Understanding demographic and clinical features of abdominal discomfort patients and the potential of serum miRNA-210 expression for the screening of colorectal cancer patients as fluid biopsy

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A thesis submitted in the partial fulfilment of the requirements for the degree of Master of Science in Biochemistry

Department of Physiology, Biochemistry and Pharmacology

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This is to certify, that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have made.

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DEDICATED TO MY RESPECTED AND BELOVED FAMILY AND TEACHERS

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Contents

List of Figures

List of Tables

Abbreviations

AMP	Adenosine Monophosphate
AMPK	AMP Activated Protein Kinase Pathway
APC	Adenomatous Polyposis Coli
BAG1	Bcl-2-Associated Athanogene-1
BAK1	BCL2-Antagonist-Killer 1 Protein
BAX	Bcl-2-Associated X Protein
BCL2	B-Cell Lymphoma 2 (Bcl-2) Protein Family
BIRC5	Baculoviral IAP Repeat Containing 5 Gene
BMP3	Bone Morphogenetic Protein 3
BRAF	B-Raf Proto-Oncogene
CDC25A	Cell Division Cycle 25A
CDC6	Cell Division Cycle 6 Protein
CDH1	Cadherin 1
CDK2	Cadherin 2
CDK6	Cyclin-Dependent Kinase 6
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CEA	Carcinoembryonic Antigen
CK	Casein Kinase
CMCH	Chittagong Medical College Hospital
CRC	Colorectal Cancer
CSCR	Center for Specialized care and Research
CVASU	Chattogram Veterinary and Animal Science University
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27B1	Cytochrome P450 Family 24 Subfamily B Member 1
EMT	Epithelial-Mesenchymal Transition
ERK1	Extracellular Signal-Regulated Kinase 1

FAP	Familial Adenomatous Polyposis
FIT	Fecal Immunochemical Tests
FOBT	Fecal Occult Blood Tests
Gaps	Gtpase Activating Protein
Gefs	Guanine Exchange Factors
GSK	Glycogen Synthase Kinase
Gtpase	GTP-Binding Proteins
IAP	Inhibitor Of Apoptosis
IBD	Inflammatory Bowel Diseases
IGF	Insulin-Like Growth Factor
IGF2	Inhibit The Secretion Of Insulin Like Growth Factor 2
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LGR	Leucine-Rich Repeat-Containing G-Protein Coupled Receptor
LINE-1	Long Interspersed Nucleotide Element -1
LRP5	Lipoprotein Receptor-Related Protein 5
LS	Lynch Syndrome
MAPK	MEK-Mitogen-Activated Protein Kinase
MCM2	Mini chromosome Maintenance 2
MCM4	Mini chromosome Maintenance 4
MCM7	Mini chromosome Maintenance 7
Mirna	Microrna
MLH1	Mutl Homolog 1
Mrna	Messenger RNA
MSH2	Mutl Homolog 2
Mtor	Mammalian Target Of Rapamycin
NDRG4	N-Myc Downregulated Gene 4
PDCD4	Programmed Cell Death
PI3K	Phosphotidyleinositol-3-Kinase

PI3KCA	Phosphotidyleinositol-3-Kinase Catalytic Alpha Subunit
PMS2	Postmeiotic Segregation Increased 2
PTEN	Phosphatase And Tensin Homolog
RSPO	R-Spondins
SNAIL1	Snail Family Transcriptional Repressor 1
SPRY2	Sprouty Homolog 2
SSP	Sessile Serrated Polyps
TCF-4	Transcription Factor 4
TGFBR2	Transforming Growth Factor Beta Receptor 2
TRCP1	Transducing Repeat-Containing Protein
UTR	Untranslated Region
VDR	Vitamin D Receptor
ZNRF3	Zinc and Ring Finger 3

Abstract

Colorectal cancer (CRC) has become one of the deadliest cancer all over the world because of its poor prognosis and high mortality rate. For CRC diagnosis, colonoscopy is considered as the most efficient method though it is an invasive process. Establishing blood biomarker as fluid biopsy can be a better option of colonoscopy. The present study was conducted to understand demographic and clinical features of abdominal discomfort patients and the potential of serum micro RNA 210 expression for the screening of colorectal cancer patients. In this study we have collected information about sociodemographic features (Age, sex, food habit, physical activity level, smoking and alcohol consumption, family history of cancer, daily sunlight exposure) of patients who comes to doctor with abdominal discomfort and were suggested to do colonoscopy. 52.4% of them were male and 47.6% were female. Mean age was 48±22 years. No significant association were noted within CRC with smoking and alcohol consumption. Almost 66% patients were physically inactive and 61.9% had sunlight exposure of about less than 30 min. They also had co-diseases such as IBD (35.7%) and diabetes (26.2%). Most common symptoms were abdominal pain and fatigue. After confirmed diagnosis, blood was collected from CRC patients (N=9) to know miRNA-210 expression in the serum, and then compared this expression with control group. As miRNA-210 is an oncogenic miRNA, its expression in CRC patients were 16 times higher than healthy individual and also increased gradually as the disease progressed to advanced stages. This expression was also negatively associated with patient's serum vitamin D status. Higher vitamin D level in serum lower the expression of miRNA-210. In summary, our data suggests that circulating miRNA-210 has the potential to become a marker for fluid biopsy for CRC diagnosis.

Keyword: CRC, MiRNA -210, Colonoscopy, Vitamin D, Blood Biomarker

Chapter- Ⅰ: Introduction

Cancer is the second leading cause of death in humans, following cardiovascular disease (Greuter et al., 2020) and colorectal cancer (CRC) is the third most commonly diagnosed cancer and also second most death causing cancer, globally (Theo et al., 2020). CRC comprises both colon and rectum cancer (Hossain et al., 2022) and approximately 9.4% of cancer related deaths are now caused by CRC (Ferlay et al., 2020). Every year more than 1.2 million people are newly diagnosed with CRC and this number continue to increase (Sung et al., 2015). Due the technological advancement such as colonoscopic screening of large intestine, the rate of CRC related death is now decreasing in developed country, however, that rate is still increasing in under developed countries like Bangladesh (Sung et al., 2015).

There are some risk factors are associated with the increasing rate of CRC such as age, sex, co-diseases (Inflammatory bowel disease, Lynch syndrome), family history, vitamin D deficiency and also some lifestyle related factors such as diet, smoking, alcohol consumption, lack of sun exposure and physical inactivity (Ren et al., 2015). Another reason of high death rate of CRC is because of mostly asymptomatic until advanced stage. Death rate of CRC can be reduced thorough surgery if it can be detected in earlier stage or as an adenomatous polyp (Kawamura et al., 2014).

Early detection of colonic cancers is a challenging task as because clinical symptoms develop slowly. Patients with colorectal cancer have usually presented with abdominal pain, alteration of bowel habit, loss of weight, vomiting, frequently with colic, anorexia, bleeding per rectum, lump, indigestion and acute-chronic obstruction (Hossain T, 2007) Colonoscopy is now widely used as CRC diagnosis gold standard method which is showing so far high sensitivity and specificity but it is expensive and also an invasive process which have significant risk of perforation (Ren et al., 2015). Among noninvasive technique guaiac-based fecal occult blood tests (FOBT), and fecal immunochemical tests (FIT) are mostly used but they have very low sensitivity and specificity (Sung et al., 2008). Now researchers are focused on developing new screening method which will be noninvasive and will also surpass the limitation of FIT and colonoscopy such as improves sensitivity and specificity, not as expensive as colonoscopy and ability to facilitate faster detection. Last few decades several

molecules have been investigated as potential biomarker to detect CRC at early stage

and among them only microRNA emerged as one of the potential candidate because of its availability in bloodstream and urine (Duran et al., 2020; Eldaly et al., 2020; Xu et al., 2017)

MicroRNA (miRNA) are short non coding single stranded RNA having 18-25 nucleotide in length that regulate target gene expression at the post-transcriptional level (Bartel et al., 2004). They attached at the 3' untranslated region (UTR) of the target mRNA and cause translational repression by degrading mRNA molecules (Zhang et al., 2007). Depending on the function of miRNA on their target gene, they can be classified as oncogene or tumor suppressor genes (Melo et al., 2011). Up regulation of oncogenic miRNA trigger tumorigenesis by reducing tumor suppressor proteins production; on the other hand, up regulation of tumor suppressor miRNA suppresses tumorigenesis by reducing expression of oncogenic miRNA (Kong et al., 2012).

Recent studies showed almost 450 miRNA's are linked with CRC; mir-210 is one of the oncogenic miRNA which has specific target and alter pathway's such as PTEN/PI3K/AKT pathway, Wnt pathway, ERK1/2 pathway etc and leads to the development of CRC (Chen et al., 2015). MiRNA also alter protein such as KRAS, p53, extracellular matrix regulators as well as epithelial-mesenchymal transition (EMT) transcription factors, Programmed cell death 4 (PDCD4), Transforming growth factor beta receptor 2 (TGFBR2), Sprouty homolog 2(SPRY2), cell division cycle 25A (CDC25A) which are related to various physiological process such as cell proliferation, metastasis, reduced apoptosis, cell cycle progression, invasion (Thomas et al., 2015).

Previous other studies revealed the changes in the expression of miRNA-210 in CRC patients. As it is an oncogenic miRNA its expression in blood is expected to be increased in CRC gradually as the diseases progress to advanced stage. Its availability in blood can make it a promising candidate for CRC screening as it can be easily collected from patients prior to any kind of treatment (Brase et al., 2010).

Specific objectives

1. To assess the socio-demographic and lifestyle behavior of the patients diagnosed with lower digestive disorders.
2. To assess the overall clinical history, laboratory findings, comorbidities and associated risk factors of the lower digestive disordered patients.

3. To assess the serum biomarkers (miRNA) and vitamin D of colorectal cancer patient.

Chapter - Ⅱ: Review of literature

2.1. Colorectal cancer

Colorectal cancer (CRC) is now one of the most common cancers worldwide. Every year, with between one and two million new cases being diagnosed, thus making CRC the third most common cancer and the fourth most common cause of cancer-related death, with 700,000 deaths per year. CRC includes colon cancer and rectum cancer where lesion can be formed from recto-sigmoid junction to rectum., Most of the CRC incident happened is colon (71%) and less in rectum (29%). It described as carcinoma, developed in the large alimentary tract which can be regional or distantly spread. (Heer and P.D, 2007).

2.2. The symptoms of CRC

The symptoms rely on growth location, mass, the extent of CRC, includes rectal bleeding, abdominal pain, alternation in bowel movement, frequency, stool diameter and diarrhoea and decrease in weight.

Symptoms are also dependent on location where that lesion or polyps originated. When carcinoma is present in ascending colon it manifested by right iliac fossa pain and mass, severe anemia, rapid weight loss, nausea, vomiting, anorexia, fainting, appendicitis. Carcinoma in descending colon manifested by alteration of bowel habit (frequency), rectal bleeding, weight loss, lower & left iliac fossa pain (Figure-2.1). Most of the cases women develop CRC in ascending colon and men in descending colon (Ahmad et al., 2020)

Carcinoma in Rectum presents by rectal bleeding, palpable mass on rectal examination, diarrhea, weight loss and sacral perineal pain (Boyle and Langman, 2000).

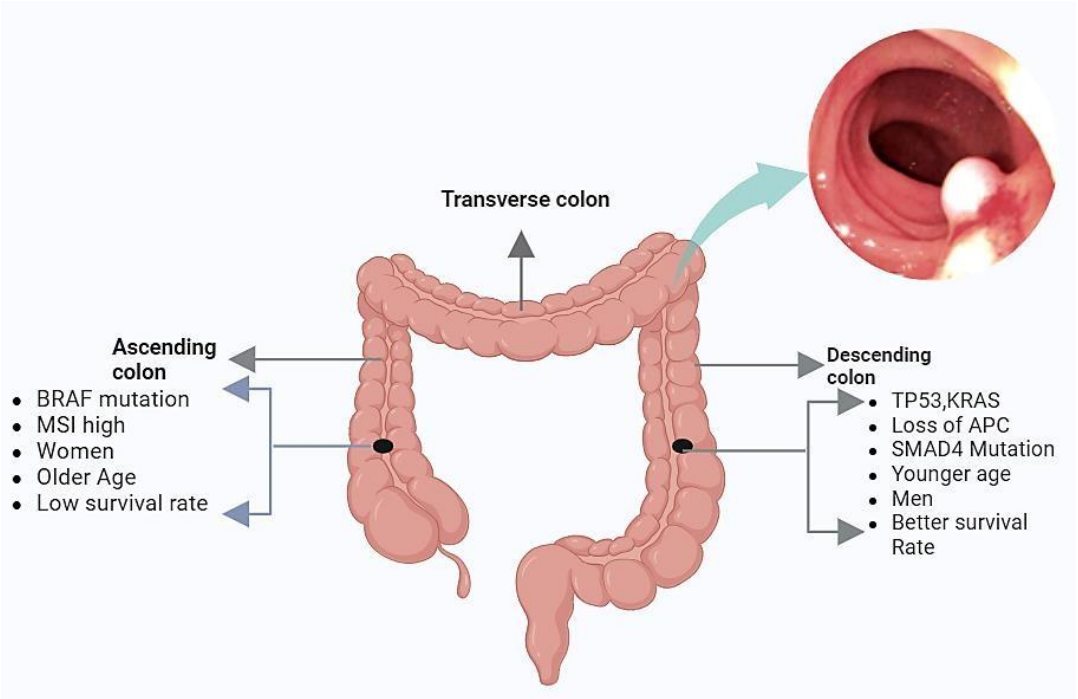


Figure 2.1: Anatomical Subtype of CRC and their associations with tumor molecular features (Ahmad et al., 2020)

2.3. Epidemiology

There is a variation in the CRC prevalence rate in Asia, Africa and Europe, Australia, New- Zealand, North America. In developing country CRC rate is comparatively higher than other developed country (figure-2.2) (Center et al., 2009). According to Globocon 2020 report CRC hold third and second position in terms of incidence and mortality rate in both male and female (figure-2.3).

