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Molecular study for the correlation between *Fusobacterium nucleatum* and colorectal cancer

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Doctorate of Philosophy in science Medical Microbiology

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جمـــهوريـــة العـــراق
وزارة التعليم العالي والبحث العلمي
جامعة بابـــــــــل
كليـــــــة الطـــــب

دراسـة جزيئية للعلاقة بين البكتـريا المغزليـــة وسـرطان القولون والمسـتقيم

أطروحة مقدمة إلى

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قَالُواْ سُبْحَٰنَكَ لَا عِلْمَ لَنَآ إِلَّا مَا عَلَّمْتَنَآ ۖ
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صَدَقَ اللهُ العَلي العَظيم

سورة البقرة الآية (٣٢)

Supervision Certificate

We certify that this thesis entitled **molecular study for the correlation between *Fusobacterium nucleatum* and colorectal cancer Diseases** is prepared under our supervision at the Department of Microbiology, college of medicine, University of Babylon, in fulfillment requirements for the degree **of Doctor of Philosophy in Science of Microbiology .**

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JASSIM 2021

Dedication

To

My father and my mother,

My Wife, and my sons

My Brothers, and all friends

JASSIM 2021

تعد البكتيريا المغزلية جزءًا من ألأحياء الطبيعية للبلعوم الفموي وأرتبط وجودها في الأمعاء بتطور سرطان القولون والمستقيم . تعززهذه البكتريا من تطور الورم وذلك عن طريق إحداث الالتهابات والاستجابة المناعية للمضيف في البيئة المايكروبية لسرطان القولون والمستقيم .

امتد جمع العينات والعمل العملي لهذه الدراسة خلال الفترة من فبراير(٢٠٢٠) إلى مارس(٢٠٢١).كان اجمالي عينات المرضى(٤٠)عينة انقسمت بين (٢٢) للذكور و(١٨) للاناث والتي تتراوح اعمارهم من(٣٠ ــ ٨٠ سنة) حيث تم جمع عشرين عينة من النسيج لمرضى سرطان القولون والمستقيم والمعاملة بالفورمالين والمثبتة بالبارافين (FFPE) وأيضا الحصول على عشرين عينة من الأنسجة الطازجة ، من المرضى الذين حضروا إلى مركز الجهاز الهضمي في مدينة الإمام الحسين (عليه السلام)الطبية ومستشفى الكفيل التخصصي ومستشفى الإمام زين العابدين في كربلاء المقدسة. تم استخراج الحمض النووي(DNA) من عينات الأنسجة لاستخدامها لاحقًا في الدراسة. ,وأيضا تم الحصول على عينات الدم من جميع المرضى الذين تم التأكد من أصابتهم بسرطان القولون والمستقيم وعند زيارتهم لمركز الاورام في مدينة الامام الحسين (عليه السلام) الطبية واستخراج (DNA) و (RNA) منها لاستخدامها لاحقا في الدراسة .

كذلك تم جمع عشرين عينة دم وخزعة طازجة من الأفراد الذين زاروا مركز الجهاز الهضمي في مدينة الإمام الحسين (عليه السلام) الطبية ، ومستشفى الكفيل التخصصي في كربلاء المقدسة كمجموعة سيطرة . تم استخراج (DNA) و (RNA) من جميع العينات لاستخدامها لاحقًا في الدراسة .

تتضمن الدراسة الحالية الكشف عن عدد النسخ للبكتريا المغزلية في مرضى سرطان القولون والمستقيم بواسطة (Quantitative Real – time PCR) وقد أظهرت النتائج أن هناك زيادة معنوية في عدد نسخ عزلات البكتريا المغزلية في المرضى عند مقارنتها مع مجموعة السيطرة .

يعتبر بروتين الالتصاق (FadA)عامل ضراوة معروف في البكتريا المغزلية مما يساهم في تسهيل الارتباط وغزو البكتيريا. أشارت الدراسة الحالية إلى أن من مجموع (٤٠) عينة للمرضى ، (٨٠٪) أعطت نتائج إيجابية لتحديد جين(*fadA*) بطول جزيئي حوالي (232 bp) .

كما تمت دراسة القابلية الوراثية لمرضى سرطان القولون والمستقيم المرتبطة بتعدد الأشكال الجيني [Micro RNA 146 SNP (rs2910164)] باستخدام تقنية نظام تضخيم الطفرة الحرارية تم تحديد نتائجه بواسطة الترحيل الكهربائي للهلام للأليلين (C وG) وا ظهرت النتائج بان الأليل C في (163 bp) بينما ألأليل G في (179 bp) ، بينما كانت نتيجة حجم (two outer primers) في (294bp) . كما

أظهرت النتائج أن معظم المرضى كانوا يحملون النوع الاصيل ، وأن هناك علاقة مؤثرة وخطيرة بين تعدد الأشكال الجيني (microRNA 146a) ومرضى سرطان القولون والمستقيم.

تضمنت الدراسة الحالية للكشف عن التعبير الجيني للأنترلوكين ١٠ والانترلوكين ١٨باستخدام real- time (PCR) في مرضى سرطان القولون والمستقيم واظهرت النتائج أن مستوى الانترلوكين ١٠وايضا الانترلوكين ١٨ ينخفض في مرضى سرطان القولون والمستقيم عند مقارنته بمجموعة السيطرة .

الجزء الثاني يتضمن دراسة الارتباط بين الجنس والعمر والإقامة ودرجة الإصابة بسرطان القولون والمستقيم وأظهرت النتائج عدم وجود فروق ذات دلالة إحصائية بين الذكور والإناث ، كما أشارت النتائج إلى أن خطر الإصابة بالمرض يبدأ في سن الأربعين ويزيد ما بين (٤٥-٦٥)سنة لكلا الجنسين. كما أظهرت النتائج بأنه لاوجود لفرق معنوي بين سكان الحضر والريف المصابين بسرطان القولون والمستقيم.

اظهرت نتائج الدراسة الحالية فروقًا بين مراحل الاصابة بالسرطان لمرضى سرطان القولون والمستقيم وفقًا لمستوى البكتريا المغزلية حيث كانت الدرجة الثانية أعلى من الانواع الآخرى لمرضى سرطان القولون والمستقيم .

Summary

Fusobacterium was considered as part of the normal flora of the oropharynx and the presence of *Fusobacterium nucleatum* in the gut is associated with the development of colorectal cancer (CRC). *Fusobacterium nucleatum* promotes tumor development by inducing inflammation and host immune response in the microenvironment.

The samples collection and the practical work of the present study extended through the period from February 2020 to the March 2021. A total of (40) Patients with confirmed CRC were recruited in the study includes (Male 22 and female 18). Age range (30-80) years .

Twenty Samples were collect from colorectal cancer (CRC) tissue patients in formalin fixed paraffin embedded (FFPE) and Twenty fresh tissue biopsy were obtained from patients who attended to gastrointestinal center in Imam Hussein medical city, Al-Kafeel Specialist Hospital and Imam Zain Al-Abidin Hospital in Holy Karbala. DNA was extracted from tissue samples in order to be used later in the study. Also blood samples were obtained from all patients with confirmed CRC and when they visited the oncology center in Imam Hussein Medical City, DNA and RNA was extracted from all samples in order to be used later in the study .

Twenty of blood and fresh biopsy samples were collected from individuals who visit the gastrointestinal center in Imam Hussein medical city, and Al-Kafeel Specialist Hospital in Holy Karbala as a control group . DNA and RNA was extracted from all samples in order to be used later in the study . The present study include detection the copy number of *Fusobacterium nucleatum* in CRC patients by Quantitative Real – time PCR **.** The results show that there was significant increase in copy number of *Fusobacterium nucleatum* isolates in patients when compared with control group.

Summary

The Adhesion protein *fadA* is a knownas virulence factor in *Fusobacterium nucleatum* contributing to early attachment and invasion of bacteria . The present study indicated that eighty percent give positive results for identification of *fadA* gene with molecular size 232bp.

The genetic susceptibility to CRC patients also study in associated with gene polymorphisms Micro RNA 146 SNP (rs2910164) SNP by using ARMS PCR technique .The amplicon were identify by gel electrophoresis as the allele C ,G . Were allele C appeared in163bp while product size of G allele in 179bp ,while the product size two outer primers 294bp. Also the results showed that the most patients were carrying genotype ,and there is a significant risky association between microRNA 146a gene polymorphisms and colorectal cancer patients.

The present study use to detect the expression of IL-10 ,IL-18 mRNA gene by using Real- time PCR (Relative gene expression) in CRC patients and the results found that the level of IL-10 ,IL-18 decrease in CRC Patients when compared with control groups.

The second part includes studying the correlation of gender , age , residence and grade of colorectal cancer disease. The results showed that there was no significant different between males and females ,also the results indicate that the risk of developing the disease begins at the age of 40 years and increases between 45-65 years for both sexes. Also the results showed a no significant difference between urban and rural residents with colorectal cancer patients . The results of the present study found difference between histologic grade of colorectal cancer patients according to the level of *Fusobacterum nucleatum*, the moderately differentiated grade II is a higher than other type of CRC disease.

1 .1 . Introduction :

Colorectal cancer (CRC)was the third most common cancer type worldwide and the second leading cause of human cancer associated mortality in 2020 (World Health organization., 2021).The pathogenesis of colorectal cancer is complex in terms of environmental factors, epidemiological studies have indicated that high-fat diet, obesity and a western lifestyle increase the risk of colorectal cancer (Mafiana *et al* .,2018). *Fusobacterium nucleatum* was considered as part of the normal flora of the oropharynx formerly, but lately its pathogenic role is found especially as a promote of periodontitis and its association with intestinal diseases has been demonstrated (Griffen *et al* .,2012).

Fusobacterium nucleatum is a genus of gram-negative anaerobic bacteria. It may act as a main anchor of biofilms that can induce periodontitis (Okuda *et al* ., 2012), vaginitis (Machado *et al* ., 2015) and other infections (Sanmillan *et al* ., 2013).
Although it is still unclear whether *Fusobacterium nucleatum* is the passenger or driver of colorectal cancer many studies have concluded that *Fusobacterium nucleatum* is a novel risk factor for colorectal cancer development and progression, as well as a determinant affecting patient survival outcomes (Mehta *et al* ., 2017) .

In colorectal adenoma, an early event in CRC development, *Fusobacterium nucleatum* is found to be enriched in comparison with surrounding normal tissue suggesting an essential role of *Fusobacterium nucleatum* in the early onset of CRC in recent years, a large number of studies have indicated that the intestinal flora is closely associated with the occurrence of colorectal cancer (Yamashiro *et al* ., 2017).

Fusobacterium nucleatum adheres to and invades the intestinal mucosa through its surface adhesion factors and virulence proteins, and ultimately promotes the occurrence and development of colorectal cancer (Han *et al* ., 2015). It has previously been identified that the absolute copy number of *Fusobacterium nucleatum* in CRC tissues may be used as an indicator to evaluate the prognosis of patients with colorectal cancer (Yamaoka *et al* ., 2018).

Recent studies have demonstrated that *Fusobacterium nucleatum* is not only associated with the development of colorectal cancer , but also promotes chemotherapeutic resistance in colon cancer via Toll-like receptor 4 (TLR4)/ nuclear factor kappa-B (NF-κB) pathway-induced autophagy (Zhang *et al* ., 2019).

Additionally, significantly larger proportions of *Fusobacterium* has been detected from feces of adenoma and colorectal cancer patients in comparison to healthy controls, distal metastasis of colonic cancer was also found to be colonized with *Fusobacterium nucleatum* and other assembled microbes investigators have also shown that tumor proliferation and cancer growth could be reduced via decreasing the load of *Fusobacterium nucleatum* by antibiotic treatment metronidazole (Bullman *et al* ., 2017).

In terms of genetic factors, the majority of colorectal cancer cases exhibit genomic instability, including microsatellite and chromosomal instability. In addition, gene mutations, including tumor suppressor gene inactivation and oncogene activation, serve an important role in the pathogenesis of colorectal cancer (Dienstmann *et al* .,2017). *Fusobacterium nucleatum* is associated with certain epigenetic phenotypes of colorectal cancer high degrees of microsatellite instability and CpG (5'- cytosine - phosphate -guanine - 3') island methylation phenotype (CIMP) (Mitsuhashi *et al* ., 2015).

Metagenomics and transcriptional analyses have revealed that compared with adjacent normal tissues, the enrichment of Bacteroidetes and Firmicutes is decreased in human CRC tissues, but the enrichment of *Fusobacterium nucleatum* is significantly increased (Yamamura *et al* ., 2017).

Aims of the study:

Study the role of the *Fusobacterium nucleatum* in colorectal cancer patients and gene susceptibility at molecular level .

So the objective of this study include :

1. Genomic DNA extracted from CRC fresh biopsy and FFPE to the patients groupe and control group.
2. Collection of blood samples from patients with CRC and control groups, DNA and RNA was extracted from all samples.
3. Detection the copy number of *Fusobacterium nucleatum* in CRC patients by Quantitative Real – time PCR .
4. Detection the Adhesion protein *fadA* as a known virulence factor in *Fusobacterium nucleatum*.
5. Study the genetic susceptibility to colorectal cancer patients in associated with gene polymorphisms Micro RNA 146a SNP by using ARMS PCR technique.
6. Detection the expression of IL10 ,IL18 mRNA genes by using real- time PCR (Relative gene expression) in CRC patients and compared with control group.

1.2 . Literature Review:

1. 2 . 1 . A heterogeneous genus of *Fusobacterium* :

Fusobacterium is a cylindrical shaped, gram-negative, non-spore-forming, strictly anaerobic genus. Although *Fusobacterium* is part of the normal microbiome, recent findings indicated that increased *Fusobacterium* levels have been detected in various inflammatory (Sekizuka *et al*., 2017).There are 14 species in *Fusobacterium*, such as *Fusobacterium. necrophorum* inhabitant of the alimentary tract and being responsible for Lemierre' syndrome and *Fusobacterium varium* found in the ulcerative colitis . Among them, *Fusobacterium. nucleatum* is one of the key pathogens which plays a role in oral plaque formation, due to its adhesive ability, serving as a bridge organism during colonization and biofilm formation (Kolenbrander *et al* ., 2006).

Although numerous studies suggest that *Fusobacterium nucleatum* strains might vary in their virulence potential, it is has been wondered that some *Fusobacterium nucleatum* strains can acquire genes through horizontal transfer and obtain increased virulence potential from different species and strains regardless of the mechanism in which *Fusobacterium nucleatum* achieves its virulence, evidence points to the positive correlation of this *Fusobacterium nucleatum* toward CRC malignancy (Ang *et al* ., 2016).

Fusobacterium nucleatum is a Gram-negative, anaerobic oral commensal bacterium that is associated with a variety of human diseases, including periodontal disease(Chaushu *et al*., 2012) Alzheimer's disease (Sparks *et al*., 2012), brain abscess (Kai *et al*., 2008), cardiovascular disease (Genco *et al* ., 2002), miscarriage (Chanomethaporn *et al* ., 2018) and inflammatory bowel disease (Strauss *et al* .,2011). Recently, *Fusobacterium nucleatum* has been proposed to be associated with colorectal cancer (Sears *et al* ., 2018).

Fusobacterium nucleatum promotes the occurrence of colorectal cancer through several virulence mechanisms colonization, invasion, and modulation of host immune response (Bullman *et al* ., 2017) .

Fusobacterium nucleatum bacteria interact with each other by expressing a variety of different virulence factors, and can adhere to many different mammalian cell types, including epithelial and endothelial cells, polymorphonuclear neutrophils, monocytes, erythrocytes, fibroblasts, and natural killer (NK) cells (Liu *et al* ., 2019).

1.2. 2 . Pathogenesis:

1.2.2.1. Adhesion and invasion of *Fusobacterium nucleatum* into host tissue cells :

Fusobacterium nucleatum is aninvasive organism the bacterium invades host with the aid of a surface adhesion molecule called *fadA*, which is abundantly expressed on *Fusobacterium nucleatum* (Han *et al*.,2005). The *fadA*, a membrane protein, encoded by *Fusobacterium nucleatum* binds to E-cadherin on the epithelial cell surface and leads to E-cadherin phosphorylation and internalization. This, in turn, activates β-catenin signaling pathways and consequently leads to inflammation and tumorigenesis gene transcription such as nuclear factor kappa-B (NF-κB) , cellular Myelocytomatosis (c-Myc), lymphoid enhance factor/T cell factor (Rubinstein *et al* ., 2013).

Moreover they detected higher *fadA* expression levels in CRC tissue compared with normal tissue. Reliably, the expression of oncogenes, inflammatory genes and Wnt signaling pathway genes(*Wnt* gene) are increased in CRC cells under modulation of purified FadA as an invasive organism that survives inside host cells, *Fusobacterium nucleatum* is also capable of releasing RNA into the host cell cytoplasm that is detected by cytosolic retinoic acid-inducible gene 1 (RIG-1), triggering activation of NF-κB and activating inflammatory genes and oncogenes (Lee *et al* ., 2014).

Fusobacterium nucleatum also has a strong ability to induce co-aggregation and shuttle unrelated microbes in particular *Streptococcus* and *Campylobacter* into host cell via Fap2, a large membrane protein present in *Fusobacterium nucleatum* (Warren *et al* ., 2013).The shuttled microbes are known to have weak binding ability to host cells or they are non-invasive being shuttled into the host cells by *Fusobacterium nucleatum* , the toxic effects on endothelial cells become multifold (Coppenhagen-Glazer *et al* .,2015).

It's recently reported that *fadA* can up-regulate Wnt/β-catenin modulator Annexin A1 expression through E-cadherin (Rubinstein *et al* ., 2019). *fadA* can also bind to vascular endothelial cadherin (VE-cadherin), which is a linker molecule on endothelial cells (Vander Haar *et al* ., 2018).

This combination alters the integrity of the endothelium, increases the permeability of the endothelium, and allows the bacteria to overcome the blood brain barriers, placental barriers, and colonize different parts of the body (Fardini *et al* ., 2011). Outer membrane vesicles (OMVs) from *Fusobacterium nucleatum* can degrade E-cadherin, thus promoting bacterial invasion and tumor metastasis(Fardini *et al*., 2019).

In addition, *Fusobacterium nucleatum* also has two other outer membrane proteins, *fap2* and *RadD*. The lectin *fap2* can bind galactose/N-acetylgalactosamine (Gal-Gal NAc), a polysaccharide overexpressed in CRC. This binding of *fap2* facilitates colonization of *Fusobacterium nucleatum* and explicates fusobacteria abundance in CRC (Abed *et al* ., 2016). *RadD* can mediate communication between *Fusobacterium nucleatum* and other bacterial species, contributing to the formation of multispecies biofilms(Kaplan *et al*., 2010) which has been shown to be associated with proximal colon cancer (Yu *et al*., 2016).

Though limited literature has proved a direct relationship between *Fusobacterium nucleatum* colonized in gingival sulcus and colorectal cancer tumorigenesis, there might be some connections between oral flora and gut flora, as well as connections

between oral infectious diseases and intestinal diseases such as colorectal cancer combined detection of fecal and oral microbes may also enhance prediction of adenomas or colorectal cancer in addition to other risk factors, it is possible that altering intestinal *Fusobacterium nucleatum* abundance could be approached by targeting oral *Fusobacterium nucleatum* (Flemer *et al*.,2017).

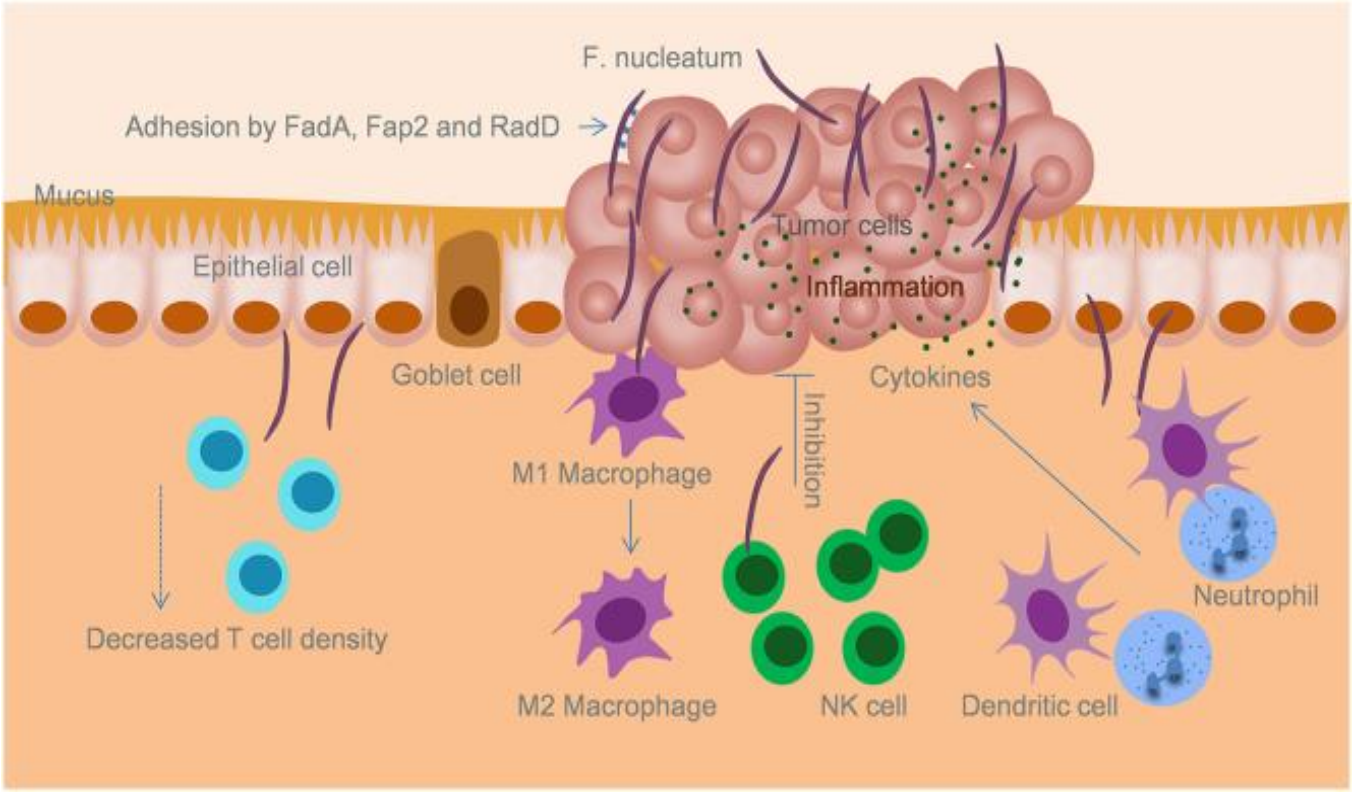
In addition to the pathogenic ability of *Fusobacterium nucleatum* to co-aggregate, invade and alter the host immune system, the bacterium may also play a role in pathogenicity through the release of tissue irritants and proteases. *Fusobacterium nucleatum* produces and releases copious amounts of butyrate, proprionate and ammonium ions, which are common tissue irritants that can inhibit the proliferation of fibroblasts and compromise wound healing (Ohkusa *et al*., 2003).

