0.90) had no independent prognostic value with respect to this end point.

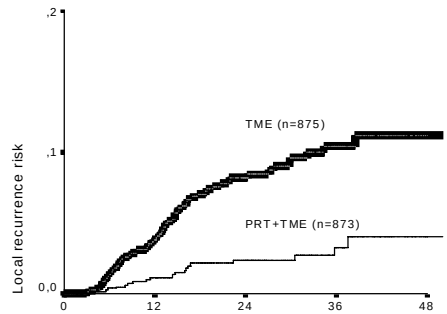
Univariate subgroup analyses showed that preoperative radiotherapy reduced the risk of local recurrence significantly in patients who had tumours with an inferior margin less than or equal to 5 cm (P=0.05) or 5.1 to 10 cm (P<0.001) from the anal verge (Table 4). Radiotherapy had no significant effect on tumours located 10.1 to 15 cm from the anal verge (P=0.17). For TNM stage II and III tumours, preoperative radiotherapy had a significant beneficial effect (P=0.01 and P<0.001, respectively), which was not observed for TNM stage I and IV tumours (P=0.15 and P=0.25, respectively). However, tests for interaction among the tumour location, TNM stage, and treatment-group assignment in a multivariate analysis showed no significant interaction between tumour location and treatment-group assignment (P=0.08) or between the TNM stage and treatment-group assignment (P=0.61), suggesting that the treatment effect did not differ among the subgroups analysed (data not shown).



No. at risk

Radiotherapy plus surgery	897	741	435	192	41
Surgery alone	908	744	454	207	42

Figure 1. Rates of overall survival in the population of 1805 eligible patients, according to treatment group. At two years, the rate of overall survival was 82.0% in the group assigned to radiotherapy and surgery and 81.8% in the group assigned to surgery alone (P=0.84).



	873	691	407	170	30
	875	688	406	173	37

Figure 2. Rates of local recurrence in the population of 1748 eligible patients who underwent macroscopically complete local resection, according to treatment group. At two years, the rate of local recurrence was 2.4% in the group assigned to radiotherapy and surgery and 8.2% in the group assigned to surgery alone (P<0.001).

Distant recurrence

The rate of distant recurrence at two years was 14.8% in the group assigned to radiotherapy and surgery and 16.8% in the group assigned to surgery alone (P=0.87). The hazard ratio for distant recurrence in the surgery-only group as compared with the radiotherapy-plus-surgery group was 1.02 (95% CI, 0.80 to 1.30).

Overall recurrence

The overall rate of recurrence (the rate of local recurrence and distant recurrence) at two years was 16.1% in the group assigned to radiotherapy and surgery and 20.9% in the group assigned to surgery alone (P=0.09). The hazard ratio for any recurrence in the surgery-only group as compared with the radiotherapy-plus-surgery group was 1.21 (95% CI, 0.97 to 1.52).

Table 3. Results of multivariate Cox regression analysis of local recurrence among the 1748 eligible patients with a macroscopically complete local resection.*

Variable	Hazard ratio	95% CI	
Treatment group			P<0.001
Radiotherapy and surgery	1.00	2.05-5.70	
Surgery alone	3.41		
Distance of tumour from anal verge			P=0.03
10.1-15 cm	1.00		
5.1-10 cm	2.13	1.13-4.01	P=0.02
≤5 cm	2.78	1.22-6.31	P=0.02
Type of resection			P=0.90
Low anterior	1.00		
Abdominoperineal	1.15	0.59-2.24	P=0.68
Hartmann†	1.16	0.42-3.25	P=0.78
TNM-stage			P<0.001
I	1.00		
II	3.44	1.26-9.39	P=0.02
III	9.69	3.89-24.2	P<0.001
IV (distant metastases but complete local resection)	16.2	5.40-48.6	P<0.001

* A variable was included in the multivariate analysis if the P-value in the univariate analysis was less than 0.10. Patients with missing data were excluded from the analysis of local recurrence. Twenty-eight patients without a tumour (TNM stage 0) were excluded from the multivariate analysis because they were not at risk for local recurrence. CI denotes confidence interval and TNM tumour-node-metastasis.

† A Hartmann resection is a low anterior resection without the construction of an anastomosis.

DISCUSSION

In this trial, we evaluated the efficacy of short-term preoperative radiotherapy combined with standardised total mesorectal excision in patients with resectable rectal cancer. We found that radiotherapy before total mesorectal excision can improve local control of disease.

Reported rates of local control after surgery for rectal cancer vary widely. In studies of conventional, nonstandardised surgery, usually with a minimal follow-up of five years, rates of local recurrence have been 15 to 45%.[3-5] By contrast, surgeons who specialise in total mesorectal excision report local-recurrence rates of 7% or less.[9-11] The low rate of local recurrence in the group assigned to total mesorectal excision only in our study (8.2% at two years) demonstrates that similar excellent results can be achieved by other surgeons at multiple centres after they are trained in the procedure.

We found that preoperative radiotherapy further reduced the two-year rate of local recurrence from 8.2% to 2.4%, an indication of the value of preoperative radiotherapy when used in conjunction with standardised surgery. In the Swedish Rectal Cancer Trial (SRCT), the reduction in the rate of local recurrence at five years from 27% in the surgery-

Table 4. Results of univariate log-rank analyses of two-year rates of local recurrence among the 1748 eligible patients with a macroscopically complete local resection, according to selected prognostic variables.*

Variable	Radiotherapy plus surgery		Surgery alone		
	no. of patients at risk	Local recurrence at 2 yr (%)	no. of patients at risk	Local recurrence at 2 yr (%)	
Overall	873	2.4	875	8.2	P<0.001
Sex					
Male	555	2.5	557	7.2	P<0.001
Female	318	2.2	318	9.8	P<0.001
Distance of tumour from anal verge					
10.1-15 cm	262	1.3	271	3.8	P=0.17
5.1-10 cm	372	1.0	350	10.1	P<0.001
≤5 cm	237	5.8	253	10.0	P=0.05
Type of resection					
Low anterior	577	1.2	603	7.3	P<0.001
Abdominoperineal	248	4.9	232	10.1	P=0.02
Hartmann†	47	3.2	39	10.7	P=0.18
TNM-stage					
I	265	0.5	244	0.7	P=0.15
II	251	1.0	241	5.7	P=0.01
III	298	4.3	324	15.0	P=0.001
IV (distant metastases but complete local resection)	47	10.1	48	23.8	P=0.25

* Patients with missing data were excluded from the analysis of local recurrence. Twenty-eight patients without a tumour (TNM stage 0) were excluded from the multivariate analysis because they were not at risk for local recurrence. In a Cox proportional-hazards analysis of age (as a continuous variable), the hazard ratio for local recurrence at two years was 0.99 (95% CI, 0.95-1.04; P=0.77) in the group of 873 patients assigned to radiotherapy and surgery and 1.01 (95% CI, 0.99-1.04; P=0.21) in the group of 875 patients assigned to surgery alone. TNM denotes tumour-node-metastasis.

† A Hartmann resection is a low anterior resection without the construction of an anastomosis.

only group to 11% in the radiotherapy-plus-surgery group improved the rate of overall survival at this time point from 48% in the surgery-only group to 58% in the combined-treatment group.[7] An effect of preoperative radiotherapy on overall survival has not yet been detected in our trial, probably because of the small number of local recurrences and the short follow-up. However, we believe that a median follow-up time of 24.9 months is sufficient to detect the effect of preoperative radiotherapy on local recurrences, 55% to 80% of which occur during the first 2 years after surgery, with the peak rate at 6 to 12 months.[4,21,22]

The beneficial effect of preoperative radiotherapy in our trial was observed for all tumour locations 15 cm or less from the anal verge and for all TNM stages. However, in a univariate subgroup analysis, the effect was not significant in patients who had tumours with an inferior margin more than 10 cm from the anal verge and in patients who had TNM stage I or IV tumours. Nevertheless, multivariate tests indicated that the treatment effect probably did not differ among subgroups defined according to tumour location, TNM stage, and treatment assignment. Therefore, considering the difficulties involved in predicting the location of tumours high above the anal verge and in determining the TNM stage preoperatively, the decision not to irradiate before surgery should be carefully considered.

Preoperative radiotherapy does not result in "down-staging"[23] and is therefore not suitable

for locally advanced tumours. To avoid short-term irradiation of such tumours, we advocate accurate preoperative imaging (e.g. computed tomography or magnetic resonance imaging). This lack of down-staging explains why short-term preoperative radiotherapy has no effect on sphincter preservation, which is often an end-point in conventional trials of long-term radiotherapy.

Concern has been expressed about the side effects of hypofractionated radiation.[24] In the Stockholm I trial[25] and Imperial Cancer Research Fund trial,[26] postoperative mortality was higher among patients who received radiotherapy than among those who did not. In both trials, a suboptimal irradiation technique increased the treated volume considerably. In the SRCT, postoperative mortality did not increase with radiation, provided that radiotherapy was optimal.[27] In our trial, there was no difference in in-hospital mortality between the two groups. In the SRCT, however, there was more incontinence among patients who underwent preoperative irradiation and subsequently underwent a sphincter-preserving surgery.[28]

In conclusion, total mesorectal excision can significantly decrease the risk of local recurrence of resectable rectal cancer. This result was achieved in a large, multicentre trial that included extensive instruction and quality control of the surgical technique. In this large group of patients who underwent standardised surgery, short-term preoperative radiotherapy further reduced the risk of local recurrence.

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7

Mechanisms of oncogenesis in colon versus rectal cancer

E. Kapiteijn[1], G.J. Liefers[1], L.C. Los[1], E. Klein Kranenbarg[1], J. Hermans[2], R.A.E.M. Tollenaar[1], Y. Moriya[4], C.J.H. van de Velde[1], J.H.J.M. van Krieken[3]

Departments of Surgery[1] and Medical Statistics[2], Leiden University Medical Centre, Leiden; Department of Pathology, University Medical Centre St. Radboud, Nijmegen[3], The Netherlands; Department of Surgery, National Cancer Centre Hospital, Tokyo, Japan[4]

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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).

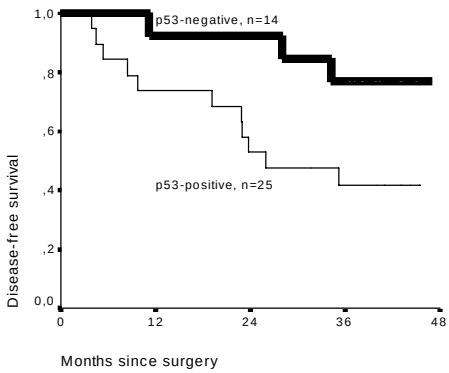
Table 4. Results of positive immunohistochemical marker expression for 77 colorectal cancer patients.*

	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.



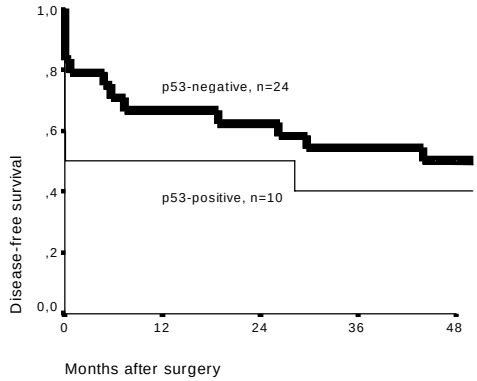


Figure 1. Kaplan Meier curve of 39 (out of 42) rectal cancer patients with regard to negative and positive p53 immunohistochemical expression (P=0.008).

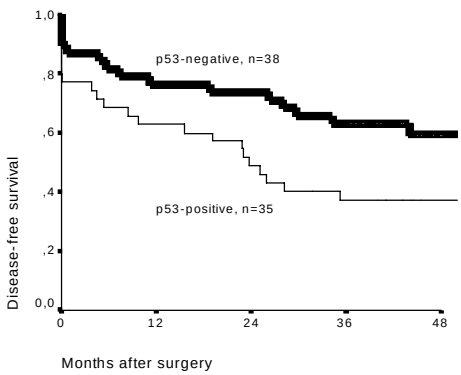


Figure 2. Kaplan Meier curve of 34 (out of 35) colon cancer patients with regard to negative and positive p53 immunohistochemical expression (P=0.37).

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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8

Diploid, microsatellite stable rectal carcinomas show different molecular phenotypes

Molecular profiling of sporadic rectal carcinomas by microsatellite, immunohistochemical, *p53* mutational and gene expression array analyses

E. Kapiteijn[1], W.J.F. de Leeuw[2], R.W. van der Gaag[1,2], P.J.J. Verkuijlen[3], J. Wijnen[3], N. Kuipers-Dijkshoorn[2], A.M. Cleton-Jansen[2], J.H.J.M. van Krieken[4], C.J.H. van de Velde[1], R.A.E.M. Tollenaar[1], C.J. Cornelisse[2], J.M. Boer[3], H. Morreau[2]

Departments of Surgery[1], Pathology[2], Human and Clinical Genetics[3], Leiden University Medical Centre, Leiden; Department of Pathology[4], University Medical Centre St. Radboud, Nijmegen, The Netherlands

Submitted

INTRODUCTION

In The Netherlands, approximately 25% of new cases of colorectal cancer comprise rectal cancers. 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[1] and 18q[2] allelic loss, p53 accumulation,[3] β-catenin expression,[3] c-myc expression[4] and aneuploidy.[5] Right-sided tumours are more often mucinous,[6] diploid[5] and of the MicroSatellite Instability (MSI) phenotype.[7] Furthermore, clinical behaviour is different. In rectal cancer local recurrence is the major problem and in colon cancer distant metastasis.

The development of genetic instability is supposed to be an important event in the multi-step evolution of colorectal cancer resulting in genetic alterations in both proto-oncogenes and tumour suppressor genes.[8,9] Two major mechanisms of genomic instability have been identified. Chromosomal instability (CIN) is characterised by gross chromosomal segregation abnormalities and is commonly detected as aneuploidy.[10] Loss or gain of genetic material at specific chromosomal regions may thereby result in altered allele ratios. The majority (85-90%) of sporadic colorectal carcinomas arise through chromosomal instability.[11]

Alterations at DNA microsatellite repeat units in tumours, so-called microsatellite instability (MSI), is a second form of genetic instability.[12] MSI occurs in hereditary non-polyposis colorectal cancers (HNPCC) and is caused by germline mutations in DNA mismatch repair (MMR) genes.[13] MSI also occurs in 10-15% of sporadic colorectal tumours,[14] mainly by somatic inactivation of hMLH1.[15]

In colorectal cancer, at least two different molecular pathways appear to be affected: the APC/β-catenin (Wnt) pathway, often linked to CIN, and the DNA mismatch repair (MMR) pathway, the latter often with inactivation of TGF-β-RII.[16] These pathways are not totally independent, but show cross talk and mutations in genes (*APC*,[17] *TGF-β-RII*,[18] *axin*[19]) of both pathways. We analysed MSI, loss of heterozygosity (LOH) and ploidy status to study genetic instability of sporadic rectal cancer. Additional immunohistochemical, *p53* mutational and expression array analyses were performed to provide a more detailed molecular profile of cancers without evidence for genetic instability (diploid, MSI-stable). Cases were obtained from a large, randomised trial investigating the role of preoperative radiotherapy in combination with standardised Total Mesorectal Excision (TME)-surgery.[20]

METHODS

Tissue specimens

Formalin-fixed, paraffin-embedded tissue of resected rectal adenocarcinomas of 81 patients, were obtained from a large prospective trial investigating the role of 5x5 Gy short-term preoperative radiotherapy in the treatment of primary rectal cancer in combination with total mesorectal excision (TME)-surgery (TME-trial). In total, 1530 patients were included from 84 Dutch hospitals. In the trial, radiotherapy, surgery and pathology were standardised.[20] The 81 patients were randomised in the trial during the first year; their samples were selected from the 12 largest pathology laboratories to avoid too many different fixation methods.

MSI/LOH-analysis

Tumour and normal cell populations were microdissected separately with a needle under microscopic observation from 10 serial paraffin sections (10 μm thick). For DNA-isolation,

a method described by Isola et al.[21] and Kersemaekers et al.[22] was used.

MSI analysis was done for 12 different microsatellite loci distributed over 10 chromosomes (BAT25, BAT26, D5S346, D2S123 and D17S250) and 2 alternative loci (BAT40, D13S175) of the NCI workshop on MSI in colorectal cancer[23] plus 5 additional loci (D3S2456, D8S1130, D15S1232, D16S752, TP53). All primer sequences for microsatellite repeat markers can be obtained from the Genome Database (http://www.gdb.org).

Fluorescent PCR and data analysis were performed essentially as described earlier.[24-26] MSI was recorded when novel peaks or peaks shifts appeared in tumour DNA cases when compared with matched DNA cases of normal cells. MSI-high tumours were defined as having instability of two or more markers of the NCI reference panel or as having MSI in >30% of all markers tested.[23] MSI-low tumours were defined as having instability of one marker of the reference panel. Cases showing no instability in any of the markers tested were classified as MSI-stable. Loss of heterozygosity (LOH) was recorded when the ratio of the peak heights of the tumour and normal alleles was <0.59 or >1.7. The tumour of a patient was defined as LOH-positive if LOH was found in one of the possible 9 heterozygous markers tested.

DNA ploidy analysis

The pepsin-digestion method of Hedley et al[27] was used for nuclear isolation from 50 μm paraffin sections. Propidium iodide was used as a DNA stain. DNA content was measured on a FACSCalibur flow cytometer (Becton Dickinson, Mountainview, CA).[28]

Immunohistochemical analysis

The following markers were investigated: E-cadherin (Zymed Laboratories), α–, β– and γ-catenin (Transduction laboratories), EpCAM (Centocor), p16 (Pharmingen), Cyclin D1 (Neomarkers), MDM2 (Neomarkers), p53 (Novocastra Laboratories), $p21^{waf1}$ (Oncogene research products), Ki-67 (Dako), Bcl-2 (Boehringer Mannheim), hMLH1 and hMSH2 (Oncogene research products) and hMSH6 (Transduction laboratories). For most antibodies, the sections were first boiled in citrate buffer (pH 6.0) for 25 minutes. For hMLH1 and hMSH2, paraffin sections were first boiled in EDTA. For EpCAM the sections were pretreated with trypsin (0.1% trypsin with 0.1% calcium chloride), pH 7.4 at 37 °C for 20 minutes. After overnight incubation with the primary antibody in 1% phosphate buffered saline/ bovine serum albumin (1% PBS-BSA), the secondary biotin-conjugated antibody and a tertiary complex of streptavidin-avidin-biotin-conjugated to amino-9-ethyl-carbazole (AEC) or 3',3'-diaminobenzidine (DAB) were applied.

For each marker a scoring system was chosen after initial screening of the variation of expression of each marker, or taking systems used in the literature or former studies of our group into account.[3,29-31] Membranous staining of E-cadherin, α-, γ-catenin and EpCAM, was scored according to the following categories: severe loss (0-49%), moderate loss (50-89%), loss only at the infiltrating front of the tumour (90-99%) and no loss (100%). β-catenin was scored as membranous expression/no obvious nuclear expression, nuclear expression in the infiltrating front of the tumour or nuclear expression all over the tumour. p16, cyclin D1 and MDM2 were scored according to the following classification: no nuclear staining/staining in a few nuclei, focal nuclear staining or >10% nuclear staining all over the tumour. Nuclear p53, p21 and Ki-67 were scored as 0-25%, 26-75% or 76-100% expression.

Bcl-2, hMLH1, hMSH2 and hMSH6 were scored according to the following categories: - negative, +/- in case of faint or doubtful staining, or + positive.

***p53* mutation analysis**

Frozen tissue sections were stained with H&E and trimmed in order to select for tumour tissue. DNA was extracted from 20 serial frozen tissue sections (20 μm thick) by way of the Promega genomic DNA purification kit (Promega). The DNA yield was determined by spectrophotometry and the DNA was analysed by *p53* Genechip assay (Affymetrix) as described by Wen et al.[32] To account for any variations that occurred during the assay, each sample batch was processed with human placental DNA as a wild-type control (Affymetrix). Any sequence mismatch present in case DNA was identified by comparison to the control placental DNA.

