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Chemokine-Mediated Migration of Colon Cancer Cells

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DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



Chemokine – Mediated Migration of Colon Cancer Cells

Amr A. Al-Haidari



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DOCTORAL DISSERTATION

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To be defended at Lilla Aula MFC, Jan Waldenströmsgata 5, SUS, Malmö on the
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Abstract Colorectal cancer (CRC) is the third most common cancer worldwide. The cause of the majority of death cases is believed to be the end result of distant organ metastasis. The mechanisms behind cancer cell metastasis are not fully understood but accumulating data suggest that enhanced tumor cell capacity to respond to different chemotactic stimuli and overexpression of adhesion molecules are essential for the spread of tumor cells. Within tumor microenvironment, chemokines and their receptors are key players in the tumorigenesis and metastasis of CRC. The aim of this thesis is to investigate the mechanism of colon cancer cell migration mediated by chemokine signaling. We found, for the first time, that colon cancer cells express CCR4 and stimulation by its respective ligand, CCL17, induced colon cancer cell migration. Interestingly, targeting CCR4 by CCR4 antibody/antagonist substantially decreased CCL17-induced colon cancer cell migration. Moreover, we found that migration of colon cancer cells was dependent on RhoA, HMG-CoA reductase, miR-155-5p, and HuR. Inhibition of RhoA, HMG-CoA reductase, and miR-155-5p by different meanings including ROCK inhibitor, simvastatin, AntagomiR-155-5p, and target site blockers significantly reduced CCL17-induced colon cancer cell migration mediated via CCR4. Our novel findings also show that miR-155-5p is heavily implicated in the regulation of CCL17-induced colon cancer cell migration via direct binding and positive regulation of RhoA and HuR proteins under cancer cell stress conditions. Our data uncover new mechanisms that can aid in better understanding of colon cancer cell migration and provide potential strategies for antagonizing colon cancer metastasis.		
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Chemokine – Mediated Migration of Colon Cancer Cells

Amr A. Al-Haidari



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قال الله تعالى :

﴿ يَرْفَعُ اللَّهُ الَّذِينَ ءَامَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا
الْعِلْمَ دَرَجَاتٍ ۗ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

سورة المجادلة

*In Ever Loving Memory
of my father*

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Tables of Content

Abbriveations.....	I
List of original papers.....	III
Chapter One: History of cancer	1
Chapter Two: Colon structure and physiology	3
Structure of the colon.....	5
Histology of the colon.....	5
Physiological function of the colon.....	6
Chapter Three: Colon cancer at a glance	7
Introduction.....	9
Epidemiology.....	9
Etilology	10
Risk factors	10
Genetic factors	10
Non-Genetic factors	10
Colon cancer staging.....	10
Clinical features of colon cancer.....	11
Molecular considerations in colon cancer	11
Chromosomal instability	11
Microsatellite instability	12
CpG island methylator phenotype.....	13
Cancer stem cells in colon cancer	13
Colon cancer therapy	14
Chapter Four: Chemokines in tumor biology	15
Introduction.....	17
Inflammation and cancer.....	17
Chemokine/Chemokine receptors in colon cancer metastasis	18
Molecular aspects of colon cancer metastasis from chemokine point of view	19
Tumor cell migration biology	19
Chapter Five: Methodology	23
Cell models used in the study	25
Assesment of protein and gene expression.....	25
Assesment of colon cancer cell proliferation	25
Apoptosis of cancer cells	25
Evaluation of cancer cell migration	25
Protein analysis: Westernblot and activation assays	26
MicroRNAs transfection	26

Bioinformatics analysis of binding sites	26
RNA immunoprecipitation	27
Chapter Six: CCL17/CCR4 axis in colon cancer cell migration	29
Aim	31
Introduction.....	31
Results and discussion	32
Chapter Seven: Role of HMG-CoA Reductase in Colon Cancer Cell Migration	39
Aim	41
Introduction.....	41
Results and discussion	41
Chapter Eight: Role of MiR-155-5p in colon cancer cell migration	47
Aim	49
Introduction.....	49
MicroRNAs and miR-155	49
AU-rich elements	50
Human antigen R.....	50
Results and discussion.....	51
Chapter Nine: Thesis conclusions.....	59
Populärvetenskaplig sammanfattning	61
Supplementary figures appendix I	62
Supplementary tables appendix II.....	65
References	67

Abbreviations

ACase	Adenyl cyclase
Ago2	Argonaute 2
AKT	Protein kinase B
APC	Adenomatous Polyposis Coli
AREs	AU-rich elements
Bcl2	B-cell lymphoma 2
BSA	Bovine serum Albumin
BRAF	B-Raf murine sarcoma viral oncogene homolog B
Cdc42	Cell division control protein 42
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
Co-IP	Co-immunoprecipitation
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CSC	Cancer stem cell
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELAV	Embryonic lethal abnormal vision
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal–regulated kinase
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FIT	Fecal immunochemical test
FOBT	Fecal occult blood test
FPP	Farnesyl pyrophosphate synthase
FTase	Farnesyl transferase
FXR-1	Fragile X mental retardation syndrome-related protein
GALT	Gut-associated lymphoid tissue
GDP	Guanosine diphosphate
GGTase	geranylgeranyltransferase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCRs	G-protein-coupled receptors
GPP	Geranylgeranyl pyrophosphate synthase
GTP	Guanosine triphosphate
GW182	GW-bodies or P-bodies
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNPCC	Hereditary non-polyposis colon cancer

HuR	Human antigen R
IBD:	Irritable bowel disease
KRAS	Kirsten rat sarcoma
NK	Natural killer cells
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDC	Macrophage–derived Chemokine
miRNAs	MicroRNAs
miRNPs	Microribonucleoprotein
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MSI	Microsatellite instability
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS	Nitric oxide synthases
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
P53	Transformation-related protein 53
QRT-PCR	Quantitative Reverse transcription polymerase chain reaction
Rac	Ras-related C3 botulinum toxin substrate
RBP	RNA binding proteins
Rho	Ras homolog protein
RIP	RNA Immunoprecipitation
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
siRNA	Small interference RNA
Snail	Zinc fingerprotein SNAIL
SOX9	Transcription factor SOX-9
TARC	Thymus and activation regulated chemokine
TH	T-helper cells
TIA-1	T-cell intracytoplasmic antigen
TNF α	Tumor necrosis factor alpha
Treg	T regulatory lymphocytes
TS	Target site
TSB	Target site blocker
TTP	Tristetraprolin
uPA	Urokinase-type plasminogen activator
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site

List of Original Papers

- I.** **Amr A. Al-haidari**, Ingvar Syk, Karin Jirström, and Henrik Thorlacius. CCR4 mediates CCL17 (TARC)-induced migration of human colon cancer cells via RhoA/Rho-kinase signalling. *Int J Colorectal Dis.* (2013) 11:1479-87
- II.** **Amr A. Al-Haidari**, Ingvar Syk, Henrik Thorlacius. HMG-CoA reductase regulates CCL17-induced colon cancer cell migration via geranylgeranylation and RhoA activation. *Biochem Biophys Res Commun.* (2014) 446(1):68-72
- III.** **Amr A. Al-Haidari**, Ingvar Syk, Henrik Thorlacius. MiR-155-5p positively regulates CCL17-induced colon cancer cell migration by targeting RhoA. *Oncotarget* 8 (2017)14887e14896.
- IV.** **Amr A. Al-Haidari**, Anwar Algaber, Raed Madhi, Ingvar Syk, and Henrik Thorlacius. MiR-155-5p controls colon cancer cell migration via post-transcriptional regulation of Human Antigen R (HuR). *Cancer Lett.* (2018) 421:145-151

History of Cancer

Cancer is not a new-era born disease. In fact, cancer has been around for thousands of years. The first cancer case description in the history was found in the survived Edwin Smith Papyrus, ca. 1600 Before century (BC) [1]. The description in the papyrus was believed to provide a solid insight of Ancient Egyptian civilization medical knowledge as early as 2500 B.C. This fascinating 4.68-meter length text describes abnormal ulcer on the breast and the use of surgery to remove it. However, the document also mentioned that: “there is no treatment”. In ca. 460–370 BC, Hippocrates also described various types of cancer as Karkinos, a Greek word referred to crabs and crayfish [2]. The description came from the superficial appearance of cancer with many branched veins that makes it looks like crab. Later on, Cesus (ca. 25 BC - 50 AD) was the first to name this malignant disease as cancer, coming from Latin word, which also means crab. Another early evidence of documenting cancer came from a well-known physician called Avicenna, an Islamic philosopher and arguably the most influential philosopher of the pre-modern era. He described the first detailed surgical intervention of radical excision of cancerous tissue in his

mother book called The Canon of Medicine [3]. In the eighteenth century, the microscope was invented in which doctors and scientists were able to understand much more about cancer. The first autopsy, removal of small piece of cancerous tissue after death, for investigation, was first made by Giovanni Morgagni in 1761 [4]. This has led to the emergence of new science called oncology, the study of cancer. The English surgeon Campbell De Morgan in 1871 was the first to demonstrate the spread of tumor through lymph nodes to other body organ sites [5]. The nineteenth century uncovered the science of new oncology. The use of modern microscopies has allowed for better understanding of tumor tissue. The father of cellular pathology, Rudolf Virchow, was the first to correlate microscopic pathology to illness of cancer and therefore, it becomes easier for surgeons to define the surgical intervention extent according to the tissue pathological findings [6]. In the twentieth century, technology has revolutionized every single aspect not only in science in general but also in cancer in particular, and the most significant advancements in cancer research were born in the current century.

Colon Structure and Physiology

Contents

1. Structure of the colon
2. Histology of the colon
3. Physiological function of the colon

1. Structure of the colon

The large intestine, also referred as colorectum, represents the last part of the gastrointestinal tract system. It consists of four main segments: the cecum, colon, rectum, and the anal canal. The colon is about 1.5 – 1.8 m long therefore it constitutes most of the length of the large intestine and it is divided into four sections: the ascending colon, transverse colon, descending colon, and sigmoid colon (**Figure 1A**). The small intestine meets the large intestine at the cecum and the ascending colon extends from the cecum and ends at the hepatic flexure near the right inferior margin of the liver. The transverse colon extends from the hepatic flexure to the splenic flexure where the colon descends the abdominal wall to form the descending colon to rim the pelvis and turns medially and inferiorly to form the S-shaped sigmoid colon.

2. Histology of the colon

Colon is composed of four tissue layers (**Figure 1B**): the mucosa (epithelium, lamina propria, and muscularis mucosae), the submucosa, the muscularis propria (inner circular muscle

layer, intermuscular space, and outer longitudinal muscle layer), and the serosa [7]. The mucosa represents the innermost layer and composed of simple columnar epithelial cells which are arranged in a layer to form the luminal surface. Mucosa contains goblet cells. These cells are more predominant in colon than in the small intestine and function mainly to produce mucus that lubricates the inner wall of the bowel for easily passage of solid colonic content. Unlike the small intestine, the mucosa of the colon lack villi structures. The submucosa consists of moderately dense connective tissue of blood and lymphatic vessels as well as nerve plexuses. The muscularis propria or (externa) has two layers; inner circular and outer longitudinal layers. Muscularis propria functions through providing rhythmic waves of contraction to move food through the colon. The last layer of the colon is an outermost layer called serosa. It is composed of loose connective tissue - covered by the visceral peritoneum and contains blood vessels, lymphatics and nerves.

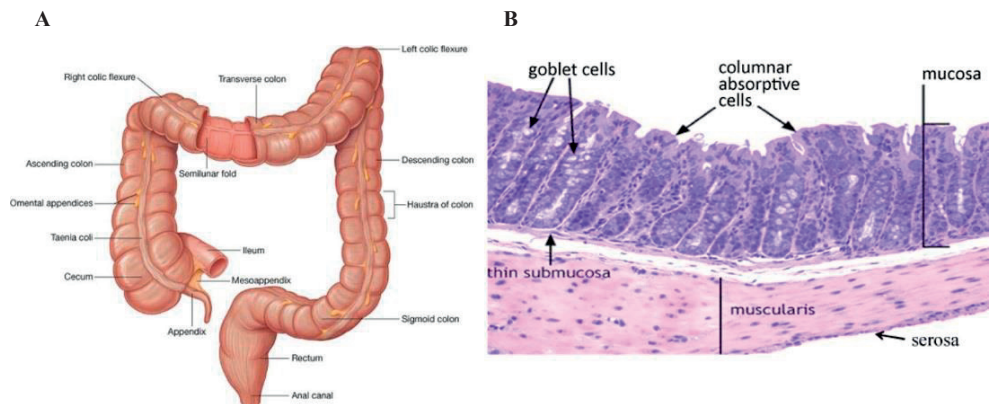


Figure 1. *A*, Basic schematic illustration of the colon. Adapted from Drake RL, Vogl W, Mitchell AWM, et al. *Gray's Atlas of Anatomy* (Elsevier); 2008. *B*, Histological structure of the colon stained with eosin-hematoxylin stain. Retrieved from Deltabase histology atlas, Deligen Inc. 2000-2006.

3. Physiological function of the colon

The main function of the colon is to absorb water from the stool required for other metabolic processes as well as electrolytes [8]. Colon principally recovers sodium and chloride from the stool by the exchange of bicarbonate and potassium, thus colon plays a significant role in the intestinal hemostasis. In addition, colon absorbs essential vitamins produced by the gut bacterial flora. For example, vitamin K is exclusively produced by the gut flora and it is vitally important for proper blood clotting [9]. Because intestinal lumen contains a massive number of bacterial flora, it is therefore exposed

to a low degree of inflammation [10]. The nature of this ecosystem in human body makes the colon harbours one of the biggest immune systems called Gut-associated lymphoid tissue (GALT). The colon epithelium is protected from external pathogens and microorganisms by a dense network of immune cells located in the lamina propria including macrophages, dendritic and lymphoid cells. Imbalance in the gut microflora or impairment of the GALT system triggers different immune responses and predisposes the colon to different inflammatory bowel diseases (IBDs).

Colon Cancer at a Glance

Contents

1. Introduction
2. Epidemiology
3. Colon cancer staging
4. Clinical features of colon cancer
5. Molecular considerations of colon cancer
6. Cancer stem cells in colon cancer
7. Colon cancer therapy

1. Introduction

Colorectal cancer, CRC, (also known as colon cancer, rectal cancer, or bowel cancer) is the development of abnormal growth in parts of the large intestine. It initiates as a proliferative growth called polyps [11]. At this stage polyps tend to grow in a slow rate and histologically called dysplastic adenoma. Because of polyp's low progression rate, their hyperplastic stage may take several years to develop. Over time, mutations start to be accumulated throughout the stages of development until shaping up the malignant carcinoma. Approximately 25% of patients have genetic familial history while the vast majority of colon cancer cases are sporadic [12]. Adenomatous Polyposis Coli (APC) gene is a tumor suppressor gene and mutations in APC have been well documented in many CRCs. It is believed that these mutations are more likely to be the initiating events of colon tumorigenesis [13]. More than 35% of Colon cancer cases are located at the sigmoid part of the colon hence called colorectal cancer [14]. The metastatic potential of colon cancer is identified by the ability of cancer cells to interact and communicate with the tumor microenvironment [15]. In metastasis, malignant cells acquire specific characteristics that make them capable to metastasize. Such characteristics include enhanced cell adhesion to endothelial cells, increase cell migration in response to chemotactic signals released by the target organs, and higher response to growth stimuli [16]. Within tumor microenvironment, chemokines and their receptors are key players in the tumorigenesis and metastasis [17; 18]. The distinct tropism for metastatic sites of different types of cancers was appreciated by the discovery of chemokines and chemokine receptor's roles in cancer biology which provide a concrete evidence not only in their role in overall metastasis but also in site – specific metastasis [19; 20]. Studies of cell cytoskeletal reorganization during cancer cell movement have provided better insights of the molecular aspects

in the metastatic biology of cancer. For example, Rho GTPases family has been heavily implicated in cancer cell metastasis [21; 22]. Various types of cancer express different chemokine/chemokine receptors and the pattern that shapes this expression could provide some clues on the metastatic behaviour of cancer cells [23; 24]. Moreover, the introduction of microRNAs (miRNAs) in cancer biology has revolutionized our understanding of many complex mechanisms that regulate cancer cell metastasis.

2. Epidemiology

CRC is a major health problem and represents one of the most common causes of cancer – related deaths in men and women mainly in the industrial world. In Europe, CRC is the third most prevalent cancer and the second leading cause of death among cancer patients [25; 26; 27]. Around 90% of CRC mortality cases are due to the spread of primary tumor to other distant organs in a complex multi-step process called metastasis leading to failure of organs function [28; 29]. If metastasis occurred, the 5-year survival rate after surgical intervention falls from 95% to less than 10% [27]. Different screening programs have been widely implemented for early detection and prevention to those who are at high risk of developing CRC and resulted in a significant reduction among CRC deaths worldwide. These screening programs involve testing for pre-cancerous colorectal polyps or early-stage cancer before well-defined symptoms appeared and before the disease has a chance to grow or spread, and while treatment is easier to implement, feasible, and more likely to be successful. Faecal occult blood (FOBT) or faecal immunochemical test (FIT) by far remains the most popular screening test for CRC. Upon positive results, sigmoidoscopy or colonoscopy might be indicated to confirm presence of cancerous or inflammatory findings, however; these screening tests are limited by their

invasiveness, low specificity and sensitivity [30]. Nevertheless, the global incidence of CRC is expected to increase to more than 2.2 million new cases and 1.1 million deaths by 2030 [31]. The most common screening options up-to-date is summarized in (**Supplementary table 1**).

2.1. Etiology

The primary cause of CRC is believed to be potential mutations that target oncogenes, tumor suppressor genes, and genes involved in DNA repair process [32]. These mutations result in either gain-of-function or loss-of-function of different major proteins involved in the regulation of vital cellular processes such as proliferation, apoptosis, and cell migration [33; 34]. About 70 – 75% of CRC cases are sporadic, i.e.; due to non-inherited gene mutations, while 25 – 30% are found in patients with family history of CRC [12].

2.2. Risk factors

The risk factors associated with increased incidence of CRC can be classified into two main categories: Genetic and non-genetic factors.

2.2.1. Genetic factors

Genetic factors account for about 20-25% of CRCs. This include some recognized adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC) also known Lynch syndrome [35].

2.2.2. Non-genetic factors

These factors include: age, life style habits for instance; red meat, low-fiber intake, heavy alcohol consumption, smoking, low physical activity, and obesity [36]. Chronic inflammatory conditions such IBD including ulcerative colitis and Crohn's disease have relatively higher risk (2 –15 fold) to develop CRC and therefore individuals within this category are recommended to be screened for CRC more frequently regardless their ages [37; 38; 39].

