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The role of CysLT1R in mouse models of colorectal cancer

Sayeh Savari



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DOCTORAL DISSERTATION

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Abstract <p>Cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) are potent pro-inflammatory lipids derived from arachidonic acid and mediate their effect through CysLT1R and CysLT2R. There is a strong correlation between long-standing inflammatory bowel disease where these pro-inflammatory mediators are abundant and colorectal cancer. We have shown that LTD₄ via its receptor CysLT1 induces expression of proteins associated with colorectal cancer and promotes proliferation, survival and migration in intestinal epithelial cells. In addition, we have demonstrated that that high expression of CysLT1R in colorectal adenocarcinomas predicts poor prognosis in patients.</p> <p>In the presented papers in this thesis we investigated the role of CysLT1R in different mouse models of colorectal cancer. In the mouse xenograft model of colon cancer, we were able to observe a reduced tumor growth in response to CysLT1R antagonist treatment. The inhibition of the tumor growth was accompanied with changes in proliferation and apoptosis as determined by reduced Ki-67 expression, increased expression of p21^{WAF/Cip1}, cleaved caspase 3 and caspase-cleaved keratin 18. An impaired tumor angiogenesis was also demonstrated by detection of increased levels of VEGF and reduced vessel size. We also investigated the role of CysLT1R in 1) FAP/sporadic colorectal cancer by crossing ApcMin⁺ mice with mice lacking CysLT1R expression and in 2) colitis-associated colorectal cancer by employing the AOM/DSS-model on mice lacking CysLT1R expression. Interestingly, a reduced polyp formation in a gender-specific manner could be observed in both models. CysLT1R knockout female mice, but not male mice exhibited a reduced polyp formation in the small intestine and colon, respectively. Also, a decreased nuclear expression of β-catenin within the epithelial tumor compartment was determined for CysLT1R mutant female mice in both models. However, the mechanism of tumor progression in FAP/sporadic colorectal cancer and in colitis-associated colorectal cancer might differ as indicated by reduced tumor expression of COX-2 and reduced serum levels of PGE₂ in the female double mutant (CysLT₁R^{-/-} Apc^{Min/+}) mice, whereas AOM/DSS-treated female single mutant (CysLT₁R^{-/-}) mice demonstrated increased serum levels of PGE₂. In conclusion, the presented mouse models of colorectal cancer further strengthen our previous <i>in vitro</i> findings and highlight the prospect of CysLT1R as an alternative therapeutic approach.</p>		
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To my family

Table of contents

Table of contents	7
List of papers	9
Abbreviations	11
Introduction	15
Background.....	17
The intestinal tract and its barrier function	17
The pathogenesis of the intestine	18
Inflammatory bowel disease	18
Colitis associated colorectal cancer	19
Sporadic and hereditary colorectal cancer	20
Eicosanoids	25
The 5-LOX pathway	26
Eicosanoids and cancer.....	30
Present investigation	35
Aim	35
Materials and methods	35
Results and discussion	39
CysLT1R antagonists inhibit tumor growth in a xenograft model of colon cancer (Paper I)	39
CysLT1R expression influences intestinal polyp incidence in a gender-specific manner in the $Apc^{Min/+}$ mouse model (Paper II).....	40
The relative expression of the inflammatory receptor CysLT1 affects tumorigenesis in a colitis-associated colorectal cancer model (Paper III)	41
Summary	42
Populärvetenskaplig sammanfattning	43
Acknowledgements	45
References	47

List of papers

Paper I (Published)

CysLT1R antagonists inhibit tumor growth in a xenograft model of colon cancer. **Sayeh Savari**, Minghui Liu, Yuan Zhang, Wondossen Sime and Anita Sjölander. PLoS One. 2013 Sep 5;8(9):e73466. doi: 10.1371/journal.pone.0073466.

Paper II (Manuscript)

*CysLT1R expression influences intestinal tumorigenesis in a gender-specific manner in the *Apc^{Min/+}* mouse model.* **Sayeh Savari**, Naveen Kumar Chandrashekar, Janina Osman, Kishan Bellamkonda, Desiree Douglas, Gunilla Jönsson, Maria Juhas and Gedas Greicius, Sven Petterson and Anita Sjölander.

Paper III (Manuscript)

The relative expression of the inflammatory receptor CysLT1 determines the tumor incidence in a colitis-associated colorectal cancer model. **Sayeh Savari**, Janina Osman, Naveen Kumar Chandrashekar, Kishan Bellamkonda, Desiree Douglas, Gunilla Jönsson, Maria Juhas and Anita Sjölander.

Abbreviations

5-ASA	5-aminosalicylic acid
AA	araidonic acid
AC	adenylyl cyclase
AJCC	American Joint Committee on Cancer
AOM	azoxymethane
APC	adenomatous polyposis coli
BrdU	bromodeoxyuridine
CAC	colitis-associated colorectal cancer
CD	Crohn's disease
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
cPLA ₂	cytosolic phospholipase A ₂
CRC	colorectal cancer
CREB	cAMP response element-binding protein
CT	computed tomography
CysLT	cysteinyl leukotriene
CysLT1R	cysteinyl leukotriene receptor 1
CysLT2R	cysteinyl leukotriene receptor 2
CysLTR	cysteinyl leukotriene receptor
DAG	1,2-diacylglycerol
DSS	dextran sulfate sodium
Dvl	dishevelled
EGF	epidermal growth factor
EP2	prostaglandin E receptor 2

EP4	prostaglandin E receptor 4
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FLAP	5-lipoxygenase activating protein
Fz	frizzled
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanine nucleotide diphosphate
GEF	GDP/GTP exchange factor
GPCR	G-protein coupled receptor
GSK-3 β	glycogen synthase kinase 3-beta
GTP	guanine nucleotide triphosphate
HNPCC	hereditary nonpolyposis colorectal cancer
HPETE	hydroperoxyeicosatetraenoic acid
IBD	inflammatory bowel disease
IESC	intestinal epithelial stem cell
IL-6	interleukin-6
IP ₃	inositol 1,4,5-triphosphate
LEF/TCF	lymphoid enhanced factor/T-cell factor
LOH	loss of heterozygosity
LOX	lipoxygenase
LRP5/6	lipoprotein-related protein 5 or 6
LT	leukotriene
LTA4	leukotriene A4
LTB4	leukotriene B4
LTC4	cysteinyl leukotriene C4
LTD4	cysteinyl leukotriene D4
LTE4	cysteinyl leukotriene E4
Min	murine intestinal neoplasia
MRP1	multidrug resistance-associated protein 1

MSI	microsatellite instability
MUC2	mucin 2
NF- κ B	nuclear factor- κ B
NSAIDS	non-steroidal anti-inflammatory drugs
p90 ^{RSK}	p90 ribosomal S6 kinase
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLC- β	phospholipase C- β
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
SRS	slow-reacting substance
STAT3	signal transducer and activator of transcription 3
TCC	transitional cell carcinoma
TNF- α	tumor necrosis factor-alpha
UC	ulcerative colitis
UICC	International Union Against Cancer
VEGF	vascular endothelial growth factor

Introduction

The first recorded descriptions of cancer are from the ancient Egypt (approx. 3000 BC). The word cancer originates from the findings of the Greek physician Hippocrates (460-375 BC), whom probably perceived the outgrowth of tumors similar in appearance to crabs. The Greek term for crab was later translated by the Roman physician Celsus (25 BC-50 AD) to the Latin *cancer* (1). Although tremendous progress has been made in the treatment of cancer, from the breakthrough of surgery during the 19th century and today's use of drugs as adjuvant to surgery and radiation therapy combined with early detection possibilities, cancer still remains the second major cause of lethality worldwide (2). The understanding that various factors such as chemical carcinogens, ionizing radiation and viruses can cause non-inherited cancer by inducing genetic damage, resulted eventually in the pinpointing of genes that were pivotal in cancer development. In the 1970's the scientists classified these genes in two families, referred to as proto-oncogenes and tumor suppressor genes (3). Accumulation of mutations in these genes is believed to cause cellular alterations that convey growth advantage and clonal expansion in analogy of Darwinian evolution, and subsequently cancer development. The essential cellular alterations that are required for neoplastic transformation, referred to as hallmarks of cancer and includes sustained proliferative and replicative ability, evasion of growth suppression and cell death, and induction of angiogenesis and invasion/metastasis (4). More recently, reprogramming of energy metabolism and evasion of immune destruction have emerged as new hallmarks of cancer. The inflammatory milieu has also been appreciated as one of the enabling characteristics of cancer, fostering several of these hallmarks (5). One of the most established connections between inflammation and cancer is between long-standing inflammatory disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD) and colorectal cancer (CRC) (6, 7).

We have previously shown that the pro-inflammatory lipid derived cysteinyl leukotriene D₄ (LTD₄) activation of the cysteinyl leukotriene receptor 1 (CysLT1R) induces proliferation, survival and migration in intestinal epithelial cells (8, 9). Moreover, high CysLT1R expression in colorectal adenocarcinoma tissues of Dukes' B patients has been associated with poor survival prognosis (10).

The foremost interest was to investigate the role of the CysLT1R in colorectal cancer development, taking into account the tumor microenvironment and employing mouse models.

