

FACULTY OF MEDICINE AND HEALTH SCIENCES

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List of Abbreviations

ACF	Aberrant crypt foci
AE	Advancing edge
AFAP	Attenuated familial adenomatous polyposis
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
AP1	Activator protein 1
AKT	Protein kinase B
ASR	Adjusted standardized residual
ATP	Adenosine triphosphate
AXIN2	Axis inhibition protein 2
BAX	Bcl-2-associated X protein
BCL	B-cell lymphoma 2
Bim	Bcl-2-interacting mediator of cell death
BRAF	B-Raf proto-oncogene serine/ threonine kinase
CI	Confidence Interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
COX-2	Cyclooxygenase-2
CpG	Cytosine preceding guanine
CRC	Colorectal cancer
CT	Computed tomography
CXCL12	Chemokine (C-X-C motif) ligand 12
DCC	Deleted in colon cancer
dMMR	Deficient MMR
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptors
ELK1	ETS Like-1 protein
EMT	Epithelial-to-mesenchymal transition
EndoG	Endonuclease G
EPCAM	Epithelial cellular adhesion molecule
FAP	Familial adenomas polyposis
FOX03a	Forkhead box O-3a
FZD10	Frizzled-10
GDP	Guanosine diphosphate
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
H & E	Haematoxylin and eosin
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
H-score	‘histo’ score
IHC	Immunohistochemistry
IRS	Immunoreactivity score
ITBCC	International Tumour Budding Consensus Conference
KRAS	Kirsten rat sarcoma
LN	Lymph node
LOH	Loss of heterozygosity
LRP	Low density lipoprotein receptor-related protein 1
LS	Lynch Syndrome

MAP	MYH-associated polyposis
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MEK	MAPK/ERK-activating kinase
MGMT	Methylguanine methyltransferase
MLH-1	MutL homolog 1
MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
MRI	Magnetic resonance imaging
MSH	MutS homolog
MSI	Microsatellite instability
MSS	Microsatellite stability
MUTYH	MutY DNA glycosylase
ncRNAs	Non-coding ribonucleic acids
NF-KB	NF-kappaB
NGS	Next Generation Sequencing
NHS	National Health Services
NKD1	Naked Cuticle 1
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
PET	Positron emission tomography
p-ERK	Phosphorylated extracellular-signal-regulated kinase
PGE2	Prostaglandin E2
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP_2	Phosphatidylinositol 4,5 biphosphate
PIP_3	Phosphatidylinositol 3,4,5- triphosphate
pMMR	Proficient MMR
PMS2	PMS1 Homolog 2
POLD1	DNA polymerase delta 1
POLE	DNA polymerase epsilon
PPAP	Polymerase proofreading-associated polyposis
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
QMC	Queens Medical Centre
RTKs	Receptor tyrosine kinases
SD	Standard deviation
SE	Standard error
SMAC	Second Mitochondria-derived Activator of Caspase
tBid	Truncated BH3 interacting-domain death agonist
TCF	T-cell factor
TGF-β	Transforming growth factor beta
TMA	Tissue microarray
TNM	Tumour-node-metastasis
TP53	Tumour protein p53
TSR	Tumour-stroma ratio

Abstract

Background: Colorectal cancer (CRC) is a heterogeneous disease with genetic and biochemical dysregulation background. The dysregulation happens in a few important signalling pathways, including Ras signalling, p53 signalling and Wnt/β-catenin signalling. This will lead to the malignant transformation of a normal cell to a cancer cell. Cancer cells are mainly caused by the alterations of multiple tumour suppressor genes and oncogenes. Research analysis was conducted to identify a better survival rate and prognosis outcome on colorectal tumours, especially in this study where the expression of tumour protein 53 (*TP53)* and B-cell lymphoma 2 (*Bcl-2)* biomarkers via immunohistochemical staining were targeted.

Methods: Tissue microarray and manual histopathological scoring were conducted in this study, with a cohort of 1000 cases. Statistical results were also reported to enable analysis of the estimated H-score. Quartiles were used to determine the cut-off points of the H-score. *TP53* and *Bcl-2* staining were only defined when the core staining is more than 15% of tumour cells. *TP53* expression, *Bcl-2* expression and the proportion of tumour epithelium to stroma in colorectal carcinoma were studied in a series of 880 patients (382 females; 498 males). Its relation to multiple clinicopathological variables and survival probability were also assessed. The involvement of *TP53* and *Bcl-2* mutations in the pathogenesis of colorectal cancer and their prognostic impact were highlighted. Correlations on tumour-stroma ratio (TSR) and the survival probability can also be determined by statistical test (p-value), with p-value < 0.05 showed their significance. Immunohistochemistry was used to assess the number of peritumoural lymphocytes for an objective manner, according to standardized histopathology.

Results: High *TP53* and low *Bcl-2* expression were identified as good prognostic factors, providing a favourable outcome, with p-value < 0.05. The median score for *TP53* was 30, with 436 (49.55%) of tumours showed high expression. Whilst there were 498 (56.59%) tumours that had low expression of *Bcl-2*, with a median score of 10. By comparing tumour and normal CRC samples, *TP53* was highly expressed in tumour-derived CRC samples. Whereas *Bcl-2* was observed with similar expression to normal samples, predominantly because it has a lower expression within tumour cells. Low tumour-stroma ratio (TSR) was detected with lower survival probability on *TP53* biomarker for the identification of CRC patients, providing with a favourable outcome.

Several reliable methods were given to have a better prognostic impact and treatments on CRC.

Conclusion: Treatment of cancer is directly based on their stage. To provide a proper cancer treatment for the matching patient at the right time, this study is crucial to give a beneficial outcome on CRC cells and a chance of treatment success.

1. Introduction and Background

1.1 Overview of Colorectal Cancer

Colorectal cancer (CRC) is a form of cancer that can usually be found in the rectum and colon. It originates from the development of abnormal colonic epithelial cells of inflamed mucosa (Kuipers *et al.*, 2015). This abnormal growth gives rise to an early adenoma (<1cm polyps' size) and may then develop into an advanced adenoma (>1cm polyps' size). When the polyps become larger, the risk of developing CRC will increase significantly (Bonnington & Rutter, 2016).

CRC is the third most common cancer in men after lung and prostate cancers and the second most common case in women after breast cancer (Brody, 2015). In 2012, there were 746,000 cases in men (10.0% of total cancers) and 614,000 cases in women (9.2% of total cancers) diagnosed with CRC globally, showing a high incidence of morbidity in CRC patients (Kolligs, 2016). By comparing CRCs to the other types of cancers in the United Kingdom (2012), an average of 19,286 (13.4% of the total cancers) CRC cases were diagnosed in males, and 15,036 (10.9% of the total cancers) CRC cases were discovered in females (Office for National Statistics, 2014). Due to the rate displayed indicating the increased significance of the prevalence of CRCs, further study is required to understand the cause and risk factors associated with the disease. This could lead to treatment strategies that may lower the incidence of CRC.

1.2 Causes & Risk factors

CRC cells mostly begin as benign polyps that grow on the inner lining of the colon or rectum (Marley & Nan, 2016). Benign polyps can be classified into two subgroups: adenomatous and hyperplastic. Hyperplastic polyps are a non-cancerous growth of extra cells that project out from tissues inside the body, and only occur in areas where the body has repaired damaged tissue, especially along the digestive tract (Jain & Chetty, 2009; Janjua *et al.,* 2015). Adenomatous polyps (adenomas) develop when there is an increased risk of metachronous and synchronous development of colorectal malignancies. When the adenomatous process invades through the muscularis mucosa, the polyp is then defined as a malignant process and may cause visible protrusions of the colonic mucosa into the lumen of the colon (Hall, 2015). It is formed when there is a loss of cellular replication control, differentiation, and apoptosis (Wong, 2011).

Adenomas are associated with dysplasia where the classification of adenomas are based on the amount of dysplasia present (Myers & Arora, 2021). Dysplastic cells (dysplasia) show cellular changes that suggest a loss of reproductive and growth mechanisms. Adenomas with low-grade dysplasia can show nuclear changes, whilst adenomas with high-grade dysplasia can have both cellular and nuclear changes, with invasive cancer (pre-cancerous polyp) (Hall, 2015). Once polyp is in severe dysplasia, it increases the risk of evolution into adenocarcinoma (malignant adenoma) and cancer (**Figure 1.1**) (Hall, 2015).

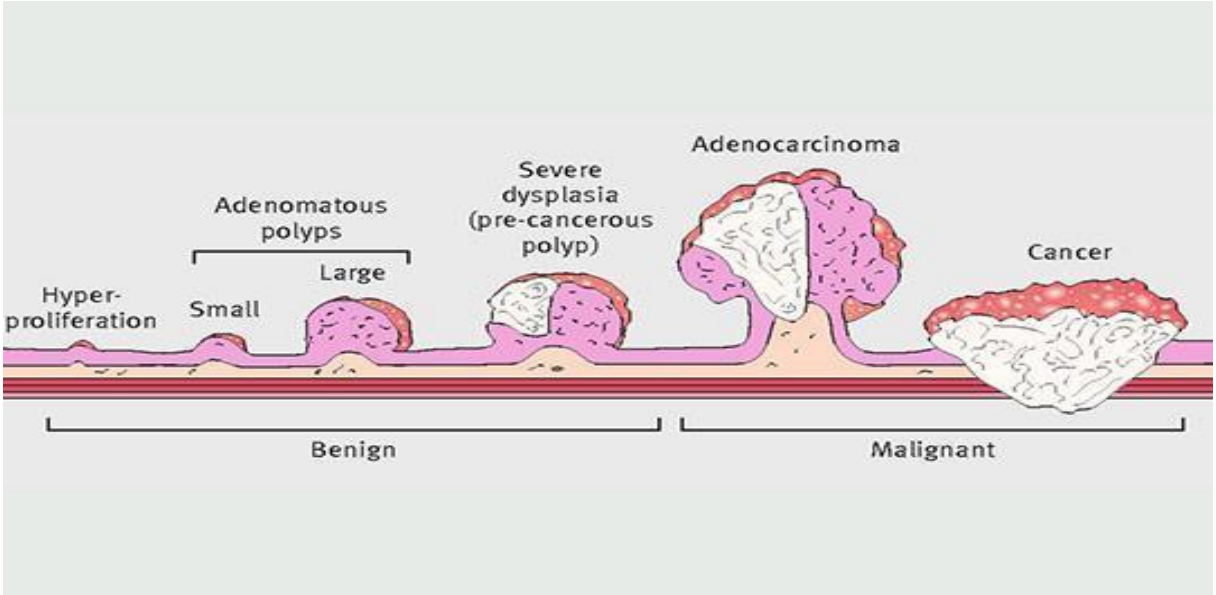


Figure 1.1: Transition of benign polyp to malignant cancer cell (Thrumurthy *et al.*, 2016)

The incidence rate of developing CRC is influenced by modifiable and non-modifiable risk factors (Wang *et al.*, 2019). These factors explore the role of heritable colon cancer syndromes and genetics, along with medical comorbidities that influence the risk (Peterson, 2015). Non-modifiable risk factors such as ageing, hereditary and genetic factors are uncontrollable, as these are mainly caused by the epigenetic and genetic changes in their colorectal epithelial cells, resulting in the development of cancer. Whilst modifiable risk factors (lifestyle-related behaviours) associated with CRC include obesity, smoking and high alcohol consumption can be controlled by managing healthy diets and lifestyles (Boden-Albala & Sacco, 2000).

By understanding the cause, pathogenesis, and risk factors susceptible to the invasion and inflammation of CRC, further assessments governing the approach on diagnosis

and treatments specific to the symptoms of CRC cases are highly required to hinder the prognosis.

1.3 Symptoms, Diagnostics and Treatments

CRC does not cause any symptoms (which is undetected) at an early-stage and can only be diagnosed when the disease has progressed into advanced stages (stage II and beyond). Possible signs of CRC in stage II or stage III include, rectal bleeding (with bright red blood), blood in the stool, unexplained weight loss, a changing bowel habit (at least three months) and abdominal pain/ cramps (Arroll, 2011). Blood loss with low red blood cell counts (anaemia) and the presence of tumour markers (commonly carcinoembryonic antigen) in CRC can be diagnosed through a blood test.

However, when vigorous symptoms of CRC metastasis such as jaundice, breathing difficulties, blurry vision, bone fractures and chronic headaches were detected, the presence of liver protein (alpha fetoprotein, AFP) will then be investigated by an AFP tumour marker test, since the liver serves as the dominant metastatic site for patients with CRC. Thus, the existence of AFP is therefore served as tumour marker for stage IV colon cancer, where spreading of them from the colon to multiple distinct organs are indicated (Nagpal *et al.*, 2016). This AFP tumour marker test cannot be used alone and is usually conducted with other tests to monitor patients that have been diagnosed with CRC, this is because tumour marker level can sometimes be normal in CRC patients and abnormal for other circumstances that are not associated with cancers. Hence, AFP tumour marker test can only help diagnose cancer when it is used with the other test, to improve the value of diagnostic testing. Other than that, a stool test could be used to diagnose the cancer when blood traces are present within the stool (Ostrow, 1990).

Several CRC screening strategies involves diagnostic colonoscopy, proctoscopy, biopsy [gene test and microsatellite instability and mismatch repair (MSI/MMR) testing], stool deoxyribonucleic acid (DNA) test and imaging test (Geiger & Ricciardi, 2009). In depth, biopsy samples obtained after screening or diagnostic test (colonoscopy/ proctoscopy) are sent for lab testing to secure a better identification and classifications of cancer cells (Bubendorf *et al.*, 2017). If the biopsy samples are identified as metastatic tumour cells, a genetic test can be performed to determine a more suitable targeted therapy on specific genes and proteins. To look for suspicious metastatic areas of colorectal tumour-cells and how far the tumour has spread, imaging tests such

as computed tomography (CT) scan, ultrasound, magnetic resonance imaging (MRI) scan, chest x-ray, positron emission tomography (PET) scan and angiography can also be used as important imaging tools to create pictures of the insides of the body (Half *et al.*, 2009).

When CRC cells are tested with a higher level of genetic changes (MSI) by biopsy, further testing is needed to discover the changes in DNA MMR genes (proficient/ deficient MMR). With the combination of tests, patients with MSI/MMR changes are identified as those with Lynch Syndrome. Patients with sporadic CRCs do not have a high level of MSI/MMR changes. Hence, a prognosis of disease outcome and a better treatment option for CRC patients can be determined (Kawakami *et al.*, 2015).

The United Kingdom healthcare system, National Health Services (NHS) (2020) indicates that CRCs are mostly treated by the surgeries, and can also be cured by the chemotherapy, biological treatments (targeted therapies) and radiotherapy depending on the patient's condition. Small adenomas (< 1cm) that have tubular growth patterns discovered at an early stage can be fully cured on the treatment given and halt the abnormal cells from re-occurring (Requena *et al.*, 2017). However, most adenomas have a mixture of growth patterns: tubular and villous, which are called as tubulovillous adenomas. These growth patterns have a higher risk of developing CRC. A complete cure has not always been possible in CRC especially within the more advanced stages (tubulovillous/ villous adenomas) (adenomas > 1cm) and sometimes CRC may re-appear during these stages (Cooper, 2000; Mishra *et al.*, 2013). This suggests a combination of treatments, where radiation therapy is being used to shrink the tumour cells and relieve the symptoms before surgeries are carried out, owing to the increased chance for complete removal (Baskar *et al.*, 2012).

Chemotherapy drugs can also be administered by taking a combination of medicines either orally or intravenously after the surgery. This would minimize the risk of recurrence and metastasis of colorectal tumour-derived cells (Eccles & Welch, 2007). Furthermore, targeted therapies such as panitumumab and cetuximab are meant to inhibit ligand binding to epidermal growth factor receptors (EGFR)-proteins located on the surface of the epithelial cells, and suppress the growth of the tumours (Moriarity *et al.*, 2016). Hence, by knowing the causes, symptoms and their affected pathways, better treatment outcomes and the prognosis of cancers can be evaluated.

1.4 Chromosomal Instability (CIN)

Three molecular pathways have been recognized in colorectal carcinogenesis: chromosomal instability (CIN), MSI, and the CpG Island Methylator Phenotype (CIMP) pathways. These three pathways are not mutually exclusive, with some tumours revealing features of multiple pathways. Particularly, in MSI tumours showing varying overlapping degrees of CIN, whilst CIMP-positive CRCs often results in high MSI, due to the presence of human mutL homolog 1 (MLH-1) promoter methylation (Ogino & Goel, 2008).

CIN tumours are commonly observed in 70% to 85% of sporadic CRC cases, with no family history or apparent genetic predisposition noted in these cases. They exhibit microsatellite stability (MSS) and mainly occur in the tumours that are found proficient with DNA MMR (pMMR) (Yamagishi *et al.*, 2016). Various genes such as Kirsten rat sarcoma (*KRAS)*, B-Raf proto-oncogene, serine/threonine kinase *(BRAF),* deleted in colon cancer (*DCC*), *TP53* and cyclooxygenase-2 (*COX-2)* are found to be implicated in the pathogenesis of CRCs, whereas adenomatous polyposis coli (*APC*) gene mutation is commonly seen in sporadic CRC cases with CIN/ pMMR status. These genes can activate the Wnt/β-catenin signalling pathway, leading to an uncontrollable growth in tumour cells (Armaghany *et al.*, 2012).

CIN is a type of genomic instability, in which chromosomes are unstable, either in the entire chromosome or specific parts that have been deleted or duplicated (Wang *et al.*, 2017). Genetic instabilities in CIN phenotype that have high genetic changes (mutations) can cause an unequal distribution of DNA strands being divided into the daughter cells during mitosis (Varetti *et al.,* 2014). These genetic mutations can change the amino acid sequence of the protein encoded by DNA and alter the mechanism of several cellular pathways (Baudot *et al.,* 2010). Mutations of genes that are involved in mitosis followed by the observation of chromosome behaviour are known as chromosome segregation. In normal cells, usually 1% of cell divisions will have errors occurring during chromosome segregation, whereas in CIN, the error for chromosome segregation during cell division increases to approximately 20% (Thompson & Compton, 2011). Impairment of the mitotic spindle assembly checkpoint can cause chromosomal mis-segregation and aneuploidy through the premature separation of sister-chromatids (Potapova & Gorbsky, 2017).

CIN phenotype is defined as the defect that involves gene duplications, deletions, and chromosome rearrangements (Vargas-Rondón *et al.*, 2018). It can lead to both numerical (numerical aneuploidy) and structural chromosomal changes (structural aneuploidy) (Orr *et al.*, 2015). Numerical aneuploidy is a process where gains or losses of the whole chromosome leads to an incorrect number of chromosomes. Whereas structural aneuploidy happens when there is no change in the number of chromosomes, however there is still the existence of the gains, losses, or translocations of either part or multiple chromosomes that are being observed (Tijhuis *et al.,* 2019). Differences between both numerical and structural aneuploidy is that numerical aneuploidy results from the chromosomal mis-segregation during mitosis, whilst structural aneuploidy results in the loss of heterozygosity and affects tumour suppressor genes (Potapova & Gorbsky, 2017).

1.5 Microsatellite instability (MSI)

MSI pathway is a condition of genetic hypermutability (predisposition to mutation), that results from the bodies with MMR-deficient (dMMR) mechanism inside the tumour cells (Boland & Goel, 2010). Those that have dMMR mechanism are unable to repair mis-corporation errors during DNA replication, this may result in frame-shift mutations, thus yielding non-functional proteins (changes in amino acids) (Hsieh & Yamane, 2008). These protein malfunctions can lead to an increase in the rate of gene mutations, mostly insertion-deletion (indel) mutations and prompt the loss of MMR protein expression (Sjursen *et al.*, 2016).

Although dMMR and MSI are distinct phenomena, they are both still considered as synonymous (Kawakami *et al.*, 2015). There are approximately 12% to 15% of all CRCs shown in dMMR/ high MSI status. Of these cases, only 3% of them are discovered with Lynch Syndrome (LS); and 12% of CRCs are categorized in the sporadic cases of MSI (Newton *et al.*, 2014). In depth, sporadic tumours were observed in dMMR/ MSI phenotypes, with *BRAF* mutation and the epigenetic silencing of *MLH-1* gene that leads to the somatic loss of MMR function. (Chen *et al.,* 2017; Simpkins *et al.*, 1999). The presence of *BRAF* mutation and *MLH-1* gene methylation are rarely seen in LS. Definitive diagnosis of LS with dMMR/ MSI status can only be determined by the germline mutations within specific MMR genes (Evrard *et al.*, 2019). These specific genes are MutS homologs (*MSH-2, MSH-6*), *MLH-2* and *EPCAM*, which mutation of each gene can promote tumorigenesis.

Although LS and sporadic cases of MSI share the same dMMR mechanism, both can still be distinguished from the other. This is because dMMR/ MSI sporadic tumours both reveals the methylation of *MLH-1* gene promoter and the presence of *BRAF* mutation in codon 600, while LS works only on specific genes. However, there are also cases where either *MLH-1* gene methylation or *BRAF* mutation were present in sporadic CRCs, this circumstance will inevitably be classified as MSI-showing degrees of CIN.

1.6 CpG Island Methylator Phenotype (CIMP)

CRC with CIMP represents an aetiologically and clinically distinct group (Mojarad *et al.*, 2013). It is categorized by 'epigenetic instability' in CRC taxonomy, and is usually *BRAF*-mutated and dMMR (MSI-high tumours). CIMP-positive tumours are often associated with poor tumour differentiation, high-grade proximal tumours and elderly persons (Issa, 2004). It serves as a potential confounding factor and is used as an emerging biomarker for CRC cells, due to its distinct clinicopathologic, molecular features and precursor lesions (Dahlin *et al.*, 2010; Zlobec *et al.*, 2011).

In CRCs, CIMP happens through an epigenetic instability pathway and is characterised by vast promoter hypermethylation of cytosine preceding guanine (CpG) island sites (Mojarad *et al.*, 2013). CpG islands are regions in the genome that are rich in CpG dinucleotides on the promoter sites (Han & Zhao, 2009). In cancer cells, CpG islands may be unmethylated or aberrantly hypermethylated, leading to transcription inactivation (switching off tumour-related genes or tumour suppressor genes) and deregulation of gene expression, which results in tumorigenesis (Ehrlich, 2009).

Genes that are identified in the CIMP pathway usually have key functions in the tumour-derived cells. As such, CpG island methylation that is the primary cause of the decay of chemokine (C-X-C motif) ligand 12 (*CXCL12*) expression in several cancers, can promote angiogenesis and the metastatic property of CRC cell lines by triggering different effects of cell signalling pathways (Itatani *et al.*, 2016). This chemokine, *CXCL12* expression can be increased in CRC due to the defect in gene regulation by DNA methylation (Wendt *et al.*, 2006).

1.7 Young-Onset Colorectal Cancer

Young-onset CRC is a 'heterogeneous disease' that possesses a strong hereditary component (Silla *et al.*, 2014). It accounts for 2% to 5% of all CRCs and can be

categorized into two distinct subtypes, sporadic and inherited, in which most of them are sporadic cases (MSS) with a lack of DNA repair mechanism abnormalities (You *et al.*, 2020). Individuals with young-onset CRC have distinctive histological features, stages of tumour presentation and site of tumour location (Ahnen *et al.*, 2014). The onset of CRC at this age group occurs mostly in the distal colon and rectum (Myers *et al.*, 2013). Since the knowledge regarding the molecular features of 'heterogeneity' young-onset CRCs is limited, these can be further researched, by comparing the molecular profile of young- and late- onsets CRCs (Ballester *et al.*, 2016).

In an evaluation of Chang et al. (2012), 75 CRC patients aged more than 40 years old participated in the study. 17 patients (22%) were diagnosed with hereditary cancer syndromes; whilst 13 patients (17%) were found with dMMR and 4 patients (5%) were identified with germline genetic disorders, predisposing to CRCs. Other 58 patients (78%) were diagnosed either with chronic inflammatory bowel disease or other genetic issues. From this study, the histologic features of young-onset CRCs were associated with poor prognosis, but the overall and disease-free survival rates were similar to the patients over 40 years old. However, relatively higher rates of metastases and locoregional recurrence were reported (Chang al. *et al.*, 2012). These two outcomes were later supported, with 59.4% of patients under 40 years old and 61.1% of patients older than 40 years old, who were identified in the five years cancer survival rate (Yeo *et al.* 2013). Despite presenting a more advanced stage in the early-onset CRC, the data series shows equivalent survival rates between the youngsters and the elders. Young CRC patients have a poorer outcome compared to the elders (Da Fonseca *et al.*, 2012). Therefore, further investigation is needed by comparing the genomic and transcriptomic profiles of sporadic cases in the early-age-of-diagnosis and lately-age-of-diagnosis CRC.

Patients with young-onset CRC have no known predisposing genetic risk factors, late diagnosis, and poor outcomes (Dozois *et al.*, 2008). Whilst individuals with predisposing hereditary syndrome can increase the likelihood of developing CRC based on a person's genetic makeup (Jasperson *et al.*, 2010).

