Prostanoids and Colorectal Cancer

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To Klara and Wilma

ABSTRACT

Tumor disease is a main cause of death in Western countries and a most common malignancy is colorectal cancer (CRC). Growing tumors are dependent on interactions among several different cells as well as signaling pathways. Many tumors display increased expression of the enzyme cyclooxygenase-2 (COX-2) in conjunction with changes in tissue levels of prostanoids. However, COX-2 expression is usually unevenly distributed among cells in tumor tissue and several cell clones display little or no COX-2 expression. A frequent change of prostanoid metabolism in CRC is increased PGE_2 production, which appears to be involved in several different steps of tumor progression. Prostanoids bind to receptors on cell membranes with subsequent activation of different intracellular signaling pathways. Therefore, a general aim of this work was to evaluate changes in expression of prostanoid receptors and related factors involved in prostanoid metabolism in human CRC suggesting possible specific targets for interventions on prostanoid metabolism to attenuate progression. This aim was partly performed by analyses with realtime-PCR of tumor and normal colon tissue samples from human CRC obtained at surgery. Uneven distribution of COX-2 expression, as confirmed by IHC, could hypothetically be explained by gene silencing following DNA methylation. Therefore, methylation analysis of the COX-2 promoter was also performed. Furthermore, our patients received short-term pre-operative treatment with non-selective COX-inhibition (indomethacin) to evaluate changes in gene expression related to prostanoid levels determined by microarray.

Prostanoid receptor expression was decreased in tumor tissue and reduced concentration of prostanoids had no negative effect on tissue expression of most prostanoid receptors. By contrast, tumor tissue expression of the EP_2 subtype receptor showed negative prediction of patient survival. Methylation of COX-2 promoter sequences did not explain the lack of COX-2 expression in tumor tissue cells. Short-term pre-operative treatment with indomethacin was followed by pronounced alterations of gene expression in both tumor and normal colon tissue. Several differences in expression of genes known to regulate COX-2 expression, including transcriptional factors, occurred in relationship to COX-2 in tumor tissue. Our observations suggest that prostanoid metabolism is complex in CRC and involves several hundred genes in different cell types. Overall, alterations in prostanoid metabolism are related to tumor stage progression as supported in different studies. These observations may offer therapeutical targets in addition to treatment with conventional COX inhibitors for chemoprevention of CRC, since such long-term treatment may be associated with considerable side effects in patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är en av de vanligaste dödsorsakerna i västvärlden. Begreppet cancer innefattar ungefär 200 olika sjukdomar där tjocktarmscancer är en av de vanligaste, som drabbar omkring 5000 svenskar varje år. Män och kvinnor drabbas i ungefär lika stor utsträckning och samband finns mellan tjocktarmscancer och livsstil samt till inflammationssjukdomar i tarmen. En tumör kan uppkomma i flera olika vävnader; i tjocktarmen är det framför allt i epitelcellagret (skiktet närmst tarminnehållet) som tumören har sin uppkomst. I en tumör finns det ett flertal olika celltyper utöver de maligna epitelcellerna, bland annat celler från immunförsvaret. Cellerna meddelar sig med varandra och påverkar varandra genom olika signalsubstanser. Några av dessa substanser benämns prostanoider, som är fem olika signalsubstanser som bildas med hjälp av två olika varianter av enzymet Cyclooxygenas, COX-1 och COX-2, från fettsyran Arakidonsyra. Dessa prostanoider förekommer ofta i större utsträckning än normalt i tumören. Studier har visat att genom att sänka halten prostanoider i tumören med en blockering av enzymet COX minskas tillväxten och spridningen. Men prostanoiderna är inblandade i en mängd normala fysiologiska processer i kroppen. En blockering medför därför biverkningar som exempelvis blödande magsår och hjärtproblem. Det är därför viktigt att hitta faktorer nedströms COX för att få en mer specifik behandling med färre biverkningar.

Prostanoiderna påverkar tumören genom att binda till receptorer på cellernas yta. Dessa receptorer kan vara lämpliga som behandlingsmål och därför har vi valt att studera skillnader i uttryck av prostanoid receptorer. Vi har använd oss av normal tjocktarm och tumörvävnad erhållit från operation av tjocktarmscancerpatienter samt från tjocktarmscancerpatienter som behandlats med en COX hämmare tre dagar innan operation. Skillnader i uttryck har studerats genom att mäta variationer i mängd RNA i de olika vävnaderna. RNA är steget mellan generna och proteinet och mäts framför allt med analysmetoden PCR. Resultaten tyder på att receptorerna minskar i antal i tumörvävnaden, men också att en av receptorerna (EP_2) är relaterad till överlevnad hos patienterna. Med en COX hämmande behandling innan operation kunde vi se en förändring av receptor uttryck samt en mängd andra gener. Detta analyserades med en avancerad DNA-teknik där hela det mänskliga genomet studeras samtidigt. Denna metod användes också för att analysera skillnader mellan tumörer med en hög halt COX-2 jämfört med tumörer med en låg halt COX-2. Detta är intressant då en hög halt COX-2 försämrar överlevnaden hos patienterna. Mängden COX-2 i tumörer varierar mellan patienter, men kan även vara olika inom en och samma tumör. För att klargöra variationen av COX-2 halten studeras regleringen utav COX-2 genen, vilket visade förändringar i många faktorer. Sammantaget visar resultaten i denna avhandling att ett flertal faktorer som påverkar prostanoid metabolismen är förändrade vid tjocktarmscancer.

ORIGINAL PAPERS

This thesis is based on results reported in the following papers, which will be referred to in the text by their roman numerals.

I **Gustafsson A**, Hansson E, Kressner U, Nordgren S, Andersson M, Wang W, Lönnroth C and Lundholm K. (2007) EP1-4 subtype, COX and PPARgamma receptor expression in colorectal cancer in prediction of disease-specific mortality. *International Journal of Cancer*: 121, 232-240

II **Gustafsson A**, Hansson E, Kressner U, Nordgren S, Andersson M, Wang W, Lönnroth C and Lundholm K. (2007) Prostanoid receptor expression in colorectal cancer related to tumor stage, differentiation and progression. *Acta Oncologica*; 46: 1107-1112

III **Gustafsson A**, Hansson E, Kressner U, Nordgren S, Andersson M, Lönnroth C, Lagerstedt KK and Lundholm K. (2010) Receptor and enzyme expression for prostanoid metabolism in colorectal cancer as related to tumor tissue PGE2. *International Journal of Oncology*; 36(2):469-78

IV **Gustafsson Asting A**, Carén H, Andersson M, Lönnroth C, Lagerstedt KK and Lundholm K. COX-2 gene expression in colorectal cancer tissue related to regulating factors and promoter methylation status.
Submitted

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ABBREVIATIONS

AA	arachidonic acid
AKT	v-akt murine thymoma viral oncogene homolog 1 / PKB – protein kinase B
AP-1	activating protein 1
AP-2	activating enhancer binding protein 2
APC	adenomatous polyposis coli
ANOVA	analysis of variance
ATF	activating transcription factor
B-ATF	B-cell-activating transcription factor / basic leucine zipper transcription factor, ATF-like
bp	base pair
C	cytosine
cAMP	cyclic adenosine/adenylate monophosphate
cDNA	complementary DNA
CDX-2	caudal type homeobox 2
C/EPB	CCAAT/enhancer binding protein
COX	cyclooxygenase
CpG	cytosine-guanine dinucleotide
CRC	colorectal cancer
CRE/-B	cAMP response element/-binding
CRTH2	Chemoattractant receptor-homologous molecule expressed on TH2 cells, DP2 receptor
Ct	cycle treshold
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxynucleoside triphosphates
DP	D prostanoid
ECM	extracellular matrix
ELK-1	ETS-like gene 1
EP	E prostanoid
ERK	extra-cellular signal-regulated kinase / MAPK1
FAP	familial adenomatous polyposis
FC	fold change
FOS	FBJ murine osteosarcoma viral oncogene homolog
FP	F prostanoid
G	guanine
GAPDH	glyceraldehyd-3-phosphate dehydrogenase
G**D**P/G**T**P	guanosine 5´-**di/tri**phosphate
GPCR	G-protein coupled receptor
GPR44	G protein-coupled receptor 44, DP2 receptor
HNPCC	hereditary non-polyposis colon cancer
HPGD	15-hydroxyprostaglandin dehydrogenase / 15-PGDH
IBD	inflammatory bowel disease
IFN	interferon
IHC	immunohistochemistry
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	interleukin
iNOS	nitric oxide synthase 2, inducible
IP	I prostanoid
IP_3	inositol 1,4,5-triphosphate
JDP	jun dimerization protein
JNK	jun terminal kinase
JUN	jun oncogene
K	kinase
K/H-RAS	Kirsten/Harvey rat sarcoma

5-LOX	5-lipoxygenase
LT	leukotriene
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAPK	mitogen activated protein kinase
MEK	MAPK kinase/ERK kinase
MMP	matrix metalloproteinase / matrix metallopeptidase
mPGES	microsomal prostaglandin E synthase
mRNA	messenger RNA
N	normal colon tissue
NFAT/c	nuclear factor of activated T-cells/cytoplasmic
NF-ϰB	nuclear factor-ϰB
ns	non significant
NSAID	nonsteroid anti-inflammatory drug
p21	protein 21
p53	protein 53
PCR	polymerase chain reaction
PEA3	polyoma enhancer activator 3
PG	prostaglandin
PI3K	phosphoinositide 3-kinases
PKC	protein kinase C
PPAR	peroxisome proliferative receptor
qPCR	quantitative realtime PCR
RAF	rapidly accelerated fibrosarcoma
RIN	RNA integrity number
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
RXR	9-*cis* retinoic acid receptor
SEM	standard error of the mean
SP1	specificity protein 1, transcription factor
T	tumor tissue
TCF-4	transcription factor 4
TNFα	tumor necrosis factor α
TP	T prostanoid
TXA	thromboxane A
U	uracil

INTRODUCTION

Cancer is a main cause of death in Western countries and colorectal cancer (CRC) is one of the most common malignancies. CRC is mainly adenocarcinoma, originating from epithelial cells. A lot of information exists that indicates that cancer is a multistage genetic and epigenetic disease. Therefore, a majority of cancer studies have focused on examining critical genetic changes for tumor progression. In this context, it is important to highlight that malignant tumors consist of heterogenous cellular entities and disease progression is possible due to involvement of several different cell types beyond genetically disturbed cancer cells. Tumors are dependent on interactions between several different cells, as well as signaling pathways [1]. The main subject of this dissertation is based on the suggestion of a link between COX enzymes, i.e. prostanoid production, and tumor progression [2-5]. Several studies have confirmed the involvement of COX enzymes and its metabolites, prostanoids, in colorectal cancer [4, 6-14].

Prostanoids

Prostanoids are a subgroup of eicosanoids and consist of prostaglandins (PGD, PGE, PGF, and PGI) and thromboxane (TXA). Prostaglandins were first discovered in the 1930's during research on reproductive biology by Ulf von Euler. Hence, the name prostaglandin origins from the prostate gland. In 1982, Sune Bergström, Bengt Samuelsson and John Vane received the Nobel Prize in Physiology or Medicine for their research on prostaglandins. Bergström had isolated prostaglandins and elucidated their structure, Samuelsson had elucidated the mechanisms of the biosynthesis and the pathways of prostaglandins metabolism, while Vane discovered prostacyclin and that aspirin inhibited the synthesis of prostaglandins [15].

Prostanoids are bioactive lipids that are important for a large number of normal physiological processes in various tissues. They are involved in relaxation and contraction of smooth muscles, regulation of blood clotting, maintenance of renal homeostasis, modulation of immune responses, inhibiting and stimulating neurotransmitter release, regulating secretion and motility in the gastrointestinal tract as well as protection of the gastrointestinal mucosa [4, 16]. In pathological conditions, prostanoids can promote inflammation, swelling, pain, and fever. Prostanoids in cancer are also known to affect induction of growth factors and enzymes, apoptosis, immunosuppression, angiogenesis, proliferation and invasion of tissues [4].