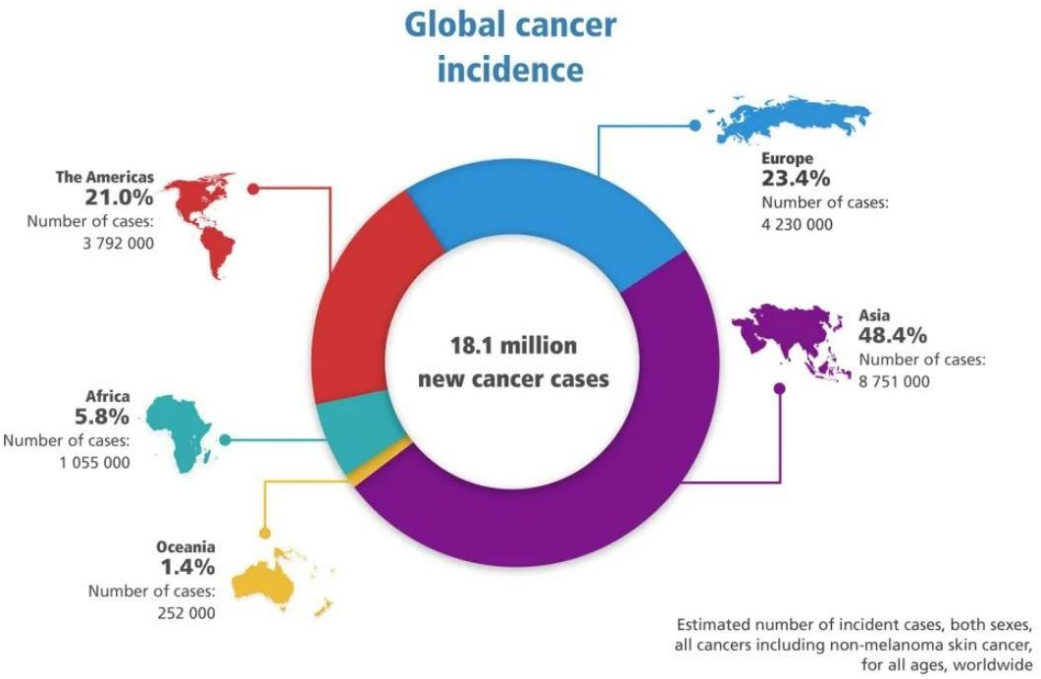


Figure 2.2: Global cancer incidence (Bray et al.,2018)

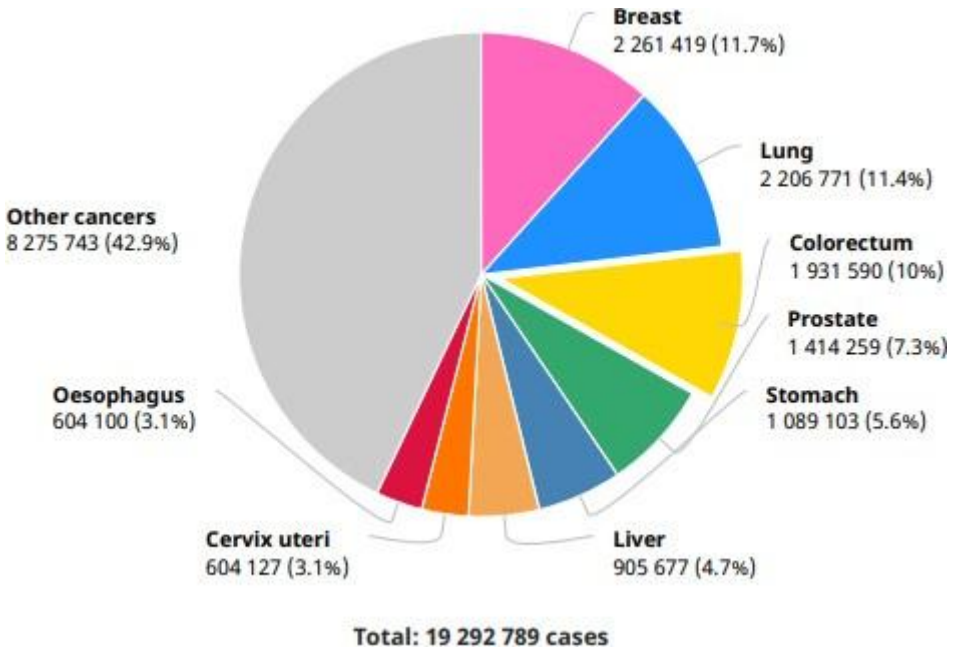


Figure 2.3: CRC incident in both sex (Bray et al.,2018)

2.4. Histopathology

Maximum colorectal cancer developed through adenocarcinomas which was initially adenomas or adenomatous polyps (Bosman et al., 2010).

Histopathologic examination can determine non-specific lesson, hyperplastic polyps, tubular adenoma, tubule villous adenoma, high grade dysplasia, adenocarcinoma. Among all of this hyperplastic polyps are mostly diagnosed. Some mutation including K-ras mutation in hyperplastic polyps also cause morphological appearance with dysplastic features of an adenoma. (Julien et al., 2012). Histopathological result can be three type – well differentiated, moderately differentiated and poorly differentiated (Figure- 2.4) (Julien et al., 2012). Grade of CRC depends on the histopathological result.

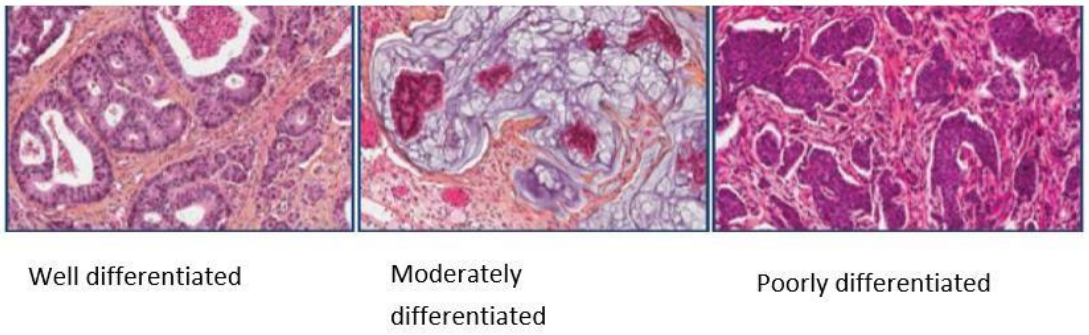


Figure2.4: Histologic feature of adenocarcinoma (Julien et al., 2012).

2.5. Development of CRC

CRC develop slowly and doesn't show symptoms until it reaches to a certain size. Most of the colon tumor develops through a multistep process which involves histological, morphological and genetic changes over time (figure 2.5).

CRC initially develops from polyps which mainly grows from intestinal mucosa by protruding into the intestinal lumen (American Cancer Society 2011). There are many type of polyps, two of these has potential to become malignant from benign condition- adenomas and sessile serrated polyps (SSP).

Most of the Adenomas polyps are characterized by dysplasia (abnormal organization of cell) and often contain long filamentous structure which known as villous or tubulovillous. Villous or tubulovillous adenomas are larger in size while SSPs are flat

like with serrated or saw toothed glands (Conteduca et al., 2013 and Yamane et al., 2014).

The risk for developing CRC from adenomas mostly dependent on polyp size. Almost 70% of CRC developed from adenomatous polyp and rest 25-30% developed from SSPs (Conteduca et al., 2013 and Yamane et al., 2014).

The size of polyps gradually increases when proliferation started inside of it and then genetic mutations or epigenetic changes also started which termed as cytologic and histologic dysplasia. With the course of time DNA damage increases and a high grade dysplasia develop which have higher degree of risk for progression to invasive carcinoma (Frank et al., 2007).

A series of genetic or epigenetic changes are responsible for the histological function of polyp to cancer. Inherited mutation in MLH1, MSH2, PMS2 and APC gene are associated with 5% case of CRC (Figure 2.5,B) (Nagy et al., 2009 and Kinzler et al.,1996).

There are mainly two pathways which leads to the development of CRC.

First one is the chromosomal instability pathway which is associated with adenomas that observed in 65%-70% of all sporadic cancers. This pathway starts with the mutation in APC gene that alter chromosomal segregation during cell division. Then second mutation occur in KRAS gene which is an oncogene having downstream effect on cell growth, differentiation, motility and survival. These two mutation make p53 to loss its function which act as a regulator of transcription and apoptosis that finally results to carcinogenesis (Pino et al, 2010). On the other side SSPs started to develop with the mutation in BRAF gene which affect growth signaling and loss of apoptosis (Yamane et al 2014). An important epigenetic alteration occur in SSPs base CRC is hyper methylation of MLH1 promoter region. This inhibit gene transcription which impacts regulation of growth promoting genes function (Kang et al., 2011).

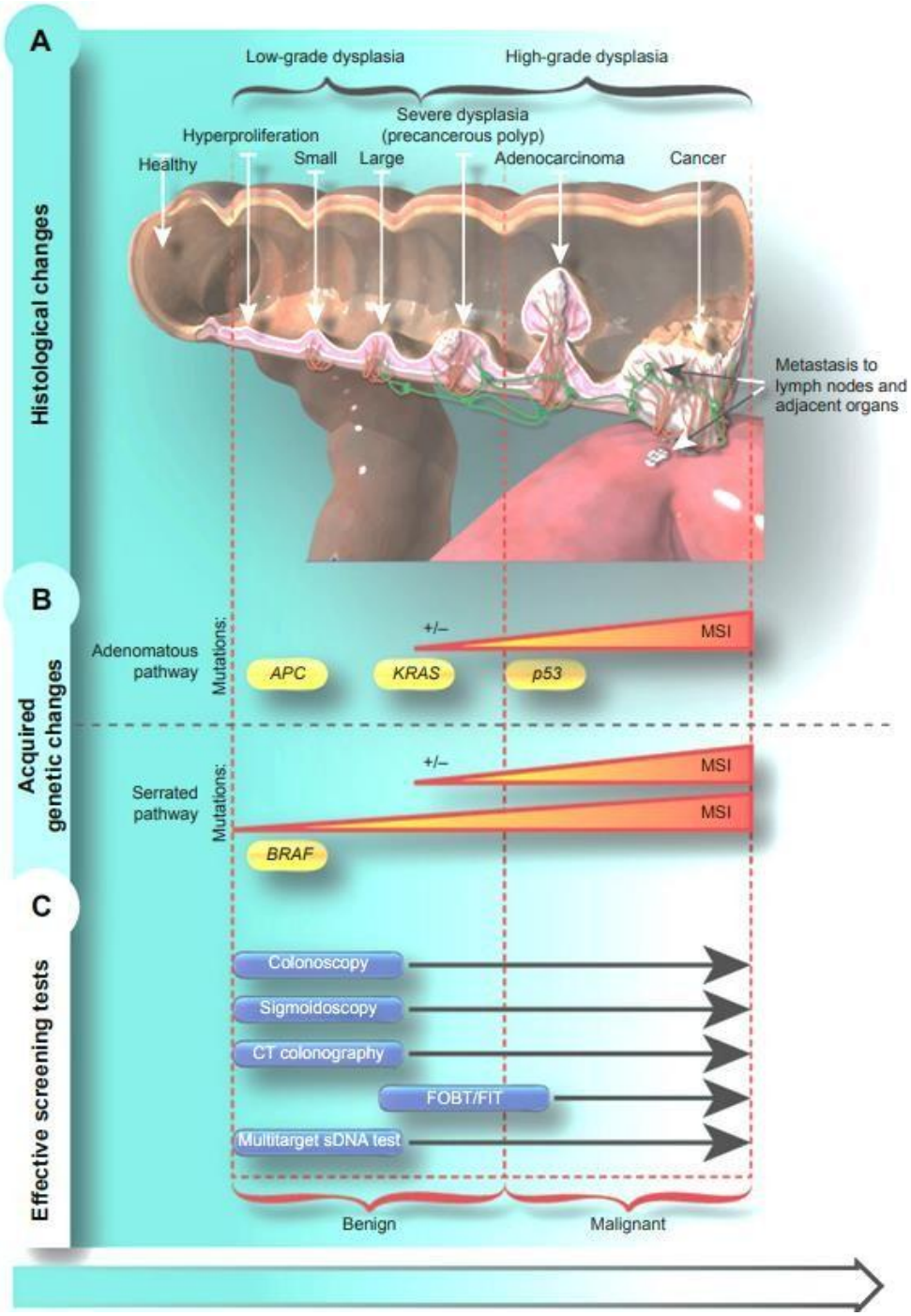


Figure 2.5: CRC development and screening methods

(A) Histological changes of CRC; (B) acquired genetic changes of CRC; (C) effective screening tests for CRC. The temporal development of CRC is indicated from left to right in each panel. Includes methylation of BRAF, KRAS, BMP3, and NDRG4 genes. (Data from O'Brien et al., 2006)

Second pathway is microsatellite instability pathway which caused by DNA repair gene. Any mutation in DNA repair gene leads to uneven replication of repetitive DNA sequence in short non coding regions which increase susceptibility to additional genetic mutation and leads to microsatellite instability (Figure 2.6). This can occur in both adenomas and SSPs (O'Brien et al., 2006).

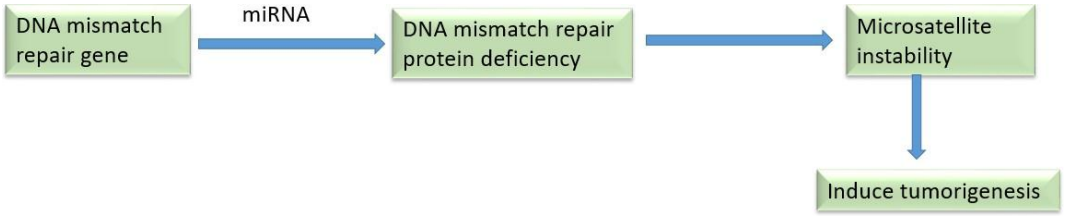


Figure 2.6: MSI induced CRC development (O'Brien et al., 2006)

2.6. Pathogenesis of CRC

CRC is highly related to lifestyle and diet, genetic predisposition, and the presence of diseases such as inflammatory bowel diseases (IBD). Epidemiological studies also showed the engagement of multiple environmental and dietary circumstances, for instance, alcohol, tobacco smoking, food rich in fat and scarce in fiber, obesity and a stationary style of living (Le Marchand et al., 1997).

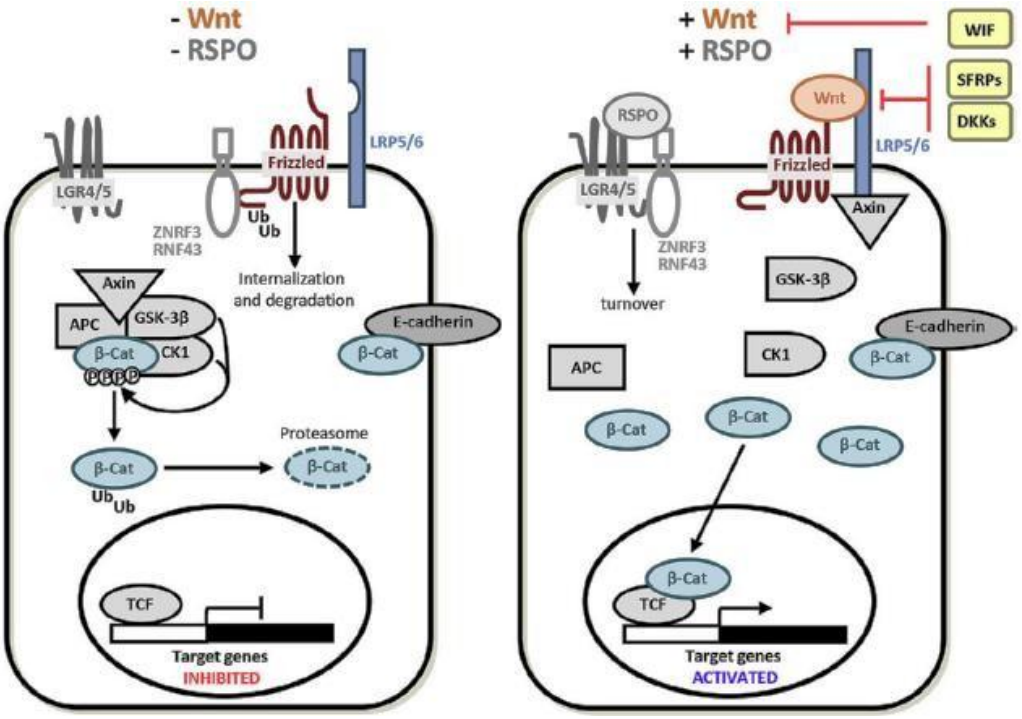
Some other factors which play protective role such as Physical activity (Slattery et al., 2004), extended-duration treatment with low-dose aspirin medicine (Rothwell et al., 2010).

