IMMUNOHISTOCHEMISTRY FOR MHL1 AND MSH2: A METHOD FOR IDENTIFYING MISMATCH REPAIR GENE IN NEPALESE COLORECTAL CANCER PATIENTS

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ABBEREVIATIONS

%	Percentage
µ	Micro
µg	Microgram
µl	Microlitre
3’	Three Prime
5’	Five Prime
APC	Adenomatous Polyposis Coli
bp	Base-Pair
CIN	chromosomal instability
cm	Centimetre
CRC	Colorectal Cancer
Dam	DNA adenine methylation
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra-Acetic Acid
EPCAM	Epithelial cell adhesion molecule
FAP	Familial Adenomatous Polyposis
G	Guanine
GTBP	G/T mismatch-binding protein
hMLH1	Human MutL homolog 1
hMLH3	Human MutL homolog 3
hMSH2	Human MutS homolog 2
hMSH3	Human MutS homolog 3
hMSH6	Human MutS homolog 6
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
hPMSH1	Human post meiotic segregation 1

hPMSH2	Human post meiotic segregation 2
IARC	International Agency for Research on Cancer
ICG	International Collaborative Group
IDLs	insertion/deletion loops
IHC	Immunohistochemistry
kb	Kilobases
LS	Lynch syndrome
MIN or MSI	microsatellite instability
MLH1	mutL Homolog 1
MLPA	Multiplex Ligation-dependent Probe Amplification
MMR	Mismatch Repair
mRNA	Messenger RNA
MSH2	mutS Homolog 2
MSH6	MutS homolog 6
MSI	Microsatellite Instability
MSI-H	High microsatellite instability
MSI-L	Low microsatellite instability
NCI	National Cancer Institute
NCR	National Cancer Registry
PCR	Polymerase Chain Reaction
PMS1	Postmeiotic Segregation 1
PMS2	Postmeiotic Segregation 2
PMS2	post-meiotic segregation increased 2
RFLP	Restriction Fragment Length Polyorphism
TSGs	Tumour suppressor genes
WHO	World Health Organization
α	Alpha

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Immunohistochemistry for MHL1 and MSH2: a method for identifying mismatch repair gene in Nepalese colorectal cancer patients

ABSTRACT

Lynch syndrome (LS) (previously known as Hereditary nonpolyposis colorectal cancer (HNPCC) syndrome) is caused by mutations in mismatch repair (MMR) genes. LS is an autosomal dominant condition accounting for 2–5% of all colorectal carcinomas. Mismatch repair genes contain mainly four genes they are MLH1, MSH2, PMS2 and MSH6. Colorectal cancers with DNA mismatch repair gene mutations characteristically display a high rate of replication errors in simple repetitive sequences detectable as microsatellite instability (MSI). Monoclonal antibodies against hMLH1 and hMSH2 are commercially available, those two DNA mismatch repair proteins accounts for most LS cancers. This study pursued to investigate the potential utility of these antibodies in determining the expression status of these proteins in paraffin-embedded formalin-fixed tissue and to identify key technical protocol components associated with successful staining. Colorectal cancer sample of 43 patients of Nepal were examined and immunohistochemistry was used to determine which tumours lacked expression MLH1 and MSH2 gene. Out of 43 patient 18.6% (8 of 43) of the sample showed abnormal staining pattern for hMLH1 antibody and 11.6%(5 of 43) of sample showed abnormal staining pattern for hMSH2 antibody only 9.30% (4 of 43) sample showed abnormal staining pattern for both antibody. The key protocol point associated with successful staining was an antigen retrieval step involving heat treatment with Tris-EDTA heated at 121^0C for 2 minutes. Tumours with mismatch repair defect were frequently found at the age less than 50 years ($p<0.05$) than tumours with no mismatch repair defect. This study demonstrates the potential utility of immunohistochemistry in detecting LS probands and identifies key technical components for successful staining. Immunohistochemistry can be done in local laboratories to find mismatch repair proteins defect on the selected cases before referring for the expensive molecular test.

Keywords: Lynch syndrome, Hereditary nonpolyposis colorectal cancer, mismatch repair defect, colorectal cancer, paraffin-embedded formalin-fixed tissue, Monoclonal antibodies, Antigen

CHAPTER I

INTRODUCTION

1.1 Colorectal cancer

Cancer is the condition involving unregularly grow in cell. In cancer, cells divide and grow uncontrollably, forming malignant tumours. If not treated, the tumour can cause problems as it spreading into normal tissues nearby, causing pressure on other body structures and spreading to other parts of the body through the lymphatic system or bloodstream. Not all tumours are cancerous. Benign tumours do not grow uncontrollably, do not invade neighbouring tissues, and do not spread throughout the body. But malignant tumours are made up of cancer cells. They usually grow faster than benign tumours, spread into and destroy surrounding tissues and spread to other parts of the body. There are over 200 different known cancers that afflict humans, because there are over 200 different types of body cells. To determine the cause of the cancer is very complex, however there are various factors which can cause cancer. Some of them are radiation, reactive oxygen, environment pollution, obesity, alcoholism, smoking etc. There is a small percentage of cancer which is hereditary which are transmitted from their parent.

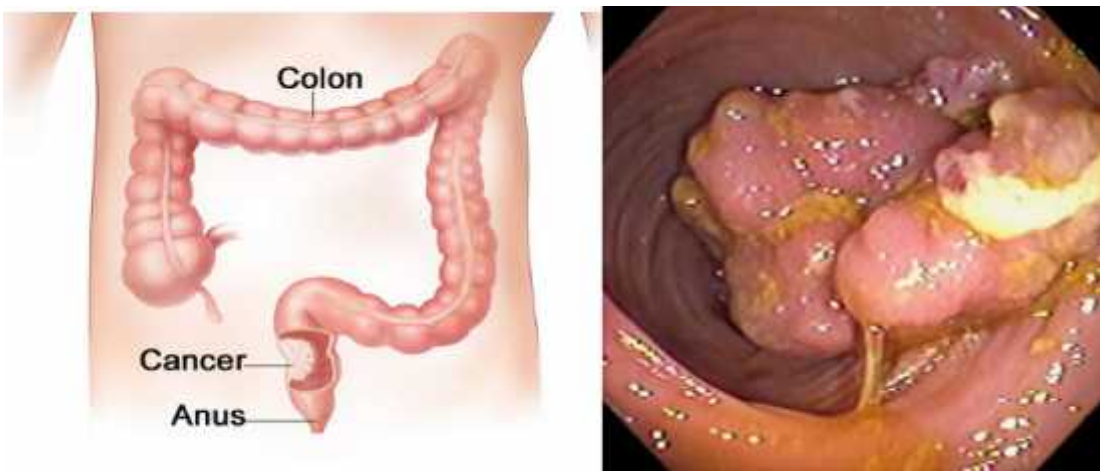


Figure1.1: Showing colon and colorectal cancer polyp

Cancer develops in the colon or the rectum is colorectal cancer (CRC). The colon and rectum are parts of the digestive system, also called the gastrointestinal. Colorectal cancer usually develops slowly over a period of 10 to 15 years (Kelloff *et al*., 2004). The tumour typically begins as a noncancerous polyp. A polyp is a growth of tissue that develops on the lining of the colon or rectum that can become cancerous. Certain kinds of polyps, called adenomatous polyps or adenomas, are the most likely to become

cancers, though fewer than 10% of adenomas progress to cancer (Levine and Ahnen, 2006).

Once cancer forms in the large intestine, it can grow through the lining and into the wall of the colon or rectum. Cancers that have invaded the wall can also penetrate Blood or lymph vessels, which are thin channels that carry away cellular waste and fluid. Cancer cells typically spread first into nearby lymph nodes, which are bean-shaped structures that help fight infections. Cancerous cells can also be carried in blood vessels to the liver or lungs, or can spread in the abdominal cavity to other areas, such as the ovary. The process through which cancer cells travel to distant parts of the body through blood or lymphatic vessels is called metastasis (Colorectal Cancer Facts & Figures, 2011-2013).

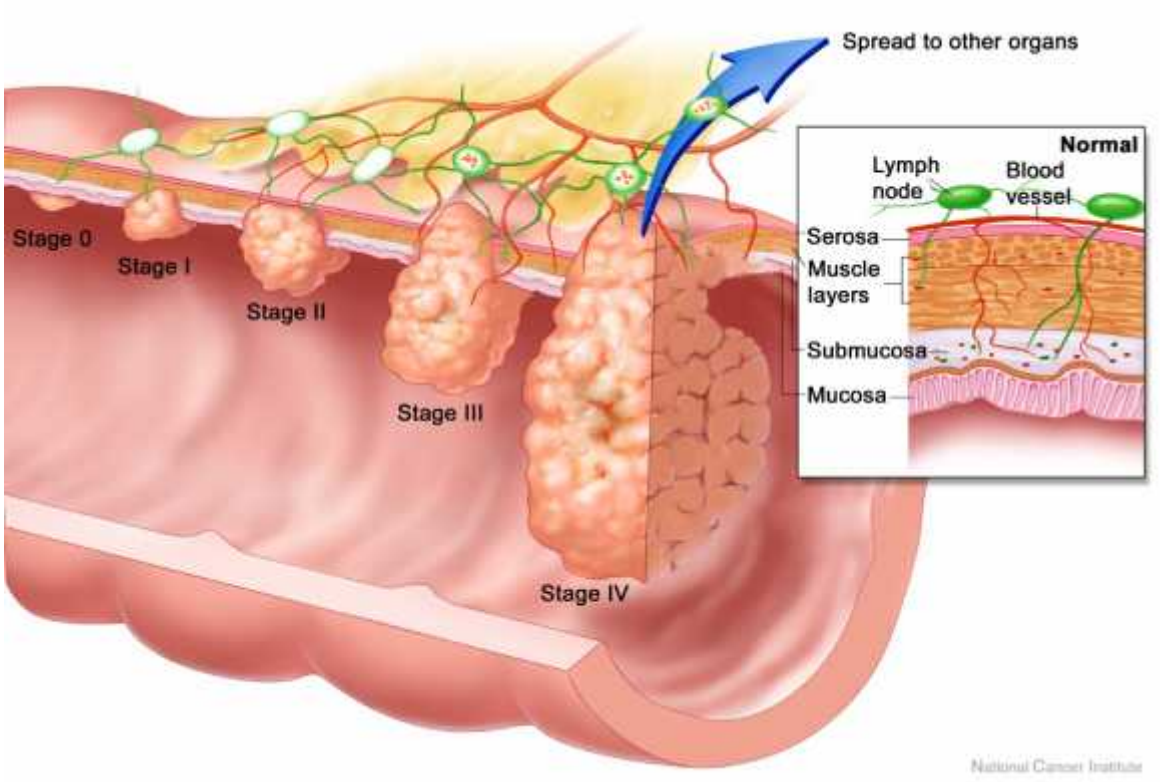


Figure 1.2: colorectal cancer growth (National cancer institute: http://www.cancer.gov/)

1.1.1 Epidemiology

Colorectal cancer (CRC) is the second most common form of cancer in Europe, and the most commonly diagnosed cancer. It has the second highest CRC incidence and mortality among 38 European countries

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women. Estimated new cases and deaths from CRC in 2014: (American Cancer Society) New cases: 136,830. Deaths: 50,310.

About 75% of patients with CRC have sporadic disease which is not inherited from their parent and 25% of patients have inherited from their parent as one of the parents has

the CRC. Colorectal tumours present with a broad spectrum of neoplasms, ranging from benign growths to invasive cancer and are predominantly epithelial-derived tumours (i.e., adenomas or adenocarcinomas).

1.1.1.1 Epidemiology of CRC in Nepal

Due to poor cancer registry system the exact cancer data is unknown for Nepal. The incidence of cancer continues to increase every year. The most common types of cancers in Nepal are the cancers of cervix uteri, lung, head & neck, breast, stomach, lymphoma and leukaemia. Lung cancer is the commonest in both genders in Nepal. This could be due to lack of awareness and higher prevalence of smoking. A major number of cancer patients die due to lack of treatment facilities in Nepal.

One study done by Pradhanananga *et al.*, (2009) found that colon represented 1.6 % and rectal cancer 2.4 % of the total cancer causes. In a further detailed report on 245 of the rectal cancer cases from the multi-hospital statistics, the colorectal cancer patients represented, 1.85 % of the total number cancers, men to women ratio 1,1: 1. The group was more commonly originating from lower to middle economics, age being generally younger (median age 46.5), with 26.55 % of the patients diagnosed below 34 years of age and totally 45.11 % diagnosed below 44 years of age.

CRC is one of the most common cancers in Nepal. CRC among Nepalese young adults accounts for a high incidence (28%) of all CRC cases. Although right sided colonic cancer has been increasing, rectum is the commonest site. There is also an increasing trend for diagnosis at earlier stages of the disease which can be treated with curative intent (Kansakar and Singe, 2012).

1.1.2 Pathology

Pathologists have classified the lesions into the following three groups:

- Nonneoplastic polyps (hyperplastic, juvenile, hamartomatous, inflammatory, and lymphoid polyps), which have not generally been thought of as precursors of cancer.
- Neoplastic polyps (adenomatous polyps, and adenomas).
- Cancers.

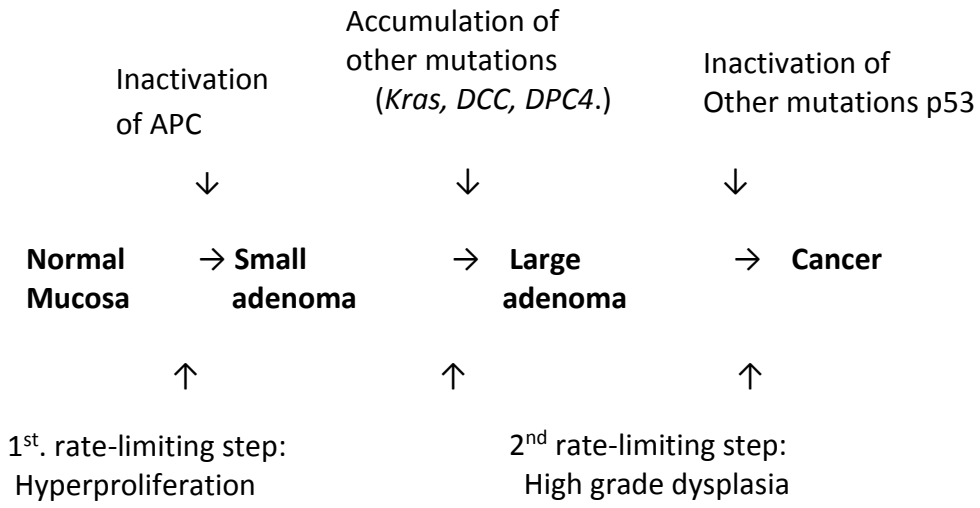
According to the Epidemiologic studies the personal history of colon adenomas places has an increased risk of developing colon cancer (Neugut *et al.,* 1993). More than 95% of CRCs are carcinomas, and about 95% of these are adenocarcinomas. It is well recognized that adenomatous polyps are benign tumours that may undergo malignant transformation. They have been classified into three histologic types, with increasing malignant potential: tubular, tubulovillous, and villous. While there is no direct proof

that most CRCs arise from adenomas, adenocarcinomas are generally considered to arise from adenomas, (Shinya *et al.,* 1997)

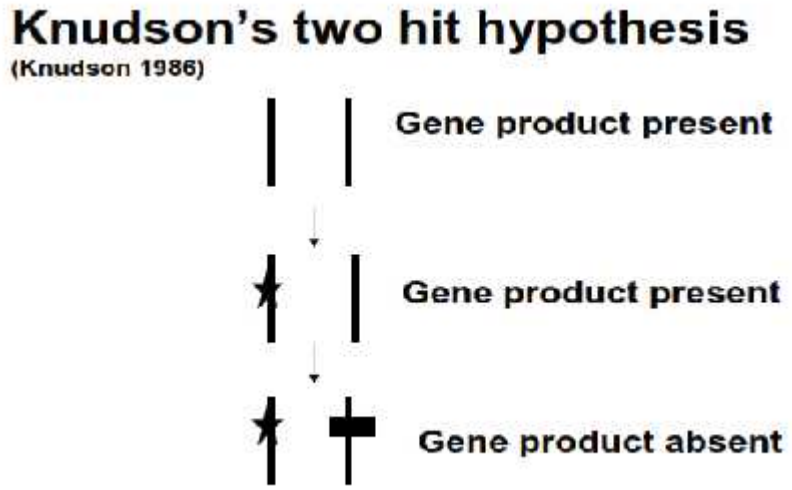
1.1.3 Molecular biology

The majority of CRCs develop from adenomas. The progression from an adenoma to a cancer passes through a series of defined histological stages referred to as the adenoma-carcinoma sequence (Vogelstein *et al.,* 1988). The colorectal tumour initiation requires several different somatic changes before a cell can develop into a carcinoma (Kinzler *et al.,* 1996).

Main events in the adenoma-carcinoma sequence
(Vogelstein *et al.,* 1988; Boland *et al.,* 1995)



The mismatch repair genes encode protein products recognizing and correcting errors that arise when DNA is replicated (Leach *et al.,* 1993). According to the two hit model of carcinogenesis, the second event in addition to the inherited mutation is expected to be an acquired mutation in the normal allele (Knudson, 1986; Hemminki *et al.,* 1994). If both the first and second hits are truncating mutations, the gene product for the gene is absent in the tumour cells (Thibodeau *et al.,* 1996).



1.1.4 Risk Factors

1.1.4.1 Age

Young people also can develop the CRC but there is a high chance of causing cancer after 50 years

1.1.4.2Personal history of colorectal polyps or colorectal cancer

If a person has adenomatous polyps (adenomas) in the colon or rectum in his history then he has a high risk of developing colorectal cancer. The chances of this happening are greater if you had your first colorectal cancer when you were younger.

1.1.4.3 Personal history of inflammatory bowel disease

People who have had inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's for many years often develop dysplasia. These cells can change into cancer over time.

1.1.4.4 Family history of colorectal cancer or adenomatous polyps

About 20% of people suffering from CRC have their family member affected by this disease. Those with a history of colorectal cancer in one or more first-degree relatives (parents, siblings, or children) are at increased risk. The risk is about doubled in those with a single affected first-degree relative. It is even higher if the first-degree relative was diagnosed when they were younger than 45, or if more than one first-degree relative is affected.

1.1.4.5 Inherited syndromes

About 5% to 10% of people who develop colorectal have mutation in inherited gene. The 2 most common inherited syndromes linked with colorectal cancers are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

1.1.4.6 Lifestyle-related factors

Several lifestyle-related factors have also been linked to colorectal cancer. It include diets, Physical inactivity, Obesity, Smoking, Heavy alcohol use etc.

1.2 Mismatch Repair Genes

The main function of the DNA repair system is to eliminate the mismatch of base-base insertions and deletions that appear as a consequence of DNA polymerase errors during DNA replication in the cell. Mismatch repair genes serve functions relating to genetic stabilization, such as correcting errors in DNA synthesis, ensuring fidelity of genetic recombination or participating in the initial steps of apoptotic responses to different classes of DNA damage. The first studies on mismatch repair genes were developed in Escherichia coli (E. coli) (Iyer *et.al*, 2006). MutS and MutL are the homo-oligomers responsible for initiating repairs on mismatches in E. coli. MutS is responsible for recognizing the mismatch and recruiting MutL to the mismatch location, thus starting the downstream activities (Modrich, 2006). In mammal cells possess two homologous MutS that work as heterodimers, in which MutSα is formed by the MSH2-MSH6 complex and MutSβ by MSH2-MSH3 (Kolodner and Marsischky, 1999). So in mammalian cell mainly there are four gene involved in mismatch repair, mainly MLH1, MSH2 PMS2 and MSH6.

The MSH2-MSH6 complex represents 80% to 90% of the cellular level of MSH2 and its function is to recognize the mismatch of base-base insertions and deletions, to contain one or two unpaired nucleotides, but it is also capable of recognizing large deletions and insertions (Jiricny, 2000). The MSH2-MSH3 complex is responsible for recognizing and repairing insertions and deletions from two to eight nucleotides (Ohmiya *et.al,* 2001)

MLH1 is located at chromosome 3p21 and contains 19 exons that span over 58 kb, with the MLH1 protein containing 756 amino acids. Exons 1 to 7 contain a region that is highly conserved in the MLH1 and PMS1 genes of yeast (Han *et al.,* 1995). Today, more than 250 different germline mutations have been identified and MLH1 is the most important gene in lynch syndrome (Peltomäki and Vasen, 2004).

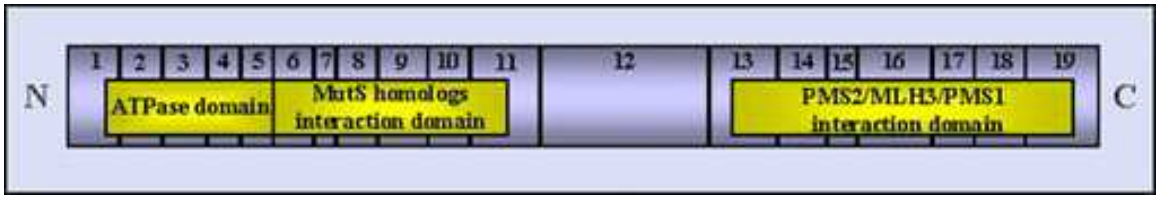


Figure 1.3: MLH1 Protein. Numbers in boxes indicate the exon. The three boxes inside represent the ATPase domain, the MutS homologs interaction domain and the PMS2/MLH3/PMS1 interaction domain; C: Carboxyl-terminal; N: Amino-terminal (Atlas of Genetics and Cytogenetics in Oncology and Haematology: http://atlasgeneticsoncology.org/Genes/GC_MLH1.html)

The MSH2 gene is located at chromosome 2p16 and contains 16 exons spanning over 73kb. The MSH2 protein has been found to contain 934 amino acids (Kolodner *et al.,* 1994). Together MSH2 and MLH1 are responsible for more than 64% of the cases of germline mutations in lynch syndrome (Liu *et al.,* 1996).

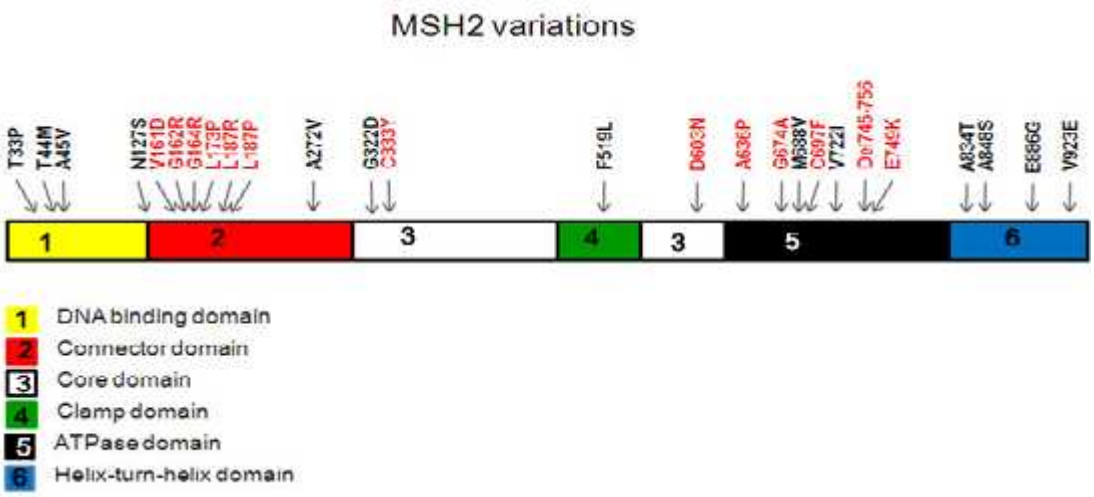


Figure 1.4: MSH2 Protein (DNA Mismatch Repair and cancer group http://www.helsinki.fi/bioscience/mmrandcancer/msh2variants.html)

The MSH6 gene is located at chromosome 2p15 and contains 10 exons and is approximately 20kb in size. The MSH6 protein, also known as GTBP, is a 153kDa protein consisting of 1360 amino acids (Acharya *et al*., 1996). Recently, germline mutations in this gene were recognized as a frequent cause of atypical Lynch syndrome (Wu *et al.,* 1999).

