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M. Sc. In Biochemistry Science and Technology

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GRADUATE SCHOOL OF
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**INVESTIGATION OF MDM2 AND RYBP GENE EXPRESSION
LEVELS IN COLORECTAL CANCER**

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IN
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**BY
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**Investigation of MDM2 and RYBP Gene Expression Levels in
Colorectal Cancer**

**M. Sc. Thesis
in
Biochemistry Science and Technology
University of Gaziantep**

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Amina FARHAN

ABSTRACT

INVESTIGATION OF MDM2 AND RYBP GENE EXPRESSION LEVELS IN COLORECTAL CANCER

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M. Sc. in Biochemistry Science and Technology

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Colorectal cancer (CRC) is known as a major cause of morbidity and mortality throughout the world including Turkey. Murine double minute 2 (MDM2) an oncogene is a critical negative regulator of the tumor suppressor p53. Ring1 and YY1 binding protein (RYBP) is a member of the Polycomb group (PcG) and regulates cell growth through PcG-dependent and -independent mechanisms. The objectives of this study were to investigate the expression of MDM2 and RYBP genes in normal and tumor colon / rectum tissues of the Turkish patients diagnosed with CRC. Also, to test the relation of MDM2 and RYBP expression with clinico pathological features such age, gender and to elucidate the correlation between MDM2 and RYBP expression in CRC tissues. Paired tissues tumor-normal (T-N) from 43 patients with CRC included in this study were examined by using qRT-PCR technique. Statistically there was no significant difference between normal tissues (3.60 ± 2.56) and tumor tissue (3.84 ± 2.70) in the terms of RYBP gene expression ($p > 0.05$). The same result for MDM2 expression was found in which no significant difference between normal tissues (3.20 ± 1.79) and tumor tissues (3.36 ± 2.16), ($p > 0.05$). None of the pathological features measured with the alteration (high and low) of gene expression for both RYBP and MDM2 were had a relation. Also, there was no correlation between the RYBP and MDM2 gene expression ($r = 0.158$, $p = 0.308$). Taken together, these data suggest that despite the fact that MDM2 plays as an oncogene and RYBP plays as tumor suppressor gene or maybe as oncogene depending on the tumor type, but both genes have no role in our patient's outcome.

Key Words: colorectal cancer, MDM2 gene, RYBP gene, qRT-PCR.

ÖZET

KOLOREKTAL KANSERDE RYBP VE MDM2 GEN EKSPRESYON DÜZEYLERİNİN ARAŞTIRILMASI

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Kolorektal kanser, Türkiye de dahil olmak üzere dünya genelinde önemli bir morbidite ve mortalite nedeni olarak bilinen bir kanser türüdür. Mouse double minute 2 (MDM2), bir tümör süpresör gen olan p53'ün kritik bir negatif regülatörü olan onkogendir. Ring1 ve YY1 bağlayıcı proteini (RYBP) ise Polykomb grubunun (PcG) bir üyesi olan ve PcG'ye bağımlı ya da bağımsız mekanizmalarla hücre büyümesini düzenleyen bir proteindir. Bu çalışmanın amacı, kolorektal kanser tanısı konulan Türk hastalarının normal ve tümör kolon/rektum dokuları arasındaki MDM2 ve RYBP mRNA ekspresyon düzeylerini araştırmaktır. Aynı zamanda kolorektal kanserli hastaların dokularında MDM2 ve RYBP ekspresyonunun yaş, cinsiyet gibi klinik patolojik özellikler ile ilişkisini test etmek ve MDM2 ve RYBP ekspresyonu arasındaki ilişkiyi aydınlatmaktır. qRT-PCR tekniği kullanılarak yapılan çalışmamızda, kolorektal kanser tanısı konulmuş olan 43 hastadan elde edilen tümörlü ve normal dokular kullanılmıştır. Normal dokular (3.60 ± 2.56) ve tümörlü dokular (3.84 ± 2.70) arasında RYBP gen ekspresyonu açısından istatistiksel olarak anlamlı fark bulunmamıştır ($p > 0.05$). MDM2 ekspresyonu için de normal dokular (3.20 ± 1.79) ile tümörlü dokular (3.36 ± 2.16) arasında benzer sonuçlar elde edilmiştir ($p > 0.05$). Her iki genin ekspresyon düzeyi (yüksek ve düşük) ile klinik patolojik veriler arasında istatistiksel olarak hiçbir ilişki bulunmamıştır. Yapılan çalışmalar sonucunda RYBP ve MDM2 gen ekspresyonu arasında korelasyon tespit edilmemiştir ($r = 0.158$, $p = 0.308$). Bu veriler birlikte ele alındığında, MDM2'nin bir onkogen ve RYBP'nin tümör tipine bağlı olarak tümör baskılayıcı gen ya da onkogen olarak rol oynamasına rağmen, çalışma grubumuzu oluşturan kolorektal kanserli hastalarla hem MDM2 hem de RYBP ekspresyon düzeyleri arasında bir ilişki olmadığı sonucuna varılmıştır.

Anahtar Kelimeler: kolorektal kanser, MDM2 gen, RYBP gen, qRT-PCR.

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LIST OF SYMBOLS/ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
Cm	Centimeter
Plagl 1	Pleiomorphic adenoma gene-like 1
NHL	Non-Hodgkin Lymphoma
A2MR	Alpha-2-Macroglobulin Receptor
ALL	Acute Lymphocytic Leukemia
AML	Acute myeloid leukemia
AJCC	American Joint Committee on Cancer
TP53	Tumor suppressor gene
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
B-CLL/SCL	Chronic Lymphocytic Leukemia/Small Cell Lymphoma
BMI1	B-Cell-Specifics Moloney Murine Leukemia Virus Integration Site-1
BRAF	A proto-oncogene B-Raf
BRCA1	Breast Cancer 1
CBX6	Chromobox 6
CBX7	Chromobox 7
CBX8	Chromobox 8
CD4+	Cluster of differentiation 4
CLL	Chronic Lymphocytic Leukemia
CML	Chronic myeloid leukemia.
CIN	Chromosomal instability

CLL	Chronic lymphocytic leukemia
DAD1	Defender Against Cell Death 1
DFS	Death Free Survival
DSBs	DNA Doublestrand Breaks
EIF4E3	Eukaryotic Translation Initiation Factor 4E Member 3
EIF4E3	Eukaryotic Translation Initiation Factor 4E Member 3.
EMT	Epithelial-Mesenchymal Transition
EZH1	Enhancer of zeste homolog 1
EZH2	Enhancer of zeste homolog 2
FAM48A	Family with Sequence Similarity 48, Member A
FANK1	Fibronectin Type III And Ankyrin Repeat Domains
FIGO	Federation of Gynecology and Obstetrics
FOXP1	Forkhead Box P1
GADD153	Growth Arrest and DNA Damage-Inducible Protein GADD153
GBE1	1,4-Alpha-Glucan Branching Enzyme 1
GBM	Glioblastoma Multiform
Gli1	Glioma-Associated Oncogene
GPR27	G Protein-Coupled Receptor 27
GXYLT2	Glucoside Xylosyltransferase 2
HCC	Hepatocellular carcinoma
HL	Hodgkin's lymphoma
HR	Homologues Recombination
IL12A	Interleukin 12A
JNK-AP1	The c-Jun N-terminal kinases -Activator Protein
KLF4	Kruppel like factor 4
KRAS	A proto-oncogene K-RAS
LC	Lung Cancer
MFH	Malignant Fibrous Histiocytoma

MED4	Mediator Complex Subunit 4
MEL18	Mouse Melanoma cells 18
MFH	Malignant Fibrous Histiocytoma
MIR133A	MicroRNA 133a-2
MMR	Miss Match Repair
MSI	Microsatellite Instability
MDS	Myelodysplastic syndrome
NSCLC	Non-Small Cell Lung Cancer
PH1	Pleckstrin Homology Domain-Containing Protein 1
PHC2	Polyhomeotic-Like Protein 2
PHF19	PHD Finger Protein 19
PROK2	Prokineticin 2
PLL	Prolymphocytic Leukemia
Rnf2	Ring Finger Protein 2
RT-qPCR	Real Time-quantity Polymerase Chain Reaction.
SAS	Subvalvular Aortic Stenosis
SHQ1	Small nucleolar RNAs of the box H/ACA family quantitative accumulation protein 1
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
STS	Soft Tissue Sarcoma
TFIID	Transcription factor II D
TNM	Tumour Node Metastasis
CD	Crohns Disease
TSGs	Tumor Suppressor Genes
UC	Ulcerative Colitis
ZEB1	Zinc Finger E-Box Binding Homeobox 1
ZEB2	Zinc Finger E-Box Binding Homeobox 2

G1	Gap 1
miRNA	MicroRNAs
RNase d H ₂ O	RNase distilled free water
dNTP	Deoxynucleotide three phosphate
SPSS	Statistical Package for the Social Sciences
χ^2	Chi square
P value	Probability value
UTR	Untranslated region
μ L	Microliter
OD	Optical Density
Ng	Nanogram
cDNA	Complementary DNA
Ct	Threshold cycle
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
SD	Standart Deviation

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is thought to be a result of several genetic changes such as cell growth, death, even senescence and DNA repair genes and it is known to occur after exposure to carcinogens. Mutations may be hereditary or due to oxygen causes. Generally, the incidence of cancer increases with age, because as people get older, they are more likely to encounter various genetic bruises that progress to cell transformation. Oncogene activation and tumor suppressor gene inactivation, or several genetic alterations involving DNA repair genes and apoptosis genes are required for complete transformation of a cell (Knudson, 2002; Ayhan, 2014).

Although malignant tumors generally differ in terms of macroscopic, microscopic, and genetic material, they share some characteristic features that determine their growth and biological behavior among themselves (Hanahan, 2000; Hanahan, 2011). Cancer, whether genetically or by environmental factors, is considered a genetic disease because it is primarily concerned with the genetic material of the cell. At the heart of the main mechanisms in carcinogenesis is the "non-lethal genetic bruising" in the cell. This DNA damage (ie, mutation) may be followed by environmental factors such as chemicals, radiation, viruses, or may be inherited by germ cells (Ayhan, 2014).

These mutations that cause malignant transformation can be classified according to the cellular mechanisms they affect;

- Activation of proto-oncogenes promoting growth and cell proliferation
- Inactivation of tumor suppressor genes that control cell proliferation
- Programmed cell death, prevention of apoptosis
- Inactivation of DNA repair enzymes

1.2 Colorectal Cancer

Abdominal pain, loss of appetite and blood in stool are the most common symptoms of colorectal cancer. The most common tests used in colorectal cancer screening are occult blood tests in feces and colonoscopy (Smith, 2010).

Understanding the molecular pathogenesis of colorectal cancer in recent years has led to the idea that some molecules may be a marker for screening for this cancer. Methyl vimentin was found in the stool of 53-84% of patients with colorectal cancer and this has been used in colorectal cancer screenings in the United States. This test is based on the detection of an incorrectly methylated vimentin gene in colorectal cancer cells. Adenomatous polyposis coli (APC), a proto-oncogene K-RAS (KRAS) and tumor suppressor gene (TP53) gene mutations in stool DNA tests, and mutations in a proto-oncogene B-Raf (BRAF), Microsatellite Instability (MSI) and Miss Match Repair (MMR) genes are clinically important genes in colorectal cancer diagnosis (Itzkowitz, 2008; Ahlquist, 2010).

1.2.1 Anatomy of Colorectum

The large intestines are approximately 150 cm in length and 4-6 cm in diameter and it is starting from the ileocecal valve and ending in an instant. The large intestines are divided into three part; cecum, colon (ascendant, transverse, descending and sigmoid colon) and rectum. They are located in the abdominopelvic space in the form of Ω (inverse U), and they are neighbors with many organs such as the liver, spleen, stomach, duodenum, small intestines, kidneys, ureters and bladder. The cecum is a large blind structure which is the origin of the large intestine and all surfaces are covered with the peritoneum. Because of its larger diameter, the cecum is the most common site of colon rupture in cases where distal obstruction is present (Fakıoğlu, 2008; Canca, 2009). Ascendant colon is located on the front face of the second base of the duodenum. The ascending colon is retroperitoneal and the anterior and lateral sides are covered with peritoneum. Transverse colon divides into two parts, upper and lower. This separation occurs by hanging in the pancreas and left kidney capsule with mesocosc. This formation also leads to a significant barrier in the case of intra-abdominal spread of the

infection. Descending colon has the thickest muscle layer and the narrowest lumen and continues from the splenic flexure to the left iliac sa. It is deeper than the ascending colon and its front side and both side faces are covered with peritoneum. Sigmoid colon is the continuation of the descending column. All surfaces are covered with peritoneum. The sigmoid column has a long sediment. It attaches to the back wall of the inverted V-shaped bait with the mezzanine (Keith L Moore, 1985; Fakioğlu, 2008; Canca, 2009). The rectum is the last part of the column, starting from the third sacral vertebra line and extending to the anorectal ring at the lower part of the tail's throat.

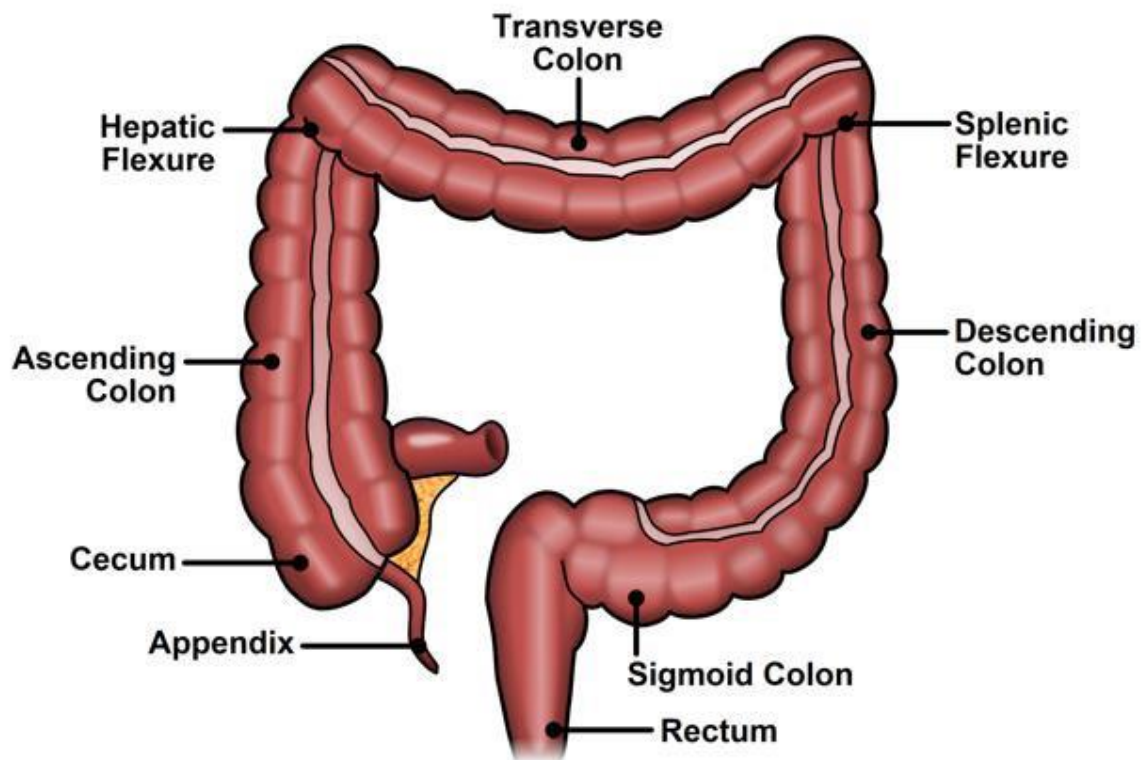


Figure 1. 1 Anatomy of large intestine (<https://www.fascrs.org/patients/disease-condition/diverticular-disease-expanded-version-0>)

1.2.2 Histology

At any organ in the body, histology represents the most important factor and that is due to its ability to determine the staging of both disease and treatment. Colorectal histology consists of 4 layers: Mucosa, submucosa, muscularis and serosa.

