Astrocyte elevated gene-1 in relation to colorectal cancer development and radiotherapy response

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“Man bewegt nichts, wenn man sich selber nicht bewegt”

Blumentopf

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Abstract

The incidence and death rate for colorectal cancer (CRC) decreased during the last decades as a result of improved diagnosis and treatment. However, CRC is still the third most common cancer in the world, and is responsible for about 700 000 deaths per year worldwide. Therefore, it is important to understand the mechanisms of the disease, and to find molecular markers in order to further improve prognosis, and to develop new treatment strategies. Astrocyte elevated gene-1 (AEG-1), encoded by the *MTDH* gene, is upregulated in a variety of cancers. AEG-1 is involved in cell survival, proliferation, migration, invasion, metastasis, angiogenesis, and apoptosis.

The aim of this thesis was to investigate the role of AEG-1 in CRC development and the impact of AEG-1 on the response of radiation treatment. The AEG-1 expression, analysed in different CRC patient cohorts in paper I and III, was increased in the tumour tissue compared with the normal mucosa, and higher in the lymph node and liver metastases. Expression analyses in normal and cancer cell lines confirmed these results. In paper II, sequencing of the complete coding sequence of the *MTDH* gene in 356 patients revealed 50 single nucleotide variants of which 29 were novel. Eight exonic variants were detected, including three frameshift variants which were probably pathogenic, and two missense variants located in functional protein regions. There was no correlation of the *MTDH* variants or AEG-1 expression with the patient survival. In paper III, we also investigated the impact of AEG-1 on the response to radiation treatment. AEG-1 knockdown decreased the cellular survival upon radiation in several colon cancer cell lines. The AEG-1 expression was furthermore analysed in patients, which were randomised to either surgery alone or preoperative radiotherapy (RT), followed by surgery. The rectal cancer patients with high AEG-1 expression treated with RT had a significantly higher risk of developing distant recurrence and had a worse disease free survival, likely due to the metastasis promoting properties of AEG-1. In paper IV, the impact of AEG-1 knockdown and radiation on migration and invasion was analysed in colon cancer cell lines *in vitro* and in a novel zebrafish model *in vivo*. AEG-1 knockdown decreased migration and invasion, and radiation-enhanced migration and invasion in the cell lines tested.

In conclusion, our data suggest that AEG-1 is involved in CRC development, while *MTDH* gene variants probably not have a high clinical importance in CRC. Furthermore, AEG-1 is a promising radiosensitising target and a valuable prognostic marker in CRC. We further showed that AEG-1 knockdown inhibits migration and invasion, as well as radiation-enhanced cell migration and invasion.

Populärvetenskaplig sammanfattning

Kolorektalcancer är en av de vanligast förekommande cancerformerna i världen. Incidensen och dödligheten i kolorektalcancer har under de senaste årtiondena minskat, mycket på grund av en förbättrad vård och tidigare diagnos. Trots detta är kolorektalcancer fortfarande den tredje vanligaste cancerformen i världen, med uppskattningsvis 700 000 dödsfall per år världen över. Därför är det viktigt att förstå sjukdomens bakomliggande mekanismer, samt att hitta molekyllmarkörer som kan användas för att förbättra prognosen och för att utveckla nya behandlingsstrategier. AEG-1 (Astrocyte elevated gene-1) som kodas av MTDH genen, är en så kallad onkogen vilken uppregleras vid cancer och som reglerar cellers överlevnad, utveckling, metastasering, angiogenes och svaret på behandling.

Syftet med denna avhandling var att undersöka AEG-1s roll i utvecklingen av kolorektalcancer, samt om AEG-1 påverkar svaret på strålningsbehandling. Analys av mRNA- och proteinuttryck i olika grupper av kolorektalcancerpatienter visade en ökning av AEG-1 i tumörvävnaden jämfört med normal mucosa, det högsta uttrycket hittades i metastaser. MTDH genen sekvenserades i 356 patientprover varpå 29 nya genvarianter hittades. Däremot fanns de ingen korrelation mellan dessa nya MTDH varianter, uttrycket av AEG-1 och överlevnad. Vi studerade därför om uttrycket av AEG-1 påverkade svaret på strålningsbehandling. Genom att slå ut AEG-1 uttrycket, s.k. ”knockdown”, minskade överlevnaden i celler som utsatts för strålning i flera olika cancercellinjer. Därefter undersöktes AEG-1 uttrycket i patienter som hade genomgått antingen enbart kirurgisk behandling eller strålning före det kirurgiska ingreppet. Patienter som behandlades med strålning och vars AEG-1 uttryck var högt, visade sig ha en förhöjd risk att utveckla återfall samt uppvisade en sämre överlevnad, troligtvis beroende på AEG-1s främjande effekt på metastaseringen. Slutligen analyserades effekten av att slå ut AEG-1 på migration och invasion i både kolorektalcancerceller *in vitro* och i zebrafiskar *in vivo*. Att slå ut uttrycket av AEG-1 minskade inte enbart migrationen och invasionen i cellinjer, utan även den strålningsinducerade migrationen och invasionen.

Sammanfattningsvis har vi funnit att uttrycket av AEG-1, på både mRNA- och proteinnivå, är involverat i utvecklingen av kolorektalcancer. De olika genvarianterna av MTDH verkar inte ha en klinisk relevans vid kolorektalcancer. AEG-1 proteinet kan däremot vara en lovande prognostisk markör i kolorektalcancer. Vi har också funnit att minskat uttryck av AEG-1 hämmar migrationen och invasionen vid kolorektalcancer, samt även hämmar den strålningsinducerade migrationen och invasionen.

List of publications

- I. Gnosä S***, Shen YM*, Wang CJ, Zhang H, Stratmann J, Arbmänn G, Sun XF. Expression of AEG-1 mRNA and protein in colorectal cancer patients and colon cancer cell lines. *J Transl Med.* (2012) 10:109.
- II. Gnosä S***, Ticha I*, Haapaniemi S, Sun XF. *MTDH* genetic variants in colorectal cancer patients. Manuscript, Re-submitted to *Sci Rep.* (2015).
- III. Gnosä S**, Zhang H, Brodin VP, Carstensen J, Adell G, Sun XF. AEG-1 expression is an independent prognostic factor in rectal cancer patients with preoperative radiotherapy: a study in a Swedish clinical trial. *Br J Cancer.* (2014) 111:166-173.
- IV. Gnosä S**, Capodanno A, Jensen LDE, Sun XF. AEG-1 knockdown in colon cancer cell lines inhibits radiation-enhanced migration and invasion *in vitro* and in a novel *in vivo* zebrafish model. Manuscript (2015).

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List of publications outside the thesis

Stratmann J, Wang CJ, **Gnosa S**, Wallin Å, Hinselwood D, Sun XF, Zhang H. Dicer and miRNA in relation to clinicopathological variables in colorectal cancer patients. BMC Cancer (2011) 11:345.

Ticha I, **Gnosa S**, Lindblom A, Liu T, Sun XF. Variants of the PPARG gene and their clinicopathological significance in colorectal cancer. PLoS One (2013) 8:e83952.

Pathak S, Meng, WJ, Zhang H, **Gnosa S**, Nandy SK, Adell G, Holmlund B, Sun XF. Tafazzin protein expression is associated with tumorigenesis and radiation response in rectal cancer: A study of Swedish trial on preoperative radiotherapy. PLoS One (2014) 9:e98317.

Table of Contents

Abstract.....	V
Populärvetenskaplig sammanfattning	VII
List of publications.....	IX
List of publications outside the thesis	X
List of abbreviations	3
Introduction.....	7
Colorectal cancer (CRC)	8
Anatomy of the colon and the rectum	8
Risk factors for CRC - sporadic, familial, and hereditary	10
Colorectal carcinogenesis	12
Metastasis	14
Prognosis and prediction	18
Treatment	21
Surgery.....	21
Chemotherapy.....	22
Radiotherapy (RT).....	23
Targeted therapy.....	25
Treatment-enhanced metastasis	25
Astrocyte elevated gene-1 (AEG-1).....	27
Aims.....	33
Materials and Methods.....	35
Cancer models and tissue samples	35
Cell lines.....	35
Zebrafish.....	37
Patients.....	38
Methods.....	41
Irradiation of cell lines and zebrafish embryos	41
DNA, RNA and protein extraction	42
Polymerase chain reaction (PCR).....	42
Capillary Sanger DNA sequencing.....	43
Quantitative PCR (qPCR) and Reverse transcription PCR	44
Immunohistochemistry (IHC).....	45

Western blot.....	46
Cytokine antibody array	46
Colony forming assay	46
WST proliferation assay	47
Boyden chamber assay	47
Zebrafish invasion assay.....	48
Statistical analyses	49
Results and Discussion.....	51
Alterations of AEG-1 in relation to CRC development	51
AEG-1 mRNA and protein expression in CRC patients and colon cancer cell lines.....	51
<i>MTDH</i> genetic variants in CRC patients and colon cancer cell lines.....	53
AEG-1 alterations in relation to clinicopathological and biological variables.....	55
AEG-1 and RT	58
Evaluation of AEG-1 as a radiosensitising target.....	58
Potential of AEG-1 as a prognostic marker after RT	58
Impact of AEG-1 in radiation-enhanced metastasis	59
The zebrafish as a novel model to study radiation-enhanced invasion	61
MMP secretion and expression in relation to the AEG-1 expression and radiation.....	61
Conclusions	63
Future perspectives	65
Acknowledgements.....	67
References	69

List of abbreviations

5-FU	5-fluorouracil
ABCC11	ATP-binding cassette sub-family C member-11
AEG-1	Astrocyte elevated gene-1
AJCC	American Joint Committee on Cancer
AKR1C2	Aldo-keto reductase family 1 member-C2
ALDH3A1	Aldehyde dehydrogenase 3 family member-A1
Angptl 2	Angiopoietin-like-2
Angptl 4	Angiopoietin-like-4
APC	Adenomas polyposis coli
ATM	Ataxia–telangiectasia-mutated
Bad	BCL2-associated agonist of cell death
BCCIP α	BRCA2 and CDKN1A interacting protein alpha
BCL-2	B-cell CLL/lymphoma-2
BCL-xl	B-cell lymphoma-extra large
BRAF	B-Raf proto-oncogene
CAM	Cell adherence molecules
CBP	CREP binding protein
CCD	Charged coupled device
CD8	Cluster of differentiation-8
CDK2	Cyclin-dependent kinase-2
cDNA	Complementary DNA
c-Myc	Cellular myelocytomatosis oncogene
COSMIC	Catalogue of Somatic Mutations in Cancer
CPEB1	Cytoplasmic polyadenylation element binding protein-1
CRC	Colorectal cancer
CREB	cAMP response element-binding protein
CSC	Cancer stem cells
CYP2B6	Cytochrome P450 family 2 subfamily B polypeptide-6
dbSNP	Single Nucleotide Polymorphism Database
DCC	Deleted in colorectal carcinoma
ddNTP	Dideoxynucleotide triphosphates
DIL	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine
dNTPs	Deoxynucleotide triphosphates
DPYD	Dihydropyrimidine dehydrogenase
DSB	Double strand breaks
ECM	Extracellular matrix
EGF	Endothelial growth factor
EGFP	Enhanced GFP
EGFR	Endothelial growth factor receptor
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial to mesenchymal transition
ERK	Extracellular regulated MAP kinase
FAP	Familial adenomatous polyposis
FFPE	Formalin fixed and paraffin-embedded
FGFR	Fibroblast growth factor receptor
FOXO	Forkheadbox
FXYP-3	FXYP domain containing ion transport regulator-3

G-CSFR	Granulocyte colony-stimulating factor receptor
GFP	Green fluorescence protein
Gy	Gray
Ha-Ras	Harvey rat sarcoma virus oncogene
HCC	Hepatocellular carcinoma
HGVS	Human Genome Variation Society
HIV-1	Human immunodeficiency virus-1
hMLH1	MutL homolog-1
HMOX1	Heme oxygenase-1
hMSH2	MutS homolog-2
HNPPC	Hereditary non-polyposis colon cancer
HRP	Horseradish peroxidase
HSP90	Heat shock protein 90kDa
ICAM-1	Intercellular adhesion molecule-1
IGFR-1	Insulin-like growth factor receptor-1
IHC	Immunohistochemistry
IKK	I κ B kinases
IL-1	Interleukin-1
IL-8	Interleukin-8
JAK1	Janus kinase-1
K-Ras	Kirsten sarcoma viral oncogene homolog
Lef	Lymphoid enhancer-binding factor
Lox	Lysyl oxidase
LYRIC	Lysine-rich CEACAM1 co-isolated
MAPK	Mitogen-activated protein kinase
MDM2	MDM2 proto-oncogene
Met	Met proto-oncogene
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MSI	Microsatellite instability
mTOR	Mechanistic target of rapamycin
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor- κ B
NLS	Nuclear localization signal
NOS	Nitric oxide synthase
N-Ras	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small cell lung cancer
PCR	Polymerase chain reaction
PDK	3-phosphoinositide-dependent protein kinase
PE	Plating efficiency
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PLZF	Promyelocytic leukaemia zinc finger
PTEN	Phosphatase and tensin homolog
PTU	1-phenyl-2-thiourea
qPCR	Quantitative PCR
RhoA	Ras homolog family member A
RISC	RNA induced silencing complex
RNAi	RNA interference

ROCK	Rho-associated kinase
Rrs1	Ribosome biogenesis regulator homolog
RT	Radiotherapy
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF	Surviving fraction
shRNA	Small (or short) hairpin RNA
siRNA	Small inhibitory RNA
SMAD2	Smad family member-2
SMAD4	Smad family member-4
SND1	Staphylococcal nuclease and tudor domain containing-1
STAT3	Signal transducer and activator of transcription-3
TCF	Transcription factor
TGF- β	Tumour growth factor beta
TIMP	Tissue inhibitors of metalloproteinases
TME	Total mesorectal excision
TNF- α	Tumour necrosis factor alpha
Top1	DNA topoisomerase I
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UBN-1	Ubinuclein-1
VEGF	Vascular endothelial growth factor
YY1	Yin-yang-1
β -catenin	Cadherin-associated protein beta

Introduction

The oldest written records mentioning human cancer are two old Egyptian papyri, also known as the Smith and Ebers papyri. The first papyrus was possibly written by the physician Imhotep around 1500-1600 BC and describes a tumour of the anterior chest. The name cancer was introduced by the Greek Hippocrates of Kos (ca. 460-360 BC), who described ulcerating and non-healing lumps as *karkinos*, the Greek term for crab, later translated to the Latin word *cancer* (1). Nowadays, cancer is the name of a collection of around 100 distinct diseases, that are characterized by an abnormal, uncontrolled growth of cells with the potential to invade normal tissue and spread to distant organs (2).

In 2012, there were 14.1 million new cases of cancer and 8.2 million cancer related-deaths, making the disease responsible for every 8th death worldwide (3, 4). Cancer is a disease of the genome and develops by stepwise transformation of normal cells to highly malignant derivatives, driven by successive genetic and epigenetic alterations accumulating over the lifetime (5-7). These genetic alterations can include point mutations, amplifications, deletions, or rearrangements that can be inherited, induced by DNA damaging carcinogens, or occur during DNA replication. Epigenetic alterations include DNA methylation and histone modifications. Critical steps in the tumour initiation are activation of proto-oncogenes and inactivation of tumour suppressor genes by genetic alteration. Mutated proto-oncogenes, called oncogenes, are dominant in nature and drive cancer progression by overexpression or hyperactivation. Tumour suppressor genes are recessive in nature and they are either deleted or inactivated on both of the alleles in cancer (8, 9). Another class of critical genes involved in cancer development are genes of the DNA repair system. A defect in DNA repair increases the rate of point mutations and chromosomal abnormalities and it is believed that in many tissues, a deregulation of the DNA repair system is needed to develop cancer (10).

