OPTIMIZATION OF IMMUNOHISTOCHEMISTRY: TESTING FOR MLH1 AND MSH2 PROTEINS IN COLON TISSUE

By

SITI NUR FADZILAH BT MUHSAIN

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ABBREVIATIONS

%	Percent
APC	Adenomatous Polyposis Coli
BAX	BCL2-Associated X Protein
c-ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2.
CRC	Colorectal Cancer
DAB	Diaminobenzidine
DCC	Deleted in Colorectal Cancer
DNA	Deoxyribonucleic acid
FAP	Familial Adenomatous Polyposis
H&E	Haematoxylin and Eosin
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IGF2R	Insulin-like Growth Factor 2 Receptor
IHC	Immunohistochemistry
MLH1	human mutL homolog 1
MMR	Mismatch Repair
MSH2	human mutS homolog 2
MSH3	mutS homolog 3
MSH6	mutS homolog 6
MSI	Microsatellite Instability

MSI-H	Microsatellite Instability High
MSI-L	Microsatellite Instability Low
MSS	Microsatellite Stable
°C	Degrees Celsius
PCR	Polymerase Chain Reaction
PMS1	Postmeiotic Segregation Increased 1
PMS2	Postmeiotic Segregation Increased 2
ras	Rat Sarcoma Viral Oncogene
TGFßRIII	Transforming Growth Factor Beta Receptor
UH	University Hospital

ABSTRAK

Optimisasi Kaedah Immunohistokemistri: Penentuan Protin MLH1 Dan MSH2 Di Dalam Tisu Usus Besar

Lebih kurang 15% daripada kanser usus besar terhasil melalui laluan genetik yang dipanggil 'microsatellite instability' (MSI). Ia berlaku akibat kerosakan di dalam gen membaiki salah padanan atau 'mismatch repair genes' (MMR). Majority kanser MSI terhasil akibat kerosakan atau mutasi di dalam gen MLH1 dan MSH2. Gen-gen ini boleh dikenalpasti melalui kaedah immunohistokemistri (IHC). Kaedah ini telah dibuktikan sensitif dan spesifik apabila dibandingkan dengan analisis molekular MSI. Walaupun begitu, sehingga kini tiada literatur mengenai kajian MSI di Malaysia dan secara spesifiknya menggunakan kaedah IHC. Sebelum kaedah ini boleh digunakan di dalam kajian, ia perlu dioptimisasikan terlebih dahulu. Dalam kajian ini, optimisasi IHC telah dijalankan menggunakan antibodi poliklonal MLH1 dan MSH2 daripada Biovision. Kaedah ini dijalankan ke atas sampel kolon yang normal yang telah ditetapkan menggunakan formalin dan dibenamkan ke dalam paraffin. Sistem penentuan sekunder Vectastain Elite ABC Kit dan DAKO EnVision Kit telah digunakan. Prosedur ini telah dijalankan menggunakan kepekatan antibodi primer dan suhu yang berbeza. Prosedur 'antigen retrieval' juga dibuat menggunakan citrate buffer pH6 atau Tris-EDTA pH 9. Keputusan menunjukkan, inkubasi spesimen pada suhu bilik menggunakan kepekatan antibodi yang berbeza gagal menghasilkan tanda

atau 'stain' pada nuklei. 'Staining' menggunakan sistem DAKO EnVision juga masih tidak memuaskan, walaupun 'stain' latarbelakang dikurangkan. Penggunaan prosedur 'antigen retrieval' menggunakan ketuhar gelombang mikro tidak menunjukkan kemajuan. Apabila diinkubasikan pada 4°C, 'stain' latarbelakang yang tidak spesifik telah didapati. Untuk memastikan sistem penentuan sekunder berfungsi, IHC dijalankan dengan menggunakan antibodi poliklonal C-erbB2 telah digunakan ke atas tisu kanser payudara. Keputusan yang didapati memuaskan. Penulis membuat kesimpulan bahawa antibodi MLH1 dan MSH2 daripada Biovision mungkin tidak sesuai dan tidak boleh digunapakai. Maka kaedah optimisasi IHC untuk mengesan ekspresi protin MMR harus selanjutnya dijalankan menggunakan antibodi primari yang lain

ABSTRACT

Microsatellite instability (MSI) accounts for the development of about 15% of all colorectal cancers. It is characterized by mutations in mismatch repair (MMR) genes that render cells unable to detect errors during DNA replication. Mutations in the MMR genes, MLH1 and MSH2 are responsible for the majority of CRC with MSI. These MMR proteins can be detected by immunohistochemistry (IHC). IHC has proved sensitive and specific when compared to molecular analysis of MSI. Nevertheless, generally, there is no published literature as yet on studies of MSI in Malaysia, and specifically using IHC. Before any IHC staining procedure can be done, it has to be optimized. In this study, attempts were made to optimize IHC staining of MLH1 and MSH2 proteins using polyclonal rabbit antibody by Biovision on formalin-fixed paraffin-embedded sections of normal colon, taken from resected margins of colectomy specimens. Either Vectastain Elite ABC Kit or DAKO EnVision Kit was used as secondary detection system. The staining procedure was done using different dilutions of the primary antibodies and incubated for different durations at different temperatures. Antigen retrieval procedures were introduced using citrate buffer pH 6 or Tris-EDTA buffer pH 9. The results showed that incubation of sections at room temperature using different dilutions of antibody failed to produce any nuclear staining. Staining with DAKO EnVision was still unsatisfactory, although background staining was reduced. Introduction of antigen retrieval procedure using microwave did not improve results. When incubated at 4°C, non-specific background staining was produced. To ensure that the secondary detection system actually worked, staining using polyclonal rabbit C-erbB2 was done

on breast cancer tissue using Vectastain Kit. The result was satisfactory. In conclusion, MLH1 and MSH2 antibodies by Biovision may not be authentic. Thus, further optimization of IHC for detecting MMR protein expression should be done using a different batch of antibodies.