The PMS2 locus at chromosome 7p22 contains 15 exons and is 16kb in size (Nicolaides et al., 1994).And PMS1 genes is located in the chromosomes 2q31-q33, with 16 Kb and 15 exons. They account for 5% of all cases of Lynch syndrome (Nakagawa *et al.,* 2004)

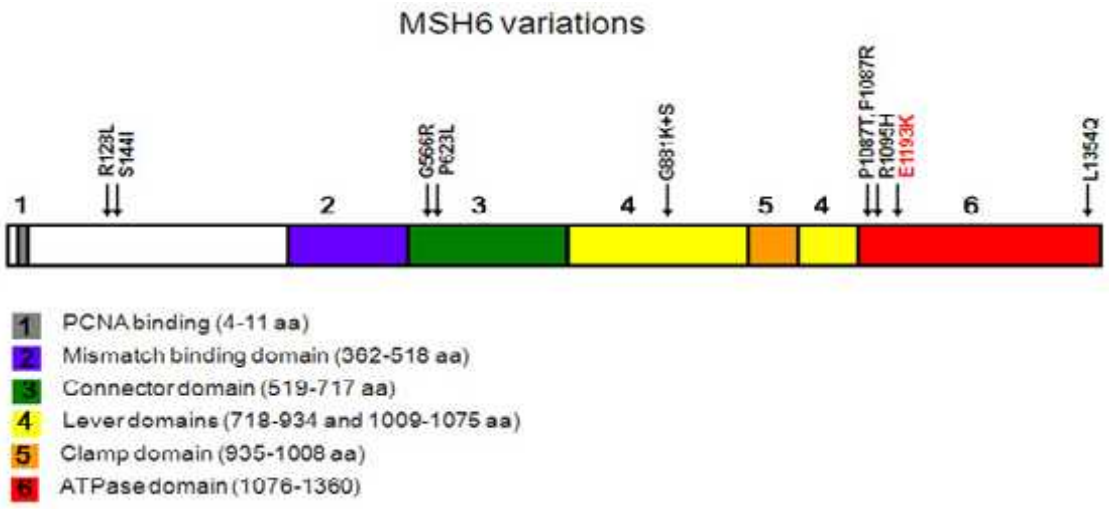


Figure 1.5: MSH6 Protein (DNA Mismatch Repair and cancer group http://www.helsinki.fi/bioscience/mmrandcancer/msh2variants.html)

1.3 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumours. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.

The presence of MMR gene products may be analysed by using antibodies against MLH1, MSH2, MSH6 and PMS2 proteins. Staining can be performed on sections from formalin fixed, paraffin embedded tissue blocks containing tumour tissue and normal adjacent mucosa. The MLH1, MSH2, MSH6 and PMS2 staining patterns in normal cells are nuclear. Expression is particularly prominent in the epithelium of the digestive tract as well as in testis and ovary. Staining is evaluated using normal epithelial cells, stromal cells or lymphocytes in the same slide as controls. Loss of nuclear staining in tumour cells and normal staining of the internal control indicates loss of expression of MMR protein.

The peroxidase-labelled antibody method, introduced in 1968, was the first practical application of antibodies to paraffin-embedded tissues and overcame some of the limitations of earlier fluorescence antibody methods (Nakene *et al.,* 1968). These

pioneering studies using enzyme labels instead of fluorescent dyes opened the door to the development of modern methods of immunohistochemistry.

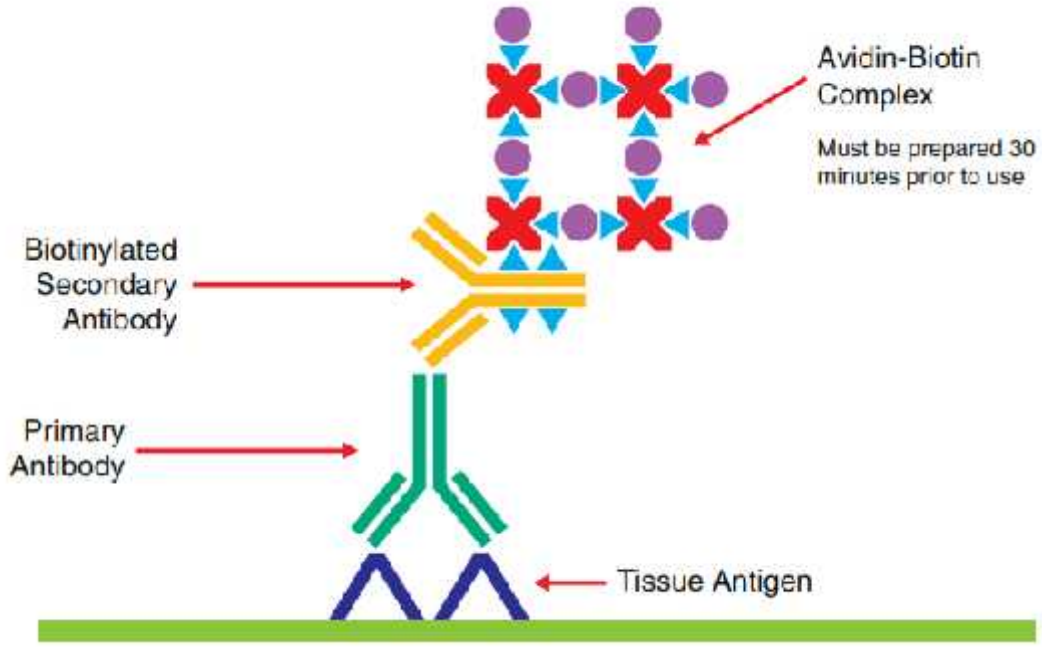


Figure 1.6: Biotin-avidin immunoenzyme technique (Immunohistochemical Staining Methods, Education Guide, 2009)

In 1981 a new generation of immunohistochemical methods emerged with the advent of the avidin-biotin methods, which remains widely used today (Hsu *et al.,* 1981). All avidin-biotin methods rely on the strong affinity of avidin or streptavidin for the vitamin biotin. Streptavidin (from *Streptomyces avidinii*) and avidin (from chicken egg) both possess four binding sites for biotin. The biotin molecule is easily conjugated to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (Heras *et al.,* 1995).

1.3 Hypothesis

As the main cause of CRC is due to the mutation in the mismatch repair (MMR) gene i.e. MLH1, MSH2, PMS2 and MSH6. And mutation of the gene results in the loss of production of protein.so there must be loss of protein MLH1 and MSH2 in CRC patient of Nepal.

1.4 Objectives

1.4.1 General objective

Using immunohistochemistry for identifying mismatch repair genes MHL1 and MSH2 in Nepalese colorectal cancer patients. To evaluate the tumour tissues from familial CRC

subjects by mean of immunohistochemistry method for formalin-fixed paraffin-embedded (FFPE) tissues.

1.4.2 Specific objectives

1. To collect paraffin embedded tissue block.
2. To optimization primary antibody concentration and Incubation time of primary antibody.
3. To perform immunohistochemical staining of the slide by using two antibody i.e. hMLH1 and hMSH2
4. To compare the clinical data with the hMLH1 and hMSH2 expression in the CRC patient
5. Conformation of MLH1 and MSH2 mutation by DNA sequence analysis.

1.5 Rationale

Cancer is the second most frequent cause of death after cardiovascular disease. Colorectal cancer (CRC) is the second most common form of cancer in Europe, and it also the one of the main cancer in Nepal. In Nepal also many people die due to the CRC before being diagnosis. The prevalence of CRC in Nepal is not much different than other countries. Due to the lack of reliable method of diagnosis some people die without knowing the cause of the disease, as up to now the base line data are also not created yet. Many molecular techniques are available for the diagnosis of the CRC as most of them are very expensive and among them most of them are not available in our country. The introduction of the reliable, rapid, cost effective and accurate method molecular methods such as immunohistochemistry for the diagnosis of CRC as well as other cancer would be effective and helpful method. Immunohistochemical analysis of hMLH1 and hMSH2 gene can be useful for determining the Lynch syndrome which account for the colorectal carcinomas. However, genetic analysis of MSI status is time consuming and expensive, and needs specialized equipment which is difficult to perform in lab of poor source (Ward *et.al*, 2001). But in context of Nepal, the diagnosis of CRC is confined to physical examination, general personal history, cytological examination done by eosin and haematoxylin examination and some other lab test that cannot be entirely trusted. Therefore this method can be preliminary steps towards the use of reliable molecular assay in the diagnosis of CRC as well as other cancer.

In the developing country like Nepal the cost of diagnosis is another important criterion that should be consider. Immunohistochemistry assay fulfil this need to some extend along with the reliable diagnosis. As immunohistochemistry cost less than a quarter of the price of MSI testing done with the PCR and gel electrophoresis method (Dietmaier *et al.,* 1997). IHC for mismatch repair gene could be introduced in most local laboratories and included as a routine test for selected cases of colorectal carcinomas

1.6 Scope/Justification

Immunohistochemistry assay has been applied efficiently in the diagnosis of several of cancer patients and analysis of several of protein expression in the cell. IHC testing is effective for detecting tumours resulting from MMR deficiency. The antibody of hMLH1 and hMSH2 has demonstrated to be more than 92% sensitivity (Shagi, 2008) for the identification tumour that rises in individuals with a germline line mutation.

In context of Nepal, a reliable diagnostic procedure for the diagnosis of cancer is yet to be introduced. IHC can be the reliable technique for the diagnosis of various cancers in Nepal. This technique can be reliable and rapid technique for the diagnosis of several of cancer patient of Nepal.

CHAPTER II

LITERATURE REVIEW

DNA mismatch repair is a system for recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases that can arise during DNA replication and recombination, as well as repairing some forms of DNA damage (Iyer *et al.,* 2006). The basic features of this system have been highly conserved during evolution. Mismatch repair (MMR) is the major post-replicative DNA repair system, which increases replication fidelity up to 1000-fold (Modrich & Lahue, 1996), which is critical for keeping mutation rates at an acceptably low level. In addition to recognizing replication generated mismatches, MMR proteins also recognize mismatches in hetero duplex recombination intermediates. Mismatch recognition in recombination intermediates can elicit a repair process that leads to a genetically detectable gene conversion event, or can trigger an anti-recombination activity that prevents the recombination event from going to completion. The anti-recombination activity of MMR proteins promotes genome stability by inhibiting interactions between diverged sequences present in a single genome or derived from different organisms.

MMR removes primary replication errors that escaped DNA polymerase proofreading, such as base-base mismatches and small insertion/deletion loops (IDLs), which are most easily formed in long repetitive sequences, i.e. in microsatellites. Thus, defects in MMR induce the mutator phenotype characterized by changes in the microsatellites length, termed microsatellite instability (MSI). MSI is an established biomarker for MMR dysfunction in tumor cells (Umar *et al.,* 2004).

The postreplication DNA mismatch repair (MMR) pathway is responsible for the maintenance of DNA fidelity upon replication (Jiricny *et al.,* 2000). MMR captures errors in the newly synthesized DNA strand that are missed by the polymerase proofreading and lowers the mutation frequency by a factor of 100-1000-fold as compared to MMR deficient cells (Eshleman *et al.*, 1995). In humans, accumulation of mutations is a critical step in carcinogenesis. Loss of a single allele of one of the mismatch repair proteins causes Hereditary Non-Polyposis Colon Cancer (HNPCC), a form of cancer that accounts for 1-5% of all cases of colon cancer (Peltomäki, 2001). HNPCC is caused by inherited mutations in MMR genes. Most HNPCC families have germline mutations in the hMSH2 (2p22-p21) and hMLH1 (3p21) genes. Mutations in the other known MMR genes hMSH6 (2p16), hMSH3 (5q11-q12), hPMSH2 (7p22), hPMSH1 (2q31), and hMLH3 (14q24) are either rare or non-existent in HNPCC families (Wood *et al.,* 2001).

2.1 Hereditary Cancer

It is estimated that about 20% of all CRC worldwide has a hereditary component. There are over 1.2 million new cases of CRC every year (de la Chapelle, 2004). Hereditary cancer syndromes are often the result of inheriting germ line mutations in genes that play a role in tumourogenesis. Some of these syndromes, such as HNPCC, are also known to have associated cancers that develop in other organs, such as the endometrium (Garber & Offit, 2005; Lynch *et al.,* 2006; Cunningham *et al.,* 2010).

Genes that predispose individuals to cancer tend to affect cell death pathways, cell cycle regulation pathways or DNA repair pathways. Germ line mutations in oncogenes are generally non-viable as the resultant phenotype is too severe. However, cancers involving inherited mutations in Tumour suppressor genes (TSGs) and stability genes are more common (Fletcher and Houlston, 2010). Cancer predisposing genes that are highly penetrant follow a single-gene Mendelian pattern of inheritance. Mutations in these genes tend to be physiologically recessive but they are inherited in a dominant fashion, via Knudson's two hit hypothesis. A mutation will be inherited in one allele but the individual eventually acquires the mutation in the second allele. Once both alleles are non-functional this will give rise to cancer (Knudson, 1971; Knudson, 2001; Frank, 2004, Fletcher and Houlston, 2010).

The effect of the hereditary components in CRC is dependent on the penetrance of the particular genes that are involved (Cunningham *et al.,* 2010, Fletcher and Houlston, 2010). CRC may seem familial but may be chance clustering since it is a common disease. A seemingly sporadic case may be hereditary but may have been misclassified due to small family size, poor diagnostics or incomplete family history (de la Chapelle, 2004; Jasperson *et al.,* 2010).

CRC syndromes, such as Familial Adenomatous Polyposis (FAP) and HNPCC, have been vital to the understanding of CRC since several highly penetrant genes have been identified in these syndromes. These two CRC syndromes are the most common hereditary variants of CRC, accounting for approximately 1% and 5% of all CRC cases respectively. The genes implicated in these syndromes are also involved in sporadic cases of CRC and their identification has led to better understanding of sporadic and hereditary CRC (de la Chapelle, 2004; Jasperson *et al.,* 2010).

2.1.1 Familial Adenomatous Polyposis

FAP is a hereditary CRC syndrome characterised by hundreds to thousands of adenomas that present in the colon of affected individuals. These adenomas usually develop in childhood and there is practically a 100% lifetime risk that at least one of the adenomas will develop into a carcinoma. Of all the CRC cases, FAP is estimated to account for about 0.2%-1% (de la Chapelle, 2004; Gryfe, 2009).

By the time of adolescence, the polyps are usually identified throughout the colon and, thereafter, increase in size and numbers. About half of FAP patients develop adenomas by 15 years of age and 95% by age 35 years (Petersen *et al.,* 1991)

FAP is caused by germ-line mutations in the tumour suppressor gene Adenomatous Polyposis Coli (APC) gene. Most FAP patients have a family history of colorectal polyps and cancer, however, 25-30% of them are "de novo", without clinical or genetic evidence of FAP in family members [Bisgaard *et al.,* 1994; Rozen *et al.,* 2001]. FAP is inherited in an autosomal dominant manner, via the two-hit hypothesis (Gryfe, 2009), and the reported incidence varies from 1 in 7,000 to 1 in 22,000 live births, with the syndrome being more common in Western countries(Campbell *et al.,*1994). Additionally, somatic mutations of the APC gene are known to be involved in sporadic CRC, leading to this gene being considered the 'gatekeeper' of neoplasia in the colorectal tract (Gryfe, 2009). It has also been noticed that APC mutations occurs early in carcinogenesis, often being the initiator of the adenomatous process (de la Chapelle, 2004)

2.1.2 HNPCC/ Lynch syndrome

HNPCC is a group of hereditary cancer syndromes that frequently manifest in the colorectal tract. HNPCC is the most common form of hereditary CRC, with an incidence estimated to be between 2% to 5% of all new CRC cases diagnosed annually (Lynch *et al.,* 2006). When pathogenic mutations in the MMR genes are detected, HNPCC is reclassified as Lynch Syndrome (Vasen *et al.,* 2007; Cunningham et al., 2010). Most in the field use the term Lynch syndrome (LS) as a preferred synonym over HNPCC.

In NHPCC, (Lynch *et al.*, 1993), most patients do not have an unusual number of polyps. NHPCC accounts for about 3% to 5% of all CRCs. NHPCC is an autosomal dominant syndrome characterized by an early age of onset of CRC, excess synchronous and metachronous colorectal neoplasms, right-sided predominance, and extracolonic tumours. NHPCC is caused by mutations in the DNA MMR genes, namely MLH1, MSH2, MSH6, and PMS2. Mutations of the EPCAM gene that result in hypermethylation and silencing of MSH2 have also been described. The average age of CRC diagnosis in NHPCC mutation carriers is 44 to 52 years (Hampel *et al.,* 2008; Vasen *et al*., 2005) and 71 years in sporadic CRC. (Jemal *et al.,* 2010) Even though NHPCC is characterized by an early age of onset of CRC, in mutation-positive families when probands were excluded and both affected and non-affected relatives were ascertained, the average age at diagnosis of CRC was reported to be 61 years. (Hampel *et al.,* 2005)

Families with HNPCC are often identified by an increased number of cases of CRC, endometrial cancer and a number of other cancers that are associated with HNPCC (ovaries, small bowel, hepato biliary tract, pancreas, upper uroepithelial tract, brain, kidney and stomach). HNPCC is relatively common for a lethal, autosomal dominant disease. However, HNPCC generally does not affect the reproductive fitness of a carrier

before they have children, leading to the mutations being transferred to children (Frank, 2004; Garber and Offit, 2005; Lynch *et al.,* 2006).

2.1.3 Features of HNPCC/ Lynch syndrome

LS is in contrast to the high number of polyps observed in other hereditary CRC syndromes such as FAP where hundreds to thousands of polyps are observed in the colon. Therefore, polyps (or adenomas) are observed in LS but there are significantly fewer polyps, and the few that develop are more likely to develop into carcinomas (Jasperson *et al.,* 2010). It can also be noted that LS is a highly penetrant disease, indicating that an MMR mutation carrier has a higher lifetime risk of developing colonic or extra-colonic tumours than the general population (Lynch *et al.,* 2006).

Patients with LS can have synchronous and metachronous colorectal neoplasms and other primary extracolonic malignancies. Synchronous tumours are present at the same time whereas metachronous tumours are present within 10 years of each other. It is estimated that about 30% of patients will develop a second primary CRC within 10 years of developing the first primary CRC (Lynch *et al.,* 2006).LS mutation carriers have an increased risk of developing colon adenomas, and the onset of adenomas appears to occur at a younger age than in non-mutation carriers from the same families. [De Jong et.al, 2004] Unlike patients with sporadic cancers, whose cancer develops most often in the left side of the colon, approximately two-thirds of LS cancers develop in the right side of the colon, defined as proximal to the splenic flexure. It is also known that progression from adenoma to carcinoma is accelerated in LS patients. An adenoma may develop into a carcinoma within 2 years of development whereas this process usually takes about 8 years in sporadic CRC (Lynch *et al.,* 2006). This is a feature of LS that makes identification and frequent screening of carriers very important.

In the general Caucasian population, the average age for sporadic CRC development is about 65. However, the average age of cancer development is about 45 years in HNPCC patients (Lynch *et al.,* 2006). It is this feature which the Bethesda criteria uses to determine if an individual should be sent for further investigation.

Between 70% and 85%, of colonic tumours develop in the proximal colon, with the estimated lifetime risk of CRC between 25% and 75% in carriers (de la Chapelle, 2004; Jasperson *et al.,* 2010). The risk of developing the other extra-colonic cancers is not as high as CRC but identification of people with MMR mutations can allow those individuals to detect these cancers early (Vasen *et al.,* 2007). In addition, the risk of developing an extra-colonic cancer increases with age (Watson *et al.,* 2008).

The tumours in LS patients tend to be poorly differentiated and contain an excess of mucoid and signet-ring cells. The tumours also display an excess of infiltrating

lymphocytes among the cells which has been linked to the improved prognosis in LS due to the lymphocytes suspected of having cytotoxic effects (Lynch *et al.,* 2006; Boland *et al.,* 2008; Boland and Goel, 2010).

MSI is an indicator that the MMR genes are non-functional. A tumour that is deficient of its MMR enzymes exhibit MSI as they are unable to correct the errors in the microsatellites. Since the errors are not corrected, the mutations accumulate within the cell. It is estimated that approximately 12% to 17% of all CRCs are deficient in the MMR proteins (Boland and Goel, 2010; Cunningham *et al.,* 2010).

Interestingly, MSI in CRC has been found to be a good prognostic factor and molecular marker. Tumours that have a high frequency of MSI maintain their diploid state but also have certain other features. These features include a more proximal colon localization, poor differentiation, contain tumour infiltrating lymphocytes, are less likely to invade and are less likely to contain mutations in KRAS or p53 (Popat *et al.,* 2005; Boland and Goel, 2010).

2.1.4 Criteria for defining HNPCC families

The research criteria for defining HNPCC families were established by the International Collaborative Group (ICG) meeting in Amsterdam in 1990, and are known as the Amsterdam criteria (Vasen *et al.,* 1991).

2.1.4.1 Amsterdam criteria:

- One member diagnosed with CRC before age 50 years.
- Two affected generations.
- Three affected relatives, one of them a first-degree relative of the other two.
- FAP should be excluded.
- Tumours should be verified by pathological examination.

These criteria provide a general approach to identifying HNPCC families, but they are not considered comprehensive; a number of families who do not meet these criteria, but have germline MMR gene mutations, have been reported. [Peltomäki *et al.,* 1997; Beck *et al.,* 1997]

To address these issues and to improve the diagnosis of HNPCC clinically, the ICG developed revised criteria in 1999; these are known as Amsterdam criteria II. (Vasen *et al.,* 1999).

2.1.4.2 Amsterdam criteria II:

There should be at least three relatives with a HNPCC-associated cancer (CRC or cancer of the endometrium, small bowel, ureter, or renal pelvis).

- One should be a first-degree relative of the other two.
- At least two successive generations should be affected.
- At least one should be diagnosed before age 50 years.
- FAP should be excluded in the CRC cases.
- Tumors should be verified by pathological examination.

Although these criteria are among the most stringent used to identify potential candidates for microsatellite and germline testing, it must be cautioned that by definition, familial CRC type X includes families meeting Amsterdam criteria but in whom there is no evidence of MSI.