Cancer formation follows the Darwinian evolution and the cancer development is based on two processes: continues heritable changes of the genome in individual cells, and natural selection by the environment of the resultant phenotype (2). The process of natural selection fosters cells with an enhanced survival and proliferation compared with the neighbouring cells. Several regulatory circuits control different physiological functions, such as cell proliferation and survival that are cell type and organ specific. These physiological functions are summarised as the “Hallmarks of cancer”, proposed by Hanahan and Weinberg in 2000, and include independency to growth signals, insensitivity to anti-growth signals, avoidance of programmed cell death (apoptosis), limitless replication potential, persistent angiogenesis, and invasion and

metastasis of tissues and distant organs (7). All of these six physiological functions dictate malignant growth and are acquired irrespective of the order by most cancers. Recently, four new characteristics have been added to the traditional six cancer hallmarks proposed by Hanahan and Weinberg, that are avoidance of the immune response, deregulated cellular energetics, tumour promoting inflammation, and genome instability and mutations (11).

Colorectal cancer (CRC)

Colorectal cancer (CRC) is the third most common form of cancer in men and the second in women worldwide with together around 1.36 million new incidences reported in 2012 (3). About 700 000 estimated deaths from CRC, make it the fourth most common cause of cancer related death worldwide accounting for about 8.5%. More than half of the cases (55%) occurred in developed regions (3). In Sweden, CRC is the third most common cancer amongst men and women and amongst 58 726 new cancer cases reported in 2011, 6 162 accounted for CRC (12). The incidence rate for colon cancer increased slightly for both sexes between 1980 and 2011, while the incidence for rectal cancer, which represents about 30% of all CRC cases, was stable (12, 13). During the last three decades, the 5-year survival rates increased both in colon cancer (around 7%) and rectal cancer (around 20%), and is currently for colon cancer around 65% and for rectal cancer around 63% (12). This increase is probably due to earlier diagnosis and improved treatments.

Anatomy of the colon and the rectum

The large intestine consists of the colon, the rectum, and the anus, and builds the final section of the gastrointestinal tract (Figure 1). The large intestine is starting with the caecum in the right side of the abdomen, where it is connected to the terminal ileum via the ileocecal sphincter, and ends with the anus (14). The large intestine is 1.5 m in length, with a surface area of around 25 m² and can be divided in several segments including the caecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anus (14, 15). The colon is located in the peritoneal cavity, while the rectum lies within the pelvis (13). The main function of the colon includes water and electrolyte absorption, as well as short chain fatty acid formation. The rectum mainly stores the faeces (13). Like the rest of the gastrointestinal tract, the large intestine is made out of four layers (Figure 1). The innermost layer is the mucosa,

which consists of an epithelial layer containing the Lieberkühn crypts and lymphoid nodules and is surrounded by the muscularis mucosae. The next layers are the submucosa containing blood vessels, nerves and connective tissue, and the muscularis externa consisting of two layers of smooth muscle cells. The last layer is built by the serosa, consisting of connective tissue covered with squamous cell epithelium (15).

During the last two decades, there has been a discussion about whether CRC should be considered as one single disease, or three distinct diseases, namely proximal colon cancer, distal colon cancer, and rectal cancer, because of the differences in their embryogenesis, morphology and physiology that might lead to different tumour developments (16-18). The proximal colon, for example, originates from the embryonic midgut and is perfused by the superior mesenteric artery, whereas the distal colon and the rectum originates from the embryonic hindgut and is perfused by the inferior mesenteric artery (13, 18). Other differences include the pH of the produced mucinous, crypt length, apoptotic index, and metabolic protein production (19-23).

Figure 1. Anatomy of the large intestine and histology of the intestinal wall.

Risk factors for CRC - sporadic, familial, and hereditary

Several risk factors for CRC development have been identified which can be divided into two groups: risk factors that can be controlled, including lifestyle and environment, and those that cannot be controlled, such as age and hereditary factors (24). Around 70-75% of all CRC cases are sporadic without familial or hereditary background, and CRC is therefore considered to be a lifestyle and environmental disease (25, 26). Some indications for the importance of the lifestyle and the environment come from migration studies. Japanese offspring who have migrated to the US, showed a similar high CRC incidence as the US population, but a 3-4 times higher incidence compared with the Japanese population (27). A major risk factor for CRC is the diet. It was shown that increased consumption of red meat, and food high in animal fat and fibres increased the risk of developing CRC (28, 29). Other lifestyle-related CRC risk factors are low physical activity, obesity, smoking, and high alcohol consumption (24). The likelihood of developing CRC increases with age. In Sweden, around 65-70% of all the CRC patients are diagnosed when 65 years of age or older. However, the age of diagnosis is decreasing mainly due to lifestyle changes, higher carcinogenic exposure and better diagnostic tools (12, 30).

Around 20% of all CRC cases occur in persons with a family history of CRC (31). Individuals with a first-degree relative diagnosed with CRC at age 50 years or older have a 2-3 fold increased risk to develop CRC (32). Furthermore, having one first-degree relative that developed CRC under age 45 years, or having two first-degree relatives diagnosed with CRC, increases the risk to develop CRC by 3-6 fold (33). However, no specific gene *loci* have been identified in those patients and it is believed that low penetrance susceptibility genes might be involved (34).

Approximately 5-10% of all CRC cases are connected to highly penetrating inherent mutations (24). The two most common syndromes are called hereditary non-polyposis colon cancer (HNPCC) also known as the Lynch syndrome, accounting for 2-4% of all CRC cases, and adenomatous polyposis coli, usually called familial adenomatous polyposis (FAP), which accounts for around 1% (35, 36). The molecular basis of HNPCC are germline mutations in the mismatch repair (MMR) genes, that involve in 90% of the cases the *mutS homolog-2* (*hMSH2*) and *mutL homolog-1* (*hMLH1*) genes (37). Several tools have been developed to identify HNPCC, including analyses of the familial history and age (Amsterdam I and II), microsatellite instability (MSI), and sequences of MMR genes (37-39). Individuals with HNPCC are prone to develop cancer especially in the colon and endometrium in early age (37).

HNPCC patients develop adenomas in a similar frequency as healthy individuals, but the increased mutation rate results in a faster progression towards malignancy (Figure 2) (40, 41). Patients with FAP on the contrary, develop hundreds to thousands of adenomas until their 30s, and due to the large number of adenomas the chance to develop CRC is very high (Figure 2) (42). FAP is an autosomal dominant inherited disease which is characterised by a deletion or mutation of the “gatekeeper” gene *adenomas polyposis coli* (*APC*) (43).

Figure 2. Sporadic colorectal tumourigenesis is a multistep process. The development of CRC from the normal epithelium to adenoma, carcinoma and finally metastasis is characterised by a series of mutational events in tumour suppressor genes and oncogenes (adapted from Vogelstein and Fearon (9)). HNPCC patients have an increased mutation rate and progress faster towards malignancy, while FAP patients develop a large number of adenomas during their lifetime.

Colorectal carcinogenesis

The concept of tumourigenesis as a multistep process, was shown in a model for CRC formation developed by Fearon and Vogelstein in 1990 (9). They described the process of tumourigenesis in three phases: initiation, promotion, and progression from pre-existing adenomas (benign and slow growing tumours), into carcinomas and metastatic disease through specific genetic events (Figure 2). Around 80-85% of all sporadic CRC follow this pathway that is often referred to as the “canonical” or “suppressor” pathway (36).

The initial step from the normal- to hyper-proliferative epithelium is a result of the activation of the Wnt signalling pathway by the loss or mutation of the *APC* tumour suppressor (5q21), found in around 70-80% of all colorectal tumours (5, 9). The canonical Wnt signalling pathway plays a central role in the biology of the colonic crypt. Stem cells in the bottom of the colonic crypt receive Wnt signals from the surrounding stroma cells. These signals stimulate the release of the cadherin-associated protein beta (β -catenin) from the APC-axin complex, and prevent β -catenin phosphorylation by the glycogen synthase kinase 3 beta (GSK-3 β) and subsequent degradation. Subsequently, β -catenin accumulates in the nucleus, associates with the transcription factor (TCF)/ lymphoid enhancer-binding factor (Lef), and drives transcription of genes involved in cell proliferation. Moreover, it prevents the differentiation of the resulting progenitor cells. While the undifferentiated progenitors migrate upwards to the intestinal lumen, the Wnt signals decrease, and the cells differentiate and stop to proliferate (44). When APC is non-functional due to genetic loss or mutation, GSK-3 β will not be able to phosphorylate β -catenin, and thereby overcomes degradation. This leads to uncontrolled cell proliferation in the intestinal lumen and the formation of adenomas (45).

In the model proposed by Fearon and Vogelstein, the CRC tumourigenesis is driven by DNA hypomethylation, activation of the *kirsten sarcoma viral oncogene homolog* (*KRAS*) proto-oncogene (12q12.1), and loss of function of *deleted in colorectal carcinoma* (*DCC*)/ *smad family member-4* (*SMAD4*), *smad family member-2* (*SMAD2*) (18q), and *TP53* (17p13.1) tumour suppressor genes (9). However, the described alterations in this model are not restricted to any specific phase of colorectal tumourigenesis and they might appear in a different order (9).

DNA hypomethylations have been detected in CRC, whilst other specific DNA regions show to be hypermethylated (46-48). DNA methylations are covalent modifications of cytosine within CpG dinucleotides, often organised in larger clusters close to the promoter regions (49). It is known that DNA methylations influence gene transcription, while the exact mechanisms are

still to be validated (50). DNA methylation patterns are balanced by methylation and demethylation and exposure to carcinogens might interfere with the process, resulting in inhibited tumour suppressor gene expression (51).

KRAS mutations are found in around 30-50% of all colorectal tumours (52). K-Ras is a member of the Ras family and encodes for a membrane bound G protein involved in extracellular signal transduction. K-Ras is involved in cell proliferation and survival via the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways (53).

Allelic loss at chromosome 18q has been detected in around 70% of all CRC cases (54). Among the genes identified at this locus, three have been found to be involved in the CRC tumorigenesis including *DCC*, *SMAD2* and *SMAD4*. *DCC* is a transmembrane receptor of the Ig superfamily of netrins, which is believed to be involved in cellular adhesion and apoptosis (55). *SMAD2* and *SMAD4*, both tumour suppressor genes have been found to be mutated in 10% and 15% of all colorectal tumours respectively (56). SMADs belong to a family of intracellular mediators and *SMAD2* and *SMAD4* are important in the anti-proliferative tumour growth factor beta (TGF- β) signalling (57).

The first tumour suppressor gene to be identified was *TP53*, which is inactivated in around 50% of all human cancers and in 50% of all CRC (58, 59). The cellular level of the p53 protein is mainly regulated by its degradation via the *MDM2* proto-oncogene (*MDM2*), an E3 ubiquitin protein ligase. Thereby, *MDM2* binds to p53 and catalyses its polyubiquitination, a label for proteasomal degradation (60). The *MDM2*-p53 interaction is regulated by a negative feedback loop, where p53 binds to the promotor region of *MDM2*, attracts the transcriptional machinery, and initiates its expression (60). A central step in the p53 activation is the destabilisation of the *MDM2*-p53 interaction, by phosphorylation or acetylation of p53 on the N-terminal *MDM2* binding domain (61, 62). In the classical tumour suppressor model for p53, activation occurs in response to DNA damage and cellular stress, such as DNA strand breakage, telomere erosion, and hypoxia. Once activated, p53 binds to the DNA as homodimer of dimers, and regulates the transcription of genes mainly involved in apoptosis and growth arrest (63-65). The majority of cancer-related *TP53* mutations (around 85%) are detected in the DNA binding domain of p53 (66). Interestingly, there is a correlation between the *TP53* mutational status and the immunohistological staining of the p53 protein, suggesting a prolonged half-life of the mutant protein (67).

Another pathway, responsible for around 15-20% of all CRCs, involves the DNA repair apparatus and is referred to as the “mutator” or MSI pathway (68, 69). In the 1990s, Perucho

and his team used a PCR technique with random primer and electrophoretic separation of the products to search for new tumour suppressor genes. Thereby, they compared the amplification signatures of matched DNA samples from colorectal tumours and adjacent normal colonic tissues (70-72). They found deletions in almost all tumours, but detected a slight difference in the electrophoretic migration pattern of some bands in around 12% of the cases. Further analyses showed that these bands contained small repetitive sequences (microsatellites), mainly consisting of polyadenine (70). Similar findings were achieved by the group led by Thibodeaus, who found mutations in the $[CA]_n$ sequence in around 28% of the analysed samples and referred to this phenotype as MSI. Subsequently, several studies have shown that these changes in the repetitive sequences are due to a defect in the DNA MMR system (68, 73). In long repetitive sequences such as the microsatellites, the DNA polymerase is prone to incorporate wrong nucleotides, and/or the wrong amount of nucleotides. The function of the MMR system is to detect those errors and repair them. If the MMR system is non-functioning, a wrong incorporated nucleotide can lead to frameshift mutations, which can result in a truncated and non-functional protein (68). Microsatellite sequences have been identified in several genes involved in colorectal carcinogenesis, including genes that regulate cell proliferation, cell cycle, apoptosis, DNA repair, and MMR (74). MSI tumours are classified into MSI-high and MSI-low, depending on the amount of mutated microsatellite marker of a defined panel (75).

Metastasis

Metastasis is the spread of cancer cells to surrounding tissues and distant organs. Metastases are responsible for around 90% of all cancer-related deaths and the most common organs for CRC metastases are the liver (~70%) and the lungs (10-20%) (76, 77). Approximately 25% of all CRC patients have a metastatic disease when the cancer is first diagnosed, and a high number of patients will have undetectable micro-metastases. Moreover, another 25% of all CRC patients will develop metastatic tumours during the course of the disease (78).

The process of tumour metastasis involves a series of interrelated events that lead to the growth of cancer cells at secondary sites (Figure 3). First, the cancer cells have to detach from the primary tumour and invade into the surrounding tissue through the basement membrane and extracellular matrix (ECM). The detached cells intravasate as they penetrate the lymphatic and/or vascular circulation. In the circulation, the cancer cells have to survive anoikis and shear forces, adhere at the secondary site, and extravasate into the vascular basement membrane and

ECM. Finally, these cells proliferate at the distant organ and form micro- and macro-metastases (76).

Epithelial cells can invade in a multicellular way as a strand or a cluster. However, single migrating and invading cells are often observed (79). Characteristic for these single migrating and invading cells are the loss or change of their polarisation, cell-cell and cell-ECM adhesion, and a mesenchymal like morphology. This cellular change is called epithelial to mesenchymal transition (EMT), and was first described by Lillie in the 1900s in chickens (80, 81). EMT can be induced by different signals including TGF- β , endothelial growth factor (EGF), and hypoxia, and involves the deregulation of E-cadherin, pro-migratory small GTPases and transcription factors (82-84).

Figure 3. Interrelated events lead to tumour metastasis. From left to right: tumour cell detach from the primary tumour, invade the surrounding tissue through the basement membrane and extracellular matrix (ECM), and some of the single migrating cells perform epithelial to mesenchymal transition (EMT). Invading cells intravasate as they penetrate the lymphatic and/or vascular circulation, and adhere to the vascular wall at the secondary site where they extravasate into the vascular basement and ECM. At the distant site, the cells might grow to micro- and macro-metastases.