Gene array expression analysis

Frozen tissue sections were stained with H&E and trimmed in order to select for tumour tissue. RNA was extracted from 20 serial frozen tissue sections (20 μm thick) by way of Trizol reagent (Gibco BRL Life Technologies). 10.0-20.0 μg of total RNA was labelled and hybridised to oligonucleotide arrays (Affymetrix, Oxford, UK), according to the manufacturer's recommendations, essentially as described earlier.[33] Briefly, we used the Affymetrix G110 array, which contains probe sets of 16-20 perfect match (PM) and mismatch (MM) 25-mer oligonucleotide pairs representing 1700 genes. Absolute analysis and pair-wise comparisons of arrays were made in the Microarray Analysis Suite (Affymetrix). Absolute analysis yields Average Difference (AvgDiff) values representing the level of gene expression calculated as the average difference between PM and MM hybridisation. In addition, the Presence Call algorithm qualifies the signal as Present (P), Absent (A) or Marginal (M) gene expression. In the comparison analysis, the Fold Change represents the ratio of gene expression between two samples. T-test and self-organising map clustering (16 nodes) were performed using the DataMining Tool software (Affymetrix).

***hMSH2/hMSH6* mutation analysis**

Genomic DNA isolation, polymerase chain reaction (PCR) amplification, denaturing gradient gel electrophoresis (DGGE) mutation analysis, and nucleotide sequence determination were performed in tumour DNA, as previously described.[34,35] In short, the general strategy was to amplify by the PCR each of the 16 *hMSH2* exons and 10 *hMSH6* exons, and to analyse these products by GC-clamped DGGE. Exons exhibiting altered migration patterns were sequenced to determine the molecular nature of the variant.

Data analysis

For statistical analysis of the data we used SPSS statistical software (version 9.0 for Windows, SPSS, Chicago). Chi-square tests were used to compare proportions. Comparison of mean values between groups were made using Student's t-tests. A P-value of 0.05 or less was considered statistically significant.

Cluster analysis of gene array expression analysis

We extracted tables (rows of genes, columns of individual array hybridisations) of scaled

AvgDiff values for the tumours. Before clustering and display, the genes and arrays were centred by subtracting the median of all values measured for that gene or array, and rows and columns were normalised so their magnitudes were close to 1.0. We applied the Eisen hierarchical clustering algorithm separately to the tumours and genes using the Pearson correlation coefficient as the measure of similarity and average linkage clustering and displayed the clusters by TreeView.[36]

RESULTS

Patient and tumour characteristics

The analysed series consisted of 42 irradiated and 39 non-irradiated patients. Mean age of the patients was 62.5 years (range 29-84). The clinicopathological characteristics were equally distributed among the randomisation groups, apart from a larger number of mucinous tumours in the irradiated group (P=0.048), as was also shown for the whole trial population.[37]

Microsatellite instability

Screening of the 81 randomly selected rectal cancers, revealed only 1 tumour to be MSI-high (1%) with instability in the mono- and dinucleotide markers D5S346, TP53, BAT25 and BAT26. Eighty tumours were MSI-stable. However, 11 of these tumours (14%) showed MSI in the tetranucleotide markers; MSI was found in 2 tumours for D3S2456 (2%), in 5 tumours for D8S1130 (6%), in 1 tumour for D15S1232 (1%) and in 3 tumours for D16S752 (4%). These tumours were classified as MSI-stable since alterations in tetranucleotide markers do not seem to have any biological relevance.[38] In concordance with this, no significant associations were found between tetranucleotide instability and clinicopathological characteristics, irradiation or prognosis.

We examined an additional series of patients of the TME-trial for a possible association of MSI status with particular subgroups of rectal cancer patients. In a subgroup of young patients ($\leq$ 45 years, n=22), all tumours were MSI-stable. However, 5 tumours showed tetranucleotide instability (23%), which is not significantly more than in the random patient group (14%, P=0.24, Table 1). In a patient group with synchronous tumours (more than one carcinoma in the colorectum, n=10), 1 tumour showed tetranucleotide instability (10%) and 9 tumours did not show instability at all (90%). The primary tumours of 8 patients who developed local recurrences in the follow-up were all MSI-stable without tetranucleotide instability (100%).

Loss of heterozygosity

The majority of tumours of the random patient group showed LOH with at least one marker (60/81, 74%), while 21 showed no LOH (26%) in any of the 9 possible heterozygous markers. The highest frequencies of LOH were detected at the TP53 (58%) and D5S346 (42%) loci. LOH for the other markers appeared as follows: D2S123 (9%), D3S2456 (19%), D8S1130 (36%), D13S175 (29%), D15S1232 (38%), D16S752 (16%) and D17S250 (23%). Of the 60 LOH-positive tumours, 53 were MSI-stable and 7 showed tetranucleotide instability. Of the 21 LOH-negative tumours, 16 were MSI-stable, 4 tumours showed tetranucleotide instability and one was MSI-high (Table 1). Comparison of tumour characteristics of the LOH-negative vs. LOH-positive random groups demonstrated that 3/4 mucinous tumours present in our series, were LOH-negative (P=0.022; all mucinous

tumours were MSI-stable). There was no association between radiotherapy or prognosis and LOH-status.

Table 1. MSI/LOH status of the tumours in the random patient group and subgroups.

	Random patient group n=81	≤ 45 yrs patient group n=22	Synchronous tumour group n=10	Local recurrence group n=8
MSI-stable without tetranucleotide instability				
-LOH-	16 (20)	7 (32)	2 (20)	-
-LOH+	53 (65)	10 (45)	7 (70)	8 (100)
MSI-stable with tetranucleotide instability				
-LOH-	4 (5)	-	-	-
-LOH+	7 (9)	5 (23)	1 (10)	-
MSI-high				
-LOH-	1 (1)	-	-	-
-LOH+	-	-	-	-

DNA ploidy

DNA ploidy status was investigated in 19 of the 21 LOH-negative tumours and in 16 of the 60 LOH-positive tumours as controls. Ten out of 19 LOH-negative tumours were diploid (53%), while only 3 out of 16 LOH-positive tumours showed diploidy (19%, P=0.04, Table 2). The only MSI-high tumour was aneuploid. No significant associations were found between ploidy status and clinicopathological characteristics, irradiation or prognosis.

As expected, the majority of LOH-positive tumours (81%) were DNA-aneuploid but also 47% of the LOH-negative tumours. We decided to rely on the DNA ploidy data as a marker for CIN, since ploidy status has been shown to reflect genome-wide instability.

Table 2. Random patient group, n=81; 35 tumours analysed for ploidy.*

	LOH-negative, n=19		LOH-positive, n=16	
	diploid	aneuploid	diploid	aneuploid
MSI-stable without tetranucleotide instability	8 (42)	6 (31)	1 (6)	10 (62)
MSI-stable with tetranucleotide instability	2 (11)	2 (11)	2 (13)	3 (19)
MSI-high	-	1 (5)	-	-

* Percentages have been calculated for the LOH-negative and LOH-positive group separately.

Immunohistochemical analysis of the diploid vs. aneuploid tumours

In order to further investigate tumours without gross genetic instability (diploid, MSI-stable), we compared the diploid and aneuploid tumours for immunohistochemical expression profiles (Table 3). There was only a significant difference in Ki-67 (lower in diploid tumours, P=0.048) and p53 expression (lower in diploid tumours (<76-100%), P=0.026).

Table 3. Immunohistochemical analyses of the random patient group; diploid vs. aneuploid tumours.*, †

	Total n=35	Diploid n=13	Aneuploid n=22	P
E-cadherin				0.24
-0-49%	9 (26)	5 (38)	4 (19)	
-50-89%	7 (21)	4 (31)	3 (14)	
-90-99%	10 (29)	2 (15)	8 (38)	
-100%	8 (24)	2 (15)	6 (29)	
α-catenin				0.38
-0-49%	10 (31)	5 (42)	5 (25)	
-50-89%	4 (13)	-	4 (20)	
-90-99%	13 (41)	5 (42)	8 (40)	
-100%	5 (16)	2 (17)	3 (15)	
β-catenin nuclear				0.46
-membranous/no nuclear	7 (21)	4 (31)	3 (14)	
-at invasive front	13 (38)	3 (23)	10 (48)	
-all over tumour	14 (41)	6 (46)	8 (38)	
γ-catenin				0.09
-0-49%	3 (9)	1 (8)	2 (10)	
-50-89%	11 (33)	4 (31)	7 (35)	
-90-99%	11 (33)	2 (15)	9 (45)	
-100%	8 (24)	6 (46)	2 (10)	
EpCAM				0.40
-0-49%	3 (9)	1 (8)	2 (10)	
-50-89%	3 (9)	-	3 (14)	
-90-99%	12 (35)	4 (31)	8 (38)	
-100%	16 (47)	8 (62)	8 (38)	
p16				0.51
-negative/few nuclei	16 (48)	6 (46)	10 (50)	
-focal positivity	2 (6)	-	2 (10)	
->10% positivity all over tumour	15 (45)	7 (54)	8 (40)	
Cyclin D1				0.81
-negative/few nuclei	28 (82)	11 (85)	17 (81)	
-focal positivity	4 (12)	1 (8)	3 (14)	
->10% positivity all over tumour	2 (6)	1 (8)	1 (5)	
MDM2				0.06
-no/few nuclei	23 (68)	6 (46)	17 (81)	
-focal positivity	5 (15)	2 (15)	3 (14)	
->10% positivity all over tumour	6 (18)	5 (38)	1 (5)	
p53				0.026
-0%	6 (17)	1 (8)	5 (23)	
-1-25%	2 (6)	2 (15)	-	
-26-75%	7 (20)	5 (38)	2 (9)	
-76-100%	20 (57)	5 (38)	15 (68)	
p21				0.11
-0%	17 (52)	4 (31)	13 (65)	
-1-25%	11 (33)	7 (54)	4 (20)	
-26-75%	5 (15)	2 (15)	3 (15)	
-76-100%	-	-	-	
Ki-67				0.048
-0%	-	-	-	
-1-25%	3 (10)	3 (27)	-	
-26-75%	18 (58)	5 (45)	13 (65)	
-76-100%	10 (32)	3 (27)	7 (35)	
Bcl-2				0.17
-negative	13 (41)	7 (58)	6 (30)	
-+/-	16 (50)	5 (42)	11 (55)	
-positive	3 (9)	-	3 (15)	
hMSH6				0.42
-negative	-	-	-	
-+/-	3 (9)	2 (15)	1 (5)	
-positive	31 (91)	11 (85)	20 (95)	

* Some numbers do not equal 35, since a few stainings were not successful.

† Because of rounding, percentages may not total be 100.

p53 mutation and immunohistochemical analysis

Fresh frozen material was available of 5/13 tumours without evidence for genetic instability (diploid, MSI-stable; of these tumours 4 did not show LOH (19, 54, 119, 157) and 1 tumour showed LOH in D8S1130 (126)). Two aneuploid tumours were studied in parallel. In 2 of the 5 diploid, MSI-stable cases, *p53* mutations were found in exon 5. Diploid tumour 54 showed a mutation in codon 175, cgc to cac, and tumour 126 demonstrated a mutation in codon 176, tgc to ttc. The two aneuploid tumours did not show a *p53* mutation.

Immunohistochemical p53 investigation corresponded well with *p53* mutation analysis; p53 expression of 76-100% was associated with a *p53* mutation and lower expression was related to a wild type *p53* status, also in irradiated tumours. In 5/13 diploid tumours high p53 expression (76-100%) was present suggesting the presence of a *p53* mutation.

Expression array analysis

For seven tumours, we analysed the expression of 1700 cancer-related genes using Affymetrix G110 oligonucleotide arrays. The tumour set comprised five tumours without evidence for genetic instability (diploid, MSI-stable), two of which carried a *p53* mutation, and the two aneuploid tumours without a *p53* mutation.

Two-way hierarchical clustering generated a tree with two subgroups, separating the diploid *p53* wild type tumours (19, 119, 157) from the diploid *p53* mutant (54, 126) and the aneuploid tumours (190, 258). However, no reliable clusters of consistently up or down regulated genes could be found in these comparisons (data not shown). Next, we analysed the diploid tumours for differential expression between *p53* mutant vs. wild type status. We selected genes with three methods: pairwise comparison, t-test and self-organising maps, and found 16 upregulated and 3 downregulated genes in common (Table 4). The results were confirmed by hierarchical clustering of the five diploid tumours, which resulted in two subgroups, separating the diploid *p53* wild type tumours (19, 119, 157) from the diploid *p53* mutant tumours (54, 126). Examples of clusters of up- and downregulated genes are shown in Figure 1. The proliferation marker Ki-67 mRNA is upregulated in the cluster analysis in the *p53* mutated tumours, which was confirmed by immunohistochemistry in these tumours using a anti-Ki-67 monoclonal antibody. Looking back at our immunohistochemistry data of the 35 tumours from Table 3, is was found that tumours with a high expression of p53 (76-100%, suggesting a *p53* mutation), also showed a significant higher expression of Ki-67 (26-75% or 76-100%, P=0.003) than tumours less likely to carry a *p53* mutation (<76-100% expression).

We also looked at the so-called “Presence Calls” in the Affymetrix expression array for the presence or absence of mismatch repair gene transcripts (Table 5). The Presence Call algorithm qualifies the signal as Present, Absent, or Marginal gene expression. A 29-year old patient with a diploid tumour was identified with an Absent call for *hMSH2/hMSH3* mRNA expression. Immunohistochemistry of hMLH1, hMSH2 and hMSH6 (Figure 2) in this tumour confirmed the loss of hMSH2. In agreement with the expression array the hMLH1 protein was present, whereas the hMSH6 protein showed loss immunohistochemically. *hMSH6* mRNA was scored positive in the expression array.

Microsatellite analysis of this tumour however, did not show MSI using an expanded international defined marker set. Analysis for *hMSH2/hMSH6* mutations using tumour material resulted negative.

Table 4. Selection of differentially expressed genes in *p53* mutated diploid rectal tumours.

Probe Set G110	Accession #	Gene Description	Mean FC	SD FC	P-value	Note
Increased in *p53* mutated diploid rectal tumours						
1616_at	D14838	Fibroblast growth factor 9 (FGF-9)	4.9	1.1	0.001	
904_s_at	L47276	Alpha topoisomerase truncated-form	8.6	5.3	0.001	
1973_s_at	V00568	c-myc	2.7	0.4	0.002	
1515_at	Consensus	HG4074-HT4344 Rad2	3.1	0.7	0.002	
842_at	U48251	Protein kinase C-binding protein RACK7	3.1	0.7	0.002	A in *p53*wt
282_at	L16782	Putative M phase phosphoprotein 1 (MPP1)	5.1	1.5	0.006	A in *p53*wt
572_at	M86699	Human kinase (TTK)	6.7	4.2	0.007	
349_g_at	D14678	Kinesin-related protein	4.8	1.6	0.009	
893_at	M91670	Ubiquitin carrier protein (E2-EPF)	3.1	1	0.013	
1229_at	U78556	Cisplatin resistance associated alpha protein	2.5	0.9	0.014	
1592_at	J04088	DNA topoisomerase II (top2)	2.3	0.5	0.015	
1990_g_at	U43746	Breast cancer susceptibility (BRCA2)	4.8	1.2	0.016	A in *p53*wt
2042_s_at	M15024	c-myb	9.8	10.8	0.017	
1721_g_at	U65410	Mad2 (hsMAD2)	7.4	2.6	0.037	
170_at	U51096	Homeobox protein Cdx2	4.1	0.5	0.04	
975_at	Y13115	Serine/threonine protein kinase SAK	3.6	0.4	0.045	A in *p53*wt
Decreased in *p53* mutated diploid rectal tumours						
239_at	M63138	Cathepsin D (catD) gene, exons 7, 8, and 9	1.7	0.2	0.007	
608_at	M12529	Apolipoprotein E mRNA	31.8	31.1	0.042	A in *p53*mt
767_at	AF001548	Chromosome 16 BAC clone CIT987SK-A-815A9	4.1	2	0.044	

Differentially expressed genes were selected by each of three analysis methods in the Affymetrix DataMiningTool: (1) Genes that were consistently increased or decreased in all six pairwise comparisons between three *p53* wild type tumours and two *p53* mutant tumours. (2) T-test to identify up- and downregulated genes. (3) Self-organising map cluster of genes that were upregulated in *p53* mutant tumours. Abbreviations: FC, Fold change; A, absent.

Table 5. HC-G110 expression array absolute calls for selected genes.

			Diploid				Aneuploid		
Accession #	Probe Set G110	Gene	Tumour 19 (RT+) (*p53*wt)	Tumour 54 (RT-) (*p53*mt)	Tumour 119 (RT+) (*p53*wt)	Tumour 126 (RT+) (*p53*mt)	Tumour 157 (RT+) (*p53*wt)	Tumour 190 (RT+) (*p53*wt)	Tumour 258 (RT-) (*p53*wt)
U73737	1017_at	hMSH6	P	P	P	P	P	P	P
U03911	860_at	hMSH2	P	P	P	P	A	P	P
U61981	1719_at	hMSH3	P	P	P	P	A	P	P
U07418	1850_at	hMLH1	P	P	P	P	P	P	P
AF001359	1944_f_at	hMLH1 alternatively spliced	A	A	A	A	A	A	A

*p53*mt: *p53* mutant
*p53*wt: *p53* wild type

Figure 1. Gene expression patterns related to *p53* mutation status of diploid rectal tumours.
Two-dimensional hierarchical clustering was applied to the expression data from 1700 Affymetrix probe sets across five tumour samples. Two main branches are apparent in the dendrogram, separating the *p53* mutant (*p53*mt) from the *p53* wild type (*p53*wt) tumours. Two subclusters of upregulated (A, red) and downregulated (B, green) genes in *p53* mutant tumours are shown. The colour in each cell of the tables reflects the mean-adjusted expression level of the gene (row) and tumour (column). (for full-colour figures, see page 177+179)

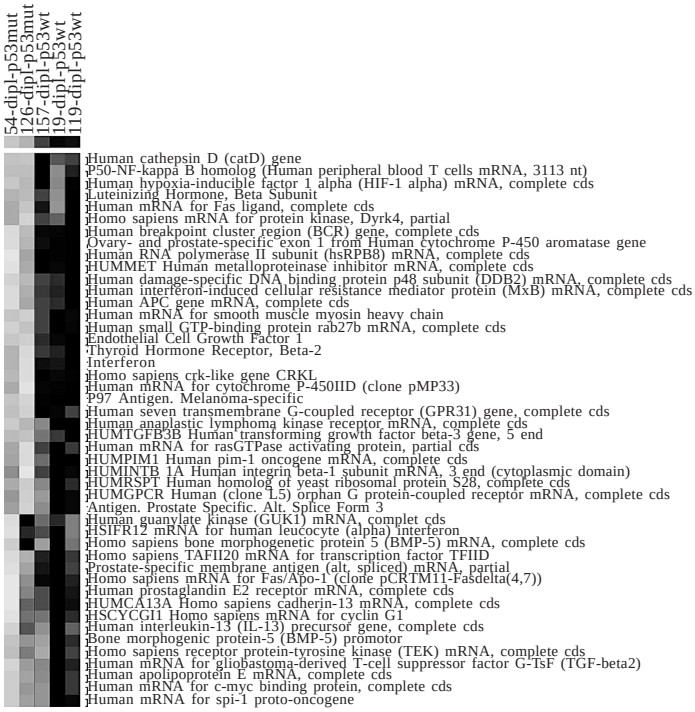
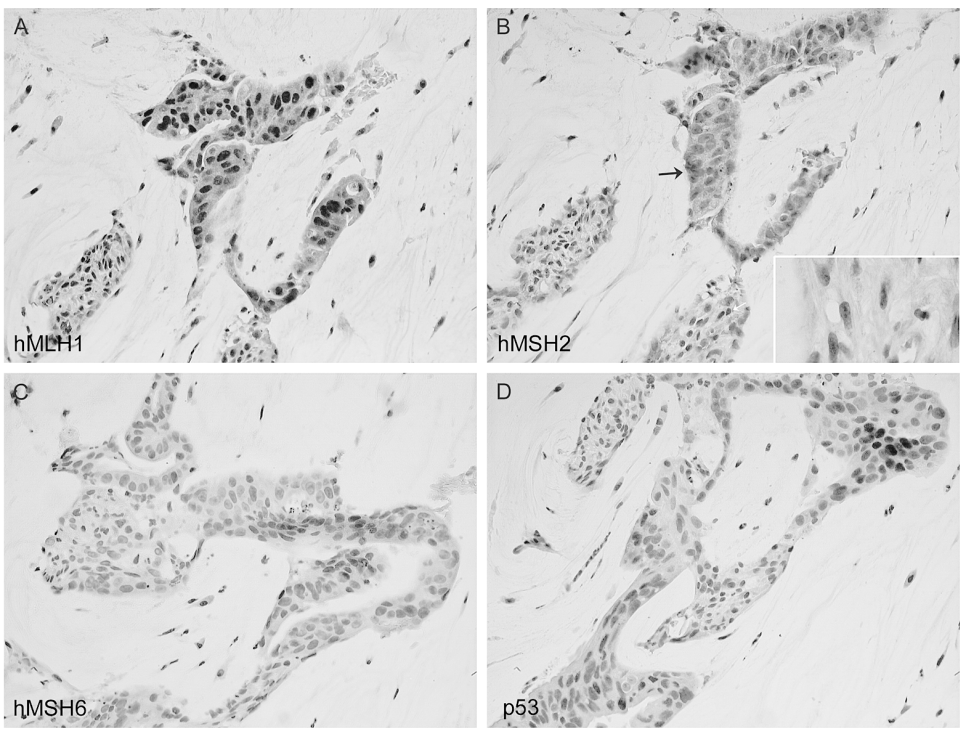


Figure 2. Immunohistochemistry of hMLH1 (A), hMSH2 (B), hMSH6 (C) and p53 (D) in a 29-year old patient (tumour 157) in whom a *hMSH2/hMSH3* expression deficit was found with oligonucleotide array analysis. (for full-colour figure, see page 179)



DISCUSSION

We studied genetic instability in sporadic rectal cancers using MSI, LOH, ploidy, immunohistochemical, *p53* mutational and gene expression array analysis.

