Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

FARMACOLOGIA E ONCOLOGIA MOLECOLARE

CICLO XXVI

COORDINATORE Prof. Antonio Cuneo

Immunohistochemical Detection of DNA Mismatch Repair Proteins Abnormalities in Sudanese Colorectal Cancer Patients

Settore Scientifico Disciplinare BIO/14

Dottorando	**Tutore**
Dott. Yosef Mohamed Azzam Yosef Mohamed Zakout	Prof. Giovanni Lanza

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Dedication

I dedicate this work to my parents, teachers, and friends.

Acknowledgments

My thanks go to all who helped me to complete this modest effort.

I am especially grateful to my supervisor, ***Prof. Giovanni Lanza***, for his expert supervision, patience, and valuable comments.

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Abbreviations

5-FU	5-fluorouracil
BAT 25	Big adenine tract 25
BAT 26	Big adenine tract 26
CC1	Cell conditioning 1
CC2	Cell conditioning 2
CI	Confidence interval
CRC	Colorectal cancer
CRCs	Colorectal cancers
CT	Computed tomographic
dMMR	Deficient mismatch repair
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EXO I	Exconuclease I
FFPE	Formalin fixed, paraffin-embedded
FOBTs	Fecal occult blood tests
HG	High grade
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
IHC	Immunohistochemistry
LG	Low grade
LIGI	Ligase I
MgCl2	Magnesium chloride

MLH1	MutL homolog 1
MMR	Mismatch repair
MMR protein -	Mismatch repair protein negative
MMR protein +	Mismatch repair protein positive
MR	Magnetic resonance
MSH2	MutS protein homolog 2
MSH6	MutS protein homolog 6
MSI	Microsatellite instability
MSI-H	Microsatellite instability- high
MSI-L	Microsatellite instability- low
MSS	Microsatellite-stable
n	Number
NCI	National cancer institute
PCNA	Proliferating cellular nuclear antigen
PCR	Polymerase chain reaction
pMMR	Proficient Mismatch repair
PMS2	Postmeiotic segregation increased 2
Pol δ	Polymerase δ
RFC	Replication factor C
RPA	Replication protein A
SEPT9	Septin 9
µm	Micrometre

Abstract

Background: The current study aimed to assess DNA mismatch repair (MMR) proteins abnormalities among Sudanese colorectal cancer (CRC) patients, mainly by immunohistochemistry (IHC).

Methods: CRC cases were retrieved from the records of two Histopathology laboratories in Khartoum, Sudan. The total number of included cases was 42. Sections were cut and stained by immunohistochemical method to assess the abnormalities of four MMR proteins (MLH1, MSH2, MSH6 and PMS2) using anti-MLH1, MSH2, MSH6 (mouse monoclonal antibodies) and anti-PMS2 (rabbit monoclonal antibody). Microsatellite instability (MSI) analysis using mainly BAT 25 & 26 was performed for cases that showed negative or inadequate staining results for any MMR protein by IHC.

Results: Of the study population, 25 (59.5%) were males and 17 (40.4%) were females. Their ages ranged between 20-85 years (the age of 4 patients was not provided). The mean age was 56.1 year, and 12 (31.5%) of the CRC patients were among the age groups younger than 50 years.

Of the 42 included cases, 34 (80.95%) were MMR protein positive for all MMR proteins under assessment, 3 (7.14%) MSH2 inadequate, and 1 (2.38%) MSH6 inadequate.

Abnormal MMR proteins expression was found in 4 (9.5%) cases. Of these, 2 (50%) were MSH2&MSH6 negative and 2 (50%) were MLH1&PMS2 negative.

Regarding MSI results, the three cases that were MSH2 inadequate and positive for the rest by IHC showed stable results with both BAT 25& 26. The case that was MSH6 inadequate, showed stable results with both BAT 25&26.

The 2 cases with MSH2&MSH6 negative results were unstable with both BAT 25&26. Of the two cases that were MLH1&PMS2 negative, one case showed non-evaluable results with both BAT 25&26 while the other case was unstable with BAT 26 and not evaluable with BAT 25.

Conclusion: In this study, the percentage of MMR protein negative cases in Sudanese CRC patients appears to be relatively low compared to what has been generally reported in certain studies done in different countries. Furthermore, MLH1&PMS2 and MSH2&MSH6 abnormal expression detected by IHC seems to be the most common form of MMR proteins abnormalities in Sudanese CRC

patients. Concerning the results of IHC, MLH1 and MSH2 seem to be the most inactivated MMR genes in Sudanese CRC patients.

Riassunto

Obiettivo: Lo scopo di questo studio è di valutare, principalmente con l'immunoistochimica, le alterazioni della espressione delle proteine del DNA mismatch repair (MMR) sui pazienti sudanesi affetti dal cancro colorettale (CRC).

Metodi: I casi di CRC di questo studio provengono dagli archivi di due laboratori istopatologici a Khartoum in Sudan. Il numero totale dei casi è 42. Mediante colorazioni immunoistochimiche (IHC) è stata valutato lo status del sistema di riparazione del DNA, mediante lo studio della espressione di 4 proteine: MLH1, MSH2, MSH6 e PMS2. A questo scopo sono stati utilizzati anticorpi monoclonali di topo, anti- MLH1, anti-MSH2, anti-MSH6 e un anticorpo monoclonale di coniglio, anti-PMS2. Per i casi che hanno evidenziato, attraverso l'IHC, la perdita di espressione di almeno una delle proteine del MMR o che sono risultati inadeguati alle colorazioni, è stata valutata l'instabilità dei microsatelliti (MSI), utilizzando principalmente il BAT 25 e il BAT 26.

Risultati: Dei casi studiati, 25 (59,5%) erano maschi e 17 (40,4%) femmine, di età compresa tra i 20 e gli 85 anni (per 4 pazienti l`età non è stata indicata), con una età media di 56,1 anni. Inoltre, 12 (31,5%) pazienti con CRC hanno un'età inferiore ai 50 anni. Dei 42 casi studiati, 34 (80,95%) erano positivi per tutte le proteine del MMR analizzate , 3 (7,14 %) sono risultati inadeguati per l'analisi di MSH2 e 1 (2,38%) per MSH6. In 4 casi (9,5%) è stata rilevata la perdita di espressione di proteine del MMR, in particolare 2 casi (50%) sono risultati negativi per MSH2 ed MSH6 e 2 sono risultati negativi per MLH1 e PMS2. Per quanto riguarda i risultati dell'instabilità, i 3 casi risultati inadeguati con l'IHC per MSH2 ed il caso inadeguato per MSH6, sono risultati tutti stabili sia per il BAT 25 che per il BAT 26. I 2 campioni negativi per MSH2 ed MSH6 hanno mostrato instabilità sia per il BAT 25 che per il BAT 26; dei due casi MLH1 e PMS2 negativi, uno è risultato inadeguato per l'analisi di entrambi i BAT, mentre l'altro ha evidenziato instabilità per il BAT 26 mentre non è stato possibile valutarlo per il BAT 25.

Conclusioni: In questo studio, la percentuale dei casi negativi per le proteine del MMR dei pazienti sudanesi con CRC sembra essere relativamente bassa rispetto a quanto generalmente riportato in alcuni studi condotti in vari paesi. Inoltre, nella nostra casistica, le forme più frequenti di anomalia delle proteine del MMR sono

risultate due: la mancata espressione di MLH1 - PMS2 e quella di MSH2-MSH6. Considerando i risultati ottenuti con l'IHC, si può concludere che nei pazienti sudanesi con CRC, MLH1 ed MSH2 sembrano essere i geni del MMR più frequentemente inattivati.

List of contents

List of tables

List of figures

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1. Introduction and literature review

1.1. Epidemiology of CRC:

Worldwide, CRC was the third most common cancer in 2008 with 1.233 million newly diagnosed cases (9.7% of the total) and about 608.000 deaths, which makes it the fourth cause of cancer deaths (Ferlay et al., 2010a).

In United States, 142.82 new cases of CRC was estimated in 2013 makes it the third most common cancer with approximately 50.830 deaths (Siegel et al., 2013).

In Europe, CRC was the most common cancer in 2008 constituting 436,000 cases, 13.6% of the total cancers and the second cause of cancer death constituting 212,000 deaths, 12.3% of the total (Ferlay et al., 2010b).

In Sudan, CRC represents 6.4% of the total number of malignant tumors and the second most common malignant gastrointestinal tumor (33.69%) after esophageal cancer (37.7%) during the period from 2000 to 2004 according to a published data from one pathology center in Khartoum (El Hassan et al., 2008).

1.2. Clinical features of CRC:

Symptoms of CRC include alteration in bowel habit, bleeding from the rectal, abdominal pain, intestinal obstruction (Kyle et al., 1991), diarrhoea (MacArthur and Smith, 1984) and anaemia (Rizk and Ryan, 1994; Saidi et al., 2008).

One of the early symptoms include abdominal pain or/and vomiting, while the more localised symptoms of the rectal and weight loss are usually related with long delay (MacArthur and Smith, 1984).

1.3. Risk factors for CRC:

Several studies found that there are different risk factors associated with the development of CRC. Such as increasing age and family history (Ballinger and Anggiansah, 2007). The rate of CRC elevates throughout the fifth decade and reaches its most extreme at the age 75; however, there are various cases in more youthful individuals each year (Mihajlovic-Bozic, 2004).

Other risk factors were reported such as smoking and consumption of alcohol (Mihajlovic-Bozic, 2004; Sanjoaquin et al., 2004; Cappellani et al., 2013), notably the higher level of alcohol intake (Cho et al., 2004; Shimizu et al., 2003) and the daily alcohol consumption (Wu et al., 1987) .

Less consumption of high-fibers grains and vegetables increases the risk of fatal colon cancer (Thun et al., 1992).

Aspirin might decrease the risk of CRC in women (Giovannucci et al., 1995; Cook et al., 2013). Another finding connected the regular use of aspirin to a decreased risk in males for developing CRC (Giovannucci et al., 1994).

Other factors such as obesity and less physical activity measured also as risk factors (Giovannucci, 2003). Furthermore, dietary system that is rich in fat and red meat considered as factors increase the risk (Mihajlović-Božić, 2004).

1.4. Screening for CRC:

Screening for CRC is important for the early detection of the disease (O'Carroll et al., 2013). There are different tests used for CRC screening, including fecal occult blood tests (FOBTs), stool DNA tests, colonoscopy, flexible sigmoidscopy, magnetic resonance (MR), computed tomographic (CT) colonography, double contrast barium enema, capsule endoscopy (Flitcroft et al., 2012), and faecal pyruvate kinase isoenzyme type M2 (Tonus et al., 2012).

Furthermore, methylated septin 9 (SEPT9) was described as a sensitive biomarker for CRC from peripheral blood, and the detection of this biomarker in plasma was found as a dependable method of screening for left and right sided colon cancers (Tóth et al., 2012).

Regarding the relationship between screening with faecal-occult-blood test testing and mortality, several studies showed a lower mortality from CRC in the screening groups compared to control groups (Kronborg et al., 1996; Hardcastle et al., 1996; Shaukat et al., 2013).

In comparison to no screening, both incidence and mortality were found to be reduced, in variable percentages, in three different screening methods namely

colonoscopy, fecal immunochemical test and low-sensitivity guaiac fecal occult blood test (Barouni et al., 2012).

Furthermore, CRC cases in the control group are at a more advanced stage compared to CRC cases detected by screening (Kewenter et al., 1994).

1.5. DNA MMR system:

DNA MMR is a process that corrects mismatches that appear during DNA replication and getaway proofreading process (Kunkel and Erie, 2005). Therefore, this system removes any base-base mismatches as well as insertion-deletion loops occur during replication of DNA as a result of DNA polymerase slippage (Peltomäki, 2001).