CHAPTER 1: INTRODUCTION

Colorectal cancer (CRC) is a commonly diagnosed cancer in both men and women. The incidence of CRC varies greatly throughout the world. Generally, it is high in western developed countries, low in developing countries, intermediate but rising rapidly in urban societies of Eastern Asia (World Cancer Report, 2003). In Malaysia, CRC is ranked as one of the ten leading cancers. This disease has become increasingly important as a public health concern (Lim, 2002).

The progression of CRC could be attributed to at least two major pathways. The majority of CRC develop through the adenoma-carcinoma sequence pathway. However, since this pathway was proposed by Vogelstein and Fearon in 1990, an alternative pathway, involving microsatellite instability (MSI) pathway has been identified. Up to 15 % of all CRCs are due to MSI, also known as replication error (RER). It is characterised by defects in mismatch repair (MMR) gene that render the cells incapable of detecting and repairing errors. The majority of the MSI cancers are due to mutations in the MLH1 and MSH2 genes (Boland et al, 1998).

MSI cancers have a better prognosis and respond differently to chemotherapy (Boland et. al, 1998). Therefore, according to Lindor et al., 2002, there are three good reasons to determine the MSI status of CRCs: 1) as a screening tool for hereditary nonpolyposis colorectal cancer (HNPCC); 2) as a prognostic marker and 3) as a

predictor of therapeutic strategies. The availability of commercial antibodies for MLH1 and MSH2 offers an alternative strategy to molecular methods for identifying MMR deficient cancers. Detection of the proteins using immunohistochemistry has proved to be highly sensitive, of good predictive value and relatively low cost when compared to the traditional molecular analysis of MSI (Stone et al, 2001).

Despite the growing significance of CRC, there has been little molecular background data generated based on the Malaysian population. Thus far, there is no published literature on MSI status in CRC in Malaysia. Accordingly, this study was conducted to serve as an initial step to collect data on MSI status in CRC cases in Penang, Malaysia. It is hoped, in the future this information will help to better manage colorectal cancer patients in Malaysia generally, and determine the prevalence of HNPCC for proper genetic counselling.

Thus, the objective of the study is to optimize the IHC method and determine the best conditions for immunohistochemical detection of MMR proteins, which subsequently could be adopted to assess the MSI status of CRC patients in Penang.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Colorectal Cancer In Malaysia.

Colorectal cancer (CRC) is the third commonest cancer and the fourth most frequent cause of cancer deaths worldwide (Weitz et. al, 2005). The World Cancer Report, 2003 estimated that 945,000 new cases occur each year, with 492,000 deaths. The incidence varies greatly throughout the world; it is high in the Western countries low in developing countries and intermediate but rising rapidly in urban societies of Eastern Asia. Nevertheless, despite improvements in medical and surgical provision, there has been comparatively little change in mortality from colorectal cancer over the past 40 years (Beart et al, 1995).

Like many other parts of the world, CRC has become increasingly important in Malaysia, inline with the development and progress that has been achieved by the population (Kandasami et al, 2003). CRC is the commonest cancer of the alimentary tract (Lim, 2002). According to the Second National Cancer Registry (Lim and Yahya, 2003), colon and rectum cancers ranked among the top ten of most common cancers in Malaysia. When both were combined, they account for 14.2% of male cancers (Figure 2.1) and 10.1% of women cancers (Figure 2.2). This makes CRC the most common cancer in men. The male to female ratio for colon cancer is 0.98:1.

Although the incidence is nearly comparable, the frequency in males is rising more rapid after the age of 60 (Figure 2.3). In rectal cancer, the prevalence in males is more prominent, with proportion of 1.26:1. The occurrence increase in males at age of 50 years onwards (Lim and Yahya, 2003), (Figure 2.4).

Malaysia is a diverse mix of several ethnic races which include Malays (58%), Chinese (24%) and Indian (8%), (World Factbook, 2005). Nevertheless, the National Cancer Registry noted that the incidence of CRC is highest in Chinese when compared to other races, Malays and Indians (Tables 2.1 and 2.2). This statistic has not changed much since being reported by King and Kutty, 1965. In a survey of biopsy specimens from six large state hospitals as well as University Hospital (UH) over a period of 4 years, they noted 135 (77.59%) colon cancers among Chinese, 22 among Indians (12.64%) and 17 among Malays (9.77%). In a 5 year study in UH from 1993, Ghee, 2001 reviewed that out of 199 patients with CRC, the Chinese accounted for 69%, Malays 20% and Indians 10%. They reported that 33% of the tumours occurred in the rectum, 24% sigmoid and 16% in caecum. Lim et. al, 1997 reported that from 98 of CRC patients referred to Institute of Oncology, Kuala Lumpur General Hospital, Chinese formed 65%, Malays 27%, Indians 6% and others 2%. According to the National Cancer Registry 2003 Report, (Lim and Yahya, 2003), Chinese males had a 5.1 higher frequency of colon cancer than Malay males whereas Chinese females had a 4.6 higher incidence of CRC than Malay females. Regarding rectal cancer, Chinese males had 2.8 higher incidence than Malays males and Chinese females had 3.4 times higher risk than Malay females. Thus, this is very worrying,

especially in Penang, where the Chinese populations is the highest than any other parts of Malaysia (Penang Statistic, 2001).