2.6.1 Wnt/β-Catenin Signaling Pathway

Activation of the Wnt/β-Catenin Signaling Pathway is regarded as the initial event for most CRC. This pathway is associated with the maintenance of self-renewal proliferative capacity of healthy intestine. Wnt factors are family of glycoprotein which control β-catenin transcriptional activity by regulating its accumulation and its localization.

In normal epithelial cell, in absence of Wnt factors β-catenin found to be bounded with E-cadherin which is a transmembrane protein responsible for intracellular adhesion at adherens juncton's structures. β-catenin rapidly bound in a destruction complex which formed by the products of the APC and AXIN ½ genes and then phosphorylated by casein kinase (CK)Iα and glycogen synthase kinase (GSK)-3α/β, which are serine/threonine kinases. This leads to poly ubiquitination of β-catenin by β-TRCP1 ubiquitin ligase and sub sequent proteasomal degradation. As a result, in absence of free β-catenin in cytosol and nucleus a group of four members of free transcriptional regulators called T cell factors are bound to their target genes and actively repress them (Figure 2.7A).

Plasma membrane has receptor for Wnt factors which formed by a member of frizzled family and LRP5 or 6. When Wnt factors binds to its receptor it inhibits phosphorylation of β-catenin which causes an increase of β-catenin in cytosol (Clevers et al., 2012). Nuclear β-catenin then interacts with DNA bound TCF (in colon, TCF-4) and restrict its activity of gene suppression (Li et al., 2012). Wnt/β-catenin pathway can be blocked by the action of some inhibitor or elimination of Frizzled receptor which mediated by the action of RNF43 and RNF3 E3 ubiquitin ligases. R-Spondins (RSPO)1–4 are secreted proteins that, on binding to their leucine-rich repeat-containing G-protein coupled receptor (LGR) 4/5/6 receptors at the plasma membrane of stem cells, cause the formation of complexes between RNF43/ZNRF3 and LGR proteins that are endocytosed and degraded at lysosomes. In this way, RSPOs reduce Frizzled-LRP turnover and potentiate Wnt/β-catenin signaling, which causes increased proliferation (Figure 2.7 B) (Carmon et al., 2011; De Lau et al., 2011).



(A) (B)

Figure 2.7: Wnt/β-catenin signaling pathway (Barbachano et al., 2018)

(A) In absence of Wnt factor and (B) in presence of Wnt factor

Mutation or loss of APC (>80% CRC), AXIN ½ (1%-2%), CTNNB 1/ β-catenin leads to the inhibition of ubiquitination of β-catenin and degradation and the accumulation of free β-catenin. This step is considered as the initial step of colorectal tumorigenesis (Madan et al., 2015).

In colon tissue, Wnt factors maintain the proliferation of stem cells that give rise to transit amplifying cells. Aberrant activation of the pathway due to mutation results in differentiation arrest, and restoration of the proliferation of TA cells, and the generation of an adenoma or polyp.

2.6.2. Ras Pathway

Ras proteins are ubiquitously expressed in almost all type of cell and considered one of the main reason of cancer because of their role in cellular signaling pathways (McCormick and frank., 1995). They are encoded by K-ras gene (proto oncogene) which identify the transition of benign condition to malignancy. They activated after binding to GTP by guanine exchange factors (GEFs) and GTPase activating

protein(GAPs) (McCormick et al., 1990). They act as molecular switch after activation and start stimulating various cellular process such as proliferation and division by turning on their target protein. Mutation in k ras gene restricted GTPase activity which disrupt GTP-GDP cycle resulting in hyper activated ras driven signaling in cells. In k-ras gene mutation usually occurs in codon 12 and 61 of their highly conserved coding sequence (Quinlan et al., 2009). Because of mutation in codon 12, glycine is converted to valine which leads to inactivation of GTPase domain of ras by GAP. On the other side mutation in codon 61 suppress Ras GTP hydrolysis as its main function was to stabilize the transition state of GTP hydrolysis (Janakiraman et al., 2010)).

Cancer is initiated when ras pathway and other different signal pathway engaged alongside. Mutation k ras gene initiated colorectal cancer when wnt activity correlated with MAPK signaling (Horst et al., 2012).

2.6.3. The p53 System

P53 gene is located at on short arm of chromosome 17 (17p13.1). It mainly functions as transcription factor that translated various stress signals such as DNA repair, cell cycle, apoptosis.

P53 is closely associated with CRC. Almost 43% CRC occurrence have p53 mutation (IARC TP53 database, R20; https://p53.iarc.fr/TP5 3SomaticMutations.aspx accessed on 1 April 2021). In CRC mutation occurs in p53 gene at 257 codons specially mutation in codons R175, G245, R248, R273 and R282 are considered as the five main hotspot (Muller et al., 2013).

All of these codon contain CpG dinucleotides. When methylation occurs in the cytosines of CpG dinucleotide, a transition occurs (G:C→ A: T) which might a reason of this high prevalence of mutation in this codon (Curtin and N. J., 2012).

Through AMP activated protein kinase pathway (AMPK), cyclin dependent kinase inhibitor 1A (CDKN1A) is upregulated by p53 which play a major role in cellular cycle. Various studies found that in 79% CRC cases p21(Tumor suppressor gene) lack its functionality and as a result of it, expression of p53 is increased (Ogino et al., 2009). P53 combined with cycloocygenase-2, increase inflammatory response and cell propagation in CRC (Swamy et al., 2003).

2.6.4. Other Pathway

APC (Adenomatous polyposis coli) gene acts as tumor suppressor and associated with phosphoinositide-3-kinase signaling pathway. Through kinase cascade mutated APC gene stimulate various nuclear transcriptional factor.

Mutation in Phosphotidyleinositol-3-kinase catalytic alpha subunit(PI3KCA) gene induce call advancements through AKT pathway. A study designed by Liao et al., (2012) showed that survival period of patient with PI3KCA mutation CRC increased after the use of aspirin for long term. Though the exact reason behind this is still not clears but it theorized that extreme methylation of long interspersed nucleotide element -1(LINE-1) is correlated with chromosomal instability (Cordaux et al., 2009).

2.7. Aetiology and Risk factors

The aetiology of of CRC is complicated because it involves both environmental and genetic factors (Wallin, 2011). There are two types of colorectal malignancy, sporadic CRC and inherited CRC. In sporadic CRC there is no genetic linkage which normally caused by age, lifestyle and environmental factors without involving any kind of genetic changes (Kang et al., 2011).

Some factors contribute to CRC as rick factors to CRC including sex, age, prior disease such IBD, style of living, family history and medicinal background (Sankaranarayanan et al., 2011). Some other lifestyle related factors such as food habit, obesity, physical inactivity, smoking, alcohol consumption also contributes to develop CRC (Hagger et al., 2009).

2.7.1. Hereditary

Genetic disorders which inherited as autosomal dominant condition al increase the chance of developing colorectal cancer. This includes Familial adenomatous polyposis(FAP) and also lynch syndrome (LS) (Yurgelun et al., 2017).

Hereditary nonpolyposis colorectal cancer is also an autosomal dominant syndrome which is highly prevalent than familial adenomatous polyposis and adenocarcinoma in the colon. LS also has relation with developing colorectal cancer and other form of malignancy. LS have a mutation in the allele of a mismatch repair gene and other allele has somatic inactivation in the CRCs via mutation, loss of heterozygosity or epigenetic silencing by hyper-methylation of promoter.

When CRC develops from LS, it is characterized through an early incidence and a predominant tumor at right side of colon. 60% of women who carries mutation in LS, cancer spread outside of colon and raised risks of tumor generation as in the ovary, liver, gall bladder, small intestine etc. (Yurgelun et al., 2017).

2.7.2. Medical History

Several studies linked CRC with various other diseases such as IBD, type 2 diabetes mellitus.

The mechanism of IBD is probably due to chronic mucosal inflammation, increased cell turnover which ultimately increase rate of sporadic mutation. Various studies showed that people with IBD have 3time greater chance to develop CRC than people with no IBD (Peterson., 2015).

On the other side, Type 2 diabetes mellitus also contribute in the process of developing CRC. People with type 2 diabetes mostly develop proximal colon cancer. In cancer cell, insulin like growth factors are overexpressed with specific IGF receptors. IGF increase cell progression also suppress apoptosis. Insulin promote cancer by stimulating insulin receptors and lowering the amounts IGF binding proteins in the body which increases free unbound IGF. As a result of this Ras-Raf-MEK-Mitogen-activated protein kinase (MAPK) pathway and the phosphotidyleinositol-3-kinase(PI3K)-AKT mammalian target of rapamycin (mTOR) pathway activated. Both of this proteins are involved with cell development and proliferation (Avgerinos et al., 2019; Moschos et al., 2002)

2.7.3. Age

CRC affect almost all age. Several studies showed, CRC susceptibility increases with age. (DePinho and R.A., 2000). According to world cancer report 2007 probability of developing CRC increases after 40s. Probability of young people getting cancer is only 2-8%.

2.7.4. Behavioral and lifestyle factors

2.7.4.1. Diet

It is well established that diet is a possible reason behind almost all health problems, which even may lead to cancer.

In 2001, Terry et al. claimed that there is relation between the decrease of in-taking green vegetables and CRC occurrence. Green vegetables contain vitamin C and E, fiber, folate, selenium, and also many phyto chemical which may play protective role against CRC. A meta-analysis showed that frequent consumption of red meat (5times in a week) higher (13%) CRC development chance (Johnson et al, 2013). On the other hand white meat such such as fish and poultry is safe and not associated with CRC risk (Aykan et al., 2015; Bradbury et al., 2020).

2.7.4.2. Exercise

Several studies showed that routine exercise may reduce the chance of CRC development approximately 25% in male and female. Exercise has an effect on prostaglandins level which reduce stomach transportation time and enhance the role of the immune system (Fung et al., 2013; Samad et al.,2005; Wolin et al., 2007).

2.7.4.3. Obesity

Obesity is also increase the chance of developing CRC. According to Moghaddam et al., (2007) showed people with BMI higher than 30 had twenty percent more chance of forming CRC.

2.7.4.4. Tobacco

Tobacco contain various carcinogenic compounds such as polycyclic hydrocarbons, nitrosamines, and Heterocyclic amine. Long term consumption of these carcinogenic compounds increases the possibility of developing malignancy after. Studies showed that there is an increased risk of having neoplasm and progress the colorectal tumor extension, and raise the CRC predisposition (Hagger et al., 2009).

2.7.4.5. Vitamin D

In recent year many studies analyzed the relation between Dietary intake and vitamin D status in CRC incidence and mortality. CRC cells response to vitamin D metabolites depends on the level of calcitriol (precursor) and the expression of VDR.

2.7.4.5.1. Calcitriol

Calcitriol, is the active form of vitamin D, its intracellular concentration is dependent on the activity of CYP27B1 and CYP24A1.

Several studies have been proposed that expression of CYP27B1 is upregulated in well differentiated and moderately differentiated human colorectal tumor while downregulated in poorly differentiated tumor (Tangpricha et al.,2001). On the other side CYP24A1 found to be very low in healthy colon epithelial cells and overexpressed in colorectal tumor. Its expression is comparatively more upregulated at poorly differentiated tumor then well and moderately differentiated tumors. Its expression strongly correlates with markers such as Ki67, MCM2, MCM4 MCM7 and CDC6 which are considered as proliferative markers. This high level CYP24A1 leads to a decrease of calcitriol (Bareis et al., 2002, Horyath et al., 2010).

Calcitriol is involved with various cellular activity such as cell proliferation, differentiation, apoptosis and angiogenesis. It's anti-proliferative effect on CRC involves several pathways. Calcitriol increase the expression of cyclin dependent kinase inhibitors, CDKN1A and CDKN1B (Scaglione et al., 2000). These two inhibit CDK2 and CDK6 and caused G1 phase arrest. It also active transforming growth factor β1 (TGF β1) and inhibit the secretion of insulin like growth factor 2 (IGF2) by stimulating the insulin-like growth factor-binding protein-6 (Oh et al., 2001).

In cell differentiation, calcitriol also has effect. In colorectal adenoma it increases the activity of alkaline phosphatase by stimulating which is a marker for cell differentiation and found in the brush border of colon mucosa. Calcitriol increase alkaline phosphatase activity by stimulating activator protein-1 activator, a proto-oncogene through protein kinase C α- and mitogen activated protein kinase (MAPK)-dependent mechanism. It also stimulates the expression of E-cadherin (CDH1) and inhibit Wnt/B1 pathway (Figure 2.8) (Palmer et al., 2001).

In colorectal adenoma and colorectal cancer, calcitriol induces apoptosis by up-regulating the pro-apoptotic proteins BAK1 (BCL2-antagonist/killer 1) and BAX (BCL2-associated X protein) and by down-regulating the expressions of anti-apoptotic proteins BAG1, BIRC5 (baculoviral IAP repeat containing 5) and BCL2 (B-cell CLL/lymphoma 2) (Diaz et al., 2000)

2.7.4.5.2. VDR

In CRC VDR levels becomes higher in well differentiated tumor than in poorly differentiated tumor (Palmer et al., 2001). In CRC cells expression of VDR is

downregulated by binding of transcription factor SNAIL1 binds to three E-boxes in the proximal human VDR gene promoter and blocks the induction of E cadherin expression. As a result, translocation of β-catenin from the nucleus to the adherens junctions at the plasma membrane stopped and Wnt/β-catenin signaling remains active (Figure 2.8) (Larriba et al., 2009).

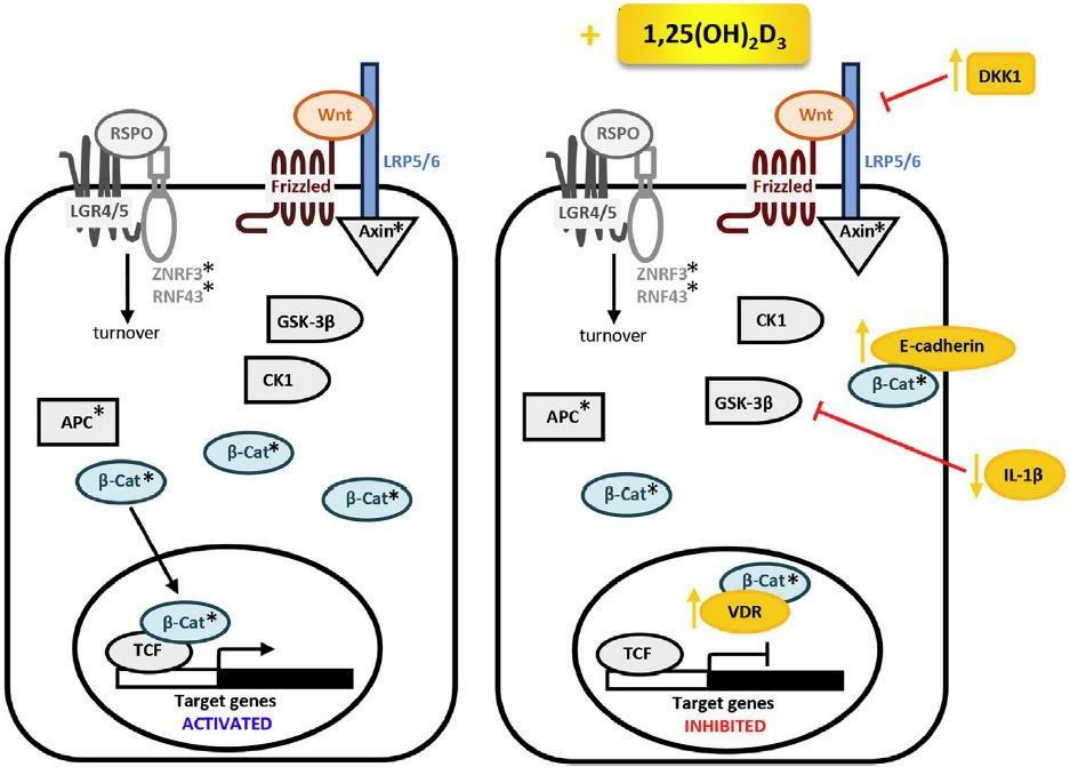


Figure 2.8: Action of VDR on uptake of calcitriol (Barbachano et al., 2018).