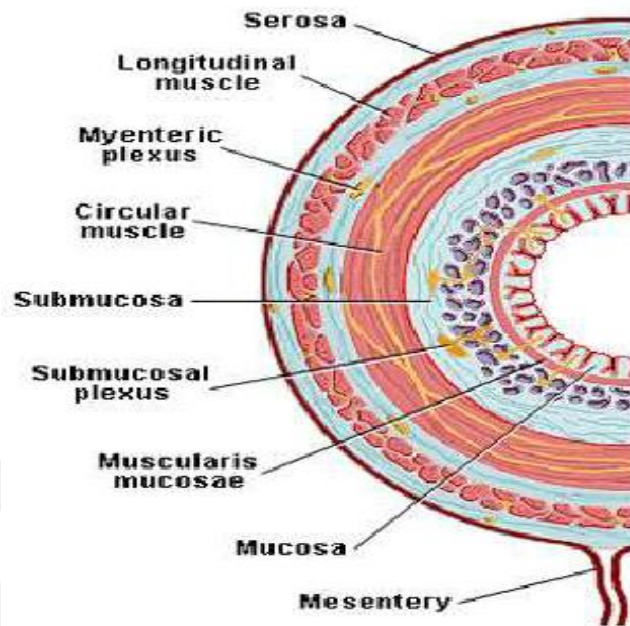


Figure 1. 2 Histology of large intestine (https://www.researchgate.net/publication/277237697_Applications_and_limits_of_raman_spectroscopy_in_the_study_of_colonic_and_pulmonary_malformations/figures?lo=1)

Mucosa consists of surface epithelium, crypt, lamina propria and lamina muscularis mucosa. There is no villus in this part of the canals. Surface consists of simple columnar or cuboidal epithelium. Hold the intestinal glands a large number of goblet and absorptive cells are characterized by a small number of enteroendocrine cells. Among epithelial cells, T lymphocytes are present. Lamina propria contains a loose collection of fibroblasts, vessels, nerves, smooth muscle and inflammatory cells. Lymphatics are limited in the lower third of the lamina propria. Normally present inflammatory cells are consisting of lymphocytes, plasma cells, mast cells, eosinophils and histiocytes. Muscular mucosa is a thin muscle layer. It separates the mucosa from the deeper submucosa. Submucosa; The cellular content of the lamina propria is also found in the submucosal stroma. Two neural plexuses are located in the submucosal region. These; Meissner submucosal plexus and deep submucosal plexus. Submucosal arterioles include venules and lymphatics.

Muscularis mucosa is thin cover mediate between mucosa and submucosa. Apparently, it roles to support local stirring at the mucosal surface, to improve the process of secretion and the absorption of nutrients.

Serosa is the outermost layer of the colon in inadequate with portions of the colon situated in the retroperitoneum. It is a layer of mesothelium and immediate adjacent fibroelastic tissue. The sub serosa is the layer of connective tissue between the serosa and the muscularis propria (Fleming et al., 2012).

1.2.3 Cancer Epidemiology in Turkey and World

Cancer epidemiology has shown that the incidence of all cancers varies from person to person, from society and time to time, and that this change is related to environmental, individual genetic factors and social habits. The incidence and mortality of colorectal cancer (CRC) vary significantly around the world (Eddy,1990).

According to the Ministry of Health in 2014, the frequency of colorectal cancer is 3 rd in both males and females in our country. The incidence in all age groups was 22.8 in one hundred for men; in women it was reported as 13.8 in one hundred because men are also seen to be about 1.5 times more frequently than women (Ministry of Health, 2017).

According to the World Health Organization's 2012 data, there are a total of 14.1 million new cases of cancer in the world, 8.2 million deaths from cancer, and 32.6 million people living with cancer diagnosed within 5 years. The worldwide death rate from cancer is steadily increasing, and it is estimated that this rate will be about 12 million in 2030, which doubles in 2008 (Ferlay, 2014; Tavossoli, 2014).

About one million CRC are diagnosed every year worldwide, and five hundred thousand patients lose their lives due to CRC (Swiderska, 2014).

Colorectal cancer is most commonly seen in industrialized countries such as Western Europe, North America, New Zealand, Australia, where a wide geographical area is observed. The CRC increases with the increase of development and westernization (Anne, 2007 and Cancer Fact, 2015). In addition, it is stated that urban areas have a higher risk of developing CRC than rural areas (Cancer Fact, 2015).

1.3 Aetiology of Colorectal Cancer

Risk factors for colon cancer are diet, age, environmental factors, adenoma or carcinoma tumor in the patient, family history and predisposing diseases such as inflammatory bowel disease (Sasche, 2002).

1.3.1 Age

The incidence of colon cancer increases after the age of 45 and 90% is seen in people over 50 years (Sasche, 2002; Parkin, 2002). There are many theories about the increase of cancer with age. Some of them are telomere biology, senescence, and adult stem-cell regulation and accumulating of genetic events in ageing tissues (Falandry, 2014).

1.3.2 Diet

Epidemiological studies have shown that a diet rich from oil and red meat increases colorectal adenoma and cancer risk, while fruit, vegetables and fiber foods reduce risk (Sasche, 2002; Parking, 2002).

A limited number of studies have shown that the antioxidant vitamins A, C, E and beta carotene reduce the risk of colon cancer. (Slattary, 1999; Eraslan, 2007). Acetylsalicylic acid and non-steroidal anti-inflammatory drugs are known to reduce the incidence of colon cancer about 22-33% by reducing prostaglandin synthesis (Slattary, 1999; Eraslan, 2007).

1.3.3 Inflammatory Bowel Disease

The relationship between inflammatory bowel diseases and colon carcinoma is known. The risk of developing colon cancers is increasing in proportion to the duration of disease and the size of the colitis. The presence of advanced ulcerative colitis for more than 10 years increases the incidence of colon carcinoma. The rate of multicentrism is higher in these cancers than in other colon carcinomas and the prognosis is worse. Patients with pancolitis have a higher risk of developing cancer (Vignali, 2014).

1.3.4 Other Risk Factors

It is known that smoking increases the risk of developing colorectal adenoma and carcinoma. Because cigarette smoking is causing the decline in miRNA levels that are important markers in cancer (Huang, 2014).

Body/abdominal fat or obesity increases the risk of colorectal cancer and physical activity decreases colon cancer risk. Accumulation of body/abdominal fat is associated with abnormal secretion of adipokines, resulting in either insulin resistance or metabolic syndrome. Adiponectin is a unique adipokine secreted from abdominal fat tissue and has anti-diabetic, anti-atherosclerotic and anti-inflammatory effects. Decreased adiponectin levels increase the risk of colon cancer. As adiponectin levels decrease in obesity, thus obesity increases the risk of colon cancer (Eraslan, 2007; Otake, 2010).

Studies have shown that diabetes mellitus and insulin resistance increase the risk of developing colorectal adenoma and associated carcinoma (Yoon, 2015).

1.4 Spread of Colorectal Cancer

Approximately 20% of the patients diagnosed with colorectal cancer have distant metastases at the time of diagnosis. Colorectal cancers spread by direct invasion, implantation, lymphatic and hematogenous pathways. Colon cancer usually spreads to the depth of the wall. They may invade neighboring organs after full-layer involvement in the intestinal wall. Intraperitoneal implantation leads to peritoneal carcinomatosis. The most common metastatic regions are; regional lymph nodes, liver, lung and peritoneum. Colon cancer is most commonly lymphatic spread (25-40%). Approximately half of the patients with complete invasion depth on the intestinal wall have lymph node involvement. Since the venous drainage of the digestive system is via the portal system, hematogenous spread is most common in liver. Then the lung, bone and brain become, respectively. The first metastatic sites frequently become lung (Chapuis,1985; Shepherd,1997).

1.5 Stage of Colorectal Cancer

Colorectal cancer staging: The TNM system is built on 3 crucial parts of data which are:

- **Tumor (T)** is the stage since we can detect through it if the tumor has developed within the fence of the intestine, also if it has any evidence in the adjacent zones
- **Lymph node (N)** has a shape in the form of bean, considered as group of protective cells (immune system) often cancer initiates the spread from this node.
- **Metastasis (M)** is the last stage where the tumor extent to other part of the body for example, colorectal cancer can range to any place in the body and the frequent spots lung and liver.



Table 1. 1 According to the American Joint Committee on Cancer (AJCC) T-stage, N-stage, M-stage of CRC (Dukes et al.,1958)

T Primary Tumor		N Lymph Node	
T0	no confirmation about the presence of incipient tumor	N0	No stretch of the cancer cell to the adjacent lymph node
Tis	Carcinoma in situ or intramucosal carcinoma	N1	Cancer cell set up in 1-3 nearby lymph node
T1	Submucosa is invaded by tumor	N1a	Cancer cell found in one lymph node
T2	Muscularis propria is invaded by tumor	N1b	Cancer cell originate in 2-3 lymph node
T3	Subserosa be invaded by tumor through muscularis propria	N1c	Small deposits of cancer cells are found in areas of fat near lymph nodes, but not in the lymph nodes themselves
T4a	Tumor infiltrate to the shallow of the visceral peritoneum	N2	Cancer cell are found in 4 or more nearby lymph node
T4b	Tumor straightly attack additional organ	N2a	Range about 4-6 cell cancer close to the lymph node
		N2b	Spread of cancer cell to more than 7 nearby lymph nodes
M- Distant Metastasis			
MX	In this stage cannot be evaluate		
M1	No distant metastasis		
M1a	Metastasis limited to one organ or site		
M1b	Metastases in more than one organ/site		

Table 1. 2 Stage I-IV according to the American Joint Committee on Cancer (AJCC) (Edge and Compton, 2010)

Stage	T	N	M
0	Tis	N0	M0
I	T1,T2	N0	M0
IIa	T3	N0	M0
IIb	T4a	N0	M0
IIc	T4b	N0	M0
IIIa	T1-T2, T1	N1/N1c, N2a	M0
IIIb	T3-t4a, T2-T3, T1-T2	N1/N1c, N2a, N2b	M0
IIIc	T4a, T3-T4a, T4b	N2a, N2b, N1-N2	M0
VIa	Any T	Any N	M1a
VIB	Any T	Any N	M1b

1.6 Important Genetic Changes in Colorectal Cancer

Colorectal cancer occurs as a result of the accumulation of a series of genetic changes leading to the disruption of the molecular mechanisms controlling normal tissue growth. The first changes in carcinogenesis are highly sensitive events affecting the normal balance between cell growth and programmed cell death (apoptosis) and histopathological findings. The genetic changes required for the epithelial cell to enter the neoplastic process are explained by two mechanisms that are fundamentally different. One of these is allelic loss and chromosomal instability (CIN) with aneuploidy (genomic instability), and the other is a manifestation of some complex DNA mutations and diploid increase (Microsatellite instability-MSI). Genomic instability plays an important role in the formation of adenomas and carcinomas by creating a condition that is sufficiently transformed into a mutated cancer cell. In 1990, the model that Fearon and Vogelstein used to describe the events at the molecular level that play a role in the colorectal cancer tumorigenesis shed light on further research (Fearon et al., 1990).

According to Fearon and Vogelstein's models;

1- Colorectal tumors occur as a result of mutational activation of oncogenes and mutational inactivation of tumor suppressor genes.

2- At least 4 or 5 genetic mutations are required for malignant tumor formation. Less quantitative changes are sufficient for benign tumor formation.

3- It is accepted that genetic changes occur in a sequence that follows each other, but the accumulation of these genetic changes is more important than their occurrence in determining the biologic characteristics of the tumor (Fearon, 1990;Fershaw, 2012).

1.6.1 Investigator Factors in CRC Carcinogenesis.

Coupled with the typical karyotypic abnormalities observed in CIN tumors is the accumulation of a characteristic set of mutations in specific tumor suppressor genes and oncogenes (Table 1.3). These mutations activate oncogenic pathways that are critical for colorectal cancer pathogenesis. It is not clear whether CIN creates the appropriate environment for the accumulation of these mutations or vice versa, although a role for APC in the establishment of the CIN phenotype has been recently proposed. Nevertheless, the combination of these mutations in colorectal cancer in the setting of CIN has been historically designated the “chromosomal instability pathway” (Jass, 2007).

Table 1. 3 Investigator factors in CRC carcinogenesis

Gene	Chromosomal location	Function of gene product
Oncogenes		
KRAS	12p12	Cell proliferation, survival, and transformation
CTNNB1	3p22	Regulation of Wnt pathway target genes that promote tumor growth and invasion
PIK3CA	3q26	Cell proliferation and survival
Tumor suppressor genes		
APC	5q21	Inhibition of Wntless/Wnt signaling; cytoskeletal regulation
TP53	17p13	Cell cycle arrest, apoptosis induction
SMAD4, SMAD2	18q21	Intracellular mediators of the TGF- β pathway
DCC	18q21	Cell surface receptor for netrin-1

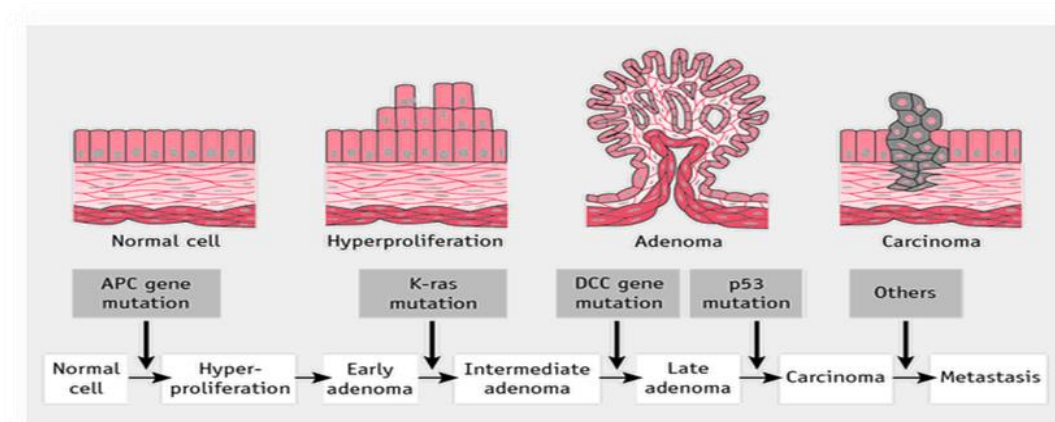


Figure 1. 3 Colorectal carcinogenesis model (<https://www.memorangapp.com/flashcards/158919/Molecular+Pathology+of+Colorectal+Cancer+6%2F2/>)

1.7 Classification of Colorectal Cancer

1.7.1 Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis (FAP) is a rare disease in autosomal dominant colorectal cancers (CRC) and accounts for less than 1% (8000) of patients with CRC (Burt, 1990). Malignant transformation has a key role in revealing the molecular basis of colorectal cancers because of their potency.

In the case of classic FAP syndrome, adenomatous polyp development which covers the entire mucosa surface like carpet in early period between 100-1000 is observed. At least 100 polyp diagnostics are required for FAP diagnosis. Multiple adenomas can be located anywhere in the digestive tract. Polyps are usually adenomatous and become prominent during adolescence or early adulthood. On average, disease progression is seen in 95% of FAP-like polyps at 35 years of age and the risk of developing CRC is 95%. For this reason, follow-up colonoscopy is recommended (Petersen, 1999).

Although germline APC mutation is the main cause of FAP syndrome, no APC mutation has been identified in patients with FAP in rate 12-20%. MYH is a base excision repair gene preventing mutations from products of oxidative damage, particularly the oxidized guanine lesion 8-oxodG. So MYH mutations that may lead to polyps (Chapelle, 2004; Filipe, 2009). In addition, more than 25% of FAPs were identified by Nano-germline mutation and characteristic autosomal dominant inheritance analysis (Galiatsatos, 2006).

1.7.2 Hereditary Non-Polyposis Colon Cancer (HNPCC)

The most common inherited conditions are hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch syndrome, and (FAP). The genes responsible for these forms of inherited colorectal cancer have been identified. HNPCC is caused by germline mutations in genes involved in the DNA mismatch repair (MMR) system, namely the MutL protein homolog 1 (MLH1), MutS protein homolog 2 (MSH2), MutL protein homolog 6 (MHL6) or the spore meiotic segregation increased 2 (PMS2) genes, leading to microsatellite instability (Papadopoulos et al., 1994; Lynch et al., 2003). Carriers of

gene mutations in the MMR genes have a 50–80% lifetime risk of developing CRC. Inheritance is autosomal dominant and accounts for 2-5% of CRC (Jeter et al., 2006; Kwak and Chung, 2007). Clinical features of HNPCC are multiple generations affected with CRC at an early age (mean, approximately 45 years) with a predominance of right-sided tumors (approximately 70 percent proximal to the splenic flexure). There is an excess of synchronous and metachronous colorectal cancers, as well as an excess of extracolonic cancers, most frequently carcinoma of the endometrium. Further, HNPCC also displays specific histopathological features, such as poor differentiation, excess of mucinous or signet ring histology and lymphoid infiltration (Jass et al., 1998). Personal and family cancer history, molecular testing of CRC tumor specimens for MSI and germline MMR gene mutation analysis should be performed to identify persons in risk.

