THE UNIVERSITY OF HULL

Humoral Immunity In Colorectal Cancer: Evaluation of the anti-p53 and anti-hTERT auto-antibody responses

Being a Thesis submitted for the degree of Doctor of Medicine in the University of Hull

By

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Preoperative serum vascular endothelial growth factor-a is a marker for subsequent recurrence in colorectal cancer patients. *Dis Colon Rectum.* 2009 May;52 (5):993-9.

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Poster Presentations

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Declaration

The planning, experimental design and execution of studies and technical work involved in this thesis were all performed by the candidate unless otherwise stated.

No part of this thesis has been submitted in support of an application for any degree or qualification in any other institute of learning

Additional work carried out during the MD period

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Synopsis

Carcinogenesis is a multi-factorial and multi-aetiological process involving suppressions, alterations and re-activation of key biomolecular markers. Some of these changes are recognised by the humoral system and are known as Tumour Associated Antigens (TAA) against which the humoral system is able to mount an auto-antibody response. Cancer cells are subject to two key mortality barriers (M1 & M2) as described in the "2-hit" hypothesis which are overcome by p53 dysfunction (M1) and hTERT re-expression (M2). These two molecular events generate TAA which are recognised by the humoral immune system with a corresponding auto-antibody response. The aim of this thesis was to investigate the significance of the humoral anti-p53 auto-antibody and anti-hTERT auto-antibody responses in colorectal cancer (CRC). This was performed by evaluating all published literature (1979-2009) on anti-p53 auto-antibody response and its association with p53 mutation to provide the largest cumulative sample size to date spanning 30 years. A critical review was performed of all anti-p53 auto-antibody studies in CRC, followed by an investigation into the long-term prognostic significance (minimum 5 years follow-up) of anti-p53 auto-antibody in CRC. The second aim of this thesis was to optimise a method of detecting anti-hTERT in CRC patients and correlate this with anti-p53 auto-antibody in order to investigate the significance of a joint humoral response against the two key TAA responsible for CRC immortality.

The overall prevalence of anti-p53 auto-antibody was 18.4% (3292/17,859) in all cancers and 2.2% (88/3,946) in normal/benign disease controls. The anti-p53 auto-antibody presence in all published cancers reports was plotted against the reported p53 mutational rates in individual cancers and showed partial correlation (R^2=0.5, correlation=0.7) between anti-p53 auto-antibody presence and p53 mutation. Anti-p53 was present in 21.5% (786/3,653) in all CRC only studies, and 19.9% (479/2,409) in CRC studies using ELISA. Anti-p53 was not associated with clinico-pathological factors or prognosis in majority of the studies. Only 4 studies associate anti-53 auto-antibody with adverse clinico-pathological parameters, mostly in selective groups. The weaknesses of these studies are discussed. This association leads to anti-p53 association with adverse prognosis but only in selective analysis. The prognostic

significance is observed in univariate analysis but lost in multivariate analysis when stronger traditional prognostic factors are incorporated.

This thesis initially compared serum with plasma anti-p53 auto-antibody titres and excluded plasma titres from further analysis due to the potential contamination by non-specific binding leading to falsely elevated levels (17-73% variation) of anti-p53 auto-antibody. Serum anti-p53 auto-antibody was present in 21.7% (20/92) CRC patients and 0% (0/20) controls. There was no association with age (p=0.750), sex (p=0.468), Dukes' / TNM stage (p=1.000), T- (p=0.900), N- (p=0.912), M-stage (p=0.632), location (p=0.175), differentiation (p=0.117) or mucinous component (p=0.699). The median follow-up was 97 months with median DFS and OS of 73 months and 62 months respectively. Dukes' / TNM stage, T-, N-, M-stage were prognostic indicators in univariate DFS and OS analysis. Only Dukes'/TNM stage remained an independent prognostic indicators in multivariate analysis (p=0.001). Anti-p53 auto-antibody did not display prognostic significance in univariate or multivariate analysis of OS or DFS.

Anti-hTERT auto-antibody has only been reported once in the literature, using molecular recombination to develop hTERT antigen. This thesis optimisation processes aimed develop a method of detecting anti-hTERT using less restrictive technology, and further development of a WB or ELISA to allow mass detection of serum anti-hTERT. The first step aimed to isolate hTERT using a streptavidin immuno-affinity column with biotinylated anti-hTERT to capture hTERT from cancer cell lysates. This was unsuccessful and further attempts were made at identifying hTERT using Western blot (WB). Five different anti-hTERT antibodies and a multitude of WB conditions were trialled in duplicate (>100 WB), each with multiple ECL exposures. hTERT was not identified. The reason for this was narrowed down in the final experiments to the lack of specificity of the primary antibodies available. The raising of a sufficiently specific anti-hTERT antibody is required to isolate hTERT antigen.

Early CRC detection is vital in improving outcomes. The humoral response to TAA in carcinogenesis could enable earlier identification of CRC and impact prognosis.

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List of Abbreviations

ACOSOG	American College of Surgeons Oncology Group
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
APS	Ammonium Persulphate
ALT	Alternative Lengthening of Telomeres
APER	Abdomino-perineal Resection
ASCO	American Society of Clinical Oncology
ATM kinase	Ataxia Telangectasia Mutated kinase
Bcl-2	B-cell lymphoma 2
BMPR1A	Bone Morphogenic Protein Receptor 1A
BRCA-1	Breast Cancer Type-1 Susceptibility Protein
BRRS	Bannayan-Riley-Ruvalcaba Syndrome
CEA	Carcino-Embryonic Antigen
Ceq	Cell equivalents
CH	Chronic Hepatitis
CIN	Chromosomal Inactivation
CONTEM	Contact Radiotherapy and Transanal Endoscopic Microsurgery
CoV	Co-efficient of Variation
COX-2	Cytochrome Oxidase subunit II
CpG	Cytosine Guanine islands
CRC	Colorectal Cancer
Diff	Differentiation
DNA	De-oxyribose Nucleic Acid
DTT	Dithiothreitol
ECL	Enhanced Chemo-Luminescence
EGFR	Endothelial Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbence Assay
EUROQOL	European Quality of Life questionnaire
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FISI	Faecal Incontinence Severity Index

FIQOL	Faecal Incontinence and Quality of Life (Rockwood)
FOXTROT	Fluoropyrimidine, Oxaliplatin and Targeted receptor Pre-operative Therapy for high risk colon cancer
FU	Follow-up
GTP	Guanosine Triphosphate
HAD	Hospital Anxiety & Depression Scale
HCC	Hepatocellular Carcinoma
HHT	Hereditary Haemorrhagic Telangectasia
HPV	Human Papilloma Virus
HRP	Horse-radish Peroxidase
hTAP	human Telomerase Associated Protein
hTR	human Telomerase Ribonucleic acid
hTERT	human Telomerase Reverse Transcriptase
IARC	International Agency for Research on Cancer
IBD	Inflammatory Bowel Disease
ICONE	International Contact Radiotherapy Evaluation Group
IHC	Immuno-histo-chemistry
IP	Immuno-precipitation
JP	Juvenile Polyposis
kRAS	Kirsten Rat Sarcoma viral oncogene homolog
LC	Liver Cirrhosis
LR	Local Recurrence
LV	Leucovorin
MAPK	Mitogen Activated Protein Kinase
MDM2	Murine Double Minute protein 2
MHC	Major Histocompatability Complex
MMR	Mismatch Repair
MSI	Microsatellite Instability
NI	Not Included
NSCCG	National Study of Colorectal Cancer Genetics
OD_{450nm}	Optical Density at 450 nanometer
OS	Overall Survival
PAGE	Polyacrylamide Gel Electrophoresis

PBS	Phosphate Buffered Saline
PI3K	Phosphinositol-3-kinase
PJS	Peutz-Jegher-Syndrome
PTEN	Phosphate and Tensin Homolog
PVDF	Polyvinylidene Fluoride
QOL	Quality of Life
R^2	Correlation co-effecient
SCTAT	Sex Cord Tumours with Annular Tubules
SDS	Sodium Dodecyl Sulphate
STK 11	Serine-threonine Kinase enzymes
SV40	Simian Vacuolating Virus 40
TAA	Tumour Associated Antigen
TEM	Transanal Endoscopic Microsurgery
TGF β	Transforming Growth Factor β
TRAP	Telomerase Repeat Amplification Protocol
TREC	Transanal Endoscopic Microsurgery in Early Rectal Cancer
UC	Ulcerative Colitis
V	Volt
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
5-FU	5-Fluorouracil
2-βME	2-β-mercapto-ethanol

1 Chapter 1: Introduction

Section 1.1 introduces colorectal cancer (CRC), **Section 1.2** describes the mechanisms of p53 genetic mutation and potential pathways of anti-p53 auto-antibody induction and **Section 1.3** demonstrates the relevance of telomerase re-activation in cancer cell immortality.

1.1 Introduction to Colorectal Cancer

The epidemiological significance of CRC is discussed, followed by pathways of carcinogenesis. CRC treatment is outlined with emphasis on current trials attempting to resolve controversies in adjuvant therapy which is one of the most intensively researched aspects of CRC treatment.

1.1.1 Epidemiology

CRC is the third most common cancer in the United Kingdom after breast and lung cancer. Incidence of CRC increases dramatically after 60 years of age and 85% of new diagnosis occurs in this group (Office of National Statistics, 2009, Information Services Division Scotland, 2009, Welsh Cancer Intelligence and Surveillance Unit, 2010, Northern Ireland Cancer Registry, 2006). The lifetime risk of CRC in 2009 was 1:16 in males and 1:20 in females (Office of National Statistics, 2009). The all cancer prevalence in the UK in 2008 was 3.3% (approximately 2,000,000 patients). CRC was the second most prevalent cancer with an estimated 250,000 diagnoses, next to breast cancer with 550,000 diagnoses (Office of National Statistics, 2009). Worldwide, CRC

is the third most common cancer after lung and breast cancer (Horner et al., 2008, International Agency for Research on Cancer, 2005). The majority of CRC (> 60%) are diagnosed in developing countries with significant differences in incidence between individual countries, ranging from < 5 / 100,000 in Africa to > 45 / 100,000 in Australia / New Zealand (Ferlay et al., 2007, International Agency for Research on Cancer, 2005). This is attributed to dietary, environmental and cultural differences, rather than genetic factors, as migrants moving from low risk regions (Asia) to high risk regions (USA) demonstrate a rapid increased in CRC risk which almost doubles in the second generation (Flood et al., 2000). Furthermore, traditionally low-incidence CRC countries such as Japan which have recently assimilated a high-fat, low-fibre "Western" diet have demonstrated a rapid increase CRC incidence over the last 20 years (Marchand, 1999, Koyama and Kotake, 1997).

1.1.2 Aetiology

The exact aetiology of CRC is unknown. The majority of CRC (70 – 90%) is known as "sporadic", which probably occurs due to a multi-factorial combination of environmental and as yet unclassified genetic factors (**Section 1.1.6.4**). The remaining 15% of CRC is attributed to inherited familial conditions and non-malignant diseases which predispose to CRC. The major inherited diseases are Hereditary Non-Polyposis Coli (HNPCC), Familial Adenomatous Polyposis (FAP) and less commonly Peutz-Jehgers Syndrome (PJS), Juvenile Polyposis (JP) and Cowden Disease. The main non-malignant disease leading to non-sporadic CRC is Inflammatory Bowel Disease (Burstein and Fearon, 2008). These disease conditions are discussed in more detail later (**Section 1.1.6.1** to **Section 1.1.6.5**). The potential molecular pathways of

carcinogenesis are discussed first as there are significant differences in carcinogenesis mechanisms of sporadic CRC (chromosomal inactivation, CIN) and non-sporadic CRC (mismatch repair, MMR gene). Both mechanisms are shown in the adenoma-carcinoma sequence.

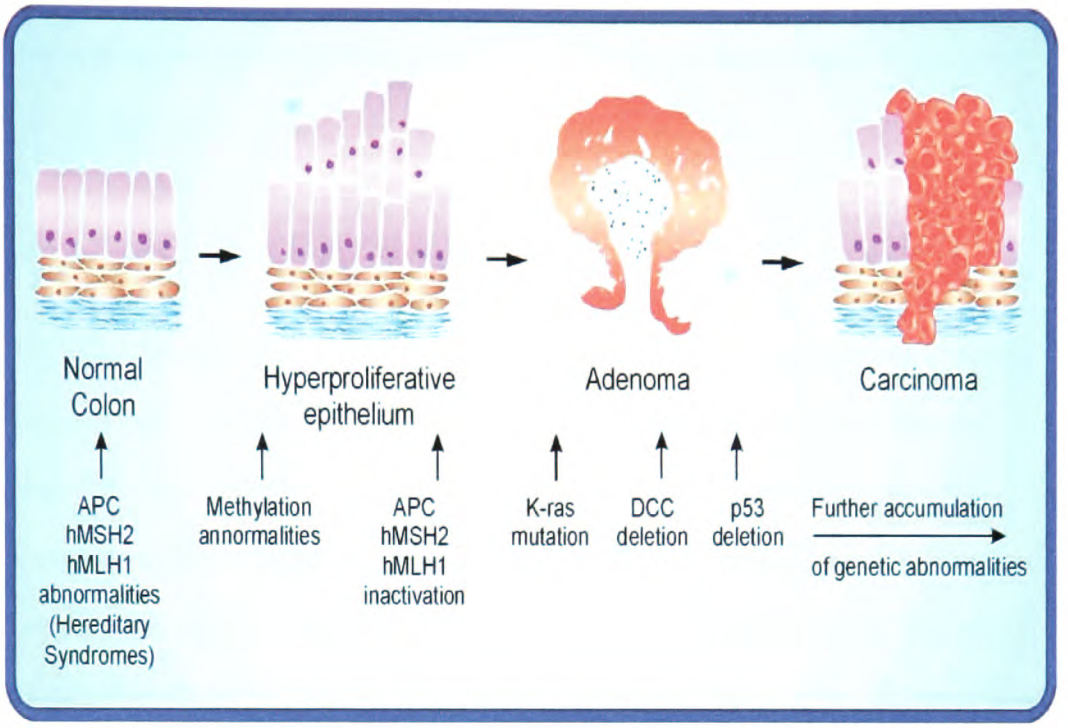
1.1.3 Molecular Basis of Carcinogenesis: Adenoma-Carcinoma Sequence

Sporadic CRC is thought to arise from adenomas as supported by observational evidence in **Table 1.1** and hallmarked in the adenoma-carcinoma sequence (**Figure 1.1**) (Fearon and Vogelstein, 1990, Vogelstein et al., 1988).

1.1.4 Mechanisms of Carcinogenesis : CIN and Methylation

Chromosomal instability (CIN) is the major carcinogenesis pathway in sporadic CRC and occurs in 85% of sporadic CRC (Markowitz and Bertagnolli, 2009). CIN induces carcinogenesis occurs by (a) inactivation of tumour suppressor genes (APC, p53, TGF-β) and/or (b) activation of oncogenic genes (RAS, BRAF). The sequence of events is postulated in the adenoma-carcinoma pathway (**Figure 1.1**). The remaining 15% of sporadic CRC occurs through mismatch repair (MMR) defects caused by methylation of cytosine residues of the MMR promoter regions. In sporadic CRC, methylation typically occurs in Cp-G rich islands of the *h*MLH1 (an MMR gene) promoter, resulting in *h*MLH1 silencing and MMR defects with characteristic micro-satellite instability (MSI) appearance (**Section 1.1.6.1**) (Veigl et al., 1998, Herman et al., 1998). The MMR defects of sporadic CRC are a due to age-acquired somatic mutation, rather than inheritance of a defective MMR gene which occurs in non-sporadic CRC (Weisenberger et al., 2006, Issa, 2004, Liu et al., 1995).

Figure 1.1: Adenoma-carcinoma sequence



http://radiographics.rsnajnls.org/content/vol24/issue1/images/large/g04jae18c1x.jpeg

Table 1.1: Observational evidence supporting adenoma to carcinoma progression

Adenoma prevalence correlates with carcinoma prevalence with the average patient age 5 years younger than the carcinoma patient
Adenoma distribution in the colon is similar to that of CRC distribution
Adenoma tissue often accompanies CRC and vice versa
Adenomas are present in up to 30% CRC specimens
Larger adenomas develop increasing cellular atypia compared to smaller adenomas
Sporadic adenomas are microscopically identical to adenomas in FAP which have 100% malignancy risk of CRC

(Steele, 2009)

1.1.5 CIN : Tumour-suppressor gene inactivation

The main tumour suppressor genes inactivated by CIN are Adenomatous Polyposis Coli (APC), p53 and Transforming Growth Factor- β (TGF-β).

1.1.5.1 Tumour-suppressor Inactivation : APC "Gate-keeper"

APC, also known as "the gate-keeper" of the adenoma-carcinoma sequence, is among the first steps in preventing CRC carcinogenesis development in the pre-adenoma stage (Half et al., 2009). Hence, APC mutation is a key event in the entry into the adenoma-carcinoma sequence (Goss and Groden, 2000). The wild-type APC protein functions include cell adhesion, signal transduction and transcriptional activation and β-catenin degradation. β-catenin is an oncoprotein transcription factor which promotes Wnt signalling, cellular activation and expression of other proto-oncogenes (Half et al., 2009). The APC proteolytic complex down-regulates β-catenin thus functioning as a tumour suppressor. APC mutation leads to unregulated β-catenin expression and inappropriate Wnt pathway signalling which in turn promotes unregulated cell growth. APC mutation is the hallmark of Familial Adenomatous Polyposis (FAP) (**Section 1.1.6.2**). The importance of APC is emphasised by the inevitable progression to CRC in FAP.