Butyrate, although known to be anti- tumorgenic and anti-inflammatory when properly metabolized by aerobic respiration, can also act as a histone deacetylase inhibitor in circumstances, such as in a biofilm or a tumor, where anaerobic respiration is inefficient at fully metabolizing butyrate. The metabolic intermediate, butyric acid, can enter the host cell's nucleus where it acts as a histone deacetylase inhibitor which results in epigenetic regulation (Leonel & Alvarez-Leite, 2012).

Butyric acid can result in hyper-acetylation and maintenance of less condensed chromatin structure, suppresses nuclear factor kappa-B a pro-inflammatory transcription factor that plays a key role in eliciting the host's immune response to non-self antigens and can also aid in the activation of genes implicated in apoptosis, cell differentiation and cell cycle arrest in cancer cell (Leonel & Alvarez-Leite, 2012).

The degradation of host proteins by *Fusobacterium nucleatum* enzymes may also contribute to the tissue damage and inflammation seen in many of the diseased sites inhabited *Fusobacterium nucleatum* .Through the release of effector proteins such as the serine protease, the bacterium is capable of degrading native host proteins, including

Immunoglobulin A, a key player in the immediate response of the host innate and adaptive immune system. Furthermore *Fusobacterium nucleatum* produces a large amount of the toxic metabolite hydrogen sulfide (H_2S). It has been demonstrated that hydrogen sulfide damages epithelial cells, increases the permeability of mucosa and is associated with the modification and release of hemoglobin in erythrocytes, endotoxin induced inflammation and the apoptosis of smooth muscles cells and fibroblasts (Yoshida *et al*.,2010).



Figure(1-1): Adhesion, invasion, inflammation and immune suppression mediated by *Fusobacterium nucleatum* in CRC . The bacterium binds to colon epithelium through *fadA, fap2* and *RadD* (Jiao *et al* .,2019).

1.2.2.2 . Mechanisms of *Fusobacterium nucleatum* pathogenesis in CRC.

The *Fusobacterium nucleatum* invades the human epithelial cell by a protein *fadA* which is in two major forms the first form is the intact pre-*fadA* consisting of 129 amino acids that is anchored to the membrane, and the second form is the secreted mature *fadA* consisting of 111 amino acids that are secreted outside of bacterium.When m*fadA* combines with pre-*fadA*, the pre-*fadA*-m*fadA* is internalized, and *fadAc* is activated . The internalization of the pre-*fadA* and mFadA complex ensures the bacterium binds to and invades host epithelial cells (Xu*etal*.,2007).

Furthermore, *fadA* binds to vascular endothelial cadherin (VE-cadherin) , a cell-cell junction molecule identified as the endothelial receptor for *fadA*. *fadA* binding causes VE-cadherin to relocate and increases the endothelial permeability, which then facilitates *Fusobacterium* and other bacteria species to penetrate into the blood stream (Fardini *et al* ., 2011).

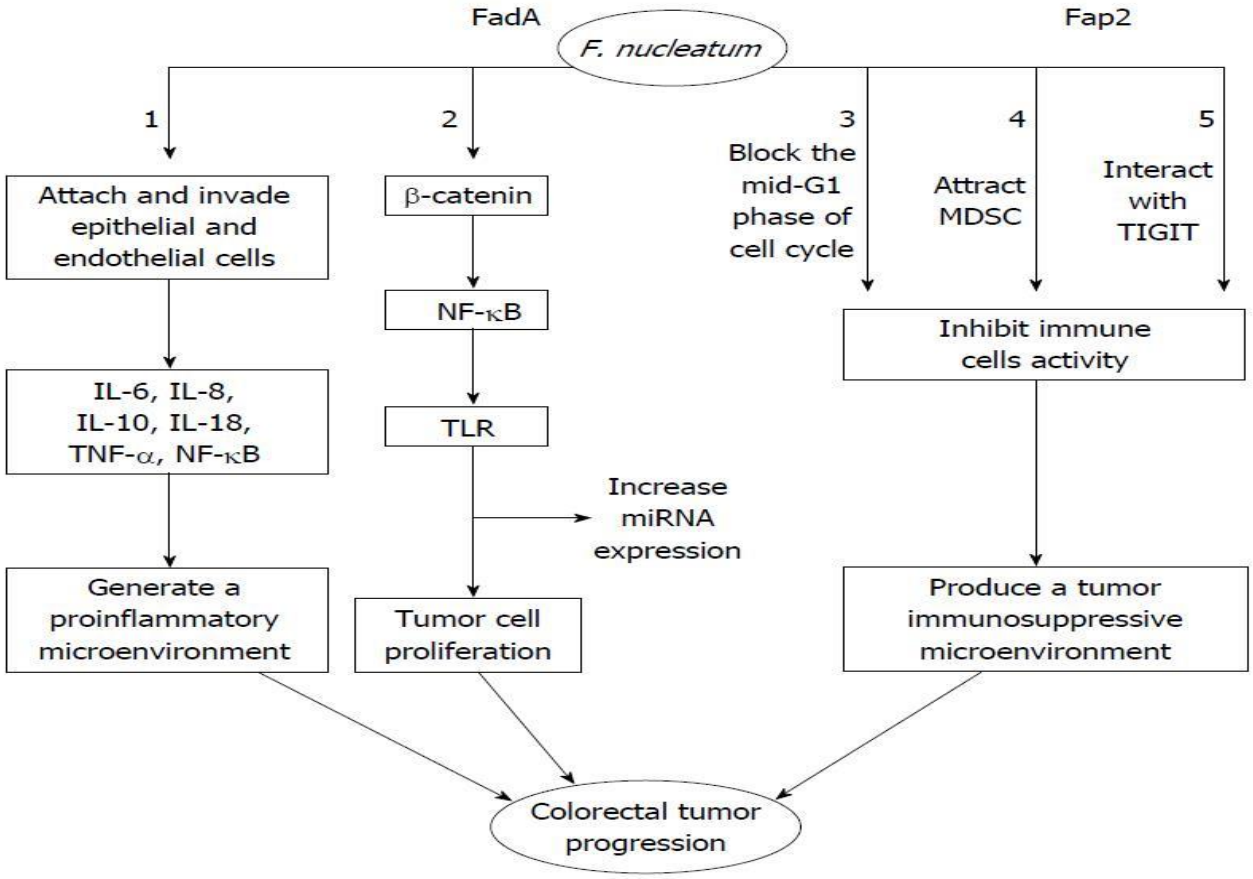
The cell surface protein *fadA* is a key virulence factor in *Fusobacterium nucleatum* which regulates adhesion and invasion of the bacterium. The expression of *fadA* gene in human CRC specimens was significantly higher than that in adjacent normal tissues (Rubinstein *et al* ., 2013). This protein enables *Fusobacterium nucleatum* to bind E-cadherin in CRC and epithelial cells, activate the β-catenin pathway, and induce the expression of transcription factors lymphoid enhancer factor (LEF)/T cell factor (TCF) which promote tumor cell growth (Chen *et al* ., 2017).

The host endothelial receptor for *fadA* is the vascular endothelial cadherin which is a member of the cadherin family it is encoded by the human gene *CDH5* . The CDH5 receptor is required for *Fusobacterium nucleatum* to adhere and invade endothelial cells (Fardini *et al* ., 2011).

Fusobacterium nucleatum invasion induces the production of cytokines such as interleukin-8 (IL-8), which is regulated by the MAPK signaling pathway but

independent of Toll-like receptor (TLR), Nucleotide Binding Oligomerization Domain Containing 1(NOD-1), NOD-2 and (NF-κB) signaling. The bacterium promotes the expression of several inflammatory genes such as NF-κB and cytokines, including IL-6, IL-8 and IL-18 (Rubinstein *et al* .,2013).

The bacterium also promotes the release of inflammatory cytokines particularly IL-10 and tumor necrosis factor-α (TNF-α) in a pro-inflammatory microenvironment that accelerates colorectal tumor progression (Quah *et al* .,2014).



Figure(1-2): Mechanism of *Fusobacterium nucleatum* pathogenesis in colorectal cancer (Shang and Hong ., 2018).

1.2.3 . Carcinogenic mechanisms of *Fusobacterium nucleatum*:

A number of studies have been conducted to study the carcinogenicity of *Fusobacterium nucleatum*. There are three biomolecules that are located on the surface of bacterium including lipopolysaccharides (LPS), *fadA* and *Fusobacterium nucleatum* auto-transporter protein 2 which are involved in and promote the occurrence of colorectal cancer figure (1-3),(Zhang *et al* ., 2018)

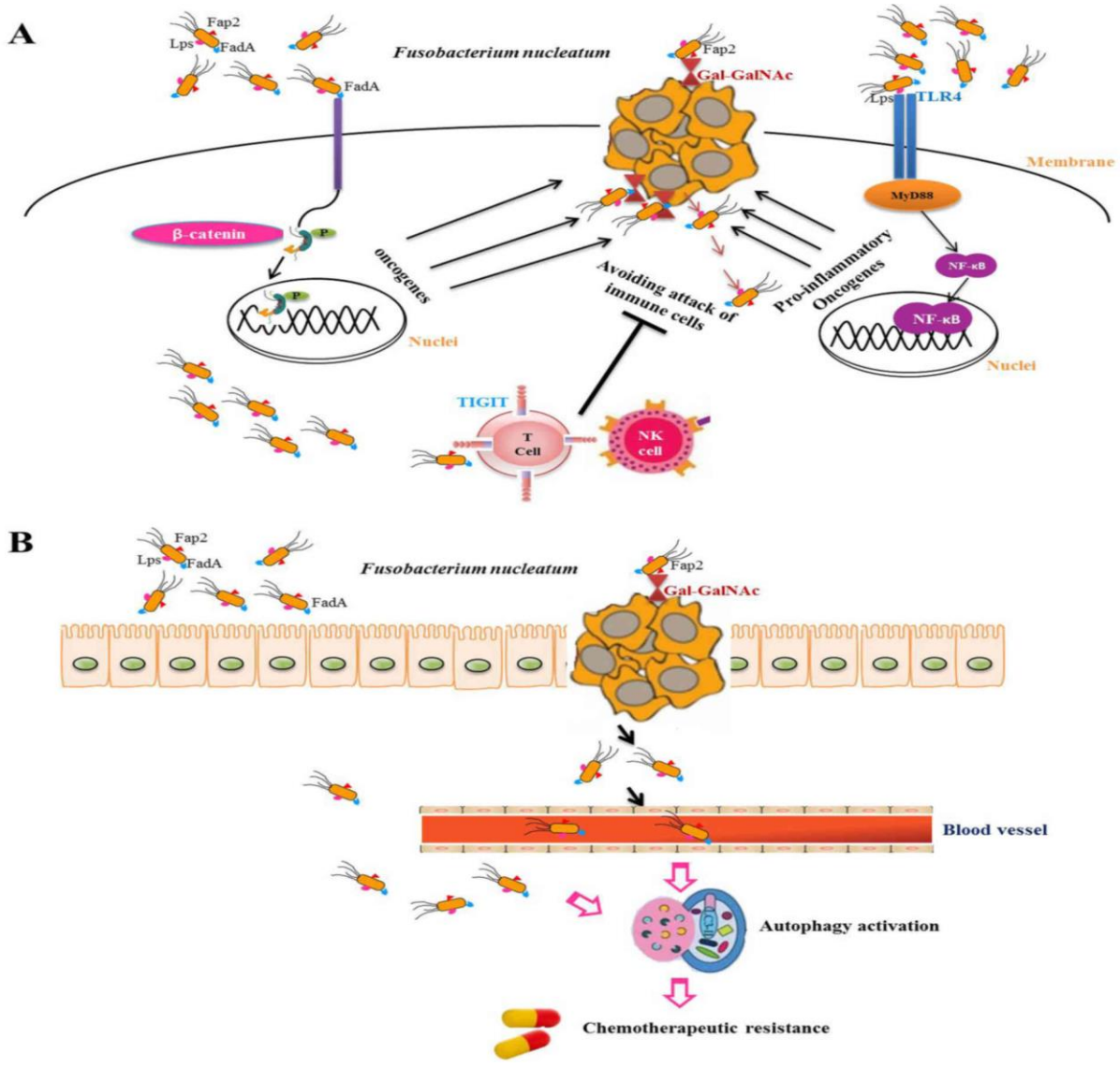
Therefore, it has been speculated that *Fusobacterium nucleatum* promotes the occurrence and development of colon tumors by down regulating the T-cell-mediated adaptive immune antitumor effects. The presence of *Fusobacterium nucleatum* is negatively correlated with the density of CD^{3+} T cells in colorectal cancer tissues (Mima *et al*., 2015).

Fusobacterium nucleatum promotes the release of pro-inflammatory cytokines, including interleukin 8 (IL-8), IL-10 and tumor necrosis factor-α (TNF-α) (Quah *et al* .,2014). In addition, studies have demonstrated that the bacterium promotes the mRNA expression of TNF-α, IL-1β, IL-6, IL-8, prostaglandin-endoperoxide synthase 2 (PTGS2), nuclear factor κ-β and matrix metallopeptidase 3, and the expression of these genes is induced in mouse and human cells treatment with *Fusobacterium nucleatum* (Trinchieri *et al*.,2012). The generation and release of these inflammatory cytokines constitutes to the immune microenvironment for the occurrence of colorectal cancer (Taniguchi and Karin ., 2018).

The bacterium adsorption and invasion into mucosa is a prerequisite for its role in carcinogenesis (Fardini *et al* .,2011). A *fadA* virulence protein is expressed on the surface of *Fusobacterium nucleatum* . This virulence protein is highly conserved for the bacterium (Han *et al* ., 2005).

Some studies have revealed the oncogenic mechanism of bacterium and identified FadA as a potential diagnostic and therapeutic target for CRC. Notably, another study demonstrated that *Fusobacterium nucleatum* activated the β-catenin signaling pathway

via lipopolysaccharide-mediated Toll-like receptor 4(TLR4)/Protein activated kinase 1(PAK1) in CRC . TLR4 activates the β-catenin signaling pathway, forming intestinal tumors, while PAK1 is associated with colorectal cancer progression and metastasis (Wu *et al* ., 2018).



Figure(1-3): Potential mechanism of *Fusobacterium nucleatum* in colorectal cancer (Yang *et al* ., 2019).

Therefore, *fadA* virulence protein cannot fully explain the selection specificity of bacterium .The highly expressed polysaccharide, D-galactose-β-N-acetyl-D-galactosamine (Gal-Gal NAc), is present in CRC tissues. The biosynthesis of Gal-Gal NAc is increased gradually from normal mucosa, adenoma to CRC tissues. Gal-Gal NAc binds to *fap2*, another protein on *Fusobacterium nucleatum* , leading to high enrichment of bacterium in CRC tissues (Arnold *et al* .,2017).

Another study on *fap2* demonstrated that *Fusobacterium nucleatum* binds to the T cell immunoreceptor with Ig and tyrosine-based inhibitory motif domains (TIGIT) via *fap2* and that TIGIT is widely present on the surface of natural killer and T cells, thereby preventing immune cells from attacking CRC cells (Gur *et al*., 2015).

Fusobacterium nucleatum also inhibited the activity of human T cells by blocking the G1 phase of the cell cycle. This study suggested the presence of an bacterium - dependent tumor immune escape mechanism revealed that the bacterium activated NF-κβ signaling via the innate immune pathway [TLR4/MYD88 innate immune signal transduction adaptor (MYD88)] and miRNA-21, and increased the proliferation of CRC cells and the development of tumors. Compared with non-neoplastic colon tissues, the bacterium DNA and miRNA-21 levels are significantly increased in CRC tissues, and the levels were even higher in advanced CRC tissues. This indicated that patients with both *Fusobacterium nucleatum* DNA and high miRNA-21 exhibit a higher risk of poor prognosis. This study revealed that the bacterium regulates the expression of miRNAs, but the specific mechanism that is not yet understood (Yang *et al*., 2017).

Another study identified that *Fusobacterium nucleatum* is associated with Microsatellite instability (MSI)–high, and B-Raf proto-oncogene serine/threonine kinase (BRAF) mutations. In CRC molecular typing, CpG island methylation phenotype (CIMP) and MSI are mainly identified in the right hemi colon, which may be related to the high enrichment of *Fusobacterium nucleatum* , but the specific mechanism needs to

be studied in more detail . Therefore, provided strong support for the pathogenic role of intestinal microbial components in CRC (Mima *et al* ., 2016).

The *Fusobacterium nucleatum* may be used as a diagnostic marker for preventing colorectal cancer recurrence and as a prognosis predictor. In addition, a series of experiments revealed that the bacterium selectively decreases miRNA-18a /4802 expression levels through the innate immune pathway (TLR4/MYD88) and subsequently activates cell autophagy via Unc-51 like autophagy activating kinase 1 to become resistant to chemotherapeutic drugs. This drug resistance was overcome by the autophagy blocker chloroquine (Yu *et al* .,2017).

1.2.4 . *Fusobacterium nucleatum* induce epigenetic change in tumor cell :

The high load of *Fusobacterium nucleatum* was associated with cancer tissues was related to CpG [5'—C (cytosine) —phosphate—G (guanine) —3'] island methylation phenotype (CIMP) status, high MSI and Human mut L homolog 1(MLH1) hypermethylation (Mima *et al* ., 2012) and up-regulating expression of microRNA-21. Interestingly, these specific molecular features of colorectal cancer occur mostly in the ascending colon (Phipps *et al* ., 2015).

The most common colonization site of *Fusobacterium nucleatum* is the GI tract. This might indicate some association between *Fusobacterium nucleatum* biogeography and the colonic mucosal microenvironment. CIMP is characterized by simultaneous hypermethylation of numerous CpG [5'—C (cytosine) —phosphate—G (guanine) —3'] islands surrounding the promoter regions of several genes. The high level of methylation of CpG [5'—C (cytosine) —phosphate—G (guanine) —3'] island indicates chronic inflammation and an aggravated immune response (Leung *et al* ., 2015).

Microsatellite instability is the somatic accumulation of length variations in repetitive DNA sequences. It has been established that defects in the DNA mismatch

repair (MMR) pathway lead to disincorporation, insertions and deletions in microsatellite in this repetitive DNA sequences. MSI is frequently observed in both hereditary and sporadic CRC (Westdorp *et al* ., 2016). Inflammatory state and reactive oxygen stress produced by *Fusobacterium nucleatum* may contribute to epigenetic silencing of the MMR protein MLH1 and reduction of its enzymatic activity, which leads to MSI colorectal cancer (Nosho *et al* ., 2016).

MicroRNAs, a small non-coding RNA functioning in RNA silence and regulating post-transcriptional gene expression, rapidly emerging as promising diagnostic and therapeutic targets, may be involved in the progression of cancer as well (Toiyama *et al* ., 2014). Studies suggest that *Fusobacterium nucleatum* might raise the level of microRNA-21 in tumor cells via epigenetic regulation during macrophage inflammatory response (Yang *et al* ., 2017).

It would be a possible explanation that CRC cells with frame-shift mutations in the absence of normal mismatch repair function produce a mutation-associated neo-antigen, which may activate anti-tumor immunity and enhance the effect of programmed cell-death ligand 1 (PD-L1) blockade. Some studies have indicated the relationship between gut microbiota and checkpoint immunotherapy . The impact of *Fusobacterium nucleatum* on checkpoint inhibitors still remains unclear (Sivan *et al* ., 2015).

1.2.5. *Fusobacterium nucleatum* and Immunity in CRCs:

1.2.5.1. *Fusobacterium nucleatum* and Innate Immunity :

Colorectal cancer display different degrees of infiltrating immune cells, essential dynamic populations of the tumor microenvironment (Grizzi *et al*.,2018). The host immune response is closely related with clinical disease behavior (Malesci *et al*.,2017).

Recently, it has been shown that the overload of *Fusobacterium nucleatum* elicits high levels of bacterium specific antibodies in colorectal cancer patients, implying that bacterium may escape host humeral immune responses by developing inside host cells (Xue *et al*.,2018).

Macrophages constitute the first line of defense against infecting pathogens. It has been reported that some aggressive intracellular bacteria can survive and disseminate in the cytoplasm of colonized macrophages (Halstead *et al* .,2010) . Immunosuppressive effect of *Fusobacterium nucleatum* by activating M2 polarization of macrophages through a Toll like receptor 4 (TLR4)dependent mechanism (Chen *et al*.,2018).

Although it has been shown that *Fusobacterium nucleatum* infection rapidly induces inflammation and macrophage infiltration in gingival tissues. In CRC tissues, *Fusobacterium nucleatum* - high was not significantly associated with cluster of differentiation (CD163+) M2 macrophage density but significantly with CD68+ tumor-infiltrating macrophages. Considering differences in the tumor-immune microenvironment of carcinomas with high or low MSI (Hamada *et al*., 2018).

The association of *Fusobacterium nucleatum* with immune response might differ by tumor MSI status. Using samples from rectal and colon cancer patients and measured *Fusobacterium nucleatum* DNA in tumor tissue and examined the association between the bacterium status and the density of cluster of differentiation 3(CD3+) ,(CD8+) , and Forkhead box P3 (FOXP3+)transcription factor regulatory T cell (Reg T cells) lymphocytes in strata of tumors by Microsatellite status. Considering differences in the tumor immune microenvironment between microsatellite instability - high and non- MSI high carcinomas, they concluded that in CRCs *Fusobacterium nucleatum* and Microsatellite status may interact to mediate anti- tumor immune reactions (Hamada *et al*., 2018) .