Recent studies also suggest that repression of VDR at post transcriptional level may also be caused by microRNA.

2.8. Prognostic Factors

The prognostic factors help clinicians to anticipate the disease most expected outcome which promote medical settlement and helps to decide proper therapy. The prognosis CRC depends on several factors.

2.8.1. Carcinoembryonic antigen (CEA)

Though Carcinoembryonic antigen is introduced for its diagnosis and prognosis uses in CRC some researches showed that individuals with a higher level of CEA preoperatively have a more unsatisfactory consequence than patients with lower

concentrations (Grande et al., 2008) and rise of carcinoembryonic antigen before operation and the extent of rising has a parallel association with heightened probability of frequency and decrease survival rate (Wigger et al., 1988).

2.8.2. Laboratory test

Several laboratory test such as complete blood count, glucose level, electrolyte in serum are also suggested for patients with colonic malignancy.

2.8.3. Pathological stage of CRC

To stage cancer, mostly used common method is TNM staging. "American Joint Committee on Cancer (AJCC)" proposed TNM staging, where T stands for tumor initial growth, N stands for node and M stands for metastasis that is coupled to form grade classifications (Fleming et al., 1997). To decide on the prerequisite of neo-adjuvant therapy in the malignancy, evaluating proper rectal grading framework is important (Brown et al., 2005). Table 2.1 is depecting staging system of colorectal cancer.

Table 2.1: staging system of Colorectal Cancer (Weisenberg et al., 2019).

The TNM staging of CRC	Indication	Description
Primary tumor (pT)	TX	primary tumor cannot be assessed.
	T0	No evidence of primary tumor
	Tis	Carcinoma is in situ, intra-mucosal carcinoma with the involvement of lamina propria with no extension through muscularis mucosae.
	T1	tumor invades submucosa through the muscularis mucosa but not reached to muscularis propria.
	T2	tumor reached to muscularis propria.
	T3	tumor invades throughout the muscularis propria into the pericolorectal tissues.
	T4a	tumor invades through the visceral peritoneum (including gross perforation of the bowel through the tumor and continuous invasion of the tumor through areas of inflammation to the surface of the visceral peritoneum)
	T4b	tumor directly invades or adheres to other adjacent organs.
	N0	lymph node is not involved yet.
	N1a	Spread through 1 regional lymph node

Regional lymph nodes (pN)	N1b	Spread through 2 - 3 regional lymph nodes
	N1c	No positive regional lymph nodes. But there are tumor deposits in the subserosa, mesentery or non-peritonealized pericolic or perirectal tissues.
	N2a	Metastasize to 4 - 6 regional lymph nodes
	N2b	Involve seven or more regional lymph nodes
Distant metastasis (pM)	M0	no distant metastasis by imaging
	M1a	cancer spreads to one organ or site (metastasis) but not reached to peritoneum
	M1b	confirmed metastasis to 2 or more sites or organs without peritoneal metastasis
	M1c	confirmed metastasis to the peritoneal surface alone or with other site or organ metastases

2.9. Current CRC screening methods.

The diagnosis of CRC mostly relies on the pattern complaining. For CRC diagnosis, doctors usually suggest colonoscopy, CT colonography. Colonoscopic biopsy is performed to obtain histopathological confirmation. Advantages and disadvantages of these procedure are depicted in the Table 2.2.

Table 2.2: Common diagnostic procedures used for CRC diagnosis

Tool	Blood or stool	Sensitivity (%)	Specificity (%)	Advantages	Limitations	Citation
Colonoscopy	Invasive	75–93	100	•Well validated and widely accepted •High sensitivity and specificity	•Need expertise to perform/ interpret • Invasive •Low compliance •Risk of intestinal perforation and bleeding	Lin et al., 2016; Wallender et al., 2007

Sigmoidoscopy	Invasive	77–84	84	•Less extensive bowel preparation	• Requires expertise to perform • Invasive • Risk of intestinal perforation and bleeding •Not as thorough as colonoscopy	Sung et al, 2003; Graser et al, 2009;
Fecal occult blood test (FOBT)	Stool	50	91–98	•Inexpensive • Can be performed at home	•Low sensitivity • Requires repeated testing	Malila et al., 2008; Elsafi et al., 2015
Fecal immunochemical test (FIT)	Stool	93	90	•Inexpensive • Can be performed at home •High sensitivity	• Not as sensitive to colorectal neoplasia	Elsafi et al., 2015; Katsoula et al., 2017
Cologuard	Stool	92–98	90	•Inexpensive • Can be performed at home	Not as sensitive to colorectal neoplasia	Imperiale et al, 2014; Lidgard et al, 2013
Carcinoembryonic antigen (CEA)	Blood	74–80	70–95	• Easy to perform	•Cannot detect early stage CRC	Thomas et al., 2015;

					•No standardized cutoff values	Duffy MJ, 2001
Epi pro Colon	Blood	66–68	91	• Easy to perform	• Low sensitivity	Yan et al., 2016

2.10. Molecular markers

The following table (table 2-3), illustrates the makers that reviewed by Colussi et al., (2013) in their study, so it provides the choice of possibly with using multiple molecular markers, to predict the behaviour of the disease in newly cancer-diagnosed patients. Hopefully, it will help to define the therapeutic strategies.

Table 2.3: The molecular markers and implications for disease behavior (Colussi et al., 2013)

Gene	Effect on Disease
CDK8 overexpression	Poor prognosis
K-ras cod. 12 mutation	Metastatic disease; poor prognosis; increased cancer-specific mortality p-AMPK Better survival among p-ERK positive
p53 expression	Better survival among non-obese
p21 loss	Better survival for patients >60 yrs
COX-2-positive tumours	Increased cancer-specific mortality
18q	Loss in non-MSI → decreased survival No loss → 5-year survival 96%
PI3KCA mutations	Increased survival among chronic aspirin users
Line-1 Hypomethylation	The young age of onset and increased cancer and overall mortality
HIF1	High colorectal cancer-specific mortality
Cathepsin B expression	High colorectal cancer and overall mortality

MSI	Better prognosis and survival than CIN/MSS
Cyclin-D1 overexpression	Low colon cancer and overall mortality
BRAF V600E	High cancer-specific mortality
MiR-203	Poor survival among stage IV Caucasians patients and stages I and II CRC patients
MiR-21	stage IV CRC poor prognosis
Interleukin-6	Increased CRC development risk, advanced CRC stage, and a worse prognosis
C-reactive protein.	Notably, in lean individuals; it is associated with increased risk of developing colorectal cancer

2.10. MicroRNAs

MicroRNAs are short noncoding single stranded RNA which control the expression of gene at post transcriptional level. They are 18-25 nucleotide in length and target mRNA to inhibit translation process of making protein. They act as a part of post transcriptional gene regulatory network (Pasquinelli et al., 2012).

2.10.1. Biogenesis of miRNA

During the canonical microRNA biogenesis; Firstly, the transcription of the micro RNA gene into an initial microRNA transcript (pri-miRNA) by RNA polymerase II action (Lee et al., 2004). The pri-micro RNA constitutes of one or more sequential units that can assemble hairpin structures, the stem of this structure is made of complementary-nucleotides that comprise the ~22 nucleotide mature and star miRNA sequences. The hairpin structures serve as substrates for the RNAse III enzyme Drosha (Lee et al., 2003), which, besides the help of its binding partner, "DGCR8" (Kwon et al., 2016), cleaves off the stem-loop with a two nucleotide offset, leaving the precursors miRNA (pre-miRNA) stem-loop. After that, pre-miRNA is carried out of the nucleus by Exportin-5 effect (Bohnsack et al., 2004).

Within the cytosol, the Dicer enzyme splits the pre-miRNA by eliminating the loop sequence, leaving a double-stranded RNA. A molecule called the duplex (miRNA miRNA*), with a 2-nucleotide offset at the 3p-ends (Hutvagner et al., 2001). The miRNA*, or passenger strand in the canonical miRNA biogenesis pathways, is degraded, leaving a ~22 nucleotide long mature miRNA which strives biological function. Deep sequencing of miRNAs displays that the majority of miRNA genes

follow this mature-star pattern expressing the 5p or the 3p-strand. However, some miRNA genes express similar read counts for both strands (co-mature pattern). The mechanism by which how one strand is identified over the other is not determined yet (Ameres et al., 2013).

Figure (2.9) illustrating different process stages. Pri-miRNA includes hairpin stem-loop and 5p- and 3p- primary transcript arms. After cleavage of drosha within the nucleus, pre-miRNA includes the hairpin stem-loop. Then transportation of Pre-miRNA into the cytosol; here, the dicer cleaves off the loop sequence, leaving the hairpin stem. The incorporation of the mature miRNA follows them into miRISC complex, and the degradation of miRNA* (Flarmark et al., 2016)

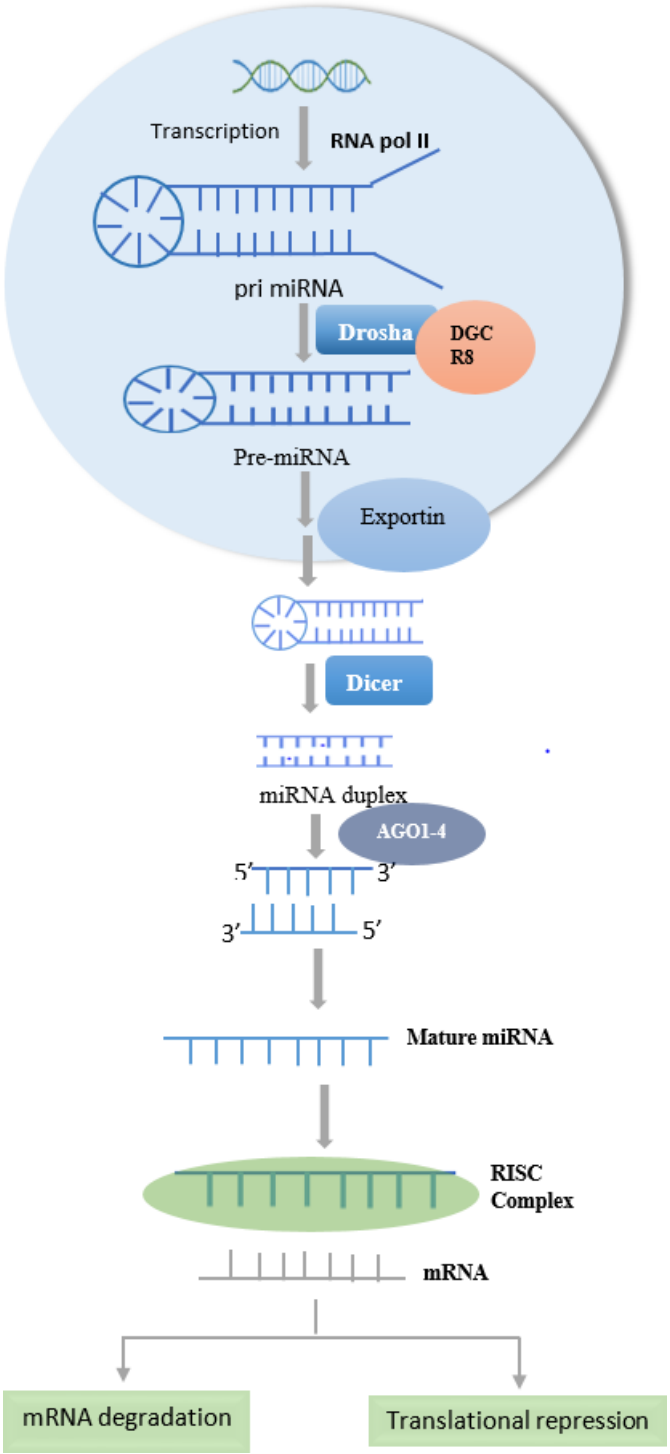


Figure 2.9: Structure of miRNA Canonical miRNA biogenesis pathway (Flarmark et al., 2016).

2.10.2. MiRNAs function

MiRNA acts on mRNA in two ways, Firstly, as post transcriptional modulators where they guide silencing protein complex to their target mRNA before translation started and secondly as gene expression modulator where they target genes which show 2 to fold alteration in their expression in corresponding protein level (Baek et al., 2008). Though miRNA plays a significant role in gene expression but they alone are not sufficient to stop a particular gene expression entirely (Bartal et al., 2009).

Since a single miRNA can target several mRNA and in contrary, a single mRNA may serve as target sites for several miRNA's, there's supposed to be an regulatory network of miRNA which control these gene expression.

Recently a new mechanism has been suggested which describe how cellular miRNA compete with endogenous RNA. Some RNA molecules have several number of target sites for miRNA and therefore can bind a lot of miRNA which are available in the cell, leaving very few available to suppress the rest RNA with those target sites (Salmena et al., 2011).

Concisely, to suppress a mRNA, availability of miRNA at single time also act as an important factor. The number of RNA containing attaching region for a specific miRNA in the cell will influence their ability to suppress specific mRNA. This way all RNA molecules that have same miRNA target site would challenge with each other for repression and produce an elaborate competitive endogenous RNA regulatory networks (Salmena et al., 2011).

2.10.3 MiRNA as biomarker

Several studies proposed miRNA as biological biomarker in different types of cancer and plays role in almost all aspect of cancer (Berindan et al., 2014). They can be released from cancer cell and they are stable in blood and tissue and also possible to detect them from samples even after long storage period (Meng et al., 2013). There are several methods by which miRNA can be extracted and quantified in samples easily (Landgraf et al., 2007; Shenoy et al., 2014). There are several ways to classify clinical biomarker for example, intracellular or extracellular, if extracellular then their level of invasiveness ranging from invasive, minimal invasive or non-invasive.

In invasive way sample collected from tissue for example in breast cancer tissue sample is collected from breast for biopsy so that breast cancer biomarker s can be analyzed (Drooger et al., 2015). On the contrary noninvasive biomarkers are those which can be collected from bloodstream and urine.

Availability of miRNA in bloodstream and stability of them in circulation makes them strong candidate as biomarker. Several studies on CRC have found the association of miRNA expression with CRC initiation. MiRNAs can act in two ways, either as oncogenic miRNA or tumor suppressor miRNA. A study done on 200 cases of different CRC stage, found out several miRNA expression level in patients (Schee et al., 2013). Same research claimed that CRC has a definite miRNA expression chart though there is no miRNA biomarker which clinically approved till now. List of some miRNA which are upregulated and downregulated in CRC with their targets, chromosomal location and their role in CRC are presented in table 2.4 and table 2.5 respectively.