In our series of 81 rectal carcinomas, a MSI-high phenotype was observed in only one tumour, indicating that this phenotype is rare in rectal cancer. Even when we separately analysed specific subgroups previously reported to show higher incidences of MSI (young patients and tumour multiplicity),[39,40] we did not find more rectal tumours to be MSI-high. Our results are in concordance with other studies, which showed low incidence of MSI in distal tumours compared to proximal tumours.[38]

We identified replication errors in 11 tumours (14%) solely in tetranucleotide markers. These tumours were classified as MSI-stable.[23] One possible explanation for the instability solely in tetranucleotide markers could be the proposed higher baseline mutation rate of tetranucleotide repeats.[41] One group of tetranucleotide alterations (AAAG)n seems to be particularly susceptible to these alterations in non-HNPCC tumours of different types, including lung, bladder and head and neck cancer.[42] This different type of MSI has been termed Elevated Microsatellite Alterations at Selected Tetranucleotide (EMAST) repeat instability.[23] In our series, 6 of the 11 tumours with tetranucleotide instability showed instability in the EMAST repeats (D8S1130 and D15S1232). These alterations, however, do not seem to have any biological relevance.[38]

In total, 74% of our tumours showed LOH with the highest frequencies for the markers TP53 and D5S346 (APC), confirming previous reports[11,43] and rendering selection bias in our panel of rectal tumours unlikely. Not surprisingly, LOH-positive tumours (81%) more frequently showed aneuploidy than LOH-negative tumours (47%, P=0.05). The discrepancy that 47% of LOH negative tumours were aneuploid may be attributed to the limited number of loci (n=12) investigated and to possible heterogeneity in tumours with regard to LOH and ploidy status.[44]

We identified three distinct phenotypes in our rectal cancer series. The first phenotype comprised the single MSI-high, aneuploid tumour (1/35). Cancers of the second phenotype were MSI-stable and showed aneuploidy in 21/35 tumours investigated for ploidy. The third phenotype combined a MSI-stable status with diploidy in 13/35 tumours. The existence of this last group suggests that a substantial number of the rectal cancers were not driven by the MSI or CIN pathway and that a third pathway might exist. Also other recent studies of colorectal and rectal tumours showed subsets of MSI-stable carcinomas with diploid DNA content or without LOH.[45-48] The latter[48] defined a subgroup of apparently stable near-diploid chromosomes and stable microsatellites (Microsatellite And Chromosome Stable (MACS)). These MACS tumours were often of early-onset with a high frequency of chromosome 18q imbalances. Seventy-nine percent of these MACS tumours were located in the distal colon. In our series of 13 diploid, MSI-stable tumours, the mean age was 56.3 years, which was nearly significantly different from the mean age in the total group of 81 tumours (mean age 62.5 years, P=0.07).

Further analysis of the diploid tumours without evidence of genetic instability (diploid, MSI-stable) indicated that these tumours did not comprise a homogeneous group, but showed different molecular phenotypes. Five out of 13 diploid tumours were likely to carry a *p53* mutation. Our data and those of others indicate that mutation of *p53* by itself may not be sufficient for chromosomal instability.[49] In concordance with our results, it has been

demonstrated that *p53* mutations occur in diploid MSI-positive tumours[49] and that aneuploid cell lines without *p53* mutations exist.[50] Other molecular mechanisms thought to be involved in CIN are changes in mitotic checkpoint genes (*Bub1*),[51] failure of DNA-damage checkpoints (*ATM*)[52] and the JC-virus.[53] In addition, it was recently demonstrated that APC is related to polarity, asymmetric division and aneuploidy and thus can cause CIN.[54]

Gene array expression analysis of five tumours without evidence for genetic instability (diploid, MSI-stable) and two aneuploid also MSI-stable tumours showed heterogeneous results. The most reliable expression differences were found between the *p53* mutant and wild-type diploid, MSI-stable tumours. Using four different methods of analysis a number of differentially expressed genes were found in these two groups indicating the molecular heterogeneity of these tumours. Gene products such as *BRCA2* and the *Cisplatin resistance associated alpha protein*, involved in tumourigenesis and therapy resistance respectively, were differentially expressed. However, the impact of these data have to be confirmed in a larger series of tumours. Differences in *Ki-67* mRNA expression (a proliferation marker) between *p53* wild type and mutated diploid, MSI-stable tumours, identified by two-way hierarchical clustering, were confirmed using immunohistochemistry. This observation might also explain the relative higher expression of *c-myc* mRNA identified in the *p53* mutated tumours in view of its role in cell proliferation.[55]

A diploid tumour from a 29-year old patient showed a *hMSH2/hMSH3* mRNA expression deficit with loss of hMSH2 and hMSH6 at the protein level. The combination of retained *hMSH6* at the RNA-level, but loss at the protein-level when hMSH2 is lost, has been reported before in mice.[56] Also in endometrial tumours from HNPCC patients with *hMSH2* mutations, loss of the hMSH6 protein was seen.[31] Microsatellite analysis and *hMSH2/hMSH6* mutation analysis of this tumour did not show MSI nor a mutation. Our results of loss of *hMSH2/hMSH3* both at the RNA and the former also at the protein level without MSI is hard to explain, but might occur as a late effect during tumourigenesis. A *hMSH3* germline mutation being responsible for early onset of rectal cancer with concomitant loss of *hMSH2* expression seems unlikely in view of the redundant function of *hMSH3*.[57]

In conclusion, as others we identified a group of rectal tumours without evidence of gross genetic instability by molecular analysis with microsatellite markers and flow cytometry. Other authors subdivided these type of tumours already on basis of their age of onset or chromosome 18q abnormalities. We show that tumour heterogeneity in this class of tumours can also be defined by other molecular characteristics such as *p53* mutation status and differential expression profiles.

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9

p53 expression in human rectal tissue after radiotherapy: upregulation in normal mucosa versus functional loss in rectal carcinomas

C.A.M. Marijnen[1,2], E. Kapiteijn[2], I.D. Nagtegaal[2,3], A.A. Mulder-Stapel[3], C.J.H. van de Velde[2], P.I. Schrier[1], L.T.C. Peltenburg[1], J.H.J.M. van Krieken[4]

Departments of Clinical Oncology[1], Surgery[2] and Pathology[3], Leiden University Medical Centre, Leiden; Department of Pathology[5], University Medical Centre St. Radboud, Nijmegen, The Netherlands

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INTRODUCTION

The high local recurrence rate is a major problem in rectal cancer. Preoperative radiotherapy (RT) has been shown to be useful in reducing the number of local recurrences.[1] However, a major disadvantage of preoperative RT is the over-treatment of a subset of patients. Therefore prognostic markers for the tumour response to RT are needed. The tumour suppressor gene p53 has been extensively studied for its prognostic value. In several studies, overexpression of the p53 protein has been shown to correlate with patient survival,[2-4] a finding that has not been confirmed in other studies.[5-8]

One of the functions of p53 in normal cells is to respond to DNA damage by causing either cell cycle arrest or by forcing damaged cells to go into apoptosis. Mutations in the *p53* gene lead to a functionally inactive protein. The *p53* gene is one of the most commonly inactivated genes in cancer[9] and plays an important role in the multistage development of colorectal cancer.[10] The stability of the p53 protein is regulated by binding to MDM2, a protein that degrades p53 and consequently inactivates the transcriptional function of p53.[11-12] Because of this regulation, wild type (wt) *p53* is highly unstable, with a half-life of minutes, and therefore hard to detect by immunohistochemistry (IHC). Mutations in *p53* prevent degradation by MDM2, allowing stabilisation and detection of the protein by IHC. Interpretation of IHC is complicated because other genetic alterations like frameshift mutations or deletions can lead to truncation or complete loss of p53, which precludes detection with IHC. Furthermore, MDM2 overexpression can prevent detection of wild type *p53*. Therefore, negative staining for p53 indicates either wild type p53 or a non-functional gene.

After ionising irradiation the half-life of wt *p53* increases significantly because of phosphorylation of the protein, which inhibits degradation of the protein by MDM2[13] and thus allows its detection by IHC. Accumulation of wt *p53* normally leads to transcription of several downstream target genes, such as $p21^{waf1}$ and GADD45.[14-15]

The induction of the CDK inhibitor $p21^{waf1}$ after ionising radiation leads to a G1 growth arrest, thus allowing the cell to repair the damage.[16] Apart from induction by wt *p53*, activation of the $p21^{waf1}$ gene can also occur through mechanisms independent of p53.[17] TGF-β, the BRCA1 gene products and Nerve Growth Factor are examples of factors that promote $p21^{waf1}$ transcription by p53-independent mechanisms.[18-20] In addition to a role in the repair process, $p21^{waf1}$ has an important function during differentiation of cells.[21]

In cell lines, the effects of ionising radiation on the expression of p53 and $p21^{waf1}$ have extensively been studied. After ionising radiation a rapid increase of wt *p53* is observed, normalising within 48-72 hours.[22] A subsequent increase of $p21^{waf1}$ expression is found in cells with wt *p53*, however, this is not observed in cells with inactive *p53*.[16] In normal intestinal tissue of irradiated mice, a rapid increase of p53 as well as of $p21^{waf1}$ positive cells is reported.[23] In $p53^{-/-}$ mice no increase in $p21^{waf1}$ was observed after irradiation, indicating that wt *p53* is mandatory for upregulation of $p21^{waf1}$.

Presence of wt *p53*, however, does not guarantee a functional intact pathway. Induction of p53 after irradiation without upregulation of $p21^{waf1}$ has been reported,[24] suggesting disruption of the pathway downstream of p53. Expression of $p21^{waf1}$ after irradiation can thus be used as an indicator of defects in the pathway downstream of p53.

The relationship between p53 and $p21^{waf1}$ after irradiation has been investigated in the normal intestinal mucosa of mice,[23] but little is known about tumours *in vivo*. We therefore evaluated the direct effect of ionising radiation on the expression of p53 and $p21^{waf1}$ in

normal mucosa and rectal carcinoma *in vivo*, by analysing a large number of tumours of rectal cancer patients participating in a randomised trial. One half of the patients received short-term preoperative RT within one week followed by surgery, and the other half underwent surgery only. This trial disclosed a unique series of samples serving as an *in vivo* model for the functional activity of the p53 protein.

By careful evaluation of expression patterns of both p53 and p21 we suggest new criteria for determination of *p53* mutations on IHC. Furthermore, we show that in tumours with *p53* wild type the downstream pathway is often disrupted.

METHODS

Patients and treatment

All tumours used for analysis were derived from rectal cancer patients, randomised in a large multicenter trial in which the effect of short-term, preoperative RT (5x5 Gy) in combination with total mesorectal excision (TME) surgery was investigated.[25] They were randomised to either RT followed by surgery or surgery alone. The patients assigned to preoperative RT received a total dose of 25 Gy in 5 fractions during 5-7 days. Irradiated patients in whom the interval between RT and operation exceeded 8 days were excluded from analysis. Standardised routine pathologic examination was performed in the laboratories of the referring hospitals as described by Quirke et al.[26] Tumour staging was performed using the Tumour-Node-Metastasis (TNM) classification.[27]

Tumours

The expression of p53 and $p21^{waf1}$ was evaluated in tumour samples from the first 103 patients entered in the trial from the 12 hospitals contributing the most patients. Of these patients, 51 received preoperative RT. To compare the expression of p53 before and after RT in individual tumours, 32 pretreatment biopsies of irradiated patients were collected, analysed for p53 expression and compared with the corresponding irradiated tumour specimen. The other 19 biopsies were either not available or too small to analyse.

To evaluate the kinetics of p53 degradation after ionising radiation, we analysed p53 expression in tumour and normal tissue with varying intervals between the last fraction of RT and surgery of 1, 3, 5 or 7 days. Because most patients underwent surgery after 3 days, we additionally stained 53 samples to extend the different groups to 20 samples. Only 15 patients had an interval of 7 days in the trial, leading to 75 tumours in total. Both tumour and normal tissue were stained for all samples.

Colorectal tumours are considered mucinous when mucin covers more than 50% of the microscopically observed areas.[28] From the literature, it is known that mucinous tumours are more often wild type *p53*.[29] To evaluate the effect of RT on the expression of p53 in wt tumours, we additionally analysed all tumours with 90-100% mucinous areas from patients randomised in the trial.

Immunohistochemistry

Tissue samples of the primary tumours were fixed in 4% phosphate-buffered formalin, dehydrated and embedded in paraffin. Tissue sections of 4 μm were cut and mounted onto 2% 3-aminopropyltriethoxysilane (APES) pre-coated slides. Serial sections were stained with hematoxylin and eosin or processed for immunohistochemistry.

p53 and p21^{waf1} expression were assessed by immunohistochemical investigation with the following antibodies: anti-p53 (mAb NCL-p53-DO-7, Novocastra Laboratories Ltd., Newcastle, United Kingdom) and anti-p21 (WAF 1 (Ab-1), Oncogene Research Products, Cambridge, Massachusetts). In brief, sections were deparaffinised in xylene and rehydrated. Endogenous peroxidase activity was blocked by 1% hydrogen peroxide for 20 minutes. For non-enzymatic epitope retrieval, 0.01 M citrate buffer (pH 6.0) was used. After overnight incubation with the primary antibody (dilutions: p53 1/2000, p21^{waf1} 1/250) in 1% phosphate-buffered saline/bovine serum albumin (1% PBS-BSA), the secondary biotin-conjugated antibody and a tertiary complex of streptavidin-avidin-biotin conjugated to 3-amino-9-ethyl-carbazole (AEC) or 3',3'-diaminobenzidine (DAB) were applied. Finally, the sections were counterstained with haematoxylin. Incubation with PBS instead of the primary antibody served as a negative control.

Scoring

All slides were evaluated semi-quantitatively and independently by two investigators (CAMM and EK). Sections that were categorised discrepantly were discussed together with an independent investigator (JHJMvK). Nuclear p53 and p21^{waf1} staining were scored in tumour tissue in the following categories: 0%, 1-5%, 5-15%, 16-25%, 26-75% and >75%. Normal mucosal tissue was scored when present in the same block. p53 expression in normal mucosa was scored in the same categories as the tumour tissue. For p21^{waf1}, normal mucosal tissue was scored as totally positive, apical cells positive or totally negative. Since some mucinous tumours contain relatively few tumour cells, p53 was only scored in three categories in these tumours: 0%, 1-25% and 26-100% positive cells.

In order to analyse the correlation between different variables, p21^{waf1} was regarded positive if more than 5% of the tumour cells stained positive. p53 in tumours was divided in three categories: 0% (negative), 1-25% (low) and >25% (positive) to evaluate the influence of RT on the expression of p53.

Data collection and statistics

All data were entered in a database and analysed with Mann-Whitney tests to compare quantitative and ordered variables and with Student's t-tests to analyse differences in normally distributed data between the two groups. Chi-square tests were used to compare proportions. A two-sided P-value of 0.05 or less was considered statistically significant.

RESULTS

Patient characteristics

The mean age was 62 years in the irradiated group and 63 years in the unirradiated group. Thirty-one percent of the irradiated patients had a TNM stage III tumour, vs. 40% of the unirradiated patients. There was no difference in the distribution of gender, type of operation or tumour type in both treatment arms.

p53

To assess the influence of RT on p53 expression *in vivo*, we examined 103 rectal tumours and normal mucosa. Nuclear expression of p53 was observed in both irradiated and non-irradiated tumours (Figure 1A). A complete absence of p53 after RT was observed in 8

tumours (Figure 1B), whereas irradiated normal mucosa as well as stromal tissue showed widely distributed p53 staining (Figure 1C). p53 expression in tumour tissue in samples from both treatment arms is displayed in Figure 2. In the non-irradiated group, slightly more tumours were found with 1-5% or 6-15% of the cells expressing p53, while in the irradiated group more tumours expressed p53 in 76-100% of the cells. These findings suggest that tumours with low p53 expression (1-25%) might contain wt p53 that can be upregulated by irradiation. When the whole group was evaluated this difference could no longer be observed (P=0.39), because of the small numbers of tumours in these categories.

p53 expression in normal mucosa was determined when present, which was in 38 samples of the irradiated group and in 28 of the non-irradiated group. Only one non-irradiated normal mucosa sample showed p53 expression in >5% of the cells, whereas this was present in 36/38 (95%) of the irradiated normal mucosa samples (P<0.001, Table 1). This clearly demonstrates upregulation of p53 in normal tissue after irradiation.

To evaluate the kinetics of p53 upregulation after RT *in vivo*, we selected tumours and normal mucosa of 75 patients with an interval between RT and surgery of 1, 3, 5 or 7 days. The percentage of p53-positive tumours ranged between 55% and 80% and did not vary significantly between the different intervals.

In all normal mucosal tissue samples p53 expression was still observed up to 7 days after the last fraction of RT. There was, however, a decrease in the percentage of positive cells over time.

Table 1. Distribution of p53 expressing cells in normal mucosa of irradiated and non-irradiated patients.

	RT+TME n=38		TME n=28		P
p53	n	%	n	%	
0%	0	-	12	43	<0.001
1-5%	2	5	15	54	
6-15%	7	18	-	-	
16-25%	2	5	-	-	
26-75%	26	68	1	3	
76-100%	1	3	-	-	

p53 in biopsies

Since the percentage of p53-positive tumour cells over the various categories in irradiated tumours was not different from that for unirradiated tumour samples, we compared p53 expression before and after irradiation in individual tumours by evaluating the 32 available preoperative biopsies. The distribution of the p53 expression in the biopsies as well as in the corresponding irradiated tumours is given in Table 2. Five negative biopsies had corresponding irradiated p53-negative tumours, indicating that these tumours represent non-functional p53. Seven biopsies with negative or low p53 expression, showed upregulation of p53 in the corresponding tumours after irradiation, suggesting the presence of wt *p53*. 19 biopsies showed p53 expression in more than 25% of the cells. Since the patients had not been irradiated at the time of biopsy, this is most likely due to mutant *p53*.

Table 2. Relation between p53 expression in non-irradiated biopsies and corresponding irradiated tumours.*

	Tumour		
Biopsy	0%	1-25%	26-100%
0%	5	1	3
1-25%	0	0	4
26-100%	0	2	17

* Numbers in the cells represent numbers of tumours.

p53 in mucinous tumours

To evaluate the effect of RT on p53 expression in a group of tumours that most probably contained wild type *p53*, we analysed p53 expression in all 100% mucinous tumours in the trial. Results are depicted in Figure 3. In the non-irradiated group, 11 of 18 mucinous tumours showed low p53 expression (1-25%) vs. 12 of 52 non-mucinous tumours (61% vs. 23%), suggesting that wt *p53* is frequently present in mucinous tumours. Eighteen of the 24 irradiated mucinous tumours were more p53-positive compared to only 4 of 18 of the non-irradiated mucinous tumours, suggesting upregulation of wt *p53* after RT in this group.