According to Khor and Gan, 1992, the high incidence of CRC seen in Chinese population could be reflective of their higher proportion in the urban areas. They suggested that more people in the urban areas are seeking better treatment. This may account for some of the high incidence seen. Furthermore, they also suggested the Chinese community is the most affluent and more likely to have a western life-style.

As reviewed by Weitz et. al, 2005, CRC is strongly linked to dietary carcinogens. In their study, Khor and Gan, 1992 also indicated an increase in the prevalence of hypercholesterolaemia and obesity among urban adults. Thus, this could also explain the high incidence of CRC seen in Chinese population.

Figure 1.2.1(a) Ten most frequent cancers in males, Peninsular Malaysia 2003

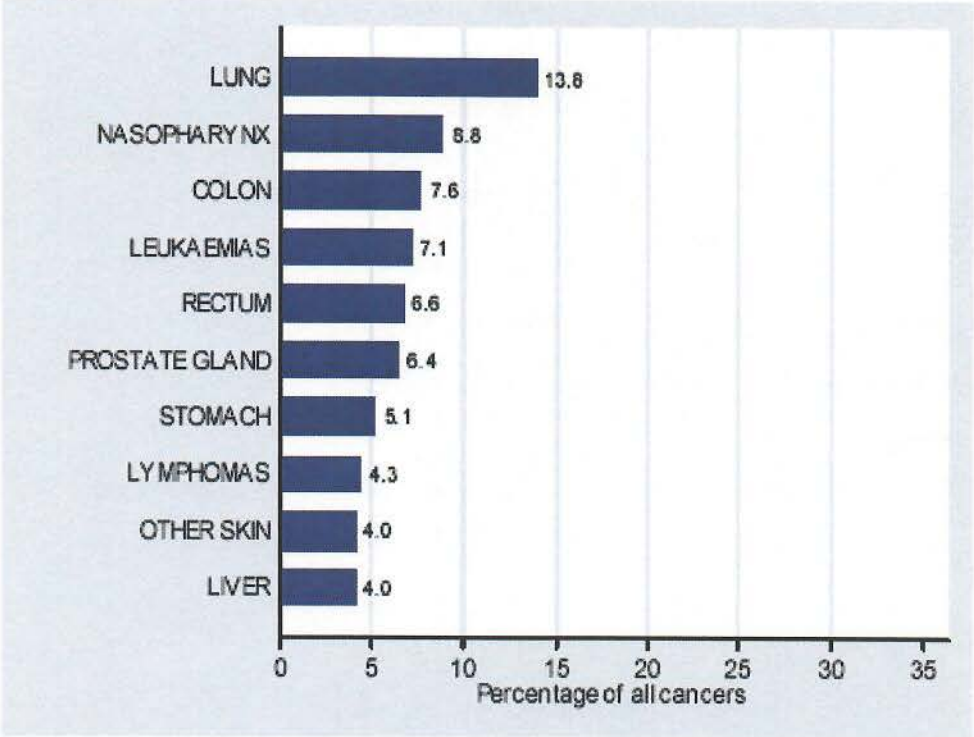

Figure 2.1 : The Ten Most Frequent Cancers In Males, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003)

Figure 1.2.1(b) Ten most frequent cancers in females, Peninsular Malaysia 2003

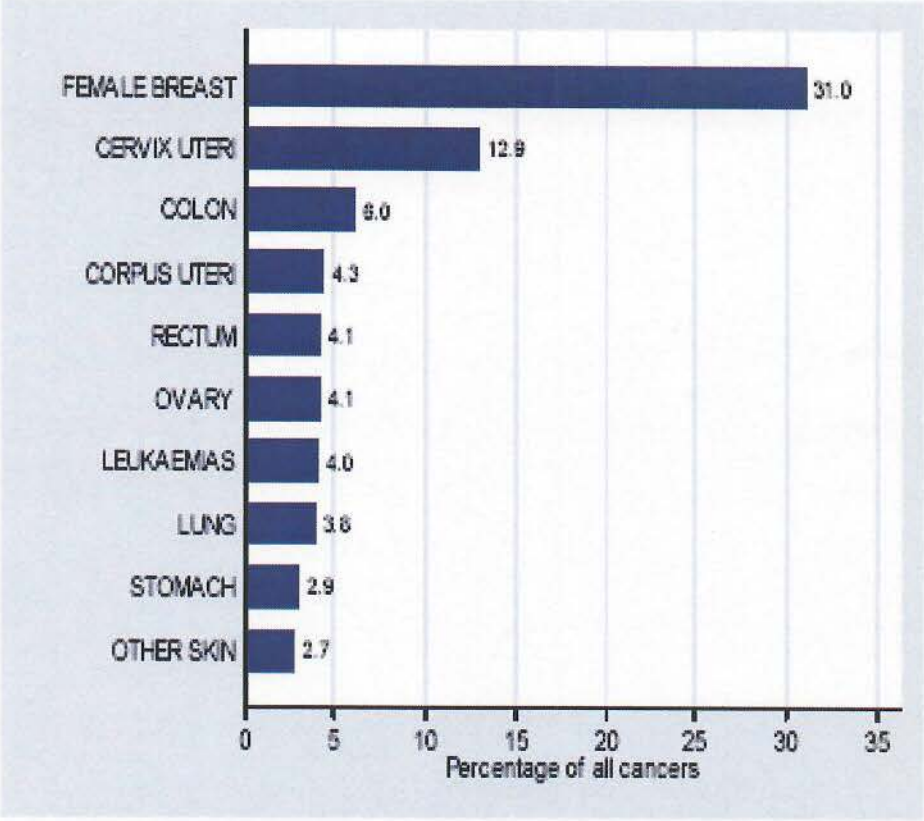

Figure 2.2 : The Ten Most Frequent Cancers In Females, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003)