3. Colon cancer staging

CRC histological staging becomes the gold standard staging system which offers valuable evaluation about the extent of the disease for clinicians, oncologists, and surgeons (**Figure 2**)

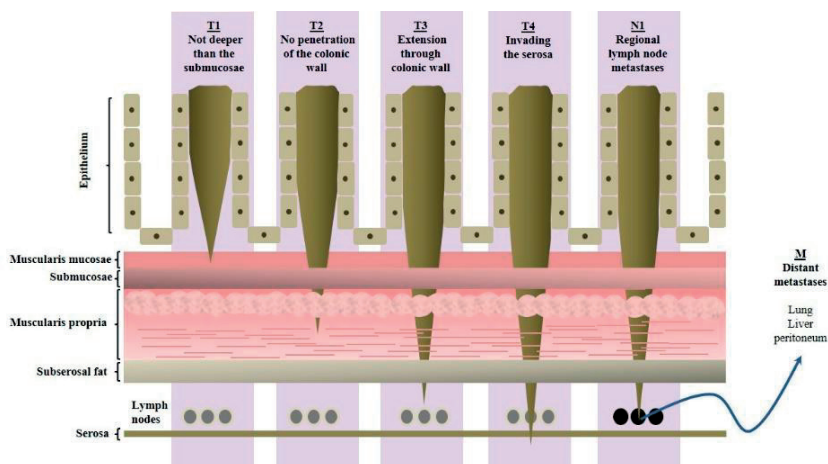


Figure 2. Schematic illustration of Histological staging in colorectal cancer.

[40]. The staging of CRC was first proposed by Lockhart-mummery in 1926 based on operative finding of patients with rectal cancer [41; 42]. In 1932, Dukes provided more detailed staging based on the relationship of rectal cancer patient's survival and the degree of tumor penetration in the intestinal wall and lymph nodes metastasis [43]. This classification has been further developed and modified by Kirkland, Astler, and Collier [44] and widely spread for many years until the American Joint Committee for Cancer (AJCC) has established the TNM staging system (Tumor, Node, Metastasis) in 1973 based on the primary tumor, regional lymph nodes involved, and distant metastasis [45] and becomes the most popular staging system in clinical practice worldwide which provides critical clues on selective therapeutic decisions and prognosis (**Supplementary table 2**).

4. Clinical features of colon cancer

Usually CRC symptoms are less common in early phases and more prominent when the disease has already been established and detectable. Many cases show clinical presentation at intermediate or advanced stages [46; 47; 48]. The most commonly reported symptoms in CRC is summarized in **Table 3**.

5. Molecular considerations in colon cancer

CRC has been molecularly recognized as a heterogeneous disease and therefore introduced potential challenges in developing effective therapy [49; 50]. The term "Adenoma-carcinoma sequence" is usually used to describe the molecular sequential events that lead to CRC carcinogenesis (**Figure 3**) [51]. The mechanism of CRC carcinogenesis is quite well known which arise from one or a combination of three different mechanisms; (i) chromosomal instability (CIN), (ii) microsatellite instability (MSI), and (iii) CpG island methylator phenotype (CIMP) [52; 53]. These mechanisms

Table 3. Common CRC clinical features
Symptom duration
Early CRC < 4 weeks (33%), > 4 weeks (77%) Advanced CRC < 4 weeks (19%), > 4 weeks (81%)
Hematological observations
Fecal blood Rectal bleeding Anemia [†]
Physical observations
Abdominal pain Weight loss Decreased appetite Anorexia
Change in bowel habits
Constipation Altered stools Diarrhea Mucus in stool
Others
Fatigue and General malaise Nausea or vomiting Rectal pain Obstruction

[†] Anemia: hemoglobin of, 13.4 g/dl (male) and, 12.3 g/dl (female).

are characterized by accumulation of somatic mutations in specific tumor suppressor genes and oncogenes and epigenetic changes leading to abnormal increase or decrease in signal transduction activity, and aberrant proteins function.

5.1 Chromosomal instability (CIN)

CIN is considered the classical pathway of CRC carcinogenesis. It is associated with traditional adenomas and account for 70% of sporadic CRCs where the vast majority of cases

begin with acquisition of mutations in APC gene which affects chromosome segregation during cell division and regulates differentiation, adhesion, polarity, migration, and apoptosis [54]. APC has been shown to be an essential component of the Wntless/Wnt signaling pathway. Disruption of Wnt signaling by APC mutation has been well linked to the development of CRC [34; 55]. Another critical event following APC mutation in the CIN pathway is Kirsten rat sarcoma (KRAS) mutation. KRAS constitutes 30% of all sporadic CRCs and plays a major role in regulating different cellular functions [56]. Point mutation in codons 12, 13, and to a lesser extent codon 61, renders KRAS constantly active and therefore activates major downstream pathways such as Mitogen-activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K). The effect of these signaling pathways on cell proliferation, survival, apoptosis, cell cycle, and migration is well documented to be KRAS-mediated malignant transformation [57]. In later stages of CRC, the loss of P53 function represents a universal hallmark and a key step in CRC tumorigenesis. P53 is defined as the “Guardian of the genome” where it regulates hundreds of genes involved in the regulation of cell cycle, apoptosis, angiogenesis, cell survival,

immune response, and cell migration [54; 58]. Mutation in P53 has been reported to be mutated in 50% of all human cancers and in 75% of CRC cases implicating its role in adenoma-carcinoma sequence transition [59].

5.2. Microsatellite instability (MSI)

Another important category of genetic alteration found in adenoma-carcinoma sequence and associated with the carcinogenesis of CRC is MSI [53]. Inactivation or mutation in any of the DNA mismatch repair genes during DNA damage, for example; DNA replication errors might lead to what so-called Microsatellite instability [60]. These genes including; (MSH2, MLH1, PMS1, PMS2, MSH6, or MSH3). It is characterized by abnormal increased/decreased length of oligonucleotides repeats and therefore creates potential mutation that is usually inheritable [61]. The incidence of microsatellite instability has been shown to be 15% in adenomas and up to 25% in CRC overall [62]. Studies have also reported that dysplastic (pre-malignant) lesions from ulcerative colitis patients contain potential microsatellites and therefore it can be counted as an early event of adenoma-carcinoma sequence in ulcerative colitis-associated CRC [63].

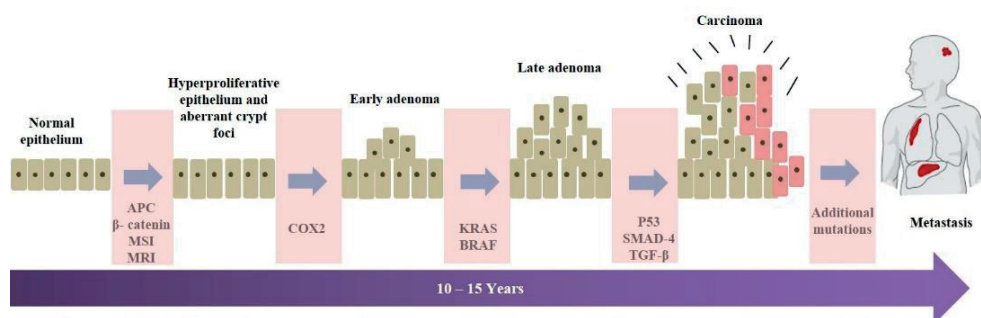


Figure 3. Basic illustration of histomolecular events in Adenoma-Carcinoma sequence.

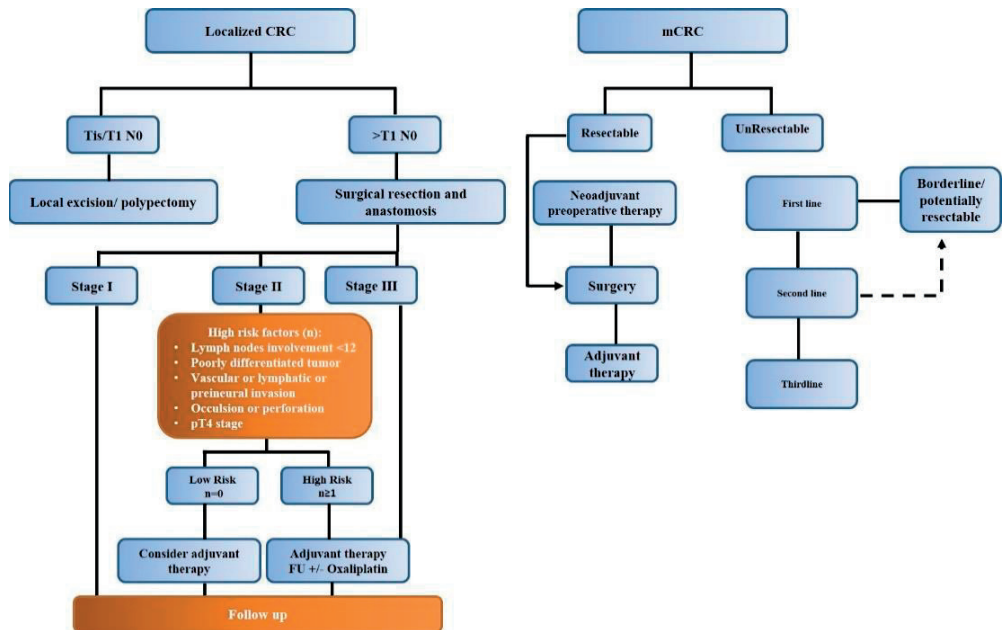


Figure 4. Colon cancer treatment algorithm adapted from Schmoll, H. *et al. Ann Oncol.* (2012)

5.3. CpG island methylator phenotype (CIMP)

Epigenetic changes are another common feature of CRC carcinogenesis. The interesting point in epigenetic approach of CRC is its mechanism. Epigenetic changes can combine between the effects of point mutations and MSI [64]. For example, Mutation in BRAF seems to be a precursor event in the CIMP tumors. The BRAF V600E mutation is strongly correlated with the hypermethylation of the mismatch repair gene MLH1 promoter in 18.7% of sporadic CRC while other studies confirmed MLH1 hypermethylation in almost 80% of MSI-H sporadic CRC cases [52; 65]. CRC arises from serrated adenomas has been attributed to BRAF mutation and DNA methylation and belongs to this category of CRC carcinogenesis.

6. Cancer stem cells in colon cancer

Differentiated epithelial colonic cells are subjected to continuous turnover throughout life. Epithelial tissue hemostasis is maintained by a subset of self-renewing undifferentiated multipotent progenitor stem cells. These cells located at the bottom of the crypt in the proliferative zone and responsible for all epithelial cell types generation. Two models have been proposed for the theory of stemness in CRC. The stochastic model which proposed that the heterogeneity of colon cancer stem cells (CSCs) results from multiclonal origin of the tumor. In other words, each cancer cell within the tumor bulk mass is tumorigenic. In contrast, the CSC model suggests that colon carcinoma cells arise from single multipotent stem cells that generate tumors containing multiple heterogeneous cell

types [66]. It is widely accepted that colon CSCs display characteristics that protect them against many existing therapeutic modalities such as slow proliferation, maintain of quiescent phase, self-renewal and capabilities to metastasize. Target therapies even in synergistic combinations have failed to cure advanced stages CRC patients. One convincing reason is that these agents are usually targeting the bulk of the tumor mass, composed mainly of proliferating cancer cells, while leaving behind few CSCs which are in the quiescent phase and have the ability to re-initiate tumor recurrence and/or metastases [67].

7. Colon cancer therapy

As I have mentioned earlier, CRC is a heterogeneous disease, therefore, the therapeutic modalities vary and options for effective treatment are dependent on several factors, however, the most determinant factor for critical therapeutic decision is the stage of the disease at diagnosis (**Figure 4**). In general, Surgery is the best intervention choice to remove the cancer in early cancer phases, however, as cancer stages advanced the surgical removal of cancer becomes

challenging. For example, in stages II, III, or IV neoadjuvant preoperational chemotherapy might be introduced to shrink the tumor and to assist better less invasive surgical removal of the cancer and selected margins. Another therapeutic option is adjuvant chemotherapy. These drugs are usually administered as a single or in combination regimens and applied after surgery in advanced stages or in non-resectable mCRC which help to kill tumor cells and to improve symptoms as well as to increase survival [68]. The most commonly used chemotherapeutic agents are summarized in (**Supplementary Table 3**). Radiotherapy is another therapeutic modality, however; it is often applied in rectal cancer [69]. Recent advancements in the treatment of colon cancer have introduced personalized medicine. This type of targeted therapy is based on the molecular profile of each cancer patient, for instance; RAS or BRAF mutation and MSI status in CRC patients [70]. In this context, it is worth noting that RAS mutated CRC patients do not benefit from anti-EGFR targeted therapy, thus RAS status can direct the therapeutic algorithm to another treatment regimen.

Chemokines in Tumor Biology

Contents

1. Introduction
2. Inflammation and cancer
3. Chemokine/chemokine receptors in colon cancer metastasis
4. Molecular aspects of colon cancer metastasis from chemokine point of view
5. Tumor cell migration biology

1. Introduction

Chemokines are group of small molecular weight (8 – 12 kDa) chemoattractant cytokines which initially discovered because their interaction with chemokine receptors was found to regulate trafficking of leukocytes to sites of inflammation and recirculation in secondary lymphatics [71], thus play a central role in the biology of cell migration. More than 40 chemokines and 20 functionally signaling chemokine receptors have been identified up to date [72]. Structurally, chemokines classified based on conserved N-terminus cysteine residues into four main groups, two major groups; CXC, and CC, also called alpha and beta chemokines respectively, and two minor groups; C, and CX3C. Chemokines usually exhibit 25 – 70% sequence identity and exist as monomers in their active state [73; 74]. Chemokines can also be classified according to their function into two main classes. First, inflammatory chemokines, which are involved in regulation of immune system, for example; trafficking immune cells to the site of inflammation. Secondly, homeostatic chemokines, which control leukocyte homing and lymphocyte recirculation under physiological conditions [71]. Chemokines

function through signaling of seven transmembrane G protein coupled receptors (GPCR). GPCRs are plasma transmembrane proteins which consist of three subunits; $G\alpha$, $G\beta$, and $G\gamma$ and transduce signals through cycling between GDP and GTP forms to activate downstream targets [75] (Figure 5). Some chemokine receptors bind multiple chemokines while others have exclusive chemokine receptor/ligand interactions. For example, CXCL12 has the only identified chemokine receptor CXCR4 [76].

2. Inflammation and cancer

It is well established that one of the most important malignant transformation factors is the inflammation [77]. Around more than 150 years ago, Virchow noticed that tumor tissues from sites of chronic inflammation were heavily manifested by inflammatory cells [78]. This observation had led to the conclusion that inflammation is a driven-force of neoplastic disease. Thereafter, it was well appreciated by significant epidemiological evidences which suggested that around 25% of all tumors are due to chronic inflammation [79; 80; 81]. Persistent inflammation such as those present in chronic

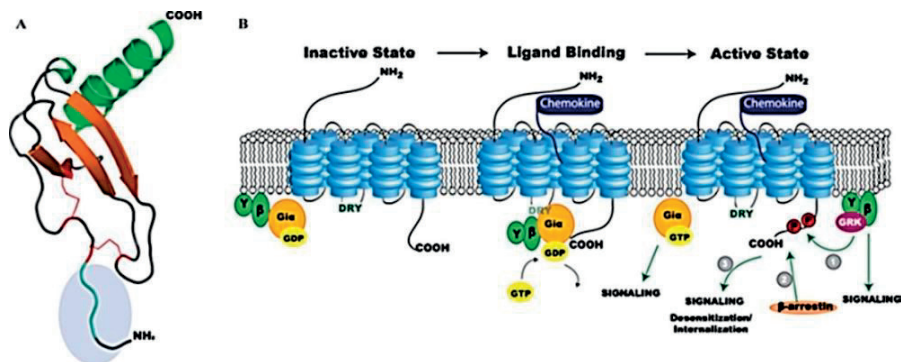


Figure 5. Chemokine and chemokine receptor structure and its classical activation. *A.* Chemokine structure consists of alpha helix and beta sheets. *B.* Classic signaling of G-protein coupled receptors. Adapted from Morgan O'HAYRE et al. *Biochem. J.* (2008)

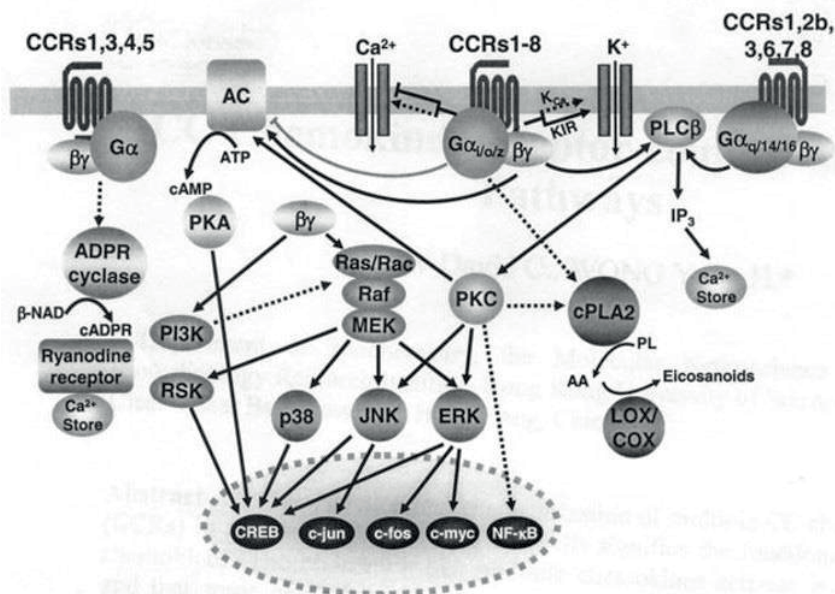


Figure 6. Intracellular signaling pathways activated by CC chemokine receptors. Adapted from New DC and Wong Yung H. *acta biochimica et biophysica sinica* (2003)

inflammatory conditions have been strongly linked to carcinogenesis, for example; IBD; (Crohn's disease and ulcerative colitis) and CRC [82], chronic reflux esophagitis resulting in Barrett's esophagus and esophageal carcinoma [83], viral hepatitis B and C or alcoholic liver cirrhosis and hepatocarcinoma [84], cervical infection by human papillomavirus and cervical cancer [85]. The tissue injury resulted from physical, chemical, biological or infectious stimuli, triggers sequential events of highly orchestrated inflammatory response. Failure or unresolved inflammation may disrupt the inflammatory microenvironment and results in alterations in oncogenes/ tumor suppressor genes and post-translational modifications in key cell signaling proteins involved in cell cycle, DNA repair and apoptosis [86; 87].

