

**PATTERN OF hMLH1, hMSH2 AND hMSH6 EXPRESSIONS
AND CLINICAL CHARACTERISTICS IN MALAYSIAN
COLORECTAL CARCINOMA CASES**

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**FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

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COLORECTAL CARCINOMA CASES**

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FAMM, MBBS, MPath, FRCPA, MIAC

**THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF MEDICINE**

DEPARTMENT OF PATHOLOGY

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2011

I declare that this thesis records the results of research carried out by me, that it is my own composition and that it has not been submitted previously for a higher degree in any University.

Khoo Joon Joon

PREFACE

“Each disease has a nature of its own; none arises without its natural cause”

Greek physician Hippocrates, born 460 BC – died 357 BC.

DEDICATION

This work is dedicated especially to my family – my husband Chee Khin who has been my main source of support and endless encouragement and my two lovely children, Cheau Wern and Ming Wei who are my constant source of joy, pride and inspiration.

SPECIAL DEDICATION

I would like to make a special dedication of this work to my parents, Mr. & Mrs. Khoo Thean Hock; both of whom I love dearly. I am very proud they have brought up all their children by example and given us everything without expecting anything in return. I am who I am today because of them. God bless them.

UNIVERSITY MALAYA

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PATTERN OF hMLH1, hMSH2 AND hMSH6 EXPRESSIONS AND CLINICAL CHARACTERISTICS IN MALAYSIAN COLORECTAL CARCINOMA CASES

Field of Study: Anatomical Pathology

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ABSTRACT

Introduction and Objectives:

Many environmental factors and genetic risk factors have been implicated in the development of colorectal carcinomas. Several genetic mechanisms of tumorigenesis had been suggested i.e. the suppressor and the mutator pathways. The suppressor pathway constitutes inactivation of tumour suppressor genes: p53, APC (adenomatous polyposis coli) and DCC (deleted in colorectal cancer) genes or the activation of proto-oncogene: k-ras. The mutator pathway consists of inactivation of mismatch repair (MMR) genes which results in inability to repair mismatched DNA (deoxyribonucleic acid) bases during replication.

The aims of the study were to determine the incidence and pattern of mismatch repair defect by immunohistochemistry in a sample of Malaysian colorectal carcinoma cases and correlate this to the clinical and pathological features. Additionally, the relationship between p53 over-expression and the mismatch repair status of the tumours were analysed.

Materials and Methods:

The clinical and demographic characteristics of 298 patients with colorectal carcinomas and the histomorphology of their tumours were studied. Analyses of the mismatch repair genes as well as mutation of the tumour suppressor gene were determined by immunohistochemical methods using antibodies against hMLH1, hMSH2 and hMSH6 proteins; and p53 respectively.

Results:

The ratio of male to female patients with colorectal carcinomas was 1.26:1. Their age ranged from 25 to 91 years (mean of 61 years). There was an overall predominance of left sided lesion (69.5%). Forty three out of 298 cases (14.4%) showed abnormal staining

pattern for at least one mismatch repair proteins with majority of cases (65.1%) showing single hMLH1 loss. About half of the colorectal carcinomas (50.7%) were associated with p53 over-expression. 92.7% of tumours with p53 over-expression did not have any mismatch repair defect (MMR-d) and 74.4% of MMR-d tumours did not show any p53 over-expression ($p<0.001$). Tumours with mismatch repair defect were located frequently at the right side of colon ($p<0.001$) while tumours showing p53 over-expression were significantly left sided ($p<0.001$).

MMR-d tumours were more likely poorly differentiated carcinomas ($p<0.001$), produced larger amounts of mucin ($p=0.007$), showed exophytic growth ($p=0.007$) and were bigger in size ($p=0.002$) than tumours with no mismatch repair defect. However, there was no significant difference in age at presentation, gender, race or survival for patients with MMR-d tumours compared to patients without the defect.

Discussion and Conclusion:

In this study there were 14.4% of colorectal carcinoma cases with mismatch repair defect, which was comparable with that found worldwide (7 to 20%).

The patients with MMR-d colorectal carcinomas had distinct clinical and pathological features. Immunohistochemical staining for MMR-d should be done on these selected cases. This information on the MMR-d status will definitely help clinicians in their management of the patients.

There was a significant inverse correlation between loss of MMR-d protein and p53 over expression. MMR-d tumours and tumours with p53 over-expression also arose in significantly different anatomical sites. This supported the suggestion that there are at least two different pathways of colorectal carcinogenesis: the suppressor gene pathway and MMR gene inactivation (mutator) pathway.

CORAK DAN KEJADIAN hMLH1, hMSH2 DAN hMSH6 SERTA CIRI-CIRI KLINIKAL PESAKIT-PESAKIT DENGAN KOLOREKTAL KARSINOMA DI MALAYSIA

ABSTRAK

Pendahuluan dan tujuan kajian

Banyak faktor persekitaran dan faktor risiko genetik terlibat dalam perkembangan karsinoma kolorektal. Beberapa mekanisme genetik tumorigenesis telah dicadangkan termasuk '*suppressor*' dan '*mutator pathways*'. *Suppressor pathway* merupakan inaktivasi gen *suppressor p53*, *APC*, dan *DCC* atau aktivasi *proto-oncogen*, *k-ras*. *Mutator pathway* terdiri dari inaktivasi gen *mismatch repair* yang tidak mampu memperbaiki basa *DNA* yang salah dan tidak se-cocok atau sepadan. Tujuan kajian ini adalah untuk menentukan kejadian dan corak mutasi pada gen *mismatch repair DNA* dalam kes-kes karsinoma kolorektal dan melibatkannya dengan ciri-ciri klinikal dan patologi serta membandingkannya dengan kejadian mutasi *p53* dalam kes-kes kanser ini.

Bahan kajian dan metodologi

Ciri-ciri klinikal dan demografi 298 pesakit yang menghidapi penyakit karsinoma kolorektal dikaji. Ciri-ciri *histomorphology* kanser mereka dipelajari. Analisisa gen *mismatch repair* dan mutasi gen *suppressor* ditentukan dengan melaksanakan ujian imuno-histokimia untuk *hMLH1*, *hMSH2* dan *hMSH6*; dan *p53* masing-masing.

Keputusan

Nisbah pesakit lelaki dengan pesakit wanita dengan karsinoma kolorektal adalah 1.26:1. Usia mereka adalah dalam lingkungan 25-91 tahun (purata = 61 tahun). Kebanyakan kes karsinoma kolorektal terdapat di sebelah kiri usus besar (69.5%). Empat puluh tiga daripada

298 kes-kes (14.4%) menunjukkan corak luar biasa dan tidak mempunyai sekurang-kurangnya satu protein *mismatch repair*. Kebanyakannya (65.1%) menunjukkan kehilangan *hMLH1* tunggal. Lebih kurang setengah daripada karsinoma kolorektal (50.7%) dikaitkan dengan mutasi *p53*. 92.7% daripada kanser dengan mutasi *p53* tidak memiliki kecacatan *mismatch repair* dan 74.4% daripada kanser dengan *MMR-d* tidak menunjukkan mutasi *p53* ($p < 0.001$). Kes-kes kanser dengan kecacatan *mismatch repair* sering terdapat di sebelah kanan usus besar ($p < 0.001$) sedangkan kes-kes kanser yang menunjukkan mutasi *p53* didapati di sebelah kiri usus besar ($p < 0.001$). Kes-kes kanser *MMR-d* lebih mungkin mempunyai karsinoma yang berdiferensiasi buruk ($p < 0.001$), menghasilkan lebih mucin ($p = 0.007$), mempunyai pertumbuhan exophytic ($p = 0.007$) dan lebih besar ($p = 0.002$) daripada kes-kes kanser yang tidak ada kecacatan gen *mismatch repair*. Namun begitu, tidak ada perbezaan dalam usia presentasi, bangsa, jantina atau kehidupan (survival) untuk pesakit-pesakit dengan kanser *MMR-d* apabila dibandingkan dengan pesakit-pesakit tanpa kecacatan gen ini.

Perbincangan dan Kesimpulan:

Dalam kajian ini ada 14.4% kes-kes karsinoma kolorektal dengan kecacatan gen *mismatch repair*. Ini boleh dibandingkan dengan laporan-laporan terbitan di seluruh dunia (7 sampai 20%). Para pesakit dengan kanser *MMR-d* memiliki ciri-ciri klinikal dan patoloji yang berbeza dan istimewa. Ujian imuno-histokimia untuk *MMR-d* harus dilakukan pada kes-kes yang terpilih. Informasi berkenaan status *MMR-d* pasti akan membantu doktor dalam rawatan mereka.

Terdapat hubungan terbalik antara kehilangan *MMR-d* protein dan mutasi *p53*. Kes-kes kanser *MMR-d* dan kes-kes kanser dengan mutasi *p53* juga muncul di tempat anatomi

berbeza. Hal ini menyokong pendapat bahawa ada dua *pathway* yang berbeza dalam pertumbuhan kanser kolorektal iaitu '*suppressor*' dan '*mutator pathways*'.

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TABLE OF CONTENTS

CHAPTER	Page
1 INTRODUCTION AND OBJECTIVES OF STUDY	
1.1 Cancer	
1.1.1 Genetics and Cancer	2
1.1.2 Cancer and Cell Cycle	7
1.1.3 Germline and Somatic Mutations	9
1.1.4 Sporadic and Hereditary Cancers	9
1.1.5 DNA Mismatch Repair Genes	11
1.2 Colorectal Carcinoma	
1.2.1 Incidence	14
1.2.2 Risk factors	20
1.2.3 Clinical Presentation	22
1.2.4 Investigations and Management	22
1.3 Genetics and Colorectal Carcinomas	
1.3.1 Genetic Pathways	26
1.3.2 Hereditary Colorectal Carcinomas	26
1.3.3 Sporadic Colorectal Carcinomas	29
1.3.4 Mismatch Repair Genes	30
1.3.5 Tumour Suppressor Gene: p53	34
1.4 Objectives of Study	
1.4.1 Tumours with MMR defects	35
1.4.2 Tumours with p53 over-expression	36
1.4.3 Summary of Objectives	37

2	METHODOLOGY	
2.1	Material	
2.1.1	Patients	39
2.1.2	Clinical Presentation	39
2.1.3	Outcome	40
2.2	Character of Tumours	
2.2.1	Anatomical Location	41
2.2.2	Multiple tumours	41
2.2.3	Size of tumours	41
2.2.4	Growth appearance	42
2.2.5	Tumour Staging	44
2.3	Histopathological features	46
2.3.1	Histological Type and Grade	46
2.3.2	Mucin production and Mucinous Cancers	49
2.3.3	Tumour advancing pattern	51
2.3.4	Lymphocytic response	54
2.3.5	Tumour necrosis	56
2.4	Staining procedure	
2.4.1	Haematoxylin and Eosin stain	57
2.4.2	Immunohistochemical stain	57
2.5	Statistical Analysis	66

3	RESULTS	
3.1	Introduction	69
3.2	Demographics	
3.2.1	Racial Distribution	71
3.2.2	Gender	73
3.2.3	Age	73
3.3	Mismatch Repair Defect Tumours	
3.3.1	Staining pattern and Incidence	75
3.3.2	MMR-d tumours and Race	76
3.3.3	MMR-d tumours and Gender	81
3.3.4	MMR-d tumours and Age	82
3.4	Family History	
3.4.1	Family history of carcinomas	86
3.4.2	Family history and Age	88
3.4.3	Family history and MMR-d tumours	90
3.5	Character of Tumours	
3.5.1	Multiple tumours	91
3.5.2	Anatomical Location	94
3.5.3	Tumour growth appearance	96
3.5.4	Size of tumours	99
3.6	Histopathological Features	
3.6.1	Histological type and grade	104
3.6.2	Mucin production	107

3.6.3	Tumour advancing pattern	109
3.6.4	Lymphocytic response	111
3.6.5	Degree of necrosis	116
3.7	Staging of tumours	
3.7.1	TNM Staging	120
3.7.2	Depth of invasion	121
3.7.3	Lymph nodes status	124
3.8	Tumour suppressor gene: p53	
3.8.1	Staining pattern	127
3.8.2	P53 and MMR-d tumours	127
3.8.3	P53 and Anatomical location of tumour	128
3.8.4	P53 and Staging	128
3.9	Survival	
3.9.1	Survival of patients with CRC	130
3.9.2	Survival and Staging	131
3.9.3	Survival and MMR-d tumours	132
3.9.4	Survival and p53 status	135
3.9.5	Survival and other pathological features	137
3.10	Treatment	142
3.11	Summary of Results	143

4	DISCUSSION	
4.1	Colorectal Carcinomas : Demographics	
4.1.1	Incidence	150
4.1.2	Racial Distribution	152
4.1.3	Age and Gender	154
4.1.4	Anatomical Location of Tumours with Race and Age	156
4.2	MMR-d Colorectal Carcinomas	160
4.2.1	Immunohistochemical testing vs. PCR testing	160
4.2.2	Incidence of MMR-d tumours	163
4.2.3	Demographics of MMR-d tumours	
4.2.3.1	Age	166
4.2.3.2	Race	169
4.2.3.3	Gender	171
4.2.4	Family History	174
4.3	Character of Tumour	
4.3.1	Multiple tumours	181
4.3.2	Anatomical Location	183
4.3.3	Growth Appearance	184
4.3.4	Size of tumours	186
4.4	Histological Features	
4.4.1	Histological Type and Grade	187
4.4.2	Mucin production	189
4.4.3	Tumour advancing pattern	190
4.4.4	Lymphocytic response	191

4.4.5	Necrosis	195
4.5	Staging	
4.5.1	Lymph nodes status	197
4.5.2	Staging and MMR-d tumours	198
4.6	Survival	201
4.6.1	Anatomical Location	201
4.6.2	MMR-d tumours	202
4.6.3	Tumour advancing pattern	204
4.7	P53 positive colorectal carcinomas	
4.7.1	Introduction: p53	206
4.7.2	P53 and MMR-d Colorectal carcinomas	207
4.7.3	P53 and Anatomical location of tumours	208
4.7.4	P53 and Survival	209
4.8	Management of Colorectal carcinomas	
4.8.1	Surgery	211
4.8.2	Adjuvant Chemotherapy	212
4.8.3	New Treatment and Trials	213
4.9	Limitations of Study	214
5	CONCLUSION AND FUTURE DIRECTIONS	216
	APPENDICES	219
	REFERENCES	232

List of Figures

	page
1. Figure 1.1	6
2. Figure 1.2	8
3. Figure 1.3	17
4. Figure 1.4	19
5. Figure 1.5	23
6. Figure 2.1	43
7. Figure 2.2	48
8. Figure 2.3	50
9. Figure 2.4	51
10. Figure 2.5	53
11. Figure 2.6	55
12. Figure 2.7	60
13. Figure 2.8	61
14. Figure 2.9	62
15. Figure 2.10	65
16. Figure 3.1	71
17. Figure 3.2	72
18. Figure 3.3	74
19. Figure 3.4	77
20. Figure 3.5	83
21. Figure 3.6	100
22. Figure 3.7	105
23. Figure 3.8	106
24. Figure 3.9	117
25. Figure 3.10	121
26. Figure 3.11	122
27. Figure 3.12	123
28. Figure 3.13	130
29. Figure 3.14	132
30. Figure 3.15	133
31. Figure 3.16	135
32. Figure 3.17	137

33. Figure 3.18	Survival curve for patients categorised according to border patterns	138
34. Figure 3.19	Survival curve of patients categorised according to peri-tumoural lymphocytic response	139
35. Figure 3.20	Survival curve of patients categorised according to Crohn-like lymphocytic response	140
36. Figure 4.1	Age standardised incidence per 100,000 by gender and country for colorectal carcinomas	151
37. Figure 4.2	Population pyramid by age and gender, Malaysia, 2006	155
38. Figure 4.3	Oestrogens, oestrogen receptors and prevention of colon tumours	173
39. Figure 4.4	Colorectal carcinoma with tumour-infiltrating lymphocytes	192

List of Tables		page
1. Table 1.1	Selective list of activated oncogenes	3
2. Table 1.2	Inherited Cancer Syndromes	10
3. Table 1.3	Ten Principle causes of deaths in Malaysian hospitals	16
4. Table 1.4	Mismatch repair genes and mutation frequencies in HNPCC	32
5. Table 3.1	hMLH1, hMSH2 and hMSH6 protein expression in MMR-d colorectal cancers	76
6. Table 3.2	The different races with colorectal carcinomas by their mismatch repair status	78
7. Table 3.3	Distribution of patients by race: analysis of MMR-d tumours among Malay vs. Non-Malay patients	79
8. Table 3.4	Distribution of patients by race: analysis of MMR-d tumours among Chinese vs. Non-Chinese patients	80
9. Table 3.5	Distribution of patients by race and gender: analysis of MMR-d tumours among Malay vs. non-Malay female and male patients	81
10. Table 3.6	Distribution of patients by gender: analysis of MMR-d tumours among male vs. female patients	82
11. Table 3.7	Distribution of patients by age: analysis of MMR-d tumours among patients aged 50 and below vs. patients aged above 50 years	84
12. Table 3.8	Distribution of patients by age: analysis of MMR-d tumours according to age group	85
13. Table 3.9	Patients with family history of carcinomas	87
14. Table 3.10	Patients with family history of malignancies by age group	89
15. Table 3.11	Patients with metachronous non-colorectal carcinomas	92
16. Table 3.12	Distribution of patients by their presentation as single tumour or multiple tumours against their MMR staining pattern	93
17. Table 3.13	Frequencies of location of colorectal carcinomas in patients by ethnic race	94
18. Table 3.14	Distribution of colorectal cancers by location and MMR status	95
19. Table 3.15	Distribution of colorectal carcinomas by different growth patterns and location of tumours	97
20. Table 3.16	Distribution of colorectal carcinomas by location and tumour growth pattern	98
21. Table 3.17	Distribution of colorectal carcinomas by growth pattern and mismatch repair status	99
22. Table 3.18	Distribution of colon cancers by growth pattern and size of tumours	101
23. Table 3.19	Distribution of colorectal carcinomas by size of tumours and mismatch repair status	102
24. Table 3.20	Distribution of colorectal carcinomas by size of tumours and amount of mucin	103
25. Table 3.21	Distribution of colorectal carcinomas by mucin production and mismatch repair status	109
26. Table 3.22	Distribution of colon cancers by tumour location and border pattern	110

27. Table 3.23	Association of peri-tumoural and Crohn-like lymphocytic response and other pathological parameters	112
28. Table 3.24	Distribution of tumours by their lymphocytic response (peri-tumoural) and MMR-status	114
29. Table 3.25	Distribution of tumours by their lymphocytic response (Crohn-like) and MMR-status	116
30. Table 3.26	Distribution of colorectal carcinomas by size and degree of necrosis present	118
31. Table 3.27	Distribution of colorectal carcinomas by MMR status and degree of necrosis present	119
32. Table 3.28	Number of lymph nodes isolated and involved compared between MMR-d and MSS groups	125
33. Table 3.29	Distribution of cases of colorectal cancers: comparing different stages to the MMR staining pattern	126
34. Table 3.30	Distribution of colon cancers by p53 staining and mismatch repair status	127
35. Table 3.31	Distribution of colon carcinomas by p53 staining and location of tumours	128
36. Table 3.32	Distribution of cases by tumour stage: analysis of p53 staining patterns in early vs. late stage tumours	129
37. Table 3.33	Clinical variables with mismatch repair status of tumours	144
38. Table 3.34	Histological variables with mismatch repair status of tumours	146
39. Table 3.35	Multivariate analysis of predictors of loss of mismatch repair proteins	147
40. Table 4.1	Association between age at onset of colorectal cancer and mismatch repair gene mutations	168

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli
APES	aminopropyltriethoxysilane
ASR	age standardised incidence rate
CA	cytosine-adenine
CAT scan	Computerised axial tomography scan
CEA	Carcinoembryonic antigen
CI	Confidence interval
CRC	Colorectal carcinoma
CRM	Circumferential resection margins
DAB	diaminobenzidine
DCC	deleted in colonic carcinoma
DNA	deoxyribonucleic acid
DPX	Dibutyl phthalate xylene
E. coli	Escherichia coli
e.g.	for example
ER	oestrogen receptor
5-FU	5-fluorouracil
FAP	familial adenomatous polyposis or familial polyposis coli
IHC	Immunohistochemical staining
H & E stain	Haematoxylin and eosin stain
hMSH2	human mutator S homologue 2
hMLH1	human mutator L homologue 1
hMSH6	human mutator S homologue 6
hPMS2	human postmeiotic segregation 2
hPMS1	human postmeiotic segregation 1
HNPCC	hereditary non-polyposis colorectal carcinoma
HPF	high power field
HRT	hormone replacement therapy
i.e.	that is

IHC	immunohistochemistry
MCC	Mutated in colorectal cancer
MMR	Mismatch repair
MMR-d	Mismatch repair defect
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSI-L	Microsatellite instability low
MSI-H	Microsatellite instability high
MSS	Microsatellite stable
Mut	mutator
MUTYH	mutator Y homologue (E.coli)
N.S.	Not significant
OR	odds ratio
PAWS	Predictive Analytic software
PCR	polymerase chain reaction
pg.	page
PTM	Pretreatment module
PT solution	Pretreatment module TM
RER	Replication error
SEER	Surveillance, Epidemiology and End Results
SSCP	Single-stranded conformation polymorphism
SPSS	Statistical Package for the Social Sciences
TG	thymine-guanine
TIL	Tumour-infiltrating lymphocytes.
TME	Total mesorectal excision
TNM	Primary tumour, regional nodes and distant metastasis.
WHO	World Health Organisation

LIST OF APPENDICES

Appendix 1:	Clinical Criteria for diagnosis of HNPCC	219
Appendix 2:	Clinical questionnaire form	221
Appendix 3:	Definitions of TNM and TNM Staging	224
Appendix 4:	Haematoxylin and eosin staining	226
Appendix 5:	Preparation for: 3-APES treated slides	227
Appendix 6:	Immunohistochemistry staining	228
Appendix 7:	Electronic-Database Information (Internet sites)	230
Appendix 8:	CRC Pathologist worksheet	231

CHAPTER 1: INTRODUCTION AND OBJECTIVES OF STUDY

CONTENTS:

1.1: CANCER

1.1.1: GENETICS AND CANCER

1.1.2: CANCER AND CELL CYCLE

1.1.3: GERMLINE AND SOMATIC MUTATIONS

1.1.4: SPORADIC AND HEREDITARY CANCERS

1.1.5: DNA MISMATCH REPAIR GENES

1.2: COLORECTAL CARCINOMA

1.2.1: INCIDENCE

1.2.2: RISK FACTORS

1.2.3: CLINICAL PRESENTATION

1.2.4: INVESTIGATIONS AND MANAGEMENT

1.3: GENETICS AND COLORECTAL CARCINOMAS

1.3.1: GENETIC PATHWAYS

1.3.2: HEREDITARY COLORECTAL CARCINOMAS

1.3.3: SPORADIC COLORECTAL CARCINOMAS

1.3.4: MISMATCH REPAIR GENES

1.3.5: TUMOUR SUPPRESSOR GENE: P53

1.4: OBJECTIVES OF STUDY

1.4.1: TUMOURS WITH MMR DEFECTS

1.4.2: TUMOURS WITH P53 OVER-EXPRESSION

1.4.3: SUMMARY OF OBJECTIVES

CHAPTER 1: INTRODUCTION AND OBJECTIVES OF STUDY

1.1: CANCER

1.1.1: GENETICS AND CANCER

Cancer is the common term for all malignant neoplasms (Kumar, Abbas et al. 2004) and is characterized by uncontrolled cell proliferation. The physiological equilibrium between cell division and cell death is regulated by various genes which can be broadly classified into proto-oncogenes and tumour suppressor genes. Growth-promoting proto-oncogenes encode proteins which stimulate cell division to replace cell loss and damage, whereas growth-inhibiting tumour suppressor genes encode proteins which slow down progression through the cell cycle or induce cell death to regulate overall cell numbers, thus preventing tumour formation.

Gain-of-function mutations in proto-oncogenes convert these into oncogenes which encode proteins that induce cancer formation. Activation of proto-oncogenes into oncogenes may result from three mechanisms: point mutations resulting in constitutively acting protein products, gene amplification of DNA segments leading to over-expression of encoded proteins, or chromosomal translocations causing inappropriate gene expression. More than 100 oncogenes have been discovered, and common examples include *ABL*, *BCL-2*, *ERB-B*, *MYC*, *RAF*, *K-RAS* and others as shown in Table 1.1 (Hyde 2009).

Table 1.1: Selective list of activated oncogenes

Gene	Location	Tissue	Function
ABL	9q34	chronic myeloid leukemia	Non-receptor tyrosine kinase
BCL-2	18q21	leukemia, follicular lymphoma	cytoplasmic /mitochondrial antiapoptotic protein
ERB-B	17q21	breast, lung, colon, ovarian, glioblastoma, medulloblastoma	Receptor tyrosine kinase (ECG receptor)
ETS	11q23	lymphoblastic leukemia, lymphoma, breast	Transcription factor
FES	15q26	promyelocytic leukemia	Non-receptor tyrosine kinase
FOS	14q24	osteosarcoma, skin	Transcription factor (heterodimerizes with Jun)
INT-2	11q13	breast	Fibroblast growth factor-3 (FGF-3)
MET	7q31	kidney	Receptor tyrosine kinase binds hepatocyte growth factor (scatter factor)
MYC	8q24	leukemia, breast, colon, stomach, lung, neuroblastoma, glioblastoma	Transcription factor (heterodimerizes with Mad or Max)
RAF	3p25	liver, lung	cytoplasmic serine/threonine kinase
H-RAS	11p15	bladder, breast and skin breast, lung, head and neck, ovarian, pancreas	GTP-binding protein
K-RAS	12p12	pancreas	GTP-binding protein
N-RAS	1p13	multiple myeloma	GTP-binding protein
REL-B		multiple myeloma	Nuclear protein (NF6B subunit)
SIS	22q12	T-cell leukemias and lymphomas	Growth factor receptor (PDGF, B chain)
SRC	20q12	multiple tumour types	Non-receptor tyrosine kinase

(Source : Cancer Genetics in Introduction to Genetics Principles. New York, McGraw-Hill, Hyde, 2009)

In contrast, oncogenic mutations in tumour suppressor genes are generally loss-of-function mutations. Tumour suppressor genes encode proteins which regulate cell-cycle check points or stimulate apoptosis. Thus loss-of-function mutations in tumour suppressor genes result in suppression of apoptosis or unregulated progression through the cell cycle, bringing about tumour growth. Tumour suppressor genes in many cancers have deletions or point mutations which prevent production of proteins or lead to production of non-functional proteins. Since generally one copy of a tumour-suppressor gene is sufficient to control proliferation, both alleles must be lost or inactivated to promote tumour development. Examples of tumour suppressor genes include RB1, p53, APC, BRCA1, BRCA2 and NF1.

According to Knudson's 'multi-hit' hypothesis of oncogenesis, a minimum of two genetic "hits" or mutations are required for oncogenesis – the activation of an oncogene (mutation of a proto-oncogene) followed by the inactivation of a tumour suppressor gene (Hyde 2009). These result in homeostasis disruption and hyperplasia or cell proliferation. Malignant progression relies on the accumulation of further mutations which may be due to environmental influences, mutagenic chemicals, ionizing radiation, or viral infections involving tumour-inducing retroviruses and viral oncogenes.

The complex genetic pathway to cancer, which incorporates Knudson's multi-hit hypothesis, is epitomised in the molecular pathogenesis of colorectal carcinoma. Benign tumours of the large intestine develop initially as a result of inactivation of the tumour suppressor gene: adenomatous polyposis coli (*APC*) gene as illustrated in Figure 1.1 (Snustad and Simmons 2010), causing dysplasia of intestinal epithelium and development of early adenomas. Next, activation of the K-ras oncogene and inactivation of tumour

suppressor genes on chromosome 18q induce development into late-stage adenoma. One of the final steps in carcinogenesis involves inactivation of the p53 tumour suppressor gene which is required for development of colorectal carcinoma. Finally, inactivation of other tumour suppressor genes leads to a carcinoma gaining certain aggressive characteristics, such as metastatic potential.

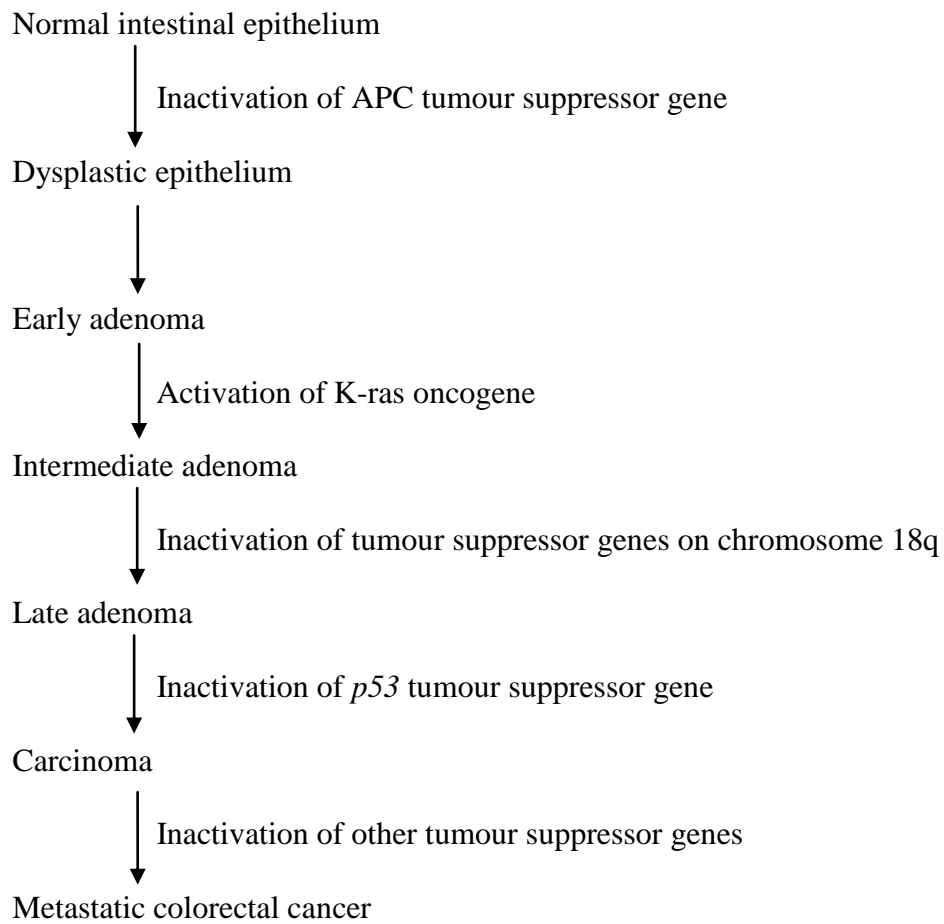


Figure 1.1: Pathway to metastatic colorectal carcinoma (Snustad and Simmons 2010).

1.1.2: CANCER AND CELL CYCLE

The cell cycle consists of periods of growth, DNA synthesis and cell division. The length of the cell cycle and duration of each of its components are controlled and determined by specific internal and external chemical signals. The transition and progression of each phase require integration of these signals and precise responses to them. Incorrect sensing of chemical signals or improper signal response will result in inappropriate progression through the cell cycle and the potential for malignant transformation.

The different phases of the cell cycle include the G1, S, G2 and M phases, with 'checkpoints' between each phase that either stop or allow progression of each phase to the next. At a checkpoint, progression through the cell cycle is halted until a critical process such as DNA synthesis is completed or until damaged DNA is repaired. When these conditions are satisfied, the cell cycle may progress. Cancer formation results from deregulation of these cell cycle checkpoints due to genetic defects described above in Chapter 1.1.1.

The *START* checkpoint, located in the mid-G1 phase (Figure 1.2), plays an important part in oncogenesis. The cell receives both internal and external signals at this checkpoint to determine when it is appropriate to progress to the S phase of the cell cycle.

Cells in which the *START* checkpoint is dysfunctional are more prone to malignant transformation. Where DNA damage has occurred within a cell, it is important that entry into the S phase is delayed to allow for damaged DNA to be repaired, otherwise replication of the damaged DNA will ensue and defects will be passed on to subsequent cell generations.

Normal cells are programmed to pause at the *START* checkpoint to ensure repair is completed before DNA replication commences. If the *START* checkpoint is dysfunctional, cells move into S phase without repairing damaged DNA. Over a series of cell cycles, mutations that result from the replication of unrepaired DNA may accumulate and cause further deregulation of the cell cycle. A clone of cells with a dysfunctional *START* checkpoint may then proceed to become malignant.

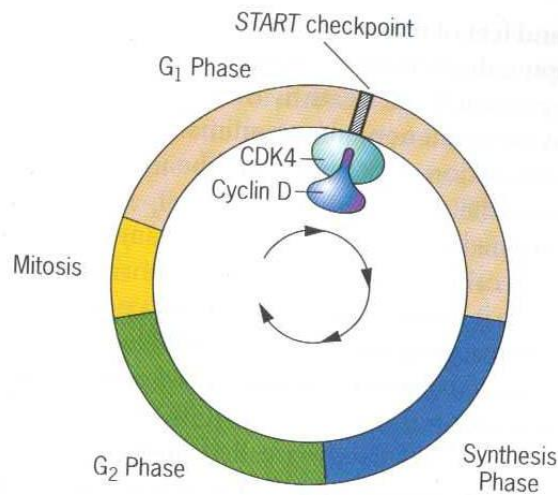


Figure 1.2: Schematic diagram of *START* checkpoint in the mammalian cell cycle (Snustad and Simmons 2010).

1.1.3: GERMLINE AND SOMATIC MUTATIONS

Genetic defects involved in oncogenesis may be either inherited or acquired mutations. These are known as germline or somatic mutations respectively. Germline mutations are present in all cells from conception and are hereditary. Somatic mutations arise in a single differentiated cell post-conception, and are not hereditary. Both germline and somatic mutations play important roles in oncogenesis.

1.1.4: SPORADIC AND HEREDITARY CANCERS

More than 90% of tumours occur as a result of spontaneous random mutations. These are caused by an accumulation of somatic mutations leading to a progressive sequence of cell cycle deregulation, benign hyperplasia, and malignant transformation.

Approximately 10% of all cancers are hereditary. More than twenty different inherited cancer syndromes have been identified and most are due to mutations of tumour suppressor genes rather than hereditary oncogenes (Snustad and Simmons 2010). Some of the more common inherited cancer syndromes are familial retinoblastoma, familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), neurofibromatosis, von Hippel-Lindau disease and familial breast cancers. The different hereditary cancers and tumour suppressor genes involved with the proposed function of encoded proteins are shown in Table 1.2 (Fearon 1997).

Table 1.2 : Inherited Cancer Syndromes				
Syndrome	Primary Tumour	Gene	Chromosomal Location	Proposed Portein Function
Familial retinoblastoma	Retinoblastoma	RB	13q14.3	Cell cycle and transcriptional regulation
Li-Fraumeni syndrome	Sarcomas, breast cancer	TP53	17p13.1	Transcription factor
Familial adenomatous polyposis	colorectal cancer	APC	5q21	Regulation of B-catenin
Hereditary non polyposis colorectal cancer (HNPCC)	colorectal cancer	MSH2	2p16	DNA mismatch repair
		MLH1	3p21	
		PMS1	2q32	
		PMS2	7p22	
Neurofibromatosis Type 1	Neurofibromas	NF1	17q11.2	Regulation of Ras-mediated signaling
Neurofibromatosis Type 2	Acoustic neuromas meningiomas	NF2	22q12.2	Linkage of membrane proteins to cytoskeletons
Wilms' tumour	Wilms' tumour	WT1	11p13	Transcriptional repressor
Familial breast cancer 1	Breast cancer	BRCA 1	17q21	DNA repair
Familial breast cancer 2	Breast cancer	BRCA 2	13q12	DNA repair
von Hippel-Lindau disease	Renal cancer	VHL	3p25	Regulation of transcriptional elongation
Familial melanoma	Melanoma	p16	9p21	Inhibitor of CDKs
Ataxia telangiectasis	Lymphoma	ATM	11q22	DNA repair
Bloom's syndrome	Solid tumours	BLM	15q26.1	DNA helicase
(Source: Human cancer syndromes: clues to the origin and nature of cancer. Science. Fearon ER 1997).				

1.1.5: DNA MISMATCH REPAIR GENES

As discussed earlier, cancer-causing mutations can arise exogenously or endogenously. Genetic information that may be altered endogenously includes errors or alterations in DNA metabolism such as DNA replication, recombination or repair. When errors in DNA metabolism occur during the process of DNA synthesis, many safeguard mechanisms are in place to maintain genomic stability. One of these is the DNA mismatch repair (MMR) system.

The DNA mismatch repair system was first demonstrated in bacteria more than 30 years ago (Tiraby and Fox 1973). It was shown to play an important role in maintaining genomic stability, with defects in the genetic pathway of this system leading to elevated levels of spontaneous mutations (Tiraby and Fox 1973).

The DNA mismatch repair system corrects any biosynthetic errors that occur in the genome, and thus acts as an important caretaker of the genome. It rectifies DNA mismatches generated during DNA replication and blocks DNA recombinations occurring between divergent sequences (Modrich and Lahue 1996; Harfe and Jinks-Robertson 2000) by recognising complementary base pair errors when one strand of DNA is either unpaired or incorrectly matched with a base pair on the complementary strand. It also contributes to genomic stability by controlling cell cycle checkpoints and is responsible for controlling programmed cell death in response to damaged DNA products (Li 1999; Stojic, Brun et al. 2004). Damaged cells are eliminated from progressing further in the cell cycle, preventing tumorigenesis. Inactivation of the mismatch repair system in human cells will thus lead to genomic instability that may potentially result in development of hereditary and sporadic cancers.

The mismatch repair system in the Gram-negative bacteria *Escherichia coli* (*E.coli*) possesses similarities in repair properties to that found in humans. The mismatch repair system in *E. coli* consists of proteins encoded by MutH, MutL, MutS and MutU genes. The term 'mut' is used for *mutator* because mutations in these genes result in high levels of spontaneous mutations or generalised hypermutability in bacterial strains. The human homologues of the *E.coli* MutS and MutL have been identified.

In the model of the mismatch repair system found in *E. coli*, the MutS homodimer detects and binds the mismatched base pair while MutL binds and locates methylation signals in DNA. The mismatch repair system also activates the endonuclease MutH which nicks the unmethylated strand of DNA initiating site for mismatch-provoked excision. This target mismatch repair on newly synthesised strand. While the MutS and MutL proteins in the *E.coli* are homodimers, their human counterparts function as heterodimeric complexes. MutL interacts with MutS to enhance mismatch recognition. Both possess ATPase activity. These human mismatch repair genes were named after their prokaryotic counterparts, e.g. human mutator L homologue 1 (hMLH1) and human mutator S homologue 2 (hMSH2).

Three human MutS homologues (hMSH2, hMSH3 and hMSH6) have been identified. hMSH2 interacts with either hMSH6 or hMSH3 to form hMutS α and hMutS β heterodimeric complexes respectively. Both complexes play critical roles in mismatch repair initiation.

The human MMR components homologous to *E.coli* MutL are hMLH1, hMLH3, hPMS1 and hPMS2. hMLH1 interacts with hPMS2, hPMS1 or hMLH3 to form three heterodimeric complexes: hMutL α , hMutL β or hMutL γ respectively. hMutL α is needed for mismatch repair and hMutL γ for meiosis but the function of hMutL β remains unclear.

Mismatch repair proteins play an important function in correcting biosynthetic errors and thus a critical role in cellular mechanisms that prevent tumorigenesis.

1.2: COLORECTAL CARCINOMA

1.2.1: INCIDENCE

Next to circulatory diseases, cancer is an important medical problem in both developed countries and developing countries alike. It is a leading cause of death worldwide. A recent estimate of global cancer burden suggested that there were 10.9 million new cases, 6.7 million deaths and 24.6 million persons living with cancer (within 5 years of diagnosis) worldwide in 2002 (Parkin, Bray et al. 2005).

Colorectal cancer represents one of the major causes of cancer-related morbidity and mortality in the world, especially the Western world. It is common among both males and females, and it is estimated that about 5% of the population worldwide will develop colorectal cancer in their lifetime.

The number of new cases of colorectal cancer globally has been increasing rapidly since 1975, from 500,000 cases annually to approximately one million. Worldwide, the estimated incidence of colorectal cancer ranks fourth among all cancers in men (after lung, prostate and stomach cancer) and third in women (after breast and cervical cancer) (Parkin, Bray et al. 2005).

Worldwide, colorectal cancer represents 9.4% of all incident cancers in men and 10.1% in women. However, the incidence of colorectal cancer varies in different parts of the world. In Western countries, namely North America, north, south and western Europe and Australasia, colorectal cancer represents 12.6% of all incident cancers in men and 14.1% in women. In other countries worldwide, that is excluding those above, colorectal cancer represents only 7.7% and 7.9% of all cases in men and women respectively (Boyle and Langman 2001).

Approximately 40% of colorectal cancers have localised disease at diagnosis, namely stages 0 (carcinoma-in-situ), I and II, which have excellent five-year survival rates (Jemal, Siegal et al. 2006). Prognosis worsens with advancing stage as patients with metastatic colorectal cancer have only a 5% five-year survival rate (Jemal, Siegal et al. 2006). It is estimated that nearly 400,000 deaths still occur from colorectal cancer worldwide annually and colorectal cancer is the second most common cause of death in men after lung cancer (Jemal, Siegal et al. 2006).

COLORECTAL CARCINOMA IN MALAYSIA

The Malaysian Department of Statistics (National_Cancer_Registry 2006) reported a total of 24.8 million residents of the Peninsular Malaysia in the year 2006. This was made up of almost equal numbers of males (50.6%) and females (49.4%) with Malays being the major ethnic group (54.3%). The other two major ethnic groups were the Chinese and Indians, making up 25.1% and 7.5% of the population respectively. Other minor ethnicities, namely the Punjabis, Eurasians and other immigrants, contributed to the remaining 13.1% of the population.

Cancer was the cause of 10.59% of reported deaths in public hospitals in Malaysia, making it the third major cause of death (National_Cancer_Registry 2006).

Table 1.3: Ten Principle Causes of Deaths in Ministry of Health, Malaysia (MOH) Hospitals, 2006		
	Diseases	Percentage (%)
1	Septicaemia	16.87
2	Heart Diseases & Diseases of Pulmonary Circulation	15.70
3	Malignant Neoplasms	10.59
4	Cerebrovascular Diseases	8.49
5	Pneumonia	5.81
6	Accidents	5.59
7	Diseases of the Digestive System	4.47
8	Certain Conditions Originating in The Perinatal Period	4.20
9	Nephritis, Nephrotic Syndrome & Nephrosis	3.83
10	Ill-defined conditions	3.03
	All causes	100.00

(Malaysian Cancer Statistics-Data and Figure, Peninsular Malaysia, 2006;
National Cancer Registry, Ministry of Health Malaysia)

In 2006, a total of 21,773 cancer cases were diagnosed in Peninsular Malaysia and registered in the National Cancer Registry. This comprised of 9,974 males and 11,799 females. The age-standardised incidence rate (ASR) for all cancers in Malaysia was 131.3 per 100,000. The ASR among males was 128.6 per 100,000 population and among females was 135.7 per 100,000 population. The five most common cancers among the population in Peninsular Malaysia were breast, colorectal, lung, cervix and nasopharyngeal carcinomas (Figure 1.3). Colorectal cancer was the most common cancer in males and second most common cancer in females after breast cancer.