Given the tumorigenic role of *Fusobacterium nucleatum* and its immune evasion properties, it has been suggested that the bacterium elimination might improve treatment tumors. Interestingly, it has also been reported that TIGIT is an immune-receptor inhibitory checkpoint implicated in tumor immune-surveillance. Expression of TIGIT has been found in both NK cells and T-lymphocytes. The function of TGIT in tumor immune-surveillance has been found to be similar to the programmed cell death protein 1 (PD-1) programmed death ligand 1 (PD-L1) axis in tumor immuno-suppression (Solomon *et al* .,2018).

1.2.5.2. *Fusobacterium nucleatum* and the Adaptive Immunity :

The adaptive immune system primarily consists of tumor-infiltrating lymphocytes (TILs), comprising CD8+ CTLs and CD4+ T-helper lymphocytes (Pages *et al*., 2018). It is known that CD4+ T-lymphocytes release cytokines, which in turn activate and promote stimulation of CTLs, CD8+ T cells synthetize and release perforin and granzyme B, which mediate their cytotoxic activity (Shunyakov *et al*., 2004), and consequent anti-tumor actions, (Dalerba *et al*.,2003). Cluster of differentiation (CD4+) T cells are heterogeneous in function and their subsets can abolish antigen-specific T cell responses, alike the FOXP3+ regulatoryT-lymphocytes (T regs),(Scurr *et al* .,2012).

The specificity of these markers has been investigated since CD25 and FOXP3 might also be expressed by activated CTL . In the tumor contexture, Tregs may exert different functions as they might explode anti-tumor immunity, as well as reduce pro - tumor inflammation (Fridman *et al*.,2012).

The role of Fap2 protein in the onset of CRC has been reported. The authors demonstrated that Fap2, when bound to the human inhibitory receptor, T cell immune receptor with Ig and immune-receptor tyrosine-based inhibitory motif (ITIM), T cell immune-receptor with Ig and ITIM domains (TIGIT), suppresses the cytotoxic role of natural killer (NK) cells (Guevarra *et al* .,2018).

Ftusobacerium nucleatum in 76 out of 598 (13%) colorectal cancer investigated compared with *Ftusobacerium nucleatum* - negative cases . *Ftusobacerium nucleatum* -high cases were found inversely associated with the density of CD3+ T cells. The amount of bacterium was not significantly associated with the density of CD8+ ,or FOXP3+ T cells (Mima *et al*., 2015).

Regarding the association between the gut microbiome and immunity, several studies have shown that *Fusobacterium nucleatum* may exert immunosuppressive activities via inhibiting human T cell responses to mitogens and antigens (Lee *et al*., 2018). Moreover, it has been reported that *Fusobacterium nucleatum* inhibitory protein can arrest human T-lymphocytes in the G1 phase of the cell cycle (Das *et al* ., 2019).

Fusobacterium nucleatum can also induce apoptotic cell death in peripheral blood mononuclear cells (PBMCs) and Jurkat T-lymphocytes (Rubinstein *et al* .,2013). It has been shown that *Fusobacterium nucleatum* -induced cell death is mediated through the clustering of the immune cells, which might have important implications for the pathogenesis of this bacterial species (Castellarin *et al*.,2012).

These findings suggest that *Fusobacterium nucleatum* has a suppressive modulation of the tumor-immune microenvironment. Recently, The bacterium may suppress anti-tumor immune responses by decreasing CD4+ T cell density and thymocyte selection, expression in colorectal cancer (Chen *et al*.,2018).

1.2.6.*Fusobacterium nucleatum* promotes development from Inflammation to malignancy:

The *Fusobacterium nucleatum* could enhance the development from being in inflammatory state to malignancy. This finding was first reported by Kostic and colleagues when adenomatous polyposis coli (APC) mice were introduced to human isolates of *Fusobacterium nucleatum* (Kostic *et al* ., 2013).

Higher level of inflammation and more colonic tumors were found in the *Fusobacterium nucleatum* group compared to control. This may suggest that *Fusobacterium nucleatum* induce oncogenesis downstream of the adenomatous polyposis coli *APC* gene pathway and the tumorigenesis does not depend on pre-existing colitis condition because the colitis mice did not develop colon tumors after the bacterium introduction. The higher expression of IL-1 β, IL-6, IL-8, TNF, and Matrix metalloproteinase-3 (MMP3) by human and mouse cell line while co-culturing with *Fusobacterium nucleatum* suggests an NF-κB -driven pro-inflammatory response. The elevated expression of inflammatory genes such as NF-κB, IL-6, IL-8 and IL-18 were correlated with FadA level in colorectal cancer tissues (Rubinstein *et al*., 2013).

Additionally, *Fusobacterium nucleatum* also binds to Toll-like receptor 4 (TLR4) of epithelial cells and activate the TLR4/ myeloid differentiation primary response gene 88 (MYD88) / NF-κB signaling pathway. NF-κB separated with phosphorylated IκB and bound to DNA, followed with microRNA-21 transcription. microR-21 could inhibit RAS p21 GTPase activating protein (RASA1) and therefore activate the a class of small G-protein (RAS)/ rapidly accelerated fibrosarcoma (RAF)/ mitogen-activated protein kinase-ERK kinase (MEK)/ extracellular regulated mitogen-activated protein kinase (ERK) signaling pathway (Yang *et al* ., 2017).

Thus, in the tumor-initiation period, *Fusobacterium nucleatum* exerts its tumor-promoting action through the augmentation of local inflammation. Furthermore, some indicative molecules in human colon samples, such as IL-1β, IL-6, IL-8, TNF-α, and matrix metalloproteinase-3(MMP3),are suggestive of activation of NK-κB-driven inflammation (Kostic *et al* ., 2013).

Among these expression signatures, IL-8, TNF-α and other chemokines could recruit neutrophils and macrophages, which synthesize nitric oxide (NO) and cause oxidative stress to epithelial and stromal cells. This results in DNA damage and consequently activation of *p53* gene transcription which in turn suppresses

tumorigenesis by inducing G1-S arrest, DNA repair and cell apoptosis. Moreover, *p53* gene overexpression also leads to tumor protein *p53* (*TP53*) mutation, which is a key event during colorectal cancer development. Additionally, chronic inflammation and reactive oxygen species (ROS) production cause many other mutations such as chromodomain helicase DNA binding protein 7 gene (CHD7) and CHD8, members of the chromodomain helicase / Adenosine triphosphate (ATP) - dependent chromatin remodeling family and genomic instability, all of which would accelerate colorectal cancer development (Tahara *et al* ., 2014) .

1.2.7.Roleof Interleukin(IL10) andInterleukin(IL18)incolorectal cancer:

1.2.7.1 . Interleukin (IL10):

Several cytokines that modulate the immunologic response have been implicated in the development of cancer . Interleukin-10 (IL-10) is a multifunctional cytokine involved in both innate and adaptive immune response, and a wealth of evidence supports its regulatory role in carcinogenesis and tumor growth . In addition, increased circulating IL10 has been shown in patients diagnosed with different malignancies, such as hepatocellular carcinoma, autoimmune cancers, and leukemia (Namazi *et al* .,2018).

Studies have shown that two family members (IL-10 and IL-22) are closely related to CRC, suggesting that they are potential therapeutic prospects. IL-10 was first discovered to be secreted by Th2 cells in mice in 1989, and since it inhibited the synthesis of IL-2 and IFN-γ, it was primarily represented as secreted cytokine synthesis inhibitory factor (CSIF). First discovered as IL-10-related T cell-derived inducible factor (IL-TIF), it can be produced by most of the lymphocyte subsets, which mainly are innate lymphoid cells(ILC3),Th17 and Th22 (Ouyang *et al*.,2019).

In recent decade, accumulating evidence has supported the hypothesis that the promoter region of IL-10 polymorphisms correlates with genetic susceptibility of CRC . However, the results from the studies were often inconsistent and inconclusive. This

inconsistency may derive from a number of issues, including limited sample size of single study, different characteristics among studies (such as ethnicity, pathological types, and sources of controls), false-positive errors, lack of power, and minor impacts of IL-10 gene polymorphisms on CRC susceptibility (Alireza *et al* ., 2018)

1.2.7.2 . Interleukin (IL18) :

Among the IL-1 family, interleukin-18 (IL-18) is one of the best characterized. The protein encoded by the IL-18 gene, is essential for the response to the pathogens and activation of host defense mechanisms (Scott *et al* .,2018). IL-18 has been shown to be a mediate product of activation by nucleotide-binding oligomerization domain-like receptors (NOD-like receptor) pyrin domain-containing protein 3 (NLRP3) inflammasome/caspase-1 signaling pathway (Xie *et al*., 2014).

IL-18 is not only the critical regulators of signaling pathways in a variety of immune responses but also involved in antitumor process in a variety of cancers. Recently, aberrant low expression of IL-18 has been identified in some digestive system cancers, such as esophageal cancer and oral squamous cell carcinoma (Li *et al* ., 2018).

Interleukin 18 was originally discovered as a factor that enhanced IFN-γ production from anti-CD3-stimulated Th1 cells, especially in the presence of IL-12. Upon stimulation with IL-12, T cells develop into IL-18 receptor (IL-18R) expressing Th1 cells, which increase IFN-γ production in response to IL-18 stimulation. Therefore, IL-12 is a commitment factor that induces the development of Th1 cells. In contrast, IL-18 is a pro-inflammatory cytokine that facilitates type 1 responses .IL-18 is a cytokine that stimulates various cell types and has pleiotropic functions (Yasuda *et al* ., 2019) .

Although IL-18 also acts on non-polarized T cells, NK cells, NKT cells, B cells, DC and macrophages to produce IFN-γ in the presence of IL-12. Moreover, IL-18 without IL-12 but with IL-2 induces Th2 cytokine production from $CD4^+$ NKT cells, NK cells, and even established Th1 cells. Furthermore, IL-18 with IL-3 induces mast cells and basophils to produce IL-4 and IL-13. IL-18 stimulates both innate immunity and acquired immunity (Nakanishi *et al*., 2018).

The source of IL-18 was initially demonstrated to be from Kupffer cells, which constitutively express pro-IL-18. In addition, LPS binding to TLR4 induces the production of IL-18 via the activation of caspase-1. In contrast, upon stimulation with LPS, DC or macrophages increase their transcription of pro-IL-18 mRNA and subsequently their production of pro-IL-18, which is then processed by caspase-1 to be secreted as mature IL-18. In addition to these IL-18 producing cells, pro-IL-18 is produced by a wide variety of other cells, including keratinocytes, intestinal epithelial cells, and osteoblasts suggesting it has an important pathophysiological role in health and disease. Like other cytokines, IL-18 shows its pleiotropic action depending on its cytokine milieu figure (1-4) ,(Yasuda *et al* ., 2019).

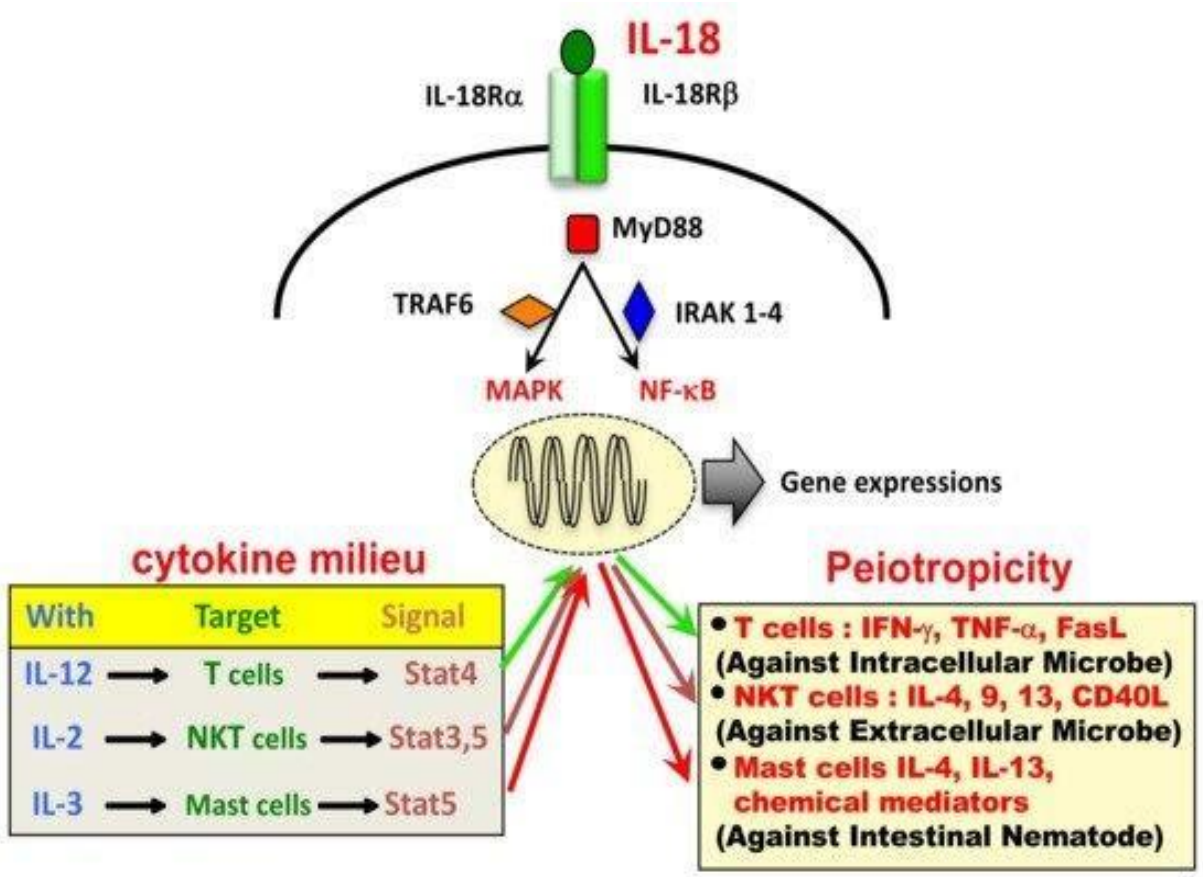


Figure (1-4) : Pleiotropic action of IL-18 depends on its cytokine milieu (Yasuda *et al* ., 2019).

Interleukin-18 is an essential pro-inflammatory and immune regulatory cytokine . In clinical analyses, mRNA and protein expressions of IL-18 were decreased in tissues of colon cancer patients. This decreased expression of IL-18 was significantly correlated with the tumor size . Patients with IL-18-positive tumors had a better survival rate than patients with IL-18-negative tumors. Moreover, up-regulation of IL-18 inhibited colon cancer cell proliferation. The decreased expression of IL-18 in colon cancer was associated with prognosis and tumor proliferation. IL-18 may be considered a novel tumor suppressor and a potential therapeutic target for colon cancer patients. (Xiaodong *et al* ., 2020).

1.2. 8 . *Fusobacterium nucleatum* resistance chemotherapy :

Colorectal cancer is the third most common cancer and the second leading cause of cancer-related death worldwide (Siegel *et al*., 2013). In advanced CRC patients, the purpose of chemotherapy is to shrink tumor size, reduce tumor growth, and inhibit tumor metastasis. In general, active cytotoxic drugs, including 5-fluorouracil (5-FU) and capecitabine, inhibit the enzyme activity of thymidylate synthase during DNA replication (Walko and Lindley *et al* .,2005).

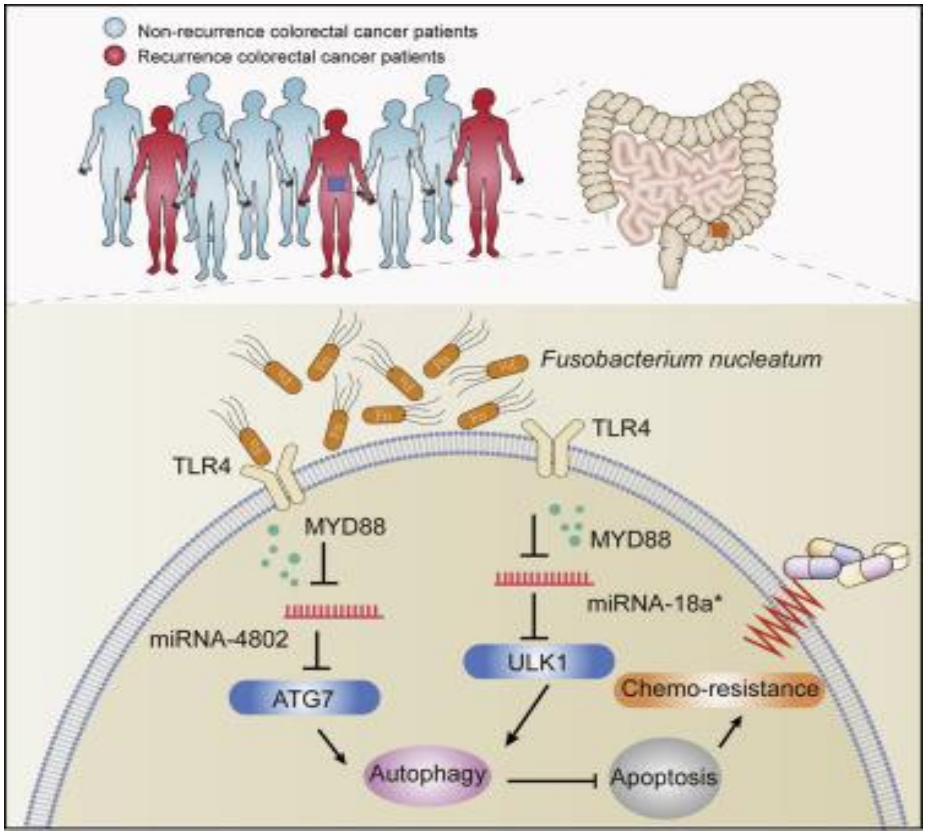
Oxaliplatin, another chemotherapy drug, inhibits tumor cell growth and causes cell G2 phase arrest by covalently binding DNA and forming platinum-DNA adducts (Kelland *et al* .,2007). The combination of these chemotherapeutic agents is widely used in the treatment of CRCs (Cartwright *et al*., 2012). The majority of patients with advanced colorectal cancer are initially responsive to the combined chemotherapy. However, the patients eventually experience tumor recurrence due to drug resistance, and the 5 year survival rate is lower than 10% in advanced CRC patients (Dahan *et al*., 2009). Unfortunately, colon cancer patients are generally not responsive to novel immune checkpoint therapy (Zou *et al*., 2016).

Thus, it is of paramount importance to elucidate the mechanism of chemotherapy resistance in CRC patients. Cancer chemoresistance results from a complex interplay between gene regulation and the environment.The microbiota is linked to CRC initiation and progression via affecting intestinal inflammation (Man *et al*., 2015), tumor-related signaling pathways (Schwabe and Jobin *et al*., 2013). The mouse studies have shown that the gut microbiota may modulate local immune responses and in turn affect chemotherapy (Viaud *et al*., 2013) and immunotherapy (Sivan *et al.*, 2015).

Human gut microbiota are linked to inflammatory cytokine production (Schirmer *et al*., 2016). Two groups have shown that the abundance of *Fusobacterium nucleatum* is gradually increased from normal tissues to adenoma tissues and to adenocarcinoma

tissues in colorectal carcinogenesis (Castellarin *et al*., 2012). Moreover, the amount of the bacterium in CRC tissues is associated with shorter survival (Mima *et al*., 2016).

In addition, *Fusobacterium nucleatum fap2* may recognize the host Gal-Gal NAc and help this bacterium localize abundantly in colon cancer epithelial cells. The amount of bacterium is increased in CRC patients with recurrence post-chemotherapy, compared with those with non-recurrence post-chemotherapy. The demonstrated that the bacterium plays a critical role in mediating colorectal cancer chemoresistance in response to small drug chemotherapeutics via a selective target loss of miR-18a and miR-4802, and activation of the autophagy pathway shown in figure (1-5) (Abed *et al*., 2016).



(Figure 1-5): Mechanistically, *Fusobacterium nucleatum* targeted TLR4 and MYD88 innate immune signaling and specific microRNAs to activate the autophagy pathway and alter colorectal cancer chemotherapeutic response. (Chung *et al*., 2017).

1.2.9. Treatment of *Fusobacterium nucleatum* positive CRC :

Fusobacterium nucleatum invasion into the mucosa promotes the release of inflammatory cytokines and is involved in the formation of the immune microenvironment for the occurrence of CRC (Taniguchi *et al* ., 2018). Due to the inflammatory basis of CRC, anti-inflammatory agents may be candidates for treating or preventing colorectal cancer (Lasry *et al* ., 2016).

Non-steroidal anti-inflammatory drugs (NSAIDs) are non-selective inhibitors of prostaglandin-endoperoxide synthase2 (PTGS2) (Huang *et al* ., 2017). PTGS2 is highly expressed in many tumor types, including CRC . Furthermore, *Fusobacterium nucleatum* promotes the expression of PTGS2 (Dharmani *et al* .,2011). NSAIDs may serve a role in the prevention of colorectal cancer. A large-scale demographic observation study from 1991 reported that the use of NSAIDs reduces the risk of fatal colorectal cancer (Rothwell *et al* ., 2010).

Retrospective studies have demonstrated that NSAID treatment is associated with a decreased risk of recurrence of colorectal polyps and tumor (Tsoi *et al* ., 2018). Patients who use low-dose aspirin for >5 years exhibit a decreased overall risk for developing colorectal cancer by 40–50% and NSAIDs have a positive effect on advanced (Meyerhardt *et al* ., 2014). Furthermore, non-steroidal antitumor drug therapy inhibits a tumor-promoting pathway by inhibiting Wnt signaling (Bos *et al* .,2006).

According to a previous report, prostaglandin E receptor 2 (PTGER2) increases the expression of NF-κβ-targeted pro-inflammatory genes in neutrophils. The expression of TNF-α and IL-6, PTGS2, chemokine (C-X-C motif) ligand 1 , Wnt and other cytokines in tumor lesions are significantly higher in PTGER2-enriched compared with PTGER2-knockout mice (Aoki *et al* ., 2015).