3. Chemokine/chemokine receptors in colon cancer metastasis

A strong body of evidence indicates that chemokines regulate multiple aspects of tumor cell biology, including proliferation, survival, angiogenesis and migration [88]. Colon cancer cells can also express chemokine receptors, including CXCR3, CXCR4 and CCR6 and respond to specific chemokines [71]. For example, it has been demonstrated that CXCR3 is expressed on colon cancer cells and mediate lymph node and lung metastasis [89]. These findings underline the importance of studying the expression and function of chemokine receptors in colon cancer cells for better understanding the molecular mechanisms controlling CRC metastasis. Several studies have linked between chemokines /chemokine receptors expression and metastasis due to the fact that cancer cells

express chemokine receptors which enable them to migrate to distant sites based on chemokine ligands gradient released by the target organs. For example, there is a strong evidence showing that CXCR4/CXCL12 axis is involved in lung, bone, and lymph nodes metastasis in several lines of cancers, and in abdominal lymph nodes and liver metastasis in colorectal carcinoma in particular [90]. Other studies indicated that CXCR3 expression promotes colon cancer metastasis to lymph nodes [89]. Moreover, previous reports also predicted lymph node metastasis in colorectal carcinoma by the expression of chemokine receptor 7 (CCR7) [91] while CCR6 and CCL20 were found to be significantly upregulated in liver metastasis of CRC [92; 93].

4. Molecular aspects of colon cancer metastasis from chemokine point of view

Upon activation, chemokine receptors mediate their effect through GPCRs which shuttle the active signal from GDP to GTP-form and activate subsequent enzymes including adenylyl cyclase (ACase), phosphoinositide-specific phospholipase C β (PLC) and phosphoinositide 3-kinase (PI3K). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to Diacylglycerol (DAG) and inositol triphosphate (IP₃). IP₃ triggers intracellular increase of Calcium and DAG activates protein kinase C (PKC) [94]. This signaling cascade activates pathways that have been shown to be critically involved in the regulation of variety of cellular processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription [95]. Several lines of evidence indicate that chemokines and chemokine receptors have a crucial role in the survival of colon cancer cells under stressed conditions such as hypoxia and serum starvation [96]. Such unfavourable tumor microenvironment conditions, along with increase release of cytokines from stromal and tumor cells, upregulate chemokines/chemokine receptor

expression and activate some transcriptional factors, resulting in increased cell migration and inhibition of apoptosis [97]. It is important to take into consideration that although these signaling pathways might be common between chemokine receptors, it is worth noting that further downstream signaling might be quite different. For example, CXCR4/CXCL12 axis has been implicated in breast cancer cell migration by activation of PI3K/AKT signaling pathway [98]. CXCR4/CXCL12 axis has also been shown to activate Akt and ERK1/2 signaling pathways through β -arrestins by G-protein-independent signaling mechanism [99]. In lung adenocarcinoma, CCL20/CCR6 axis has been found to activate ERK signaling pathway while another study showed that CCR6 promotes tumor angiogenesis via the AKT/NF- κ B/VEGF pathway in CRC [100]. One reason is that signaling pathways might be governed by how strong the chemotactic stimulus is. Moreover, cell surface expression as well as specific cellular conditions could play a role in selecting the optimal subsequent cascade signaling [101]. The exact mechanism by which chemokine/chemokine receptor signaling regulates colon cancer metastasis is still poorly understood. The most common CC chemokine receptors intracellular signaling pathways involved in cell migration, survival, and proliferation are summarized in (Figure 6).

5. Tumor cell migration biology

Cancer metastasis remains the biggest challenge in cancer pathology and the hardest consequence among cancer patients. The 5-year survival rate is dramatically decreased to nearly 10% when the cancer is metastasized [102]. This multistep process is primarily dependent on the cancer cell ability to invade, migrate, and adhere to the adjacent tissues [103]. The first step in cancer metastasis is the cancer cell detachment from the primary tumor. This process is mediated by the loss of a tumor suppressor gene called E-cadherin [104]. E-cadherin is expressed at junctions between the cells and responsible for

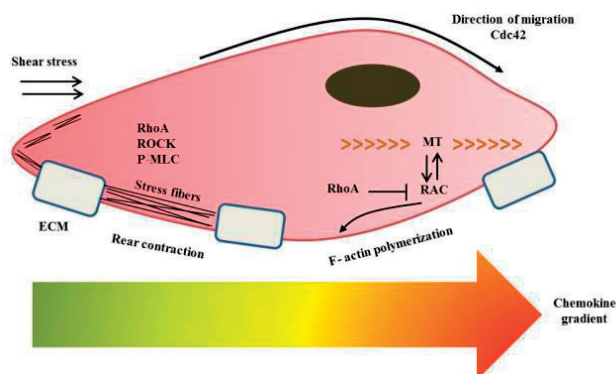


Figure 7. Basic illustration of Rho family member's interaction in directed chemotaxis.

maintaining cell-cell adhesion [105]. Reduced expression of E-cadherin has been shown in aggressive types of tumors and it is well-associated with Epithelial to Mesenchymal Transition (EMT), a state where cells acquire invasive phenotype, and E-cadherin switched to N-cadherin and promotes cell-cell-matrix adhesion instead of cell-cell adhesion [104]. Several signaling pathways have been implicated in the regulation of EMT for instance; Ras-MAPK and Wnt pathways [106]. Wnt/ β -catenin signaling pathway in this context is believed to be one of the earliest events in the cancer metastatic process [107]. Invasion is an integral part of tumor cell active migration. The invasive migration capacity of tumor cells is initiated once EMT is activated and governed by a complex process involving changes in cell-matrix adhesion and cell cytoskeleton adaptation and reorganization. Intravital imaging studies of tumor cell migration showed that changes in cell-matrix adhesion are necessary for the leading edge of the cell to adhere to the surrounded matrix allowing forward self-movement similar to an inchworm movement [108]. In fact, the invasive migration of tumor cells can be regarded as alternating cycles of adhesion and relief-of-adhesion, allowing the cell to bind, then detach

after pulling forward. This active process is fuelled by the ability of cells to interact with the extracellular matrix (ECM). In this regard, integrins, a family of cell surface receptors that are heterodimers composed of non-covalently associated α and β subunits represent a major class of adhesion molecules by which cancer cells integrate with different proteins to remodel the ECM, promoting cell passage through the stroma and enter the tissue. The expression of integrins in tumor cells, endothelial cells, and stroma cells indicate broad activities on cancer microenvironment. The blockade of integrin signaling has been demonstrated to be efficient to inhibit tumor growth, angiogenesis, and metastasis [109]. Directed tumor cell migration is mediated mainly by chemoattractants released from blood vessels or by other cell types. Once tumor cells reach blood vessels, they must enter the circulation to migrate to distant organs [110]. This process, called intravasation, requires cancer cells to penetrate the basement membrane of ECM lining the blood vessel wall and squeeze through the endothelium barrier. At the site of intravasation, cancer cells develop an amoeboid-like pseudopod structures by regulating different genes expression required for active cell mobility such as Rho family proteins [111]. Numerous

studies have shown that members of Rho family proteins including RhoA, RAC and Cdc42 cooperate to regulate the cytoskeletal changes required for migratory behaviour of the cells. For example, Cdc42 is required for the polarity of cell migration while RAC regulates membrane protrusions formation at the leading edge [112; 113]. This process is frequently accompanied by upregulation of different proteases such as metalloproteinases (MMPs) and cathepsins which are required for digesting the basement membrane or the surrounding tissue to facilitate invasive migration [103]. Furthermore, RhoA is required for the generation of actin filaments, the contractile force which is mediated by Myosin II and predominantly induced by RhoA and its downstream effector RHO-associated serine/threonine Kinase (ROCK) leading to stress fiber formation and contraction at the rear edge of the cell and allowing the cell body to slide forward (**Figure 7**) [114]. Tumor cell migration can be classified into two main categories; individual and collective tumor cell migration [115]. Individual tumor cell migration is dependent on single cell dissemination where cancer cell utilizes the EMT phenotypic capabilities or amoeboid form of migration. EMT cell migration type is heavily dependent on integrins and MMPs and predominantly found in connective tissue tumors such as gliomas, fibrosarcomas, and epithelial carcinomas following progressive differentiation [116].

In contrast, amoeboid tumor cell migration uses integrin and protease-independent mechanisms to navigate rather than degrade the ECM barrier [117; 118]. The deformable shape of this type of cells allows them to migrate at 10-30-fold higher velocities than those observed in EMT migration mechanism [118]. This type of migration is characteristic feature of many neuroendocrine tumors, kidney, prostate, small-cell lung carcinomas, and many hematological malignancies including lymphomas and leukemic cells [118]. On the other hand, collective tumor cell migration seems to be the most efficient mechanism by which epithelial carcinomas including colon cancer cells migrate and circulate in the vessels. This type of migration creates a powerful large-sized, heterogeneous contractile body of cells that allows efficient cell movement and ensures survival in the circulation and support mechanical arrest at the endothelium of the distant organs where tumor cell extravasation process takes place [119]. Collective migration provides the required autocrine signaling of promigratory factors and proteases as well as protecting cells from the immune system attack [120]. Hence the heterogeneity of the migratory cells represents a significant tool for the cells to move as a functional unit. Therefore, understanding the dynamics of cancer cell migration would allow the development of effective anti-metastatic agents.

Methodology

Contents

1. Cell models lines used in the study
2. Assessment of protein and gene expression
3. Evaluation of cancer cell proliferation
4. Apoptosis of cancer cells
5. Evaluation of cancer cell migration
6. Protein analysis; Western blot and activation assays
7. MicroRNAs transfection
8. Bioinformatics analysis of binding sites
9. RNA immunoprecipitation assay

1. Cancer cell model

During my PhD studies, I have used two main colon cancer cell models; the human epithelial colon adenocarcinoma cell line HT-29 which was obtained from American Type Culture Collection and primary colon cancer cells which was established in our laboratory at Skåne University Hospital called AZ-97 and isolated from a 76-year-old female patient undergoing surgical resection as previously described [121]. Cells were maintained at optimal growth conditions in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C and 5% CO₂.

2. Assessment of protein and gene expression

2.1. Flow cytometry

In my study, I used flow cytometry to assess cell surface expression of chemokine receptors. Colon cancer cell lines, HT-29 and AZ-97, were detached gently with 0.25% trypsin-EDTA in phosphate buffer saline (PBS) when reaching 80% confluence. The Cells then incubated with 1 µg of human FcγR inhibitor for 15 min to block Fc receptors and reduce nonspecific binding. Cells were incubated with 10 µg PE labelled anti-human CXCR4 or CCR8, FITC labelled anti-human CCR4 or CCR6, and APC labelled anti-human CXCR3 antibodies and incubated for 90 min at room temperature protected from light. For intracellular staining, cells were fixed in 0.25% paraformaldehyde and incubated for 1 h at 4°C. Cells were washed and permeabilized in 1ml 0.2% Tween20 and incubated at 37°C for 15 min. Cells then treated and stained as surface expression protocol. Thereafter, cells were washed twice and resuspended in 0.4 ml final volume FACS buffer and analyzed using BD FACSCalibur. Unstained cells were served as negative control. Histograms were made using CellQuest software with assessment of 20 000 events per sample.

2.2. Quantitative real time polymerase chain reaction (qRT-PCR)

For gene expression studies, I have used qRT-PCR. The total RNA of colon cancer cells from different experimental settings was isolated and followed by concentration and purity determination using Nano Drop spectrophotometer at 260 nm absorbance and the integrity of RNA samples was confirmed by 1%

agarose gel electrophoresis. Reverse transcription was conducted on (0.1 - 2.5 µg) of total RNA. QRT-PCR was conducted using a DNA-intercalating Syber green dye. The mRNA reference sequences were used to design primers using web-based primer design tools of national center of biotechnology information. The Primer sequences used in this study are listed in (table 5.) Relative expressions to control housekeeping gene U6/ beta actin were determined using 2^{-ΔΔCT} method.

3. Colon cancer cell Proliferation assessment

In this thesis, I have evaluated colon cancer cell proliferation under different treatment conditions, for example, with or without CCL17 (100 ng/ml), simvastatin (25 and 50 µM), AntagomiR-155 (25 – 200nM), AntagomiR-Ctrl or target site blockers for 24, 48, and 72 h at 37°C (5% CO₂). Quantitative determination of proliferation was performed using either CCK-8 colorimetric kit or Fluorescence based methods. In experiments where proliferation is not of major concern, I used trypan blue exclusion assay to assess viability of tumor cells under certain treatment, for example, after microRNAs transfection.

4. Apoptosis of colon cancer cells

In this experiment, I have used flow cytometry based-annexin V staining to assess HT-29 colon cancer cell apoptosis. Tumor cells were incubated either with or without simvastatin (25 and 50 µM) for 24 h. To detect apoptosis, cells were stained by annexin V and number of early apoptotic cells were counted and expressed as the percentage of annexin V positive cells. propidium iodide according to manufacturer's recommendations. Cells were analyzed, and the

5. Tumor cell migration evaluation

The core investigations of the ability of colon cancer cells to migrate in vitro as part of key mechanisms of tumor cell metastasis was made using migration assays. In this method, I have tested the chemotactic responses of HT-29 and AZ-97 colon cancer cells using 24-well cell migration boyden chambers with 8 µm pore size inserts. The colon cancer cells were serum starved overnight and resuspended in serum free DMEM with 0.5% BSA and loaded in the inserts. DMEM with 10% FBS with or without CCL17 was added in the lower chambers and incubated for 12, 24, and 48h at 37°C (5% CO₂). Non-migrated cells were removed by cotton

Table 5. Primers sequences used for mRNAs chemokine receptors gene expression			
Chemokine receptor	Sequences of primers		Genbank accession Number
			Product length (bp)
CCR4	Forward 5'- AAGCAAGCTGCTTCTGGT-3'	Reverse 5'- TCAGTCATGGACCTGAGC-3'	NM_005508
CCR6	Forward 5'-CTAGCGGAGTTCAGCAAAC-3'	Reverse 5'-TCAGCCACACATTGCTCTTC-3'	NM_004367
CCR8	Forward 5'-GTGGCCTCTGAAGATGGTGT-3'	Reverse 5'-GCCTTGGTCTTGTGTGGTT-3'	NM_005201
CXCR3	Forward 5'-TCAGCTTTGACCGCTACCTGAACA-3'	Reverse 5'-TGGGAAGTTGTATTGGCAGTGGGT-3'	NM_001504
CXCR4	Forward 5'-TGGGCAGTTGATGCCGTGGC-3'	Reverse 5'-CGATGGCCAGGTAGCGGTCC-3'	NM_001008540
RhoA	Forward 5'-AGAGGTGTATGTGCCACAGTGTT-3'	Reverse 5'-AGGCGATCATAATCTTCGCCCA-3'	NM_001664.2
RhoB	Forward 5'-GCCATAAGCGAACTTGTGCTGT-3'	Reverse 5'-AGCGTACAAGTGTGGTCAGAATGC-3'	NM_004040.2
RhoC	Forward 5'-AGCGGAAGCCTTGACTTCATCTCA-3'	Reverse 5'-TCACCAGTCTTTTCGGATTGCAG-3'	NM_175744.4
β-actin	Forward 5'-AGAGCCTCGCCTTGCCGATCC-3'	Reverse 5'-CACATGCCGAGCCGTTGTTCG-3'	NM_001101
Hsa-MiR-155-5p	Forward 5'- GGGTTAATGCTAATCGTGATAGGGGT -3'		MIMAT0000646
U6 snRNA	Forward 5'-GCTTCGGCAGCACATATACTA-3'	Reverse 5'- CGAATTTGCGTGCATCCTTG-3'	NR_004394.1

swabs from the upper surface of the insert and cells on the lower surface of the insert membrane were fixed in ice-cold 100% methanol and stained with 0.5% crystal violet. In separate experiments, cells were treated the same way as above except that they were also pre-incubated for 30 min with different concentrations of anti-CCR4 antibody, CCR4 antagonist, Rho kinase inhibitor Y-27632 or geranylgeranyl transferase inhibitor GGTI-2133, simvastatin, and AntagomiR-155-5p. All migrated cells were counted microscopically in at least 5 different fields. Migration index was then calculated as the ratio of the number of migrated cells divided by the number of cells in the control wells.

6. Protein analysis: activation assays and Western blot

In my research, I have approached experimental protein research investigations through two main methodologies; ELISA based techniques and immunoblotting. For RhoA activation assay, RhoA-GTP activity was measured using the G-LISA RhoA activation assay according to manufacturer's instructions. protein concentration was determined using Precision Red Advanced Protein Assay. For quantitative detection of active RhoA, 1 mg/ml of protein was used, and the absorbance was read at 490

nm using a microplate ELISA reader. In Western blot experiments, cells were starved 24h and transfected with AntagomiR-155-5p, AntagomiR-Ctrl, Target site blocker (TSB1) and TSB-Ctrl for 24h. Next day, the cells were processed, and 10 µg of protein-cytoplasmic fraction was immunoblotted using specific monoclonal anti-HuR antibody and anti-lamin A as an internal control to avoid nuclear contamination and to normalize protein loading.

7. MicroRNAs transfection

HT-29 colon cancer cells at 60% confluency were starved overnight and on the next day cells were plated in a 6-well culture plate. Cells were reverse transfected with AntagomiR-155-5p (25-200 nM) or AntagomiR-ctrl for 24h in Opti-MEM reduced serum media according to the transfection kit's manufacturer instructions. After 24h, cells were harvested and expression of miR-155-5p and RhoA as well as HuR mRNAs were analyzed by qRT-PCR. The PCR primers used are listed in (Table 5).

8. Bioinformatic analysis of binding sites

RNAhybrid web-based bioinformatics target prediction algorithm was used to predict binding sites for miR-155-5p at the 3'-UTR of RhoA and HuR mRNA (<http://bibiserv.techfak.unibielefeld.de/rna>

hybrid). However, based on evidences of AU-rich elements (AREs) role in stabilizing mRNA targets, we used complementary base-pairing approach between the AREs in the 3' UTR of RhoA and HuR mRNAs and miR-155-5p seeding region. We took into consideration specific ARE motifs such as AUUUA and AUUA motifs, and therefore our analysis was limited to these sites. To assess the function of the binding sites, we designed (TSBs) to bind selectively to sequences overlapping with the miR-155-5p ARE sites in the 3'-UTR of RhoA and HuR mRNAs. The blockers were synthesized as fully phosphorothiolated Locked Nucleic acids in the DNA sequences to increase their affinity and selectivity for the target. Under serum starved conditions, the target site blockers TSB1 of RhoA; 5'-TTAATAATCATAGITGGCTTCT-3' and TSB2 of RhoA; 5'-GTAATCTTAGGTAAATTATAGA-3', TSB1 of HuR; 5'-TTAATATATATCTTAAAGGAAAT-3' and TSB2 of HuR; 5'-TTAATGGICTTAAATGCAAAGT-3' and TSB1 Ctrl of HuR 5'-TAACACGICTATACGCCA-3' were co-transfected with the AntagomiR-155-5p in different concentrations in HT-29 colon cancer cells. RhoA and HuR mRNAs levels were quantified using qRT-PCR. The potential target was functionally validated using RNA immunoprecipitation (RIP) assays.

