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HYPOXIA-ASSOCIATED BIOMARKERS IN RECTAL CANCER TREATED BY PREOPERATIVE RADIOTHERAPY OR CHEMORADIOTHERAPY

by

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ABSTRACT

Eija Korkeila:

Hypoxia-associated biomarkers in rectal cancer treated by preoperative radiotherapy or chemoradiotherapy

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Rectal cancer (RC) is a common malignancy. Preoperative radiotherapy (RT) is usually given to patients with T3-T4 tumours. The aim of the study was to assess hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CA IX), ezrin and glucose transporter-1 (GLUT-1) expression as predictors of disease-free survival (DFS) and disease-specific survival (DSS) in RC treated by preoperative radiotherapy (RT) or chemoradiotherapy.

Diagnostic biopsies (n=80) and corresponding operative samples from 178 consecutive RC patients, treated by short- (n= 77) or long-course RT with (n=37) or without (n=10) chemotherapy or no treatment preoperatively (n=54), were analysed for HIF-1α, CA IX, ezrin and GLUT-1 using immunohistochemistry (IHC). Tumour regression grade (TRG) was analysed after long-course RT.

In operative samples, negative/weak (N/W) CA IX staining intensity was associated with favourable DFS (p=0.003) and DSS (p=0.034). After long-course RT, negative HIF-1α expression was linked to longer DSS (p= 0.001) and N/W GLUT-1 (0.066) with longer DFS than with positive or strong expression of these markers. Moderate/strong (M/S) ezrin expression in biopsies was associated with unfavourable DFS (p=0.027) and DSS (p= 0.002). In multivariate analysis, M/S CA IX intensity in operative samples was an independent predictor of poor DFS and DSS. Excellent TRG was linked to N/W CA IX (p=0.057), ezrin (p=0.012) and GLUT-1 (p=0.013) in operative samples. In multivariate model with all four markers, CA IX intensity in operative samples independently predicted DSS.

In conclusion, positive HIF-1α expression and M/S expression of CA IX and GLUT-1 in operative samples as well as M/S ezrin in biopsies were related to unfavourable disease outcome. CA IX intensity in operative samples independently predicted DFS and DSS in multivariate analysis. Moderate/strong CA IX intensity is a powerful predictor of poor disease outcome in RC.

Key words: rectal cancer, chemotherapy, radiotherapy, prognosis, predictive factor, tumour regression, hypoxia-inducible factor-1α, carbonic anhydrase IX, ezrin, GLUT-1

TIIVISTELMÄ

Eija Korkeila:

Hypoksiaan liittyvät biologiset merkkiaineet leikkausta edeltävällä sädehoidolla tai kemosädehoidolla hoidetussa peräsuolisyövässä

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Peräsuolensyöpä on yleinen pahanlaatuinen kasvain. Leikkausta edeltävä sädehoito annetaan yleensä T3-T4-kasvaimille. Tutkimuksella pyrittiin selvittämään, voidaanko kasvaimen hapenpuutteeseen liittyvillä biologisilla merkkiaineilla arvioida peräsuolisyövän ennustetta leikkausta edeltävän sädehoidon tai kemosädehoidon jälkeen. Tällaisia merkkiaineita ovat hapenpuutteen vaikutuksesta aktivoituva HIF-1α, hiilihappoanhydraasi IX (CA IX), sokerin kuljetukseen solussa osallistuva GLUT-1 sekä solun tukirankaproteiini ezrin.

Tutkimukseen otettiin 178 potilasta, jotka olivat saaneet ennen leikkausta lyhyen (n=77) tai pitkän sädehoidon (n=10), pitkän sädehoidon ja solunsalpaajahoidon (n=37) tai ei mitään hoitoa (n=54). Lisäksi osalta leikkausta edeltävää sädehoitoa saaneelta potilaalta tutkittiin hoitoja edeltävät, diagnostiset näytteet (n=80). Tutkimuksessa käytettiin immunehistokemiallisia värjäysmenetelmiä. Kasvaimen regressiota (TRG) arvioitiin pitkän sädehoidon jälkeisistä näytteistä.

Leikkausnäytteissä negatiivinen/heikko CA IX intensiteetti liittyi sekä pidempään tautispesifiseen (p=0.034) että tautivapaaseen elinaikaan (p=0.003) ja pitkän sädehoidon jälkeen HIF-1α-negatiivisuus pidempään tautispesifiseen (p=0.001) sekä negatiivinen/heikko GLUT-1 pidempään tautivapaaseen elinaikaan (p=0.066). Voimakas ezrin-ilmentymä diagnostisissa näytteissä liittyi lyhyempään tautivapaaseen ja tautispesifiseen (p=0.027 ja p=0.002) ennusteeseen. Monimuuttuja-analyysissä vahva CA IX intensiteetti leikkausnäytteissä ennusti itsenäisesti huonompaa tautivapaata ja tautispesifistä selviytymistä. Erinomainen TRG liittyi negatiiviseen/heikkoon CA IX- (p=0.057), ezrin- (p=0.012) ja GLUT-1 -ilmentymään (p=0.013) leikkausnäytteissä. Kun kaikki neljä merkkiainetta analysoitiin yhdessä monimuuttuja-analyysissä, CA IX intensiteetti leikkausnäytteissä ennusti itsenäisesti tautispesifistä elinaikaa.

Voimakas CA IX-ilmentymä leikkausnäytteissä ja positiivinen HIF-1α- ja vahva GLUT-1-ilmentymä pitkän sädehoidon jälkeisissä leikkausnäytteissä sekä vahva ezrin-ilmentymä diagnostisissa näytteissä liittyivät epäsuotuisaan ennusteeseen. Monimuuttuja-analyysissä kohtalainen/voimakas CA IX intensiteetti leikkausnäytteissä ennusti itsenäisesti huonompaa tautivapaata ja tautispesifistä elinaikaa. CA IX on vahva biologinen merkkiaine peräsuolisyövässä.

Avainsanat: peräsuolisyöpä, solunsalpaajahoito, sädehoito, ennuste, ennusteellinen tekijä, kasvaimen regressio, hapenpuutteen vaikutuksesta aktivoituva tekijä 1α, hiilihappoanhydraasi IX, ezrin, GLUT-1.

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ABBREVIATIONS

[18F]	fluorine-18
Ab	antibody
AIDS	acquired immunodeficiency syndrome
AJCC	American Joint Committee on Cancer
AKT	protein kinase of protein kinase B family
APC	adenomatous polyposis coli gene
APR	abdominoperineal resection
AR	anterior resection
ASCO	American Society for Clinical Oncology
ATP	adenosine triphosphate
BCL-2	B cell lymphoma-2 protein
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CA IX	carbonic anhydrase IX
Ca^{2+}	calcium ion
CA19-9	membrane-associated glycoprotein
CAT	catalogue
CC	colon cancer
CEA	carcinoembryonic antigen
CI	confidence interval
CIN	chromosomal instability
Cl^{-}	chloride ion
COX-2	cyclo-oxygenase-2
CRC	colorectal cancer
CRM	circumferential margin
CT	computerised tomography
DFS	disease-free survival
DNA	deoxyribonucleic acid
DSS	disease- specific survival
E. coli	Escherichia coli
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ERM	ezrin-radixin-moesin
ESA	erythropoiesis-stimulating agent
ESMO	European Society for Medical Oncology
EUS	endorectal ultrasound
FAP	familial adenomatous polyposis
FOB	faecal occult blood
FULV	fluorouracil-leucovorin combination
G	differentiation grade

GLUT-1	glucose transporter-1
Gy	Gray
HE	haematoxylin-eosin
HER2	human epidermal growth factor receptor 2
HIF-1	hypoxia-inducible factor-1
HIF-1α	hypoxia-inducible factor -1α
HIF-1β	hypoxia-inducible factor -1β
HIV	human immunodeficiency virus
hMLH-1	human mutL homolog 1
hMSH-2	human MutS homolog 2, colon cancer, non-polyposis type 1
HNPCC	hereditary non-polyposis colorectal cancer
HR	hazard ratio
HRE	hypoxia-response element
HRP	horseradish peroxidase
ICC	intra-class correlation coefficient
IgG	Immunoglobulin G
IHC	immunohistochemistry
K^+	potassium ion
kDa	kilodalton
KM	Kaplan-Meier method
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LDH	lactate dehydrogenase
LR	likelihood ratio
LVFU2	continuous infusion of fluorouracil combined to leucovorin
M	metastasis, metastatic
M/H	moderate/high
mAb	monoclonal antibody
MAP	MYH-associated polyposis
MAPK	mitogen-activated protein kinase
MLH1	mutL homolog 1 colon cancer, nonpolyposis type 2 (E. coli)
mM	millimoles per litre
mmHg	millimetre of mercury
MMR	mismatch repair
MRI	magnetic resonance imaging
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSH6	mutS homolog 6 (E. coli)
MSI	microsatellite instable/instability
MSI-H	high level of MSI
MSI-L	low level of MSI
MSS	microsatellite stable
mTOR	mammalian target of rapamycin
MYH	myosin, heavy chain 1, skeletal muscle, adult
N	lymph node
N/W	negative/weak

Na^+	sodium ion
NPV	negative predictive value
NSAID	non-steroidal anti-inflammatory agent
O_2	oxygen pressure
OR	odds ratio
p53	tumour protein 53
pCR	pathologic complete response
PDGF	platelet-derived growth factor
PET	positron emission tomography
PI3K	phosphoinositide-3-kinase
PMS2	post-meiotic segregation increased 2 (Saccaromyces cerevisiae)
pO_2	partial pressure of oxygen
PPV	positive predictive value
pRb	retinoblastoma pathway
RAF	v-raf-1 murine leukaemia viral oncogene homolog
RAS	rat sarcoma
RC	rectal cancer
RNA	ribonucleic acid
RT	radiotherapy
SE	sensitivity
SOS	son of sevenless homolog
SP	specificity
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
T	depth of tumour invasion
TCA	tricarboxylic acid cycle
TME	total mesorectal excision
TNM	tumour-node-metastasis classification system
TRG	tumour regression grade
TS	thymidylate synthase
U251	human glioma cell line
U2OS	human osteosarcoma cell line
UICC	Union for International Cancer Control
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	Von Hippel- Lindau protein
γH2A.X	phosphorylated form of histone H2A.X
κ	kappa

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles and unpublished data. The publications are referred to in the text by Roman numerals I-V:

I Korkeila E, Talvinen K, Jaakkola PM, Minn H, Syrjänen K, Sundström J and Pyrhönen S. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. Br J Cancer 2009; 100(6):874-80.

II Korkeila E, Jaakkola PM, Syrjänen K, Sundström J and Pyrhönen S: Preoperative radiotherapy down-regulates the nuclear expression of hypoxia-inducible factor-1α (HIF-1 α) in rectal cancer. Scand J Gastroenterol, 2010; 45(3): 340-348.

III Korkeila E, Syrjänen K, Bendardaf R, Laulajainen M, Carpén O, Pyrhönen S and Sundström J: Preoperative radiotherapy modulates ezrin expression and its value as a predictive marker in patients with rectal cancer. Hum Pathol 2011; 42(3): 384-392.

IV Korkeila E, Jaakkola PM, Syrjänen K, Pyrhönen S and Sundström J: Pronounced tumour regression after radiotherapy is associated with negative/weak glucose transporter-1 expression in rectal cancer. Anticancer Res 2011; 31 (1): 311–315.

V Korkeila E, Talvinen K, Jaakkola PM, Minn H, Syrjänen K, Sundström J and Pyrhönen, S. Reply: Expression of carbonic anhydrase IX suggests poor response to therapy in rectal cancer. Br J Cancer 2009; 101 (2): 373–373 *(letter)*.

1. INTRODUCTION

Colorectal cancer (CRC) is a common malignancy with rising incidence in the western world. Annually about 1000 patients are diagnosed with rectal cancer (RC) in Finland. The most important prognostic factors of RC include the number of metastatic and examined lymph nodes, the depth of tumour invasion and the involvement of the circumferential margin (CRM) [1, 2].

Preoperative short-course radiotherapy (RT), utilised in the treatment of T3-tumours, has been shown to improve local disease control [3, 4]. Long-course RT, preferably combined with fluorouracil-based chemotherapy, is used to treat locally advanced T4-tumours or tumours with threatened CRM involvement. Long-course RT is recommended preoperatively but it may be administered postoperatively, if the tumour has been under-staged prior to the operation or CRM is found to be involved [5].

Large tumours frequently have necrotic regions caused by lack of oxygen. However, tumours have hypoxic areas regardless of their size, stage, differentiation grade and the location [6]. Hypoxia starts a cascade of events, including the activation of hypoxia-response element (HRE). The key protein in tissue response to oxygen deficiency is hypoxia-inducible factor-1α (HIF-1α). HIF-1α consists of two protein subunits, HIF-1α and HIF-1β [7-9]. HIF-1α expression is oxygen dependent [7]. In hypoxia, HIF-1α stabilises, followed by dimerisation with the β-unit [8]. HIF-1α stabilisation leads to the activation of several genes involved in angiogenesis, glycolysis, invasion, metastasis, apoptosis, pH regulation and growth factor signalling [7, 8, 10-15].

The downstream targets of HIF-1α include glucose transporter-1 (GLUT-1) and carbonic anhydrase IX (CA IX) [7, 10, 13]. The mammalian target of rapamycin (mTOR) is a key regulator of HIF-1α protein synthesis in many cancers [16]. Rapamycin, a protein kinase that inhibits mTOR activity, is shown to act as an up-stream regulator of HIF-1α in hypoxia [17]. Ezrin belongs to the ezrin-radixin-moesin (ERM) family of proteins [18, 19], which function, besides of cross-linking the plasma membrane and the cytoskeleton [20-23], also in signal transduction [24-26]. Ezrin expression is necessary for metastatic behaviour of cancer cells [27] and ezrin-related metastasis is shown to be linked to mTOR pathway [28]. Blocking this pathway with rapamycin is found to inhibit experimental lung metastasis in vivo [28]**.** Thus, the regulation of mTOR and ezrin as well as mTOR, ezrin and HIF-1α are interrelated.

Hypoxia is associated with resistance to treatment and unfavourable disease outcome in several solid tumours [29-32]. Preoperative radiotherapy is shown to improve local disease outcome and to be better tolerated than postoperative RT [3, 33]. Chemotherapy is usually combined with long-course RT in the treatment of locally advanced tumours, aiming at enhancing response and diminishing radioresistance [30, 34, 35].

2. REVIEW OF THE LITERATURE

2.1. GENERAL ASPECTS OF COLORECTAL CANCER

2.1.1. Epidemiology of CRC

Colorectal cancer (CRC) is a common malignancy in the western world. Annually there are over one million new cases of CRC worldwide, accounting for 9% of all cancers [36]. In Europe, the United States, and elsewhere in the western world the incidence rates are higher than in other parts of the world, corresponding to 10-13% of all cancers [37, 38]. In Finland, CRC is the third most common cancer. More than 1500 new cases of colon cancer and about 1000 new cases of RC are diagnosed annually [39]. Increased survival into advanced age is mainly responsible for the growing incidence of CRC [40]. CRC is rare at young age, albeit patients with genetic alterations are at risk for CRC at a younger age [41]. Only about 2% of patients are younger than 40 years, whereas two thirds of patients are at or above age 65 at diagnosis [39].

2.1.2. Aetiology of CRC

2.1.2.1. Sporadic CRC

2.1.2.1.1. Carcinogenesis

Carcinogenesis involves growth signalling between cancer cells and non-malignant stromal cells [42], as well as activation of several pathways, e.g. SOS-RAS-RAF-MAPK [43] and disruption of others, e.g. pRb pathway [44]. Tumours also have the ability to generate growth factors [42]. Over-expression of receptors, e.g. epidermal growth factor receptor (EGFR) enables a tumour to hyper-response to growth signals from its environment [42]. Extracellular receptors, integrins, link the cancer cell to the extracellular matrix (ECM) [45]. The integrins and ligand-activated growth receptors have the ability to activate the SOS-RAS-RAF-MAPK cascade [45]. RAS can mediate mitogenic signals without normal up-stream activation and it is shown to be mutated in many cancers, including CRC [43]. The signalling pathways in cancer are complex, with cross-talking linkages to other pathways [42]. The hallmarks of cancer also include sustained angiogenesis, the ability to resist or inhibit programmed cell death, apoptosis, and the ability to invade and metastasise, features that

separate benign tumours and cancer from each other [42]. Malignant transformation can proceed through the adenoma-carcinoma sequence or the serrated pathway.

2.1.2.1.2. Adenoma-carcinoma sequence

CRC developing through the adenoma-carcinoma sequence [46, 47] involves a series of events in which a benign adenoma transforms into a malignant tumour via intermediate steps of premalignant states [42]. This multistep process requires oncogene activation and loss of tumour suppressor genes [46, 48].

2.1.2.1.3. Serrated adenoma pathway

Serrated colorectal carcinomas are considered to develop from hyperplastic polyps and serrated adenomas through the serrated neoplasia pathway [49, 50]. Oncogenic BRAF mutations are characteristic to this pathway [50]. Serrated cancers represent microsatellite stable (MSS) or microsatellite instable-low (MSI-L) phenotype [49, 51]. In IHC analysis, they are more frequently HIF-1α positive than conventional CRCs [49]. Serrated carcinomas account for about 10-15% of all CRCs [50] and have a predilection to the caecum and rectum [51].

2.1.2.1.4. Genomic instability in sporadic CRC

CRC carcinogenesis involves two forms of genomic instability: microsatellite instability (MSI) and chromosomal instability (CIN) [42]. The inefficiency of DNA mismatch repair system (MMR) to fix errors in DNA replication probably results to changes in the number of repeats during DNA replication, which leads to the instability of microsatellite loci [52]. High level of MSI (MSI-H) has been identified in about 10% of sporadic CRC [48]. CIN or MSI was found in about 60% of tumours in a study comprising tumour samples from patients with sporadic CRC [48]. A Finnish study of patients with advanced CRC, investigating thymidylate synthase (TS) and two MMR proteins, hMLH1 and hMSH2, showed that patients with low TS and deficient MMR had significantly shorter DFS than patients with high expression of these markers [52].

