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Evaluation of the Role of *KRAS* gene Polymorphism, Serum IL-17A and Tissue CD33 in Prognosis of patients with Colorectal Carcinoma

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Medical Microbiology

By

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قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا
عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ
الْحَكِيمُ

صدق الله العلي العظيم

(البقرة الآية ٣٢)

Dedication

To My father, my mother, Sisters and Brothers, as well as to all my friends, I would like to express my deep appreciation for your real support which made me reach this far. Without you, the mission would be impossible.

OSAMA ABD ALI

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In the name of Alllah, the most gracious the most merciful

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Last but not least, I would like to dedicate this humble work to the Dr. Humadi Al-Hillaly' soul who inspired me to achieve my dream.

OSAMA ABD ALI

Supervisor certificate

I certify that this thesis (**Evaluation of the Role of *KRAS* gene Polymorphism, Serum IL-17A and Tissue CD33 in Prognosis of patients with Colorectal Carcinoma**) has been prepared under my supervision at the department of Microbiology, College of Medicine/University of Al-Qadisiyah as partial fulfillment of the requirement of the degree of Master of Science in Medical Microbiology.

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Summary

Summary

Colorectal cancer is a malignant tumor of epithelial cells origin affecting the large intestine. This is the most common tumor affecting the gastrointestinal tract and this is of favorable prognosis provided that it is diagnosed and treated in early stage. Currently it constitutes about 9% of all cancer in both gender. In Iraq, according to the Iraqi cancer registry, the incidence of colorectal cancer was 4.89% of all malignancy and it is the seventh cancer related death.

The aim of this study is collection of blood samples along with tissue sample. Three technique were used which are Enzyme linked immunosorbent assay for IL17A concentration measurement, indirect immunofluorescent assay to score tumor infiltrating CD33+ myeloid derived suppressor cells and Asymmetric real time polymerase chain reaction DNA melting analysis technique to measure the expression of *KRAS* gene polymorphism.

The case control study done on 40 formalin fixed paraffin embedded tumor tissue were sectioned to 3 micrometer and placed on positively charged slides and prepared for immunofluorescent technique, along with blood sample from the same patients. The whole blood sample was for the molecular study and serum sample was for the enzyme linked immunosorbent assay.

According to the immunoflourescent staining technique, the result of CD33 was significantly high with high grade adenocarcinoma ($p<0.0001$). High serum concentration of IL17A was significantly elevated in patients rather than the healthy control group.

KRAS gene mutations, TGT mutation was significantly higher in patient group than in control group (25.0% and 0.0, respectively, $p= 0.02$) and odd ratio foe such position 14.11 and etiological fraction was 0.23.

Summary

In conclusion. Such a study clarified that the KRAS gene mutation in codon 12 is higher than in codon 13 which means that such patients with wild type tumor (non- mutant) with get benefit from the anti EGFR therapy and have a good overall survival, on contrast with those whom mutant KRAS, such patients will face a worse overall survival and resist anti EGFR therapy. IL17A is another parameter that involved in this study. High serum concentration of IL17A is seen in patient with high grade carcinoma, which means that high level of such interleukin could be a good predictive marker for grade three carcinoma and indicative for tumor progression. Another predictive marker for worse prognosis and poor response to chemotherapy is CD33+ MDSCs, such phenotype presented in high percentage in patient with grade 2 and grade 3, which means that increasing of the percentage is associated with increasing progression of the tumor.

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List of abbreviations

CRC	Colorectal cancer
IL17	Interleukin 17
MDSC	Myloid deriver suppresser cells
PCR	Polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
IF	Immunofluorescent
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
LOH	Loss of heterozygosity
MMP	Matrix multiple metalloprotease
EPCs	Endothelial progenitor cells
VEGF	Vascular endothelial growth factor

Chapter one
Introduction
&
Literature review

1- Introduction.

Colorectal cancer is the most common malignant tumour of the lower gastrointestinal tract (colon and rectum) of epithelial cells origin, perhaps the only cancer that start as a benign adenomatous poly, which can last a few years to become a malignant. Colorectal cancer considered the fourth most common malignant affecting the gastrointestinal tract. Epidemiologically, nearly 1,200,000 new colorectal cancer cases occur globally, which represent 10% of all incident malignant tumours(Nash, Gimbel et al. 2010). In Iraq colorectal cancer is considered the fourth most common malignancy affecting the lower gastrointestinal tract that affecting both genders. Based on Iraqi cancer registry 2014, it is believed that 812 (7.12%) and 689 (4.86%) new cases of colorectal affecting the male and female respectively.

Factors suggestive of a genetic contribution to colorectal cancer include the fallowing; (1) a strong family history of colorectal cancer or polyp; (2) multiple primary cancers in a patient with colorectal cancer; (3) the existence of other cancers within the kindred consistent with known syndromes causing inherited risk of CRC such as Endometrial cancer; and (4) early age at diagnosis of CRC. Hereditary CRC is the most commonly inherited in an autosomal dominant pattern, although two syndromes are inherited in an autosomal recessive pattern (MUTYH-associated polyposis and NTHL1). At least three validated computer models are available to estimate the probability that an individual affected with cancer carries a pathogenic variant in a mismatch repair genes associated with lynch syndrome, the most common inherited CRC syndrome. These include MMRpro, MMRpredict and PREMM5 (PREdiction model for gene mutation) prediction models. Individuals with a quantified risk of 2.5% or greater on PREMM5 or 5% or greater on MMRpro and MMR predict are recommended for genetic evaluation referral and testing.(Doubeni, Laiyemo et al. 2012)

Through a systemic review of diet and cancer, the colorectal cancer 2011 report showed that there is convincing evidence for decreased CRC risk with increase intake of total dietary fibre. This concept has been in existence for more than decades and extensive case control and cohort studies have evaluated the relationship between CRC and fibre. For example a large prospective study in the European prospective investigation into cancer and nutrition indicated an approximately 40% lower risk of CRC through the result of these studies are in consistent, a meta-analysis stated an approximately 10% lower risk of colorectal adenoma per 10 g/day increase in fibre(Hubbard and Grothey 2013).

It has become clear that the risk for developing cancer arising substantially as a result of poorly regulated chronic inflammatory response. Increasing medical and scientific data point to balance of pro- and anti- inflammatory immune events within the body may confer outcome of human health and disease. In this context a high prevalence of some immune cells with tumor microenvironment may enhance tumor development through there cytokines and growth factors. In this review myeloid derived suppressor cells play an important role in tumor progression, aggressiveness and tumor neovascularization. Interleukin 17 another parameter in such study which has a role in tumor progression but the mechanism of such function still unclear. KRAS gene polymorphism has been studied, mutation in KRAS gene in patient with stage III and stage IV colorectal cancer may give an indication of chemotherapeutic resistance(Levi, Kark et al. 2011).

The objective of present study are;

1- To find out the significant deference in the expression of the three investigated parameters (KRAS gene, IL17 and CD33).

2- To correlate the expression of the parameters with the clinic-pathological variables which are grade, stage and lymph node involvements.

3- to assess the prognostic significance of the IL17A and CD33 with the most expressed genotype of the KRAS gene.

2. Literature review

2.1 Colorectal Cancer

2.1.1 Definition

Colorectal adenocarcinoma refer to the malignancy of the epithelial cell origin, it's considered the most common cancer that affecting the lower gastrointestinal tract (colon and rectum) and a major contributor to morbidity and mortality worldwide, perhaps it's the only cancer that start as benign adenomatous polyp, it takes years to become malignant through a sequence of genetic mutations influenced by environmental factors. (http://www.londoncanceralliance.nhs.uk/media/83350/lca_colorectalclinicalguidelines2014.pdf).

2.1.2. Epidemiology

Colorectal cancer incidence varies around the world. Nearly 1,200,000 new CRC cases occur globally, which represent 10 % of all incident malignant tumors (Vincent T. devita cancer principle and practice 10th edition, chap. 57 p 1167.). In Iraq, CRC is considered the fourth most common malignancy of the lower GI that affecting the both genders. Colorectal cancer comes after bronchus and lung, urinary bladder and leukemia in case of male gander, on the other hand, it comes after breast cancer, Leukemia and brain & other CNS cancer in case of female gender. Based on Iraqi cancer registry 2014, it is believed that 812 (7.12%), 689 (4.86%) new cases of CRC affecting the male and female, respectively.

2.1.3 Etiology and risk factors

Mutation in specific genes can lead to the onset of colorectal cancer, as

happens in other types of cancer. Those mutations can appear in oncogenes, tumor suppressor genes and genes related to DNA repair mechanism(Fearon and Vogelstein 1990). Depending on the origin of the mutation, colorectal carcinomas can be classified as sporadic, inherited and familial.

Point mutations, which appear during life, are not associated with inherited syndromes and only affect individual cells and their descendants. Cancers derived from point mutations are called sporadic cancers, and account for 70% of all colorectal cancers. The molecular pathogenesis of sporadic cancer is heterogeneous as mutations can target different genes(Fearon and Vogelstein 1990).

However, approximately 70% of CRC cases follow a specific succession of mutations that is then translated into a specific morphological sequence, starting with the formation of an adenoma and ending in the carcinoma state. The first mutation occurs in adenomatous polyposis coli (APC), a tumor suppressor gene, triggering the formation of non-malignant adenomas, also called polyps. Approximately 15% of those adenomas are expected Tobe promoted to the carcinoma state within a period of ten years. This APC mutation is followed by mutations in KRAS, TP53 and, finally, DCC(Fearon and Vogelstein 1990, Lynch and De la Chapelle 2003).

Inherited cancers account for just 5% of all CRC cases. Those cancers are caused by inherited mutations that affect one of the alleles of the mutated gene, meaning that a point mutation in the other allele will trigger the apparition of the tumor cell and, subsequently the carcinoma In order to generate a more accurate classification of inherited cancers, two groups, namely polyposis and non-polyposis forms have been establ--ished. The polyposis variant mainly involves familial adenomatous polyposis (FAP), which is characterized by the formation of multiple Potentially malignant polyps in the colon (Lynch and De la Chapelle 2003).

In contrast, hereditary non-polyposis colorectal cancer (HNPCC) is related to mutations in DNA repair mechanisms. The main cause of HNPCC is Lynch syndrome، which is caused by inherited mutations in one of the alleles coding for DNA repair proteins such asMSH2, MLH1, MLH6, PMS1 and PMS2. Lynch syndrome can be found in 2%–3% of all colorectal cancer cases, and is therefore the most common syndrome in the HNPCC group(Lynch and De la Chapelle 2003, Umar, Boland et al. 2004). Familial colorectal cancer accounts for approximately 25% of all cases and is also caused by inherited mutations، although they are not classified as inherited cancers per se since they cannot be included in any inherited cancer variant (Stoffel and Kastrinos 2014**).**

Worldwide, the probability of suffering from colorectal cancer is about 4%–5%. Furthermore، many personal traits or habits are considered to be risk factors as they increase the chances of developing polyps or colorectal cancer. The main risk factor for colorectal cancer is age: past the fifth decade of life, the risk of developing CRC is markedly increased, while the onset of colorectal cancer below the age of fifty is rare(apart from inherited cancers)(Levin, Lieberman et al. 2008).

In addition to age, there are other inherent risk factors that cannot be modified. A personal history of colorectal cancer or inflammatory bowel disease (IBD)—the risk in patients with ulcerative colitis is increased by 3.7% (Eaden, Abrams et al. 2001), while people suffering from Crohn's disease have a 2.5% higher risk of developing colorectal cancer(Canavan, Abrams et al. 2006)—are also important risks for colo--rectal cancer development.

The chronic inflammation found in IBD often produces an abnormal cell growth known as dysplasia. Although dysplastic cells are not yet malignant, they have more chances of becoming anaplastic and developing into a tumor. Another risk factor that can be included in this Group is the presence of a positive familial history of CRC in relatives, Especially those relatives under fifty years of age at diagnosis. An Increased risk due to familial history can be derived from inherited mutations or the environment (Johns and Houlston 2001).

Some other risk factors, which are related to lifestyle, can be reduced by implementing modest lifestyle changes in terms of dietary and physical activity habits. For instance, it is thought that sedentary lifestyle can increase the risk of developing colorectal cancer, although this relationship between colorectal cancer and inactivity is not completely defined. However, it has been proved that moderate physical activity increases metabolic rates and gut motility and, in the long term, increases metabolic efficiency and reduces blood pressure (Mármol, Sánchez-de-Diego et al. 2017).

A sedentary lifestyle is also related with obesity, another important risk factor for colorectal cancer. Remarkably, this increased risk is linked to both food intake and increased levels of visceral adipose tissue (VAT), a hormonally active component of total body fat that can promote the

development of colorectal cancer through the secretion of pro-inflammatory cytokines, which leads to an inflammatory situation in the colon and rectum, insulin resistance and modulation of metabolic enzymes such as adiponectin or lectin(Martinez-Useros and Garcia-Foncillas 2016).

In this context, diet is strongly linked to the risk of colorectal cancer such that unhealthy nutritional habits increase the chances of developing colorectal cancer by up to 70%(Willett 2005).

For instance, red meat releases heme groups in the intestine, which enhance the formation of carcinogenic N-nitroso compounds as well as cytotoxic and genotoxic aldehydes by lip peroxidation(Bastide, Pierre et al. 2011)،and meat cooked at high temperatures produces heterocyclic amines and polycyclic hydrocarbons after digestion, both of which are considered to be potential carcinogens(Santarelli, Pierre et al. 2008).

Furthermore, smoking and alcohol consumption have also been shown to increase CRC risk. In the case of alcohol consumption, acetaldehyde (the main metabolite of ethanol) has been described as carcinogenic by increasing the risk of developing colorectal cancer among populations depending on polymorphisms of alcohol metabolism enzymes(Pöschl and Seitz 2004). However, the relationship between alcohol consumption and CRC has not yet been totally elucidated. Tobacco smoking, in turn, can increase the chances of suffering from CRC by up to 10.8% (Botteri, Iodice et al. 2008) due to the high content in carcinogens such as nicotine, the metabolites of which can easily reach the intestine and generate polyps (Botteri, Iodice et al. 2008, Cross, Boca et al. 2014). Although smoking increases CRC risk, a significant relationship has only been found for long-term smokers, whether they have stopped smoking or not (Liang, Chen et al. 2009).

2.1.4 Molecular pathway of colorectal cancer

Genomic instability is an important feature underlying colorectal cancer. The pathogenic-mechanisms leading to this situation can be included in three different pathways, namely chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP).The CIN pathway, which is also considered the classical pathway since it represents the cause of up to 80 -85 % of all CRC cases (Grady and Carethers 2008), is characterized by imbalances in the number of chromosomes، thus leading to aneuploydic tumors and loss of heterozygosity (LOH).

The mechanisms underlying CIN include alterations in chromosome segregation, telomere dysfunction and DNA damage response، which affect critical genes involved in the maintenance of correct cell function, such as APC, KRAS، PI3K and TP53 amongst others. APC mutations cause the translocation of B-catenin to the nucleus and drive the transcription of genes implicated in tumorigenesis and invasion, whereas mutations in KRAS and PI3K lead to a constant activation of MAP kinase, thus increasing cell proliferation. Finally, loss-of-function mutations in TP53, which encodes for p53, the main cell-cycle checkpoint, cause an uncontrolled entry in the cell cycle (Pino and Chung 2010).

The RAS gene family is among the most studied and best characterized of the known cancer-related genes. Of the three human ras isoforms, KRAS is the most frequently altered gene, with mutations occurring in 17%–25% of all cancers. Particularly, approximately 30%–40% of colon cancers carry a KRAS mutation. KRAS mutations in colon cancers have been associated with a poorer survival and increased tumor aggressiveness. Additionally, KRAS mutations in colorectal cancer lead to resistance to select treatment strategies.

The detection of KRAS mutations has been associated with decreased response rates to select chemotherapeutic agents. Therefore, KRAS mutational status is a critical factor when considering the use of targeted therapies. The association of KRAS gene mutation and response to therapy was first reported in patients with metastatic colorectal cancer, who were treated with anti-epidermal growth factor receptor (EGFR) agents. Lievre et al. were the first to report the link between the KRAS gene mutation and decreased response to anti-EGFR agent(Fearon and Vogelstein 1990).

The KRAS oncogene is mutated in approximately 35%-45% of colorectal cancers, and KRAS mutational status testing has been highlighted in recent years. The most frequent mutations in this gene, point substitutions in codons 12 and 13, were validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the KRAS mutational status of tumor samples has become an essential tool in managing patients with colorectal cancers.

2.1.5. Histological grading system of colorectal cancer

A number of grading systems for colorectal cancer have been suggested but a single widely accepted and uniformly used standard for grading is lacking. Most systems stratify tumors into 3 or 4 grades as follows:

I. Grade 1 Well differentiated (>95% gland formation)

II. Grade 2 moderately differentiated (50-95% gland formation)

III. Grade 3 poorly differentiated (<50% gland formation)

IX. Grade 4 Undifferentiated (no gland formation or mucin; no squamous or neuroendocrine differentiation). Despite a significant degree of inter-observer variability(Chandler and Houlston 2008), histologic grade has been shown to be an important prognostic factor in many studies(Cho, Chun et al. 2009, Derwinger, Kodeda et al. 2010) with strong correlation between poor differentiation and adverse outcome.17 while some studies have stratified grade into a two-tiered low- and high-grade system, a three- or four-tier system is more commonly used for gastrointestinal carcinomas.

2.1.6 Staging system of colorectal carcinoma

The Dukes staging system is a classification system for colorectal cancer (figure 1). This system is now mainly of historical interest as it has largely been replaced by the TNM staging system. It is not recommended for clinical practice.(Edge and Cancer 2010)

Dukes A: invasion into but not through the bowel wall (90% 5 year survival)

Dukes B: invasion through the bowel wall but not involving lymph nodes (70% 5 year survival)

Dukes C: involvement of lymph nodes (30% 5 year survival)

Dukes D: widespread metastases.