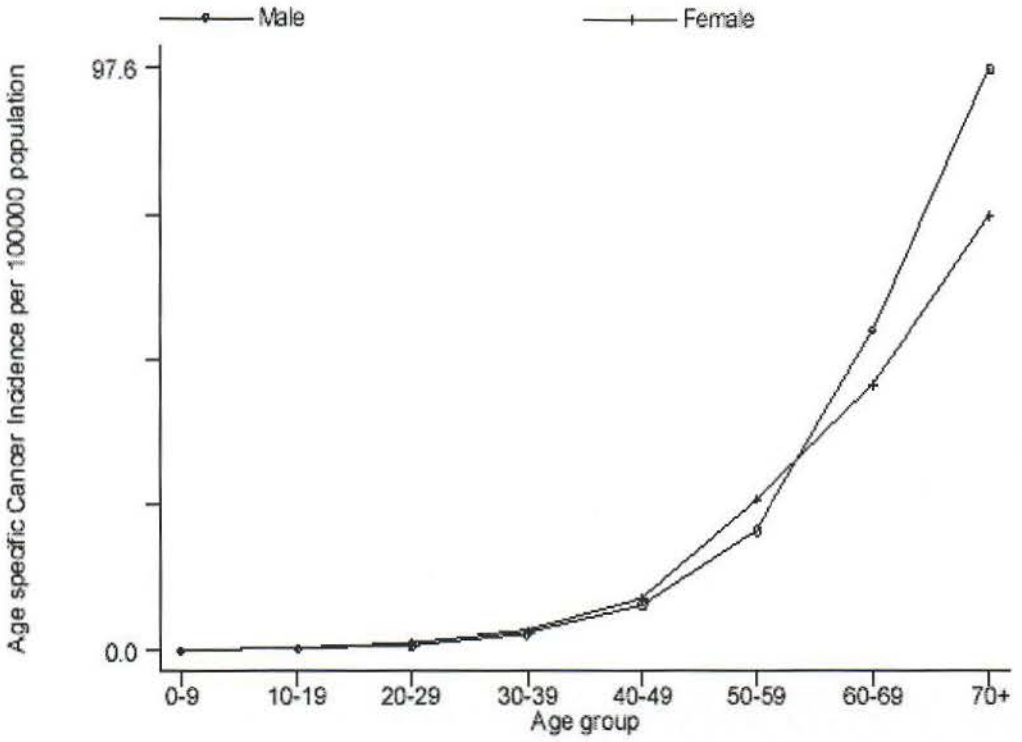

Figure 2.3 : Colon Age Specific Cancer Incidence per 100,000 Population, By Sex, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003)

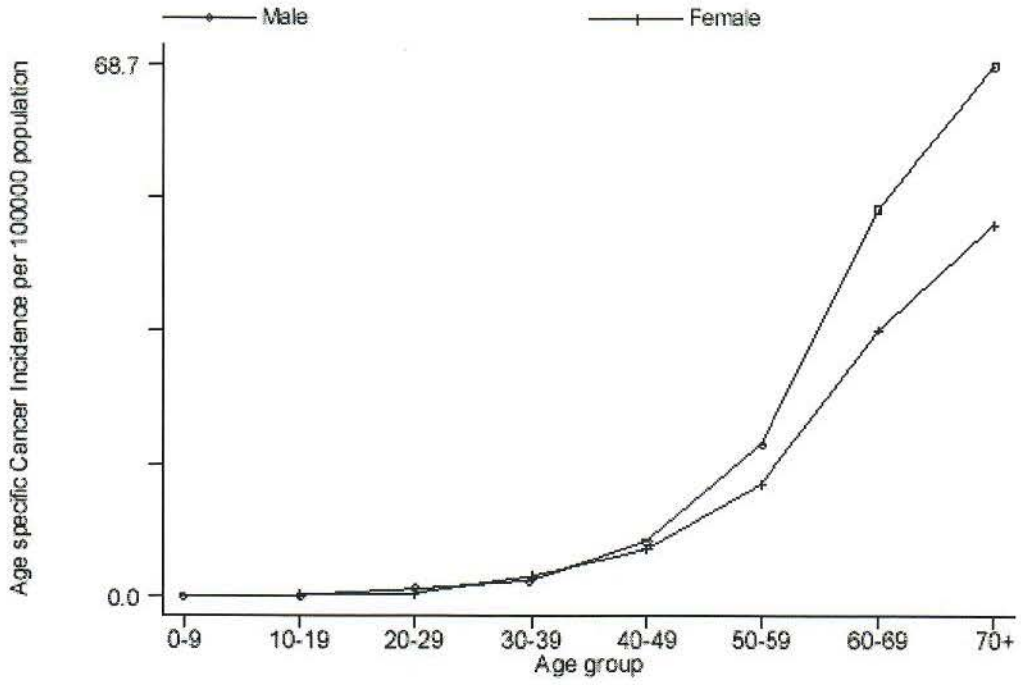

Figure 2.4 : Rectum Age Specific Cancer Incidence per 100,000 Population, By Sex, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003)

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	0.1	0.4	0.7	2	4	11.4	25.6	43.4	0.7
	Chinese	0	0.4	1	4	14	31.5	86.7	190.4	2.2
	Indian	0	0	0	0.7	3.4	11.5	38.1	30.3	0.7
Female	Malay	0.1	0.2	1.6	2.1	5.9	14.8	21.1	30.6	0.7
	Chinese	0	0.2	1	4.9	13.5	40	80.8	136.2	2
	Indian	0	0	0	1.4	6.7	20	24.5	20.4	0.5

Table 2.1: Colon Age Specific Cancer Incidence per 100,000 Population, By Ethnicity and Sex, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003).

