بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Immunohistochemical Expression of Kras, Ck20, P53 and Bcl-2 Tumor Markers and Molecular Detection of Kras Gene Mutation in Colorectal Tumors among Sudanese Patients

الأفراز المناعى النسيجي الكيميائي لواسمات الاورام Kras، Ck20، P53 وBcl-2 والكشف الجزيئي عن الطفرة لجين Kras في اورام القولون والمستقيم بين المرضى السودانيين

A thesis Submitted in Fulfillment of the Degree of PhD in Medical Laboratory Science (Histopathology and Cytology)

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2017

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قَالَ تَعَالَىٰ:

﴿ ٱقۡرَأۡ بِٱسۡمِ رَبِّكَ ٱلَّذِي خَلَقَ ۝١ خَلَقَ ٱلۡإِنسَٰنَ مِنۡ عَلَقٍ ۝٢ ٱقۡرَأۡ وَرَبُّكَ ٱلۡأَكۡرَمُ ۝٣ ٱلَّذِي عَلَّمَ بِٱلۡقَلَمِ ۝٤ عَلَّمَ ٱلۡإِنسَٰنَ مَا لَمۡ يَعۡلَمۡ ۝٥ ﴾

صدق الله العظيم

العلق: ١ ــ ٥

Dedication

To my parents.

To my brothers.

To my wife and children.

To my teachers.

To my friends.

Acknowledgment

First of all, I would like to express my deepest gratitude and thanks to Allah, whose help was the main factor in the accomplishment this work.

I can't find the letters or the words that can express my sincere gratitude, to my supervisor **Prof. Ahmed Abdelbadia,** who kindly offered his fruitful guidance, constructive criticism generous help, effective impetus and endless support through this work. The accomplishment of this study could have never reached its destination without his indispensable supervision. I hope I could express my greatest thanks to the Co-Supervisors Dr. **Mai Abdul Rahman Masri and Dr. Mohammed Siddig Abd El Aziz,** who kindly offered me too much of their efforts, precious time, sincere advice, rays of light that shines darkness around me and guidance for the fulfillment of this work. Surely I can't find the words to express my true feelings towards them. I thank them for their sincere help, guidance and remarkable experience.

My thanks to the all staff in the research laboratory, college of laboratory Science, (Sudan University of Science and Technology) and center of excellence, department of zoology, faculty of science, university of Khartoum, for their unlimited help, also thanks to any person helped me in this research.

My thanks are also extended to Dr. Abu-Elgasim Abass, Dr. Talal Ali Alqalah, Dr. Abbas Elsafi, Mr. Salah Eldeen Jumaa, Miss. Fatima Majzoob, Miss. Nahla Gafer, Miss. Rania Ahmed and Eng. Ali Mahdi Al-Omari, for their support to complete this research.

Finally, to my caring and loving wife, whose support encouraged me during the rough time. I am especially indebted to my country (Republic of

Yemen) for the support they have afforded me, to finish my study and complete my dissertation.

Abstract

This is a retrospective, analytical, case control study which was conducted at Ibn Sina hospital and Soba teaching hospital in Khartoum state during the period from April 2014 to December 2016. The study was aimed to detect the immunohistochemical expression of Kras, Ck20, P53 and Bcl-2 tumor markers and molecular detection of kras gene mutation in colorectal tumors among Sudanese patients using immunohistochemistry and PCR-RFLP.

One hundred and fifty blocks previously diagnosed as colorectal tumors were selected for this study. Included; one hundred malignant colorectal tumor blocks and fifty benign colorectal tumor blocks.

The distribution of gender in the study population revealed that 44(44%) were males and 56(56%) were females among malignant tumor. While 35(70%) were males and 15(30%) were females among the benign tumor. Distribution of age group were found as follow, less than 50 years were 41(41%) and 59(59%) were more than 50years among the malignant tumor, while 29(58%) were less than 50 years and 21(42%) were older than 50 years in the benign c tumor. According to the site of tumor the study found 59(59%) of malignant tumor were in the rectum, while 36(72%) of benign tumor were in the colon, with significant relation between the characteristics data (gender, age and site of tumor) and histopathological diagnosis ($P < 0.05$).

Most of malignant tumors were moderately differentiated and well differentiated 92(92%) and only 8(8%) were poorly differentiated tumors. Positive immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 had a significant relation ($P= 0.000$), with histopathological diagnosis represented 61%, 52%, 31% and 58% in malignant tumors respectively. While, in benign

tumors expression was noticed in two markers only with lower percentages (2% in Bcl-2 and 22% in Ck20).

The present study found insignificant relation between immunohistochemical expression of p53, Kras, Bcl-2 and Ck20 and cancer grade ($p > 0.05$).

The molecular detection of Kras gene mutation gave 26% of malignant tumors showed the mutant genotype of codon12 and 9% had the mutant codon13 genotype, while none of the benign tumors showed mutant genotype of both codons, with significant relation between Kras gene mutations and histopathological diagnosis ($P=0.000$). In contrast the study found insignificant relation between Kras gene mutations and cancer grades ($P > 0.05$).

The immunohistochemical expression of Kras was positive in 69.2% of codon 12 mutant samples and 55.6% of codon 13 mutant samples. However, the relation between Kras expression and mutant samples was significant with codon 12 (p. value 0.000) and insignificant with codon 13 ($P= 0.174$). The immunohistochemical expression of P53 and Ck20 was significantly related to codon 12. On the other hand, Bcl-2 was significantly related to codon 13 mutant samples ($P <0.05$).

The study concluded that there is a significant relation between immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 as well as Kras gene mutations (codon 12 and 13) with histopathological diagnosis of colorectal tumors and insignificant with cancer grade.

المستخلص

اجريت هذه الدراسة التراجعية التحليلية الحالة والحالة الضابطة في مستشفى أبن سينا ومستشفى سوبا الجامعي في ولاية الخرطوم في الفترة من أبريل 2014 الي ديسمبر2015. هدفت الدراسة الي الكشف عن الأفراز المناعي النسيجي الكيميائي لواسمات الاورام Kras، Ck20، P53 وBcl-2 والكشف الجزيئي عن الطفرة لجين Kras في أورام القولون والمستقيم بين المرضى السودانيين، أستخدمت تقنيات الكشف المناعي الكيميائي النسيجي وتفاعل البلمرة المتسلسل (PCR-RFLP).

تم جمع مائة وخمسون قالب نسيجي لعينات شخصت مسبقا بأورام القولون والمستقيم. مائة عينة كانت مشخصة بسرطان القولون والمستقيم وخمسون عينة كانت مشخصة بأورام حميدة للقولون والمستقيم

أظهرت الدراسة أن توزيع الجنس في الأورام الخبيثة للقولون والمستقيم كانت في النساء 56(56%) وفي الرجال44 (44%) بينما كانت النسبة للرجال 35 (70%) والنساء15 (30%) في الأورام الحميدة. وجدت الدراسة ان الأشخاص الذين تجاوزت اعمارهم 50 سنة في الأورام الخبيثة هم الاكثر بنسبة 59(59%) وكانت أقل ظهوراً في الاشخاص الذين اعمارهم تقل عن 50 سنة بنسبة 41(41%). بينما في الأورام الحميدة ظهرت أكبر في الاشخاص الذين تقل أعمارهم عن 50 سنه بنسبة 29(58 %) وكانت اقل في الاشخاص الذين أعمارهم أكثر من 50سنه بنسبة21(42%).أما من حيث توزيع وجود الورم كانت في الأورام الخبيثة أكثر في المستقيم بنسبة59 (59%) بينما في الأورام الحميدة أكثر في القولون بنسبة36(72%)، وجدت الدراسة علاقة ذات دلالة أحصائية بين الجنس والعمر ومكان الورم مع تشخيص الورم ($P < 0.05$).

معظم خلايا الأورام الخبيثة كانت متوسطة وجيدة التمايز 92%، بينما 8% منها كانت ضعيفة التمايز.

وجدت الدراسة علاقة ذات دلالة أحصائية بين التعبير المناعي للواسمات P53 وKras وBcl-2 وCK20 مع اورام القولون والمستقيم ($P=0.000$) بنسبة 61% و52% و31% و58% في الاورام الخبيثة على التوالي.

وجدت الدراسة انه لا توجد علاقة ذات دلالة أالحصائية بين التعبير المناعي للواسمات P53 وKras وBcl-2 وCk20 ودرجة تمايز السرطان ($P > 0.05$).

اظهر الكشف الجزيئي لطفرة لـ Kras بنسبة 26% في الكودون 12 هي الاكثر في سرطان القولون والمستقيم بينما في الكودون 13 بنسبة 9%، علي عكس الاورام الحميدة حيث لم تظهر الطفرات الجينيه فيها مع وجود علاقة ذات دلالة أحصائية بين طفرة جين الـ Kras والتشخيص النسيجي ($P < 0.05$).

واظهرت الدراسة انه لاتوجد علاقة ذات دلالة أحصائية بين الطفرات الجينيه لـ Kras ودرجة تمايز السرطان ($P <0.05$).

كان التعبير المناعي لـ Kras إيجابيا في 69.2% من عينات طفرة كودون 12 و (55.6%) من عينات طفرة كودون 13. وجدت الدراسة علاقة ذات دلالة أحصائية بين التعبيرالمناعي لـ Kras وكودون 12 ($P= 0.000$). وكان التعبير المناعى لواسمات p53 و Ck20 مرتبطا مع طفرة كودون 12. و Bcl-2 مرتبطا مع طفرة كودون13 ($P <0.05$) .

خلصت الدراسة الى وجود علاقة ذات دلالة أحصائية بين التعبير المناعي للواسمات P53 و Kras و Bcl-2 و Ck20 فضلا عن الطفرات الجينية لـ Kras (كودون 12,13) مع التشخيص النسيجي في أورام القولون والمستقيم ولاتوجد علاقة ذات دلالة أحصائية مع درجة تمايز السرطان.

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Abbreviation	Full name
APC	Adenomatous polyposis coli
Bcl-2	B-cell lymphoma -2
CEA	Carcinoembryonic antigen
CIN	Chromosomal in stability
CK20	Cytokeratin 20
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CT	Computed tomography
DAB	Di amino benzidinetetrahydrochloride
DATP	Deoxyadenosine triphosphate
DCTP	Deoxycytidine triphosphate
DGTP	Deoxy guanosine triphosphate
DNA	Deoxyribo nucleic acid
DPX	Di styrene plasticizer xylene
DTTP	Deoxythymidine triphosphate
EGFR	Epidermal growth factor receptor
FAP	Familial adenomatous polyposis
FOBT	Faecal occult blood tests
GDP	Guanosine diphosphate
GTP	Guanosine triphosphates
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene
LOH	Loss of heterozygosity

MEK	Methyl ethyl ketone
MRI	Magnetic resonance imaging
NSAIDs	Non-steroidal anti-inflammatory drugs
P53	Protein 53
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PSC	Primary sclerosing cholangitis
RFLP	Restriction fragment length polymorphism
TBE	Tries buffer EDTA
TNM	Tumor-lymph node-metastasis
TP53	Tumor protein 53

CHAPTER ONE
INTRODUCTION

Colorectal cancer (CRC) is defined as the cancerous growths in the colon, rectum and appendix. It is also referred to as colon cancer or large bowel cancer (Center *et al*., 2009). Classically there are two types of CRC, sporadic and familial (hereditary) cases with a percentage of familial CRC of 20–25% (Dela *et al*., 2004). Colorectal cancer (CRC) has high incidence and mortality worldwide. In 2012, CRC was the second most prevalent cancer among males (9%) and the third among females (8%) (Siegel *et al*., 2012). Colorectal cancer is the third most commonly diagnosed cancer in the world; there are 1.23 million clinically diagnosed new cases of colorectal cancer and 608,000 people suffered from the disease worldwide. It is the second most common cause of cancer in women and the third most common in men with it being the fourth most common cause of cancer death after lung, stomach, and liver cancer (Ferlay *et al*., 2010).

The broad ethnic and climatic diversity of Sudan makes it in many ways a microcosm of Africa (Hamad, 2006). Colorectal cancer was one of the commonly diagnosed cancers in Sudan during 2009-2010 (Saeed *et al*., 2014). The disease was the third leading cause of death, the prevalence of cancer cases has dramatically increased in Sudan in recent years and cancer is ranked as the major cause of death in most instances (Ahmed *et al*., 2014). Although the exact reason behind this increase is not known, it partially could be attributed to exposures to common and local carcinogens and the change in lifestyles (Elamin *et al*., 2015).

The 5-year survival rate of colon cancer has risen as the integrated treatment with surgery, radiotherapy and chemotherapy has been applied for

patients. However, the primary cause of death in the patients after treatment is metastasis, which was thought to be affected by multiple factors (Plesec, 2009; Fulton, 2009).

Developing countries have lower rates, particularly Africa and Asia. In 2006, about 21% of the world population was covered by population-based cancer registries, with sparse registration in Asia (8% of the total population) and in Africa (11%) (Ferlay, *et al*., 2010).

Kirsten rat sarcoma **(**KRAS**)** is a proto-oncogene located at 12p12.1 that encodes a 21-kDa GTP-binding protein. Kras is frequently mutated during the very early stages of colorectal cancer development (35% - 42%) of colorectal cancers and advanced adenomas present mutations on this proto-oncogene (Leslie, *et al*. 2002). When it is bound to GTP, the ras protein is active. This protein is involved in many different processes. It activates a large number of transduction signal pathways, among them the mitogen-activated protein kinases pathway. It has been demonstrated that mutant Kras promotes hyperplastic growth in the colonic epithelium (signaling through MEK) (Haigis *et al*., 2008).

The most challengers in oncology are that of patient selection for therapy with molecularly targeted agents. The importance of the Kras mutation status as a discriminating biomarker used in treatment selection is becoming increasingly recognized (Karapetis *et al*., 2006). Kras is reported as an important determinant of response or resistance to anti EGFR antibodies (Shuangshoti, 2011). Carcinogenesis and development of colorectal cancer involve changes of several genes such as Kras and P53. The accumulative effects of such genes may play a more important role during carcinogenesis than the order of change in these genes (Wang *et al*., 1998). The rate of mutations in Kras gene (predominantly in codon 12) was 46.4% with G>A

transitions and the G>T transversion. Both mutations are most commonly observed and can be used in molecular diagnosis of the colorectal cancer (Dobre *et al*., 2013).

IHC may compliment PCR in the detection of Kras mutation (Elsabah and Adel, 2013). The Kras mutations revealed high mutation frequency (67%) in rectal cancers from females and thus implicates that this subset is the least likely to respond to anti EGFR therapies. Although concurrent Kras mutations remain as rare findings, it was suggested that repeated Kras targeting may occur during colorectal cancer progression (Jonsson *et al*., 2009).

P53 is a tumor suppressor gene on the short arm of chromosome 17 that encodes a protein that is important in the regulation of cell division. Although the full role of P53 in the normal and neoplastic cell is unknown there is evidence that the gene product is important in preventing the division of cell containing damaged DNA (Finkelstein *et al*., 1996).

Bcl-2 proto-oncogene is an inhibitor of apoptosis and may therefore permit the accumulation of genetic alteration that influence cell division and potentially contribute to tumor development, the Bcl-2 gene is located at chromosome 18q21 and its product is a 24 KD protein localized to the nuclear envelope endoplasmic reticulum and outer mitochondrial membranes (Zavrides *et al*., 2005). Bcl-2 may play an important role in the early stage of adenoma-carcinoma sequence (Qasim *et al*., 2012).