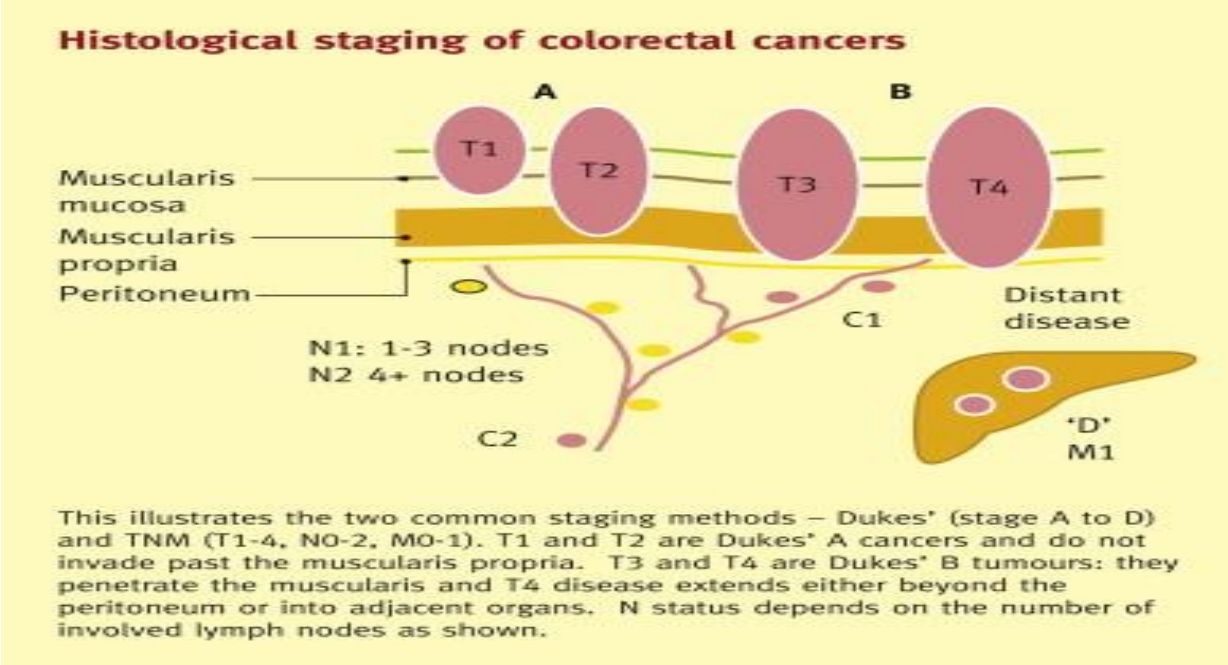


Figure 1-1: Dukes staging system of colorectal adenocarcinoma.

2.1.7 Adenoma to carcinoma sequence

The epithelium of the normal colon undergoes continuous renewal. At the base of glandular invagination of the colonic mucosa, called crypts, a pool of rapidly diving intestinal stem cells (ISCs) sustains the homoeostatic regeneration of the epithelium throughout a lifetime (Clevers 2013). (Figure 1-2) illustrates the sequences of adenoma carcinoma from normal epithelial till metastasis. Over the past decade, signals that regulate ISC renewal and proliferation have been extensively characterized: WNT, EGFR/MAPK and NOTCH signaling promote the undifferentiated proliferative state of ISCs in the niche, whereas BMP and TGF-beta signaling induce cytostasis and differentiation(Clevers 2013). The elevated division rate of ISCs increases their probability to acquire mutations during DNA replication(Vogelstein, Papadopoulos et al. 2013).

Additional environmental factors such as lifestyle, diet and microbiota can also greatly influence the transformation of the epithelium (Bishehsari, Mahdavinia et al. 2014). The most common genetic events in CRCs are

alterations that inactivate the tumor suppressor gene APC**.** This triggers the constitutive activation of WNT signaling and imposes a continuous stem like self-renewing state at the onset of tumorigenesis, giving rise to benign outgrowths of the epithelium known as adenomas.

Genetic experiments performed in mouse models support the hypothesis that Apc mutation in ISCs represents the origin of intestinal polyps(Barker, Ridgway et al. 2009, Tetteh, Basak et al. 2016), although chronic inflammation or dysregulation of BMP signaling has been shown to help convert non-stem cells into CRC-initiating cells(Schwitalla, Fingerle et al. 2013, Davis, Irshad et al. 2015).

A small fraction of adenomas become progressively aggressive through acquisition of additional driver mutations, which mainly affect three additional signaling pathways(Network 2012, Seshagiri, Stawiski et al. 2012): (a) the MAPK pathway is often hit by activating mutations in KRAS, BRAF orPIK3CA and provides cell autonomous mito-genic and pro-survival stimuli to cancer cells; (b) the p53 pathways inactivated by mutations in the eponymous protein, or less commonly in ATM, facilitating acquisition of genomic instability; and (c) the TGF-beta pathways frequently silenced by loss-of-function mutations inTGFBR2, SMAD4, SMAD2 or SMAD3, which bypasses the suppressive effects of high TGF-beta levels present in the tumor microenvironment (Fearon 2011).

Pioneer studies by Eric Fearon and Bert Vogelstein correlated these mutations with pathologically classifiable stages of adenoma malignancy and suggested a linear progression model, in which the compounding of the four mentioned pathway mutations associated with development of aggressive adenocarcinomas(Fearon and Vogelstein 1990, Fearon 2011).

Acquisition of these mutations is a slow process, and consequently, the development of invasive CRC often takes decades(Jones, Chen et al. 2008, Vogelstein, Papadopoulos et al. 2013).

Of note, the linear progression model based on four stepwise genetic alterations represents simplification, as not every tumor carries genetic alterations in these four pathways or develops through the equivalent sequence of events. Moreover, full-blown CRCs have a riche and complex mutational landscape that expands well beyond mutations in the four driver pathways(Network 2012, Seshagiri, Stawiski et al. 2012).

Due to the acquisition of chromosomal instability or defects in the DNA mismatch repair system, tumors accumulate hundreds or even thousands of genetic alterations. Some of these are passenger mutations, as they do not confer advantages to tumor cells, but others drive the biology of the cancer and therefore give selective advantage. Beyond the context of the linear progression model, the role of many of these mutations remains poorly understood. Together, these issues of complexity and heterogeneity impinge upon the functional analysis of CRC and complicate the development and application of therapeutic approaches.(Grady and Carethers 2008).

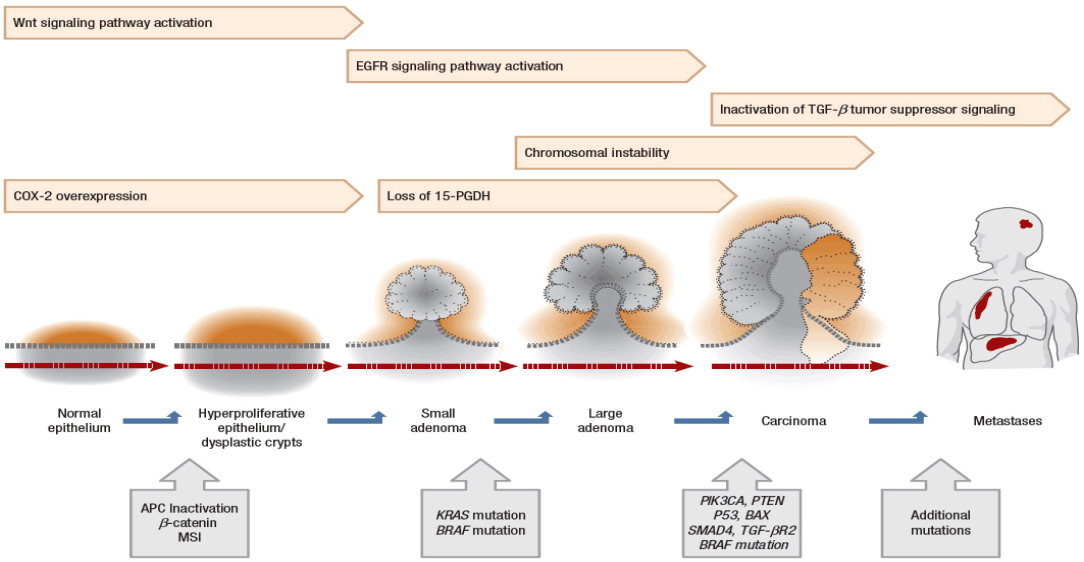


Figure 1-2: Sequences of adenoma to carcinoma

2.1.8 Hereditary adenomatous polyposis VS. Lynch syndrome

Lynch syndrome and Familial Adenomatous Polyposis (FAP) are two major inherited tumor syndromes predisposing to colorectal cancer (Jasperson, Tuohy et al. 2010). FAP is inherited through a germline mutation in the *APC* gene, which, upon a second somatic hit, results in the formation of hundreds to thousands of adenomatous polyps (polyposis) in the colonic mucosa of mutation carriers(Half, Bercovich et al. 2009). The multiple clinically detectable lesions illustrate that *APC* germline mutations lead to a strong increase of the adenoma initiation rate in the colorectum (Jass 1995, Half, Bercovich et al. 2009).In contrast to FAP, polyposis is absent in Lynch syndrome, the most common hereditary colorectal cancer syndrome in adults, which is caused by germline mutations of DNA mismatch repair (MMR) genes(Mecklin, Aarnio et al. 2007, Boland and Goel 2010).

Although some studies found an increased adenoma incidence in Lynch syndrome mutation carriers, adenomatous polyps in Lynch syndrome were only slightly more prevalent than in the unaffected population(De Jong, Morreau et al. 2004, Lynch, Snyder et al. 2015). This observation has suggested that Lynch syndrome-causing MMR gene germline mutations do not increase the adenoma initiation rate, but rather accelerate the progression of pre-formed adenomas, which have developed independently from MMR deficiency, into invasive cancer(Fearon 2011, Lynch, Snyder et al. 2015).

Therefore, Lynch syndrome was long regarded as a prime example of an inherited tumor predisposition that does not act through enhanced tumor initiation(Fearon 2011, Lynch, Snyder et al. 2015). MMR deficiency, accordingly, has commonly been believed to be a secondary event, and somatic mutations of MMR genes were thought to occur after the formation of polyps that had been caused by *APC* mutations or other events occurring independently from MMR deficiency. Various observations of Lynch syndrome pathogenesis have been interpreted as supportive of this concept: these include the existence of polyps with retained or partial expression of MMR proteins 9 found in some Lynch syndrome patients(Walsh, Buchanan et al. 2012, Shia, Stadler et al. 2015).

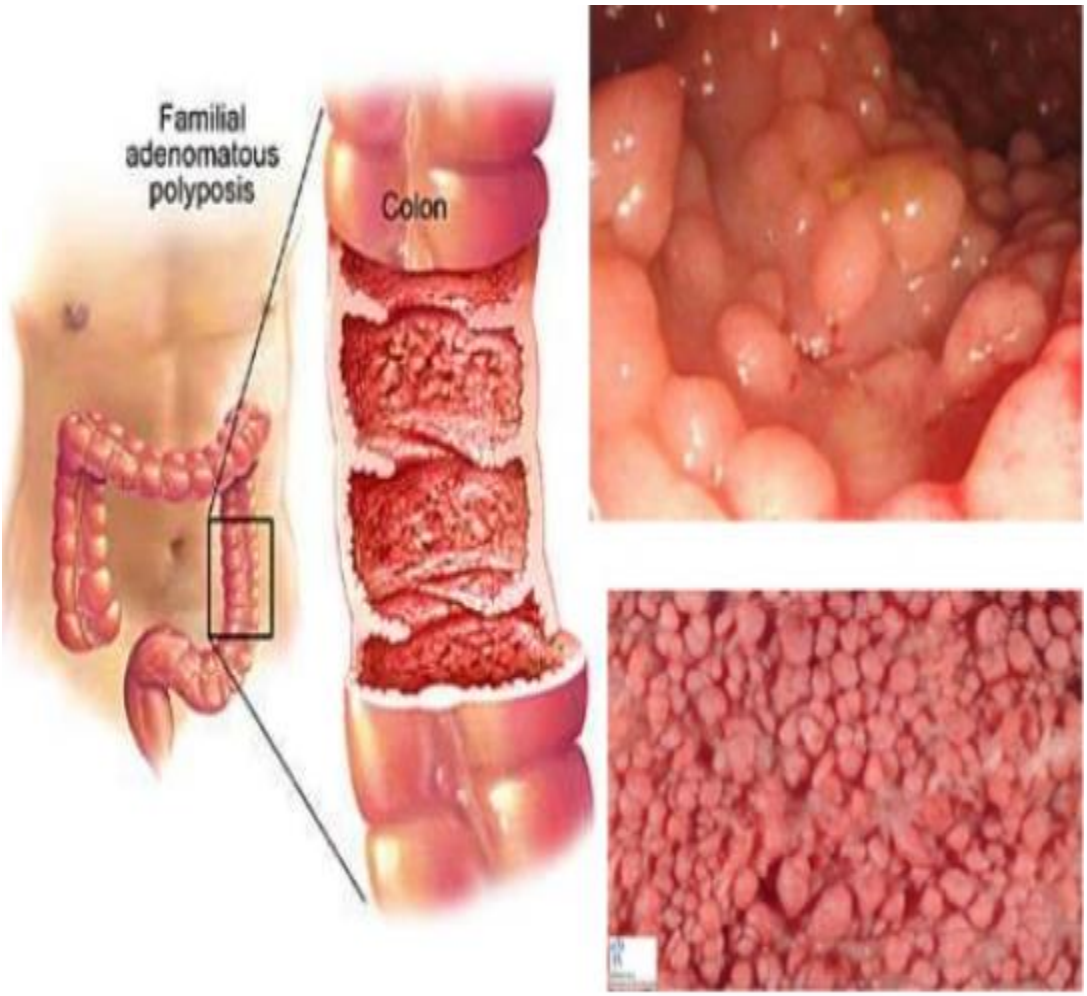


Figure 1-3 : Adenomatous polyposis syndrome

Moreover, correlation of MMR deficiency with the higher grade and bigger size of the adenomas seemed to further corroborate the role of MMR deficiency as a "non-initiating" event in Lynch syndrome-associated colorectal carcinomas(De Jong, Morreau et al. 2004, Halvarsson, Lindblom et al. 2005).

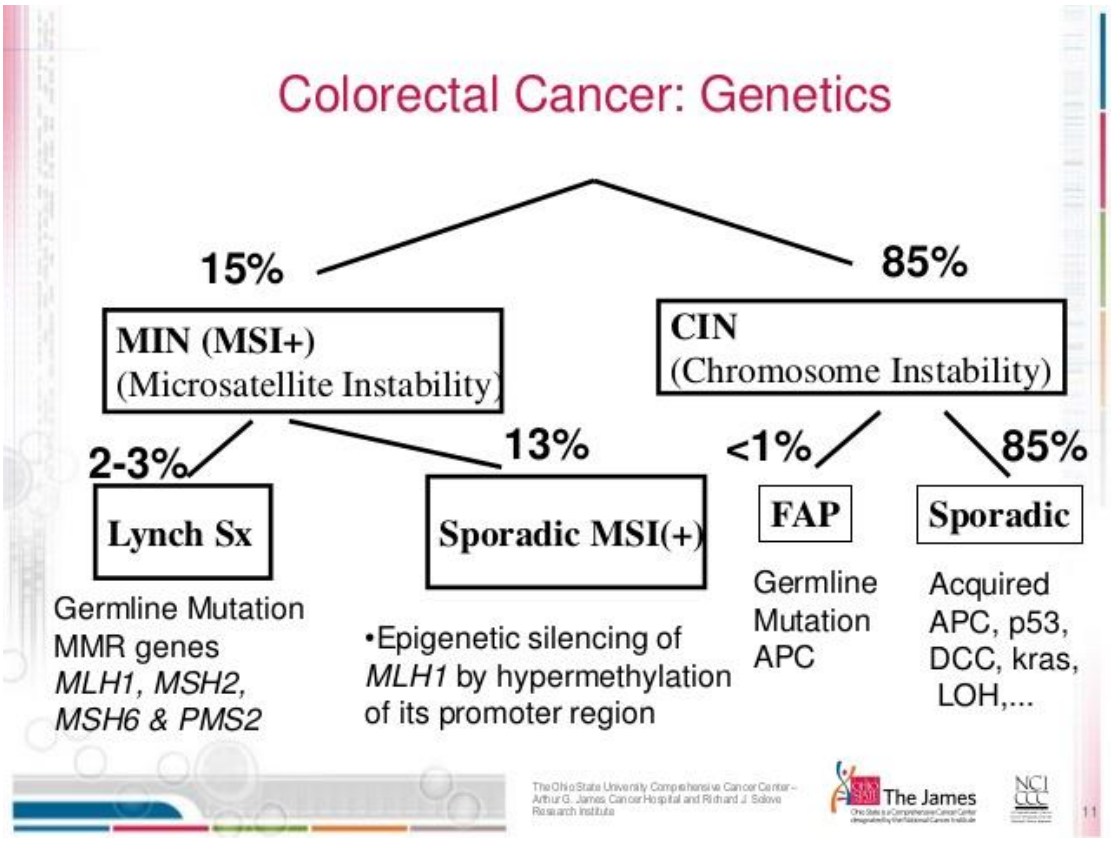


Figure 1-4 : genetic of lynch syndrome.

2.1.9 Sporadic colorectal carcinoma

Sporadic CRCs occur in patients who have a median age of 70–75 years, and approximately 70% of CRCs develop in the distal colon. Many Differences in clinic-pathologic features exist between the proximal and distal colons. Genetically, sporadic CRCs develop by the accumulation of a series of abnormalities in tumor suppressor genes and oncogenes. Several investigators have postulated the adenoma-carcinoma sequence theory, in which *APC* mutation serves as an initiating event, followed by the accumulation of multiple mutations of genes, such as Kirsten RAS (*KRAS*), *Sma-* and *Mad-related protein 4*(*SMAD4*), and *TP53(Fearon and Vogelstein 1990, White 1998, Polakis 2000)*. According to this model, at least seven distinct mutations are required for CRC pathogenesis.