Background

The intestinal tract and its barrier function

The intestinal tract is a tubular construct composed of three tissue layers. The outermost layer with sheets of innervated smooth muscle, the stromal layer in the middle and the innermost epithelial layer. Moreover, the epithelial monolayer of the intestine is the largest mucosal surface in mammals, covering a surface area of 400 m². In addition to water- and nutrition absorption it functions as a barrier, separating the mammalian host from the external environment. The small intestine is subdivided into the duodenum, jejunum and ileum. The absorptive area of the small intestine is profoundly increased by luminal protrusions, villi and invaginations into the submucosa, crypts of Lieberkün. The large intestine on the other hand is composed of a flat epithelium with only crypts and is roughly subdivided in cecum, colon and rectum/anus. Notably, the intestinal epithelium is continually renewed every ~ 5 day by proliferation of intestinal epithelial stem cells (IESCs) at the base of the crypts. This creates an upward movement of proliferative progenitor cells and shedding of differentiated cells at the top of villi/crypts (11, 12) (Figure 1).

There are four types of terminally differentiated cell types. Majority being absorptive enterocytes, which have a brush border termed microvilli at their apical surface that is of assistance in their digestive task. The other three differentiated cells are secretory and part of maintaining the barrier or digestive function. The luminal secretion of antimicrobial proteins and mucins and by Paneth cells and goblet cells, respectively, provides a physical and biochemical barrier. Conversely, the secretion of hormones such as serotonin and secretin by enteroendocrine cells regulates digestion. Paneth cells differ from the other cell types in that they reside in the crypts whilst the rest reside in the luminal part of the small intestine. However, the crypts of the large intestine in mammals lack Paneth cells. The differentiated cells of the large intestine reside in the upper one third of the crypt epithelium, while the proliferating compartment makes up the rest (11, 12).

Mucins are highly glycosylated proteins and they are the first line of defense, not only against microbes but also mechanical- and chemical stress. Mucin 2 (MUC2) is a secreted gel forming mucin and the most prominent in the mucosal layer of the intestine. It is secreted by differentiated goblet cells and is responsible for their characteristic morphology with apparent subapical granules (13). The importance of

MUC2 has been emphasized in *Muc2^{-/-}* mice, which develop spontaneous colitis and are predisposed to CRC (14-16).

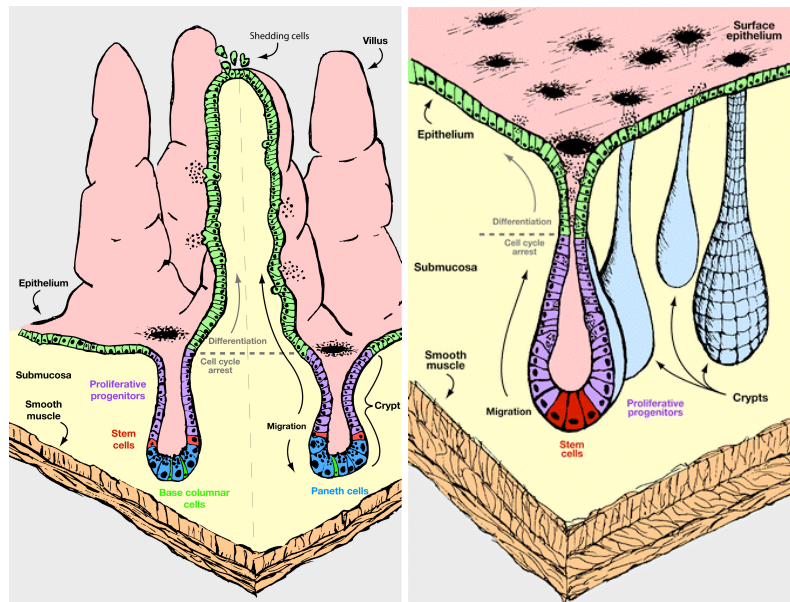


Figure 1. The structure of the small intestine (left) and large intestine (right). Adapted from Clevers, H *et al.*, 2004.

The pathogenesis of the intestine

Inflammatory bowel disease

IBD is a chronic inflammatory condition of the intestine. The two major forms of IBD are UC and CD. UC may affect the mucosal lining of the colon in a continuous pattern, initiating in the rectum and involving part of or the entire colon. Crohn's disease may affect the whole intestinal wall and can occur in patches anywhere along the gastrointestinal tract. Although they are distinct diseases they share clinical symptoms such as diarrhea, abdominal pain, gastrointestinal bleeding and weight loss (17). The pathogenesis of IBD have a genetic basis and genome-wide association studies have identified loci that overlap and others that are unique for either UC or CD (18). The role of lipid inflammatory mediators has been implicated and one of the overlapping genetic risk factors for UC and CD is *PTGER4*, the prostaglandin E

receptor 4 (EP4), which acts as a receptor for the lipid inflammatory mediator prostaglandin E2 (PGE2) (19, 20). However, IBD patients have demonstrated relapse receiving non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of the cyclooxygenase (COX) enzyme, responsible for production of prostanoids such as PGE2. Accordingly, mice that were either deficient in PTGER4 (*Ptger4^{-/-}*) or received NSAIDs exhibited increased susceptibility in the dextran sulfate sodium (DSS)-induced model of colitis (21). An increased susceptibility has also been observed in DSS treated mice deficient in COX-1 (*Ptgs1^{-/-}*) and COX-2 (*Ptgs2^{-/-}*) (22). Another group of lipid inflammatory mediators that has been implicated in the pathogenesis of IBD are the leukotrienes (LTs). Increased levels of leukotriene B4 (LTB4) and cysteinyl leukotriene E4 (LTE4) have been observed in the colonic mucosa and in the urine of patients with active IBD(23, 24). In addition, animal models of colitis have shown that both pharmacological inhibition and deletion of the gene encoding 5-LOX, the enzyme responsible for production of leukotrienes, reduces the severity of the disease (25-27).

Colitis associated colorectal cancer

There is an established connection between inflammation and cancer (5, 28), and perhaps one of the best characterized is between IBD and colorectal cancer, referred to as colitis-associated colorectal cancer (CAC). Although CAC represents only 1-2% of all cases of colorectal cancer (29) (Figure 2), these patients have a high mortality rate and an approximate 50% 5-year overall survival have been estimated for CAC patients (30). Crohn *et al.* were the first to describe the increased risk of developing colorectal cancer in IBD patients in 1925 (31). Population-based studies have estimated that IBD patients, compared to age-matched healthy individuals have a 2- to 5- fold increased risk of developing colorectal cancer (32). Family history of colorectal cancer, early onset and concomitant primary sclerosing cholangitis are some of the established risk factors of developing colorectal cancer in IBD patients. Other risk factors are duration, extent and severity of inflammation of the affected intestinal mucosa (33). Treatment of IBD patients with the anti-inflammatory agent 5-aminosalicylic acid (5-ASA) has demonstrated a reduced risk of developing CAC (34-36). In line with these studies is the reduction of dysplastic colon lesions in 5-ASA treated mice in the azoxymethane (AOM)-DSS model of CAC (37).

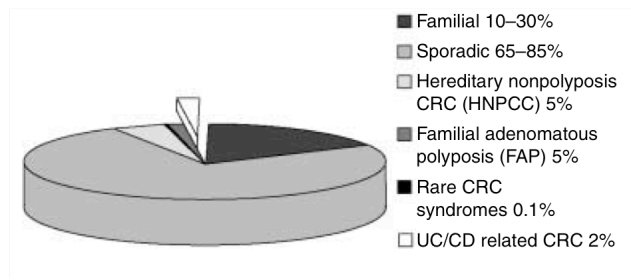


Figure 2. Prevalence and etiology of colorectal cancer (CRC) in the general population. Adapted from P. Munkholm, 2003.

Sporadic and hereditary colorectal cancer

CRC is the third most prevalent cancer, affecting men and women almost equally and the fourth leading cause of all cancer-related deaths worldwide (38). The majority of all CRC cases are either sporadic (65-85%) or hereditary (10-30%) (39)(Figure 2).

Pathogenesis of CRC (CAC vs. sporadic CRC)

The pathogenesis of sporadic/familial CRC shares many similarities with CAC, independently of evident inflammatory disease in the former case. In general, CRC develops from dysplastic precursor lesion and the sequence of progression that follows includes formation of adenoma and subsequently carcinoma (40). Although an alternative sequence have been suggested for CAC development including various degree of dysplasia without the formation of the adenoma (Figure 3). The adenoma, a distinct area of neoplasia can easily be removed by endoscopic polypectomy, whereas the dysplastic lesions in CAC patients can be polypoid/nonpolypoid localized or multifocal, which could require the removal of the entire colon-rectum. These morphological changes are accompanied with a specific sequence of molecular alterations acquired throughout the tumor progression. Many of these alterations are common features for sporadic CRC and CAC, such as development of aneuploidy (chromosomal instability), microsatellite instability (MSI), DNA methylation, activation of the oncogene *KRAS*, increased expression of *COX-2*, allelic deletion and eventual loss of function of *p53*, adenomatous polyposis coli (*APC*), and *DCC/DPC4* (41) (Figure 3). However, there are some differences between sporadic CRC and CAC in the frequency and timing at which these molecular alterations occur. For example, allelic deletion of *APC* is one of the earliest events in the pathogenesis of sporadic CRC while occurring later and less frequent in CAC (42-44). It has been estimated that nearly 70% of sporadic tumors harbor allelic *APC* mutations, while approximately 30% of colitis-associated tumors seem to harbor the same mutation

(45, 46). On the other hand, loss of function mutation of p53 is more common and occurs early in the progression of CAC. Approximately 50-80% of colitis-associated tumors have been shown to have allelic deletion of *p53*, and loss of p53 function is an important genetic factor in the malignant progression of CAC (47).