Synthesis of prostanoids

There are three series of prostanoids based on the number of double bonds in their side chain. Serie 2 prostanoids, with two double-bonds, are predominant in humans since its precursor fatty acid, Arachidonic acid (AA), is abundant in humans [16]. AA is released from cell membrane phospholipids through the action of phospholipase A2 and is converted in three different pathways to either prostanoids, hydroperoxyeicosatetraenoic (HpETEs) or epoxyeicosatrienoic acids (EETs). Prostanoids are produced through the conversion of AA by cyclooxygenase (COX) to the intermediates PGG_2 and PGH_2, which in turn is converted by specific synthases to different prostanoids. The prostanoid produced is depending on cell type and enzymes present in the cell (Fig 1) [17]. PGE_2 is produced by three different isoforms of PGE_2 synthase; cytosolic PGE synthase (cPGES) and two membrane-bound synthases called microsomal PGE_2 synthase-

1(mPGES-1) and microsomal PGE_2 synthase-2 (mPGES-2). Microsomal PGES-1 is an inducible form coupled to COX-2 preferentially compared to COX-1 [18, 19].

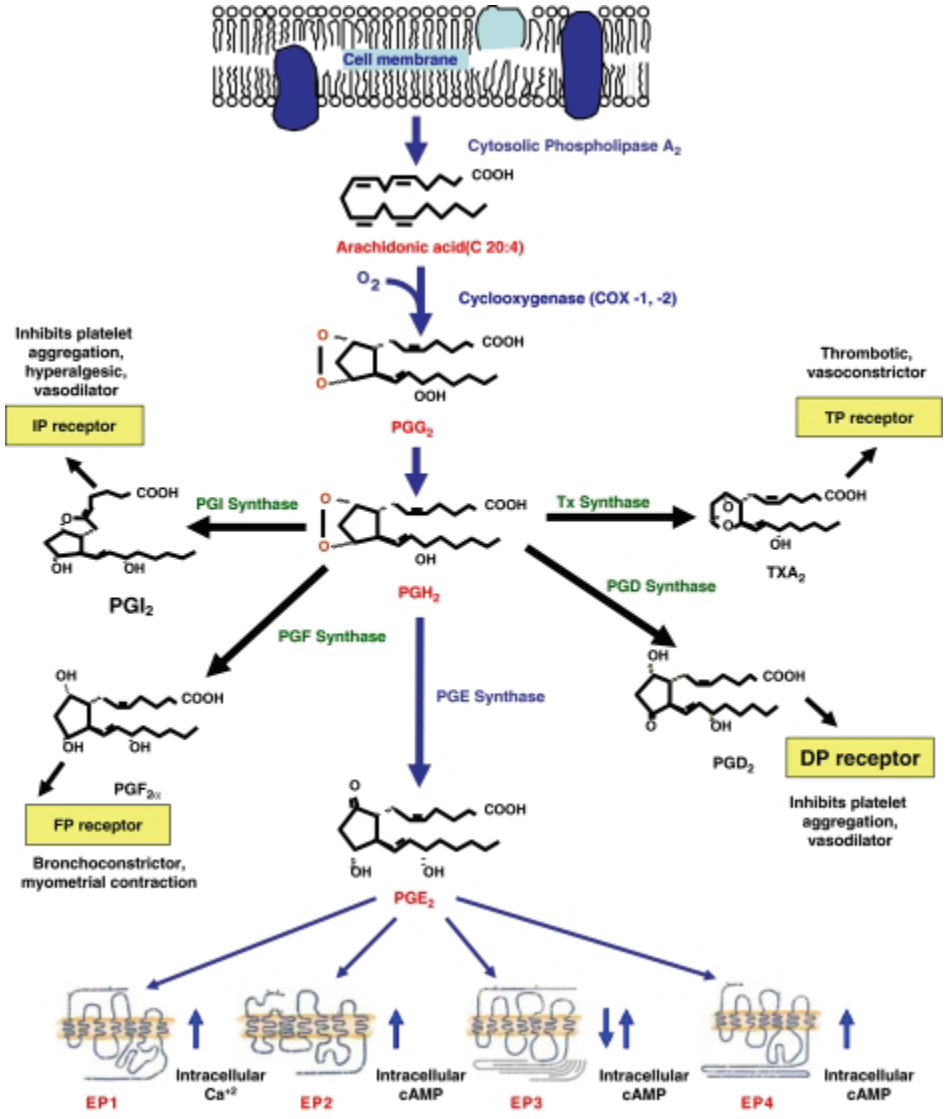

Fig 1. Biosynthesis of prostanoids with corresponding enzymes and receptors. (Adapted by permission from Nature Publishing Group: British Journal of Pharmacology [17], copyright 2006).

Prostanoids need to cross the cell membrane to exert some biological action. It is unclear how this is performed in detail. Different theories are provided about PG transport to the extracellular environment; diffusion driven by pH and the membrane potential, involvement of the multidrug resistance-associated protein 4 (MRP4) and by prostaglandin transporter (PGT) [20-22]. However, prostanoids are chemically unstable molecules (PGG, PGH, PGI and TXA) with approximate half-life of 30 s to a few minutes and even though other PGs are more stable they are metabolized quickly. Therefore, prostanoids are thought to have mainly autocrine and paracrine actions [16]. Metabolic clearance of prostanoids is assumed to be a two-step process [23, 24]. First, prostanoids need to be transported across the cell membrane to be degraded,

which is an energy-dependent uptake performed by PGT [22]. PGT is broadly expressed in many cell types and co-ordinately regulated with COX [25]. Then, degradation occur inside the cell by the enzyme 15- hydroxyprostaglandin dehydrogenase (HPGD), which has little or no action in human blood [20, 23].

Cyclooxygenase

There are two known genes located on different chromosomes that produce COX; *PTGS1* on chromosome 9 and *PTGS2* on chromosome 1 [26]. The simple view of the two COX genes is that *PTGS1* is constitutively transcribed to COX-1 and *PTGS2* is inducible transcribed to COX-2. However, new findings imply a constitutive expression of COX-2 in brain and renal tissue as well as a contribution to inflammation by COX-1 derived PGs [27]. The difference between the transcription of the two genes depends on the promoter where *PTGS2* (COX-2) is tightly regulated by transcription factor response elements including a TATA-sequence, a NF-IL-6 motif, two AP-2 sites, three SP1 sites and two NF-$\varkappa$B sites, a CRE motif, and an E box that can be stimulated by growth factors and cytokines [28-30]. The activation of intracellular signaling pathways induces the recruitment of specific transcription factors to promoter elements which trigger *PTGS2* transcription. By contrast, the promoter for *PTGS1* (COX-1) has similarities with housekeeping genes, rich in guanine (G) and cytosine (C) [31].

There are several known polymorphisms in *PTGS2*, but most of them seem to have no effect on transcription of the gene [32-35]. However, one known polymorphism (-765G>C) in the *PTGS2* promoter region disrupts the binding site of stimulatory protein 1 (SP1) resulting in reduced promoter activity with 30 % [36]. This polymorphism (-765G>C) has been reported to be over-represented in patients with gastric adenocarcinoma [37]. Epigenetic and post-transcriptional modulation as well as regulation at protein level may influence the expression of COX-2 [38].

Prostanoid receptors

Prostanoid receptors are G-protein coupled receptors (GPCR) that transverse the cell membrane with a seven-transmembrane α-helix structure. Each of the prostanoid receptor show selective ligand-binding specificity, but the affinity varies [16]. Some cross-binding may occur. For example, EP_1 receptor has the highest affinity for PGE_2 but could bind $PGF_{2\alpha}$ as well as PGD_2 [39]. Ligand binding to prostanoid receptors causes a conformational change in the GPCR that allows it to act as a guanine nucleotide exchange factor, which activates G proteins by exchanging GDP for GTP.

Different prostanoid receptors are associated with different G-proteins in their carboxy tail and hence, activate different signaling pathways. Alternative splicing of the carboxy-terminal region after the seventh transmembrane domain can occur and creates various receptor isoforms. Today, eight human EP_3 receptor isoforms have been identified that differ only in their carboxy tail and have almost identical ligand-binding specificities [40]. Alternative splicing may impact on G-protein coupling specificity and thereby activation of signaling pathways [41]. The two main signaling pathways activated by GPCRs are the cAMP and the Phosphatidylinositol (IP_3/DAG) signaling pathway [42].

Derivates of prostanoids activate nuclear receptors called peroxisome proliferative receptors (PPARs). Three subtypes have been identified; PPARα, PPARγ, and PPARδ. These receptors are ligand-activated transcription factors that act as heterodimers with 9-*cis* retinoic acid receptor (RXR) and regulate transcription. The receptors are differently distributed among tissues, where PPARγ is the main receptor in colon tissue [43]. PPARγ is activated by a derivate of PGD_2, 15-deoxy-$\delta^{12,14}$-PGJ_2, and has a suggested role as a tumor suppressor in tumor progression [44].

Colon tissue

The primary physiological function of the colon is to store and concentrate fecal material before defecation. The colon contains a lot of bacteria that metabolise undigested polysaccharides to assist breakdown and fermentation. Colon tissue consists of four layers; mucosa, submucosa, muscularis externa, and serosa. The mucosa contains crypts that enlarge the absorptive area and secrete mucus and fluids with ions for osmotic absorptions of water. The crypts consist of a single layer of epithelial cells such as colonocytes, goblet, Paneth, and endocrine cells [45]. The lower third of the crypt constitutes of newly generated epithelial cells that undergo two or more divisions when migrating towards the top. All epithelial cells in the crypt epithelium origin from stem cells at the base of the crypt and have an approximate life span of five to six days [46]. Surrounding the crypts is lamina propria and beneath is a thin muscle layer, muscularis mucosae. The lamina propria consists of connective tissue that is fibroblasts and extracellular matrix (ECM), and small blood vessels (endothelial cells), nerve fibres, and various immune cells (like macrophages and lymphocytes). In contrast to the rest of the digestive tract, the colon lamina propria lacks lymphatic vessels [46]. The ECM is a mixture of fibrillar proteins (collagen, fibrin), glycoproteins, proteoglycans, cytokines, and growth factors that support cell adhesion and transmit signals [47]. It provides structural support as well as information to cells in response to stimuli. The ECM adapts easily to various signals during developmental and pathological processes such as cancer. The balance between ECM synthesis and remodeling is tightly regulated and may be essential for maintenance of tissue integrity [1]. The submucosa is another layer of connective tissue with a nerve network called submucous plexus. Followed by a circular and a longitudinell muscle layer, muscularis externa [45]. The outer layer is the serosa, which consists of an epithelium (the mesothelium), connective tissue, and ECM [46].

Wound healing and inflammation

Upon injury of tissue a set of complex biochemical events are activated to repair damage. The repairing process can be divided into four overlapping phases; hemostasis, inflammation, proliferation, and remodelling. The first phase involves platlet aggregation followed by removal of bacteria and debris by inflammatory cells. In the proliferative phase angiogenesis, collagen deposition, granulation tissue formation, and wound contraction occur, while the last phase involves remodelling of collagen and apoptosis of cells that appear in excess [47]. Several different cell types are activated during tissue repair with different mediators like cytokines, chemokines, and prostanoids. For example, neutrophils and monocyte/macrophages are important for wound cleaning. Fibroblasts make granulation tissue of fibronectin and collagen, while endothelial cells re-vascularise the damaged area [47]. The tissue repair process has similarities with tumor progression in many ways, such as re-vascularisation and migration of inflammatory cells.