Failure in MMR system will most likely affect functions and structure of the cell ensuing in tumorigenesis, immortalization, malignant transformation, and/or degenerative diseases (Conde-Pérezprina et al., 2012). MSI is the term that used to describe the form of genomic instability that related to DNA MMR defective in tumors (Boland et al., 1998).

1.5.1. MSI-High (MSI-H) and MSI-Low (MSI-L):

Five microsatellites markers were validated and recommended for detecting MSI CRC in 1997 in the National Cancer Institute (NCI) sponsored conference entitled "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition". This panel includes mononucleotide repeats: Big adenine tract (BAT) 25, BAT-26, and dinucleotide repeats: D5S346, D2S123, and D17S250 (Boland et al., 1998). This panel is known as the Bethesda panel (Vilar and Gruber, 2010).

Colorectal cancers (CRCs) are called MSI-H if there is instability in 2 or more of the five microsatellite markers, but those with instability in only one of the five markers are called MSI-L and share the phenotype of microsatellite-stable (MSS) tumors, while MSI-H tumors have distinctive pathological and clinical phenotype (Boland et al., 1998). However, expanded panel composed of 10 markers were evaluated (Mead et al., 2007).

In 2002, one more workshop was held at the NCI in Bethesda to revise and improve the Bethesda Guidelines. The participants in this workshop suggested

including more mononucleotide markers in order to increase the sensitivity of the panel (Umar et al., 2004).

One of the proposed panels is to use a pentaplex panel of 5 quasimonomorphic mononucleotide repeats, which includes BAT-25, BAT-26, NR-21, NR-22, and NR-24, and provides a precise assessment of tumor MSI status with 100% specificity and sensitivity and obviate the need to compare to normal DNA (Suraweera et al. 2002).

Bacher et al. (2004) described another method for MSI analysis. In their system, they use five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). They compared the results of MSI analysis in 153 CRCs using their new MSI Multiplex System to the results obtained using a panel composed of 10 MSI markers, and they found that there is 99% concordance between the two methods and the accuracy was almost 100% in detecting MSI-H cases.

Patil et al. (2012) examined the same previous panel (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) regarding the need of control DNA to detect MSI status. They found that this panel is able to accurately detect 95.2% of MSS CRC cases and all MSI-H CRC cases without using normal DNA.

Brennetot et al. (2005) found that detecting of MSI-H can be done by BAT-26 and BAT-25 analysis even if the DNA sample contains low tumor DNA (5-10%). Moreover, MSI can be detected using one marker, BAT 26, but this method can be used to screen CRCs for MMR deficiency but cannot differentiate between different degrees and types of instability (de la Chapelle, 1999).

Moreover, BAT-26 was used for MSI analysis in different studies (Chai et al., 2004; Jover et al., 2004, Samowitz et al., 2001). Nevertheless, Oh et al. (2012) found that using BAT-26 and BAT-25 for MSI analysis seems not to provide an accurate assessment of MSI status, mainly in MSI-L cases.

1.5.2. Hereditary nonpolyposis colorectal cancer (HNPCC) and MMR:

HNPCC (also known as Lynch syndrome) is caused by mutations in MMR genes; furthermore, a noteworthy percentage of sporadic cancer is connected with loss of MMR (Hsieh and Yamane, 2008).

In HNPCC, hMLH1 and hMSH2 are the most frequently affected genes. Deficient expression of these genes makes the cell at risk to the accumulation of several molecular defects, a circumstance that can be assessed by the instability in segments of base repeats in the genome identified as MSI (Silva et al., 2005).

1.5.3. MMR, MSI and CRC:

According to the data reported from different articles, MSI (notably the high levels) is present in almost 15% of CRC (Boland et al., 1998; Vilar and Gruber, 2010; Hamilton, 2013). This is due to germline mutation in one of the MMR genes (mainly MLH1, MSH2, MSH6 or PMS2) or MLH1 epigenetic silencing (Vilar and Gruber, 2010). Of this 15% CRCs with MSI, 12% are due to acquired hypermetylation of the MLH1 gene promoter while 3% are related to HNPCC (Boland and Goel, 2010).

Furthermore, MSI is present in over 90% of HNPCC patients in which MSI caused by defects in DNA MMR (Søreide, 2007).

1.5.3.1. Pathological and clinical features of MSI CRC:

CRCs with MMR-defective that causes MSI have characteristic pathological and clinical features as shown in different studies. They tend to be in the ascending colon (Jover et al., 2004), have a lower risk of recurrence (Yoon et al., 2011; Sinicrope et al., 2011), at stage that is less advanced, mucinous, poorly differentiated and have pattern of expansive growth more often compared to microsatellite stable (MSS) CRCs (Benatti et al., 2005).

Xiao et al. (2013) examined 1,941 CRC patients and found that poorly differentiated CRC (PD) was more frequent among MSI CRC cases compared to MSS CRC cases representing 23.6% and 4.2% respectively.

However, another study by Ashktorab et al. (2005) showed a different finding regarding the histologic tumor grade. In their study, MSI analysis was performed using 5 MSI markers (BAT25, BAT26, D2S123, D5S346 and D17S250) in 51 CRC cases from African Americans. Of those, 22 (43%) showed MSI-H, and most of them were well differentiated.

Moreover, CRCs with MSI-H demonstrate association with infiltration of a dense local lymphocyte, less incidence of metastasis to distant organ, and proximal localization of the tumor (Kloor et al., 2013). Additionally, MSI CRCs have a better

prognosis compared to MSS CRCs (Popat et al., 2005; Pino and Chung, 2011; Kloor et al., 2013).

Jensen et al. (2009) detected MSI in (13.8%) of the examined CRC cases. They found that MSI tumors were associated with lesser risk of death (hazard ratio (HR) = 0.4; 95% CI: 0.2–0.9; P = 0.02) and recurrence (HR = 0.3; 95% CI: 0.2–0.7; P = 0.0007) compared to MSS tumors in multivariate analysis.

Ward et al. (2001) studied MSI and the clinicopathological characteristics of sporadic CRC. They reported that 33 (10.6%) tumors out of 310 were MSI-H. Additionally, they found that these MSI-H cases were more likely to be mucinous, high grade, and to harbor elevated numbers of both intraepithelial and peritumoral lymphocytes. Furthermore, these MSI-H cases were more likely to occur among females and to be right sided.

Ziadi et al. (2013) detected 6 (13.8%) CRC tumors with MSI-H out of 44 CRC cases. These MSI-H cases were more likely to have medullary pattern, to be poorly differentiated, and to harbor elevated numbers of peritumoral lymphocytes. Regarding lymph node involvement, tumor location and stage of disease, they found no significant difference between MSI-H and MSI-L/MSS cases.

CRCs with MSI or dMMR thought to have no or poorer benefit from adjuvant 5-fluorouracil (5-FU) based chemotherapy compared to CRCs with MSS (Laghi and Malesci, 2012; Pino and Chung, 2011; Benatti et al., 2005). However, a study found that any advantage of 5-FU among dMMR stage III tumors is suggested to be limited to tumors with suspected germline mutations compared to sporadic CRCs (Sinicrope et al., 2011).

1.5.4. IHC for detection of MMR status:

IHC is a technique where monoclonal and polyclonal antibodies are used to identify certain components in cells or tissues (antigens), and the antibody binding site is being recognized by labelling of the primary antibody or a suitable secondary antibody with a marker such as enzyme, colloidal gold, radioactive elements, fluorescent dye, and then being seen under either ordinary or fluorescent microscope (Duraiyan et al., 2012).

CRCs with mutations in DNA MMR gene show elevated rate of replication errors in simple repetitive sequences demonstrable as MSI, in which the majority are due to somatic MMR dysfunction in addition to a subset occur due to germline mutations (Stone et al., 2001). The availability of antibodies to MMR proteins has offered an alternative method to molecular techniques for recognizing dMMR CRCs (Stone et al., 2001). IHC can be a specific, sensitive, fast, and cost-effective method for identifying MMR defects (Lindor et al., 2002).

Additionally, the other important advantage of IHC is that it could direct and guide genetic analysis by detecting the affected gene based on the absence of protein expression in IHC (Zhang and Li, 2013; Rigau et al., 2003).

Lindor et al. (2002) compared between IHC and MSI analysis regarding the phenotyping of CRC. In their study, 1,144 CRC cases were examined for deficiency of DNA MMR using MSI analysis and immunohistochemical detection of MSH2 and MLH1 proteins. MSI-H was detected in 350 (30.6%) by MSI analysis. Of these MSI-H cases, 323 showed loss of expression by IHC of either MLH1 or MSH2 (228 and 98 respectively). They concluded that IHC for MLH1 and MSH2 has sensitivity of 92.3% and specificity of 100% for dMMR screening. Furthermore, they found that the normal expression of MMR proteins by IHC has 96.7% predictive value for MSS/MSI-L, and it reaches 100% in regard abnormal IHC for MSI-H.

Jover et al. (2004) reported that IHC appears to provide a reliable technique to detect most dMMR CRCs. They performed microsatellite analysis in 172 cases of CRCs by polymerase chain reaction (PCR) using BAT-26, and IHC for MLH1 and MSH2. MSI was assessed in 13 (7.6%) cases and all these cases showed negative staining by IHC for MLH1 or MSH2.

Marcus et al. (1999) compared between the results of MSI analysis and IHC for MLH1 and MSH2 proteins in 72 CRC tumors. Of those cases, 38 were MSI-H and 34 were MSS. All MSS cases showed intact IHC staining with MLH1 and MSH2, while loss of expression of MLH1 and/or MSH2 was detected in 37 of 38 MSI-H tumors. They concluded that IHC can differentiate accurately between MSS and MSI-H tumors.

Cunningham et al. (2001) examined MMR status among 257 CRC patients by MSI testing and IHC for MSH2, MSH6, and MLH1. Defective MMR was detected in 51 (20%) cases, which demonstrated MSI-H. All these MSI-H cases showed loss of expression for one or more of the assessed MMR proteins.

Stone et al. (2001) performed MSI analysis and IHC for MMR proteins (MSH2 and MLH1) using monoclonal antibodies in 46 CRC cases. MSI was detected in 23 cases. Of these 23 cases, 22 showed loss of expression of one of the assessed MMR proteins. MSS was detected in 23 CRC cases and all of them showed positive staining with both antibodies

Ruszkiewicz et al. (2002) found that IHC provides an alternative relatively inexpensive and fast technique to assess the status of MSI compared to MSI analysis. Furthermore, they found that the sensitivity and specificity of IHC are 92% and 99.8% respectively when assessed against MSI analysis. However, they reported that it has to be accepted that using IHC technique only; would miss a small fraction of MSI cases.

The validity of IHC regarding MMR proteins in CRC was examined by Overbeek et al. (2008). In their study, 100 molecularly assessed CRC cancer cases were stained for 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2) and then were examined by 7 pathologists from 5 different laboratories. Of those pathologists, 2 have experience in Interpretation of IHC results for DNA MMR proteins. The authors found that IHC is a valid method to recognize sporadic MSI CRC patients as well as those patients at risk for HNPCC when the IHC stained sections are assessed by experienced pathologists. Furthermore, they reported that MSI test is to be performed for those cases with aberrant or indefinite staining results by IHC in order to verify the presence of defective MMR.

Boardman et al. (2007) studied the frequency of defective MMR among Alaska native patients with CRC by IHC (MLH1, MSH2, and hMSH6), and MSI analysis. In their study, 329 CRC cases were studied. Of these cases, MSI and loss of expression of MMR proteins were detected among 46 (14%). Of these 46 cases, loss of MLH1, MSH2, and MLH1/MSH2 was detected in 42 (91%), 3 (7%), and 1 (2%) respectively.

Shia et al. (2009) reported that using a two-antibody panel composed of MSH6 and PMS2 is as effective as the four-antibody panel (MLH1, MSH2, MSH6 and PMS2) in detecting the abnormalities of DNA MMR protein.