The expression of p53 was correlated with various clinical features of patients with colorectal cancer. The combined use of these histopathological markers appeared to be a promising tool in predicting the prognosis of patients with this type of cancer (He *et al*., 2010). Patient stratification by combined P53/Bcl-2 phenotype provides stage independent prognostic information in colorectal cancer (Watson *et al*., 2005). The immunohistochemical reactivity

of Bcl-2 and p53 together was suggested as a better independent prognostic indicator than the status of either biomarker alone. Thus, the determination of both Bcl-2 and p53 status, using appropriate techniques increases the ability at the time of biopsy to identify those patients with aggressive subtypes of colorectal adenocarcinoma (Manne *et al*., 1997). It might be possible, however, that various tumor related factors may profoundly affect the impact of P53 and Bcl-2 immunostaining patterns on prognosis and that more information about these factors is needed to identify reliably relevant subtypes of colorectal cancer (Tollenaar *et al*., 1998). The P53 over expression was associated with non-mucinous colorectal carcinomas, the histological grade of colorectal adenocarcinomas and high proliferative activity (Georgescu *et al*., 2007).

This immunohistochemical expression of Ck20 marker is suitable for the localization and therapy checks. The levels of Ck20 reflect the success of surgery, radiotherapy and chemotherapy on the patients (Warren and Shields, 1997). Ck20 expression is restricted to a few organ systems. Almost all cases of colon carcinoma (95-100%) are found positive for Ck20 (Barrett *et al*., 2000). Cytokeratin 20 (Ck20) is a described polypeptide with molecular weight of 48.5 kDa and an isoelectric point at pH 5.66. This protein is encoded by a gene located on chromosome 17q21.2 (Schweizer *et al*., 2006; Bragulla and Homberger, 2009).

1.2. Rationale

The association between gene mutation, expression of proteins and colorectal tumors can pave the way towards new ways of early diagnosis, prevention, treatment and follow up. This study used advanced technology to detect Kras gene mutation by molecular techniques and immunohistochemical expression of Kras, Ck20, P53 and Bcl-2. Many studies were conducted to detect Kras, Ck20, P53 and Bcl-2 expression and kras gene mutation worldwide, but no such studies were carried out in Sudan according to my knowledge. This is the first study in Sudan addressing this topic, hoping to add to the needed knowledge for better assessment of the genetic bases associated with colorectal cancer in Sudanese. Findings can be used also to monitor patients with colorectal cancer and follow up of the disease in Sudan. Thus, current study is an attempt to set genetic baseline for a group of data concerning evaluation and prevention with colorectal cancer in Sudan.

1.3. Objectives

1.3.1. General objective:

To detect the immunohistochemical expression of Kras, Ck20, P53 and Bcl-2 tumor markers and molecular detection of kras gene mutation in colorectal tumors among Sudanese patients

1.3.2. Specific objectives:

- To detect immunohistochemical expression of Kras, Ck20, p53 and Bcl-2 tumor markers in colorectal tumors.
- To detect Kras gene mutations (codon12 and codon13) in colorectal tumors using PCR-RFLP techniques.
- To correlate immunohistochemical expression of Kras, Ck20, p53 and Bcl-2 tumor markers with histopathological diagnosis and cancer grades.
- To correlate immunohistochemical expression of Kras, Ck20, p53 and Bcl-2 tumor markers with Kras gene mutations (codon12 and codon13).
- To correlate the characteristics data of patients with colorectal tumors.

CHAPTER TWO
LITERATURE REVIEW

2.1 Anatomy, physiology and histology overview

2.1.1 The large intestine:

The large intestine which is about 1.5m (5ft) long and 6.5cm (2.5inch.) in diameter in living humans and cadavers, extends from the ileum to the anus. It is attached to the posterior abdominal wall by its mesocolon, which is a double layer of peritoneum. Structurally, the four major regions of the large intestine are the cecum, colon, rectum, and anal canal (Tortora and Derrickson, 2014).

2.1.2 The cecum

The large intestine begins in a blind pouch called the cecum. Attached to the lower end of the cecum is a tubular organ called the appendix (Thompson, 2015).

2.1.3 The colon

The colon has layered bowel wall with a mucosa (epithelium, lamina propria, muscular is mucosae), submucosa, muscular is propria, and serosa or adventitia. Because the function of the colon is to absorb water and electrolytes its, mucosal architecture is different from that of the small bowel. The colon epithelium does not have villi; it has straight invaginations called crypts, which line up like “test tubes in a rack”. The crypt epithelium mostly consists of goblet cells, each with a single large mucin vacuole, but a few absorptive cells also are present. The submucosa of the colon is composed of loose connective tissue and contains large-caliber vessels as well as

Meissner's nerve plexus of parasympathetic ganglion cells and sympathetic neurons (Reinus and Simon, 2014).

The ascending colon extends up toward the liver. The colon makes a sharp turn at the right colic (hepatic) flexure. The transverse colon passes below the liver, stomach, and spleen. The colon turns sharply downward at the left colic (splenic) flexure. The descending colon extends downward along the left side of the abdominal cavity. The sigmoid colon forms an S shape down to the rectum (Thompson, 2015).

2.1.4 The rectum:

The rectum is about 15 cm (6 inch.) in length and lies anterior to the sacrum and coccyx. The terminal 2–3 cm (1 inch.) of the large intestine is called the anal canal. The mucous membrane of the anal canal is arranged in longitudinal folds called anal columns that contain a network of arteries and veins. The opening of the anal canal to the exterior, called the anus, is guarded by an internal anal sphincter of smooth muscle (involuntary) and an external anal sphincter of skeletal muscle (voluntary). Normally these sphincters keep the anus closed except during the elimination of feces (Tortora and Derrickson. 2014).

2.2 Colorectal tumors

2.2.1 Benign colorectal tumors:

The tumor typically begins as noncancerous polyps. A polyp is the growth of tissue that develops on the lining of the colon or rectum that can become cancerous (Levine and Ahnen, 2006).

There are three major types of colorectal adenomas: tubular, villous, and tubule villous (Cash, 2012). In adenomatous polyp the nuclei are usually hyperchromatic, enlarged, cigar shaped and crowded together in palisade pattern. The hyperplastic polyps contain an increased number of glandular

cells with decreased cytoplasmic mucus, but lack nuclear hyperchromatic, stratification, or atypia (Cappell, 2008).

Colonic polyps are of two main types; non neoplastic polyps and adenomatous polyps. Non neoplastic polyps are benign lesions with no malignant potential. Hyperplastic (metaplastic) polyps are the most common (90%) non neoplastic polyps; generally, remain small and asymptomatic. No specific therapy required, but they can be difficult to distinguish from neoplastic polyps and so are commonly removed. Juvenile polyps (typically in children younger than 10 years) are highly vascular and common, so they should be removed. Adenomatous polyp's benign lesions, but have significant malignant potential; precursors of adenocarcinoma. Can be one of three types of adenoma; tubular (most common; up to 60% to 80% of cases) smallest risk of malignancy. Tubulovillous-intermediate risk of malignancy and villous-greatest risk of malignancy (Agabegi and Agabegi, 2016).

2.2.2 Colorectal cancer (CRC):

Colorectal cancer (CRC) incidence and mortality rates vary markedly around the world. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females, with 1.4 million new cases and almost 694,000 deaths estimated to have occurred in 2012. The highest incidence rates are in Australia and New Zealand, Europe, and North America, and the lowest rates are found in Africa and South-Central Asia (Ahnen and Macrae, 2010).

Colorectal cancers are almost all adenocarcinomas, which tend to form bulky exophytic masses or annular constricting lesions. The majority of colorectal cancers are thought to arise from malignant transformation of an adenomatous polyp (tubular, tubule villous, or villous adenoma) or serrated polyp (hyperplastic polyp, traditional serrated adenoma, or sessile serrated

adenoma) (Papadakis *et al*., 2016). CRC is histologically divided into several types, suggested by World Health Organization, with adenocarcinoma, mucinous adenocarcinoma and signet ring cell cancer being the most common subtypes in decreasing order (Ghanipour, 2014).

Colorectal cancer starts when the process of the normal replacement of lining cells goes away. Mistakes in mucosal cell division occur frequently. When this occurs, these cells begin to divide independently of the normal checks and balances that control growth. As these abnormal cells grow and divide, they can lead to growths within the colon called polyps. Polyps vary in type, but many are precancerous tumors that grow slowly over the course of years and do not spread. As polyps grow, additional genetic mutations further destabilize the cells and can make the cells more bizarre. When these precancerous tumors change direction (growing through the tube rather than into the middle of it) and invade other layers of the large intestine (such as the submucosa or muscular layer), the precancerous polyp has become cancerous. In most cases this process is slow, taking at least 8 to 10 years to develop from those early aberrant cells to a frank cancer (Herbs, 2016).

2.2.2.1 Pathogenesis of colorectal cancer:

Colorectal cancer development results from the accumulation of multiple genetic mutations arising from two major pathways: chromosomal instability and microsatellite instability. In chromosomal instability, mutations or deletions of portions of chromosomes arise, with loss of heterozygosity (LOH) and inactivation of specific tumor suppressor genes. In LOH, one allele of a gene is deleted but gene inactivation only occurs when a subsequent unrelated mutation affects the other allele. While microsatellite instability, involves germline mutations in one of six genes encoding enzymes involved in repairing errors that occur normally during DNA replication

(DNA mismatch repair); replication errors accumulate and can be detected in ‘microsatellites’ of repetitive DNA sequences. They also occur in important regulatory genes, resulting in a genetically unstable phenotype and accumulation of multiple somatic mutations throughout the genome that eventually lead to cancer (Walker *et al*., 2014).

In most CRC patients, the progression of normal colonic mucosa to invasive cancer requires several molecular changes. The estimated time interval of the malignant transformation from normal mucosa through adenomatous polyp into an invasive carcinoma is about 5-10 years in most cases. This long time-interval provides the ground for early detection and even prevention of CRC as was shown by the national polyp study (Mazeh *et al*., 2013).

Most develop as a result of a stepwise progression from normal mucosa to adenoma to invasive cancer. The progression of colorectal cancers is controlled by the accumulation of abnormalities in a number of critical growth-regulating genes. These include APC mutation and loss, Kras mutation, Smad2/4 loss, and TP53 mutation and loss, and altered DNA methylation with progression to carcinoma. CDK8 has recently been found to regulate gene expression in the proliferation of colorectal cancer, and it also regulates the WNT/beta-catenin signaling pathway involved in many colon cancers. Microsatellite instability and chromosomal instability (CIN) are frequently detected in colon cancers. A third group, MACS (Microsatellite and Chromosomal Stable), is also recognized. CIN indicates loss of heterozygosity (LOH) in a number of cancer-related genes, though the underlying mechanisms are not well understood. About 15% of sporadic colorectal cancers show MSI and 50% exhibit LOH (Kumar and Clark, 2012).

2.2.2.2 Complicated disease:

Colorectal tumors may manifest with complications such as obstruction, perforation, or significant bleeding. These presentations are generally related to more advanced disease and may preclude a complete staging work-up or potential neoadjuvant therapy. Unless patients are unstable or critically ill or the tumor is unresectable, the tumor should be appropriately resected (Bope and Kellerman, 2016).

2.2.2.3 Metastatic disease:

Surgical therapy is also available for metastatic disease in certain situations. Metastatic liver lesions amenable to resection can be addressed at the time of colon resection or after the patient has healed from colectomy (Bope and Kellerman, 2016).

2.2.2.4 Prognosis:

Among gastrointestinal cancers, colorectal cancer has the best overall prognosis. For non-metastatic disease, the 5-year survival rate ranges from 50% to 95%, depending on the extent of lymph node involvement. For metastatic disease, newer therapies, given in succession, can achieve a median overall survival time of more than 2 years. The key remains early detection by screening, which can improve population-level outcomes (Ivor, 2016).

2.2.2.5 Grade of colorectal cancer:

The tumor cells and the tumor tissue showed be looked under a microscope. It is an indicator of how quickly a tumor is likely to grow and spread. If the cells of the tumor and the organization of the tumor's tissue are close to those of normal cells and tissue, the tumor is called "well-differentiated." These tumors tend to grow and spread at a slower rate than tumors that are "undifferentiated" or "poorly differentiated," which have abnormal-looking cells and may lack normal tissue structures. Based on these

and other differences in microscopic appearance, doctors assign a numerical "grade" to most cancers. The factors used to determine tumor grade can vary between different types of cancer (National Cancer Institute, 2013).

CRC is classified according to the tumor lymph node-metastasis (TNM) staging system, which is most widely used and last revised. There are several different tumor grading system based on architectural and/or cytological features, these describe the level of cell differentiation within the tumor, commonly through separation into four groups: well differentiated (grade 1); moderately differentiated (grade 2); poorly differentiated (grade 3); and, undifferentiated (grade 4). Although, this grading system has been questioned, as it has not reached widespread acceptance, however high tumor grade is a prognostic unfavorable pathological factor, as are lymphatic- and vascular invasion by the tumor, absence of tumor an infiltrating lymphocytic response and venous vessel invasion (Ghanipour, 2014).

2.2.2.6 Causes of colorectal cancer:

The underlying cause of all types of cancers is changes in certain genes in a cell that lead to uncontrollable growth. These genetic changes may be inherited or acquired during the course of a person's life, usually for unknown reasons. An uncommon form colorectal cancer called hereditary nonpolyposis colorectal cancer is due to inheriting faculty gene. Another condition called familial adenomatous polyposis (FAP) increase the risk of colorectal cancer. In FAP, the colon is lined with polyps will eventually become cancerous. In the most cases, the faulty is inherited from a parent, but is about a quarter of cases of FAP the faulty gene appears spontaneously (Wendy, 2008).

2.2.2.7 Risk factors of colorectal cancer:

A number of factors increase the risk of developing colorectal cancer. Recognition of these has impact on screening strategies. However, 75% of all

cases occur in people with unknown predisposing factors (Papadakis *et al*., 2016). The development of colorectal cancer is related to a number of factors, including age, diet, activity, environmental exposures, family history, and genetics (Bope and Kellerman, 2016).

2.2.2.7.1 Age:

An older age is the most important risk factor and is associated not only with a higher prevalence of polyps but also with multiple polyps, severe dysplasia, and larger adenoma size (Gyawali, 2012). The incidence of colorectal cancer rises sharply after age 45 years, and 90% of cases occur in persons over the age of 50 years (Papadakis *et al*., 2016). Ninety percent of colorectal cancers are diagnosed after the age of 50 years, and fewer than 5% of cases are diagnosed before the age of 40. The peak incidence of diagnosis is in the seventh decade of life (Bope and Kellerman, 2016).

2.2.2.7.2 A family history of colorectal cancer:

Can be obtained in 20% of patients who do not fulfill the criteria for HNPCC. In these families, when one or two first-degree relatives are affected. The risk is even higher if relatives were affected at an early age (Walker *et al*., 2014).

2.2.2.7.3 Personal history of adenoma or colon cancer:

The risk increases with the number of adenomas; from 2% to 6% of patients with colon cancer have synchronous colon cancer and 1.1% to 4.7% have metachronous colon cancer (Ficalora, 2013).

2.2.2.7.4 Dietary increase risk factors:

Red meat, high saturated fat and protein content, carcinogenic amines formed during cooking. Also high fecal bile acid and fatty acid levels, may affect colonic prostaglandin turnover (Walker *et al*., 2014).

2.2.2.7.5 Non-dietary risk factors for colorectal cancer:

These include medical conditions like colorectal adenomas, long-standing extensive ulcerative colitis or Crohn's colitis especially if associated with primary sclerosing cholangitis (PSC), ureter sigmoidostomy, acromegaly, and pelvic radiotherapy. Others risk factor like obesity and sedentary lifestyles may be related to diet, smoking, alcohol (weak association), cholecystectomy (effect of bile acids in right colon) and type 2 diabetes (hyperinsulinemia). While the use of aspirin or NSAIDs (COX-2 inhibition) and perhaps statins associated with reduced risk (Walker *et a*l., 2014).

2.2.2.7.6 Race:

African Americans have an increased risk of developing colon cancer than other races (Gyawali, 2012). The rate of colon cancer was also increased 4 to 20 times in Crohn disease or ileocolitis. A family history of colon cancer is a risk factor, and a personal history of female genital or breast cancer carries by two folds increased risk of colon cancer (Ficalora, 2013).