Other investigators have described another route to colorectal carcinogenesis through serrated polyps (Grady and Carethers 2008, Bettington, Walker et al. 2013).Presently, three major distinct genetic pathways to CRC have been postulated. Approximately 70% of sporadic CRCs develop along the CIN pathway. These cancers are characterized by the accumulation of numerical or structural chromosomal abnormalities, resulting inaneuploid karyotype, frequent loss-of-heterozygosity(LOH) at tumor suppressor gene loci, and chromosomal rearrangements(Grady and Carethers 2008).

Moreover, CIN tumors are distinguished by the accumulation of mutations in specific oncogenes and tumor suppressor genes [e.g., *APC*, *KRAS*, phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), *SMAD4*, and *TP53*], thereby activating pathways critical for carcinogenesis. Another important pathway is the MSI pathway, caused by dysfunction of DNA MMR genes. MSI is found in15% of sporadic CRCs. Unlike Lynch syndrome that is caused by germ-line mutations of MMR genes, such as MutL homolog 1 (*MLH1*) (32% of cases), MutS homolog2 (*MSH2*) (39%), post meiotic segregation increased 2(*PMS2*) (15%), and *MSH6* (14%) [18], MMR deficiency in sporadic CRCs is due Mainly to silencing of the MMR genes, mostly *MLH1* (>80% of cases), by promoter hyper methylation (Herman, Umar et al. 1998, Weisenberger, Siegmund et al. 2006).

Usually, expression is lost in the case of MLH1 and MSH2 and their binding partners (MSH6and PMS2, respectively). Classification of MSI is based on altered size of various mono- and di-nucleotide repeat Sequences, such as BAT25, BAT26, D2S123, D5S346, andD17S250, known as the Bethesda panel (Boland, Thibodeau et al. 1998, Umar, Boland et al. 2004).Altered size of at least two of the five microsatellite

Panel markers is defined as MSI-high (MSI-H). Sporadic MSI-H is associated with CIMP. Most MSI-H CRCs are diploid or near diploid and LOH is rare. CRCs with one abnormal marker in the panel are termed MSI-low (MSI-L), and their clinical significance is controversial.

MSI-L is often grouped with microsatellite-stable (MSS) tumors. MSI-H tumors frequently have frame shift mutations in those genes that contain small runs of nucleotide repeats in exon-coding regions, such as transforming growth factor-β receptor2 (*TGFBR2*), insulin-like growth factor 2 receptor(*IGF2R*), E2F transcription factor 4, p107/p130-binding(*E2F4*), *MSH6*, *MSH3*, and caspase 5 (*CASP5*)(Markowitz, Wang et al. 1995, Yamamoto, Sawai et al. 1997).

An (A) 10 repeat of the *TGFBR2* gene is mutated in 80% of MSI-H CRCs. In MSI-H tumors, *APC* and *BRAF* are often mutated, but *KRAS* mutation is rare. Sporadic MSI-HCRCs, as well as those with Lynch syndrome, are characterized by right-sided location, mucinous or medullary type, and presence of tumor-infiltrating lymphocytes, earlier Stages, and better prognoses (Bettington, Walker et al. 2015).The third pathway, designated as CIMP, is characterized by a widespread CpG island methylation(Toyota, Ahuja et al. 1999).

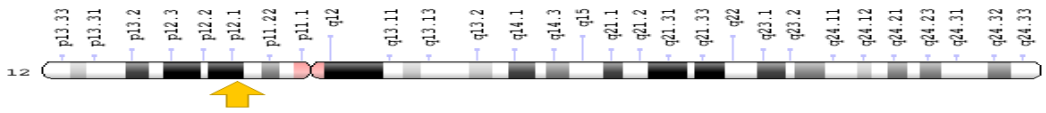
2.2.1 The *KRAS* gene

The KRAS provides instructions for making a protein called K-RAS that is part of a signaling pathway known RAS/MAPK pathway. The protein relays signals from outside the cell to the cell's nucleus. These signals instruct the cell to grow and proliferate or to mature and take on specialized function (differentiate)(Peeters and Jay Price 2010). The K-RAS protein is a GTPase, which means it converts a molecule called GTP into another molecule called GDP. In this way the K-RAS protein acts like a switch that is turned on and off by the GTP and GDP molecules. To

transmit signals, it must be turned on by attaching to the molecule of GTP. The K-RAS protein is turned off when it converts GTP to GDP. When the protein is bound to GDP, it does not relay signals to the cell's nucleus. The KRAS gene belongs to a class of genes known as oncogene. When mutated, oncogene have the potential to cause normal cells to become cancerous(Vigil, Cherfils et al. 2010).

(https://ghr.nlm.nih.gov/gene/KRAS).

Cytogenetic location: 12p 12.1, which is the (P) arm of chromosome 12 at position 12.1. Molecular location: base pair 25,204,789, to 25, 251, 003 on chromosome 12 (Homo Sapiens Annotation Release 109, GR Ch 38.p12) (NCBI). Credit: Genome Decoration Page / NCBI



2.2.2 *KRAS* gene signaling and CRC

The RAS gene was initially identified as a viral gene homologous to protein and is widely expressed in most human cells. As a small GTPase, encoded by 189 amino acids, and contain four coding exons and a 5' non coding exon(Malumbres and Barbacid 2003). KRAS is a membrane – anchored guanosine triphosphate / guanosine diphosphate (GTP/GDT) – binding KRAS is involved in intracellular single transduction and mainly responsible for EGFR signaling activation. The exchange of the active GTP-bound state is tightly controlled by GTPase activating proteins and guanine nucleotide exchange factors. Under normal physiological condition, upstream signal activate wild –type KRAS by promoting the exchange of bound GDP for GTP. This process is transient because of

GAP- mediated GTP hydrolysis. However, this process becomes altered when the KRAS gene is mutated(Vigil, Cherfils et al. 2010).

Mutated KRAS is found in 35% - 45% of CRC, and codon 12 and 13 are two hotspots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13. Other mutations in codon 61, 146 and 154 occur less frequently in CRC, accounting for 5% of all mutation type. Refereeing to the catalogue of somatic mutations in cancer database, more than 5000 mutation have been found in the KRAS gene in the CRC samples(Adelstein, Dobbins et al. 2011).

2.2.3 KRAS as a prognostic factor

The prognostic value of KRAS mutation was in dispute. Tow canonical trials have demonstrated that the KRAS mutation may be prognostic of treatment outcome for patient with CRC(DeSantis, Lin et al. 2014). The Kristin Ras in Colorectal Cancer Collaborative Group Study (RASCAL study) with 2721 patient samples collected from 13 different nations indicated that the presence of a KRAS mutation increased the risk of recurrence and death, especially in a guanine and thymine mutation(Mascaux, Iannino et al. 2005). Moreover the expended RASCAL II study suggested that the prognostic role of the KRAS mutation limited only to a glycine to valine mutation, was found in 8.6% of all patient and had a statistically significant effect on failure free survival and overall survival(Abrams, Meyer et al. 2014).

2.3.1 Tumor microenvironment

The immunosuppressive tumor microenvironment represents not only one of the key factors stimulating tumor progression but also a strong obstacle for efficient tumor immunotherapy. Immuno-suppression was found to be associated with chronic inflammatory mediators including cytokines, chemokines and growth factors produced by cancer and stroma cells(Wang, Lu et al. 2015).

Long-term intensive production of these factors induces the formation of myeloid-derived suppressor cells (MDSCs) representing one of the most important players mediating immunosuppression. Moreover, MDSC should not only inhibit anti-tumor immune reactions but also directly stimulate tumor growth and metastasis. Therefore, understanding the mechanisms of their generation, expansion, recruitment and activation is required for the development of novel strategies for tumor immunotherapy(Gabrilovich, Ostrand-Rosenberg et al. 2012).

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature myeloid cells consisting of precursors for granulocytes, macrophages or dendritic cells (DCs) that are accumulated during chronic inflammation and tumor progression(Raychaudhuri, Rayman et al. 2015). These cells show a broadly distinct phenotype. In mice, MDSCs express both CD11b and Gr1 markers and consist of two major subsets: polymorph nuclear Ly6G+Ly6Clo (PMN) and monocytic Ly6G,Ly6Chi (M) cells(Youn, Collazo et al. 2012). MDSCs derive from the bone marrow hematopoietic precursor cells through the pathologic modulation of myelopoiesis induced by constantly produced inflammatory mediators and exhibit remarkable immunosuppressive and tumorigenic activities(Arina and Bronte 2015).

These functions include (i) a deprivation of amino acids arginine and cysteine, which are essential for T cell proliferation and anti-tumor reactivity(Marvel and Gabrilovich 2015);(ii) a production of nitric oxide (NO) and reactive oxygen species (ROS) that causes the nitration of T cell receptors (TCR) and chemokines important for T cell migration or inducing apoptosis of T cells and NK cells; (iii) an intensive production of interleukin (IL)-10 and transforming growth factor (TGF)-_1 inhibiting immune effector cell functions; (iv) an upregulated expression of programmed death-ligand 1 (PD-L1) which can drastically downregulate an anti-tumor T cell-mediated reactivity via interaction with PD-1 receptor expressed on T cells(Waight, Netherby et al. 2013).

A reduction of the TCR _-chain expression playing an important role in coupling the TCR-mediated antigen recognition to diverse signal transduction pathways(Allavena, Sica et al. 2008); (vi) a secretion of angiogenic factors promoting tumor neovascularization, and (vii) a production of growth factors, matrix metalloproteinase and cytokines stimulating tumor growth and skewing immune reactions towardsTh2 type and activation of regulatory T cells (Tregs) . Therefore, MDSCs can be considered a Major players in tumor-mediated immunosuppression (Ichikawa, Williams et al. 2011).

2.3.2 MDSCs Generation and Expansion during Tumor Progression.

Numerous reports published during the last decade described a strong correlation between the development of chronic inflammatory conditions in the tumor microenvironment and generation and expansion of MDSCs(Schlecker, Stojanovic et al. 2012).

Tumor cells are able to produce a variety of inflammatory mediators including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor(G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), TGF-_, tumor necrosis factor (TNF)-_, IL-1_, IL-6, and IL-10(Raber, Thevenot et al. 2014).The effect of all these factors is combinatorial and dose-dependent. Furthermore, tumor cells can induce the production of these factors by fibroblasts and immune cells in the tumor stroma(Maenhout, Van Lint et al. 2014).

Altogether, these inflammatory factors can modulate myeloid cells in the tumor microenvironment, and having them delivered distantly to hematopoietic organs can change normal myelopoiesis and skew the differentiation of myeloid cells in favor of MDSCs(Schulz, Perdiguero et al. 2012). GM-CSF is considered as a major growth factor driving myelopoiesis, whereas further differentiation to granulocytes or macrophages is mediated by G-CSF or M-CSF, respectively(Ostrand-Rosenberg and Sinha 2009).These growth factors have been shown to be expressed in tumor lesions(Franklin, Liao et al. 2014). Tumor-derived GM-CSF has been demonstrated to play a major role in the generation of MDSCs both in vivo and in vitro. Moreover, it has been reported that the effect of GM-CSF is dose-dependent: it slow concentrations in the absence of IL-4 resulted in the generation of MDSCs and immature DCs from bone marrow precursors in vitro, whereas in high concentrations, it induced the development of neutrophils and mature DCs(Schulz, Perdiguero et al. 2012).

Importantly, GM-CSF and IL-6 allowed a rapid and efficient generation of MDSCs with a strong tolerogenic activity from precursors present in mouse and human bone marrow(Thevenot, Sierra et al. 2014).VEGF and TGF-_ have also been demonstrated to be involved in the regulation of hematopoiesis. Both growth factors are produced in high concentrations by many tumor types and display a strong impact on the MDSC generation and expansion(Qian and Pollard 2010). It has been demonstrated that VEGF secreted by tumor cells interfered with the proliferation, differentiation and maturation of immature granulocyte-macrophage progenitors, causing an inhibition of DC maturation and activation as well as a development of immunosuppressive tumor-associated macrophages (TAMs)(Yang, Huang et al. 2008).

In combination with VEGF, TGF-_ prevented DC maturation, polarized myeloid cells towards immunosuppressive cells in the tumor microenvironment and participated in the induction of TAMs. Numerous publications have described a significant increase in the frequency of circulating M-MDSCs and PMN-MDSCs in patients with melanoma and other tumor entities that strongly correlated with tumor burden(Cortez-Retamozo, Etzrodt et al. 2012). Furthermore, circulating-MDSCs have been reported to provide a negative impact on survival and inversely correlate with the presence of functional antigen-specific T cells in patients with advanced melanoma(Qian and Pollard 2010).

High frequencies of PMN-MDSC correlate with poor prognosis in patients with breast or colorectal cancer. The MDSC frequency in cancer patients increased during tumor development. However, 3–4 weeks after surgical resection of the tumor, the frequency of these cells decreased. These findings are consistent with the fact that the generation of MDSCs is due to the higher production of inflammatory factors secreted mostly by the tumor(Pan, Ma et al. 2009).

2.3.3 MDSC Recruitment into the Tumor Site

Chemokines are small (8–14 kDa), structurally related chemotactic cytokines that regulate trafficking of various cells (including leukocytes) through interactions with specific seven-transmembrane protein-coupled receptors. Fifty endogenous chemokines that bind 20 receptors have been described(Palomino and Marti 2015). Chemokines are considered to be key drivers in the development of inflammatory diseases and cancer. The pattern of chemokines involved in MDSC migration into the tumor microenvironment seems to be dependent on the MDSC subset (monocyte or polymorph nuclear) and on the tumor model.

The role of chemokine (C-C motif) ligand (CCL) 2 and its receptors in the attraction of M-MDSCs has been well described(Homey, Müller et al. 2002). In particular, it has been demonstrated that an accumulation of M-MDSCs in several mouse tumor models occurred via an interaction between CCL2 and its receptors, chemokine (C-C motif) receptor (CCR) 2, 4,and 5(Lesokhin, Hohl et al. 2011). Moreover, melanoma-infiltrating-MDSCs displayed CCR2-dependent immunosuppressive activities in the presence of GM-CSF. In the transplantable prostate cancer mouse model, it has been recently demonstrated that CCL2-CCR2interaction plays a pivotal role in the recruitment of bone marrow-derived myeloid cells to the blood and their subsequent migration into the tumor site(Izhak, Wildbaum et al. 2012).

The production of CCL2 but also chemokine (C-X-C motif) ligand (CXCL) 8 (also known asIL-8), and CXCL12 can be induced by PGE2 resulting in a dramatic MDSC accumulation in the ovarian and gastric cancer microenvironment(Kalinski 2012). In contrast, the expression of CXCL12 has been found to reduce MDSC recruitment in breast cancer mouse model(Williams, Harata-Lee et al. 2010). Other investigators reported a dominating role of CCL3, CCL5 and CX3CL1 but not CCL2 in the migration of M-MDSC or an importance of CXCL-1 (also known as KC), CCL5 and CCL7 in the MDSC enrichment in mouse colon and liver carcinoma models. Recently, it has been published that CCL5 strongly activated hypoxia-inducible factor (HIF)-1_ signaling cascades leading to the upregulation of VEGF expression(Wang, Liu et al. 2014). Importantly, both HIF-1_ and VEGF are considered to play a key role in MDSC generation and functions(Gama, Shirk et al. 2012).

Interestingly, comparing various transplantable tumor mouse models,(Nywening, Belt et al. 2017) observed that MDSC migration into the tumor site could be mediated by different chemokine. Therefore, the migration of different MDSC subsets into the tumor site can be strongly determined by the histology and the spectrum of chemokines produced by particular tumors.

2.3.4 MDSCs stimulate tumor progression

There is growing evidence that MDSCs are not only induced, recruited and activated by tumor-derived factors but can also directly support tumor development, neovascularization and metastasis(Gabrilovich, Ostrand-Rosenberg et al. 2012). These cells were demonstrated to produce VEGF and basic Fibroblast growth factor (bFGF) to promote tumor neoangiogenesis (Kujawski, Kortylewski et al. 2008). MDSCs also participated in tumor neovascularization together with Vascular endothelial progenitor cells (EPCs), which are found in different tumor models(Friedlander, Dorrell et al. 2007). Moreover, it has been found that MDSCs could even directly incorporate into tumor endothelia, displaying endothelial cell morphology and expressing VEGFR2, a marker for endothelial cells(Filipazzi, Bürdek et al. 2012).

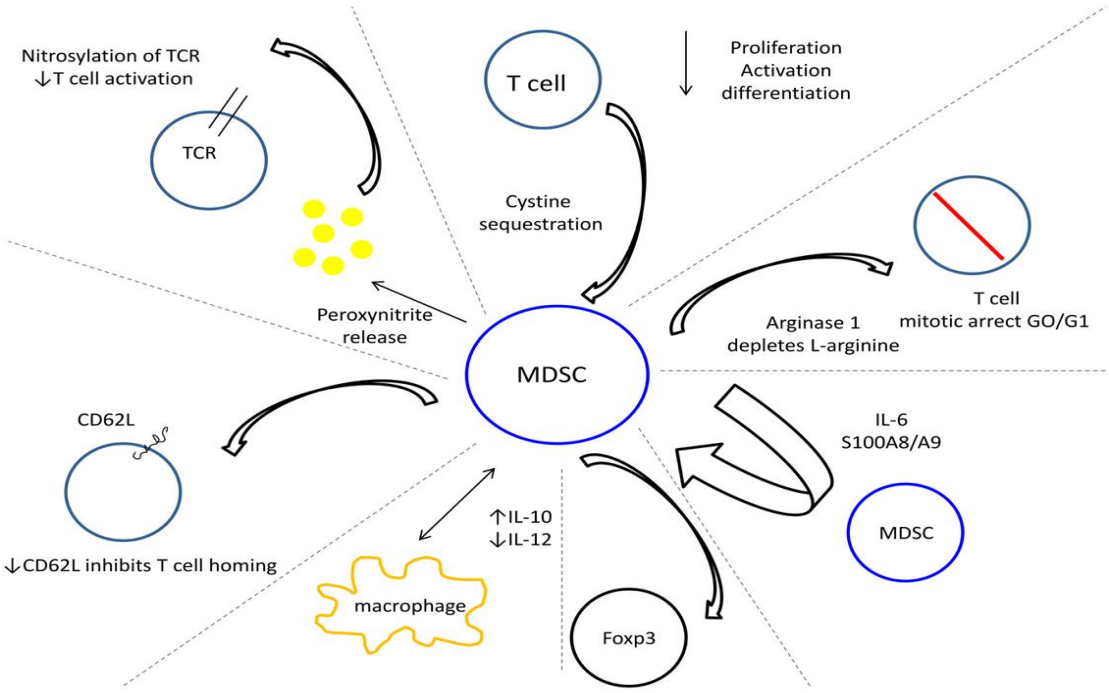


Figure 1-5: Myeloid derived suppressor cells use multiple mechanisms to dampen anti-tumor immunity.