Therefore, NSAIDs and PTGER2 antagonists may be candidates for the prevention and treatment of *Fusobacterium nucleatum* positive CRC. The bacterium is one of the

most common gram-negative bacteria therefore antibiotics against bacterium are used to treat *Fusobacterium nucleatum* positive colorectal cancer (Bullman *et al* .,2017).

Transplanted *Fusobacterium nucleatum* infected colon cancer cell allografts into mice, maintained the animals for several weeks and then treated them with erythromycin or metronidazole. The bacterium is resistant to erythromycin but sensitive to metronidazole according to the drug sensitivity test. The results demonstrated that compared with the erythromycin resistant *Fusobacterium nucleatum* group, the volume and number of tumors is significantly reduced in mice treated with metronidazole this indicated that antibiotic intervention may be used as a potential treatment for bacterium positive patients with CRC. Therefore, the development of targeted antibiotics with a narrow spectrum may selectively eliminate pathogens while maintaining the balance of flora and avoiding the side effects caused by the use of broad spectrum antibiotics (Haak *et al* ., 2019).

It has been reported that berberine reverses the imbalance of intestinal microbiota caused by *Fusobacterium nucleatum* , thereby reversing the growth of colorectal cancer *in vivo* (Yu *et al* ., 2015). Tumor immunotherapy is a promising area, with significant progress being made in tumor molecular biology, particularly with regards to the use of immune checkpoint inhibitors, such as programmed cell death 1 (PDCD1) inhibitors (Bever *et al* ., 2017).

The efficacy of checkpoint inhibitors depends on the patient's gut microbiota complex interactions between the gut microorganisms and the immune system limit the effects of PDCD1 inhibitors (Bhatt *et al* ., 2017). According to the OncoKB classification system, pembrolizumab is a Food and drug association-approved drug for MSI-high solid tumors (level 1) and *Fusobacterium nucleatum* is associated with MSI-high, CIMP and BRAF mutations (Chakravarty *et al* ., 2017).

Therefore, PDCD1 inhibitors may exhibit anticancer effects on *Fusobacterium nucleatum* positive CRC. However, immune checkpoint inhibitors have a number of

side effects, including hepatitis, diarrhea and enterocolitis, resulting from the complex interactions between host genetics, immune responses, the environment and microbes . Therefore, the use of immune checkpoint inhibitors has very strict indications (Cramer *et al* ., 2017).

Since *Fusobacterium nucleatum* binds to TIGIT, an immune cell inhibitory receptor, through Fap2 to avoid attacks of immune cells, the development of an anti-Fap2 antibody may be beneficial for the antitumor immune response (Gur *et al* .,2015). The inhibition of miRNA-21 suppresses metastasis of CRC cells through modulation of programmed cell death 4 (Nedaeinia *et al* ., 2017).

The host-pathogen protein-protein interactions (HP-PPIs) and identified that *Fusobacterium nucleatum* and CRC related proteins have 186 interactions, including 103 host proteins and 76 *Fusobacterium nucleatum* pathogenic proteins. Therefore, the development of drugs targeting HP-PPIs may be used to treat *Fusobacterium nucleatum* -positive colorectal cancer (Kumar *et al* ., 2016) .

In view of the important role of TLR4 in *Fusobacterium nucleatum* carcinogenesis, it may be possible to develop a drug for the treatment of *Fusobacterium nucleatum* -positive tumors for patients with recurrent CRC, in addition to combination chemotherapy, the benefit of autophagy blocking agents or *Fusobacterium nucleatum* inhibitors requires investigation in future studies (Wu *et al* ., 2018).

Evidence suggest a potential relationship between *Fusobacterium nucleatum* and resistance to 5-fluorouracil chemotherapy which is a standard treatment for advanced CRC patients and oxaliplatin and no response to immunotherapy (Temraz *et al* ., 2019). The advanced of colorectal cance patients who received standard 5- fluorouracil-based adjuvant chemotherapy, that a high *Fusobacterium nucleatum* abundance is an independent risk factor for recurrence (Zhang *et al* ., 2019).

The patients with low levels of *Fusobacterium nucleatum* benefit from adjuvant chemotherapy more than those with high *Fusobacterium nucleatum* levels, in terms of disease-free survival, and it may be an effective adjuvant approach for preventing CRC metastasis and chemotherapy resistance (Yan *et al*.,2017). Other studies also investigated pathways that are activated by *Fusobacterium nucleatum* and could be targets for treatment of bacterium related CRC (Haruki *et al* ., 2020).

The development of anti-*fap2* antibodies could be used to treat *Fusobacterium nucleatum* positive tumors and may allow restoration of antitumoral immune detection and response . Moreover, *Fusobacterium nucleatum* enriched tumors demonstrate increased myeloid cells, this treatment would block myeloid cell migration and differentiation and can drive myeloid infiltration and intratumoral function, reducing inflammation and the development of CRC (Brennan *et al* ., 2016) .

In addition to these strategies, modification of the tumor microenvironment by methods which stimulate the patient's own immune system to fight tumor cells by fecal transplantation is a novel and important topic that will shape colorectal cancer management in the future (Chen *et al* ., 2019 ; Fong *et al* ., 2020) .

2. Materials and Methods:

2.1. Instruments and Chemical:

2.1.1. Instrument

Table (2.1) Laboratory Instruments and Equipment used in this study

Instruments	Company	Country
Autoclave, microtome	Stermite	Germany
Conventional PCR	**5** prime- Techne - UK	United Kingdom
Cooling block	Biobase	China
Deepfreeze	Indisit	Turkey
DNA extraction tubes 100 µl., qPCR tubes ,Pipette tips with filters .	Eppendrof	Germany
Digital camera	Samsung	Korea
Gel electrophoresis	Clever	USA
Heat block 80°C ,56 °C	Biobase	China
Hood	Labogene	Denmark
Microcentrifuge ,	Capp	Denmark
Microcentrifuge tubes 1.5 ml , 2ml	Eppendrof	Germany
Micropipettes 5-50 µl ,100-1000 µl , 0.5 –10 µl	Eppendrof	Germany
Plastic test tubes 10 ml ,EDTA tubes 5 ml,	Concord	Italy
Refrigerator	Afco	Jordan
Real-time PCR device	Cfx96. Biorad	USA
Screw capped bottles 100 ml.	Hirschman	Germany
Ts-Thermoshaker , shaker	Biometra	Germany
UV-trans illuminator	Clever	USA
Vortex mixer	Gilson	Germany
Water bath	H H 2	China

2.1.2. Chemicals :

The Chemicals and Kits that have been used in the project were listed in table (2-2).

Table (2-2) Commercial and kits used in the present study

Chemical name	Supplying company
Agarose	Bio-Basic/ Canada
DNA Ladder	Promega / USA
Ethiedium bromide	Promega / USA
Ethanol 95–100%	Biosolve / USA
Genomic DNA Mini Kit (FFPE)	Promega / USA
Genomic DNA Mini Kit (blood)	Promega / USA
Genomic RNA Mini Kit(blood)	Geneaid / Korea
Genomic DNA Mini Kit (Fresh biopsy)	Promega / USA
Isolation kit of *Fusobacterium nucleatum*(qPCR)	GPS / Spain
Nuclease free water	GPS / Spin
Proteinase k	Promega / USA
Phosphate-buffered saline (PBS)	Promega / USA
T.B.E 10 × buffer	Geneaid / Korea
Tris EDTA buffer	Bio Basic / Canada

2.2. Subjects and Methods :

2.2.1 . Patients

A case-control study was conducted in Imam Hussein medical city, Al-Kafeel Specialist Hospital and Imam Zain Al-Abidin Hospital in Holy Karbala between February 2020 to the March 2021. Individuals visited these centers with a suspected of colorectal cancer were undergone physical examinations, standardized colonoscopic examinations and histopathological examination.

Total of 40 Patients with confirmed CRC by two consensus histopathological reports were recruited in the study includes (Male 22 and female 18) age range (30-80) years. Twenty tissue samples were obtained from formalin fixed paraffin embedded (FFPE) colorectal cancer (CRC) patients and the other twenty samples were taken from fresh biopsy .

DNA extraction were done to all samples in order to be for molecular study.

Two ml of blood were obtained from all patients after they were confirmed to have colorectal cancer and when they visited the oncology center in Imam Hussein Medical City by puncture of the vein and placing it in Ethylenediaminetetraaceticacid (EDTA) tubes. DNA was extracted from EDTA tubes for molecular study, and RNA was also extracted for gene expression study.

2.2.2. Control group

Twenty individuals with the clinical diagnosis of hemorrhoid who visited the gastrointestinal center in Imam Hussein medical city, and Al-Kafeel Specialist Hospital in Holy Karbala were agreed to participate in our study. Colonoscopy were done to those individuals as a diagnostic workup and their finding revealed negative endoscopy apart from bleeding hemorrhoid. Colonic biopsy was taken from all those control twenty patients. At the same time , Blood samples were obtained from all

patients . DNA and RNA extraction from these samples were done in order to be used for molecular study.

2.3. Inclusion criteria:

Patients with colorectal cancer who meet the diagnostic criteria and were diagnosed as colorectal cancer by two consensus histopathological examination.

2. 4. Exclusion criteria:

- Those who currently have intestinal infection.
- Those who had used antibiotics or microecological agents within 2 months before enrollment.
- Patients who received adjuvant chemoradiotherapy before sampling.
- Those who suffer from chronic diseases such as hypertension, heart disease, and diabetes.
- Those who refuse to participate in our study.

2.5. Ethical Approval :

The necessary ethical approval was obtained by verbal consent from patients. This study was approved by the committee of publication ethics at College of Medicine, Babylon Province, Iraq, under the reference No. BMS /0203/ 016.

2.6. Molecular Analysis :

2.6.1. Genomic DNA extraction

2.6.1. A. Genomic DNA Mini Kit for blood :

The DNA extraction from fresh whole blood specimens were carried out according to the manual of manufacturer of Promega company **:**

The Genomic DNA extracted kit for blood contents show in Table (2-3) .

Table (2-3) DNA extraction kit for blood contents

Content	Volume
Cell Lysis Buffer (CLD)	2.5ml
Column Wash Solution (CWD)	17ml
Binding Buffer (BBA)	3ml
Proteinase K (PK) Solution	250µl
Nuclease-Free Water	13ml
Collection Tubes	1pack
Binding Columns	1pack

Protocol :

1. The blood sample was mixed for at least 10 minutes in a shaker at room temperature.

2. A volume of 20µl of Proteinase K(PK) Solution was added into a 1.5ml microcentrifuge tube.
3. A volume of 200µl of blood was added to the tube containing the Proteinase K (PK) Solution, and was briefly mix.
4. A volume of 200µl of Cell Lysis Buffer (CLD) was added to the tube. The tube was covered and was mixed by vortexing for at least 10 seconds.
5. The tube was incubated 10 minutes at 56°C .
6. While the blood sample was incubated , the ReliaPrep binding column was placed into an empty Collection Tube.
7. The tube from the heating block was removed. A volume of 250µl of Binding Buffer (BBA) was added.
8. The tube was caped, and was mixed by vortexing for 10 seconds with a vortex mixer.
9. The contents of the tube was added to the Relia Prep binding column, and was caped it and placed it in a microcentrifuge tube.
10. The tube was placed in the centrifuge for 1 minute at 14000 rpm .
11. The binding column was placed into a fresh collection tube.
12. A volume of 500µl of column wash solution (CWD) was added to the column.
13. The tube was placed in a centrifuge for 3 minutes at 14000 rpm and flow through was discarded.
14. The Step 11 were repeated twice for a total of three washes.
15. The column was placed in a clean 1.5ml microcentrifuge tube .
16. A volume of 100µl of elution was added to the column .
17. The tube was placed in a centrifuge for 1 minutes at 14000 rpm .
18. The Relia Prep binding column was discarded, and the elution tube containing DNA was kept at -20°C.

2.6.1. B . Genomic DNA Mini Kit for FFPE:

The DNA extraction from FFPE specimens were carried out according to the manual of manufacturer of Promega company **:**

The Genomic DNA extracted Kit for FFPE contents show in Table (2-4) .

Table (2-4) DNA extraction kit for FFPE contents

Content	Volume
Mineral Oil	5ml
Blue Dye	100µl
Lysis Buffer (LB)	2 × 1ml
Proteinase K (PK)	250 µl
BL Buffer	3.25ml
Wash Solution	3ml
RNase A	200ml
Elution Buffer	15ml
Collection Tubes	1package
FFPE Binding Columns	1package

Preparation of solutions :

Wash Solution:

Ten reaction size: Twelve ml of 95–100% ethanol was added to the bottle containing 3ml of concentrated Wash Solution.

Hundred reaction size: One hundred twenty ml of 95–100% ethanol was added to the bottle containing 30ml of concentrated Wash Solution.

After adding ethanol, the bottle has been marked to confirm this step. This reagent is stable at 22–25°C when capped tightly.

Lysis Buffer :

Ten reaction size: Ten µl of Blue Dye was added to each vial of Lysis Buffer (LBA); vortex to mix.

Hundred reaction size: contents of Blue Dye vial was added to 30ml of Lysis Buffer (LBA) ,vortex to mix.

Preparation of FFPE Sections :

1. Twenty µm sections were cut from FFPE blocks using a microtome. When extracting nucleic acid from tissue sections that have been applied to microscope slides, a sterileblade is used to scrape the sections from the slide.
2. The sections were place in a 2ml microcentrifuge tube.

Done deparaffinization of FFPE samples using mineral oil.

Deparaffinization Using Mineral Oil : (Lin J *et al.*, 2009).

1. A volume of 500 µl of mineral oil was added to the sample .
2. The sample was incubated at 80°C for 1 minute .
3. The sample was placed in a mixing vortex .

Sample Lysis :

1. A volume of 200µl lysis buffer was added to the sample.
2. The sample was placed in the centrifuge at 10,000 rpm × g for 15 seconds at room temperature. Two phases were formed, a lower blue (aqueous) phase and an upper (oil) phase.
3. A volume of 20µl of proteinase K directly was added to the lower blue phase. The lower phase has been mixed by pipetting .
4. The sample was incubated 56°C for 1 hour.
5. The sample was incubated 80°C for 4 hours.
6. The sample is left to cool to room temperature. Then the sample was placed in Centrifuge briefly at room temperature to collect condensation.

Optional storage:

After incubating at 80°C, samples may be stored overnight at 4°C. If samples are stored at 4°C .The sample is left to warm to room temperature prior to adding RNase and proceeding with the protocol.

RNase treatment :

1. A volume of 10µl of RNase was added directly to the lysed sample in the lower blue phase. The lower phase was mixed by pipetting .
2. Incubation was done at room temperature for 5 minutes.

Nucleic Acid Binding :

1. A volume of 220µl of BL buffer was added to the lysed sample.
2. A volume of 240µl of ethanol (95–100%)was added .
3. The sample was placed in a mixing vortex .
4. The sample was placed in the centrifuge at 10,000 rpm × g for 15 seconds at room temperature. Two phases were formed, a lower blue (aqueous) phase and an upper (oil) phase

5. For each sample processed a binding column was placed in one of the collection tubes provided .
6. The lower blue phase (aqueous) has been transferred entire of the sample, including any precipitate that may have formed, to the binding column/collection tube assembly, and cap the column. The remaining mineral oil was discard.
7. Discard were flowthrough , the binding column was inserted into the Collection Tube.

Column washing and Elution :

1. A volume of 500µl of wash solution (with ethanol added) was added to the binding column. The column was covered.
2. The sample was placed in the centrifuge at 10,000 rpm × g for 30 seconds at room temperature .
3. The flowthrough was discarded , the binding column has been reinserted into the same collection tube used for nucleic acid binding .
4. A volume of 500µl of wash solution was added to the binding column. The column was covered.
5. The sample was placed in the centrifuge at 10,000 × g for 30 seconds at room temperature.
6. The flowthrough is eliminated , and the binding column has been reinserted in to the collection tube used for nucleic acid binding .
7. The cap has been opened on the binding column, and centrifuge the Binding column/collection tube assembly at 16,000 × g for 3 minutes at room temperature to dry the column .
8. Transfer the binding column were to a clean 1.5ml microcentrifuge tube , the collection tube was discard.
9. A volume of 30–50µl of elution buffer was added to the column, and cap the column.

10. The sample was placed in the centrifuge at 16,000 × g for 1 minute at room temperature. The binding column was discard and remove .
11. The microcentrifuge tube were covered , and store the eluted DNA at -20 °C

2.6.1. C . Genomic DNA Mini Kit for fresh biopsy :

The DNA extraction from fresh biopsy specimens were carried out according to the manual of manufacturer of Promega company **:**

The Genomic DNA extracted Kit for fresh biopsy contents show in table (2-5) .

Table (2-5) DNA extraction kit for fresh biopsy contents

Component	Volume
Binding Columns	1pake
Collection Tubes	1pake
Cell Lysis Buffer (CLD)	3ml
Proteinase K (PK) Solution	250ml
Binding Buffer (BBA)	3ml
Column Wash Solution (CWD)	17ml
Nuclease-Free Water	13ml
RNase A Solution (4mg/ml)	250ml

Protocols:

1. The sample was cut into smaller pieces with a scalpel before we began to work.
2. A volume of 160µl of phosphate buffered saline (PBS) to each sample to be processed was added, and mix by vortexing.
3. The sample was homogenized using a rotary homogenizer.

4. A volume of 20µl of Proteinase K (PK) Solution was added to the homogenized sample.
5. A volume of 200µl of Cell Lysis Buffer (CLD) was added to the tube. Cap and mix by vortexing for at least 10 seconds.
6. Incubation was done at 56 ° C for 30 minutes to 2 hours.
7. A volume of 20µl of RNase A Solution was added to each sample, mix by vortexing for 10 seconds and place microcentrifuge tube at 56°C for 10 minutes.
8. The tube was removed from the heating block. A volume of 250 µl of Binding Buffer (BBA) was added then the tube was cap and mixed with a vortex for 10 seconds using a vortex mixer.
9. A ReliaPrep ™ binding column was placed inside a collection tube for each sample. The liquid portion of the sample was transferred onto the binding column, column cap and placed in a centrifuge.
10. The binding column was placed in a centrifuge for one minute at full speed. Then it was confirmed that the solution had completely passed through the membrane. If the analyzer is still visible on top of the membrane, the column is centrifuged for another minute.
11. The collection tube containing the flux was removed, and the liquid was disposed of as a hazardous waste.
12. The binding column was placed in a new collection tube. A volume of 500 µl of column wash solution (CWD) was added to the column, and placed in the centrifuge for 2 minutes at maximum speed.
13. Step 12 was repeated twice, for a total of three washes.
14. The column was placed into a clean 1.5 mL microcentrifuge tube.
15. A volume of 100 µl Nuclease-Free Water was added to the column. It was placed in a centrifuge for one minute at full speed.
16. The Relia Prep bind column is discarded. Genomic DNA can be placed at 4 °C for short term storage or -20 ° C for long term storage.

2.6. 2. Genomic RNA extraction :

2.6.2. A. Genomic RNA Mini Kit for blood :

The RNA extraction from fresh whole blood specimens were carried out according to the manual of manufacturer of Geneaid company **:**

The Genomic RNA extracted kit for blood Component show in Table (2- 6).

Table (2-6) RNA extraction kit for blood contents

Component	Volume Final
RBC Lysis Buffer	10 ml
RB Buffer	2ml
DNase I Reaction Buffer	200 µl
W1 Buffer	2 ml
Wash Buffer (Added Ethanol)	1.5 ml (6 ml)
RNase-free Water	1 ml
RB Column	4
2 ml Collection Tube	8

Protocols:

1. A volume of 300 µl of whole human blood was added to a sterile 1.5 ml microcentrifuge tube. Mixed by inversion.
2. A volume of 1 ml of RBC Lysis Buffer was added to the tube and incubated on ice for 10 minutes (briefly vortex twice during incubation).
3. Centrifuge at 3,000 x g for 5 minutes then remove the supernatant completely.

4. A volume of 400 µl of RB Buffer was added, and the tube was incubated at room temperature for 5 minutes.
5. A volume of 400 µl of Wash Buffer was added to the RB Column. Ethanol was confirmed to be added to the RB Column and centrifuge at 14000 x g for 30 seconds. Flow- through was discarded and the RB Column was placed in the 2 ml collection tube.
6. DNase 1 solution was prepared in a 1.5 ml microcentrifuge tube (RNase-free) as follow:

Table (2-7) The DNase I prepared contents

Content	Volume
D Nase 1	5 µl (2 U/µl)
DNase 1 Reaction Buffer	45 µl
Total Volume	50 µl

7. By pipette DNase 1 solution was mixed carefully.
8. A volume of 50 µl DNase 1 solution was added into the RB column and Incubated for 15 minutes at room temperature (20-30ºC).
9. A volume of 400 µl of W1 Buffer was added into the RB Column, centrifuge at 14000 x g for 30 seconds. Flow-through was discarded then the RB Column was placed in the 2 ml collection Tube.
10. A volume of 600 µl of Wash Buffer was added into the RB Column. Centrifuge at 14000 x g for 30 seconds .
11. A volum of 600 µl of Wash Buffer was added into the RB Column, Centrifuge at 14000 x g for 30 seconds . The RB Column was placed back in

the 2 ml collection tube and centrifuge at 14000 x g for 3 min to dry the column.

12. The dried RB Column was place in a clean 1.5 ml microcentrifuge tube.
13. A volume of 50 µl of RNase-free Water was added into the column. It was left for at least 1 minute to ensure that the RNase-free water was absorbed. Centrifuge at 14,000 x g for 1 minute to elute the purified RNA.
14. DNA digestion in Solution the DNase 1 reaction in a 1.5 ml microcentrifuge tube (RNase-free)was prepare as follows :

Table (2-8) The DNase I reaction contents

Content	Volume
RNA in RNase-free Water	1-40 µl
DNase I	0.5 µl/µg RNA
DNase I Reaction Buffer	5 µl
RNase-free Water	Added to final
Total Volume	50 µl

15. By pipette DNase 1 solution was mixed carefully.
16. The microcentrifuge tube was incubated at 37ºC for 15-30 minutes.
17. The tube containing RNA was kept at -20 °C.