Additionally, Hall et al. (2010) found that using a two-antibody panel composed of PMS2 and MSH6 has 100% sensitivity and specificity compared to the four-antibody panel test (MLH1, MSH2, MSH6 and PMS2), with absence of false positives or negatives. They concluded that the four-antibody panel should be replaced by the two-antibody panel for screening for MMR deficiency by IHC.

Furthermore, the usefulness of using the 2 antibodies (MSH6 and PMS2) in CRC was confirmed by Mojtahed et al. (2011).

Moreover, O'Regan et al. (2013) achieved similar findings in their study. They suggested that the initial use of two antibodies (MSH6 & PMS2) followed by the other two antibodies (MLH1& MSH2) ,if there is loss of expression in the initially used antibodies, could have the same efficiency of using the four antibodies (MLH1, MSH2, MSH6 and PMS2) to detect loss of expression of MMR gene protein.

Regarding HNPCC, Caldés et al. (2004) studied the sensitivity of MSI analysis and IHC for MSH2, MSH6, and MLH1 among tumors obtained from carriers of known MMR gene mutation. They studied the germline mutations in MSH2, MSH6, and MLH1 in 58 samples from HNPCC families. Of them, 28 were found with a real mutation and all of them showed loss of expression by IHC for at least one of the examined MMR proteins. MSI-H was found in 27 of the 28 cases with a real mutation. Sensitivity by IHC was 100% compared to 96% by MSI analysis for detection of MMR deficiency among carriers of a real pathogenic mutation in MMR. Furthermore, they found that IHC can be used to predict the expected gene to harbor the mutation for MSH2, MSH6, and MLH1.

Hendriks et al. (2003) found that the sensitivity of IHC in identifying MMR deficiency among carriers of a pathogenic mutation in MMR is 89% compared to 93% by MSI analysis. Furthermore, they found that IHC have rightly forecasted the MSH2, MSH6 and MLH1 mutation in 92%, 75% and 48% of the cases in that order.

Another study by Shia et al. (2005) found that IHC has 79% sensitivity and 89% specificity regarding the prediction of the germline mutation, compared to 97% sensitivity and 83% specificity for MSI analysis. Moreover, they found that the low sensitivity of IHC is particularly due to the low sensitivity of this method concerning the detection of MLH1 gene mutation.

However, Mangold et al. (2005) mentioned that MLH1 mutation is indicated by weak positive staining of MLH1. This observation increases the ability of IHC in predicting MLH1 mutation carriers. In this concept, they found negative staining of MLH1 by IHC in 66% of carriers of MLH1 mutation. This percentage of predicting MLH1 mutation elevated to 98% when the pathologist in the study took into account the weak staining of MLH1 as an indicator of MLH1 mutation. Furthermore, all tumors (100%) with MSH2 mutation showed negative MSH2 staining.

1.5.4.1. Interpretation of IHC results for DNA MMR proteins:

In IHC, MLH1, MSH2, MSH6 and PMS2 protein antibodies give an idea about the MMR system functionality (Vilar and Gruber, 2010).

Loss of expression of any of these proteins indicates the presence of dMMR and detects the gene that is most probably to harbor a germline mutation or inactivated due to another cause (Vilar and Gruber, 2010).

Positive staining by IHC for MMR protein defined as the presence of nuclear staining in any malignant cells, while the adjacent normal epithelium of the colon and lymphocytes serve as internal controls (Watson et al., 2007).

The sections considered negative for MMR protein when all malignant cells show complete loss of staining for the examined MMR protein while there is a nuclear staining in the adjacent normal cells (Watson et al., 2007).

Some examples of positive and negative IHC staining for MMR proteins are shown in **Figures 2&3.**

Mutation of MSH2 often result in simultaneous loss of MSH2/MSH6 by IHC, while mutation of MLH1 often result in simultaneous loss of MLH1/PMS2, but mutation of MSH6 or PMS2 often result in loss of MSH6 or PMS2 only (Shia, 2008).

The Interpretation of IHC results for MMR proteins (MLH1, MSH2, MSH6, and PMS2), the inactivated gene, and their association to MSI status are as follows (Vilar and Gruber, 2010):

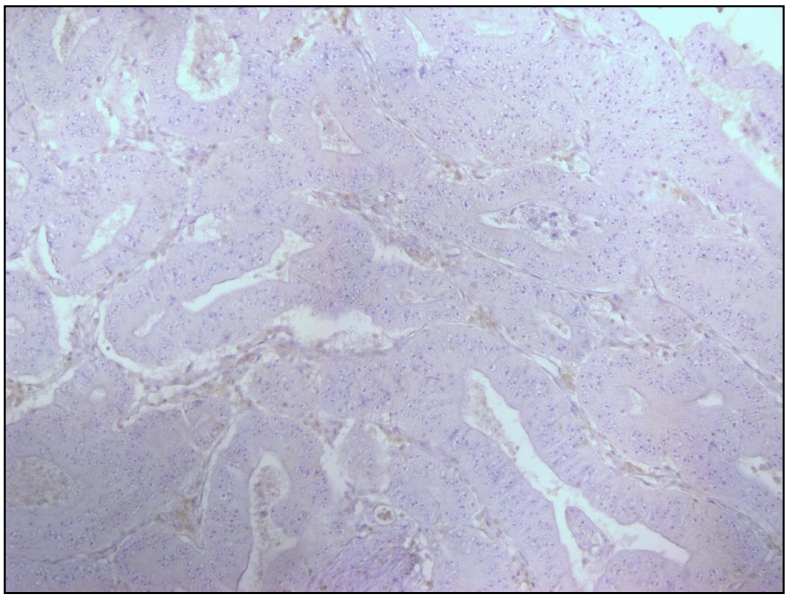
A- MLH1, MSH2, MSH6 and PMS2 positive:
- Interpretation: Intact or proficient MMR (pMMR).
- Inactivated gene: none.
- MSI status: MSS.

B- MSH2&MSH6 negative and MLH1&PMS2 positive:
- Interpretation: dMMR.
- Inactivated gene: MSH2.
- MSI status: MSI.

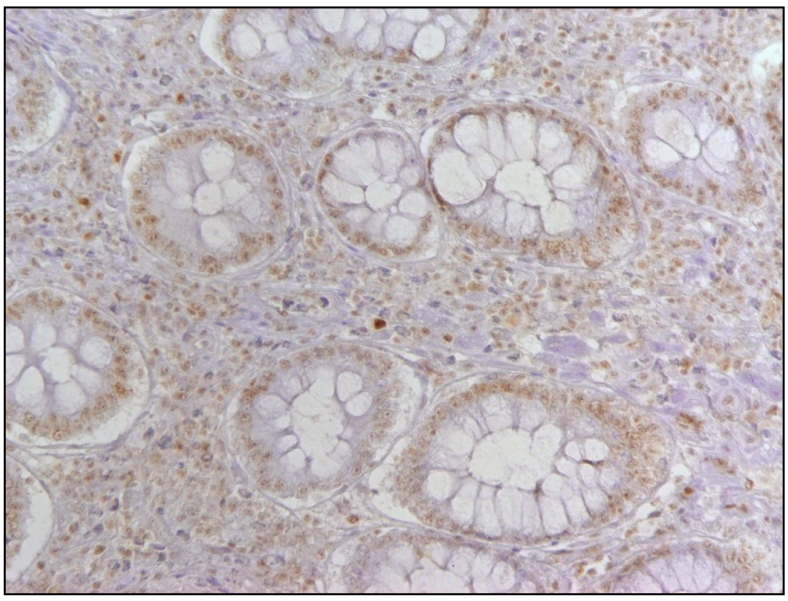
C- MLH1&PMS2 negative and MSH2&MSH6 positive:
- Interpretation: dMMR.
- Inactivated gene: MLH1.
- MSI status: MSI.

D- PMS2 negative and the others are positive:
- Interpretation: dMMR.
- Inactivated gene: PMS2.
- MSI status: MSI.

E- MSH6 negative and the others are positive:
- Interpretation: dMMR.
- Inactivated gene: MSH6.
- MSI status: MSI or MSS (Vilar and Gruber, 2010).



(a)



(b)

Figure 1: Immunohistochemical staining results of PMS2. **(a)** PMS2 negative by IHC in malignant cells, shown at 100x. **(b)** Positive in normal cells in the same case, shown at 200x. (Source: from the current study).

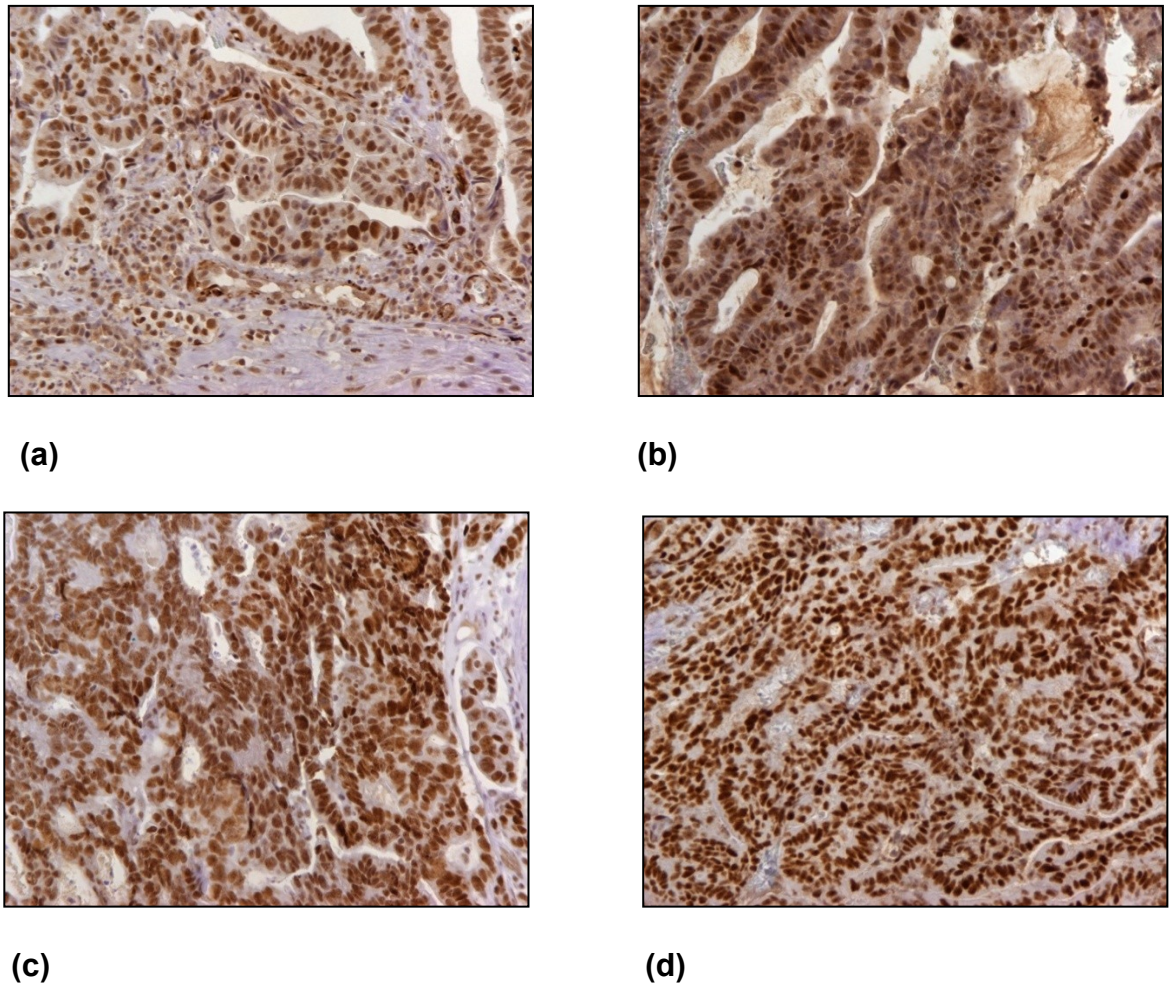


Figure 2: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 in cancer cells (shown at 200x). **(a)** MSH2 positive, **(b)** MSH6 positive, **(c)** MLH1 positive and **(d)** PMS2 positive. (Source: from the current study).