Table 2.4: MiRNA upregulated in CRC

MiRNA	Chromosomal Location	Target gene	Function	Clinical application	References
MiR-21	17q23.2	PDCD4, TIAM1, SPRY2, PTEN, CDC25A, hMSH2, ITGB4,CCL20	Angiogenesis, EMT, Invasion, Migration, Proliferation, Apoptosis	Potential diagnostic and prognostic biomarker.	(Li et al., 2014; Sun et al., 2013; Asangani et al., 2008; Chen et al., 2015; Bunney et al., 2010)
MiR-92a	13q13	PTEN, SMAD2, SMAD4, TGFBR2	Metastasis, Proliferation, EMT, Invasion.	Potential screening biomarker	Tsuchida et al., 2011; Lin et al., 2013; Hayashit

					a et al., 2005
MiR-135 a,b	12q23, 1q32.1	APC, hMLH1,hMSH2	Proliferation	Biomarker clinical stage analysis.	Wu et al., 2014; Nagel et al., 2008
Mir-155	21q21.3	PTPRJ, TP53INP1, MSH2, MSH6, MLH1, FOXO3a, HuR,E2F2	Angiogenesis, Invasion, Proliferation, Stemness, Drug resistance, Genome instability	Tumor biomarker for diagnosis and prognostic assessment.	Herbst et al., 2014; Xue et al., 2013
Mir-224	Xq28	SMAD4, p21, PHLPP1, PHLPP2, GSK-3b	Metastasis, Proliferation, Tumorigenesis ,Chemo-radio sensitivity	Predictive marker for relapse after surgery	Ling et al., 2016; Olaru et al., 2013; Liao et al., 2013
MiR-214	1q24. 3	PTEN, PDLM2	Inflammation		Polytarchou et al., 2015
MiR-210	11p15.5	Cdc 25B,cdc25C, cyclin D_1, cyclin D_2, cyclin F	Inflammation Cell proliferation apoptosis		He et al., 2013; Tagscherer et al., 2016
MiR-182/503	7q32.2/ Xq26	FBXW7	Malignant transformation		Li et al., 2014

Table 2.5: miRNA downregulated in CRC

MiRNA	Chromosomal Location	Target gene	Function	Clinical application	References
Let-7	Multiple members (3,9,11,19,21,22)	KRAS	Proliferation	tumor suppressor in the CRC treatment	Saridaki et al., 2014
MiR-143/145	5q32-33	IGF1R, CD44, KLF5, KRAS, BRAF	Proliferation, invasion, migration, apoptosis, angiogenesis, chemo-resistance,	treatment and prognosis marker	Cordes et al., 2009; Su et al., 2014.
MiR-148	7p15. 2	cholecystokinin-2 receptor	Proliferation, apoptosis	biomarker and a therapeutic tool	Takahashi et al., 2012; Song et al., 2012.
MiR-34	1p36.22	E2F1, SIRT1, CD24, SRC, PAR2, AXIN2, KIT, ZNF281, IL6R, FMNL2, E2F5, LMTK3, HMGB1, MYC, KITLG	Proliferation, invasiveness, metastasis, apoptosis, chemo-resistance		Akao et al., 2011; Jiang et al., 2017.
MiR-126	9q34.3	PI3K, VCAM-1, CXCR4, VEGFA, IRS1, RhoA	Proliferation, invasion, migration, cell cycle, angiogenesis, hematopoiesis	biomarker for earlier detection	Guo et al., 2008; Bu et al., 2015.

MiR-26b	2q35	TAF12, PTP4A1, CHFR, ALS2CR2, FUT4	Proliferation, apoptosis, invasiveness, metastasis, migration,		Li et al., 2017
MiR-7	9q21.32	EGFR, RAF-1	proliferation		Suto et al., 2015
MiR-194	11q13.1	P21	Proliferation, apoptosis, invasion, migration, cell cycle		Wang et al., 2015; Zhao et al., 2014.

Flatmark et al (2016) explained several possible reason behind this situation. First of all, the biology of miRNA is highly complicated and so their mechanism of action. A miRNA may have several target sites on several gene and their mechanism of action also vary from tissue to tissue. To become a biomarker moderate fold change will not be enough. There must be a substantial fold change between the disease and the normal state and the variance must be consistent. In most of the miRNA literature has some contradiction such as same miRNA in same tissue sometime expressed as upregulated ad sometime as downregulated in different studies. One of the possible reason can be use of different detection techniques. The qRT-PCR and microarray are more reliable techniques for miRNA expression level analysis.

Table 2.6: Different detection techniques of miRNA

Technology	**Advantages**	**Limitations**	**Citations**
qPCR	• Current gold standard for sensitivity and specificity	No genome-wide coverage	Chen et al., 2005; Redshaw et al., 2013; Balcells et al., 2011;
Microarray	• Commercially available reagents • Genome-wide coverage	• Specific probes • Specialized equipment	Draghici et al., 2006; Sato et al., 2009;

		• Lack of reproducibility between platforms • Difficult data normalization	Meyer, et al., 2012;Wu et al., 2013; Pradervand et al., 2009; Rao et al., 2008; Wang, 2013)
NGS	• Genome-wide coverage •Multiple samples may be run in parallel • Promotes novel miRNA discovery • Can detect polymorphism	• Complicated, non-standardized data analysis	Li J, et al., 2015; Ansorge WJ., 2009; Chatterjee A, et al., 2015; Malone and Oliver, 2011; Park et al., 2016
Isothermal amplification	• No need for thermocycling equipment • Can improve existing qPCR, microarray, and NGS methods	• Disadvantages are technique specific	Graser et al., 2009
Exponential amplification	• High sensitivity	• May require a nicking enzyme, which complicates primer design	Zhao et al., 2015; Jia H, et al., 2010
Rolling circle amplification	• 1 primer • Can be optimized for linear or exponential amplification	Requires 2 enzymes (polymerase and ligase) • Initial denaturation not performed at room temperature	Tian et al., 2015;Li et al., 2013;Sun et al., 2012;Ge et al., 2014;Drmanac et al., 2010

Duplex-specific nuclease signal amplification	• High specificity	• Enzyme is not readily available	Yin et al., 2012; Pang et al., 2016; Wang Q, et al., 2015; Shuai HL et al., 2017
Hybridization chain reaction	• No polymerase	• Linear amplification only	Dirks and Pierce 2004; Miao et al., 2016; Miao et al., 2016; Ge et al., 2014; Yang et al., 2012; Bi S, et al., 2016; Cheglakov et al., 2015
Near-infrared technology	• No autofluoresence • Minimal photobleaching • No tedious treatment of sample before or after the test	Lanthanide probes are not yet commercially available and must be optimized	Zhong Y, et al., 2011;Miao P, et al., 2016;Shah P, et al.,2014;He, H., 2014; He H, et al., 2012

2.11. miRNA-210

The location of miRNA210 is on chromosome 11p15.5. This function as a regulator of several biological process which may dependent or independent on hypoxia. It acts as an oncomir (oncogenic miRNA) in the process of carcinogenesis and its upregulation reduces proliferation, cell cycle progression and colony formation. It also functions as a potent inducer of apoptosis and generate ROS (Figure 2.10) (Sabry et al., 2018)

miR-210 acts in an anti-tumorigenic manner and decrease proliferation by increased amount of cells in the G2/M phase of cell cycle (arrest) (Bertero et al., 2012; Nakuda et al., 2011). Its overexpression reduces expression of Cyclin D1 and Cyclin D2 (Zheng et al., 2013). For cell cycle arrest, miR-210 target E2F3 which plays a crucial role in proliferation (Kong et al., 2007). Some other molecules which also plays a role in cell cycle arrests are PIK1, Cyclin F, CDC25B (cell division cycle 25B) and Fam83D (Family with sequence similarity 83member D) (He et al., 2013). Overexpression of mir-210 also cause increase in ROS generation which also induce cell arrest. Elevated ROS level repressed cyclin B /cdk1 complexes by inducing an inhibitory disulfide bond (Burhans et al., 2009).

Expression of mi210 positively regulate ROS generation (Chen et al., 2010). In cancer cell it reduces oxygen consumption and increases glycolysis through regulation of Fe-S cluster scaffold protein ISCU, SDHD a subunit of the succinate dehydrogenase complex, COX10, a subunit of cytochrome c oxidase and NDUFA4 a subunit of the NADH dehydrogenase1 alpha sub complex (Chen et al., 2010; Puissegur et al., 2011)

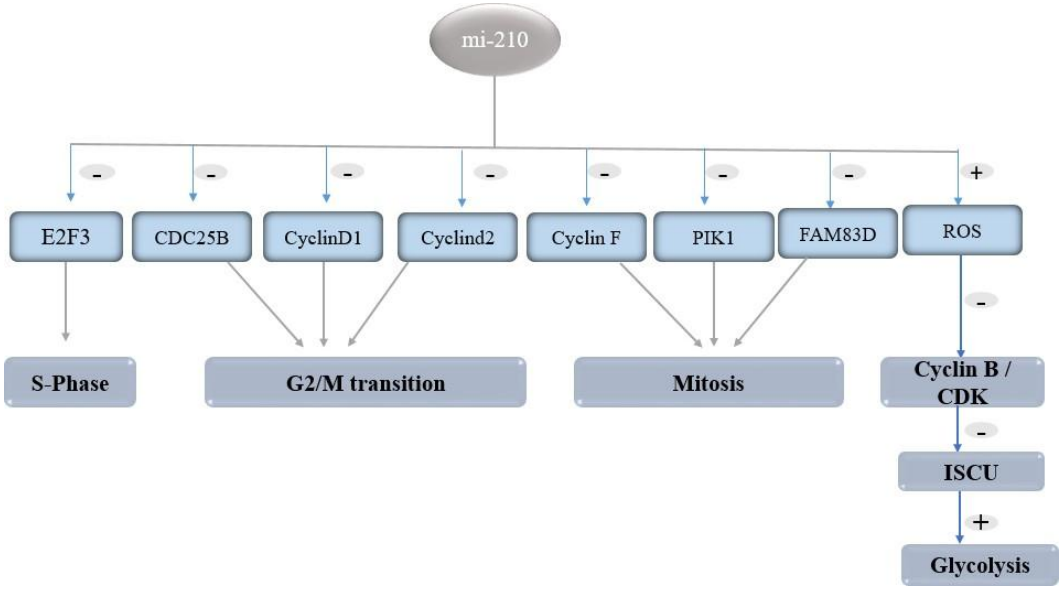


Figure 2.10: Mechanism of action of miRNA-210 in CRC (Bertero et al., 2012; Nakuda et al., 2011; Kong et al., 2007; Burhans et al., 2009; Huang et al., 2007; Luo et al., 2013)

Overexpression of miR-210 also leads to decrease in cellular phosphorylated AKT level that negatively regulate FOXOs, a transcription factors associated with the regulation of the transactivation of a series of genes involved in cell cycle control. AKT signaling pathway is also associated with ROS induced apoptosis (Huang et al., 2007; Luo et al., 2013). In this pathway ROS induced oxidation of the inhibitory protein thioredoxin. As a result ASK1 and the downstream stress kinase JNK and p38 get activated which latter induce cell death (Ray et al., 2012).

Chapter III: Materials and Methods

3.1. Study area

This study was conducted in two tertiary hospitals of Chattogram of Bangladesh namely Center for Specialized Care and Research (CSCR) and Chittagong Medical College Hospital (CMCH). The collected blood samples were analyzed at the 'Department of Physiology, Biochemistry and Pharmacology (Post-graduate Lab)' and 'Poultry Research and Training Center (PRTC) of Chattogram Veterinary and Animal Science University(CVASU).

3.2. Ethical approval

This study was ethically approved by ethical committee of university (CVASU). Before taking any information, a written consent was taken from each patient and from patient's guardian when patients were not in the state of talking.

3.3 Data collection

A cross sectional study was carried out for a period of 12 months from June 2022 to June 2023 on the patients, who undergone colonoscopy due to abdominal discomfort suggested by the physician. these patients were named as colorectal patients. For this purpose, a questionnaire was constructed by the study of literature and consult with the gastrointestinal specialist. The information includes are socio-demographic information, lifestyle behavior, history of medication, family history. A total of 42 patients were included for this study out of 58 patients attended to the hospitals because of insufficient information during fill up the questionnaire

3.4. Sample collection and preservation

Upon detailed investigation with colonoscopy and histopathology of biopsy materials, 16 of the 42 colorectal patients were diagnosed positive for colorectal cancer (CRC). Two mL of venous blood was collected from 9 CRC patients and 3 non-cancer colorectal patients as control. The collected blood was transferred to vacutainer without anticoagulant for serum preparation. The blood containing vacutainers were kept into the ice box and transported to the Department of Physiology, Biochemistry and Pharmacology, CVASU within 2 hrs. of collection. For serum separation, the vacutainers containing blood were then kept at room temperature for 2 hrs. Then the clotted blood was centrifuged at 820 × g for 10 min at 4 ºC. The resulting supernatant serum was then transferred to 1.5 mL centrifuge tubes followed by further centrifugation at 16,000 ×g for 10 min at 4 ºC, to completely remove any cell debris.

The cleared supernatant serum was then transferred to a new 1.5 mL tube and preserved at -80 ºC until analysis.

3.5. Measurement of vitamin D in serum

The preserved serum was thawed on ice. After it comes to normal temperature, we measured serum vitamin D level of CRC patients (N=9) by using Anbio 25-(OH) VD rapid quantitative test kit (#2023092510, Xiamen Biotechnology, China)

3.6.mi-210 expression analysis

3.6.1. MiRNA extraction

Total RNA was extracted from the preserved serum samples using Trizol LS Reagent (#10296028, Invitrogen, Carlsbad, CA) following the manufacturers protocol. Briefly, for phase separation, in a 1.5-mL micro-centrifuge tube, 750µL of Trizol LS and 200µL of tri-chloromethane were added with 250µL of serum. Vortexed it for 10 second and then incubated on ice for 15 min. When incubation period is over, again centrifuged for 15 min at 12000 × g and then collected the organic phase (upper phase) in another tube. In the next step, 0.5 mL of isopropanol was added in the tube and again incubate on ice for 10 min for nucleic acid precipitation. After precipitation, the tube was then again centrifuged at 12000 × g for 10 min. The supernatant was discarded and 1 mL of 70% ethanol was added for ethanol wash, the ethanol was carefully discarded without disturbing the nucleic acid pellet after and centrifugation at 7500 × g for 10 min. The pellet was then briefly air dried and 30 µL of RNase free water was added for elution of RNA. For long term storage this RNA can be stored at -80 ºC.

3.6.2. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

3.6.2.1 Reverse transcription

The extracted RNA was reverse transcribed using MMLV Reverse Transcriptase (#2641A, Takara, Japan) following the manufacturers instruction using the following gene specific stem-loop primers (Khanbabakhani 2019). RNU6B was used as internal control.