2.1.2.2. Familial and hereditary CRC

CRC is most commonly sporadic but about 10-15% of cases are familial [53]. The hereditary conditions include hereditary non-polyposis colorectal cancer (HNPCC or Lynch disease) and familial adenomatous polyposis (FAP), which are inherited in autosomal dominant manner [54]. People, who have a first-degree relative with CRC, are at two-fold risk of getting the disease [55]. The

risk is more pronounced, if the relative is diagnosed at an early age and if other first-degree relatives are also affected [55].

FAP accounts for less than 1 % of all CRCs [40]. FAP patients have multiple adenomas and an associated high risk of malignancy at young age [54] linked to a mutation in APC- tumour suppressor gene [56]. HNPCC accounts for about 2-4% of CRC [53]. The syndrome has a high penetrance and involves the development of CRC at an early age [57]. HNPCC is associated with an increased lifetime risk of 80% of predominantly right-sided CRC and an increased lifetime risk of 30-60% of endometrial cancer [54, 58, 59]. Mutation carriers also have an increased risk for small bowel, urothelial and ovarian cancers as well as brain tumours [26]. The genetic background of HNPCC comprises germ line mutations in the MMR genes (MLH1, MSH2, MSH6 and PMS2) [56, 58, 60].

The distinction of sporadic CRC from familial or hereditary cancers is not clear-cut but rather sliding [56], presumably with less influence of lifestyle, nutritional and environmental factors on hereditary than sporadic forms of cancer [56]. Several workshops have formulated criteria for identifying people at the highest risk of CRC and extending screening methods, genetic testing and counselling at their disposal. These clinical diagnostic tools include the Amsterdam criteria [61] to reveal possible HNPCC **(Table I)** and the Bethesda Guidelines to find families with indications for genetic (MMR) testing [58].

2.1.2.3. Predisposing conditions

Patients with ulcerative colitis and Crohn's disease [60, 62], polyps, previous malignancy of the large bowel or radiotherapy to a pelvic malignancy are at increased risk of CRC [46, 63].

2.1.2.4. Nutritional and lifestyle factors

High incidence of CRC is possibly modified by western sedentary lifestyle, including obesity, diabetes, and dietary habits [40]. However, the influence of these factors on the risk of RC is somewhat different from that of colon cancer (CC). Body mass index is not associated with an increased risk of RC [64]. Active physical exercise may modestly decrease the risk of CC but not RC [65]. Red and processed meat and alcohol consumption is associated with an increased risk of RC [66, 67], whereas fish intake may have a protective effect [66]. No clear association of dietary antioxidant vitamins and carotenoids with CRC risk has been found [68]. Short duration of sleep is shown to increase the risk of colorectal adenoma, and hence, colorectal neoplasia [69].

Table I. Revised ICG-HNPCC Criteria (Amsterdam Criteria II)*

There should be at least 3 relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis)
One should be a first-degree relative of the other 2
At least 2 successive generations should be affected
At least 1 person should be diagnosed before the age of 50
Familial adenomatous polyposis should be excluded in the CRC case(s) if any
Tumours should be verified by pathological examination

**Reprinted from Gastroenterology,Vol 116, Vasen H, Watson P, Mecklin JP, Lynch H and the ICG-HNPCC: New Clinical Criteria for Hereditary Nonpolyposis Colorectal Cancer (HNPCC, Lynch syndrome) Proposed by the International Collaborative Group on HNPCC, pages 1453-1456,1999,© 2010 with permission from Elsevier.*

2.1.2.5. Other risk factors

Use of acetylsalicylic acid compounds and oral contraceptives reduce the risk of CRC [70, 71]. Chemically induced RC is shown to be inhibited in male rats by giving exogenous testosterone during carcinogen administration, implicating that hormonal manipulation may have an effect on RC risk in men [72]. Currently there is no clear evidence of an association of CRC with viral infection, but AIDS patients have a 3-4 fold increased risk of CRC, possibly caused by an oncogenic protein related to HIV infection [73]. Human papilloma virus may be associated with the risk of CRC [74]. Interestingly, observations in animal studies have shown that colonisation of the large intestine with certain E. coli strains induces DNA damage in vivo, implying that these E. coli strains could be involved in sporadic colorectal carcinogenesis [75].

2.2. RECTAL CANCER

2.2.1. Signs and symptoms of disease

Symptoms of RC include a change in the bowel habits or the consistency of the stools, faecal blood or dark stools, narrow thread-like or mucous faeces, abdominal or pelvic discomfort or bloating, urgency, incompleteness of emptying the bowel, obstructive symptoms or increased flatulence. General

symptoms like tiredness, pain, nausea, vomiting, weight loss or anaemia may be present. However, the disease may give only obscure or slight symptoms.

2.2.2. Diagnostic and screening procedures

The diagnosis of suspected RC is based on digital rectal examination, colonoscopy and histological biopsies. A magnetic resonance imaging (MRI) is performed to define the local extent and resectability of the disease and a computerised tomography (CT) to rule out distant metastasis. CRCs developing from benign adenomas through the adenoma-carcinoma sequence [46, 47] offer an opportunity for screening. Faecal occult blood test (FOB) is a generally used method [76, 77]. In the screening programmes, a 16% reduction of mortality from colorectal cancer has been shown [78]. In Finland, a gradually expanding screening programme was started in 2004, including individuals 60-69 years old randomised into screening and control arms. FOB is tested twice a year and in case of a positive faecal occult blood test, a colonoscopy is performed [79]. In this screening programme after the first screening interval, an invasive CRC was reported to be diagnosed in 8.2% of the FOB positive population [80]. However, among the screened FOB negative patients, the proportion of interval cancers was found to be 27.3% [80].

2.2.3. Pathology and classification

2.2.3.1. Histopathology

Tumours up to 15 cm from the anal verge are considered rectal carcinomas [5]. The majority of them (85-90%) are classified as conventional adenocarcinomas [81]. About 8-10% of tumours are mucinous cancers, defined by the composition of ≥50% of extracellular mucin [82]. CRC associated with serrated adenoma tends to produce mucin and harbour KRAS and BRAF mutations [49, 50]. Other types of tumours can be found in the rectum albeit rarely, including angiosarcomas [83], melanomas [84], squamous cell carcinomas [85], as well as neuroendocrine carcinomas, the incidence of which is rising [86].

2.2.3.2. Handling and evaluation of the specimen

The specimen is examined macroscopically by the surgeon and the pathologist for the completeness of the dissected mesorectum. The resection margins are inked and the specimen is fixed in formalin and thinly sliced. The pathologist measures the narrowest proximal, distal and circumferential margins. The

mesorectal fat is sliced and carefully examined for lymph nodes, using fat clearance techniques as needed to detect a sufficient amount of lymph nodes [87].

2.2.3.3. Circumferential margin

The resected tissue is macroscopically examined to assure that the mesorectum is excised completely [88]. Proximal and distal margins are also measured [88]. Moreover, a clear CRM is critical to achieve a curative resection. The radial resection margin encompasses the vessels, lymph nodes and lymphatics of the rectum, surrounded by fascia. CRM less than 1 mm is associated with a higher local relapse rate [4], and an increased risk is reported to be present with CRM less than 2 mm [89]. Also, tumour location closer than 5 cm from the anal verge increases the probability of positive CRM [89]. Abdominoperineal resection is associated with a higher rate of intraoperative perforation [90], positive CRM, higher local recurrence rate of 22%, and more unfavourable prognosis than anterior resection [91].

2.2.3.4. Lymph node evaluation

The number of examined lymph nodes strongly affects the patient's prognosis [1]. According to the ESMO clinical recommendations, a minimum of twelve lymph nodes is to be examined for adequate staging [5]. However, after long-course preoperative treatment it is not always possible to collect the required number of lymph nodes [92-94]. In general, the more lymph nodes are examined, the more adequate is the staging [1, 95]. The impact of the number of examined lymph nodes was studied in T3N0 rectal cancer [1]. When 8-12 lymph nodes were examined, a patient's hazard ratio (HR) for death was reduced to 0.81 and if at least 13 lymph nodes are examined, it dropped to 0.68, as compared to patients with 1-7 lymph nodes examined [1].

2.2.3.5. Tumour differentiation grade

Tumour differentiation grade (G) **(Table II)** is assessed from the diagnostic biopsies and the operative samples. However, the size of biopsy may be insufficient to reliably determine the differentiation grade. Also, preoperative RT may affect tumour differentiation grade and after a complete response to preoperative treatment it is not feasible to assess.

2.2.3.6. Perineural and vascular invasion

The interactions of cancer cells and their surrounding microenvironment play an important role in cancer growth and progression as well as in tumour response to treatment [96]. Vascular invasion is shown to predict cancer-specific mortality and local recurrence in RC [97]. Perineural tumour invasion also affects the patient's prognosis, and may help to identify patients with node-negative tumours in need of adjuvant therapy [98].

Table II. WHO tumour classification

Criterion	Differentiation category	Numerical grade [a]	Descriptive grade
> 95 % with gland formation	Well-differentiated	1	Low
50-95 % with gland formation	Moderately differentiated	2	Low
>0-49 % with gland formation	Poorly differentiated	3	High
High level of microsatellite instability[b]	Variable	Variable	Low

[a] The category "undifferentiated carcinoma" (grade 4) is reserved for carcinomas with no gland formation, mucin production or endocrine, squamous or sarcomatoid differentiation; [b] MSI-H

MSI-H microsatellite instability-high

Reprinted from Hamilton SR, Bosman FT, Boffetta P, Ilyas M, Morreau H, Nakamura SI, Quirke P, Riboli E and Sobin LH: Carcinoma of the colon and rectum in Bosman FT, Carneiro F, Hruban RH and Theise ND (editors). WHO Classification of Tumours of the Digestive System, 4th edition. International Agency for Research on Cancer, Lyon, 2010, page 138, © 2010 with permission from the World Health Organization.

2.2.4. Tumour response to preoperative treatment

Preoperative treatment provokes morphological changes in both cancer tissue and the surrounding stromal cells. These changes can be identified by the appearance of tumour fibrosis and inflammatory cells, increased mucin production and mucin pools, accompanied by eosinophilia, nuclear pyknosis and necrosis as well as cytoplasmic vacuolisation of cancer cells [99, 100]. Damaged cells do not die immediately after radiation, but divide 1-2 times before their mitotic death, occurring by necrosis or apoptosis [101].

2.2.4.1. Methods for predicting response to treatment

Several methods have been used to predict response to radiotherapy, including approaches for detecting DNA variability (e.g. DNA sequencing), RNA expression measurements (e.g. microarray) as well as methods for studying genetic alterations (e.g. gene deletions, amplifications) or protein expression (e.g. IHC, Western blot) [102]. In rectal cancer, high TS expression and persistent positive lymph nodes after preoperative chemoradiotherapy have been found to be associated with unfavourable prognosis [103]. Tumour suppressor gene p53 mutations have been detected by complete direct sequencing of exons 2 to 10 and compared with p53 IHC from the preoperative biopsies, showing p53 genotype but not p53 IHC to predict response to preoperative treatment and survival [104]. Furthermore, CEA level falling below 5ng/ml after preoperative chemoradiotherapy has been found to be coupled with complete clinical and pathologic response as well as survival [105].

2.2.4.2. Tumour regression grade

Tumour regression grade (TRG) is used to estimate the response to given preoperative treatment. TRG is analysed in HE-stained sections of the tumour after long-course RT. This assessment is generally based on a scale by Mandard and colleagues, further modified by Dworak, Rödel and colleagues [99, 106, 107]. The response of the tumour is assessed using three to five main categories, depending on the scale used [99, 106, 107], with the response varying from complete to no regression. Pathologic complete disappearance of the tumour is achieved in 0-29% [100, 107-112] of tumours. Good tumour regression is shown to associate with favourable disease outcome [107, 108, 112].

2.2.4.3. Imaging in predicting response to treatment

Imaging of hypoxia could give additional prognostic information and help in estimating the response to radiation and the effect of novel therapies [113]. The resolution of the current routine imaging methods is not sufficient to measure biological response in tumours [114]. Vital cancer cells may persist after clinical and pathological complete response [114]. Thus, additional methods could be helpful in defining the amount of truly vital cancer cells. [18 F]Fluorodeoxyglucose positron emission tomography (FDG-PET) has been studied to evaluate radiation response in solid tumours, including rectal cancer. A favourable disease outcome has been observed with negative FDG-PET after preoperative chemo-RT in locally advanced rectal cancer [115]. MRI imaging can detect the CRM, reduction in tumour size and nodal down-staging

in rectal cancer. However, radiation-associated fibrosis may be one confounding factor in imaging these post-treatment tumour parameters [116]. The ability of MRI to detect pCR in rectal cancer after chemoradiation has been evaluated in a prospective study, showing MRI to underestimate tumour response to therapy in about two thirds of patients [117].

2.2.5. Staging

RC staging is based on the assessment of the depth of tumour invasion through the basal layer and the bowel wall (T), the regional lymph node involvement (N) and the presence or absence of distant metastases (M) [118] as depicted in **Tables III-V**.

2.2.5.1. Preoperative staging

Preoperative staging helps to define the patient's risk of disease recurrence as well as the need and type of preoperative treatment. To assess the local extent of the disease, a digital rectal examination and an endorectal ultrasound (EUS), a magnetic resonance imaging (MRI) **(Figure 1a and b)**, or a computerised tomography (CT) scan of the rectum are performed. MRI interpretation tends to over-stage T1 and T2 lesions [87], while EUS is the most accurate method to define the local invasion of a T1-T2 tumour [119]. However, in nodal staging, EUS is not markedly superior to MRI and CT [87]. Furthermore, the size and shape of lymph nodes is not a reliable method to rule out metastases in rectal cancer, since even small nodes of 2-3 mm can be metastatic [87, 120].

To rule out distant metastases, a CT of the chest and abdomen and chest x-ray are performed. A positron-emission tomography (PET) scan may be considered in selected cases. A complete colonoscopy is needed pre- or postoperatively to detect possible synchronous tumours in the colon [5].

2.2.5.2. Pathology report and postoperative staging

In many centres, a structured pathology report is used to clarify the assessment of the pathological stage. A structured pathology report includes information on the proximal, distal and circumferential margins of the tumour, the depth of invasion, the number of metastatic and examined lymph nodes, tumour differentiation grade and the presence or absence of extramural venous invasion [5] **(Table VI)**. The complete report also comprises information

of perineural and vascular invasion, and the presence or absence of vital cancer cells. A tumour regression grade may also be assessed. Thus, the pathologist estimates the extent of the disease, the accuracy of preoperative imaging, the quality of surgery and the effect of preoperative therapies [87].

Postoperative staging is based on the pathological findings of the tumour specimens, using the International Union Against Cancer (UICC) [118] **(Tables III-V)** or American Joint Committee on Cancer (AJCC) [121] tumour classification tables. In case of complete tumour regression, postoperative tumour grade is not assessable.

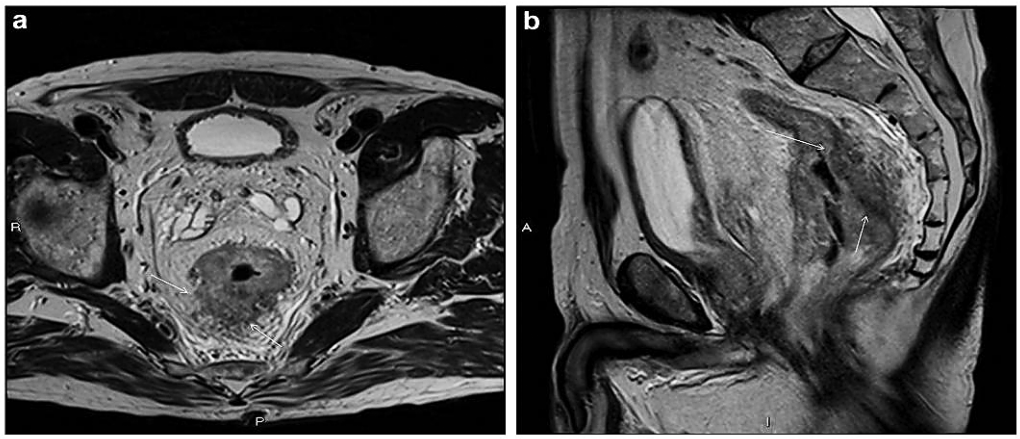

Figure 1a and b. Axial and sagittal MRI scan of advanced rectal cancer (arrows) (with patient's permission).

Table III. TNM Clinical Classification*

T-Primary Tumour	**N-Regional Lymph Nodes**
Tx Primary tumour cannot be assesses T0 No evidence of primary tumour Tis[1] Carcinoma in situ: intraepithelial or invasion of lamina propria T1 Tumour invades submucosa T2 Tumour invades muscularis propria T3 Tumour invades subserosa or into the non-peritonealized pericolic or perirectal tissues T4 Tumour directly invades other organs or structures and/or perforates visceral peritoneum T4a Tumour perforatesvisceral peritoneum T4b Tumour directly invades other organs or structures[2,3] Notes: 1. Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa 2. Direct invasion in T4b includes invasion of other organs or segments of the colorectum by way of the serosa, as confirmed on microscopic examination, or for tumours in a retroperitoneal or subperitoneal location, direct invasion of other organs or structures by virtue of extension beyond the muscularis propria. 3. Tumour that is adherent to other organs or structures, macroscopically, is classified cT4b. However, if no tumour is present in the adhesion, microscopically, the classification should be pT1-3, depending on the anatomical depth of wall invasion.	Nx Regional lymph nodes cannot be assessed N0 No regional lymph node metastasis N1 Metastasis in 1-3 regional lymph nodes N1a Metastasis in 1 regional lymph node N1b Metastasis in 2-3 regional lymph nodes N1c Tumour deposit(s), i.e. satellites*, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis N2 Metastasis in 4 or more regional lymph nodes N2a Metastasis in 4-6 regional lymph nodes N2b Metastasis in 7 or more regional lymph nodes Note: *Tumour deposits (satellites), i.e. macroscopic or microscopic nests or nodules, in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extracellular spread (V1/2) or a totally displaced lymph node (N1/2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded as N1c. If a nodule is considered by the pathologist to be totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as a lymph node in the final pN determination.