A third set of clinical criteria that can be used to identify HNPCC families is the revised Bethesda guidelines. (Umar *et al.,* 2004). The criteria was expanded to improve sensitivity in identifying families. The Bethesda guidelines are the least stringent for identifying families with germline mutations in one of the MMR genes. Because of lack of specificity for HNPCC, the Bethesda guidelines are utilized to identify individuals whose colorectal tumors should be tested for MSI and/or IHC, rather than to identify families that meet clinical criteria for HNPCC.

2.1.4.3 Revised Bethesda Guidelines for Testing of Colorectal Tumors for MSI:

- CRC diagnosed in an individual younger than 50 years.
- Presence of synchronous, metachronous colorectal, or other LS-associated tumors.*
- CRC with MSI-high (MSI-H) pathologic associated features diagnosed in an individual younger than 60 years. [Note: Presence of tumour-infiltrating lymphocytes, Crohn-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.]
- CRC or LS-associated tumour* diagnosed in at least one first-degree relative younger than 50 years.
- CRC or LS-associated tumour* diagnosed at any age in two first-degree or second-degree relatives.

*LS-associated tumors include colorectal, endometrial, stomach, ovarian, pancreatic, ureter and renal pelvis, biliary tract, and brain tumors; sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome; and carcinoma of the small bowel. (Umar *et al.*, 2004; Laghi *et al.,* 2004) Data from the Cancer Family Registry suggest that breast and prostate cancer may also be considered in the spectrum of LS-associated tumours. (Win *et al.,* 2012)

Research has included CRC families who do not meet Amsterdam criteria for LS and/or in whom the colorectal tumors are microsatellite stable (MSS). A number of these families have been found to have mutations in MSH6. (Miyaki *et al.,* 1997; Akiyama *et al.,*1997; Wu *et al.,* 1999; Kolodner *et al.,* 1999; Plaschke *et al.,* 2004) While the clinical significance and implications of these findings are not clear, these observations suggest that germline mutations in MSH6 may predispose to late-onset familial CRCs that do not meet Amsterdam criteria for LS and tumors that might not necessarily display MSI.

2.2 DNA Mismatch Repair

2.2.1 MMR in Escherichia coli

Mechanisms and functions of mismatch correction are best understood in Escherichia coli. In Escherichia coli, MMR increases the accuracy of DNA replication by 20–400-fold (Schaaper *et al.,* 1993). MMR is directed by the state of adenine methylation of GATC sequences. Since DNA adenine methylation (Dam) occurs after replication, an unmethylated newly synthesized strand is temporarily paired with a fully methylated parental strand, which provides a strand discrimination signal for MMR in E. coli (Lahue & Modrich, 1989). Initiation of MMR occurs via mismatch recognition and binding of a MutS homodimer followed by binding of a MutL homodimer. In E. coli MutL serves to couple mismatch recognition with downstream MMR events. Interactions between MutL, MutS, and ATP are believed to result in translocation of the MutS-MutL complex away from the mispair, leading to the activation of the MutH endonuclease (Allen *et al.,* 1997; Hall & Matson, 1999). After activation, MutH introduces a nick in the nascent strand of the nearest hemi-methylated GATC sequences. MutL helps to load DNA helicase II (UvrD) at the nicked GATC site and UvrD unwinds DNA from the nick toward and past the mismatch (Hall *et al.,* 1998). Removal of the error-containing DNA strand is facilitated by one of four single-stranded, DNA-specific exonucleases (RecJ, ExoI, ExoVII, ExoX) depending on the polarity of the reaction (Viswanathan & Lovett, 1998; Burdett *et al.,* 2001). The resulting single-stranded gap, is stabilized by single-strand binding protein (SSB) and filled by DNA polymerase III holoenzyme. The remaining nick is closed by DNA ligase and Dam methyltransferase finishes the MMR pathway in E. coli by methylating the newly synthesized strand (Rasmussen *et al.,* 1998).

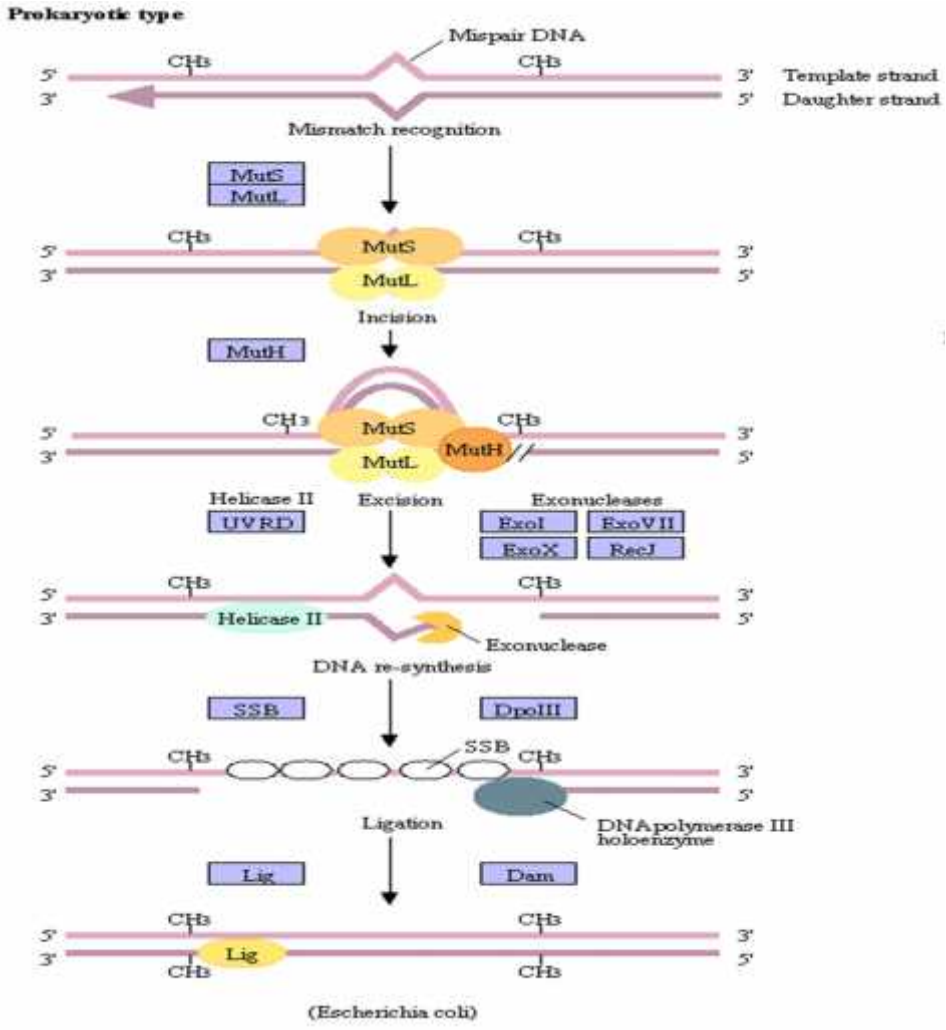


Figure 2.1: Schematic model of MMR in E.coli (KEGG Pathway: mismatch repair)

2.2.2 MMR in Eukaryotes

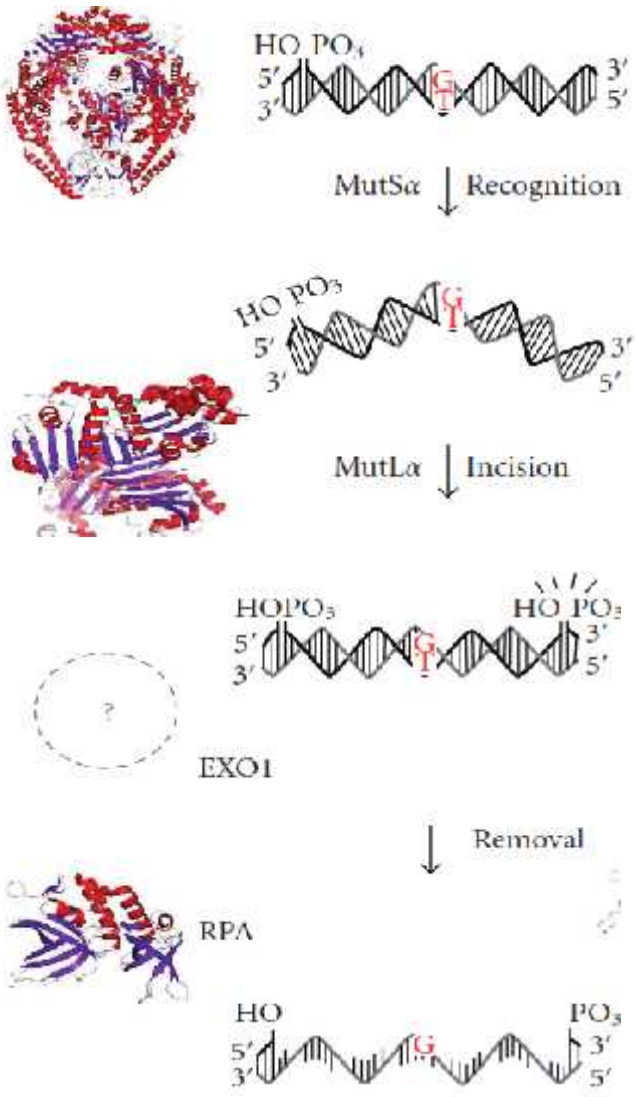
In eukaryotic cells also has two complex heterodimers which is homologues to MutS of E. coli- in which MutSα is formed by the MSH2-MSH6 complex and MutSβ by MSH2-MSH3(Kolodner *et al.,*1999). Other six homologues to MutS are presence in eukaryotic cells (Peltomäki, 2005).

The MSH2-MSH6 complex represents 80% to 90% of the cellular level of MSH2 and its function is to recognize the mismatch of base -base insertions and deletions, to contain one or two unpaired nucleotides, but it is also capable of recognizing large deletions and insertions (Jiricny, 2000)

Mispaired bases in DNA are recognized by the heterodimeric complexes, MSH2-MSH6 and MSH2-MSH3 (MutS homologs) (Acharya *et al.,* 1996; Guerrette *et al.,* 1998, Genschel *et al.,* 1998). Analysis of mismatch binding specificities of the human hMSH2-hMSH6 and hMSH2-hMSH3 complexes showed that they were overlapping but not identical. The hMSH2-hMSH6 complex recognizes base:base mispairs and insertion/deletion mispairs of up to 8 unpaired bases. In comparison, hMSH2-hMSH3

has a high affinity for insertion/deletions of 2-8 unpaired bases, weak affinity for single-nucleotid insertion/deletion mispairs, and do not bind base:base mispairs (Drummond *et al.,* 1997; Genschel *et al.,* 1998). The predominant DNA-binding protein in the hMSH2-hMSH6 complex appears to be hMSH6 when binding to a mismatched oligonucleotide (Matton *et al.,* 2000). Hence, the hMSH2-hMSH6 complex appears to provide the predominant mismatch-binding activity in human cells (Genschel *et al.,* 1998; Marra *et al.,* 1998). The hMSH3 protein is believed to compete with hMSH6 for the available hMSH2, as the interacting regions of hMSH2 with hMSH3 and hMSH6 are identical (Guerrette *et al.,* 1998).

In eukaryotic cells also have three complex homologues to MutL of E. coli: MLH1-PMS2 (MutLα), MLH1-PMS1 (MutLβ) and MLH1-MLH3 (MutLγ). MutLα is the most active of these complexes in humans and supports repairs initiated by the MutS complex (Iyer *et al.,* 2006)



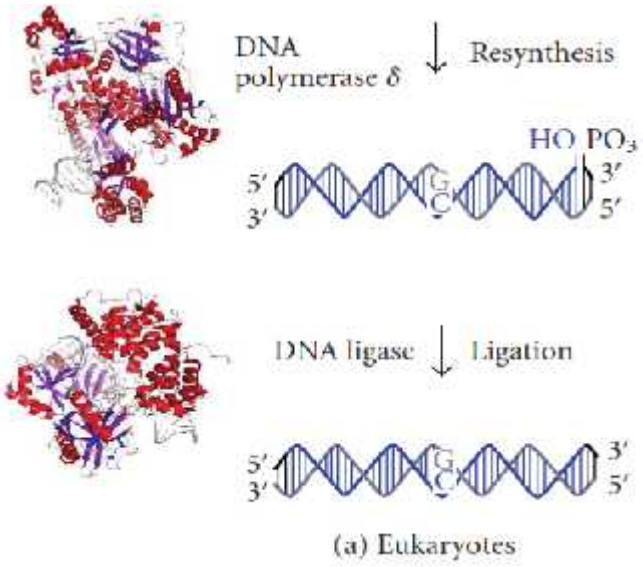


Figure 2.2: A schematic representation of MMR pathway models of Eukaryotic MMR (Kenji Fukui, 2010)

(A DNA mismatch is generated by the misincorporation of a base during DNA replication. MutSα recognizes base-base mismatches and MutLα nicks the 3 -side of the mismatched base on the discontinuous strand. The resulting DNA segment is excised by the EXO1 exonuclease, in cooperation with the single-stranded DNA-binding protein RPA. The DNA strand is resynthesized by DNA polymerase δ and DNA ligase 1).

2.3 Mismatch Repair Genes

HNPCC is known to be caused by a loss of function in one of the MMR genes, also known as the 'mutator phenotype'. There is usually one of four genes that are commonly mutated in HNPCC and they are MLH1, MSH2, MSH6 or PMS2. Of these four genes, MLH1 and MSH2 are the most commonly mutated genes in HNPCC, accounting for about 40% and 50% of all HNPCC cases, respectively (Jasperson *et al.,* 2010). These two genes are the focus of this study because they are the most commonly mutated genes. A summary of the contribution towards HNPCC from each MMR gene can be seen in Table

Percentage of HNPCC/LS cases caused by mutation in each MMR gene (Peltomaki, 2005)

Gene	Percentage of HNPCC case
MLH1	40%
MSH2	50%
MSH6	~10%
PMS2	<1%

2.3.1 MLH1

The MLH1 locus had been known to be involved in HNPCC through linkage studies and it is a highly penetrant gene (Nyström-Lahti *et al.,* 1994, Kolodner *et al.,* 1995). Interestingly, each of the major genes in HNPCC has been found to be associated with specific tumours. Families with MLH1 mutations tend to have a higher incidence of CRC than families with mutations in the other genes (Kolodner *et al.,* 1995; da Silva *et al.,* 2009)

Characterization of MLH1 was an important step in understanding HNPCC as it gave further insights into the aetiology of HNPCC and allowed for enhanced mutation detection. After characterizing MLH1, Kolodner *et al.* (1995) performed mutation screening in a large family who were shown to have possible MLH1 mutations by linkage analysis. Using direct DNA sequencing of the gene, it was found that an insertion led to a frame shift mutation which produced a truncated MLH1 protein and it was this mutation that was responsible for HNPCC in that family.

Kosinski *et al.* (2010) determined that the dimerization of MLH1 and PMS2 occurs via their C-terminal domains and involves residues 531 to 549 and 740 to 756 in MLH1 and residues 679 to 699 and 847 to 862 in PMS2.

Herman *et al.* (1998) reported that hypermethylation of the 5-prime CpG island of the MLH1 gene is found in most sporadic primary colorectal cancers with MSI and that this methylation was often, but not invariably, associated with loss of MLH1 protein expression. Hitchins *et al.* (2007) found evidence that the epimutation was transmitted from a mother to her son but was erased in his spermatozoa.

Gazzoli *et al.* (2002) reported a case of HNPCC where methylation of one allele was present in the blood, but both alleles were methylated in the tumour. The pathogenesis suggested a constitutional MLH1 epimutation. Hesson *et al*. (2010) indicate that many cases with a similar pathogenesis have been reported but that the epimutation may not be present in the germ line. However, those individuals are predisposed to HNPCC by way of methylation of the MLH1 locus in somatic tissue, proposed to be caused by cis-acting elements in the same region as MLH1.

MLH1 mutations have been associated with the entire spectrum of malignancies associated with LS.[Peltomäki,2003] The lifetime risk of CRC in MLH1 mutation carriers is estimated to be 41% to 68%.(Choi *et al.,* 2009;Bonadona *et al.,*2011; Hampel *et al.,*2005) The lifetime risk of endometrial cancer is estimated to be approximately 40%. (Bonadona *et al.,* 2011; Vasen *et al.,* 1996) Muir-Torre syndrome is less commonly associated with MLH1 mutations than are MSH2 mutations. (Kastrinos *et al.,* 2008)

2.3.2 MSH2

Similarly to MLH1, MSH2 is highly penetrant, but mutations in MSH2 have been found to be more associated with extra-colonic tumours and Muir-Torre features (Kolodner *et al.,* 1994; da Silva *et al.,* 2009).

Germ line mutations in MSH2 tend to lead to abolition of the protein. These mutations include premature stop codons, altered splice sites or rearrangements (Boland *et al.,* 2008). However, epimutations have also been discovered. Terminal mutations in the EPCAM gene, located upstream of MSH2, lead to an abnormal EPCAM-MSH2 fusion transcript and MSH2 promoter methylation (Hesson *et al.,* 2010). This EPCAM mutation leads to reduced expression of the MSH2 gene. This EPCAM mutation confers the same risk of tumour development as MSH2 mutations in familial cancer (Hesson *et al.,* 2010).

The lifetime risk of colon cancer associated with MSH2 mutations is estimated to be between 48% and 68%.(Choi *et al.,* 2009;Bonadona *et al.,*2011; Hampel *et al.,*2005) MSH2 mutations were reported in 38% to 54% of LS families (Quehenberger *et al.,*2005; Barnetson *et al.,*2006)

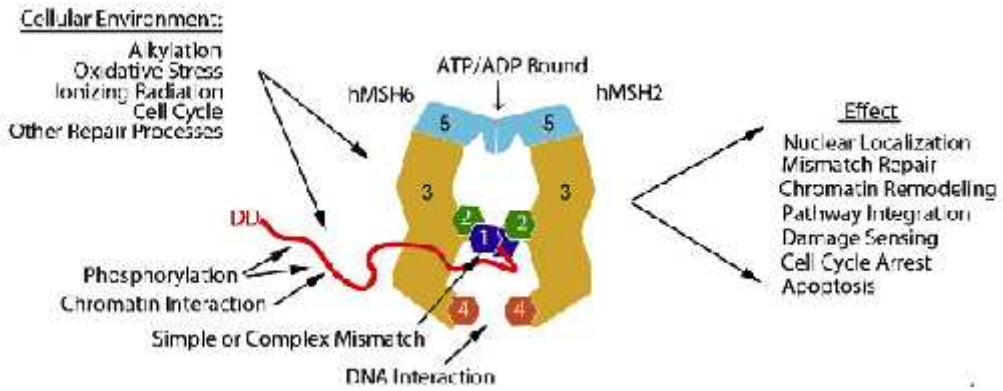


Figure 2.3: hMutSα in DNA binding configuration. Environmental influences (cellular environment) and subsequent effects on hMutS are briefly listed (adapted from Warren *et al*., 2007)

2.3.3 MSH6

The mean age of onset of CRC in families with MSH6 mutations is 56 years, as opposed to 45 years in classical HNPCC. This attenuated phenotype is the result of compensation provided by MSH3 in the cell (Acharya *et al.,* 1996; da Silva *et al.,* 2009; Talseth-Palmer *et al.,* 2010). In addition to later age of onset, women in families with MSH6 mutations tend to develop endometrial cancers more frequently, with a 71% lifetime risk by age 70 (Peltomäki, 2005; da Silva *et al.,* 2009; Talseth-Palmer *et al.,* 2010).

Although MSH6 is responsible for roughly 10% of cases, it is estimated that many cases involving MSH6 are overlooked. One reason for under-representation in these families is due to lack of routine testing of MSH6 in many laboratories. Another reason for under-representation is that families with MSH6 mutations have an attenuated phenotype that does not fulfil the Amsterdam criteria, or the Bethesda guidelines. There are reports that there is a high incidence of MSH6 mutation carriers in Amsterdam negative families (Boland *et al.,* 2008; da Silva *et al.,* 2009; Sjursen *et al.,* 2010).

2.3.4 PMS2

PMS2 gene is crucial in the repair system.PMS2 has been found to be mutated in families with HNPCC but is reported in less than 1% of all HNPCC cases. PMS2 is however, also thought to be under-represented due to the attenuated phenotype of older age of onset and weak family history (da Silva *et al.,* 2009).

Paradoxically, PMS2 mutations are also thought to lead to childhood onset of tumours when two non-functional alleles are inherited. One challenge facing PMS2 management is the limited number of observed cases, which is one reason why there is no clear phenotype described for this gene (Boland *et al.,* 2008; da Silva *et al.,* 2009).

Shimodaira *et al.* (2003) found that PMS2 interacted with and stabilized the apoptotic protein p73 and that the interaction caused redistribution of PMS2 to the nuclear compartment. Yuan *et al*. (2002) noted that the MLH1 and MSH2 proteins interact with PMS1, PMS2, and MSH6 proteins, and that missense mutations in specific regions of MLH1 have been shown to lead to defects in protein-protein interactions with PMS2.

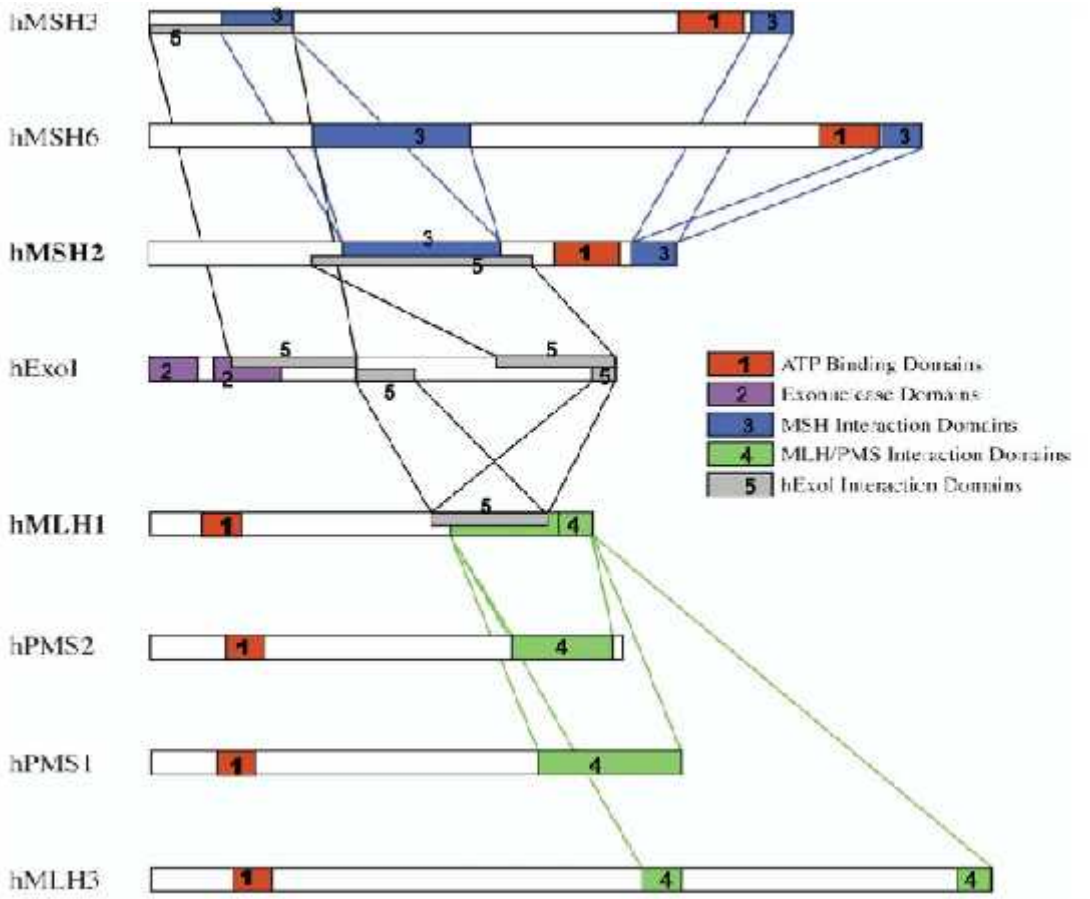


Figure 2.4: Interaction among the DNA MMR proteins. Each of the MutS homologues (MSH3, MSH6, and MSH2) interacts as a heterodimer with the MutL homologues (MLH1, PMS2, PMS1, and MLH3), acting as heterodimers, and the exonuclease, ExoI. Mutations that occur in the interactive domains may abrogate the ability of these proteins to interact and function in DNA MMR, but in some instances, the mutations may not lead to destabilization and loss of the protein product. The interactive domains among the MutS homologues, among the MutL homologues, and between one another are illustrated here. (Adapted from Boland and Fishel, 2005)

2.3.5 Recurrent and Founder Mutations

These above four MMR genes, MLH1, MSH2, MSH6 and PMS2, are the main genes involved in the aetiology of HNPCC. There is a wide range of mutations found in HNPCC families. However, it has been found that occasionally the same mutations occur in families that are unrelated. There are two explanations for these occurrences. The first explanation is that these mutations are recurrent mutations that occur within gene 'hot spots'. These hot-spots are prone to mutations, which often appear de novo in individuals (De la Chapelle, 2004; Lynch *et al.,* 2006).