Gene	Sequence
mi210	5´- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACTCCATC-3´
RNU6B	5´- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACAAAAATAT-3´

This procedure was divided in two steps. In first step, 2.5 µL (20µM) of stem loop primer was added with 9.5 µL of extracted RNA and which then heated at 70 ºC for 3 min. After this step, the content was immediately placed it on ice for cooling. In the next step, we have added 4 µL reaction buffer, 1 µL RNAse inhibitor (#2313A, Takara, Japan), 1 µL MMLV Reverse Transcriptase, 2 µL dNTP (#4030, Takara, Japan) sequentially. Then mixed everything by gently pipetting up and down. Then, the content was incubated at 4ºC for 60 min. The reverse transcriptase enzyme was then inactivated by incubating the content at 70 ºC for 15 min

3.6.2.2. Quantification mi-210 by qPCR

Once cDNA is prepared, next step was qPCR. For qPCR, first we prepared a reaction mixture by adding 7.5µL of 2xTB green (#RR420A, Takara, Japan), 0.75µL primer forward and reverse primers (0.5 µM final concentration for each) and 4.75µL water, mixed it well by tapping and spin down and then 2µL diluted (1:20) cDNA product was added in the PCR tube and again mixed it well. After mixing everything together total volume was 15µL and then placed it into Applied biosystem7500 Fast real time PCR system. Temperature was set as followed:

Stage	Temperature	Duration	No of cycle
Initial denaturation	95 ºC	3 sec	1
Denaturation	95 ºC	30 sec	40
Annealing	60 ºC	30sec	
Extension	60 ºC	1 min	

The primer sequence used in this qPCR is given below(Tafsiri et al., 2016):

Primer Name	Sequence
Mi-210 forward	5'-CTGTGCGTGTGACAGCGG-3'
SNUB6 forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
Universal Reverse	“GTGCAGGGTCCGAGGTAT”

The mi-210 expression was calculated by the ΔΔ Ct method using SNUB6 as internal control:

ΔΔ Ct = (Ct $_{Target}$ – Ct $_{U6}$) $_{Cancer}$ - (Ct $_{Target}$ – Ct $_{U6}$) $_{Control}$. (Livak et al.,2001)

Δ Ct $Ratio$= $(E_{target})^{\Delta ct\ target}$ /$(E\ reference)^{\Delta ct\ reference}$

Δ ct $_{target}$ = Ct $_{control}$ - Ct $_{cancer}$

Δ ct $_{reference}$ = Ct $_{control}$ - Ct $_{reference}$

2-ΔΔCt

Ratio= 2-ΔΔCt

ΔΔ Ct =Δ Ct $_{reference}$ – Δ Ct $_{target}$

3.6.2.3. Confirmation of the qPCR product by electrophoresis

After qPCR, electrophoresis was done for the amplified products to confirm the products length. The qPCR product was mix with 6 × loading dye and loaded into a 2% agarose gel containing ethidium bromide. The electrophoresis was then performed in presence of 1 × TAE buffer. The expected product length for mi-210 and SNUB6 were confirmed by comparing the migration with 1000 bp ladder in gel.

3.7. Statistical analysis

Collected data were entered MS Excell -2013, sorted out and then exported to statistical package for social science 16 .0 software. Descriptive studies were performed including percentage, mean, SD and graph. T test was performed for vitamin D and miRNA level of expression between healthy and CRC subjects. Co-relation was performed between Vitamin-D and miRNA level of expression. $P<0.05$ was considered significant differences between groups.Graphs were generated with Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA. And Figure were made by using Biored.

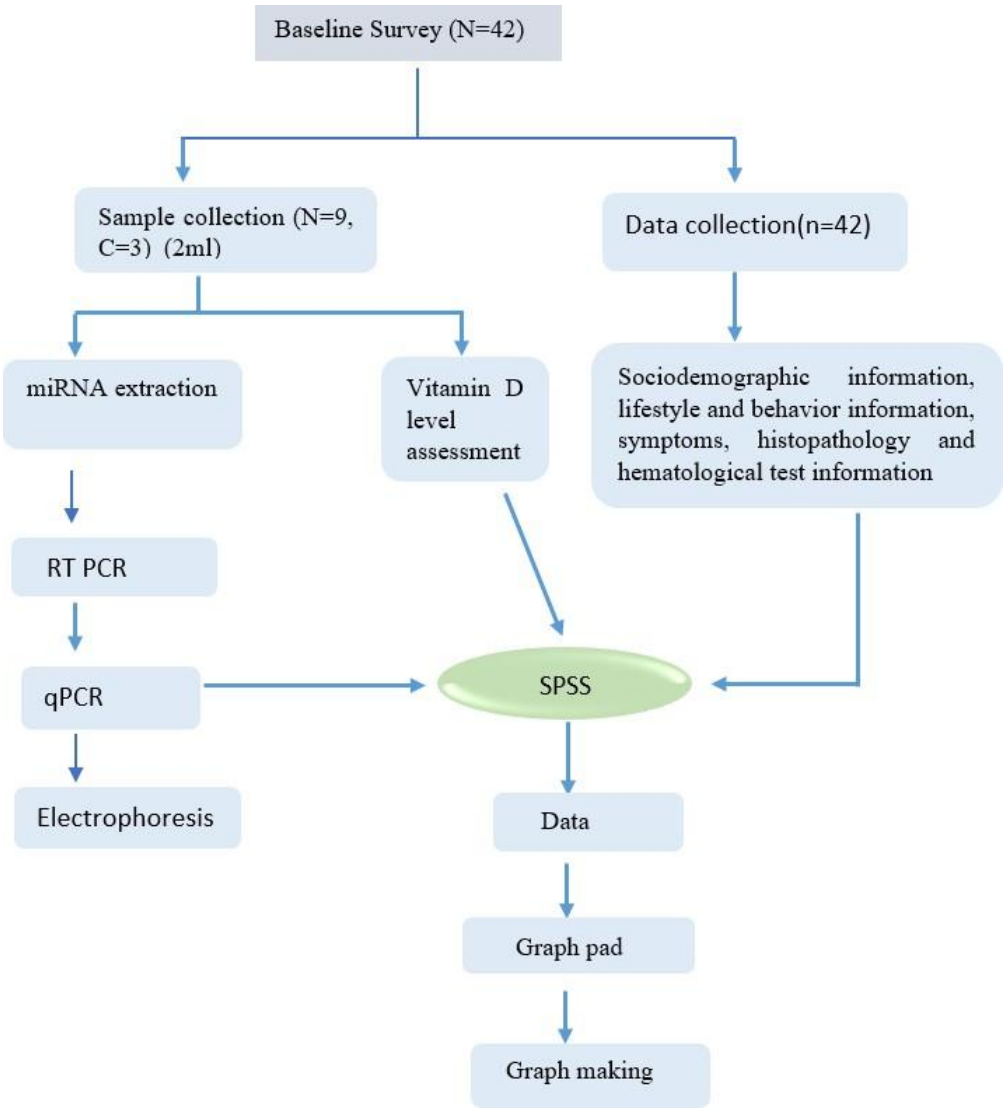


Figure 3.1: The workflow of this study

Chapter IV: Results

4.1 Demographic and clinical features of colorectal patients

4.1.1. Socio-demographic Characteristics

In this study, we collected a total of 42 colorectal patients' data. Among these patients 52.4 % of our patients were male and 47.6 % were female. Most of them comes from rural area (50%) followed by from urban area (42.9%), slum (4.8%) and overseas (2.4%) respectively. Our 26.2 % of the patients were not doing any kind of job, 23.8 % were job holder, 4.3 were farmers, 7.1% were retired, and 26.2 % were categorized as others (Freelancer and daily worker).

In our study working hour was categorized into three groups, where 31% of our patients were from group 1 (1-5 hrs.), 50% from group 2 (6-10 hrs.) and 19% were from group 3 (11-15 hrs.).

In terms of education 28.6% completed primary study level, 23.8% were illiterate, 23.8 % completed honors,14.3% completed secondary school examination and 9.5 % completed higher secondary examination

Table 4.1: Demographic characteristics of patients for baseline study (N=42)

Variable	Category	Number	Percentage
Age	20 to39 years	20	47.61
	40 to 59 years	14	33.33
	60 to 79 years	8	19.04
Sex	Male	22	52.4
	Female	20	47.6
Residency type	Urban	18	42.9
	Slum	2	4.8
	Rural	21	50
	Overseas	1	2.4
Occupation	Not doing any job	11	26.2
	Job holder	10	23.8
	Farmers	6	14.3
	Retired	3	7.1
	Businessman	1	2.4
	Others	11	26.2
Working hour	1-5 hrs.	13	31
	6-10 hrs.	21	50
	11-15 hrs.	8	19

Educational status	Illiterate	10	23.8
	Primary	12	28.6
	SSC	6	14.3
	HSC	4	9.5
	Honors and above	10	23.8
Family structure	Nuclear	25	59.5
	Joint	17	40.5
Religion	Muslim	37	88.1
	Hindu	5	11.9
	Christian	0	0
	Buddhist	0	0
	Other	0	0

Among these 42 patients, 59.5% of our patients came from nuclear family and 40% came from joint family, 88.1 % were Muslim and only 11.9 % were Hindu.

4.1.2. Food habit

We categorized the colorectal patients into 6 categories according to their preferred foods where we got maximum percentage for fish (73.8%) followed by fruits and vegetables (61.9%), dry fish (38.1 %), junk food (35.7 %) and lean meat (31.0 %).

Table 4.2: Food habit of the patients (CRC and others) (N=42)

Variable	Category	Numbers	Percentage
Preferred Food	Fish	31	73.8
	Fruits and vegetables	26	61.9
	Dry fish	16	38.1
	Junk food	15	35.7
	Red meat	14	33.3
	Lean meat	13	31.0

[Note: Most of the patients provided opinion on foods of multiple categories.]

4.1.3. Smoking and alcohol consumption

From these 42 patients, we took data about their smoking and alcohol consumption habit. About 76.2 % of the patients never smoked, 9.5 % were former smoker and 14.3 % are current smoker. In terms of alcohol consumption, 59.5 % never consumed

alcohol,19 % were former consumer, 14.3 % were occasional consumer and 4.8 % are current consumer.

Table 4.3: Smoking and alcohol consumption status of the patients (CRC and others) (N=42)

Variable	Category	Number	Percentage
Smoking status	Never smoked	32	76.2
	Current smoker	6	14.3
	Former smoker	4	9.5
Alcohol consumption status	Never consumed	26	59.5
	Former consumer	8	19
	Occasional consumer	6	14.3
	Current consumer	2	4.8

4.1.4. Sleeping pattern

We also asked them about their sleeping pattern. Among 42 patients, 16 (38.1%) were having proper 8 hr. sleep, 15 (35%) were having 6hr. sleep, 2 (4.8%) patients were getting 4hr. sleep and 9 (21.4%) were getting less than 4hr. sleep.

Table 4.4: Sleeping pattern of patients of the patients (CRC and others) (N=42)

Sleeping pattern	Number	Percentage
8 hr.	16	38.1
6 hr.	15	35.7
4 hr.	2	4.8
Less than 4 hr.	9	21.4

4.1.5. Physical exercise

Physical activity level and physical exercise habit was also included in this study. 22(52.38%) patients of this study are doing sedentary work (majority of time spends sitting) and 20 (47.6%) of them doing non-sedentary work. But only 5(12.2%) of them are doing physical exercise regularly, 8(19.5%) are doing it once or twice in a week,

1(2.4%) is doing 4 to 5 times in a week and 28(66.6%) are not doing any kind of physical exercise in their daily life.

Table 4.5: Physical activity of patients (N=42)

Variable	Category	Number	Percentage
Physical activity level	Sedentary work	22	52.38
	Non sedentary work	20	47.6
Routine physical exercise	Everyday	5	12.2
	4 to 5 times in a week	1	2.4
	Once or twice in a week	8	19.5
	Never	28	66.6

4.1.6. Symptoms

Among all clinical features abdominal pain and fatigue is the most common which was 32 (76.2%) patients followed by rapid weight loss, rectal bleeding and altered bowel movement which were 19 (45.2%), 16 (38.1%) and 16 (38.1%) respectively.

Table 4.6: Symptoms of the patients (CRC and others) (N=42)

Clinical Features	Number	Percentage
Abdominal pain or lump	32	76.2
Fatigue	32	76.2
Rapid weight loss	19	45.2
Rectal bleeding	16	38.1
Altered bowel movement	16	38.1

Note: Most of the patients provided opinion on multiple categories.

4.1.7. Other medical condition

Diseases such as diabetes, hypertension, asthma and inflammatory bowel diseases (IBD) have role in the occurrences of colorectal diseases including colorectal cancer (CRC). About 26.2% of our patients had diabetes. 38.1% had hypertension, 14.3% had asthma and 35.7% had IBD.

Table 4.7: Others co-disease of patients having lower digestive tract disorder

Category	N	Percent
Hypertension	16	38.1
IBD	15	35.7
Diabetes	11	26.2
Asthma	6	14.3

Note: Some of the patients provided opinion on multiple categories.

4.1.8. Family history

As family history also play an important role in the occurrences of colorectal diseases including CRC almost 31% of our patients had first degree relatives who had cancer before.

Table 4.8: Family history of the patients

Variables	category	Number	Percent
Relative having cancer	First degree relatives	13	31
	Second degree relatives	0	0
Type of cancer	Stomach cancer	1	2.4
	lung cancer	4	9.5
	Liver cancer	1	2.4
	rectal	2	4.8
	others	4	9.5

[other* includes breast cancer cervical cancer and neck cancer]

4.1.9. Daily sunlight exposure

Since sunlight exposure is necessary for synthesis of vitamin D and it was reported that vitamin D level has significant role in the prevention of colorectal disease, we took the data of sunlight exposure. About 57.1% of the colorectal patients them have sunlight exposure of less than 30 min. per day, 9.5% have 30 to 60 min. per day, 16.7% have 60 to 120 min per day and 11.9% had more than 120 min sunlight exposure

Table 4.9: Daily sunlight exposure of the patient (CRC and others) (N=42)

Variables	category	Number	Percentage
Sunlight exposure	Less than 30 min	26	61.9
	30 to 60 minutes	4	9.5
	1 to 2 hours	7	16.7
	More than 2 hours	5	11.9
	Less than 30 min	0	0

4.2. Patient type based on Colonoscopic findings

Based on colonoscopy result we devided the colorectal patients in two main groups- diseases (73.8%) and healthy (26.2%). Again diseases group was subdivided into 5 groups: polyp (16.7%), colitis (9.5%), adenocarcinoma (38.1%), haemorrhoid with anal fisher (7.1%) and ulcer (2.4%). Adenocarcinoma patients were the highest among all group.

Table 4.10: Patient category based on Colonoscopic findings (N=42)

Variable	Sub category		N	Percentage
Patients type (n=42)	Disease	Adenocarcinoma	16	38.1
		Polyp	7	16.7
		Colitis	4	9.5
		Haemorrhoids with anal fisher	3	7.1
		Ulcer	1	2.4
	Healthy	Healthy	11	26.2

4.2.1. Age and gender

Out of 42 colorectal patients, a total of 16 were diagnosed as cases of colorectal cancer (Adenocarcinoma) patients based on colonoscopy followed by biopsy. We looked for the age and gender distribution of CRC patients

A total of 16 cases of colorectal cancer patients from all ages and both sex were included in this study. The age range was from 25 to 78 years. Maximum patient, 6(37.5%) we get from 20-39 age range group, followed by 6(37.5%) from 40-59 year's group and

4(25%) from 60-79 year's group respectively. Among these 16 patients, 6(37.5%) were male and 10(62.5%) were female.