1.1.5.2 Tumour-suppressor Inactivation : p53 "Guardian of the Genome"

Mutation of the p53 gene is one of the final events in the adenoma-carcinoma sequence (**Section 1.2**). Other mechanisms of p53 inactivation are discussed (**Section 1.2.2**) and induction of anti-p53 auto-antibody response is outlined (**Section 1.2.5**).

1.1.5.3 Tumour-suppressor Inactivation : TGF-β

TGF-β is a family of proteins with various functions including anti-proliferation at G1 stage of cell cycle, and induction of apoptosis (Blobe et al., 2000). TGF-β induced apoptosis is thought to occur via the SMAD or the DAXX pathways (Salomoni and Dyer, 2005, ten Dijke and Hill, 2004). TGF-β is inactivated in 30% of CRC through TGFBR2 mutation, usually at the TGFBR2-kinase domain. TGF-β function is also indirectly inactivated by mutations of its regulators such as SMAD4 (Wood et al., 2007). Deletions in SMAD4 and another TGF-β regulator, BMPR1A, are found in 40% of JPS patients (van Hattem et al., 2008). TGF-β inactivation occurs in the final steps of the adenoma-carcinoma sequence, during transition from adenoma to high-grade dysplasia or invasive carcinoma (Compton, 2003, Grady et al., 1998).

1.1.5.4 Oncogene Activation : KRAS

Kirsten Rat Sarcoma viral oncogene homolog (KRAS) is a family of genes coding for small GTPases that are involved in cellular signal transduction. KRAS mutations occur in 20 – 40% cancers and are up to 90% specific for neoplasia (Markowitz and Bertagnolli, 2009). KRAS protein activation occurs primarily through gene mutation. This activates the GTPase pathway signalling leading to RAS production and activation of the Mitogen-activated protein kinase (MAPK) pathway (Rajagopalan et al., 2002). MAPK is a signal transduction pathway, which when activated leads to binding of growth factors. These signals activates serine / threonine-specific kinases which regulate cell functions and ultimately promote cell proliferation (Pearson et al., 2001).

1.1.5.5 Oncogene Activation : BRAF

The BRAF gene encodes for serine / threonine protein kinases which also involves signal transduction and activation of the MAPK pathway (Davies et al., 2002). Inherited BRAF mutations cause cardio-facio-cutaneous syndrome, a disease characterized by heart defects, mental retardation and a distinctive facial appearance. Acquired BRAF mutation activation occurs in 13% of CRC (Rajagopalan et al., 2002). BRAF mutations in particular are also linked with hyperplastic polyp syndrome with an increased CRC risk (**Table 1.2**)(O'Brien, 2007).

1.1.5.6 Oncogene Activation : PI3K

Phosphatidylinositol 3-kinase (PI3K) is another group of enzymes involved in signal transduction and growth promotion which is up-regulated in 30% of CRC. PI3K activation occurs through mutation of the gene itself, or mutations of its regulators such as its inhibitor pTEN, or its upstream activator AKT. The roles of PTEN, PI3K and AKT are shown schematically in **Figure 1.3.** The most recent uncovered clinical relevance of PI3K mutation is that, like that of kRAS and BRAF mutations, CRC containing these mutations is unresponsive to EGFR-based immunotherapy (Amado et al., 2008, Karapetis et al., 2008).

1.1.6 Non-sporadic CRC : MMR Defects

MMR defects are less important than CIN in sporadic CRC, accounting for only 15% - 25% of sporadic CRC (usually through hMLH1 silencing). However, MMR defects are the main pathway of carcinogenesis in non-sporadic or familial CRC.

1.1.6.1 Non-sporadic CRC : HNPCC

Hereditary Non-Polyposis Coli (HNPCC) accounts for 2 – 5% of all CRC and is the biggest cause of non-sporadic CRC. HNPCC is autosomal dominant, with an average age of CRC presentation of 45 years and a life-time CRC risk of 80% (Lynch et al., 2008). HNPCC is characterised by defective MMR genes which normally correct errors in base-sequence pairing during DNA replication. Wild-type MMR gene function is therefore tumour-suppressive. The most common MMR defects (90%) occur in hMSH1, hMSH2, hMSH6, hPMS2 and hMSH3 (Di Fiore et al., 2004, Papadopoulos and Lindblom, 1997). Defective MMR function is characterised by microsatellite instability (MSI) which is detectable by DNA sequencing of tumour tissue. Microsatellites are normally occurring short DNA repeats (usually 5 nucleotide sequences) which contain non-coding DNA. MMR defects lead to defective base-pairing resulting in changes in the microsatellite sequence and length, known as MSI (Clark, 2009). MSI is not exclusive to HNPCC as MSI can occur in up to 25% of sporadic CRC as a result of acquired methylation silencing rather than inherited MMR defects.

1.1.6.2 Non-sporadic CRC : FAP

FAP is the less common of the two major inherited CRC syndromes, accounting for 1% of CRC. Patients with FAP have an almost inevitable progression to CRC by the fourth decade of life (Cancer Research UK, 2010). FAP is autosomal dominant and caused by mutation of the tumour-suppressor gene APC located on the short arm of chromosome 5q21 (Clark, 2009). Patients with FAP have thousands of polyps

throughout the colon giving the colonic mucosa a typical "carpet"-like appearance. FAP is also associated with extra-colonic tumours of ectodermal origin (epidermoid cysts, central nervous system tumours), mesodermal origin (desmoids and connective tissue tumours), and most importantly to the gastrointestinal surgeon - adenomas and carcinomas of endodermal origin (gastric, duodenal, biliary, thyroid and adrenal) (Trainer, 2009). Intensive screening is advocated with annual flexible sigmoidoscopy at age 13 – 15 years, progressing to annual colonoscopy above the age of 20 years. Prophylactic colectomy is offered when FAP is detected on colonoscopy, or if FAP is diagnosed by predictive gene testing (even with absence of polyps) due to its almost inevitable progression to CRC (Gammon et al., 2009, Dunlop, 2002a).

1.1.6.3 Non sporadic CRC : Other inherited conditions

Several other lesser known inherited diseases are associated with CRC and an increased frequency of non-gastrointestinal cancers (**Table 1.2**). The majority of these are autosomal dominant and involve mutations of the cell cycle proliferation regulators.

Table 1.2: Other familial conditions leading to non-sporadic CRC

	Defect; Prevalence	Description
MutY Human (MYH)-associated polyposis	Autosomal recessive; MYH chromosome 1p; 1/200 heterozygote carrier	Similar in appearance to FAP, but later age at presentation (47 years). Almost 100% CRC risk but at a later age of 60 years (40 years in FAP) (Clark, 2009). Upper GI polyps in 33% (Half et al., 2009).
Peutz-Jeghers Syndrome	Autosomal dominant; STK11 Chromosome 19p13 mutation in 50-90%; 1/8000 – 1/250,000	Life time risk: any GI cancer (60%), Breast (40%), CRC (20%), gastric (5%) and pancreatic. Over 90% develop small bowel polyps. Ovarian SCTAT is classical feature. Main mortality due to small bowel polyp intussusceptions and multiple laparotomies (Westerman et al., 1999).
Juvenile Polyposis	Autosomal dominant; SMAD4 mutation; 1/100,000, lesser extent BMPR1A, PTEN	Gastrointestinal cancer risk 50%. Median age of CRC diagnosis is 42 years with 68% CRC risk at age 60 years. Associated with Hereditary Haemorrhagic Telangectasia (HHT) (Gallione et al., 2006, Howe et al., 1998, Howe et al., 2004).
PTEN Hamartoma Tumour Syndromes	Autosomal dominant; PTEN, chromosome 10q22 in 80% CS and 60% BRRS; 1/200,000	Includes Cowden disease and Bannayan-Riley Ruvalcaba syndrome (BRRS). GI polyps in 60% - gastric (75%), colon (66%), oesophagus (66%). Lifetime cancer risk: Breast (40%), endometrial (10%), thyroid (10%) (Eng, 2003, Lachlan et al., 2007).

STK 11 serine-threonine kinase; SCTAT Sex cord tumours with annular tubules

1.1.6.4 Non-sporadic CRC : Unclassified family preponderance

Familial preponderance accounts for up to 15% - 30% of CRC but these are labelled "sporadic" as they do not fulfil the diagnostic criteria for familial CRC and do not possess an identifiable inherited mutation. Individuals with a first-degree relative with CRC have 1.7 times increased risk of developing CRC, which increases with the number of relatives affected (Lichtenstein et al., 2000, Strate and Syngal, 2005). Patients with two first-degree relatives with CRC and one first-degree relative diagnosed with CRC < 45 years have 3 – 6 fold increase in relative risk for CRC even in the absence of detectable genetic defects (Dunlop, 2002b, Houlston et al., 1990). The ongoing National Study of Colorectal Cancer Genetics (NSCCG) aims to identify the unclassified family preponderances and related biomarkers (Penegar et al., 2007). The NSCCG examines family histories and serum and tumour samples from 20,000 CRC patients, using relatives-by-marriage or friends as controls. The pattern of familial history and CRC risk is correlated with several genetic markers, including p53, MSI, hMLH1, hMSH2 and hMSH6.

1.1.6.5 Non-sporadic CRC : Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory gastrointestinal conditions. These are mainly Ulcerative Colitis (UC) and Crohns' disease (CD). IBD accounts for 1 – 2% of CRC and has the highest CRC risk among the non-malignant disease groups (Winawer et al., 2005, Choi and Zelig, 1994). The reported life-time risk of CRC in IBD varies due to differences in study methodology and population, IBD severity and treatment (e.g. short-term isolated enteritis vs.

long-term severe pancolitis) and referral centre bias (Zisman and Rubin, 2008). A previous meta-analysis estimated overall 3.7% CRC prevalence in UC; which increased with disease duration - 2% at 10 years, 8% at 20 years and 18% at 30 years (Eaden et al., 2001). A more recent UK database reported a lower CRC probability (2.5% at 20 years of UC) which again increased with disease duration - 7.6% at 30 years and 10.8% at 40 years (Rutter et al., 2006). The most likely reason for this decreasing CRC risk in IBD is more intensive screening, more aggressive therapy at diagnosis with more effective maintenance/ remission treatments, and possibly more aggressive prophylactic colectomy in patients with colonic dysplasia (Clark, 2009).

1.1.7 Prognostic factors in CRC

The major adverse prognostic indicators in CRC are advanced stage (which involves tumour depth, node involvement and metastases), poor differentiation and mucinous/ signet cell morphology. Lymphovascular invasion and tumour budding have been added recently.

1.1.7.1 Dukes' / TNM Stage

The American Joint Committee on Cancer (AJCC) / International Union Against Cancer (UICC) TNM staging system supersedes that of the previous Dukes' and modified Astler-Collier staging systems. The advantage of the TNM stage is that each aspect of CRC – depth of penetration (T), nodal stage (N) and presence of metastases (M), are assessed independently making this a more discriminating system (**Table 1.3**). TNM stage is the strongest independent predictor of survival. The 5-year overall survival by TNM and corresponding Dukes' stage is shown (**Table 1.4**).

Table 1.3: AJCC / UICC TNM Stage Definitions

T	
Tx	Cannot be assessed
T0	No evidence of tumour
Tis	Carcinoma in situ (intramucosal)
T1	Invades submucosa
T2	Invades muscularis propria
T3	Invades subserosa or non-peritonealised peri-colic / peri-rectal tissue
T4	Directly invades other organ / structures or perforates visceral peritoneum
N	
NX	Cannot be assessed
N0	No lymph node metastases
N1	Metastases in 1 – 3 lymph nodes
N2	Metastases in ≥ 4 lymph nodes
M	
MX	Cannot be assessed
M0	No distant metastases
M1	Distant metastases

Table 1.4: 5-year survival by TNM and Dukes' stage

TNM Stage	5 Year Survival	Dukes Stage
Stage 0 (Tis, T1 N0 Mo)	**> 90%**	-
Stage I (T2 N0 M0)	**80 – 85 %**	Dukes' B1
Stage II (T3, N0, M0)	**70 – 75 %**	Dukes' B2
Stage II (T4, N0, M0)	**70 – 75 %**	Dukes' B3
Stage III (T2, N1 – 3, M0)	**70 – 75 %**	Dukes' C1
Stage III (T3, N1 – 3, M0)	**50 – 65 %**	Dukes' C2
Stage III (T4, N1 – 3, M0)	**25 – 45 %**	Dukes' C3
Stage IV (any T / N, M1)	**< 3%**	Dukes' D

1.1.7.1.1 T – stage

T-stage assesses the depth of tumour penetration with early T-stage (T1 / T2) predicting better prognosis (**Table 1.4**). The prognostic influence of T-stage does not increase in a uniform linear stepwise progression, but increases almost exponentially, especially when associated with co-existing nodal involvement. For example, in node negative CRC, early progression (T1 → T2) only reduces 5-year survival by <5% whereas late progression (T3→T4) reduces 5-year survival by 5 – 15%. Furthermore, when nodal involvement (N1-3) is present, T2→T4 progression exponentially reduces survival by up to 50%. Hence, T1 and T2 node-negative CRC are often grouped together in studies as there is minimal survival difference but allow larger sample numbers for more robust statistical analysis. Complete serosal penetration (T4) is an extremely strong adverse prognostic factor, especially when combined with nodal or peritoneal disease, and indicates tumour escape and high probability of micrometastases.

1.1.7.1.2 N – stage

Nodal spread occurs via the lymphatic chain which runs alongside the regional vascular supply. Nodal involvement is associated with poor prognosis **Table 1.4** with 5-year survival in node negative disease of 70-85% which decreases to 25-75% (depending on T-stage) in node positive disease. Nodal status is staged by number of nodes involved, and hence staging accuracy is a function of the number of nodes available for examination. A minimum of 12 – 15 lymph nodes are required to accurately predict N- stage (Tepper et al., 2001, Scott and Grace, 1989).

Another important feature of nodal assessment is the differentiation between N3 and M1 disease. Regional node involvement outside the anatomical region should be considered metastatic disease, even in the absence of radiological evidence of distant metastases (Compton, 2003). Similarly, central (e.g. para-aortic) or proximal (e.g. external and common iliac) nodal involvement should also be considered metastatic and not nodal disease. The accurate differentiation between proximal / central regional node within anatomical distribution, nodal involvement outside anatomical region and metastatic disease is important to avoid misinterpretation of results as discussed in light of one of the major studies of anti-p53 auto-antibody in CRC (**Section 3.5.2.1**).

1.1.7.1.3 M – stage

The most common metastases at presentation is in the liver (Raftery, 2002). Significant improvements in surgical techniques of liver resection result in <5% operative mortality, and 5- year and 10- year survival of 25 - 40% and 20 - 25% respectively (Rees et al., 2008, Shah et al., 2007, Tomlinson et al., 2007, Wei et al., 2006). Similarly, major advances in adjuvant therapy for unresectable liver metastases have also been demonstrated, initially with 5-FU, followed by oxaliplatin and irinotecan, and most recently with addition of EGFR, VEGF and COX2 inhibition (Cassidy, 2007, Van Cutsem et al., 2007, Cunningham et al., 2004, Goldberg et al., 2004). Despite these improvements, M1 remains the strongest adverse prognostic indicator. The factors discussed next are of less prognostic significance than TNM stage, but are still recognised prognostic markers and were used for survival analysis in this thesis.

1.1.7.2 Mucinous and signet cell CRC

Mucinous CRC is a morphological description characterized by excess abundant extracellular mucin which is associated with more advanced tumours (Sung et al., 2008, Halvorsen and Seim, 1988). Signet cell carcinoma is characterized by the presence of signet cells which resemble signet rings, due to a large mucinous vacuole which displaces the nucleus to the periphery. Historically, mucinous and signet cell carcinomas were considered different morphological manifestation of the same CRC-type as they shared certain characteristics such as younger patients with nodal metastases and advanced disease at presentation (Nagtegaal and Quirke, 2008, Tung et al., 1996, Nozoe et al., 2000). The most recent multi-centre study compared 19 patients with signet cell CRC with 5792 non signet CRC between 1993 – 2007 (Mizushima et al.). All 19 signet cell CRC patients had ≥ T3 stage with 74% (14 / 19) nodal involvement and 38% (7 / 19) metastases at presentation. The resulting 5-year survival was 24% (signet cell) compared with 58% (poorly differentiated / mucinous) and 78% (well / moderately differentiated) which proves a significant adverse prognostic influence of signet cell CRC, beyond that of mucinous CRC. Similarly, 2 recent studies reported median survival of 33 months in signet cell compared with 68 months in mucinous and 151 months in non-mucinous CRC. The 5-year survival rates were 27% (signet cell), 51% (mucinous) and 69% (non-mucinous) (Song et al., 2009, Sung et al., 2008). However, mucinous type is still used as a prognostic marker due to its association with advanced stage CRC, although signet cell carcinoma has greater significance. Both factors are sufficiently important to justify inclusion into Royal College of Pathologists dataset for colorectal cancer (Williams et al., 2007).