9. RIP assay

RIP allows the identification of subsets of RNAs including microRNAs associated with RNA-binding proteins and provides information about intracellular changes in the composition of miRNA-ribonucleoprotein (miRNPs). Thus, RIP can be used to identify RNAs that have related functions and are potentially co-regulated via association to RNP complexes [122], the RNA-silencing machinery and therefore being targets of cellular miRNAs [123]. RIP assay was performed using the EZ-Magna RIP kit following the manufacturer's protocol. Cells were scraped off 24h after transfection with either AntagomiR-155-5p or AntagomiR ctrl. Cells were then lysed in complete RIP lysis buffer containing protease inhibitor cocktail, after which 100 µl of whole cell extract was incubated with RIP buffer containing magnetic beads conjugated with an anti-Ago2 antibody or Ctrl-IgG antibody and rotated for 3h at 4°C. After several washes samples were incubated with Proteinase K with shaking to digest proteins at 55°C. RNA was then isolated, and concentrations were measured using a NanoDrop spectrophotometer. The co-immunoprecipitated (co-IP) RNA, including microRNA:mRNA complexes, were analyzed by qRT-PCR to measure relative enrichment of miR-155-5p and mRNAs in Ago-2 immunoprecipitates.

CCL17/CCR4 axis in Colon Cancer Cell Migration

Contents

1. Aim
2. Introduction
3. Results and discussion

1. Aim

To understand how CCL17/CCR4 axis plays a role in mediating colon cancer cell migration and therefore promotes colon cancer metastasis.

2. Introduction

CRC remains one of most aggressive cancers, and currently becomes the second leading cause of cancer-related death cases in Europe [102]. The cause of the vast majority of death cases are believed to be the end result of distant organ metastasis [124; 125]. The mechanisms behind cancer cell metastasis are not fully understood but accumulating data suggest that enhanced tumor cell capacity to respond to different chemotactic stimuli and overexpression of adhesion molecules are essential for the spread of tumor cells [88].

CCR4 is a key receptor that regulates chemokine-dependent immune homeostasis and is predominantly expressed on regulatory T cells and Th2 cells [126]. In tumor microenvironment, tumor cells exploit the accumulation of CCR4-expressed regulatory T cells to escape from the immune surveillance [127]. Several lines of investigations have reported that CCR4 is implicated in some hematologic malignancies, such as adult T-cell leukemia, acute myeloid lymphoblastic leukemia and chronic lymphocytic leukemia [128]. Moreover, functional CCR4 expression has been also reported on gastric and breast cancer cells [129; 130]. In the context of breast cancer, it is worth noting that high expression of CCR4 in patient's biopsies was associated with reduced overall survival in breast cancer patients [129]. One study has shown that CCR4 is expressed in 17% of primary gastric cancers and is associated with a poor patient's prognosis [130]. Another report indicated that only CCR4-positive cancer cells were able to migrate to the lung in the metastatic 4T1 mouse mammary carcinoma model [131].

Chiara Berlato et al. found that CCR4 is expressed among patient's samples of renal carcinoma, orthotopic mouse RCC model as well as different renal cancer cell lines. Interestingly, the study demonstrated that antagonizing CCR4 was not associated with reduce CCR4-positive infiltrating leukocytes, however, anti-CCR4 antibody was able to alter the phenotype of the infiltrating leukocytes and tumor-associated macrophages and increased the number of neutral killer cell NK [132]. Although these emerging evidences suggest a role of CCR4 in gastric, renal and mammary carcinogenesis, the role of CCR4 in promoting colon cancer metastasis remains elusive. CCR4 functions through signaling by two main ligands; chemokine (C-C motif) ligand CCL17 (also known as thymus and activation-regulated chemokine, TARC), and CCL22 (also known as macrophage-derived chemokine, MDC). TARC was first cloned in 1996 and thereafter designated the name of CCL17 [133]. It is expressed constitutively in the thymus tissue and transiently in stimulated peripheral blood mononuclear cells. CCL22 is another CCR4 ligand which shares 37% amino acid sequence identity with CCL17. Like CCL17, CCL22 is expressed in the thymus and myeloid cells [133] and both have been linked tightly to the regulation of different immune cells migration such as T lymphocytes, monocytes, NK cells, and dendritic cells, therefore, they are believed to play a central role in the progression of diverse pathologies ranging from autoimmune diseases to allergies and tumor growth [133; 134; 135]. CCL17 and CCL22 have been reported to be secreted by various types of tumors and have been shown to be elevated in cancer patient's serum. In this respect, one study found that increase serum CCL17/CCL22 ratio was associated with poor survival [132]. Furthermore, previous reports implicated CCL17 and CCL22/CCR4 axis in promoting cancer

metastasis of breast, prostate, gastric, and esophageal cancer [135; 136; 137]. In contrast, Studies involving CCL-17 gene therapy have shown significant colon cancer regression upon viral-transduced CCL17 to the tumor tissue of murine colon carcinoma model because of increased accumulation of immune-infiltrating T cells particularly CD8 T cells to the tumor microenvironment [138]. This is in line with previous findings showed that tumor regression in RCC murine model, because of inhibition of CCR4-infiltrated leukocytes, was associated with phenotype shift of tumor-associated immune cells [132]. It is becoming clear that CCL17/CCR4 axis is involved in the progression of some malignancies; however, the molecular mechanism of how CCL17 promotes or suppresses the carcinogenesis of colon cancer remains to be unraveled. Based on the considerations above, we hypothesized that CCR4 might play a role in mediating migration of colon cancer cells. Thus, understanding the mechanism of how CCL17/CCR4 axis works to promote colon cancer metastasis might propose

new approaches to develop anti-metastatic therapy of colon cancer.

3. Results and discussion

Chemokine receptors have been implicated in tumor growth and distant organ metastasis [88]. In our cell models, we have demonstrated high expression of CXCR3, CXCR4, and CCR6 as well as CCR4 within mRNA levels (**Figure 8A**), thus confirming earlier data where some specific chemokine receptors such as CXCR3, CXCR4, and CCR6 have been shown to be expressed in colon cancer cells [71]. Interestingly, we found that CCR4 was among the highly expressed chemokine receptors in HT-29 cell line and AZ-97 colon cancer cells within protein levels both in cell surface and intracellularly (**Figure 8B&C**). CCR4 has been shown to be expressed in breast, gastric, renal, and leukemic cells [128; 129; 130; 132]. However, to our best knowledge, this is the first report that colon cancer cells express high levels of CCR4 suggesting an important role of this receptor. Given the well-known role of chemokine receptors in cancer

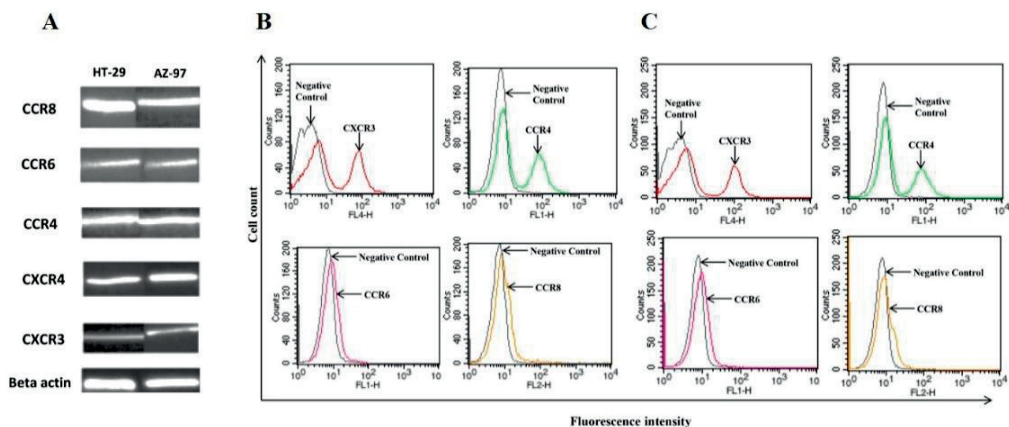


Figure 8. Chemokine receptors expression profile in colon cancer cells. A, Gene expression of chemokine receptors mRNAs in HT-29 colon cancer cells. All gene expression products were at the expected length as summarized in Table 5. B, Cell surface expression. C, Intracellular expression of chemokine receptors. The expression profile of AZ-97 colon cancer cells was similar to HT-29 cancer cells.

metastasis, we addressed a question whether CCR4 could be a functional receptor in colon cancer cells. For this purpose, we used migration assays to evaluate the role of CCR4 in cancer cell migration under CCL17 stimulation. CCL17 provoked increased HT-29 and AZ-97 colon cancer cells migration in a dose-dependent manner (**Figure 9**). Moreover, blocking CCR4 with either blocking antibody or using CCR4 antagonist, significantly decreased CCL17-induced colon cancer cell migration, indicating that migration of colon cancer cells is mediated by CCR4 (**Figure 10**). Providing that migration of colon cancer cells in these settings is dependent on CCR4, we have further extended our studies to uncover the signaling mechanism by which colon cancer cells exploit CCR4 for active migration. To this end, we hypothesized that Rho protein family might be involved in the signaling of CCL17/CCR4 – induced colon

cancer cell migration. Challenging colon cancer cells with CCL17 upregulated Rho (A, B, and C) mRNAs and increased both total and active RhoA protein (**Figure 11**). In addition, co-incubation of colon cancer cells with Rho-kinase inhibitor, Y-27632, and geranyltransferase inhibitor, GGTI-2133, abolished CCL17-triggered colon cancer cell migration (**Figure 12**). Altogether indicate that migration of HT-29 and AZ-97 colon cancer cells is mediated by CCR4. To our understanding, some of chemokine receptors might signal tumor growth besides their capabilities to stimulate cell migration. Therefore, we asked whether CCL17/CCR4 axis has a role in colon cancer proliferation. Proliferation results up to 72 hours showed no difference between CCL17-stimulated and non-stimulated colon cancer cells suggesting the CCL17/CCR4 axis has no role in colon cancer growth (**Figure 13**). Chemokine

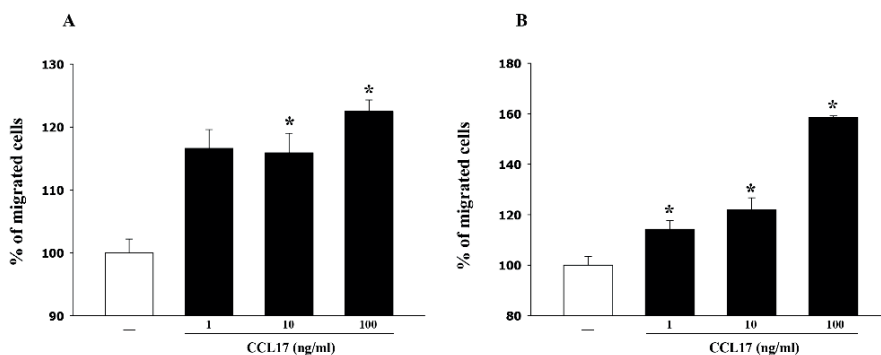


Figure 9. CCL17-induced migration of colon cancer cells. Migration of colon cancer cells was induced by 24 h stimulation with or without 1, 10, and 100 ng CCL17 in **A**, HT-29 and **B**, AZ-97. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represents mean \pm SEM and $n=4$. * $P<0.05$ versus negative control

expressed by various types of tumors including colon cancer [72; 88; 96]. Increase tumor metastasis and poor patient's survival have been linked to aberrant expression of these receptors [139; 140]. The role of CCR4 in mediating immune cell migration has been well documented and it is believed to be exclusively found in myeloid and lymphoid cells because it is predominantly expressed by leukemic cells and T lymphocytes and associated with

hematological malignancies [128; 141], however; CCR4 role in the metastasis of solid tumors remains largely ambiguous. Our findings show that CCR4 is highly expressed in colon cancer cells and well matched to their mRNAs content. This is in line with other findings where CCR4 has been shown to be expressed in breast, renal, prostate and gastric cancer [129, 130, 132, 136]. In our cell models, we found an interesting chemokine receptor's expression profile

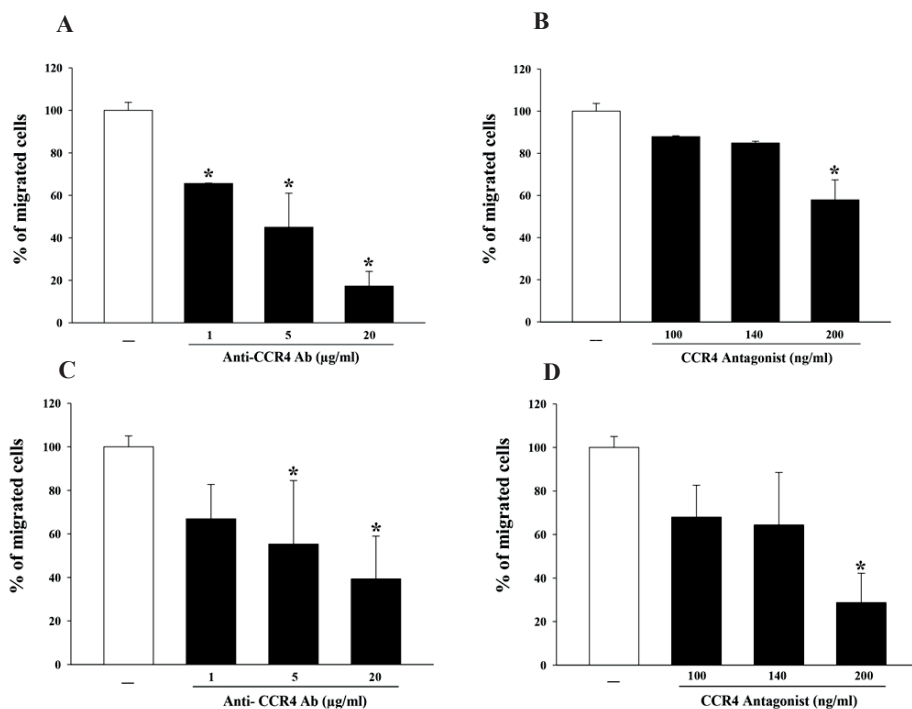


Figure 10. Inhibition of CCL17-induced migration of colon cancer cells is mediated via CCR4. Migration of colon cancer cells was induced by 24 h stimulation with or without 100 ng CCL17 and tumor cell migration was inhibited by pre-incubation of tumor cells with different doses of CCR4 antagonist/ antibody in HT-29 cells **A & C** and AZ-97 cells **B & D**. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represents mean \pm SEM and $n=4$. * $P<0.05$ versus negative control

intracellularly. This might be explained by desensitization phenomena where cell receptors get internalized because of overwhelming activation by their respective ligands. Thus, providing type of protection mechanism against overstimulation [142]. The chemotactic capability of tumor cells is considered as a key mechanism by which cancer cells spread to distant organs. Previous findings reported that CCR4 promotes tumor growth, and the metastasis of breast cancer cells to the lung was

mediated by CCR4 [129; 131]. One study showed that CCR4 is expressed in 12 gastric cancer cell lines and was associated with poor patient's Survival [130]. Another study demonstrated that the expression of CCR4 in renal cell carcinoma cell lines was able to induce cell migration in response to CCL17 stimulation [132]. In addition, studies involved in prostate cancer showed that different prostate cancer cell lines as well as prostate cancer tissues from patients express CCR4, and stimulation with

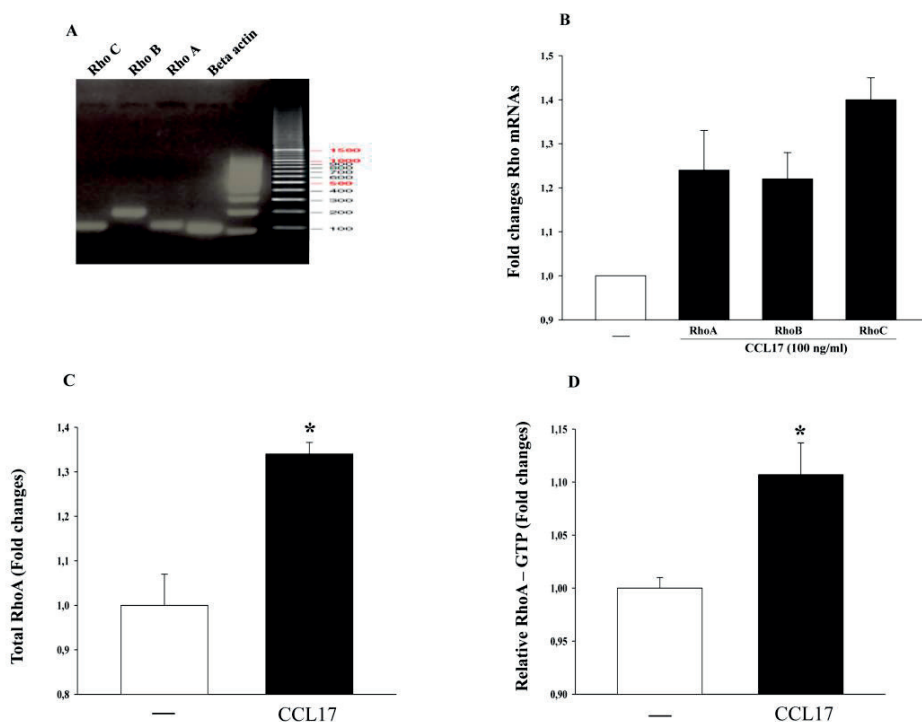


Figure 11. CCL17-provoked RhoA activation in HT-29 colon cancer cells. **A**, CCL17-induced upregulation of Rho proteins mRNAs after CCL17 stimulation in serum-free media for 24 h. **A**, Gel electrophoresis shows respective bands at their expected length. **B**, Relative expression of Rho A-C mRNAs levels expressed as Fold change over control. **C & D**; Levels of total RhoA and active RhoA-GTP relative quantification. Serum-free media served as negative control. Data represent mean ± SEM and n=4. *P<0.05 versus control cells.

either CCL17/CCL22 evoked prostate cancer cell migration while treatment with CCR4 antagonist abolished CCL17/CCL22-induced tumor cell migration [136]. Consistently, our data show that migration of colon cancer cells is mediated through CCL17/CCR4 axis and inhibiting CCR4 function by specific antagonist or blocking antibody, yield in clear reduction in colon cancer cell migration indicating that CCR4 is a functional receptor in colon cancer cells. The chemotactic capacity of tumor cells is governed by the expression of chemokine receptors. Previous studies in athymic xenograft tumor model of breast cancer lung metastasis showed

that high CCL17 levels in the lung is associated with increase migration of CCR4-expressed cancer cells to the lung. Knocking down CCR4 by the use of small interference RNA (siRNA) resulted in reduced chemotactic activity, lung tumor nodules, and angiogenesis induced by CCR4-positive cells [129]. In contrast, another study found that CCR4-positive breast cancer cell lung metastasis is dependent, to a very large extent, on the recruitment of CCR4-positive (T reg) cells [131]. Whether T reg cells are implicated in the metastasis of colon cancer remains to be elucidated.