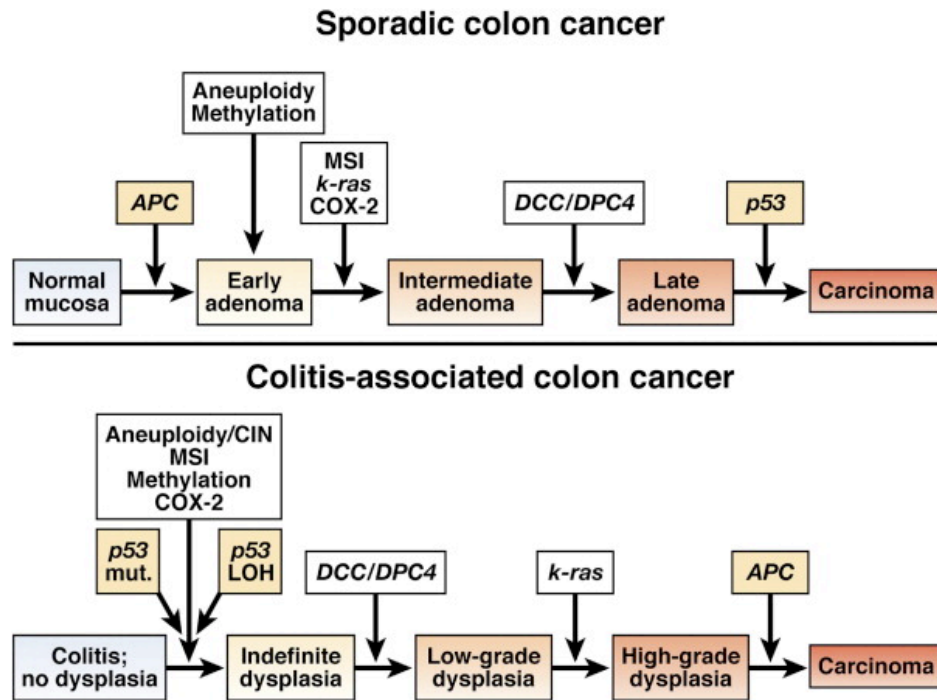


Figure 3. Molecular pathogenesis of sporadic colorectal cancer (top) and colitis-associated colorectal cancer (bottom). Adapted from Itzkowitz *et al.*, 2011.

Even though chronic inflammation might not be required for initial pathogenesis of sporadic CRC, there are evidences that inflammation could be of importance for perpetuation of the disease. Tumors of CRC display increased inflammatory infiltration of immune cells and expression of proinflammatory cytokines. In general, high frequency of tumor infiltrating lymphocytes is associated with good prognosis (48). The subtype of tumor infiltrating lymphocyte has also been shown to have prognostic value. Low CD4⁺:CD8⁺ and high CD3⁺:Foxp3⁺ ratio of tumor infiltrating lymphocytes are considered conveying a better clinical outcome and higher 5-year survival for colorectal patients (49, 50). In addition to producing mutagens such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that may

induce DNA damage and facilitate tumor progression (51), the infiltrating immune cells can also secrete inflammatory cytokines. The best-characterized cytokines in promotion of CAC are tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (52, 53). IL-6 signaling via signal transducer and activator of transcription 3 (STAT3), and TNF- α signaling via nuclear factor- κ B (NF- κ B) have been implicated in the pathogenesis of CAC (41). Activation of these transcription factors induces expression of genes such as *Bcl2* or *Bcl-xL* that suppresses apoptosis, *Cyclin D1* or *c-Myc* that mediate proliferation, and vascular endothelial growth factor (*VEGF*) that promotes angiogenesis (40).

FAP and the Wnt/ β -catenin signaling pathway

Inherited forms of colorectal cancer harbor germline gene mutations and include familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC), hamartomatous polyposis and other more rare variants. There is a difference between hereditary and familial colorectal cancer, the latter comprises a predisposition but without a defined genetic basis. It is estimated that the lifetime risk of developing familial colorectal cancer increases by approximately 20% with affected first and/or second degree relatives while reaching 80-100% in hereditary cases (54).

The autosomal dominant inherited disorder FAP was first described by Lockhart-Mummery in 1925 (55). FAP is characterized by hundreds to thousands of colorectal small adenomatous polyps that usually emerges in the second-third decade of life and if not dealt with surgically, progresses to colorectal cancer (56). Positional cloning has verified germline mutations of the tumor suppressor *APC* gene responsible for FAP (57-59). The majority of these are mutations that result in truncated APC protein with aberrant function. Colorectal adenomas of FAP patients have shown to harbor an additional (somatic) mutation or display loss of heterozygosity (LOH) of the second *APC* allele (60, 61).

The APC protein regulates the activity of β -catenin via the canonical Wnt signaling pathway. β -catenin has several subcellular localizations, at the cell membrane where it maintains cell-cell adhesions together with E-cadherin, in the cytoplasm where it is tightly regulated by a complex of proteins or in the nucleus where it has a transcriptional activity. The canonical Wnt pathway is involved in physiological processes such as embryonic development but also in various cancers, including colorectal cancer. The cytoplasmic β -catenin is in the absence of Wnt ligands targeted by the destruction complex including APC, glycogen synthase kinase 3- β (GSK-3 β), Axin and other proteins. Subsequently, this leads to the phosphorylation, ubiquitination and finally proteasomal degradation of β -catenin. In contrast, upon Wnt binding to the G-protein coupled receptor Frizzled (Fz), the scaffold protein Dishevelled (Dvl) is recruited, which leads to phosphorylation of the co-receptor lipoprotein-related protein 5 or 6 (LRP5/6) and further recruitment of Axin and GSK-3 β . Prevented assembly of the destruction complex allows activation of β -

catenin and its translocation to the nucleus where it together with transcriptional factors such as lymphoid enhanced factor/T-cell factor (LEF/TCF) can drive transcription of oncogenic genes such as *Cyclin D1* or *c-Myc* (62) (Figure 4).

Approximately 90% of sporadic colorectal cancers harbor activating mutations in the Wnt pathway. Other mutations that result in constitutive active Wnt signaling are those occurring in β -catenin, Axin, GSK-3 β but these are significantly less frequent (~10%) than APC mutations (~80%) (63). Activation of the Wnt pathway is important for adenoma initiation but insufficient by itself to drive carcinogenesis. Accordingly, it has been demonstrated that activation of additional signaling pathways, such as those mediated by KRAS and nuclear localization of β -catenin are required for CRC progression (64).

There are alternative ways of achieving nuclear accumulation of β -catenin, without an activating mutation in the Wnt pathway. Interestingly, LTD4 and PGE2 stimulation of CysLT1R and prostaglandin E receptor 2 (EP2), respectively, have been demonstrated to induce β -catenin nuclear accumulation and transcriptional activity in colon cancer cells and stimulate growth (65, 66).

The role of PGE2 in colorectal cancer has been further established using a mouse model of FAP. Crossbreeding with mice lacking expression of COX-2 (*Ptgs2*) and the PGE2 receptor EP2 (*Ptgerep2*) has demonstrated attenuation in intestinal polyp formation (67, 68). Several animal models exist for FAP. The first described was the murine intestinal neoplasia (Min) model, which developed multiple benign adenomas predominantly in the small intestine (69). The gene responsible for this phenotype was later identified as the murine homolog of the *APC* gene (70). Gene targeting strategies has led to additional *Apc* knockout models such as *Apc1638N* and *Apc Δ 716* (71, 72).

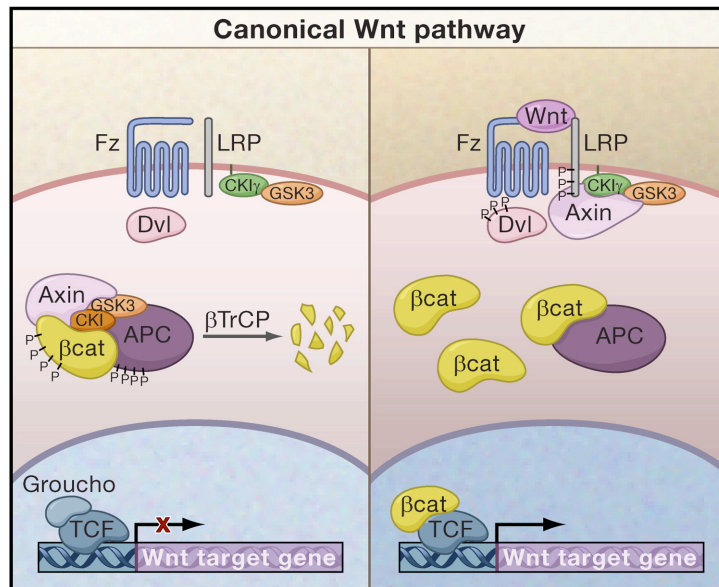


Figure 4. Canonical Wnt/ β -catenin signaling pathway. Adapted from H. Clevers, 2006.