Table 4.11: Age and gender distribution of CRC patients (N=16)

Age Group	Male	Female	Total
20 to39 years	2(12.5%)	4 (25%)	6(37.50%)
40 to 59 years	4(25%)	2(12.5%)	6(37.50%)
60 to 79 years	-	4(25%)	4(25%)
Total	6(37.5)	10(62.5%)	16(100%)

4.2.2. CEA level

Carcinoembryonic antigen (CEA) is a blood biomarker for CRC. Among these16 CRC patients the blood CEA level is available for 9 patients. The maximum CEA level was 93ng/ml and minimum was 2.91 ng/mL, while normal range was 0-2.5ng/mL.

Table 4.12: CEA level of CRC patients (N=9)

Variable	Maximum (ng/mL) (n=9)	Minimum (ng/mL) (n=9)	Average (ng/mL)	Normal Range(ng/mL)
Carcinoembryonic antigen	93	2.91	23.31	0 to 2.5

4.2.3. Histopathology result of biopsy material

Among these 16 CRC patients, histopathology results were available for 9 patients. On the basis of histopathology result, 11.1% patients histopathologic feature of their adenocarcinoma was well differentiated,77.7% were, moderately differentiated and 11.1% were poorly differentiated.

Table 4.13: Histopathological feature of CRC (N=9)

Histologic feature of adenocarcinoma	Number	Percentage (%)
Well differentiated	1	11.1
Moderate differentiated	7	77.7
Poorly Differentiated	1	11.1

4.2.4. Location of lesion

From colonoscopic examination we found out 37.5% of our patient's lesion were in recto sigmoid junction, 50% patients had in ascending colon and 12.5% had at rectum.

Table 4.14: Location of Lesion (N=8)

Location	Number	Percentage (%)
Ascending colon	4	50%
Descending colon	-	
Transverse colon	-	
Sigmoid	-	
Recto sigmoid Junction	3	37.5%
Rectum	1	12.5%

4.3. MiRNA level expression

We compared the mi-210 expression level in serum of 9 CRC with that of 3 non-CRC colorectal patients (as control) by RT-qPCR and the data are presented as bar plot in Figure 4.1. We found that the expression of mi-210 in the serum of CRC patients were around 16 times higher than the control group. The data are presented as average of 9 CRC patients and 3 non-CRC colorectal patients. The SNUB6 was considered as internal control. This finding suggest that mi-210 expression level can be considered as bio-marker for the detection of CRC patients. Figure 4.2 shows the amplification plot and Figure 4.3 shows the gel image of target amplified gene. Here marker was 1000 base pair in length and our expected miRNA length was within 20-25 base pair. The detail calculation for quantification in presented in Table 4.15.

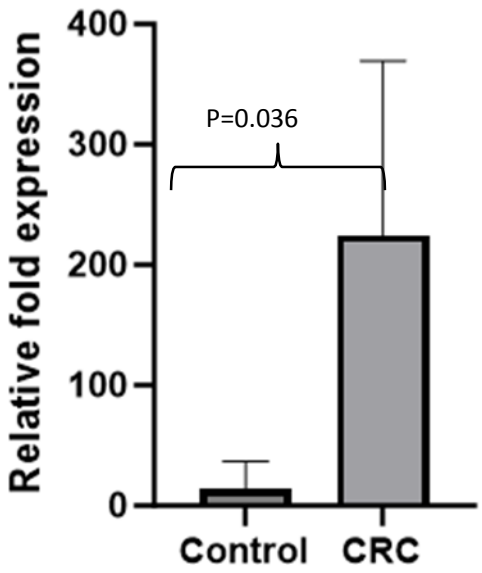


Figure 4.1: miRNA-210 expression level in control and CRC patients. The data are presented as average of 9 CRC patients and 3 non-CRC colorectal patients. The SNUB6 was considered as internal control.

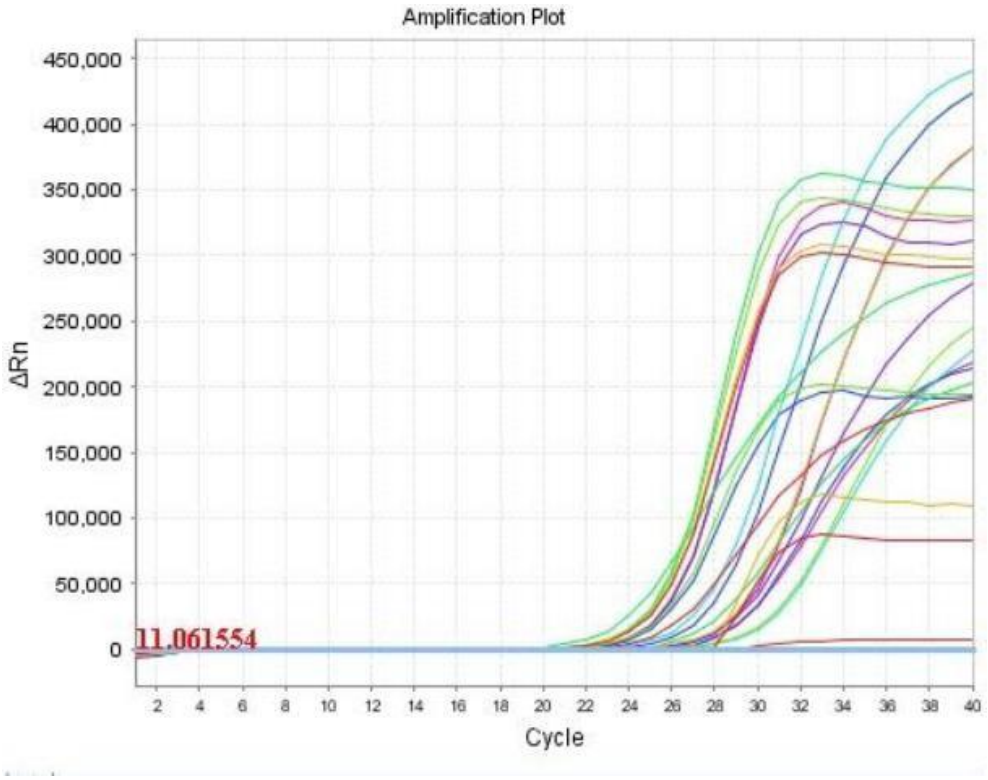


Figure 4.2: Amplification curve of mi-20 and SNUB 6 in qPCR

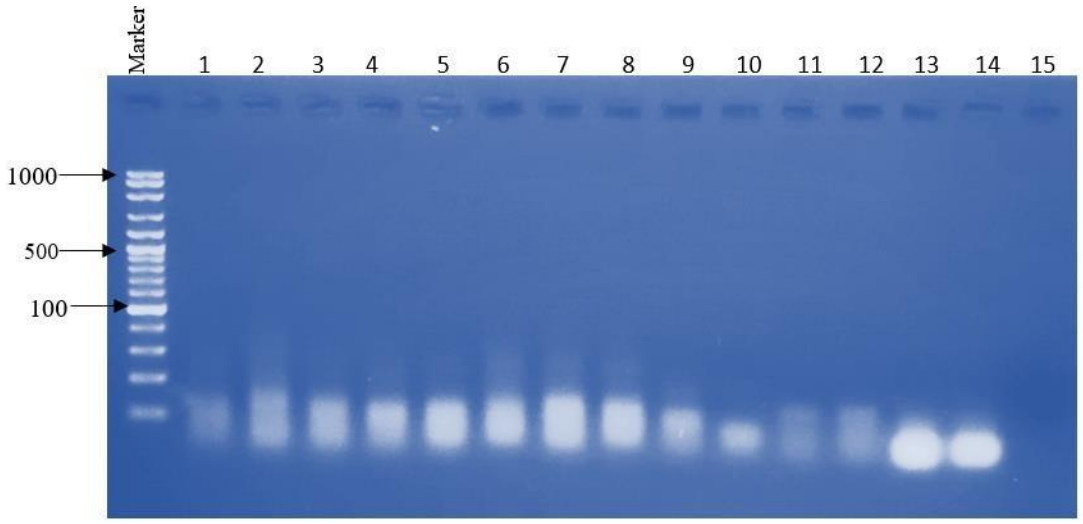


Figure 4.3: First column had DNA ladder (100bp in length). 1st-9th are CRC sample, 10th-12th are control group (mi-210). 13th column had sample for CRC (U6) and 14th column had sample for control (U6) and 15th column is the negative control.

Table 4.15: Quantification of gene expression by ^-ΔΔCT method.

Sample	Target	CT	Target	CT	ΔCT	ΔΔCT	2^-ΔΔCT	Relative fold change	Average
CT1	mi-210	32	RNUB6	34	-2	-3.333	10.079	40.31	14.18
CT2		33		31	2	1	0.5	2	
CT3		36		32	4	4	0.0625	0.25	
CA1		29.3		36	-6.7	-6.7	103.96	415	224.40
CA2		28.5		34	-5.5	-5.5	45.254	181	
CA3		31		36	-5	-5	32	128	
CA4		29		34	-5	-5	32	128	
CA5		28		35	-7	-7	128	512	
CA6		29		34	-5	-5	32	128	
CA7		29.33		34	-4.6	-4.6	25.45	101.82	
CA8		30		36	-6	-6	64	256	
CA9		27.6		33	-5.4	-5.4	42.22	168.89	

4.3.1. miRNA-210 expression level in different grades of CRC

Figure 4.4 plots the mean of expression level of mir-210 indicating between the control group and the patients with different colorectal malignancy grades: Grade Ⅰ, grade ⅠⅠ, grade ⅠⅠⅠ and grade IV. Grading can be done on the basis of histopathological image: highly differentiated (grade I), moderately differentiated (grade II), Poorly differentiated (grade III) and undifferentiated (grade IV). As we can see miRNA-210 is upregulated in CRC comparison with the control group, the expression level is also increasing with the cancer progression.

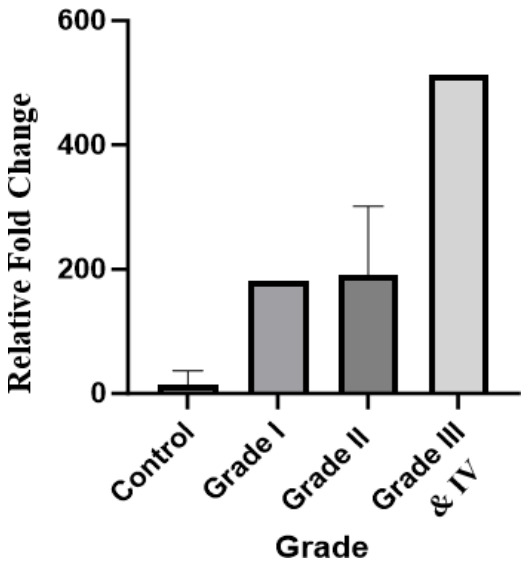


Figure 4.4: miRNA-210 expression level changes in control and different grade of CRC patients (Grade I-1, GradeII-7, Grade IV-1)

From this graph it could be seen that there's a constant increase in miRNA expression as the grade are going higher.

4.3.2. Vitamin D level in CRC patients

To co-relate serum vitamin D and miRNA level, pearson co-relate test was done.

Table 4.16: Correlation between level of serum vitamin D and miRNA-210 expression (N=12)

		miRNA
Vitamin-D	Pearson Correlation	-.478
	p	.116

Here we found Vitamin D level negatively regulate miRNA level. In CRC group, vitamin D level is lower and miRNA level is higher on the other side in control group vitamin D level is higher and miRNA level is lower.

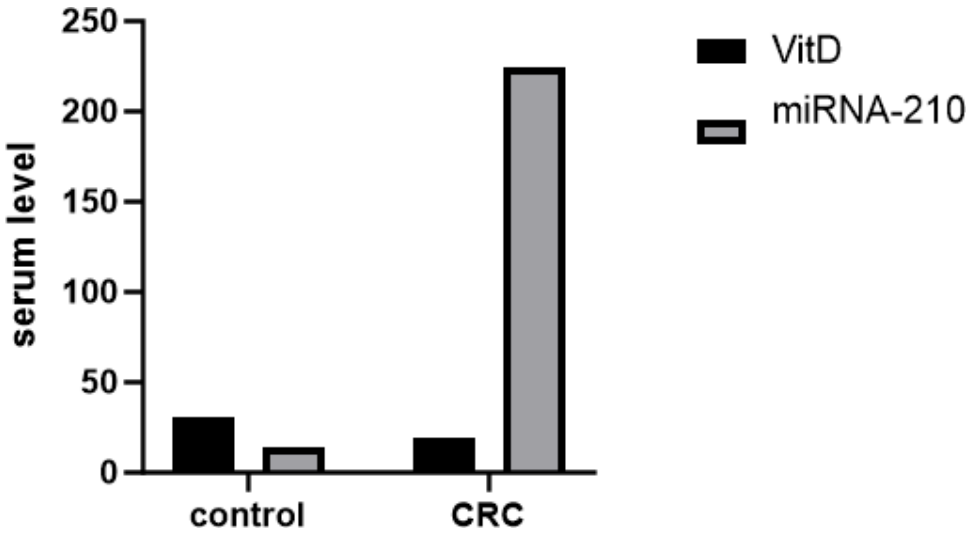


Figure 4.5: Relative fold changes in terms of vitamin-d level and miRNA-210 in both.

In CRC patients, maximum vitamin D level was 33.74 and minimum vitamin D was 12.39ng/mL. On the other hand, in control group maximum vitamin D level was 33.84 ng/mL and minimum was 26.42ng/mL.

Table 4.18: Vitamin D and miRNA-210 expression in CRC and control (T test)

Variable	Patient Type	N	Maximum	Minimum	Mean± SD	P value
VitD Level (ng/mL)	Case	9	33.82	12.39	21.82±5.95	0.017
	Control(Healthy)	4	33.84	26.32	30.90±3.26	
miRNA	Case	9	512	101	224.38±145.0	0.036
	Control(Healthy)	3	40	0.25	14.19±22.64	

Chapter V: Discussion

Colorectal cancer has emerged as a worldwide concern due to its increasing mortality rate. This mortality rate might be reduced if it can be diagnosed at early stage. Not only late diagnosis but also additional factors known as risk factors also contribute in the process of CRC development. Thses factors can be either non modifiable risk factors (sex, age, race, co diseases, family history) or modifiable risk factors (food habit, smoking, alcohol consumption, obesity). This study primarily aimed to understand sociodemographic and lifestyle-related information of patients presenting with abdominal discomfort subsequently diagnosed with adenocarcinoma and the potential of serum miRNA-210 expression for the screening of colorectal cancer patients as fluid biopsy. We also tried to establish a correlation between miRNA-210 expression level as the the diseases progress to advanced stage (grade) and their vitamin-D status.

Risk factors which are associated with CRC can be either non modifiable risk factors (sex, age, race, co diseases, family history) or modifiable risk (Lifestyle).Among non modifiable risk factors sex and age are well studied. A study conducted by Steele et al., (2014) revealed that the risk of developing CRC increases after 50 years (Steel et al., 2014). Our finding is also simillar with this study. The mean age of these 42 cases was 48±28 years. The age distribution ranged from 20-76 years. Mean age of the patients (16) who was diagnosed with CRC later is 47.5±22 years. In our study we got 6 patients who were within 20 to 39 years. This results can be compared with the other study in South and South East Asia, where CRC has been reported to occur with a great frequency in young patients (<40 years old) (Chan et al., 2010).