Furthermore, MDSCs were demonstrated to promote tumor invasion and metastasis by two mechanisms: (i) elevated production of multiple matrix metalloproteinase (MMPs), playing a major role in matrix degradation, and chemokines to create a pre-metastatic environment(DeNardo, Barreto et al. 2009), and(ii) fusion with tumor cells' MDSCs promoting the metastatic process (Huysentruyt, Mukherjee et al. 2008).

Indeed, MDSCs have been shown to infiltrate pre-invasive cancer lesions and to be enriched at the invasive frontier of human cancers. In these lesions, MDSCs were able to produce S100A8 and S100A9 induced by VEGF and TGF-_(Hiratsuka, Watanabe et al. 2006). S100A8/A9 inflammatory proteins have been found not only to attract MDSCs into the tumor microenvironment and enhance their immunosuppressive activity but also to promote the activation of MAPK and NF-_B signaling pathways in tumor cells, stimulating thereby the tumor progression(Sinha, Okoro et al. 2008).

2.3.5 Neutralizing Immunosuppression Induced by MDSCs.

Possibility to decrease MDSC numbers and/or immunosuppressive activities leading to the tumor growth delay and the survival prolongation was already demonstrated both in animal models and in cancer patients(Kao, Ko et al. 2011).One of the key targets in preventing MDSC formation is SCF(Ko, Rayman et al. 2010). The knockdown of SCF with siRNA and inhibition of SCF signaling by anti-c-kit antibodies or with tyrosine kinase inhibitors like sunitinib and sorafenib have been demonstrated to reduce MDSC frequencies in the human bone marrow cells in vitro as well as in murine models of colon and Lewis lung carcinoma that was associated with enhanced anti-tumor reactivity, tumor regression and prolonged survival. In addition, sunitinib has been shown to reverse the MDSC accumulation in patients with renal cell carcinoma(RCC) resulting in the restoration of Th1 cells and a decrease in regulatory T cells(Ko, Zea et al. 2009).

This beneficial effect of sunitinib effect was also detected in the murine RCC model correlated with the suppression of MDSC functions(Ko, Rayman et al. 2010). It has been also reported that the selective pharmacologic inhibition of CSF1Rsignaling resulted in the decreased tumor angiogenesis associated with reduced recruitment of MDSCs into the tumor site(Priceman, Sung et al. 2010).

The prevention of MDSC trafficking towards tumor lesions is based on the targeting of tumor-derived chemokines. Prostate and breast carcinomas, melanomas, colorectal cancer and Lewis lung carcinoma were found to produce various chemokines (including CCL2,CCL3, CCL4, CCL5, etc.), which were described to attract MDSCs and to maintain their suppressive activity (Williams, Harata-Lee et al. 2010).

Direct CCL2 targeting(Zollo, Di Dato et al. 2012) or the inhibition of its production has been reported to decrease the frequency of tumor-infiltrating MDSCs, to restrict neoangiogenesis and to suppress the growth of transplantable tumors. Once migrated into the tumor microenvironment, MDSCs may affect anti-tumor reactivity of T and NK cells by various mechanisms(Umansky, Sevko et al. 2014). Among them, the activation of inducible NOsynthase (i-NOS) and arginase (ARG)-1 plays a key role. Production catalyzed by I-NOS was not demonstrated (i) to induce a nitration of T cell receptors in situ(De Sanctis, Solito et al. 2016); (ii) to target distinct signaling pathways resulting in the inhibition of cytokine production required for T cell functions; (iii) and to mediate T cell apoptosis(Umansky and Schirrmacher 2001). The activation ARG-1 induced a deprivation of L-arginine, which is not produced by T cells and is critical for protein synthesis(Bronte and Zanovello 2005). Importantly, the blockade of the activity of phosphodiesterase (PDE)-5 has been reported to increase intracellular concentrations of cyclic guanosine monophosphate (cGMP) resulting in the inhibition of both iNOS and ARG-1activities.

Based on these observations, PDE-5 inhibitors such as sildenafil, tadalafil and vardenafil have been proposed for the inhibition of MDSC immunosuppressive functions(Capuano, Rigamonti et al. 2009). The chronic sildenafil administration with the drinking water was reported to cause a significant reduction in the NO production and in the expression of ARG-1 associated with the restoration of tumor-specificCD8 T cell responses and a significantly prolonged survival of tumor-bearing mice(Meyer, Sevko et al. 2011).

2.3.6 MDSC immunosuppression

There is growing evidence that MDSCs are not only induced, recruited and activated by tumor-derived factors but can also directly support tumor development, neovascularization and metastasis(Gabrilovich, Ostrand-Rosenberg et al. 2012) These cells were demonstrated to produce VEGF and basic fibroblast growth factor (bFGF) to promote tumor neoangiogenesis(Du, Lu et al. 2008, Kujawski, Kortylewski et al. 2008). MDSCs also participated in tumor neovascularization together with vascular endothelial progenitor cells (EPCs), which are found in different tumor models(Du, Lu et al. 2008). Moreover, it has been found that MDSCs could even directly incorporate into tumor endothelia, displaying endothelial cell morphology and expressing VEGFR2, a marker for endothelial cells(Yang, DeBusk et al. 2004).

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2.3.7 IL 17 secretion and its role in tumor

IL-17 is an inflammatory cytokine produced by a wide variety of leukocytes, including T cells natural killer cells (NK cells), lymphoid tissue inducer-like cells (LTi-like cells), and neutrophils(Cua and Tato 2010). Among these cells IL-17 is reported to be predominantly produced by activated CD4+ T cells (Th17 cells). It is generally accepted thatTh17 cells are induced from naive CD4+ T cells by IL-6, IL-1β, TGF-β, and IL-23, which upregulate the expression of retinoic acid receptor-related orphan receptor-γt(RORγt) via activation of signal transducer and activator

of transcription-3 (Stat3) and interferon regulatory factor 4(IRF4) (Huber, Brüstle et al. 2008).

Other transcriptional factors such as RORa, basic leucine zipper Transcription factor (Batf), Runx1, and aryl hydrocarbon receptor (AHR) Can also induce Th17 cell polarization when coordinated with RORγt(Hirahara, Ghoreschi et al. 2010). Moreover, the regulation mechanism of IL-17 production in Th17 Cells is affected by other inflammatory immunocytes and related cytokines. For instance, human inflammatory dendritic cells (inf. DCs), derived from monocytes, can stimulate autologous memory CD4+ T cells to produce IL-17(Segura, Touzot et al. 2013). IL-17 expression is elevated in several human tumors, such as ovarian cancer, cervical cancer, breast cancer, hepatocellular carcinoma, esophageal cancer, gastric cancer, and CRC(Liu, Duan et al. 2011). But the underlying mechanism of IL-17 in tumor initiation and progression is not completely clear yet. Some researchers propose that IL-17 promotes tumor initiation and progression through suppressing antitumor immune response.

For instance, CD8+ T cells are polarized towards an IL-17 secreting (Tc17) fate in the presence of both TGF-β and IL-6, resulting in losing their cytotoxic ability and promoting tumorigenesis(Nam, Terabe et al. 2008).

In gastric cancer, activated monocytes promote the development of Tc17 cells via IL-6, IL-1β, and IL-23, resulting in the production of the chemokine CXCL12 by tumor cells, which promotes MDSCs-mediated immunosuppression(Zhuang, Peng et al. 2012).Other investigators demonstrate that IL-17 can enhance tumor progression through angiogenesis. IL-17 induces fibroblasts and tumor cells to produce a variety of angiogenic factors, includingPGE1, PGE2, VEGF, keratinocyte-derived chemokine (KC), and macrophage inflammatory protein-2 (MIP-2), which promotes angiogenesis in the tumor(Numasaki, Fukushi et al. 2003). In breast cancer, the angiogenic factors CXCL8, MMP-2, MMP-9, and VEGF are induced by IL-17 and associated with poor prognosis(Benevides, Cardoso et al. 2013).

Analogously, IL-17 has been demonstrated to selectively promote the secretion of an array of angiogenic chemokines from NSCLC, such as CXCL1, CXCL5, CXCL6, and CXCL8 [37].The molecule mechanism involved in the pro tumor activity of IL-17 is considered to be mediated by inflammation associated signaling pathways. Transfecting IL-17 into hepatocellular carcinoma cells significantly promotes neoangiogenesis, neutrophils recruitment, and tumor growth viaAKTdependent-6/JAK2/STAT3 signaling pathway *in vivo*(Gu, Li et al. 2011*)*.IL-17 induces IL-6 production, which in turn activates Stat3and promotes cancer cells survival(Wang, Yi et al. 2009).

Further studies on the molecular mechanism of IL-17 inducing tumor promotion are required in the future. Besides, some studies suggest that IL-17 can inhibit tumor growth. In the tumor initiating model, IL-17 deficient mice are more susceptible to developing lung melanoma, and adoptive T-cell therapy with tumor-specific Th17 cells prevents tumor development by eliciting a remarkable activation of tumor-specific CD8+T cells(Martin-Orozco, Muranski et al. 2009).

Study in hematopoietic cancer shows that IL-17 inhibits the tumor growth in a CTL dependent manner (Benchetrit, Ciree et al. 2002).Murine Meth-A fibro sarcoma cells transfected with the hIL-17 gene can promote CD4+ and CD8+ T-cells-mediated antitumor activity(Hirahara, Nio et al. 2001). Interestingly, hIL-17-gene-transfected Chinese hamster ovary (CHO) cells show a significant decrease in metastatic potential to the lung by directly reducing the invasiveness of CHO cells and enhancing NK activity(Oshiro, Kohama et al. 2012). This evidence indicates thatIL-17 may have partial antitumor effect through promoting immune response in the tumor initiation stage.

Based On these findings, we propose that the pro-tumor activity of IL-17 in CRC microenvironment may exert in several aspects: (1) promoting tumor elicited inflammation which facilitates the proliferation and survival of malignant cells, (2) forming an immunosuppressive tumor microenvironment by chemo attracting immunosuppressive cells and cytokines, (3) suppressing cytotoxic cells-mediated immune-surveillance against tumor, (4) fostering tumor angiogenesis to promote tumor growth and metastasis, and (5) inducing cancer-initiating cells, which facilitates tumor malignant progression and escaping from host immune surveillance. Although major evidence considers IL-17 as a promoter in CRC progression, there is still controversy. For instance, Kryczek and colleagues have demonstrated that tumor growth is enhanced in subcutaneous transplanted model and lung metastases model in IL-17−/− mice(Kryczek, Wei et al. 2009).

However, Ngiow et al. fail to reproduce the same results and they conclude that tumor growth has no difference between IL-17-deficient mice and control WT mice after3 independent sources of MC38 cells inoculated subcutaneously(Teng, Bowman et al. 2015).

Analogously, it has been reported that IL-17promotes the expression of the tight junction protein-Claudine in T84 cells via ERK MAPK activation in intestine(Kinugasa, Sakaguchi et al. 2000).Whereas, another study has shown that adenoma-linked barrier deterioration leads to microbial products invasion and triggers IL-23/IL-17-mediated tumor growth in CPCAPC mouse model(Grivennikov, Wang et al. 2012). In summary, despite the existing controversy presumably derived from the different models, most investigators appreciate IL-17 as a promoter in CRC progression. Table 1 summarizes studies with IL-17 which describe its possible antitumor role in CRC.

2.3.8 IL-17 as a Clinical Prognostic Indicator for Human CRC.

Tumor progression is affected by the complicated interaction of tumor cells, stromal cells, immune cells, and related cytokines in tumor microenvironment. IL-17 produced by epithelial cells and immune cells plays an important role in CRC development. Increased IL-17 concentration is detected in serum of CRC patients compared with healthy donors, which is inversely correlated with p53 expression. Moreover, it is proposed that IL-17 may act as a valuable tumor marker in patients with CRC and that concomitant expression of p53 and VEGF may provide further information about tumor features(Radosavljevic, Ljujic et al. 2010). Analogously,IL-17 producing cells induced by microbial dysbiosis are increased in intestinal mucosa of CRC patients, indicating that IL-17 producing cells may be a promising sensitive prognostic indicator for CRC.

Interestingly, an early increase of IL-17 expression in the premalignant stage and its dynamic change in tumor microenvironment throughout the adenoma-carcinoma sequence is associated with the progression of adenomas toward CRC(Cui, Yuan et al. 2012). These clinical studies indicate that IL-17 plays a critical role in the human CRC progression, which deserves to be studied further. Elevated IL-17 expression level in serum and tissue of CRC patients suggests that it may contribute to predicting cancer prognosis accompanied with another existing panel of molecular prognosticator.

Chapter Two
Material
&
Methods

2. Material and Methods

2.1Equipment's and Apparatuses:

The equipment's and apparatus, which were used throughout the study, are listed in table (2-1) with producing supplier and their origin.

Table (2-1): Equipment's and Apparatuses that used in the study.

Apparatus	Company	Origin
Incubator	JRAD	China
Microwave oven	Gosonic	China
Balance	KERN	Germany
AURA TM PCR Cabinet	Labnet	Italy
Microspin 12, High-speed Mini-centrifuge	Bio San	Germany
V-1 plus, Personal Vortex for tubes	Digsystem	Germany
Electrophoreses	CBS, Scientific	U.S.A
Microspin	Biosan	Lativa
Combi-spin	Biosan	Lative
Centrifuge	Hettich	Germany
Deep freeze	Sanyo	Japan
Micropipettes	Slamed	U.S.A
NanoDrop(Quantus^{tm}fluorometer)	promega	U.S.A
Water distiller	GFL	Germany

Tips (blue, yellow, white)	AFCO	Jordan
Automatic Elisa Reader	PARA medicaly	Italy
SaCycler-96 Real Time PCR SYSTEM	sacace	Italy
Eppendorf bench centrifuge	Hermle	Germany
Gel tube	AFCO	Jordan
EDTA tube	AFCO	Jordan
Plain Tubes	AFCO	Jordan
Graduated cylinder		
Coplin jar		
Slide holder		
Tissue pape		
Leica fluorescent microscope	Leica	Germany

2.1.1 The Chemical Materials and Kits:

The chemical and kits were used in this study are listed in table (2-2) with the producing supplier and the origin.

Table (2-2): Chemical materials and kits that used in the study.

Material	Company	Origin
Agarose	Conda	U.S.A
Red stain	Intron	Korea
Loading dye	Intron	Korea
TBE buffer 10 X	Conda	U.S.A
DNA extraction kit	Zymo	U.S.A
Human IL-17A Elisa MAX^{TM}Deluxe Set	BioLegend	U.S.A
Probe	IDT	U.S.A
Taq man master mix	IDT	U.S.A
Primary, mouse anti-human monoclonal Ab	biorbyte	U.S.A
Secondary FITC labelled Ab	biorbyte	U.S.A
Antibody diluent	Daku	Denmark
Positive charge slides	Daku	Denmark
Absolute alcohol		
Xylene		
Phosphate buffer saline		
Aqueous mounting media		

2.2 Subjects:

2.2.1 Patients and Control Groups:

A case control study has been constructed and consists of a total (40) patients which divided into (male n=25 and female=15) with age ranged (26–82years) . This study material were collected from Gastroenterology and Liver diseases teaching hospital from March 2018 to the end of May 2018, all patient case sheet have been recorded.

Paraffin embedded tumour tissue and normal donor colonic tissue that involve in this study were sectioned to thickness of (3um) and spread on positively charged slide for indirect immune-fluorescent technique. This study used the WHO grading system of colorectal carcinoma and modified Duck's staging system of colorectal carcinoma.

Five milliliter of blood were collected from the patients and healthy control then immediately transferred 2ml in to EDTA tube and 3ml into gel tube allowed to coagulate at room temperature then centrifuge at 3000 rpm for 5 mina nd the serum was divided into aliquots in eppendorf tubes until estimation and stored at(-20°C) until assay.

2.2.3 Indirect immunofluorecent technique for detection of CD 33 positive myeloid derived suppressor cells on formalin fixed paraffin embedded tissue FFPE.

A- Principle

Immunofluorescence is an antigen-antibody reaction where there antibodies are labelled with fluorescent dye and the reaction is visualised using fluorescent microscopy. Fluorochrome are dyes that absorb ultra-violate rays and emit visible light, this process is called fluorescence. Commonly used fluorochromes are Acridine orange, fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocaynate (TRITC). However, when FITC is excited by blue light it will emit green colour. Phycoerythrin emit an orange colour. The fluorochromes commonly used in immunofluorescent are fluorescein isothiocaynate and tetramathyl rhodamine isothiocyante. (Sawant, Kshar et al. 2013)

B- Preparation of tissue section and reagents

1-Paraffin embedded tissue were sectioned to the thickness of 3-4 micrometre, placed on positive charge slide and left overnight at room temperature to dry.

2- fifty ml of 20X concentrated detergent wash buffer were diluted into 1000 ml of distilled water.

3- Primary antibody was diluted to 1: 100 for CD33 monoclonal Ab.

4- Secondary antibody FITC labelled was diluted to 1:100.