Changes in the cell-cell and cell-matrix adherence are important in the detachment of the cells from the primary tumour and in invasion, intravasation and extravasation (85). Cell-cell signalling and adherence in endothelial cells are mainly accomplished by cell junction complexes consisting of tight junctions, gap junctions, adherence junctions, and desmosomes (86). Tight junctions act as semipermeable gates for ions, water, solutes, and cells, between the lumen and the extracellular space (87). Gap junctions are intercellular channels that control diffusion of ions and small molecules, and are mainly composed of connexins, a family of transmembrane proteins (88). Adherence junctions and desmosomes substantially contribute to cellular adherence and motility. The adhesion in these junctions is accomplished by factors called cell adherence molecules (CAMs). CAMs can be divided into four main groups: cadherines, immunoglobulin superfamily, selectins, and integrines, and are often found to be deregulated in epithelial cancers (86, 89-93).

Once detached from the tumour, single cells are able to migrate and invade within the tissue. The tumour cell migration and invasion are multistep processes, similar to events found during embryonic morphogenesis, wound healing, and immune-cell trafficking (79). First, the migrating cell changes its shape and becomes polarised and elongated. A finger-like protrusion called pseudopod is formed at the leading edge and interacts with the ECM via focal contacts. These focal contacts consists of the CAM integrin that binds the ECM intercellular and intracellular actin filaments via a multi-protein complex. In the next step, pro-proteases like matrix metalloproteinase (MMP) 1, 2, and 9 are recruited to the focal contacts. Activation of the MMPs leads to focalised proteolysis of specific parts of the ECM that generates a track for the cell (94). The movement of the cell is then facilitated via actomyosin contraction. In the last step, the focal contacts at the trailing edge are disassembled and recycled (79).

The MMPs have been of special interest during the last years. It was shown in multiple studies that the expression of MMPs increased substantially in the majority of malignant tumours compared with their normal counterpart (95-99). MMPs consist of 18 structurally related members of zinc endopeptidases that are either secreted or membrane bound (94). The expression of MMPs is induced by a variety of signals, including cytokines, growth factors, and cell-cell and cell-ECM interactions (100-103). Most MMPs are secreted into the intercellular space and proteolytically activated by cleaving of the pro-peptide and exposing the catalytic site (104). Inhibitors of MMPs, called tissue inhibitors of metalloproteinases (TIMPs), are able to bind MMPs intercellular, and inhibit their activation or activity (103). MMPs are involved in intravasation, extravasation, metastasis, neovascularisation, and tumour growth (105). Many pharmacological studies were aimed to develop drugs to inhibit cellular

migration and invasion by blocking MMPs; however, those inhibitors have failed and it remains a challenging task to achieve therapeutically meaningful outcomes by targeting these proteins (106).

Once the invaded cells reached a blood vessel, they intravasate by facilitating molecular changes and interactions with macrophages, allowing them to pass the endothelial cell barrier. Tumour-associated blood vessels, built during tumourigenesis, are likely to facilitate the intravasation due to their poor architecture and the lack in epithelial adherence (107). Arrived in the blood circulation the cells can disseminate widely throughout the body. However, to be able to survive in the circulation, the tumour cells have to overcome several barriers like anoikis and the stress from shear forces (108).

When the microvasculature of distant organs is reached by the disseminated cells, they attach at the vascular wall, proliferate, and extravasate (109, 110). However, the architecture of the epithelium in these vessel is highly functional and the exact mechanisms of penetration is probably different compared with the intravasation (111). Several factors have been identified that disturb the vessels, induce permeability, and subsequently allow the cells to invade. These factors are secreted from the cancer cells and include, among others, angiopoietin-like-4 (Angptl 4), angiopoietin-like-2 (Angptl 2), vascular endothelial growth factor (VEGF) and several MMPs (107).

Once the cells are in the distant organ parenchyma, they face a foreign microenvironment, consisting of different growth factors, ECM constituents and different types of stromal cells, in which they have to survive and persist. The cells often survive as micrometastases in the new environment, without significant increase in size and net gain or loss in overall cell number. These micrometastases can sustain for up to several years before growing to detectable macrometastases (112, 113).

During all steps of metastasis, the cells are attacked by the immune system. However, by interacting with tumour-associated macrophages, tumour-associated neutrophils, myeloid-derived suppressor cells, and regulatory T cells, the cancer cells can survive the journey to the distant site through different mechanisms, including the suppression of natural killer cells and cluster of differentiation 8-positive (CD8⁺) T cells (114).

Several studies indicated that there is a preference of organs for metastases, dependent on the origin of the primary tumour (110). Already in the 1880s, Paget proposed the hypothesis that the interactions of the tumour cells with their environment prone the cells to metastasise to certain organs with a similar (approved) environment. This postulation is also known as the “seed and soil” hypothesis, meaning that the seed (the tumour cells) can only grow when the

soil is compatible (milieu of certain organs) (115). Today, some studies suggest that pre-metastatic niche, induced by cells of the immune system, prime the tumour cells to metastasise to a certain organ (116). However, the cells have to make several adaptations, even in similar environments to be able to sustain in the foreign tissue.

Prognosis and prediction

Prognosis is the medical term used to indicate the probable course and outcome of a disease and may give an estimation for the aggressiveness of a cancer. It is widely measured by the probability to stay alive or without disease recovery for a certain amount of time after the cancer diagnosis, or the start of an adjuvant treatment. A threshold of five years is commonly used for the survival or cancer re-growth, but depending on the treatment and disease, shorter or longer time periods might be considered (117). The best estimation for prognosis in CRC is the pathological stage. Several staging systems have been developed during the years. In the 1930's, Dukes established a staging system for rectal cancer (later applied also to colon cancer) that included three stages: stage A, in which the tumour growth is limited to the wall of the rectum with no metastases in the lymph node; stage B, in which the tumour extend into the extrarectal tissue without metastases in the regional lymph nodes; stage C, in which the tumour has spread to the regional lymph nodes (118). Later Turnbull *et al.* added stage D for tumours with distant metastases (119). A more differentiated staging system was introduced by the American Joint Committee on Cancer (AJCC) based on the local tumour depths of invasion (T1-4), the presence and amount of lymph node metastases (N0-2), and the presence of distant metastases (M0-1) (120). Today, the clinicians use the 7th version of the TNM staging published in 2009 (Table 1) (121). In addition to the tumour stage, other prognostic factors for CRC have been discovered such as blood or lymphatic vessel invasion, surgical margins, and tumour grade (well-, moderately-, poorly- or un-differentiated) (122). Tumour staging, together with these histopathological features, give rather good estimations of the likely course of the disease. However, no or little information about the outcome of a treatment is offered by these factors (123).

In the last several years, the attention has been focused on the identification of biological characteristics with improved prognostic and especially predictive values. These biological characteristics, also called biomarker, include genetic and epigenetic alterations, mRNA and protein expression, and posttranslational modifications (123).

Table 1: Dukes and TNM classification for CRC (adapted from Engstrom *et al.* (124) and Edge *et al.* (121)).

Dukes staging	Stage grouping and TNM staging system	Comments	Prognosis (5 year)
-	0 Tis, N0, M0	Tis: Tumour <i>in situ</i>	
A	I T1, N0, M0 T2, N0, M0	T1: Tumour has grown through the muscularis mucosa into the submucosa N0: No regional lymph node metastasis M0: No distant metastasis T2: Tumour has grown into the muscularis externia	~92%
B	IIA T3, N0, M0 IIB T4a, N0, M0 IIC T4b, N0, M0	T3: Tumour has grown through the muscularis externia into the serosa T4a: Tumour has grown through the serosa T4b: Tumour has grown through the serosa and is attached, or invades other organs	~82%
C	IIIA T1, T2, N1, M0 or T1, T2, N2a, M0 IIIB T3-T4a, N1, M0 or T2-T3, N2a, M0 or T1, T2, N2b, M0 IIIC T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0	N1: Metastasis in one-three regional lymph nodes N2a: Metastasis in four-six regional lymph nodes N2b: Metastasis in more than seven regional lymph nodes	~60%
D	IVA AnyT, Any N, M1a IVB AnyT, Any N, M1b	M1a: Metastasis in one distant organ M1b: Metastasis in more than one distant organ	~8%

A prognostic biomarker provides information, about the patients overall outcome and can be useful to select for a certain treatment. A predictive biomarker provides information, about the likelihood to response to a clinical treatment (123, 125). So far, there are only few biomarkers used in the routine clinical practice for CRC and most of them include genetic markers. The prognostic and predictive value of mutations in the *KRAS* and *neuroblastoma RAS viral oncogene homolog (NRAS)* gene at the codons 12 and 13 (exon 2) as well as 61 (exon 3) have been evaluated in several studies. It was shown that patients with a mutation in *KRAS* have a significant lower benefit from endothelial growth factor receptor (EGFR) target therapy with the monoclonal antibodies cetuximab and panitumumab (126-128). Similarly, patients with a mutation in the *B-Raf proto-oncogene (BRAF)* did not respond to the anti-EGFR treatment (129). EGFR is thought to signal via the Ras-Raf-MAPK signalling pathway, leading to proliferation. Mutations in *KRAS* or *BRAF* genes leads to the constative activation of this signalling pathway and the upstream inhibition of EGFR in patients with *KRAS* or *BRAF* mutated tumours has no effect (130). Moreover, a MSI-high genotype in CRC has been shown to have both a prognostic value and a predictive value for 5-Flourouracil treatment (131, 132). However, intratumoural (different types of cell clones within a tumour) and intertumoural (differences between tumours of different patients) heterogeneity make treatment predictions very challenging (133, 134). Several clones within a tumour might be eradicated by a certain treatment, while other clones might be resistant against the same treatment. Whether the cell is resistant to a certain treatment depends thereby on several factors (like the *KRAS* and *BRAF* mutations for anti-EGFR treatment), which might be different within the tumour, and differ among patients. The personalised treatment, based on the specific tumour phenotype and genotype, is considered as the future in oncological treatment, also called personalised treatment. However, to be able to treat cancer in a personalised setting, more knowledge about resistance mechanisms, as well as more precise predictive and prognostic biomarkers are needed (135).

Treatment

The choice for the optimal treatment for CRC patient's depends on several factors, including tumour location, stage, genotype for *RAS* mutations and recurrence. The treatment options can be mainly categorised in four groups: surgery, chemotherapy, radiotherapy (RT), and targeted therapy. The main goal of all anti-cancer treatments, apart from surgery, is the decrease in tumour growth and ultimately its eradication. It has been postulated that most cancers are hierarchically organised, containing a few cells that act as cancer stem cells (CSCs). These CSCs have the same characteristics as the normal stem cells including, self-renewal and differentiation, and sustain the cancer (136). It is thought that CSCs have to be targeted to be able to successfully eradicate the tumour (137).

Surgery

The main goal of CRC surgery is to curatively remove the tumour-bearing bowel segment in order to restore the bowel continuance and assure the best quality of life for the patient (138). For locally resectable colon cancer, the preferred surgical technique is colectomy with removal of regional lymph nodes, *en bloc* if possible. The location of the tumour dictates whether only a part of the colon (left or right sided hemicolectomy) or the entire colon (subtotal or total colectomy) is resected (124). Total colectomy is considered if the patient has a synchronous tumour (left and right colon) or if the patient is diagnosed with FAP or HNPCC (139). For rectal cancer, the type of surgery depends on the localisation and stage of the tumour. Early stage rectal cancer can be removed by polypectomy, transanal local excision or, for selected tumours, by transanal endoscopic microsurgery (124). For more advanced tumours, which do not meet the criteria for local surgery, a transabdominal resection is performed. The surgical method of choice for transabdominal resection is the total mesorectal excision (TME), with removal of the mesorectum including vasculature, lymphatic structures, and fatty tissue *en bloc*, through sharp dissection with preservation of the autonomous nerves. An abdominoperineal resection, including the resection of the rectosigmoid, the rectum, and the anus, is performed when the tumour involves the anal sphincter of the levator muscle (140). The pelvic localisation of the rectum complicates the total removal substantially. To be curative, the margins of resected tissue must be tumour-free and any organs or structures attached to the tumour must be resected *en bloc*. Therefore, skills of the surgeon have a major impact on the outcome of the surgery (138).

Chemotherapy

To reduce the risk of recurrence after surgery, adjuvant chemotherapy is given to both colon and rectal cancer. In rectal cancer, chemotherapy is given together with RT as a neo-adjuvant treatment to reduce the tumour size before surgery. For unresectable CRC, chemotherapy is used as a palliative care to reduce suffering and pain, and to prolong life. Many chemotherapeutic agents target cycling cells with the rationale that cancer cells replicate more frequent. One strategy to inhibit cell growth is to target the DNA, thereby inhibiting the replication that eventually results in apoptosis or necrosis. The main chemotherapeutic agents used for CRC treatment are 5-fluorouracil (5-FU in combination with folinic acid), leucovorin, capecitabine, oxaliplatin and irinotecan (124, 140).

5-FU and capecitabine are both metabolised *in vivo* to a monophosphate derivate. These build a complex with the thymidylate synthase and block the function of the enzyme. As a result, the pool of deoxythymidine monophosphate, a building block of the DNA, is reduced, while deoxyuridine monophosphate is increased in the cell. This inhibits DNA replication and cell division, and leads consequently to DNA damage. A strategy to increase the effect of 5-FU is to increase the duration of the monophosphate derivate binding to the thymidylate synthase. One way to do this is to increase the levels of the cofactor 5, 10-methylene-tetrahydrofuran. In a clinical setting 5-FU is given together with folinic acid or leucovorin, which are converted to 5,10-methylene-tetrahydrofuran *in vivo* (141).

Oxaliplatin is a platinum based chemotherapeutic with a 1, 2-diaminocyclohexane (DACH) carrier. Oxaliplatin binds to guanine in the DNA and forms DNA intra- and inter-strand crosslinks that disturbs DNA replication and eventually lead to apoptosis (141). The pro-drug irinotecan is converted *in vivo* to SN-38, which binds and inhibits the DNA topoisomerase I (Top1) that is part of the DNA replication complex and catalyses the DNA unwinding and reannealing. The binding of SN-38 to Top1 inhibits the reannealing of the DNA, leading to the collusion of the DNA replication complex with the replication fork and consequently results in replication stop and DNA double-strand breaks (DSB) (142).

Several combinations of the above mentioned chemotherapeutics are applied to increase the effect and overcome possible resistances. The most common combinations are 5-FU, leucovorin and oxaliplatin (FOLFOX); 5-FU, leucovorin and irinotecan (FOLFIRI); and capecitabine and oxaliplatin (CapeOx) (124, 140). Unfortunately, chemotherapy is not tumour cell specific and therefore has a number of side effects including hair loss, nausea, vomiting, and low blood counts.

Radiotherapy (RT)

As for chemotherapy, the DNA is the main molecular target for RT. Since the discovery of the x-rays by Röntgen in 1895 and the natural radioactivity by Becquerel in 1896, radiation has been used in multiple medical applications (143). Grubbé was the first to treat cancer (breast cancer) with the newly discovered x-rays in 1896 (144). Nowadays, RT for rectal cancer is applied by an external beam consisting of γ -rays, electromagnetic high-energy radiation produced by a linear accelerator. These waves or photons have the ability to remove electrons from atoms and are therefore called ionising radiation. The biological effect of radiation is mainly attributed to the effect on the DNA, even though radiation also affects membranes and proteins. The DNA can be damaged directly by the photons, but mainly indirectly by free-radicals, produced in the near surrounding of the DNA. The resulting damages of the DNA include base/sugar damage, single-strand breaks and DSB all of which can be clustered in complex DNA damaging sites (145). However, the cytotoxic effect is mainly due to the DNA DSB. When DNA DSB occur, the cell is arrested in the cell cycle via the activation of the kinases ataxia–telangiectasia–mutated (ATM) or ataxia–telangiectasia and Rad3 related (ATR), and subsequent reduced cyclin-dependent kinase activity leading to G₁-S, intra S, and G₂-M cell cycle arrest (146). DNA DSB lesions are repaired by either of two DNA DSB repair mechanisms: the fast, but error prone, non-homologues end joining, or the slow, but more precise, homologues recombination. Damages too complex to be repaired lead to chronic DNA damage signals that trigger cell death by apoptosis or senescence (146).