Founder mutations have significant impact for individuals and molecular genetic diagnosis (Lynch *et al.,* 2006). As a first step in molecular diagnosis, those individuals from that population can be tested for the founder mutation as it provides a first line target for mutation detection, rather than whole gene sequencing. Targeted mutation analysis will save costs and facilitate better management (Lynch *et al.,* 2006).

2.3.6 Functions of the MMR Genes

The MMR gene products do not function independently of one another. Instead, they interact together in a complex pathway which is responsible for correcting errors and even play a role in apoptosis if damage is not repaired.

The MMR system is responsible for detecting and correcting DNA replication errors that occur on the newly synthesized strand of DNA. DNA polymerase occasionally makes a mistake when synthesising DNA and inserts an incorrect nucleotide. This can lead to a base mismatch or an insertion-deletion loop (Boland and Goel, 2010).

Within the cell, the predominant heterodimer for detecting mismatches on the DNA strand is known as MutSα. The heterodimer MutSα is made up of MSH2 and MSH6 proteins. MutSα is able to bind to base/base mismatches as well as 1bp insertion-deletion loops and repair them (Peltomäki, 2005).

A second mismatch recognition protein known as MutSβ consists of MSH2 and MSH3. The MutSβ complex functions to effectively eliminate 2-8bp unpaired nucleotides, which form an insertion deletion loop that the complex recognizes. The MutSα and MutSβ vary in their affinities due to the MSH6 or MSH3 protein bound to MSH2, with the MSH3 complex being able to bind to larger insertion-deletions loops than the MSH6 complex (Peltomäki, 2005; Boland and Goel, 2010).

The functions of the MutL complexes are not as well-known or understood as the MutS complexes.The MutLα complex, which consists of MLH1 and PMS2, is also vital to the functioning of the MMR system. This complex is considered important in bridging mismatch recognition with downstream repair processes, such as long-patch excision (Peltomäki, 2005; Boland and Goel, 2010).

The MLH1 protein is also able to dimerize with PMS1, forming the MutLβ complex. This complex has been shown to suppress mutagenesis in Saccharomyces cerevisiae (yeast), but the function of this complex has not been fully elucidated in humans (Boland and Goel, 2010). Similarly, the MutLγ complex, composed of MLH1 and MLH3, suppresses insertion-deletion loops in yeast but has an unknown function in humans (Boland and Goel, 2010).

2.4 Genetic Testing for HNPCC/LS

2.4.1 MSI Testing

CRC is associated with two forms of genetic instability: chromosomal instability (CIN) and microsatellite instability (MIN or MSI) (Sieber *et al.,* 2003). In contrast to the molecular basis underlying CIN, which is not precisely defined although it may well involve mutant APC, as APC is involved in spindle formation and correct chromosomal segregation, MSI is almost always associated with defective function of a small number of proteins, the products of the MMR genes MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2. (Lonov *et al.,* 1993; Thibodeau *et al.,* 1993)

Based on their MSI status, tumours can be divided into three categories: (i) those that show no MSI, termed microsatellite stable (MSS); (ii) tumours in which MSI is found at a high frequency (defined as ≥29%) of microsatellites tested (MSI-H); and (iii) tumours showing MSI at a low frequency (MSI-L) of microsatellite markers tested (1–28%, equivalent to 1 to 2 out of 10 markers).Laiho *et al.,*2002 and Halford *et al.,*2002 has shown that if enough microsatellite markers are tested, eventually one is found to be unstable, and hence all or almost all tumours exhibit at least MSI-L if enough microsatellites are analysed. Previous studies have also shown that MSI-L tumours appear phenotypically similar to the MSS tumours, so they are often categorized together with the MSS group. Thus, MSIH is due to defective MMR and can be considered as either 'Lynch syndrome-associated MSI' occurring due to inheritance of a MMR gene mutation, or 'sporadic MSI' occurring almost always due to hypermethylation of the MLH1 promoter. However, there is evidence that a tumour phenotype manifesting MSI-L may be associated with sporadic colorectal tumorigenesis and a cancer predisposition condition distinct from LS. (Jass, 2007; Jass *et al.,* 2000)

MSI occurs in approximately 12-17% of all CRC's. Cases of sporadic CRC with MSI tend to be in older patients who, additionally, do not show familial clustering of CRC. MSI in sporadic CRC is often the result of biallelic methylation of MMR genes, typically MLH1 (Boland and Goel, 2010). However, MSI is present in more than 90% of cases of HNPCC due to the deficiency of the MMR proteins (Boland *et al.,* 2008; Boland and Goel, 2010).

MSI testing allows one to determine whether a microsatellite has expanded and can be performed simply and cheaply using PCR techniques (Boland and Goel, 2010). The National Cancer Institute (NCI) has endorsed a panel of five microsatellite markers which include two mononucleotide microsatellites (BAT25 and BAT26) and three dinucleotide microsatellites (D2S123, D5S346 and D17S250) (Vasen *et al.,* 2007; Pineda *et al.,* 2010; Boland and Goel, 2010). The tumour DNA is compared to the blood DNA to determine if the microsatellites show expansions or deletions. If there are no differences among any microsatellites, then the tumour is microsatellite stable. If one marker shows a change in

size, then the tumour has low MSI (MSI-L). If two or more markers are unstable, then the tumour is said to have high MSI (MSI-H) (Pineda *et al.,* 2009; Boland and Goel, 2010).

MSI testing has been shown to be very sensitive for predicting the presence of MMR mutations. This testing can be very useful in families that have a high probability of having HNPCC, even when protein expression seems normal or in individuals who meet the Bethesda criteria to determine whether they should proceed to genetic analysis. The protein could be detected using IHC, but it may be non-functional due to missense mutations. MSI testing can be cost effective as it determines whether a tumour is caused by MMR deficiency before sequencing the MMR genes (Vasen *et al.,* 2007).

MSI testing also has disadvantages for testing. MSI testing requires a high proportion of tumour tissue in order to get good results. MSI testing has also been regarded as not being useful for MSH6 mutation carriers due to the lower level of MSI seen in their tumours. Furthermore, MSI may be acquired through somatic mutations in sporadic tumours, which would yield a false positive for inherited MMR gene testing (Pineda *et al.,* 2010).

2.4.2 Immunohistochemistry

IHC is the process of using antibodies, prepared against specific proteins, to determine if the protein is present in tissue. This process is achieved by labelling an antibody, then allowing it to bind to proteins in tissue. If the protein is not present, the antibodies cannot bind and are washed away (Strachan and Read, 2004). IHC has been an important histological tool for the molecular diagnosis of HNPCC.

When using IHC for the detection of loss of MMR, antibodies against MLH1 and MSH2 are usually used. However, to get a more complete result, antibodies against MSH6 and PMS2 should also be used (Vasen *et al.,* 2007). Since MSH2 and MLH1 are obligatory components in the MMR system, absence of these proteins leads to instability of their protein partners. If the MLH1 protein is absent, the IHC shows the loss of staining for both MHL1 and PMS2 and similarly, if MSH2 is absent, for MSH2 and MSH6 (Peltomäki, 2005; Boland *et al.,* 2008).

IHC has been shown to be very sensitive for detecting MMR expression but there is a higher rate of false negatives than with MSI testing. These false negatives are the result of detecting non-functional proteins or protein fragments. However, IHC allows one to detect which proteins are abolished, which can be used to direct mutation testing. This has been thought to be particularly useful in MSH6 mutation carriers (Lynch *et al.,* 2006; Vasen *et al.,* 2007). IHC has a lower cost and is also widely available in many pathology laboratories (Pineda *et al.,* 2010).

IHC also has several limitations which can affect its usefulness. These limitations include a lower sensitivity for detecting MLH1 expression. IHC does not distinguish between

germ line mutations or hyper-methylation as a mechanism for loss of expression. It can also be challenging to interpret the results from IHC staining, especially if there are no internal controls (Lynch *et al.,* 2006; Pineda *et al.,* 2010).

Both MSI testing and IHC have strengths and limitations but both have been shown to be very useful for detecting loss of MMR function in a tumour. Thus either test can be offered to patients as a first step to determine MMR status. However, there seems to be consensus that IHC is the more beneficial as it can direct mutation testing. (Boland and Goel, 2010).

2.4.3 Direct Sequencing and MLPA

Individuals that fulfil the Bethesda criteria can be screened using MSI testing or IHC but the ideal course of action is to sequence their DNA and determine if they harbour any germ line mutations in the MMR genes. Sequencing the MMR genes is the gold standard method for mutation detection and can be complemented by MLPA analysis (Boland and Goel, 2010).

Sequencing of the exons of these genes is most useful when using primers that include a portion of the intron/exon boundary. This allows the entire exon to be sequenced as well as the splice sites, where mutations are known to occur (Boland and Goel, 2010). However, some pathogenic mutations are small and may be missed by sequencing.

MLPA allows for the detections of large mutations that include the deletion or duplication of whole exons. If an exon is deleted and the breakpoint occurs outside of the sequencing primer binding site, then the sequencing analysis will not show any mutation as only the wildtype allele is sequenced and not the mutant allele. MLPA allows for the quantification of each exon, thus determining if an exon is missing or duplicated. Large deletions are not rare in HNPCC, with more than a third of mutations in MSH2 believed to be large exon deletions (Schouten *et al.,* 2002; Boland and Goel, 2010). Furthermore, the mutations detected by MLPA are almost certainly pathogenic as they affect large portions of coding DNA (Lynch *et al.,* 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Sample

The tissue embedded paraffin block sample was collected from B. P. Koirala Memorial Cancer Hospital (BPKMCH) from Bharatpur, Chitwan, Nepal. The sample has been approved by observing the haemoxylene and eosine staining slide. The clinical data was also taken in reference for proving the block contains the cancer tissue.

3.2 Optimization

3.2.1 Primary antibody concentration

Various concentrations of antibody were prepared by using various dilutions of the primary antibody as 1:50, 1:100 and 1:200.

3.2.2 Incubation time

The slide with the various antibody concentrations was incubated at various time intervals as 1 hours, 2 hours and overnight.

3.3 Cutting, mounting and fixing the section

The paraffin embedded block was sectioned in to 4 µm (microns) length (Leica RM4425). Before the slide was sectioned in to the 4 µm (microns) length the slide was kept at -20 for about 4 hours to make the slide fine.

After making the section the tissue section was mounted on the positively charged or APES (amino-propyl-tri-ethoxy-silane) coated slides (DakoCytomation, Glostrup,Denmark). After mounting the slide was kept dried to remove any water that may be trapped under the section. This was done by leaving the slide at room temperature overnight.

For the proper fixing the tissue in the positively charge slides were incubate at 60^0c in medite OTS 40 thermoplate for one hours.

3.4 Deparaffinizing and rehydrating the section

3.4.1 Materials and reagents

Xylene ,100% ethanol,95% ethanol,80% ethanol,70% ethanol,50% ethanol, Distilled water and TBS

3.4.2 Method

The slide was immersed twice with Xylene for 5 minutes each time. And then it was immersed twice with 100% Ethanol for 2 minutes each time after that it was again Immerse once with 95%, 90%, 80% and 70% Ethanol for 2-2 minutes respectively. After that the slide was immersed once with distilled water for 5 minutes. And it was immersed once with TBS for 5 minutes. Then slide was immersed again in distilled water for 5 minutes

Note: The section were not allowed to dry at any time until ready to perform antigen retrieval Drying will cause nonspecific antibody binding and therefore high background staining.

3.5 Endogenous peroxidase inhibition

3.5.1 Materials and reagents

3% H_2O_2 (Hydrogen Peroxide) and Distilled Water

3.5.2 Method

The slide was immersed in Hydrogen Peroxide Buffer for 10 minutes at room temperature. And then the slide was immersed in distilled water for 5 minutes.

Note: As the 3% H_2O_2 was prepared by mixing Methanol and Hydrogen Peroxide. They must be store in 4^0C and before using it must be bring in to the room temperature. Once it is prepared it can be run for around 80 slides only.

3.6 Antigen retrieval

For antigen retrieval the slide was immersed in Tris-EDTA pH 9.0 and was heated at 121^0C for 2 minutes by using biocare medical decloaking chamber. And then it was cooled on bench top for 30 minutes. Then the slide was immersed in distilled water for 5 minutes.

3.7 Immunohistochemical staining

The slide was put in coverplate and the coverplate with slide was kept in cassette base which was kept in Shandon Sequenza.

3.7.1 Blocking

The slide was first washed with TBS for three times and then Peroxidase blocking reagent (Dako Cytomation) was used to block endogenous peroxidase activity and then the ultra-blocker (dako.co, Denmark) about 200 µl was applied and kept the slide at room temperature for 5 minutes.

3.7.2 Application of primary antibody (anti human mouse antibody)

The slide was washed with TBS for three times and then 200 µl of 1:200 diluted antibodies (dako.co, Denmark, initial concentration hMLH1- 78.1 mg/L, hMSH2- 23.7mg/L) was applied and it was incubated at 4^0C for overnight. In case of negative control 200 µl of distilled water was used.

3.7.3 Application of secondary antibody

The slide was washed with TBS for three time and then secondary antibody ((DAKO Envision detection kit, peroxidase/DAB, Rabbit/Mouse) was applied and kept for 1 hours at room temperature. Then the slide was again rinsed with TBS for three times and slides was taken out of the coverplate of sequenza.

3.7.4 Application of Chromogen:

Excess buffer was removed from slide and freshly prepared DAB chromogen was added in the tissue section on the slide and kept at room temperature for about 5-10 minutes. Then the slides were washed in running tap water for 5 minutes.

Note: DAB chromogen must be prepare 1 hour earlier only. And it must be kept in 4^0C and it should not be kept in direct contact with light.

3.7.5 Counterstaining

The slide were dipped in hematoxylene for 10 dip after that the slide were washed in running tap water till the total hematoxylene is removed. And the slide was immerse in the TBS for 3 minutes.

3.8 Dehydrating

The slide was immersed in distilled water for 3 minutes and the slide was immersed in 70%, 80% and 95% Ethanol for 2-2 minutes respectively. And the slide was immersed twice with 100% Ethanol for 2 minutes each time then the slide was immersed twice with Xylene for 5 minutes each time.

3.9 Stabilizing with mounting medium

The slide was mounted with DPX and kept in room temperature for about 2 hours before observing in the microscope.

3.10 Viewing the staining under the microscope

The slides were observed under the light microscope (Olympus Cx31). And loss of expression of protein hMLH1 and hMSH2 were analysed. And pictures were taken by using Olympus DP72 (model Bx61TRF-FL-CCD) at 200 and 400X

3.11 Immunohistochemical Scoring.

All samples had a negative control slide (no primary antibody) of an adjacent section to assess the degree of nonspecific staining. Positive controls included appendix biopsy sample known to exhibit high levels of each marker. Nuclear immunostaining of normal epithelial cells, lymphocytes, and stromal cells served as internal positive controls. If the internal control cells were negative or only weakly stained, the stain was repeated.

And the immunohistochemical scoring is given by comparing with the internal control. The percentage score and Intensity score are given as follow:

Score	Percentage of staining
	0%(no tumour cell immunopositivity)
1	1-10% positive tumour cells
2	11-50% positive tumour cells
3	51-80% positive tumour cells
4	More than 80% positive tumour cells

(Adopted from Barrow *et al.,* 2010)

Score	Intensity
0	No
1	Very week
2	Week
3	Similar to control

(Adopted from Muller *et al.,* 2001)

Total score (T) is obtained by multiplying % scoring with intensity.

3.12 PCR amplification and sequencing

3.12.1 DNA extraction

DNA was extracted from paraffin embedded tissue sample by using Qiagen (QIAamp DNA FFPE Tissue Kit) and Geneall (genomic DNA FFPE kit) commercial available kit. Firstly the paraffin block with tissue was sectioned into 10 µm thin sections by using microtome and they were put in the 1.5 ml eppendrof tube and the paraffin was removed by using xylene. After this kit protocol (Qiagen and Geneall) was followed.

3.12.2 PCR amplification/sequencing

The DNA, after extraction from paraffin embedded tissue was loaded for the PCR reaction. For this 3µl (100ng) of DNA was loaded for 25 µl of total PCR reaction. The PCR is run with different annealing temperature according to the primer used. For

hMLH1 six set of primer was used and for hMSH2 seven set of primer was used. The amplified fragment got after the PCR were send for the sequencing.

3.13 Statistics

Statistical analyses were performed using GraphPad Prism v. 5 (GraphPad Software, Inc.). The association between age, gender, types Of Biopsy, tumour Diameter, diagnosis, tumour grade and site of tumour, and the association between immunohistochemistry result of hMLH1 and hMSH2 were examined using $\chi 2$ or Fisher's exact test for categorical variables and analysis of variance for continuous variables. P value of less than 0.05 was used as the criterion for statistical significance.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Immunohistochemistry scoring

Immunohistochemistry scoring of 43 samples by hMLH1 and hMSH2 is given in the following **table 4.1** and **table 4.2** respectively. Semi quantitative scoring system was used for our research which has been widely accepted and used in previous studies done by Mittal *et al.,* (2008).Based on the scoring obtained the immunohistochemistry, table was divided in to three group they are negative, moderate and positive. During scoring the percentage scoring (% score) is given according to the percentage number of cell that is stained. The percentage (%) of positivity was reported as has been previously described in the ICD-HNPCC study. Intensity number (I score) is given by comparing with the internal control i.e. staining with lymphocyte. The intensity (I) of immunoreaction of the nuclear compartment of the malignant epithelial cells was measured on 0-3 scale. This scale was based on comparison of intensity of reactivity of the tumour cells with the positive control cells. A score of 0 indicated no reactivity; a score of 3 denoted an intensity of tumour cell reactivity equivalent to positive control cells (Barrow *et al.,* 2010).

Total score (T) is obtained by multiplying % scoring with intensity. After getting T score the patient is divided in to three groups i.e. Negative, Moderate and Positive which is shown in **figure 4.1** for hHMLH1 and **figure 4.2** for hMSH2.

4.1.1 Immunohistochemistry Scoring of hMLH1 protein

No	Lab Number	hMLH1			
		% score	I score	T score	pos/neg
1	656	0	3	0	neg
2	1499	1	2	2	neg
3	5729	4	3	12	pos
4	5411	3	3	9	pos
5	487	4	3	12	pos
6	4095	4	3	12	pos
7	5362	4	3	12	pos
8	5238	4	3	12	pos
9	4319	4	3	12	pos
10	1872	4	3	12	pos

11	1933	1	1	1	neg
12	679	4	3	12	pos
13	427	4	3	12	pos
14	1660	4	3	12	pos
15	1581	4	3	12	pos
16	279	4	3	12	pos
17	1317	3	3	9	pos
18	5237	3	3	9	pos
19	1871	3	3	9	pos
20	513	3	3	9	pos
21	247	4	3	12	pos
22	827	3	3	9	pos
23	3455	4	3	12	pos
24	1737	3	2	6	pos
25	380	3	3	9	pos
26	431	4	3	12	pos
27	1470	2	2	4	neg
28	5718	1	3	3	neg
29	1516	4	3	12	pos
30	1592	3	2	6	pos
31	5407	4	2	8	pos
32	1751	3	2	6	pos
33	3450	4	2	8	pos
34	463	4	3	12	pos
35	1960	3	3	9	pos
36	3710	2	2	4	neg
37	1576	2	1	2	Neg
38	179	4	2	8	pos
39	851	3	3	9	pos
40	1332	4	2	8	pos
41	4134	3	2	6	pos
42	149	4	3	12	pos
43	2938	2	2	4	neg

Table 4.1: Immunohistochemistry Scoring of hMLH1 protein (%=Percentage of staining, I= intensity, pos=positive for Immunohistochemistry, neg= negative for Immunohistochemistry)

hMLH1

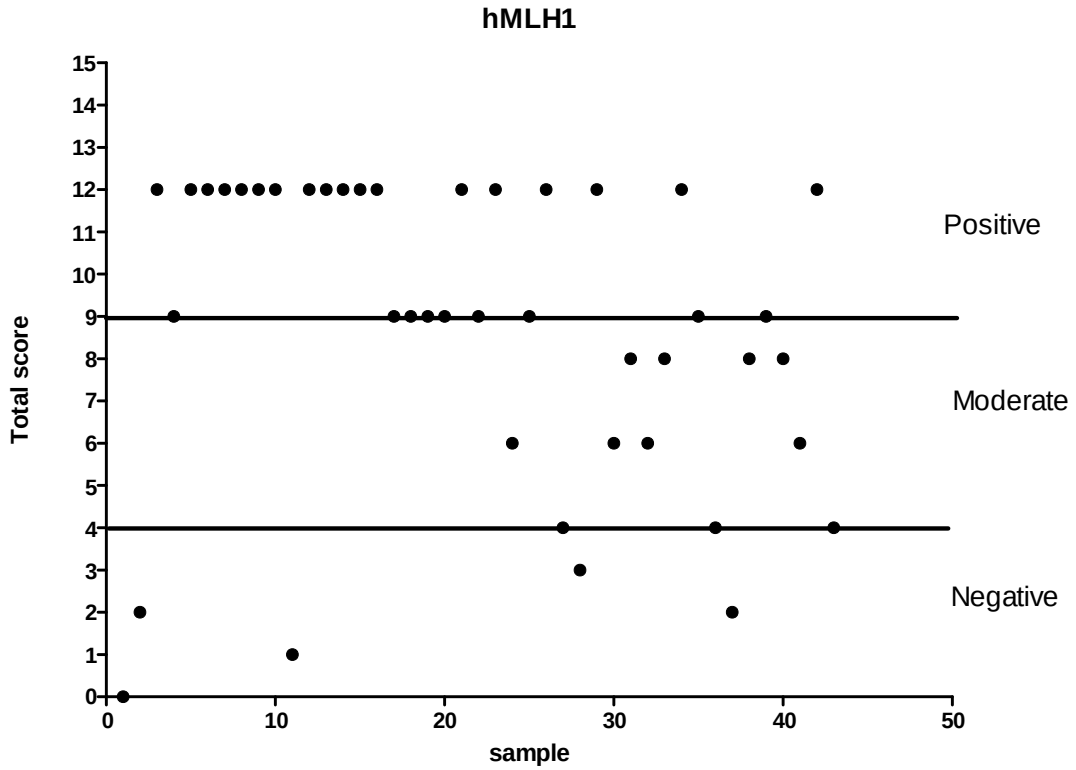


Figure 4.1: Total score obtain after immunohistochemistry of hMLH1 antibody

By evaluation of the total score obtained after immunohistochemistry of hMLH1 protein, out of 43 samples 18.6% (8 of 43) sample has the score less than 4 and they are the negative sample as they have the loss of expression of protein hMLH1. 39.53% (17 of 43) sample has the score between 4-9 and they are listed in the moderate staining. And the score more than 9 is listed in positive sample and 41.86% (18 of 43) sample has the total expressed protein.

