SCHOOL OF LIFE SCIENCES

JAWAHARLAL NEHRU UNIVERSITY

NEW DELHI-110067

INDIA

CERTIFICATE

This is to certify that the research work embodied in this thesis entitled "**Exploring the role of microRNA dysregulation in the pathogenesis of Ulcerative Colitis**" submitted for the award of Degree of Doctor of Philosophy has been carried out by **Ms. Swati** under the guidance and supervision of **Dr. Jaishree Paul** and **Dr. Niti Puri** at the School of Life Sciences, Jawaharlal Nehru University, New Delhi- 110067, India.

This work is original and has not been submitted so far, in part or in full for the award of any other degree or diploma of any other university

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Exploring the role of microRNA dysregulation in the pathogenesis of Ulcerative Colitis

Thesis submitted to

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By

SWATI

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Abbreviations and Symbols

AIEC	Adherent invasive *Escherichia coli*
α	Alpha
5-ASA	5-amino salicylic acid
ANOVA	Analysis of variance
APC	Antigen presenting cell
AMO	Anti-miRNA oligonucleotide
ASO	Anti-sense oligonucleotides
ATG16L	Autophagy-related protein 16-1
β-ME	Beta mercaptoethanol
CO_2	Carbon dioxide
CARD9	Caspase recruitment domain containing protein 9
CCR	C-C chemokine receptor
CLDN	Claudin
CRC	Colorectal cancer
cDNA	Complementary DNA
CD	Crohn's Disease
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
CMV	Cytomegalovirus
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DEPC	Diethyl pyrocarbonate

DNMT	DNA methyl transferase
DDW	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
EtBr	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
ECCO	European Crohn’s and colitis organization
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
FP	Forward primer
γ	Gamma
GEO	Gene expression omnibus
GRN	Gene regulatory network
GWAS	Genome wide association studies
GDNF	Glial cell derived neurotrophic factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gm	Gram
HIV	Human immunodeficiency virus
IKKα	IκB Kinase
IRAK	IL-1 receptor-associated kinase-1
Ig	Immunoglobulin
IRGM	Immunity Related GTPase M
IBD	Inflammatory Bowel Disease
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukine

IMF	Intestinal myofibrofblast
κ	Kappa
Kg	Kilogram
LPS	Lipopolysaccharide
LNA	Locked nucleic acid
LB	Luria Bertani
MIP	Macrophage inactivating peptide
MST1	Macrophage stimulating 1
MMP	Matrix metalloproteinases
µg	Microgram
µL	Microliter
MiRNA	MicroRNA
Mg	Milligram
mM	Milli molar
MQ	Milli Q
MAPK	Mitogen activated protein kinase
MMLV	Moloney Murine Leukemia Virus
MUC2	Mucin 2
MDR1	Multi drug resistance 1
MS	Multiple sclerosis
ng	Nano gram
NK	Natural killer
NI	Non-inflamed
NSAID	Nonsteroidal anti-inflammatory drugs
NFκB	Nuclear factor κ B

NOD	Nucletide oligomarization domain
PRR	Pattern recognition receptor
PenStrep	Penicillin-Streptomycine
PBMC	Peripheral blood mononuclear cell
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PS	Phosphorothioate
PCR	Polymerase chain reaction
PSC	Primary sclerosing cholangitis
RORγt	RAR- related orphan receptor gamma t
RP	Reverse primer
RPM	Revolution per minute
RA	Rheumatoid arthritis
RNAse	Ribonuclease
RNA	Ribonucleic acid
RISC	RNA inducing silencing complex
STAT3	Signal transducer and activator of transcription 3
SCCAI	Simple clinical colitis activity index
SNP	Single nucleotide polymorphism
SDM	Site directed mutagenesis
snoRNA	Small nucleolar RNA
SOCS1	Suppressor of cytokine signaling
SLE	Systemic lupus erythematosus
TAB2	TGF beta associated kinase 2
Th	T helper

TGN	Thioguanine nucleotides
TNBS	Trinitrobenzenesulfonic acid
TBE	Tris/Borate/EDTA
TP53	Tumor protein 53
TRAF6	TNF receptor-associated factor-6
TLR	Toll Like receptor
TGFβ	Transforming growth factor beta
TNFAIP3	Tumor necrosis factor alpha induced protein 1
TNFα	Tumor necrosis factor beta
Treg	T Regulatory cell
UC	Ulcerative Colitis
UTR	Untranslated region

Table of Contents

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic and idiopathic inflammatory disorder of gastrointestinal tract. Ulcerative colitis (UC) and Crohn's Disease (CD) are the two clinical subtypes of IBD. The typical symptoms of IBD include rectal bleeding, diarrhea, fever, loss of appetite and abdominal pain. UC and CD share the underlying cause of pathogenesis but these forms can be differentiated on the basis of their clinical representation and their characteristic colonoscopic features. In UC, the inflammation initiates from the rectum and progresses in upward direction in a continuous manner affecting the colon at varying degree. Any region of gastrointestinal tract can be affected in case of CD, with a discontinuous pattern of inflammation that is responsible for its characteristic skip lesions. In case of UC, the inflammation is superficial in nature while in CD inflammation is transmural in nature affecting the deeper layers of the intestine.

The highest incidence and prevalence rates of IBD are found in western countries such as North America and Europe therefore initially IBD was considered to be a disease of western world. But the past few decades have shown a gradual rise in IBD incidences even in the developing countries. The adoption of western culture and rapid industrialization is considered as one of the main reason behind the increasing cases of IBD in developing countries. In India, UC is the prevalent form of IBD.

IBD results from a complex interplay of microbial, genetic and environmental factors. It is hypothesized that a dysregulated immune response is generated against the commensal flora of the gut in genetically susceptible individuals under an influence of environmental factors. 163 genetic loci for IBD have been identified through genome wide association studies (GWAS), some of which overlap for UC and CD while others are specific for each subtype. Mutation within any of the susceptibility gene can either render an individual susceptible to IBD or it may provide protection against IBD development. Environmental factors including smoking, diet, sanitation, appendectomy, geographical location, race, ethnicity contributes to IBD onset. Dysbiosis in the commensal gut flora is another contributing factor in IBD pathogenesis. Reduced diversity in the microbiome has been reported in the fecal samples obtained from IBD patients with respect to healthy individuals.

Despite our current hypothesis regarding the genetic basis of IBD, the monozygotic twin studies have suggested a concordance rate of 10-15% for UC and 30-35% for CD. While

according to the GWAS, UC and CD account for 16% and 23% heritability respectively. These reports leave gaps in our apprehension of IBD heritability but also highlight the contribution of environmental and epigenetic factors in IBD development and progression. The epigenetic factors such as microRNA work at the interface of genetic and environmental factors and altered miRNA signatures have been reported from UC and CD patients.

MiRNA are short in length (18-22 nucleotide long), single stranded, non-coding, RNA molecules which binds within the 3'UTRs of their target mRNA to inhibit their protein synthesis either by degrading the mRNA or by translational inhibition. Mutation within the genomic region coding for miRNA affects the miRNA expression and also the miRNA mediated regulation of gene expression. Distinct miRNA signatures have been reported from the blood and mucosal tissues of both UC and CD patients and site-specific alteration in the expression of miRNA has also been reported from UC patients. The functional analysis has linked these altered miRNAs with the major inflammatory pathways contributing to IBD pathogenesis. MiRNAs play key role in the regulation of development, proliferation and differentiation of Th17 and Treg cells which have pro-inflammatory and anti-inflammatory roles during inflammatory response respectively. MiRNA can directly modulate the expression levels of molecules that have significant roles in Toll like receptor (TLR) signaling and pattern recognition receptors (PRR) induced signaling which are considered as the integral part of innate immune response.

NFκB signaling pathway is a major inflammatory pathway. During steady state, regulatory molecules such as TNFAIP3 and IKKα resolve the inflammatory response through negative feedback regulation. But during IBD, an exaggerated and aberrant inflammatory response is triggered which persist for longer duration leading to more severe diseased state. Some of the signaling molecules that are integral part of NFκB pathway also happen to be potential gene targets for miRNAs and the expression of these signaling molecules are often modulated by miRNAs thereby, influencing the inflammatory response.

In, the present study we looked at the miRNA signatures of the inflamed and non-inflamed colonic regions of UC patients. Preliminary functional analysis was carried out to identify the biological relevance of altered miRNA expression during UC.

The specific objectives of our study were:

1. To investigate the miRNA profile of inflamed and non-inflamed colonic mucosa of UC patients.
2. Identification of potential gene targets for miRNA showing altered expression in the inflamed colonic mucosa.
3. To evaluate the expression of dysregulated miRNAs and their respective gene targets in UC patients.
4. Functional analysis of dysregulated miRNA through *in vitro* studies.

LITERATURE REVIEW

1.1 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a gastrointestinal inflammatory disorder which clinically demonstrates in two forms, Ulcerative Colitis (UC) and Crohn's Disease. These two forms of IBD are characterized on the basis of their clinical, pathological, endoscopic and radiological features. UC and CD share common risk factors and both display chronic intestinal inflammation with intermittent cycles of relapse and remission but they can be well differentiated on the basis of their clinical representation. The characteristic features of UC and CD are described in **Figure 1**. IBD normally commences during the second or third decade of life and in majority of patients it progresses into a chronic and relapsing disease **(Xavier and Podolsky (2007)**. Initially IBD was considered to be a disease of western world but in recent decades its incidences have risen up even in the developing countries both in pediatric and adult populations leading to increased global burden of IBD. The etiology of IBD still remains elusive but it is hypothesized that it develops due to inappropriate immune response generated against the commensal flora of gut in genetically susceptible individuals with an influence of environmental factors (**Chapman and Pekow 2015)**. The innate and adaptive immunity play pivotal role in regulating the inflammatory response during IBD. Under steady state, the inflammatory response is down regulated to maintain the gut homeostasis, but in IBD patients the mucosal immune response remains activated due to which inflammation persists for longer duration eventually resulting in chronically inflamed intestine. **Figure 2** describes the major histological features observed in UC and CD.

1.2 Major subtypes of IBD

1.2.1 Ulcerative Colitis

In UC, mucosal inflammation begins from rectum and progresses proximally in a continuous manner affecting the gastrointestinal tract to a varying degree. Based on the extent of inflammation, UC has been divided into five subtype, i) Proctitis (affecting only rectum), ii) recto sigmoiditis also known as proctosigmoiditis (involving rectum and sigmoid colon), iii) distal colitis (where inflammation extends up to transverse colon) iv) extensive colitis affecting large area of colon and v) pancolitis (involving whole colon) **(Satsangi, Silverberg et al. 2006) (Figure 3).**

During the diseased state, massive infiltration of neutrophils in the crypts and lamina propria are observed which results in formation of micro abscesses. Patients diagnosed with UC are at higher risk of developing dysplasia and adenocarcinomas therefore they require lifelong regular endoscopic surveillance. Also, UC patients are prone to develop colorectal cancer (CRC) after 8-10 years of diagnosis and the risk for CRC development depends upon the duration and extent of UC , patients with pancolitis are more prone to develop CRC **(Eaden, Abrams et al. 2001, Jess, Rungoe et al. 2012, Bopanna, Ananthakrishnan et al. 2017).**

1.2.2 Crohn's Disease

Any region of the gastrointestinal tract can be affected in case of CD but the terminal ileum and perianal regions are affected most commonly. It shows a discontinuous inflammation throughout the intestine and gives it the characteristic skip lesions; also, the inflammation is transmural in nature affecting the deeper layers of intestinal tract. One of the characteristic features of CD is macrophage aggregation forming non-caseating granulomas and thickening of submucosa giving the bowel. a cobblestone appearance due to deep ulcers and tissue swelling. In addition to this, CD patients encounter additional problems such as formation of fistula, abscesses and strictures **(Khor, Gardet et al. 2011, Zhang and Li 2014).** CD is also characterized in different types based on the extent of inflammation **(Figure 4)**.

1.3 Other types of IBD

1.3.1 Indeterminate colitis

Indeterminate colitis defines those cases of IBD where a conclusive diagnosis for UC and CD cannot be made using the standard colonoscopy and histologic parameters. Of the total cases, around 10-15% cases of IBD fall in this category **(Guindi and Riddell 2004).**

1.3.2 Pediatric IBD

In IBD patients, 25-30% of UC patients and 20% of CD patients are younger and less than 20 years of age **(Baldassano and Piccoli 1999**). Over the time, the incidence of pediatric IBD have risen up worldwide; however, the accurate estimates of worldwide incidence rates are lacking **(Murch, Baldassano et al. 2004).** Apart from the specific

sign and symptoms of IBD, pediatric IBD manifests some nonspecific symptoms such as anorexia, delayed puberty, fatigue and decreased linear growth **(Diefenbach and Breuer 2006)**.

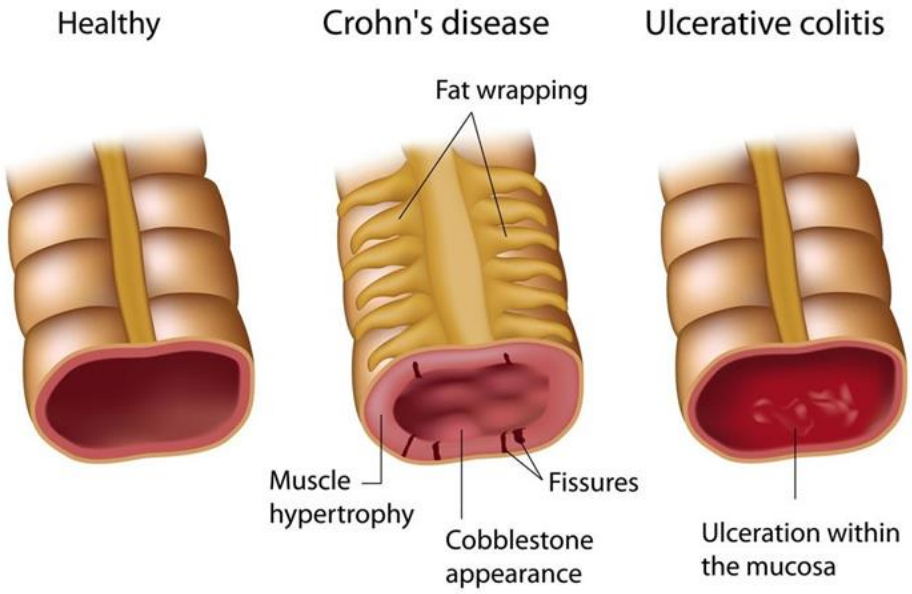


Figure 1: Characteristic features of Ulcerative colitis and Crohn's Disease (https://www.bowelcanceraustralia.org/bowel-diseases)

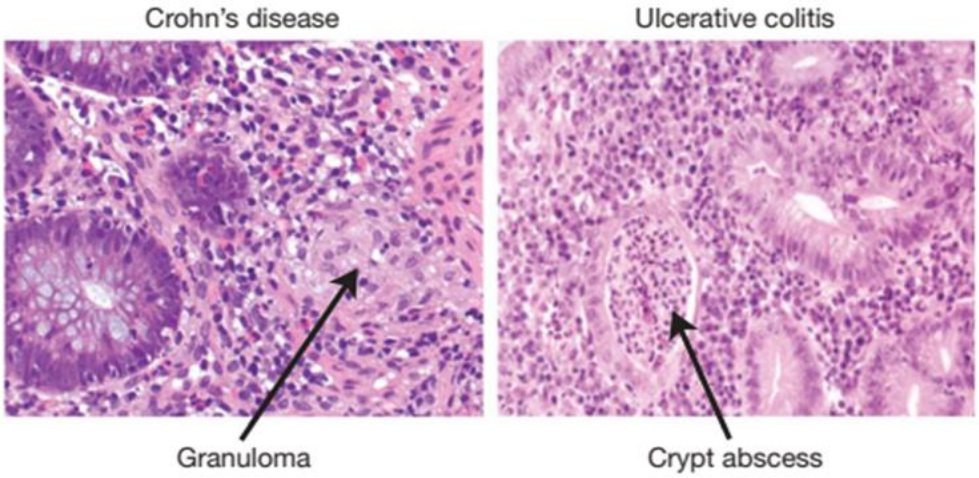


Figure 2: Histological hallmarks of Crohn's Disease and Ulcerative Colitis (Xavier and Podolsky 2007)

1.4 Sign and Symptoms of IBD

The symptoms of IBD depend upon the severity and location of inflammation. The major sign and symptoms commonly observed in UC and CD are given below.

- Abdominal pain
- Diarrhea
- Rectal bleeding
- Weight loss

Some individuals suffer from extra intestinal manifestation such as:

- Arthritis
- Skin problems and mouth sores
- Eye inflammation affecting vision

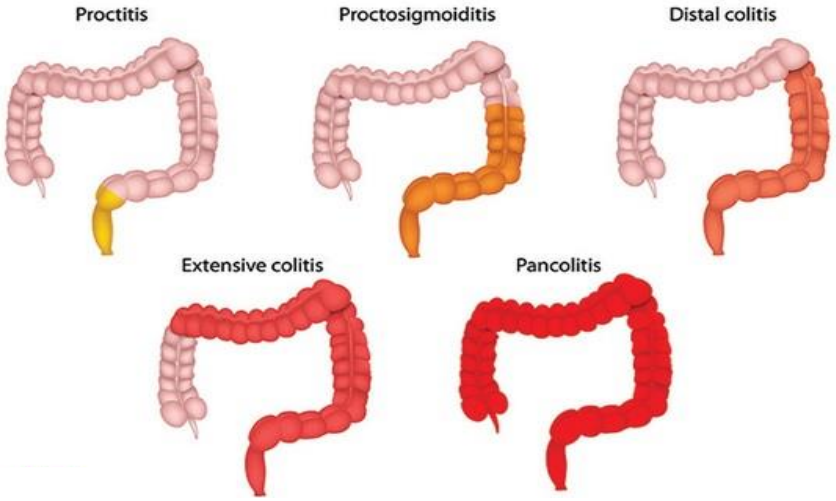


Figure 3: Types of Ulcerative colitis based on disease extent

(Adapted from https://www.ibdrelief.com)

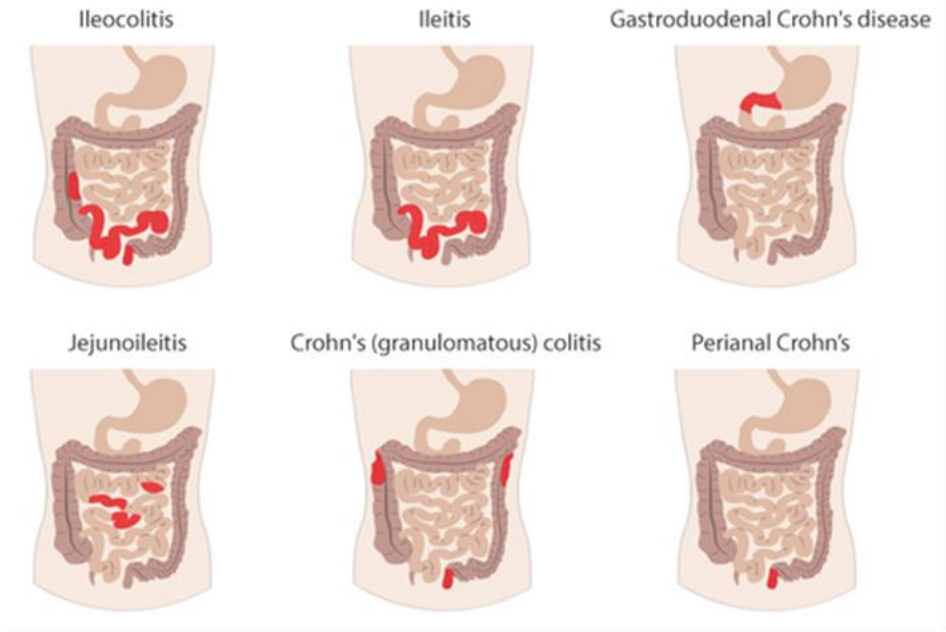


Figure 4: Types of Crohn's Disease based on disease extent

(Adapted from https://www.ibdrelief.com)

1.5 Treatment of IBD

Despite improvement in our understanding the etiology of IBD past few years, the complete cure for the disease still remains elusive. Therefore, the primary goal for therapeutic interventions is to improve the quality life of patients by inducing and maintaining remission so as to prevent disease relapse. The major therapeutic approaches currently used for IBD treatment are discussed below.

1.5.1 Anti-inflammatory drugs

1.5.1.1 5-amino salicylic acid (5 ASA)

5-ASA is effectively used for the induction **(Feagan and Macdonald 2012)** and maintenance of remission **(Wang, Parker et al. 2016)** in mild and moderate UC patients. 5-ASA helps in resolving inflammation by several different mechanisms such as inhibition of nuclear factor kappa-B (NFkB) pathway **(Egan, Mays et al. 1999)**, induction of oxidative stress to suppress the intestinal epithelial cell injury as well as apoptosis **(Dallegri, Ottonello et al. 1990) (Sandoval, Liu et al. 1997),** inhibition of leukotrienes production in colon **(Lauritsen, Laursen et al. 1986)**. A dose of 2.4 to 4.8 gm 5-ASA per day is considered to be effective in the treatment of mild and moderate active distal UC and since 5-ASA has similar mode of action as peroxisome proliferator-activated receptor-γ (PPAR-γ), it probably acts as a chemopreventive measure to reduce the colorectal cancer risk in UC patients **(Triantafillidis, Merikas et al. 2011).**

1.5.1.2 Corticosteroids

Corticosteroids are the second major treatment therapy used to induce remission in active UC and CD patients. Prednisolone, methylprednisolone, and budesonide are the most widely used corticosteroids to treat moderate to severe IBD patients. Corticosteroids work by inhibiting the activation of nuclear factor kappa-B (NFkB) and its downstream pro-inflammatory cytokine production. They also reduce the recruitment of immune cells and expression of adhesion molecules at the site of inflammation thereby, resolving inflammation **(Sales-Campos, Basso et al. 2015)**. Although corticosteroids are one of the most effective therapeutic measures to treat the acute exacerbation in IBD, prolonged use of corticosteroids have some serious side effects such as osteoporosis, osteonecrosis, increased susceptibility to infections and myopathy **(Carter, Lobo et al. 2004)**.

1.5.2 Immunomodulators

An inappropriate immune response triggered under influence of environmental factors is one of the major causes of IBD development, therefore immunomodulation is one of the ways to induce remission and prevent disease relapse in IBD patients. Immunomodulators used for IBD treatment are thiopurines (6-mercaptopurine and azathioprine), methotrexate, cyclosporine and tacrolimus **(Triantafillidis, Merikas et al. 2011)**.

1.5.2.1 Immunosuppressant

Azathioprine and 6-mercaptopurine are immunosuppressants and their mode of action includes the inhibition of T-lymphocytes and natural killer cells proliferation and activation **(Zenlea and Peppercorn 2014)**. Azathioprine is a pro-drug which is converted to 6-mercatopurine by glutathione S-transferase, which is further metabolized in subsequent steps to yield the final pharmacological active product 6-thioguanine nucleotides (6-TGNs). Then the assimilation of these 6-TGNs occurs into DNA or RNA as purine antagonists to carry out the cytotoxic and immunosuppressive effects **(Nielsen, Coskun et al. 2015)**. Recommended doses for azathioprine is 2 to 2.5 mg/kg bodyweight and for 6-mercatopurine is 1 to 1.5 mg/kg bodyweight and these are found to effectively treat about 40% of IBD cases post 5 years of treatment. According to studies, concurrent use of aminosalicylates and azathioprine is reported to be much more beneficial to IBD patients **(Triantafillidis, Merikas et al. 2011)**.

Methotreaxate is an antagonist of folic acid and possess anti-inflammatory and anti-apoptotic properties. It is effectively used to induce remission and prevent relapse in active CD patients who fail to respond against 5-ASA and mercaptopurines. But there are not enough reports to suggest role of methotrexate in treatment and maintenance of remission in UC **(Carter, Lobo et al. 2004)**.

Cyclosporine is a calcineurin inhibitor and it ameliorates the secretion of pro-inflammatory lymphokines **(Zenlea and Peppercorn 2014)**. It has been effectively used for inducing remission and delaying colectomy in steroid refractory UC cases **(Cohen, Stein et al. 1999**) but it is does not induces remission in CD patients successfully **(Feagan, McDonald et al. 1994)**. Tacrolimus is another immunosuppressive drug and gets easily absorbed in intestine than cyclosporine therefore; it is a better choice for

steroid refractory UC patients. It also acts by inhibiting T-cell activation and proliferation **(Matsuoka, Saito et al. 2015)**.

1.5.2.2 Anti-TNF therapy

TNFα is a pro inflammatory cytokines and has major role in IBD pathogenesis. Anti TNF therapy is a major advancement for management of steroid dependent IBD patients and also for fistulating CD patients **(Bernstein 2015)**. Infliximab is a human murine chimeric monoclonal antibody specific for TNFα and is used for treatment of CD and occasionally for UC as well. Infliximab is even effective in treating the fistula in CD. Infliximab efficiently binds with the soluble and membrane bound form of TNFα and subsequently inhibits the biological activity of this cytokine. Adalimumab and golimumab are some other TNFα blockers used for CD treatment **(Sales-Campos, Basso et al. 2015)**.

1.5.3 Modulators of intestinal microbiota

Human gut is home to about 10^{14} bacteria with approximately 1000 different species and this gut microbiota has a very important role to play in IBD pathogenesis. An abnormal immune response generated against gut commensal flora in genetically susceptible individuals is responsible for chronic intestinal inflammation. Besides this, dysbiosis in the gut flora is also responsible for IBD development. IBD patients reported to have increased population of bacteroides, *Escherichia coli* and enterococcus and reduced population of lactobacilli and bifidobacteria **(Nishida, Inoue et al. 2018)**. Therefore, modulation of intestinal flora through use of antibiotics or probiotics could be beneficial for IBD patients. The primary goal of this therapeutic strategy is to restore the balance between gut microflora and prevent inflammation.

1.5.3.1 Antibiotics

Metronidazole and ciprofloxacin are the most frequently used antibiotics for the secondary complications such as abscesses, perianal fistula and also for maintaining remission in CD patients. Rifaximin is another drug used to treat active luminal disease involving colon. Besides these, clarithromycin and anti-tuberculous regimens have also been considered in clinical trials. (**Nitzan, Elias et al. 2016)**. There are not enough evidences to support the role of antibiotic for UC treatment. Antibiotic combination

therapy with amoxicillin, metronidazole and tetracycline are under clinical trial to determine whether they can help in inducing and maintain remission in UC patients **(Ohkusa, Kato et al. 2010)**.

1.5.3.2 Probiotics

Probiotics are living microorganisms that are safe for human consumption and provide health benefits when taken in adequate amount. They provide beneficial effects by their antimicrobial activity, suppressing bacterial growth by inducing or modulating immune response, enhancing the barrier activity and suppressing T cell proliferation **(Damaskos and Kolios 2008)**. *E.coli* Nissle 1917 is one of the most effective probiotic used for maintaining remission in UC patients and is considered as effective as mesalazine **(Kruis, Fric et al. 2004)**. A recent meta-analysis suggested the effectiveness of probiotics in IBD. It reports that a combination of Lactobacillus as a probiotic with a prebiotic maintains remission in UC patients more effectively while a combination of *Streptococcus boulardii*, Lactobacillus, and VSL#3 probiotics could provide beneficial effects in CD **(Ganji-Arjenaki and Rafieian-Kopaei 2018)**.

1.5.3.3 Prebiotics

Prebiotics are the food substances that are not hydrolyzed or absorbed in the small bowel, specifically promotes the growth of beneficial bacteria and also improves the gut flora to a healthier composition **(Looijer-van Langen and Dieleman 2009)**. Oligofructose, inulin, and galacto-oligosaccharides selectively promote the growth of bifidobacteria and lactobacilli in colon which improves the barrier function. Bifidobacteria can potentially metabolize the fructooligosaccaharides with the help of enzyme β-fructosidase. To date, there are not enough evidences to support beneficial effects of prebiotics in maintaining remission CD and the reports on UC are also very limited **(Bernstein 2015).**

1.5.3.4 Fecal Microbiota Transplantation (FMT)

Considering the role of intestinal microflora in IBD pathogenesis, restoration of the normal gut flora has become one of the therapeutic approaches to treat IBD. Fecal microbiota transplantation (FMT) is one such therapy and has shown some encouraging outcomes in management of IBD patients. FMT has shown successful results in inducing

remission in mild to moderate UC patients but the long-term management and safety still remains undefined **(Paramsothy, Paramsothy et al. 2017)**. Besides this, since IBD patients are prone to get *Clostridium difficile* infection where fecal microbiota transplantation is considered very effective in treatment of recurrent and refractory *Clostridium difficile* infections. **(Syal, Kashani et al. 2018)**. There have been limited studies reporting the efficacy of FMT in management of CD and the results are not conclusive **(Suskind, Brittnacher et al. 2015, Vaughn, Vatanen et al. 2016).**

1.6 History of IBD

1.6.1 Ulcerative Colitis

UC and CD have been distressing people for a long time before modern diagnosis and treatment came into practice to distinguish them. The first subtype of IBD characterized as a distinct identity was Ulcerative Colitis. Sir Samuel Wilks in 1859 introduced the term ulcerative colitis, to describe a condition that is well understood as UC today. Ulcerative colitis became an accepted term in general medical vocabulary in 1888 when Sir William Hale White of London published reports describing the cases of ulcerative colitis and also stated that the disease defied from other known causes such as tubercle, dysentery and typhoid **(Mulder, Noble et al. 2014)**. 1909 is considered as influential year in UC history because in January 1909 a symposium was held in Royal Society of Medicine in London where hundreds of cases of UC were presented and discussed. The meeting also threw some light on the sign and symptoms of UC, risk factors and the treatment strategies employed for UC management **(Allchin 1909)**. In the same year, sigmoidoscopy was demonstrated as a vital procedure for colon evaluation and diagnosis by John Percy Lockhart-Mummery **(Mummery 1909)**. The understanding of UC by the medical community gradually increased from 1910 to 1950s. The detailed demonstration of familial disposition was understood, UC association with polyps and colorectal cancer was explored and the randomized clinical trials started following World War II **(Mulder, Noble et al. 2014)**. A major breakthrough was coincidental discovery of sulfasalazine by physician Nanna Svartz while treating his patients for arthritis observed that the patients also got symptomatic relief from UC as well **(Svartz 1948, Kirsner 1998)**. Later in 1960s immunosuppressive therapy was introduced as an effective treatment approach for

UC. In the past six decades genome wide association studies and molecular biology has helped in understanding the etiology and pathogenesis of IBD and also in designing biological drugs for disease management.

1.6.2 Crohn's Disease

CD was identified as a separate entity from UC in1932 in a publication by Crohn et al and this publication is recognized as a significant report in understanding CD. Through this publication it was also learnt that some initial cases reported by Wilks in 1859 were of CD but not UC **(Crohn, Ginzburg et al.,1952)**. Prior to 1932, there were few reports by Morgagni and Dalziel where they described CD by other names such as regional ileitis or regional enteritis and also described the symptoms and histological features associated with the disease **(Dalziel 1989, Mulder, Noble et al. 2014)**. But Crohn's article is recognized as a milestone in CD history as it was a descriptive article about CD defining its symptoms such as necrotizing and cicatrizing inflammation in the terminal ileum, transmural nature of inflammation and also its associated complications like strictures and fistulas **(Crohn, Ginzburg et al. 1952)**. Later on, it was learnt that CD could occur throughout the gastrointestinal tract and the characteristic feature of CD i.e. skip lesions were identified by Charles Wells **(Wells 1952)**. Following World War II, the random clinical trial began for CD and its epidemiology was studied in details and it was found that CD was more prevalent in industrialized countries and its incidences were rising continuously **(Farmer, Hawk et al. 1975, Garland, Lilienfeld et al. 1981)**. The paramount historical events in the history of UC and CD are given in **Figure 5.**

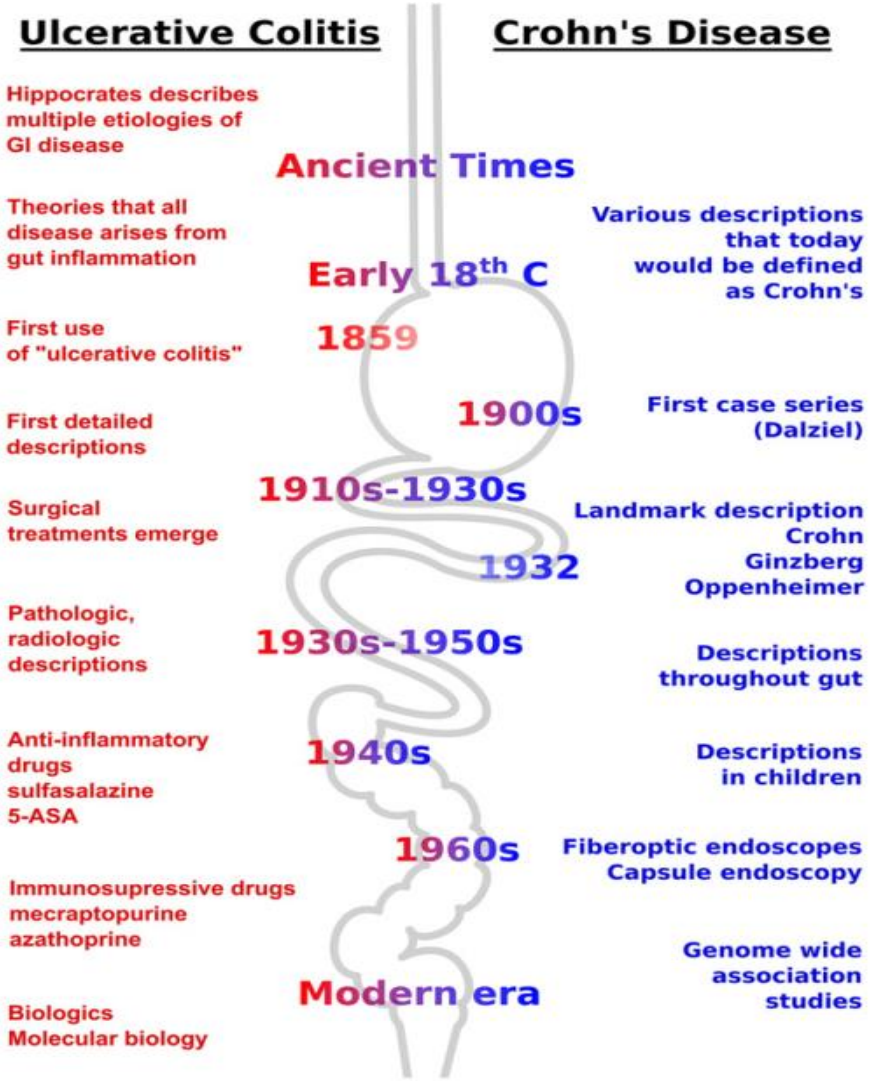


Figure 5: Major historical events in the history of IBD (Mulder, Noble et al. 2014)

1.6.3 IBD history in India

In India, IBD gained attention only after 1980s that is after the availability of endoscopic procedures. Before that, UC was confused with infectious colitis and CD that were the most prevalent forms of the disease **(Ray 2016)**. The first case of UC in India was reported in late 1930s and approximately after thirty years the first case of CD was reported **(Makharia, Ramakrishna et al. 2012)**.

1.7 Epidemiology of IBD

Initially, IBD was considered to predominate in the western world as its incidence and prevalence was high in the western countries such as North America, Europe but with the adoption of western culture and industrialization the incidence and prevalence rates for IBD started rising even in the developing countries which has increased the global burden of IBD **(Bernstein and Shanahan 2008**) **(Figure 6)**. According to several studies, the peak incidences of IBD occur in second and fourth decade of life and persist for several decades. Also the incidences of IBD varies not only with the geographical location but also get influenced by the race and ethnicity of people.

1.7.1 Global Scenario

IBD is found to be more common in Northern world than Southern part of the world and it is more common in the Caucasian population than non-Caucasian population. The incidence of IBD is highest in western countries such as North America, North Europe, the United Kingdoms and Australia while countries such as Africa, South America, and Asia including China reported lower incidence rate **(Lakatos 2006)**. An initial increase in the incidence of UC was reported in Northern Europe and North America from 1939 to 1964 but then it decreased till 1979 and became stable thereafter. CD cases increased from 1960 and plateaued after 1975 but increased worldwide recently **(Ray 2016)**. Europe has a highest annual incidence rate for UC (24.3 per 10^5 persons per year) while North America has highest annual incidence rate for CD (20.2 per 10^5 persons per year. Similarly, the highest prevalence of both UC and CD are reported form Europe (505 and 322, per 100,000 persons per year respectively **(Ponder and Long 2013)**. The incidence rates of IBD varies with the different geographical regions with UC incidence rates ranging from 0.6-24.3 per 10^5 individuals in Europe, 0.1-6.3 per 10^5 individuals in Asia

and the Middle East and 0-20.2 per 10^5 individuals in North America. CD incidence rate ranges from 0.3-12.7 per 10^5 individuals in Europe, 0.04-5.0 per 10^5 individuals in Asia and the Middle East and 0-20.2 per 10^5 individuals in North America. Similarly, prevalence rate for UC ranges from 4.9-505 per 10^5 individuals in Europe, 4.9-168.3 per 10^5 individuals in Asia and the Middle East and 37.5-248.6 per 10^5 individuals in North America and CD prevalence rate ranges from 0.6-322 per 10^5 individuals in Europe, 0.88-67.9 per 10^5 individuals in Asia and the Middle East and 16.7-318.5 per 10^5 individuals in North America **(Molodecky, Soon et al. 2012)**. Over the several past decades, the epidemiology of pediatric IBD has also been evaluated, showing significant rise in the incidence and prevalence rates. In a study from US, the IBD incidence rate was reported to be 5 to 11 per 100,000 children with rate of diagnosis as 4.56 per 100,000 for CD and 2.14 per 100,000 for UC with the prevalence rate of pediatric CD and UC as 43 and 28 per 100,000 children respectively **(M'Koma 2013)**. In a similar study from Canada, a rise in the pediatric IBD cases was reported from 5 to 11 per 100,000 children with rate of diagnosis as 4.56 per 100,000 for CD and 2.14 per 100,000 for UC **(Benchimol, Guttmann et al. 2009)**. **Table 1** summarizes the incidence and prevalence rates of IBD in different countries.

The Asia pacific region is still considered as a lower incidence area for IBD but these lower incidence rates are often confusing as to whether it is because of poor diagnostic awareness or a true lower incidence rate. Most of the recent studies showed a significant rise in the incidence of UC in Asians and a similar but relatively lower rise in CD incidence. And these higher rates are probably due to the adoption of western culture and better sanitary condition **(Ahuja and Tandon 2010)**. Several studies have pointed out the increased prevalence in different countries such as increase in prevalence from $2.9/10^5$ individuals in 1986 to $13.5/10^5$ individuals in 1998 was reported from Japan **(Thia, Loftus et al. 2008).**

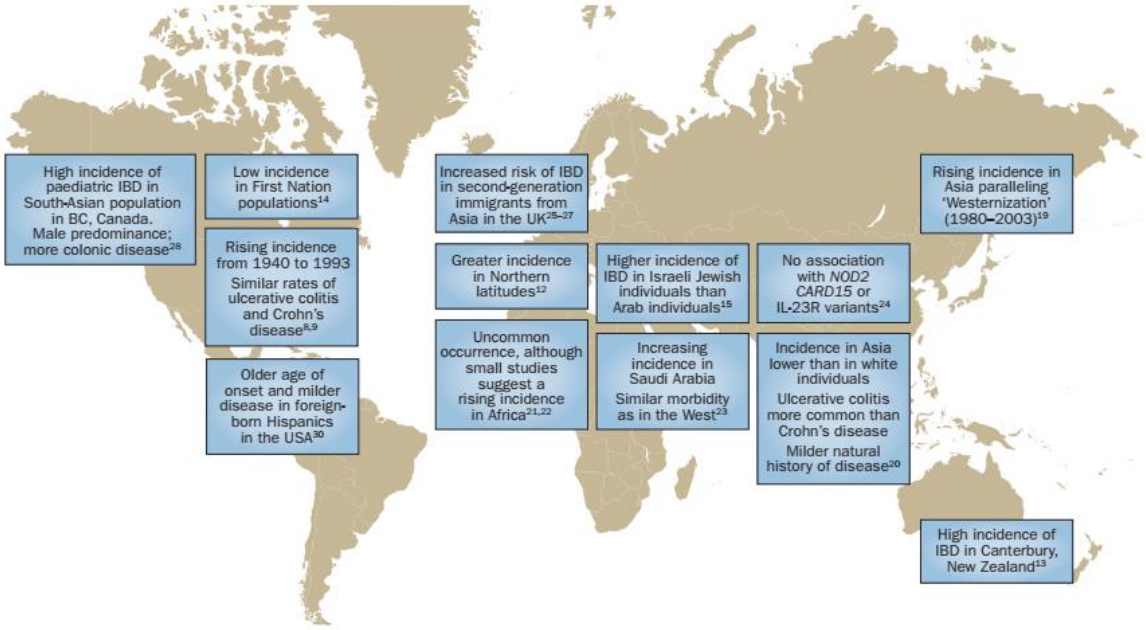


Figure 6: Global map of IBD incidences. (Ananthakrishnan 2015)

In South Korea, the prevalence of IBD changed from $7.57/10^5$ individuals in 1997 to $30.87/10^5$ individuals in 2005 **(Yang, Yun et al. 2008)**. In Hong King UC prevalence was reported to rise from $2.26/10^5$ individuals in 1997 to $6.30/10^5$ individuals in 2006 **(Lok, Hung et al. 2008)**. Similarly, marked increase in CD prevalence from $1.3/10^5$ individuals in 1990 to $7.2/10^5$ individuals in 2004 was reported from Singapore **(Thia, Luman et al. 2006)**.

1.7.2 Indian Scenario

Two population-based studies have been reported from India looked at the load of UC in India. The first population-based study was performed by Khosla *et al* in Haryana including 21,971participants and he reported a prevalence rate of $42.8/10^5$ individuals **(Khosla, Girdhar et al. 1986)**. In the second study from Punjab, the incidence rate was reported to be $6.02/10^5$ individuals per year and prevalence of rate of $44.3/10^5$ inhabitants and these rates were found to be highest from the Asian subcontinent **(Sood, Midha et al. 2003)**. There is lack of systemic studies for CD in India but there is one study by Das *et al* where he analyzed the data from three IBD clinics in India and suggested a cumulative rise in CD incidence from less than 5000 in 1987 to 21,061 in 2001. He also proposed that most of the CD patients in India are young individual with a mean age of 34.5 years, CD had a male preponderance and delayed diagnosis as compared to western countries **(Das, Ghoshal et al. 2009)**. In India, the cumulative IBD burden came out to be 1.4 million in 2010 **(Figure 7)**, which is second highest in the world after USA (1.64) suggesting that although India has a prevalence rate lower than western countries with a population more than 120 million, the cumulative IBD burden is largest in India **(Kedia and Ahuja 2017)**.

1.7.3 Studies on epidemiology of migrant population

Studies on migrants from one geographical area to another provide scope for studying the interaction between environmental and genetic factors in disease pathogenesis. Studies on migrant epidemiology suggest that South Asian migrants in UK have higher incidence rate for UC as compared to the indigenous UK population **(Probert, Jayanthi et al. 1992)**. In a similar study they found that although the incidences of CD were rising in

both the European and South Asian population but the incidence were significantly higher in the Europeans as compared to South Asian migrants **(Jayanthi, Probert et al. 1992)**.

Table 1: The incidence and prevalence of IBD among different countries (Ng, Shi et al. 2018)

	Incidence per 100 000 person-years				Prevalence per 100 000			
	Crohn's disease		Ulcerative colitis		Crohn's disease		Ulcerative colitis	
	Lowest estimate	Highest estimate	Lowest estimate	Highest estimate	Lowest estimate	Highest estimate	Lowest estimate	Highest estimate
North America	6·30 (California, USA)	23·82 (Nova Scotia, Canada)	8·8 (Olmsted County, USA)	23·14 (Nova Scotia, Canada)	96·3 (California, USA)	318·5 (Nova Scotia, Canada)	139·8 (Quebec, Canada)	286·3 (Olmsted County, USA)
Eastern Europe	0·40 (Chisinau, Moldova)	14·6 (Veszprém, Hungary)	0·97 (Romania, Nationwide)	11·9 (Veszprém, Hungary)	1·51 (Romania, Nationwide)	200·0 (Hungary, Nationwide)	2·42 (Romania, Nationwide)	340·0 (Hungary, Nationwide)
Northern Europe	0·0 (Greenland, Nationwide)	11·4 (Funen, Denmark)	1·7 (Tartu, Estonia)	57·9 (Faroe Islands, Nationwide)	24·0 (Kuopio, Finland)	262·0 (Southeast, Norway)	90·8 (Leicestershire, UK)	505·0 (Southeast, Norway)
Southern Europe	0·95 (Vukovarsko-Srijemska, Croatia)	15·4 (Casteltermini, Italy)	3·3 (Zagreb, Croatia)	11·47 (Caceres, Spain)	4·5 (Vukovarsko-Srijemska, Croatia)	137·17 (Ciudad Real, Spain)	14·5 (Vukovarsko-Srijemska, Croatia)	133·9 (Zadar, Croatia)
Western Europe	1·85 (Guadeloupe and Martinique islands, France)	10·5 (Central, Netherlands)	1·9 (Puy-de-Dome, France)	17·2 (Central, Netherlands)	28·2 (Tuzla, Bosnia and Herzegovina)	322·0 (Hesse, Germany)	43·1 (Tuzla, Bosnia and Herzegovina)	412·0 (Hesse, Germany)
Eastern Asia	0·06 (Kunming, China)	3·2 (South Korea, Nationwide)	0·42 (Xian, China)	4·6 (Seoul, South Korea)	1·05 (Taiwan, Nationwide)	18·6 (Japan, Nationwide)	4·59 (Taiwan, Nationwide)	57·3 (Japan, Nationwide)
South-eastern Asia	0·14 (Kinta Valley, Malaysia)	0·41 (Brunei, Nationwide)	0·15 (Manila, Philippines)	0·68 (Kinta Valley, Malaysia)	2·17 (Kinta Valley, Malaysia)	2·17 (Kinta Valley, Malaysia)	6·67 (Kinta Valley, Malaysia)	6·67 (Kinta Valley, Malaysia)
Southern Asia	0·09 (Colombo and Gampaha, Sri Lanka)	3·91 (Hyderabad, India)	0·69 (Colombo and Gampaha, Sri Lanka)	6·02 (Punjab, India)	1·2 (Colombo and Gampaha, Sri Lanka)	1·2 (Colombo and Gampaha, Sri Lanka)	5·3 (Colombo and Gampaha, Sri Lanka)	44·3 (Punjab, India)
Western Asia	0·94 (Riyadh, Saudi Arabia)	8·4 (Southern Israel, Israel)	0·77 (Trakya, Turkey)	6·5 (Southern Israel, Israel)	50·6 (Southern Israel, Israel)	53·1 (Beirut, Lebanon)	4·9 (Trakya, Turkey)	106·2 (Beirut, Lebanon)
South America	0·0 (District of Colón, Panama)	3·50 (São Paulo, Brazil)	0·19 (Piauí, Brazil)	6·76 (São Paulo, Brazil)	0·9 (São Paulo, Brazil)	41·4 (Southwest, Puerto Rico)	4·7 (São Paulo, Brazil)	44·3 (Barbados, Nationwide)
Oceania	12·96 (Geelong, Australia)*	29·3 (Geelong, Australia)*	7·33 (Geelong, Australia)*	17·4 (Geelong, Australia)*	155·2 (Canterbury, New Zealand)	197·3 (Barwon, Australia)	145·0 (Canterbury, New Zealand)	196·0 (Barwon, Australia)
Africa	5·87 (Constantine, Algeria)	5·87 (Constantine, Algeria)	3·29 (Constantine, Algeria)	3·29 (Constantine, Algeria)	19·02 (Constantine, Algeria)	19·02 (Constantine, Algeria)	10·57 (Constantine, Algeria)	10·57 (Constantine, Algeria)

*Geelong has the lowest and highest estimates because of reporting in time periods ranging from 2007 to 2013.

Table: **Range in incidence and prevalence of inflammatory bowel disease since 1990 stratified by geographic regions**

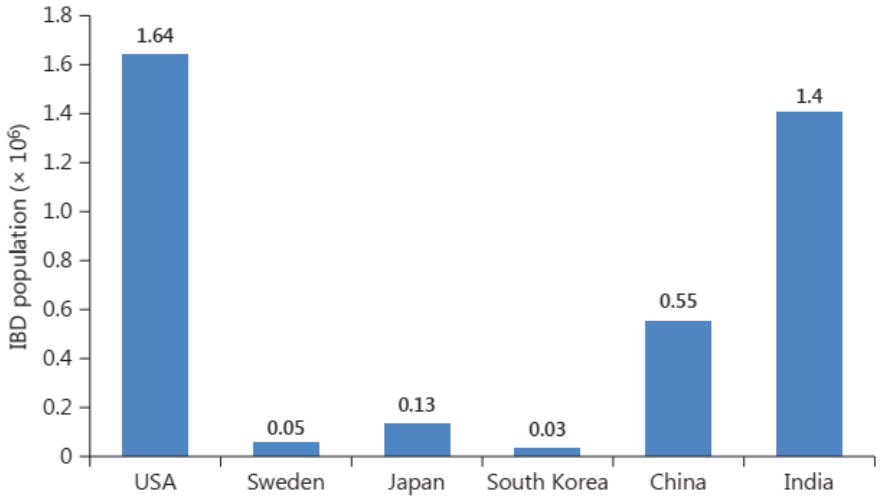


Figure 7: IBD burden in different countries. (Kedia and Ahuja 2017)

1.8 Factors contributing to the pathogenesis of IBD

The pathogenesis of IBD has not been completely understood till date but the current understanding of IBD pathogenesis is that altered immune response due to dysbiosis of gut microflora in genetically susceptible individuals. These individuals are then predisposed to develop IBD under the influence of environmental triggers **(Kim and Cheon 2017)**. The pathogenesis of IBD and involved risk are given in **figure 8.**

1.8.1 Microbial factors

Human GI tract acts as a reservoir to trillions of bacteria with majority of them residing in the intestinal tract. The microbial ecology of healthy human GI tract is summarized in **Figure 9**. In healthy individuals, the intestinal flora exerts several benefits like prevent the colonization of pathogenic bacteria, help in digesting the otherwise indigestible

complex oligosaccharides, supplementing essential vitamins and maintaining gut homeostasis along with the intestinal immune system **(Hillman, Lu et al. 2017)**. Therefore, dysbiosis in the commensal gut flora, that leads to aberrant or inappropriate immune response against the persistent commensal flora plays pivotal role in IBD pathogenesis. Recent progression in microbial studies notably culture independent molecular techniques have contributed immensely in understanding the structure and functioning of intestinal dysbiosis.

Dysbiosis is defined as alterations or disturbance in the normally persistent bacterial diversity of gut microflora and it has been strongly associated with IBD. The demonstration of dysbiosis leading to aberrant immune response during IBD has been shown in **Figure 10**. Environmental triggers like smoking and most importantly dietary habits play profound role in shaping the gut microbiome and it could affect the growth and composition of gut microbiome **(Brown, DeCoffe et al. 2012, Alhagamhmad, Day et al. 2016)**. Significant differences have been observed in the gut microbiota of IBD patients compared to healthy individuals. The healthy human gut is dominated by *Firmicutes* and *Bacteroidetes* as compared to *Actinobacteria* and *Proteobacteria* that are present in lesser numbers **(Qin, Li et al. 2010)** while during IBD, *Firmicutes* and *Bacteroidetes* are reduced and *Actinobacteria* and *Proteobacteria* are significantly increased **(Frank, St Amand et al. 2007, Morgan, Tickle et al. 2012)**. The most defined alteration in CD includes reduction in *Firmicutes* notably *Faecalibacterium prausnitzii* and increase in *Proteobacteria* specially *Escherichia coli* and *Bacteroids* **(Fujimoto, Imaeda et al. 2013)**. *Faecalibacterium prausnitzii* are commensal bacteria of gut and exerts anti-inflammatory response through production of immunomodulating protein **(Quevrain, Maubert et al. 2016)**. The dominant butyrate producing bacteria in the gut are *Clostridia* specially group IV and XIV.