$p21^{waf1}$

To evaluate the effect of radiotherapy on the expression of $p21^{waf1}$ in rectal cancer *in vivo* we compared irradiated and non-irradiated tumours and normal mucosa. Nuclear expression of $p21^{waf1}$ was observed in irradiated tumours and in non-irradiated tumours. $p21^{waf1}$ expression in tumours in both treatment arms is displayed in Figure 4, demonstrating similar $p21^{waf1}$ expression in both treatment arms. Normal mucosa was present in 29 samples of each treatment arm and showed widely distributed $p21^{waf1}$ staining in 97% (28 of 29 of the irradiated cases and was negative in 76% (22 of 29) of the unirradiated cases ($P<0.001$). Occasionally, the unirradiated mucosa showed some $p21^{waf1}$ positive cells in the upper part of the crypts. This suggests that radiotherapy induces $p21^{waf1}$ expression in normal cells, but has no influence on the $p21^{waf1}$ expression in rectal tumour cells.

Relationship between p53 and $p21^{waf1}$

To investigate whether $p21^{waf1}$ expression in vivo is dependent on the p53 status, we analysed the relationship between p53 and $p21^{waf1}$. This relationship for irradiated and non-irradiated tumour tissue is represented in Table 3. In the irradiated group, none of the eight p53-negative tumours showed $p21^{waf1}$ expression, in line with the functional absence of p53. Of the 36 irradiated tumours positive for p53, 9 (25%) were also positive for $p21^{waf1}$. In the unirradiated group 33 tumours were positive for p53, of which 6 (18%) were also positive for $p21^{waf1}$. These percentages for $p21^{waf1}$ positivity in irradiated and unirradiated tumours show that upregulation of $p21^{waf1}$ by p53 after radiotherapy is not very common.

In normal mucosa, 28 samples in each group could be analysed for both $p21^{waf1}$ and p53 expression. Of the unirradiated samples, 20 of 28 were negative for $p21^{waf1}$ and p53 expression, however, of the irradiated samples 26 of 28 were positive for both p53 and $p21^{waf1}$ expression. This indicates that in normal tissue expression of p53 and $p21^{waf1}$ is clearly increased after irradiation.

The status of p53 expression in untreated biopsies in relation to the corresponding irradiated tumours is shown in Figure 5. In this figure, we included expression of $p21^{waf1}$ as a marker for the functionality of p53. Of the 7 tumours in Table 2 with probably wild-type *p53*, only two tumours showed upregulation of $p21^{waf1}$, and 5 were negative for $p21^{waf1}$ (Figure 1D-1F).

Of the 19 tumours with a p53-positive biopsy, 17 showed p53 positivity in the tumour, indicating the presence of mutant *p53*. Three of these tumours showed $p21^{waf1}$ expression (Figure 1G-1I). Although the numbers were small, these results indicate that the presence of wt *p53* does not necessarily lead to upregulation of $p21^{waf1}$, while the presence of mutant *p53* does not exclude $p21^{waf1}$ overexpression.

Table 3. Relationship between p53 and p21 expression in irradiated and non-irradiated tumours.*

	Irradiated tumours	
	p21 negative	p21 positive
p53 negative	8	0
p53 low	3	2
p53 positive	27	9
	Non-irradiated tumours	
	p21 negative	p21 positive
p53 negative	6	1
p53 low	9	3
p53 positive	27	6

* Numbers represent numbers of tumours.

DISCUSSION

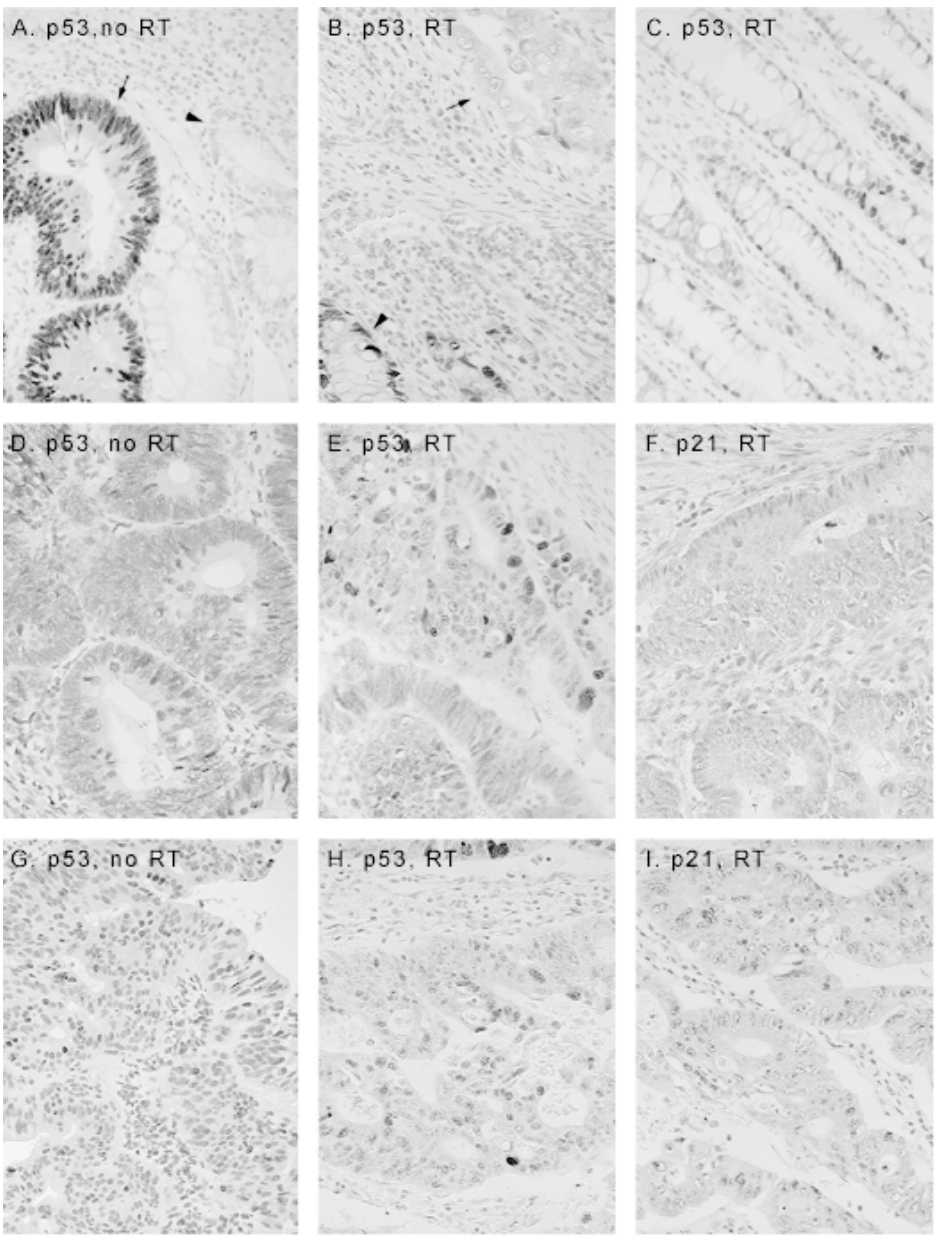
This study was undertaken to evaluate the *in vivo* effect of radiotherapy on the expression of p53 and $p21^{waf1}$ in normal rectal mucosa and rectal carcinoma.

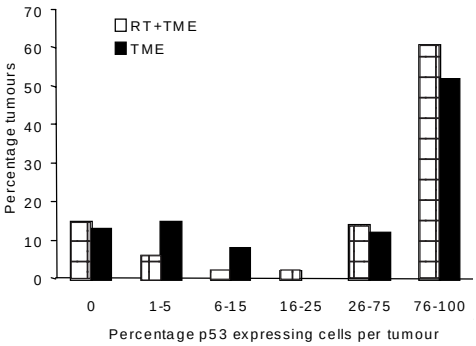
For the first time, we demonstrate that in normal cells p53 as well as $p21^{waf1}$ are upregulated in humans in vivo after short-term preoperative radiotherapy. In tumour cells however, no difference in the expression of p53 or $p21^{waf1}$ in rectal tumours could be observed between the irradiated and non-irradiated groups. These results indicate that p53 protein in rectal tumours does not respond to irradiation, suggesting a very high frequency of p53 abnormalities. We conclude that the p53-$p21^{waf1}$ pathway is disrupted in nearly all tumours, but that there are different underlying mechanisms.

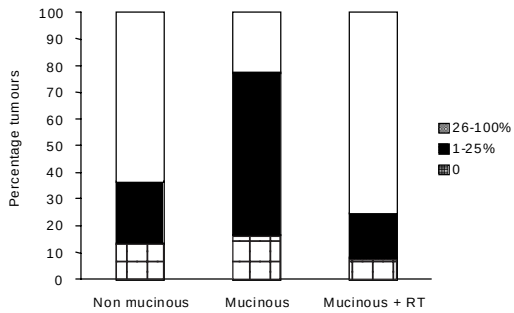
p53 in normal mucosa

In normal cells, p53 can generally not be detected by immunohistochemistry, whereas stabilised p53 can be detected. Stabilisation may occur either through mutation (in cancer) or through phosphorylation of the protein (e.g. after radiotherapy). All irradiated normal mucosa samples showed overexpression of p53, confirming that *in vivo* wt *p53* is upregulated after irradiation. We detected p53 expression 7 days after the last fraction of radiotherapy, which is even later than the reported p53 expression found in the large intestine of mice (3 days after irradiation).[23]

Figure 1. Expression of p53 and p21 in tumour biopsies, tumours and normal mucosa.
A: Non-irradiated sample, showing p53-positive tumour cells (→), whereas normal mucosal cells (►) are p53-negative. **B:** Irradiated sample, showing a tumour completely negative for p53 (→), with p53-positive normal mucosa cells (►) and stromal cells. **C:** p53-positive normal mucosa after irradiation.
D, E, F: Samples from the same patient. **D:** Non-irradiated tumour biopsy with 1-25% of the cells p53 positive. **E:** Corresponding irradiated tumour, showing >25% of the cells p53 positive, indicative for wild type *p53*. **F:** Same tumour as E, showing no $p21^{waf1}$ staining in tumour cells, indicative for a disrupted pathway. Stromal cells are clearly positive.
G, H, I: Samples from the same patient. **G:** Non-irradiated tumour biopsy with >25% of cells p53 positive, indicative for mutant *p53*. **H:** Corresponding irradiated tumour, showing >25% of the cells p53 positive. **I:** Same tumour as H, showing $p21^{waf1}$ positive cells throughout the tumour, indicating a p53 independent upregulation of $p21^{waf1}$. RT: irradiated, no RT: non-irradiated. (for full-colour figure, see page 181)







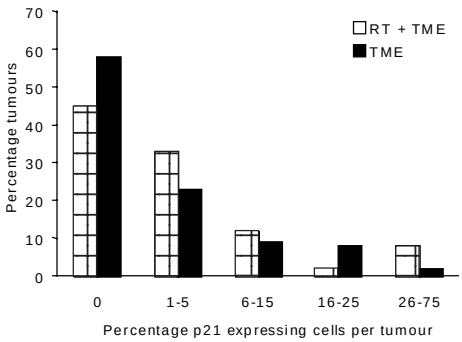


Figure 2. Distribution of percentage of p53 expressing cells over the treatment arms. No difference is observed (P=0.39). RT+TME: radiotherapy followed by surgery, TME: surgery only.

Figure 3. Distribution of p53 expression in non-mucinous, mucinous and irradiated mucinous tumours. A shift from low p53 expression in the mucinous tumours towards p53 positivity in the irradiated tumours is observed, indicative for the upregulation of wild type *p53* after irradiation.

Figure 4. Distribution of $p21^{waf1}$ expressing cells over the treatment arms. No difference is observed (P=0.36). RT+TME: radiotherapy followed by surgery, TME: surgery only.

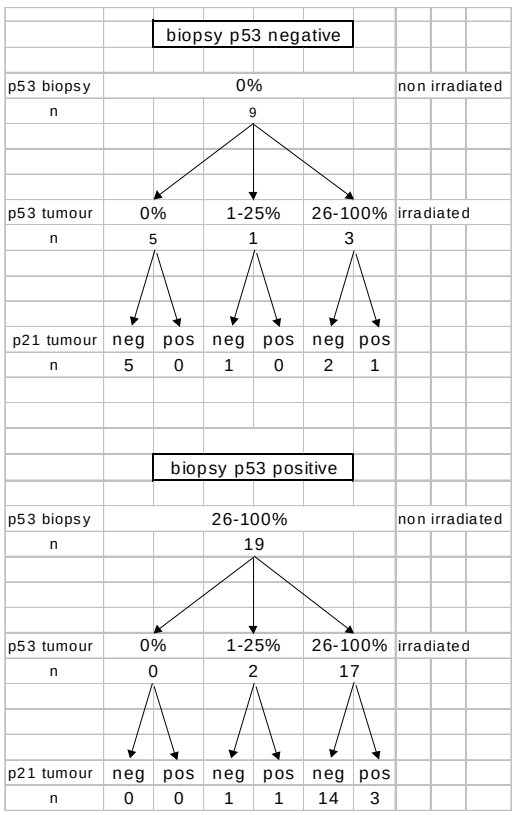


Figure 5. Flow chart demonstrating the relationship between the p53 expression in untreated biopsies and the irradiated tumours. $p21^{waf1}$ expression in the corresponding tumours is displayed.

p21^{waf1} in normal mucosa

In unirradiated mucosa, we observed expression of p21^{waf1} in cells located in the upper part of the crypts. This has been described before and this apical expression is thought to be limited to differentiated and non-proliferating cells.[21] After radiotherapy expression of p21^{waf1} in normal mucosa was upregulated throughout the crypt and demonstrates upregulation of p21^{waf1} by wt *p53* after ionising irradiation. These data confirm that *in vivo* a functional p53-p21^{waf1} pathway can be demonstrated using IHC.

p53 in tumours

The clear upregulation of wt *p53* in normal mucosa suggests that tumours in the irradiated group completely negative for p53 do not represent tumours with functional p53. Because no significant difference was found in the number of p53-negative tumours in both treatment arms, we propose that all tumours completely devoid of p53-positive cells contain either a frameshift or truncating *p53* mutation or have MDM2 overexpression.

Previously we have shown that p53 positivity by immunohistochemistry in nonirradiated carcinomas almost always represents mutated *p53*.[30] In the irradiated group however, p53-positive tumours can either have mutated or upregulated p53. Because the number of p53-positive tumours was not significantly different between both treatment arms, it is likely that in the irradiated group high p53 expression was in the great majority of cases caused by mutation and not by radiation-induced upregulation of wt *p53*. The slight difference between both treatment arms in the distribution of tumours with low p53 expression, suggests that tumours expressing p53 in 1-25% of the cells might contain wt *p53*. In conclusion, we propose that tumours completely negative for p53 or showing p53 overexpression represent tumours with non-functional p53, and p53 expression in 1-25% of the cells is indicative for wt *p53*. This means that in our study, in 84% of the tumours p53 was non-functional, a higher percentage than usually reported for colorectal cancer (40-80%).[4,31] The results of p53 expression in mucinous tumours are in agreement with this hypothesis. The higher number of unirradiated mucinous tumours with low p53 expression vs. the relatively low frequency in the irradiated group, confirms the assumption that tumours with low p53 expression (1-25% of the cells positive) represent tumours with wild type protein, that becomes upregulated after radiotherapy.

The observed upregulation after irradiation in p53 negative biopsies, seems in contrast with the conclusion that p53-negative samples contain mutated *p53*. This might be explained by the small size of these samples, preventing the detection of p53-positive cells in biopsies. In a study with 5x5 Gy preoperative radiotherapy, the irradiated as well as the unirradiated group showed p53 positivity in the tumours, while the biopsies were negative.[4] This observed increase in the non-irradiated tumours confirms that biopsies can be too small to reliably indicate p53 status. Another study comparing nonirradiated biopsies with irradiated surgical samples, showed no increase in the expression of p53 after radiotherapy.[5] Apart from the size of the biopsies, this might be explained by the fact that in this study the median interval between radiotherapy and surgery was 14 days, allowing the degradation of radiation-induced stabilised wt *p53*.

p21^{waf1} in tumours

Our results indicate that the expression of p21^{waf1} in tumour tissue does not change after radiotherapy. In contrast, loss of p21^{waf1} expression after radiotherapy in initially positive colorectal tumours has been described.[32] However, the average interval between radiotherapy and surgery in that study was 12 weeks, which might allow for outgrowth of a subset of p21^{waf1} negative tumour cells.

The expression of p21^{waf1} observed in tumours completely negative for p53 indicates that p21^{waf1} transcription in tumours is not always dependent on p53, as has been described before.[18-20,33] In addition to this, we observed that upregulation of wt *p53* by irradiation does not necessarily lead to increased expression of p21^{waf1}. On basis of these arguments it must be concluded that a high percentage of rectal cancer tumours contain *p53* mutations or show a failure in the signaling downstream of p53. Consequently, the number of rectal carcinomas with functionally active p53 is very limited.

In the literature, the overexpression of p53 in colorectal carcinomas varies between 40% and 80%.[2,4,7,8,31,32] The variation may be explained by patient selection and by the various cut-off points used for p53 positivity. Furthermore, none of the studies differentiate between tumours absolutely negative for p53 and tumours with a low number of positive cells, thus obscuring *p53*-mutated tumours in a group that is usually considered wild type. This might explain the contradictory results concerning the prognostic value of p53 overexpression. We suggest that the downstream pathway of p53 is often disrupted in *p53* wild type tumours, complicating interpretation even further. Therefore, we believe that the value of p53 as prognostic marker requires reconsideration. Using this new information we will develop an assay for p53 assessment that we will use to study the prognostic value of p53 in rectal cancer.

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10

Loss of EpCAM expression is associated with increased local recurrence risk and low microvessel count with increased distant recurrence risk in rectal cancer

E. Kapiteijn[1], I.D. Nagtegaal[2], B.E. van der Worp[3], A.A Mulder-Stapel[1,2], C.J.H. van de Velde[1], R.A.E.M. Tollenaar[1], J.H.J.M. van Krieken[3]

Departments of Surgery[1] and Pathology[2], Leiden University Medical Centre, Leiden; Department of Pathology[3], University Medical Centre St. Radboud, Nijmegen, The Netherlands

Submitted

INTRODUCTION

In colorectal carcinoma there is an accumulation of genetic changes in a preferential order in which specific oncogenes and tumour suppressor genes take part. In the initiation of colorectal cancer, genomic instability plays an important role in the accumulation of these genetic changes.

In the progression of colorectal tumours, microenvironmental interactions are important. Loss of cell adhesion leads to a reorganisation of epithelial cells and enables invasion and metastasis.[1] In cell-cell adhesion, E-cadherin is associated with the actin cytoskeleton via cytoplasmic proteins, including α-, β-, and γ-catenins, which together form the cadherin/catenin complex.[2] The epithelial cell adhesion molecule (EpCAM) has been shown to affect in vitro expression of the intercellular adhesions mediated by cadherins.[3] Furthermore, angiogenesis has been described as vital for tumour growth and expansion; influx of new blood vessels may facilitate dissemination to distant sites.[4,5]

Complete resection remains the best chance for cure in colorectal cancer. The results of traditional rectal cancer surgery however, are discouraging with a high percentage of local recurrence and large variability between surgeons.[6,7] Two important factors that have been reported to improve local control and survival are standardised Total Mesorectal Excision (TME)-surgery[8] and preoperative radiotherapy.[9] Many studies have been performed to find biological parameters that identify a higher degree of aggressiveness, independent of known clinical and pathological features. Such parameters may have additional value to improve treatment strategies.

In this study, the aim was to analyse the influence of irradiation on the expression of cell adhesion molecules and microvessel count and to investigate the prognostic value of these factors in rectal cancer. Rectal cancer cases were obtained from a large, prospective trial in which the additional role of preoperative radiotherapy was investigated in combination with TME-surgery. In this trial, radiotherapy, surgery and pathology were standardised and provides optimal conditions for studying prognostic markers.[10]

METHODS

Patients

Ninety-seven rectal cancer patients who had undergone a macroscopically curative resection with or without preoperative radiotherapy, were analysed. These patients were included in a multicentre trial in which randomisation took place for preoperative radiotherapy of 5x5 Gy followed by standardised TME-surgery or TME-surgery alone. The 97 patients were randomised in the trial during the first year; their samples were selected from the 12 largest pathology laboratories to avoid too many different fixation methods.