CAHPTER TWO

OBJECTIVES

2. Objectives

The aims of this study were to assess the frequency of DNA MMR proteins abnormalities among Sudanese CRC patients mainly by detection of four MMR proteins expression (MLH1, MSH2, MSH6, and PMS2) by immunohistochemical method, and to determine the type of abnormal MMR proteins expression among Sudanese CRC patients.

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and methods

3.1. Study samples:

In this study, CRC cases were retrieved from the records of two Histopathology laboratories in Khartoum, Sudan. Paraffin sections were cut from paraffin wax embedded tissue blocks. The total number of included cases was 42.

3.2. Laboratory procedures:

3.2.1. Cutting:

Special slides for IHC (SuperFrost® plus, DIAPATH), as well as ordinary frosted end slides were used. Using a rotary microtome, 3 & 10 µm sections were cut.

3.2.2. Immunohistochemical analysis:

The sections were examined for MMR protein expression of MLH1, MSH2, MSH6 and PMS2 using anti-MLH1, MSH2, MSH6 (mouse monoclonal antibodies-CELL MARQUE, USA) and anti-PMS2 (rabbit monoclonal antibody-CELL MARQUE, USA). IHC was performed using a fully automated slide preparation system (BenchMark XT, Ventana, USA), and the staining of 2 sections was repeated again using another fully automated slide preparation system (BenchMark ULTRA, Ventana, USA).

Positive staining defined as the presence of nuclear staining in any percentage of malignant cells, while nuclear staining in adjacent normal cells serve as internal positive control. Negative staining defined as the complete absence of nuclear staining in malignant cells while normal cells show positive nuclear staining. MMR protein positive cases also indicated that the cases are proficient MMR (pMMR), and MMR protein negative cases indicated that the cases are dMMR (Vilar and Gruber, 2010).

Immunohistochemical staining in some sections was inadequate to provide a good evaluation. In such instances, immunohistochemical analysis was repeated in another section, when available, from the same case. The repetition was performed using a prolonged time of antigen retrieval, and 2 inadequately stained sections were repeated for MSH2 and MSH6 using the two fully automated slide preparation systems. Sections were treated in an oven, at 60-65^0 C - up to 30

minutes approximately, before repeating the Immunohistochemical analysis with the antigen retrieval modification several. In BenchMark XT, the time of treatment in cell conditioning 1 (CC1) for antigen retrieval was increased to 90 minutes for MSH2, MLH1, and MSH6. For PMS2, the time in cell conditioning 2 (CC2) for antigen retrieval was increased to 84 minutes. In BenchMark ULTRA, treatment in CC1 was done for 92 minutes for both MSH2 and MSH6. After repetition, a number of sections were evaluable. However, the immunohistochemical staining remained inadequate to allow a good evaluation in other sections. Therefore, MSI analysis was performed for these cases, which remained inadequate, as well as for those cases that showed negative staining results for any MMR protein.

3.2.3. Extraction of DNA:

The areas with tumor cells were labelled. Then, DNA was extracted from formalin-fixed, paraffin-embedded tissues from unstained sections, hematoxylin & eosin stained sections or immunohistochemical-stained sections of different thicknesses and number, vary from 3-10 µm of 1-3 sections. The extraction was done using the kit “QIAamp® DNA FFPE Tissue” (Qiagen®) according to the manufacturer instructions with some minor modifications.

3.2.4. MSI analysis:

BAT26 and BAT25 microsatellite markers were used mainly. Polymerase chain reaction (PCR) was done using the extracted DNA in a final volume of 6 µl, 1X Buffer, dNTPs (250 µM each), primers (0.1 µM each), MgCl2 (3.75 mM) and 1 unit of Taq polymerase (Roche). For PCR reaction, the first step was denaturation at 95 ° C for 5 minutes before the addition of the polymerase, then 35 cycles (30 seconds at 95 ° C, 30 seconds at the annealing temperature and 30 seconds at 72 ° C), and 10-minute final extension at 72 ° C.

The PCR products were marked with fluorochrome. In which, the primer of BAT 25 was labelled with fluorochrome NED, while the primer of BAT 26 was labelled with FAM. Each sample was added to formamide and standard (ROX 500), and then transferred to capillary automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The analysis was conducted using the program GenMapper V4.0 for data processing. Electropherograms can recognize MSI CRC by the presence of new shorter peaks due to the shortening of the adenine repeats in cancer cells (Vilar

and Gruber 2010). In some instances, the electropherograms was not definite to provide a clear MSI results for one or more of the used MSI markers. Such results were considered non-evaluable.

CHAPTER FOUR

RESULTS

4. Results

In this study, we aimed to assess the MMR proteins abnormalities in Sudanese CRC patients. MMR proteins were assessed by IHC in 42 Sudanese CRC patients.

Table 1 & Figure 3 show the distribution of the study population by gender, 25 (59.5%) were males, and 17 (40.4%) were females. Male to female ratio was 1.47:1. The ages of the study population were between 20-85 years (the age of 4 patients was not provided), and the mean age was 56.1 year. The majority of the study population were among age groups 50-59 and 60-69 years representing 7 (18.4%) for each, followed by 70-79 and 80-89 constituting 6 (15.7%) for each, followed by age groups 20-29, 30-39, and 40-49 representing 4 (10.5%) for each as shown in **Table 2 & Figure 4.** Furthermore, the ages of 12 (31.5%) of the CRC patients were less than 50 years.

Regarding the grade of differentiation (assessed in sections stained by either hematoxylin & eosin or IHC and obtained from one block of each case), 11 (26.1%) of the cases were poorly differentiated and classified as high grade (HG), comparing to 31 (73.8%) well/moderately differentiated classified as low grade (LG) as shown in **Table 3 & Figure 5.**

Table 4 & Figures (for selected cases) 6, 8-16 show the MMR proteins expression by IHC, and MSI status. Of the 42 included cases, 34 (80.95%) were MMR protein positive for MLH1, PMS2, MSH2 and MSH6, 4 (9.5%) MMR protein negative (2 (4.76%) MLH1&PMS2 negative and 2 (4.76%) MSH2&MSH6 negative), 3 (7.14%) MSH2 inadequate and positive for the rest, and 1 (2.38%) MSH6 inadequate and positive for the others.

Regarding MSI results, the three cases that were MSH2 Inadequate and positive for the rest by IHC showed stable results with both BAT 25 & 26. The two cases that showed MSH2&MSH6 negative results were unstable with both BAT 25 & 26. Of the two cases that were MLH1&PMS2 negative, one of them showed non-evaluable results with BAT 25 & 26, while the other case was unstable with BAT 26 and not evaluable with BAT 25. The case that was MSH6 Inadequate and positive for the others showed stable results with both BAT 25 & 26.

Immunohistohhemical staining results in some sections were inadequate for allowing a good evaluation. In such instances, the immunohistochemical analysis was repeated in another section using a prolonged time of antigen retrieval. For

one case, there was no additional section to be re-stained. After repetition, a number of sections were evaluable as the example shown in **Figure 17**. However, the immunohistochemical staining results remained inadequate to provide a good evaluation in other sections.

Concerning the MMR status based on the immunohistochemical results, MMR protein negative cases (which considered dMMR) were detected among 4 (9.5%) of the included cases as shown in **Table 5 & Figure 7**.

Immunohistochemical patterns of these four dMMR cases were 2 (50%) MLH1/PMS2 negative, and 2 (50%) MSH2/MSH6 negative, as shown in **Table 6.**

All MMR protein negative cases were among males. In which 4 (16%) of the males were MMR protein negative while no MMR protein negative cases were detected among females as shown in **Table 7.**

All MMR protein negative cases were LG, which refer to well/moderately differentiated CRC, as shown in **Table 8.**

Table 1: Description of study population by gender.

Gender	*n*	*%*
Male	25	59.5
Female	17	40.4
Total	42	100*

*The total percentage was rounded to the nearest tenth.

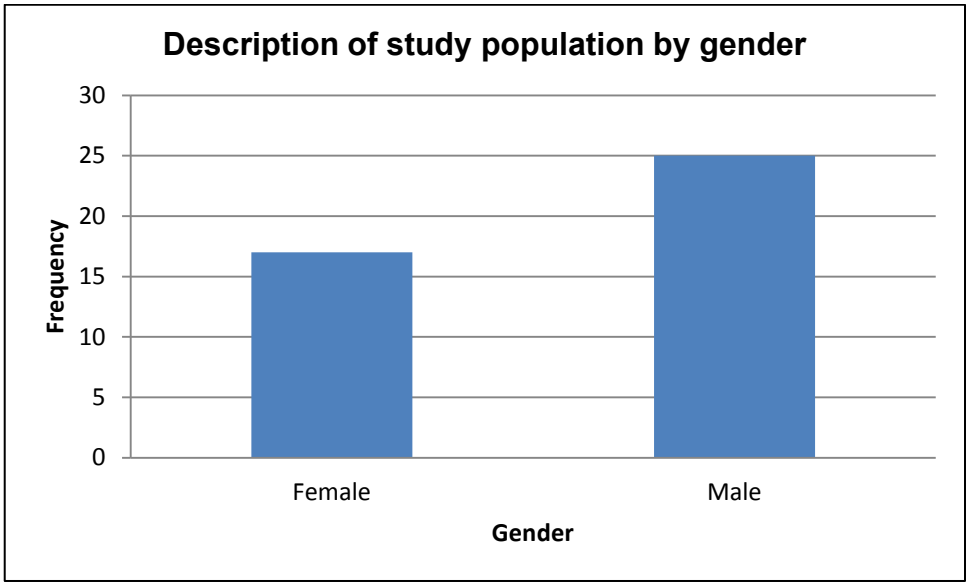


Figure 3: Description of study population by gender.

Table 2: Description of study population by age.

Age (Years)	Male	Female	Total	%
20-29	2	2	4	10.5
30-39	2	2	4	10.5
40-49	2	2	4	10.5
50-59	5	2	7	18.4
60-69	5	2	7	18.4
70-79	4	2	6	15.7
80-89	3	3	6	15.7
Total	23	15	38*	100**

* The age of 4 patients was not provided.

** The total percentage was rounded to the nearest tenth.

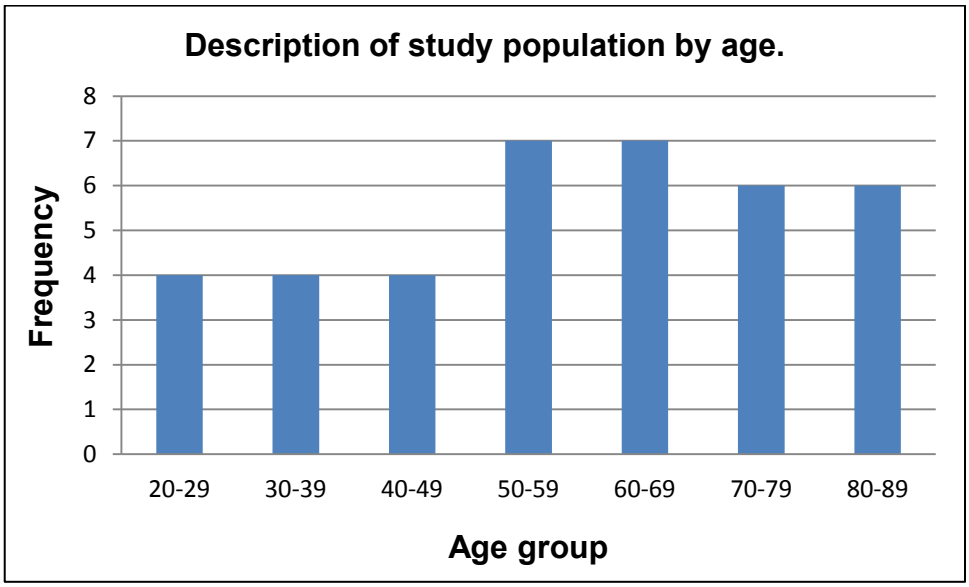


Figure 4: Description of study population by age.