Inflammation is a response to tissue damage or microbe invasion and is mediated by several different cell types and mediators. In normal tissue innate immune cells, like macrophages and mast cells, are present. At site of injury, those cells start to secrete factors to attract other cells. Neutrophils are the first cells to migrate to an inflammatory site. During progress of inflammation a signaling network is activated that attracts and activates lymphocytes, leukocytes, and other inflammatory cells [48]. When the damage is removed, cells shift to a tissue repair process and inflammatory cells execute apoptosis. Disruption of the regulation of the inflammatory response can lead to chronic inflammation, which is dominated by macrophages, lymphocytes, and plasma cells that secrete a great number of growth factors and cytokines as well as reactive oxygen and nitrogen species that may cause DNA damage [49]. This may lead to continuous tissue damage and subsequent neoplasia. Globally, about 15% of cancer cases are related to infectious agents [50].

Colorectal cancer

Colorectal cancer (CRC) constitutes of two separate cancer types, colon cancer and rectal cancer, which share several characteristics. There are three variants of CRC where two are hereditary; familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). Sporadic cancer is the third variant and counts for the majority of cases (about 80%) and is the one considered in the present work. CRC is the third most common cancer type and the second cause of cancer-related deaths worldwide. In Sweden there are more than 5000 new cases of CRC every year and some risk factors for CRC include a diet high in fat and meat, obesity, sedentary lifestyle, and tobacco smoking [51]. Also, there is a 10-fold greater risk of CRC when linked to inflammation of the colon [48].

Progression of colorectal cancer

The cause of CRC is unclear and like all cancers it starts with growth due to failure in regulation of cell proliferation. In proliferation each cell passes through the cell cycle, which consists of four strictly regulated phases; G_1, S, G_2, and M. Alterations in tumor growth, beyond cell cycle control, is apoptosis which is disturbed in many cancers [52]. There are several ways that regulation of proliferation and apoptosis can fail; however, these are not the only explanation for cancer. Weinberg and Hanahan described the hallmarks of cancer as; self-sufficency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis [53].

A model for describing the genetic tumor progression in CRC is the Vogelstein model (the Vogelgram), which was introduced 1990 [54]. Mutation of the tumor suppressor *APC* that is a key protein in the Wnt signaling pathway is suggested as the start of CRC (Fig 2). This mutation is also assumed to be the reason behind the hereditary variant of CRC called FAP. Wild type APC protein forms a complex with axin and GSK-3β (kinas) upon Wnt signaling. The protein complex promotes degradation of β-catenin, which is an intracellular signaling molecule. If not degraded, β-catenin can enter the nucleus and bind to TCF/LEF transcription factors, which starts transcription of genes such as *MYC* (c-Myc, a proto-oncogene) and possibly *PTGS2* (COX-2) [55-57]. Another way to disturb transcription of genes is methylation. Aberrant methylation of CpG islands, regions rich in guanines (G) and cytosines (C), is an early event in cancer [58].

Methylation is an important epigenetic silencing function maintained by DNA methyltransferases (DNMTs) [59].

Transition from adenoma to dysplastic tissue may be caused by several genetic changes as well as changes in the adenoma microenvironment. KRAS activation due to mutation is found in 40-50% of adenomas and carcinomas and leads to a constant signal to the nucleus for division [60]. Another well-known mutation is in the *DCC* gene (**d**eleted in **c**olorectal **c**arcinoma). The DCC protein has a possible roll in cell-environment interactions [61]. A mutation that occurs in the majority of CRC cases appears in the tumor suppressor *p53* gene, which regulates phases of the cell cycle at wild type [62].

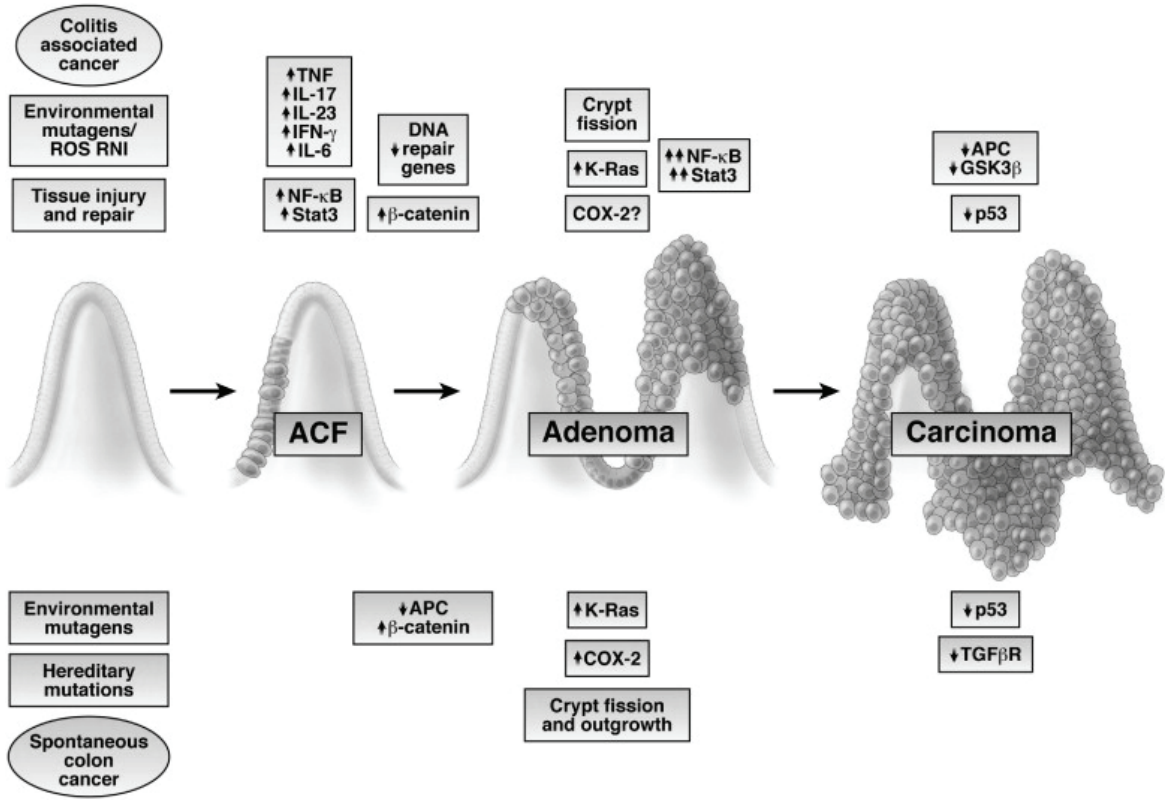

Fig 2. Transition from normal colon tissue to tumor tissue involves several different factors. (Adapted by permission from Elsevier: Gastroenterology [63], copyright 2010).

The progression of CRC is not only a genetic event. Changes in tissue microenvironment and expression of growth factors and other signaling factors are also of major importance [1, 4]. A tumor is not dividing, mutated epithelial cells only. It also consists of the vascular, inflammatory, and other activated stromal cell. Interactions among such cells with the stroma and soluble molecules favour cell proliferation, movement, and differentiation [1]. Fibroblasts which are the predominant cell in stroma are responsible for synthesis and remodelling of the stroma as well as production of many paracrine growth factors that regulate cell proliferation, morphology, cell survival, and cell death. In tumor tissue, fibroblasts have a disorganized growth pattern and enhanced proliferation as well as high production of collagens, hyaluronates, and epithelial growth factors. Recent data display that fibroblasts can promote neoplastic progression in

combination with inflammatory cells [64]. However, this depends on the type of gene mutated in adjacent epithelial cells [1]. In colon, deletions on chromosomes in stromal cells may predispose to carcinogenic conditions [65]. Another important stroma interaction is the formation of blood vessels, called angiogenesis. The tumor needs to stimulate angiogenesis to ensure influx of nutrients and oxygen as well as efflux of waste products and carbon dioxide when it grows larger than ~2 mm^3 [66].

Stages of colorectal cancer

Classification according to Dukes was first proposed by Dr. C.E. Dukes in 1932 and describes the stages of colorectal cancer as: A – tumor confined to intestinal wall, B – tumor invading through the intestinal wall, C – with lymph node(s) involvement and D – with distant metastasis [67]. The most common current staging system is TNM (Tumors/Nodes/Metastases) system [68]. Dukes staging was used in the present work and can be converted to approximate TNM staging (Table 1).

Table 1. Approximate relationships between Dukes staging and TNM classification.

Dukes	TNM
A	T1, N0, M0
B	T2/T3, N0, M0
C	T2/T3/T4, N1/N2, M0
D	T2/T3/T4, N1/N2, M1

Treatment of colorectal cancer

Screening may be performed by colonoscopy and detection of haemoglobin (F-Hb) in feces to decrease CRC incidence and mortality. The curative treatment for CRC is surgery. By offering neo-adjuvant chemotherapy or adjunct chemotherapy to patients with advanced tumor stages, survival in CRC has increased. In rectal cancer, preoperative radiotherapy has reduced the risk for local recurrence. An important observation in CRC research was that NSAIDs reduced death rates from CRC [2]. There is now an accumulation of evidence that NSAIDs decrease tumor growth in CRC and may prevent tumorigenesis [69-73]. NSAID acts through inhibition of COX with some exceptions like sulindac sulfone [74]. However, the precise mechanism(s) by which NSAIDs exert anti-carcinogenic actions remains unclear. Several different mechanisms and targets have been suggested that can be divided into two groups; COX-dependent and COX-independent. Indomethacin is a classic unspecific COX inhibitor that may act through decreased angiogenesis, activation of PPARγ, and inhibition of telomerase activity [75-77].

Several other agents are candidates for chemoprevention of CRC; targeting signal transduction, epigenetic modulation, and anti-inflammation. Some well-known therapeutic agents for cancer are used worldwide such as EGF receptor inhibitors (Erlotinib, Cetuximab) and anti-VEGF antibodies (Bevacizumab) [78, 79]. Several other agents have effects on colon tumorigenesis in animal models and are now in clinical trials; for example PPARγ agonists (rosiglitazone and pioglitazone) and anti-inflammatory agents directed towards EP_{1-4} (ONO-8711) and NF-κB (Bortezomib, Curcumin, Tea polyphenols, Statins, NSAIDs) [79].

AIM

Metabolites from arachidonic acid (AA) have many actions in pathological conditions ranging from inflammation to tumor progression. Previous analyses at our laboratory indicated that PGE_2 was a frequently changed metabolite in CRC from AA. One possibility to inhibit actions of prostanoids is by blocking the rate limiting enzyme for synthesis of prostanoids, COX. However, prostanoids are required in tissue for normal metabolism and complete inhibition may damage barrier functions. Therefore, the general aim of this dissertation was to evaluate changes in prostanoid receptors and enzymes expression in human CRC to find and suggest more specific targets for interventions on prostanoid metabolism in progression of CRC.

Specific aims:

Paper I - To evaluate changes in PGE_2 receptors, PPARγ and COX-1/COX-2 gene expression in human colon cancer related to normal colon tissue, tumor progression, and pathological factors such as differentiation and patient survival.

Paper II - To evaluate changes in PG receptor gene expression in human colon cancer related to normal colon tissue, tumor progression, and pathological factors such as differentiation and patient survival.

Paper III – To evaluate by short-term COX-inhibition the relationship between prostanoid production and the expression of corresponding receptors and other genes that are dependent on high PGE_2 in CRC.

Paper IV- To study expression of known transcription and external cell factors involved in COX-2 induction and to evaluate if DNA methylation explains altered COX-2 expression in CRC.