5- Absolute ethanol was diluted in distilled water to 95%, 70% and 30%.

C. Indirect immunofluorescence procedure

1- Dewax the paraffin embedded tissue section by placing the slide in hot air oven at 70 c for one hour, then immersed the slide in xylene, alcohol and distilled water containing jars as the following

A- Xylene for 5 min

B- 95 % ethanol alcohol for 5 min

C- 70 % ethanol alcohol for 5 min

D- 30% ethanol alcohol for 5min

E- Distilled water for 5 min

2- The slide then tipped over a tissue paper to remove the ruminant distilled water.

3- Pin pen was used to circle the tissue in order to prevent the diluted antibody not to spill out the slide.

4- Humid chamber was prepared and the slide placed in it, the diluted primary antibody was added, than incubated at 37c for one hour.

5- After first incubation, the slide was washed with phosphate buffer saline and tipped over tissue, then secondary antibody was added and incubated at 37c for one hour (this step was performed in dark field).

6- After second incubation, the slide was washed with phosphate buffer saline and tipped over highly absorbable tissue paper to remove the ruminant antibody (this step was performed in dark field).

7- two drops of aqueous mounting media were added to the slide and covered with coverslip.

8- The slide was stored at 4 c overnight.

9- The CD33 positive MDSCs were visualised under the fluorescent microscope in dark room.

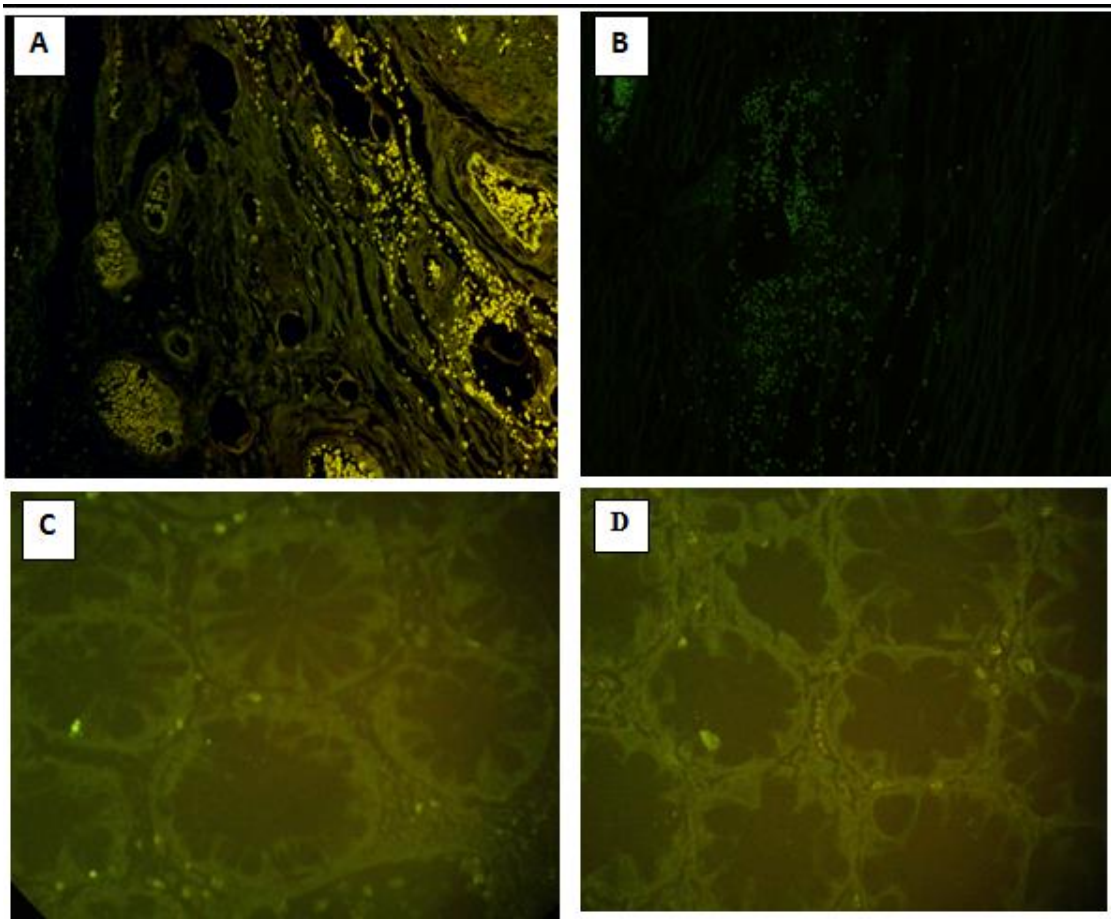

Figure: (2-3) Immunoflourecent staining of tumor infiltrating CD33^{+} cells (MDSCs) in colorectal adenocarcinoma section stained by FITC flourochrome (Green color) with dark background. (A, and B) tumor infiltrating MDSCs (CD33^{+}) expression within tumor tissue which. (C, and D) Immunoflourecent staining of infiltrating CD33^{+} cells (MDSCs) in normal resection margin. Magnification power (10X) for both A, and B, and (40x) for both C, and D.

2.3.1 Measurement Of Human IL-17A Elisa MAX^{TM}Deluxe Set.

A. Principle

This assay employs the quantitative sandwich enzyme immunoassay techniques. The micro ELISA plate has been pre-coated with an antibody specific to IL 17A. Then antigen is bound to immobilized capture antibody, standard and samples are pipetted into the well and any IL17A present is bound by the immobilized antibody .After removing any unbound substance by simple washing procedure. Biotin - conjugated antibody specific for IL17A is added to the wells. After washing, Avidin conjugated Horse radish peroxidase (HRP) was add to

each microplate well and then incubated and then wash to remove any unbound Avidin -enzyme reagent, substrate solution was added specific to the enzyme in the well. The color intensity produced is directly proportional to the amount of IL17A bound in the initial step. The enzyme-substrate reaction is terminated by the addition of a stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wave length of 450nm .The OD value is proportional to the concentration of IL17A and then calculate the concentration of IL17A in the sample by comparing the OD of the samples to the standard curve.

B. Reagent preparation

a. Coating Buffer A (5X): 5X Coating Buffer has been diluted to 1X with deionized water. For one plate, 2.4 mL 5X Coating Buffer has been diluted in 9.6 mL deionized water**.**

b. Capture Antibody (200X): Capture Antibody 200 X has been diluted in 1X Coating Buffer A. For one plate, 60 µL Capture Antibody has been diluted in 12 mL 1X Coating Buffer.

c. Assay Diluent (5X): has been diluted 5X Assay Diluent A to 1X with PBS (pH 7.4). 12 mL 5X Assay Diluent A has been diluted in 48 mL PBS.

d. Detection Antibody (200X): the Detection Antibody 200x has been diluted in 1X Assay Diluent. For one plate, 60 µL Detection Antibody has been diluted in 12 mL 1X Assay Diluent A.

e. Avidin-HRP(1000X): Avidin-HRP 1:1000 has been diluted in 1X Assay Diluent A. For one plate, 12 µL Avidin-HRP has been diluted in 12 mL 1X Assay Diluent A.

f. lyophilized Human IL-17A standard has been reconstituted with 0.2 mL of 1X Assay Diluent A to make 60ng/ml standard solution .the reconstituted standard has been allowed to sit for 15-20 minutes at room temperature, then gently mixed prior to making dilutions.

g. 1,000 µL of the top standard has been prepared at a concentration of 250 pg/mL by adding 4.2µL of reconstituted stock solution to 995.8 µL 1X Assay Diluent A.

h. TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A with Substrate Solution B. the two components were mixed immediately prior to use. For one plate 5.5 mL Substrate Solution A has been mixed with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

C. Procedure

Day 1.

100 µL of this Capture Antibody solution has been added to all wells of a 96-well plate provided in this set. Seal plate and incubate overnight between 2°C and 8°C.

Day2.

1- The plate was washed 4 times block the plate by adding 200µL 1X Assay Diluent A per well. The plate has been sealed and incubate at RT for 1 hour with shaking at 500 rpm on a plate shaker. All subsequent washes should be performed similarly.

2- The plate was washed 4 times, 100µL diluted standards and samples has been added to appropriate wells.

3- The plate sealed and incubate at RT for 2 hour with shaking.

4- The plate was washed 4 times, 100 µL of diluted Detection Antibody solution has been added to each well, the plate was sealed and incubated at RT for 1 hour with shaking.

5-the plate was washed 4 times with Wash Buffer. 100 µL of diluted Avidin-HRP solution has been added to each well. The plate sealed and incubated at RT for 30 minutes with shaking.

6- The plate was washed 5 times soaking for 30 seconds to 1 minute for each wash. 100 µL of freshly mixed TMB Substrate Solution has been added to each well and incubated in the dark for 30 minutes.

7- 100 µL of Stop Solution has been added to each well. The absorbance has been red at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

D. Calculation of Results

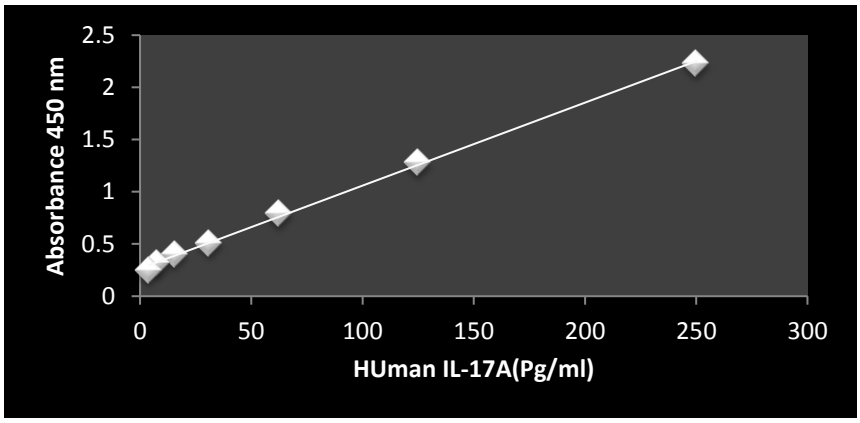

Figure (2-2): Standard curve for determination of Human IL-17.

2.3.2 Molecular Biological Studies:

2.3.2.1 DNA Extraction

A. Principle

The *Quick-gDNA*™ Blood MiniPrep is a simple procedure for the rapid isolation of total DNA (e.g., genomic, mitochondrial, viral) from a variety of biological sample sources. This product has been optimized for maximal recovery of ultra-pure DNA without RNA contamination and is compatible with whole blood (fresh or stored), serum, and plasma. For

processing, simply add the specially formulated Genomic Lysis Buffer to a sample, vortex, and transfer the mixture to the supplied Zymo-Spin™ Column. There is no need for organic denaturants or Proteinase K digestion because of the unique chemistries featured in the kit. Instead, the product features *Fast-Spin* technology to yield high-quality, purified DNA in just minutes (see below). PCR inhibitors are effectively removed during the purification process. DNA purified using the *QuickgDNA*™ Blood MiniPrep is suitable for PCR, nucleotide blotting, DNA sequencing, restriction endonuclease digestion, bisulfite conversion/methylation analysis, and other downstream applications.

Table (2-3): Kit Contents of ZYMO Research , and cat.No. D3072 & D3073.

***Quick-gDNA*™ Blood MiniPrep** (Kit Size)	**D3072** (50 preps.)	**D3073** (200 preps.)	**Storage Temperature**
Genomic Lysis Buffer*	50 ml	2x 100ml	Room Temp.
DNA Pre-Wash Buffer**	15 ml	50 ml	Room Temp.
g-DNA Wash Buffer	50 ml	100 ml	Room Temp.
DNA Elution Buffer	10 ml	2x 10 ml	Room Temp.
Zymo-Spin™ IIC Columns	50	200	Room Temp.
Collection Tubes	100	400	Room Temp.
Instruction Manual	1	1	-

B. Protocol

Buffer Preparation

◄*Recommended*: Add beta-mercaptoethanol (user supplied) to the Genomic Lysis Buffer to a final dilution of 0.5%(v/v) i.e., 250 µl per 50 ml *or* 500 µl per 100 ml.

Protocol

The following is for the purification of DNA from 100 µl whole blood, serum or plasma(the volumes can be adjusted up to 200 µl (max.) depending on your requirements).Fresh, frozen, or preserved blood (in

EDTA, citrate, or heparin) can be used. If material cannot be processed immediately, the sample can be "stabilized" for later processing (as noted below) although the immediate processing of blood samples is recommended.

1. Add 400 µl of Genomic Lysis Buffer to 100µl of blood, serum, or plasma(4:1). Mix completely by vortexing 4-6 seconds, then let stand 5-10 minutes at room temperature.1

2. Transfer the mixture to a Zymo-Spin IIC™ Column2 in a Collection Tube. Centrifuge at13,000 rpm for one minute. Discard the Collection Tube with the flow through.

3. Transfer the Zymo-Spin™ IIC Column to a new Collection Tube. Add 200µlof DNA Pre-Wash Buffer to the spin column. Centrifuge at 13,000 rpm for one minute.

4. Add 500µl of g-DNA Wash Buffer to the spin column. Centrifuge at 13,000 rpm for one minute.

5. Transfer the spin column to a clean microcentrifuge tube. Add ≥50 µl DNA Elution Buffer or water to the spin column3. Incubate 2-5 minutes at room temperature and then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20ºC for future use.

2.3.3 Determination of DNA Concentration:

DNA concentration was determined by two ways:

1. Nano Drop microvolume Quantitation of Nucleic Acid:-

A. Protocol

1. Add 200 µl from Tris-EDTA (TE) to 3,800 from D. water the mix 4000 µl, pull 10 µl ignore it and add 10 µl from dye (DNA dye)
2. Pull 200 µl of the mix for each sample.
3. The series of the following tubes are prepared as follows:

	Blank	Standard	Sample
Mixture	200 µl	200 µl	200 µl
DNA Extraction		2 µl	2 µl

4. Make vortex for second to mix.
5. Leaves on rake at room temperature for 5 min.
6. Extracted the value from the device immediately.

2. Gel Electrophoresis to Analyze DNA Quality:-

This way done according to the sambrook and russel [(1)].Agarose gel electrophoresis was used to assess the purity and mobility of purified DNA.

1- An agarose solution was prepared by dissolve 0.5g of agarose powder in 50 ml of 1x TBE in the (100 ml) flask, agarose was melted in hot block until the solution became clear.
2- The agarose solution was made cool to about (50- 55^0C), swirling the flask occasionally to cool evenly.
3- Red stain (2.5µl) was added to the warm gel then sealed the ends of the casting tray with two layer of tape.
4- The combs were placed in the gel-casting tray.
5- Meltedagarose solution was poured into the casting tray.
6- The agarose was allowed to solidify at room temperature, the comb pulled out carefully and the tape was removed. The gel was placed onto the electrophoresis chamber that was filled with TBE (1x) buffer.
7- DNA samples (5µl)were mixed with (3µl) DNA loading buffer and loaded in agarose gel wells.

8- The agarose gel electrophoresis was completed at 70V, 65Amp for 1hour.The DNA was observed by viewed under UV transilluminator.

2.3.4 Genotyping of KRAS gene codon 13 by symmetric pcr technique (RT-PCR).

Principle:

Asymmetric Real Time Polymerase Chain Reaction is performed in case of DNA melting Analysis with TaqMan probes(Huang, Liu et al. 2011). The resulting single stranded amplicon present in the incubation medium can hybridize with the probes. The spreading of the probe on the temperature leads to spatial separation of the fluorophore and the quencher and as a consequence to the buildup of fluorescence. The subsequent thermal denaturation of the complex lead to the release of the probe and its folding into a random coil and, consequently to the quenching of the fluorescence. Appropriate melting curve allow one to discriminate entirely complementary homoduplexes and partially complementary heteroduplexes. In the latter case there is mismatch at the site of mutation that lead to destabilization of the complex and a decrease in the melting temperature. Thus the symmetric melting peak indicates the wild type sequence in the initial DNA sample, while the additional peak at the left at the lower temperature indicates the presence of another (mutant) sequences(Krypuy, Newnham et al. 2006, Vossen, Aten et al. 2009, Erali and Wittwer 2010).

A- Preparation of Primers:

KRAS gene was investigated by IDT (Integrated DNA Technologies company, Canada).The lyophilized primers were, dissolved in free

DdH2O to give a final concentration of (100 pmol/µl) as stock solution and the stock was kept at (-20) to prepare (10 pmol/µl) concentration as work primer suspended, (10 µl) of the stock solution (90µl) of the free DdH2O water was added to reach a final volume (100 µl).

B- Primers Selection:-

1- Materials of genotyping positive strand for KRAS gene as shown in table (2-4)[0].

Table (3-4): Primers used in study.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- AGG CCT GCT GAAAA TGA CTG - 3'		
Reverse	5'-TTG GATCAT ATT CGTCCA CAA - 3’		
Cy5 (Probe 1)	5'- CTT GCC TAC GCC ACC AGC TCC AACT-BHQ2 - 3’		

The RT-PCR mixture For genotyping positive strand as shown in table (2-5).

Table (2-5):The mixture of working solution.

Component	20 µL (Final volume)	Final concentration
GoTaq Probe qPCR Master Mix	10 µL	
Probe	0.2 µL	0.1µM
Forward primer	0.4µL	0.02µM
Reverse primer	0.4µL	0.2µM
Nuclease-free water	4.0µL	
DNA Sample Volume	5µL	1pg-100ng

2 - Materials of genotyping negative strand for KRAS gene as shown in table (2-6)[0].

Table (2-6): Primers used in study.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- AGG CCT GCT GAAAA TGA CTG - 3'		
Reverse	5'-TTG GATCAT ATT CGTCCA CAA - 3’		
ROX(Probe2)	5'- AGT TGG AGC TGG TGG CGT AGG CAAG -BHQ2 - 3’		

The RT-PCR mixture For genotyping negative strand as shown in table (2-7).

Table (2-7):The mixture of working solution.