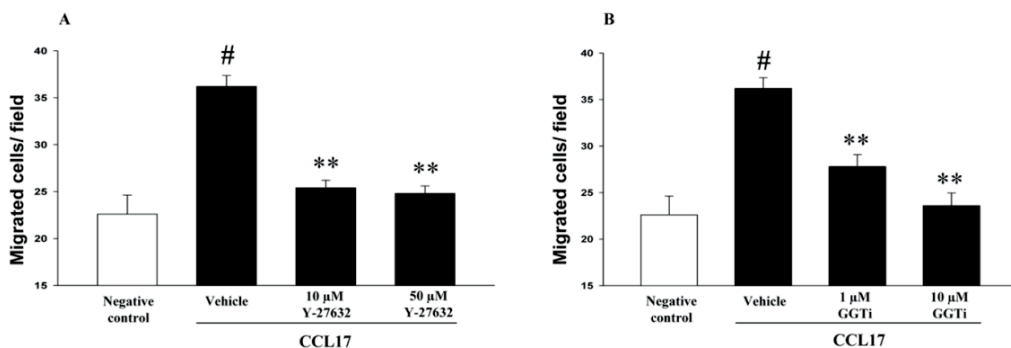


Figure 12. CCL17-induced colon cancer cell migration is regulated by Rho-Kinase and geranylgeranylation. HT-29 cancer cells were preincubated with **A**, Rho-kinase inhibitor (Y-27632, 10 and 50 μ M) and **B**, geranylgeranyl transferase inhibitor (GGTI-2133, 1 and 10 μ M) and allowed to migrate for 24 h in response to 100ng CCL17 stimulation. Data represents mean \pm SEM and n=8. # P <0.05 versus negative control and ** P <0.05 versus vehicle + CCL17

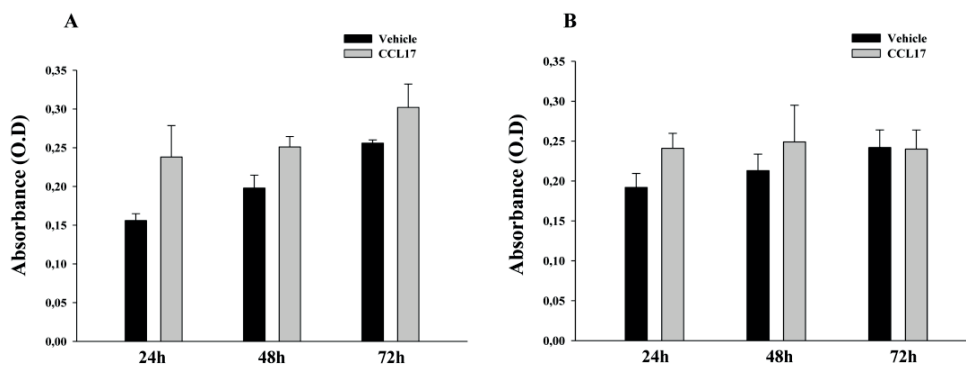


Figure 13. CCL17 has no role in HT-29 colon cancer cell proliferation. A, HT-29 cells. B, AZ-97. Colon cancer cells were stimulated with 100ng CCL17 or vehicle up to 72 h with no significant observation in HT-29 cell proliferation. Data represent mean \pm SEM and $n=5$.

Rho subfamily is a group of small GTPases that belongs to Ras-related small GTP-binding family of proteins. The mesenchymal and amoeboid structural elements and dynamics of the cell are mainly orchestrated by RhoA, RhoB, RhoC, Rac1/2 and Cdc42 [111]. These Rho proteins function as molecular switches that cycle between inactive GDP form and active GTP-bound state and then activate downstream signaling. Rho proteins have been shown to play a central role in key aspects of oncogenic process such as cell proliferation, migration, apoptosis, invasion and survival [113; 114]. Of interest, RhoA which regulates cell cytoskeleton reorganization, focal adhesion and actin stress fiber formation is required for active cell migration [143]. Persistent activation of RhoA has been found in many tumors including colon cancer and associated with poor patient's prognosis [144; 145]. Because of its critical role in cancer cell migration as the driving force of active cell cytoskeleton reorganization, we sought to establish a concrete evidence on its role in regulating CCL17/CCR4-induced colon cancer cell migration. Our findings not only

showed increased expression of Rho (A-C) mRNAs in colon cancer cells, but also RhoA protein found to be activated during CCL17 stimulation. Interestingly, inhibition of ROCK, a downstream effector of RhoA, substantially decreased CCL17-induced chemotaxis of colon cancer cells. Our data are consistent with some depletion studies on RhoA/ROCK showed strong dependence of cancer cells on their effects in migration in response to chemotactic stimuli [146; 147; 148]. Pre-clinical studies reported that inhibition of ROCK results in dramatic reduction in tumor progression and metastasis in peritoneal dissemination, orthotopic breast and pancreatic cancer as well as hepatoma lung metastases models [149; 150]. One supporting mechanism by which RhoA mediates this type of signaling is through activation ERK1/2 signaling pathway [151]. In a model of LPS-induced tumor cell migration, Martina Stähle et al. found that ERK pathway is essential for RhoA-dependent tumor cell migration [152]. Another study found that inhibition of PI3K/Akt pathway suppresses invasion of gastric cancer cells mediated via RhoA [153].

Taken together, these findings indicate that RhoA is a critical player not only in the migration of different cancer cells but also in the migration of colon cancer cells. RhoA must undergo isoprenylation; lipid post-translational modification, in order to facilitate its translocation from the cytoplasm to the cell membrane where it binds the GTPase complex and thereby activate ROCK for downstream signaling [154]. Isoprenylation process are mediated by two major enzymes; geranylgeranyltransferase (GGTase) and farnesyltransferase (FTase), which catalyzes the attachment of pyrophosphate groups to cysteine residues in Rho proteins required for lipid post-translational modification [155]. In cancer, hyper activation of these enzymes is a pre-requisite for atypical activation of Rho proteins. Therefore, we have examined the role of geranylgeranylation in CCL17-triggered colon cancer cell migration. It was found that co-incubation of colon cancer cells with GGTI-2133 drastically decreased CCL17-evoked migration of colon cancer cells, suggesting that geranylgeranylation regulates the chemotactic response of colon cancer cells to CCL17. Notably, previous studies showed that geranylgeranylation inhibition potentially decreased the migration and invasion of

different cancers including colon cancer cells [145; 156; 157]. In a murine model of prostate cancer metastasis, suppression of isoprenoid biosynthetic pathway results in reduction of whole body tumor burden, and significantly slowed the development of tumors, and prolonged overall survival as compared to vehicle treated animals [158].

Cell proliferation is an essential step by which tumor starts growing and increases its size. We examined the proliferation ability of both colon adenocarcinoma cell lines, HT-29 and AZ-97, under the stimulation of CCL17 to understand how CCR4 can regulate tumor growth. No significant proliferation was observed when cells treated by 100 ng/ml CCL17 for 3 days suggesting that CCL17/CCR4 signaling is not crucial for tumor growth. Our findings were consistent with Lee et al.'s findings [130] in gastric cancer and Ji-Yu et al's in breast cancer in which CCR4 did not show a potential role in tumor growth [129]. In contrast, one study using murine model of breast cancer demonstrated that CCR4 increased tumor growth in vivo but not in vitro [159]. This can be explained by the complex cancer-host interaction in vivo and due to the fact that many factors present in the tumor microenvironment can influence tumor growth.

Role of HMG-CoA Reductase in Colon Cancer Cell Migration

Contents

1. Aim
2. Introduction
3. Results and discussion

1. Aim

To examine the role of Three-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) in CCL17/CCR4-induced colon cancer cell migration

2. Introduction

HMG-CoA reductase, is an enzyme that catalyzes the rate limiting step of cholesterol biosynthesis from mevalonate (Figure 14) [160]. Cholesterol biosynthetic pathway is very important to the cells because it provides some proteins the required lipid moiety groups required for normal biological activity [161]. Among the vital proteins involved in regulating cell migration is the small Rho GTPases protein family. This family must undergo isoprenylation process, a post-translational lipid modification required for many protein-protein interactions and plasma membrane translocation of Ras and Rho proteins to exert their functions [161].

Isoprenylation process is catalyzed by two major prenyltransferases: FTase and GGTaseI which covalently attach farnesyl and geranylgeranyl group to the carboxyl-terminal cysteine of proteins terminated by CAAX motif (C is a cysteine, A usually aliphatic amino acid, and X is serine, methionine, cysteine, alanine, or glutamine if the protein is farnesylated or leucine/isoleucine if the protein is geranylgeranylated) [162]. HMGCoA inhibitors, Statins, have received a global attention when they have been demonstrated to exhibit pleiotropic therapeutic effects beyond its cardio-preventing activity. Besides statins role in lowering blood cholesterol, accumulating evidences indicated that statins also exert an antitumor effect in various types of cancers including colon cancer [163; 164; 165]. Interestingly, statins have been shown to reduce CRC incidence with anti-metastatic properties [166; 167; 168]. It has been suggested that inhibition of mevalonate biosynthesis by statins, blocks RhoA and its

effector ROCK which results in decreased tumor cell migration and invasion [169; 170]. One retrospective case-control study of 1309 patients diagnosed with CRC showed that statin users were less likely to develop advanced colon cancer stages with lower metastases [171]. However, controversy findings indicated that statins are not correlated with increase survival rate in patients with colon cancer [172]. Previous data pointed that simvastatin may exert antiproliferative, proapoptotic and anti-metastatic effects on several human malignancies including colon cancer but the exact mechanism is still not fully understood and remains to be elucidated. In previous work, we have showed that CCR4 is expressed and mediate colon cancer cell migration via RhoA/ROCK signaling and dependent on geranylgeranylation [173]. In this paper, we link for the first time between HMG-CoA and its downstream intermediates with CCL17/CCR4 axis signaling in colon cancer and highlight the mechanism by which RhoA involved in colon cancer metastasis. This study also indicates that simvastatin could be a potential therapeutic agent against colon cancer metastasis.

3. Results and discussion

HMG-CoA reductase inhibitors, statins, are drugs prescribed for hypercholesterolemic patients. It has become interestingly obvious that statins exert multiple effects beyond their cholesterol-lowering effect by blocking isoprenoid intermediates such as farnesyl transferase and geranylgeranyl transferase [145; 174]. These intermediates are required for lipid posttranslational modification of small proteins such as GTPases, mainly Rho family, for their intracellular signaling functions. Accumulating data suggested that simvastatin has anti-proliferative, anti-metastatic, and apoptotic properties and plays a potential role in the regression of various types of cancer including colon cancer [163; 165]. The inhibitory effect of

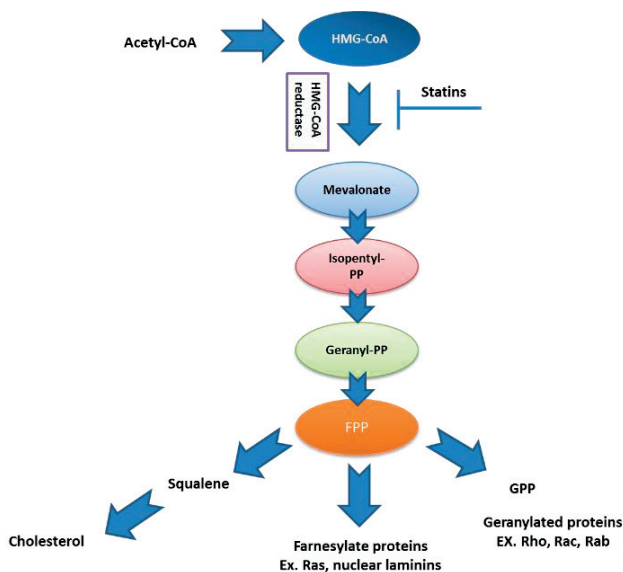


Figure 14. Cholesterol biosynthetic pathway. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by statins impairs the isoprenoids synthesis and cholesterol.

statins are believed to be due to its pleotropic effect. Statins affect nitric oxide (NOS), a critical enzyme in the physiological and pathophysiological responses of the vascular endothelium [175] which has a major role in inflammation. Also, statins have been shown to reduce some inflammatory markers including C-reactive protein and nuclear factor kappa-beta (NF- κ B), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase (MMP) secretion, and platelet aggregation by decreasing thromboxane A2 biosynthesis [176; 177; 178]. Thus, statins exhibit immunomodulatory and anti-inflammatory effect. Moreover, the pleotropic impact of statins seems to be influenced by their distinct physiochemical properties. For example, hydrophilic statins such as Simvastatin and Fluvastatin can enter cells by passive diffusion, whereas hydrophobic statins such as Pravastatin and Rosuvastatin can only

enter the cells via cell membrane protein transporters [179]. This nature of pharmacodynamics may alter statins half-life and potency and therefore might explain the potential effect of statins on many disorders beyond just cardiovascular diseases. Increasing evidence shows that chemokine receptors and chemokines have crucial roles in colon cancer pathogenesis particularly in colon cancer cell migration. Tumor cell migration is a critical step in the metastasis of cancer cells and the inhibitory effect of statins could counteract the metastasis of CRC by suppression of tumor cell migration. Previous studies have demonstrated the role of different statins in reducing cell migration ability through inhibition of RhoA in different tumors [157; 170; 180; 181], however, no one has linked between simvastatin and its downstream effects with chemokine-chemokine receptor signaling in colon cancer. We have previously shown that

CCL17/CCR4 axis is implicated in the regulation of colon cancer cell migration through RhoA signaling [173]. In this study, inhibition of HMG-CoA reductase by Simvastatin reduced CCL17-induced colon cancer cell migration in a dose-dependent manner (**Figure 15 A**). Our findings confirmed earlier data in which simvastatin found to exert significant migration and invasion inhibition of breast, hepatic, renal, and prostate cancer cells, supporting that simvastatin is an effective inhibitor of tumor cell migration [182; 183; 184; 185]. Interestingly, simvastatin did not induce apoptosis nor decreased proliferation of colon cancer cells within 24h suggesting that HMG-CoA is implicated directly in CCL17-induced migration of colon cancer cells (**Figure 15 B & C**). Several studies have documented the ability of statins to induce apoptosis in colon cancer cells including simvastatin [165; 186]. Others also demonstrated that simvastatin augments some chemotherapeutic drugs to induce apoptosis in colon cancer cells [187; 188]. In our study, we found that simvastatin does not induce a significant apoptosis of HT-29 colon cancer cells

in 24h although we have used high concentrations of simvastatin. Several studies showed that simvastatin may induce cell cycle arrest by downregulation of the anti-apoptotic gene Bcl2 within 48h in different cancer cell models including HT-29 colon cancer cell lines [186; 189; 190; 191]. Tumor cell proliferation is an important event in tumor pathogenesis. It has been reported that simvastatin inhibits tumor growth and induces apoptosis in different types of malignancies. Smaller tumor volumes were also observed after simvastatin treatment in previous preclinical studies [165; 192; 193]. Interestingly, we have not observed any significant decrease in tumor cell proliferation at least within 24h. These results suggested that simvastatin seems to reduce invitro colon cancer cell migration independently from its pro-apoptotic effect at least within the timeline of simvastatin treatment.

Mevalonate is not only critical for the synthesis of cholesterol but is also an important precursor of isoprenoid intermediates, including FPP and GPP for cell membrane translocation of Rho family members, including RhoA, RAC,

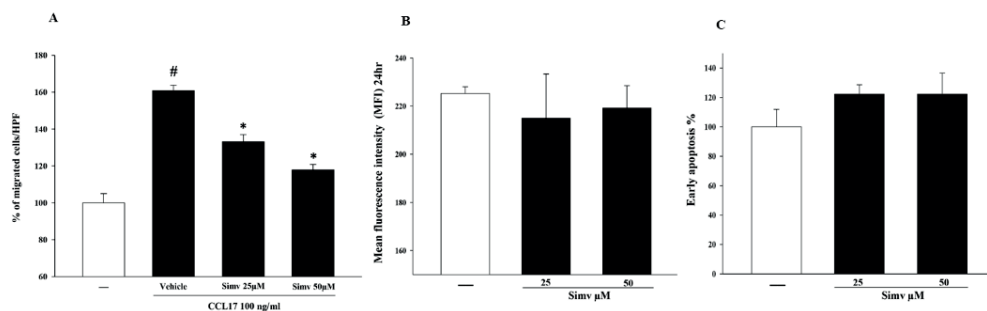


Figure 15. Simvastatin independently inhibits CCL17- induced colon cancer cell migration from its pro-apoptotic effect. A. Simvastatin reduced CCL17-induced HT-29 cell migration in a dose-dependent manner in 24 h. B and C; simvastatin (25 and 50 μM) has no effect on proliferation and apoptosis in 24 h. Proliferation was assessed using Calcein AM fluorescence assay and early apoptosis was evaluated using Annexin V/FITC-PI assay as described in the methodology chapter. The number of early apoptotic cells was counted and expressed as the percentage of annexin V positive and PI negative cells. Data represents mean ± SEM and n = 4. #P < 0.05 versus negative control and *P < 0.05 versus vehicle + CCL17.