In the 1920s, it was shown that the effect of radiation on cell growth has a bigger effect when given in small daily fractions over days instead of a single dose. Later it was generally accepted that the patient outcome improves with fractionated doses (147, 148). The success of fractionated treatment can be described by the 4 R's of radiobiology, **R**epair of sublethal DNA damage, **R**edistribution of the cells in the cell cycle, **R**eoxygenation of previously hypoxic areas, and cell **R**epopulation (149).

The amount of repaired sublethal damage after radiation is depending on the ability of the cell to activate their repair mechanisms and to induce cell cycle arrest. In many cancer cells, DNA repair and cell cycle control pathways are deregulated, making those cells more sensitive to radiation (150). Furthermore, the sensitivity depends on the phase of the cell cycle in which the cell is at the time of radiation. Cells in the S-phase have been shown to have the highest survival after radiation, whereas cells in the G₂ or M phase are most sensitive. The fractionated treatment allows the cells to repair their damage, move through the cell cycle and be “hit” in a

more sensitive cell cycle phase. Due to the increased proliferation rate of cancer cells, this effect will be higher in cancer tissue than in the normal tissue (151). The influence of oxygen on cell survival upon radiation has already been observed as early as 1904 by Hahn (152) and Schwarz (153). Under hypoxic conditions, the effect of radiation on the survival of cells can be 2-3 times higher compared with oxygenated cells. Tumours usually have hypoxic areas due to poor vascularisation and vascular architecture, therefore a single dose of radiation would have a lower effect in these areas. However, angiogenesis is induced after radiation and former hypoxic areas in the tumour become re-oxygenated, and sensitivity will increase for the following fractions (154). Radiation leads to a massive loss of cells, but triggers the proliferation of surviving CSC that repopulate the tumour. The amount of newly produced cells after radiation can even exceed the amount of cells before the treatment and therefore, it is important to eradicate all CSCs within the tumour (155).

Later, the intrinsic **R**adiosensitivity of the different types of cancer was suggested as the 5th R of radiobiology (150). The intrinsic radiosensitivity is depending on factors regulating mainly DNA repair, cell cycle progression, and apoptosis.

In the last 40 years, several European trials showed a reduced local recurrence rate for rectal cancer patients after neoadjuvant RT (156-160). The Swedish rectal cancer trial was one of two big European clinical trials evaluating the benefit from short-course preoperative radiotherapy treatment for rectal cancer. The Swedish rectal cancer trial was the first and only study to show a significant decreased local recurrence and an improved overall survival after preoperative RT (156, 161). However, a Dutch group using the same treatment regime, though performing TME, confirmed the decrease in local recurrence, yet could not show an increased overall survival (157). Thereafter, several clinical trials evaluated whether pre- or post-operative RT given in a short- and in a long-course in combination with chemotherapy could achieve a better outcome (159, 160, 162). Two systems of RT have shown clear clinical evidence with improved local control and sphincter preservation: preoperative short-course RT and preoperative long-course chemoradiotherapy (161, 163). The administered radiation dose and the time interval depends on the stage and localisation of the tumour. Primary resectable tumours are usually treated by a short-course preoperative RT. A total dose of 25 Gy is given in five fractions over one week followed by surgery within one week. For primary non-resectable tumours, the long-course chemoradiotherapy is generally used. The given dose is between 45-50 Gy in 25 fractions over five weeks, followed by surgery after a gap of six to 10 weeks, whenever possible (164). Although, improved radiation margins, resulting from higher resolution imaging, and radiation from different angles, leading to a dose maximum in the

tumour, has decreased the dose deposited in the normal tissue, there are still many side effects from radiation in rectal cancer (165). Moreover, there is a wide variation in the response to RT in rectal cancer and many patients experience recurrence, especially distant recurrence due to differences in the intrinsic radiosensitivity (166). A big challenge is to find factors that enable to select for patients that will benefit from the treatment and/or need an alternative treatment. Some factors have been identified that have the potential to predict the radiation outcome in rectal cancer including p53, p21, and survivin, but those factors have not been evaluated for a clinical application (166, 167). Furthermore, more markers are needed to account for the big variety between the different patients and tumours.

Targeted therapy

Research on the molecular basis of CRC, and thereby increased knowledge about the pathophysiological mechanisms leading to CRC, brought in the new area of targeted therapy. Targeted therapy includes monoclonal antibodies and small-molecular drugs that are designed to target a specific molecule. Today, there are at least three monoclonal antibodies in clinical use against CRC often combined with chemotherapy. The monoclonal antibodies cetuximab and panitumumab inhibit EGFR by binding to its extracellular domain and thereby blocking its activation, whereas the antibody bevacizumab is designed to inhibit VEGF (168). Additionally, several antibodies against important signalling pathways in CRC have been developed, including the Wnt/ β -catenin and Nuclear factor- κ B (NF- κ B), and are under evaluation in clinical trials (169).

Treatment-enhanced metastasis

All cancer treatments currently used in clinical practice including surgery, chemotherapy, and RT have proven their effect in large clinical trials including several hundreds to thousands of patients, but they may also create phenotypes and niches that facilitate metastatic spread (170). Already in the 1940s, it was shown in a mouse model that radiation of a subcutaneous tumour resulted in size reduction, but increased metastases in the lungs (171). Since then, several studies have analysed the effect of radiation-enhanced metastasis, mainly using *in vitro* cell culture models and *in vivo* mouse models. Two distinct effects have been established: increased localisation of metastases in irradiated tissue, and increase in metastases upon local tumour radiation (170, 172, 173).

The effect of increased metastasis in radiated normal/secondary tissue has been observed in patients, and was studied in mouse models (173, 174). It was observed that a dose related aggregation of cancer cells at the radiated tissue is increased immediately after radiation, but sometimes also months later (173). Possible mechanisms for this effect could be localised immunosuppression by reduced natural killer cell activity or local tissue damage at the radiated site leads to extracellular re-modelling and formation of a pre-metastatic niche (175-178).

Increased metastasis upon local tumour radiation has been observed in several models. Many mechanisms have been postulated, including direct alterations of the tumour cell activities and changes of the local normal tissue containing tumour associated cells at the tumour site (172, 173, 179).

Countless studies have been performed in cell lines and mouse models showing the activation of several signalling pathways involved in invasion and metastasis, upon direct radiation of cancer cells (172, 179). One important observation was the morphological change of irradiated cancer cells that showed a mesenchymal phenotype typical to EMT. Several signalling pathways in different cell types have been found to induce EMT upon radiation, including NF- κ B, TGF- β , PI3K/ v-akt murine thymoma viral oncogene homolog (Akt)/ mechanistic target of rapamycin (mTOR), and granulocyte colony-stimulating factor receptor (G-CSFR)/ Janus kinase-1 (JAK1) / Signal transducer and activator of transcription 3 (STAT3) (180-184). Another important observation was the increased MMP expression and secretion (especially MMP-2 and MMP-9) after radiation both in experimental models as well as in patients (185-188). Many studies showed that knockdown and inhibition of MMP-9 could abolish the radiation-enhanced invasion (187, 188). However, similar studies on MMP-2 did not show any effect on radiation-enhanced invasion, but it was shown that the phosphatase and tensin homolog (PTEN) status might be of importance (187, 189, 190). The MMP secretion was linked to the activation of the PI3K/Akt signalling pathway and subsequent NF- κ B or mTOR activation (188, 189). Moreover, several other signalling pathways have been identified that are involved in radiation-enhanced invasion, including insulin-like growth factor receptor-1 (IGFR-1) and subsequent PI3K/Akt, ras homolog family member A (RhoA) and Rho-associated kinase (Rock) activation, K-Ras and c-Raf and ATM-NF- κ B-MET proto-oncogene (MET) (190-192).

Tumours are heterogeneous masses of cells consisting of tumour cells and tumour-associated cells and radiation has been shown to stimulate tumour-associated cells to boost the invasion of the tumour cells. However, radiation often acts as a double-edged sword on the tumour-associated cells. Blood vessels supply the tumour with important cytokines, nutrients and

oxygen, and represent gates for the tumour cells to reach out to the rest of the body. Radiation has an apoptotic effect on endothelial cells of the blood vessels and therefore an anti-angiogenic effect. However, there are several pro-angiogenic factors released by the tumour cells upon radiation including VEGF and nitric oxide synthase (NOS) that stimulate the formation of new blood vessels (193-195). The pro-angiogenic effect of the radiated cancer cells might counteract the endothelial cell death (179). Similar is the effect on the inflammatory and immune system. Radiation kills the pro-metastatic macrophages and leukocytes present in the tumour environment, but induces inflammation (114, 179). Pro-inflammatory factors released by lymphocytes, including tumour necrosis factor alpha (TNF- α) and interleukin-1 (IL-1), recruit leukocytes and macrophages to the radiation site and increase the blood vessel permeability (179, 196).

Astrocyte elevated gene-1 (AEG-1)

Astrocyte elevated gene-1 (AEG-1), was originally identified as a human immunodeficiency virus-1 (HIV-1), gp120 and TNF- α inducible gene in human foetal astrocytes by Fisher *et al.* in 2002 (197, 198). Thereafter, other groups using different experimental settings identified AEG-1 as a protein localised at tight junctions (199) and the nucleus (200), and called it lysine-rich CEACAM1 co-isolated (LYRIC) and 3D3/LYRIC, respectively. Another study attempted to identify cell surface molecules that mediate metastasis from mouse breast cancer, found a domain of LYRIC involved in lung metastasis and that protein was named metadherin or MTDH derived from metastasis adhesion protein (109).

The gene coding for AEG-1, called *MTDH*, comprises of 12 exons/11 introns, and is located at chromosome 8q22 (201). The AEG-1 mRNA consist of 7667 nucleotides with a large 3'untranslated region [GeneBank reference sequence NM_178812.3]. The gene encodes for a lysine-rich, highly basic 582 amino acid protein with a molecular mass of 64 kDa and a half-life of about 20 h (201-203). Northern blot analyses indicated multiple AEG-1 transcripts and Western blot analyses revealed several proteins with a molecular mass ranging from 65 to 80 kDa and 35 to 20 kDa, possibly due to alternative transcription starting sites, alternative splicing, and/or post-translational modifications, like ubiquitination (201, 203). The protein contains several predicted domains and motifs, including a transmembrane domain (amino acid (aa) 51-72) and three nuclear localisation signals (NLS-1, NLS-2, NLS-3; aa79-91, 432-451, and 561-580, respectively; Figure 4) (109, 199-201). The membrane topology of AEG-1 was considered both type Ib and II, both single membrane spanning proteins, but with a different

orientation. Class I proteins have the NH₂- terminus exposed to the exterior site and the COOH-terminal exposed to the cytoplasmic site, while it is the other way around for class II proteins (109, 199). The three NLS are shown to have different functions in coordinating the localisation of AEG-1. The NLS-1 has been shown to primarily regulate the nucleolar localisation, NLS-3 the nuclear localisation, and the NLS-2 the cytoplasmic localisation (203). Additionally, several protein-protein interaction sites have been discovered, including CREP binding protein (CBP, aa 1-70), yin-yang-1 (YY1, aa 71-100), BRCA2 and CDKN1A interacting protein alpha (BCCIP α , aa 72-169), NF- κ B (aa 101-205), staphylococcal nuclease and tudor domain containing-1 (SND1, aa 101-205 and aa 386-470), a lung homing domain (aa 378-440), and promyelocytic leukaemia zinc finger (PLZF, aa 487-582) (109, 204-208). Furthermore, several post-translational modification sites were predicted, including phosphorylation, ubiquitination, and acetylation, while only the phosphorylation of AEG-1 at Ser298 was experimentally validated and showed biological significance (209, 210).

The protein has been localised in the cell membrane, cytoplasm, endoplasmic reticulum, nucleus and nucleolus (200, 201, 203). However, the biological significance of AEG-1 at the varying locations is not clearly understood.

Figure 4. Schematic overview of the AEG-1 protein with functional regions and protein binding sites (adapted from Emdad et al. (211)).

Since AEG-1 has been identified as a metastasis mediating factor in 2004, it has become an intensively studied molecule in the context of cancer (109). The AEG-1 mRNA and/or protein expression has been intensively analysed in a large variety of cancers, including oesophageal squamous cell carcinoma (212), gastric cancer (213), CRC (214), hepatocellular carcinoma (HCC) (202), non-small cell lung cancer (NSCLC) (215), neuroblastoma (216), breast cancer

(217), prostate cancer (203), and renal cell carcinoma (218). In these studies, the AEG-1 expression was markedly increased in the tumour tissue compared with the matched non-neoplastic tissues, and gradually increased as the disease progresses. In ovarian cancer, an AEG-1 exon 11 skipping variant was detected at higher levels in the cancer tissue compared with benign ovarian epithelial cells (219). Sequencing analyses in blood samples from breast and ovarian cancer patients identified two polymorphisms in the *MTDH* gene (c.1353G>A, rs2331652, and c.1679-6 T>C) connected with breast cancer susceptibility and one (-470 G>A) with ovarian cancer susceptibility (220, 221). However, whether mutations in *MTDH* attenuates the protein function or are of importance in cancer development have not been validated.

The impact of AEG-1 up-regulation has been evaluated in multiple studies, and it was shown that AEG-1 mediates oncogenesis via the regulation of cell survival, proliferation, anchorage independent growth, migration, invasion, metastasis, chemoresistance, radioresistance, and angiogenesis (222). Furthermore, high AEG-1 expression correlated with a worse patient outcome in most of the studied cancers (223). The expression of AEG-1 can be deregulated by several ways in cancer. Studies in breast cancer and HCC have linked genomic amplification of AEG-1, common for chromosome 8q22, with increased AEG-1 expression (202, 224). Another process called RNA interference (RNAi) has shown to regulate the expression of AEG-1. Thereby, small 21-25nt long RNA molecules called micro RNAs (miRNA), bind to the mRNA, and either inhibit expression or initiate degradation (225). Several of these miRNAs have been identified that target AEG-1 including miR-375, miR-217, miR-153, miR-136, miR30a, and miR-26a (226-232). It has been furthermore shown that the binding of the cytoplasmic polyadenylation element binding protein-1 (CPEB1) to the 3'UTR of the AEG-1 mRNA increases translation, while monoubiquitination of the AEG-1 protein increases its stability in cancer (203, 233, 234). In 2006, Lee *et al.* (235) published the first activation pathway of AEG-1, in which AEG-1 was activated by the Harvey rat sarcoma virus oncogene (Ha-Ras) through the PI3K/Akt signalling pathway leading to the binding of the cellular myelocytomatosis oncogene (c-Myc) to the *MTDH* promoter (Figure 5). Activation or up-regulation of any of these components can also increase the expression of AEG-1.