2.2.2.8 Clinical features of colorectal cancer:

Symptoms suggestive of colorectal cancer include changes in bowel habit with looser and more frequent stools, rectal bleeding, tensemus and symptoms of anemia. Looser and more frequent stools, with or without abdominal pain, are common symptoms of left-sided colonic lesions. Rectal and sigmoid cancers often bleed; blood being mixed in with the stool. Presentation with constipation with hard stools is not a risk factor for colon cancer. A rectal or abdominal mass may be palpable. Cancers arising in the caecum and right colon are often asymptomatic until they present as an iron deficiency anemia. Cancer may also present with intestinal obstruction (Kumar and Clark, 2012).

Early cases are essentially asymptomatic and are typically identified by screening. Advanced cases can manifest with bowel obstruction or perforation, frank rectal bleeding, weight loss, abdominal pain, and ascites due to hepatic or peritoneal metastases. Cancers associated with the mismatch repair pathway have certain typical features: They are right-sided, occur in younger patients, are more common in women, are poorly differentiated, and are locally advanced but without significant lymph node spread (Ivor, 2016).

2.2.2.9 Diagnosis:

Malignant potential can be determined by the size (the larger the polyp, the greater the malignant potential), histologic type, atypia of cells, shape sessile (flat, more likely to be malignant) versus pedunculated (on a stalk) (Agabegi and Agabegi, 2016).

The history, physical examination, and judicious use of both laboratory and radiologic tests are important in diagnosing CRC. Methods for diagnosing CRC are similar to those used to detect adenomatous polyps. Colonoscopy is more accurate than barium radiographic studies for the detection of colorectal neoplasms of all sizes and has the advantage of enabling the clinician to detect synchronous cancers and to obtain tissue for histologic analysis. Endoscopic ultrasound combines high-frequency ultrasonography with video endoscopy. It is superior to CT and allows an accurate determination of the degree of invasion and detection and sampling of enlarged lymph nodes. Endoscopic ultrasound is also highly sensitive for the detection of rectal cancer recurrence after local resection or low anterior resection. MRI using either end rectal or phased array coils can also provide accurate local staging of rectal cancer (Goldman and Schafer, 2016).

Double contrast barium enema can visualize the large bowel but is now superseded by CT colonography. Endoanal ultrasound and pelvic MRI are

used for staging rectal cancer. Chest, abdominal and pelvic CT scanning to evaluate tumor size, local spread and liver and lung metastases contributes to the tumor staging. PET scanning is useful for detecting occult metastases and for evaluation of suspicious lesions found on CT or MRI. Serum carcinoembryonic antigen (CEA) is of little use for primary diagnosis and should not be performed as a screening test. It is useful for follow-up; rising levels suggest recurrence (Kumar and Clark, 2012).

Faecal occult blood tests (FOBT) are used for mass population screening and are of value in hospital or general practice. Guaiac based feacal occult blood testing using rehydrated or no rehydrated stool specimens in people aged 50 to 80 decreases mortality from colorectal cancer. The fact that occult gastrointestinal bleeding is more common in benign conditions and the fact that bleeding is not universal in adenomas and early cancers, results in relatively low sensitivity and specificity of FOBT for CRC detection (Mazeh *et al*., 2013; Kumar and Clark, 2012).

The feaces for specific cancer-related DNA or RNA is appealing allowing DNA and RNA screening. However, technical complexity as well as the lack of specific CRC molecular markers prevented the development of a robust, commercially available, fecal DNA screening kit (Mazeh *et al*., 2013).

Immunohistochemistry (IHC), or immunocytochemistry, is a method for localizing specific antigens in tissues or cells based on antigen–antibody recognition; it seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level. Antibody molecules cannot be seen with the light microscope or even with the electron microscope unless they are labeled or flagged by some method that permits their visualization (Dabbs, 2013).

Immunohistochemistry (IHC) plays an important role in the differentiation of tumor types, assessing aggressiveness and metastasis origin recognition. Although molecular analysis is increasingly gaining more ground, many therapeutic protocols are still based on histological types and immunohistochemical phenotypes (Zlatian *et al*., 2014).

Polymerase chain reaction (PCR) has become the cornerstone of molecular diagnostic tools, including those developed for Kras mutation testing. PCR assays are highly sensitive and can be easily automated. PCR assays are thus well-suited for large-scale, high-throughput diagnostic testing. For Kras mutation testing, however, standard PCR assays are not sufficient. The main requirement for conclusive Kras genotyping by PCR assay is the ability to discriminate between different mutant alleles and wild type. There are two main challenges to achieving a conclusive result: one is the heterogeneity of the testing material, and the other is differences in the detection limits for distinct mutations. Depending on the tissue analyzed, the amount of tumor versus non-tumor area is variable and heterogeneous, resulting in a template mixture in which wild-type and mutant DNA are not present in equimolar amounts (Tollenaar *et al*., 1998).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis is a widely applied method to detect gene mutations, which allows distinguishing mutant-type and wild-type sequences via destructing or generating enzyme restriction sites through PCR and subsequent electrophoresis separation of differential fragments. Compared to other methods, PCR-RFLP offers a simple operation, higher sensitivity and reproducibility, and no complex equipment requirements. For Kras exon-2 mutations, the sensitivity of the PCR-RFLP method was at least 0.1 %. More

importantly, it is preferentially suitable to detect point mutations (Li *et al*., 2016).

2.2.2.10 Treatment of colorectal cancer:

Treatment should be undertaken by multidisciplinary teams working in specialist units. About 80% of patients with colorectal cancer undergo surgery (often laparoscopically), though fewer than half of these survive more than 5 years. The operative procedure depends on the cancer site. Long-term survival relates to the stage of the primary tumor and the presence of metastatic disease. Long-term survival is only likely when the cancer is completely removed by surgery with adequate clearance margins and regional lymph node clearance (Kumar and Clark, 2012).

2.2.2.10.1 Surgery:

Total resection of tumor is the optimal treatment when a malignant lesion is detected in the large bowel. An evaluation for the presence of metastatic disease, including a thorough physical examination, biochemical assessment of liver function, measurement of the plasma CEA level, and a CT scan of the chest, abdomen, and pelvis, should be performed before surgery. When possible, a colonoscopy of the entire large bowel should be performed to identify synchronous neoplasms and/or polyps (Kasper *et al*., 2016).

Surgery can be elective rather than emergency, and is probably associated with a decrease in morbidity and mortality. Local trans anal surgery is very occasionally used for early superficial rectal cancers. Surgical or ablative treatment of liver and lung metastases prolongs life where treatment is technically feasible and the patient is fit enough to undergo the treatment (Papadakis *et al*., 2016).

Types of surgical resections performed also depend on location and extent of tumor. Right, transverse, or left hemicolectomy; wide sigmoid

resection; or a low anterior resection with end-to-end anastomosis for proximal rectal cancers may be performed. For distal rectal tumors, when unable to spare the sphincter, abdominoperineal resection with permanent colostomy is often necessary. An alternative procedure for tumors 2 to 5 cm from the anal verge is a coloanal anastomosis (Ashar *et al*., 2016).

For colon cancer, treatment of cancers above the peritoneal reflection usually involves partial colectomy, unless the extent of metastatic disease necessitates systemic chemotherapy. Principles of surgical oncology, including establishing wide surgical margins, should be followed to optimize outcomes. In experienced hands, a laparoscopic procedure is acceptable. While in Rectal cancer, treatment involves abdominoperineal resection or, if the lesion is higher in the rectum, a low anterior resection that preserves sphincter function and does not require colostomy. Another approach for low rectal tumors includes initial chemotherapy and radiation followed by a segmental rectum resection and the formation of a colon anal anastomosis (Wolfsthal, 2012).

Morbidity and mortality related to surgery have decreased due to a more accurate pre-operative investigation, modern anesthetic and surgical techniques. Open, laparoscopic or robotic approaches of CRC surgery are used. Principles of operation of CRC are resection of the tumor affected bowel segment with an adequate margin (Ledel, 2016).

2.2.2.10.2 Chemotherapy and radiotherapy:

Chemotherapy and radiotherapy have been demonstrated to improve overall and tumor-free survival in selected patients with colorectal cancers depending on stage. In stage I; Because of the excellent 5-year survival rate (90–100%), no adjuvant therapy is recommended. Regarding stage II (node-negative disease), the 5-year survival rate is approximately 80%. A significant

survival benefit from adjuvant chemotherapy has not been demonstrated in most randomized clinical trials for stage II colon cancer. For stage III (node-positive disease), with surgical resection alone, the expected 5-year survival rate reduced reaching 30–50%. Thus postoperative adjuvant chemotherapy significantly increases disease-free survival as well as overall survival up to 30% and is recommended for all fit patients. In stage IV (metastatic disease), approximately 20% of patients have metastatic disease at the time of initial diagnosis, and an additional 30% eventually develop metastasis. A subset of these patients has limited disease that is potentially curable with surgical resection (Papadakis *et al*., 2016).

2.2.2.10.3 Radiation therapy:

Radiation therapy may be used as curative or palliative treatment of large bowel cancers. In general, it is employed much more commonly in treating rectal versus colonic primaries. Current recommendations for adjuvant radiation to the tumor bed following colon cancer resection include positive margins and localized perforation. Some authorities also advocate its use in colon cancers at particularly high risk of local recurrence (T4, T3N1-2 tumors in the ascending or descending colon), but that recommendation is not universally accepted. Irradiation may still play a role in treating colon cancer metastases to bone, brain, liver, and lung, as well as in cases of bleeding, obstruction, and locally advanced unrespectable disease. A major use for radiation in the definitive treatment of large bowel adenocarcinoma involves perioperative therapy for respectable rectal cancer. It is also commonly employed with chemotherapy for unrespectable locally advanced invasive tumors, which occasionally may downsize and be surgically removed after therapy. As with colon primaries, irradiation may be used to palliate bleeding,

obstruction, or selected metastases from rectal cancers (Goldman and Schafer, 2016).

2.2.2.11 Prevention and screening:

2.2.2.11.1 Dietary prevention:

Dietary prevention epidemiologic studies have reported correlations between CRC and obesity, smoking, inactivity, excessive alcohol use, and diets high in fat and low in fruits, vegetables, and fiber. These observations suggest that lifestyle modifications may decrease CRC risk (Goldman and Schafer, 2016).

Screening of high-risk patients those with inflammatory bowel disease, familial adenomatous polyposis, or hereditary nonpolyposis colorectal cancer) and those with a significant positive family history should begin at an earlier age and occur more frequently (Bope and Kellerman, 2016).

Secondary prevention aims to detect and remove lesions at an early or pre-malignant stage. Several potential methods exist: Population-based screening of people over the age of 50 years by regular Faecal occult blood (FOB) testing reduces colorectal cancer mortality and increases the proportion of early cancers detected. The sensitivity and specificity of these tests need to be improved. Colonoscopy remains the gold standard but is expensive and carries risks; many countries lack the resources to offer this form of screening. Flexible sigmoidoscopy is an alternative option and has been shown to reduce overall colorectal cancer mortality by approximately 35% (70% for cases arising in the recto sigmoid). It is recommended in the USA every 5 years in all persons over the age of 50 (Walker *et al*., 2014).

2.3 Tumor markers:

Tumor markers are chemicals produced by the body in response to the development of a tumor. When they are present in the body, they “mark” or

indicate the presence of a tumor. Tumor markers can be used to determine whether certain cancers have spread or recurred after initial treatment. Currently, genetic tests can only determine whether an individual has a predisposition to cancer. Genetic testing cannot determine the presence of a growing tumor in the body. Mutations associated with colon cancer can sometimes be found in cells removed from the colon along with the faeces (Ireland, 2011).

2.3.1. P53:

P53 protein is encoded by the gene TP53, which is involved in the development of many human cancers, in which TP53 gene mutations were found. Bad functioning of P53 is required for tumor progression. It can be activated by genotoxic damage, activation of oncogenes, telomere erosion, loss of stromal support and deprivation of nutrients or oxygen, cases in which this command the cell to enter apoptosis, thus removing from the proliferating cell population (Zlatian *et al*., 2014). Under normal conditions, the expression of P53 protein is kept at extremely low level. In response to multiple cellular stresses, p53 rapidly accumulates in the nucleus. P53 exerts its pro apoptotic function when cellular DNA damage is severe and repair is impossible. On the other hand, P53 promotes G1 cell cycle arrest in the early stage of DNA damage response. A P53 mutation is final step in the conversion of adenoma to carcinoma. The frequency of P53 abnormalities increases with the progression of the lesion (Qasim *et al*., 2012).

P53 is tumor suppressor gene that plays a key role in the control of the cell cycle, alteration of this suppressor gene is a common event in colorectal cancer and has been associated with adverse postoperative outcome and poor survival (Zavrides *et al*., 2005).

2.3.2. Bcl-2:

Bcl-2 protein is encoded by a 25-kDa oncogene that inhibits apoptosis named bcl-2. Bcl-2 overexpression results in a 14–18 translocation. Bcl-2 family members play important roles in tumor initiation and progression, but also in response to chemotherapy, their level of expression being a cancer prognostic factor. It has been developed antitumor chemotherapy that target Bcl-2 family members (Zlatian *et al*., 2014).

Bcl-2 gene is seen in normal lymphocytes, but its mutant form with characteristic translocation was first described in B-cell lymphoma and hence the name Bcl-2. It is also seen in many other human cancers such as that of breast, thyroid and prostate. Mutation in Bcl-2 gene removes the apoptosis-inhibitory control on cancer cells, thus more live cells undergoing mitosis contributing to tumor growth (Mohan, 2015).

Bcl-2 inhibits apoptosis and prolongs the survival of variety of cells, in addition to its role in the progression of cells division. All these actions will potentiate tumor growth. In the large bowel, Bcl-2 protein has been localized to the epithelial cells at the base of crypts, where stem cell proliferation takes place. Studies have shown that Bcl-2 expression is higher in colorectal adenomas than adenocarcinoma. Many studes have examined the value of the immunohistochemical expression of Bcl-2 protein in colorectal cancer, but results have been contradictory (Qasim *et al*., 2012).

The bcl-2 oncogene is a known inhibitor of apoptosis that may allow the accumulation and propagation of cells containing genetic alterations (Zhao *et al*., 2005)

2.3.3. Cytokeratin 20 (CK20):

Cytokeratin 20 is a member of the type I cytokeratin family, with a molecular weight of approximately 48.5 kDa and sharing only about 50%

amino acid sequence homology with other type I cytokeratin. Immunohistochemical studies with highly specific antibodies showed that CK20 expression in normal tissues is limited to epithelial cells of the gastrointestinal (GI) tract, the urothelium and to Merkel cells, and that this profile is maintained in malignant tumors of these cells. In colorectal cancer, CK20 protein is expressed in 90–95% of cases by conventional immunohistochemistry (IHC), depending on laboratory protocols. Both the specificity of CK20 antibodies and the restricted CK20 expression make CK20 a valuable diagnostic marker. Accordingly, CK20 IHC is a useful diagnostic tool for the classification of tumors, especially in the case of distant metastasis where the tissue of origin is unknown, as well as for the detection of disseminated tumor cells (Lassmann *et al*., 2002).

Cytokeratin 20 tumor marker is expressed in pancreatic, gastric and colorectal cancers. Further, CK20 expression has been shown in normal colonic epithelial cells as well as in cells of patients with stomach cancers. The expression of CK20 has been shown to be almost entirely restricted to gastric and intestinal epithelium, urothelium and Merkel cells (Teama and Agwa, 2010).

2.3.4. KRAS:

Kirsten rat sarcoma **(**Kras**)** is a proto-oncogene that encodes a small guanosine triphosphate (GTP) guanosine diphosphate (GDP) binding the protein involved in the regulation of the cellular response to many extracellular stimuli. Kras is located at 12p12.1, spans approximately 38 kb, and encodes a 188–amino acid residue with a molecular weight of 21.6 kDa. Kras normally functions in signal transduction cascades initiated by the binding of the epidermal growth factor receptor (EGFR), hepatocyte growth factor, and insulin like growth factor to their receptors. When activated, wild-

type Kras binds GTP, this resulting in a conformational change that allows the protein to bind and activate over 20 known downstream effectors, including Raf, Braf, mTOR, MEK1 and 2, ERK, AKT, and PIK3CA. These downstream effectors exert many different effects, including apoptosis suppression, promotion of cell growth, cell transformation, angiogenesis, migration, and differentiation (Dinu *et al*., 2014).