Tumor staging and treatment

Colorectal cancer can be diagnosed with colonoscopy or sigmoidoscopy and tumor biopsy. Other imaging tests such as computed tomography (CT) colonography could be used to evaluate possibility of distant metastasis (73, 74). Staging of the disease is the strongest predictor of survival and of importance for the determination of treatment strategy. The most common is the TNM (Tumor-Node-Metastasis) staging system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (75). The TNM system assigns a stage based on the degree of invasion of the intestinal wall by the primary tumor (T), the degree of lymphatic node involvement (N) and degree of metastasis (M). Surgery is the main curative treatment in localized colorectal cancer. Although in node-positive (stage II-III) disease, preoperative neoadjuvant treatment such as short-course radiotherapy or long-course chemoradiotherapy is recommended (73, 74). In colorectal cancer patients with potentially resectable liver-only or lung-only metastases palliative chemotherapy could be beneficial in terms of survival. Targeted treatment with EGFR monoclonal antibodies such as cetuximab has been one the major advances in the management of metastatic disease in colorectal cancer patients with wild-type *KRAS* (76).

Eicosanoids

Eicosanoids derived from the Greek word *eicosa* meaning 20, describes an important class of lipid mediators derived from 20-carbon polyunsaturated fatty acids. The main eicosanoid precursor in most mammalian systems is arachidonic acid (AA), which belongs to the ω -6 family of essential polyunsaturated fatty acids with 4-*cis* double bonds (20:4 ω 6) (77). AA resides predominantly at the second carbon position of membrane phospholipids and its release is mainly initiated by cytosolic phospholipase A₂ (cPLA₂) in response to mechanical stress or specific extracellular stimuli (78). Cytosolic AA is metabolized via three enzymatic pathways. The generated eicosanoids are prostanoids (prostaglandins and thromboxanes), synthesized via the COX pathway, leukotrienes and hydroxyl eicosatetraenoic acids (HETEs), which are generated via the lipoxygenase (LOX) pathway and epoxides via the cytochrome P-450 epoxygenase pathway (Figure 5).

In the early 1930s the vasodepressor and smooth muscle-stimulating activity of “prostaglandins” was discovered. However, the structure and origin of these compounds were not reported until 30 years later by Bergström and Samuelson, for which they were rewarded with the Nobel prize in 1982 (79). These two scientists contributed also to the elucidation of the 5-LOX pathway and the discovery of cysteinyl leukotrienes as the slow-reacting substance (SRS). These compounds were responsible for the smooth muscle contraction in the perfusate of guinea pig lung treated with cobra venom that was observed in 1938 by Feldberg and Kellaway. They were also later found to be the mediators in asthma and other types of immediate hypersensitivity reactions, referred to as slow-reacting substance of anaphylaxis (SRS-A) (80, 81). In addition to their role in acute systemic inflammatory responses (78), the role of eicosanoids in the maintenance of intestinal homeostasis (82) and their dysregulated functions in pathological conditions such as chronic inflammation and cancer is well established (83, 84).

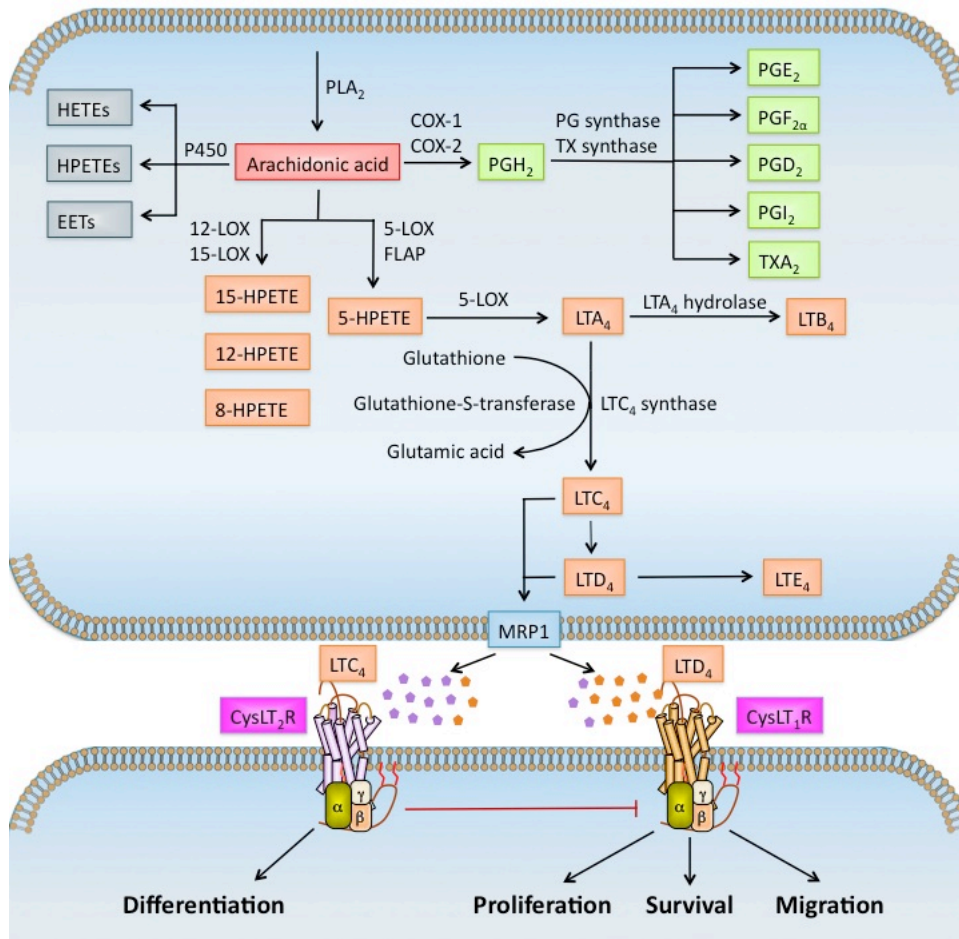


Figure 5. Arachidonic acid metabolism and cysteinyl leukotriene signaling. Adapted from Savari *et al.*, 2014.

The 5-LOX pathway

Leukotrienes

In contrast to prostaglandins, which are produced by most cells, the production of LTs is predominantly constricted to leukocytes such as mast cells, eosinophils, basophils and macrophages. The term *leukotriene* denotes the cell type (*leukocytes*) in which the originally were identified and the presence of three conjugated double bonds (*trienes*) (85).

The mammalian lipoxygenase pathway consists of three enzymes with ability of catalyzing the insertion of an oxygen molecule into AA, at positions 5, 12 or 15 and

generating hydroperoxyeicosatetraenoic acid (i.e. 5-, 12- or 15-HPETE)(77). The 5-lipoxygenase pathway (5-LOX) is involved in the biosynthesis of leukotrienes. The catalytic task of 5-LOX is facilitated 5-lipoxygenase activating protein (FLAP), which does not exert any enzymatic activity. The unstable 5-HPETE undergoes immediate dehydration, yielding leukotriene A₄ (LTA₄). Depending on the cellular context, three metabolic fates are possible for LTA₄: hydrolysis, conjugation with glutathione or transcellular metabolism. Hydrolysis by the cytosolic LTA₄ hydrolase generating LTB₄, a potent neutrophil chemoattractant and promoter of leukocyte adhesion to vascular endothelium. The integral membrane protein LTC₄ synthase conjugates reduced glutathione to LTA₄ to produce cysteinyl leukotriene C₄ (LTC₄). After carrier-mediated transport to the extracellular *milieu* by transporters such as multidrug resistance-associated protein 1(MRP1), LTC₄ undergoes cleavage of the glutamic acid moiety to form LTD₄ by the catalytic action of γ -glutamyl transpeptidase. LTD₄ is further metabolized by dipeptidase through cleavage of the glycine moiety to yield cysteinyl leukotriene E₄ (LTE₄) (Figure 5). The last three mentioned derivatives of LTA₄ are collectively termed *cysteinyl leukotrienes* (CysLTs) due to a common cysteine residue (78, 86, 87). Although 5-LOX is predominately expressed by leukocytes such as neutrophils, eosinophils, macrophages and mast cells, the expression of LTC₄ synthase and LTA₄ hydrolase are expressed more ubiquitously and CysLTs can be produced by nonleukocytes via uptake of exogenous LTA₄, a process referred to as transcellular metabolism (88). As previously stated, CysLTs have been shown to induce airway mucus secretion, increased vascular permeability, eosinophil chemotaxis, and bronchoconstriction (89-92).

Leukotriene receptors

Early pharmacological studies of the smooth muscle contractile properties of different cysteinyl leukotrienes and blocking of contractions with different antagonists indicated the possibility of at least two receptors (93-95). Eventually these CysLT receptors (CysLTRs) received their nomenclature based on their sensitivity to “classical” antagonists including montelukast, zafirlukast, pranlukast and MK571. Accordingly, CysLT1R is sensitive to classical antagonists while the effects of cysteinyl leukotriene receptor 2 (CysLT2R) are not inhibited by these antagonists (96). Cloning and characterization of the human CysLT1 and CysLT2 receptors revealed their position on chromosomes X (Xq13–Xq21) and 13 (13q14), respectively (97, 98). These receptors are seven transmembrane G-protein-coupled receptors (GPCRs) and CysLT-ligand binding induces conformational changes resulting in G-protein activation, including GDP to GTP exchange, GTP hydrolysis and intracellular events such as increase in cytosolic concentration of Ca²⁺ (99).