** According to TNM Classification of Malignant Tumours, International Union Against Cancer, 7th Edition, Wiley–Blackwell, Singapore, 2010. Reprinted with permission © 2010 John Wiley and sons Ltd. All rights reserved.*

Table IV. Stage grouping of rectal cancer*

Stage 0	Tis	No	M0
Stage I	T1,T2	N0	M0
Stage II	T3,T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1,N2	M0
Stage IIIA	T1,T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3,T4a	N1	M0
	T2,T3	N2a	M0
	T1,T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3,T4a	N2b	M0
	T4b	N1,N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

** M0=no distant metastases, M1 distant metastases present. According to TNM Classification of Malignant Tumours, International Union Against Cancer, 7th Edition, Wiley–Blackwell, Singapore, 2010. Reprinted with permission © 2010 John Wiley and sons Ltd. All rights reserved.*

Table V. TNM classification of distant metastasis (M)*

M0	No distant metastasis
M1	Distant metastasis
	M1a Metastasis confined to one organ (liver, lung, ovary, non-regional (lymph node (s)) M1b Metastasis in more than one organ or the peritoneum

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Table VI. The structured attachment of pathology report used in Turku University Hospital

Tumour diameter	(mm)
Distance from visceral peritoneum	(mm)
Lateral/circumferential margin	(mm)
Proximal margin	(cm)
Distal margin	(cm)
Histological grade	I/II/III
Depth of penetration	pT1 invasion of submucosa pT2 invasion of muscularis propria pT3 through muscularis propria into subserosa pT4 invasion of visceral peritoneum/lateral margin
Vascular/lymphatic invasion	yes/no
Metastatic/Examined lymph nodes	(number M/E)
Tumour regression grade	1)<25% 2) 2-50% 3)>50% 4) 100%

2.2.6. Surgical treatment of localised rectal cancer

2.2.6.1. Anterior resection and abdominoperineal resection

The goal of RC surgery is to achieve a macroscopically as well as microscopically radical resection (R0) with negative distal and circumferential margins [122]. Two primary modes of operation, anterior resection (AR) and abdominoperineal resection (APR) are used. AR preserves the continuity of the intestine, while APR comprises the pelvic and the perineal incisions and necessitates a colostomy. Low AR is usually feasible for tumours of the upper and mid rectum [122]. It may also be possible when operating distal carcinomas, unless the sphincter mechanism is involved [122]. Usually a protecting temporary stoma, however, is required.

Sufficient proximal, distal and CRM margins are prerequisites for achieving local disease control. For tumours of the upper and mid rectum, a macroscopic distal margin of 4-5 cm is preferred, while 1-2 cm margin may be acceptable for low-situating cancers [87, 122, 123].

2.2.6.2. Total mesorectal excision technique

The surgical technique of choice is total mesorectal excision (TME), which involves the removal of the mesorectum intact, including the lymphovascular tissue [124], in order to obtain a sufficient CRM and improved local control [4]. The local recurrence rate varies from 12-15% to 30-40% after conventional surgery [124-126], while it is 5-15 % after TME [124, 125].

2.2.6.3. Laparoscopic surgery

The interest in less invasive operative procedures is increasing due to anticipated earlier recovery and better physical functioning [127, 128]. Early reports from ongoing trials have not shown increased local recurrence rates or worse disease outcome [129, 130].

2.2.6.4. Local excision

Local excision of RC can be considered for patients with superficial T1-adenocarcinomas with no adverse histologic or clinical features [1, 2, 4, 97, 98, 103, 107, 108, 131-142] (**Table VII**). Surgery is generally performed using transanal excision involving the whole rectal wall to the perirectal fat with at least a 1 cm lateral margin [122]. Local excision is also an option for those patients, who have

severe comorbidities or metastatic disease or who refuse APR[143]. However, even selected elderly patients tolerate and benefit from major curative surgery[144].

Table VII. Adverse histopathologic and clinical features in rectal cancer

Advanced postoperative stage (TNM)	Dukes CE, 1958 Kuo LJ et al, 2007 Liersch et al, 2007 Peeters K et al, 2007
Poorly differentiated histology	McDermott et al, 1984
Presence of lymphovascular invasion	Compton et al, 2000 Tilney et al, 2009
Tumour level (low)	Peeters K et al, 2007
Presence of perineural invasion	Liebig et al, 2009
Examination of less than 12 lymph nodes	Tepper JE et al, 2001 Kapiteijn et al, 2001 Swanson et al, 2003 Edler D et al, 2007
Involved circumferential margin	Adam et al, 1994 Hall et al 1998 Nagtegaal 2008
Poor response to preoperative treatment	Ruo et al, 2002 Rödel et al, 2005
Tumour involving the surface of the specimen	Compton et al, 2000
Clinical obstruction or perforation	Griffin et al, 1987
Intraoperative tumour perforation	Zirngibl H et al, 1990
High level of CEA	Compton et al, 2000

2.2.7. Oncological treatments of rectal cancer

2.2.7.1. General aspects of radiotherapy

Local recurrences were previously a major problem after RC, deteriorating the patients' quality and quantity of life. RT is used to improve local control and/or operability and to allow sphincter preservation [145]. RT is given before or after surgery, with or without chemotherapy. Several advantages are associated with preoperative radiotherapy, including patient compliance and treatment tolerability, tumour down-staging, operability and possible sphincter preservation. Possible disadvantages include side-effects of treatment and over-treatment due to the limitations of imaging in estimating tumour stage [33]. CT-based dose planning is used, encompassing 3-4 radiotherapy fields and 6-24 megavoltage photons with daily fractions of 1.8 Gy to a total dose of 50.4 Gy in 6 weeks (long-course RT) or 5 fractions of 5Gy in a week (short-course RT) [146]. The RT portals cover the primary tumour and the adjacent lymph nodes, reaching from the border of the first sacral and the fifth lumbar vertebra down to the obturator foramina, anteriorly from the hip joints to the sacral bone [3]. Radiotherapy plan of a rectal cancer patient is depicted in **Figures 2a and b.**

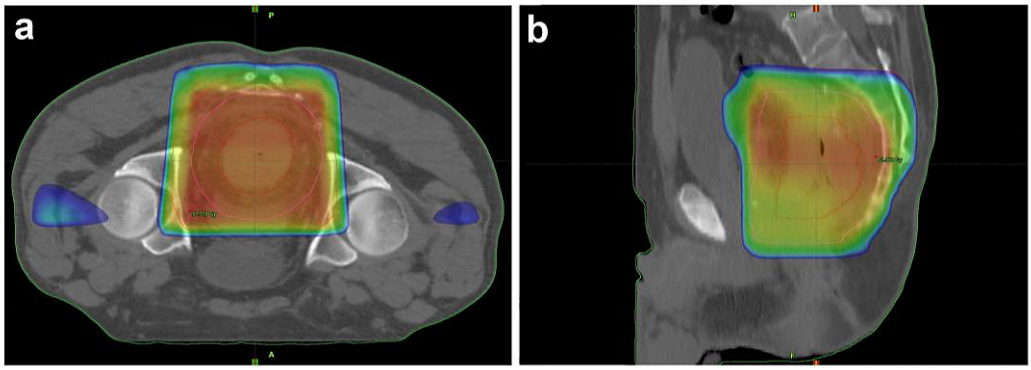


Figure 2 a-b. Radiotherapy plan in the treatment of rectal cancer (patient in a prone position): a, axial b, sagittal scans (with patient's permission).

Patients with T3 tumours and tumours with suspected metastatic lymph nodes are usually treated with short-course preoperative RT, whereas T4, very large or fixed tumours or tumours with predicted CRM involvement are generally treated with long-course RT [5, 147]. Patients with T1-T2 tumours may be treated without preoperative therapy [147]. There is still controversy about the treatment and clinical behaviour of proximal or upper rectal tumours [148]. A

British study showed that patients with early disease (T2N0) and tumours in the upper rectum were less likely offered neoadjuvant therapy by multidisciplinary team [149]. Subgroups of patients with high risk upper RC may be identified by the depth of tumour infiltration, lymph node status and tumour differentiation grade [131].

Precise RT planning diminishes the risk of possible side effects, which include diarrhoea, frequent defecation, incontinence, urgency and soiling, sexual dysfunction, and bladder irritation [3]. Interestingly, the risk of prostate cancer, after RT for rectal cancer, is substantially reduced [150].

2.2.7.2. Preoperative short-course radiotherapy

The Swedish Rectal Cancer Group showed that preoperative short-course RT improved local disease control and survival. Local relapse rate decreased from 27% to 11% and five-year overall survival increased from 48% to 58% [3]. The rate of side effects and surgical complications did not increase significantly after preoperative RT [3]. The results were reported to persist after median follow-up time of 13 years [151]. The Dutch Colorectal Cancer Group confirmed that preoperative RT further reduced the risk of local recurrence significantly from 8.2% to 2.4% even after TME- technique was utilised in surgery [4]. After median follow-up of 6 years, the difference persisted between the two groups but no survival advantage was seen [141]. Patients with positive nodes, tumours of the mid rectum or tumours with negative CRMs benefited most from preoperative RT [141]. The relative risk reduction of local recurrences in the preoperative radiotherapy group was 71 % at 2 years and 49% at 5 years as compared with the surgery group [4, 141].

2.2.7.3. Preoperative chemoradiotherapy

RT is generally combined with fluorouracil-based chemotherapy (fluorouracil or capecitabine), if not contraindicated, based on the results of two EORTC trials [152, 153]. Fluorouracil (FU) is a fluoropyrimidine analogue, given either as a bolus treatment in 4-5 day-cycles (Mayo regimen) [154] or via continuous intravenous infusion [110]. Capecitabine is an oral fluoropyrimidine precursor that is activated by a three-step enzymatic cascade, including conversion to active FU by thymidine phosphorylase. Thymidine phosphorylase is more active in the tumour than in normal tissues, resulting in about three-fold concentration of FU in the tumour, as compared to normal tissue [155]. Capecitabine is given concomitantly and continuously with RT [156].

Preoperative chemoradiotherapy is utilised in the treatment of locally advanced rectal cancer, showing infiltration to the adjacent structures or threatened CRM. After preoperative treatment, an inoperable carcinoma may turn operable and even pCRs are seen [108]. The EORTC-trial reported chemoradiotherapy to improve local disease control, irrespective of timing [152]. Sauer and collegeaues compared preoperative with postoperative chemoradiotherapy in the treatment of stage II and III RCs in a randomised study [33]. Preoperative chemoradiotherapy was shown to improve local control but not overall survival of patients with T3-T4 tumours, as compared to postoperative combined treatment [33]. This trial demonstrated a lower local relapse rate and less acute toxicity in the preoperative as compared with the postoperative treatment arm [33]. The Polish study randomised patients to receive either short-course radiotherapy or long-course chemoradiotherapy preoperatively. In contrary to the EORTC trial, no increase in survival, local control or late toxicity was found after chemoradiotherapy, as compared with short-course radiotherapy, albeit a down-staging effect was seen [157].

In the study by Guillem and colleagues, excellent response after preoperative treatment was shown to predict improved disease outcome [158], which may reflect tumour biology sensitive to therapeutic interventions [109]. Since most studies have not shown survival advantage after preoperative treatment, despite improved local disease outcome, chemotherapy intensification could be feasible. In fact, in locally advanced RC, oxaliplatin in combination with capecitabine in the preoperative setting was shown to be efficient and result in excellent tumour regression in a phase II study [159]. The Dutch Colorectal Cancer Group reported a high R0-resection rate of 81% in a phase I-II study of locally advanced rectal cancer, treated by capecitabine and oxaliplatin combined to preoperative radiotherapy [160].

Sphincter-preservation has been one of the focuses in several studies investigating the effects of preoperative chemoradiotherapy. Randomised trials have so far not shown an increased rate of sphincter-saving surgery after CRT [161].

2.2.7.4. Time interval between radiotherapy and surgery

Timing of surgery after preoperative treatment is critical, when considering down-staging of the tumour or possible sphincter-saving procedures. The effects of radiotherapy on cancer tissue are not seen immediately [162] but cancer cells may appear morphologically intact shortly after radiation [163]. The

Lyon trial randomised patients in two groups: short (2 weeks) or long (4 weeks) interval between the end of RT and the subsequent operation. This trial showed that a long interval resulted in increased down-staging and complete pathologic remission (pCR) rate, as compared with a short interval [162]. The Dutch Colorectal Cancer Group study found no tumour down-staging, if the interval between RT and surgery was less than ten days [92]. A waiting time of at least 8 weeks was found to be the only factor predicting pCR in a non-randomised study by Kalady and colleagues [164]. In their study, pCR was also associated with improved local control and survival [164]. Even longer intervals of 12 weeks have been reported [165].

Currently, short-course RT is given over five days, with surgery on the following week. The time interval between long-course RT and surgery is about 6-8 weeks. Recently, delayed surgery up to 6-8 weeks after short-course preoperative RT was also shown to result in substantial tumour regression [166]. A randomised trial is under way to evaluate this treatment strategy in resectable rectal cancer [166].

2.2.7.5. Postoperative radiotherapy and chemoradiotherapy

Postoperative RT delivered 30-60 days after surgery to a total dose of 40 Gy, was found to reduce and postpone local relapses in RC [167]. Postoperative RT in combination with fluorouracil-based chemotherapy, was shown to reduce local recurrences significantly [168], and was recommended by the National Cancer Institute as standard treatment after resection of stage II-III rectal cancer [169]. According to the current European guidelines, chemoradiotherapy should preferably be given preoperatively, but postoperative treatment is adequate, when CRM turns out positive or the tumour has been under-staged preoperatively and the patient has thus not received prior chemoradiotherapy [5].

2.2.7.6. Novel agents in chemoradiation

2.2.7.6.1. Epidermal growth factor receptor inhibitors

Cetuximab is a monoclonal antibody to epidermal growth factor (EGFR). It regulates cellular proliferation, survival, and differentiation [170]. In head and neck cancer, cetuximab given concomitantly with RT was shown to improve locoregional disease control and overall survival, as compared to RT alone [171]. Cetuximab combined with preoperative chemoradiotherapy in locally advanced rectal cancer was demonstrated to result in an overall 9.1% pCR rate

[170], which is not superior to previous reports with chemotherapy and radiation. Cetuximab is known to cause G1 cell cycle arrest [172], thereby its concurrent use with radiotherapy may revoke the additive effect of FU [170]. Resistance to anti-EGFR-therapy may also be involved [173]. Currently, cetuximab is not recommended in combination with chemoradiotherapy outside clinical trials in rectal cancer [170]. Panitumumab, a fully human monoclonal antibody to EGFR, is being investigated in the preoperative treatment setting in rectal cancer [174].

2.2.6.7.2. Bevacizumab

Vascular endothelial growth factor (VEGF) inhibitor bevacizumab was shown to be beneficial in metastatic colorectal cancer [175]. A phase II trial examined bevacizumab in combination with capecitabine and preoperative radiotherapy in locally advanced rectal cancer and showed a promising pathologic complete response rate but also major wound healing complications [176]. Bevacizumab is currently not indicated in the treatment of localised rectal cancer.

2.2.7.7. Adjuvant chemotherapy

In CRC, postoperative adjuvant chemotherapy is usually delivered according to the current standard recommendations, adjusted for a patient's individual recurrence risk. Adjuvant therapy is considered for patients with stage III and high risk stage II cancer [177] **(Table VII)**.

FU combined to levamisole [178] and later on to leucovorin became the standard adjuvant treatment in stage III CC [179]. A regimen of continuous infusion FU with leucovorin (LVFU2) was then shown to be less toxic than the bolus regimen [180]. Again, capecitabine was shown to be an effective alternative to fluorouracil-leucovorin combination (FULV) in the adjuvant setting in a non-inferiority trial [181]. The Mosaic study demonstrated an improved overall survival in stage III CC with the addition of oxaliplatin to FULV (72.9% vs. 68.7%). DFS at five years was 66.4% for the oxaliplatin treatment group, as compared with 58.9% for the FULV group [182]. However, there is somewhat less evidence supporting the use of adjuvant chemotherapy in RC than in CC [5, 122, 183]. Nevertheless, adjuvant treatment is generally used in RC along the same principles as in CC. EORTC- 22921 trial showed no significant benefit of adjuvant chemotherapy after preoperative treatment for the whole study population. However, good prognosis patients, who responded to preoperative treatment, had longer DFS after adjuvant chemotherapy than patients who did not receive adjuvant chemotherapy [184]. Interestingly, a meta-analysis of five randomised Japanese trials reported improved overall survival

and DFS for RC patients with uracil-tegafur adjuvant chemotherapy [185]. In western countries, this agent is not currently used due to associated severe toxicity. In Japanese population, severe gastrointestinal toxicities appear to be less frequent for an unknown reason [185].

2.2.8. Multidisciplinary approach

The treatment of rectal cancer patients is preferably discussed in a multidisciplinary team, involving representatives from colorectal surgery, pathology, medical oncology, radiotherapy and radiology to ensure adequate treatment planning [186].

2.2.9. Current treatment recommendations

According to the European Society for Clinical Oncology (ESMO) Clinical recommendations [5], surgery alone is the treatment of choice of T1-T2N0 favourable cancers. Generally, preoperative short-course RT is recommended to most patients with T3-tumours, with surgery by total mesorectal excision technique on the following week. Preoperative chemoradiotherapy is recommended for locally advanced T4-tumours and T3-tumours with threatened CRM margin involvement, followed by radical surgery 6-8 weeks later. Postoperative chemoradiotherapy can be used, if CRM is positive or the tumour has perforated or was under-staged preoperatively, and thus, no treatment has been given preoperatively. Adjuvant chemotherapy can be considered for stage III and high risk stage II RC [5]. After preoperative combined therapy, treatment decisions should rather be based on the pre-treatment than the postoperative pathological stage [122].