2.6.2. B . Measuring RNA with Nano Drop :

Introduction:

Measuring the amount and purity of purified RNA is crucial for determining the amount of each sample to use in q RT-PCR. NanoDrop Spectrophotometers (NDS), such as the one below, are very convenient instruments for assessing RNA quantity and quality.

Procedure:

1. Samples of RNA as well as water used to separate them were prepared on ice to a spectrophotometer.
2. The sample reader was blank with molecular grade water and dried with Kimwipe
3. Following the program instructions, 2 µl of water was loaded to initialize the system.
4. The computer setting was changed to RNA.
5. Two µl of sample was loaded and the Measure button was clicked.
6. After the reading was completed, the A260/A280 and A260/A230 ratios plus the amount of RNA recovered (ng/µl) were recorded.
7. The sample reader was wiped by Kimwipe clean and dry between samples and the steps were repeated.

Interpreting the Results:

1. A lower than expected concentration of RNA indicates low cell numbers in the sample, poor homogenization of samples, or too much volume of water used in the elution step of RNA purification.
2. Very pure RNA will have an **A260/A280** ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A260/A280 ratio is likely due

to mixing phases when removing the upper aqueous phase or is also more common in samples with a very low yield of RNA.

3. The **A260/A230** ratio should also be above 2.0. A low A260/230 ratio indicates contamination with the wash solutions, chaotropic salts, phenols or protein. A low A260/A230 ratio is most likely due to contamination of the samples with washing buffers during the Mini spin washes.

2 .6. 3. PCR amplification:

2.6. 3. A .Copy number:

The sensitive detection and quantification of *Fusobacterium nucleatum* was achieved by special kit (The Fus Nuc MONODOSE dtec-qPCR Test) provided by (GPS, Spain). This kit allows the amplification of a target region from a DNA template by using specific oligonucleotides and the copy number of each sample was calculated according to standard curve method. Briefly, Standard Curve .

Table (2-9) The *Fusobacterium nucleatum* monodose kit content

Content of kit
DNA template : Target , Primers , Labelled probe , dNTPs , BSA , Polymerase ,Buffer . **Internal control :** Standard template , Standers buffer .

Serial Dilution were prepared by the following steps :

1. Five tubes were filled by 900 µl of DNAse/RNAse free water and labeled as 2 to 6.
2. The Standard Template (RED CAP), it's reconstituted with 120 µl of Standard buffer (BLACK CAP) and vortex thoroughly, label as number 1

3. A volume of 100 µl of diluted Standard Template (RED CAP) was pipetted into tube 2.
4. Vortex thoroughly .
5. A volume of 100 µl was pipetted from tube 2 into tube 3 .
6. Vortex thoroughly .
7. Steps 3 and 4 were repeated to the tubes 4 to 6 to complete serial dilution .

Table (2-10) The Stander curve serial dilution

Stander curve serial dilution	Copies / µl	copies in 5 µl
Standard Template (RED CAP)	2×10^5	10^6
Tube 2	2×10^4	10^5
Tube3	2×10^3	10^4
Tube 4	2×10^2	10^3
Tube 5	2×10	10^2
Tube 6	2	10

RT-qPCR conditions was summarized by table (2-11).

Table (2-11): The RT-qPCR conditions of *Fusobacterium nucleatum*

Step		Time	Temperature
Initial denaturation		1 minutes	95°C
Denaturation	40 Cycles	10 second	95°C
Annealing		1 minutes	60°C
Extension		1 minutes	72°C

Interpretation of results :

One fluorogenic signal should be collected during this step by using the FAM channel. The number of copies in the sample can be calculated based on the regression

$$Coy\ number = 10^{\frac{ct-Yinter}{slope}}$$

2.6.3. B. Detection of virulence gene (*fad A*) by PCR technique :

DNA was used as a template for specific PCR to detacted the *fad A* gene .

A pair of specific primer were used for the amplification of a fragment gene as shown in table (2- 12) .

A single reaction mixture contained 2.5 µl of down stream primer , 5 µl of DNA extraction , 5 µl of master mix and 5 µl of nuclease free water to obtain a total volume 20 µl . The amplicon were run in 1.5 % agarose gel for 60 minute, the PCR conditions to detect *fadA* genes shown in table (2- 13) .

Table (2-12): Primers sequences and PCR product (bp) to detect *Fusobacterium nucleatum fadA* genes .

Type of Primer	Sequence $5^- \rightarrow 3^-$	PCR product (bp)	References
FadA-F	CACAAGCTGACGCTGCTAGA	232 bp	(Lopez -Nguyen *et al.*, 2020)
FadA-R	TTACCAGCTCTTAAAGCTTG		

Table (2-13): PCR conditions to detect *fadA* genes

Step	Cycles	Temperature	Time
Denaturation	30	95°C	30 second
Annealing		56°C	30 second
Extension		72°C for	30 second

2.6.3. C . Detection of microRNA146a gene polymorphism :

The polymorphism in MicroRNA 146a (rs2910164) was typed by using Amplification Refractory Mutation system –polymerase chin reaction method (ARMS- PCR).

The ARMS method consist of two complementary reaction : one containing an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus and the other one containing a mutant – specific primer and does not amplify normal DNA .

The reaction was employed a antisense primer and one of the two allelic specific (G / C) sense primer .

ARMS- PCR Component : The reaction mixture for detection of MicroRNA 146a gene polymorphism is listed in the table (2 - 14) .

Table (2- 14): ARMS-PCR mixtures

PCR mixtures	
Master Mix	12.5 µl
Forward inner primer (C allele)	1 µl
Reverse inner Primer (G allele)	1 µl
Forward outer primer	1.2 µl
Reverse outer primer	1.2 µl
Template DNA	3 µl
Nuclase-Free Water	5.1 µl
Total volume	25 µl

2.6.3. D . ARMS- PCR :

In this study, ARMS-PCR assay was used to identify SNP polymorphism (rs2910164) of MicroRNA 146a in patients with CRC. Primers were newly designed by using BatchPrimer3 (You *et al*., 2008) as shown in table (2-15), PCR conditions to identify MicroRNA 146a as shown in table (2-16).

Table (2-15): Primers sequences and PCR product to identify SNP polymorphism (rs2910164) of MicroRNA 146a in patients with CRC .

Bacteria	Type of Primer	Sequence ($5^- \rightarrow 3^-$)	PCR product (bp)	Ref.
ARMS-PCR Micro RNA 146a	Forward inner primer (C allele)	GGGTTGTGTCAGTGTCAGACGTC	163bp	design in this study
	Reverse inner primer (G allele):	TCCCAGCTGAAGAACTGAATTTGAC	179bp	
	Forward outer primer	CTGCATTGGATTTACCAGGCTTTT	294bp	
	Reverse outer primer	CTTCAGAGCCTGAGACTCTGCCTT		

Table (2-16): PCR conditions of MicroRNA 146a

Step	Cycles	Temperature	Time
Initial denaturation	1	94°C	45 second
Denaturation			
Annealing	40	60 °C	45 second
Extension		72°C	45 second
Final extension	1	72 °C	10 mint

2.6.3. E . Gene expression of IL10 and IL18 :

Real-time PCR (RT-PCR) : After collection of blood samples from patients and control individuals, the total RNA was collected with Blood/Cell RNA Mini Kit (Geneaid) according to the manufacturer's protocol.

The real-time qPCR reactions were performed by using specific primers and probs targeting reference gene GAPDH and the sequence of target genes IL10 and IL18 shown in table (2-17). Conversion the total RNA to cDNA and amplification of DNA was done according to instructions provided by GoTaq Probe 1-Step RT-qPCR System (Promega), where RT-qPCR Mixture and conditions were summarized in tables (2-18) and (2-19), where the final volume of RT-qPCR reaction was 20 µl and relative expression fold was calculated by delta delta method (2^-(ΔΔCt) according to , (Livak and Schmittgen., 2001).

Table(2-17):Primers sequences and PCR product (bp) to detect genes expression

Bacteria	Type of Primer	Sequence	PCR product (bp)	Ref.
1L10	IL10-F	GTGATGCCCCAAGCTGAGA	138	Giulietti *et al.*, 2001
	IL10-R	CACGGCCTTGCTCTTGTTTT		
	IL10- Probe	[6-FAM] CCAAGACCCAGACATCAAGGCGCA[TAM]		
IL18	IL18-F	CCAAGGAAATCGGCCTCTATT	70	design in this study
	IL18-R	CATACCTCTAGGCTGGCTATCT		
	IL18- Probe	[6-FAM] ACTGATTCTGACTGTAGAGATAATGCACCC [TAM]		
GAPDH	GAPDH-F	GGAAGCTTGTCATCAATG	35	Poole *et al.* (2014)
	GAPDH-R	CCCCACTTGATTTTGGAG		
	GAPDH-Probe	ATCACCATCTTCCAGGAGCGAG [BHQ1] [JOE]		

Table (2-18): RT-qPCR Mixture

Component	Volume
GoTaq Probe qPCR Master Mix	10 µl
RT Mix for 1-Step RT-qPCR	0.5 µl
Forward primer	1µl
Reverse primer	1µl
Probe	1µl
RNA Template to cDNA	5 µl
Nuclease-Free Water	1.5 µl
Total volume	20 µl

Table (2-19): RT-qPCR conditions

Step	Cycles	Temperature	Time
Reverse Transcreption	1	45 °C	15 minutes
Initial denaturation	1	95°C	2 minutes
Denaturation		95°C	15 seconds
Annealing	40	60°C	1minutes
Extension			

2. 7. Agarose Gel Electrophoresis:

Agarose gel was prepared by dissolving 1.5% of agarose powder in 100ml of TBE buffer (pH:8) in boiling water bath, allowed to cool to 50^{o}C and ethidium bromide at the concentration of 0.5mg/ml was added.

The comb was fixed at one end of the tray for making wells used for loading DNA sample.

The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min.

The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covered the surface of the gel, 5µl of DNA sample was transferred in to the wells in agarose gel, and in one well, we put the 5µl DNA ladder mixed with 1µl of loading buffer.

The electric current was allowed at 60 volt for 80min. UV transilluminater in 280 nm was used for the observation of DNA bands, and gel was photographed using a digital camera (Deuk *et al* ., 2019) .

2.8. Allele frequency:

Allele frequency was estimated according to Hardy –Weinberg equilibrium which include: Allele frequency $= \frac{\text{Homogenes} \times 2 + \text{Heterogenes}}{\text{No of sample} \times 2}$

(Romero *et al* ., 2015) .

2. 9. Statistical Analysis:

Genetic analysis was performed by using Chi-square(x2) test. P values less than (0.05) is considered. Statistical analysis was performed by using SPSS 19 version. Data were expressed as (mean ± SD). The normality of the distribution of all variables was assessed by the student's ANOVA test and Pearson correlation analyses that have been used to determine the significant difference between the groups. Finally, a method T-test was used to find out the variables and calculate the results to some groups in the study.

3. Results and Discussion:

3.1. Molecular Detection of *Fusobacterium nucleatum* by Quantitative Real – time PCR:

The presence of *Fusobacterium nucleatum* DNA was detected in abundance relative total number of bacteria in 40 patients tissue samples with colorectal cancer and 20 normal tissue sample as a control group .

Fusobacterium bacterium positivity was detected in 33 (82.51%) patients with CRC as shown in table (3-1) and in 13 (65 %) of control sample . *Fusobacterium nucleatum* was not detected in 7 (17.5%) from patients group and in 7 (35%) in control group and the negative result may be due to the low level of DNA tissue samples , tissue fixation method and storage time has been shown to effect on *Fusobacterium nucleatum* qPCR positivity or non–detectable *Fusobacterium* DNA copies .

The fixation process chemically alters the nucleic acid in a sample by inducing covalent DNA cross- linking fragmentation (Olert *et al* ., 2001).

Table (3-1) : Percentage distribution of *Fusobacterium nucleatum* detected from colorectal cancer (CRC) by q PCR assay.

Results	Patient	Control	Total
Positive	33 (82.5%)	13 (65%)	46 (76.7%)
Negative	7 (17.5%)	7 (35%)	14 (23.3%)
Total	40 (100%)	20 (100%)	60 (100%)
P value	< 0.0001*	0.180	< 0.0001*

* represent a significant difference at p≤ 0.05.

The results show that there was significant increase in copy number of *Fusobacterium nucleatum* isolates in patients with CRC when compared with control group and the range of CT value according to the copy number range from (30.23 ± 1.6) in control and (27.86 ± 3) in patient groups as shown in (table 3-2) .

Table (3-2) : Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum* between Control and Patient groups.

Results	Control	Patient	P value
Cq	30.2369 ± 1.6	27.8624 ± 3	0.017*
Copy number	367.5985 ± 179.09	3993.4909 ± 1433	

* represent a significant difference at p≤ 0.05

The results of the present study was similar with (Claudio *et al* ., 2018) that indicated *Fusobacterium nucleatum* play a causal role in colorectal carcinogenesis ,and also may be involved in cancer initiation and progression stimulating and proliferation of colorectal cancer

Andrew *et al* ., (2019) found observed that *Fusobacterium nucleatum* was significantly more abundant in the colorectal cancer tumor tissue compared to the matched surrounding mucosa .

Studies provide evidence that *Fusobacterium nucleatum* mediated increased gut inflammation and chemoresistance , through immune signaling and autophagy activation , explains the poorer prognosis for CRC patients in different positions along the colon was determined using qPCR , *Fusobacterium nucleatum* was detected in samples from 60 % patient and 18% in control group(Yu *et al* ., 2017)

Melike and Yumuk ., (2021) found the rate of *Fusobacterium nucleatum* in Frozen CRC tissues was 86.7% . While the rate of *Fusobacterium nucleatum* in FFPE colorectal cancer tissue was 13% the different rate may be due to the tissue preparation used for detection *Fusobacterium nucleatum* .

The varying rate of *Fusobacterium nucleatum* in CRC tissue from different part of the world might also be due to the characteristics of the study population demographic ,environmental or genetic factors (Melike and Yumuk ., 2021).

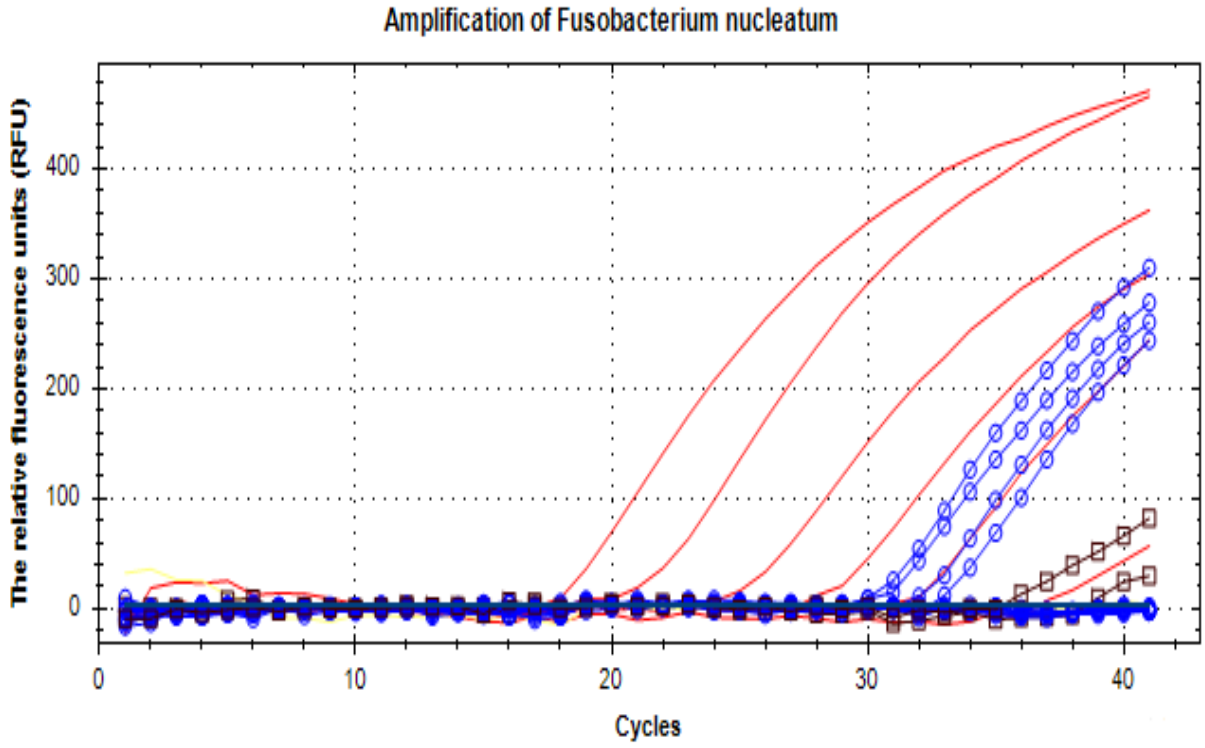


Figure (3-1):

Identification and quantification of *Fusobacterium nucleatum* copy number by qPCR assay. This is the first run for 15 samples only, represent a standard curve amplification, represents amplification of patient samples. represents amplification of Control samples.

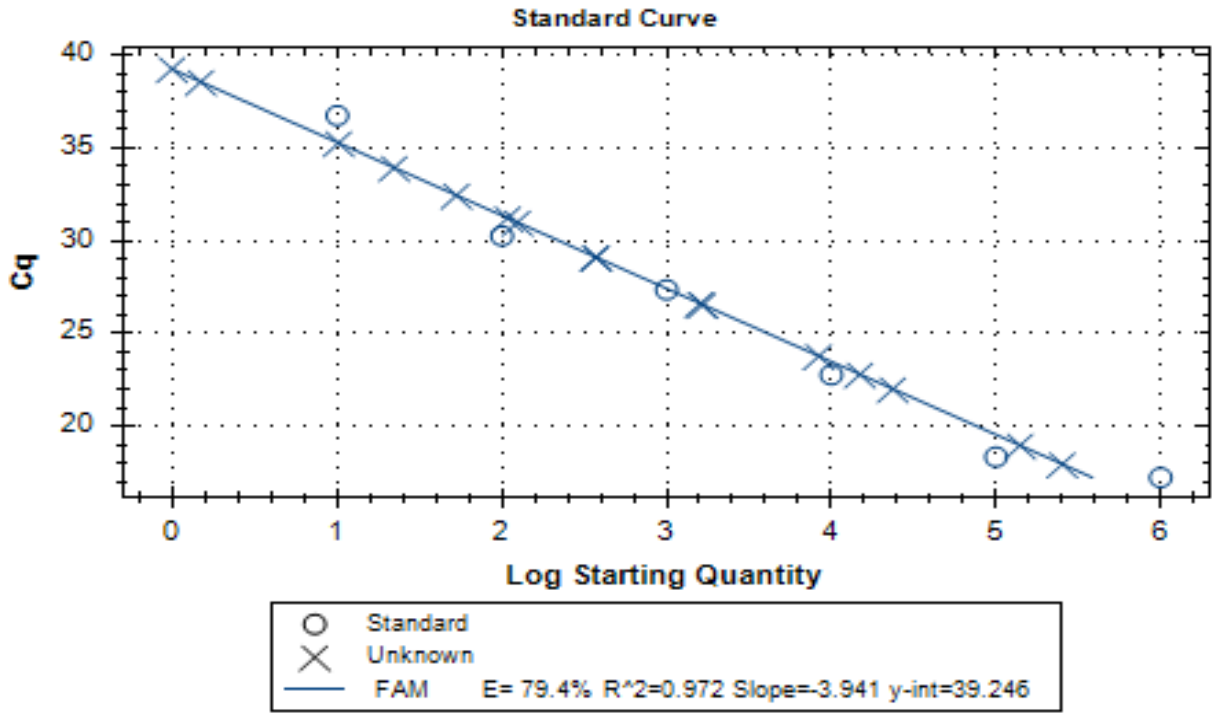


Figure (3-2): Amplification standard curve for quantification of *Fusobacterium nucleatum* copy number by qPCR assay.

Also the results found the some control group contain low copy number of *Fusobacterium nucleatum* DNA this may be due to that *Fusobacterium* was found as a normal flora in the gut and colon .

3.2. Gender of patients

A total from 40 samples ,it was found that 22 samples were male and 18 female for patients group, while for control group they were 20 sampls 10 male and10 female The results of the current study showed that the percentage value of colorectal cancer was no significant different between male and female table (3-3) . Also the result was found that there was no significant results between the copy number of *Fusobacterium nucleatum* male and female as shown in table (3-3).

Table (3-3) Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum* between Control and Patient groups according to Gender:

Results	Control		P value	Patient		P value
	Male (N= 7)	Female (N= 6)		Male (N=17)	Female (N=16)	
Cq	29.46±0.7	31.1433±0.58	0.06	27.7153±0.82	28.0188±0.67	0.0777
Copy numbe	580.6114±320	119.0833±15.2		5287.8147±2507	2618.2719±1290	

represent a significant difference at p≤ 0.05.

Murphy *et al* (2011) explained that men are more likely to have risk for CRC than women, this due to hormonal and other risk factors, which disagreed with current study.

Jose Valery *et al* (2020) appear that there was not any difference based on the gender in colorectal cancer screenings ,other factor may influence on the rate like insurance status ,and relatively young age .

The results of the present study was consonant with (Hassan *et al* ., 2021) which indicated that there is no significant difference between the male and female in colorectal cancer cases .

The results of the present study disagreed with (White *et al.,* 2018) results who indicated that males have more risk than female. This may be due to that men are more likely to have high a diet in red and processed meat, behavior consumers of alcohol , and smoking . Men also have a greater propensity to deposit visceral fat which is associated with increased risk of colorectal cancer.

The results of the present study was disagreed with (Kang *et al*, 2017) who indicated that the a percentage rate of women with CRC more than that of men**.**

3. 3 . Age of patients :

A total from 40 patients samples and 20 control group ,the range of age about 30 - 80 years and the results found that the high number of colorectal cancer cases increased in age 45- 65 years, also the copy number of *Fusobacterum nucleatum* in this age group are increased, while incidence rate of the CRC in other age was extremely rare and the result show there was significant different age group as show in table (3 -4).