CysLT1R has high affinity for LTD₄, while CysLT2R has a weaker but equal affinity for LTD₄ and LTC₄ (97, 98). The expression patterns of these receptors are tissue

dependent but not mutually exclusive. Higher expression of the human CysLT1R can be observed in the spleen, peripheral blood leukocytes and less expression in lung, small intestine, pancreas and placenta and little or no expression in the liver, colon, kidney, skeletal muscle, thymus, ovary, testis, heart and brain. In addition to high human CysLT2R expression in the spleen and peripheral blood leukocytes, high expression of CysLT2R has also been observed in the heart, adrenal gland and brain (99). The human CysLT1R shares 38% amino acid identity with the human CysLT2R, and 87% with the mouse CysLT1R (98, 100). Molecular cloning and characterization of the mouse CysLT1R have revealed two potential isoforms resulting from alternative splicing (100, 101). Kanaoka *et al.* have reported the generation of mice with a disrupted coding region of the *CysLT1R* gene that is common for both isoforms (102). A comparison between the human and mouse CysLTRs is described in Table 1. The presence of additional CysLT receptors such as GPR17, P2Y12 and CysLTER has also been proposed (103-105). LTB₄ mediates signaling via two GPCRs, BLT1 and BLT2 (106, 107). BLT1 is a LTB₄ high affinity receptor highly expressed in peripheral blood leukocytes while BLT2 is a LTB₄ low affinity receptor and present in most human tissues, with the highest expression in spleen, liver, ovary, and peripheral blood leukocytes (108, 109). By mediating the effects of LTB₄, the receptors BLT1 and BLT2 play an important role in the host immune defense and the pathogenesis of inflammatory diseases (109). In polymorphonuclear leukocytes such as neutrophils, LTB₄ induces chemotactic response (106, 110), adherence to the endothelium (111), production of superoxides (112), release of lysosomal enzymes (113), phagocytosis of bacteria (114) and increased survival (115).

Table 1. Human and mouse cysteinyl leukotriene receptors. Adapted from Singh *et al.*, 2010.

Receptors	Human CysLT ₁	Human CysLT ₂	Mouse CysLT ₁	Mouse CysLT ₂
Genebank Acc.	AH119711, AF133266	AF254664	AC021992	-
Chromosome	Xq13-q21	13q14.2	XD	14
Agonist	LTD ₄ >> LTC ₄ > LTE ₄	LTD ₄ = LTC ₄ > LTE ₄	LTD ₄ >> LTC ₄ > LTE ₄	LTD ₄ = LTC ₄ > LTE ₄
Antagonist	montelukast, zafirlukast, pranlukast, BAY u9773	BAYu9773	MK571	BAYu9773
Primary coupling	G _{αq}	G _{αq}	G _{αq}	G _{αq}
Primary expression	spleen, lung, smooth muscle	heart, pulmonary vein, adrenal medulla, placenta	skin, lung	spleen, thymus, adrenal gland, small intestine

G-protein coupled receptors

CysLT1R and CysLT2R belong to the rhodopsin family of the seven-transmembrane GPCRs (116). These receptors function as GDP/GTP exchange factors (GEFs) and binding of the extracellular domain to a specific ligand induces a conformational change and promote the release of guanine nucleotide diphosphate (GDP) from the G_α subunit of a specific intracellular heterotrimeric G-protein complex (117, 118). G-proteins are members of the GTPase superfamily and are vaguely attached in the

inner plasma membrane. The $G\alpha$ and $G\beta\gamma$ subunits are detached from each other following the release of GDP and binding of guanine nucleotide triphosphate (GTP), which more or less becomes instantly hydrolyzed and allowing the two subunits to re-associate, hence allowing the heterotrimeric complex to become once again activated (119). The G-proteins are classified based on the α -subunits, and based on their amino acid sequence similarity they are classified as four family members, termed s, i, q, and 12 (119). G-proteins are also historically classified according to their sensitivity to pertussis-toxin and ADP-ribosylation of their $G\alpha$ -subunit, which renders them incapable of associating with the receptor. In general, members of the $G\alpha_i$ family are considered sensitive, whereas members of the G_q and G_{12} families are regarded pertussis toxin insensitive (120). Although both the $G\alpha$ and $G\beta\gamma$ subunits separately can activate different effector molecules, the G-protein effector specificity is considered to be determined by the $G\alpha$ subunit. For example, the G_{α_q} and the G_{α_i} family are coupled to receptors that all can activate phospholipase C- β (PLC- β) which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG), which releases Ca^{2+} from endoplasmic reticulum and activates isozymes of protein kinase C (PKC), respectively (121). The G_{α_s} family has been shown to directly stimulate all mammalian adenylyl cyclase (AC) subtypes whereas certain members of the G_{α_i} family inhibit some AC subtypes and their production of cyclic AMP (122). The different $G\alpha$ subunits are generally considered to associate with specific receptor families, although there are some exceptions such as in the case of CysLT1R (123). LTD₄-induced CysLT1R activation of the monocytic cell lines U937 and THP-1 and the intestinal epithelial cell line Int407 has demonstrated coupling to both PTX insensitive G_{α_q} and PTX sensitive G_{α_i} subunits (124-126). Termination of GPCR signaling, referred to as desensitization is either initiated through homologous desensitization or heterologous desensitization. The former refers to a situation in which only the activated GPCR becomes desensitized while the latter refers to desensitization of one GPCR due to activation of another, heterologous GPCR. The best characterized homologous desensitization is G-protein coupled receptor kinase (GRK)-dependent and initiated when proteins such as arrestins bind to GRK-phosphorylated GPCRs and causing either G-protein uncoupling or receptor internalization (117). In terms of CysLT1R desensitization both types have been encountered. Heterologous desensitization of CysLT1R has been observed in the monocyte/macrophage cell line U937 by series of inflammatory mediators such as LTB₄- induced activation of BLT1, whereas homologous desensitization and arrestin-clathrin-dependent internalization has been demonstrated in the intestinal epithelial cell line Int407 (127, 128).

Eicosanoids and cancer

Leukotriene B₄

LTB₄ has not only been implicated in inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), arthritis and IBD but also in cancers including pancreatic, breast, melanoma, lymphoma and head- and neck- carcinoma (129-134). LTB₄ stimulation has been shown to promote proliferation in the colon cancer cell lines HT-29 and HCT-115 in a time- and concentration-dependent manner (135). Increased expression of BLT1 has been observed in human colon cancer tissues and the inhibition of the LTB₄ signaling pathway have demonstrated reduced proliferation and induction of apoptosis in the colon cancer cell lines Caco-2 and HT-29 (136). Another study has shown that the LTB₄ antagonist LY293111 can potentiate the cytotoxic and tumor growth inhibiting effects of gemcitabine in a xenograft model of colon cancer (137).

Prostaglandin E₂

PGE₂ is the major prostanoid found in several cancers, including CRC, and often conveys poor prognosis. The mechanisms by which PGE₂ has been shown to promote epithelial tumorigenesis include 1) induction of tumor epithelial cell proliferation, survival, and migration/invasion and 2) establishment of a tumor microenvironment where tumor growth and metastatic spread is facilitated by immunosurveillance inhibition and angiogenesis induction (83).

PGE₂ has been shown to increase the tumor burden in *Apc*^{Min/+} and AOM-treated mice (138, 139). However, selective inhibition of PGE₂ via deletion of *mPGES-1*, a PGE₂ terminal synthase in *Apc*^{Δ14/+} mice significantly reduced both the size and number of intestinal tumors (140). Deletion of the *mPGES-1* gene resulted also in ~90% reduction in the colon tumor load of AOM-treated mice, which was associated with expansion of FoxP3-positive regulatory T cells within the colon-draining mesenteric lymph nodes (141). In addition, global *Cox-2* gene disruption and pharmacological inhibition of COX-2 have resulted in reduced intestinal tumor formation in *Apc*^{Min/+} mice (142, 143). Accordingly, tumors were not formed in AOM-treated *Cox-2*^{-/-}-knockout mice. Although, genetic deletion of either *Cox-1* or *Cox-2* did not have any impact on the tumor formation in the AOM/DSS mouse model of CAC (144).

Increased expression of COX-2 has been demonstrated in human colorectal adenocarcinomas and a correlation between increased adenoma prostanoid levels and adenoma size has been established in FAP patients (10, 145). Additionally, treatment with NSAIDs and selective COX-2 inhibitors have been shown to reduce the size and number of intestinal polyps in FAP patients (146-148). Also, patients receiving the selective COX-2 inhibitor celecoxib, after colonoscopic removal of sporadic colorectal adenomas have shown diminished adenoma recurrence (149).

Cysteinyl leukotrienes

Increased expression of CysLT1R has been documented in several human cancers, including bladder transitional cell carcinoma (TCC), neuroblastoma, brain-, prostate-, breast-, and colorectal cancer (10, 150-154). Elevated CysLT1R tumor expression has been associated with poor prognosis in breast cancer and CRC. The expression of CysLT1R in colorectal adenocarcinoma tissues has been positively correlated with cell survival factors such as COX-2 and Bcl-xL (10). However, low nuclear CysLT1R:CysLT2R expression is considered to mediate good prognosis and higher overall survival in CRC (155). Compared to intestinal epithelial cells, higher expression level of CysLT1R (10, 156) and lower expression level of CysLT2R has been observed in colon cancer cell lines (156).

Interestingly, LTC₄ mediated CysLT2R activation in intestinal epithelial cells have been shown to negatively regulate the plasma membrane expression of CysLT1R by inducing internalization of the CysLT1R:CysLT2R heterodimer complex(128). LTD₄ mediated CysLT1R signaling in intestinal epithelial cells has been demonstrated to induce the expression of COX-2, β -catenin, and Bcl-2, which are positively correlated with CRC progression(157). In addition, the increased expression of β -catenin was shown to be mediated by phosphatidylinositol 3-kinase (PI3K)-GSK-3 β signaling (158). Moreover, LTD₄-mediated CysLT1R activation in intestinal epithelial cells via cAMP response element-binding protein (CREB) and p90 ribosomal S6 kinase (p90^{RSK}) signaling has been shown to promote survival and proliferation, respectively, while enabling migration via PI3K-Rac signaling (8, 9) (Figure 6).