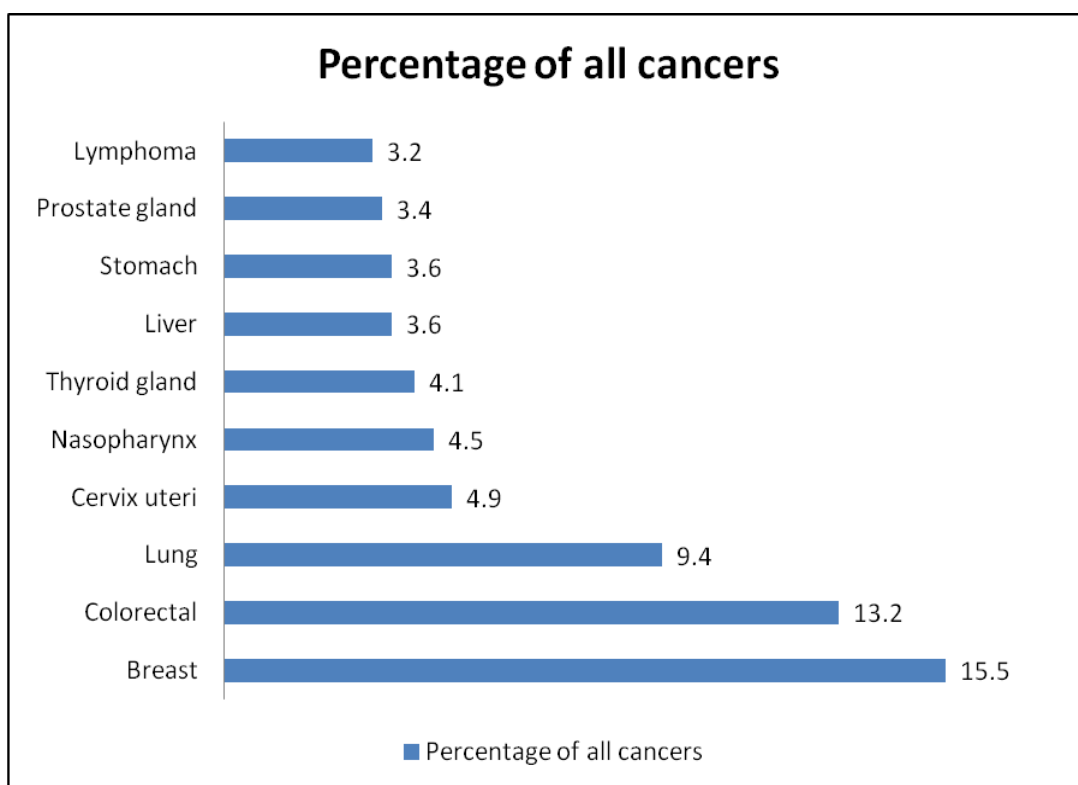


Figure 1.3: Ten most common cancers, Peninsular Malaysia, 2006.

(Source: Malaysian Cancer Statistics-Data and Figure, Peninsular Malaysia, 2006;
National Cancer Registry, Ministry of Health Malaysia)

In the age demographic of fifty years old and over, colorectal cancer was the predominant cancer in males and the second most common among females.

A total of 2,866 colorectal cancer cases were registered with the National Cancer Registry in 2006 and this represented 13.2% of all cancer cases. The incidence of colorectal cancer increased with age; with an overall ASR of 18.4 per 100,000 (Figure 1.4). The incidence was higher among males where the ASR was 21.6 per 100,000 compared to females with an ASR of 15.4 per 100,000. It was also reported that the incidence was highest amongst Chinese where the ASR was 21.4 per 100,000 population and lower in the other 2 major races: Indians and Malays, with an ASR of 11.3 per 100,000 and 9.5 per 100,000 respectively.

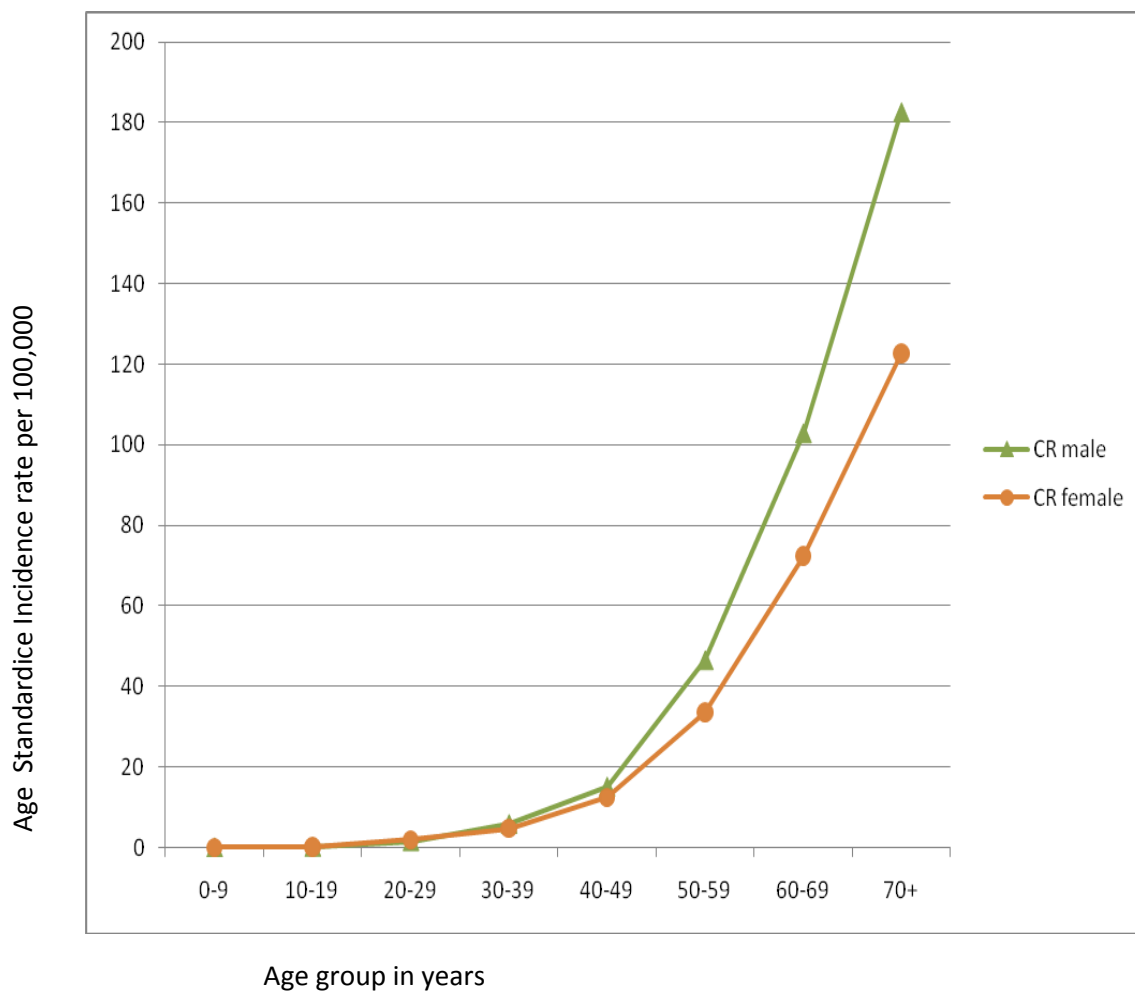


Figure 1.4: Colorectal cancer - Age specific cancer incidence per 100,000 population by gender, Peninsular Malaysia, 2006.

(Source: Malaysian Cancer Statistics-Data and Figure, Peninsular Malaysia, 2006;

National Cancer Registry, Ministry of Health Malaysia)

1.2.2: RISK FACTORS

A number of environmental and genetic risk factors for the development of colorectal cancer have been identified. These include age, diet, physical inactivity, smoking, inflammatory bowel disease and genetic factors.

DIET

The intestinal epithelium is exposed to various types of ingested food. Increased consumption of dietary fibre in the form of fruits, vegetables and cereals has been said to have a protective effect against the formation of colorectal cancer (Graham, Dayal et al. 1978). A high-fibre diet increases faecal bulk and decreases bowel transit time, thus reducing the duration of contact time of the colonic mucosa to any potential exogenous toxins or carcinogens. Some fibres may bind with various reactive compounds and thus have direct antitoxic effects against carcinogens (Ferguson and Harris 1996).

Conversely, a diet rich in red or processed meat or high in animal fat is postulated to increase the risk of developing colorectal cancer. It has been found that diets high in protein and fat but low in fibre content are associated with formation of hydroxyl radicals in faeces (Erhardt, Lim et al. 1997). This may lead to oxidative injury to the DNA of colonic epithelial cells and subsequent neoplastic transformation.

Substantial alcohol consumption has also been linked to the development of adenomas and carcinomas due to abnormal DNA methylation (Sandler, Lyles et al. 1993).

PHYSICAL ACTIVITY AND SMOKING

Epidemiological studies have highlighted that men who are physically active have a lower risk of developing colorectal cancer (Giovannucci and Willett 1994). However, smoking and occupations associated with inhalation of dusts and fumes increase the risk of colorectal cancer. This is more common in males than females, thus explaining the higher ASR of colorectal carcinoma in males.

INFLAMMATORY BOWEL DISEASE

Patients with inflammatory bowel disease, including both ulcerative colitis and Crohn colitis, are well known to have a higher risk of developing colorectal cancer than the general population. The risk of developing colorectal carcinoma correlates closely with the duration of the disease. The risk of cancer only starts after a disease duration of 10 years and the risk rises by about 10% per decade. Chronic inflammation is a common basis for epithelial carcinogenesis. Carcinomas develop from mucosa that has undergone a series of morphological changes culminating in invasive carcinoma. Dysplasia is a pre-cursor to carcinoma in inflammatory bowel disease.

GENETIC FACTORS

Whilst environmental factors play a role as catalysts in genetically susceptible individuals, there are a number of hereditary factors that increase the likelihood of the development of colorectal carcinomas. There are various familial forms of colorectal cancers that include familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC) and other groups of patients with sporadic cancers whose strong family histories do not fulfil any criteria of known familial syndromes.

1.2.3: CLINICAL PRESENTATION

Presenting symptoms of colorectal cancer may include abdominal pain, per rectal bleeding, symptoms of anaemia or change in bowel habit. The initial symptoms may be vague and non-specific. A small percentage of patients present with relatively minimal symptoms on diagnosis. Patients with colorectal carcinoma present differently depending on the location and size of the tumour. Large tumours on the left side of the colon may cause a change in bowel habit including constipation, watery diarrhoea or tenesmus. Tumours on the right side of the colon are often asymptomatic but large tumours may cause intestinal obstruction. Advanced tumours may present with constitutional symptoms including weakness, loss of appetite and weight loss.

1.2.4: INVESTIGATIONS AND MANAGEMENT

A thorough history and physical examination of the patient that includes a per-rectal examination is crucial in helping the clinician make a diagnosis of colorectal cancer. Colonoscopy and biopsy of suspicious lesions for histopathological examination is the gold standard in the detection of colorectal cancer. Alternatively, procto-sigmoidoscopy and/or an air contrast barium enema in addition to a plain abdominal X-ray can be performed. This may sometimes reveal a typical apple core lesion (Figure 1.5). A double-contrast barium enema or CT colonography can help provide a radiographic diagnosis in cases where colonoscope cannot reach the tumour for some reasons for example partially obstructing cancer, tortuous colon or poor preparation. It is essential at the same time to examine the entire colon for presence of synchronous tumours. Other investigations may include stool

samples for occult blood and serum tumour markers such as CEA, which may be raised in colon cancers.



Figure 1.5: Barium enema shows apple core appearance (arrow) indicating filling defect of tumour in the colon.

The role of the pathologist in helping clinicians manage the patient with colon cancer centres on the histopathological examination of the biopsy of the lesion seen on either colonoscopy or proctoscopy. This will guide a plan of management for the patient. A detailed histopathological report of the specimen should include tumour type, grade, depth of invasion, lymphovascular involvement or lymph node metastasis and the surgical margins. The immunohistochemical staining patterns of the mismatch repair genes, hMLH1, hMSH3 and hMSH6 and p53 tumour suppressor gene are usually not included in the histopathological report.

Once the biopsy report has been confirmed, the stage of the tumour is determined. Staging of the tumour to ascertain the local and distant extent of the disease is carried out by physical examination particularly for ascites, hepatomegaly or lymphadenopathy, as well as investigations such as CT scan or MRI of the abdomen and pelvis and chest imaging.

Surgical resection is still the main form of treatment for colorectal carcinomas with or without chemotherapy and radiotherapy. Surgery may be in the form of a hemicolectomy, anterior resection or abdominoperineal resection depending on the site of the tumour.

The aim of postoperative (adjuvant) chemotherapy is to eradicate micrometastases and thus reduce the likelihood of tumour recurrence. Since the mid 1990s, it is generally recommended that patients with stage III disease should be treated with adjuvant chemotherapy as this has been shown to reduce the risk of tumour recurrence and mortality (Cutsem, D'Hoore et al. 2008). Combination chemotherapy consisting of 5-fluorouracil/Capecitabine, leucovorin and oxaliplatin under the name of FOLFOX; is a widely accepted regimen for patients with stage III disease.

Radiotherapy is used neo-adjuvantly and adjuvantly for certain patients with colorectal carcinoma, in particular rectal cancers. Pre-operative radiotherapy aims to shrink advanced tumours to allow surgical resection and can improve the chance of sphincter-preserving surgery in low-lying tumours. Radiotherapy also helps reduce local recurrence. Disadvantages of radiotherapy include the risk of damage to surrounding structures, including irradiation of the small intestine.

1.3: GENETICS AND COLORECTAL CARCINOMAS

1.3.1: GENETIC PATHWAYS

Genetic alterations may evolve in two distinct pathways; namely the suppressor and the mutator pathways (Sutherland, Haine et al. 1998). The suppressor pathways constitute the activation of proto-oncogenes and inactivation of tumour suppressor genes, leading to aneuploid tumour clones. This pathway is seen in familial adenomatous polyposis (FAP) and in most cases of sporadic colorectal carcinomas. On the other hand, the mutator pathway consists of inactivation of mismatch repair (MMR) genes which result in microsatellite instability (MSI). This occurs in hereditary non-polyposis colorectal cancer (HNPCC) and in about 12 to 16% of all sporadic colorectal cancers (Kim, Jen et al. 1994).

1.3.2: HEREDITARY COLORECTAL CARCINOMAS

Although environmental factors play an important role in the aetiology of colorectal cancer, genetic factors also have a significant input. A family history of colorectal cancer is an important element in the history of a patient newly diagnosed with colorectal cancer. Many patients have an affected relative who either can be a first degree (parent, sibling or child) or a second degree (grandparent, aunts or uncles) relative. About 20 % of colorectal carcinomas have a genetic basis (Giardello, J.D. et al. 2001) or 80% of colorectal carcinomas occur spontaneously. Indeed, approximately 25% of patients with colorectal cancer give a positive family history (Fisher and Daniels 2007; Mayer 2009). Patients with one or more first degree relatives with colorectal carcinoma but who do not fulfil the criteria for any specific genetic syndromes like FAP or HNPCC would have about twice the risk of developing colorectal carcinoma as an individual without any family history (Fuchs,

Giovannucci et al. 1994). This risk significantly increases in the fourth decade and rises further with age (Fuchs, Giovannucci et al. 1994). If the individual has more than one first-degree relative or if the relative's cancer occurred before the age of 55 years (St. John, McDermott et al. 1993), the risk increases even further.

It is estimated that 5-10% of all cases of colorectal cancer have a hereditary component (Lynch and de la Chapelle 2003). They can be classified into two groups: autosomal dominant syndromes with high penetrance and cancers with familial clustering with a multifactorial mode of inheritance. The mode of inheritance of the latter group is also autosomal dominant but with low penetrance.

The more common highly penetrant autosomal dominant syndromes include familial adenomatous polyposis (FAP) and its variants such as Gardner's syndrome and Turcot's syndrome; hereditary non-polyposis colorectal carcinoma (HNPCC), Peutz-Jeghers syndrome, juvenile polyposis syndrome and MUTYH associated polyposis syndrome.

HEREDITARY NON-POLYPOSIS COLORECTAL CARCINOMA

Hereditary non-polyposis colorectal cancer is the most common hereditary colorectal cancer. It accounts for 6-13% of all colorectal cancers. It has an autosomal dominant inheritance with 80% penetrance. It is caused by mutations in DNA mismatch repair genes. The penetrance is greater in males and hMSH2 mutation carriers (Vasen, Wijnen et al. 1996; Dunlop, Farrington et al. 1997; Vasen, Stormorken et al. 2001).

Hereditary non-polyposis colorectal cancer differs from sporadic colorectal cancer in its clinical presentation. It has a younger age of onset with a mean of 45 years (Hamilton and Aaltonen 2000), and may present with synchronous or metachronous tumours (Jeong, Chessin et al. 2006). Affected individuals are at increased risk of developing other cancers

including endometrial carcinoma, small bowel carcinomas, renal and urethral cancers, gastric cancers and ovarian carcinomas. Tumours are usually located in the proximal colon. Germline mutations occur in mismatch repair genes namely: hMSH2, hMLH1, PMS1, PMS2 and hMSH6. These tumours show high levels of instability at short tandem repeat sequences, known as *microsatellite instability high* (MSI-H). Several diagnostic criteria have been established for the diagnosis of HNPCC (Appendix 1). Currently, in the modified Amsterdam criteria (Amsterdam II Criteria) HNPCC is defined by presence of HNPCC-associated cancers in at least three family members in two successive generations with one affected member diagnosed before the age of 50 years (Vasen, Watson et al. 1999). HNPCC-associated cancers include colorectal cancer and cancers of endometrium, small bowel, ureter and renal pelvis.

FAMILIAL ADENOMATOUS POLYPOSIS

Familial adenomatous polyposis is a hereditary condition that progresses inevitably to colon carcinoma. It is inherited in an autosomal dominant fashion with a high penetrance of 90%. The FAP-associated gene is known as the APC gene (adenomatous polyposis coli). Patients with the APC gene begin to develop numerous polyps (adenomas) after puberty until the entire colon eventually becomes carpeted with thousands of polyps. Affected patients inevitably develop colon cancer by the fourth decade of life. They also have an increased risk of developing adenomas at other gastrointestinal sites, namely the stomach, duodenum and small intestine. It is also associated with other extra-colonic malignancies such as papillary thyroid cancer, sarcomas and brain tumours.

1.3.3: SPORADIC COLORECTAL CARCINOMAS

Most colorectal carcinomas occur sporadically without any evidence or association of familial or inheritable syndromes. The ‘adenoma-carcinoma sequence’ proposed by Fearon and Vogelstein (Kinzler and Vogelstein 1996) is widely accepted and supported by various observations.

Most sporadic colorectal carcinomas are believed to originate from mutational inactivation of the APC suppressor gene. Other sporadic colorectal carcinomas may arise from somatic mutations of the ‘Mutated in Colorectal Cancer’ (MCC) gene, which is located close to the APC gene. The evolution of adenomas into carcinomas may be accompanied by inactivation–mutation of the ‘Deleted in Colorectal Cancer’ (DCC) gene on chromosome 18 and also the p53 tumour suppressor gene found on chromosome 17. Additional genetic alterations may occur, including activation of proto-oncogenes such as c-myc and k-ras.

The second pathway involves genetic mutations in the DNA mismatch repair genes. About 7-20% of sporadic colorectal carcinomas are due to defects in the mismatch repair gene (Ionov, Peinado et al. 1993; Cunningham, Kim et al. 2001; Chapusot, Martin et al. 2003; Wright and Stewart 2003).

SPORADIC MSI-H COLON CARCINOMA

Sporadic MSI-H colorectal carcinoma is another type of cancer which differs from HNPCC in certain key aspects. These patients do not fulfil the criteria for HNPCC. However, the colorectal carcinomas have high microsatellite instability (MSI-H). Young et al. (Young, Simms et al. 2001) found that patients with sporadic MSI-H colorectal carcinomas presented at a later age as compared with HNPCC. They also found that MSI-H sporadic colorectal cancers affected females more frequently than males. Their findings suggested

that familial (HNPCC) and sporadic MSI-H cancers evolved through different and independent pathways but converged with respect to the pattern of mismatch repair deficiency.

In their study, all cases of sporadic cancers lacked hMLH1 staining while there was a range of mismatch repair protein staining patterns seen in HNPCC cases. Sporadic MSI-H cancers had a higher frequency of features such as poor differentiation, proximal location and mucinous histology which differentiated them from common sporadic colorectal carcinomas. Approximately 90% of sporadic MSI-H tumours were located in the proximal colon compared to 60% of HNPCC. Peri-tumoural and tumour-infiltrating lymphocytes were more frequently seen in HNPCC as compared to sporadic MSI-H colorectal carcinomas.

1.3.4: MISMATCH REPAIR GENES

Defects in the DNA mismatch repair system result in tumour progression. Tumours arising from these mutations exhibit microsatellite instability (MSI), an accumulation of single nucleotide mutations and alterations in the length of repetitive sequences found throughout the genome.

There are several types of repeated DNA sequences in the human genome including satellites, minisatellites, telomeric families and microsatellites. A microsatellite is a short sequence or runs of one to six dinucleotides that is repeated in a tandem array. The most common nucleotide repeats are CA (cytosine-adenine) or TG (thymine-guanine) on the complementary DNA strand. The repeated sequences are located adjacent to each other.

The number of repeats can be anywhere between two to a few hundred in a given genomic location, meaning that there can be several hundreds of alleles in a microsatellite location.

When a defect occurs in the gene regulating DNA repair, replication errors result. This is reflected by widespread variations in short, repeating sequences of DNA microsatellites. Tumours exhibiting this replication error (RER) phenotype are said to have microsatellite instability tumour phenotype.

Microsatellite instability is seen in hereditary non-polyposis colorectal cancers and a subset of sporadic colorectal carcinomas caused by germline mutations in DNA mismatch repair genes.

If tumours exhibit microsatellite instability in at least 30% of loci studied, they are referred to as MSI-high tumours, whereas if fewer than 30% of loci are involved, they are known as MSI-low tumours. MSI-low tumours resemble tumours with no mismatch defect (or microsatellite stable) in most aspects. Microsatellite instability has been found not only in tumours in the colon but also in tumours of the stomach, endometrium and ovary.

Molecular testing is the gold standard for assessing the DNA mismatch repair competency. This involves extracting DNA from the tumour and normal tissue and then performing polymerase chain reaction amplification and gel electrophoresis of a few chromosomal loci and comparing the microsatellite sequences. Many microsatellite markers are available for molecular testing, namely mononucleotide markers: BAT25, BAT26, BAT40, BAT34C4; dinucleotide markers: D5S346, D17S250, ACTC, D18S55, TP53, D18S61, D18S49, D18S34 and D10S197 and the penta-mono-tetra compound marker MYCL. Generally, a panel of five microsatellite markers is recommended for use. If two or more of the five markers demonstrate instability, the tumour is considered to be MSI-high. If only one

marker demonstrates instability, then it is considered MSI-low. If none of the microsatellite markers demonstrate instability, the tumour is considered microsatellite stable. However, molecular testing is extremely time consuming, labour intensive and expensive. It is also not widely available in most laboratories. An alternative method of detecting mismatch repair defect is by using immunohistochemical tests for mismatch repair proteins; namely against hMLH1, hMSH2, hMSH6 and hPMS2.

More than 90% of hereditary non-polyposis colorectal carcinomas are associated with germline mutations of one of the mismatch repair genes, most frequently hMLH1 or hMSH2 (Table 1.4).

Table 1.4 Mismatch repair genes and mutation frequencies in HNPCC		
Gene	Frequency (%)	Location
hMLH1	49	3p21
hMSH2	45	2p15
hPMS2	4	7p22
hPMS1	1	2p32
hMSH6	1	2p15
hMSH3	0	5q11-13

Source: (Petrias and Frankel 2009)

HNPCC patients are generally heterozygous for a normal and mutant allele of one of the MMR genes. Two hits of the DNA mismatch repair genes are required to cause a phenotypic effect. Inactivation of the wild-type allele occurs early in carcinogenesis whilst the inactivation of the normal allele may occur as result of somatic deletions, point

mutations or promoter methylation. The loss of a post-replicative DNA mismatch repair system would increase the mutation rates to over 100 to 600 fold above normal. In the absence of effective DNA repair, the tumour possesses the mutator phenotype.

Sporadic carcinomas are colorectal cancers arising in patients with no family history of colon cancer. 12-20% of these sporadic cancers were shown to have microsatellite instability (Ionov, Peinado et al. 1993; Cunningham, Kim et al. 2001; Chapusot, Martin et al. 2003; Wright and Stewart 2003). This group of patients with microsatellite instability tumours share similar characteristics to patients with HNPCC tumours.

In more than 90% of sporadic MSI tumours, the mismatch repair defect is due to inactivation of hMLH1. The loss of hMLH1 expression is mostly due to bi-allelic methylation of the hMLH1 promoter. On the other hand, hMSH2 has not been found to be prone to hypermethylation.

Most MSI-high cancers are generally diploid or near diploid and carry fewer p53 mutations (Cottu, Muzeau et al. 1996; Lengauer, Kinzler et al. 1997; Eshleman, Casey et al. 1998). Mutations in mismatch repair gene cause a subset of colorectal cancer cases.

Supporting evidence from various studies (Aarnio, Sankila et al. 1999; Millar, Pal et al. 1999) demonstrate that carriers of mismatch repair mutations have an increased risk of developing colorectal cancer compared to the general population and when these patients develop colorectal carcinomas, the tumours also behave differently from patients with microsatellite stable tumours. They demonstrate better response to adjuvant 5-fluorouracil-based chemotherapy (Hemminki, Mecklin et al. 2000) and have better prognosis and survival rates with lower risk of metastasis (Lim, Jeong et al. 2004). Thus, there is benefit in identifying patients with tumours that are microsatellite unstable or MSI-H in order to

manage them accordingly with appropriate therapy including screening for mutations in other family members. It is important to study the constellation of phenotypic features of microsatellite instability tumours to identify characteristics which could predict tumours with mismatch repair defects. Some histopathological features are more frequently seen in tumours with mismatch repair defects and thus could be used as positive predictors of MSI-H tumours. Tumours with such features could then be selected for testing either by immunohistochemistry or polymerase chain reaction for mismatch repair defects.

1.3.5: TUMOUR SUPPRESSOR GENE: P53

P53 is a DNA-binding protein that inhibits tumour development. It is encoded by the tumour suppressor gene TP53, and regulates tumorigenesis by inducing apoptotic mechanisms in cells that are damaged by extensive DNA mutations. This allows cells with accumulated mutations that would progress to malignant transformation to be removed by apoptosis before cell proliferation occurs.

The p53 protein plays numerous roles in the cell. Besides its role in initiating programmed cell death, it is also implicated in control of cellular proliferation, differentiation, DNA repair and synthesis. Lack of p53 will result in loss of normal growth regulatory activity, loss of regulation of proliferation of cells and decreased likelihood of apoptosis of damaged cells. Consequently, genetic instability and mutations will not be eliminated or removed. Thus, p53 deletions or mutations are oncogenic.

P53 mutations tend to be associated with advanced stages of colorectal carcinomas suggesting that the allelic deletions demonstrate a higher tendency to nodal metastasis and vascular or lymphatic invasion.

1.4: OBJECTIVES OF STUDY

1.4.1: TUMOURS WITH MMR DEFECTS

The main aim of this study was to delineate and compare the characteristics of colorectal cancers with and without mismatch repair defects. To date there are no large scale studies on the incidence or prevalence of mismatch repair defects in colorectal carcinomas in Malaysia.

In this study, immunohistochemical staining was performed against hMLH1, hMSH2 and hMSH6 because majority of the familial and non-familial (sporadic) colorectal carcinoma with mismatch defect were due to hMLH1 or hMSH2 (Liu, Parsons et al. 1996; Peltomaki and Vasen 1997; Herman, Umar et al. 1998; Wheeler, Loukola et al. 2000; Potocnik, Glavac et al. 2001; Yamamoto, Min et al. 2002) and a small minority due to hMSH6 (Wu, Berends et al. 1999). At the start of the study the significance of hPMS2 mutation or defect was not well tested. Furthermore commercial antibodies to hPMS2 were also not readily available then. Using the immunohistochemical method against the mismatch repair proteins: hMLH1, hMSH2 and hMSH6, we identified colorectal cancers with mismatch repair defects in a cohort of patients in Malaysia and also studied the pattern of mismatch repair defect present.

CLINICAL FEATURES

This study also identified clinical or pathological characteristics that could be positive predictors of patients with mismatch repair defect tumours. We studied the age, gender, race, past medical histories and family histories of these patients together with tumour stage, site and gross appearance in this group of patients. The aim was to identify

significant associations of any of these features with mismatch repair defect colorectal carcinomas.

PATHOLOGICAL FEATURES

We examined the histological features of tumours, namely the grade (differentiation), amount of mucin present, degree of necrosis, lymphocytic response (peri-tumoural and Crohn-like) and type of infiltrative border to identify phenotypical features which were more frequently associated with mismatch repair defect tumours.

SURVIVAL

The survival of patients with mismatch repair defect tumours was compared to that of patients with microsatellite stable tumours.

1.4.2: TUMOURS WITH P53 OVER-EXPRESSION

Immunohistochemistry was also performed against the p53 protein to identify p53 mutations. The aim was to delineate the group of tumours with p53 over-expression from those without p53 expression and study their clinical and pathological features, namely the staging of tumours, location, lymphocytic response and the patient survival.

Data analysis was also performed to identify correlations between these clinical and pathological features including the incidence of co-existent MMR defects among tumours which stained positive for the p53 protein.

1.4.3: SUMMARY OF OBJECTIVES

In summary, the aim of this study was to examine the incidence of mismatch repair defect in colorectal carcinoma cases; evaluated by immunohistochemical expression of hMLH1, hMSH2 and hMSH6 in a series of unselected consecutive colorectal carcinomas in the Malaysian population; and to correlate this to the clinical and pathological features of the tumours.

The study objectives may be summarised as follows:-

1. To study the incidence of mismatch repair defect in incidental cases of colorectal carcinoma in a local setting in Malaysia by using immunohistochemical staining against hMLH1, hMSH2 and hMSH6 in a series of 298 colorectal carcinoma cases
2. To study the clinical features of colorectal cancer with mismatch repair defects
3. To analyse the histological features of colorectal cancer with mismatch repair defects
4. To compare significant clinico-pathological differences in colorectal cancer patients with and without mismatch repair defects
5. To compare the survival rates of patients with and without mismatch repair defects
6. To study the incidence of p53 over-expression in incidental unselected colorectal carcinomas and in tumours with mismatch repair defects
7. To compare significant clinico-pathological differences in colorectal cancer patients with and without p53 over-expression.

CHAPTER 2: METHODOLOGY

CONTENTS:

2.1: MATERIAL

2.1.1: PATIENTS

2.1.2: CLINICAL PRESENTATION

2.1.3: OUTCOME

2.2: CHARACTER OF TUMOURS

2.2.1: ANATOMICAL LOCATION

2.2.2: MULTIPLE TUMOURS

2.2.3: SIZE OF TUMOURS

2.2.4: GROWTH APPEARANCE

2.2.5: TUMOUR STAGING

2.3: HISTOPATHOLOGICAL FEATURES

2.3.1: HISTOLOGICAL TYPE AND GRADE

2.3.2: MUCIN PRODUCTION AND MUCINOUS CANCERS

2.3.3: TUMOUR ADVANCING PATTERN

2.3.4: LYMPHOCYTIC RESPONSE

2.3.5: TUMOUR NECROSIS

2.4: STAINING PROCEDURE

2.4.1: HAEMATOXYLIN AND EOSIN STAIN

2.4.2: IMMUNOHISTOCHEMICAL STAIN

2.5: STATISTICAL ANALYSIS

CHAPTER 2: METHODOLOGY

2.1: MATERIAL

2.1.1: PATIENTS

This retrospective study included 298 patients with histologically proven diagnosis of colorectal carcinoma, who were operated on in a major tertiary hospital in the southern state of Johor, Malaysia during a period of 4 years from January 2004 to December 2007. The surgical specimens were received in the Department of Pathology, Sultanah Aminah Hospital, Johor Bahru.

2.1.2: CLINICAL PRESENTATION

The clinical presentations, past histories and family histories of the patients were reviewed. Hospital records were assessed and each patient was interviewed in detail during clinical follow-up visits. Standardised questions were asked during this interview and a standard clinical proforma was used for data entry (Appendix 2: Clinical questionnaire form).

The patients were asked if they had a history of previous malignancies (colorectal or non-colorectal cancers) before their current presentation. This was confirmed by a review of hospital clinical records. The patients were also asked if they had a family history of cancer, and if so, their relationship with those family members and the type of malignancy. In some instances, phone calls were made to interview the patients who did not present for follow-up and information obtained was also recorded in the clinical questionnaire forms. The patients' medical histories including a history of inflammatory bowel disease were also noted.

Information regarding the site of tumour, clinical stage at time of diagnosis, relapse of cancer and tumour metastasis was obtained from hospital clinical records and also transcribed into the clinical questionnaire forms.

2.1.3: OUTCOME

The length of survival was calculated from the date of first presentation to the final follow-up date (if living) or date of death (if applicable). This was confirmed by reviewing national birth and death registry records from the Malaysian National Registration Office of Records.

2.2: CHARACTER OF TUMOURS

2.2.1: ANATOMICAL LOCATION

Tumour sites were divided into caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. They were also grouped as right-sided for tumours proximal to and including the splenic flexure and left sided for those located distal to the splenic flexure.

2.2.2: MULTIPLE TUMOURS

The presence of multiple neoplasms in patients were classified into synchronous or metachronous tumours. Synchronous tumours were defined as tumours occurring in a patient at different locations at the same time. Metachronous tumours were defined as carcinomas occurring more than 6 months before or after the index cancer, usually at a different location. Metachronous tumours may be the same or a different type of tumour to the index case but must not be a metastatic lesion of the primary carcinoma. Patients from which this information could not be obtained were excluded from this part of the study.

2.2.3: SIZE OF TUMOURS

The size of the tumour was measured across three dimensions and the largest cross-sectional diameter (in centimetres) was recorded as the tumour size for the purpose of this study. In the event where more than one malignant lesion was present, the measurement of the larger tumour was used for the purpose of this study.

Tumours with a diameter larger than or equal to 5.0 cm were considered as large tumours and small tumours were less than 5.0 cm in diameter.

2.2.4: GROWTH APPEARANCE

The growth appearance of the tumour was described according to the macroscopic appearance of the colectomy specimens. The tumour was described as exophytic if tumour growth protruded beyond the mucosal surface in a polypoidal or fungating fashion (Figure 2.1A). The tumour was considered non-exophytic if it invaded deep into the mucosa forming an ulcerating or flat surface (Figure 2.1B). In the non-exophytic growth pattern, no external protruding growth into the lumen was seen.

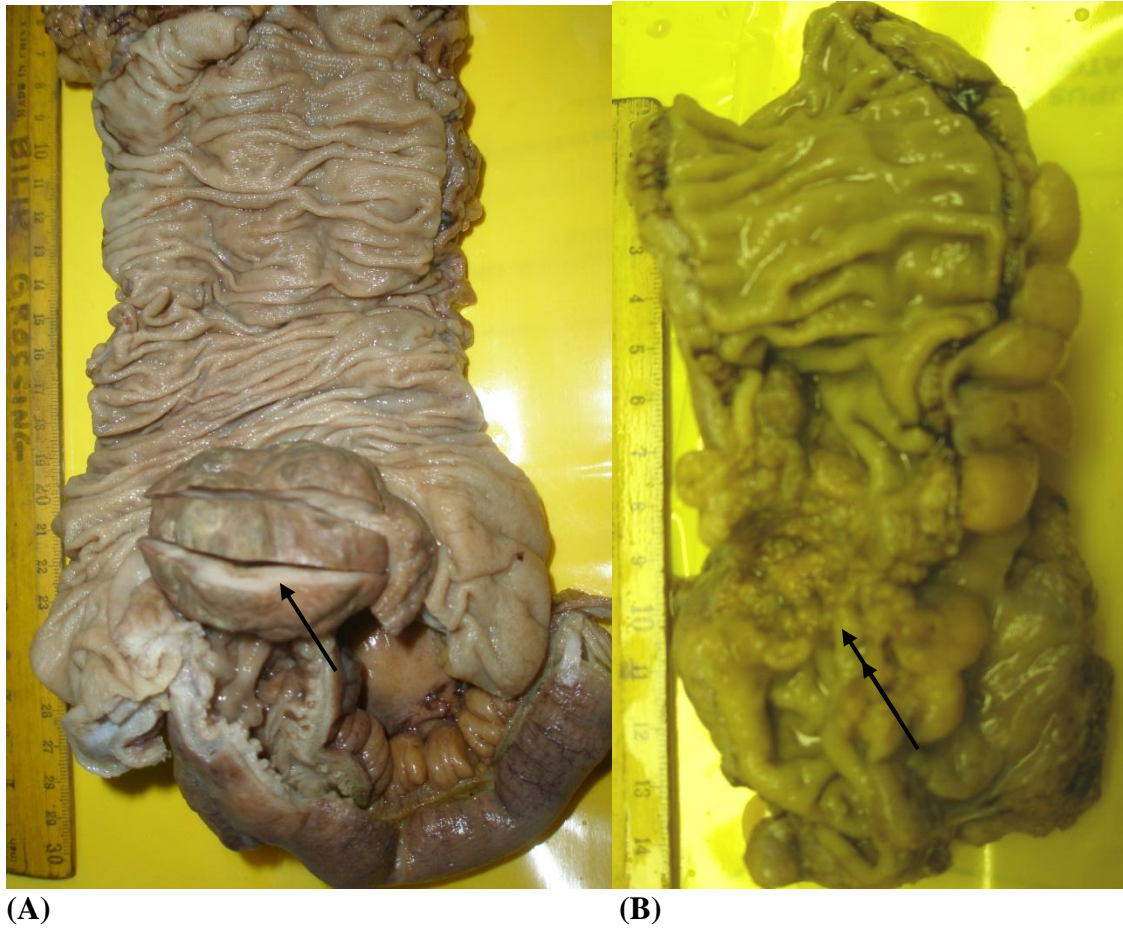


Figure 2.1:

A: Picture of an exophytic colorectal carcinoma: a large polypoid tumour mass seen at the caecum (arrow).

B: Picture of a non-exophytic colorectal carcinoma: an ulcerated tumour mass seen at distal part of colon (arrow with double arrow head).

2.2.5: TUMOUR STAGING

Staging of the tumour was performed using the TNM classification of tumours of the colon and rectum as recommended by the American Joint Committee on Cancer (AJCC), (Edge, Byrd et al. 2010).

This classification is based on the degree of local tumour invasion; number of regional lymph nodes involved and presence of distant metastasis. Local invasion of tumour into the submucosa was classified as T1, invasion into muscularis propria as T2, invasion through the muscle into the pericorectal tissues as T3 and invasion beyond the surface of visceral peritoneum or direct invasion into or adherence to other organs or structures as T4. The number of lymph nodes resected and their status were obtained from the respective histopathological reports. Tumours were classified as N0 when regional lymph nodes isolated were not involved and N1 when one to three lymph nodes were involved. If more than three nodes were positive, the tumour was classified as N2. When no nodes were isolated from the specimen the status of the nodes was deemed unknown or Nx. Further information regarding the presence of distant metastases was obtained from clinical notes and results of radiological investigations. Tumours without distant metastases were classified as M0 and classified as M1 when distant metastases were present.

Tumour size was not taken into consideration when determining the stage of colorectal carcinomas. Stage 0, or carcinoma-in-situ, was defined as tumour limited to the lamina propria, Stage I tumours included tumours which invaded the muscularis propria with no lymph node involvement or metastasis (T1-2, N0, M0). Tumours which progressed further into the subserosa or into the non-peritonealised pericolic and perirectal tissues and / or spread to adjacent organs and structures without exhibiting lymph node involvement or

distant metastasis were classified as Stage II tumours (T3-4, N0, M0). Tumours with involvement of regional lymph nodes without distant metastasis were classified as Stage III tumours (T1-4, N1-2, M0). Tumours with distant metastases were classified as Stage IV tumours (T1-4, N0-2, M1). Appendix 3 summarises the TNM classification and the staging used by the AJCC.

2.3: HISTOPATHOLOGICAL FEATURES

The histopathological reports of the tumours and of any previous malignancies were reviewed. Tumour stage was ascertained from the clinical notes or from histopathological reports.

The original microscopy slides were also reviewed to determine:

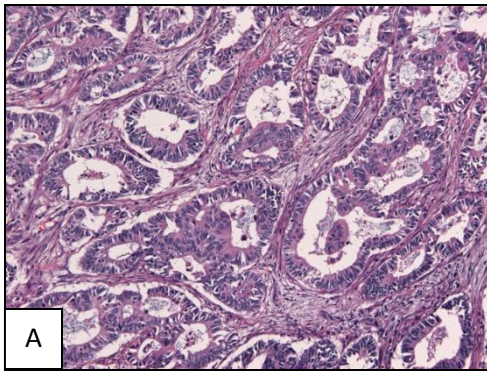
- a. Histological type and grade of tumour
- b. Amount of mucin production
- c. Characteristics of the tumour-advancing front (infiltrative or expansive)
- d. Type of lymphocytic response around the tumour (if present), and
- e. Degree of necrosis (if present) – minimal or marked.

2.3.1: HISTOLOGICAL TYPE AND GRADE

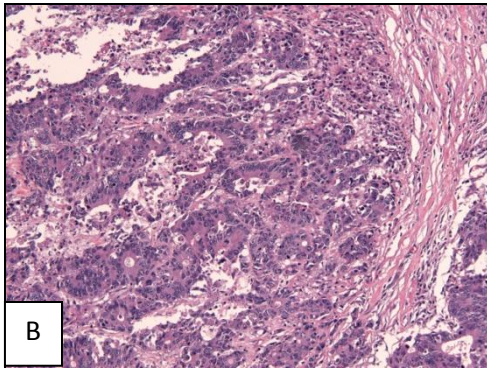
Typing and grading of tumours were performed according to the World Health Organisation (WHO) tumour classification system (Hamilton and Aaltonen 2000).

The tumours were defined as adenocarcinomas when glandular formations were present. Grading of the tumours was based on the proportion of glandular structures seen on histopathological examination. When the lesion demonstrated areas that were heterogenous in differentiation, grading was performed on areas with the least glandular structures i.e. the more poorly differentiated areas. Well differentiated adenocarcinomas (Grade 1) were defined as lesions with glandular structures comprising more than 95% of the tumour, while moderately differentiated cancers (Grade 2) were comprised of 50-95% glandular structures. Poorly differentiated adenocarcinomas (Grade 3) were defined as those with glandular structures between 5-50% and undifferentiated cancers (Grade 4) were defined as

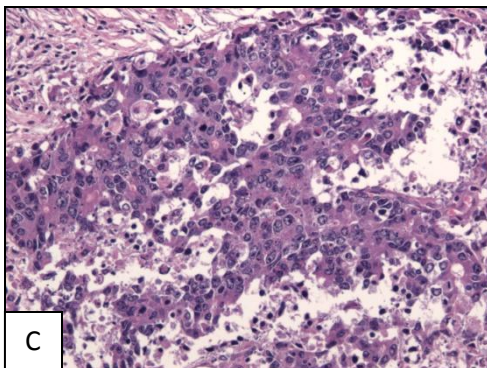
tumours with less than 5% glandular structures. In our study, due to the limited number of cases, poorly differentiated and undifferentiated tumours were grouped together for analysis. Figure 2.2 shows the various grades of differentiation of colorectal carcinoma.



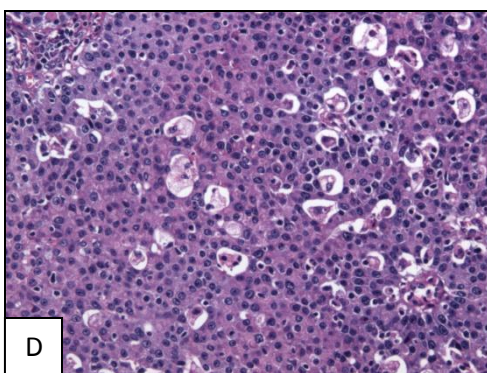
A: Well differentiated adenocarcinoma, (H&E stain, original magnification x 100).