1.7.3 Sporadic Colorectal Cancer

75% of colorectal cancers are sporadic CRC. Although sporadic colorectal cancer is classified as a genetic disorder caused by somatic mutations, it is affected by cancer formation and development, local colonic environment, the patient's genetic background and environmental factors. Different approaches are used to identify genes that cause genetic predisposition for the reason that sporadic cases are not a specific inheritance pattern. To date, some biomarkers have been identified that genetic and epigenetic changes can be effective in the initiation, development and metastasis of cancer. Nevertheless, in the development of sporadic CRC, a multifactorial disease of both hereditary and environmental factors, effective susceptibility genes and biomarkers that can be used for early diagnosis have not been fully identified to cover the entire population (Fearon, 2011).

1.7.4 Familial Colorectal Cancer

Non-syndromic or familial CRC is generally defined as clustering of CRC that is distinguished from the hereditary syndromes. Familial CRC is a heterogeneous condition that includes patients with unrecognized hereditary syndromes and patients with seemingly sporadic forms that aggregate in families. In these patients the molecular mechanism has not been established. Probably a combination of environmental and

inherited genetic factors (common, low-penetrance, genetic alterations) play a role in the development of CRC in these families. Intensive colonoscopic surveillance is offered to high-risk individuals from families with Lynch syndrome; reduced CRC mortality has been demonstrated in these individuals. Colonoscopy surveillance is already offered to people with moderate risk due to a family history (FH) of CRC, but evidence supporting reduced mortality is lacking (Armellao, 2014).

1.8 Mouse Double Minute2 (MDM2)

The MDM2 (mouse double minute 2) gene was first obtained from chromosomes of "double minute" (non-centromeric small chromatin particles) in spontaneously transformed mouse cell lines. These constructs contain three genes in the size of 1-2 megabases (Mb). The second of these genes is called MDM2 (Freedman et al., 1999). The MDM2 gene was identified as an oncogene in 1991 when it caused tumor formation when transferred to mouse cells. In later studies, a protein of 90 kilodaltons (kD), which can complex with the p53 tumor suppressor protein, is observed. When this protein sequence was removed, it was understood to be the product of the MDM2 gene. It was also found that excessive expression of MDM2 inhibited p53 mediated activation. In 1992, human double minute 2 (HDM2), the homologue of MDM2, was mapped in the 12q13 / 14 region of the chromosome (Schlott et al., 1997). The most important task of the MDM2 gene is to inhibit functions of p53 by binding it. However, MDM2 has also many functions independently of p53 (Ganguli and Wasylyk, 2003).

Additional information demonstrating that MDM2 works as an oncogene is obtained by observing overexpression of MDM2 in breast epithelium during lactation in mice (Lundgren et al., 1997). In mice, normal development and differentiation in the mammary gland is inhibited by the formation of high amounts of MDM2. In addition, studies in tissue culture have shown that MDM2 clones are cooperating with the activated Ras (Rat sarcoma) gene, mediating the immortalization and transformation of fibroblasts (REF) in rat embryos (Finlay et al., 1993). The MDM2 gene can also be classified as an oncogene by looking at its functions in human tumors. MDM2 is determined by extreme amounts of tumorigenic human tumors.

1.8.1 MDM2 Gene Structure and Protein Zones

The MDM2 gene is approximately 25 kb in size and contains at least 12 exons (Montes de Oca Luna et al., 1996). There are two promoters, P1 and P2. The P1 promoter is in front of the first exon and does not contain a sequence to which p53 can be ligated. The P2 promoter is in the first intron and contains the sequence to which p53 is ligated. Both promoters are rich in the number of guanine (G) and cytosine (C) nucleotides, and both promoters are present in multiple promoter regions observed in promoters rich in G and C (Jones et al., 1996). In many cell lines, isoforms of mRNA of MDM2 and proteins of MDM2 are found. For example, in 3T3-DM (3T3 double minute) cells, MDM2 has five different MDM2 polypeptides originally cloned ranging in size from 57 to 90 kilodaltons (kDa) (Olson, 1993). In addition, at least seven MDM2 transcripts have been identified in human and mouse cells (Fakharzadeh et al., 1991; Oliner et al., 1992). These transcripts arise from the use of "splice" (splice: the process of integrating exons by cutting introns from RNA) regions in the interior of MDM2.

The MDM2 protein consists of an average of 491 amino acids. MDM2 protein contains a variety of conserved regions from Zebra to human (Marechal et al., 1994). The first conserved region is the p53 binding region located between amino acids 19-102. This region is necessary for MDM2 to neutralize the activation function of transcription factor p53 protein (Chen et al., 1995). The "nucleotide localization sequence" (NLS) region, located between amino acids 181-185, is required for the mediation of MDM2 between the nucleus and the cytoplasm. The next conserved region is the "acidic" site containing residues of aspartic acid and glutamic acid, located between amino acids 221-272. This region mediates the interaction of MDM2 with ribosomal protein L5 and 5S ribosomal RNA (rRNA) (Marechal et al., 1994). This acidic site is followed by the "zinc finger" region located between amino acids 305-322. The last conserved region of the MDM2 protein is the "ring finger" region located between amino acids 438-478 at the carboxyl end. This region contains two "zinc finger" structures that link MDM2 to downstream in-vitro and specific RNA sequences (Elenbaas et al., 1996).

1.8.2 MDM2 and P53 Relationship

The MDM2 protein plays a fundamental role in regulating the function of the p53 protein. P53 is a cell cycle control agent that is required for normal growth of the cell and it inhibits cell division. The p53 protein, the product of the p53 gene, stops cell division when DNA is damaged and saves time for the cell to repair damage. If this damage is too great, the cell leads to apoptosis, ie programmed cell death (Lowe et al., 1994). In normal cells, p53 and MDM2 are expressed in equilibrium. P53 increases the expression of MDM2, its own inhibitor, to maintain this balance in the cell. Some damage to p53 and MDM2, when a cell receives damage or when it receives a signal in the cell division pathway, allows p53 to remain active by inhibiting the interaction between these two proteins. However, when the MDM2 gene is overexpressed, the degradation of the p53 protein is excessive and p53 is not functional (Roth et al., 1998; Freedman et al., 1998). While the MDM2 protein regulates the function of the p53 protein negatively, both proteins act in a path called the “autoregulatory feedback loop”. This pathway involves first binding and activating the p53 protein to the MDM2 gene, and then activating the MDM2 protein to inhibit the p53 protein (Piette et al., 1997).

MDM2 is first activated by p53 in an established study using the heat sensitive form of p53. The expression of MDM2 is increased at a suitable temperature for p53 function. P53 directly binds to the p53 binding domain of MDM2 to increase this expression. Activation of MDM2 with p53 has also been demonstrated in cell line studies. After exposure of cells to ultraviolet (UV) radiation, increases in MDM2 transcripts and protein levels have been shown to be due to the presence of p53 protein in cells (Reinke et al., 1997). All these studies show that p53 activates the expression of the MDM2 gene in response to DNA damage under the right physiological conditions.

MDM2 has been shown to be capable of inhibiting the apoptosis inducing functions of p53 and stopping the cell cycle of it in G1 phase. And these has also been demonstrated in mammalian cell culture transfection studies and in mouse studies (Chen et al., 1996). Studies in mouse embryos have shown that embryos without MDM2 gene activity die

early in their development (Momand et al., 2000). Inhibition of P53 functions by MDM2 is an essential factor in the early stages of embryonic development in mice. This result indicates that p53 and MDM2 need balanced expression to maintain normal development of the cells.

1.8.3 Inhibition of MDM2 and P53 Association

Modifications that inhibit the interaction between p53 and MDM2 when a cell becomes damaged or receives a signal in the cell division pathway are phosphorylation of P53, phosphorylation of MDM2 and the inhibition of MDM2 function by an alternate reading frame protein (p14ARF).

Phosphorylation of P53: The results of the DNA damage in the studies revealed that p53 was phosphorylated from the 15th amino acid by DNA-dependent protein kinase (DNA-PK), and thus, it was observed that MDM2 could not bind to p53 and that p53 was activated (Shieh et al., 1997). It has also been observed that p53 is phosphorylated and activated by the 15th amino acid serine by the Ataxia telangiectasia mutated (ATM) protein (genetic product responsible for a genetic disease, Ataxia telangiectasia mutated) as a result of DNA damage and ionizing radiation (Banin et al., 1998). ATM is a protein that regulates cellular responses that occur in DNA breaks. It is believed that this protein phosphorylates p53 in the event of cellular damage and breaks the P53-MDM2 association, thereby helping to save time to the cell for repair (Canman et al., 1998). In a further study, phosphorylation of p53 from the 15th amino acid serine resulting from the ionizing radiation resulted in CK1 (Casein Kinase 1) or CK1-like enzymes phosphorylating p53 from the 18th amino acid threonine (Thr) and It is said that p53 could not bind to MDM2 and remained active (Sakaguchi et al., 2000).

Phosphorylation of MDM2: There are regions for the phosphorylation of MDM2 in the MDM2 which are responsible for p53 binding and ubiquitination (Hay et al., 2000). Phosphorylation of MDM2 from the 17th serine amino acid by DNA-dependent protein kinase (DNA-PK), which is active in double-stranded DNA breaks, prevents MDM2 from forming complex with p53 and thus activates p53 (Mayo et al., 1997).

Inhibition of MDM2 function by p14ARF protein: p14ARF, a protein synthesized by alternative reading frame from p14INK4a gene (Chin et al., 1998). The product of the P14ARF gene inhibits MDM2 by binding to MDM2 and plays a role in the activation of p53. The binding of P14ARF to MDM2 prevents MDM2 from interacting with p53 and also stimulates the degradation of MDM2 (Pomerantz et al., 1998; Zhang et al., 1998).

1.8.4 Inhibition of P53 Functions by MDM2

In the previous conducted studies, it was determined that the MDM2 protein inhibited the tumor suppressor function of p53 protein by several different mechanisms. In the first mechanism, MDM2 blocks the functions of p53 by linking to the region where p53 is used as transcription activator (Lin et al., 1994; Lu et al., 1995). MDM2 competes with the transcription factor complex TFIID to be able to bind to this region. This sequence masks the region used to activate transcription of p53 and inhibits the function of p53 (Thut et al., 1997).

Another mechanism by which MDM2 inhibits p53 activity is to negatively regulate the level of p53 protein. In various cell lines, when p53 and MDM2 are co-transfected, the amount of p53 protein is reduced compared to single-cell transfection of p53. This reduction in the level of the P53 protein stems from the ability of MDM2 to inhibit p53 by reducing the half-life of p53 (Kubbuhat et al., 1997; Haupt et al., 1997). MDM2 binds p53 to the nucleus of the cell and migrates to the cytoplasm together with p53, inducing the degradation of p53 by proteosomes (Roth et al., 1998). Alternatively, the MDM2 protein activates E3 ubiquitin ligase activity using the "zinc finger" region at the carboxyl end and acts directly on the inhibition of p53 protein (Honda et al., 1997).

The last mechanism by which MDM2 blocks p53 function is to inhibit the acetylation of p53. Coactivators such as P300 are attached to the amino terminus of p53 and provide acetylation from the lysine amino acid at the carboxyl end. This acetylation accelerates the attachment of p53 to DNA in a specific sequence. MDM2 inhibits p300 mediated acetylation of p53, inhibiting the transcriptional activating function of p53 (Ito et al., 2001). MDM2-p300 complex also plays a role in MDM2-mediated cleavage of p53 (Grossman et al., 1998).

1.9 Ring1 YY1 Biding Protein (RYBP) Gene

The inheritable silencing of sets of genes is responsible, at least in part, for the maintenance of the determined state of the wide variety of cell types found in multicellular organisms. Genetic analysis in *Drosophila* originally identified a number of genes, the Polycomb group (PcG) of genes, whose products are essential for the maintenance, but not the initiation, of the transcriptionally repressed state of developmentally relevant genes. The products of the Polycomb group (PcG) of genes are necessary for the maintenance of transcriptional repression of a number of important developmental genes, including the homeotic genes. The PcG genes encode a structurally diverse group of proteins, which form large complexes arising from their mutual interaction through protein motifs conserved from flies to man. One member of the PcG gene family is Ring 1 and YY1 binding protein (RYBP) gene. RYBP is a Protein Coding gene. RYBP has many synonyms. The most commonly used are AAP1, DEDAF and YEAF1 (Bienz and Muller, 1995; Kennison, 1995). RYBP is located on chromosome 3 and has a length of 7215 bp. RYBP gene has 1 transcript (splice variant), 99 orthologous, 1 prologue, is a member of 1 Ensemble protein family and is associated with 44 phenotypes. This transcript has 4 exons, is annotated with 12 domains and features, is associated with 132 variations. The RYBP gene product is the RYBP protein (<https://www.genecards.org/cgi-bin/carddisp.pl?gene=RYBP>).

1.9.1 RYBP Protein

RYBP was first identified in 1999. The human RYBP protein contains 228 amino acids, and its structure includes a conserved Npl4 Zinc finger (NZF) motif at the N-terminus, a central region that is characteristically enriched with arginine and lysine residues and a C-terminal region enriched with serine and threonine amino acids (Tan et al., 2017). The N-terminus of this protein is evolutionarily conserved among different species, including *Homo sapiens*, *Mus musculus* and *Drosophila melanogaster* (Arrigoni et al., 2006). RYBP is a multifunctional protein, which binds several transcriptional factors and

components of polycomb repressive complex 1 (PRC1), and is associated with development, as well as apoptosis and cancer.

1.9.2 The Roles of RYBP in Development

RYBP transcripts are expressed mainly in the developing central nervous system, as well as in the branchial arch, forelimb buds, tail bud and hindgut at mouse embryonic day 9.0. Furthermore, RYBP knockout mice exhibited lethality at the early post-implantation stage (Pirity et al., 2005), and homozygous null mutant *Drosophila* died progressively, with 43% dying during embryogenesis and 44% during larval/pupal development (Gonzalez et al., 2008). An important study associated with RYBP function in nerve development was reported that RYBP plays a dose-dependent role in central nervous system development. RYBP heterozygous null embryos exhibited aberrant brain development, including disrupted neural tube closure, forebrain overgrowth and exencephaly (Pirity et al., 2005). In further investigations of the underlying mechanisms, they also demonstrated that RYBP impaired the differentiation of pluripotent embryonic stem cells (ESCs) to mature neural cell types, including neurons, astrocytes and oligodendrocytes, through up-regulation of the neural marker Paired box protein 6 (Pax6) and down-regulation of pleiomorphic adenoma gene-like 1 (Plagl1). Furthermore, RYBP is located specifically in the ganglion and inner nuclear cell layers of the neuroretina during mouse eye development. Dysregulated RYBP expression resulted in retinal coloboma, malformed lenses, defects in anterior eye development and corneal neovascularization, so RYBP plays critical roles in mouse eye development.

Additionally, RYBP is also important for both cardiac and germ cell development (Ujhely et al., 2014). In terms of cardiac development, the absence of RYBP in ESCs blocked cardiac differentiation to contractile cardiomyocytes, possibly through regulation of the expression of *Plagl1*, *Isl1* and *Tnnt2* genes. Furthermore, these impaired phenotypes are rescued by ectopic expression of RYBP using a lentivirus vector. In contrast to the active function in development, the expression of RYBP and its binding protein YY1 were gradually decreased during C2C12 myoblast differentiation, accompanied by miR-29 overexpression, so RYBP acts as a repressor of skeletal myogenesis (Zhou., 2012).