1.1.7.3 Tumour Differentiation

The association between poor differentiation and advanced stage is also sufficient to warrant tumour differentiation as a core item in microscopic assessment of CRC (Williams et al., 2007).There are 4 grades of differentiation which and most clinical studies group into a 2-tier classification system (**Table 1.5**). Grade 1 (well differentiated) and Grade 2 (moderately differentiated) are grouped into a "Low Grade" tier, whereas Grade 3 (poorly differentiated) and Grade 4 (undifferentiated) are grouped into a "High Grade" tier. Heterogeneous tumour tissue containing multiple degrees of differentiation and inter-observer variability are acknowledged weaknesses of this system. The 2-tier system described above reduces the inter-observer error and increases diagnostic accuracy without markedly biasing survival data of groups. The 2-tier differentiation was also used in this thesis for this reason, and to obtain larger groups for analysis **(Section 4.3.3 & Section 4.4.5).** New trials incorporate mandatory quality control measures of pathological assessment such as attendance at educational sessions in pathological assessment, re-evaluation of the pathological assessments by a central committee, and random assessment of quality of pathological assessment from participating centres (Sebag-Montefiore et al., 2009, Ota and Nelson, 2007a, Kapiteijn et al., 1999).

Table 1.5: Grade of tumour differentiation

Grade 1: Well differentiated
Grade 2: Moderately differentiated
Grade 3: Poorly differentiated
Grade 4: Undifferentiated

1.1.7.4 Other pathological factors : Lymphovascular invasion and Tumour Budding

Several pathological factors have been described more recently, but are considered less important than the factors described previously. Lymphovascular invasion is a adverse prognostic factor with a recent study demonstrating decreased 5-year disease-free survival from 91% to 63% in CRC with lymphovascular invasion (Wang et al., 2009). Tumour budding is another prognostic factor linked to more aggressive tumours and potential lymph node metastases (Ogawa et al., 2009). Recent studies confirm the independent adverse prognostic significance of tumour budding in multivariate analysis and others associate tumour budding with liver and peritoneal metastases. One recent large study reported decreased 5- and 10-year survival in Stage II CRC with high grade tumour budding compared to low-grade tumour budding. The 5-year survival rates decreased from 94% to 74%, and 10-year survival decreased from 90% to 68% in patients with high-grade tumour budding. (Nakamura et al., 2008). Furthermore, these was no survival difference between Stage II CRC with high-grade tumour budding and Stage III CRC.

These factors are especially important in staging CRC treated by local excision as strong prognostic factors such as T-stage and N-stage cannot be assessed on local excision specimens. The use of pathological criteria such as mucinous and signet cell morphology and poor differentiation, lymphovascular invasion and tumour budding are especially important in these cases to predict CRC stage and aggressiveness and the requirement for further surgery or adjuvant treatment.

1.1.8 P53 mutation in CRC

The prognostic value of the anti-p53 auto-antibody response was examined in this thesis. As anti-p53 auto-antibody production occurs partly in response to p53 mutation (**Section 1.2.5 & Section 3.4**), the reported prognostic significance of the p53 mutation itself requires examination. There is discrepancy in the literature with regards to the prognostic value of p53 mutations in CRC but most studies report no prognostic significance. (Bouzourene et al., 2000, Ahnen et al., 1998, Borresen-Dale et al., 1998, Hirvikoski et al., 1999, Tortola et al., 1999). The conclusions from all studies are severely hampered by several factors. These studies are inadequately powered for survival analysis, surrogate markers of p53 mutation such as p53 protein immuno-histochemical detection (IHC) detection are used, CRC stage heterogeneity and confounding bias of the sporadic association between p53 mutation and advanced stage CRC. Finally, the prognostic significance of each p53 mutation type may vary as it is probable that different mutations affect different p53 functions and will thus have a variable influence of prognosis. Several studies have specifically linked p53 mutations in exon 7, codon 245, conserved areas and L3 structural domains as independent prognostic indicators but these are not reproduced (Russo et al., 2002, Samowitz et al., 2002, Goh et al., 1999, Iniesta et al., 1998, Jernvall et al., 1997)

The TP53-CRC Collaborative group produced the largest pooled review of p53 mutations in CRC (Russo et al., 2005). This analysis included 3583 CRC patients from 25 study groups in 17 countries. The median follow up for survival analysis in all groups was a respectable 58 – 61 months. p53 mutation was strongly associated with CRC stage, nodal involvement, poor differentiation, lymphatic invasion (in

rectal CRC) and lympho-venous invasion (in distal and rectal CRC) – all of which are independent prognostic indicators, which was further confirmed by highly significant survival effect ($p < 0.001$ or increased Odds ratio, OR = 1.3 – 7.2) for each clinico-pathological parameter in this review. Multivariate analysis demonstrated a non-significant trend toward decreased overall survival with p53 mutation in exon 5 - only in proximal CRC (Relative Risk, RR 1.36); and p53 amino acid loss - only in distal CRC (RR 2.52). Exon 5 mutations have previously been reported to confer poor prognosis in lung cancer but this was refuted in other studies (Huang et al., 1998, Vega et al., 1997). As such, p53 mutations are of little prognostic value. However, an interesting group analysis was performed in this study which is discussed next.

1.1.9 p53 mutation and adjuvant therapy

5- fluorouracil (5-FU) has been the mainstay of adjuvant treatment in CRC for > 40 years. *In vitro* studies have shown that 5-FU induced apoptosis is partly p53 dependant with decreased *in vitro* cell-kill recorded in p53 mutated cells treated with chemo-radiation (Bunz et al., 1999). Human trials also report decreased 5-FU efficacy as demonstrated by minimal or no survival benefit in Stage III CRC patients with p53 mutation receiving 5-FU based adjuvant treatment (Liang et al., 2002, Elsaleh et al., 2001). The TP53-CRC Collaborative Group review supported this finding in sub-group analysis of adjuvant chemotherapy. Only Dukes' C CRC were included in this analysis thus removing the effect of varying Dukes' stage on survival. These patients were then divided by p53 status thus reducing the bias caused by association between p53 mutation and other poor prognostic indicators such as lymphovascular invasion. Each group was then further divided by adjuvant chemotherapy provision (**Table 1.6**).

Table 1.6: Summary Overall Survival of Dukes' C patients

	Proximal RR (95%CI)	Distal RR (95%CI)	Rectum RR (95% CI)
Wild Type p53			
No Chemotherapy	1.00	1.00	1.00
Chemotherapy	0.61 (0.43 – 0.87)	0.35 (0.14 – 0.86)	0.55 (0.43 – 0.71)
	P = 0.006	P = 0.022	p = 0.006
Mutant p53			
No chemotherapy	1.00	1.00	1.00
Chemotherapy	0.61 (0.22 – 0.68)	1.15 (0.49 – 2.7)	0.78 (0.57 – 1.06)
	P = 0.006	NS	NS

RR Relative Risk; (Russo et al., 2005)

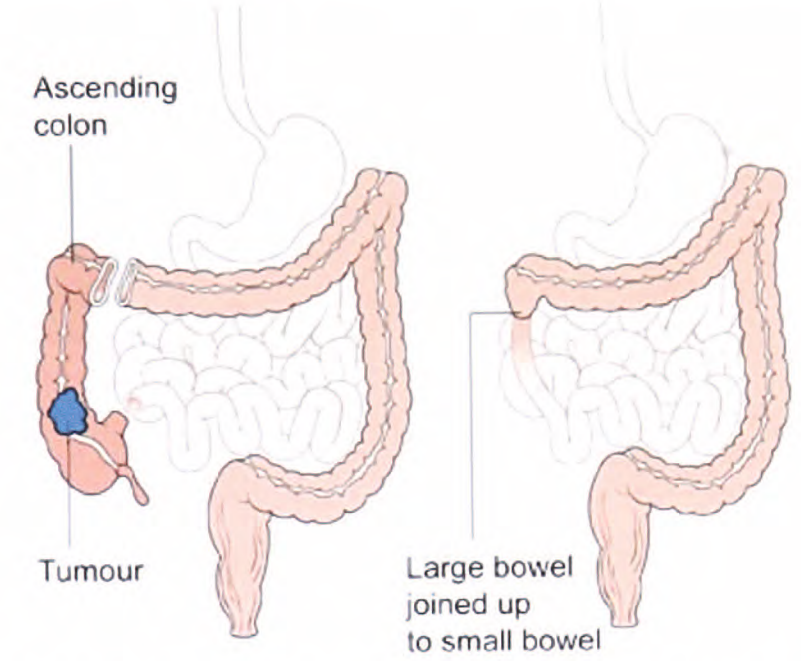
This shows that when other prognostic indices are adjusted for, patients with wild-type p53 obtain significant benefit with chemotherapy whereas this survival benefit is not observed in those with mutant p53. This survival difference was observed in the proximal and rectal CRC, but not in distal CRC. This is most likely due to under-powering in the distal CRC group. The average number of patients in the proximal and rectal CRC groups were 183 (range: 98 – 305 per group) compared to only 28 patients (range: 20 – 39 per group) in distal CRC. Despite these low numbers, there was already a trend towards improved overall survival with wild-type p53 ($p = 0.022$), suggesting the survival difference by p53 status observed in proximal and rectal CRC, would also be observed in a distal CRC group with larger sample size.

In summary, p53 mutation, when displaying prognostic significance, is always adverse and usually associated with stronger adverse prognostic factors such as advanced stage. Specific types of p53 mutation display have been related to poor prognosis but the complexity and diversity of p53 function hampers efforts to isolate a single mutation type, or function, which could independently predict prognosis.

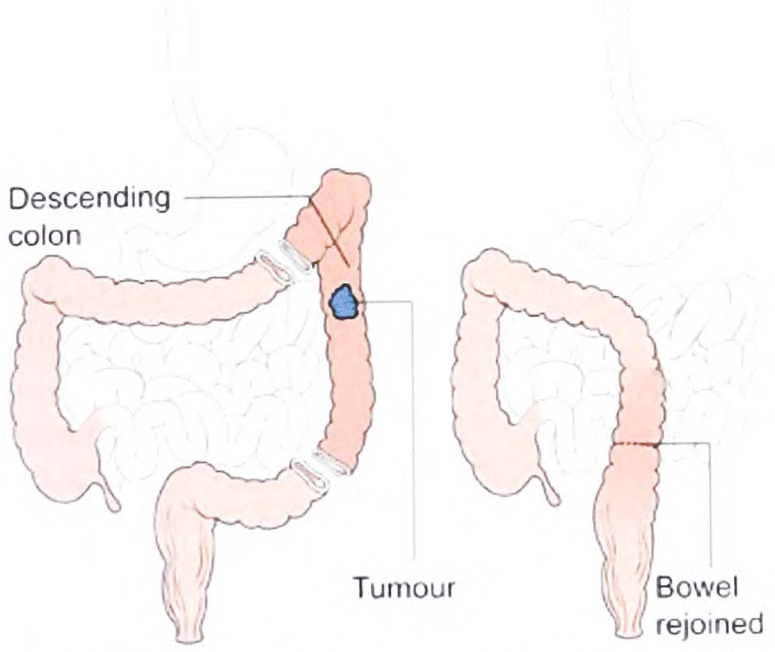
1.1.10 Surgical Treatment of CRC

Surgery for CRC is dependant on surgical intent of curative or palliative. Palliative procedures usually involve local transanal excision, endoscopic ablation or stenting, surgical defunctioning stoma or bypass procedures. The curative procedures are more radical and aim to remove the tumour, along its vascular supply and the closely associated lymphatic chain. This technique is known as "high tie" and results in resection of a devitalised segment of colon which is much larger than the tumour itself. There is no proven survival benefit of this technique (Lange et al., 2008). However, N-stage which is a strong prognostic predictor and also an indication for chemotherapy, is dependant on the number of nodes available for examination. This is best provided by the "high-tie" technique (Titu et al., 2008). Furthermore, approximately 15% lymph node involvement can "skip" sequential local lymph nodes and display node involvement node higher up the lymphatic chain. Thus whilst non-"high tie" technique benefits by less extensive resection, the specimen provides only local nodes for assessment and potentially misses "skip" nodes leading to understaging. Hence, "high-tie" is the recommended technique, provided it can be performed safely with no immediate surgical risk. The common curative surgical procedures are shown in **Figure 1.2**. (Permission obtained. Taken from CancerHelpUK, the patient information website of Cancer Research UK: www.cancerhelp.org.uk) These demonstrate the "high-tie" resection, as can be seen by the large devitalised colonic segment excised which is much larger compared to the smaller CRC segment.

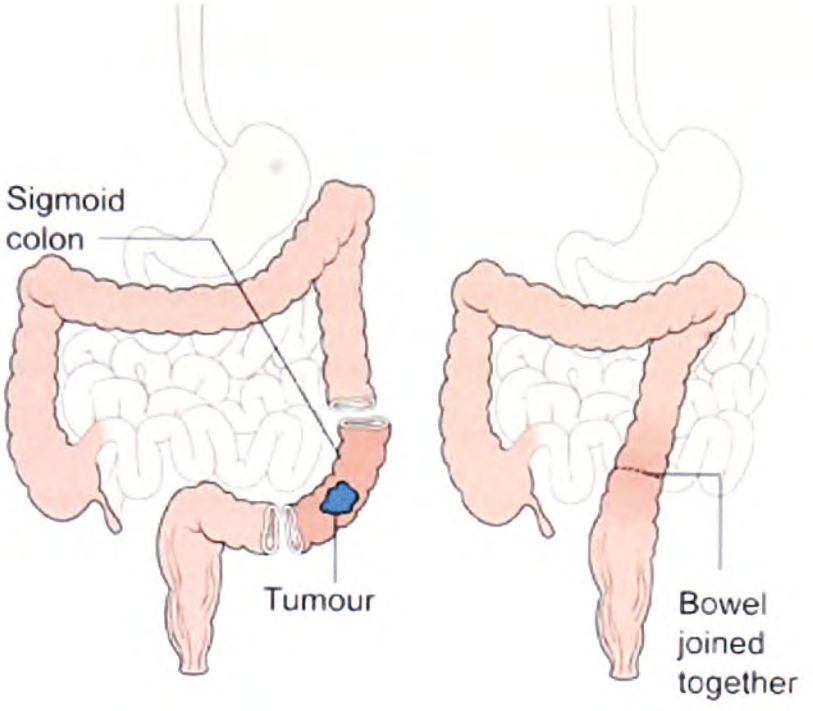
Figure 1.2: Commonly performed CRC curative resections using high-tie of supplying vasculature. Permission obtained (www.cancerhelp.org.uk)



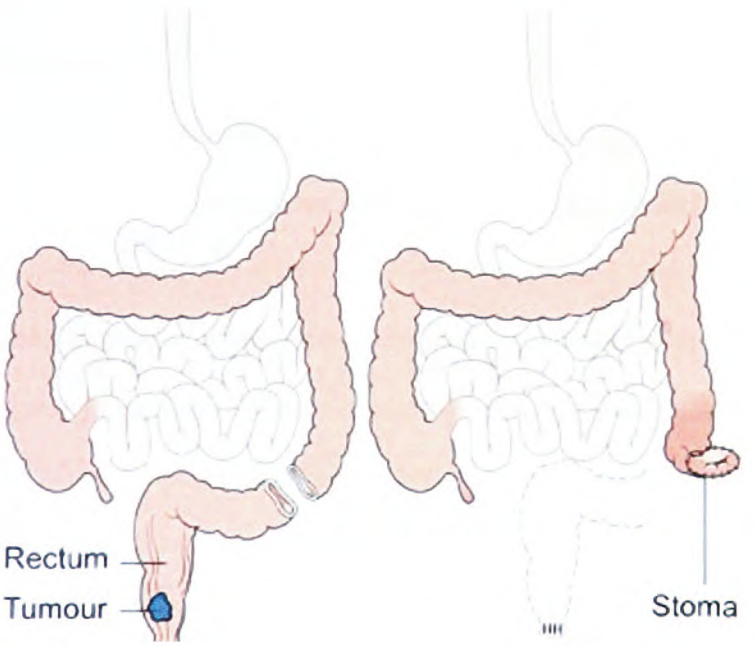
a) Right hemicolectomy for proximal (caecal and ascending colon) CRC.



a) Left hemicolectomy for descending colon CRC.



c) Anterior resection for sigmoid or upper rectal CRC



d) Abdomino-perineal resection (APER) or Miles' procedure for low rectal tumours where anastomasis is not possible or suitable.

1.1.11 (Neo) Adjuvant treatment in CRC

Adjuvant treatments represent one of the most costly and intensively researched aspects of CRC treatment. Neo-adjuvant treatment is only offered to rectal cancer patients. The mesorectal fascia is a fascial envelope which covers the rectum, surrounding mesorectal fat and lymph nodes. Mesorectal fascial tumour involvement is a poor prognostic indicator (Bernstein et al., 2009). Neo-adjuvant therapy in rectal cancer reduces tumour bulk and sterilises peri-rectal nodes leading to decreased mesorectal fascia involvement and hence lower local recurrence rates (Sebag-Montefiore et al., 2009, Kapiteijn et al., 1999, Martling et al., 2001). This radiation benefit does not extend to colon cancer which does not possess a circumferential fascia and radiation risks unnecessary toxicity (Peeters et al., 2005).