METHODOLOGICAL CONSIDERATIONS

Study design

Tumor and normal colon biopsies were continuously collected from patients operated for primary CRC during various time periods at three Swedish hospitals (1988-1990 Uppsala/Falun, 51 patients without collection of normal colon tissue), 1998-2002 in Uddevalla (150 patients, tumor and normal colon tissue) and 2001-2004 in Gothenburg (23 patients, tumor and normal colon tissue, preoperative indomethacin treatment). Tissue biopsies were taken immediately after colon resection from the tumor and adjacent normal colon tissue (around 10 cm away from the macroscopic tumor margin) and collected in a biobank. Exclusion criteria for patients were pre-operative radiation and prescribed drugs that affects prostaglandin metabolism, such as acetylsalicylic acid etc.

Paper I

All patients from Uppsala/Falun and every second patient from Uddevalla were selected from the biobank for RNA extraction. All samples that showed degraded RNA were excluded from further analysis, which was particularly true for normal colon tissue. Remaining 99 tumor biopsies and 27 adjacent normal colon biopsies were analyzed with reverse transcription PCR (RT-PCR) for COX-1, COX-2, EP receptors and PPARγ receptor expression. Localization of protein expression of EP receptors and COX were visualized by immunohistochemistry.

Paper II

A total number of 62 patients were included in this study. The same patients that were used in paper I were used when possible. In addition to the 48 patients from paper I the placebo treated patients from paper III (14 patients) were included. Adjacent normal colon biopsies (29 from Uddevalla and 14 from Gothenburg) as well as two well-differentiated human colon adenocarcinoma cell lines (HT-29 and HCA-7) were used. Gene expression of prostanoid receptors for PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 were analyzed with quantitative realtime RT-PCR (qPCR).

Paper III

Twenty-three patients were randomized to receive either indomethacin (n = 9, Confortid, 50mg x 2 and 40 mg Nexium daily) or sham-treatment (n = 14, 40 mg Nexium daily) for three days before surgery. All patients were operated at Sahlgrenska University Hospital, Gothenburg, between 2001 and 2004. Two cell lines (HT-29 and HCA-7) were treated with indomethacin (8.4 μM) or saline for 14 days in vitro. Differences in gene expression after COX inhibition were studied with microarrays. Pooled tumor RNA from 6 patients who received indomethacin treatment was run against pooled tumor RNA from 6 patients who received placebo treatment. Similar principles were used for normal colon tissue and HCA-7 cells. Prostanoid receptor expression, mPGES-1, COX-1, COX-2, and HPGD gene expression were analyzed with qPCR.

Paper IV

Microarrays were performed to evaluate differences in gene expression related to high versus low prostaglandin expression. Twenty patients were selected based on COX-2 gene expression in tumor tissue according to results from the 48 patients (Uddevalla) in paper I and II. The selection was based on the degree of COX-2 gene expression in the tumor tissue and the material was divided into two groups; 10 patients with high and 10 patients with low COX-2 gene expression. Both tumor and adjacent normal colon tissue were used from the same patient. RNA from each group was pooled and microarrays were run as follows:

Tumor tissue High COX-2 Versus Low COX-2	Normal colon tissue High COX-2 Versus Low COX-2	High COX-2 Tumor tissue Versus Normal colon tissue

DNA from tumor and normal colon tissue from the 20 patients were extracted and used for methylation analysis of two areas of the COX-2 promoter region.

Cell lines

Two different human adenocarcinoma cell lines were used in paper I and III. HT-29 displayed low intrinsic PGE_2 expression and HCA-7 showed high intrinsic PGE_2 expression. HCA-7 cells expressed several prostanoid receptors (DP2, $EP_{1\text{-}4}$, FP, TP) while HT-29 cells expressed only some (DP2, $EP_{1/4}$, FP, TP).

Gene expression analysis

The step between DNA and protein is RNA, which is transcribed from DNA and translated to proteins. The transcription of DNA results in precursor mRNA which is processed into mRNA by splicing. The mRNA molecule is comparatively unstable and may be degraded rapidly after translation; halftime is 30 min in eukaryotes [80]. Measurement of mRNA expression in tissue may be a means to measure the increase or decrease in protein production at specific conditions and specific events such as tumor progression. There are some weaknesses in measurements of mRNA expression to define the changes of a specific receptor / enzyme; first, mRNA is unstable and easily degraded and second, post-translational modulation may occur. To partly overcome these obstacles the quality of the mRNA has been rigorously checked and only RNA with a RNA Integrity Number (RIN) above 6.0 (or ratio of 1.5 between 18S and 28S RNA) have been used. Post-translational modulations are a bias that has to be considered.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) technique was described in the mid 1980's by Mullis et al. and is used to amplify specific DNA sequences [81]. PCR is based on thermal cycling where a cycle involves three steps; (1) denaturation of DNA/amplicons (PCR copies), (2) annealing of

template and primers, (3) extension of DNA. Theoretically, 10^9 copies of specific DNA sequences have been produced after about 30 cycles of PCR. Two sequence specific oligonucleotide primers are needed; one forward (P1) and one reverse (P2) to get the specific amplification of the DNA sequence of interest. The primers bind to denatured DNA. Copies of the DNA sequence are produced during extension in the presence of a heat-stable DNA polymerase and deoxynucleoside triphosphates (dNTPs) (Fig. 3).

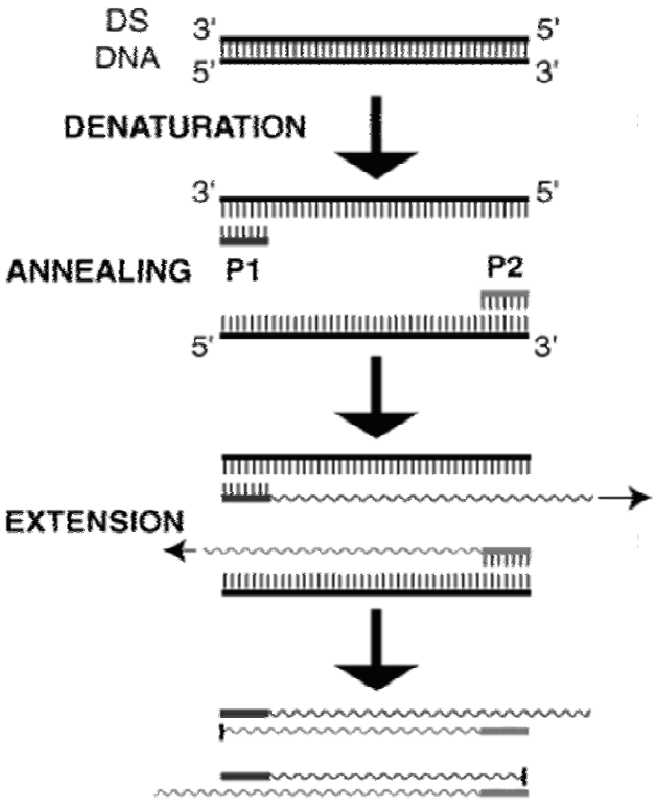

Fig 3. The polymerase chain reaction with denaturation of double stranded DNA followed by annealing with primers and extension.

Reverse transcriptase PCR (RT-PCR)

We mainly used reverse transcriptase PCR which is a method to detect the expression of specific genes. The principle is the same as for PCR. However, a first separate step where mRNA are transformed to cDNA is included. The RNA strand is converted to cDNA by the use of the enzyme reverse transcriptase and oligo-dT primers that are mRNA selective by binding to the poly A-tails. Primers are designed to generate products spanning over exon-exon boundaries as a second step to ensure that it is only cDNA and not genomic DNA in the PCR.

The Agilent 2100 Bioanalyzer was used for size determination, quantification, and quality control of RNA and DNA. It is a microfluid-based platform that is developed from capillary electrophoresis. RNA/DNA samples are loaded to chips containing interconnected sets of micro-channels that are used for electrophoretically driven separation of nucleic acid fragments. The results are displayed in an electropherogram for each sample as well as in a gel-like image. RIN is a computer based algorithm that is extracted from a number of characteristic features

from the Bioanalyzer electropherogram and is used to ensure repeatability of experiments. RIN is also a measurement of RNA quality in an ascending scale (1-10) [82].

Real-time PCR

Quantitative real-time PCR (qPCR) is a method were fluorescent molecules are incorporated to or bind to the DNA strand. The fluorescence is measured for each cycle and the signal is proportional to the initial amount of PCR product. Two different types of qPCR have been used in this dissertation, Taqman® (ΔCt, paper III) and LightCycler® 1.5 (relative standard curve, paper I, II and III). Two methods can be used, either the standard curve method or the comparative Ct (cycle threshold) method, to get a relative quantification of the results obtained by qPCR.

A standard curve is prepared for each primer pair using serial dilutions of a calibrator cDNA (standard) when using the standard curve method. The standard is used in every PCR run and all samples are related to the amount of standard and the standard curve. In the comparative Ct method, Ct is defined as the number of PCR cycles required for the fluorescent signal to cross the treshold (i.e. exceed background level). Ct levels are inversely proportional to the amount of target cDNA in the sample (low Ct = great amount of cDNA). A standard is also used in every PCR run in the comparative Ct method, but no standard curve is produced. In contrast to the relative standard method that is calculated by the LightCycler 1.5 software, the comparative Ct method is calculated according to following:

Ct target gene – Ct reference gene = ΔCt normalized to reference gene

ΔCt sample – ΔCt standard = ΔΔCt normalized to standard

$$\mathbf{2}^{-\Delta\Delta Ct} = \text{expression level of gene}$$

A reference gene (endogenous control) is used to normalize the input of amount of cDNA and PCR efficiency. It is important that the reference gene has a constant expression level in the tissue of interest. Usually the choice of reference gene is housekeeping genes, which has strong promoters and are transcribed efficiently and continuously. We performed a test with Taqman human endogenous control plate (Applied Biosystem) to find a suitable reference gene. Samples from four different patients (Dukes A, B, C, and D), tumor and normal colon tissue, were used at two plates (Fig. 4).

Glyceraldehyd-3-phosphate dehydrogenase (GAPDH) was used as reference gene, since its expression was at almost the same level in tumor and in normal colon tissue and the standard deviation between the samples were modestly different.