Kirsten rat sarcoma viral oncogene homologue is one of the RAS oncogenes (in addition to HRAS and NRAS) and accounts for most of the AS mutations in cancer. RAS functions in intracellular signal transduction downstream of transmembrane receptor TKs such as EGFR (Yousef and Jothy, 2014).

Extracellular binding of ligands to transmembrane receptors like the EGFR causes activation of the downstream signal transduction cascade to the nucleus. In the first step the intracellular tyrosine kinase domain of the EGFR is phosphorylated which in turn induces a transient activation of the RAS protein. While in its inactive state, RAS is bound to guanosine diphosphate (GDP), activation occurs by the conversion of GDP to guanosine triphosphate (GTP) (Heinemann *et al*., 2009).

Rat sarcoma (Ras) is a superfamily of G proteins, specifically small GTPases. There are three main gene members of its human subfamily, Kras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue) located on 12p12.1 encoding GTPase Kras, NRAS (neuroblastoma rat sarcoma viral oncogene homologue) located on 1p13.2 encoding GTPase NRas (N-Ras), and HRAS (v-Ha-ras Harvey rat sarcoma viral oncogene homologue) located on 11p15.5 encoding GTPase HRas (H-Ras). These Ras proteins are localized to the plasma membrane and function to transmit signals from a cell surface receptor, such as a receptor tyrosine kinase like Kit, to downstream signaling

pathways, such as the MAP kinase and I3K/Akt/ mTOR pathways. Ras proteins are activated by binding guanosine triphosphate, or GTP. The RAS superfamily of genes contains the most commonly mutated oncogenes in human cancer, found in up to one-third of human malignancies (Hosler and Murphy, 2014).

2.4 KRAS gene mutation:

Kras gene mutations increase its tyrosine kinase activity, thereby promoting cell transformation, aggressive tumors and resistance to chemotherapy and anti-EGFR-targeted biological therapies. Activating Kras gene mutations have been detected in approximately 35-45% of CRCs, and these mutations are associated with poor therapeutic responses. (Ghanipour, 2014).

The most common Kras mutations (approximately 90%) are found in codons 12 and 13. They are activation mutations, leading to continuous activation of downstream pathways. Mutant, activated forms of Kras proteins have an impaired intrinsic GTPase activity, which renders the protein resistant to inactivation by regulatory GTPase-activating proteins. Mutations of the Kras gene have been identified in tissues from both colonic adenoma and carcinoma cases, but at much lower frequencies in adenoma tissues than in carcinoma tissues. Kras mutation has been suggested to be associated with proliferation and decreased apoptosis. (Elsabah and Adel, 2013).

The RAS gene family is among the most studied and best characterized of the known cancer-related genes. Of the three human ras isoforms, KRAS is the most frequently altered gene, with mutations occurring in 17% to 25% of all cancers. KRAS mutations in colon cancers have been associated with a poorer survival and increased tumor aggressiveness. Additionally, KRAS mutations in colorectal cancer lead to resistance to select treatment strategies.

The detection of Kras mutations has been associated with decreased response rates to select chemotherapeutic agents. Therefore, Kras mutational status is a critical factor when considering the use of targeted therapies. The association of KRAS gene mutation and response to therapy was first reported in patients with metastatic colorectal cancer, who were treated with anti-epidermal growth factor receptor (EGFR) agents. The first to report the link between the Kras gene mutation and decreased response to anti-EGFR agents (Arrington *et al*., 2012).

The 95% of Kras mutations occur in codons 12 and 13 while mutations in codons 61, 146 and other are less frequent in CRC, accounting for 5% of all mutations (Vincenzi *et al*., 2015). The Kras mutational status testing has been highlighted in recent years. The most frequent mutations in this gene, point substitutions in codons 12 and 13, were validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the Kras mutational status of tumor samples has become an essential tool in managing patients with colorectal cancers (Dinu *et al*., 2014). Although a wild type Kras gene is a negative predictor of EGFR targeted therapeutic response, recent studies have indicated a wild type BRAF genotype is also required for anti-EGFR based therapeutic responses (Baskin *et al*., 2014).

There are three RAS genes in the human genome; Kras, Hras and Nras. Approximately 15-20% of all human neoplasms contain RAS mutations. Mutations in the Kras gene are detected in 35-45% of CRCs, whereas NRAS and HRAS mutations are only found in 1-3% of CRCs. KRAS mutations are frequent in pancreatic, colorectal, biliary tract, and lung cancers. The most common Kras mutations in CRCs are found in codons 12 (~77% of mutations) and 13 (~20% of mutations) in exon 2 of the gene (Domagała *et al*., 2012).

The most frequent types of Kras mutations in colorectal cancers are G-to-A transitions and G-to-T trans versions. The codons 12 and 13 code for two adjacent glycine residues located in the proximity of the catalytic site of RAS. Different Kras mutations result in an exchange of different amino acids at these catalytic sites, and therefore, may be responsible for the different levels of intrinsic GTPase activity reduction (Heinemann *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design:

This is a retrospective analytical case control study aimed to detect the immunohistochemical expression of Kras, Ck20, P53 and Bcl-2 tumor markers and molecular detection of Kras gene mutation in colorectal tumors among Sudanese patients

3.2. Study area:

This study was conducted in Khartoum state in Ibn Sina hospital and Soba teaching hospital during the period April 2014 to December 2016.

3.3. Sampling:

One hundred and fifty blocks previously diagnosed as colorectal tumors. They include, one hundred blocks were malignant tumors and the remaining fifty blocks were benign tumors, were included in this study the patient's data were collected from patient's files.

3.4. Sample processing:

3.4.1. Histopathology tissue processing

The sections of 5µm in thickness were obtained from each formalin fixed paraffin wax embedded tissue using rotary microtome.

3.4.2. Immunohistochemical staining

Four sections of 5µm in thickness were obtained from each formalin-fixed paraffin embedded tissue block using rotary microtome. Sections were immunostained using monoclonal antibodies by new indirect techniques as follows: Sections on coated slides were dewaxed in hot plate oven and cleared in two changes of xylene for two minutes. Sections were then hydrated through ethanol (100%, 90%, 70%, 50%) and water two minutes for each.

Slides were retrieved by water bath heat retrieval technique and treated with hydrogen peroxide for fifteen minutes. After that sections were washed in phosphate buffer saline (PBS) (pH 7.4) for five minutes and treated with protein blocker solution for fifteen minutes. Sections were treated with anti-p53, Ck20, Bcl-2 and kras primary antibodies for thirty minutes, then rinsed in PBS before being treated with secondary polymer conjugate for thirty minutes and rinsed in PBS. Slides were treated with DAB for seven minutes then washed in PBS for five minutes. For the staining step, sections were counter stained in Mayer's hematoxylin for one minute washed and blued in running tap water before they were dehydrated through ascending concentrations of ethanol (50%,70%,90%,100%). Sections were finally cleared in xylene and mounted in DPX (Bancroft and Gamble, 2008).

3.4.3 Molecular techniques

3.4.3.1. DNA Extraction:

From each paraffin block, small sections of 20 μm thick were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut with new gloves and new disposable microtome blade.

All subsequent necessary procedures were taken for preventing genetic contamination. Genomic DNA from FFPE tissue sections was isolated using the QIA amp DNA Kit (Qiagen) according to the manufacturers protocols. Excess paraffin of the sample block was trimmed and cut up into sections (20μm thick). Prepared sections were immediately placed in a 1.5 ml micro centrifuge tube and one ml of xylene was added to the sample. The lid was closed and vortexed vigorously for 10 seconds. The tube was centrifuged at full speed for 2 mins at room temperature (25°C). The supernatant was removed by pipetting and the pellets were kept. One ml of absolute ethanol (100%) was added to the pellet, mixed by vortexing and centrifuged at full

speed for 2 mins at room temperature. The supernatant was removed by pipetting and the pellet was preserved. The tube was opened and incubated at room temperature or up to 37°C for 30 mins or until all residual ethanol has evaporated. The pellet was suspended in 180 µl ATL Buffer. Twenty microliters of proteinase k were added and mixed by vortexing. The mixture was then incubated at 56°C for 1 hour and then incubated at 90°C for another 1 hour. The tube was briefly centrifuged to removed drops from the inside of the lid before 200 µl of AL buffer were beaded to the sample and mixed thoroughly by vortexing. Absolute ethanol (200 µl) was added and mixed again thoroughly by vortexing. The tube was briefly centrifuged to remove drops from the inside of the lid then the entire lysate was transferred to the QIA ampMinElute column (in a 2 ml collection tube). The lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIA ampMinElute column was placed in a clean 2 ml collection tube and the collection tube was discarded containing the flow-through. The QIA ampMinElute column was opened and 500 µl of AW1 buffer were added without wetting the rim. The lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIA ampMinElute column was placed in a clean 2 ml collection tube. and 500 µl of AW2 buffer were added without wetting the rim. The lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIA ampMinElute column was placed in a clean 2 ml collection tube and centrifuged at full speed (20000 x g; 14000 rpm) for 3 minute. To collect DNA, the QIA ampMinElute column was placed in a clean 1.5 ml micro centrifuge tube.50 µl buffer ATE was applied to the center of the QIA ampMinElute column membrane. The lid was closed and incubated at room temperature for 1 minute and centrifuged at full speed (20000 x g; 14000 rpm) for 1 minute (Dobre, *et al*., 2013; QIAGEN, 2012).

3.4.3.2. Measuring DNA quality and quantity

Concentration and purity of DNA was measured using a Nano drop Spectrophotometer (ND1000 Spectrophotometer, Nano drop Technologies). The absorbance (A) was measured at wave lengths of 260 and 280 nanometers (nm) and the spectral measurements were done using operating software. The DNA concentration was measured as ng /µL. The purity (indication of the quality) was indicated by the ratio calculated from the reading of the wave length 260 and 280 nm. The genomic DNA concentrations of the 150 specimens included in the study group were between 50 ng/µL– 1200 ng/µL and 260 nm/280 nm ratio was between 1.63 and 2.18 among the 150 specimens.

3.4.4.3. PCR-RFLP

The PCR reactions were contained 1X reaction buffer, 1.5 mM magnesium chloride, 0.2 mm deoxy nucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 1 mM of each primer, 1.5 units of platinum Taq DNA polymerase and 200 ng to 500 ng of genomic isolated DNA in 25 µL total volume. The primers were used to detect codon 12 and 13 mutations, were synthesized by Vivantis and the sequences were used according to data published by (Kubrusly *et al*., 2002).

(Sense) ACTGAATATAAACTTGTGGTAGTTGGACCT,

(Antisense)TAATATGTCGACAAAACAAGATTTACCTC.

PCR was performed in a thermos cycler for 45 cycles; each cycle was performed by a denaturation step at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for 30 seconds. For codon 13, the PCR reactions contained the same components except for the primers concentration (1.25 mM) and 57°C annealing temperature. The sequences for primers for codon 13 were:

(sense) 5’-GTACTGGTGGAGTATTTGATAGTGTATTAA-3’

(antisense) 5’-GTATCGTCAAGGCACTCTTGCCTAGG-3’ (Hatzaki *et al*., 2001).

Restriction digestion was performed for positive PCR reactions. For codon 12 the restriction enzyme MvaI (BstNI) (Thermo) were used. After amplification the135 bp fragments were overnight digested by MvaI (10 U enzyme and 12μL PCR product). The wild-type fragment is cleaved in two fragments with sizes of 106 and 29 base pairs (Kubrusly *et al*., 2002) and the mutant case has both alleles, normal and mutant.

For codon 13, the HaeIII (Thermo) were used as restriction enzyme. After amplification, 12.5 μL PCR products (159 base pairs) and 10 U HaeIII were incubated overnight. The wild-type allele was cleaved into three fragments of 85, 48 and 26 pair base while the mutant allele was cleaved into two fragments of 85 and 74 bp. In both codons, electrophoresis analysis was performed using 2% agarose gel. Stained with ethidium bromide and photographed by ultraviolet trans illuminator. The digested fragment sizes were determined using standard size marker 100 bp or 50 bp DNA Ladder.

3.5. Results interpretation:

All quality control measures were adapted during sample collection and preparation. The immunohistochemical expression and molecular results were assessed by research group and confirmed by an expertise pathologist.

3.6. Statistical analysis:

The data were analyzed using SPSS computer program, frequency, means and chi-square values were calculated.

3.7. Ethical consideration:

Before the study was conducted the proposal of the study was ethically approved by ethical committee of the Sudan University of Science and

Technology, (college of medical laboratory science) and ministry of health research committee. An official written permission to conduct the study was obtained by the investigator from responsible authorities.

CHAPTER FOUR

RESULTS

A total of 150 paraffin blocks previously diagnosed as colorectal tumors were collected in the study. According to the histopathological diagnosis, 100 patients were classified as malignant tumors and 50 as benign tumors. All tissue samples were included in the study and investigated for the P53, Kras, Bcl-2 and Ck20 by immunohistochemical technique and Kras gene mutations (codon12 and codon13) using PCR-RFLP molecular technique.

As shown in figure 4.1, the distribution of study population according to the gender represented 44 males (44%) and 56 females (56%) among malignant tumor. While 35 (70%) were male and 15 (30%) were female among the benign tumor.

The distribution of study population according to the age at presentation, characterized 41 (41%) who were less than 50 years old and 59 (59%) were more than 50 years old among the malignant tumor. Whereas, 29 (58%) were less than 50 years old and 21 (42%) were more than 50 years in the benign tumor (Figure 4.2).

According to the site of tumor, colon site was lower presented 41% than rectal 59% across malignant tumor. On the other hand, the majority of benign tumors were in the colon site of the digestive tract 72%, while rectal site presented only 28% the benign tumor (Figure 4.3).

When samples were distributed according to cancer grade, the majority of malignant tumors were either moderately differentiated 51% or well differentiated 41%. While, 8% of the samples were noticed as poorly differentiated tumors (Figure 4.4).

Table 4.1 shows the relation between the immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 and histopathological diagnosis. Samples with positive immunohistochemical expression of p53, Kras, Bcl-2 and Ck20 in malignant tumors tissues represented 61(61%), 52(52%), 31(31%), and 58(58%) respectively. While only Bcl-2 (2%), and Ck20 (22%) indicated positive immunohistochemical reactions among the benign tumor. With significant relation between immunohistochemical expression and histopathological diagnosis (p. value 0.000).

Twenty-six percent of malignant tumors showed the mutant genotype of codon12 in comparison to wild type 74%. None of the benign tumors revealed the mutant genotype. The Kras gene codon 12 mutations was significantly correlated with malignant tumor (p. value 0.000). Similar significant relation was obtained for the Kras gene mutation at codon13 as the detected mutations represented 9% of the malignant tumor, while none of the benign tumors revealed the mutant genotype (p. value 0.029) (Table 4.2).

Table 4.3 shows the relation between the immunohistochemical expression of p53, Kras, Bcl-2 and Ck20 and cancer grade, the immunohistochemical expression of P53 in positive most majority was 31 (61%), in moderately differentiated. While the negative was 20 (39%) in moderately differentiated. With insignificant relation between the immunohistochemical expression of P53 and cancer grade (p. value 0.776). Furthermore, the immunohistochemical expression of Kras in positive most majority was 27 (53%), in moderately differentiated tumor. While the negative was 24 (47%) in moderately differentiated. With insignificant relation between the immunohistochemical expression of Kras and cancer grade (P. value 0.057). In addition, the immunohistochemical expression of Bcl-2 in positive most majority was 21 (41%), in moderately differentiated.

While the negative was 32 (59%), in well differentiated. With insignificant relation between the immunohistochemical expression of Bcl-2 and cancer grade (P. value 0.070). Furthermore, the immunohistochemical expression of Ck20 in positive most majority was 33 (65%), in moderately differentiated. While the negative was 18 (35%) in moderately differentiated. With insignificant relation between the immunohistochemical expression of Ck20 and cancer grade (P. value 0.123).

