

Sudan University of Science and Technology

College of Graduate Studies

**Immunohistochemical Detection of Alfa1-antitrypsin among
Sudanese Patients with Colorectal Tumors**

الكشف النسيجي الكيمائي المناعي لالفا انتى تريبتسين لدى المرضى
السودانيين باورام القولون والمستقيم

**A dissertation submitted for partial fulfillment of the requirements of the degree of
M.Sc in medical laboratory sciences (histopathology and cytology)**

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بسم الله الرحمن الرحيم

قال تعالى:

فَإِذَا بَلَغْنَا أَجَلَهُنَّ فَأَمْسِكُوهُنَّ بِمَعْرُوفٍ أَوْ فَارِقُوهُنَّ بِمَعْرُوفٍ
وَأَشْهِدُوا ذَوِي عَدْلٍ مِّنْكُمْ وَأَقِيمُوا الشَّهَادَةَ لِلَّهِ ذَلِكَ يُوعِظُ بِهِ مَنْ
كَانَ يُؤْمِنُ بِاللَّهِ وَالْيَوْمِ الْآخِرِ وَمَنْ يَتَّقِ اللَّهَ يَجْعَلْ لَهُ مَخْرَجًا
﴿٢﴾ وَيَرْزُقْهُ مِنْ حَيْثُ لَا يَحْتَسِبُ وَمَنْ يَتَوَكَّلْ عَلَى اللَّهِ فَهُوَ حَسْبُهُ
إِنَّ اللَّهَ بَالِغُ أَمْرِهِ قَدْ جَعَلَ اللَّهُ لِكُلِّ شَيْءٍ قَدْرًا ﴿٣﴾

صدق الله العظيم

سورة الطلاق الآيات 2-3

Dedication

To.....

My father..... who spend his life for us

To.....

My mother.....who support me every moment

To.....

My other family members.....whoseencouragement me

To.....

My memory of my friends and colleague

Amna, Hadeel, Mashier, Esra and Eman

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Abstract

This is a hospital based case control study conducted in national public health laboratory during the period from December 2016 to April 2017, the study aimed to detect the expression of Alfa1-antitripsin in the diagnosis of colorectal tumors.

A total of 40 formalin fixed paraffin blocks previously diagnosed as colorectal tumors, 20(50.0%) samples were benign colorectal and 20(50.0%) samples were malignant colorectal tumors were selected for this study. One section of three microns was cut from each block and stained by immunohistochemical method (avidin biotin technique) for Alfa1-antitripsin detection. The data obtained were analyzed using SPSS computer program version 20.

The patient ages ranged between 26-80 years with mean age of 53 years, most of them 22(55.0%) were above 50 years and the remaining 18(45.0%) were less than 50 years.

Regarding gender patient sex revealed that 12(30.0%) patients were female and 28(70.0%) patients were male.

Alfa1-antitripsin was positive in 21(52.0%) samples, while 19(47.0) samples were negative. From positive result 11(27.5%) benign colorectal samples while 10(25.0%) malignant colorectal samples with insignificant correlation between Alfa1-antitripsin and colorectal tumors (P.value 0.752).

The study concluded that there is no relation between expression of Alfa1-antitripsin and colorectal tumors.

المستخلص

اجريت هذه الدراسة التحليلية الأسترجاعية فيالمعمل القومي للصحة العامةخلالالفترةمن ديسمبر 2016 إلى ابريل 2017 لدراسة دورألفا-1 أنتي تريبيين في تشخيص اورام القولون والمستقيم.