Table (3-4): Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum* between Control and Patient groups according to Ag :

Age Groups	Control		Patient		
	Cq	Copy number	Cq	Copy number	P value
<45	30.71±0.25	149.87±22.96014	27.8±0.94	3096.9778±1886	0.017*
45-65	31.2050±0.59	116.4200±38.91	27.7±0.73	4981.3060±2205	0.012*
>65	29.8175±0.69	512.0413±285.427	28.4±1.27	1071.57±549.9	0.391
P value	0.522		0.920		

* represent a significant difference at p≤ 0.05.

From table (3-4) the results indicate that the risk for developing the disease begins at the age of 40 years and increases between 45-65 years for both sexes. Age can be

considered an equally relevant risk factor for men and women. This study like with another study (Net Editorial Board., 2021)which indicated the risk colorectal cancer increases as people get older .

The results of the current study was similar with (Martin *et al*., 2016) that showed that the risk classification based on age and sex for colorectal cancer patients was with an rang age of 50-70 year.

The results of present study are in consonant with (Hassan *et al*. 2021) who found that the colorectal cancer rises with age. Incidence rates increased over 45 age groups and higher than under 45 age groups .

The results of the present study was disagreed with (Vuik *et al.,* 2019) which indicated that the incidence increased in age group 30-39 years in both sexes with colorectal cancer

3. 4 . Residence of patients :

The results of the present study found no significant difference between urban and rural residents with colorectal cancer patients as shown in table (3-5) .

Table (3-5): Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum* between Control and Patient groups according to Residence.

Results	Control		P value	Patient		P value
	Urban (9)	Rural (4)		Urban (21)	Rural (12)	
Cq	30.43 ± 0.37	29.8 ± 1.33	0.677	28.0290 ± 0.6	27.5708 ± 0.94	0.692
Copy number	219.7467±70	700.2 ± 1156		3838.0357±1935	4265.5375±2121	

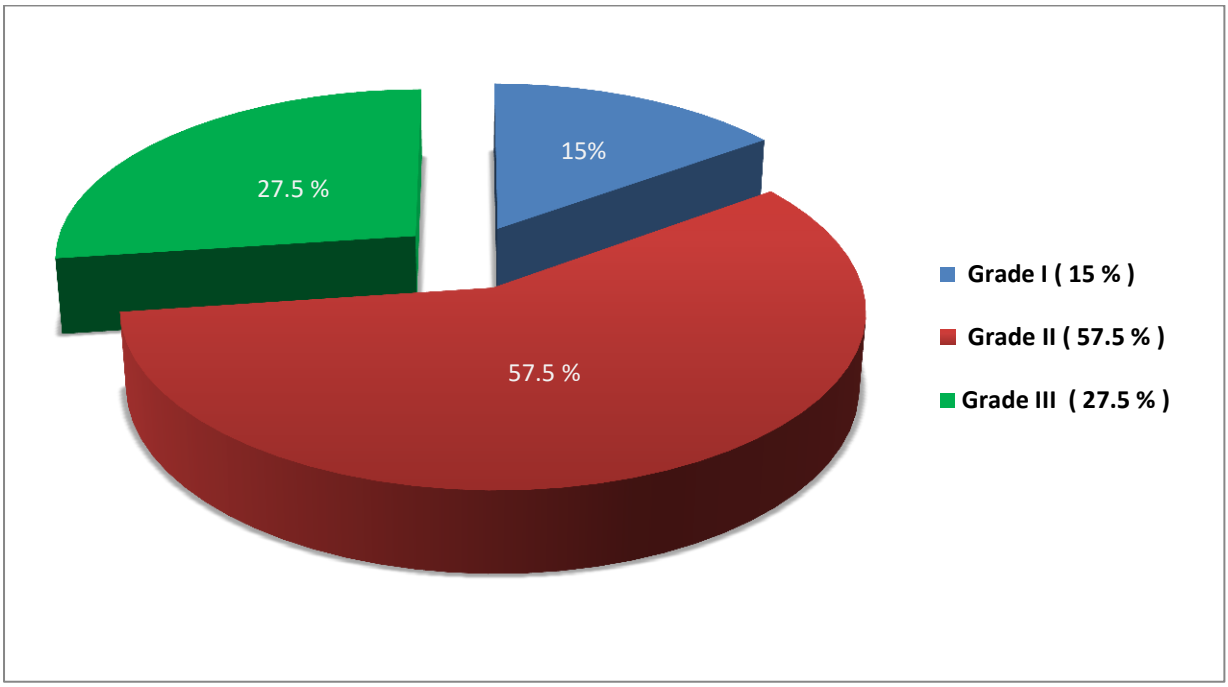
represent a significant difference at p≤ 0.05.

The results of the present study was disagreed with (Denggui *et al* ., 2018) which indicated that the colorectal cancer in Shijiazhuang (urban) were considerably higher than in Shexian (rural) in both men and women.

The results of the present study was consonant with (Jane *et al* ., 2018) which indicated there is no differences between the rural and urban populations with colorectal cancer.

3.5. Distribution of patients according to Grade of colorectal cancer :

The results of the present study found difference between histologic grade of colorectal cancer patients according to the level of *Fusobacterum nucleatum* include the well differentiated grade I , moderately differentiated grade II poorly differentiated grade III and majority of patients were in grade II (57.5 %), as shown in Figure(3-3).



Figure(3-3) Distribution of grade at CRC diagnosis in patients

Distribution of patients according to grade of colonic adenocarcinoma was as follows:

1. There were 6 patients (15 %) in grade I disease.
2. There were 23 patients (57.5 %) in grade II disease.
3. There were 11 patients (27.5 %) in grade III disease.

The results of the present study consonant with (Yongyu *et al*., 2020), who showed that *Fusobacterum nucleatum* is present in metastases and evaluated the relationship between the amount of bacterium and different clinicopathological features such as histological grade, cancer stage, location, depth of invasion, and node metastasis. The high levels of *Fusobacterum nucleatum* were significantly associated with moderately differentiated. The result is as follows well differentiated (4.9%) , moderately differentiated (75.4 %) and poorly differentiated (19.6 %).

The results of the present study was disagreed with (Shu *et al* ., 2021) which indicated that the high levels of *Fusobacterum nucleatum* were significantly associated with poor tumor differentiation in CRC patients.

The results of the present study consonant with (Yuan *et al* ., 2016) which found that the high levels of *Fusobacterum nucleatum* were significantly associated with moderately differentiated (58.4 %) in colorectal cancer patients

3.6. Molecular Identification of virulence gene *fadA Fusobacterium* adhesion A:

A total from 40 patients samples ,80% give positive results for identificationof *fadA* gene with molecular length about 232bp when compared with allelic ladder as shown in figure (3-7),while 20% of total samples give negative result as shown in table (3-6) .

Table (3-6):Distribution of *fadA* gene in the screened population (Patients).

Result	Number	Percentage	P value
Positive	**32**	**80**	**< 0.0001***
Negative	**8**	**20**	

* represent a significant difference at p<0.05.

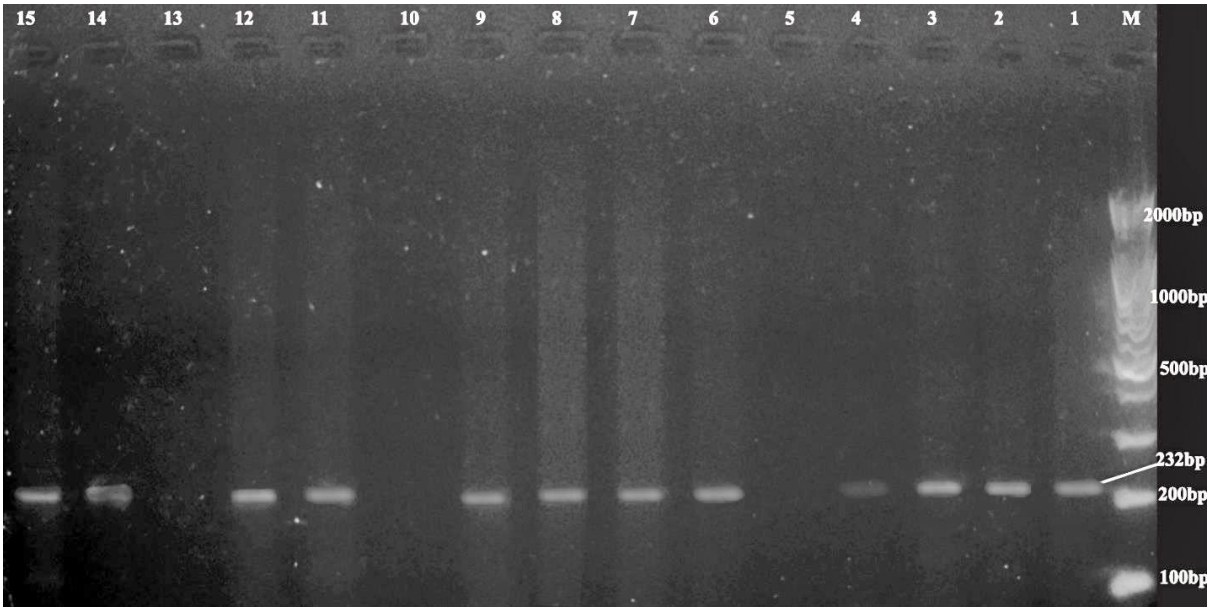


Figure (3-4): Agarose gel electrophoresis of Uniplex-PCR products obtained by using *fadA*-specific primer. Lanes (1-15)represent the identified *fadA* gene products, in exception of lanes (5, 10) and (13) Negative. Lane M represent 100bp DNA ladder. Note: Product size for *fadA* gene is 232bp.

FadA is a small ligand present on the surface of bacterium, which has been shown to bind to E-Cadherin and activates β-catenin signaling in human cancer xenografts of mice models . Thus, *fadA* binding is directly involved in host cell binding and invasion of *Fusobacterium* (Umana *et al* ., 2019).

The results of the present study similar with (Maryam *et al* .,2021) which indicated that presence of *Fusobacterium nucleatum* in human colorectal specimens has been

demonstrated by RNA-sequencing the bacterium has been shown to express high levels of virulence factors such as *fadA*, *fap*2 . Also the published data suggest that *Fusobacterium nucleatum* may be a prognostic biomarker of colorectal cancer risk, and hence raises the potential of antibiotic treatment of the bacterium for the prevention of colorectal cancer.

Huang *et al* (2016) found that Nine of the 13 samples proved positive for *fadA*, which demonstrated that the virulence factor could be detected in most *Fusobacterium nucleatum* strains while the negative result may be due to possible that the bacterium DNA concentration of the other negative samples is not with in PCR detection range resulting in the decreased PCR positivity rate.

The results of the present study similar with (Pin *et al* .,2020) ,as this study provided evidence that *Fusobacterium nucleatum* induced DNA damage and cell growth in CRC through *fadA*-dependent activation of the E-cadherin/β-catenin pathway leading to up-regulation of checkpoint kinase 2 (Chk2) is a multifunctional enzyme that has been shown to be central to cell cycle arrest and apoptosis by DNA damage.

The negative result in this study may due to that the strain negative for *fadA* gene or the gene found but not expression or contain other genes responsible for adhesion like Fap2 .The percentages of *fadA* gene in the screened population patients as shown in figure (3- 5).

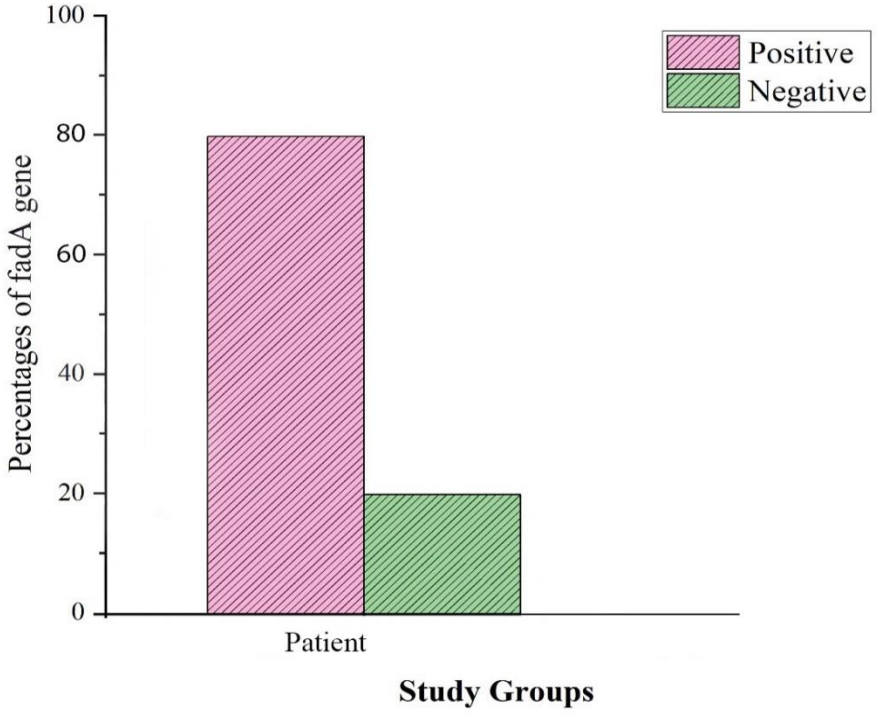


Figure (3-5): Distribution of *fadA gene* in the screened population (Patients).

3.7. Detection of genetic Susceptibilityto CRC in Associated with gene polymorphisms

3. 7. A. Micro RNA 146a SNP (rs2910164) :

Blood sample from 40 patient with CRC and 20 sample from control were subjected for DNA extraction and detection of (micro RNA 146 a) SNP by using ARMS PCR technique .The product of the results of ARMS were identify by gel electrophoresis as the allele **C**, **G** .Where allele **C** appeared in163bp the product size of **G** allele in 179bp ,while the product size of two outer primers 294bp as show in figure(3-6) .

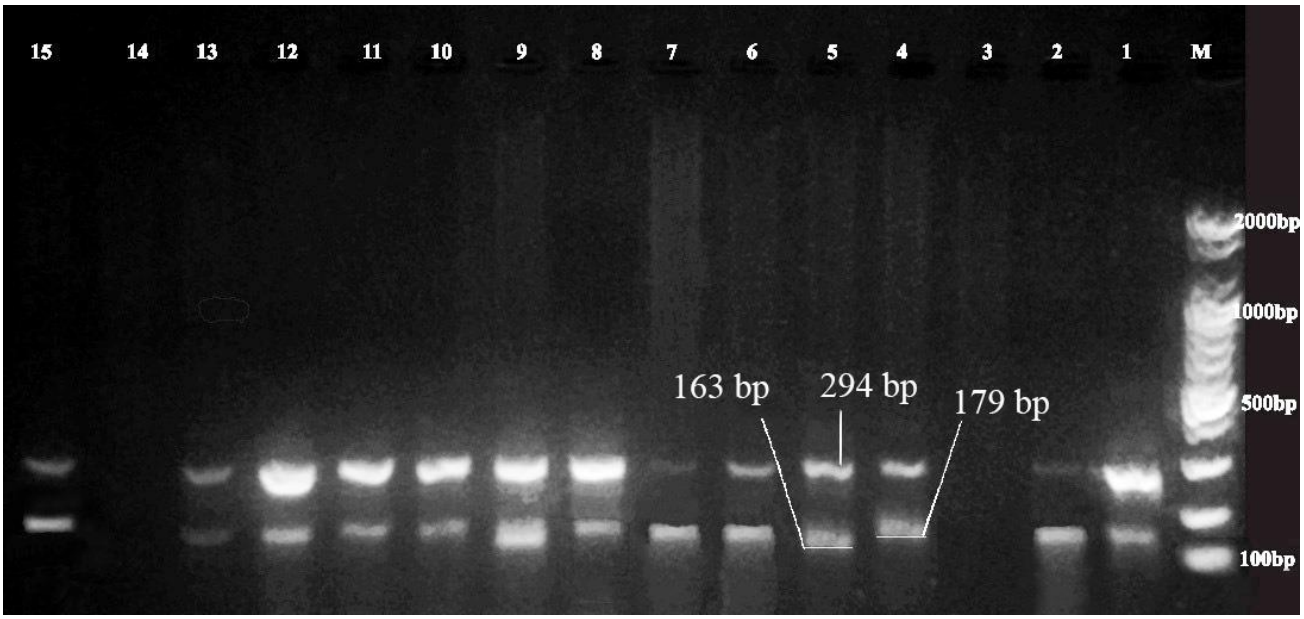


Figure (3-6): Agarose gel electrophoresis of ARMS-PCR products obtained by using rs2910164 SNP-specific primer. Lanes (1-15) represent the identified rs2910164 SNP products, in exception of lane (3) and (14) Negative, Lane M represent 100bp DNA ladder.

The result revealed that the homozygous **CC** genotype was found in 12 patient group and 14 in control group where heterozygous group **CG** was found in 6 control and 28 patient group and the homozygous genotype GG was not detected observed in control and patient group as show in table (3-7).

Table (3-7): Micro RNA 146a (rs2910164) SNP Genotypes distribution frequencies in the screened population (Control and Patients).

SNP	Genotypes	Allele Frequency	Controls	Patient	P Value	OR (95% CI)
rs2910164	C/C	26 (0.43)	14 (0.7)	12 (0.3)	0.0046*	1.00
	C/G	34 (0.57)	6 (0.03)	28 (0.7)	-----	5.82 (1.62-20.91)
	G/G	0 (0)	0 (0)	0 (0)		-----
	P value	< 0.0001*	< 0.0001*	< 0.0001*		

* represent a significant difference at p<0.05

Moreover **C** allele frequency was detect in 34 control group and 52 in patient group where as **G** allele frequency was 6 in control group and 28 in patient group as show in table (3-8).

Table (3-8): Micro RNA 146a (rs2910164) SNP Allele frequencies in the screened population (Control and Patients).

SNP	Allele Frequency	Frequency	Controls	Patient	P Value	OR (95% CI)
rs2910164	C	86 (0.72)	34 (0.85)	52 (0.65)	0.017*	1.00
	G	34 (0.28)	6 (0.15)	28 (0.35)		3.05 (1.14-8.146)
	P value	< 0.0001*	< 0.0001*	< 0.0001*		

* represent a significant difference at p<0.05.

The results in this study showed that the most patients were carrying wild-type. The CG heterozygous genotype with significant difference between patients and control group P < 0.05 and (OR =5.82 ,95% CI).

Regarding the allele frequency ,allele **C** was higher than allele **G** among control and patients with significant difference between patients and control (OR=1.00 ,95% CI) ,since allele **G** occur in low frequency ,thus possibility of appearance of the risky genotype GG is absent or reduce as show infigure (3-7).

Single nucleotide polymorphisms in miRNA could affect the stability and biological function of miRNA and their influence the regulation of target gene (rs2910164) **C** locus in miRNA -146a could influence the survival of CRC by

regulating the cell apoptosis and the expression ofcyclogenase-2 (Zhang *et al*.,2019).

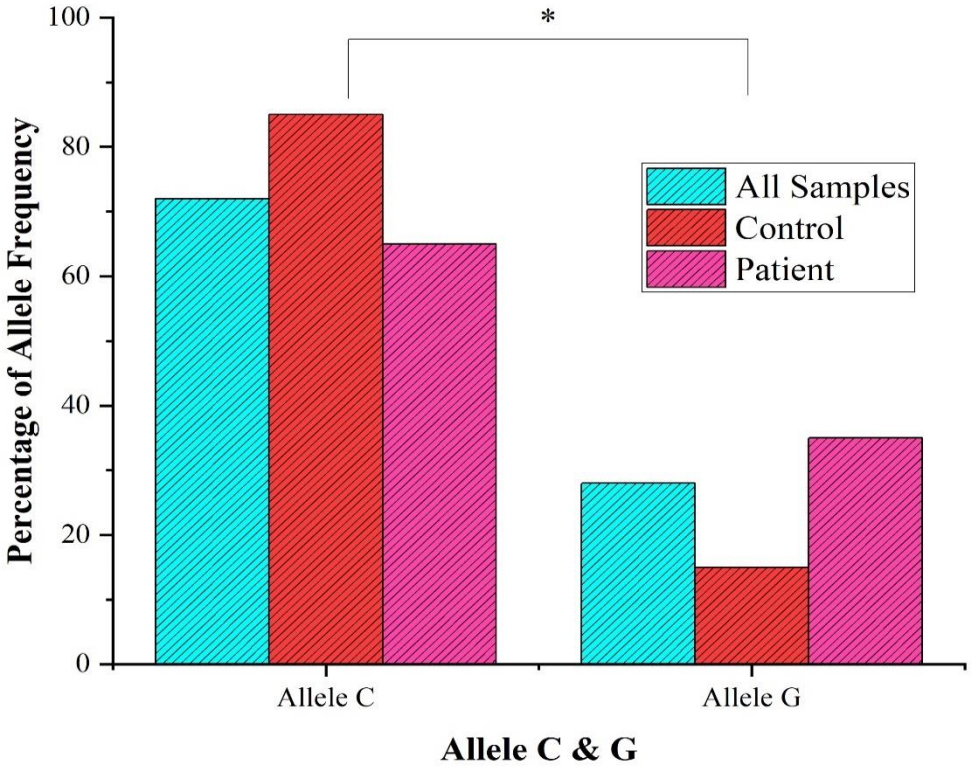


Figure (3-7): Percentage of Allele frequencies distribution in the screened population (Control and Patient).

In this study ,there is a significant risky association between microRNA 146 a gene polymorphisms and colorectal cancer patients .

micro RNAs (miRNAs) consist of 22 nucleotide and belong to a class of highly conserved single strand RNAs that epigenetically regulate protein translation through binding to the three un translated region of target mRNA and mediated either mRNA degradation or translation repression (Gao *et al* ., 2018).

Some mi RNA associated SNP have been reported to involve the development of cancer by influencing the biological function or repression of level of micro RNA(Wang *et al* ., 2012).

Park *et al* (2020) found that miRNA -146a SNPs **GG** genotype and the **CC** genotype may be linked to higher risk of gastric cancer and colorectal cancer while the **CG** genotype may be protective against digestive system in European population .