1.9.3 The Roles of RYBP in the Apoptosis

Apoptosis is an evolutionarily conserved cell suicide process that is stimulated in response to a variety of stimuli (Ashkenazi and Dixit, 2012), and plays critical roles in different biological processes and diseases, including embryonic development and cell differentiation, normal cell turnover and immunological processes, as well as neurodegenerative diseases and various types of cancer (Elmore, 2007). One of our understanding mechanisms of apoptosis induction is through death receptors, such as CD95/FAS (Schulze-osthoff, 1998). The cytoplasmic tail of CD95 can interact with several death effector domain (DED)-containing proteins to form the death-inducing signaling complex (DISC), which enhances CD95-mediated apoptosis (siegel et al., 2000). These DED-containing proteins residing in the cytoplasmic DISC includes FADD, caspase-8 and/or caspase-10. RYBP not only interacts with FADD, caspase-8 and caspase-10, but also augments the formation of DISC, promoting CD95-mediated apoptosis (Zheng et al., 2001). In addition, RYBP can directly interact with another DED-containing DNA-binding protein (DEDD) in the nucleus, resulting in the diffuse distribution of DEDD in the nucleoplasm and facilitating DEDD mediated apoptosis through activation of caspase-6. Collectively, RYBP regulates apoptosis in both the nucleus and the cytoplasm through DED-containing proteins. Moreover, through constructing a cytoplasm-located RYBP mutant (RYBPmut), has enhanced potential to promote tumor apoptosis and inhibit tumor cell proliferation via p53-dependent and caspase 8-dependent mechanisms compared with wild-type RYBP. In addition, RYBP not only plays a pivotal role in the interaction between Hip1 protein interactor (Hippi) and caspase 8 (Stanton et al., 2001), but also enhances Hippi-mediated apoptosis via caspase 8. Intriguingly, RYBP not only interacts directly with apoptin, but also partially colocalizes with this protein in the nucleus of tumor cells (Oorschot et al., 2004). In this dimer, transient RYBP overexpression has a similar function to apoptin in that it specifically induces apoptosis in tumor cells, but not in normal and untransformed cells. Additionally, RYBP both interacts with and up-regulates fibronectin type III and ankyrin

repeat domains 1 (FANK1) protein in tumor cells to induce apoptosis via the c-Jun N-terminal kinases -Activator Protein signaling pathway JNK-AP1.

In mammals, RYBP also binds to and inhibits the function of E3 ubiquitin ligase mouse double minute 2 (MDM2) in the proteasomal degradation of p53 (Chen et al., 2009). It is well established that p53 plays critical roles in the regulation of cell cycle arrest, programmed cell death, apoptosis and the prevention of tumor progression (Amaral et al., 2010; Muller and Vousden, 2012). RYBP was shown to exert its pro-apoptotic activity via the regulation of the MDM2-p53 loop (Chen et al., 2009). In *Drosophila*, RYBP overexpression induced apoptosis in imaginal discs cells via a mechanism that was dependent on the pro-apoptotic reaper, Hid and Grim proteins, as well as dFADD and DREDD (mammalian homologues of FADD and caspase-8, respectively) (Gonzalez and Busturia, 2009). Furthermore, under stress conditions, RYBP-induced apoptosis required the epigenetic adaptor Trithorax Group (trxG), which not only induced apoptosis, but also promoted reaper protein expression. Thus, stress-induced apoptosis requires the co-function of RYBP and trxG.

1.10 The Aims of The Study

There are three aim of this study:

1. To test the mRNA levels of MDM2 and RYBP gene expression between normal and tumor tissues in patients diagnosed with colorectal cancer.
2. To examine if there is relation between MDM2 and RYBP gene expressions and the clinicopathological features as age, gender, tissues type, smoking, lymph node, stage, metastasis and others disease (independent factor or not) in the patient's group.
3. To elucidate if there is correlation between MDM2 expression and RYBP expression in CRC tissues.



CHAPTER 2

LITERATURE REVIEW

The role of the MDM2 as human oncogene was confirmed after recognizing the frequent aberration of the 12q13-14 chromosome in which MDM2 gene locate in a vary types of cancer including more than one third of human soft tissue sarcoma (STS) (Turc-carel et al., 1986; Paul S. Meltzer, Jankowski and Paola Dal Cm., 1991).

According to previous data and clues about the aberration at this locus(12q13-14), a list of various subtypes of sarcoma and carcinoma specimens were studied to detect the genetic modification, and they found that one third of forty-seven used samples of sarcoma's type were had amplification in this mentioned locus. However, none of the examined carcinoma (colorectal, gastric) specimens has amplification or overexpression of transcript levels (Oliner et al., 1992).

Reifenberger and his colleagues (1993) has investigated that MDM2 gene characterized with the next highest occurrence of amplification and overexpression in malignant gliomas after the EGFR, and that after examining 157 primary brain tumor and finding that MDM2 was amplified and over-expressed in 8-10 % of glioblastoma and anaplastic astrocytoma. However, He et al. (1994), has confirmed this study via establishing about 30 cell lines from such brain tumors and revealed overexpression of MDM2 mRNA with amplification in two of thirty cases (6.7%).

Due to the importance of modification of MDM2 and TTP53, both were studied in 24 human STS in which alteration of TP53 either by deletion, mutation or overexpression was observed in 8 of 24 and the another one-third of 24 was had amplification of MDM2 which concomitant with overexpression of its product in the same tumors of one-third. This finding boosts the supposition that both of gene was not seen in the same tumors and both constitute an alternative mechanism to inactivate the growth factor in the same controlling pathway (Leach et al., 1993). To test the relation between the MDM2 and

pathological features in osteosarcoma samples (including 16 primary, 11 metastatic, and 1 local recurrence) were studied and found that tumors had amplification of MDM2 in metastatic or more than primary one. In addition, amplification was happened in sarcoma deficient detectable TP53 alterations (Ladanyi et al., 1993).

Because the role of MDM2 was not investigated in leukemia, Bueso-Ramos and coworker (1993), worked on this type of cancer by using highly sensitive technique RT-PCR and 64 samples including patients diagnosed with different type of leukemia including chronic Lymphocytic Leukemia (CLL), Acute myeloid leukemia (AML), Acute Lymphocytic Leukemia (ALL), Myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML). Over-expression of MDM2 mRNA was detected in 34 of 64 used samples and none of those (overexpressed samples) were contain amplification so this result suggests that mechanism other than amplification is responsible for the expression.

Another experience related to investigating the level of MDM2 mRNA overexpression in various type of leukemia was done by Quesnel et al. (1994). Since this group also found overexpression of MDM2 mRNA of leukemia but in small cases about 9/66 (14%) patients tested including 3/9 ALL, 3/24 AML, 2/4 myelomas, 1/1 prolymphocytic Leukemia (PLL), but 0/2 CML, 0/2 Non-Hodgkin Lymphoma (NHL) and 0/21 MDS. It is worth to note that also in this study none of the patients who overexpressed MDM2 had TP53 mutation or TP53 gene transcript and it appeared that over expression of MDM2 is associated with poor prognosis.

Another study has conducted by Florenes et al. (1994) to determine the association between MDM2 amplification and mRNA expression in sarcoma tissues containing different histological subtype of (68) sample, as well as 26 human xenografts in nude mice and 2 human sarcoma cell line. Detection of amplification of MDM2 was seen in ten of all sample inclusive of (three liposarcomas, two malignant schwannomas, two fibrosarcoma, one non-classified sarcoma, one hemangiopericytoma, and one in the OSA cell line). Overexpression of MDM2 mRNA was shown in all nine cases with increasing in a copy number of the MDM2 gene and 3 cases without amplification, indicating and substantiating that overexpression also could be elevated by mechanisms

other than amplification.

Watanabe and his coworker (1994) studied expression of MDM2 gene in neoplastic B cells for both type of leukemia Non-Hodgkin Lymphoma (NHL) and CLL. Sixty patients with leukemia were included in their study. In 17 of 60 patients were showed MDM2 mRNA overexpression at levels more than (10-fold) higher than what observed when compared with non-tumor cases. Other 19 patients also were showed the MDM2 mRNA expression but in twofold to fivefold higher expression than that in normal. In addition, MDM2 overexpression was detected more frequently in patients in advanced clinical stages suggesting its role in tumor genesis and/or disease.

Enhanced translation has been discovered as a novel means of regulating the MDM2 gene expression and activate its function as carcinogenesis. It was observed high levels of both MDM2 and TP53 wild type in the human choriocarcinoma cell lines in which enhanced translation of MDM2 mRNA was the reason (Landers et al., 1994).

Nakayama and his colleagues (1995), sought to know whether the amplification and overexpression of MDM2 is restricted to metastatic type of osteosarcoma as testified by Oliner, Flores, and Leadley, and that via examining 107 sarcoma sample (bone and soft tissue sarcoma) and 8 lipomas, also they attempted to confirm the relation between MDM2 and TP53 alternation in the same control pathway. Ten of one hundred and seven sarcoma cases were shown amplification including 3 osteosarcomas, 3 Malignant Fibrous Histiocytoma (MFH) and lip sarcoma. Interestingly, none of the osteosarcoma samples were presented in metastatic type and the all three amplified and overexpressed MDM2 gene were existed in primary tumor which was contrary to what discovered in earlier report. Furthermore, MDM2 mRNA overexpression was appeared in six of ten amplified sarcoma of cases and in all the other tumors without amplification. Similarly, to what investigated in previous studies related to existence of both MDM2 and TP53 alternations are so excessive in the same tumor progression pathway.

In New York (1993), Manchetti and his colleagues has explored that TP53 overexpression could take place (accumulate) in tumor cells without any deletion or mutation, by using a wide panel of non-small cell lung carcinoma (53 specimens and consists of different histological subtype with different stage of cancer development). In

1995, the same group used the same panel (same tissues) of NSCLC to study the over-expression and amplification of MDM2 gene and then found that MDM2 levels anomaly were shown in small numbers just in three of examined NSCLC samples which already recognized by possessing a strong accumulation of TP53 protein in the absence of mutation. In addition, tumors showed positive presence of MDM2 were classified as adenocarcinoma so this may refer that MDM2 prefer existing in specific subtypes of NSCLC (Marchetti et al., 1995).

Bueso-ramos and his colleagues (1996) have reported the existence of MDM2 mRNA overexpression in 24 (73%) out of 33 examined cases of human primary breast carcinoma as compared with normal breast tissue in the absence of MDM-2 gene amplification. In addition, it has observed that 69% of breast carcinomas that overexpressed the MDM-2 mRNA had detectable nuclear TP53 protein.

Five alternatively spliced MDM2 gene transcripts (MDM2-A, -B, -C, -D, and -E) that are lacking TP53 binding domain sequences have been observed while studying the role of alternative and aberration of MDM2 mRNA expression in ovarian and bladder cancer, where the higher incidence of this splice variants were found in the advanced stage and low grade (Sigalas et al., 1996).

To explore the discrepancy between the overexpression of MDM2 gene with and without amplification, it has been tested about 129 soft tissue tumors for amplification of the MDM2 by using Southern technique and also used 39 sample of 129 for examining MDM2 gene over-expression. They found that 14 of 114 samples have been shown amplification in malignant tumor, and none in the benign tumors, also 21 out of 39 in malignance tumor shown MDM2 overexpression without amplification. In conclusion, MDM2 gene overexpressed in all tumor possess amplification and in ten tumors didn't exhibit any amplification suggesting that overexpression of MDM2 could be done by means other than amplification (Patterson et al., 1997).

Tanner et al. (1997), has sought to examine whether pre-therapeutic MDM2 mRNA expression in 90 patients diagnosed with ovarian cancer and received chemotherapy with cyclophosphamide and carboplatin. They reached to the result that MDM2 mRNA expression was positively correlated with increasing the time of survival after

chemotherapy (835 days for patients expressed MDM2 mRNA and 171 days for patient who did not) in the patients with Federation of Gynecology and Obstetrics (FIGO) stage (3 and 4 who were received chemotherapist after surgery. Remarkably, MDM2 mRNA was an independent prognostic marker in addition to FIGO stage, grade and histologic type.

Matsumoto et al., (1998), suggested that MDM2 was an oncogene, independent of TP53 gene status. Alternatively spliced transcripts of MDM2 also was reported in 7 of 16 human glioblastoma cells (GBM primary cell) and in the established GBM cell lines (MDM2- LN229a, -LN229b, -LN18, -G116, and -G150) (Raus et al., 1999).

The significant of MDM2 mRNA expression as an important prognostic value was detected in 35 of 81 Taiwan' patients diagnosed with NSCLC basing on using RT-PCR. In which they found that patients delecTable MDM2 mRNA expression were had median survival (1,549 days) longer than those did not. Interestingly, MDM2 mRNA has considered as an independent prognostic factor of other clinical characteristics, such as histological type, nodal involvement, tumor stage, and age. Also was more suiTable prognostic factor than MDM2, T53 protein expression and T53 mutation (Heng et al., 2000).

A cohort of (38) invasive breast cancers and (9) normal breast specimens also were analyzed for the same aim, in which the presence of aberrant expression products of MDM2 in breast cancer specimens was correlated with a shortened overall patient survival and associated with more an aggressive disease (Lukas et al., 2001).

Margarita Sanchez-Beato et al. (2004), studied a large panel of proteins including (The B-cell-specifics Moloney Murine Leukemia virus integration site-1 (BMI1), Mouse Melanoma cell 18 (MEL18), Pleckstrin Homology domain-containing protein 1(PH1), Ring Finger protein 2(RNF2), RING1and RYBP which are belong to the polycomb group (PcG)in 321 Hodgkin's lymphoma (HL) and in reactive lymphoid tissues as control via utilizing tissues microarrays technique. They discovered unusual finding specially to what related of over expressing of both RYBP and BMI1expression in tumor cells in comparison with control once and the most surprising result was abnormal

expression of RYBP in fifty five percent of all HL cases and was correlated with shorter survival rate and unfavorable response treatment.

A comprehensive analysis including gene dosage, expression, and gene ontology (GO) has been carried out to detect the genes involved in the genetic changes events during the carcinogenesis and chemoradio-resistance of cervical cancers. 104 locally advanced cervical cancer and an independent cohort of 41 patients for validation the gene expression profile were included in this study. By performing statistic test, they recognized 29 recurrent gains and losses and 3 losses (3p,13q,21q) linked with poor outcome after chemoradiotherapy. Also, they discovered the genes affected during alteration and the biological process such as (apoptosis, metabolism, macromolecule localization, translation, and transcription, which were driven by the alteration of those genes. However, they found RYBP, 1,4-Alpha-Glucan Branching Enzyme 1 (GBE1) on the deleted 3p and Family with Sequence Similarity 48 Member A (FAM48A), Mediator Complex Subunit 4 (MED4) on the deleted 13q were associated with the result at both the gene dosage and expression level and were satisfactorily validated in the independent cohort (Lando et al., 2009).

Assessment the genes, which involve in prostate cancer process offers the basis for understanding the factor affected disease and treatment. In 2010 another group also discovered a small deletion about 21.2 % of the area harbor genes function as tumor suppressor gene like deletion of 3P14 chromosome carrying Forkhead Box P1 (FOXP1), Eukaryotic Translation Initiation Factor 4E Member 3 (EIF4E3), G Protein-Coupled Receptor 27 (GPR27), Prokineticin 2 (PROK2), RYBP, H/ACA Ribonucleoprotein Assembly Factor (SHQ1) and Glucoside Xylosyltransferase 2 (GXYLT2) and that by analyzing the DNA copy number, mRNA expression, and 218 prostate cancer tumors (Taylor et al., 2010).

In another work performed by Sasaki et al. (2011), increased levels of RYBP and enhancer of zeste homolog 2 transcripts (EZH2) which boosted levels of trimethylation H3K27 were observed in patients with adult T-cell leukemia/lymphoma cells more than CD4⁺ T cells from healthy volunteer and that due to the alteration of microRNA which negatively regulated its target and sited into the untranslated region 3 (3UTR) of

genes in while studying the role of the polycomb members in ATL cells and this high expression level was associated with poor prognosis referring the role of this genes in tumor genesis and progression.

Li and his colleagues (2013), also studied a well-known members of the polycomb group which are playing a serious role in histone-mediated epigenetics modification and involved in tumorigenesis progression in glioblastoma multiform (GBM) samples compared to normal brain. There was a remarkable differences in the expression of this members in GBM in which enhancer of zeste homolog 2 transcripts (EZH2), PHD Finger Protein 19 (PHF19), Chromobox Protein Homolog 8 (CBX8) and Polycomb Homolog 2 (PBC2) were over-expressed in GBM samples while RYBP was belong to the down regulation expression group Chromobox 7 (CBX7), Chromobox 6 (CBX6), EZH1 and RYBP.

Lando et al. in 2013, also reported in another work on the same subject (detection the genes involving in cervical cancer) what they observed about the repeated loss of 3p12–p14 containing lots of important genes responsible for important biological process like apoptosis, cell proliferation in cervical squamous cell carcinoma by using microarray, and they found the frequent loss was 3p taking place in 61% of 92 invasive carcinomas, in only 2% of 43 high-grade intraepithelial lesions chromosomal instability (CIN2/3), and in 33% of 6 CIN3 lesions adjacent to invasive carcinoma. RYBP was among eight of the genes within this region which stickily down-regulated during the loss of this region confirming their involving in neoplastic progression.