B: Moderately differentiated adenocarcinoma, (H&E stain, original magnification x 100).



C: Poorly differentiated adenocarcinoma, (H&E stain, original magnification x 200).



D: Undifferentiated carcinoma, (H&E stain, original magnification x 200).

Figure 2.2: Grades of differentiation of colorectal carcinomas

2.3.2: MUCIN PRODUCTION AND MUCINOUS CANCERS

Mucinous carcinomas were defined when more than 50% of the tumour area comprised of secretory mucin. Signet ring cell carcinomas were defined when more than 50% of the tumour cells had intracellular mucin with a *signet ring appearance*. By convention, both mucinous carcinomas and signet ring cell carcinomas (Figure 2.3) were considered poorly differentiated adenocarcinomas.

The amount of mucin present in the tumour was also determined. The mucin content was defined as the amount of extracellular mucin in the tumour and was categorized as less than 10%, 11-50% or more than 50% (mucinous carcinomas). Signet ring carcinomas were classified together with tumours producing more than 50% mucin.

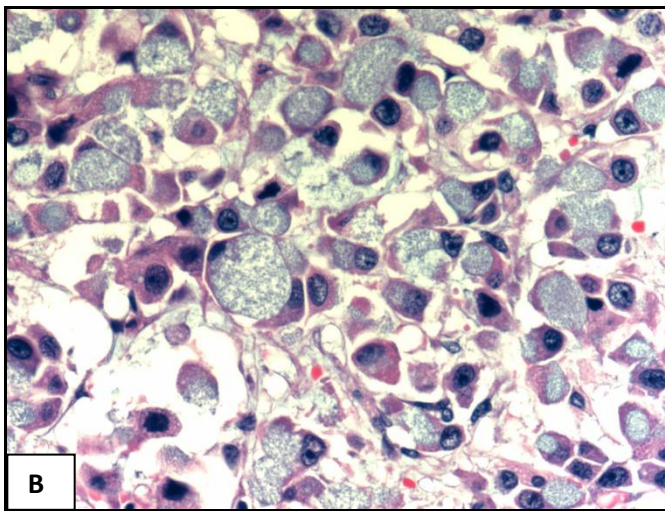
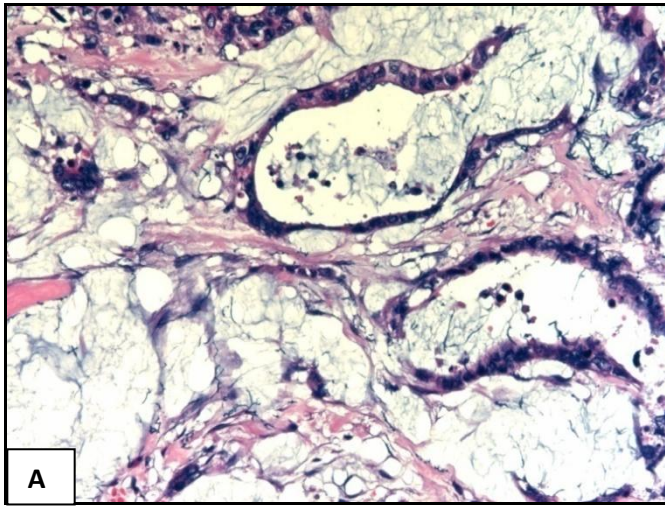


Figure 2.3:

A: Mucinous carcinoma with lakes of mucin present in the tumour (H & E stain, original magnification x 800).

B: Signet ring carcinoma: Mucin is present intracellularly (H & E stain, original magnification x 400).

2.3.3: TUMOUR ADVANCING PATTERN

Colorectal cancer infiltrates the bowel wall in two main patterns of invasive growth depending on the characteristics of its advancing margins. This is illustrated in Figure 2.4 below.

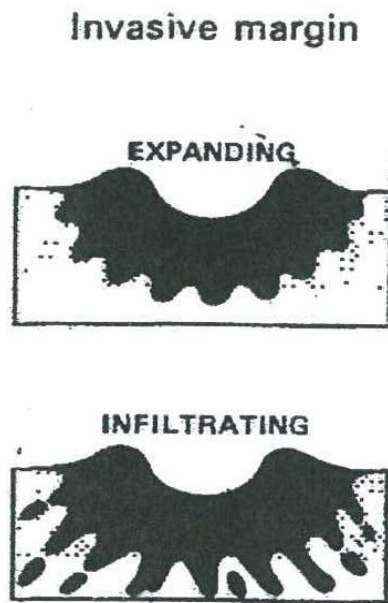


Figure 2.4: Schematic diagram of the invasive border of tumours: expanding vs. infiltrating. Source: (Jass, Love et al. 1987).

The tumour was defined as expanding with a circumferential growth pattern if the margins were reasonably well circumscribed from normal tissue. The limits of the advancing borders of an expanding tumour were easily visible by gross inspection of haematoxylin and eosin stained slides, where adjacent host tissue was easily discernible from the basophilic appearance of the malignant tumour even with the naked eye. Microscopic examination of an expanding lesion would show a well-defined smooth advancing front

between tumour and host muscular tissue (Figure 2.5A). As opposed to this, tumours were defined as diffusely infiltrative when the tumour and its host tissue could not be differentiated by examination of the slide with the naked eye. This was confirmed histologically by the presence of tumour glands or individual tumour cells dissecting into the muscular tissue or mesenteric fat (Figure 2.5B). Perineural invasion was frequently encountered in tumours with this growth pattern.

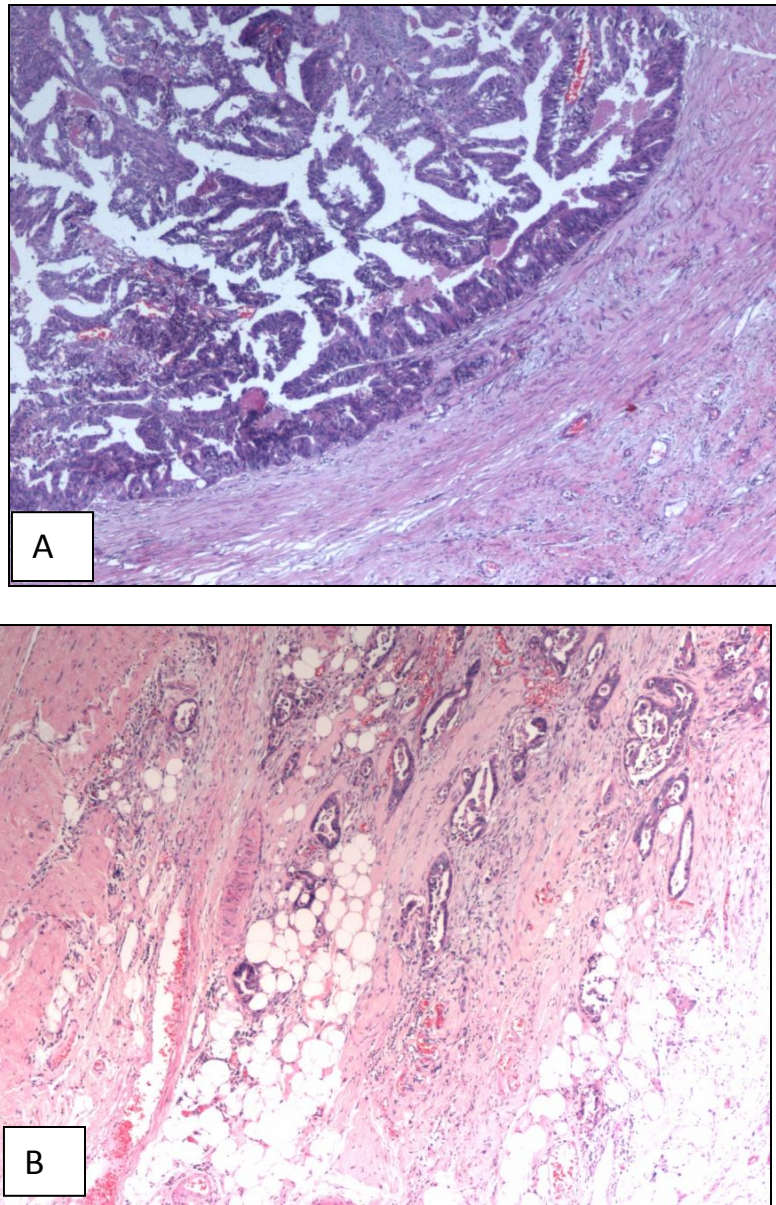


Figure 2.5:

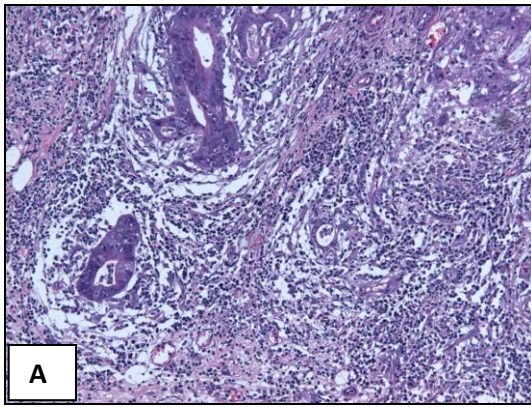
A: Tumour with an expansive border with distinct outline from adjacent normal muscular propria (H & E stain, original magnification X 40).

B: Tumour with infiltrating borders; infiltrates and streams into the serosal fat beyond the muscular layer (H & E stain, original magnification X 40).

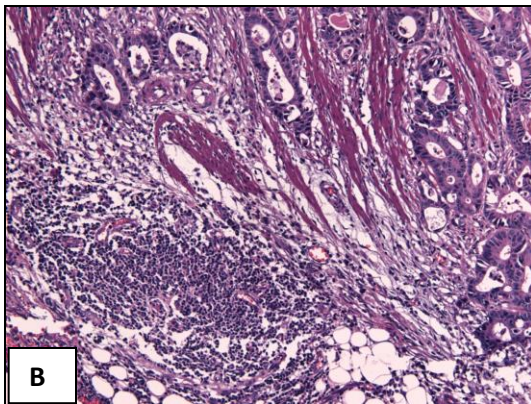
2.3.4: LYMPHOCYTIC RESPONSE

The form of lymphocytic response seen was categorised according to the classification described by Jass et al (Jass, Ajioka et al. 1996) into minimal peri-tumoural lymphocytic, marked peri-tumoural lymphocytic, and Crohn-like lymphoid response. Peri-tumoural lymphocytic response was defined when a cap of lymphocytes or lymphocytic cuff was present in the loose connective tissue at the deepest point of tumour penetration (Figure 2.6A). The slides were reviewed to assess if there was minimal or a conspicuous and distinctive peri-tumoural cap or cuff of lymphocytes around the advancing front of the tumour. The former was classified as minimal and the latter classified as marked peri-tumoural lymphocytic response.

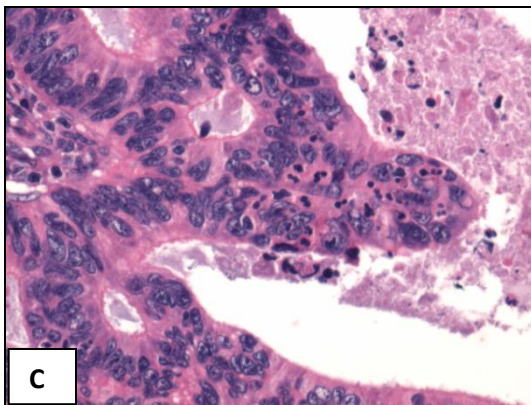
The tumour was considered to have Crohn-like lymphoid response (Figure 2.6B) when nodular or discrete lymphoid aggregates with or without germinal centres were present at the advancing front of the tumour, usually more than 1 mm beyond the advancing tumour front (Graham and Appelman 1990). Occasionally, the tumour was associated with tumour-infiltrating lymphocytes, i.e. lymphocytes present within the tumour. Brisk lymphocytic response was considered to be present when lymphocytes were seen infiltrating the tumour and there were more than two lymphocytes present in the tumour per high power field (Figure 2.6C). However, this form of lymphocytic response classification was not used in this study.



A: The tumour is surrounded by lymphocytic cuff all around. This is peri-tumoural lymphocytic response. H & E stain, original magnification x100.



B: Infiltrative tumour with a Crohn-like lymphoid aggregate (follicle). H & E stain, original magnification x100.



C: Lymphocytes seen infiltrating within the tumour. This is tumour-infiltrating lymphocytes. H & E stain, original magnification x400.

Figure 2.6: Lymphocytic response in colorectal carcinoma.

2.3.5: TUMOUR NECROSIS

Colorectal cancer has been known to be associated with dirty necrosis. Dirty necrosis is defined as the presence of cellular debris with numerous inflammatory cells within the glandular lumina. However, in this study, instead of identifying dirty necrosis, the tumour was examined for the degree of necrosis present.

Tumours with confluent areas of necrosis of more than two low power fields (at 4x10 magnification of Olympus BX41 microscope with diameter of 4.5 mm) were considered as tumours with *marked necrosis* and those with involvement of less than two low power fields as tumours with *minimal necrosis*.

2.4: STAINING PROCEDURE

2.4.1: HAEMATOXYLIN AND EOSIN STAIN

All routine slides were previously sectioned at 3 to 4 um thickness and stained with Haematoxylin and Eosin stain (H & E stain) using the Leica Autostainer XL (Appendix 4). This was carried out with a control slide (usually a section of an appendix) which was run together with each batch of staining. Old slides for the study were retrieved and reviewed. In instances where slides were missing from the stores, a re-cut was done from the original paraffin block of the tumour and the section stained with routine Haematoxylin and Eosin stain in the autostainer.

2.4.2: IMMUNOHISTOCHEMICAL STAIN

Two blocks of 10% formalin-fixed, paraffin wax-embedded colorectal carcinoma tissue in the study were selected per case studied to include a region of normal mucosa adjacent to the carcinoma in one of the blocks selected.

Chapusot et al (Chapusot, Martin et al. 2002) showed that the immunohistochemical assessment status of tumours differed when different sampled areas were used, due to tumour heterogeneity. Thus, they suggested that at least two or more samples of tissue from the tumour should be taken from different areas of the cancer for analysis in order to accurately assess the mismatch repair status of the tumour by immunohistochemistry.

Two blocks of tumour tissue were used and a total of five sections were cut from each block to perform immunohistochemistry for each mismatch repair proteins (namely hMLH1, hMSH2 and hMSH6). The fourth section was used for immunohistochemistry staining for the p53 protein which was also performed for all the case studies. A section

from each block was used for negative staining. A negative control case was one where the primary antibody was omitted during the immunohistochemistry staining procedure. Altogether, ten sections were cut and stained correspondingly by IHC.

Tissue sections were cut into 3 um slices and mounted on glass microscopy slides which were pre-treated with 3-aminopropyltriethoxysilane (Appendix 5). The slides were deparaffinised by treatment with heated Pretreatment moduleTM (PTM) Deparaffinization and Heat Induced Epitope Retrieval solution to optimise the antibody-antigen reaction. Non-specific background staining due to endogenous peroxidase was reduced by treatment with hydrogen peroxide block. The primary monoclonal antibodies were used with optimal dilution as listed below:-

- (i) hMLH1 clone G168-15 (catalogue number 551091) at 1:40 dilution
- (ii) hMSH2 clone G219-1129 (catalogue number 556349) at 1:100 dilution
- (iii) hMSH6 clone GTBP-44 (catalogue number 610918) at 1:800 dilution

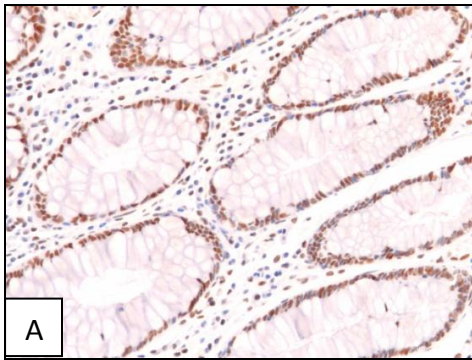
All the above antibodies to mismatch repair proteins (hMLH1, hMSH2 and hMSH6) were from BD Pharmingen.

- (iv) p53 (catalogue number LV-RM-9105-S) at 1:100 dilution.

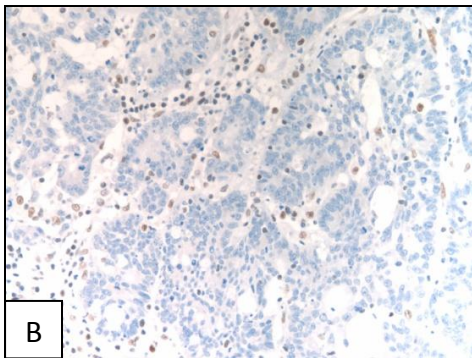
The specific primary antibody was located by a universal secondary antibody polymer formulation. The amino acid polymer was conjugated to horseradish peroxidase and the Fab fragments of goat anti-rabbit and goat anti-mouse. The polymer complex was then visualized with diaminobenzidine tetrachloride solution by adding the DAB chromogen plus. Appendix 6 lists the immunohistochemistry staining procedure used.

MISMATCH REPAIR PROTEIN STAINING

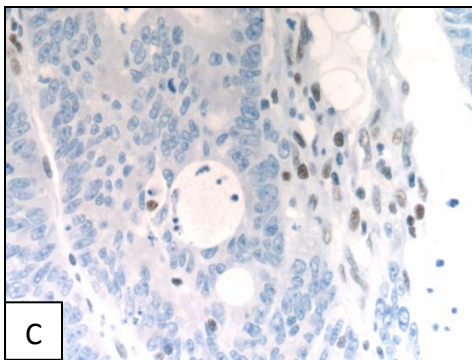
Two known cases of colorectal carcinoma that had previously stained positive for all 3 proteins: hMLH1, hMSH2 and hMSH6 were considered as intact tumours and were used as controls for tumours with no mismatch repair defect. These controls were used for each batch of staining. Positive nuclear staining of more than 10% of tumour cells was considered positive for protein expression for the mismatch repair proteins: hMLH1, hMSH2 and hMSH6. Loss of expression was recorded when all malignant cells showed absent nuclear staining or when less than 10% of tumour cells showed positive nuclear staining. This is needed to be demonstrated in the presence of preserved nuclear staining in the external positive case control as well as in normal epithelial cells and lymphocytes (internal control) in the case itself. Tumours with loss of expression of one or more proteins were considered to be tumours with mismatch repair defects (MMR-d) while tumours with intact expression for all three proteins were considered to be intact tumours with no loss of mismatch repair defect. Figures 2.7 to 2.9 show the various staining patterns for the mismatch repair proteins: hMLH1, hMSH2 and hMSH6 in colorectal carcinomas, lymphocytes and normal colonic epithelium (internal control).



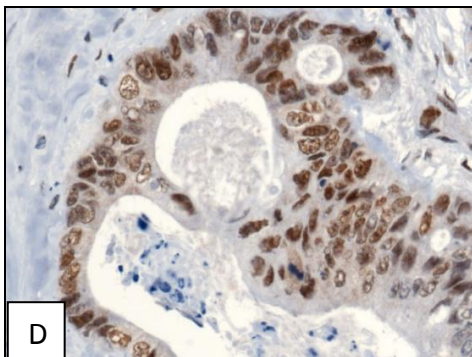
A: The normal colonic glands stained up for hMLH1. (IHC stain with hMLH1, original magnification x 200).



B: The lymphocytes around the tumour stained up for hMLH1. MMR-d tumour showed no reaction to this protein stain. (IHC with hMLH1, original magnification x 200).

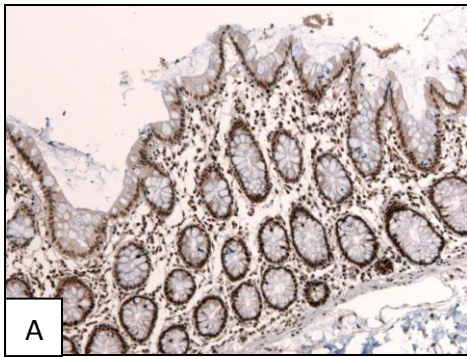


C: Higher magnification showing lymphocytes reactive to hMLH1. (IHC with hMLH1, original magnification x 400).

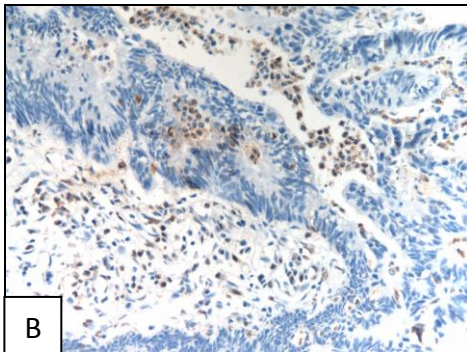


D: Tumour glands showed nuclear positive reaction to hMLH1 in an intact tumour. (IHC with hMLH1, original magnification x 400).

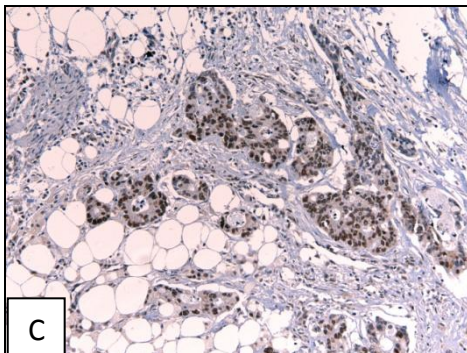
Figure 2.7: Patterns of staining for hMLH1 protein.



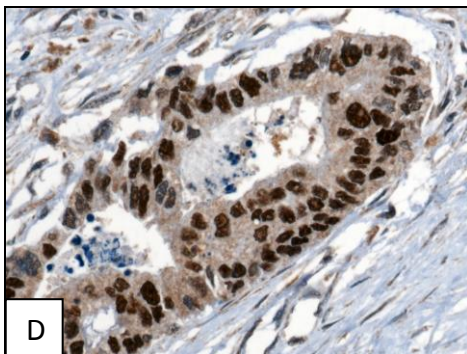
A: The normal colonic glands stained up for hMSH2 (IHC stain with hMSH2, original magnification x 100).



B: The lymphocytes around the tumour stained up for hMSH2. This MMR-d tumour was negative for hMSH2. (IHC with hMSH2, original magnification x 200).

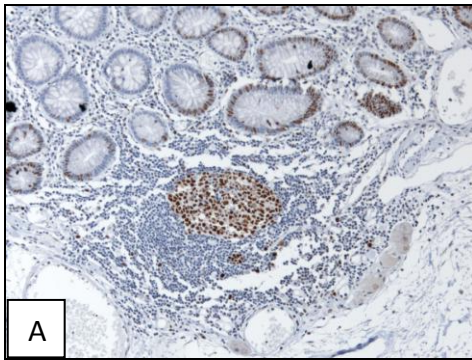


C: Low power field showed malignant glands reactive to hMSH2 in an intact tumour. (IHC with hMSH2, original magnification x 100).

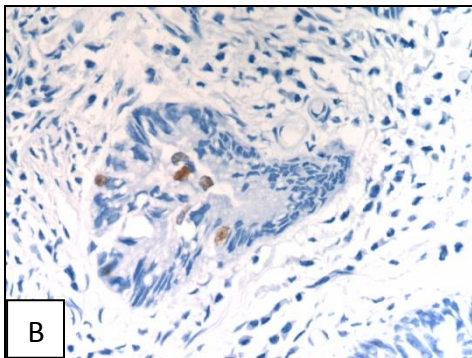


D: High power field showed tumour glands with nuclear positive reaction to hMSH2. (IHC with hMSH2, original magnification x 400).

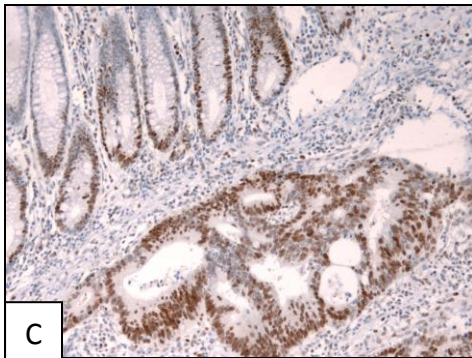
Figure 2.8: Patterns of staining for hMSH2 protein.



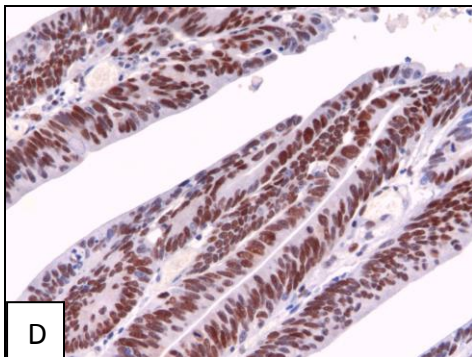
A: The normal colonic glands and the lymphoid follicles stained up for hMSH6 (IHC stain with hMSH6, original magnification x 100).



B: The lymphocytes within the tumour stained up for hMSH6 but the tumour cells were negative in a MMR-d tumour. (IHC with hMSH6, original magnification x 400).



C: Low power field showed normal colonic glands (above) and malignant glands (below) were reactive to hMSH6 in an intact tumour. (IHC with hMSH6, original magnification x 100).



D: High power view showed tumour glands with nuclear positive reaction to hMSH6. (IHC with hMSH6, original magnification x 200).

Figure 2.9: Patterns of staining for hMSH6 protein.

P53 STAINING

P53 mutations can be detected easily by routine immunohistochemistry. Single-stranded conformation polymorphism (SSCP) and DNA sequencing analyses could have been done to identify specific types of mutation of p53 but immunohistochemistry is a more simple and cost-effective method which is widely available and readily used in most laboratories. In fact, studies by Leahy (Leahy, Salman et al. 1996) and Gervaz (Gervaz, Bouzourene et al. 2001) suggested that p53 abnormalities detected at the protein level by immunohistochemistry provided better prognostic discrimination than those detected by SSCP analysis at the gene level.

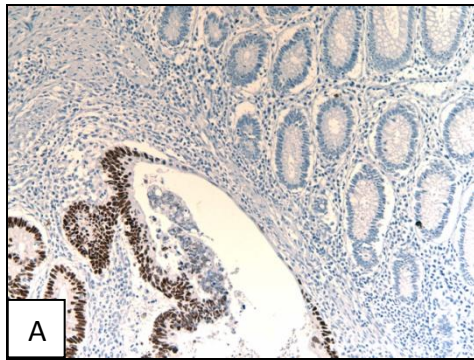
Thus in this study, mutations in p53 were detected using immunohistochemistry. P53 mutation was detected by presence of nuclear accumulation and cytoplasmic staining was not considered as positive staining.

False positive staining sometimes occurred as a result of altered regulation of the wild-type protein. Similarly, false negative staining appeared occasionally with some missense mutations and short gene deletions.

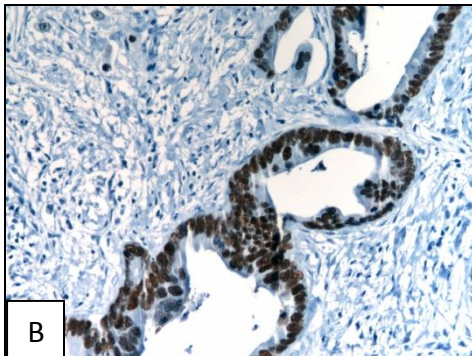
There have been wide variations in the cut-offs used to define p53 positivity in various studies. The proportion of tumour cells with positive nuclear staining used to delineate tumours as p53 positive range from 10% (Bosari, Viale et al. 1995; Manne, Weiss et al. 1998), to 20% (Ward, Meagher et al. 2001), to 50% (Edmonston, Cuesta et al. 2000; Jourdan, Sebbagh et al. 2003). .

In this study, we defined p53 positivity using a cut-off value of 10% of tumour cells with positive nuclear staining (Feeley, Fullard et al. 1999; Gafa, Maestri et al. 2000; Gervaz, Bouzourene et al. 2001; Sinicrope, Rego et al. 2006) because this value demonstrated the

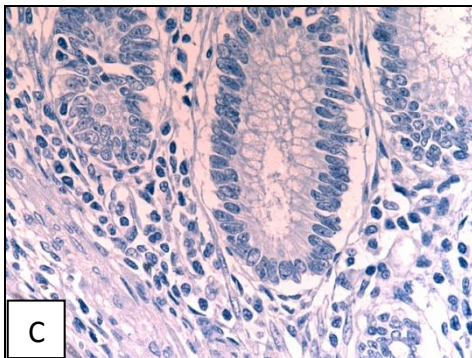
highest concordance between immunohistochemical detection of nuclear accumulation of p53 and point mutations of p53 gene detected by SSCP analysis (95% of point mutations were detected) (Grizzle, Myers et al. 1998). P53 staining was recorded as negative when there was only cytoplasmic staining with no nuclear staining or when the nuclear staining was 10% or less. Figure 2.10 shows the reactivity pattern to the p53 stain used. Nuclear staining for p53 protein is only detected in malignant cells and is not seen in normal epithelial cells of the intestinal mucosa.



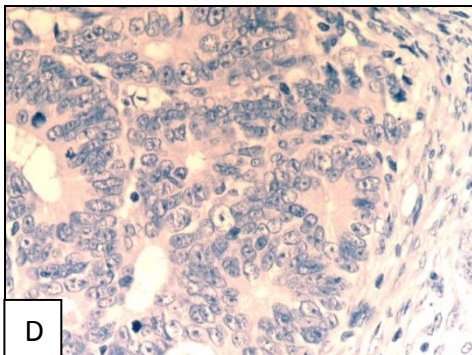
A: The normal colonic glands were negative to p53 (above) but the malignant glands (below) showed nuclear reactive staining to p53 (IHC stain with p53, original magnification x 100).



B: Higher power showed tumour glands with strong nuclei staining for p53 (IHC stain with p53, original magnification x 200).



C: The normal colonic glands were negative to p53 (IHC stain with p53, original magnification x 400).



D: The tumour cells showed cytoplasmic pale brown staining. This was regarded as negative staining (IHC stain with p53, original magnification x 400).

Figure 2.10: Patterns of p53 staining.

2.5: STATISTICAL ANALYSIS

Statistical analysis was performed using the Predictive Analytic Software (PASW), Statistical version 18.0 software program, formerly known as Statistical Package for the Social Sciences (SPSS). The variables were compared using Pearson's Chi-square test, student's t-test or the Mann-Whitney U test, according to the data type. Statistical significance was defined as a *p*-value of less than 0.05. Kaplan and Meier survival curves were plotted. Comparisons of survival rates and their statistical significance were tested using the log rank test where $p < 0.05$ was considered significant.

Multivariate analysis was performed using logistic regression to determine independent predictors of loss of mismatch repair proteins in the tumours.

CHAPTER 3: RESULTS

CONTENTS:

3.1: INTRODUCTION

3.2: DEMOGRAPHICS

3.2.1: RACIAL DISTRIBUTION

3.2.2: GENDER

3.2.3: AGE

3.3: MISMATCH REPAIR DEFECT TUMOURS

3.3.1: STAINING PATTERN AND INCIDENCE

3.3.2: MISMATCH REPAIR DEFECT TUMOURS AND RACE

3.3.3: MISMATCH REPAIR DEFECT TUMOURS AND GENDER

3.3.4: MISMATCH REPAIR DEFECT TUMOURS AND AGE

3.4: FAMILY HISTORY

3.4.1: FAMILY HISTORY OF CARCINOMAS

3.4.2: FAMILY HISTORY AND AGE

3.4.3: FAMILY HISTORY AND MISMATCH REPAIR DEFECT TUMOURS

3.5: CHARACTER OF TUMOUR

3.5.1: MULTIPLE TUMOURS

3.5.2: ANATOMICAL LOCATION

3.5.3: TUMOUR GROWTH APPEARANCE

3.5.4: SIZE OF TUMOUR

3.6: HISTOPATHOLOGICAL FEATURES

3.6.1: HISTOLOGICAL TYPE AND GRADE

3.6.2: MUCIN PRODUCTION

3.6.3: TUMOUR ADVANCING PATTERN

3.6.4: LYMPHOCYTIC RESPONSE

3.6.5: DEGREE OF NECROSIS

3.7: STAGING OF TUMOURS

3.7.1: TNM STAGING

3.7.2: DEPTH OF INVASION

3.7.3: LYMPH NODES STATUS

3.8: TUMOUR SUPPRESSOR GENE: P53

3.8.1: STAINING PATTERN

3.8.2: P53 AND MISMATCH REPAIR DEFECT TUMOURS

3.8.3: P53 AND ANATOMICAL LOCATION OF TUMOUR

3.8.4: P53 AND STAGING

3.9: SURVIVAL

3.9.1: SURVIVAL OF PATIENTS WITH CRC

3.9.2: SURVIVAL AND STAGING

3.9.3: SURVIVAL AND MMR- D TUMOURS

3.9.4: SURVIVAL AND P53 STATUS

3.9.5: SURVIVAL AND OTHER PATHOLOGICAL FEATURES

3.10: TREATMENT

3.11: SUMMARY OF RESULTS

CHAPTER 3: RESULTS

3.1: INTRODUCTION

There were a total of 304 cases of unselected consecutive colorectal carcinoma diagnosed during the study period from January 2004 to December 2007. Five cases were excluded from the series where the diagnosis of colorectal cancer was confirmed only on biopsy but no subsequent surgical resection of the tumour was performed within the specified study period. One known case of confirmed familial adenomatous polyposis (FAP) was excluded from the study. In this autosomal dominant disease the mutated allele is a germline mutation of the APC allele inherited from the affected parent. The patient starts to develop adenomas and then colorectal carcinoma when the second APC allele becomes mutated or lost. As we are looking at tumours with mismatch repair defect and not with mutated APC gene, we decided to exclude this case of FAP. Altogether 298 patients diagnosed with colorectal carcinomas were included in this study. In these cases, surgical resection of the colon occurred between the period January 2004 and December 2007 and the diagnosis was confirmed as 'colorectal carcinoma'.

From the 298 patients, 40 patients cannot be traced. Their clinical notes were unrecoverable and they were lost to follow-up. The Malaysian National Registration Office of Records could provide the status of these patients but clinical details that include the past history and family history were not available for these patients to be included in this part of the study. The remaining 258 patients were followed up between 1 to 54 months from the time of presentation, with a mean follow-up period of 18.1 months. At the end of the study

period, 146 patients were still alive, while a total of 112 patients had died between 1 to 45 months from the time of presentation.

All histopathological reports were available for analysis but in 3 cases the Haematoxylin and Eosin stained slides were missing from the storage room and had to be re-cut. As all paraffin blocks were still stored in the laboratory this did not pose any problems and the cases were still included in the study.

3.2: DEMOGRAPHICS

3.2.1: RACIAL DISTRIBUTION

Malaysia is a multi-ethnic society, populated by Malays, Chinese, Indians, Eurasians and other minority ethnic groups. According to the Department of Statistics Malaysia, Malays comprised more than half the population of Peninsular Malaysia (54.3%) in the year 2006 followed by Chinese (25.1%) and Indians (7.5%) (Figure 3.1).

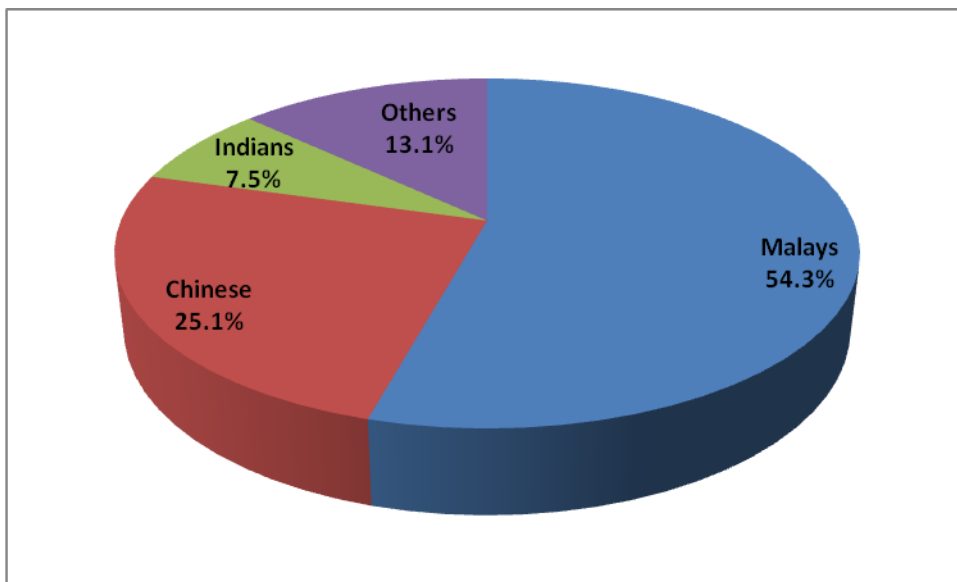


Figure 3.1: Distribution of ethnic groups in Peninsular Malaysia in year 2006.

Source:(Department_of_Statistics_Malaysia 2007).

In this study, demographic analysis demonstrated a predominance of Chinese patients with colorectal carcinoma. There were 139 Chinese patients out of the total of 298 patients (46.6%), followed by 134 Malay patients (45.0%), 19 Indian patients (6.3%) and 6 patients of other races (2.0%) [Figure 3.2]. The hospital records for all patient admissions between the years studied (2004 to 2007) showed a predominance of Malays (60.0%), followed by

Chinese (20.8%), Indians (11.9%) and others (7.3%). There was a significant difference that Chinese were more likely to have colorectal carcinomas than Malays ($p=0.001$, 95% CI 1.568 to 5.679, OR 2.984).



Figure 3.2: Patients with colorectal cancer by race and gender.

Considering that Malays make up the majority of the population (54.3%) in Malaysia (National_Cancer_Registry 2006), patients were classified into two groups of Malay and non-Malay patients for analysis. There were 134 Malay and 164 non-Malay patients from a total of 298 colorectal cancer cases.

3.2.2: GENDER

In this study there were 166 male (55.7%) and 132 female (44.2%) patients with colorectal carcinoma, giving a male to female ratio of 1.26:1. Among Chinese patients, the male to female ratio was 1.40:1, Malay patients 1.20:1 and Indians 1.11:1. Evidently, the Chinese demonstrated a higher male predominance than the other races.

3.2.3: AGE

The age at presentation of the patients in this study ranged between 25-91 years. Approximately two-thirds of the patients (62.0%) were above the age of 60 years at presentation. The peak incidence was in the sixth and seventh decades of life. Only 54 out of 298 patients (18.1%) presented before or at the age of 50 years whereas 81.9% of patients were above the age of 50 years.

The median age of presentation was 62.0 years and the mean age of presentation was 61.0 years with a standard deviation of 13.0 years (Figure 3.3).

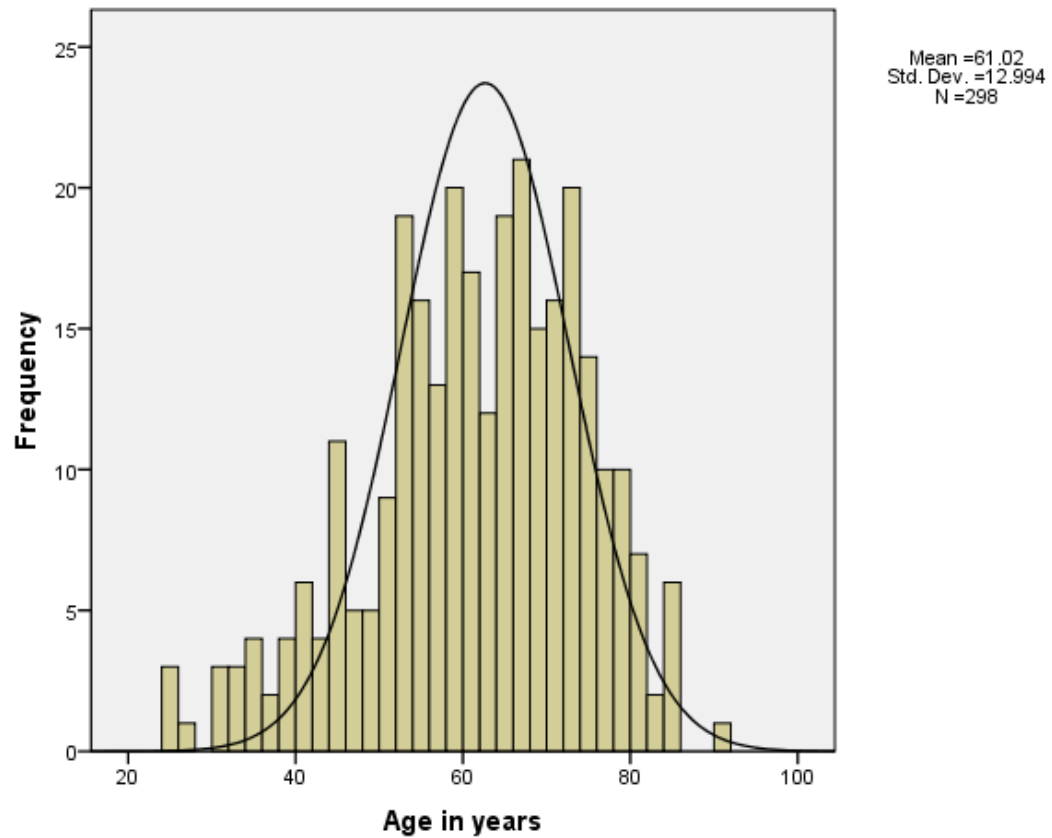


Figure 3.3: Distribution of patients' age (in years).

The mean age at presentation of colorectal carcinomas differed slightly for males and females. Males presented at a slightly older age with a mean of 61.1 years while females presented at 60.1 years, but this difference was not statistically significant ($p = 0.934$).

3.3: MISMATCH REPAIR DEFECT TUMOURS

3.3.1: STAINING PATTERN AND INCIDENCE

In two cases, the immunohistochemistry staining for hMLH1 was repeated because the lymphoid tissue acting as internal control and the test tumour cells did not stain up. Repeat staining showed positive staining in the lymphocytes and the tumour cells in both cases.

Using immunohistochemical staining against hMLH1, hMSH2 and hMSH6 in all 298 cases, the tumours with mismatch repair defects were able to be identified. 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. They were labelled as mismatch repair defect tumours.

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 (Table 3.1). None of the cases lost all three MMR proteins.

TABLE 3.1:

hMLH1, hMSH2 and hMSH6 protein expression in mismatch repair defect colorectal cancers (n=43).

Immunohistochemistry results	No. of tumours
hMLH1negative/ hMSH2 positive / hMSH6 positive	28 (65.1%)
hMLH1 positive/ hMSH2 negative / hMSH6 positive	2 (4.70%)
hMLH1 positive/ hMSH2 positive / hMSH6 negative	6 (14.0%)
hMLH1 positive / hMSH2 negative / hMSH6 negative	7 (16.3%)
Total	43 (100%)

Two hundred and fifty-five patients had intact protein expression by immunohistochemical staining analysis for all the 3 antibodies: hMLH1, hMSH2 and hMSH6 and were recorded as intact tumours with no loss of mismatch repair defect or non-MMR-d tumours.

3.3.2: MISMATCH REPAIR DEFECT TUMOURS AND RACE

The racial breakdown of the 43 cases of mismatch repair defect tumours in the study read as follows: 55.8% (24 cases) were from Malay patients, 41.9% (18 cases) from Chinese patients, and 2.3% (1 case) from an Indian patient (Figure 3.4).