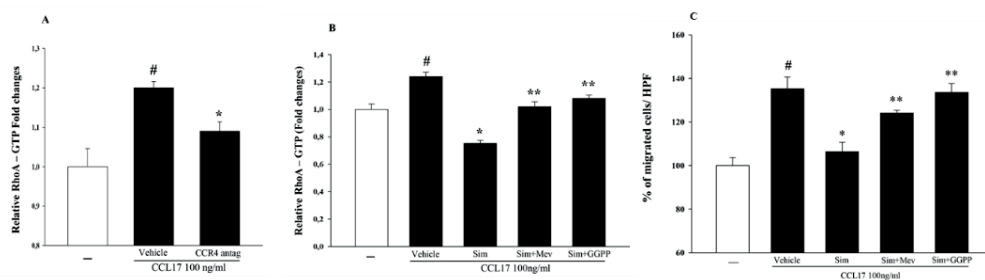


Figure 16. RhoA activation requires geranylgeranylation to mediate CCL17/CCR4-induced colon cancer cell migration. **A**, CCR4 regulates CCL17-induced RhoA activation in colon cancer cells. HT-29 colon cancer cells were pretreated with CCR4 antagonist (200 ng/ml) for 30 min and then stimulated with 100ng CCL17 for 10 min. **B and C**; Adding isoprenoids intermediates; mevalonate (100 μ M) and GGPP (10 μ M) rescued Simvastatin-induced inhibition of CCL17-triggered RhoA activation and colon cancer cell migration. Data represent mean \pm SEM and $n = 4$. # $P < 0.05$ versus negative control and * $P < 0.05$ versus vehicle + CCL17; ** $P < 0.05$ versus CCL17 + simvastatin.

and Cdc42, required for their biological activity to activate downstream signaling [161]. In this regard, aberrant expression of RhoA has been associated with poor prognosis in patients with CRC [144]. Indeed, simvastatin inhibited active RhoA in CCL17-induced cell chemotaxis and this was associated with significant decrease of colon cancer cell migration (Figure 16). Interestingly, our previous findings showed that targeting isoprenylation intermediates such as geranylgeranyl transferase by its specific inhibitor GGTI-2133 reduced CCL17-induced colon cancer cell migration [173]. These results were comparable to those obtained by using ROCK inhibitor, Y-27632, in similar experimental settings as a result of CCL17-triggered RhoA activation (Figure 12A & B). This has led to an important question whether HMG-CoA and GPP could mediate CCL17/CCR4 axis-provoked activation of RhoA and thereby enhance colon cancer cell migration. Emerging data indicated that simvastatin mediates its antitumor effect through inhibition of HMG-CoA reductase intermediates such as mevalonate and geranylgeranyl transferase [189], however, no studies have linked this to

chemokine/chemokine receptor signaling in colon cancer. Therefore, we first examined if CCR4 regulates RhoA activation. It was found that inhibition of CCR4 significantly reduced CCL17-induced RhoA activation (Figure 16A) indicating a functional role of CCR4 in regulating RhoA activity in colon cancer cells. Next, we tested whether this activation is linked to mevalonate pathway. Interestingly, co-incubation of colon cancer cells with mevalonate or GPP reversed the effect of simvastatin-evoked inhibition of colon cancer cell migration and RhoA activity (Figure 16B & C) suggesting that HMG-CoA reductase and geranylgeranylation constitute critical components in the regulation of CCL17-induced RhoA activation in colon cancer cells. Several reports indicated that GPP reversed the inhibitory effect of simvastatin-induced inhibition of RhoA activation not only in cancer cell migration but also in proliferation and angiogenesis [174; 194]. Our findings were also consistent with this observation in which migration inhibition due to simvastatin treatment recovered when mevalonate and geranylgeranyl pyrophosphate (GGPP) were added (Figure 16B & C) confirming some earlier results in which

addition of GPP and FPP reversed the effect of simvastatin-induced migration inhibition of different tumor cell lines [145; 195]. One study showed that overexpression of GPP contributes to lung cancer metastasis and low survival [196]. Interestingly, Although Farnesyl transferase represents another meaning of targeting isoprenoid such as FPP and might be more selective and less toxic to normal cells than GGTase; we have used the geranylation pathway to study the effect of targeting isoprenoid intermediates on colon cancer cell migration. The reasons behind that are: (i) the cancer cell model used in the study, HT-29 colon adenocarcinoma cell, has a wild type KRAS which can switch to geranylated form in case farnesylated form is blocked. This indicates one mechanism by which oncogenic RAS activate another pathway in CRC-chemotherapy resistant cells [197]. In this context, it is worth noting that synergistic combination of chemotherapeutic drugs such as Cetuximab/irinotecan or Doxorubicin and simvastatin overcame drug-resistant colon cancer cells originated from

mutated form of KRAS/BRAF in xenograft models and sensitizes cells for effective chemotherapy in CRC [187; 188; 198], (ii) RhoA has been shown to be well correlated with patient's poor prognosis and it undergoes geranylation rather than farnesylation [199]; therefore, targeting geranylation pathway would be of interest in this scenario.

Taken together, this paper showed that targeting HMG-CoA reductase using simvastatin significantly impaired CCL-17-provoked colon cancer cell migration. In addition, simvastatin reduced CCL-17-induced RhoA activation while addition of mevalonate and/or GGPP recovered colon cancer cell migration and RhoA activation, suggesting that CCL17/CCR4- induced tumor cell migration via RhoA activation is mediated by HMG-CoA reductase. Thus, our findings provide a new insight on how HMG-CoA reductase regulates chemokine/chemokine receptor-induced colon cancer cell migration and using statins might have significant impact in antagonizing colon cancer metastasis.

Role of MIR-155-5p in Colon Cancer Cell migration

Contents

1. Aim
2. Introduction
 - 2.1. MicroRNAs and miR-155
 - 2.2. AU-rich elements (AREs)
 - 2.3. Human antigen R (HuR)
3. Results and discussion

1. Aim

To understand how microRNA-155 regulates colon cancer cell migration under stressed conditions.

2. Introduction

2.1. MicroRNAs

MicroRNAs (miRNAs) are ~ 22 –25 non-coding nucleotide RNAs that post-transcriptionally regulate gene expression. Around 30% of protein-coding genes in human genome is under tight regulation by miRNAs [200; 201]. These short-run nucleotide molecules are believed to be evolutionary conserved between different species because they regulate a broad range of vital cellular functions such as cell proliferation, differentiation, survival, migration, and apoptosis [202]. Due to the nature of miRNAs roles, different miRNAs can exert their function on a single gene while single miRNA can act on several gene targets [203]. In general, miRNAs negatively regulate their mRNAs through direct target recognition by perfect or imperfect base-pair binding leading either to inhibition of translation or complete target degradation. This process is mediated by argonaute-2 protein (Ago-2), the RNA-induced silencing complex (RISC) catalytic subunit and the core decay machinery of RISC complex [204; 205]. MiR-155 was previously reported to be overexpressed in different cancers including colon cancer [206; 207] and high expression was correlated with poor prognosis in CRC patients [207]. Several investigations have reported that miR-155 is implicated in tumor cell migration and invasion [207; 208; 209] and many of worth published reports support the notion that miR-155 functions as a stress and an oncogenic miRNA in some human tumors including colon cancer [206; 210]. Like all miRNAs that post-transcriptionally regulate gene expression, miR-155 must bind its own targets by perfect or imperfect base-pairing and associate with Ago

proteins in RISC in order to exert its function by triggering either mRNA targets degradation or inhibition of their translation [204]. However, a growing body of evidence revealed that microRNAs, contrary to their traditional role, could potentially upregulate their targets either directly or indirectly in response to distinct cofactors such as specific cell type, AU-rich elements (AREs) in the 3'-UTR, and stress cellular conditions for instance; serum starvation. Therefore, they have different regulatory profiles in different types of cancers [211; 212]. Accumulating data suggest that miR-155 could upregulate tumor necrosis factor alpha (TNF α) in RAW 264.7 cells [213]. MiR-155 has also been shown to exert positive regulation through enhancing TNF α translation in response to endotoxic shock [214]. Other miRNAs also demonstrated translation activation as a result of microRNA-target binding. For example, one report showed that synthetic miR-cxcr4 and let-7 activate their targets translation upon placing the target sites in the 3'-UTR of a reporter gene in serum starved HeLa and HEK293 cells [215]. MiR-21 has also been demonstrated to upregulate Bcl-2 in pancreatic cancer [216] and miR-106b was found to upregulate RhoA [217] and more recently, miR-183 showed to positively regulate PSA in prostate cancer cells [211].

RhoA is one of the most extensively studied Rho GTPases family members which involved in different vital tumor cellular functions such as proliferation, membrane trafficking, cytoskeletal organization, and actin stress fiber formation [113]. RhoA has been well-documented to be overexpressed in various tumor types including colon cancer [144]. Accumulating evidence showed that RhoA has a crucial role in active colon cancer cell migration, and abolishing RhoA significantly decreased cancer cell metastasis. In our previous studies, we found that CCL17, the respective ligand of CCR4, directly

contributed to colon cancer cell migration and the migration was mediated by activation of RhoA [173]. However, no studies up-to-date approached how miR-155 regulates chemokine-dependent colon cancer cell migration in stressed conditions such as serum starvation. For this purpose, we knocked down miR-155-5p and evaluated its function in serum-deprived colon cancer HT-29 cells.

2.2. AU-rich elements (AREs)

The stability of mRNAs is a critical factor that controls gene expression at the post-transcriptional level. One of the well-identified destabilizing cis-acting elements present in the 3'-untranslated region (UTR) of short-lived mRNAs, including cytokines and proto-oncogenes, is the AREs [218]. However, not all AREs exhibit equivalent destabilizing functions because AREs are regulated by specific binding activities for instance; those exerted by RNA binding proteins (RBPs) and noncoding RNAs such as miRNAs [219; 220], which determine the faith of the mRNAs. AREs were first described in 1986 as a DNA sequence act as decay elements and regulate the stability of mRNAs [221]. AREs can be classified into three main classes: (i) Class I AREs contain several dispersed pentamer AUUUA, tetramer AUUA, or nonamer UUAUUUA (U/A) (U/A) motifs such as those found in c-fos mRNA. (ii) Class II AREs contain several overlapping AUUUA motifs and exemplified by GM-CSF ARE. (iii) Class III AREs contains no AUUUA pentamer such as those found in c-jun ARE [213; 222]. Although these AREs can act as a decay machinery for many mRNAs, several reports indicated that cellular conditions could be an integral determinant part that influences gene expression under AREs and miRNAs interactions [212; 213]. For example, TNF α can be targeted by Tristetraprolin (TTP) with the microRibonucleoprotein (microRNP) in the destabilizing RBP, and miR-16 in association

with Ago-protein complex leading to TNF α degradation, however; it is translationally activated in quiescent cell conditions if miRNAs target the AREs [213].

2.3. Human antigen R (HuR)

HuR is a nuclear RBP and a member of the embryonic lethal abnormal vision (ELAV) family of RBPs that contains specific RNA recognition motifs which binds AREs-bearing mRNAs [223]. These AREs are usually located in the 3'-UTR of target transcripts, and binding to HuR often results in mRNA stabilization and/or increased translation [224; 225]. HuR is expressed ubiquitously and predominantly localized in the nucleus where it shuttles from the nucleus to the cytoplasm to allow its RNA targets processing [226]. Aberrant expression of HuR in the cytoplasm has been associated with numerous cancers including colon cancer [226; 227] and persistent cytoplasmic translocation was also correlated with poor prognosis [228; 229; 230]. HuR has been shown to play an important role in cancer metastasis through regulating major genes involved in the metastatic process such as Snail, Cox2, MMP-9, uPA, and EGFR [223]. It is therefore well documented how HuR stabilizes oncogenic mRNAs as a part of post-transcriptional regulation, however; how HuR being regulated by miRNAs during cancer cell stress to pursue its biological function remains poorly understood. HuR was identified earlier as a stress de-repressor of miRNA function, for example; it has been shown that HuR inhibits the interaction between miRNA and their targets by uncoupling the miRNPs from the targets and mobilizing them from P-bodies in amino acid-starved human hepatoma cells [231]. However, the role and mechanism of MiR-155 in regulating colon cancer cell migration via HuR under stress conditions are not known. To this end, we hypothesized that miR-155-5p might regulate stress-induced and ARE-dependent

migration of colon cancer cells via RhoA and HuR.

3. Results and Discussion

The discovery of microRNAs has revolutionized our understanding of cell biology and different cellular functions both in health and disease. Despite all the efforts being applied for the treatment of colon cancer, the 5 – years survival rate remains low when the disease spread to distant organs [124]. MiR-155 has been shown to be overexpressed in colon cancer and to play a central role in tumor cell migration [206; 207]. Herein we report for the first time that chemokine CCL17- induced tumor cell

migration is mediated through miR-155-5p by direct positive regulation of RhoA GTPase in stressed colon cancer cells. RhoA mRNA was drastically decreased when miR-155-5p knocked down by antagomiR-155-5p (**Figure 17A & B**). This has lead us to address an important question whether miR-155-5p could regulate tumor cell migration since RhoA is one of the key players in tumor cell migration and it is overexpressed in colon cancer and associated with poor prognosis in CRC patients [144]. Indeed, knocking down miR-155-5p in starved cancer cells has significantly reduced CCL17 – induced HT-29 colon cancer cell migration without affecting tumor cell viability, as indicated by trypan blue exclusion assay, suggesting that transfection

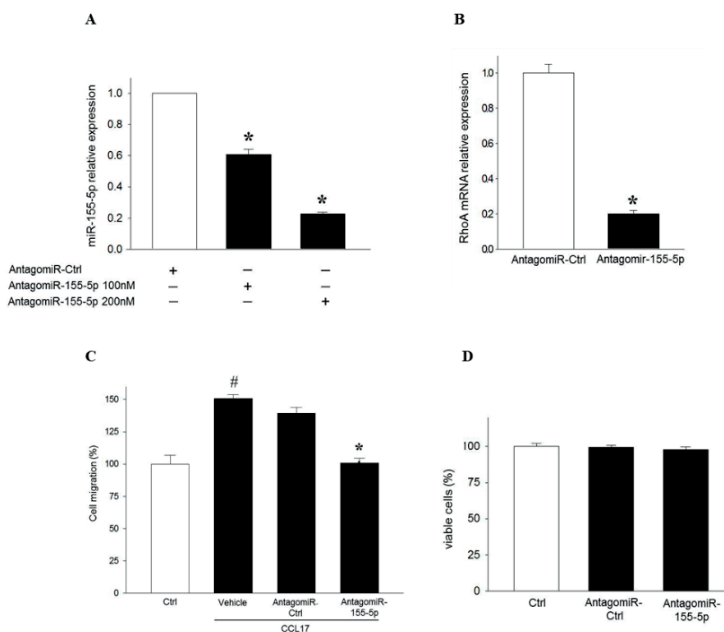


Figure 17. miR-155-5p regulates colon cancer cell migration. **A.** transfection by AntagomiR-155-5p for 24h dose-dependently inhibits miR-155-5p expression and **B.** RhoA mRNA in serum starved HT-29 colon cancer cells. QRT-PCR was used for relative quantification. * $P < 0.05$ versus AntagomiR ctrl. **C.** AntagomiR-155-5p (200 nM) decreased CCL17-induced HT-29 colon cancer cell migration. **D.** viability of HT-29 colon cancer cells transfected with AntagomiR-155-5p (200 nM) or AntagomiR ctrl. All assays were performed in quadruplicate. # $P < 0.05$ versus negative ctrl and * $P < 0.05$ versus AntagomiR ctrl. All data are expressed as mean \pm SEM and $n = 4$.

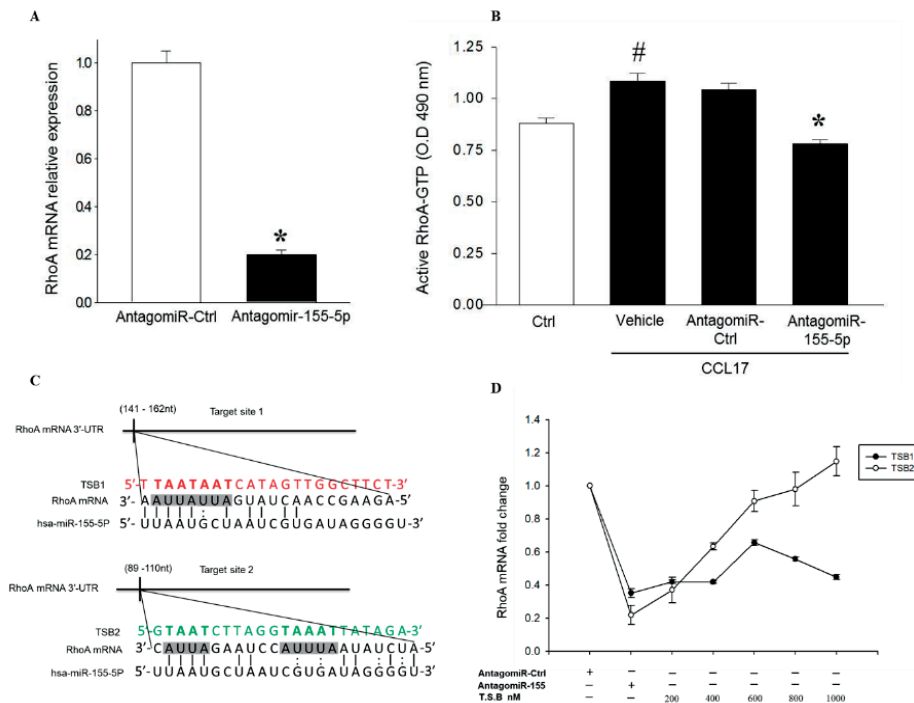


Figure 18. AntagomiR-155-5p reduces RhoA expression and activity in colon cancer cells by direct binding. **A**, transfection by AntagomiR-155-5p (200 nM) downregulates RhoA mRNA expression in serum starved HT-29 colon cancer cells. Relative expression was demonstrated using qRT-PCR. **B**, AntagomiR-155-5p (200 nM) reduced CCL17-induced RhoA activation in serum starved HT-29 colon cancer cells. Data represent mean \pm SEM and $n = 4$. # $P < 0.05$ versus negative ctrl and * $P < 0.05$ versus AntagomiR ctrl. **C**, RhoA is a direct target of miR-155-5p. Predicted target site 1 of miR-155-5p in RhoA mRNA 3'-UTR sequence containing an ARE (AUUA) motif in shaded box. The seeding region of miR-155-5p complementary to AUUA was blocked using TSB1, red sequence. Target site 2 of miR-155-5p in the RhoA mRNA 3'-UTR sequence is depicted in shaded boxes containing two AREs motifs, AUUA and AUUUA, blocked by TSB2, green sequence. **D**, TSB2 dose-dependently reversed the effect of AntagomiR-155-5p on RhoA mRNA expression in serum starved HT-29 colon cancer cells. Data represent mean \pm SEM and $n = 3$.

process was not toxic to the cells and no cell death has been observed within 24h of transfection (**Figure 17C & D**). Altogether, these results suggest that inhibition of miR-155-5p suppresses metastatic behavior of colon cancer cells in vitro under serum starvation condition. RhoA is a Rho GTPase that is regarded as proto-oncogene and often overexpressed in several types of cancers including colon cancer and plays an important role as a molecular switch in transducing extracellular signals to actin and microtubule cytoskeleton [114]. We hypothesized that miR-155-5p should directly target the gene that regulates cytoskeleton organization. Therefore, we assessed RhoA mRNA and RhoA activity under CCL17 stimulation when treated by antagomiR-155-5p. Interestingly, RhoA mRNA and active RhoA were also decreased by antagomiR-155-5p consistent with the migration results suggesting that there is a direct positive correlation between miR-155-5p and RhoA in HT-29 colon cancer cells in serum starvation condition and decrease migration of cancer cells in respect to CCL17 signaling could be regulated by miR-155-5p (**Figure 18A & B**).

