Experimental Radiosensitization and Molecular Prediction of Chemoradiotherapy Response in Rectal Cancer

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1 Preface

1.1 Acknowledgements

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Years of preparations.
Months of waiting.
Days to remember.
Hours until sunrise.
Minutes of terror.
Seconds of glory.
Big time is here.

David Carson

1.2 Abbreviations

ATCC	American Type Culture Collection
ATM	ataxia telangiectasia mutated
5-FU	5-fluorouracil
APR	abdominoperineal resection
CI	combination index
CDK	cyclin dependent kinase
CRM	circumferential resection margin
CRT	chemoradiotherapy
CT	computerized tomography
DMSO	dimethyl sulfoxide
dsb	double strand break
DW-MRI	diffusion weighted magnetic resonance imaging
Gy	Gray, unit for radiation dose
HE	hematoxylin-eosin
EGFR	epidermal growth factor receptor
FITC	fluorescein isothiocyanate
HAT	histone acetyl transferase
HDAC	histone deacetylase
IHC	immunohistochemistry
HR	homologous recombination
IORT	intraoperative radiotherapy
IR	ionizing radiation
ip	intraperitoneal
iv	intravenous
LAR	low anterior resection
LARC	locally advanced rectal cancer
LARC-RRP	locally advanced rectal cancer – radiation response prediction
LOOCV	leave one out cross validation
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NACT	neoadjuvant chemotherapy
NHEJ	non-homologous endjoining
pCR	pathological complete response
PLS	partial least squares
RT	radiotherapy
SAHA	suberoylanilide hydroxamic acid
sc	subcutaneous
ssb	single strand break
TNM	tumor, node, metastasis
TRG	tumor regression grade
TSA	trichostatin A
VEGF	vascular endothelial growth factor

1.3 List of papers

I Folkvord S, Flatmark K, Seierstad T, Røe K, Rasmussen H, Ree AH. Inhibitory effects of oxaliplatin in experimental radiation treatment of colorectal carcinoma: does oxaliplatin improve 5-fluorouracil-dependent radiosensitivity? *Radiother Oncol,* 2008, 86 (3):428-34

II Folkvord S, Ree AH, Furre T, Halvorsen T, Flatmark K. Radiosensitization by SAHA in experimental colorectal carcinoma models – *in vivo* effects and relevance of histone acetylation status. *Int J Radiat Oncol Biol Phys*, 2009, 74 (2):546-52

III Ree AH, Folkvord S, Flatmark K. HDAC2 deficiency and histone acetylation. *Nat Genet,* 2008, 40 (7):812-3

IV Folkvord S, Flatmark K, Dueland S, Wijn R, Grøholt KK, Nesland JM, Hole KH, Boender PJ, Johansen M, Giercksky KE, Ree AH. Prediction of response to preoperative chemoradiotherapy in rectal cancer by multiplex kinase activity profiling. *Manuscript*.

2 General introduction

2.1 Rectal cancer

2.1.1 Epidemiology

Colorectal cancer is the third most frequent cancer in both genders combined, after prostate and breast cancer. Around 30 % of all colorectal cancer cases are diagnosed in the rectal anatomical site, defined as tumors with the inferior margin within 16 cm from the anal verge as assessed by rigid rectoscopy. In 2007, 1111 new cases of rectal cancer were diagnosed in Norway with an age-adjusted (world) incidence rate per 100 000 persons-years of 15.4 and 10.7 for men and women, respectively (1). The incidence rates have been stable over the last ~20 years (Figure 1), however, due to an aging population and increased survival after treatment, the prevalence has increased from 6883 on the 31st December 1997 to 9259 persons on the 31st December 2007 (1). The incidence rates in Norway are among the highest in the world (2, 3), but the survival rates are also among the highest (4, 5). Five-year survival of persons diagnosed during 1998-2002 was 58.2% and 63.8% for men and women, respectively, an increase from 1978-1982 which was 44.2% and 47.4% (1). The increased survival rates may be attributed to advances in surgical techniques, centralized surgery, more effective (neo-)adjuvant oncologic treatment, as well as improved general patient care reducing treatment complications.

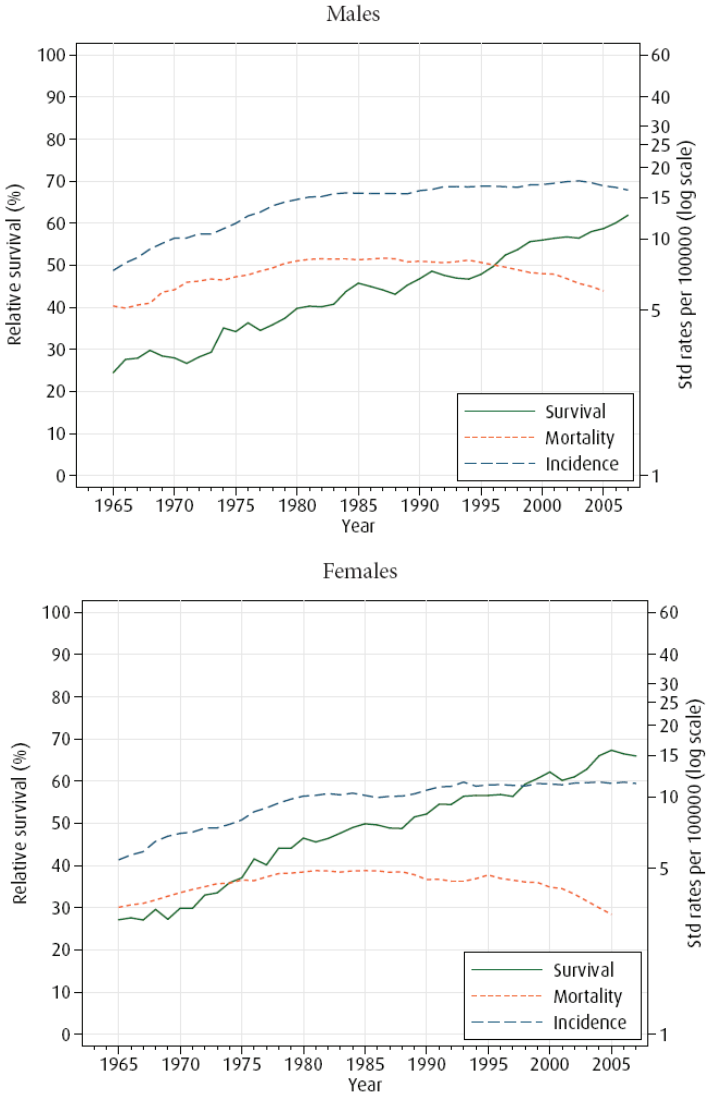

Figure 1: Trends in age-standardised 5-year survival proportions and incidence and mortality rates for rectal cancer 1965-2007. With permission from the Cancer Registry of Norway, 2009 (6).

2.1.2 Risk factors and prevention

Risk factors for rectal cancer are mainly similar to those of colon cancer, with a few exceptions. Dietary factors that are associated with increased risk include high intake of red and processed meat and alcohol consumption of more than 30 g/day. High dietary intake of fish, calcium, milk, vitamin B, vitamin D, selenium, and ever use of oral contraceptives have been shown to be protective for rectal cancer. High body mass index and little physical exercise are associated with colon cancer, but is negligible for rectal cancer (3). Persons with a first degree family relative with colorectal cancer have a two-fold risk of developing bowel cancer compared to the general population. Furthermore, persons with certain autosomal dominant conditions (hereditary non-polyposis colorectal cancer or familial adenomatous polyposis) constitute 5-10% of all colorectal cancers (3). Screening with fecal occult blood testing has been demonstrated to reduce mortality rates by 15-33%, whereas screening with flexible colonoscopy, computerized tomography (CT), and molecular screening in feces or blood is under investigation (7).

2.1.3 Staging

The TNM (Tumor, Node, Metastasis) classification is used to describe the anatomic extent of cancer growth (Table 1), in which T1-T4 designate increasing local growth of the primary tumor and N and M indicate the presence (N1, N2, M1) or absence (N0, M0) of metastatic lymph nodes or distant metastasis, respectively.

Table 1. Staging of rectal cancer used both clinically and pathologically (8, 9)

T0	No evidence of primary tumor
Tis	Carcinoma *in situ:* intraepithelial or invasion into lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa or non-peritonealized perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes
M0	No distant metastasis
M1	Distant metastasis

Other useful staging systems are frequently used clinically (Dukes' A-D, International Union Against Cancer – UICC stage 0-IV), but these classifications are today based on defined TNM stages. Almost all rectal tumors are adenocarcinomas originating from the glandular epithelium of the rectum and they are classified by histologic grade; low, intermediate and high differentiation, in which the less differentiated tumors tend to be more aggressive with less favorable prognosis (10). Rectal cancers may also be graded by the distance from the anal verge as high (upper 1/3 of rectum), medium (middle rectum), or low (lower 1/3 of rectum) which influences treatment (surgical techniques, radiation fields) as well as treatment outcome with lower cancers having higher risk of disease recurrence (11). An involved circumferential resection margin (CRM, see below) or CRM of less than 2 mm are highly associated with local recurrence and poor survival (12, 13), thus, CRM status is crucial for treatment and prognosis and are therefore reported during staging of the disease.

2.1.4 Locally advanced rectal cancer – LARC

An exact definition of LARC has not unambiguously been established (14), and the term has also changed during recent years due to increasing knowledge of risk factors of local failure and improvement of disease staging with the use of magnetic resonance imaging (MRI) (15, 16). The classic definition is a rectal tumor that by clinical or radiological assessment grows through the rectal wall, or has lymph node spread to an extent precluding complete removal by surgery alone. Resectable LARC is defined more strictly as tumors with penetration through the entire rectal wall, or with evidence of involved pelvic nodes, but still a non-threatened CRM based upon preoperative MRI and without distant metastasis (3), whereas non-resectable LARC usually comprises fixed tumors for which surgery is not possible without leaving tumor tissue within the pelvis. The latter tumors may after neoadjuvant treatment become resectable (17). The locally advanced tumors constitute at least 50% of all rectal carcinomas including the primary non-resectable tumors which constitute about 10-15% of all cases (18).

In current clinical practice in Norway, preoperative CRT is given to patients with primary resectable and non-resectable tumors, i.e all T4 tumors, T3 tumors with MRI predicted CRM ≤ 3 mm or any T stage with N+ tumors with MRI predicted CRM ≤ 3 mm (19, 20). These criteria

were used for inclusion in the LARC-RRP study (described below), also including patients with resectable metastases (usually a single liver or lung metastasis). In this thesis, rectal cancers in which preoperative CRT with a curative intention is indicated constitute the definition of LARC.

2.1.5 Treatment of LARC

Surgical treatment

Surgery is the main treatment for rectal cancer with the aim of achieving radical resection margins. When feasible, low anterior resection (LAR) is performed. The tumor with margins is removed *en bloc* and a colorectal or coloanal anastomosis is made. A temporary diverting stoma may be performed in order to relieve the anastomosis during the postoperative period. When LAR is not feasible, especially in tumors located close to the anal verge, an abdominoperineal resection (APR) is performed. The rectum is removed and a permanent terminal colostomy is made. In some cases, Hartmann's procedure is performed for tumor removal. The distal rectal stump is closed and left in the pelvis, and a permanent terminal colostomy is made (21). If the tumor has invaded neighboring organs (e.g. bladder, vagina, uterus, prostate, coccyx), curative surgery may require partial or total resection of these organs or even pelvic exenteration including cystectomi (22).

Total mesorectal excision (TME) was introduced by Heald *et al.* in 1982 (23) and the technique was further improved in 1993 (24). TME has been shown to reduce local recurrence and improve survival (25), and is now considered the gold standard in rectal cancer surgery. The mesorectum refers to the fatty connective tissue layer 2-3 cm in thickness with associated vessels, lymphatics and lymph nodes, which surrounds the rectum and is enveloped by a fascia (the mesorectal fascia). TME refers to total surgical removal of this soft tissue envelope, but the resection can also be partial (PME) for tumors situated in the upper rectum. TME was implemented as the standard surgical technique in Norway in 1993 as part of a national project (26), and improved results on a national basis have been reported (27).

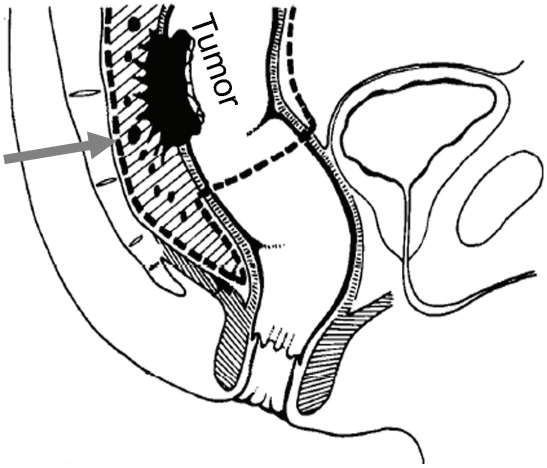

Figure 2. Sagittal section of the rectum illustrating a locally advanced rectal cancer with lymphatic metastatic spread in a male. The broken line shows the surgical dissection plane using the TME technique. Grey arrow indicates the shortest distance from tumor to the mesorectal fascia, i.e. CRM status. Adapted from British Journal of Surgery 1982 with permission (23).

Neoadjuvant radiotherapy (RT)

Since surgery is the principal treatment, any supplementing oncologic treatment is *adjuvant* (literally *to help*) to surgery. Often, adjuvant treatment is given after surgery, however, in the case of LARC, it may be applied before and is thus called *neoadjuvant* therapy.

The rationale for combining radiotherapy (RT) and surgery is based on the observation that surgery has its limitation in the periphery of the tumor due to the need of preserving vital structures adjacent to the lesion. On the other hand, RT has its main effect in the margins of the tumor, and less effect in the central part of the tumor due to hypoxia-induced radioresistance and the huge amount of tumor cells needed to be killed. The surgical limitation in the periphery of the tumor is demonstrated by the association between histopathological involvement of resection margins (CRM positivity) and local recurrence rates, metastasis, and overall survival in patients only receiving surgery with TME technique (20, 28). Hence, tumor sterilization of the surgical periphery with adjuvant RT has been used for a long time, and two prospective randomized trials conducted in the 1980s demonstrated improved outcome with the use of postoperative RT in LARC (29, 30). At Memorial Hospital for Cancer and Allied Diseases in

New York, Stearns and colleagues established preoperative RT in the late 1950s (31, 32), which resulted later in a long-lasting discussion whether RT should be given pre- or postoperatively. A randomized controlled German trial found preoperative RT to be superior to postoperative RT with respect to local control and toxicity, but overall survival was not significantly different between the two groups (33). A systematic review concluded that adjuvant RT decreases the risk of local failure by 50-70% if given preoperatively, but 30-40% if given postoperatively in doses of more than 30 Gy, and there is strong evidence that preoperative RT improves survival by 10%. Furthermore, in patients presenting with a surgically non-resectable tumor, preoperative RT can cause tumor regression allowing subsequent radical surgery in a substantial proportion of patients (34). It has also been suggested that preoperative RT facilitates sphincter-preserving procedures by decreasing the size of the tumor (33, 35), however, this was not significant in a Cochrane systematic review (36). Thus, preoperative RT is now considered standard treatment in LARC at least in the European health community (37-39).

RT may be given as a short course (5 Gy in 5 consecutive days, also called “Swedish style RT” after a Swedish phase III trial (40)) or more protracted courses with 1.8-2 Gy per fraction 5 days a week for 5-6 weeks to total doses of 45-50.4 Gy. The optimal schedule is still a matter of controversy, however, studies indicate that long course RT regimens are superior to the short course schedules with respect to downstaging, free resection margins, local recurrences, sphincter preservation, and probably late normal tissue toxicity (41). The protracted course is also easier to combine with chemotherapy. On the other hand, the short course schedule has less surgery delay, is generally less expensive, more convenient for the elderly and others with transportation difficulties, and is easier to complete for patients with poor compliance.

Adjuvant chemoradiotherapy (CRT)

Adenocarcinoma of the rectum is a radiosensitive tumor, and RT has the potential to eradicate the disease as a sole modality. Radical curative doses for adenocarcinoma at other sites would normally be considered to be greater than 70 Gy. However, this dose is too high because rectal and small-bowel toxicity would be unacceptable (42). If chemotherapy is given together with RT, it may potentiate the effect of RT. This radiosensitizing effect of the chemotherapeutic

agent will be discussed in detail later. Additionally, but not equally important in concomitant CRT in the treatment of LARC, it is claimed that undetected circulating tumor cells, or micrometastasis, outside the surgical specimen and the radiation field, may be eradicated by chemotherapy (43).