In another study done by Krohn et al. (2013), they sought to know the relation between the deletion of 3p13 with phenotype of prostate cancer in which this deletion constitute about 20% of it and that by mapping the 3p13 deletion locus utilizing single nucleotide polymorphism (SNP) array analysis of 72 prostate cancers which revealed small deletion at 3p13 in 14 (19%) of the tumors, including a putative tumor suppressors gene FOXP1, RYBP, and SHQ. mRNA expression analysis assured that all the genes on 3p13 chromosome were downregulated by this deletion and hence this inactivation of this tumor suppressor genes (TSGs) considered as aggressive molecular subsets of positive ETS-related gene (ERG+) prostate cancers.

Due to the unchanged survival rate in patients with ileal carcinoids which is popular malignant neuroendocrine neoplasms of the gastrointestinal tract. Resesrcher examined the profile of this type of tumor (ileal carcinoids) to get the complete sight of risk factor, genetic alteration and transcriptional changing. Increased risk of ileal carcinoids was correlated with (SNP) of Interleukin 12A (IL12A) and Defender against Cell Death 1(DAD1), while down-regulation of MicroRNA133a-1 (MIR133A) was increase tumor development. However, the major of the tumor type characterized by deletion the chromosome 18 and chromosomes 3p, 11q, and 13. RYBP located on the 3p chromosome and other genes located on the another chromosome were chosen as candidate to be novel target for therapy (Nilsson et al., 2013).

Wang et al. (2014), was the first group who studied the contribution role of RYBP in HCC progression, relation of the variable levels of RYBP with rate survival and RYBP ability to serve as inducing factor for both apoptosis and increasing the sensitize to chemotherapy by using 400 pairs of human hepatocellular carcinoma (HCC) tissues and matched noncancerous samples in both vivo and vitro. They observed a significant down-regulation of RYBP mRNA levels expression in HCC tissue compared with noncancerous tissue suggesting the actual involving role of RYBP in HCC occurrence, and this low expression of RYBP in HCC tissues clinically was interpreted as important independent predictor factor of poor prognosis. In vitro they noted that the applied expression of RYBP contributed to inhibit cell growth and invasion, enhanced RYBP tumor cell specific killing function (apoptosis), and amplified the responsive ability to the chemo sensitivity of the cell, while knockdown of RYBP led to opposite result. Moreover, in vivo the stimulating expression of RYBP by both cisplatin (anticancer' activity) and adenovirus-mediated RYBP expression blocked HCC tumor growth and increase sensitize of HCC tissue to the conventional chemotherapy. All the mentioned above indicate that activating RYBP ability as tumor suppressor gene in cancer cells provides an effective and safe therapeutic approach to HCC therapy.

Voruganti and his collageous (2015), has conducted the experiments seeking to determine the role of RYBP mRNA expression in (NSCLC) in 100 pairs of patients diagnosed with (NSCLC) by performing qRT-PCR. The result showed that RYBP

mRNA expression was significantly down-regulated in NSCLC specimens, as compared to that of matching adjacent normal lung tissues. To confirm this result they also examined the RYBP mRNA expression in new collection of the human NSCLC tissue samples 441 patients . Of these cases, more than half of the cases (232/441) exhibited high expression of RYBP mRNA expression, while the rest of cases (209/441) showed low RYBP mRNA expression. This down-regulation of RYBP was associated with poor prognosis. Moreover, adenoviral delivery of RYBP induced the sensitize of NSCLC cells to chemotherapy in vivo. As well, RYBP expression was prompted by paclitaxel, the agent used in chemotherapeutic. However, they observed that by executing high expression of RYBP, cell survival and colony formation were inhibited and apoptosis process was induced and the chemo sensitivity in NSCLC was increased, while opposite effects were showed by knockdown of RYBP. All that suggest that RYBP could be critical candidate and a novel target for therapy in patients with lung cancer.

Epithelial-mesenchym transition (EMT) represents the important biological characteristics of HCC. Zhu' group has attempt to investigate the role of a putative member of PcG (RYBP) in HCC by analyzing 20 pairs of HCC tissues and matched adjacent normal tissues via performing RT-qPCR and western blot. They found that both RYBP mRNA and protein expression were remarkably lower in HHC tissue when compared with normal tissue. Moreover, in another 216 HCC archival paraffin embedded tissues, they have tested the relation of RYBP with clinicopathological characterization and prognosis, in addition they has investigated the role of both transcription inhibitor of E-cadherin Zinc, Finger E-Box Binding Homeobox 1 (ZEB1) and Zinc Finger E-Box Binding Homeobox 2 (ZEB2) which known as critical marker of EMT using immunohistochemical analysis. The result was that (60 of 216) cancerous tissues were positive for RYBP and the rest were negative and the negative expression of RYBP was associated with tumor size, metastasis and with poor prognosis, all that indicate that RYBP is involved in HHC. However, for ZEB1 (159 of 216) were positive in cancerous tissues and (57 of 216) were negative and for ZEB2 expression and (153 of 216) were high expressed and (81) low expressed in which this confirms the involving of the both transcription factor in patients with HCC and were associated with distant metastatic as with RYBP. In conclusion statistical analysis of the association between

the expression of RYBP and both ZEB1 and ZEB2 also showed a negative correlation, meaning that in the time that the levels of RYBP was down-regulated, the increasing of expression of both ZEB1 and ZEB2 transcription factor was observed suggesting that that RYBP involve in the regulation of tumor EMT process (Zhu et al., 2016).

Zhou and his collagous (2017), conducted the experiment to know if the RYBP also has role in breast cancer, employing tumor and peritumoral tissues breast samples and cell lines by performing qPCR and western blot. They found that RYBP mRNA and protein were down-regulated in cancer tissues and in several breast cancer cell lines compared with control tissues in which down-regulation of RYBP was correlated with poor prognosis. In addition, they enforced RYBP expression in the breast cell line which exhibited the lowest expression of RYBP and found that overexpression of RYBP decreased cell proliferation, migration, and invasion ability significantly suggesting that patients with high RYBP expression had significantly longer death free survival (DFS) than those with low RYBP expression.

Zhao et al. (2017), has conducted the experiment seeking to know the mechanism underlying the down-regulation of RYBP expression in (HCC) which was observed in the previous studies using a normal liver cell line, tumor cell lines, and hepatocellular carcinoma tissue samples as models. They performed DNA methylation analysis to detect if the reduced expression of RYBP is related to or not with down-regulation and performed another technique which is mapping to an important transcription factor on the RYBP promoter region like specificity protein 1 (SP1) which acts as oncogene and Kruppel like factor 4 (KLF4) functions as tumor suppressor gene which were already observed in some type of cancer. They found that both mRNA and protein level of RYBP is increased by up-regulation of KLF4 expression suggesting that KLF4 positively regulate RYBP expression and decreased via up-regulation of oncogene SP1 indicating that SP1 negatively regulate RYBP expression. In addition, down-regulation of RYBP was not due to either promoter sequence variation/polymorphisms or CpG dinucleotide methylation. Moreover, a lower RYBP level in HCC tumor tissues is statistically associated with a larger tumor size, poor differentiation and an increased risk of distant metastasis in HCC patients. In conclusion dysregulation of the levels of tow

transcription factor (SP1 AND KLF4) may account for variations of RYBP levels in pathologist condition.

Zhu et al. (2017), who previously investigated the positive role of RYBP in HCC metastasis also attempted to investigate the correlation between RYBP single nucleotide polymorphism (SNP) and HCC to be the first group who reported this relation and that by recruiting a couple of 1.100 cases including (HCC patients and control (non-HCC) from chins populations who haven't receive chemotherapy or radiation before sample collecting and applied genotype study for five RYBP SNP rs12956, rs2118593, rs17009699, rs4676875, rs4532099. By applying multivariate logistic regression analysis they found that among the five candidate of RYBP SNP just two (rs12956, rs2118593) were had a significant difference between HCC cases and control ($p>0.05$). In addition, they observed that HCC patients with TT genotype rs12956 had decreased risk of HCC suggesting that it acts as protective of HCC while the genotype of rs2118593 had the increased risk of HCC indicating it as another important prognostic factor of HCC.

Dinglin and his collageous (2017), studied the role of RYBP in Lung Cancer (LC) progression and metastasis, recruiting 149 patients with lung cancer LC in this study. The clinical stage of tumors, metastasis status, survival time, presence of epidermal growth factor receptor (EGFR) mutation, and RYBP expression levels were measured. Both up-regulation and down-regulation of RYBP were experimentally performed in LC cell lines and in nude mice. Down regulation of RYBP was observed in the LC tissues compared with normal tissues and this low RYBP expression was associated with a more severe clinical stage, high mortality, high risk metastasis and poor survival.

CHAPTER 3

MATERIAL AND METHOD

3.1 Material

3.1.1 Sample Collection

In this study, 43 normal tissue (control sample) and 43 tumor tissue were collected from the same patients (total 86 specimens) who were diagnosed with colorectal cancer and underwent surgical operation between 2017 and 2018 in the General Surgery Department of the Gaziantep University Hospital within ethical conditions. Tissues directly frozen in liquid nitrogen and stored at -80 C. At this ethnic point all patients were given the volunteer' forms and after their signature, samples were taken and used in this research. Approval of ethical committee from Gaziantep, Turkey, was received at (13.03.2017) and this project (Project No: FEF.YLT.17.13) was supported by the Scientific Research Projects Department of Gaziantep University. Invitrogen by Thermo Fisher Scientific Pure Link RNA mini Kit was used to isolate RNA (Cat.no. 12183018A, 12183025, USA). Applied biosystems by Thermo Fisher Scientific was utilized for (High Capacity cDNA Reverse Transcription Kit) (REF: 4368814, Lithuania). TaqMan Gene Expression Assays also used for the final step Real Time- PCR including (gene ex assay, Master Mix, RNase free water) (Ref: 4346907, USA).

- Gene expression assay (genes of interest and internal control)
- RYBY (Taq Man THERMO FISHER 4331182, Assay ID Hs00393028_m1).
- MDM2 (Taq Man THERMO FISHER 4331182, Assay ID Hs 00540450-s1).
- GABDH (Taq Man THERMO FISHER 4331182, Assay ID Hs02786624_g1)

3.2 Method

3.2.1 RNA Isolation

3.2.1.1 Steps of Lyses and Homogenization

1. 2 ml sterilized Eppendorf tubes were numbered with 1,2,3.....43, according to the number of patient and coded with T for tumor tissue and N for normal tissue , after that every tube was added 10 μ L 2-mercaptoethanol ,1000 μ lyses buffer and pellet
2. Samples were taken out freezer and put them on dry ice to prevent them thawing, and were cut with both scalpel and tongs in a dish (each dish for every tissue shall be thrown after the cutting is completed and start by cutting another sample to prevent contamination) and immediately after samples were cut then they were transferred about (1 mg) to prepared tubes and frozen for ten minutes to help performing best breaking of the samples to be ready for the next step (homogenization).
3. Tubes were placed into the homogenizer device (Tissue Lyser) and the last was set on the (10 minutes, 50 volts), the device immediately was run at the room temperature until the cells spherule were scattered in the lyses and became ready lysate for the centrifuge. Proceed homogenization may take more than one time (once) depending on the toughness or thickness of samples. The pellets were removed from tubes and lysate (underwent centrifuge for 5 minutes at 26.000*g.
4. Transfer the lysate into 1.5 ml new clean free RNAs tubes.

3.2.1.2 The Steps of Binding, Washing and Elution

1. For each one volume of homogenate, cell one volume of 70% of ethanol was added.
2. Vortex was done to mix the lysate completely and to separate any obvious sediment that may form after adding ethanol.

3. About 700 μL of the sample (containing any remaining precipitate) was transferred to the Spin Cartridge with the Collection Tube.
4. Centrifuge at $12,000 \times g$ for 15 seconds at room temperature was done. The flow-through which formed after centrifuge was disposed, and the Spin Cartridge again placed back into the same Collection Tube.
5. Steps 3-4 was repeated till the entire of sample was processed.
6. 700 μL of (Wash Buffer I) was added to the lysate existed in the Spin Cartridge and was Centrifuged at $12,000 \times g$ for 15 seconds at room temperature and then both of the flow –through and collection tube were thrown out and new collection tube placed into the spin Cartridge.
7. 500 μL of Wash Buffer II with ethanol was added also to lysate existed in the Spin Cartridge.
8. Centrifuge at $12,000 \times g$ for 15 seconds at room temperature was achieved, the flow-through was removed and reinsert the Spin Cartridge into the same Collection Tube.
9. Steps 7-8 were repeated just for one time.
10. To dry the membrane with bound RNA Again, the step of centrifuge the Spin Cartridge at $12,000 \times g$ for 1-2 minutes has done and collection tube has been replaced with recovery tube.
11. 30–100 (50 μL) of RNAase free water was added to the center of Spin Cartridge.
12. For one minute at the room temperature Spin Cartridge tubes were incubated.
13. The RNA was eluted from the membrane into the Recovery tube by done the Centrifuge of lysate existed in the Spin Cartridge for 2 minutes at $\geq 12,000 \times g$ at room temperature.
14. Purified RNA was stored at -80°C to proceed it in next the downstream application.

3.2.1.3 Spectrophotometric Analysis

The concentration of RNA samples was measured by spectrophotometry device at 260-nm wavelength densities. Pure preparations of RNA have OD₂₆₀/OD₂₈₀ values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible. The OD A₂₆₀/OD A₂₃₀ ratio indicates the presence of organic contaminants; Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants that will interfere with downstream applications (Warburg and Christian, 1942; Glasel, 1995).

3.2.1.4 Dilution of Samples

The concentration of all samples was not equalled (some has a high or low concentration) so to unify this concentration the samples was diluted 30 ng / μ L by using distilled water according to the formula which determines how much I need of both RNA sample and distilled water.

3.2.2 cDNA Synthesis

Standard ingredients of master mix for cDNA synthesis were shown in the Table 3.1.

Table 3. 1 Standard ingredients of master mix for cDNA synthesis

Ingredients	Volume μ L for per reaction in kit with RNase inhibitor
10 $\times$ RT Buffer	2.0
25 $\times$ dNTP Mix (100 mM)	0.8
10 $\times$ RT Random Primers	2.0
MultiScribe™ Reverse Transcriptas	1.0
RNase Inhibitor	1.0
Nuclease-free H ₂ O	3.2
Total master mix Reaction	10.0

Total volume for per reaction	20 μ L (10 μ L master mix and 10 μ L RNA).
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3.2.2.1 Steps for cDNA synthesis

1. The needed volume of components was calculated to prepare the required number of reactions.
2. Kit components were kept on the ice for thawing.
3. Master mix was prepared from the mentioned components shown in the Table above with accounting for one to two additional reactions to avoid the loss of volume that occurs during transferring the reagents.
4. 10 μ L of the reverse transcription master mix and 10 μ L of the amount of RNA was added to 0.5 -ml PCR tubes labeled with sample number on the side.
5. Place and incubate the tubes contain reaction at the following conditions of temperatures and time using a PCR thermal cycler (Table 3.2).
6. The outcome of cDNA may be analyzed immediately by real-time PCR or stored at 2 to 6 °C for short time or at -15 to -25 °C for long time.

Table 3. 2 Conditions of PCR running

Setting	Step 1	Step 2	Step 3	Step 4
Temperature	20	37	85	4
Time	10 minutes	120	5	Infinitive

3.2.3 Quantitative Real Time-PCR (qRT-PCR)

Using qRT-PCR in the molecular diagnosis ascribed to it's the high sensitivity. In which, this technique used to amplify and simultaneously quantify a specific region of the DNA molecule. (Abbott et al., 1988; Syvänen et al., 1988). Also it used to quantify and determine the level of nucleic acid (mRNA) expression in tumor and normal tissues for

each gene (RYBP, MDM2) (Bustin, 2002), using cDNA as substrate in the majority of qPCR gene expression experiments. TaqMan Primer Probe for both (genes of interest and internal control) was used as gene expression assay. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (endogenous control or housekeeping) characterized by its ability to express in wide and diversity of human tissues and cells with slight differences of their expression levels, often it should be not changed (be constant) under the disease condition, consequently was chosen to be used as a normalizer of mRNA analysis data for both gene of interest during quantitative RT-PCR (Eswards et al., 1985; Winer et al., 1999; Vandesompele et al., 2002). Software programs as Applied Biosystems (StepOne & StepOnePlus Real-Time PCR Systems) was used as a device to data analyzing .

During conducting qRT-PCR the exact amount of nucleic acid can be determined by following the accumulated fluoresce signals, which are emitted from sample during the amplification. However, there is also a lot of fluorescence located in background in which we need to avoid them that we can get a glean meaningful information from signals; this is where the C_t comes in. C_t represents the spot where reaction curve intersects with threshold line and this C_t correlates with the number of copy gene and has inverse relationship with the nucleic acid (the greater levels of C_t , the lowest amount of nucleic acid and vice versa) (Wang and Meddrano, 2005).