جمع اربعون قالب شمعي لهذهالدراسةمغمورةفيالبرافينتمتشخيصهامسبقا كعينات أورام للقولون والمستقيم, منها 20(50.0%) عينة مشخصة بسرطان القولون والمستقيم و20(50.0%) عينة مشخصة كأورام حميدة.قطعت القوالب بسمكثلاثة مايكرون وصبغت بطريقة الكشف النسيجي الكيميائي المناعي باستخدام تقنية الافيدين بايوتين.وحلت البياناتالتيتماالحصولعليهاباستخدامبرنامج الحزمة الاحصائية للعلوم الاجتماعية إصدار20.تراوحتأعمار المرضييين26-80سنة لمتوسط عمر 53 عاما،ومعظمأعمار المرضي تركزت فوق 50عاما 22 (55.0%) وكانت أعمار البقية أقل من 50 عاما 18 (45.0%). أما توزيع جنسالمرضي فكان 12 (30.0%) إناثو 28 (70.0%) ذكور.

ظهر التعبير الإيجابي لألفا-1 أنتي تريبيين وسط مجتمع الدراسة في 21(25.0%) عينة ونتيجة سلبية في 19 (47.0%) عينة. من مجموع 21 عينة والتي أعطت نتيجة إجابية كان منها 11 (27.5%) عينة في الأورام الحميدة و 10 (25.0%) في الأورام الخبيثة مع وجود عدم علاقة بين ألفا-1 أنتي تريبيين و أورام القولون والمستقيم(القيمة الاحتمالية تساوي 0.752). خلصت الدراسة إلى عدم وجود علاقة بين افراز ألفا-1 أنتي تريبيين وأورام القولون والمستقيم.

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List of Abbreviations

CT	Computed tomography
CRCs	Colorectal cancers
DAB	3,3- diaminobenzidine tetra hydrochloride
DPX	Distyrene plasticizer xylene
FAP	Familial adenomatous polyposis
HNPCC	Hereditary non polyposis colon cancer
HPP	Hyperplastic polyposis
MAP	MUTYH-associated polyposis
PJS	Peutz-Jeghers syndrome
JPS	Juvenile polyposis syndrome
MRI	Magnetic resonance imaging
α_1 -AT	Alpha-1 antitrypsin
ERUS	Endorectal ultrasound
FDG	Fluorodeoxyglucose
PET	Positron emission tomography
SPSS	Statistical package for social sciences
VEGF	Vascular endothelial growth factor
AFP	Alfa 1-fetoprotein
TGF	Transforming growth factor
CD	Cluster of differentiation
IBD	Inflammatory bowel disease

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Chapter one

Introduction

1.1 Introduction:

Colorectal carcinoma is the most common malignancy of the gastrointestinal tract (Kulaylat, 2015).

Colorectal cancer is a major cause of morbidity and mortality throughout the world. It is the third most common cancer worldwide and the fourth most common cause of death. Countries with the highest incidence rates include Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk include China, India, and parts of Africa and South America (Hagger and Boushey, 2009). Colorectal cancer in Sudan was found less in young patient below the age 40 and 50 years old, highest incidence of patients were between the ages of 41 and 60 years (Taha, *et al.* 2015)

Several risk factors are associated with colorectal cancer include family history, heredity, such as polyposis and hereditary no polyposis (Amersi, *et al.* 2005).

Diagnosis of colorectal cancers includes colonoscopy, computed tomography (CT or CTC), magnetic resonance imaging (MRI), endorectal ultrasound (ERUS) (De Rosa, *et al.* 2015).

Treatment of colorectal cancer depends on several factors, such as race, age, and stage at diagnosis (Amersi, *etal.* 2005). Surgery is the first treatment option for early stage to provide cure, adjuvant chemotherapy

with the aim of eradicating any micrometastatic residual disease following surgery. Targeted therapies with constant and careful monitoring for early detection and treatment of toxicities, along with best supportive care (Kordatou, *etal* .2014).

Alpha-1 antitrypsin (α_1 -AT) is a serum glycoprotein and it is a member of the serine protease inhibitors (serpins) family. There are many types of malignant cells are expressed and secreted α_1 -AT. Alpha-1 antitrypsin in colorectal carcinoma is related to the invasive and non-invasive capacity and thus it may serve as a biological marker for prognosis of colorectal carcinomas at relatively early stages (El-Akawi, *etal*. 2013).