Immunohistochemistry

From formalin-fixed, paraffin embedded tissue blocks 4 µm sections were cut and mounted on 2% 3-aminopropyltriethoxysilane (APES) pre-coated slides. Sections were deparaffinised in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 minutes. Immunohistochemical investigation was performed with the following antibodies: E-cadherin (1:1000, Zymed Laboratories, San Francisco, CA, USA), α-catenin (1:1000, Transduction laboratories, Lexington, KY, USA), β-catenin (1:20000, Transduction laboratories, Lexington, KY, USA), γ-catenin (1:8000, Transduction

laboratories, Lexington, KY, USA), EpCAM (1:2500, Centocor, Malvern, PA, USA) and CD31 (1:400, Dako, Glostrup, Denmark). For E-cadherin, α-, β- and γ-catenin and CD31, the sections were first boiled in citrate buffer (pH 6.0) for 25 minutes. For EpCAM the sections were pretreated with trypsin (0.1% trypsin with 0.1% calcium chloride), pH 7.4 at 37 °C for 20 min. After overnight incubation with the primary antibody in 1% phosphate buffered saline/bovine serum albumin (1% PBS-BSA), the secondary biotin-conjugated antibody and a tertiary complex of streptavidin-avidin-biotin-conjugated to amino-9-ethyl-carbazole (AEC) or 3',3'-diaminobenzidine (DAB) were applied. Finally, the sections were counterstained with hematoxylin. Incubation with PBS instead of the primary antibody served as a negative control. Positive controls were included in each staining session. In addition, in most slides normal colorectal tissue served as a positive internal control. The same area of the tumour was used for the various stains, so there was no sampling problem with respect to the comparison of staining patterns.

Analysis of staining patterns

The E-cadherin, α-catenin, β-catenin, γ-catenin and EpCAM slides were independently assessed by two observers (EK and JHJMvK) and in case of discrepancy, discussed until agreement was reached.

For each marker a scoring system was developed after initial screening of the variation of expression of each marker and taking systems used in the literature into account.[11,12] Membranous staining patterns of E-cadherin, α-catenin, γ-catenin and EpCAM, were scored according to the following categories: severe loss (0-49% expression), moderate loss (50-89% expression), loss only at the infiltrating front of the tumour (90-99% expression) and no loss (100% expression). β-catenin was scored as membranous expression/no obvious nuclear expression, nuclear expression in the infiltrating front of the tumour or nuclear expression all over the tumour.

For microvessel count analysis (CD31), image analysis was performed using the Zeiss vision KS400 image analysis system. Images were recorded by a three-chip CCD camera (DXC-950P, Sony) mounted on top of a conventional light microscope (Axioskop, Zeiss). Five hot spots were selected at x40 and/or x100 magnification by EK after consultation with JHJMvK. Finally, the fields selected were scanned at x200 magnification. Microvessel count was analysed by the computer and expressed as the amount of microvessel perimeter per square millimetre. The mean value of the 5 measurements per sample was taken as microvessel count for that sample.

To investigate the reproducibility of our staining technique and microvessel count analysis, 20 CD31 slides of our series were stained a second time using CD31 as antibody and investigated with another computer-aided image system by BEvdW. There was a significant correlation between the series of EK and BEvdW (Pearson correlation coefficient 0.5, P=0.04), implying reasonably good reproducibility of the staining technique and microvessel count analysis.

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. Marker expression was dichotomised on the basis of frequency

tables to achieve a 50%-50% distribution as close as possible; cases with an expression below the cut-off were referred to as loss of expression, and above the cut-off as no loss or preserved expression. For microvessel count, the mean was taken as cut-off for low- vs. high-vascularisation. Chi-square tests were used to compare proportions. Comparison of mean values between two groups were made using Student's t-tests. Univariate recurrence and survival analyses were carried out by using the Kaplan-Meier method and differences between groups were compared by the log-rank test. The Cox proportional hazards model was used for multivariate analysis; variables with a P-value of less than 0.1 in the univariate analysis were included in the multivariate analysis. A P-value of 0.05 (two-sided) or less was considered statistically significant.

RESULTS

Patients

The analysed series consisted of 46 irradiated and 51 non-irradiated patients, who underwent a macroscopically curative resection. Clinical and pathological characteristics were equally distributed among the randomisation groups, apart from more mucinous tumours (P=0.035) and a worse differentiation grade in the irradiated group (P=0.02, Table 1). These differences were also present in the whole trial population.[13] Mean follow-up of patients still alive was 47 months (range 35-56 months). Of the 97 patients, 25 patients died. Five patients developed local recurrences, all with distant recurrence at the time of presentation of local recurrence or later in the follow-up. Eighteen patients developed distant recurrence alone.

Table 1. Clinical and histopathological data according to randomisation group, n (%).†

	Total (n=97)	RT+TME (n=46)	TME (n=51)	P
Gender				0.82
-male	56 (58)	26 (57)	30 (59)	
-female	41 (42)	20 (43)	21 (41)	
Age (yrs)				0.66
-mean	62.7	62.2	63.2	
-range	29-84	29-83	37-84	
WHO classification				0.035
-adenocarcinoma	90 (93)	40 (87)	50 (98)	
-mucinous carcinoma	7 (7)	6 (13)	1 (2)	
Differentiation grade				0.02
-well/moderate	79 (81)	33 (72)	46 (90)	
-poor/undifferentiated	18 (19)	13 (28)	5 (10)	
Tumour infiltration				0.55
-circumscribed	62 (64)	28 (61)	34 (67)	
-diffuse	35 (36)	18 (39)	17 (33)	
Lymphoid reaction				0.30
-none/few	85 (88)	42 (91)	43 (84)	
-moderate/extensive	12 (12)	4 (9)	8 (16)	
Eosinophylic infiltration				0.89
-none/few	68 (70)	33 (72)	35 (69)	
-moderate	21 (22)	9 (20)	12 (24)	
-extensive	8 (8)	4 (8)	4 (8)	
TNM stage				0.23
-I	24 (25)	15 (33)	9 (18)	
-II	36 (37)	15 (33)	21 (41)	
-III	37 (38)	16 (35)	21 (41)	

† Because of rounding, percentages may not total 100.

Randomisation group (Table 2)

Adhesion

The cut-off points for E-cadherin, α- and γ-catenin were determined at 0-89% vs. 90-100% expression to obtain a 50%-50% distribution as close as possible. For EpCAM the cut-off point was 90-99% vs. 100%. Irradiated tumours showed more nuclear β-catenin all over the tumour as compared to non-irradiated tumours (P=0.007).

Microvessel count

We used mean microvessel count as cut-off; 41 tumours had a low microvessel count and 50 a high microvessel count. The mean microvessel count was significantly lower in irradiated tumours (P=0.03).

Table 2. Results of adhesion marker expression and microvessel count according to randomisation group.*,†

	Total (n=97) positive cases / n (%)	RT+TME (n=46) positive cases / n (%)	TME (n=51) positive cases / n (%)	P
E-cadherin				0.17
-0-89%	32 (33)	12 (26)	20 (39)	
-90-100%	65 (67)	34 (74)	31 (61)	
α-catenin				0.21
-0-89%	40 (41)	22 (48)	18 (35)	
-90-100%	57 (59)	24 (52)	33 (65)	
β-catenin nuclear				0.02
-membranous/no nuclear	15 (15)	6 (13)	9 (18)	
-only at infiltrating front	26 (27)	7 (15)	19 (37)	
-all over tumour	56 (58)	33 (72)	23 (45)	
γ-catenin				0.95
-0-89%	41 (44)	20 (44)	21 (44)	
-90-100%	52 (56)	25 (56)	27 (56)	
-not analysed	4	1	3	
EpCAM				0.76
-0-99%	48 (49)	22 (48)	26 (51)	
-100%	49 (51)	24 (52)	25 (49)	
Microvessel count				0.03
-mean	7.31	6.70	7.82	
-range	2.92-13.05	2.92-11.57	3.47-13.05	
-not analysed	6	4	2	

* There are missing cases, since some stainings were not successful.

† Because of rounding, percentages may not total 100.

Tumour characteristics

Adhesion

Loss of E-cadherin expression, was associated with the presence of a moderate/extensive lymphoid reaction (P=0.046) and advanced TNM-stage (P=0.048). Furthermore, absence of nuclear β-catenin expression was related to the mucinous phenotype (P=0.007). Tumours with loss of EpCAM expression showed more often a growth pattern of diffuse tumour infiltration (P=0.005).

Microvessel count
A low microvessel count was associated with diffuse tumour infiltration (P=0.001) and the presence of an extensive eosinophyl reaction (P=0.03).

Mutual associations between adhesion and microvessel count
Preserved E-cadherin expression was associated with nuclear expression of β-catenin either at the invasive front or all over the tumour (P=0.05). Furthermore, preserved α-catenin was associated with preserved γ-expression (P=0.004). No other mutual associations were found between adhesion expression profiles and between adhesion and microvessel count.

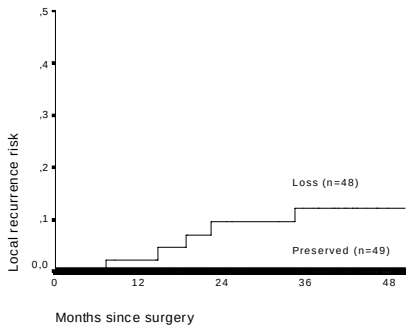
Prognosis (Table 3)
Adhesion
Loss of EpCAM expression was significantly associated with local recurrence (loss: 12% vs. preserved: 0%, P=0.015, Figure 1). No association was found between EpCAM expression and distant recurrence (P=0.61). A minor effect of loss of EpCAM expression was seen on overall survival (66% vs. 80%, P=0.08). Since the number of local recurrences was low (n=5), we did not perform a multivariate Cox analysis for local recurrence risk.
Microvessel count
Low microvessel count was associated with an increased distant recurrence risk (low: 33% vs. high: 5%, P=0.04, Figure 2), and probably due to this, low microvessel count was also associated with a lower survival rate (72% vs. 85%, P=0.02, Figure 3). No association was found between microvessel count and local recurrence (P=0.73). For distant recurrence risk, multivariate Cox regression showed that only TNM-stage (P=0.005) was an independent predictor; microvessel count was not an independent predictor for distant recurrence when corrected for TNM-stage (P=0.11). For overall survival, multivariate Cox regression showed that TNM-stage (P<0.001) and gender (P=0.004) were independent predictors; microvessel count was not significant in this analysis (P=0.21).

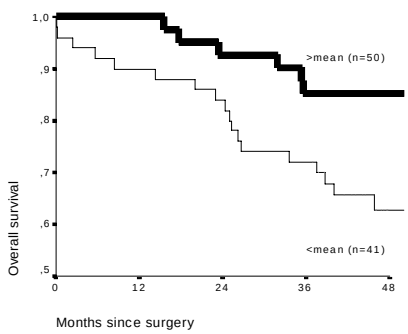
Remarkably, in Figure 2 showing distant recurrence risks of patients with low and high microvessel count, the curves for low and high microvessel count diverge up to 36 months, while after 36 months the curves converge. It seems that distant recurrences in the high microvessel count group occurred later in the follow-up. However, we have to be careful with this conclusion since the curves become less reliable after longer follow-up as lower numbers of patients are at risk and the numbers of events are low. In addition, the difference between low and high microvessel count was still significant for the whole period of follow-up.

Separate analysis of irradiated vs. non-irradiated tumours revealed non-significant associations between EpCAM-expression and local recurrence risk for irradiated tumours (P=0.16) and non-irradiated tumours (P=0.27). Also, no significant association was found between microvessel count and distant recurrence risk for irradiated tumours (P=0.12) and non-irradiated tumours (P=0.065). These non-significant associations in our subgroup analysis are probably due to the low numbers of events in the separate randomisation groups.

Table 3. Results of univariate log rank analyses for the risk on local recurrence, distant recurrence and overall survival.

	Local recurrence (4 years)	P	Distant recurrence (4 years)	P	Overall survival (4 years)	P
E-cadherin		0.21		0.31		0.52
-0-89%	10%		32%		68%	
-90-100%	3%		23%		77%	
α-catenin		0.30		0.43		0.84
-0-89%	3%		23%		74%	
-90-100%	8%		24%		73%	
β-catenin nuclear		0.19		0.41		0.39
-no	13%		27%		71%	
-infiltrating front	0%		15%		85%	
-all over tumour	6%		32%		69%	
γ-catenin		0.09		0.23		0.37
-0-89%	12%		29%		66%	
-90-100%	2%		23%		75%	
EpCAM		0.015		0.59		0.08
-0-99%	12%		22%		66%	
-100%	0%		29%		80%	
Microvessel count		0.73		0.04		0.02
-≤mean	7%		33%		63%	
->mean	5%		18%		85%	





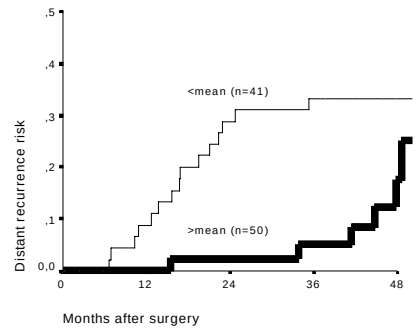


Figure 1. Local recurrence risk of 97 rectal cancer patients with loss vs. preserved EpCAM expression (P=0.015).

Figure 2. Distant recurrence risk of 91 (out of 97) rectal cancer patients with low vs. high microvessel count (P=0.04).

Figure 3. Overall survival of 91 (out of 97) rectal cancer patients with low vs. high microvessel count (P=0.02).

DISCUSSION

In this study, we analysed the influence of irradiation on the expression of cell adhesion molecules and microvessel count, and investigated the prognostic value of these aspects in rectal cancer. Since cell adhesion and angiogenesis are important components in the process of invasion and metastasis, we focussed on these two issues. Cases were obtained from a randomised trial investigating the role of preoperative radiotherapy in combination with standardised surgery. Standardisation of treatment is a prerequisite to optimally study aspects of prognostic markers in rectal cancer, especially since the surgeon has been shown to be an important factor for outcome.[6,7]

Nearly all components of the cadherin/catenin complex were investigated, which plays an essential role in intercellular adhesions. We found an association between radiotherapy and the presence of nuclear β-catenin. Nuclear localisation of β-catenin can be the result of mutations in *APC* or *β-catenin* itself, and is an indication of a disruption of the wnt/wingless signaling pathway.[14] It is difficult to speculate on this finding, but perhaps irradiation can affect components of the wnt/wingless signaling pathway which might lead to nuclear localisation of β-catenin. We did not find an association between nuclear β-catenin and prognosis. In concordance with our results, Gunther et al.[15] did not detect an association between nuclear β-catenin expression and the occurrence of distant metastases.

Our study did not show any association between expression of E-cadherin, α–, β–, and γ–catenin and prognosis. However, a relationship between E-cadherin expression and metastasis has been suggested for colorectal tumours.[16] Nevertheless, other studies did not find an association between E-cadherin and metastasis; such studies have shown metastatic tumour cells to be strongly E-cadherin positive.[17,18] Associations between loss of α-catenin and invasion, metastasis or poor prognosis have been reported in colorectal carcinomas.[19,20] Another study found no association between loss of α-catenin expression and worse prognosis.[21] For γ-catenin, most studies showed no or few loss of expression in colorectal cancers.[16,22] One study however, showed decreased γ-catenin expression to be associated with increasing severity of dysplasia in adenomas.[23]

EpCAM is an important component in cell-cell adhesion and seems to have a central role in this process since it affects intercellular adhesions mediated by cadherins.[3] In normal and cancerous intestinal tissue, EpCAM is generally strongly expressed.[24] We found an association between loss of EpCAM expression and local recurrence. The number of local recurrences was only 5 in our series, representing a major decrease in local recurrence rate by the introduction of TME-surgery in our multicentre trial.[25] Although the number is low, we consider our finding of EpCAM predicting local recurrence of substantial value, since all the 5 primary tumours of patients with local recurrence showed loss of EpCAM expression. In addition, when we compared the tumours of patients with a local recurrence vs. the tumours with also loss of EpCAM expression (i.e. < 99% expression) but without local recurrence, we found that in the local recurrence group 3/5 (60%) tumours showed severe/moderate loss of EpCAM vs. 14/43 (33%) in the no local recurrence group with loss of EpCAM.

Angiogenesis has been described as vital for tumour growth and expansion.[4,5] We found an association between irradiation and low microvessel count. Radiation therapy has been shown to injure both endothelial cells and the basement membrane of microvessels, mainly by increased permeability and fibrosis.[26] On the other hand, the response of tumours to

irradiation depends on the distribution of oxygen which is determined in part by the architecture of the vascular network in tumours. Retrospective analysis of cervical and nasopharyngeal carcinomas revealed that vascular density was related to results of radiotherapy, larger vascular density being associated with prolonged survival.[27,28] Vascular density determination can therefore be helpful in recognising the cases for which adjuvant treatment may be useful. In our study we did not investigate expression profiles which could predict the response to preoperative radiation since biopsies are not representative for assessment of the microenvironment of tumours (i.e. the invasive front). Future research of our group with preoperative biopsies will reveal which factors predict the response to radiotherapy in rectal cancer.

We found low microvessel count to be associated with a higher distant recurrence risk and worse overall survival for the total group of tumours, although it was not an independent predictor for these outcomes and separate analysis in irradiated and non-irradiated tumours did not reveal significant associations between microvessel count and distant recurrence. Recently, we showed that preoperative radiotherapy significantly reduces local recurrence risk when combined with total mesorectal excision, but does not have an effect on distant recurrence risk.[25] We cannot be absolutely sure whether the association between microvessel count and distant recurrence for the total group of tumours has been influenced by the effect of radiotherapy on microvessel count. However, this seems highly unlikely since we found an association between low microvessel count (which can be induced by radiotherapy as we show in this paper) and more distant recurrence; an outcome probably not influenced by preoperative irradiation.

Several interacting factors control angiogenesis. The host defense, represented by e.g. T lymphocytes, mononuclear phagocytes and natural-killer cells, is dependent on the tumour blood supply,[4] and tumour-associated macrophages have been shown to induce neoangiogenesis.[29] Our finding of high microvessel count being associated with less distant recurrence and better overall survival, might be attributable to the fact that by increasing the contact surface between circulating blood and the tumour (i.e. high microvessel density), the opportunity for an immunological response becomes greater. We did not detect an association between high microvessel count and extensive lymphoid or eosinophilic infiltration, but other immune cells may have a more prominent role in the response against the tumour and influence recurrence and survival rates, as has been shown in another paper of our group.[30]

Our findings of microvessel count being a favourable prognostic factor are supported by results of Lindmark et al.,[31] who found that a high microvascular count predicted a longer survival time in colorectal cancer. However, this study was criticised for its methodology.[31] Most other studies demonstrated that vessel count was associated with metastasis or worse prognosis,[32-35] although there were also studies in which no association was found.[36,37] The only prospective study of the effect of microvessel density by Vermeulen et al.[38] showed that high intratumoural microvessel density was significantly associated with shorter survival and haematogenous metastasis.

Controversy concerning the role of microvessel count may arise from different methodologies utilised in assessing microvessel counts.[39] We defined microvessel count as the total perimeter of all vessels in one microscopic field. Although we did not use Chalkley counting,[40] a method which suggests the validity for comparing angiogenesis for prognostic

purposes between different centres, we think that we followed most guidelines of the proposed standard method for intratumoural microvessel density as described by Vermeulen et al.[41] In addition, the most observer-dependent step still remains with Chalkley counting; i.e. the selection of vascular hot spots. This was as much as possible ruled out by defining the hot spots by two observers (EK and JHJMvK) for all tumours in our study. Furthermore, our microvessel counts of 20 samples were reanalysed and confirmed by BEvdW, who used a different image analysis system, indicating that our results are reliable. Our findings and that of Lindmark et al. however, do need further investigation. We have already started further investigations to analyse microvessel counts at the invasive front of rectal tumours.[42]

In conclusion, we demonstrate that molecules involved in adhesion and angiogenesis provide prognostic information in a series of rectal cancer patients with well documented, prospectively collected data from a randomised trial in which treatment was standardised. Loss of EpCAM expression was associated with increased local recurrence risk and low microvessel count with increased distant recurrence risk. Examining multiple mechanisms in colorectal oncogenesis is a useful approach to dissect the complexity of genetic alterations thereby uncovering the role, timing and prognostic value of such alterations. This provides a better understanding of colorectal tumour behaviour and may contribute to improved therapy.