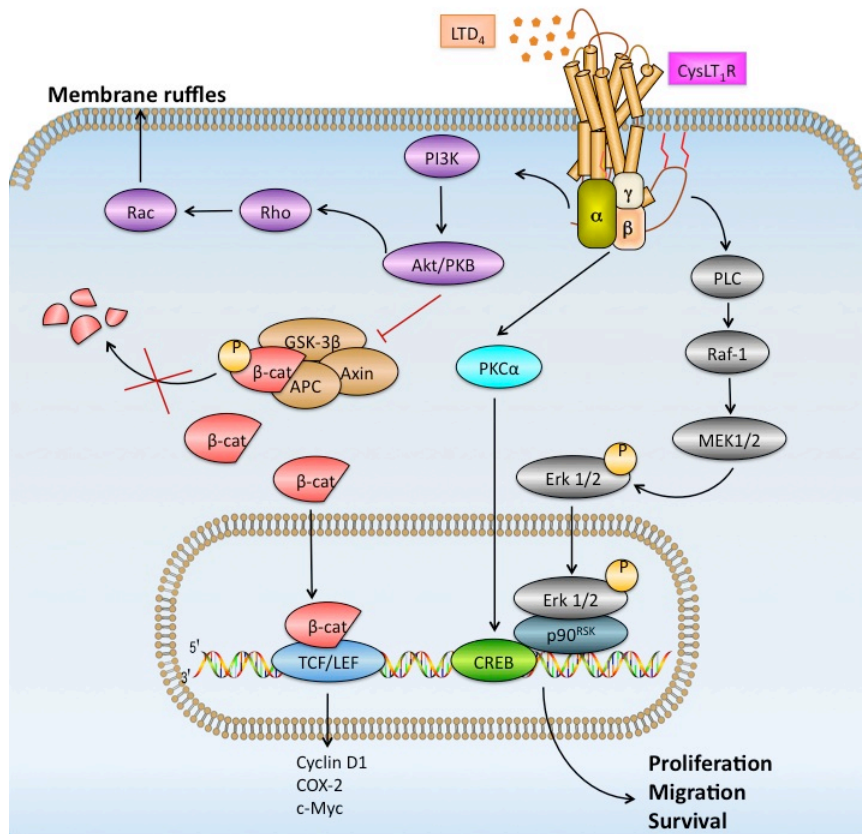


Figure 6. LTD4 signaling via CysLT1R in colorectal cancer. Adapted from Savari *et al.*, 2014.

The expression of COX-2 has been documented in various colon cancer cell lines (159) and has been shown to be upregulated via LTD4-mediated CysLT1R activation and Erk-1/2 signaling in the colon cancer cell line Caco-2, subsequently increasing the expression of the anti-apoptotic protein Bcl-2 and thus promoting survival (160). Furthermore, LTD4-mediated CysLT1R activation in the colon cancer cell line HCT-116 has been shown to induce proliferation and migration, accompanied with nuclear accumulation of β -catenin and increased transcription of the GSK-3 β / β -catenin signaling pathway associated genes including *MYC* and *CCD1* (65).

A potential anti-tumor mechanism of IFN- α could be regulation of the CysLT2R promoter activity and expression (161), and LTC4-mediated CysLT1R activation has been shown to induce differentiation as demonstrated by increased intestinal alkaline phosphatase activity (156) and suppression of epidermal growth factor (EGF)-induced migration (161) in the colon cancer cell line Caco-2.

CysLT1R antagonists have been used in studies of inflammatory diseases including rheumatoid arthritis, atherosclerosis and Alzheimer's disease (162-164). The CysLT1R antagonists montelukast, pranlukast, zafirlukast and the 5-lipoxygenase inhibitor zileuton are currently used to treat asthmatic patients and have shown to reduce bronchial constriction, coughing, bronchial inflammation and the risk of asthmatic exacerbations (165). The CysLT1R antagonists have also been used in the studies of several cancers. In a variety of human urological cancer cell lines (*e.g.*, renal cell carcinoma, bladder cancer, prostate cancer, and testicular cancer) and in neuroblastoma cell lines, the CysLT1R antagonist montelukast has been shown to induce apoptosis (150-152, 166). In the colon cancer cell lines Caco-2 and SW480, the CysLT1R antagonist ZM198,615 has demonstrated reduced proliferation (167). There are several reports on dual inhibition of the COX- and LOX-pathway and augmented anti-tumor effects. A more pronounced decrease in proliferation has been observed in the colon cancer cell lines Caco-2 and HT29 when these cells were treated with the COX-2 selective inhibitor celecoxib in combination with either the 5-LOX inhibitor MK886 or CysLT1R antagonist LY171883. The combined treatment also resulted in induction of apoptosis as evidenced by caspase-3 activation and increased Bax:Bcl-2 expression ratio, whereas either of these inhibitors alone could elicit did any significant effect (168). Dual inhibition of the COX-2 and 5-LOX activity with celecoxib and AA861, respectively, has also demonstrated additional anti-tumor growth effect in a cigarette smoke-promoting mouse xenograft model of CRC. Moreover, the combined treatment resulted in greater inhibitory effect on proliferation and angiogenesis than the individual compounds (169).

Present investigation

Aim

The overall aim of the presented studies has been to investigate the role of CysLT1R in colorectal cancer *in vivo*, using different mouse models.

Materials and methods

Chemicals

The CysLT1R antagonists ZM198,615 (ICI-198,615) and montelukast were from AstraZeneca (London, England, UK) and Cayman Chemicals Co. (Ann Arbor, MI, USA), respectively. Azoxymethane (AOM) and dextran sulfate sodium (DSS) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and MP Biomedicals (Santa Ana, CA, USA), respectively.

Cell culture

The human colon adenocarcinoma cell lines HCT-116 (ATCC® No. CCL-247), SW-480 (ATCC® No. CCL-228) and HT-29 (ATCC® No. HTB-38) were obtained from American Type Culture Collection (Manassas, VA, USA). The HCT-116 cells and HT-29 were cultured in McCoy's 5A medium, whereas SW-480 was cultured in RPMI 1640. The cells were maintained in a humidified incubator at 37°C with 5% CO₂, in the presence of 10% fetal bovine serum (FBS) 55 µg/ml streptomycin, 55 IU/ml penicillin, and 1.5 µg/ml fungizone.

Animals

Athymic nude mice (BalbC nu/nu) were purchased from Taconic Europe A/S (Ry, Denmark) and C57BL/6J-Apc^{Min/+} founder mice from The Jackson Laboratory (Bar Harbor, ME, USA). The CysLT1R knockout mice on a C57BL/6N background were kindly provided by Prof Frank Austen (Harvard Medical School, Brigham and Women's Hospital, Boston MA, USA). Single-mutant (CysLT1R^{+/-}, CysLT1R^{-/-} and Apc^{Min/+}) and double-mutant mice (CysLT1R^{+/-} Apc^{Min/+} and CysLT1R^{-/-} Apc^{Min/+}) and their control littermates were established and maintained at Lund University Animal Facility, Lund. The breeding colony and offspring were genotyped for

CysLT1R and/or *Min* by PCR assays. All animal experiments were conducted according D.Nr. M205-10 (Paper I), M262-12 (Paper III) or M262-13 (Paper II), approved by the Regional Ethical Committee for Animal Research at Lund University, Sweden.

Experimental setup

Female 6- to 8-week-old athymic nude mice (BalbC nu/nu) received subcutaneous injections with 2.5×10^6 HCT-116, SW-480 or HT-29 cells in two flanks per mouse (n = 6-9 mice/group). These mice received daily i.p. injections with either DMSO, ZM198,615 or montelukast (5 mg/kg) once the tumors were palpable. Moreover, mice were inoculated with either DMSO, ZM198,615 or montelukast pretreated HCT-116 cells (50 μ M for 30 min) and received continued treatment from the day of inoculation. The female nude mice were sacrificed 21 days post-inoculation of colon cancer cells and had their tumors either fixed in 10% buffered formalin or snap frozen in liquid nitrogen.

Gender matched 6- to 8-week-old single mutant mice (*CysLT1R*^{+/-} and *CysLT1R*^{-/-}) and their control littermates (C57BL/6N) received AOM (10 mg/kg, i.p.) followed one week later by two 5 day-cycles of 2% DSS in the drinking water with an intermediate recovering period of two weeks. The control mice received vehicle (0.9% NaCl, i.p.) and drinking water without DSS. The mice were sacrificed either after the second cycle of DSS or 60 days after the AOM/vehicle injection. Gender matched double-mutant mice (*CysLT1R*^{+/-} *Apc*^{Min/+} and *CysLT1R*^{-/-} *Apc*^{Min/+}) and their control littermates (*Apc*^{Min/+}) were sacrificed at 14 weeks of age. Approximately half of the animals had their colon (single and double-mutant mice) and/or the small intestine (double-mutant mice) excised, fixed flat in 10% buffered formalin and subsequently evaluated for tumor count/size using a dissection microscope (2X). The entire colon and/or the distal small intestine were finally embedded in paraffin. The remaining animals had their colon and/or small intestine snap frozen in liquid nitrogen.