So far, many downstream signalling pathways activated by AEG-1, as well as interacting proteins, have been discovered by which AEG-1 exerts its oncogenic function (Figure 5). The first discovered signalling pathway was the NF- κ B pathway. The NF- κ B transcription factor family consists of important mediators for inflammation processes, immune and stress responses, and apoptosis. The NF- κ B proteins consists of different heterodimers which can be

activated via two signalling pathways: the canonical and the non-canonical pathway. In resting cells, NF- κ B is bound to its inhibitors I κ B (α , β , ϵ and ζ) or p100. Upon activating signals, I κ B kinases (IKKs) phosphorylates the inhibitors I κ B or p100, and thereby targeting them for degradation. The released NF- κ B heterodimer translocate to the nucleus, where it is transcriptionally active (236). The genes activated by NF- κ B include anti-apoptotic genes (e.g. *B-cell lymphoma-extra-large (BCL-xl)*, *Survivin* and *B-cell CLL/lymphoma-2 (BCL-2)*), genes that regulate the cell cycle (e.g. *cyclin 1*, *c-Myc* and *cyclin-dependent kinase-2 (CDK2)*) and genes involved in migration and invasion (e.g. *MMP-2*, *MMP-9*, *intercellular adhesion molecule-1 (ICAM-1)*, *selectin E* and *inducible NOS*) (237, 238). It was shown in HeLa and human glioma cells that ectopic expression of AEG-1 leads to the degradation of inhibitor I κ B α and increases the DNA binding of the transcriptional activator p50/p65 complex of NF- κ B leading to the expression of NF- κ B downstream targets involved in cell growth, survival, and invasion. Furthermore, the AEG-1 expression was increased and the protein was phosphorylated by the IKK β at Ser298 after treatment with the NF- κ B activator TNF- α . This resulted in the interaction with the p65 subunit of NF- κ B and nuclear translocation (204, 205, 210). Additionally, it was shown that AEG-1 functions as a bridging factor between NF- κ B and the cAMP response element-binding protein (CREB) to induce interleukin-8 (IL-8) transcription, a promotor of angiogenesis, cell growth and migration (205, 239).

The second discovered major pathway activated by AEG-1 is the PI3K/Akt signalling pathway (240). The PI3 kinase constitutes of a regulatory subunit p85 and a catalytic isoform p110. The auto-phosphorylation of several receptor tyrosine kinases (e.g. EGFR, VEGF, IGFR-1, fibroblast growth factor receptor (FGFR), and Met) attracts the regulatory subunit of PI3K and results in allosteric activation of the catalytic subunit (241). Furthermore, PI3K can be activated directly via G-coupled protein receptors, like Ras (242). The activated kinase generates the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphat (PIP₂) at the inner site of the cell membrane that leads to the translocation of Akt to the inner membrane and activation via 3-phosphoinositide-dependent protein kinase (PDK) 1 or 2 phosphorylation. Activated Akt translocate to the cytoplasm and nucleus, and activates downstream targets involved in cell survival, cell cycle progression, growth, migration and angiogenesis. The signalling of PI3K to Akt is regulated by PTEN, a tumour suppressor gene that dephosphorylates PIP₃ to PIP₂ (243). Overexpression of AEG-1 activates PI3K/Akt and c-Myc expression in primary human foetal astrocytes and thereby inhibits serum starvation-induced apoptosis. In this study, the Akt downstream targets BCL2-associated agonist of cell death (Bad) and p21 were down-regulated, while MDM2 was upregulated (240).

Other studies showed that inhibition of AEG-1 induced apoptosis via down-regulation of the Akt activity and subsequent upregulated forkhead box (FOXO)3a and FOXO1 activity in prostate and breast cancer cells, respectively (244, 245). AEG-1 up-regulation in human umbilical vein endothelial cells resulted in increased VEGF expression and in human glioma cells increased VEGF promoter activity, both via Akt, resulting in increased angiogenesis (246). Furthermore, AEG-1 has been shown to upregulate cyclin D1 and downregulate p27 in an Akt-dependent way in oesophageal cancer (212). However, the exact activation mechanism of Akt by AEG-1 is still unknown.

Other major signalling pathways activated by AEG-1 are the Wnt and the MAPK signalling pathways by either activation of LEF-1 or extracellular regulated MAP kinase (ERK), or down-regulation of APC (202). A study in CRC showed a positive correlation between the AEG-1 expression and the nuclear β -catenin expression. Furthermore, overexpression of AEG-1 in the SW480 colon cancer cell line increased nuclear β -catenin accumulation (247). The MAPK signalling pathway links extracellular signals to processes that regulate growth, proliferation, differentiation, migration, and apoptosis. Thereby, a stimulus (e.g. stress, growth factor, or cytokines) activates a G-coupled receptor, which in turn activates a cascade of three kinases (MAPKKK, MAPKK and MAPK) (248). Inhibitors of the MAPK signalling pathways have been shown to abolish the AEG-1 induced invasion and anchorage-independent growth in HCC cells (202). Apart from the activation of multiple signalling pathways, AEG-1 interacts with a variety of proteins and multiple protein complexes including CBP, BCCIP α , NF- κ B, SND1, PLZF, ribosome biogenesis regulator homolog (Rrs1), β -catenin, and ubinuclein-1 (UBN-1) and stimulates tumourigenesis (211).

The AEG-1 expression has been correlated with metastasis in several types of tumours and AEG-1 up-regulation increased migration and invasion in a large variety of cancer cells, as well as metastasis formation *in vivo*. AEG-1 has been shown to modulate the expression of MMP-2 in glioma and osteosarcoma, and MMP-9 in glioma, prostate cancer, and NSCLC. Furthermore, it was shown that AEG-1 up-regulation induces EMT by several mechanisms, such as Wnt/ β -catenin and p38 MAPK activation (202, 204, 228, 240, 244, 246, 247, 249-252). In addition to all these tumourigenic properties of AEG-1, gene expression profiles of AEG-1 knockdown breast cancer cells and up-regulation HCC revealed deregulation of several genes, including *aldehyde dehydrogenase 3 family, member-A1 (ALDH3A1)*, *Met*, *heat shock protein 90kDa (HSP90)*, *heme oxygenase-1 (HMOX1)*, *dihydropyrimidine dehydrogenase (DPYD)*, *cytochrome P450, family 2 subfamily B polypeptide-6 (CYP2B6)*, *aldo-keto reductase family 1 member-C2 (AKR1C2)* and *ATP-binding cassette sub-family C member-11 (ABCC11)*. All of

these genes are involved in resistance to various chemotherapeutics, such as 5-FU, doxorubicin, paclitaxel, cisplatin and 4-hydroxycyclophosphamide (202, 224). Furthermore, Zhao *et al.* showed in 2012 that AEG-1 affects survival, DNA DSB repair, and cell cycle distribution after radiation in the cervical cancer cell line SiHa (253).

Figure 5. An overview of AEG-1 signalling pathways. AEG-1 has been shown to be activated via Ha-Ras, PI3k/Akt and c-Myc (blue), and contributes through several signalling pathways and proteins (orange) to different aspects of oncogenesis (white).

Aims

- I. To investigate the role of AEG-1 in CRC development
- II. To evaluate the impact of AEG-1 on radiation treatment response

Materials and Methods

Cancer models and tissue samples

It is very challenging to select models for cancer research, especially for the complex mechanisms of carcinogenesis and treatment response. The choice for a model depends thereby on the research question, the availability, as well as the knowledge about the application.

Cell lines

The first successful attempt of studying the behaviour of cells *in vitro* is attributed to Harrison and his colleagues in 1907, who cultured isolated tadpole tissue in a hanging drop of lymph for up to four weeks (254). Based on these findings, Burrow and Carrel were the first in 1911 to culture and subculture mammalian cells. One of those cell lines explanted from a chicken embryo heart fragment was subcultured over a hundred times and even outlived Carrel by two years (255-257). The implication of tissue culture on cancer research were obvious, and a lot of research was dedicated to be able to study cancer *in vitro* (258). However, it took almost 40 years until Gey for the first time in 1951 successfully established a human cell line, which was called HeLa (259). The cell line originated from a cervical cancer biopsy specimen of **Henrietta Lacks** and is currently the most widespread human cell lines (260). Nowadays, cancer cell lines are important tools to study the biology of cancer and for the development of anticancer drugs (261). The cell line models have several advantages, such as the unlimited amount of material, high reproducibility, low cost, and high throughput. However, there are also several limitations with that model and especially the representation and maintenance of the genetic diversity of a tumour are difficult, if not impossible to maintain in a cell culture.

In the studies I-IV we used the KM12C, KM12SM, KM12L4a, SW480, SW620, and HCT116 colon cancer cell lines, as well as the CCD-18Co normal colon fibroblast cell line and the CCD-841CoN normal colon epithelial cell line.

The KM12C cells derived from a patient with stage II colon cancer. The KM12SM cells where “SM” stands for spontaneous metastasis, arose from spontaneous liver metastasis of injected KM12C cells into the caecum of nude mice. The KM12L4a cells were produced by repeated intrasplenic injection of the KM12C cells and harvesting of the liver metastases in nude mice (262, 263). “L” stands for liver and “4” for four repetitions of intrasplenic injection. The KM12

cell lines were kindly provided by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Centre, Houston, TX).

The SW480 cell line was established from a primary colon adenocarcinoma, whereas the SW620 derived from a lymph node metastasis, taken from the same patient one year later (264). The HCT116 cell line was one out of three subpopulations of malignant cells isolated from a primary cell culture of human colonic carcinoma (265).

The CCD-18Co fibroblast cell line originated from a colonic mucosal biopsy of a two month-old human infant and was a kind gift of Dr. Richard Palmqvist (Umeå University, Sweden) (266). The CCD-841-CoN epithelial cell line originated from a five month-old human infant and was a kind gift of Dr. Liang Xu (University of Kansas, Lawrence, KS) (267).

The KM12C, KM12SM, KM12L4a, SW480, SW620, CCD-18Co and CCD-841-CoN cell lines were maintained at 37°C and 5% CO₂ in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% heat inactivated foetal bovine serum. For the KM12 cell lines, 2% vitamin solution and 0.5% L-glutamine were added, while for the SW480 and SW620 the medium was supplemented with 1% L-glutamine. The HCT116 cell line was maintained in McCoy's 5A medium supplemented with 10% heat inactivated foetal bovine serum at 37°C and 5% CO₂.

A simple and inexpensive way to study the involvement of a protein in cellular mechanisms is to inhibit or overexpress that protein expression in a cell line. The inhibition of the protein expression, also called gene knockdown can be achieved by several approaches. A common way to knockdown a gene in a eukaryotic cell is based on the findings by Fire and colleagues in Mello's laboratory in 1998 (268). They showed for the first time that the injection of a sense-antisense mixture of RNA, covering a segment of a nonessential myofilament protein mRNA (*unc-22*) in *Caenorhabditis elegans*, resulted in a highly effective interference with the endogenous gene activity (268). The process behind, called RNAi, can regulate gene expression by inhibiting the translation or by degrading the target mRNA via endonucleases. The basic process can be divided into three steps. Firstly, a long double stranded RNA, which is expressed or introduced into the cells, is processed into a small RNA duplex. Secondly, the duplex is unwound and assembled into the RNA induced silencing complex (RISC) or miRNP and thirdly, the complex binds to the target RNA leading to either RNA cleavage or transcriptional repression (269).

The transfection of a cell with a specific designed double-stranded RNA allows to specifically inhibit the translation of the protein of interest. The cells can be transfected with a transient approach, in which the protein expression is manipulated only for a certain time period, or by

a stable approach, where the protein expression is continually inhibited. In paper III, we inhibited the AEG-1 protein expression both transient and stable in colon cancer cell lines. For the transient AEG-1 knockdown, we transfected the KM12C, KM12L4a, SW480, SW620, and HCT116 cell line with a pool of four small inhibitory RNAs (siRNA) targeting the AEG-1 mRNA. A scrambled non targeting siRNA was used as a negative control. The knockdown was confirmed by qPCR and Western blot. For the production of stable AEG-1 knockdown cells used in paper III and IV, we transfected the SW480, SW620, and HCT116 cell lines with different DNA plasmids containing two genes for selection (*Puromycin resistance* and the *Green fluorescence protein (GFP)* gene), as well as an anti-AEG-1 small (or short) hairpin RNA (shRNA), or a non-targeting shRNA under a viral promoter. After Puromycin selection, single cells expressing GFP were expanded and regularly tested for AEG-1 knockdown by qPCR and Western blot. For AEG-1 up-regulation, the KM12C and KM12L4a cell lines were transiently transfected either with a plasmid containing the AEG-1 cDNA under viral promoter control, or an empty vector as a negative control.

Zebrafish

Animal models are widely used in cancer research mainly to study carcinogenesis, invasion and metastasis, but also used for treatment development, toxicity and resistance studies. Several animal models, mainly mouse models, have been developed during the years for haematopoietic malignancies and solid tumours (270). However, their relevance to human cancer, especially for treatment development is questionable. A breakthrough in cancer research was in the late 1960s when for the first time a human tumour cell lines was transplanted into immunodeficient mice, a process called xenotransplantation (271, 272). The development of mice lacking T, B, and natural killer cells (NSG mice) allowed superior engraftments of healthy and malignant human cells directly from the patient (273). Engraftments of human tumours in mice (either subcutaneously or orthotopic) is considered the golden standard model and has several advantages, especially to examine the therapeutic response to drugs and radiation (274). However, despite the advantages of the model, there are several disadvantages: nude mice husbandry is very expensive and requires specialised facilities, the process from implantation to endpoint measurement can take several months, and it is very difficult to image single cells (275). To overcome those shortcomings various attempts have been made to develop non-mammalian animal models (276-278). A promising model is the Zebrafish (*Danio rerio*). The Zebrafish model has several advantages compared with the

classic murine models that include the availability of large number of offspring, the possibility to make the transplantations at embryonic stages, the fish permeability to small molecules, the external development, the availability of transparent lines, the absence of immune rejection in early stages, small number of cells required for transplantation, and a fast readout (279). The first xenotransplantation of human cells into the zebrafish embryo has been reported in 2005 (280). This work has been followed by several studies implanting a diverse range of human cell lines and primary tumours into the zebrafish embryo at different sites. The model has been used to study invasiveness and metastatic behaviour (278, 280), angiogenesis (278, 281) and response to anticancer therapies (282, 283). Despite the advantages and possibilities with the zebrafish model, there are also several limitations such as the lack of knowledge about the niche structures and micro-environmental cues, the disproportional ratio between human cells (large) and zebrafish organs and vessels (small), the different maintenance temperature between the zebrafish and the cell lines (28°C vs 37°C), different organs, and fewer possibilities for orthotopic transplantation (279).

The transgenic Tg(fli1:EGFP)^{y1} zebrafish strain, with enhanced GFP (EGFP) labelled endothelial cells (284), were used and maintained according to standard protocols at the zebrafish facility at Linköping University. Zebrafish were mate overnight in a breeding aquarium, and eggs were collected in the morning immediately after spawning. Subsequently the eggs were cleaned, sorted, and incubated in E3 medium supplemented with 1-phenyl-2-thiourea (PTU) at 28.5°C in humidified ambient air until injection.

Patients

A common way to study biomarkers in solid tumours is to analyse tissues taken from the tumour by biopsy or surgery. These samples can either be directly cultured or stored for long time, e.g. frozen or formalin fixed and paraffin-embedded (FFPE), for further analyses. Subsequently DNA, RNA, and proteins can be isolated and analysed in different cells (e.g. tumour and stromal cells), different tissues (normal, adjacent, tumour, and metastasis), and at different sampling time points (e.g. biopsy, surgery, recurrence). Furthermore, the achieved data can be correlated with clinicopathological features, e.g. gender, age, tumour size, stage, differentiation, and survival. However, there are several issues that have to be considered when interpreting the results from patient tumours. The patient population might not be representative, and values such as ethnic groups, gender or age might be over- or under-represented. Confounding factors such as social status, diet, smoking, alcohol consumption,

and physical activity might be unknown. Furthermore, it can be difficult to identify from which part of the tumour the sample originated (core or border) or to get information about the cellular environment (e.g. close to a blood or lymph vessel). Additionally, tumour cell subpopulations might be under- or over-represented, if not the entire tumour is available.

In paper I and II, we used samples from unselected CRC patients. Tissues were available from the normal mucosa, adjacent mucosa, primary tumour, lymph node metastases, and liver metastases. The samples were collected from the University Hospital Linköping or Vrinnevi Hospital in Norrköping between 1983 and 2004. The samples were either FFPE or snap frozen and stored at -70°C for DNA and RNA extraction. Clinical information about the patient gender and age as well as tumour localisation, stage, and differentiation were collected from the patient records (Table 2). Survival data were obtained from the Swedish National board of Health and Welfare (Socialstyrelsen) and the patients were followed up until the year 2013.