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11

Summary and conclusive remarks

SUMMARY

The general introduction in **Chapter 1** presents clinical aspects and molecular backgrounds of (colo)rectal cancer. In this thesis, we have focused on both of these aspects since understanding of the molecular background of rectal cancer can provide useful information for the determination of clinical strategies. The results are mainly obtained from a trial performed by the Dutch ColoRectal Cancer Group "Total mesorectal excision with or without preoperative radiotherapy in the treatment of primary rectal cancer" (TME-trial).

PART I: ADVANCES IN TREATMENT

Local recurrences (LR) have been a major problem in the treatment of rectal cancer. A high incidence of local recurrence (15-45%) is associated with conventional, non-standardised procedures, which consists of blunt dissection of the rectal fascia and often results in incomplete removal of mesorectal tissue. **Chapter 2** describes a population-based study of local recurrence rates in curatively resected patients with rectal cancer, diagnosed between 1988 and 1992, in the west Netherlands. The first objective was to make an inventory of the overall local recurrence rate after non-standardised conventional surgery, inter-institutional local recurrence rate variability, and correlations between patient- and tumour-related factors and local recurrence rate. A second objective was to investigate the compliance to guidelines for postoperative radiotherapy. The overall local recurrence rate was 22.5% with a range of 9-36% between the 12 hospitals. These differences were not significant. Dukes' Astler-Coller stage, tumour location and residual tumour were significant independent prognostic factors for the risk of local recurrence. Indications for postoperative radiotherapy were Dukes' Astler-Coller B2 and C tumours, positive surgical margins and tumour spill, but compliance to these guidelines was only 50%. However, no significant difference in local recurrence rate was found between patients treated according to the guidelines and those not treated according to the guidelines. In conclusion, this study shows a high local recurrence rate with conventional surgery and variability in local recurrence rate between the participating hospitals. Furthermore, it confirms that the risk of local recurrence in primary rectal cancer is dependent on Dukes' Astler-Coller stage, tumour location and residual tumour. Lastly, this study contributes to the discussion about the feasibility of guidelines for postoperative radiotherapy.

To improve results of surgery, various additional treatments, such as radiotherapy, chemotherapy and immunotherapy, have been applied. The Swedish Rectal Cancer Trial (SRCT) was the first trial to show that better local control, achieved with preoperative radiotherapy, resulted in improved survival. A major problem of published studies on adjuvant therapy however, is that surgery has not been standardised. Furthermore, quality control of the surgical technique by standardised pathological examination of the specimen is absent in most studies. In Europe, TME has become the preferred standard of operative management for rectal cancer. Adjuvant therapies should now be re-examined based upon a platform of standardised, optimal surgery and pathology. In **Chapter 3** we studied the current European trials in which TME-surgery is intentionally performed. Most of these trials are still in progress or have too short follow-up, so definitive results, apart from interim-analyses, are not known yet. The TME-trial however, has already shown that performing a large, multicentre trial with quality control of both surgery and pathology is feasible.

Reports on improved local control after short-term 5x5 Gy preoperative radiotherapy

and TME-surgery have led to the conduction of the TME-trial, in which the effect of TME surgery with or without short-term preoperative radiotherapy was evaluated. However, any benefit regarding a reduced local recurrence rate and possible improved survival must be weighed against potential adverse effects. The study in **Chapter 4** was undertaken to assess the acute side effects of short-term, preoperative radiotherapy in rectal cancer patients undergoing TME and to study the influence of 5x5 Gy on surgical parameters, postoperative morbidity and mortality. We analysed 1530 Dutch patients entered in the TME-trial of which 1414 were evaluable. Toxicity during radiotherapy hardly occurred. Irradiated patients had 100 ml more blood loss during the operation ($P<0.001$) and showed more perineal complications ($P=0.008$) in case of an abdominoperineal resection. The total number of complications was slightly increased in the irradiated group ($P=0.008$). No difference was observed in postoperative mortality (4.0% vs. 3.3%) or in the number of reinterventions. In conclusion, preoperative hypofractionated RT is a safe procedure in patients treated with TME surgery, despite a slight increase in complications when compared to TME surgery only.

Local control and survival of rectal cancer have been improved by the introduction of the TME-technique. In addition to the surgical technique, hospital volume and specialisation can be important prognostic factors. In **Chapter 5** the effect of training in TME-surgery was assessed on short- and long-term outcomes in rectal cancer in the TME-trial and outcomes were compared with results from a former randomised trial (Cancer Recurrence And Blood transfusion (CRAB) trial), in which conventional surgery was performed without quality control. We analysed the eligible, preoperatively non-irradiated, curatively operated patients. The influence of hospital volume was investigated in both trials, while the role of hospital specialisation was analysed only in the TME-trial. We corrected for differences in clinicopathological characteristics by means of multivariate analyses and to ensure valid comparisons, only events occurring within 2 years of surgery were analysed for long-term outcomes. Hospital volume was analysed as a continuous factor. Local recurrence rate decreased from 16.3% in the CRAB-trial to 8.6% in the TME-trial, and type of surgery (conventional (CRAB-trial) vs. TME (TME-trial)) was an independent predictor for local recurrence ($P=0.002$). Type of surgery was also an independent predictor for overall survival ($P=0.019$) with a higher survival rate in the TME-trial. Higher hospital volume was significantly associated with lower distant recurrence ($P=0.006$) and higher overall survival ($P=0.011$) in the CRAB-trial. However, in the TME-trial hospital volume and specialisation were not of significant value for short- and long-term outcomes. In conclusion, training of surgeons with TME-surgery, leads to improved long-term outcome of rectal cancer patients without volume- or specialisation-related differences.

In **Chapter 6**, the outcome of the main objective of the TME-trial is reported: is short-term preoperative radiotherapy still beneficial in rectal cancer patients undergoing TME? The combination of these treatment modalities was never investigated. Between January 1996 and December 2000, 1861 Dutch and foreign patients with resectable rectal cancer were randomly assigned to preoperative radiotherapy of 5x5 Gy followed by TME or to TME alone. Of the 1861 randomised patients, 1805 were eligible. The 2-year overall survival rate for the 1805 eligible patients was 82.0% in the RT+TME group and 81.8% in the TME group ($P=0.84$). For the 1748 patients who underwent a macroscopically local complete resection, 2-year local recurrence rate was 5.3%. The 2-year local recurrence

rates were 2.4% in the RT+TME group and 8.2% in the TME group (P<0.0001). In conclusion, in a setting of standardised TME-surgery, short-term preoperative radiotherapy still has a beneficial effect on local recurrence risk.

PART II: NEW INSIGHTS IN MOLECULAR BIOLOGY

Observations from other studies support the theory that development of left- and right-sided colorectal cancers may involve different mechanisms. In this study, different genes involved in tumourigenesis of colon vs. rectal cancers, were investigated, and their prognostic value was analysed. **Chapter 7** compares a series of colon cancers with standardised treated rectal cancers obtained from the pilot-study of the TME-trial, with regard to different genes involved in tumourigenesis of colorectal cancer. Mutation and expression profiles were investigated and related to tumour site and prognosis. *APC* mutation analysis of the mutation cluster region showed truncating mutations in 18 of 22 rectal tumours (82%), but presence of an *APC* mutation was not related to nuclear β-catenin expression (P=0.75). Rectal cancers showed significant more nuclear β-catenin than colon cancers (65% vs. 40%, P=0.04). *p53* mutation analysis corresponded well with p53 immunohistochemistry (P<0.001) and with this, rectal cancers showed significant more p53 expression than colon cancers (64% vs. 29%, P=0.003). In rectal cancers a significant correlation was found between positive p53 expression and worse disease-free survival (P=0.008), but not in colon cancers. Cox regression showed that p53-expression (P=0.03) was an independent predictor for disease-free survival in rectal cancers. This study shows that rectal cancers may involve more nuclear β-catenin in the APC/β-catenin pathway than colon cancer and/or nuclear β-catenin may have another role in rectal cancer independent of APC. The p53-pathway seems to be more important in rectal cancer, in which p53 expression also has independent prognostic value. When prognostic markers are investigated in larger series, differences in biological behaviour between colon and rectal cancer should be considered.

In **Chapter 8** we investigated molecular profiles of sporadic rectal cancers using 12 microsatellite markers and DNA ploidy analysis in order to classify tumours in terms of genetic instability. Screening of 81 rectal cancers revealed one tumour with high frequency of microsatellite instability (MSI). The majority of tumours (74%) showed loss of heterozygosity (LOH) for at least one marker. Most of the LOH-positive tumours (81%) and 47% of the LOH-negative tumours were aneuploid. The data indicate that chromosomal instability (CIN) rather than microsatellite instability (MSI) plays a role in rectal cancers. We found a subset of rectal tumours without hallmarks of gross genetic instability (n=13). Five of these diploid, MSI-stable tumours, of which 4 did not show LOH, were further characterised for *p53* mutation status and expression of 1700 cancer-related genes, and compared to two aneuploid tumours. Clustering of gene expression profiles revealed that the *p53* mutant diploid tumours seemed more similar to the *p53* wild type aneuploid tumours than to the *p53* wild type diploid tumours. Within the diploid tumour subset, differential gene expression patterns related to *p53* mutation status were found. The expression analysis also revealed a lack of mRNA expression of *hMSH2* and *hMSH3* in a diploid tumour originating from a 29-year-old patient. In addition, hMSH2 and hMSH6 were lost at the protein level. No mutation was detected in *hMSH2* and *hMSH6*. Since this tumour was MSI-stable, the loss of expression of these mismatch repair genes may be a late event. In conclusion, as others we identified a group of rectal tumours without evidence of gross genetic instability

by molecular analysis with microsatellite markers and flow cytometry. We show that tumour heterogeneity in this class of tumours can be defined by molecular characteristics, such as *p53* mutation status and differential expression profiles.

In **Chapter 9**, the influence of radiotherapy on the expression of p53 and $p21^{waf1}$ was investigated in normal mucosa and rectal carcinomas in patients from the TME-trial. In vitro, ionising radiation of epithelial cells leads to upregulation of wild type *p53* and subsequent induction of $p21^{waf1}$. The effect of radiotherapy on the expression of these proteins in patients is unknown. p53 and $p21^{waf1}$ expression was determined in 51 irradiated and 52 non-irradiated patients using immunohistochemistry. In normal mucosa, both p53 and $p21^{waf1}$ were strongly upregulated after radiotherapy, compared with the expression in unirradiated normal tissue ($P<0.001$). In tumour cells, no significant difference in the expression of p53 or $p21^{waf1}$ was found in the irradiated vs. the non-irradiated group. In the few rectal tumours with wt *p53*, induction of p53 after radiotherapy did not necessarily lead to upregulation of $p21^{waf1}$. These findings demonstrate that in normal mucosa a functional p53-$p21^{waf1}$ pathway is present, whereas in tumour cells it is defective in almost all cases due to either *p53* mutation or down- or upstream disruption in tumours with wild type *p53*. Therefore, we believe that the role of p53 expression as a single prognostic marker in rectal cancer needs reconsideration.

In the process of invasion and metastasis, cell adhesion and angiogenesis are important. In **Chapter 10** we investigated 97 rectal tumours from the TME-trial to analyse the influence of irradiation on the expression of cell adhesion molecules and microvessel count, and to examine the prognostic value of these factors. Immunohistochemical expression of E-cadherin, α-, β-, γ-catenin, EpCAM and CD31 were investigated in patients who had undergone surgery with or without preoperative radiotherapy. Irradiated tumours showed more nuclear β-catenin expression ($P=0.004$) and a lower microvessel count ($P=0.03$). No other differences were found between irradiated and non-irradiated tumours. Loss of EpCAM expression was significantly associated with local recurrence ($P=0.015$) for the total group of tumours. Low microvessel count was associated with an increased distant recurrence risk ($P=0.04$) and lower overall survival ($P=0.02$). The overall results of this study show that loss of EpCAM expression is associated with increased local recurrence risk and low microvessel count with increased distant recurrence risk in rectal cancer. Furthermore, irradiation has an influence on nuclear β-catenin expression and microvessel count.

CONCLUSIVE REMARKS

In the last decades, major advances have been made in the treatment of rectal cancer by the introduction of new surgical techniques and additional technical improvements (e.g. staplers). During the last years, quality assurance of surgery has become an important topic in rectal cancer treatment. Quality assurance is of major importance for standardisation of treatment in (neo)adjuvant therapy studies and for improvement of outcomes.

The introduction of TME-surgery has led to a major reduction in local recurrence rates and improved survival. We showed that short-term preoperative radiotherapy gives a further reduction in local recurrence rate when standardised TME-surgery is used. TME-based operations are now established as the standard of care for rectal cancer, and should form the basis for trials concerning the role of (neo)adjuvant therapy.

In general, it is thought that high volume and specialist care produces superior results to

low volume and non-specialist care, especially for those less frequent forms of cancer and in technically difficult operations, like those for rectal cancer. However, limiting the performance of rectal cancer surgery to surgeons who work in specialised centres or to only those general surgeons who perform more than a certain volume is impractical in view of the prevalence of rectal cancer. The concentration process can also take place within one hospital surgical unit with 1-3 surgeons performing rectal cancer surgery. This has been demonstrated in the TME-trial, in which training in TME-surgery to surgeons who are dedicated to oncology, has led to improved outcome without volume- or specialisation-related differences.

Quality assurance of the surgical technique requires besides training, adequate knowledge of the anatomy of the organs and nerves in the pelvis and other related structures. Furthermore, standardisation in the description of operations and reporting of pathology specimens should be implemented as important features of quality control. In addition, a multidisciplinary approach provides the best care for patients, since the access and use of standardised and up-to-date therapy is better organised. Similarly, patients participating in clinical trials generally experience a survival advantage over non-participating patients, which is probably due to standardised treatment.

Within the TME-trial structuralisation and audit of rectal cancer treatment has led to improvement of treatment results and this infrastructure provides optimal conditions for conducting future rectal cancer trials. The successor trial of the TME-trial, the Preoperative Radiotherapy and/Or adjuvant Chemotherapy combined with Tme surgery in Operable Rectal cancer (PROCTOR)-trial, is currently investigating the role of postoperative chemotherapy in TME-treated patients. However, it is of utmost importance that outside the setting of trials, standardisation of treatment is also applied and sustained. Population-based cancer registries, covering an increasing proportion of the world's population, are an invaluable source of data for this goal.

In addition to clinical improvements, the molecular biology of colorectal cancer will be unravelled even more in the coming years. New techniques in cancer research comprise genome-wide analysis techniques such as chromosome painting, comparative genomic hybridisation, high-throughput analysis of LOH, serial analysis of gene expression (SAGE) and expression microarray analysis. These techniques are now accelerating the high resolution of aberrations in human tumours. By these new techniques identification of affected genes, elucidation of their functions and associations of these genes with tumour progression will be disentangled by which the tumourigenesis of colorectal cancer will be more fully understood. Furthermore, these techniques can help to predict sensitivity or resistance of individual patients to adjuvant therapy. Hereby, individual patients can be offered their own most "suitable" therapy. This "tailor-made" therapy will emerge most likely in the next decade for several diseases.

In the TME-trial, the criteria for analysis of individual risk factors as stated by R.A.E.M. Tollenaar in his thesis were completely met.[1] The criteria of uniform collection of clinical findings according to strictly defined criteria, detailed documentation and standardisation of therapeutic procedures, uniform collection of macroscopic and histological tumour characteristics, standardised documentation of the course of the disease and lastly, evaluation of the data using multivariate statistical methods, were all fulfilled. The TME-trial with its unique and thorough setup, still offers a challenge to future investigators and will certainly

provide more answers to questions concerning the molecular biology, prognostic factors and the mechanisms of radiation-induced damage in tumour cells. In addition, more clinical outcomes of the TME-trial will be known in the coming years, such as the long-term side-effects of preoperative radiotherapy and the influence of irradiation on overall survival. However, the most important objective of this trial has already been achieved; improvement of the treatment for rectal cancer patients with much lower local recurrence rates as compared to a decade ago when conventional surgical techniques were applied. This thesis has dealt with clinical and molecular aspects of rectal cancer and shows that by investigating the combination of these aspects in a large randomised multicentre trial, advances in treatment and new insights in molecular biology have been obtained.

REFERENCES

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Samenvatting en afsluitende opmerkingen
Lijst van deelnemers TME-trial
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SAMENVATTING

De algemene introductie in **Hoofdstuk 1** geeft een overzicht van de klinische en moleculaire aspecten van (colo)rectale tumoren. In dit proefschrift is de aandacht gericht op beide aspecten, omdat het onderzoeken van de moleculaire achtergrond van rectumkanker nuttige informatie kan verschaffen voor het bepalen van klinische strategieën. De resultaten in dit proefschrift zijn met name gebaseerd op de data van een grote gerandomiseerde studie, uitgevoerd door de Dutch ColoRectal Cancer Group: "Totale Mesorectale Excisie met of zonder preoperatieve radiotherapie in de behandeling van het primair rectumcarcinoom" (TME-studie).

DEEL I: VOORUITGANG IN DE BEHANDELING

Lokale recidieven zijn een groot probleem in de behandeling van het rectumcarcinoom omdat ze ernstig invaliderende symptomen veroorzaken en moeilijk te behandelen zijn. Conventionele, niet-gestandaardiseerde chirurgische procedures zijn geassocieerd met een hoge incidentie van lokaal recidieven (15-45%). Deze technieken bestaan uit stompe dissectie van de rectale fascie met vaak een incomplete resectie van de tumor en het achterlaten van mogelijk tumordragend weefsel. **Hoofdstuk 2** beschrijft een populatie-studie in de IKW-regio van lokaal recidiefpercentages in curatief geopereerde patiënten met een rectumcarcinoom, gediagnosticeerd tussen 1988 en 1992. Het eerste doel van deze studie was het inventariseren van het lokaal recidiefpercentage na niet-gestandaardiseerde conventionele chirurgie. Tevens werd de variatie in interinstitutionele recidiefpercentages en correlaties tussen patiënt- en tumorgerelateerde factoren en lokaal recidiefpercentage bestudeerd. Een tweede doel was te onderzoeken wat de therapietrouw was met betrekking tot de richtlijnen voor postoperatieve radiotherapie. Het totale lokaal recidiefpercentage was 22.5% met een variatie van 9-36% tussen de 12 ziekenhuizen. De verschillen tussen de ziekenhuizen waren statistisch niet significant. Dukes' Astler-Coller stadium, tumorlocatie en de aanwezigheid van tumorresidu waren significante onafhankelijke prognostische factoren voor het risico op een lokaal recidief. De indicaties voor postoperatieve radiotherapie waren Dukes' Astler-Coller B2 en C tumoren, positieve chirurgische marges en tumor-"spill" tijdens de operatie. Deze richtlijnen werden maar in 50% van de patiënten opgevolgd. Opvallend genoeg werd er geen verschil in lokaal recidiefpercentage gevonden tussen patiënten die volgens de richtlijnen waren behandeld en patiënten die niet volgens de richtlijnen waren behandeld. Samenvattend toont deze studie een hoog lokaal recidiefpercentage met conventionele chirurgie en variabiliteit in het lokaal recidiefpercentage tussen de participerende ziekenhuizen. Verder wordt in deze studie bevestigd dat het risico op een lokaal recidief bij het primaire rectumcarcinoom afhankelijk is van Dukes' Astler-Coller stadium, tumorlocatie en de aanwezigheid van een tumorresidu. Tenslotte zet deze studie vraagtekens bij het opvolgen van richtlijnen voor postoperatieve radiotherapie.

Om de resultaten van chirurgie voor rectumkanker te verbeteren zijn verschillende adjuvante therapieën toegepast, zoals radiotherapie, chemotherapie en immunotherapie. De Swedish Rectal Cancer Trial (SRCT) was de eerste studie die liet zien dat een verbeterde lokale controle, als gevolg van preoperatieve radiotherapie, resulteerde in een verbeterde overleving. Een groot probleem van de tot nu toe uitgevoerde adjuvante therapie studies is echter dat chirurgie niet gestandaardiseerd werd uitgevoerd. Verder is kwaliteitscontrole van de chirurgische techniek door middel van een gestandaardiseerd pathologisch onder-

zoek van het resectiepreparaat in de meeste studies niet verricht. In Europa is de TME-techniek de standaard geworden voor de operatieve behandeling van het rectumcarcinoom. Adjuvante therapie studies moeten nu herhaald worden tegen een achtergrond van gestandaardiseerde chirurgie en pathologie. In **Hoofdstuk 3** hebben we de Europese studies, waarin de intentie bestond om TME-chirurgie uit te voeren, onderzocht. De meeste van deze studies zijn nog steeds in de inclusiefase of hebben een te korte follow-up, waardoor definitieve resultaten, uitgezonderd resultaten van interim-analyses, nog niet bekend zijn. De TME-studie heeft echter al laten zien dat het uitvoeren van een grote, multicenter studie met kwaliteitscontrole van zowel chirurgie als pathologie, mogelijk is.