5-fluorouracil (5-FU) has been used in colorectal cancer therapy and in combination with RT for decades (44, 45), and has been extensively studied in CRT regimens for the last two decades as adjuvant treatment in LARC. Three randomized controlled trials have all concluded that the addition of 5-FU to adjuvant RT in LARC improves local control and increases respectability, but without influencing survival or rate of sphincter preservation (18, 46, 47), with the exception of one trial showing increased disease-free survival in the most advanced non-resectable cancers (18). Importantly, these studies also show increased but tolerable acute toxicity (14). A newly reported Cochrane systematic review concluded that, compared to preoperative RT alone, preoperative CRT enhances histopathological response and improves local control in resectable LARC but does not benefit disease-free or overall survival. The effects of preoperative CRT on functional outcome and quality of life are not completely understood and should be addressed in future trials (48).

Capecitabine is an oral pro-drug that is converted to 5-FU in tissues, and it has potential advantages to intravenously (iv) administered 5-FU which will be discussed later. Capecitabine has been proven to be as effective as 5-FU in metastatic colon cancer with favorable toxicity in phase III trials (49). Emerging data from phase II trials of neoadjuvant regimens in LARC, in which capecitabine has been substituted for 5-FU, are encouraging (50), and a randomized phase III trial is still recruiting patients (NSABP R-04 study) (51).

In summary, the standard treatment protocol used in Norway for LARC is preoperative RT, 50 Gy in daily 2 Gy fractions (Monday through Friday) with concomitant 5-FU based chemotherapy, which is either 5-FU (400 mg/m^2) 30 minutes before fractions 1, 2, 11, 12, 21 and 22 combined with 100 mg Leucovorin® ("Nordic schedule") or capecitabine (825 mg/m^2) twice daily Sunday to Friday during RT. The RT is usually given with three-dimensional conformal treatment planning with 23 fractions of 2 Gy to the clinical target volume (tumor and

threatened lymph nodes) followed by a boost of 2 fractions of 2 Gy to the macroscopic tumor, resulting in a total dose of 50 Gy. Surgery is usually performed 6-8 weeks after the last radiation dose to allow for tumor shrinkage and the patient to recover (19). At our institution, each patient is evaluated by a multidisciplinary team composed of surgeons, oncologists and radiologists. In addition, histopathologists and specialist nurses should be included.

2.1.6 The clinical trials LARC-RRP and PRAVO

Currently, at the Norwegian Radium Hospital, two clinical trials are ongoing with relevance for the work conducted in this thesis. The LARC-RRP study (Locally Advanced Rectal Cancer – Radiation Response Prediction) is a phase II trial (ClinicalTrials NCT00278694) that started to enroll patients in October 2005. Eligible patients have primary LARC, and the treatment protocol includes two 2-weekly cycles of neoadjuvant chemotherapy (NACT) (oxaliplatin 85 mg/m^2 day 1, 5-FU 500 mg/m^2 with folinic acid 60 mg/m^2 day 1 and 2 – "Nordic FLOX"), and 4 weeks after start of NACT RT is given as described for standard treatment with the use of capecitabine 825 mg/m^2 twice daily on RT days and with the addition of oxaliplatin 50 mg/m^2 once weekly during RT (Figure 3). Tumor biopsies are sampled before start of treatment and are snap frozen immediately. Patients are examined before, during, and after CRT with magnetic resonance spectroscopy (MRS) as well as standard MRI. The surgical specimens are examined by an experienced pathologist, and the radiation response is classified by tumor regression grade (TRG) 1-5, in which TRG 1 represents pathological complete response (no tumor cells left) and TRG 5 corresponds to no radiation response with differential grades of histopathological tumor responses between these extremes (52). Patient follow-up takes place at the institution, and data will be compiled for 5 years postoperatively. The tumor biopsies are hematoxylin-eosin (HE) stained for validation of tumor content, and the biopsies will be analyzed further with *ex vivo* MRS and gene expression and kinase activity microarray with the aim of finding predictive molecular and metabolic tumor profiles of response.

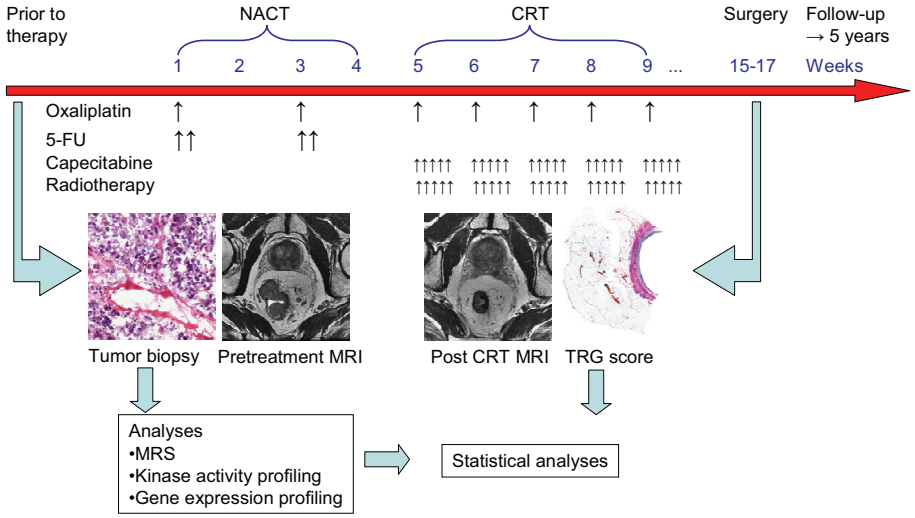

Figure 3. Time line of treatment and selected examinations in the LARC-RRP study. Figure made by author.

The Pelvic Radiation and Vorinostat (PRAVO) study is a phase I trial (ClinicalTrials NCT00455351) in which vorinostat, a histone deacetylase (HDAC) inhibitor, also called suberoylanilide hydroxamic acid (SAHA)[1] and licensed in the US as Zolinza™, is combined with pelvic RT in advanced cancers with palliative intent. Patients with advanced pelvic cancers (mostly rectal and colon primaries, but also one gastric primary) have received palliative RT to macroscopic tumor with a fractionation dose of 3 Gy to a total dose of 30 Gy with concomitant oral vorinostat in sequential escalating doses. RT is given 3 hours after administration of vorinostat, and tumor biopsies are sampled at approximately the same time point and before treatment for correlative laboratory analyses (e.g. detection of histone acetylation). Toxicity and tumor response are monitored. The first patient was included in February 2007, and the accrual is about to finalize at the time this is written. The rationale for combining vorinostat with RT has resulted from the interest of our research group in combining HDAC inhibitors with RT (paper II and III). A PRAVO II study is currently being planned in preparation in which LARC patients will receive vorinostat in addition to standard 5-FU based CRT. In the view of new molecular targeting agents in neoadjuvant treatment of LARC, this

[1] Vorinostat is the oral clinically used form of SAHA. In experimental settings SAHA was used, whereas vorinostat was given to patients in the PRAVO study.

study was the first to combine an HDAC inhibitor with RT with basis from pre-clinical evidence.

2.1.7 Prognostic factors and response evaluation

The terms prognostic and predictive factors may be somewhat overlapping, however, prognostic factors define the risk of recurrence or death of a standard treatment, whereas predictive factors indicate the likelihood of response to a given therapy and may therefore select patients for individualized therapy (53). Some prognostic factors may function as surrogate end point in clinical trials, as these factors are highly correlated to overall survival or disease-free survival. Surrogate end points are used since trial end points based on overall survival requires waiting for a minimum of 5 years to observe reliable results (54).

TNM stage is historically the most important prognostic factor in most cancer types. Importantly, TNM staging may be clinically or radiologically assessed before or after (prefix y) neoadjuvant therapy or histopathologically staged by use of the surgical specimen following surgery (prefix p). Tumor depth, namely T stage, is important as T1 and T2 tumors have excellent long time survival, whereas T3 tumors, which make up 80% of rectal tumors seen in clinical practice (55) and T4 have more variable prognoses, thus, the newest edition of the TNM classification suggests an optional expansion of classifying extramural spread (9, 55, 56). Norwegian men diagnosed during 1998-2002 with rectal cancer had a 5 year survival of 86.2%, 66.0% and 10.5% for localized (T1-2), regional (T3-4), and metastatic stage (M1), respectively (1). Lymph node metastases and the number of involved lymph nodes are independent prognostic factors for survival with prognosis deteriorating when more than 4 lymph nodes are involved as assessed histopathologically (20, 54).

CRM is the shortest distance from tumor to the non-peritonealised bare area of the rectum located both anteriorly and posteriorly, i.e. the anatomical plane the surgeon dissects through during TME surgery. After the introduction of the concept of CRM more than 20 years ago (12, 57), CRM has been shown to be an even more significant prognostic factor than TN stage, as involvement strongly predicts local recurrence, development of distant metastases, and overall survival (58, 59). A minimum distance of 1 mm appeared to discriminate between high (85%)

and low risk (3%) with respect to local recurrence, however, the number of millimeters are still under debate, and in general, it can be stated that the larger the CRM, the better the prognosis (59). CRM is correlated to TN-stage, tumor size, differentiation grade, vascular invasion, degree of stenosis and ulcerative tumor growth patterns, distance to anal verge, surgical technique and age, and most of these factors are also correlated to prognosis (59). Preoperative high resolution MRI can predict CRM with high accuracy (60, 61) and can therefore be used to select patients for neoadjuvant treatment. Macroscopic (R2) or microscopic (R1) presence or absence (R0 resections) of residual disease, i.e. tumor in the margin of the surgical specimen, is also highly correlated to local recurrence and survival (62).

The degree of response after CRT is also important for prognosis. Pathological complete response (pCR) has been associated with increased overall survival in prospective studies (63, 64), however, even though the addition of 5-FU to neoadjuvant treatment in LARC increased the rate of pCR, overall survival was not improved, thus it has been claimed that modest increase in pCR does not impact on survival (54). The interval between CRT and surgery, tumor size, and how assiduous the pathologists search for viable tumor cells influence the rate of pCR, and if there are tumor cells left in the specimen, there exists no method to assess the true viability of these cells. Since the majority of patients treated with preoperative CRT do not achieve pCR, TRG scoring systems have been developed based on the amount of residual tumor cells and the extent of fibrosis in the bowel. The first scoring system was developed for oesophageal cancer following CRT using a five-point scale (65), and different variations of this system has been employed for rectal cancer (52, 66-68). In the LARC-RRP study, TRG scoring system after Bouzourene was used (Table 2), and TRG have been shown to be independent prognostic indicator for long-term local tumor control and disease-free survival (52, 63). Since different TRG systems are used, comparison between trials has been difficult. Hence, a suggestion of a standardized four-point scale TRG has been proposed recently (69).

Table 2. Tumor Regression Grade – TRG, from Bouzourene *et al.* (52)

TRG1	Absence of residual tumor and fibrosis extending through the different layers of the rectal wall, i.e. pCR
TRG2	Presence of residual tumor cells, ranging from one single tumor cell to tumor cells scattered throughout the fibrosis
TRG3	Increase in the number of residual cells compared to TRG2, but fibrosis still dominates
TRG4	Residual tumor outgrowing the fibrosis
TRG5	Absence of any tumor regression (no fibrosis)

2.1.8 Molecular response prediction

The varying response to neoadjuvant CRT indicates tumor heterogeneity among LARC patients. It is not known why such large differences in response occur, but the phenomenon may reflect diverse intrinsic biology of tumors or yet undetected external factors. CRT delays surgery by several months, is expensive, has acute and long-term side effects, and may increase perioperative morbidity (36, 48, 70), thus, the possibility to predict response either before or in the early stages of treatment may spare poorly responding patients from undergoing treatment that is not beneficial. Patients with no response after CRT might probably go straight to surgery omitting CRT or be candidates for alternative or more intensive CRT regimens (71), and patients with a complete remission would benefit from omitting surgery (72). At present, no reliable technique (radiological or clinical) for predicting tumor response after CRT is available (42, 73), and it has been concluded that pre-treatment T stage, N stage, histological grade, differentiation, age and gender do not reliably predict histological response to CRT (70). Extensive research based on histological and molecular assessment of pre-treatment tumor biopsy specimens or blood samples have been employed in order to elucidate factors that may allow for response prediction.

Pretreatment serum level of carcinoembryonic antigen (CEA) of >2.5 ng/ml has been associated with lower pCR rates, and in conjunction with high tumor circumferential extent (>60%) and tumor distance of >5 cm from the anal verge, response to CRT has been predicted using these factors (74). The tumor suppressor protein p53 is the most extensively studied single molecular predictor of response to CRT in LARC. Only 4 out of 18 studies utilizing immunohistochemistry (IHC) found that p53 expression significantly predicted response (70), however, IHC can not always discriminate between wild-type and mutant *TP53* gene.

Assessment of *TP53* mutations by gene sequencing analysis has revealed association between p53 gene mutations and response rates (75, 76). High endogenous expression of the cell cycle inhibitor protein p21by IHC correlated to good response following CRT (77), however, contrary to this finding, pretherapeutic p21-positive biopsies were shown to be associated with poorer disease-free and overall survival compared to p21-negative tumors (78), hence, its role as predictor in this setting remains controversial.

EGFR is expressed in 50-70% of colorectal carcinomas and upregulation of the protein is associated with aggressive tumor growth, poor prognosis, and resistance to radiation in pre-clinical and clinical studies (79, 80). Accordingly, low EGFR expression in pretreatment biopsies is associated with favorable response and prognosis (81, 82). Even so, contradictory results also exist for this marker. A high gene copy number of *EGFR* was associated with tumor regression following a cetuximab-containing CRT regimen (83) and, secondly, the combined analysis with low EGFR and high VEGF was recently shown to predict radioresistant tumors with high accuracy (84). VEGF alone (in plasma or IHC-based) has not shown correlation to tumor response, but high expression was an indicator of poor disease-free survival linked to distant metastasis (85, 86). In a single study, growth hormone receptor overexpression was significantly associated with poor tumor response (87).

DNA damage repair factors may be of importance in predicting radiosensitivity. Microsatellite instability, which is a defect in the DNA mismatch repair pathway, is found in 95% of hereditary and 15% of sporadic colorectal cancers, and is of importance for prognosis (88). Three studies did not show any predictive value of microsatellite instability for treatment response in LARC (70), but high expression of the DNA double-strand break (dsb) repair proteins Ku70 and Ku86 was associated with decreased response to CRT in a single study (89). Low telomerase activity has in pre-clinical experiments been related to radiosensitivity, and pre-treatment tumors with high telomerase reverse transcriptase protein stain have been shown to be radioresistant, which was also correlated to recurrence and survival (90).

The ability to evade apoptosis is thought to be central in both tumorigenesis and resistance to cytotoxic drugs and radiation. The level of spontaneous apoptosis in pretreatment rectal cancer

biopsies has been evaluated in seven studies. In six of those, a high apoptotic index was associated with good response after CRT (70). Furthermore, survivin, an inhibitor of apoptosis, was inversely correlated to the apoptotic level, and was also correlated to disease-free survival and metastasis development (91). The pro-survival protein Bcl-2 and pro-apoptotic Bax were of no predictive value (70).

As radiation and cytotoxic drugs mainly kill proliferating cells, the proliferative index has been evaluated in pretreatment biopsies. Mitotic activity, as assessed by mitotic cell fraction, proliferating cell nuclear antigen, or the proliferative marker Ki-67, has shown different results, and a review concluded that proliferating index unlikely predicts response to CRT in LARC (70, 92-94). A high flow-cytometric S-phase fraction in aneuploid tumors was shown to be associated with local recurrence, but tumor response was not evaluated and no correlation was found in diploid tumors (95). Thymidylate synthase and thymidine phosphorylase are thought to be important for 5-FU based therapy, and gene expression was found to be lower in responders compared to non-responders following 5-FU based CRT (96).

A wide range of other factors have been tested with no or only weak correlation to tumor response following CRT. The most important are COX-2, caspase-8, APC, DCC, cleaved CK-18, APAF-1, MLH-1, MSH2, and MIB-1 (78, 84, 93, 97, 98).