The Quasar-I trial which randomised Stage II CRC patients to post-operative observation or chemotherapy reported superior disease control in colon cancer patients with node-negative disease. It was suggested that neo-adjuvant chemotherapy in colon cancer may reduce nodal involvement, eradicate micrometastases earlier, reduce peri-operative tumour cell shedding and be more effective than in post-operative patients who have concurrent immunological and surgical stress. This forms the rationale for trials of neo-adjuvant therapy in colon cancer. This is investigated by the FOxTROT (Fluoropyrimidine, Oxaliplatin & Targeted Receptor pre-Operative Therapy for colon cancer) trial, which additionally also introduces a concurrent chemo-immunotherapy trial arm using an Endothelial Growth Factor Receptor (EGFR) inhibitor, Panitumumab.

The addition of EGFR inhibitors represents one of the major researched aspects of combined immuno-chemotherapy. However, ongoing trials (e.g. FOxTROT) have had to be re-designed following the announcement by the American Society of Clinical Oncology (ASCO) and Food and Drug Administration (FDA) that from 2009, all patients receiving EGFR monoclonal antibodies require proof of wild-type kRAS as patients with mutated kRAS did not benefit from EGFR inhibition (Oncogenetics.Org, 2009). Trials using monoclonal EGFR may sustain yet another setback as there is increasing evidence that genes downstream of EGFR (e.g. PI3K, BRAF) which are mutated will similarly reduce efficacy of these EGFR inhibitors. Firstly, this will not only substantially impact future trial recruitment but will also confound retrospective analysis of EGFR-inhibition efficacy (Linot et al., 2010). Secondly, this demonstrates the need for continuous advancement development in biomolecular research, especially in a process as complex and multi-factorial as CRC.

1.1.12 The future of adjuvant therapies in CRC

Adjuvant therapies have undergone an interesting expansion from its previous role as an aide to "gold-standard" treatment of CRC treatment, which is surgery, to a more active role in treating CRC in conjunction with minimally invasive surgical technologies. This combined approach may be suitable in a significant proportion of patients who are deemed high surgical risk due to severe co-morbidity, or fit patients who are willing to accept a potentially increase in risk of recurrence in return for avoidance of mortality and morbidity of open surgery or stoma formation. Transanal Endoscopic Microsurgery (TEM) is one such local excision technique which provides an alternative to open surgery for low rectal cancer which would otherwise require

APER and permanent colostomy. Only 1 RCT comparing TEM and open surgery has been published and this was insufficiently powered (n=20 in each group) for survival analysis (Winde et al., 1996). The remaining literature whilst favouring TEM, are retrospective case controls studies and significantly biased by case selection (Suppiah et al., 2008).

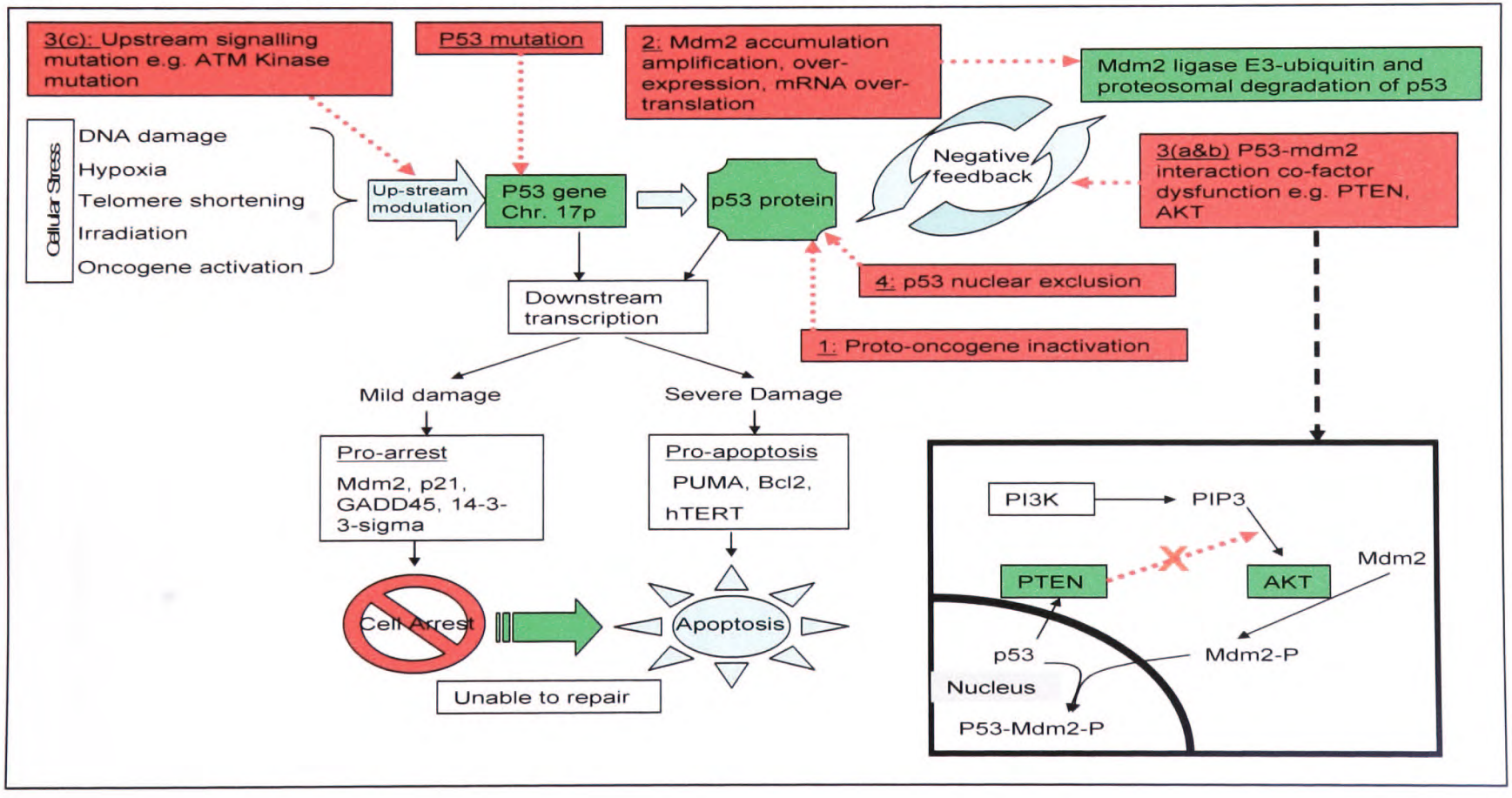
The combined local therapy and neo-adjuvant therapies are explored on both sides of the Atlantic. TEM and (neo)adjuvant treatment is explored in the proposed TREC (Transanal Endoscopic Microsurgery in Early rectal Cancer) trial, a Phase II / III trial which uses pre-operative SCRT and TEM in the UK; and American College Of Surgeons Oncology Group (ACOSOG) Z6041 Trial in the USA which uses pre-operative long-course chemoradiation with local transanal excision (Ota and Nelson, 2007b). The International Contact Radiotherapy Evaluation (ICONE) group have also launched the CONTEM (Contact X-Ray and Transanal Endoscopic Microsurgery) which uses contact radiotherapy, transanal excision and chemotherapy. The limitation of this trial is the availability of specialised radiotherapy equipment required.

This "trade-off" approach where recurrence rates are sacrificed for lower immediate morbidity rates in these trials represent the shifting paradigm that local recurrence rates, once considered the most important outcome measure, is no longer the sole predictor of oncological success. Almost all new trials evaluating CRC treatments also incorporate measures of gastrointestinal function (e.g. Faecal Incontinence and Severity Index), psychological functioning (e.g. Hospital Anxiety and Depression scale) and quality of life measures which combine daily function and psychological scores (e.g. Faecal Incontinence and Quality of Life score) as part of trial outcomes.

1.2 p53 : Introduction

The p53 gene is located on the distal band of short of the short arm of chromosome 17p13.1 (IARC, 2008). It consists of approximately 20,000 base pairs spread over 11 exons (Lutz and Nowakowska-Swirta, 2002). It was initially discovered in 1979, as a protein binding to a viral oncogene, Simian Vacuolating 40 (SV40) large T-antigen, and hence thought to be an oncogene itself (Levine, 2009, Wiman, 2006, Lane and Crawford, 1979). It has since established its role as a critical tumour-suppressor gene (Staples et al., 2008, Finlay et al., 1989). p53 inactivation predisposes to malignant transformation in rodent models and in human clinical syndromes such as Li-Fraumeni syndrome which is characterized by p53 germline mutation (Schwarzbraun et al., 2009, Donehower et al., 1992, Eliyahu et al., 1989). The tumour suppressive role of p53 is so crucial that it is referred to as "guardian of the genome" (Bueter et al., 2006). It is the most common mutation found in cancers and is present in half of all solid tumours thus emphasising its importance in protecting cell lines from carcinogenesis. The frequency of mutation varies in individual cancers ranging from 5-12% in cervical and haemo-poietic malignancies to 40-50% in colorectal and ovarian cancer (IARC, 2008). Additionally, the remaining cancers with no detectable p53 mutation are still thought to have dysfunctional p53 caused by mechanisms other than mutation (Staples et al., 2008). As almost all recent advances in CRC treatment have been in molecular biology (e.g. Endothelial Growth Factor Receptor) and chemo-immunotherapy, p53 and its diverse function, may yet again be catapulted to the forefront. The normal p53 gene function (**Section 1.2.1**) and mechanisms of p53 inactivation (**Section 1.2.2**) are described and summarised in **Figure 1.3**

Figure 1.3: Normal p53 function highlighted in green boxes and blue figures as described in **Section 1.2.1.** Mechanisms of p53 inactivation shown in red boxes and described in **Section 1.2.2.**



1.2.1 p53 gene and function

p53 acts as a tumour suppressor by preventing propagation of defective cells. It is up-regulated in response to cellular stress or damage by various upstream factors such as DNA damage or radiation (**Figure 1.3**). Activated p53 modifies downstream gene expression and co-factor transcription, which in conjunction with p53, lead to growth arrest (e.g. p21 / WAF) or apoptosis (e.g. PUMA) (Vousden and Lu, 2002). The p53 gene encodes for a 393 amino-acid 53 kDA phospho-protein which is divided into 3 domains – an amino (-NH2) terminal region (approximately amino acids 1-100), a central "core" domain (amino acids 100-300) and a terminal carboxyl (-COOH) region (amino acids 320-360) (Schlichtholz et al., 1992). Almost all mutations are harboured in the central "core" which contains the DNA-binding regions. Hence, it is likely that p53 dysfunction caused by mutations occurs by modifications in this central "core" p53-DNA binding complex. Interestingly, the central mutated core which harbours majority of the mutations is the least immunogenic. The anti-p53 auto-antibody recognises epitopes of the 2 terminal regions which are possessed by both the wild-type and mutant p53 protein and harbour the least mutations (Saleh etal., 2004, Schlichtholz et al., 1992). The implications of recognition of terminal epitopes on anti-p53 auto-antibody production are discussed in **Section 1.2.5.1**.

Wild-type p53 protein is intra-nuclear and has a short half-life of 5- 30 minutes, primarily due to negative feedback regulation by Murine Double Minute 2 (Mdm2) (Moll and Petrenko, 2003, Iwakuma and Lozano, 2003). Mdm2 has an ubiquitin-dependant E3 ligase which targets wild-type p53 for active nuclear and cytoplasmic proteosome-mediated degradation (Shirangi et al., 2002, Haupt et al., 1997).

Upregulated p53 binds to the Mdm2 promoter leading to upregulated Mdm2 transcription. The Mdm2 gene product inhibits p53, resulting in a classic negative feedback loop which controls p53 expression and function. p53 and various co-factors of the mdm2-p53 interaction constitute a dynamic negative feedback loop which maintains low levels of intra-nuclear p53 (**Figure 1.3**) (Staples et al., 2008, Kubbutat et al., 1997).

In normal cells, excessive cellular stress threatens DNA stability which leads to upstream modifications and post-translational alterations in p53 protein (e.g. phosphorylation) which impairs the p53-Mdm2 interaction. This results in inability of Mdm2 to degrade p53 and hence increases p53 levels. In addition, Mdm2 can also undergo post-translational modifications (e.g. ATM kinase phosphorylation), or self-ubiquinate to target itself for degradation (Fang et al., 2000, Honda and Yasuda, 2000). These mechanisms lead to p53 accumulation which then triggers a transcription cascade of downstream factors leading to cell arrest or apoptosis (**Figure 1.3**). Cell cycle arrest provides the cell time to repair damaged DNA. Cells unable to repair damage after a certain amount of time has lapsed are directed towards apoptosis by shifts in the balance between pro-arrest and pro-apoptotic factors. Alternatively, in cases of severe damage, the cell is pushed directly towards apoptosis. These safe-guards prevent the propagation of potentially defective cells. In mutated cells, the disruption of p53 gene transcription function and subsequent production of an inactive mutant p53 protein allows cells to escape this cellular arrest / apoptosis controls. This allows unregulated propagation of abnormal cells and a predisposition to malignant transformation. Normal p53 function is summarised in the green boxes in **Fignre 1.3**.

1.2.2 p53 Inactivation : Mutation

The most common cause of p53 inactivation is mutation. Mutations of other co-factors and other mechanisms have been observed in different cancers and are also able to indirectly affect p53 function leading to p53 dysfunction despite presence of the wild-type p53. p53 inactivation by mutation most frequently occurs within the p53 core (amino acids 100-300) whilst 70% occur at "hot spots" - amino acids 132-142, 151-159, 172-179, 237-249 and 272-286 (Iacopetta, 2003, Levine et al., 1991). The International Agency for Research on Cancer (IARC) TP53 database also reports the most frequent mutations to occur at codons 175, 245, 248, 249, 273 and 282; and is further corroborated by another international database spanning over 25 years, the UMD-TP53 mutation database (IARC, 2008, Soussi, 2008). The most common type of mutation is mis-sense (73%) followed by frame shift (9%), non-sense (8%), silent (4%) and others (6%) (IARC, 2008).

1.2.3 Mechanisms of p53 Indirect inactivation

p53 is also inactivated by mechanisms other than mutation. Some of the major pathways of indirect inactivation are described below, which include up- and down-stream modulation. These mechanisms in relation to p53 are shown in **Figure 1.3** and described in the red text boxes.

1.2.3.1 Indirect inactivation : Proto-oncogenes

Proto-oncogenes are proteins that predispose to tumour formation. These proteins are able to bind to p53 protein and induce p53 protein degradation. Human Papilloma Virus (HPV) 16 / 18 E6 protein which causes cervical cancer is one such proto-oncogene which explains the relatively low incidence of p53 mutation in cervical cancer. The rate of p53 mutation was 6% compared to >25% in most solid tumours as calculated in this thesis which included all published p53 reports over 30 years (**Figure 3.1**) (Soussi, 2008, Crook et al., 1991, Scheffner et al., 1990). In animal models, the SV40 large-T-antigen is able to inactivate wild-type p53 (Dong et al., 1994).

1.2.3.2 Indirect inactivation : Mdm2 over-expression

a) Mdm2 is a p53 negative regulator, and Mdm2 over-expression leads to p53 suppression and reduces the cell ability to trigger the pro-arrest/ apoptotic pathway in the event of cellular damage (Chene, 2003, Momand et al., 1992). Mdm2 over-expression can occur by gene amplification, gene over-expression or mRNA over-transcription (Soussi, 2008, Oren et al., 2002). Mdm2 over-expression is classically observed in soft tissue sarcomas (Leach et al., 1993, Oliner et al., 1992). Interestingly, instead of a decrease in p53 expression as would be expected due to rise in its inhibitor (Mdm2), the levels of both Mdm2 and p53 expression are increased. This suggests Mdm2 may have a separate p53-independent oncogenic mechanism (in addition to p53 suppression) which promotes tumour growth and overrides cellular arrest mechanisms despite increasing levels of p53 tumour suppressor protein.

1.2.3.3 Indirect inactivation : Co-factors of the p53-Mdm2 loop

p53 is also inactivated by alterations in co-factors of the p53-mdm2 negative feedback loop such as AKT Kinase, PTEN and ATM Kinase. Alterations in these co-factors have been associated with other tumour suppressors' inactivation, e.g. BRCA1, Bcl-2, TGF-β, and CRC syndromes such as PTEN Hamartoma Syndrome (**Table 1.2**).

a) AKT kinase phosphorylates Mdm2 and induces migration of phosphorylated-Mdm2 into the nucleus where it inactivates p53. Hence, AKT over-expression can lead to p53 suppression (Ogawara et al., 2002, Gottlieb et al., 2002).

b) PTEN is upregulated with p53 activity in response to stress. PTEN reduces AKT activity which results in decreased Mdm2 phosphorylation and intranuclear migration. Intranuclear Mdm2 acts in the classic negative feedback loop within the nucleus (Soussi, 2008, Mayo and Donner, 2002). Mutated PTEN is inactive and unable to inhibit AKT. This promotes continuous Mdm2 phosphorylation and intranuclear migration leading to continuous p53 degradation thus reducing p53 tumour suppressive ability (Choi et al., 2006).

c) Other co-factors occur upstream of the p53 –Mdm2 interaction and are responsible for p53 induction. Ataxia Telangectasia Mutated (ATM) kinase phosphorylates p53 and Mdm2 in response to irradiation. The phosphorylation of Mdm2 and p53 reduces the efficacy of the negative feedback loop resulting in p53 increase and cell cycle arrest/ apoptosis. Mutations of ATM kinase prevent this response allowing irradiated cells to escape cellular growth constraints.