Publicaties over verbeterde lokale controle na kortdurende 5x5 Gy preoperatieve radiotherapie en TME-chirurgie hebben geleid tot het opzetten van de TME-studie. In deze trial werd het effect van TME-chirurgie met of zonder kortdurende preoperatieve radiotherapie geëvalueerd. Belangrijk bij het onderzoeken van dit effect is dat enig voordeel, dat wordt bereikt met betrekking tot een reductie in lokaal recidiefpercentage en een mogelijke verbetering in overleving, moet worden afgewogen tegen potentiële bijwerkingen. In **Hoofdstuk 4** werden de acute bijwerkingen van kortdurende 5x5 Gy preoperatieve radiotherapie geëvalueerd in rectumcarcinoom patiënten die een TME ondergingen. Tevens werd de invloed van 5x5 Gy bestudeerd op chirurgische parameters, postoperatieve morbiditeit en mortaliteit. We analyseerden 1530 Nederlandse patiënten uit de TME-studie; hiervan waren 1414 patiënten evalueerbaar. Het optreden van toxiciteit tijdens het toedienen van de radiotherapie vond maar zelden plaats. Bestraalde patiënten hadden 100 ml meer bloedverlies tijdens de operatie ($P<0.001$) en toonden meer perineale complicaties ($P=0.008$) als ze een abdominoperineale resectie hadden ondergaan. Het totaal aantal complicaties was verhoogd in de bestraalde groep ($P=0.008$). Er werd geen verschil gevonden in postoperatieve mortaliteit (4.0% vs. 3.3%) en in het aantal reïnterventies. Preoperatieve, gehypofractioneerde radiotherapie kan veilig gegeven worden bij patiënten die TME-chirurgie ondergaan, ondanks een iets hoger complicatie percentage in bestraalde patiënten.

Lokale controle en overleving in rectumcarcinoom patiënten zijn verbeterd door de introductie van de TME-techniek. Naast de chirurgische techniek kunnen ziekenhuisvolume- en specialisatie ook belangrijke prognostische factoren zijn. In **Hoofdstuk 5** werd het effect van training in TME-chirurgie bestudeerd op korte en lange termijn uitkomsten in rectumcarcinoom patiënten uit de TME-studie. De uitkomsten werden vergeleken met resultaten van een eerdere gerandomiseerde studie (Cancer Recurrence And Blood transfusion (CRAB)-studie), waarin patiënten conventionele chirurgie ondergingen zonder kwaliteitscontrole. De invloed van het ziekenhuisvolume werd in beide studies onderzocht, terwijl de rol van ziekenhuis specialisatie alleen werd onderzocht in de TME-studie. We analyseerden uit beide studies de patiënten die aan de inclusiecriteria voldeden, die niet voorbestraald waren en die een in opzet curatieve operatie hadden ondergaan. Er werd gecorrigeerd voor verschillen in clinicopathologische karakteristieken door middel van multivariate analyses. Voor de lange termijn uitkomsten werden alleen waarnemingen binnen 2 jaar na chirurgie geanalyseerd om een betrouwbare vergelijking te waarborgen. Het lokaal recidiefpercentage daalde van 16.3% in de CRAB-studie naar 8.6% in de TME-studie, waarbij het type chirurgie (conventioneel (CRAB-studie) vs. TME (TME-studie)) een onafhankelijke voorspeller was voor het optreden van een lokaal recidief ($P=0.002$). Het type chirurgie was ook een onafhankelijke voorspeller voor de totale overleving ($P=0.019$) met een langere

overleving in de TME-studie. In de CRAB-studie was een groter ziekenhuisvolume significant geassocieerd met een lager afstandsrecidief risico (P=0.006) en een langere totale overleving (P=0.011). In de TME-studie waren ziekenhuisvolume- en specialisatie niet van significante voorspellende waarde voor korte en/of lange termijn uitkomsten. Uit deze studie kan geconcludeerd worden dat het trainen van chirurgen in de TME-techniek heeft geleid tot verbeterde lange termijn uitkomsten van rectumcarcinoom patiënten zonder volume- of specialisatiegerelateerde verschillen.

In **Hoofdstuk 6** wordt een van de belangrijkste uitkomsten van de TME-studie beschreven. Van zowel kortdurende preoperatieve radiotherapie van 5x5 Gy als TME was aangetoond dat ze de lokale controle bij het resectabel rectumcarcinoom verbeteren. De combinatie van deze behandelingsmodaliteiten was echter nooit eerder onderzocht. In de TME-studie werden tussen januari 1996 en december 1999 in totaal 1861 Nederlandse en buitenlandse patiënten met een resectabel rectumcarcinoom gerandomiseerd tussen preoperatieve radiotherapie van 5x5 Gy gevolgd door TME (n=924) of TME alleen (n=937). In de TME-studie werden standaardisatie en kwaliteitscontrole van radiotherapie, chirurgie en pathologie doorgevoerd. Van de 1861 gerandomiseerde patiënten voldeden er 1805 aan de inclusiecriteria. De 2-jaars totale overleving voor de 1805 patiënten die aan de inclusiecriteria voldeden, bedroeg 82.0% in de radiotherapie groep en 81.8% in de TME alleen groep (P=0.84). Voor de 1748 patiënten die een macroscopisch lokale complete resectie hadden ondergaan, bedroeg het 2-jaars lokaal recidiefpercentage 5.3%. De 2-jaars lokaal recidiefpercentages waren 2.4% in de radiotherapie groep en 8.2% in de TME alleen groep (P<0.001). De introductie van de TME-techniek in een grote multicenter studie heeft geleid tot een substantiële daling in het lokaal recidiefpercentage. In combinatie met gestandaardiseerde chirurgie heeft kortdurende preoperatieve radiotherapie nog steeds een gunstig effect op het lokaal recidief risico.

DEEL II: NIEUWE INZICHTEN IN MOLECULAIRE BIOLOGIE

Verscheidene studies hebben aangetoond dat bij de ontwikkeling van links- en rechtszijdige colorectale tumoren verschillende mechanismen betrokken zijn. In **hoofdstuk 7** werd een serie colontumoren vergeleken met gestandaardiseerd behandelde rectumtumoren uit een “pilot”-studie, die vooraf ging aan de TME-studie. De mutatie- en expressieprofielen van verschillende genen werden onderzocht en gerelateerd aan de tumorlocatie en prognose. *APC* mutatie analyse van de mutatie cluster regio liet truncerende mutaties in 18 van de 22 rectumtumoren (82%) zien. Het optreden van een *APC* mutatie was niet gerelateerd aan het voorkomen van nucleair β-catenine expressie (P=0.75). Rectumtumoren lieten significant vaker nucleair β-catenine zien dan colontumoren (65% vs. 40%, P=0.04). p53 immunohistochemie kwam goed overeen met *p53* mutatie analyse (P<0.001) en was significant vaker positief in rectumtumoren dan in colontumoren (64% vs. 29%, P=0.003). In de rectumgroep werd een significante associatie gevonden tussen positieve p53 expressie en een verminderde ziekte-vrije overleving (P=0.008), maar niet in de colongroep. p53-expressie was een onafhankelijke voorspeller voor ziekte-vrije overleving in de rectumgroep in de multivariate analyse (Cox regressie model, P=0.03). Concluderend blijkt uit deze studie dat bij rectumtumoren mogelijk meer nucleair β-catenine in de APC/β-catenine route is betrokken dan in colontumoren. Nucleair β-catenine heeft mogelijk ook een andere rol in

rectumkanker onafhankelijk van APC. De p53-pathway lijkt een grotere rol te spelen in rectumtumoren, waarbij p53 expressie ook een onafhankelijke prognostische waarde heeft. Als prognostische markers worden onderzocht in grote patiëntenseries, moet men rekening houden met verschillen in biologisch gedrag tussen colon- en rectumtumoren.

Hoofdstuk 8 beschrijft een studie waarin we moleculaire profielen van rectumtumoren uit de TME-studie hebben onderzocht met behulp van microsatelliet analyse, flow cytometrie, immunohistochemische, *p53* mutatie en genexpressie analyses. Van de 81 rectumtumoren was er slechts één tumor die een hoge frequentie van microsatelliet instabiliteit liet zien (MSI-high, 1.2%). De meeste tumoren lieten verlies van heterozygositeit (LOH) zien van tenminste één marker (74%). Flow-cytometrie toonde dat de meeste LOH-positieve tumoren (81%) en 47% van de LOH-negatieve tumoren aneuploid waren. Deze data indiceren dat chromosomale instabiliteit (CIN) belangrijker is in rectumcarcinomen dan microsatelliet instabiliteit (MSI). We identificeerden een groep tumoren zonder tekenen van genetische instabiliteit (n=13). Vijf van deze diploïde, MSI-negatieve tumoren, waarvan er 4 geen LOH toonden, werden verder gekarakteriseerd voor *p53* mutatie status en expressie van 1700 kankergerelateerde genen, en vergeleken met twee aneuploïde tumoren. Binnen de diploïde tumor groep werden differentiële genexpressie patronen gevonden die gerelateerd waren aan *p53* mutatie status. De genexpressie analyse toonde tevens een gebrek aan mRNA expressie van *hMSH2* en *hMSH3* in de diploïde tumor van een 29-jarige patiënt. Bovendien werd in deze tumor verlies van hMSH2 en hMSH6 op eiwitniveau gezien. Er werden geen mutaties gevonden in *hMSH2* en *hMSH6.* Daar deze tumor MSI-stabiel was, is het verlies van expressie van de mismatch repair genen mogelijk secundair opgetreden. Resumerend, hebben wij met behulp van een moleculaire analyse met microsatelliet markers en flow cytometrie, net als andere onderzoekers, een groep rectumtumoren geïdentificeerd zonder aanwijzingen voor genetische instabiliteit. We tonen dat heterogeniteit in deze tumoren gedefinieerd kan worden op basis van moleculaire karakteristieken, zoals *p53* mutatie status en differentiële expressie profielen.

In **Hoofdstuk 9** werd de invloed van bestraling op de expressie van p53 en $p21^{waf1}$ onderzocht in normale mucosa en tumorweefsel van patiënten uit de TME-studie. In vitro is aangetoond dat ioniserende bestraling van epitheliale cellen tot opregulatie leidt van wild type (wt) *p53* en daardoor tot de inductie van $p21^{waf1}$. Het effect van radiotherapie op de expressie van deze eiwitten was niet eerder onderzocht in tumoren van patiënten (in vivo). p53 en $p21^{waf1}$ expressie werd in 51 bestraalde en 52 niet-bestraalde patiënten onderzocht met behulp van immunohistochemie. Zowel p53 als $p21^{waf1}$ waren sterk opgereguleerd in bestraalde normale mucosa vergeleken met de expressie in niet-bestraald normaal weefsel ($P<0.001$). In tumorcellen werden geen significante verschillen gevonden in de expressie van p53 en $p21^{waf1}$ tussen bestraalde en niet-bestraalde tumoren. In het lage aantal rectumtumoren met wt *p53* leidde de inductie van p53 na bestraling niet noodzakelijk tot opregulatie van $p21^{waf1}$. De resultaten in deze studie laten zien dat in *normale* mucosa een functionele p53-$p21^{waf1}$ route aanwezig is, terwijl in *tumor* cellen deze route defect is in bijna alle patiënten ten gevolge van hetzij een *p53* mutatie, hetzij een verstoring “downstream” van p53 in tumoren met wild type *p53*. Op basis van deze resultaten concluderen wij dat de rol van p53 expressie als enkelvoudige prognostische marker in rectumtumoren heroverwogen moet worden.

Bij het proces van invasie en metastasering zijn de verstoring van celadhesie en het

optreden van vaatnieuwvorming belangrijk. In **Hoofdstuk 10** onderzochten we 97 rectumtumoren om de invloed van bestraling op de expressie van celadhesie moleculen en de mate van microvascularisatie te analyseren. Tevens werd de prognostische waarde van deze factoren bestudeerd. De immunohistochemische expressie van E-cadherine, α-, β-, γ-catenine, EpCAM en CD31 werd onderzocht in patiënten die TME-chirurgie hadden ondergaan met of zonder preoperatieve radiotherapie. In bestraalde tumoren werd meer nucleair β-catenine (P=0.004) en een lagere mate van microvascularisatie (P=0.03) gezien dan in niet-bestraalde tumoren. Er werden geen andere verschillen gevonden tussen bestraalde en niet-bestraalde tumoren. Verlies van EpCAM expressie was significant geassocieerd met het optreden van een lokaal recidief (P=0.015) voor de hele groep tumoren. Verder was een lagere mate van microvascularisatie geassocieerd met een verhoogd risico op afstandsrecidieven (P=0.04) en een lagere overleving (P=0.02). Concluderend laten de resultaten van de bestraalde en niet-bestraalde patiënten samen zien dat verlies van EpCAM expressie geassocieerd is met een grote kans op een lokaal recidief en dat een lagere mate van microvascularisatie voorspellend is voor het optreden van afstandsrecidieven in rectumkanker. Verder vonden we dat bestraling invloed heeft op de expressie van nucleair β-catenine en de mate van microvascularisatie.

AFSLUITENDE OPMERKINGEN

In de afgelopen jaren is er een grote vooruitgang geboekt in de behandeling van het rectumcarcinoom door de introductie van nieuwe chirurgische methodes. Kwaliteitscontrole van chirurgie is een belangrijk onderwerp geworden in de behandeling van rectumtumoren. Deze kwaliteitscontrole is van groot belang voor de standaardisatie van de behandeling in (neo)adjuvante therapie studies en voor het verbeteren van de resultaten.

De introductie van de TME-techniek heeft geleid tot een grote reductie in het lokaal recidiefpercentage en een verbeterde overleving. De TME-studie toonde dat kortdurende preoperatieve radiotherapie een verdere reductie geeft in het lokaal recidiefpercentage bij gestandaardiseerde TME-chirurgie. De TME-techniek wordt momenteel als de standaard van zorg gezien voor het rectumcarcinoom en zou ook de standaard moeten zijn in studies die de rol van (neo)adjuvante therapie onderzoeken.

Over het algemeen wordt gedacht dat een hoog volume aan procedures en gespecialiseerde zorg tot betere resultaten leiden dan een laag volume en niet-gespecialiseerde zorg, met name wat betreft minder frequent voorkomende kankersoorten en technisch moeilijk uit te voeren operaties, zoals die voor het rectumcarcinoom. Het beperken van chirurgie voor rectumkanker tot gespecialiseerde chirurgen in een beperkt aantal centra of tot algemeen chirurgen die een bepaald volume aan procedures halen, is echter niet haalbaar met het oog op de hoge prevalentie van rectumkanker. Het concentratieproces kan ook binnen een ziekenhuis plaatsvinden waarbij 1-3 chirurgen rectumchirurgie uitvoeren. Goede resultaten met deze opzet zijn bereikt in de TME-studie, waarin training in de TME-techniek aan chirurgen die geïnteresseerd zijn in de oncologie, heeft geleid tot verbeterde uitkomsten zonder volume- of specialisatiegerelateerde verschillen.

Naast training is voor de kwaliteitsverbetering van de chirurgische techniek ook adequate kennis van de anatomie van organen en zenuwen in het bekken vereist. Verder moet standaardisatie in de beschrijving van operaties en pathologische beoordeling van het preparaat geïmplementeerd worden als belangrijke onderdelen van de kwaliteitscontrole van de

behandeling. Tevens levert een multidisciplinaire benadering de beste zorg op voor patiënten doordat de toegang en het gebruik van gestandaardiseerde en "up-to-date" therapie beter georganiseerd is. Ten slotte tonen patiënten die deelnemen aan klinische trials over het algemeen een betere overleving in vergelijking met patiënten die buiten studieverband behandeld worden, hetgeen waarschijnlijk het gevolg is van de gestandaardiseerde behandeling in trials.

Binnen de TME-studie heeft de structurering en toetsing van de behandeling van het rectumcarcinoom geleid tot verbeterde uitkomsten. Deze infrastructuur verschaft optimale condities voor het uitvoeren van toekomstige studies. De opvolger van de TME-studie, de Preoperatieve Radiotherapie en/Of adjuvante Chemotherapie gecombineerd met Tme chirurgie in Operabele Rectumkanker (PROCTOR)-studie, onderzoekt momenteel de rol van postoperatieve chemotherapie in TME-behandelde patiënten. Het is echter van groot belang dat ook buiten het verband van klinische trials, de standaardisatie van behandeling wordt doorgevoerd. Kankerregistraties vormen een bron van grote waarde om dit te bereiken.

Naast de klinische verbeteringen, zal in de komende jaren de moleculaire biologie van colorectale tumoren nog meer ontrafeld worden. Nieuwe technieken in het kankeronderzoek bestaan uit analysetechnieken van het totale genoom, zoals chromosoom "painting", comparatieve genomische hybridisatie, seriële analyse van genexpressie (SAGE) en "microarray" genexpressie analyse. Deze technieken versnellen momenteel het opsporen van genetische afwijkingen in humane tumoren. Door deze nieuwe technieken zal ook de identificatie van aangedane genen, alsmede de functie en associaties van deze genen met tumorprogressie, nog meer ontward worden waardoor de tumorgenese beter begrepen wordt. Verder kunnen deze technieken helpen bij het voorspellen of individuele patiënten mogelijk sensitief of resistent zijn voor adjuvante therapieën. Hierdoor kan aan individuele patiënten een eigen, passende therapie worden aangeboden. Deze "tailor-made" therapie mogelijkheden zullen waarschijnlijk in de komende jaren beschikbaar komen voor verschillende ziekten.

In de TME-studie werd aan alle criteria voldaan voor de analyse van individuele risico factoren bij patiënten met kanker, zoals beschreven in het proefschrift van R.A.E.M. Tollenaar.[1] Het uniform verzamelen van klinische bevindingen volgens strikt gedefinieerde criteria, gedetailleerde documentatie en standaardisatie van therapeutische procedures, uniforme collectie van macroscopische en histologische tumorkarakteristieken, gestandaardiseerde documentatie van het verloop van de ziekte en ten slotte, evaluatie van de data met multivariate statistische methoden, werden alle doorgevoerd. De TME-studie met zijn unieke en degelijke opzet, biedt ook in de toekomst nog veel uitdaging voor onderzoekers en zal zeker nog meer antwoorden gaan geven op vragen met betrekking tot de moleculaire biologie, prognostische factoren en mechanismen van stralingsgeïnduceerde schade in tumorcellen. Tevens zullen er meer klinische uitkomsten van de TME-studie bekend worden in de komende jaren, zoals de lange termijn bijwerkingen van preoperatieve bestraling en de invloed van radiotherapie op de totale overleving. Een van de belangrijkste doelen van de trial is echter reeds bereikt; de verbetering van de behandeling van rectumcarcinoompatiënten door de invoering van de TME-techniek met significant lagere lokaal recidiefpercentages vergeleken met een tiental jaar geleden. In dit proefschrift zijn de klinische en moleculaire aspecten van het rectumcarcinoom onderzocht en wordt geïllustreerd dat door het gecombineerd onderzoeken van deze aspecten in een grote gerandomiseerde multicenter trial, vooruitgang in de behandeling en nieuwe inzichten in de moleculaire biologie

van het rectumcarcinoom zijn verkregen.