One of the most intriguing molecular developments has been the use of pretreatment gene expression profiling. At present, four studies have evaluated gene expression of pretherapeutic rectal cancer biopsies as a predictive tool of CRT response (71, 99-101). Ghadimi *et al.* found in a cohort consisting of 30 patients that a set of 54 genes could predict T-level downstaging with 78% sensitivity and 86% specificity, but there was no correlation to TRG. A second study by Watanabe *et al.* used TRG as response criterion, and found discriminating expression of 33 genes in 35 patient samples. A prediction model was made of 17 patients (testing set) and an accuracy of 82% of predicting poor versus good responders was found. In a third study, Kim *et al.* were able to discriminate pCR (TRG 1) from partial responders (TRG 2-4) in a total of 46 patients, using a similar approach as Watanabe, and finding a prediction accuracy of 87% in the 15 validation test samples. In a study by Rimkus *et al.*, 43 patients were predicted to be either

good responders (< 10% tumor cells in the surgical specimen) or partial/poor responders (> 10% tumor cells in the resected material) with 71% sensitivity and 86% specificity by the use of 42 discriminating genes. Notably, the response criteria were different in the three studies. Nevertheless, in the future, gene expression profiling may assist in response prediction, although validation in larger, independent studies is required.

A clinically useful predictive test would be simple to perform, easy and fast to evaluate, would give reproducible results, and would have a high sensitivity and specificity. Potential predictors need to be validated in prospective clinical trials followed by interventional studies, i.e. studies that include a therapeutic decision based on the results of the predictive test (53). In conclusion, none of the above-mentioned molecular markers has been found useful, mainly because no marker has shown high enough sensitivity and specificity for being capable of detecting responders or non-responders. Furthermore, the markers have not yet been rigorously tested on a large scale. At present, existing molecular data is not sufficiently reliable but the possibility of combining clinical, radiological, and molecular information to tailor treatment for LARC patients is intriguing. A novel methodological platform for molecular prediction is suggested in paper IV.

2.2 Radiation

The discoveries of X-rays in 1895 by Wilhelm Conrad Röntgen and of natural radioactivity by Henry Becquerel a few months later were a breakthrough that paved the way for a new era in science and cancer treatment. As early as in 1896, Emil Grubbé treated an advanced ulcerated breast cancer with X-rays in Chicago. In 1898, Marie Curie discovered a natural source of γ-rays, namely radium, which was the only source of γ-rays for cancer treatment for 20 years (102). In the beginning, single large doses were used, however, in the 1920s, side-effects of radiation became apparent. Claude Regaud sterilized ram testes with radiation and discovered that a single dose caused necrosis of the skin, however, if radiation was delivered in small daily fractions over a period of weeks, the animal could be sterilized with a minimal scrotal skin reaction. In this way, fractionated RT was born. Furthermore, in 1912, it was discovered that skin reactions were less severe if the radiation source was pressed tightly to the skin (102). Later the direct correlation between radiosensitivity and access of oxygen was discovered (103, 104). In 1955, Thomlinson and Gray proposed that oxygen levels within a tumor decreased with the distance from the capillary within a tumor and that radioresistant hypoxic cells might re-grow if allowed to re-oxygenate after radiation therapy (105). These initial and other later scientific efforts formed the basis of the safe and effective RT being used today, which is the second most effective treatment modality in cancer after surgery (106, 107). Even though RT still has a role as monotherapy for some cancer presentations, it is more commonly used as a component of multimodality managements, and more than half of all cancer patients require RT during at least one stage of their care (106).

Ionizing radiation consists of subatomic particles (e.g. alpha and beta particles, neutrons) or electromagnetic waves (photons) in the short wavelength end of the electromagnetic spectrum (X-rays and γ-rays) and is energetic enough to detach electrons from atoms or molecules (ionizing them). RT is the medical use of ionizing radiation as part of cancer treatment and may be used in a curative or palliative setting. The source of ionizing radiation for use in external beam RT is most commonly a linear electron accelerator (linac) (Figure 4). Until quite recently, radioactive material like cobalt-60 was used, which is still important in developing countries and for research purposes. The absorbed dose of ionizing radiation in tissues is measured in Gy

(Gray), and 1 Gy is the absorption of 1 Joule per kilogram of water. The old term rad is still in use, and 100 rad is equal to 1 Gy.

The ability to delineate normal and adjacent tumor tissue by CT/MRI/PET scans has improved during recent years. Furthermore, the development of two- and three-dimensional treatment planning, intensity-modulated RT and image-guided RT has provided the basis for better ensuring accurate beam placement to allow higher doses to be applied within the target volume while simultaneously protecting adjacent normal organs (102).

Figure 4. The author is preparing for irradiation of mice at the Radiation Department, Norwegian Radium Hospital, using a linac.

2.2.1 Radiobiology

As high-energy radiation traverses a cell, it mainly interacts with electrons, ejecting some of them from atoms (ionization) and raising others to higher energy levels within an atom or molecule (excitation). These resulting secondary electrons may excite or ionize other nearby atoms, giving rise to a cascade of ionizing events. Ionization and excitation lead to breakage of chemical bonds and formation of broken molecules (107). A 1-Gy X-ray dose results in 10^5

ionization events per cell, producing 1-2000 single-stranded breaks (ssb) and 40 dsb of the DNA. Cells undergo a critical period after irradiation, which determines their fate: death, repaired damage, or continued growth and division without complete repair (106). Most decisive of cell fate is the induction of both ssb and dsb in DNA but damages to the cell membrane and other structures may as well be important. These cellular events initiate very complex signaling cascades on the molecular level that result in a variety of responses, including cell cycle arrest, DNA repair and apoptosis (programmed cell death) (Figure 5) (108). Another phenomenon is the bystander effect, whereby irradiated cells exert effects on neighboring unirradiated cells, thought to be mediated by diffusible substances (109, 110). Hence, not only the tumor cells but also the tumor microenvironment (e.g. the host stromal component within a tumor) contribute to radiation responsiveness (106).

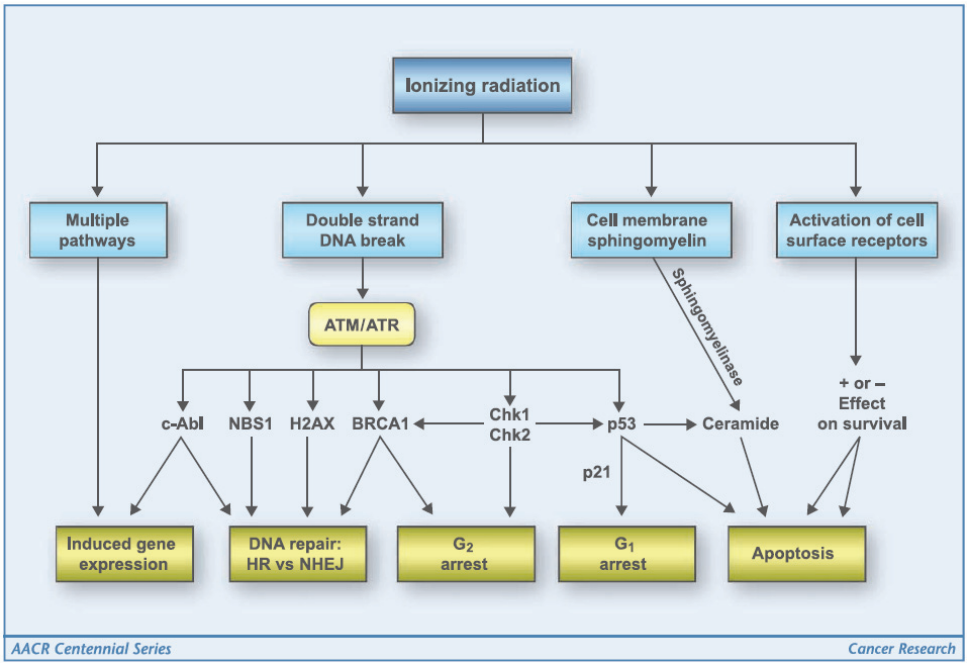

Figure 5. A simplified overview of some of the cellular pathways involved in response to ionizing radiation. Reprintet with permission from Cancer Research 2009 (106).

Cellular radiosensitivity is influenced by intrinsic factors, such as phase of the cell cycle, activation of apoptotic programmes, DNA break repair proficiency and accumulation of genetic

mutations in oncogenes and tumor-suppressor genes, and extrinsic factors such as oxygen, nutrients and metabolic waste elimination (102). To selectively kill tumor cells and spare normal tissue, the response of tumor tissue must be greater than that of normal tissue for the same radiation dose, i.e. a favorable therapeutic index (Figure 6).

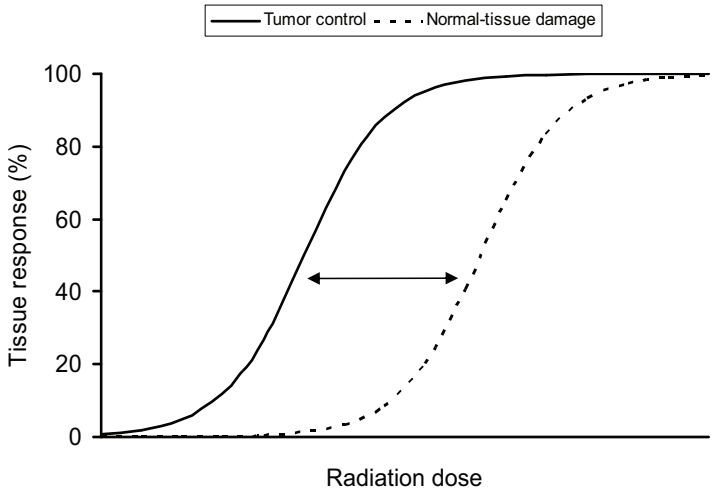

Figure 6. Therapeutic index (arrow) as function of radiation dose. A favorable outcome implies that the response of tumor tissue is greater than that of normal tissue (large therapeutic index), in contrast, similar responses of tumor tissue and normal tissue implicate an unfavorable outcome (small therapeutic index). Figure made by author.

As Claude Regaud discovered at an early stage, a series of fractionated radiation doses, instead of using one single large dose, amplify the therapeutic differential between normal tissue and the tumor for several reasons. These are easily recollected as the 4 Rs, as described by Withers: Repair, repopulation, redistribution and reoxygenation (111). DNA repair capacity differs between tissues, and in general, normal tissues like connective tissue, kidney, and spinal cord are capable of greater repair than malignant tissues if allowed enough time after a radiation dose. Thus, by spacing dose fractions by at least 6 hours (normally 24 hours), the DNA damage recovery in normal tissue is greater than that of tumor. Because cell killing is logarithmic rather

than linear, the difference in each day's effect is amplified exponentially. During an extended course of RT, cells that survive irradiation proliferate (repopulate) and thereby increase the number of cells that must be killed when the next dose is given. The rate of repopulation in the average tumor is less than that of normal tissues. This regenerative response allows normal tissue (e.g. mucosa) to tolerate a larger dose over time. Redistribution, or reassortment, is based on the differential sensitivity to radiation in different phases of the cell cycle. Cells that survive a first dose of radiation tend to be in a resistant phase of the cell cycle and within a few hours, they may progress into a more sensitive phase. Late-responding normal tissue tends to be essentially static in the division cycle, in a phase that allows repair. Cell cycle with relation to radiation will be discussed later.

The response of cells to ionizing radiation is strongly dependent on oxygen (103). Without oxygen, the free radicals induced by ionizing radiation will react with free protons, thereby "neutralize" the effect of the free radicals (107), i.e. hypoxia causes relative radioresistance. Tumors larger than 1 mm in diameter become partially hypoxic due to poor vascularization (112), whereas normal tissues are normoxic. After a single dose of radiation, the surviving tumor cells are mainly from the hypoxic cell fraction. Subsequent to radiation, the hypoxic fraction falls, i.e. the tumor is reoxygenized, by means of different mechanisms, resulting in increased radiosensitivity (107).

These 4Rs modify the response of a tissue to repeated doses of radiation and are responsible for the increased therapeutic index. A fifth R was proposed by Steel et al, namely intrinsic radiosensitivity (113). Different tumors and different tissues respond in a varying degree to the same single dose of radiation, i.e. are harboring different radiosensitivity, which should be considered together with the above mentioned 4 Rs as a determinant of response.

In conventional RT, daily fractions of 1.8-2.0 Gy to a weekly dose of 9.0-10 Gy are normally used. More than one fraction per day (hyperfractionation) with at least 6 hours in between to allow sufficient repair in normal tissues may also be used (102).

Cell cycle and radiation

A cell proliferates by performing an orderly sequence of events, in which it duplicates its DNA content and divides in two identical daughter cells. This cycle of events is known as the cell cycle. The cell cycle is divided into four defined phases: S, M, G_1 and G_2 phase. During the S (synthesis) phase, DNA synthesis occurs and in M (mitosis) phase, chromosome segregation and cell division take place. Between S- and M-phases are the two regulatory "gap" phases, G_1 and G_2 which are important for cell cycle regulation (114).

Cell cycle checkpoints are mechanisms by which the cell actively halts progression through the cell cycle until it can ensure than an earlier process, such as DNA replication or mitosis, is complete (115). The G_1 phase contains an important restriction point before the cell enters S phase. If conditions are unfavorable, the cell can delay progress through G_1 or even enter a specialized resting state known as G_0. The cell can remain permanently in G_0 or until growth signals tell it to recommence cell division (114). Ionizing radiation causes breaks in the phosphodiester bonds in the backbone of the DNA helix. When two of these breaks are close to each other, but on opposite DNA strands, a dsb is present and the cell faces a particularly challenging situation for repair. To accomplish this, an arrest of cell cycle progression is rapidly engaged through the activation of ATM (ataxia telangiectasia mutated), which is a kinase that phosphorylates numerous substrates upon activation. Among them, the transducer kinases CHK1 and CHK2 increase the level of p53, this in turn increases transcription of the gene for p21, an inhibitor of cyclin dependent kinases (CDK). The Cyclin E/CDK2 complex promotes G_1-S phase transition through phosphorylation of the retinoblastoma protein (which in turn releases transcription factors necessary for entry into S phase), and inhibition of these events by p21 leads to G_1 phase arrest and allows the cell to repair the DNA damage before DNA replication is to occur in S phase. The G_2 checkpoint prevents cells to enter mitosis when the cell experiences DNA damage when in G_2 phase or when G_1 checkpoint fails (such as in cells defective in p53). ATM, through CHK1, inhibits a family of phosphatases called cdc25, that normally activate CDK1 at the G_2/M boundary (Figure7) (115).

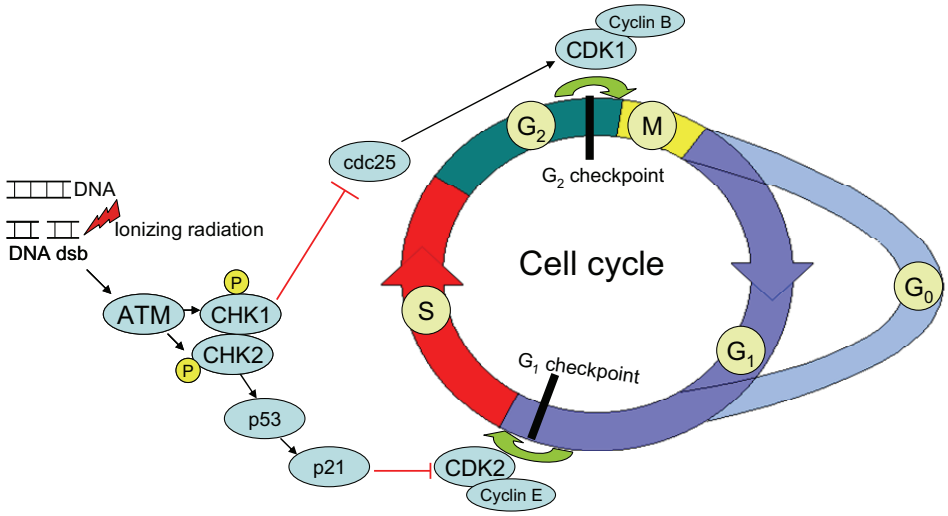

Figure 7. Simplified overview of the cell cycle and checkpoint activation after DNA damage. Given functional checkpoints, ionizing radiation induces both G_1 and G_2 phase arrest. Figure made by author.

There are several aspects of how the cell cycle and its regulation are important with respect to ionizing radiation. Mutations in genes encoding factors of the regulatory pathways, as described above, are major contributors to the development of cancer (116). After radiation, normal cells are allowed to stop cycling to ensure DNA repair, whereas cancer cells harboring defect checkpoint activation will continue to cycle, passing through mitosis with unrepaired DNA breaks and lethal chromosomal aberrations, and progressing into mitotic cell death (also known as mitotic catastrophe), which is considered the most important death mechanism caused by ionizing radiation (108). Furthermore, the radiosensitivity of cells varies considerably as they pass through the cell cycle. Cells in G_2, M, and G_1 phases are relatively radiosensitive compared to cells in S phase, and this principle is used in both fractionated RT and experimentally with radiosensitizers to synchronize cells in a relatively radiosensitive phase of the cell cycle before radiation is applied (107).