1.8 Hereditary nonpolyposis colorectal cancer (HNPCC)/ Lynch Syndrome (LS)

Hereditary CRC syndrome that is caused by germline mutations, accounts for 3% to 5% of all CRC cases. It falls into two subgroups: hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (Giardiello, 2011).

HNPCC patients develop adenomas at the same frequency as the normal population but display a dramatic increase in the adenoma-to-carcinoma transition (Baucom & Wise, 2012). Whilst, FAP patients have plenty of benign adenomas, in which their numbers of adenomas are directly proportional to the probability of tumour development (Markowitz & Winawer, 1997).

Although LS, also known as HNPCC, is a type of inherited cancer that has contributed to the genetic predisposition of cancers such as endometrial cancer, ovarian cancer, stomach cancer and small intestine cancer. In CRC, LS has appeared in 13% of sporadic cases. It still stands a higher lifetime risk (50 to 70% risk) to get other types of cancers in LS patients (Jasperson *et al.*, 2010). Clinicians indicate that out of 3 of every 100 (3%) colon cancers or endometrial cancers are triggered by LS (Haraldsdottir *et al.*, 2016), which specifies people who have LS have significantly increased risk of developing CRCs.

As LS can be passed from generation to generation, genetic and epigenetic alterations (loss of protein expression) observed in DNA dMMR adenomas [compromised to *MSH2*, *MSH6, MLH-1, PMS1 Homolog 2 (PMS2)* and epithelial cellular adhesion molecule *(EPCAM)*], were reported to be responsible for tumour-initiating and promoting events (Ruiz-Bañobre & Goel, 2019). There are also hyperplastic benign polyps and benign adenomas in LS patients. Hyperplastic polyps in LS patients do not show signs of MSI, whereas adenomas in LS patients, show dMMR/ MSI status, with an increased dysplasia and risk of CRC cancer (Chen *et al.*, 2017; Conteduca *et al.*, 2013). Previous study also suggests that the emergence of adenomas (without MMR mutation) in lately-onset CRC and LS are considered synchronous, though the carcinogenesis adenoma-to-carcinoma progression was gradually accelerated in LS patients within 2 to 3 years (Møller *et al.*, 2017). This becomes highly conscious as the incidence of LS has rapidly increased the risk of colonic tumours in recent years. This cancer linked to the syndrome usually develops when individuals are relatively young.

1.9 Familial adenomatous polyposis (FAP)

FAP is an autosomal dominant inherited pre-malignant disease, characterized by numerous adenomas in the epithelium of the large intestine (Murgic *et al.*, 2014). It begins the transition from benign adenomas to malignant cancer cells, when no proper treatment is given during early-age onset CRC (Half *et al.*, 2009). Inheritance FAP is the most common and severe polyposis that is caused by a tumour suppressor gene

mutation. It is divided into two main subtypes, classic FAP and attenuated FAP (AFAP) (Claes *et al.*, 2011). The reported incidences for both FAPs, accounts less than 1% (0.003% to 0.014%) of the total CRC cases (Yorke *et al.*, 2020). It is caused by the germline mutation of adenomatous polyposis coli (*APC)* gene (chromosome 5) in both classic FAP and AFAP. This mutation can affect the normal growth and function of cells, which then leads to the formation of colon polyps. Individuals with AFAP may develop polyps later in life compared to people with classic FAP. Individuals with AFAP also possess a lower lifetime risk with fewer polyps (20-100) compared to individuals with classic FAP (Mitchem & Hall, 2016). On the other hand, polyps ranging from hundreds to thousands are being observed in individuals with classic FAP, which drives a higher lifetime risk of CRCs compared to individuals who have AFAP (Jasperson *et al.*, 1993).

1.10 *MUTYH* (MYH)-associated polyposis (MAP)

MYH-associated polyposis (MAP) is an inherited condition identified by the growth of multiple adenomas (Venesio *et al.*, 2012). This condition, is a milder form of FAP, also called autosomal recessive familial adenomatous polyposis, as it is manifested by the inheritance of both parents (carriers) who have the prevalence of autosomal recessive conditions imputable to mutY DNA glycosylase (*MUTYH)* gene mutation (Leoz *et al.*, 2015). This gene encodes DNA repair enzyme (MUTYH glycosylase) which is embroiled in both base excision repair and oxidative DNA damage repair pathway. Mutation of this enzyme can cause tumorigenesis, as failures on correcting errors in DNA replication could change the pairing of guanine-adenine to guanine-cytosine (Ide & Kotera, 2004; Kairupan & Scott, 2007). Mispairing of DNA nucleotide bases will increase the likelihood of cell overgrowth, resulting in colon polyps and CRCs.

MAP originates from the mutation of *MUTYH* gene on chromosome 1 (Aretz *et al.*, 2014). It is a possible diagnosis found in patients with multiple adenomatous polyps. This MAP phenotype is found to be similar to the FAP and AFAP germline phenotypes, with none of the alterations in *APC* gene shown (Sokic-Milutinovic, 2019). Patients with MAP could grow many adenomas in the rectum and colon, which could then lead to the progression of the severe stage of cancer. At this advanced stage, surgery may be required to minimize the risk (Hall, 2015). By comparing MAP and FAP, people with FAP or AFAP are diagnosed with fewer polyps (lower than 20) at a younger age, whilst polyps in MAP usually grow much later during adulthood (Half *et al.*, 2009).

1.11 PPAP

PPAP is a new, dominantly inherited, highly penetrant syndrome of oligo adenomatous polyposis and the early-age-of-diagnostic CRC/ endometrial cancer (Church, 2014). It is relevant to the germline missense mutations in the exonuclease (proofreading) domains of two DNA polymerases genes, DNA polymerase epsilon (*POLE)* and DNA polymerase delta *(POLD1*) in hereditary CRC predisposition (Spier *et al.*, 2015). PPAP which has the loss of proofreading capability, is caused mainly by dysfunctional polymerases, and can lead to multiple mutations throughout the genomes (Church, 2014). Furthermore, PPAP can be presented as MSS/ CIN phenotype, with pMMR (Glaire *et al.*, 2017; Kawakami *et al.*, 2015). *POLE* or *POLD1*-exonuclease domain mutated tumours are different from those that are mutator phenotype (caused by dMMR) in autosomal dominant genetic disorder, LS. This is mainly because the proportion of MMR/ MSS holds between PPAP and LS are completely different, with LS in all individuals are dMMR/ MSI-high status, as well as pMMR/ MSI-low status in PPAP (Nojadeh *et al.*, 2018).

1.12 Mechanisms of CRC carcinogenesis

Most CRCs happen sporadically due to the epigenetic modifications and the genetic mutations of the human genome (Bardhan & Liu, 2013). Epigenetic modifications can enhance genomic mutagenesis and instability, whereas genetic mutations can modify several epigenetic controls (Hong, 2018). Recently, next general sequencing (NGS) has revealed genetic mutations collaborated with epigenetic modifications in various types of cancers (Park *et al.*, 2011). There is crosstalk between both mechanisms (You & Jones, 2012). These mutations (epigenetic modifications and genetic mutations) can alter histone modification, cytosine methylation and nucleosome organization (Miller & Grant, 2013). They can also trigger the progression from normal mucosa towards carcinomas by affecting the signalling pathways involved in CRC (Okugawa *et al.*, 2015).

Meanwhile, DNA MMR genes attributed to epigenetic silencing can enhance genomic instability and the mutations of tumour suppressor genes and oncogenes (Shen & Laird, 2013). Like other types of cancers, colon or rectal cancers (colorectal carcinomas) can occur when the alterations on tumour suppressor genes, target oncogenes or genes related to DNA repair mechanisms, are present to promote the progression of tumours (de Leon & Percesepe, 2000).

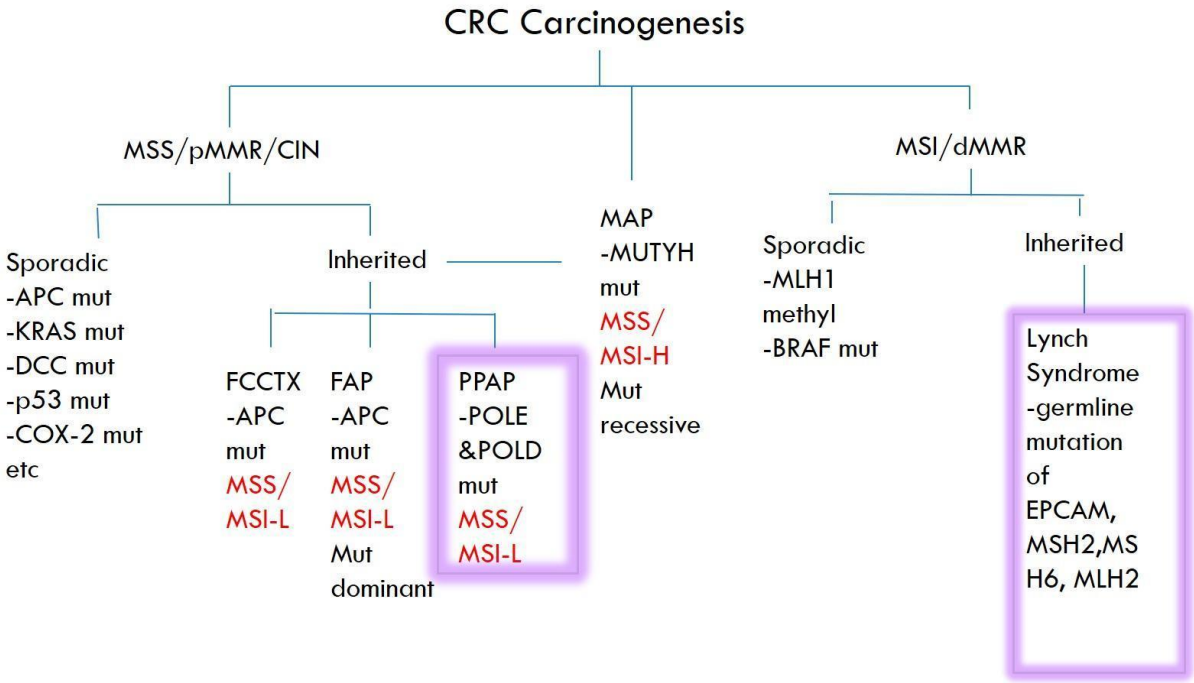


Figure 1.2: Target genes and microsatellite stability in CRC carcinogenesis. CRC-colorectal cancer; MSS-microsatellite stability; MSI- microsatellite instability; pMMR- proficient mismatch repair gene; dMMR- deficient mismatch repair gene; CIN- chromosome instability; mut- mutation; FAP- Familial adenomatous polyposis; PPAP- Polymerase Proofreading-associated polyposis; MAP- MUTYH-associated polyposis

Colorectal carcinomas can be classified as sporadic (70%), familial (25%), and inherited (5%) (Mármol *et al.*, 2017). This becomes pertinent when considering that CRCs arise from either one or a combination of different pathogenic mechanisms (CIN, CIMP and MSI), which could alter the important signalling pathways [*TP53*, transforming growth factor-beta (TGF-β), mitogen-activated protein kinase (MAPK/PI3K)] caused by mutated genes (Hong, 2018).

As shown in **Figure 1.2**, CIN pathway that serves as the major pathway of sporadic CRC is triggered when the tumours have the acquisition of *APC* mutations, along with an inactivation of tumour suppressor gene *TP53* and the mutational activation of *KRAS* gene (Gonzalez *et al.*, 2017). Loss of heterozygosity (LOH) and aneuploidy also serves as critical roles in CIN tumours, that may also be involved in FAP cases associated with germline mutations in the *APC* gene (Tariq & Ghias, 2016). Tumours with the dMMR/ MSI in CRCs, serve as the hallmark condition in LS (Boland & Goel, 2010). As

stated above, LS can be identified by the mutations of various genes (*MSH2, MSH6, MLH2 and EPCAM*), whilst in sporadic MSI cases, only *MLH-1* and *BRAF* genetic and epigenetic alterations can be observed. Hypermethylation of MMR genes can trigger MSI pathway and are found associated with the CIMP pathway (McCabe *et al.*, 2019). This CIMP pathway is specified by the promoter hypermethylation of tumour suppressor genes, which mainly of them are *MLH-1* and methylguanine methyltransferase (MGMT) genes (Wajed *et al.*, 2001).

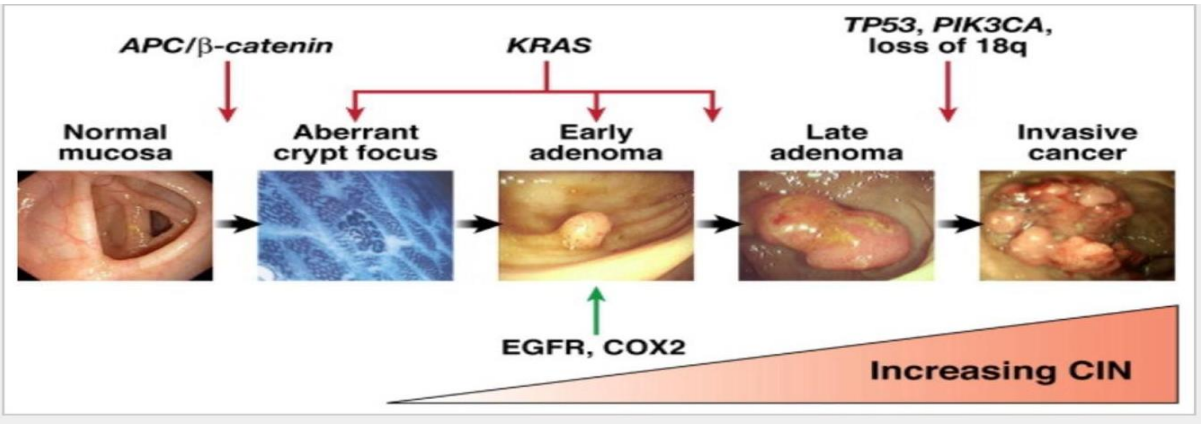


Figure 1.3: Stepwise genetic model of colorectal carcinogenesis. With the initial step of aberrant crypt foci (ACF) formation, it triggers Wnt signalling pathway and results in adenomatous polyposis coli (*APC)* gene mutation. Progression to larger adenomas and early carcinomas involve activation of proto-oncogene *KRAS* mutation, tumour protein 53 (*TP53)* mutation and loss of heterozygosity at chromosome 18q. Mutation of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA)* frequently occurs throughout colorectal cancer in adenoma-carcinoma sequence. Chromosomal instability (CIN) has been recognised in normal mucosa and increases in high level alongside each other during tumour progression (Pino & Chung, 2010).

Mutations in some oncogenic genes [*KRAS*, *BRAF*, phosphatase and tensin homolog *(PTEN)*, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA)*] and the modifications in non-coding ribonucleic acids (ncRNAs) such as miRNA/ lncRNA, are contributed to the CRC carcinogenesis process. They can serve as useful predictive markers for better identification, prognosis and treatment selection (Lizarbe *et al.*, 2017). This may result in the progression from benign adenomatous polyps to advanced adenocarcinomas (**Figure 1.3**), when those gene mutations are diagnosed with aberrant genomic copy numbers and higher gene expression levels (Hong, 2018). By understanding the mechanism of CRC carcinogenesis, it gives an idea on how CRC carcinogenesis happens in sporadic, familial, and inherited cases.

1.12.1 *APC* gene

Genomic alterations harbouring the CIN subtype of CRC primarily starts from the protein-truncating mutations on the *APC* suppressor gene (Zhang & Shay, 2017). This *APC* gene is located in chromosome 5q21-q22. In normal cells, the *APC* gene in charge of suppressing the canonical Wnt pathway is found responsible for controlling the differentiation of cells (Schneikert & Behrens, 2007). As such, the mutated *APC* gene that is found in 80% of FAP germline mutation can alter the Wnt pathway and leads to an uncontrollable cell growth (Yedid *et al.*, 2016).

The Wnt signalling pathway is the main mediator of tissue repair and homeostasis (Whyte *et al.*, 2012). It is believed that the hyperactivation of the Wnt pathway is being initiated and driven in most CRC cases (Schatoff *et al.*, 2017). Mutation in genes such as axis inhibition protein 2 (*AXIN2),* Naked cuticle 1 *(NKD1),* transcription factor 7-like 2 *(TCF7L2) and* (sex determining region Y) -box 9 (*SOX9)* genes are associated with aberrant Wnt activation. They can stabilize β-catenin in tumorigenesis (Shang *et al.*, 2017). This comes to an extent where the signalling pathway can regulate cell differentiation and proliferation in the intestine, thereby fosters growth, differentiation and apoptosis to maintain tissue-specific stem cell compartment and the initiation of carcinogenic mechanisms (Flanagan *et al.*, 2018).

The activated Wnt/ β-catenin pathway suggests that the Wnt ligand binds on the cell surface of seven-pass transmembrane Frizzled-10 (FZD10) receptor and low-density lipoprotein receptor-related protein (LRP) receptor complexes. This binding will lead to the disruption of the β-catenin destruction complex [*APC*, AXIN, glycogen synthase kinase 3beta (GSK3β), casein kinase 1 (CK1)] and the membrane recruitment of key scaffold proteins [(AXIN, dishevelled (DVL)) (MacDonald *et al.,* 2009). It activates Dvl in humans through the activation of casein kinases, which subsequently inhibits the formation of *APC*-β-catenin complex and suppresses the ability of *APC* gene to prevent uncontrollable growth of cells (Stamos & Weis, 2013). Free β-catenins will accumulate within the cytoplasm and flow into the nucleus to form complexes with the T-cell factor (TCF) family and other transcription factors. With higher amounts of β-catenins bound to the transcription factors, it can lead to cell proliferation and differentiation (Zwezdaryk *et al.*, 2016).

However, the Wnt pathway and EGFR signalling play a major role in cell proliferation and embryonic development (Paul *et al.*, 2013). Dysregulation of these two pathways

can lead to tumorigenesis with a poor prognosis (Pohl *et al.*, 2017). In depth, Wnt ligands can activate EGFR signalling through the FZD10 receptor and form EGFR-β-catenin complex, whereas EGFR signalling can regulate β-catenin localization and stability through receptor tyrosine kinase-PI3K/Akt pathway. This cross-communication between EGFR and Wnt/β-catenin pathway demonstrates that the aberrant accumulation of β-catenin triggered by EGFR activation can promote metastasis and the invasion of tumour-derived cells (Hu & Li, 2010).

EGFR is a transmembrane protein receptor of the four ErbB family members. It selectively binds to seven different ligands upon activation (EGF, TGFα) (Carrión-Salip *et al.*, 2012). High EGFR expression present in most human epithelial cancers can serve as a target for cancer treatment (Sigismund *et al.*, 2018). Prostaglandin E2 (PGE2), a product of *COX-2* activity can also promote colon cancer development by either activating EGFR or the formation of EGFR-β-catenin complex in the Wnt pathway (Sun & Li, 2018). Thus, both mutated EGFR and APC genes can stabilize β-catenin in tumorigenesis, and promote colorectal tumours. Since both Wnt and EGFR signalling pathways correspond to β-catenin, this brings an understanding where β-catenin expression levels are highly concerned on cancer cells.

The activation of Wnt/ β-catenin pathway and upregulation of PGE2 levels will allow EGFR to transactivate through extracellular stimuli. In the meantime, the mutation of EGFR that correlates with EGFR activation is found in resistance to anti-EGFR treatment (Gazdar, 2009; Hu & Li, 2010). Mutation on the main downstream effector of the EGFR pathway such as *BRAF*, *KRAS* and *PIK3CA* genes that signals both in MAPK and PI3K/AKT pathways will also serve as strong predictors for anti-EGFR resistance (Zhao *et al.*, 2017). Even though there is effective usage of anti-EGFR antibodies such as cetuximab and panitumumab in therapies treatment, the cell development and invasion of tumour-derived cells in lately-onstage CRC are still uncontrollable (Di Fiore *et al.*, 2010). Thus, the anti-EGFR and drug resistance triggered by mutated *BRAF*, *KRAS* and *PIK3CA* genes can promote EGFR signalling pathway and *APC* gene mutations, as well as the Wnt/ β-catenin pathway.

1.12.2 KRAS, PIK3CA & BRAF

Aside from *APC* mutation, mutated *KRAS*, *BRAF* and *PIK3CA* genes also play a predominant role in the transition of adenomas to adenocarcinomas (Armaghany *et al.*, 2012). There is about 30%- 50% of colorectal tumours that have *KRAS* mutation, 10%

of colorectal tumours have *BRAF* mutation and 15%-20% of colorectal tumours have *PIK3CA* mutation (Dankner *et al.*, 2018; Yoon *et al.*, 2014). Mutant *KRAS* and *BRAF* genes can activate the MAPK signalling pathway that controls important cell functions in tumour-derived cells (Hymowitz & Malek, 2018). Differences between both genes are clearly shown, with the *KRAS* gene needing RAS activation to trigger the MAPK signalling pathway; whilst the *BRAF* gene is independent of RAS activation. The *PIK3CA* gene can be triggered in multiple signalling pathways (PI3K/ AKT/ mTOR pathway and MAPK pathway) with or without RAS activation.

Prevalence of *KRAS* mutation can be observed on codon 12 or codon 13 (exon 2) (Yoon *et al.*, 2014). It triggers K-Ras protein on the signalling RAS/MAPK pathway (Wan *et al.*, 2019). K-Ras protein relays signals from outside the cell to the nucleus, this signal can instruct the cell to proliferate, grow and differentiate in CRCs (Zenonos, 2013). CRC cells derive from normal colonic mucosa by stepwise mutations can transfer normal epithelial cells to malignant tumour cells (Hong, 2018). GTPase, an enzyme that can either binds to guanosine triphosphate (GTP) or hydrolyses it to guanosine diphosphate (GDP), acts as a molecular switch in many fundamental cellular pathways (Tripathi *et al.,* 2016). To transmit signals, Ras can be activated by binding to GTP molecules, and it can also be inactivated when it converts from GTP to GDP. When Ras-GTP is formed, this enzyme allows *KRAS* gene to activate the MAPK signalling pathway, through the binding of growth factor receptors and the extracellular region of receptor tyrosine kinases (RTKs) (Du & Lovly, 2018). Downstream activation of MAPK signalling will trigger the progression and invasion of tumour-derived colorectal cells (Blaj *et al.*, 2017). In *KRAS* wild-type tumour cells, EGF binds to EGFR, which then triggers the activation of GTP-bound form of RAS and leads to the phosphorylation of specific MAPK/ERK-activating kinase (MEK) into phosphorylates extracellular-signal-regulated kinase (p-ERK) (Mebratu & Tesfaigzi, 2009). Additionally, the involvement of GTP-bound RAS in cancer was found contributing to the expression of MAPK target genes through ETS Like-1 protein (ELK1) and activator protein 1 (AP1) transcription factors, would potentially promote tumour progression (Adiseshaiah *et al.*, 2008). This gain-of-function mutation of *KRAS* will cause an increase in the signalling of transcriptional activation, which leads to the aberrant stimulation of MAPK signalling and enhancing further cell invasion and metastasis (Brandt *et al.*, 2019).

Simultaneously, *BRAF* mutation encodes a member of the Raf kinase family (Bond & Whitehall, 2018; Dankner *et al.*, 2018). It provides instruction to make a protein that transmits chemical signals from outside the cell to the nucleus and triggers the same RAS/MAPK pathway as *KRAS* and *PIK3CA* genes. Overactivation of *BRAF* can also result in aberrant MAPK signalling (Paton *et al.*, 2020). The most common *BRAF* point mutation is *BRAF V600E* (GAG to GTG, which results in a valine to glutamic acid transition at position 600). This mutation can activate the MAPK pathway independent of RAS activation (Oikonomou *et al.*, 2014).

PIK3CA is a catalytic unit of PI3K mutation that can promote cell invasion and differentiation (Castellano & Downward, 2011). This *PIK3CA* mutation in CRC cases is normally reported in exon 9 and exon 20 (Mei *et al.*, 2016). Its functions relate to the ability of class I PI3K, to activate protein kinase B (AKT) in the PI3K/ AKT/ mTOR pathway. There are three classes of PI3K: class I PI3Ks, AKT and mTOR. Class I PI3K is a dimeric enzyme that has a catalytic and a regulatory subunit. The catalytic subunit comprises of four isoforms: p110α, p110β, p110γ and p110δ. These isoforms will bind with regulatory subunits; as for p110α, p110β and p110δ, the standard regulatory subunit is p85, whilst in p110γ, the regulatory subunit is p101 (Arcaro & Guerreiro, 2007). *PIK3CA,* is a gene that encodes p110α, and is frequently mutated in various cancers (Ligresti *et al.*, 2009). p110α has gain-of-function mutation in enzymatic and MAPK/ ERK signalling activity in oncogenic cells, subsequently binds to p85 in the absence of RAS binding (Castellano & Downward, 2011).