The patient samples analysed in paper III were from the South-East Swedish Health Care region that participated in the randomised Swedish rectal cancer trial of preoperative RT between 1987 and 1990 (156). Patients with stage I-III rectal cancer were randomised for surgery alone (anterior resection or abdominoperineal excision) or preoperative RT followed by surgery. During surgery some of the patients were diagnosed with metastases. These patients have not been included for survival analyses in paper III. The total radiation dose was 25 Gy, given in 5 fractions (5x5 Gy). Surgery was performed after a median of 3 days (range, 0-11 days). Clinical information about the patient's gender and age as well as tumour localisation, stage, differentiation, and recurrence were collected from the patient records (Table 2). Survival data were obtained from the Swedish National board of Health and Welfare (Socialstyrelsen) and the patients were followed up until the year 2004.

The use of all the patient material was approved by the Regional Ethical Review Board in Linköping and an informed consent document was signed by all the participants to the study.

Table 2: Clinicopathological variables of the patients from paper I-III

Characteristics	Paper I		Paper II	Paper III	
	Fresh-frozen	FFPE		Non-RT	RT
Patient number	156	158	356	78	64
Diagnosis (year)	1983-2003		1989-2004	1987-1990	
Gender					
Male	103	88	190	46	40
Female	53	70	166	32	24
Age at diagnosis (years)	34-94	42-95	35-95	45-80	36-85
Tumour location					
Colon	98	77	203	-	-
Rectum	57	81	153	78	64
Tumour stage					
I	20	25	44	20	16
II	73	61	150	22	25
III	34	40	108	32	17
IV	18	31	54	4	6
Differentiation					
Well	19	9	32	5	4
Moderately	91	98	222	56	40
Poorly	40	50	98	17	20

Varied numbers in the variables are due to missing data

Methods

Irradiation of cell lines and zebrafish embryos

Cell cultures and zebrafish embryos were irradiated with single doses, between 2-10 Gy, with a 6 MV photon spectra using a linear accelerator. The cells and zebrafish embryos were positioned below 3 cm acryl glass, 105 cm from the photon source (the distance from the photon source to the acryl glass-surface was 100 cm). The dose rate at the position of the cells was 4.8 Gy/min and the field size at SSD was 30 x 30 cm (Figure 6). An unirradiated negative control was included in each radiation experiment and treated in the exact same way, apart from radiation.

Figure 6. *Experimental set-up for cell and zebrafish embryo radiation.*

DNA, RNA and protein extraction

The careful extraction of DNA and especially RNA and protein is essential for all following molecular analyses. The total DNA and RNA yield should be maximised, while contamination and degradation should be minimised. The successful isolation of nucleic acids requires the effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of nucleases (DNase and RNase), and low contamination with salts, lipids, proteins and DNA (in RNA samples) or RNA (in DNA samples) (285). Moreover, a high integrity and high concentrations are required for many applications. The correct handling and storage of the samples will increase the stability. This is particularly important for RNA, since it is very susceptible to degradation.

In paper I, we extracted RNA from fresh frozen tissue and colon cancer cell lines using the TRIzol reagent and RNeasy extract Kit according to the manufacturer's instructions. The concentration, purity and integrity of RNA were measured by NanoDrop and Bioanalyzer Agilent, and the samples were stored at -70°C.

In paper II we extracted genomic DNA from fresh frozen tissue and colon cancer cell lines using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions. The DNA concentration and purity was measured by NanoDrop and the integrity by agarose gel-electrophoresis, and the samples were stored at -70°C.

The procedure for protein isolation depends on the tissue and cell type, the localisation of the protein (membrane bound, cytoplasmic, nuclear), and the research question. In paper I-IV proteins for Western blot analyses were extracted using analysis buffer containing 150 mM NaCl, 2% Triton, 0.1% SDS, 50 mM Tris pH 8.0, and 10% protease inhibitor cocktail. The lysates were centrifuged to separate the dissolved proteins from the cell fragments and stored at -20°C.

Polymerase chain reaction (PCR)

In many cases, not the entire extracted DNA is of interest, but only particular genes or sequences. The polymerase chain reaction (PCR) is a very powerful and revolutionary technique to amplify a particular sequence out of small amounts of DNA. It was developed in the 1980s by Mullis, who was rewarded for his work on the PCR with the Nobel Prize in Chemistry in 1993 (286). The PCR is based on the amplification of a template DNA and requires four main components: a heat resistant DNA polymerase, deoxynucleotide triphosphates (dNTPS), small oligonucleotides called primers, and the template DNA. The

PCR consists of three steps. The first step separates the original DNA-double strand into single-strands using high temperatures (around 90°C), also called melting. In the second step, the sample is cooled and the primer will anneal to the target DNA sequence. The temperature is specific for each primer pair and depends on several physicochemical variables. In the last step, the elongation, the temperature rises up to 72°C, which is the optimal temperature for the heat-resistant DNA polymerase TaqPol that is isolated and named after the thermophile bacterium *Thermus aquaticus*. The TaqPol takes the primers as a starting point to synthesise a sequence-specific complementary strand to the target DNA using dNTPs (dATP, dGTP, dCTP, dTTP) as building units (287). The copy number doubles with each repetition of these three steps and the final number of copies will be 2^{n+1} , while n is the amount of cycles. The PCR technique has been further developed and several variations have been established.

Capillary Sanger DNA sequencing

The genome and its sequence have been a main focus in biomedical research over the last decades, especially in cancer research. The completion of the human genome sequencing project in 2003 was a milestone for biomedical research and capillary Sanger sequencing played an essential role (288). Sanger developed the DNA sequencing in the 1970s and was rewarded for this work with his second Nobel Prize in Chemistry in 1980. The method is based on the terminated chain reaction, similar to the PCR described above, but uses radioactively or fluorescently labelled primer and dideoxynucleotide triphosphates (ddNTPs: ddATP, ddGTP, ddCTP, ddTTP) as DNA chain terminators (289). In the beginning of DNA sequencing, the samples were aliquoted into four PCR reactions, each containing one ddNTP and the labelled primer. The random incorporation of the ddNTPs led to the synthesis of DNA fragments with various length. Each of the four reactions were then separated by gel electrophoresis in an individual lane, visualised by autoradiography or UV light, and the sequence was manually read from the gel. In the 1980s, a major development was done by Hood and his colleagues, who fluorescently labelled the ddNTPs. The labelling of each of the four ddNTPs with a fluorescent dye of different emission wavelength allowed to perform the terminated chain reaction with all four ddNTPs in a single reaction (290). The separation by automated capillary gel electrophoresis with on column detection, automated injection, and the multiplexing of this approach increased the capacity of this method significantly (291, 292).

In paper II, we analysed the coding region of the *MTDH* gene by direct Sanger DNA sequencing in 356 CRC tumour samples and three colon cancer cell lines. We therefore pre-

amplified the sequencing fragments from the isolated DNA by PCR and confirmed the amplification by gel electrophoresis. BigDye Terminator v3.1 Ready Reaction Mix was used for sequencing reaction and separation was performed on ABI 3500 genetic analyser. The collected data were analysed using sequence analyser software. The obtained sequences were compared with a public available reference sequence (NM_178812.3), and mutations were described according to the nomenclature system recommended by the Human Genome Variation Society (HGVS) (293). Designation of the genomic alterations in the *MTDH* gene is based on the GenBank reference sequences NM_178812. Mutations which were not described in the literature, the Single Nucleotide Polymorphism Database (dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>, accessed in June, 2015) (294), or in the Catalogue of Somatic Mutations in Cancer (COSMIC, <http://www.sanger.ac.uk/cosmic>, accessed in June, 2015) (295), were considered as novel. Exonic variants were evaluated by widely used programs for prediction of possible interference with the function, structure, or stability of the protein: Mutation Taster (<http://www.mutationtaster.org>; Ensembl transcript ENST00000336273, NM_178812; GRCh37/ Ensembl 69), SIFT and GVG D as a part of commercial Alamut 2.0 (Interactive Biosoftware, Roven, France), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>; UniProt peptide Q86UE4), PROVEAN (<http://provean.jcvi.org/index.php>; Human GRCh37/ Ensemble 66), and MUpro (<http://mupro.proteomics.ics.uci.edu>).

Quantitative PCR (qPCR) and Reverse transcription PCR

The quantitative PCR (qPCR), allows to detect and measure the amount of copies produced during each cycle of PCR in real time. Two common methods are applied to detect PCR products. The first method uses a non-specific fluorescence dye like SYBR® green that intercalates with any double-stranded DNA. Increased amplification will lead to an increased fluorescence signal from the dye, allowing to quantify the total amount of double-stranded DNA. However, this method also detects unspecific PCR products (296). The second method is based on the 5' endonuclease activity of the TaqPol and a nonexpendable probe specially designed to hybridise in close proximity to the forward or reverse primer, which is labelled with a fluorescent dye as a reporter and a quencher (297). During the annealing phase, both the primer and the probe bind to the template, while the fluorescence signal from the dye is inhibited by the quencher. During the elongation step, the TaqPol synthesises the complementary DNA strand and degrades the probe. The fluorescent reporter dye is released

from the quencher and its specific emission can be detected. The fluorescence is continuously measured during the PCR and the signal intensity increases proportional to the PCR product. To be able to quantify RNA by qPCR, a complementary DNA (cDNA) strand has to be produced. Reverse transcription PCR uses a retroviral RNA dependent DNA polymerase called reverse transcriptase that produces cDNA from RNA. The reaction consists of dNTPs, RNA, reverse transcriptase and random hexamers or oligo DNA primer, which bind either randomly to the RNA or to the poly a tail of mRNA (298).

In the studies I-IV the High Capacity cDNA Reverse Transcription Kit was used to reverse transcribe RNA using the MultiScribe™ Reverse Transcriptase according to the manufacturer's instructions. The relative expression levels of AEG-1 mRNA in CRC patient material and colon cancer cell lines were determined by standard curve with TaqMan™® Gene Expression Fast Master Mix in Applied Biosystems 7900HT Fast Real-Time PCR system according to the manufacturer's instructions. To adjust for differences in cDNA input, the expression levels were normalised to the housekeeping gene *GAPDH*.

Immunohistochemistry (IHC)

Since its discovery in 1941, protein detection in tissue sections via antibody binding is widely used in clinical applications for diagnosis, prognosis, and prediction (299). In principle, a specific antibody binds to an antigen (e.g. protein) and is visualised either directly or indirectly by a fluorophore or a peroxidase-conjugated antibody. A big advantage of immunohistochemistry (IHC) is that protein expression in different parts of the tissue as well as in the subcellular compartments (cytoplasm, nucleus) can be distinguished.

In paper I and III, the FFPE tissue samples were first deparaffinised and the antigen retrieved using the PT-link. Then, the endogenous peroxidases and unspecific binding sites were blocked before the incubation of the tissue sections with the primary antibody. Thereafter, the slides were incubated with a secondary antibody, which was conjugated with horseradish peroxidase (HRP). The HRP-coupled antibodies were visualised using hydrogen peroxide and 3,3'-diaminobenzidine tetrachloride and the specimens were counterstained with haematoxylin. The IHC staining was evaluated by two independent observers based on the proportion of positively stained cells, the staining intensity, and localisation, without knowledge of clinicopathological and biological information.

Western blot

Western blot is an immunological method developed in the 1970s that allows to identify specific proteins from a complex protein mixture that can be used to compare the expression between different samples (300-302). The technique is divided in three parts: protein separation, transfer to a solid support, and protein visualisation using a specific primary and secondary antibody. In paper I-IV, we used denaturing conditions to separate the proteins according to the size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to a PVDF membrane and unspecific binding sites were blocked. A primary antibody, specific for a certain protein, was subsequently added followed by the incubation with a HRP-conjugated secondary antibody, specific for the primary antibody (303). The protein of interest was then indirectly visualised by the oxidative reaction of luminol catalysed by the HRP and captured by a charged coupled device (CCD) camera. To adjust for differences in total protein loading, the protein levels were normalised to the housekeeping protein β -actin. Detailed Western blot procedures are described in the respective publications.

Cytokine antibody array

In paper IV, we used a commercial available human cytokine antibody array. The principle of this array is the same as for Western blot. However, the antibodies specific for the secreted proteins, were covalently linked to a membrane. The membrane was then incubated with the conditioned medium of differently treated cell lines, whereby the secreted proteins attach to the specific antibody on the membrane. Subsequently, the medium was washed away and a secondary biotinylated antibody binds to the attached proteins. In the last step, the membranes were incubated with a HRP-conjugated streptavidin and the visualisation was the same as in the Western blot. A negative control and a positive control were included on each membrane.

Colony forming assay

The colony forming assay is the golden standard to evaluate cell survival and proliferation in cell culture models upon radiation (304). Single cell suspensions were seeded in 6 well plates, radiated after attachment, and incubated until sufficiently large colonies were formed. After fixation and staining, the colonies were counted and the plating efficiency

($PE = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\%$) and the surviving fraction ($SF = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times PE}$)

were calculated. The SF is used as a measurement to evaluate the effect of radiation on the viability of the different cell lines. It is common to use the SF after 2 Gy radiation to distinguish between radiation resistant (>50%) and radiation sensitive cell lines (<50%). In paper III, we used the colony forming assay to evaluate the effect of radiation on AEG-1 knockdown and overexpressing colon cancer cell lines. The cells were grown between six and 10 days after radiation and clones of a minimum of 30 and 50 cells, respectively, were counted as one colony.

WST proliferation assay

The WST proliferation assay is based on the cleavage of the WST-1 tetrazolium salt to formazan by the mitochondrial NAD(P)H-dependent oxidoreductases and dehydrogenases, only present in metabolically active cells (305). In paper IV, we used the WST proliferation assay to evaluate whether the reduced radiation-enhanced invasion and migration in the SW480 and HCT116 AEG-1 knockdown cell lines, was due to cell death. Therefore, the cells were seeded 24 h before radiation with 0 Gy or 7.5 Gy, and incubated for 24 h. The WST-1 was added and the formation of formazan was quantified by measuring spectrometrically the absorbance at 450 nm.

Boyden chamber assay

The Boyden chamber assay (or Transwell assay) is a cell migration assay, originally introduced by Boyden to study leukocyte chemotaxis in the 1960s (306). It consists of 2 medium containing chambers that are separated by a porous membrane. Usually, the cells are seeded in the upper chamber in low serum medium and migrate vertical trough the membrane into a lower chamber containing higher serum levels. Cells that have passed the membrane can be fixed, stained, and quantified under a microscope. To be able to study invasion, a modified version of the Boyden chamber can be used, in which the membrane facing the upper chamber is coated with an ECM like matrigel, collagen, or fibronectin before seeding the cells (307). Only cells able to degrade the ECM proteins will be able to migrate through the membrane (Figure 7A). In paper IV, we analysed the impact of AEG-1 and radiation on migration and invasion in several colon cancer cell lines. For the latter experiments we did not apply a serum gradient in order to study only the radiation effect, and radiated the cells with either 0 Gy or

7.5 Gy. Additionally, we analysed the impact of AEG-1 in the radiation enhanced migration and invasion (Figure 7A).

Zebrafish invasion assay

In paper IV, we evaluated whether the zebrafish model can be used to study radiation enhanced invasion and whether AEG-1 is involved in this process. We therefore implanted 1,1'-diiodo-3,3',3',3'-tetramethylindocarbocyanine (DIL) labelled AEG-1 knockdown, negative control, or non-transfected SW480 cell lines in the perivitelline space of 48 h old transgenic Tg(fli1:EGFP)^{y1} zebrafish embryos (284). After the injection, the zebrafish embryos with labelled cells in the circulation were excluded. The embryos were then radiated with doses between 0-10 Gy and incubated at 28.5°C in humidified ambient air. The amount of cells posterior to the anal opening were visualised under a fluorescence microscope and counted (Figure 7B).