DNA damage and repair following radiation

Ionizing radiation induces several types of damage to DNA, including ssb, dsb, DNA-protein cross-link, base damage, intra-strand cross-link and inter-strand cross-link. Most of these aberrations are successfully repaired by different mechanisms. However, the incidence of cell killing is only strongly correlated to the amount of DNA dsb (107) and, secondly, if dsb is left unrepaired or repaired improperly, dsb causes chromosomal aberrations such as translocations, amplifications, or deletions, which may be lethal or result in oncogenic transformation (117). A cell's ability to survive following radiation is highly dependent on intact dsb repair systems (118), which is also exemplified by patients harboring mutations in genes involved in these repair systems, e.g. patients with ATM or NBS1 mutations, who are prone to cancer development and have severe normal tissue reactions following RT (107). Based on these observations, it is generally believed that dsb lesions are critical for cell killing by radiation.

At the site of a dsb, a multiprotein complex is rapidly formed. This can be visualised by immunostaining and is known as ionizing radiation-induced foci. It is not known in detail how dsb is recognized but ATM has a particular affinity for DNA ends, and this end-binding activity suggests that ATM might be a sensor for DNA dsb (117). Histone H2AX is rapidly phosphorylated by ATM (resulting in γH2AX) along the dsb site, and this protein assists in the assembly of other repair proteins (among them the NBS1 protein) (119). There are two major pathways for repairing dsb; homologous recombination (HR) is a highly accurate process that requires large regions of homologous sequence as a template, whereas non-homologous DNA endjoining (NHEJ) simply joins broken ends together, thereby often generating deletions, insertions, or base pair substitutions. As HR requires a homologous sequence of DNA (a sister chromatid) to repair the dsb, this repair pathway is taking place only in late S-phase, M-phase and in the G_2-phase. NHEJ is the main pathway in the G_0-, G_1- and early S-phase (Figure 8) (117).

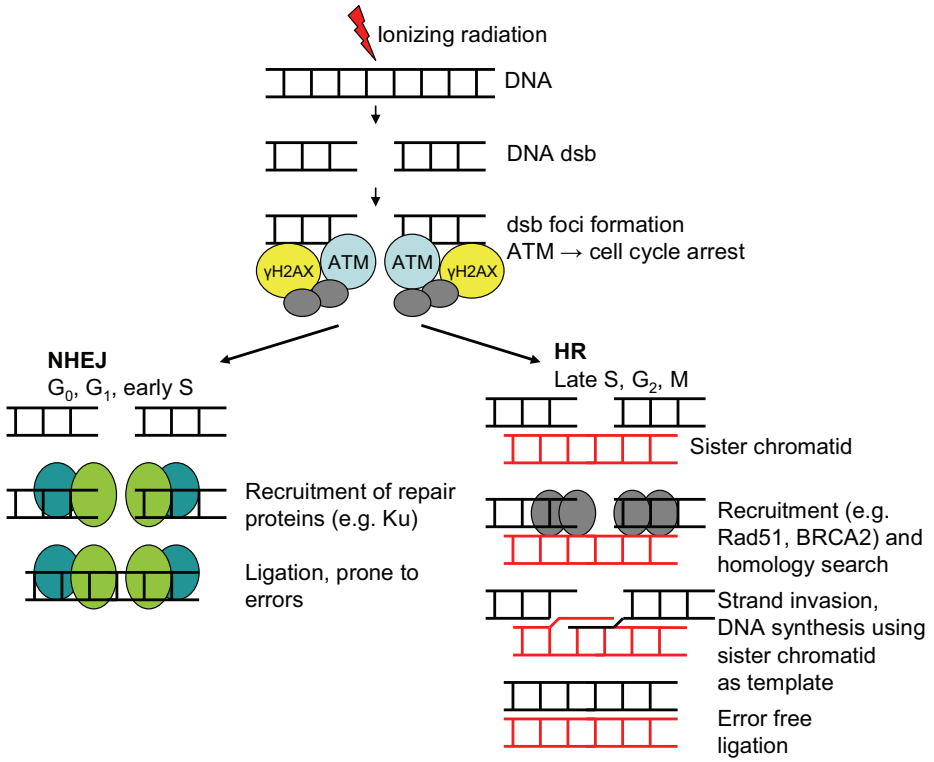

Figure 8. Non-homologous endjoining (NHEJ) and homologous recombination (HR) are the two main pathways involved in DNA dsb repair following ionizing radiation. Figure made by author.

Tumor cells often harbor defects in DNA dsb repair genes and these mutations give rise to genomic instability and facilitate cancer development (115). Following radiation exposure, tumor cells are more prone to lethal unrepaired DNA damage than that of normal cells. Cell cycle regulation and DNA dsb repair are tightly coordinated through p53, and if cells are not repaired efficiently, p53 may induce apoptotic cell death. Important for cell death following radiation is the slow death response of mitotic catastrophe, where cells may die within 1-2 turns of the cell cycle related to misrejoining during mitosis caused by persistent dsb (108, 120). In addition, certain irradiated cells will remain metabolically active but incapable of division, a situation called senescence or sustained cell cycle arrest (108).

Radiosensitivity

Radiosensitivity is described by the radiation dose required to produce a defined level of cell inactivation (107). In 1956, Puck and Marcus developed an assay to quantify cell killing – the cell survival assay, also called clonogenic survival assay (121). This technique is based on the fact that one reproductively viable cultured cell can divide infinitely and generate a large colony of cells, and cells that fail to form self-sustaining colonies may undergo limited numbers of cell divisions forming undetectable microcolonies, remain as single cells or disappear. The most extensively used model to describe radiation effects in cells is the linear-quadratic formula, defining cell survival as $S = (e^{-[\alpha d + \beta d^2]})^N$, in which S = surviving fraction, d = dose per fraction, α represents the linear initial slope of the line (at 0 dose). β is a measure of the downward curvature of the line at higher doses (the quadratic component of cell killing), and N is the number of fractions. Using a single fraction, the formula therefore is $S = e^{-[\alpha d + \beta d^2]}$ (122). A cell survival curve is a plot of the surviving fraction as function of radiation dose. The survival curves are normally plotted on a logarithmic scale (Figure 9).

The linear quadratic model assumes that lethal radiation damage is created in either of two ways: as a consequence of a single ionizing event (e.g. a dsb) or of two separate sublethal ionizing events that interact pairwise to create lethal damage (e.g. two closely ssb). In the latter case, the damage is considered repairable if allowed enough time between the two sublethal events. Thus, the amount of lethal damage from the last component is dependent on repair rate of sublethal damage. α and β can be interpreted as the probability of inducing the first and the second form of damage, respectively, and the α/β value represents the dose at which the two types of damage are equal. At doses less than α/β, the first type of damage predominates, and at doses above, the second type of damage predominates. Tissues possessing small α/β are said to have more sparing capacity than those with higher α/β values. This means that a reduction in dose per fraction will reduce cell kill relatively greater in low α/β-value tissues in tissues with high α/β-values (123). The explanation is mainly that tissues with a low α/β also have a long repair half-time, which is the time required between fractions for half of the maximum possible repair to take place (107).

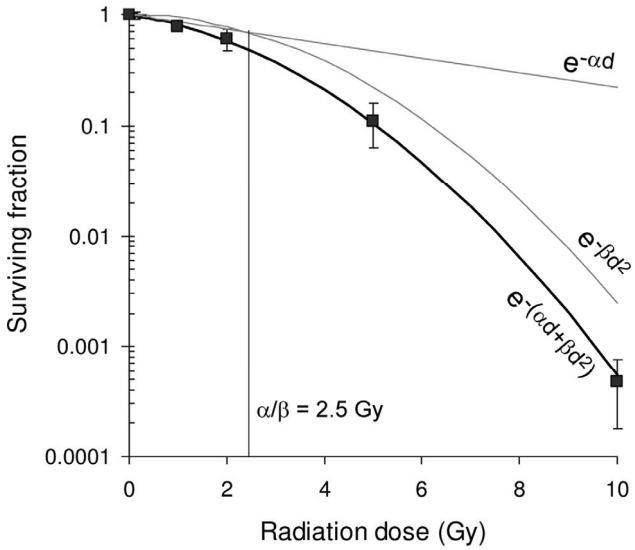

Figure 9. Clonogenic survival of the colon carcinoma cell line RKO (squares), mean of three independent experiments and bars representing ± one standard deviation. The black curved line shows the linear quadratic relationship between cell survival (surviving fraction) and radiation dose, plottet on a log-linear scale, and fits well into survival of RKO cells following a single fraction of radiation. The curved grey line and the linear grey line indicate the two components of cell killing. At the intersection between these two lines, the two components of cell killing is equal, and this occurs at a dose of 2.5 Gy, i.e. $\alpha/\beta = 2.5$ Gy (indicated by the vertical linear line). Data partly from paper III, figure made by author.

In normal tissues with rapidly proliferating cells (acute responding tissues, e.g. intestinal epithelium, bone marrow and skin), the α/β is relatively high (as for tumors), and severity of acute side effects increases with increasing dose and is seen during or within a few weeks after treatment. These effects are transient compared to responses seen in late-responding tissues (such as subcutaneous tissue, brain, kidney, liver, intestinal wall), in which adverse effects are seen months or years after RT and are often progressive. The severity of acute effects increases with increasing dose (as cell kill of tumor tissue), while late side-effects are more sensitive to changes in fraction size (124). For late responding normal tissues, the expected biological equivalent dose (BED) is described as $BED = D \times [1 + d/(\alpha/\beta)]$, where D is the total radiation dose and d is the dose per fraction. Using this formula, one can calculate the change in total dose necessary to achieve equal tissue response when the dose per fraction is varied. By

optimizing fractionation schedules, it is possible to spare late responding tissues with a relatively smaller effect on tumor cell kill, thereby increasing the therapeutic index (107, 123).

2.3 Chemoradiotherapy (CRT)

2.3.1 Rationale for combining RT with chemotherapy

In a clinical setting, the aim of combining chemotherapy with RT is to improve local control. The introduction of a second modality (chemotherapy) potentially increases both tumor cell kill and normal tissue toxicities, and in order to evaluate benefits, the concept of therapeutic index is essential. Therapeutic gain is only achieved if the combined-modality regimen results in improved tumor response under comparable levels of toxicity. Otherwise, the improved effect could have been obtained by merely increasing the radiation dose (107).

In 1979, Steel and Peckham proposed several potential advantages of combined chemotherapy and RT (125). *Spatial cooperation* describes the situation in which disease in some particular anatomical site that is missed by one therapeutic agent is dealt with adequately by another. By this modality, RT aims to control local disease, whereas chemotherapy aims to control disease outside the radiation field, e.g. distant micrometastasis. *Toxicity independence* refers to when two partially effective anticancer agents can be given without any requirement for reducing the individual doses and because of independent differential and acceptable toxicity profiles, additive improvement in therapeutic result can be expected (effect of agent A + B). For neither of the mentioned mechanisms, interaction between the modalities is needed. *Protection of normal tissues* refers to a possible agent that allows a greater dose of radiation to be given than would have been tolerated otherwise. This will be therapeutically beneficial only if tumor cells are not similarly protected. At last, *enhancement of tumor response* is the situation in which administration of one agent apparently increases the effect of another or in which the effect of a combination appears to be greater than would be expected. For both of the two latter mechanisms, interaction between concomitantly administered modalities will take place. Enhanced tumor response may also be caused by an indirect interaction by killing subpopulations resistant to the other modality and may, therefore, not necessarily change the survival curve as seen below.

In cell survival curves derived from clonogenic experiments, interaction between modalities would mean a change in the shape of the curve. If the steepness of the dose-response curve is increased (bending down), the response is enhanced (radiosensitization) and in the case of protection, the curve is bending up. If chemotherapy results in a fixed amount of cell kill, but no interaction occurs, the dose-response curve will shift to a lower surviving fraction without changing its shape. To more easily compare the shape of curves, the curve of the combined chemotherapeutic agent together with radiation is usually normalised to the cytotoxic effect of the chemotherapeutic agent alone (Figure 10) (107). An important limitation using the clonogenic assay is that it relies on established tumor cell lines (since normal cells are difficult to grow in cultures and if they do, they are not considered normal any more) and can not be used to assess effects in normal tissues. Secondly, the above mentioned non-interactive mechanisms of advantages in combined chemotherapy-radiotherapy treatment can only be assessed in animal or clinical trials (102).

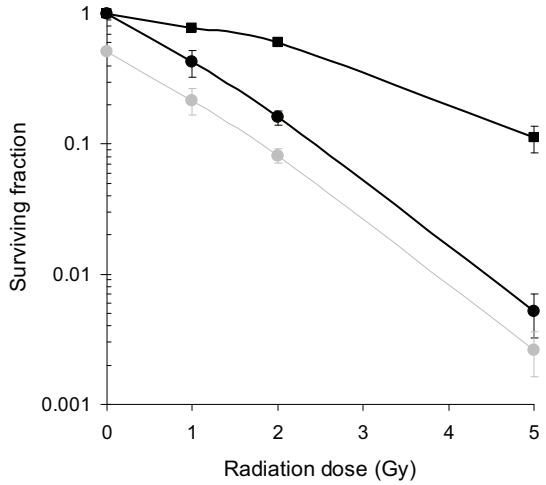

Figure 10. Cell survival curve of RKO cells exposed to radiation only (squares) or pretreated with 100 nM Trichostatin A (TSA) for 18 hours before radiation exposure (circles). Mean of 3 independent experiments, and bars representing one standard deviation. The cytotoxic effect of TSA alone was 49 ± 5% (mean ± one standard deviation) (gray line). The TSA-treated radiation curve is normalized to this effect, which makes it easier to see a downward bending of the curve, i.e. that exposure of TSA before radiation is applied sensitizes the cells to the effect of radiation. Data from paper III, figure made by author.

A new framework for considering drug-radiation combinations has been suggested after Steel and Peckham, encompassing five mechanisms (126). *Spatial cooperation* is mentioned above, and important for this mechanism is full-dose of both modalities. The modalities are typically administered sequentially, as in the case with NACT given before CRT in the LARC-RRP study. Only clinical trials or metastatic animal models can demonstrate this effect. *Cytotoxic enhancement* is the strategy described above in which the *in vitro* clonogenic survival curve is modulated and tumor cells are sensitized to radiation. For this mechanism, the drug must be present at time of or in proximity to radiation. *Biological cooperation* refers to strategies that target distinct cell populations, such as a drug that target the radioresistant hypoxic fraction of the cells in a tumor. *Temporal modulation* is an approach to enhance the tumor response to fractionated therapy, and typically, the two modalities should be delivered concomitantly or in a rapidly alternating schedule. Candidate drugs could for instance be inhibitors of DNA repair selective for tumor cells. *Normal tissue protection* was also proposed by Steel and has been discussed above. An example is stimulation of stem-cell proliferation in early-responding tissues.

Interaction between chemotherapy and RT may be explained by several mechanisms, and for most agents several interactions apply at the same time (43):

- DNA damage may be caused by both chemotherapy and RT, and a non-lethal ssb may become more difficult to repair if, for instance, a cisplatin adduct is close to the ssb.
- Chemotherapy can inhibit post-radiation damage repair. DNA synthesis and repair share common pathways, which provide the rationale for using DNA synthesis inhibitors, such as 5-FU, together with radiation.
- Administered concurrently, RT and chemotherapy often target different phases of the cell cycle, such as 5-FU targeting the S phase cells and radiation targeting the other cell cycle phases.
- Some drugs are also able to synchronize the cells within the cell cycle, to allow increased efficacy of subsequent RT (also called cell cycle pooling).
- Targeting of hypoxic cell fraction in a tumor with a drug has been discussed, but also shrinking of the tumor mass will increase perfusion and oxygenation of the cells in a tumor and reduce the radioresistant cell fraction.

- Repopulation of rapidly proliferating tumors is usually mediated by overactivation of growth factors or downstream signalling pathways, and agents that target S phase of the cell cycle (5-FU) as well as growth factor pathways (EGFR inhibitors) may effectively prevent tumor cell repopulation thereby sensitize tumor cells to radiation.
- Inhibition of prosurvival and "poor prognosis" markers, e.g. EGFR inhibition.