The experiment for every gene was performed in duplicate. The data from qRT-PCR was used to calculate the value of mRNA expression levels in tumor tissue relative to normal based on following formula. Fold gene expression = $2^{-(\Delta\Delta C_t)}$ (Livak et al., 2001). To calculate the relative fold gene expression level, it could be presented as: Calculate the average C_t values for each gene in each measured sample (C_t Threshold).

- Calculate the ΔC_t (delta C_t) for each sample by using the average C_t values

- $\Delta C_t = C_t (\text{gene of interest}) - C_t (\text{housekeeping gene})$

- Now calculate the $\Delta\Delta C_t$ relative to the ΔC_t Control average.

- $\Delta\Delta C_t = \Delta C_t (\text{tumor sample}) - \Delta C_t (\text{normal sample})$

- Finally, to work out the fold gene expression, it must be to do 2 to the power of negative $\Delta\Delta C_t$: $2^{-(\Delta\Delta C_t)}$.

Table 3. 3 Standard required reagents for RT-PCR reaction

Ingredient	Per reaction (μL)
Gene ex assay	1 μL
Master mix	10 μL
RNase d H2o	7 μL
Total reaction for mix	18 μL

The total volume of reaction for each well in the 96 plate is 20 μL (2 μL cDNA and 18- μL mix).

3.2.3.1 Steps for Real Time PCR

1. The needed volume of components was calculated to prepare the required number of reactions with accounting 1 to 2 additional reaction per gene.
2. Kit components were put on the ice for thawing.
3. Three single tubes were prepared and labeled according to the type of gene assay (MDM2, RYBP and GAPDH).
4. All the components were added to the labeled tubes.
5. After preparing mix briefly, tubes underwent the vortex.
6. Per gene was performed duplicate.
7. Carefully, 18 of mix has withdrawn and pipit it into each well of 96 well reaction plate.
8. 2 μL of cDNA was added to the 18 μL of prepared mix into each well of 96 well reaction plate (pipetting up and down tow time to ensure mix).
9. The plate was sealed carefully by optical adhesive film to avoid forming any air bubbles.
10. The plate was loaded into Real-time PCR system Thermal Cycling Block (bio systems step one pulse) and the RT-PCR was set up and run.

3.3 Statistical Analysis

Statistical analyses were performed using SPSS version 22.0 software program for (IBW) windows, USA). Student's T-test was used to compare the difference of the mRNA expression of RYBP and mRNA expression of MDM2 normalized to GAPDH between the tumor and normal tissues. Whereas the χ^2 test was applied for the comparison of dichotomous variables (determining the relation between the gene expression (high and low) with all the clinical pathological features. Spearman test was applied to examine the correlation between the expression of MDM2 and RYBP.

P<0.05 was considered to indicate a statistically significant difference.

CHAPTER 4

RESULTS

4.1 Demographic and Clinical Outcomes of The Patients

All data related to pathological features and other information were obtained from the medical records of patients in which the information of patients including (age, gender, tissue type, stage, lymph node, metastasis, smoking and other diseases) as described in the Table 4.1. Forty- three paired samples were included in this study. The mean age of colorectal cancer patients included in the study was between $53.65 \pm$. 26 out of 43 (60,5%) was 50 years old and above, while 17 out of 43 (39,5 %) was younger than 50 years. When the patients were examined in terms of gender, 29 of the 43 patients (67,5 %) were male and 14 of the 43 patients (32,5 %) were female. When the patients were examined in terms of stage, 23 of the 43 patients (53,5 %) were stage I and stage II, 20 of the 43 patients (46,5 %) were stage III and stage IV. In addition, 10 of the patients had metastasis, while the rest were without metastasis. None of the patients had any alcohol consumption. While 27 of the patients (62,79 %) had no smoking habits, the rest of the patients (37,20 %) had a habit of smoking cigarettes. Tissue types of 26 patients (60,50 %) were colon, while tissue types of 17 patients (39,50 %) were rectum. 19 of the patients (44,2 %) had lymph nodes, while the rest (55,8 %) had no lymph nodes. In addition, 21 patients (48,9 %) had chronic diseases besides colorectal cancer.

Table 4. 1 The general distribution of clinical pathologic features related to patients with colorectal cancer

Characterizes		Patients (n:43)	
		N	N (%)
Age	≥50 age	26	60,5%
	<50 age	17	39,5%
Gender	Female	14	32.6%
	Male	29	67.4%
Smoking habit	Yes	27	37,2%
	No	16	62,8%
Alcohol	Yes	43	100%
	No	0	0%
Tissue type	Colon	26	60.5%
	Rectum	17	39.5%
Metastasis	With	10	23.3%
	Without	33	76.7%
Stage	I- II	23	53,5%
	III-IV	20	46,5%
Lymph node	Exist	19	44.2%
	Not exist	24	55.8%
Chronic disease	With	21	48.8%
	Without	22	51.2%

4.2 The Concentration of RNA Measured by Spectrophotometry After Isolation in Both Tumor and Normal Samples

After the isolation of RNA in the entire eighty-six colorectal samples, including tumor and normal tissues, have successfully achieved. The measured concentrations of RNA in all the samples by spectrophotometry device were given in the Table 4.2.

Table 4. 2 The concentration of RNA after isolation in both tumor and normal tissues (OD: Optical Density)

Sample number		RNA concentration	OD 260/280
1	T	921.008	2.249
	N	845.623	2.257
2	T	46.102	2.32
	N	4.67	1.096
3	T	144.511	1.648
	N	48.482	2.352
4	T	33.506	2.097
	N	18.06	1.482
5	T	116.157	2.023
	N	29.085	2.133
6	T	478.883	2.002
	N	32.6	1.93
7	T	112.11	2.121
	N	139.022	1.954
8	T	140.932	1.99
	N	81.396	2.002
9	T	94.706	2.081
	N	146.549	2.066
10	T	530.11	2.139
	N	244.83	2.139
11	T	116.94	2.166
	N	436.21	0.986
12	T	205.33	2.086
	N	138.9	2.133
13	T	226.9	2.154
	N	50.77	1.961
14	T	380.63	2.214
	N	657.41	2.185
15	T	182.59	2.136
	N	378.55	2.191
16	T	30.25	1.624
	N	230.69	2.141
17	T	39.19	2.053
	N	17.17	2.422
18	T	80.47	2.156
	N	456.87	1.913
19	T	76.2	2.035
	N	154.66	1.843
20	T	127.38	1.885
	N	110.84	2.1
21	T	271.33	2.147
	N	151.72	2.185
Sample number		RNA concentration	OD 260/280
22	T	467.05	1.626
	N	108.09	2.255
23	T	59.15	2.08
	N	71.75	2.197
24	T	1298.69	2.155
	N	1222.54	2.154
25	T	802.05	2.193
	N	101.67	2.253
26	T	30.03	2.092
	N	27.98	2.124
27	T	410.24	2.35
	N	96.15	2.641
28	T	1001.89	2.204
	N	336.12	2.25
29	T	408.8	2.209
	N	148.33	2.367
30	T	1363.4	2.151
	N	570.21	2.237
31	T	329.38	2.277
	N	694.07	2.227
32	T	343.34	2.229
	N	158.96	2.237
33	T	560.83	2.209
	N	951.58	2.166
34	T	50.8	2.188
	N	44.25	2.151
35	T	127.15	2.302
	N	615.74	2.22
36	T	556.43	2.213
	N	914.11	2.209
37	T	96.17	2.364
	N	39.06	2.188
38	T	758.12	2.199
	N	33.03	1.911
39	T	668.82	2.216
	N	56.22	2.24
40	T	30.29	2.001
	N	118.39	2.344
41	T	33.07	1.176
	N	56.94	2.358
42	T	1395.46	2.175
	N	157.49	2.175
43	T	160.41	2.362
	N	164.12	2.378

4.3 Analysis of RYBP and MDM2 Expression in Colorectal Cancer Tissues

1) To investigate the expression profile of RYBP and MDM2 in colorectal cancer, and determine if there is a correlation between the alteration (up-regulation or down-regulation) of these two genes with colorectal cancer incidence, this study performed, relative qRT-PCR in 43 pairs of colorectal cancer tissues (normal and tumor). The ΔC_t values of the mRNA expression for each gene alone (RYBP, MDM2) with housekeeping (GAPDH) in every recruited sample was calculated by using the C_t value from both tumor and normal tissues of the same patients as shown in the Figure 4.1, 4.2, 4.3 respectively.

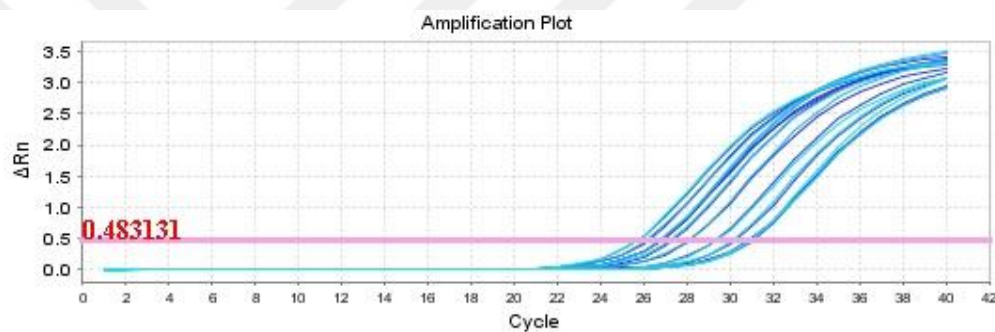


Figure 4. 1 Image of RYBP expression in Real-Time

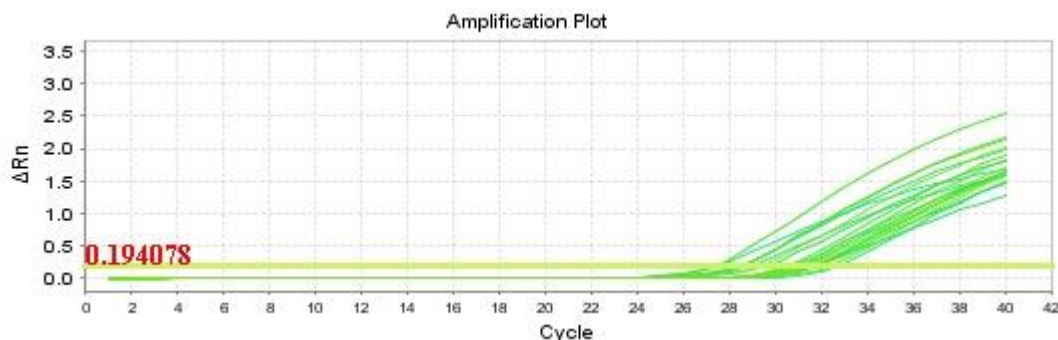


Figure 4. 2 Image of MDM2 expression in Real-Time

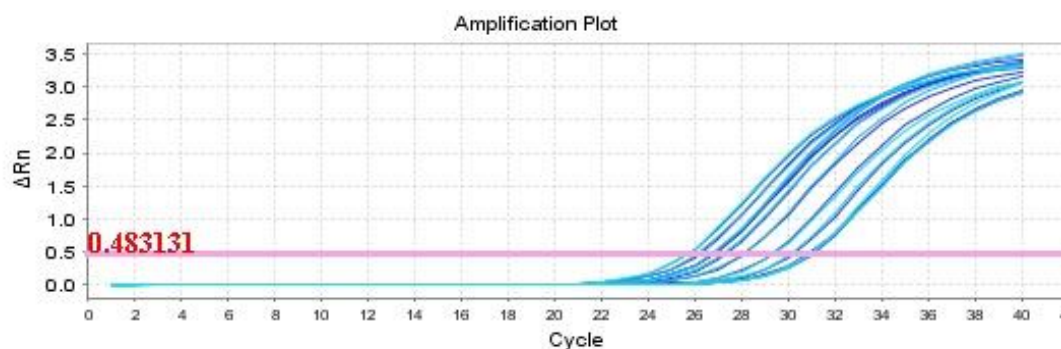


Figure 4. 3 Image of GAPDH expression in Real-Time

Student's T-test was applied using ΔC_t values to compare the difference of the mRNA expression of RYBP normalized to GAPDH and mRNA expression of MDM2 normalized to GAPDH between the tumor and normal tissues. The mean $\Delta C_t \pm SD$ value for RYBP gene was calculated as 3.60 ± 2.56 for normal tissues and 3.84 ± 2.70 for tumor tissues as shown in the Figure 4.4. Statistically, there was no significant difference between RYBP gene expression in tumor and normal tissue in the patients with colorectal cancer ($p = 0.673$).

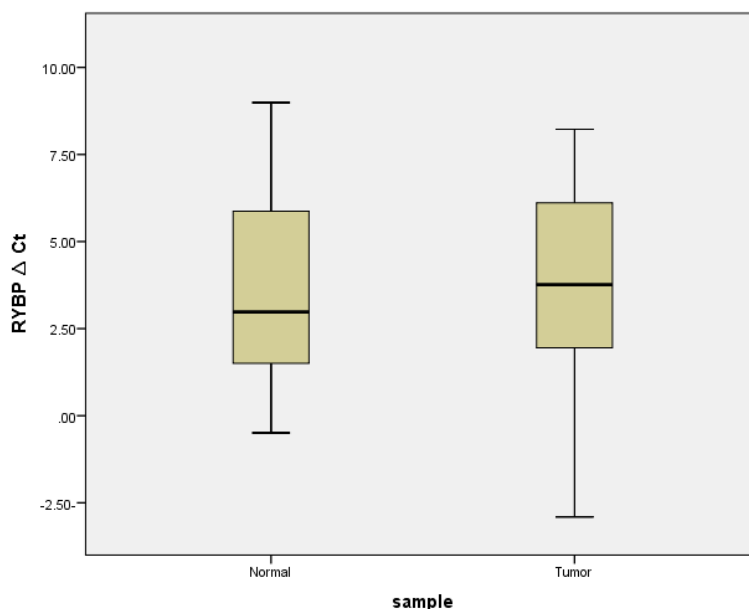


Figure 4. 4 Box plot of RYBP gene expression between normal and tumor tissues

while the mean $\Delta Ct \pm SD$ value for the MDM2 gene was calculated as 3.20 ± 1.79 for normal tissues and 3.36 ± 2.16 for tumor tissues as shown in Figure 4.5. The same finding was observed for MDM2, in which statistically no significant difference between tumor and normal tissues in patients with colorectal cancers so no relation between expression of the MDM2 gene and colorectal cancer MDM2 ($p= 0.721$).

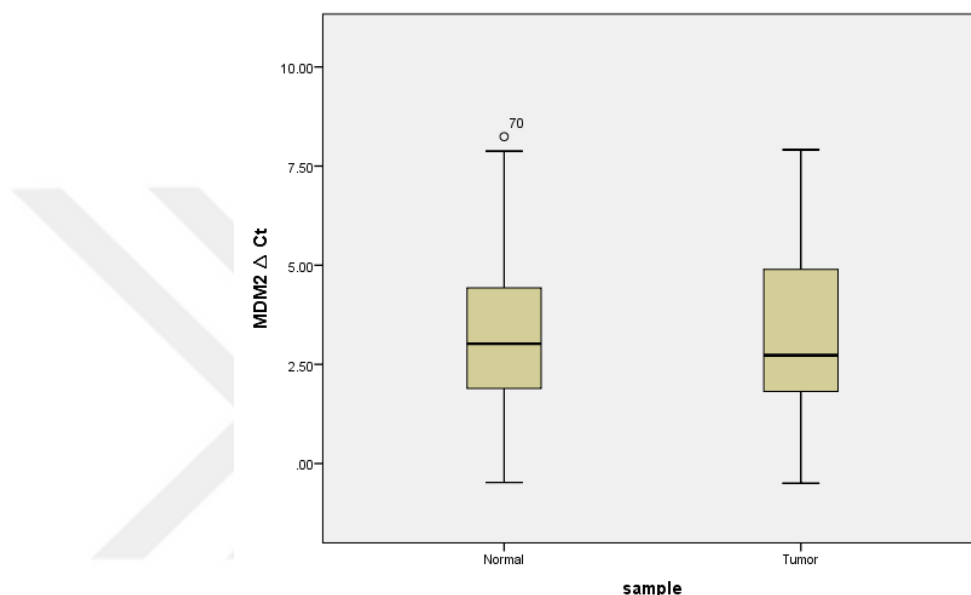


Figure 4. 5 Box plot of MDM2 gene expression between normal and tumor tissues

After calculating mRNA expression for each single gene (RYBP, MDM2). A comparison step of mRNA expression in both tumor and normal in the same patient had done to determine the change (how many times the gene expression in tumor tissues increased or decreased in comparison with normal tissues, in which the last has fold change $2^{-\Delta\Delta CT} = 1$ and that depending on the formula) $2^{-\Delta\Delta CT}$.