1.2.3.4 Indirect inactivation : Nuclear exclusion

Extrusion of p53 into cytoplasm has been observed in certain tumours such as breast (Moll et al., 1992) and colon (Bosari et al., 1995), and neuroblastoma and malignant melanoma (Weiss et al., 1995, Moll et al., 1995). It is thought the nuclear extrusion of p53 protein prevents p53 from performing its functions which are primarily through intra-nuclear interactions.

1.2.4 p53 : Loss of Protective & Gain of Oncogenic Function

p53 mutation is the most common mechanism of p53 dysfunction. The mutant p53 has impaired DNA-binding and transcription abilities resulting in impaired cellular regulation. This is referred to as "loss of function" (Levine, 2009, Olivier et al., 2009). Furthermore, the mutant p53 is able to complex with, and inactivate, wild-type p53 in cell culture thus exhibiting a "dominant negative" effect on the "loss of function". Finally, certain mutant conformational changes can result in acquisition of new pro-oncogenic abilities, known as "gain of function". This includes transcription of tumour growth promoting factors such as MYC and VEGF (Sigal and Rotter, 2000); or disruption of other pro-apoptotic factors such as p73 (Wiman, 2006, Strano et al., 2000).

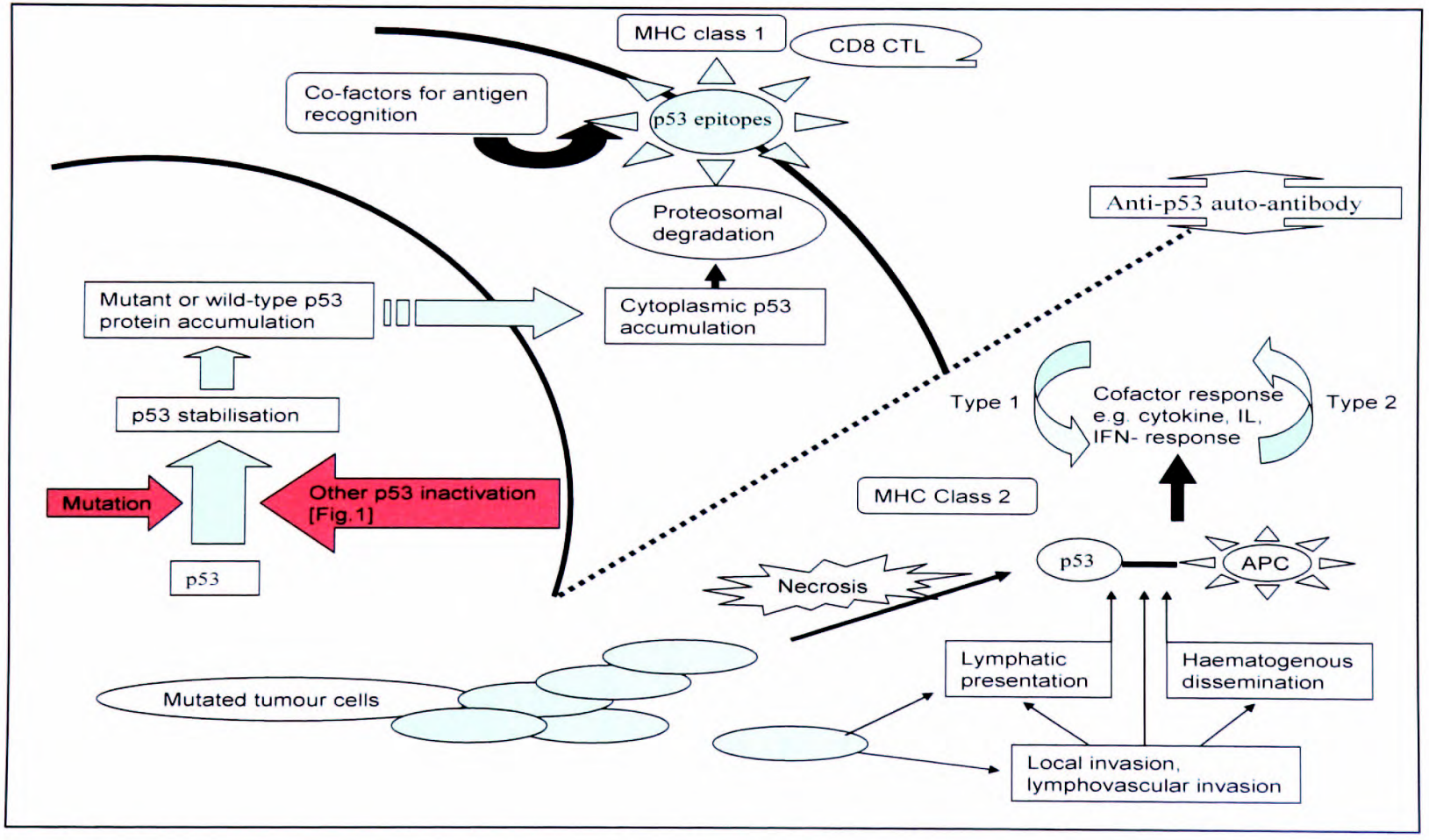
These alterations in function not only promote tumour growth but may induce chemo-resistance with subsequent impact on prognosis. Hence, p53 mutations not only cause loss of tumour suppressive capacity, but can also additionally become pro-oncogenic, which allows these mutated cells to avoid both, p53-dependant and p53-independent apoptosis, resulting in unregulated propagation culminating in carcinogenesis.

1.2.5 Anti-p53 auto-antibody

Anti-p53 auto-antibody was first reported by Crawford et al in 1982 in 9% (14/155) of patients with breast cancer (Crawford et al., 1982). Further interest in anti-p53 auto-antibody declined due to the lack of accurate quantification methods and no observable clinical relevance. Research into the auto-antibody was invigorated in the 1990s when the role of p53 in carcinogenesis was increasingly recognized. The exact cause of induction of anti-p53 auto-antibody production is unknown but is thought to be associated with the presence of p53 mutation and p53 protein over-expression (**Figure 1.4**).

Anti-p53 auto-antibody is not normally present as the wild-type p53 protein is intra-nuclear, expressed at low concentrations, not expressed on surface of cells and hence escapes detection by the immune system (Bargonetti and Manfredi, 2002, Haupt et al., 1997). In abnormal cells, p53 protein is stabilised mainly through p53 gene mutation, but can also be stabilised by other mechanisms such as proto-oncogene-binding or inhibition of negative regulatory pathways (**Section 1.2.3**). This causes high intra-nuclear p53 protein accumulation which then escapes into the cytoplasm. The resulting high cytoplasmic p53 levels increase the likelihood of p53 being degraded by proteosomes and presented on cell surfaces to be recognised by T-cells in a MHC I response (Bueter et al., 2006). The auto-antibody recognises epitopes on the terminal regions of the protein, and hence auto-antibody production can be triggered by either the wild-type or the mutant p53, provided sufficiently high levels of these immuno-dominant epitopes at present at the cell surface (Schlichtholz et al., 1994).

Figure 1.4: Proposed mechanisms of anti-p53 auto-antibody induction



Another possible antigen presentation mechanism is where cancer cells containing high cytoplasmic concentrations of p53 undergo necrosis and release p53 into the blood and lymphatic system. These antigens are captured Antigen Presenting Cells (APC) in their normal scavenging role and presented as MHC class II response (Bueter et al., 2006).

1.2.5.1 Increased p53 levels induce the auto-antibody response

Mutation alone is insufficient to trigger the immune response as evidenced by several observations. Firstly, only 20-50% of patients with detectable p53 mutations produce detectable auto-antibodies. This is attributed to the type of mutation. For instance, missense mutations are more likely to produce a stable mutant p53 protein which is more likely to accumulate to sufficient levels to increase the likelihood of antigen presentation. Other mutations such as non-sense, frameshift and deletions often lead to truncated mRNA and unstable protein sequences which are less likely to accumulate and induce auto-antibody production (Forslund et al., 2001). Secondly, anti-p53 auto-antibodies most frequently recognise terminal epitopes but not the central domain with the majority of mutations (Saleh et al., 2004, Lubin et al., 1995a, Schlichtholz et al., 1992). This suggests mutation is not essential for anti-p53 auto-antibody production. Thirdly, large SV40 T-antigen stabilises p53 protein leading to accumulation of the wild-type protein which also induces auto-antibody production. These observations suggest that humoral response is triggered by elevated p53 protein levels rather than specifically directed at a mutated sequence (Saleh et al., 2004, Schlichtholz et al., 1994, Legros et al., 1994, Lubin et al., 1993). Mutation simply happens to be the most common mechanism of p53 accumulation.

1.2.5.2 Differences in humoral response and presence of p53 mutation

There are discrepancies between the presence of mutation, protein expression and auto-antibody production which can be attributed to the methods of detection. Historically, direct sequencing for mutations localize most mutations to exons 5-8 and to a lesser extent 4,9,10. Many studies subsequently screened only these areas leading to substantial screening bias as it is now known that 10% mutations occur outside these areas (Chang et al., 2005, Forslund et al., 2001). p53 protein expression is subject to sampling/biopsy errors, and older studies have different immuno-histochemical, fixation, parafinisation, antigen and antibody retrieval and observer scoring techniques. Finally, the auto-antibody was initially detected using immunoblots or in-house ELISA. This led to a vast range of reported frequencies within individual cancers because of different cut-off values used (**Table 3.1**). Although commercial ELISA are now widely available, auto-antibody detection can still vary depending on different manufacturers (**Table 3.2**) (Rohayem et al., 1999).

Finally, the differences in the individual's immune systems cannot be ignored. The humoral response is dependant on an individual's unique MHC presentation. Patients with similar cancers and same mutations do not necessarily mount the same immune response (Angelopoulou et al., 1997). Also, anti-p53 auto-antibody titres increase and decrease reflecting tumour load. However, patients who are sero- positive at diagnosis do not generally sero-convert following curative complete resection i.e. the system has been primed (Metcalfe et al., 2000, Lubin et al., 1995b). Conversely, patients who are sero-negative do not develop an auto-antibody response, despite disease progression and metastases. This is discussed in more detail in **Section 3.5.4**.

1.3 Anti-hTERT auto-antibody in Colorectal Cancer

Anti-hTERT auto-antibody is a humoral response to re-induction of hTERT which is the main component in reactivation of telomerase activity which is required for CRC cell immortalisation. The importance of hTERT and telomerase activity in normal and cancer cell immortality is discussed (**Section 1.3.4**). The results of the only study to detect anti-hTERT auto-antibody are summarised (**Section 1.3.6**). First, normal cellular mortality and the importance of DNA telomere length in mortality is discussed (**Section 1.3.1**), followed by the function of telomerase enzyme in telomere elongation (**Section 1.3.3**) and its impact on overcoming mortality.

1.3.1 Cellular mortality

Cells have a finite reproductive capability as demonstrated by *in-vitro* human fibroblasts studies. Hayflick showed that human fibroblasts entered a period of reduced replication after a certain number of population doublings (pd) suggesting the cells had reached a mortality barrier (Hayflick and Moorhead, 1961). Another elegant experiment compared senescence in population doublings of normal diploid cell *in-vitro*, with population doublings of its frozen counterparts which were frozen earlier on before senescence was reached. On thawing at a later date, the frozen cells resumed cell replication until it reached the same number of population doublings as had been reached by its non-frozen counterparts, showing that this mortality barrier was due to an internal mechanism controlling the number of population doublings, rather than the simple passage of time i.e. biological age, not chronological age. (Hayflick, 1965).

Watson et al had previously described the mechanism of DNA replication (Watson, 1972). The semi-conservative nature of eukaryotic DNA replication leads to progressive loss of terminal DNA sequences due to the mechanism of action of DNA polymerase which only acts in the 5' → 3' direction. Thus, each replication cycle results in loss of terminal DNA sequences potentially leading to loss of vital DNA encoding for vital genes. This problem is overcome by telomeres.

Telomeres are terminal DNA repetitive tandem 6 base sequences (TTAGGG) which do not code for gene products. Telomeres are 10kbp long consisting of approximately 1500 – 1800 of these TTAGGG repeats (Kim et al., 1994). Telomeres protect DNA as these non-coding telomeric DNA sequences are shortened during cell replication, thus preventing loss of vital coding DNA. Telomeres are eroded at 50 -200 bp per population doubling in human lymphocytes and fibroblast studies (Allsopp et al., 1992, Janknecht, 2004). As discussed before, cells eventually reach a period of senescence after a certain number of population doublings caused by an internal mechanism. Olovnikov postulated that this semi-conservative DNA replication model, with its resulting progressive loss of terminal DNA sequences leading to shorter unstable DNA, was responsible for the cellular senescence and growth arrest observed in these studies. The proposed term for this regulation of cell replication was the "mitotic clock" (Olovnikov, 1973).This postulation was only supported much later when the telomeric sequence was discovered, initially in protozoa, then in humans, thus enabling telomere length to be measured (Blackburn and Gall, 1978). Subsequent work confirmed telomeric shortening occurred with each DNA replication, and this also predicted the replicative capacity in human fibroblasts (Allsopp et al., 1992).

1.3.2 Telomerase Enzyme

Telomerase is a eukaryotic ribonuclear RNA-dependant reverse transcriptase enzyme which functions to re-construct telomeres and increase telomere length. Kim et al showed that this ability to elongate telomerase led to indefinite proliferation not only in normal cells, but was also a necessary requirement for cancer cells. Rapid growing cancer cells are subject to the similar growth constraints of normal cells and would also reach a period of senescence i.e. mortality (Kim et al., 1994). Re-establishment of telomere length by telomerase activity allowed the cancer cells to proliferate indefinitely. Hence, telomerase was also known as the "immortality" enzyme.

Telomerase enzyme consists of 3 sub-units: a RNA template (hTR), human telomerase associated protein (hTAP) and human telomerase reverse transcriptase (hTERT) (Nakamura et al., 1997, Harrington et al., 1997, Feng et al., 1995). The hTR is complementary to the human telomeric TTAGGG sequence, and forms the template for telomere elongation. hTERT is the catalytic component and the rate-determinant of telomerase activity. The mechanism of telomere elongation is described (**Section 1.3.3**). Telomerase was first described in Protozoa Tetrahymena (Greider and Blackburn, 1985). Telomerase activity peaks in utero, at 18 – 21 weeks gestation and is repressed to almost undetectable levels, except in proliferative self-renewing cells which require a low but indefinite proliferative rate, such as lymphocytes, trachea and bronchi, basal cells of the epidermis and intestinal crypt cells (Forsyth et al., 2002, Shay and Wright, 2005). Telomerase is re-expressed at higher levels in 90% of all cancers making it a sensitive marker for neoplasia (Masutomi et al., 2000).

1.3.3 Telomerase Mechanism of Action

A variety of protein sites on the telomerase enzyme are involved and the exact motion in telomere elongation is unknown. However, the mechanism can be simplified as follows (**Figure 1.5**).