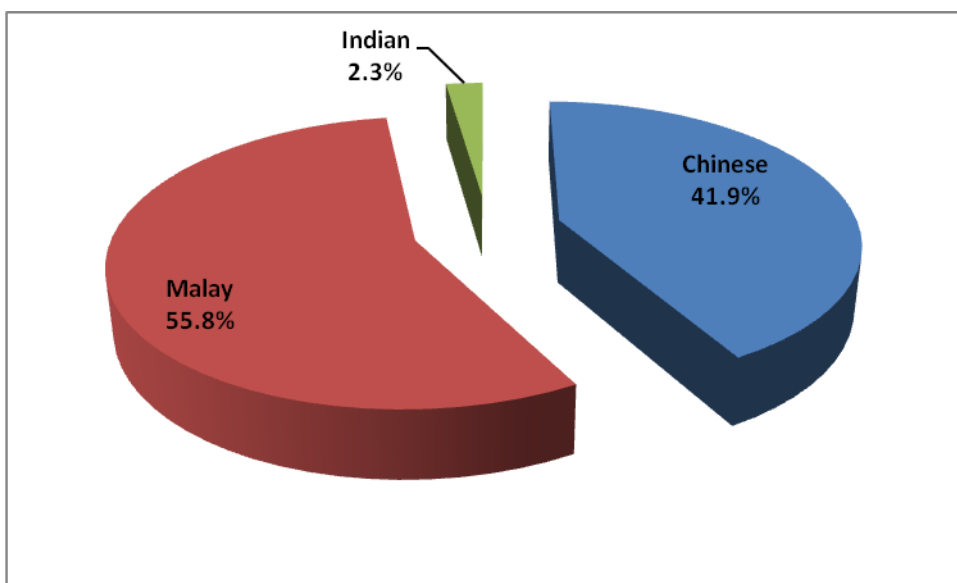


Figure 3.4: Distribution of patients with mismatch repair defect tumours by ethnic groups.

Although Chinese was the predominant race of patients presenting with CRC in the study, it was found that Malay patients had the highest proportion of mismatch repair defect tumours. 17.9% of Malay patients with colorectal carcinomas had mismatch repair defect tumours as compared to 12.9% of Chinese patients and 5.3% of Indian patients (Table 3.2).

Table 3.2: The different racial groups with colorectal carcinomas according to their mismatch repair status.

	MMR status		Total
	MMR-d	Non-MMR-d	
Race Chinese	18 12.9%	121 87.1%	139 100.0%
Indian	1 5.3%	18 94.7%	19 100.0%
Malay	24 17.9%	110 82.1%	134 100.0%
Others	0 0.0%	6 100.0%	6 100.0%
Total	43 14.4%	255 85.6%	298 100.0%

In the group of Malay patients there were 24 cases (17.9%) with mismatch repair defect tumours as compared to 19 cases (11.6%) of mismatch repair defect tumours in non-Malay patients (Table 3.3). However, this difference did not reach statistical significance ($p = 0.122$).

Table 3.3: Distribution of patients by race: analysis of mismatch repair defect tumours among Malay vs. non-Malay patients.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Race	Malays	134 (45.0%)	24 (55.8%)	110 (43.1%)	0.122	1.66 (0.87-3.19)
	Non-Malays	164 (55.0%)	19 (44.2%)	145 (56.9%)		

On the other hand, as Chinese patients were the predominant racial group in this study, subjects were also compared as part of Chinese and non-Chinese groups. There were 18 Chinese patients with mismatch repair defect tumours out of 139 Chinese patients (12.9%) compared to 25 non-Chinese patients with mismatch repair defect tumours out of 159 non-Chinese patients (15.7%). There was no statistical difference found between Chinese and non-Chinese patients with colorectal carcinomas for mismatch repair defect tumours ($p=0.497$, Table 3.4).

Table 3.4: Distribution of patients by race: analysis of mismatch repair defect tumours among Chinese vs. non-Chinese patients.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Race	Chinese	139 (46.6%)	18 (41.9%)	121 (47.4%)	0.497	1.25 (0.65-2.41)
	Non-Chinese	159(53.4%)	25 (58.1%)	134 (52.6%)		

The proportion of mismatch repair defect tumours was further analysed according to both gender and race combined. Analysis of female patients alone revealed a significantly higher proportion of mismatch repair defect tumours (19.7%) among Malay females when compared to non-Malay females (7.00%, $p=0.031$). This suggested that mismatch repair defect tumours were more likely to occur in Malay females than non-Malay females [odds ratio (OR) = 3.24, 95% confidence interval (CI) =1.07-9.80]. However, this difference was not observed in the analysis of male patients alone. The proportion of mismatch repair defect tumours did not differ significantly between Malay male patients (16.4%) and non-Malay male patients (15.1%) [$p=0.808$, Table 3.5].

Table 3.5: Distribution of patients by race and gender: analysis of MMR-d tumours among Malay vs. non-Malay female and male patients respectively.

Factor	Category	MMR status			<i>P</i> value	Odds Ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Gender	Race					
Female	Malay	All females (n=132) 61	MMR-d group (n=17) 12	Non-MMR-d group (n=115) 49	0.031	3.24 (1.07-9.80)
	Non-Malay	71	5	66		
Male	Malay	All males (n=166) 73	MMR-d group (n=26) 12	Non-MMR-d group (n=140) 61	0.808	1.11 (0.48-2.57)
	Non-Malay	93	14	79		

3.3.3: MISMATCH REPAIR DEFECT TUMOURS AND GENDER

Within the cohort of patients with mismatch repair defect tumours, 26 were males (60.5%) and 17 were females (39.5%). On the other hand, there were 140 male patients (54.9%) and 115 female patients (45.1%) in the non-MMR-d group. This is illustrated in Table 3.6. Statistical analysis using the Pearson Chi-square test revealed no significant difference when comparing the proportion of male to female patients between the two groups ($p = 0.497$).

Table 3.6: Distribution of patients by gender: analysis of mismatch repair defect tumours among male vs. female patients.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Gender	Females	132 (44.3%)	17 (39.5%)	115 (45.1%)	0.497	1.26 (0.65-2.43)
	Males	166 (55.7%)	26 (60.5%)	140 (54.9%)		

3.3.4: MISMATCH REPAIR DEFECT TUMOURS AND AGE

Patients with mismatch repair defect tumours presented at a younger age [mean age and standard error (SE) = 58.9 ± 2.2 years, median age of 60.0 year] than patients with Non-MMR-d tumours (mean age and standard error = 61.4 ± 0.8 years, median age of 63.0 years). However, this difference was not statistically significant ($p=0.292$, Figure 3.5).

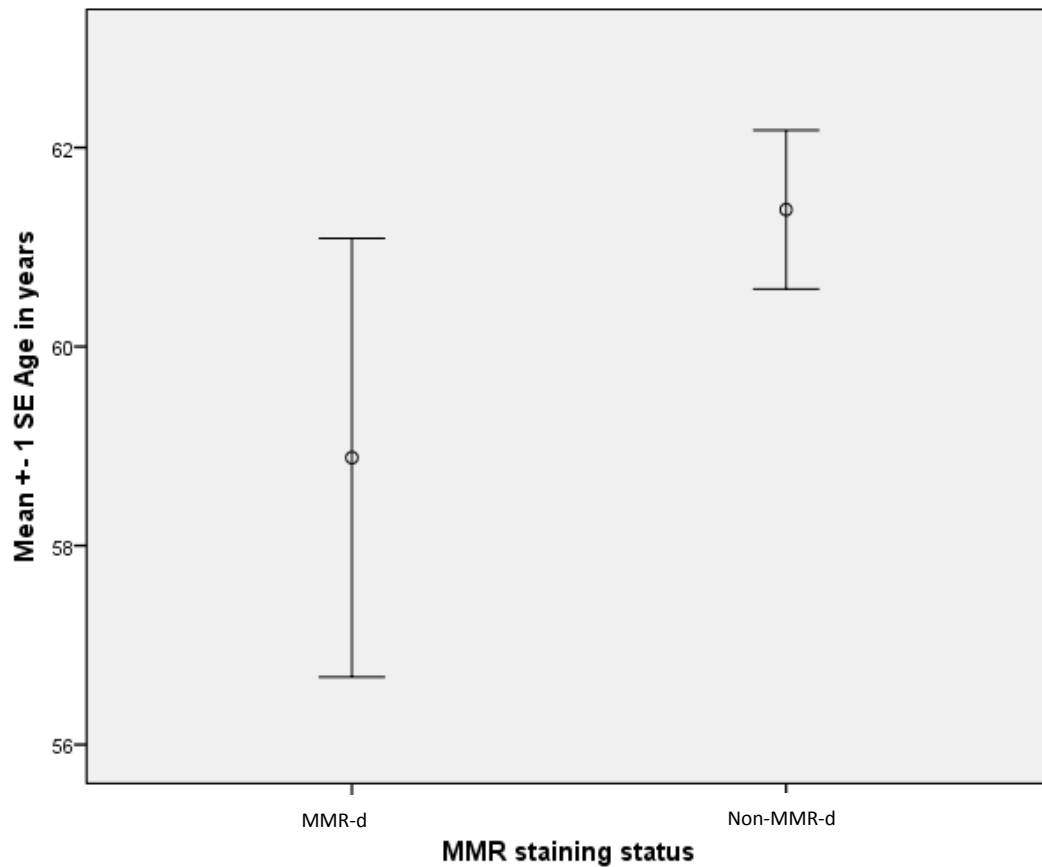


Figure 3.5: Distribution of patients by age: mean age of presentation with one standard error by MMR staining status.

In this study of 298 patients, there were 53 patients who were 50 years and below. In this younger age group, there were 11 patients with tumours showing mismatch repair defects. 25.6% of patients with mismatch repair defect tumours were 50 years old or less as compared to 16.9% of patients with non-MMR-d tumours (Table 3.7). This difference did not reach statistical significance ($p = 0.148$).

Table 3.7: Distribution of patients by age: analysis of mismatch repair defect tumours among patients aged 50 and below vs. patients aged above 50.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Age (in years)	≤ 50	53	11 (25.6%)	42(16.5%)	0.148	1.74 (0.82-3.73)
	> 50	245	32 (74.4%)	213 (83.5%)		

Further analysis of the data stratified according to age demonstrated an increasing trend towards higher proportions of mismatch repair defect tumours in younger age groups. This is shown in Table 3.8.

Overall, 43 out of 298 cases (14.3%) were mismatch repair defect tumours. There was a higher percentage of mismatch repair defect tumours in the younger age groups, namely in patients less than 31 years old (2 of 6 cases or 33.3%) and in the 31-40 age group (4 of 17 cases or 23.5%). The percentage of MMR defect tumours decreased with age, constituting 16.7% (5 out of 30 cases) in the 41-50 age group, 13.1% in the 51-60 age group; and 12.2% in the age group between 61 to 70 years old. There were only 7.1% of patients above the age of 80 years who had mismatch repair defect tumours (Table 3.8).

Table 3.8: Distribution of patients by age: analysis of mismatch repair defect tumours according to age group.

Age range	No. of cases	No of MMR-d tumours	Percentage
<31	6	2	33.3%
31- 40	17	4	23.5%
41-50	30	5	16.7%
51-60	84	11	13.1%
61-70	82	10	12.2%
71-80	65	10	15.4%
>80	14	1	7.1%

3.4: FAMILY HISTORY

3.4.1: FAMILY HISTORY OF CARCINOMAS

A full family history was obtained from a total of 160 patients. 40 patients were lost to follow-up and unable to be contacted. Another 98 patients had passed away before the start of the study and contact with their families was not successful.

A total of 25 out of these 160 patients (15.6%) had a family history of previous cancer in one or more first degree relatives. These included a family history of cancers involving the lower gastrointestinal tract, stomach, oesophagus, endometrium, cervix, breast, kidney, prostate, brain as well as haematological malignancies (Table 3.9).

Table 3.9: Patients with family history of carcinomas

No.	Age of onset (yrs)	Gender	Race	MMR status	Family member: cancer
1	51	F	Chinese	Non-MMR-d	Sister: Colorectal cancer
2	61	M	Malay	Non-MMR-d	Father: Gastric cancer
3	63	F	Malay	Non-MMR-d	Mother: Gastric cancer Sister: Breast cancer
4	71	M	Chinese	Non-MMR-d	Mother: Cervical cancer
5	57	F	Chinese	Non-MMR-d	Brother: Gastric cancer, Sister 1: Cervical cancer Sister2: Colorectal cancer
6	76	F	Chinese	Non-MMR-d	Son: Renal cell cancer
7	56	M	Chinese	Non-MMR-d	Brother: Colorectal cancer
8	45	F	Malay	MSH2, MSH6	Father: died of cancer Brother: Colorectal cancer Sister: Colorectal cancer
9	52	M	Chinese	Non-MMR-d	Sister 1: Gastric cancer, Sister2: Breast cancer
10	52	M	Malay	Non-MMR-d	Sister: Breast cancer
11	46	F	Malay	MLH1	Father: Colorectal cancer, Sister: Colorectal cancer Brother: Gastric cancer
12	56	F	Chinese	Non-MMR-d	Mother: Endometrial cancer
13	42	F	Chinese	Non-MMR-d	Sister: Breast cancer
14	53	F	Indian	Non-MMR-d	Father: Oesophageal cancer, Brother: Colorectal cancer
15	38	M	Malay	Non-MMR-d	Father: Colorectal cancer Brother: Colorectal cancer Sister: Colorectal cancer
16	45	M	Indian	Non-MMR-d	Mother: Gastric cancer
17	65	M	Chinese	Non-MMR-d	Brother: Prostate Cancer
18	81	F	Chinese	Non-MMR-d	Son: Colorectal cancer
19	60	M	Chinese	Non-MMR-d	Mother: Colorectal cancer
20	55	M	Malay	Non-MMR-d	Sister 1: Colorectal cancer, Sister 2: Leukaemia
21	35	M	Malay	Non-MMR-d	Father: Colorectal cancer
22	47	M	Chinese	Non-MMR-d	Sister1: Colorectal cancer, Sister 2: Cervical cancer
23	68	M	Chinese	Non-MMR-d	Sister: Cervical and colorectal cancer
24	56	F	Malay	Non-MMR-d	Mother: gynaecological cancer (site unclear)
25	31	M	Malay	Non-MMR-d	Brother: Intracranial malignancy

All the relatives of the 25 patients with positive family histories were first degree relatives. Fifteen patients had one relative with a history of cancer while ten had more than one relative with a history of cancer. Fourteen cases had a positive family history of colorectal carcinomas and a further three cases with HNPCC-associated cancers, namely cancers of the endometrium, small bowel or kidney. Thus, a total of 17 cases (10.6%) were with positive family history of colorectal carcinomas or HNPCC-associated cancers. Two patients (case 11 and 15) fulfilled the Amsterdam Criteria II (Appendix 1) for hereditary non-polyposis colorectal carcinoma, while another case (case 8) did not fully fulfil the criteria but was highly suspected of it.

One patient (case no 23, Table 3.9) had a sibling (sister) suffering from multiple metachronous tumours that included colorectal carcinoma and cervical carcinoma.

There were 8 patients with family members with non-HNPCC-associated cancers which included cancers of the prostate, stomach, oesophagus, breast, cervix, cerebral tumour and haematological malignancy.

3.4.2: FAMILY HISTORY AND AGE

Patients with a family history of a first degree relative with cancer presented at a significantly younger age than those patients without any family history of cancer. The mean age of these patients was 54.4 years compared to 61.5 years in patients without a family history of cancer. This difference was statistically significant ($p=0.013$).

Of the total of 160 patients from whom a family history with or without malignancies was obtained, 31 patients were aged 50 years or below and 129 aged above 50 years. A higher percentage of patients who presented younger had positive family history: 8 out of 31

patients (25.8%) presenting at age 50 years or below had a positive family history of malignancy as compared to only 13.2% (17 out of 129 patients) presenting older than 50 years of age with a positive family history of malignancy. Conversely, a higher proportion of patients with family history (32.0%) presented earlier (at age 50 years or younger) as compared to 17.0% of patients presenting at the same age but with no family history (Table 3.10). However, analysis using Fisher's exact test showed that the difference between these two groups was not statistically significant ($p=0.099$).

Table 3.10: Distribution of patients with positive family history of malignancy by age group.

Factor	Category	All patients (n=160)	No family history (n=135)	Family history (n= 25)	<i>P</i> value	Odds ratio (95% CI)
Age (in years)	≤50	31 (19.4%)	23 (17.0%)	8 (32.0%)	0.099	2.29 (0.88- 5.94)
	> 50	129 (80.6%)	112 (83.0%)	17 (68.0%)		

3.4.3: FAMILY HISTORY AND MISMATCH REPAIR DEFECT TUMOURS

Only two cases with a positive family history of malignancy (case no. 8 & 11, Table 3.9) were found to have mismatch repair defect tumours. The MMR staining was defective for hMLH1 in one case, and for both hMSH2 and hMSH6 in the other. It was interesting to note that both these patients with mismatch repair defect tumours had two or more family members with colorectal carcinomas and both presented in their mid-forties.

One of the patients (case no. 11) had three immediate relatives with GIT malignancies, two of which were colorectal carcinomas. Both the father and sister had colorectal carcinomas while her brother had gastric carcinoma. Although we did not have records of the age of presentation of the relatives but the patient herself presented at a young age (46 years old). She fulfilled the criteria for HNPCC. The other patient (case no. 8) was also in her forties (45 years old) when she presented with malignancy. Both her two siblings had colorectal carcinomas. However, we could not confirm there were 2 generations involved with malignancies in her case. Nonetheless, the patient was highly suspected of having HNPCC. It was interesting to note that both the cases (no. 8 & 11) were Malay females and in their forties.

3.5: CHARACTER OF TUMOUR

3.5.1: MULTIPLE TUMOURS

The records of 236 patients were studied to determine whether they had multiple malignant tumours (synchronous or metachronous) before or at presentation. From a total of 298 patients, 40 cases were lost to follow up and another 12 cases were excluded as their history and past clinical records were not available. Of the remaining 236 patients, 34 patients (14.4%) were found to have multiple malignancies, ten of whom had synchronous malignant colorectal carcinomas (4.2%) and 24 with metachronous malignant tumours (10.2%). Eight patients had metachronous colorectal carcinomas (at a different site and time to the index tumour) while the remaining sixteen had metachronous non-colorectal carcinomas which included prostate carcinomas (3 cases), gynaecological malignancy (cervical -3 cases, endometrial -2 cases, ovarian -2 cases), breast carcinomas (3 cases) and renal, bronchogenic and gastric carcinomas (one case each) (Table 3.11).

Only twenty five patients (8.3% of cases) had associated benign adenomas in their colectomy specimen. This may not be reflective of the true situation as we did not look into their scope findings or their barium enema images. Hence benign adenomas were excluded as multiple tumours in this study.

Table 3.11: Patients with metachronous non-colorectal carcinomas

No	Age of onset (yrs)	Gender	Race	MMR status	Metachronous non-colorectal carcinoma
1	64	F	Malay	hMSH6	Cervical carcinoma
2	71	F	Chinese	Non-MMR-d	Cervical carcinoma
3	79	F	Malay	Non-MMR-d	Cervical carcinoma
4	45	F	Malay	hMSH2 & hMSH6	Ovarian carcinoma
5	45	F	Chinese	Non-MMR-d	Ovarian carcinoma
6	46	F	Malay	hMLH1	Endometrial carcinoma
7	89	F	Malay	Non-MMR-d	Endometrial carcinoma
8	79	M	Malay	Non-MMR-d	Prostate cancer
9	69	M	Chinese	Non-MMR-d	Prostate cancer
10	74	M	Malay	Non-MMR-d	Prostate cancer
11	54	F	Malay	Non-MMR-d	Breast cancer
12	77	F	Chinese	Non-MMR-d	Breast cancer
13	55	F	Chinese	Non-MMR-d	Breast cancer
14	63	M	Chinese	Non-MMR-d	Gastric cancer
15	53	M	Malay	Non-MMR-d	Bronchogenic carcinoma
16	58	F	Malay	Non-MMR-d	Renal cell carcinoma

MULTIPLE TUMOURS AND MISMATCH REPAIR DEFECT TUMOURS

Of the 34 patients with synchronous or metachronous tumours, there were nine patients (26.5%) with mismatch repair defect tumours. This rate was higher than that observed among patients with single tumours (24 of 202 patients or 11.9%). Conversely, 9 out of 33 patients (27.3%) with mismatch repair defect tumours as compared to 25 of 203 patients (12.3%) with non-MMR-d tumours presented with multiple (synchronous or metachronous) tumours. This difference was statistically significant ($p=0.032$, Table 3.12). Patients with mismatch repair defect tumours had an odds ratio of 2.67 of developing synchronous or metachronous tumours (95% CI was 1.12-6.41).

Table 3.12: Distribution of patients by their presentation as single or multiple tumours against their MMR staining pattern.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=236)	MMR-d group (n=33)	Non-MMR-d group (n=203)		
Multiplicity of tumours	Single tumours	202 (85.6%)	24 (72.7%)	178 (87.7%)	0.032	2.67 (1.12-6.41)
	Multiple tumours	34 (14.4%)	9 (27.3%)	25 (12.3%)		

Nine cases with MMR-d colorectal carcinomas presenting with multiple tumours were made up 3 cases with synchronous colorectal carcinomas, 3 with metachronous colorectal carcinomas and another three with metachronous non-colorectal carcinoma.

3.5.2: ANATOMICAL LOCATION

In this study, the majority of the 298 unselected consecutive colorectal carcinomas were localised to the left side. Two hundred and seven cases (69.5%) were left sided lesions (defined as distal to but not including the splenic flexure). Most of the tumours were on the left side and all the races showed similar predilection for that site: 70.1% of Chinese patients, 70.5% of Malay patients, 63.2% of Indian patients and 50.0% of patients of other races had left sided colorectal carcinomas (Table 3.13).

Table 3.13: Frequencies of location of colorectal carcinomas in patients by ethnic race.

		Race				Total
		Chinese	Indian	Malay	Others	
Site	Left side	98 70.5%	12 63.2%	94 70.1%	3 50.0%	207 69.5%
	Right side	41 29.5%	7 36.8%	40 29.9%	3 50.0%	91 30.5%
Total		139 100.0%	19 100.0%	134 100.0%	6 100.0%	298 100.0%

ANATOMICAL LOCATION AND MMR-D TUMOURS

Out of the 43 cases of mismatch repair defect tumours, 26 were right-sided tumours (60.5%), in contrast to only 17 on the left side of the colon (39.5%). Mismatch repair defect sporadic colorectal carcinomas were found to significantly localise to the right side of the colon. Almost two-thirds (60.5%) of mismatch repair defect tumours occurred in the right side of colon; while only 25.5% of intact tumours were right-sided. Conversely, 28.6% of right-sided tumours were mismatch repair deficient, compared with only 8.20% of left sided tumours ($p < 0.001$, Table 3.14). Right-sided tumours had an odds ratio of 4.47 (95% CI 2.28-8.76) for being mismatch repair deficient compared to left sided tumours.

Table 3.14: Distribution of colorectal cancers by location and MMR status.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Site	Left	207 (69.5%)	17 (39.5%)	190 (74.5%)	<0.001	4.47 (2.28-8.76)
	Right	91 (30.5%)	26 (60.5%)	65 (25.5%)		

Sixteen out of 28 cases of hMLH1 defective tumours (57.1%) were localised to the right side while ten cases of either hMSH2 or hMSH6 defective tumours (10 out of 15 cases or 66.7%) were right-sided tumours. There was no significant difference between the types of mismatch repair defect protein loss in tumours located on the right side of the colon.

ANATOMICAL LOCATION AND AGE

In this study, the mean age of patients with right-sided tumours was lower than the mean age of patients with left sided tumours. The mean age of patients with right sided tumours was 59.2 ± 1.5 years while the mean age for patients with left sided tumours was 61.8 ± 0.9 years. However this was not statistically significant ($p=0.120$).

3.5.3: TUMOUR GROWTH APPEARANCE

The majority of colorectal carcinomas in this study were endophytic tumours with deep ulceration. There were 199 cases (66.8%) with endophytic growth pattern while the remaining 99 cases (33.2%) were polypoidal or exophytic in growth, and protruded into the lumen.

TUMOUR GROWTH APPEARANCE AND ANATOMICAL LOCATION

One hundred and forty cases (70.4%) of the tumours with endophytic growth were found in the left side of colon (distal colon) [Table 3.15].

Table 3.15: Distribution of colorectal carcinomas by different growth patterns and location of tumours.

Factor	Category	Growth pattern:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	Exophytic (n=99)	Endophytic (n=199)		
Site	Left side	207 (69.5%)	67 (67.7%)	140 (70.4%)	0.637	1.13 (0.67-1.91)
	Right side	91 (30.5%)	32 (32.3%)	59 (29.6%)		

Although there were more endophytic tumours (59 cases) than exophytic tumours (32 cases) among right sided tumours in this study, there was no statistical difference between right and left sided tumours with regards to tumour growth appearance ($p=0.637$).

Table 3.16 shows the distribution of colorectal carcinomas by each specific location, namely: caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum, and the tumour growth appearance. In all sites, tumours were predominantly endophytic. The descending colon (79.2%) and the sigmoid colon (69.4%) had the highest proportions of tumours with endophytic lesions. Exophytic lesions were seen most commonly in the transverse colon (40.9%), followed by the caecum (35.7%) and the rectum (35.5%), but even in these sites; remained less common than their endophytic counterparts.

Table 3.16: Distribution of colorectal carcinomas by location and tumour growth pattern.

Factor	Category	Tumour growth pattern		
		All cases (n=298)	Exophytic	Endophytic
Location	Ascending colon	41 (13.8%)	13 (31.7%)	28 (68.3%)
	Caecum	28 (9.4%)	10 (35.7%)	18 (64.3%)
	Descending colon	24 (8.0%)	5 (20.8%)	19 (79.2%)
	Rectum	121 (40.6%)	43 (35.5%)	78 (64.5%)
	Sigmoid colon	62 (20.8%)	19 (30.6%)	43 (69.4%)
	Transverse colon	22 (7.4%)	9 (40.9%)	13 (59.1%)

TUMOUR GROWTH APPEARANCE AND MMR STATUS

Although approximately 2/3 of the tumours were mainly endophytic lesions and only 1/3 were exophytic lesions, a significant percentage (22.2% or 22 of 99 cases) of exophytic tumours had mismatch repair protein loss as compared to only 10.6% (21 of 199 cases) of endophytic tumours ($p=0.007$, OR = 2.42, 95% CI 1.26-4.67, Table 3.17). Conversely, more than 2/3 (69.8%) of the non-MMR-d tumours were endophytic while less than 1/3 of non-MMR-d tumours were exophytic. This showed that mismatch repair defect tumours were significantly associated with an exophytic growth appearance.

Table 3.17: Distribution of colorectal carcinomas by growth pattern and mismatch repair status.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Growth pattern	Exophytic	99 (33.2%)	22 (51.2%)	77 (30.2%)	0.007	2.42 (1.26-4.67)
	Endophytic	199 (66.8%)	21 (48.8%)	178 (69.8%)		

3.5.4: SIZE OF TUMOUR

The mean size of all tumours was 4.8 cm with a standard deviation of 2.1 cm. Tumour size ranged from 1.0 cm to 17.0 cm (Figure 3.6).

The majority of tumours (199 cases, 66.8%) were smaller than 5.0 cm in diameter, while approximately one-third of cases had a diameter larger than or equal to 5.0 cm.

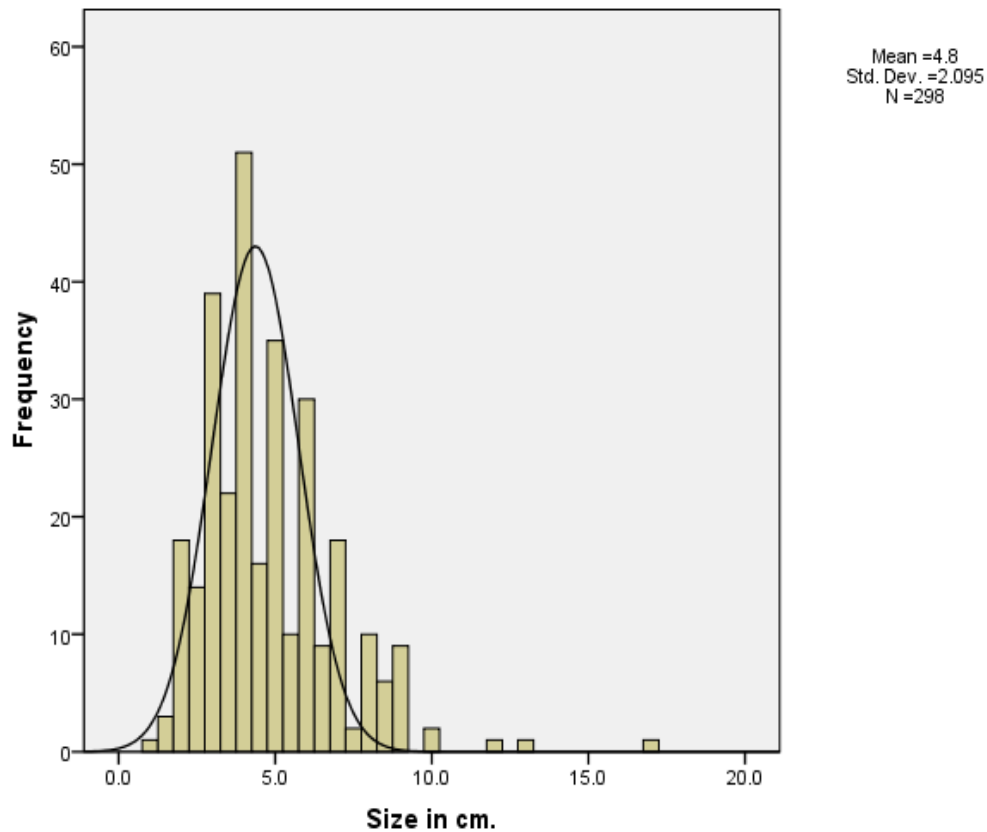


Figure 3.6: Distribution of colorectal cancers by size (diameter in cm).

SIZE OF TUMOURS AND GROWTH APPEARANCE

Larger tumours (defined as tumours with a diameter of 5.0 cm and above) were found to be significantly associated with an exophytic growth appearance. A significantly higher proportion (41 out of 99 cases) of large tumours were exophytic as compared to 58 out of 199 cases of small tumours, (41.4% vs. 29.1%, $p=0.034$). Conversely, 70.9% of small tumours (defined as tumours with a diameter of less than 5.0 cm) appeared endophytic (Table 3.18).

Table 3.18: Distribution of colon cancers by growth pattern and size of tumours.

Factor	Category	All patients (n=298)	Growth pattern		P value	Odds Ratio (95% CI)
			Exophytic (n=99)	Endophytic (199)		
Size	Small (<5cm)	199 (66.8%)	58 (58.6%)	141 (70.9%)	0.034	1.72 (1.04- 2.84)
	Large (≥5cm)	99(33.2%)	41 (41.4%)	58 (29.1%)		

SIZE AND MMR-D TUMOURS

The mean sizes of MMR-d tumours and non-MMR-d tumours were 5.7 ± 2.4 cm and 4.7 ± 2.0 cm respectively. Mismatch repair defect tumours were larger than non-MMR-d tumours by a mean difference of 1.0 cm (95% CI 0.27-1.81).

When the means of the tumours with and without mismatch repair defect were compared, tumours with mismatch repair protein defects were found to be significantly larger ($p=0.009$) compared to non-MMR-d tumours.

Conversely, more than half of the MMR-d tumours were larger than 5.0 cm (53.5 %) as compared to only 29.8% of the non-MMR-d tumours ($p=0.002$, Table 3.19). This difference reached statistical significance. The odds ratio was 2.71 (95% CI 1.41-5.22). Thus, MMR-d tumours were significantly associated with larger sized tumours.

Table 3.19: Distribution of colon cancers by size of tumours and mismatch repair status.

Factor	Category	All patients (n=298)	MMR status:		<i>P</i> value	Odds Ratio (95% CI)
			MMR-d group (n=43)	Non-MMR-d group (n=255)		
Size	Small (<5.0cm)	199 (66.8%)	20 (46.5%)	179 (70.2%)	0.002	2.71 (1.41- 5.22)
	Large (≥5.0cm)	99(33.2%)	23 (53.5%)	76 (29.8%)		

Larger tumours with diameter of 5.0 cm and above were significantly associated with a higher amount of mucin (more than 10%) compared to smaller tumours ($p=0.011$). The larger tumours had an odds ratio of 2.02 with a 95% confidence interval of 1.17-3.51 (Table 3.20).

Table 3.20: Distribution of patients with colorectal carcinomas by size of tumours and amount of mucin in the tumour.

Factor	Category	All patients (n=298)	Amount of mucin:		<i>P</i> value	Odds Ratio (95% CI)
			$\leq 10\%$ (n=228)	$> 10\%$ (n=70)		
Size	Small ($<5.0\text{cm}$)	199 (66.8%)	161 (70.6%)	38 (54.3%)	0.011	2.02 (1.17-3.51)
	Large ($\geq 5.0\text{cm}$)	99 (33.2%)	67 (29.4%)	32 (45.7%)		

SIZE AND OTHER FEATURES

Tumour size, however, did not have any significant associations with tumour location ($p=0.203$), stage ($p=0.406$), grade ($p=0.227$), invasive border pattern ($p=0.174$) nor expression of p53 staining in the tumours ($p=0.306$). Size of tumours did not have any influence on survival rates ($p=0.952$) either.

3.6: HISTOPATHOLOGICAL FEATURES

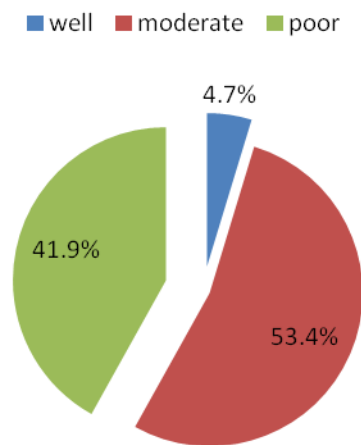
3.6.1: HISTOLOGICAL TYPE AND GRADE

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. The latter group include mucinous carcinomas, signet-ring carcinomas and undifferentiated carcinomas.

HISTOLOGICAL GRADE AND MMR STATUS

Only 34 out of 255 patients (13.3%) with non-MMR-d tumours had poorly differentiated carcinomas. This was in stark contrast to the group of patients with mismatch repair defect tumours: 18 out of 43 cases (41.9%) of mismatch repair defect tumours were poorly differentiated colorectal carcinomas (Figure 3.7). This revealed a statistically significant positive correlation between mismatch repair defects and poorly differentiated tumours as compared to patients with non-MMR-d tumours ($p < 0.001$, OR 4.68, 95% CI 2.31–9.47). The majority of the patients with non-MMR-d tumours had well or moderately differentiated tumours (221 patients, 86.7%).

Mismatch repair defect tumours



Non- Mismatch repair defect tumours

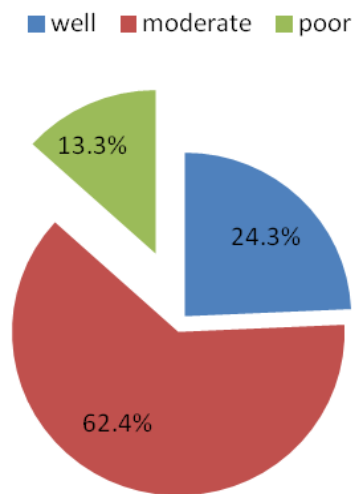


Figure 3.7: Distribution of tumour grade (differentiation) in MMR-d and Non-MMR-d colorectal carcinomas.

MEDULLARY HISTOLOGY

Some colon cancers have medullary histology; characterised by trabecular to sheet-like growth of tumour cells with abundant eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli and no appreciable glandular formation. In the review of slides two cases with undifferentiated carcinomas were found to have medullary histology (Figure 3.8A). They were composed of sheets of monomorphic cells with no glandular differentiation. Both showed loss of either one of the mismatch repair proteins namely hMLH1 (Figure 3.8B) or hMSH2.

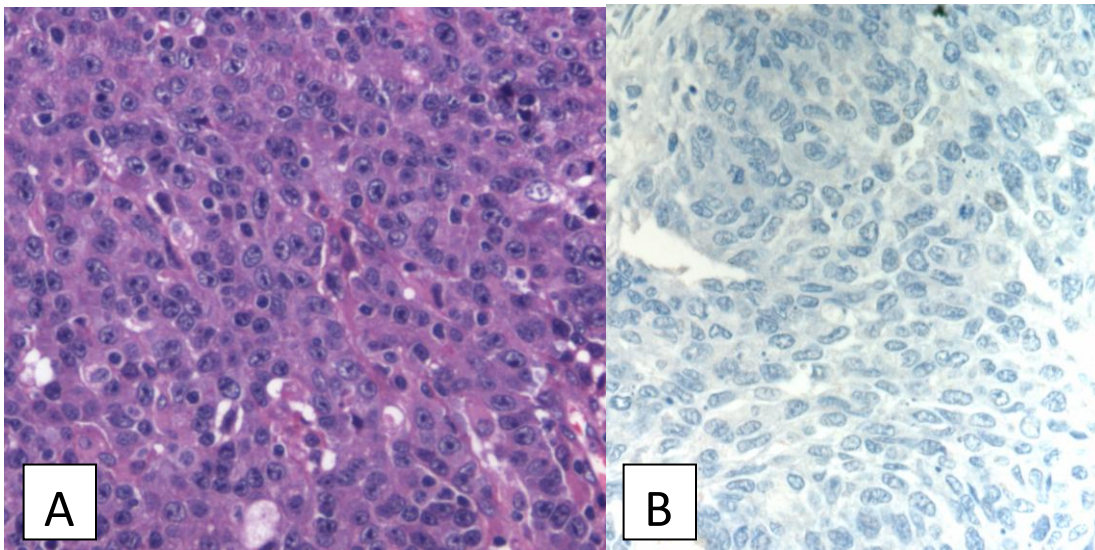


Figure 3.8: Undifferentiated carcinoma

A: shows medullary histology in an undifferentiated carcinoma (H & E stain, original magnification X100).

B: shows the tumour cells with no reaction to hMLH1 protein by IHC method (IHC with hMLH1 antibody, original magnification X200).

GRADE AND TYPE OF MMR DEFECT

Out of the 18 patients with mismatch repair defect tumours which were poorly differentiated, 11 had tumours that were hMLH1 defective (39.3% of hMLH1 defective tumours) and 7 were either hMSH2 or hMSH6 defective tumours (or 46.7% of hMSH2 or hMSH6 deficient tumours). Thus, a higher proportion of hMSH2 or hMSH6 defective tumours were found to be poorly differentiated carcinomas. As the number of cases was small, statistical evaluation cannot be done.

3.6.2: MUCIN PRODUCTION

The amount of mucin in each tumour was evaluated and recorded as less than 10%, 11 to 50% or more than 50% (mucinous carcinomas) as described in Chapter 2.3.2.

Most tumours (228 cases or 76.5%) had minimal mucin, that is, less than 10% mucin. 41 cases (13.7%) involved tumours producing between 10% and 50% mucin and 28 cases (9.40%) had more than 50% mucin present.

MUCINOUS CARCINOMAS AND MMR STATUS

Comparison of data according to the type of carcinoma, namely adenocarcinomas (glandular formation with less mucin production) vs. mucinous carcinomas (tumours where there were more than 50% mucin production) we found that there were 273 patients (91.6%) with adenocarcinoma of the colo-rectum, 22 patients (7.3%) with mucinous carcinomas and only three patients (1.00%) with signet ring cell carcinomas. Due to the small number of cases, mucinous carcinomas were combined with signet ring cell carcinomas and classified as 'mucinous type carcinomas' with mucin production more than

50% (extra-cellular or intracellular) giving a total of 25 patients with ‘mucinous type carcinomas’.

11.6% of patients (or 5 out of 43 patients) with mismatch repair defect tumours had mucinous type carcinomas as compared to 7.8% of patients with non-MMR-d tumours (20 patients out of 255). Although a higher proportion of mucinous type carcinomas was observed among mismatch repair defect tumours, this was not found to be statistically different ($p=0.379$).

MUCIN PRODUCTION AND MMR STATUS

Most tumours (202 out of 255 cases, 79.2%) with intact MMR protein staining had less than 10% mucin and only 53 cases (20.8%) of intact tumours had more than 10% mucin. In contrast, 17 out of 43 cases (39.5%) of mismatch repair defect tumours produced more than 10% mucin (Table 3.21).

Data analysis using the Mann-Whitney U test demonstrated a significant difference in amount of mucin production between patients with mismatch repair defect tumours and non-MMR-d tumours ($p=0.007$). Hence, excessive mucin production was found to be significantly associated with MMR-d tumours.

Table 3.21: Distribution of colorectal carcinomas by mucin production and mismatch repair status.

Factor	Category	MMR status:			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Mucin production	Minimal ($\leq 10\%$ mucin)	228 (76.5%)	26 (60.5%)	202 (79.2%)	0.007	2.49
	Marked ($> 10\%$ mucin)	70 (23.5%)	17 (39.5%)	53 (20.8%)		(1.26-4.93)

In summary, although there was no significant association between mucinous histology and mismatch repair defect tumours, there was a significant association between excessive amount of mucin present and tumours with mismatch repair defects.

3.6.3: TUMOUR ADVANCING PATTERN

Tumours were classified as expansive or infiltrative based on the pattern of tumour advancement as described by Jass et al (Jass, Ajioka et al. 1996). Overall, there were slightly more tumours (161 cases, 54.0%) with expansive borders than there were cases of infiltrating borders (137 cases, 46.0%).

Comparisons were made to identify differences between tumour advancing patterns between right and left sided tumours. Out of 207 left sided tumours, there were 109 cases of colorectal cancers (52.7%) with expansive borders and 98 cases (47.3%) with infiltrating

borders. There was no significant difference between advancing tumour patterns for left sided tumours.

Right sided tumours had a higher proportion of tumours with expansive borders compared to left sided tumours. Out of 91 right sided tumours, there were 52 cases (57.1%) of expansive tumours and 39 cases (42.9%) of infiltrating tumours (Table 3.22). However, this difference was not statistically significant either ($p=0.474$).

Table 3.22: Distribution of colon cancers by tumour location and border pattern.

Factor	Category	All cases (n=298)	Location		P value	Odds Ratio (95% CI)
			Left	Right		
Borders	Expanding	161 (54.0%)	109 (52.7%)	52 (57.1%)	0.474	1.20 (0.73- 1.97)
	Infiltrating	137 (46.0%)	98 (47.3%)	39 (42.9%)		

TUMOUR ADVANCING PATTERN AND MMR STATUS

Twenty nine out of 43 cases (67.4%) of mismatch repair defect tumours had expansive borders. The remainder of the mismatch repair defect tumours (14 cases) had infiltrative borders. This meant about a third of mismatch repair defect tumours had infiltrative borders. By comparison, intact tumours had almost equal numbers of expansive (132 cases, 51.8%) and infiltrative borders (123 cases, 48.2%) respectively. Data analysis revealed a non-significant trend towards an association between mismatch repair defect tumours and expansive borders ($p=0.056$).

3.6.4: LYMPHOCYTIC RESPONSE

PERITUMOURAL LYMPHOCYTIC RESPONSE

There were 216 tumours (72.5%) with marked peri-tumoural lymphocytic response but only 82 cases (27.5%) with minimal lymphocytic response. The presence of a marked peri-tumoural lymphocytic response around a tumour was not associated with many of the characteristics of the tumour studied. Specifically, it showed no association with localisation of tumour (right sided vs. left sided, $p=0.770$), gross appearance of tumour (exophytic vs. endophytic lesion $p=0.537$) or the size of the tumour ($p=0.372$). It was also not significantly associated with either the depth of invasion ($p=0.336$) or tumour stage ($p=0.057$) (Table 3.23).