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LIIST VAN DEELNEMERS TME-TRIAL, DUTCH COLORECTAL CANCER GROUP (DCRCG)
Deelnemers Nederland

Chirurgen: A.B. Bijnen, P. de Ruiter, Medisch Centrum Alkmaar, ALKMAAR; B. van Ooijen, Algemeen Christelijk Ziekenhuis Eemland Lokatie de Lichtenberg, AMERSFOORT; D. van Geldere, R.P.A. Boom, Ziekenhuis Amstelveen, AMSTELVEEN; R.P. Bleichrodt, S. Meyer, Academisch Ziekenhuis Vrije Universiteit, AMSTERDAM; R.M.J.M. Butzelaar, E.Ph. Steller, Sint Lucas Andreas Ziekenhuis, Lokatie Lucas, AMSTERDAM; W.F. van Tets, A.C.H. Boissevain, Sint Lucas Andreas Ziekenhuis, Lokatie Andreas, AMSTERDAM; F.A.N. Zoetmulder, F. van Coevorden, Antoni van Leeuwenhoekziekenhuis, AMSTERDAM; F.J. Sjardin, BovenIJ Ziekenhuis, AMSTERDAM; J.F.M. Slors, Academisch Medisch Centrum, AMSTERDAM; W.H. Bouma, J.G.J. Roussel, Gelre Ziekenhuizen, Lokatie Lukas Ziekenhuiscentrum Apeldoorn, APELDOORN; J.H.G. Klinkenbijl, E.J. Spillenaar Bilgen, Ziekenhuis Rijnstate, ARNHEM; Ph.M. Kruyt, W.K. de Roos, Stichting Ziekenhuisvoorzieningen Gelderse Vallei Lokatie Ziekenhuis Gelderse Vallei Bennekom, BENNEKOM; E.J.R. Slingenberg, P.D. de Rooij, Sint Ziekenhuis Lievensberg, BERGEN OP ZOOM; M.A.J.M. Hunfeld, Rode Kruis Ziekenhuis, BEVERWIJK; A.L.A. Meersman, Maasziekenhuis, BOXMEER; J.K.S. Nuytinck, Ignatius Ziekenhuis Breda, BREDA; R.M.P.H. Crolla, Ziekenhuis de Baronie, BREDA; J. van der Bijl, Atrium Brunssum, Atrium Heerlen, BRUNSSUM/HEERLEN; G.W.M. Tetteroo, IJsselland Ziekenhuis, CAPELLE A/D IJSSEL; L.P.S. Stassen, P.W. de Graaf, Reinier de Graaf Groep Lokatie Reinier de Graaf Gasthuis, DELFT; W.A.H. Gelderman, F.G.J. Willekens, Bosch Medicentrum Lokatie Groot Ziekengasthuis, DEN BOSCH; I.P.T. van Bebber, E.J. Carol, Stichting Carolus-Liduina-Lindelust Ziekenhuis Lokatie Carolus Ziekenhuis, DEN BOSCH; G.W. Kastelein, H. Boutkan, Stichting Juliana Kinderziekenhuis/Rode Kruis Ziekenhuis Lokatie Rode Kruis Ziekenhuis, DEN HAAG; Ch. Ulrich, B.C. de Vries, Medisch Centrum Haaglanden Lokatie Westeinde, DEN HAAG; H.J. Smeets, J.M. Heslinga, Stichting Bronovo-Nebo, Ziekenhuis Bronovo, DEN HAAG; W.H. Steup, P.V.M. Pahlplatz, Ziekenhuis Leyenburg, DEN HAAG; P. Heres, J.A. van Oijen, Stichting het van Weel-Bethesda Ziekenhuis, DIRKSLAND; M. van Hillo, Stichting Talma Sionsberg, DOKKUM; R.J. Oostenbroek, K.G. Tan, Albert Schweitzer Ziekenhuis Lokatie Dordwijk, DORDRECHT; H.C.J. van der Mijle, Christelijk Ziekenhuis Nij Smellinghe, DRACHTEN; R. Looijen, Christelijk Ziekenhuis Nij Smellinghe, DRACHTEN; H.J.T. Rutten, J.J. Jakimowicz, Catharina Ziekenhuis, EINDHOVEN; O.J. Repelaer van Driel, P.H.M. Reemst, Diaconessenhuis Eindhoven, EINDHOVEN; E.J.Th. Luiten, R.F.T.A. Assmann, Sint Annaziekenhuis, GELDROP; C.M. Dijkhuis, Oosterscheldeziekenhuis, GOES; R.T. Ottow, Het Groene Hart Ziekenhuis Lokatie Bleuland, GOUDA; T. Wiggers, J.T.M. Plukker, Academisch Ziekenhuis Groningen, GRONINGEN; E.J. Boerma, R. Silvis, Kennemer Gasthuis Lokatie Deo, HAARLEM; J.H. Tomee, Stichting Streekziekenhuis Coevorden-Hardenberg Lokatie Röpcke Zweers, HARDENBERG; G.J.M. Akkersdijk, Spaarne Ziekenhuis, HEEMSTEDE; C.G.B.M. Rupert, de Tjongerschans, Ziekenhuis, HEERENVEEN; G.J.C.M. Niessen, G. Verspui, Elkerliek Ziekenhuis Lokatie Helmond, HELMOND; J.H. Kroesen, J.W. Juttmann, Ziekenhuis Hilversum, HILVERSUM; J.W.D. de Waard, M.W.C. de Jonge, Westfries Gasthuis Lokatie Sint Jan, HOORN; D.B.W. de Roy van Zuidewijn, W. Dahmen, Medisch Centrum Leeuwarden Lokatie Zuid, LEEUWARDEN; R. Vree, J.A. Zonnevylle, Diaconessenhuis, LEIDEN; C.J.H. van de Velde, R.A.E.M. Tollenaar, LUMC, LEIDEN; P.A. Neijenhuis, S.A. da Costa, S.K. Adhin, Rijnland Ziekenhuis Lokatie Sint Elisabeth, LEIDERDORP; F.J. Idenburg, Medisch Centrum Haaglanden Lokatie Antoniushove, LEIDSCHENDAM; H. van der Veen, IJsselmeerziekenhuizen Lokatie Zuiderzeeziekenhuis, LELYSTAD; C.E.A.M. Hoynck van Papendrecht, IJsselmeerziekenhuizen Lokatie Zuiderzeeziekenhuis, LELYSTAD; C.G.M.I. Baeten, M.F. von Meyenfeldt, G.L. Beets, Academisch Ziekenhuis Maastricht, MAASTRICHT; T. Wobbes, Academisch Ziekenhuis Nijmegen St. Radboud, NIJMEGEN; E.D.M. Bruggink, L.J.A. Strobbe, Canisius-Wilhelmina Ziekenhuis, NIJMEGEN; O.J. van West, R.A.J. Dörr, Pasteurziekenhuis, OOSTERHOUT; C.D. van Duyn, Ziekenhuis Bernhoven Lokatie Oss, OSS; J.W.M. Bol, Th.A.A. van den Broek, Waterlandziekenhuis, PURMEREND; J.M.H. Debets, R.J.A. Estourgie, Laurentius Ziekenhuis, ROERMOND; H.W.P.M. Kemperman, Ziekenhuis Franciscus, ROOSENDAAL; H.F. Veen, W.F. Weidema, C.J. van Steensel, Ikazia Ziekenhuis, ROTTERDAM; F. Logeman, A.A.E.A. de Smet, Sint Clara Ziekenhuis, ROTTERDAM; A.W.K.S. Marinelli, Daniel den Hoed Kliniek, ROTTERDAM; J.H. Driebeek-van Dam, Havenziekenhuis, ROTTERDAM; W.R. Schouten, P.P.L.O. Coene, Academisch Ziekenhuis Rotterdam Dijkzigt, ROTTERDAM; M.A. Paul, Zuiderziekenhuis, ROTTERDAM; J.J. van Bruggen, Schieland Ziekenhuis, SCHIEDAM; E.J. Mulder, Antonius Ziekenhuis, SNEEK; R. den Toom, A.J. van Beek, Ruwaard van Putten Ziekenhuis, SPIJKENISSE; S.J. Brenninkmeyer, G.P. Gerritsen, TweeSteden ziekenhuis,

TILBURG; H.J.M. Oostvogel, J.A. Roukema, Sint Elisabeth Ziekenhuis, TILBURG; E.B.M. Theunissen, Mesos, Medisch Centrum Lokatie Overvecht, UTRECHT; L.W.M. Janssen, A. Hennipman, Universitair Medisch Centrum Utrecht, UTRECHT; A.J.M. van Wieringen, Mesos, Medisch Centrum Lokatie Oudenrijn, UTRECHT; A. Pronk, P. Leguit, Diakonessenhuis, UTRECHT; F.A.A.M. Croiset van Uchelen, R.M.H. Roumen, Sint Joseph Ziekenhuis, VELDHOVEN; C.L.H. van Berlo, J.F.M. Reinders, Sint Maartens Gasthuis, VENLO; C.D.G.W. Verheij, Sint Elisabeth Ziekenhuis, VENRAY; J.H. ten Thije, Ziekenhuis Walcheren, VLISSINGEN; W. van Overhagen, I.H. Oei, Reinier de Graaf Groep Lokatie Diaconessenhuis Voorburg, VOORBURG; E.M.G. Leerkotte, J.W.A. van Luijt, TweeSteden ziekenhuis, WAALWIJK; H.C.M. Verkooyen, J.A.L. Jansen, Sint Jans-Gasthuis, WEERT; J. Merkx, J.P. Vente, Hofpoort Ziekenhuis, WOERDEN; H. de Morree, Stichting Oosterscheldeziekenhuizen, ZIERIKZEE; P.J.J. van Rijn, 't Lange Land Ziekenhuis, ZOETERMEER; W.F. Blom, Albert Schweitzer Ziekenhuis Lokatie Zwijndrecht, ZWIJNDRECHT.

Pathologen: J.P.A Baak, Medisch Centrum Alkmaar, ALKMAAR; H. Barrowclough, Algemeen Christelijk Ziekenhuis Eemland Lokatie de Lichtenberg, AMERSFOORT; G.J.A. Offerhaus, Academisch Medisch Centrum, AMSTERDAM; G. Brutel de la Riviere, Sint Lucas Andreas Ziekenhuis Lokatie Sint Lucas, AMSTERDAM; M.L.F. van Velthuysen, Antoni van Leeuwenhoekziekenhuis, AMSTERDAM; B.A. van de Wiel, Sint Lucas Andreas Ziekenhuis Lokatie Andreas, AMSTERDAM; H.H. Oushoorn, BovenIJ Ziekenhuis, AMSTERDAM; E. Bloemena, Vrije Universiteit, AMSTERDAM; Th.A.J.M. Manschot, Gelre Ziekenhuizen Lokatie Lukas Ziekenhuiscentrum Apeldoorn, APELDOORN; J.M. Wiersma-van Tilburg, Ziekenhuis Rijnstate, ARNHEM; V. Potters, Stichting Ziekenhuis Lievensberg, BERGEN OP ZOOM; H.V. Stel, Ziekenhuis Gooi-Noord, BLARICUM; J. Los, Ignatius Ziekenhuis, BREDA; G.W. Verdonk, Atrium Brunssum, BRUNSSUM; C. van Krimpen, S.H. Sastrowijoto, E.M. van der Loo, Stichting Diagnostisch Centrum Stichting Samenwerkende Delftse Ziekenhuizen, DELFT; H.A. Meijer, Bosch Medicentrum Lokatie Groot Ziekengasthuis, DEN BOSCH; P. Blok, Ziekenhuis Leyenburg, DEN HAAG; C.J. Tinga, Stichting Bronovo-Nebo, Ziekenhuis Bronovo, DEN HAAG; E.C.M. Ooms, Medisch Centrum Haaglanden Lokatie Westeinde, DEN HAAG; C.M. Bruijn-van Duinen, Ziekenhuis Leyenburg, DEN HAAG; J.W. Steffelaar, Stichting Juliana Kinderziekenhuis/Rode Kruis Ziekenhuis Lokatie Rode Kruis Ziekenhuis, DEN HAAG; P.J. Westenend, Pathologisch Laboratorium voor Dordrecht en omstreken, DORDRECHT; I.W.N. Tan-Go, H.M. Peters, Stichting Pathologische Anatomie en Medische Microbiologie, EINDHOVEN; E.J.M. Ahsmann, Stichting Laboratoria Goudse Ziekenhuizen, GOUDA; J.F. Keuning, Stichting Pathologisch Anatomisch Laboratorium Kennemerland, HAARLEM; K. van Groningen, Spaarne Ziekenhuis, HEEMSTEDE; P.H.M.H. Theunissen, Atrium Heerlen, HEERLEN; F.J.J.M. van Merrienboer, Elkerliek Ziekenhuis Lokatie Helmond, HELMOND; G. Freling, Ziekenhuis Bethesda, HOOGEVEEN; A.J.K. Grond, Laboratorium voor de Volksgezondheid in Friesland, LEEUWARDEN; M.C.B. Gorsira, Diaconessenhuis, LEIDEN; J.J. Calame, Rijnland Ziekenhuis Lokatie Sint Elisabeth, LEIDERDORP; E.A. Neefjes-Borst, IJsselmeerziekenhuizen Lokatie Zuiderzeeziekenhuis, LELYSTAD; J.W. Arends, Academisch Ziekenhuis Maastricht, MAASTRICHT; A.P. Runsink, Streeklaboratorium "Zeeland", MIDDELBURG; C.A. Seldenrijk, Stichting Sint Antonius Ziekenhuis, NIEUWEGEIN; J.H.J.M. van Krieken, Academisch Ziekenhuis Nijmegen St. Radboud, NIJMEGEN; M. Mravunac, Canisius-Wilhelmina Ziekenhuis, NIJMEGEN; W.S. Kwee, Laurentius Ziekenhuis, ROERMOND; H. van Dekken, Daniel den Hoed Kliniek, ROTTERDAM; J.C. Verhaar, Stichting Pathan, ROTTERDAM; N.A.L. van Kaam, Stichting Pathan, ROTTERDAM; H. van Dekken, Academisch Ziekenhuis Rotterdam Dijkzigt, ROTTERDAM; R.W.M. Giard, Sint Clara Ziekenhuis, ROTTERDAM; H. Beerman, Zuiderziekenhuis, ROTTERDAM; A.A.M. van der Wurff, Sint Elisabeth Ziekenhuis, TILBURG; M.E.I. Schipper, Universitair Medisch Centrum Utrecht, UTRECHT; H.M. Ruitenberg, Diakonessenhuis, UTRECHT; R.F.M. Schapers, Stichting Pathologisch Laboratorium, VENLO; A.P. Willig, Sint Jans-Gasthuis, WEERT; A.G. Balk, Stichting Ziekenhuis De Heel, ZAANDAM.

Radiotherapeuten: E.H.J.M. Rutten, Medisch Centrum Alkmaar, ALKMAAR; D. Gonzalez Gonzalez, G. van Tienhoven, Academisch Medisch Centrum, AMSTERDAM; B.J. Slotman, J.A. Langendijk, Academisch Ziekenhuis Vrije Universiteit, AMSTERDAM; G.M.M. Bartelink, B.M.P. Aleman, Antoni van Leeuwenhoekziekenhuis, AMSTERDAM; A.H. Westenberg, Arnhems Radiotherapeutisch Instituut, ARNHEM; J. Pomp, Reinier de Graaf Gasthuis, DELFT; C.C.E. Koning, R.G.J. Wiggenraad, Medisch Centrum Haaglanden Lokatie Westeinde, DEN HAAG; F.M. Gescher, Ziekenhuis Leyenburg, DEN HAAG;

J.J.F.M. Immerzeel, A.C.A. Mak, Radiotherapeutisch Instituut Stedendriehoek en Omstreken, DEVENTER; J.G. Ribot, H. Martijn, Catharina Ziekenhuis, EINDHOVEN; D.F.M. de Haas-Kock, Stichting Radiotherapeutisch Instituut Limburg, HEERLEN; G. Botke, A. Slot, Radiotherapeutisch Instituut Friesland, LEEUWARDEN; E.M. Noordijk, Leids Universitair Medisch Centrum, LEIDEN; Ph. Lambin, Academisch Ziekenhuis Maastricht, MAASTRICHT; J.W.H. Leer, J. Hoogenhout, Academisch Ziekenhuis Nijmegen Sint Radboud, NIJMEGEN; P.C. Levendag, P.E.J. Hanssens, Daniel den Hoed Kliniek, ROTTERDAM; G.S.J. Bunnik, K.A.J. de Winter, dokter Bernard Verbeeten Instituut, TILBURG; J.J. Batterman, H.K. Wijrdeman, Universitair Medisch Centrum Utrecht, UTRECHT; J.M. Tabak, M.F.H. Dielwart, Zeeuws Radiotherapeutisch Instituut, VLISSINGEN.

Deelnemers andere landen: J.C. Pector, Institut Jules Bordet, BRUSSEL, BELGIË; J. van de Stadt, Universite Libre de Bruxelles, Hospital Erasme, BRUSSEL, BELGIË; P.T. Phang, J.K. MacFarlane, St. Paul's Hospital, VANCOUVER, CANADA; R.J. Heald, B.J. Moran, The North Hampshire Hospital, BASINGSTOKE, ENGELAND; P. Teniere, Hopital Charles Nicolle, ROUEN, FRANKRIJK; J.R. Delpero, Institut J. Paoli & I. Calmettes, MARSEILLE, FRANKRIJK; B. Sastre, Hopital Sainte-Marguerite, MARSEILLE, FRANKRIJK; B. Nordlinger, C. Penna, Chu Ambroise Pare, BOULOGNE-BILLANCOURT, FRANKRIJK; B. Gerdes, B. Stinner, Klinikum der Philips-Universität, MARBURG, DUITSLAND; P. Delrio, V. Parisi, Inst. Nazionale per lo studio e la cura dei tumori, NAPELS, ITALIË; S. Pucciarelli, Universita Di Padova, PADOVA, ITALIË; J. Guimaraes dos Santos, Instituto Portugues de Oncologica do Porto, PORTO, PORTUGAL; A. Nihlberg, O. Bendtsen, Falu Lasarett, FALUN, ZWEDEN; G. Lindmark, Helsingborgs lasarett AB, HELSINGBORG, ZWEDEN; A. Törnqvist, T. Hallgren, Centralsjukhuset, KARLSTAD, ZWEDEN; R. Sjödahl, O. Hallbook, University of Linköping, LINKÖPING, ZWEDEN; M. Bohe, H. Jiborn, Malmö, Allmäna Sjukhuset, MALMÖ, ZWEDEN; E. Nilsson, Lasarettet i Motala, MOTALA, ZWEDEN; H. Krook, G. Arbman, Landstinget i Östergötland, NORRKÖPING, ZWEDEN; Örnsköldsvik Hospital, J. Rutegard, ÖRNSKÖLDSVIK, ZWEDEN; B. Sandzén, Umeå University Hospital, UMEÅ, ZWEDEN; L. Pahlman, W. Graf, B. Glimelius, Akademiska Sjukhuset, UPPSALA, ZWEDEN; K. Smedh, Centralhospital, VÄSTERÅS, ZWEDEN; K. Johansson, Västerviks sjukhus, VÄSTERVIK, ZWEDEN.

PUBLICATIES

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I.D. Nagtegaal, C.G.S. Gaspar, L.T.C. Peltenburg, C.A.M. Marijnen, E. Kapiteijn, C.J.H. van de Velde, R. Fodde, J.H.J.M. van Krieken. Radiation-induced changes in human rectal tumor and normal tissue. *Submitted*

CURRICULUM VITAE

Ellen Kapiteijn werd geboren op 7 november 1970 te Sassenheim. Zij behaalde in 1989 het diploma Atheneum B aan de Rijnland Scholengemeenschap te Sassenheim. In 1989 werd begonnen met de studie Geneeskunde aan de Universiteit Leiden. Tijdens haar studie vervulde zij meerdere student-assistentschappen bij de vakgroepen Heelkunde, Immunohematologie, Anatomie en Immunologie. Van september 1994 tot februari 1995 verbleef zij als student in Bristol, Engeland voor stages Heelkunde en Interne Geneeskunde in het kader van het European course Credit Transfer System (ECTS). Haar afstudeerproject (Dr. J.G.A. Houbiers en Dr. R.A.E.M. Tollenaar) en keuzeco-schappen (Drs. G.J. Liefers, Dr. R.A.E.M. Tollenaar en Prof. Dr. J.H.J.M. van Krieken) bracht zij door op de afdelingen Heelkunde en Pathologie, alwaar onderzoek werd gedaan naar prognostische factoren bij het colorectaal carcinoom. Het doctoraalexamen van de studie Geneeskunde werd behaald in 1995 en het artsexamen in 1997. Van 1 april 1997 tot 1 mei 2001 werkte zij als artsonderzoeker en studiecoördinator van de TME-studie op de afdelingen Heelkunde en Pathologie van het LUMC onder de leiding van Prof. Dr. C.J.H. van de Velde, Dr. R.A.E.M. Tollenaar, Prof. Dr. J.H.J.M. van Krieken en Dr. H. Morreau, hetgeen heeft geresulteerd in dit proefschrift. Op 1 mei 2001 is zij gestart met de opleiding tot chirurg in het Rijnland ziekenhuis te Leiderdorp/Alphen aan de Rijn.