2.3.2 5-fluorouracil/capecitabine

5-FU is a halogenated pyrimidine nucleoside analogue and its metabolites impede nucleic acid synthesis through thymidylate synthase inhibition, thereby depleting the pool of nucleotide triphosphates and causing inhibition of DNA synthesis. Furthermore, the metabolites are also incorporated into RNA and DNA, thus inhibiting both transcription and protein synthesis (127). The drug has been used in treatment for colorectal cancer for decades (128), and is also extensively used with radiation. It is often combined with folinic acid since this drug enhances and prolongs the inhibition of thymidylate synthase caused by 5-FU. When 5-FU is given concomitantly with RT, a lower and non-cytotoxic concentration is typically used, and radiosensitization has been demonstrated in experimental models (129, 130). Interaction between ionizing radiation and 5-FU is caused by several mechanisms (131). 5-FU kills radioresistant S phase cells, and the drug causes increase in tumor blood flow and abrogation of radiation-induced G_2-arrest. Depletion of thymidine also inhibits repair of DNA dsb. Sensitization is enhanced in cells defective in the G_1 checkpoint (e.g. mutated *TP53*) since these cells progress into S phase after radiation and are then killed by 5-FU. Preclinical studies suggest that 5-FU should be present for a prolonged period before, during, and after radiation for optimal radiosensitization (129). However, 5-FU, which is administered intravenously, and its active metabolites have short half lives (132), which led to the search of oral prodrug alternatives.

Capecitabine is a fluoropyrimidine carbamate that is converted to 5-FU in a 3-step enzymatic reaction *in vivo*, the final step involving thymidine phosphorylase. Thymidine phosphorylase has higher concentrations in many tumor types compared to normal tissue and, secondly, radiation itself can selectively induce thymidine phosphorylase activity in tumor tissue (133). Toxicity of 5-FU primarily involves rapidly proliferating tissues and includes epithelial

ulceration throughout the gastrointestinal tract (mucositis, esophagitis, gastritis, colitis with diarrhea, nausea/vomiting) and myelosuppression (128). Capecitabine has correspondingly gastrointestinal side effects, but less myelosuppression, and may cause painful swelling and erythema in the hands and under the feet, as well as fatigue (134).

2.3.3 Oxaliplatin

Cisplatin has been used for anticancer therapy since the 1970s and has improved the prognosis in ovarian and especially testicular cancer. However, cisplatin has little effect in colorectal cancer and is associated with severe side effects (renal toxicity, nausea, neurotoxicity). In an attempt to overcome these side effects, less toxic platinum analogues were developed, and as a result, carboplatin and, later, oxaliplatin were discovered (135). As previously discussed, oxaliplatin was found to be effective in combination with 5-FU in metastatic colorectal cancer, and disease-free survival was improved when oxaliplatin was added to 5-FU based adjuvant treatment in stage II and III colon cancer (136). Importantly, oxaliplatin increases both gastrointestinal and myelosuppressive side effects caused by 5-FU and sensory neuropathy is common in patients receiving this drug (135).

Oxaliplatin shows similar chemical behaviour and has comparable mechanisms of action as other platinum derivatives. Its antitumor effects are thought to be related to Pt-DNA adducts, but irreversible binding to proteins may also contribute. DNA adducts are formed in guanine and adenine, and since Pt may attach to two different nucleotide bases, both intra- and interstrand links are created. This in turn will modify the three-dimensional DNA structure, which inhibits normal DNA synthesis and repair. Binding of oxaliplatin to proteins will normally inactivate their function and enzymes important in repair of the DNA adducts may covalently bind to oxaliplatin and impair their function (137). If substantial DNA damage persists, it may ultimately lead to activation of the apoptotic pathway and cell death (138). Furthermore, the effect of oxaliplatin and 5-FU in combination has in *in vitro* and *in vivo* experimental models shown synergistic effects, also in 5-FU resistant cell lines (137).

Cisplatin acts as radiosensitizer (139) and is a commonly used drug in CRT for malignant diseases other than rectal cancer. The increased combined effects are caused by radiation-

induced enhancement of cellular uptake of the drug, inhibition of DNA repair, cell cycle perturbations, and production of more lethal DNA damage (43). This and the above mentioned increased effect of oxaliplatin seen in clinical trials in colon cancer led to the hypothesis that oxaliplatin as a novel chemotherapeutic agent in LARC could improve preoperative CRT results, both in terms of local control and prevention of distant metastases (140). Oxaliplatin has been incorporated in treatment of LARC in many studies, both as NACT, adjuvant chemotherapy and concomitantly with 5-FU based CRT. In the latter, the main aim is to increase local control by sensitizing cells to radiation (141). However, at least in the beginning of the work of this thesis, preclinical evidence of oxaliplatin as radiosensitizer was scarce, which led to questioning whether oxaliplatin might act as radiosensitizer in preclinical models (paper I).

2.3.4 Histone deacetylase inhibitors

Cancer has traditionally been considered to be a disorder of genetic defects, such as gene mutations and deletions or chromosomal abnormalities that result in loss of function of tumor-suppressor genes or gain of function or hyperactivation of oncogenes. However, gene expression may be altered by epigenetic changes, which have been shown to be crucial to the onset and progression of cancer (142). Epigenetics is the study of heritable changes in gene expression that are not due to any alteration in the DNA sequence (143). Examples are chromatin remodelling causing changes in the access of transcription factors to genes and DNA methylation, in which methyl group are bound to cytosine residues, thereby silencing the gene without changing the genetic code.

The packaging and compaction of DNA into chromatin is critical for essentially all DNA metabolic processes, including transcription, replication and repair. The primary repeating unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around an octameric structure composed of two copies of the four core histones; H2A, H2B, H3 and H4. These are dynamic structures that can be altered by at least three different processes; incorporation of histone variants (e.g. γ-H2AX important for DNA repair), replacement, repositioning, or removal of nucleosomes by chromatin remodelling complexes that alter access to DNA, and finally, chromatin structure can be modified by coordinated post-translational

modifications on histone residues such as acetylation of lysines, serine phosphorylation, methylation of lysines and arginines, or polyribosylation and ubiquitylation (144). Most of these modifications are reversible, and since the changes are not necessarily heritable, it has been questioned whether these modifications are truly epigenetic (145).

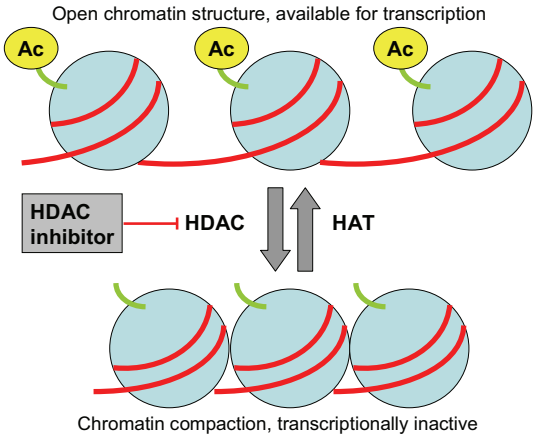

Figure 11. Function of HDAC inhibitors. DNA (red) is wrapped around a histone octamer (blue). Lysine residues (green) on histones are targets for acetylation. The opposing effects of histone acetyl transferases (HATs) and histone deacetylases (HDACs) regulate gene expression through transferral or removal of acetyl groups (yellow) to histones, respectively, leading to changed accessibility of regulatory proteins to DNA. HDAC inhibition will lead to hyperacetylated histones. Figure made by author.

The most studied of these modifications is histone acetylation, which is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) as shown in Figure 11. Generally, histone acetylation promotes a more relaxed chromatin structure, allowing transcriptional gene activation (145, 146). However, specific patterns of histone acetylation and deacetylation are influenced by other histone modifications, such as methylation. Together, these post-translational modifications generate a “histone code” and additionally, cross-talk between histone modifications and DNA methylation also occurs, demonstrating the complexity of transcriptional regulation by histone acetylation (147). Importantly, HDACs also have many non-histone protein substrates that regulate cell proliferation and death and therefore, the alternative name “lysine deacetylases” instead of HDAC has been suggested.

Acetylation can either increase or decrease function or stability of these proteins. 18 HDAC enzymes have been identified. Class I HDACs (HDAC1, 2, 3 and 8) are localized in the nucleus and ubiquitously expressed, whereas class II HDACs (HDAC4, 5, 6, 7, 9 and 10) are localized both in the nucleus and cytoplasm and are tissue-restricted in expression. These two classes have a zinc ion at the catalytic pocket and are inhibited by hydroxamates, as will be discussed below. Class III HDACs, also called sirtuins, are not inhibited by these compounds. Class IV HDAC consists of HDAC11 (146).

HDACs are required for cell cycle progression and are aberrantly recruited to promoter regions of tumor-suppressor genes in many cancers, thereby repressing those. In addition to recruitment of HDACs to specific loci, different HDACs are overexpressed in many tumor types, such as HDAC2 and HDAC3 in colon cancers. Correspondingly, global hypoacetylation of histone H4 is a common hallmark of tumors (142). During the last decade, targeting HDACs with inhibitors has become an emerging therapeutic concept. In the 1970s, sodium butyrate was observed to cause morphological reversion of a transformed cell phenotype and later, the compound was found to be an HDAC inhibitor. Today, numerous HDAC inhibitors are available and may be classified according to their chemical structures or which HDACs they inhibit: Hydroxamates (e.g. TSA and SAHA which inhibit class I and II), benzamides (e.g. MS-275 which inhibits class I), cyclic peptide (e.g. depsipeptide inhibits class I) and the aliphatic acids (e.g. valproate and butyrate which inhibit class I and II). The quest for inhibitors of specific HDACs is ongoing, and the only one found is tubacin, which inhibits HDAC6 only, and is responsible for acetylation of tubulin with no effect on histones. Whether or not these specific inhibitors will have lower toxicity profiles while maintaining anticancer activity remains to be determined (142).

Expression levels of 7-10% of all genes were altered using a twofold change as a cut-off value in various cell lines following incubation with HDAC inhibitors from all 4 classes mentioned above, and the compounds were found to induce about as many genes as were repressed (146). The therapeutic potential of HDAC inhibitors stems from their capacity to selectively induce cell death in tumor cells at concentrations that are minimally toxic to the host. Apoptosis is induced by both the extrinsic and intrinsic apoptotic pathway due to alteration of the pro- and

antiapoptotic balance or due to activation of specific proteins upstream of these pathways. Most known HDAC inhibitors induce G_1 arrest, which is mediated through p53 independent activation of p21 and inhibition of DNA synthesis proteins, whereas G_2 arrest is a much rarer event. The compounds also have anti-angiogenic effects caused by downregulation of pro-angiogenic genes (142).

HDAC inhibitors are used in clinical trials as single agent therapy, in combination with chemotherapeutic agents, and recently, in combination with RT (148). The potentially favorable combinatory use with RT is based on preclinical data. All tested HDAC inhibitors have shown radiosensitization *in vitro* and for four of the drugs, this has also been demonstrated *in vivo* (for MS-275, Valproic acid, depsipepside and CBHA). Paper II includes the fifth of these, namely SAHA. Based on *in vitro* experiments, a correlation between histone hyperacetylation and enhancement of radiosensitivity was suggested, and hyperacetylation should be present both before and sustained some time after radiation exposure to induce optimal sensitization. Furthermore, histone acetylation was proposed as a molecular marker in the design of treatment protocols when combining HDAC inhibitors and RT to comply with the *in vitro* findings (149). The role of histone acetylation as a molecular marker is further exploited in paper II.

No radiosensitization by HDAC inhibitors has been observed in fibroblasts, normal breast, and intestinal cells, suggesting a selective effect on tumor cells. The mechanisms of enhanced radiation response are only partially understood. Inhibition of DNA dsb repair as measured by prolonged γH2AX foci following radiation is detected, whereas repair of other types of DNA damage has not been identified. This may be due to decreased levels of DNA dsb repair proteins (Ku70, Rad51) but could also be due to acetylation of DNA repair proteins (ATM, Ku70, p53). The number of DNA dsb is increased following combinatory exposure, suggesting that change in chromatin structure makes cells more vulnerable to form dsb. Cell cycle arrest as described above may also contribute, but low nontoxic concentrations of HDAC inhibitors that do not induce cell cycle effects have a radiosensitizing effect as well (149-151).

SAHA

SAHA and TSA are similar in chemical structures, and both bind to the zinc ion in the catalytic pocket of class I and II HDACs and act as non-specific pan-HDAC inhibitors. TSA is a naturally occurring antifungal compound and was the first high-affinity inhibitor to be identified. TSA is quite cytotoxic and is unstable under *in vivo* conditions, thus this compound is not recommended for treatment of patients, although the compound has proven to be important in studying HDAC inhibition effects *in vitro* (149, 152). In contrast, SAHA alters cellular acetylation patterns and causes growth arrest and death in a wide range of transformed cells, both *in vitro* and in animal tumor models, and at concentrations that are non-toxic to normal cells (148). *In vitro* experiments suggest tumor cell lines to be 10-fold more sensitive to SAHA-induced cell death than normal fibroblasts (153). Among the numerous novel HDAC inhibitors being tested in clinical trials, SAHA is the only compound approved by the US Food and Drug Administration (October 2006; for treatment of cutaneous T-cell lymphoma). The most common adverse effects by SAHA are fatigue, diarrhea, thrombocytopenia and dehydration (152).

SAHA regulates expression of 2-10% of all genes. Mostly studied is the induction of p21, which induces G_1 phase arrest. SAHA causes changes in the promoter region of p21, whereas no change has been found in genes not regulated by SAHA, hence, suggesting selectivity in altering gene expression (154). SAHA also down-regulates expression of thymidylate synthase, consequently, exposure to SAHA prior to 5-FU induced synergistic effects between these two compounds in colorectal carcinoma cells (155). Increased acetylation levels of transcription factors (p53, HIF-1α, E2F) and cytoplasmic proteins (α-tubulin, cortacin, heat shock protein 90) may also contribute in SAHA-induced cell cycle arrest, cell death, and the inhibition of tumor growth seen in many xenograft models, although the specific mechanism of action is not known. Interestingly, SAHA given to carcinogen-treated rats and mice reduces tumor incidence, multiplicity, and volume. SAHA has shown synergistic activity in combination with a wide range of chemotherapeutic agents (e.g. kinase inhibitors, cytotoxic agents) (148). *In vitro*, SAHA radiosensitizes cell lines from various cancers and has been shown to increase the number of radiation-induced DNA dsb and repair of dsb as assessed by γH2AX foci (156). This is probably mediated by inhibition of the NHEJ pathway, apparently by reducing the expression

of repair related genes such as Ku70, Ku80, Rad50, DNA-PK, and Rad 51. Acetylation of p53 helps stabilizing the protein following DNA damage and may contribute to radiosensitization by pooling cells in the G1 cell cycle phase. Cell cycle arrest has already been mentioned as a possible mechanism (151). Presently, five clinical trials of the combined treatment with RT and SAHA are registered in addition to the PRAVO study (157).

3 Aims of the study

Preoperative chemoradiotherapy (CRT) in locally advanced rectal cancer (LARC) is intended to shrink intrapelvic tumor to facilitate complete surgical removal, with the intention to improve local tumor control. However, the effect of CRT may vary from complete response to lack of objective response; also, preoperative CRT postpones surgery and has adverse effects. Thus, the two most important challenges in preoperative CRT in LARC are to improve treatment efficacy and to predict response to enable treatment stratification.

The main objectives of this work were:

I To assess potential radiosensitizing effects of oxaliplatin and SAHA in relevant colorectal carcinoma cell line and xenograft models (paper I and II)

II To investigate the relevance of HDAC2 deficiency for radiosensitization by HDAC inhibition in colorectal carcinoma cell lines (paper III)

III To determine whether kinase activity profiles in pretreatment tumor biopsies can predict tumor response to preoperative CRT in LARC (paper IV)

4 Summary of papers

Paper I: ***Inhibitory effects of oxaliplatin in experimental radiation treatment of colorectal carcinoma: Does oxaliplatin improve 5-fluorouracil-dependent radiosensitivity?***

The aim of this investigation was to study potential radiosensitizing properties of oxaliplatin in colorectal carcinoma cell lines in the absence and presence of 5-FU, and to examine whether the addition of oxaliplatin to capecitabine and radiation might improve radiation-induced tumor growth inhibition in a colorectal carcinoma xenograft model.

In vitro, a 2-hour pre-exposure of oxaliplatin to two different cell lines enhanced the effect of radiation in a concentration-dependent manner. Combining oxaliplatin with post-radiation exposure to 5-FU enhanced the radiosensitization seen with 5-FU alone. Oxaliplatin exposure caused delayed G_2/M phase arrest, however, since this occurred after radiation was applied, a possible cell cycle pooling in these radiosensitive cell cycle phases could not explain the radiosensitization observed *in vitro*.