Table 3.23: Association of peri-tumoural and Crohn-like lymphocytic response and other pathological parameters

Parameters		PTL +ve n, %	PTL -ve n, %	<i>p</i> value	CLR +ve n, %	CLR -ve n, %	<i>p</i> value
Site	Right	67 (31.0%)	24 (29.3%)	0.770	80 (29.5%)	11 (40.7%)	0.227
	Left	149 (69.0%)	58 (70.7%)		191 (70.5%)	16 (59.3%)	
Size	Small**	141 (65.3%)	58 (70.7%)	0.372	180 (66.4%)	19 (70.4%)	0.678
	Large*	75 (34.7%)	24 (29.3%)		91 (33.6%)	8 (29.6%)	
Gross app	Exophytic	74 (34.3%)	25 (30.5%)	0.537	90 (33.2%)	9 (33.3%)	0.990
	Endophytic	142 (65.7%)	57 (69.5%)		181 (66.8%)	18 (66.7%)	
Stage	Low	124 (57.4%)	37 (45.1%)	0.057	146 (53.9%)	15 (55.6%)	0.867
	High	92 (42.6%)	45 (54.9%)		125 (46.1%)	12 (44.4%)	
Grade	low	184 (85.2%)	62 (75.6%)	0.052	223 (82.3%)	23 (85.2%)	0.705
	high	32 (14.8%)	20 (24.4%)		48 (17.7%)	4 (14.8%)	
Borders	Expanding	120 (55.6%)	41 (50.0%)	0.390	147 (54.2%)	14 (51.9%)	0.812
	Infiltrative	96 (44.4%)	41 (50.0%)		124 (45.8%)	13 (48.1%)	
Necrosis	Minimal	144 (66.7%)	53 (64.6%)	0.741	173 (63.8%)	24 (88.9%)	0.009
	Marked	72 (33.3%)	29 (35.4%)		98 (36.2%)	3 (11.1%)	
Mucin	Minimal	172 (79.6%)	56 (68.3%)	0.039	212 (78.2%)	16 (59.3%)	0.027
	Marked	44 (20.4%)	26 (31.7%)		59 (21.8%)	11 (40.7%)	
p53	negative	112 (51.9%)	35 (42.7%)	0.157	128 (47.2%)	19 (70.4%)	0.022
	positive	104 (48.1%)	47 (57.3%)		143 (52.8%)	8 (29.6%)	

PTL= peri-tumoural lymphocytic response, CLR = Crohn-like lymphocytic response

Large* = tumours more than or equal to 5 cm diameter; Small ** = tumours smaller than 5 cm diameter

Stage low = Stage 1 & 2; Stage high = Stage 3 & 4

Grade high =poorly differentiated; Grade low = well & moderately differentiated

Mucin marked = ≥ 10 % mucin present; Mucin minimal = <10 % mucin present

p < 0.05 is significant

Histologically, the presence of peri-tumoural lymphocytic response occurred irrespective of the grade of the tumour ($p=0.052$), the type of invasive border pattern ($p=0.390$) or degree of necrosis ($p=0.741$). Almost three quarters of the tumours had a marked peri-tumoural lymphocytic response regardless of the expression or accumulation of p53 ($p=0.157$).

The majority of the tumours that exhibited marked peri-tumoural lymphocytic response (79.6%) showed minimal mucin production (<10% mucin present), with only 20.4% showing marked mucin production. This can be compared with tumours with minimal lymphocytic response which had a greater percentage of marked mucin production at 31.7% (26 out of 82 cases). This relationship between lymphocytic response and mucin production was statistically significant ($p=0.039$), and it can be concluded that the presence of marked peri-tumoural lymphocytic response was significantly associated with minimal production of mucin. Tumours with abundant mucin production were less likely to have a peri-tumoural lymphocytic response.

Twenty eight cases (65.1%) of MMR-d tumours had marked peri-tumoural lymphocytic response. 73.7% of intact tumours had marked lymphocytic response (Table 3.24). The peri-tumoural lymphocytic response was not found to be significantly different between the MMR-d tumours and non-MMR-d tumours ($p=0.242$).

Table 3.24: Distribution of tumours by their lymphocytic response (peri-tumoural) and MMR-status.

Factor	Category	All patients (n=298)	MMR status		<i>P</i> value	Odds ratio (95% CI)
			MMR-d group (n=43)	Non-MMR-d group (n=255)		
Peri-tumoural lymphocytic response	Minimal	82 (27.5%)	15 (34.9%)	67 (26.3%)	0.242	0.67 (0.34-1.32)
	Marked	216 (72.5%)	28 (65.1%)	188 (73.7%)		

CROHN-LIKE LYMPHOCYTIC RESPONSE

There were 271 cases (90.1%) with conspicuous Crohn-like lymphocytic response. Twenty seven cases (9.9%) did not demonstrate any response. Similar to the peri-tumoural lymphocytic response, conspicuous Crohn-like lymphocytic response occurred independent of various features of the tumour, namely: tumour site, growth appearance, size, staging and depth, invasive border pattern and grade (Table 3.23).

Crohn-like lymphocytic response was also found to be associated with minimal mucin production. A significant number of tumours (212 cases, 78.2%) with conspicuous Crohn-like lymphocytic response had minimal (<10%) mucin present in the tumours, with a smaller percentage (59.3%) of cases without Crohn-like lymphocytic response having minimal mucin production ($p=0.027$). This can be translated into the fact that 40.7% of cases without Crohn-like lymphocytic response had abundant (>10%) mucin production as

compared to about half that proportion (21.8%) in cases with Crohn-like lymphocytic response. In addition, it was found that the presence of conspicuous Crohn-like lymphocytic response was significantly associated with marked tumour necrosis. There was a higher proportion of marked necrosis in cases with conspicuous Crohn-like lymphocytic response (98 out of 271 cases, 36.2%) than in cases without Crohn-like lymphocytic response (11.1% of). This difference was statistically significant ($p=0.009$).

Nuclear accumulation of p53 was seen in more than half of the cases (52.8%) with Crohn-like lymphocytic response but only in 8 out of 27 cases or 29.6% of tumours with no Crohn-like lymphocytic response. Conversely 70.4% of cases with no such lymphocytic response did not have any p53 abnormality detected by immunohistochemistry. Therefore conspicuous Crohn-like lymphocytic response in tumours was found to be significantly associated with p53 over-expression ($p=0.022$).

However, there was no significant association between tumours with Crohn-like lymphocytic response with respect to their mismatch repair status. Crohn-like lymphocytic response was equally likely to be present in MMR-d tumours (88.4%) compared to non-MMR-d tumours (91.4%) [$p=0.564$, Table 3.25].

Table 3.25: Distribution of tumours by their lymphocytic response (Crohn-like) and MMR status.

Factor	Category	All patients (n=298)	MMR status		P value	Odds Ratio (95% CI)
			MMR-d group (n=43)	Non- MMR-d group (n=255)		
Crohn-like lymphocytic response	Inconspicuous	27 (9.1%)	5 (11.6%)	22 (8.6%)	0.564	1.39 (0.49-3.90)
	Conspicuous	271 (90.9%)	38 (88.4%)	233 (91.4%)		

In this study, peri-tumoural lymphocytic response was found to correlate with the presence of conspicuous Crohn-like lymphocytic response. 93.1% or 201 cases (out of 216 cases) with marked peri-tumoural lymphocytic response also showed co-existing presence of conspicuous Crohn-like lymphocytic response ($p=0.039$).

3.6.5: DEGREE OF NECROSIS

About two-thirds of colorectal cancer cases (197 cases, 66.1%) had minimal necrosis while another 101 cases (33.9%) demonstrated marked necrosis (Figure 3.9).

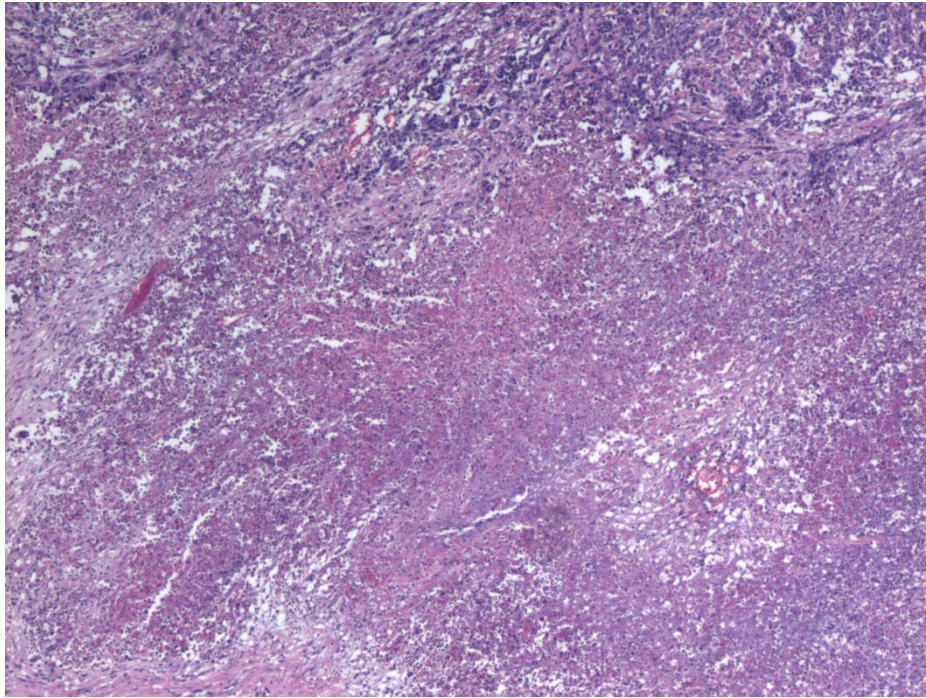


Figure 3.9: Colorectal carcinoma with extensive necrosis, seen replacing the tumour (Haematoxylin and eosin stain, original magnification x 100).

NECROSIS AND SIZE

It was found that larger tumours were more strongly associated with confluent necrosis (marked necrosis) compared to smaller tumours. Approximately half of the cases (49 out of 99 cases, 49.5%) with large tumours, i.e. diameter of at least 5.0 cm had presence of marked necrosis compared to only 26.1% (52 out of 199) of small tumours (less than 5.0 cm in diameter) [Table 3.26]. Larger tumours more than or equal to 5.0 cm in diameter had a significantly higher rate of necrosis compared to small tumours ($p<0.001$).

Table 3.26: Distribution of colorectal carcinomas by size and degree of necrosis present.

Factor	Category	All CRC cases (n=298)	Size of tumours		<i>P</i> value	Odds Ratio (95% CI)
			Small (< 5cm)	Large (≥ 5cm)		
Necrosis	Little	197 (66.1%)	147 (73.9%)	50 (50.5%)	<0.001	2.77 (1.67- 4.59)
	Marked	101 (33.9%)	52 (26.1%)	49 (49.5%)		

NECROSIS AND MMR STATUS

Although only approximately one-third (33.9%) of all colon cancer cases had marked necrosis, more than half of the mismatch repair defect cases (24 out of 43 cases, 55.8%) were associated with marked necrosis. Tumours with absent or minimal necrosis were more frequently non-MMR-d tumours. 90.4% of tumours with no or minimal necrosis were

tumours with no mismatch repair deficiency. Conversely, 69.8% of non-MMR-d tumours had minimal or no necrosis (Table 3.27).

Mismatch repair defect tumours were significantly more likely to have marked necrosis than non-MMR-d tumours. 55.8% of mismatch repair defect tumours had marked necrosis compared to 30.2% of intact tumours without the defect ($p=0.001$).

Table 3.27: Distribution of colorectal carcinomas by MMR status and degree of necrosis present.

Factor	Category	MMR status			<i>P</i> value	Odds ratio (95% CI)
		All patients (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
Necrosis	Little	197 (66.1%)	19 (44.2%)	178 (69.8%)	0.001	2.92 (1.51-5.64)
	Marked	101 (33.9%)	24 (55.8%)	77 (30.2%)		

3.7: STAGING OF TUMOURS

3.7.1: TNM STAGING

There were 11 cases (3.69%) where staging could not be performed as no lymph nodes were isolated and thus the nodal status was unknown (Nx). Of the remaining 287 cases, there were 36 patients (12.5%) with Stage I, 108 patients (37.6%) with Stage II, 129 patients (44.9%) with Stage III and 14 patients (4.9%) with Stage IV colorectal cancer at the time of operation. Liver was the main metastatic site in those patients with stage IV disease (10 out of 14 patients, 71.4%). Three other patients had lung metastasis while another had peritoneal seedling including ovarian metastasis.

The majority of patients with colorectal carcinomas (82.5%) had either stage II or III cancer (Figure 3.10).

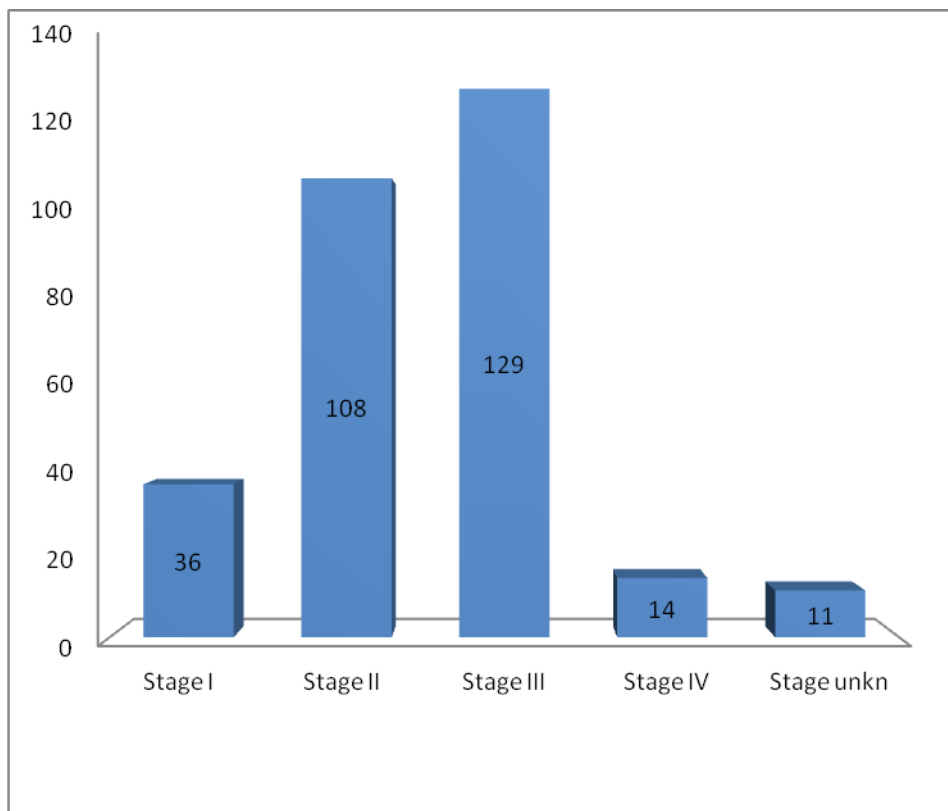


Figure 3.10: Distribution of cases of colorectal carcinoma by staging.

3.7.2: DEPTH OF INVASION

In this study, the majority of colorectal carcinomas (246 out of 298, 82.6%) were found to have invaded through the muscularis propria into the subserosa (T3) or perforated through the visceral peritoneum (T4). 38 out of 43 cases (88.4%) of tumours expressing mismatch repair defects were T3 or T4 tumours. Only 5 cases (10.2%) were T2 tumours and none were T1 tumours (Figure 3.11). A significantly higher number of tumours with mismatch repair defects were found to demonstrate deeper local invasion as compared to tumours with intact protein expression (Mann-Whitney test, $p=0.039$). Mismatch repair defect

tumours were associated with a higher degree of local invasion even though they were more frequently associated with an earlier stage at presentation (Figure 3.12).

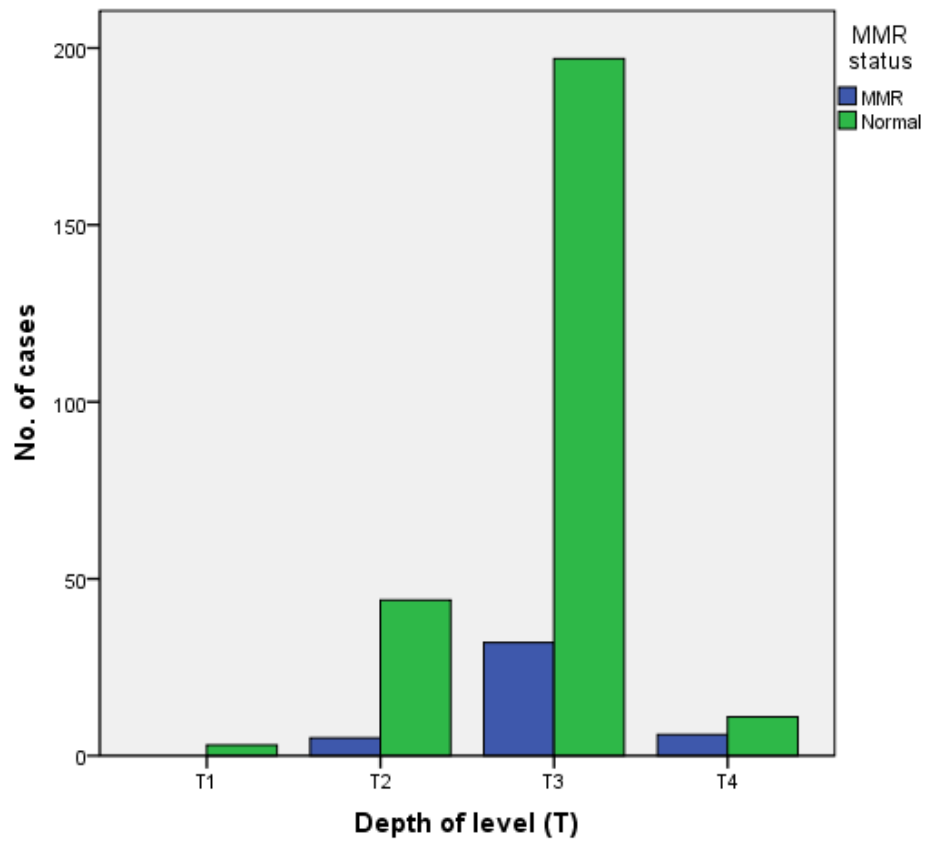


Figure 3.11: Distribution of colon cancer cases by depth of tumour invasion (T) and mismatch repair staining pattern.

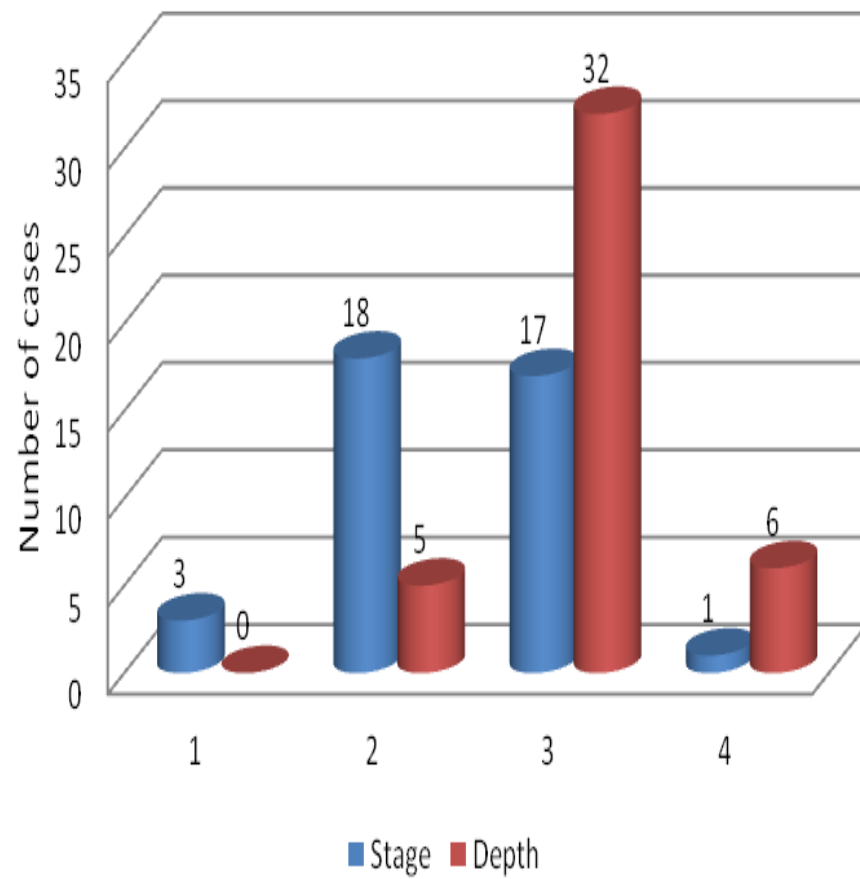


Figure 3.12: Distribution of colon cancers with mismatch repair defect by stage and depth of invasion.

3.7.3: LYMPH NODES STATUS

Unfortunately, in 11 cases (four cases of mismatch repair defect tumours and seven cases of non-MMR-d tumours), lymph node retrieval was not performed and as a result the status of the nodes was not evaluated. As the specimens were received between the years 2004 to 2007, they had already been discarded and it was not possible to re-examine them to look for lymph nodes.

In the 11 cases where the nodes were not assessed, they were classified as unknown or Nx. Out of the remaining 287 patients, between 1 to 79 nodes were removed with a mean of 8.8 lymph nodes isolated per case.

144 patients (50.2%) were found to have no lymph node involvement (N0), leaving 143 patients (49.8%) with lymph node involvement, either N1 (when one to three lymph nodes were involved by carcinoma) or N2 (when more than 3 lymph nodes were involved). In patients with positive lymph node status, a mean of 3.4 lymph nodes were involved by tumour.

18 out of 39 patients (46.2%) with mismatch repair defect tumours had nodal infiltration, compared to 125 out of 248 patients with non-MMR-d tumours (50.4%). Thus, there were relatively more patients with lymph node metastases if their tumours had no loss of mismatch repair defect, although this was not found to be statistically significant ($p=0.622$). There was also no significant difference in the degree of lymph node involvement between the mismatch repair defect tumour group and patients with non-MMR-d tumours ($p=0.746$, Table 3.28).

Table 3.28: Number of lymph nodes isolated and involved compared between two groups: MMR-d and non-MMR-d group.

		All cases (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)
Nodes staging (n=298)	N0	144	21 (%)	123 (85.4%)
	N1	101	13 (12.9%)	88 (87.1%)
	N2	42	5 (11.9%)	37 (88.1%)
	Nx	11	4	7
Cases with nodes involved		143	18 (46.2%)	125 (50.4%)
Nodes involved (n=136)	Mean no. of LN	3.4	3.3	3.4
	(range)	(1-18)	(1-10)	(1-18)

The tumour stage was known for 39 patients with mismatch repair defect tumours and 248 patients with non-MMR-d tumours as lymph nodes were not available for assessment in 11 cases.

53.8% of patients (or 21 out of 39 patients) with mismatch repair defect tumours presented in Stage I or II as compared to 46.2% of patients with mismatch repair defect tumours presenting at Stage III or IV. With regards to Stage III tumours, 45.2% of patients with non-MMR-d tumours presented in this stage, an almost similar proportion to that of patients with mismatch repair defect tumours(43.6%). 13 out of 248 patients (5.2%) with non-MMR-d tumours developed metastasis at presentation but only one out of 39 patients (2.6%) with MMR-defective tumours presented in Stage IV (Table 3.29). It appeared that

patients with mismatch repair defect tumours presented at earlier stages and fewer were associated with metastasis as compared to patients with intact tumours, but there was no significant difference between the two groups when comparing the stage of tumours at presentation ($p=0.622$).

Table 3.29: Distribution of cases of colorectal cancers: comparing different stages to the MMR staining pattern.

Category	MMR status			<i>P</i> value	Odds ratio (95% CI)
	All patients (n=287)	MMR-d group (n=39)	Non-MMR-d group (n=248)		
Stage I	36	3 (7.7%)	33 (13.3%)	0.622	1.19 (0.60.-2.33)
Stage II	108	18 (46.2%)	90 (36.3%)		
Early Stage	144	21 (53.8%)	123 (49.6%)		
Stage III	129	17 (43.6)	112 (45.2%)		
Stage IV	14	1 (2.6%)	13 (5.2%)		
Late Stage	143	18 (46.2%)	125 (50.4%)		

3.8: TUMOUR SUPPRESSOR GENE: P53

3.8.1: STAINING PATTERN

There were almost equal numbers of tumours with p53 positive staining and p53 negative staining. One hundred and fifty one cases (50.7%) of colorectal cancers showed accumulation of p53 expression whereas 147 cases (49.3%) were p53 negative.

3.8.2: P53 AND MISMATCH REPAIR DEFECT TUMOURS

Analysis of the relationship between p53 and MMR status of the tumours studied showed that almost three quarters of mismatch repair defect tumours (74.4%) did not demonstrate nuclear staining for p53. Furthermore, 92.7% of tumours with p53 over-expression were found to be intact tumours with no loss of MMR protein (Table 3.30). Mismatch repair defect tumours were significantly associated with poor expression of p53 ($p < 0.001$, OR 3.54, 95% CI 1.71-7.34).

Table 3.30: Distribution of colon cancers by p53 staining and mismatch repair status.

Factor	Category	MMR status			<i>P</i> value	Odds ratio (95% CI)
		All cases (n=298)	MMR-d group (n=43)	Non-MMR-d group (n=255)		
p53 staining	Positive	151 (50.7%)	11 (7.3%)	140 (92.7%)	<0.001	3.54 (1.71-7.34)
	Negative	147 (49.3%)	32 (21.8%)	115 (78.2%)		

3.8.3: P53 AND ANATOMICAL LOCATION OF TUMOUR

78.8% percent of p53 positive tumours were located in the left side of colon (distal to but not including the splenic flexure) as compared to only 21.2% on the right side (Table 3.31). Tumours distal to the splenic flexure were significantly more likely to have p53 over-expression as compared to tumours located proximally ($p<0.001$).

Table 3.31: Distribution of colon carcinomas by p53 staining and location of tumours.

Factor	Category	P53 staining:		<i>P</i> value	Odds ratio (95% CI)
		All cases (n=298)	p53 positive		
Site	Left	207 (69.5%)	119 (78.8%)	<0.001	2.49 (1.50-4.16)
	Right	91 (30.5%)	32 (21.2%)		

3.8.4: P53 AND STAGING

There was no correlation between tumour stage and the accumulation of p53 over-expression. There were almost equal numbers of cases with p53 positive staining and negative staining among tumours presenting at an early stage (Stage I and II tumours) and at an advanced stage (Stages III and IV) (Table 3.32).

Table 3.32: Distribution of cases of by tumour stage: analysis of p53 staining patterns in early vs. late stage tumours

Category	P53 staining:			<i>P</i> value	Odds ratio (95% CI)
	All patients (n=287)	p53 positive (n=145)	p53 negative (n=142)		
Stage I	36	23	13	0.262	1.30 (0.82-2.07)
Stage II	108	45	63		
Early Stage	144	68 (47.2%)	76 52.8%)		
Stage III	129	71	58		
Stage IV	14	6	8		
Late Stage	143	77(53.8%)	66 (46.2%)		

3.9: SURVIVAL

3.9.1: SURVIVAL OF PATIENTS WITH CRC

Forty patients were lost to follow-up after their initial surgery. Data regarding survival of these patients were not obtained. From the data available from the other 258 patients, the overall mean survival was 31.2 ± 1.6 months (Figure 3.13).

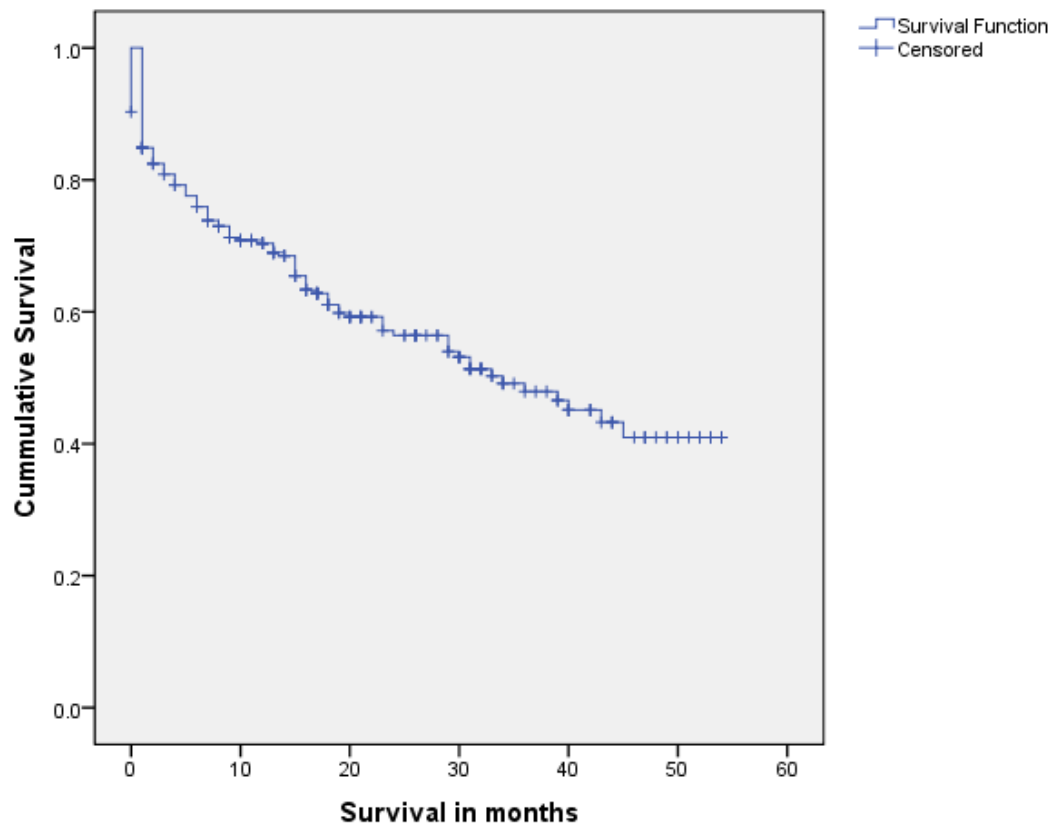


Figure 3.13: Survival curve for all patients with colorectal carcinomas

(Censored data represents patients alive at their last follow up visits).

3.9.2: SURVIVAL AND STAGING

One of the most important features that was found to affect prognosis and hence the survival of patients with colorectal cancer was their lymph node status. Patients with colon cancers with lymph node metastases i.e. Stages III or IV had a worse prognosis than those with earlier stages.

The mean survival of patients presenting with early stage cancer (Stages I or II) was 34.9 ± 2.2 months while those with late stage cancer (Stages III or IV) had a mean survival of 26.0 ± 2.1 months. Analysis with the log rank test showed that this difference was statistically significant ($p=0.010$). Clearly, patients presenting at an earlier stage (either Stage I or II) had a longer survival than those who presented at a later stage.

On the whole, patients survived longer when there was no lymph node involvement. The cumulative survival of 50% of patients with nodal metastases was approximately 18 months (figure 3.14), but that of patients free from nodal involvement was close to 4 years, confirming that nodal involvement drastically decreased patient survival.

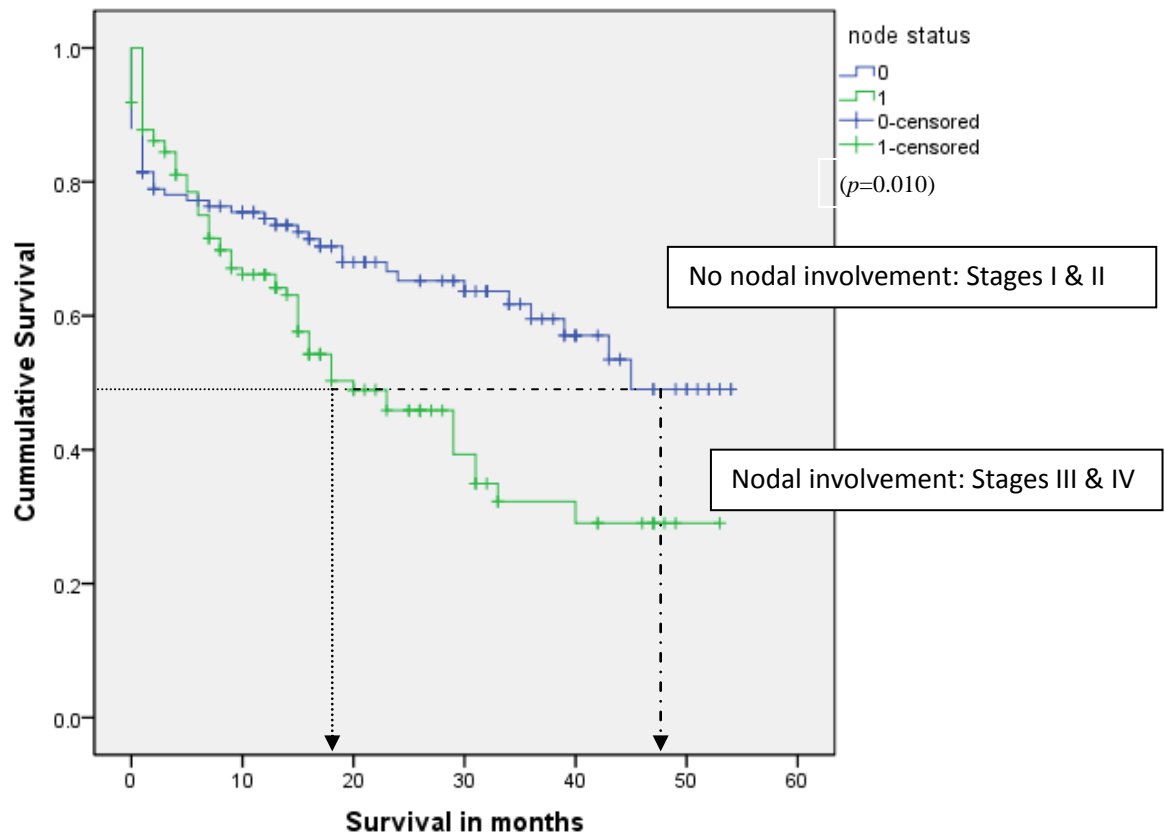


Figure 3.14: Survival curves for patients categorised according to nodal status. Arrows show cumulative survival of 50% of patients.

3.9.3: SURVIVAL AND MMR-D TUMOURS

The mean survival of patients with mismatch repair defect tumours was 31.4 ± 4.6 months compared with mean survival of patients with non-MMR-d tumours at 31.0 ± 1.6 months. Although patients with mismatch repair defect tumours had a slightly longer mean survival

time compared to patients with MSS tumours, this was not statistically significant ($p=0.615$, Figure 3.15).

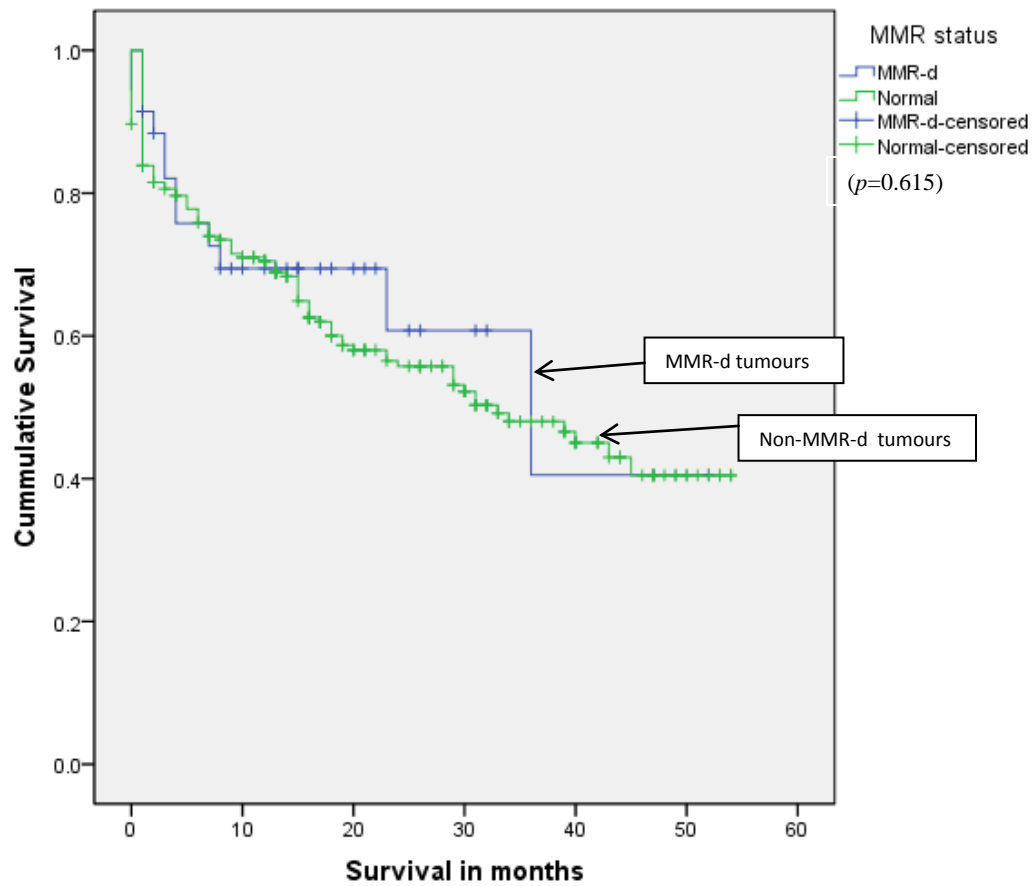


Figure 3.15: Survival curves for patients categorised according to mismatch repair status.

There were 177 cases of colon carcinomas when rectal tumours were excluded from the study. Twenty-nine cases did not have clinical notes and the status of the patients was unknown. These were excluded. In the remaining sample of 148 patients with colon cancers, 27 cases had loss of mismatch repair protein by IHC testing and 121 had normal protein pattern. The clinical outcomes in relation to their mismatch repair status for this remaining group of patients were studied. The mean survival of patients with mismatch repair defect by IHC was 32.7 months was longer as compared to those with normal protein pattern of 29.9 months. However, this was not significant with a probability value of 0.377.

3.9.4: SURVIVAL AND P53 STATUS

This study found that patients with tumours expressing p53 did not demonstrate any significant difference in survival rates compared to patients with p53 negative tumours (Figure 3.16, $p=0.741$). Patients with p53 positive tumours had a mean survival of 31.1 ± 2.2 months, whereas the mean survival of patients with p53 negative tumours was 31.4 ± 2.2 months.

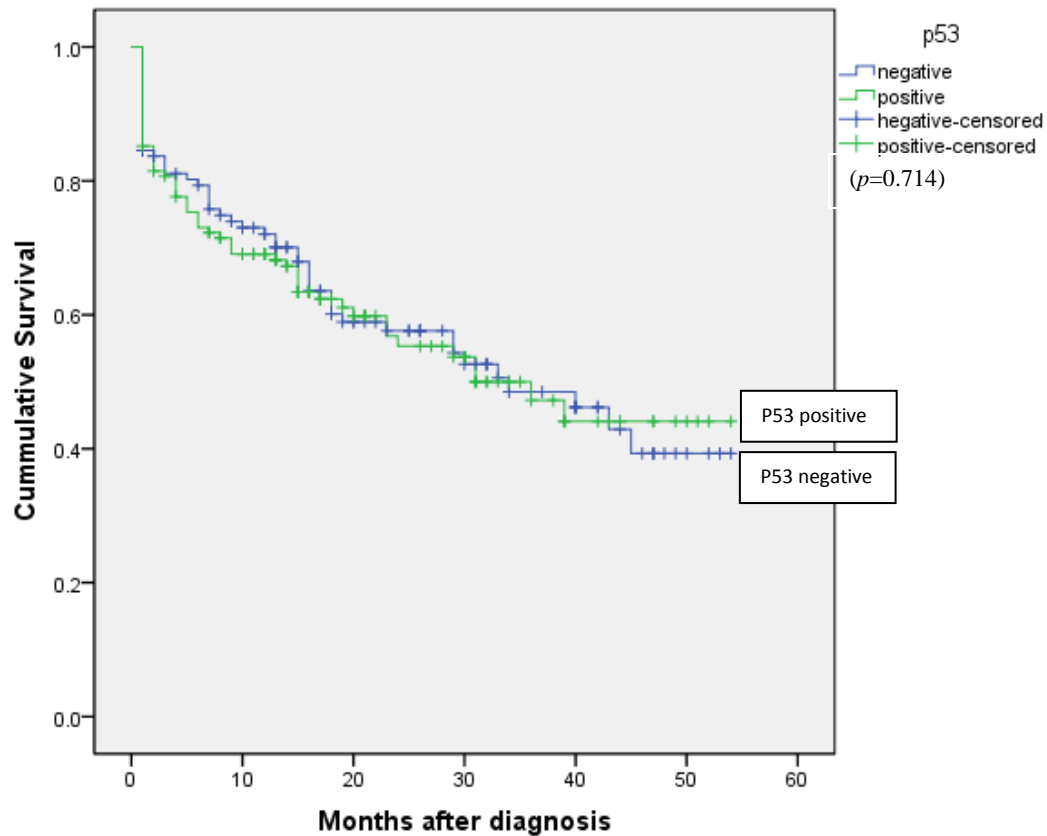


Figure 3.16: Survival curves for patients with categorised according to p53 tumour staining patterns.

One hundred and twenty-one cases of rectal tumours were excluded and the remaining 148 patients with known clinical status were studied. The clinical outcomes for these patients with colon cancers were compared in relation to their p53 expression by IHC testing. The mean survival of patients with p53 over-expression was 27.7 months was shorter as compared to a longer survival of 30.8 months for those with negative p53 expression. However, this was not significant ($p=0.495$).

3.9.5: SURVIVAL AND OTHER PATHOLOGICAL FEATURES:

ANATOMICAL LOCATION

Comparison of survival of patients with left versus right sided tumours (without taking MMR-d status into consideration) revealed no significant difference (Figure 3.17). The mean age of survival for patients with left sided tumours was 30.6 ± 1.8 months, and for patients with right sided tumours was 32.2 ± 3.0 months. Survival rates among patients with right sided tumours were slightly better but this was not significantly different ($p=0.724$).

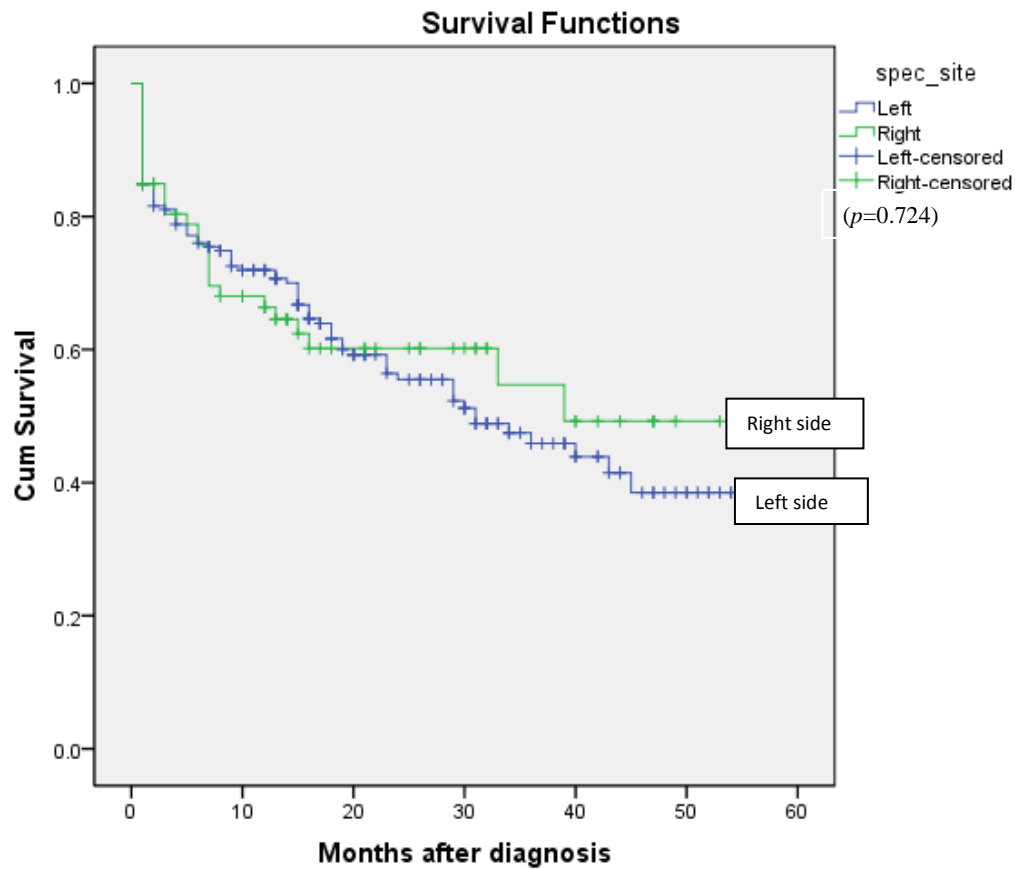


Figure 3.17: Survival curve of patients categorised according to tumour location.