4.1.2 Immunohistochemistry Scoring of hMSH2 protein

No	Lab Number	hMSH2			
		% score	I score	T score	pos/neg
1	656	3	2	6	pos
2	1499	1	2	2	neg
3	5729	4	3	12	pos
4	5411	4	3	12	pos
5	487	3	3	9	pos
6	4095	4	3	12	pos
7	5362	3	3	9	pos
8	5238	4	3	12	pos

9	4319	4	3	12	pos
10	1872	4	3	12	pos
11	1933	1	2	2	neg
12	679	4	3	12	pos
13	427	3	3	9	pos
14	1660	4	3	12	pos
15	1581	4	3	12	pos
16	279	4	3	12	pos
17	1317	3	3	9	pos
18	5237	3	3	9	pos
19	1871	3	3	9	pos
20	513	4	3	12	pos
21	247	3	3	9	pos
22	827	3	3	9	pos
23	3455	4	3	12	pos
24	1737	3	3	9	pos
25	380	4	2	8	pos
26	431	4	3	12	pos
27	1470	2	2	4	neg
28	5718	2	3	6	pos
29	1516	3	3	9	pos
30	1592	3	2	6	pos
31	5407	4	3	12	pos
32	1751	3	3	9	pos
33	3450	3	3	9	pos
34	463	4	2	8	pos
35	1960	3	3	9	pos
36	3710	3	3	9	pos
37	1576	3	2	6	pos
38	179	4	3	12	pos
39	851	3	3	9	pos
40	1332	0	3	0	neg
41	4134	3	3	9	pos
42	149	3	3	9	pos
43	2938	0	3	0	neg

Table 4.2: Immunohistochemistry Scoring of hMSH2 protein (%=Percentage of staining, I= intensity, pos=positive for Immunohistochemistry, neg= negative for Immunohistochemistry)

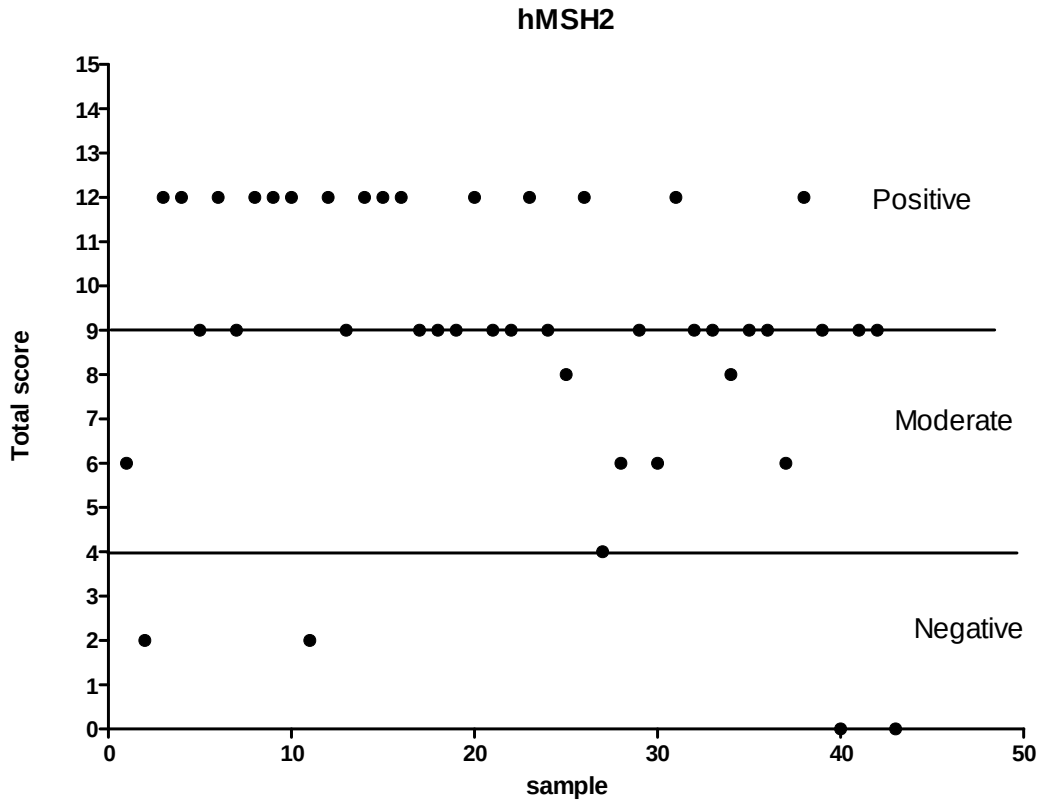


Figure 4.2: Total score obtain after immunohistochemistry of hMSH2 antibody

By evaluation of the total score obtained after immunohistochemistry of MSH2 protein, out of 43 samples 11.6% (5 of 43) sample has the score less than 4 and they are the negative sample as they have the loss of expression of protein hMSH2.and most of the sample i.e. 53.49% (23 of 43) sample has the score between 4-9 and they are listed in the moderate staining .And the score more than 9 is listed in positive sample and 34.88% (18 of 43) sample has the total expressed protein.

Our study showed only one sample has the score zero in scoring obtain after the immunohistochemistry by hMLH1 antibody but for hMSH2 antibody there are two sample that has the score zero. Semi quantitative scoring system was used for the scoring, in the method percentage (scored on a scale of 0-4) of staining is multiplied with the intensity (scored on a scale of 0-3) of the staining to produce total score of 0 to 12 and the score less than 4 is regarded to be negative and score more than 4 is regarded to be positive.

According to Allred *et al.* (1998) they have used Allred score which is semi quantitative system that takes into consideration the proportion of positive cells (scored on a scale of 0-5) and staining intensity (scored on a scale of 0-3). The proportion and intensity were then summed to produce total scores of 0 or 2 through 8. A score of 0 -2 was regarded as negative while 3 - 8 as positive 2.

Specific staining as described by Mc Carthy, Jr *et al,* (1985) the immunohistochemical scored is done as semi quantitative fashion incorporating both the intensity and the distribution of specific staining. The evaluations were recorded as percentages of positively stained tumour cells in each of the five intensity categories denoted as zero (no staining), 1+ (weak but detectable), 2+ (mildly distinct), 3+ (moderately distinct) and 4+ (strong). For each tissue a value designated as HSCORE was derived by summing up the percentages of cells staining at each intensity multiplied by the weighted intensity of staining. An HSCORE of less than 50 was established as negative, between 51 to 100 as mild (weak positive), 101 to 200 as moderate (intermediate positive),

4.2 Immunohistochemistry

Protein expression for hMLH1 and hMSH2 antigens was evaluated in 43 colorectal cancer tissue samples. Normal expression of the respective antigen was reported when there was nuclear staining in normal and cancer colon epithelium. Abnormal gene expression resulted in absent nuclear staining in cancer epithelium. Staining was successful in all cases though hMLH1 and hMSH2 staining was often of a heterogeneous pattern whereby many tumour cells nuclei were positively stained.

4.2.1 Picture

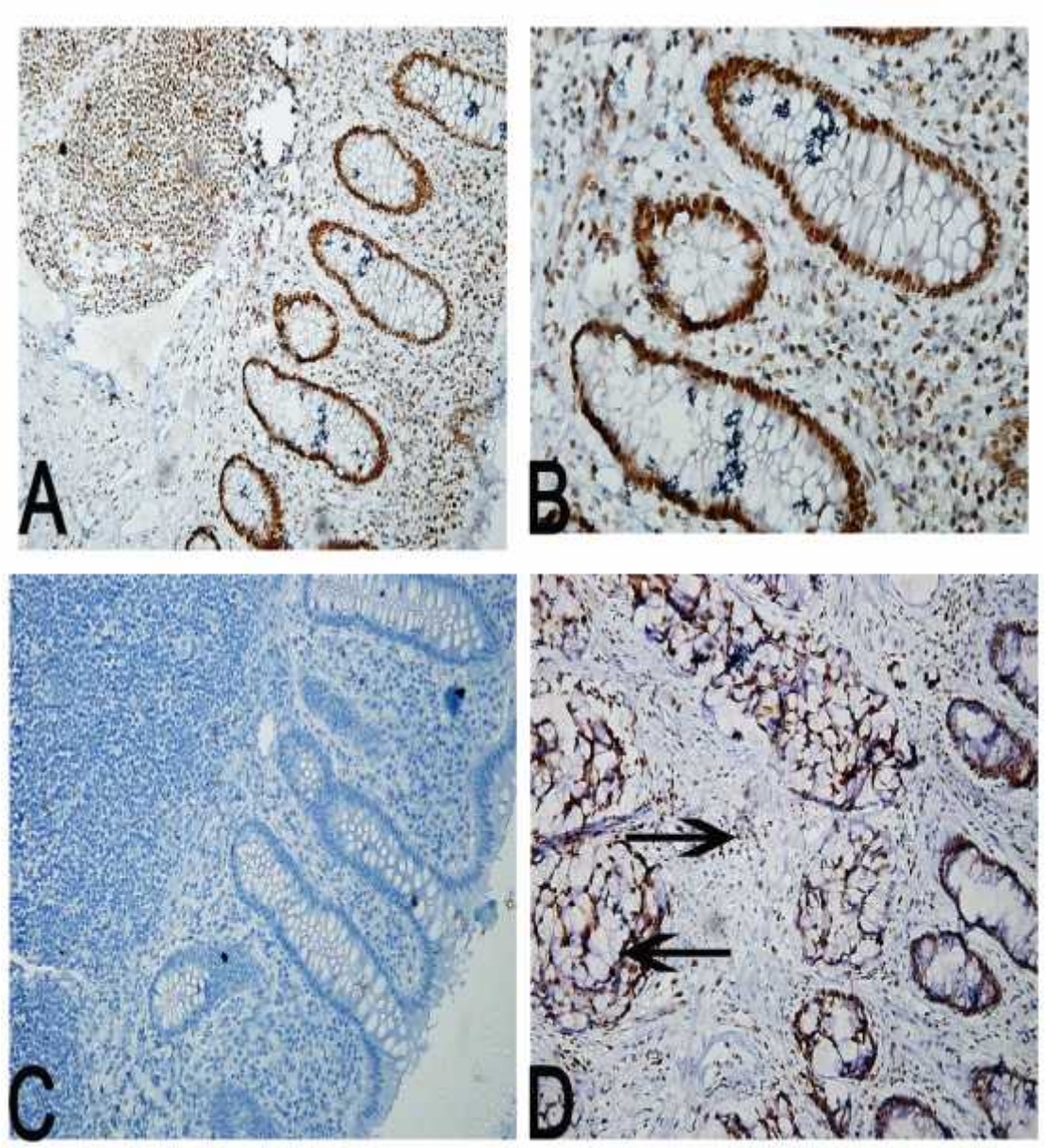


Figure 4.3: Immunohistochemical staining with hMLH1 for the mismatch repair proteins in normal and tumour tissue**. (A)** (Control positive) Normal appendix mucosa epithelium reactive to hHMLH1. IHC stain with hMLH1, original magnification x 200. **(B)** (Control positive) Normal appendix mucosa epithelium reactive to hMLH1. IHC stain with hMLH1, original magnification x 400 **(C)** (Control Negative) Normal appendix mucosa epithelium stained with Haematoxylin. IHC was carried out without primary hMLH1 antibody, original magnification x 200. **(D)** Colorectal carcinoma showing positive reaction to hMLH1. IHC staining with hMLH1, original magnification x 200 (for case 1516). The arrow showing lymphocytes acted as internal control and cancer tissue.

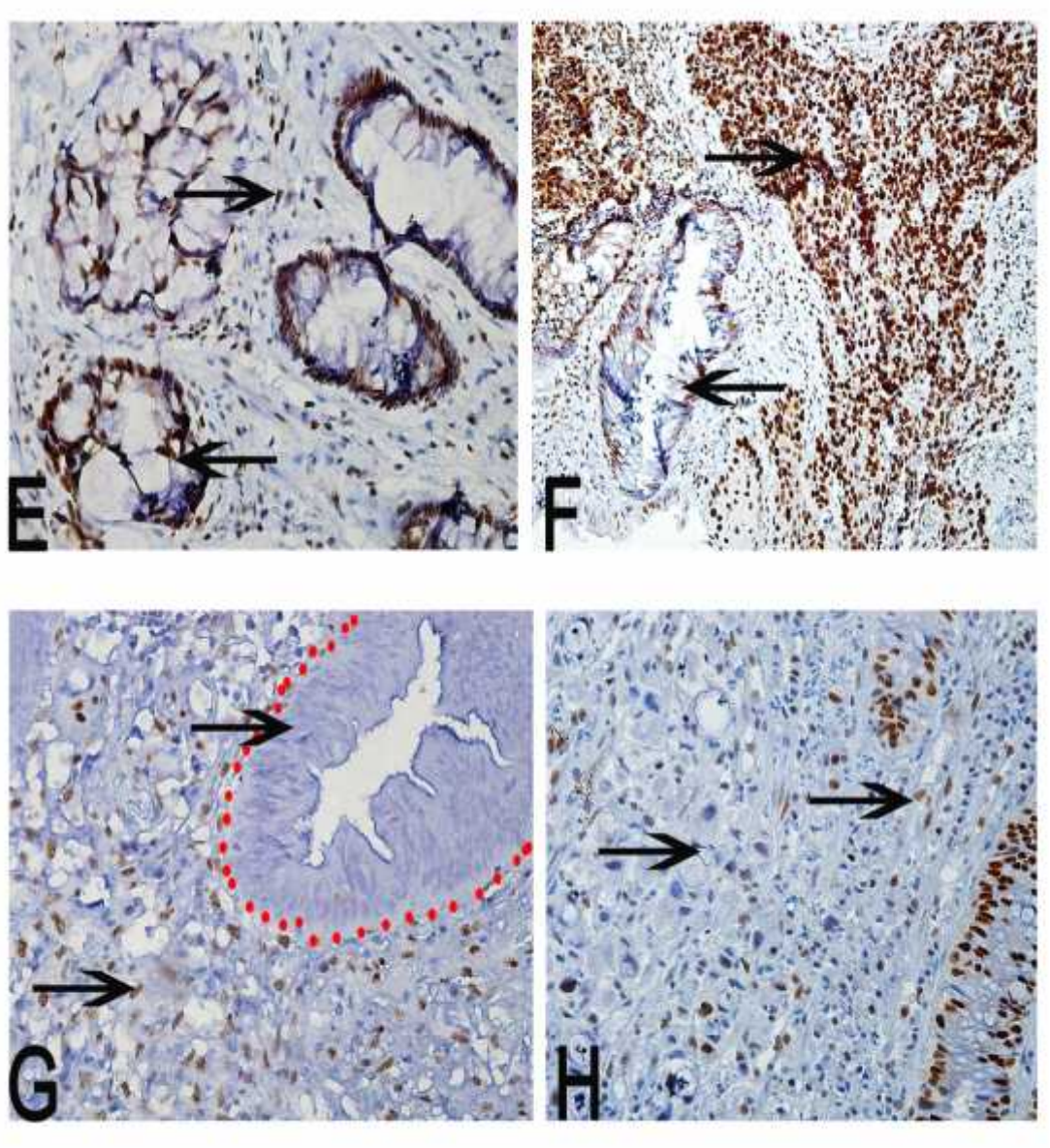


Figure 4.4: Immunohistochemical staining with hMLH1 for the mismatch repair proteins in tumour tissue **(E)** Colorectal carcinoma showing positive reaction to hMLH1. IHC staining with hMLH1, original magnification x 400 (for case 1516). **(F)** Colorectal carcinoma showing positive reaction to hMLH1. IHC staining with hMLH1, original magnification x 200 (for case 697). **(G)** Colorectal carcinoma showing negative reaction to hHMLH1. IHC staining with hMLH1, original magnification x 400 (for case 1499).Area circled by red dot shows the cancer tissue which lacks the staining with hMLH1. **(H)** Colorectal carcinoma showing negative reaction to hMLH1. IHC staining with hMLH1, original magnification x 400 (for case 5718). The lymphocytes (arrow) were reactive to hMLH1 and acted as internal control.

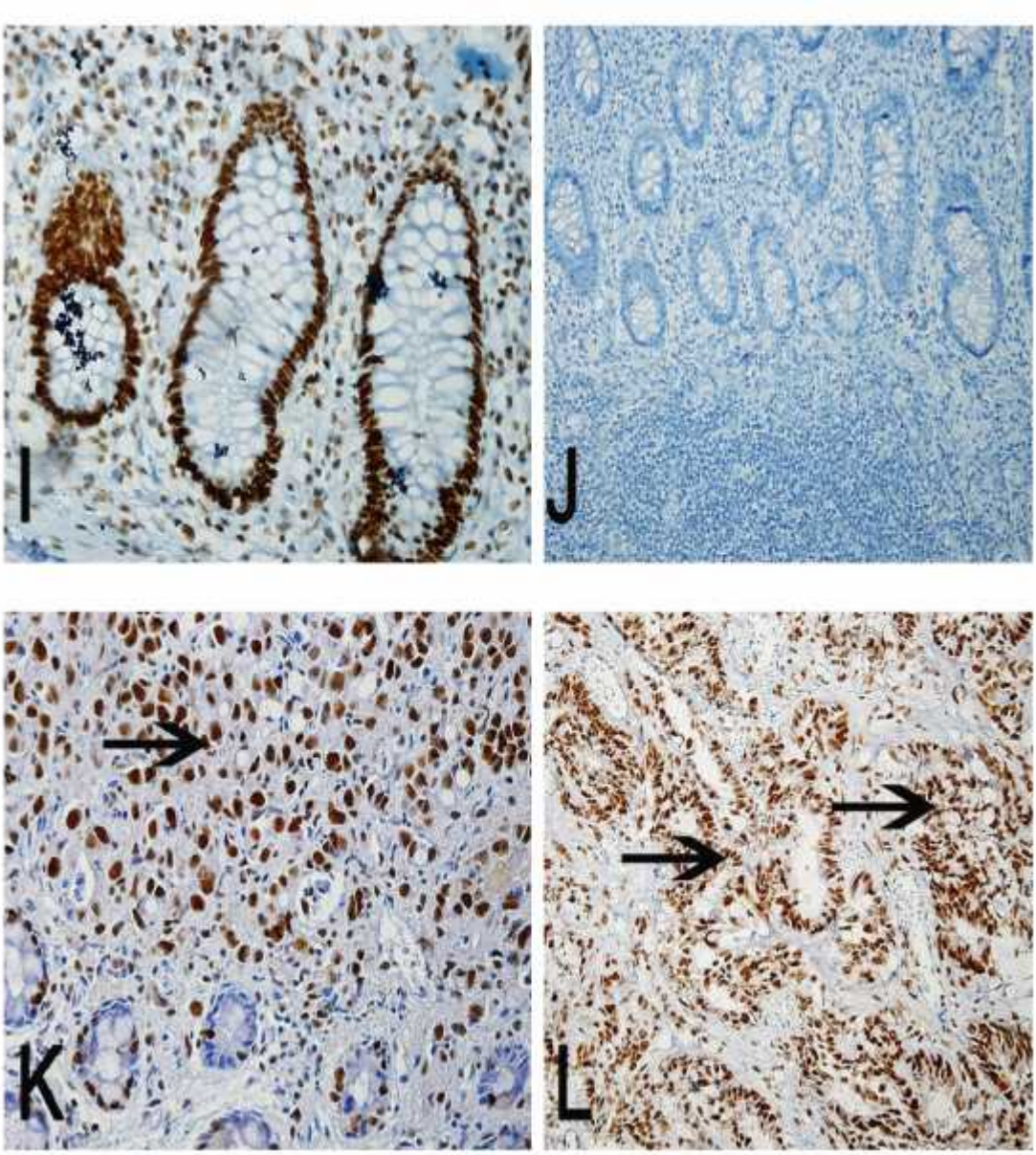


Figure 4.5: Immunohistochemical staining with hMSH2 for the mismatch repair proteins in normal and tumour tissue. **(I)** (control Positive) Normal appendix mucosa epithelium reactive to hMSH2. IHC stain with hMSH2, original magnification x 400. **(J)** (Control Negative) Normal appendix mucosa epithelium stained with Haematoxylin. IHC was carried out without primary hMSH2 antibody, original magnification x 200. **(K)** Colorectal carcinoma showing positive reaction to hMSH2. IHC staining with hMSH2, original magnification x 400 (for case 656). **(L)** Colorectal carcinoma showing positive reaction to hMSH2. IHC staining with hMSH2, original magnification x 200 (for case 656).The arrow showing lymphocytes acted as internal control and cancer tissue.