2.2.10. Surveillance

2.2.10.1. Tumour markers

Carcinoembryonic antigen (CEA) is the first and most widely used tumour marker in CRC surveillance [187], known to be up-regulated by hypoxia in cancer cells [188]. Normal CEA does not rule out cancer recurrence and values exceeding upper normal limits may not reflect recurrent disease [189]. However, the highest CEA values are obtained from patients with metastatic disease [189]. Membrane-associated glycoprotein CA19-9 is not recommended for CRC

surveillance [189]. Serum lactate dehydrogenase (LDH) level has been used as a prognostic marker in CRC. Removal of the primary tumour is followed by a decline in serum LDH within a week [190]. High serum LDH levels are associated with tissue LDH-5 expression by IHC [190]. LDH is known to be affected by various conditions, e.g. hypothyroidism, infection and renal insufficiency, and is not routinely used to monitor CRC.

2.2.10.2. ASCO and ESMO recommendations for follow-up

According to the American Society for Clinical Oncology (ASCO) recommendations for the use of tumour markers, the evaluation of CEA is recommended preoperatively, if it would help in staging and treatment planning. Further, the assessment of CEA postoperatively every three months for three years is recommended by ASCO, if the patient would be eligible for surgery or chemotherapy of metastatic disease. No routine use of novel tumour markers is recommended due to insufficient data [191]. According to the ESMO Clinical Practice Guidelines, a completion colonoscopy should be performed within the first year of diagnosis, as well as medical history and rectosigmoidoscopy, if feasible, every 6 months for 2 years. Thereafter, history and colonoscopy along with the resection of colonic polyps every 5 years are recommended [5]. CEA testing is recommended before treatment, but no directives for post-operative clinical examinations and laboratory tests are given since their value is unknown. Postoperative imaging of the liver and lungs is recommended at 1 and 3 years after curative surgery. A Cochrane intervention review analysis concerning follow-up strategies for patients with non-metastatic colorectal cancer was recently performed. This analysis showed intensive follow-up to be associated with improved all-cause survival, but the optimal follow-up regimen is not known [192].

Lynch syndrome carriers with DNA mismatch repair gene mutation are offered colonoscopies and vaginal ultrasound at 2-3 years intervals [193], including personal counselling [58].

2.2.11. Prognosis

2.2.11.1. Adverse prognostic factors

The prognosis of RC depends on the radicality of the operation and the tumour's TNM-stage. Lymph node metastases worsen disease outcome considerably [1]. A positive CRM is a strong predictor of both local disease

recurrence and distant metastasis [2]. Histopathologic and clinical features associated with adverse disease outcome [1, 2, 4, 97, 98, 103, 107, 108, 131-142] are listed in **Table VII**. Precise surgical and pathological procedures ensure adequate staging and postoperative treatment decisions, leading to best possible disease outcome. Optimal, radical surgery is a cornerstone in the treatment of RC and it cannot be compensated by postoperative oncological treatments [194].

2.2.11.2. Survival by stage

The presence of lymph node metastasis adversely affects disease outcome [1]. The 5-year relative and observed survival probability of RC patients in Finland [195] is depicted in **Table VIII**.

Table VIII. The 5 year survival data of rectal cancer patients diagnosed in 2000-2007 in Finland.*

Extent of Disease	Relative Survival proportion/ Males	Observed Survival probability/ Males	Relative Survival proportion/ Females	Observed Survival probability/ Females
Localised disease	84,1%	67,6 %	87,2%	73,4%
Regional disease	67,7%	56,0%	64,8%	55,9%
Distant disease	18,9%	17,1 %	18,0%	14,9%

** Finnish Cancer Registry (personal communication, 2010).*

2.3. TUMOUR HYPOXIA

2.3.1. General aspects of hypoxia

Hypoxia refers to insufficient levels of oxygen in blood or tissue, which also results in reduced adenosine triphosphate (ATP) production [6]. There are several mechanisms, which can cause tissue hypoxia [6, 196] **(Table IX)**. ATP is a key mediator of energy in cells. Abated ATP production causes changes in Na^+-K^+ channels, membrane depolarisation, enhancement of cellular uptake of Cl^-, swelling of cells, increased Ca^{2+}-concentration and decreased pH in the cytoplasm, leading to tumour cell acidosis [6]. Anoxia in turn refers to complete lack of oxygen in blood or tissue. Hypoxia induces programmed cell death, or apoptosis, both in normal and malignant tissue [6].

Table IX. Causes of hypoxia with examples of associated clinical conditions.*

Cause	Example
Low oxygen partial pressure	Pulmonary diseases High altitude sickness
Reduced ability of blood to carry oxygen	Anemia Methemoglobinemia Carbon monoxide poisoning
Reduced tissue perfusion Impaired oxygen transport	Sepsis, hypotension, dehydration Capillary microthrombosis in sepsis Arterial thrombosis or embolism
Impaired diffusion 1) Decreased oxygen tension gradient 2) Increased diffusion distance 3) Increased vascular permeability	Excessive fluid loading, tissue oedema Capillary microthrombosis in sepsis
Inability to use cellular oxygen	Cyanide poisoning Endotoxins associated with sepsis

* Modified. Based on *Höckel M, Vaupel P. Tumor Hypoxia: Definitions and Current Clinical, Biologic, and Molecular Aspects. J Natl Cancer Inst 93(4): 266-276, 2001 and Leach RM, Treacher DF. ABC of Oxygen. Oxygen Transport-2. Tissue Hypoxia. BMJ 317: 1370-1373, 1998.*

2.3.2. Angiogenesis and hypoxia

A tumour cannot grow beyond a few millimetres without the formation of new blood vessels [197], angiogenesis. Several mechanisms of angiogenesis are involved in neovascularisation. New capillaries can sprout out of the existing vessels, which starts by endothelial cell activation, a process stimulated by angiogenic growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [198, 199]. New vessels can also be formed by splitting of the pre-existing vessels or the tumour can grow along them, enabling avascular growth [199]. Even tumour cells per se can dedifferentiate to endothelial phenotype and thus provide a collateral vascular system to the tumour [199].

2.3.3. Tumour vasculature, oxygenation and glycolysis

New tumour vessels are tortuous, disorganised and dilated, branch abnormally and are often more permeable than normal blood vessels, resulting in uneven blood flow [200]. Inadequate vasculature, insufficient blood flow and uncontrolled rapid growth result in hypoxia in the tumour [7, 200]. Large tumours

frequently have necrotic areas caused by anoxia. However, tumours have hypoxic areas regardless of their size, stage, differentiation grade and location[6].

Under hypoxic conditions, cells turn to lactic acid production instead of oxidative phosphorylation from tricarboxylic acid cycle (TCA) to produce energy [201], a phenomenon called anaerobic glycolysis or Pasteur Effect [202]. However, even in an environment with normal oxygen tension, cancer cells are inclined to use anaerobic glycolysis as a source of energy (Warburg effect) [203]. Since aerobic glycolysis is known to be associated with higher ATP production [203], it is not clear why tumours tend to use anaerobic glycolysis. One reason for this tendency could be that purine and pyrimidine pathway precursors are produced during anaerobic glycolysis, thus providing DNA building material for tumour growth [204].

2.3.4. Measuring hypoxia

Tumour oxygenation has been measured by computerised polarographic electrode systems [31]. In general, tumours tend to have lower partial oxygen tension (pO_2) than the tissue they are originated from, and the oxygenation status deteriorates along with disease recurrence [204]. In contrast to pO_2 of 40-50mmHg in normal tissues, values as low as 5-10 mmHg have been measured in tumours [30].

2.3.5. Activation of the hypoxia response element and HIF-1α

Hypoxia starts a cascade of events, including the activation of hypoxia-response element (HRE). The key protein in tissue response to oxygen deficiency is hypoxia-inducible factor-1α (HIF-1α). HIF-1α is expressed in the nuclei of almost all superficial epithelial cells in the normal colorectal mucosa. The same staining pattern is seen in adenomas. However, in malignant tumours HIF-1α is mostly present in the nuclei of cancer cells around necrotic areas [205].

HIF-1 consists of two protein subunits, HIF-1α and HIF-1β [7-9]. HIF-1β is constitutively expressed, whereas HIF-1α expression is oxygen-dependent [7]. In hypoxia, HIF-1α stabilises, followed by dimerisation with the β-unit [8, 206]. HIF-1α binds to HRE, which is a special DNA sequence [14, 15, 207].

In normoxic conditions, HIF-1α is bound to Von Hippel-Lindau protein (VHL), which is a tumour suppressor gene [7]. Binding results in HIF-1α degradation within minutes after hypoxia is resolved [208], by an enzymatic mechanism involving prolyl hydroxylation [206, 209]. HIF-1α up-regulation may also occur independently of the oxygen tension in the tumour due to defects of its degradation pathway, e.g. VHL protein mutations [210], oncogenic activation leading to increased HIF mRNA transcription, or intracellular hypoxia caused by increased oxygen consumption [211]. HIF-1α stabilisation leads to the activation of several genes involved in angiogenesis, glycolysis, invasion, metastasis, apoptosis, pH regulation and growth factor signalling [7, 10]. Many of these genes are more abundant in malignant than in normal tissues [10, 212, 213].

2.3.6. Association of HIF-1α with PI3-AKT and mTOR

In breast cancer cell lines, phosphoinositide-3-kinase (PI3K)-inhibitor LY294002 decreased the expression of HIF-1α, showing that HIF-1α activation is regulated by the PI3K/AKT pathway [210]. In VHL mutant renal cancer cell line, the PI3K inhibitor was shown to down-regulate the expression of HIF-1α, in spite of the stabilisation of HIF-1α. This implies that HIF-1α is also regulated by VHL-independent pathways [210].

Mammalian target of rapamycin (mTOR) is a key regulator of HIF-1α protein synthesis in many cancers [16]. Activation of receptor tyrosine kinases including AKT, HER2 and EGFR, leads to increased mTOR activity [16, 214], in turn inducing HIF-1 α [9, 16, 215]. Rapamycin, a protein kinase that inhibits mTOR acitivity is shown to act as a regulator of HIF-1α in hypoxia [17]. Ezrin is a protein which acts as a cross-linker between the cell membrane and the cytoskeleton [18, 19, 21]. Ezrin expression is necessary for metastatic behaviour in cancer cell lines [27] and ezrin-related metastatic behaviour in turn is shown to be linked to mTOR pathway [28]. Blocking the mTOR pathway with rapamycin is found to inhibit experimental lung metastasis in vivo [28]. Thus, the regulation of mTOR and ezrin as well as mTOR, ezrin and HIF-1α are interrelated **(Figure 3).**

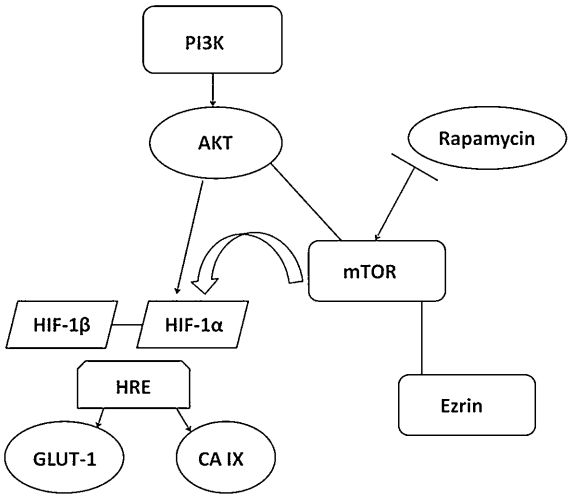


Figure 3. The association of HIF-1α with PI3K, mTOR and ezrin. Modified, based on Wang G et al, 1993; Semenza G, 2000; Pakkanen R, 1998; Hudson C et al, 2002; Kivelä A et al, 2001; Harris A, 2002, Brambilla D et al, 2009; Niggli V et al, 2008; Fehon R et al, 2010.

2.3.7. Down-stream targets of HIF-1α

2.3.7.1. Vascular endothelial growth factor (VEGF)

Blood vessels as well as lymphatic vessels have an essential role in tumour metastasis [216]. In normal tissues there is a balance between the factors that induce and inhibit angiogenesis [204]. Hypoxia induces HIF-1α and thereby promotes the up-regulation of vascular endothelial growth factor (VEGF), which leads to neoangiogenesis [217]. This concept is called the angiogenic switch [9], with HIF-1α as the key player turning the switch on. VEGF up-regulation also results in increasing vascular permeability, which elevates the interstitial pressure in the tumour and deteriorates drug delivery to tissues [204].

VEGF-A, often termed as VEGF, belongs to the VEGF-PDGF - family [218]. The expression of VEGF-A and VEGF-C is shown to be increased in CRC [219] and the physiological effects of VEGF are mediated by its receptors VEGFR-1 and VEGFR-2 [219]. VEGFR-3 receptor was reported to be expressed in the endothelial cells of blood and lymphatic vessels [220] and the expression of VEGFR-3 mRNA to be positive in most CRCs [219].

2.3.7.2. Carbonic anhydrase IX (CA IX)

Carbonic anhydrases are zinc-containing metalloenzymes that are widely present in the gastrointestinal tract. They are important enzymes in the production of saliva, gastric acid and bile and several other physiological functions [11]. Carbonic anhydrase IX (CA IX) is a transmembrane glycoprotein [221], which is expressed on protein level only in some tissues, like the epithelia of bile and pancreatic ducts, gallbladder, gastric mucosa and duodenum [222]. The staining weakens in small intestine progressively towards colon [223]. In normal colorectal epithelium, CA IX staining is very weak or absent, confined only to occasional cryptal cells [11]. Instead in CRC, CA IX is significantly over-expressed, showing membranous staining pattern [12]. CA IX expression is thus detectable in CRC, but not in corresponding non-cancerous tissue [12, 221].

Cancer cells tend to produce lactic acid [203], which results in acidic environment, in order to maintain optimal acidic pH and malignant biological functions [224]. CA IX catalyses the reversible chemical reaction in which carbon dioxide is hydrated to carbonic acid and further to bicarbonate ion and proton [221, 225-227]. Thus, CA IX is an important enzyme for cancer cells in hypoxic and normoxic conditions [226, 228] in the regulation of acid-base balance [229]. Most hypoxic tumours have acidic pH values of 6 [230]. Maintaining an acidic environment enhances tumour invasion and relates to poor disease outcome [204, 225]. Carbonic anhydrases may also have a role in signal transduction [224].

2.3.7.3. GLUT-1

GLUT-1 is one of the down-stream targets of HIF-1α [10]. GLUT-1 is widely expressed in tissue-blood barriers [231] and at variable levels in many tissues [232, 233]. In the mucosa of normal colon, there is a gradient of GLUT-1 staining from the surface epithelium to the base of the crypts [205]. The staining is more intense in CRC than in normal mucosa [205]. In cancer tissue, GLUT-1 staining is primarily membranous [234, 235], but a simultaneous cytoplasmic staining is seen along with the dominantly strong membranous staining [236]. GLUT-1 is over-expressed in several malignancies, e.g. cancers of the colon [237], and pancreas [238].

Under hypoxic conditions, cells switch to use lactate production instead of oxidative phosphorylation to produce energy [202]. Hypoxia leads to the stabilisation of HIF-1α [8, 206, 209], which results in the activation of its down-stream targets including GLUT-1 [10, 227]. Glucose is actively transported into the cell by transporter proteins [239]. GLUT-1 is the main carrier of glucose in malignant

tumours [231]. In addition to hypoxia, GLUT-1 is also regulated by inhibitors of oxidative phosphorylation [240].

2.3.7.4. Ezrin

Ezrin belongs to the ezrin-radixin-moesin (ERM) family of proteins [18, 19]. The ERM proteins function as cross-linkers between the plasma membrane and the cytoskeleton [21, 22].

Ezrin, also known as cytovillin, is widely expressed in several normal and malignant tissues of both epithelial and non-epithelial origin [23, 241]. In normal colorectal mucosa, ezrin is mainly expressed on the apical cell membrane in a polarised fashion, whereas in cancer cells, the staining is predominantly cytoplasmic [242]. Staining is also less intense in normal colorectal mucosa than in carcinoma [243].

Ezrin has an active C-terminal, which is connected to the actin cytoskeleton and an N-terminal, located to the plasma membrane [21]. It is present in the cytoplasm in its inactive form [244]. Stimulation leads to ezrin phosphorylation and translocation to the ruffles, which in turn increases motility and proliferation [18, 19, 241, 245, 246]. In cell cultures and in tumour samples, ezrin is distributed both underneath the plasma membrane and in the cytoplasm, where it links transmembrane proteins, especially adhesion molecules to actin cytoskeleton [20, 247, 248]. Ezrin is also involved in the control of cell morphology, motility, proliferation and survival, as well as in signal transduction by protein kinase C, Rho-kinase, EGFR, Src, mTOR and PI3K/AKT pathways [24-26].

2.3.8. Effect of hypoxia on resistance to treatment

The key mechanism for malignant progression as well as resistance to therapy in hypoxia is the selection of neoplastic cells insensitive to apoptosis [31]. Well-oxygenated cells sensitive to radiotherapy are killed, and resistant hypoxic cells continue to proliferate and are thus selected to survive [101]. Apoptosis is an essential mechanism in the response to radiotherapy and chemotherapy [249].