1.2 Objective

- To detect the expression of Alfa1-antitrypsin in colorectal tumors using immunohistochemistry and correlate the histopathological diagnosis.

Chapter Two

Literature Review

2. Literature Review:

2.1. Scientific back ground:

Colorectal cancer is the third most common cancer among both men and women. A colorectal cancer begins when normal cells in the lining of the colon or rectum change and grows uncontrollably, forming a mass called a tumor (Khan, *etal.* 2013).

Colorectal cancer is one of the most prevalent and incident cancers worldwide, and is one of the most deadly (Binefa, *etal.* 2014).

Colorectal cancer (CRC) is one of the most common cancers in the Western world .The lifetime risk for developing CRC is ~6%, with a 5-year survival of ~55%.Most of patients are diagnosed with CRC above the age of 70 years (Van Wezel, *etal.*2012).

2.2. Anatomy and physiology of colon and rectum:

Colon is divided into four portions: ascending, transverse, descending, and sigmoid. Ascending colon lies on the right side of the abdomen, transverse extend from the hepatic flexure to the splenic flexure, descending colon lies in vertical position on the left side of abdomen and sigmoid meaning S shaped it is downward below the iliac crest (Thibodeau and Patton, 2003).

Rectum is measure 13-18 in length,The rectum has intraperitoneal and extra peritoneal components and is divided into upper, middle and lower parts (Kulaylat, 2015)

2.3. Abnormalities of colon and rectum :

2.3.1. Inflammation of colon and rectum:

Inflammatory bowel disease (IBD) encompasses a range of intestinal pathologies, the 2 major clinical entities ulcerative colitis and Crohn's disease show overlapping features and a similar symptom profile which can include; diarrhea, rectal bleeding, abdominal pain and weight loss (Jurjus, *et al.* 2016).

2.3.2. Precancerous changes of colon and rectum:

Polyps any mass protruding arise from the mucosal layer of gastrointestinal organ, colorectal polyps may be histologically classified as neoplastic and hyperplastic (Shussman and Wexner, 2014)

Colorectal adenoma is the most frequent precancerous lesion (Conteduca, *et al.* 2013). Adenomas classify into tubular, tubulovillous and villous (Bujanda, *et al.* 2010).

2.3.3. Malignant tumors of colon and rectum:

2.3.3.1. Adenocarcinoma:

Adenocarcinoma of the colon is the most common histopathological type of colorectal carcinoma (Tumwine, *et al.* 2012). Adenocarcinoma count about 90% of all colorectal cancer and have sub types: mucinous adenocarcinoma, signet ring cell and medullary carcinoma. The mucinous major about 10 percent of adenocarcinoma, signet ring cell less than 1 percent while medullary extremely rare (Fleming, *et al.* 2012).

2.3.3.2. Carcinoid and neuroendocrine tumors:

Carcinoid tumors are most commonly found in the gastrointestinal tract. The management of these lesions depends upon the size of the lesion, involvement of the muscularis, location, and presence of metastatic disease (Chung and Hunt, 2006).

2.3.3.3. Other colorectal cancer:

2.3.3.3.1. Lymphoma:

Colorectal lymphoma is rare and it accounts less than 1% of all colorectal malignancy. Most patients with colorectal lymphoma treated by chemotherapy (Quayle and Lowney, 2006)

Primary lymphoma of the colon is a rare tumor of the gastrointestinal (GI) tract and comprises only 0.2-1.2 % (Tauro, *etal.* 2009).