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	0	0.1	0.8	2	5.3	16.3	34.4	43.4	0.9
	Chinese	0	0	0.7	1.5	8.9	24.9	67.1	103.8	1.4
	Indian	0	0	1.3	0.7	6	10.1	47.6	36.4	0.8
Female	Malay	0	0.1	0.5	2.2	5.1	11.2	22.4	23.7	0.6
	Chinese	0	0	0	2.8	9	19.2	54.3	82.1	1.2
	Indian	0	0	0	1.4	3.3	15.7	8.2	51	0.5

Table 2.2 : Rectum Age Specific Cancer Incidence per 100,000 Population, By Ethnicity and Sex, Peninsular Malaysia, 2003.

(Adapted from Lim and Yahya, Second National Cancer Registry Report, 2003).

2.2 Pathogenesis of Colorectal Cancer

Most colorectal cancers develop from normal epithelium through worsening degrees of adenomatous dysplasia. In 1990, Fearon and Vogelstein proposed the pathogenesis of colorectal cancer through the adenoma- carcinoma sequence pathway (Figure 2.5). The adenoma-carcinoma sequence is the basis for development of CRC (Lynch and Hoops, 2002, and Kondo and Issa, 2004). Basically, colorectal carcinogenesis can be explained by two pathways: the gatekeeper (or the suppressor pathway) and caretaker (or the mutator phenotype pathway). These two pathways are different because they result from mutations in different cancer genes (Kinzler and Vogelstein, 1997, and Rodriguez-Bigas et. al, 1997).

In the first pathway, mutational inactivation of both alleles of a tumour suppressor gene is needed and activated oncogenes are also involved (Rodriguez-Bigas et. al,1997). One of the critical steps in this pathway is mutation of the adenomatous polyposis coli (APC) gene (Powell et. al, 1992, Groden et.al, 1991 and Kinzler et. al, 1991). APC mutations are important in early cell transformation and known as "gatekeeper gene" (Kinzler and Vogelstein, 1998). Mutations in APC will lead to a truncated APC protein (Miyoshi et. al, 1992) and are detected in 75% of sporadic CRC (Miyake et.al, 1994). Nevertheless, germline mutations in APC can cause familial CRC (Miyake et. al, 1994). The APC gene product exerts its tumour suppressor actions through intracellular signaling, control of cell proliferation and interactions with the cytoskeleton, possibly by affecting the rate of cell division or

apoptosis (Baeg et. al, 1995, Su et.al,1993, Rubinfeld et.al, 1993 and Browne et.al, 1994). Other tumour suppressor genes involved are deleted colon cancer (DCC), Smad4 and p53. Oncogenes such K-ras, c-myc, c-neu and C-erb2 are also involved (Calvert and Frucht, 2002). This pathway will lead to an aneuploid tumour. Cells that contain the inactivated tumour suppressor genes and activated oncogenes will have growth advantage over neighbouring cells (Weitz et.al, 2005 and Rodriguez-Bigas et. al, 1997).

The second pathway is characterised by mutations of genes that maintain genetic stability, the mismatch repair (MMR) genes (Lynch and de la Chapelle, 2003). Tumours resulting from this pathway are diploid and have microsatellite instability (MSI) (Rodriguez-Bigas et. al, 1997). Besides the oncogenes and tumour suppressor genes in the gatekeeper pathway, further tumour suppressor genes such as the TGFβRIII, IGF2R and BAX are mutated in this pathway (Lynch and de la Chapelle, 2003, Lynch and Hoops, 2002, and Jass et.al, 2002). Nevertheless, tumours arising from this pathway do not have any territorial advantage over neighbouring cells (Rodriguez-Bigas et. al, 1997).

However, according to Jass et. al, 2002, both pathways might not be completely separated. This is because APC gene can also act as a caretaker and MMR genes can affect cell proliferation. Additional pathways could also exist, such as the serrated pathway, distinct carcinogenesis pathway for flat and depressed colorectal neoplasms and carcinogenesis for inflammatory bowel disease (Krok and Lichtenstein, 2004,

Jass et. al, 2002, Soetikno et. al, 2003, and Itzkowitz and Yio, 2004). Epigenetic changes in DNA methylation, loss of imprinting, histone acetylation, modifier genes (peroxisome proliferators-activating receptor gene and cyclooxygenase-2 gene also seem to be involved (Kondo and Issa, 2004, Rao et. al, 2004, Sinicrope and Gill, 2004, and Harman et. al, 2004).

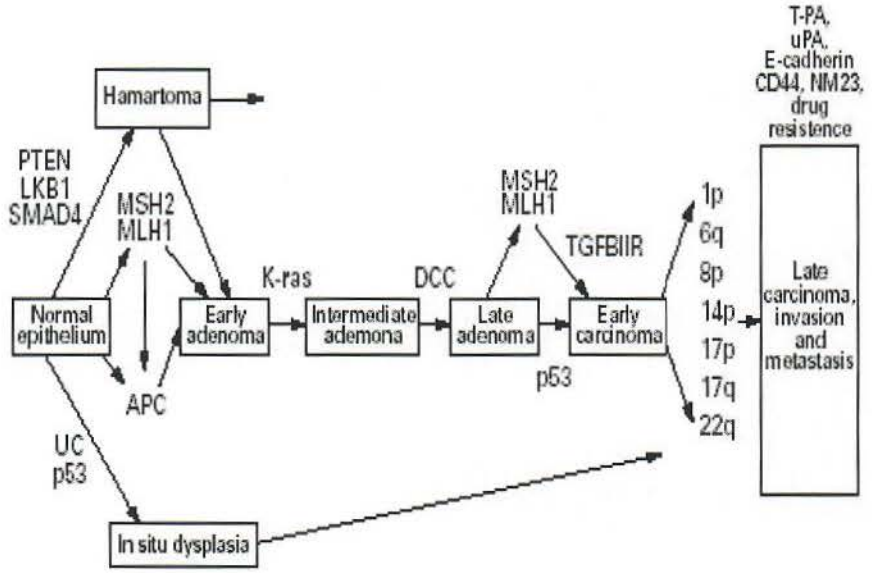

Figure 1 Genetic pathways of colorectal carcinogenesis.