Component	20 µL (Final volume)	Final concentration
GoTaq Probe qPCR Master Mix	10 µL	
Probe	0.2 µL	0.1µM
Forward primer	0.4µL	0.2µM
Reverse primer	0.4µL	0.02µM
Nuclease-free water	4.0µL	
DNA Sample Volume	5µL	1pg-100ng

C- RT-PCR Cycling program:-

Table (2-8): amplification program of KRAS gene.

Step	Temp. (°C)	Time	Cycle
pre denaturation	95°C	10 min	1
	95°C	15 sec	45
Annealing/Extension	55.0 °C	10sec	
Detection(Scan)	72.0 °C	15 sec	
Melting curve	55 -95°C	15 sec	75

2.3.5 Preparation of Tris-Borate EDTA Buffer (TBE10X) (TBE buffer):

1XTris Boric EDTA buffer prepared by dilution the stock solution (TBE10X) buffer by using 100 ml of 10X TBE to 900 ml of distilled water to prepared 1 litter.

2.4 Statistical Analyses

The SPSS software (version 23) as used for statistical analysis. The difference of gender, smoking status, alcohol status and mutational group were examined by pearson chi-square numerical data were presented as mean ± SD. One way ANOVA test was used to compare between the tumor differentiation groups and mutational status. P value less than 0.05 were considered significant.

Chapter Three
Results
&
Discussion

3. Results & Discussion

3.1 Distribution of patients and control subjects according to age and gender

Mean age and age range of patients and control subjects are shown in table 3.1. Indeed, mean age of study group was higher than that of control group, 52.1±15.57 years versus 43.7 ±14.85 years; however, the difference was not significant from statistical perspective (P = 0.051), as shown in table 3.1. this finding is mandatory, since lack of significance with respect to age is essential in such case control study in order to avoid possible bias arising from in-equality in age between study and control groups.

The frequency distribution of patients with colorectal carcinoma according to 10 years age intervals is shown in figure 3.1. Patients between 20 and 29 years of age accounted for 7 out 40 (17.5 %), patients between 30 and 39 years of age accounted for 3 out 40 (7.5 %), patients between 40 and 49 years of age accounted for 3 out 40 (7.5 %), patients between 50 and 59 years of age accounted for 8 out 40 (20.0 %), patients between 60 and 69 years of age accounted for 15 out 40 (37.5 %) and patients who were 70 years or older accounted for 4 out 40 (10.0 %). Accordingly, majority of cases were seen above 50 years of age, as shown in figure 3.1.

Table 3.1: Mean age and age range in control and study groups.

Characteristic	**Control** ***n*** **= 20**	**Study** ***n*** **= 40**
Mean age ± SD (years)	43.7 ±14.85	52.1±15.57
Range (min.-max.)	21 - 80	22 – 81
t	-2.000	
P *	0.051 NS	

SD: standard deviation; *n*: number of cases; *: Independent samples t-test (equal variance was assumed); NS: not significant.

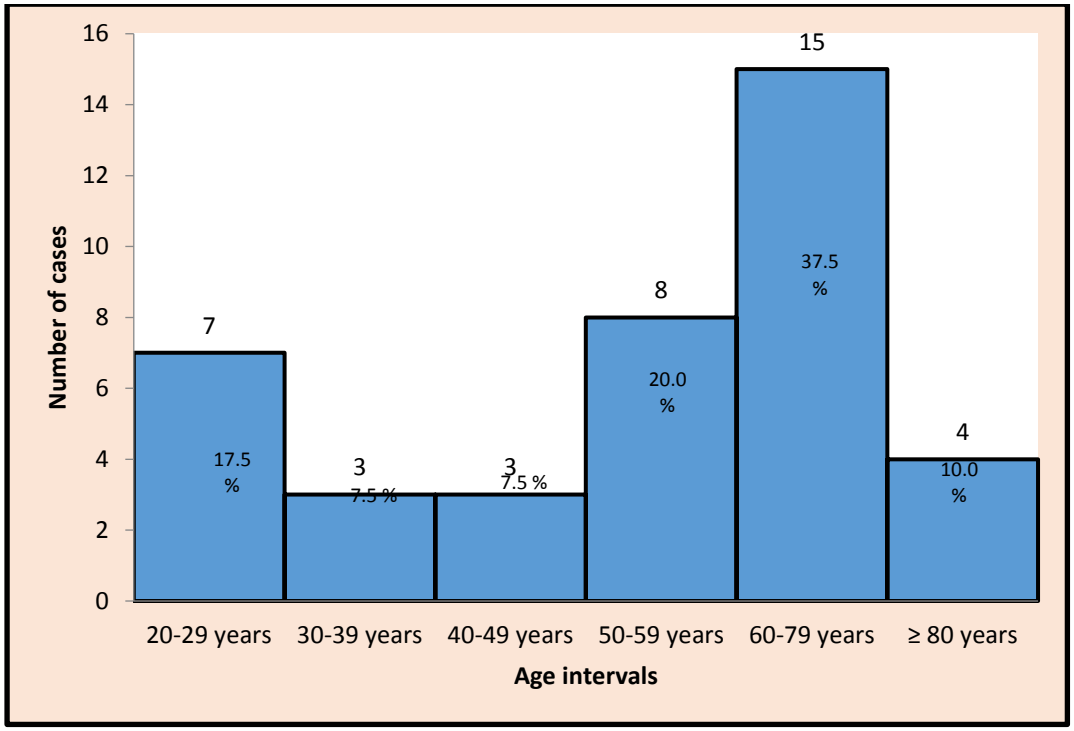

Figure 3.1: Histogram showing the number and percentage of patients according to 10 years age intervals.

The distribution of patients and control subjects according to gender is shown in table 3.2. Study groups included 25 men and 15 women, 62.5 % versus 37.5 %, respectively; while control group included 11 men and 9 women, 55.0 % versus 45.0 %, respectively. These finding implies that colorectal carcinoma is more common in men. In addition, it appeared that the difference in distribution of patients and control subjects with respect to gender is statistically insignificant (P = 0.576), as shown in table 3.2.

Table 3.2: Distribution of patients and control subjects according to gender.

Gender	**Control group** ***n* = 20**	**Study group** ***n* = 40**	χ^2	***P* ***
Male	11 (55.0 %)	25 (62.5 %)	0.312	0.576 NS
Female	9 (45.0 %)	15 (37.5 %)		

***n*: number of cases; *: chi-square test; NS: not significant at P ≤ 0.05**

previous literature mentioned that the prevalence of colorectal cancer per 100,000 is 202 in men, and 231 in women, amounting to 10 and 8% , respectively.(Lutz *etal.*,2003). Men have higher incidences of colon and especially of rectal cancer than women (Matanoski *etal.*,2006, Wei *etal.*,2004). Beyond 50 years of age, CRCs occur in men typically 4 to 8 years earlier than in women (Brenner *etal.*,2007). Thus, these studies support the result of the current study.

Incidence of CRC at specific subsites varies considerably with age,and gender (Troisi *etal.*,1999), perhaps due to different environmental, lifestyle or socioeconomic factors. For example, a large retrospective cohort study in Ohio showed that women presented with proximal colon cancers at a significantly higher age than men(Woods *etal.*,2006). In some studies, age-related incidence of cancer was higher in the proximal colon compared to distal sites (Rapp *etal.*,2005) but showed different patterns in men and women. In another study, based on 28 US cancer registries, the male-to-female ratio increased as the site shifted from the proximal to the distal colorectum. (Matanoski *etal.*,2006).

Several studies indicated that women are less likely than men to develop CRC at all ages. (Ries *etal.*,2000). The risk of CRC increases with age; the median age at diagnosis for colon cancer is 68 in men and 72 in women; for rectal cancer it is 63 years of age in both men and women (Howlader *etal.*,2016). As a result of rising CRC incidence rates in younger age groups coincident with declining rates in older age groups, the proportion of cases diagnosed in individuals younger than age 50 increased from 6% in 1990 to 11% in 2013(Surveillance *etal.*,2016).

Most of these cases (72%) occur in people who are in their 40sCRC trends reflect patterns in older age groups, among whom the majority of cases occur, masking trends in young individuals. From 2009 to 2013, CRC incidence rates decreased by 4.6% per year in individuals

65 years of age and older and by 1.4% per year in individuals 50-64, but increased by 1.6% per year in adults younger than 50 (Surveillance *etal.*,2016). Notably, the increase in young adults followed a decade of rapid declines during the late 1970s and early 1980s. Reasons for the rise in young age groups are unknown, but may reflect an increased sedentary lifestyle and a higher prevalence of obesity and/or unfavorable dietary patterns in children and young adults. Regarding the gender, CRC incidence rates are approximately 30% higher in men than in women, while mortality rates are approximately 40% higher.

Reasons for the gender disparity are not fully understood, but partly reflect differences in exposures to risk factors (e.g., cigarette smoking) and sex hormones, as well as complex interactions between these influences.(Murphy *etal.*,2011) other studies have found increased risk(Gunter *etal.*,2008) or no association (Lin *etal.*,2013).

The study conducted by Farin shown of developing colorectal cancer increases with advancing age. More than 90% of the people diagnosed with the disease are older than 50, with the average age at the time of diagnosis being 64. In the United States it is the most common cancer in the population older than 75. People between the ages of 65 and 85 are six times more likely to develop colorectal cancer than people younger than 50.8 Patients older than 70 present mostly with early-stage disease, whereas younger patients, usually in their 40s, present with much more aggressive disease for a given stage of presentation (Farinetal.,2005).

3.2 Distribution of patients and control subjects according to bad habits smoking

Being enrolled in the list of factors that are associated with colorectal carcinoma, bad habits of smoking was studied and the results are shown in table 3.3. There was insignificant difference (P = 0.264) in rate of

smoking between study and control groups, 55.0 % versus 70.0% respectively.

Table 3.3: Bad habits in patients and control groups.

smoking	**Control group** ***n* = 20**	**Study group** ***n* = 40**	χ^2	***P*** *
Smokers	14 (70.0 %)	22 (55.0 %)	1.250	0.264 NS
Non smokers	6 (30.0 %)	18 (45.0 %)		

***n*: number of cases; *: chi-square test; NS: not significant at P ≤ 0.05; S: significant at P ≤ 0.05**

Cigarette smoking are considered as major risk factors for gastrointestinal cancer, including colorectal cancer (Jung *etal.* 2014). As a result of cumulative evidence from epidemiological studies, colorectal cancer has been listed as a cancer site with 'sufficient evidence' for human linked to carcinogen of cigarette smoking (Cogliano *et al.*, 2011, Giovannucci *et al.*, 2001). Another previous epidemiological studies conducted in Korea did not find clear associations (Shin *et al.*, 2011, Jee *et al.*, 2004).

A health insurance-based cohort study did not find any association between smoking and colorectal cancer in both men and women.(Jee *etal.*,2004) However, the most recent health insurance-based cohort study suggested marginal elevated risk for distal colon and rectal cancer among male former smokers(Shin *etal.*,2011). In a Korean cohort study with an elderly population, former smokers showed a higher risk of colorectal cancer (Kim *etal.*,2006) The result conducted by Sooyoung Former smokers showed a non-significantly elevated risk of colorectal cancer (Sooyoung *etal.*,2015)

The present study did not show an association between smoking status and colorectal cancer risk similar to the findings of earlier studies from India and Malaysia (Iswarya *etal.*,2016, Ramzi *etal.*,2014). However, the association between smoking and CRC was not observed in

tthe American Veterans' Study (Heineman et al, 1994) with over a 20-year follow-up. In the past four decades, summarised the results from several studies, which support the hypothesis that smoking is a risk factor of CRC, showing that smoking slightly increases the risk of CRC mortality by 25%–40% when comparing smokers with nonsmokers (Botteri *et al*, 2008; Liang *et al,* 2009).

From tobacco smoke could have a role in the aetiology of human CRCs (Alexandrov et al, 1996; Phillips, 2002). Recently, new biomedical evidence on nicotine, a major active component of cigarette smoke, suggests that this chemical might serve to promote colorectal cell proliferation (Cucina et al, 2012) , inhibit apoptosis in colon cancer (Cucina et al, 2012), and enhance colon cancer cell migration (Wei et al, 2011).

The contaversary between such studies concerning the effect of smoking on CRC might be related to sample size that included in such study. Age at initiating smoking, smoking duration, and amount of daily cigarette smoking were all shown to be positively associated with increased CRC risk (Qaseem et al, 2012).

3.3 Pathological characteristics of colorectal carcinoma

The main pathological characteristics related to the malignant colorectal tumor such as grade, stage and lymph node (LN) involvement are shown in table 3.4. The distribution of patients according to grade was as following: 4 (10 %), 14 (35 %) and 22 (55 %) as grade I, II and III respectively. The distribution of patients according to stage was as following: 1 (2.5 %), 7 (17.5 %), 23 (57.5 %) and 9 (22.5 %), as stage I, II, III and IV respectively. Positive LN involvement was seen in 31 out of 40 (77.5 %) and all patients with positive LN had 3 or more positive LNs; median positive LN number was 4 (3) and it ranged from 0 to 10 LNs, as shown in table (3.4).

Table 3.4: Pathological characteristics of colorectal tumor in study groups.

Characteristic	*n*	%	Median (IQR)	Range (min. - max.)
Grade				
I	4	10		
II	14	35		
III	22	55		
Stage				
A	1	2.5		
B	7	17.5		
C	23	57.5		
D	9	22.5		
LN				
Positive	31	77.5	4 (3)	10 (0 -10)
Negative	9	22.5		

3.4 IL-17 level and CD33 expression in control and study groups.

The variable IL-17 was not normally distributed according to Kolmogorov-Smirnov test and hence median and inter-quartile range were used to describe central tendency and dispersion instead of mean and standard deviation. Median IL-17 was significantly higher in study group than in control group ($P < 0.001$), 12.13 (9.73) pg/ml versus 0.41 (0.67) pg/ml, respectively, as shown in figure 3.2.

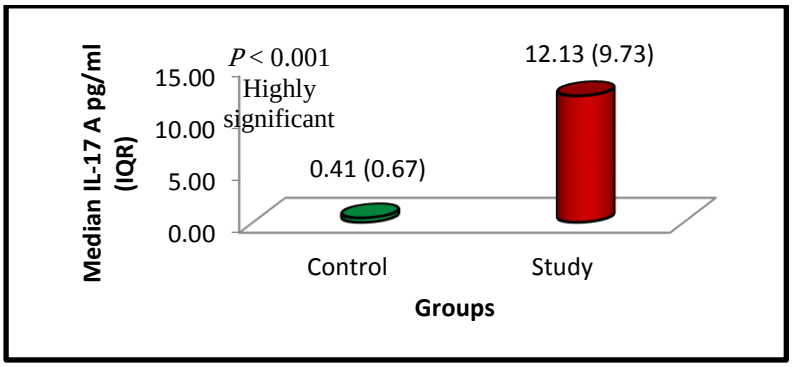

Figure 3.2: Median IL-17 level in control and study groups.

The variable CD33 was not normally distributed according to Kolmogorov-Smirnov test and hence median and inter-quartile range

were used to describe central tendency and dispersion instead of mean and standard deviation. Median CD33 was significantly higher in study group than in control group ($P < 0.001$), 73.00 (5.0) % versus 4.50 (3.75) %, respectively, as shown in figure 3.3.

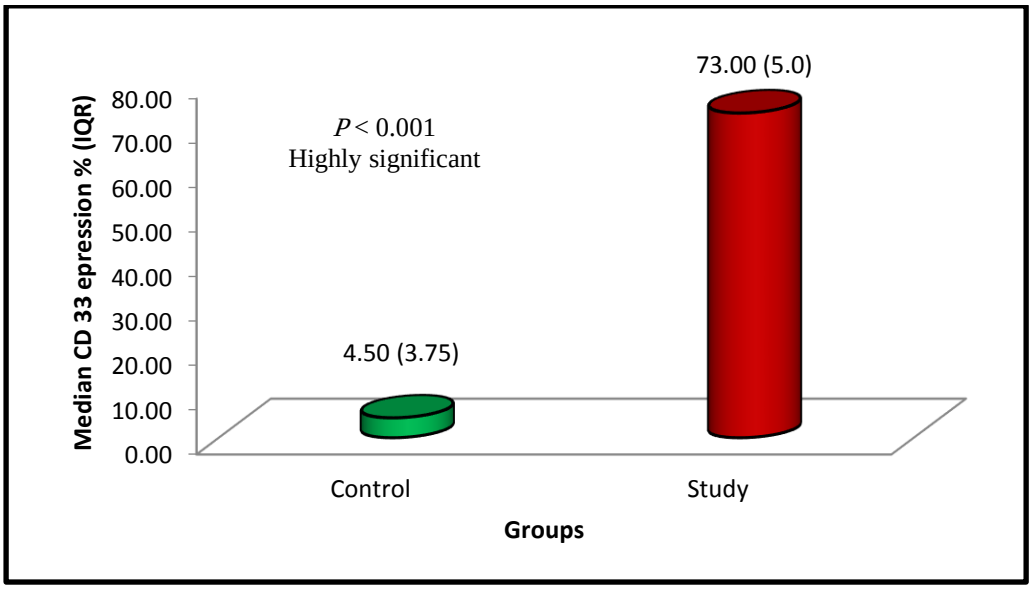

Figure 3.3: Median CD33 expression in control and study groups.

Present study indicated that IL-17 could have a tumor promoting activity. Moreover, the majority of studies consider that IL-17 acts as a promoter in tumor initiation and progression. Particularly, the ablation of IL-17A can inhibit the progression of spontaneous intestinal tumorigenesis in ApcMin/+ mice(Chae, Gibson et al. 2010). In accordance with studies in other cancers, growing evidence has shown that IL-17 can also promote tumor progression in CRC.