In normal cells, cytosolic PI3Ks are recruited to plasma membranes by upstream signals. It is activated by RTKs or G-protein coupled receptors (GPCRs), and their main role is to convert phosphorylate phosphatidylinositol 4,5 biphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Cheng *et al.*, 2009). PIP_3 can control tumour cell growth and serves as a lipid second messenger molecule formed by PI3K, in response to RTKs signalling. It will recruit the proteins that have the pleckstrin homology domain to cellular membranes (Stratikopoulos & Parsons, 2016). The PI3K/ AKT/ mTOR pathway will be activated when the pleckstrin homology domain of AKT binds with Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2. As Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2 are restricted to the plasma membrane, it results in the translocation of AKT to the plasma membrane (Manna & Jain, 2015). Pyruvate Dehydrogenase Kinase 1 (PDK1) also has a pleckstrin homology domain that binds to Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2,

thus a translocation of PDK1 is also retrieved upon PI3K activation (Komander *et al.*, 2004). When AKT and PDK1 are activated at the same time, PDK1 will phosphorylate AKT on threonine 308, causing partial activation of AKT. Full activation of AKT will only be triggered when phosphorylation of serine 473 is possessed by the target of rapamycin complex (TORC) 2 complex of the mTOR protein kinase (Dangelmaier *et al.*, 2013; LoPiccolo *et al.*, 2008). mTOR is a key protein in the pathway that can upstream and downstream AKT (Laplante & Sabatini, 2012). It is active in two multiprotein complexes, TORC 1 and TORC2. It regulates protein synthesis for cell growth, angiogenesis and proliferation. An increased activity of this pathway is associated with CRCs and often are resistant to cancer therapies (Zhou & Huang, 2010).

In Class I PI3Ks of normal cells, the phosphatase *PTEN* will hydrolyse PIP_3 to PIP_2, and act as a catalytic antagonist of the PI3K pathway (Papa & Pandolfi, 2019). The balance of PI3K and *PTEN* therefore serves as an important regulator in cellular responses. In cancer cells, *PTEN* that antagonises PI3K signalling is absent from many tumours. RTKs signalling will be dysregulated and PIP_3 will maintain a higher rate as compared to PIP_2, leading to CRCs. This is mainly caused by an increased activation of the PI3K pathway, which promotes uncontrollable cell growth, differentiation, and proliferation (Chalhoub & Baker, 2009). In addition, overexpression of *EGFR* that triggers the Wnt/ β-catenin pathway can also activate PI3K pathway in CRCs, and promotes tumorigenesis by driving cell growth and transformation (Paul *et al.*, 2013).

RAS-dependent PI3K activation is directly linked to the activation of Akt (Castellano & Downward, 2011). As stated above, Akt is able to promote cell growth, it can also play an essential role in the regulation of apoptosis. Akt can phosphorylate Bad, a proapoptotic member of the *Bcl-2* family, and triggers Bad to bind with 14-3-3 inactive complexes. This binding will prevent Bad from heterodimerizing Bcl-X_L and may contribute to the development of CRCs through a different number of mechanisms (Hata *et al.,* 2015).

1.12.3 Loss of 18q

Many reports have revealed that genetic alterations such as *TP53*, *BRAF* or *KRAS* mutations are associated with poor prognosis in CRCs (Armaghany *et al.*, 2012). Another common alteration in CRC is the loss of chromosomal regions, where tumour-suppressor genes are somatically inactivated. This becomes relevant when

considering the LOH analyses have discovered the allelic loss of chromosomal arms (5q, 7q, 8p, 17p, 18q, and 22q) (Xu *et al.*, 2003). In contrast, loss of chromosome 18q, is a common event (70%) in CRCs, in which approximately 50% of them are large, late-stage adenomas and 10% of them are early-stage adenomas (Martinez-Lopez *et al.*, 1998). The tumour suppressor gene, deleted in colon cancer (*DCC*) has its locus on chromosome 18q in CRC cases (Armaghany *et al.*, 2012). The *DCC* protein that is expressed in the goblet cells of normal mucosa, can regulate cell growth through the recognition of extracellular signals. Inactivation of *DCC* will lead to the loss of normal adhesion in the colonic mucosa, in which *DCC* expressions are seen greatly reduced in 85% of CRC cell lines. However, specific genes targeted on allelic deletions have not yet been determined, where the evidence supporting the role of *DCC* gene in CRC is not well established (Mamot *et al.*, 2003).

1.12.4 DNA polymerase epsilon (*POLE)* & DNA polymerase delta 1 (*POLD1)*

POLE, is a DNA polymerase gene that displays the main proofreading function and encodes the catalytic subunit of Polε (Logan *et al.*, 2018). In cancer, errors form in improper proofreading within enzyme complexes (Polε) are used to synthesize the leading strand (5'-3' direction) during DNA replication. This leading strand will result in an increased mutation rate as the exonuclease repair of Polε is affected (Avhandling, 2008). Similarly, *POLD1* serves the same function as polymerase gamma protein (Polδ), that synthesizes the lagging strand (5'-3' direction) at the replication fork during DNA replication (Ganai & Johansson, 2016). Germline *POLD1* mutation predisposes to CRCs will exhibit increased mutation rate, when *POLD1* defects in DNA repair during DNA replication. This will result in an increased base substitution and production of DNA double-strand breaks (Nicolas *et al.*, 2016).

There is some evidence showing *POLD1* and *POLE* gene mutations that were involved in the sporadic CRC/ endometrial cancer, while *POLD1* shows fewer CRC cases compared to *POLE* (Palles *et al.*, 2013). A study from the Cancer Genome Atlas has indicated that 7 cases of 224 CRCs (3%) had *POLE* proofreading (exonuclease) domain mutations and all the cases were hypermutated in MSS/ MSI-L status. However, research by Yamaguchi et al. (2019) revealed that MSI-positive (high MSI) will also appear in *POLE* and *POLD1* genes through epigenetic silencing of the *MLH-1* gene. This is further evidenced by the bisulphite sequencing of the tumours that

associate with hypermethylated regions, where whole genome sequencing of the high MSI tumour is diagnosed to have pathogenic germline frameshift mutation in MMR.

Germline pathogenic variants in *POLE* and *POLD1* exonuclease domain can corroborate with PPAP on the loss of proofreading function. There are two specific germline 'hotspot' mutations that can cause carriers to develop CRCs or multiple colorectal adenomas (Palles *et al.*, 2013). These mutations are found related to DNA polymerase genes, *POLE* (p. Leu424Val) and *POLD1* (p. Ser478Asn), which precisely serves as a role on the hotspot missense mutation in both genes respectively (Briggs & Tomlinson, 2013). From the analysis on the National Centre for Biotechnology Information (NCBI), the hotspot mutation of *POLE* (p. Leu424Val) can be found in chromosome 12 exon 13, while hotspot mutation of *POLD1* (p. Ser478Asn) appears in chromosome 9 exon 11. Deficient proofreading repair in DNA replication is defined as the cause of tumour-derived *POLE/POLD1* CRC cells due to the presence of pathogenic germline mutations.

Cancer is initiated by several DNA damages, tumour suppressor genes, DNA repairing genes and proto-oncogenes. From the mechanism of CRC carcinogenesis (**Section 1.12**), several genes in CRCs have been reported in the progression of cancer. In this study, *TP53* and *Bcl-2* genes will be the main focus, to determine the favourable clinicopathologic parameters and the prognostic value of *TP53* and *Bcl-2* expression on CRCs. The connection between both genes requires further research in order to have a clearer understanding on how the genes are being regulated in the apoptotic pathways (**Figure 1.4**) of CRC.

1.13 Tumour protein 53 (*TP53)*

The tumour suppressor gene, *TP53* is one of the commonly mutated genes found in various types of cancers, including CRC. It maintains the homeostasis of the cell cycle and cell death (Liu & Di Wang, 2019). In response to several stress factors such as DNA damage, oncogenes, ultraviolet radiation, oxidative free radicals or the deficiencies in growth factors, *TP53* is activated. Activated *TP53* can suppress downstream target genes and regulate apoptosis, DNA repair, cell cycle arrest, angiogenesis and metastasis on tumour-derived colorectal cells (Toma-Jonik *et al.*, 2019). There are around 34% of proximal and 45% of distal colon tumours cases developing this *TP53* mutation, with a majority of them are missense mutations found

at ‘hotspots’ (exon 4-8 in chromosome 17), suggesting an oncogenic role of mutated p53 by gain-of-function mutations (Freed-Pastor & Prives, 2012; Li *et al.*, 2015). Inactivation of *TP53* is also involved in the malignant transformation of colorectal adenoma to cancer. This is due to the loss of another allele and the appearance of point mutation in one of the alleles (Haber & Fearon, 1998). Predominantly, mutant p53 in either one of the mechanisms (losing another allele/ point mutation in one of the alleles) can induce nuclear factor kappa B (NF-KB) activation and may contribute to CRCs (Nakayama & Oshima, 2019). Research by Nakayama & Oshima (2019) shows that the presence of *TP53* missense mutation and the loss of wild-type p53 serves as a vital role in the late-stage of tumorigenesis, as they can spread tumours to distant organs and promote the transition of adenoma-carcinoma, through the activation of both oncogenic and inflammatory pathway.

1.14 B-cell lymphoma 2 (*Bcl-2*)

B-cell lymphoma 2 *(Bcl-2)* is a member of *Bcl-2* family regulator proteins that affect cell death, either in promoting (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis (Marie Hardwick & Soane, 2013). It is a key regulator of apoptosis, which dysregulation can cause several pathological consequences including the development of cancer (Elmore, 2007). *Bcl-2* protein overexpression in 90% of follicular lymphomas cases were induced by the t(14;18) (q32;q21) chromosomal translocation (Masir *et al.*, 2009). The increased levels of *Bcl-2* will extend the survival of B-cells, and the accumulation of cells will likely be the targets for oncogenic events and transformation. In depth, *Bcl-2* localizes at the outer membrane of mitochondria that serves the essential role in inhibiting the activation of pro-apoptotic proteins and promoting cellular survival (Lindsay *et al.,* 2011). The release of proapoptotic factors are controlled by anti-apoptotic protein Bcl-2, which is found responsible for the caspase activation in the apoptosis pathway. However, aberrant *Bcl-2* expression can also be seen in various types of cancers including CRC, breast cancer, prostate cancer and lung cancer. This is typically because of the under-expression of pro-apoptotic genes and overexpression of anti-apoptotic genes observed in that specific type of cancer, which can inhibit cell death and enhance the progression of tumour-derived cells (Marie Hardwick & Soane, 2013).

1.15 *TP53* and *Bcl-2* connection in Colorectal Carcinogenesis

The occurrence of CRC is a multiphase procession. It involves many biomarkers, where p53 abnormal is one of the essential and familiar incidents from the transition of advanced adenoma to cancer. Recent studies have demonstrated that p53 abnormal was discovered in more than 50% of human tumour cases, and is closely related to the development, occurrence, infiltration, and metastasis of CRC (Munro *et al.,* 2005; P. Wang *et al.*, 2017). A low level of *TP53* expression was caused by missense-type gain-of-function mutations and the loss of wild-type *TP53* has been strongly associated with cell invasion and proliferation (Rivlin et al., 2011). Conversely, these findings also suggest that enforced *TP53* expression promotes cell death (apoptosis) in cells, which could be directly linked to its tumour suppressive functions (Chen, 2016).

Apoptosis, besides having a dominant role in controlling and regulating tumour development, occurrence, and treatment of CRC, it can also be examined as an irreversible process with caspase activation performing cell death or caspase-independent death pathways (Elmore, 2007; Hassan *et al.*, 2014). Evidence on the incidence of cancer cases were found to be correlated to the disturbance of balance between apoptosis, cell proliferation and the loss of control on normal apoptosis (Shan & Li, 2002). In depth, the apoptosis-related gene (*Bcl-2* family) that comprises anti-apoptotic and pro-apoptotic proteins plays a pivotal role in the apoptosis pathway.

TP53 is characterised as a major mediator of cell cycle arrest, in response to diverse cellular stresses. The choice between the cellular responses can be influenced by many factors, including *TP53* co-activators and the type of cells and stresses derived (Bieging *et al*., 2014). Interestingly, both anti-apoptotic and pro-apoptotic members of the *Bcl-2* family can result in robust mitochondrial outer membrane permeabilization (MOMP) and inevitably activating caspase cascade, in the response to apoptotic stress (Kale *et al.,* 2018). As a hallmark of cancer with overexpression of anti-apoptotic genes and under-expression of pro-apoptotic genes, tumour-derived p53 mutant defective in the transcription ability can bind DNA in a sequence-specific manner and impairs apoptosis induction to promote tumour development (Rivlin *et al.,* 2011). These changes can enhance genomic instability, resistance to apoptosis, aberrant cell cycle, invasion, metastasis, and inflammation.

Activated *TP53* stimulates two vital apoptotic pathways, mitochondrial pathway and death receptor pathway through a wide range of signals, to trigger apoptosis cell death

(Elmore, 2007). The extrinsic, death receptor pathway, is activated through the induction of genes that encode three transmembrane proteins [Fas, death receptor 5 (DR5) and P53 Apoptosis Effector Related to PMP22 (PERP)] on the cell surface, which results in triggering the caspase cascade activation and induce apoptosis (Haupt *et al.,* 2003). Whereas the intrinsic, mitochondrial pathway is dominated by the *Bcl-2* family of proteins, that is composed of anti-apoptotic members including *Bcl-2*, Bcl-X_L and Myeloid cell leukaemia 1 (Mcl-1) (contains BH1-4 domains), and the pro-apoptotic members. The pro-apoptotic members fall into two categories: (i) the MOMP effectors, *Bcl-2*-associated X protein (BAX) and BAK (contain BH1-3 domains), (ii) BH3-only protein that has a single BH3 domain (L.Omonosova & C.Hinnadurai, 2008; Shamas-Din *et al.,* 2013). This pro-apoptotic activity corresponds to the ability to perform as a transcription factor which serves as the main function of *TP53* (Ozaki & Nakagawara, 2011). In this study, the cytosolic and mitochondrial (intrinsic) p53 apoptotic pathway triggered by mitochondria and apoptotic stress stimuli, remains as the main focus to enhance transcription-independent apoptosis, leading to an irreversible cellular damage (Wong, 2011).

In an immediate response to apoptotic stress, cytoplasmic p53 is translocated to the mitochondria (Ozaki & Nakagawara, 2011). It can either inhibit or activate the multi-domain anti-apoptotic and proapoptotic *Bcl-2* family members, and can result in taking control in the amount of cytochrome c being released from mitochondria to the cytosol (Endo *et al*., 2006). Swelling of mitochondrial matrix caused by *TP53* that contributes to the secretion of cytochrome c, is triggered by the death stimuli either directly or through the transduction of *Bcl-2* family, such as BAX and BAK (Schuler *et al.*, 2000). This sheer power of death stimulus in the transcription-independent apoptotic pathway of *TP53* can dictate mitochondria to release apoptogenic factors. It will lead to an activation of caspase cascade, along with directing the cell to the death process (**Figure 1.4**) (Gupta *et al.*, 2009).

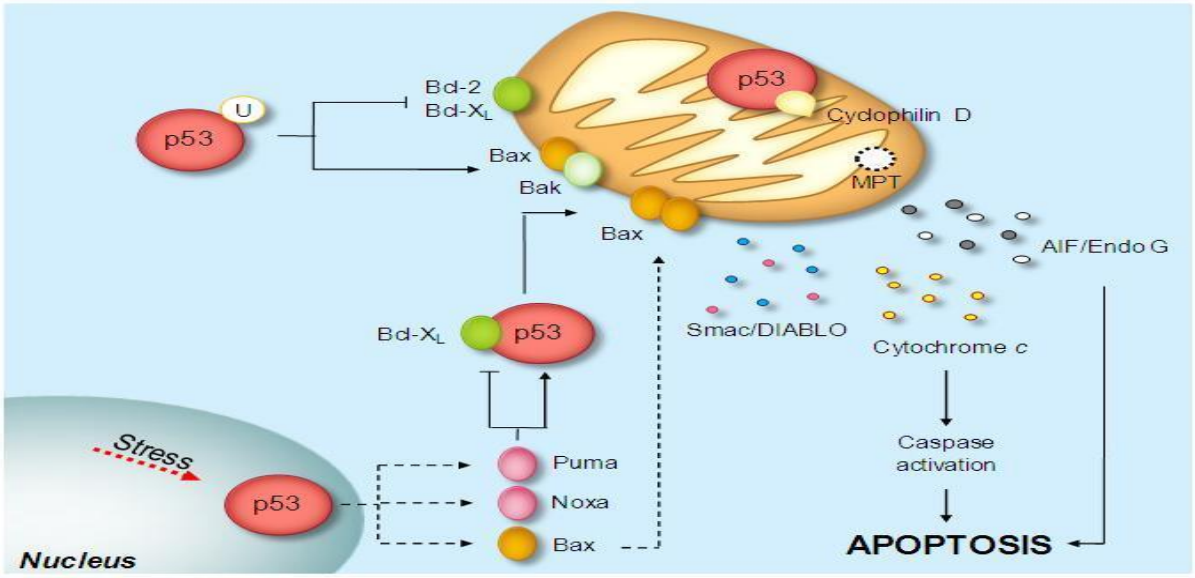


Figure 1.4: Cytosolic and mitochondrial p53 apoptotic pathway (Amaral *et al.*, 2010).

Intriguingly, upregulation of BH3-only proteins can occur either in the transcriptional or the post-translational status, in response to apoptotic-stress (Elkholi *et al.*, 2011). These proteins can be divided into two subclasses, 'activators' and 'de-repressors', depending on how they react to cell death **(Figure 1.5)**. 'Activators' of BH3-only proteins such as truncated BH3 interacting-domain death agonist (tBid) and Bcl-2-interacting mediator of cell death (Bim) can respond by direct trigger on MOMP through BAX and BAK oligomerization. Whilst 'de-repressors' including BAX, phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and p53 upregulated modulator of apoptosis (PUMA) proteins, can inhibit the anti-apoptotic members (BAX/ tBid from Bcl-X_L and BAK from Mcl1) and trigger the release of pro-apoptotic *Bcl-2* family members (Elkholi *et al.*, 2014). This 'indirect' modification suggests that anti-apoptotic (also known as 'pro-survival') *Bcl-2* proteins can adopt functions through direct-interaction with BH3-only proteins and pro-apoptotic effectors BAX/BAK, to sequester anti-apoptotic proteins away from BAX/ BAK (Campbell & Tait, 2018). Yet, both 'activators' and 'depressors' of BH3-only proteins share the similarity on activating pro-apoptotic proteins of *Bcl-2* family, to conduct DNA fragmentation, chromatin condensation and dismantling of cells.

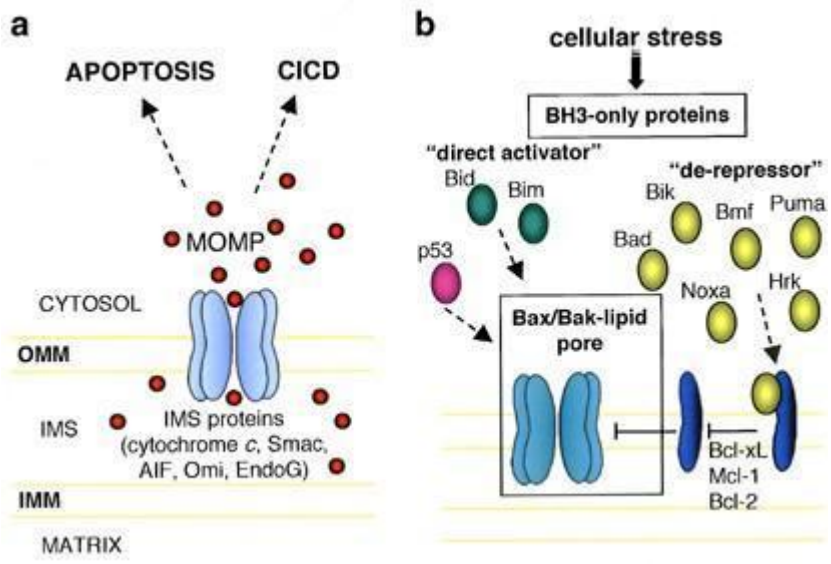


Figure 1.5: (a) Representation of mitochondrial outer membrane permeabilization (MOMP). Cellular stress activates the mitochondrial pathway and leads to the permeabilization of outer mitochondrial membrane (OMM). It allows the release of numerous intramembrane space proteins and caspase activation. (b) The Bax/ Bak-lipid pore model. Activators of BH3-only proteins can induce pore formation in OMM, whilst depressors can block the formation of pores in OMM during apoptosis (Chipuk *et al.*, 2006).

BAX, NOXA and PUMA are pro-apoptotic proteins that are retrieved from *Bcl-2* family members and are presented as the transcriptional targets of *TP53*. This is because their high expression pro-apoptotic protein levels can trigger the release of cytotoxic stimuli during p53 activation. BAX regulator that belongs to the *Bcl-2* protein family is targeted in a variety of cellular activities including cancer and apoptosis (Campbell & Tait, 2018).

In healthy cells, the *Bcl-2* family of protein that is localized at the outer membrane of mitochondria, plays an essential role in promoting cellular survival and the inhibition of pro-apoptotic proteins (Adams & Cory, 2007). This shows that inactive monomeric form of BAX appears in the cytosol or periodically within mitochondrial membranes, can shift to dimeric form and produces heterodimeric/ homodimeric state of anti-apoptotic *Bcl-2* proteins (Jeng *et al.*,2018). Contrary, these findings also suggest that active BAX in cancer cells can shift the balance towards pro-apoptotic members in the *Bcl-2* family, when the cellular levels of activator molecules exceed the neutralizing capacity of antiapoptotic members (Danial, 2007). When higher amounts of pro-apoptotic members are being released, they verify cell death via BAX/BAK-lipid pore formation

at the outer mitochondrial membranes and induce mitochondrial membrane permeabilization, subsequently secreting cytochrome c to the cytosol (Green & Kroemer, 2004).

Cytochrome c that binds to apoptotic protease activating factor 1 (APAF-1) can promote oligomerization and binds to pro-caspase 9, forming a complex known as apoptosome (Bratton & Salvesen, 2010). At apoptosome, pro-caspase 9 is stimulated through dimerization and results in the activation of downstream effector caspases, caspase-3 and caspase-7 (Parrish *et al.*, 2013). Activated caspase-3 can trigger caspase-2 to induce MOMP and strengthen BAX/BAK-lipid pore formation for apoptosis, through the activation and cleavage of pro-apoptotic *Bcl-2* family protein, Bid (Bouchier-Hayes, 2010). Moreover, caspase-3 has also been observed to release effectors such as endonuclease G (EndoG) and apoptosis-inducing factor (AIF) that are functional in caspase-dependent and caspase-independent death pathways (Arnoult *et al.*, 2003). These effectors can operate on the oligonucleosomal DNA fragmentation and induce chromatin condensation, to promote apoptosis, which work independently from Bak/ *TP53* (Sevrioukova, 2011).

Mitochondria that generate adenosine triphosphate (ATP) via oxidative phosphorylation also has a negative effect. Of which, the production of cytosolic cytochrome c from mitochondria will disable energy production and instigates proteolytic cascade, to dismantle the cells (Gupta *et al.*, 2009). This will only happen when there is release of cytochrome c and an absence of functional apoptosome, where cells that are supposed to undergo apoptosis, failed to undergo cell death and still maintains in a high metabolism for a couple of days before dying (Ferraro *et al.*, 2008).

The mitochondrial *TP53* pathway, an alternative of cytosolic p53 apoptotic pathway, can also stimulate Bax activation. There are similarities with Bax activation in these two pathways (mitochondrial and cytosolic *TP53* pathway), along with the differences between them of the *Bcl-2* family getting involved (Wolff *et al.*, 2008). Thoroughly, pathways can be clearly shown, with mitochondrial *TP53* pathway involving the *Bcl-2* members, whilst the cytosolic TP53 pathway does not.

Inactive cytosolic *TP53* held by pre-existing soluble p53-Bcl-X_L complex in the cytosolic pathway, can be activated upon stress stimuli. This comes to an extent when nuclear

TP53 transcriptionally triggers PUMA expression, in response to DNA damage. PUMA serves as a key mediator in the cytosolic proapoptotic p53 pathway, by freeing inactive cytosolic p53 from its p53-Bcl-X_L complex and binds with Bcl-X_L. Hence, free *TP53* has been used to activate BAX/BAK and caspase cascade via MOMP (Chen, 2016). However, PUMA can only reverse the apoptosis-suppressing effect of wild-type Bcl-X_L, but not mutant Bcl-X_L. This finding implies that the binding of *TP53* and mutant Bcl-X_L does not trigger the apoptotic cascade formation (Wolff *et al.*, 2008).