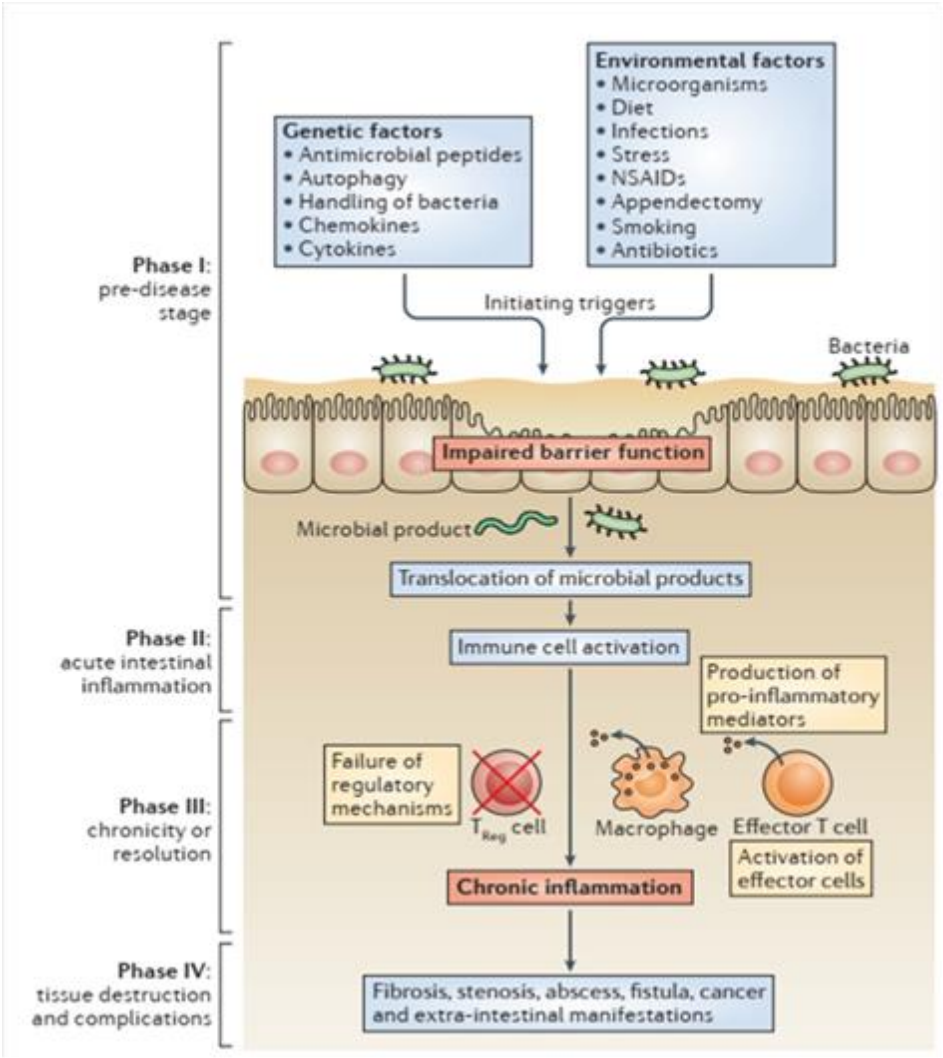


Figure 8: Immunopathogenesis of IBD and the major factors involved (Neurath 2014)

Butyrate in gut is required for maintaining gut epithelium integrity and it also suppresses NFkB signaling to exert anti-inflammatory response **(Segain, Raingeard de la Bletiere et al. 2000, Vanhoutvin, Troost et al. 2009)**. A reduction in *Clostridium* cluster along with *Dialister invisus* is observed in CD patients with respect to healthy individuals **(Joossens, Huys et al. 2011)**. While the beneficial bacteria are depleted, a marked increase in the pathogenic bacteria such as Bacteroidetes, *Ruminococcus gnavus*, *Pseudomonas* and Enteroccocal species, *Clostridium perfringens* and *Bacteroides fragilis* has been reported in CD patients **(De Hertogh, Aerssens et al. 2008, Alhagamhmad, Day et al. 2016)**. Recently, *Proteobacteria* especially adherent invasive *Escherichia coli* (AIEC) have been identified as a causative agent for IBD pathogenesis. AIEC was abundant in the inflamed ileum of 22% CD patients and 36% of terminal ilea of postsurgical CD patients. It was very rarely present in the colonic tissues of CD patients and not at all identified in UC patients suggesting their specific association with ileal CD. AIEC can easily invade the epithelial cells and can survive and proliferate within the macrophages which might result in defective autophagy **(Wallace, Zheng et al. 2014)**. The data supporting contribution of fungal microbiota and viruses in the pathogenesis of IBD remains obscure. Sokol H *et al* in 2017 conducted a study where faecal microbiota of 235 patients was compared with 38 healthy subjects for the bacterial and fungal population present in it. He reported a great rise in the population of Basidiomycota in IBD particularly during flares, while significant reduction in Ascomycota particularly *Malassezia sympodialis* and *Saccharomyces cerevisiae*. *Candida albicans* during IBD flares as compared to the remission patients both in proportion and absolute numbers. *Saccharomyces cerevisiae* was suggested to possess anti-inflammatory properties as it could stimulate the production of IL-10 **(Sokol, Leducq et al. 2017)**. Expansion of *Caudovirales* bacteriophage has also been reported in IBD patients as compared to healthy subjects **(Sartor and Wu 2017)**. Similarly, cytomegalovirus (CMV) virus is present in 70% of IBD patients but the contribution of CMV in IBD pathogenesis or its aggregation still remains a matter of debate **(Sun, Nava et al. 2011)**.

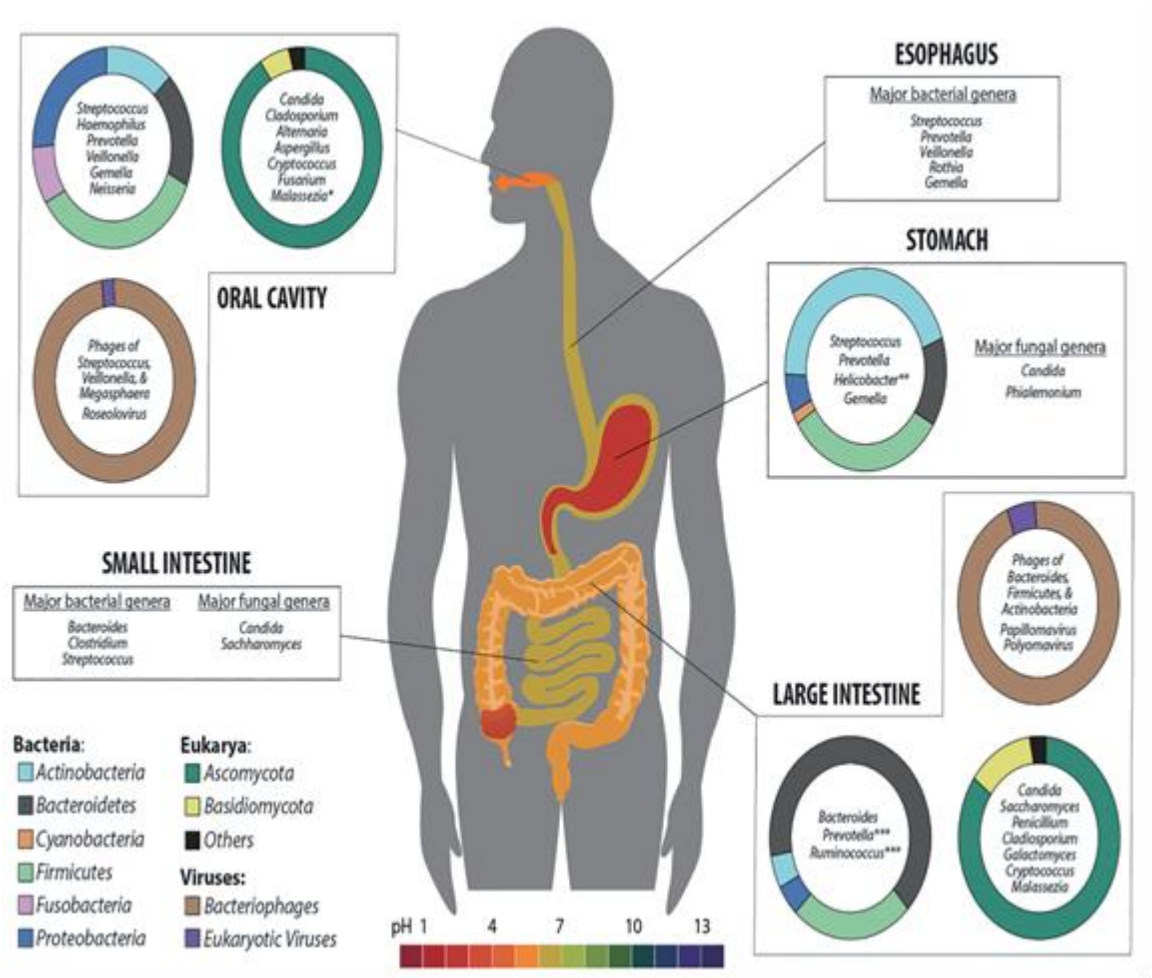


Figure 9: Microbial composition of human gastrointestinal tract (Hillman, Lu et al. 2017)

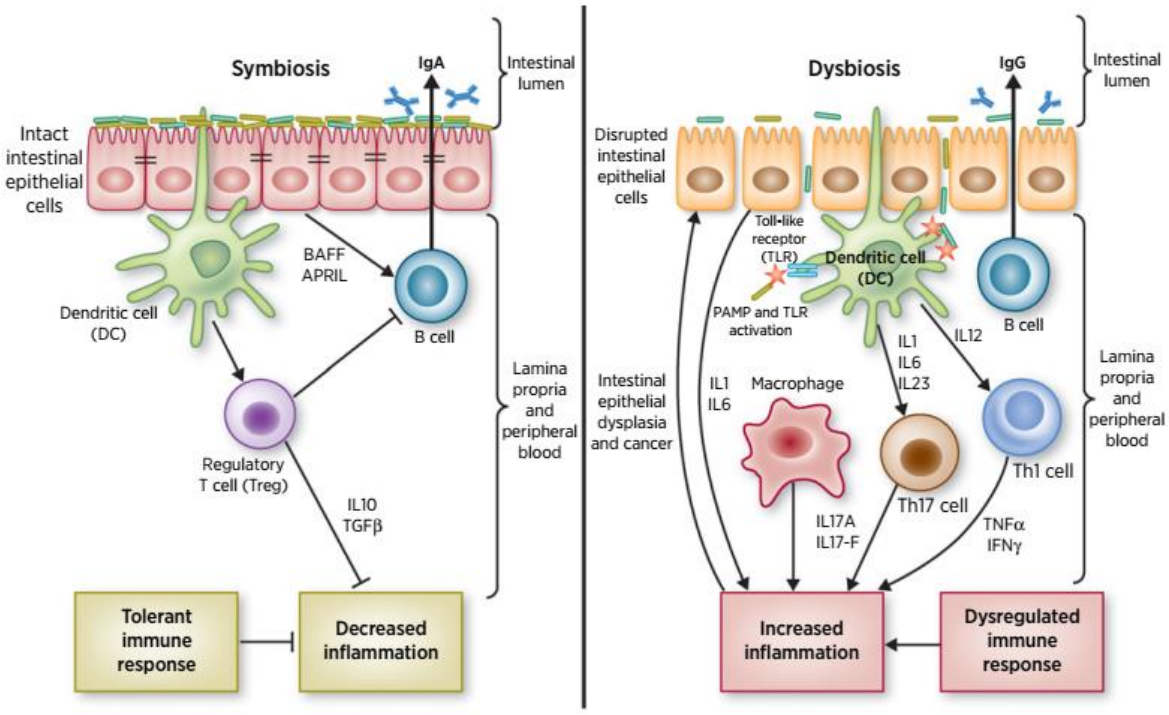


Figure 10: Changes in immune response due to Gut microbiota under steady state (left panel) and during diseased condition (right panel) (Thomas, Izard et al. 2017)

1.8.2 Environmental factors

Environmental triggers play considerable role in IBD pathogenesis. The incidence and prevalence rates of IBD are race and ethnicity dependent indicating the critical role of environmental factors. As on today, the increasing incidence of IBD in developing countries is mainly attributed to the environmental triggers. Factors such as diet, smoking, breastfeeding and childhood hygiene plays pivotal role in shaping the intestinal microflora and also in developing the immune system which later on plays significant role in providing protection against infections.

1.8.2.1 Diet

The significant changes in dietary habits such as adoption of westernized food low in fiber content and rich in saturated fatty acids has contributed greatly to the rising incidence of IBD in developing countries. The consumption of dietary fiber, fruits and vegetables is strongly associated with IBD. Intake of soluble fiber from fruits and vegetables but not from cereal and whole grains has been inversely correlated with CD risk however, with very low association with UC **(Ananthakrishnan, Khalili et al. 2013)**. In a Canadian population, higher consumption of monounsaturated and saturated fats increased the risk for CD while an inverse relationship was reported with carbohydrate consumption **(Amre, D'Souza et al. 2007)** and a similar association is reported in UC with monounsaturated and polyunsaturated fat consumption **(Geerling, Dagnelie et al. 2000)**. Consumption of high fat diet especially rich in cholesterol and animal fat is also linked with increase IBD risk **(Reif, Klein et al. 1997)**.

1.8.2.2 Smoking

Smoking exhibits divergent, more specifically opposite association with UC and CD. In UC the active cigarette smokers are at lower risk than the former smokers and cigarette cessation enhances the risk of disease development as can be observed within first year of cigarette cessation **(Lakatos, Szamosi et al. 2007, Higuchi, Khalili et al. 2012)**. While in CD both the active and former smokers are at high risk of CD, facing a more aggressive disease and cigarette cessation ameliorates the disease within one year of cessation **(Cosnes, Carbonnel et al. 1999, Cosnes, Beaugerie et al. 2001)**. One of the

most unexplained enigma of IBD pathegenesis is why smoking exerts opposite association with UC and CD risk development. Smoking exert these beneficial effects probably by modulation of cellular immunity, enhancing mucus secretion from goblet cells and altering the release of cytokines including as IL-8, IL-10 and IL-1β in IBD patients **(Sher, Bank et al. 1999, Molodecky and Kaplan 2010)**.

1.8.2.3 Appendectomy

Similar to smoking, appendectomy shows divergent association with UC and CD pathogenesis. Appendectomy in early age (before 10) reduces the risk of UC development but its role in CD is less clear **(Molodecky and Kaplan 2010)**. While some studies suggested that appendectomy is not a risk factor for CD **(Reif, Lavy et al. 2001)** others have demonstrated an initial risk for CD following appendectomy **(Andersson, Olaison et al. 2003)**.

1.8.2.4 Hygiene hypothesis

The hygiene hypothesis states that the reduced childhood exposure to enteric pathogens is one of the major contributing factors for increased autoimmune disorders in late life. Improved sanitation and hygiene during childhood reduces the exposure to enteric flora which results in weak or inappropriate immunologic response to newly encountered antigens **(Gent, Hellier et al. 1994)**. A meta-analysis including 23 studies demonstrated a negative association of *Helicobacter pylori* infection with CD and UC. *H. pylori* is suggested to provide protection against IBD by enhancing the expression of Foxp3 which is involved in the development and functioning of Treg cells **(Luther, Dave et al. 2010)**. Others factors such as number of siblings, exposure to pets during childhood, intake of unpasteurized milk and family size have also been negatively associated with UC or CD **(Ananthakrishnan 2015)**.

1.8.2.5 Breastfeeding

The protective role of breastfeeding has been demonstrated in many studies. A meta-analysis conducted by Klement *et al* showed protective role of breastfeeding for both CD and UC **(Klement, Cohen et al. 2004)**. Although protective role of breastfeeding has been reported in several studies, conflicting results for increased risk of CD development

with breastfeeding **(Baron, Turck et al. 2005)** have also been reported. Breastfeeding provide tolerance to microflora and food antigens. Additionally, lactoferrin present in breast milk possess antimicrobial, antiviral and anti-inflammatory properties **(Molodecky and Kaplan 2010)**.

1.8.2.6 Medication

Use of antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs) increases the disease risk by altering the gut microbiome and disrupting the intestinal barrier respectively. Use of antibiotics is found to be more common in IBD patients more importantly in CD patients **(Card, Logan et al. 2004)**. Similarly, use of NSAIDs elicited the disease activity both in UC and CD and also triggers the disease flares **(Felder, Korelitz et al. 2000)**. According to a meta-analysis conducted in 2008 oral contraceptives may increase the risk for CD and UC. Prolonged use of oral contraceptives increased the risk of CD and upon discontinuing the pills the effects got reversed **(Cornish, Tan et al. 2008)**. The estrogen present in oral contraceptives enhances the proliferation of macrophages and attributed to its thrombogenic properties and it plays pathogenic role in IBD **(Cutolo, Capellino et al. 2006)**.

1.8.2.7 Vitamin D

Vitamin D deficiency has been reported in newly diagnosed cases of IBD. In CD patients, a greater disease activity and lower quality of life was observed due to vitamin D deficiency **(Ulitsky, Ananthakrishnan et al. 2011)**. Supplementation of vitamin D to remission patients reduced the risk of disease relapse. Women with highest quartile of plasma vitamin D level were at a reduced risk of CD **(Molodecky and Kaplan 2010)**.

1.8.2.8 Lifestyle: sleep and stress

Psychological stress induces gut inflammation by increasing the pro-inflammatory cytokines production, activation of macrophages and modulation of gut microflora and intestinal permeability. Psychological stress, anxiety and depression have been strongly associated with increased disease risk **(Ananthakrishnan, Khalili et al. 2013)**. In IBD patients, depression induces disease relapse, make the person unresponsive to immunosuppressant and reduces the quality life **(Bernstein, Singh et al. 2010)**.

Disturbed sleep is also associated with IBD, impairment in sleep and lack of enough sleep increases risk for both UC and CD **(Ananthakrishnan, Long et al. 2013, Ananthakrishnan, Khalili et al. 2014)**.

1.8.3 Immunological factors

Human gastrointestinal tract harbors the most complex and precise immune system, preventing the invasion and colonization of harmful pathogens while remaining tolerant to inhabitant commensal bacteria and food antigens at the same time. The immune balance between host and microbiota is crucial for healthy gut development, mounting immunity against pathogens and maintaining intestinal integrity. Any alteration or breakdown in gut immune homeostasis results in disease conditions like IBD **(Maloy and Powrie 2011)**. The very first and immediate physical barrier for intestinal pathogens is the mucous layer that covers intestinal epithelium. Mucous is secreted by goblet cells and has two layers, the sterile inner firm layer with strong antimicrobial properties and an outer loose layer with mucin and commensal bacteria with diluted antimicrobial properties. The importance of mucous in providing protection against bacterial invasion and intestinal inflammation was highlighted in a study where *MUC2* knockout mice developed spontaneous colitis and was at higher risk of developing colorectal cancer **(Velcich, Yang et al. 2002, Van der Sluis, De Koning et al. 2006)**. Differential expression of mucin genes has been reported in CD patients **(Buisine, Desreumaux et al. 1999)** and in active UC patients where altered glycosylation of MUC2 has been observed which resulted in disease worsening **(Larsson, Karlsson et al. 2011)**. Under physiological conditions several immune cells such as macrophages, natural killer cells (NK), T and B lymphocytes, mast cells, neutrophils, eosinophils and dendritic cells coexist in the lamina propria of intestine. However, under inflammatory condition, there is increased infiltration of activated immune cells to the intestinal mucosa in large numbers and high level of inflammatory mediators and chemokine receptors such as CCR5, CCR6 and CCR9 and adhesion molecules ICAM-1 and L selectin are expressed by these activated cells **(Xu, Liu et al. 2014)**. Antimicrobial peptides including α and β defensins, cathelicidin and elafin secreted by Paneth cells constitute the chemical barrier responsible in maintaining homeostasis between host mucosa and commensal flora.

Decreased level of α defensins (HD5 and HD6) and also β defensins have been reported in CD patients which is responsible for defective antimicrobial activity in colon of CD patients **(Wehkamp, Harder et al. 2003, Wehkamp, Salzman et al. 2005)**.

In CD, marked elevation of Th1 derived cytokines IL-2, IL-12 and IFNγ **(Bamias, Sugawara et al. 2003)** are observed while UC is characterized by infiltration of Th2 lymphocytes and cytokines IL5, IL13 and TGFβ **(Targan and Karp 2005)**. Besides Th1 and Th2 cell population, Th17 and regulatory T cells (Treg) are also found at the intestinal mucosa where Th17 cells provide protection against the invading pathogens by proinflammatory response and Treg cells help in restraining the effector Th17 response. Therefore, maintaining the balanced population of Th17 and Treg is crucial for maintaining gut homoeostasis. The naïve $CD4^+$ T cells can differentiate into Th17 or Treg cells under the influence of key mediators such as cytokines IL-6, TGFβ and transcription factors like RORγt and FoxP3 **(Omenetti and Pizarro 2015)**. IL-6 and TGFβ are the main cytokines to drive the Th17 differentiation of but besides IL-6 and TGFβ, IL-23 is crucial for maintaining or stabilizing the Th17 response. IL-6 and TGFβ induces the expression of IL-23R on Th17 cells providing them responsiveness to IL-23. Absence of IL-23R results in decreased cell number of Th17 cells. IL-23 can also induce the increased production of proinflammatory cytokines through activated macrophages and DCs as they express IL-23R. IL-23 expressions are higher in the inflamed colonic mucosa where it induces the secretion of proinflammatory cytokines (**Liu, Yadav et al. 2011)**. IL23R has also been introduced as susceptibility gene for IBD through Genome wide association studies **(Duerr, Taylor et al. 2006)**. IL-22 also plays a paramount role in maintaining balanced population of Th17 and Treg cells in GI tract and it also induces differentiation of Th17 cells along with TGFβ. IL-22 is upregulated in the ileal and colonic lesions of CD patients **(Monteleone, Monteleone et al. 2005)**. Elevated expression of other Th17 produced cytokines IL-17A, IL-17F and IL-21 are reported from the inflamed colonic mucosa and serum of active IBD patients **(Fujino, Andoh et al. 2003, Seiderer, Elben et al. 2008)**. In addition to this, IL-21 cytokine belonging to the IL-2 family of cytokines and preferably expressed by $CD4^+$ T cells shows significant higher expression in the inflamed colonic regions of IBD patients **(Monteleone,**

Monteleone et al. 2005, Yamamoto-Furusho, Miranda-Perez et al. 2010). IL-21 exerts its proinflammatory actions most probably by inducing the production of Th-17A and IFNγ. In IBD patients, IL-21 enhances the cytotoxicity of NK cells, triggers production of proinflammatory cytokine form T cells and also renders the $CD4^+$ cells differentiation into Th17 cells. IBD patients also exhibit significantly higher expression of IL-21R positive cells in the inflamed colonic mucosa **(Monteleone, Monteleone et al. 2005, Fina, Sarra et al. 2008)**. The $CD4^+CD25^+Foxp3^+$ suppressor T cells or regulatory T cells (Tregs) maintain the self-tolerance in intestine and also restrain the effector Th17 response. The most convincing study to highlight the role of Tregs in inflammation was conducted in 1993 where adoptive transfer of Tregs prevented colitis development in mice **(Powrie, Leach et al. 1993)**. Complete depletion of Tregs by Foxp3 deficiency results in immunodysregulation, polyendocrinopathy, enteropathy and Xlinked syndrome (IPEX). The organ most affected during this condition is the intestine, highlighting the mandatory role of Tregs in GI tract **(Mayne and Williams 2013)**. The Tregs mediated suppression mechanisms include production of TGFβ, IL-10 and IL-35 cytokines and immunoregulation with direct interaction with Antigen Presenting Cells (APCs) and T cells. Several studies have supported the notion that imbalance in Th17 and Treg population in the intestine might contribute to IBD development. In IBD patients, Th17 cells and derived proinflammatory cytokines IL-17, IL-6, IL-21 and IL-23 show increased expression while Tregs and their derived cytokine IL-10 and TGF-β1 show decreased expression **(Eastaff-Leung, Mabarrack et al. 2010, Geng and Xue 2016)**.

Role of B cells has also been explored in the pathogenesis of IBD patients. Higher activation of B cells is observed in CD patients. Also the B cells from CD patients express surface TLR2 and produce higher levels of IL-8 and these higher level of TLR2 and IL8 correlate positively with the disease activity **(Noronha, Liang et al. 2009)**. In addition to this, B cells produce altered amount of antibodies IgG, IgM, and IgA in IBD patients **(MacDermott, Nash et al. 1981)**.

1.8.4 Genetic factors

The role of genetic factors in IBD pathogenesis is supported by multiple evidences such as familial aggregation and twin studies. A higher concordance rate in monozygotic twins in CD (30-35%) than UC (10-15%) suggests that the derived inheritance in CD is higher than UC. Multiple disease comparative analysis revealed that more than 50% of IBD susceptibility genes are also associated with other autoimmune and inflammatory diseases like genes *MST1*, *IL2*, *CARD9* and *REL* are associated with UC and primary sclerosing cholangitis (PSC) **(Khor, Gardet et al. 2011)**. Advancement in techniques such as DNA sequencing has enabled many Genome Wide Association Studies (GWAS) to identify the genetic loci and new single nucleotide polymorphisms (SNPs) that contribute to IBD susceptibility. Nucleotide-binding oligomerization domain containing 2 (NOD2) was the first susceptibility gene identified for CD. NOD2 variant in Dendritic cells (DC) of CD patients were found to be defective in autophagy induction **(Loddo and Romano 2015).** One of the recent studies included 75000 IBD patients where 163 susceptibility loci for IBD were identified. Out of these 163 genetic loci, 110 were associated with both UC and CD while 30 were unique for CD and 23 for UC **(Loddo and Romano 2015)**. The different susceptibility genes associated with UC and CD are given in **Figure 11**.

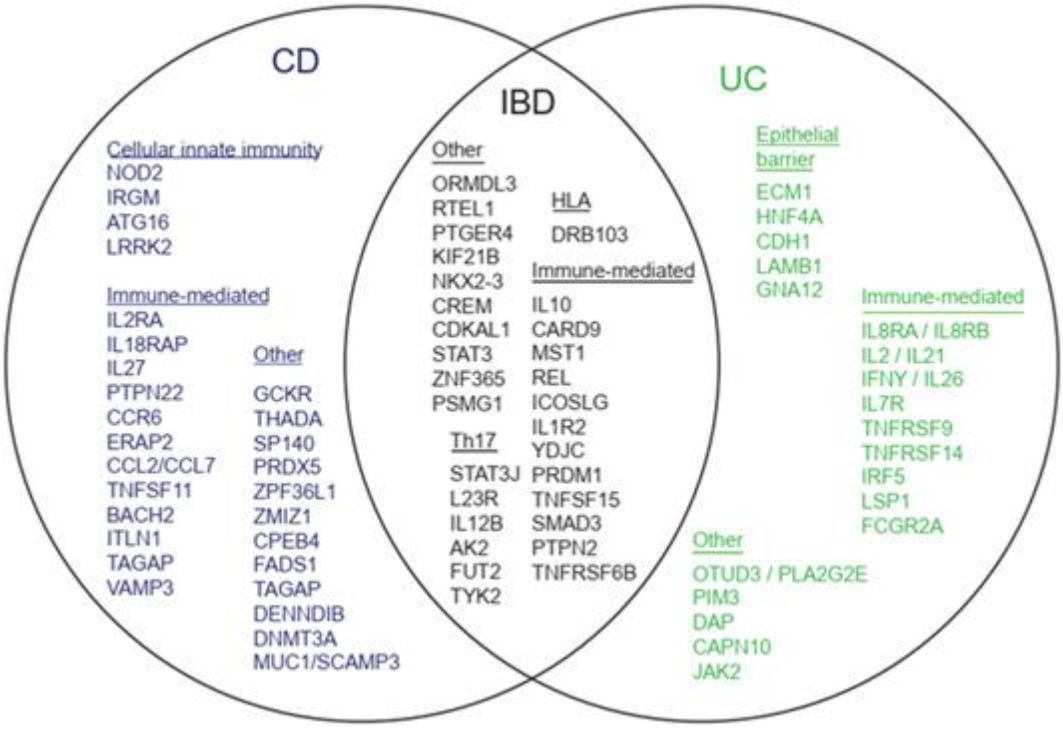


Figure 11: Genetic loci identified for UC and CD (Ek, D'Amato et al. 2014)

1.8.5 The Epigenetic factors

Besides the involvement of microbial, environmental and genetic factors, significant efforts have been made in understanding the role of epigenetic regulators including as DNA methylation, modification of histone proteins and miRNA in the pathogenesis of IBD. A number of studies have reported the different DNA methylation patterns and altered miRNA expression in IBD patients but enough information regarding the role of histone modification are still lacking. Hypermethylation of several gene promoters is known to be associated with IBD patients. Hypermethylation of gene promoters for *E-cadherin, GDNF* (Glial cell derived neurotrophic factor), *CDH1* (Epithelial Cadherin), and *MDR1* (Multi Drug Resistance) have been observed in UC patients **(Yi and Kim 2015)**. Also, a tenfold higher incorporation of methyl group has been observed in active UC patients **(Gloria, Cravo et al. 1996)**. A genome wide DNA methylation profiling study of ileal CD patient reported differential DNA methylation in genes responsible for immune response and susceptibility genes of CD **(Scarpa and Stylianou 2012)**. MicroRNA have gained focus in past few years due to their role in modulation of inflammatory response in various inflammatory and autoimmune disorders like multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, psoriasis, and various cancers. Over 1800 miRNAs have now been identified that are functionally active and each miRNA has the efficiency to bind and target multiple gene transcripts and these miRNAs are members of complex gene regulatory networks (GRNs) **(Kozomara and Griffiths-Jones 2011)**. Around 60% of human proteins encoding genes are regulated by miRNA.

MiRNA actively regulate several biological processes such as development, cell differentiation, proliferation and apoptosis. Any alteration in miRNA expression could result in diminished cellular functioning and impaired downstream gene regulation which implicates the role of miRNA in disease pathogenesis. Distinct miRNA expression has been reported both in the colonic mucosa and peripheral blood of IBD patients and some of these miRNAs have been implicated to regulate the expression of genes involved in major inflammatory pathways such as NFkB and MAPK signaling **(Dalal and Kwon 2010)**.

1.9 Role of MiRNA in pathogenesis of different diseases

MiRNA are single stranded, conserved, small sized (18-22 nucleotide long) non-coding RNA molecules. A mature miRNA is produced in a two-step cleavage process of primary miRNA (pri-miRNA) and then loaded into the effector protein complex, the RNA inducing silencing complex (RISC), which directs the miRNA to target mRNA **(Figure 12)**. The mature miRNA binds within the 3'UTR of target mRNA and inhibits the protein production either by its degradation or by translational inhibition **(Perron and Provost 2008)**.

1.9.1 MiRNA in Rheumatoid Arthritis (RS)

RA is a chronic inflammatory and auto-immune disorder with a prevalence rate of approximately 1% worldwide. RA is characterized mainly by synovial hyperplasia, affects the lining of joints causing inflammation that eventually results in bone erosion and joint deformities. Distinct miRNA signatures with deregulated expression of miR15a, miR16, miR146a, miR124a, miRNA155, miR203 and miR-346 have been demonstrated in the tissues of RA patients **(Singh, Massachi et al. 2013)**. Overexpression of few miRNA including miR-132, miR-16, miR-223 and miR-146a has been identified in the synovial fluid and blood plasma obtained from RA patients as compared to healthy individuals **(Murata, Yoshitomi et al. 2010)**. MiR-188 is downregulated in the synovial tissues and in synovial fluid of RA patients and is also predicted to target the genes hyaluronan binding protein KIAA1199 and collagens COL1A1 and COL12A1 **(Ruedel, Dietrich et al. 2015)**. Downregulated expression of several miRNAs namely miR-99a, miR-125b, miR-100, miR-152, miR-214 and upregulation of miR-223 within the macrophages of RA patients as compared to osteoarthritis patients has also been identified **(Chen, Papp et al. 2016)**.

1.9.2 MiRNA in Multiple Sclerosis

MS is an autoimmune neurological disorder affecting mainly the brain and spinal cord and is diagnosed usually in the second and forth decade of life. In MS, immune response is generated against the myelin sheath that results in inflammation and impaired transmission of nerve impulses **(Singh, Massachi et al. 2013)**. Altered miRNA expression is observed in the active MS lesions as well as in the blood plasma of MS

patients. Overexpression of miR-146a, miR-146b, miR-21 and miR-155 is observed in the peripheral blood mononuclear cells (PBMCs) of relapsing remitting MS patients. miR-27a is upregulated in MS patients under disease relapse as compared to the healthy controls and remitting patients and is suggested to inhibit the Th17 cell differentiation **(Chen, Papp et al. 2016)**. Similarly, miR-326, miR-155 and miR-34a are upregulated in the active MS patients where they target the CD47 (a membrane protein) whose expression is significantly lower in the active MS patients as compared to healthy controls. miR-326 regulates the Th17 cell differentiation by negatively targeting the expression of Et-s1 gene. **(Du, Liu et al. 2009)**. All these recent studies suggest the possible contribution of miRNA in pathogenesis of MS.

1.9.3 MiRNA in systemic lupus erythematosus (SLE)

SLE is a systemic autoimmune disorder affecting multiple organs including skin, lungs, joints, nervous system and serous membrane. Despite the pivotal role of adaptive immune response in auto-immune disorders, the innate immune response plays significant role in SLE pathogenesis and negative regulation of innate response by miRNA is well recognized in recent years. MiR-146a acts as negative regulator of NFkB pathway by targeting the genes TNF receptor-associated family-6 (TRFA6) and IL-1 receptor-associated kinase-1 (IRAK1) shows significant lower expression in the SLE patients **(Tang, Luo et al. 2009)**. Likewise miR-148a, miR-21, miR-126 and miR-29b are upregulated within the PBMCs and CD4+ Tcells where they target the different entities of adaptive immune response. MiR-21 and miR-148a seems to target *DNMT1* that contributes to the DNA hypermethylation in T cells. MiR-21 also regulates the RAS-MAPK-ERK signaling upstream of *DNMT1* in T cells **(Ballestar, Esteller et al. 2006).**

1.9.4 MiRNA in psoriasis

Psoriasis is a chronic and relapsing inflammatory condition generated due to an overactive autoimmune response. The marked pathological features of psoriasis include hyperkeratosis with parakeratosis, vascular dilatation and accelerated epidermopoiesis. A group of study has revealed altered miRNA expression within the PBMCs and immune cells of psoriasis patients. While miR-99a, miR-125b and miR-181a showed significant

down regulation in PBMCs of psoriasis patients, miR-142-3p, miR-146a, miR-155, miR-224 and miR-378 showed significant upregulation in their expression. Within the Th17 cells, miR-223 was upregulated and miR-193b was downregulated. Similarly, higher miR-125b expression is reported in the Treg cells **(Lovendorf, Mitsui et al. 2015)**.

1.10 Altered miRNA expression in IBD

In the past few years, several studies have reported the differential expression of miRNA in the colonic tissues as well in blood of IBD patients.

1.10.1 Differential expression of miRNAs in colonic tissues of IBD patients

The first ever study to investigate the altered expression of miRNAs in IBD patients was carried out in 2008, where Wu *et al* reported differential expression of eleven miRNA including miR-21, miR-192, miR-23a, miR-126 and many other in the colonic mucosa of UC patients. He also observed that miR-192 was able to target and regulate the expression of Macrophage Inflammatory Peptide-2α (MIP2α), which is a chemokine expressed by epithelial cells **(Wu, Zikusoka et al. 2008)**. After that several studies have been carried out and reported the differential expression of miRNA in the UC as well as CD patients. Five miRNAs, including miR-19b, miR-629, miR-23b, miR-106 and miR-191 exhibit altered expression in the CD patients with miR-23b, miR-106 and miR-191 being upregulated and miR-19b and miR-629 being significantly down regulated. Additionally, miRNA also display region specific differential expression in the gut **(Wu, Zhang et al. 2010)**. Fasseu et al examined the miRNA expression of active and quiescent CD patients with healthy controls.

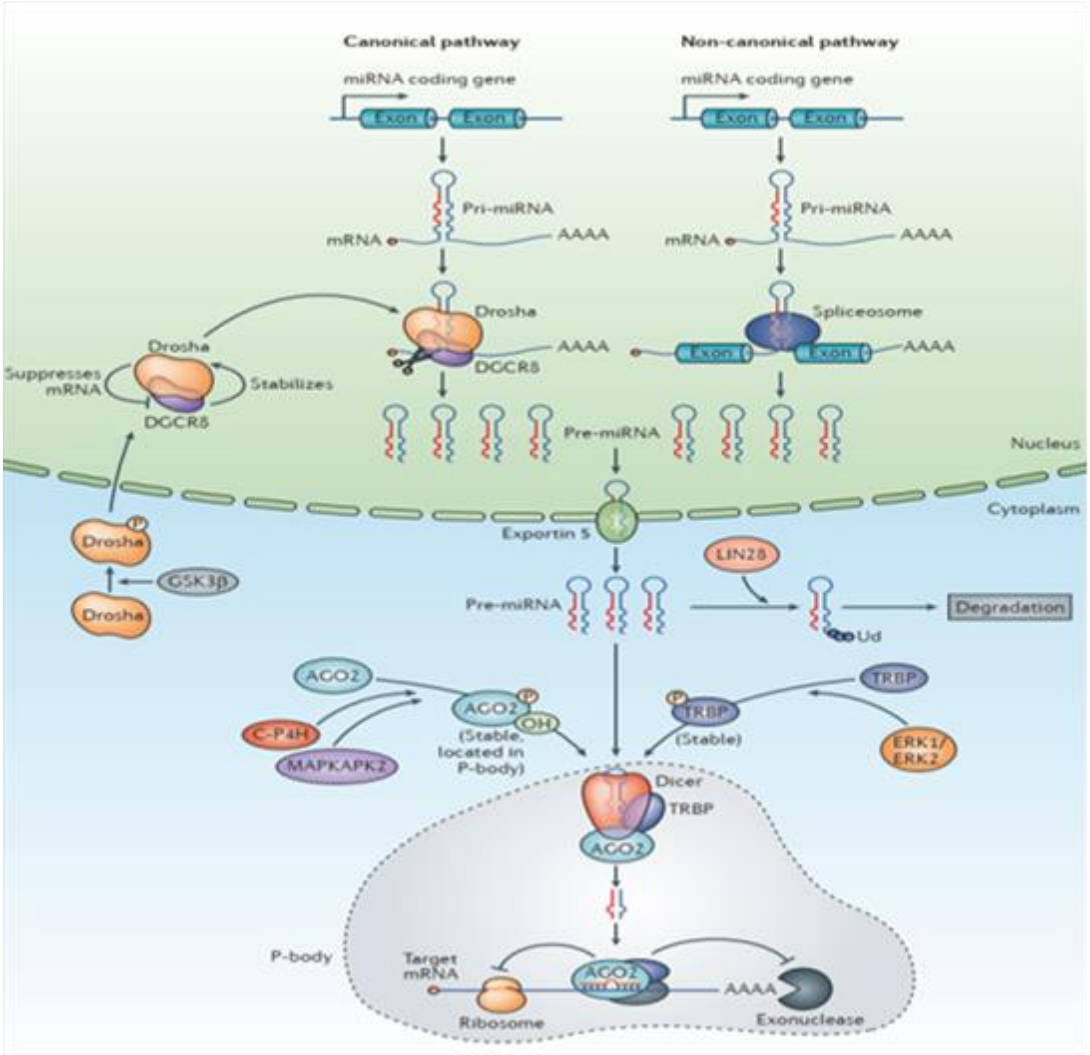


Figure 12: The canonical and non-canonical pathways for miRNA biogenesis (Li and Rana 2014).

They demonstrated upregulation of total twenty three miRNAs, out of which five miRNAs including miRs-9, miR-126, miR-130a, miR-181c and miR-375 were upregulated in the active CD while remaining eighteen miRNAs including miR-21, miR-155, miR-126, miR-150 and many others were upregulated even in the quiescent CD tissues **(Fasseu, Treton et al. 2010)**. The miRNA expression profile of active UC patients differs significantly not just from healthy controls but also from the miRNA expression profile of inactive UC patients **(Van der Goten, Vanhove et al. 2014)**. Altered expression of 20b, miR-98, miR-125b-1*, and let-7e* has also been reported in UC patients and are suggested to be developed as biomarkers for UC **(Coskun, Bjerrum et al. 2013)**. The two clinical forms of IBD, UC and CD are associated with a distinct and specific expression pattern of miRNAs in the tissues/body fluids and set of these miRNAs such as miR-19a, miR-21, miR-31, miR-101, miR-146a, and miR-375 might be used as biomarkers to distinguish between UC and CD **(Schaefer, Attumi et al. 2015)**. Downregulation of miR-10a is reported in the inflamed colonic mucosa of CD and UC patients is inversely correlated with increased expression of DC driven proinflammatory cytokine and increased Th1 and Th17 response **(Wu, He et al. 2015)**. MiR-126 is upregulated in UC patients and it is localized in the endothelial cells, similarly, miR-21 is upregulated in UC patients as compared to healthy controls and CD patients and is expressed by the macrophages and T cells in the lamina propria **(Thorlacius-Ussing, Schnack Nielsen et al. 2017)**.

1.10.2 Differential expression of miRNAs in body fluids of IBD patients

Circulating miRNAs may be developed as potential biomarkers and used as a non-invasive alternative to the current diagnostic methods of IBD. Seven miRNAs from peripheral blood mononuclear cells including miR-1909-3p, miR-212-3p, miR-933, miR-129-5p, miR-885-5p, miR-619-3p and miR-874-3p are significantly upregulated in the colonic CD patients as compared to UC with miR-874 exhibiting the strongest differential expression **(Mohammadi, Kelly et al. 2018)**. Eleven miRNA from blood of CD patients showed significant upregulation in their expression as compared to healthy controls and similarly, six miRNAs from blood of UC patients exhibited significantly higher expression in comparison to healthy controls **(Paraskevi, Theodoropoulos et al.**

2012). Eight miRNAs from the blood of active UC patients including miRs-28-5p, miR-103-2*, miR-149*, miR-151-5p, miR-340*, miR-505*, miR-532-3p, and miR-plus-E1153 exhibited significant altered expression as compared to healthy controls and these miRNAs could also distinguish between UC and CD **(Wu, Guo et al. 2011)**. Significant higher expression of miR-223 has been observed in the serum of both CD and UC patients and this higher miR-223 expression was positively correlated with the disease activity in IBD patients **(Wang, Zhang et al. 2016)**.

1.11 MicroRNA as epigenetic regulator of inflammation during in IBD

The innate and adaptive immune response play central role in providing protection against the pathogens and also in maintaining the gut homeostasis. Emerging evidences have demonstrated the role of miRNA in regulating the innate and adaptive immune response, including regulation of T and B cell differentiation and function **(Lu and Liston 2009)**. The innate immune response includes the TLR activation by stimuli such as lipopolysaccharide (LPS). Upon activation, the TLR binds the adaptor protein Myd88 and activates the downstream signal transduction pathways such as NFkB and MAPK signaling which eventually regulates the transcriptional activation of various cytokines like TNFα, IFN-γ, IFN-β. Various genes involved in these inflammatory pathways are actively regulated by miRNAs. TNFα, well known for its role in IBD pathogenesis is highly upregulated in UC patients. MiR-19a negatively regulates the expression of TNFα and an inverse correlation between the expression of miR-19a and TNFα has been observed in UC patients and mouse model of colitis **(Chen, She et al. 2013)**. Role of miR-146a in maintaining intestinal barrier function and integrity has also been explored. Under physiological condition, miR-146a is expressed within the intestinal tissues of mice where it regulates the expression of a subset of genes involved in maintaining the gut homeostasis. MiR-146a regulates the expansion of intestinal T cell population including Tregs, Th17 cells and also controls the expression of intestinal cell adhesion molecules, antimicrobial peptides and inflammatory cytokines. Loss of miR-146a functions ameliorated the colitis development in mice and these findings correlated well with the elevated miR-146a expression in UC patients **(Runtsch, Hu et al. 2015)**. MiR-874 shows significant upregulation in the colonic CD patients in comparison to UC

patients and it binds within the 3'UTR of autophagy gene *ATG16L1* and suppresses its expression **(Mohammadi, Kelly et al. 2018)**. Similarly, miR-141 targets the expression of chemokine CXC ligand 12β (CXCL12β) and also inhibits the CXCL12β driven localization of leukocytes at the site of inflammation. Significant downregulation of miR-141 and upregulation of CXCL12 is observed in the colonic tissues of colitis mouse models and also in active CD patients **(Huang, Shi et al. 2014)**. Higher miR-155 expression is seen in the intestinal myofibroblasts (IMFs) derived from colonic mucosa of UC patients and it can efficiently target the 3' UTR of *SOC1* (Suppressor of Cytokine Signaling) which is an STAT-induced STAT inhibitor (SSI) and works downstream of cytokine receptors to ameliorate the cytokine signaling. Inhibition of miR-155 in UC derived IMFs resulted in enhanced SOCS1 expression and demolished proinflammatory response while silencing of *SOCS1* in IMFs exaggerated the inflammation by inducing the expression of IL-6 and IL-8 **(Pathak, Grillo et al. 2015)**. The Th17/IL-23 pathway plays a remarkable role in the onset of IBD. Claudin-8 (CLDN8) which forms the backbone of intestinal barrier junction has been identified as a potential target for IL-23 signaling and downregulation in *CLDN8* expression has been observed in inflamed colonic mucosa of IBD patients and in trinitrobenzene sulphonic acid (TNBS) colitis model. Loss of IL-23 function restored the *CLDN8* expression in mice and this cross talk between IL-23 and intestinal barrier found to be mediated by miR-223. Also, the increase miR-223 expression correlated with the decrease *CLDN8* expression in IBD patients **(Wang, Chao et al. 2016)**. Downregulation of miR-141 has also been correlated with the increased CXCL5 expression in the active UC patients **(Cai, Chen et al. 2017)**. CXCL5 is known to be involved in UC pathogenesis by recruiting neutrophils to the site of inflammation **(Z'Graggen, Walz et al. 1997).** Decreased miR-141 enhances the CXCL5 expression which might be responsible for the activation of AKT signaling and upregulation of MMP2 and MMP9 **(Cai, Chen et al. 2017)**. Downregulation of miR-19a is correlated with a subsequent increased expression of its target gene SOCS3 in active CD patients. MiR-19a is suggested to regulate the SOCS3 driven chemokine production in intestinal epithelial cells which is altered during CD **(Cheng, Zhang et al. 2015)**.

1.11.1 Role of miRNA in IBD associated colorectal cancer development

Longstanding inflammation in UC patients results in severe complication i.e. colorectal cancer (CRC) and the risk for CRC development depends upon the duration and extent of disease. MiRNA have been implicated in the progression of inflammation associated CRC development. Differential expression of miR-31 has been studied at different stages of UC i.e. quiescent UC, active UC, neoplastic tissues and dysplastic tissues. The expression of miR-31 is reported to be progressively higher in the progression of normal colonic tissues into inflamed tissues and was highest in the cancer tissues. MiR-31 expression was not changed between the dysplastic tissues as compared to cancer tissues which suggest that elevation in miR-31 expression might be an early event in the transformation of neoplastic tissues **(Olaru, Selaru et al. 2011)**. This study suggested that monitoring the expression levels of miR-31 could be used as a diagnostic marker for neoplastic progression. Differential expression of miRNA has also been reported in CD associated cancer. Upregulation of miR-181a, miR-146b-5p, miR-17 and let-7e is reported in tissues progressing from neoplasia to dysplasia while downregulation of miR-17, miR-143 and let-7e was observed in tissues progressing from dysplasia to cancer. Out of these miRNAs miR-17 and let-7e regulated the expression of E2F1, which is an upstream regulator of TP53 gene **(Kanaan, Rai et al. 2012)**.

1.12 SNP discoveries in miRNA and the associated IBD risk

Single nucleotide polymorphism (SNP) around the miRNA processing sites can enhance or impair the biogenesis and function of miRNA. The sequence variation within the seed region of miRNA alters the binding affinity for its target and also drives the generation of new miRNA with altered binding capacity for new target mRNAs. Similarly, SNPs within the target site-sequence may delete the binding site for miRNA and result in altered gene expression. Sequence variation within the miRNA and their role in disease pathogenesis has been explored through a variety of studies **(Sun, Yan et al. 2009)**. The association of four SNPs rs2910164, rs11614913, rs3746444 and rs2292832 within the pre-miRNAs mir-146a, mir-196a, mir-499 and mir-149 with increased IBD and IBD associated CRC risk has been studied in Chinese population. The SNPs within miR-146a and miR-149 resulted in significantly reduced miR-146a and miR-149 expression in IBD

patients. The risk for IBD was significantly increased for rs2910164 in mir-146a with GC and CC genotypes as compared to GG genotype. Similarly for rs2292832 miR-149, IBD patients with T allele expressed lower level of miR-149 and were at higher IBD rick as compared to C allele. In case of mir-196a rs11614913, individual with CC genotype were at higher IBD-CRC risk as compared to individuals with TT genotype **(Zhu, Li et al. 2016)**. The IBD risk associated with rs2910164 and rs11614913 in miR-146a and miR-196a2 respectively has also been investigated in Greek population. The CC genotype and C allelic frequencies in case of rs2910164 miR-146a were significantly higher in CD patients but not in UC suggesting that rs2910164 miR-146a contribute to genetic susceptibility for CD but not UC. In case of rs11614913 in miR-146a, the TT genotype and T allele provided protection against UC **(Gazouli, Papaconstantinou et al. 2013)**.

A synonymous variant of IRGM gene which is a susceptibility gene for CD was found to be in a strong linkage disequilibrium with a deletion polymorphism. Based on the hypothesis that polymorphism could hamper the miRNA mediated suppression of target mRNA, the binding of miR-196 with IRGM was investigated and it was found that miR-196 significantly altered the expression of IRGM protective allele (c.313C) but not targeted the risk associated allele (c.313T) **(Brest, Lapaquette et al. 2011)**. IL-23 is well known to be a key player in IBD pathogenesis as it plays critical role in the Th1-IFN-γ inflamatory axis in the intestine and also known to inhibit the deveolment of FoxP3+ Treg cells which have important role in controling the inflammatory response. Genetic variant of IL-23 gene are associated with increased IBD risk, rs10889677 polymorphism within the 3' UTR of IL-23 results in substitution of C in the wild type allele with an A. These changes in the 3' UTR result in loss of binding affinity for miR-let-7e and let-7f which targets the wild type IL-23. Due to the loss of miRNA binding site, the rs10889677 variant exhibited higher IL-23 expression, which was suggested to contribute to chronic inflammation during IBD through sustained IL-23 signaling **(Zwiers, Kraal et al. 2012)**.

1.13 Functional characterization of miRNA

Functional characterization of miRNAs is essential to understand the contribution of miRNA in disease pathogensis. Identification of potential gene targets and validation of

mRNA:miRNA inetraction forms the foundation for recognising the involvement of miRNA in biological pathways. Since each miRNA possess potential target sites for multiple genes, validating potential gene target becomes too laborious and time consuming to perform manually. Computational aproaches employing bioinformatic target prediction tools help in narrowing down the potential gene targets for experimental validation. Currently available prediction tools employ different computational approaches ranging from modelling of physical miRNA:mRNA to incorporating machine learning. Several tools with a distinct approach to predict putative gene targets for miRNAs are available but there are four common parameters which forms the basis of miRNA target prediction: seed complementarity, free energy, conservation and site accessibility **(Peterson, Thompson et al. 2014)**. The seed sequence for miRNA counts the first 2-8 nucleotides starting form 5'end of miRNA and for most of the prediction tools the miRNA and corresponding target mRNA forms the Watson and Crick (WC) match. Based on the algorithms, there are different types of seed matches that form between the miRNA and target mRNA **(Brennecke, Stark et al. 2005, Krek, Grun et al. 2005, Lewis, Burge et al. 2005)**.

i) 8 mer: Perfect WC match from 2-8 nucleotides in miRNA seed region with an A across the first miRNA nucleotide.

ii) 6 mer: Perfect WC match beetween miRNA seed and target mRNA for six nucleotides

iii) 7 mer-m8: Perfect WC match from nucleotides 2–8 of the miRNA seed

iv) 7mer-A1: Perfect WC match from nucleotides 2–7 of the miRNA seed with an A across from the miRNA nucleotide 1.

Conservation analysis focus on the maintenance of 3'UTR sequence, 5'UTR sequence or miRNA across different species. Usually the miRNA sequence in the seed region is more conserved than the non seed region. There have been increasing interest in studying the conservation of genomic regions flanking the miRNA gene and target mRNA gene **(Peterson, Thompson et al. 2014)**. Free energy accounts for the stability of miRNA and target mRNA bond. In case the bond between miRNA seed region and complementary

3'UTR sequence is stable it has a lower free energy, and it is considered to be the true target of miRNA. The region of high and low energy between miRNA seed and target mRNA can be inferred by the hybridization of miRNA and mRNA which is an indicator of their binding strength **(Yue, Liu et al. 2009)**. Site accessibility refers to how easily a miRNA can find the complementary sequence within 3'UTR and bind with it. Since mRNA attain a secondary structure following transcription, it can hinder the binding of miRNA with target mRNA. The miRNA first binds with the short accessible sequence within the mRNA which is followed by unwinding of mRNA secondary structure and subsequently, complete binding of miRNA with mRNA occurs **(Long, Lee et al. 2007)**. The amount of energy required to make the target site accessible to miRNA can be evaluated to identify a gene as miRNA true target. The presently available bioinformatic tools for target prediction utilize different combination of the four basic parameters to identify any gene as miRNA target. **Table 2** summarizes the parameter employed by different tools for target prediction.