In intestinal tumor bearing model, the tumor size is significantly reduced in IL-17 gene-knockout mice compared with wide-type (WT) mice, and anti-IL-17A monoclonal antibody treatment results in decreased tumor size in the WT mice(Oshiro, Kohama et al. 2012). *In vitro*, IL-17 and TNF-α synergistically promote carcinogenesis by stimulating glycolysis and growth factor production by CRC cells(Straus 2013). In colitis-associated cancer model, tumorigenesis and inflammatory cytokines including IL-6, IFN-γ, and TNF-α are markedly

decreased in IL-17-deficient mice compared with WT mice, suggesting that IL-7 plays an pivotal role in promoting CRC initiation in colitis-associated cancer(Hyun, Han et al. 2012).

Enterotoxigenic bacteroides fragilis (ETBF), a human colonic commensal bacterium, can promote colonic tumorigenesis in APC mutant mice via Stat3 activation and Th17-cell polarization, and further blocking IL-17 or IL-23R can significantly reduce tumor formation *in vivo(Wu, Rhee et al. 2009)*. It has been reported that epithelial barrier deterioration results in microbial pathogen invasion and microbial products release are driving IL-23/IL-17 axis activation and promoting tumor growth and progression in CPCAPC mice(Wu, Rhee et al. 2009). Moreover, IL-17/G-CSF/Bv8 axis has been reported to promote VEGF independent CRC tumor angiogenesis *in vivo(Chung, Wu et al. 2013)*. A study has suggested that CRC tissue-derived Foxp3+ IL- 17+ cells have the capacity to induce cancer-initiating cells *in vitro (Yang, Wang et al. 2011)*.

Based on these findings, we propose that the pro-tumor activity of IL-17 in CRC microenvironment may exert in several aspects: (1) promoting tumor elicited inflammation which facilitates the proliferation and survival of malignant cells, (2) forming an immunosuppressive tumor microenvironment by chemo-attracting immunosuppressive cells and cytokines, (3) suppressing cytotoxic cells-mediated immunosurveillance against tumor, (4) fostering tumor angiogenesis to promote tumor growth and metastasis, and (5) inducing cancer-initiating cells, which facilitates tumor malignant progression and escaping from host immune surveillance.

Whereas, another study has shown that adenoma-linked barrier deterioration leads to microbial products invasion and triggers IL-23/IL-17-mediated tumor growth in CPCAPC mouse model(Grivennikov,

Wang et al. 2012). Despite the existing controversy presumably derived from the different models, most investigators appreciate IL-17 as a promoter in CRC progression. A previous study conducted by L. LI., et al.2016 came in an agreement with present study since they demonstrated that the expression of IL-17A was increased in Vδ2 T cells in colorectal cancer patients.

A previous literatures mentioned that Tumor progression is affected by the complicated interaction of tumor cells, stromal cells, immune cells, and related cytokines in tumor microenvironment. IL-17 produced by epithelial cells and immune cells plays an important role in CRC development. Increased IL-17 concentration is detected in serum of CRC patients compared with healthy donors. Moreover, it is proposed that IL-17 may act as a valuable tumor marker in patients with CRC and that concomitant expression of p53 and VEGF may provide further information about tumor features(Radosavljevic, Ljujic et al. 2010). Furthermore, elevated Th17 cells have been observed in more than 80% of human sporadic colon cancer tissues, indicating that IL-17 expression may be one of potential biomarkers for the future development of a new prognostic “test set” for sporadic CRC(Le Gouvello, Bastuji-Garin et al. 2008). Univariate and multivariate analysis reveal that 5-year survival rate is 72.41% in the 26 cases with lower IL-17 expression and 38.08% in the 26 cases with higher IL-17 expression, proposing that IL-17 is an independent prognostic factor for overall survival and IL-17 producing cells may facilitate development of CRC by fostering angiogenesis via stimulation of VEGF production by cancer cells(Liu, Duan et al. 2011). Interestingly, an early increase of IL-17 expression in the premalignant stage and its dynamic change in tumor microenvironment throughout the adenoma-carcinoma sequence is associated with the progression of adenomas toward CRC(Cui, Yuan et al. 2012). Elevated IL-17 expression

level in serum and tissue of CRC patients suggests that it may contribute to predicting cancer prognosis accompanied with another existing panel of molecular prognosticator.

Furthermore, in a study done by Mohsen, *et at*., 2015 mention that high expression of IL-17 showed significant association with TNM stage, but no significant association was found with other clinical factors. Lin et al. indicated that the IL-17 mRNA level was higher in CRC than in adenoma and non-tumor tissue. They found that histological differentiation, Dukes staging (early stage), TIN infiltration, and better survival were linked to IL-17 expression(Lin, Xu et al. 2015). However, the findings of Lin et al. is completely different from the findings of Liu et al. that showed that high expression of IL-17 was associated with high microvessel density and poorer OS but was not significantly related to differentiation, or histological type(Liu, Duan et al. 2011).

A growing evidence has shown that IL-17 can also promote tumor progression in CRC. In intestinal tumor bearing model, the tumor size is significantly reduced in IL-17 gene-knockout mice compared with wide-type (WT) mice, and anti-IL-17A monoclonal antibody treatment results in decreased tumor size in the WT mice(Oshiro, Kohama et al. 2012). *In vitro*, IL-17 and TNF-α synergistically promote carcinogenesis by stimulating glycolysis and growth factor production by CRC cells(Straus 2013).

It has been reported that epithelial barrier deterioration results in microbial pathogen invasion and microbial products release are driving IL-23/IL-17 axis activation and promoting tumor growth and progression in CPCAPC mice(Grivennikov, Wang et al. 2012). Similarly, another study has presented that a subset of Foxp3+ IL-17+ T cells in CRC tissue suppress the tumor-specific CD8+ T cells and attenuate the antitumor immune response(Ma and Dong 2011). Based on these findings, the

protumor activity of IL-17 in CRC microenvironmentmay might be exert in several aspects: (1) promoting tumorelicited inflammation which facilitates the proliferation and survival of malignant cells, (2) forming an immunosuppressive tumor microenvironment by chemoattracting immunosuppressive cells and cytokines, (3) suppressing cytotoxic cells-mediated immunosurveillance against tumor, and (4) fostering tumor angiogenesis to promote tumor growth and metastasis.

However even present study demonstrated that there were inverse relationship between IL-17 and all of tumor histopathological parameters, while major evidence considers IL-17 as a promoter in CRC progression. However this contraversary could be explained on a study focused on the intrinsic role of endogenous IL-17 in CRC has demonstrated that CD4+ T-cell-derived IL-17 promotes spontaneous intestinal tumorigenesis in ApcMin/+ mice, suggesting that IL-17 plays an important role in CRC initiation(Chae, Gibson et al. 2010). In colitis-associated cancer model, tumorigenesis and inflammatory cytokines including IL-6, IFN-γ, and TNF-α are markedly decreased in IL-17-deficient mice compared with WT mice, suggesting that IL-7 plays an pivotal role in promoting CRC initiation in colitis-associated cancer(Hyun, Han et al. 2012).

There is still controversy. For instance, Kryczek and colleagues have demonstrated that tumor growth is enhanced in subcutaneous transplanted model and lung metastases model in IL-17−/− mice (Kryczek, Wei et al. 2009). In summary, despite the existing controversy presumably derived from the different models, most investigators and according to present data IL-17 could be consider as a promoter in CRC progression.

Concerning CD33+ cells (MDSCs) that infiltrating tumor tissue. Many studies have highlighted the role of MDSCs in cancer patients immune suppression(Kusmartsev and Gabrilovich 2006, Gabrilovich and Nagaraj 2009). - MDSCs represent a homogeneous cell population that is significantly elevated in the peripheral blood of CRC patients, while it was found at a relative high density in CRC tumor tissues compared with healthy control. These data show that MDSCs expansion could be involved in CRC development, and suggest that the tumor tissue microenvironment might serve to promote MDSC expansion. Present data confirm previous studies demonstrating a dramatic expansion of MDSCs during tumor progression, infection, and even following immunization.

The present study observed an expansion of MDSCs among tumorous tissues from patients with CRC. The increased local MDSC population correlated with advanced disease stages and tumor lymph node metastases in CRC patients

The TME contains a mixture of cells of lymphoid and myeloid lineages, comprising of both innate and adaptive immune cells. Tumor-infiltrating myeloid cellular populations include granulocytes (neutrophils, basophils, and eosinophils), tumor-associated macrophages, IMC, and MDSC(Hanahan and Coussens 2012). Expansion of infiltrating lymphocytes in cancers is often associated with antitumor response and improved clinical outcome(Angell and Galon 2013). While cancer treatment strategies like adoptive T cell therapies, which involve transferring in vitro expanded autologous cytotoxic T cells to eradicate tumor cells(Vignali and Kallikourdis 2017). Elevated levels of circulating MDSC inversely correlated with lymphocyte count in CRC patients(Ohki, Shibata et al. 2012). In a previous conducted found that, that expansion of peripheral MDSCs in CRC patients correlated with

advanced stage and histological grade, which suggested their role in tumor progression (Toor, Khaja et al. 2016); however, no such correlation was observed in the levels of circulating myeloid cells in PBC(Toor, Khaja et al. 2017). They, found that CRC patients with poorly differentiated tumors have significantly higher levels of MDSCs than those with well-defined tumors and also breast cancer patients, regardless of their tumor histological presentation. Therefore, our data suggest that myeloid cell levels in tumor burden is reflected by the tumor grade and disease stage.

A previous study done by(Chanan-Khan, Cramer et al. 2016), mentioned that Circulating MDSCs were significantly elevated in CRC patients with regional and distant metastases compared with HDs. However, there were no differences in GMCs levels between patients with different tumor stages. Another studies in CRC and cutaneous melanoma, have also shown higher levels of MDSCs in metastatic cancers compared with control group (Zhang, Wang et al. 2013, Stanojevic, Miller et al. 2015).

There are studies, however, which reported significant differences in levels of myeloid cells between healthy group and patients with low stage cancers, and between patients with different cancer stages(Diaz-Montero, Salem et al. 2009, Sun, Zhou et al. 2012) and these data came in agreement with result of present study; this discrepancy could be due to the number of patients and type of techniques used in the experiments. Previous studies have highlighted the immunosuppressive functions of neutrophils and myeloid cells, which would facilitate tumors to evade local immune response, and a role of MDSCs in invasion and metastases is emerging from ongoing research(Pillay, Tak et al. 2013, Bronte, Brandau et al. 2016). Tumor cells secrete several soluble factors such as Interleukin 6 and GM-CSF, which influence bone marrow and help in

expansion of myeloid cells. These cells accumulate in TME and produce factors such as VEGF, matrix metallopeptidase 9 (MMP9), and transforming growth factor beta (TGF-β), which support angiogenesis and tumor growth and eventually invasion and metastases of tumor cells(Marvel and Gabrilovich 2015, Moses and Brandau 2016). Thus, present results suggest a role of immunosuppressive MDSCs in cancer invasion and metastases.

Grading of tumors depends upon histological differentiation of tumor cells compared to normal cells, and it represents how quickly a tumor can grow and spread. Histological grade has been shown to be stage-independent prognostic variable in CRC patients; high-grade CRC patients with poorly or undifferentiated cancer cells have worse disease prognosis compared to low-grade CRC patients with well or moderately differentiated tumor cells(Compton 2003, Ueno, Kajiwara et al. 2012). Our work shows that in high-grade CRCs, MDSCs may play important role in tumor-mediated immune suppression and suggest their involvement in tumor progression and this result supported by previously conducted study done by (Salman, *et al*., 2016). In conclusion, our study shows that MDSCs was expanded in CRC patients. Importantly, expansion of MDSCs correlated with tumor stage and histological grades, thereby identifying these cells as key players among others in CRC patients. Better understanding of their characteristics should aid in therapeutic strategies to target immunosuppressive pathways employed by tumors.

Infiltration of these cells in the tumor host is promoted by the tumor microenvironment, and tumor-associated expansion of MDSCs contributes to tumor escape from the immune system. The decline of immune function in the tumor leads to ineffective tumor treatment outcomes, due to the insufficient activity of antigen-specific antitumor

responses and the possible extension of immune tolerance in the tumor host. Many studies have addressed MDSCs in solid tumors, but little is known about MDSCs in CRC. In this study, we investigated tumor-infiltrating MDSCs in CRC patients, and demonstrating the clinical significance of MDSCs in CRC. MDSCs represent 20%-30% of normal bone marrow cells and 1%-4% of all nucleated cells in the space(Stanojevic, Miller et al. 2015).

Present study showed that the CD33+ was present at a very low proportion in the colonic tissue healthy population. CD33+ MDSCs are identified as a population of myeloid cells at earlier stages of differentiation(Kusmartsev and Gabrilovich 2006, Sica and Bronte 2007). In CRC, these CD33+-MDSCs displayed characteristics of immature myeloid cells, expressing high levels of CD33, CD11b, CD18 and CD1a. MDSCs in tumors might alter their expression of functional molecules according to the tumor microenvironment. the accumulation of MDSCs in tumorous tissues might be because of recruitment from the peripheral blood to tumors and aggregation in situ. A recent study discovered that CXCR2-expressing MDSCs could be tracked from the peripheral blood to the colonic mucosa according to their CXCR2 ligands, and these MDSCs were found to promote colitis-associated tumorigenesis in a mouse model of colitis-associated cancer (Cui, Kryczek et al. 2013, OuYang, Wu et al. 2015).

Correlations between the tumor burden and immune cells have been explored in cancer patients(Rotstein, Blomgren et al. 1985, Frey and Monu 2006). In a previously conducted study, they observed that the percentages of circulating MDSCs and Tregs were significantly decreased in patients after undergoing resection, whereas the percentages of CTL and NK cells were unchanged, indicating that immunosuppression is weakened by decreasing the MDSC and Treg cell populations in the CRC

patients after reducing the tumor burden. (Sinha, Okoro et al. 2008, Xiang, Poliakov et al. 2009).

In an in vitro experimental system, the CRC cell lines SW480 and SW620 could induce CD33+CD11b+HLA-DR− MDSCs from CD33+ PBMCs. These tumor-induced MDSCs express high levels of immune inhibitory molecules, including TGF-β, IDO, IL-10, Arg-1, iNOS and NOX2, and could strongly suppress the proliferation of OKT3- stimulated CD4+ and CD8+ T cells. Accordingly, these data indicate that the CRC cells induce functional MDSCs in vitro. Thuds, present study confirm and came in agreement with previous reports of other types of cancer cells (Mace, Ameen et al. 2013, Waight, Netherby et al. 2013).

Because both IL-17 and CD33 were not normally distributed, log transformation was used to normalize their distribution in order to carry out Pearson correlation and the result was shown in figure 3.4. Although, the correlation between log IL-17 and log CD33 was positive (r = 0.284), in statistical terms it was not significant (P = 0.075). However, this value of 0.075 is not so far from 0.05 and increasing sample size may produce significant correlation. The explanation for such positive correlation between IL-17 and CD33 + cells (MDSCs), could be explained according to the studies that they clearly demonstrate that innate γδT cells are the major source of IL-17 in human CRC. γδT17 cell activation is triggered by IL-23, which is highly expressed in human CRC tissues. The source of IL-23 is mainly from tumor-infiltrating inf-DCs, which is activated by microbial pathogen invasion as a consequence of tumorous epithelial barrier deterioration. These γδT17 cells not only secrete large amounts of IL-17 but also other cytokines including IL-8, GM-CSF, and TNF-α. More importantly, the in vitro studies demonstrate that tumor-infiltrating γδT17 cells chemoattract PMN-MDSCs and further expand and provide survival advantage for them to maintain immune suppressive activity via

secretion of these cytokines, also they demonstrate a strong and positive correlation between tumor-infiltrating γδT17 cells and advanced clinicopathological features including TNM stages, tumor sizes, and lymphatic and vascular invasions of poor clinical outcome. Taken together, these findings suggest that innate γδT17 cells contribute to human CRC development and progression.

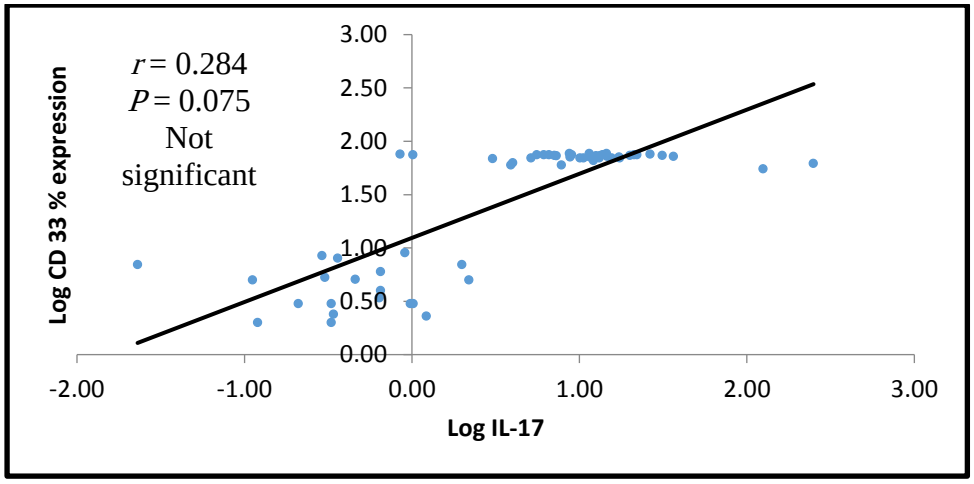

Figure 3.4: Correlation between Log IL-17 and Log CD33.

3.5 KRAS genotype frequency and percentage in control and study groups.

The following genotypes related to KRAS, GC (wild genotype) and AC, AT and GT (mutant genotypes) were studied and the results are shown in table 3.5. The rate of the wild genotype (GC) was 24 (60.0 %) and 15 (75.0 %), in study and control groups, respectively. The mutant genotype (AC) was more frequent in study group than in control group, 8 (20.0 %) versus 3 (15.0 %), respectively; however the difference did not reach statistical significance (P = 0.724).