The **GG** genotype was not detected in patient and control this may be due to this genotype detect on normal case only ,or this SNP no found or detect ,this could be due to limited sample size ,or effect other environmental factor or geographic variation between patient and control .

miRNA -146a is one of the common microRNA which are of great importance for the roles of posttranscriptional regulator investigation have suggest that miRNA-146a is important for process of innate immune response and inflammation ,in which it acts as a vital negative regulator (Lu *et al* ., 2020).

The results of the present study similar with(Chen *et al* ., 2014) that identified that **G** allele in rs2910164 polymorphism might not influence the initiate digestive system cancer (DSC).

Mohamed *et al* (2019) found that the miRNA -146a (C → G) mutation could increase the expression of miR -146a and lead to immune suppression ,so the SNP could increase the risk of (DSC) .

3. 7. B. Distribution of Micro RNA rs2910164 SNP according to Age:

The results of distribution of Micro RNA rs2910164 SNP between control and patient according to age show that there is a significant different between allele frequency and age group in patient and control in age group 45-65 ,while their no significant different between patient and control in other age group , this result show in table (3- 9) .

Table (3-9) : Micro RNA 146a (rs2910164) SNP distribution frequencies in the screened population (Control and Patients) according to Age .

Age	Genotypes	Controls	Patients	OR (95% CI)	P value
< 45	CC	3 (60)	5 (45)	1.00	0.500
	CG	2 (40)	6 (54.5)	1.79 (0.20 -15.89)	
	GG	0 (0)	0 (0)	------	
45-65	CC	2 (100)	5 (20.8)	1.00	0.015*
	CG	0 (0)	19 (79.2)	0.714 (0.447-1.141)	
	GG	0 (0)	0 (0)	-----	
>65	CC	9 (69.2)	2 (40)	1.00	0.255
	CG	4 (30.8)	3 (60)	3.37 (0.40-28.75)	
	GG	0 (0)	0 (0)	----	

* represent a significant difference at p≤ 0.05.

The result of the current study is similar with (Greg *et al* ., 2020) indicated that single nucleotide polymorphisms (SNPs) in some gene are associated with increased adverse drug reactions (ADRs) risk, multimorbidity, and frailty in older people .These protein expression declined significantly with age in human peripheral blood mononuclear cells.

Furthermore numerous traits, such as cardiovascular diseases and cancer, are associated with age . However, these associations vary across races and ethnicities, suggesting an interplay between age and the genetic background in determining the trait . In addition, cellular stress response is a crucial biological process which modulates the damage to cells by activating repair signaling pathways with aging, both cellular stress response and repair pathways decline, which could be a trigger for

age-associated pathology (Wang *et al* ., 2021) . This result is consonant with the current study.

The study presented by (Yuhan *et al* .,2018) that the tumor suppressor p53 prevents early death due to cancer development. However, the role of p53 in aging process and longevity has not been well-established. In humans, single nucleotide polymorphism with either arginine (R72) or proline (P72) at codon 72 influences p53.

3. 7. C. Distribution of Micro RNA rs2910164 SNP according to Sex:

The results of distribution of Micro RNA rs2910164 SNP between control and patient according to sex show that there is no significant different between allele frequency and sex group in patient and control this result show in table (3- 10)

Table (3-10) : Micro RNA 146a (rs2910164)SNP distribution frequencies in the screened population (Control and Patients) according to Sex

Sex	Genotype	Controls	Patients	OR (95% CI)	P value
Female	C/C	6 (75)	5 (26.3)	1.00	0.027*
	C/G	2 (25)	14 (73.7)	7.25 (0.98-53.56)	
	G/G	0 (0)	0 (0)	------	
Male	C/C	8 (66.7)	7 (33.3)	1.00	0.068
	C/G	4 (33.3)	14 (66.7)	4.97 (0.95-26.0)	
	G/G	0 (0)	0 (0)	-----	

* represent a significant difference at p<0.05.

Chen *et al* (2018) found that there was no statistically significant difference in genotype distribution of the SNP and the sex status which similar with present study.

The results of the present study was consonant with (Carrie *et al*., 2012) that indicated no sex differences in the effects of autosomal single nucleotide polymorphism was analysis of more than 50,000 men and women .

Jiakai *et al* (2021) found that miR-146a rs2910164 polymorphism did not influence the risk of colorectal cancer even in subgroup analysis such as sex , in the future, more case control studies are needed to confirm our results.

Tomohiro *et al* (2016) hypothesized that the molecular mechanism of liver metastasis in CRC is caused by dramatic changes of gene signatures affected by miR-146a polymorphism and found there is no different between genotypes and sex .

3. 8. Gene expression of Interleukin 10(IL10) by using real- time PCR :

A total from 40 blood patients samples ,RNA was extracted to study the gene expression of IL-10 by using real- time PCR (Relative gene expression) (2-ddcT) methods in this method the level of expression of IL-10 gene in test samples as well as in control samples normalize with house - keeping for test samples ,as shown in figure (3 – 8).

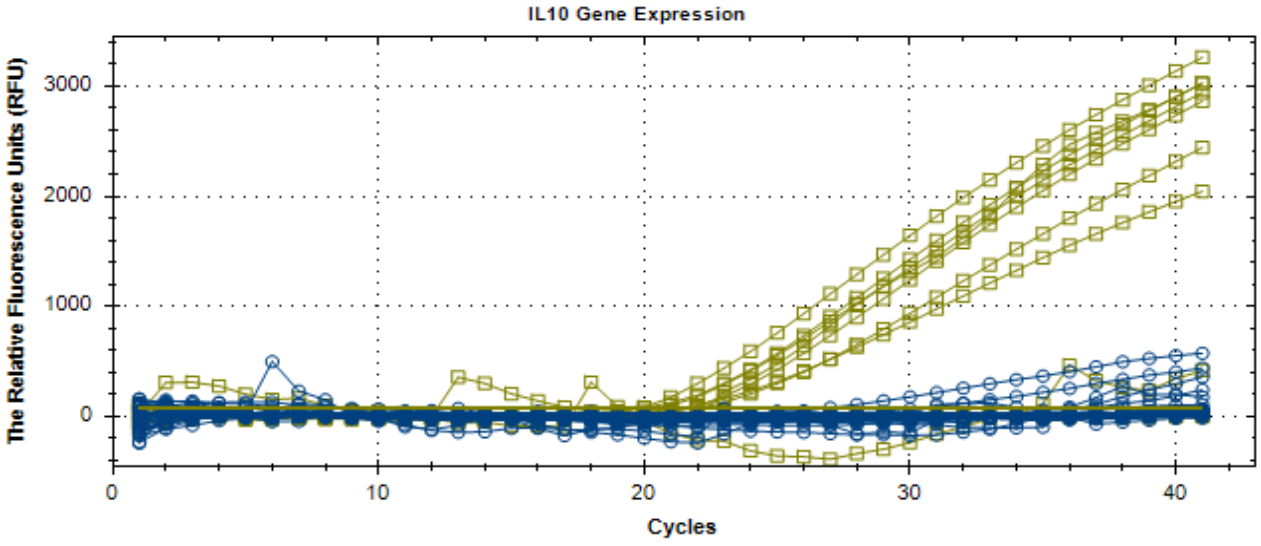


Figure (3-8): IL10 Gene expression level. This is the first run for 10 samples (Reference GAPDH) and 13, 33 samples (Control+ CRC Patients), represents amplification of Reference gene (GAPDH), represents amplification of samples (Control + CRC Patients).

The present study found that the expression of IL-10 gene very low in the CRC Patients when compared with control group. So expression of the gene is decrease in comparison with control set which increase more than (1.5) fold in compared with tumor tissues as shown in figure(3-9) and table (3-11).

Table (3-11): IL 10 Fold Gene Expression among Control and CRC Patients versus the reference gene (GAPDH).

Groups	N	Expression levels (2^-(ΔΔCt))	
		Mean	SD
Control	13	1.7	1.59
Patient	33	0.37	0.60
P value	< 0.0001*		

* represent a significant difference at p≤ 0.05.

The result in this study show that the value of level of IL-10 in CRC patients less than 1 , so expression is decrease of the gene in comparison with control set as shown in figure (3- 9).

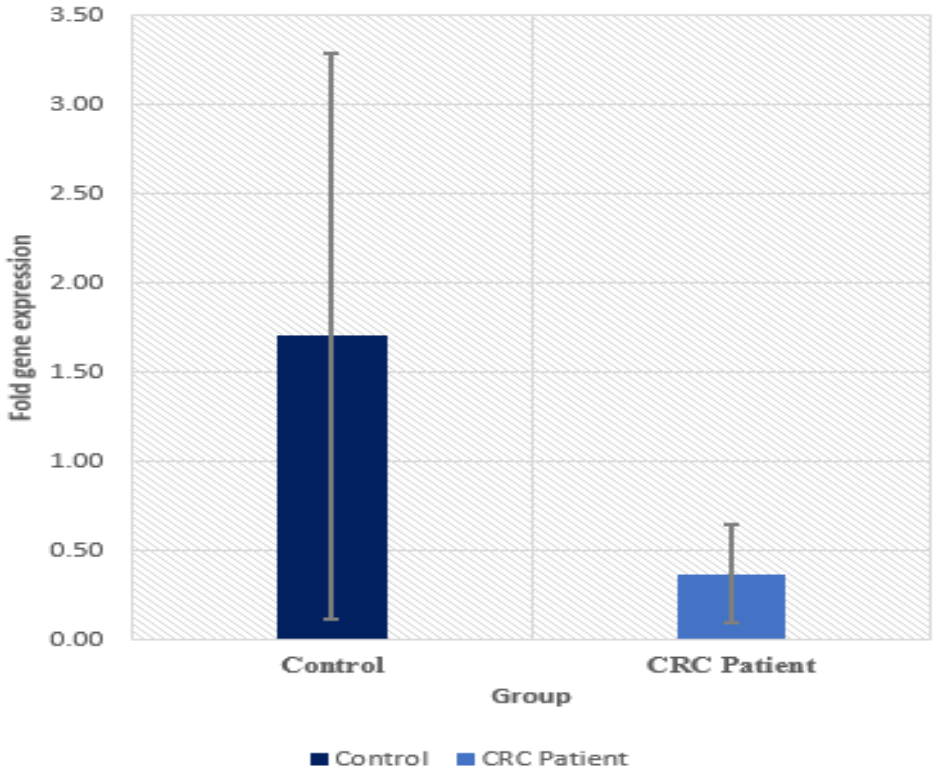


Figure (3- 9): IL 10 Fold Gene Expression among Control and CRC Patients versus the reference gene (GAPDH)s

IL-10 is crucial immunosuppression agent ,and the lack of IL -10 R in colorectal tissue could cause sever spontaneous colitis ,which poses a risk for CRC initiation (Shouval *et al* ., 2014).

Baosong *et al* (2019) found that the expression level of IL-10 was found lower in patient after CRC surgery than before ,and patient with recurrence CRC after surgery had significantly higher level of IL-10 ,this indicating that IL-10 can be a prognostic biomarker in CRC .

IL-10 expression is regulated at the transcriptional and post-transcriptional levels ,IL-10 appears to have considerable importance in the development of human cancer and it immune escape so it serve as a biomarker for prognostic diseases and for treatment (Maria *et al* ., 2020).

The results of the present study consonant with (Shabnam *et al* ., 2017) which indicated that (IL-10) level were significantly lower in CRC patients than in controls

(P = 0.04). colorectal cancer patients with worse prognosis at the time of diagnosis tend to have higher levels of circulating IL-10 than those with better prognosis (P = 0.008). Receiver operating characteristics curve analysis demonstrated that IL-10 levels in the sera of CRC patients can be used as a prognostic biomarker in CRC patients . Therefore, lower IL-10 levels were associated with higher risk of the disease, its higher levels were associated with a poorer prognosis .

The results of the present study was disagreed with (Yong *et al* .,2021) which indicated IL-10 is produced not only by immune cells but also by cancer cells themselves many studies have examined the ability of IL-10 to suppress antitumor immunity for example, IL-10 secreted by peritoneal monocytes down regulates cytokine production and T-cell proliferation in ovarian cancers patients with more advanced CRC have higher serum IL-10 levels and serum IL-10 has been shown to affect the prognosis of colon cancer patients.

Andrzej *et al* (2009)found the serum cytokine levels in patients with CRC characterized by the stimulated production of monocyte / macrophage pro-inflammatory cytokines in the presence of normal circulating levels of IL-10. The level of IL-10 was not significantly different from the normal value. The CRC patients had a significantly lower level of IL-10 than the Ulcerative colitis (UC) patients. The cytokine levels in the CRC patients showed no significant differences (0.6255).

The results of the present study was disagreed with (Braham *et al* ., 2017) which indicated the Interleukin 10 is considered an immune modulator cytokine, showing both antitumor and pro-tumor characteristics. Its role in the pathogenesis and progression of colorectal cancer depends on microenvironmental milieu mean serum IL-10 levels were significantly higher in CRC patients than in controls. CRC patients with worse prognosis at the time of diagnosis tend to have higher levels of circulating IL-10 than those with better prognosis

3.9. Gene expression of Interleukin18(IL-18) by using real- time PCR :

A total from 40 blood patients samples and 20 blood control samples ,RNA was extracted to study the gene expression of IL-18 by using real- time PCR (Relative gene expression) (2-ddcT) technique in this method the level of expression of IL-18 gene in test samples as well as in control samples normalize with house - keeping gene for test samples ,as shown in figure (3-10) .

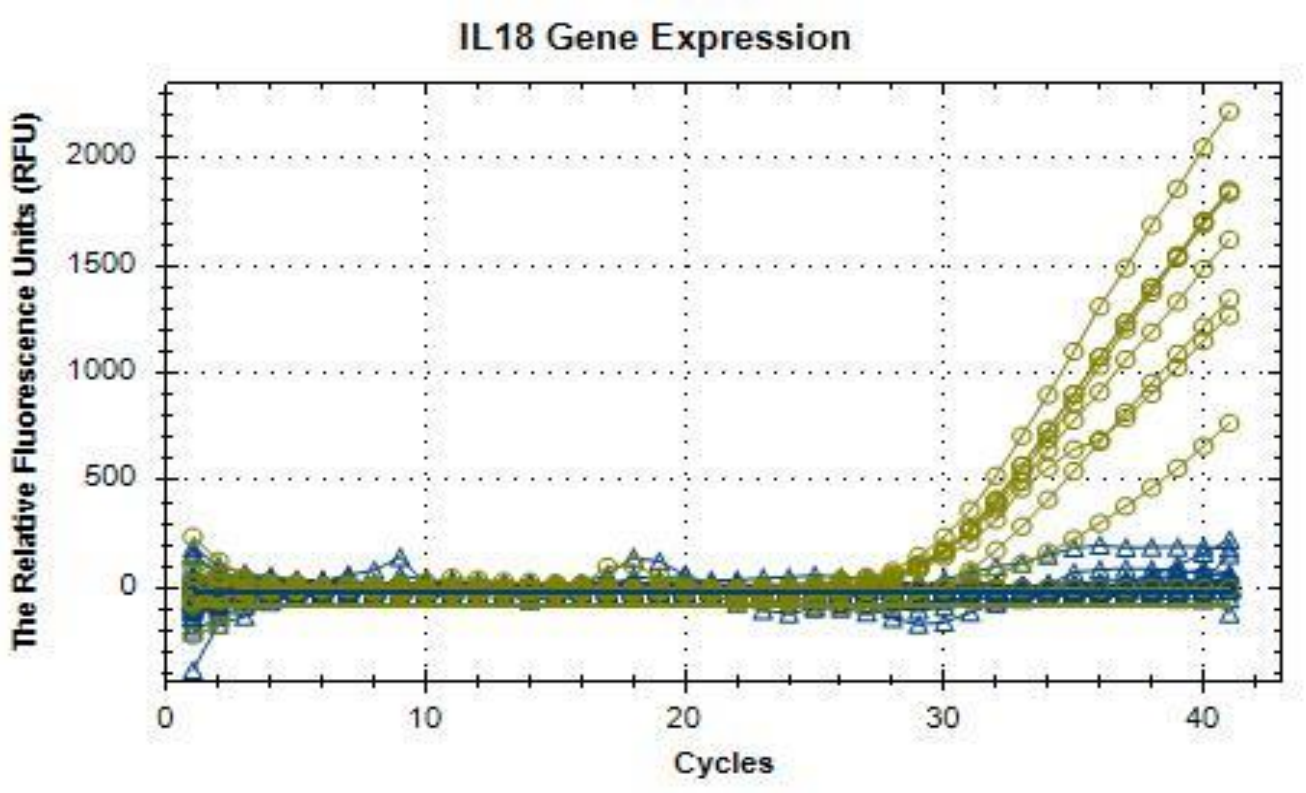


Figure (3-10): IL-18 Gene expression level. This is the first run for 10 samples (Reference GAPDH) and 5 samples (Control+CRC Patients), represents amplification of Reference gene (GAPDH), represents amplification of samples (Control + CRC Patients).

The results found that the expression of IL-18 mRNA gene were very low in the CRC Patients when compared with control group as shown in Table (3-12) .

Table (3-12): IL- 18 Fold Gene Expression among Control and CRC Patients versus the reference gene (GAPDH).

Groups	N	Expression levels (2^-(ΔΔCt))	
		Mean	SD
Control	**13**	**3.05**	**2.79**
Patient	**33**	**0.22**	**0.05**
P value	**< 0.0001***		

* represent a significant difference at p≤ 0.05

The result in this study show that the value of level of fold gene expression of IL18 in CRC patients less than (1) , so expression of the gene is decrease in comparison with control set which increase more than 2-fold in compared with tumor tissues as shown in figure (3-11).

Among the IL-1 family, interleukin18 (IL-18) is one of the best characterized. The protein encoded by the IL-18 gene, located at 11q23.1, is essential for the response to the pathogens and activation of host defense mechanisms. IL-18 has been shown to be a mediate product of activation by NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome /caspase-1 signaling pathway. It is secreted mainly by macrophages and dendritic cells and stimulates interferon-γ (IFN-γ) production by natural killer (NK) cells and thymus-dependent lymphocyte(T cells) (Yasuda *et al* ., 2019).

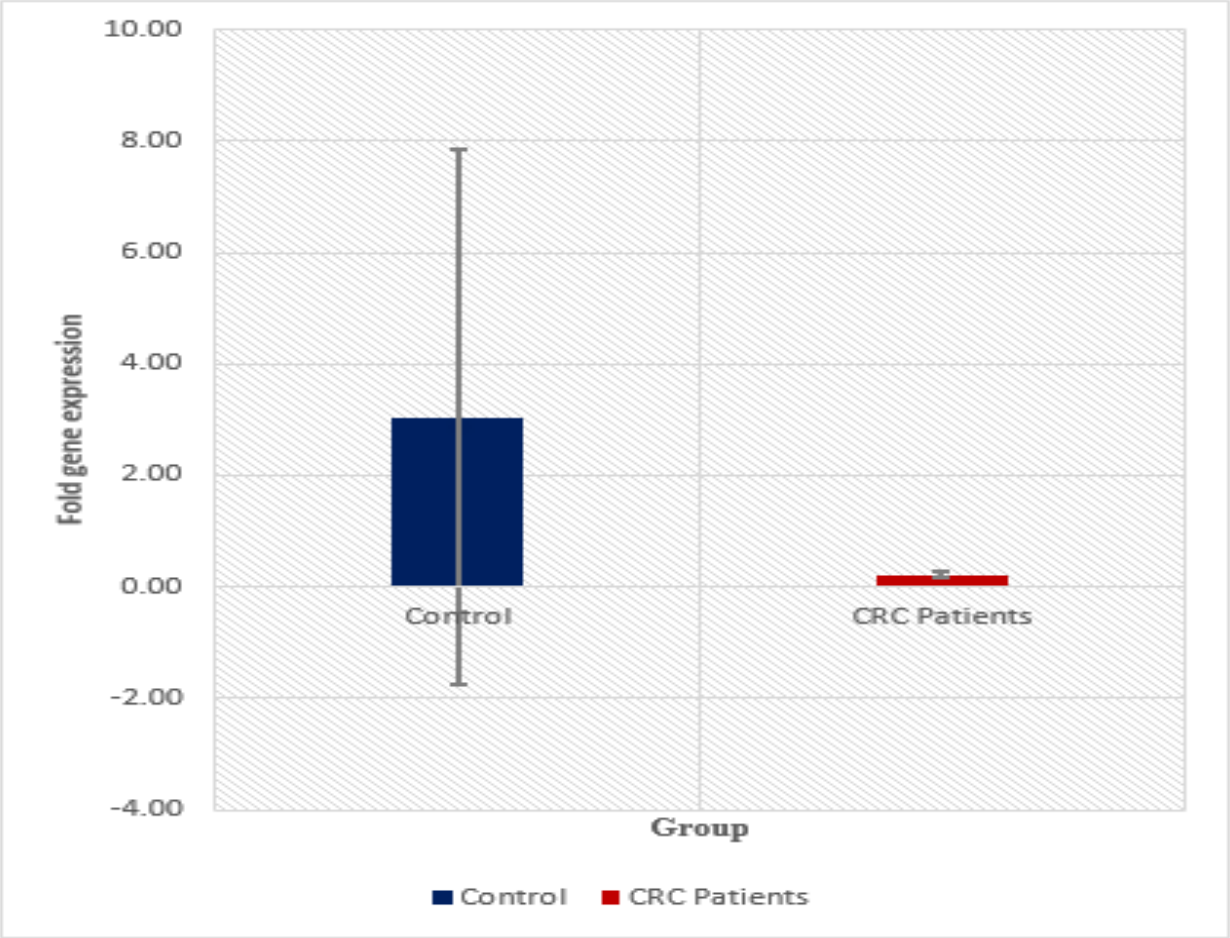


Figure (3-11): IL -18 Fold Gene Expression among Control and CRC Patients versus the reference gene (GAPDH)

Interleukin-18 , also known as interferon-gamma inducing factor is a protein which in humans encoded by the *IL-18* gene. The protein encoded by this gene is a proinflammatory cytokine., IL-18 can modulate both innate and adaptive immunity and its dysregulation can cause autoimmune or inflammatory diseases. Recently found , aberrant low expression of IL-18 has been identified in some digestive system cancers, such as esophageal cancer and oral squamous cell carcinoma (Li *et al* .,2018).