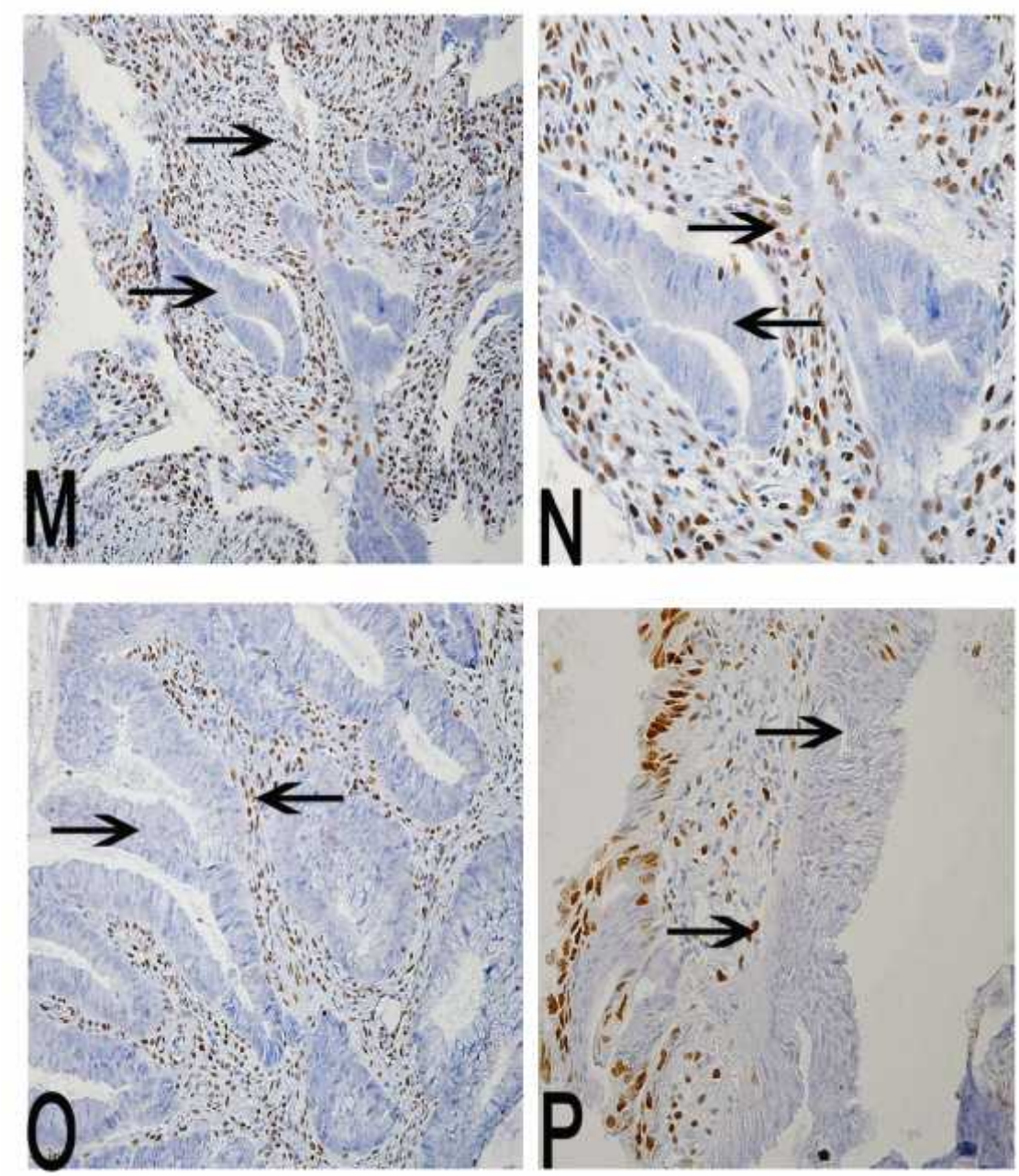


Figure 4.6: Immunohistochemical staining with hMSH2 for the mismatch repair proteins tumour tissue. **(M)** Colorectal carcinoma showing negative reaction to hMSH2. IHC staining with hMSH2, original magnification x 200 (for case 2938). **(N)** Colorectal carcinoma showing negative reaction to hMSH2. IHC staining with hMSH2, original magnification x 400 (for case 2938). **(O)** Colorectal carcinoma showing negative reaction to hMSH2. IHC staining with hMSH2, original magnification x 200 (for case 1332). **(P)** Colorectal carcinoma showing negative reaction to hMSH2. IHC staining with hMSH2, original magnification x 400 (for case 1933) the tissue show the squamous cell carcinomas. The arrow showing lymphocytes acted as internal control and cancer tissue.

Protein expression of hMLH1 and hMSH2 antigens was evaluated in tissue samples from 43 Nepalese CRC patients. The nuclei of normal epithelial cells, lymphocytes, and other stromal cells stained positively with monoclonal antibodies against hMLH1 and hMSH2 gene products. Eight out of 43 tissue samples (18.6%) showed absence of nuclear staining for hMLH1 and five out of 43 tissue samples (11.6%) showed loss of nuclear staining for hMSH2 in cancer epithelium cells. And four out of 43 tissue samples (9.3%) show the loss of nuclear staining for both hMLH1 and hMSH2 because of absence of the protein.

Our data provide the immunohistochemistry analysis of two proteins i.e. hMLH1 and hMSH2 of colorectal cancer patient of Nepalese patient. hMLH1 and hMSH2 is the important proteins which are involved in the mismatch repair of the DNA which may have the wrong sequence put during the replication by the DNA polymerase. Out of 43 patient 18.6% of the sample show the loss of protein hMLH1 and 11.6% of sample show the loss of expression of protein hMSH2 and among the 43 sample only 9.30% (4 of 43) sample show the loss of expression of both protein. In similar experiment done by Zahary *et al.* (2012) Protein expression of hMLH1 and hMSH2 antigens were evaluated in tissue samples from 34 Malaysian Lynch syndrome patients. Three out of 34 tissue samples (8.8%) showed absence of nuclear staining for hMLH1 and four out of 34 tissue samples (11.8%) showed loss of nuclear staining for hMSH2 in cancer epithelium cells.

And other similar work done by Zhoo, (2013) Of a total of 298 cases of colorectal carcinomas, 255 cases (85.6%) demonstrated normal nuclear expression for all mismatch repair proteins namely hMLH1, hMSH2 and hMSH6, while 43 cases (14.4%) showed abnormal staining patterns for at least one of the three mismatch repair proteins. Among mismatch repair defect tumours, 28 showed complete loss of hMLH1, 7 cases with loss of both hMSH2 and hMSH6, 6 cases of loss of hMSH6 and 2 cases of loss of hMSH2.And none of the cases show loss of all three MMR proteins.

Immunohistochemical analysis method is rapid, cost effective and reliable method for diagnosis of cancer and other genetic diseases in country like Nepal where other molecular technique are not available and reliable for the people. According to the Ward *et al*. (2001) immunohistochemical analysis of hMLH1 and hMSH2 expression is a rapid, cost-effective, and accurate method for the assessment of MMR status in colorectal adenocarcinomas. However, genetic analysis of MSI status is time consuming and expensive, and needs specialized equipment which is difficult to perform in lab of poor source.

Although immunohistochemistry is an extremely reliable tool for the detection of mutations that result in truncation and/or degradation of the antigen, it cannot distinguish between cells expressing variants of proteins carrying missense mutations

that inactivate, but do not destabilize, the protein and cells expressing wild-type polypeptides (Truninger *et al*.,2005).

According to Mangold *et al.(*2005) most hMSH2-mutant colorectal tumours are expected to show absent hMSH2 expression by IHC. And in another study done by Salahshor *et al*.(2001) and Peltomaki *et al*.(2004) find that more than one-third of the mutations in hMLH1 are missense mutations that may result in mutant proteins that are catalytically inactive but antigenically intact. Thus, on IHC, these mutant proteins may result in a false-normal staining pattern.

The loss of expression of protein hMLH1 and hMSH2 may not be due to mutation in the gene only but it may be due to the epigenetic inactivation as recent studies done by Cunningham *et al.* (1998) indicate that epigenetic inactivation of the hMLH1 gene by promoter methylation is the most prevalent mechanism of mismatch repair deficiency in sporadic colorectal tumours. Therefore, hypermethylation of the hMLH1 promoter region leading to transcriptional silencing of the hMLH1 gene is the probable cause of the loss of hMLH1 expression in the large majority of hMLH1 negative.

According to Marcus *et al.* (1999) Loss of immunohistochemical expression of hMSH2 is almost exclusively caused by an underlying germline mutation. In contrast to Thibodeau *et al.* (1998), loss of hMLH1 expression might either reflect a germline mutation or a somatic inactivation of hMLH1, which in the majority of cases is caused by epigenetic silencing through promoter hypermethylation.

Loss of expression of two protein hMLH1 and hMSH2 can result in loss of function of the MMR protein as in their functional state, the MMR proteins form heterodimers formed by MSH2-MSH6 forming the functional complex, MutSα (Acharya *et al.*,1996) and second MLH1-PMS2 forming MutLα (Kadyrov *et al.,*2006). Their abnormalities can result in proteolytic degradation of their dimer and consequent loss of both the obligatory and secondary partner proteins. The reverse, however, is not true. When mutation occurs in genes of the secondary proteins, i.e., MSH6 and PMS2, there may not be concurrent loss of the obligatory proteins, hMSH2 and hMLH1. This is because the function of the secondary proteins may be compensated by other proteins, such as MSH3, MLH3, and PMS1. As a result, mutations of hMLH1 or hMSH2 often cause concurrent loss of hMLH1/PMS2 or hMSH2/MSH6, respectively, by IHC, whereas mutations of PSM2 or MSH6 often cause isolated loss of PMS2 or MSH6 only.

But according to Hall *et al.* (2010) IHC testing for PMS2 and MSH6 alone has been suggested for the MMR protein. This strategy relies on the binding properties of the MMR heterodimer complexes, by which gene mutation and loss of hMLH1 and hMSH2 invariably result in the degradation of PMS2 and MSH6, respectively, but the converse is not true. The authors do not suggest a definitive algorithm after the finding of an IHC-deficient tumour. However, given the predominance of hMLH1 and MSH2

mutations in LS, the authors suggest that a PMS2-deficient tumour should be investigated for either hMLH1 hypermethylation (utilizing BRAF mutations status as a proxy) or germline hMLH1 mutation analysis. Similarly, MSH6 deficiency would generally result in hMSH2 germline testing as a first step. This strategy has not been validated or widely adopted in clinical practice.

IHC can be useful for testing the MSI of the tumour by protein expression using monoclonal antibodies of the hMSH2 and hMLH1. Loss of expression of these proteins appears to correlate with the presence of MSI and may suggest which specific MMR gene is altered in a particular patient (Thibodeau *et al.,* 1996; Cawkwell *et al.,* 1999; Lindor *et al.,* 2002).

IHC can be used for evaluation of the microsatellite instability status of the tumours rather than using molecular testing that consists of polymerase chain reaction (PCR) and gel electrophoresis to examine the DNA sequences; as the molecular testing was an expensive and time consuming test and not easily available. Lindor *et al.,(*2002) tested over 1000 colorectal cancers for DNA mismatch repair deficiency with both methods namely PCR and IHC detection for hHMLH1 and hMSH2 and showed that IHC was 92.3% sensitive and 100% specific for screening DNA mismatch repair defects. IHC was also able to suggest which gene was defective, which was not possible with PCR testing.

Dietmaier *et al.* (1997) estimated that immunohistochemistry cost less than a quarter of the price of MSI testing done with the PCR and gel electrophoresis method. IHC for mismatch repair gene could be introduced in most local laboratories and included as a routine test for selected cases of colorectal carcinomas. This could be followed with confirmatory genetic testing and further screening of family members for possible carriers.

4.3 Histopathologic Evaluation

4.3.1 Characteristics of Patients and CRCs in Relation to hMLH1 Status

Characteristic	N	hMLH1 proficient	hMLH1 deficient	P value
All patients (%)	43 (100%)	35(81.39)	8(18.69)	
Age (years)				
Mean (SD)	57.488(13.73)	60.06(12.59)	46.25(13.60)	
Median	58	60	40.50	
Range	30-85	30-85	35-69	

< 40	4	1	3	0.0086 (S)
40-49	10	7	3	
50-59	9	9	0	
60-69	10	8	2	
≥ 70	10	10	0	
Gender, no. (%)				
Male	27(62.79)	20(57.14)	7(87.5)	0.2230(NS)
Female	16(37.21)	15(42.86)	1(12.5)	
Types Of Biopsy, no. (%)				
Minor	42(97.67)	34(97.14)	8(100)	0.6286(NS) (Chi square)
Major	1(2.33)	1(2.86)	0(0)	
Tumour Diameter (mm), no. (%)				
≤ 20	31(72.09)	26(74.29)	5(62.5)	0.0806 (NS) (chi square)
21-50	9(20.93)	8(22.86)	1(12.5)	
>50	3(6.98)	1(2.85)	2(25)	
Diagnosis, no. (%)				
Adenocarcinomas	41(95.35)	34(97.14)	7(87.5)	0.3411(NS)
Squamous cell carcinomas	2(4.65)	1(2.86)	1(12.5)	
Tumour Grade, no. (%)				
Well differentiated	28(65.11)	25(71.43)	3(37.5)	0.1237(NS) (chi-square)
Moderately differentiated	10(23.25)	6(17.14)	4(50)	
Poorly differentiated	5(11.63)	4(11.42)	1(12.5)	
Site Of Tumour, no. (%)				
Rectum	26(60.47)	22(62.86)	4(50)	0.0652(NS) (chi-square)
Caecum	3(6.98)	3(8.57)	0	
Sigmoid colon	2(4.65)	0	2(25)	
Descending colon	1(2.32)	1(2.86)	0	
Ascending Colon	2(4.65)	2(5.71)	0	
Anal Canal	9(20.93)	7(20)	2(25)	

Table 4.3: Patient clinical characteristics related to the hMLH1(S=significant, NS= not significant)

Patient clinical characteristics related to the hMLH1 are presented in above **Table 4.3.** Among the 43 patient analysed, 27 are men and 16 are women. The mean age of the patient is 57.488 with the standard deviation of 13.73, the patient have the median age of 58 years (range, 30-85). The patient having the loss of expression of hMLH1 protein have the mean of 46.25 with the standard deviation of 13.60 and median age was 40.50 years (range, 35-69). The loss of expression of protein hMLH1 didn't show any significant correlation with the sex, Tumour Diameter, Diagnosis, Tumour Grade and Site of Tumour. But it showed the significant correlation with the age of the patient ($p< 0.05$).

The probable reason behind the non-significant relation between those parameter and hMLH1 protein expression may be due to low sample size i.e. less than 300. However the age of the patient show the significant correlation with the expression of the hMLH1 protein even at the low sample size (n=43).

4.3.2 Characteristics of Patients and CRCs in Relation to hMSH2 Status

Characteristic	N	hMSH2 proficient	hMSH2 deficient	P value
All patients (%)	43 (100%)	38(88.37)	5(11.63)	
Age (years)				
Mean (SD)	57.488(13.73)	58.53(13.12)	49.60(17.33)	
Median	58	58	38	
Range	30-85	30-85	35-70	
< 40	4	1	3	0.0011(S)
40-49	10	10	0	
50-59	9	9	0	
60-69	10	9	1	
≥ 70	10	9	1	
Gender, no. (%)				
Male	27(62.79)	23(60.5)	4(80)	0.6354(NS)
Female	16(37.21)	15(39.5)	1(20)	
Types Of Biopsy, no. (%)				
Minor	42(97.67)	37(97.37)	5(1000	0.7136(NS) (chi-square)
Major	1(2.33)	1(2.63)	0	
Tumour Diameter (mm), no. (%)				
≤ 20	31(72.09)	28(73.68)	2(40)	0.3015(NS)

21-50	9(20.93)	7(18.42)	2(40)	(chi-square)
>50	3(6.98)	3(7.89)	1(20)	
Diagnosis, no. (%)				
Adenocarcinomas	41(95.35)	37(97.37)	4(80)	0.2215(NS)
Squamous cell carcinomas	2(4.65)	1(2.63)	1(20)	
Tumour Grade, no. (%)				
Well differentiated	28(65.11)	26(68.42)	2(40)	0.1059(NS) (chi-square)
Moderately differentiated	10(23.25)	7(18.42)	3(60)	
Poorly differentiated	5(11.63)	5(13.16)	0	
Site Of Tumour, no. (%)				
Rectum	26(60.47)	23(60.52)	3(60)	0.5997(NS) (chi-square)
Caecum	3(6.98)	3(7.89)	0	
Sigmoid colon	2(4.65)	1(2.63)	1(20)	
Descending colon	1(2.32)	1(2.63)	0	
Ascending Colon	2(4.65)	2(5.26)	0	
Anal Canal	9(20.93)	8(21.05)	1(20)	

Table 4.4: Patient clinical characteristics related to the hMSH2(S=significant, NS= not significant)

Patient clinical characteristics related to the hMSH2 are presented in above **Table 4.4.** Among the 43 patient analysed, 27 are men and 16 are women. The mean age of the patient is 57.488 with the standard deviation of 13.73, the patient have the median age of 58 years (range, 30-85). The patient having the loss of expression of hMSH2 protein has the mean of 49.60 with the standard deviation of 17.33 and median age was 38 years (range, 35-70). The patient that didn't show the loss of expression has the mean age of 58.53 with the standard deviation of 13.12and median age of 58 year (30-850. The loss of expression of protein hMSH2 also didn't show any significant correlation with the sex, Tumour Diameter, Diagnosis, Tumour Grade and Site of Tumour. But it showed the significant correlation with the age of the patient ($p< 0.05$).

The probable reason behind the non-significant relation between those parameter and hMSH2 protein expression may be due to low sample size i.e. less than 300. However the age of the patient show the significant correlation with the expression of the hMSH2 protein even at the low sample size (n=43).

In a similar study done by Cheryl *et al.* (2003) found that there was no significant difference between the hHMLH1 and hMSH2 groups with the age and sex of the 458 patient. But the tumour grade shows the significant with the expression of hMLH1 and hMSH2. And pathologic features and stage have been correlated with immunohistochemical expression of the mismatch repair proteins hMSH2 and hMLH1.

According to Chapusot *et al.* (2003) the sex, location and size of the patient show significant with the expression of protein hMLH1 and hMSH2 but no significant link was noticed between the age of the patients (more or less than 60 years) and the expression of the two MMR proteins.

According to Barton *et al.* (2013) there is a correlation between younger age and loss of expression of MSH2 was significant. But there was no association between age and loss of hMLH1 expression. With all age groups taken together, loss of either hMLH1 or hMSH2 was interrogated relative to morphologic features. There was a significant association between loss of expression and tumour stage, tumour grade, mucinous or medullary differentiation and larger tumour size.

Recent study by Martinez-Uruena *et al.* (2013) showed that there is a relationship between the hMLH1 and the risk of sporadic colorectal cancer and that this variant appears to be related with the cases with focal IHC activity more than with the complete absence of the hMLH1 protein in the tumour tissue.

According to Kaug *et al.* (2011) in Malaysia studied 148 patients with CRC and found a significant association between abnormal MMR gene protein expression and proximal colon cancers, mucinous, signet ring and poorly differentiated morphology.

4.4 Expression of hMLH1 and hMSH2

4.4 1 Age

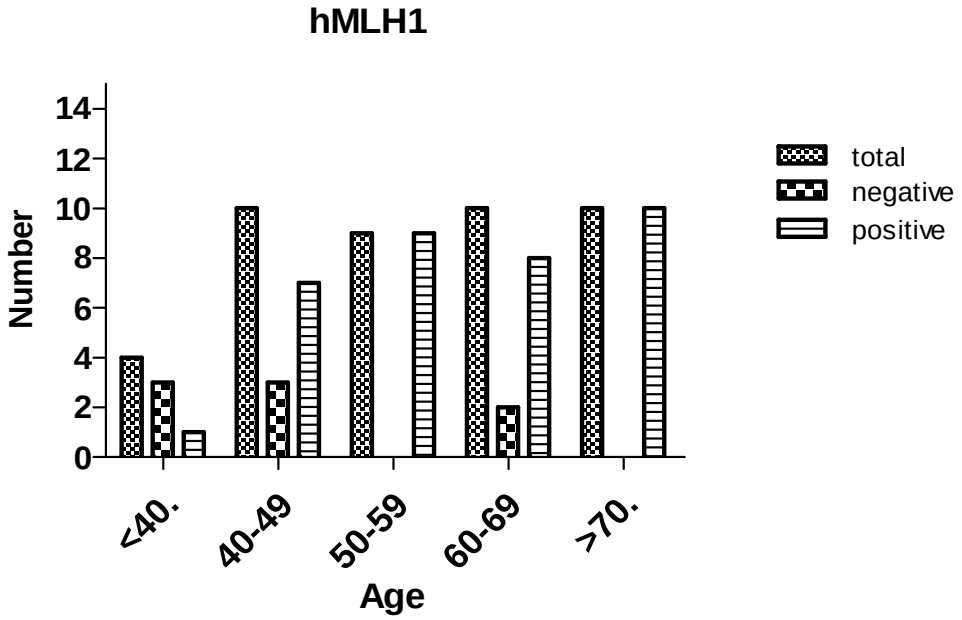


Figure 4.7: Graph showing relation between age group of patient and expression of hMLH1 protein

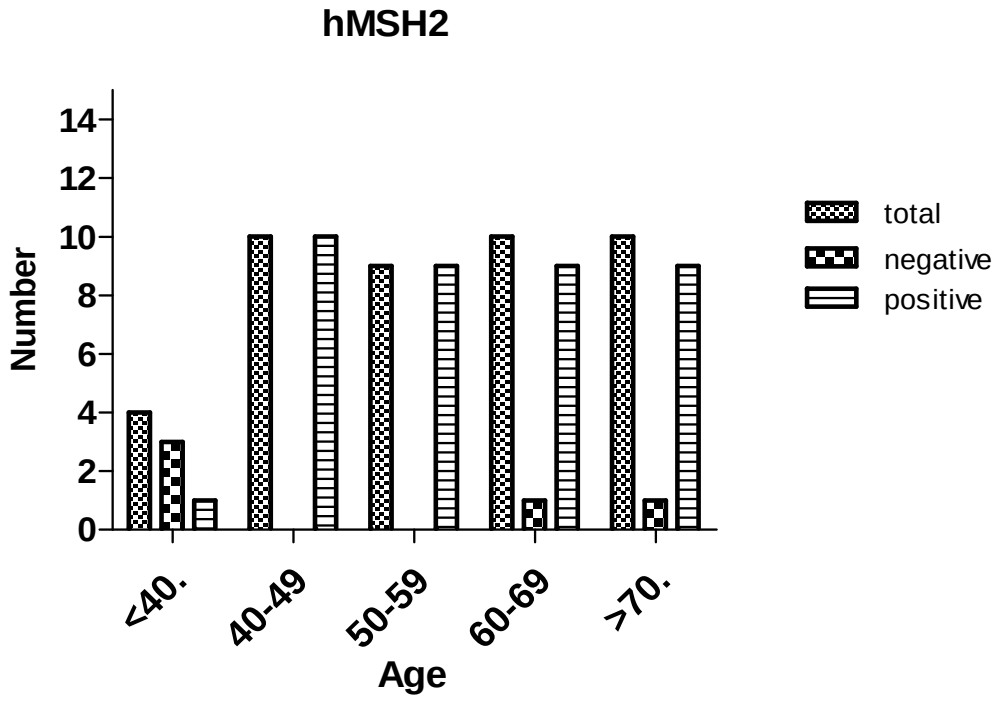


Figure 4.8: Graph showing relation between age group of patient and expression of hMSH2 protein

Out of 43 CRC patient 4 patients are of age less than 4o years of age among them 75% show the loss of expression of hMLH1 and hMSH2 protein. And 10 patient are of age group between 40-49 years among them 30% of them show the loss of expression of protein hMLH1 and none of them show the loss of expression of protein hMSH2.9 patient are of age group 50-59 and those patient doesn't have the loss of expression of both Protein. Among the patient of age group from 60-69, 10 patient are included and 20% patient show the loss of expression of hMLH1 and only 10% show the loss of expression of protein hMSH2. Age group more than 70 years doesn't show the loss of expression of hMLH1 protein but 10% patient show the hMSH2 negative.

In the similar work done by Barton *et al.* (2013) showed loss of hMSH2 in 14% of tumours in patients younger than 40 where in our work 75% (3 of 4) of tumour shows mutation of patient less than 40 years but Yoon *et al.* (2011) shows only 5% across all ages.