Besides these four basic characteristic features, some new additional features have also been incorporated with the advancement of miRNA:mRNA interaction and their characterization. These additional features include target abundance which monitors the number of sites present in the 3'UTR, local AU content referring to the A and U nucleotides present across the seed region and GU wobble in seed region which takes into account the pairing of G with U instead of C **(Peterson, Thompson et al. 2014)**.

Table 2. Parameters employed by different bioinformatic tools for target prediction

(Yue, Liu et al. 2009, Peterson, Thompson et al. 2014)

Program	Supported Organism	Seed Match	Free Energy	Conservation	Site accessibility	Website
miRanda	Human, Mice, Rat	+	+	+		http://www.microrna.org/microrna/releaseNotes.do
miRanda-mirSVR		+	+	+	+	http://www.microrna.org/
TargetScan	Mammals, Worms, Flies	+		+		http://www.targetscan.org/
DIANA-microT	Human	+	+	+	+	http://diana.cslab.ece.ntua.gr/
MirTarget	Any	+	+	+	+	http://cbit.snu.ac.kr/~miTarget/
TargetMiner		+	+	+	+	http://www.isical.ac.in/~bioinfo_miu/targetminer20.html
PicTar	Nematodes, vertebrate, flies	+	+	+	+	http://pictar.mdc-berlin.de/
RNAhybrid	Any	+	+			http://bibiserv.techfak.unibielefeld.de/rnahybrid/
SVMicrO	Any	+	+	+	+	http://compgenomics.utsa.edu/svmicro.html

1.14 MiRNA in therapeutics and IBD management

MiRNA play remarkable role in IBD pathogenesis by regulating several signaling pathways associated with inflammation. Lines of evidences have suggested altered miRNA expression in serum and colonic mucosa of IBD patients. In the circulation, miRNAs exist either in membrane vesicles like exosomes **(Gallo, Tandon et al. 2012)** or high-density lipoproteins **(Vickers, Palmisano et al. 2011)** which provide them protection from degradation against RNAses and make them more stable than mRNA and proteins. Besides colonic mucosal tissues and body fluids, altered miR-223 and miR-155 has recently been reported in fecal samples from IBD patients and their expression correlates well with the C-reactive protein and fecal calprotein which are considered as clinical parameters to evaluate disease activity in IBD. These findings suggest that miRNAs can be considered as potential tools for development of non-invasive diagnostic biomarkers for IBD **(Schonauen, Le et al. 2018)**.

MiRNAs play both the pro-inflammatory and anti-inflammatory role in IBD pathogenesis by modulating different biological processes such as NFkB activation, affecting the membrane integrity and autophagy. All these studies have paved the ways to investigate and specifically target these miRNAs as therapeutic approaches to treat IBD patients. The current treatment strategies employed for IBD have limited efficiency and significant side effects therefore novel therapeutic interventions are required for the efficient disease management. Three therapeutic approaches have been developed to specifically target miRNA: anti sense oligonucleotides (ASOs), miRNA sponges and small molecular inhibitor **(Li and Rana 2014) (Figure 13)**. For the miRNAs, exerting pro-inflammatory response, oligonucleotide based therapies to neutralize or block miRNA actions have been developed for different diseases. Anti-miRNA oligonucleotides (AMOs) are perfectly complimentary nucleotides being developed to block the activated miRNAs during diseased condition so that the downstream effector gene can effectively carry out its function and the inflammatory response can be resolved **(Esau 2008)**. However, these DNA oligonucleotides are liable to get degraded by nucleases, therefore, to overcome this issue and to increase the potential of AMOs, modifications such as the 2'-O-methyl RNA (2'-OMe) and phosphorothioate (PS) are introduced in their backbone. But, the 2'-OMe

modification could not provide complete resistance to nucleases and the PS modification compromised with the binding affinity of these DNA nucleotides. The locked nucleic acids (LNAs) are one such modification where the 2'-O and 4'-C are connected via a methylene bridge. The LNAs are the most prominent and efficient in providing better binding affinity and nuclease resistance to AMOs both *in vitro* and *in vivo* **(Lennox and Behlke 2011)**. Although the oligonucleotide based approaches have the therapeutic potential, some of them persuade toxicity either due to eliciting the autoimmune response or due to specific chemical modifications. The chemically modified AMOs also face poor tissue distribution when there is no carrier involved and this could be due to rapid excretion in urine, negative charge being repelled from entering the membrane and poor stability in body fluids and tissues. To overcome these issues liposomes, hydrogel or nanoparticle based approaches are being explored.

The miRNA sponges employ mRNA molecules bearing multiple artificial complementary binding sites for miRNA. These miRNA sponges can specifically sequester miRNAs when overexpressed in a cell which eventually augment the target mRNA expression. *In vitro* application of miRNA sponges has been explored but the *in vivo* application has been limited to transgenic animals only **(Ebert and Sharp 2010)**. In vivo testing of some of the miRNA mimics have been done in mouse model of colitis to investigate the efficacy and potential of miRNA mimics in controlling inflammatory response. MiR-310a promotes the differentiation of Th17 cells and its expression is found significantly higher in the inflamed tissues and PBMCs of TNBS induced colitis mouse as well in IBD patients. Administration of anti-miR-301a in the mice ameliorated the inflammatory response in inflamed mucosa by decreasing the expression of pro-inflammatory cytokines IL-17 and TNFα **(He, Shi et al. 2016)**. Similarly, miR-210 exhibit higher expression in TNBS induces colitis mice where it exerts its pro-inflammatory effects by targeting hypoxia-inducing factor 1 alpha (HIF1α). Administration of LNA-anti-miR-210 in TNBS induced colitis mice decreased the colitis response in mice **(Bakirtzi, Law et al. 2016)**. Increased miR-31 expression is seen in TNBS induces colitis mice and IL-10 knockout mice where it targets the IL-25 expression and promotes the inflammatory response by interfering with the IL-12/23 axis.

Administration of anti-miR-31 in mice decreased the Th1/Th17 mediated inflammatory response by restoring IL-25 expression **(Shi, Xie et al. 2017)**.

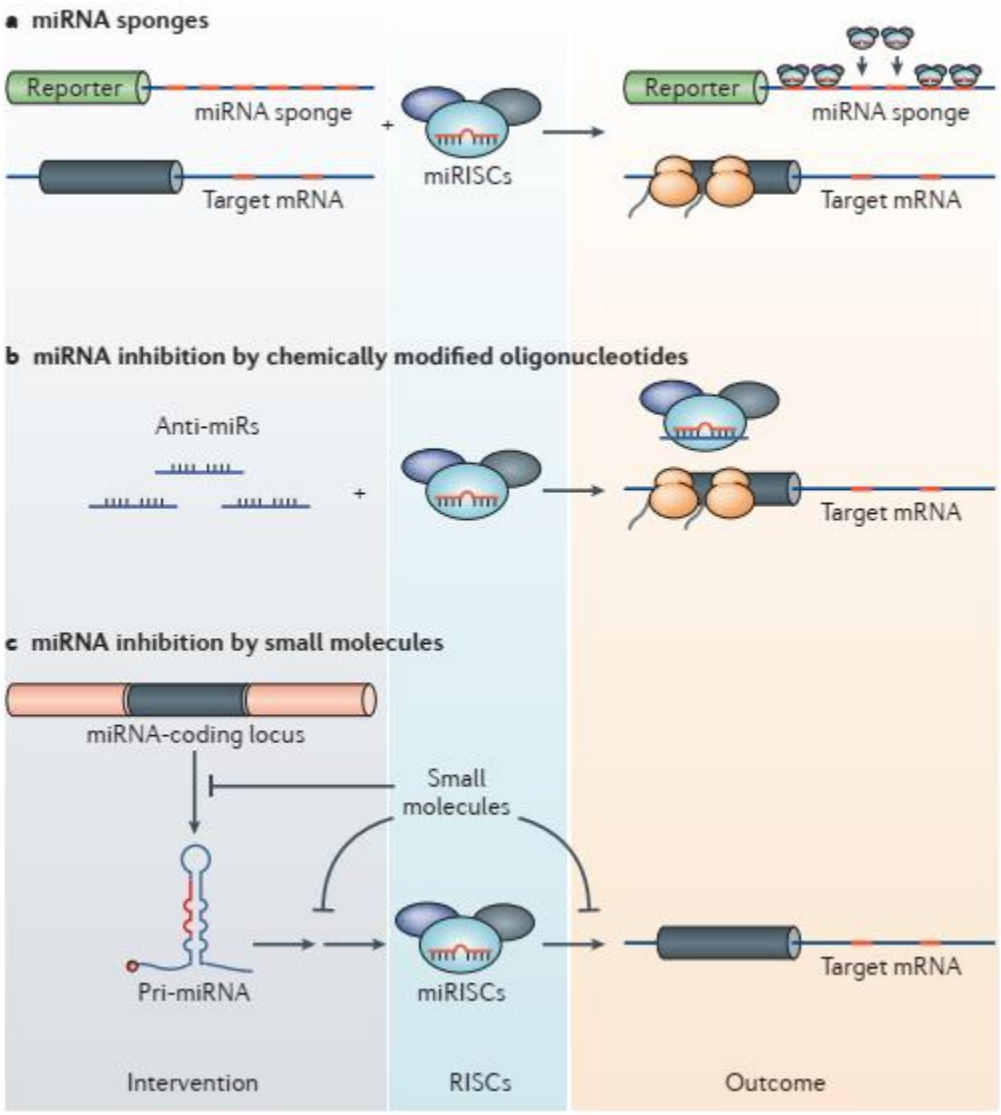


Figure 13: MiRNA based therapeutic approaches (Li and Rana 2014)

MATERIALS & METHODS

2.1 Materials

2.1.1 Ethical approval

Ethical approval to carry out the present study was taken from the ethics committee of *All India Institute of Medical Sciences, New Delhi (*Reference No- T-290/23.06.2015, RT-7/27.01.2016) and also from the *Institutional Ethics Review Board, JNU, New Delhi* (Reference Number 2016/Student/93). Informed consent was taken from all the subjects included in the study.

2.1.2 Sample Collection

Colonic mucosal pinch biopsies were collected from patients reporting to Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi. Samples were collected from Ulcerative colitis patient and non-IBD control subjects.

2.1.2.1 UC Patients

UC patients undergoing colonoscopy were screened for sign and symptoms of UC following the standard protocols and biopsies were collected from affected/inflamed region of colon under supervision of gastroenterologist. For the study involving paired samples, biopsies were collected from the colonoscopically inflamed as well as non-inflamed region of UC patients. The disease activity was measured using SCCAI score (**Walmsley, Ayres et al. (1998) (Table 3)**. UC patients who showed a mild and moderate disease activity were included in the study. Diagnosis of UC was confirmed through endoscopic and histological examination by following ECCO guidelines **(Dignass, Eliakim et al. 2012)**.

2.1.2.2 Controls

Controls were individuals who did not show any inflammatory disorder of gastrointestinal tract and IBD symptoms. All the controls were age and sex matched with patients. Biopsy samples were collected in RNA later solution and stored in -80 deep freezer until RNA isolation was carried out. Demographic and clinical features of all the study subjects are enlisted in **Table No. 4**

2.1.2.3 Inclusion and exclusion criteria for samples

Inclusion criteria for cases:

- Age should be greater than or equal to 18 years.
- Documented/confirmed cases of UC as defined by standard clinical criteria.
- Patients willing to participate.

Exclusion criteria for cases:

- Pregnant women.
- Patients with human immunodeficiency virus (HIV), Hepatitis B virus infection.
- Patients not willing to participate.

Controls:

- Sex and age matched with cases.
- Willing to participate in study

Table 3: The SCCAI score basics (Walmsley, Ayres et al. 1998)

Symptoms	Score
Bowel Frequency (Day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel Frequency (Night)	
1-3	1
4-6	2
Urgency of defecation	
Hurry	1
Immediate	2
Incontinence	3
Blood in stool	
Traces	1
Occasionally frank	2
Usually frank	3
General Well Being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extra colonic features	1

Table 4. Demographic features of Study subjects

Characteristics	UC Patients	Controls (n)
No. of samples	48	30
Sex (M/F)	32/16	24/6
Age (Yr.), mean±SD (Range)	37.02±11.87(20-65)	38.25±13.91(21-64)
Disease duration (Yr.), mean±SD (Range)	4.906±5.327(1-26)	
Disease Extent		
Proctitis	8(16.67%)	
Proctosigmoiditis	12(25%)	
Left sided colitis	17(35.42%)	
Pancolitis	11(22.92%)	
Medication		
Mesalamine	31(64.57%)	
Azathioprine	5(10.42%)	
Steroids	0	0
Smoking		
Yes	5(10.42%)	
No	43(89.52%)	
Appendectomy (Y/N)	0/48 (0%)	

2.1.3 Tools used for Target Prediction

- miRBase
- TargetScan
- MIRDB
- Mirna.org
- Pictar
- DIANA-MicroT
- MirTarBase
- RNA22

2.1.4 Reagents used in gel electrophoresis

Agarose powder (Lonza, ME USA)

TBE buffer (5X Stock and 0.5X working)

Ethidium Bromide (Sigma Aldrich) (50mg/ml stock)

2.1.5 Reagents used in RNA isolation

mirVana miRNA isolation kit (Ambion INC,TX78744,USA)

Ethyl alcohol (Sigma Aldrich)

2.1.6 Reagents and instruments for RNA quantity check

Nuclease free water

NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA).

2.1.7 Reagents and instruments for RNA quantity check

DPEC treated MQ water

Agarose powder (Lonza, ME USA)

TBE Buffer (5X Stock and 0.5X working)

Ethidium Bromide (Sigma Aldrich)

Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States).

2.1.8 Microarray

RNA Samples were submitted to Affymetrix for miRNA profiling using GeneChip ® miRNA 4.0 Array.

2.1.9 Reagents used in Reverse Transcription

RNAse free DPEC water

MicroRNA specific stem loop primers

Random hexamer primer

5X Reaction buffer

MMLV reverse transcriptase (Thermo fisher Scientific, USA)

10mM dNTP mix (Fermentas, USA)

RiboLock (40U/μL)

2.1.10 Reagents used in real time PCR

SYBR green Master Mix (Affymetrix, USA)

Forward and reverse primers (For target genes)

Forward primer for miRNA

Reverse universal primer for miRNA

96 well plates (Biorad, USA)

Optical adhesive cover (Applied Biosystems)

CFX96 Touch^{TM} Real Time PCR Detection System (Biorad, USA)

MQ water

2.1.11 Bacterial strain and Plasmid

E. coli strain DH5α

pBABE puro retroviral vector (For cloning miRNA)

pMIR-REPORT-miRNA Expression Reporter Vector (For cloning 3'UTR of target gene).

2.1.12 Culture Media

2.1.12.1 Luria Bertani (LB) Broth

Tryptone: 10g

Sodium chloride: 10g

Yeast extract: 5g

The above contents were dissolved in 1litre of double distilled water (DDW) and pH adjusted to 7.0 followed by autoclaving.

2.1.12.2 Luria Bertani (LB) Agar

0.9% w/v Bacto-Agar was added to LB broth and autoclaved. Filter sterilized Ampicillin 100µg/ml was added to the LB agar at lukewarm temperature (~55^{o}C). Plates were prepared in laminar flow hood.

2.1.12.3 NZY broth

NZ amine (casein hydrolysate): 10 g

Yeast extract: 5g

NaCl: 5g

Deionized water was added to a final volume of 1 liter.

pH was adjusted to 7.5 using NaOH and autoclaved.

Following filer-sterilized supplements were added just prior to use:

1M $MgCl_2$: 12.5 ml

1M $MgSO_4$: 12.5 ml

20% (w/v) glucose (or 10 ml of 2 M glucose): 20 ml

2.1.13 Site Directed Mutagenesis

QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene).

PfuUltra™ High Fidelity DNA polymerase (2.5 U/µl)

10× reaction buffer

Dpn I restriction enzyme (10 U/µl)

QuikSolution™ reagent

dNTP mix

XL10-Gold® ultracompetent cells

XL10-Gold® β-mercaptoethanol mix (β-ME)

Thermo cycler (Eppendorf)

Forward and Reverse primers for mutagenesis

Forward primers for sequencing

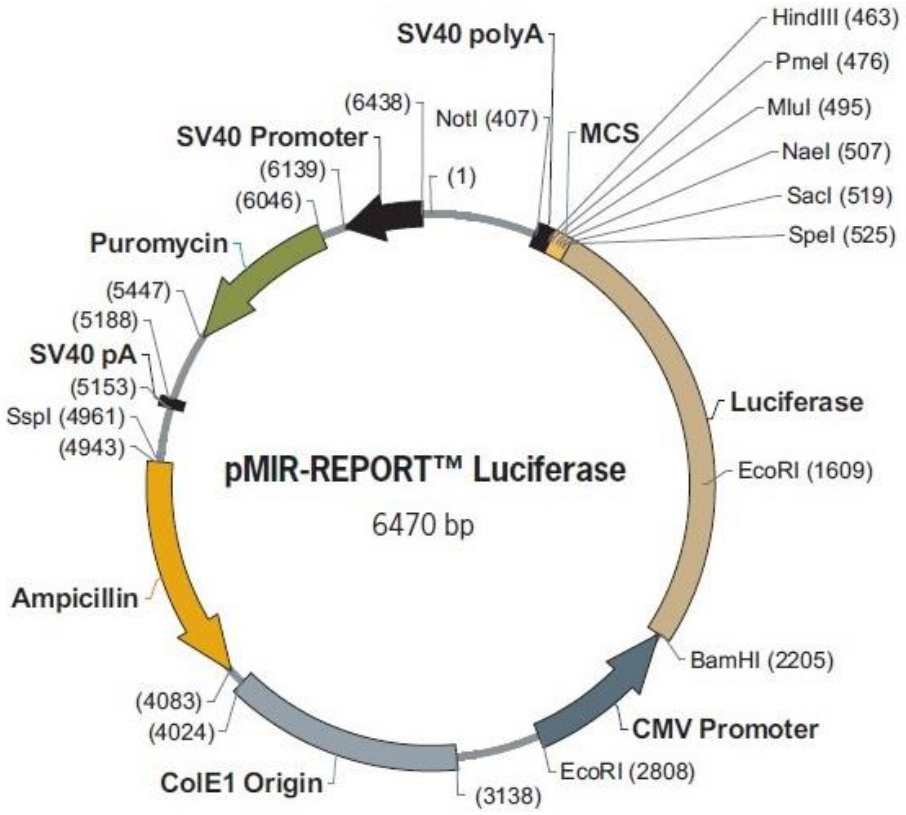


Figure 14: Plasmid map of pMIR-REPORT-miRNA Expression Reporter Vector

2.1.14 Plasmid Isolation

2.1.14.1 Reagents used in manual method

Solution I: 50mM Glucose, 25mM Tris HCl pH 8.0,10mM EDTA pH8.0

Solution II: 1% SDS, 0.2 N NaOH

Solution III: 3M Potassium acetate, 5M Glacial acetic acid

Isopropanol

70% Ethanol

$T_{10}E_1$RNase (pH-8.0)

2.1.14.2 For transfection

GSure™ Plasmid mini prep kit (GCC Biotech, India)

2.1.15 Animal cell culture

HT29 cell line (NCCS Pune, India)

Dulbecco's Modified Eagle Medium (DMEM) (Gibco)

Fetal bovine serum (Gibco)

Penicillin-Streptomycin (10000 U/ml) (Sigma-Aldrich)

Trypsin-EDTA (1X) (Sigma-Aldrich)

1X Phosphate Buffer Saline (PBS)

Cell culture flasks

6, 12 and 96-well plates (Corning)

Serological pipettes (2ml and 10ml) (Corning)

CO_2 incubator and cell culture laminar flow hood

2.1.16 Transfection

Lipofectamine 2000

Opti-MEM media

2.1.17 Dual Luciferase Reporter Assay

Dual-Glo® Luciferase Buffer

Dual-Glo® Luciferase Substrate

Dual-Glo® Stop & Glo® Buffer

Dual-Glo® Stop & Glo® Substrate

Varioskan™ Flash Multimode Reader (Thermo Scientific,USA)

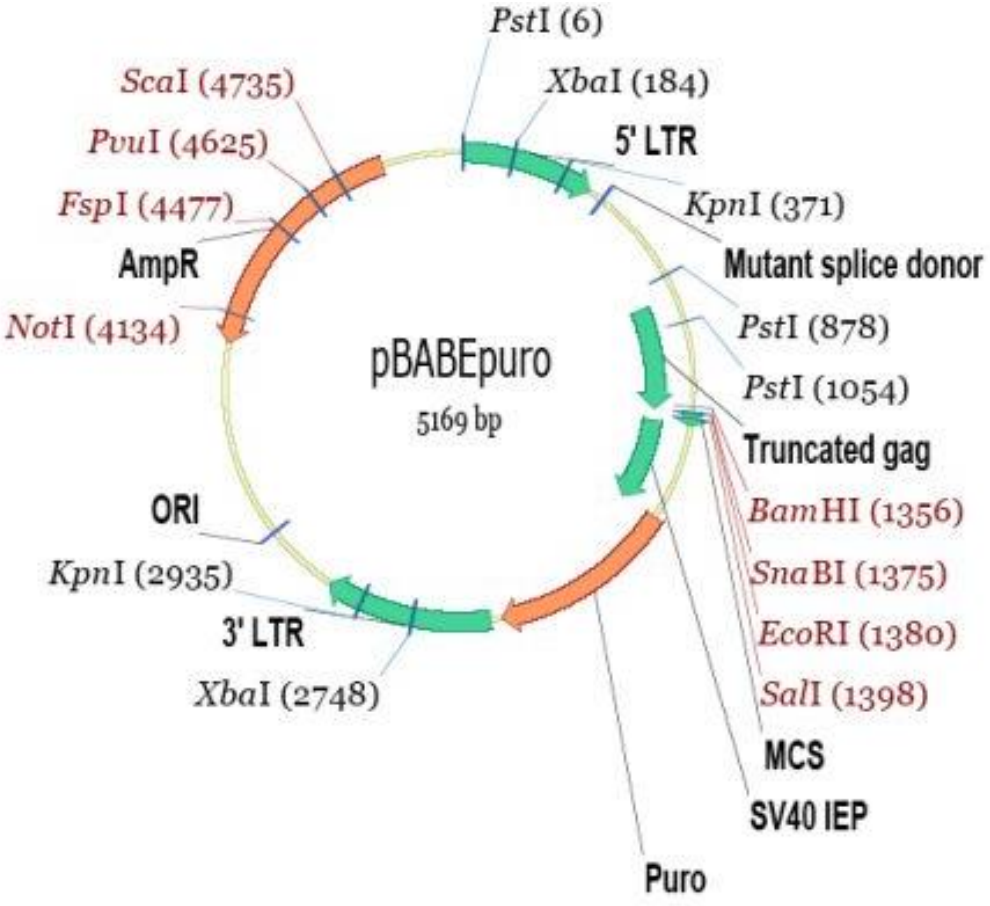


Figure 15: Plasmid map of pBABE puro retroviral vector

2.2 Methods

2.2.1 Target Prediction

Target prediction for differentially expressed miRNAs was carried out by miRWalk database (http://www.ma.uniheidelberg.de/apps/zmf/mirwalk/) which simultaneously searches several other databases **(Dweep, Sticht et al. 2011)**. Additionally, TargetScan, MIRDB, mirna.org, PicTar, DIANA-MictoT, RNA22, MirTarBase were also used for target prediction and genes picked by at least three tools were chosen for quantitative real time (qRT-PCR) validation. Further, biological relevance of dysregulated miRNA expression was studied by finding the signaling pathway involved, using mirPath v.3: DIANA TOOLS.

2.2.2 Primer designing

2.2.2.1 Reverse Transcription of miRNA: To reverse transcribe the miRNA, stem loop primers were designed so as to increase the transcript length upto 90bp **(Chen, Ridzon et al. 2005)**. MiRNA sequence was obtained from miRBase (http://www.mirbase.org/) to design the microRNA specific cDNA primers. 7-8 nucleotides complementary to the 3'end of miRNA sequence were added to the 5' end of stem loop sequence to design the reverse transcription primers for different miRNAs. For qRT- PCR, forward primers were designed from the same sequence obtained from miRBase (http://www.mirbase.org/) and universal reverse primer designed from stem loop was used for all microRNAs. The sequences of primers used for reverse transcription and qRT-PCR of miRNAs are enlisted in **Table 5**.

2.2.2.2 Reverse Transcription for miRNA Target genes: Random hexamer primer was used to reverse transcribe the target genes of miRNAs. For qRT-PCR analysis, gene specific forward and reverse primers were designed for each target gene and Gapdh was used as internal reference gene for normalization. The sequences of primers used for qRT-PCR of target genes are given in **Table 6**.

2.2.2.3 Cloning primers for miRNAs and 3'UTR of Target genes: Genomic sequence containing the sequence for different miRNA was downloaded from Ensembl genome browser (https://www.ensembl.org) along with a flanking region of about 200bp both at 5' and 3' end. Forward and reverse primers were designed from the sequences flanking the seed region of miRNA binds with the 3'UTR or target gene. BamH1 and EcoR1

restriction sites were inserted at the 5' end of forward and reverse primer. MiRNAs were cloned in pBABE puro vector which is a retroviral vector derived from Moloney murine leukemia virus (MMLV). 3'UTR sequence of target genes was downloaded from Ensembl genome browser (https://www.ensembl.org). Forward and reverse primers were designed from the sequence flanking the region complementary to the seed region of microRNA i.e. the microRNA binding site. Sac1 and Mlu1 restriction sites were inserted at the 5' end of forward and reverse primer. All the primers used for cloning of miRNAs and respective target genes are given in **Table 7**.

2.2.2.4 Site Directed Mutagenesis primers: The genomic sequences obtained from Ensembl genome browser (https://www.ensembl.org) were used as templates for designing site directed mutagenesis primers. The desired mutations in the seed region of miRNAs were introduced manually in the seed region of microRNA. Three nucleotides in the seed region were substituted to hamper the binding with 3'UTR of target gene. All the primers were checked for the presence of hair-pin loop or dimers using Primers express software v3.0 (Applied Biosystems, California, USA). The primers used for the site directed mutagenesis of miRNAs are enlisted in **Table 8**.

2.2.3 RNA Isolation and quality check

For RNA isolation, tissues were homogenized in a tissue homogenizer and total RNA was isolated using mirVana miRNA isolation kit (Ambion, Thermo Fisher Scientific, USA) following the manufacturers protocol. To assess the quality of RNA, 1µg of RNA was loaded on 1.5% agarose gel along with 1.5% 1X RNA loading dye and gel was observed for two clear bands of 28S and 18S **(Figure 15)**. For Cell line, cells well grown at 90% confluency and transfected with desired plasmids. RNA was isolated after 48hrs of transfection using mirVana miRNA isolation kit (Ambion, Thermo Fisher Scientific, USA).

2.2.4 Microarray

The array included three pairs of colonic mucosal biopsied obtained from inflamed and non- inflamed region UC patients. Total RNA extracted from the samples were submitted to Affymetrix for quality check using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States) and microRNA profiling using GeneChip ® miRNA 4.0

Array which is designed to interrogate all mature microRNA sequences in miRBase Release 20. The differentially expressed miRNAs were statistically assessed by one way ANOVA paired method. P value of 0.05 was considered significant and fold change of 1.5 was taken as cut off for upregulation and -1.5 for downregulation of microRNA in a given sample.

2.2.5 Reverse Transcription

800ng of RNA was used to synthesize the cDNA. 1µl of primer (miRNA specific primer for miRNA and random hexamer for genes) was added to RNA and DPEC treated MQ was used to give a final volume of 12.5ml. Tubes were tapped gently and incubated at 65^0C for 5 minutes followed by immediate chilling at ice. After that, 4µL of 5X reaction buffer, 2µL of 10mM dNTP mix, 1µL of MMLV (40U/µL) reverse transcriptase and RiboLock (40U/µL) were added to the tubes. The tubes were incubated at room temperature for 10 minutes to allow the binding of primer. Reverse transcription carried out by incubating the mixture at 42^0C for 1 hour and then the mixture was transferred to 70^0C (for 10 minutes) to terminate the reverse transcriptase activity.

2.2.6 Quantitative Real time PCR

The quantitative real time PCR (qRT-PCR) reaction was prepared by adding 7 µL of MQ water, 1µL of forward and reverse primer each (4pmole/ µL), 10µL of SYBR Green PCR master mix (Affymetrix, USA) and 1µL of cDNA. The reaction was briefly spun at 2000rpm for 2 minutes and transferred to thermo cycler. (Biorad)

The reaction conditions were:

Stage 1- 50ºC/min (1 cycle)

Stage 2- 95ºC/10min (1 cycle)

Stage 3- 95ºC /15sec, 60 ºC/1min (40 cycles)

U6 snoRNA and GAPDH were used as internal reference for miRNA and target genes respectively. The relative expression was analyzed using comparative threshold cycle method (n fold=$2^{-\Delta\Delta ct}$ method) (Schmittgen and Livak 2008). The statistical analysis was done using unpaired, two way student's t-test and a p value <0.05 were considered significant.

Table 5: Primers used for reverse transcription (RT) and qRT-PCR for microRNAs

Name	Primer	Sequence (5'-3')
Reverse Universal Primer		GTGCAGGGTCCGAGGT
hsa-miR-125b-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGTCACAAGT
	Forward	TCCCTGAGACCCTAACTTG
hsa-miR-223-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGTGGGGTAT
	Forward	TGTCAGTTTGTCAAATACCC
hsa-miR-194	RT	
	Forward	
hsa-miR-155-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCCCTAT
	Forward	TTAATGCTAATCGTGATAGG
hsa-miR-200a-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACATCGTT
	Forward	TAACACTGTCTGGTAACGAT
Sno RNA U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGAAAATATG
	Forward	CAAATTCGTGAAGCGTTCCA

Table 6: Primers used for qRT- PCR for target genes.

Name	Primer	Sequence (5'-3')
STAT3	Forward	GGCTTTTGTCAGCGATGGAG
	Reverse	ATTTGTTGACGGGTCTGAAG
TRAF3	Forward	CAGAGGTTGTGCAGAGCAGT
	Reverse	CCGGTATTTACACGCCTTCT
IKKα	Forward	CGGCTTCGGGAACGTCTG
	Reverse	GCCTTTACAACATTGGCATGG
TRAF6	Forward	CCTTTGGCAAATGTCATCTGTG
	Reverse	CTCTGCATCTTTTCATGGCAAC
GAPDH	Forward	GCTCCTCCTGTTCGACAGTCA
	Reverse	GCAACAATATCCACTTACCAG

Table 7: Primers used for cloning of miRNAs and their respective gene targets

Name	Primer	Sequence
miR-125b	Cloning F.P	CGAGCTCCCTCTCCTACCAAGCAG
miR-125b	Cloning R.P	GACGCGTGTCCATGGATGGTTCTG
miR-223	Cloning F.P	CGCGGATCCCTTCAGGATCTCTCTTCTGG
miR-223	Cloning R.P	CCGGAATTCCCCTGGTGTCCTCAGAT
TRAF6	Cloning F.P	CGAGCTCGCTAGGATTAGAAGTCACAGTG
TRAF6	Cloning R.P	GACGCGTCTCTGATTTGGCCTCTCTG
IKKα	Cloning F.P	CGAGCTCGGGATTGTGTATCTGTGCTTC
IKKα	Cloning R.P	GACGCGTCGATGATAGAGGTCCACAGTCC
A20	Cloning F.P	CGAGCTCCCTCTCCTACCAAGCAG
A20	Cloning R.P	GACGCGTGTCCATGGATGGTTCTG

Table 8: Primer sequences used for site directed mutagenesis of miR-125b and miR-223

Name	Primer	Sequence
miR-125b	F.P	CGCTCCTCTCAGTCAAGGAGACCCTAAC
miR-125b	R.P	GTTAGGGTCTCCTTGACTGAGAGGAGCG
miR-223	F.P	CACTCCATGTGGTAGAGTGTACTTTTGTCAAATACCCC
miR-223	R.P	GGGGTATTTGACAAAAGTACACTCTACCACATGGAGTG

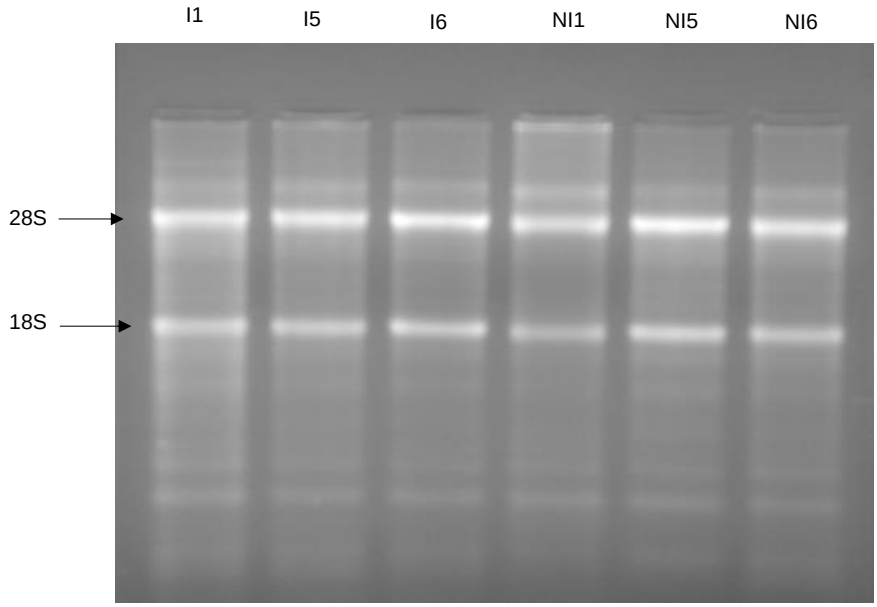


Figure 16: Quality and integrity of RNA on agarose gel: Total RNA isolated from biopsy samples were checked for RNA integrity. Two clear bands of 28S and 18S rRNA were observed. I denote the RNA isolated from Inflamed colinic mucosa of UC patients and NI denotes RNA form Non-Inflamed colonic regions.

2.2.7 Cloning of miRNA and 3' UTR

The DNA sequence having miRNA sequence and flanking region was cloned in pBABE puro retroviral vector within the BamH1 and EcoR1 restriction sites. Similarly, 3'UTR of genes were cloned in pMIR-REPORT-miRNA Expression Reporter Vector downstream the luciferase gene. Sac1 and Mlu1 were used to clone the target genes.

2.2.8 Site Directed Mutagenesis

1. The site directed mutagenesis was carried out following the manufacturer's protocol.
2. The reaction mixture was consisted of following components:

Plasmid template: 100ng

10X reaction buffer: 5µL

Forward primer: 125ng

Reverse primer: 125ng

dNTP: 1µL

Quick solution: 3.5µL

MQ: To give a final volume of 50 µL

Pfu Ultra HF DNA polymerase (2.5U/µL): 1µL

The reaction mixture was kept in thermos cycler at following reaction conditions:

Stage1: 95^{o}C for 2 minutes

Stage 2:

95^{o}C for 50s
65^{o}C for 50s
68^{0}C for 16min
} 18 Cycles

Stage 3: µL for 7min.

3. 10µL of PCR product was run on agarose gel to confirm the product size.
4. Rest of the PCR (40µL) product was digested with 1µL of Dpn1 and incubated overnight at 37^{0}C.
5. 4µL of digested product was transformed in ultra-competent cells following manufacturer's protocol.
6. Plasmid was isolated from the colonies obtained and further analyzed for expected mutations by sequencing.

7. Glycerol stocks were prepared for the mutants and stored at -80^{0}C.

2.2.9 Sequencing

The pBABE plasmids carrying desired mutations were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequencing reactions were prepared using ABI sequencing kit v1.1 (Applied Biosystems). Sequences were analyzed using Bioedit Sequence Alignment Editor. The sequencing primers are giver in **Table 9.**

Table 9: List of primers used for sequencing

Name	Primer	Sequence
pBABE	5' Sequencing Primer	CTTTATCCAGCCCTCAC
pMIR	5' Sequencing Primer	AGGCGATTAAGTTGGGTA

2.2.10 Plasmid isolation

Plasmids were isolated from bacterial colonies by alkaline lysis method described by Ehrt and Schnappinger 2003(Ehrt and Schnappinger 2003). Single bacterial colony was inoculated in 2ml LB media with Ampicillin and incubated at 37^{0}C with continuous shaking at 220rpm. On the next day, the bacterial growth obtained was pelleted at 13000 rpm for 1minute at 4^{0}C. The cell pellet was resuspended in 100µL of Solution I by with vortexing. 200µL of Solution II was then added to carry cell lysis and alkaline denaturation of DNA. 300µL of Solution III was then added to neutralize the Solution II and tubes were centrifuged at 13000 rpm for 10 minutes. 250µL isopropanol was added to the supernatant collected and incubated for 10 minutes at room temperature. The tubes were then centrifuged at 13000 rpm for 10 minutes and the pellet obtained was washed with 70% ethanol followed by air drying for 20-30 minutes. Plasmids were then dissolved in 40µL of $T_{10}E_1$RNase and quantified by nano spectrophotometer. For the purpose of

transfection, plasmids were isolated using GCC Biotech kit following manufacturer's protocol.

2.2.11 Animal Cell Culture

HT29 Human colon adenocarcinoma cell line was cultured in Dulbecco's Modified Eagle Medium with high glucose (DMEM) (Gibco, Invitrogen) supplemented with 5% heat inactivated fetal bovine serum (FBS) and 5% Penicillin-Streptomycine (PenStrep) (Thermo fisher Scientific). Cells were incubated at 37^0C and 5% CO_2 and culture media was changed 2-3 times a week. Cells were passaged regularly as and when reached confluency of 90%.

2.2.12 Plasmid transfection and LPS treatment

The transfection of the plasmid was carried out using the manufacturer's protocol. HT29 cells were seeded at a density of 1×10^6 cells a day before transfection in 12 well plates for co-expression and downstream pathway analysis. Next day when the cells reached a confluency of 80-90%, cells were washed with incomplete DMEM twice (without antibiotic and serum). Lipofectamine 2000 and plasmids were diluted in serum free DMEM and added to the cells dropwise after proper mixing. After 6 hours of transfection, the serum free media was replaced with complete DMEM and cells were further incubated at 37^oC. For co-expression and pathway analysis, plasmid carrying miRNA and 3'UTR sequences of target gene were co-transfected in 12 well plates. While for dual luciferase reporter assay plasmid carrying miRNA and 3' UTR of target gene were co-transfected along with pRL-TK plasmid. The optimized amount of plasmids and Lipofectamine 2000 are given in **Table 10.** For NFkB pathway activation LPS treatment was given to the cells at a concentration of 100ng/ml for 6 hours.

Table 10: Optimized amount of Lipofectamine and plasmid DNA

Plates	Lipofectamine/well (µg)	Amount of plasmid/well (µg/ml)	DMEM (ml)
12 well	4 µl	1.6 µg	1ml
96 well	0.2 µl	0.2 µg	0.1ml

2.2.13 Dual Luciferase Reporter assay

1. 96-well plates containing HT29 cells were removed from incubator and equilibrated to room temperature.
2. 75µl of Dual-Glo® Luciferase Reagent was added to each well in 96-well plate containing 75 µl of DMEM and mixed well.
3. Firefly luminescence was measured after 20 minutes in Thermo Fisher luminometer.
4. 75µl of 1:100 Dual-Glo® Stop & Glo® Reagent was added to each well.
5. *Renilla* luminescence was measured after 20 minutes in Thermo Fisher luminometer.
6. Ratio of luminescence from experimental reporter to control reporter was calculated.

2.2.14 Gene-expression and pathway analysis

The 12 well plates were taken out of the incubator and total RNA was isolated from the HT29 cells. After cDNA synthesis, the gene expression was investigated by performing qRT-PCR for respective target genes. For the pathway analysis, qRT-PCR was performed for p65 and downstream pro-inflammatory cytokines IL-8 and IL-1β.

RESULTS

OBJECTIVE 1

3.1 To investigate the miRNA profile of inflamed and non-inflamed colonic mucosa of UC patients.

MiRNA signatures during inflamed state are significantly different from the healthy state where they promote the disease pathogenesis by regulating the expression of genes involved in major inflammatory pathways including NFkB pathway or genes crucial for maintaining the membrane integrity. There are ample of evidences to support the altered miRNA expression during IBD as compared to healthy individuals. But the studies reporting the expression of miRNA within the inflamed and non-inflamed colonic mucosa of UC patients are very sparse. Therefore, we investigated the miRNA expression profile within the inflamed and non-inflamed colonic mucosal region of same UC patients.

3.1.1 MiRNA profiling by Microarray

To investigate the disease specific expression profile of miRNA during UC, mucosal pinch biopsies from the inflamed and non-inflamed colonic region of UC patients were collected and total RNA isolated from the biopsy samples was submitted to Affymetrix for quality assessment and microarray profiling. The differentially expressed miRNAs were statistically calculated by one way ANOVA paired method and p value of 0.05 was considered as significant. A fold change of 1.5 was set as cut off for upregulation and -1.5 for down regulation of a specific miRNA.

3.1.2 Generation of heat map with the hierarchical clustering

The two way hierarchical clustering was done for showing the differential expression of microRNA in the samples, represented as heat map **(Figure 17)**. Clustering for samples and miRNA is shown through the dendrogram trees, plotted on the left. Each column represents a sample and each row represents microRNA. The color scale illustrates the relative expression of microRNA, green color represents down regulation and red color represents up regulation. The clustering has helped to identify novel miRNA signatures in distinguishing group of samples like inflamed vs. non-inflamed and also to identify miRNA with similar pattern of expression across samples as shown in the subsequent paragraph.

3.1.3 MiRNA expression in the inflamed *vs* non-inflamed mucosa

The microarray analysis revealed that the miRNA profile of inflamed colonic pockets differs significantly from the non-inflamed pockets in UC patients. We had selected three paired samples for microarray (NI1, I1, NI5, I5, NI6, I6) out of which two pairs (NI1, I1 and NI6, I6) showed significant changes in microRNA expression profile while in the third paired sample (NI5 and I5) we did not observe significant changes between the inflamed and non-inflamed colonic mucosa, which could be probably due to the mild disease activity in the selected patient. Some of the microRNAs showed upregulation with a fold change of more than 10 such as miR-4538, miR-4521, miR-138-5p, miR-708-5p, miR-17-3p, miR-212-3p, miR-424-3p, miR-874-3p, miR-25-5p, miR-148b-3p, miR-501-5p, miR-224-4p and miR-223-3p. MicroRNA such as miR-552-3p, miR-196b-3p, miR-378d-5p, miR-192-5p, miR-215-5p, miR-194-3p, miR-422a, miR-200a, miR-147b, miR-200b-3p, miR-572 and miR-279-5p exhibited significant downregulation in the inflamed colonic mucosa. The top 44 differentially regulated microRNAs have been enlisted in **Table 11** with their respective fold changes. MiR-223, miR-125b, miR-138 and miR-155 showed higher expression in the inflamed colonic mucosa with a fold change of 13.01, 2.56, 35.16 and 2.33 respectively. Similarly, miR-378d, miR-200a and miR-194 showed significant down regulation in the inflamed colonic mucosa with a fold change of -4.06, -2.14 and -2.31 respectively.

3.1.4 Selection of candidate microRNAs

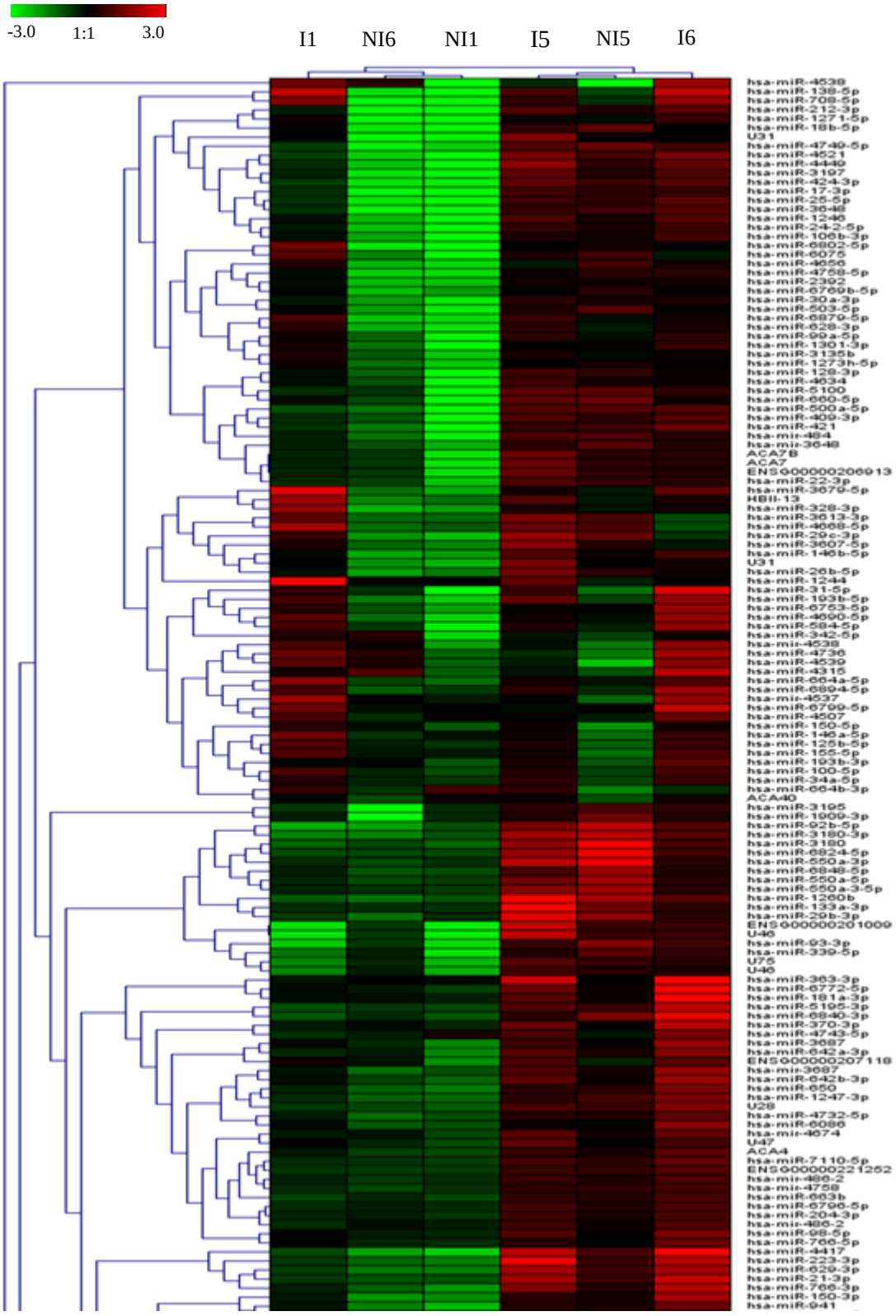
The microarray demonstrated significant alterations in the expression of a panel of miRNAs between the inflamed and non-inflamed colonic mucosa of UC patients as shown in the **Figure 17**. From the microarray findings we selected few candidate miRNAs for further validation through qRT-PCR and subsequently carried out their functional characterization. The basis of selecting candidate miRNA was to look for the miRNAs which have previously shown to be associated with pathogenesis of other inflammatory or autoimmune diseases. Some of these miRNA exhibited altered expression in a previous study conducted in our laboratory where microarray analysis was carried out to compare the miRNA profile of UC patients with non IBD controls (GEO accession number GSE99632). Subsequently, we performed the target prediction

for the selected miRNAs. MiRNAs shown to target the genes involved in major inflammatory pathways were selected as our candidate miRNAs for further investigation. Differential expression of miR-223 was seen in UC and this miRNA also known to regulate the intestinal inflammation **(Iborra, Bernuzzi et al. 2013, Neudecker, Haneklaus et al. 2017)**. MiR-125b and miR-223 both were upregulated in the inflamed mucosa in our microarray analysis and they have been previously shown to get upregulated during UC. MiR-125b is also studied as a link between UC and colorectal cancer suggesting the critical role of this microRNA in disease pathogenesis. MiR-155 was upregulated with a fold change of 2.33 and it is well studied in cancer pathways. MiR-194 and miR-378d were significantly down regulated in microarray. Differential expression of some of these microRNA has been reported in other autoimmune diseases. However, the spatial expression of these microRNAs in the inflamed and non-inflamed mucosa was investigated in our study. Therefore, we selected these microRNAs for further studies. We also included miR-155 keeping in mind its critical role in cancer and other diseases.

Table 11: Top differentially expressed miRNAs in the inflamed colonic mucosa of UC patients as demonstrated by microarray analysis

MicroRNA	F.C*	Expression	MicroRNA	F.C	Expression
hsa-miR-138-5p	35.16	Upregulated	hsa-miR-125b-5p	2.56	Upregulated
hsa-miR-708-5p	34.7	Upregulated	hsa-miR-148a-5p	2.42	Upregulated
hsa-miR-212-3p	24.06	Upregulated	hsa-miR-155-5p	2.33	Upregulated
hsa-miR-4538	26.12	Upregulated	hsa-miR-21-5p	1.81	Upregulated
hsa-miR-4521	17.69	Upregulated	hsa-miR-196b-3p	1.50	Upregulated
hsa-miR-17-3p	15.28	Upregulated	hsa-miR-491-5p	3.92	Upregulated
hsa-miR-424-3p	14.17	Upregulated	hsa-miR-552-3p	-7.82	Downregulated
hsa-miR-874-3p	13.47	Upregulated	hsa-miR-196b-5p	-7.6	Downregulated
hsa-miR-25-5p	13.41	Upregulated	hsa-miR-378d-5p	-4.06	Downregulated
hsa-miR-223-3p	13.01	Upregulated	hsa-miR-141-3p	-3.03	Downregulated
hsa-miR-1271-5p	11.96	Upregulated	hsa-miR-215-5p	-2.56	Downregulated
hsa-miR-148b-3p	11.31	Upregulated	hsa-miR-192-5p	-2.52	Downregulated
hsa-miR-501-5p	11.26	Upregulated	hsa-miR-194-3p	-2.31	Downregulated
hsa-miR-224-3p	10.46	Upregulated	hsa-miR-200a-3p	-2.14	Downregulated
hsa-miR-21-3p	8.46	Upregulated	hsa-miR-378a-3p	-2.04	Downregulated
hsa-miR-146b-5p	6.83	Upregulated	hsa-miR-200b-3p	-1.7	Downregulated
hsa-miR-149-5p	6.24	Upregulated	hsa-miR-4649-5p	-1.61	Downregulated
hsa-miR-31-5p	4.04	Upregulated	hsa-miR-299-5p	-1.59	Downregulated

***F.C denotes fold change**



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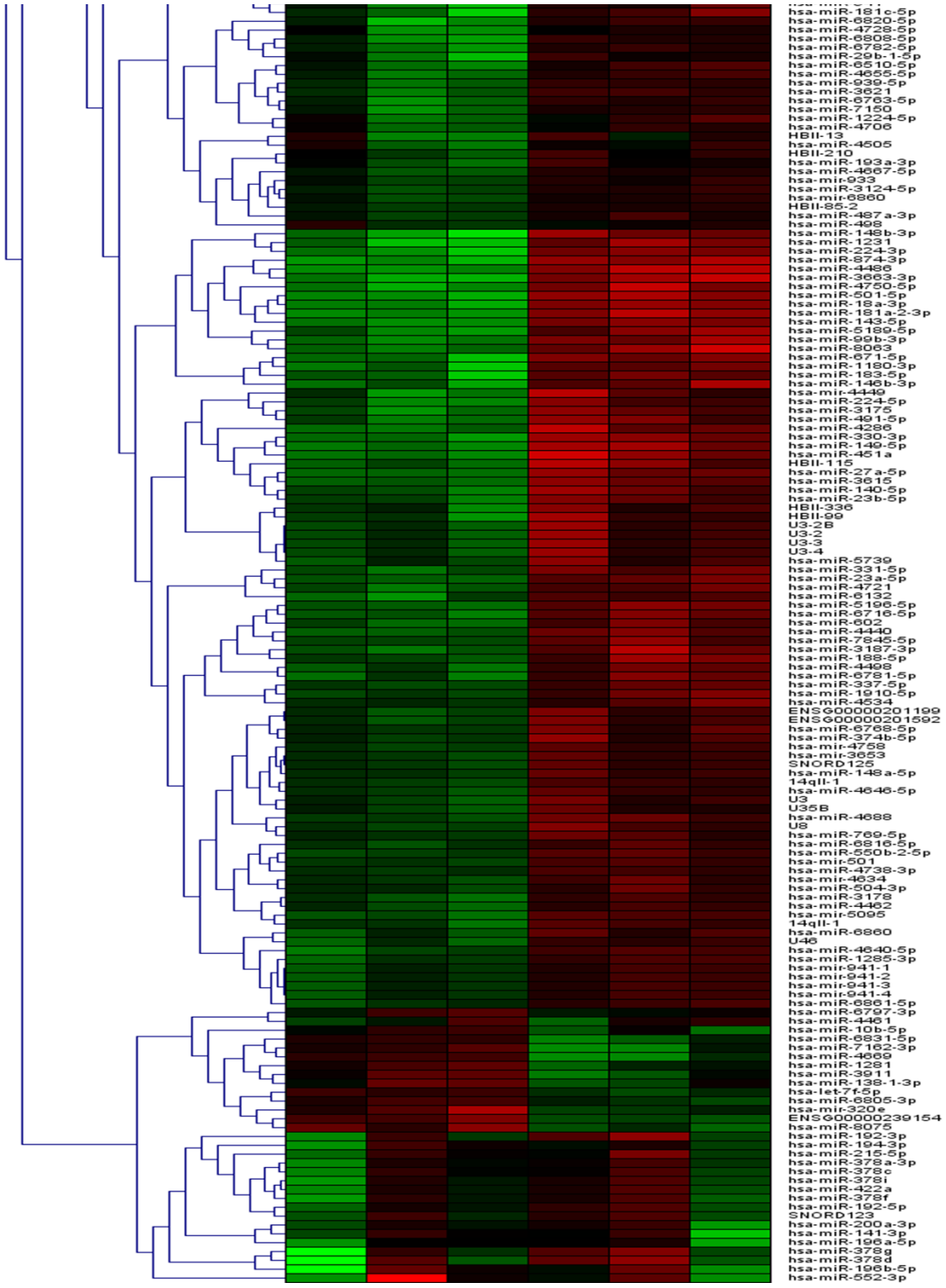


Figure 17: Heat map showing the differential expression of microRNAs in the inflamed and non-inflamed colonic mucosa of UC patients: The microRNA profile in the inflamed colonic mucosa differs significantly from the non-inflamed. I denotes inflamed, NI denotes non-inflamed.