The mutant genotype (AT) was equally frequent in study and control groups, 2 (5.0 %) versus 1 (5.0 %), respectively (P = 0.724). The mutant genotype (GT) was more frequent in study group than in control group, 6 (15.0 %) versus 1 (5.0 %), respectively; however the difference

did not reach statistical significance (*P* = 0.394), as shown in table 3.5. Overall, the frequency of mutant genotypes was more in study group than in control group, 40 % versus 25 %, respectively, but the difference was not significant in statistical terms (P = 0.251), as shown in table 3.6.

The risk of mutant KRAS genotype in association with colorectal cancer was assessed in terms of Odds ratio which was 2.0 (95 % confidence interval of 0.61 - 6.60). This implies that persons harboring mutant KRAS genotypes are at two fold risk of developing colorectal carcinoma than general population and the etiologic fraction (EF) of these mutant genotypes collectively accounted for 0.38, as shown in table 3.6. The results of the HRMA (amplification and melting curve) represented in figure (3-5) showed the melting curve results of only 35 samples. An advantage of performing HRMA analysis on a real time PCR machine with HRM capability, is that the PCR amplification and HRM analysis are performed in the one run and the results are available for analysis at the end of the run. While the figure (3-6) showed the difference between two types of KRAS mutation melting curve.

Table 3.5: KRAS genotypes in control and study groups.

KRAS genotype		**Control group *n* = 20**	**Study group *n* = 40**	***P* ***
Wild	GC	15 (75.0 %)	24 (60.0 %)	Reference
Mutant	AC	3 (15.0 %)	8 (20.0 %)	0.724 NS
	AT	1 (5.0 %)	2 (5.0 %)	1.000 NS
	GT	1 (5.0 %)	6 (15.0 %)	0.394 NS

***n*: number of cases; *: Fischer exact test; NS: not significant**

Table 3.6: Mutant and wild type KRAS genotypes according to groups.

KRAS genotype	Control group *n* = 20	Study group *n* = 40	χ^2	*P* *	OR	95% CI	EF
Wild	15 (75.0 %)	24 (60.0 %)	1.319	0.251 NS	2.0	0.61 - 6.60	0.38
Mutant	5 (25.0 %)	16 (40.0 %)					

***n*: number of cases; *: chi-square test; NS: not significant; OR: Odds ratio; CI: confidence interval; EF: etiologic fraction.**

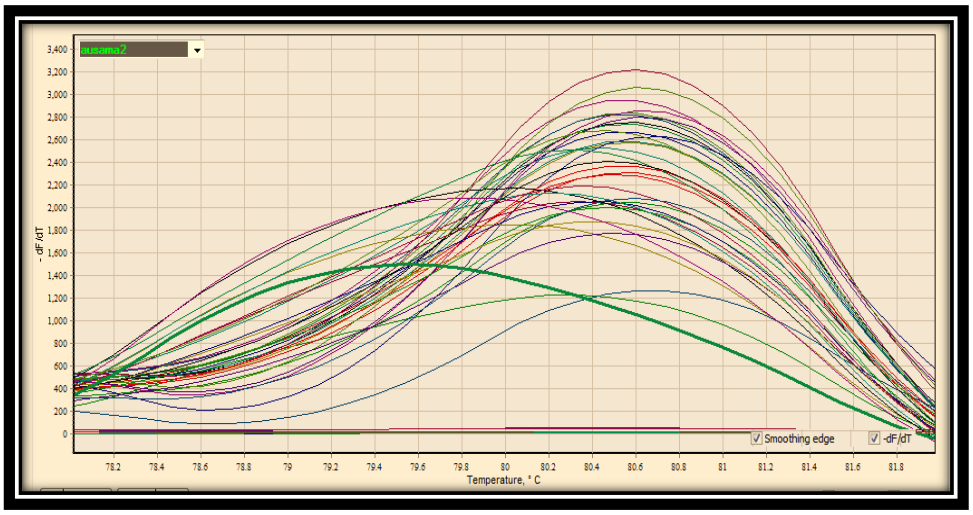

Figure (3-5); different melting peaks for mutation scanning of KRAS gene.

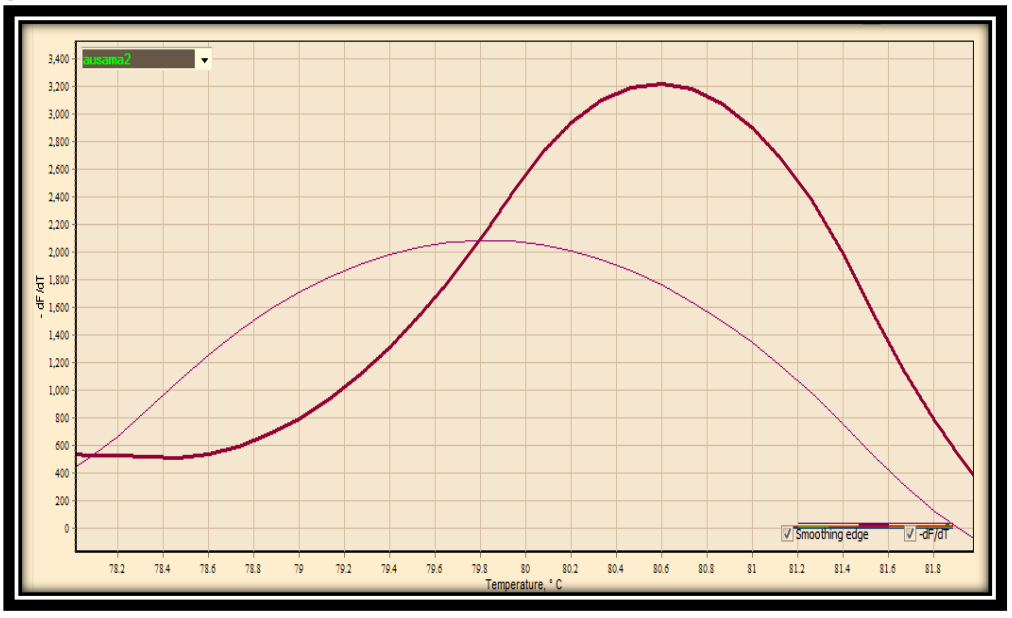

Figure (3-6); two types of melting curves (1) wild type (2) 12AC mutantion

In this study the aim is to develop a highly sensitive method for detecting somatic mutations of KRAS gene for genotyping CRC patients. The high resolution melting analysis technique (HRMA) applied to mutation scanning is often implemented in high-resolution format. Upon the completion of amplification, PCR products are subjected to the melting procedure in the presence of fluorescent probes. Characteristic changes in DNA melting curves indicate the presence of mismatched bases in the duplexes. and, therefore, the presence of mutations (Krypuy, Newnham, Thomas, Conron, & Dobrovic, 2006).

Since TaqMan probes are present in the incubation medium at both steps of the analysis (amplification and melting curve) the analysis can be carried out in the closed tube format. This is a 1.5–2-h assay in a single tube without any intermediate or additional procedures that minimizes not only time and labor expenditures, but also the probability of the cross contamination of samples, which is the most important, so The HRMA method is much more sensitive than Sanger sequencing (Krypuy et al., 2006). Our results agreed with Emelyanova et al., (2015) and Jones et al., (2017) who revealed that codon 12 AC mutation were in a higher frequency in patients followed by the codon 13 mutation.

3.6 Association between pathological characteristics and KRAS mutant genotype.

There was no significant association between grade of disease, stage of disease and LN status with mutant KRAS genotype, as shown in tables 3.7, 3.8 and 3.9. In addition, there was no significant correlation between mutant KRAS genotypes and both IL-17 and CD33, as shown in table 3.8.

Table 3.7: Association between grade of tumor and KRAS mutant genotype.

Grade	Total	Mutant	Mild	*P* *
I	4	2 (50.0 %)	2 (50.0 %)	1.000 NS
II	14	6 (42.9 %)	8 (57.1 %)	
III	22	8 (36.4 %)	14 (63.6 %)	

***: Fischer exact test (low grade I versus intermediate and high grade II and III); NS: not significant**

Table 3.8: Association between stage of tumor and KRAS mutant genotype.

Stage	Total	Mutant	Wild	*P* *
I	1	0 (0.0 %)	1 (100.0 %)	0.960 NS
II	7	4 (57.1 %)	3 (42.9 %)	
III	23	7 (30.4 %)	16 (69.9 %)	
IV	9	5 (55.6 %)	4 (44.4 %)	

***: Fischer exact test (Stages I and II versus stages III and IV); NS: not significant**

Table 3.9: Association between lymph node status and KRAS mutant genotype..

LN	Total	Mutant	Wild	*P* *
Positive	31	14 (45.2 %)	17 (54.8 %)	0.272 NS
Negative	9	2 (22.2 %)	7 (77.8 %)	

***: Fischer exact test; NS: not significant**

Tumorigenesis and tumor progression in CRC result from multiple genetic and epigenetic abnormalities. These epigenetic changes may affect the survival of CRC patients (Dobre M. et al., 2015). The results of correlation between KRAS mutations and pathological characteristics like stage and grade showed an agreement with many studies who had proved that there is no significant correlation between the stage and grade

characteristic and the KRAS mutation (Shaukat et al., 2012; Roa, Sánchez, Majlis, & Schalper, 2013; Emelyanova et al., 2015;). However it showed disagreement with (Yoon et al., 2014) and Jones et al., (2017)who reported that codon 13 mutations associated with outcome in stage III.

3.7 Median IL-17 level and CD33 expression in study group according to KRAS genotype

The results in table (3.10) showed insignificant correlation between the IL-17 and CD33 with the KRAS genotype mutant and wild type frequencies (p= 0.334 and p= 0.539, respectively).

Table 3.10: Median IL-17 level and CD33 expression in study group according to KRAS genotype.

Variable	Mutant	Wild	*P* *
IL -17A	10.77 (8.73)	12.85 (11.69)	0.334 NS
CD 33	73.5 (5.00)	73.00 (5.00)	0.539 NS

***: Mann Whitney U test; NS: not significant**

Mutations in the KRAS oncogene are often associated with distinct clinical and pathological characteristics. The most important one among such mutations is the G12A. in fact that the association mechanism between interleukins and K-ras remains unknown, activation of the RAS-ERK-MAPK pathway has been shown to trigger various immunological responses (Shaukat et al., 2012).

Our result disagree with (Petanidis, Anestakis, Argyraki, Hadzopoulou-Cladaras, & Salifoglou, 2013) who indicated that interleukin expression is influenced by KRAS signaling and specific interleukins play an oncogenic promoter role in colorectal cancer.

3.8 Correlation of IL-17 to tumor pathological characteristics

Present study showed that IL-17 was not correlated to grade and stage of disease; however it was negatively correlated to lymph node involvement in a significant way, as shown in table 3.11.

Table 3.11: Correlation of IL-17 to tumor pathological characteristics

Characteristic	IL 17A	
	r	***P*** *
Stage	-0.060	0.714 NS
Grade	-0.204	0.206 NS
LN	-0.353	0.026 S

***: Spearman correlation test; NS: not significant at $P \leq 0.05$; S: significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$**

IL17A secreted by TH17 cells, innate lymphoid cells exerts pro-inflammatory proprieties essential to the host protection against extracellular pathogens. On the other hand,it has recently been reported that IL17 producing cells may facilitate development of colorectal carcinoma by fostering angiogenesis via promoting VEGF production from cancer cells. This proposes IL17 as a novel indicator of prognosis in the patients with colorectal carcinoma and could serve as a novel therapeutic target for colorectal carcinoma (Inés etal.,2014).

. Lymph node (LN) informs prognosis and is a key factor in deciding further management, particularly adjuvant chemotherapy. As such, the lymph node has had a role in colorectal cancer staging from the earliest classification systems. Its importance in prognosis has been borne out by successive classification systems and is reflected in all contemporary staging systems, in particular the widely used tumor node metastasis

(TNM) staging system, developed and maintained by the Union for International Cancer Control and American Joint Committee on Cancer (AJCC)(Fang etal.,2014, Dienstmann etal.,2015).

3.9 Correlation of CD 33 to tumor pathological characteristics

CD 33 showed highly significant positive correlation to stage, grade and lymph node involvement which indicates that it correlated with poor prognosis. The correlation of CD33 expression with stage, Grade and lymph node of the colorectal cancer showed a significant positive correlation (0.001, 0.001 and 0.001, respectively).

In other word, a significantly higher levels of local CD33 in CRC patients compared with control and that level increased positively with the increment in stage, grade and L.N.

Table 3.12: Correlation of CD 33 to tumor pathological characteristics

Characteristic	CD 33	
	r	***P*** *
Stage	0.648	<0.001 HS
Grade	0.688	<0.001 HS
LN	0.741	<0.001 HS

***: Spearman correlation test; NS: not significant at $P \leq 0.05$; S: significant at $P \leq 0.05$; HS: highly significant at $P \leq 0.01$**

CD33 are commonly identified as the marker for the Myeloid-derived suppressor cells. And recent studies suggested that myeloid-derived suppressor cells (MDSCs) play important roles in cancer patients. MDSCs could inhibit anti-tumor immunity by suppressing T cell and NK cell functions (Hoechst et al., 2009). likely by increasing the production of arginine, reactive oxygen species (ROS), and nitric oxide (NO) and by

inducing Treg cells and TGF-β secretion to mediate T cell suppression (Zhang et al., 2013). Moreover, a role of CD33 in cancer patients is not well defined, and very few studies have reported their levels in peripheral blood.

present results are agreed with (Jewett et al., 2016) who found that CRC patients with high-grade, high-stage tumors had significantly higher levels of circulating CD33. And with (Zhang et al., 2013) who also found the increment in CD33% with CRC. This finding contrasted with the phenotypic characteristics of MDSCs observed in other cancers, such as breast cancer, indicating that MDSCs are heterogeneous in different cancers (Yu et al., 2013).

Conclusion
&
Recommendation

Conclusions:

1. On the basis of the current study, significant over productions of all investigated parameters (KRAS mutations, CD33, IL17A) seems to have potential role in colorectal cancer progression and worse prognosis when compared with healthy people.
2. This study have found that all the investigated parameters were associated with moderate to high grade carcinoma.
3. When this study correlate the investigated parameters, KRAS gene which found mutated in all stage 4 patient along with increased level of serum IL17A in such patients, CD33+MDSCs where found in a very high percentage in patient with poorly differentiated carcinoma which associated with increased level of IL17A in such grade.
4. This study may clarify that IL17A along with CD33+MDSCs could be a very important predictive marker for poorly differentiated adenocarcinoma and treatment responsiveness.
5. This study could not find a correlation between these three parameters with any stage of the tumor, it might be due to small simple size.

Recommendations

Recommendation:

1. Further study based on reasonable number of colorectal cancer patients sample needed to solidify the outcome of the current data, and get more information about distribution and expression pattern of CD33+MDSCs, serum concentration of IL17A, KRAS gene mutation status.

2. Further study is required to find a correlation between the investigated parameters with colorectal cancer staging, and in which stage is the highest expressions.

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وزارة التعليم العالي
والبحث العلمي
جامعه القادسية
كليه الطب

تقييم دور تعدد الاشكال الجينية ل KRAS ومستوى IL17 في مصل الدم والتعبير النسجي لCD33 في تحديد مآل المرضى المصابين بسرطان القولون

رسالة مقدمه الى
مجلس كلية الطب ـجامعة القادسية
كجزء من متطلبات نيل درجة الماجستير
في علم الاحياء المجهرية الطبية

من قبل

اسامة عبد علي نصر

بكالوريوس في تقنيات التحليلات المرضية/بغداد (٢٠١٣)

بأشراف

أ.م.د.أبراهيم عبد المجيد مصطفى التميمي

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الخلاصة

الخلاصة

سرطان القولون و المستقيم هو مرض خبيث ينشأ من الخلايا الطلائيه المبطنه للامعاء الغليظه. هذا النوع من السرطان من الانواع الاكثر شيوعا اللذي يصاب به الجهار الهضمي بنسبة ٩% لكلا الجنسين. في العراق، و حسب التسجيل السرطاني هذا المرض يتمثل بنسبة ٤,٨٩% بين كل انواع السرطان.

الدراسات الحديثه استنتجت من خلال دراسه البيئه الداخليه للسرطان انه يوجد عوامل اما تساعد على نشر المرض او تثبيطه و من هذه العوامل ، MDSCs +CD33 & IL17A. هذه العوامل من الممكن ان يكون لهذه العوامل دور اما في نشر المرض او توقفه بواسطه افراز مواد تأثر على نمو المرض.

الهدف من الدراسه الحاليه هو معرفه الفائده المستقبليه للجين KRAS في الورم المنتشر وغير المنتشر و ربط الجين مع MDSCs & IL17 A و معرفة مدا نسبه هذه العوامل و تأثيرها في درجة و مرحلة الورم.

نوع هذه الدراسه هو مقارنه بين المرضى المصابين و السليمين حيث تم جمع ٤٠ عينه من هولاء المرضى و تقطيعها الى 0.3 جزء من المليمتر و وضعها على شريحه زجاجيه خاصه وتم تصبيغها بالصبغه الفلوريسنت الخضراء و ايضا تم اخذ عينات دم من نفس المرضى للاخذ بنضر الاعتبار عمل الطرق المناعيه لقياس IL17A و الطرق الاحيائيه الجزيئيه لقياس نسبة KRAS gene.

حسب صبغة الفلوريسنت الخضراء، أنه نسبة CD33 كانت عاليه في المرضى المصابين بالمرض ذو المرحله المتقدمه (٠،٠٠١). IL17A ايضا كان بنسبه عاليه في جمع المرضى على عكس الغير المصابين حيث كانت نسبهم طبيعيه.

اما نسبة KRAS gene فكانت الطفره TGT الاعلى من بين المرضى المصابين على عكس الغير مصابين (٠،٠٠٢).

بالاستنتاج، النسبه العاليه للصبغه المناعيه CD33 و نسبة تركيز IL17A مع KRAS gene من الممكن ان تزيد من انتشار المرض و قوته و والتقليل من عمر المريض. تجب الاخذ بنضر الاعتبار ان هذه العوامل من المحتمل ان تكون معلمات مستقبليه تعطي انطباعا عن مدى تقبل المريض للعلاج الكيميائي.