Figure 2.5: Schematic Diagram of Progression of Colorectal Cancer

(Adapted from Houlston, 2001)

The majority of CRC cases are sporadic. Nevertheless, genetic and environmental factors can play important roles in the development of CRC (Table 2.3) (Weitz et. al, 2005). However, about 20% of sporadic CRC patients are also predicted to have some element of familial risks (Lynch and de la Chapelle, 2003). Thus, family history should always be obtained when assessing a patient suspected with CRC (Mitchell et. al, 2004).

Approximately 5 to 10% of all CRC cases develop as a result of hereditary syndromes. The two main hereditary CRCs are hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (Weitz et. al, 2005, Sack and Rothman, 2000). Syndromes such as Peutz-Jeghers syndrome, juvenile polyposis syndrome and Cowden syndrome are also associated with an increased risk of this hereditary CRC (Half and Bresalier, 2004).

HNPCC is the most common form of hereditary CRC (Lynch and de la Chapelle, 1999). In HNPCC patients, there are mutations in mismatch repair (MMR) genes which result in a state called microsatellite instability (MSI) (Lindblom et. al,1993, Lengauer et. al, 1998, Boland et.al, 1998, Peltomaki et. al,1993, Leach et. al, 1993, and Papadopoulos et.al, 1994). HNPCC affected persons have a very high risk for CRC but they do not develop hundreds of adenomas as seen in FAP. Although they only have few polyps, these polyps are very likely to progress to cancer and the rate of progression is rapid. This is because the polyps start with mutations in the MMR genes and transform rapidly when APC mutation occur (Calvert and Frucht, 2002).

FAP is the rarest colon cancer syndrome, which is responsible for about 0.5% of CRC (Rutsgi, 1994 and Burt and Samowitz, 1988). As reviewed by American National Health Institutes, 2004, Calvert and Frucht, 2002, and Weitz et al, 2005, FAP is an autosomal dominant disease. Germline mutations of APC gene can be identified in affected individuals (Kinzler et.al, 1991, Groden et. al, 1991, Spirio et. al, 1993, Brensinger et.al, 1998, Soravia et. al, 1998, Pedemonte et. al, 1998, and Sieber et. al, 2002). FAP patients can develop more than 100 colorectal adenomas, but their rate of transition is low. However, if left untreated, these adenomas can give rise to CRC, usually before the age of 40 years (Half and Bresalier, 2004, and Weitz et.al, 2005).

In 1991, the Amsterdam Criteria (Table 2.4) was established by International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer to ensure that researchers investigating the genetic basis of HNPCC were studying families or individuals with the same syndrome (Grady, 2003 and Vasen et. al, 1999). However, when genetic basis of HNPCC were clinically relevant, the Amsterdam Criteria was modified and replaced by the Bethesda Guidelines, (Table 2.4) (Umar et. al, 2004).

2.3 Microsatellite Instability

According to American National Cancer Institute, MSI cancer is responsible for 10% to 15% of all CRC. It can be seen in majority of hereditary nonpolyposis colorectal cancer (HNPCC) (Aaltonen et. al, 1993 and Thibodeau et. al, 1993) and some sporadic cases (10%-15%) (Somawitz et. al, 1995, Kim H et. al, 1994, Liu et. al,

1995, Bubb et. al, 1996, and Syngal et. al, 1998). MSI cancers are characterized by mutations of mismatch repair (MMR) genes (Peltomaki, 2001). In humans, at least six different MMR proteins are required, namely, MLH1, MSH2, PMS1, PMS2, MSH6 and MSH3 (Papadopoulos et. al, 1994, Chung and Rutsgi, 1995, Papadopoulos et. al, 1995). The majority of the mutations in CRC affect MLH1 (human mutL homolog 1) and MSH2 (human mutS homolog 2) (Eshleman and Markowitz, 1996, Peltomaki and Vasen, 1997, Boland et. al, 1998, and Lynch and de la Chapelle, 1999).

In HNPCC, one MMR mutation is inherited and the other one occurs somatically, whereas in sporadic CRC both MMR mutations occur somatically (Calvert and Frucht, 2002, Liu et. al,1996 and Wheeler et. al, 2000). Inactivation of MMR and APC (adenomatous polyposis coli) proteins will produce a raised mutation rate in HNPCC. Nevertheless, it is also likely that MMR mutation have a direct role in initiation of HNPCC (Liu et al, 1995). In sporadic MSI cancers, APC remain the initiator of the tumour and therefore MMR mutations are rather involved in the progression of CRC (Kinzler and Vogelstein, 1998). MSI may be a late event in the adenoma and carcinoma sequence and is not directly related to the increased mutation rate (Thomlinson et al, 1996).

The primary role of MMR proteins is to eliminate mismatches and insertion-deletion loops that may arise during DNA replication as a result of DNA polymerase slippage (Buermeyer et.al, 1999, Jiricny and Nystrom-Lahti, 2000, and Peltomaki, 2001).

MLH1 and MSH2 proteins are the integral components of mismatch repair (Mitchell et. al, 2002).