OBJECTIVE 2

3.2 Identification of potential gene targets for miRNA showing altered expression in the inflamed colonic mucosa.

Functional characterization of miRNAs is necessary to understand their contribution in the disease pathogenesis and target prediction is one initial step in miRNA functional characterization. We employed TargetScan, MIRDB, DIANA Tools, PicTar, microRNA.org, for predicting the gene targets and further the involvement of genes in biological pathways was studied with the help of mirPath v.3: DIANA TOOLS. The four basic parameters namely seed conservation, complementarity, site accessibility and free energy are used in different set of combinations by available target prediction tools therefore it is suggested to select the target genes which are picked at least by three bioinformatics tools. A single miRNA can target several genes involved in different signaling pathways but for our study, we chose only those target genes that are involved in inflammatory pathways directly or indirectly.

3.2.1 Potential gene targets for miR-125b as shown by target prediction tools

The potential gene targets for miR-125b as emerged out using the target prediction tools included TRAF6 (TNF receptor associated factor 6), TNFAIP3 (TNF alpha induced protein 3) also referred to as A20, STAT3 (signal transducer and activator of transcription 3), TP53 and IL6R (Interleukin 6 receptor) **(Table 12).** A20 was picked up as a target of miR-125b by three target prediction tools: TargetScan, DIANA-microT-Tarbase, and microrna.org. TargetScan and DIANA microT-CDS revealed an 8 mer site in the 3'UTR of A20 at transcript position 497-504 targeted by miR-125b **(Figure 18).** TRAF6 was also picked up as a target by three prediction tools: TargetScan, DIANA-microT-CDS and microrna.org. It also bears an 8mer target site for miR-125b in its 3'UTR at transcript position 1276-1283 as shown by TargetScan **(Figure 19).** Both TRAF6 and A20 are key molecules involved in the canonical NFkB pathway and a hyperactive NFkB is responsible for the acute inflammation during UC therefore, we selected these two genes as potential targets for miR-125b. STAT3 was picked as a target by three bioinformatics tools TargetScan, MIRDB and DIANA-microT-CDS, bearing 8mer binding site for miR-125b at transcript position 1532-1539 **(Figure 20)**. STAT3 acts as a positive regulator for

Th17 cell differentiation and during UC, an exaggerated Th17 response leading to overproduction of Th17 derived cytokine is observed **(Egwuagu 2009, Galvez 2014)**.

3.2.2 Potential gene targets for miR-155

The putative targets for miR-155 included TRAF3 (TNF receptor associated factor 3), SOCS1 (Suppressor of Cytokine Signaling 1), TAB2 (TGF beta associated kinase 2) and STAT3. Out of which only TRAF3 was picked by three prediction tools: microRNA.org, DIANA-microT-CDS and TargetScan **(Table 13)**. TRAF3 has a 7mer-m8 complementary binding site for miR-155 in its 3'UTR at transcript position 5523-5529 as shown by Target Scan and DIANA-microT-CDS **(Figure 21)**. TRAF3 is involved in the non-canonical NFkB pathway that is mainly dedicated for organogenesis and T cell differentiation, but we still selected it for our study to find out its possible role in inflammation during UC. TAB2, which was picked by TargetScan and MIRDB is an important molecule of canonical NFkB pathway but it was not selected for further analysis as it was picked only by two tools. Similarly, SOCS1 and STAT3 were shown as potential miR-155 target only by single tool therefore, we proceeded further only with TRAF3

3.2.3 Potential gene targets for miR-223

The potential gene targets for miR-223 included TAB3 (TGF beta associated kinase 3), ICAM1 (Intercellular Cell Adhesion Molecule 1) and IKKα (IkB Kinase alpha) and all of these three genes are shown to be involved in intestinal inflammation. ICAM1, a cell adhesion molecule was identified as miR-223 target by DIANA-microT-CDS and microRNA.org while TAB3, a signaling molecule involved in NFkB pathway was identified as miR-223 target only by DIANA-microT-CDS. TargetScan, microRNA.org and DIANA-mirTarBase displayed IKKα as gene target for miR-223 and according to TargetScan, IKKα showed a 7mer-m8 site in its 3' UTR for miR-223 at transcript position 917-923 **(Table 14) (Figure 22)**. We selected IKKα as potential gene target for miR-223, it is generally perceived to play major role in regulating non-canonical NFkB pathway but it is also responsible for resolving the acute inflammatory response by terminating the pro-inflammatory cytokine signaling generated due to prolonged NFkB activation **(Lawrence and Bebien 2007)**.

3.2.4 Potential gene targets for miR-378d

The genes identified as putative targets for miR-378d included TRAF3, IRAK3 (Interleukin-1 receptor associated kinase 3), SOCS2 and ATG2B (Autophagy related 2B) and PTEN (Phosphatase and tensin homolog) **(Table 15).** SOCS2 and ATG2B were picked by a single tool i.e. MIRDB and similarly IRAK3 and PTEN were identified only by DIANA-microT-CDS. Therefore, we selected TRAF3 as a potential miR-378d target as it was identified by more than three prediction tools including TargetScan, microRNA.org, DIANA-microT-CDS, MIRDB. A 8mer site in the 3'UTR of TRAF3 was identified for miR-378d from transcript position 4484-4491 as shown by TargetScan **(Figure 23)**.

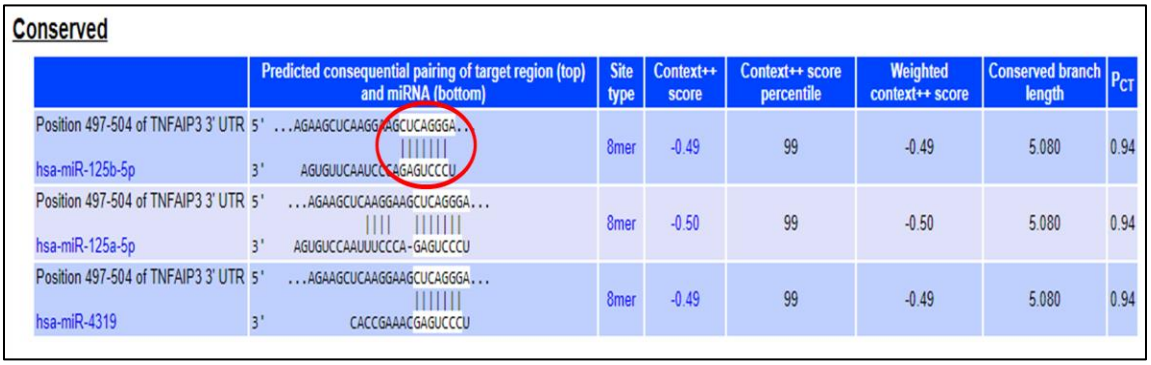
3.2.5 Potential gene targets for miR-194

The putative targets for miR-194 included TRAF3, TRAF6, IRAK3, TAB2 and CXCR3 (Chemokine receptor 3). TRAF3, IRAK3 and CXCR3 were identified as miR-194 targets by a single prediction tool, TargetScan **(Table 16)**. TAB2 and TRAF6 both are key signaling molecules of NFkB pathway and were picked by two prediction tools (TAB2 by TargetScan, DIANA-microT-CDS and TRAF6 by TargetScan, MIRDB) **(Figure 24)**. Although TRAF6 was identified as a target of miR-194 by only two prediction tools we still selected it for further validation because TRAF6 bear binding sites for both miR-125b and miR-194 at different positions in its 3' UTR. Therefore, we were interested in finding out if miR-194 targets TRAF6.

Table 12: Target genes identified for miR-125b by different prediction tools.

Target gene	Prediction tool	Biological function
TRAF6	TargetScan, DIANA-MICROT, mirna.org	Adapter molecule involved in NFkB pathway
TNFAIP3	TargetScan, MirTarBase, mirna.org	Negative feedback in NFkB pathway
STAT3	TargetScan, , DIANA-microT-CDS, MIRDB	Promotes Th17 differentiation
IL6R	TargetScan, MIRDB	Cell growth and differentiation
TP53	DIANA-microT-CDS	Tumor suppressor gene

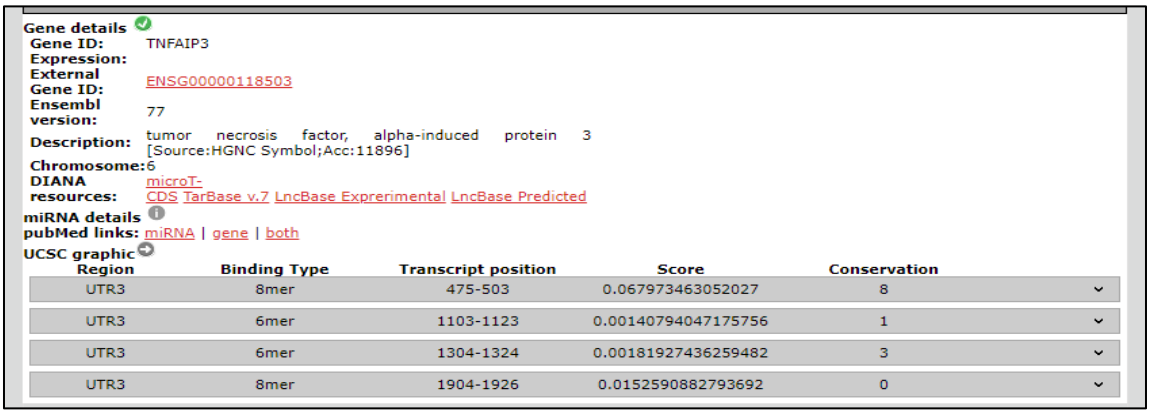
(a)



Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 497-504 of TNFAIP3 3' UTR hsa-miR-125b-5p	5' ...AGAAGCUCAAGGAAGCUCAGGGA... 3' AGUGUUCAAUCCCAGAGUCCCU	8mer	-0.49	99	-0.49	5.080	0.94
Position 497-504 of TNFAIP3 3' UTR hsa-miR-125a-5p	5' ...AGAAGCUCAAGGAAGCUCAGGGA... 3' AGUGUCCAAUUUCCCA-GAGUCCCU	8mer	-0.50	99	-0.50	5.080	0.94
Position 497-504 of TNFAIP3 3' UTR hsa-miR-4319	5' ...AGAAGCUCAAGGAAGCUCAGGGA... 3' CACCGAAACGAGUCCCU	8mer	-0.49	99	-0.49	5.080	0.94

(b)

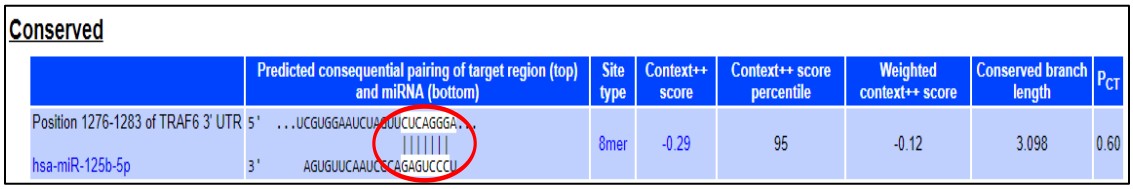


Gene details
Gene ID: TNFAIP3
Expression:
External Gene ID: ENSG00000118503
Ensembl version: 77
Description: tumor necrosis factor, alpha-induced protein 3 [Source:HGNC Symbol;Acc:11896]
Chromosome: 6
DIANA resources: microT-CDS TarBase v.7 LncBase Exprerimental LncBase Predicted
miRNA details
pubMed links: miRNA | gene | both
UCSC graphic

Region	Binding Type	Transcript position	Score	Conservation
UTR3	8mer	475-503	0.067973463052027	8
UTR3	6mer	1103-1123	0.00140794047175756	1
UTR3	6mer	1304-1324	0.00181927436259482	3
UTR3	8mer	1904-1926	0.0152590882793692	0

Figure 18: Binding sites in 3′UTR of A20 for miR-125b. a) 3′UTR of A20 contain binding sites for miR-125b-5p at position 497-504 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000237289.4&taxid=9606&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1#miR-125-5p) b) miR-125b binding sites within the 3'UTR of A20 were also shown by DIANA-microTCDS.(http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microT_CDS/results&keywords=ENSG00000118503&genes=ENSG00000118503%20&mirnas=&descr=&threshold=0.7).

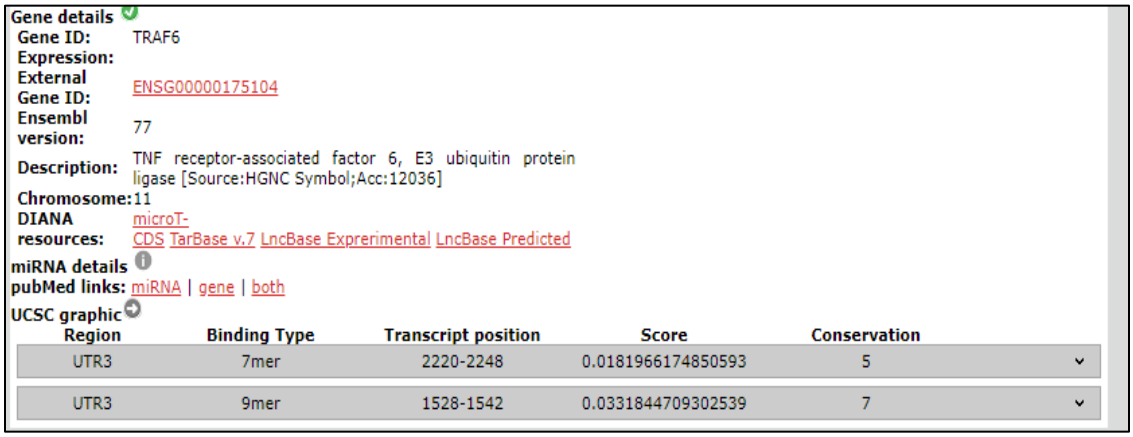
(a)



Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 1276-1283 of TRAF6 3' UTR hsa-miR-125b-5p	5' ...UCGUGGAAUCUAGUUCUCAGGGA... 3' AGUGUUCAAUCCCAGAGUCCCU	8mer	-0.29	95	-0.12	3.098	0.60

(b)

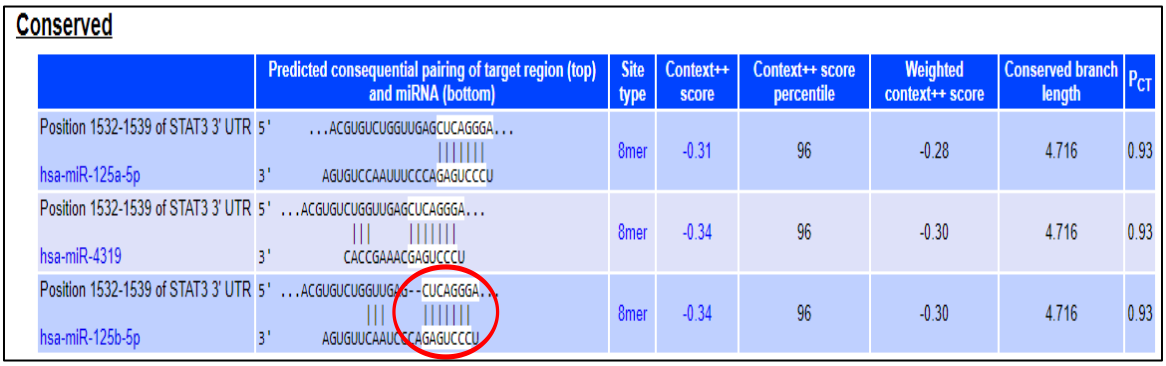


Gene details

Gene ID: TRAF6
Expression:
External Gene ID: ENSG00000175104
Ensembl version: 77
Description: TNF receptor-associated factor 6, E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:12036]
Chromosome: 11
DIANA resources: microT-CDS TarBase v.7 LncBase Exprerimental LncBase Predicted
miRNA details
pubMed links: miRNA | gene | both
UCSC graphic

Region	Binding Type	Transcript position	Score	Conservation
UTR3	7mer	2220-2248	0.0181966174850593	5
UTR3	9mer	1528-1542	0.0331844709302539	7

Figure 19: Binding sites in 3′UTR of TRAF6 for miR-125b. a) 3′UTR of TRAF6 contains binding sites for miR-125b-5p at position 1276-1283 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000526995.1&taxid=9606&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1#miR-125-5p) b) miR-125b binding sites within the 3'UTR of A20 were also shown by DIANA-microT-CDS.(http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microT_CDS/results&keywords=ENSG00000175104&genes=ENSG00000175104%20&mirnas=&descr=&threshold=0.7)



Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 1532-1539 of STAT3 3' UTR hsa-miR-125a-5p	5' ...ACGUGUCUGGUUGAGCUCAGGGA... 3' AGUGUCCAAUUUCCCAGAGUCCCU	8mer	-0.31	96	-0.28	4.716	0.93
Position 1532-1539 of STAT3 3' UTR hsa-miR-4319	5' ...ACGUGUCUGGUUGAGCUCAGGGA... 3' CACCGAAACGAGUCCCU	8mer	-0.34	96	-0.30	4.716	0.93
Position 1532-1539 of STAT3 3' UTR hsa-miR-125b-5p	5' ...ACGUGUCUGGUUGAG--CUCAGGGA... 3' AGUGUUCAAUCCCAGAGUCCCU	8mer	-0.34	96	-0.30	4.716	0.93

Figure 20: Binding sites in 3′UTR of STAT3 for miR-125b. 3′UTR of STAT3 contains binding sites for miR-125b-5p at position 1532-1539 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000585517.1&taxid=9606&members=&showcnc=0&shownc=0&showncf1=&showncf2=&subset=1#miR-125-5p)

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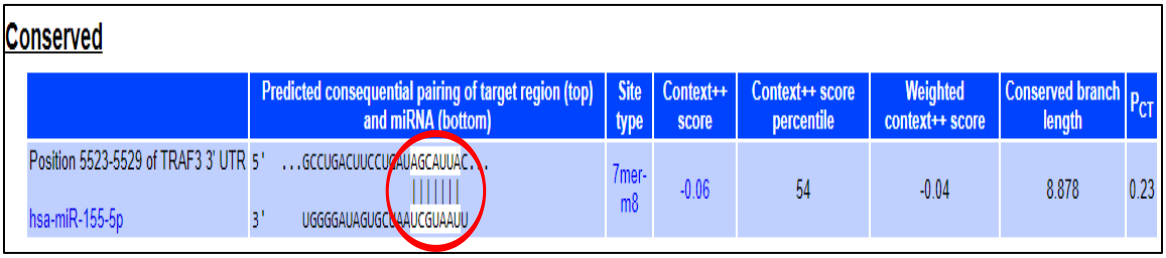
Table 13: Genes identified as potential gene targets for miR-155

Target gene	Prediction tool	Biological function
TRAF3	microRNA.org, DIANA-microT-CDS, TargetScan	Signaling molecule in non- canonical NFkB pathway
TAB2	TargetScan, MIRDB	Signaling molecules in NFkB pathway
STAT3	DIANA-Tarbase	Th17 cell differentiation
SOCS1	TargetScan	Negative feedback in receptor cytokine signaling

(a)



(b)

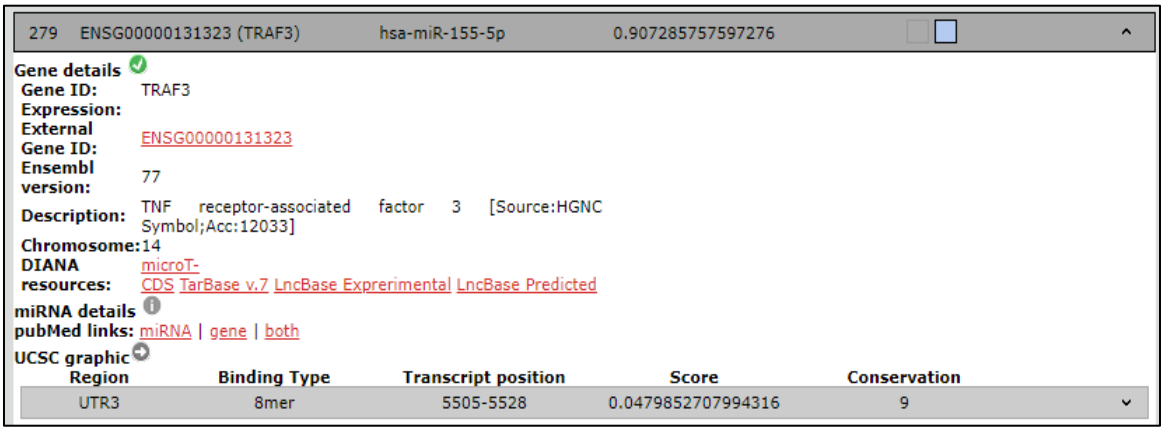
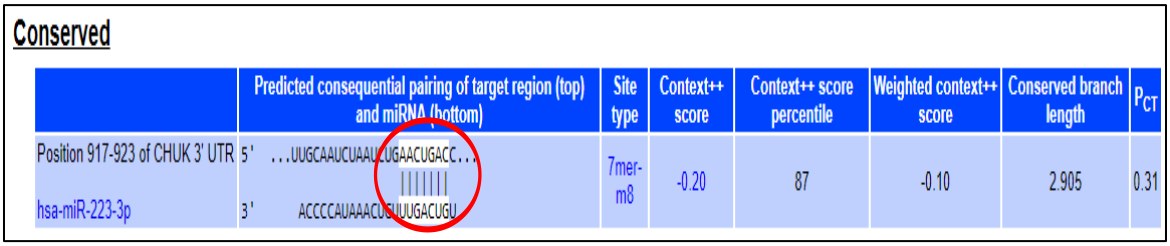


Figure 21: Binding sites in 3′UTR of TRAF3 for miR-155. a) 3′UTR of TRAF3 contains a 7mer-m8 binding sites for miR-155-5p at position 5523-5529 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000560371.1&taxid=9606&members=miR-155-5p&showcnc=0&shownc=0&subset=1) b) miR-155 binding sites within the 3'UTR of TRAF3 was also shown by DIANA-microT-CDS. (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/results&keywords=ENSG00000131323&genes=ENSG00000131323%20&mirnas=&descr=&threshold=0.7&page=2)

Table 14: Genes identified as potential targtes for miR-223

Target gene	Prediction tool	Biological Function
TAB3	DIANA-microT-CDS	Signaling molecule in NFkB pathway
IKKα	TargetScan, DIANA TOOLS-mirTarbase, microRNA.org	Negative feedback in canonical NFkB pathway
ICAM1	DIANA-microT-CDS, microRNA.org	Cell adhesion molecule

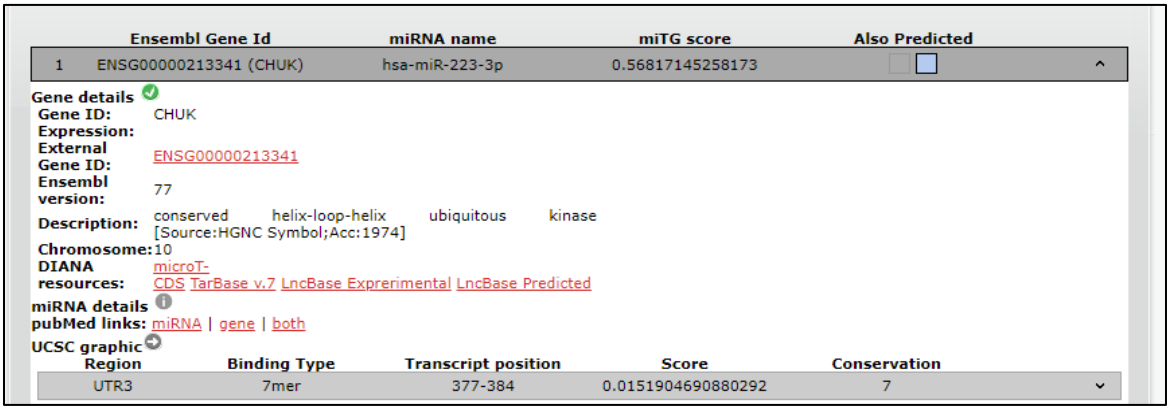
(a)



Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 917-923 of CHUK 3' UTR hsa-miR-223-3p	5' ...UUGCAAUCUAAUCUGAACUGACC... 3' ACCCCAUAAACUGUUUGACUGU	7mer-m8	-0.20	87	-0.10	2.905	0.31

(b)



	Ensembl Gene Id	miRNA name	miTG score	Also Predicted
1	ENSG00000213341 (CHUK)	hsa-miR-223-3p	0.56817145258173	

Gene details
Gene ID: CHUK
Expression:
External Gene ID: ENSG00000213341
Ensembl version: 77
Description: conserved helix-loop-helix ubiquitous kinase [Source:HGNC Symbol;Acc:1974]
Chromosome: 10
DIANA resources: microT-CDS TarBase v.7 LncBase Exprerimental LncBase Predicted
miRNA details
pubMed links: miRNA | gene | both
UCSC graphic

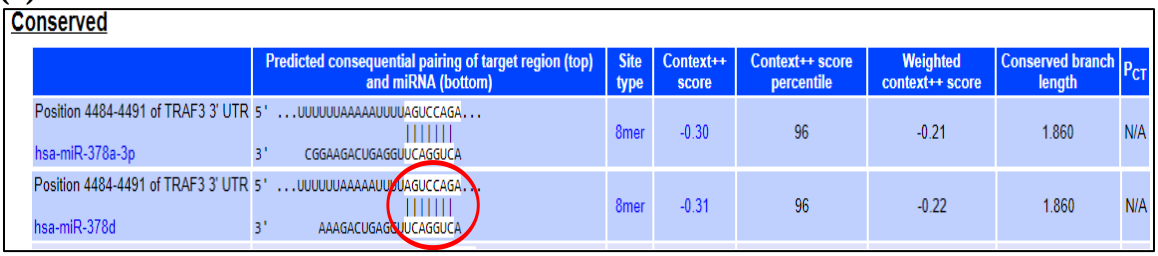
Region	Binding Type	Transcript position	Score	Conservation
UTR3	7mer	377-384	0.0151904690880292	7

Figure 22: Binding sites in 3′UTR of IKKα/CHUK for miR-223. a) 3′UTR of IKKα contains (a) 7mer-m8 binding sites for miR-223 at position 917-923 as shown by target scan. (b) miR-223 binding site within the 3'UTR of IKKα was also shown by DIANA-microT-CDS.

Table 15: Genes identified as potential targets for miR-378d

Target gene	Prediction tool	Biological function
TRAF3	microRNA.org, DIANA-microT-CDS, MIRDB, TargteScan	Signaling molecule in non canonical NFkB pathway
IRAK3	DIANA-microT-CDS	Negative regulator of TLR signaling
PTEN	DIANA-microT-CDS	Indirectly involved in inflammation
SOCS2	MIRDB	Negative regulator of receptor signaling
ATG2B	MIRDB	Formation of autophagosomes

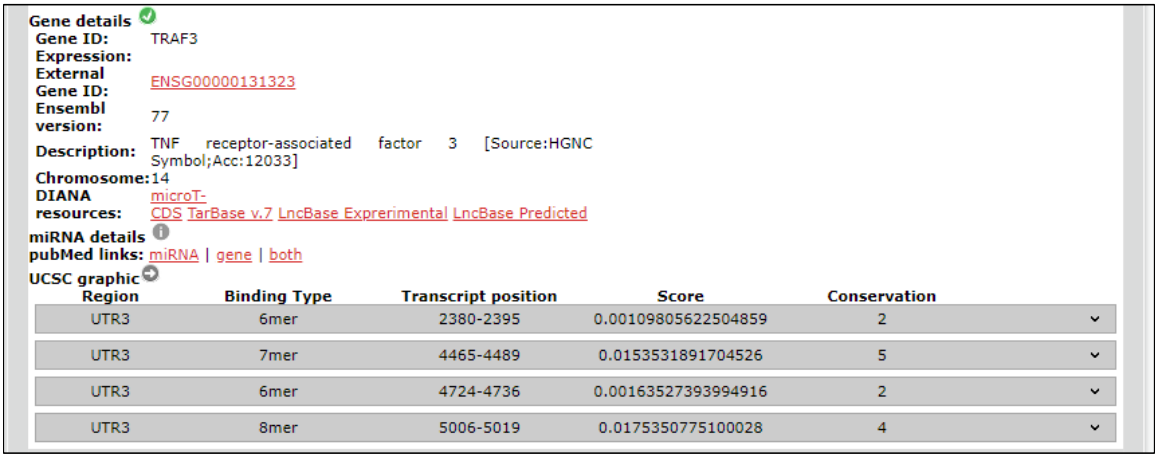
(a)



Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 4484-4491 of TRAF3 3' UTR hsa-miR-378a-3p	5' ...UUUUUUAAAAAUUUUAGUCCAGA... 3' CGGAAGACUGAGGUUCAGGUCA	8mer	-0.30	96	-0.21	1.860	N/A
Position 4484-4491 of TRAF3 3' UTR hsa-miR-378d	5' ...UUUUUUAAAAAUUUUAGUCCAGA... 3' AAAGACUGAGGUUCAGGUCA	8mer	-0.31	96	-0.22	1.860	N/A

(b)



Gene details
Gene ID: TRAF3
Expression:
External Gene ID: ENSG00000131323
Ensembl version: 77
Description: TNF receptor-associated factor 3 [Source:HGNC Symbol;Acc:12033]
Chromosome: 14
DIANA resources: microT-CDS TarBase v.7 LncBase Exprerimental LncBase Predicted
miRNA details
pubMed links: miRNA | gene | both
UCSC graphic

Region	Binding Type	Transcript position	Score	Conservation
UTR3	6mer	2380-2395	0.00109805622504859	2
UTR3	7mer	4465-4489	0.0153531891704526	5
UTR3	6mer	4724-4736	0.00163527393994916	2
UTR3	8mer	5006-5019	0.0175350775100028	4

Figure 23: Binding sites in 3′UTR of TRAF3 for miR-378d. a) 3′UTR of TRAF3 contains a 8mer binding site for miR-378d at position 4484-4491 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000560371.1&taxid=9606&members=miR-378-3p&showcnc=1&shownc=1&subset=1) b) miR-378d binding site within the 3'UTR of TRAF3 was also shown by DIANA-microT-CDS.(http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microT_CDS/results&keywords=ENSG00000131323&genes=ENSG00000131323%20&mirnas=&descr=&threshold=0.7&page=3)

Table 16: Potential gene targets emerged from Bioinformatic anaysis for miR-194

Target gene	Prediction tool	Biological function
TRAF6	TargetScan, MIRDB	Adaptor molecule in NFkB pathway
TAB2	TargetScan, DIANA-microT-CDS	Signaling molecule of NFkB pathway
CXCR3	TargetScan	Chemokine receptor
IRAK3, TRAF3	TargetScan	Signaling molecule of NFkB pathway

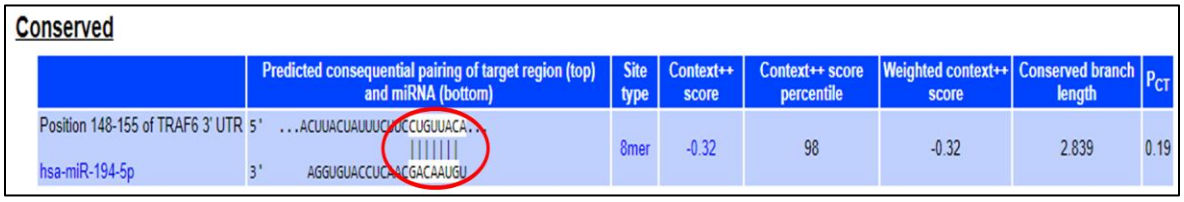


Figure 24: Binding sites in 3′UTR of TRAF6 for miR-194. a) 3′UTR of TRAF6 contains a 8mer binding site for miR-194 at position 148-155 as shown by target scan. (http://www.targetscan.org/cgibin/targetscan/vert_72/view_gene.cgi?rs=ENST00000526995.1&taxid=9606&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1#miR-194-5p)

OBJECTIVE 3

3.3 To evaluate the expression of dysregulated miRNAs and their respective gene targets in UC patients.

The altered miRNA expression obtained from microarray data was further validated by performing qRT-PCR with large number of samples. We carried out qRT-PCR to validate the differential expression of miR-125b, miR-223, miR-155, miR-194, miR-378d and their respective target genes. qRT-PCR for miRNAs was carried out in two categories i) UC vs Control and ii) inflamed vs non-inflamed colonic mucosa. For UC vs controls, we included 20 healthy controls and 30 UC patients and for the inflamed vs non-inflamed analysis we included eight paired samples.

3.3.1 Expression of miR-125b and its potential target genes

The qRT-PCR analysis showed a significant upregulation in the expression of miR-125b in UC patients as compared to non-IBD controls. We observed a 9.85 folds higher expression in UC patients as compared to the non-IBD controls (p=0.004). Similarly in the paired samples, we observed a significantly higher expression of miR-125b in the inflamed colonic mucosa as compared to the non-inflamed region (p=0.004) and non IBD controls (p=0.0001). These findings validated our microarray results. We did not observe significant expression change between non-IBD control and non-inflamed mucosa of UC patients for miR-125b (p=0.165) **(Figure 25).** We also investigated the expression of TRAF6 and STAT3 that are potential targets of miR-125b. TRAF6, a key signaling molecule in the canonical NFkB pathway, exhibited a significant downregulation with a fold change of 0.35 in UC patients as compared to non IBD controls (p=0.03) showing an inverse relation with the miR-125b expression **(Figure 26).** We observed a downregulation in the expression of STAT3 in UC patients with 0.25 fold change as compared to no IBD controls but the change was not significant (p=0.62) **(Figure 26)**. We did not perform qRT-PCR for A20 as it has already been investigated previously with the same set of samples in our laboratory where we found a decreased A20 expression in UC patients at the protein level **(Majumdar, Ahuja et al. 2017)**.

3.3.2 Expression of miR-155 and its potential target gene TRAF3

In the qRT-PCR analysis, miR-155 showed significant upregulation in the UC patients with a fold change of 23.89 as compared to non-IBD controls (p=0.006). In the paired samples also, we found significantly higher expression of miR-155 in the inflamed colonic mucosa as compared to non-inflamed mucosa of UC patients (p=0.028) and non-IBD controls. These findings validated our microarray results **(Figure 27)**. The expression levels were similar in the control and non-inflamed regions with no significant change (p=0.75). The target gene of miR-155 i.e. TRAF3 also showed significant upregulation in its expression in the UC patients as compared to non IBD controls (p=0.039) with a fold change of 1.47 indicating that it may not be targeted by miR-155 during UC rather it is targeted by some other miRNA **(Figure 28)**.

3.3.3 Expression of miR-223 and its potential target gene IKK alpha

In accordance with the microarray results, miR-223 showed a significant upregulation in its expression in UC patients as compared to the non-IBD controls (fold change 8.63) in the qRT-PCR analysis (p=0.012). Interestingly, in the paired samples, miR-223 showed a significant upregulation in inflamed as well as in the non-inflamed colonic regions of UC patients when compared to non-IBD controls (p=0.023). The non-inflamed samples also exhibited significantly higher miR-223 levels as compared to non IBD control samples (p=0.003) which indicates that miR-223 upregulation is independent of inflammation **(Figure 29)**. IKKα, a potential target for miR-223 showed a significant 0.40 folds down regulation in the UC patients as compared to non-IBD controls. This proves an inverse correlation of IKKα expression with the increased expression of miR-223 (p=0.014) **(Figure 30).**

3.3.4 Expression of miR-194 and its potential target gene TRAF6

In the qRT-PCR analysis, miR-194 expression was found to be upregulated in the UC patients with respect to the non-IBD controls, whereas the microarray data exhibited significant downregulation in the inflamed colonic mucosa of UC patients. Although in real time analysis we observed an upregulation (2.40 folds) however this change was not significant (p=0.20). Expression of TRAF6 targeted by miR-194 was significantly downregulated (0.35 folds) in UC patients as compared to healthy controls (p=0.027)

(Figure 31). We did observe an inverse relation between microRNA and target gene expression but the expression results for miR-194 were not conclusive as we obtained different trend in microarray and qRT-PCR.

3.3.5 Expression of miR-378d and its potential target gene TRAF3

Microarray demonstrated a significant downregulation in miR-378d expression while in qRT-PCR analysis, a significant (13.81 folds) higher expression was observed in UC patients in comparison to controls (p=0.020). In addition to this we found an increased miR-378d expression in the inflamed colonic mucosa as compared to non-inflamed regions of UC patients. We did not observe significant changes in the miR-378d expression levels between control and non-inflamed colonic mucosa of UC patients **(Figure 32)**. TRAF3, the putative target gene for miR-378d also exhibited significant upregulation in UC patients as compared to non-IBD controls (p=0.039), showing no inverse correlation with miR-378d expression and also suggesting that TRAF3 is not targeted by miR-378d during UC **(Figure 33)**.

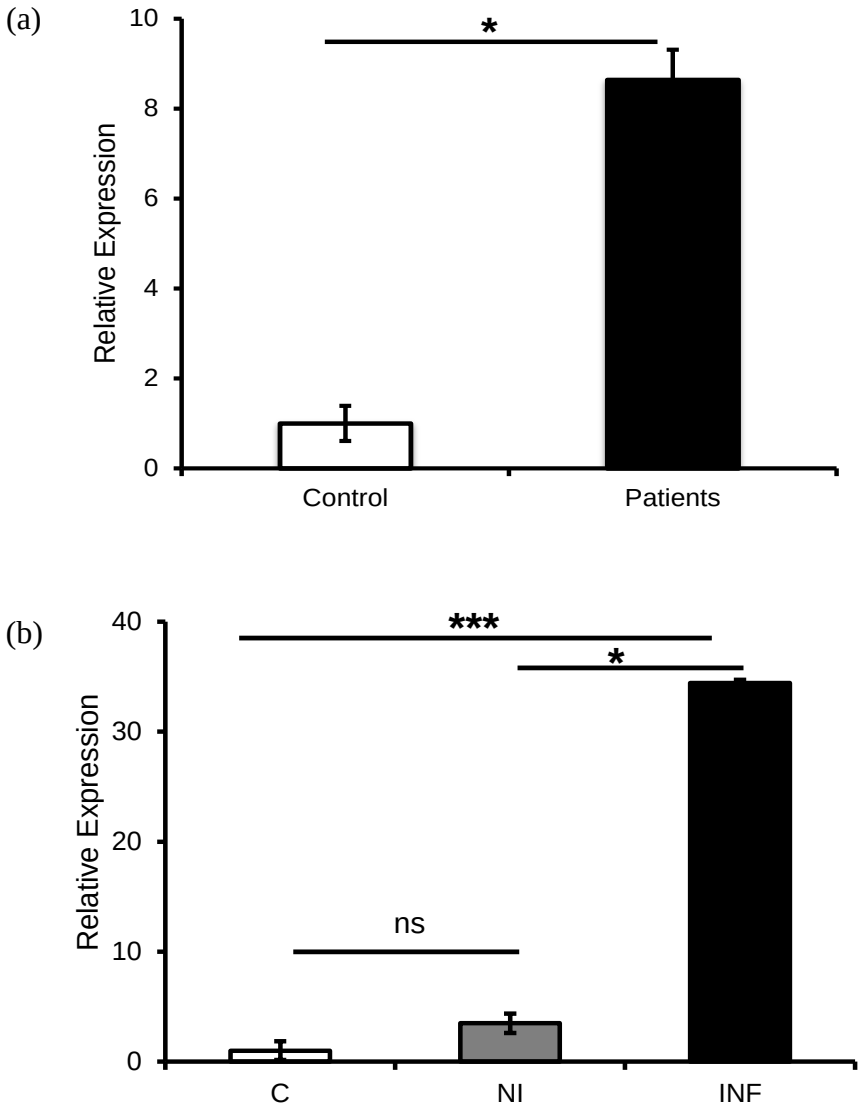


Figure 25: Relative expression of miR-125b in UC patients: qRT-PCR for miR-125b was performed in UC vs control (n=20 for controls and n=30 for patients) and for inflamed vs non-inflamed regions of UC patients (n=8). a) miR-125b exhibited significantly higher expression in UC patients as compared to controls with a change of around 9.85 folds b) miR-125b expression levels were significantly higher in the inflamed colonic mucosa as compared to non-inflamed and controls. U6 was used as internal reference. NI denotes non-inflamed, INF denotes inflamed. *denotes the level of significance. *$p<0.05$, ***$p<0.001$. NS-Not Significant. Bars represent Standard Error (SEM).

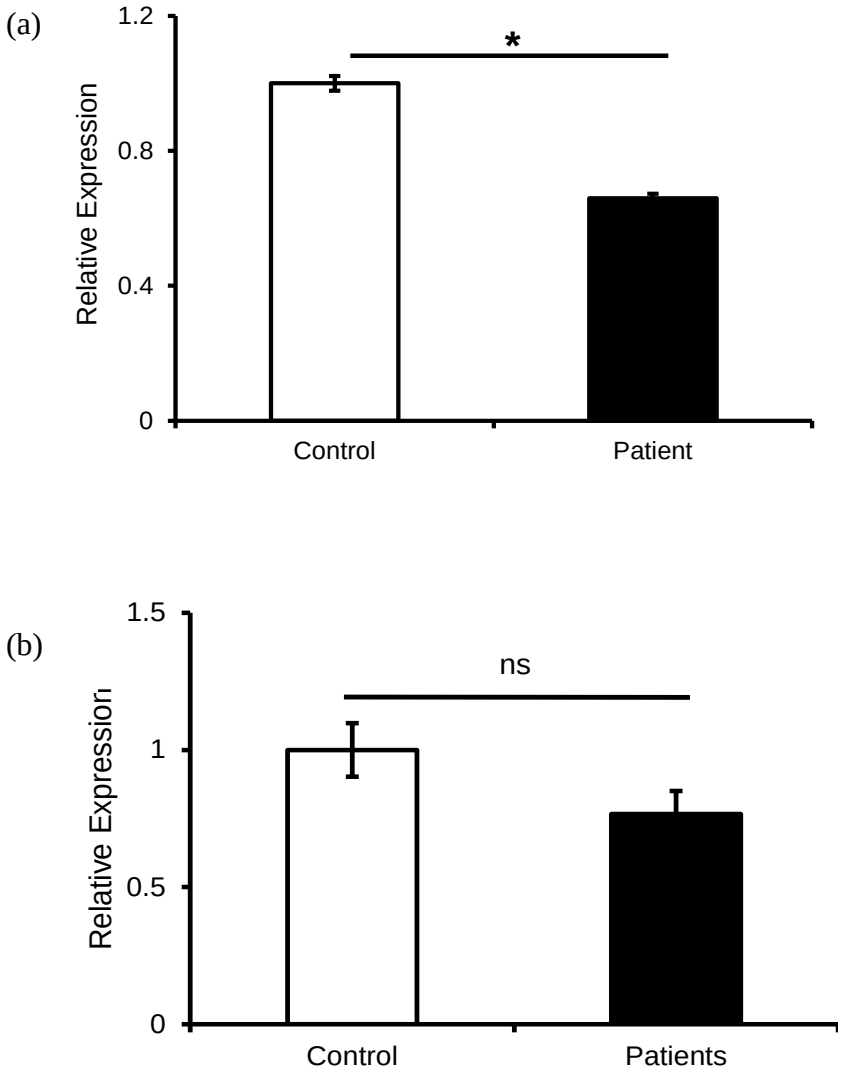


Figure 26: Relative expression of miR-125b target genes in UC patients: qRT-PCR for TRAF6 and STAT3 was performed in 20 controls and 30 patients (a)TRAF6 exhibited significantly lower expression in UC patients as compared to controls (b)STAT3 did not show significant changes in expression in UC patients as compared to controls. GAPDH was used as internal reference. * denotes the level of significance. *$p<0.05$. Bars represent Standard Error (SEM). ns denotes Not Significant.

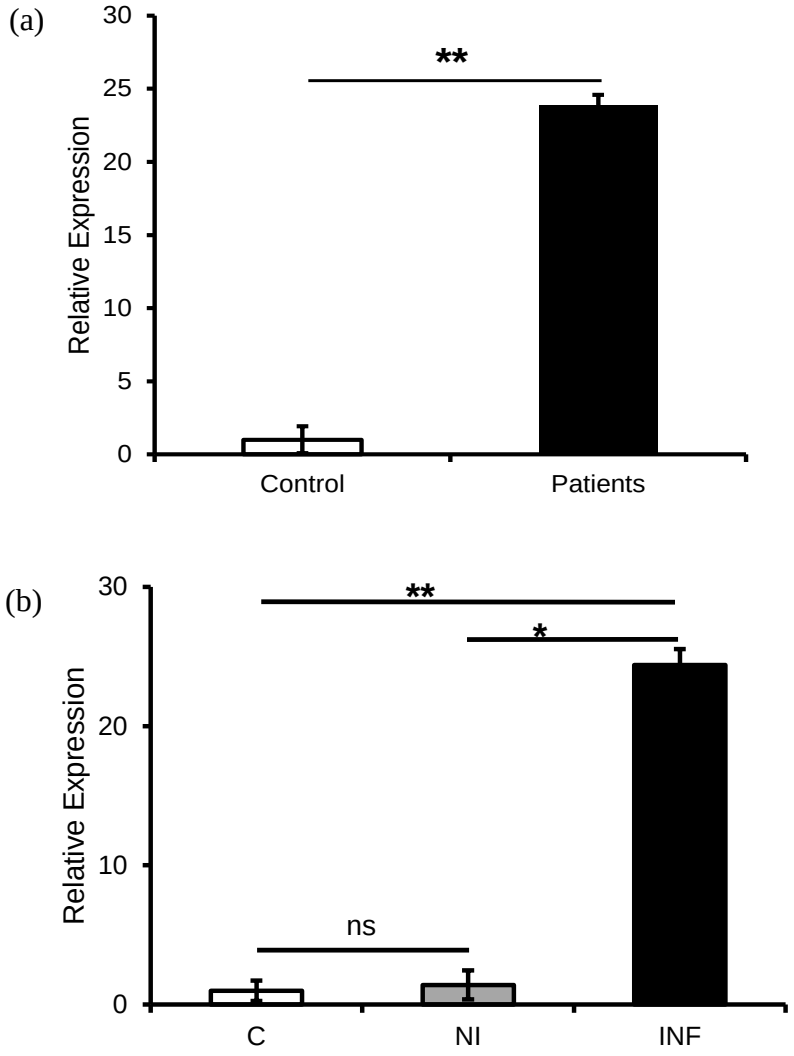


Figure 27: Relative expression of miR-155: qRT-PCR for miR-155 was performed in UC vs control (n-20 for controls and n=30 for patients) and for inflamed vs non-inflamed regions of UC patients (n=8). U6 was used as internal reference. a) miR-155 showed significantly higher expression in UC patients as compared to controls with a change more than 20. (b) miR-155 expression levels were significantly higher in the inflamed colonic mucosa as compared to non-inflamed and controls. NI denotes non-inflamed, INF denotes inflamed. * denotes the level of significance. *p<0.05, **p<0.01. Bars represent Standard Error (SEM). ns denotes Not Significant.

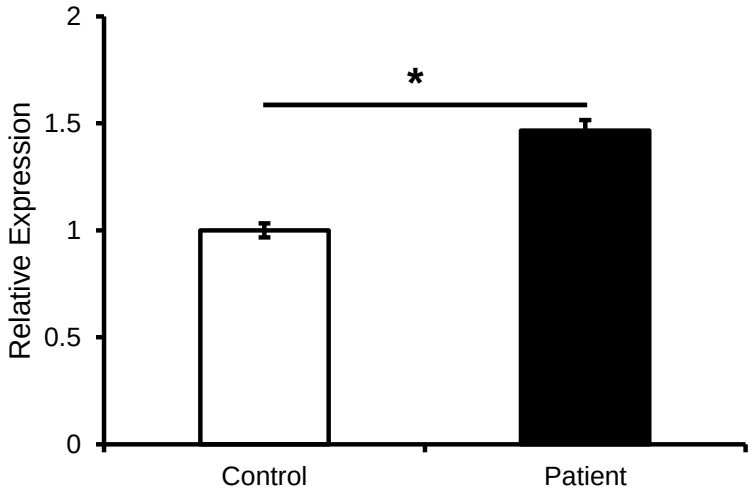


Figure 28: Relative expression of TRAF3 in UC patients: qRT-PCR for TRAF3 was performed in 20 controls and 30 patients. TRAF3 exhibited significant 1.47 folds upregulation in UC patients as compared to non IBD controls. GAPDH gene was used as internal reference. * denotes the level of significance. *$p<0.05$. Bars represent Standard Error (SEM).

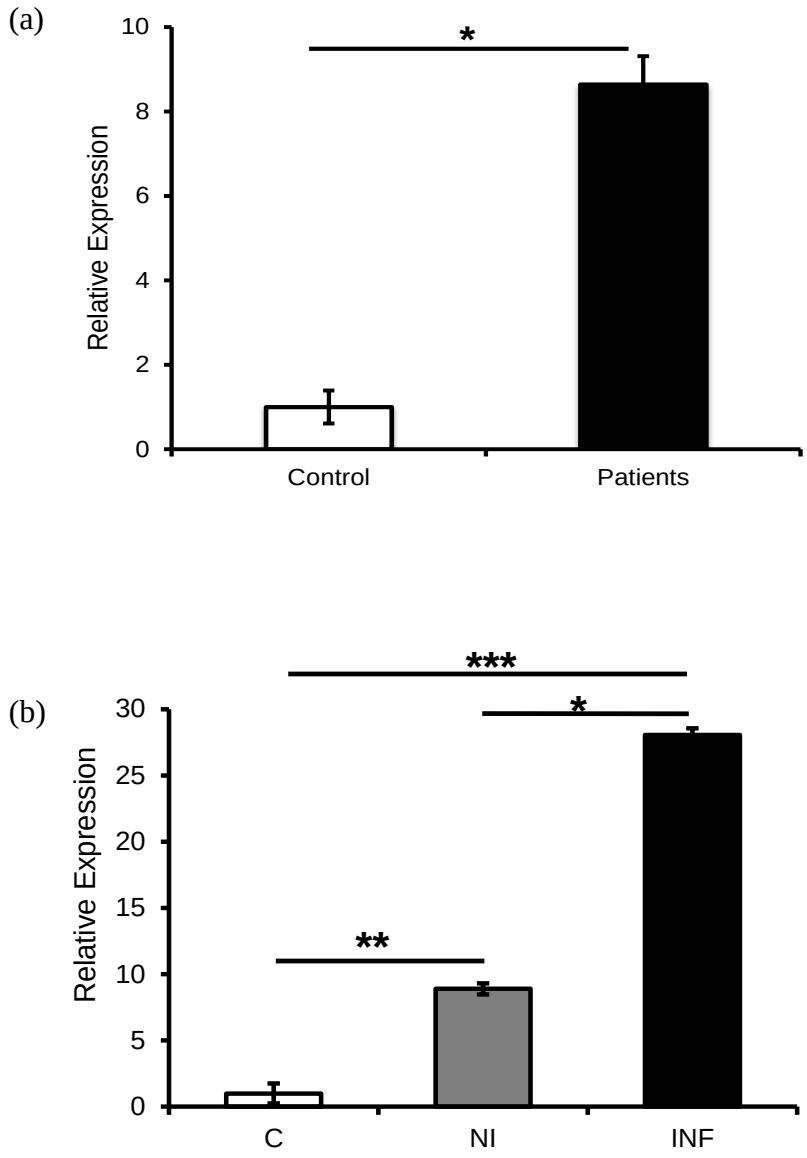


Figure 29: Relative expression of miR-223 in patients and controls: qRT-PCR for miR-223 was performed in UC vs control (n-20 for controls and n=30 for patients) and for inflamed vs non-inflamed regions of UC patients (n=8). U6 was used as internal reference. a) miR-223 showed significantly higher expression in UC patients as compared to controls b) miR-223 expression levels were significantly higher in the inflamed as well as in non-inflamed mucosa as compared to controls. C denotes control, NI denotes non-inflamed, INF denotes inflamed. NS denotes Not Significant. * denotes the level of significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Bars represent Standard Error (SEM).