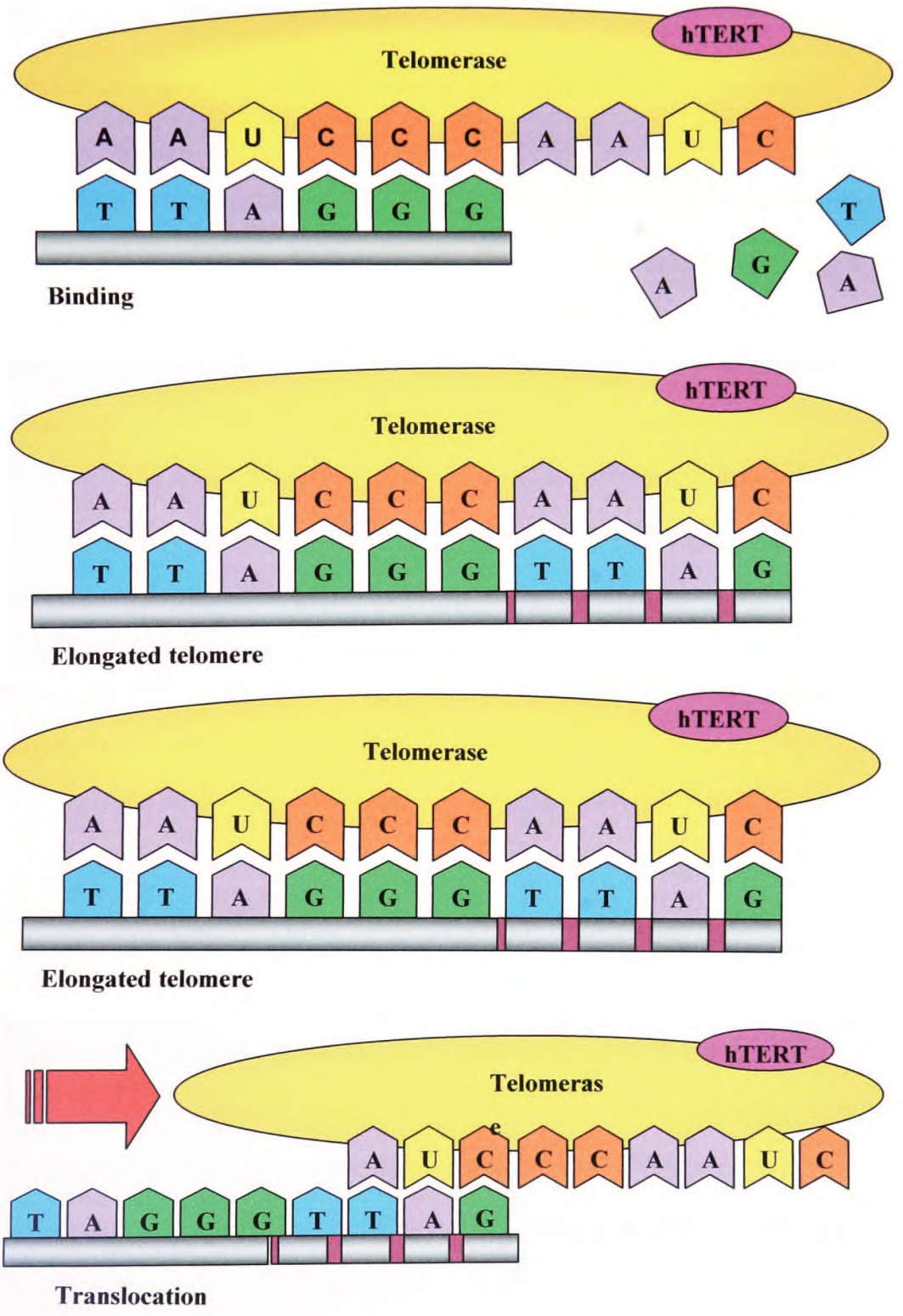
The first stage is "**Binding**", where the hTR template attaches to the complimentary TTAGGG terminal telomere sequence of the DNA. The hTR template overhangs beyond the telomere end.

The second stage is "**Elongation**" where complementary nucleotides are attached to the overhanging hTR sequence. The nucleotides are arranged and secured with the DNA deoxyribose backbone.

Finally, "**Translocation**" occurs, where the telomerase enzyme and hTR component slides forward onto the next terminal TTAGGG sequence.

The process is repeated, starting with "Binding", resulting in progressive elongation of the telomere.

Figure 1.5: Telomerase mechanism of action



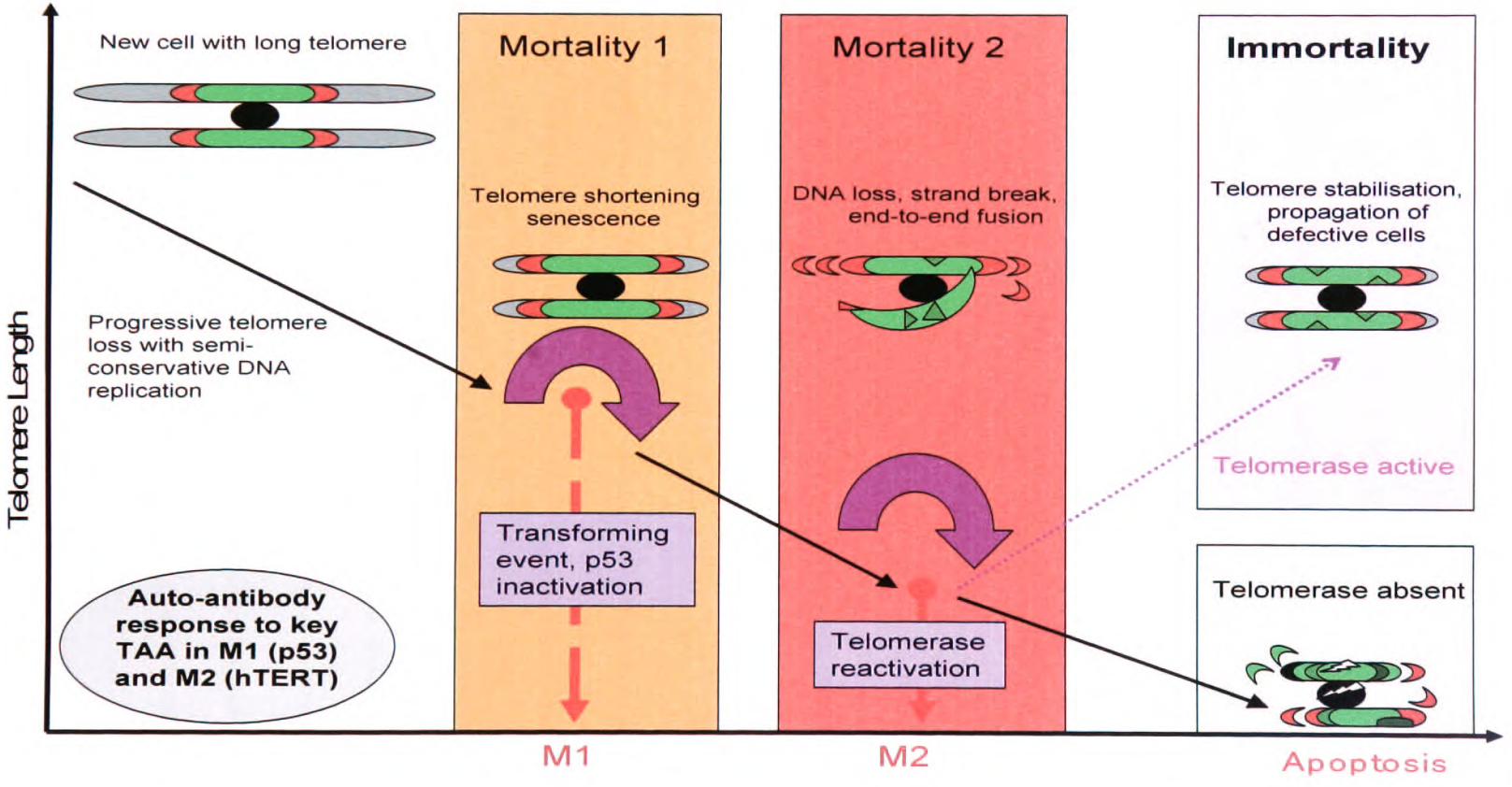
1.3.4 Telomerase "Immortality" and Carcinogenesis

Normal cells have a finite replicative capacity determined by the internal mitotic clock caused by telomere shortening every DNA replication cycle. The cells then reach a period of "senescence", characterised by slow replication or cell growth arrest. Human fibroblasts display this "senescence" after 40 – 70 population doublings. This "senescence" was overcome by transfection of these cells with proto-oncogenic viruses such as SV40 large T-antigen, HPV16 E6 and HPV16 E7 (Ozer, 2000). These transformed cells were able to continue a further 20 – 30 population doublings. This is a substantial (approximately 50%) increase in replicative potential considering senescence occurs at 70 population doublings.

Following this additional population doublings, the cells enter a "crisis" phase which is characterised by DNA loss and cell death. Newbold showed that human dermal fibroblasts exposed to carcinogens were subject to this "crisis" (Newbold, 2002). In rare instances ($< 10^6$ cells), a single clone emerges from this phase to demonstrate a selective growth advantage over the other cells. This suggested another mechanism, in addition to oncogenic transformation to overcome senescence, was required for transformed cells to continue indefinite proliferation or "immortality". This indefinite proliferation would drive clonal selection in progression of neoplastic tissue. The importance of telomerase in overcoming "crisis" was hinted at by the presence of the active telomerase enzyme in human cancer tissue, cancer cell lines and *in-vitro* transformed cell lines (but not normal human cells) using a modified PCR known as Telomerase Repeat Amplification Protocol (TRAP) (Hirose et al., 1997).

Wright et al suggested a "2-hit" hypothesis, introducing the term Mortality 1 (M1) for the initial period of "senescence" which is overcome by transformation of tumour-suppressors; and Mortality 2 (M2) for the second mortality "crisis" barrier characterised by cell death (Wright et al., 1989). The importance of telomere length and telomerase activation in the 2-hit hypothesis is explained as follows. The aging cells have increasingly shorter telomeres because of semi-conservative DNA replication. Following a certain number of population doublings ("aging") the telomeres become critically short hence endangering vital DNA coding sequences. Certain telomeric changes, such as destabilisation of the telomere cap is recognised by cell growth regulators such as p53 (Hemann et al., 2001). This recognition is similar to recognition of DNA breaks. Senescence is initiated as a protective mechanism against propagation of these defective cells. This barrier is termed the Mortality 1 (M1) barrier. M1 can be overcome by inactivation of p53, or other tumour-suppressor genes ("Transformation"). The transformed cells are able to continue replications until telomeres are critically short, resulting in chromosomal destabilisation and end-to-end fusion. The resulting dicentric chromosomes and DNA breakage now promote apoptosis, not senescence, resulting in the second mortality barrier (M2). Re-initiation of telomerase activity stabilises telomere length and overcomes M2. This is required for even transformed cells to achieve immortality as human dermal fibroblasts exposed to carcinogens (SV40 large-T, HPV E16) are unable to overcome M2 (Newbold, 2002). In landmark trials, cells similarly exposed, but with addition of hTERT, resulted in immortality (Hahn et al., 1999, Hahn et al., 2002). Other studies showed introduction of a mutant hTERT which inactivate telomerase in tumours resulted in telomere shortening and transformed cancer cell death (Hahn et al., 1999).

Figure 1.6: Telomere length, Mortality Barriers and Immortality



1.3.5 Alternative Lengthening of Telomeres

Telomerase length and immortality is not solely dependant on telomerase activity as shown by 2 observations (Kim et al., 1994). Firstly, in mice (mTR) knock-out studies, the mice cells were able to activate an alternative mechanism of telomere length maintenance. Secondly, not all in-vitro immortalised cell lines (e.g. with SV-T40 large antigen) have detectable telomerase activity.

Alternative Lengthening of Telomeres (ALT) is a mechanism of maintaining telomere length without telomerase, and was first described in humans by Bryan et al. (Reddel, 2000, Bryan et al., 1995). The main mechanism is likely to be DNA recombinational as analysis of ALT dependant human cells show heterogeneous telomere length ranging from undetectable to abnormally long (Bryan et al., 1997a, Henson et al., 2002). Although up to 50% of SV4-immortalised fibroblasts used ALT, most epithelial and mesothelial cell lines are telomerase positive (Perrem et al., 2001). Furthermore, human tumours and human tumour derived cell lines display only 7% ALT (Bryan et al., 1997b). The high frequency of ALT in cell lines caused by tumour-suppressive DNA viruses suggest p53 suppression may activate ALT. Alternatively, ALT may be repressed by telomerase enzyme positivity as shown by low proportion of ALT in human telomerase positive cancer lines. This ALT mechanism is not explored in this thesis. However, this section is included as there is intense research into ALT, as its presence threatens the use of anti-telomerase therapy in cancer.

1.3.6 Anti-hTERT auto-antibody

Masutomi et al first described a technique of producing purified hTERT using recombinant technology (Masutomi et al., 2000). The recombinant hTERT protein was confirmed by WB. The WB confirmed a 127 kDa protein band in lysates of cells transfected by anti-hTERT antibody, but not in non-transfected cells. Secondly, *in vitro* procedures were used to transcribe hTR, which when combined the hTERT resulted in telomerase activity detected by TRAP. This purified hTERT was then tested in WB for the presence of anti-hTERT auto-antibodies. Serum samples from 18 healthy volunteers were compared with serum from 30 hepatocellular (HCC) patients, 16 liver cirrhosis (LC) patients, 7 patients with chronic hepatitis (CH) and 12 patients with other malignancies (colorectal, gastric and lung) in WB using the recombinant hTERT embedded in the membrane. Serum from patients with HCC and various malignancies produced a 127kDa band whereas no protein detection occurred with HCC, LC or normal or normal volunteers. The authors confirm this as anti-hTERT as it reacted to hTERT and was not present in non-cancer patients.

The levels of anti-hTERT auto-antibody were then measured in an ELISA. The serum anti-hTERT auto-antibody titres in HCC and other malignancies were significantly higher than normal controls. Furthermore, there was significant step-wise increase in anti-hTERT from CH to LC, and LC to HCC, suggesting and increased telomerase activity with carcinogenesis. No further analysis was performed on this pilot work, and neither have there been any published reports since then.

1.4 Thesis Summary

This thesis aims to investigate the clinical significance and the correlation between the humoral response (anti-p53 and anti-hTERT auto-antibody) against the two key events in carcinogenesis resulting in Tumour Associated Antigens (p53 mutation and hTERT re-expression) required for cancer cell immortality. The methods used are described (**Chapter 2**). The significance of anti-p53 auto-antibody in cancer and in CRC is evaluated (**Chapter 3**). The long-term prognostic significance of anti-p53 auto-antibody is then investigated in a cohort of patients with minimum 5 years follow-up (**Chapter 4**). The optimisation of a method to detect anti-hTERT auto-antibody is described with suggestions for future improvement (**Chapter 5**).

2 Chapter 2: Materials and Methods

Section 2.1 describes patient recruitment, **Section 2.2** describes ELISA validation, **Section 2.3** describes statistical methods used to investigate the significance of anti-p53 auto-antibody in CRC and **Section 2.4** describes WB optimisation in attempts to isolate anti-hTERT auto-antibody.

2.1 Subject Recruitment and Sample Collection

Blood samples were obtained following ethical approval for research purposes from patients with colorectal carcinoma (CRC) treated at Hull and North East Yorkshire NHS Trust, and from 28 controls (Kumar R, LEC 05/02/091; Rao VS LEC 10/03/213). Pre-operative sera from ninety two patients who were treated with curative intent for biopsy-proven CRC between 1996 and 2001 were available for analysis (Chin KF, LEC 03/98/41, O'Hara R, LEC 07/09/102). All bloods were collected in pre-assessment clinic 7-10 days prior to surgery. Each sample of whole blood was collected for simultaneous processing into serum and plasma.

2.1.1 Serum and plasma preparation

A sample of whole blood (7ml) was taken into BD Vacutainers® (BD Bioscience, Oxford, UK) and refrigerated for 30 minutes. The sample was then centrifuged at 2000*g* for 10 minutes at room temperature. The supernatant was pipetted out to obtain the serum

sample. Sera was divided into 500µl aliquots in 1.5ml polypropylene tubes and stored at -80°C until analysis. Plasma was derived from whole blood collected in BD Vacutainers® ethylenediaminetetraacetic acid (EDTA) tube which prevents clotting and consumption of coagulation factors. The whole blood was refrigerated for 30 minutes and centrifuged at 1500*g* for 5 minutes. The supernatant (plasma) was similarly pippeted into 500µl aliquots in 1.5ml polypropylene tubes and stored at -80°C until analysis. Repeated freeze-thaw cycles of serum and plasma were avoided.

2.1.2 Patient data collection

Sera were available for 92 CRC patients treated between 1996 and 2001 and 28 non-cancer controls of similar age and sex. Parameters recorded were patient demographics, date of diagnosis defined by date of biopsy, surgical procedure undertaken, final pathological staging and duration of follow-up (defined as time to death or time last seen in clinic / hospital). Death was recorded in hospital case notes. In addition to this, due to the relatively high probability of death in patients in this series (elderly population, malignant disease with major surgery and long follow-up, up to 10 years in some) and the possibility of non-hospital death (e.g. in hospice or community) where death may not be recorded in hospital case notes, the electronic patient information system was checked and the general practitioners were contacted where doubt existed. This was especially valuable and identified 16 patients who had moved away, died at home / residential home and those with recurrent disease deemed as "palliative only" and were no longer followed up by the surgical teams. Causes of death were recorded as that registered on death

certificates. Although this allowed greater completion of information, the cause of death in these circumstances was not issued by the cancer clinician. This could lead coding errors as non-cancer related death could be erroneously attributed to cancer (as patients were already in nursing / residential homes suggesting non-cancer co-morbidity). Alternatively, death in hospices due to end-stage cancer could be coded as non-cancer death (e.g. bronchopneumonia). The GP / health care worker notes of the final events preceding death were not available and even if so, the retrospective information could still lead to potential inaccurate cancer death coding.