- $2^{-\Delta\Delta CT} > 1$ means that gene expression in tumor tissues is higher than normal tissues
- $2^{-\Delta\Delta CT} < 1$ means that the gene expression in tumor tissues is lower than normal tissues
- $2^{-\Delta\Delta CT} = 1$ means that the gene expression is equal in both tumor and normal tissues.

The mRNA expression levels of RYBP were up-regulated in 20 of 43 cases (46.51%) and down-regulated in 23 of 43 (53.49%). Whereas MDM2 mRNA level expression was found overexpressed in 21 of 43 (48.84 %) and low expressed in 22 of 43 (51.16 %).

2) Relation of MDM2 gene expression with clinico pathological parameters analyzed by Chi-square test. In this work, 17 of 43 patients were younger than 50 age, in which in the 17 patients the MDM2 expression was detected in low expression in 8 (47.1%) and high in the rest 9 (52.9%) patients, while 26 of 43 patients were detected to be 50 years old and above, in which within this 26 patients, high expression of MDM2 gene expression was shown in 12 of 26 (46.2%) patients and low expression in (14 of 26) (53.8%) patients. Statistically no association between the age and MDM2 expression ($p=0.663$) as elucidated in Table 4.3.

Table 4. 3 Relationship between MDM2 expression and age

			MDM2 expression		Total
			Low	High	
Age	Younger than 50	Number of patients	8	9	17
		Age (%)	47.1%	52.9%	100.0%
		MDM2 expression (%)	36.4%	42.9%	39.5%
		Total (%)	18.6%	20.9%	39.5%
	50 years old and above	Number of patients	14	12	26
		Age (%)	53.8%	46.2%	100.0%
		MDM2 expression (%)	63.6%	57.1%	60.5%
		Total (%)	32.6%	27.9%	60.5%
Total		Number of patients	22	21	43
p value *		0.663			

* Pearson Chi-Square Test

As it has shown in the Table 4.4 which elucidated the relationship between MDM2 expression and gender. For the link between the gender with the MDM2 expression among the 43 patients, (14 of 43) was female and (29 of 43) was male. within the female group, the number of female patients who had low expression of MDM2 was (6 of 14)

42.9% and (8 of 14) was high expression 57.1%. While the number of male' patients at low expression was (16 of 29) 55.2% and at high expression was (13 of 29) 44.8%.

Table 4. 4 Relationship between MDM2 expression and gender

			MDM2 expression		Total
			Low	High	
Gender	Female	Number of patients	6	8	14
		Gender (%)	42.9%	57.1%	100.0%
		MDM2 expression (%)	27.3%	38.1%	32.6%
		Total (%)	14.0%	18.6%	32.6%
	Male	Number of patients	16	13	29
		Gender (%)	55.2%	44.8%	100.0%
		MDM2 expression (%)	72.7%	61.9%	67.4%
		Total (%)	37.2%	30.2%	67.4%
Total		Number of patients	22	21	43
p value *		0.449			

* Pearson Chi-Square Test

As shown in the Table 4.5 colon tissues forms 26 of 43 of the taken sample, while rectum forms 17 of 43 samples. Within colon tissues, MDM2 gene expression was detected at low levels in (14 of 26), while 12 of 26 of the same tissues expressed high levels of the MDM2 gene in high levels. On the other hand, within the 17 rectum tissues 8 of 17 tissues had low expression of MDM2 and the rest of 9 rectum tissues had high MDM2 expression. Statistically, there was no relation between the expressions of MDM2 with tissues types ($p = 0.961$)

Table 4. 5 Relationship of MDM2 expression with tissues type

			MDM2 expression		Total
			Low	High	
Tissue type	Colon	Number of patients	14	12	26
		Colon (%)	53.8%	46.2%	100.0%
		MDM2expression (%)	63.6%	57.1%	60.5%
		Total (%)	32.6%	27.9%	60.5%
	Rectum	Number of patients	8	9	17
		Rectum (%)	47.1%	52.9%	100.0%
		MDM2 expression (%)	36.4%	42.9%	39.5%
		Total (%)	18.6%	20.9%	39.5%
Total		Number of patients	22	21	43
p value *		0.961			

* Pearson Chi-Square Test

Smoking is the other parameter, which its relation with MDM2 expression was studied. The numbers of non-smoker (27 of 43) were more than smokers (16 of 43). In non-smoker' group low and high expression of MDM2 was detected in a number of patients (13 of 27) and (14 of 27) respectively. While in the smoker group the number of patients with MDM2 low expression was (9 of 16), while those with MDM2 high expression (7 of 16) ($p=0.607$) indicating that there is no relation of the colorectal cancer incidence with smoking habit as explained in the Table 4.6.

Table 4. 6 Relationship of MDM2 expression with smoking

			MDM2 expression		Total
			Low	High	
Smoking	Non-Smoker	Number of patients	13	14	27
		Non-Smoker (%)	48.1%	51.9 %	100,0%
		MDM2 expression (%)	59,1%	66,7%	62,8%
		Total (%)	30,2%	32,6%	62,8%
	Smoker	Number of patients	9	7	16
		Smoker (%)	56,3%	43,8%	100,0%
		MDM2 expression (%)	40,9%	33,8%	37,2%
		Total (%)	20,9%	16,3%	37,2%
Total		Number of patients	22	21	43
p value *		0.607			

*Pearson Chi-Square Test

As elucidated in the Table 4.7 (10 of the 43) studied cases of patients with colorectal cancer were detected with metastasis and the rest of 33 cases were without metastasis. Within the first ten cases (4) patients were found with MDM2 low expression (40.0%) and (6) patients found with MDM2 high expression (60.0%). On the other hand, in the cases does not have metastasis (18) of patients were displayed low expression (54.5%) and (15) patients (45.5%) displayed high expression.

Table 4. 7 Relationship of MDM2 expression with metastasis

			MDM2 expression		Total
			Low	High	
Metastasis	With metastasis	Number of patients	4	6	10
		With metastasis (%)	40,0%	60,0%	100,0%
		MDM2 expression (%)	18,2%	28,6%	23,3%
		Total (%)	%9,3	14,0%	23,3%
	Without metastasis	Number of patients	18	15	33
		Without metastasis (%)	54.5%	45.5%	100.0%
		MDM2 expression (%)	81.8%	71.4%	76.7%
		Total (%)	41.9%	34.9%	76.7%
Total		Number of patients	22	21	43
p value *		0.420			

* Fisher's Exact Test

Depending on the TNM stage there are four main stage including (the depth of tumor and the lymph node and the distant of tumor). We gathered stage I and II in one group and stage III and IV in another group. In our patients the first group were found in 23 of 43 and the second group were found in 20 of 43. Statistically there is no relation between the stages and MDM2 ($p = 0.541$). As shown in the below Table 4.8.

Table 4. 8 Relationship of MDM2 expression with stages

			MDM2 expression		Total
			Low	High	
Stage	Stage I-II	Number of patients	13	10	23
		Stage I and II (%)	56.5%	43.5%	100.0%
		MDM2 expression (%)	59.1%	47.6%	53.5%
		Total (%)	30.2%	23.3%	53.5%
	Stage III-IV	Number of patients	9	11	20
		Stage III and IV (%)	45.0%	55.0%	100.0%
		MDM2 expression (%)	40.9%	52.4%	46.5%
		Total (%)	20.9%	25.6%	46.5%
Total		Number of patients	22	21	43
p value *		0.541			

*Fisher's Exact Test

The lymph node was observed in 19 of 43, while not observed in the rest 24 cases. In the lymph node' group (9) of patients displayed high expression (47.4%), while (10) displayed low expression (52.6%). In the other 24 cases, the number of patients with high expression was (12) and that was similar to those with low expression (50.0%), ($p=0.864$) as shown in the Table 4.9.

Table 4. 9 Relationship of MDM2 expression with lymph node

			MDM2 expression		Total
			Low	High	
Lymph node	With Lymph node	Number of patients	10	9	19
		With Lymph node (%)	52.6%	47.4%	100,0%
		MDM2 expression (%)	45.5%	42.9%	44,2%
		Total (%)	23.3%	20.9%	44,2%
	Without Lymph node	Number of patients	12	12	24
		Without Lymph node (%)	50.0%	50.0%	100,0%
		MDM2 expression (%)	54.5%	57.1%	55,8%
		Total (%)	27.9%	27.9%	55.8%
Total		Number of patients	22	21	43
p value *		0.864			

*Pearson Chi-Square Test

As indicated in Table 4.10, (21 of the 43) patients had a chronic disease besides colorectal cancer, while 22 had no other chronic disease besides cancer. (10 of 22) of those with chronic disease had low expression of MDM2 (47.6%), and the rest of 11 had high expression of MDM2 (52.4%), While in the group without other disease 10 patients had high expression and 12 had low expression. There is no statistical significance between MDM2 gene expression and other chronic diseases ($p = 0.650$).

..

Table 4. 10 Relationship of MDM2 expression with other disease

			MDM2expression		Total
			Low	High	
Other disease	With	Number of patients	10	11	21
		With other disease (%)	47.6%	52.4%	100,0%
		MDM2 expression (%)	45.5%	52.4%	48,8%
		Total (%)	23.3%	25.6%	48,8%
	Without	Number of patients	12	10	22
		Without other disease (%)	54.5%	45,5%	100,0%
		MDM2 expression (%)	54.5%	47.6%	51,2%
		Total (%)	27.9%	23.3%	51,2%
Total		Number of patients	22	21	43
p value *		0.650			

*Pearson Chi-Square Test

Individuals which are younger than 50 form 17 of 43 of the samples, while at 50 years old and above form 26. In the group includes individuals with age younger than 50, the ratio of low and high expression levels of RYBP was (47,1%,52.9%) respectively. Whereas, the low and high expression levels of RYBP was (57,7%, 42,3%) respectively in the group of patients 50 years old and above. Statistically, no relation between RYBP expressions with age ($p=0.494$), as shown in the Table 4.11.

Table 4. 11 Relationship of RYBP expression with age

			RYBP expression		Total
			Low	High	
Age	Younger than 50	Number of patients	8	9	17
		Age (%)	47,1%	52,9%	100,0%
		RYBP expression (%)	34,8%	45,0%	39,5%
		Total (%)	18,6%	20,9%	39,5%
	50 years old and above	Number of patients	15	11	26
		Age (%)	57,7%	42,3%	100,0%
		RYBP expression (%)	65,2%	55,0%	60,5%
		Total (%)	34,9%	25,6%	60,5%
Total		Number of patients	23	20	43
p value *		0.494			

* Pearson Chi-Square Test.

Females represent 14 and male the rest of 29 of the studied cases. Among the female group, RYBP gene expressed at low levels in eight individuals and at high levels in six individuals. Within male group RYBP, expression was shown to be expressed at low expression in fifteen individuals and at high expression in fourteen once ($p = 0.739$) that means that there is no relation between gender and RYBP expression. As explained in the below Table 4.12.

Table 4. 12 Relationship of RYBP expression with gender

			RYBP expression		Total
			Low	High	
Gender	Female	Number of patients	8	6	14
		Gender (%)	57.1%	42.9%	100.0%
		RYBP expression (%)	34.8%	30.0%	32.6%
		Total (%)	18.6%	14.0%	32.6%
	Male	Number of patients	15	14	29
		Gender (%)	51.7%	48.3%	100.0%
		RYBP expression (%)	65.2%	70.0%	67.4%
		Total (%)	34.9%	32.6%	67.4%
Total		Number of patients	23	20	43
p value *		0.739			

* Pearson Chi-Square Test

As shown in the Table 4.13 tissues types in the patients divided into two group. Colon type contains 26 individual and rectum type contains 17 once. Colon' group has appeared low rate expression of RYBP (53,8%) in 14 of 26 and high rate expression 46.2% in (12 of 26), While Rectum' group has appeared high expression in the eight patients of seventeen with ratio (47.1%) and low expression in nine of seventeen with ratio (52.9%). According to the p value ($p = 0.233$) no relation between tissue type and RYBP expression.

Table 4. 13 Relationship of RYBP expression with tissue type

			RYBP expression		Total
			Low	High	
Tissue type	Colon	Number of patients	14	12	26
		Colon (%)	53,8%	46.2%	100,0%
		RYBP expression (%)	60,9%	60.0%	60,5%
		Total (%)	32,6%	27.9%	60,5%
	Rectum	Number of patients	9	8	17
		Rectum (%)	52.9%	47.1%	100,0%
		RYBP expression (%)	39.1%	40.0%	39,5%
		Total (%)	53.6%	46.5%	39,5%
Total		Number of patients	23	20	43
p value *		0.233			

*Pearson Chi-Square Test

In the non-smoker group 17 of 27 had low expression and 10 had high expression, while, in smoker group 6 individuals had low expression and 10 once had high expression. Statistically, there was no correlation ($p=0.106$). As shown in the Table 4.14.

Table 4. 14 Relationship of RYBP expression with smoking habit

			RYBP expression		Total
			Low	High	
Smoking	Smoker	Number of patients	6	10	16
		Smoker (%)	37.5%	62.5%	100.0%
		RYBP expression (%)	26.1%	50.0%	37.2%
		Total (%)	14.0%	23.3%	37.2%
	Non-smoker	Number of patients	17	10	27
		Non-smoker (%)	63.0%	37.0%	100.0%
		RYBP expression (%)	73.9%	50.0%	62.8%
		Total (%)	39.5%	23.3%	62.8%
Total		Number of patients	21	22	43
p value *		0.106			

*Pearson Chi-Square Test

Depending on the TNM stage there are four main stage including (the depth of tumor and the lymph node and the distant of tumor). We gathered stage I and II in one group and stage III and IV in another group. In our patients the first group were found in 23 of 43 and the second group were found in 20 of 43. Statistically there is no relation between the stages and RYBP ($p = 0.298$). As shown in the below Table 4.15.

Table 4. 15 Relationship of RYBP expressions with stages

			RYBP expression		Total
			Low	High	
Stage	Stage I-II	Number of patients	14	9	23
		Stage I-II (%)	60.9%	39.1%	100.0%
		RYBP expression (%)	60.9%	45.0%	53.5%
		Total (%)	32.6%	20.9%	53.5%
	Stage III-IV	Number of patients	9	11	20
		Stage III-IV (%)	45.0%	55.0%	100.0%
		RYBP expression (%)	39.1%	55.0%	46.5%
		Total (%)	20.9%	25.6%	46.5%
Total		Number of patients	23	20	43
p value *		0.298			

*Fisher' Exact Test

As shown in the Table 4.16. (19 of 43) patients diagnosed with existing lymph node and 24 of those did with not existing. Among (19) cases, low rate of expression (63.2%) was found in twelve patients and the high rate of expression (36.8%) in the other seven cases. Whereas, in the other 24 cases, expression of RYBP was found at low levels (45.8%) in 11 one and at high levels (54.2%) in 13 one. There is no statistically significant ($p=0.258$) thus, there is no relation between the expression of RYBP and existence of lymph node.

Table 4. 16 Relationship of RYBP expression with lymph node

			RYBP expression		Total
			Low	High	
Lymph node	With	Number of patients	12	7	19
		With Lymph node (%)	63.2%	36.8%	100.0%
		RYBP expression (%)	52.2%	35.0%	44.2%
		Total (%)	27.9%	16.3%	44.2%
	Without	Number of patients	11	13	24
		Without Lymph node (%)	45.8%	54.2%	100.0%
		RYBP expression (%)	47.8%	65.0%	55.8%
		Total (%)	25.6%	30.2%	55.8%
Total		Number of patients	23	20	43
p value *		0.258			

*Pearson Chi-Square Test

Metastasis is found in 10 of the all the collected sample and in the ten cases six patients exhibited high expression of RYBP and 4 low expression, while in the group doesn't have metastasis 19 cases of them showed low expression and 14 one high expression. As shown in below the Table 4.17.