2.3.3.3.2. Leiomyosarcoma:

Leiomyosarcoma arising in the colorectal is a rare smooth muscle tumor that occurs primarily in the sigmoid and transverse colon accounting for less than 1% of all malignancies of colon and rectum. It is most frequent occurring in middle-aged patients between 50 and 70 years of age with an equal male: female ratio. The histological criteria show interlacing bundles of spindle-shaped smooth-muscle cells, as well as cellular pleomorphism, vasoinvasion, large hyperchromatic nuclei with prominent nucleoli and presence of bizarre tumor cells (Faraj, *etal.* 2015)

2.3.3.3.3. Malignant melanoma:

Primary anorectal melanoma is a rare, major about 1% of all anorectal carcinomas. Present in fifth or sixth age especially on women and prognosis is very poor and almost die because of metastases (Van, *etal.* 2008).

2.4. Staging of colorectal cancer:

Staging of colorectal cancer is the strongest predictor of survival and accurate staging also is critical for appropriate patient management and meaningful clinical research (Compton and Greene, 2004).

2.5 Signs and symptoms of colorectal cancer:

Colorectal cancer has primary symptoms include rectal bleeding, change in bowel habit and abdominal pain. They may also present with systemic

symptoms – anorexia, significant weight loss, fatigue and symptoms of anaemia – features usually suggestive of advanced disease (Smith, *etal.* 2006).

Most important symptoms of colorectal cancer are weight loss and rectal bleeding (Adelstein, *etal.* 2011).

2.6. Risk factors of colorectal cancer:

2.6.1. Family history:

Colorectal carcinoma usually occurs in one of three patterns: sporadic, inherited, or familial. Sporadic.20% to 25% of cases occur in patients who have other family members who have also had colon cancer (Amersi, 2005).

2.6.2.Dietary factors

Dietary factors that increase the risk of CRC include low fruit, vegetable, or fiber intake, high red meat or saturated fat consumption, and exposure to caffeine (Lin, 2009).

Vegetable fiber and total fiber play very important roles in protecting against colorectal cancer. Soluble and insoluble fibers were inversely associated with only colorectal cancer and colon cancer(Song,*etal.* 2015).

2.6.3. Genetic factor

Alterations in single genes increased risk of colorectal cancer includehereditary nonpolyposis colorectal cancer (HNPCC), familial adenomatous polyps (FAP), MUTYH-associated polyposis (MAP),hamartomatous polyps are the primary lesions in Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). Finally, hyperplastic polyposis (HPP) is an unusual condition that has a substantial cancer risk and must be distinguished from the other conditions(Jasperson, *etal.* 2010).

2.6.4. Tobacco smoking

Tobacco smoking showed a 20% increased risk of colorectal cancers (Johnson,*et al.* 2013).

2.6.5. Alcohol consumption

There is a strong association between alcohol consumption and increased risk for colorectal cancer and it is stronger among men than women (Fedirko,*etal.* 2011).

2.6.6. Side-effects of medical interventions

It includes pelvic irradiation, cholecystectomy, and ureterocolic anastomosis after major surgery of the urinary and intestinal tracts (Lin, 2009).

2.7. Diagnosis of colorectal cancer:

2.7.1. Colonoscopy:

Colonoscopy is the best choice for screening high-risk populations, as it allows simultaneous detection and removal of preneoplastic lesions (Del, *etal.* 2015). Colonoscopy is considered the standard for colon evaluation and it is time-consuming (Yee, 2002).

Colonoscopy allows for greater diagnostic specificity and sensitivity compared with other types of examinations and it plays an important role in the prevention and removal of adenomatous polyps (Lee,*etal.* 014).

2.7.2. Fecal occult blood test:

Fecal occult blood test (FOBT) screening improves detection of colorectal cancer at the first screening round (Paimela, *etal.* 2010).

2.7.3. Barium enema:

Barium enema is an equivalent option for colorectal cancer screening of the general population and high risk patients (Seth, 2000).

2.7.4. Imaging techniques:

2.7.4.1. Computed tomography scan (CT scan):

Computed tomography is not routinely performed for detection of colon cancer, CT is useful for detect tumor into adjacent organs or distant metastases, staging, treatment planning and follow-up (Horton, *etal.* 2000).