4.4.2 Gender

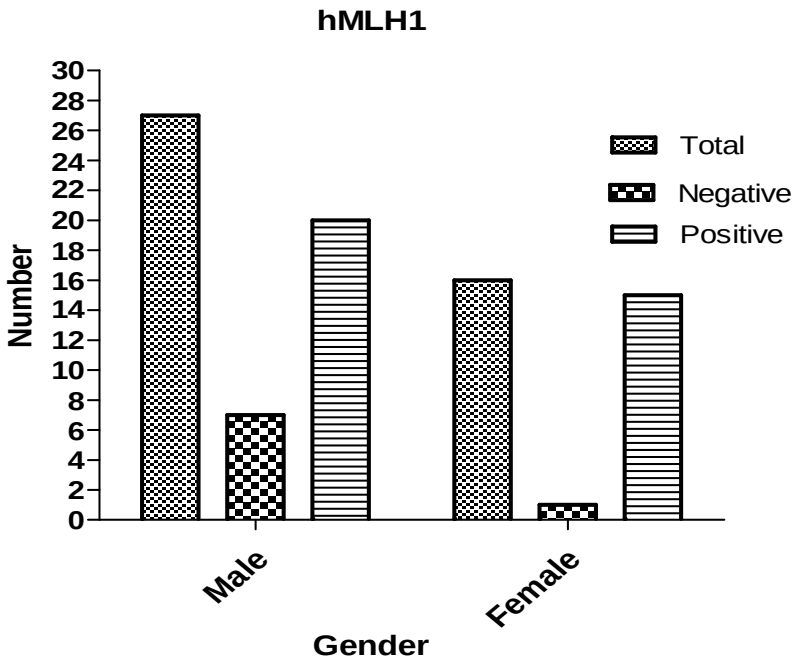


Figure 4.9: Graph showing relation between gender of patient and expression of hMLH1 protein

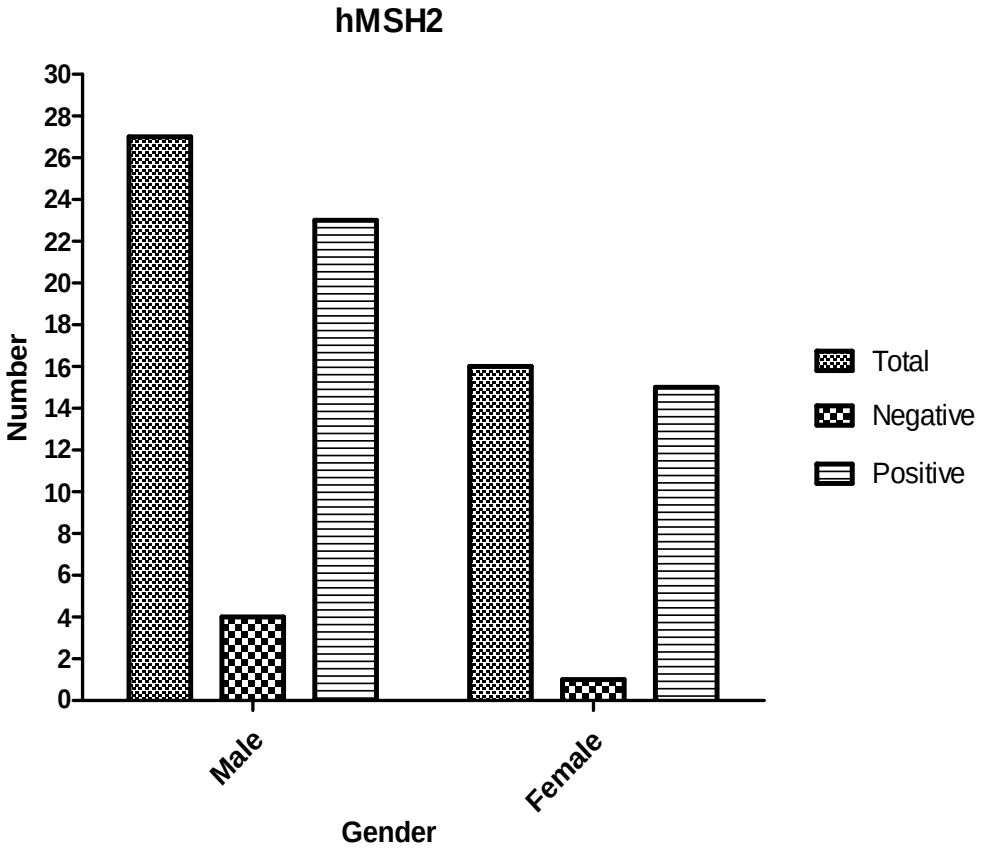


Figure 4.10: Graph showing relation between gender of patient and expression of hMSH2 protein

BY analysis of expression of protein hMLH1 and hMSH2 with the gender of the patient. It was evaluated that the occurance of CRC is high in in the male than female with the ratio of 1.6875:1. The loss of expression of both proteins was found to be more in male than female.

Out of male 25.97% (7 of 27) of them show the loss of expression of Protein hMLH1 and only 14.81 % (4 of 27) of them show the loss of expression of protein hMSH2. In female 6.25% (1 of 15) of them show the loss of expression of hMLH1 and hMSH2 protein.

In a similar work done by Truninger *et al.* (2005) found that female show more MMR deficiency than male. Also work done by Wright and Stewart (2003) also show that female show more MMR deficiency which is different than our result. But works did by Kim *et al.* (2011) show similar result with our result.

4.4.3 Types of Biopsy

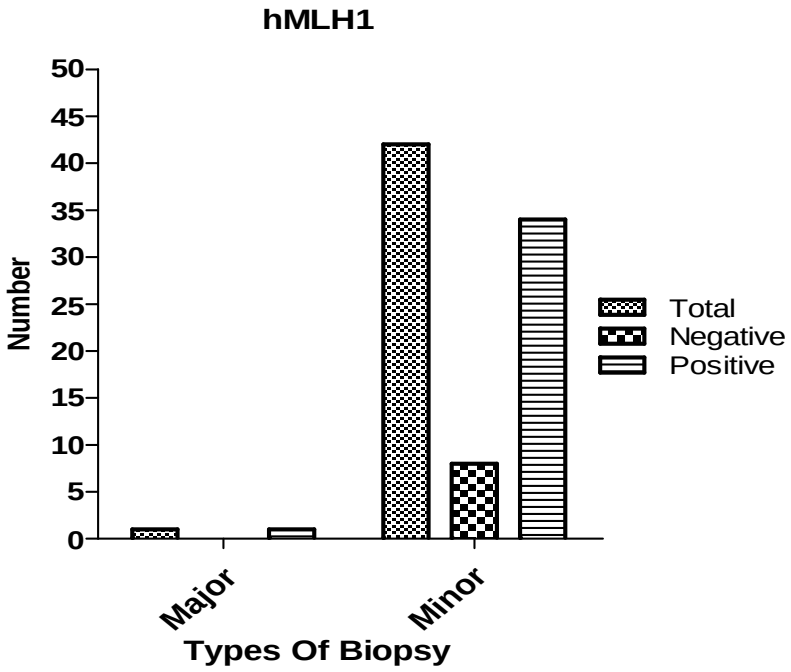


Figure 4.11: Graph showing relation between types of biopsy of patient and expression of hMLH1 protein

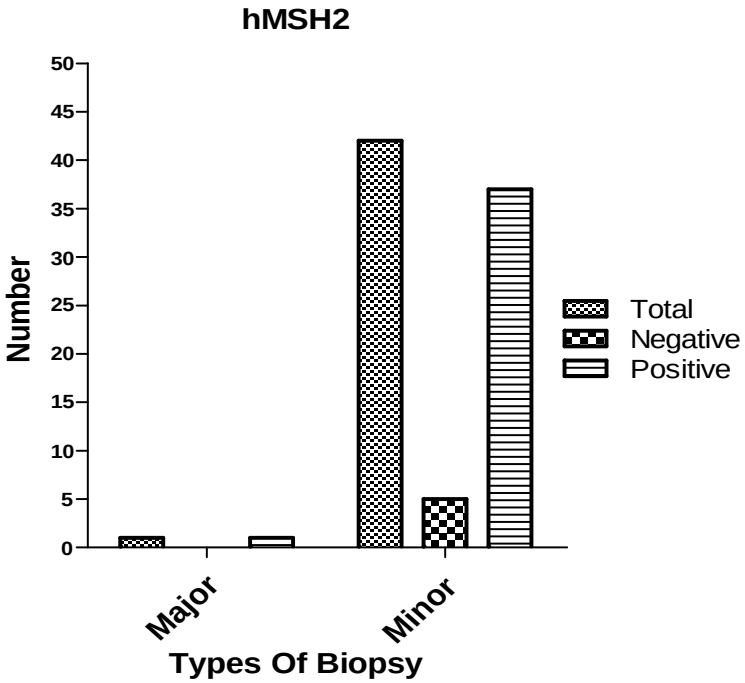


Figure 4.12: Graph showing relation between types of biopsy of patient and expression of hMSH2 protein

Major and minor criteria are used to establish the diagnosis, based on the microscopic appearance of slides stained using haematoxylin and eosin. Major criteria include an infiltrative glandular growth pattern, an absence of basal cells and nuclear atypia in the form of nucleomegaly and nucleolomegaly. Minor criteria include intraluminal wispy blue mucin, pink amorphous secretions, mitotic figures, intraluminal crystalloids, adjacent high-grade prostatic intraepithelial neoplasia, amphophilic cytoplasm and nuclear hyperchromasia.

BY analysis of expression of protein hMLH1 and hMSH2 with the types of biopsy of the patient. It has been evaluated that most of the patient has the minor tumour tissue (42 of 43) where only one patient has the major tumour tissue. In our study we found that loss of expression of both proteins was found in minor tumour tissue only.

4.4.4 Tumour Diameter (mm)

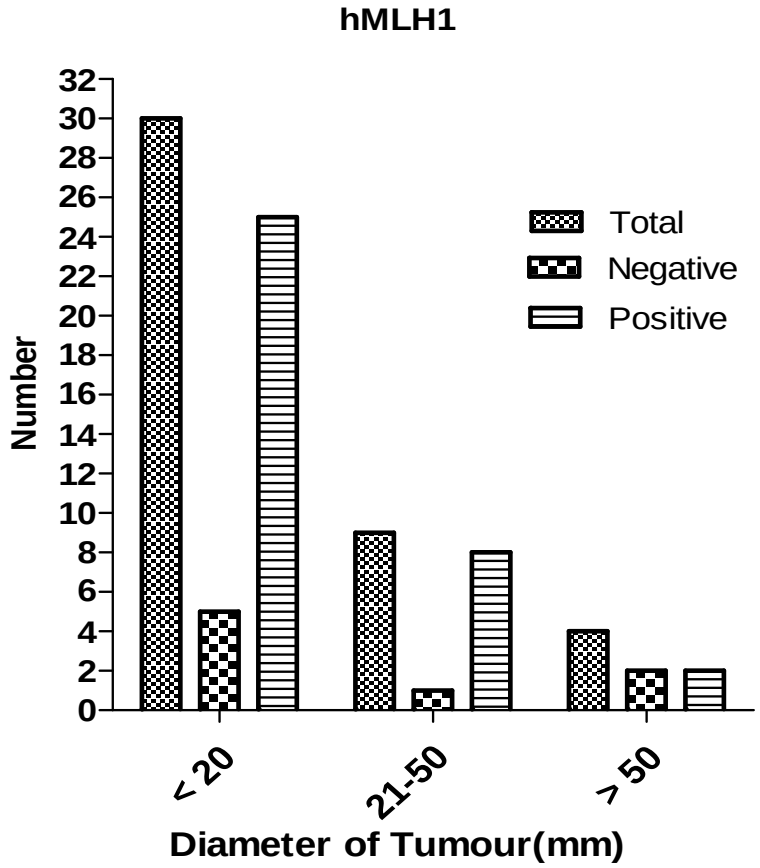


Figure 4.13: Graph showing relation between diameter of tumour of patient and expression of hMLH1 protein

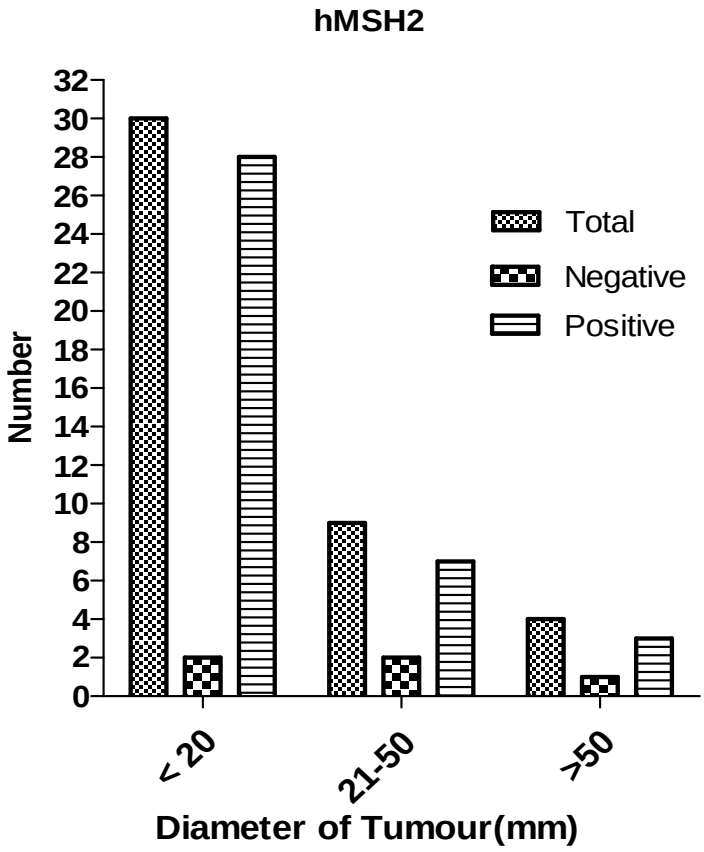


Figure 4.14: Graph showing relation between diameter of tumour of patient and expression of hMSH2 protein

By evaluation of expression of protein hMLH1 and hMSH2 with the diameter of tumour it was found that among 43 sample 30 sample has the diameter less than 20mm and among them only five sample shows the negative staining with hMLH1 protein and two samples show the negative staining with the hMSH2 protein. Only nine samples has the tumour diameter between 20- 50 mm among them only one sample show the loss of expression of protein hMLH1 and two sample show the loss of expression of hMSH2 protein. Four sample has the tumour more than 50 mm, two sample show the negative staining with hMLH1 and one sample show the negative to hMSH2.

In the similar work done by KHOO Joon-Joon (2013) mismatch repair protein defects was found in tumours which are significantly larger. Chapusot *et al.* (2003) found that tumours with microsatellite instability were significantly associated with poorer differentiation.

4.4.5 Diagnosis

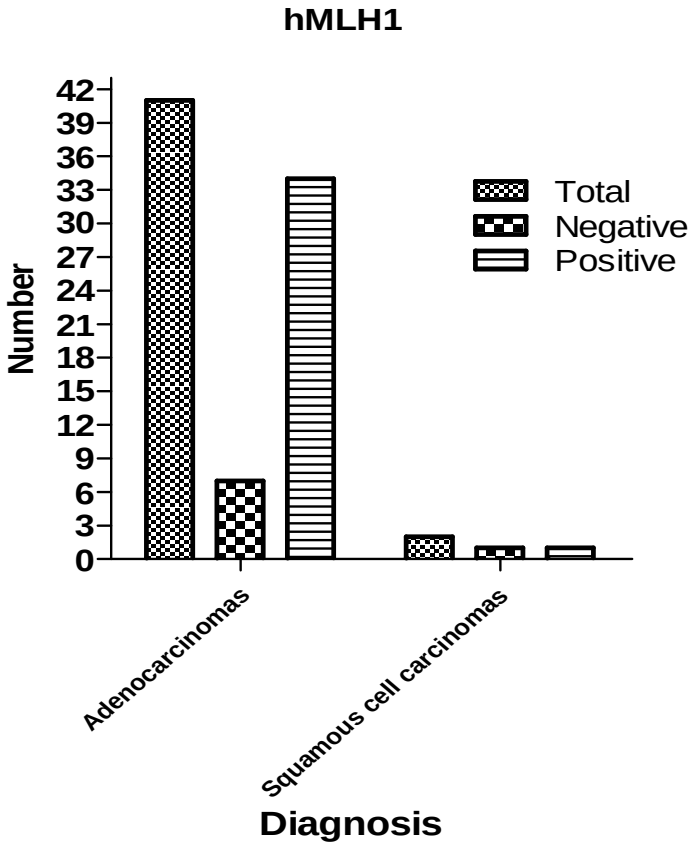


Figure 4.15: Graph showing relation between diagnosis of tumour of patient and expression of hMLH1 protein

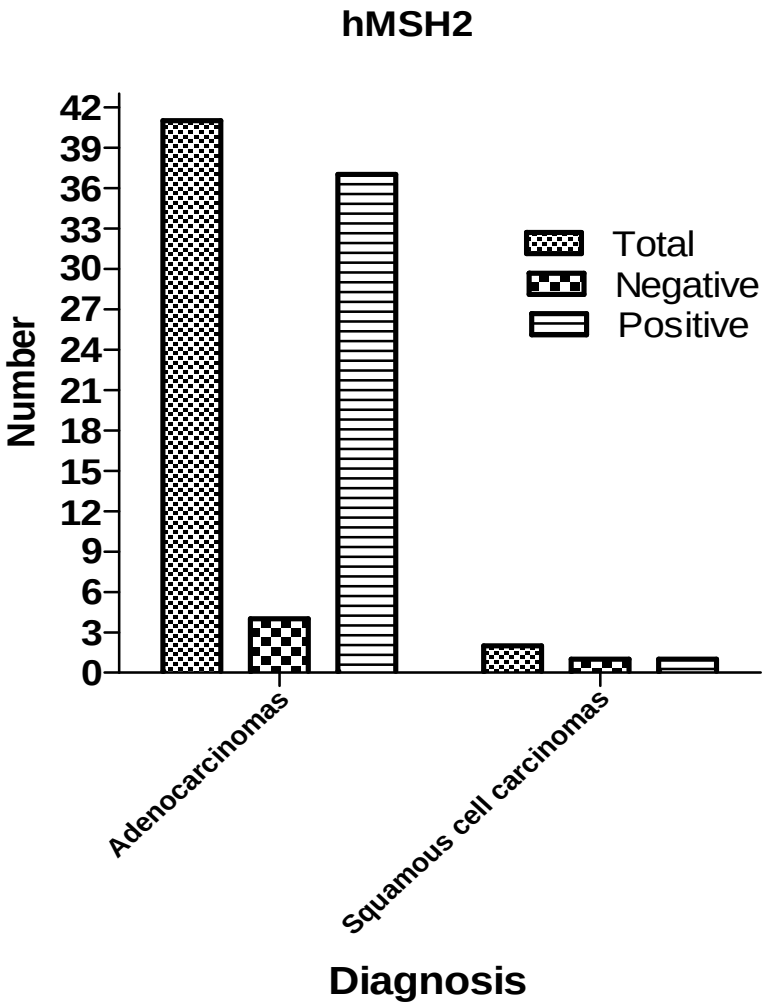


Figure 4.16: Graph showing relation between diagnosis of tumour of patient and expression of hMSH2 protein

By analysing the diagnosis of the patient with the expression of hMLH1 and hMSH2 protein it was found that most of the patient(41 of 43) has the adenocarcinomas tumour and among them 87.5 %(7 of 8) of hMLH1 deficiency patient has the loss of expression of hMLH1 protein. And 80% (4 of 5) of hMSH2 deficiency patient has the loss of expression of hMSH2 protein. Only two patients has the Squamous cell carcinomas among them one patient has the loss of expression of both protein.

In the similar work done by Kaur *et al.* (2011) Majority of carcinomas were histologically typed as classical adenocarcinoma (134/150 cases, 89.3%). The others constituted 11 cases of mucinous carcinoma, 3 signet ring carcinomas and 2 neuroendocrine carcinomas. Among them 14.9 % (20/134) cases of classical adenocarcinoma demonstrated absent expression of MMR protein. Two of 3 (66.7%) signet ring carcinomas and 6 of 11 (54.5%) mucinous carcinomas showed absent MMR protein.

4.4.6 Tumour Grade

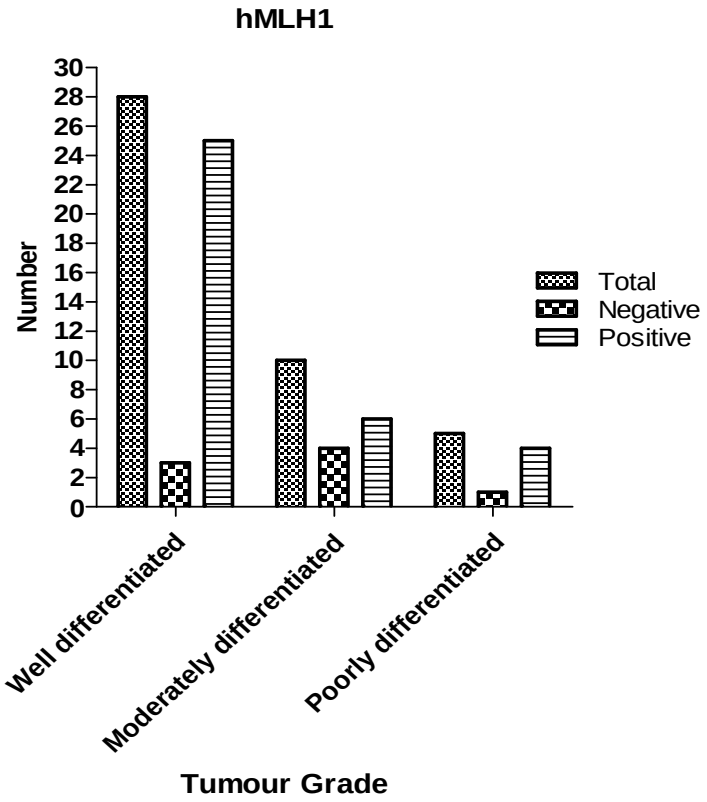


Figure 4.17: Graph showing relation between tumour grade of patient and expression of hMLH1 protein

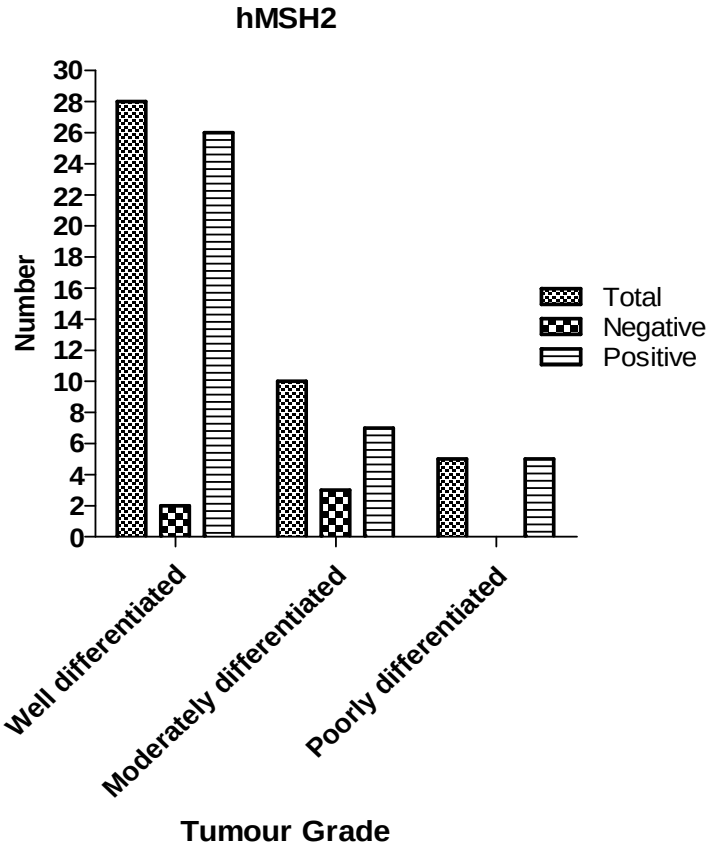


Figure 4.18: Graph showing relation between tumour grade of patient and expression of hMSH2 protein

By evaluation of expression of protein hMLH1 and hMSH2 with the tumour grade , it was found that 65.11% (28 of 43) sample were well differentiated tumour and out them three sample show negative for protein hMLH1 protein and two sample show negative for hMSH2 protein. 23.25% (10 of 43) sample were moderately differentiated tumour and out of them four sample show the negative for hMLH1 and three sample show negative for hMSH2 protein.11.63% (5 of 43) sample were poorly differentiated tumour and out of them one sample show negative for hMLH1 protein and none of the sample show negative for hMSH2 protein.