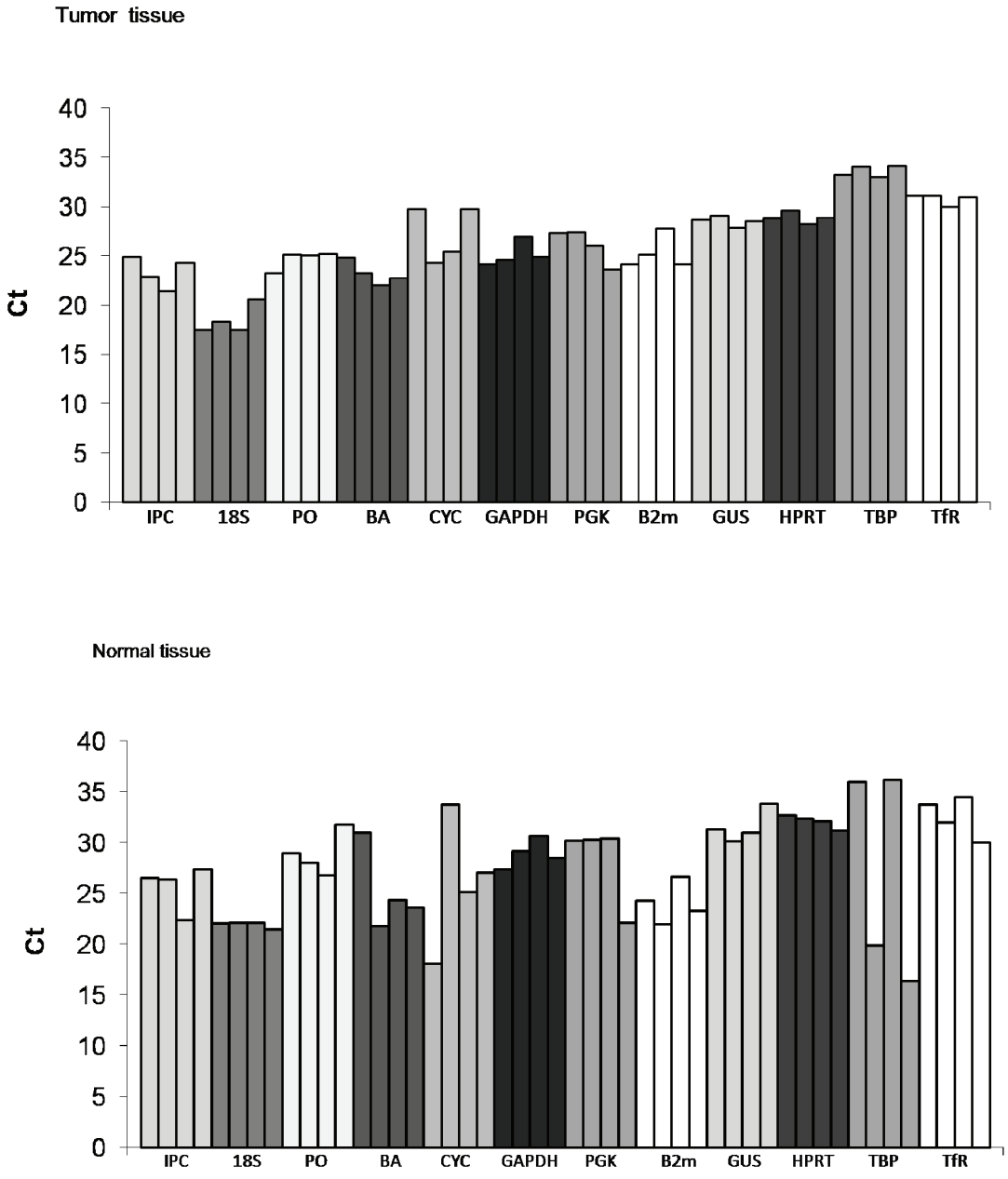

Fig 4. Ct-values of reference genes in order at the diagram; IPC – internal positive control, 18S – 18 S rRNA, PO – acidic ribosomal protein, BA – β-aktin , CYC - Cyclophilin, GAPDH - glyceraldehyd-3-phosphate dehydrogenase, PGK - Phosphoglycerokinase, B2m – β2 microglobulin, GUS – β-glucronidase, HPRT – Hypoxanthine ribosyl transferase, TBP – TATA binding protein TfIID, and TfR – transferring receptor.

Microarray Analysis

Microarray analysis was applied for assessment of the expression of several genes in parallel and nowadays several different platforms have been created for applications within various areas of research [83]. There are two major microarray techniques, one-color and two-color arrays. One-color arrays are based on hybridization of one source of DNA/RNA while two-color arrays have

two different sources of DNA/RNA. Two-color gene expression arrays from Agilent were used. The principle of microarray technique is hybridization of fluorescence-labelled DNA/RNA (Cy 3 and Cy 5) to probes that are bound on a surface. The two different fluorescence-labelled DNA/RNA samples are competing for the same probe. The fluorescence intensities are measured at each probe by laser emission. In the computer software program for image analysis (Feature Extraction, Agilent Technologies), a grid is placed on the array to verify the location of each probe. Thereafter, the ratios of fluorescence intensities among the probes are calculated. In the software program Genespring (Agilent Technologies) normalization, quantification, and further data analysis was performed.

DNA methylation analysis

Methylation in promoter regions of a gene is known to silence the gene expression. One of the most common ways to analyze DNA methylation is the bisulfite-modified DNA technique, which makes it possible to detect methylated cytosines with DNA sequencing [84]. This is possible since bisulfite deaminates unmethylated cytosines into uracil while methylated cytosines are protected. In a following PCR analysis unmethylated cytosines converted to uracils are amplified as thymine while methylated cytosines are amplified as cytosine. PCR was performed with primers without any preference for methylation and followed by DNA sequencing.

Immunohistochemistry

Immunohistochemistry (IHC) is a method to detect and localize protein expression in tissue and in cells with specific antibodies. Thin sections of the tissue are exposed to specifically labelled antibodies. Visualisation of the antibody can be accomplished in different ways. In this dissertation, IHC was used to visualize the expression of COX and subtype EP_{1-4} receptors in tumor and normal colon tissue (Paper I).

PGE_2 analysis

Prostaglandin E_2 [^{125}I] assay system (Amersham LIFE SCIENCES) was used to measure the amount of PGE_2 in tissue. In this assay PGE_2 is converted to the more stable methyl oximate derivative by methoxyamine hydrochloride. The assay is based on competition between unlabelled PGE_2 (methyl oximated) and a fixed quantity of ^{125}I-labelled PGE_2 (methyl oximate derivate) for binding sites on an antibody specific for methyl oximated PGE_2. Separation of bound and unbound PGE_2 is performed with a second antibody bound to magnetic polymer particles and separated by centrifugation. The amount of radioactive ligand bound is inversely proportional to the concentration of added non-radioactive ligand and is determined by interpolation from a standard curve [85].

Statistical analysis

Survival analysis was used to check that our patients were representative for expected survival among patients with CRC related to tumor stage according to the Kaplan-Meier curve. Statistical testing was performed with the log rank technique and alive patients were censored. The gene expression results from RT-PCR are presented as mean ± SEM and $p<0.05$ was considered statistically significant in two-tailed tests.

In paper I it was assumed that sample means and proportions are observations from a Normal distribution and that the calculated standard errors are good estimates of the standard deviations of these Normal distributions since sample size is large [86]. Hence, parametric tests were used on this material. Factorial ANOVA was used to compare the gene expression of prostanoid receptors and enzymes at different variables, as Dukes stage and differentiation. Regression analyses were used to study the relationship between variables such as gene expression, survival, differentiation, etc. Multivariate regression analysis was performed according to standard regression analysis in Statview 5.0.1 (SAS Institute Inc.) with disease-specific mortality as dependent factor.

In paper II a moderate sample size was used; therefore, calculations of differences between parameters were performed with non-parametric tests (U test / Kruskal-Wallis and Mann-Whitney). The advantage is that the only assumption about the distribution of the data is that the observations can be ranked. The disadvantage is less power with the risk of type-I error. However, non-parametric testing is usually appropriate in moderate sample sizes.

In paper III the sample size required to detect any differences between indomethacin and placebo treated patients was determined with test of power with significance level $\alpha=0.05$ and β at 0.80, which represent 1 - probability of detecting a significant difference at the α level.

In paper IV the selection of patients to the two groups was done according to a graphical distribution of COX-2 gene expression in a histogram with 48 patients. Statistical analysis of microarray data was performed in Genespring GX7.3.1/GX9.0/GX10 (Agilent Technologies) and a fold change 1.5 of log2 ratio was considered statistically significant.

RESULTS AND DISCUSSION

Prostanoid synthesis is a pathway of major interest in prevention of CRC since its enzymes and metabolites have aberrant expression in epithelial cancers [87]. Several studies have confirmed actions of PGE_2 as well as other prostanoids in CRC progression [4, 6-14]. Indeed, our investigations confirm that production of prostanoids as well as corresponding receptor signaling in tumor tissue is critical for CRC progression. The production of prostanoids is rate limited by COX-2, which has a scattered expression in tumor tissue that may represent different cell clones or different local tissue conditions at the cellular level. Differences in the expression of genes known to regulate COX-2 including transcriptional factors are seen among tumors with different COX-2 expression (paper IV). Short-term treatment with a non-selective COX-inhibitor, indomethacin, changed gene expression strikingly in CRC tumor tissue (FC 1.5: ↓ 623, ↑ 541, paper III). This suggests that prostanoid metabolism is complex in CRC and involves several hundred genes and different cell types.

Prostanoids

Prostanoids in CRC became interesting with the report of reduced adenomas in patients on NSAID medication [2]. Measurements of arachidonic acid metabolites in tumor tissue display an imbalance in prostanoid production during tumor progression. The major metabolite of COX-2 is PGE_2, which is increased in most CRC tumors and has been accredited to affect all the hallmarks of cancer [4]. However, changes occur in concentrations of other prostanoids as well [88, 89]. Elevated levels of PGD_2 and TXA_2 have been observed as well as reduced levels of $PGF_{2\alpha}$ and PGI_2 [90-92]. Most reports have focused on PGE_2 and have left remaining products of COX without detailed considerations in CRC. Other prostanoids may exert some of the tumor promoting actions performed by COX-2 metabolites even though PGE_2 is the major metabolite. For example, TXA_2 is involved in angiogenesis and development of tumor metastases [93]. $PGF_{2\alpha}$ may activate potentially oncogenic pathways such as the β-catenin transcription and it also promotes neoplastic epithelial proliferation [94, 95]. Contradictory, PGI_2 displays anti-cancerogenic effect in a murine cell model [96]. Depending on which receptor PGD_2 may activate it exerts different actions; DP1 anti-inflammatory, DP2 pro-inflammatory, and PPARγ is tumor suppressing, and thereby has PGD_2 both anti- and pro-cancerogenic effects [97-99].

One of the other pathways derived from arachidonic acid, which is known to be involved in CRC is via the enzyme 5-lipoxygenase (5-LOX), resulting in the leukotrienes (LTs). LTs are among other mediators of acute inflammatory responses and are predominantly produced by inflammatory cells. One possible action of LTs in CRC may be affecting the expression of adhesion molecules needed for interactions between endothelial and inflammatory cells [100]. Compared to prostanoids much less is known about LTs in cancer. However, 5-LOX has enhanced expression in human cancer tissue and there is evidence that LTs are involved in regulation of apoptosis as well as promoting cell proliferation [30, 101]. Also, expression of a LT receptor, $CysLT_1$, correlates negatively with patient survival and activation of the receptor leads to increased production of PGE_2 [102].

It can be assumed that PGE_2 is the main prostanoid involved in tumor progression since mPGES-1 like COX-2 is up-regulated in most cancers [4]. There are many similarities in activation of COX-2 and mPGES-1, but some differences in regulation seem to occur [19]. Transfection of mPGES-1 together with COX-2 results in faster growth, increased cell aggregation, and abnormal cell morphology [103]. This indicates that induction of mPGES-1, i.e. induction of PGE_2 production, may be driving PG-related inflammation and tumor progression.

Degradation and transportation of prostanoids are affected during tumor progression in addition to changes in production. The enzyme responsible for degradation of prostanoids HPGD showed overall lower expression in tumor tissue than normal colon tissue (paper III). This agrees with findings by others reporting down-regulation of HPGD in CRC [104]. Moreover, the proteins PGT and MRP4, believed to be responsible for transportation of prostanoids across the cell membrane, show decreased and increased expression respectively in CRC [105]. This was not observed in tumor tissue with high COX-2 where increased expression of PGT, but not of MRP4, was detected (unpublished data, paper IV).

Cyclooxygenase

It seems that induction of COX-2 is a key-factor behind progression of epithelial cell transformation to invasive cancer in colon mucosa. Indeed, an aberrant expression of COX-2 is seen in most CRC even though it shows uneven expression and distribution in tumor tissue [106, 107]. Therefore, it is of great relevance to determine what regulates the induction of aberrant expression of COX-2 in specific cells. Little is known about initiating events that trigger COX-2 induction in cancer, although COX-2 is regulated by multiple signaling pathways and transcription factors. A predominant role in COX-2 regulation has been assigned to RAS transmitted signals via the RAS-RAF-MEK-ERK and RAS-AKT-MEKK1-JNKK-JNK cascades, which activate the transcription factors PEA3, C/EPB and AP-1 [108].