The advantages of CT are that it presents minimal risk to patients, has a short procedure time (about 10 minutes) (Yee, 2002).

2.7.4.2. Ultra sound (US):

Abdominal ultrasound presents high sensitivity and specificity in the diagnosis of colon cancer (Martinez, *etal.* 2005).

2.7.4.3. Positron emission tomography (PET) scan:

Positron emission tomography (PET) with ¹⁸F-fluorodeoxy, (FDG) is a functional imaging modality that provides mapping of glucose metabolism in the whole body and highly accurate in detecting early localized tumor recurrence with high sensitivity and specificity (Nachar, 2002).

2.7.4.4. Magnetic resonance imaging (MRI):

MRI is the recommended modality for initial staging, due to its high accuracy for the definition of localization, determining the total extension and the relationship of the tumor to the peritoneal reflection (Kekelidze, *etal.* 2013).

2.7.5. Immunohistochemistry:

Immunohistochemistry plays an important role in differentiating tumor types, assessment of aggressiveness and recognizing of metastasis origin use many marker for this aim such as P53, BCL2, E-cadherin, CD44, α 1-fetoprotein (AFP), VEGF receptor (VEGF-R), TGF β -RI and TGF β -RII, PCNA (Mihalache and Rogoveanu, 2011)

2.7.6. Tumor marker:

2.7.6.1. Alpha-1 Antitrypsin:

Human alpha-1 antitrypsin (AAT), also known as alpha1 proteinase inhibitor (α 1-Pi) and SERPINA1 (Serine Protease Inhibitor, group A, member 1), is a circulating glycoprotein whose main function is to inhibit neutrophil elastase and other serine proteases in blood and tissues (Pérez-Holanda, *et al.* 2014).

Alpha-1 antitrypsin (α ₁-AT) is a member of the serine protease inhibitors (serpins) family. Liver cells are the major source of synthesis and secretion of (α ₁-AT) into the blood. Moreover, it has been demonstrated that α ₁-AT is expressed and secreted by many types of malignant cells. Studies have indicated that serum levels of (α ₁-AT) increase in a good number of malignant diseases(El-Akawi, *etal.* 2013).

Studies have indicated relationship between AAT deficiency and the development of colorectal cancer. On the one hand, a recent retrospective large study from the UK registry of patients with severe AAT deficiency confirmed a higher prevalence of ulcerative colitis than would be expected in the general population (Holanda and Blanco, 2016).

2.8. Management of colorectal cancer:

Treatment options are dependent on the stage of the disease, the performance status of the patient, and increasingly the molecular makeup of the tumor(Stintzing,2014).

The management of CRC should be a multi-modal approach according to tumor localization, extent and biology, and patient factors. Although optimal surgical resection is still the mainstay of curative treatment, optimal multi-modal treatments could maintain long-term survival, quality of life and even cure in selected patients(Nakayama, *etal.* 2014).

Chapter Three

Materials and Methods

3. Materials:

3.1. Materials:

Archive tissue blocks previously diagnosed as colorectal tumors were used in this study.

3.2. Methods:

3.2.1. Study design:

This is analytical retrospective hospital based case control study aimed to detect the role of alfa1- antitrypsin in diagnosis of colorectal tumors.

3.2.2. Study samples:

Forty colorectal tissue formalin fixed paraffin blocks were obtained from tissues previously diagnosed as colorectal tumors at national public health laboratory during the period from December 2016 to April 2017. Patient identification data including age and sex was obtained from patient`s file.

3.2.3. Sample processing:

Section of 3 μ in thickness was obtained from each formalin fixed paraffin wax embedded tissue using rotary microtome, then dewaxed in oven.