Table 3: Description of grade of differentiation.

Grade of differentiation	*n*	*%*
Poorly differentiated (HG)	11	26.1
Well/Moderately differentiated (LG)	31	73.8
Total	42	100*

*The total percent was rounded to the nearest tenth.

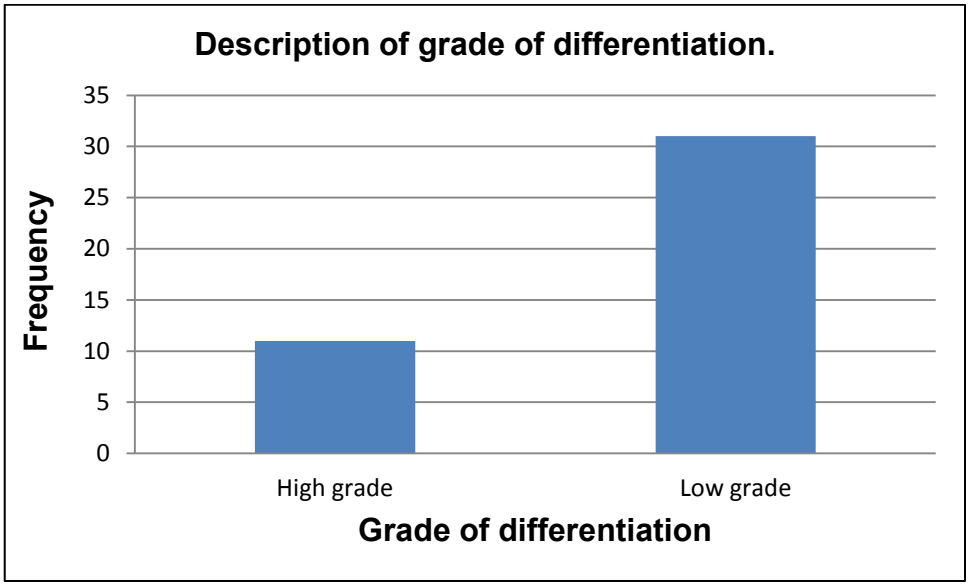


Figure 5: Description of grade of differentiation.

Table 4: MMR proteins expression by IHC, and MSI status.

Expression of MMR protein	*n*	%	MSI analysis	
			BAT 25	BAT 26
MLH1&PMS2 negative (MMR protein - cases)	2	4.76	1- Not evaluable 2- Not evaluable	Unstable Not evaluable
MSH2&MSH6 negative (MMR protein - cases)	2	4.76	Unstable	Unstable
All positive (MMR protein + cases)	34	80.95	-	-
MSH2 Inadequate / MSH6,MLH1, PMS2 positive	3	7.14	Stable	Stable
MSH6 Inadequate/ MSH2, MLH1, PMS2 positive	1	2.38	Stable	Stable
Total	42	100*	-	

*The total percentage was rounded to the nearest tenth.

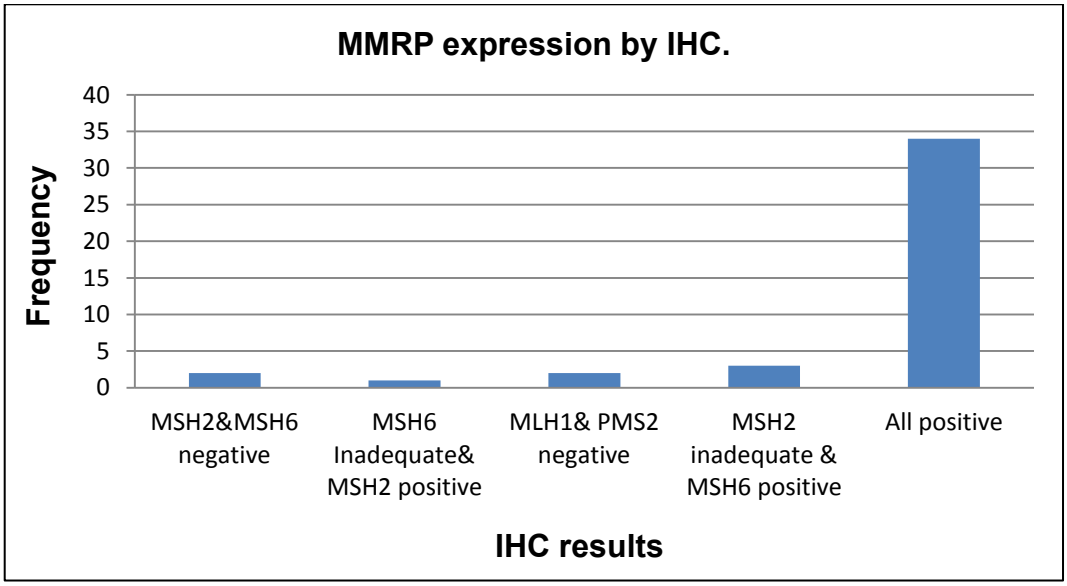


Figure 6: MMR proteins expression by IHC.

Table 5: Description of study population by MMR status.

MMR status	*n*	*%*
pMMR / Insufficient evidences of dMMR	38	90.4
dMMR	4	9.5
Total	42	100*

*The total percentage was rounded to the nearest tenth.

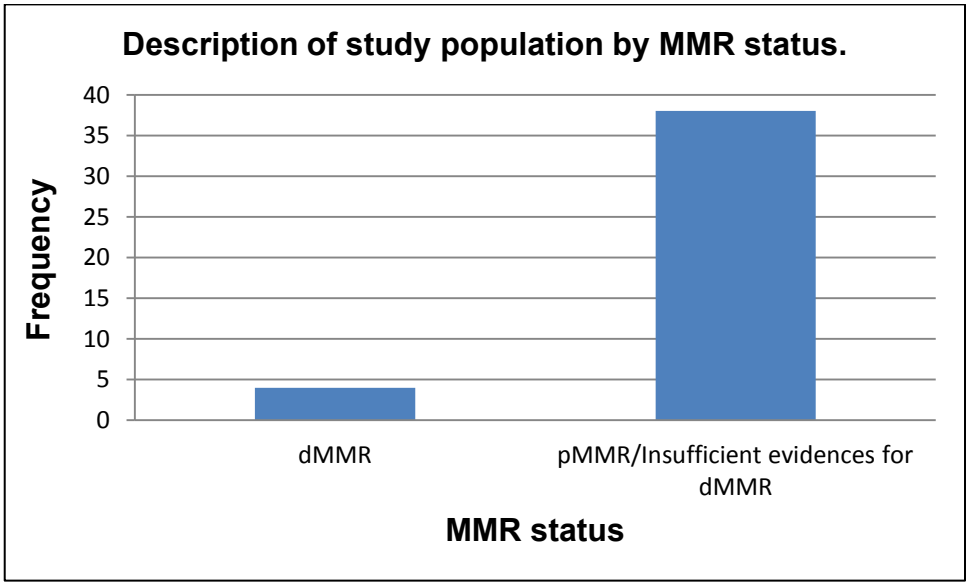


Figure 7: Description of study population by MMR status.

Table 6: Immunohistochemical patterns of dMMR CRCs.

Immunohistochemical patterns of dMMR cases	*n*	*%*
MLH1/PMS2 negative	2	50
MSH2/MSH6 negative	2	50
Total	4	100

Table 7: Description of study population by MMR protein expression and gender.

MMR protein status	Male		Female		Total
	n	*%*	*n*	*%*	
MMR protein -	4	16	0	0	4
MMR protein + /Insufficient evidences of MMR protein -	21	84	17	100	38
Total	25	100	17	100	42

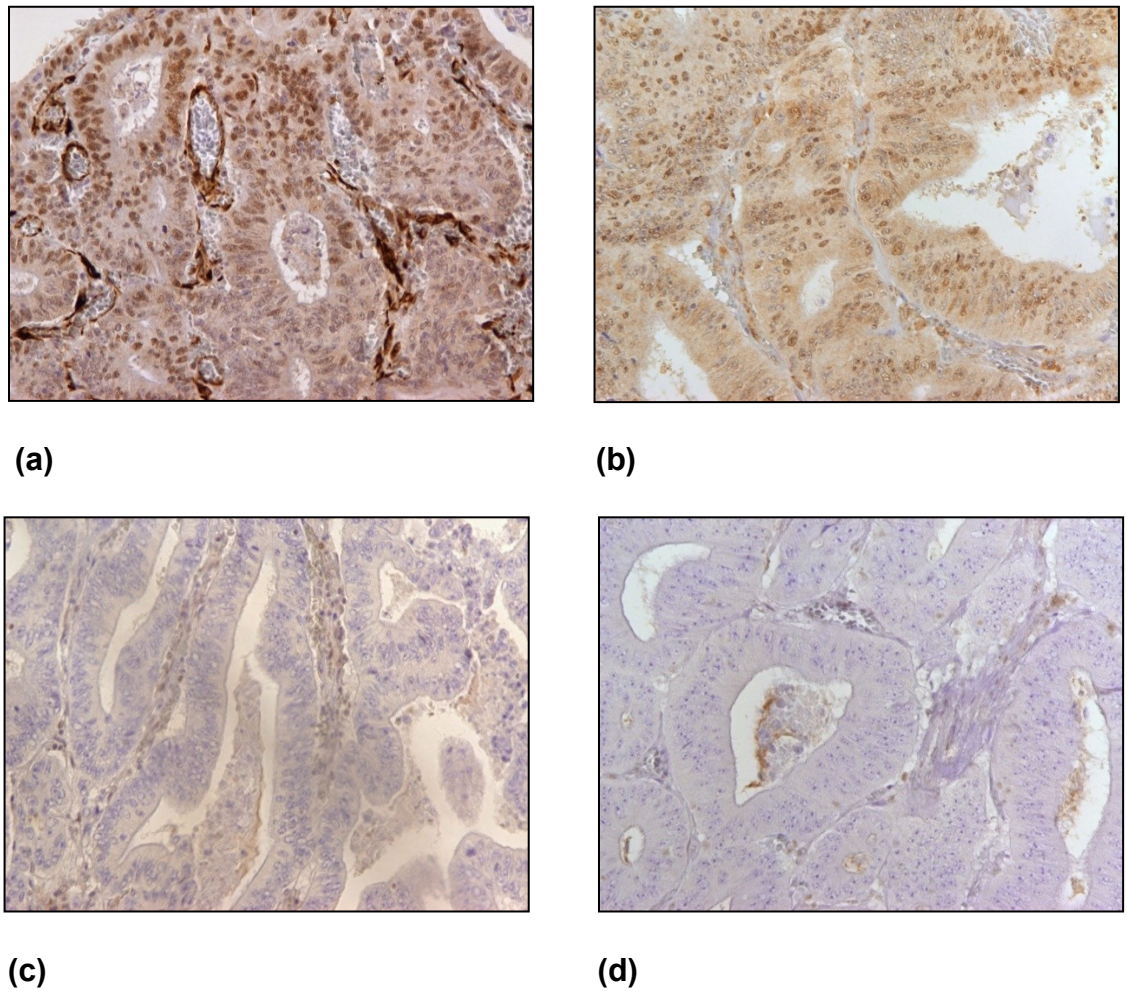
Table 8: Description of study population by MMR protein expression and grade of differentiation.