To determine whether RhoA is a direct target of miR-155-5p we made bioinformatics analysis and found 5 target sites using RNA hybrid in the 3'-UTR of RhoA (**Figure 18S**). However, existing evidence showed that AREs present in the 3'-UTR of mRNAs could play a central role in regulating mRNA stability and translation. Therefore, we focused on analyzing AREs in RhoA sequence and found a putative AREs binding site complementary to the seeding region of miR-155-5p (**Figure 18C**). By blocking this site using target site blocker, RhoA was significantly recovered its mRNA up-regulation profile when knocking down miR-155 suggesting that AREs have a key role in regulation of RhoA mRNA in serum starvation condition (**Figure 18D**). The miR-155-5p:RhoA mRNA binding was experimentally verified using RIP assay. Strikingly, we found that serum starved cells were enriched by miR-155-5p and RhoA mRNA in Ago2 protein and knocking down miR-155-5p decreased miR-155-5p and RhoA mRNA indicating a positive direct association between miR-155-5p and RhoA in serum starved cells (**Figure 19**).

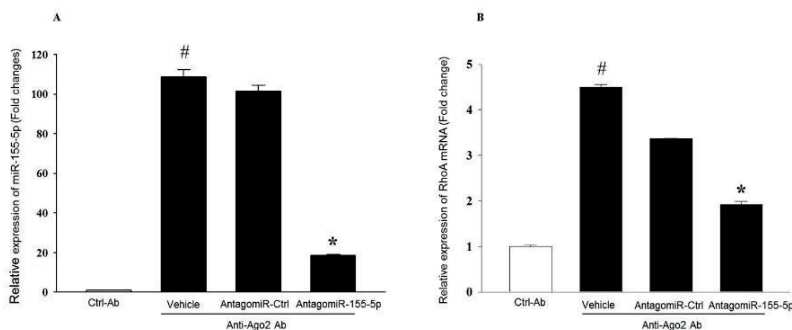


Figure 19. MiR-155-5p is associated with RhoA mRNA in Ago2 immunoprecipitates. The amount of miR-155-5p and RhoA mRNA was measured in input RNA used for the RIP assays by qRT-PCR. AntagomiR-155-5p decreased relative enrichment of **A**, miR-155-5p and **B**, RhoA mRNA in Ago2 immunoprecipitates. Data are expressed as mean \pm SEM and $n = 4$. Data are expressed as fold change compared to anti-IgG Ctrl Ab. # $P < 0.05$ versus ctrl-Ab and * $P < 0.05$ versus anti-Ago2-AntagomiR ctrl treated cells.

The theory that overexpression of miRNAs negatively regulates their targets remains controversial. One report showed that miR-155 negatively regulates RhoA in breast cancer [232]. However, accumulating data suggest that miR-155 could also upregulate other mRNAs such as TNF α in RAW 264.7 cells [233]. It is also worth noting that some miRNAs have been shown to have distinct different pattern of regulation for the same targets in different tumors. For example, miR-21 has been demonstrated to upregulate Bcl-2 in pancreatic cancer and downregulates Bcl-2 in breast cancer and glioblastoma [216]. MiR-106b has also been shown to upregulate RhoA [217] and miR-122 enhances the expression of HCV RNA replication in HepG2 cells [234]. All these evidences suggest that miRNAs regulation profile could be cell-type specific. Other interesting published findings were attributed to cellular conditions such as serum starvation in which miRNAs can be switched from translation repression to translation activation [213]. Vasudevan et al. showed increased translation activation of synthetic miR-cxcr4 and let-7 targets upon placing the target sites of these miRNAs in the 3'-UTR of a reporter gene in serum starved HeLa and HEK293 cells [235]. Similar evidences reported that miRNAs can activate their targets translation when cell enter quiescent state, also refers to G0 phase, in cell cycle arrest conditions such as cell high confluence or serum starvation [213]. TNF α increased translation by direct binding to miR-369-3 was documented on serum starved HEK293 cells by shifting the regulatory role of AREs upon direct binding of miR-369-3 to AUUUA and AUUA motifs in 3'-UTR of TNF α mRNA and therefore recruiting Ago2 protein to mediate translation activation [213]. This growing line of investigations indicated that the presence of AREs in the 3'-UTR of mRNAs could act as a stabilizing or destabilizing factor that initiates translation or increase half-life or degrades mRNAs depending on AREs binding

to either microRNAs or RBPs under serum starvation conditions. In the former case, we found that RhoA mRNA 3'-UTR has several ARE sites mainly AUUA, and three sites contain AUUUA. Interestingly, one of them was complementary to the seeding region of miR-155-5p with 4'-mer perfect binding and the other has an imperfect binding at its centered site. Previous reports indicated the importance of the centered sites of miRNAs in augmenting the miRNA binding to their targets through imperfect binding [236]. By blocking these motifs using target site blocker, we were specifically able to restore the regular RhoA mRNA up-regulation profile when knocking down miR-155 suggesting that these AREs have a significant role in regulation of RhoA mRNA in serum starvation condition. In such conditions, levels of Ago2-RISC repressive components including GW182 can be altered and failure to maintain GW182 interaction with the RISC complex leads to lose repression function and initiate target upregulation [237]. These data are consistency with our findings in which HT-29 colon cancer starved cells showed enrichment of RhoA mRNA and miR-155-5p expression in Ago2 protein. It is important to note here that miRNAs could act on multiple gene targets and whether miR-155-5p could negatively regulate GW182 and therefore initiates the translation activation remains to be elucidated. Increase translation can also depend on AREs-binding proteins such as Hur, TIA-1, and TTP by direct or indirect miRNA binding [238]. MiR-155 has been shown to increase TNF α mRNA transcription stability via Hur in liver alcohol-induced macrophage activation [233]. The authors suggested that miR-155 could regulate Hur either directly or indirectly to stabilize TNF α mRNA. Hur protein is overexpressed in colon cancer and it also might have some role in providing additional stabilizing role to RhoA mRNA in this context. Considering the key role of HuR in cancer, several reports indicated that HuR plays a

significant role in regulating tumor cell migration [239]. Therefore, it was of great interest to examine the role of miR-155 in regulating colon cancer cell migration through HuR in our next study. We found that AntagomiR significantly reduced HuR mRNA in serum starved HT-29 cells while knocking down miR-155-5p in normal serum conditions upregulates HuR mRNA (**Figure 20A and B and supplementary figure 1**). Moreover, AntagomiR-mediated knock down of miR-155-5p decreased serum-dependent cell migration without affecting colon cancer cell viability, suggesting that miR-155-5p is an important regulator of serum-induced directed colon cancer cell migration (**Figure 20C and supplementary figure 2**). This finding extends on a previous study showing that overexpression of miR-155 in HT-29 cells leads to increase random migration [240]. Indeed, this

adds colon cancer cells to a growing list of cancer cells, including breast, hepatocellular, renal, and pancreatic cancer cells, which exhibits miR-155-dependent migration [208; 241; 242; 243]. Interestingly, increased expression of miR-155 is associated with higher frequency of distant metastases in patients with CRC [240]. Thus, miR-155-dependent tumor cell migration might explain at least in part the increased risk of metastasis in patients with high expression of miR-155. Furthermore, we observed that AntagomiR-induced knock down of miR-155-5p also impaired HuR protein expression, suggesting that miR-155-5p positively regulates HuR in serum starved colon cancer cells (**Figure 21**). It is widely accepted that miRNAs are considered as gene translation inhibitors; however, a growing line of evidences demonstrate that several miRNAs can induce increased RNA translation in cells under

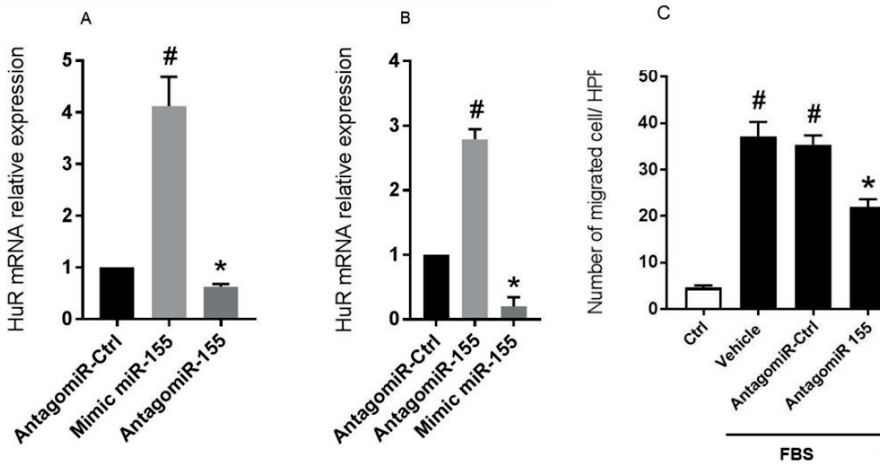


Figure 20. AntagomiR-155-5p reduces HuR mRNA expression and migration of colon cancer cells. HuR mRNA relative expression in **A.** serum-starved and **B.** serum-grown HT-29 colon cancer cells transfected by AntagomiR-Ctrl, AntagomiR-155-5p (25 nM) and miR-155 mimic. Relative expression was demonstrated by qRT-PCR. **C.** Migration of HT-29 cells was inhibited by targeting miR-155-5p by AntagomiR-155. Data represents mean \pm SEM and (n = 4). #P < 0.05 versus AntagomiR-ctrl or Ctrl and *P < 0.05 versus mimic or AntagomiR Ctrl.

quiescence-like conditions, for instance; high cell confluence or serum starvation [213; 235]. Besides cellular conditions, different cell types have been documented to respond differently to specific miRNAs. For example, Bcl-2 has been shown to be upregulated in pancreatic carcinoma cells but downregulated in breast and glioblastoma cancer cells when targeted by miR-21 [216]. The observation of dependent translation of some miRNAs targets is supported by our findings showing that inhibition of miR-155-5p in non-serum starved colon cancer cells triggered increased expression of HuR mRNA (**Figure 20B**). Taken together, these findings indicate that miR-155-5p plays a pro-carcinogenic role in serum starved colon cancer cells via promotion of HuR-dependent cell migration in response to chemotactic stimulation. The current study represents the first report to show that miR-155-5p regulates colon cancer cell migration via HuR. HuR has been reported to be overexpressed in various types of tumors, including colon cancer and plays an important function in regulating many mRNAs that encode proteins involved in cell proliferation, apoptosis, and differentiation [225; 244]. As described above, we found that miR-155-5p positively regulates HuR mRNA expression and function in serum starved colon cancer cells. Positive regulation of mRNA translation by miRNAs has been shown to be related to 3'-UTR-AREs target sites and miRNAs interaction during cell cycle arrest [215; 235]. To verify whether HuR is a direct target of miR-155-5p, we performed bioinformatics analysis and found five target sites that contain AREs complementary to miR-155-5p seeding sequence (**Supplementary figure 3**), however; our work was focused on ARE motifs specifically (AUUA and AUUU) in the HuR mRNA sequence considering that published data have shown that AREs present in the 3'-UTR of mRNAs could play a central role in activating RNA translation [245]. We found two

regions of interest, Target site 1 (TS1) and Target site 2 (TS2) that were complementary to the seeding region of miR-155-5p and have the ARE motifs AUUA and AUUU (**Figure 21A**). Therefore, we have designed specific blockers targeting these ARE sites at 3'-UTR of HuR mRNA. Interestingly, we found co-incubation with one specific blocker targeting the TS1 ARE motifs (AUUA and AUUU), which has more binding interactions with miR-155-5p than TS2, dose-dependently reversed AntagomiR-155-5p-induced inhibition of HuR mRNA and protein expression as well as HT-29 colon cancer cell migration without affecting tumor cell proliferation suggesting that this specific ARE region of 3'-UTR of HuR mRNA is a functional target of miR-155-5p in serum starved colon cancer cells (**Figure 21B, C, D, and E**). Previous reports indicated that binding to centered sites is as important as binding to seeding sites in augmenting miRNA and mRNA target interactions. To confirm the target site, we also co-transfected HT-29 cells with mimic miR-155 and TSB1 in normal serum conditions (**Supplementary figure 4**) and found increased HuR mRNA expression. Moreover, co-incubation with TSB1 recovered both HuR protein expression and tumor cell migration (**Figure 21 C and D**) indicating that this site is a potential binding site. We have also used anti-lamin A to exclude if this effect was mediated through nuclear contamination of HuR during cytoplasmic extraction. Indeed, there was no detection of Lamin A in the cytoplasm (**Supplementary figure 5**) suggesting that only cytoplasmic HuR was mediating the effect of tumor cell migration. Thus, this study identifies a novel target site regulating translational activation of HuR mRNA by miR-155-5p. These results are in line with our findings showing that serum starved HT-29 colon cancer cells exhibited enrichment of HuR mRNA and miR-155-5p levels in the Ago2 protein indicating functional relation between miR-155-5p and HuR (**Figure 22**). MiR-155 has been

demonstrated to increase TNF α mRNA stability and transcription via HuR in activated macrophages [233]. The overlapping in ARE-BPs and ARE-Binding microRNAs has been documented previously to stabilize different transcripts. For example; miR-3134 has been found to bind HuR and stabilizes HuR-regulated transcripts such as SOX9, VEGFA, ARE motifs

that not only can target HuR directly but also can compete with TTP, a translation repression-mediated RNA-BP, to the ARE binding sites on HuR preventing HuR degradation. Another speculated scenario would be that miR-155-5p might negatively regulate TTP since miRNAs act on multiple gene targets therefore relief the repression induced by TTP upon binding HuR.

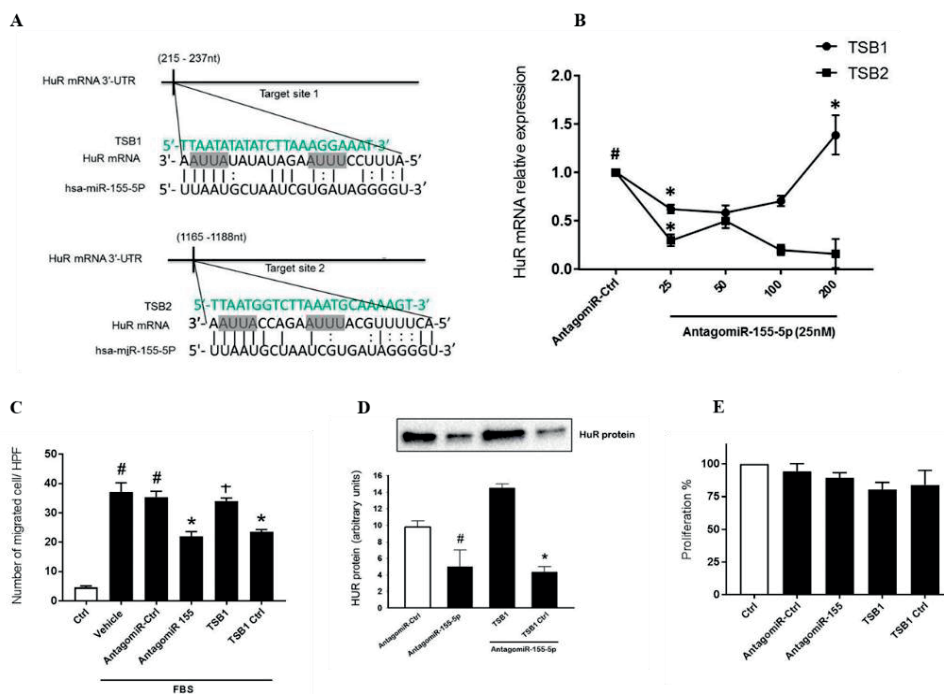


Figure 21. HuR is a functional target of miR-155-5p. **A.** Predicted target sites of miR-155-5p in HuR mRNA 3'-UTR sequence containing ARE motifs (AUUA, AUUU) is depicted in shaded box. The seeding region of miR-155-5p complementary to ARE motifs was blocked using TSBs, green sequences as described in the methodology chapter. **B.** TSB1 dose-dependently reversed the effect of AntagomiR-155-5p on HuR mRNA expression in serum starved HT-29 colon cancer cells. **C.** Effect of blocking TSB1 on the migration of starved HT-29 cancer cells after transfection with AntagomiR-Ctrl, AntagomiR-155-5p (25 nM), TSB1, and TSB1 Ctrl. **D.** Blocking TSB1 recovered cytoplasmic expression of HuR protein determined by western blot. Protein fractions were normalized to total protein and quantitative assessment of HuR protein in each fraction is illustrated as a histogram in arbitrary unit. **E.** Proliferation of HT-29 cancer cells after transfection with AntagomiR-155-5p (25 nM), AntagomiR-ctrl, TSB1 and TSB Ctrl. Data are expressed as mean \pm SEM and $n = 4$. # $P < 0.05$ versus Ctrl or AntagomiR-Ctrl and * $P < 0.05$ versus TSB1 or AntagomiR-155-Ctrl.

Such mechanisms could be cell specific or because of cellular regulation during stress conditions. It is also quite interesting to point out that GW182 and FXR-1, which are key proteins in miRNA-mediated translation repression/activation and part of the RISC complex, promotes miRNA induced translation in G0 phase, a condition which can be achieved by serum starvation [213; 237], and whether miR-155-5p could regulate GW182 and FXR-1 and thereby initiate translation activation remains to be uncovered. Knowing that HuR protein and RhoA as well as miR-155-5p are overexpressed in colon cancer cells and miR-155-5p directly and positively regulates RhoA and HuR, it would be of great interest to target

miR-155-5p in early metastatic stages of colon cancer where stress conditions are at highest demands for the tumor to improve the understanding of mechanisms regulating colon cancer cell migration and metastasis. In summary, we have provided a new insight on how miR-155-5p regulates RhoA and HuR in tumor cell migration and showed that miR-155-5p regulates RhoA and HuR positively by direct binding in serum starved colon cancer cells and this regulation was mediated by AU-rich elements present in 3'-UTR of their mRNAs. In addition, antagonizing miR-155-5p had a potential impact on tumor cell migration which could be a potential strategy to prevent colon cancer metastasis.