3.2.4. Staining Method:

Immunohistochemical staining:

Paraffin sections were immunostained using avidin biotin technique. Sections were put in oven and cleared in two change of xylene for two minutes, then rehydrated through descending concentration of ethanol (100%, 90%, 70% and 50%) and water two minutes for each, then antigen retrieved by water path for forty minutes, then treated with 3% hydrogen peroxide and methanol solution for fifteen minutes, then washed in phosphate buffer saline (pH7.4) for five minutes. Then treated with Alfa1-antitrypsin primary antibody for twenty minutes, then rinsed in phosphate buffer saline then binding of antibody detected by

incubating for twenty minutes with biotin followed by fifteen minutes with streptavidin (Thermo kit), then the sections were washed in three changes of phosphate buffer saline, then treated with substrate and 3,3-diaminobenzidine tetra hydrochloride (DAB) chromogen for seven minutes, then washed in phosphate buffer saline, then counterstained in Mayer's haematoxylin for one minute, then washed and blued in running tap water, then dehydrated through ascending concentration of ethanol (50%, 70%, 90%, 100%), then cleared in xylene and mounted in DPX mountant (Bancroft and Marilyn, 2008).

3.2.5. Result interpretation:

All quality control measures were adopted during sample staining for immunohistochemical results assessment. Positive and negative controls were used to confirm location of positivity of alfa1 antitrypsin expression.

3.2.6. Statistical analysis:

Data were analyzed using SPSS version 20 computer program. Frequencies, means and Chi-square value were calculated.

3.2.7. Ethical considerations:

Hospital administration agreements were taken ethically for archive samples and patient data collection.

Chapter Four

Result

4. Results:

The study involved forty blocks, previously diagnosed as colorectal tumors were used in this study, 20(50.0%) samples were benign colorectal tumors and 20(50.0%) samples were malignant colorectal tumors (table 4.1). The patient sex revealed that 12(30.0%) patients were female and 28(70.0%) patients were male (table 4.2). The patient ages ranged between 26-80 years with mean age 53 years, most of them 22(55.0%) were above 50 years and the remaining 18(45.0%) were less than 50 years (table 4.3). Positive expression of Alfa1-antitrypsin among study population is 21/40 sample, positive expression of Alfa1-antitrypsin among benign 11/20 and in malignant 10/20, negative expression of Alfa1-antitrypsin among study population is 19/40 sample, the negative expression of Alfa1-antitrypsin seen in malignant 10/20 and in benign 9/20 with insignificant correlation between Alfa1-antitrypsin and colorectal tumor (P. value 0.752), (table 4.4).

Table (4.1): Distribution of histopathological diagnosis among study population:

Diagnosis	Frequency	Precent
Benign	20	50
Malignant	20	50
Total	40	% 100

Table (4.2): Distribution of sex among study population:

Sex	Frequency	Precent
Male	28	70%
Female	12	30%
Total	40	100%

Table (4.3) Distribution of age groups among study population:

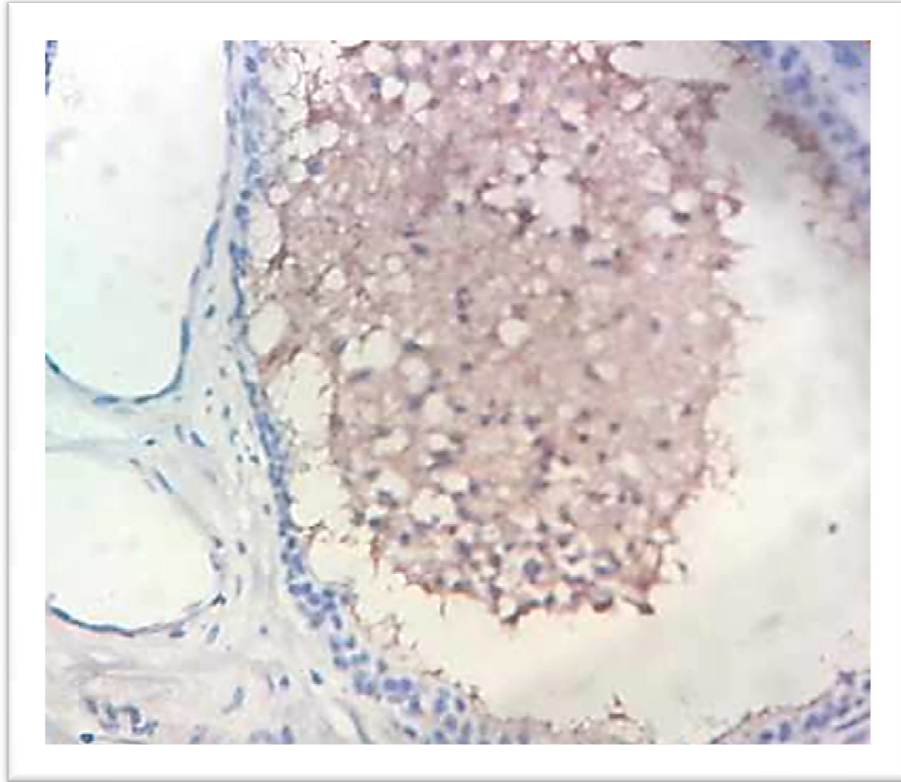
Age group(years)	Frequency	Percent
50>	18	45.0%
50<	22	55.0%
Total	40	100%

Table (4.4): Expression of Alfa1-antitrypsin among study population:

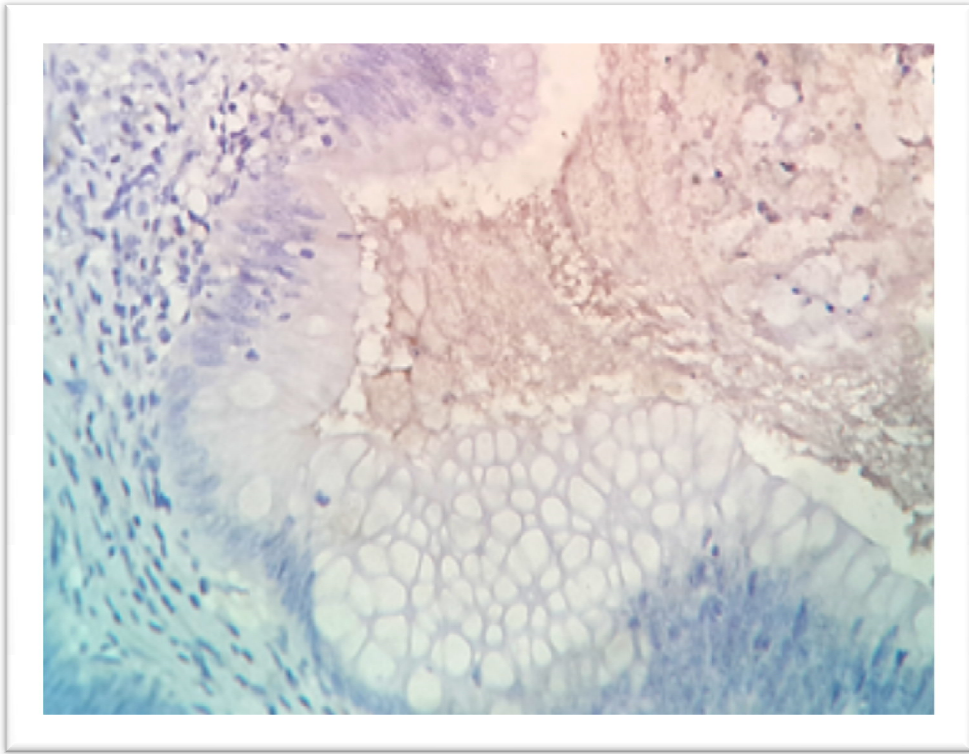
Expression	Frequency	Percent
Positive	21	52.5%
Negative	19	47.5%
Total	40	100%

Table (4.5): Relation between Alfa1-antitrypsin expression and histopathological diagnosis:

Histopathological Diagnosis	Expression of Alfa1- antitrypsin		Total	P.value
	Positive	Negative		
Benign	11(27.5%)	9(22.5%)	20(50.0%)	0.752
Malignant	10(25.0%)	10(25.0%)	20(50.0%)	
Total	21(52.5%)	19(47.5%)	40(100 %)	



Photograph (4.1): Positive expression of Alfa1-antitrypsin in non-malignant colorectal (40x).