In the *in vivo* model, mice bearing bilateral xenografts were treated with daily 2-Gy radiation fractions Monday through Friday for two consecutive weeks and were given oral capecitabine on the same days as RT was applied. Oxaliplatin was administered once weekly (Mondays). Capecitabine alone did not inhibit tumor growth, but when combined with oxaliplatin, growth inhibition was significant and comparable to the inhibition seen with radiation alone. Capecitabine enhanced radiation-induced tumor tumor growth delay but the addition of oxaliplatin to this regimen did not significantly improve the effect.

Taken together, this work demonstrates *in vitro* radiosensitizing effect of oxaliplatin with or without 5-FU, however, this effect was not seen in the *in vivo* situation, questioning the rationale for incorporating oxaliplatin into 5-FU based CRT regimens in LARC.

Paper II: ***Radiosensitization by SAHA in experimental colorectal carcinoma models – in vivo effects and relevance of histone acetylation status***

In this work, SAHA was evaluated for its ability to induce histone acetylation and to enhance radiation effects in *in vitro* and *in vivo* colorectal carcinoma models. Furthermore, the general assumption that histone hyperacetylation at time of radiation exposure is required to obtain optimal combinatory effects, was questioned.

In vitro, SAHA caused rapid induction of H3 and H4 acetylation, which was lost within 3 hours after removal of the compound. Radiosensitization, as assessed by clonogenicity, was observed when the radiation was applied at time of H3 and H4 hyperacetylation following SAHA exposure. Furthermore, radiosensitization was also seen, but to a lesser degree, when radiation was applied 3 hours after SAHA removal, on restoration of baseline histone acetylation.

In vivo, increased xenograft histone acetylation was detected 0.5-3 hours after intraperitoneal (ip) injection of SAHA in two xenograft models. No accumulation was seen following daily SAHA injections for 5 days. Fractionated RT combined with daily SAHA treatment caused significantly increased tumor growth delay compared to radiation alone, whereas SAHA monotherapy had no or only a modest effect on tumor growth. Radiation exposure 12 hours after SAHA injection, when histone acetylation levels were restored to baseline levels, also caused radiosensitization *in vivo* and surprisingly, in one of the xenograft models, a significantly improved effect compared with the situation when radiation was applied at maximal histone acetylation was observed. Acetylation of p53 and tubulin was detected with similar kinetics as for histones.

In conclusion, pre-exposure to SAHA induced both *in vitro* and *in vivo* radiosensitization. Hyperacetylation at the time of radiation exposure did not seem to be a permissive requirement for radiosensitization, suggesting that the usefulness of measuring histone acetylation as a biomarker in determining optimal treatment schedules of HDAC inhibitors in combination with RT is debatable.

Paper III: ***HDAC2 deficiency and histone acetylation***

This paper was a correspondence to the Nature Genetic letter by Ropero *et al.* in 2006 (158). The authors had hypothesized that an HDAC2 inactivating mutation in colon cancer cells may predict response to HDAC inhibitors. The background was their finding that histones H3 and H4 were not hyperacetylated following a 24-hour incubation with 250 nM TSA in *HDAC2*-mutated cell lines (RKO and Co115) compared to wild-type *HDAC2* cell lines. Furthermore, the *HDAC2*-mutated cell lines were resistant to TSA with respect to apoptosis, cell cycle arrest and xenograft growth. The authors concluded that cell lines with mutated *HDAC2* may show impaired responsiveness to TSA, which may have potential relevance for the pharmacogenetic selection of cancer patients to be treated with HDAC inhibitors.

Our research group was intrigued by these results and wanted to use this model to study the relevance of histone acetylation for radiation effect of HDAC inhibitors. The intention was to expose *HDAC2*-mutated colorectal carcinoma cell lines to HDAC inhibitors to observe whether radiosensitization was induced or not in cells that had lost the propensity of histone hyperacetylation upon TSA exposure. However, when reexamining the results from Ropero et al, we found that TSA (100 and 250 nM) induced transiently increased hyperacetylation of H3 and H4, which was restored to baseline levels after ~12-18 hours of continuous TSA exposure. This was shown in both RKO cells at our laboratory, newly purchased RKO cells from American Type Culture Collection (ATCC) and RKO cells kindly provided from Ropero's research group. Among the three RKO variants, only RKO cells from Ropero's research group were deficient in HDAC2 expression. To verify the identity of the RKO cell lines and to exclude inadvertent contamination or mislabeling, DNA fingerprinting analysis of the three RKO variants was performed. This examination revealed that the three cell lines most likely were from the same origin but that the cell line from Ropero harbored distinct discrepancies when compared to the other two variants.

TSA treatment caused histone hyperacetylation as well as radiosensitization of RKO cells regardless of HDAC2 status and consequently, we believe that HDAC2 status need not be taken into account when investigating HDAC inhibitors as radiosensitizers in colorectal carcinoma.

Paper IV: ***Prediction of response to preoperative chemotherapy in rectal cancer by multiplex kinase activity profiling***

Histological tumor response of rectal cancer to preoperative CRT varies from complete remission to no objective response. Activity of kinase signaling pathways has been shown to be predictor of radioresponse. This study aimed to investigate whether intrinsic kinase activity profiles of pretreatment tumor samples by use of a novel peptide array platform might predict the individual tumor responses to preoperative CRT in LARC.

67 LARC patients were treated with two neoadjuvant cycles of oxaliplatin and 5-fluorouracil and subsequent CRT consisting of oxaliplatin, capecitabine, and radiotherapy before surgery was performed 6-8 weeks after CRT completion. Prospectively collected pre-treatment tumor biopsies were analyzed using microarrays with kinase substrates and the resulting substrate phosphorylation patterns were correlated with the individual tumor responses to the preoperative treatment as assessed by histomorphologic tumor regression grade (TRG).

73% and 15% were scored as good responders (TRG 1–2) or intermediate responders (TRG 3), respectively, whereas 12% were assessed as poor responders (TRG 4–5). The ability to predict response to therapy was evaluated using leave-one-out cross-validation from phosphorylation patterns of 144 peptide substrates in the array using PLS-DA as classifier. In a subset of 7 poor responders and 12 good responders, treatment outcome was correctly predicted for 95%. When building a prediction model from these samples, correct prediction of 85% in the remaining patient samples was found. In tumors from poor responders, peptide substrate phosphorylation indicated significantly higher kinase activity mediated by signaling pathways implicated in radioresistance.

Multiplex kinase activity profiling may assist in predicting tumor response to preoperative CRT in LARC. However, because the number of patients, especially poor responders, was limited, the ultimate robustness and clinical applicability of the analytical approach and the resulting classifier need to be evaluated in larger, independent studies.

5 Methodological considerations

Cell cultures

In vitro cell cultures were used to study clonogenicity and biological effects of treatment with radiation and chemotherapy. All cell lines were originally derived from colorectal adenocarcinomas (Table 3).

Table 3. Characterization of colorectal cell lines

Cell line	Derived from	Anatomical site	Patient (sex/age)	Differentiation	*TP53* status	Established (year)	Reference
HT29	Primary	Rectosigmoid	Female/44	Moderate	Mutated	1964	(159)[1]
HCT116	Primary	Colon	Male/-	Poor	Wild-type	~1981	(160)[1]
RKO	Primary	Colon	-	Poor	Wild-type	~1983	(161)[1]
SW620	Lymph node	Colon	Male/51	-	Mutated	1971-5	(162)[1]
Co115	Primary	Ascending colon	Female/77	Poor	Mutated	1976	(163)

[1]Information was also found at www.atcc.org.

Working with cell lines has obvious advantages: They grow continuously, have an unlimited lifespan, allow the performance of various experiments and based on their clonal origin, apparently have homogenous and specific phenotype and genotype. In addition, they are easily available and culture conditions are simple and easily standardized among different laboratories. However, cell lines in general are prone to genotypic, karyotypic and phenotypic drift during prolonged culture. In addition, sub-populations may arise by the selection of specific, more rapidly growing sub-clones that may result in inter- and intralaboratory cell line heterogeneity (164). Such changes make cell lines more and more different from their origin. Moreover, the origin of a cell line is often an aggressive and fast-growing tumor, which may not represent normal more slow-growing cancers in patients. Cell cultures also lack the normal microenvironment and importantly, cell-cell interactions between tumor and stromal cells are absent. Finally, a human tumor consists of many heterogeneous cancer cells with various phenotypes and genotypes. These features must be considered when translating pre-clinical cell line-based treatment effects into a clinical setting.

Cell line clonogenicity

The classic assay from 1956 essentially tests every cell for its ability to undergo unlimited proliferation and reflects all modes of cell death, also when cells divide for a few cycles before dying. Compared to other faster and less laborious assays, it is considered the best assay to study radiation effects *in vitro* (165). Moreover, this assay has been shown to predict the response of drugs seen in animal xenograft models and to some degree clinical response (166, 167). Still, it has to be kept in mind that effects *in vitro* do not necessarily translate into an effect of the same direction or magnitude *in vivo*. Typical problems are that drug concentrations *in vitro* are often higher than those that can be achieved *in vivo* and that the drugs are not homogeneously available to tumor cells *in vivo*. Furthermore, cell-cell interactions and microenvironmental factors known to be important for radiation sensitivity and resistance are more or less lacking (168).

Experiments are performed in essentially two different modes: Cells may be plated before treatment, or cells are treated in dishes and subsequently plated in appropriate dilutions to assess clonogenic ability (169). For the experiments performed in this thesis, I used the first variant to avoid counting the cells more than once, which reduces the variation of the number of seeded cells between treatment modalities within the same experiment. However, the limitation is that the cells can not be treated too long because radiation must be applied after cells have attached to the bottom of the plates and before the cells start to divide. Radiation was always applied 24 hours after seeding, which allowed enough time to pre-treat cells but not allow cells to divide.

Xenografts

Subcutaneous growth of human tumor cell lines in immunodeficient athymic nude mice is a well established and accepted model, in which the tumor cells, in contrast to the situation in *in vitro* experiments, can interplay with the microenvironment (e.g. connective tissue, vasculature), hence, the model is considered more “natural” than monolayer growth of tumor cells in tissue flasks with unlimited access to nutrients. However, it may be discussed how “natural” colorectal cancer cells are when growing in subcutaneous tissue in a mouse.

Orthotopic models exist in which colorectal cancer cells grow in their organ of origin (170), but measuring radiation effects in such models is technically difficult.

Figure 12. An athymic female nude mouse bearing bilateral HCT116 xenografts in the rear flanks. Picture made by author.

Since establishment in 1975, xenograft models have contributed substantially in screening of treatment effects (171). Because mice with defective immune system do not reject the foreign tissue, the tumors usually grow unchecked unless stopped by effective treatment. However, xenografts do not behave like naturally occurring tumors in humans, e.g. they do not metastasize to other tissues. Xenografts, at least those used in the experiments of this thesis, have been established from cell lines being cultured for various time periods *in vitro*. Two concerns have been raised using xenograft models in treatment screening. Firstly, the treatment tested may appear effective in mice but work poorly in humans, and secondly, treatment with little effect in mice will not be further investigated and consequently, effective treatment may be missed. However, these models are the best available and ethically acceptable models we have. There is correlation between effects seen in xenograft models and human cancer, but without absolutely prediction of effects seen in the clinic (172). Thus, as for *in vitro* experiments, caution should be made when interpreting findings from xenograft experiments and only clinical trials can give the true answer.

Flow cytometry analysis

Cell cycle profiles were analyzed by means of flow cytometry. Cells were stained the propidium iodide fluorochrome, which binds to DNA. When excited by laser at 488 nm light,

propidium iodide can be detected using a 562-588 nm filter. DNA content in each cell can then be measured in thousands of cells per second by flow cytometry. Cells in G_1 phase have two copies of each chromosome, whereas cells in G_2/M phase have four copies (Figure 13). Cells in S phase have between two and four copies of their DNA.

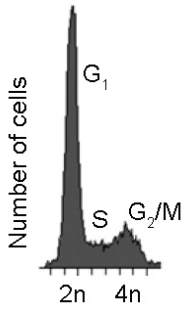

Figure 13. Cell cycle profile of HT29 cells as detected by flow cytometry. Number of cells as function of DNA content is shown. DNA content of 2n corresponds to two copies of each chromosome (G_1 and G_0 phase cells), whereas the double amount of DNA corresponds to cells in the G_2/M phase as DNA replication has occurred during S phase. Data from paper I, figure made by author.

Western blot immunostaining

To detect protein expression, total protein lysates from cell cultures or xenografts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies before incubation with a horseradish peroxidase-linked secondary antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Radiation

Whereas high-energy radiation from ^{60}Co (Cobalt) sources was delivered to cells, both ^{60}Co and a linear accelerator were used in the animal experiments. The dose rate used in paper I was lower than that of used in paper II because our institution received a new Cobalt source in the meantime. The linear accelerator was used because no Cobalt source was available for a 9-month period. For *in vitro* experiments, up to 4 plates could be irradiated simultaneously by placing the plates in room temperature in the radiation field 80 cm from the source. Clinically relevant doses of 2 and 5 Gy were used. In the animal experiments, mice were placed so the

tumor was at least 10 mm inside the radiation field while the rest of the mouse was outside. Accurate radiation dose deposition was ensured by using both mini-ionization chambers and thermoluminescence dosimetry, and daily 2-Gy fractions were given Monday through Friday, similar to the fractionated RT given in a clinical setting.

Figure 14. Anesthetized mice were aligned along the radiation field so that the tumors were kept inside the radiation field while the rest of the body was placed outside to minimize radiation deposition to other parts of the mouse (left). A 20 mm polystyrene build-up bolus was placed on top of the tumors to give homogeneous and maximal dose deposition within the target volume (right). Eye ointment was applied because mice do not close eyes during anesthesia. Picture made by author.

In paper I, radiation was only applied to one side (unilaterally), whereas the contralateral tumor was the unirradiated control. This led to two problems. First, abscopal effects might have influenced growth of the unirradiated tumor. Second, the unirradiated tumor grew much faster than the irradiated and the mouse had to be killed before a 5-fold increase of the irradiated tumor was reached. Thus, in paper II, both sides of the mouse were irradiated by simply turning the mouse around, whereas control mice were not irradiated at all.

Tumor growth delay

In 1969, Barendsen and colleagues developed the tumor growth delay assay by using rats (173). By measuring tumor volume changes as function of time, radiation effects with and without a potential enhancer of the radiation effect can be quantified by looking at the time needed for a tumor to reach a specific volume, e.g. a doubling of the pretreatment volume. The difference in time needed to reach this volume in presence of a drug is defined as tumor growth delay.

This assay reflects the effect of treatment on clonogenic as well as non-clonogenic cells with proliferative activity. A limitation is that it does not measure those few clonogenic cells that survive after high-dose irradiation. Whether these few cells are killed or not determines the outcome of RT in a curative setting (174). An alternative animal tumor model has been suggested to be used in order to detect killing of these few resistant cells, namely the TCD_{50} assay developed by Suit in 1966. In this assay, groups of animals bearing tumors of the same size are irradiated with different doses with and without the agent of interest and the mice are observed in a sufficient time period thereafter with respect to recurrence. By plotting the proportion of tumors that are locally controlled as a function of radiation dose, the dose at which 50% of the tumors are locally controlled (TCD_{50}) can be determined. Theoretically, a single surviving proliferative cell left in the xenograft will then subsequently cause recurrence (102, 168). However, this assay needs a large number of mice, which raises animal ethical challenges. The assay is labor-demanding, expensive and difficult to perform using fractionated radiation. Additionally, curation by CRT alone is not yet the aim in LARC. This justifies measurement of tumor growth delay as the assay of choice when studying potential radiation response enhancement in LARC.

Chemotherapy

For *in vitro* studies, the chemotherapeutic agents were diluted in culture medium from frozen stock solutions immediately before addition to cell cultures. In the animal experiments, capecitabine was suspended in Arabic gum and then given to the mice orally by the use of a gavage. Oxaliplatin was given iv diluted in 5% glucose and SAHA was given ip diluted in dimethyl sulfoxide (DMSO). All agents were freshly made before use. For the animal experiments, the potential radiosensitizers were given daily or weekly, according to the protocols, in order to imitate the clinical situation as closely as possible. Capecitabine was given immediately before radiation and during anesthesia to minimize discomfort for the mice.