Figure 7. Schematic presentation of A) *In vitro* migration and invasion assay and B) *In vivo* zebrafish invasion assay.

Statistical analyses

In paper I, we used the Student's *t*-test to analyse the differences in AEG-1 mRNA expression between different tissues. Student's *t*-test or one-way ANOVA method was used to analyse the relationship between the AEG-1 mRNA and clinicopathological variables. In paper III and IV the Student's *t*-test was applied for the *in vitro* and *in vivo* studies to test for difference between the treatment groups. In paper I and III, McNemar's or Chi-square test was applied to examine the differences in AEG-1 expression between different tissues analysed by IHC, as well as the association with clinicopathological or biological variables. In paper I, the Cox's Proportional Hazard Model was used to test the relationship between the AEG-1 expression and the patient survival. In paper III, the Log-rank test was used to examine the relationship of the AEG-1 expression with the relative risk for distant recurrence and the patient disease-free survival. In paper III, the Stratified log-rank test was used for examining independency of AEG-1 expression from tumour stage in the relative risk of distant recurrence and disease-free survival (308). Survival curves were computed according to the Kaplan-Meier method.

All cell line experiments were performed at least 3 times and data were presented as the mean $\pm$ standard deviation. All data were analysed by the statistical program STATISTICA (SatSoft, Tulsa, OK) or Excel (Microsoft, Redmond, WA). All tests were 2 tailed, and a p-value less than 0.05 was considered as significant.

Results and Discussion

Alterations of AEG-1 in relation to CRC development

AEG-1 mRNA and protein expression in CRC patients and colon cancer cell lines

AEG-1 was shown to be up-regulated in several kinds of cancers, including oesophageal squamous cell carcinoma (212), gastric cancer (213), HCC (202), NSCLC (215), neuroblastoma (216), breast cancer (217), prostate cancer (203), and renal cell carcinoma (218), compared with normal cells and the matched non-neoplastic tissue. However, data about the expression of AEG-1 in CRC was largely unknown. Therefore, we analysed the AEG-1 mRNA and protein expression in different tissues from CRC patients and colon cancer cell lines in **paper I** (309).

The AEG-1 protein localisation and expression was evaluated by IHC in CRC patient samples from the distant normal and adjacent normal mucosa, primary tumour, and metastasis in the regional lymph nodes and liver. We found cytoplasmic and nuclear AEG-1 staining in all tissues analysed, whereby strong nuclear expression was found in more than half of the tumours, and strong cytoplasmic expression in every third tumour. The AEG-1 protein has been detected before at multiple cell localisations, including cell membrane, cytoplasm, endoplasmic reticulum, nucleus, and nucleolus (200, 201, 203). It was suggested that AEG-1 isoforms, produced by alternative transcription starting sites, splicing and/or posttranslational modifications like ubiquitination, alter the localisation of the AEG-1 protein (201, 203). However, other studies analysing the AEG-1 expression in CRC showed no or only slight nuclear expression (214, 247, 310, 311). The differences in AEG-1 localisation between our study and the other studies performed in CRC, might be due to different antibodies and staining protocols used. These antibodies have different epitopes within the protein and might therefore detect different isoforms of the protein. In addition, we speculated that the different staining pattern might be due to the different ethnical background of the analysed patients (European vs Asian). However, a study by Casimiro *et al.* (312) analysed the AEG-1 protein expression in a Portuguese CRC population and found only a low percentage (<5%) of CRC cases with nuclear AEG-1 expression. Further studies are needed in order to clarify whether the different localization of AEG-1 might play a different role in tumorigenesis.

The AEG-1 expression did not differ between the distant normal mucosa and the adjacent mucosa, neither in the cytoplasm nor in the nucleus, and was therefore considered as one group

for further analyses to increase the sample size. The AEG-1 protein expression in the primary tumour was higher compared with the normal mucosa, in both the cytoplasm and in the nucleus. To evaluate whether the increased AEG-1 protein expression in the tumour is due to increased AEG-1 transcription, we analysed the AEG-1 mRNA expression in 156 primary tumours, and the corresponding normal mucosa from fresh frozen CRC patient samples. The results showed a significant increase of the AEG-1 mRNA expression in the primary tumour compared with the corresponding normal mucosa, in accordance with reports in HCC (202), and CRC (247, 310). AEG-1 up-regulation both at mRNA and protein level in CRC indicates that the oncogenic function of AEG-1 is likely related to transcriptional up-regulation. Furthermore, AEG-1 protein expression was higher both in lymph node metastasis and in liver metastasis, compared with the primary tumour.

In **paper III**, we examined the AEG-1 protein expression and localisation by IHC in a cohort of rectal cancer patients, who participated in the randomised Swedish rectal cancer trial of preoperative RT. The samples included tissues from the distant and adjacent normal mucosa, primary rectal adenocarcinoma, and lymph node metastasis. Among the 158 patients analysed, 83 underwent surgery alone and 75 received RT followed by surgery. For this study, we used a different anti-AEG-1 antibody as in **paper I**, the reason for this change will be discussed later. This antibody has been shown by Western blot to detect only two bands at 65 kDa and 75 kDa. AEG-1 expression was detected in the cytoplasm in all the sites, but nuclear expression only in some samples from the distant normal mucosa and the adjacent normal mucosa. Similar to the results obtained in **paper I**, we found in both the non-RT and RT group that the AEG-1 expression in the primary tumour was significantly higher compared with the distant normal mucosa and with the adjacent mucosa. AEG-1 expression in the lymph node metastasis was elevated compared with the primary tumour, although the difference did not reach statistical significance.

In **paper III**, we also compared the AEG-1 protein expression in five colon cancer cell lines *versus* a normal colon cell line and a colon fibroblast cell line by Western blot. The AEG-1 expression was higher in three cancer cell lines compared with the normal cell line and the fibroblast cell line, sustaining the up-regulation of AEG-1 in cancer. To further evaluate the impact of the AEG-1 expression on metastasis, we analysed the mRNA and protein expression levels in the SW480 primary colon cancer cell line and its lymph node metastatic counterpart SW620, as well as from the KM12C primary colon cancer cell line and its experimental metastatic counterpart KM12SM and KM12L4a cell lines in **paper I**. The SW620 cell line had higher AEG-1 mRNA and protein expression compared with the SW480 cell line. On the

contrary, there was no statistically significant difference in the AEG-1 mRNA and protein expression among the KM12C, KM12SM and KM12L4a cell lines. The bands detected by Western blot were at 65 kDa and 75 kDa in all of the cell lines as well as a band around 35 kDa in the SW480 and KM12C cell lines. The varied bands might be due to alternative transcription starting sites, splicing and/or posttranslational modifications like ubiquitination (201, 203). To our knowledge, no other study analysing AEG-1 in CRC has found a band as low as 35 kDa by Western blot and that might be an explanation for the different staining patterns found by IHC (214, 247, 310). The higher expression of AEG-1 found in samples from lymph node and liver metastases in both patients and cell lines confirms the importance of AEG-1 in CRC metastasis, and is in line with other studies performed in CRC (214, 247, 310). Taken together, the AEG-1 mRNA and protein expression are increased in CRC compared with the normal mucosa, independently of RT, and further elevated in metastatic lesions. These results suggest an important role of AEG-1 in CRC development and metastasis.

***MTDH* genetic variants in CRC patients and colon cancer cell lines**

As we showed in **paper I** and **III**, AEG-1 is involved in CRC development. Several studies have established AEG-1 as an oncogene, and different mechanisms have been suggested to explain the up-regulation of AEG-1 in cancer (223). Moreover, many oncogenes involved in colorectal carcinogenesis have been shown to have genetic alterations, including point mutations, insertions, deletions, and gene amplification (9, 313, 314). However, it is unknown whether mutations in the *MTDH* gene appear during carcinogenesis.

We therefore determined in **paper II** the frequency and the spectrum of *MTDH* variants by direct Sanger DNA sequencing of the complete coding sequence of the *MTDH* gene in 356 tumour samples from CRC patients. We found 50 single nucleotide variants, eight of the variants were exonic and 42 were in a non-coding region adjacent to an exon. No variant was detected in the SW480, SW620 and HCT116 cell lines. Among all the variants, there were four novel exonic variants (c.533delA, c.977C>G, c.1340dupA and c.1731delA) and 25 novel intronic variants adjacent to exons.

The impact of the intronic variants is difficult to evaluate. Introns are non-coding regions between the exons that can have multiple functions. They can contain *cis*-regulatory elements that control the transcription of nearby genes, splicing regulatory elements and/or non-coding RNA elements, while they can also be without function (315, 316). The determination of these elements and the impact of mutations is very challenging.

Among the eight exonic variants, four were missense (c.160G>A, p.V54M; c.232G>T, p.A78S; c.949A>G, p.T317A, and c.977C>G, p.T326S), one silent (c.1353G>A, p.K451K), and three frame shift mutations (c.533delA, p.N178Tfs34; c.1340dupA, p.K448Efs7, and c.1731delA, p.A578Pfs29). To evaluate whether the exonic variants had an effect on the protein structure or function, six *in silico* prediction tools were used. The *in silico* prediction analyses revealed that four of these variants c.232G>T (p.A78S), c.533delA (p.N178Tfs34), c.1340dupA (p.K448Efs7) and c.1731delA (p.A578Pfs29), were deleterious. The variants, c.533delA (p.N178Tfs34), and c.1340dupA (p.K448Efs7), lead to a truncation of the protein, while the variant, c.1731delA (p.A578Pfs29), is predicted to lead to protein prolongation. The frameshift mutations were all heterozygote and only detected each in one patient. Even though the three-dimensional structure of AEG-1 is not completely solved, a transmembrane domain, three putative nuclear localisation signals as well as several protein interaction regions have been identified (199, 203). The variant, c.232G>T (p.A78S), found in 35 patients, is located in the extended nuclear localisation region between aa 78 and 130, that has been shown to regulate the nucleolar localization of AEG-1, and in the YY1, BCCIP and PLZF binding region (203, 211). The variant, c.160G>A (p.V54M), even though not predicted to be deleterious, is located in the transmembrane domain, which spans the aa 51 to 72, as well as in the in the CBP and PLZF binding region (211). Transmembrane domains consist of hydrophobic aa that can interact with the fatty acyl groups of the membrane lipids and anchor the protein to the membrane (8). The variant, c.160G>A (p.V54M), leads to an amino acid substitution from Valine to Methionine, which contain both hydrophobic site chains, thus the effect on the membrane interaction might be at most marginal. However, it would be interesting to analyse, whether the two missense variants have an impact on the protein function and localisation. The exonic variants, c.949A>G, p.T317A and c.977C>G, p.T326S, are not predicted to be deleterious and are not located in any of the described functional regions of the AEG-1 protein. We also analysed the corresponding normal mucosa, whenever available, to be able to identify whether the exonic mutations are hereditary or appeared during carcinogenesis. Only the variant, c.533delA (p.N178Tfs34), was not detected in the corresponding normal mucosa and we therefore considered it as a somatic mutation. The exonic variants, c.160G>A (p.V54M), c.232G>T (p.A78S), c.949A>G (p.T317A), c.977C>G (p.T326S) and c.1353G>A (p.K451K), were also detected in the corresponding normal mucosa and therefore considered to be germline mutations.

No conclusion could be drawn about impact of *MTDH* mutations on the AEG-1 mRNA or protein expression because of the low frequency of mutations and the small amount of matching cases.

Taken together, **paper II** was the first study to analyse *MTDH* mutations in tumour tissue, and 29 novel *MTDH* variants were detected. Three frameshift variants in tumour tissue were probably pathogenic, and two variants were detected in functional protein regions. The only variant found exclusively in the tumour was the variant, c.533delA, suggesting that mutations in the *MTDH* gene are not frequently acquired during colorectal carcinogenesis.

AEG-1 alterations in relation to clinicopathological and biological variables

The variants, c.232G>T (p.A78S), c.382-50C>T, c.1048+131T>G and c.1353G>A, were more frequent in patients <72 years-old compared with the patients ≥72 years-old. The intronic variant, c.1048+82delA, was only detected in tumours located in the colon but not those located in the rectum. Interesting, in **paper I** we found a lower AEG-1 mRNA expression in the colon compared with the rectum, but whether the variant c.1048+82delA influences the transcription or mRNA stabilization has to be experimentally validated. The significant difference in *MTDH* mutational status and mRNA expression between the colon and the rectum supports the idea of different diseases. However, the protein expression of AEG-1 did not differ between the colon and the rectum in **paper I**, and no other study analysing the AEG-1 protein expression in CRC has found a difference between the two locations (214). No further correlation between *MTDH* genetic variants and clinicopathological variables were found in **paper II** in agreement to a study conducted in breast cancer by Liu *et al.* (220).

In **paper I**, AEG-1 mRNA and protein expression decreased from stage I to stage III tumours and was increased in stage IV compared with stage III tumours. We speculated that subclones with high AEG-1 expression within the stage III tumours might invaded into the lymph nodes, leaving a higher amount of low-expressing AEG-1 cells in the primary site. Furthermore, we found that the AEG-1 expression was higher in lymph node metastases compared to stage III tumours. However, no difference of the AEG-1 protein expression among the rectal cancer patients with different stages, in both unirradiated and radiated treatment groups were observed in **paper III**. Other studies analysing the AEG-1 expression in CRC found a gradual increase of the expression from stage I-IV tumours, and a higher expression of tumours with metastases in the lymph nodes compared with tumours without metastases (214, 247, 310, 311).

High AEG-1 protein expression was correlated to a worse patient survival in several cancer types, including CRC (202, 203, 212-218), however, we could not find a correlation between the AEG-1 mRNA or protein expression and the patient survival in **paper I**. We hypothesized that the differences between the studies might be due to the different antibody, staining protocols and/or grading systems used in our study compared with the other studies conducted on CRC. Therefore we changed the antibody in **paper III** to a commonly used anti-AEG-1 antibody as described earlier. However, with the changed antibody, we could not find a correlation of the AEG-1 expression with the patient survival in the untreated rectal cancer patients. It remains unclear as to why we could not find a correlation of the AEG-1 protein expression to the patient survival. We used two patient cohorts and two anti-AEG-1 antibodies, though several factors of biological and technical nature can influence the results of IHC, and might be the reason for the discrepancy between our studies and the other studies performed in CRC, such as the study populations, the follow-up processes of the patients, the quality of the samples, the staining protocol and the grading systems.

The patient cohorts analysed in **paper I** and **paper III** have been used to evaluate multiple proteins at our laboratory in the past. Analyses between the AEG-1 protein expression and the expression of the multiple biological factors revealed several correlations. In **paper I**, cytoplasmic AEG-1 protein expression in the primary tumour was positively correlated with the expression of phosphorylated NF- κ B/p65 at Ser 536, p73, and Rad50, as well as with the apoptotic rate analysed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Nuclear AEG-1 expression was also positively correlated with phosphorylation of NF- κ B/p65 at Ser 536, p73, and Rad50, but not to the apoptotic rate. It has been reported that the phosphorylation of NF- κ B/p65 at Ser 536 is induced by the IKK α and β , which are activated by TNF- α , leading to p65 translocation to the nucleus (317). One of the initial studies on AEG-1 showed already that the expression increases upon TNF- α treatment (198). Later, treatment with TNF- α showed to induces AEG-1 phosphorylation at Ser298 by the kinase IKK β (210). Furthermore, TNF- α treatment or AEG-1 up-regulation increased the interaction with the NF- κ B p65 subunit and nuclear translocation (198, 204, 205, 210). We could confirmed the increased AEG-1 expression and translocation to the nucleolus upon TNF- α treatment in the SW480 cell line (unpublished data).