Photograph (4.2): Positive expression of Alfa1-antitrypsin in benign colorectal (40x).

Chapter Five

Discussion

5. Discussion:

The present study involves forty cases of colorectal tumors were used for immunohistochemical detection of Alfa1-antitrypsin.

Regarding the age of study population the study revealed that most of patients were more than 50 years. This result compatible with Rudy and Zdon. (2000), they reported that number of patient was increased in patient from 40 to 50 years and increased more with patient than 80 years of age.

The study revealed that most of patients are men, with female: male ratio 1:2, differences in sex hormones might explain the lower female: male ratio. This result agrees with Murphy *et al.* (2011), they reported that colorectal cancer incidence and mortality rates are about 30% to 40% higher in men than in women. Also Boyle and Leon (2002) reported that colorectal cancer incidence are higher in men than in women. The expression of Alfa1-antitrypsin in colorectal tumors were positive in 11 (27.5%) in benign tumors, while in malignant tumors 10 (25.0%) cases were positive, this result explain there was no significant difference between Alfa1- antitrypsin and histopathological diagnosis of colorectal tumors (P. value 0.752). This result disagree with study of Karashima *et al.* (1990), who reported that the degree of Alfa 1-antitrypsin positive tumor cells was significantly higher in advanced cases than in early cases of colorectal cancer.

Chapter Six

6. Conclusion and recommendation

6.1 Conclusion:

On basis of this study we conclude the follow:

- The age of the colorectal tumors patient in our study is commonly more than 50 years.
- The sex of the colorectal tumors patient in our study is more in male than female.
- There is no association between Alfa1-antitrpsin and colorectal tumors.

6.2 Recommendation:

On basis of this study we recommend the follow:

- Further studies should be done with large sample size to detect another adhesion molecule in colorectal tumors.

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Appendices

Instrument and Material:

Instrument:

Disposable gloves.

Rotary microtome.

Microtome knives.

Coplin jars.

Oven.

Staining racks.

Coated slides.

Water path.

Cover glass.

Dako pen.

Humidity chamber.

Materials:

Mayer`s haematoxylin.

Xylene.

Ethyl alcohol (absolute, 90%, 70%, 50%).

Distill water.

Peroxidase blocker.

Primary antibody (Alfa 1 antitrepsin) .

Secondary antibodies (biotinylated secondary antibody).

3.3 di amino benzidine tetra hydrochloride in substrate buffer.

DPX mounting media.

Phosphate (PH7.4) component:

Solution A (0.2 M sodium di hydrogen orthophosphate, 3.12g di sodium hydrogen orthophosphate, 100ml DW).

Solution B (0.2 M sodium di hydrogen orthophosphate, 2.83g di sodium hydrogen orthophosphate, 100ml DW) (9.5ml from solution A + 40.5ml solution B).

Citrate buffer(PH6.8) component:

Solution A (0.2 M sodium di hydrogen orthophosphate, 2.83g di sodium hydrogen orthophosphate, 100ml DW).

Solution B (2.1g citric acid, 100ml DW) (72.7ml from solution A +22.8ml from solution B).

Mayer`s haematoxylin component:

Haematoxylin powder 1 gm

Potassium alum or ammonium alum 50gm

Sodium iodate 0.2 gm

Citric acid 1 gm

Chloral hydrate 50gm

Distilled water 1000ml

Ammoniated water:

Concentrated ammonia 0.05ml

Tap water 99.95ml