Several factors and signaling pathways involved in COX-2 regulation were studied (Table 2, Fig 5). Some factors that elsewhere have been reported to affect COX-2 expression had no change in gene expression among tumors with different COX-2 mRNA expression (paper IV) [31, 109]. However, the cytokines IL-1β and IL-6, which play important roles in cancer development, were increased at high COX-2 mRNA expression in tumors and in normal colon tissue. This supports a role for PGE_2 in shifting the tumors immune response from anti-tumor T_H1 to immunosuppressive T_H2 response resulting in down-regulation of TNF-α, IFN-γ and IL-2 as well as up-regulation of IL-4, IL-10 and IL-6 [110]. Moreover, the receptor that mediates the immunosuppressive signal of IL-10, IL-10 receptor α, is up-regulated at high COX-2 in tumors (unpublished data, paper IV).

A major transcription factor complex that is changed at high COX-2 expression in tumors is the activating protein-1 (AP-1) complex (Table 2). In response to stimuli AP-1 forms homo- or heterodimers composed of members from the JUN and FOS families. AP-1 is related to the transcription of genes exerting various biological effects whereas many are considered hallmarks of cancer. *KRAS* mutations and the Wnt signaling pathway (*APC* or *CTNNB1* (β-catenin) mutations) that are common in CRC can activate AP-1 [111]. RAS activation of AP-1 is mediated via RAS-RAC-MEKK1-JNKK-JNK signals [108]. Activated AP-1 (dimers of FOS and JUN

family members) interacts with ATF and binds to the CRE motif in *PTGS2* (COX-2) promoter [30, 108]. In normal colon tissue the AP-1 complex is not changed as observed in tumor tissue (Table 2). In agreement, it has been proposed that AP-1 is highly activated during late progression of CRC in rats [112]. Furthermore, transcription of AP-1 components is regulated by other transcription factors affected by COX-2 expression. For example, decrease of ELK-1 at high COX-2 mRNA in tumor tissue (Table 2) may affect transcription of the AP-1 component FOS [109].

Table 2. Changes in gene expression of factors involved in regulation of COX-2 gene expression at high tissue intrinsic COX-2 (FC 1.5).

Factor	Tumor tissue	Normal colon tissue
ATF3 (AP-1)	↑	↑
C/EBP-δ	↑	↑
c-FOS (AP-1)	↑	ns
FOS-B (AP-1)	↑	↑
CREB	ns	↑
JDP1 (AP-1)	ns	↑
JDP2 (AP-1)	↑	ns
JUND (AP-1)	↑	ns
c-MAF (AP-1)	↑	ns
IκBα (NF-κB)	↑	ns
TCF4	↑	ns
DNA MTase 3A	↑	ns
IL1β	↑	↑
IL6	↑	↑
IL6 receptor	↑	ns
iNOS	ns	↑
Protein kinas Cβ1	↑	ns
TNF-α	↑	ns
AP-2γ	↓	ns
B-ATF (AP-1)	ns	↓
ELK-1	↓	ns
CDX2	↓	ns
NFATc1/NFAT2	ns	↓
p53	↓	ns
PEA3/ETV4	↓	ns
PPARγ	ns	↓

Induction of COX-2 expression is mediated by growth factors, oncogenes, and cytokines that partly act via protein kinase C (PKC) and RAS-mediated signaling (Fig 5) [109]. Transcription factors activated by RAS-mediated signaling are PEA3 and C/EPB-δ via ERK [108]. PKC and C/EPB-δ gene expression were increased at high COX-2 gene expression while PEA3 gene expression was decreased (Table 2). Several factors involved in RAS signaling were investigated as well as *HRAS* and *KRAS* (unpublished data), but no difference in expression could be found

between high and low COX-2 expressing tumors (paper IV). C/EBP transcription factors can in response to IL-1β bind to NF-IL-6 element and PEA3 can be activated by Wnt signaling, which activates TCF-4 as well [108, 113]. In contrast to PEA3, TCF-4 was increased at high COX-2 gene expression in tumors (Table 2).

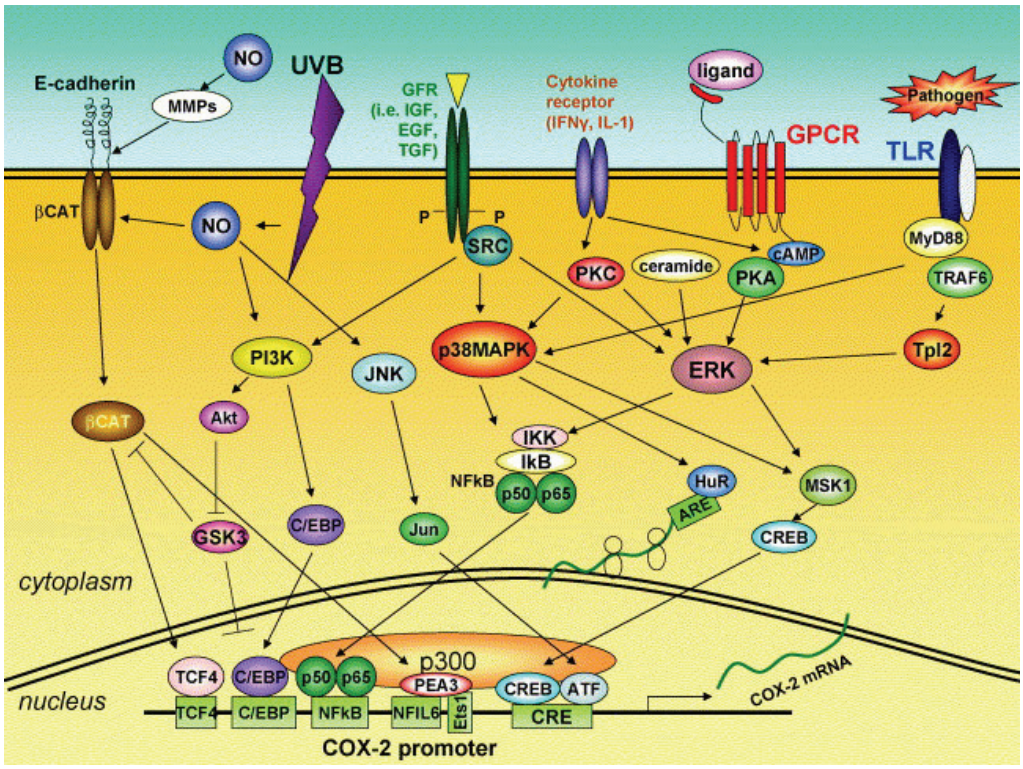

Fig 5. Intracellular signaling pathways involved in the induction of COX-2 expression. The CRE site in the promoter binds AP-1, which also can be components from the JUN and FOS families. (Adapted by permission from Elsevier: The International Journal of Biochemistry & Cell Biology [31], copyright 2006).

The COX-2 promoter has binding sites for several transcription factors. One well known in breast cancer is AP-2γ, which was reduced in CRC with high COX-2 gene expression (Table 2). This transcription factor may function as a tumor suppressor since it up-regulates p21, involved in cell cycle control [114, 115]. Another transcription factor with increased expression in normal colon tissue with high COX-2 is CREB (Table 2), which is a central regulator of COX-2 expression and over-expression of CREB is linked to poor prognosis and metastases [31, 116]. The multi-component transcription factor NFAT regulates COX-2 gene expression in Jurkat human leukemic T-cells and one of these factors, NFAT2, was reduced in normal colon mucosa with high COX-2 (Table 2) [109]. Yet, another transcription factor controlling COX-2 gene expression is CDX-2, which is an intestine specific tumor suppressor that interacts with NF-κB and down-regulates COX-2 promoter activity [117]. As shown in table 2, it was reduced in tumor tissue with high COX-2 expression. Gene expression of the regulatory transcription factor NF-κB did not follow the gene expression of COX-2. The only NF-κB family member with changed expression at high COX-2 was IκBα, which inhibits NF-κB (Table 2) [109].

There is conflicting evidence about the quantity of COX-2 expression as well as its distribution in CRC, possibly depending on different analytical techniques and investigated tissue. However, the results differ on cells in tumor tissue that express COX-2 even when the same technique (IHC) and the same tissue (CRC) are used [6-13, 89, 118]. Some reports indicate that COX-2 is mainly visible in macrophages while we observed that COX-2 protein was visible in epithelial cells, muscle layer, adipose tissue and submucosa (paper I) [7]. COX-2 as well as other growth factors are expressed unevenly in tumor tissue with locally high concentrations recognized as 'hot spots' [107]. Some neoplastic cells do not express COX-2 at all (20-60%) and some patients showed weak or absent COX-2 expression in tumor tissue [87, 119]. Yet, others have low levels with constitutive COX-2 expression in normal colonic epithelium [120]. Apparently, the expression of COX-2 is affected both intercellularly and individually.

Moreover, gene expression studies of COX-2 also differ in results [121] (paper I). The possible explanation for this may be tumor localization since COX-2 distribution shows a pronounced prevalence for rectum [122]. A common adjuvant treatment of rectum cancers is pre-operative radiation and such patients were excluded from the study in paper I. Another explanation for the divergent expression of COX-2 in paper I may be changes in the expression pattern in normal colon mucosa [9]. This suggestion was supported by our findings in paper IV, where transcription factors and expression of factors involved in COX-2 regulation were changed in normal colon mucosa at high gene expression of COX-2 (Table 2).

In summary, the regulation of COX-2 transcription is complex and involves several different genes and appears to be cell specific. COX-2 is expressed early on in tumorigenesis and factors found in normal colon tissue with high COX-2 expression might therefore be more significant for understanding the induction of COX-2 expression. There were several changes in transcription factors known to regulate COX-2 expression, especially components of AP-1. However, transcription factors are multifunctional since they transcribe several different genes involved in several different processes and hence, might not be suitable as drug targets. A strength of our study of COX-2 in human tumor tissue is the display of several different genes in one experiment, which reduce technical and biological errors. More than 6000 genes (3086↑ 3031↓) showed different expression in tumors with high COX-2 compared to tumors with low COX-2 gene expression. A limitation may be that our results were obtained from pooled tumor respectively normal colon tissue with a lack of individual changes of the different factors. From frozen tumor tissue selection of 'hot spots' of COX-2 expression is not possible. However, our patients were grouped according to tumor expression of COX-2 that was individually checked by PCR analyses (paper I) and should contain and reflect characteristics of the cells in COX-2 expression regions.

Prostanoid receptors

The specific action of different prostanoids in tumor tissue depends on different cell expression of prostanoid receptors as well as on the specific production of prostanoids. Reports on the expression of prostanoid receptors in human CRC are sparse in the literature, except for EP receptors. Therefore, expression of all prostanoid receptors was studied to get a more complete evaluation. Several prostanoid receptors showed changed expression in tumor tissue compared to

normal colon tissue. In general, prostanoid receptors were decreased (Table 3). The exception was TXA_2 receptor (TP) expression, which was increased (Table 3). Interestingly, TP receptor expression was high in Dukes A and D, conceptually connected to angiogenesis and development of tumor metastases [93]. On the contrary, in azoxymethane-induced (AOM) mouse model the disruption of FP, IP, and TP receptors did not affect colon tumor formation, suggesting that these receptors may not be involved in CRC appearance and progression [110]. Over-expression or disruption of PGD_2 synthase in mice may reduce and accelerate tumor growth respectively, indicating anti-tumor activity of PGD_2. However, genetic disruption of DP1 receptor had no effect on tumor growth [5, 110]. All our patients showed PGD_2 receptor down-regulation (DP1, DP2, and PPARγ, Table 3, paper I and II). The anti-tumoral effects of PGD_2 might be attributed to PPARγ that show decreased expression in CRC patients (paper I).

Table 3. Observed changes in prostanoid receptors gene expression in human colorectal tumors (Dukes A-D) compared with normal colon tissue from the same patients.