Studies have shown that PUMA interacts with *Bcl-2* family anti-apoptotic members can promote apoptosis by selectively displacing *TP53* from anti-apoptotic Bcl-X_L via allosteric mechanism (Edwards *et al.*, 2013). This clearly verifies that the 'indirect' model of apoptosis in cancer cells is involved in the BAX oligomerization and the activation of mitochondrial dysfunction, when BAX/BAK is being triggered. It is considered that the release of mitochondrial pro-apoptotic proteins, second mitochondria-derived activator of caspase (SMAC), cytochrome c, and apoptotic-inducing factor can account for caspase activation and cell death (Kalkavan & Green, 2018). However, NOXA pro-apoptotic proteins that can trigger the BAX/BAK regulator work less effectively compared to PUMA in p53-mediated apoptosis. It can only antagonize Mcl-1 and A1 proteins to induce apoptosis. This shows that PUMA are more functional compared to NOXA as it can bind to all anti-apoptotic members and perform both Mcl-1 stabilization and apoptosis activation (Van Delft & Huang, 2006). It is proposed that the functional overlap between PUMA and NOXA in apoptosis caused by DNA damage, reveals that to some extent they may cooperate in the progress of apoptosis.

Therefore, within the cytosolic and mitochondrial p53 pathway in colorectal carcinogenesis, mutated *TP53* can activate *Bcl-2* gene to promote cell death. However, if the activation of *Bcl-2* gene is abnormal (mutated *Bcl-2*), it appears to be an early event on colorectal tumorigenesis and triggers tumour progression. Thus, to identify the prognosis of colorectal tumours, several analyses have been performed. It has been reported that elevated *TP53* expression (high mutated-*TP53*) was significantly and positively correlated with a low level of *Bcl-2* family expression (low mutated-*Bcl2*) on overcoming apoptosis resistance, suggesting a good survival in colorectal patient (Chan *et al.*, 2000). Studies by Jiang and Milner (2003) suggests that neither mutated *Bcl-2* nor mutated p53 require Bax and caspase-2 as important apoptotic mediators,

and can be activated by targeting *Bcl-2* in colorectal carcinoma cells. In CRC carcinogenesis, mutation of *TP53* and *Bcl-2* genes have resulted in a high apoptotic/ proliferation ratio, which can commonly be identified in high-grade tumours (Chan *et al.*, 2000). This significantly shows that the phenotypic features of MSI-high tumours are correlated with an increased tumour infiltrating lymphocytes, indicating a high immune response and favourable survival rates (Sinicrope *et al.*, 2007).

Though in this study, analysis is not done on PPAP, one of the hereditary disorders in CRC cases. In fact, a total of 20% of CRC cases were accounted for in hereditary genetic disorders such as Lynch syndrome, FAP and chronic intestinal inflammation. Susanti *et al.* (2018) has clarified a simple and rapid screening test for LS. It is to refine the diagnostic tool that is being used and to find out the comparison between youngsters and elders, either genomics or transcriptomics of them. PPAP is added to refine the easy and speedy screening diagnostic tool in low-income countries. Despite using high-cost NGS, it has become consistent where PPAP, one of the inherited-type syndromes cannot be diagnosed clinically. However, there are articles that mention the similarity of the survival rates between the youngsters and elders in CRC cases, despite having a higher risk of distant metastases and the incidence of recurrence in the youngsters (Chang *et al.*, 2012; Yeo *et al.*, 2013). This leads to the comparison between the genomics or transcriptomics early- and lately- onset of CRCs, where hereditary cases need to be excluded, so that sporadic cases can be tested in the early- and late- onset CRCs.

Nonetheless, the gene analysis of this project has changed from DNA *POLE and* DNA *POLD1* to *TP53* and *Bcl-2* genes. Yet, the main approach of colorectal carcinogenesis is still the same, which is caused by the alterations in oncogenes, tumour suppressor genes and DNA genes. In CRC, colorectal carcinogenesis arises when there is a progressive accumulation of multiple genetic mutations. They can trigger the transition of normal mucosa to benign adenoma to severe dysplasia and finally to frank carcinoma. To examine the prognosis effect of colorectal tumours, *TP53* and *Bcl-2* immunohistochemically stained tumour/ normal colorectal tissues have been validated using CaseViewer 2.4 (3DHISTECH) digital image analysis, to generate H-score manually. This scoring can assess the extent of nuclear immunoreactivity in tumour/ normal CRC cells.

1.16 Coronavirus- Slight change on the project evaluation

By understanding how accelerated carcinogenesis works in the CRC status, it shows there are lots of factors that are enhancing the proliferation of CRC cells. Nevertheless, due to the restrictions around the coronavirus outbreak, the Biodiscovery Institute at the University of Nottingham has new regulations on not allowing students to access the lab. This 'moved to work from home' policy is giving the supervisors a hard moment on trying to make a slight change on this project so that students can continue to do their analysis at home, but not affecting the overall analysis. In fact, a focus on this accelerated carcinogenesis is needed to change from PPAP to mutated *TP53* gene and *Bcl-2* overexpression found in solid tumours.

2. Rational and Objectives of this study

The project changed direction, despite not affecting the overall analysis of accelerated colorectal carcinogenesis. The main approach of this project remains the same, which is the investigation of accelerated colorectal carcinogenesis caused by the alterations in different oncogenes, tumour suppressor genes and DNA genes. Therefore, to further validate the mechanisms and potentials of *TP53* and *Bcl-2* biomarkers on CRC, manual histopathological review of tumour and normal samples on these genes were also demonstrated. This analysis is thereby being conducted to compare the expression level in tumour and normal colorectal tissues, and to have an overall nuclear and cytoplasm expression view on different sites of colorectal tissues with the following objectives:

- Comparing survival rates between the young and olds
- Comparing survival rates on tumour samples in 3- and 5-years survival time
- Clinicopathological significance on highest and lowest expressors of *TP53* and *Bcl-2* biomarkers
- Relationship between both *TP53* and *Bcl-2* biomarkers
- Measure on the genetic effects of nuclear and cytoplasmic expression between both biomarkers at different stages of CRC

3. Materials and Methods (reproducible to the detailed)

3.1 Case Cohort

A cohort of 1000 cases of CRC sequentially collected between July 2008 to September 2014 was used to construct a Tissue MicroArray (TMA). Blocks of formalin-fixed paraffin-embedded tissue were retrieved from the Diagnostic Archive of the Department of Histopathology, Queens Medical Centre (QMC), Nottingham University Hospital NHS TRUST. Access to tissue was granted by Nottingham Sciences Biobank under ethical approval 05/Q/1605/66. The demographic and pathological details were retrieved by a senior research assistant.

3.2 Tissue microarray (TMA)

Colorectal TMA followed by standard protocols were prepared by the same senior research assistant as described below. The donor blocks (invariably stored formalin-fixed, paraffin-embedded tissue blocks) containing CRCs were retrieved and sectioned to produce haematoxylin and eosin (H&E) standard microscopic-stained slides. An experienced pathologist examined the slides and marked the area of interest on the H&E slides, to locate the areas on the corresponding paraffin block to be cored, which is commonly an area of cancer. A tissue microarrayer (Beecher Instruments, Wisconsin, USA) was used to obtain a tissue core from a donor block and transferred to the recipient block. This Beecher Instruments arraying device was used to produce circular cores that were 0.6-mm in diameter. A total of ~1000 patient cases were made into 15 TMA blocks; four 0.6-mm in diameter small-tissue cores (1^{st} core from the luminal surface of tumour, 2^{nd} core from the centre of tumour, 3^{rd} core from the deep margins of tumour and the 4^{th} core from the adjacent normal colonic epithelium) were collected from each representative case and organised in TMAs. Three cores were obtained from different areas of the tumour-derived cells, to verify that the samples collected were sufficient to represent for the tumours, as intratumor heterogeneity is commonly seen in the colon. TMA blocks were then cut into 4-µm sections and transferred onto slides (Surgipath Xtra Adhesive, Leica, Germany). It was used for immunohistochemical staining to determine the protein expression patterns for *TP53* (Leica MCL-L-p53-D07) and *Bcl-2* antibodies (Roche 05986826001) **(Figure 3.1).** Immunohistochemistry was performed by the pathology department at QMC using standard techniques. Immunohistochemically stained TMA sections on glass slides

were scanned at X20 magnification using 3D histech scanner (CaseViewer 2.4 software).

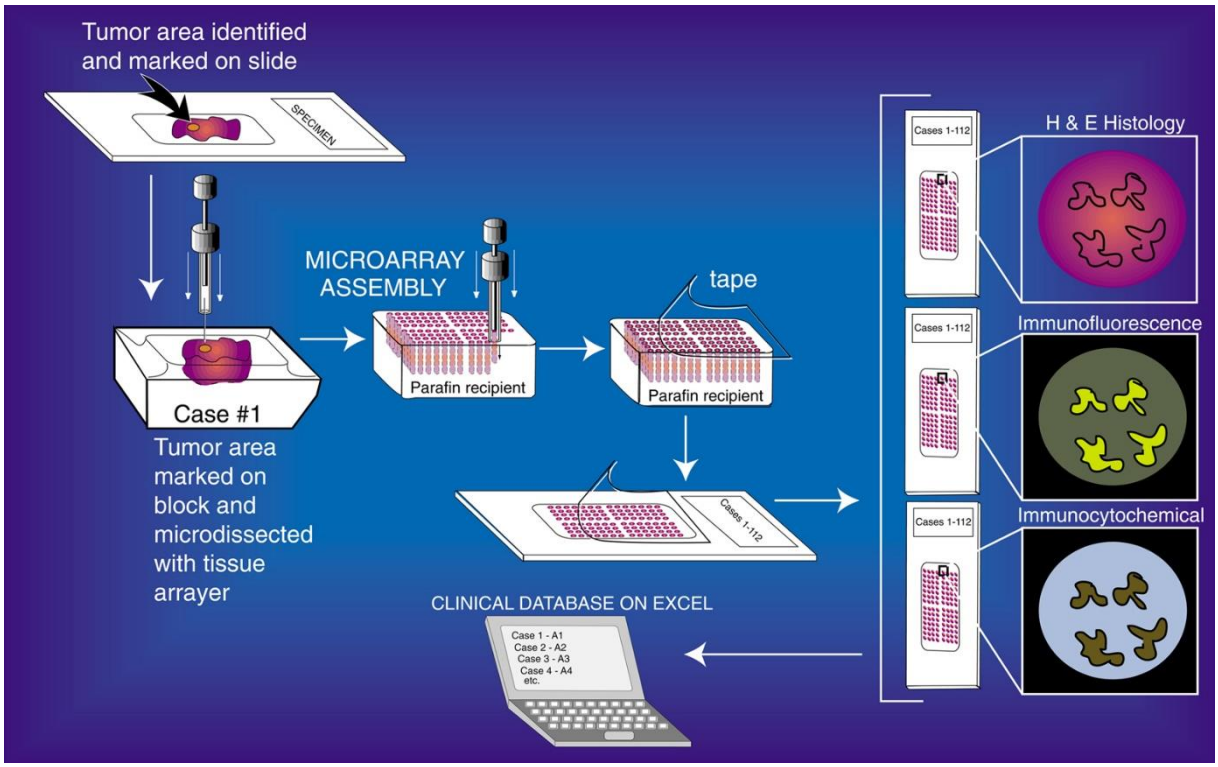


Figure 3.1: Tissue microarray manufacturing process (Adapted from http://apps.pathology.jhu.edu/blogs/pathology/tissue-microarrays)

3.3 Histopathological Evaluation of scores

Manual histopathological review was undertaken using CaseViewer 2.4, 3DHISTECH's advanced slide viewing software on these colorectal tissue slides. *TP53* and *Bcl-2* staining across the entire tumour section was assessed by a pathologist and was blinded to other biomarker and pathological data, along with their mutational status. The pathologist also assessed the number of peritumoural lymphocytes on H&E sections, in strict accordance with The Nuclear Forensics International Technical Working Group (ITWG) guidelines. The peritumoural lymphocytes were categorized into two groups (inconspicuous and conspicuous), and the associations with the biomarkers (*TP53* and *Bcl-2*) were assessed using statistical tests.

Immunohistochemistry results were further evaluated to assign an H-score ('histo' score) to tumour samples, recording the average nuclear and cytoplasmic staining intensity on four-category scales: 0 (negative), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong staining). The percentage of positive cells were also recorded with 0 (no positive cells), 1 (<15% of positive cells), 2 (15-50% of positive cells), 3 (51-

80% of positive cells) and 4 (> 80% of positive cells). A score of 0-12 can be retrieved when the categories of the percentage of positive cells were multiplied with the categories of the intensity of staining. They were then categorized in immunoreactivity score (IRS) classification with 0 (negative, score 0-1), 1 (positive, weak expression, score 2-3), 2 (positive, intermediate expression, score 4-8), 3 (positive, strong expression, score 9-12). The percentage of cells at each staining intensity is calculated, and H-score was assigned with the formula below:

$$[1 \times (\%\ \text{cells}\ 1+) + 2 \times (\%\ \text{cells}\ 2+) + 3 \times (\%\ \text{cells}\ 3+)]$$

A modified H-score was calculated as the outcome of the manually scored percentage of positive tumour cells (range 0-300) and average staining intensity score. It gives a more relative weight to a higher-intensity cytoplasmic and nuclear staining in a given tumour sample, to assess the extent of immunoreactivity. *TP53* and *Bcl-2* positive staining were only defined/ identified when it was more than 15% of the tumour cells, which cores with staining less than 15% was disregarded. The immunoscore illustrated to be quantitative, reproducible, and robust. The functionality of immunohistochemistry scoring method is quite limited, not only its ability to improve the predictive or prognostic value of tumour markers, but also its reproducibility. The reproducibility of *TP53* and *Bcl-2* scores was similar to the 15% cut-off. Although the greatest interobserver agreement was 0% cut-off, the distribution of *TP53* and *Bcl-2* expression shows that it will be essential to evaluate a complete range of scores.

Each of the cores was scored individually at different areas of the tumour and a mean score was used for each representative cases. Tumour H-score was compared with normal sample H-score on their expression of *TP53* and *Bcl-2* biomarkers. Manual scoring on the epithelial (tumours) and stroma compartments was performed on each immunohistochemistry (IHC) stained sections, to determine immune cell counts and cell staining intensity. Tumour-stroma percentage (can also be manifested as tumour-stroma ratio, TSR) has also been measured. It is an estimate of proportion of epithelial and stromal cells, which act as a promising prognostic parameter that stratifies solid tumours into stroma-low and stroma-high based on the amount of the stroma scored. Tumour-stroma has been observed in suppressing host immune responses against tumour cells. When tumour-stroma is high in percentage, it has a weakened immune system against cancer/ cancer treatment and results in promoting tumour-derived cells (high tumour %). Relations on TSR and the survival probability can be determined by

statistical test (p-value), with p-value< 0.05 shows their significance. Over the decades, there is an extensive use of IHC, however it is still lack of standardization, even aggravated in the era of targeted therapy. So, Nordic Immunohistochemical Quality Control (NordiQC) has been established to assess the analytical phases of the laboratory IHC quality. It is an international academic proficiency testing used to identify an improved staining of IHC module, and to achieve better IHC results, that is sufficient for diagnostic use. In order to follow the quality control guidelines, where efficacy, precision and accuracy of scoring were increased, a large cohort (888 patients) was used with multiple tests being established using Statistical Package for the IBM Social Sciences (SPSS) software.

3.4 Statistical Analysis

All data was analysed using SPSS 26.0 for Windows 10 (SPSS Inc., Chicago, IL, USA). Normality of the data followed by normal distribution were given as a form of mean ± standard deviation. Three quartiles (Q1, Q2, Q3) were used to determine their cut-off points in their normal distribution. The second quartile (Q2) manifests as the median (50%) of the data set. First quartile (Q1) serves as the lowest expression value of H-score (1-25% of the data set), whereas third quartile (Q3) as the highest expression value of H-score (75-100% of the data set). Paired t-test was also being conducted on different sites of tumours, to test for heterogeneity. There was also analysis using Chi-square test (X^2 test) to assess the association between *TP53* expression, demographic characteristics of the patients and their histopathological features. A low p-value (or a large chi-square statistic relative to its degree of freedom) shows the evidence of heterogeneity of intervention effects. Kaplan-Meier was also used to examine the survival distribution in a certain period of time, as well as survival curve analysed by a log-rank test for significance. Survival was estimated as the ratio of the observed survival (where all deaths considered as events) to the expected survival of the patients. Likewise, cox regression was also utilized to explore multivariate factors in evaluating standard prognostic factors in CRC. For quantitative data, paired t-test was applied to obtain 95% confidence interval (CI) and p-value, to assess the relationships between the comparable factors. P-value less than 0.05 was considered as significantly difference. When the adjusted standardized residuals (ASR) is higher than 1.96 (conventionally 2.0) and p-value significantly correlated, it shows that the clinic-

pathological variables in the tumour cell are significantly larger than expected if the null hypothesis is true.

4. Results

4.1 Cohort Characteristics

The patient cohort consisted of 1000 patients but due to some cores being disregarded (staining less than 15%), the final cohort consisted of 880 patients. The demographic and clinic-pathological results of the cohort are as below (**Table 4.1.1**). The data includes: age, gender, 3 years survival, 5 years survival, survival status, synchronous metastases, recurrence, tumour site, primary tumour stage, final stage, number of lymph nodes harvested, metastasised, vascular invasion, grade, perineural invasion, intramural lymphovascular invasion, tumour edge, tumour budding, peritumoural lymphocytes, DNA mismatch repair status (MLH-1, PMS-2, MSH-2 and MSH-6).

Parameters		Number (%)	Parameters		Number (%)
Sex	Female	382 (43.4)	Synchronous metastasis	Absent	781 (88.8)
	Male	498 (56.6)		Present	99 (11.3)
Age	Less than 50	59 (6.7)	Recurrence	No	626 (71.1)
	50 or more	821 (93.3)		Yes	254 (28.9)
3-years survival	Alive	648 (73.6)	Survival status	Alive	555 (63.1)
	Dead	232 (26.4)		Dead	325 (36.9)
5-years survival	Alive	387 (44)	Tumour edge	Infiltrative	353 (52.9)
	Dead	493 (56)		Pushing	314 (47.1)
Final Stage	I	150 (17)	Tumour budding score	Low	441 (67.1)
	II	353 (40.1)		High	216 (32.9)
	III	279 (31.7)	Peritumoural lymphocytes	Inconspicuous	475 (70.9)
	IV	98 (11.1)		Conspicuous	195 (29.1)
T-stage	I	69 (7.8)	MLH-1	Proficient	718 (88.4)
	II	98 (11.1)		Deficient	94 (11.6)
	III	459 (52.2)	PMS-2	Proficient	760 (87.4)
	IV	254 (28.9)		Deficient	110 (12.6)
Regional Lymph Nodes	N0	508 (59.4)	MSH-2	Proficient	863 (99.1)
	N1	215 (25.1)		Deficient	8 (0.9)
	N2	132 (15.4)	MSH-6	Proficient	854 (98.5)
N2	Uninvolved	508 (59.4)		Deficient	13 (1.5)
	Involved	347 (40.6)	MMR status	Proficient	746 (84.8)
Metastasis	No	781 (88.8)		Deficient	124 (14.1)
	Yes	99 (11.3)	Perineural invasion	Absent	683 (80.5)
Vascular Invasion	Absent	445 (51.4)		Present	165 (19.5)
	Present	420 (48.6)	Intramural lymphovascular invasion	Absent	566 (69.1)
Grade	Low	823 (93.6)			
	High	56 (6.4)			

Site	Right	416 (47.3)	Present	253 (30.9)
	Left	464 (52.7)		

Table 4.1.1: Demographic and clinic-pathological results of the cohort

The features of the analysed cohort were similar to what might be expected and showed that there had not been a bias introduced by the lost cores. There was a slightly higher proportion of stage I cases than may be expected from an historical cohort, but this is due to the NHS bowel cancer screening program (BCSP) introduced in 2006 onwards where CRC mortality was improved by earlier detection of CRC cases (Koo et al., 2017), thus there was a slight shifting to an early-stage cancer detection.

4.2 Correlations between *TP53* nuclear gene expression and clinicopathological parameters

Of the 1000 cases in the cohort series, p53 expression could be evaluated in 880 (88%) cases (at least one site of the tumour is being measured) while no cores were assessable in 120 (12%) cases. The nuclear expression of all cores was evaluated by H-score following *TP53* nuclear immunostaining guidelines (**Figure 4.2.1**) and a mean score derived from the assessable cores (three sites of tumour) was allocated to each case. Across the cohort, *TP53* expression was not normally distributed. The median score was 30 and this was therefore used as the cut-off point for low (H-score: 1-30) and high expression (H-score: 31-300). *TP53* nuclear staining (with a median cut-off point) was associated with 3-years survival status (p=0.046), survival status (p=0.036), MMR status (p< 0.001; MLH-1 and PMS-2, both p<0.001), regional lymph nodes (p= 0.018), N2 (p= 0.025) and site (p=0.002) (**Table 4.2.2**).

A. Negative (H-score: 0) | B. 1+ stained (H-score: 1-100) | C. 2+ stained (H-score:101-200) | D. 3+ stained (H-score: 201-300)

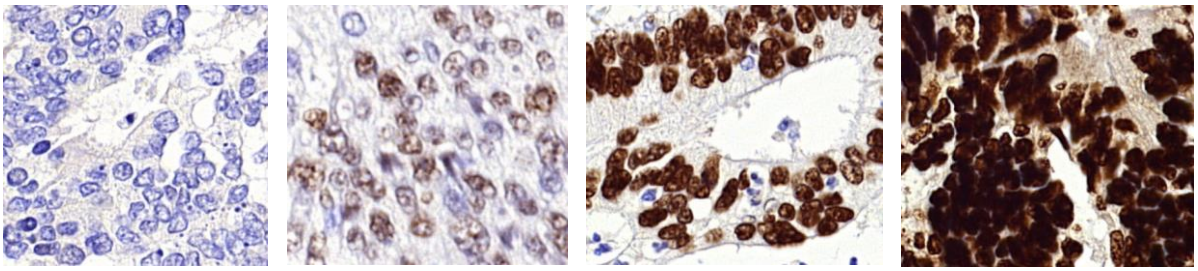


Figure 4.2.1: *TP53* nuclear immunostaining guidelines, a. Negative *TP53*, b. 1+ low *TP53*-stained, c. 2+ moderate *TP53*-stained, d. 3+ high *TP53*-stained

In order, to have a clear view on whether low/ high expression could have a significant and huge impact on those clinicopathological variables (**Table 4.2.3**), a comparison using extreme cut-offs on highest (H-score: 151-300) and lowest expressors (H-score: 0-10) of *TP53* biomarker was carried out. Significant correlations were observed in highest expression of *TP53* with poor 3-years survival status (p=0.005), survival status (p=0.015), MMR status (p< 0.001; MLH-1 and PMS-2, both p< 0.001), regional lymph nodes (p=0.022), N2 (p=0.01), recurrence (p=0.026), primary tumours stage (p=0.024), vascular invasion (p=0.016), tumour edge (p=0.025) and site (p< 0.001). Simultaneously, none of the lowest expressors of *TP53* had correlations with all those clinicopathological variables shown in **Table 4.2.3**. As none of the clinic-pathological variables are related, the survival probability of lowest TP53 expressors does not need to be determined.

Survival rates changed from borderline significance (p=0.051) to not significantly different (p=0.682) when period-of-time change from 3 to 5 years. Low *TP53* nuclear expression (survival probability: 76.6%) displayed a better survival in 3-years survival period compared to high *TP53* nuclear expression (survival probability: 70.6%). However, their survival curves (survival probability range: 43.5% to 44.5%) were overlapping with each other in 3-years and 5-years survival period, which showed a bad patients' survival when the survival time to death changed from 3-years to 5-years (**Fig. 4.2.2 a-b**). Higher nuclear expressions of *TP53* were significant (p= 0.005) in 3-years survival time, but not significant (p=0.193) in 5-years survival time. This result on the highest *TP53* nuclear expression showed that there was prognostic significance in 3-years survival, but the significance was lost in 5-years survival. In the meantime, by comparing the survival probability between 3-years and 5-years on highest nuclear *TP53* expressors, a lower survival rate can be concluded on both time-periods, suggesting a low survival benefit **(Fig. 4.2.2 c-d).** In multivariate cox regression, *TP53* nuclear expression was not correlated with lymph node (LN) stage, metastasis, vascular invasion, grade, perineural invasion and primary tumour stage (T) in 5 years survival time (HR=1.00, p=0.315), but was correlated in 3 years survival time (HR=1.26, p=0.015) **(Table 4.2.1).** HR is often calculated in clinical trials to measure the survival of CRC patients at a point time given. HR lower than 1 shows a drop in event hazard (smaller risk), but an increase in survival length. This showed that there is consequently insufficient evidence to conclude that a prognostic effect of *TP53* in CRC patients is

present, revealing *TP53* nuclear expression, unlikely to be an independent factor in colonic cancers on 5-years period of time, and likely to be an independent factor on 3-years survival period. It also firmly exhibits that *TP53* nuclear expression has decreased in their survival length, along with a higher risk in 3-years survival period.