Considering the relation between kras gene mutations and cancer grade, the mutant genotype frequency of codon 12 in poorly, moderately and well differentiated tumors was 0.0%, 12 (23.5%) and 14 (34%) respectively. With insignificant relation between kras gene mutation codon12 and cancer grade (P. value 0.112). Moreover, codon13 was mutant in 0.0%, 5(10%) and 4 (10%) respectively. With insignificant relation between Kras gene mutation at codon13 and cancer grade (P. value 0.650) (Table 4.4).

The immunohistochemical expression of kras was positive in 27.4% of the non-mutant codon 12 samples and 69% of the mutant samples. While 8 samples were mutant among negatively expressed Kras samples. With significant relation between kras immunohitochemical expression and Kras gene mutation codon12 (p. value 0.000). Also the expression of Kras was positive in 47 (33.3%) of the non-mutant codon 13 samples and 5 (55.6%) of the mutant samples. While 4 samples were mutant among negatively expressed Kras samples. With insignificant relation between kras immunohitochemical expression and Kras gene mutation codon13 (P. value 0.174) (Table 4.5).

The table 4.6 shows the comparison between immunohitochemical expression of P53, Bcl-2 and Ck20 and Kras gene mutations. Codon 12 mutant genotype was found in 17 samples of the P53 positive samples, with

significant relation between immunohitochemical expression of p53 and kras gene mutation codon12 (p 0.005). In addition, positive p53 in codon13 was 56 (40%) in wild type and 5 (55.5%) in mutant. while in negative 85 (60%) in wild type and 4 (44.5%) in mutant. With insignificant relation between immunohitochemical expression of P53 and kras gene mutation codon13 (P. value 0.348).

The immunohistochemical expression of Bcl-2 the positive was 25 (20%) in wild type and 7 (27%) in mutant in codon12. while in negative 99 (80%) in wild type and 19 (73%) in mutant in codon12. With insignificant relation between immunohitochemical expression of Bcl-2 and Kras gene mutation codon12 (p. value 0.444). also positive Bcl-2 in codon13 was 27 (19%) in wild type and 5 (55.5%) in mutant. while in negative 114 (81%) in wild type and 4 (44.5%) in mutant. With significant relation between immunohitochemical expression of Bcl-2 and Kras gene mutation codon13 (p. value 0.010).

The immunohistochemical expression of Ck20 the positive in codon12 was 50 (40%) in wild type and 19 (73%) in mutant. while in negative 74 (60%) in wild type and 7 (27%) in mutant, with significant relation between immunohitochemical expression of Ck20 and kras gene mutation codon12 (P. value 0.002). On the other hand, positive Ck20 in codon13 was 65 (46%) in wild type and 4 (44.5%) in mutant. while in negative 76 (74%) in wild type and 5 (55.5%) in mutant, with insignificant relation between immunohitochemical expression of Ck20 and Kras gene mutation codon13 (P. value 0.923).

According to the gender, 44% of the malignant tumors were males and 56% were female. While the majority of benign tumors were among males

70%. Females were found to be significantly at greater risk of getting malignant tumors (P. value 0.003).

According to the age group, patients were slightly older at presentation among malignant tumor 41 (41%) were less than 50 years old and 59 (59%) were more than 50 years). While, the opposite was among patients with benign tumors 29 (58%) were less than 50 years and 21 (42%) were more than 50 years in benign. With significant relation between the elder age group and malignant histopathological diagnosis (P. value 0.049).

Concerning the site of the tumor, colon site was less represented among malignant tumors (41%) when compared to rectal site tumors (59%). While the majority of benign tumors were from colon site (72%). Concerning the rectal site benign tumor presented only 28%. with significant relation between the rectal site of tumor and malignant histopathological diagnosis (P. value 0.000) (Table 4.7).

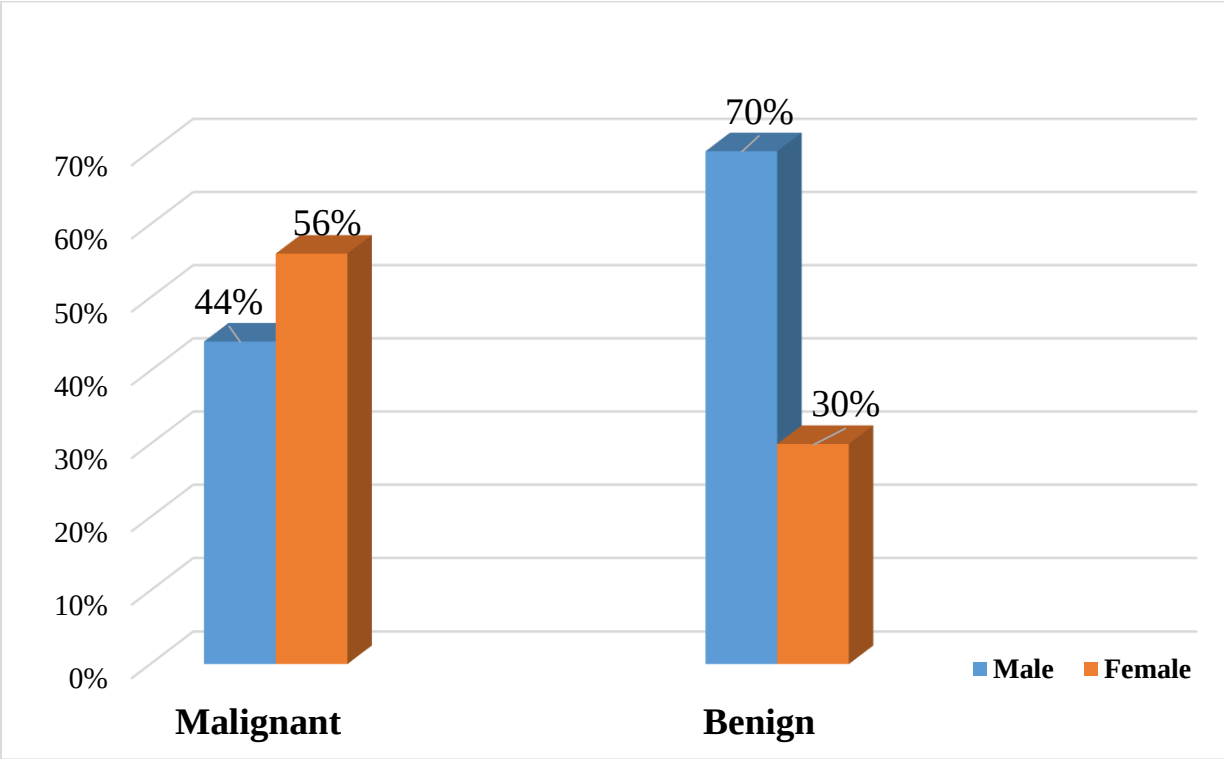


Figure (4.1): Distribution of study population according to gender.

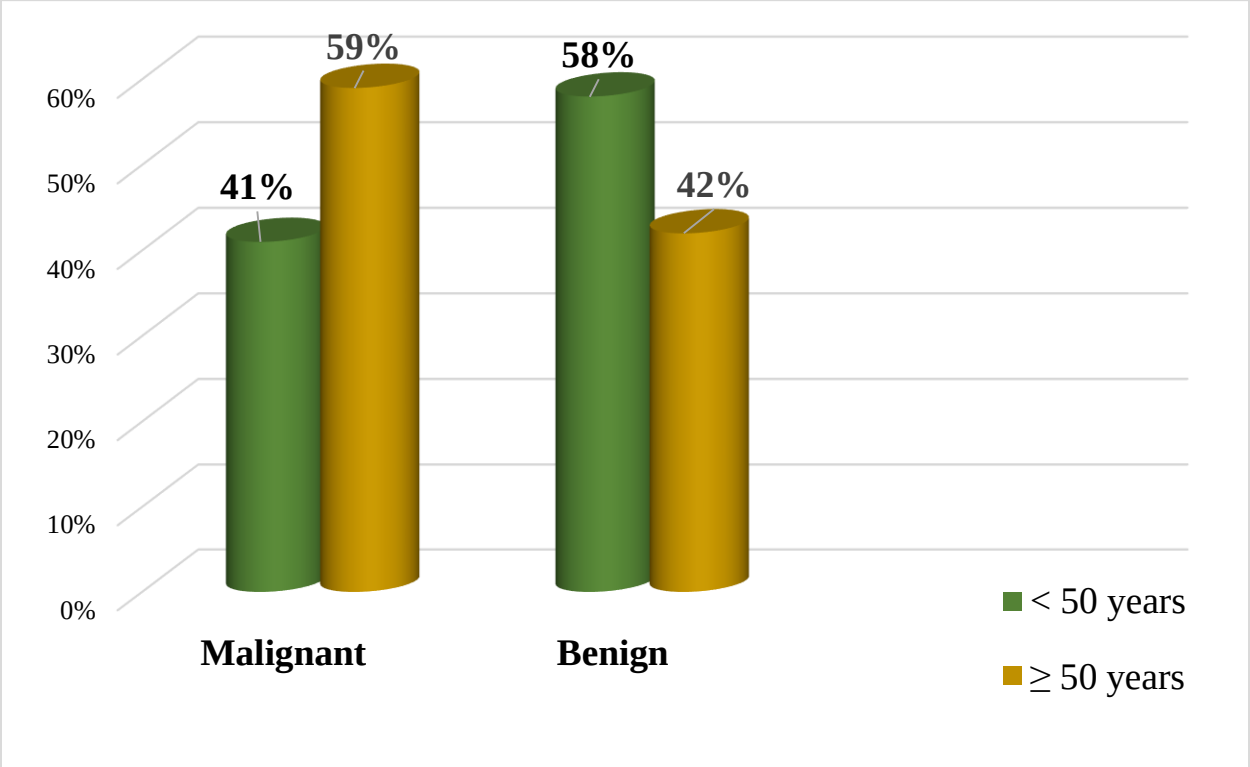


Figure (4.2): Distribution of study population according to age group.

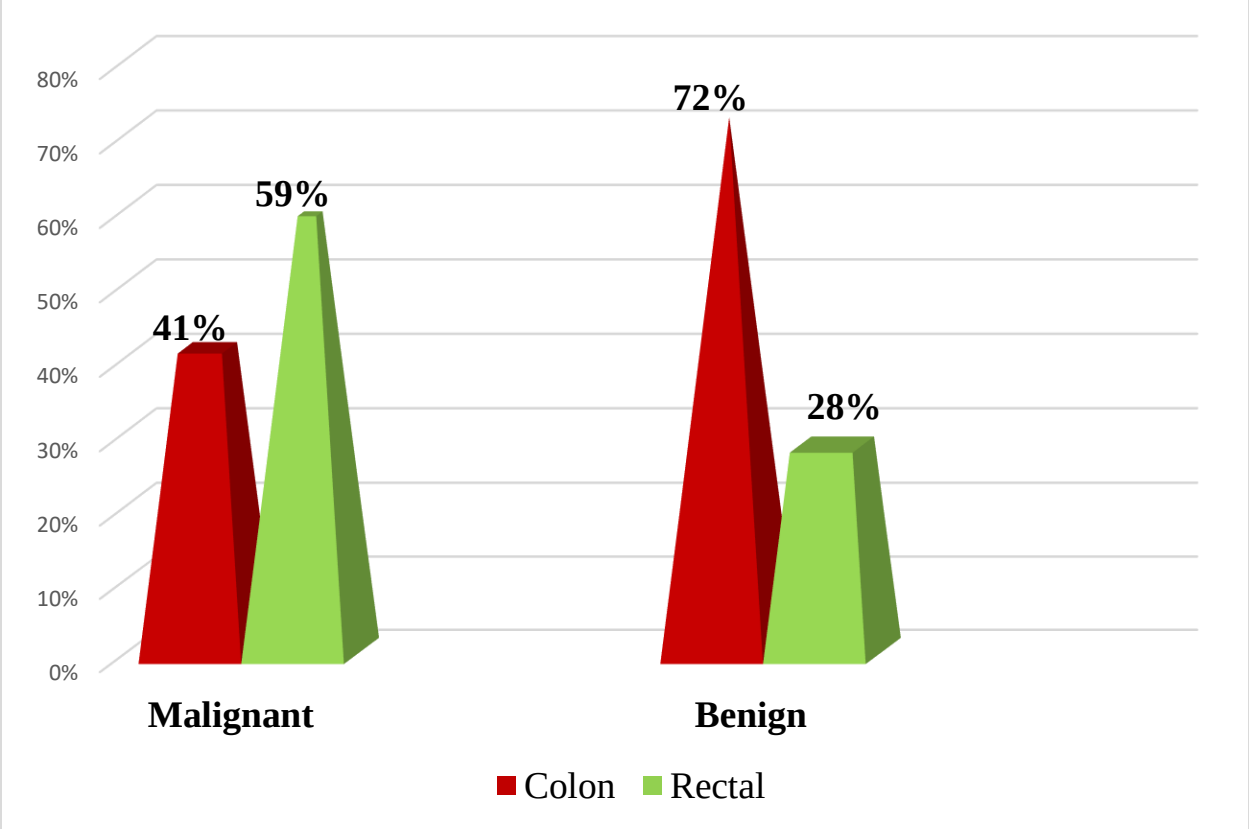


Figure (4.3): Distribution of study population according to the site of tumor.

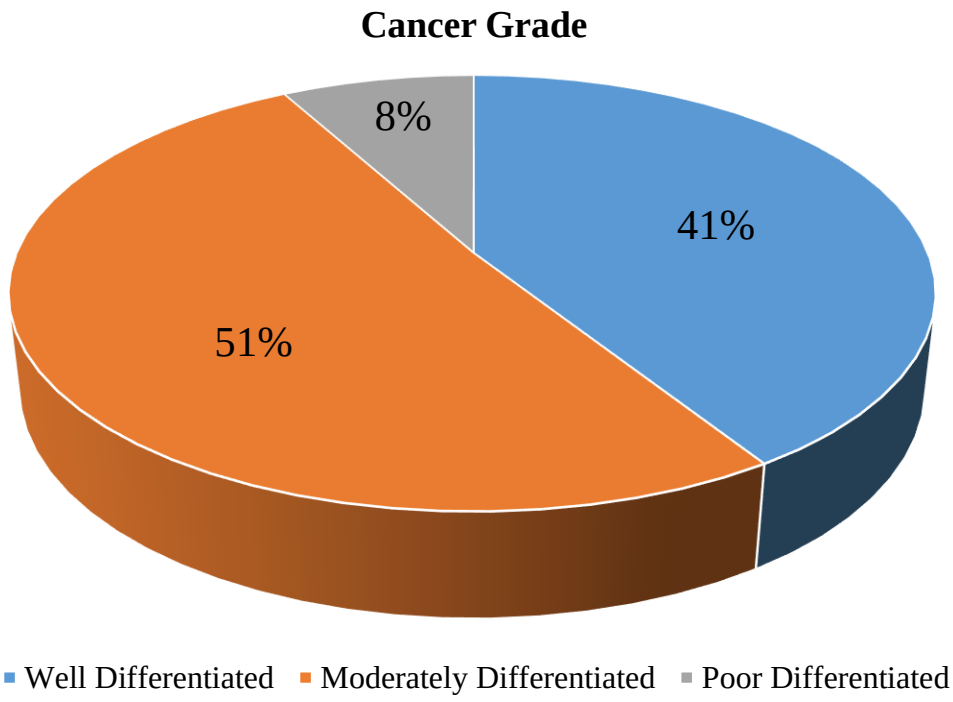


Figure (4.4): Distribution of case study population according to cancer grade.

Table (4.1): Relation between P53, Kras, Bcl-2 and Ck20 immunohistochemical expression and histopathological diagnosis.