2.2 Enzyme Linked Immunosorbent Immunoassay (ELISA)

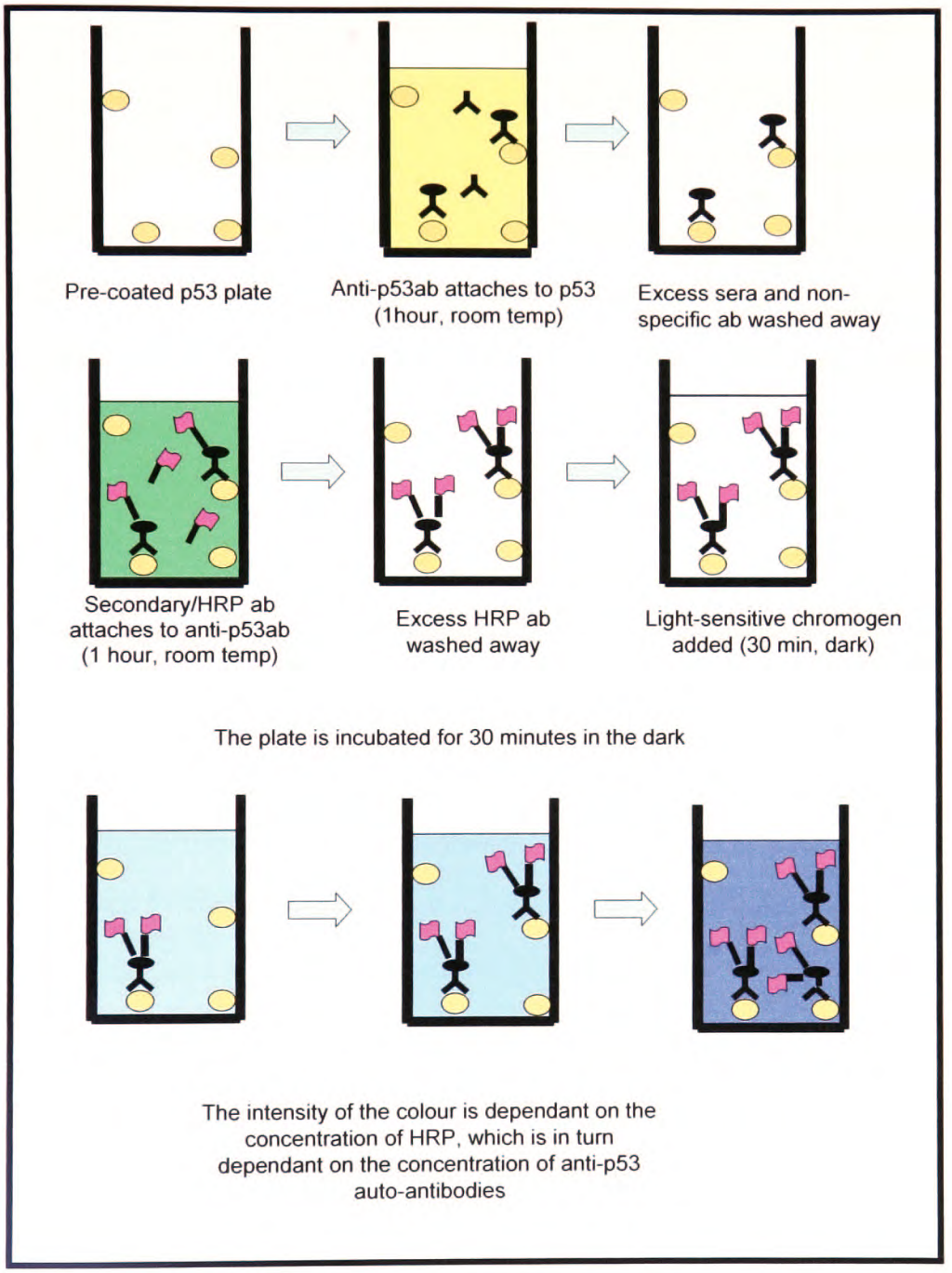
Enzyme Linked Immunosorbent Immunoassay (ELISA) is an immunological technique used to semi-quantitatively assess for presence of antibody/protein of interest (Adler et al., 2009, Engvall and Perlmann, 1971).

2.2.1 ELISA principles

The ELISA principles are illustrated in **Figure 2.1**. A suitable antigen which binds to the antibody of interest is fixed on an ELISA plate. The test sample containing the antibody is poured into these wells and the antibody binds to the immobilised antigen. The excess solution and non-specifically bound antibodies are washed away. A secondary antibody, which targets the species-specific portion of the primary antibody is poured in. The secondary antibody is conjugated to a detection enzyme such as horse-radish peroxidase. The excess solution is washed away along with non-specifically bound secondary

antibodies. This leaves the antigen, bound to the primary antibody of interest, which is in turn attached to the secondary HRP-conjugated antibody. A suitable detection reagent is added. The HRP enzymatically oxidises the detection reagent leading to a change in colour of the solution over a 5 – 30 minutes. The optical density (OD) of each well is read on a plate spectrometer. The colour intensity is dependant on the amount of HRP, which is in turn dependant on the amount of secondary antibody and hence, the amount of the primary antibody. By using positive and negative controls, the primary antibody titres can be measured using sample OD. In this thesis, the antibody of interest (anti-p53 auto-antibody) lies between the p53 antigen and the HRP- anti-human HRP.

Figure 2.1: Principle of anti-p53 auto-antibody ELISA



(Ab = antibody; HRP = horse-radish peroxidise)

2.2.2 ELISA protocol for Measurement of Anti-p53 Auto-antibody

Anti-p53 auto-antibody titres were measured using a commercially available ELISA - p53 ELISA PLUS (Autoantibody) Kit (Cat. No. QIA53, Calbiochem, Darmstadt, Germany). The 96-well ELISA microplates were pre-coated with recombinant human wild-type p53 protein, to which any anti-p53 auto-antibodies within serum samples would be expected to bind. The manufacturer's protocols were used (User protocol QIA53. Rev.19, May 2005). Subjects' sera were diluted 1:100 and incubated alongside the provided calibrator samples at dilutions of 1, 0.67, 0.5, 0.33 and 0.16; a negative control and 2 blank wells for 1 hour at room temperature. All samples were assayed in duplicate at 100µl/well. After incubation, the plate was washed five times with the manufacturer's wash buffer to remove non-specifically bound antibodies, leaving anti-p53 auto-antibodies bound to the plate. The detection reagent, a goat-HRP anti-human polyclonal antibody (100µl), was added to each well to bind to any captured human anti-p53 auto-antibody. The plate was incubated for a further 1 hour at room temperature, followed by another five washes to remove the excess unbound HRP detection reagent. The manufacturer provided Substrate solution (3, 5, 5 tetramethylbenzidine, TMB) was added to the plate and incubated for 30 minutes in the dark. The HRP enzymatically converts the chromogenic TMB substrate from colourless to blue. The reaction was stopped with 50µl of the provided stop solution (2 M Hydrochloric Acid, HCl) which turns the reaction mix yellow. The optical density was measured at 450nm (OD_{450nm}) using a 620nm reference filter in order to compensate for possible differences in the material of the microtitre plate (Labsystems, Multiskan MS, Finland).

2.2.3 ELISA plate configuration

A sample ELISA plate is shown in **Figure 2.2**. Note the following:

1. Decreasing colour intensity from rows A to H, consistent with decreasing auto-antibody concentration of the calibrators i.e. Row A 1.0 U, Row B: 0.67; Row C: 0.5

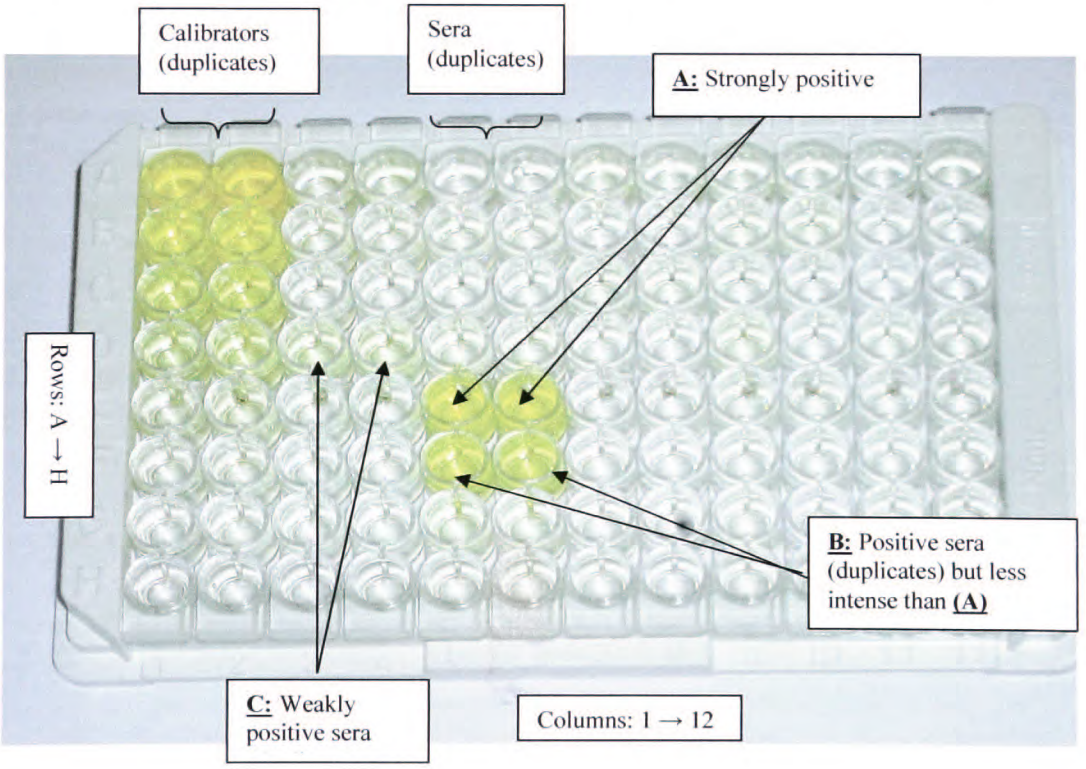
2. **A:** Strongly positive sera sample

B: Positive but not as strong as sample A, suggesting auto-antibody presence, but at lesser concentration to sample A

C: Weak colour intensity. Visually, this intensity is between calibrators D (0.33 U) and E (0.16). Accurate quantification of is performed by means of a photo-spectrometer

3. All paired samples have similar colour intensity demonstrating accuracy of technique, i.e. no pipetting inaccuracies or sample variation

Figure 2.2: Typical ELISA plate: Vertical rows (A-H); Horizontal columns (1-12).

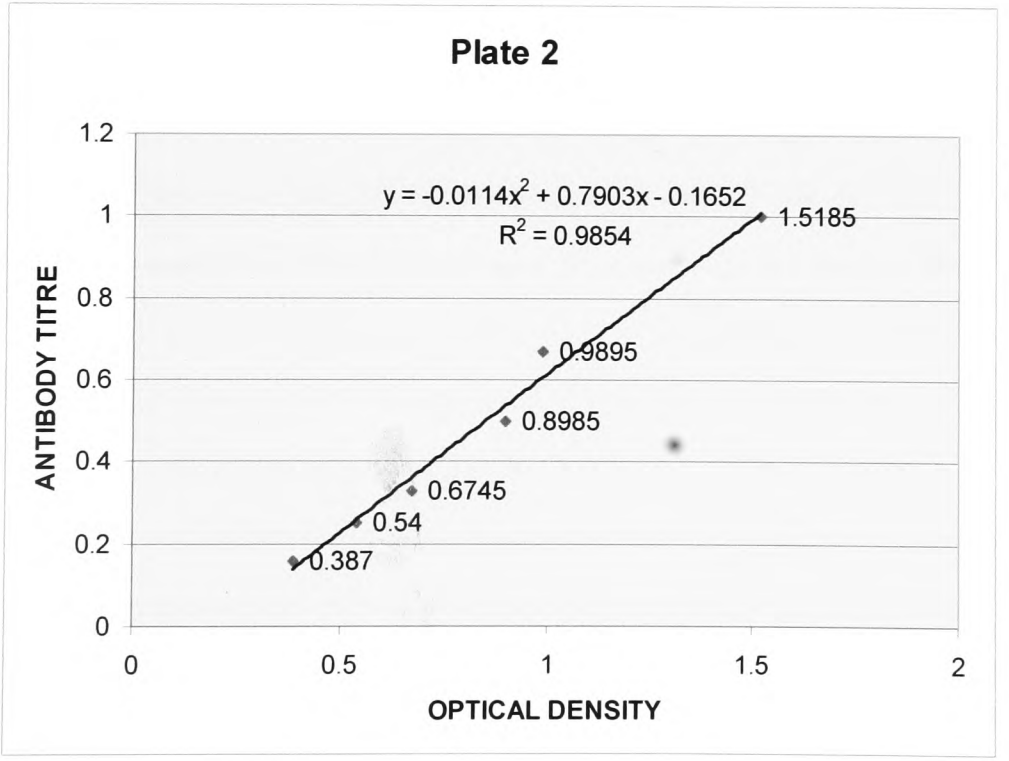


2.2.4 ELISA Calibration Curve

Calibration graphs were constructed for each plate by plotting the calibrator dilutions, which represent the relative antibody titres (neat calibrator, 1 = 100U auto-antibody/100µl), on the *y*-axis. The OD_{450nm} of each calibrator dilution is plotted on the *x*-axis. A "line-of-best-fit" was generated with a resulting quadratic equation (Microsoft® Excel 2002) **(Figure 2.3)** The auto-antibody concentration is either read from the graph, or more accurately, by substituting the OD_{450nm} of the sample into the equation obtained from the line-of-best-fit. Example from plate 2:

Auto-antibody titre $(y) = 0.0114(x^2) + 0.7903(x) - 0.1652$, where $x = OD_{450nm}$ sample

Figure 2.3: Calibration curve generated using calibrator dilutions and corresponding OD_{450nm}



2.3 Statistical analysis

Statistical advice was obtained from Rachel Waddington-Clarke, a statistician at the University of Hull. The Null Hypothesis was that there was no difference in clinico-pathological parameters or survival in colorectal cancer patients with anti-53 auto-antibody and those without. All tests were two-tailed and significance was set at $\alpha = 0.05$. Analysis was performed using the Statistical Package for the Social Sciences, SPSS, and v.14 for Windows (SPSS Inc, Chicago, USA).

2.3.1 Assessment of ELISA technique / methodology

The OD_{450nm} of all samples were recorded. Accuracy of each ELISA plate was assessed by measuring the Co-efficient of Variation (CoV) of duplicate samples and the correlation co-efficient (R^2) of the lines-of-best-fit. A CoV > 20% indicates a difference of >20% in duplicate OD_{450nm} of a sample suggesting erroneous technique.

2.3.2 Categorical data

Categorical data was divided into nominal groups (e.g. anti-p53 auto-antibody positive vs. negative) or ordinal groups (age <70 and age > 70) and analysed using the Chi square test ($\chi 2$) for presence or absence of anti-p53 auto-antibody. Data in each category (Categories: Control vs. patient; age <70 vs. age >70; sex; recurrence) were mutually exclusive, meaning a value can only belong to one group or the other. Additionally, when individual group numbers were small, the groups were collapsed in a rational fashion based on biological parameters to obtain larger sample sizes. $\chi 2$ analysis was performed for anti-p53 correlation with categorical variables. Fisher's Exact test was used when frequency in a group was < 5.

2.3.3 Measurement of Prognosis

The follow-up distribution was assessed and the appropriate statistical tests chosen.

2.3.3.1 Follow up Distribution

Kolmogrov-Smirnov analysis showed the follow-up period to be to be skewed ($p = 0.015$, **Figure 4.5**), thus limiting survival analysis to non-parametric tests - Mann Whitney U-test (comparing 2 means) and Kruskall-Wallis test (comparing > 2 means). Survival analysis is calculated using the time it takes for an individual to reach a pre-defined end-point such as time to recurrence or metastases. In this study, the end-points used were Overall survival (OS) and Disease-Free Survival (DFS). Individuals who were lost to follow-up were appropriately censored.

2.3.3.2 Kaplan-Meier Survival Curve

Kaplan-Meier survival analysis displays possible survival differences in various groups within a single variable (e.g. Dukes A-D) over time. Significance is calculated using a log-rank test. The curve displays the cumulative probability of reaching the defined end points (OS and DFS) from a pre-determined starting point (date of diagnosis). As a test of validity of this data, Kaplan-Meier analysis was first performed on known CRC prognostic factors, and compared with current literature. Seven clinico-pathological factors were incorporated into univariate Kaplan-Meier survival analysis – Dukes' stage, TNM stage, T-stage, N-stage, M-stage, Location and Differentiation. Once the validity this patient cohort was confirmed, survival analysis curves were performed for anti-p53 in OS and DFS. A limitation of Kaplan-Meier

analysis is that ability to analyse only a single variable at a time. In order to assess significance of multiple factors at the same time within the same sample, a multivariate analysis model, Cox Proportional Hazards Regression Analysis was used.