4.3 Correlations between *TP53* cytoplasmic gene expression and clinicopathological parameters

881 (88.1%) of 1000 cases were analysed on *TP53* cytoplasmic tumour-derived CRC tissue, whereas 119 (11.9%) cases were not valid for molecular analysis. The *TP53* cytoplasmic expression of all cores was evaluated by H-score following immunostaining guidelines **(Figure 4.3.1)**. For immunohistochemistry analysis on *TP53* cytoplasmic staining, their expression did not follow a normal distribution, hence a median cut-off point was given for low (H-score: 0-1) and high expression (H-score: 2-300) by using the SPSS software. Similar to the *TP53* nuclear staining observation (with a median cut-off point), significant correlation was discovered on 5-years survival status (p=0.037), site (p=0.002), MMR status (p=0.014; MLH-1 and PMS-2, both p=0.003) and tumour budding (p=0.023) on *TP53* cytoplasmic staining (median cut-off point) (**Table 4.2.2**).

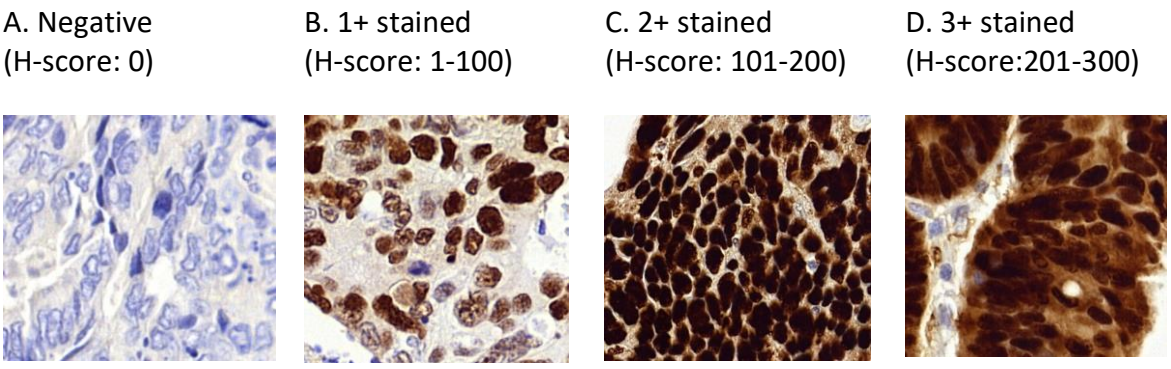


Figure 4.3.1: *TP53* cytoplasmic immunostaining guidelines, a. Negative *TP53*, b. 1+ low *TP53*-stained, c. 2+ moderate *TP53*-stained, d. 3+ high *TP53*-stained

Survival probability changed from not significantly different (p=0.756) to a borderline significance (p=0.058). This reassured that there were no correlations between the survival rates and *TP53* cytoplasmic expression in 3-years and 5-years period of time, suggesting a bad prognosis outcome (**Fig. 4.2.2 e-f**). Low *TP53* cytoplasmic

expression had a similar survival rate (survival probability: 74%) with high *TP53* cytoplasmic expression (survival probability: 72%) in 3-years survival time. Nevertheless, in the 5-years survival period, low *TP53* cytoplasmic expression (survival probability: 42.1%) had poor survival rate when compared with high *TP53* cytoplasmic expression (survival probability: 50.9%). In *TP53* cytoplasmic expression multivariate regression, significant association can also not be seen in those multiple variables in 3-years (HR=1.00, p=0.431) and 5-years survival period (HR=1.00, p=0.169) **(Table 4.2.1).** This displayed a trustable result where *TP53* cytoplasmic expression does not serve as an independent factor of improved 3-years survival and 5-years survival. It also shows that *TP53* cytoplasmic expression has maintained its survival length and risk of CRC patients in neither 3-years nor 5-years survival period.

Table 4.2.1 Results of Multivariate Cox Regression Model: Effects of TP53 gene expression, LN stage, Grade, Metastasis, Vascular Invasion, Perineural Invasion and Primary-tumour stage

	3 years				5 years			
Variables	SE	HR	P-value	95% CI	SE	HR	P-value	95% CI
TP53								
LN stage	0.099	1.11	0.364	(0.901, 1.327)	0.071	1.2	0.01	(1.046, 1.384)
Metastasis	0.159	2.41	<0.001	(1.783, 3.324)	0.128	2.35	<0.001	(1.826, 3.081)
Vascular Invasion	0.168	1.14	0.426	(0.822, 1.589)	0.115	0.96	0.728	(0.767, 1.203)
Grade	0.204	1.36	0.004	(1.212, 2.702)	0.167	1.36	0.063	(0.983, 1.893)
Perineural Invasion	0.161	1.35	0.065	(0.981, 1.847)	0.123	1.20	0.138	(0.943, 1.527)
Primary-tumour Stage (T)								
T2	0.478	0.38	0.05	(0.154, 1.001)	0.259	0.65	0.092	(0.388, 1.073)
T3	0.372	0.28	0.001	(0.132, 0.569)	0.198	0.54	0.002	(0.363, 0.788)
T4	0.151	0.49	<0.001	(0.373, 0.673)	0.109	0.64	<0.001	(0.519, 0.794)
Nuclear expression	0.001	1.26	0.015	(1.000, 1.003)	<0.001	1.00	0.315	(1.000, 1.001)
TP53								
LN stage	0.098	1.12	0.234	(0.927, 1.360)	0.071	1.22	0.005	(1.060, 1.399)
Metastasis	0.158	2.37	<0.001	(1.734, 3.223)	0.127	2.32	<0.001	(1.807, 2.976)
Vascular Invasion	0.168	1.14	0.423	(0.823, 1.590)	0.115	0.97	0.766	(0.772, 1.210)
Grade	0.204	1.8	0.004	(1.209, 2.690)	0.167	1.39	0.049	(1.002, 1.926)
Perineural Invasion	0.162	1.33	0.077	(0.970, 1.827)	0.123	1.20	0.124	(0.949, 1.536)
Primary-tumour Stage (T)								
T2	0.478	0.38	0.043	(0.149, 0.972)	0.260	0.65	0.102	(0.393, 1.088)
T3	0.373	0.28	0.001	(0.135, 0.580)	0.198	0.55	0.002	(0.371, 0.806)
T4	0.151	0.49	<0.001	(0.366, 0.662)	0.109	0.64	<0.001	(0.518, 0.793)
Cytoplasmic expression	0.162	1.00	0.431	(0.998, 1.005)	0.002	1.00	0.169	(0.995, 1.001)

Notes: Significant p-values (< 0.05) are highlighted in red. HR >1 is highlighted in blue.
Abbreviations: 95% CI, 95% Confidence Interval; HR, hazard ratio; SE, standard deviation; LN stage, Lymph Node stage.

Table 4.2.2 Associations between *TP53* gene expression and clinicopathological variables

Clinicopathological criteria	*TP53* nuclear staining			*TP53* cytoplasmic staining		
	Low (%)	High (%)	X^2 (p-value)	Low (%)	High (%)	X^2 (p-value)
Age at diagnosis (years)						
≤ 50	33 (7.4) (ASR= 0.9)	26 (6) (ASR= -0.9)	0.76 (0.384)	47 (6.7) (ASR= -0.1)	12 (6.9) (ASR= 0.1)	0.01 (0.935)
> 50	411 (92.6) (ASR= -0.9)	410 (94) (ASR= 0.9)		656 (93.3) (ASR= 0.1)	163 (93.1) (ASR= -0.1)	
Gender						
Female	201 (45.3) (ASR= 1.1)	181 (41.5) (ASR= -1.1)	1.26 (0.261)	304 (43.2) (ASR= 0.0)	76 (43.4) (ASR= 0.0)	0.002 (0.965)
Male	243 (54.7) (ASR= -1.1)	255 (58.5) (ASR= 1.1)		399 (56.8) (ASR= 0.0)	99 (56.6) (ASR= 0.0)	
3-years survival status						
Alive	340 (76.6) (ASR= 2.0)	308 (70.6) (ASR= -2.0)	3.99 (0.046)	520 (74) (ASR= 0.5)	126 (72) (ASR= -0.5)	0.28 (0.597)
Dead	104 (23.4) (ASR= -2.0)	128 (29.4) (ASR= 2.0)		183 (26) (ASR= -0.5)	49 (28) (ASR= 0.5)	
5-years survival status						
Alive	194 (43.7) (ASR= -0.2)	193 (44.3) (ASR= 0.2)	0.03 (0.864)	296 (42.1) (ASR= -2.1)	89 (50.9) (ASR= 2.1)	4.36 (0.037)
Dead	250 (56.3) (ASR= 0.2)	243 (55.7) (ASR= -0.2)		407 (57.9) (ASR= 2.1)	86 (49.1) (ASR= -2.1)	
Synchronous Metastases						
Absent	396 (89.2) (ASR= 0.4)	385 (88.3) (ASR= -0.4)	0.173 (0.677)	622 (88.5) (ASR= -0.7)	158 (90.3) (ASR= 0.7)	0.46 (0.497)
Present	48 (10.8) (ASR= -0.4)	51 (11.7) (ASR= 0.4)		81 (11.5) (ASR= 0.7)	17 (9.7) (ASR= -0.7)	
Recurrence						
No	322 (72.5)	304 (69.7)	0.84 (0.36)	502 (71.4)	124 (70.9)	0.02 (0.885)

	(ASR= 0.9)	(ASR= -0.9)		(ASR= 0.1)	(ASR= -0.1)	
Yes	122 (27.5)	132 (30.3)		201 (28.6)	51 (29.1)	
	(ASR= -0.9)	(ASR= 0.9)		(ASR= -0.1)	(ASR= 0.1)	
Survival status						
Alive	295 (66.4)	260 (59.6)	4.38 (0.036)	443 (63)	110 (62.9)	0.002 (0.969)
	(ASR= 2.1)	(ASR= -2.1)		(ASR= 0.0)	(ASR= 0.0)	
Dead	149 (33.6)	176 (40.4)		260 (37)	65 (37.1)	
	(ASR= -2.1)	(ASR= 2.1)		(ASR= 0.0)	(ASR= 0.0)	
Tumour edge						
Infiltrative	171 (50.4)	182 (55.5)	1.70 (0.192)	283 (51.2)	69 (61.1)	3.68 (0.055)
	(ASR= -1.3)	(ASR= 1.3)		(ASR= -1.9)	(ASR= 1.9)	
Pushing	168 (49.6)	146 (44.5)		270 (48.8)	44 (38.9)	
	(ASR= 1.3)	(ASR= -1.3)		(ASR= 1.9)	(ASR= -1.9)	
Tumour budding score						
Low	235 (70.1)	206 (64)	2.84 (0.092)	376 (69.1)	65 (58)	5.18 (0.023)
	(ASR= 1.7)	(ASR= -1.7)		(ASR= 2.3)	(ASR= -2.3)	
High	100 (29.9)	116 (36)		168 (30.9)	47 (42)	
	(ASR= -1.7)	(ASR= 1.7)		(ASR= -2.3)	(ASR= 2.3)	
Peritumoural lymphocytes						
inconspicuous	238 (69.4)	237 (72.5)	0.78 (0.379)	395 (71.4)	79 (68.1)	0.51 (0.474)
	(ASR= -0.9)	(ASR= 0.9)		(ASR= 0.7)	(ASR= -0.7)	
conspicuous	105 (30.6)	90 (27.5)		158 (28.6)	37 (31.9)	
	(ASR= 0.9)	(ASR= -0.9)		(ASR= -0.7)	(ASR= 0.7)	
MMR Status						
proficient (MSS/MSI-L)	354 (79.7)	392 (89.9)	18.93 (< 0.001)	585 (83.2)	159 (90.9)	8.54 (0.014)
	(ASR= -4.2)	(ASR= 4.2)		(ASR= -2.5)	(ASR= 2.5)	
deficient (MSI-H)	85 (19.1)	39 (8.9)		111 (15.8)	13 (7.4)	
	(ASR= 4.3)	(ASR= -4.3)		(ASR= 2.8)	(ASR= -2.8)	
MLH-1						
proficient	342 (84.2)	376 (92.6)	13.91 (<0.001)	561 (86.7)	155 (95.1)	8.92 (0.003)

	(ASR= -3.7)	(ASR= 3.7)		(ASR= -3.0)	(ASR= 3.0)	
deficient	64 (15.8)	30 (7.4)		86 (13.3)	8 (4.9)	
	(ASR= 3.7)	(ASR= -3.7)		(ASR= 3.0)	(ASR= -3.0)	
PMS-2						
proficient	363 (82.7)	397 (92.1)	17.49 (<0.001)	596 (85.6)	162 (94.2)	9.12 (0.003)
	(ASR= -4.2)	(ASR= 4.2)		(ASR= -3.0)	(ASR= 3.0)	
deficient	76 (17.3)	34 (7.9)		100 (14.4)	10 (5.8)	
	(ASR= 4.2)	(ASR= -4.2)		(ASR= 3.0)	(ASR= -3.0)	
MSH-2						
proficient	433 (98.6)	430 (99.5)	1.95 (0.162)	689 (99)	172 (99.4)	0.28 (0.598)
	(ASR= -1.4)	(ASR= 1.4)		(ASR= -0.5)	(ASR= 0.5)	
deficient	6 (1.4)	2 (0.5)		7 (1)	1 (0.6)	
	(ASR= 1.4)	(ASR= -1.4)		(ASR= 0.5)	(ASR= -0.5)	
MSH-6						
proficient	429 (97.9)	425 (99.1)	1.85 (0.174)	682 (98.6)	170 (98.3)	0.08 (0.78)
	(ASR= -1.4)	(ASR= 1.4)		(ASR= 0.3)	(ASR= -0.3)	
deficient	9 (2.1)	4 (0.9)		10 (1.4)	3 (1.7)	
	(ASR= 1.4)	(ASR= -1.4)		(ASR= -0.3)	(ASR= 0.3)	
Final Stage						
I	75 (16.9)	75 (17.2)	3.29 (0.35)	113 (16.1)	37 (21.1)	5.87 (0.118)
	(ASR= -0.1)	(ASR= 0.1)		(ASR= -1.6)	(ASR= 1.6)	
II	190 (42.8)	163 (37.4)		295 (42)	59 (33.7)	
	(ASR= 1.6)	(ASR= -1.6)		(ASR= 2.0)	(ASR= -2.0)	
III	130 (29.3)	149 (34.2)		215 (30.6)	62 (35.4)	
	(ASR= -1.6)	(ASR= 1.6)		(ASR= -1.2)	(ASR= 1.2)	
IV	49 (11)	49 (11.2)		80 (11.4)	17 (9.7)	
	(ASR= -0.1)	(ASR= 0.1)		(ASR= 0.6)	(ASR= -0.6)	
Primary tumour-stage (T)						
T1	36 (8.1)	33 (7.6)	1.62 (0.656)	53 (7.5)	16 (9.1)	2.02 (0.569)
	(ASR= 0.3)	(ASR= -0.3)		(ASR= -0.7)	(ASR= 0.7)	

T2	49 (11) (ASR= -0.1)	49 (11.2) (ASR= 0.1)		75 (10.7) (ASR= -0.9)	23 (13.1) (ASR= 0.9)	
T3	239 (53.8) (ASR= 1.0)	220 (50.5) (ASR= -1.0)		374 (53.2) (ASR= 1.2)	84 (48) (ASR= -1.2)	
T4	120 (27) (ASR= -1.2)	134 (30.7) (ASR= 1.2)		201 (28.6) (ASR= -0.3)	52 (29.7) (ASR= 0.3)	
Regional Lymph Nodes-stage						
N0	274 (63.1) (ASR= 2.2)	234 (55.6) (ASR= -2.2)	8.08 (0.018)	417 (60.9) (ASR= 1.4)	92 (54.8) (ASR= -1.4)	2.1 (0.35)
N1	107 (24.7) (ASR= -0.3)	108 (25.7) (ASR= 0.3)		165 (24.1) (ASR= -1.0)	47 (28) (ASR= 1.0)	
N2	53 (12.2) (ASR= -2.7)	79 (18.8) (ASR= 2.7)		103 (15) (ASR= -0.7)	29 (17.3) (ASR= 0.7)	
N2						
uninvolved	274 (63.1) (ASR= 2.2)	234 (55.6) (ASR= -2.2)	5.05 (0.025)	417 (60.9) (ASR= 1.4)	92 (54.8) (ASR= -1.4)	2.1 (0.148)
involved	160 (36.9) (ASR= -2.2)	187 (44.4) (ASR= 2.2)		268 (39.1) (ASR= -1.4)	76 (45.2) (ASR= 1.4)	
Distant Metastasis						
No metastasis	395 (89) (ASR= 0.2)	386 (88.5) (ASR= -0.2)	0.04 (0.839)	622 (88.5) (ASR= -0.7)	158 (90.3) (ASR= 0.7)	0.46 (0.497)
With metastasis	49 (11) (ASR= -0.2)	50 (11.5) (ASR= 0.2)		81 (11.5) (ASR= 0.7)	17 (9.7) (ASR= -0.7)	
Vascular Invasion						
Absent	237 (53.9) (ASR= 1.4)	208 (48.9) (ASR= -1.4)	2.1 (0.148)	356 (51.4) (ASR= -0.1)	88 (51.8) (ASR= 0.1)	0.01 (0.927)
Present	203 (46.1) (ASR= -1.4)	217 (51.1) (ASR= 1.4)		337 (48.6) (ASR= 0.1)	82 (48.2) (ASR= -0.1)	
Grade						

Low	417 (93.9) (ASR= 0.4)	406 (93.3) (ASR= -0.4)	0.13 (0.722)	653 (93) (ASR= -1.4)	168 (96) (ASR= 1.4)	2.08 (0.149)
High	27 (6.1) (ASR= -0.4)	29 (6.7) (ASR= 0.4)		49 (7) (ASR= 1.4)	7 (4) (ASR= -1.4)	
Perineural invasion						
absent	346 (80.8) (ASR= 0.2)	337 (80.2) (ASR= -0.2)	0.05 (0.824)	551 (81.3) (ASR= 0.8)	132 (78.6) (ASR= -0.8)	0.63 (0.428)
present	82 (19.2) (ASR= -0.2)	83 (19.8) (ASR= 0.2)		127 (18.7) (ASR= -0.8)	36 (21.4) (ASR= 0.8)	
Intramural lymphovascular Invasion						
absent	299 (71.9) (ASR= 1.7)	267 (66.3) (ASR= -1.7)	3.03 (0.082)	458 (69.1) (ASR= -0.1)	107 (69.5) (ASR= 0.1)	0.01 (0.923)
present	117 (28.1) (ASR= -1.7)	136 (33.7) (ASR= 1.7)		205 (30.9) (ASR= 0.1)	47 (30.5) (ASR= -0.1)	
Site						
right	233 (52.5) (ASR= 3.1)	183 (42) (ASR= -3.1)	9.74 (0.002)	352 (50.1) (ASR= 3.1)	65 (37.1) (ASR= -3.1)	9.39 (0.002)
left	211 (47.5) (ASR= -3.1)	253 (58) (ASR= 3.1)		351 (49.9) (ASR= -3.1)	110 (62.9) (ASR= 3.1)	

Significant p-values are highlighted in red, Primary tumour stage p-values refers to the association between *TP53* and size, extent of the tumours. Lymph Node stages N0, N1, N2 shows the regional lymph nodes metastasis in the cohort: N0 refers to no regional lymph nodes metastasis, N1 refers to regional lymph nodes metastasis.

*Abbreviations: X^2, Chi-square; ASR, adjusted standardized residual. ASR more than 2 or less than -2, are highlighted in blue.

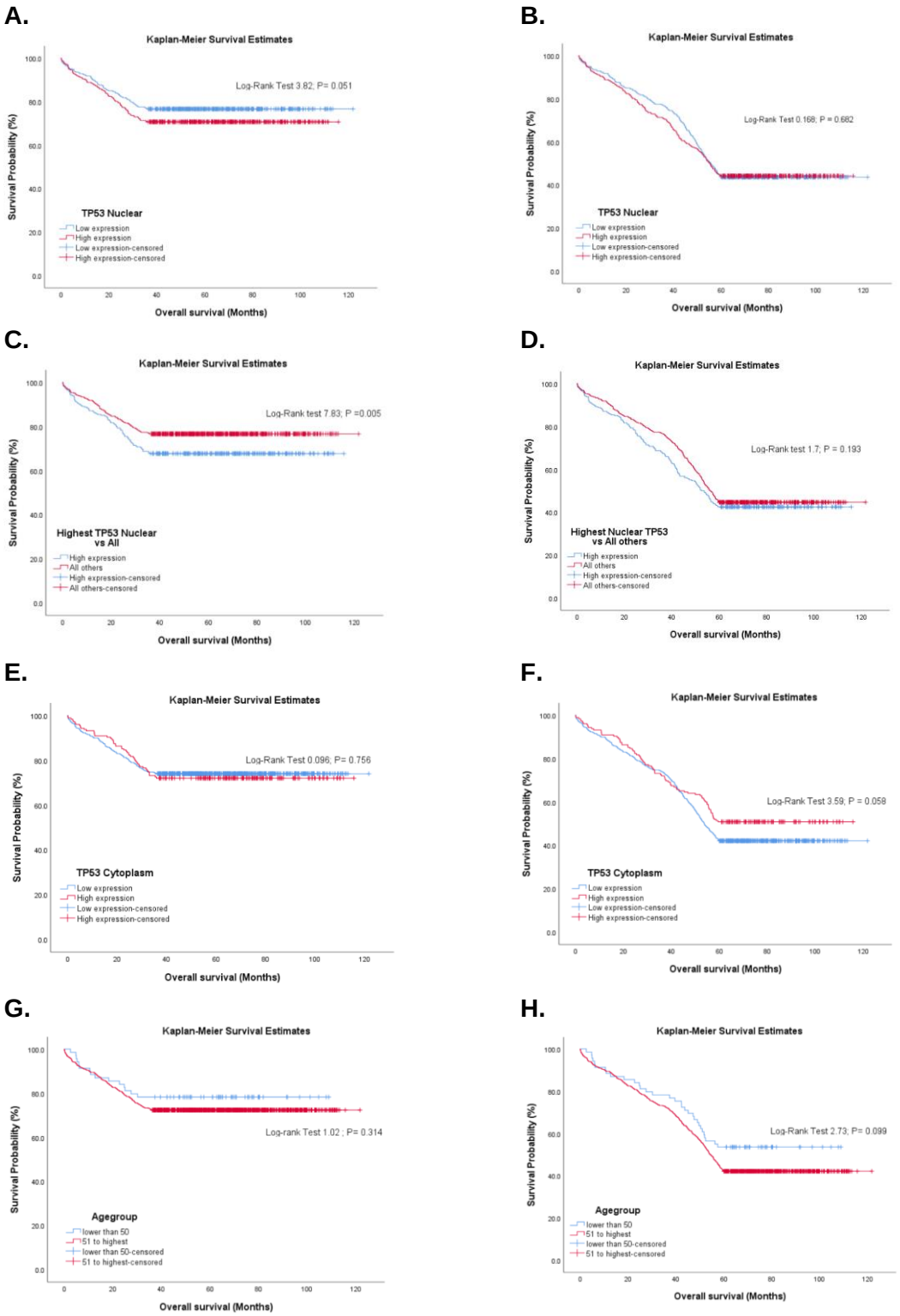


Figure 4.2.2 Survival probability of *TP53* expression and age group, **a**. Survival plot on low/high *TP53* nuclear expression in 3-years survival time, **b.** Survival plot on low/ high *TP53* nuclear expression in 5-years survival time, **c.** Survival plot on highest nuclear expressors/ all others of *TP53* biomarker in 3-years' time-period, **d.** Survival plot on highest nuclear expressors/ all others of *TP53* biomarker in 5-years' time-period, **e.** Survival plot on low/high *TP53* cytoplasmic expression in 3-years survival time, **d.** Survival plot on low/high *TP53* cytoplasmic expression in 5-years survival time, **g.** Survival plot on young and old patients in 3-years survival time, **h.** Survival plot on young and old patients in 5-years survival time.

Table 4.2.3 Associations between highest and lowest expressors of *TP53* nuclear staining and its clinicopathological significance.