For mismatch recognition, the MSH2 proteins will form a heterodimer with MSH6 or MSH3. The resulting complexes are called hMutSα and hMutSß respectively. This is dependant on whether base-base mispairs or insertion deletion loops are to be fixed (Kolodner and Das Gupta, 2000, and Kolodner, 1996). In other instances, MLH1 and PMS2 will form a heterodime (hMutLα), and organize the interaction between the mismatch recognition complex and other proteins required for the mismatch repair (Wheeler and Bodmer, 2000) (Figure 2.6). These proteins are DNA polymerase δ, replication protein A, proliferating cell nuclear antigen (PCNA), replication factor C, exonuclease 1, FEN1 and DNA polymerase δ and ε associated exonuclease (Syngal et. al, 1999). MLH1 may also heterodimerize with MLH3 and PMS1. According to Lipkin et.al, 2000, MLH1-MSH3 complex functions mainly in the repair of insertion deletion loops. However, the role of MLH1-PMS1 is yet to be determined (Raschle et. al, 1999 and Leung et. al, 2000). Once the DNA mismatch has been identified, and the MutS and MutS heterodimer complexes have combined with it, activated exonuclease will then mediate the degradation of the DNA (Sancar, 1999).

As has been noted, mutations in MLH1 and MSH2 cause the majority of MSI cancers. So far, more than 200 allelic variants have been identified for each gene, and majority of these can be pathogenic in CRC (Mitchell et al, 2002). The MSH2 gene is located at chromosome 2p21 (Peltomaki et. al, 1993 a, and Leach et al, 1993) whereas

MLH1 is located at 3p21-23 (Lindblom et. al, 1993), both areas have been identified to be important in HNPCC. Akiyama et. al, 1997, Wijnen et. al, 1999 and Wu et. al, 1999 reported an increased mutations in the MSH6 gene in recent years. In MSI sporadic CRC, MSI-H tumors are due to inactivation of MLH1 gene (Kuismanen et al, 2000). Nevertheless, in MSI-L, both MLH1 and MSH2 do not seem to be associated (Thibodeau et. al, 1998, and Percesepe et. al, 1998).

Panel 1: Risks factors and causes

Sporadic colorectal cancer (88-94%)

Older age

Male sex

Cholecystectomy

Ureterocolic anastomosis

Hormonal factors: nulliparity, late age at pregnancy, early menopause

Environmental factors

Diet rich in meat and fat, and poor in fibre, folate and calcium

Sedentary lifestyle

Obesity

Diabetes mellitus

Smoking

Previous irradiation

Occupational hazards (eg. asbestos exposure)

High alcohol intake

Personal history of sporadic tumours

History of colorectal polyps

History of colorectal cancer (risk is 1.5%for second such cancer in first 5 years)

History of small bowel, endometrial, breast or ovarian cancer

Familial colorectal cancer (20%)

First or second degree relatives with this cancer, criteria for hereditary colorectal cancer not fulfilled:

- One affected first-degree relatives increases risk 2.3 fold
- Two or more first-degree relatives increases risk 4.25 fold
- Index case < 45 years increases risk 3.9 fold
- Familial history of colorectal adenoma increases risk 2 fold

Colorectal cancer in inflammatory bowel disease (1-2%)

Ulcerative colitis

Crohn's colitis

Hereditary colorectal cancer (5-10%)

Polyposis syndrome: familial adenomatous polyposis (FAP), Gardner's syndrome, Turcot's syndrome, attenuated adenomatous polyposis coli, flat adenoma syndrome

Hereditary nonpolyposis colorectal cancer (HNPCC)

Hamartomatous polyposis syndromes (Peutz- Jeghers syndrome, juvenile polyposis syndrome, Cowden syndrome)

Table 2.3: Risk Factors Associated With Sporadic and Hereditary Colorectal Cancer (Adapted from Weitz et. al, 2005)

Bethesda Gudelines

- Colorectal cancer diagnosed in patient who is younger than 50 years
- Presence of synchronous, metachronous colorectal, or other HNPCC-associated tumours, irrespective of age*
- Colorectal cancer with the MSI-H† histology ‡diagnosed in a patient who is younger than 60 years§
- Colorectal cancer diagnosed in one or more first degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under the age 50 years
- CRC diagnosed in two or more first or second degree relatives with HNPCC-related tumours, irrespective of age
-

Amsterdam I and II

- One individual diagnosed with colorectal cancer (or extracolonic HNPCC-associated tumours)¶ before age 50 years
- Two affected generations
- Three affected relatives, one a first-degree of the other two
- FAP should be excluded
- Tumours should be verified by pathological examination

*HNPCC-related tumours include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, billiary trac, and brain(usually glioblastoma as seen in Turcot's syndrome) tumours sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel. †MSI-H=microsatellite instability-high in tumours refer to changes in two or more of the five US National Cancer Institute-recommended panels of microsatellite markers.‡Presence of tumour-infiltrating lymphocyte, Crohn's -like lymphocytic reaction, mucinous/signet- ring differentiation, or medullary growth pattern. §No consensus was reached among the workshop participants on whether to include the age criteria in guideline 3 above: participants voted to keep younger than 60 years in the guidelines. ¶Amsterdam II criteria: extracolonic HNPCC-associated tumours also considered.

Table 2.4: Bethesda Guidelines for detection of patients at risk for HNPCC who should undergo microsatellite instability testing of tumour (only one criterion need be fulfilled), and Amsterdam I and II criteria for clinical definition of HNPCC patients (all criteria must be met).

(Adapted from Weitz et. al, 2005)

Figure 1. Human DNA MMR protein complexes and their preferences for different types of mismatches, shown before and after correction (see text).