Grade of diffrentiation	MMR protein -		MMR protein + /Insufficient evidences of MMR protein -	
	n	*%*	*n*	*%*
Poorly differentiated (HG)	0	0	11	29*
Well/Moderately differentiated (LG)	4	100	27	71
Total	4	100	38	100

***The value was rounded to the nearest tenth.

CASE 1 (IHC)

MLH1/PMS2 negative



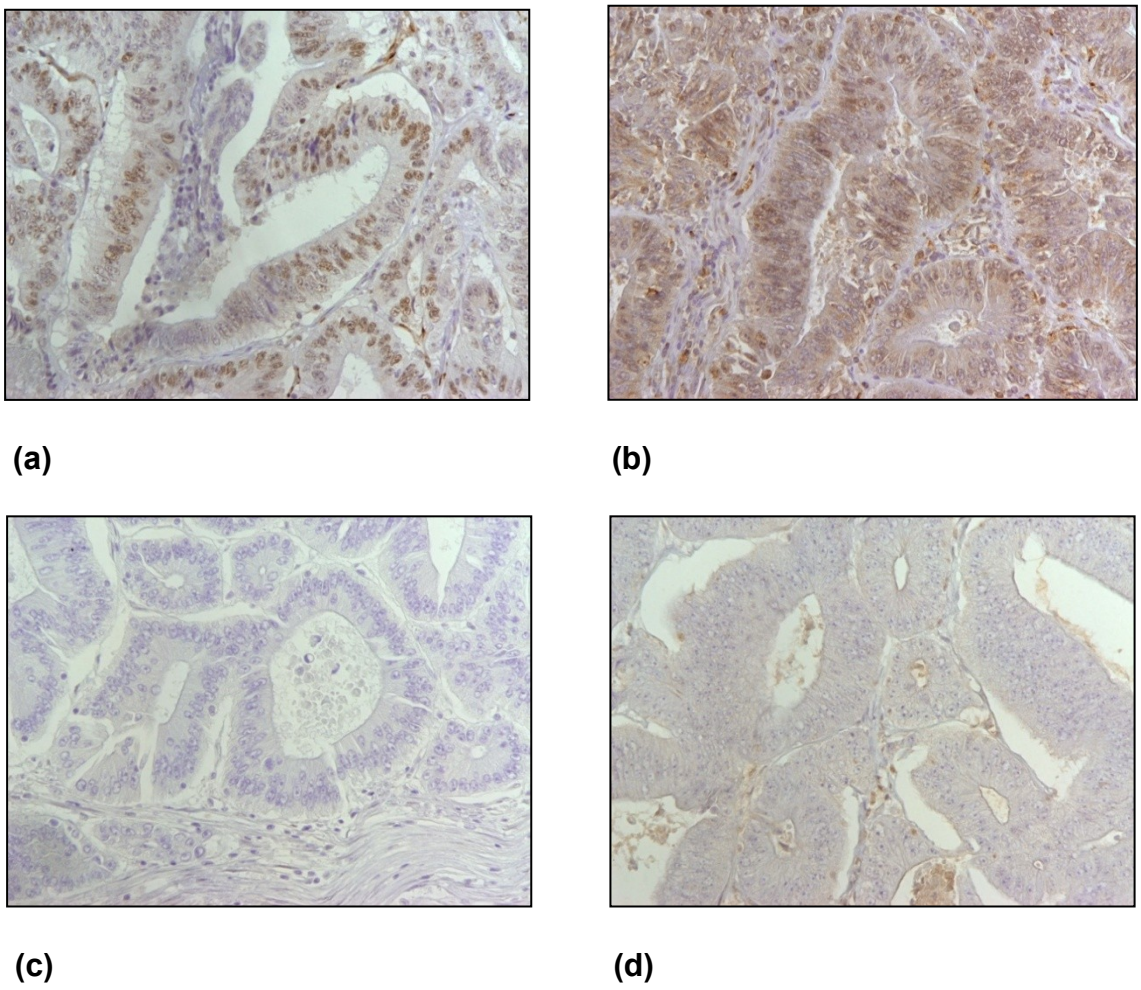
(a) **(b)**

(c) **(d)**

Figure 8: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 positive, **(b)** MSH6 positive, **(c)** MLH1 negative, and **(d)** PMS2 negative.

CASE 2 (IHC)

MLH1/PMS2 negative



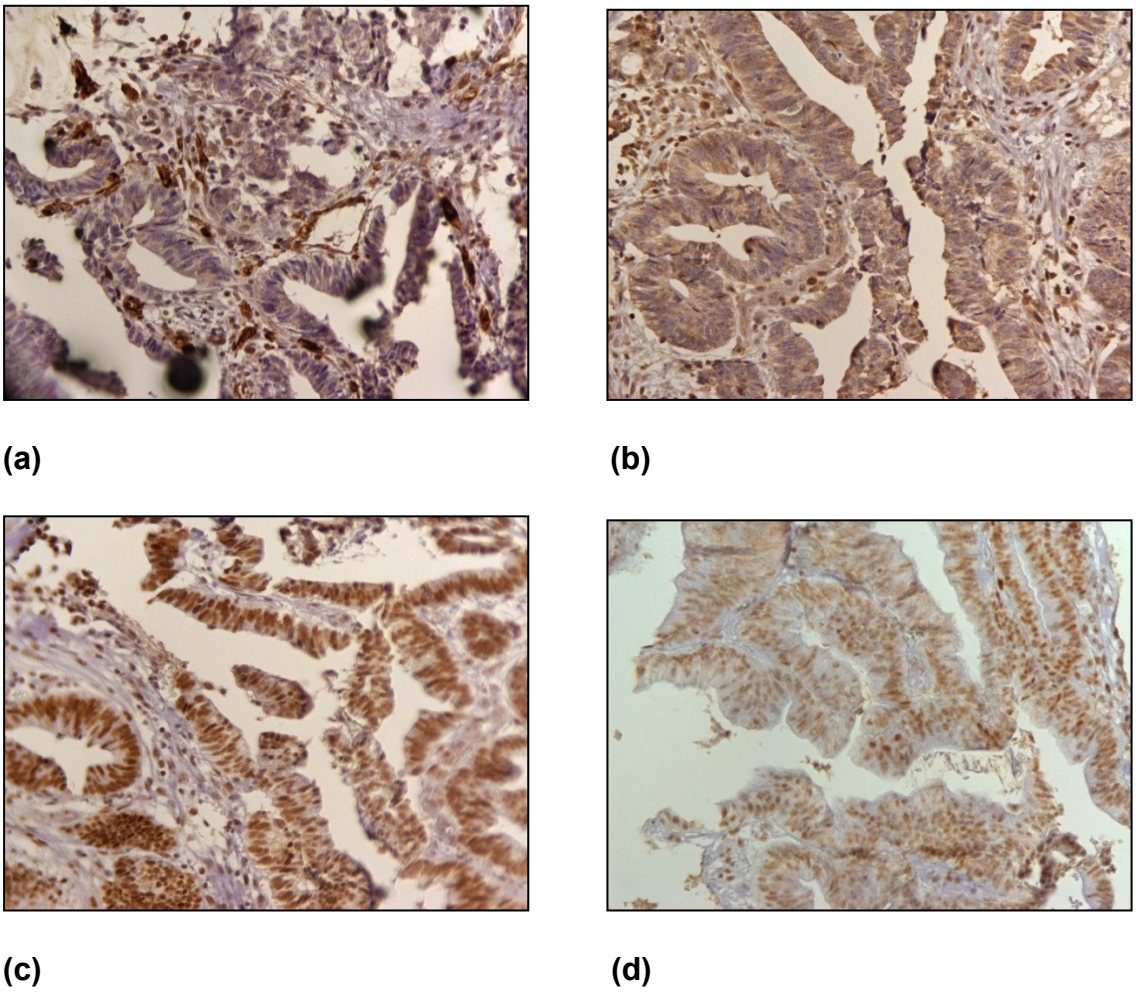
(a) **(b)**

(c) **(d)**

Figure 9: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 positive, **(b)** MSH6 positive, **(c)** MLH1 negative, and **(d)** PMS2 negative.

CASE 3 (1- IHC)

MSH2/MSH62 negative

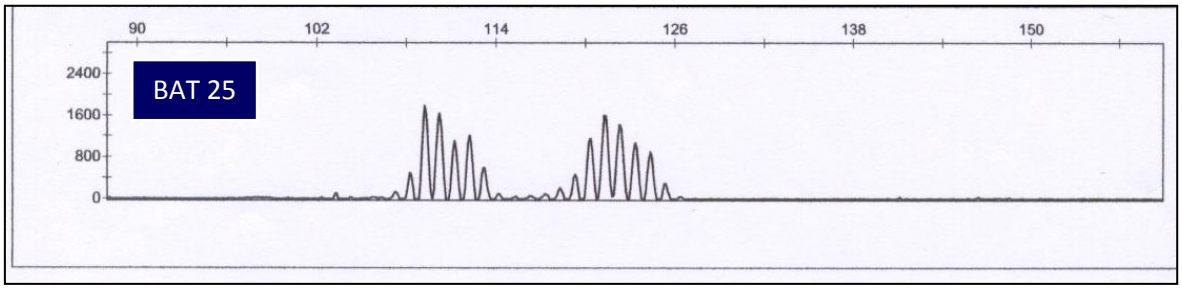


(a) **(b)**

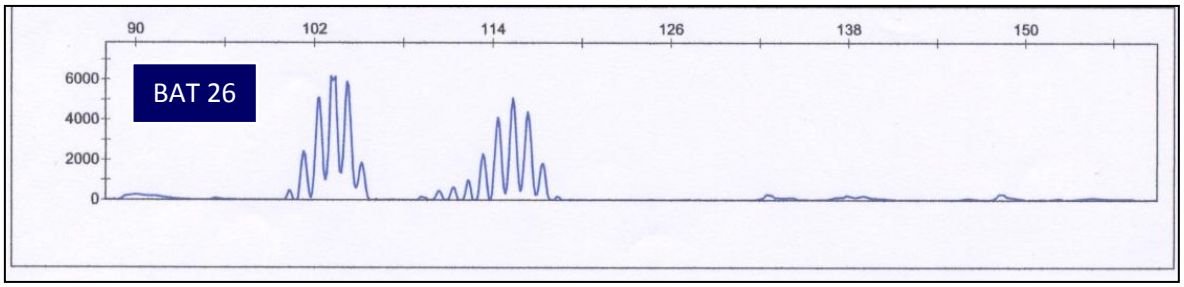
(c) **(d)**

Figure 10: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 negative, **(b)** MSH6 negative, **(c)** MLH1 positive, and **(d)** PMS2 positive.