Based on the correlation between AEG-1 and these factors, we speculated in **paper I** that AEG-1 might be involved in a p53 independent apoptotic pathway, whereby AEG-1 interacts with NF- κ B/p65 leading to p73 activation and Noxa induction after genotoxic treatment (318). ATM, together with NF-kappa-B essential modulator (NEMO), degrades I κ B α and activates

NF- κ B in response to DNA-damage, while Rad50, a member of the MRN-complex, is essential for ATM activation (319, 320). So far no other study could find a correlation between AEG-1 and p73 or Rad50. Several factors, however, make it unlikely to detect such a complex signalling by IHC in an unselected patient cohort. The suggested apoptotic pathway was believed to be activated by genotoxic treatment in a p53 independent manner. Since around 50% of all CRC patients have a mutated *TP53* gene, the suggested pathway would only be active in half of the patients; unfortunately, we do not have any information about the *TP53* status in our sample set (59). Additionally, patients with low stage tumours did not receive neoadjuvant genotoxic treatment and underwent surgery directly. That significantly reduces the number of patients with tumours that fulfil the framework for the suggested pathway. Besides, the time interval from the last day of the treatment until the day of surgery is unknown to us, but based on the clinical practise, it can take several days to weeks. The expression of p73, however, has been shown in cell line studies to increase shortly after genotoxic treatment like radiation, but does not sustain over several days and the protein expression of Rad50 did not alter upon DNA damage in several cell line models (206, 321-323). Furthermore, there was only a small number of matching cases with the data about expression of AEG-1 and p73 and the apoptotic rate. The proposed pathway remains therefore highly speculative and the reason for the correlations found have to be experimentally validated in further studies.

In the sample set of the patients that participated in the randomised Swedish rectal cancer trial of preoperative RT analysed in **paper III**, different factors have been analysed, but data about phosphorylation of NF- κ B/p65 at Ser 536, p73, Rad50, and the apoptotic rate were not available. In this sample set AEG-1 was negatively correlated with the Mac30 expression and positively with the Ki-67 expression in the non-RT group. Furthermore, AEG-1 was positively related to the expression of FXD domain containing ion transport regulator 3 (FXD-3) and Lysyl oxidase (Lox), only in tumours treated with RT. High AEG-1 expression was related to Livin expression in both the non-RT and RT groups. None of these factors have been found before to correlate with AEG-1 and the nature for these correlations remains to be experimentally validated.

Taken together, the results from **paper I, II, and III**, show that *MTDH* variants, AEG-1 mRNA and/or protein expression in untreated patients did not correlated to the survival. AEG-1 protein expression positively correlated with phosphorylated NF- κ B/p65 at Ser 536 confirming the interaction of these two proteins as previously reported.

AEG-1 and RT

Evaluation of AEG-1 as a radiosensitising target

AEG-1 has been shown to mediate chemoresistance in HCC and breast cancer cells (202, 224). However, whether AEG-1 is involved in radiation response has only been validated in the cervical cancer cell line SiHa, where AEG-1 knockdown resulted in a decreased survival, G2/M phase arrest, and apoptosis after radiation (253). In **paper III**, we down- and up-regulated the AEG-1 expression in five colon cancer cell lines, and measured the survival after radiation by clonogenic assay. The KM12L4a, SW480, and SW620 cell lines, showed the highest survival after radiation and are also often regarded as radioresistant cell lines (324-326). Transient AEG-1 knockdown in those cell lines lowered survival after 2 Gy radiation compared with the negative control cells. In the KM12C cell line, the survival increased in the AEG-1 knockdown cells after 2 Gy radiation compared with the negative control, whereas no change was seen in the HCT116 cell line. Both of these cell lines had a high radiation sensitivity: only 28% and 33% of the KM12C and HCT116 cells, respectively, were able to form colonies after 2 Gy radiation. These results were confirmed in stable AEG-1 knockdown cells of SW480, SW620, and HCT116 the cell lines. Interestingly, among the tested cell lines the KM12C and HCT116 cell line had the highest endogenous AEG-1 protein expression. However, AEG-1 up-regulation had no effect on the clonogenic survival of the KM12C and KM12L4a cell lines. Although the predictive role of AEG-1 in the rectal cancer patients has to be further investigate, knockdown of AEG-1 can sensitize resistant colon cancer cell lines to radiation.

Potential of AEG-1 as a prognostic marker after RT

Despite improved treatment protocols and surgical methods, the frequency of local recurrence and distant recurrence in rectal cancer after neoadjuvant and adjuvant treatment is still very high (327). The cytotoxic effect of the current anti-cancer treatments are not specific for the tumour mass and are accompanied by several side effects, which might even lead to the patient's death (328-330). Nowadays, the most challenging task for the oncologists is to diminish the health risks, improve the quality of life, and to eradicate the tumour. Therefore, there is a need for new and more valuable prognostic markers to improve the patient outcome after the primary treatment. We analysed the AEG-1 expression in a patient cohort from the Swedish rectal cancer trial of preoperative RT in **paper III** (156). As mentioned before, we could not find any correlation between AEG-1 and the patient survival or recurrence in the

non-RT patients, or a difference in AEG-1 expression between the non-RT and RT patient groups. In the group of patients treated with RT, however, we found that tumours with a high AEG-1 expression had a higher risk of developing recurrence, especially distant recurrence, compared with the tumours with low AEG-1 expression. Furthermore, high AEG-1 expression was related to worse disease-free survival. A similar trend was obtained when we analysed the all-cause mortality and the cancer-specific mortality. Stratified log-rank analyses showed that the statistical significance remained, independent of the tumour stage. However, the group of patients with AEG-1 negative tumours was very small, with only 13 patients in the RT group, and further studies are needed to confirm these findings in a larger patient cohort. In summary, the increased AEG-1 protein expression was independently related to high risk for distant recurrence and worse disease-free survival in rectal cancer patients treated with preoperative RT.

Impact of AEG-1 in radiation-enhanced metastasis

The impact of AEG-1 on invasion, migration, and metastasis has been shown in several studies for different kinds of cancers, and we found in **paper I** that the expression of AEG-1 was increased in liver metastases compared with the primary CRC tumours (109, 204, 224, 247). Hence, we speculated that the increased distant recurrence rate after radiation in high AEG-1 expressing tumours found in **paper III** could be due to the metastasis promoting properties of AEG-1. In **paper IV**, we therefore analysed the involvement of AEG-1 in migration and invasion in colon cancer cell lines by Boyden chamber assay. Stable AEG-1 knockdown cells had a lower migration compared to the negative control cells in the SW480, SW620, and HCT116 cell lines. Similar to the migration, stable AEG-1 knockdown cells of all three cell lines had a lower invasion compared with negative control cells in all the analysed cell lines. These results confirmed the involvement of AEG-1 in colon cancer cell migration and invasion, as previously observed by Zhang *et al.* (247). However, the question remains why AEG-1 correlated with distant recurrence, only in the patients treated with RT but not in the untreated patients. In **paper III**, we analysed the AEG-1 expression by tissue microarray in three sections taken from different localisations of each tumour block. However, a tumour is a heterogeneous mass of different cell populations, and it might be possible that the samples from the non-treatment group underrepresent the tumour heterogeneity and AEG-1 negative graded tumours actually contain positive AEG-1 cells that facilitate metastasis (331). In the RT group, however, radiation acts as a selection pressure in a similar way as described by the Darwinian evolution

theory, and lowers the tumour heterogeneity (331-333). In this group, the samples from the tissue microarray might be more representative for the entire tumour and negative graded tumours do not have positive colonies. Since we only found a small amount of negative AEG-1 expressing tumours for both groups, this effect might have a big impact on our sample set. A larger sample group might correct for the tumour heterogeneity.

Furthermore, it might be possible that radiation induces metastasis and AEG-1 is essential for this process in rectal cancer. It has been shown earlier that radiation changes the tumour environment and enhances metastasis *in vitro*, as well as *in vivo*, by different mechanisms (170).

We therefore evaluated in **paper IV** whether radiation enhances migration and invasion in colon cancer cells, and whether AEG is involved in radiation-enhanced migration and invasion. Data about radiation-enhanced migration and invasion in colon cancer are rare and inconclusive. It was shown that cellular migration was diminished in the HCT116 cell line in a dose dependent manner in two different studies (334, 335). Another study showed that out of three colon cancer cell lines, only the HCT116 cell line had an increased matrigel invasion upon 4 Gy radiation (336). However, we found, a significant increase of migration upon radiation in the SW480, SW620 and HCT116 cell lines. Additionally, we found that the invasion increased upon radiation in the SW480 cell line. To evaluate whether AEG-1 is involvement in radiation-enhanced migration and invasion, we evaluated the migration of the SW480 and HCT116 AEG-1 knockdown and negative control cells upon radiation treatment. The results showed a significant increase of migration in the negative control cells for both SW480 and HCT116 cell lines, similar to the non-transfected cells. Radiation did not increase the migration of the HCT116 AEG-1 knockdown cells, while the migration increased in the SW480 AEG-1 knockdown cells upon radiation, suggesting independent pathways involved in radiation-enhanced migration. Since radiation-enhanced invasion was only seen in the SW480 cell line, we evaluated the impact of AEG-1 knockdown on radiation-enhanced invasion in the SW480 AEG-1 knockdown and negative control cells. The invasion in the AEG-1 knockdown cells was only slightly increased upon radiation without reaching significance, while a significant increase in invasion upon radiation was observed in the negative control cells. This was confirmed in different AEG-1 knockdown clones with the same AEG-1 shRNA and with a different AEG-1 shRNA. To evaluate whether the reduced amount of migrating and invading cells upon radiation was due to reduced cell viability, we performed a WST-1 proliferation assay. No difference in cell viability, 24 h after radiation, was observed in the AEG-1 knockdown cells compared with the negative control cells for both the SW480 and HCT116

cell lines. Taken together, these findings support our hypothesis that AEG-1 is involved in radiation-enhanced migration and invasion, and might explain the findings in the patient material from **paper III**.

The zebrafish as a novel model to study radiation-enhanced invasion

To further investigate the process of radiation-enhanced invasion, we developed an embryonic zebrafish model in **paper IV**, for testing the effects of radiation on early stages of tumour cell invasion and dissemination. The dissemination of SW480 cells in the tail of the fish increased in a dose dependent manner, with a significant increase after 10 Gy radiation showing that the zebrafish model is a great supplementation to the already existing models to study early events in radiation-enhanced invasion. Next, we injected the SW480 AEG-1 knockdown and negative control cells into the zebrafish embryo and radiated them with either 0 Gy or 10 Gy. As for the non-transfected cells we found in the SW480 negative control cells a significant increase in the disseminated cells into the tail upon radiation. However, only a small and non-significant increase was observed upon radiation for the SW480 AEG-1 knockdown cells. The results from the zebrafish embryo model confirm our results *in vitro*, whereby the invasion can be enhanced by radiation, and AEG-1 knockdown can inhibit this process.

MMP secretion and expression in relation to the AEG-1 expression and radiation

It has been reported that both MMP-2 and MMP-9 are up-regulated after radiation in HCC and glioblastoma (185, 188). Furthermore, it has been shown that AEG-1 knockdown inhibits invasion and decreases the MMP-9 expression in colon cancer cell lines (337). To evaluate whether MMPs are activated via AEG-1 after radiation, we performed a cytokine antibody array including MMPs and MMP inhibitors, growth factors and receptors, and EMT marker to evaluate possible downstream effectors of AEG-1. The results showed that MMP-8, MMP-9, and EGF increased slightly, or did not change upon radiation in the SW480 negative control cell line while they were decreased in the irradiated SW480 AEG-1 knockdown cells. To evaluate whether AEG-1 influences the MMP-9 secretion via the mRNA expression, we analysed the MMP-9 mRNA expression levels after radiation. Indeed, the MMP-9 mRNA levels were decreased in the SW480 AEG-1 knockdown cells compared with the negative control cells in both the unirradiated and radiated samples. Radiation slightly increased the MMP-9 expression in the negative control cell line, whereas there was no change in the AEG-1

knockdown cell line. It was previously shown that radiation activated MMP-9, and binding of AEG-1 on the MMP-9 promotor involves NF- κ B (188, 338). Based on our results, we speculate that AEG-1 inhibits the MMP-9 expression and thereby influences the radiation enhanced invasion. However, it remains to be evaluated whether radiation and AEG-1 also influence the activation of MMP-9 in the intercellular space and whether other factors are involved in AEG-1 mediated cell invasion upon radiation.

Conclusions

Based on the findings in paper I-IV, the following conclusions can be drawn:

- AEG-1 is upregulated on the mRNA and protein level during CRC development (**paper I** and **paper III**)
- Genetic variants of the *MTDH* gene are probably not of high clinical importance in CRC (**paper II**)
- The AEG-1 protein expression is a promising marker to discriminate rectal cancer patients treated with preoperative RT regarding the risk of distant relapse (**paper III**)
- AEG-1 is a promising radiosensitising target in CRC (**paper III**)
- AEG-1 knockdown inhibits migration and invasion, as well as radiation-enhanced cell migration and invasion in colon cancer, perhaps via MMP-9 down-regulation (**paper IV**)
- The zebrafish model can be used to study early events in radiation-enhanced invasion (**paper IV**)

Future perspectives

Hundreds of laboratories around the globe participate in the chase for predictive and prognostic biomarkers to improve the management of cancer. A multiplicity of molecules have been identified for treatment prediction and patient prognosis by several approaches. Unfortunately, the number of markers that have been applied for clinical practices is rather small, even though a need for such applications is immense. The main reason for this unbalance is the rigorous analytical and clinical validation of the markers. In our study, many questions remain to be answered about the impact of AEG-1 in CRC development and radiation response.

- We found a different staining pattern of AEG-1 by IHC in the CRC patients compared with other studies. This is probably because of the detection of several AEG-1 isoforms by using different staining protocols and antibodies. It would be interesting to evaluate the exact reason for the different staining pattern and furthermore, the impact of AEG-1 isoforms in tumourigenesis and treatment response.
- Although we showed that exonic *MTDH* variants are rare in CRC, it would be interesting to further investigate the protein localisation, protein-interaction, and signalling of these variants and whether they might influence the function of AEG-1 in carcinogenesis and/or treatment response.
- We showed that high AEG-1 expression correlates with worse prognosis only in patients treated with RT. Since the number of patients with low AEG-1 expression was very small in both groups, it is necessary to confirm the results in a larger cohort. Moreover, the treatment regime for rectal cancer has changed since the Swedish rectal cancer trial of preoperative RT was conducted. Therefore, analyses of the AEG-1 expression in a more recent clinical trial, for example the Stockholm III trial, would be needed to determine the value of the AEG-1 expression as a prognostic marker for rectal cancer patients.
- AEG-1 has been shown to be involved in radiation-enhanced migration and invasion *in vitro* and *in vivo*, and MMP-9 has been found as a possible downstream target. However, it remains to be solved in which signalling cascade AEG-1 is involved upon radiation, leading to increased migration and invasion, and whether AEG-1 knockdown also decreases the MMP-9 activity.
- Several studies have shown that AEG-1 would be a good target for cancer treatment and it has been discussed a long time to develop specific inhibitors for AEG-1.

However, even though the crystal structure has not been solved, it does not seem that AEG-1 is a classical druggable target. No catalytic unit has been discovered and the classical binding-pocket approach cannot be applied, though it has been suggested that AEG-1 acts as a bridging factor. It might be more likely that a successful attempt to target AEG-1 is to disrupt the protein-protein binding with an interaction partner. Therefore, further structural analyses of protein complexes are needed to design specific inhibitors and inhibit the AEG-1 mediated oncogenesis.

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