In a similar study done by Cheryl *et al*. (2003) found that most of the poorly differentiated tumour has loss of expression of hMLH1 (70%) compared with only 22.2% for hMSH2.

In the research done by Chapusot *et al.* (2003) in the tumour that lack the expression of MMR protein were poorly differentiated. Eight (50%) of the 16 poorly differentiated cancers showed loss of MMR protein expression, whereas only 29 (11.3%) of the 257 moderately or well-differentiated tumours lacked the normal nuclear signal.

In the similar work done by KHOO Joon-Joon (2013) the majority of the colorectal carcinomas (246/298, 82.6%) were classified as well to moderately differentiated carcinomas while only 52 cases (17.4%) were classified as poorly differentiated carcinomas. where 41.9% of mismatch repair defect tumours were poorly differentiated colorectal carcinomas.

In the similar work done by Kaur *et al.* (2011) in which 109 (72.7%) of moderately differentiated cancers, 20 (13.3%) well differentiated and 8 (5.3%) poorly differentiated. Among them Fifty per cent (4/8 cases) of poorly differentiated cancers showed absent MMR protein expression compared to 13.8 per cent (15/109) of moderately differentiated tumours and 15.0 per cent (3/20) of well differentiated tumours

4.4.7 Site of Tumour

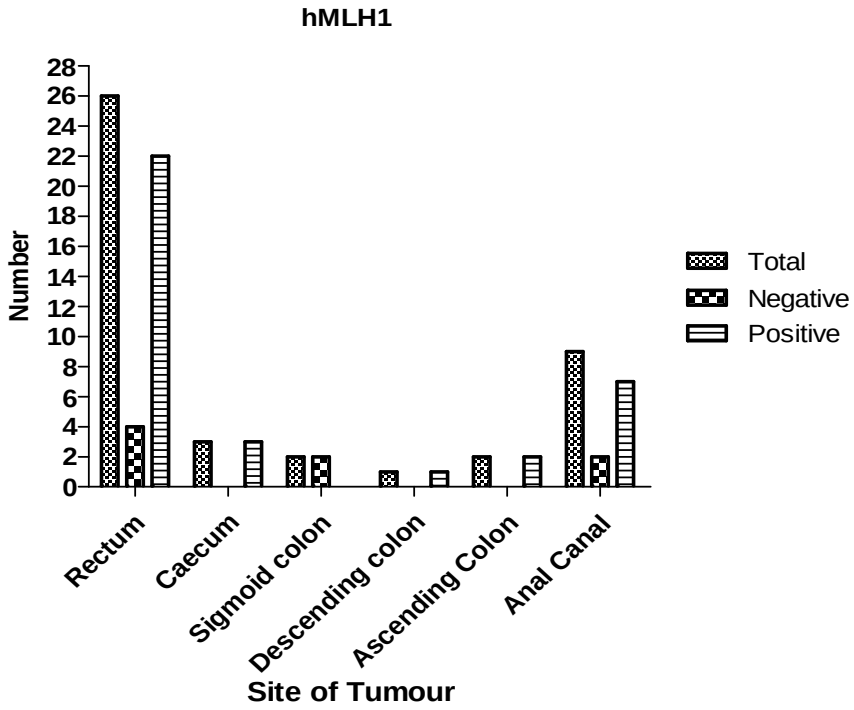


Figure 4.19: Graph showing relation between site of tumour of patient and expression of hMLH1 protein

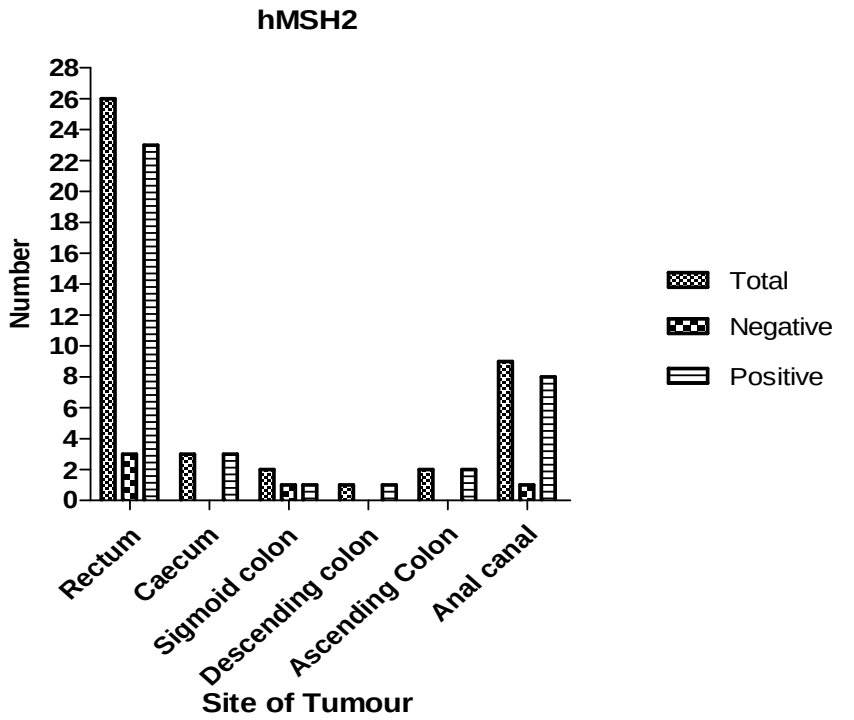


Figure 4.20: Graph showing relation between site of tumour of patient and expression of hMSH2 protein

By evaluation of the expression of protein hMLH1 and hMSH2 with the site of tumour present show, 60.47% (26 of 43) of sample has a tumour at the rectum and among them four sample show negative for hMLH1 protein and three sample show negative for hMSH2 protein. Only 6.98% (3 of 43) sample has the tumour in caecum and none of them show the negative for both the protein. 4.65% (2 of 43) sample has the tumour in sigmoid colon and both sample show negative for hMLH1 protein and only one show negative for hMSH2 protein. 2.32 % (1of 43) tumour has the location in descending colon and 4.65% (2 of 43) tumour are from ascending colon and the sample from both site doesn't show the negative for both the protein HMLH1 and MSH2. 20.93% (9 of 43) of the tumour are from anal canal, among them two sample show the negative for hMLH1 and one sample show negative for hMSH2 protein.

According to Chapusot *et al.* (2003) one (1.2%) of the 85 carcinomas was located in the rectum did not express one or both proteins. Where in other research done by Jung *et al.* (2012) Loss of hMSH2 expression was more strongly associated with colonic than rectal tumours. In the similar work done by de la Chapelle, 2004 and Jasperson *et al.,* (2010) found that between 70% and 85%, of colonic tumours develop in the proximal colon, with the estimated lifetime risk of CRC between 25%
and 75% in carriers.

4.5 Comparison with different ethnic groups

Ethnic group	Total	hMLH1 proficient	hMLH1 deficiency	P value	hMSH2 proficient	hMSH2 deficiency	P value
Brahmin/ Chhetri	23	19	4	0.8447 (NS)	20	3	0.9563 (NS)
Kirati	3	3	0		3	0	
Newar	1	1	0		1	0	
Tamang	2	2	0		2	0	
Magar	2	1	1		2	0	
Chaudhari	6	5	1		5	1	
Gurung	1	1	0		1	0	
Tharu	1	1	0		1	0	
Mandal	2	1	1		2	0	
Damai	2	1	1		1	1	
Total	43	35	8		38	5	

Table 4.5: Loss of expression of hMLH1 and hMSH2 proteins among different ethnic groups of Nepal. (NS= not significant)

The different ethnic group that are available in Nepal is compared with the loss of expression of protein hMLH1 and hMSH2 in the colorectal cancer patient as shown in table 4.5. Among the 43 patient analysed most of them are Brahmin and chhetri (23 of 43) among them four patient show the loss of expression of hMLH1 and three of them show the loss of expression to hMSH2 protein. Other ethnic groups like Kirati, Newars, Tamangs, Magars, Damai, Gurung, Tharu, Mandal and Chaudhari are less in number. In Magar ethnic group out of 2 colorectal patient one show the negative result to hMLH1 protein. And in Chaudhari there were six colorectal cancer patient and out of them only one shows the negative to hMLH1 and hMSH2. And in Mandal and Damai there 2-2 patients respectively and only one show negative to hMLH1 in Mandal where in Damai one patient show negative to both proteins.

The loss of expression of protein hMLH1 and hMSH2 didn't show any significant correlation with different ethnic groups of Nepal ($p>0.05$). The probable reason behind the non-significant relation between those parameter with hMLH1 and hMSH2 protein expression may be due to low sample size i.e. less than 300.

4.6 PCR Amplification

For the further confirmation of the mutation of the two genes hMLH1 and hMSH2 we must perform the PCR amplification and we should do sequencing of the gene. To conform our IHC result we also tried to do PCR of the DNA which we have extracted from the formalin fixed tissue embedded paraffin block sample in which we have performed IHC and found the loss of expression of the protein. We have extracted DNA from the tissue that was embedded in the paraffin block by using the two kits Qiagen (QIAamp DNA FFPE Tissue Kit) and Geneall (genomic DNA FFPE kit) but we only got the degraded DNA as shown in **figure 4.21** in which we cannot perform the PCR reaction.

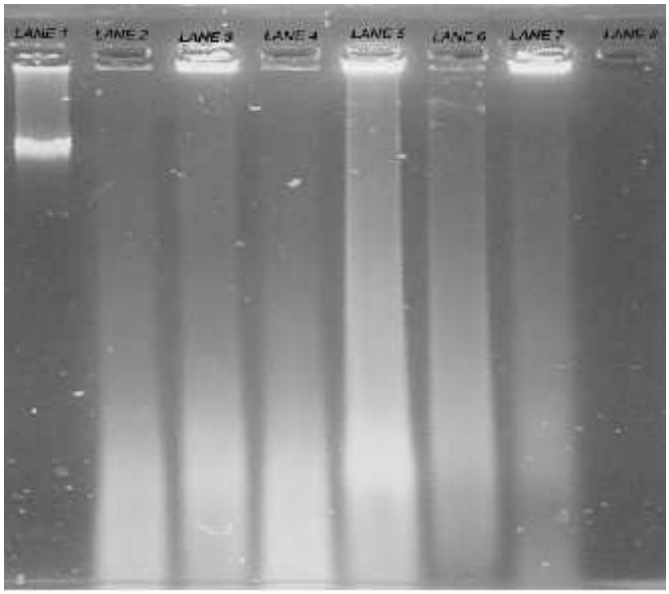


Figure 4.21: Gel picture of extracted DNA

(Run at 1% agarose gel, 100v for 45 minutes)

Lane 1=DNA extracted from the fresh tissue (as a positive control)

Lane 2-7=DNA extracted from our paraffin embedded tissue sample

Lane 8= Negative control

The degraded DNA that we got we tried to run the PCR reaction but we could not get the amplified fragment in the gel as shown in **figure 4.22.**

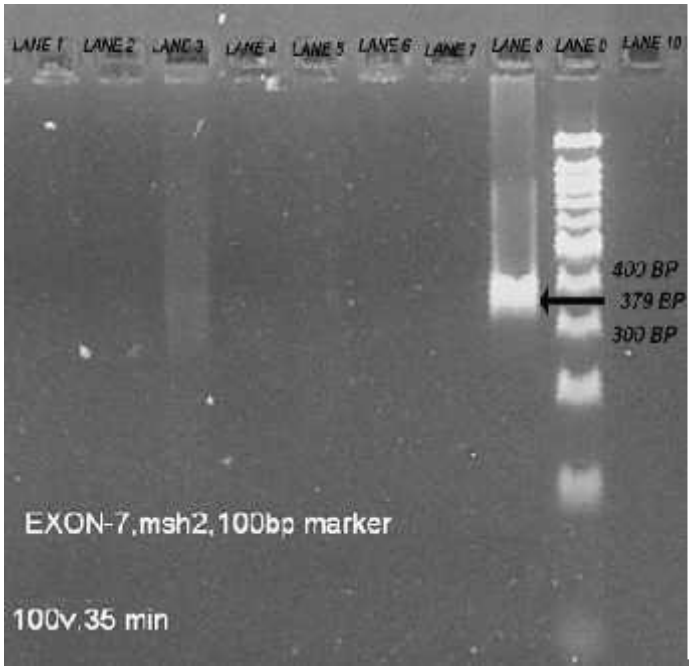


Figure 4.22: Gel picture of the PCR product

(Run at 2.5% agarose, 100v for 35 min)

Lane 1-7= PCR product of our CRC sample

Lane 8= Positive control

Lane 9= 100 bp Marker (1st base, Singapore)

Lane 10= Negative control

The DNA that we have extracted from the paraffin embedded tissue were found to be degraded, this may be due to the inappropriate storage in formalin for long period in B. P. Koirala Memorial Cancer Hospital (BPKMCH). According to Koshiba *et al.* (1993) tissue stored of formalin fixatives resulted in degradation of DNA at room temperature but not at 4°C. The degradation also occurred in formalin when the pH or the salt concentration was low, or the formic acid level was high.

According to Gillio-Tos *et al.* (2007) the penetration rate of 10% formalin is approximately 1 mm/h, the tissue should not be exposed to the fixative for longer than necessary, as this can cause damage to nucleic acids. Extensive cross-links between proteins in the tissues and DNA fragmentation can be found when fixing is performed for too long, leading to lower quality DNA.

According to Kelly ,(2006) the formalin-induced cross-linking effectively preserves structural morphology, but it is extremely detrimental to subsequent DNA analysis because crosslinked bases stall polymerases and DNA-DNA crosslinks can inhibit denaturation. In addition, the pH of formalin solutions drops over time due to the formation of formic acid, increasing the rate of apurinic/apyrimidinic site formation and subsequent fragmentation.

According to Pairedera *et al*, (2013) formalin fixation not only leads to covalent bonds between proteins but also cross-links between proteins and nucleic acids. In addition, it induces methylol modification of nucleobases. Unbuffered fixative solutions are known to cause acid-driven hydrolytic fragmentation of nucleic acids. Therefore, standard DNA extraction protocols generally yield low quantities of impure and sometimes highly fragmented DNA that cannot be amplified by PCR.

CHAPTER V

SUMMARY

Colorectal carcinoma (CRC) is the most common gastrointestinal malignancy in the older population, but it is also quite frequent among young adults in developing countries. However there is a lower incidence in south Asian countries in the past few decades. Most of the CRC develop from the adenomas and to develop the adenomas to cancer it require a series of stages referred to as the adenoma-carcinoma sequence. The colorectal tumour initiation requires several different somatic changes before a cell can develop into a carcinoma. The mismatch repair genes encode protein products recognizing and correcting errors that arise when DNA is replicated. Mutation in the MMR protein result in the mis-function of these protein later which cannot function in recognizing and correcting the errors that arise in the replication.

To find the mutation in the MMR protein by using PCR technique and electrophoresis can be expensive and time consuming. As this technique can be replaced by IHC technique which is reliable and effective in country like Nepal. The two antibody hMLH1 and hMSH2 can be used for the detection of loss of expression of MMR protein in the tumour cell. As other protein PMS2 and MSH6 detection is not necessary for the detection of MMR protein. This is because there are other secondary proteins which can replace the PMS2 and MSH6 and form the functional complex, such as MSH3, MLH3, and PMS1. So PMS2 and MSH6 antibodies don't have the capability of detecting most abnormalities in hMLH1 and hMSH2. As a result, mutations of hMLH1 or hMSH2 often cause concurrent loss of hMLH1/PMS2 or hMSH2/MSH6, respectively, by IHC, whereas mutations of PSM2 or MSH6 often cause isolated loss of PMS2 or MSH6 only.

In our present study, 43 samples were assayed, among them 18.6% of sample show the loss of expression of hMLH1 and 11.6 % of sample show the loss of expression of protein hMSH2 and 9.3% of sample show loss of expression of both protein. In a correlation of the loss of expression of hMLH1 and hMSH2 with the various histopathological evaluation like age, sex, tumour grade, tumour diameter, diagnosis and site of tumour it was found that only age show significant result (<0.05) with loss of expression of hMLH1 and hMSH2. But other data were statistically not significant because of relatively small number of cases in the present study.

During running the IHC every time appendix slide as external control is also run with a positive and negative control to fine whether the process is correct of not and for evaluation the sample the internal control as lymphocytes are used . The percentage and intensity of the staining is evaluated by comparing it with the internal control.

CHAPTER VI

CONCLUSION

Although there are many other molecular tools that are applied in the diagnosis of the cancer there is always a need of a technique that is precise, cost effective, reliable and time subsiding. As IHC assay meet all these needs to more or less extent. The large application of this approach is the result of a number of advantages provided by IHC when compared to other technique. As the 18 samples can be run at the single time, with results being available within the 24 hours. It allows the study of several proteins at a single reaction. IHC have the high sensitivity and specificity, which can be used for detection of various of protein expressed in the cell. As IHC cannot be used for the diagnosis but it can be used for the research proposes also.

From the present investigation, the use of IHC assay was observed to be more effective to be used as a reliable tool in the diagnosis of the cancer. IHC can be used for evaluation of the microsatellite instability status of the tumours rather than using molecular testing that consists of polymerase chain reaction (PCR) and gel electrophoresis to examine the DNA sequences; as the molecular testing was an expensive and time consuming test and not easily available. The result suggests that most of the negative staining is shown by the patient less than 50 years which is different than the result of other country. CRC in young adults is more frequently reported from developing countries, in which the incidence is much higher from that reported in developed countries.

As the high frequency of CRC can be due to the ignorance of the family member and also due to the lack of knowledge regarding the disease. Thus provided the necessary information the rate of many such cancers can be decreased or even nullified. IHC assay is an essential tool in deterring the loss of expression of MMR protein in the CRC cancer. It can be used in all laboratories performing diagnostic as well as a confirmation tool.

There has always been a need of extensive study on effective, safe and cheap and reliable molecular diagnostic methods in our country. This study aims to fulfil the same objective through at the preliminary state. This finding encourages us for further investigation and research work on various aspects of CRC diagnosis.

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APPENDICES

Appendix 1: Preparation of chemical

95% Ethanol (100 ml)

Ethanol	95ml
Distilled Water	5ml

90% Ethanol (100 ml)

Ethanol	90ml
Distilled Water	10ml

80% Ethanol (100 ml)

Ethanol	80ml
Distilled Water	20ml

70% Ethanol (100 ml)

Ethanol	70ml
Distilled Water	30ml

3% H_2O_2(250ml)

H_2O_2(30%)	25ml
Methanol	225ml

Appendix 2: PCR condition

Appendix 2.1: MLH1 primer

Exon	F	R	size
1	gttgagaaatttgactggcattc	gttaagtcgtagcccttaagtg	337
2	ggcactattgtttgtatttggag	catctgcaaaagcctagtttcc	290
8	ggagataaggttatgatgtttcag	cacacataatatcttgaaaggttcc	261
12	gctccatttggggacctgtat	gaataaaggaggtaggctgtac	517
17	gagtggcagataggagcacaa	cttatcatctttatcattccagatc	301
18	gtagtctgtgatctccgtttag	gagatgggcaagtttcatctc	272

Appendix 2.2: MSH2

Exon	F	R	size
2	cagtgcttgaacatgtaatatctc	actgcgaccagccaaactgc	384
5	gaactggatccagtggtataga	tagctcctttataagcttcttcag	301
6	gtaaggttttcactaatgagcttg	tatgtactctgtacagttaaatgg	314
7	gagctgatttagttgagacttac	caccaccaccaactttatgagg	379
8	ggatcaaatgatgcttgtttatct	cacaaaggtgctacaattagatac	317

12	cggcttatatctgtttattattcag	cctttctaatgttaagaactggg	435

Appendix 2.3: Thermocycling condition

	Cycles	Temp	Time
Initial Denaturation	1 Cycle	95°C	7 min
Denaturation	30 Cycles	94°C	1 min
Annealing		*	1 min
Extension		72°C	2 min
Final Extension	1 Cycle	72°C	3 min
Hold	1 Cycle	4°C	

Appendix 2.4: Annealing temperature of different exons

MSH2

Exons	**Annealing temperature**
2	**58.7**
5	**56.4**
6	**56.6**
7	**55.3**
8	**55.7**
12	**51.4**

MLH1

Exons	**Annealing temperature**
1	**55.3**
2	**55.3**
8	**59.5**
12	**61.5**
17	**57.2**
18	**59.5**

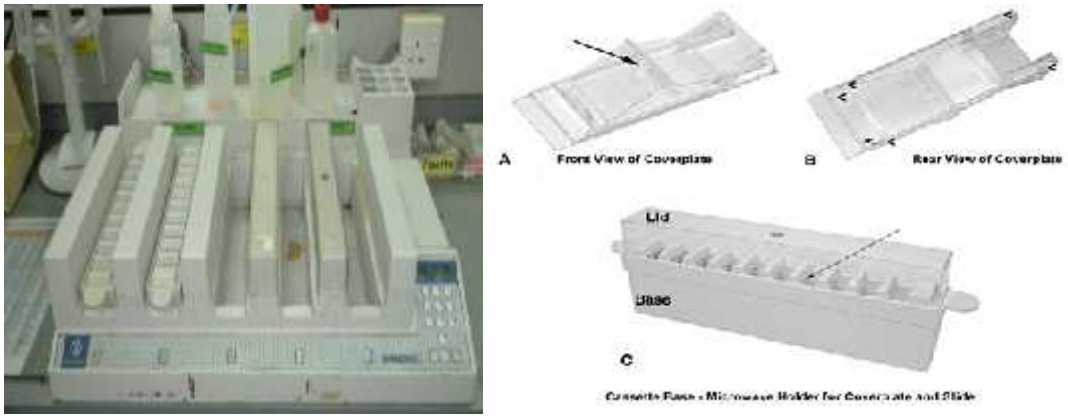
Appendix 2.5: PCR Reaction Mixture

Components	25 µl Reaction
Template	3 µl
10mM dNTPs each	0.5 µl
10µM Forward primer	0.5 µl

10µM Reverse primer	0.5µl
25 mM $MgCl_2$	1.5
5 X Reaction Buffer	5 µl
5U/ µl DNA polymerase	0.25 µl
Nuclease free water	Upto 25µl

Appendix 3: Picture

Microtome



Shandon Sequenza

Pressure cooker (biocare medical decloaking chamber)

Laminar flow

Slide and slide holder