Immunohistochemical expression		Histopathological diagnosis				P. value
		Malignant		Benign		
		Number	Percent	Number	Percent	
P53	Positive	61	61.0%	0	0.0%	**0.000**
	Negative	39	39.0%	50	100.0%	
	Total	100	100%	50	100%	
Kras	Positive	52	52.0%	0	0.0	**0.000**
	Negative	48	48.0%	50	100.0	
	Total	100	100%	50	100%	
Bcl-2	Positive	31	31.0%	1	2.0%	**0.000**
	Negative	69	69.0%	49	98.0%	
	Total	100	100%	50	100%	
Ck20	Positive	58	58.0%	11	22.0%	**0.000**
	Negative	42	42.0%	39	78.0%	
	Total	100	100%	50	100%	

Table (4.2): Relation between Kras gene mutations (codon12 and codon13) and histopathological diagnosis.

Kras gene mutations		Histopathological diagnosis				P. value
		Malignant		Benign		
		Number	Percent	Number	Percent	
Codon 12	Wild type	74	74.0%	50	100.0%	0.000
	Mutant	26	26.0%	0	0.0%	
	Total	100	100%	50	100%	
Codon 13	Wild type	91	91.0%	50	100.0%	0.029
	Mutant	9	9.0%	0	0.0%	
	Total	100	100%	50	100%	

Table (4.3): Relation between P53, Kras, Bcl-2 and Ck20 immunohistochemical expression and cancer grade.

Immunohistochemical expression		Cancer grade						P. value
		Well differentiated		Moderately differentiated		Poorly differentiated		
		No	%	No	%	No	%	
P53	Positive	26	63.5%	31	61.0%	4	50.0%	0.776
	Negative	15	36.5%	20	39.0%	4	50.0%	
	Total	41	100%	51	100%	8	100%	
Kras	Positive	24	58.5%	27	53.0%	1	12.5%	0.057
	Negative	17	41.5%	24	47.0%	7	87.5%	
	Total	41	100%	51	100%	8	100%	
Bcl-2	Positive	9	22.0%	21	41.0%	1	12.5%	0.070
	Negative	32	78.0%	30	59.0%	7	87.5%	
	Total	41	100%	51	100%	8	100%	
Ck20	Positive	22	54.0%	33	65.0%	3	37.5%	0.123
	Negative	19	46.0%	18	35.0%	5	62.5%	
	Total	41	100%	51	100%	8	100%	

Table (4.4): Relation between the kras gene mutations (codon12 and codon13) and cancer grade.

Kras gene mutations		Cancer grade						P. value
		Well differentiated		Moderately differentiated		Poorly differentiated		
		No	%	No	%	No	%	
Codon 12	Wild type	27	66.0%	39	76.5%	8	100.0%	0.112
	Mutant	14	34.0%	12	23.5%	0	0.0%	
	Total	41	100%	51	100%	8	100%	
Codon 13	Wild type	37	90.0%	46	90.0%	8	100.0%	0.650
	Mutant	4	10.0%	5	10.0%	0	0.0%	
	Total	41	100%	51	100%	8	100%	

Table (4.5): Relation between Kras immunohistochemical expression and Kras gene mutations (codon12 and codon13).

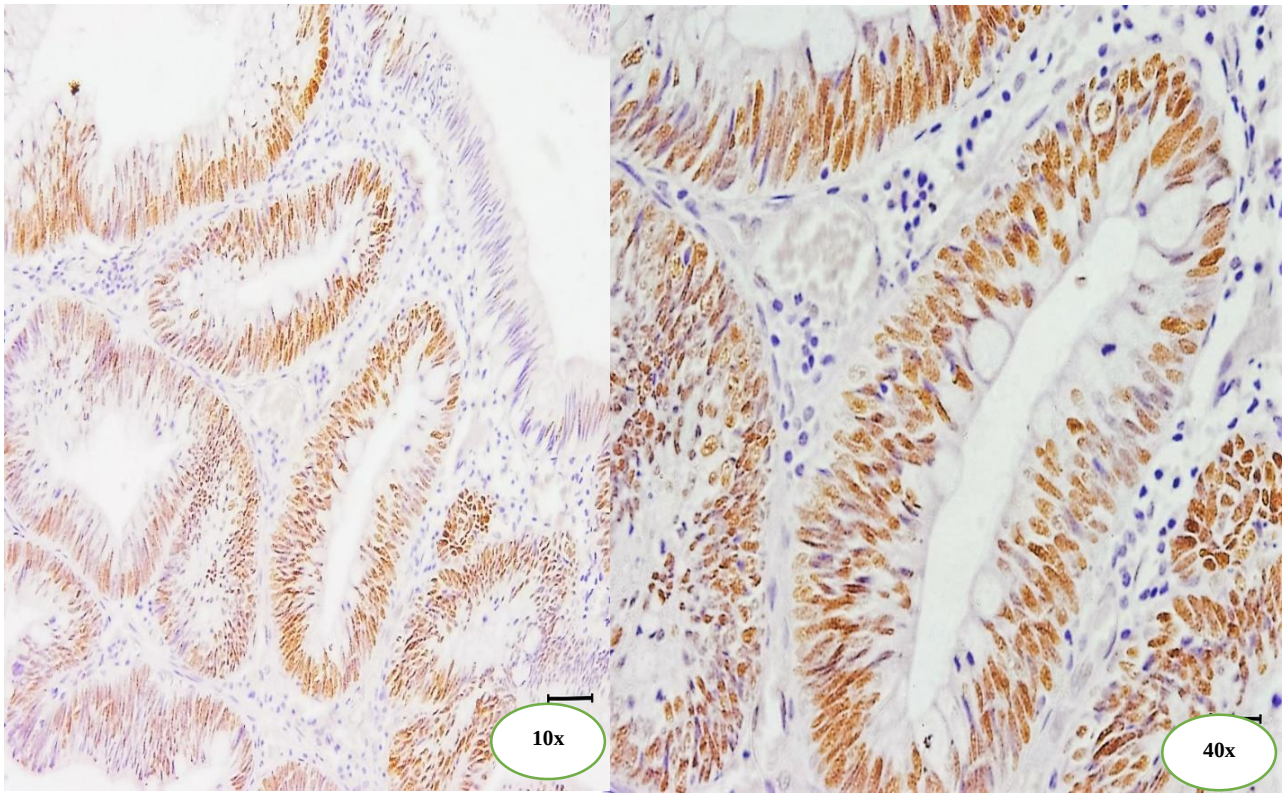
Immunohistochemical expression		Kras gene mutations							
		Codon12				Codon13			
		Wild type		Mutant		Wild type		Mutant	
		No	%	No	%	No	%	No	%
Kras	Positive	34	27.4%	18	69.2%	47	33.3%	5	55.6%
	Negative	90	72.6%	8	30.8%	94	66.7%	4	44.4%
	Total	124	100%	26	100%	141	100%	9	100%
P. value		**0.000**				**0.174**			

Table (4.6): Relation between P53, Bcl-2 and Ck20 immunohistochemical expression and Kras gene mutations (codon12 and codon13).

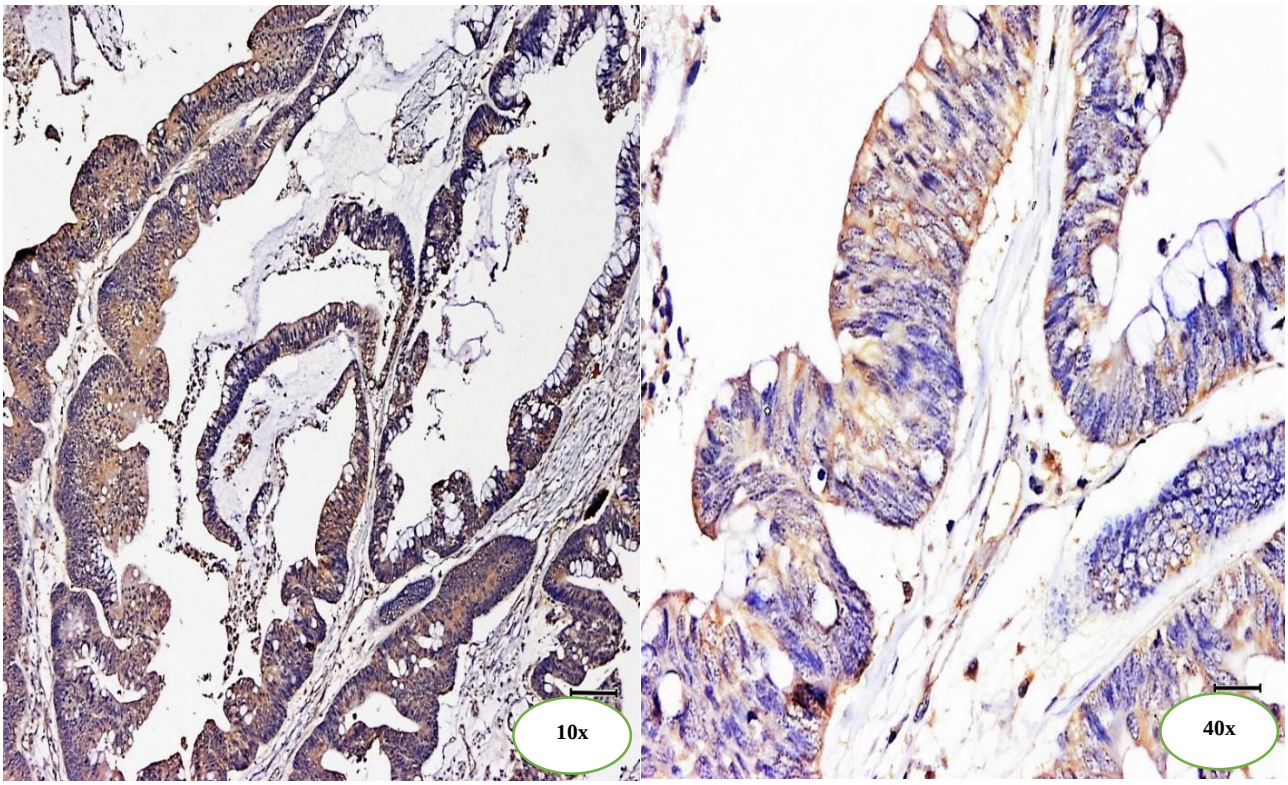
Immunohistochemical expression		kras gene mutations							
		Codon12				Codon13			
		Wild type		Mutant		Wild type		Mutant	
		No	%	No	%	No	%	No	%
P53	Positive	44	35.4%	17	65.0%	56	40.0%	5	55.5%
	Negative	80	64.6%	9	35.0%	85	60.0%	4	44.5%
	Total	124	100%	26	100%	141	100%	9	100%
P. value		0.005				0.348			
Bcl-2	Positive	25	20.0%	7	27.0%	27	19.0%	5	55.5%
	Negative	99	80.0%	19	73.0%	114	81.0%	4	44.5%
	Total	124	100%	26	100%	141	100%	9	100%
P. value		0.444				0.010			
Ck20	Positive	50	40.0%	19	73.0%	65	46.0%	4	44.5%
	Negative	74	60.0%	7	27.0%	76	54.0%	5	55.5%
	Total	124	100%	26	100%	141	100%	9	100%
P. value		0.002				0.923			

Table (4.7): Relation between characteristics data and histopathological diagnosis among study population.

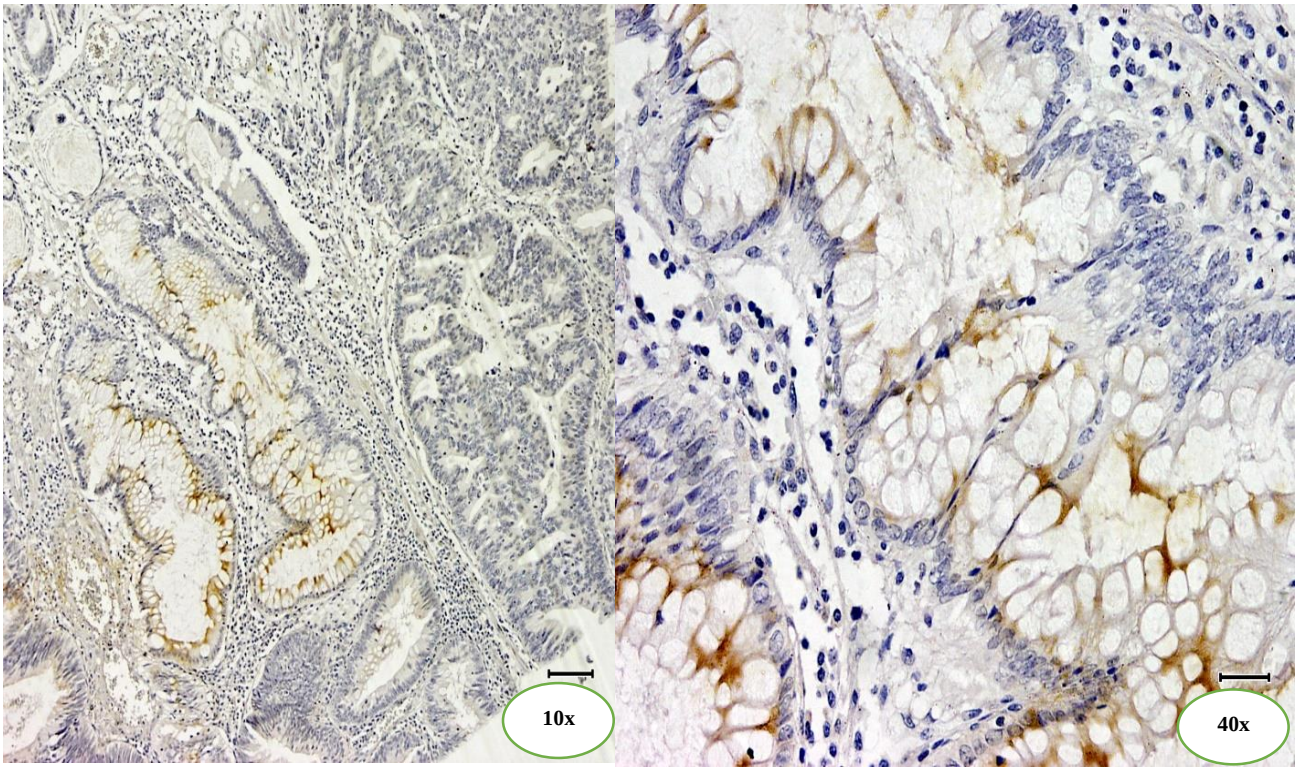
Characteristics data		Histopathological diagnosis				P. value
		Malignant		Benign		
		Number	Percent	Number	Percent	
Gender	Male	44	44%	35	70%	**0.003**
	Female	56	56%	15	30%	
	Total	100	100.0%	50	100.0%	
Age	< 50	41	41%	29	58%	**0.049**
	≥ 50	59	59%	21	42%	
	Total	100	100.0%	50	100.0%	
Site of tumor	Colon	41	41%	36	72%	**0.000**
	Rectum	59	59%	14	28%	
	Total	100	100.0%	50	100.0%	



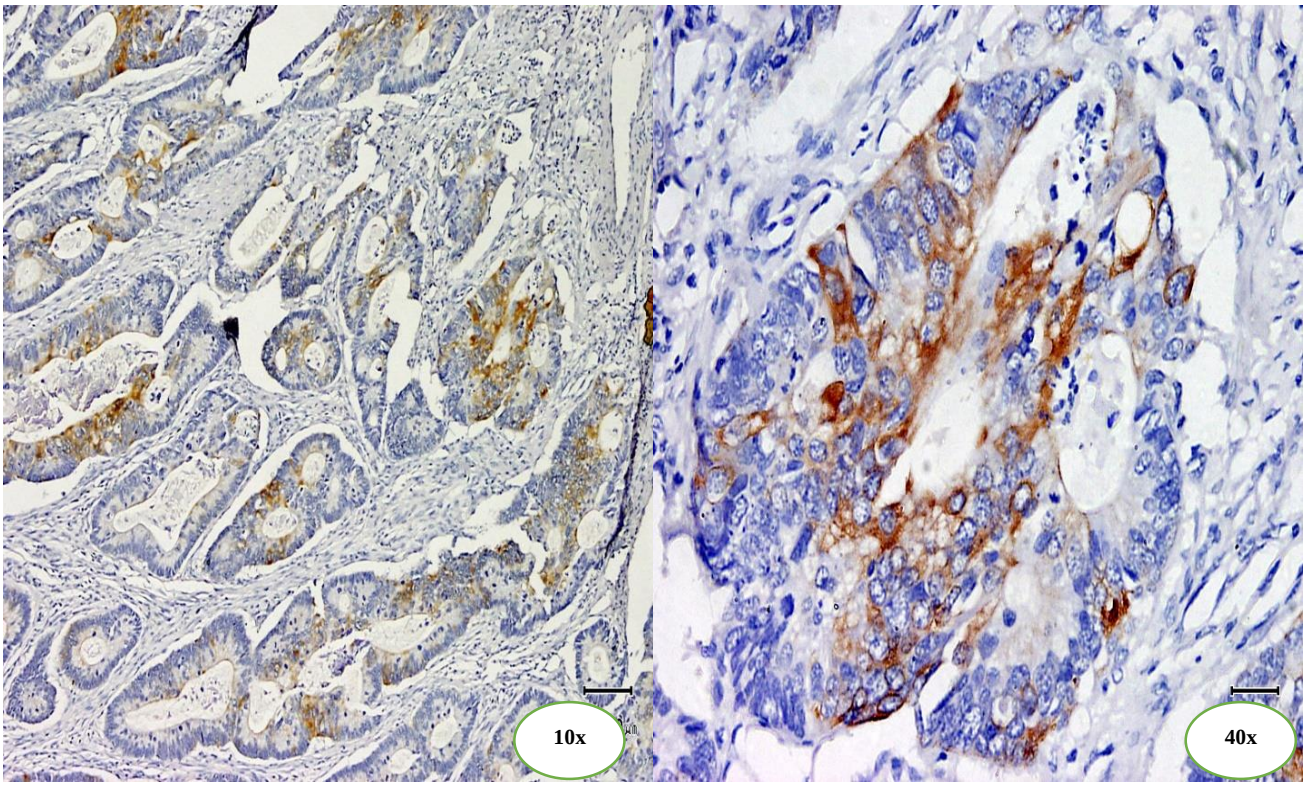
Microphotography (4. 1): Well differentiated colorectal cancer show positive P53 expression (10X and 40X).