2.3.8.1. Radiotherapy and hypoxia

2.3.8.1.1. Oxygenation and reoxygenation

Radiosensitivity depends on various factors; including cell cycle phase, tumour type and size, and most importantly, oxygen tension [101]. Radiation causes DNA damages to cancer cells by formation of free radicals. Irreparable lethal double-strand breaks cause cancer cell death by apoptosis. Apoptosis, or programmed cell death is regulated by pro- and antiapoptotic factors, which in turn are regulated by HIF-1α [250]. Cancer cells that have suffered sublethal damages can escape apoptosis, especially in hypoxic conditions [250]. Oxygen is important for radiosensitivity due to its participation in free radical generation, free radical damage fixation and limiting chemical repair [101]. Cancer cells can also be injured by the effect of radiation on the microvasculature, supporting stroma or parenchymal cells [101]. Poorly oxygenated tissues are less sensitive to radiation than well oxygenated tissues, thus impairing the therapeutic effect: a three-fold higher radiation dose is required to achieve the same biologic effect as in normoxic conditions [251]. Moreover, oedema, frequently present in malignant tissues during radiotherapy may deteriorate the hypoxic conditions, further aggravating radioresistance [252]. Thus, oxygen is the most important and potent radiosensitiser [253, 254].

Reoxygenation is seen in tumour tissue after exposure to radiation. In mouse mammary carcinoma, the oxygen levels in tumour tissue before radiation have been shown to resemble those of advanced cancer, but after radiation a significant improvement in oxygenation has been found. Reoxygenation is particularly evident after very low pO_2 [255]. Early reoxygenation has been studied in animal tumour models, where increased oxygen pressure has been observed starting 3-4 hours after radiation and being elevated for at least 24 hours after radiation [253]. Reogygenation may occur due to decreased interstitial pressure and better oxygen diffusion after irradiation caused by a loss of tumour cells, resulting in increased radiosensitivity [253].

Proliferating cells are more prone to radiation damages than cells in resting phase [101]. Fractionated treatment schedule enhances the possibility of targeting proliferating cells [101, 253]. Repopulation, or accelerated proliferation and repair of sublethal damages also take place in cells after RT, implicating that RT is best given without any additional breaks in the treatment [101].

2.3.8.1.2. Nitroimidazoles and anti-inflammatory drugs in radiosensitation

Nitroimidazoles, including metronidazole and misonidazole, can mimic the effect of oxygen and have therefore been studied for their possible radiosensitising impact. Due to associated toxicites it has not been possible to use them at effective doses. However, a meta-analysis of the trials revealed slightly improved local control with the use of nitroimidazoles [256]. Cyclo-oxygenase-2 (COX-2) is an enzyme that catalyses the formation of prostaglandin H2 from arachidonic acid [257]. COX-2 over-expression has been shown to associate with poor prognosis in many cancers [254] and COX-2 inhibitors to induce apoptosis in colon cancer cell lines [257]. In tumour model studies, COX-2 inhibitors and other tested non-steroidal anti-inflammatory agents (NSAID) have been found to increase pO_2, leading to enhanced tumour radiosensitivity. These findings implicate that NSAIDs can act as radiosensitisers [254]. However, COX-2 inhibitors are found to increase the risk of cardiac events and all NSAIDs the risk of upper gastrointestinal tract problems, thus restraining their exploitation as radiosensitisers [258].

2.3.8.1.3. Chemotherapeutic agents in radiosensitisation

The addition of fluorouracil to postoperative radiotherapy has been found to improve disease outcome in rectal cancer as compared to radiotherapy alone [168, 259]. An increase in the rate of pCR and a decrease in local relapses has been demonstrated with the use of preoperative chemoradiotherapy when compared to radiotherapy alone [153]. The benefit of chemotherapy can be explained by its systemic effects, but favourable tumour regression and a decline in local disease recurrences points out the possibility of fluorouracil acting as a radiosensitiser [259].

Cisplatinum, which forms DNA adducts, leading to increased apoptosis through the activation of several signalling pathways [260], has been shown to act as a radiosensitiser [261]. Oxaliplatin is a platinum analogue currently used in the treatment of colorectal cancer, both in the adjuvant and the metastatic setting [182]. Oxaliplatin has been reported to increase the efficacy of radiation even when the doses are diminished, as long as a threshold dose is maintained [262]. In phase II studies, oxaliplatin combined with capecitabine and radiotherapy preoperatively, has been shown to evoke excellent responses in rectal cancer [159].

Some agents are toxic to hypoxic cells only, which are resistant to conventional cytotoxic treatments. These drugs include chemotherapeutic agent mitomycin C and a more selective drug tirapazamine [263], which brings about single and

double strand DNA damages more lethal than those produced by ionising radiation [264]. In locally advanced cervical cancer, tirapazamine in combination with radiation and weekly cisplatin has, however, been found to associate with more severe toxicity than anticipated [265].

2.3.8.1.4. Oxygen manipulation

Hyperbaric oxygen

Since oxygen is critical to the efficacy of treatment, hyperbaric oxygen should in theory potentiate the benefit of radiotherapy. This rationale has been studied in malignant glioma, showing longer median survival with than without hyperbaric oxygen [266]. This approach, however, is not routinely available and does not take into account other factors related to hypoxia, including impaired blood flow in tumour vessels [267].

Treatment of anaemia

Lower than 120 g/L haemoglobin level, has been shown to associate with increased pelvic recurrence in cervical cancer. However, correction of anaemia by blood transfusions and maintaining the haemoglobin level ≥120g/L has been observed to improve survival rates for these patients [268]. Average weekly haemoglobin levels have also been shown to be coupled with disease outcome in cervical cancer [269]. Erythropoiesis-stimulating agents (ESAs) have been variably used in the treatment of anaemic cancer patients, but concerns of its safety were brought about by the observations of increased risk of thromboembolism [270]. A meta-analysis of controlled ESA oncology trials was carried out and no significant influence of ESAs on disease progression or survival was found but an increased risk for thromboembolic events was revealed [271]. Moreover, the activation of hypoxia-induced pathways have been shown to occur independently, irrespective of the patient's haemoglobin levels [272], further emphasising the complexity of the effects of tumour hypoxia and its regulation.

2.3.8.3. Chemotherapy and hypoxia

The mechanisms of chemoresistance are not well known [273]. One mechanism involved in hypoxia-related chemoresistance could be linked to HIF-1α, which is shown to regulate VEGF [204]. HIF-1α activation leads to the up-regulation of VEGF, which in turn increases vascular permeability. VEGF up-regulation elevates interstitial pressure and causes impaired drug delivery to tissues [204]. Hypoxia appears to render cells resistant to chemotherapy [263]. In malignant

colon, ovarian and liver cell lines, oxygen deprivation before and during etoposide and oxaliplatin treatment have been found to result in down-regulation of proapoptotic proteins and drug resistance to etoposide. These results implicate hypoxia to lead to apoptosis inhibition [273]. Loss of tumour-suppressor gene p53 or over-expression of apoptosis inhibitor BCL-2 is known to inhibit apoptosis in hypoxic cervical cancer tissue [274]. Clinical studies have shown that over-expression of HIF-1α is associated with unfavourable outcome in several human cancers, linking hypoxia with chemoresistance [10, 29].

2.3.8.4. Hypoxia, response to therapy and disease outcome

Hypoxia, oncogene activation or loss of function of suppressor genes result into increased activation or decreased degradation of HIF-1α [9], leading to its over-expression in many cancers, including CRC [205]. HIF-1α has a central role in tumour progression, resistance to treatment and disease outcome [9]. A significantly worse DFS and overall survival has been observed with hypoxic cervical cancers, as compared with normoxic tumours [30]. Also, larger and thus more hypoxic tumours tend to be accompanied by more frequent parametrial and lymphovascular involvement than well oxygenated tumours [30]. In clinical studies concerning head and neck or cervical cancer, poor tumour oxygenation has been associated with increased tumour invasion, metastasis, unfavourable disease outcome [29, 275] or resistance to radiotherapy [6, 9, 276]. HIF-1α expression has also been shown to be linked to Duke's stage [277] and lymph node status in rectal cancer [277]. Patients, whose tumours express HIF-1α strongly, have shorter overall [277, 278] and local relapse-free and disease-free survival [277, 278]. Previous studies on the prognostic significance of HIF-1α, CA IX, GLUT-1 and ezrin with regard to outcome of human tumours are depicted in **Table X.** In general, strong expression is related to unfavourable disease outcome [27, 30, 227, 242, 272, 275, 277-294]. However, in renal cancer, low CA IX expression and absence of VHL mutation have been shown to be related to more advanced tumours and unfavourable outcome [285-288].

Table X. HIF-1α, CA IX, ezrin and GLUT-1 as prognostic/predictive markers in human malignancies in previously published studies.

Biomarker	Type of cancer	Prognostic/predictive significance of high expression	Reference
CA IX	Head and neck cancer	Unfavourable	Bache et al, 2006
	Non-small cell lung cancer	Unfavourable	Giatromanolaki et al, 2001 Swinson et al 2003
	Breast cancer	Unfavourable	Brennan et al, 2006 Generali et al, 2006
	Renal cell cancer	Favourable	Bui et al, 2003, 2004 Atkins et al, 2005 Patard et al, 2008
HIF-1α	Cervical cancer	Unfavourable	Höckel M, 1996 Birner et al, 2000
	Head and neck cancer	Unfavourable	Lu X et al, 2006 Koukourakis M et al, 2002
	Rectal cancer	Unfavourable	Theodoropoulos G et al, 2006
Ezrin	Colorectal cancer	Unfavourable	Elzagheid et al, 2008
	Head and neck cancer	Unfavourable	Mhavec-Fauceglia P et al, 2007
	Ovarian cancer	Unfavourable	Köbel M et al, 2006
	Malignant melanoma	Unfavourable	Mäkitie et al, 2001
GLUT-1	Rectal cancer	Unfavourable	Brophy et al, 2009 Cooper et al, 2003
	Colorectal cancer	Unfavourable	Haber et al, 1998
	Gastric cancer	Unfavourable	Kawamura 2001
CA IX & GLUT-1	Bladder cancer	Unfavourable	Hoskin et al, 2003

3. AIMS OF THE STUDY

Hypoxia is shown to have an impact on disease outcome and resistance to treatment [29, 30]. Hypoxia activates HIF-1α, leading to the activation of its down-stream targets CA IX and GLUT-1 [7, 8]. The focus of this study is to evaluate hypoxia-associated biological markers HIF-1α, CA IX and GLUT-1, as well as cell skeleton protein ezrin, also linked to HIF-1α, in RC treated by preoperative RT or chemoradiotherapy. The aim of the study is on one hand to compare by IHC the expression of these biomarkers in the diagnostic biopsies with the expression in the operative samples, and on the other hand in relation to disease outcome. In addition, the aim is to evaluate histopathologic features in all tumour samples and tumour regression grade in the operative samples after long-course RT and compare them to the results of IHC stainings.

The specific aims of the study are

1) To analyse the expression of the key protein in hypoxia, HIF-1α in the diagnostic biopsies and operative samples after preoperative radiotherapy or chemoradiotherapy, the impact of preoperative treatment on HIF-1α expression and the association of HIF-1α expression with disease outcome and tumour regression grade.

2) To evaluate the expression of CA IX, regulated by HIF-1α, in the diagnostic biopsies and operative samples after preoperative radiotherapy or chemoradiotherapy, as well as the influence of preoperative treatment on CA IX expression and the association of CA IX expression with tumour regression grade and survival.

3) To study the expression of cell skeleton protein ezrin, also linked to HIF-1α, in the diagnostic biopsies and operative samples after preoperative radiotherapy or chemoradiotherapy, the impact of preoperative treatment on ezrin expression and ezrin expression in relation to tumour regression grade and survival.

4) To investigate the expression of GLUT-1, a downstream target of HIF-1α, in the diagnostic biopsies and operative samples after preoperative radiotherapy or chemoradiotherapy, the effect of preoperative treatment on GLUT-1 expression and the relation of GLUT-1 expression to disease outcome and tumour regression grade.

4. PATIENTS AND METHODS

4. 1. PATIENTS

This retrospective, non-randomised study included 178 consecutive RC patients treated at Turku University Hospital according to the standard treatment protocols in the years 2000-2009. Patients with metastatic disease, those with upper rectal cancer or superficial tumours treated by excision only were excluded and only adenocarcinomas were included, in order to build up a biologically and therapeutically homogenous patient population. Preoperatively, the patients were staged by CT or MRI and digital examination of the rectum, CT of the abdomen and x-ray or CT of the chest. Since 2005, rectal cancer treatments in this hospital have been planned by a multidisciplinary team. The number of patients in each study is depicted in **Table XI** and the patient population described in detail in the original publications I-IV and in **Table XII**.

Table XI. Number of patients and tumour samples in each study

Study/Biomarker		**Number of patients/tumour samples**				
		Preoperative biopsies	**Operative samples**			
			Total	Short-course RT	Long-course RT	Control
I	**CA IX**	80	166	75	37	54
II	**HIF-1α**	79	168	75	39	54
III	**Ezrin**	76	176	76	46	54
IV	**GLUT-1**	78	175	76	46	53

Table XII. The clinical characteristics of the patients in the study (n=178).

Variable	n (%)
Patient gender	
Male	106 (60)
Female	72 (40)
Type of preoperative treatment	
None	54 (30)
Short-course radiotherapy (RT)	77 (43)
Long-course RT, no chemotherapy	10 (6)
Long-course RT with chemotherapy	37 (21)
Preoperative T	
T1-T2	33 (18)
T3	55 (31)
T4*	47 (26)
TX†	43 (24)
Preoperative N	
N+‡	74 (42)
N0	41 (23)
Ns§	24 (13)
NX†	39 (22)
Preoperative G	
G1	22 (12)
G2	53 (30)
G3	14 (8)
GX†	89 (50)
Postoperative stage	
Stage I	44 (25)
Stage II	56 (31)
Stage III	76 (43)
No vital cancer	1 (<1)
Stage not assessable	1 (<1)

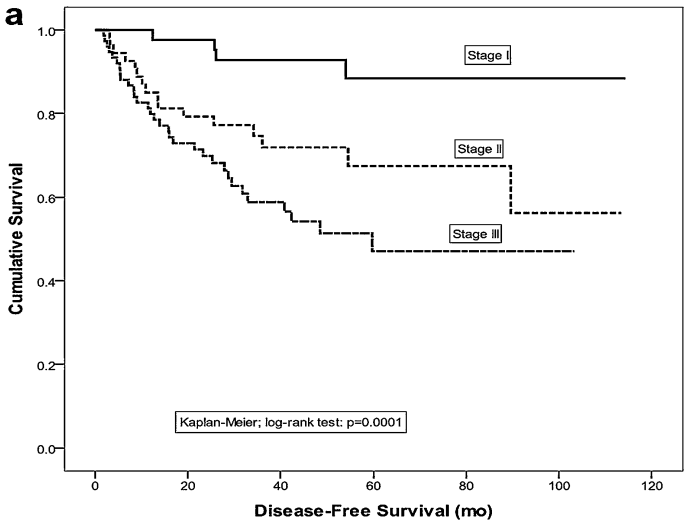
** including T3 tumours with threatened CRM involvement, † not defined, ‡ lymph nodes visualised in imaging, § lymph nodes visualised in imaging, suspected metastatic. RT= radiotherapy*

The patients were treated with short-course (5x5 Gy) (n=77) or long-course radiotherapy (50.40 Gy) (n=47) or received no preoperative treatment (n=54), depending on the stage and localisation of the tumour and following the standard treatment protocols [5, 147], based upon the judgment of the surgeon and the multidisciplinary team. Long-course RT was given with (n=37) or without (n=10) concomitant chemotherapy. Postoperative adjuvant chemotherapy was given to patients with lymph node positive or high-risk lymph node negative tumours, according to the standard practice [185]. As a control group (n=54), we studied a third series of patients who had not

received any treatment prior to surgery. Postoperative RT, chemoradiotherapy or adjuvant chemotherapy were given to eligible control group patients when indicated (19/54, 35%). After completion of the treatment protocols, patients were scheduled for further follow-up at the Department of Surgery. The median follow-up time of the patients was 35 months and the mean follow-up time 40 months (range 2-114 months).

The mean age of the patients was 67 years (median 69 years, range 34-92 years). All tumours in this study were adenocarcinomas, of them 4% were classified as mucinous. Of the operations, 96 (54%) were performed by AR. Since the introduction of the multidisciplinary team in 2005, the proportion of AR was higher, 63%. Surgery was macroscopically radical in 175/178 (98%). The CRM was ≤1 mm in 23/125 (18%), and more than 1mm but ≤2 mm in 7/125 (6%) of the patients. The number of examined lymph nodes was less than 12 in 88 (50%) and at least 12 in the rest of the operative specimens. The number of metastatic lymph nodes was <4 (N1- tumours) in 47/178 (26%) and ≥4 (N2-tumours) in 25/178 (14%) of the tumours. The percentage of patients diagnosed before year 2005 was 39%. During follow-up, 12 patients were diagnosed with locally relapsed and 43 patients with metastatic disease. Of the metastatic disease patients, 4 patients also had verified local relapses concurrently at 3 years follow-up. The survival data (updated until 2010, May) of the patients are depicted in **Table XIII**. The survival curves of the patients with regard to postoperative stage are shown in **Figures 4a and b**. The mean disease-free survival (DFS) was 56 months for stage I, 41 months for stage II and 33 months for stage III patients. The mean disease-specific survival (DSS) was 60 months for stage I, 51 months for stage II and 44 months for stage III patients. The mean DFS was 26 months for patients with CRM<2 mm and 36 months for those with CRM≥2 mm, and the mean DSS were 34 months and 44 months, respectively. The mean DSS for patients with only local relapse was 41 months and for those with distant relapse 40 months.

The study protocol was approved by the ethics committee of the Hospital District of Southwest Finland and the collection and use of archival tissue material by the National Authority for Medico-Legal affairs. The study was conducted in accordance with the Declaration of Helsinki.