Prostanoid receptor	Tumor tissue
DP1[II]	↓
DP2[II]*	↓
EP1[I]	ns
EP2[I]	↓
EP3[I]	ns
EP4[I]	↓
FP[II]	↓
IP[II]	↓
PPARγ[II]	↓
TP[II]	↑

[I] N (n=26), T (n=99), ANOVA analysis

[II] N (n=43), T (n=62), Mann-Whitney analysis

*Also known as CRTH2 and GPR44

The prostanoid receptor and transcription factor PPARγ was decreased at high COX-2 in normal colon tissue (Table 2) and showed reduced expression in tumor tissue compared to normal colon tissue (Table 3). This agrees with the hypothesis that PPARγ is recognized as tumor suppressor and involved in transcription of regulators of the cell cycle as well as apoptosis. PPARγ is transcribed to three different mRNA transcripts that translate into the same protein [43]. Our primers used in qPCR were designed to amplify all three transcripts. Reduction in PPARγ expression is associated with an increase in β-catenin which increases the transcriptional activity of TCF-4 and hence, the target genes of Wnt signaling such as *c-MYC* and *CCND1* (cyclin D) [56, 57]. This happens only when cells have two wild-type *APC* alleles, which is usually not the case in CRC where both alleles of *APC* often is mutated [55]. It is therefore possible that PPARγ exerts its tumor suppressor activities by another pathway. A connection between Wnt signaling and COX-2 may be PPARγ. COX-2 may either regulate β-catenin via PPARγ or β-catenin may regulate transcription of the COX-2 gene [123]. Another possible tumor suppressing activity of PPARγ could be down-regulation of PPARδ expression [108]. There is evidence for involvement of two additional PPAR receptors, PPARα and PPARδ, in inflammation and CRC. In contrast to

PPARγ, PPARδ seems to promote tumorigenesis [108]. Even though PPARα and PPARδ are mainly expressed in other tissues than colon [43] their expressions were decreased in tumor tissue versus normal colon tissue at high COX-2 expression (unpublished data paper IV). Effects of PPARα and PPARδ reduction on COX-2 were not evaluated, but a link is not yet described.

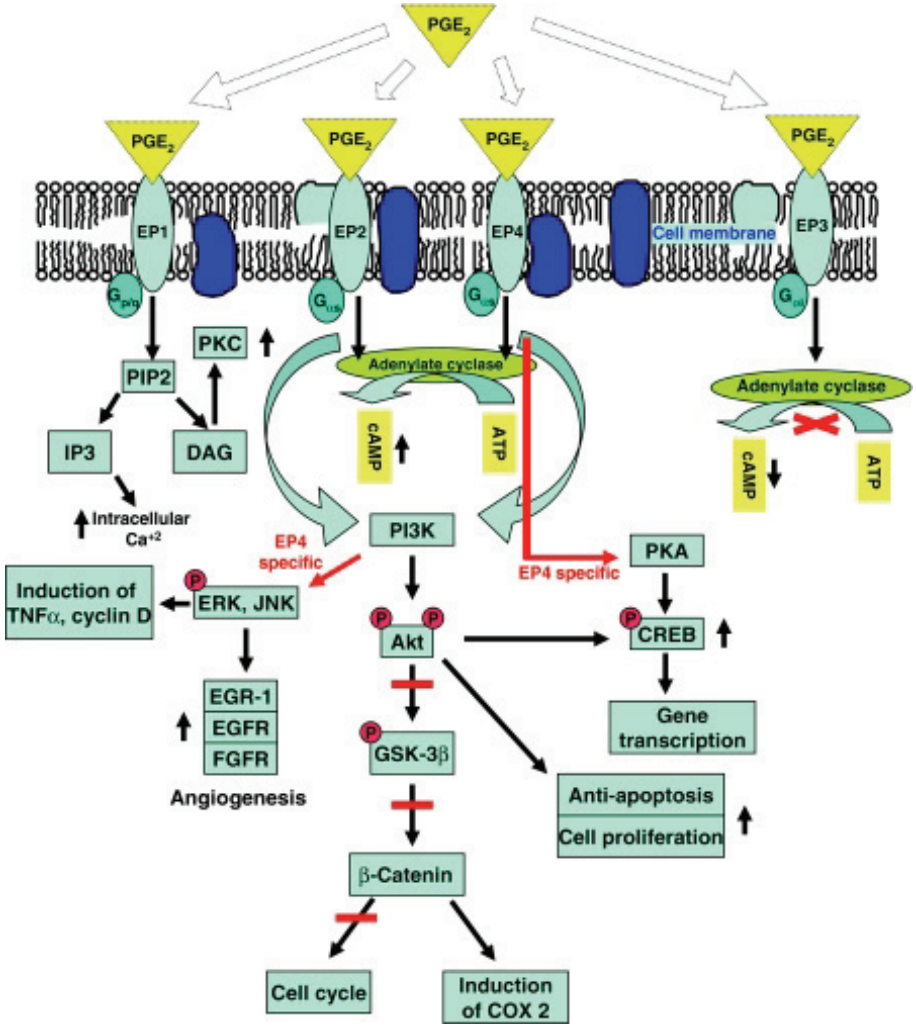

Fig 6. Signaling pathways of the EP_{1-4} receptors. (Adapted by permission from Nature Publishing Group: British Journal of Pharmacology [17], copyright 2006).

A major prostanoid promoting cell growth and survival signaling in CRC is PGE_2 that acts via the four EP receptors [4]. Genetic deletion/inhibition and the use of antagonists/agonists in mice demonstrated that EP_1, EP_2 and EP_4, but not EP_3, reduced colon adenoma formation [124-126]. On the other hand, mRNA expression studies in rats and mice displayed various changes in expression level of the different receptors [126, 127]. It remains uncertain how experimental animal results compare to human CRC [128]. We found no significant change in EP_{1-4} receptor expression among tumors with either different stage according to Dukes, although EP_2 and EP_4 receptor expression were significantly lower in tumor tissue compared to normal colon tissue (Table 3). Decreased receptor expression could be due to increased ligand availability as well as negative feedback mechanisms [16, 126]. Multivariate regression analysis with disease-specific mortality as dependent factor indicated EP_2 as a negative predictor for survival even though EP_2

and EP_4 receptor expressions were decreased. One way that PGE_2 signaling, via EP_2 receptor, can promote tumorigenesis is by increasing nuclear accumulation of β-catenin and subsequently the transcriptional activity [63] or that EP_2 may signal tumor promotion in some cells or tissue compartments.

Regulation of prostanoid receptor expression was not evaluated. However, the promoters for the different prostanoid receptors share common features with the COX-2 promoter such as the TATA-box, SP-1 binding site, AP-2, and AP-1 binding sites [16]. More important, imbalance in prostanoid receptor expression results in activation of different signaling pathways which may contribute to tumor progression (Fig 6) [4, 17]. Another possibility that may impair prostanoid signaling is genetic changes of the receptors, although genetic variability in prostanoid receptors (EP_2, EP_4) is not regarded related to the risk of colorectal adenoma [129].

Immunohistochemistry was used to visualize where EP receptors were localized in tumor tissue. EP_1 and EP_2 subtype receptor protein were highly present in tumor epithelial cells, with considerably less staining in the stroma of tumor tissue. The opposite occurred for EP_3 and EP_4 receptor protein that was mostly seen in the submucosa of tumors. The EP_3 receptor protein was occasionally seen in tumor cells while the EP_4 receptor protein was almost not visible at all in tumor cells (paper I). This expression pattern is different from that found in normal colon tissue by Takafuji et al. who did not describe protein expression of EP_1 in colonocytes. EP_2 and EP_3 receptors were found at epithelial cells and EP_4 receptor protein was found on mononuclear cells scattered throughout the lamina propria. In addition, epithelial cells expressed EP_4 receptor protein in a universal manner [130]. Perhaps there is plasticity also in EP receptor proteins among different cell types at tumor progression.

Table 4. Relative estimates of receptor expression in established tumor cell lines from CRC.

Prostanoid receptor	HCA-7[A]	HT-29[B]
	Relative to CRC specimens	
EP_1	2%	433%
EP_2	720%	0
EP_3	14%	0
EP_4	742%	87%
DP1	0	0
DP2	4%	7%
FP	1%	1%
IP	0	0
TP	4%	3%

[A] = PGE_2 in cell medium at confluence 120 – 130 pg/ml
[B] = PGE_2 in cell medium at confluence <2.5 pg/ml

A strength of our study is that prostanoid receptor expression analyses were performed on human CRC specimens achieved at operations for cure, while reported experiments are usually performed on cultured cells that may be more or less selected. Also, there might be discrepancies

between cultured cells and growing human tumors since cell cultures lack the important interactions with stroma [64]. Accordingly, prostanoid receptors differ a lot between tumor tissue and two common CRC cell lines (HT-29 and HCA-7, table 4), which displays individual expression patterns.

Seen together, our findings suggest that prostanoid receptor subtypes play important roles in intestinal tumorigenesis. Imbalances in receptor expression may mediate altered prostanoid signals that could initiate metastatic spread, affect tumor angiogenesis, cell proliferation, apoptosis and immune reactions [4]. Thus, alterations in prostanoid receptors are significant parts of tumor progression and may offer therapeutical advantages over upstream treatment with COX inhibitors for chemoprevention of CRC. However, it is presently not possible to point out specific receptor profiles that could effectively attenuate tumor progression.

Wound healing and inflammation

Wound healing and inflammation have been implicated as initial steps of tumorigenesis. A difference between malignant and benign conditions is that wound healing and inflammation are self-limited [50]. Inflammation is limited by anti-inflammatory factors that are secreted closely after onset of pro-inflammatory factors. However, cells that are initiated, i.e. with irreversible DNA alterations as in tumors, can be promoted at exposure of factors released from the site of wounding or chronic inflammation [49]. An increased incidence of tumor formation at the sites of scar tissue and in areas of chronic damage has been found. Inflammatory responses are present in both tissue repair processes and tumorigenesis and may establish a link between the two [1].

The immune response in inflammation and tumors is supposed to oppose infection and tumor growth. However, sometimes it may have the opposite effect [131]. Cytokines and chemokines secreted at inflammation may influence survival, growth, mutation, differentiation, and mobility of surrounding cells partly by controlling interactions and communications between cells [50]. Cytokines and chemokines attract immune cells like macrophages, dendritic cells, and lymphocytes, which in turn induce production of additional cytokines and chemokines resulting in recruitment and infiltration of more cells that are supposed to be a positive factor for the host [50, 131]. Induction of cytokines and chemokines may also be stimulated by hypoxia, which is a state of reduced oxygen tension [50]. Even though tumors recruit immune cells and the production of cytokines and chemokines are high the immune response seems defect. The tumor microenvironment contains many different factors that contribute to systemic inflammation associated with cancer. T cells and macrophages are required for inflammation and perhaps tumor progression, as well as for anti-cancer immunity. A paradox in tumors is that immune cells, like activated macrophages that are supposed to protect from foreign cells, may instead promote the action of tumor growth [63, 131]. Tumor cells, for instance, can use the same pathway for invasion as immune cells may use for effects against tumors [50]. A pro-inflammatory cytokine made by activated macrophages as well as by malignant cells is TNF-α, which was increased at high COX-2 in tumor tissue (Table 2) [132]. TNF-α is a major mediator of inflammation with dual actions as destruction of blood vessels and induction of angiogenic growth factors [50]. High TNF-α expression in tumor tissue may facilitate invasion and metastasis, two important

features of tumor progression [133]. Other factors involved in inflammation and hypoxia, characterized by increased expression at high COX-2, were inducible nitric oxide synthase (iNOS2) and hypoxia inducible factor (HIF1a, unpublished results, paper IV).