Table 4. 17 Relationship of RYBP expression with metastasis

			RYBP expression		Total
			Low	High	
Metastasis	With	Number of patients	4	6	10
		With metastasis (%)	40.0%	60.0%	100.0%
		RYBP expression (%)	17.4%	30.0%	23.3%
		Total (%)	9.3%	14.0%	23.3%
	Without	Number of patients	19	14	33
		Without metastasis (%)	57.6%	42.4%	100.0%
		RYBP expression (%)	82.6%	70.0%	76.7%
		Total (%)	44.2%	32.6%	76.7%
Total		Number of patients	23	20	43
p value *		0.473			

*Fisher Exact Test

Statistically, there was no relation between existing of chronic disease with cancer occurrence ($p = 0.639$). Low levels of RYBP expression found in the ratio (57.1%) and high expression in the ratio (42.90%) in the group of cancer patients with chronic disease. On the other hand, the number of the patients expressed low expression 11 was equal to those who expressed high expression in the group of cancer' patients without chronic disease. As shown in the Table 4.18.

Table 4. 18 Relationship of RYBP expression with chronic disease beside the cancer

			RYBP expression		Total
			Low	High	
Other disease	With	Number of patients	12	9	21
		With other disease (%)	57.1%	42.9%	100.0%
		RYBP expression (%)	52.2%	45.0%	48.8%
		Total (%)	27.9%	20.9%	48.8%
	Without	Number of patients	11	11	22
		Without other disease (%)	50.0%	50.0%	100.0%
		RYBP expression (%)	47.8%	55.0%	51.2%
		Total (%)	25.6%	25.6%	51.2%
Total		Number of patients	23	20	43
p value *		0.639			

*Pearson Chi-Square Test

The last aim in this study is the correlation between these two genes. Spearman Test from the correlation list was applied as shown in Figure 4.6 to elucidate the correlation between the levels of MDM2 and RYBP gene expression in the patients with colorectal cancer in which there was no correlation between the expression of MDM2 and RYBP ($r=0.159, p=0.308$).

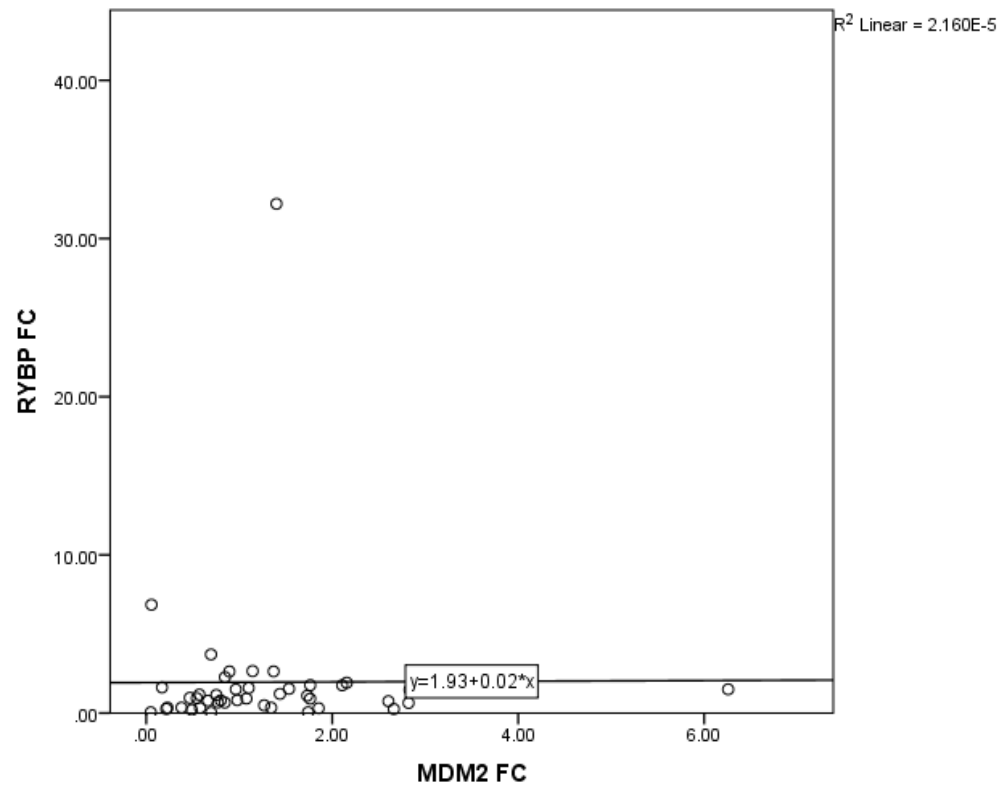


Figure 4. 6 Scatter plot of correlation between MDM2 and RYBP gene expression in the colorectal cancer patients ($r = 0.159$, $p = 0.308$). fc= fold change

CHAPTER 5

DISCUSSION

As a member of PcG family, RYBP is a transcription repressor and dependable of RYBP function as apoptotic-inducing ability, RYBP expression has been shown to be involved in variety of human cancer including hepatocellular carcinoma (Wang et al., 2014), breast cancer (Zhou et al., 2016; Kenny et al., 2017), non-small cell lung cancer (Voruganti et al., 2015). However, there is no study investigating the role of RYBP in CRC in the literature. For this reason, we aimed to elucidate the relationship between CRC and RYBP expressions in this study. Although RYBP mRNA expression levels were up-regulation at 20 cases (46,51 %) and down-regulation at 23 cases (53,9%) of 43 tumor cases, there was no statistically significant difference ($p=0,673$). So, there was also no relation between RYBP expressions and colorectal cancer incidence. As our study was restricted to a small number of samples, it could be a reason for what we got in our result. On the other hand, transcription factors located on the RYBP' promoters represent one important way to regulate RYBP levels as evidenced in the hepatocellular cancer that alteration of KLF4 binding site functions as repressor and SP1 binding site functions as activator may be accountable for the discrepancy of RYBP levels in the pathological conditions (Zhao et al., 2017). Moreover, recent investigations have demonstrated that existing a number of microRNAs binding site in the 3' UTR of both dRYBP and RYBP also represent another way to control RYBP levels. However, the role of microRNAs in the down-regulation of RYBP have been experimentally proved in the melanoma miR-29, miR-1, miR-206 by (Zhou et al., 2012). So, studying the transcription factors located in the promoter of the gene and microRNA, which plays a role in RYBP expression levels in colorectal cancer type in the future studies, could be a powerful tool to detect the reasons of alteration of RYBP in CRC. Moreover, undefined isoform result of the most important mechanism (alternative splicing) that all genes undergo during the expression could be another possible reason.

In addition, the relation between the RYBP expression and clinico pathological feature has analyzed by performing Chi-square test. None of the pathological features were had relation with RYBP expression. Our result regarding to age, gender, stage, metastasis, lymph node was agreement with hepatocellular carcinoma (Wang et al., 2014; Zhu., 2017), non-small cell lung cancer (Voruganti., 2015) except that in the study done by Zhu, that they have significant difference with metastases and by Voruganti who have relation with gender and smoking and that was contrast of us.

In contrast to our result, previous studies found that alteration levels of RYBP have switched between up-and under regulation were a cause of disease in the various type of cancer. Down-regulation levels of RYBP have observed by many groups related to the improvement of a number of different type of cancers, inclusive hepatocellular carcinoma (Wang et al., 2014; Zhao et al., 2017; Zhu et al., 2017), in lung cancer (Voruganti et al., 2015; Dinglin et al., 2017), in breast cancer (Buas et al., 2015; Zhou et al., 2016) and glioblastoma (Li et al., 2013). Because RYBP placed on the chromosome 3p, integrative genomic profile had been conducted and showed that the down regulation of this gene was result of the recurrent deletion of 3P arm in cervical cancer (Lando et al.,2009; Lando et al., 2013), in ileal carcinoid (Adresson, 2009) and prostate cancer (Taylor et al., 2010; Krohn et al., 2013). Moreover, the poor prognosis has been associated with cancer patients including loss of 3P than those do not have. Some of the searches mentioned above used in addition to the patient's sample both cell lines taken from the same tumor and non –tumor tissue samples and mouse xenograft to study what are the effects of decreasing or increasing the levels of RYBP in cancer type and with what were associated. They have proven the inverse relation between the decreased levels of RYBP with survival rate (shorter survival rate), diseases severity (increase the seriousness of disease) and were associated with poor prognosis in cervical cancer (Lando et al.,2009); hepatocellular (Wang et al.,2014; Zhao et al.,2017; Zhu et al 2017), lung cancer (Voruganti et al., 2015; Dinglin et al., 2017), breast cancer (kenny et al.,2017) as well as glioblastoma (Li et al.,2013). However, the increasing levels of RYBP have proven to be harmful to cancer cells and increased survival rate by either inducing apoptosis process which inhibits tumor growth and progression as reported in ileal carcinoids (Andresson et al., 2009), hepatocellular cancer (Wang et al., 2014) and

NSCLC (Voruganti et al., 2015) or via decreasing cell proliferation as reported in lung cancer (Voruganti., 2015; Dinglin et al., 2017) and glioblastoma (Li et al., 2013). Moreover, increasing levels of RYBP in tumor cells was associated with increasing the sensitivity of tumor cell to the chemotherapy. In agreement with the effective role of increasing RYBP expression dosage whether endogenously or ectopically in reducing DNA repair activity in tumor cells, (Ali et al., 2018) also have confirmed the defensive capacity of RYBP by impeding the proteins of homologous recombination (HR) repair pathway and stimulate the cancer cells to DNA damage.

In contrast to what detected in studies related to the beneficial role of increasing expression of RYBP in tumor cells to inhibit it from prognosis, other groups have investigated the detrimental side of increasing RYBP expression levels in classical Hodgkin' lymphoma and T-cell lymphoma via analyzing a comprehensive data of human cancer including both tumor and its counterpart (non-tumor) to investigate the role of PcG proteins. Further, the positive expression of RYBP in leukemia and lymphoma was correlated with more aggressiveness and poor prognosis. All that indicates that RYBP can act as a tumor suppressor gene or maybe as an oncogene depending on the tumor type. However, further studies have to be under investigation in the future direction to help to elucidate the alteration of RYBP expression levels in CRC (Sanchez-Beat et al.,2004;Saski et al.,2011; Seitzet al.,2011).

The product of the MDM2 gene has been studied since 1987 and shown to be one of the more oncogenic factors which are responsible for cell' transformation when overexpressed in different types of cancers reviewed in (Momand et al.,1998). In addition to its role in prognostic factors and implication for chemotherapy (Rayburn et al., 2005). Within the frame of colorectal cancer type and due to the capability of MDM2 to bind and inactive the function of P53 (guardian of genome), this tow couple of gene has been studied in different levels of gene including, MDM2 proteins levels (Hao et al., 1998; Abdel-Fattah et al., 1999), amplification of MDM2 gene (Forslund et al.,2008; Sugano et al.,2010) and although MDM2 mRNA expression was examined simultaneously with P53and p14ARF as an important regulatory pathway (p53/MDM2/p14ARF) of apoptosis in primary CRC (Kondo et al., 2008). To our

knowledge the MDM2 mRNA expression in the patients with colorectal cancer in solo case has not been tested yet (without comparing with any partner). In our result MDM2 mRNA levels were shown to be overexpressed in 21 of 43 (48.84%) of the patients with CRC and low expressed in 22 of 43 (51.16 %) in the same group of the patients. Statistically, there was no significant difference between MDM2 gene expression in tumor and normal tissue in the patients with colorectal cancer, hence no relation between expression of MDM2 gene and colorectal cancer ($p= 0.721$). None of the clinical pathologic features, which were examined in our study including age, gender, invasion, lymph node, metastasis, smoking and chronic disease were had an association with MDM2 expression. Our result related to the correlation of MDM2 with colorectal cancer was agreement with the MDM2 proteins levels study done by (Hao et al., 1998) and in contrast to those had relation with CRC such of protein levels (Abdel-Fattah et al., 1999), MDM2 amplification with CRC (Forslund et al., 2008; Sugano et al., 2010), and MDM2 mRNA expression levels (Kondo et al., 2008). Regarding to the stages and metastasis also was in the line with MDM2 protein levels (Hao et al., 1998; Abdel-Fattah et al., 1999), and contrast to MDM2 mRNA expression in CRC (Kondo et al., 2008) and MDM2 amplification in CRC (Forslund et al., 2008; Sugano et al., 2010) with the most significant point of the difference which should be to shed light that decreased levels of MDM2 was linked to advanced stage in study done by (Kondo et al., 2008; Sugano et al., 2010), while the result relating to the age, gender, tissue type and lymph node was in the consistent with MDM2 amplification (Forslund, 2008; Sugano et al., 2010).

We can give some explanation for what we didn't have relation might be due to the heterogeneity feature which colorectal tumors characterized by (Smith et al., 2002; Samowitz et al., 2007). Also, it could be due to a small number of studied samples. Or because we didn't study our experiments on the different subtype of colorectal cancer (Oliner et al., 1992). Moreover, because of the origin of which the tissues are taken, the type of stresses of which these tissues are exposed and vulnerable, in addition, some types of tissue vary according to the pattern of gene expression (Onel and Cordon-Cardo, 2004). Also, the result may be ascribed to the undefined isoform which result of the most important mechanism (alternative splicing) that all genes undergo during the

expression as reported by (Sigalas., 1996; Matsumoto., 1998; Kraus et al., 1999; Lukas., 2001). Regardless of the cancer type, our result is consistent with the finding in cervical cancer (Kessis et al., 1993), ovarian cancer (Hashiguchi et al., 2001), soft tissue sarcoma (MFH) (Reid et al., 1996), soft tissue sarcoma Rhabdomyosarcoma (Leuschner et al., 2003), head and neck (Millon et al., 2001) and thyroid cancer(zou., 1997).

However, in contrast to our result, MDM2 gene has been shown to abnormally up-regulated in more than 40 type of cancers and tumor cell lines and associated with poor prognosis and short survival rate (Rayburn et al., 2005) by different mechanisms including amplification with the highest frequency of occurrence in of soft tissue sarcoma 20% (Oliner et al., 1992) and its different histological subtypes (Forus et al., 1993; Ladenyi et al., 1993; Leach et al 1993; Florenes et al., 1994; Nakayama et al ., 1995; Patterson et al., 1997). In addition, to human malignant glioma (8-10%) (Reifenberger et al.,1993); in lung cancer (Marchetti et al et al., 1995). Also, overexpression of MDM2 was observed in the absence of amplification as reported by (M. Saeed Sheikh et al., 1993; Bueso-ramos et al., 1996) in breast cancer and bladder cancer (Lianse et al., 1994), melanoma (Polsky et al., 2001) and in leukemia (Ridge et al., 1993; Bueso-Ramo et al.,1994; Watanabe et al., 1994; Quesnel et al., 1994).

Moreover, overexpression was carried out by enhanced translocation in mouse tumor cells (Berberich et al., 1994), enhanced translation in choriocarcinoma cell line (Landers et al., 1997) Also, over expression of MDM2 gene was observed independent of P53 function via MDM2 alternatively splicing variance mechanism (AS) in urothelial ovarian and bladder (Sigalas et al. 1996), human astrocytes neoplasm (Matsumoto et al. 1998) and glioma (Kraous et al., 1999).

unexpected, although over expression of MDM2 mRNA was associated with poor prognosis in the type of cancer mentioned above, it has been investigated to be as an independent prognostic factor for survival rate. In other words, over expression of MDM2 mRNA levels in some patients represented as indicator for early diagnosis, therapeutic and then increasing median survival rate more than those patients who had MDM2 low expression as reported in ovarian cancer (Tanner et al., 1997), NSCLC cancer (Heng et al., 2000), and Soft Tissue Sarcoma (Tuberet et al., 2000).

CHAPTER 6

CONCLUSION

In this study we have sought to investigate the relation of MDM2 and RYBP gene expression levels in tissues taken from patients with colorectal cancer, association of gene expression with parameters including (age, gender, smoking, metastasis, lymph node, stages and other disease) and if there is correlation between these two gene during the expression. We have got the following results. No difference between the MDM2 and RYBP mRNA expression in normal and tumor tissues. None of the parameters recruited in this study was had relation neither with MDM2 nor with RYBP. No correlation between the MDM2 and RYBP expression. Taken together, these data suggest that despite the fact that MDM2 plays as an oncogene and that RYBP plays as tumor suppressor gene and maybe as oncogene depending on the tumor type, but both genes have no role our patient's outcome.

Future direction:

Working on documentation of data on CRC at all levels including different groups such as group with different histological subtype of the colorectal cancer, group with different cancer development stages and another types of group.

Further research should be pursued to identify the factors involve in the mechanism responsible for gene expression in colorectal cancer in different levels of colorectal cancer for both genes.

As RYBP is a member of PcG which is known to involve the gene expression and hence maintain chromatin in the (off or on) so it is important to perform experiment including the other proteins in this family to determine, which can bind to and regulate the levels RYBP positively or negatively.

Undergo the specimens for sequencing to determine which isoform we have in our experiments.



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