TUMOUR ADVANCING PATTERN

The survival of patients with tumours with expansive borders was far superior than that of patients whose tumours had diffuse infiltrating borders (Figure 3.18). The mean duration of survival was 35.0 ± 2.1 months for patients with expansive tumours but only 26.8 ± 2.1 months for patients with diffuse infiltrating tumours. This difference was statistically significant ($p=0.014$).

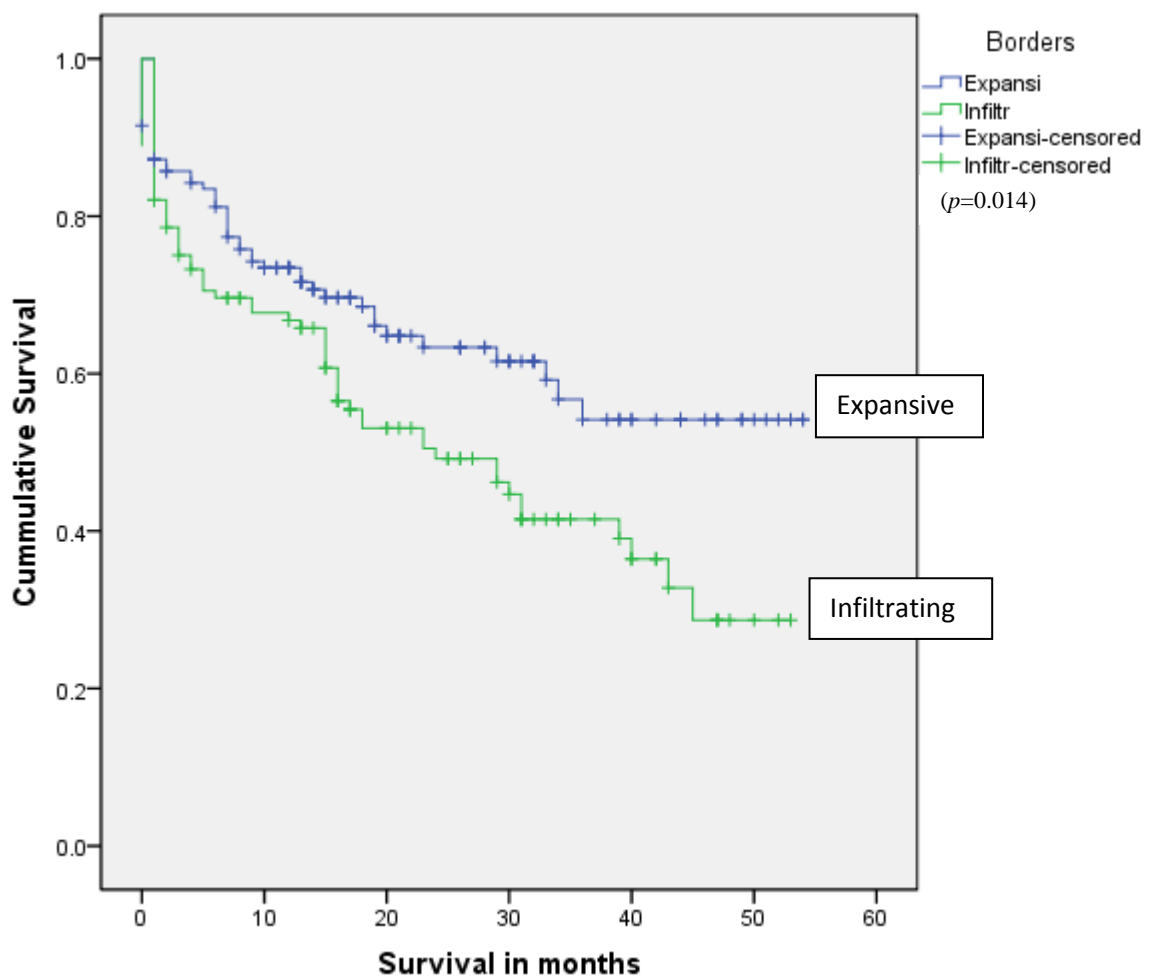


Figure 3.18: Survival curve for patients categorised according to border patterns (expansive vs. infiltrating).

LYMPHOCYTIC RESPONSE

Patients with tumours showing marked peri-tumoural lymphocytic response had a longer mean survival of 32.0 ± 1.8 months as compared to those with minimal or little lymphocytic response (27.7 ± 3.1 months, Figure 3.19). However this difference was not statistically significant ($p=0.293$).

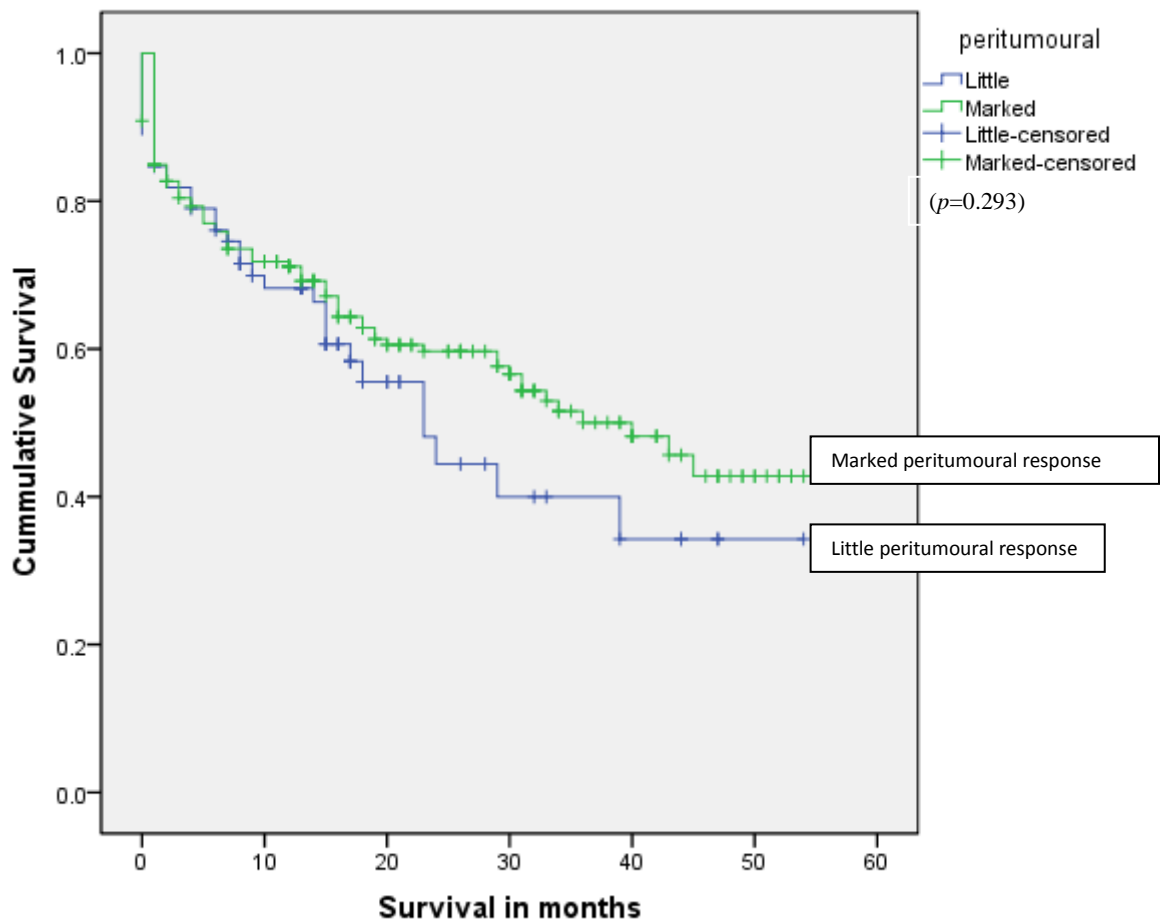


Figure 3.19: Survival curve of patients categorised according to peri-tumoural lymphocytic response.

Similarly, patients with conspicuous Crohn-like lymphocytic response also survived longer with a mean survival time of 31.2 ± 1.6 months compared to 29.3 ± 4.9 months in patients with no Crohn-like lymphocytic response (Figure 3.20). However, this difference also did not reach statistical significance ($p=0.698$).

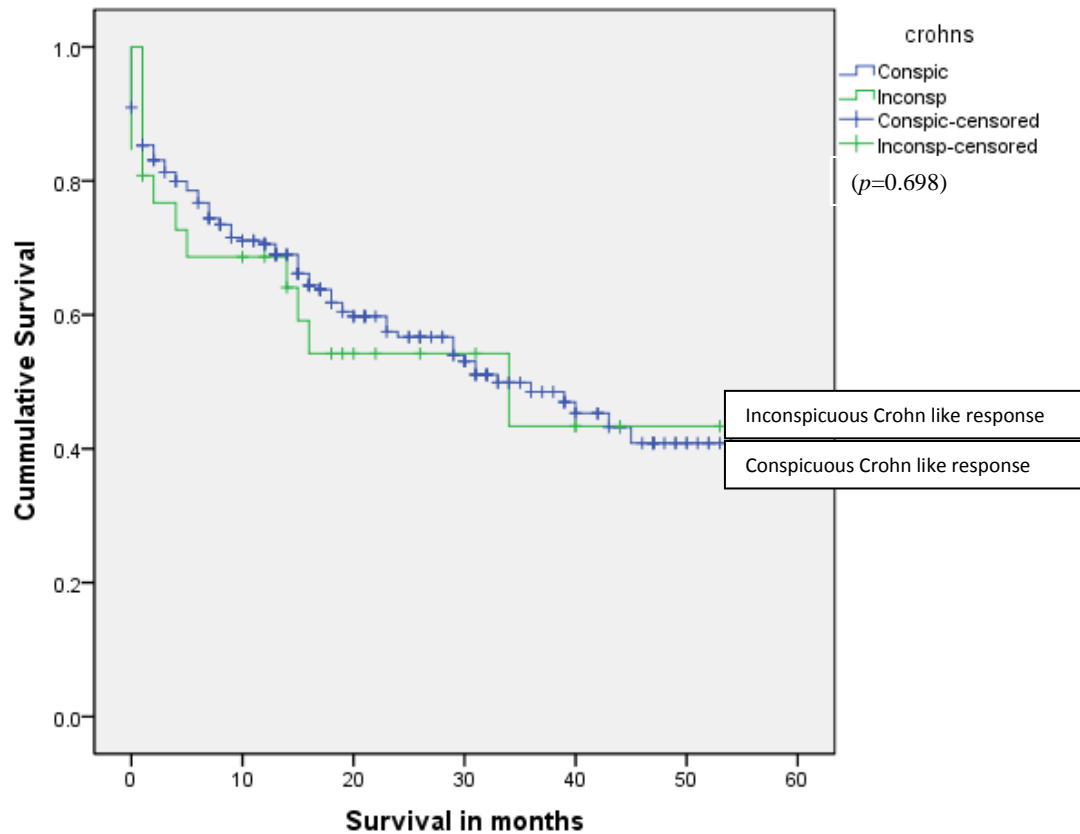


Figure 3.20: Survival curve of patients categorised according to presence of Crohn-like lymphocytic response.

OTHER FEATURES

We found that survival was also independent of other features including tumour growth appearance (exophytic or endophytic), tumour size, amount of mucin production and degree of necrosis.

3.10: TREATMENT

All 298 patients included in the study had surgical resection of the cancer: hemicolectomy, anterior resection or abdominoperineal excision. However, this study had not looked at the resection margins of the cases nor studied recurrences and their clinical outcome.

We were only able to document 145 patients who had completed various types of chemotherapy, nine of which had additional pelvic radiation. Majority of the patients (94 out of 145 cases, 64.8%) had fluorouracil (5-FU) combined with leucovorin (folinic acid) in either the Mayo Clinic regimen or the De Gramont colon cancer chemotherapy regimen.

The remaining other patients were on various chemotherapy: Folfox4 (oxaliplatin), Folfiri (leucovorin, fluorouracil, irinotecan), oral capecitabine or a combination of the chemotherapy drugs.

3.11: SUMMARY OF RESULTS

Patients with mismatch repair defect CRCs were equally likely to be of either gender ($p=0.497$) with no difference in their age of presentation ($p=0.148$) as compared to the group with non MMR-d tumours. They did not present at an earlier stage ($p=0.622$) but were found to be significantly associated with either synchronous or metachronous carcinomas ($p=0.032$).

Their tumours were found frequently at the right side ($p<0.001$), were larger ($p=0.002$) and had an exophytic pattern of growth ($p=0.007$).

Table 3.33 summarised the association of different clinical variables with mismatch repair status of the tumours.

Table 3.33: Clinical variables with mismatch repair status of tumours

Clinical Parameters	Category	All patients	MMR status MMR-d gp	Non MMR-d gp	P value	Odds ratio (95% CI)
Gender	Male	166	26	140	0.497	1.26
	Female	132	17	115		(0.65-2.43)
Age	≤ 50 years	53	22	42	0.148	1.74
	>50 years	245	32	213		(0.82-3.73)
Family history (n=160)	With fly h/o	25	2	23	NA	
	Without fly h/o	135	15	120		
Staging	Stage I-II	144	21	123	0.622	1.19
	Stage III-IV	143	18	125		(0.60-2.33)
Multiple tumours	Single tumours	202	24	178	0.032	2.67
	Multiple tumours	34	9	25		(1.12-6.41)
Location	Left side	207	17	190	<0.001	4.47
	Right side	91	26	65		(2.28-8.76)
Tumour growth	Exophytic	99	22	77	0.007	2.42
	Endophytic	199	21	178		(1.26-4.67)
Size of tumour	Small (<5cm)	199	20	179	0.002	2.71
	Large (≥5cm)	99	23	76		(1.41-5.22)
Survival in mths		31.2	31.4	31.0	0.615	

gp= group, fly= family, h/o= history of, ND= not done, mths= months.

Colorectal carcinomas with mismatch repair defect were more likely to be poorly differentiated tumours ($p<0.001$), produced more mucin ($p=0.007$) and had marked degree of necrosis ($p=0.001$) as compared with tumours with no such defect. They also had a non-significant trend towards an expansive border ($p=0.056$) than an infiltrative border. However, they did not have any association with any lymphocytic response whether peritumoural or Crohn like pattern. There was a significant inverse relation with p53 overexpression ($p<0.001$).

Table 3.34 summarised the histopathological variables with MMR-d tumours and non-MMR-d tumours.

Table 3.34: Histological variables with mismatch repair status of tumours

Histological Parameters	Category	All patients	MMR status		P value	Odds ratio (95% CI)
			MMR-d gp	Non MMR-d gp		
Histological grade	Mod-well diff	246	25	221	<0.001	4.68
	poorly diff	52	18	34		(2.31-9.47)
Mucin production	Minimal ($\leq 10\%$)	228	26	202	0.007	2.49
	Marked (>10%)	70	17	53		(1.26-4.93)
Tumour advancing pattern	Expanding	161	29	132	0.056	0.52
	Infiltrating	137	14	123		(0.262-1.026)
Peritumoural lymphocytic resp	Minimal	82	15	67	0.242	0.67
	Marked	216	28	188		(0.34-1.32)
Crohn-like lymphocytic resp	Minimal	27	5	22	0.564	1.39
	Marked	271	38	233		(0.49-3.90)
Degree of necrosis	Little	197	19	178	0.001	2.92
	Marked	101	24	77		(1.51-5.64)
P53 staining	Positive	151	11	140	<0.001	3.54
	Negative	147	32	115		(1.71-7.34)

gp= group, mod= moderate, diff= differentiated.

MULTIVARIATE ANALYSIS

The different variables that showed statistical significance after univariate analysis namely anatomical location, tumour shape, size, histological grade, mucin production, necrosis and p53 expression; were selected for multivariate analysis to determine independent covariates that were associated with loss of mismatch repair protein.

Four independent variables were significantly associated with loss of expression of one of the mismatch repair protein tested i.e. hMLH1, hMSH2 or hMSH6.

Poor differentiation in tumour grade had the strongest association with loss of expression of the mismatch repair proteins (OR=5.917, 95% CI 2.174-16.129). Right sided location, exophytic growth and poor p53 expression were the three other independent predictors of loss of expression of hMLH1, hMSH2 or hMSH6 (Table 3.35).

Table 3.35: Multivariate analysis of predictors of loss of mismatch repair proteins (using logistic regression model).

Feature	<i>p</i> value	Odds Ratio	95% Confidence limits
Poor differentiation	<0.001	5.92	2.17-16.13
Right sided	0.011	2.70	1.25-5.81
Exophytic growth	0.014	2.56	1.21-5.44
Poor p53 expression	0.046	2.27	1.01-5.09
Large-size	0.061	2.11	0.97-4.59
Marked necrosis	0.079	0.50	0.23-1.08
Mucin production	0.069	3.66	0.90-14.86

CHAPTER 4: DISCUSSION

CONTENTS:

4.1: COLORECTAL CARCINOMAS: DEMOGRAPHICS

4.1.1: INCIDENCE

4.1.2: RACIAL DISTRIBUTION

4.1.3: AGE AND GENDER

4.1.4: ANATOMICAL LOCATION OF TUMOURS WITH RACE AND AGE

4.2: MMR-d COLORECTAL CARCINOMAS

4.2.1: IMMUNOHISTOCHEMICAL TESTING VS. PCR TESTING

4.2.2: INCIDENCE OF MMR-d TUMOURS

4.2.3: DEMOGRAPHICS OF MMR-d TUMOURS

4.2.3.1: AGE

4.2.3.2: RACE

4.2.3.3: GENDER

4.2.4: FAMILY HISTORY

4.3: CHARACTER OF TUMOUR

4.3.1: MULTIPLE TUMOURS

4.3.2: ANATOMICAL LOCATION

4.3.3: GROWTH APPEARANCE

4.3.4: SIZE OF TUMOURS

- 4.4: HISTOLOGICAL FEATURES**
 - 4.4.1: HISTOLOGICAL TYPE AND GRADE**
 - 4.4.2: MUCIN PRODUCTION**
 - 4.4.3: TUMOUR ADVANCING PATTERN**
 - 4.4.4: LYMPHOCYTIC RESPONSE**
 - 4.4.5: NECROSIS**
- 4.5: STAGING**
 - 4.5.1: LYMPH NODES STATUS**
 - 4.5.2: STAGING AND MMR-d TUMOURS**
- 4.6: SURVIVAL**
 - 4.6.1: ANATOMICAL LOCATION**
 - 4.6.2: MMR-d TUMOURS**
 - 4.6.3: TUMOUR ADVANCING PATTERN**
- 4.7: P53 POSITIVE COLORECTAL CARCINOMAS**
 - 4.7.1: INTRODUCTION: P53**
 - 4.7.2: P53 AND MMR-d COLORECTAL CARCINOMAS**
 - 4.7.3: P53 AND ANATOMICAL LOCATION OF TUMOURS**
 - 4.7.4: P53 AND SURVIVAL**
- 4.8: MANAGEMENT OF COLORECTAL CARCINOMAS**
 - 4.8.1: SURGERY**
 - 4.8.2: ADJUVANT CHEMOTHERAPY**
 - 4.8.3: NEW TREATMENT AND TRIALS**
- 4.9 LIMITATIONS OF THE STUDY**

CHAPTER 4: DISCUSSION

4.1: COLORECTAL CARCINOMAS: DEMOGRAPHICS

4.1.1: INCIDENCE

In the year 2002, carcinomas of colon and rectum accounted for more than one million new cases, making up 9.4% of total cancer cases worldwide. However, there was a wide variation in incidence of colorectal cancer worldwide. The highest incidence rates of more than 40 per 100,000 were in North America, Australia/New Zealand, Western Europe and Japan while the incidence in Asia especially South East Asia was low at about 12.5 and 9.9 per 100,000 for males and females respectively (Figure 4.1) (Parkin, Bray et al. 2005).

In the less developed countries and regions, the age standardised rate (ASR) for colorectal carcinomas was as low as 2.3 per 100,000 for males in Middle Africa while in developed countries like Japan or Australia the ASR was as high as 49.3 per 100,000 for males.

The vast epidemiological differences in these different regions and countries for colorectal carcinomas could be explained by different environmental exposures as well as different dietary habits, lifestyle and culture in these areas. It may also be related to the development of the country which translates to an association with a Westernised diet and lifestyle.

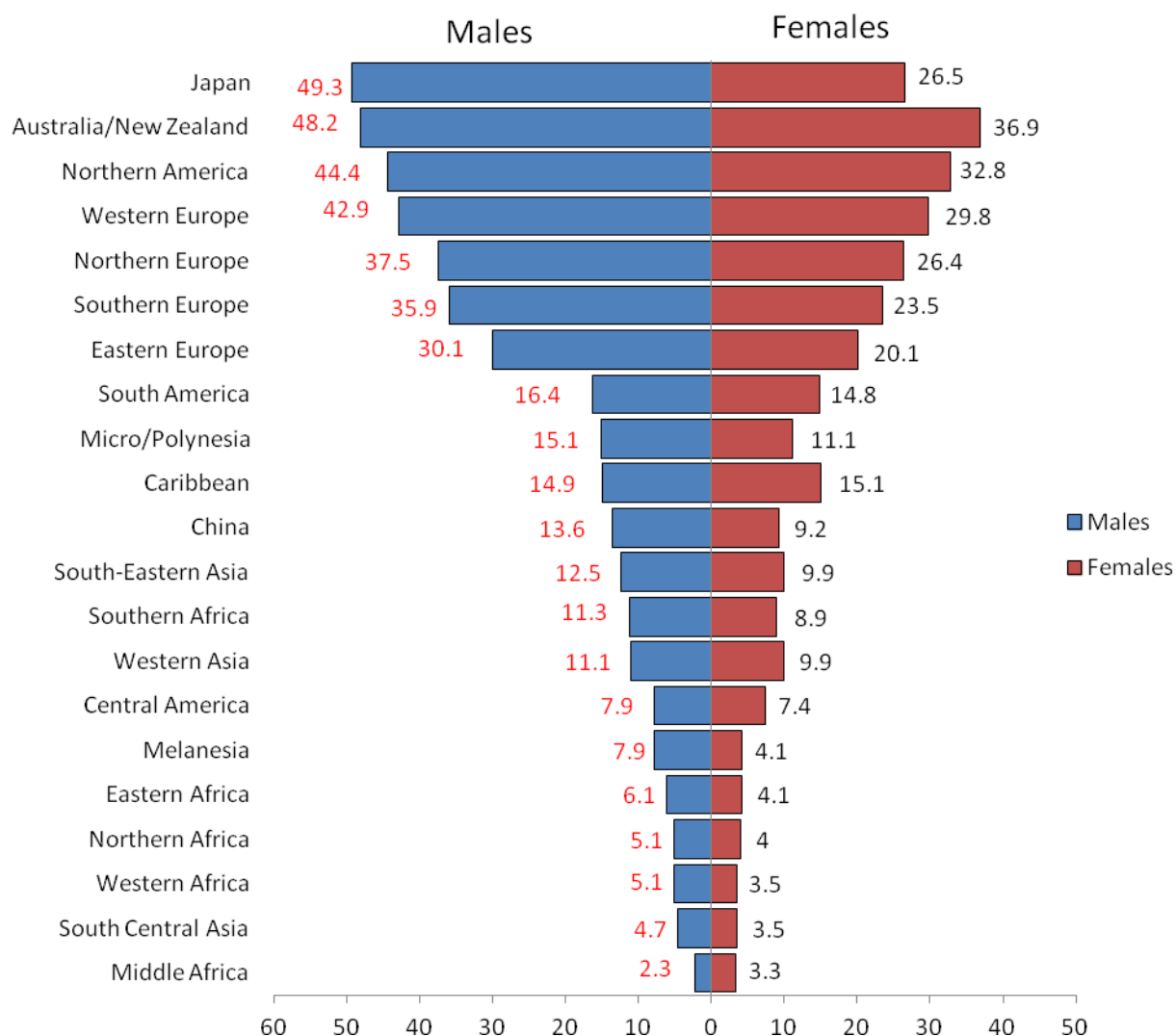


Figure 4.1: Age standardised incidence per 100,000 by gender and country for colorectal carcinomas.

(Source: Parkin, D. M., F. Bray, et al. (2005).

In Malaysia the age standardised incidence rate (ASR) for colorectal carcinomas was actually higher than the rate reported for the South East Asian region. The overall ASR in Malaysia was 18.4 per 100,000 for the year 2006 (National_Cancer_Registry 2006). The

incidence rate was higher for Malaysian males (21.6 per 100,000) as compared to Malaysian females at 15.4 per 100,000. This can be partially explained by the rapid development Malaysia has enjoyed especially over the last two decades. With Western influences and a higher standard of living, people have slowly embraced a Westernised lifestyle and diet. This pattern of Western lifestyle includes smoking, alcohol and high meat consumption and fast food with little vegetable content; which have all been found to contribute or are associated to the development of colorectal cancer.

4.1.2: RACIAL DISTRIBUTION

In addition to various environmental influences, there may be racial differences between people exposed to the same environmental surroundings. People of different races inherit different sets of genes and have different dietary habits and cultural practices.

Malaysia is a multi-racial country. The Malaysian population is predominantly made up of Malays (54.3%) followed by Chinese (25.1%), Indians (7.5%). Other races make up 13.1% of the remaining population (Department_of_Statistics_Malaysia 2007).

The Malaysian National Cancer Registry 2006 reported that, 52.4% patients with colorectal cancer nationwide were made up of Chinese, followed by Malay, 42.7% and Indian, 5% (National_Cancer_Registry 2006). The age standardised rates for colorectal carcinomas were 15.5 and 9.5 per 100,000 for Malay males and Malay females respectively in Malaysia. This was higher among both Chinese males and Chinese females at 28.0 and 21.4 per 100,000 respectively. The ASRs for Indians were almost similar to that of Malay patients: 12.4 and 11.3 per 100,000 for Indian males and Indian females respectively.

Chinese patients had a higher risk of developing colorectal carcinomas than Malays or Indians in Malaysia.

Similar to that reported by the Malaysian National Cancer Registry (National_Cancer_Registry 2006) where Chinese were noted to have higher incidence of colorectal carcinomas, this study also found there was a predominance of Chinese patients with colorectal carcinomas: 46.6% of the patients studied were Chinese compared to 45.0% Malays and 6.3% Indians. This was significant when it was compared to the hospital admission for the period studied ($p=0.001$, 95%CI 1.568 to 5.679).

The higher number of Chinese patients with colorectal carcinoma may be explained by their relatively urbanised lifestyle and westernised diet compared to Malays or Indians in the country. Chinese people settled in urbanised regions and lead a more modern Westernised type of lifestyle than other populations of Malaysia. They are concentrated in higher economic and developed cities in Malaysia. Their relative financial stability also allowed them to afford richer meat content in their daily diet. This pattern of distribution of colorectal carcinomas was also seen globally where a rising incidence of colorectal cancers has been reported in populations or countries undergoing rapid economic development and a Westernised diet and lifestyle. (Parkin, Bray et al. 2005).

4.1.3: AGE AND GENDER

The incidence of colorectal carcinomas increased with age. Colorectal carcinomas are rare before the age of 40 years. The rise in colorectal cancer incidence per 100,000 population with age was seen all over the world, including in Malaysia. The Malaysian National Cancer Registry reported a sharp rise of colorectal carcinomas from the age of 50 years onwards (Figure 1.4, pg. 19). Males have a higher age-specific colon cancer incidence than females.

The census population of Peninsular Malaysia in the year 2006 was 24.8 million people with a proportion of 50.6% males to 49.4% females giving a male to female ratio of 1.02:1 (Department_of_Statistics_Malaysia 2007).

This study found more males than females with colorectal carcinomas with a male to female ratio of 1.26:1. In the group of Malay patients, the male to female ratio was 1.20:1 and this male predominance was even more obvious among Non-Malays with a ratio of 1.31:1. This was despite the fact that there were generally more females than males in the older age group in Malaysia (more than 65 years of age) (Figure 4.2).

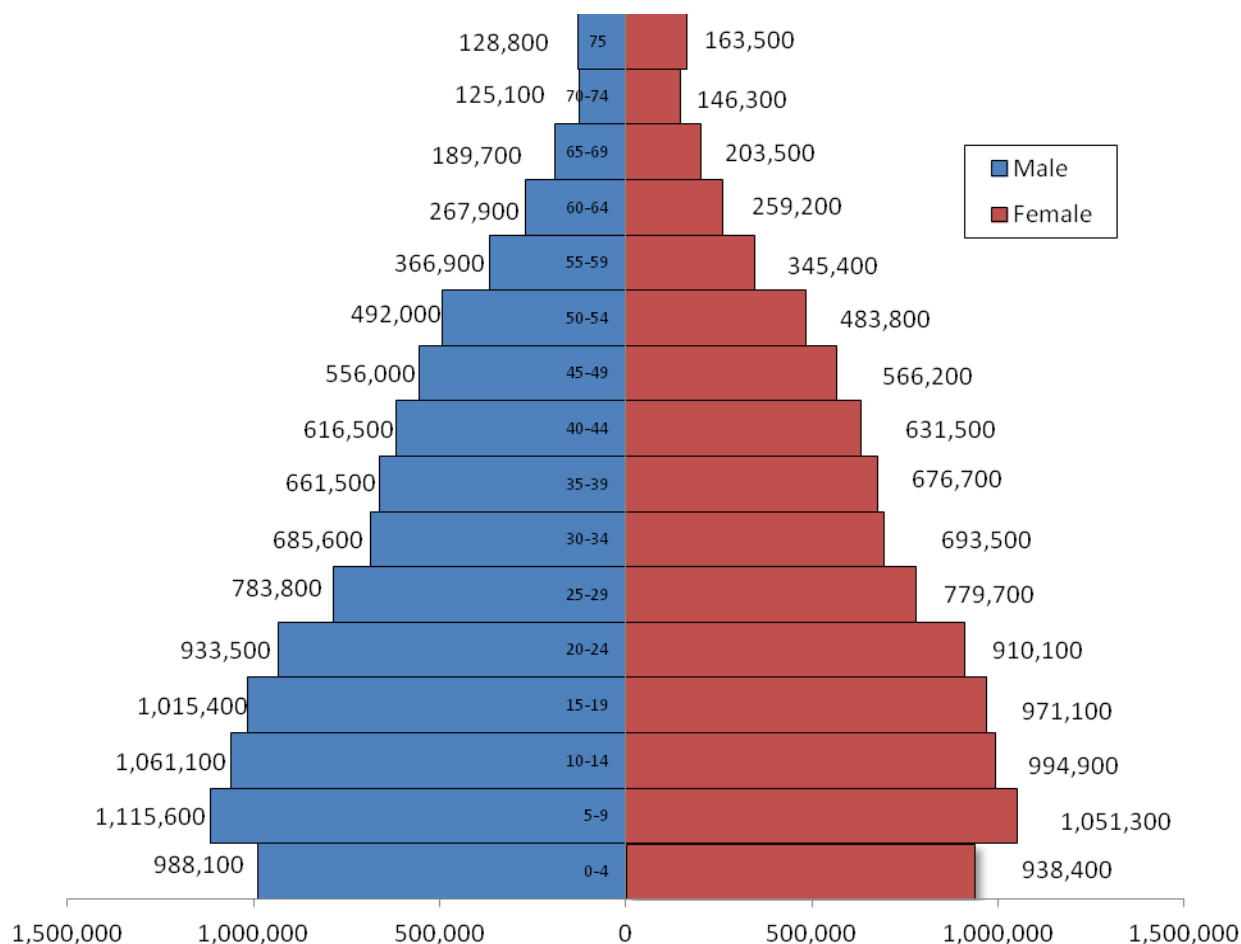


Figure 4.2: Population pyramid by age and gender, Malaysia, 2006.

Source: (National_Cancer_Registry 2006).

Patients with colorectal carcinomas presented at an older age. In this study the mean age of 61.0 years and median age of 62.0 years at presentation was found in the older age group but was much younger than what was reported from the United States Surveillance, Epidemiology, and End Results (SEER) study (Thomas and Sobin 1995) of American patients with colorectal cancer. The median age of presentation for colorectal cancer in their report was 70 years of age. This was a much older age of presentation than that found in this study. The reasons for this discrepancy were not immediately apparent but it could be postulated that the United States is a more developed country with better socio-economic status and better health facilities, thus giving rise to greater survival into old age who may suffer from colorectal carcinomas. This may lead to a higher median age of presentation for colorectal carcinomas

4.1.4: ANATOMICAL LOCATION OF TUMOURS WITH RACE AND AGE

The United States Surveillance, Epidemiology, and End Results (SEER) study [Appendix 7] between the years of 1975 to 1994 showed that colorectal cancers mainly arise in the proximal colon (38.8%) and the remaining 61.2% divided almost equally in the distal colon (29.6%) and rectum (28.5%) (Troisi, Freedman et al. 1998). Since 1980s, there had been a trend showing increasing incidence of right sided colon cancers and a decreasing incidence of left sided colon cancers. This change may reflect the increasing use of flexible colonoscopy and faecal occult blood test screening as well as an increasing population of elderly people. The incidence of right sided colon cancers increased with the age of the patients especially in women (Troisi, Freedman et al. 1998).

During embryologic development the right colon arises from the midgut while the left colon arises from the hindgut. The right and left colons are exposed to different luminal contents of faecal matter that include free oxygen radicals and chemicals broken from food products that are potentially carcinogenic. The left colon as compared to the right colon maybe exposed to more carcinogens that are implicated in carcinogenesis such as heterocyclic amines (breakdown from cooked meat) and reactive oxygens. In addition, the right and left colons have different blood supplies. The right colon (proximal to the splenic flexure) is supplied by the superior mesenteric artery and the left colon by the inferior mesenteric artery. Meguid et al (Meguid, Slidell et al. 2008) proposed that the risks of developing cancer differed for the right and left sided cancers because of the different courses of embryologic development and the exposure to different luminal contents and different blood supplies of the right and left colon.

The site of colon cancers reflects the differences in risk factors, gender and racial differences and the patient's age. It had been noted that in low risk areas or countries such as the African countries; carcinomas localised to the right side while carcinomas of the distal colon were more frequently seen in the high risk countries such as Japan, Australia and the United States (Fenoglio-Preiser, Perzin et al. 1990).

Similar to the epidemiology in United States, colorectal carcinomas in Malaysian patients had also been also noted to localise to the left side. Similarly in this study, the colorectal carcinomas were predominantly found on the left side of colon (69.5%).

In this study, patients of all races showed similar predilection to the left side. However, the Surveillance, Epidemiology and End Results (SEER) program started in 1973 by the National Cancer Institute in the United States found that there were differences in

occurrences of colorectal carcinomas among the Caucasian and African-American patients in the United States when separately assessed by gender and anatomic location. In their study, the proximal colon was defined to include the caecum, ascending, transverse and descending colon while the distal colon started from the sigmoid and included the rectum. They found that the African-American males and females had higher rates of proximal colon carcinomas compared to Caucasian males and females. On the other hand Caucasian males demonstrated higher rates for distal colorectal carcinomas than the African-American males. No reason was apparent from the differences in race and gender to the location of the tumour (Nelsonn, Persky et al. 1999).

However, our study did not reveal the differences as reported by SEER. Most of the tumours (69.5%) were on the left side. No ethnic group had any specific anatomical location of tumour. 70.1% of Chinese patients, 70.5% of Malay patients and 60.0% of Indians or other races had colorectal carcinomas on the left side of colon.

In the United States Surveillance, Epidemiology, and End Results (SEER) (Thomas and Sobin 1995) for the years 1973 to 1987 the age of presentation for patients with right sided tumours also differed from those with left sided tumours. They reported that the median age of presentation for patients with colorectal carcinomas on the right side was older than the left side. Adenocarcinomas on the right side presented at median age of 72 years as compared to the left sided at 69 years. On the other hand, our study found the converse to be true. Patients with right sided tumours presented at a non-significantly earlier age than those with left sided tumours (mean of 59.2 years compared to 61.8 years, $p=0.120$). Some patients with tumours on the right side may be familial cases; which presented at an earlier age. The other reason for the difference in ages between this study in Malaysia and the

results reported from United States probably lie in the status of development of the countries. United States is a developed country where the population enjoy a better socio-economic status, including better health facilities and hence a larger proportion of the population surviving into old age as compared to developing countries including Malaysia. This would have led to higher numbers of older surviving people in the United States who may go on to develop colorectal carcinomas.

4.2: MMR-d COLORECTAL CARCINOMAS

Colorectal carcinomas arise through several pathways, one of which is defect in the mismatch repair gene. The genes for mismatch repair are hMLH1, hMSH2, hMSH6, hMSH3, hPMS1 and hPMS2. Microsatellite instability (MSI) can arise because of germline mutation of one of the alleles followed by somatic mutation or from methylation followed by inactivation of the promoter gene hMLH1. High levels of defective mutations in these genes can lead to colorectal carcinomas.

4.2.1: IMMUNOHISTOCHEMICAL TESTING VS. PCR TESTING

The main aim of this study was to determine the pattern of mismatch repair defects in colorectal carcinomas in Malaysian patients by studying unselected consecutive colorectal carcinomas and correlating to their clinicopathological features and survival.

Immunohistochemistry (IHC) was used to evaluate the mismatch repair status of the tumours rather than using the polymerase chain reaction (PCR) and gel electrophoresis to examine the DNA sequences; as the latter method was an expensive and time consuming test which was not readily available in most laboratories in Malaysia. Previous studies (Dietmaier, Wallinger et al. 1997; Cawkwell, Gray et al. 1999; Dieumegard, Grandjouan et al. 2000; Stone, Robertson et al. 2001; Lindor, Burgart et al. 2002; Valentini, Armentano et al. 2006) had found the use of IHC to be a good alternative and highly specific to assess the status of mismatch repair in these tumours. Lindor et al (Lindor, Burgart et al. 2002) tested over 1000 colorectal cancers for DNA mismatch repair deficiency with both methods namely PCR and IHC detection for hMLH1 and hMSH2. Their study showed that IHC was 92.3% sensitive and 100% specific for screening DNA mismatch repair defects. The

predictive value of IHC for microsatellite stable or low-level microsatellite instability (MSI-L) was 96.7% and the predictive value of abnormal IHC staining was 100% for a high frequency microsatellite instability (MSI-H) gene. IHC distinguished MSI-H from MSI-L and MSS but it did not distinguish MSI-L from MSS. However, an advantage IHC had over PCR testing was it was able to suggest which gene was defective. This was not possible with PCR testing for microsatellite stability.

Another advantage of IHC over PCR testing was the cost. Debniak et al (Debniak, Kurzawski et al. 2000) estimated that immunohistochemistry cost less than a quarter of the price of MSI testing done with the PCR and gel electrophoresis method. IHC is easy to perform and requires minimal expertise. The technique is readily available in many laboratories, hence IHC for mismatch repair protein could be introduced and be potentially included as a routine test in the histopathology report for all colorectal carcinomas fairly easily. This would delineate the subset of patients who require further genetic testing and also possible screening of family members for carriers. As these patients have an increased risk for multiplicity of tumours (metachronous or synchronous cancers) they would also require closer and longer term follow-up. It had also been found that tumours with microsatellite instability or defective mismatch repair genes respond differently to various chemotherapeutic agents including 5-fluorouracil and irinotecan (Ribic, Sargent et al. 2003; Carethers, Smith et al. 2004; Bertagnolli, Niedzwiecki et al. 2009; Jover, Zapater et al. 2009). Hence the mismatch repair status of the tumour would help guide the type of chemotherapy given to this group of patients.

FALSE NEGATIVE STAINING

False negative staining with any of the mismatch repair proteins could occur during the process of immunohistochemistry testing. Edmonston et al (Edmonston, Cuesta et al. 2000) suggested that weak staining was most probably associated with problems in fixation, especially when both hMLH1 and hMSH2 stains were affected. Formalin fixation of more than 24 hours had also been shown to result in a significantly decreased stain, especially for hMLH1 (Monzon, Kovatich et al. 1999).

Others (Marcus, Madlensky et al. 1999) found no alteration of immunohistochemistry staining for hMLH1 and hMSH2 when they used archived unstained sialinated slides kept between 1 to 2 years of age compared to those that were freshly sectioned. Most surgical specimens received in our laboratory were processed between 24 to 48 hours of fixation. We did not encounter any problems with immunohistochemistry staining for other markers. Moreover, we used freshly prepared tissue sections in this study (less than 12 hours after sectioning) for immunohistochemistry staining to avoid any problems in loss of staining due to oxidation. Using these methods, we minimised the likelihood for errors/false negatives during the immunohistochemistry staining process.

The monoclonal antibody hMLH1 was very sensitive to over-fixation of tissue and preparation differences practised in different institutes. In the process of immunohistochemistry staining done in this study; precautions were taken and procedures were manually carried out. Hence over-staining was not seen in any of our cases, even for hMLH1 stains.

4.2.2: INCIDENCE OF MISMATCH REPAIR DEFECT TUMOURS

Of the 298 cases of colorectal carcinomas in our series, there were 43 cases (14.4%) with loss of one or more mismatch repair proteins. These results were comparable with those published previously in other countries, in which the proportion of colorectal carcinomas with mismatch repair defects ranged from 7 to 20% (Ionov, Peinado et al. 1993; Cunningham, Kim et al. 2001; Percesepe, Borghi et al. 2001; Chapusot, Martin et al. 2003; Wright and Stewart 2003; Jover, Paya et al. 2004; Lin, Lin et al. 2011).

TYPE OF MMR PROTEIN LOSS

Mismatch repair defects in colon cancers were commonly reported to be hMLH1, hMSH2 or hMSH6 defects. hMSH2 defects usually resulted from germline mutations (Mangold, Pagenstecher et al. 2005) while hMLH1 could be due to germline or somatic hypermethylation of its promoter (Cunningham, Christensen et al. 1998; Thibodeau, French et al. 1998). Similarly, Asian investigators (Lin, Lin et al. 2011) found that the majority of their sporadic CRC tumours showed loss of hMLH1 protein expression followed by loss of hMSH2 protein. In sporadic colorectal carcinomas, the predominant loss was reported to be hMLH1 as a result of promoter methylation (Herman, Umar et al. 1998; Lin, Lin et al. 2011) while hereditary non-polyposis colorectal cancers could arise from either hMLH1, hMSH2 germline mutations (Liu, Parsons et al. 1996; Peltomaki and Vasen 1997) or hMLH1 promoter region methylation (Wheeler, Loukola et al. 2000; Potocnik, Glavac et al. 2001; Yamamoto, Min et al. 2002). Wu et al (Wu, Berends et al. 1999) reported that a small minority of HNPCC cases could be caused by defects in hPMS2 or hMSH6 that could have resulted from germline mutations. It was beneficial to distinguish familial from sporadic

colon cancers because management for the patients and their family members differed between the two groups. However, immunohistochemistry testing that helped delineate the group of tumours with mismatch repair defect did not differentiate somatic from germline mutation. Further testing that included BRAF mutation assays and test for methylation of hMLH1 could be done to distinguish sporadic colon cancer from familial CRC (Lynch syndrome). When the tumour was found to be hMLH1/hPMS2 defective, BRAF mutation analysis or test for methylation could be done (Sharma and Gulley 2010; Geiersbach and Samowitz 2011). BRAF mutation was found in nearly 91% of sporadic CRCs with MSI-H (Davies, Bignell et al. 2002) but not in CRCs of patients with Lynch syndrome (Ikenoue, Hikiba et al. 2003; French, Sargent et al. 2008). Methylation testing was an alternative test but was a more technically challenging test than BRAF mutation assay. hMLH1 methylation was typically found in sporadic CRCs lacking hMLH1 expression and was only found in 1.6% of CRCs in patients with Lynch syndrome (Farina-Sarasqueta, van Lijnschoten et al. 2010).