Microphotography (4.2): Well differentiated colorectal cancer show positive Kras expression (10X and 40X).



Microphotography (4.3): Moderately differentiated colorectal cancer show positive Bcl-2 expression (10X and 40X).



Microphotography (4.4): Moderately differentiated colorectal cancer show positive Ck20 expression (10X and 40X).

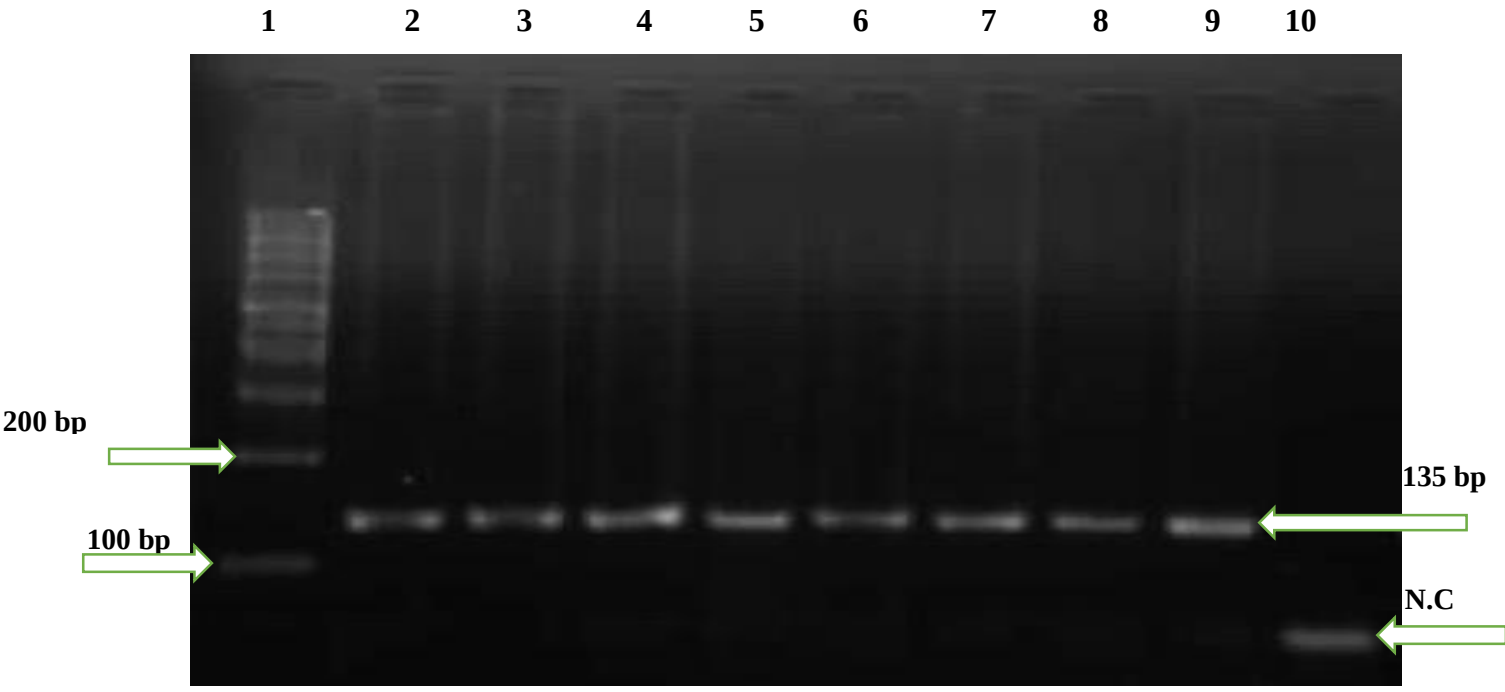


Figure (4. 5): Gel electrophoresis of PCR products of codon 12 (band at 135 bp). Lane 1; DNA ladder 100 pb. Lanes 2-9; samples PCR products of codon 12 band at 135 bp; Lane 10; negative control.

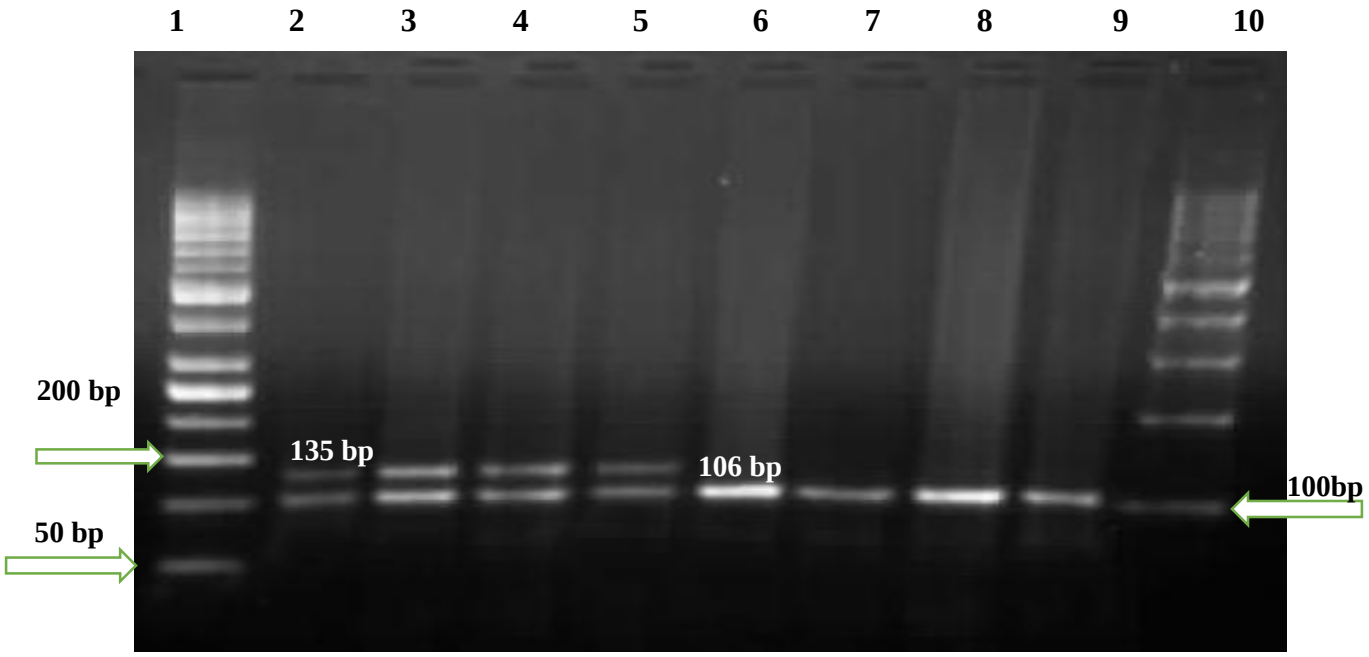


Figure (4.6): Gel electrophoresis of PCR product in 2% agarose after digestion with BstNI (Thermo) restriction enzyme. Lane 1; DNA ladder 50 pb. Lane 10; 100 bp DNA ladder. Lanes 2-5; mutant genotype (bands at 135, 106 and 29 bp). Lanes 6-9; wild type genotype (bands at 106 and 29 bp).

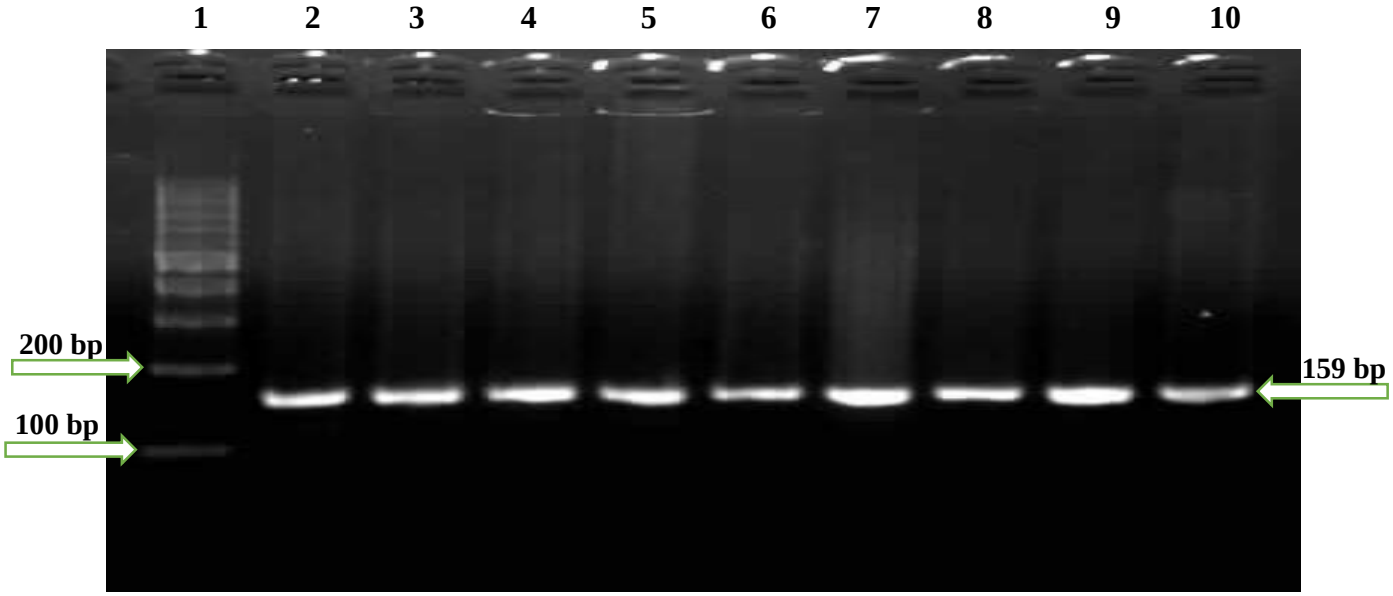


Figure (4.7): Gel electrophoresis of PCR products of codon 13 (band at 159bp). Lane 1; DNA ladder 100 pb. Lanes 2-9; samples PCR products of codon 13 band at 159 bp.

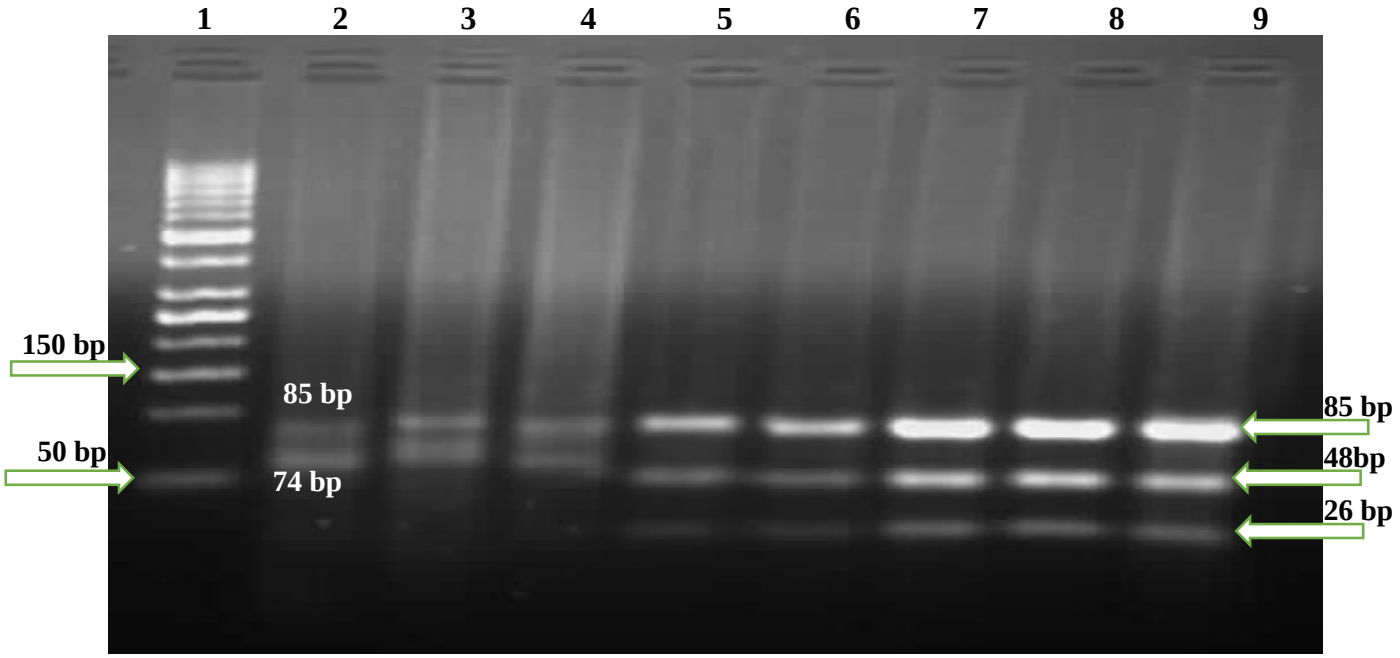


Figure (4.8): Gel electrophoresis of PCR product in 2% agarose after digestion with HaeIII (Thermo) restriction enzyme. Lane 1; DNA ladder 50 pb. Lanes 2-3; mutant genotype (bands at 85and 74 bp). Lanes 6-9; wild type genotype (bands at 85, 48 and 26 bp).

CHAPTER FIVE

DISCUSSION

Colorectal cancer (CRC) is the second most common cancer in females and the third in males with 1.2 million annual new cases worldwide (Mazeh *et al*., 2013).

Cancers of the gastrointestinal tract are of the most rewarding interfaces in translational medicine, with recent work particularly on colorectal cancer leading to greater understanding of the genetic mechanisms leading to cancer and the development of novel targeted therapies **(**Warrell *et al*., 2010).