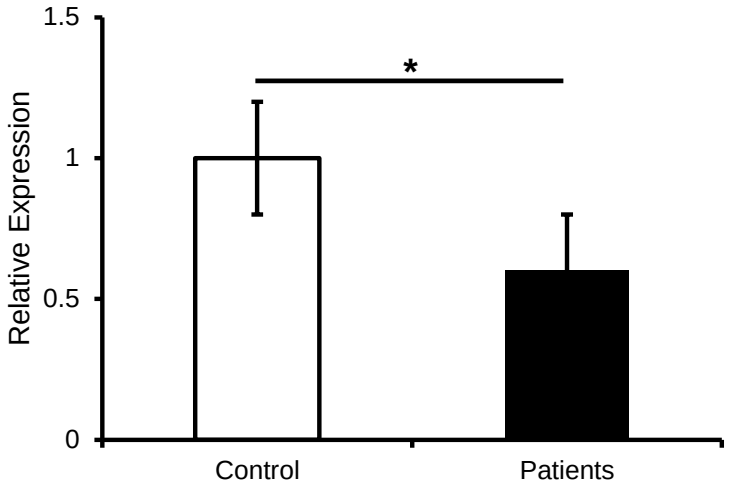


Figure 30: Comparison of expression IKKα expression in patients and controls: qRT-PCR for IKKα was performed in 20 controls and 30 patients. GAPDH gene was used as internal reference. IKKα showed significant downregulation in UC patients as compared to controls * denotes the level of significance. *p<0.05. Bars represent Standard Error (SEM).

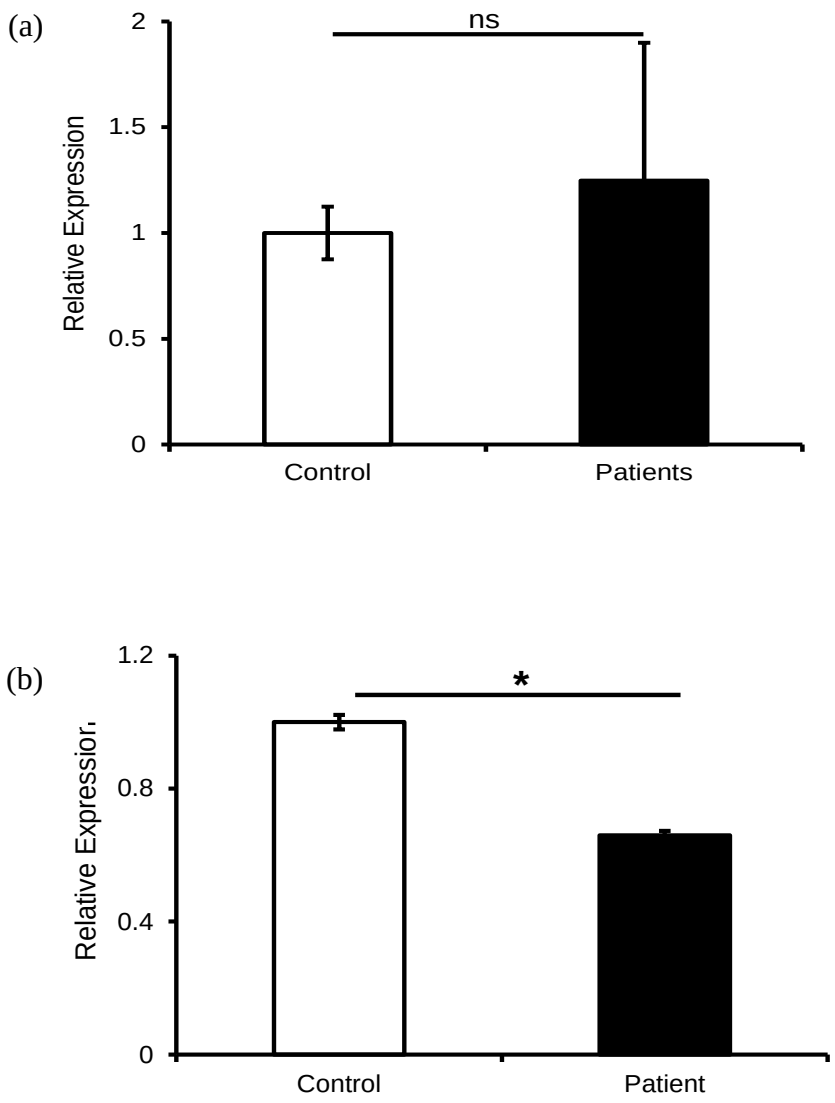


Figure 31: Relative expression of miR-194 and TRAF6 in UC patients: qRT-PCR for miR-194 and its target gene TRAF6 was performed in UC vs control (n-20 for controls and n=30 for patients). a) miR-194 showed upregulation in UC patients as compared to non IBD controls but the change was not significant. U6 was used as internal reference for miR-194. b) TRAF6 expression was significantly upregulated in UC patients. GAPDH was used as internal reference gene for TRAF6. * denotes the level of significance. *p<0.05. NS denotes Not Significant. Bars represent Standard Error (SEM).

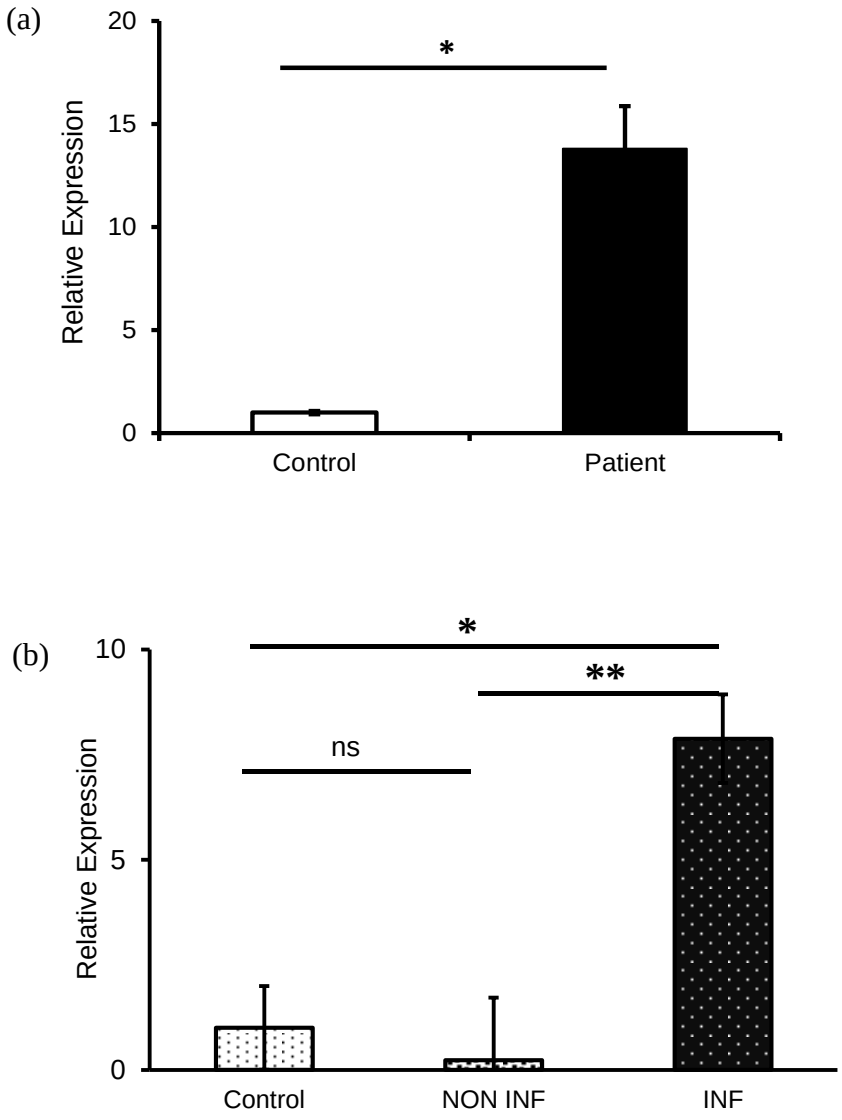


Figure 32: Relative expression of miR-378d in patients and controls: qRT-PCR for miR-378d was performed in UC vs control (n-20 for controls and n=30 for patients) and for inflamed vs non-inflamed regions of UC patients (n=8). U6 was used as internal reference. a) miR-378d showed significantly higher expression in UC patients as compared to controls b) miR-378d expression levels were significantly higher in the inflamed regions as compared to non-inflamed mucosa and controls. C denotes control, NI denotes non-inflamed, INF denotes inflamed. NS denotes Not Significant. * denotes the level of significance. *$p<0.05$, **$p<0.01$. Bars represent Standard Error (SEM).

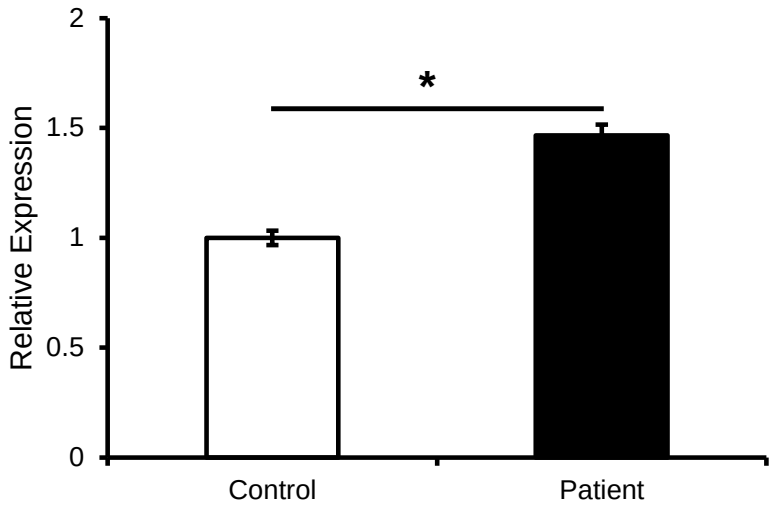


Figure 33: Comparison of expression TRAF3 expression in patients and controls: qRT-PCR for TRAF3 was performed in 20 controls and 30 patients. GAPDH gene was used as internal reference. TRAF3 exhibited significant upregulation in UC patients as compared to controls. * denotes the level of significance. *$p<0.05$. Bars represent Standard Error (SEM).

OBJECTIVE 4

3.4 Functional analysis of dysregulated microRNA through in vitro studies.

In order to carry out the functional analysis of selected microRNAs which were dysregulated in disease condition, in vitro studies were carried out. We selected miR-125b and miR-223 with their respective gene targets because these two miRNAs showed significant upregulation in UC vs. controls and also in the inflamed vs non-inflamed colonic mucosa of UC patients when expression analysis were carried out both by microarray and qRT-PCR analysis of the samples. Additionally, the target genes of the above miRNAs, TRAF3 and IKKα are known to play crucial roles in inflammatory signaling pathway. Both of these genes exhibited down regulation in UC patients showing an inverse correlation with miR-125b and miR-223 expressions respectively. Therefore miR-125b with TRAF6 and A20; and miR-223 with IKKα were selected for the validation of mRNA:miRNA interaction through *in vitro* studies.

3.4.1 Cloning and mutant generation for miR-125b and miR-223

In order to carry out functional analysis, we cloned a 401 bp fragment containing miR-125b coding sequence with its flanking region in pBABE-puro vector. The pBABE-puro vector contains the bacterial origin of replication, ampicillin-resistance gene, and puromycin resistance gene for the growth of infected mammalian cells. The presence of miR-125b sequence in plasmid was confirmed by colony PCR. A single desired band of 401bp was obtained in agarose gel electrophoresis as shown in **Figure 34**. We further sequenced the plasmid to confirm the presence of desired miR-125b insert. After confirming the presence of miR-125b insert in plasmid, we used this cloned plasmid as a template to insert mutation in the seed region of miR-125b with site directed mutagenesis. Three nucleotides within the seed region of miR-125b were substituted with new nucleotides to block its binding with target mRNA. The presence of mutation within seed region was confirmed by sequencing the derived plasmid **(Figure 35)**. Similarly, we cloned a 434 bp fragment containing miR-223 coding sequence with its flanking region in pBABE-puro vector following the same protocol as miR-125b. The presence of desired insert in plasmid was confirmed with colony PCR and sequencing and the cloned plasmid with desired insert was used as template to generate miR-223 mutant bearing mutation in its seed region **(Figure 36)**.

3.4.2 Cloning of TRAF6, TNFAIP3 and IKKα

The microRNA binding sequences present within the 3'UTRs of TRAF6, TNFAIP3 and IKKα were cloned along with their flanking regions in pMIR-REPORT-miRNA Expression Reporter Vector. Cloning of each gene was confirmed with colony PCR, obtaining single desired band of our interest. The presence of desired insert was also confirmed by sequencing the cloned plasmid.

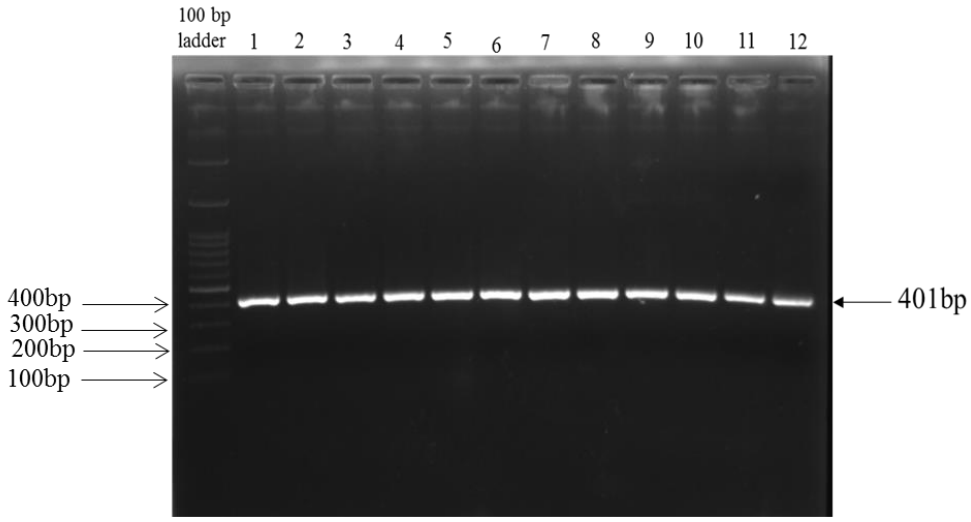
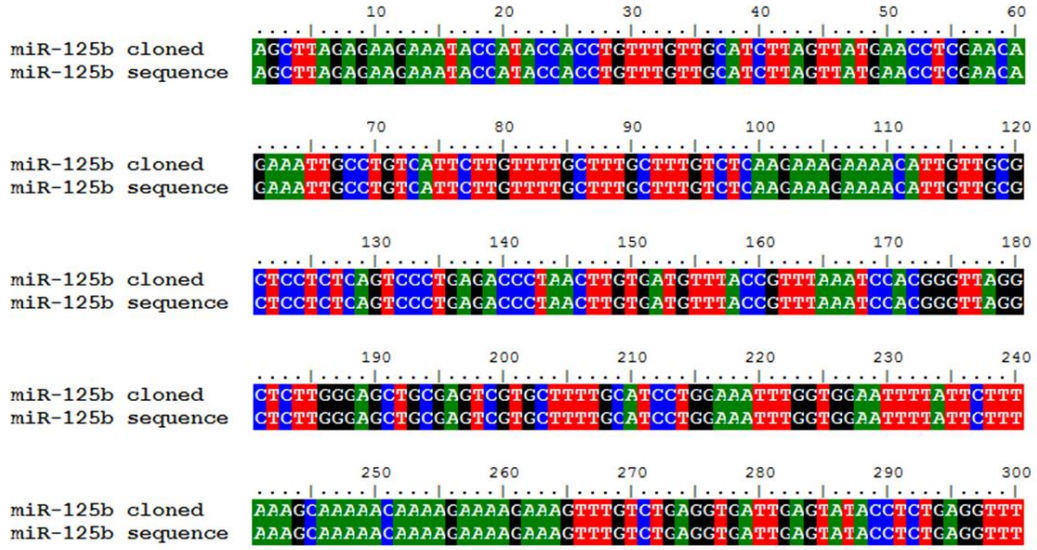


Figure 34: Colony PCR for miR-125b. Presence of miR-125b insert in pBABE-puro vector backbone was confirmed by colony PCR and showed a single band of 401bp which confirmed the presence of miR-125b in pBABE-puro. Lane 1 contains the 100 bp ladder and Lane 1-12 shows the presence of miR-125b insert in the colonies obtained after transformation.

a. Wild type miR-125b



b. Mutant miR-125b

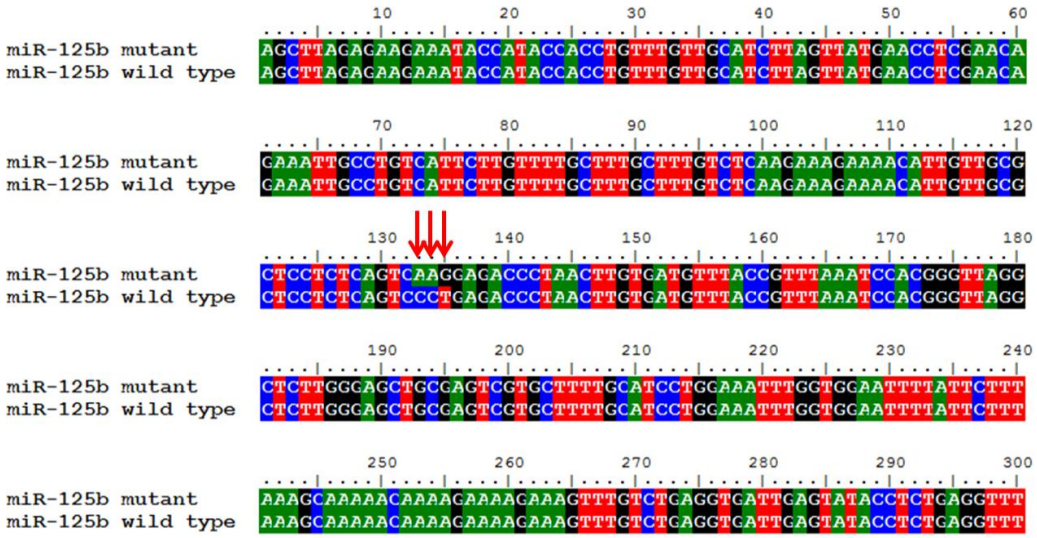
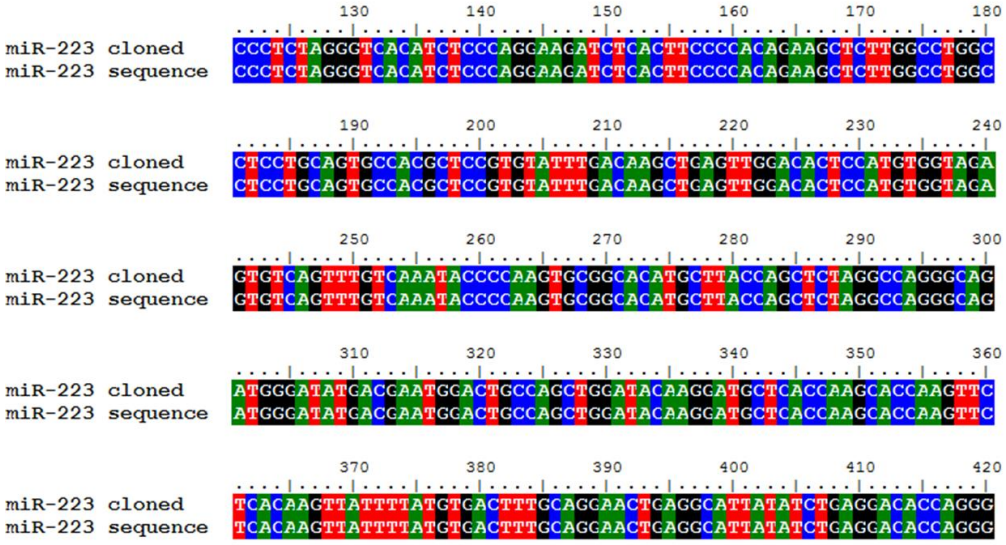


Figure 35: Cloning and mutant generation miR-125b mutant: miR-125b was cloned in pBABE-puro vector and the cloning was confirmed by sequencing a) sequence alignment of cloned 125b with miR-125b gene sequence obtained from Ensembl b) Sequence alignment of mutant miR-125b bearing mutation in seed region with wild type miR-125b.

a. Wild type miR-223



b. Mutant miR-223

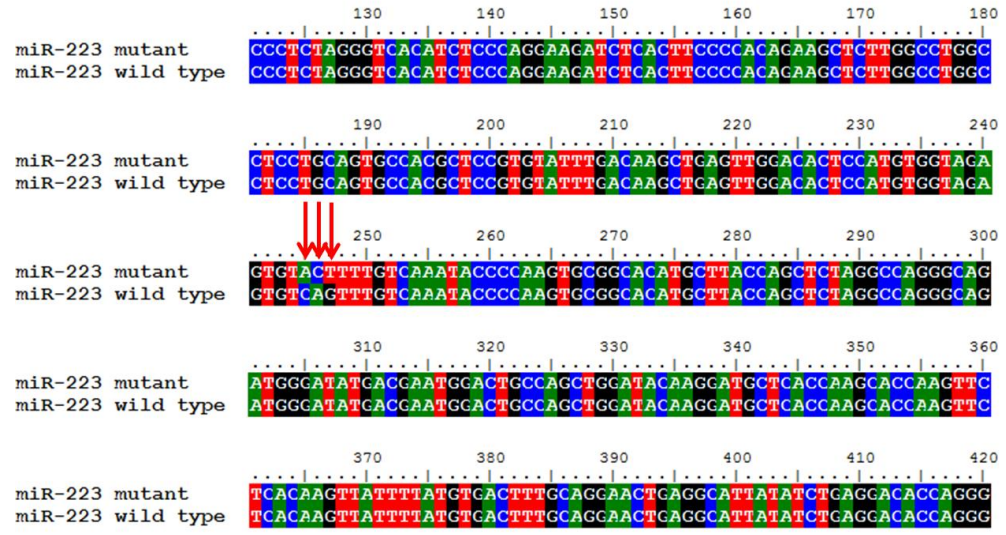


Figure 36: Cloning and mutant generation for miR-223 mutant: miR-223 was cloned in pBABE-puro vector and the cloning was confirmed by sequencing a) sequence alignment of cloned 223 with miR-223 gene sequence obtained from Ensembl b) Sequence alignment of mutant miR-223 bearing mutation in seed region with wild type miR-223.

3.4.3 Validation of miRNA and target gene interactions

MiR-125b and miR-223 showed upregulated expression in the UC patients while their respective target genes TRAF6, A20 and IKKα displayed downregulation. To study the biological relevance of these miRNA upregulation, we first validated the interaction between the miRNAs and their target genes by performing gene expression assay and dual luciferase reporter assay and subsequently investigated the expression levels of p65 and downstream activation of pro-inflammatory cytokines IL-8 and Il-1β in the presence of miR-125b and miR-223.

3.4.3.1 miR-125b negatively regulates the expression of A20 by targeting its 3'UTR

HT29 cells were co-transfected with cloned vectors *pBABE-miR125b* and *pMIR-A20-3'UTR* to study the interaction between miR-125b and A20. Untransfected HT29 cells and pBABE-puro vector without miR-125b insert were used as positive control and pBABE-puro with mutant miR-125b was used as negative control. In the gene expression assay, we observed a significant decrease in A20 expression with a fold change of 4.41 in the presence of wild type miR-125b ($p>0.0001$) and this reduced A20 expression was restored to a significant level when the mutated form of miR-125b bearing mutation in seed region was present ($p=0.02$) **(Figure 37).** Similarly, in dual luciferase reporter assay, the relative renilla luciferase activity was 2.84 folds lower in the presence of wild type miR-125b ($p=0.0002$) that correlated with the A20 expression in the presence of miR-125b. In the presence of mutated miR-125b the renilla luciferase activity again increased to a significant level (1.52 folds increased) as compared to the wild miR-125b ($p>0.0001$) **(Figure 38)**.

3.4.3.2 Effect of miR-125b and A20 interaction on NFkB pathway

We observed a suppression of A20 expression in the presence of miR-125b which is significantly upregulated during UC and also in inflamed colonic pockets of UC patients. To understand the contribution of miR-125b upregulation in inflammation during UC, the expression of p65 and downstream pro-inflammatory cytokines IL-8 and IL-1β was investigated after overexpression of miR-125b and A20 in HT29 cells. A20 acts as a feedback regulator in NFkB pathway where it negatively regulates the NFkB activation to resolve inflammation. We expected an increased expression of p65 and proinflammatory

cytokines in the absence of A20. In the presence of miR-125b, an increased p65 expression with 1.55 folds was observed where A20 expression was masked by miR-125b while in the presence of mutant miR-125b with dysfunctional seed region, the expression of p65 was again suppressed to a significant level (0.5 folds).

3.4.3.2.1 p65 is upregulated in absence of A20

A20 responsible for resolving inflammation by negatively regulating the expression of NFkB pathway is targeted by miR-125b. To investigate the effect of miR-125b upregulation and miR-125b-A20 interaction on NFkB pathway, we co-transfected *pBABE-miR125b* along with *pMIR-A20* in HT29 cells. After 48 hours, the cells were stimulated with LPS for six hours to activate the NFkB pathway and the p65 expression was evaluated by performing qRT-PCR. *pBABE-puro* vector without miR-125b insert was used as positive control and *pBABE-puro* with mutant miR-125b was used as negative control. In the presence of wild type miR-125b where it could target the expression of A20, we observed a 1.55 folds higher p65 expression ($p=0.03$) and this increased p65 expression was again significantly repressed in the presence of mutant miR-125b where it was no longer able to target A20 ($p=0.03$) **(Figure 39).**

3.4.3.2.2 Expression of pro-inflammatory cytokines IL-8 and Il-1β is increased in the absence of A20

We evaluated the expression of pro-inflammatory cytokines IL-8 and IL-1β to check whether increased p65 expression in the presence of miR-125b correspond to higher pro-inflammatory cytokine expression or not. In accordance with p65, IL-8 expression was increased to a significant level (2.0 folds) in the presence of miR-125b where it inhibited the expression of A20 ($p=0.019$). In the presence of mutant miR-125b the IL-8 expression was again decreased to a significant level where A20 was able to carry out its negative regulation ($p=0.04$). Similarly, IL-1β exhibited significant upregulation in the presence of miR-125b where it inhibited the expression of A20 with a fold change of 5.02 ($p=0.0019$). However, in the presence of mutant miR-125b, we observed 3.01 fold reduced IL-1β expression ($p=0.04$) probably due to the regulatory effect of A20 **(Figure 40).**

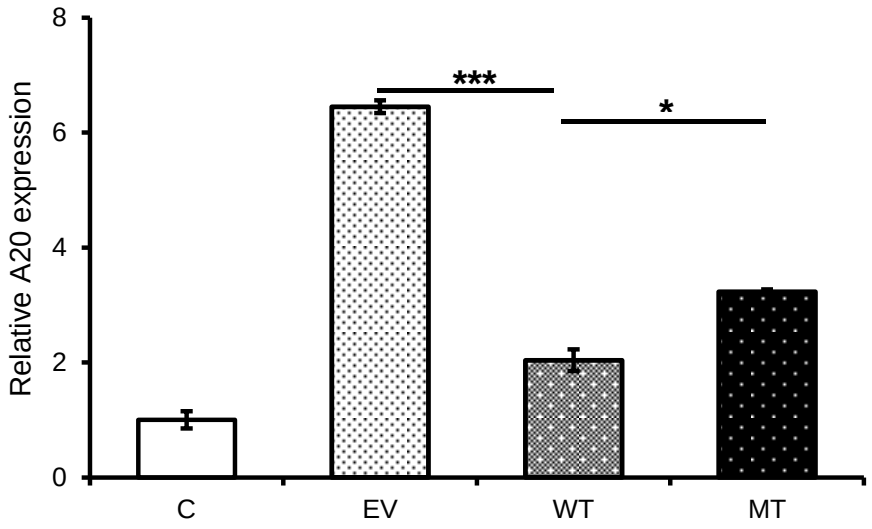


Figure 37: A20 expression in presence of miR-125b: miR-125b and A20 were co-transfected in HT29 cells for 48 hours. After 48 hours RNA was isolated and reverse transcribed to investigate the mRNA expression of A20 through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was derived with respect to the untransfected control group.*$p \leq 0.05$, ***$p \leq 0.001$. C-Control; EV-Empty vector; WT-Wild type; MT-Mutant. Bars represent Standard Error (SEM).

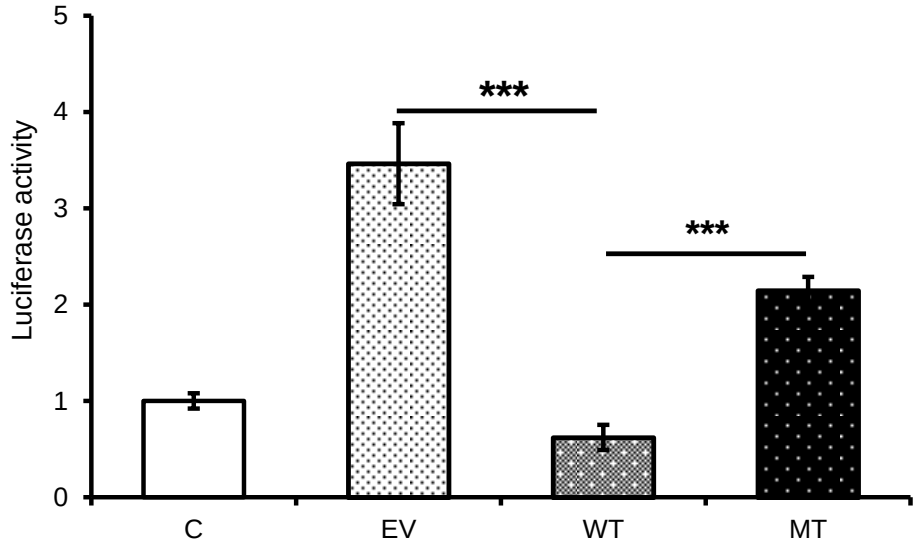


Figure 38: Validation of miR-125b and A20 interaction through dual luciferase reporter assay: miR-125b and A20 were co-transfected in HT29 cells along with pRL-TK control plasmid for 24 hours. After 24 hours the dual luciferase reporter assay was performed. p values less than 0.05 were statistically significant. Significance was estimated with respect to the untransfected control group.***$p \leq 0.001$. Results are expressed as fold change that represents normalized ratio of firefly luciferase to renilla luciferase with the normalized ratio of control. C-Control; EV-Empty vector; WT-Wild type; MT-Mutant. Bars represent Standard Error (SEM).

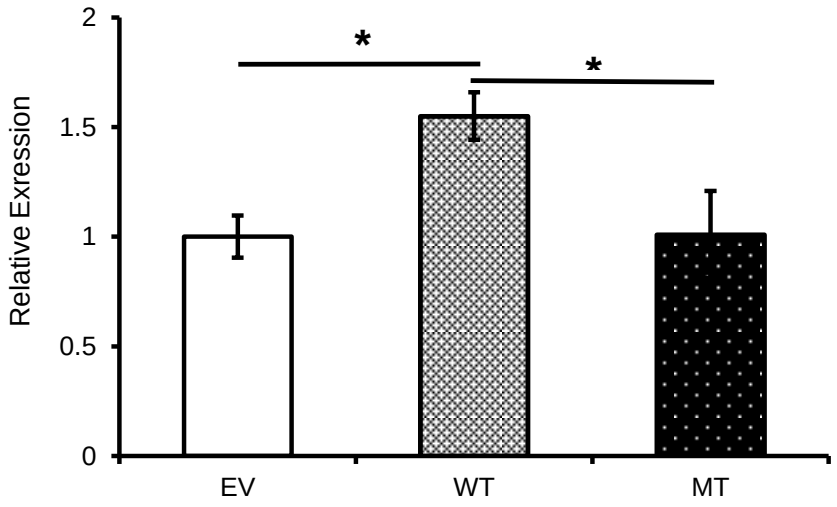


Figure 39: p65 expression in presence of miR-125b. MiR-125b and A20 were co-transfected in HT29 cells for 48 hours and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of p65 through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE empty vector control group.*$p \leq 0.05$. Bars represent Standard Error (SEM).

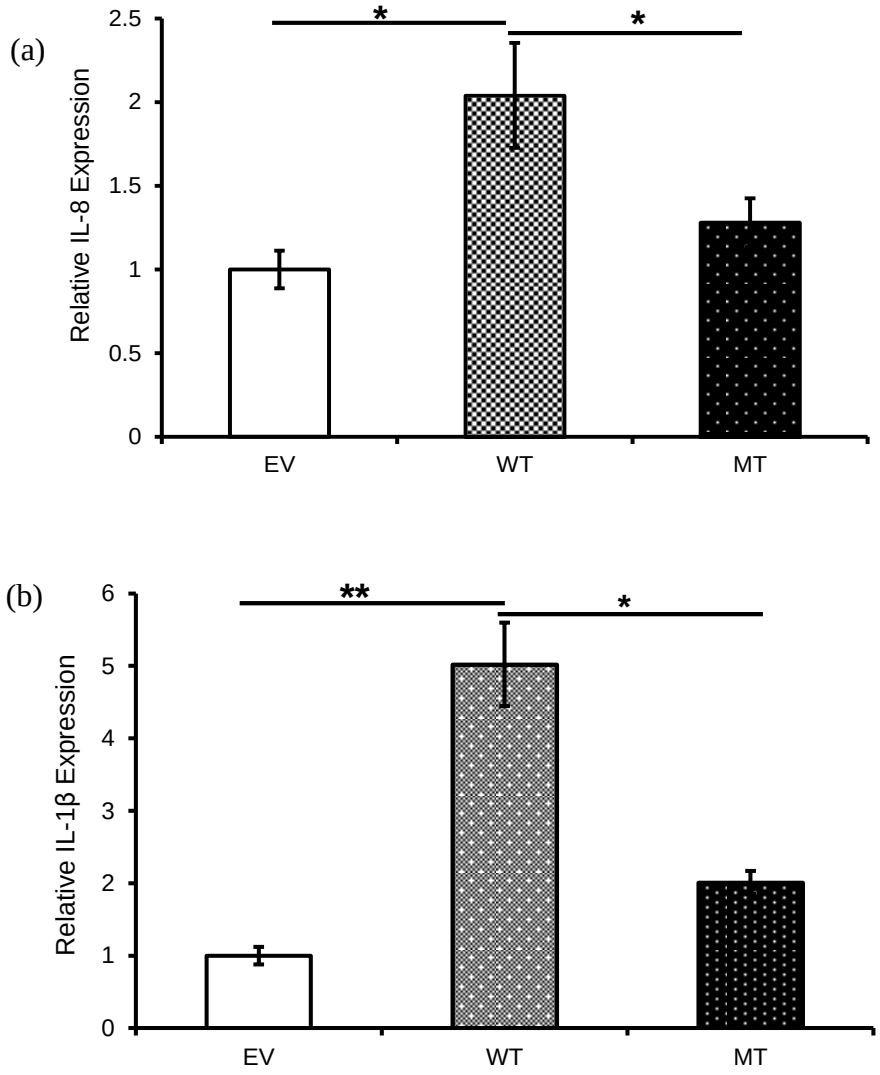


Figure 40: IL-1β expression in presence of miR-125b. miR-125b and A20 were co-transfected in HT29 cells for 48 hours and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of IL-8 and IL-1β through qRT-PCR. Both IL-8 (Panel a) and IL-1β (Panel b) showed significant rise in their expression in the miR-125b overexpressed cells where it suppressed the expression of A20. While in the presence of mutant miR-125b their expression showed a marked decrease. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE-puro empty vector control group.*$p \leq 0.05$, **$p < 0.01$. EV= Empty vector; WT=Wild type; MT= Mutant. Bars represent Standard Error (SEM).

3.4.4 miR-125b negatively regulates TRAF6 expression by targeting its 3'UTR

The bioinformatic tools predicted putative binding sites for miR-125b within the 3'UTR of TRAF6 gene. We validated the interaction between miR-125b and TRAF6 by performing gene expression assay and dual luciferase reporter assay. To study the interaction between miR-125b and TRAF6, we transfected the HT29 cells with *pBABE-miR-125b and pMIR-TRAF6-3'UTR*. The untransfected HT29 cells and pBABE-puro vector without miR-125b insert were used as positive control and pBABE-puro with mutant miR-125b was used as negative control. In the gene expression assay, the wild type miR-125b potentially reduced the expression of TRAF6 to a significant level by 1.74 folds (p=0.0152) while in the presence of mutated miR-125b the TRAF6 expression was restored to a significant point (0.65 folds) as compared to the wild type miR-125b (p=0.0225) **(Figure 41)**. Similarly in the dual luciferase reporter assay, the luciferase activity that corresponds to the expression of TRAF6, significantly decreased in the presence of wild type miR-125b by 6.95 folds (p=0.0032) which again showed a significant increased expression in the presence of mutated miR-125b by 1.47 folds (p=0.0187) (**Figure 42).**

3.4.4.1 Effect of miR-125b upregulation on NFκB pathway

In the patient samples, miR-125b and TRAF6 exhibited an inverse correlation with respect to their expression pattern with miR-125b being significantly upregulated and TRAF6 downregulated. Through gene expression and dual luciferase reporter assay we validated the interaction between them. To explore the biological relevance of miR-125b upregulation during UC, we investigated the expression of NFκB active subunit p65 and its downstream pro-inflammatory cytokines IL-8 and IL-1β after co-transfecting the HT29 cells with miR-125b and TRAF6 followed by LPS stimulation. Since we observed a downregulated expression of TRAF6 in UC patients, we were expecting an increased pro-inflammatory response in the presence of wild type miR-125b where it could negatively regulate the expression of TRAF6. As expected, we observed an increased expression of p65 and subsequent increase in pro-inflammatory cytokines IL-8 and IL-1β, in the presence of wild type miR-125b which could successfully target the TRAF6 and

regulate its expression while in the presence of mutated form of miR-125b, the expression levels of p65 and pro-inflammatory cytokines reduces to a significant level.

3.4.4.1.1 p65 exhibited decreased expression in the absence of TRAF6

TRAF6 is one of the key signaling molecules involved in NFκB signaling pathway and it showed a downregulated expression in UC patients. To investigate the effect of miR-125b and TRAf6 interaction on NFκB pathway, we co-transfected the HT29 cells with *pBABE-miR-125b* and *pMIR-TRAF6-3'UTR.* After 48 hours, the cells were stimulated with LPS for six hours to activate the NFkB pathway and the p65 expression was evaluated by performing qRT-PCR. *pBABE-puro* vector without miR-125b insert was used as positive control and *pBABE-puro* with mutant miR-125b was used as negative control. In the presence of wild type miR-125b where it could target the expression of TRAF6, we observed 3.0 folds higher p65 expression (p=0.0004) while in the presence of mutated form of miR-125b, the p65 expression was again repressed to a significant level with 1.5 folds change (p=0.047) **(Figure 43).**

3.4.4.1.2 Pro-inflammatory cytokines showed increased expression in the absence of TRAF6

The impact of miR-125b and TRAF6 interaction on NFκB pathway was studied by looking at the expression of p65. We also checked the expression of pro-inflammatory cytokines IL-8 and IL-1β in order to study the consequences of miR-125b overexpression in HT29 cells. We co-transfected the HT29 cells with *pBABE-miR-125b* and *pMIR-TRAF6-3'UTR.* After 48 hours, the cells were stimulated with LPS for six hours to activate the NFkB pathway and the expression of IL-8 and IL-1β was evaluated by performing qRT-PCR. *pBABE-puro* vector without miR-125b insert was used as positive control and *pBABE-puro* with mutant miR-125b was used as negative control. The expression of both IL-8 (p=0.047) and Il-1β (0.0003) significantly increased in the presence of miR125b where it negatively regulates the expression of TRAF6. Similarly, as observed with p65, in the presence of mutated miR-125b, IL-8 (p=0.0354) and IL-1β (0.0326) showed decreased expression by 1.25 fold and 1.78 folds respectively (**Figure 44).**

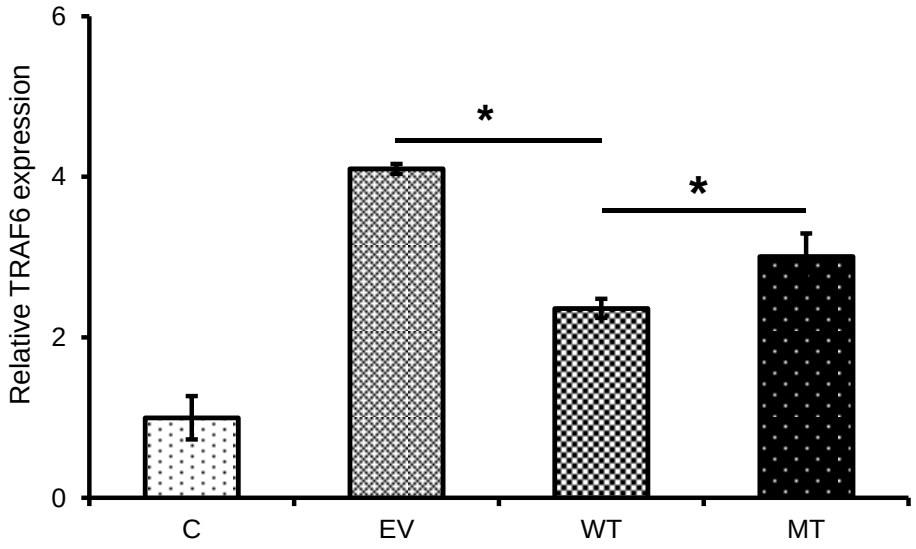


Figure 41: TRAF6 expression in presence of miR-125b: miR-125b and TRAF6 were co-transfected in HT29 cells for 48 hours. After 48 hours RNA was isolated and reverse transcribed to investigate the mRNA expression of TRAF6 through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the untransfected control group.*$p \leq 0.05$. C= Control; EV= Empty vector; WT=Wild type; MT= Mutant. Bars represent Standard Error (SEM).

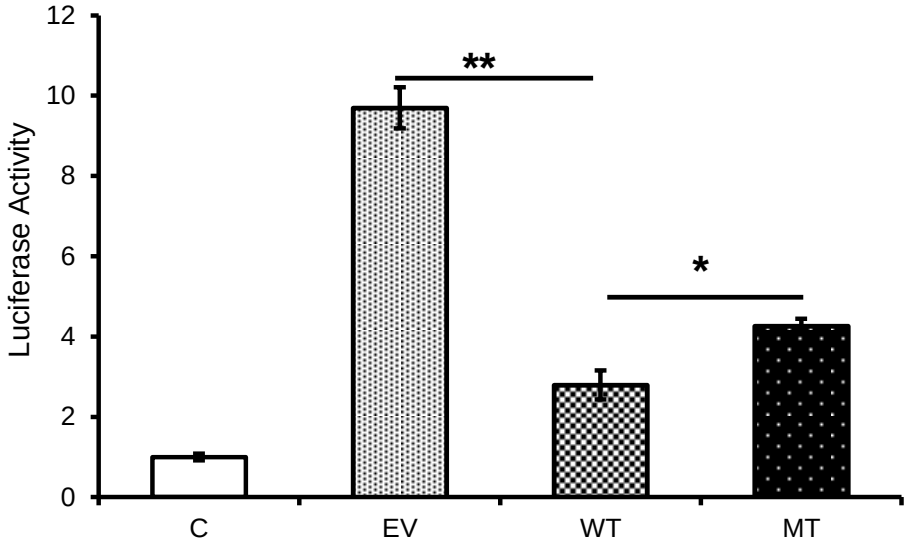


Figure 42: Validation of miR-125b and TRAF6 interaction through dual luciferase reporter assay: miR-125b and TRAF6 were co-transfected in HT29 cells for 24 hours along with pRL-TK control plasmid which was used as internal reference. After 24 hours the dual luciferase reporter assay was performed. p values less than 0.05 were statistically significant. Significance was estimated with respect to the untransfected control group.*$p \leq 0.05$, **$p \leq 0.01$. Results are expressed as fold change that represents normalized ratio of firefly luciferase to renilla luciferase with the normalized ratio of control. C= Control; EV= Empty vector; WT=Wild type; MT= Mutant. Bars represent Standard Error (SEM).

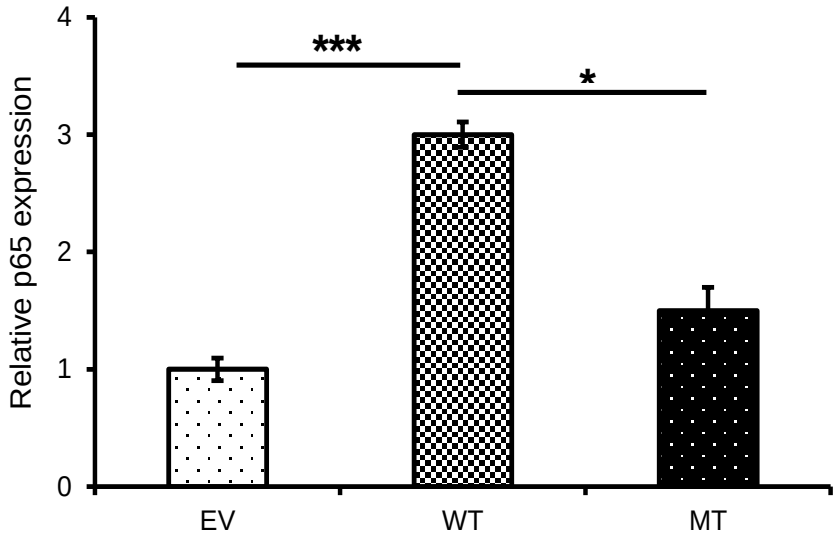


Figure 43: p65 expression in absence of TRAF6. miR-125b and TRAF6 were co-transfected in HT29 cells for 48 hours and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of p65 through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE puro empty vector control group. *$p \leq 0.05$, **$p < 0.01$,***$p \leq 0.001$. Bars represent Standard Error (SEM).

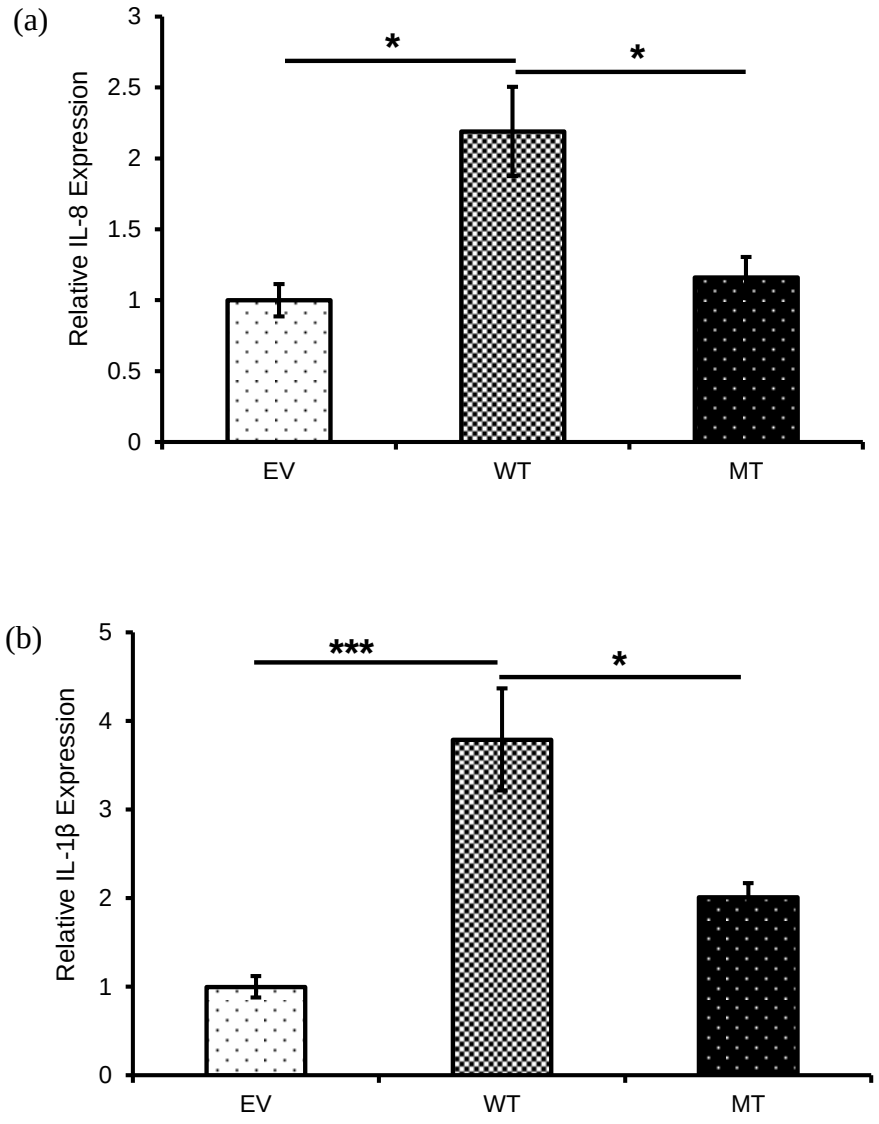


Figure 44: IL-8 and IL-1β expression in presence of miR-125b. MiR-125b and TRAF6 were co-transfected in HT29 cells for 48h and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of IL-8 and IL-1β through qRT-PCR. Both IL-8 (Panel a) and IL-1-β (Panle b) showed increased expression in in the presence of miR-125b which subsequently reduced in the presemce of mutamt miR-125b which failed to suppress the expression of TRAF6. GAPDH was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE-puro empty vector control group. *$p \leq 0.05$, ***$p \leq 0.001$. Bars represent Standard Error (SEM).

3.4.5 miR-223 targets the 3'UTR of IKKα

HT29 cells were cotransfected with cloned vectors *pBABE-miR-223* and *pMIR-IKKα-3'UTR* to study the interaction between miR-223 and IKKα. Untransfected HT29 cells and pBABE-puro vector without miR-223 insert were used as positive control and pBABE-puro with mutant miR-223 was used as negative control. In the gene expression assay, we observed a 4.16 fold decrease in IKKα expression in the presence of wild type miR-223 and this reduced IKKα expression was restored to a significant level (2.78 folds) when the mutated form of miR-125b bearing mutation in seed region was present (p=0.02) **(Figure 45).** Similarly, in the dual luciferase reporter assay, we observed significant reduction in renilla luciferase expression to about 2.78 folds, in the presence of miR-223 that corresponds to the lower IKKα expression (p=0.0004). Again in the presence of mutant miR-223 the renilla luciferase expression was increased to a significant level (p=0.0007) (2.0 folds) probably due to inactive miR-223 that was unable to target the IKKα gene **(Figure 46).**

3.4.5.1 Effect of miR-223 and IKKα interaction on NFκB pathway

We observed an upregulation in miR-223 expression in the inflamed colonic mucosa of UC patients as compared to the non-inflamed and healthy controls, while its target gene IKKα exhibited significant down regulation in UC patients. This increased miR-223 expression could be one of the contributing factors to inflammation during UC. Therefore, in order to study the role of miR-223 upregulation in inflammation, we co-transfected miR-223 and IKKα in HT29 cells and studied the expression of p65 and its downstream pro-inflammatory cytokines, IL-8 and IL-1β. Since, IKKα negatively regulates the NFκB pathway, we hypothesized an increased p65 and pro-inflammatory cytokines expression in the presence of miR-223 where it could target the IKKα 3'UTR and suppress its expression and its downstream regulatory effects. We observed a significant rise in the p65 as well as pro-inflammatory cytokines, IL-8 and IL-1β expression in the presence of miR-223 and absence of IKKα. While in the control conditions where miR-223 was present, the p65 and pro-inflammatory cytokines expression was again reduced to a significant level.