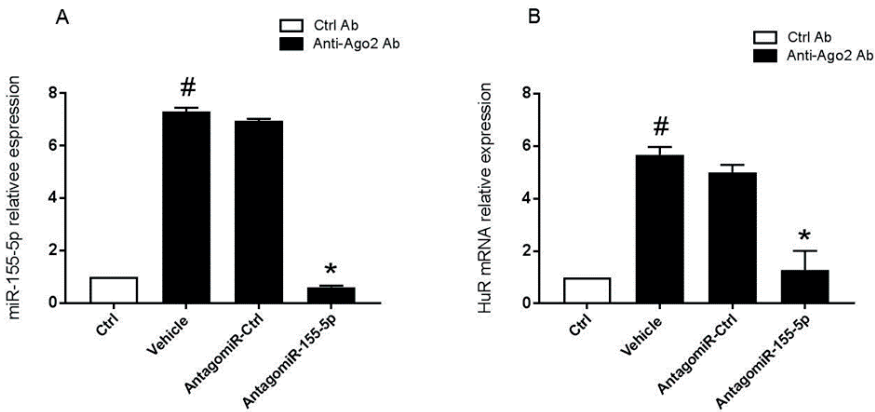


Figure 22. MiR-155-5p is associated with HuR mRNA in Ago2 immunoprecipitates. AntagomiR-155-5p decreased relative enrichment of A. miR-155-5p and B. HuR mRNA in Ago2 immunoprecipitates. Data are expressed as mean ± SEM and n = 5 and represented as fold change compared to anti-IgG ctrl and U6 was used as a housekeeping gene. #P < 0.05 versus Ctrl Ab and *P < 0.05 versus anti-Ago2-antagomiR-Ctrl

Thesis Conclusions

- CCR4 is a functional receptor that signals colon cancer cell migration.
- CCL17-induced colon cancer cell migration is mediated by RhoA protein and ROCK.
- RhoA geranylgeranylation is required for active cell migration.
- HMG-CoA mediates CCL17/CCR4-dependent colon cancer cell migration.
- MiR-155-5p positively regulates colon cancer cell migration by direct binding to RhoA and HuR-AREs
- Cancer stress conditions may alter traditional regulation profile exerted by miR-155-5p.
- Targeting CCR4, mevalonate pathway, and miR-155-5p might be effective ways to antagonize colon cancer metastasis.

Populärvetenskaplig sammanfattning

Koloncancer är en av de vanligaste cancertyperna i världen. Trots att det finns flera sätt att behandla cancer på räknas det som en av de dödligaste på grund av spridningen till andra organ. Om cancer spridits till andra organ minskar chansen för överlevnad bland cancerpatienterna. Vår förståelse för hur cancer sprider sig, särskilt hur det regleras på molekylärnivå, kan göra det möjligt att studera molekyler som är dysreglerade och därmed kan användas för tidig upptäckt av cancer, eller som prognostiska biomarkörer för att kunna utveckla behandling mot tumören. Koloncancer kan klassificeras i huvudsak två kategorier baserade på riskfaktorer: genetiskt- och sporadiskt koloncancer. Genetisk koloncancer har gener som är kopplade till koloncancer, till exempel Hereditär Nonpolyposis Colon Cancer (HNPCC) och Familjär adenomatös polypos (FAP). Sporadisk koloncancer utgör upp till 70 % av alla koloncancer sjukdomar. Denna cancertyp nedärvs inte till nästa generation som den hereditära gör, dock är generna aktiverade på grund av riskfaktorer bland annat ålder, inflammatoriska tarmsjukdomar, låg fysisk aktivitet, högt fettintag och alkoholkonsumtion. För att kunna spridas behöver koloncancer små inflammatoriska mediatorer som kallas chemokiner. När det finns en inflammation i kroppen utsöndrar cellerna chemokiner för att signalera till de vitabloodkropparna via receptorer, så kallade chemokinreceptorer, så att de kan hitta och bekämpa infektionen eller inflammationen. Studier har visat att chemokiner

spelar en viktig roll för cancerspridningen. De kan stimulera receptorer som finns på cancercellerna, liknande de som hittas på vitabloodkroppar, och därför kan cancercellerna ta sig till andra organ. Dessutom har det visats i några studier att immunceller som har infiltrerats i tumörer kan producera en massiv inflammatorisk respons via chemokinreceptorsignalering vilket i sin tur kan aktivera tillväxt av tumören. Syftet med min avhandling var att studera mekanismen bakom migrationen av koloncancer celler och hur cancer celler svarar på chemokinstimulering och vilka andra molekyler som kan reglera cancer cellsmigrationen under chemokine-inducerad koloncancer cell migration. I min studie har jag undersökt fyra huvudsakliga spelare som skulle kunna ha betydelse för migrationen av cancer celler: RhoA och HMG-CoA som båda är enzymer, microRNA-155 som är icke-kodande RNA och HuR protein. RhoA är en väldigt viktig molekyl som reglerar cellers förflyttning. HMG-CoA är ett annat enzym vilket spelar en roll i kolesterolbiosyntesen, dock visade det sig att det enzymet kan leda till att RhoA binds till plasmamembranet genom att en fettgrupp tillsätts i en process, så kallad geranylationsprocess, så att RhoA blir funktionellt aktivt. HuR är ett RNA bindande protein som visade sig kunna stabilisera gener involverade i cellmigrationen. Dessutom fann jag att miR-155 hade en tydlig roll på cellmigration genom att kontrollera RhoAs och HuRs uttryck och funktion.

Supplementary figures

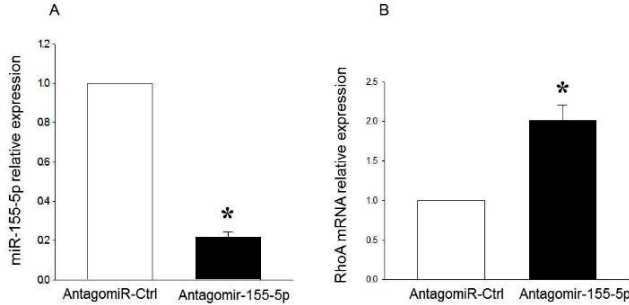


Figure 1. Effect of miR-155-5p on RhoA. A, HT-29 colon cancer cells were grown in 10% serum and miR-155-5p was knocked down using AntagomiR-155-5p (200 nM) or AntagomiR control (200 nM) and relative expression was demonstrated using qRT-PCR. B, RhoA mRNA expression in response to miR-155-5p knockdown in serum-grown HT-29 cells was demonstrated by qRT-PCR. Data represents mean ± SEM and (n = 4). *P < 0.05 versus AntagomiR control.

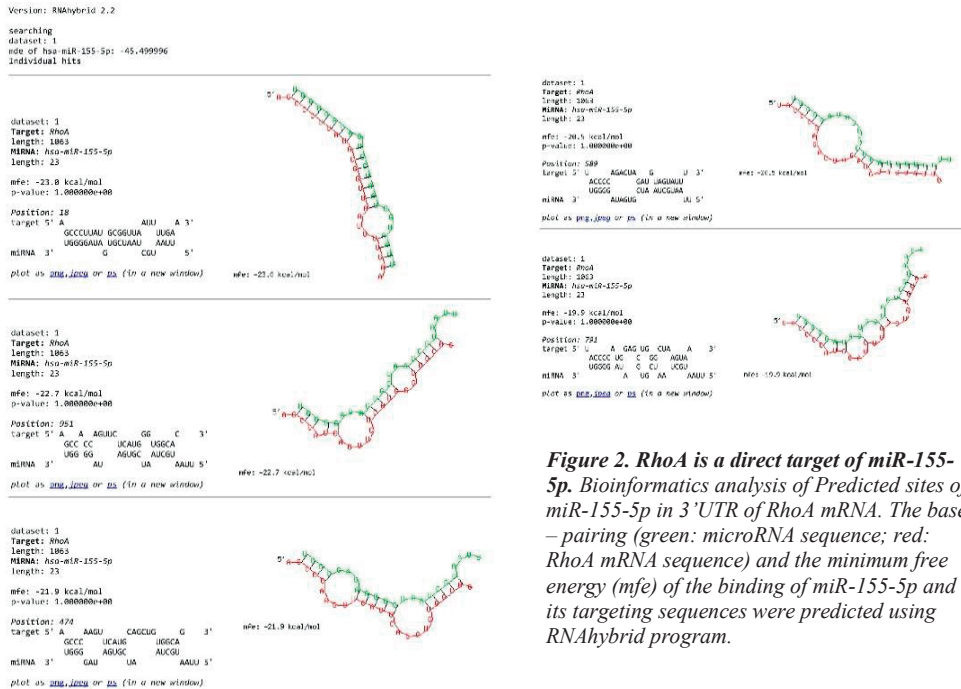


Figure 2. RhoA is a direct target of miR-155-5p. Bioinformatics analysis of Predicted sites of miR-155-5p in 3'UTR of RhoA mRNA. The base – pairing (green: microRNA sequence; red: RhoA mRNA sequence) and the minimum free energy (mfe) of the binding of miR-155-5p and its targeting sequences were predicted using RNAhybrid program.

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3utr_human -t /var/bibiserv2/anonymous/rnahybrid/23/14/09/bibiserv2_201
11-23_140952_QHgz/rnahybrid_input_target_rna_sequences_.file
searching
dataset: 1
mfe of hsa-miR-155: -45.499996
Individual hits
-----
dataset: 1
target: HuR
length: 1208
miRNA : hsa-miR-155
length: 23
mfe: -20.6 kcal/mol
p-value: 1.000000e+00
position 272
target 5' G      UG      GG      A 3'
      UCCC UC GA UGGU UGA
      GGGG AG CU AUCG AUU
miRNA 3' U      AU UG A      UA      5'
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target: HuR
length: 1208
miRNA : hsa-miR-155
length: 23
mfe: -20.5 kcal/mol
p-value: 1.000000e+00
position 923
target 5' C      U      UG      C      A 3'
      AUCCCC UC CG      ASCG UUAG
      UGGGGG AG GC      UCGU AAUU
miRNA 3'      U      U      UAA      5'
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dataset: 3
target: HuR
length: 1208
miRNA : hsa-miR-155
length: 23
mfe: -19.4 kcal/mol
p-value: 1.000000e+00
position 794
target 5' C      A      AUACU      U 3'
      GCCCC      CAC AUUG      GUUG
      UGGGG      GUG UAAUC      UAAU
miRNA 3'      AUA      C      G      U 5'
-----
dataset: 4
target: HuR
length: 1208
miRNA : hsa-miR-155
length: 23
mfe: -18.8 kcal/mol
p-value: 1.000000e+00
position 506
target 5' C      U      C      UCAGUCCCG      U      3'
      ACC CCUG UCAC      GGC
      UGG GGAU AGUG      UCG
miRNA 3'      CUA      UAAUU 5'
-----
dataset: 5
target: HuR
length: 1208
miRNA : hsa-miR-155
length: 23
mfe: -18.4 kcal/mol
p-value: 1.000000e+00
position 561
target 5' G      G      CCA      UG      C      3'
      GCCC U      GCG      GGC
      UGGG A      UGC      UCG
miRNA 3'      G      UAG      UAA      UAAUU 5'

```

Figure 3. HuR is a direct target of miR-155-5p. Bioinformatics analysis of Predicted sites of miR-155-5p in 3'UTR of RhoA mRNA. The base – pairing between miR-155-5p sequence and HuR mRNA sequence were aligned according to the the minimum free energy (mfe) of miR-155-5p required to bind its target sequences. Target sites were predicted using RNAhybrid program.

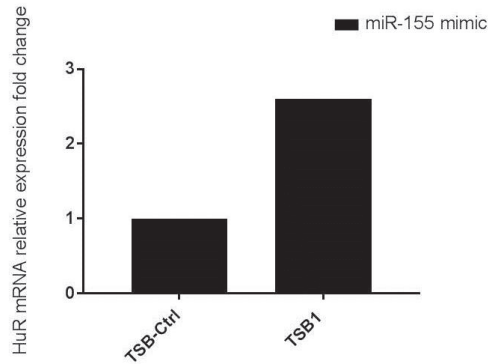


Figure 4. Effect of TSB1 on HuR mRNA in normal serum conditions. HT-29 colon cancer cells were grown in 10% serum and co-transfected with miR-155-5p mimic (25 nM), TSB1, and TSB-Ctrl as described in materials and methods. Relative expression was demonstrated by qRT-PCR. Data are represented as mean \pm SEM and (n = 4).

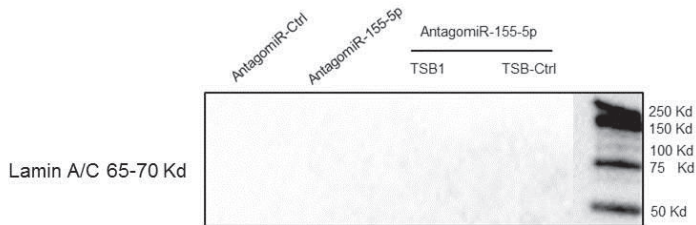


Figure 5. Cytoplasmic fraction of HuR protein expression. Cytoplasmic samples show that the extraction process has no nuclear contamination as indicated by the nuclear marker lamin A/C.

Supplementary tables

Table 1. Screening tests recommended for CRC

Screening method	Benefits	Limitations	Time interval
Fecal occult blood (FOBT)	<ul style="list-style-type: none"> • Low cost • Sampling is done at home • No bowel preparation required • Dose not required sedation • Non-invasive 	<ul style="list-style-type: none"> • Low specificity • and sensitivity • Multiple samples are needed • Colonoscopy is indicated upon positive results 	Annual
Fecal Immunochemical test (FIT)	<ul style="list-style-type: none"> • Low cost • Sampling is done at home • No bowel preparation required • Dose not required sedation • Non-invasive 	<ul style="list-style-type: none"> • Low specificity • and sensitivity • Multiple samples are needed • Colonoscopy is indicated upon positive results 	Annual
Stool DNA test	<ul style="list-style-type: none"> • Sampling is done at home • Single sample is needed • No bowel preparation required • Dose not required sedation • Non-invasive 	<ul style="list-style-type: none"> • Low specificity • and sensitivity • High cost • Colonoscopy is indicated upon positive results 	Uncertain
Double-contrast Barium Enema	<ul style="list-style-type: none"> • Can usually visualize all the colon • No need to sedation 	<ul style="list-style-type: none"> • Full bowel preparation • Low specificity • Can not remove polyps in Realtime • Exposure to low dose of radiation • Colonoscopy is indicated upon positive results 	5 years
Computed Tomographic Colonography (CTC)	<ul style="list-style-type: none"> • Non-invasive • Quick • Can visualize the entire colon • No sedation required 	<ul style="list-style-type: none"> • Full bowel preparation • Can not remove polyps in Realtime • Exposure to low dose of radiation • Colonoscopy is indicated upon positive results 	5 years
Flexible Sigmoidoscopy	<ul style="list-style-type: none"> • Quick • No sedation required • Minimum bowel preparation 	<ul style="list-style-type: none"> • Can only visualize one third of the colon • Can not remove large polyps • Slightly effective when combined with stool based tests • Small risk of infection or bowel tearing • Colonoscopy is indicated upon positive findings 	5 years
Colonoscopy	<ul style="list-style-type: none"> • High sensitivity and specificity • Can visualize the entire colon • Can remove polyps, obtain biopsies and detect other diseases 	<ul style="list-style-type: none"> • Full bowel preparation is needed • Sometimes sedation is required • Risk of infection or bowel tearing • High cost 	5 years

Table 2. TNM Classification for Colon Cancer (American Joint Committee on Cancer AJCC and Dukes staging)

Stages	T	N	M	Dukes Stages
Stage 0	T0/Tis	Nx/N0	M0	-----
Stage I	T1	N0	M0	A
	T2	N0	M0	B1
Stage II	T3	N0	M0	B2
	T4	N0	M0	B2
Stage III	T1/T2	N1/N2	M0	C1
	T3/T4	N1/N2	M0	C2
Stage IV	Any T	Any N	M1	D

Tis; carcinoma in situ; Cancer cells are found only in the epithelium or lamina propria, which are the top layers lining the inside of the colon or rectum.

Tx: The primary tumor cannot be evaluated.

T0 (T plus zero): There is no evidence of cancer in the colon or rectum.

T1: The tumor has grown into the submucosa

T2: The tumor has grown into the muscularis propria

T3: The tumor has grown through the muscularis propria and into the subserosa.

T4: The tumor has grown into the surface of the visceral peritoneum or has attached to other organs or structures.

NX: The regional lymph nodes cannot be evaluated.

N0: There is no spread to regional lymph nodes.

N1: There are tumor cells found in 1-3 regional lymph node or tumor cells found in the structures near the colon that do not appear to be lymph nodes.

N2a: There are tumor cells found in 4 or more regional lymph nodes.

M0 (M plus zero): The disease has not spread to a distant part of the body.

M1: The cancer has spread to 1 other parts of the body beyond the colon or rectum.

Table 3. Commonly used medications for colorectal cancer.

First line	Second line	Third line
FOLFOX bevacizumab FOLFOX FOLFIRI +bevacizumab Capecitabine Bevacizumab +FU/LV FU FU/LV Cetuximab, irinotecan FOLFIRI Bevacizumab	FOLFOX +bevacizumab Cetuximab, irinotecan Capecitabine FOLFOX FOLFIRI Bevacizumab, capecitabine Bevacizumab +FU/LV FOLFIRI +cetuximab Bevacizumab Irinotecan	FOLFIRI +bevacizumab FOLFOX +bevacizumab Capecitabine FOLFIRI Panitumumab Bevacizumab, irinotecan FOLFIRI +cetuximab FOLFOX Bevacizumab, capecitabine Cetuximab

Abbreviations: FOLFOX, fluorouracil, leucovorin, and oxaliplatin; FOLFIRI, fluorouracil, leucovorin, and irinotecan; SD, standard deviation; FU, fluorouracil; LV, leucovorin.

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د/ عمرو عبدالوهاب الحيدري

Colon cancer is one of the hardest healthcare challenges of our time. The heterogeneity of the disease creates a deep dilemma in front of the existing therapeutic modalities. Understanding the mechanisms by which colon cancer spreads to distant organs is a key in developing new strategies to win our war against cancer.

يعتبر سرطان القولون أحد أكبر التحديات التي تواجه النظام الصحي في عصرنا الراهن. تكمن مشكلة سرطان القولون في التعدد النوعي والنمطي لهذا المرض والذي بدوره يشكل عوائق أمام الأشكال العلاجية المختلفة حالياً. إن الفهم العميق لآلية الإنتشار العضوي لسرطان القولون يعتبر أهم مفاتيح الحلول لتطوير إستراتيجيات جديدة تساعدنا على القضاء على سرطان القولون

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