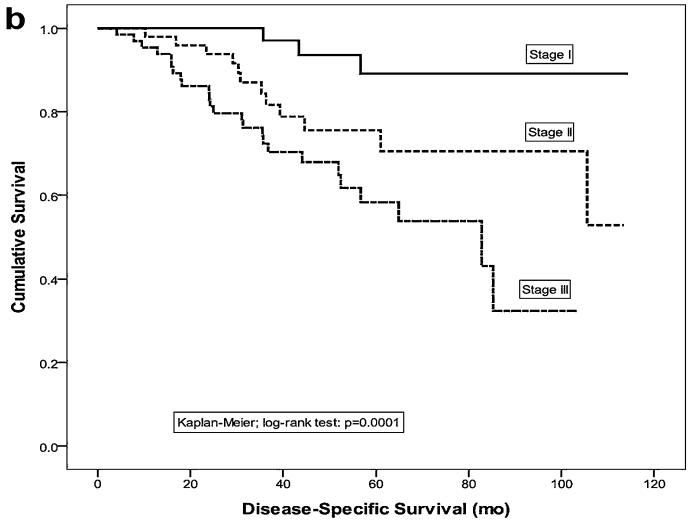


Figure 4. a) Disease-Free Survival and b) Disease-Specific Survival with regard to postoperative stage.

Table XIII. Disease outcome of the patients at the latest follow-up (May 2010)

Disease outcome	**n (%)**
Alive without cancer	105 (59)
Alive with cancer	12 (7)
Died of cancer	40 (23)
Died of other causes	21 (12)

4. 2. METHODS

4.2.1. Immunohistochemical (IHC) stainings

4.2.1.1. IHC staining procedure

The dilutions of the primary antibodies were optimised by a series of pilot stainings. The pre-treatments were chosen by the recommendations of the manufacturers. In each case, the most representative blocks were selected, cut to 5µm sections. The specimens were pre-treated in microwave oven twice for 7 minutes in 10 mM sodium citrate buffer, pH 6.0, and subjected to IHC staining with the following antibodies: polyclonal rabbit anti-Human CA IX (antibody 15086, Abcam, Cambridge, UK), diluted to 1:8000 concentration (I); monoclonal mouse anti-human HIF-1α (BD Transduction Laboratories San Jose, CA, CAT# 610958), diluted to 1:100 concentration (II); mouse monoclonal IgG antibody to human ezrin (clone 3 C12)[295] (III), provided by Antti Vaheri, final dilution 0.3 mg/ml, and polyclonal rabbit anti-human GLUT-1 (Alpha Diagnostic International, rabbit anti-human GLUT-1 IgG, affinity pure, San Antonio, TX, USA), and diluted to 1:1000 (IV). To detect the immunoreactions, a Power Vision poly-HRP IHC Detection system (Immunovision Technologies Co, Burlingame, CA, USA), was used.

4.2.1.2. Evaluation of the IHC staining

The IHC staining of the samples was evaluated by two observers (EK and JS), blinded to all clinical data and radiological findings. Light microscopes with 4x and 10x objectives (EK) and 5x and 10x objectives (JS) were used for evaluation. The analyses of the IHC staining of CA IX, HIF-1α, ezrin and GLUT-1 are described in detail in publications I-IV.

4.2.1.2.1. CA IX (I)

IHC staining in the operative samples was graded using three approaches: 1) general grouping of the cases into positive or negative, 2) proportion of positive staining, and 3) staining intensity. The slides were assessed as negative if the proportion of positive carcinoma cells in the section was less than 10%. For positive cases, the proportion of positively stained carcinoma cells was analysed using three categories: 1) 10-25%, 2) 26-50%, and 3) over 50%. The staining intensity was defined as 0 = negative, 1 = weak, and 2 = moderate and 3 = strong staining intensity. Weak staining intensity was defined as a faint positive staining in the cytoplasm and occasional staining in the cell membranes impossible to detect at a small magnification (objectives

4x-5x). The staining was assessed as moderate or strong, if the positive reaction in the cell membranes could easily be identified at a small magnification (objectives 4x–5x). In preoperative biopsies, only positive/negative staining and staining intensity were assessed.

4.2.1.2.2. HIF-1α (II)

HIF-1α staining was graded using three approaches: 1) general grouping of the cases into positive or negative, 2) proportion of positive staining in cancer cells, and 3) staining intensity. For positive cases, the number of visual fields in 10x10 grid containing carcinoma cells was first calculated in the HE-slides with a small magnification (objectives 4-5x). The proportion of fields with stained cancer cell nuclei was further calculated in relation to the fields containing carcinoma cells, giving a percentage of positive fields. Staining intensity was then evaluated using three categories: 0 = negative, 1 = weak, 2 = moderate and 3= strong staining intensity. In weak staining intensity there was a faint positive staining in the nucleus. The staining was assessed moderate or strong, if the positive reaction in the nuclei could be easily identified, corresponding to the staining intensity seen in the nuclei of renal carcinoma cells, which was used as a positive control. From the preoperative biopsies, only positive/negative staining and staining intensity were assessed, because of the small size of the samples.

An immunoreactive staining score (IRS) was calculated in order to take both the percentage and the intensity of staining into account. The proportion of positively stained cells was given scores as follows: 0 = 0-4%, 1= 5-10%, 2=11-29%, 3=30-59% and 4=60% and over. The product of the percentage score and intensity grade (0-3) was calculated, giving a score (IRS) of 0-12.

4.2.1.2.3. Ezrin (III)

Ezrin was evaluated by analysing the staining intensity, using four categories: negative, weak (1+), moderate (2+) and strong (3+), corresponding to the staining intensity in the adjacent lymphocytes. There was a weak membranous staining in normal rectal epithelium, concentrated at the apical cell surface, whereas in RC, the staining pattern was mostly cytoplasmic. However, in some tumours concurrent focal membranous staining was also seen.

4.2.1.2.4. GLUT-1 (IV)

GLUT-1 staining was primarily membranous, but a concurrent focal cytoplasmic staining was seen in some cases. The analyses were based on the membranous staining, graded by two approaches: the percentage of positive

tumour cells and staining intensity. Staining intensity was graded as 0 if negative, 1 if weak, 2 if moderate, and 3 if strongly positive. The proportion of positive cells was given scores in the following way: 0 = 0%, 1 = 0-2%, and 2 = 3-9%, 3 =10-25%, 4 = 26-50% and 5 >50%. The staining in the biopsies was only assessed as negative or positive, in addition to intensity.

4.2.1.2.5. HE-staining

HE- staining was performed according to the standard laboratory protocols.

4.2.2. Western Blot analysis (WB) (I, III)

4.2.2.1. Confirming CA IX expression in WB (I)

To confirm the IHC results of CA IX staining, a small fresh tissue material from CC patients (n = 4) was further studied for the detection of CA IX protein in WB. CA IX primary antibody (ab15086, Abcam, Cambridge, UK) was diluted 1:1000 for western detection. Horseradish peroxidase conjugated anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) and Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, U.S.A.) were used according to the manufacturers' instructions for visualisation of the signal.

4.2.2.2. Ezrin in relation to radiation by WB (III)

Two different cell lines with well characterised ezrin expression pattern [296, 297] were analysed to test whether ezrin expression is directly regulated in response to radiation. Human osteosarcoma cells (U2OS) express wild-type p53 [298], whereas p53 is mutated in human glioma cells (U251) [299]. The cultured cells were incubated, then irradiated and analysed by immunoblotting with ezrin 3C12 mAb [295], p53 (DO-1) Ab, anti-phospho-histone H2A.X (Ser 139) Ab (Upstate, Temecula, CA, USA) and α-tubulin mAb (Sigma-Aldrich, St Louis, MO, USA) using enhanced chemiluminescence detection (Amersham Biosciences, Uppsala, Sweden).

4.2.3. Tumour regression grade evaluation (I-IV)

Tumour regression grade (TRG) was analysed from HE-stained sections of the operative samples after long-course RT according to the modified Dworak and Rödel scales, using three categories: poor, moderate and excellent response [106, 107] (study I). The response was assessed as poor, if only minimal or no tumour regression was seen and there was a dominant tumour mass left (Dworak 0-1). When only a few tumour cells or tumour cell groups were left in the primary

tumour, lymph nodes or perirectal fat, the response was assessed as moderate (Dworak 2). The response was defined as excellent if there were only very few or no tumour cells left (Dworak 3-4). In case of moderate and excellent response, a total of 2-8 separate histological slides were studied to confirm the regression grade. Examples of the different degrees of TRG are presented in **Figure 5.**

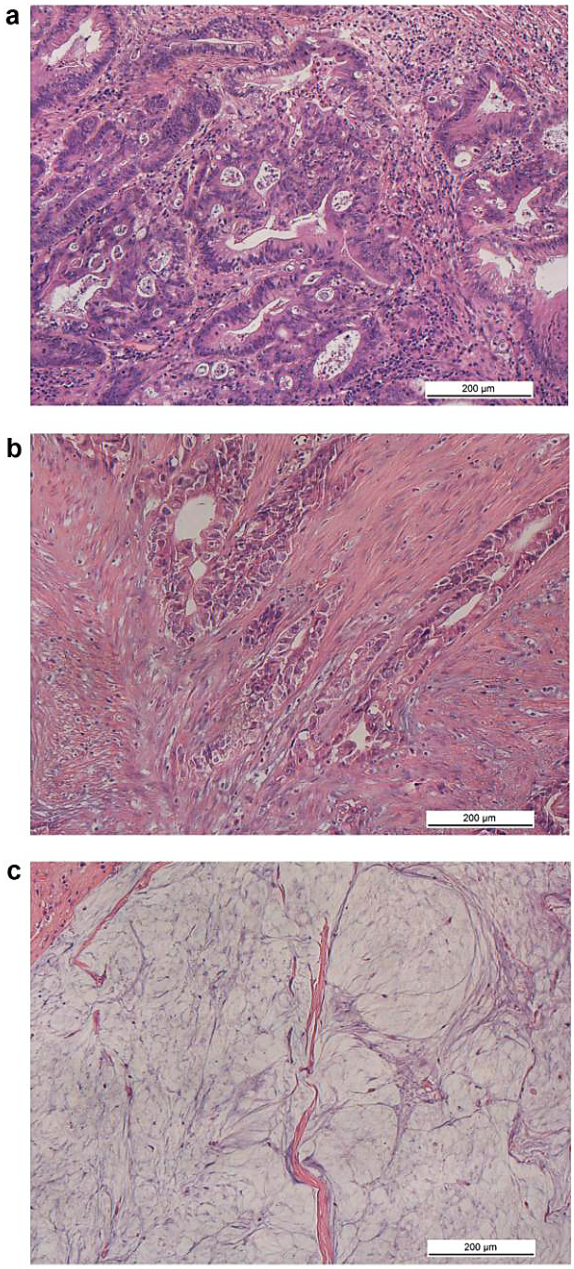


Figure 5. Tumour regression after preoperative long-course radiotherapy: A) poor, B) moderate, and C) excellent tumour regression.

4.2.4. Statistical analysis (I-IV)

All statistical analyses were run using SPSS® (SPSS, Inc., Chicago, USA) and STATA™ (Stata Corp., College Station, TX, USA) software packages (SPSS for Windows, version 17.0.2 and STATA/SE 11.1). Frequency tables were analysed using the χ^2-test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. Differences in the means of continuous variables were analysed using Mann-Whitney's test or Kruskal-Wallis test for two and multiple independent samples, respectively. Concordance of IHC staining between preoperative biopsies and operative samples was analysed using non-parametric paired-samples test (Wilcoxon signed ranks test, or McNemar test for dichotomous (+/-) variables. Inter-observer reproducibility of the IHC assessments was tested using regular (Cohen's) kappa and weighted kappa. To calculate the latter, the ICC (intra-class correlation coefficient) test was used, with parallel mode and two-way random model, using consistency assumption and average measures option to interpret the ICC (95%CI). The inter-observer reproducibility of all CA IX assessments was excellent (with κ value 0.988 for CA IX+/-, 0.931 for staining proportion and 0.892 for staining intensity), for HIF-1α good (with κ value 0.963 for HIF+/-, 0.864 for staining intensity and 0.843 for HIF percentage), for ezrin good (with κ value 0.671) and for GLUT-1 moderate to good (with κ value 0.500 for the percentage of positive cells, 0.500 for staining intensity and 0.587 for GLUT-1 +/-).

To analyse ezrin as a predictor of DSS, performance indicators [sensitivity (SE), specificity (SP), positive (PPV) and negative (NPV) predictive value] were calculated using the contingency tables, and 95% CI calculated with the exact method.

Univariate survival analysis for DFS, and DSS was based on the Kaplan-Meier method, where stratum-specific outcomes were compared using log-rank (Mantel-Cox) statistics. To adjust for covariates, Cox proportional hazards regression model was used, co-variates being entered in a stepwise backward manner. All statistical tests were two-sided and declared significant at *p*-value <0.05 level.

5. RESULTS

5.1. GENERAL ASPECTS OF IHC STAINING (I-IV)

HIF-1α staining was primarily confined to the nuclei of cancer cells, while CA IX and GLUT-1 staining patterns were predominantly membranous, and ezrin staining mostly cytoplasmic.

HIF-1α, CA IX, ezrin or GLUT-1 expression in the operative samples were not related to patient age, gender, preoperative T (the depth of tumour invasion), preoperative N (nodal status), the type or radicality of the operation, postoperative stage, postoperative tumour differentiation (G), the number of metastatic (1-3 vs. ≥4) or examined lymph nodes (< 12 or ≥12), CRM or vessel invasion. In the multivariate models, the number of metastatic lymph nodes was a significant predictor of DSS in HIF-1α and ezrin analysis and of DFS in CA IX analysis. In HIF-1α analysis, preoperative treatment group predicted DFS. Unfavourable disease outcome with regard to IHC analysis and treatment group is shown in **Table XIV. Table XV** depicts IHC staining in the preoperative RT group biopsies and control group operative samples (excluding those patients who had received postoperative RT).

5.2. CA IX (I, V)

CAIX expression was positive in 49% of the biopsies and 44% of all the operative samples. There was a significant difference in CA IX staining intensity of the tumours in the different treatment categories (p=0.006). Staining intensity was moderate/strong in 10/55 (18%) of the preoperative biopsies in short-course RT group, and 6/25 (24%) in the long-course RT group. Staining intensity in the operative samples was moderate/strong in 18/75 (24%) of the short-course RT group, 7/8 (87%) of the long-course RT only (without chemotherapy), 8/29 (27%) of the chemoradiotherapy group, and 16/54 (30%) of the control group. In all, positive CA IX expression in 18/38 (48 %) biopsies turned into negative and negative expression in 12/40 (30 %) biopsies turned into positive in the operative samples, while the expression in 48/78 (61%) biopsies remained constant. CA IX expression in the preoperative biopsies and the operative samples were not significantly different (p= 0.268 for CA IX +/- and p= 1.000 for CA IX intensity).

Table. XIV. Unfavourable disease outcome with regard to IHC analysis.

IHC	Expression	Sample	Unfavourable outcome/group	Significance
HIF	positive	operative sample	DSS, long-course RT with or w/o chemotherapy	**0.001**
CA IX	moderate/strong staining intensity	operative sample	DFS, study population	**0.001**
			DSS, study population	**0.009**
			DFS, short-course RT	**0.004**
			DFS, long-course chemoradiotherapy	**0.041**
Ezrin	moderate/strong staining intensity	preoperative biopsy	DFS, study population	**0.027**
			DSS, study population	**0.002**
		operative sample	DSS, long-course RT with or w/o chemotherapy	**0.0001**
GLUT-1	moderate/strong staining intensity	operative sample	DFS, long-course RT with or w/o chemotherapy	**0.066**

RT=radiotherapy, w/o =without

Table XV. Immunohistochemical analyses in the biopsies of the short-course and long-course radiotherapy group patients and the operative samples of the control group patients *

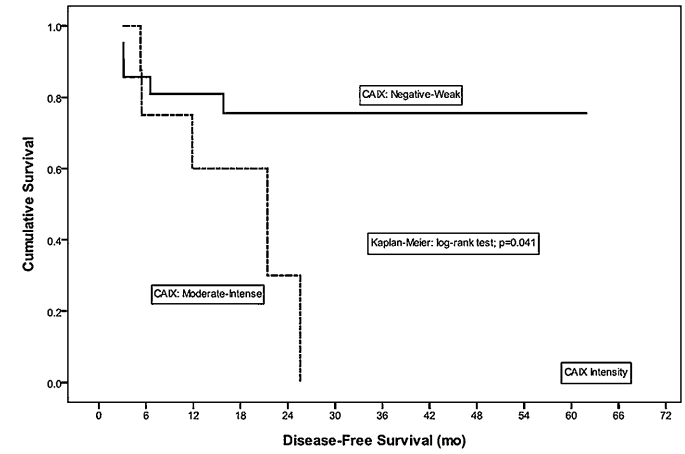
Variable	**Sample type**	**Immunohistochemistry**		**Significance †**
CAIX staining intensity		**Negative/Weak intensity n (%)**	**Moderate/Strong intensity n (%)**	
Short-course RT	biopsy	45 (82)	10 (18)	p=0.065
Long-course RT	biopsy	19 (76)	6 (24)	
Control	operative	26 (63)	14 (34)	
HIF-1α		**Negative n(%)**	**Positive n (%)**	
Short-course RT	biopsy	18 (32)	38 (68)	p=0.084
Long-course RT	biopsy	6 (25)	18 (75)	
Control	operative	20 (49)	20 (49)	
Ezrin staining intensity		**Negative/Weak n (%)**	**Moderate/Strong n (%)**	
Short-course RT	biopsy	50 (93)	4 (7)	p=0.0001
Long-course RT	biopsy	12 (52)	11 (48)	
Control	operative	20 (49)	20 (49)	
GLUT-1 staining intensity		**Negative/Weak intensity n(%)**	**Moderate/Strong intensity n (%)**	
Short-course RT	biopsy	35 (62)	21 (37)	p=0.014
Long-course RT	biopsy	11 (48)	12 (52)	
Control	operative	13 (32)	27 (66)	

** Control group patients who had received postoperative radiotherapy or chemoradiotherapy (n=13) are excluded from this table. † IHC in the biopsies of the preoperative treatment groups are compared to the operative samples in the control group.*

The results of IHC staining were confirmed by WB with CRC tissue samples. Three out of four CRC samples showed strong bands with molecular weights of 50 and 56 kDa, in concordance with CA IX positivity in the IHC slides. However, in the normal colorectal mucosa and in one tumour sample only a faint band of 50 kDa was seen. These samples were CA IX negative in the corresponding IHC slides.