3.4.5.1.1 p65 expression was upregulated in the absence of IKKα

The upregulated miR-223 and downregulated expression of IKKα could be one of the contributing factors to chronic inflammation observed during UC. Since NFκB is one of the major inflammatory pathways, we investigated the effect of miR-223 and IKKα interaction on p65 expression levels. *pBABE-miR-223* and *pMIR-IKKα* were co-transfected in HT29 cells for 48 hours, then the cells were stimulated with LPS for six hours to activate the NFκB pathway and the p65 expression was evaluated by performing qRT-PCR. *pBABE-puro* vector without miR-223 insert was used as positive control and *pBABE-puro* with mutant miR-223 bearing mutation in seed region was used as negative control. We observed that p65 expression was significantly higher with an increase of 0.55 folds (p=0.0023), in the presence of wild type miR-223 where it blocked the expression of IKKα. While in the presence of mutant miR-223 which is not able to target IKKα, we observed a decreased p65 expression (0.50 folds) to a significant level (p=0.029) **(Figure 47)**

3.4.5.1.2 Pro-inflammatory cytokines showed decreased expression in the absence of IKKα

To study the consequences of increased p65 expression on inflammation in the absence of IKKα, we investigated the expression of downstream pro-inflammatory cytokines IL-8 and IL-1β. The HT29 cells transfected with *pBABE-puro-miR-223* and *pMIR-IKKα* were stimulated with LPS for six hours to activate the NFκB signaling and expression of downstream pro-inflammatory cytokines IL-8 and IL-1β was evaluated by performing qRT-PCR. Similar to the trend of p65 expression, we observed an increased IL-8 (p=0.026) and IL-1β (p=0.0019) expression in the presence of wild type miR-223 with a fold change of 2.04 and 5.02 respectively In the presence of mutated miR-223, both IL-8 (p=0.044) and IL-1β (0.0402) exhibited significant decreased expression with a change of 0.76 and 3.01 folds respectively **(Figure 48)**

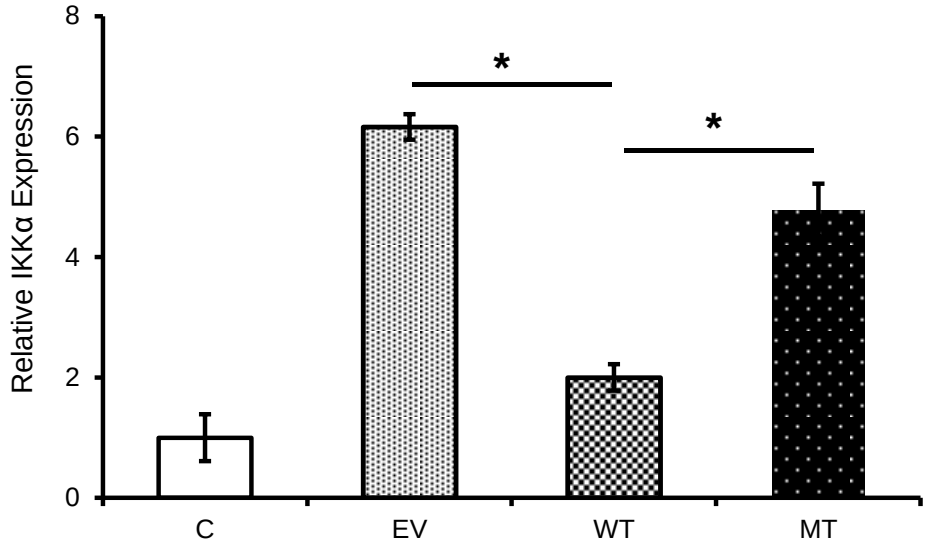


Figure 45: IKKα expression in presence of miR-223: miR-223 and IKKα were co-transfected in HT29 cells for 48 hours. After 48 hours RNA was isolated and reverse transcribed to investigate the mRNA expression of IKKα through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the untransfected control group. *$p \leq 0.05$. Bars represent Standard Error (SEM).

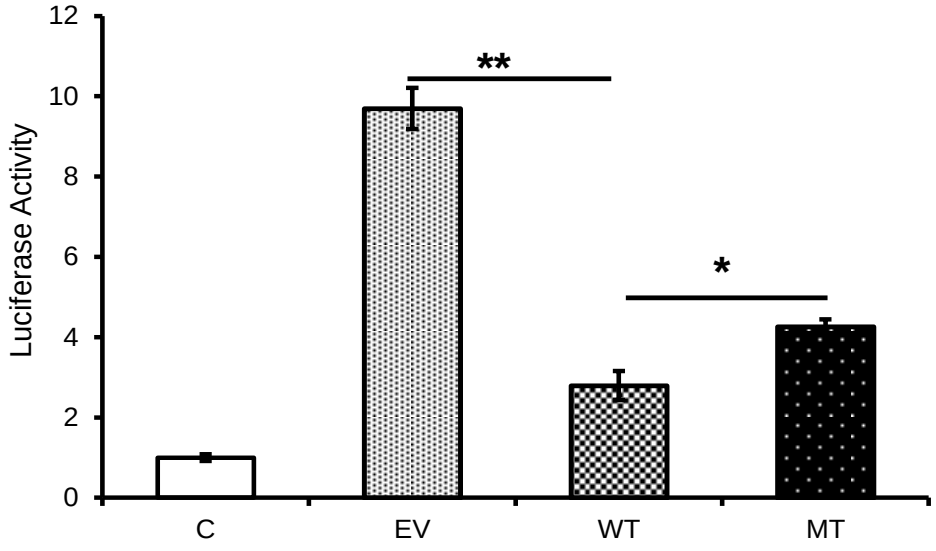


Figure 46: Validation of miR-223 and IKKα interaction through dual luciferase reporter assay: miR-223 and IKKα were co-transfected in HT29 cells along with pRL-TK control plasmid which was used as internal reference, for 24 hours. After 24 hours the dual luciferase reporter assay was performed. p values less than 0.05 were statistically significant. Significance was estimated with respect to the untransfected control group. *$p \leq 0.05$, **$p \leq 0.01$. Results are expressed as fold change that represents normalized ratio of firefly luciferase to renilla luciferase with the normalized ratio of control. C= Control; EV= Empty vector; WT-Wild type; MT-Mutant. Bars represent Standard Error (SEM).

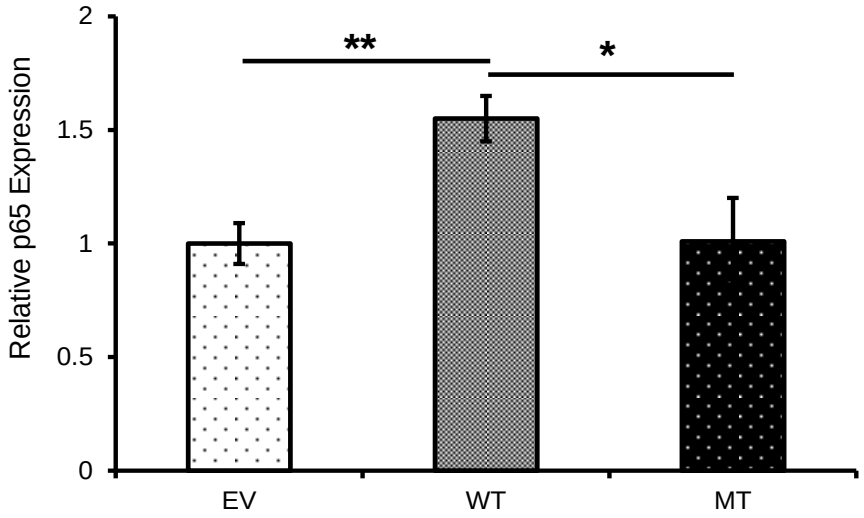


Figure 47: p65 expression in presence of miR-223. miR-223 and IKKα were co-transfected in HT29 cells for 48 hours and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of p65 through qRT-PCR. GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE-puro empty vector control group. *$p \leq 0.05$, **$p < 0.01$. EV- Empty vector; WT-Wild type; MT-Mutant. Bars represent Standard Error (SEM).

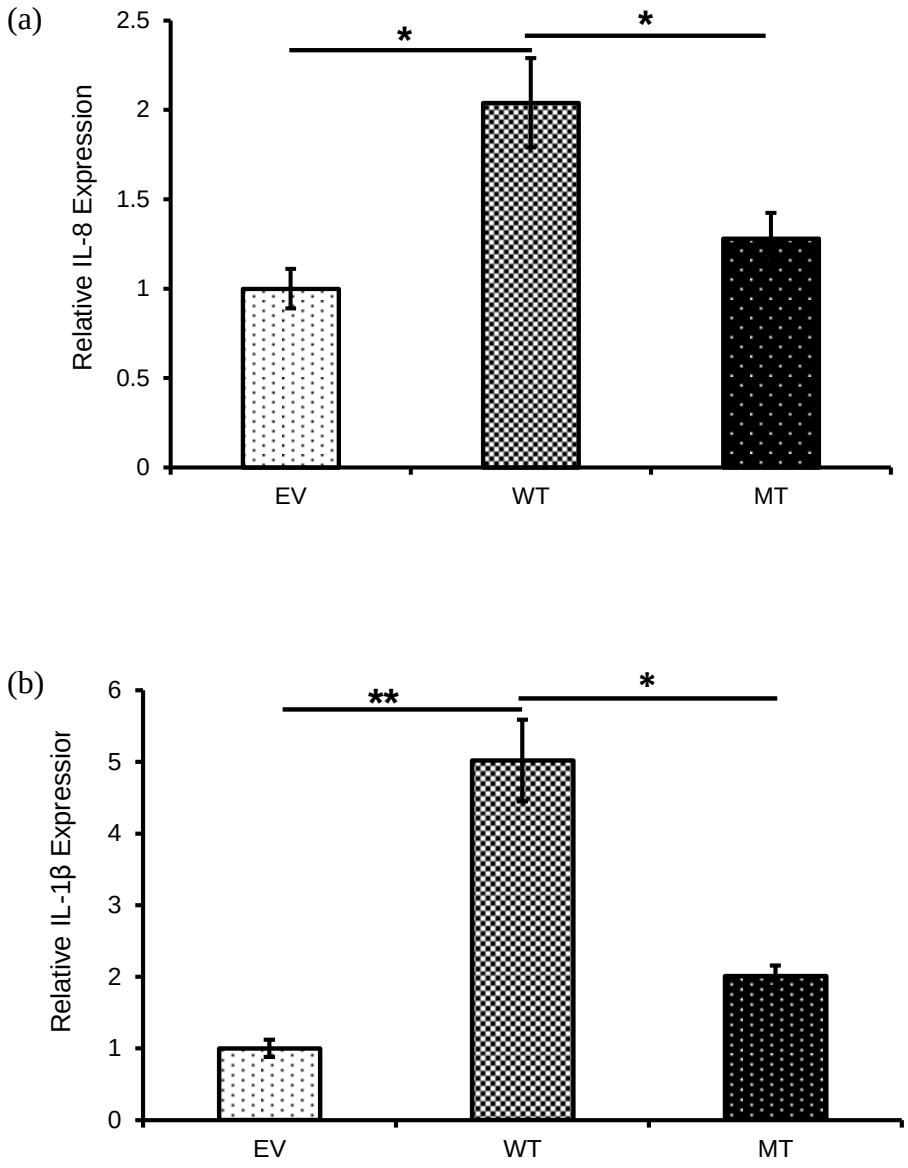


Figure 48: IL-1β expression in presence of miR-223. MiR-223 and IKKα were co-transfected in HT29 cells for 48 hours and stimulated with 100ng/ml of LPS. After 6 hours of stimulation RNA was isolated and reverse transcribed to investigate the mRNA expression of IL-8 and IL-1β through qRT-PCR. (a) IL-8 GAPDH gene was used as an internal reference. p values less than 0.05 were statistically significant. Significance was estimated with respect to the pBABE-puro empty vector control group.*$p \leq 0.05$, **$p < 0.01$. EV= Empty vector; WT-Wild type;MT-Mutant. Bars represent Standard Error (SEM).

DISCUSSION

MiRNA exert their regulatory functions by restraining the protein synthesis of protein coding genes either by translational inhibition or by carrying out the degradation of target mRNA. About 30% of mammalian functionally related genes are controlled by miRNAs **(Ambros 2003)**. Since miRNA harbor these regulatory capacities, a number of different miRNAs have been found to be associated with vital biological processes and any alteration in the expression of these miRNA hampers these critical pathways which eventually results in severe outcomes such as malignancies and various other pathological conditions including IBD **(Taganov, Boldin et al. 2006, Png, Halberg et al. 2011, Ng, Song et al. 2012, Zhang, Bill et al. 2012, Cao, Zhou et al. 2017)**. Also, the epigenetic modulation of miRNAs through DNA methylation or histone acetylation has resulted in altered miRNA expression in cancer cells where the restoration of mRNA expression ameliorated the tumor growth and metastasis **(Lujambio, Calin et al. 2008)**. Additionally, recent deep sequencing experiments and bioinformatic tools have been able to identify genetic variations including SNPs within the miRNA genes. This can affect the processing and functioning of miRNA that contributes to the disease development either by increasing the susceptibility towards the disease or by playing protective role. SNPs within miR-196a-2 gene (rs11614913) have been found to be negatively associated with UC while rs3746444 SNP within miR-499 gene showed a positive association with UC in north Indian population **(Ranjha, Meena et al. 2017)**. A large number of evidences has supported the fact that miRNA exhibit altered expression levels in UC, CD and even in pediatric IBD during different disease stages where this altered expression has been studied in colonic tissues, serum and also in oral fluids **(Zahm, Thayu et al. 2011, Paraskevi, Theodoropoulos et al. 2012, Iborra, Bernuzzi et al. 2013, Schaefer, Attumi et al. 2015)**.

It is established that miRNA signatures in UC patients are different from healthy individuals. Differential expression of eleven miRNAs was reported during UC in comparison to healthy controls **(Wu, Zikusoka et al. 2008)**. Site-specific alteration in miRNA expression has also been explored in UC patients where six miRNAs showed downregulation and one miRNA exhibited upregulated expression within the rectosigmoid area of UC patients with respect to ascending colon **(Ranjha, Aggarwal et al. 2015)**. But the miRNA expression profile within the inflamed and non-inflamed

colonic mucosa of same UC patients has not been studied so far. Therefore, we investigated the miRNA expression profile in the inflamed and non-inflamed colonic mucosal biopsy specimens within the same UC patients.

Microarray analysis revealed that the miRNA signatures within the inflamed colonic region of UC patients differ significantly from the non-inflamed ones. Among the miRNAs which showed upregulated expression, we considered **miR-125b** and **miR-223** as two very important miRNA because upregulation of these two miRNA have previously been reported in UC patients **(Fasseu, Treton et al. 2010)**. In a study carried out by *Nishida et al*, miR-125b was found to be directly associated with cancer progression and poor prognosis of colorectal cancer in patients which suggested that miR-125b could prove as a prognostic marker for colorectal cancer (CRC) **(Nishida, Yokobori et al. 2011)**. Similarly, miR-223 has also been identified to play a crucial role in regulating the differentiation of intestinal dendritic cells and macrophages that are required to maintain the intestinal homeostasis, and miR-223 is known to exert these regulatory effects by directly targeting the C/EBPβ gene **(Zhou, Xiao et al. 2015)**. Recently, the oncogenic properties of miR-223 during colorectal cancer have also been reported where higher expression of miR-223 was found in human colon cancer tissues as compared to normal colon tissues. MiR-223 enhanced the cancer progression by targeting p120 catenin **(Liu, Zhang et al. 2017)**.

Another important miRNA that was upregulated within the inflamed colonic mucosa in microarray was **miR-155**. MiR-155 is known to contribute to the chronic inflammation during UC by targeting FOXO3a (Forkhead box O transcriptional protein) which results in exaggerated pro-inflammatory response by suppressing the expression of IκBα, the inhibitory subunit of p65. Higher miR-155 expression has been observed in the inflamed colonic regions of UC patients with respect to healthy individuals **(Min, Peng et al. 2014, Beres, Szabo et al. 2016)**. Besides its role in UC, miR-155 has a pivotal role in various cancers. An upregulated expression of miR-155 has been seen in breast cancers and also enhances the cell proliferation, tumor invasion and angiogenesis by targeting the tumor suppressor gene von Hippel-Lindau (VHL) **(Iorio, Ferracin et al. 2005, Kong, He et al. 2014)**. Next miRNA that showed significant upregulation in the inflamed

regions of UC patients in our study was **miR-138** which was earlier reported to be upregulated in the active UC patients as compared to inactive UC patients **(Van der Goten, Vanhove et al. 2014)**. Not much is known about the contribution of miR-138 in inflammation and the studies regarding the role of miR-138 in UC are also very limited. But there are reports regarding the tumor suppressive role of miR-138 during cervical cancer and colorectal cancer. MiR-138 has shown downregulated expression in cervical cancer tissues while its overexpression resulted in decreased cell proliferation, tumor invasion and migration **(Zhou, Fei et al. 2016)**. Similarly, miR-138 exhibited decreased expression in the human CRC tissues. The decreased expression of miR-138 was found to be associated with decreased cancer cell proliferation and transition from G1 to S phase by blocking the expression of PDL-1 gene, a ligand that binds with PD1 receptor **(Zhao, Yu et al. 2016)**.

Among the upregulated miRNAs, we observed increased expression of hsa-miR-708-5p and hsa-miR-212-3p in inflamed mucosa with a fold change more than twenty. These two miRNA have been earlier shown to be upregulated in the active UC patients as compared to inactive UC patients and healthy individuals **(Van der Goten, Vanhove et al. 2014)**. While there are very few reports on the role of miR-212 whereas miR-708 has been studied in detail and has been shown to be associated with various cancers where it was found to play both the oncogenic or tumor suppressive roles. It shows upregulated expression in bladder urothelial carcinomas (**Song, Xia et al. 2010)** and CRC tissues as compared to adjacent healthy tissues. MiR-708 exerts its oncogenic effects by amelioarting cell apoptosis and increasing tumor invasion and targeting the CDKN2B gene (Cyclin Dependent Kinase Inhibitor 2B) in colorectal cancer cells **(Lei, Zhao et al. 2014)**. The tumor suppressive behavior of miR-708 has been reported in breast cancer, ovarian cancer, prostate cancer, renal-cell carcinoma **(Saini, Yamamura et al. 2011, Saini, Majid et al. 2012, Lin, Yeh et al. 2015)**. Lower miR-708 expressions are reported from primary breast cancer cells and even in metastatic breast cancer cells as compared to normal breast cells where suppression of miR-708 resulted in increased metastasis (**Ryu, McDonnell et al. 2013)**. Among the downregulated expression of miRNAs, miR-378d and miR-194 were considered important as they were also detected in a previous study

carried out in our laboratory, where the miRNA profile of UC vs controls was investigated (GEO accession no. GSE99632). MiR-194 downregulation has earlier been seen in colonic tissue biopsies from UC patients with respect to controls (**Van der Goten, Vanhove et al. 2014, Zahm, Hand et al. 2014)**. In one of the study, decreased miR-194 levels were reported from colorectal cancer tissues and it was found to be associated with poor prognosis of colorectal cancer. MiR-194 was found to play tumor suppressive roles by targeting MAP4K4 (mitogen-activated Protein Kinase) expression which in turn regulates the MDM2 (human homologue of the mouse double minute 2) expression. Overexpression of miR-194 improved cell apoptosis rate and decreased the cell proliferation and tumor invasion **(Wang, Shen et al. 2015)**. MiR-378d has not been explored in great detail in context with IBD therefore, we selected it for our study to find its possible role in the pathogenesis of UC.

The microarray results revealed differential expression for a panel of miRNAs in the inflamed colonic mucosa vs. non-inflamed mucosa and it was not feasible for us to validate all of these miRNAs. Therefore, we selected few miRNAs that are somewhat known to be involved in inflammatory pathways during IBD. We attempted to identify their putative gene targets and carried out validation through qRT-PCR. Four miRNAs namely **miR-125b**, **miR-223**, **miR-378d** and **miR-194** were chosen for further investigation through target prediction and qRT-PCR because these four miRNAs were also altered in a previous study where UC vs control miRNA profiling was carried out in our laboratory (GEO accession no. GSE99632). **MiR-155** and **miR-138** were also selected keeping in mind that they have been earlier reported to get altered during UC and also have been linked with various cancers. From the microarray results we concluded that the miRNA profile within the inflamed colonic mucosa differs significantly from the non-inflamed mucosa and the changes in expression depends upon the disease severity. The moderate UC patients show more pronounced difference in the miRNA expression as compared to the mild patients.

Due to their ability to regulate gene expression, miRNAs play critical role in the management of different biological pathways including the major inflammatory pathways such as Toll like receptor signaling. For example, IL-1 signaling has been implicated as a

link between inflammation and cancer **(Taganov, Boldin et al. 2006, Ceppi, Pereira et al. 2009, Iliopoulos, Jaeger et al. 2010)**. To explore the contribution of altered miRNAs to chronic inflammation during UC, we first identified the potential gene targets for dysregulated miRNAs as revealed by our microarray data and then validated their expression patterns in UC patients by performing qRT-PCR. The identification of gene targets was carried out by employing bioinformatic tools for miRNA target prediction such as TargetScan, MIRDB, microrna.org, PicTar, DIANA-microT-CDS, MirTarBase.

Applying target porediction tools, TRAF3 was selected as potential gene target for miR-155 and miR-378d as it possessed binding sites for both these miRNA in its 3'UTR. TRAF3 is an adaptor molecule and it is involved in non-canonical NFκB pathway. It regulates the NFκB activation by physically interacting with the specific sequences present in the N-termianl of NFκB-inducing kinase (NIK) which is the key component of non-canonical NFκB pathway for carrying out its degradation **(Liao, Zhang et al. 2004)**. Also, higher TRAF3 mRNA and protein levels are reported from serum and inflamed colonic mucosa of both UC and CD patients as compared to healthy individual which suggests the possible role of TRAF3 in IBD pathogenesis **(Shen, Qiao et al. 2013)**. In our microarray data, miR-155 had shown a significant higher expression while miR-378d exhibited a significant downregulation, therefore, we were keen to know which of these miRNAs is actually regulating TRAF3 expression and to find out this, we performed qRT-PCR studies to quantify expressions of miR-378d, miR-155 and TRAF3 in UC patients. In accordance with microarray, miR-155 showed significant higher expression in the inflamed colonic mucosa as compared to non inflamed as well as non IBD controls

In qRT-PCR however, interestingly TRAF3 also showed significant higher expression in UC patients as compared to non IBD controls. Since, both the miRNA and TRAF3 showed upregulation in UC patients we concluded here that TRFAF3 expression might not be regulated by miR-155 in UC patients. However, in case of miR-378d we observed contradictory expression pattern in microarray and qRT-PCR. While we observed a significant downregulation in miR-378d expression in microarray analysis, but qRT-PCR analysis yielded significant upregulated expression in the UC patients as compared to the non IBD controls and similar pattern was observed in the inflamed

colonic mucosa as compared to non inflamed mucosa and non IBD controls. The small sample size in microarray could be one of the reasons for these expression differences and such opposite expression patterns in microarray and qRT-PCR are reported earlier also where miR-98 showed higher expression in the active UC mucosa in microarray while in the qRT-PCR it was highly expressed in the inactive mucosa of UC patients **(Coskun, Bjerrum et al. 2013)**.

However, miR-194 expression pattern observed in microarray was also validated through qRT-PCR. Although we found reduced miR-194 expression in the colonic tissues from UC patients with respect to non-IBD controls, the change in expression level was not significant even with large sample size. The qRT-PCR results for miR-194 were not conclusive as we also observed high inter sample variation for this particular miRNA though we used the same number of samples that were used for analyzing other miRNAs.

In case of miR-125b, we selected TRAF6, A20 and STAT3 as its potential gene targets as all these three genes bears binding sites for miR-125b in their 3' UTRs. TRAF6 and A20 are directly involved in inflammatory response as they are important part of canonical NFκB pathway. A20 is a zinc finger protein and it acts as a negative regulator in the NFκB pathway **(Heyninck, De Valck et al. 1999, Wertz, O'Rourke et al. 2004)**. Mice deficient in A20 were shown to develop extreme inflammation and tissue damage in various organs including intestine. Also, upon injecting with LPS the A20 deficient mice died within 2 hours while the normal mice survived the LPS injection **(Lee, Boone et al. 2000)**. Earlier studies on the CD40 and TNFR2 triggered NFκB activation was inhibited by A20 by its interaction with TRAF2. It also inhibits the TNF and IL-1 triggered NFκB activation in HEK 293 cells **(Song, Rothe et al. 1996)**. Decreased A20 expression has been reported at protein level in UC patients **(Majumdar, Ahuja et al. 2017)** and both at mRNA and protein level in pediatric UC **(Zheng and Huang 2011)**. TRAF6 is an E3 ubiquitin ligase which is having a role in the synthesis of 'Lys-63'-linked-polyubiquitin chains that are conjugated to different proteins with the help of UBE2N and UBE2V1. Reports are available regarding the contribution of TRAF6 in chronic inflammation during colitis but these reports are not conclusive. In one of the report TRAF6 is seen to provide protection against DSS induced colitis independent of

TLR signaling. The TRAF6 deletions in intestinal epithelial cells lead to exacerbated colitis in mice while the deletion of Myd88/TRIF did not change the inflammatory response indicating that TRAF6 protected the mice against colitis, in a manner which was independent of TLR signaling in epithelial cells **(Vlantis, Polykratis et al. 2016)**. Another study conducted in IBD patients, increased TRAF6 levels were reported in plasma, peripheral blood mononuclear cells and also in the inflamed colonic mucosa of UC and CD patients as compared to healthy individuals **(Shen, Qiao et al. 2013)**. STAT3 is a transcription factor which belongs to the STAT protein family. STAT3 activation in response to wide range of cytokines and growth factors results in its phosphorylation, dimerization and migration to nucleus which leads to gene expression of molecules essential for different biological pathways including suppression of cytokine production, pro and anti-apoptosis, cell growth, regulatory cytokine production and cell motility **(Wang, Niu et al. 2004, Choi and Han 2012, Teng, Ross et al. 2014)**. It is reported that the intestinal T cells from CD patients display constitutive expression of phosphorylated STAT3 and STAT3 regulated protein SOCS3 as compared to healthy individuals **(Lovato, Brender et al. 2003)**. The macroscopically inflamed tissues of UC patients have also shown marked increase in activated STAT3 in comparison the non-inflamed tissues. Similarly the lamina propria mononuclear cells and activated T lymphocytes obtained from UC and CD patients had higher number of activated STAT3 **(Musso, Dentelli et al. 2005)**

The qRT-PCR results for miR-125b validated our microarray results as we observed significant upregulated expression of miR-125b in the inflamed colonic mucosa of UC patients as compared to non-inflamed mucosa and non-IBD controls. Its expression was also higher in UC patients as compared to non-IBD controls. The higher miR-125b expression in the inflamed colonic mucosa suggests that miR-125b contributes to the chronic inflammation observed during UC. Our qRT-PCR findings for miR-125b supported previous report where increased miR-125b expression was seen in UC patients and it was also proposed as an important diagnostic marker for UC **(Coskun, Bjerrum et al. 2013)**. Higher miR-125b expressions are also seen in tissues obtained from gastric cancer patients as compared to non-cancerous gastric tissues where higher miR-125b

levels lead to gastric cancer malignance progression. Also miR-125b was associated with poor prognosis and trastuzumab resistance in HER-2 positive gastric cancer patients **(Sui, Jiao et al. 2017)**. Another probable target of miR-125b, the STAT3 whose expression levels were reduced in the colonic tissues of UC patients as compared to non-IBD controls but the change in expression levels were not significant even with large sample size. Therefore we did not pursue further validation for miR-125b and STAT3 through *in vitro* studies.

In our study TRAF6, chosen as another target of miR-125b by target scan was found to be significantly downregulated in the colonic tissues of UC patients as compared to non IBD controls and from these results we concluded that miR-125b might be negatively regulating the TRAF6 expression during UC and the higher miR-125b expression is responsible for the decreased TRAF6 expression in UC patients. And this interaction between miR-125b and TRAF6 could be contributing to the elevated inflammatory response during UC.

A20, another probable target of miR-125b was earlier shown to exhibit reduced protein expression in UC patients as compared to non-IBD controls in our own laboratory **(Majumdar, Ahuja et al. 2017)**. The A20 mRNA levels were reported to be higher in UC patients in this study and this could be probably due to presence of miR-125b binding sites in its 3'UTR and miR-125b probably regulating its expression through translational inhibition. Therefore we directly validated the miR-125b and A20 interaction through *in vitro* studies. Since, A20 works as a negative regulator of NFκB signaling and helps in resolving inflammation we conclude here that due to miR-125b targeted negative regulation of A20 it exhibits reduced protein levels in UC patients.

The interactions between miRNA and their respective genes were validated by performing dual luciferase reporter assay and gene expression assay in HT29 cells. The miRNA cloned in pBABE-puro vector and target gene cloned in pMIR-REPORT were transfected in HT29 cells and the expression of target gene was monitored in the presence and absence of miRNA. We observed a marked decrease in A20 and TRAF6 mRNA expression in the cells co-expressed with miR-125b along with A20-3'UTR and TRAF6-3'UTR in comparison to the cells coexpressed with empty pBABE-puro vector without

miR-125b insert. In order to reconfirm the role of miR-125 in the interaction with A20 and TRAF6, mutants were generated in the seed region of miR-125. In the presence of mutant miR-125b possessing mutation in its seed region, the A20 and TRAF6 expression was again restored to a significant level. The dual luciferase assay also exhibited reduction in luciferase levels in cells coexpressing wild type miR-125b with A20-3'UTR or TRAF6-3'UTR whereas this effect reversed in the cells coexpressed with mutated form of miR-125b. From these results we concluded that miR-125b effectively targets the 3'UTR of A20 and TRAF6 genes and negatively regulates their expression. This finding also validated the inverse expression patterns for miR-125b with A20 and TRAF6 that we had observed in UC patients.

After confirming the interaction between miR-125b with A20 and TRAF6, we wanted to see whether the interaction between miR-125b with A20 and TRAF6 affects the NFκB inflammatory pathway. To prove this we investigated the expression of p65 sub unit of NFκB along with pro-inflammatory cytokines IL-8 and IL-1β after transfecting the HT29 cells with miR-125b and A20-3'UTR or TRAF6-3'UTR. LPS treatment was given to the cells to activate the downstream NFκB signaling. As we expected, the expression of p65 and pro-inflammatory cytokines IL-8 and IL-1β was higher in the HT29 cells co-transfected with the wild type miR-125b along with A20-3'UTR as compared to the cells co-transfected with A20-3'UTR along with empty pBABE-puro vector without miR-125b insert. While the cells co-transfected with mutated forms of miR-125b along with A20-3'UTR showed decreased p65 and IL-8 and IL-1β expression. From our results we therefore concluded that LPS triggered activation of NFκB signaling is negatively regulated by A20 and it subsequently helps in the resolution of inflammation under steady state. Also, the miR-125b mediated downregulation of A20 acts as a contributing factor to the increased inflammatory response observed during UC. Our study support the earlier observations where A20 was shown to control inflammatory response mediated through inhibition of NFκB **(Pujari, Hunte et al. 2013)**.

In case of TRAF6 also, we observed marked increase in the expression of p65 and pro-inflammatory cytokines IL-8 and IL-1β in cells transfected with wild type miR-125b along with TRAF6-3'UTR as compared to the cells coexpressed with empty pBABE-

puro vector and TRAF6-3'UTR. The expression of p65 and pro-inflammatory cytokines were reduced to a significant level when the cells were co-transfected with mutated form of miR-125b and TRAF6-3'UTR. These results indicated that suppression of TRAF6 expression in presence of miR-125b resulted in increased pro-inflammatory response therefore, it was concluded that miR-125b contributes to UC pathogenesis by down regulating TRAF6 expression which in turn leads to elevated inflammatory response during UC. Role of TRAF6 in mediating negative regulation of NFκB pathway has not been reported till date and further detailed studies are needed to understand at what point in the pathway of NFκB activation, TRAF6 acts and how it regulates the inflammatory response.

Increased miR-223 expression observed in microarray was also validated by performing qRT-PCR in large number of samples where miR-223 exhibited higher expression in the inflamed as well as the non-inflamed colonic tissues of UC patients as compared to non IBD controls. It is inferred from the qRT-PCR that the higher miR-223 expression observed during UC must be independent of inflammation and its higher expression does not contribute to the inflammation during UC. Since miR-223 expression was high during UC irrespective of inflammation, this miRNA could be considered as a potential diagnostic marker for UC to assess the disease development. MiR-223 has been previously reported to contribute to the pathogenesis of IBD. It enhances the pro-inflammatory cytokines production by suppressing the IκBα through downregulation of transcription factor FOXO3 **(Kim, Kwon et al. 2016, Wang, Zhang et al. 2016)**. Higher miR-223 expression was also seen in prostate cancer tissues as compared to non-cancerous tissue and overexpression of miR-223 in prostate cancer cell line resulted in tumor invasion, increased cell proliferation and reduced cell apoptosis **(Wei, Yang et al. 2014)**. In our study, IKKα, the target gene for miR-223 showed significantly reduced expression in colonic tissues of UC patients as compared to non-IBD controls. Since IKKα was identified as a target gene for miR-223, we wanted to investigate whether the upregulated expression of miR-223 is responsible for decreased IKKα expression in UC patients. Therefore, we validated the interaction between miR-223 and IKKα through *in vitro* studies by performing dual luciferase reporter assay and gene expression assay. In

gene expression assay, a marked decrease in expression of IKKα was observed in cells coexpressing wild type miR-223 along with IKKα-3'UTR with respect to cells coexpressing pBABE-puro empty vector without any miR-223 insert. The IKKα expression again increased to a significant level in cells coexpressing mutant form of miR-223 bearing mutation in its seed region along with IKKα-3'UTR. In consistence with the gene expression assay, we observed marked decrease in luciferase levels in the cells where miR-223 was coexpressed along with IKKα-3'UTR as compared to the cells with empty pBABE-puro vector coexpressed with IKKα-3'UTR. The luciferase level was restored significantly when IKKα-3'UTR was coexpressed with mutant form of miR-223. From the gene expression and dual luciferase reporter assay we concluded that IKKα is a potential target for miR-223 and it negatively regulated its expression. These findings also suggest that the upregulation of miR-223 observed in UC patients could be responsible for the marked decrease in IKKα.

We next investigated whether the miR-223 targeted decrease in IKKα affects the NFκB mediated inflammatory response. MiR-223 was coexpressed in HT29 cells along with IKKα-3'UTR and the cells were stimulated with LPS to activate the NFkB inflammatory pathway. Expression of p65 and expression of downstream pro-inflammatory cytokines IL-8 and IL-1β were monitored by performing qRT-PCR. Both p65 and pro-inflammatory cytokines (IL-8 and IL-1β) showed higher mRNA levels in the cells coexpressed with miR-223 and IKKα-3'UTR as compared to the cells with empty pBABE-puro vector without miR-223 insert and IKKα-3'UTR. Again the cells coexpressed with mutant form of miR-223 and IKKα-3'UTR showed decreased level of both p65 and pro-inflammatory cytokines. From these results we concluded that the negative regulation of NFκB signaling is mediated by IKKα, thereby controlling the inflammatory response. These findings are in consistence with the previous studies where the role of IKKα in resolving NFκB mediated inflammatory response was explored **(Lawrence, Bebien et al. 2005, Lawrence and Bebien 2007)**. We also concluded that the significant upregulation of miR-223 in UC patients contributes to UC pathogenesis by suppressing the expression of IKKα that has a critical role in controlling inflammation. Lastly we concluded that under steady state, both A20 and IKKα rescue the cells from

exaggerated pro-inflammatory response through negative feedback regulation of NFκB (Figure 50). But during UC, these regulatory functions of A20 and IKKα are hampered by miR-125b and miR-223 that effectively modulate their expression and as a result A20 and IKKα fail to restore the normal homeostasis. Since, both miR-125b and miR-223 contributes to UC pathogenesis and are altered in UC patients, these miRNAs could be considered as candidates for developing diagnostic biomarkers for UC to access disease development.

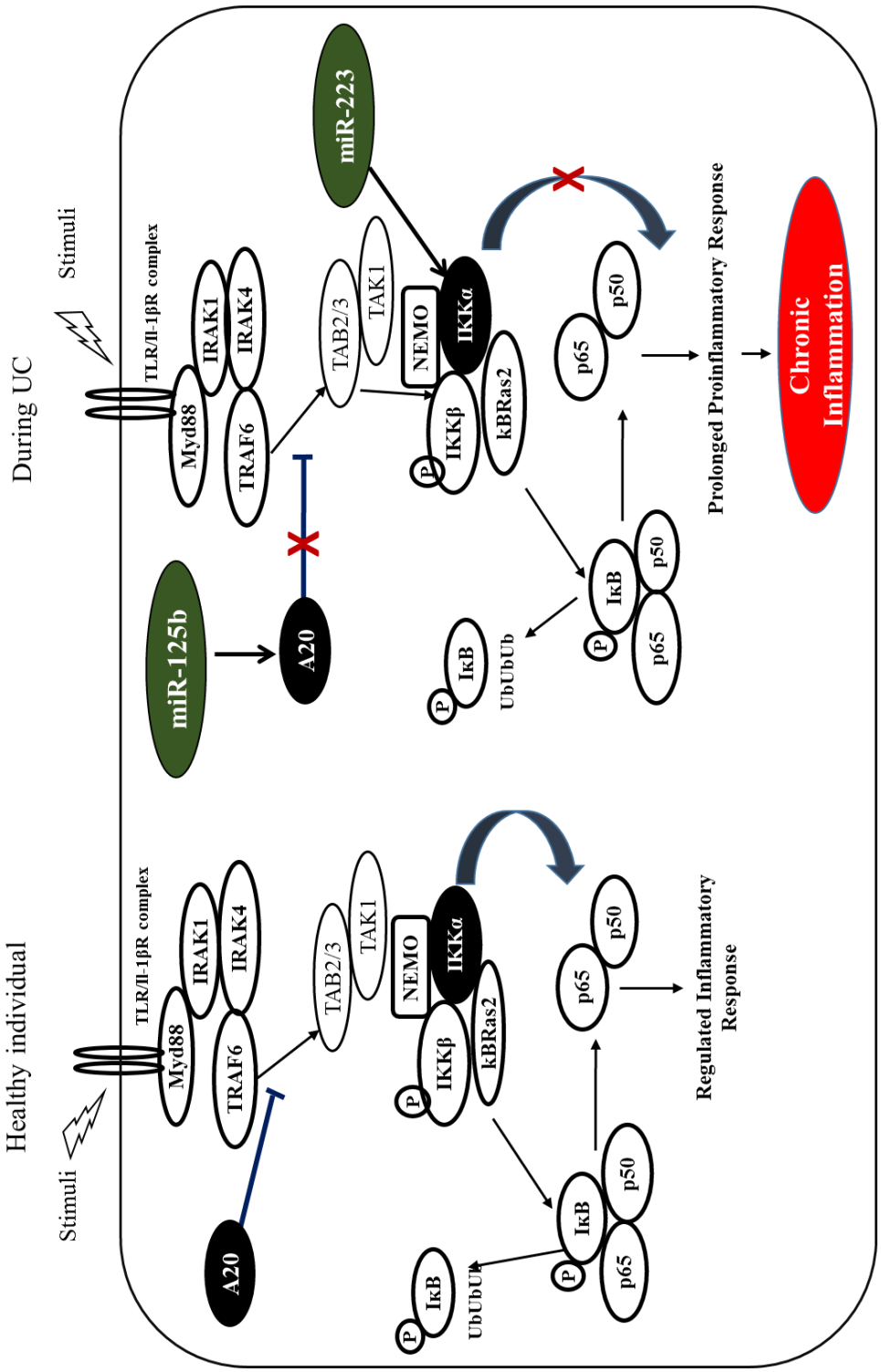


Figure 50: Proposed mechanism showing the role of miRNAs in UC pathogenesis. The TLR mediated activation of NFκB induces the pro-inflammatory response. To maintain the intestinal homeostasis genes such as A20, IKKα and TRAF6 resolve the inflammatory response through negative feedback mechanism. But under disease state such as UC, miR-125b and miR-223 gets upregulated and these miRNAs negatively regulates the genes A20, TRAF6 and IKKα. The miRNA mediated downregulation of A20 and IKKα disturbs the negative feedback regulation carried out by these genes which aggravates the inflammatory response.

SUMMARY

Inflammatory bowel disease is a chronic and relapsing inflammatory disorder of intestine which is idiopathic in nature. It manifests in two major clinical subtypes, ulcerative colitis (UC) and Crohn's disease (CD). The etiology of IBD is not understood but a complex interplay of genetic, microbial and environmental factors is known to result in disease pathogenesis and development. The current understanding of IBD pathogenesis states that, in genetically susceptible individuals an aberrant and exaggerated immune response is generated against the gut microbiota under influence of environmental factors **(Bouma and Strober 2003, Zhang and Li 2014)**. Rectal bleeding, abdominal pain, fever, weight loss and loss of appetite are typical symptoms of IBD while some patients face the extra intestinal manifestations such as arthritis, mouth sores and eye inflammation. The primary goal of current treatment procedures for IBD works on improving the quality life of patient by inducing and maintaining remission in IBD patients. Anti-inflammatory drugs, immunomodulators, anti-TNF therapy, modulators of intestinal flora and antibiotics are employed for IBD treatment. UC and CD share the basic underlying cause of pathogenesis but these two forms can be differentiated on the basis of clinical representations, histological features and colonoscopic observations. UC begins from rectum and progress continuously in upward direction affecting the colon upto a varying degree. The inflammation in case of UC is continuous in manner and show superficial nature. On the basis of inflammation extent UC is characterized as i) proctitis (involving only the rectum), ii) rectosigmoiditis (involving rectum and sigmoid colon), iii) distal colitis (inflammation upto transverse colon) and iv) pancolitis (where whole colon is inflamed) CD can affect any area of the gastrointestinal tract and the inflammation in CD is discontinuous in nature giving it the characteristic skip lesions appearance. In case of CD, the inflammation is transmural in nature which affects the underlying deep layers of the intestine **(Hendrickson, Gokhale et al. 2002, Satsangi, Silverberg et al. 2006)**.

The highest incidence and prevalence rates for IBD are reported from the western countries Europe and North America. Europe has reported a highest annual incidence rate for UC (24.3 per 10^5 per person years) while North America has highest annual incidence rate for CD (20.2 per 10^5 per person years). Similarly, the prevalence of both UC and CD are highest in Europe (505 and 322, per 100,000 per person years respectively **(Ponder**

and Long 2013). In India, the first case of UC was reported in late 1930s and approximately after thirty years the first case of CD was reported **(Makharia, Ramakrishna et al. 2012)**. The first population based in Haryana reported a prevalence rate of $42.8/10^5$ individuals **(Khosla, Girdhar et al. 1986)**. Another study from Punjab, reported the incidence rate of be $6.02/10^5$ per year and prevalence of rate of $44.3/10^5$ inhabitants and these rates were found to be highest form the Asian subcontinent **(Sood, Midha et al. 2003)**.

IBD is a multifactorial disease and microbial, genetic and environmental factors contribute to the pathogenesis of disease. Microbial factors have considerable role in disease pathogenesis as any alterations in the normal gut microbiota can result in disease development. Lesser numbers of *Firmicutes* and *Bacteroidetes* and higher numbers of *Actinobacteria* and *Proteobacteria* have been reported from the fecal samples of IBD patients as compared to healthy individuals **(Qin, Li et al. 2010, Morgan, Tickle et al. 2012)**. Environmental factors such as diet, smoking, sanitation, breast feeding, appendectomy, geographical location, race and ethnicity adds to the predisposition of IBD **(Molodecky and Kaplan 2010)**. The complex interaction between genetic and environmental factor leads to IBD development.

MicroRNAs have recently emerged as critical regulators of inflammation during IBD as they possess the ability to target and regulate the expression of gene involved in major inflammatory pathways associated with disease pathogenesis. Altered miRNA expression has been reported from the blood and mucosal tissues of both UC and CD patients. The site specific alterations in the expression profile of miRNAs have also been reported form UC patients. These alterations in miRNA expression can affect the expression levels of their target genes that subsequently influence the inflammatory response during the disease. In the present study, we investigated the miRNA profile of the inflamed and non-inflamed colonic mucosal region of UC patients and carried out preliminary functional analysis to study the biological relevance of altered miRNA expression.

The results from present studies are summarized below:

1. The miRNA expression profile within the inflamed colonic mucosal region of UC patients differs significantly from the non-inflamed colonic mucosa. These differences in the miRNA expression patterns are more pronounced in the moderate UC patients, while in the mild patients the changes were not comparable. We infer from here that the changes miRNA signatures depend upon the disease severity.
2. MiR-125b, miR-223, miR-155 and miR-138 showed significant higher expression within the inflamed colonic mucosa with respect to the non-inflamed colonic mucosa while miR-378d and miR-194 were down regulated in the inflamed colonic regions to a significant level as compared to the non-inflamed ones.
3. The *in silico* target prediction analysis showed that all these miRNAs which show altered expression in inflamed colonic mucosa bears binding affinity for genes involved in major inflammatory pathways.
4. TRAF6 and A20 bear complementary binding sites for miR-125b within their 3' UTRs at different transcript positions. TRAF6 is an adaptor molecule present downstream of Toll like receptors (TLRs) and it is a crucial molecule required in canonical NFκB pathway. Similarly, A20 has a pivotal role in resolving the NFκB mediated inflammatory response through negative feedback mechanism thereby, maintaining the intestinal homeostasis.
5. IKKα possess complementary binding sites for miR-223 within its 3' UTR. IKKα is a signaling molecule having major regulatory role in the non-canonical NFκB signaling but it has also been reported to negatively regulate the inflammatory response generated through the canonical NFκB pathway.
6. In the qRT-PCR analysis, we observed a significantly upregulated expression of miR-125b within the inflamed colonic mucosa obtained from UC patients as compared to the non-inflamed colonic tissues and non IBD controls. While its target TRAF6 showed a significantly reduced expression within the colonic mucosal tissues of UC patients as compared to the non-IBD controls. Thus, miR-125b and TRAF6 showed inverse relationship with respect to their expression patterns suggesting that miR-125b negatively regulates the TRAF6 expression

7. MiR-223 showed significantly higher expression within the inflamed as well as non-inflamed colonic mucosal tissues from UC patients as compared to the non IBD controls. The higher miR-223 expression within the non-inflamed regions suggests that its expression is independent of disease activity. IKKα, the gene target for miR-223 showed significant down regulation within the mucosal tissues of UC patients as compared to the non-IBD controls. The inverse expression pattern between miR-223 and IKKα indicates that IKKα expression is negatively regulated by miR-223.
8. The interactions between miRNAs with their respective target genes were validated through *in vitro* studies by over expressing the miRNA with its target gene in HT29 cells and monitoring the target gene expression.
9. The dual luciferase and gene expression assays showed that TRAF6 and A20 exerts decrease in their expressions in the presence of miR-125b, while a mutation within the seed region of miR-125b restored the TRAF6 and A20 expression. These results made it evident that miR-125b negatively regulates the TRAF6 and A20 expression and the increased miR-125b expression in UC patients is responsible for the subsequent decrease in TRAF6 and A20 expression during UC.
10. Similarly IKKα showed a marked decrease in its expression in the presence of miR-223 and mutation within the seed region regained the IKKα expression to a significant point. Our results showed that IKKα expression is efficiently regulated by miR-223 and in UC patients the decreased IKKα expression is due to its negative regulation by miR-223.
11. We next investigated the effect of miR-125b and miR-223 overexpression and subsequent downregulation of their target genes on the inflammatory response generated through NFκB signaling. For this we overexpressed the HT29 cells with the 3'UTRs of TRAF6, A20 and IKKα along with their miR-125b and miR-223 respectively. The cells were then stimulated with LPS to activate the TLR mediated NFκB pathway and the expression of p65 and pro-inflammatory cytokine (IL-8 and IL-1β) was monitored.
12. We observed a marked increase in the expression of p65 and pro-inflammatory cytokines (IL-8 and IL-1β) in the HT29 cells overexpressed with the 3'UTR of

TRAF6 and A20 along with miR-125b as compared to the cells overexpressed with the empty vector without miR-125b insert or mutated form of miR-125b.

13. Similarly the cells overexpressed with the 3' UTR of IKKα along with miR-223 resulted in significant higher levels of p65 and pro-inflammatory cytokine with respect to the cells with empty vector without miR-223 insert. Also, mutation within the seed region of miR-223 resulted in increase in p65 and pro-inflammatory cytokines (IL-8 and IL-1β) to a significant level.
14. Form our study we propose that the upregulated expressions of miR-125b and miR-223 mediate the down regulation of TRAF6, A20 and IKKα genes responsible for worsening the inflammatory response in UC patients. Both miR-125b and miR-223 contribute to disease pathogenesis by negatively regulating the expression of genes A20 and IKKα which otherwise helps in the resolution of inflammation and maintenance of intestinal homeostasis.
15. Increased expression of miR-223 in the inflamed as well as non-inflamed colonic mucosa of UC patients suggests that the expression of this miRNA is independent of disease activity. Therefore, miR-223 could be developed as a biomarker for UC to access the disease development in case of UC.
16. We also propose that miR-125b and miR-223 could be considered as potential candidate miRNAs for diagnostic and therapeutic intervention for UC as they contribute to the disease pathogenesis in significant ways.

REFERENCES

- Ahuja, V. and R. K. Tandon (2010). "Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences." J Dig Dis **11**(3): 134-147.
- Alhagamhmad, M. H., A. S. Day, D. A. Lemberg and S. T. Leach (2016). "An overview of the bacterial contribution to Crohn disease pathogenesis." J Med Microbiol **65**(10): 1049-1059.
- Allchin, W. H. (1909). "A Discussion on "Ulcerative Colitis.": Introductory Address." Proc R Soc Med **2**(Med Sect): 59-75.
- Ambros, V. (2003). "MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing." Cell **113**(6): 673-676.
- Amre, D. K., S. D'Souza, K. Morgan, G. Seidman, P. Lambrette, G. Grimard, D. Israel, D. Mack, P. Ghadirian, C. Deslandres, V. Chotard, B. Budai, L. Law, E. Levy and E. G. Seidman (2007). "Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children." Am J Gastroenterol **102**(9): 2016-2025.
- Ananthakrishnan, A. N. (2015). "Epidemiology and risk factors for IBD." Nat Rev Gastroenterol Hepatol **12**(4): 205-217.
- Ananthakrishnan, A. N., H. Khalili, G. G. Konijeti, L. M. Higuchi, P. de Silva, C. S. Fuchs, J. M. Richter, E. S. Schernhammer and A. T. Chan (2014). "Sleep duration affects risk for ulcerative colitis: a prospective cohort study." Clin Gastroenterol Hepatol **12**(11): 1879-1886.
- Ananthakrishnan, A. N., H. Khalili, G. G. Konijeti, L. M. Higuchi, P. de Silva, J. R. Korzenik, C. S. Fuchs, W. C. Willett, J. M. Richter and A. T. Chan (2013). "A prospective study of long-term intake of dietary fiber and risk of Crohn's disease and ulcerative colitis." Gastroenterology **145**(5): 970-977.
- Ananthakrishnan, A. N., H. Khalili, A. Pan, L. M. Higuchi, P. de Silva, J. M. Richter, C. S. Fuchs and A. T. Chan (2013). "Association between depressive symptoms and incidence of Crohn's disease and ulcerative colitis: results from the Nurses' Health Study." Clin Gastroenterol Hepatol **11**(1): 57-62.
- Ananthakrishnan, A. N., M. D. Long, C. F. Martin, R. S. Sandler and M. D. Kappelman (2013). "Sleep disturbance and risk of active disease in patients with

Crohn's disease and ulcerative colitis." Clin Gastroenterol Hepatol **11**(8): 965-971.