Clinicopathological criteria	*TP53* nuclear staining			*TP53* nuclear staining		
	Low (%)	All (%)	X^2 (p-value)	High (%)	All (%)	X^2 (p-value)
3-years survival status						
Alive	226 (76.1)	418 (72.3)	1.44 (0.23)	199 (67.7)	449 (76.6)	8.05 (0.005)
Dead	71 (23.9)	160 (27.7)		95 (32.3)	137 (23.4)	
Survival status						
Alive	194 (65.3)	357 (61.8)	1.06 (0.302)	169 (57.5)	386 (65.9)	5.91 (0.015)
Dead	103 (34.7)	221 (38.2)		125 (42.5)	200 (34.1)	
MMR Status						
proficient (MSS/MSI-L)	251 (84.5)	491 (84.9)	1.06 (0.59)	275 (93.5)	471 (80.4)	27.49 (< 0.001)
deficient (MSI-H)	44 (14.8)	79 (13.7)		16 (5.4)	108 (18.4)	
MLH-1						
proficient	238 (87.5)	476 (89)	0.38 (0.536)	260 (95.6)	458 (84.8)	20.51 (<0.001)
deficient	34 (12.5)	59(11)		12 (4.4)	82 (15.2)	
PMS-2						
proficient	255 (86.4)	501 (87.9)	0.37 (0.541)	278 (95.5)	482 (83.2)	26.47 (<0.001)
deficient	40 (13.6)	69 (12.1)		13 (4.5)	97 (16.8)	
Regional Lymph Nodes-stage						
N0	174 (59.6)	332 (59.5)	0.85 (0.655)	152 (53.3)	356 (62.5)	7.63 (0.022)
N1	77 (26.4)	136 (24.4)		78 (27.4)	137 (24)	
N2	41 (14)	90 (16.1)		55 (19.3)	77 (13.5)	
N2						
uninvolved	174 (59.6)	332 (59.5)	0.001 (0.98)	152 (53.3)	356 (62.5)	6.56 (0.01)
involved	118 (40.4)	226 (40.5)		133 (46.7)	214 (37.5)	
Recurrence						

No	206 (69.4)	417 (72.1)	0.74 (0.389)	195 (66.3)	431 (73.5)	4.98 (0.026)
Yes	91 (30.6)	161 (27.9)		99 (33.7)	155 (26.5)	
Primary tumours-stage (T)						
T1	25 (8.4)	44 (7.6)	3.55 (0.314)	18 (6.1)	51 (8.7)	9.41 (0.024)
T2	25 (8.4)	73 (12.6)		37 (12.6)	61 (10.4)	
T3	159 (53.5)	296 (51.2)		138 (46.9)	321 (54.8)	
T4	88 (29.6)	165 (28.5)		101 (34.4)	153 (26.1)	
Vascular Invasion						
Absent	144 (48.6)	298 (52.8)	1.36 (0.243)	130 (45.6)	315 (54.3)	5.79 (0.016)
Present	152 (51.4)	266 (47.2)		155 (54.4)	265 (45.7)	
Tumour edge						
Infiltrative	123 (54.9)	228 (51.8)	0.57 (0.45)	130 (59.1)	223 (49.9)	5.01 (0.025)
Pushing	101 (45.1)	212 (48.2)		90 (40.9)	224 (50.1)	
Site						
right	151 (50.8)	264 (45.7)	2.1 (0.147)	114 (38.8)	302 (51.5)	12.79 (<0.001)
left	146 (49.2)	314 (54.3)		180 (61.2)	284 (48.5)	

Significant p-values (< 0.05) are highlighted in red. Primary tumour stage p-value refers to the association between *TP53* and size and/ or extent of the tumour. Regional lymph nodes N0, N1, N2 show the presence of cancer in nearby lymph nodes: N0 refers to no cancer in nearby lymph nodes; N1, N2 refers to the location and number of lymph nodes that have cancer. The higher the number after the N, the higher the number of lymph nodes consist cancer.
*Abbreviations: X^2, Chi-square.

4.4 Correlations between *Bcl-2* cytoplasmic gene expression and clinicopathological parameters

Out of 1000 cases, a total of 882 (88.2%) cases were examined for molecular analysis on *Bcl-2* cytoplasmic staining tumour-derived colorectal cells. The *Bcl-2* cytoplasmic expression of all cores was evaluated by H-score following immunostaining guidelines (**Figure 4.4.1**) and a mean score derived from the assessable cores was allocated to each case. The H-score of *Bcl-2* cytoplasmic expression was not normally distributed in a bell curve shape; hence a median cut-off point was set using SPSS software to differentiate high (H-score: 11-300) and low (H-score: 1-10) expressions. A significant association in *Bcl-2* cytoplasmic staining (median cut-off) was derived from better 3-years survival status (p=0.021), better 5-years survival status (p=0.041), better survival status (p=0.008), final stage (p=0.001) and primary tumour stage (p< 0.001) (**Table 4.4.2**). Much the same as *TP53* nuclear staining observation, a throughout analysis was conducted on the comparison of the levels on highest (H-score: 6-300) and lowest (H-score: 0-5) cytoplasmic expressors of *Bcl-2* biomarker (**Table 4.4.3**). Significant correlations were determined in lowest expressors of *Bcl-2* with 3-years survival status (p=0.022), survival status (p=0.016), MMR status (p=0.018; MLH-1 p=0.017, PMS-2 p=0.032), vascular invasion (p=0.01), final stage (p=0.003), primary tumours stage (p=0.001) and grade (p=0.021). Conversely, 5-years survival status (p=0.014), peritumoural lymphocytes (p=0.023), final stage (p=0.04) and primary tumours stage (p=0.008) were significantly related with the highest expression of *Bcl-2*. High Bcl-2 expressors will have an increase in the number of patients with inconspicuous peritumoural lymphocytes (74%). By comparing with both high and low *Bcl-2* cytoplasmic expressors, a similarity can be concluded on the positive correlations of the results in 3-years and 5-years survival periods, where final stage and primary tumours stage were both found significantly correlated.

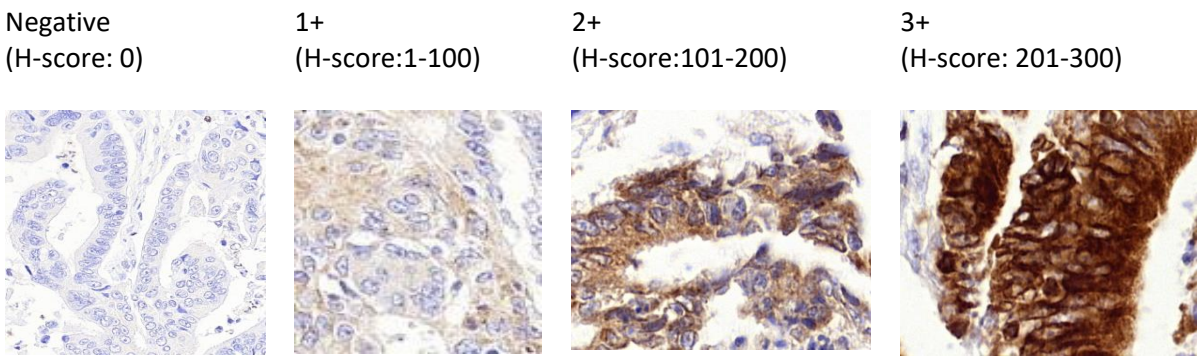


Figure 4.4.1: *Bcl-2* cytoplasmic immunostaining guidelines, a. Negative *Bcl-2*, b. 1+ low *Bcl-2*-stained, c. 2+ moderate *Bcl-2*-stained, d. 3+ high *Bcl-2*-stained

Survival rates were significant (p=0.014) in 3-years survival time, but not significant (p=0.213) in 5-years survival time on the relationship between the survival probability and *Bcl-2* cytoplasmic expression **(Fig. 4.4.2 a-b)**. High *Bcl-2* expression showed a higher survival probability (77.6%) in 3-years survival time, but lower survival probability (40.1%) in 5-years survival time compared to low *Bcl-2* expression that had a survival probability of 70.7% in 3-years survival time and 47% in 5-years survival time. To compare the survival rates on the highest and lowest Bcl-2 expressors versus all other expressors of biomarkers, four survival plots were revealed in **Fig. 4.4.2 c-f**. Through the survival plot of the lowest expressors, significant correlations (p=0.014) was observed in 3-years survival time, and no correlation (p=0.805) was determined in 5-years survival time **(Fig. 4.4.2 c-d)**. Lowest expressors of *Bcl-2* had a lower survival probability (69.8%) when comparing to all other expressors (76.6%) in 3-years survival time, but their survival probability (43.5%-44.5%) can be identified overlapping/similar with each other in 5-years survival time. Meanwhile, survival rates in the highest *Bcl-2* expression had no association with 3-years and 5-years survival time **(Fig. 4.4.2 e-f).** In *Bcl-2* cytoplasmic multivariate regression, there was no significantly correlation (HR=1.00, p=0.159) found between *Bcl-2* cytoplasmic expression and those multiple variables (LN stage, metastasis, vascular invasion, grade, perineural invasion and T-stage) in 3-years survival time (**Table 4.4.1**). Yet, no correlation (HR=1.00, p=0.174) can be found between them in the 5-years survival period. This showed that *Bcl-2* cytoplasmic expression does not present as an independent factor of improved 3-years and 5-years survival. It also shows that *Bcl-2* cytoplasmic expression has maintained their survival length and risk in 3-years survival period and 5-years survival period.

4.5 Correlations between *Bcl-2* nuclear gene expression and clinicopathological parameters

Similar to *Bcl-2* cytoplasmic staining, 882 (88.2%) out of 1000 cases were present for *Bcl-2* nuclear immunohistochemistry analysis on tumour-derived colorectal cells. As the H-score of *Bcl-2* nuclear expression was not normally distributed, a median cut-off point was set as low expression (lowest thru H-score: 0.000) and high expression (H-score: 0.001 and above) by the SPSS software. *Bcl-2* nuclear expression was significantly associated with 5-years survival status (p=0.001) and regional lymph nodes (p=0.017) (**Table 4.4.2**). Concurrently, survival plot on *Bcl-2* nuclear expression was significantly correlated (p=0.002) in 5-years survival time, but not related (p=0.281)

in 3-years survival time (**Fig. 4.4.2 g-h**). Low expression of *Bcl-2* nuclear expression had a lower survival probability neither in 3-years (73.3%) nor 5-years survival time (42.5%) by comparing them to high *Bcl-2* nuclear expression.

Meanwhile, on multivariate cox regression, *Bcl-2* nuclear expression was not significant difference (HR=0.91, p=0.611) on those multiple variables in 3-years survival period, but significantly correlated (HR=0.73, p=0.036) in 5-years survival time (**Table 4.4.1**). Results can be concluded that *Bcl-2* nuclear expression does not present as an independent factor of improved 3-years, but an independent factor in 5-years survival period. It also shows that *Bcl-2* nuclear expression has increased in their survival length, along with a lower risk in (HR= 0.91) 3-years and (HR= 0.73) 5-years survival period.

Table 4.4.1. Results of Multivariate Cox Regression Model: Effects of Bcl-2 gene expression, LN stage, Grade, Metastasis, Vascular Invasion, Perineural Invasion and Primary-tumour stage

	3 years				5 years			
Variables	SE	HR	P-value	95% CI	SE	HR	P-value	95% CI
Bcl-2								
LN stage	0.097	1.11	0.271	(0.920, 1.347)	0.071	1.21	0.006	(1.057, 1.395)
Metastasis	0.16	2.36	<0.001	(1.725, 3.230)	0.130	2.24	<0.001	(1.738, 2.890)
Vascular Invasion	0.166	1.13	0.456	(0.817, 1.567)	0.115	0.96	0.697	(0.764, 1.197)
Grade	0.195	2.34	<0.001	(1.593, 3.428)	0.167	1.53	0.012	(1.099, 2.118)
Perineural Invasion	0.161	1.32	0.085	(0.962, 1.811)	0.122	1.19	0.152	(0.938, 1.514)
Primary-tumour Stage(T)								
T2	0.529	0.31	0.025	(0.108,0.860)	0.268	0.52	0.014	(0.305, 0.872)
T3	0.389	0.25	<0.001	(0.115, 0.529)	0.199	0.50	<0.001	(0.337, 0.738)
T4	0.152	0.48	<0.001	(0.357, 0.647)	0.109	0.62	<0.001	(0.503, 0.770)
Cytoplasmic expression	0.004	1.00	0.159	(0.988, 1.002)	0.002	1.00	0.174	(0.999, 1.006)
Bcl-2								
LN stage	0.097	1.11	0.291	(0.916, 1.340)	0.071	1.21	0.008	(1.051, 1.388)
Metastasis	0.16	2.33	<0.001	(1.703, 3.194)	0.130	2.22	<0.001	(1.723, 2.864)
Vascular Invasion	0.166	1.13	0.478	(0.812, 1.559)	0.115	0.94	0.609	(0.753, 1.181)
Grade	0.195	2.34	<0.001	(1.598, 3.438)	0.167	1.48	0.018	(1.071, 2.057)
Perineural Invasion	0.161	1.34	0.067	(0.979, 1.841)	0.123	1.21	0.126	(0.948, 1.536)
Primary-tumour Stage (T)								
T2	0.527	0.29	0.017	(0.101, 0.800)	0.265	0.55	0.025	(0.328, 0.928)
T3	0.39	0.24	<0.001	(0.112, 0.517)	0.200	0.49	<0.001	(0.333, 0.727)
T4	0.151	0.48	<0.001	(0.359, 0.649)	0.109	0.62	<0.001	(0.502, 0.770)
Nuclear expression	0.183	0.91	0.611	(0.637, 1.304)	0.15	0.73	0.036	(0.544, 0.980)

Notes: Significant p-values (< 0.05) are highlighted in red. HR <1 are highlighted in blue.
Abbreviations: 95% CI, 95% Confidence Interval; HR, hazard ratio; SE, standard deviation; LN stage, Lymph Node stage.

Table 4.4.2 Associations between *Bcl-2* gene expression and clinicopathological variables

Clinicopathological criteria	*Bcl-2* nuclear staining			*Bcl-2* cytoplasmic staining		
	Low (%)	High (%)	X^2 (p-value)	Low (%)	High (%)	X^2 (p-value)
Age at diagnosis (years)						
≤ 50	56 (6.8) (ASR= 0.3)	3 (5.6) (ASR= -0.3)	0.12 (0.731)	32 (6.4) (ASR= -0.4)	27 (7) (ASR= 0.4)	0.13 (0.721)
> 50	772 (93.2) (ASR= -0.3)	51 (94.4) (ASR= 0.3)		466 (93.6) (ASR= 0.4)	357 (93) (ASR= -0.4)	
Gender						
Female	361 (43.6) (ASR= -0.4)	25 (46.3) (ASR= 0.4)	0.15 (0.699)	211 (42.4) (ASR= -1.0)	175 (45.6) (ASR= 1.0)	0.904 (0.342)
Male	467 (56.4) (ASR= 0.4)	29 (53.7) (ASR= -0.4)		287 (57.6) (ASR= 1.0)	209 (54.4) (ASR= -1.0)	
3-years survival status						
Alive	607 (73.3) (ASR= -1.0)	43 (79.6) (ASR= 1.0)	1.05 (0.307)	352 (70.7) (ASR= -2.3)	298 (77.6) (ASR= 2.3)	5.36 (0.021)
Dead	221 (26.7) (ASR= 1.0)	11 (20.4) (ASR= -1.0)		146 (29.3) (ASR= 2.3)	86 (22.4) (ASR= -2.3)	
5-years survival status						
Alive	352 (42.5) (ASR= -3.5)	36 (66.7) (ASR= 3.5)	12.004 (0.001)	234 (47) (ASR= 2.0)	154 (40.1) (ASR= -2.0)	4.17 (0.041)
Dead	476 (57.5) (ASR= 3.5)	18 (33.3) (ASR= -3.5)		264 (53) (ASR= -2.0)	230 (59.9) (ASR= 2.0)	
Synchronous Metastases						
Absent	738 (89.1) (ASR= -0.8)	50 (92.6) (ASR= 0.8)	0.64 (0.424)	446 (89.6) (ASR= 0.2)	342 (89.1) (ASR= -0.2)	0.06 (0.813)
High	90 (10.9) (ASR= 0.8)	4 (7.4) (ASR= -0.8)		52 (10.4) (ASR= -0.2)	42 (10.9) (ASR= 0.2)	
Recurrence						
No	592 (71.5)	38 (70.4)	0.03 (0.859)	353 (70.9)	277 (72.1)	0.17 (0.683)

	(ASR= 0.2)	(ASR= -0.2)		(ASR= -0.4)	(ASR= 0.4)	
Yes	236 (28.5)	16 (29.6)		145 (29.1)	107 (27.9)	
	(ASR= -0.2)	(ASR= 0.2)		(ASR= 0.4)	(ASR= -0.4)	
Survival status						
Alive	519 (62.7)	37 (68.5)	0.74 (0.389)	295 (59.2)	261 (68)	7.095 (0.008)
	(ASR= -0.9)	(ASR= 0.9)		(ASR= -2.7)	(ASR= 2.7)	
Dead	309 (37.3)	17 (31.5)		203 (40.8)	123 (32)	
	(ASR= 0.9)	(ASR= -0.9)		(ASR= 2.7)	(ASR= -2.7)	
Tumour edge						
Infiltrative	342 (52.5)	8 (38.1)	1.68 (0.195)	192 (52.5)	158 (51.5)	0.07 (0.797)
	(ASR= 1.3)	(ASR= -1.3)		(ASR= 0.3)	(ASR= -0.3)	
Pushing	310 (47.5)	13 (61.9)		174 (47.5)	149 (48.5)	
	(ASR= -1.3)	(ASR= 1.3)		(ASR= -0.3)	(ASR= 0.3)	
Tumour budding score						
Low	434 (67.3)	14 (77.8)	0.88 (0.348)	239 (66)	209 (69.4)	0.87 (0.35)
	(ASR= -0.9)	(ASR= 0.9)		(ASR= -0.9)	(ASR= 0.9)	
High	211 (32.7)	4 (22.2)		123 (34)	92 (30.6)	
	(ASR= 0.9)	(ASR= -0.9)		(ASR= 0.9)	(ASR= -0.9)	
Peritumoural lymphocytes						
inconspicuous	466 (71.1)	13 (65)	0.36 (0.551)	271 (73.4)	208 (68)	2.43 (0.119)
	(ASR= 0.6)	(ASR= -0.6)		(ASR= 1.6)	(ASR= -1.6)	
conspicuous	189 (28.9)	7 (35)		98 (26.6)	98 (32)	
	(ASR= -0.6)	(ASR= 0.6)		(ASR= -1.6)	(ASR= 1.6)	
MMR Status						
proficient (MSS/MSI-L)	701 (84.7)	45 (83.3)	0.22 (0.894)	412 (82.7)	334 (87)	4.12 (0.128)
	(ASR= 0.3)	(ASR= -0.3)		(ASR= -1.7)	(ASR= 1.7)	
deficient (MSI-H)	125 (15.1)	9 (16.7)		84 (16.9)	50 (13)	
	(ASR= -0.3)	(ASR= 0.3)		(ASR= 1.6)	(ASR= -1.6)	
MLH-1						
proficient	672 (88)	46 (85.2)	0.36 (0.548)	400 (86)	318 (90.1)	3.09 (0.079)
	(ASR= 0.6)	(ASR= -0.6)		(ASR= -1.8)	(ASR= 1.8)	
deficient	92 (12)	8 (14.8)		65 (14)	35 (9.9)	

	(ASR= -0.6)	(ASR= 0.6)		(ASR= 1.8)	(ASR= -1.8)	
PMS-2						
proficient	715 (86.6) (ASR= 0.3)	46 (85.2) (ASR= -0.3)	0.08 (0.774)	421 (84.9) (ASR= -1.6)	340 (88.5) (ASR= 1.6)	2.48 (0.115)
deficient	111 (13.4) (ASR= -0.3)	8 (14.8) (ASR= 0.3)		75 (15.1) (ASR= 1.6)	44 (11.5) (ASR= -1.6)	
MSH-2						
proficient	819 (99) (ASR= 0.6)	53 (98.1) (ASR= -0.6)	0.39 (0.531)	490 (98.6) (ASR= -1.3)	382 (99.5) (ASR= 1.3)	1.69 (0.194)
deficient	8 (1) (ASR= -0.6)	1 (1.9) (ASR= 0.6)		7 (1.4) (ASR= 1.3)	2 (0.5) (ASR= -1.3)	
MSH-6						
proficient	810 (98.4) (ASR= 0.2)	53 (98.1) (ASR= -0.2)	0.02 (0.877)	486 (98.4) (ASR= -0.1)	377 (98.4) (ASR= 0.1)	0.004 (0.951)
deficient	13 (1.6) (ASR= -0.2)	1 (1.9) (ASR= 0.2)		8 (1.6) (ASR= 0.1)	6 (1.6) (ASR= -0.1)	
Final Stage						
I	146 (17.6) (ASR= 0.5)	8 (14.8) (ASR= -0.5)	1.57 (0.666)	66 (13.3) (ASR= -3.7)	88 (22.9) (ASR= 3.7)	15.46 (0.001)
II	334 (40.3) (ASR= -1.1)	26 (48.1) (ASR= 1.1)		215 (43.2) (ASR= 1.6)	145 (37.8) (ASR= -1.6)	
III	259 (31.3) (ASR= 0.3)	16 (29.6) (ASR= -0.3)		167 (33.5) (ASR= 1.7)	108 (28.1) (ASR= -1.7)	
IV	89 (10.7) (ASR= 0.8)	4 (7.4) (ASR= -0.8)		50 (10) (ASR= -0.6)	43 (11.2) (ASR= 0.6)	
Primary tumours-stage (T)						
T1	65 (7.9) (ASR= -0.9)	6 (11.1) (ASR= 0.9)	1.95 (0.582)	25 (5) (ASR= -3.8)	46 (12) (ASR= 3.8)	19.43 (< 0.001)
T2	94 (11.4) (ASR= 0.9)	4 (7.4) (ASR= -0.9)		47 (9.4) (ASR= -1.8)	51 (13.3) (ASR= 1.8)	
T3	434 (52.4) (ASR= -0.7)	31 (57.4) (ASR= 0.7)		274 (55) (ASR= 1.6)	191 (49.7) (ASR= -1.6)	

T4	235 (28.4) (ASR= 0.7)	13 (24.1) (ASR= -0.7)		152 (30.5) (ASR= 1.8)	96 (25) (ASR= -1.8)	
Regional Lymph Nodes-stage						
N0	483 (60) (ASR= -0.8)	34 (65.4) (ASR= 0.8)	8.15 (0.017)	290 (59.2) (ASR= -0.8)	227 (61.9) (ASR= 0.8)	0.64 (0.728)
N1	193 (24) (ASR= -1.4)	17 (32.7) (ASR= 1.4)		124 (25.3) (ASR= 0.6)	86 (23.4) (ASR= -0.6)	
N2	129 (16) (ASR= 2.7)	1 (1.9) (ASR= -2.7)		76 (15.5) (ASR= 0.3)	54 (14.7) (ASR= -0.3)	
N2						
uninvolved	483 (60) (ASR= -0.8)	34 (65.4) (ASR= 0.8)	0.59 (0.442)	290 (59.2) (ASR= -0.8)	227 (61.9) (ASR= 0.8)	0.63 (0.429)
involved	322 (40) (ASR= 0.8)	18 (34.6) (ASR= -0.8)		200 (40.8) (ASR= 0.8)	140 (38.1) (ASR= -0.8)	
Distant Metastasis						
No metastasis	738 (89.1) (ASR= -0.8)	50 (92.6) (ASR= 0.8)	0.64 (0.424)	447 (89.8) (ASR= 0.5)	341 (88.8) (ASR= -0.5)	0.21 (0.648)
With metastasis	90 (10.9) (ASR= 0.8)	4 (7.4) (ASR= -0.8)		51 (10.2) (ASR= -0.5)	43 (11.2) (ASR= 0.5)	
Vascular Invasion						
Absent	421 (51.7) (ASR= -1.5)	33 (62.3) (ASR= 1.5)	2.22 (0.136)	243 (49.7) (ASR= -1.8)	211 (55.8) (ASR= 1.8)	3.21 (0.073)
Present	393 (48.3) (ASR= 1.5)	20 (37.7) (ASR= -1.5)		246 (50.3) (ASR= 1.8)	167 (44.2) (ASR= -1.8)	
Grade						
Low	769 (93) (ASR= -1.5)	53 (98.1) (ASR= 1.5)	2.16 (0.142)	458 (92) (ASR= -1.8)	364 (95) (ASR= 1.8)	3.23 (0.071)
High	58 (7) (ASR= 1.5)	1 (1.9) (ASR= -1.5)		40 (8) (ASR= 1.8)	19 (5) (ASR= -1.8)	
Perineural invasion						
absent	647 (81.1)	41 (80.4)	0.02 (0.904)	389 (80.7)	299 (81.5)	0.08 (0.778)

	(ASR= 0.1)	(ASR= -0.1)		(ASR= -0.3)	(ASR= 0.3)	
present	151 (18.9)	10 (19.6)		93 (19.3)	68 (18.5)	
	(ASR= -0.1)	(ASR= 0.1)		(ASR= 0.3)	(ASR= -0.3)	
Intramural lymphovascular Invasion						
absent	534 (68.7)	37 (80.4)	2.8 (0.094)	315 (68.9)	256 (69.9)	0.099 (0.753)
	(ASR= -1.7)	(ASR= 1.7)		(ASR= -0.3)	(ASR= 0.3)	
present	243 (31.3)	9 (19.6)		142 (31.1)	110 (30.1)	
	(ASR= 1.7)	(ASR= -1.7)		(ASR= 0.3)	(ASR= -0.3)	
Site						
right	397 (47.9)	26 (48.1)	0.001 (0.977)	248 (49.8)	175 (45.6)	1.55 (0.213)
	(ASR= 0.0)	(ASR= 0.0)		(ASR= 1.2)	(ASR= -1.2)	
left	431 (52.1)	28 (51.9)		250 (50.2)	209 (54.4)	
	(ASR= 0.0)	(ASR= 0.0)		(ASR= -1.2)	(ASR= 1.2)	

Significant p-values (< 0.05) are highlighted in red. Primary tumour stage p-value refers to the association between *TP53* and size and/ or extent of the tumour. Regional lymph nodes N0, N1, N2 show the presence of cancer in nearby lymph nodes: N0 refers to no cancer in nearby lymph nodes; N1, N2 refers to the location and number of lymph nodes that have cancer. The higher the number after the N, the higher the number of lymph nodes consist cancer.