CASE 3 (2- MSI analysis)



(a)

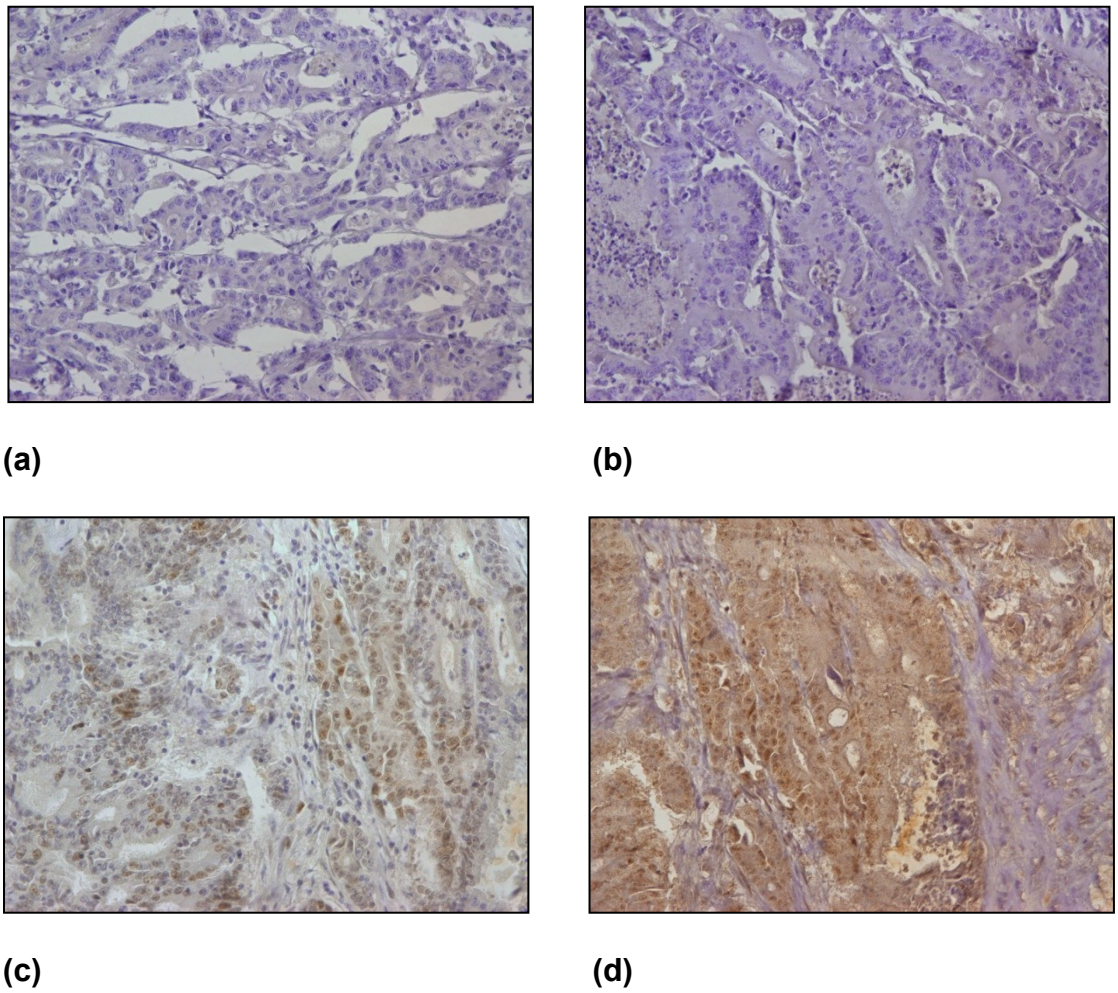


(b)

Figure 11: Electropherograms for BAT25 and BAT26 from CRC tissue. **(a)** BAT 25 unstable, **(b)** BAT 26 unstable.

CASE 4 (1- IHC)

MSH2/MSH62 negative

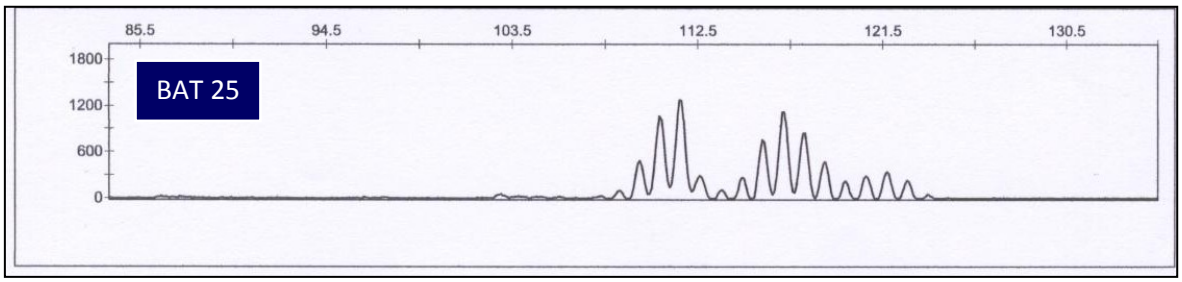


(a) **(b)**

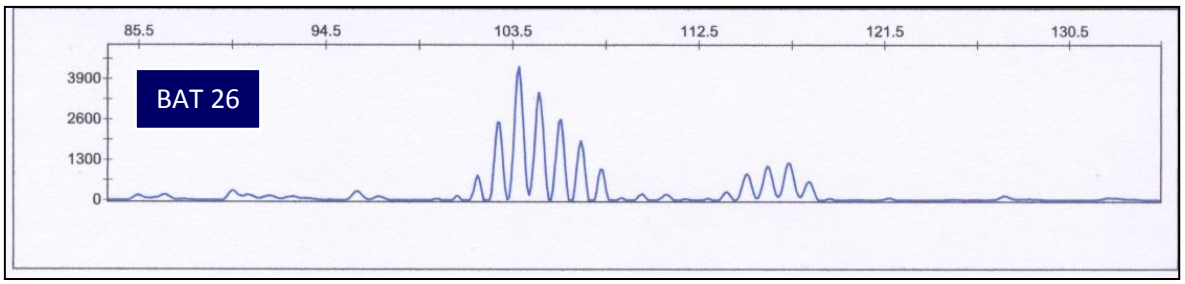
(c) **(d)**

Figure12: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 negative, **(b)** MSH6 negative, **(c)** MLH1 positive, and **(d)** PMS2 positive.

CASE 4 (2- MSI analysis)



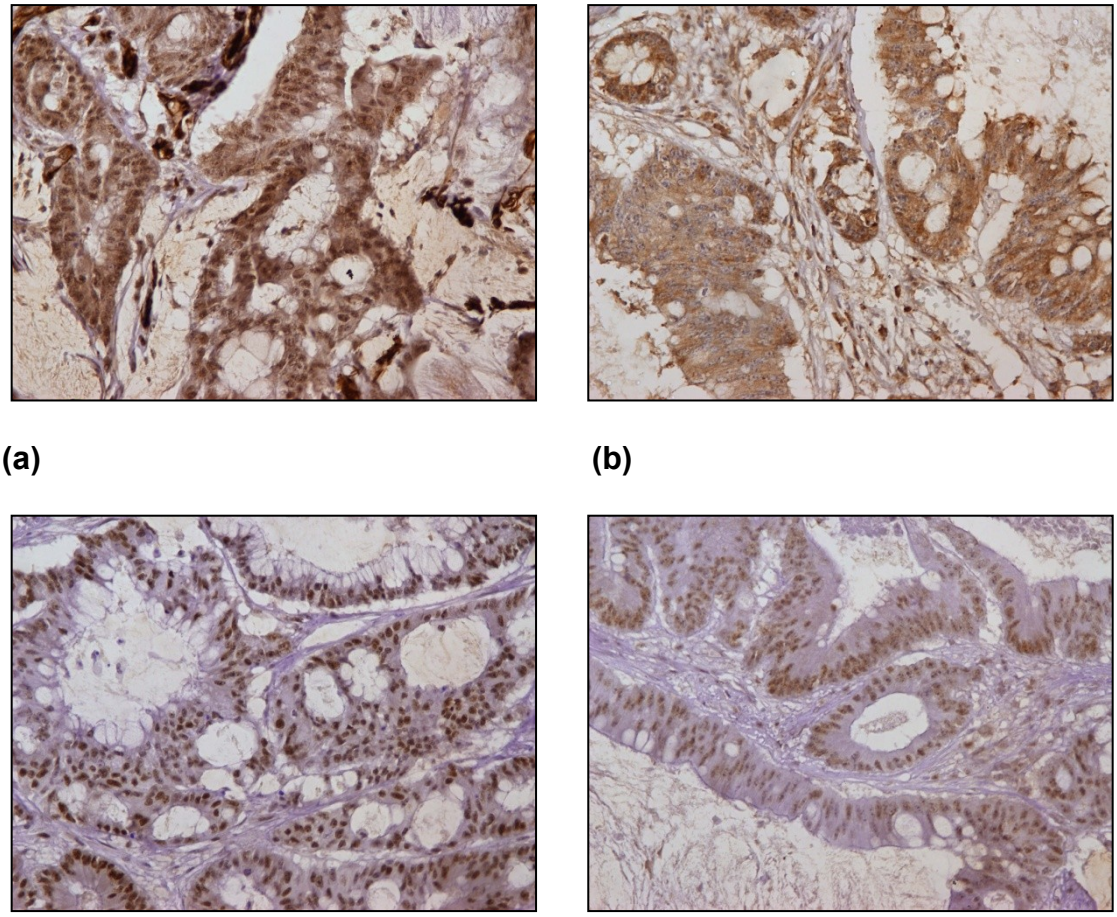
(a)



(b)

Figure 13: Electropherograms for BAT25 and BAT26 from CRC tissue. **(a)** BAT 25 unstable, **(b)** BAT 26 unstable.

MSH6 Inadequate results by IHC



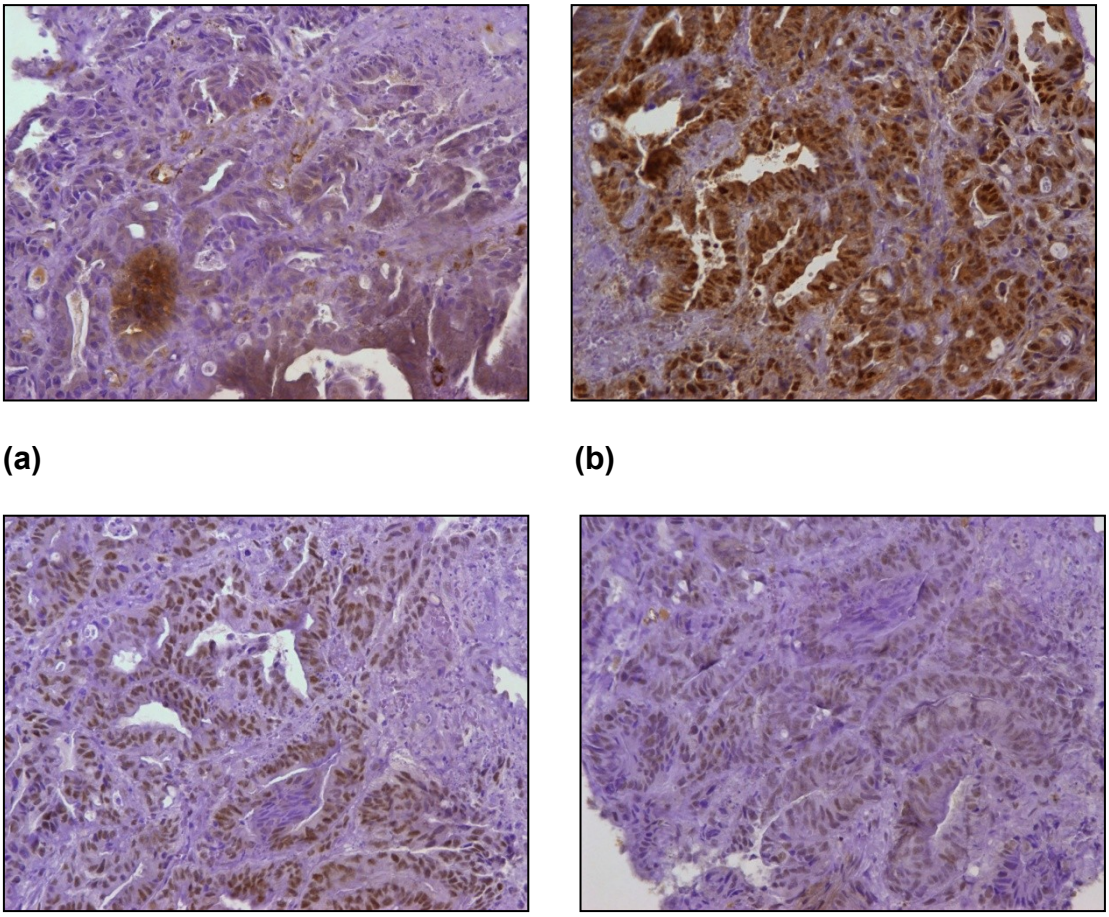
(a) **(b)**

(c) **(d)**

Figure 14: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 positive, **(b)** MSH6 inadequate, **(c)** MLH1 positive, and **(d)** PMS2 positive.