- Andersson, R. E., G. Olaison, C. Tysk and A. Ekbom (2003). "Appendectomy is followed by increased risk of Crohn's disease." Gastroenterology **124**(1): 40-46.
- Bakirtzi, K., I. K. Law, X. Xue, D. Iliopoulos, Y. M. Shah and C. Pothoulakis (2016). "Neurotensin Promotes the Development of Colitis and Intestinal Angiogenesis via Hif-1alpha-miR-210 Signaling." J Immunol **196**(10): 4311-4321.
- Baldassano, R. N. and D. A. Piccoli (1999). "Inflammatory bowel disease in pediatric and adolescent patients." Gastroenterol Clin North Am **28**(2): 445-458.
- Ballestar, E., M. Esteller and B. C. Richardson (2006). "The epigenetic face of systemic lupus erythematosus." J Immunol **176**(12): 7143-7147.
- Bamias, G., K. Sugawara, C. Pagnini and F. Cominelli (2003). "The Th1 immune pathway as a therapeutic target in Crohn's disease." Curr Opin Investig Drugs **4**(11): 1279-1286.
- Baron, S., D. Turck, C. Leplat, V. Merle, C. Gower-Rousseau, R. Marti, T. Yzet, E. Lerebours, J. L. Dupas, S. Debeugny, J. L. Salomez, A. Cortot and J. F. Colombel (2005). "Environmental risk factors in paediatric inflammatory bowel diseases: a population based case control study." Gut **54**(3): 357-363.
- Benchimol, E. I., A. Guttmann, A. M. Griffiths, L. Rabeneck, D. R. Mack, H. Brill, J. Howard, J. Guan and T. To (2009). "Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data." Gut **58**(11): 1490-1497.
- Beres, N. J., D. Szabo, D. Kocsis, D. Szucs, Z. Kiss, K. E. Muller, G. Lendvai, A. Kiss, A. Arato, E. Sziksz, A. Vannay, A. J. Szabo and G. Veres (2016). "Role of Altered Expression of miR-146a, miR-155, and miR-122 in Pediatric Patients with Inflammatory Bowel Disease." Inflamm Bowel Dis **22**(2): 327-335.
- Bernstein, C. N. (2015). "Treatment of IBD: where we are and where we are going." Am J Gastroenterol **110**(1): 114-126.

- Bernstein, C. N. and F. Shanahan (2008). "Disorders of a modern lifestyle: reconciling the epidemiology of inflammatory bowel diseases." Gut **57**(9): 1185-1191.
- Bernstein, C. N., S. Singh, L. A. Graff, J. R. Walker, N. Miller and M. Cheang (2010). "A prospective population-based study of triggers of symptomatic flares in IBD." Am J Gastroenterol **105**(9): 1994-2002.
- Bopanna, S., A. N. Ananthakrishnan, S. Kedia, V. Yajnik and V. Ahuja (2017). "Risk of colorectal cancer in Asian patients with ulcerative colitis: a systematic review and meta-analysis." Lancet Gastroenterol Hepatol **2**(4): 269-276.
- Bouma, G. and W. Strober (2003). "The immunological and genetic basis of inflammatory bowel disease." Nat Rev Immunol **3**(7): 521-533.
- Brennecke, J., A. Stark, R. B. Russell and S. M. Cohen (2005). "Principles of microRNA-target recognition." PLoS Biol **3**(3): e85.
- Brest, P., P. Lapaquette, M. Souidi, K. Lebrigand, A. Cesaro, V. Vouret-Craviari, B. Mari, P. Barbry, J. F. Mosnier, X. Hebuterne, A. Harel-Bellan, B. Mograbi, A. Darfeuille-Michaud and P. Hofman (2011). "A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease." Nat Genet **43**(3): 242-245.
- Brown, K., D. DeCoffe, E. Molcan and D. L. Gibson (2012). "Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease." Nutrients **4**(8): 1095-1119.
- Buisine, M. P., P. Desreumaux, V. Debailleul, L. Gambiez, K. Geboes, N. Ectors, M. P. Delescaut, P. Degand, J. P. Aubert, J. F. Colombel and N. Porchet (1999). "Abnormalities in mucin gene expression in Crohn's disease." Inflamm Bowel Dis **5**(1): 24-32.
- Cai, M., S. Chen and W. Hu (2017). "MicroRNA-141 Is Involved in Ulcerative Colitis Pathogenesis via Aiming at CXCL5." J Interferon Cytokine Res **37**(9): 415-420.
- Cao, B., X. Zhou, J. Ma, W. Zhou, W. Yang, D. Fan and L. Hong (2017). "Role of MiRNAs in Inflammatory Bowel Disease." Dig Dis Sci **62**(6): 1426-1438.

- Card, T., R. F. Logan, L. C. Rodrigues and J. G. Wheeler (2004). "Antibiotic use and the development of Crohn's disease." Gut **53**(2): 246-250.
- Carter, M. J., A. J. Lobo, S. P. Travis and B. S. o. G. Ibd Section (2004). "Guidelines for the management of inflammatory bowel disease in adults." Gut **53 Suppl 5**: V1-16.
- Ceppi, M., P. M. Pereira, I. Dunand-Sauthier, E. Barras, W. Reith, M. A. Santos and P. Pierre (2009). "MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells." Proc Natl Acad Sci U S A **106**(8): 2735-2740.
- Chapman, C. G. and J. Pekow (2015). "The emerging role of miRNAs in inflammatory bowel disease: a review." Therap Adv Gastroenterol **8**(1): 4-22.
- Chen, B., S. She, D. Li, Z. Liu, X. Yang, Z. Zeng and F. Liu (2013). "Role of miR-19a targeting TNF-alpha in mediating ulcerative colitis." Scand J Gastroenterol **48**(7): 815-824.
- Chen, C., D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak and K. J. Guegler (2005). "Real-time quantification of microRNAs by stem-loop RT-PCR." Nucleic Acids Res **33**(20): e179.
- Chen, J. Q., G. Papp, P. Szodoray and M. Zeher (2016). "The role of microRNAs in the pathogenesis of autoimmune diseases." Autoimmun Rev **15**(12): 1171-1180.
- Cheng, X., X. Zhang, J. Su, Y. Zhang, W. Zhou, J. Zhou, C. Wang, H. Liang, X. Chen, R. Shi, K. Zen, C. Y. Zhang and H. Zhang (2015). "miR-19b downregulates intestinal SOCS3 to reduce intestinal inflammation in Crohn's disease." Sci Rep **5**: 10397.
- Choi, H. J. and J. S. Han (2012). "Overexpression of phospholipase D enhances Bcl-2 expression by activating STAT3 through independent activation of ERK and p38MAPK in HeLa cells." Biochim Biophys Acta **1823**(6): 1082-1091.
- Cohen, R. D., R. Stein and S. B. Hanauer (1999). "Intravenous cyclosporin in ulcerative colitis: a five-year experience." Am J Gastroenterol **94**(6): 1587-1592.

- Cornish, J. A., E. Tan, C. Simillis, S. K. Clark, J. Teare and P. P. Tekkis (2008). "The risk of oral contraceptives in the etiology of inflammatory bowel disease: a meta-analysis." Am J Gastroenterol **103**(9): 2394-2400.
- Coskun, M., J. T. Bjerrum, J. B. Seidelin, J. T. Troelsen, J. Olsen and O. H. Nielsen (2013). "miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis." World J Gastroenterol **19**(27): 4289-4299.
- Cosnes, J., L. Beaugerie, F. Carbonnel and J. P. Gendre (2001). "Smoking cessation and the course of Crohn's disease: an intervention study." Gastroenterology **120**(5): 1093-1099.
- Cosnes, J., F. Carbonnel, F. Carrat, L. Beaugerie, S. Cattan and J. Gendre (1999). "Effects of current and former cigarette smoking on the clinical course of Crohn's disease." Aliment Pharmacol Ther **13**(11): 1403-1411.
- Crohn, B. B., L. Ginzburg and G. D. Oppenheimer (1952). "Regional ileitis; a pathologic and clinical entity." Am J Med **13**(5): 583-590.
- Cutolo, M., S. Capellino, A. Sulli, B. Serioli, M. E. Secchi, B. Villaggio and R. H. Straub (2006). "Estrogens and autoimmune diseases." Ann N Y Acad Sci **1089**: 538-547.
- Dalal, S. R. and J. H. Kwon (2010). "The Role of MicroRNA in Inflammatory Bowel Disease." Gastroenterol Hepatol (N Y) **6**(11): 714-722.
- Dallegri, F., L. Ottonello, A. Ballestrero, F. Bogliolo, F. Ferrando and F. Patrone (1990). "Cytoprotection against neutrophil derived hypochlorous acid: a potential mechanism for the therapeutic action of 5-aminosalicylic acid in ulcerative colitis." Gut **31**(2): 184-186.
- Dalziel, T. K. (1989). "Thomas Kennedy Dalziel 1861-1924. Chronic interstitial enteritis." Dis Colon Rectum **32**(12): 1076-1078.
- Damaskos, D. and G. Kolios (2008). "Probiotics and prebiotics in inflammatory bowel disease: microflora 'on the scope'." Br J Clin Pharmacol **65**(4): 453-467.

- Das, K., U. C. Ghoshal, G. K. Dhali, J. Benjamin, V. Ahuja and G. K. Makharia (2009). "Crohn's disease in India: a multicenter study from a country where tuberculosis is endemic." Dig Dis Sci **54**(5): 1099-1107.
- De Hertogh, G., J. Aerssens, K. P. Geboes and K. Geboes (2008). "Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease." World J Gastroenterol **14**(6): 845-852.
- Diefenbach, K. A. and C. K. Breuer (2006). "Pediatric inflammatory bowel disease." World J Gastroenterol **12**(20): 3204-3212.
- Dignass, A., R. Eliakim, F. Magro, C. Maaser, Y. Chowers, K. Geboes, G. Mantzaris, W. Reinisch, J. F. Colombel, S. Vermeire, S. Travis, J. O. Lindsay and G. Van Assche (2012). "Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis." J Crohns Colitis **6**(10): 965-990.
- Du, C., C. Liu, J. Kang, G. Zhao, Z. Ye, S. Huang, Z. Li, Z. Wu and G. Pei (2009). "MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis." Nat Immunol **10**(12): 1252-1259.
- Duerr, R. H., K. D. Taylor, S. R. Brant, J. D. Rioux, M. S. Silverberg, M. J. Daly, A. H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, T. Dassopoulos, A. Bitton, H. Yang, S. Targan, L. W. Datta, E. O. Kistner, L. P. Schumm, A. T. Lee, P. K. Gregersen, M. M. Barmada, J. I. Rotter, D. L. Nicolae and J. H. Cho (2006). "A genome-wide association study identifies IL23R as an inflammatory bowel disease gene." Science **314**(5804): 1461-1463.
- Dweep, H., C. Sticht, P. Pandey and N. Gretz (2011). "miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes." J Biomed Inform **44**(5): 839-847.
- Eaden, J. A., K. R. Abrams and J. F. Mayberry (2001). "The risk of colorectal cancer in ulcerative colitis: a meta-analysis." Gut **48**(4): 526-535.
- Eastaff-Leung, N., N. Mabarrack, A. Barbour, A. Cummins and S. Barry (2010). "Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease." J Clin Immunol **30**(1): 80-89.

- Ebert, M. S. and P. A. Sharp (2010). "MicroRNA sponges: progress and possibilities." RNA **16**(11): 2043-2050.
- Egan, L. J., D. C. Mays, C. J. Huntoon, M. P. Bell, M. G. Pike, W. J. Sandborn, J. J. Lipsky and D. J. McKean (1999). "Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity." J Biol Chem **274**(37): 26448-26453.
- Egwuagu, C. E. (2009). "STAT3 in CD4+ T helper cell differentiation and inflammatory diseases." Cytokine **47**(3): 149-156.
- Ehrt, S. and D. Schnappinger (2003). "Isolation of plasmids from E. coli by alkaline lysis." Methods Mol Biol **235**: 75-78.
- Ek, W. E., M. D'Amato and J. Halfvarson (2014). "The history of genetics in inflammatory bowel disease." Ann Gastroenterol **27**(4): 294-303.
- Esau, C. C. (2008). "Inhibition of microRNA with antisense oligonucleotides." Methods **44**(1): 55-60.
- Farmer, R. G., W. A. Hawk and R. B. Turnbull, Jr. (1975). "Clinical patterns in Crohn's disease: a statistical study of 615 cases." Gastroenterology **68**(4 Pt 1): 627-635.
- Fasseu, M., X. Treton, C. Guichard, E. Pedruzzi, D. Cazals-Hatem, C. Richard, T. Aparicio, F. Daniel, J. C. Soule, R. Moreau, Y. Bouhnik, M. Laburthe, A. Groyer and E. Ogier-Denis (2010). "Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease." PLoS One **5**(10).
- Feagan, B. G. and J. K. Macdonald (2012). "Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis." Cochrane Database Syst Rev **10**: CD000543.
- Feagan, B. G., J. W. McDonald, J. Rochon, A. Laupacis, R. N. Fedorak, D. Kinnear, F. Saibil, A. Groll, A. Archambault, R. Gillies and et al. (1994). "Low-dose cyclosporine for the treatment of Crohn's disease. The Canadian Crohn's Relapse Prevention Trial Investigators." N Engl J Med **330**(26): 1846-1851.

- Felder, J. B., B. I. Korelitz, R. Rajapakse, S. Schwarz, A. P. Horatagis and G. Gleim (2000). "Effects of nonsteroidal antiinflammatory drugs on inflammatory bowel disease: a case-control study." Am J Gastroenterol **95**(8): 1949-1954.
- Fina, D., M. Sarra, M. C. Fantini, A. Rizzo, R. Caruso, F. Caprioli, C. Stolfi, I. Cardolini, M. Dottori, M. Boirivant, F. Pallone, T. T. Macdonald and G. Monteleone (2008). "Regulation of gut inflammation and th17 cell response by interleukin-21." Gastroenterology **134**(4): 1038-1048.
- Frank, D. N., A. L. St Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz and N. R. Pace (2007). "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases." Proc Natl Acad Sci U S A **104**(34): 13780-13785.
- Fujimoto, T., H. Imaeda, K. Takahashi, E. Kasumi, S. Bamba, Y. Fujiyama and A. Andoh (2013). "Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease." J Gastroenterol Hepatol **28**(4): 613-619.
- Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba and Y. Fujiyama (2003). "Increased expression of interleukin 17 in inflammatory bowel disease." Gut **52**(1): 65-70.
- Gallo, A., M. Tandon, I. Alevizos and G. G. Illei (2012). "The majority of microRNAs detectable in serum and saliva is concentrated in exosomes." PLoS One **7**(3): e30679.
- Galvez, J. (2014). "Role of Th17 Cells in the Pathogenesis of Human IBD." ISRN Inflamm **2014**: 928461.
- Ganji-Arjenaki, M. and M. Rafieian-Kopaei (2018). "Probiotics are a good choice in remission of inflammatory bowel diseases: A meta analysis and systematic review." J Cell Physiol **233**(3): 2091-2103.
- Garland, C. F., A. M. Lilienfeld, A. I. Mendeloff, J. A. Markowitz, K. B. Terrell and F. C. Garland (1981). "Incidence rates of ulcerative colitis and Crohn's disease in fifteen areas of the United States." Gastroenterology **81**(6): 1115-1124.
- Gazouli, M., I. Papaconstantinou, K. Stamatis, A. Vaiopoulou, C. Zeglinas, I. Vassiliou, G. Giokas and C. Tzathas (2013). "Association study of genetic

variants in miRNAs in patients with inflammatory bowel disease: preliminary results." Dig Dis Sci **58**(8): 2324-2328.

- Geerling, B. J., P. C. Dagnelie, A. Badart-Smook, M. G. Russel, R. W. Stockbrugger and R. J. Brummer (2000). "Diet as a risk factor for the development of ulcerative colitis." Am J Gastroenterol **95**(4): 1008-1013.
- Geng, X. and J. Xue (2016). "Expression of Treg/Th17 cells as well as related cytokines in patients with inflammatory bowel disease." Pak J Med Sci **32**(5): 1164-1168.
- Gent, A. E., M. D. Hellier, R. H. Grace, E. T. Swarbrick and D. Coggon (1994). "Inflammatory bowel disease and domestic hygiene in infancy." Lancet **343**(8900): 766-767.
- Gloria, L., M. Cravo, A. Pinto, L. S. de Sousa, P. Chaves, C. N. Leitao, M. Quina, F. C. Mira and J. Soares (1996). "DNA hypomethylation and proliferative activity are increased in the rectal mucosa of patients with long-standing ulcerative colitis." Cancer **78**(11): 2300-2306.
- Guindi, M. and R. H. Riddell (2004). "Indeterminate colitis." J Clin Pathol **57**(12): 1233-1244.
- He, C., Y. Shi, R. Wu, M. Sun, L. Fang, W. Wu, C. Liu, M. Tang, Z. Li, P. Wang, Y. Cong and Z. Liu (2016). "miR-301a promotes intestinal mucosal inflammation through induction of IL-17A and TNF-alpha in IBD." Gut **65**(12): 1938-1950.
- Hendrickson, B. A., R. Gokhale and J. H. Cho (2002). "Clinical aspects and pathophysiology of inflammatory bowel disease." Clin Microbiol Rev **15**(1): 79-94.
- Heyninck, K., D. De Valck, W. Vanden Berghe, W. Van Criekinge, R. Contreras, W. Fiers, G. Haegeman and R. Beyaert (1999). "The zinc finger protein A20 inhibits TNF-induced NF-kappaB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-kappaB-inhibiting protein ABIN." J Cell Biol **145**(7): 1471-1482.
- Higuchi, L. M., H. Khalili, A. T. Chan, J. M. Richter, A. Bousvaros and C. S. Fuchs (2012). "A prospective study of cigarette smoking and the risk of inflammatory bowel disease in women." Am J Gastroenterol **107**(9): 1399-1406.

- Hillman, E. T., H. Lu, T. Yao and C. H. Nakatsu (2017). "Microbial Ecology along the Gastrointestinal Tract." Microbes Environ **32**(4): 300-313.
- Huang, Z., T. Shi, Q. Zhou, S. Shi, R. Zhao, H. Shi, L. Dong, C. Zhang, K. Zeng, J. Chen and J. Zhang (2014). "miR-141 Regulates colonic leukocytic trafficking by targeting CXCL12beta during murine colitis and human Crohn's disease." Gut **63**(8): 1247-1257.
- Iborra, M., F. Bernuzzi, C. Correale, S. Vetrano, G. Fiorino, B. Beltran, F. Marabita, M. Locati, A. Spinelli, P. Nos, P. Invernizzi and S. Danese (2013). "Identification of serum and tissue micro-RNA expression profiles in different stages of inflammatory bowel disease." Clin Exp Immunol **173**(2): 250-258.
- Iliopoulos, D., S. A. Jaeger, H. A. Hirsch, M. L. Bulyk and K. Struhl (2010). "STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer." Mol Cell **39**(4): 493-506.
- Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J. P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini and C. M. Croce (2005). "MicroRNA gene expression deregulation in human breast cancer." Cancer Res **65**(16): 7065-7070.
- Jayanthi, V., C. S. Probert, D. Pinder, A. C. Wicks and J. F. Mayberry (1992). "Epidemiology of Crohn's disease in Indian migrants and the indigenous population in Leicestershire." Q J Med **82**(298): 125-138.
- Jess, T., C. Rungoe and L. Peyrin-Biroulet (2012). "Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies." Clin Gastroenterol Hepatol **10**(6): 639-645.
- Joossens, M., G. Huys, M. Cnockaert, V. De Preter, K. Verbeke, P. Rutgeerts, P. Vandamme and S. Vermeire (2011). "Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives." Gut **60**(5): 631-637.
- Kanaan, Z., S. N. Rai, M. R. Eichenberger, C. Barnes, A. M. Dworkin, C. Weller, E. Cohen, H. Roberts, B. Keskey, R. E. Petras, N. P. Crawford and S. Galandiuk (2012). "Differential microRNA expression tracks neoplastic progression in

inflammatory bowel disease-associated colorectal cancer." Hum Mutat **33**(3): 551-560.

- Kedia, S. and V. Ahuja (2017). "Epidemiology of Inflammatory Bowel Disease in India: The Great Shift East." Inflamm Intest Dis **2**(2): 102-115.
- Khor, B., A. Gardet and R. J. Xavier (2011). "Genetics and pathogenesis of inflammatory bowel disease." Nature **474**(7351): 307-317.
- Khor, B., A. Gardet and R. J. Xavier (2011). "Genetics and pathogenesis of inflammatory bowel disease." Nature **474**(7351): 307-317.
- Khosla, S. N., N. K. Girdhar, S. Lal and D. S. Mishra (1986). "Epidemiology of ulcerative colitis in hospital and select general population of northern India." J Assoc Physicians India **34**(6): 405-407.
- Kim, D. H. and J. H. Cheon (2017). "Pathogenesis of Inflammatory Bowel Disease and Recent Advances in Biologic Therapies." Immune Netw **17**(1): 25-40.
- Kim, H. Y., H. Y. Kwon, H. T. Ha Thi, H. J. Lee, G. I. Kim, K. B. Hahm and S. Hong (2016). "MicroRNA-132 and microRNA-223 control positive feedback circuit by regulating FOXO3a in inflammatory bowel disease." J Gastroenterol Hepatol **31**(10): 1727-1735.
- Kirsner, J. B. (1998). "Historical origins of medical and surgical therapy of inflammatory bowel disease." Lancet **352**(9136): 1303-1305.
- Klement, E., R. V. Cohen, J. Boxman, A. Joseph and S. Reif (2004). "Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis." Am J Clin Nutr **80**(5): 1342-1352.
- Kong, W., L. He, E. J. Richards, S. Challa, C. X. Xu, J. Permuth-Wey, J. M. Lancaster, D. Coppola, T. A. Sellers, J. Y. Djeu and J. Q. Cheng (2014). "Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer." Oncogene **33**(6): 679-689.
- Kozomara, A. and S. Griffiths-Jones (2011). "miRBase: integrating microRNA annotation and deep-sequencing data." Nucleic Acids Res **39**(Database issue): D152-157.

- Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel and N. Rajewsky (2005). "Combinatorial microRNA target predictions." Nat Genet **37**(5): 495-500.
- Kruis, W., P. Fric, J. Pokrotnieks, M. Lukas, B. Fixa, M. Kascak, M. A. Kamm, J. Weismueller, C. Beglinger, M. Stolte, C. Wolff and J. Schulze (2004). "Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine." Gut **53**(11): 1617-1623.
- Lakatos, P. L. (2006). "Recent trends in the epidemiology of inflammatory bowel diseases: up or down?" World J Gastroenterol **12**(38): 6102-6108.
- Lakatos, P. L., T. Szamosi and L. Lakatos (2007). "Smoking in inflammatory bowel diseases: good, bad or ugly?" World J Gastroenterol **13**(46): 6134-6139.
- Larsson, J. M., H. Karlsson, J. G. Crespo, M. E. Johansson, L. Eklund, H. Sjovall and G. C. Hansson (2011). "Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation." Inflamm Bowel Dis **17**(11): 2299-2307.
- Lauritsen, K., L. S. Laursen, K. Bukhave and J. Rask-Madsen (1986). "Effects of topical 5-aminosalicylic acid and prednisolone on prostaglandin E2 and leukotriene B4 levels determined by equilibrium in vivo dialysis of rectum in relapsing ulcerative colitis." Gastroenterology **91**(4): 837-844.
- Lawrence, T. and M. Bebien (2007). "IKKalpha in the regulation of inflammation and adaptive immunity." Biochem Soc Trans **35**(Pt 2): 270-272.
- Lawrence, T., M. Bebien, G. Y. Liu, V. Nizet and M. Karin (2005). "IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation." Nature **434**(7037): 1138-1143.
- Lee, E. G., D. L. Boone, S. Chai, S. L. Libby, M. Chien, J. P. Lodolce and A. Ma (2000). "Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice." Science **289**(5488): 2350-2354.
- Lei, S. L., H. Zhao, H. L. Yao, Y. Chen, Z. D. Lei, K. J. Liu and Q. Yang (2014). "Regulatory roles of microRNA-708 and microRNA-31 in proliferation, apoptosis and invasion of colorectal cancer cells." Oncol Lett **8**(4): 1768-1774.

- Lennox, K. A. and M. A. Behlke (2011). "Chemical modification and design of anti-miRNA oligonucleotides." Gene Ther **18**(12): 1111-1120.
- Lewis, B. P., C. B. Burge and D. P. Bartel (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.
- Li, Z. and T. M. Rana (2014). "Therapeutic targeting of microRNAs: current status and future challenges." Nat Rev Drug Discov **13**(8): 622-638.
- Liao, G., M. Zhang, E. W. Harhaj and S. C. Sun (2004). "Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation." J Biol Chem **279**(25): 26243-26250.
- Lin, K. T., Y. M. Yeh, C. M. Chuang, S. Y. Yang, J. W. Chang, S. P. Sun, Y. S. Wang, K. C. Chao and L. H. Wang (2015). "Glucocorticoids mediate induction of microRNA-708 to suppress ovarian cancer metastasis through targeting Rap1B." Nat Commun **6**: 5917.
- Liu, L., C. Zhang, X. Li, W. Sun, S. Qin, L. Qin and X. Wang (2017). "miR-223 promotes colon cancer by directly targeting p120 catenin." Oncotarget **8**(38): 63764-63779.
- Liu, Z., P. K. Yadav, X. Xu, J. Su, C. Chen, M. Tang, H. Lin, J. Yu, J. Qian, P. C. Yang and X. Wang (2011). "The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity." J Leukoc Biol **89**(4): 597-606.
- Loddo, I. and C. Romano (2015). "Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis." Front Immunol **6**: 551.
- Lok, K. H., H. G. Hung, C. H. Ng, K. C. Kwong, W. M. Yip, S. F. Lau, K. K. Li, K. F. Li and M. L. Szeto (2008). "Epidemiology and clinical characteristics of ulcerative colitis in Chinese population: experience from a single center in Hong Kong." J Gastroenterol Hepatol **23**(3): 406-410.
- Long, D., R. Lee, P. Williams, C. Y. Chan, V. Ambros and Y. Ding (2007). "Potent effect of target structure on microRNA function." Nat Struct Mol Biol **14**(4): 287-294.

- Looijer-van Langen, M. A. and L. A. Dieleman (2009). "Prebiotics in chronic intestinal inflammation." Inflamm Bowel Dis **15**(3): 454-462.
- Lovato, P., C. Brender, J. Agnholt, J. Kelsen, K. Kaltoft, A. Svejgaard, K. W. Eriksen, A. Woetmann and N. Odum (2003). "Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease." J Biol Chem **278**(19): 16777-16781.
- Lovendorf, M. B., H. Mitsui, J. R. Zibert, M. A. Ropke, M. Hafner, B. Dyring-Andersen, C. M. Bonefeld, J. G. Krueger and L. Skov (2015). "Laser capture microdissection followed by next-generation sequencing identifies disease-related microRNAs in psoriatic skin that reflect systemic microRNA changes in psoriasis." Exp Dermatol **24**(3): 187-193.
- Lu, L. F. and A. Liston (2009). "MicroRNA in the immune system, microRNA as an immune system." Immunology **127**(3): 291-298.
- Lujambio, A., G. A. Calin, A. Villanueva, S. Ropero, M. Sanchez-Cespedes, D. Blanco, L. M. Montuenga, S. Rossi, M. S. Nicoloso, W. J. Faller, W. M. Gallagher, S. A. Eccles, C. M. Croce and M. Esteller (2008). "A microRNA DNA methylation signature for human cancer metastasis." Proc Natl Acad Sci U S A **105**(36): 13556-13561.
- Luther, J., M. Dave, P. D. Higgins and J. Y. Kao (2010). "Association between Helicobacter pylori infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature." Inflamm Bowel Dis **16**(6): 1077-1084.
- M'Koma, A. E. (2013). "Inflammatory bowel disease: an expanding global health problem." Clin Med Insights Gastroenterol **6**: 33-47.
- MacDermott, R. P., G. S. Nash, M. J. Bertovich, M. V. Seiden, M. J. Bragdon and M. G. Beale (1981). "Alterations of IgM, IgG, and IgA Synthesis and secretion by peripheral blood and intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease." Gastroenterology **81**(5): 844-852.
- Majumdar, I., V. Ahuja and J. Paul (2017). "Altered expression of Tumor Necrosis Factor Alpha -Induced Protein 3 correlates with disease severity in Ulcerative Colitis." Sci Rep **7**(1): 9420.

- Makharia, G. K., B. S. Ramakrishna, P. Abraham, G. Choudhuri, S. P. Misra, V. Ahuja, S. J. Bhatia, D. K. Bhasin, S. Dadhich, G. K. Dhali, D. C. Desai, U. C. Ghoshal, B. D. Goswami, S. K. Issar, A. K. Jain, V. Jayanthi, G. Loganathan, C. G. Pai, A. S. Puri, S. S. Rana, G. Ray, S. P. Singh, A. Sood and D. Indian Society of Gastroenterology Task Force on Inflammatory Bowel (2012). "Survey of inflammatory bowel diseases in India." Indian J Gastroenterol **31**(6): 299-306.
- Maloy, K. J. and F. Powrie (2011). "Intestinal homeostasis and its breakdown in inflammatory bowel disease." Nature **474**(7351): 298-306.
- Matsuoka, K., E. Saito, T. Fujii, K. Takenaka, M. Kimura, M. Nagahori, K. Ohtsuka and M. Watanabe (2015). "Tacrolimus for the Treatment of Ulcerative Colitis." Intest Res **13**(3): 219-226.
- Mayne, C. G. and C. B. Williams (2013). "Induced and natural regulatory T cells in the development of inflammatory bowel disease." Inflamm Bowel Dis **19**(8): 1772-1788.
- Min, M., L. Peng, Y. Yang, M. Guo, W. Wang and G. Sun (2014). "MicroRNA-155 is involved in the pathogenesis of ulcerative colitis by targeting FOXO3a." Inflamm Bowel Dis **20**(4): 652-659.
- Mohammadi, A., O. B. Kelly, M. Filice, B. Kabakchiev, M. I. Smith and M. S. Silverberg (2018). "Differential Expression of microRNAs in Peripheral Blood Mononuclear Cells Identifies Autophagy and TGF-Beta-Related Signatures Aberrantly Expressed in Inflammatory Bowel Disease." J Crohns Colitis **12**(5): 568-581.
- Molodecky, N. A. and G. G. Kaplan (2010). "Environmental risk factors for inflammatory bowel disease." Gastroenterol Hepatol (N Y) **6**(5): 339-346.
- Molodecky, N. A., I. S. Soon, D. M. Rabi, W. A. Ghali, M. Ferris, G. Chernoff, E. I. Benchimol, R. Panaccione, S. Ghosh, H. W. Barkema and G. G. Kaplan (2012). "Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review." Gastroenterology **142**(1): 46-54 e42; quiz e30.
- Monteleone, G., I. Monteleone, D. Fina, P. Vavassori, G. Del Vecchio Blanco, R. Caruso, R. Tersigni, L. Alessandroni, L. Biancone, G. C. Naccari, T. T. MacDonald and F. Pallone (2005). "Interleukin-21 enhances T-helper cell type I

signaling and interferon-gamma production in Crohn's disease." Gastroenterology **128**(3): 687-694.

- Morgan, X. C., T. L. Tickle, H. Sokol, D. Gevers, K. L. Devaney, D. V. Ward, J. A. Reyes, S. A. Shah, N. LeLeiko, S. B. Snapper, A. Bousvaros, J. Korzenik, B. E. Sands, R. J. Xavier and C. Huttenhower (2012). "Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment." Genome Biol **13**(9): R79.
- Mulder, D. J., A. J. Noble, C. J. Justinich and J. M. Duffin (2014). "A tale of two diseases: the history of inflammatory bowel disease." J Crohns Colitis **8**(5): 341-348.
- Mummery, L. (1909). "A Discussion on Ulcerative Colitis." Proc R Soc Med **2**(Med Sect): 92-94.
- Murata, K., H. Yoshitomi, S. Tanida, M. Ishikawa, K. Nishitani, H. Ito and T. Nakamura (2010). "Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis." Arthritis Res Ther **12**(3): R86.
- Murch, S. H., R. Baldassano, H. Buller, S. Chin, A. M. Griffiths, H. Hildebrand, C. Jasinsky, T. Kong, D. Moore, M. Orsi, G. Commonwealth Association of Paediatric and Nutrition (2004). "Inflammatory bowel disease: Working Group report of the second World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition." J Pediatr Gastroenterol Nutr **39 Suppl 2**: S647-654.
- Musso, A., P. Dentelli, A. Carlino, L. Chiusa, A. Repici, A. Sturm, C. Fiocchi, M. Rizzetto, L. Pegoraro, C. Sategna-Guidetti and M. F. Brizzi (2005). "Signal transducers and activators of transcription 3 signaling pathway: an essential mediator of inflammatory bowel disease and other forms of intestinal inflammation." Inflamm Bowel Dis **11**(2): 91-98.
- Neudecker, V., M. Haneklaus, O. Jensen, L. Khailova, J. C. Masterson, H. Tye, K. Biette, P. Jedlicka, K. S. Brodsky, M. E. Gerich, M. Mack, A. A. B. Robertson, M. A. Cooper, G. T. Furuta, C. A. Dinarello, L. A. O'Neill, H. K. Eltzschig, S. L. Masters and E. N. McNamee (2017). "Myeloid-derived miR-223 regulates

intestinal inflammation via repression of the NLRP3 inflammasome." J Exp Med **214**(6): 1737-1752.

- Neurath, M. F. (2014). "Cytokines in inflammatory bowel disease." Nat Rev Immunol **14**(5): 329-342.
- Ng, R., G. Song, G. R. Roll, N. M. Frandsen and H. Willenbring (2012). "A microRNA-21 surge facilitates rapid cyclin D1 translation and cell cycle progression in mouse liver regeneration." J Clin Invest **122**(3): 1097-1108.
- Ng, S. C., H. Y. Shi, N. Hamidi, F. E. Underwood, W. Tang, E. I. Benchimol, R. Panaccione, S. Ghosh, J. C. Y. Wu, F. K. L. Chan, J. J. Y. Sung and G. G. Kaplan (2018). "Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies." Lancet **390**(10114): 2769-2778.
- Nielsen, O. H., M. Coskun, C. Steenholdt and G. Rogler (2015). "The role and advances of immunomodulator therapy for inflammatory bowel disease." Expert Rev Gastroenterol Hepatol **9**(2): 177-189.
- Nishida, A., R. Inoue, O. Inatomi, S. Bamba, Y. Naito and A. Andoh (2018). "Gut microbiota in the pathogenesis of inflammatory bowel disease." Clin J Gastroenterol **11**(1): 1-10.
- Nishida, N., T. Yokobori, K. Mimori, T. Sudo, F. Tanaka, K. Shibata, H. Ishii, Y. Doki, H. Kuwano and M. Mori (2011). "MicroRNA miR-125b is a prognostic marker in human colorectal cancer." Int J Oncol **38**(5): 1437-1443.
- Nitzan, O., M. Elias, A. Peretz and W. Saliba (2016). "Role of antibiotics for treatment of inflammatory bowel disease." World J Gastroenterol **22**(3): 1078-1087.
- Noronha, A. M., Y. Liang, J. T. Hetzel, H. Hasturk, A. Kantarci, A. Stucchi, Y. Zhang, B. S. Nikolajczyk, F. A. Farraye and L. M. Ganley-Leal (2009). "Hyperactivated B cells in human inflammatory bowel disease." J Leukoc Biol **86**(4): 1007-1016.
- Ohkusa, T., K. Kato, S. Terao, T. Chiba, K. Mabe, K. Murakami, Y. Mizokami, T. Sugiyama, A. Yanaka, Y. Takeuchi, S. Yamato, T. Yokoyama, I. Okayasu, S. Watanabe, H. Tajiri, N. Sato and U. C. A. T. S. G. Japan (2010). "Newly

developed antibiotic combination therapy for ulcerative colitis: a double-blind placebo-controlled multicenter trial." Am J Gastroenterol **105**(8): 1820-1829.

- Olaru, A. V., F. M. Selaru, Y. Mori, C. Vazquez, S. David, B. Paun, Y. Cheng, Z. Jin, J. Yang, R. Agarwal, J. M. Abraham, T. Dassopoulos, M. Harris, T. M. Bayless, J. Kwon, N. Harpaz, F. Livak and S. J. Meltzer (2011). "Dynamic changes in the expression of MicroRNA-31 during inflammatory bowel disease-associated neoplastic transformation." Inflamm Bowel Dis **17**(1): 221-231.
- Omenetti, S. and T. T. Pizarro (2015). "The Treg/Th17 Axis: A Dynamic Balance Regulated by the Gut Microbiome." Front Immunol **6**: 639.
- Paramsothy, S., R. Paramsothy, D. T. Rubin, M. A. Kamm, N. O. Kaakoush, H. M. Mitchell and N. Castano-Rodriguez (2017). "Faecal Microbiota Transplantation for Inflammatory Bowel Disease: A Systematic Review and Meta-analysis." J Crohns Colitis **11**(10): 1180-1199.
- Paraskevi, A., G. Theodoropoulos, I. Papaconstantinou, G. Mantzaris, N. Nikiteas and M. Gazouli (2012). "Circulating MicroRNA in inflammatory bowel disease." J Crohns Colitis **6**(9): 900-904.
- Pathak, S., A. R. Grillo, M. Scarpa, P. Brun, R. D'Inca, L. Nai, A. Banerjee, D. Cavallo, L. Barzon, G. Palu, G. C. Sturniolo, A. Buda and I. Castagliuolo (2015). "MiR-155 modulates the inflammatory phenotype of intestinal myofibroblasts by targeting SOCS1 in ulcerative colitis." Exp Mol Med **47**: e164.
- Perron, M. P. and P. Provost (2008). "Protein interactions and complexes in human microRNA biogenesis and function." Front Biosci **13**: 2537-2547.
- Peterson, S. M., J. A. Thompson, M. L. Ufkin, P. Sathyanarayana, L. Liaw and C. B. Congdon (2014). "Common features of microRNA target prediction tools." Front Genet **5**: 23.
- Png, K. J., N. Halberg, M. Yoshida and S. F. Tavazoie (2011). "A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells." Nature **481**(7380): 190-194.
- Ponder, A. and M. D. Long (2013). "A clinical review of recent findings in the epidemiology of inflammatory bowel disease." Clin Epidemiol **5**: 237-247.

- Powrie, F., M. W. Leach, S. Mauze, L. B. Caddle and R. L. Coffman (1993). "Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice." Int Immunol **5**(11): 1461-1471.
- Probert, C. S., V. Jayanthi, D. Pinder, A. C. Wicks and J. F. Mayberry (1992). "Epidemiological study of ulcerative proctocolitis in Indian migrants and the indigenous population of Leicestershire." Gut **33**(5): 687-693.
- Pujari, R., R. Hunte, W. N. Khan and N. Shembade (2013). "A20-mediated negative regulation of canonical NF-kappaB signaling pathway." Immunol Res **57**(1-3): 166-171.
- Qin, J., R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J. M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, H. I. T. C. Meta, P. Bork, S. D. Ehrlich and J. Wang (2010). "A human gut microbial gene catalogue established by metagenomic sequencing." Nature **464**(7285): 59-65.
- Quevrain, E., M. A. Maubert, C. Michon, F. Chain, R. Marquant, J. Tailhades, S. Miquel, L. Carlier, L. G. Bermudez-Humaran, B. Pigneur, O. Lequin, P. Kharrat, G. Thomas, D. Rainteau, C. Aubry, N. Breyner, C. Afonso, S. Lavielle, J. P. Grill, G. Chassaing, J. M. Chatel, G. Trugnan, R. Xavier, P. Langella, H. Sokol and P. Seksik (2016). "Identification of an anti-inflammatory protein from Faecalibacterium prausnitzii, a commensal bacterium deficient in Crohn's disease." Gut **65**(3): 415-425.
- Ranjha, R., S. Aggarwal, S. Bopanna, V. Ahuja and J. Paul (2015). "Site-Specific MicroRNA Expression May Lead to Different Subtypes in Ulcerative Colitis." PLoS One **10**(11): e0142869.
- Ranjha, R., N. K. Meena, A. Singh, V. Ahuja and J. Paul (2017). "Correction: Association of miR-196a-2 and miR-499 variants with ulcerative colitis and their correlation with expression of respective miRNAs." PLoS One **12**(9): e0184701.

- Ray, G. (2016). "Inflammatory bowel disease in India - Past, present and future." World J Gastroenterol **22**(36): 8123-8136.
- Reif, S., I. Klein, F. Lubin, M. Farbstein, A. Hallak and T. Gilat (1997). "Pre-illness dietary factors in inflammatory bowel disease." Gut **40**(6): 754-760.
- Reif, S., A. Lavy, D. Keter, E. Broide, Y. Niv, A. Halak, Y. Ron, R. Eliakim, S. Odes, J. Patz, A. Fich, Y. Villa, N. Arber and T. Gilat (2001). "Appendectomy is more frequent but not a risk factor in Crohn's disease while being protective in ulcerative colitis: a comparison of surgical procedures in inflammatory bowel disease." Am J Gastroenterol **96**(3): 829-832.
- Ruedel, A., P. Dietrich, T. Schubert, S. Hofmeister, C. Hellerbrand and A. K. Bosserhoff (2015). "Expression and function of microRNA-188-5p in activated rheumatoid arthritis synovial fibroblasts." Int J Clin Exp Pathol **8**(5): 4953-4962.
- Runtsch, M. C., R. Hu, M. Alexander, J. Wallace, D. Kagele, C. Petersen, J. F. Valentine, N. C. Welker, M. P. Bronner, X. Chen, D. P. Smith, N. J. Ajami, J. F. Petrosino, J. L. Round and R. M. O'Connell (2015). "MicroRNA-146a constrains multiple parameters of intestinal immunity and increases susceptibility to DSS colitis." Oncotarget **6**(30): 28556-28572.
- Ryu, S., K. McDonnell, H. Choi, D. Gao, M. Hahn, N. Joshi, S. M. Park, R. Catena, Y. Do, J. Brazin, L. T. Vahdat, R. B. Silver and V. Mittal (2013). "Suppression of miRNA-708 by polycomb group promotes metastases by calcium-induced cell migration." Cancer Cell **23**(1): 63-76.
- Saini, S., S. Majid, V. Shahryari, S. Arora, S. Yamamura, I. Chang, M. S. Zaman, G. Deng, Y. Tanaka and R. Dahiya (2012). "miRNA-708 control of CD44(+) prostate cancer-initiating cells." Cancer Res **72**(14): 3618-3630.
- Saini, S., S. Yamamura, S. Majid, V. Shahryari, H. Hirata, Y. Tanaka and R. Dahiya (2011). "MicroRNA-708 induces apoptosis and suppresses tumorigenicity in renal cancer cells." Cancer Res **71**(19): 6208-6219.
- Sales-Campos, H., P. J. Basso, V. B. Alves, M. T. Fonseca, G. Bonfa, V. Nardini and C. R. Cardoso (2015). "Classical and recent advances in the treatment of inflammatory bowel diseases." Braz J Med Biol Res **48**(2): 96-107.

- Sandoval, M., X. Liu, E. E. Mannick, D. A. Clark and M. J. Miller (1997). "Peroxynitrite-induced apoptosis in human intestinal epithelial cells is attenuated by mesalamine." Gastroenterology **113**(5): 1480-1488.
- Sartor, R. B. and G. D. Wu (2017). "Roles for Intestinal Bacteria, Viruses, and Fungi in Pathogenesis of Inflammatory Bowel Diseases and Therapeutic Approaches." Gastroenterology **152**(2): 327-339 e324.
- Satsangi, J., M. S. Silverberg, S. Vermeire and J. F. Colombel (2006). "The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications." Gut **55**(6): 749-753.
- Schaefer, J. S., T. Attumi, A. R. Opekun, B. Abraham, J. Hou, H. Shelby, D. Y. Graham, C. Streckfus and J. R. Klein (2015). "MicroRNA signatures differentiate Crohn's disease from ulcerative colitis." BMC Immunol **16**: 5.
- Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.
- Schonauen, K., N. Le, U. von Arnim, C. Schulz, P. Malfertheiner and A. Link (2018). "Circulating and Fecal microRNAs as Biomarkers for Inflammatory Bowel Diseases." Inflamm Bowel Dis **24**(7): 1547-1557.
- Segain, J. P., D. Raingeard de la Bletiere, A. Bourreille, V. Leray, N. Gervois, C. Rosales, L. Ferrier, C. Bonnet, H. M. Blottiere and J. P. Galmiche (2000). "Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease." Gut **47**(3): 397-403.
- Seiderer, J., I. Elben, J. Diegelmann, J. Glas, J. Stallhofer, C. Tillack, S. Pfennig, M. Jurgens, S. Schmechel, A. Konrad, B. Goke, T. Ochsenkuhn, B. Muller-Myhsok, P. Lohse and S. Brand (2008). "Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD." Inflamm Bowel Dis **14**(4): 437-445.
- Shen, J., Y. Qiao, Z. Ran and T. Wang (2013). "Different activation of TRAF4 and TRAF6 in inflammatory bowel disease." Mediators Inflamm **2013**: 647936.

- Shen, J., Y. Q. Qiao, Z. H. Ran and T. R. Wang (2013). "Up-regulation and pre-activation of TRAF3 and TRAF5 in inflammatory bowel disease." Int J Med Sci **10**(2): 156-163.
- Sher, M. E., S. Bank, R. Greenberg, T. C. Sardinha, S. Weissman, B. Bailey, R. Gilliland and S. D. Wexner (1999). "The influence of cigarette smoking on cytokine levels in patients with inflammatory bowel disease." Inflamm Bowel Dis **5**(2): 73-78.
- Shi, T., Y. Xie, Y. Fu, Q. Zhou, Z. Ma, J. Ma, Z. Huang, J. Zhang and J. Chen (2017). "The signaling axis of microRNA-31/interleukin-25 regulates Th1/Th17-mediated inflammation response in colitis." Mucosal Immunol **10**(4): 983-995.
- Singh, R. P., I. Massachi, S. Manickavel, S. Singh, N. P. Rao, S. Hasan, D. K. Mc Curdy, S. Sharma, D. Wong, B. H. Hahn and H. Rehimi (2013). "The role of miRNA in inflammation and autoimmunity." Autoimmun Rev **12**(12): 1160-1165.
- Sokol, H., V. Leducq, H. Aschard, H. P. Pham, S. Jegou, C. Landman, D. Cohen, G. Liguori, A. Bourrier, I. Nion-Larmurier, J. Cosnes, P. Seksik, P. Langella, D. Skurnik, M. L. Richard and L. Beaugerie (2017). "Fungal microbiota dysbiosis in IBD." Gut **66**(6): 1039-1048.
- Song, H. Y., M. Rothe and D. V. Goeddel (1996). "The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation." Proc Natl Acad Sci U S A **93**(13): 6721-6725.
- Song, T., W. Xia, N. Shao, X. Zhang, C. Wang, Y. Wu, J. Dong, W. Cai and H. Li (2010). "Differential miRNA expression profiles in bladder urothelial carcinomas." Asian Pac J Cancer Prev **11**(4): 905-911.
- Sood, A., V. Midha, N. Sood, A. S. Bhatia and G. Avasthi (2003). "Incidence and prevalence of ulcerative colitis in Punjab, North India." Gut **52**(11): 1587-1590.
- Sui, M., A. Jiao, H. Zhai, Y. Wang, Y. Wang, D. Sun and P. Li (2017). "Upregulation of miR-125b is associated with poor prognosis and trastuzumab resistance in HER2-positive gastric cancer." Exp Ther Med **14**(1): 657-663.

- Sun, G., J. Yan, K. Noltner, J. Feng, H. Li, D. A. Sarkis, S. S. Sommer and J. J. Rossi (2009). "SNPs in human miRNA genes affect biogenesis and function." RNA **15**(9): 1640-1651.
- Sun, L., G. M. Nava and T. S. Stappenbeck (2011). "Host genetic susceptibility, dysbiosis, and viral triggers in inflammatory bowel disease." Curr Opin Gastroenterol **27**(4): 321-327.
- Suskind, D. L., M. J. Brittnacher, G. Wahbeh, M. L. Shaffer, H. S. Hayden, X. Qin, N. Singh, C. J. Damman, K. R. Hager, H. Nielson and S. I. Miller (2015). "Fecal microbial transplant effect on clinical outcomes and fecal microbiome in active Crohn's disease." Inflamm Bowel Dis **21**(3): 556-563.
- Svartz, M. (1948). "The treatment of 124 cases of ulcerative colitis with salazopyrine and attempts of desensibilization in cases of hypersensitiveness to sulfa." Acta Med Scand **131**(Suppl 206): 465-472.
- Syal, G., A. Kashani and D. Q. Shih (2018). "Fecal Microbiota Transplantation in Inflammatory Bowel Disease: A Primer for Internists." Am J Med.
- Taganov, K. D., M. P. Boldin, K. J. Chang and D. Baltimore (2006). "NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses." Proc Natl Acad Sci U S A **103**(33): 12481-12486.
- Tang, Y., X. Luo, H. Cui, X. Ni, M. Yuan, Y. Guo, X. Huang, H. Zhou, N. de Vries, P. P. Tak, S. Chen and N. Shen (2009). "MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins." Arthritis Rheum **60**(4): 1065-1075.
- Targan, S. R. and L. C. Karp (2005). "Defects in mucosal immunity leading to ulcerative colitis." Immunol Rev **206**: 296-305.
- Teng, Y., J. L. Ross and J. K. Cowell (2014). "The involvement of JAK-STAT3 in cell motility, invasion, and metastasis." JAKSTAT **3**(1): e28086.
- Thia, K. T., E. V. Loftus, Jr., W. J. Sandborn and S. K. Yang (2008). "An update on the epidemiology of inflammatory bowel disease in Asia." Am J Gastroenterol **103**(12): 3167-3182.

- Thia, K. T., W. Luman and O. C. Jin (2006). "Crohn's disease runs a more aggressive course in young Asian patients." Inflamm Bowel Dis **12**(1): 57-61.
- Thomas, S., J. Izard, E. Walsh, K. Batich, P. Chongsathidkiet, G. Clarke, D. A. Sela, A. J. Muller, J. M. Mullin, K. Albert, J. P. Gilligan, K. DiGuilio, R. Dilbarova, W. Alexander and G. C. Prendergast (2017). "The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists." Cancer Res **77**(8): 1783-1812.
- Thorlacius-Ussing, G., B. Schnack Nielsen, V. Andersen, K. Holmstrom and A. E. Pedersen (2017). "Expression and Localization of miR-21 and miR-126 in Mucosal Tissue from Patients with Inflammatory Bowel Disease." Inflamm Bowel Dis **23**(5): 739-752.
- Triantafillidis, J. K., E. Merikas and F. Georgopoulos (2011). "Current and emerging drugs for the treatment of inflammatory bowel disease." Drug Des Devel Ther **5**: 185-210.
- Ulitsky, A., A. N. Ananthakrishnan, A. Naik, S. Skaros, Y. Zadvornova, D. G. Binion and M. Issa (2011). "Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life." JPEN J Parenter Enteral Nutr **35**(3): 308-316.
- Van der Goten, J., W. Vanhove, K. Lemaire, L. Van Lommel, K. Machiels, W. J. Wollants, V. De Preter, G. De Hertogh, M. Ferrante, G. Van Assche, P. Rutgeerts, F. Schuit, S. Vermeire and I. Arijs (2014). "Integrated miRNA and mRNA expression profiling in inflamed colon of patients with ulcerative colitis." PLoS One **9**(12): e116117.
- Van der Sluis, M., B. A. De Koning, A. C. De Bruijn, A. Velcich, J. P. Meijerink, J. B. Van Goudoever, H. A. Buller, J. Dekker, I. Van Seuningen, I. B. Renes and A. W. Einerhand (2006). "Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection." Gastroenterology **131**(1): 117-129.
- Vanhoutvin, S. A., F. J. Troost, H. M. Hamer, P. J. Lindsey, G. H. Koek, D. M. Jonkers, A. Kodde, K. Venema and R. J. Brummer (2009). "Butyrate-induced transcriptional changes in human colonic mucosa." PLoS One **4**(8): e6759.