Immunohistochemistry

Immunostaining of sectioned (5 μ m) paraffin-embedded tissues were performed using Dako automated slide stainer (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The immunostained slides were scanned with Aperio ScanScope CS (Aperio Technologies, Inc, Vista, CA, USA) and evaluated by two independent observers in a blinded fashion. The COX-2 and 5-LOX expression were evaluated as the percentage of positive stained cells within the tumors. The subcellular localization (membraneous, cytosolic, nuclear) was evaluated for β -catenin. MUC2 expressing goblet cells, proliferative cells with incorporated bromodeoxyuridine (BrdU), and CD45-expressing infiltrating cells were estimated by counting the number of positive stained cells within the villi/crypts (n= approx. 10) and the tumor, respectively. Apoptotic cells with expression of the caspase-cleaved

product of cytokeratin 18 (M30) and vessel formation as determined by CD31 expression, were estimated by counting positive stained cells within a predetermined area in the xenograft tumors.

Flow cytometry

CysLT1R antagonist pre-treated HCT-116 cells were subjected to cell cycle and cell death measurements using propidium iodide staining and the Annexin V-PE Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA), respectively. Single cell suspensions of colon or small intestine were obtained using gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA, USA) and stained with specific conjugated antibodies to detect T-cell subpopulations of interest. Measurements were performed with FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), and analyses were performed with FCS Express 4.0 (De Novo Software, Los Angeles, CA, USA).

Cysteinyl leukotriene and prostaglandin E2 enzyme immunoassay

Media from cultured cells and animal sera were purified using solid-phase extraction Sep-Pak Vac RC (C18-500mg) cartridges from Water Corporation (Milford, MA, USA). CysLT and PG serum levels were measured with a competitive enzyme immunoassay obtained from Enzo Life Sciences (Farmingdale, NY, USA), whereas CysLT media levels were determined using an assay from Cayman Chemical Company (Ann Arbor, MI, USA). All measurements were performed according to manufacturer's instructions.

Quantitative RT-PCR

RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was used to extract RNA, that subsequently was used for cDNA synthesis using RevertAid H Minus M-MuLV Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA). Maxima probe/ROX qPCR master mix and Taqman probes were used for amplification in Mx3005P thermocycler (Agilent Technologies Inc., CA, USA). Data were normalized against the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed with MxPro software (Invitrogen Corp, Carlsbad, CA, USA).

Western blotting

HCT-116, SW-480 and HT-29 cell lysates were prepared as previously described (9), whereas xenograft tumors were subjected to sonication. Protein separated on precast any kD™ SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) by electrophoresis were electrotransferred onto PVDF membranes. The membranes were blocked in 5% nonfat dry milk or 5% BSA in 0.05% Tween/PBS at room temperature for 1h and then incubated with primary antibody for either 1 h at room temperature or overnight at 4°C. After washing off unbound antibodies with 0.05%

TWEEN/PBS, the membranes were incubated with HRP-conjugated secondary antibodies for approximately 1 h at room temperature. Detection of proteins was made by the chemiluminescence system of Molecular Imager ChemiDoc XRS System and Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

Proliferation assay

HCT-116 cells were seeded in triplicates in 96-well plates (1500 cells/well). Following 24 h incubation (2% FBS), the cells were treated with CysLT₁R antagonists for different time points. After 90 min incubation with WST-1 reagent (Roche, Basel, Switzerland), the absorption was measured at 440 nm using the Tecan Infinite M200 plate reader.

Adhesion assay

Cells were treated with or without CysLT₁R antagonists for 30 before being seeded in 12-well plates (2.0×10^5 cells/well). After 1 h incubation the cells were fixed in 4% formaldehyde for 15 min and then stained with crystal violet (5 mg/ml in 2% ethanol) for 10 min. Incubations steps longer than 30 min were made in the cell incubator, while shorter incubations were performed at room temperature, followed by (2% SDS)-PBS washing step. The staining intensity was measured at 550 nm using the Tecan Infinite M200 plate reader.

Soft agar assay

Before seeding 1.0×10^4 cells/well in medium (2% FBS) with 0.35% agarose, 0.5 % agar/well (bottom layer) was added to 6-well plates and allowed to solidify. The medium was exchanged every third day, with or without CysLT₁R antagonist supplementation. Cell colonies were visualized after 14 days with 0.005% crystal violet and the ChemiDoc™ XRS+ System.

Statistical analysis

GraphPad Prism version 5.0a (GraphPad *Software* Inc., San Diego, CA, USA) was used for all statistical analyses. Paired or unpaired *t* test (Student's *t* test) was performed for comparison between two groups, while one-way or two-way ANOVA was used to compare more than two groups. P value ≤ 0.05 was considered significant. All data are presented as mean \pm standard error of the mean (SEM).

Results and discussion

CysLT₁R antagonists inhibit tumor growth in a xenograft model of colon cancer (Paper I)

CysLTs are pro-inflammatory lipid mediators involved in physiological conditions including mucus secretion, increased vascular permeability, eosinophil chemotaxis, and bronchoconstriction (89-92). They are also implicated in pathophysiological conditions including rheumatoid arthritis, asthma, and IBD (170-172). There is a strong correlation between the inflammatory milieu and cancer, such as IBD and colorectal cancer. In support, we have previously demonstrated that LTD₄-mediated CysLT₁R activation induces expression of proteins associated with colorectal cancer and promotes survival, proliferation and migration in intestinal epithelial cells (8, 9, 157). To investigate the effects of CysLT₁R on colon cancer growth *in vivo*, we used the HCT-116 xenograft mouse model and two different drug administration regimens of CysLT₁R antagonists (ZM198,615 or montelukast). The first regimen was established to investigate the importance of CysLT₁R in tumor initiation. Mice included in this group were subcutaneously inoculated with CysLT₁R antagonist-pretreated HCT-116 colon cancer cells and received continued daily treatment (5 mg/kg, i.p.). The second regimen aimed to address the role of CysLT₁R in tumor progression. Mice included in this group were inoculated with non-pretreated HCT-116 cells and did not receive CysLT₁R antagonist treatment (5 mg/kg, i.p.) until recordable tumor initiation. Furthermore, we performed a series of *in vitro* studies using the human colon cancer cell line HCT-116 and CysLT₁R antagonists. Both CysLT₁R antagonist administration regimens resulted in significantly reduced tumor size, which was accompanied with increased levels of p21^{WAF/Cip1} ($P < 0.01$), cleaved caspase 3, and the caspase-cleaved product of cytokeratin 18. These data were further strengthened *in vitro* by the findings of induced apoptosis and cell cycle arrest at G1 phase in the colon cancer cell line HCT-116 after CysLT₁R antagonist treatment in a dose-dependent manner, as analyzed by flow cytometry. In the same notion, montelukast has been shown to induce apoptosis in a series of human urological cancer cell lines and in neuroblastoma cell lines (150-152, 166). We were also able to detect decreased levels of VEGF ($P < 0.01$) and reduced vessel size ($P < 0.05$), the latter only in tumors established from CysLT₁R antagonist pre-treated HCT-116 cells. Our results fits well with previous findings of montelukast reducing LTD₄-mediated CysLT₁R activation and migration of endothelial cells, a process that is required for new vessel formation (173). Furthermore, *in vitro* studies with the colon cancer cell line HCT-116 demonstrated a significant reduction in the ability of these

cells to proliferate, adhere and form colonies under the influence of CysLT1R antagonists. The ability of montelukast to inhibit tumor growth was further established by using the additional colon cancer cell lines SW-480 and HT-29 in the mouse xenograft model.

CysLT1R expression influences intestinal polyp incidence in a gender-specific manner in the $Apc^{Min/+}$ mouse model (Paper II)

High expression of CysLT1R has been observed in adenocarcinomas of colorectal cancer patients and associated with poor prognosis. In the present study we investigated the role of CysLT1R in tumorigenesis by crossing the $Apc^{Min/+}$ mice with mice lacking CysLT1R expression. The female, and not the male double mutant mice exhibited a significant ($P < 0.05$) reduction of small intestinal polyp formation in a CysLT1R gene-dosage dependent manner. A gender specific tumor reduction due to a gene deletion in $Apc^{Min/+}$ mice has been previously reported. Targeted gene deletion of *Cox-2* in the intestinal epithelium of female, but not male $Apc^{Min/+}$ mice was shown resulted to reduce intestinal polyp formation (174). The female double mutant ($CysLT_1R^{-/-} Apc^{Min/+}$) phenotype was accompanied with significantly decreased intestinal polyp expression of 5-LOX and COX-2, in addition to decreased serum levels of CysLTs and PGE2. We have previously demonstrated an up-regulation of COX-2 expression in intestinal epithelial cells and colon cancer cells mediated by LTD4 stimulation of CysLT1R (157, 160). Additionally, COX-2 and 5-LOX expression in colorectal cancer specimens positively correlates with CysLT1R expression (10). We therefore speculate that global *CysLT1R* gene deletion results in decreased 5-LOX and COX expression and activity as evidenced by reduced CysLT and PGE2 serum levels. In addition, we were also able to detect reduced nuclear translocation of β -catenin within the epithelial compartment of small intestinal polyps in female double mutant ($CysLT_1R^{-/-} Apc^{Min/+}$) mice compared to wild-type littermates ($CysLT_1R^{+/+} Apc^{Min/+}$). Interestingly, deletion of *mPGES-1* that encodes the enzyme that is responsible for the production of PGE2 has been shown to inhibit nuclear translocation of β -catenin in the carcinogen azoxymethane-induced colonic lesions of mice (140). The frequency, type and site of tumor infiltrating cells have been suggested as stronger predictor of prognosis than TNM classification in colorectal cancer (175). High tumor infiltration of CD8⁺ T cells has been positively correlated with patient survival (176). In line with this, we were able to detect an increased CD3⁺CD8⁺ T cell tumor infiltration in female double mutant ($CysLT_1R^{-/-} Apc^{Min/+}$) mice compared to wild-type littermates ($CysLT_1R^{+/+} Apc^{Min/+}$). Notably, PGE2 has been demonstrated to block the activity and expansion of CD3⁺CD8⁺ T cells and thereby contributing to the evasion of tumor cells from the immune surveillance (177). We speculate that decreased expression of COX-2, and subsequent

reduced production of PGE₂, contributes to higher tumor infiltration of CD8⁺ T cells and reduced nuclear translocation of β -catenin, which combined results in the reduced tumor incidence observed in female double mutant (CysLT₁R^{-/-} Apc^{Min/+}).