In the present study, females were found to be highly susceptible to malignant tumors 56 % when compared to males 44%. These results exceed other reported frequencies in the literature in which females were only slightly more presented than males including Akkiprik *et al*., 2008; Kleist *et al*., 2014 and Menezes *et al*., 2010 who that reported females and males' ratios of 58%: 42%, 52%: 48% and 53.7% :46.3% respectively. However, some studies reported higher frequencies among males in comparison to females reporting 1.5:1 male to female ratios (Nussrat *et al*., 2011 and Al-Attraqhchi *et al*., 2015). Such disagreeing results may be due to racial and geographical factors. It is possible that lifestyle differences between the two sexes may also have an impact on histological differentiation of colorectal carcinomas (Newcomb *et al*., 2007; Wallace *et al*., 2009).

In concordance to most of the previous studies, the current study considered aging as one of the risk factors of the disease as 41% were less than 50 years old and 59% were more than 50 years old with mean age of 52.51±14.33 among the malignant tumor. This result agree with Zlatian *et al*. (2015) who found that 24% were less than 55 years and 76% were more than

55 years with a mean age of 59.7 years in an investigated group of 50 patients with colorectal adenocarcinoma. A study by Awad Elkareem and Mohammed (2016) reported that most patients were older than the age of 50 years representing 56.7% and the remaining 43.3% were younger than 50 years.

The same findings were reported in a study by Pity *et al*. (2013) who found that the mean age of the 52 patients studied was 50.2 years. Moreover, approximately 75% of colorectal cancers diagnosed after 65 years of age (Fernebro *et al*., 2004).

According to the site of tumor, the present study revealed that the colon was significantly lower presented (41%) than rectal (59%) across malignant tumor. These findings congruent with Kruschewski *et al*. (2011) who clarified that the tumor location was more in rectum (68.1%), while only 31.9% was in colon. Another study by (Kerin, 2013) reported that tumour location was 57.4% in rectum and 42.6% in colon. Contrasting results were reported by Watson *et al*. (2005) who found that malignant tumors were in the colon site in 52% of the cases and in the rectum in 39% of them.

On the other hand, the majority of benign tumors were in the colon site of the digestive tract (72%), while rectal site presented only 28% of the benign samples in this study. Similarly, across the benign tumors a study by Al-Attraqhchi *et al*. (2015) found that colorectal adenomas were more frequent in the colon site (90%) and only 10% occurred in the rectum. Inconsistency of the reported site of malignancy might be due to the fact that risk factors for colorectal cancer were suggested to unequally contribute to colon and rectal carcinogenesis (Wei *et al*., 2004). Thus, generally, the differences in the reported site of malignancy might be due to differences in sample size, environmental risk factors in addition to dietary habits.

Although, aggressive tumor behavior in patients with high grade colon tumors was thought to be a major cause of poor prognosis and mortality of colon cancer, our data revealed that colorectal malignant tumors were mostly moderately differentiated or well differentiated (92%) while 8% noticed as poorly differentiated indicating possible better outcomes among affected patients. Nevertheless, moderately differentiated tumors were found to be more likely to metastasize than well differentiated tumors (Yokoyama *et al*., 2010).

These results were comparable with Mogoanta *et al*., (2014) who reported that adenocarcinoma was moderately differentiated or well differentiated in 87% of tumors while 13% were poorly differentiated. Also, Tsamandas *et al*. (2007) clarified that about 82.1% of adenocarcinomas were well and moderately differentiated, whereas the remaining (17.9%) were poorly differentiated.

On the other hand, Kruschewski *et al*., (2011) pointed that most colorectal carcinoma tumors were poorly differentiated (52.6%). Fernebro *et al*., (2004) found that only 2% were well differentiated, 68% were moderately differentiated and 30% were poorly differentiated or undifferentiated.

In the current study four different markers were targeted for immunohistochemical analysis for their putative role in cancer prediction and prognosis. P53 protein expression has an important role in the progression of CRC, it is considered as an independent predictor of shorter overall survival in patients with completely resected CRC (Liu *et al*., 2014). On the other hand, Bcl-2 family members play important roles in tumor initiation and progression (Zlatian *et al*., 2014).

Moreover, Ck20 was considered a relevant marker for colorectal diagnosis, since its expression is restricted to gastric and intestinal epithelium,

urothelium, and Merkel cells, as well as cancers originating from these tissues (Kust *et al*., 2016). Thus, Ck20 levels can provide clinically valuable information on the postoperative prognosis of patients with colorectal cancer (Li *et al*., 2015).

The accumulative effects of such genes may play an important role during carcinogenesis (Wang *et al*., 1998). In an attempt to study the contribution all these markers to colorectal malignancy, the present study investigated the relation between P53, Kras, Bcl-2 and Ck20 immunohistochemical expression and histopathological diagnosis. Samples with positive immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 in malignant represented 61%, 52%, 31%, and 58% respectively. While only 2% Bcl2, and 22% Ck20 indicated positive immunohistochemical expression among the benign tumor, with significant relation between immunohistochemical expression and histopathological diagnosis (P. value 0.000).

In the same way a study by Petrişor *et al*. (2008) evaluated P53 and Bcl-2 markers in colorectal adenocarcinoma as 66% and 46.6% positive for P53 and Bcl-2 respectively. Furthermore 53.4% and 60% were positive for P53 and Bcl-2 respectively in rectal adenocarcinoma (Petrişor *et al*., 2008). Another study showed that 56% of tissues expressed p53 protein in the nucleus of malignant cells (Rambau *et al*., 2009). A study of Bcl-2 and P53 immunostaining expressions in colonic carcinomas by Shashi Kiran *et al*. (2016) pointed that among the 30 colorectal adenocarcinomas of the study, 56.67% and 33.33% of tumors were classified as expressing p53 and Bcl-2 respectively.

Qasim *et al*. (2012) reported that the frequency of P53 positive cases was significantly higher in colorectal carcinoma than adenoma. Furthermore,

according to Menezes *et al*. (2010) the immunohistochemical expression of p53 was 85.4% positive while the expression of Bcl-2 was positive in 31.7% of studied samples.

Although Ck20 reported high positivity in the current study (58%) Zlatian *et al*. (2014) reported higher expression values of Ck20 in most of the cases of colorectal cancer (80%). Moreover, other studies showed high frequencies of Ck20 in colorectal adenocarcinomas reaching 81% and 84% (Bayrak *et al*., 2012; Wong *et al*., 2009).

Kras positivity in this study was similar to that reported by Zlatian *et al*., (2014) who demonstrated that the Kras protein expression in colorectal cancer was identified in 52% of cases studied. Moreover, Kras cytoplasmic positivity was observed in 42.3% of cases (Elsabah and Adel, 2013).

Kras mutation were considered to have an important role in the multi-step process early in carcinogenesis. Kras mutations are highly prevalent in colorectal cancer and understanding the factors that regulate Kras expression may lead to future effective therapeutic strategy (Arrington *et al*., 2012). In the current study, the detected Kras gene mutations frequencies were within the general detection rates by most of the other studies clarifying that codon12 and codon13 were detected in more than one third of the samples (35%) with significant relation between malignant tumor and Kras gene mutation (P. value 0.000 and P. value 0.029 for codon12 and codon13 respectively). Also, as expected, among samples with Kras gene mutations, codon 12 was highly presented (74.3%) than codon 13 (25.7 %). Those findings might affect possible anti-EGFR therapy as patients with colorectal cancer harboring codon 13 mutations were reported to respond better to anti-EGFR therapy compared to patients with codon 12 mutations (Stolze *et al*., 2015).

Comparable findings were reported by Chretien *et al*. (2013) who detected Kras mutations in 38.0% of the colorectal cancer tumors. Of these, 82.4% were reported in codon12 while 17.6% were in codon 13. Similarly, 33.5% of Kras mutations were detected in another study with 82% frequency of codon 12 mutations among all detected Kras mutations (Nakanishi *et al*., 2013).

Relevant values were reported by other studies including a study by Samowitz *et al*., (2000) who reported that the Kras mutations were identified in 31.8% of tumors. Of these, 77.9% were in codon 12 and 22.1% were in codon 13. Also Jonsson *et al*., (2009) reported 39% Kras mutations in colorectal cancers tumors. A study in Egypt by El-Serafi *et al*., (2010) identified KRAS mutations in 32.2% of codon 12 and 8.9% of codon 13 in colorectal cancer. Furthermore, Stec *et al*., (2012) reported that Kras gene mutations were present in 27.8% of codon12 and 3.7% of codon 13.

In an attempt to clarify the relationship between immunohistochemical expression (P53, Kras, Bcl-2 and Ck20) and cancer grade, the study indicated that the expression of all markers was not significantly correlated with cancer grade ($P > 0.05$).

When positive P53 expression was analyzed according to the degree of differentiation of the tumor; 63.5 of % of well differentiated tumors expressed p53, slightly lowered expression was noticed among the moderately differentiated tumors 61% while only 50% of poorly differentiated tumors showed expression. Indicating that P53 (although not significant) might be correlated with poor outcome and bad prognosis. Over the past two decades, p53 has been one of the most studied presumptive prognostic markers in colorectal cancer. The lack of a clear consensus in the literature on P53 prognostic significance may be because of the use of heterogeneous study

populations, different antibodies, variations in cut-off values, patient stages included and duration of follow up (Theodoropoulos *et al*., 2009).

These findings seemed to be similar to those reported by another study by Rambau *et al*., (2009) who noticed that P53 was expressed in 65.9% of well differentiated tumors, 54.3% of moderately differentiated group and 45.5% of poorly differentiated tumors.

The same pattern was observed when Kras expression was addressed; in which 47% of well differentiated tumors were positive for the marker, 53% of moderately differentiated samples expressed the gene and only 14% expression was observed in poorly differentiated tumors.

Although Bcl-2 expression among poorly differentiated samples was again exceptionally lowered (14.3%); well differentiated tumors showed only half expression (22%) of that of moderately differentiated tumors (41%) with insignificant relation between Bcl-2 status and histological degree. Indicating that apoptotic machinery might be playing an important role in metastatic colorectal malignancies. Lack of significance was reported by Contu *et al*., (2006) who noticed that there was insignificant association between Bcl-2 status and histological degree.

Ck20 was likewise showing differential expression in the analyzed subset of samples representing 53.6% of well differentiated, 64.7% in moderately differentiated samples and 37.5% of poorly differentiated tumors.

Similarly, Menezes *et al*., (2010) found no significance when cell differentiation was correlated with P53.

Furthermore, the relation between tumor grade and Ck20 expression showed no association according to Awad-Elkareem and Mohammed (2016). Insignificant association between both Bcl-2 and P53 and histological grade of colorectal adenocarcinomas was reported by Shashi Kiran *et al*., (2016).

Kras expression was also not significantly different between the two grades according to Elsabah and Adel (2013).

Considering the relation between Kras gene mutations and cancer grade, the mutant genotype frequency of both targeted codons (codon 12 and 13), was not significantly associated with the cancer grads ($P > 0.05$).

Several authors reported lack of association between tumor grade and Kras mutation. Including Japanese patients (Kawazoe *et al*., 2015), Japanese (Kadowaki *et al*., 2015), Norway (Andersen *et al*., 1997) Chinese (Ye *et al*., 2015) and Irish patients (Morrin *et al*., 1994).

The relation between Kras mutations and immunohistochemical expression was also investigated. Kras was expressed in 69.2% of codon 12 mutant samples and 44.4% of codon 13 mutant samples. However, the relation between Kras expression and mutant samples was significant in codon 12 only.

Similarly, immunohistochemical expression of P53 and Ck20 was not related to codon 13 mutant samples and significantly related to codon 12. On the other hand, Bcl-2 was significantly related to codon 13 mutant samples.

This is an agree with Birkeland *et al*. (2012) who reported that the Kras amplification was highly significantly correlated to pathological P53 expression estimated by immunohistochemistry (P. value 0.000).

Nevertheless, Shetty *et al*., (2013) did not find correlation between Kras mutation and rate of P53 overexpression in the 62 patients tested. Also, CK20 expression was not associated with Kras mutations according to Yatabe *et al*., (2004).

The present study clarified that there was a significant relation between the characteristics data (Gender, Age and Site of tumor) and histopathological diagnosis ($P < 0.05$). The analysis also revealed that the disease occurred at

a significantly higher frequency at a later age, especially in patients with rectal cancer. Immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 was not correlated with tumor grade. P53 and Ck20 expression was not related to codon 13 mutant samples and significantly related to codon 12. On the other hand, Bcl-2 was significantly related to codon 13 mutant. These findings can be used to baseline data for diagnostic strategies for colorectal cancer among Sudanese.

CHAPTER SIX

Conclusion and Recommendations

6.1 Conclusion

On the basis of this study we concluded that:

- Most patients of colorectal tumors in this study appear to be above 50 years old of age.
- Colorectal cancer was more detected in female and the rectal was the most effective origin than colon.
- Molecular of Kras gene mutation and immunohistochemical expression of P53, Kras, Bcl-2 and Ck20 were higher in the colorectal malignant tumor compared than benign tumor.
- The incidence of codon 12 was higher than codon 13 in Kras gene mutations.
- The immunohistochemical expression of p53, Kras, Bcl-2 and Ck20 and Kras gene mutation are not related to the histological grading of colorectal tumors.

6.2 Recommendations

On the basis of this study we recommended that:

- Molecular methods for P53 and Bcl-2 gene mutations are recommended to be used in the investigation of colorectal tumors.
- Sequencing methods should be used to detect Kras gene mutation.
- Immunohistochemical panels of the Kras, Ck20, P53 and Bcl-2 tumor markers can be used in differential diagnosis for colorectal cancer, nevertheless molecular methods for kras gene mutation can be combined to refine and detect unknown rare phenotypes.

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APPENDICES

Appendix I

Materials and instruments used for processing and the staining of the specimens

Instruments:

Disposable gloves.

Rotary microtome.

Microtome knifes.

Coated slides.

Glass slides.

Cover glasses.

Dray oven.

Water bath.

Coplin jars.

Humidity chamber.

Polymerase chain reaction (PCR) machine.

Desktop micro centrifuge for eppendorf type tubes.

Gel documentation system.

Pipettes.

Freezer.

Refrigerator.

Eppendorf tube.

Vortex mixer.

Permanent marker.

Materials:

Ethyl alcohol (100, 90, 70, 50).

Xylene.

Distilled water (D.W).

Mayer's haematoxylin.

Citrate buffer (6.8PH).

Phosphate buffer (7.4PH).

Primary antibody (p53, ck20, kras and bcl-2).

Avidin.

Biotin.

Wash buffer (PBS)

Substrate buffer.

Substrate Chromogen.

Tri-Borate EDTA buffer (TBE).

Ethidium bromide.

Lysis solution.

ATE buffer.

Washing solution 1

Washing solution 2

Ethanol 100%

QIAamp DNA FFPE Tissue

QIAGEN extraction DNA.

PCR Premix kit (i-Taq).

BsuRI (HaeIII).

Mval (BstNI).

Preparation of solutions and stain

Mayer's haematoxylin:

Haematoxylin 1g.

Distilled water 100g.

Potassium Alum 50g.

Sodium iodates 0.2g.

Citric acid 1g.

Chloral hydrates 50g.

The haematoxylin, potassium alum, and sodium iodate are dissolving in the distilled water by warming and stirring.

The chloral hydrate and citric acid are added, and then mixture is boiled at five minutes, then cold and filtered.

Buffer reagents:

Phosphate buffer saline (PBS):

Six packets of PBS containing sodium phosphate dibasic, sodium phosphate monobasic and sodium chloride.

The PBS supplied in each packet sufficient for preparing 1 liter of phosphate buffer saline.

Target retrieval solution:

Citrate buffer, PH 9.0, dilution: 1:50

Blocker regents:

Peroxides Blocker regents:

Peroxides inhibiter containing hydrogen peroxide and 0.03 mol/L sodium azide. Dilution: 1:20

Preparation of TBE (250 ml):

Tris base	2.695 gm
Boric acid	1.376 gm
EDTA	0.186 gm
pH	8.3

Running buffer

TBE	25 ml
Distilled water	225

Appendix II

Kites leaflets