- Vaughn, B. P., T. Vatanen, J. R. Allegretti, A. Bai, R. J. Xavier, J. Korzenik, D. Gevers, A. Ting, S. C. Robson and A. C. Moss (2016). "Increased Intestinal Microbial Diversity Following Fecal Microbiota Transplant for Active Crohn's Disease." Inflamm Bowel Dis **22**(9): 2182-2190.
- Velcich, A., W. Yang, J. Heyer, A. Fragale, C. Nicholas, S. Viani, R. Kucherlapati, M. Lipkin, K. Yang and L. Augenlicht (2002). "Colorectal cancer in mice genetically deficient in the mucin Muc2." Science **295**(5560): 1726-1729.
- Vickers, K. C., B. T. Palmisano, B. M. Shoucri, R. D. Shamburek and A. T. Remaley (2011). "MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins." Nat Cell Biol **13**(4): 423-433.
- Vlantis, K., A. Polykratis, P. S. Welz, G. van Loo, M. Pasparakis and A. Wullaert (2016). "TLR-independent anti-inflammatory function of intestinal epithelial TRAF6 signalling prevents DSS-induced colitis in mice." Gut **65**(6): 935-943.
- Wallace, K. L., L. B. Zheng, Y. Kanazawa and D. Q. Shih (2014). "Immunopathology of inflammatory bowel disease." World J Gastroenterol **20**(1): 6-21.
- Walmsley, R. S., R. C. Ayres, R. E. Pounder and R. N. Allan (1998). "A simple clinical colitis activity index." Gut **43**(1): 29-32.
- Wang, B., Z. L. Shen, Z. D. Gao, G. Zhao, C. Y. Wang, Y. Yang, J. Z. Zhang, Y. C. Yan, C. Shen, K. W. Jiang, Y. J. Ye and S. Wang (2015). "MiR-194, commonly repressed in colorectal cancer, suppresses tumor growth by regulating the MAP4K4/c-Jun/MDM2 signaling pathway." Cell Cycle **14**(7): 1046-1058.
- Wang, H., K. Chao, S. C. Ng, A. H. Bai, Q. Yu, J. Yu, M. Li, Y. Cui, M. Chen, J. F. Hu and S. Zhang (2016). "Pro-inflammatory miR-223 mediates the cross-talk between the IL23 pathway and the intestinal barrier in inflammatory bowel disease." Genome Biol **17**: 58.
- Wang, H., S. Zhang, Q. Yu, G. Yang, J. Guo, M. Li, Z. Zeng, Y. He, B. Chen and M. Chen (2016). "Circulating MicroRNA223 is a New Biomarker for Inflammatory Bowel Disease." Medicine (Baltimore) **95**(5): e2703.
- Wang, T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D.

Pardoll and H. Yu (2004). "Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells." Nat Med **10**(1): 48-54.

- Wang, Y., C. E. Parker, B. G. Feagan and J. K. MacDonald (2016). "Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis." Cochrane Database Syst Rev(5): CD000544.
- Wehkamp, J., J. Harder, M. Weichenthal, O. Mueller, K. R. Herrlinger, K. Fellermann, J. M. Schroeder and E. F. Stange (2003). "Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis." Inflamm Bowel Dis **9**(4): 215-223.
- Wehkamp, J., N. H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R. E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R. W. Feathers, H. Chu, H. Lima, Jr., K. Fellermann, T. Ganz, E. F. Stange and C. L. Bevins (2005). "Reduced Paneth cell alpha-defensins in ileal Crohn's disease." Proc Natl Acad Sci U S A **102**(50): 18129-18134.
- Wei, Y., J. Yang, L. Yi, Y. Wang, Z. Dong, Z. Liu, S. Ou-yang, H. Wu, Z. Zhong, Z. Yin, K. Zhou, Y. Gao, B. Yan and Z. Wang (2014). "MiR-223-3p targeting SEPT6 promotes the biological behavior of prostate cancer." Sci Rep **4**: 7546.
- Wells, C. (1952). "Ulcerative colitis and Crohn's disease." Ann R Coll Surg Engl **11**(2): 105-120.
- Wertz, I. E., K. M. O'Rourke, H. Zhou, M. Eby, L. Aravind, S. Seshagiri, P. Wu, C. Wiesmann, R. Baker, D. L. Boone, A. Ma, E. V. Koonin and V. M. Dixit (2004). "De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling." Nature **430**(7000): 694-699.
- Wu, F., N. J. Guo, H. Tian, M. Marohn, S. Gearhart, T. M. Bayless, S. R. Brant and J. H. Kwon (2011). "Peripheral blood microRNAs distinguish active ulcerative colitis and Crohn's disease." Inflamm Bowel Dis **17**(1): 241-250.
- Wu, F., S. Zhang, T. Dassopoulos, M. L. Harris, T. M. Bayless, S. J. Meltzer, S. R. Brant and J. H. Kwon (2010). "Identification of microRNAs associated with ileal and colonic Crohn's disease." Inflamm Bowel Dis **16**(10): 1729-1738.
- Wu, F., M. Zikusoka, A. Trindade, T. Dassopoulos, M. L. Harris, T. M. Bayless, S. R. Brant, S. Chakravarti and J. H. Kwon (2008). "MicroRNAs are differentially

expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha." Gastroenterology **135**(5): 1624-1635 e1624.

- Wu, W., C. He, C. Liu, A. T. Cao, X. Xue, H. L. Evans-Marin, M. Sun, L. Fang, S. Yao, I. V. Pinchuk, D. W. Powell, Z. Liu and Y. Cong (2015). "miR-10a inhibits dendritic cell activation and Th1/Th17 cell immune responses in IBD." Gut **64**(11): 1755-1764.
- Xavier, R. J. and D. K. Podolsky (2007). "Unravelling the pathogenesis of inflammatory bowel disease." Nature **448**(7152): 427-434.
- Xu, X. R., C. Q. Liu, B. S. Feng and Z. J. Liu (2014). "Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease." World J Gastroenterol **20**(12): 3255-3264.
- Yamamoto-Furusho, J. K., E. Miranda-Perez, G. Fonseca-Camarillo, F. Sanchez-Munoz, R. Barreto-Zuniga and A. Dominguez-Lopez (2010). "Interleukin 21 expression is increased in rectal biopsies from patients with ulcerative colitis." Inflamm Bowel Dis **16**(7): 1090.
- Yang, S. K., S. Yun, J. H. Kim, J. Y. Park, H. Y. Kim, Y. H. Kim, D. K. Chang, J. S. Kim, I. S. Song, J. B. Park, E. R. Park, K. J. Kim, G. Moon and S. H. Yang (2008). "Epidemiology of inflammatory bowel disease in the Songpa-Kangdong district, Seoul, Korea, 1986-2005: a KASID study." Inflamm Bowel Dis **14**(4): 542-549.
- Yi, J. M. and T. O. Kim (2015). "Epigenetic alterations in inflammatory bowel disease and cancer." Intest Res **13**(2): 112-121.
- Yue, D., H. Liu and Y. Huang (2009). "Survey of Computational Algorithms for MicroRNA Target Prediction." Curr Genomics **10**(7): 478-492.
- Z'Graggen, K., A. Walz, L. Mazzucchelli, R. M. Strieter and C. Mueller (1997). "The C-X-C chemokine ENA-78 is preferentially expressed in intestinal epithelium in inflammatory bowel disease." Gastroenterology **113**(3): 808-816.
- Zahm, A. M., N. J. Hand, D. M. Tsoucas, C. L. Le Guen, R. N. Baldassano and J. R. Friedman (2014). "Rectal microRNAs are perturbed in pediatric inflammatory bowel disease of the colon." J Crohns Colitis **8**(9): 1108-1117.

- Zahm, A. M., M. Thayu, N. J. Hand, A. Horner, M. B. Leonard and J. R. Friedman (2011). "Circulating microRNA is a biomarker of pediatric Crohn disease." J Pediatr Gastroenterol Nutr **53**(1): 26-33.
- Zenlea, T. and M. A. Peppercorn (2014). "Immunosuppressive therapies for inflammatory bowel disease." World J Gastroenterol **20**(12): 3146-3152.
- Zhang, P., K. Bill, J. Liu, E. Young, T. Peng, S. Bolshakov, A. Hoffman, Y. Song, E. G. Demicco, D. L. Terrada, C. J. Creighton, M. L. Anderson, A. J. Lazar, G. G. Calin, R. E. Pollock and D. Lev (2012). "MiR-155 is a liposarcoma oncogene that targets casein kinase-1alpha and enhances beta-catenin signaling." Cancer Res **72**(7): 1751-1762.
- Zhang, Y. Z. and Y. Y. Li (2014). "Inflammatory bowel disease: pathogenesis." World J Gastroenterol **20**(1): 91-99.
- Zhao, L., H. Yu, S. Yi, X. Peng, P. Su, Z. Xiao, R. Liu, A. Tang, X. Li, F. Liu and S. Shen (2016). "The tumor suppressor miR-138-5p targets PD-L1 in colorectal cancer." Oncotarget **7**(29): 45370-45384.
- Zheng, C. F. and Y. Huang (2011). "[Expression of zinc finger protein A20 in pediatric inflammatory bowel disease]." Zhonghua Er Ke Za Zhi **49**(4): 261-265.
- Zhou, H., J. Xiao, N. Wu, C. Liu, J. Xu, F. Liu and L. Wu (2015). "MicroRNA-223 Regulates the Differentiation and Function of Intestinal Dendritic Cells and Macrophages by Targeting C/EBPbeta." Cell Rep **13**(6): 1149-1160.
- Zhou, N., D. Fei, S. Zong, M. Zhang and Y. Yue (2016). "MicroRNA-138 inhibits proliferation, migration and invasion through targeting hTERT in cervical cancer." Oncol Lett **12**(5): 3633-3639.
- Zhu, M., D. Li, M. Jin and M. Li (2016). "Association between microRNA polymorphisms and the risk of inflammatory bowel disease." Mol Med Rep **13**(6): 5297-5308.
- Zwiers, A., L. Kraal, T. C. van de Pouw Kraan, T. Wurdinger, G. Bouma and G. Kraal (2012). "Cutting edge: a variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production." J Immunol **188**(4): 1573-1577.

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ORIGINAL ARTICLE

Basic Study

MicroRNA exhibit altered expression in the inflamed colonic mucosa of ulcerative colitis patients

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Author contributions: Valmiki S and Paul J designed the study, analyzed the data and wrote the manuscript; Valmiki S performed the experiments; Ahuja V provided the samples with clinical history.

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Abstract

AIM

To investigate the miRNA expression in colonic mucosal biopsies from endoscopically inflamed and non inflamed regions of ulcerative colitis (UC) patients.

METHODS

Colonic mucosal pinch biopsies were analyzed from the inflamed and non inflamed regions of same UC patient. Total RNA was isolated and differential miRNA profiling was done using microarray platform. Quantitative Real Time PCR was performed in colonic biopsies from inflamed (n = 8) and non-inflamed (n = 8) regions of UC and controls (n = 8) to validate the differential expression of miRNA. Potential targets of dysregulated miRNA were identified by using *in silico* prediction tools and probable role of these miRNA in inflammatory pathways were predicted.

RESULTS

The miRNA profile of inflamed colonic mucosa differs significantly from the non-inflamed. Real time PCR analysis showed that some of the miRNA were differentially expressed in the inflamed mucosa as compared to non inflamed mucosa and controls (miR-125b, miR-223, miR-138, and miR-155), while (miR-200a) did not show any significant changes. In contrast to microarray, where miR-378d showed downregulation in the inflamed mucosa, qRT-PCR showed a significant upregulation in the inflamed mucosa as compared

to the non inflamed. The *in silico* prediction analysis revealed that the genes targeted by these miRNAs play role in the major signaling pathways like MAPK pathway, NF-κB signaling pathway, cell adhesion molecules which are all assciated with UC.

CONCLUSION

The present study reports disease specific alteration in the expression of miR-125b, miR-155, miR-223 and miR-138 in UC patients and also predict their biological significance.

Key words: Ulcerative colitis; Colon mucosa; MicroRNA; Microarray; qRT-PCR; *In silico* analysis

Core tip: In order to get an insight into the pathogenesis of ulcerative colitis (UC), we explored spatial expression of microRNA in the inflamed and non-inflamed region of mucosal tissue of patients. Profiling of differentially expressed miRNA was generated by microarray from three paired samples. Few significantly dysregulated microRNA were validated using qRT-PCR. The present study reports disease specific alteration in the expression of miR-125b, miR-155, miR-223 and miR-138 in UC patients and also analyzed their biological significance using *in silico* tools. MiR-223 exhibited elevated expression independent of disease activity therefore, could be a potential candidate as biomarker for UC.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a gastrointestinal disorder which is chronic and relapsing in nature and exists in two clinical forms ulcerative colitis (UC) and Crohn's disease (CD)[1]. Over the past decade, the incidence and prevalence of IBD are continuously increasing worldwide[2]. The etiology of IBD is not defined yet, but several reports in the past have hypothesized that there is complex interplay of microbial, environmental and genetic factors which predisposes an individual to develop the disease[3]. Various systematic approaches like candidate gene identification or genome wide association studies have been made to identify the genes that get dysregulated and affect the downstream signaling pathways and subsequent gene expression during disease condition. Molecular changes in the expression of several genes have been found to be assciated with IBD pathogenesis[4], and some of these genes play important roles in the major inflammatory pathways.

MicroRNA are a class of small sized (about 18-22 nucleotides in length), non coding, single stranded, endogenous mRNA which modulates the expression of their target mRNA by binding to them in their 3'UTR by either carrying out their degradation or inhibiting the translation prcess[5-8]. However, target sites for miRNA have also been reported in the 5'UTR of many genes[9]. Around 2000 mature miRNA have been reported so far and they are known to modulate the expression of about one third of human genes[10]. Recent evidences suggest that miRNAs play key role in modulating the expression of target genes involved in the pathogenesis of various diseases. According to a recent estimate almost 60% of genes in a cell are regulated by miRNAs[11].

In the past few years miRNAs have emerged as the new epigenetic regulators in IBD[12,13] also differential expression of miRNAs have been assciated with several autoimmune diseases and cancer including IBD[14]. These studies have revealed differential profiling of miRNAs in mucosal tissues of UC and CD patients thus indicating their importance as potential biomarkers[15]. Our aim in this study had been to profile the miRNAs exhibiting differential expression in inflamed and non-inflamed region of the colon tissue in UC patients. Further we attempted to assess using bioinformatics tools, the functional significance of selected miRNAs involved in the regulation of target genes. Therefore, studying these miRNAs could provide better ways for disease diagnosis and therapeutics by establishing potential biomarkers.

MATERIALS AND METHODS

Patients and tissue samples

The study included 8 UC patients and 8 non IBD controls. Colonic pinch biopsies were collected from the endoscopically inflamed as well as non inflamed regions of UC patients from the Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India. The disease activities of the samples were measured on the basis of SCCAI score. Demographic features of study subjects are enlisted in Table 1. For obtaining inflamed mucosal samples, pinch biopsies were withdrawn from the colonic regions which showed clear symptoms of UC such as loss of vascular pattern, erythema, spontaneous bleeding or ulceration while in case of non inflamed mucosal samples, biopsies were collected from colonic regions of same UC patients with no disease activity. In case of control subjects biopsies were collected from rectosigmoid area. All the controls were age and sex matched with patients. Controls included were individual attending the clinic for routine colonoscopy and were without any inflammatory disorder of intestine and without any

Table 1 Demographic features of study subject *n* (%)

Characteristics	UC patients	Non IBD control
No. of patients (total)	8	8
Sex (M/F)	3/5	2/6
Age (mean ± SD, range, yr)	39.75 ± 10.29 (25-62)	40.75 ± 11.86 (24-60)
Disease duration (mean ± SD, Range, yr)	6.35 ± 6.52 (1.5-21)	NA
Disease extent		
Proctitis	3 (37.5)	
Left sided colitis	5 (62.5)	
Medication		
Mesalamine	5 (62.5)	0
Azathioprine	3 (37.5)	0
Steroids	0	0

UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

IBD symptoms. Mucosal biopsy samples were collected in RNA later solution. Diagnosis of UC was confirmed through endoscopic and histological examination by following ECCO guidelines[16].

Ethical approval

The study has been ethically approved from the Institute Ethics Committee, All India Institute of Medical Sciences (Ref. No. T-290/23.06.2015, RT-7/27.01.2016) and Institutional Ethics Review Board, JNU (IERB Ref. No.2016/Student/93). Informed consent was taken from all the subjects included in study.

RNA isolation and quality check

Total RNA was extracted from the mucosal biopsies using mirVana miRNA isolation kit (Ambion INC, TX, United States) according to manufacturer's protcol and quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, United States). The 260/280 values were above 1.9. Quality check for RNA was assayed by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States).

miRNA microarray profiling

The array included three pairs of inflamed and non inflamed samples from UC patients. Samples were submitted to Affymetrix for miRNA profiling using GeneChip® miRNA 4.0 Array which is designed to interrogate all mature miRNA sequences in miRBase Release 20. The differentially expressed miRNA were statistically assessed by one way ANOVA paired method. *P* value of 0.05 was considered significant and fold change of 1.5 was taken as cut off for upregulation and -1.5 for downregulation of miRNA in a given sample.

Reverse transcription and quantitative real time PCR

Reverse transcription for each miRNA (800 ng) was carried out using gene specific looped primers[17], using revert aid cDNA synthesis kit (Fermentas St. Leon Rot, Germany). Sequence of primers used for reverse transcription and qRT-PCR are enlisted in Table 2. Differential expression of six selected miRNAs identified by microarray was performed using BioRad CFX96 Real Time System with C1000 Touch Thermal cylcer. (BioRad, Hercules, CA, United States) by SYBR Green method, and the cycles were as follows: initial denaturation - 94 ℃ for 2 min, denaturation - 94 ℃ for 30 s, annealing - 60 ℃ for 1 min for 40 cycles. The relative expression differences of miRNAs were normalized to internal reference U6 snoRNA and analyzed using $2^{-\Delta\Delta ct}$ method. The statistical analysis was done using unpaired, two way student's *t*-test and a P value < 0.05 were considered significant.

Table 2 List of primers used for reverse transcription and qRT-PCR

Name	Primer	Sequence (5'-3')
Reverse Universal Primer		GTGCAGGGTCCGAGGT
hsa-miR-125b-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGTCACAAGT
	Forward	TCCCTGAGACCCTAACTTG
hsa-miR-223-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGTGGGGTAT
	Forward	TGTCAGTTTGTCAAATACCC
hsa-miR-155-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACCCCTAT
	Forward	TTAATGCTAATCGTGATAGG
hsa-miR-138-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGCGGCCTGA
	Forward	AGCTGGTGTTGTGAATCAG
hsa-miR-200a-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACATCGTT
	Forward	TAACACTGTCTGGTAACGAT
hsa-miR-378d	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGTTTCTGTC
	Forward	ACTGGACTTGGAGTCAGAAA
Sno RNA U6	RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGAAAATATG
	Forward	CAAATTCGTGAAGCGTTCCA

RT primers were used to sythesize miRNA specific cDNA. For real time PCR miRNA specific forward primer and reverse universal primers were used.

Target prediction for differentially expressed miRNAs

Target prediction for differentially expressed miRNAs was carried out by miRWalk database (http://www.ma.uniheidelberg.de/apps/zmf/mirwalk/). This database simultaneously searches several other databases[18]. Additionally, TargetScan, miRBD, RNA22, DIANA, Pictar were also selected for target prediction and genes picked at least by three tools were selected for our list of potential targets. Further, involvement of these miRNAs in different biological pathways was investigated using mirPath v.3: DIANA TOOLS.

RESULTS

MiRNA microarray profiling of inflamed colonic mucosa of UC patients

To investigate the disease specific changes in the miRNA

profiling, mucosal biopsy tissues from endoscopically inflamed and non inflamed region of UC patients were submitted for microarray analysis. Microarray analysis revealed that miRNAs are differentially expressed in the inflamed colonic region of UC patients as compared to the non inflamed ones Supplementary Figure 1. We had selected three matched pairs for miRNA microarray profiling, out of which two pairs (Sample NI1, I1 and NI6, I6) exhibited dysregulated expression of several miRNAs in the inflamed mucosa which indicates the disease specific behavior of miRNAs. In the third matched pair (NI5, I5) the changes in miRNA expression were not comparable probably due to the mild disease activity in the patient.

Some of the miRNAs were upregulated with a fold change more than 10 such as miR-138, miR-708-5p, miR-212-3p, miR-4521, miR-17,-3p, miR-223,-3p, miR-21. The top 44 differentially expressed miRNAs have been enlisted in Table 3 with their respective fold changes. MiR-138 and miR-223 were upregulated more than 10 folds in the inflamed tissues and they have also been reported to be dysregulated in UC. miR-125b and miR-155 were upregulated with a fold change of 2.56 and 2.33 respectively in our study. Additionally, miR-200a and miR-378d were downregulated with a fold change of -2.14 and -4.06 respectively. We selected these six miRNAs as candidate miRNAs for further analysis because these miRNAs were reported to be dysregulated during autoimmune diseases and in various cancers. Some of these miRNAs were reported to be differentially regulated during UC but whether these miRNAs show any spatial expression within the endoscopically inflamed and non inflamed regions was not known. Also the gene targeted by these miRNAs play role in major inflammatory pathways.

Validation of differentially expressed miRNA by qRT-PCR

The differential expression of six candidate miRNAs (miR-155, miR-138, miR-125b, miR-223, miR-378d and miR-200a) was validated by performing qRT-PCR in matched pair samples from UC patients (n = 8) and non IBD controls (n = 8). The expression of miR-155, miR-138, miR-125b and miR-223 was found to be significantly upregulated in inflamed colonic mucosa of UC patients as compared to non inflamed mucosa and controls Figure 1. We found that miR-223 was significantly upregulated in the inflamed *vs* non inflamed tissues ($P < 0.05$) and in inflamed *vs* control ($P < 0.001$). Its expression was also significantly higher in the non inflamed *vs* controls ($P < 0.01$) as well. Similarly, miR-125b, was significantly higher in inflamed *vs* non inflamed ($P < 0.01$) as seen in microarray and inflamed *vs* controls ($P < 0.001$). In accordance with microarray, miR-138 exhibited increased expression in inflamed *vs* non inflamed and also in inflamed *vs* controls ($P < 0.05$) and mir-155 was also upregulated in inflamed *vs* non inflamed ($P < 0.05$) as well as in inflamed *vs* control ($P < 0.01$). In contrast to microarray results, the expression of miR-200a did not change significantly in the inflamed samples when compared with non inflamed and controls Figure 2. But interestingly, the expression of miR-200a in non inflamed mucosa was significantly lower in the non inflamed *vs* controls. As seen in microarray miRNA-378d was downregulated in the inflamed mucosa of UC patients but in qRT-PCR, it showed a significant upregulation in the inflamed mucosa as compared to the non inflamed ($P < 0.01$) and controls ($P < 0.05$). Interestingly, miR-378d showed less expression in the non inflamed mucosa as compared to the control.

The qRT-PCR results were further reanalyzed individually for each matched pair and it showed that although inter sample variation was there in each case, the expression of miR-155, miR-138, miR-223, miR-125b was upregulated in most of the inflamed samples as compared to the non inflamed ones Figure 3 and these changes were found to be statistically significant. In contrast to microarray, miR-378d showed significant upregulation in the inflamed samples as compared to the non inflamed although inter sample variation was seen in this case also. In case of miR-200a, the inter sample variation was quite high, although the changes in expression were not significant it showed upregulation in the expression in four pairs whereas downregulation in the rest four pairs Figure 2. Therefore, we could not draw a conclusive trend for this miRNA.

Prediction of potential targets by in silico prediction tool

miRNAs (miR-155, miR-138, miR-125b andmiR-223) showing significant changes in qRT-PCR analysis were examined for their biological relevance by studying their respective potential gene targets and the signaling pathways involved. *In silico* target prediction analysis revealed several putative targets which play important role in major signaling pathways. Target prediction analysis indicated biological relevance of these dysregulated miRNAs as the genes targeted by these miRNAs are involved in the major inflammatory pathways such as NF-κB signaling pathways and MAPK pathway. Potential targets of all four miRNAs have been enlisted in Table 4. Potential targets of miR-125b included TNF receptor associated factor 6 (TRAF6), TNF alpha induced protein 3 (TNFAIP3), IL6R of NF-κB signaling pathway and Signal transducer and activator of transcription (STAT3), JUN, AKT1, IRF6 and IRAK1 of TLR signaling. In addition to this, miR-125b also targets SMAD4 and cAMP responsive element binding protein1 (CREB1), SIRT5, DICER1, acetylcholine esterase (ACHE), CDK16, CCR2 and MUC1.Putative targets of miR-155 includes signaling molecules of NF-κB signaling such as TGF beta activated kinase 2 (TAB2), CARD6, RelA and TRAF3, suppressor of cytokine signaling (SCS1), KRAS, PAK2, BDNF of MAPK

Table 3 List of top 44 differentially expressed miRNAs in the inflamed colonic mucosal biopsies of ulcerative colitis patients determined by microarray

MicroRNA	F.C[1]	Expression	MicroRNA	F.C	Expression
hsa-miR-138-5p	35.16	Upregulated	hsa-miR-148a-5p	2.42	Upregulated
hsa-miR-708-5p	34.7	Upregulated	hsa-miR-155-5p	2.33	Upregulated
hsa-miR-212-3p	24.06	Upregulated	hsa-miR-21-5p	1.81	Upregulated
hsa-miR-4538	26.12	Upregulated	hsa-miR-196b-3p	1.50	Upregulated
hsa-miR-4521	17.69	Upregulated	hsa-miR-552-3p	-7.82	Downregulated
hsa-miR-4417	16.63	Upregulated	hsa-miR-196b-5p	-7.60	Downregulated
hsa-miR-17-3p	15.28	Upregulated	hsa-miR-378d-5p	-4.06	Downregulated
hsa-miR-424-3p	14.17	Upregulated	hsa-miR-141-3p	-3.03	Downregulated
hsa-miR-874-3p	13.47	Upregulated	hsa-miR-10b-5p	-2.85	Downregulated
hsa-miR-25-5p	13.41	Upregulated	hsa-miR-215-5p	-2.56	Downregulated
hsa-miR-223-3p	13.01	Upregulated	hsa-miR-192-5p	-2.52	Downregulated
hsa-miR-1271-5p	11.96	Upregulated	hsa-miR-194-3p	-2.31	Downregulated
hsa-miR-148b-3p	11.31	Upregulated	hsa-miR-422a	-2.22	Downregulated
hsa-miR-501-5p	11.26	Upregulated	hsa-miR-200a-3p	-2.14	Downregulated
hsa-miR-4486	10.86	Upregulated	hsa-miR-378a-3p	-2.04	Downregulated
hsa-miR-224-3p	10.46	Upregulated	hsa-miR-6732-5p	-1.94	Downregulated
hsa-miR-21-3p	8.46	Upregulated	hsa-miR-147b	-1.78	Downregulated
hsa-miR-146b-5p	6.83	Upregulated	hsa-miR-200b-3p	-1.7	Downregulated
hsa-miR-149-5p	6.24	Upregulated	hsa-miR-572	-1.63	Downregulated
hsa-miR-31-5p	4.04	Upregulated	hsa-miR-4649-5p	-1.61	Downregulated
hsa-miR-491-5p	3.92	Upregulated	hsa-miR-638	-1.6	Downregulated
hsa-miR-125b-5p	2.56	Upregulated	hsa-miR-299-5p	-1.59	Downregulated

[1]F.C denoted the fold change in the expression of respective miRNA in the inflamed biopsies compared to the non inflamed biopsy.

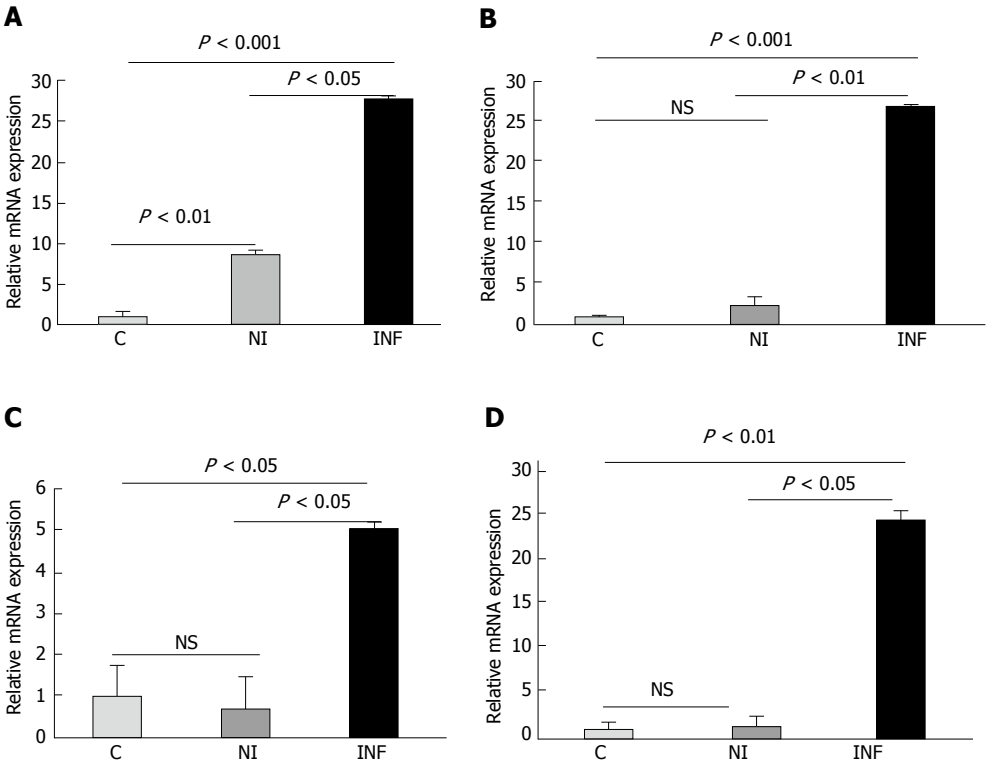

Figure 1 Differential expression of miRNAs investigated by performing real time PCR. A: miR-223; B: miR-125b; C: miR-138; D: miR-155. C denotes Control, NI denotes non-inflamed and INF denotes inflamed. NS: Not significant.

signaling, CTLA4, IL6R, nuclear factor of activated T cells 5 (NFAT5), IL17RB, Myd88, IKBKE, CTLA4, CLCN3 and APC.

Similarly, miR-138 targets genes such as CXCL8, RELA of NF-κB signaling pathway and other genes like CASP3, HDAC4, SIRT1, MAST4, SMAD4, MyD88 and CDK6. miR-223 targets STAT1, RELA and TAB3 of NF-κB signaling. The putative targets of miR-378d include TRAF3, SCS2 and MAPK1. Putative target genes of miR-200a involved in inflammatory pathways were

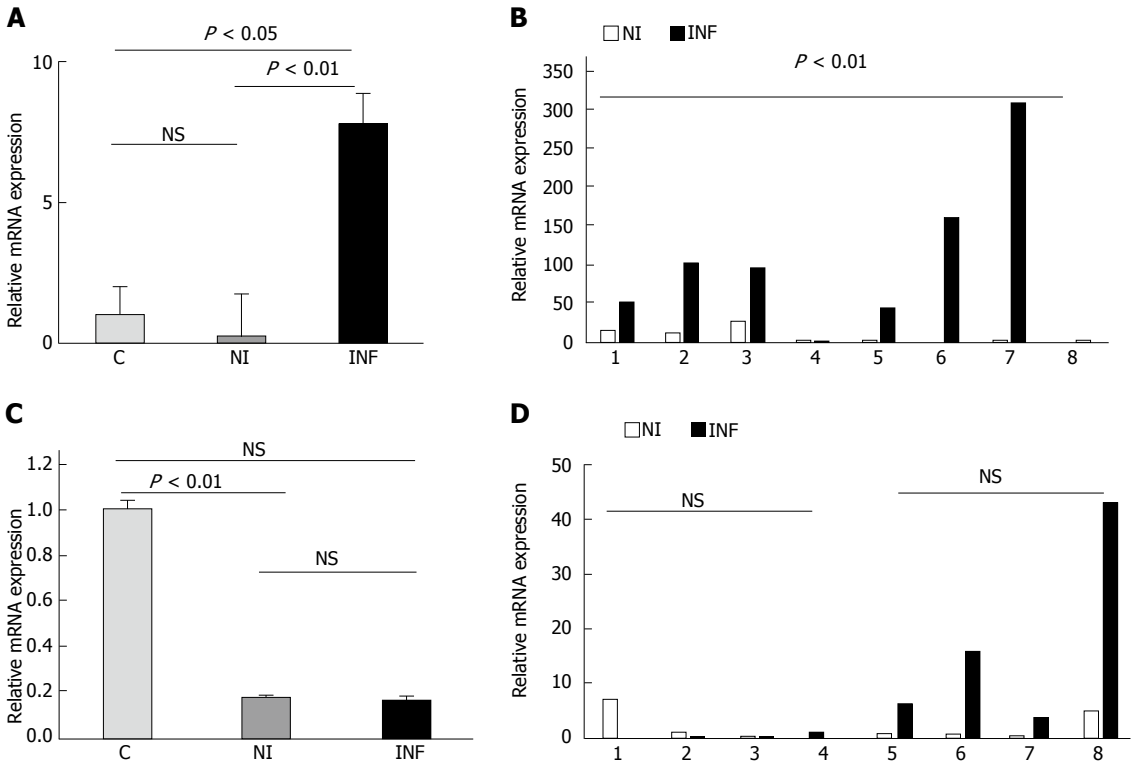

Figure 2 Real time analysis for miR-378d and miR-200a. A: miR-378d; B: miR-378d matched individual samples; C: miR-200a; D: miR-200a matched individual samples. C: Control; NI: Non inflamed; INF: Inflamed; NS: Not significant.

Table 4 List of potential targets and the pathway involved

MicroRNA	Target gene	Pathway involved
hsa-miR-155-5p	*TAB2, CFLAR, CARD11, ReLA, TRAF3*	NF-κB signaling.
hsa-miR-155-5p	*CARD6, ERBB2IP, MAPK10*	Nod like receptor signaling
hsa-miR-155-5p	*S℃S1, KRAS, PAK2, RAP1B, RFLA, TAOK1, CACNB4, BDNF*	MAPK signaling
hsa-miR-155-5p	*FOS, S℃S1, IKBKE, TRAF3, MAPK10, TAB2*	TLR signaling
has-miR-155	*NFAT5, GSK3B, INPP5D, VAV3, PIK3CA*	B cell receptor signaling
hsa-miR-138-5p	*CSNK2A2, RELA*	NF-κB signaling
hsa-miR-138-5p	*CXCL8, MAP2K7*	TLR signaling
hsa-miR-138-5p	*CLDN19, CD274, ITGAL*	Cell Adhesion Molecules.
hsa-miR-125b-5p	*MAPK14, JUN.AKT1, IRAK1, TRAF6, IRF5*	TLR signaling
hsa-miR-125b	*TNFAIP3, CSNK2A1, IRAK1*	NF-κB signaling
hsa-miR-223-3p	*STAT1, RELA*	TLR signaling
hsa-miR-223-3p	*TAB3, ERC1, PARP1, RELA*	NF-κB signaling
hsa-miR-378d	*MAPK1*	MAPK signaling
hsa-miR-378d	*TRAF3*	NF-κB signaling
hsa-miR-200a	*TAB2, TNFAIP3*	NF-κB signaling

TAB2, CXCL12, TNFAIP3, SIRT1 and TGFB2.

DISCUSSION

In the past few years, miRNAs have been reported to be dysregulated during UC, there are several studies which claimed that miRNA profile in the blood and tissue of UC patients differs from that of control individuals[19,20]. MiRNA were also reported to play critical role in mediating cross talk between CD, UC and CRC[21].

Our microarray analysis included three matched pairs from the endoscopically inflamed and non inflamed regions of UC patients. One of the pair didn't show much changes in the miRNA transcriptome of inflamed region when compared with the non inflamed one (NI5 and I5), which is more certainly due to the mild activity of disease in the patient. Other two pairs showed significant changes in the miRNA profile in the inflamed regions as compared to the non inflamed ones. We found that miR-223 and miR-138 were upregulated with a fold change of more than 10 folds in the inflamed mucosa and these miRNAs are also reported earlier to be dysregulated during UC[22-24] however the spatial expression of these miRNAs in the endoscopically inflamed and non inflamed regions are reported here for the first time. miR-125b and miR-155 mediates cross talk between UC and CRC[21] as reported earlier and also modulates the expression of genes playing role in inflammatory pathways[25]. miR-200a is earlier reported to get downregulated in UC[22]. Keeping the role of these miRNAs in mind we selected these six as our candidate miRNAs for further analysis, so that we can elucidate the disease specific behavior of these miRNA and correlate their expression with disease activity.

qRT-PCR analysis revealed that miR-223 was upregulated in inflamed tissues of UC patients, also

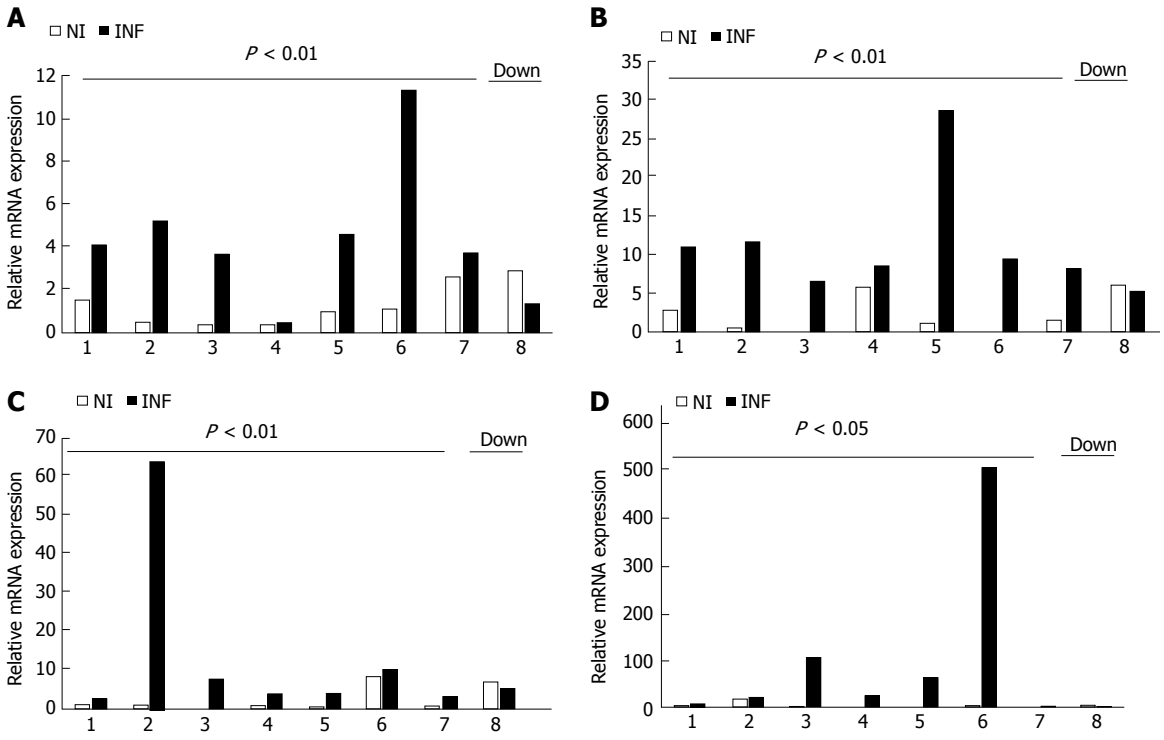

Figure 3 Differentially expressed miRNAs in the matched colonic biopsies of each ulcerative colitis patient determined by qRT-PCR analysis. A: miR- 223; B: miR-125-b; C: miR-138; D: miR-155. NI denotes non-inflamed and INF denotes inflamed. NS: Not significant.

this was the only miRNA in our study, which showed significantly higher expression in both the inflamed as well as non inflamed tissues of UC patients as compared to controls. Therefore, this miRNA could be established as a potential biomarker to differentiate between UC and controls as its expression does not depend upon disease activity. Moreover, previous studies have shown miR-223 plays a role in gastric cancer and also known to target the tumor suppressor gene EPB41L3[26,27]. Wang *et al*[28] showed that miR-223 targets claudin-8 (CLDN8) which is a tight junction protein and maintains the intestinal barrier. miR-223 targets CLDN8 thereby mediating cross talk between IL23 pathway during IBD.

In accordance with the microarray studies, miR-155 expression was also significantly higher in the inflamed *vs* non inflamed as well as inflamed *vs* controls. Its upregulation is also reported in breast cancer where it acts as an OncomiR by targeting suppressor of cytokine signaling 1 (SCS1)[29,30]. In addition to this Kong *et al*[31] claimed that miR-155 modulated the expression of tumor suppressor gene von Hippel-Lindau (VHL) and promotes tumor invasion, proliferation, angiogenesis and recruitment of proinflamatory cells in breast cancer. Expression of miR-125b was also found significantly higher in the inflamed mucosa as compared to non inflamed and controls. Since miRNAs (miR125b and miR-155) are responsible for mediating cross talk between UC and CRC[21], therefore, studying these miRNAs in detail could provide new insights in disease diagnosis and therapeutics.

In the present study, miR-138 was also highly expressed in the inflamed mucosa of UC patient. Elevated level of miR-138 expression reported here has not been shown earlier from mucosal tissue of UC patients but only from peripheral blood samples[32]. Some of the previous reports suggest tumor suppressive role of miR-138 in nasopharyngeal cancers by targeting oncogene CCDN1 and controlling tumor proliferation[33] and also downregulation of miR-138 was reported by Liu *et al*[34] in head and neck squamous cell carcinomas where it played role in promoting apoptosis and suppressing tumor invasion. Role of this microRNA, regulating the target gene of inflammatory pathway during UC is yet to be validated. In contrast with the microarray results, qRT-PCR analysis did not show significant changes in expression of miR-200a in the inflamed mucosa *vs* non inflamed mucosa of UC patients. Interestingly, miR-378d showed a significant upregulation in the inflamed mucosa as compared to the non inflamed and controls in paired samples, while our microarray data a reverse trend in the inflamed mucosa. We also investigated the expression of miR-200a and miR-378d in UC (n = 30) *vs* controls (n = 20) by qRT-PCR, where we again observed significant downregulation in miR-200a and significant upregulation in miR-378d (data not shown).

The next approach in our study was to find out the putative targets of candidate miRNAs using *in silico* target prediction tools. The potential targets of our candidate miRNAs included signaling molecules of inflammatory pathways such as MAPK signaling, NF-κB signaling and cytokine signaling pathways, which are

known to be directly asscіated with pathogenesis of IBD[35].

In conclusion, our study elucidated the spatial relationship of miR-125b, miR-155, miR-138 and miR-223 in the endoscopically involved and non involved pockets of UC patients. miR-223 showed differential expression independent of disease activity therefore, could be a potential target as biomarker for UC.

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COMMENTS

Background

MicroRNA (miRNAs) are endogenous, single stranded, short length (18-22 nucleotides), non coding RNA which modulates the expression of their target mRNA by binding to them in the 3'UTR and causing RNA degradation or translational repression. The differential expression of miRNA has been reported in different diseases including psoriasis, multiple sclerosis, and inflammatory bowel disease.

Research frontiers

Expression profile of different miRNA was investigated in the colonic mucosal samples of endoscopically inflamed and non inflamed regions of the same ulcerative colitis (UC) patient to get an insight in to the status of these miRNA in the inflamed and non inflamed pockets of UC patients. Microarray and qRT-PCR showed a spatial distribution in expression of miR-125b, miR-223, miR-155 and miR-138 in the inflamed colonic samples. Putative targets of these miRNAs included the genes involved in the major inflammatory pathways such as NF-κB pathway and MAPK signaling which showed that these miRNA might be one of the contributing factors in disease pathogenesis.

Innovations and breakthroughs

In order to get an insight into the pathogenesis of UC, the authors explored spatial distribution in expression of microRNA in the inflamed and non-inflamed region of mucosal tissue of patients. Profiling of differentially expressed miRNA was generated by microarray from three paired samples. Few significantly dysregulated miRNA were validated using qRT-PCR. The present study reports disease specific alteration in the expression of miR-125b, miR-155, miR-223 *and* miR-138 in UC patients and also analyzed their biological significance using *in silico* tools. miR-223 exhibited elevated expression independent of disease activity therefore, could be a potential candidate as biomarker for UC.

Applications

miR-223 altered expression during UC, irrespective of inflammation, therefore it could be developed as a biomarker for UC. Expression of this miRNA in colonic biopsy of UC patient could indicate toward disease status.

Terminology

SCCAI (Simple Clinical Colitis Activity Index) score is a symptoms based diagnostic tool and questionnaire to estimate the Ulcerative colitis disease severity. It takes into account parameters such as general well-being, bowel frequency, urgency, abdominal pain, rectal bleeding and extra intestinal features.

Peer-review

The authors reported miRNA exhibit altered expression in the inflamed colonic mucosa of UC patients. The article is informative and well-presented.

REFERENCES

1 **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**: 521-533 [PMID: 12876555 DOI: 10.1038/nri1132]
2 **Hisamatsu T**, Kanai T, Mikami Y, Yoneno K, Matsuoka K, Hibi T. Immune aspects of the pathogenesis of inflammatory bowel disease. *Pharmacol Ther* 2013; **137**: 283-297 [PMID: 23103332 DOI: 10.1016/j.pharmthera.2012.10.008]
3 **Chen WX,** Ren LH, Shi RH. Implication of miRNA for inflammatory bowel disease treatmernt: Systemic review. *World J Gastrointest Pathophysiol* 2014; **5**: 63-70 [PMID: 24891977 DOI: 10.4291/wjgp.v5.i2.63]
4 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185 DOI: 10.1038/nature06005]
5 **Guo H**, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010; **466**: 835-840 [PMID: 20703300 DOI: 10.1038/nature09267]
6 **Baek D**, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* 2008; **455**: 64-71 [PMID: 18668037 DOI: 10.1038/nature07242]
7 **Esteller M**. Non-coding RNAs in human disease. *Nat Rev Genet* 2011; **12**: 861-874 [PMID: 22094949 DOI: 10.1038/nrg3074]
8 **Selbach M**, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature* 2008; **455**: 58-63 [PMID: 18668040 DOI: 10.1038/nature07228]
9 **Lytle JR**, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci USA* 2007; **104**: 9667-9672 [PMID: 17535905 DOI: 10.1073/pnas.0703820104]
10 **Kozomara A**, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* 2011; **39**: D152-D157 [PMID: 21037258 DOI: 10.1093/nar/gkq1027]
11 **Friedman RC**, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; **19**: 92-105 [PMID: 18955434 DOI: 10.1101/gr.082701.108]
12 **Loddo I**, Romano C. Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis. *Front Immunol* 2015; **6**: 551 [PMID: 26579126 DOI: 10.3389/fimmu.2015.00551]
13 **Scarpa M**, Stylianou E. Epigenetics: Concepts and relevance to IBD pathogenesis. *Inflamm Bowel Dis* 2012; **18**: 1982-1996 [PMID: 22407855 DOI: 10.1002/ibd.22934]
14 **Ranjha R**, Paul J. Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. *Inflamm Res* 2013; **62**: 343-355 [PMID: 23417288 DOI: 10.1007/s00011-013-0600-9]
15 **Fasseu M**, Tréton X, Guichard C, Pedruzzi E, Cazals-Hatem D, Richard C, Aparicio T, Daniel F, Soulé JC, Moreau R, Bouhnik Y, Laburthe M, Groyer A, Ogier-Denis E. Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease. *PLoS One* 2010; **5**: [PMID: 20957151 DOI: 10.1371/journal.pone.0013160]
16 **Dignass A**, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, Mantzaris G, Reinisch W, Colombel JF, Vermeire S, Travis S, Lindsay JO, Van Assche G. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis* 2012; **6**: 965-990 [PMID: 23040452 DOI: 10.1016/j.crohns.2012.09.003]
17 **Chen C**, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005; **33**: e179 [PMID: 16314309 DOI: 10.1093/nar/gni178]
18 **Dweep H**, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform* 2011; **44**: 839-847 [PMID: 21605702 DOI: 10.1016/j.jbi.2011.05.002]
19 **Dalal SR**, Kwon JH. The Role of MicroRNA in Inflammatory

Bowel Disease. *Gastroenterol Hepatol* (N Y) 2010; **6**: 714-722 [PMID: 21437020]
20 **Schaefer JS**, Attumi T, Opekun AR, Abraham B, Hou J, Shelby H, Graham DY, Streckfus C, Klein JR. MicroRNA signatures differentiate Crohn's disease from ulcerative colitis. *BMC Immunol* 2015; **16**: 5 [PMID: 25886994 DOI: 10.1186/s12865-015-0069-0]
21 **Bai J**, Li Y, Shao T, Zhao Z, Wang Y, Wu A, Chen H, Li S, Jiang C, Xu J, Li X. Integrating analysis reveals microRNA-mediated pathway crosstalk among Crohn's disease, ulcerative colitis and colorectal cancer. *Mol Biosyst* 2014; **10**: 2317-2328 [PMID: 24949825 DOI: 10.1039/c4mb00169a]
22 **Van der Goten J**, Vanhove W, Lemaire K, Van Lommel L, Machiels K, Wollants WJ, De Preter V, De Hertogh G, Ferrante M, Van Assche G, Rutgeerts P, Schuit F, Vermeire S, Arijs I. Integrated miRNA and mRNA expression profiling in inflamed colon of patients with ulcerative colitis. *PLoS One* 2014; **9**: e116117 [PMID: 25546151 DOI: 10.1371/journal.pone.0116117]
23 **Wang H**, Zhang S, Yu Q, Yang G, Guo J, Li M, Zeng Z, He Y, Chen B, Chen M. Circulating MicroRNA223 is a New Biomarker for Inflammatory Bowel Disease. *Medicine* (Baltimore) 2016; **95**: e2703 [PMID: 26844512 DOI: 10.1097/MD.0000000000002703]
24 **Xu XM**, Zhang HJ. miRNAs as new molecular insights into inflammatory bowel disease: Crucial regulators in autoimmunity and inflammation. *World J Gastroenterol* 2016; **22**: 2206-2218 [PMID: 26900285 DOI: 10.3748/wjg.v22.i7.2206]
25 **Kim SW**, Ramasamy K, Bouamar H, Lin AP, Jiang D, Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-κB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proc Natl Acad Sci USA* 2012; **109**: 7865-7870 [PMID: 22550173 DOI: 10.1073/pnas.1200081109]
26 **Li BS**, Zhao YL, Guo G, Li W, Zhu ED, Luo X, Mao XH, Zou QM, Yu PW, Zuo QF, Li N, Tang B, Liu KY, Xiao B. Plasma microRNAs, miR-223, miR-21 and miR-218, as novel potential biomarkers for gastric cancer detection. *PLoS One* 2012; **7**: e41629 [PMID: 22860003 DOI: 10.1371/journal.pone.0041629]
27 **Li X**, Zhang Y, Zhang H, Liu X, Gong T, Li M, Sun L, Ji G, Shi Y, Han Z, Han S, Nie Y, Chen X, Zhao Q, Ding J, Wu K, Daiming F. miRNA-223 promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3. *Mol Cancer Res* 2011; **9**: 824-833 [PMID: 21628394 DOI: 10.1158/1541-7786.MCR-10-0529]
28 **Wang H**, Chao K, Ng SC, Bai AH, Yu Q, Yu J, Li M, Cui Y, Chen M, Hu JF, Zhang S. Pro-inflammatory miR-223 mediates the cross-talk between the IL23 pathway and the intestinal barrier in inflammatory bowel disease. *Genome Biol* 2016; **17**: 58 [PMID: 27029486 DOI: 10.1186/s13059-016-0901-8]
29 **Mattiske S**, Suetani RJ, Neilsen PM, Callen DF. The oncogenic role of miR-155 in breast cancer. *Cancer Epidemiol Biomarkers Prev* 2012; **21**: 1236-1243 [PMID: 22736789 DOI: 10.1158/1055-9965.EPI-12-0173]
30 **Jiang S**, Zhang HW, Lu MH, He XH, Li Y, Gu H, Liu MF, Wang ED. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. *Cancer Res* 2010; **70**: 3119-3127 [PMID: 20354188 DOI: 10.1158/0008-5472.CAN-09-4250]
31 **Kong W**, He L, Richards EJ, Challa S, Xu CX, Permuth-Wey J, Lancaster JM, Coppola D, Sellers TA, Djeu JY, Cheng JQ. Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. *Oncogene* 2014; **33**: 679-689 [PMID: 23353819 DOI: 10.1038/onc.2012.636]
32 **Fisher K**, Lin J. MicroRNA in inflammatory bowel disease: Translational research and clinical implication. *World J Gastroenterol* 2015; **21**: 12274-12282 [PMID: 26604636 DOI: 10.3748/wjg.v21.i43.12274]
33 **Liu X**, Lv XB, Wang XP, Sang Y, Xu S, Hu K, Wu M, Liang Y, Liu P, Tang J, Lu WH, Feng QS, Chen LZ, Qian CN, Bei JX, Kang T, Zeng YX. MiR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. *Cell Cycle* 2012; **11**: 2495-2506 [PMID: 22739938 DOI: 10.4161/cc.20898]
34 **Liu X**, Jiang L, Wang A, Yu J, Shi F, Zhou X. MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines. *Cancer Lett* 2009; **286**: 217-222 [PMID: 19540661 DOI: 10.1016/j.canlet.2009.05.030]
35 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]

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