MSH2 inadequate results by IHC, (IHC & MSI analysis)

1- IHC

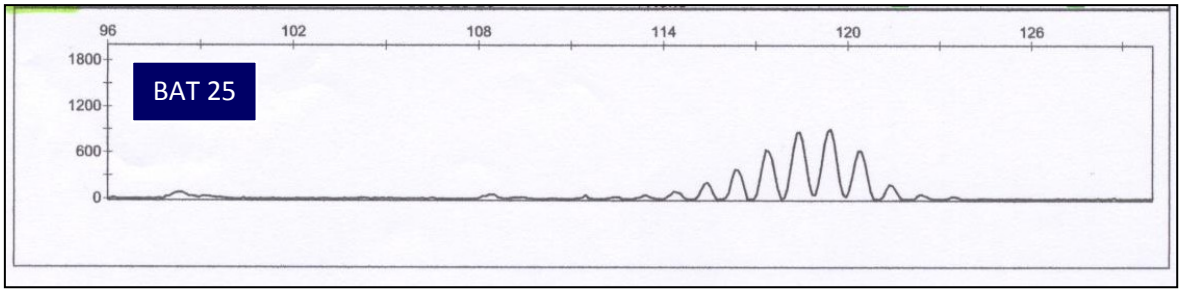


(a) **(b)**

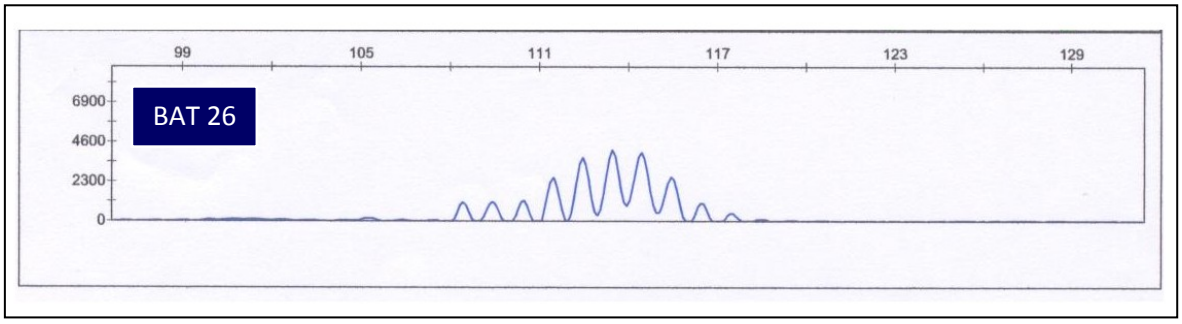
(c) **(d)**

Figure 15: Immunohistochemical staining results of MSH2, MSH6, MLH1 and PMS2 (shown at 200x). **(a)** MSH2 inadequate, **(b)** MSH6 positive, **(c)** MLH1 positive, and **(d)** PMS2 positive.

2- MSI analysis

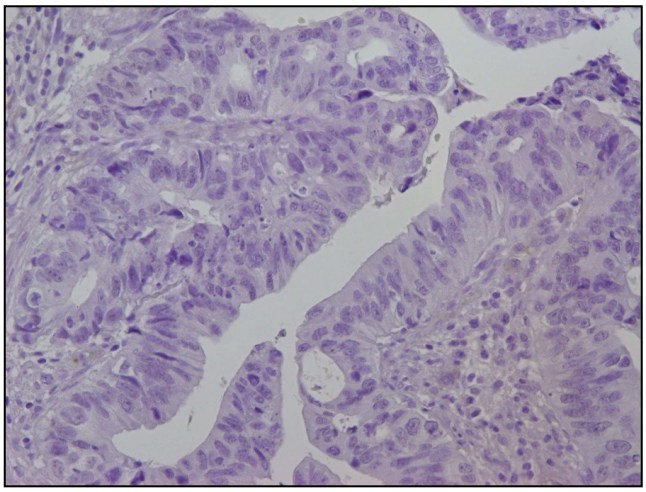


(a)

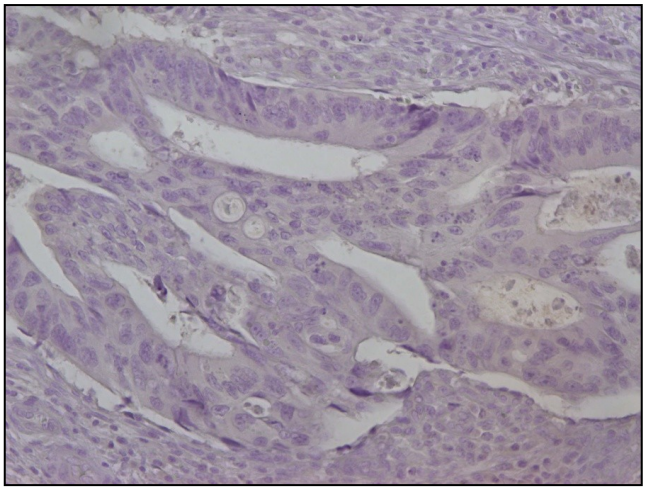


(b)

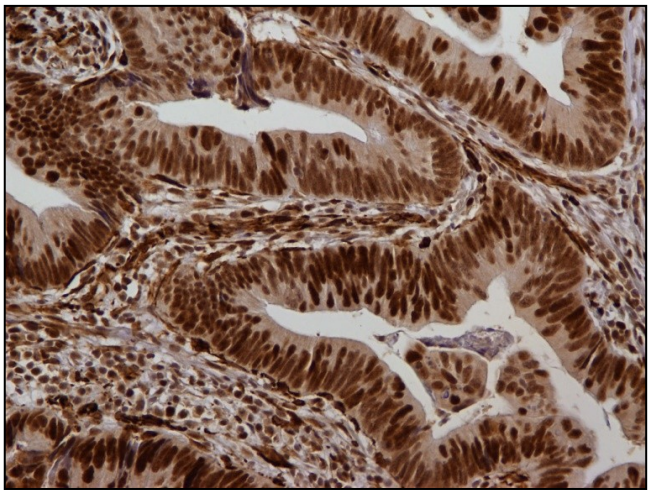
Figure 16: Electropherograms for BAT25 and BAT26 from CRC tissue. This case showed MSH2 inadequate results by IHC. **(a)** BAT 25 stable, **(b)** BAT 26 stable.



(a)



(b)



(c)

Figure 17: Immunohistochemical staining results for MSH2 (shown at 200x). **(a)** inadequate, **(b)** the same case repeated without prolonged time of antigen retrieval and still inadequate, **(c)** the same case repeated with prolonged time of antigen retrieval shows MSH2 positive staining result.

CHAPTER FIVE
DISCUSSION

5. Discussion

In this study, we aimed to assess the DNA MMR proteins abnormalities among Sudanese CRC patients. The total number of included cases was 42.

Sections were cut and assessed by IHC for the expression of 4 MMR protiens (MLH1, MSH2, MSH6, and PMS2). Furthermore, MSI analysis using mainly BAT 25 & 26 was performed for cases that showed negative or inadequate staining results by IHC.

In the current study, 4 (9.5%) MMR protein negative CRC cases were detected by IHC. Different studies showed higher percentages of MMR proteins abnormalities by IHC compared to our study. Khoo et al. (2013) found that the percentage of abnormal IHC staining for MLH1, MSH2 and MSH6 expression was (14.4%) among 298 Malaysian CRC cases. Furthermore, our finding in the current study is less than what was detected by Lanza et al. (2006) in Ferrara, Italy. In their study, 718 colorectal adenocarcinoma patients were studied. Of whom, 114 (15.9%) were found with abnormal expression of MMR protein. Of these 114 cases with abnormal expression, 18 were MSH2 negative and 96 were MLH1 negative.

Moreover, Jensen et al. (2008) in Denmark, found that 39 (14.9%) out of 262 CRC cases were MMR deficient by IHC or MSI analysis.

However, other studies found less percentages compared to our finding. A study from Spain by Jover et al. (2004) reported that the percentage of MMR-defective CRC cases was 7.6%. In their study, 172 CRC cases were studied. MSI analysis was performed by BAT 26, while IHC was performed using antibodies against MLH1 and MSH2 proteins. MSI was detected in 13 (7.6%) cases and all exhibited loss of expression of one of the two examined proteins (11 MLH1 negative and 2 MSH2 negative). De Jesus-Monge et al. (2010) examined the MMR protein expression among Hispanics from Puerto Rico. In their study, MLH1 and MSH2 protein were examined by IHC in 164 CRC cases. Of these cases, 7 (4.3%) were negative. **Table 9** shows a summary of some other studies from different countries including the percentage of MMR protein negative cases assessed by IHC. Most of these studies reported higher percentages of MMR protein negative CRC cases compared to our study. However, the result obtained by Kheirelseid et al. (2013) was almost near to our finding, while the finding of Coggins et al. (2005) was less than our finding.

Table 9. A summary of some studies regarding MMR protein expression examined by IHC.

Authors and year	Country	Number of included cases	MMR protein negative CRCs		Examined MMR proteins
			n	*%*	
Molaei et al. (2010)	Iran	343	48	14	MLH1, MSH2, PMS2, and MSH6
Erdamar et al. (2007)	Turkey	74	34	45.9	MLH1 and MSH2
Coggins et al. (2005)	England	732	57	7.78	MLH1 and MSH2
Wright and Stewart (2003)	New Zealand	458	89	19.4	MLH1 and MSH2
Jin et al. (2008)	China	146	32	21.9	MLH1, MSH2 and MSH6
Ashktorab et al. (2008)	Oman	49	8	16.3	MLH1 and MSH2
Kheirelseid et al. (2013)	Ireland	33	3	9.09	MLH1, MSH2, and PMS2
Lindor et al. (2002)	USA	1,144	326	28.4	MLH1 and MSH2

Furthermore, another study from Ghana, which is one of the West African countries, showed a high percentage (41%) of MSI-H CRC cases (Raskin et al., 2013). Kria Ben Mahmoud et al. (2012) examined the MSI status among 150 CRCs from Tunisians patients. They found that 15 % of these CRCs were MSI-H.

Regarding the grade of differentiation of MMR protein negative CRC cases in the current study, all these cases were LG, which refers to well/moderately differentiated CRC. However, this finding requires additional evaluation using a larger sample size to find out the accurate association between MMR protein negative cases and the grade of differentiation in Sudanese patients. Nevertheless, this observation opposes other published data. Lanza et al. (2006) found that MMR protein negative CRCs are characterized by poor differentiation. Furthermore, Khoo et al. (2013) reported that CRC cases with MMR defect were frequently found poorly differentiated compared to those cases with no defect in MMR.

In our study, the results of IHC in several sections were inadequate to allow a good assessment. In such instances, the immunohistochemical analysis was repeated using successive antigen retrieval by increasing the time of this step. After repetition, a number of sections were evaluable. However, the immunohistochemical staining remained inadequate to provide a good evaluation in other sections. Raskin et al. (2013) found some difficulties in their study regarding the analysis of MMR proteins by IHC, which resulted in the presence of a limited number of CRC cases with sufficient immunohistochemical staining. They attributed these difficulties in achieving a good immunohistochemical staining to the prolonged time of fixation, which makes the tissue excessively dehydrated.

CHAPTER SIX
CONCLUSIONS

6. Conclusions

The percentage of MMR protein negative cases among Sudanese CRC patients in this study is 9.5%, which appears to be relatively low compared to what has been generally reported in certain studies performed in different countries. However, detection of MMR proteins by IHC is recommended to be introduced in the referral Histopathology laboratories in Sudan due to its importance in the management of CRC patients.

In this study, MLH1&PMS2 and MSH2&MSH6 abnormal expression detected by IHC seem to be the most common form of MMR proteins abnormalities in Sudanese CRC patients. Moreover, MLH1 and MSH2 seem to be the most inactivated MMR genes in Sudanese CRC patients, concerning the results of IHC.

CHAPTER SEVEN
REFERENCES

7. References

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