A large majority of MMR-d tumours in many published reports (Stone, Robertson et al. 2001; Ward, Meagher et al. 2001; Lindor, Burgart et al. 2002; Chapusot, Martin et al. 2003; Valentini, Armentano et al. 2006) was due to loss of either hMLH1 or hMSH2 proteins.

In this study, our predominant mismatch repair gene loss was hMLH1. Twenty eight out of 43 cases or two thirds of the MMR-d CRCs were deficient for hMLH1, and 15 cases (or one third of the cases) showed either loss of hMSH2 or hMSH6 or both. One of the cases with hMLH1 was a HNPCC (case 11, Table 3.9) while another case (case 8, Table 3.9) was highly suspected of HNPCC. Her tumour had mismatch repair defect for hMSH2 and

hMSH6. The remaining cases with MMR-d tumours had no family history and were considered sporadic carcinomas.

In humans, the hMSH2 gene is located on chromosome 2p21 which was initially identified as an area for the gene involved in hereditary non-polyposis colorectal cancer. The hMLH1 gene is located on chromosome 3p21 to 3p23, an area which has also been identified as an important candidate region within large HNPCC families that were not linked to 2p21 to 2p22 (Lindblom, Tannergard et al. 1993) .

Our study found that the majority of the MMR-d tumours were defective for hMLH1 (28 out of 43 MMR-d tumours, 65.1% or 9.4% of all colorectal cases) and to a much lesser extent hMSH2 or hMSH6. However, there was no case with loss of all three proteins namely hMLH1, hMSH2 and hMSH6. Similarly, previous studies (Kakar, Burgart et al. 2003; Wright and Stewart 2003; Chai, Zeps et al. 2004) reported the rate of loss of hMLH1 in colorectal cancer to be between 8.0% and 18.6% while the rate of hMSH2 loss was between 1.0% and 2.1%.

HETERODIMERS OF MMR GENES

MMR proteins interacted in the form of heterodimers; commonly hMSH2 and hMSH6; and hMLH1 and hPMS2 (Kolodner and Marsischky 1999). Thus mutations of hMLH1 may entail concurrent loss of protein of hPMS2 and similarly hMSH2 with loss of hMSH6. This occurred through degeneration of the corresponding heterodimerizing protein partner (Wu, Berends et al. 1999; Young, Simms et al. 2001). In their study of 214 colorectal carcinomas examined for MMR protein expression (hMSH1, hMSH2, hMSH6 and PMS2) Rigau et al (Rigau, Sebbagh et al. 2003) found that there were only two combinations possible when there were loss of two proteins, namely hMLH1/hPMS2 and hMSH2/hMSH6. Other

studies found that besides hPMS2, hMLH1 could also form heterodimers with hMLH3 or hPMS1. In their study of sporadic MSI-H tumours and HNPCC, Young et al (Young, Simms et al. 2001) found that all tumours lacking hMLH1 showed absence of hPMS2 as well. The close relationship between hMLH1 and hPMS2 had also been reported by Leung et al and Ma et al (Leung, Kim et al. 2000; Ma, Xia et al. 2000). This suggested that hPMS2 may be degraded in the absence of its binding partner, hMLH1.

On the other hand, hMSH2 commonly formed heterodimers with both hMSH6 and hMSH3 as well. Young et al reported that many tumours lacking hMSH2 were also not staining up for hMSH6 (Young, Simms et al. 2001).

In this study, 7 out of 9 cases with loss of hMSH2 were defective for hMSH6 but none of the cases with loss of hMLH1 were found to be defective for hMSH2 or hMSH6. There was also no loss of all three proteins in any of the cases. hPMS2 was not tested in this study. The results of this study supported the proposal that hMSH2 could form heterodimers with hMSH6 thus forming loss of two proteins namely hMSH2/hMSH6 but hMLH1 lacked any such relationship with hMSH2 or hMSH6.

4.2.3: DEMOGRAPHICS OF MMR-d TUMOURS

4.2.3.1: AGE

Although the diagnosis of colorectal cancer at a younger age was frequent in HNPCC, most studies found that sporadic mismatch repair defect tumours were seen in all ages. Most studies did not report any significant correlation between patients with mismatch repair defect tumours and patients with intact tumours with regards to their mean presenting age. In this study, patients with MMR-d tumours presented at a slightly younger age (mean age

=58.9 \pm 2.2 years) than the patients with non-MMR-d colorectal carcinomas (mean age = 61.4 \pm 0.8 years), which was not statistically significant ($p=0.292$). The absence of mismatch repair protein in these patients was a sporadic event and thus the events leading to tumorigenesis would take more time than it would have if it were an inherited genetic defect like in HNPCC. Hence we would expect the age of presentation for these patients with MMR-d tumours to be as old as other cases of sporadic colorectal carcinomas without any loss of the mismatch repair protein. Hence, the results of our study were not unexpected. It was noted two cases of probable HNPCC (cases no. 8 and 11, Table 3.9) who both had MMR-d tumours presented in their mid forties. They were included into the group of MMR-d tumours and this might explain the slightly lowered age of presentation of the group with MMR-d tumours.

However, there were a few studies (Messerini, Vitelli et al. 1997; Molaei, Mansoori et al. 2010), which also found that patients with MMR-d tumours presented at an earlier age when compared to the patients with microsatellite stable tumours. Messerini et al studied sporadic mucinous and non-mucinous colorectal carcinomas and found that the mean age of patients with microsatellite instability was younger at 56.6 years of age when compared to their microsatellite stable cases with the mean age of 65.0 years. This was even more striking for the studies conducted by Molaei et al. They found the mean age of presentation for patients with mismatch repair defect was very much younger at 42.8 years as compared to 53.0 years for those with no mismatch repair protein defect. They reported that patients had an odds ratio of 5.95 (95% CI 2.69-13.18) of presenting at an age younger than 50 years among tumours with mismatch repair defect ($p<0.001$). It was not certain whether they had excluded the cases with HNPCC.

Similarly there were a few studies looking at the characteristics of patients selected solely on the basis of early age of onset. These studies demonstrated that there was a trend towards a higher pathogenetic mutation detection rate when the patients were diagnosed at a younger age (Table 4.1). There was a higher percentage of cases with mismatch repair protein when the age of presentation was younger. Liu et al (Liu, Farrington et al. 1995) studied 31 cases of colorectal carcinoma presenting before 35 years of age who did not have any family history fulfilling the Amsterdam criteria II and found 58% of the patients' tumours had microsatellite instability.

Table 4.1 : Association between age at onset of colorectal cancer and mismatch repair gene mutations.

Age range (years)	No. of index cases	hMLH1 mutation carrier:		hMSH2 mutation carrier:		Published references
		No. of cases	%	No. of cases	%	
<30	50	7	14	7	14	Farrington et al (Farrington, Lin-Goerke et al. 1998)
<40	12	1	8.3	1	8.3	Syngal et al (Syngal, Fox et al. 1999)
<45	38	1	2.6	2	5.3	Fornasarig et al (Fornasarig, Viel et al. 2000)

The results of our study showed that the incidence rate of mismatch repair defects occurring in colorectal cancer decreased with age of presentation (Table 3.8). There was a higher number of cases of sporadic mismatch repair defect tumours in patients presenting at a younger age compared to older patients. MMR defect-associated colon cancers occurred

frequently in early-age onset patients with or without a family history of cancer. This suggested that genetic defects namely loss of mismatch repair protein would have occurred early in the multi-step process of tumorigenesis.

4.2.3.2: RACE

Although Chinese patients were the predominant racial group with colorectal carcinomas in our study, it was found that Malay patients had the highest proportion of colorectal carcinomas with mismatch repair defects. However there was no significant difference between the major ethnic races (Malays vs. Non- Malays ($p=0.122$) and Chinese vs. Non-Chinese ($p=0.497$) for colorectal carcinomas with mismatch repair defect.

In one other study in Malaysia, Tan et al (Tan, Ng et al. 2007) studied Chinese and non-Chinese patients and found no difference in the rates of MMR-d tumours and intact tumours between the racial groups.

Similarly, most published reports suggested no differences between ethnic groups for patients with MMR-d tumours and microsatellite stable tumours. Two studies (Carethers, Smith et al. 2004; Hatch, Lightfoot et al. 2005) looking at Caucasian and African-American patients with colorectal carcinoma found no difference in frequencies in the rate of mismatch repair defects between the two racial groups. However, one report (Ashktorab, Smoot et al. 2003) found that African-American patients had a significantly higher frequency of MSI tumours than Caucasian patients.

Although the incidence rates of micro-satellite tumours did not differ for various racial groups in the same region in most reports, interestingly incidence rates of MSI-tumours had been noted to differ from country to country or region to region. Microsatellite instability

had been reported all over to occur in 7 to 20% of colon tumours (Ionov, Peinado et al. 1993; Cunningham, Kim et al. 2001; Chapusot, Martin et al. 2003; Wright and Stewart 2003). However studies done in Mediterranean region found a lower rate of 7 to 8% (Percesepe, Borghi et al. 2001; Jover, Paya et al. 2004). In a recent study in Taiwan the investigators (Lin, Lin et al. 2011) found only 6.4% of the CRC tumours to have high frequency MSI (MSI-H). This was a large scale sporadic CRC study done in an Asian population that included 1,173 sporadic CRC tumours. On the other hand, in Iran, the rate of MSI-tumours was almost double this figure: Molaei et al (Molaei, Mansoori et al. 2010) reported it to be as high as 14%.

It had been proposed that dietary, toxic or other environmental factors could be causes of epigenetic disruption of hMLH1 (such as promoting hypermethylation of the gene) in a particular population. Red meat ingestion, higher frequency of using different cooking practices that increased intake of heterocyclic amines like frying, barbequing or boiling (Wu, Shibata et al. 2001), as well as high consumption of high-grade alcoholic beverages (Slattery, Anderson et al. 2001) were possible risk factors as these were frequently practised in countries with high MSI incidence rates.

In their extensive review on mismatch repair genes and colorectal cancer, Mitchell et al (Mitchell, Farrington et al. 2002) found little ethnic or population variations from the available gene variant data. However, they cautioned a need for accurate and extensive population-based review of data before population differences in the spectrum and frequency of mismatch repair gene variants would become apparent. Perhaps the differences in incidence rates between different populations may be due to their different lifestyle as mentioned above and to a lesser extent the inherent genetic properties.

4.2.3.3: GENDER

Although there was no significant difference of MMR-d tumours for gender or race, our study found a significant Malay predilection amongst the female patients for MMR-d tumours (p value =0.031). This was not seen among male patients in our study. It was interesting to note that Malay females instead of non-Malay females were significantly associated with MMR-d tumours. The reason for this race selection only occurring in females of this ethnic group (Malay) was not apparent in this study. Perhaps a more detailed analysis and comparison of the cultural and social habits of Malay females with other racial groups in the country could be undertaken in the future to explore this finding. On the other hand, we did not find any statistical difference for gender predilection for tumours with mismatch repair defect ($p=0.497$). Male and female patients were equally likely to develop MMR-d tumours. However, many other studies (Ionov, Peinado et al. 1993; Thibodeau, French et al. 1998; Ward, Meagher et al. 2001; Wright and Stewart 2003; Chai, Zeps et al. 2004) found a strong association of tumours with mismatch repair gene defect for females. They found gender differences in the colorectal tumours with mismatch repair defects; with females more likely to develop colorectal cancers with mismatch repair defects. This had been directly attributed to the effects of oestrogens. The effect of oestrogens in females was studied by Slattery et al (Slattery, Potter et al. 2001) who showed that the risk of MSI-phenotype colon cancer was actually reduced by presence of oestrogens but increased when there was withdrawal of oestrogens. They found a reduction of risk of MMR-d in tumours when the women were pregnant or were on oral contraceptives. Similarly, use of hormone replacement therapy (HRT) resulted in a lowered

risk of MSI phenotype tumours but on the other hand former users of HRT had an elevated risk of MSI tumours after ceasing HRT. This was also noted in their earlier papers (Kampman, Potter et al. 1997). Oestrogenic exposure in women thus protected them from MSI tumours but oestrogen withdrawal in post-menopausal women increased the risk to this gene defect. It was found that women were more likely to have MSI phenotype CRC as they aged as compared to men. On the other hand, women at a younger age were less likely than younger men to have these tumours because of the presence of oestrogens.

Breivik et al (Breivik, Lothe et al. 1997) proposed that oestrogens could inhibit the pathway to colon carcinoma involving the mismatch repair gene defect when they observed that younger women had a lowered prevalence of MSI colon cancers than older women. They noted as well as others (Gaglia, Atkin et al. 1995) that women with inherited colon cancer syndromes such as HNPCC had reduced risk (by approximately 50%) of developing colorectal adenomas as compared to their male relatives.

The effects of oestrogens are linked to the oestrogen receptors in the colon. Issa et al (Issa, Ottaviano et al. 1994) showed that methylation associated inactivation of oestrogen receptors in the colon was associated with aging and that colon tumours arose from cells that had lost or reduced oestrogen receptors. Decreased circulating oestrogens will lead to reduced oestrogen receptors and this will be associated with microsatellite instability colon cancers. On the other hand, the presence of circulating oestrogen in premenopausal women or those on HRT or obese women reduced the risk of losing these oestrogen receptors and thus reduced the risk of colon tumours (Figure 4.3).

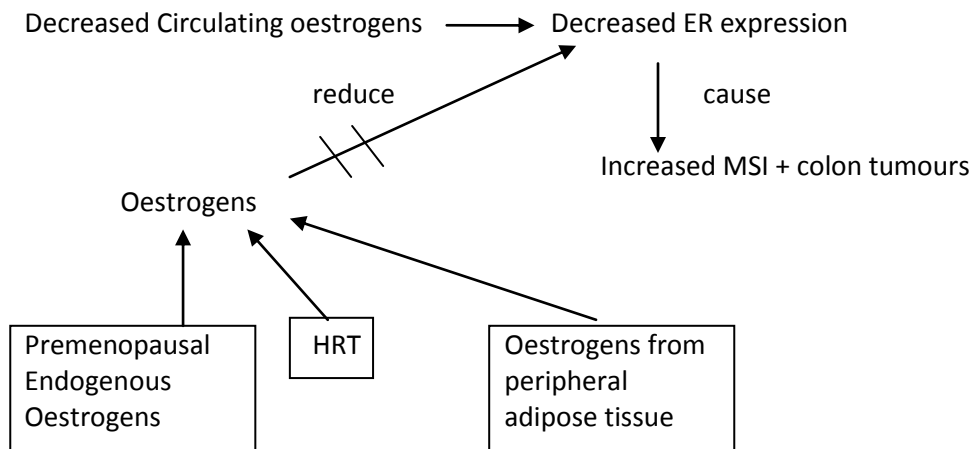


Figure 4.3: Oestrogens, oestrogen receptors and prevention of colon tumours

Source: (Slattery, Potter et al. 2001)

It was uncertain why or how oestrogen levels and oestrogen receptors were associated with MSI tumours. The roles of endogenous (reproductive status), exogenous (HRT) and metabolic (obesity-associated) oestrogens in preventing ER methylation and thus MMR genes were unclear. There had been several hypotheses about this. Slattery et al (Slattery, Potter et al. 2001) hypothesised that at least one major mismatch repair gene may be oestrogen responsive and thus loss of oestrogen could result in loss of DNA mismatch repair capacity.

Whatever the possible explanations may be, the data observed in these studies (Issa, Ottaviano et al. 1994; Gaglia, Atkin et al. 1995; Breivik, Lothe et al. 1997; Kampman, Potter et al. 1997; Slattery, Potter et al. 2001) supported the finding that hormones play an important aetiological role in colon cancer via the MSI related pathway and that oestrogens prevented MSI tumours whether endogenous, exogenous or obesity associated.

The excess of microsatellite instability colon cancers in women were explained by the excess of these tumours at an older age when there was a reduction or withdrawal of oestrogens at the time when these women became post-menopausal. In fact, there were fewer MSI phenotype tumours in pre-menopausal young women than young men.

4.2.4: FAMILY HISTORY

Aside from genetic mutations seen in familial type of colon cancers, there was evidence that people with close relatives who had colorectal carcinomas had an increased risk of colon cancers. First-degree family members of patients (parent, sibling or child) with colorectal carcinomas without any evidence of the inherited cancer syndromes namely HNPCC or FAP had approximately twice the risk of getting colon cancers compared to those without a family history (Fuchs, Giovannucci et al. 1994). They were also more likely to develop the disease at a younger age than people without family history of colon cancers. Worldwide, about 15-20% of patients with colorectal cancers had a positive family history (Lynch and Smyrk 1996; Aaltonen, Salovaara et al. 1998).

In this study, there were 25 patients out of 160 patients (15.6%) who gave positive family history of previous carcinomas in their immediate family members. This was consistent with what was found worldwide.

However, there was no statistical difference between patients with and without family history in terms of the MMR staining status of their tumours ($p=1.00$). There were only two patients with MMR-d tumours out of 25 patients (8%) who had family members with malignancies as compared to 15 patients with MMR-d tumours out of 135 patients (11.1%) who had no family history.

One of the two patients with MMR-d tumours who had a family history of cancer (case no. 11, Table 3.9) fulfilled the revised diagnostic criteria for HNPCC (Amsterdam Criteria II, Appendix I). Three relatives including the patient herself had colon carcinomas. Another fourth relative, her sibling (brother) suffered a non-HNPCC related carcinoma: gastric carcinoma. There were two generations involved: patient herself and siblings and their father. Furthermore, she presented with colon carcinoma at age of 46 years. Her tumour was found on the left side and showed loss of mismatch repair protein for hMLH1.

The other patient (case no. 8, Table 3.9) was a suspected familial HNPCC who did not meet the full criteria for diagnosis of hereditary non-polyposis colorectal cancer by Amsterdam Criteria II. This patient and two other siblings had colon carcinomas. There was no history of two consecutive generations that were affected by any HNPCC-related carcinomas but she had presented at an early age of 45 years. This patient's tumour was localised to right side and had MMR-d of hMLSH2 and hMSH6. The patient was unsure of the cause of death of her parents and could not say whether they had any malignancies in their lifetimes.

One more patient (case no. 15) in the list of patients with family histories appeared to have fulfilled the criteria for HNPCC using the Amsterdam Criteria II. He had three first degree relatives suffering colon carcinomas: his two siblings and father. He presented at an early age of 38 years old. His tumour was localised to the right side. However, his tumour was found to be intact tumour with no mismatch repair defect by immunohistochemical methods. It would be beneficial in this case to counter check this by doing a molecular test of mismatch repair gene by PCR and gel electrophoresis methods. However, our laboratory did not run the molecular test and it was not outsourced elsewhere due to the cost it might incur. Another possibility was that it may be a true case of HNPCC microsatellite stable

tumour as it was known there was a small proportion of patients with HNPCC whose tumours were microsatellite stable.

Hereditary non-polyposis colorectal carcinoma (HNPCC) or Lynch syndrome is an autosomal dominant disorder also associated with defects in mismatch repair genes leading to microsatellite instability. It accounts for 5% of all colon cancers in the Western population. Patients with this autosomal disorder may have colorectal carcinomas, endometrial carcinomas and cancers of the small intestine, ureter or renal pelvis. It is an early-onset inheritable malignancy and characterised by a few clinicopathological characteristics. HNPCC usually presents before 50 years of age.

In the most recently revised diagnostic criteria used for hereditary non-polyposis colorectal carcinoma: the Amsterdam Criteria II, there must be at least three relatives with HNPCC-associated cancer to diagnose HNPCC in a patient. This may include colorectal cancer, cancer of endometrium, small bowel, ureter or renal pelvis and at least two successive generations affected with at least one patient diagnosed before the age of 50 years and one of whom should be a first-degree relative of the other two. Familial adenomatous polyposis should be excluded and the tumours should be verified by histopathological examination (Vasen, Watson et al. 1999).

HNPCC predominantly localizes to the right side and has a decreased risk of developing rectal cancer. It was found that rectal tumours had the lowest rate (1.2 to 4.7%) of loss of expression of mismatch repair proteins (Chapusot, Martin et al. 2003; Wright and Stewart 2003). Of these MMR-d rectal cancers, only a small proportion (1/7) was from HNPCC patients (Wright and Stewart 2003).

There may be some adenomas present in HNPCC patients but they usually are not as numerous as those seen in familial adenomatous polyposis (FAP). Patients are frequently seen with development of multiple tumours (metachronous or synchronous) with an increased risk of developing extra-intestinal tumours. The colorectal carcinomas are usually poorer differentiated, with increased production of mucin associated with marked host lymphocytic infiltration in the form of peri-tumoural lymphocytic response or Crohn-like lymphocytic response. HNPCC is associated with improved survival compared to non-HNPCC colorectal cancer.

In many ways, the pathology of HNPCC tumours is similar with that of sporadic colorectal carcinomas with microsatellite instability. Some studies showed no distinction between familial and non-familial sporadic MSI carcinomas.

In our study of the 43 patients with tumours showing MMR-protein defect, there were only 2 patients with suspected or diagnosed HNPCC, by Amsterdam Criteria II. More than 95% of cases (41/43 patients) were not HNPCC but were found to have mismatch repair defect tumours. This supported the fact that MMR gene alterations associated colorectal carcinomas were not restricted to HNPCC or highly selected families with colorectal carcinomas but could also be found in sporadic colorectal carcinomas.

From a total of 298 patients, only 160 patients (53.7%) could give a full family history and this was only about half the number of cases studied. Forty patients were lost to follow-up and another 98 patients had passed away. Attempts made to contact the relatives of these patients who had passed away for more information were futile. This was mostly due to change of address or contact. Some of the relatives whom we were able to contact could not

recall whether their elderly relatives who had passed away had any immediate family members with malignancies.

In this present era, people migrate and readily move across long distance to look for work and settle down far away from their original hometown, breaking family ties and communication frequently. Families no longer live in same village or town but are spread across the globe. It is thus difficult to trace family trees and keep up with news of family members who have migrated off to distant places. Thus proper family history is getting difficult to obtain nowadays. Families also have become smaller in numbers and thus family history plays a less important role nowadays.

In our study, 25 patients gave strong family history of malignancies in their immediate family members. Twelve of them or about half were with family history of colorectal carcinomas. Nine patients had two or more family members with malignancies, two of whom had MMR-d tumours. One of the patients had 3 siblings with malignancies of different sites including colorectal carcinoma (case no. 5, Table 3.9) while another two patients had 3 family members (each), all with gastrointestinal malignancies (cases nos. 11, 15, Table 3.9). These two patients had very strong family history and the malignancies were similar to the patients. They were also very young (46 years old and 38 years old) when they presented with colorectal carcinomas. One of them (case no.11, Table 3.9) had hMLH1 defect. The number of patients whom we could get family history was small in our study and we could not prove any significant association of family history with mismatch repair defect tumours.

However, some published reports found that patients with mismatch repair defect were more likely to have a positive family history (Lothe, Peltomaki et al. 1993; Thibodeau,

Bren et al. 1993; Molaie, Mansoori et al. 2010) than patients with microsatellite stable tumours. Molaie et al in Iran had found a positive independent association between patients with MMR-d tumours and a positive family history of malignancies. In Iran, there was a high familial trend of between 29.4 to 35.1% of patients with colorectal cancers (Mahdavinia, Bishehsari et al. 2005; Azadeh, Moghimi-Dehkordi et al. 2008). Molaie's study showed 46.0% of patients with colorectal cancers had positive family history of cancers and almost half of these were with family history of colorectal carcinomas. This suggested a high familial inheritance of colorectal carcinomas in their country which was not seen in our study in Malaysia. They also found a correlation between a positive family history and mismatch repair defect tumours. However, they could not find a significant relationship between early onset of colon carcinomas (before age of 50) and positive family history.

Many patients with colorectal carcinomas having family members with malignancies did not fulfil any of the familial syndromes (FAP, HNPCC or rarer syndromes) nor have any mutation defect noted in their tumours. This familial cluster of malignancies may be pure coincidental occurrence or due to other germline mutations.

However, germline mutation was strongly suspected when there was high frequency of the same tumour or associated tumours (like endometrial and colorectal cancers) in a family or when the age of presentation was early in onset and there were multiple tumours (metachronous or synchronous) in a patient.

It was in fact noted by Campbell and Dunlop (Campbell, Spence et al. 1994; Dunlop, Farrington et al. 1997) that approximately 1% of the entire population had either two first-degree relatives affected by colon cancers or one relative affected at or younger than 45

years of age. This suggested a genetic mutation causing or bringing about tumorigenesis of colon cancers.

4.3: CHARACTER OF TUMOUR

4.3.1: MULTIPLE TUMOURS

Multiple neoplasms can be synchronous or metachronous. Synchronous tumours occurred twice as frequently as metachronous tumours in patients with colon cancers (Parkash 1977; Lasser 1978). Hereditary colorectal carcinoma was characterised by the development of multiple primaries. The presence of multiple primaries was used as one of the criteria for the diagnosis of hereditary non-polyposis colorectal carcinomas in the Bethesda Criteria (Appendix 1).

Synchronous tumours can be in the forms of adenomas and carcinomas. They are tumours occurring in a patient at different locations at the same time. Although synchronous neoplasms affected about 35.9% of the cases, some of these included adenomas (Slater, Aufses et al. 1990). Synchronous carcinomas were seen less frequently. Our study had included only synchronous and metachronous malignancies and excluded benign neoplasms like adenomas as explained in Chapter 3.4.1.

A proportion of patients with colorectal carcinomas had more than one primary cancer in the colon at the time of diagnosis (Slater, Aufses et al. 1990; Fante, Roncucci et al. 1996). The incidence rate in various studies was said to be about 1.5 to 12% (Parkash 1977; Lasser 1978; Langevin and Nivatvongs 1984; Slater, Aufses et al. 1990). Hemminki et al (Hemminki, Li et al. 2001) found that the relative risk was 2.2 for developing a second colorectal carcinoma in patients with synchronous carcinomas.

Metachronous tumours are secondary neoplasms occurring more than 6 months apart from the index cancer which must not be a metastatic lesion of the primary carcinoma.

According to Kiefer et al (Kiefer, Thorson et al. 1986) metachronous tumours occurred in 1.6% of patients with colon cancers.

Our study confirmed what others found (Cawkwell, Gray et al. 1999; Gryfe, Kim et al. 2000) that patients with MMR-d colorectal cancers were more prone to have multiple malignancies (synchronous or metachronous). Nine out of 34 patients (or 26.5%) who had multiple malignancies were with mismatch repair defect tumours as compared to 11.9% of patients with multiple malignancies who had non-MMR-d tumours ($p=0.032$). Patients with mismatch repair defect tumours had an odds ratio of 2.67 with a 95% confidence interval of 1.12 to 6.41 of developing multiple malignancies (synchronous or metachronous). Other studies (Sengupta, Yiu et al. 1997; Brown, Finan et al. 1998; Pedroni, Tamassia et al. 1999; Dykes, Qui et al. 2003; Lawes, Pearson et al. 2005) also found that the incidence of mismatch defect in multiple cancers was significantly higher than in single cancers. Patients with multiple tumours had a higher chance of having mismatch repair defect tumours and conversely patients with mismatch repair defect tumours were more likely to develop multiple tumours. This would be expected. Patients with the mismatch repair defect would be more prone to having multiple malignancies as the other tissue (in the gut as well as extra-intestinal) would be likely to have this genetic defect (mismatch repair defect) and as a result more prone to developing malignancies.

4.3.2: ANATOMICAL LOCATION

Most of the colorectal carcinomas were found in the left side of the colon. Sixty-nine point five percent of the cases were localised to the left side of colon in our study. On the other hand we showed a significant predilection of MMR-d colorectal carcinomas to the right side of the colon ($p<0.001$). Right sided tumours had an odds ratio of 4.47 with a 95% confidence interval of 2.28 to 8.76 of being mismatch repair deficient compared to left sided tumours.

Our results were similar and comparable to many other published reports (Feeley, Fullard et al. 1999; Gafa, Maestri et al. 2000; Chapusot, Martin et al. 2003; Wright and Stewart 2003; Lim, Jeong et al. 2004; Hameed, Goldberg et al. 2006; Molaei, Mansoori et al. 2010). In most studies, there was a significant predilection of MMR-defective tumours to the right side although majority of all colorectal carcinomas were predominantly found on the left side of the colon.

In one large series (Wright and Stewart 2003) it was found that not only were the MMR-d tumours located more on the right side but that the proportion of MMR-d tumours increased progressively along the proximal colon and abruptly declined after the splenic flexure where the demarcation of right and left colon lies. The reason for this was not apparent. Wright et al (Wright and Stewart 2003) found that the incidence of MMR-d tumours on the right side increased from caecum (32%) to ascending to transverse colon (41%) and then rapidly decreased on the left side from splenic flexure, reaching the lowest in the rectum (4.7%). In addition, Chapusot et al (Chapusot, Martin et al. 2003) also found that right sided location was a clinically useful positive predictor of mismatch repair status expression. It had a positive predictive value of 33%, second only to poor differentiation.

The negative predictive value was 97%. The strong association of right sided tumours with mismatch repair defect makes us more likely to perform immunohistochemical stains on right sided tumours to detect these tumours.

However, Wright (Wright and Stewart 2003) further found that hMSH2 defective tumours were similar to intact tumours in that they were more likely to be found on the left side. Wright et al and Kruschewski et al (Kruschewski, Noske et al. 2002) found that the predominant proportion of MMR-d tumours that were localised to the right side were hMLH1 defective tumours. Wright found 83% of hMLH1 defective tumours were right sided.

Our study did not find such an occurrence. We found instead that more hMSH2 or hMSH6 defective tumour cases were at the right side rather than hMLH1 defective tumours. Although Wright et al and others (Kruschewski, Noske et al. 2002; Wright and Stewart 2003) found that hMLH1 defective tumours were more likely than hMSH2 defective tumours to localise to the right side, other studies (Marcus, Madlensky et al. 1999) disputed these findings and found no difference to the type of mismatch repair protein defect in right and left colonic tumours.

4.3.3: GROWTH APPEARANCE

The appearance of colorectal carcinomas can be described as fungating or exophytic if their growth protrudes into the colonic lumen. The mass effect of tumour may cause obstruction to the luminal contents of the intestine. However, in the proximal colon, the contents may be fluid and can easily pass through the obstruction. Hence, the symptoms of obstruction are usually late in onset in the proximal tumours.

Colorectal carcinomas can also grow as ulcerative or endophytic lesions with intramural invasion. It may infiltrate the bowel wall and cause constrictive growth. If the tumour growth is annular it can cause obstruction to the contents of the intestine as well by circumferential constriction. It can infiltrate deeply into the bowel wall and cause bowel perforation and peritonitis. The ulceration is usually associated with raised, indurated and sometimes everted edges.

Tumours at the caecum and the ascending colon are usually large and exophytic tumours while those in the transverse and descending colons are usually endophytic. Fungating tumours may have papillary surface configuration. In a small proportion (~10%) of tumours there is secretion of mucin by tumour cells and this gives a mucoid appearance to some exophytic tumours located at the proximal colon.

Carcinomas in the distal colon on the other hand are more likely to be endophytic tumours. They can cause stenosis of the lumen and are called 'napkin-ring tumours'. At other times they are also known as 'string carcinomas' because of the effect they cause, which is similar to a string tied tightly around the bowel wall. They can produce constriction at the site of the tumour and dilatation proximal to the obstruction.

Most of the colorectal carcinomas (70.4%) in this study were of the endophytic type. However, a significant percentage (22.2%) of the cases of exophytic tumours had mismatch repair defect. This study showed that tumours with mismatch repair defect were significantly associated with an exophytic growth appearance ($p=0.007$). The odds ratio was 2.42 with a 95% confidence interval of 1.26 to 4.67. This was also similarly reported by others. Feeley et al (Feeley, Fullard et al. 1999) and Messerini et al (Messerini, Vitelli et al. 1997) studied the growth appearance of tumours found in MMR-d colon cancers. They

noted that there was a significant correlation between mismatch repair deficient tumours and exophytic growth. This may be also related to the association of mismatch repair deficient tumours to the right side of colon as right sided tumours were more likely to be exophytic growths.

4.3.4: SIZE OF TUMOURS

The size of the tumour was measured 3-dimensionally and the largest diameter was taken as the size of the tumour. Several studies (Feeley, Fullard et al. 1999; Gafa, Maestri et al. 2000; Chapusot, Martin et al. 2003) showed similar findings that MMR-d tumours were significantly associated with larger tumours. Our study and others (Feeley, Fullard et al. 1999) used the cut-off point of 5.0cm to differentiate large from small tumours while others used various sizes as their cut-off point: ranging from 4.0cm (16cm²) to more than 7.0cm in diameter. More than half of the MMR-d tumours in our study were larger than 5.0cm in diameter as compared to 29.8% of non-MMR-d tumours ($p=0.002$). MMR-d tumours had an odds ratio of 2.71 (with 95% confidence interval of 1.41 to 5.22) of being large in size. When the mean sizes of MMR-d tumours and intact tumours were compared; mismatch repair defect tumours were larger than non-MMR-d tumours by a mean difference of 1.0 cm ($p=0.009$). All the studies, despite the different sizes used, also found significant association between mismatch repair defect and large tumours.

Hence, MMR-d tumours were more likely to be larger tumours with an exophytic growth appearance.

In our study, larger colorectal tumours (tumours larger than or equal to 5.0cm in diameter) were also significantly associated with marked necrosis ($p<0.001$). The odds ratio was 2.77

with 95% confidence interval of 1.67 to 4.59. This would be expected as larger tumours grew beyond their blood supply and thus suffered necrosis more frequently than smaller tumours would.

4.4: HISTOLOGICAL FEATURES

4.4.1: HISTOLOGICAL TYPE AND GRADE

Forty-one point nine percent of mismatch repair defect tumours were poorly differentiated colorectal carcinomas as compared to 13.3% of MSS tumours ($p<0.001$). Patients with MMR-d colorectal carcinomas were more likely to be poorly differentiated tumours than those tumours with intact mismatch repair protein staining with an odds ratio of 4.68 (95% CI 2.31 to 9.47). This was similarly reported by many studies (Gafa, Maestri et al. 2000; Chapusot, Martin et al. 2003; Wright and Stewart 2003; Lim, Jeong et al. 2004; Hameed, Goldberg et al. 2006) when they compared mismatch repair defect tumours with intact tumours.

Chapusot et al (Chapusot, Martin et al. 2003) not only found that tumours with microsatellite instability were significantly associated with poorer differentiation but poor differentiation was the most accurate predictor of lack of MMR expression with a positive predictive value of 50% and negative predictive value of 89%. By multivariate analysis, they also demonstrated that poor differentiation was a significant independent factor associated with loss of expression of hMLH1 and hMSH2 proteins with an odds ratio that was much higher than this current study (OR=8.33, 95% CI 1.63 to 40.32).

TYPE OF MMR DEFECT

Shashidharan et al (Shashidharan, Smyrk et al. 1999) found that poorly differentiated colorectal cancers were significantly associated with hMSH2 defect and not hMLH1 defect. They found that 57% of hMSH2 defective tumours were poorly differentiated compared to 26% of hMLH1 defective CRCs. We also found that a higher proportion of hMSH2 or hMSH6 defective tumours (46.7%) were poorly differentiated compared to hMLH1 defective tumours (39.3%). Our number of cases was small and statistical evaluation unfortunately cannot be done.

In contrast to these previous reports stated, Wright et al (Wright and Stewart 2003) found that poorly differentiated carcinomas were predominantly hMLH1 defective tumours rather than hMSH2 defective tumours.

MEDULLARY CARCINOMAS

Gafa et al (Gafa, Maestri et al. 2000) further noted that a large majority (85.7%) of medullary adenocarcinomas were microsatellite instability tumours. Their results showed 20 out of 28 cases of poorly differentiated MSI-H tumours were mucinous or medullary carcinomas but only 8 cases were poorly differentiated adenocarcinomas. This suggested that the excess of poor differentiation among the MSI carcinomas was determined by the presence of medullary and mucinous carcinomas. Medullary tumours were found to be nearly always associated with microsatellite instability (Kim, Jen et al. 1994; Ruschoff, Dietmaier et al. 1997; Jass 1998; Gafa, Maestri et al. 2000).

Medullary carcinomas are characterised by trabecular to sheet-like growth of tumour cells with abundant eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli. They are generally not classified separately from ordinary adenocarcinomas but typed as poorly

differentiated or undifferentiated carcinomas. This study also did not attempt to distinguish medullary carcinomas from the poorly differentiated carcinomas but in a review of the slides, two undifferentiated tumours with features of medullary histology were noted in the study. These cases were actually found to be mismatch repair deficient as well, thus supporting the association between medullary carcinomas and mismatch repair deficient tumours as reported by other researchers. However, the numbers were small and no statistical evaluation was done in our study.

4.4.2: MUCIN PRODUCTION

In this study, there was a significant association between tumours with large amount of extracellular mucin and mismatch repair defect tumours ($p=0.007$). MMR-d tumours produced more mucin than non-MMR-d tumours. The odds ratio was 2.49 (95% CI 1.26 to 4.93).

Many reports (Kim, Jen et al. 1994; Risio, Reato et al. 1996; Messerini, Vitelli et al. 1997; Jass 1998; Gafa, Maestri et al. 2000; Ward, Meagher et al. 2001) also claimed that mucinous histology was the hallmark of MMR-defective carcinomas. In our study although a higher proportion of mucinous type carcinomas was observed in patients with MMR-d tumours: 11.6% of patients with MMR-d tumours as compared to 7.8% of patients with non-MMR-d tumours) but this was not statistically significant ($p=0.379$).

Chapusot et al (Chapusot, Martin et al. 2003) demonstrated in a univariate analysis that MMR-defective tumours were shown to be significantly associated with a distinct pattern of extracellular mucin production ($p=0.0001$) but was not significant when adjusted for other factors on multivariate analysis. Messerini et al (Messerini, Vitelli et al. 1997) studied

mucinous sporadic tumours with non-mucinous sporadic tumours as controls and found that mucinous carcinomas showed microsatellite instability more frequently than the controls. In addition they noted this was more marked when the tumours had lost two or more microsatellite alterations. This association may be because mismatch repair defects or replication errors may directly influence mucus production both in sporadic and familial cases (HNPCC). Altered mismatch repair genes may be involved in mucin synthesis or degradation resulting in increased amount of mucin in these tumours compared to stable tumours.

4.4.3: TUMOUR ADVANCING PATTERN

In earlier studies, Jass et al (Jass, Love et al. 1987; Jass, Ajioka et al. 1996) found that the character of invasive growth pattern (expanding vs. infiltrating) played an important and independent influence upon survival. In their study of over 200 cases of colon cancer, Gafa et al (Gafa, Maestri et al. 2000) also found the pattern of growth was an independent prognostic factor for disease specific survival by multivariate analysis. They found that nearly 90% of patients with tumours that had diffuse infiltration pattern died in the observation period of their study but less than a quarter (24.3%) of patients with expansive type of tumours died within the same period.

Diffuse infiltration of tumour was associated with a poorer outcome than expansive type of growth pattern in a tumour. Tumours that diffusely infiltrated surrounding tissue with irregular invasive borders would ramify beyond the limits and thus were more prone to spreading freely and metastasising as compared to tumours with expansive border that was circumscribed and walled off easily by the surrounding lymphocytic response.

We found that patients with tumours with expansive borders had better survival (mean survival = 35.0 ± 2.1 months) compared to patients with tumours with diffuse infiltrating borders (mean survival 26.8 ± 2.1 months). The p -value was 0.014.

There was also a non-significant trend towards an association between mismatch repair defect tumours and expansive borders. However, this was statistically not significant ($p=0.056$). Other published studies (Messerini, Vitelli et al. 1997; Gafa, Maestri et al. 2000; Wright and Stewart 2003; Valentini, Armentano et al. 2006) found that expansive type of tumour growth pattern was significantly associated with tumours defective for mismatch repair protein. Wright et al further added that both hMLH1 and hMSH2 defective tumours were equally as likely to be associated with an expansive type of border. They also found that the association was independent of the stage of presentation of the disease but added that an expansive-type border was less frequently seen with increasing stage of the tumour.

4.4.4: LYMPHOCYTIC RESPONSE

In response to the tumour neoantigens, lymphoid components may be produced which are intimately admixed with the tumour (Figure 4.4). These are largely CD3/CD8 co-expressing cytotoxic T cells. They are also called tumour-infiltrating lymphocytes (TIL).

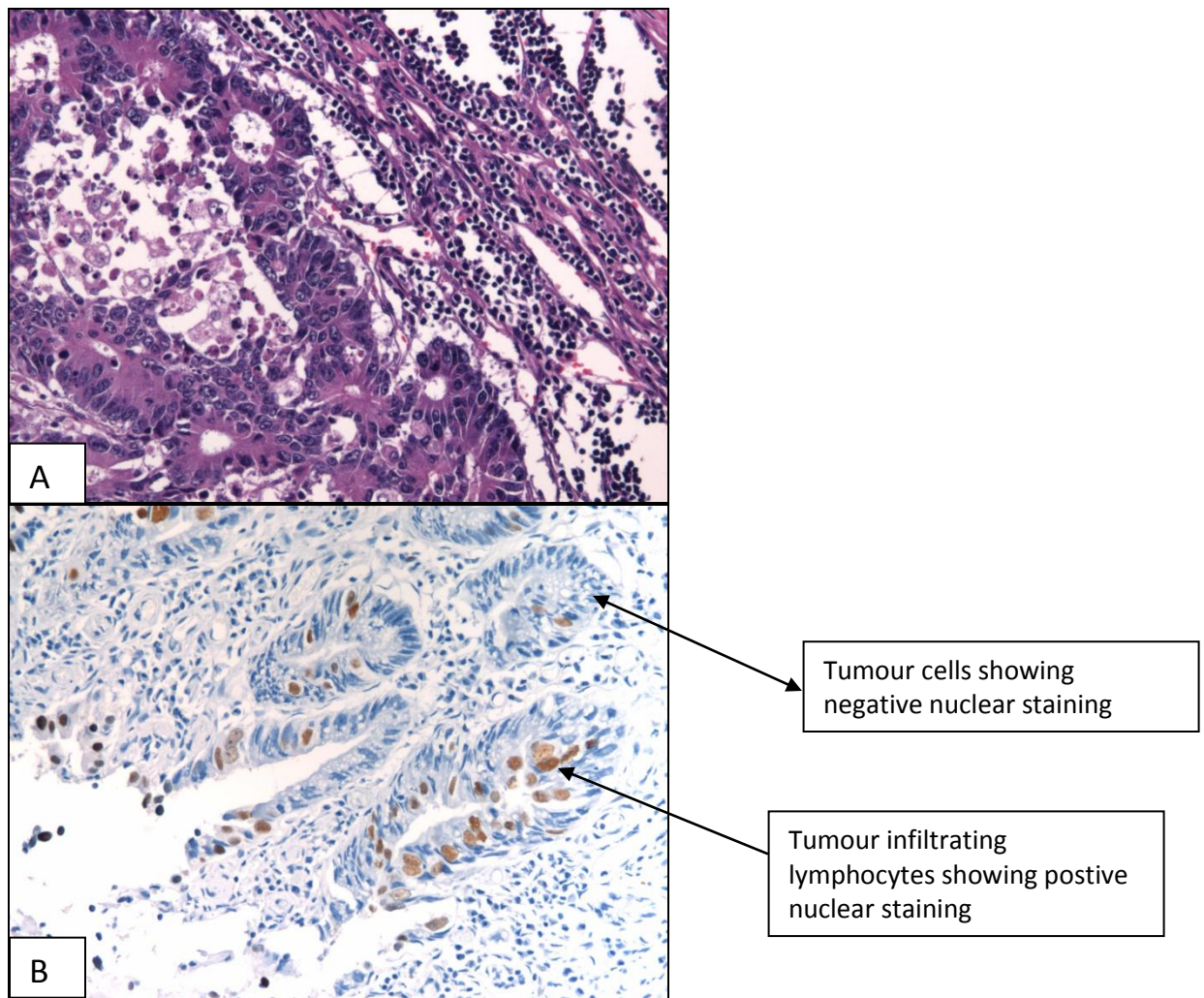


Figure 4.4: Colorectal carcinoma with tumour-infiltrating lymphocytes.