Kinase activity profiling

Sample preparation

In rectal carcinomas the tumor is normally available for biopsy sampling without causing too much discomfort for the patient and 3-5 biopsies were collected from each patient. Tumor content was assessed histologically in frozen sections, allowing microscopy guided rough dissection to remove normal tissue, increasing the relative proportion of tumor cells in the samples. Tumors are heterogeneous, usually with increased hypoxia and necrosis in the central part of a tumor compared to the peripheral part. The latter are usually more available for biopsy sampling, thus, the biopsies do not necessarily represent the inner gross mass of the tumor. Moreover, the effects on the biological samples of immediate freezing in liquid nitrogen, storage, handling, cutting and, finally, making lysates in room temperature are mainly unknown. These factors must be kept in mind when interpreting the results.

Tyrosine Kinase PamChip® Array technology

With the intention of studying kinase activity as potential predictor of CRT response, we chose the Tyrosine Kinase PamChip® Array technology (Figure 16), which allows functional comparison of biological samples that indicate the state of information flow mediated by kinases. Other methods, such as immunoblotting, IHC, mass spectroscopy, or enzyme-linked immunosorbent assay, identify phosphorylated proteins that represent the end products of kinase activity. An advantage of the technological platform is that small sample quantities are required, typically 2-5 μg total protein being sufficient. One main challenge of the used technology was that the computational tools for data analysis were at an early stage of development, and no general consensus approach to data validation existed.

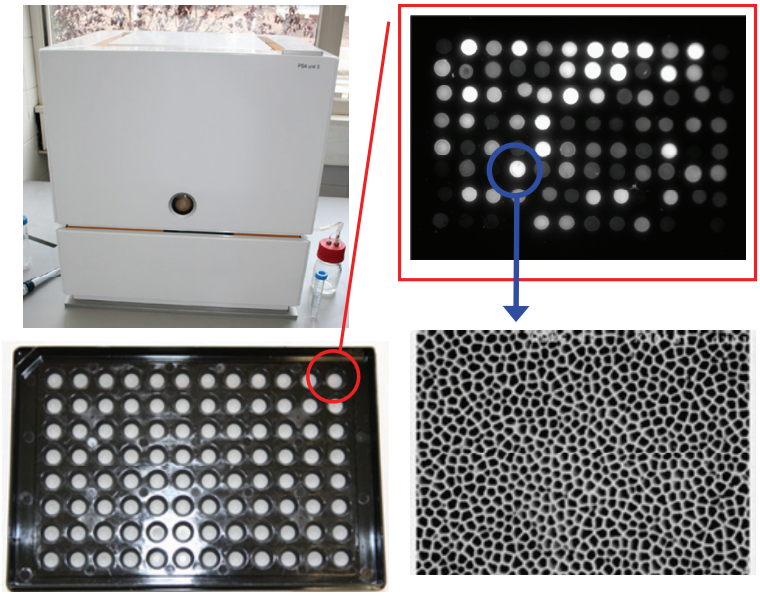

Figure 16. Tyrosine Kinase Pamchip® Array technology. The 96-well Pamchip unit (lower left) is analyzed in the PamStation® (upper left). The PamStation® consists of a pump, which pumps the sample back and forth in each array (well) of the unit, and a specialized camera detects light intensity for each of the 144 peptides (upper right) induced by FITC-conjugated phosphotyrosine antibody bound to phosphorylated peptide substrates attached to the porous structure (lower right). A high degree of phosphorylation for a specific substrate leads to high signal intensity. Figure made by author.

Data analysis

The strategy for analyzing phosphopeptide signatures of the tumor samples and correlation to TRG was developed during the analytical process. The methods were derived from previously reported gene expression analysis and other high-throughput technologies in similar settings, but had to be adapted and the analysis is thoroughly described in the manuscript. It will be briefly summarized below with some specific comments.

From each sample, the peptide substrate signal intensity was calculated by averaging technical replicates after removal of signal outliers. Correction for plate variation was necessary, and this

was performed by normalizing each peptide signal to the mean peptide signal intensity of the TRG 1-2 samples in each Pamchip®96 unit. After normalization, this variation was minimal (Figure 17).

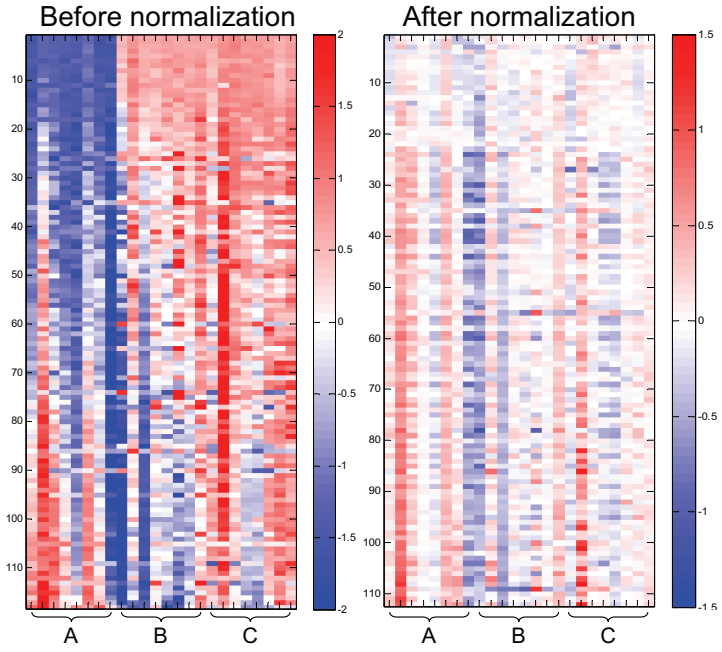

Figure 17. Color map illustrating signal intensities before (left panel) or after (right panel) normalization to mean signal intensities of good responders (TRG 1-2) within each Pamchip®96 unit. Patient samples sorted according to three of the analyzed arrays (A, B and C) are shown along the horizontal axis and peptides along the vertical axis. Red means high signal intensity and blue represents low signal intensity. Clustering according to the arrays was seen in the non-normalized panel; in particular, array A samples are different from samples on arrays B and C. After normalization, no obvious clustering was seen. Figure made by Rik de Wijn (PamGene International B.V.) and author.

A total of 67 patient samples were analyzed in 9 different units, and samples were applied blinded for TRG status. TRG 1-2 were defined as good responders, TRG 3 as intermediate responders and TRG 4-5 as poor responders. The samples were divided into two sets; a training set and a testing set. The training set consisted of 7 poor responders and 12 good responders, whereas the testing set consisted of the remaining patient samples.

TRG/Plate	Total	Plate A	Plate B	Plate C	Responder group
TRG 1	5	0	3	2	Good
TRG 2	7	3	2	2	
TRG 3	4	1	1	2	Intermediate
TRG 4	7	4	1	2	Poor
TRG 5	1	0	1	0	

Table 4. Distribution of 24 patients samples according to TRG in the three analyzed Pamchip®96 units used for the testing set.

Peptides with generally low signal intensities were left out, leaving 86 of the initial 144 peptides for further analysis. Before applying a classification method to high dimensional data, such as gene expression profiling or, as in our case, kinase arrays consisting of 86 peptides, dimension reduction is necessary. This is essential because most classification methods require the sample size (i.e. number of patients) to be larger than the number of variables. Dimension reduction basically aims to "summarize" the numerous predictors in form of a small number of new components before applying a classification method to the constructed components (175). Importantly, several complex methods for dimension reduction exist, and the below mentioned approach was chosen.

Binary treatment response class prediction was performed applying partial-least-squares discriminant analysis (PLS-DA), and a leave-one-out cross-validation (LOOCV) was used to estimate the prediction performance in the training set. With LOOCV, one sample is left out and the remaining samples are used to build a classifier, which is then used to classify the left out sample. The fraction of samples that are classified correctly provides unbiased estimates of prediction error (71). Initially, all 8 poor responders were used in the training set, however, the single TRG 5 patient was classified as good responder, and to obtain a robust classifier we chose to exclude this sample from further analysis. Finally, a classification model based on the entire training set was built and used to predict response in the testing set.

The large number of available biostatistical approaches for analyzing high dimensional data for predictive purposes indicates that there is no consensus as to which is the best method. This is an area of intensive research, and improvements can be expected in the future. Many models suffer from the risk of overfitting the data, meaning that the model classifies the training set

well, but future samples are classified poorly. However, the model used in this work is considered a relatively conservative strategy (176), and a reliable prediction can be expected given high sensitivity and specificity in an independent testing set.

6 Discussion

6.1 Future treatment options in LARC

New CRT regimens

Combination of new chemotherapeutic drugs with today's standardized 5-FU-based CRT is being extensively studied. In the majority of studies, the new combinatory regimens are derived from efficacy studies in metastatic colorectal cancer. Irinotecan was shown to improve outcome in metastatic colorectal cancer (177, 178), and in 2004, a randomized controlled trial confirmed that the combination of oxaliplatin and 5-FU was superior to the irinotecan/5-FU combination in this setting (179). Furthermore, inhibition of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) has been shown beneficial in metastatic colorectal cancer (180-182). In clinical trials for rectal cancer, all these compounds have been incorporated as part of CRT regimens (183).

New agents under clinical investigation in CRT for LARC are summarized below:

- Irinotecan: Phase II trials have found a dose-dependent improved pCR rates (0-24%) with irinotecan implemented in CRT with acceptable toxicity (184, 185), and a German study found pCR of 15% (186). A recent phase III trial reported no improved efficacy and increased toxicity of irinotecan when implemented in postoperative CRT after a 3-year follow-up (187).
- Cetuximab: The inclusion of this EGFR-targeting antibody in preoperative CRT has been studied in several phase I or II trials, but with a somewhat disappointing low pCR rate of 8% in two of the conducted studies (188, 189). It has recently been suggested that a fraction of LARC tumors, those with wild-type *K-ras* and high *EGFR* gene copy number, may be sensitive to preoperative treatment with cetuximab (83).
- Gefitinib: This tyrosine kinase inhibitor, selective for EGFR, has been shown to be well tolerated when added to 5-FU based CRT in LARC and with a pCR rate of 30% (190).
- Bevacizumab: Inhibition of VEGF signaling via this anti-VEGF antibody was shown in a phase I rectal cancer CRT study to have antivascular effects and encouraging tumor downstaging rates (191). Importantly, this antiangiogenic therapy in rectal cancer has

been associated with increased toxicity rates and higher than expected rates of colonic perforations (192). A phase III study randomizing to capecitabine and oxaliplatin with or without bevacuzimab is ongoing (193).

- Celecoxib: This selective cyclooxygenase-2 inhibitor, which influences the prostaglandin synthesis, induced rash in 50% of LARC patients, and the trial thus was terminated (194).
- Oxaliplatin: Several phase I and II studies, including the ongoing LARC-RRP study, have been conducted with oxaliplatin combined with 5-FU based CRT in LARC with relatively high pCR rates of 16-28% (183, 195-197). Randomized phase III trials are still recruiting patients (Table 5) (51, 198-200).

Table 5. Ongoing phase III trials with or without oxaliplatin in neoadjuvant CRT in LARC

Name of trial (country of origin)	Study arms	CRT (50 Gy in 2-Gy fractions)	Adjuvant chemotherapy
PETACC-6 (Germany, Europe)	Arm A Arm B	CAP CAP/OXA	CAP CAP/OXA
CAO/ARO/AIO-04 (Germany)	Arm A Arm B	5-FU 5-FU/OXA	5-FU 5-FU/OXA
ACCORD 12/0405 (France)	Arm A Arm B	CAP CAP/OXA	
NSABP R-04 (US)	Arm A Arm B Arm C Arm D	5-FU 5-FU/OXA CAP CAP/OXA	

Abbreviations: CAP – capecitabine, OXA – oxaliplatin.

Chemotherapy without RT

Neoadjuvant chemotherapy (NACT) refers to chemotherapy given without RT and before surgery, usually with full-dose combination regimens given before preoperative CRT, which is also the case in the LARC-RRP study, but chemotherapy may also be given postoperatively as adjuvant treatment. Since local recurrence rates have decreased to a level of 6-8% with new CRT regimens but without documented effect on metastatic spread and overall survival, NACT and adjuvant chemotherapy have been suggested to be the focus for the future direction in order to eradicate micrometastatic disease (201).

Chemotherapy may be an important intervention at an early stage of the disease, and at a stage where the patient is fit enough to tolerate full systemic doses of chemotherapy. It is also speculated whether NACT also induce shrinkage of primary tumor, thereby improving resectability and CRT response (202). For this reason the term "induction chemotherapy" has been proposed (17). However, NACT will delay both CRT and surgery, and in the case of cervical and head-and-nech cancer, NACT consistently failed to show any benefits in clinical trials (202). Phase II studies with NACT in rectal cancer have demonstrated promising results on local control, but effects on systemic disease have not been reported (196, 203). In ongoing phase III trials (Table 5), chemotherapy is administered postoperatively for a prolonged period of 4-5 months (198, 200). Currently, no evidence exists whether administration of postoperative chemotherapy in LARC patients with clear margins is beneficial (204).

Non-surgical approach

Since around 25% of LARC patients undergoing today's neoadjuvant treatment end up with pCR (42), a strategy of close observation without immediate surgery has been suggested as an initial approach in a highly selected patient group with no detectable residual tumor after neoadjuvant treatment (72). A research group at the University of São Paulo School of Medicine conducted a trial that included patients with resectable distal rectal cancers (also T2 tumors) to undergo neoadjuvant treatment and then omit surgery with excellent survival data (205). They further claimed that if failure after neoadjuvant treatment should be detected at a later stage, delayed surgery will be safe and will not influence survival (206). However, no reliable method for predicting pathological complete response exists, and the inclusion of low-stage tumors might have affected the good survival rates in this study. A review concludes that this "wait-and-see" policy is insufficiently documented and should only be applied in patients who are recognized to be unfit for or refuse radical surgery (207). A prospective study with a non-operative approach is ongoing in the UK, and the patients enter a rigorous program of MRI and clinical and endoscopic follow-up, with urgent surgery if any evidence of recurrence is found (42).

Intraoperative radiotherapy (IORT)

IORT allows high radiation doses to be applied locally to the tumor bed after resection while the dose-limiting structures are displaced during radiation exposure. IORT was used by our own institution until late 1990s, but was terminated because no definite effect of the treatment was seen (208). The treatment was also resource-demanding as the patients needed to be transported to the RT department under anesthesia during surgery. However, in recent years, different dedicated facilities have evolved, such as mobile IORT units that can be employed in any operating room without the need for specific shielding or room construction requirements. Even though no randomized trials have been performed, different reports are encouraging (209) and the technique may have a renaissance in the future.

6.2 Oxaliplatin and SAHA as radiosensitizers in LARC

In view of future treatment options, oxaliplatin and SAHA as potential new agents to be incorporated in preoperative CRT were evaluated in experimental colorectal carcinoma models in papers I and II. The role of the compounds as part of NACT or adjuvant chemotherapy was not assessed, as the main aim of chemotherapy given independently of RT is to eradicate micrometastatic disease, the so-called *spatial cooperation* effect, which can not be evaluated in the used model systems. Crucial for new compound to be integrated in CRT is the ability to potentiate the radiation effects in order to improve local control.

Oxaliplatin

As seen above, oxaliplatin is extensively being studied in clinical trials as potential candidate in preoperative CRT of LARC. However, scarce pre-clinical evidence of the potential benefits had been reported at the onset of these trials. In paper I, the combined oxaliplatin/radiation effects were evaluated in colorectal carcinoma cells and xenografts.

In vitro clonogenicity demonstrated that oxaliplatin acted as radiosensitizer in colorectal carcinoma cells, and this was also shown in combination with 5-FU. It should be noted that the concentration range used (5-40 μM) is beyond a clinically obtainable concentration as a 2-hour iv injection of a normal patient dose (85 mg/m^2) reaches a peak plasma concentration of 3.6 μM

(210). When adding oxaliplatin to fractionated RT and daily capecitabine in a colorectal carcinoma xenograft model, we could not detect an additional effect. This experiment harbored a couple of limitations worth to be mentioned:

- All left-sided tumors were irradiated with the contralateral tumor as unirradiated controls. This approach reduced the number of mice that had to be used in the experiment. However, the unirradiated control tumors grew faster and we had to sacrifice the mice when the unirradiated tumors reached a critical size. Four of nine oxaliplatin/capecitabine/radiation-treated xenografts did not reach a 2-fold increase before the mice had to be killed. If allowed to grow further, a statistically improved effect in presence of oxaliplatin might have been found.
- Two weekly cycles of oxaliplatin were given to the mice. In the LARC-RRP study, five cycles are given. Even if we could not detect any significant effect after two cycles in mice, more than two cycles might have caused significant radiosensitizing effects. However, mice would not tolerate such long-lasting treatment.
- The dominating effect of radiation and capecitabine might have marginalized the contribution of oxaliplatin to the overall effect.
- The oxaliplatin dose given to the mice might have been too low, however, the dose given was the maximum tolerated dose (211). The dose given to the mice (10 mg/kg) equals 35 mg/m^2 (dose/body surface area) and a normal dose of oxaliplatin given concomitantly with radiation in humans is 50 mg/m^2.
- Adding a second model would have strengthened the evaluation of potential benefits of adding oxaliplatin in this setting.
- Oxaliplatin was given iv 30 minutes before radiation exposure of practical reasons. The concentration of oxaliplatin has been found to be peaking 2 hours after ip injection in another xenograft model (212).