Inflammatory cells play important parts in tumorigenesis. This is displayed by the efficiency of anti-inflammatory therapy against tumorigenesis and by several chronic inflammatory conditions that are associated with increased risk of tumor development. Also, tumors are dependent on growth factors, activated stroma, and DNA alterations to be able to grow indefinitely [49]. A common feature in inflammation and tumorigenesis is activation and aberrant expression of the enzymes COX-2 and 5-LOX [30].

Colorectal cancer

Cancer is mainly known as a genetic disease which may be related to age, a well-known risk factor of cancer. The most obvious feature is accumulation of genetic damage and epigenetic alterations [134]. However, changes in the cellular microenvironment occur as well. Fibroblasts, the major cell in ECM, increase collagen production and are related to the expression of matrix metalloproteinases (MMPs) when tissues are ageing and fibroblasts enter senescence [1]. MMPs are proteinases that digest components of ECM and extracellular proteins. In cancer, MMP may affect the invasive activity of tumor cells as well as their ability to metastasize, which have been observed in experimental animal models [135]. The MMP family consists of around 24 members; whereof MMP1 and MMP7 were significantly increased in tumor tissue compared to normal colon tissue (Paper IV). In comparison of high and low COX-2 gene expression in normal colon tissue, MMP7 was decreased at high COX-2 levels (paper IV). Moreover, MMPs are up-regulated in almost all human tumors as well as in tumor cell lines [135].

Even though age is a risk factor, cancer is the most common reason of death before the age of 75 years [136]. For development of tumors it is necessary to elicit some kind of initiation, such as mutation in *APC*, which results in different gene expression pattern; among others an aberrant COX-2 expression and hence, a different microenvironment [137]. Accumulating evidence suggests that increased prostanoid levels, mainly PGE_2, are important for tumor progression via expression of COX-2 [138].

Progression of colorectal cancer

The Vogelgram have been a widely accepted concept for tumor progression in CRC [54]. However, it is based on genetic progression and needs some additions regarding microenvironmental changes (Fig 2). For instance, a well known tumor suppressor is p53 that regulates cell cycle activity and is often inactivated in tumors either by mutation or by cytokines [50]. Moreover, wild-type p53 inhibits the binding of TATA-binding protein (TBP) to the promoter region of *PTGS2* (COX-2) and may be a factor that reduce gene expression of COX-2 [139]. Indeed, in tumor tissue with high COX-2, expression of *p53* was lower compared to tumor tissue with low COX-2 content (Table 2). However, whether *p53* detected by microarray was wild-type or mutated is unclear. Mutations in *p53* are seen in approximately 50% of human cancers and mutant *p53* may directly or indirectly induce COX-2 expression [140, 141]. In assembly with mutations in genes like *APC*, *K-RAS* and *p53* epigenetic abnormalities like DNA

methylation drives the progression of CRC. Methylations are mediated by DNA methyltransferases (DNMTs) where DNA MTase 3A showed increased expression at high COX-2 in tumor tissue (Table 2). Several transcription factors known to transcribe COX-2 cannot bind to the promoter region when DNA is methylated [134]. However, promoter methylation of COX-2 did not seem to be the explanation for differences in COX-2 gene expression among our patients (paper IV). Others found COX-2 methylations in 10-20% of CRC cases [134].

A classic view of tumors is that tumor cells produce and secrete many of the tissue factors necessary for growth and invasion. However, a current view is also that many factors like COX-2 and MMPs are produced by the surrounding stroma, for example by fibroblasts and inflammatory cells [135]. The importance of the stroma has been confirmed as related to patient survival. Thus, tumors with more stroma than tumor cells may have significantly worse prognosis overall than tumors with high proportion of tumor cells [142, 143].

Treatment of colorectal cancer

The only clear curative treatment for CRC is surgery, which is performed in approximately 80% of patients whereas 40% will remain disease free in the long term [144]. Today, a promising way to overall control cancer is to find ways to prevent significant tumor development by screening, such as colonoscopy, for early diagnosis [145]. Treatments are multioptional since the cause and pathology can be very different within a specific cancer type. In the case of CRC, non-steroid anti-inflammatory drugs (NSAIDs) have provided hope to find effective and comparatively non-toxic treatment. However, inhibition of COX enzymes may cause severe gastrointestinal side effect. Hence, COX-2 inhibitors with a reduction in gastrointestinal events (from 4% to 2%), but with maintained benefits of NSAIDs, were introduced worldwide for treatment of CRC. Unfortunately, increased risk of adverse cardiovascular events like myocardial infarction and stroke were linked to COX-2 inhibitors [27]. A more reasonable approach may be medication with low doses of inhibitors of both COX-1 and COX-2. However, understanding the contribution of individual prostanoids and leukotrienes in the pathogenesis of cancer could enable identification of new and safer therapeutic and chemopreventive agents with benefits and few side effects [110]. Also, the effects of COX inhibition on AA metabolism considering 5-LOX and its metabolites is not fully evaluated and might be rewarding, just like combined COX and 5-LOX inhibitors [30].

Although NSAIDs are still among the most promising chemopreventive agents for cancer, cardiovascular and gastrointestinal side effects have dampened the enthusiasm for their use as chemopreventive agents. Therefore, it is crucial to evaluate whether antagonists of PGE_2 and its receptors have specificity for CRC prevention and treatment. Accordingly, preoperative short-term treatment with indomethacin, a non-specific COX-inhibitor, caused several changes of gene expression including prostanoid receptor expression (Table 5, paper III). The receptors for PGE_2 showed no significantly changed expression at indomethacin treatment even though PGE_2 is a dominating product of both COX enzymes and an important mediator of many of the actions of prostanoids in cancer (Table 5) [4].

Table 5. Gene expression of prostanoid receptors and enzymes in normal colon and tumor tissue following preoperative indomethacin treatment.

Tissue	Transcripts	Change of transcripts*
Normal	DP1	↑
Normal	DP2	↑
Tumor	IP	↓
Normal	IP	↓
Tumor	PPARγ	↓
Normal	PPARγ	↓
Normal	TP	↓
Tumor	HPGD	↓
Normal	HPGD	↓
Tumor	mPGES-1	↑
Normal	mPGES-1	↑

*Relative standard curve (units/units GAPDH),
versus placebo-treated in tumor respectively normal colon tissue

Other components of the PG pathway that were changed at short-term indomethacin treatment were mPGES-1 and HPGD. mPGES-1 showed increased expression, while HPGD, the enzyme responsible for prostanoid break-down, was decreased in tumor and normal colon tissue (Table 5). An explanation to these results could be positive feedback following indomethacin exposure [126]. Specific inhibition of PGE_2 seems the most logic way to proceed in treatment of CRC instead of inhibition of the entire prostanoid cascade. Therefore, it is important to define pathways that are deregulated in tumor tissue leading to increased levels of PGE_2.

One hypothetical method to avoid the cardiovascular side effects of COX-2 inhibition is targeting only COX-derived PGE_2 signaling that mediates most of the tumor promoting effects of COX-2. The enzyme that converts PGH_2 to PGE_2 is PGE synthase, which exist in an inducible isoform called mPGES-1. In mice, deletion of mPGES-1 reduced inflammation and pain perception. However, experimental data regarding cardiovascular side effects are inconsistent and it is possible that mPGES-1 inhibition displays similar gastrointestinal side effects as COX-2 inhibition [27]. In rheumatoid arthritis (RA) the expression of mPGES-1 is markedly up-regulated, especially in inflammatory cells. However, treatment of patients with recent biological agents as blockers of TNF-α, did not suppress mPGES-1 expression implying that other mechanisms operate to sustain inflammation independently of TNF-α [19]. Other specific treatments are directed towards the prostanoid receptors. Stimulation of the tumor suppressor PPARγ inhibits the formation of CRC in animal models and seems promising for future treatment of CRC [79]. In other diseases than cancer specific EP receptor agonists or antagonists are in use or have been tested [146]. Still several questions remain to be answered. Hypothetically, it would be interesting to inhibit EP_2 receptor based on our result in paper I that reduced EP_2 receptor expression was related to prolonged survival.

Alterations in tumor tissue of gene expression following preoperative indomethacin treatment were not entirely similar to alterations in normal colon tissue or to findings in cultured colon cancer cells. EP receptors showed no significant change in tumor tissue at short-term preoperative indomethacin treatment. However, others have found that indomethacin decreases EP_2 prostanoid receptor expression in cultured colon cancer cells [147]. In tumor tissue HPGD expression was reduced by indomethacin, while indomethacin treatment in cultured HCA-7 cells rather increased HPGD expression (Fig 7). This and other observations point towards the importance of tumor stroma [107].

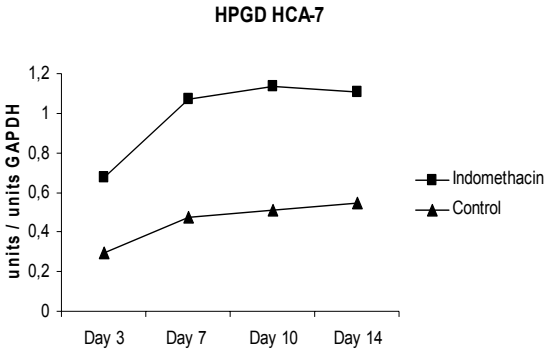

Fig 7. In tumor cells (HCA-7) expression of HPGD is increased at indomethacin treatment.

It may be possible that different medications act differently depending on the stage of cancer since cancer development may progress through alterations in control. Evidence on treatment with NSAIDs and COX-2 inhibitors support benefits of anti-inflammatory treatment at early stages of neoplastic progression. Some benefits may also occur at activation of immune responses (B and T lymphocytes) by NSAIDs at later tumor stages [1, 148]. Patients respond differently to COX inhibition depending on the extent of COX-2 expression in the tumor, which makes sense. A retrospective cohort study showed that aspirin (non-specific COX-inhibitor) specifically reduced CRC risk in subgroup of patients with tumors that expressed high levels of COX-2. In addition to prevention, regular aspirin use after diagnosis of CRC at stages I-III improved overall survival, especially among individuals with tumors that over-expressed COX-2 [110, 119]. Nevertheless, while good evidence exists suggesting that COX-2/PGE_2 pathway inhibition may be useful in the prevention of CRC, this may not be the case under all circumstances. Indeed, COX-2/PGE_2 signaling is likely to act in concert with other important signaling pathways that become deregulated in cancer, meaning that such pathways would also need to be targeted in conjunction with the COX-2/PGE_2 pathway for efficient prevention and treatment of CRC.

CONCLUDING REMARKS

Tumor tissue specimens of CRC achieved at operations were an excellent source for biological analyses of COX and prostanoid metabolism with overall results that are not always in agreement with previous published results from cell and animal experiments. Our present experiments displayed reduced expression of prostanoid receptors, except TP, in human CRC. Also, a relationship between EP_2 receptor and survival was demonstrated. COX-1 showed significantly reduced expression in tumor tissue, while gene expression of COX-2 was overall not significantly changed with a possible explanation of uneven distributions of COX-2 protein in tumor tissue where 20-60% of all neoplastic cells did not express COX-2 at all as reported by others [87]. Our results displayed changes in expression of several different transcription factors at high content of COX-2 in both tumor and normal colon tissue. Indeed, the whole genome expression changed markedly at preoperative inhibition of COX-1/COX-2 by indomethacin. Such changed genes are involved in many different actions such as immunity, regulation of transcription, and cell mobility. Seen together, our results indicated that gene expression of prostanoid receptors are changed in tumor tissue and COX-2 expression in tumor cells is affected by several different signaling pathways. It appears of great importance to understand the metabolism of prostanoids and related signaling pathways, as well as the mechanism behind induced COX-2 expression in tumor tissue for application of such information in clinical settings. A detailed understanding of cross-talk between stroma and tumor cells and related signaling pathways behind increased PGE_2 production in CRC is the next step to define future therapeutical targets in treatment of CRC.

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