Patients who had tumours with negative/weak CA IX staining intensity in the operative samples had significantly longer DFS (p=0.001) than patients who had tumours with moderate/strong staining intensity in Kaplan-Meier (KM) analysis. Likewise, DSS was significantly (p=0.009) longer among patients who had tumours with negative/weak CA IX staining intensity in the operative samples. When CA IX was analysed in the preoperative biopsies, there were no significant differences in survival (unpublished data). The control group patients, who had not received any postoperative adjuvant chemotherapy or RT (n=34), were further compared to those, who had received postoperative RT, chemoradiotherapy or adjuvant chemotherapy (n=20) and no statistically significant differences were seen in DFS or DSS. In the Cox multivariate proportional hazards regression model, only two variables were independent predictors of DFS: 1) number of metastatic lymph nodes; HR 4.44 (95% CI 1.37-14.38) (p=0.013) for recurrence if ≥4 metastatic lymph nodes, and 2) CA IX intensity in the operative samples; HR 7.54 (95% CI 2.44-23.28) (p= 0.003) for disease recurrence if moderate/strong CA IX intensity was present. In a similar Cox model, CA IX intensity in the operative samples remained the only significant independent predictor of DSS; HR 9.23 (95% CI 2.26-37.64) for dying of disease if moderate/strong CA IX intensity was present. The cumulative proportion of survivors at the 36- month follow-up time point was 83% and 35% for CA IX negative/weak and moderate/strong groups, respectively, as estimated from the survival curves.

When the treatment groups were analysed separately, most tumours (7/8, 87%) in the long-course RT group that had not been treated with concomitant chemotherapy expressed CA IX in the operative samples with moderate/strong intensity, as compared to tumours that had been treated with chemoradiotherapy (8/29, 27%). Patients who had tumours with negative/weak CA IX staining intensity in the operative samples after short-course RT or long-course chemoradiotherapy had significantly better DFS than patients, whose tumours had moderate/strong CA IX staining intensity (p=0.004 and p= 0.041) **(Figure 6a, b)**. No statistical differences in disease outcome with regard to CA IX staining intensity in the operative samples were seen in the control group or the long-course RT without chemotherapy group. (Unpublished data).



b)

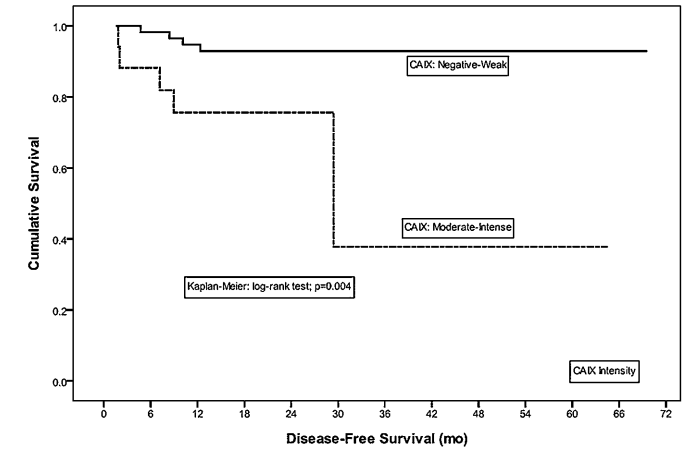


Figure 6. Disease-Free Survival in the a) long-course chemoradiotherapy and b) short-course radiotherapy groups with regard to CA IX intensity.

5.3. HIF-1α analysis (II)

HIF-1α was positive in 70% of the diagnostic biopsies and in 38% of the corresponding operative specimens after RT. After short-course RT, positive HIF-1α expression in 23/37 (62 %) biopsies turned into negative and negative expression in 5/18 (28 %) biopsies turned into positive in the operative samples. After long-course RT, positive expression in 8/18 (44 %) biopsies turned into negative and negative expression in 1/6 (17 %) biopsies turned

into positive. Thus, HIF-1α in the preoperative biopsies was down-regulated in 31/55 (56 %) and up-regulated in 6/24 (25 %) operative samples. In pair-wise comparison, the staining pattern in the biopsy-operative sample pairs was significantly different (p=0.0001 and p=0.039). After short-course RT 32% and after long-course RT 38% of the tumours were HIF-1α positive, whereas in the control group 54% of tumours were positive (p=0.046).

In univariate survival analysis, no significant differences in disease outcome were seen, when HIF-1α in the operative or preoperative samples were analysed in the whole patient population. However, long-course RT group patients whose tumours had HIF-1α negative expression in the operative sample had significantly longer (p=0.001) DSS, as compared to HIF-α positive tumours. There were no significant differences in either DFS or DSS with regard to HIF-1α in the short-course RT or the control group. In the multivariate regression model (Cox), the preoperative treatment group was the only independent predictor of DFS (p=0.007), whereas disease recurrence (p=0.015) and the number of metastatic lymph nodes ≥4 (p=0.0001) were independent predictors of poor DSS.

5.4. EZRIN (III)

Most preoperative biopsies (80%) but only half of the operative specimens (52%) were negative/weak in ezrin IHC. Ezrin expression in the biopsies and the respective operative samples was significantly different (κ=-0.010 for four-tier and κ= 0.028 for dichotomised scoring). Negative expression in 18/22 biopsies (82%) turned positive in the operative sample and positive expression in 6/54 (11%) biopsies turned negative in the operative sample.

Osteosarcoma (U2OS) and glioma (U251) cell lines were tested in WB, to study whether RT directly affects ezrin expression in cancer cells. Radiation increased expression of p53 in U2OS but not in U251 cells, whereas the expression of γH2A.X, another stress marker, was up-regulated in U251 cells. However, there were no changes in ezrin expression in either cell line.

In survival analysis, two thirds (70%) of the patients with negative/weak ezrin expression in the biopsy were alive without disease, as compared to one third (33 %) of the patients with moderate/strong ezrin expression. In Kaplan-Meier survival analysis, DFS and DSS were significantly longer (p= 0.027 and p= 0.002), if ezrin expression in the preoperative biopsy sample was

negative/weak as compared to moderate/strong expression. When analysed in samples taken after preoperative treatment, ezrin expression did not correlate with disease outcome. In the multivariate model ezrin expression did not reach statistical significance as a predictor of DFS (p=0.071). Disease recurrence (p= 0.0001) and the number of metastatic lymph nodes ≥4 (p= 0.028) were the only independent predictors of poor DSS. In a separate analysis of the long-course RT group, survival of patients, whose tumours showed negative/weak ezrin expression in the operative sample, had better DSS as compared to patients, whose tumours had moderate/strong expression (p=0.0001). No statistical differences in disease outcome were seen with regard to ezrin expression in the operative samples of the short-course RT group or the control group.

Negative-weak ezrin expression in the preoperative biopsies was a sensitive predictor of DSS (sensitivity 89.1%), with positive predictive value (PPV) of 86% for the patient being alive. High sensitivity and PPV were lost in the operative samples. The expression of ezrin was further compared with the expression of HIF-1α and CA IX. Ezrin and HIF-1α were significantly congruent in the operative samples but not in the preoperative biopsies. Low HIF-1α expression was associated with negative/weak ezrin expression with OR 4.15 (95% CI, 1.44-11.93; p=0.004). Likewise, negative/weak CA IX was linked with negative/weak ezrin with OR=6.42 (95% CI, 3.04-13.56; p=0.0001). When the three markers were compared in the preoperative biopsies, no such associations were seen.

When the three markers were analysed together (CA IX and HIF-1α in the operative samples and ezrin in the preoperative biopsies) and the panel was tested for its longitudinal predictive value of DSS, negative/weak expression of all three markers predicted survival with 31.1% sensitivity, 89.2% specificity, 90.2% PPV and 28.7% NPV. The panel lost its predictive value if ezrin expression in the operative samples was used instead of preoperative biopsies.

5.5. GLUT-1 (IV)

GLUT-1 expression was positive in 68% of the preoperative diagnostic biopsies and in 51% of the corresponding operative samples. Positive GLUT-1 expression of 33/53 (62%) preoperative biopsies remained positive, while that of 20/53 (38%) biopsies turned into negative in the operative specimens. Negative GLUT-1 expression in 7/25 (28%) biopsies turned into positive in the

operative sample, while that of 18/25 (72%) biopsies remained negative (κ=0.301, CI [0.007; 0.204], ICC= 0.319). Moderate staining intensity in 1/26 (4 %) and strong intensity in 0/7 (0%) biopsies turned into negative in the operative specimen. In the short-course RT group, staining intensities in the biopsies and the respective operative samples were significantly different (p= 0.005), but not in the long-course RT group.

In univariate analysis (KM), there were no significant differences in DFS or DSS with regard to GLUT-1 expression in the biopsies or the operative samples. No significant differences in disease outcome with regard to GLUT-1 expression were found in the short-course RT group or the control group. However, patients, who had received long-course preoperative RT and had negative/weak staining intensity in the operative specimen, had a tendency towards better DFS (p=0.066) as compared to patients, who had moderate/strong staining intensity. When CA IX and GLUT-1 expression in the operative samples were analysed together (n=165), patients who had tumours with negative/weak staining intensity of both markers had longer DFS than patients who had tumours with moderate/high intensity (p=0.005) (unpublished data).

5.6. TUMOUR REGRESSION GRADE WITH REGARD TO IHC (I-IV)

TRG was analysed in the operative tumour samples treated by long-course preoperative RT or chemoradiotherapy. In all, TRG was excellent in 9 tumours and poor/moderate in 36 tumours. TRG with regard to each IHC-analysis is shown in **Table XVI.** In the GLUT-1 study (IV), DFS (p=0.068) and DSS (p=0.024) of the patients who had excellent tumour regression were longer than those of the patients with moderate or poor response. There was one pCR (1/45, 2%) with no viable tumour cells left after preoperative long-course RT in the excellent response group. There have been no local relapses in the excellent response group, whereas in the moderate/poor response group two patients are alive with and five patients have died of locally recurrent disease.

In a further analysis, the expression of all four markers in the preoperative biopsies was compared to TRG in the operative samples **(Table XVII)**. There were no statistically significant differences with regard to IHC of any of the four markers in the biopsies in relation to tumour response. However, no excellent responses in the operative samples were seen, if CA IX intensity in the biopsy was strong (p=0.092) (unpublished data).

5.8. COMBINED ANALYSIS OF HIF-1α, CA IX, EZRIN AND GLUT-1

HIF-1α (positive/negative), CA IX (negative/weak; moderate/strong intensity), ezrin (negative/weak; moderate/strong) and GLUT-1 (negative/weak; moderate/strong staining intensity) in the operative samples were entered in a multivariate (Cox) model as only variables. In this model, moderate/strong CA IX intensity was a significant independent predictor of poor DSS (HR 2.35; 95% CI 1.167-4.74) (*p*=0.017), but none of the markers predicted DFS (data not shown).

Table XVI. Tumour regression grade with regard to the immunohistochemical (IHC) analysis of HIF-1α, CA IX, ezrin and GLUT-1 in the operative samples. The number of patients in each publication is included in parentheses.

IHC analysis/ Tumour regression	**Staining intensity**				**Significance**
	Negative n (%)	**Weak n (%)**	**Moderate n (%)**	**Strong n (%)**	
CAIX (n=36)					
Poor/Moderate	14 (50)	1 (3)	6 (21)	7 (25)	0.057
Excellent	7 (87)	0 (0)	1 (12)	0 (0)	
HIF-1α (n=39)					
Poor/Moderate	16 (52)	7 (22)	7 (22)	1 (3)	0.413
Excellent	7 (87)	0 (0)	1 (12)	0 (0))	
Ezrin (n=38)					
Poor/Moderate	1 (3)	12 (39)	8 (26)	10 (32)	0.012
Excellent	4 (50)	2 (25)	1 (12)	1 (12)	
GLUT-1 (n=45)					
Poor/Moderate	1 (3)	12 (33)	16 (44)	7 (19)	0.013
Excellent	4 (44)	2 (22)	2 (22)	1 (11)	

Table XVII. Tumour regression grade with regard to immunohistochemical analysis in the preoperative biopsies

Immunohistochemistry	**Tumour regression grade**		**Significance**
	Poor/Moderate n (%)	Excellent n (%)	
CA IX intensity (n=24)			
Negative/Weak	11 (61)	7 (39)	0.092
Moderate/Strong	6 (100)	0 (0)	
HIF (n=24)			
Positive	13 (72)	5 (28)	0.586
Negative	4 (67)	2 (33)	
Ezrin (n=23)			
Negative/Weak	7 (58)	5 (42)	0.222
Moderate/Strong	9 (82)	2 (18)	
GLUT-1 intensity (n=23)			
Negative/Weak	10 (53)	9 (47)	0.671
Moderate/Strong	2 (50)	2 (50)	

6. DISCUSSION

In spite of improved disease outcome, a significant proportion of RC patients still succumb to their disease. Consequently, more sophisticated prognostic and predictive markers are needed to direct therapeutic interventions. The aim of this study was to evaluate the ability of three investigational hypoxia-associated markers, HIF-1α, CA IX and GLUT-1, as well as ezrin, an important protein also associated to HIF-1α, to predict disease outcome in rectal cancer treated by preoperative RT or chemoradiotherapy.

The IHC staining of HIF-1 α, CA IX, ezrin or GLUT-1 in the operative samples did not correlate with established clinicopathologic factors. One explanation to the lack of correlation could be given preoperative treatment, which may influence tumour size, stage and grade as well as nodal status. Tumour differentiation grade assessed from a biopsy specimen may not be representative of the whole tumour. After long-course RT, the evaluation of tumour differentiation grade may be impossible due to the potential disappearance of most or all of vital tumour [108, 111]. Preoperative staging can also be challenging [87, 119]. Currently available imaging methods can over- or underestimate the depth of tumour invasion [87, 119]. Moreover, there are no reliable methods available as yet to sort out metastatic lymph nodes from benign, and thus, exact preoperative staging is currently challenging [120].

In order to circumvent this problem, the operative samples were on one hand compared to the pre-treatment, diagnostic biopsies and on the other hand to the operative samples of patients, who had not received preoperative therapy. Our hypothesis was that the expression of the biomarkers in the non-irradiated specimens would resemble their expression in the preoperative biopsies. As shown in **Table XV**, the IHC expression in the biopsies of the preoperative RT group patients deviated from the expression in the control group operative samples. This may partly be explained by the size of the biopsies. Generally multiple biopsies are taken to cover different parts of the tumour and to ensure sufficient tissue material for diagnosis. However, biopsies tend to be taken from the tumour surface, which may be problematic considering the potential heterogeneity of tumours. The differences in patient demographics and the tumour features between the treatment groups also need to be taken into account, keeping in mind the non-randomised retrospective approach of the current study. The control group patients had been too frail or had had too severe comorbidities to have received preoperative treatment, or they had presented with less advanced disease

that had not necessitated preoperative intervention. Control group patients, who had been under-staged preoperatively and who had received postoperative RT or chemoradiotherapy were excluded from this analysis.

6.1. CA IX (I)

The current study showed CA IX staining intensity in the operative specimens to predict adverse disease outcome in RC, which has not been reported previously. CA IX staining intensity was significantly different among the tumours in the different treatment categories, with the greatest proportion of tumours having moderate/strong CA IX intensity in the long-course RT only group. CA IX staining intensity in the operative samples was found to be a remarkable predictive factor, significantly related to both DFS and DSS.

Patients, who had tumours with negative or weakly positive CA IX staining intensity in the operative samples had better DFS and DSS than patients who had tumours with moderately or strongly positive staining intensity. The difference in DFS was evident from early postoperative period and it increased over time to about 50% at 3 years. Similar data have not been published before in RC. No such differences in survival were seen when CA IX intensity was analysed in the preoperative biopsies. In multivariate (Cox) regression model, CA IX intensity in the operative samples and the number of metastatic lymph nodes independently predicted DFS. In a similar analysis, CA IX intensity in the operative samples was the only independent predictor of DSS.

The preoperative treatment groups were then analysed separately. When the long-course RT group was divided into two categories (those with and without concomitant chemotherapy), there was a statistically significant difference in staining intensities in the operative samples between the two groups, showing more intense staining in the tumours of those patients who had not received concomitant chemotherapy. Patients, whose tumours had negative/weak CA IX staining intensity in the operative samples after short-course RT or long-course chemoradiotherapy, had significantly better DFS than patients, whose tumours had moderate/strong CA IX staining intensity (data not shown). DSS was also better for patients whose tumours showed negative/weak CA IX staining intensity in the operative samples after long-course preoperative chemoradiotherapy than for patients, whose tumours had moderate/strong CA IX staining intensity. No significant differences in survival with regard to CA IX intensity in the preoperative biopsies in any of the patient groups or the operative samples after long-course RT

without chemotherapy were seen. The findings of this study may imply that chemotherapy can act as a radiosensitiser, improve tumour oxygenation and consequently, also final treatment outcome. *CA9* is known to encode carbonic anhydrase isoenzyme CA IX [12, 226] and to be the most up-regulated gene in CRC [300]. CA IX in turn is shown to be up-regulated by HIF-1α [301]. Thus, CA IX positivity is presumptuously associated with up-regulated HIF-1α and further, tumour hypoxia [302], as well as unfavourable disease outcome [6].

Hypoxia-associated markers, including CA IX, are known to be linked with resistance to radiotherapy [30, 34]. Tumours in the short-course RT group were in general less locally advanced and hence, probably better oxygenated than tumours in the long-course RT group. The locally advanced tumours in the long-course RT group likely harboured hypoxic and radiotherapy resistant areas. After combined chemoradiotherapy, improved disease outcome has previously been reported in RC, malignant glioma and other tumour types as well [168, 169, 303]. Chemotherapy may thus revoke radioresistance, or act as a radiosensitiser. The radiosensitising effect of chemotherapy could explain the differences between the long-course RT only and the chemoradiotherapy groups in this study. However, the results of these analyses may also be affected by the small number of patients in the long-course RT only group.