Bearing these limitations in mind, as the only experimental model evaluating *in vivo* radiosensitizing effect of oxaliplatin in colorectal cancer, a conclusion that the integration of oxaliplatin into combined-modality treatment of rectal cancer is controversial can still be made. However, phase II trials, including the LARC-RRP study, report encouraging high pCR rates when oxaliplatin is implemented in preoperative CRT, but also higher toxicity rates, and results

from ongoing phase III trials will hopefully provide conclusive evidence. Preliminary results from two of these trials are expected in late June 2009.

SAHA

The exciting new group of compounds targeting histone acetylation, HDAC inhibitors, has demonstrated radiosensitizing properties. In paper II, SAHA was for the first time shown to be radiosensitizer in colorectal carcinoma cell lines as assessed by clonogenicity. Moreover, radiosensitization was observed in two colorectal carcinoma xenograft models.

To elucidate whether the concentration/dose ranges used in the experiments are relevant, a comparison to humans can be made. After a normal dose of 400 mg SAHA (vorinostat) in a person, a peak plasma concentration of 1.8 μM after 1 ½ hours is reached (213). This dose equals 232 mg/m^2 (dose/body surface area) in the author, whereas the dose of 100 mg/kg used in the mice experiments equals 357 mg/m^2. Hence, the concentrations used *in vitro* (1 μM) and *in vivo* are for practical purposes within a relevant range. However, ip injection of SAHA causes higher bioavailability than oral intake of vorinostat, which is the normal administration route in humans. The dose of SAHA injected to the mice induced histone acetylation in the xenograft, which implies that a sufficient concentration was achieved within the tumor as regards the most evident biological effect. Similarly, in the PRAVO study, tumor biopsies have been sampled 3 hours after oral intake of 100, 200, 300 and 400 mg vorinostat, with histone acetylation demonstrated at all dose levels (compared to corresponding pre-treatment biopsies; data by our research group, not yet published).

In conclusion, the results from the pre-clinical models render SAHA to be a promising candidate in preoperative treatment of LARC. For possible future integration into CRT, the effect of vorinostat must also be evaluated together with 5-FU or capecitabine. *In vitro* studies in colorectal carcinoma cell lines have demonstrated synergistic effect between 5-FU and SAHA when SAHA causes downregulation of thymidylate synthase (155). A recent phase I study has also shown that the combination of vorinostat with 5-FU and oxaliplatin (but without RT) is safe (214), and results from ongoing phase I trials will shortly provide results whether combination of vorinostat and RT is safe. Even though SAHA, with or without radiation, did

not show increased toxicity to the mice, it remains to be seen whether vorinostat increases normal tissue injury in humans as it apparently increases cancer cell radiosensitivity. Emerging data from the ongoing phase I PRAVO trial may turn out to be indicative in this regard. Preclinical experimental models evaluating combination of SAHA/5-FU/RT are also warranted. A new trial in preparation will study toxicity using preoperatively an HDAC inhibitor in combination with 5-FU in rectal cancer. If safe and indicative of being beneficial, efficacy studies may subsequently be undertaken.

Histone acetylation as biomarker for radiosensitivity

The assumption that histone hyperacetylation is a permissive requirement to obtain maximal combined HDAC-inhibitor/radiation effects was challenged in paper II. It has been suggested to use tumor histone acetylation, or mononuclear blood cell histone acetylation, as a biomarker to design *in vivo* antitumor protocols combining HDAC inhibitors and radiation. In the HCT116 xenograft model, we demonstrated that the combined SAHA/radiation effect was significantly improved when radiation was applied at restored baseline levels of histone acetylation following SAHA injection compared to when xenografts were irradiated at maximum histone acetylation, whereas the SW620 xenograft model showed similar responses in these two situations. We can not comprehensively explain the difference between the two models; however, the models harbor well-characterized molecular differences such as in *TP53* mutational status. As the delayed increased radiosensitivity was observed in one of the two xenograft models only, a conclusion about optimal timing of radiation application following drug administration can not be made. Yet, the usefulness of histone acetylation as biomarker of HDAC-inhibitors in combination with radiation is debatable. The practical implication of this finding is that exact timing of vorinostat administration relative to radiation in a future clinical setting does not seem to be as important as previously believed.

6.3 The HDAC inhibitor TSA in *HDAC2*-mutated cell lines

As detailed in the introduction, class I HDACs are ubiquitously expressed in tissues and are localized in the cell nucleus. The isoforms HDAC1, HDAC2, and HDAC3 have been investigated in colorectal cancer, and high expression is associated with aggressive tumors, low differentiation, and poor survival. In particular, HDAC2 has been shown to be an independent prognostic factor (215). 15-20% of colorectal tumors have microsatellite instability, causing *HDAC2* inactivation mutation in 20-43% of these tumors (158, 216).

In paper III, two versions of the RKO colorectal carcinoma cell line expressed HDAC2 whereas one cell line version did not. The most likely explanation is that a mutation has occurred during culturing to give rise to the HDAC2-defective subclone. This explanation has also been suggested by others (217). Loss of HDAC2 leads to resistance to HDAC inhibitor-induced apoptosis in cell lines, and it was suggested that loss of HDAC2 impaired cells' ability to acetylate histones by TSA (158). In paper III , we demonstrated that histones are hyperacetylated following SAHA and TSA treatment regardless of *HDAC2* mutational status, as has also been shown by others (216). Interestingly, cells cultured at high confluence, both HDAC2-defective RKO cells and wild-type *HDAC2* HCT116 cells, have been shown to lose the ability to become hyperacetylated following TSA exposure (216). This may be the reason for the transient histone hyperacetylation seen in paper III, as cells grew more confluently during continuous exposure, and may also explain the findings by Ropero *et al.* It has also been shown that transient siRNA-mediated repression of HDAC2 induces HDAC1, and vice versa, and that deletion of HDAC1 or HDAC2 alone does not change the phenotype, whereas deletion of both induces a range of defects (217). These findings indicate an overlapping function between these two proteins, and since both enzymes acetylate histones, and both are inhibited by TSA, it is unlikely that histones are not acetylated following TSA exposure in *HDAC2*-mutated cell lines. Our results confirm that both HDAC2-deficient and HDAC2-proficient RKO cells can be radiosensitized by TSA, and that HDAC2 status need not be taken into account when investigating HDAC inhibitors as radiosensitizers.

6.4 Prediction of CRT response in LARC by kinase activity profiling

Background

Protein kinases are enzymes that facilitate transfer of phosphate groups to amino acids upon activation, and may be tyrosine specific (tyrosine kinases) or threonine/serine specific. Protein kinase activation, and subsequent protein phosphorylation, is a key mechanism of information transfer in major signal transduction pathways relevant for cancer development (116, 218). Hence, targeting protein kinases with small-molecular inhibitors or antibodies that bind to and inactivate their function is an emerging therapeutic concept. More recently, the relevance of activated protein kinases in radioresistance and potential benefits of protein kinase inhibition in RT have been increasingly recognized (192, 219-222). One example is that increased tumor EGFR tyrosine kinase activity, or downstream signaling pathways, resulting in activation of the phosphateidylinositol-3-kinase (PI3-K)/protein kinase B (AKT), has been associated with radioresistance (219, 223). As proof of principle, Bonner *et al.* showed that cetuximab, which binds to the ligand-binding domain of EGFR, in combination with RT improved locoregional control and survival in advanced head-and-neck cancer compared to RT alone without increasing RT induced toxicity (224). In addition to theoretical considerations, these observations provide rationale for studying kinase activity profiling as a potential predictive marker of CRT response in LARC. The PamChip array technology is a novel platform that allows rapid, real-time measurements of phosphopeptide signatures generated by biological samples. PamGene International B.V. has two different microarray chips: one containing a panel of peptides with mainly serine/threonine phosphorylation sites, and one containing mainly tyrosine residues which was used in our studies (www.pamgene.com).

Tyrosine Kinase PamChip® Array technology for prediction of CRT response

In paper IV, pretreatment rectal cancer biopsies were analyzed for kinase activity, and we demonstrated that this technology may assist in predicting response to preoperative CRT. An accuracy rate of 95% in a subset of 7 poor responders and 12 good responders using LOOCV was detected, and furthermore, using a prediction model based on these 19 patients, an 85% accurate prediction was found in a testing set of 47 patients consisting of patients having intermediate or good pathological responses (TRG 1-3). This is comparable to what has been

found for gene expression analysis (71, 99-101) as prediction accuracies of 81%, 83%, 87%, and 89% by use of subsets of discriminating genes between responders and poor responders as assessed by tumor regression grade (TRG) or T-level downstaging was observed. Multiplex kinase activity is, therefore, a promising new tool in predicting response to preoperative chemoradiotherapy in rectal cancer.

The procedure of collecting biopsies, tumor content validation, and analysis is somewhat labor-demanding, but highly feasible. Patients have to undergo rectoscopy, but this is often performed as a standard procedure during pretreatment examinations. Other advantages are that the technology requires small volumes of tumor tissue.

The main challenge in this study was the finding of very few poor responding patients, which challenged the statistical adaptation. We needed all poor-responding patient samples in building the prediction model, and the testing set consisted of intermediate or good responders only.

For reasons detailed in the manuscript, a biological interpretation of the results is complicated. However, all peptides with discriminating signal intensities between poor and good responders showed that kinase activity in poor responders was relatively high. This is in agreement with the observation that kinase activation is associated with radioresistance.

Perspectives

Testing this technology in a larger scale is warranted to confirm the validity of the potential predictive tool; in particular, to validate prediction of poor responders. The variation between the PamChip®96 units necessitated normalization of the signal intensities in each Pamchip®96 array. Good responders in each unit were used to normalize signal intensities, and for future experiments, a set of reference samples are needed to predict response in a set of patient samples with unknown response, unless another normalization method is developed. We also analyzed aliquoted lysates from HCT116 in each unit but normalization to these lysates did not reduce the inter-unit variation sufficiently.

This array technology may be extended by adding small-molecular tyrosine kinase inhibitors to the samples before analysis. This may have at least two potential advantages. First, it will provide us with tyrosine kinase inhibition profiles, which may correlate to radiation response. Secondly, inter-unit variation would probably be less for the inhibition profiles since peptide phosphorylation levels would be normalized against the corresponding baseline levels, which would be measured in the same unit. If this variation was negligible, good responder samples in each Pamchip®96 unit for normalization would not be needed. A pilot experiment has been performed to select promising inhibitors and to determine appropriate concentrations (Figure 18).

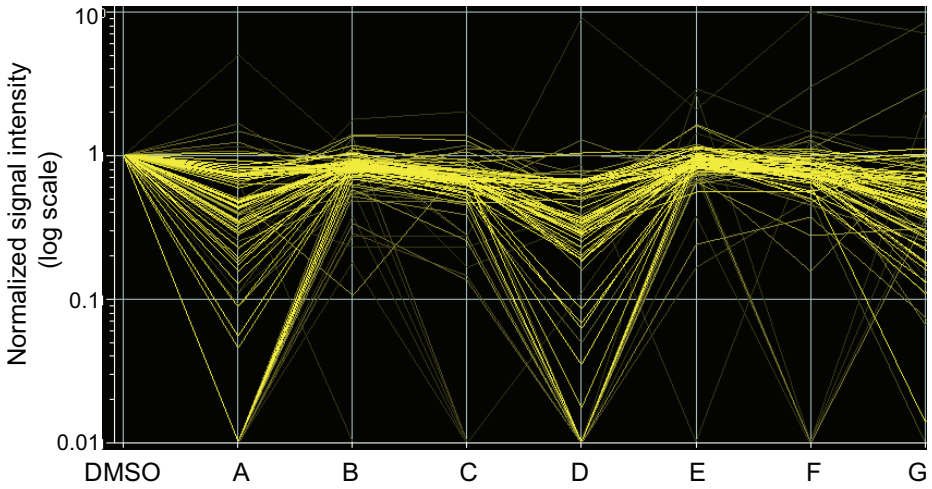

Figure 18. Phosphorylation levels (i.e. signal intensity, log scale, Y axis) of 144 peptides following incubation with 7 small-molecular tyrosine kinase inhibitors (A-G) normalized to the phosphorylation levels of untreated lysate from a rectal cancer biopsy (DMSO). Experiments and figure made by Piet J. Boender (PamGene International B.V.) and author.

The PamChip array technology may in the future also be developed to include more peptides, or a selected panel of relevant peptide substrates which may improve prediction of response. It should be noted that many new peptide array technologies are under investigation; however, paper IV is the first to evaluate this technology as a predictive tool in CRT response.

To facilitate biological interpretation, validation with well-known methods such as immunoblotting using phosphospecific antibodies or antibodies to protein kinases for detection

of phosphorylation levels of analyzed proteins, or level of kinases in patient samples or xenografts could be performed. Immunohistochemistry may also be an option. Detection of phosphoproteins may also be measured by other high-throughput methods such as sandwich enzyme-linked immunosorbent assay, mass spectrometry analysis and reverse phase protein array (225-228). These methods are different from, but complementary to the PamChip array technology, and may in the future assist in validation of our findings.

7 Conclusions

The search for new and more effective compounds to be integrated in preoperative CRT regimens in LARC has mainly taken place on the basis of drugs that have been shown to be efficacious in treatment of metastatic colorectal cancer. Such new agents should be rigorously tested in pre-clinical models, and should also be demonstrated to potentiate the effect of RT before being introduced in preoperative CRT for LARC. If the main objective of integrating a new drug is to kill micrometastatic cells or solely have a cytotoxic effect to the primary tumor (i.e. not enhance the effect of RT), the drug should be given in full-dose regimens independent of RT, either as NACT or as postoperative adjuvant treatment.

Among the potential drugs to be integrated in CRT in LARC, oxaliplatin is considered promising and several phase III trials are ongoing. However, at the time oxaliplatin was introduced in clinical trials, scarce pre-clinical evidence was available demonstrating radiosensitizing effects of oxaliplatin in colorectal carcinoma cells. We showed that oxaliplatin was a radiosensitizer in colorectal carcinoma cell lines alone and in combination with 5-FU as measured by clonogenicity. However, oxaliplatin did not convincingly increase radiosensitivity when added to fractionated RT and capecitabine in a relevant mouse model. Even though only one *in vivo* model was tested and limitations exist for the experimental design, as the only *in vivo* data available, our results question the relevance of oxaliplatin as an *in vivo* radiosensitizer. Hence, the integration of oxaliplatin into combined-modality treatment of rectal cancer is still controversial.

Several HDAC inhibitors have exhibited promising results in combination with RT in experimental models. SAHA, the only HDAC inhibitor approved by the US Food and Drug Administration for cancer treatment, performed convincingly as a radiosensitizer in *in vitro* and *in vivo* colorectal carcinoma models, rendering this drug as a potential promising supplement to current multimodal treatment strategies in rectal cancer. The current assumption that radiation must be applied at maximal histone hyperacetylation to obtain optimal effects was challenged, questioning the validity of using histone hyperacetylation as a molecular marker for radiosensitivity when combining HDAC inhibitors and RT.

Colorectal carcinoma cells with HDAC2 mutations were hypothesized to be partially resistant to histone acetylation by the HDAC inhibitor TSA, and based on this, HDAC2 status was suggested as a predictive marker of the effect of HDAC inhibitors. It was, however, demonstrated that histones were transiently acetylated by TSA irrespective of HDAC2 status. Furthermore, both HDAC2-deficient and HDAC2-expressing colorectal carcinoma cells were radiosensitized by TSA; hence, HDAC2 status needs not be taken into account when investigating HDAC inhibitors as radiosensitizers.

The possibility of predicting response to preoperative CRT in LARC would be useful for stratifying poorly responding patients to alternative treatment schedules as well as sparing non-responders from unnecessary treatment. CRT response was predicted with an accuracy of 85% based on multiplex kinase activity profiling performed on endoscopically acquired biopsy specimens. Because the number of patients, especially poor responders, was limited in the present study, larger prospective trials will be needed to confirm the validity of the present predictor.

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