*Abbreviations: X^2, Chi-square; ASR, adjusted standardized residual. ASR more than 2 or less than -2, are highlighted in blue.

Table 4.4.3 Associations between highest and lowest expressors of *Bcl-2* cytoplasmic staining and its clinicopathological significance.

Clinicopathological criteria	*Bcl-2* cytoplasmic staining			*Bcl-2* cytoplasmic staining		
	Low (%)	All (%)	X^2 (p-value)	High (%)	All (%)	X^2 (p-value)
3-years survival status						
Alive	266 (69.8)	384 (76.6)	5.21 (0.022)	223 (76.6)	427 (72.3)	1.93 (0.165)
Dead	115 (30.2)	117 (23.4)		68 (23.4)	164 (27.7)	
Survival status						
Alive	223 (58.5)	333 (66.5)	5.85 (0.016)	196 (67.4)	360 (60.9)	3.47 (0.062)
Dead	158 (41.5)	168 (33.5)		95 (32.6)	231 (39.1)	
MMR Status						
proficient (MSS/MSI-L)	309 (81.1)	437 (87.2)	8.05 (0.018)	252 (86.6)	494 (83.6)	2.11 (0.348)
deficient (MSI-H)	70 (18.4)	64 (12.8)		39 (13.4)	95 (16.1)	
MLH-1						
proficient	304 (84.7)	414 (90.2)	5.71 (0.017)	242 (90.6)	476 (86.4)	3.03 (0.082)
deficient	55 (15.3)	45 (9.8)		25 (9.4)	75 (13.6)	
PMS-2						
proficient	317 (83.6)	444 (88.6)	4.58 (0.032)	257 (88.3)	504 (85.6)	1.26 (0.262)
deficient	62 (16.4)	57 (11.4)		34 (11.7)	85 (14.4)	
Vascular Invasion						
Absent	177 (47.3)	277 (56.2)	6.69 (0.01)	160 (55.9)	294 (50.6)	2.19 (0.139)
Present	197 (52.7)	216 (43.8)		126 (44.1)	287 (49.4)	
Grade						
Low	347 (91.1)	475 (95)	5.33 (0.021)	274 (94.5)	548 (92.7)	0.96 (0.326)
High	34 (8.9)	25 (5)		16 (5.5)	43 (7.3)	
5-years survival status						

Alive	170 (44.6)	218 (43.5)	0.11 (0.743)	111 (38.1)	277 (46.9)	6.03 (0.014)
Dead	211 (55.4)	283 (56.5)		180 (61.9)	314 (53.1)	
Peritumoural lymphocytes						
inconspicuous	205 (71.4)	274 (70.6)	0.05 (0.819)	161 (65.7)	318 (74)	5.14 (0.023)
conspicuous	82 (28.6)	114 (29.4)		84 (34.3)	112 (26)	
Final Stage						
I	46 (12.1)	108 (21.6)	13.9 (0.003)	66 (22.7)	88 (14.9)	8.32 (0.04)
II	167 (43.8)	193 (38.5)		113 (38.8)	247 (41.8)	
III	128 (33.6)	147 (29.3)		83 (28.5)	192 (32.5)	
IV	40 (10.5)	53 (10.6)		29 (10)	64 (10.8)	
Primary tumours-stage (T)						
T1	19 (5)	52 (10.4)	15.99 (0.001)	36 (12.4)	35 (5.9)	11.94 (0.008)
T2	32 (8.4)	66 (13.2)		35 (12)	63 (10.7)	
T3	209 (54.9)	256 (51.1)		145 (49.8)	320 (54.1)	
T4	121 (31.8)	127 (25.3)		75 (25.8)	173 (29.3)	

Significant p-values (< 0.05) are highlighted in red. Primary tumour-stage p-value refers to the association between *Bcl-2* and size and/ or extent of the tumour. \
*Abbreviations: X^2, Chi-square.

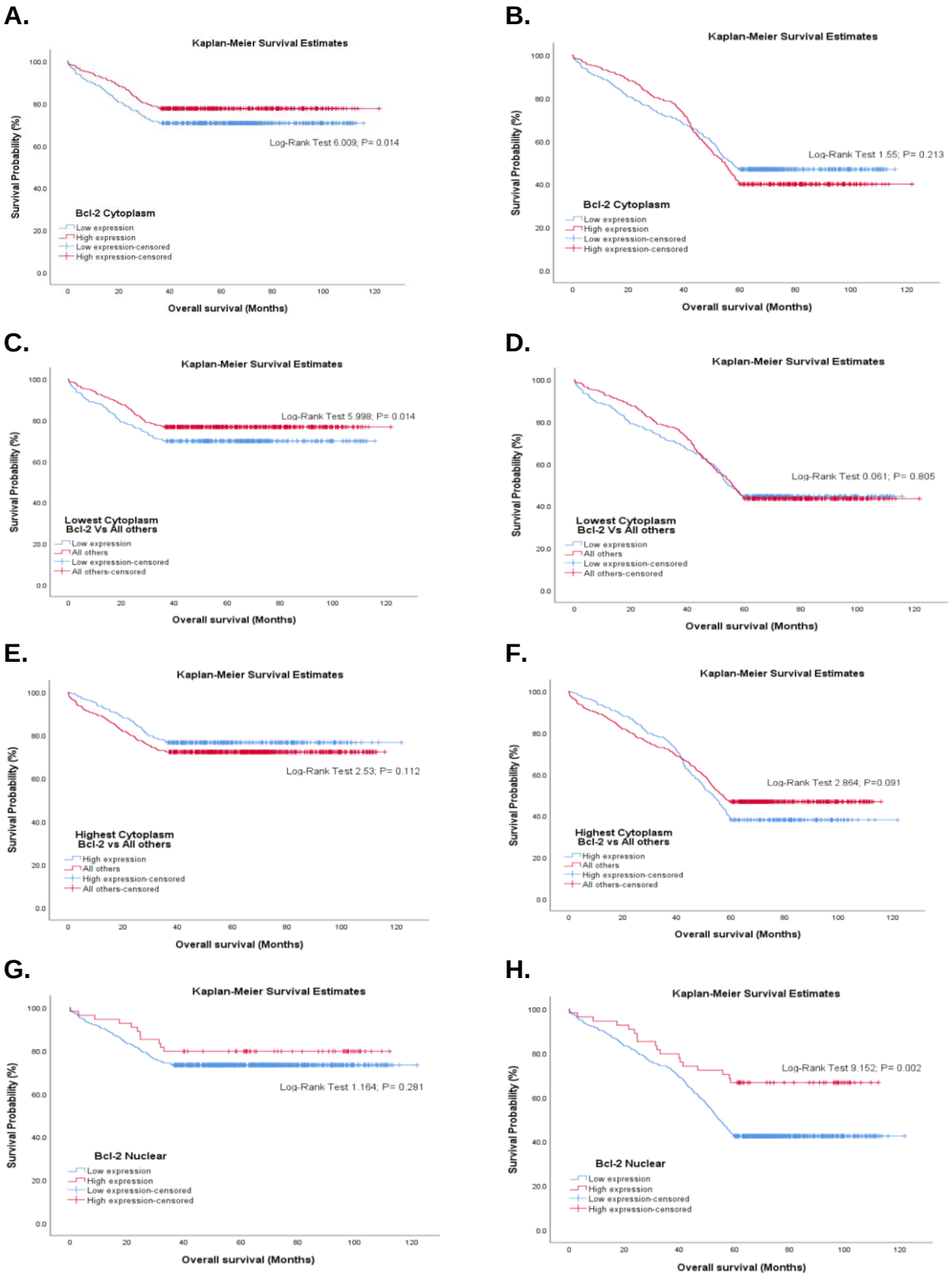


Figure 4.4.2 Survival probably of *Bcl-2* expression, **a.** Survival plot on low/high *Bcl-2* cytoplasmic expression in 3-years survival time, **b.** Survival plot on low/ high *Bcl-2* cytoplasmic expression in 5-years survival time, **c.** Survival plot on lowest cytoplasmic expressors/ all others of *Bcl-2* in 3-years' time-period, **d.** Survival plot on lowest cytoplasmic expressors/ all others of *Bcl-2* in 5-years' time-period, **e.** Survival plot on highest cytoplasmic expressors/ all others of *Bcl-2* biomarker in 3-years' time-period, **f.** Survival plot on highest cytoplasmic expressors/ all others of *Bcl-2* biomarker in 5-years' time-period, **g.** Survival plot on low/high *Bcl-2* nuclear expression in 3-years survival time, **h.** Survival plot on low/high *Bcl-2* nuclear expression in 5-years survival time.

Table 4.6. Paired t-test results on *TP53* and *Bcl-2* nuclear/cytoplasmic expression at different sites of tumour.

Gene Expression	Mean difference	95% CI	t	df	P-value	Notes
***TP53* Nuclear**						
Luminal-Central	5.12	(1.26, 8.97)	2.606	594	0.009	
AE-Central	4.31	(0.4, 8.21)	2.166	537	0.031	
AE-Luminal	-1.19	(-5.43, 3.05)	-0.551	533	0.582	Luminal = AE > Central
***TP53* Cytoplasm**						
Luminal-Central	3.43	(1.4, 5.47)	3.314	594	0.001	
Central-AE	-1.91	(-3.86, 0.036)	-1.93	537	0.054	
Luminal-AE	0.9	(-1.62,3.42)	0.705	533	0.481	Luminal = AE > Central
***BCL-2* Nuclear**						
Luminal-Central	0.04	(-0.037,0.11)	1.002	548	0.317	
AE-Central	-0.039	(-0.1, 0.02)	-1.24	466	0.217	
AE-luminal	-0.059	(-0.14,0.02)	-1.45	477	0.148	Luminal = AE = Central
***BCL-2* Cytoplasm**						
Luminal-Central	2.15	(0.37, 3.93)	2.37	548	0.018	
Central-AE	1.03	(-0.74, 2.79)	1.14	466	0.254	
Luminal-AE	2.73	(0.68, 4.77)	2.62	477	0.009	Luminal > Central = AE

Notes: Significant p-values (< 0.05) are highlighted in red.

Abbreviations: AE, advancing edge; CI, confidence interval; df, degree of freedom.

4.6 Comparisons of *TP53* and *Bcl-2* nuclear/cytoplasmic expression at different sites of the tumour

In *TP53* nuclear expression, there were significant differences between the groups of luminal and central (p=0.009), as well as advancing edge and central (p=0.031), with t-value 2.606 and 2.166 respectively. This showed that there was heterogeneous expression, but it's uncertain which part of the tumour was higher or lower. Whereas, in *TP53* cytoplasmic expression, significant association with heterogeneous expression was observed in luminal and central sites (p=0.001), with t-value 3.314. However, borderline association with the central and advancing edge (p=0.054) was also detected and directed a result where the expression effect between the groups had no differences (**Table 4.6**).

No significant associations were determined in *Bcl-2* nuclear expression, which showed a homogeneous effect. Instead, significant correlation discovered in the groups of Bcl-2 cytoplasmic expression luminal-central (p= 0.018, t-value=2.37) and luminal-advancing edge (p= 0.009, t-value=2.62) showed that there were still differences between groups, with heterogeneous effect. By comparing all these three sites of both biomarkers (*TP53* and *Bcl-2*), conclusions were drawn that the luminal expression was higher compared to the central and advancing edge in *Bcl-2* cytoplasmic expression. Although in *TP53* cytoplasmic/ nuclear expression, luminal and advancing edge expressions were higher compared to the centre of the tumour.

As shown in **Table 4.6**, the degree of freedoms was in the range of 450 to 600 on both biomarkers, this result revealed that the hypothesis test obtained was quite powerful and their estimates were precise. There was also no significant difference between the age group and the survival rates in the 3-years (p= 0.314) and 5-years (p=0.099) period of time.

4.7 *TP53* nuclear/cytoplasmic expression and association with normal and tumour samples

There was a mean difference of 90.03 with 95% CI (-98.80, -81.25) by comparing the *TP53* nuclear expression level in tumour-derived and normal colorectal cells. This outcome showed CRC patients had higher *TP53* nuclear expression (shown in **Table 4.7**) in tumour-derived cells compared to normal cells with a significant difference (p< 0.001).

In *TP53* cytoplasmic expression, there was 95% confidence that the true mean laid between -16.84 and -10.32. Mean difference of 13.58 was derived when comparing the cytoplasmic *TP53* expression in tumour-derived and normal colorectal cells with significant correlations (p< 0.001). Hence, *TP53* cytoplasmic expression level was higher in tumour-derived cells compared to normal cells as their mean was higher.

Table 4.7. Paired t-test results comparing normal and tumour samples on *TP53* and *Bcl-2* nuclear/cytoplasmic expression.

Gene Expression	N	Mean	SD	95% CI	P-value
TP53 Nuclear					
Normal	520	6.08	22.45	(-98.80, -81.25)	<0.001
Tumour	520	96.1	102.73		
TP53 Cytoplasm					
Normal	521	0.02	0.438	(-16.84, -10.32)	<0.001
Tumour	521	13.6	37.84		
BCL-2 Nuclear					
Normal	503	0.48	1.537	(-0.54, -0.27)	<0.001
Tumour	503	0.07	0.407		
BCL-2 Cytoplasm					
Normal	503	15.49	24.72	(-4.39, 0.95)	0.207
Tumour	503	17.2	22.37		

Notes: Significant p-values (< 0.05) are highlighted in red.
Abbreviations: N, sample size; SD, standard deviation; CI, confidence interval.

4.8 *Bcl-2* nuclear/cytoplasmic expression and association with normal and tumour samples

Bcl-2 cytoplasmic expression was not significantly correlated (p=0.207) with normal and tumour colorectal samples, whereas *Bcl-2* nuclear expression was significantly associated (p< 0.001) with normal and tumour colorectal cells. The true mean of the difference laid between -0.54 and -0.27 in Bcl-2 nuclear expression (**Table 4.7**) and their means were quite similar to each other, thus they were significantly correlated with similar expression level (similar means) in tumour and normal samples.

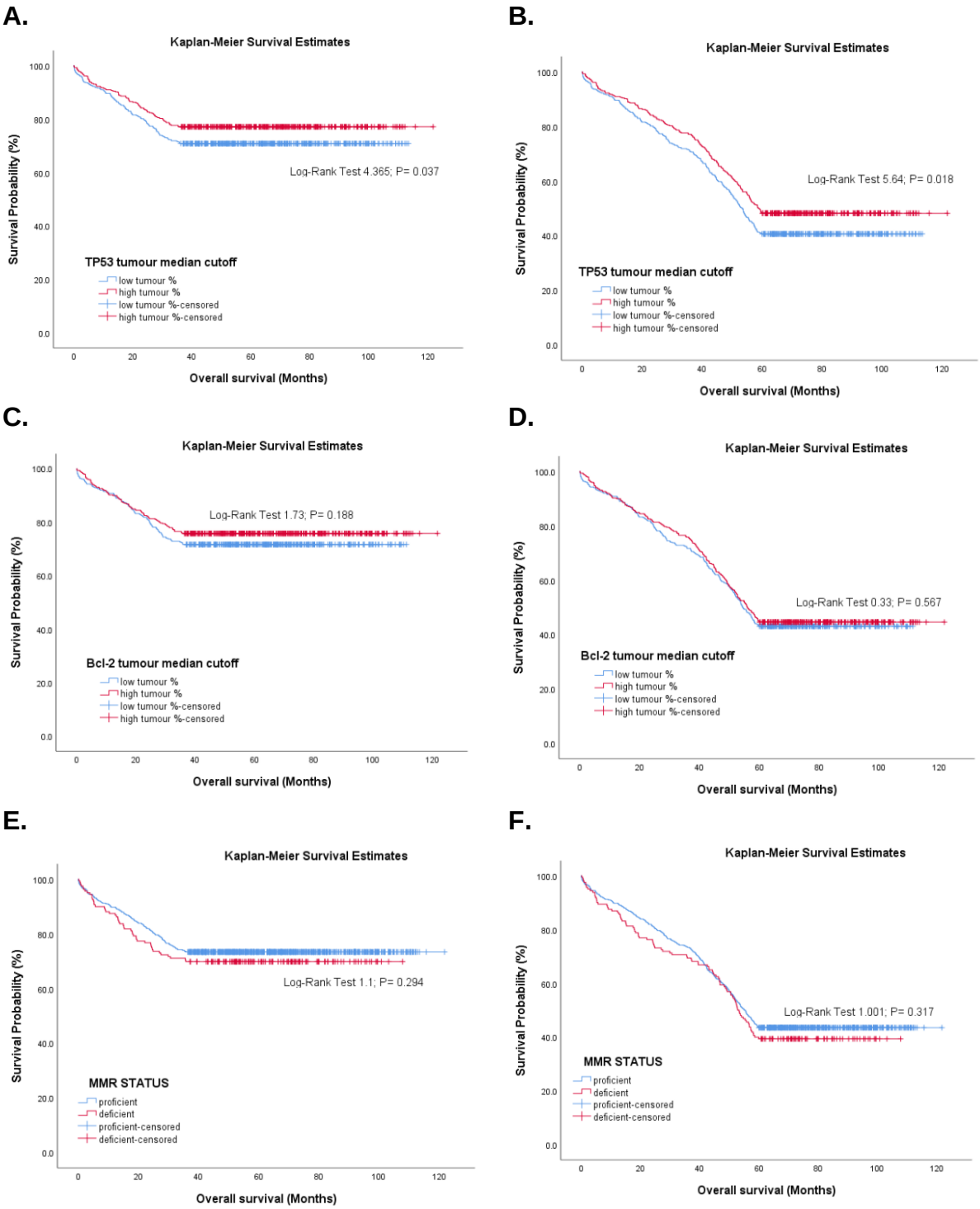


Figure 4.9 Survival probabilities of MMR status and *TP53/ Bcl-2* tumour-stroma percentage, **a.** Survival plot on *TP53* tumour-stroma percentage in 3-years survival time, **b.** Survival plot on *TP53* tumour-stroma percentage in 5-years survival time, **c.** Survival plot on *Bcl-2* tumour-stroma percentage in 3-years' time-period, **d.** Survival plot on *Bcl-2* tumour-stroma percentage in 5-years' time-period **e.** Survival plot on MMR status in 3-years' survival time **f.** Survival plot on MMR status in 5-years survival period.

4.9 Prognostic impact of tumour-stroma ratio

The multivariate regression resulted from **Table 4.9** manifested that none of the significance was observed on both biomarkers' tumour-stroma expression in several clinicopathological variables; lymph node (LN) stage, metastasis, vascular invasion, grade, perineural invasion and primary tumour stage (T). Tumour-stroma expression was not an independent factor of improved 3-years survival and 5-years survival. However, survival plots of tumour-stroma percentage were both significance in *TP53* either 3-years (p= 0.037) or 5-years (p= 0.018) survival time, but not significantly associated in *Bcl-2* biomarker in 3-years (p= 0.188) and 5-years survival period (p= 0.567) **(Figure 4.9 a-d)** owing that *TP53*, a good prognostic biomarker in CRC tumour cells.

Table 4.9. Multivariate Regression Model: Effects of tumour-stroma and MMR status on CRC samples

Variables	3 years			5 years		
	SE	HR	P-value	SE	HR	P-value
TP53						
LN stage	0.097	1.13	0.209	0.071	1.216	0.006
Metastasis	0.158	2.344	< 0.001	0.128	2.282	<0.001
Vascular Invasion	0.168	1.157	0.385	0.115	0.964	0.749
Grade	0.105	1.363	0.003	0.089	1.184	0.058
Perineural Invasion	0.162	1.328	0.079	0.123	1.19	0.159
Primary-tumour Stage (T)			<0.001			<0.001
T2	0.558	0.757	0.618	0.279	0.847	0.553
T3	0.467	1.321	0.552	0.245	1.005	0.983
T4	0.478	2.629	0.043	0.259	1.55	0.091
Tumour-stroma ratio	0.137	0.942	0.663	0.095	0.901	0.273
Bcl-2						
LN stage	0.099	1.1	0.333	0.072	1.226	0.004
Metastasis	0.163	2.478	<0.001	0.132	2.382	<0.001
Vascular Invasion	0.169	1.191	0.302	0.116	0.994	0.959
Grade	0.102	1.633	<0.001	0.089	1.338	0.001
Perineural Invasion	0.168	1.279	0.142	0.128	1.128	0.346
Primary-tumour Stage (T)			<0.001			<0.001
T2	0.528	0.288	0.018	0.271	0.54	0.023
T3	0.391	0.249	<0.001	0.202	0.509	0.001
T4	0.154	0.478	<0.001	0.11	0.622	<0.001
Tumour-stroma ratio	0.138	1.039	0.784	0.095	1.02	0.875
CRC tumours						
LN stage	0.091	1.19	0.057	0.066	1.247	0.001
Metastasis	0.147	2.488	<0.001	0.119	2.408	<0.001
Vascular Invasion	0.158	1.064	0.695	0.108	0.957	0.685
Grade	0.092	1.419	<0.001	0.078	1.255	0.003

Perineural Invasion	0.148	1.287	0.088	0.115	1.098	0.417
Primary-tumour Stage (T)			<0.001			<0.001
T2	0.601	0.965	0.953	0.277	0.915	0.748
T3	0.516	1.823	0.245	0.243	1.11	0.669
T4	0.525	3.73	0.012	0.257	1.804	0.021
MMR status	0.177	1.029	0.871	0.127	1.02	0.875

Notes: Significant p-values (< 0.05) are highlighted in red.
Abbreviations: HR, hazard ratio; SE, standard error; LN stage, Lymph Node stage.

4.10 Prognostic impact of MMR status in CRC tumours

Significant correlation was discovered between highest TP53 expression, lowest Bcl-2 expression, and MMR status ($p < 0.05$) **(Table 4.2.3, Table 4.4.3)**. As shown in **Table 4.9**, multivariate analysis showed that MMR status together with primary tumour stage, lymph node stage, grade, metastasis, vascular invasion and perineural invasion were all not associated (p-value> 0.05) in neither 3-years (HR, 1.029, p= 0.871) and 5-years (HR= 1.02, p= 0.875) period of time. Thus, there is consequently insufficient evidence to conclude that a prognostic effect of MMR status in CRC patients is present, revealing that MMR status was not an independent factor of improved survival in colonic cancers.

In **Fig. 4.9 e-f**, proficient MMR (MSS) has a higher survival probability compared to deficient MMR (MSI). There were 746 proficient MMR (84.8%) and 124 deficient MMR cases (14.1%), with a reduction of 15% and 10% in the risk of death observed in CRC patients during 3-years and 5-years survival time, respectively. In depth, MMR status was not significantly associated with the survival status in neither 3-years (p=0.294) and 5-years (p=0.317) survival time **(Figure 4.9 e-f),** which can conclude that there was no significant correlation between MMR status and survival status.

5. Discussion

There has been a drastic increase in patients of CRC all over the world. Given the advances in primary and adjuvant treatments of CRC, including chemotherapy, radiotherapy, surgery, and targeted therapies, the mortality and morbidity rate of CRC has still remained at a higher rate. CRC is a heterogeneous disease classified by the alterations in gene expression and tumour location (Testa *et al.*, 2020). Changes in the patterns of gene expression caused by genetic and epigenetic aberrations in oncogenes, tumour suppressor genes and DNA MMR genes can dysregulate several signalling pathways, including p53 signalling, RAS signalling and Wnt/β-catenin signalling (Holmes *et al.*, 2008). It will lead to the induction of proliferation, invasion and migration of tumour-derived colorectal cells and the inhibition of apoptosis (Gupta *et al.*, 2010).