The relative expression of the inflammatory receptor CysLT1 affects tumorigenesis in a colitis-associated colorectal cancer model (Paper III)

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) involves chronic inflammation of the intestinal tract. The increased risk of developing colorectal cancer in patients with ulcerative colitis was recognized by Crohn *et al.* (31) and various population-based studies have estimated a 2- to 5-fold increased risk for IBD patients of developing colorectal cancer (32). To investigate the role of CysLT₁R in colitis-associated colorectal cancer, we used CysLT₁R knockout mice and employed the AOM/DSS-model. AOM/DSS-treated female knockout (CysLT₁R^{-/-}) mice demonstrated reduced tendency of polyp formation in the colon, compared to their wild-type littermates. Although, no difference could be observed in the tumor frequency in male knockout mice, they exhibited significantly decreased number of smaller tumors (≥ 0.5 -1.0 mm, $P < 0.01$) compared to male wild-type mice. In line with previous findings of correlation between high expression of CysLT₁R and 5-LOX in colorectal adenocarcinomas, we were able to detect significantly ($P < 0.05$) decreased 5-LOX tumor expression in AOM/DSS-treated female mice lacking CysLT₁R expression (CysLT₁R^{-/-}). Also, an increased expression of 5-LOX has been demonstrated in tumors compared with normal surrounding mucosa in the colon of AOM/DSS treated mice (178). Interestingly, immunostaining revealed an increased ($P < 0.05$) expression of MUC2 in tumor surrounding villi in the colon of AOM/DSS-treated female mice lacking CysLT₁R expression (CysLT₁R^{-/-}). MUC2 is a major structural component of the gastrointestinal mucosal barrier and reduced production by goblet cells is characteristic of pre-neoplastic lesions, referred to as aberrant crypt foci (ACF) in both rodents and humans (179-181). The pivotal role of MUC2 in colorectal cancer has been demonstrated in MUC2-deficient mice, which develop invasive tumors in the intestine and the colon (15). A significantly higher membranous:nuclear ratio of β -catenin within the epithelial tumor compartment could be observed for AOM/DSS-treated female knockout (CysLT₁R^{-/-}) mice. In accordance with these observations, we have previously demonstrated that LTD₄ stimulation of CysLT₁R induces nuclear translocation of β -catenin with subsequent increased proliferation and migration (65). Nuclear accumulation of β -catenin is readily found in late adenomas and carcinomas and a prerequisite for colorectal tumor progression (64). Increased serum levels of PGE₂ were detected in female CysLT₁R mutant (CysLT₁R^{+/-} and CysLT₁R^{-/-}) mice compared to wild-type littermates. However, knockout mice have illustrated that cyclooxygenase-derived

prostanoids are not major components in colitis-associated colorectal cancer (144) but might actually have a protective role against inflammation in the colon (22, 182). In conclusion, we demonstrate that AOM/DSS treated female knockout (*CysLT₁R*^{-/-}) mice have a reduced tendency to develop tumors in the colon, which could be attributed to increased tumor epithelial membranous:nuclear ratio of β -catenin expression and increased MUC2 expression, indicative of a more differentiated phenotype.

Summary

- *CysLT₁R* antagonists inhibit tumor growth in a xenograft model of colon cancer by impairing angiogenesis and inducing apoptosis.
- Global deletion of the *CysLT₁R* affects tumorigenesis in a gender-specific manner in both the *Apc*^{Min/+} mouse model and the AOM/DSS model of colitis-associated colorectal cancer.

Populärvetenskaplig sammanfattning

Det finns ett starkt samband mellan kronisk inflammation och cancer. Ett av de mer etablerade sambanden är den mellan kronisk inflammation i tarmen och den i Sverige tredje vanligaste cancersjukdomen, nämligen tjocktarmscancer. Risken för tjocktarmscancer korrelerar med inflammationsutbredning, varaktighet och svårighetsgrad. Den inflammatoriska processen regleras av rekryterade immunologiska celler och berörd vävnad huvudsakligen via olika signaleringsmolekyler såsom leukotriener. Dessa molekyler är pro-inflammatoriska och bidrar bland annat till vätskeansamling i samband med en inflammatorisk process. De bidrar också till muskelsammandragningar och är patologiskt förknippade i det avseendet med andningssvårigheter vid astma. Leukotriener förmedlar sin effekt via inbindning till specifika cellmottagare, s.k. receptorer. Det mest potenta leukotrienet är cysteinyl leukotriene D₄ (LTD₄) och medlar främst via receptorn cysteinyl leukotriene 1 (CysLT1R). Vår forskningsgrupp har tidigare funnit att patienter med koloncancer (Dukes B typ) som har ett högt tumörvävnadsuttryck av CysLT1R har sämre överlevnadsprognos jämfört med patienter med lågt uttryck. Vi har också upptäckt att en närbesläktad receptor (CysLT2R) som också finns i tumörer men har uppvisat motsatt effekt på tumörceller och därmed positiv effekt för patienten. Vi har tidigare också i cellkulturer med olika koloncancer cellinjer sett att LTD₄ via CysLT1R ger en ökad cellöverlevnad och vandringsförmåga, s.k. migration som är en förutsättning för metastasering, d.v.s. spridning till andra vävnader och etablering av sekundära tumörer. Däremot har cysteinyl leukotriene-inducerad CysLT2R signalering påvisat celldifferentiering, något som förknippas med cellmognad, vävnadsstabilitet och integritet. Eftersom tumörbildningsförmågan beror på interaktion mellan tumörceller och omgivande celler och vävnader inte går att undersöka genom att studera enskilda celler i en cellkultur, har nästa givna steg varit att utföra djurstudier.

Jag har i mina avhandlingsarbeten undersökt betydelsen av CysLT1R i olika djurmodeller för tjocktarmscancer. Vi har kunnat visa att behandling av subkutana humana koloncancerceller med substanser som specifikt binder till CysLT1R och förhindrar dess aktivitet, s.k. antagonister ger reducerad tumör tillväxt i möss med bristfälligt cellulär immunsystem. Detta åtföljdes av en minskad celldelning och kärlbildning i tumörerna samt ökad celldöd. Genomisk förändring, s.k. mutation av *APC*-genen bärs av mellan 80-90% av alla koloncancer patienter samt är ansvarig för den familjära formen av tjocktarmscancer (familial adenomatous polyposis; FAP), d.v.s. *APC*-genen är en betydande faktor i den mänskliga etiologin för tjocktarmscancer. *APC*-genen är involverad i flera cellulära processer som reglerar

celltillväxt och celledelning. För att studera betydelsen av CysLT1R i utvecklingen av tjocktarmscancer hos dessa patienter använde vi oss av $Apc^{Min/+}$ musmodellen som har en mutation i *Apc*-genen, vilket möjliggör bildandet av flera tumörer i huvudsakligen tunntarmen. Vi kunde särskilja en könsspecifik skillnad i tumörbildningen, som var direkt beroende av CysLT1R-genuttrycket. Honor som saknade uttryck av CysLT1R hade signifikant mindre antal av tumörer i tunntarmen, i jämförelse med honor som hade oförändrad CysLT1R-uttryck. Dessa möss hade också större tumörinfiltrat av vita blodceller, subtyp CD8+ T celler, något som är gynnsamt och överensstämmer med tjocktarmscancer patienter och deras överlevnadsprognoser. Honor som saknade uttryck av CysLT1R hade också, förutom reduktion av cysteinyl leukotriener såsom LTD₄, även en minskning av prostaglandin E₂ (PGE₂) serumkoncentrationer. Detta är anmärkningsvärt med avseende på att PGE₂, och det enzym som ansvarar för dess produktion (COX-2), har påvisats i större mängder hos patienter med tjocktarmscancer. Låga doser av aspirin (acetylsalicylsyra – ASA), som hämmar bildningen av prostaglandiner har också uppvisat en skyddande effekt mot tjocktarmscancer. För att vidare verifiera betydelsen av CysLT1R använde vi möss som saknade uttryck av CysLT1R och tillämpade ett väletablerat protokoll för kemisk induktion av kolit-associerad tjocktarmscancer. Dessa djur uppvisade samma tendenser men inte i samma utsträckning som de honmöss som också saknade uttryck av APC. Vårt resultat indikerar att CysLT1R är av betydelse för utvecklingen av tjocktarmscancer i möss, möjligtvis mer i samband med avsaknad av APC uttryck. Dessa resultat lyfter också fram möjligheterna med att påverka aktiviteten hos CysLT1R som alternativ terapeutiskt mål vid behandling av patienter med tjocktarmscancer.

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