Regarding gender distribution of this study , 52.4% of patients were male and 47.6% were female. Later, 14% male and 23% of women were diagnosed with adenocarcinoma. Though most of the previous studies suggested that the CRC ratio in males is higher than in females, in our study, the CRC ratio in females was higher than males (Demb et al., 2019).

A prior study demonstrated a correlation between alcohol consumption and smoking with CRC (Driver et al., 2007). Based on alcohol consumption, the risk depends on duration, as it is a time-dependent relationship (Lin et al., 2020). In our study, only

4.8% of patients are current consumers, 14.3% are occasional consumers, 19% are former consumers, and the rest of the people (59.5%) never consumed alcohol. As most of the patients never consumed alcohol, it can not be considered as a possible reason for developing CRC. Same goes for smoking also. Many researchers consider smoking as a modifiable factor that increases CRC risk with increasing consumption (Driver et al., 2007; Gausman et al., 2020). Our findings also match this study.Regarding smoking, 14.3% of our patients are current smokers, 9.5% of patients were former smokers, and 76.2% of patients never smoked. Here, the majority of the patients never smoked. Therefore, it also can not be considered a major reason that may initiate their health risk regarding the development of CRC.

Throughout the years, numerous researchers have established food habits as a significant factor that serves a crucial role in the development of CRC. Red meat, dry fish, and processed food increase CRC risk (peterson et al., 2015); on the other hand, fish, lean meat, fruits and vegetables reduce CRC risk (Bradburry et al., 2020). In our study, fish, fruits and vegetables were preferred by majority of patients ((73.8 and 61.9%) while less than 40% of patients preferred red meat, dry fish, junk food, and lean meat. This outcome also reflects in our colonoscopic finding. Our 38.1 % of patients were later diagnosed with carcinoma, whereas nearly 26.2 % of patients were healthy. Here, one point needs to be mentioned: Many patients choose more than one preferred food.

Various studies have already proved the association of physical inactivity with CRC. According to previous data, physical exercise and and intensity are found to decrease CRC risk (Wolin et al., 2009). Our results also coincide with this finding. In our study, only 12.2 % of patients were doing physical exercise regularly, 2.4 % were doing it 4 to 5 times a week, 19.5 % were doing it once or twice a week, and 65.8 % were not doing any routine exercise. This higher percentage of physical inactivity may also serve as a reason for getting a lower percentage of healthy people (26.2%) after colonoscopy.

Another important factor regarding CRC development is sun exposure time. For sufficient vitamin D, we need proper sun exposure. Researchers showed data that for maintaining serum 25(OH)D concentration above 50 nmol/L an individual need more

than 1 hour sun exposure, and 75 nmol/L needs more than 2 hours (Patwardhan et al., 2018). However, in our study, 61.9.1% have daily sunlight exposure of about less than 30 min, 9.5% for 30 to 60 min, 16.7% for 1 to 2 hours, and 11.9% for more than 2 hours. Later when we estimate serum vitamin D level in CRC patients, average vitamin D level was lower than normal range(21.82nmol/L).

CRC is associated with both IBD and diabetes in multiple investigations. In a meta-analysis of 13 trials involving nearly 45,000 patients with IBD, the risk of CRC was shown to be approximately three times greater in people with IBD than in people with no IBD (Johnson et al., 2013). Epidemiological studies have demonstrated that individuals with type 2 diabetes mellitus have an increased risk of developing CRC (Demb et al, 2019). Moreover, a comprehensive analysis involving almost 22,000 CRC patients in the US revealed that diabetes prevalence was more significantly associated with proximal than distal or rectal cancer (Katsidzira et al., 2019). Furthermore, our study confirmed this finding. Patients who participated in this study had IBD(35.7%), hypertension (38.1%), and diabetes (26.2%).

CRC is highly related to family history (First degree relative such as parents, siblings,children). A large-scale meta-analysis involving 8091 cases of CRC in 16 studies concluded that the mean risk of CRC was almost two times higher in individuals with a family history of CRC compared to those with no family history of CRC (Johnson et al., 2013). This study also reveals that patients who have a family history are more likely to have distal colon cancer in both men and women and proximal colon cancer in men (Shin et al., 2011). Our findings are also similar to this result, where 31% of patients had a previous family history of CRC, and 50 % of them had cancer in the proximal colon, whereas 37.5 % had it in the distal colon .

According to Mohsin et al., (2021) Colonoscopic finding can be polyp, haemmorhoid, colitis, piles and adenocarcinoma. Our finding also similar with their study. In our study we get maximum patients with adenocarcinoma (38.1%). Rest of the patients had condition such as polyp, ulcer, haemorrhoid. Patients with no complication were considerred as healthy (26.2 %). All of these patients data were included in dataset

because polpy, ulcer, haemrrhoid known as the prestage of CRC. CRC can develop from these condition over time.

Rectal bleeding is commonly observed as one of the initial and frequent signs of colorectal cancer (CRC), according to several research. Though in most of the cases it happened because of hemorrhoids, rectal ulcer (Shinya et al., 1982). In our investigation, rectal bleeding was identified as the third symptom before the confirmed diagnosis of adenocarcinoma. As common symptoms, all patients whether CRC or polyps, colitis or ulcer, presented with abdominal pain and fatigue. Abdominal pain (76.2%) and fatigue (76.2%) were the most common presentation at the time of consultation. the other presenting complaints were rapid weight loss (45.2%), rectal bleeding (38.1%) and altered bowel movement (38.1%).

CEA is a non specific biomarker and glycoprotein in nature that controlled by fetal oncogene. When related genes are clustured on chromosome 19q13.2. When malignancy occurs, serum CEA level is elevated. Elevated CEA level of >5 µg/L at the time of new diagnosis of colorectal cancer is associated with poor prognosis. Patients who were diagnosed with adenocarcinoma had a minimum CEA level of 2.91 ng/ml while the maximum was 93ng/ml (normal,0-2.5).In histopathology results, 20% of our patients showed well differentiation (grade I), 70% showed moderate differentiation (gradeII), and 10% showed poor differentiation (grade III) . Our findings are also similar with the study of Kankanala et, al, where they describe CEA level increased greatly when metastasis occur and a subsequent drop in patients who undergone sergury or chemotherapy(Kankanala et al., 2022).

Concerning our investigation, it observed that the serum level of microRNA-210 expression is elevated in the cancer patients compared to the control group. With an overall fold change reach of up to 16 times higher expression in carcinoma subjects than the control. Moreover, the level is even higher with the stage progression. Referring to the figure 4.3.2, we can notice that the changes in the expression level of miRNA-210 as the diseases progress to advances stage. This study confirms the result

of several previous studies which also claims that miRNA-210 is associated with CRC and increased in metastasis level (Sabry et al., 2019; Wang et al.,2017).

The concentration of serum expression of microRNA-210 in CRC patients was prominent and reliable and correlated with advanced stages and metastasis. We have to assess its specificity to consider it as a specific biomarker for CRC. We have to increase the samples number and to assess the patients after the treatment period to compare the result before and after the treatment.

Another important finding of our experiment is the changes in serum vitamin D levels of CRC patients with the expression of CRC. Several researchers have also tried to co relate serum vitamin D levels with several other miRNAs, such as miRNA-22 and miRNA-627 (Alvarez-Diaz et al., 2012; Padi et al., 2013). Only these two miRNA previously investigated which had positive and negative effect on calcitriol uptake respectively. However, miRNA-210 exhibits a negative correlation with Serum vit D level. Any decrease in serum vitamin D level increases miRNA 210 expression in serum. In control group, the average vitamin D level is 30ng/mL, whereas in CRC patients, this level is 19 ng/mL. This suggests that Vitamin D also significantly reduces the risk of CRC by decreasing the expression of miRNA-210.

Chapter VI: Conclusion

This study helps us to understand sociodemographic and clinical feature of CRC and other CRC related intestinal condition such as polyps, ulcer and colitis This study also shows the association of miRNA-210 with CRC and its gradual increase with cancer grade and also its inversely proportional relation with serum Vitamin-D. CRC is not incurable. It's possible to treat and survival chance of the patients also can be increased if it is possible to diagnose at early stage. All the diagnostic procedure which are used now are either invasive (colonoscopy) or their specificity(FBOT) is lower and also expensive. In this scenario blood biomarker can be a good alternative of those testing. Proving the availability of using microRNA as an invasive diagnostic would help in early diagnosis and a better outcome, which would reduce the cost of treatment. Moreover, it would be such a relief to the patients who are suffering from the invasive diagnostic method.

Chapter VII: Strength and weakness

The strength of this study are-

- The data have been collected on self by producing a structured questionnaire from two hospitals where gastrointestinal patients came for checkup and interviewed by the author herself.
- The author assessed data of their socioeconomic status, demographic information, general health characteristic, laboratory parameters of blood and molecular study, the documentation, data entry, data analysis and data interpretation.

The limitation of this study-

- Major limitation of this study was small sample size which has been collected at narrowly time period.

Chapter VIII: Recommendation

To establish miRNA-210 as early blood biomarker for CRC we need to do several other investigations –

- Checking protein expression
- MSI checking through immunohistochemistry
- In-vivo investigation by inducing CRC chemically and then checking miRNA -210 expression.

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Appendices

Questionnaire:

Prevalence of Complication associated with Colorectal Cancer in Chattogram

Chattogram Veterinary and Animal Sciences University

Zakir Hossain Road, Khulshi, Chattogram 4202

A. General Information of the patient

Date of interview: Doctor's Name:		1. Patient ID: 2. Contact No:
3. Name:	4. Age:	5. Sex: i) M ii) F iii) Other
6. Address:		
7. Where do you live in? i) Urban ii) Slum iii) Rural iv) Overseas?		
8. Occupation: i) Don't do any job ii) Job holder iii) Businessman iv) Farmer v) Retired vi) Other		
9. How many hour you work everyday? i.1-5 hr ii. 6-10 hr iii. 11-15 hr		
10. Educational status: i) Illiterate ii) Primary iii) SSC iv) HSC v) Honors level and above		
11. Type of family? i) Nuclear ii) Joint		
12. Family member:		
13. Religion: i) Muslim ii) Hindu iii) Christian iv) Buddhist v) Other		
14. Attendant (If patient is severely ill):	15. Monthly income in BDT:	
16. Have you taken covid 19 vaccine? i. Yes ii. No 17. If yes then which of the foolowing it was? i. Pfier iii. Moderna ii. AstraZeneca iv. Sinopharm 18. Any other comorbid diseases: i. Diabetes ii. Hypertension iii. Asthma iv. Inflammatory bowel diseases v. Irritable bowel syndrome		

B. Lifestyle

19. What types of food you generally preferred?

i.	Red meat	iv.	dry fish
ii.	Lean meat	v.	Junk food
iii.	Fish	vi.	Fruits& vegetables

20. Please mention your smoking status:

i. Never smoked ii. Former smoker iii. Current smoker

21. What is your alcohol consumption status:
    i. Never consumed
    ii. Former consumer
    iii. Current consumer
    iv. Occasional consumer
22. How many hour you usually sleep everyday?
    i. 8 hr at night
    ii. 6 hr
    iii. 4 hr
    iv. Less than 4 hr.
23. Sun exposer time?
    i. <30 min ii.30-60 min iii. 1hr-2hr iv. more than 2 hr.
24. What about your physical activity level?
    i. sedentary work ii. non sedentary work
25. How often you do routine physical exercise?
    i. Everyday ii. 4 to 5 times in a week iii. Once or twice in a week iv. Never

C. History of medication

26. Do you take any kind of vit supplement?
    i. Yes ii. No
27. If yes then which type it is?

28. Long term (>6month)Drug exposure history:
    i. No
    ii. Aspirin
    iii. NSAIDs
    iv. statin
    v. Hormone Replacement Therapy(HRT)
    vi. Angiotensin II inhibitor
    vii. Oral antidiabetic agent (metformin)
    viii. Omeprazole (proton pump inhibitor)
    ix. others
29. Do you take regular anthelmintic on regular basis (after every 3 month)?
    i. Yes ii. No

D. Symptoms with duration

30. Which of the following Symptoms you had:
    i. rectal bleeding
    ii. Alteration of bowel movement which occurs in the form of consistency, frequency and diameter
    iii. abdominal pain or lump
    iv. fatigue
    v. Rapid weight loss

31. Do you have any other relative who had cancer?
    i. First degree relatives (parents siblings, and children)
    ii. Second degree relatives (grandparents, grandchildren, uncles, aunts, nephews, nieces, and half-siblings)
32. If yes, which type of cancer it was?
    i. Breast cancer
    ii. Stomach cancer
    iii. lung cancer
    iv. Liver cancer
    v. rectal
    vi. others
33. Do you have any previous history of Cancer?
    i. Yes ii. No
34. If Yes, then which type it was?
    i. Breast cancer
    ii. Stomach cancer
    iii. Colorectal Cancer
    iv. Liver cancer
    v. lung cancer
    vi. Others
35. Did your doctor suggested any of the following therapy?
    i. Neoadjuvant therapies (delivered before the main treatment, to help reduce the size of a tumor or kill cancer cells that have spread)
    ii. Adjuvant therapies (delivered after the primary treatment, to destroy remaining cancer cells)

E. General & local Examination:

36. Pulse:	37. Blood pressure
38. Temperature	39. Respiratory rate
40. Height (In cm):	41. Weight (Kg):
42. Abdominal Circumference (In cm):	43. BMI:

F. Colonoscopic Finding

44. What is colonoscopic Finding ?
    i. Polyp
    ii. Malignant
    iii. Both
45. Size of the lesion ?
46. Where is that lesion located?
47. Number of lession?
48. Is it Synchronous or not? i. yes ii.no
49. Is it colitis? i. Yes ii. No

G. Histopathology Finding:

50. Histopathology of the lesion ?
    i. polyp
    ii. malignancy

51. If polyp, histologic feature of each polyp?
    i. non-specific lesion
    ii. Hyperplastic polyp
    iii. tubular adenoma
    iv. tubulovillous adenoma
    v. high grade dysplasia
    vi. Adeno carcinoma
52. Location of your polyp?
    i. ascending colon
    ii. transverse colon
    iii. descending colon
    iv. Sigmoid colon
    v. Rectum
53. If malignant, is it -
    i. dysplasia
    ii. Adenocarcinoma

54. If adenocarcinoma, is it-
    i. well differentiated
    ii. Moderate differentiated
    iii. poorly differentiated

H. Staging Investigation:

55. Which of the following test were reffered by your doctor?
    i. Chest X-ray
    ii. Ultrasonography
    iii. Ct scan
56. Which stage your cancer was diagnosed:
    i. stage 1
    ii. stage 2
    iii. stage 3 (lymph node or tissue)
    iv. stage 4 (metastasis)

I. Hematology test finding:

57. CEA level –
58. CBC -
59. Serum albumin level-
60. CRP protein level-

J. TNM staging:

Ice making

Serum Separation

Centrifugation

miRNA extraction

RT PCR

Electrophoresis

Brief Biography

Jannatul Ferdous passed the Secondary School Certificate Examination in 2011 and then Higher Secondary Certificate Examination in 2013. She obtained her B.Sc. (Hon's) in Biotechnology and Genetic Engineering from The Faculty of life Science of Noakhali Science and Technology University, Noakhali, Bangladesh. Now, she is a candidate for the degree of Master of Science in Biochemistry under the Department of Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University (CVASU). She has immense interest in clinical and molecular Biochemistry and oncology.