CRC screening is a multiphase organized process (identifying target, conduct screening, confirm diagnosis and treatment coherently), in which failure in any steps will cause mortality (Wang *et al.*, 2018). It will cause a delay in the diagnostic of CRC when the screening is not able to perform accurately, hence colorectal tumours will grow to an advance stage. *TP53* serves as a common mutated gene found in tumour-derived colorectal cells, can maintain homeostasis of the cell cycle and cell death (Kastenhuber & Lowe, 2017). *TP53* mutation is an important and common occasion in CRC carcinogenesis to involve in the malignant transformation of colorectal adenoma to cancer. Not only it can promote NF-KB activation, but also metastasize to distant organs and progress to invasion carcinomas by inflammatory and oncogenic pathways (Nakayama & Oshima, 2019). The abilities of *TP53* on repairing DNA damage, regulating immune response, and regulating cell cycle will be triggered in response, as such it serves as a common suppressor gene that plays essential roles in cancer cells (Ozaki & Nakagawara, 2011). However, in cancer cells, this protein is no longer able to control its growth (cell proliferation), and results in genetically unstable cells, along with inefficient DNA repair.

TP53 abnormalities caused by gain- and loss-of-function mutations can alter gene expression level in cancer cells. Simultaneously, the nuclear versus cytoplasmic effects of *TP53* are regulated by multiple post-translational modifications [Poly (adenosine diphosphate ADP) ribosylation of *TP53*, the transcription factor, forkhead box O-3a (FOXO3a) monoubiquitylation by murine double minute 2 (MDM2)]. These

modifications can affect its interaction with other proteins, shuttling between the nucleus, cytoplasm and its biological activities. Fundamentally, poly (ADP) ribosylation of TP53 can cause nuclear accumulation, whilst FOXO3a enhances p53 cytoplasmic accumulation by triggering its nuclear export and MOMP induction (Kanai *et al.*, 2007).

In normal unstressed cells, *TP53* is a very unstable protein and present at very low cellular levels owing to the continuous degradation mediated by MDM2 (Nag *et al.*, 2013). MDM2 functions as a main cellular antagonist of *TP53* can limit *TP53* growth suppressive functions in unstressed cells. It monoubiquitinates *TP53* and serves as a crucial step in mediating the degradation by cytoplasmic and nuclear proteasomes (Wolff *et al.*, 2008). However, stress pathways such as oncogenes activation, DNA damage, telomere shortening, and hypoxia (mutation) can block its degradation and cause a rapid stabilization of *TP53* (Labuschagne *et al.*, 2018). When nuclear *TP53* induces the expression of MDM2 and inhibit the protein (MDM2) through binding and ubiquitinylation, the cellular stress that interrupts this inhibition will allow p53 to accumulate in both nucleus and cytoplasm in CRC carcinogenesis (Green & Kroemer, 2009). Thus, the interactions on post-transcriptional *TP53* modifications and proteins can affect the subcellular localization and the function of *TP53*. These nuclear and cytoplasmic accumulation triggered by *TP53* mutation in cancer cells can cause gain- or loss-of-function mutation and enhance the propagation of cells that carry damaged DNA with oncogenic mutations.

Abnormal *TP53* that trigger *Bcl-2* activation can stimulate the mitochondrial intrinsic pathway through a wide range of signals and activates caspase cascade in response to promote transcription-independent apoptosis cell death (García-Aranda *et al.*, 2018). This metabolic pathway is characterised by the permeabilization of mitochondria and can lead to a loss of *Bcl-2* expression or a gain of abnormal expression [*Bcl-2*(+)/ p53(-) phenotype]. On nuclear versus cytoplasmic expression of Bcl-2, the mechanism underlying the effect of Bcl-2 expression on prognosis is still remain unknown (Shamas-Din *et al.*, 2013). However, the process of apoptosis that involves pro- and anti-apoptotic proteins can balance the entire process of programmed cell death. Thus, analysis of Bcl-2 protein should be performed with caution and studies on the combination of pro- and anti-proteins were conducted to determine the impact of clinical outcome.

5.1 The association of *TP53* expression levels with clinic-pathological features and prognosis of CRC patients

The present study enrolled 1000 patients with colorectal tumours, but only 880 patients were investigated as the remaining 120 cores have staining less than 15%. The clinicopathological features were investigated to provide a theoretical understanding of influencing factors in the prognosis of CRC. *TP53* nuclear expression had significant differences with 3-years survival, survival status, proficient MMR status, proficient *MLH-1*, proficient *PMS-2*, the number of lymph nodes harvested and the right-sided colon **(Table 4.2.2)**. Meanwhile, in *TP53* cytoplasmic expression, positive correlations were determined in 5-years survival status, low tumour budding score, proficient MMR status, proficient *MLH-1*, proficient *PMS-2* and the right-sided colon **(Table 4.2.2)**. The similarities between *TP53* nuclear and cytoplasmic expressions have also been found with a significant association in proficient MMR status and the right-sided colon. MSS tumours characterised by a higher incidence of *TP53* mutation, have a positive association between proficient MMR, proficient *MLH-1* and proficient *PMS-2* and *TP53* expression (Lin *et al.*, 2015). The *TP53* expressions observed in the right-sided colon shows that there is a transcription-independent apoptotic activity of p53. This is because the study established by Paluszkiewicz et al. (2004) indicates that the p53 activity presents in the right-sided colon tumours can develop in a p53-independent manner, and the p53 status in cancer cells has prognostic value only in left-sided colon tumours.

In this present study, highest and lowest expressors of *TP53* have been used to compared to all other expressors of biomarkers, and the association of them on their clinicopathological features were investigated **(Table 4.2.3)**. There was no significant difference between the lowest expressors of *TP53* and the clinicopathological features. However, a positive correlation had observed in higher *TP53* nuclear expression with better tumour characteristics found on good survival rates, proficient MMR status, *MSH-1*, *PMS-2*, no metastasised, no reoccurrence, T-stage III, vascular invasion, infiltrative tumour edge and the left-sided colon**.** This showed that well to moderately differentiated tumours with increased survival have higher expression levels of p53 compared to poorly differentiated tumours (Lan *et al.*, 2007).

In addition, the lymph node stage, metastasis, vascular invasion, grade, perineural invasion, primary tumour stage and p53 nuclear expression status were independent

factors that influenced the survival rate of CRC patients in 3-years survival period **(Table 4.2.1)**. These was further clarified by the survival plot of higher TP53 nuclear expression where it was found significantly correlated in 3-years survival time with lower survival benefit compared to all other expressors of biomarkers, showing that the accumulation of p53 protein will have a favourable prognostic value in CRC **(Figure 4.2.2 c).** A previous retrospective tumour microarray study also demonstrated that p53 can be a potential biological diagnostic marker and p53 has been demonstrated to be associated with better colon cancer prognosis (Lan *et al.*, 2007; Resnick *et al.*, 2004). These results are further justified and compared to the studies by He et al. (2015) with the downregulation of *TP53* increasing the risk of expressing colorectal tumours. Thus, an ideal strategy that can reduce the deleterious mutant p53 expression and restores wild type p53 production should be approached in cancer cells, to have a better treatment for CRC.

There are also some other types of cancers such as lung squamous-cell carcinoma and glioblastoma multiforme that have higher *TP53* expression levels compared to normal tissue, which is mainly due to an overexpression of mutant-*TP53* (Wang & Sun, 2017). These show that an overexpression of mutant-*TP53* can observe in other types of cancers, including CRC. Thus, in this study, high *TP53* expression levels caused by an overexpression of mutant-TP53, can be observed when aberrant *TP53* are triggered by DNA damage and other stress signals in several types of cancer. These can enhance cell proliferation, differentiation and spreading within the body.

There are lots of studies reported a good prognosis for *TP53* mutation, but there are also some studies that show poor prognosis on it (Olivier *et al.*, 2010). Brannon et al. (2014) noted that, there was an 80% increase in the mutation rate of TP53 for advanced CRC patients who have metastasis (Baker *et al.*, 1990; Hao *et al.*, 2002). Study by Rambau et al. (2008) was using *TP53* protein in malignant cells to investigate their survival and prognosis outcome, in which poor prognosis and reduced survival rate were noted in high *TP53* expression levels (high *TP53* mutations). This shows that *TP53* mutations will only have a poor prognosis and reduced survival rate in malignant tumour cells, however when *TP53* protein was examined in different stages of colorectal tumour cells in this present study, it provided a better prognosis outcome, with an increased survival in high *TP53* expression levels. Thus, *TP53* mutations have an essential role in serving a reduced survival rate in the late stage of tumorigenesis

when invasive adenomas metastasis to distant organs. Nonetheless, study from Lanza et al. (1996) had use different stages of colorectal adenocarcinomas to determine the survival and prognosis outcome of CRC. Results with poor survival was noted in high *TP53* expression levels, these shows that p53 protein expression may be used as an indicator for the prognosis of CRC, however the exact mechanism of CRC requires further exploration. As *TP53* mutations are commonly seen in colorectal tumours, it is not possible to prevent this *TP53* protein from mutating, hence p53 protein expression should be regularly screened in different stages of CRC patients to enable individualized treatments to improve clinical outcomes. Screening at different stages of CRC patients is strongly recommended, as the exact mechanisms and pathways for different stages of colorectal tumours can be different, which results in different prognosis values.

This present study found to have a higher *TP53* nuclear expression and a better survival status in tumour-derived CRC cells. This is particularly because the enforced *TP53* expression in CRC patients promote irreversible apoptosis within cells, which regulates the tumour occurrence and development. Yet, when there is a disturbance between the apoptosis and cell proliferation, it will lose the control of normal apoptosis. As the anti-apoptotic *Bcl-2* proteins can restrain the pro-apoptotic BAX/BAK *Bcl-2* protein, it will cause an overexpression of anti-apoptotic genes and under-expression of pro-apoptotic genes. Anti-apoptotic proteins that regulate apoptosis can block the mitochondrial from releasing cytochrome c and promotes apoptosis. These show that tumour-derived p53 mutant defective in the transcription ability can bind to the DNA in a sequence-specific manner and impairs apoptosis induction. Hence, high *TP53* expression levels will cause an increase in anti-apoptotic proteins (*Bcl-2*) level and can reduce pro-apoptotic proteins *(Bcl-2)* by increasing cell death exhibit by cancer cells, which leads to a poor prognosis.

However, the defects in apoptosis caused by anti-apoptotic members will allow colorectal tumours to resist in traditional therapies such as chemotherapy and radiotherapy. This is mainly because the threshold of apoptotic cells has raised which caused them difficult to be kill in traditional therapies. It will also promote resistance to the overall immune system as the body immune system is depends on the integrity of apoptotic pathway (Hassan *et al.,* 2014). As apoptosis is critical for the development and homeostasis of the immune system, when there are defects in apoptosis caused

by high *TP53* expression, it will take control of the immune response, the deletion of immune cells recognising self-antigens, and cytotoxic killing. These defects in apoptosis will cause a disturbance between the apoptosis and cell proliferation, and leads to CRC. These show that high *TP53* expression can have a poor prognosis and has their disadvantages when it comes to anti-angiogenic therapy and the immune system of CRC patients.

Although high *TP53* expression is correlated to apoptosis, however the prognosis outcome of CRC is found dependent on *Bcl-2* family. If the anti-apoptotic proteins of *Bcl-2* family (Bcl-2 overexpression) is higher, there will be a poor prognosis outcome. These were further verified by Um, (2016), where the cellular invasiveness of cancer is reduced, when the expression levels of anti-apoptotic members are reduced, which shows that anti-apoptotic members can promote cell migration and invasion. As the result shown in this study, there is a low *Bcl-2* expression level in CRC, in which a good prognosis outcome is presented. Therefore, anti-apoptotic Bcl-2 family proteins are important regulators and are targeted by researchers as cancer therapy, to minimize their damage on tissues.

5.2 The association of *Bcl-2* expression levels with clinic-pathological features and prognosis of CRC patients

Alterations in the expression levels of apoptosis-associated proteins have been reported in several cancers, including CRC cancer. To further validate the relationship between *Bcl-2* biomarkers and the clinic-pathological variables, manual scoring and statistical analysis were performed **(Table 4.4.2)**. Results divulge *Bcl-2* nuclear expression has a significant association to 5-years survival and metastasis to regional lymph nodes, whereas *Bcl-2* cytoplasmic expression has found positively correlated with 3-years and 5-years survival, survival status, final stage, and primary tumours stage.

In this present study, *Bcl-2* that expressed in the cytoplasm does not present as an independent factor in colonic cancers in 3-years and 5-years period of time, whereas *Bcl-2* that is expressed in the nuclear can be an independent factor in 5-years survival time **(Table 4.4.1).** In the meantime, *Bcl-2* nuclear expression was the only one that shows an absent of confidence interval crosses 1, this clearly clarifies that a sufficient evidence was provided showing *Bcl-2* nuclear expression and their multivariate groups were statistically significant. Survival probability observed in **Figure 4.4.2** was

significant in 3-years survival time on *Bcl-2* cytoplasmic expression and correlated in 5-years survival time on *Bcl-2* nuclear expression. This given result is supported by the outcome study of Hoetelmans et al. (2000), showing that *Bcl-2* expression was mostly detectable in the cytoplasm initially, and mainly *Bcl-2* nuclear expression after 5 days. As such, it shows that the nuclear expression of *Bcl-2* is a slow process and the permeabilization procedure and the mild fixation are not responsible for *Bcl-2* relocalization (Hoetelmans *et al.*, 2000).

In this study, highest and lowest expressors of *Bcl-2* have been used to compared to all other expressors of biomarkers, and the association of them on their clinicopathological features were investigated **(Table 4.4.3)**. A significant association had been discovered in lowest *Bcl-2* cytoplasmic expression with better survival status, proficient MMR status, proficient *MLH-1*, proficient *PMS-2*, vascular invasion, lower grade, final stage II and T-stage III. In low-grade cancer, tumour cells with low Bcl-2 expression tend to grow slowly and are less likely to spread (McClure *et al.*, 2018). Study by Gockley et al. (2017) has the outcome showing an increased survival in low grade serous ovarian cancer compared to high grade serous ovarian cancer. Although the study is investigated in ovarian cancer, it still indicates that the adenomas in low grade cancer is less aggressive compared to high grade cancer, which shows a better prognosis outcome. In this present study, a better survival rate was also observed in *Bcl-2* MSS phenotype (*MSH-1* and *PMS-2*) in lowest *Bcl-2* (high Bax) (high Bax/*Bcl-2* ratio) cytoplasmic expression in 3-years survival period **(Figure 4.4.2 c)**. This correlation shows that a lower Bcl-2 cytoplasmic expression levels can have a better prognosis outcome, with an increased survival and serve as a good prognostic indicator in tumour-derived CRC cells. Concurrently, results have also been verified by Roh et al. (2020) that *Bcl-2* overexpression involved in tumour growth and angiogenesis can provide a poor prognosis with a higher risk in colorectal tumours.

Bcl-2 family in colorectal tissues have concentrated Bcl-2 and BAX/ BAK (Shamas-Din *et al.*, 2013). In apoptosis, BAX/ BAK protein activates cascade by releasing cytochrome c from the mitochondria and promotes cell death (Korsmeyer *et al.*, 2000). However, *Bcl-2* family can take control of apoptosis by preventing BAX/BAK from releasing cytochrome c and restricts apoptotic machinery. These show that the immunostaining for BAX/ BAK protein should also be performed, to have a clear view on the levels of pro-apoptotic proteins and its prognosis for CRC.

5.3 Comparison of TP53 and Bcl-2 expression in normal and tumour samples

By comparing tumour and normal samples of colorectal cells, a positive correlation is observed on *TP53* nuclear/ cytoplasmic expression and *Bcl-2* nuclear expression (all p< 0.001) (**Table 4.7**). These significant difference between *TP53* expression and the colorectal cells, indicates that there are differences and associations on their *TP53* expression levels in both tumour and normal samples. Study by Park et al. (2012) indicates that the mean value of expression levels in all CRC tissues is defined as a measure of expression level. Hence, tumour colorectal cells will have more *TP53* expression (higher mean) when compared to normal cells.

As the mean values in *Bcl-2* nuclear and cytoplasmic expression are quite similar in both tumour and normal samples, there is not much of a difference in their expression levels **(Table 4.7)**. However, the result of Bcl-2 nuclear expression (p-value < 0.001) shows that Bcl-2 nuclear expression is correlated in normal and tumour samples. These shows that *Bcl-2* nuclear expression may play an essential role in the colorectal normal and tumour samples, but due to its low expression level in the colorectal samples, their differences in expression levels cannot be clearly seen.

5.4 Comparison of TP53 and Bcl-2 expression on different sites of CRC tumours sample

A paired t-test (**Table 4.6**) is managed to compare and contrast the expression level in different positions of the tumour. Their results of association (p< 0.05) show that intra-tumour heterogeneity is observed within the tumours, but still unsure which expression on the sites of the tumour is higher or lower. This shows that the distinct morphological and phenotypic profiles, including gene expression, proliferation and metastasis can be presented when there is an imperfection in their DNA replication (Borisch & Stanta, 2018). By comparing their mean values, the luminal surface of tumour has a similar expression level with deep margins of tumour, but a lower expression level in the centre of tumour in both *TP53* nuclear and cytoplasmic expressions. It is also well postulated that the expression level in all sites of the tumour maintains the same in *Bcl-2* nuclear expression. Yet, in *Bcl-2* cytoplasmic expression, a higher expression level was determined in the luminal surface of tumour compared to the two sites of tumour. It is also given that in **Table 4.6**, two significance p-value (<0.05) were observed when comparing the three sites of tumour in both *TP53* nuclear and *Bcl-2* cytoplasmic expressions, showing that these two expression levels play a crucial role in tumour development.

5.5 Survival probability of TP53 with tumour-stroma ratio

The tumour stroma is a component of the tumour microenvironment that serves a vital role in regulating cancer cells (Valkenburg *et al.,* 2018). It is involved in tumorigenesis by the epithelial-to-mesenchymal transition (EMT) and stem cell-like properties in cancer cells (Roche, 2018). In this study, tumour-stroma percentage (can also manifest as tumour-stroma ratio, TSR) has been calculated. Low TSR (low tumour %) has been detected with lower survival probability ($p < 0.05$) on *TP53* biomarker for the identification of CRC patients in both 3-years and 5-years period of time (**Figure 4.9 a-d**). This is further evidenced by Eriksen et al. (2018) which indicates stroma-high tumours (low TSR) will have poor patient prognosis (adverse outcome) in CRC tumours as well as other epithelial tumours. This shows that the quantity or composition of the tumour stroma can be distributed as an essential determinant of cancer behaviour.

5.6 The association of proficient MMR, TP53 and *Bcl-2* expression levels and prognosis of CRC patients

In this study, the proficient MMR status was also investigated in both highest *TP53* nuclear expression and lowest *Bcl-2* expression. A significant correlation was observed in both expression levels, with p-value <0.05 **(Table 4.2.3, Table 4.4.3)**. This showed that in sporadic CRC, highest *TP53* and lowest *Bcl-2* expression levels present in proficient MMR tumours are mainly MSS and MSI-low. Additionally, proficient MMR was not an independent factor of improved survival in colonic cancers with poor prognosis value in CRC **(Table 4.9, Figure 4.9 e-f**). This was evidenced by the results of the article accomplished by Sinicrope & Yang (2011), of which MSI tumours have a better survival rate compared to MSS tumours. In order to have a better prognosis and predictive impact of MMR status, it is being recommended that all CRC patients in this study are not allowed to receive chemotherapy before TMA construction (Sinicrope & Yang, 2011). Although the mechanism underlying a better prognosis of deficient MMR is still not completely understood, but an observation on the immune response of enhanced host-mediated anti-tumour on CRC cells is encouraged to contribute to their clinical behaviour.

5.7 Tumour budding guidelines and factors affecting test results

Tumour budding is a predictor of lymph node metastasis, though it is a promising prognostic factor in CRC that is independent of tumour-node-metastasis (TNM) stages (Wyk *et al.*, 2019). There is a standardized, evidence-based, international procedure

proposed by International Tumour Budding Consensus Conference (ITBCC) for tumour budding assessment, provides a guideline/protocol for future reporting of tumour budding in CRC. To ensure that the correct field of the tumour budding hotspot is selected during immunohistochemical staining, 10 separate fields (20x objective) along the invasive front of H&E slide are scanned before counting tumour buds in the single selected 'hotspot' (Zlobec *et al.*, 2011). It is however, mandatory that tumour budding hotspot must be chosen accurately in immunohistochemically stained sections of CRC samples, but this selected 'hotspot' can be difficult in tissue microarray as the spatial relationship of tumour buds were not preserved (Weis *et al.*, 2018).

As the mutation/loss (non-functional) of the *TP53* gene is the most common genetic abnormality in human cancer, immunohistochemical staining for *TP53* therefore relates to the mutation status in many cases. Although reassuring that large validation studies (1000 cases) are conducted when investigating biomarkers in CRC, there are still factors that are affecting accurate results including intra-tumour tissue heterogeneity and fixation artefacts. The use of tissue microarrays can be limited due to inadequate sampling of representative tumour cells caused by tumour heterogeneity (Permuth-Wey *et al.*, 2009). Besides, tissue fixation is one of the least controlled phases in the immunohistochemical staining process as the fixation conditions (total fixation time, time to fixation, rate of fixative penetrates in different thickness and types of tissues) vary by the specimen and institution (Arima *et al.*, 2016). This can affect the reliability of the staining interpretation and validity of the study results. Although staining artefacts at the edge of the tissue sections (leading edge effects), is also known as a phenomenon in immunohistochemistry. As the cores with a lack of expression were distributed throughout the tumour blocks and located at various sections of the tumour cells, the staining artefacts are not responsible for the discrepant biomarker expression scores when sampling different areas (luminal, peripheral, and central) of tumour blocks (Permuth-Wey *et al.*, 2009). Thus, it is compulsory to discover the existence of tumour heterogeneity and demonstrate new prognostic factors in order to achieve discern treatment to improve poor prognostic at a molecular level.

6. Limitations

However, no study is completely flawless, or inclusive of all possible aspects. Manual assessment of immunohistochemistry staining has still remained a traditional method for most predictive and diagnostic decisions in pathology. This manual interpretation

of immunohistochemistry data can be time-consuming. There will also have an intra-observer error as the staining of the normal and tumour cells were assessed by an individual.

7. Conclusion

In conclusion, signalling in the stroma will promote invasion, growth, and metastasis of tumour cells. CRC patients with high *TP53* nuclear expression and low *Bcl-2* cytoplasmic expression tended to have a better survival rate in CRC patients (lower risk), and serve as favourable prognostic factors, providing a chance of treatment success. Together with stroma-low tumours (high TSR), it will bring a beneficial outcome on CRC cells.

8. Recommendations

Although the p53 gene was not sequenced in this study, p53 immunohistochemical staining often shows an accumulation of mutant p53 proteins. This close association is established mainly for tumour cells and is likely to apply for stromal p53. Stromal p53 that was not seen in this study also plays an important role in enhancing the response to radiotherapy and chemotherapy (Bar *et al.*, 2010). However, this is only being verified by Bar et al. (2010) in mouse studies, which needs to be investigated in human cancer patients. If further analysis has been accomplished and stromal p53 activation becomes a component of mechanism driving tumour regression, it will become an alternative therapeutic target and complementary approach for antiangiogenic therapy.

Cancer is manifest as a genetic disease caused by oncogenic mutations. In a similar gene-centric view, intratumoural heterogeneity (also known as intralesion genetic heterogeneity) generally defined as different tumour cell populations (with different phenotypical and molecular profiles) in the same tumour specimen has been allocated to the genetic diversity in cancer cell populations (Ramón y Cajal *et al.*, 2020). It is one of the determining factors of treatment failure and therapeutic resistance, thereby possess a poor survival in cancer patients with metastasis disease (Baliu-Piqué *et al.*, 2020). As cancer distributes as a heterogenous dynamic target, it is well postulated that patients, cell populations and lesions must be characterised thoroughly at different times (Ramón y Cajal *et al.*, 2020). This will bring tumour heterogeneity a comparable challenge to provide a proper treatment for the matching patient at the right time, thus it still remains a demand to achieve the goals of precision medicine.

Detecting new prognostic markers is essential in guiding more precise treatment to improve survival rates of CRC patients. This study based solely on both *TP53* and *Bcl-2* biomarkers are not sufficient for the investigation of colorectal carcinogenesis. Hence, more biomarkers are needed to investigate in this present study, to determine their prognosis outcome in CRC. There are studies showing the incidence of other biomarkers genetic (*BRAF, K-ras,* β-catenin and *c-Myc*) abnormalities can result in abnormal cell cycles (Li and Martin, 2016; Nagaoka *et al.*, 2016; Pikoulis *et al.*, 2016). As the treatment of the cancer is directly based on the stage of tumours, and not all treatment is right for the matching patients with CRC, ways to achieve the goal of precision medicine is strongly needed.

Clinicians often used statistical survival rates to evaluate and compare the treatment options of CRC. Through the survival statistics regression model, clinicians can provide the most beneficial treatment for the patients and weigh the benefits against the risk of treatments for CRC patients. Interestingly, the survival rate includes people in remission, that is permanent or temporary absence of the disease or those who are still receiving treatment. Hence, more specific results by obtaining both progression-free survival and disease-free survival statistics will be more beneficial for this study to evaluate better treatments.

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