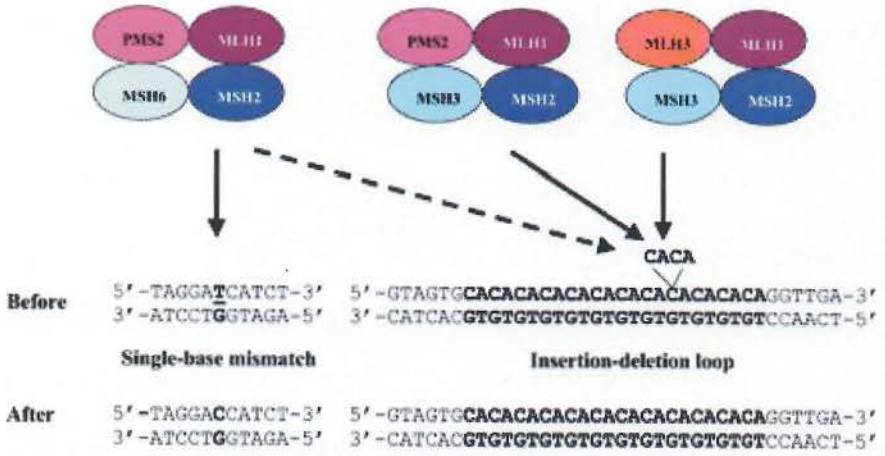

Figure 2.6: DNA Mismatch Repair

(Adapted from Peltomaki, 2001)

2.3.1 Microsatellite Instability and Clinicipathological Characteristics

As different genetic pathways exist for colorectal cancer (CRC), studies have suggested that there is a correlation between these pathways and clinicopathological features of CRC. As reviewed by de la Chapelle, 2002, correlations between phenotype of CRC and microsatellite instability (MSI) could help in the management of therapies and could serve as a prognostic marker in patients.

HNPCC and sporadic MSI positive cancers can be separated from sporadic MSS (microsatellite stable) on the basis of some clinicopathological features. Studies have reported that MSI cancers showed significant survival advantage when compared to MSS tumours. In 1998, Watson et. al demonstrated better survival of 98 HNPCC families when compared to 820 control patients with colorectal cancers. Many subsequent studies have reinforced this finding. For example, a large study by Gryfe et. al, 2000 on 607 patients also concluded that MSI status is associated with a survival advantage with a hazard ratio (HR) of 0.42.A study done by Ward et.al, 2001, based on analysis of 310 sporadic cases reported that MSI-H tumours had a trend towards prolonged recurrence free interval and improved overall survival. A study carried out by Cawkwell et.al,1999 showed improved survival although MSI cancers possessed 5.54 times higher risks of developing a metachronous CRC.

As reviewed by Boland et. al, 1998 on the National Cancer Institute Workshop on MSI, MSI-H tumours are found predominantly in the proximal colon and present in

older age group. In a population based study of 201 colorectal cancer cases, Chao et. al, 2000 had found significant association between MSI with tumours in proximal colon. The MSI tumours were more common in older age group patients (more than 70 years and 80 years). There was also a positive association between MSI and female patients. Ward et. al, 2001 also made the same conclusion that positive correlation existed between MSI and proximal (right) colon. Jernvall et. al, 1999 also found a better survival rate in MSI cancers of the proximal colon as compared to those of MSS proximal disease. They concluded that this finding was attributed to better response of this cancer to adjuvant chemotherapy. Thus, MSI status could foresee response to adjuvant chemotherapy. Nevertheless, in a randomized trial of 570 tissue specimens of which 95% exhibited MSI-H, Ribic et. al, 2003 assessed whether fluorouracil-based adjuvant chemotherapy would benefit MSI positive CRC. They found that MSI CRC had improved five-year rate of overall survival than patients with tumors exhibiting MSS cancers. Adjuvant chemotherapy improved overall survival among patients with MSS tumors. Nevertheless, it did not increase survival of patients in MSI-H tumours. Studies conducted by Carethers et. al 1999, Colella et. al, 1999 and Fink et. al,1998 reported that MSI-H tumours either hereditary or sporadic may be resistant to certain commonly used chemotherapeutic agents. Thus, information on MSI status would guide clinicians in predicting prognosis and planning treatment for patients. All the studies are summarised in table 2.5.

A prospective study by Jass et.al, 1998 on 303 cases found that tumour type (adenocarcinoma, mucinous carcinoma and undifferentiated carcinoma), tumour infiltrating lymphocytes and anatomical sites were the most significant variables discriminating MSI positive and MSI negative cancers. They concluded that pathological examinations of CRC allow identification of tumours with extensive MSI. Ward et. al, 2001 also concluded that MSI-H tumours were significantly more likely to demonstrate a mucinous phenotype, have increased number of intraepithelial lymphocytes and preferably occur in women. Alexander et. al, 2001 found that intraepithelial lymphocytosis and poor differentiation were the best discriminators between MSI-H and MSS cancers. Nevertheless, they concluded that even though histopathological assessment can be used to prioritize sporadic colon cancers for MSI studies, it has a low sensitivity (49%), thus requires further analysis for therapeutics judgment.

2.4 Evaluation of Microsatellite Instability

According to Mitchell et. al, 2002 and Jover et. al, 2004, the current gold standard for assessing microsatellite instability (MSI) is molecular MSI testing. As noted by Mitchell et. al, 2002 and de la Chapelle, 2002, the test involves extracting DNA from both normal and tumour tissue resected during surgery. The DNA is subjected to polymerase chain reaction (PCR) for amplification of different chromosomal loci that compare repeated sequence of CA dinucleotide or known as microsatellite. Then, the PCR product is separated by polyacrylamide gel electrophoresis and potential