Feng *et al* (2020) found that IL-18 was significantly lower in colon cancer tissues than normal tissues, and this down regulated expression was happened on the early stage of the disease, The data revealed that low IL-18 expression in colon cancer tissues was associated with tumor size and American Joint Committee on Cancer

(AJCC) stage and implicated that IL-18 is a prognostic factor for overall survival (OS). Furthermore, the results indicate that IL-18 markedly represses colon cancer cell proliferation.

Baosong *et al* (2019) have demonstrated that the expression levels of IL-18 in the serum of patients with CRC were statistically higher than those of the control group. And the expressions of IL-18 of patients with reoccurred CRC after the operation were significantly higher than that of patients without recurrence of CRC in thestudy group.

The results of current study disagree with (Hosseini *et al* .,2019) who indicated that the cytokine level was increased significantly in cancer patients.

The expressions of IL-10 and IL-18 in CRC patients were not statistically related with factors including age, gender and body mass index ,but were in statistical relation to factors such as the Dukes staging, tumor size, histological grades and the distant metastasis of cancer cells a gradual decrease of the expression of IL-10 and IL-18 in colorectal cancer patients surfaced after surgery and thus the expression levels of IL-10 and IL-18 in the serum seven days after the operation were statistically lower than those before the operation $P<0.05$ (Baosong *et al* .,2019).

Decision of Examination Committee

We are the examiner committee, certify that we have read this thesis entitled **Molecular study for the correlation between *Fusobacterium nucleatum* and colorectal cancer** and have examined the student (**Jassim Hamza K. Al-Masoudi**) in its content, and that in our opinion; it is accepted as a thesis for degree of **Doctor of Philosophy** in Microbiology with **Excellent** estimation

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1-Result of copy number:

Well	Target	Cq	Amount	Sample	Effeciancy	Type	Copy number	Status	Gender	Age	Location
A01	Fn	17.23	1.00E+06		79.36%±14.71	standard	1.00E+06				
A02	Fn	18.34	100000		79.36%±14.71	standard	100000				
A03	Fn	22.77	10000		79.36%±14.71	standard	10000				
A04	Fn	27.36	1000		79.36%±14.71	standard	1000				
A05	Fn	30.26	100		79.36%±14.71	standard	100				
A06	Fn	36.75	10		79.36%±14.71	standard	10				
B01	Fn	39.26			79.36%±14.71	negative					
C01	Fn	29.64		1	79.36%±14.71	unknown	273.75	Patient	Female	42	Urban
C02	Fn	31.23		2	79.36%±14.71	unknown	108.13	Patient	Male	43	Rural
C03	Fn	30.25		3	79.36%±14.71	unknown	191.69	Patient	Male	65	Rural
C04	Fn	30.25		4	79.36%±14.71	unknown	191.69	Patient	Female	67	Urban
C05	Fn	28.17		5	79.36%±14.71	unknown	646.13	Patient	Male	36	Rural
C06	Fn	28.04		6	79.36%±14.71	unknown	697.11	Patient	Male	33	Urban
C07	Fn	26.18		7	79.36%±14.71	unknown	2066.38	Patient	Male	58	Urban
C08	Fn	25.95		8	79.36%±14.71	unknown	2363.55	Patient	Male	72	Urban
C09	Fn	26		9	79.36%±14.71	unknown	2295.51	Patient	Female	62	Rural
C10	Fn	31		10	79.36%±14.71	unknown	123.68	Patient	Female	50	Urban
C11	Fn	30.5		11	79.36%±14.71	unknown	165.64	Patient	Male	49	Urban
C12	Fn	29.23		12	79.36%±14.71	unknown	347.84	Patient	Male	53	Rural
D01	Fn	28		13	79.36%±14.71	unknown	713.59	Patient	Female	36	Urban
D02	Fn	28.5		14	79.36%±14.71	unknown	532.83	Patient	Female	52	Urban
D03	Fn	26.5		15	79.36%±14.71	unknown	1714.04	Patient	Female	45	Urban
D04	Fn	21.24		16	79.36%±14.71	unknown	37030.83	Patient	Male	55	Urban
D05	Fn	22.63		17	79.36%±14.71	unknown	16439.91	Patient	Female	42	Urban
D06	Fn	23		18	79.36%±14.71	unknown	13244.17	Patient	Male	60	Rural
D07	Fn	29		19	79.36%±14.71	unknown	397.86	Patient	Female	55	Urban

D08	Fn	31		20	79.36%±14.71	unknown	123.68	Patient	Male	72	Urban
D09	Fn	26.56		21	79.36%±14.71	unknown	1655	Patient	Female	62	Rural
D10	Fn	22.79		22	79.36%±14.71	unknown	14972.87	Patient	Female	50	Urban
D11	Fn	31.23		23	79.36%±14.71	unknown	108.13	Patient	Male	49	Urban
D12	Fn	29.12		24	79.36%±14.71	unknown	370.93	Patient	Male	53	Rural
E01	Fn	29.1		25	79.36%±14.71	unknown	375.29	Patient	Female	36	Urban
E02	Fn	31.22		26	79.36%±14.71	unknown	108.76	Patient	Female	52	Urban
E03	Fn	30		27	79.36%±14.71	unknown	221.83	Patient	Male	43	Rural
E04	Fn	22.01		28	79.36%±14.71	unknown	23615.81	Patient	Male	65	Rural
E05	Fn	26.61		29	79.36%±14.71	unknown	1607.36	Patient	Female	67	Urban
E06	Fn	23.78		30	79.36%±14.71	unknown	8397.06	Patient	Male	36	Rural
E07	Fn	31.5		31	79.36%±14.71	unknown	92.35	Patient	Female	62	Rural
E08	Fn	29		32	79.36%±14.71	unknown	397.86	Patient	Female	50	Urban
E09	Fn	30.23		33	79.36%±14.71	unknown	193.94	Patient	Male	49	Urban
E10	Fn	30.22		34	79.36%±14.71	unknown	195.08	Control	Male	70	Urban
E11	Fn	30.23		35	79.36%±14.71	unknown	193.94	Control	Male	37	Urban
E12	Fn	31.15		36	79.36%±14.71	unknown	113.3	Control	Female	80	Urban
F01	Fn	29.9		37	79.36%±14.71	unknown	235.17	Control	Male	70	Urban
F02	Fn	30.8		38	79.36%±14.71	unknown	139.01	Control	Female	44	Rural
F03	Fn	30.99		39	79.36%±14.71	unknown	124.41	Control	Male	69	Urban
F04	Fn	25.9		40	79.36%±14.71	unknown	2433.61	Control	Male	70	Rural
F05	Fn	31.9		41	79.36%±14.71	unknown	73.11	Control	Female	72	Rural
F06	Fn	30.6		42	79.36%±14.71	unknown	156.24	Control	Female	67	Urban
F07	Fn	31.8		43	79.36%±14.71	unknown	77.51	Control	Female	56	Urban
F08	Fn	31.1		44	79.36%±14.71	unknown	116.66	Control	Male	35	Urban
F09	Fn	30.61		45	79.36%±14.71	unknown	155.33	Control	Female	51	Rural
F10	Fn	27.88		46	79.36%±14.71	unknown	765.41	Control	Male	74	Urban

2-Result of ARMS PCR:

G1	**< 45**
G2	**45-65**
G3	**> 65**

N	SNP	STATUS	SEX	AGE	Residence
1	C/C	Control	Male	G3	Urban
2	C/C	Control	Male	G1	Urban
3	C/C	Control	Female	G3	Urban
4	C/C	Control	Male	G3	Urban
5	C/C	Control	Female	G1	Rural
6	C/C	Control	Male	G3	Urban
7	C/C	Control	Male	G3	Rural
8	C/C	Control	Female	G3	Rural
9	G/G	Control	Female	G3	Urban
10	C/C	Control	Female	G2	Urban
11	C/C	Control	Male	G1	Urban
12	C/C	Control	Female	G2	Rural
13	C/C	Control	Male	G3	Urban
14	C/C	Control	Male	G3	Urban
15	C/G	Control	Male	G1	Urban
16	C/G	Control	Female	G3	Urban
17	C/G	Control	Male	G3	Urban
18	C/G	Control	Female	G1	Rural
19	C/G	Control	Male	G3	Urban
20	C/G	Control	Male	G3	Rural
21	C/C	Case	Male	G1	Rural
22	C/C	Case	Male	G2	Rural
23	C/C	Case	Female	G3	Urban
24	C/C	Case	Male	G1	Rural
25	C/C	Case	Female	G2	Rural
26	C/C	Case	Female	G2	Urban
27	C/C	Case	Male	G2	Urban
28	G/G	Case	Female	G1	Urban

29	C/C	Case	Male	G1	Rural
30	C/C	Case	Male	G2	Rural
31	C/C	Case	Female	G3	Urban
32	C/C	Case	Male	G1	Rural
33	C/G	Case	Male	G1	Urban
34	C/G	Case	Male	G2	Urban
35	C/G	Case	Male	G3	Urban
36	C/G	Case	Female	G2	Rural
37	C/G	Case	Female	G2	Urban
38	C/G	Case	Male	G2	Urban
39	C/G	Case	Male	G2	Rural
40	C/G	Case	Female	G1	Urban
41	C/G	Case	Female	G2	Urban
42	C/G	Case	Female	G2	Urban
43	C/G	Case	Male	G2	Urban
44	C/G	Case	Female	G1	Urban
45	C/G	Case	Male	G2	Rural
46	C/G	Case	Female	G2	Urban
47	C/G	Case	Male	G3	Urban
48	C/G	Case	Female	G2	Rural
49	C/G	Case	Female	G2	Urban
50	C/G	Case	Male	G2	Urban
51	C/G	Case	Male	G2	Rural
52	C/G	Case	Female	G1	Urban
53	C/G	Case	Female	G2	Urban
54	C/G	Case	Male	G1	Rural
55	C/G	Case	Male	G2	Rural
56	C/G	Case	Female	G3	Urban
57	C/G	Case	Male	G1	Rural
58	C/G	Case	Female	G2	Rural
59	C/G	Case	Female	G2	Urban
60	C/G	Case	Male	G2	Urban

3-Result of gene expression:

Groups	Ct1 (GAPDH)	Ct2 (GAPDH)	Ct1 (IL10)	Ct2 (IL10)
Control 1	20.08668456	21	36	32
Control 2	22	21	33	33.2
Control 3	21.9	22.5	35	35.3
Control 4	22.3665231	21	33.8	33.1
Control 5	20.67858643	20.9	33	33.3
Control 6	20.08668456	21	34	33
Control 7	21	20	37	36
Control 8	22.2	22.9	33	33.5
Control 9	22.3665231	20	37.6	36
Control 10	20.67858643	21	32	33
Control 11	22.3665231	20.9	35.56592	34
Control 12	20.67858643	21.2	38	37
Control 13	20.08668456	20.2	33	33.5
Treated 1	22.07129121	21	38.48752	38.1
Treated 2	21.45425419	22	35.81013	35.60
Treated 3	21.73496198	21	37.55632	37.20
Treated 4	23	22	37.978436	37.10
Treated 5	21	20.2	39.61625	39.20
Treated 6	22.7	21.4	37.43478	37.80
Treated 7	21.5	20.2	39.53344	39.30
Treated 8	21.7	21	36.55215	36.90
Treated 9	23.8	23.9	37.85057	37.30
Treated 10	21.9	21.5	34.9	34.20
Treated 11	22.1	22.1	38.1	38.00
Treated 12	21.45425419	21.2	37.98801	38.00
Treated 13	21.73496198	22	38.68574	37.00
Treated 14	23	23.9	40.2964	38.00
Treated 15	21	21.5	38.2	38.90
Treated 16	22.07129121	22.1	37.8	36.30
Treated 17	21.45425419	21.2	36.99814	36.10
Treated 18	21.73496198	22	33	33.90
Treated 19	23	23.9	38.8082	38.40
Treated 20	21	21.5	36	37.00
Treated 21	22.07129121	22.1	37.99777	37.30
Treated 22	21.45425419	21.2	37.978436	37.10
Treated 23	21.73496198	22	39.61625	38.00
Treated 24	23	23.9	37.43478	37.10
Treated 25	21	21.5	39.53344	39.10
Treated 26	22.07129121	22.1	36.55215	35.80

Treated 27	21.45425419	21.2	37.85057	37.50
Treated 28	21.73496198	22	34	34.93
Treated 29	23	23.9	40.99901	38.00
Treated 30	21	21.5	37.98801	38.00
Treated 31	22.07129121	22.1	38.68574	38.20
Treated 32	21.45425419	21.2	40.2964	38.12
Treated 33	22.07129121	22	38.2	37.22

Groups	Ct1 (GAPDH)	Ct2 (GAPDH)	Ct1 (IL18)	Ct2 (IL18)
Control 1	27.79	26.3	30.23	31.23
Control 2	27.70	27.12	31.30	30.34
Control 3	26.00	26.9	30.28	31.8
Control 4	25.65	25.1	33.37	32.45
Control 5	28.08	27.2	34.00	35.2
Control 6	27.79	26.2	34.50	34.9
Control 7	27.70	26.3	34.90	33.89
Control 8	26.00	25.3	33.42	34.92
Control 9	25.65	24.1	35.12	35.9
Control 10	28.08	27.55	36.28	37.2
Control 11	26.8	26.1	34.13	35
Control 12	27.33	27.3	33.18	34.25
Control 13	26.21	26.3	37.14	36.3
Treated 1	27.40	27.99	39.55	38.1
Treated 2	26.55	26.94	38.14	37.22
Treated 3	20.38	20.98	38.55	38.19
Treated 4	28.22	28.89	39.14	38.90
Treated 5	30.57	29.34	40.09	39.20
Treated 6	27.10	27.86	39.12	39.14
Treated 7	24.30	25.2	37.12	38.18

Treated 8	21.20	21.36	36.23	37.10
Treated 9	28.10	28.91	37.14	36.22
Treated 10	29.00	28.1	38.23	37.24
Treated 11	26.70	26.2	37.45	38.32
Treated 12	25.80	25.1	36.14	37.22
Treated 13	22.30	23	34.80	35.22
Treated 14	27.12	26.32	36.12	37.34
Treated 15	29.14	28.26	36.39	37.00
Treated 16	21.92	23	35.24	36.33
Treated 17	27.34	25.9	34.38	35.12
Treated 18	29.67	28.23	34.16	35.72
Treated 19	26.12	25.15	35.89	35.23
Treated 20	28.23	28.11	35.89	36.21
Treated 21	21.59	22.1	34.16	35.34
Treated 22	28.33	28.2	36.81	37.23
Treated 23	26.12	26.9	39.53	38.23
Treated 24	27.13	26.14	39.19	38.31
Treated 25	29.23	29.81	40.23	39.23
Treated 26	28.12	27.23	38.24	37.23
Treated 27	23.34	24.1	34.16	35.25
Treated 28	23.14	24.6	36.81	37.15
Treated 29	27.33	26.6	39.53	38.14
Treated 30	26.80	26.1	39.19	39.35
Treated 31	25.10	24	40.23	40.10
Treated 32	22.92	23	38.24	38.49
Treated 33	26.23	26.7	39.19	40.18

Conclusions :

1. The incidence of *Fusobacterium nucleatum* in patients with colorectal cancer disease which diagnosed by qRT-PCR copy number methods and *Fusobacterium nucleatum* infection is prevalent in human colorectal cancer (CRC) tissues .
2. The *fadA* as virulence gene was found highly among *Fusobacterium nucleatum* isolates .
3. Several potential biomarkers such as MicRNA 146a may be considered as criteria for determining CRC prognosis
4. The CG and CC genotype may be linked to higher risk of CRC and gastric cancer .
5. *Fusobacterium nucleatum* promote progression of CRC via multiple potential mechanisms.
6. The tumor tissues with *Fusobacterium nucleatum* had decrease the expression of interleukin 10 and interleukin 18 .
7. Perspective studies should be done to confirmative role of *Fusobacterium nucleatum* in colorectal cancer

Recommendations:

1. The use of genotypic methods such as pulsed –field gel electrophoresis and /or multilocus sequence typing that provides more discriminatory power .
2. Further important study to explore the relationship between *Fusobacterium nucleatum* virulence genes and their ability to survive in human and hence contribute to incidence CRC .
3. Further study to developed immunotherapy drug biological agents with minimal side effects targeting *Fusobacterium nucleatum* positive colorectal cancer .
4. Clinical and epidemiological studies should address the potential role of *Fusobacterium nucleatum* in the etiology of colorectal cancer .

Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

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Conclusions and Recommendations

Appendix

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List of abbreviations

Abbreviation	*Meaning*
APC	Adenomatous polyposis coli
ATG7	Autophagy-related protein 7
AJCC	American Joint Committee on Cancer
BRAF	B-Raf proto-oncogene serine/threonine kinase
β-catenin	Beta – catenin
bp	Base pair
BSA	Bovine serum albumin
CRC	Colorectal cancer
CpG	5'—C (cytosine) —phosphate—G (guanine) —3'
CIMP	CpG island methylator phenotype
CHD	Chromodomain helicase DNA binding protein
CDH5	Cadherins 5 - are calcium-dependent cell adhesion proteins
COX2	Cyclo-oxygenase-2
c-Myc	cellular Myelocytomatosis
CD	Cluster of differentiation
CTLs	Cytotoxic T lymphocytes
CSIF	cytokine synthesis inhibitory factor
CI	confidence interval.
CXCL1	C-X-C motif chemokine ligand 1
^{o}C	Degree Celsius
Cq	quantification cycle
DNA	Deoxyribonucleic acid
DSC	digestive system cancer
DC	Dendritic cells
EDTA	Ethelynediaminetetra acitic acid
ECM	extracellular matrix
ERK	Extracellular regulated mitogen-activated protein kinase
FASLG	Fas ligand

FIP	*Fusobacterium nucleatum* immunosuppressive protein
FOXP3	forkhead box P3
FadA	Fusobacterium adhesion A
5-FU	5-fluorouracil
Fap2	fibroblast activation protein 2
FFPE	formalin fixed paraffin embedded
Gr1	Granulocyte differentiation antigen 1
GI tract	Gastrointestinal tract
GTP	Guanosine triphosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gal-Gal NAc	Calactose /N-acetylgalactosamine
HEK293cells	Human embryonic kidney 293 cells
H2S	hydrogen sulfide
HP-PPIs	host-pathogen protein-protein interactions
IU	International unit
IARC	International agency for research on cancer
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IL	Interleukin
IG	Immunoglobulin
IBD	Inflammatory bowel disease
IFNG	Interferon Gamma
IκB	inhibitor kappa B
IL-18R	Interleukin -18 receptor
ILC3	Innate lymphoid cells
JAK	Janus kinase
KDa	kilo Dalton
LCR	Ligase chain reaction
LPS	lipopolysaccharide
LEF	lymphoid enhancer factor
MSI	Microsatellite instability
MEK	Mitogen-activated protein kinase-ERK kinase
MMR	Mismatch repair

MMP3	Matrix metalloproteinase-3
MLH1	Human mut L homolog 1
Mgcl2	Manganese chloride
MYD88	Myeloid differentiation primary response gene 88
MDSCs	Myeloid-derived suppressive cells
MAPK	mitogen-activated protein kinases
miRNA	MicroRNA
mm	Millimeter
mg	Milligram
ml	Milliliter
µl	Microliter
NF-κB	nuclear factor kappa-B
NK cell	Natural killer cell
NKT	Natural killer T cells
ng	Nanogram
Nm	Nanometer
NOD1	Nucleotide Binding Oligomerization Domain Containing 1
NLRP3	NOD-like receptor pyrin domain-containing protein 3
NSAIDs	Non-steroidal anti-inflammatory drugs
NOD- LR	nucleotide-binding oligomerization domain-like receptors
OMV	outer membrane vesicles
OR	odds ratio
OS	overall survival
PCNA	proliferating cell nuclear antigen
POMP	Polymorphic outer membrane protein
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Picogram
PH	Power of hydrogen (H^+)
p-value	probability value
PGE2	prostaglandin E2

PMN	polymorph nuclear neutrophils
PD-1	programmed cell death 1
PD-L1	Programmed death-ligand 1
PDCD1	programmed cell death 1
PTGS2	prostaglandin-endoperoxide synthase2
PTGER2	prostaglandin E receptor 2
PAK1	Protein activated kinase 1
PBMCs	Peripheral blood mononuclear cells
Reg T cells	regulatory T cell
ROS	Reactive oxygen species
RIG-1	Retinoic acid inducible gene I
RASA1	RASp21 GTPase activating protein
RAS	A class of small G-protein
RAF	Rapidly accelerated fibrosarcoma
RNA	Ribonucleic acid
Rpm	Round per minute
rs	Reference SNP
SPSS	Statistical Package for the Social Sciences
SDS-PAGM	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCARB1	The scavenger receptor class B type I gene
STAT	signal transducer and activator of transcription
TBE	Tris-Borate-EDTA buffer
TAMs	Tumor associated macrophages
TANs	Tumor associated neutrophils
TRUC mice	T-bet −/− × Rag 2−/− ulcerative colitis mice
TNF	Tumor necrosis factor
TNF-α and β	Tumor Necrosis Factor-alph and beta
TTP	Thrombotic thrombocytopenic purpura
TE	Tris-EDTA buffer
Th 17 cell	T helper 17 cells
Tregs	Regulatory T cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains

TLR	Toll-like receptor
TIF	T cell-derived inducible factor
TP53	Tumor protein p53
TCF	T cell factor
TMB	Tetra methyl benzidine
TILs	Tumor-infiltrating lymphocytes
TLT	Tertiary lymphoid tissue
TOX	Thymocyte selection-associated high mobility group box protein
Unc51	Unables protein kinase activity 51
VCAM1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle
VE-cadherin	vascular endothelial cadherin
Wnt	Wnt (gene)signaling pathways Wingless and Int-1
WBC	White blood cell
WIF	Whole-inclusion fluorescence
WHO	World Health Organization
χ^2	value of the chi-square test

References

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