The control group patients were also analysed separately. There were no differences in survival between the patients who had or who had not received any RT/chemoradiotherapy and on the other hand, between the patients who had or who had not received any RT/ chemoradiotherapy/adjuvant chemotherapy postoperatively. This may implicate that moderate/strong CA IX intensity rather predicts response to therapy than unfavourable disease outcome per se [304]. However, the number of patients is low, and thus the analysis may not have enough statistical power to show differences. Also, the control group population deviates from the preoperative treatment group patients with regard to disease stage and patient characteristics. Adjuvant postoperative chemotherapy was likewise given after preoperative treatment when implicated. Moreover, since the current study in addition is retrospective and non-randomised, a direct comparison of the groups may be somewhat questionable.

The data of the current study suggest that CA IX intensity in the operative samples is a powerful independent predictive factor, in line with the findings reported in other human malignancies. Studies have shown CA IX to be associated with adverse disease outcome in non-small cell lung cancer [290, 294], bladder [235] and breast cancer [227] as well as oligodendroglioma [305]. Instead, low

CA IX expression and absence of VHL mutation were found to be related to a more advanced tumour and worse outcome in renal cancer [288].

These findings could have important therapeutic implications in the future. Also, CA IX expression might prove to be a useful tool in selecting high-risk patients for adjuvant therapies. In fact, in renal cell cancer, a large placebo-controlled trial is ongoing to define the benefit of CA IX-targeted treatment in the adjuvant setting [306]. Investigational agent cG250 is a CA IX- specific monoclonal antibody. There are phase I and II trials underway with therapeutic high-dose radiolabelled cG250 and CA IX-loaded dendritic cells in metastatic renal cancer, as well as a large randomised study of cG250 imaging for primary renal cancer [306].

In conclusion, CA IX staining intensity in the operative sample is a powerful predictive factor in RC. Negative/weak CA IX staining intensity is associated with favourable DFS and DSS. CA IX may prove to be a useful tool in identifying patients with poor prognosis and thus in need of additional postoperative therapies.

6.2. HIF-1α (II)

To the best of our knowledge, this is the first study to report HIF-1α expression in the preoperative biopsies and operative tumour samples in RC treated with preoperative RT. The hypothesis of the study was that preoperative radiotherapy may affect tumour oxygenation and thereby, HIF-1α expression in the tumour. Indeed, in over 50% of cases, HIF-1α positive expression in the diagnostic sample turned negative in the operative specimens. Hypoxic tumours are more resistant to therapy and result in worse disease outcome than well oxygenated tumours [30, 35]. After radiation, reoxygenation is shown to be evident especially in the most poorly oxygenated tumour regions [255], probably due to the loss of tumour cells, resulting in improved oxygen diffusion and decreased interstitial pressure [255]. Improved oxygenation should also lead to the inactivation of HIF-1α and its down-stream targets, which include VEGF [217]. Mathematical models concerning the effects of antiangiogenic treatments have shown that they likely act by decreasing peritumoural oedema and interstitial pressure, thus normalising the tumour vasculature [307]. Preoperative radiotherapy may also decrease hypoxia in the tumour and its microenvironment, and thus, the expression of HIF-1α [15]. However, HIF-1α is also regulated by other mechanisms, which are independent of the tumour oxygenation status. These mechanisms involve oncogenic stimulation [211, 308] or defects in HIF-1α degradation, including VHL

protein mutations [210]. Thus, decreased HIF-1α expression may also result from other causes than improved tumour oxygenation.

There were no differences with regard to IHC in the preoperative biopsies or the operative samples analysed in the whole patient population. Patients in this study, who were treated with long-course preoperative RT and whose tumours were HIF-1α negative in the operative specimen, had significantly better DSS than patients whose tumours were HIF-1α positive. HIF-1α is recognised as a key mediator in tumour hypoxia [7-9, 16, 206, 209, 211]. It has a central role in tumour progression as well as in resistance to cancer treatment [29-31, 309]. This implies that tumours, which remain HIF-1α positive, evaluated in the operative samples after preoperative treatment, may be resistant to RT. In the short-course RT group, there were no significant differences in survival with regard to HIF-1α expression in the operative samples. This may reflect the tumours being smaller and probably less hypoxic, and thus the patients having an overall better prognosis than in the long-course RT group [30, 31, 310].

In multivariate survival analysis, disease recurrence remained the only independent predictor of DFS, while the preoperative treatment group and the number of metastatic lymph nodes were the only predictors of DSS, HIF-1α losing its predictive value. Potentially, the size of the long-course RT group was too small to have statistical power in multivariate analysis, or the number of metastatic lymph nodes may exceed the power of other predictors of poor outcome [1, 311]. There are only two previous trials examining the role of HIF-1α expression in RC, but patients in neither of these trials had received preoperative RT. Nevertheless, the results of the previous studies showed that high HIF-1α expression correlated with shorter overall survival [277, 278]. These findings are in line with the current study and trials with other malignancies [275, 312] as well.

Our study suggests that RC patients with locally advanced HIF-1α positive tumours may have an aggressive disease with rapid progression. Thereby, they should probably be followed up closely, regardless of the stage of the tumour. HIF-1α- analysis could be useful in selecting patients for adjuvant postoperative therapies. Also, HIF-1α might turn out to be an applicable therapeutic target enhancing the impact of adjuvant treatment on disease outcome [16]. In cell line studies, the effect of HIF-1α inhibition has, however, been shown to be dependent on its sequencing with radiotherapy [313]. HIF-1α may on one hand increase radiosensitivity by promoting proliferation, apoptosis, ATP metabolism and p53 activation [313]; on the other hand HIF-1α blockade may result in the destruction of tumour vasculature, which in turn

can contribute to radioresistance [313]. Although tumour cells and their signalling in vivo and in vitro may be different, these results imply that HIF-1α blockade may not be unequivocal.

In conclusion, HIF-1α expression was positive in most of the preoperative biopsies but negative in most of the operative samples, implying that preoperative treatment down-regulates HIF-1α. Importantly, DSS of the patients who had tumours with negative HIF-1α expression in the operative samples after long-course radiotherapy had favourable DSS.

6.3. EZRIN (III)

The aim of the current study was to evaluate the possible effect of preoperative treatment on ezrin expression and further, the potential influence of ezrin expression on disease outcome. We are not aware of any previous reports studying ezrin expression in the preoperative biopsies and operative samples after preoperative RT of RC.

Most of the preoperative biopsies but only about half of the operative specimens had negative/weak expression. Thus, more intense ezrin staining was seen after preoperative treatment, which could possibly be induced by radiotherapy. Radiotherapy can shrink the tumour and even complete remissions are seen after preoperative treatment varying between 0-29% [100, 107, 108, 111, 162]. Normalisation of tumour vasculature [307] or loss of tumour cells may improve its oxygenation and down-regulate the expression of various hypoxia markers, e.g. HIF-1α and CA IX (I, II). Therefore, the relation of ezrin, HIF1-α and CAIX expression was further analysed. The expression of HIF-1α and ezrin, as well as CAIX and ezrin were significantly concordant in the operative samples, but not in the preoperative biopsies. Ezrin is shown to be linked to mTOR signalling [28], which in turn controls the translation of cap-mRNA proteins, including HIF-1α[16]. It can be postulated that in response to radiation, ezrin expression is up-regulated, inducing also HIF-1α expression. HIF-1α is associated with resistance to RT [30]. Hence, increased ezrin expression due to radiation could bring about resistance to RT [314].

In this study, in WB, the cell lines responded to radiation by up-regulation of a stress-response marker but this did not affect ezrin expression (III). Ezrin expression in cultured cells, however, may be different from its expression in tissue samples [20, 241, 247, 315]. In tissues, factors that are not linked to tumour

cells per se can have an effect on how the tissues respond to injury [316]. These factors include the extracellular matrix and various soluble factors, e.g. transforming growth factors, which are known to modulate each other and the effects of injury on e.g. vascular cells [317]. Thereby they can modify the phenotype of epithelial cells [317, 318] and affect cancer growth and invasion [316]. Thus, tumour environment can modify the response of the tumour to injury caused by radiation and explain the differences seen between cell lines and histological tissue samples.

Ezrin expression in the preoperative biopsies as well as in the surgical specimens of the long-course RT group patients predicted disease outcome in this study. However, this association was not seen in the short-course RT group. It is possible that short-course RT causes rapid changes in tumour microenvironment [96], which may affect the cellular ezrin levels. During long-course RT or the interval between the end of RT and the operation, tumour environment might restore its balance and thus, re-establish the ezrin levels. Instead, surgery is performed on the following week after short-course RT, and the time interval or the treatment per se, may be too short for the tissues to recover.

Disease outcome of the patients with negative/weak ezrin IHC in the biopsy was significantly better than that of patients with moderate/strong expression. A similar trend was seen for ezrin in the operative specimens, but the difference was not statistically significant. In the long-course RT group, negative or weak ezrin expression in the operative samples was associated with excellent tumour regression and superior DSS. These findings imply that moderate/high ezrin expression in itself predicts adverse disease outcome or is associated with the response of the tumour to preoperative treatment.

Ezrin is a key regulator of HIF-1α protein synthesis in many cancers [16]. Ezrin expression is shown to be related to mTOR pathway [28], and its inhibition to significantly reduce tumour cell invasion [284]. Furthermore, mTOR inhibition is shown to inhibit experimental lung metastasis [28]. Poor survival in CRC seems to be related to intense ezrin expression [242] as well as HIF-1α expression [277, 278]. Strong ezrin expression has also been related to adverse disease outcome in epithelial ovarian cancer [284], head and neck cancer [283] and uveal malignant melanoma [291]. Thereby, therapies targeting ezrin expression could decrease HIF-1α expression, which in turn may improve the effect of preoperative therapies and disease outcome in RC. These treatment strategies could include an antibody to or inhibitor of ezrin or possibly its downstream molecules. Indeed, there are preclinical and clinical trials ongoing to study the usefulness of rapamycin and its

analogues [28]. Temsirolimus and everolimus are mTOR inhibitors currently indicated in the treatment of metastatic renal cell carcinoma.

It can be concluded that ezrin expression in the preoperative, pre-treatment biopsies and the subsequent operative specimens was significantly different, which implies that preoperative treatment modulates ezrin expression in RC. Moderate/strong ezrin expression in the preoperative samples as well as in the operative samples of the long-course RT group was linked to unfavourable disease outcome. In the multivariate analysis, however, ezrin did not reach statistical significance as an independent predictor of disease outcome. Ezrin is a promising biomarker that is associated with mTOR pathway signalling. The concept of mTOR inhibition is already exploited in some cancers.

6.4. GLUT-1 (IV)

Our hypothesis was that preoperative treatment shrinks the tumour and alters its microenvironment, which leads to improved tumour oxygenation and a change in GLUT-1 expression. There are no previous reports comparing GLUT-1 expression in the diagnostic biopsies and operative samples after preoperative RT.

In this study, GLUT-1 staining in the preoperative biopsies and the corresponding operative samples was different in that two thirds of the biopsies but only half of the corresponding operative samples were GLUT-1 positive, reflecting a change in GLUT-1 expression in the tumour during the preoperative period. In an earlier study, surgical procedure was associated with significantly increased GLUT-1 levels in RC [319], when biopsies were taken just before the operation under anaesthesia, which may also have some effect on the results. In contrast, in the current study, GLUT-1 expression levels decreased when pre-RT biopsies were compared to the samples taken at operation. This implies that preoperative treatment interferes with GLUT-1 expression in the tumour. RT destroys tumour cells, leading to down-staging, down-sizing or even complete tumour elimination [108]. Importantly, RT can also have an effect on the tumour microenvironment, especially the tumour vasculature [96, 316, 317]. Thus, it is not unexpected that preoperative treatment seems to have an effect on oxygenation and hence, also tumour metabolism.

In our study, there were no significant differences in DFS or DSS with regard to GLUT-1 expression in the biopsies or the operative samples. However, patients, who had received long-course preoperative RT and had

negative/weak staining intensity in the operative specimens, had a trend towards better DFS as compared to patients, who had moderate/strong staining intensity. This is in parallel to the findings of a previous study, showing an association of increased GLUT-1 expression in the pre-treatment biopsies with poorer response to chemoradiotherapy [279]. High GLUT-1 expression is shown to be associated with poor prognosis in other malignancies as well [280, 293]. In a combined analysis with CA IX, negative/weak intensity of both markers in the operative samples was associated with longer DFS, further emphasising the power of CA IX in predicting disease outcome in rectal cancer. The expressions of CA IX and GLUT-1 have earlier been shown to correlate with each other and to be interchangeable prognostic markers in bladder cancer [294]. However, to the best of our knowledge there are no previous reports of the combined expression of these markers with regard to survival in rectal cancer.

Malignant cells have a tendency to turn to anaerobic glycolysis as a source of energy even in well-oxygenated conditions [203] and an association between GLUT-1 expression and chemoresistance has been shown [236]. Thus, the inhibition of glycolysis could be a useful approach for therapeutic purposes in cancer [320]. Tyrosine kinase inhibitors with specificity for ATP binding sites are shown to directly inhibit GLUT-1 [321]. GLUT-1 antibodies have been tested in cancer cell lines and they have been shown to inhibit proliferation and to induce apoptosis [322].

Taken together, there was a change in the tumour's GLUT-1 expression during the preoperative period, since GLUT-1 expression was positive in two thirds of the preoperative biopsies but only half of the operative samples. Patients of the long-course RT group with negative/weak GLUT-1 expression in the operative sample had better DFS, as compared to patients with moderate/strong expression, but the difference did not reach statistical significance.

6.5. TUMOUR REGRESSION GRADE (I-IV)

Tumour regression grade was analysed in the long-course RT group operative samples. Good tumour regression was non-significantly linked to negative/weak HIF-1α and CA IX, and significantly to GLUT-1 and ezrin expression. Excellent TRG was significantly associated with favourable DFS and DSS. In the study population, there was only one pCR with no viable tumour cells left. Thus, the rate of pCR was less than previously reported [100, 107, 111, 162].

The rate of pCR depends on the strictness of the criteria and the analysis of sufficient tissue material, including the mural tumour and the lymph nodes. In

the current study, the criteria for complete pathologic regression were set tight and several slides were studied to ensure the complete disappearance of vital cancer in the tumour and lymph nodes as well. Time interval between the end of RT and surgery is shown to be the single most important factor affecting pCR [164]. The effects of RT are not visible shortly after its completion. Non-viable cancer cells may appear morphologically intact, before they undergo apoptosis [163]. Extending the interval beyond 12 weeks does not appear to increase pCR since most responses are shown to occur during 8-10 weeks after RT [164]. The controversy between the findings of the current study and most of the previously reported trials may partly be explained by the deviating time interval. In this study, waiting time varied between 4-10 weeks, being shorter among some patients who were operated at the start and longer towards the end of patient accrual.

The expression of all four markers in the preoperative biopsies was also compared with TRG in the operative samples after long-course RT, showing no significant differences between response to therapy and IHC. However, no excellent responses were seen if CA IX intensity in the preoperative biopsy was strong. Taken together, in this study, IHC in the biopsies was not a significant predictor of good response to chemoradiotherapy. This may be explained by the small number of patients, for whom biopsies were available, but the relatively small size of the biopsy specimens can also have an influence on the results.

Excellent response, with only a small remaining proportion of possibly vital cancer, was associated with favourable disease outcome. Determining complete pathologic response is challenging and analysing the degree (poor/moderate or excellent) of TRG may be helpful in estimating disease outcome and weighing the possible benefits of additional postoperative therapies to optimise prognosis. Sufficient time interval between the end of RT and surgery is important to achieve pCR.

6.6. COMBINED ANALYSIS OF HIF-1α, CA IX, EZRIN AND GLUT-1

All four markers, HIF-1α, CA IX, ezrin and GLUT-1, were tested in multivariate analysis, where CA IX intensity proved to be the only independent predictor of DSS. Albeit preliminary, this multivariate analysis suggests that CA IX indeed is a powerful predictive factor in RC. Further analyses are planned in the future to investigate the potential role of all these markers or their various combinations in predicting disease outcome in RC, using models where classical prognostic factors are included as well.

7. SUMMARY AND CONCLUSIONS

The present study concerning rectal cancer was focused on evaluating the predictive significance of hypoxia-related molecular markers HIF-1α, CA IX and GLUT-1 as well as ezrin, which is also linked to these markers. The following conclusions can be drawn from the results of this study:

1) CA IX staining intensity is a powerful predictor of disease outcome. In the present study, moderate/strong CA IX staining intensity in the operative samples was related to unfavourable DFS and DSS in rectal cancer. In multivariate analysis, CA IX intensity in the operative samples was an independent predictor of DFS. The risk for dying of rectal cancer was 9.2-fold if moderate/strong CA IX intensity was present.

2) HIF-1α expression predicts disease outcome in rectal cancer after long-course RT. In the present study, positive HIF-1α expression in the operative samples after long-course RT was associated with unfavourable DSS in rectal cancer.

3) Ezrin expression in the diagnostic pre-treatment biopsies is associated with disease outcome in rectal cancer. In this study, negative/weak ezrin expression in the biopsies was related to longer DFS and DSS. In the long-course RT group, DSS of the patients with negative/weak ezrin expression in the operative samples was also significantly better than of those patients with moderate/strong ezrin expression.

4) GLUT-1 expression is not a powerful predictive factor in rectal cancer. In the current study, patients with negative/weak GLUT-1 staining intensity in the operative specimen after long-course RT had longer DFS, as compared to patients with moderate/strong staining intensity, but the difference was not statistically significant.

5) Excellent tumour regression after preoperative long-course RT with or without chemotherapy was linked to negative or weak expression of CA IX, ezrin and GLUT-1 in the operative samples. Excellent TRG was associated with favourable DFS and DSS.

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