A: Colorectal carcinoma with tumour-infiltrating lymphocytes (Haematoxylin and eosin stain, original magnification x 200).

B: MMR-d colorectal carcinoma with tumour-infiltrating lymphocytes. Lymphocytes are present with brown nuclear staining (single arrow) within tumour cells with negative nuclear staining (double ended arrow) in a case of MMR-d tumour (IHC stain with hMLH1, original magnification x 200).

In some studies, various methods were utilised to count these tumour-infiltrating lymphocytes (TILs), including evaluation of H&E stained slides or immunohistochemical staining methods with markers for T-lymphocytes, namely CD3 immunostained slides. In those studies five consecutive fields under 40x magnification views of H & E stained slides were examined microscopically to look for tumour-infiltrating lymphocytes. The number of lymphocytes present was counted. The mean number of TIL/HPF was calculated and was considered positive result if there were more than 2TIL/HPF (Greenson, Bonner et al. 2003; Greenson, S.C. et al. 2009).

Instead of using routine H&E stained slides to study for TIL, Jass et al (Jass 2000) used the immunoperoxidase methods to stain for the lymphocytes. Jass et al (Jass 2000) and Alexander et al (Alexander, Watanabe et al. 2001) also used different values of cut-off as positive lymphocytic response namely 5 and 8 TIL per high power field respectively.

Aside from tumour-infiltrating lymphocytes, other lymphocytic responses associated with tumours included peri-tumoural lymphocytic and Crohn-like lymphocytic responses.

Our study looked into the peri-tumoural lymphocytic and Crohn-like lymphocytic responses but did not look into tumour-infiltrating lymphocytes. Neither peri-tumoural lymphocytic or Crohn-like lymphocytic response was associated with MMR-d tumours ($p=0.242$ and 0.564 respectively).

Although our study did not show any relationship between the MMR-d tumours and the lymphocytic response (either the peri-tumoural or Crohn-like lymphocytic response) other workers (Chapusot, Martin et al. 2003; Greenson, Bonner et al. 2003; Wright and Stewart 2003; Valentini, Armentano et al. 2006) showed that both peri-tumoural and Crohn-like lymphocytic responses were seen frequently in mismatch repair defect tumours. Peri-

tumoural lymphocytic response was significantly more common in these tumours with MMR protein deficient, as was Crohn-like lymphocytic response.

In addition, Wright's group (Wright and Stewart 2003) found no difference between the two mismatch repair proteins. hMLH1 and hMSH2 deficient tumours were found equally as significant to be associated with a brisk lymphocytic response. Greenson et al (Greenson, Bonner et al. 2003) studied tumour infiltrating lymphocytes and showed that positive tumour-infiltrating lymphocytes were significantly associated with high microsatellite instability tumours. The positive predictor value in their study for the MSI-H status was 30.1% and its negative predictor value was 98.6%.

LYMPHOCYTIC RESPONSE AND SURVIVAL

In our study, patients with tumours showing marked peri-tumoural lymphocytic response had longer mean survival (mean survival 32.0 ± 1.8 months) than patients with minimal lymphocytic response (mean survival 27.7 ± 3.1 months). Unfortunately this was not statistically significant ($p=0.293$) and we were not unable to demonstrate a significant association of patients with brisk lymphocytic response and a better survival.

However, earlier studies by Graham et al (Graham and Appelman 1990) and Harrison et al (Harrison, Dean et al. 1995) showed that Crohn-like lymphoid response in colorectal cancers was an independent predictor of a good outcome in patients with colorectal cancers. Presence of Crohn-like lymphoid reaction in colon cancers was more frequently seen in cases with a lowered incidence of nodal metastases and a statistically significant increase in 10-year survival. This response was evident of a favourable host response towards the tumour which attempts to limit the tumour and prevent it from spreading.

The significant association of mismatch repair tumours with marked lymphocytic response as reported in the forms of peri-tumoural lymphocytic, Crohn-like lymphocytic response or tumour-infiltrating lymphocytes demonstrated the increased host defence mechanisms in limiting these tumours. Therefore this explained why patients with MMR-d tumours showing a higher incidence of marked lymphocytic response were associated with a better outcome than patients with tumours which were microsatellite stable with less lymphocytic response.

4.4.5: NECROSIS

This study examined the amount of necrosis in colorectal cancer. We did not study the type of necrosis present (*dirty necrosis*). We found that MMR-d tumours were associated with more extensive necrosis. Tumours with marked necrosis were significantly more likely to have deficient mismatch repair protein staining by IHC ($p=0.001$).

The reason for this can be explained by the association of marked necrosis with size of tumours, as previously stated. Larger sized tumours outgrew their blood supply and suffered extensive necrosis more often than smaller tumours. As larger tumours were more frequently seen in mismatch repair deficient tumours, thus there was also more necrosis seen in MMR-d tumours.

The association between the degree of necrosis and MMR-d tumours may also suggest that MMR-d tumours were proliferating at a much faster rate compared to their blood supply. An alternative explanation may be that there were less angiogenesis initiated to support the tumour growth.

Most studies (Greenson, Bonner et al. 2003; Raut, Pawlik et al. 2004; Halvarsson, Anderson et al. 2008) specifically looked at *dirty necrosis* and not the amount of necrosis. Greenson et al (Greenson, Bonner et al. 2003) found that tumours with mismatch repair defect were significantly lacking dirty necrosis that was characteristic of colorectal carcinomas. Dirty necrosis consists of fragmented destruction of glands and cellular debris. Thus, it can be argued that poorly differentiated cancers and mucinous tumours having less gland formation would hence lack dirty necrosis. As tumours with microsatellite instability were more likely to be poorly differentiated, thus the lack of dirty necrosis in tumours with microsatellite instability may be due to their poorer differentiation. Halvarsson et al (Halvarsson, Anderson et al. 2008) in studying the clinicopathological features that identify mismatch repair defect tumours found that lack of dirty necrosis was associated with a relative risk of 7.5 for MMR-d tumours. Greenson et al demonstrated that a lack of dirty necrosis was an independent predictor of microsatellite instability, independent of tumour grade and mucin production. These findings together with other studies (Dolcetti, Viel et al. 1999; Jass 2000) showed that there was a higher rate of cell death in MSI-H tumours than stable tumours, suggesting that the mechanism of cell death in these tumours was different from that in microsatellite stable tumours. These may help to explain the different response to chemotherapy in MSI tumours and stable tumours. Different chemotherapy would target at different mechanisms of cell death and help promote cell death thus shrinking the size of the tumour.

4.5: STAGING

4.5.1: LYMPH NODES STATUS

Lymph node metastases are common in colorectal carcinomas. The staging of colon carcinomas is dependent on the status of the nodes. It was thus imperative to dissect out all lymph nodes during the processing of the resected colectomy specimens as examination of all colectomy specimens should include a careful examination of peri-colic lymph nodes. A standard resection of a colectomy specimen for colon cancer should contain between 10 to 25 lymph nodes (Petrias and Frankel 2009). Although this procedure of searching for lymph nodes is tedious and time consuming, but the yield of lymph nodes for examination to determine the presence or absence of lymph node metastases is very important. The status of the lymph nodes determines the prognosis of the patients.

In the 287 patients with lymph nodes retrieval in this study, the mean number of nodes was 8.8. This was lower than the recommended number of nodes (12) for proper assessment. The possibility of a lower yield of lymph nodes retrieval could be due to pre-operative pelvic radiation therapy in some of the cases (Sermier, Gervaz et al. 2006). Another reason was that fewer lymph nodes could be found for anterior resection of rectal carcinomas which were included in this study.

It was unfortunate in our study that lymph nodes were not found in 11 other cases. The possible reasons for this unsatisfactory processing of colectomy specimens could be due to new change-over of medical officers in training in the Pathology department who routinely processed (grossed) the specimen. Another possibility could be from the surgical department where trainee surgeons may have missed removing lymph nodes together with the colectomy specimens.

4.5.2: STAGING AND MMR-d TUMOURS

Some studies (Cunningham, Kim et al. 2001; Wright and Stewart 2003) found that patients with MMR-d colon cancers presented at a significantly earlier stage than intact tumours. Their studies found that although MMR-d tumours and intact tumours had similar levels of tumour invasion, MMR-d tumours were less likely to have lymph nodes involvement and metastases. Thus, MMR-d tumours were more likely to present at an earlier stage. This can also be explained by association of MMR-d tumours with peri-tumoural or Crohn-like lymphocytic response that may help limit the spread. Extramural vascular, lymphatic and perineural invasion were also less likely to be seen in MMR-d tumours but were frequently associated with microsatellite stable tumours. However, this study showed no statistical difference in patients with MMR-d tumours and patients with intact tumours when the staging of disease was compared ($p=0.622$). There was no difference in nodal metastases in these two groups ($p=0.746$).

Some studies (Gryfe, Kim et al. 2000; Truninger, Menigatti et al. 2005) on the other hand showed that tumours with mismatch repair defect had a lowered frequency of nodal metastases and hence a better survival. Gryfe et al (Gryfe, Kim et al. 2000) found that there was a decreased likelihood of MMR-d tumours metastasising to regional lymph nodes with an odds ratio of 0.33 and 95% confidence of 0.21 to 0.53. Others (Cunningham, Kim et al. 2001; Wright and Stewart 2003) reported that MMR-d tumours were more likely to present at an earlier stage and hence, by inference, less likely to be associated with nodal metastases.

Hemminki et al (Hemminki, Mecklin et al. 2000) found that presence of distant metastases at time of diagnosis was rare in MSI tumours. This was similarly seen in our study. Five point two percent of the patients with non-MMR-d tumours developed metastasis at presentation as compared to 2.6% of patients with MMR-d tumours in our study. The number of cases was small and it could not be statistically compared.

Hemminki et al (Hemminki, Mecklin et al. 2000) found that patients in their study with MSI colon cancers were associated with excellent survival and had overall survival of 90% at 3 years as compared to patients with intact tumours at overall survival of 62%. Wright et al (Wright and Stewart 2003) also found that when they compared MMR-d tumours against their pattern of gene defect, hMSH-2-defective colon cancers more frequently presented at an earlier stage than hMLH1-defective tumours. Half of their hMSH2-defective tumours presented Stage I compared to less than a quarter of hMLH1-defective tumours. They found that hMSH2-defective tumours were more likely to be confined to bowel wall (T1-T2) as compared to hMLH1-defective tumours. However, a few reports including our study showed no significant difference in the staging between the types of gene defect in MMR defective tumours. We found 53.8% of MMR-d tumour cases as compared to a slightly lesser proportion (49.6%) of non-MMR-d tumours were in early stages (Stage I and II) and 46.2% of MMR-d tumour cases as compared to slightly more (50.4%) non-MMR-d tumours in the later stages (Stage III & IV). There was hardly any difference in the proportion of cases found in each stages when we compared the mismatch repair defect status ($p=0.622$).

Shashidharan et al (Shashidharan, Smyrk et al. 1999) did not find any difference in nodal involvement between colon cancers with mismatch repair gene mutations and sporadic

stable tumours but found instead that lymph node involvement was more frequently seen in hMSH2 defective tumours than hMLH1 defective tumours ($p=0.03$). hMLH1 defective tumours were reported to elicit more Crohn-like lymphocytic response. This may explain the absence of nodal involvement by tumour in hMLH1 defective tumours. In their study, there was suggestion that the association of tumour-free nodes with MMR-d tumours could be more due to hMLH1 and not hMSH2 defective tumours.

4.6: SURVIVAL

Many studies have looked at patients with colorectal carcinomas to determine the possible prognostic factors for survival. The results had been conflicting and this was because of the different pathogenetic mechanisms of tumorigenesis involved in sporadic and familial types of colorectal carcinomas. Different genetic alterations such as chromosomal instability either due to inactivation of tumour suppressor gene p53 or deletion of the mismatch repair genes resulted in different clinical pathological features and types of neoplasia.

4.6.1: ANATOMICAL LOCATION

Localisation of tumour is an important factor for survival; some studies predicted that the right side had a worse prognosis compared to the left side. The reason may be due to a more delayed time to detection of the tumour and presentation if it was localised at the right side.

In our study there was no difference in survivals of patients with regards to the location of the tumours. Although we found that the survival of patients with left sided tumours was slightly better (mean survival = 30.6 ± 1.80 months) as compared to patients with right sided tumours (mean survival = 32.2 ± 3.00 months) it was not statistically significant ($p=0.724$). On the other hand, Meguid et al (Meguid, Slidell et al. 2008) demonstrated that right sided tumours had a worse prognosis than left sided tumours when they did not take into account the MMR status of the tumours. The reason for this was unclear but possible explanations included differences in time to detection of tumour, differences in embryologic origin as well as exposure to different faecal content. Tumours on the right side of the colon, because of their proximal location, manifested themselves later and detection of tumours in these

patients was frequently delayed. This may mean the tumours would have progressed to a later stage at time of diagnosis and thus had a worse prognosis.

However, tumours with mismatch repair defects were noted to be more on the right side with better prognosis and survival (Ionov, Peinado et al. 1993; Lothe, Peltomaki et al. 1993; Thibodeau, Bren et al. 1993; Kim, Jen et al. 1994) for various reasons including a more prominent lymphocytic response and less likelihood of metastasis to lymph nodes as discussed previously. This may be the reason for our results obtained with a slightly better survival for patients with right sided tumours that included many cases of mismatch repair defect tumours.

4.6.2: MMR-d TUMOURS

The mean period of survival of patients with MMR-d tumours (31.4 months) was almost the same as that of patients with intact tumours (31.0 months, $p=0.615$). However, when colon cancers were compared after separately removing rectal tumours from the group, the mean survival of patients with mismatch repair defect by IHC was better (32.7 months) compared to those with normal protein pattern (29.1 months). Nonetheless, this was not statistically significant either ($p=0.377$). There were various possible reasons to explain the difference of our findings compared to other previous published reports (Ionov, Peinado et al. 1993; Lothe, Peltomaki et al. 1993; Thibodeau, Bren et al. 1993; Kim, Jen et al. 1994; Gafa, Maestri et al. 2000; Gryfe, Kim et al. 2000; Lim, Jeong et al. 2004) that found patients with mismatch repair defect tumours demonstrated better disease specific survival than patients with microsatellite stable tumours. Many of our patients were not subjected to close follow-up and rigorous chemotherapy after diagnosis or surgery. In the data collection

we found many patients were lost to follow-up after the major surgery. Some of these patients may have resorted to traditional therapy at home while others accepted the fate of a terminal illness and seek no further treatment elsewhere. However, these were postulations and further in-depth study need to be carried out in future to look into the real possible reasons for the difference.

Gafa et al (Gafa, Maestri et al. 2000) found that patients with MSI-H tumours had a significant survival advantage even when only patients with tumours localised to the right side were included in their analysis. The prognostic significance became more evident in the subgroup of those with poorly differentiated carcinomas. In their report, the 5-year survival rate of patients with MSI-H poorly differentiated tumours was 79.2% compared to patients with MSI-L/MSS poorly differentiated tumours of 36.7% ($p<0.05$). Lim et al (Lim, Jeong et al. 2004) reported that the overall 5-year survival for patients with MSI was more than 90% but those with microsatellite stable tumours was less than 60% ($p<0.05$). This improved prognosis and longer survival were seen together with a lower number of distant metastases in these tumours. Other investigators (Hutchins, Southward et al. 2011; Sinicrope, Foster et al. 2011) also found these patients with MMR defective colorectal carcinomas had significantly reduced rates of tumour recurrences as compared to those without the defect.

Improved prognosis and better survival in sporadic MSI tumours may be due to the up-regulated immune response as demarcated by prominent peri-tumoural and Crohn-like lymphocytic responses or presence of tumour-infiltrating lymphocytes. Alternatively, it may be due to the high mutation rate of defective DNA mismatch repair genes that did not allow time for emergence of the genes contributing to tumour metastases. All these would

have prevented emergence of metastatic deposits and restricted growth of the tumour with a final better outcome and prolonged survival.

On the other hand, similar to our study, a few other studies (Feeley, Fullard et al. 1999; Hameed, Goldberg et al. 2006) found no significant difference in survival between MMR-d tumours and intact tumours. Possible reasons postulated may be that MMR-d tumours being at a proximal site may have similar behavioural features as the sporadic tumours with intact MMR pattern that were associated with a poorer outcome.

4.6.3: TUMOUR ADVANCING PATTERN

Tumours which infiltrated in an indistinctive manner splaying the muscular layer would spread further and faster compared to tumours with an expanding or circumscribed border that grew in a pushing manner. Hence the survival and outcome would be poorer for patients whose tumours display an infiltrating pattern.

Our study found there was a significant difference between the survival rates of patients with different tumour advancing patterns. Patients who had tumours with a diffuse infiltrating pattern of growth had a significantly poorer survival. Patients with tumours with expanding borders survived longer (mean survival = 35.0 ± 2.1 months) compared to patients with diffusely infiltrating borders (mean survival = 26.8 ± 2.1 months). The probability value was 0.014.

This was similarly reported by Gafa et al (Gafa, Maestri et al. 2000) where they found nearly 90% of patients with tumours having a diffuse infiltrating pattern of growth died of the disease whereas only less than a quarter (about 24%) of patients who had tumours with an expanding growth pattern died of the disease in the same period ($p < 0.0001$). They also reported that extramural vein invasion was a strong predictor of adverse outcome whereas intense lymphocytic response was associated with a better outcome.

4.7: P53 POSITIVE COLORECTAL CARCINOMAS

4.7.1: INTRODUCTION: P53

Development of colorectal cancers is driven by loss of genomic stability from the acquisition of multiple tumour associated mutations. One of the more common forms of genomic instability in colorectal cancer is chromosomal instability. This can result from loss of wild-type copy of a tumour suppressor gene such as p53 or APC gene.

P53 is a tumour suppressor gene. It stops neoplastic transformation by various ways. It activates temporary cell cycle rest (quiescence) or induces permanent cell cycle rest (senescence). Lastly, it also triggers programmed cell death (apoptosis). Damage to integrity of DNA or any form of stress can trigger off p53 response pathways. P53 acts to help maintain the integrity of the DNA. P53 thus has been rightly called the *guardian of the genome*. The wild-type p53 protein usually resides in the cell nucleus and has a short life-time. It is present in low quantities and cannot be detected by routine immunohistochemistry.

When there is homozygous loss of p53, DNA damage goes unrepaired and mutations become fixed and passed on to dividing cells. The cells then transform into malignant tumour. Both p53 alleles have to be inactivated, usually by a combination of missense mutations that inactivate the activity of p53 and a 17p chromosomal deletion that eliminates the second allele.

The majority of mutations in the p53 gene will lead to loss of the wild-type phenotype of p53 due to conformational changes as well as 17p allelic loss. This will result in increase in half-life of p53 and ensuing nuclear accumulation.

4.7.2: P53 AND MMR-d COLORECTAL CARCINOMAS

Our study together with other publications (Edmonston, Cuesta et al. 2000; Gafa, Maestri et al. 2000) found that there was significant inverse correlation between MMR-d protein and p53 over-expression. Our study showed 74.4% of mismatch repair defect tumours did not stain for p53. Conversely, 92.7% of tumours with p53 over-expression were found to be non-MMR-d tumours. This was statistically significant ($p < 0.001$). The odds ratio was 3.54 with 95% confidence interval of 1.71 to 7.34.

Sinicrope et al (Sinicrope, Rego et al. 2006) furthermore found that MSI-H tumours were more likely to have negative expression for p53 as compared to MSS/MSI-L tumours ($p < 0.001$). MSI and alterations in p53 protein expression seemed to be mutually exclusive.

All these findings supported the suggestion that there are two different pathways of colorectal carcinogenesis (Perucho 1996). The two alternative genetic pathways described in colorectal carcinogenesis are the APC gene pathway and the MMR gene inactivation pathway. One of the pathways, the APC pathway starts with a defective APC protein which is unable to promote proteolytic degradation of b-catenin thus leading to accumulation of b-catenin (Munemitsu, Albert et al. 1995). The over-expression of b-catenin results in accumulation of p53. This was possibly due to interference with its proteolytic degradation (Damalas, Ben-Ze've et al. 1999). This pathway thus results in accumulation or over-expression of tumour suppressor gene such as p53 and accounts for the majority (approximately 80%) of sporadic colorectal carcinomas. These tumours do not express microsatellite instability but instead show chromosomal instability reflected by losses of heterozygosity especially at the loci of the APC gene, chromosomal 17q and 18q. These tumours would exhibit chromosomal instability and aneuploidy. Additionally, mutations in

the APC gene and activation of other oncogenes such as k-ras had been described (Becker, Ruschoff et al. 1999).

The other pathway for colorectal carcinogenesis is the inactivation of MMR genes and this pathway accounts for 15% of all colorectal carcinomas. In these tumours, there is inactivation of both alleles of a MMR gene with possible secondary mutations in genes for growth control and apoptosis (TGF β RII, IGFR, BAX). In such tumours, there may be germline mutations of hMSH2 or hMLH1 with subsequent somatic mutation of the second allele. This would lead to loss of protein expression and may be detected by immunohistochemical staining. In some sporadic cases, inactivation results from hypermethylation of hMLH1-promoter with loss of immunohistochemical staining. All these tumours were said to be mismatch repair defect tumours which were typically p53 negative. There was thus strong correlation of microsatellite stable tumours that have no MMR gene inactivation but probable APC gene inactivation with p53 accumulation.

4.7.3: P53 AND ANATOMICAL LOCATION OF TUMOURS

P53 positive tumours were found to be significantly localised to the left side of colon, in contrast to MMR-d tumours which localised to the right side. We found 78.8% of p53 positive tumours were left sided as compared to the right side ($p < 0.001$). These findings were also noted by others (Gervaz, Bouzourene et al. 2001). This may be related to prolonged faecal exposure time in the distal colon where stool collection before defecation occurred; resulting in left sided tumours with higher incidence of p53 positive tumours. The prolonged contact with exogenous carcinogens could induce point mutations of p53 (Jones, Buckley et al. 1991). It had been found that different carcinogens present in our diet could

be linked to the higher incidence of nuclear accumulation of p53 (Ishioka, Suzuki et al. 1992; Freedman, Michalek et al. 1996). This also explained the predilection of p53 positive tumours to the left side considering the left colon has a longer faecal exposure time to different types of carcinogens.

4.7.4: P53 AND SURVIVAL

The correlation of p53 mutations in colorectal carcinomas with patient survival had been debated. In some reports, p53 protein expression was correlated with poorer survival (Leahy, Salman et al. 1996; Manne, Myers et al. 1997; Gervaz, Bouzourene et al. 2001) while others did not find this to be the case. Manne et al (Manne, Weiss et al. 1998) noted that nuclear accumulation of p53 was correlated to poorer survival in white patients with adenocarcinomas of the proximal colon but not in African-Americans. When the results of the two major ethnic races were grouped together they found no differences in survival. These findings reflect differences in the type of mutation of the p53 gene. Goh et al (Goh, Yao et al. 1995) reported that patients with point mutations in conserved regions of the p53 gene were associated with a more aggressive tumour and thus had a significantly poorer prognosis than those with base changes outside these areas. These point mutations were more related to distant organ metastases and lymphatic dissemination.

Our study did not find any difference in survival between patients with p53 positive tumours and p53 negative tumours. The mean survival of patients with p53 positive and p53 negative tumours were almost similar ($p=0.741$). However, when the patients were stratified without rectal tumours, there was a poorer survival if patients had tumours with p53 over-expression. The mean survival was 27.7 months as compared to 30.8 months for

patients with negative p53 over-expression. The probability value was not significant ($p=0.495$).

Locally, Goh et al (Goh, Ong et al. 2004) in Malaysia studied p53 staining in 116 colorectal carcinomas and did not find any difference in survival between patients with p53 positive and p53 negative tumours. Their study (Goh, Ong et al. 2004) together with that of Manne et al (Manne, Weiss et al. 1998) also did not demonstrate evidence of any difference in survival between tumours with and without p53 abnormalities when the studies included different races in a multi-ethnic population of people.

4.8: MANAGEMENT OF COLORECTAL CARCINOMAS

4.8.1: SURGERY

Definitive surgery is the main modality of treatment in most patients with or without adjuvant chemotherapy or radiotherapy. The aim of surgery is mainly curative to remove the tumours but to preserve as much normal bowel, bladder and sexual function as possible. For patients in early stages of cancer, curative surgery is possible and may involve only local excision but for others in the advanced stages cure may not be possible even with wide excision.

Thus major surgery may be associated with significant mortality and morbidity especially for elderly patients with advanced or metastatic diseases who may be unfit for surgery. For patients in advanced stages of cancer, surgery may not be curative but to relieve local symptoms like obstruction or bleeding.

A total surgical excision should adequately remove the tumour with clear margins and reduce local recurrences. This hence improves the survival of the patient.

Surgical treatment of colorectal carcinoma depends on the location of the tumour and includes hemicolectomy, anterior resection, and abdominoperineal excision. Total mesorectal excision for rectal cancers has also been performed with varying results.

In any form of surgery, clearance of the circumferential resection margins (CRM) free of tumours is a very important prognostic factor that can be manipulated by surgical treatment. Other prognostic factors such as nodal involvement, differentiation of tumour, or vascular or lymphatic involvement undoubtedly play major roles in determining the survival of the patient but cannot be altered by treatment.

4.8.2: ADJUVANT CHEMOTHERAPY

The results of reported studies had been inconsistent on the response of patients with mismatch repair deficient tumours to chemotherapy. One published study (Rosty, Chazal et al. 2001) showed that treatment with 5-fluorouracil (5-FU) patients with metastatic disease did not show any difference in response between patients with MSI tumours and patients with MSS tumours. On the other hand, another larger series (Liang, Huang et al. 2002) found that MSI status and administration of chemotherapy were independent favourable prognostic parameters and suggested that this was due to increased chemo-sensitivity of MSI tumours.

Hemminki et al (Hemminki, Mecklin et al. 2000) found that patients with MSI colorectal cancers who were treated with the same adjuvant 5-fluorouracil based chemotherapy which was the standard treatment for Stage III colon cancers performed significantly better when compared with patients with microsatellite stable tumours. These results suggested that patients with MSI tumours were potentially curable despite loco-regional lymph node metastases. It also supported the hypothesis that MSI tumours were more sensitive to 5-FU. However, several other studies, (Aebi, Fink et al. 1997; Fink, Aebi et al. 1998; Carethers, Smith et al. 2004) suggested that there was association between hMLH1/hMSH2 deficiency in cell lines and resistance to chemotherapeutic agents. These studies found that mismatch repair deficient cells were resistant to various cytotoxic drugs including 5-fluorouracil.

4.8.3: NEW TREATMENT AND TRIALS

Studies by Ruschoff (Ruschoff, Wallinger et al. 1998) and Yamamoto (Yamamoto, Itoh et al. 1999) demonstrated a reduction of proportion of hMLH1 or hMSH2 deficient cell lines exhibiting microsatellite instability when treated with nonsteroidal antiinflammatory drugs (NSAIDs). This had led to new drug trials to change the phenotypic manifestation of this mismatch repair deficiency and hence hopefully alter the course of cancers. Steinbach et al (Steinbach, Lynch et al. 2000) found that there was an actual reduction in the number of adenomas in patients with colorectal cancers when treated with NSAIDs. This may reduce the recurrences of tumours in these patients with colorectal carcinomas.

As the number of cases studied in this study was small and the types of chemotherapeutic agents used namely: fluorouracil (5-FU) combined with leucovorin (folinic acid), Folfex (oxaliplatin) or Folfiri; were varied it was difficult to obtain a statistical evaluation of patients for each type of treatment received.

4.9: LIMITATIONS OF STUDY

In our current investigation the sample of about 300 patients collected retrospectively was not large. A significantly larger cohort may improve the results of the study statistically. However, we encountered difficulty when we tried to expand the study retrospectively for a longer time period. A greater percentage of clinical notes were non-retrievable and many patients were lost to follow-up. Over the years, the contact of patients or relatives of deceased patients were lost or changed and this made our collection of data very difficult or impossible.

We realised that our investigation was limited as it was not an epidemiological study. We have concentrated on looking at a sample of patients who had been operated in our local hospital for a period of time and the data collected was mostly from clinical notes and interviews from patients or next of kin. The results thus may not have represented the Malaysian population or subpopulation. We instead had placed emphasis on histopathological methods carried out to determine the mismatch repair defects by immunohistochemical tests and compared these with the clinical data obtained.

We were aware that the clinical follow-up was for a short interval with a mean follow-up of 18.1 months. Accurate prognostic assessment was not possible. It would be more ideal if the duration of follow-up was for a longer duration. However, we were not able to carry this study for a longer time period as time of study and available finances were limited.

Immunohistochemical testing was used to detect the protein products of the mismatch repair genes and p53 gene in this study. However, we had not validated the group of patients with abnormal immune-staining with gene sequencing for p53 or molecular testing (PCR) for mismatch repair genes. Our laboratory did not have the facility for the molecular

testing or genetic testing and our limited financial resources had made it impossible to outsource the tests.

Immunohistochemistry testing for mismatch repair gene protein is robust and gave a high specificity of 100% as reported by most investigators and over 90 to 95% for sensitivity. In some of these false negative cases non-functional mismatch repair protein may retain their antigenicity and appeared as non mismatch repair defect tumours when they were actually not. These cases could be detected by molecular testing using the PCR method.

We used immunohistochemical methods to check for nuclear accumulation of p53 protein when there was presence of p53 mutation. However, immunoreactivity for p53 protein did not always indicate p53 mutations. Dysregulation of wild-type p53 protein may cause nuclear accumulation of the protein and resulted in a false positive test despite absence of mutation. In addition, not all cases of p53 mutations resulted in protein accumulation. Nonetheless, there was strong positive correlation of immunoreactivity and p53 mutations (Cordon-Cardo, Dalbagni et al. 1994) and hence the test could be used with limitations.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Colorectal carcinomas arise from the mutator and suppressor pathways that include inactivation of mismatch repair genes namely hMLH1, hMSH2, hMSH6, hPMS1 and hPMS2 and inactivation of p53, the tumour suppressor gene.

Forty-three cases or 14.4% of 298 patients with colorectal carcinoma studied showed loss of one or more mismatch repair gene. MMR-d tumours were mostly due to hMLH1 (65.1%) and to a lesser extent hMSH2 or hMSH6. About half of the colorectal carcinomas (50.7%) were found to be associated with p53 over-expression. Tumours with mismatch repair defect were inversely related to tumours with p53 over-expression. Ninety-two point seven percent of tumours with p53 over-expression were found to have intact mismatch repair whereas 74.4% of tumour with mismatch repair did not show p53 expression ($p<0.001$). Similarly, MMR-d tumours and tumours with p53 over-expression were significantly localised to different sites. Mismatch repair defect tumours were found at the right sided colon ($p<0.001$) whereas tumours with p53 accumulation were significantly localised to the left side of colon ($p<0.001$). This study showed that MMR-d tumours were not only more likely to be right sided but were larger, exophytic tumours producing mucin with large areas of necrosis. The tumours were more likely to be poorer differentiated and the patients were significantly associated with having multiple malignancies either synchronous or metachronous malignancies.

Multivariate analysis showed that four independent variables were significantly associated with loss of expression of one of the mismatch repair protein tested. Poor differentiation in tumour grade, right sided location, exophytic growth and poor p53 expression were independent predictors of loss of expression of hMLH1, hMSH2 or hMSH6 in the tumours. Our study showed patients with MMR-d tumours did not have a better survival compared to tumours with intact mismatch repair. It was also found that the tumours were not associated with any lymphocytic response (peri-tumoral or Crohn-like) and were not significantly associated with an earlier stage of disease. However, this was in contrast to many other reports (Gryfe, Kim et al. 2000; Truninger, Menigatti et al. 2005) which showed patients with MMR-d tumours had better survival as in those studies the tumours presented at an earlier stage and were associated with lymphocytic response. There were various possible reasons for the non-significant survival of these patients that were not looked into in our study. The local people generally accepted their fate readily and some resorted and preferred traditional therapy rather than seek the prescribed treatment for their tumours. There were also many patients who were lost to follow-up.

Our study showed that immunohistochemical testing for hMLH1, hMSH2 and hMSH6 could be done in a routine histopathology laboratory to detect the group of mismatch repair defect colorectal tumours associated with characteristic features as mentioned. It is important to delineate this group of patients for the management and the prognosis differs from the general group of patients with colorectal carcinomas. It can thus be proposed to include immunohistochemical testing for mismatch repair defect proteins in a routine histopathological reporting of all colorectal carcinomas or limit it to colorectal carcinomas with characteristic associated features that are highly be suspicious of mismatch repair

defect tumours. This preliminary testing can then be confirmed with a more sophisticated and expensive test, PCR test for the mismatch repair gene. On the other hand, immunostaining for p53 expression did not give any added usefulness to the report and may not be done routinely.

APPENDICES

APPENDIX 1:

Clinical Criteria for diagnosis of Hereditary Non-Polyposis Colorectal Cancer

Name of Criteria	Specific criteria	Published reference
Amsterdam	Three relatives with colorectal cancer, one of which is a first-degree relative of the other two; colorectal cancer affecting more than one generation; at least one colorectal cancer case diagnosed before age 50 years	(Vasen, Mecklin et al. 1991)
Modified Amsterdam [#]	Two colorectal cancer cases in first-degree relatives in very small families that cannot be expanded further; colorectal cancer affecting more than one generation; at least one colorectal cancer case diagnosed before age 55 years	(Bellacosa, Genuardi et al. 1996)
Amsterdam Criteria II	≥ 3 relatives with an HNPCC-associated cancer ^a and ≥ 2 successive generations affected and ≥ 1 diagnosed before age 50 years and one should be a first-degree relative of the other two. Familial adenomatous polyposis should be excluded and the tumours should be verified by histopathological examination.	(Vasen, Watson et al. 1999)
<i>(HNPCC-associated cancers^a = colorectal cancer, cancer of endometrium, small bowel, ureter or renal pelvis.)</i>		
Bethesda [#]	Individuals from families that fulfil the Amsterdam criteria	(Rodriguez-Bigas, Boland et al. 1997)
	Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extra-colonic cancers	

APPENDIX 1: Continued

Individuals with colorectal cancer, plus colorectal cancer and/or HNPCC-related extra colonic cancer and/or colorectal adenoma in a first-degree relative; at least one of the cancers diagnosed before age of 45 years and the adenoma diagnosed before age of 40 years

Individuals with colorectal or endometrial cancer diagnosed before age 45 years

Individuals with right-sided colorectal cancer with an undifferentiated histopathological pattern (solid/ cribriform) diagnosed before age 45 years

Individuals with signet-ring cell type colorectal cancer diagnosed before age 45 years

Individuals with colorectal adenomas diagnosed before age 40 years

Japanese ^{##}	Three or more colorectal cancer cases among first-degree relatives	(Fujita, Moriya et al. 1996)
	Two or more colorectal cancers among first-degree relatives and any of the following: diagnosis before age 50 years; right colon involvement; synchronous or metachronous multiple colorectal cancers; association with extra colonic malignancy	

[#] *Fulfilment of all criteria listed in any paragraph in this section is sufficient.*

^{##} *Cases can be classified as fulfilling either the first set of criteria or the second set and can be diagnosed with hereditary non-polyposis colorectal cancer if they fulfil either set of criteria.*

APPENDIX 2:

Clinical Questionnaire form

COLORECTAL CARCINOMA STUDY (PERMISSION FROM MOH :-)

NAME

--	--

IC no NEW

--

OLD

--

RNo

--

HPE

--

SEX

--

RACE

--

AGE (at presentation)

--

ALIVE

--

(DATE OF LAST F/U)

AGE (operated)

--

DEAD

--

(DATE/YEAR)

PREVIOUS CARCINOMA

YES

NO

DON'T KNOW

WHERE

WHEN

--

RELATIVE WITH CARCINOMA

YES

NO

DON'T KNOW

SPECIFY:

WHERE

WHO

PREVIOUS IBD (COLONIC SX)

YES

NO

DON'T KNOW

SPECIFY

PAST MEDICAL HISTORY

--

NSAID TX BEFORE

YES

DURATION

NO

--

DON'T KNOW

--

APPENDIX 2: Continued

PRESENTATION			
PAIN			
CHANGE BOWEL HABITS		MASS	LOA
OBSTRUCTION		BLEED	LOW
OTHERS (specify)		PERFORATION	
SURGERY			
HEMICOLECTOMY			
TOTAL COLECTOMY	ANT RESECTION		
OTHERS	AP RESECTION		
SITE OF TUMOUR			
RECTUM		CAECUM	
ASCENDING COLON		HEPATIC FLEXURE	
TRANSVERSE COLON		SPLenic FLEXURE	
DESCENDING COLON		SIGMOID COLON	
OTHERS			
(SPECIFY)			
STAGING			
T		N	
METASTASIS (AT OPERATION)			
LIVER		LUNG	
BRAIN		BONE	
OTHERS			
METASTASIS (AT FOLLOW-UP)			
LIVER		LUNG	
BRAIN		BONE	
			OTHERS

APPENDIX 2: Continued

CHEMOTHERAPY	5FU-FOLINIC ACID		
	MAYO		
	DE		
	GRAMMONT		
	FOL FOX4 (OXALIPLATIN)		
	FOL FIRI (IRINOTICAN)		
	ORAL CAPACETABIN		
COMMENTS			
Prepared by Dr Khoo JJ (Tel: 07-2190606/0177693652)			

APPENDIX 3:

Definitions of TNM

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma-in-situ: intraepithelial or invasion of lamina propria*
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into pericorectal tissues
T4a	Tumour penetrates to the surface of visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures.
Regional Lymph nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (e.g. Liver, lung, ovary, nonregional node)
M1b	Metastasis in more than one organ/site or the peritoneum

APPENDIX 3: Continued

Staging

Anatomic Stage			
Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
IIIB	T1	N2a	M0
	T3-T4a	N1-/N1c	M0
	T2-T3	N2a	M0
IIIC	T1-T2	N2b	M0
	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

(Source: AJCC Cancer Staging Manual (Edge, Byrd et al. 2010) .

APPENDIX 4:

Haematoxylin and eosin staining: Program set for Leica Autostainer XL

1. Slides are sectioned from respective paraffin blocks and placed in oven briefly for 10 seconds.
2. Slides go through a series of xylene 1 to 4 for duration of 10 seconds, 30 seconds, 4 minutes and 4 minutes respectively.
3. The slides are taken to absolute alcohol 1 and 2 for 3 minutes each.
4. They then go through a decreasing concentration of alcohol from 95% to 70% alcohol for 3 minutes each.
5. The slides are brought to water for 3 minutes and stained with Haematoxylin for 12 minutes.
6. They are washed with water again for 3 minutes and then dipped into 0.2% acid alcohol.
7. They are immersed into water again for 12 minutes and then counter-stained with eosin for 1 minute.
8. The slides are briefly washed with water for 1 second and then dehydrate with increasing alcohol starting from 70% alcohol through 90% alcohol to two-times absolute alcohol for 10 seconds, 10 seconds, 1 minute and 2 minutes respectively.
9. The slides go through 4 different troughs of xylene for 2 minutes each trough.
10. The slides are then taken out from the last (fourth) trough of xylene and immediately mounted with DPX.

APPENDIX 5:

Preparation for: 3-aminopropyltriethoxysilane (APES) treated slides

1. Wash glass slides in Detergent for 30 minutes
2. Wash glass slides in running tap water for 30 minutes
3. Wash glass slides in distilled water for 5 minutes (twice).
4. Wash glass slides in 95% alcohol for 5 minute (twice).
5. Air dry the slides for 10 minutes.
6. Immerse slides into freshly prepared 2% *3-aminopropyltriethoxysilane* in dry acetone for 5 seconds.
7. Wash briefly in distilled water twice.
8. Dry overnight at 42°C.
9. Store at room temperature. Treated slides can be kept indefinitely.

APPENDIX 6:

Immunohistochemistry staining

1. Diluted PT module solution is prepared accordingly: 15 ml. of PT module solution is made up to 1500 ml. by adding distilled water. Prepared solution is poured into the PT module instrument, pre-heated to 65°C for 10 to 15 minutes.
2. Slides are sectioned from respective paraffin blocks and placed on glass slides pre-treated with 3-aminopropyltriethoxysilane. They are labelled accordingly and put into oven (set at melting point of wax i.e. 56°C) for about 2 hours
3. The slides are taken out and arranged in a special rack and are immediately immersed into the PT module solution. The PT module solution is warmed to 100°C for 35 minutes.
4. The temperature of the PT module instrument is cooled down to 65 °C. The cooling phase takes about 15 minutes.
5. The rack and slides are taken out and flushed with Citrate Buffer Tween 20.
6. They are then treated with hydrogen peroxide for 5-10 minutes.
7. The slides are washed with Citrate Buffer Tween 20 for 3 times. Each wash takes 2 minutes.
8. The slides are treated with UV block for 5 minutes and then washed once again with Citrate Buffer Tween 20.
9. The respective primary antibody is added at the recommended dilution and incubated for 30 minutes. Step 8 is repeated.

APPENDIX 6: continuation

10. The antibody enhancer is added and incubated for 20 minutes. Step 8 is repeated.
The polymer is added and incubated for 30 minutes. Step 8 is repeated.
11. Freshly prepared DAB chromogen is added to slide and colour development is observed, which takes about 5 minutes.
12. The slides are washed with buffer and arranged in a staining rack.
13. The slides are dipped (one to two dips) into Harris Haematoxylin.
14. The slides are put under running tap water for 5 minutes.
15. The slides are dehydrated in series of alcohol in ascending order from 70% alcohol to absolute alcohol and then in xylene.
16. The slides are mounted with DPX and labelled accordingly.

APPENDIX 7:

Electronic-Database Information (Internet sites)

1. SEER Cancer Statistics Review, 1973-1994, <http://www-seer.ims.nci.nih.gov/Publications/CSR7394/>
2. Surveillance, Epidemiology, and End Results Program, <http://seer.cancer.gov/>

APPENDIX 8:

Pathologist worksheet

HPE No:			Blocks		Case No	
Date						

Diagnosis	well		T1		caecum	
	moderately		T2		transverse	
	poorly		T3		ascending	
	undifferentiate		T4		descending	
	signet ring		others (specify)		sigmoid	
	mucinous				rectum	
					rectosigmoid	
					hepatic flex	
					splenic flex	
					recurrent	

Mucin production						
Mucinous ADC or Adenocarcinoma		(ADC= adenocarcinoma)				
Lymphoid	Crohn's like inflammatory infiltrate -	Yes/No				
	Peritumoural lymphocytes -	Little /Marked				

Tumour	Infiltrative /	Expansive	necrosis little /marked			

Size of tumour		(cm)	Tumour type	
Others (if any)	No / Yes			
	Site / Size			

Lymph nodes isolated		N0	
Lymph nodes involved		N1	
		N2	
		N3	

Metastasis	M0 / M1	Site(s)	
	STAGE		

IHC staining					
	tumour	area	normal	area	comments
MLH1					
MSH2					
MSH6					
p53 (no)					

additional comments/action to take	
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