2.3.3.3 Cox Proportional Hazards Regression Analysis

This analysis calculates a hazard function of an event occurring at any particular point in time, *t*. Using individual hazard functions from different variables, the cumulative hazard (risk) of an event occurring, in a particular individual with certain prognostic factors, at a time *t*, can be calculated (Cox, 1972). In this study, the event is death, the individual is a patient with colorectal cancer, and the prognostic risk factors which could be potentially incorporated into this model were anti-p53 auto-antibody status, tumour differentiation, Duke's stage, T-stage, N-stage, and M-stage. The number of prognostic factors selected for the Proportional Hazards Model should be limited to 1 variable per 10 - 20 patients in order to reduce the risk of over-analysing the data. Over-analysing a small sample size can lead to co-linearity errors where a false positive prognostic significance is displayed when a variable is associated with other prognostic indicators, but not in itself truly independent. Hence the 7 clinico-pathological factors used in univariate analysis were reduced to four factors (10 – 20 patients / factor) for a sample of 92 patients. The 4 factors selected were anti-p53 auto-antibody (as the primary objective of this thesis) and 3 clinico-pathological parameters (Dukes'/TNM stage, T-stage and differentiation). N- and M-stage are strong prognostic factors but strongly associated with Dukes' / TNM overall stage and hence not used

2.4 Anti-hTERT Auto-antibody: Objective

The first objective was to isolate the hTERT protein to establish a positive control. This was attempted by immunoaffinity chromatography, and hTERT presence was to be confirmed by Western blot (WB). Once isolated, the hTERT could be immobilised on a nitrocellulose membrane, and probed with patient sera in using WB to detect anti-hTERT auto-antibody. Alternatively, the hTERT could be used as the antigen in ELISA (**Figure 2.1**) to quantify anti-hTERT is patients' sera.

2.4.1 Preparation of Cancer Cell Lysate

Cell concentrations of 0.5 – 1 x 10^6 cell equivalents per well were used, which was later increased to 1 x 10^8 cell equivalents per well. A cell scraper (Helena Biosciences, Gateshead, UK) was used to remove cancer cells from a confluent 75cm^2 culture flask (Sarstedt, Numbrecht, Germany). Colo205, Colo320, LoVo, Caco, TD47, MCF7 and HT29 cancer cells (see **Appendix, Section 7.3** for cell line details) were cultured in RPMI 1640 (PAA Laboratories, GmbH, Colbe, Germany), 0.3g /L L-glutamine (Sigma-Aldrich, Poole, UK), 10% Foetal calf serum (Biosera, East Sussex, UK) and 1% Penicillin / Streptomycin (Sigma-Aldrich, Poole, UK) and incubated at 37°C. Details of reagents are shown in **Appendix, Section 7.1.** The contents of the flask were poured into a 50 ml polypropylene tube and spun at 400*g* for 3 minutes. The supernatant was discarded and the pellet resuspended in 10 ml PBS. 20µl was removed and the cells were counted using an Improved Neubaeur haemcytometer. The remainder solution in the tube was centrifuged at 400*g* for 3 minutes. The supernatant was discarded again and the pellet resuspended in 1 mL Lysis buffer

Protease Inhibitor Cocktail Set III (539134, Calbiochem, Darmstadt, Germany) and incubated on ice for 30 minutes to reduce enzymatic activity which would denature proteins such as hTERT. The solution was centrifuged at 10,000*g* for 5 minutes after which the supernatant was removed and stored at -80°C until future use.

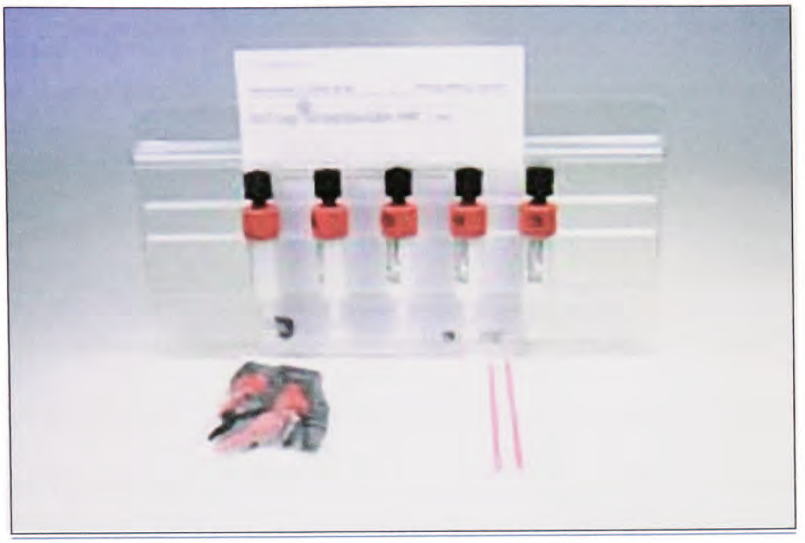
2.4.2 Principles of the Streptavidin-Biotin Interaction

Streptavidin is the 52-kDa tetrameric protein product of Streptomyces avidinii and consists quaternary subunits with a ~13-kDa β-barrel biotin binding site (Hendrickson et al., 1989). Its key biochemical feature is its ability to bind with biotin to form one of the strongest natural non-covalent bonds in the order of $\sim 10^{13\text{-}15}$ M^{-1}. This interaction was used in an immuno-affinity column to isolate hTERT. The biotinylated anti-hTERT antibody (Santa Cruz, sc-7212, **Appendix 7.7**) was passed down a streptavidin-coated agarose column (**Figure 2.4**), where it would be immobilised by the streptavidin-biotin interaction. A sample containing hTERT would then be passed into this column. The immobilised biotinylated anti-hTERT antibody should capture any hTERT in this solution. An elution buffer would then be used to break the anti-hTERT and hTERT bond, resulting in hTERT being eluted from the column.

2.4.3 Protocol for the Streptavidin-Biotinylated anti-hTERT column

The HiTrap™ Streptavidin HP columns (GE Healthcare UK Ltd, Buckinghamshire, UK) contain Streptavidin immobilised in a 6% (w/v) cross-linked agarose matrix (**Figure 2.4**). The manufacturers' recommended methods of binding a biotinylated substance were used.

Figure 2.4: HiTrap™ Streptavidin HP 1ml Column



(http://www4.gelifesciences.com/)

All steps were performed in a cold room (4°C) and every sample loaded by "drop-to-drop" technique to avoid inclusion of air into the column. The binding buffer (20mM sodium phosphate, 0.15 M NaCl, pH 7.5) was prepared and passed through a 0.45µm filter (Pall Corporation, NY, USA) to remove potential contaminants such as bacterial cell wall fragments or salt crystals. The stopper at the top of the HiTrap™ column was removed and the 10ml of the buffer was loaded into the column using a syringe and "drop-to-drop" technique at a rate of approximately 1 ml/minute, which corresponds to approximately 30 drops/minute.

The biotinylated anti-hTERT antibody was obtained by custom biotinylation of a rabbit polyclonal anti-hTERT (0.2 mg/ml;3 ml in PBS, sc-7212; Santa Cruz, Autogen Bioclear UK Ltd., Wiltshire, UK). The antibody was introduced into the column at approximately 0.1 – 0.3 ml/minute. The resulting elute was collected into serial 0.5ml polypropylene tubes. A further 10 column volumes (10ml) of binding buffer was used

to wash the column and remove unbound antibody. The elute was similarly collected in 0.5ml polypropylene tubes. The column was washed with 20% (w/v) ethanol and stored at 4°C for later use. If stored in ethanol, the HiTrap^{TM} column was re-equilibrated with 10 column volumes (10ml) of Binding buffer at a rate of 1ml/ minute.

The cell lysate/protein samples were introduced using a syringe, again using drop-to-drop technique at 0.1 ml/minute (approximately 3 drops/ minute) in the cold room (4°C). The column was washed with a further 10 column volumes (10ml) of Binding buffer at 1 ml/minute. Finally, Elution buffer (8 M Guanidine - HCl, pH 1.5) was used to break the interaction between biotinylated anti-hTERT and hTERT. The eluate containing hTERT was collected in polypropylene tubes containing NaOH 10M to neutralise the pH 1.5 of the Elution buffer and protect the protein in the elute. The column was then discarded as the relatively harsh elution conditions will potentially have damaged the streptavidin ligand within the column.

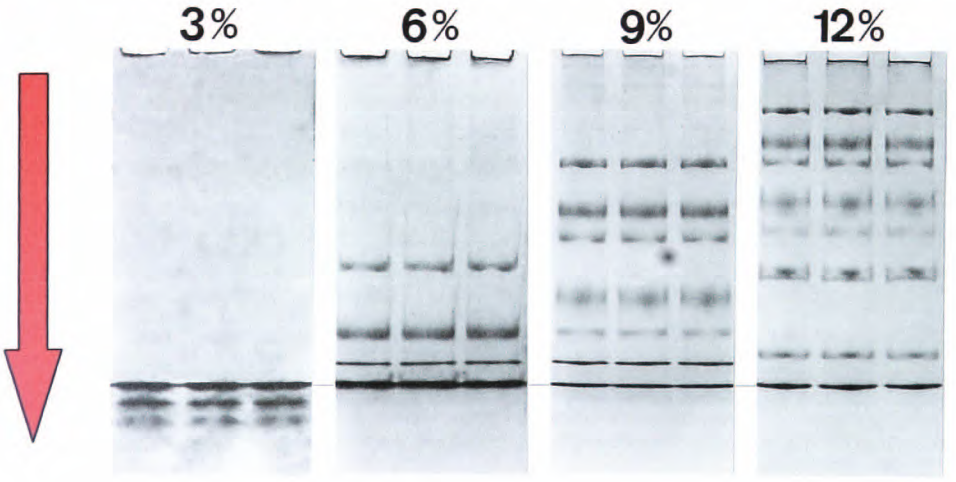
2.4.4 Principles of optimising Western Blot conditions

Western Blot (WB) was first described in 1981 to detect specific proteins within a sample (Burnette, 1981). The proteins undergo polyacrylamide gel electrophoresis (PAGE) where they are separated based on electrophoretic mobility, which is a function of charge and molecular weight i.e. the smallest molecules with highest charge move faster and furthest. These proteins are transferred onto a membrane which is probed with antibodies to detect the protein location. The protein molecular weight obtained by comparing to a pre-stained protein ladder

2.4.4.1 Acrylamide content

PAGE conditions such as acrylamide content, native vs. non-native or reducing vs. non-reducing conditions can be modified to obtain different information about the proteins loaded. The acrylamide content in separating gels can vary between 5% - 30% (w/v) which determines the gel pore size. Higher % acrylamide allows greater resolution of light weight proteins as these proteins are slowed down by the increasingly smaller pore sizes. The 12% gel (small pore size) slows the proteins thus enforcing resolution. Conversely, lower acrylamide (3%) leads to larger pore sizes where fast-moving proteins migrate and accumulate beyond the gel (**Figure 2.5**). Similarly heavy proteins require lower acrylamide content (large pores) as smaller pores would restrict migration leading to proteins accumulating at the start of the gel. The stacking gel has lower % acrylamide content than the separating gel so that the proteins concentrate or "stack" at the interface between the 2 gels prior to being resolved in the separating gel (Wittig and Schagger, 2005, Laemmli, 1970).

Figure 2.5: Effect of varying the percentage (%) acrylamide in separating gels.



Red arrow shows direction of protein migration (Hempelmann, 2003)

2.4.4.2 Native and Non-native conditions

Electrophoretic migration is dependant on the protein shape and charge:mass ratio. Proteins with different weights can still migrate anomalously into the same location due to compensatory charge and shape factors. Alternately, same molecular weights may migrate into different locations due to complex shape which slows its passage through the gel. This is reduced with Sodium Dodecyl Sulphate (SDS, $C_{12}H_{25}NaO_4S$, Mw 288.38), a detergent with two functions. Firstly, SDS denatures complex proteins into individual polypeptides; secondly, SDS applies a uniform charge per length of peptide, which renders the intrinsic charge of the protein negligible. In doing so, the protein's electrophoretic mobility is determined by its molecular weight, not its complex structure or intrinsic charge (Wittig and Schagger, 2005). SDS-PAGE is more commonly used than native PAGE. Native PAGE (without SDS) is still used when the composition and structure/function of bioactive proteins in its natural state is required e.g. metalloproteinases (Kastenholz, 2006).

2.4.4.3 Reducing and Non-reducing conditions

Complex proteins may be further broken down by boiling with reducing agents such as 2-β Mercaptoethanol (2β-ME, C_2H_6OH, Mw: 78.13) or dithithreitol (DTT, $C_4H_{10}O_2S_2$, Mw: 154.25). Reduction in this thesis was performed by boiling the protein at 99°C for 5 minutes with 10% (w/v) 2β-ME prior to loading into the stacking gel. This disrupts disulphide bridges of tertiary and quaternary proteins structures thus "reducing" complex proteins to monomeric chains. This process also ensures protein migration is based on molecular weight only.

2.4.5 Western Blot Protocol

A gel casting apparatus consisting glass plates, spacers, an assembly unit with securing screws and a water-tight base was used. The glass plates were washed in distilled H_2O and cleaned with ethanol and air-dried. The 2 glass plates were opposed with spacers in-between to create a square mould for the liquid polyacrylamide gel to polymerise. The plates were held vertically by the clamping screws with the bottom secured into the apparatus.

The separating gel was prepared (**Appendix 7.4**) in a chemical fume hood as acrylamide is potentially neurotoxic. APS and TEMED were added only when the gel-casting apparatus was ready as any delay leads to premature gel polymerisation within the flask. The liquid gel was pipetted into the space between the 2 glass plates leaving 2 cm from the top for the stacking gel. Water-saturated butanol was pipetted onto the liquid gel to reduce exposure to air as molecular oxygen inhibits polymerisation by reacting with the SO_4^- free radical.

The stacking gel (**Appendix 7.4**) was similarly prepared in the fume hood, again adding APS and TEMED last. The liquid stacking gel was pipetted and a plastic comb inserted into the square gel to mould the sample loading wells. This was left for 20 minutes. The comb was removed and the gels were ready for use.

The sample/ loading buffer were made (**Appendix 7.5**). Bromophenol blue (BPB, 3', 5', 5' 5' tetrabromophenolsulfonphtalein, $C_{19}H_{10}Br_4O_5S$, Mw 669.99) is a small negatively charged molecule used in the sample buffer as a coloured marker to migrate ahead of proteins which are usually colourless in the separation gel. This

allows visualisation of the advancing front in the gel and reduces the risk of proteins being run past the anode and lost. This also allows visualisation of anomalous directions of the protein separation which could be a result of overloaded wells or separating gel distortion.

The protein samples were mixed with the sample / loading buffer with 2β-ME (10%w/v, for non-native conditions) and boiled (99°C, 5minutes, for reducing conditions). The samples were loaded ensuring no more than 40 µl per well to avoid cross-contamination between wells. A protein ladder (10µl Benchmark™, Invitrogen) was loaded in a pre-determined well. The in-house gels were run at 200V for 60-90 minutes; precast gels were run at 100V for 45-60 minutes, or until the bromophenol blue front crossed the anode terminal.

The transfer buffer was prepared (**Appendix 7.5 & 7.6**) within 2 hours of use and stored at -20°C to reduce protein interaction/degradation when added to the separating gel. The 20% (w/v) methanol allowed the buffer to be kept at this temperature without freezing. Once separation was complete, the transfer buffer was poured into a tray with the gel.

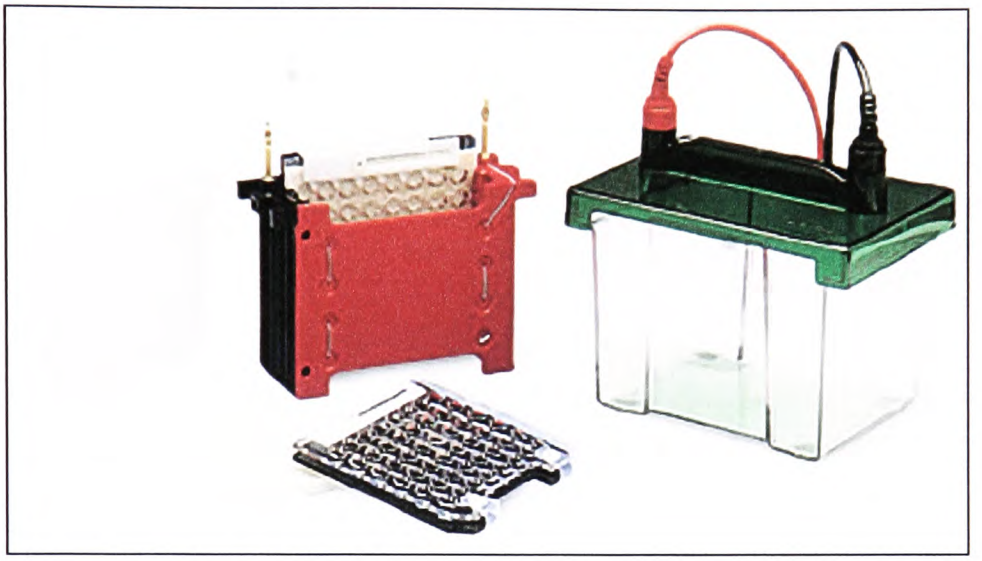
2.4.6 Protein transfer

Protein transfer was performed by electroblotting, where a voltage gradient attracts the anionic proteins away from the gel and into a suitable membrane, usually nitrocellulose or polyvinylidene fluoride (PVDF) membrane, which is performed in an electro-blotting apparatus (**Figure 2.6**). The "sandwich" cassette is assembled with the black plate down. The following materials are laid in order: 1 sponge pad, 2

filter papers, gel, nitrocellulose membrane, 2 filter papers, 1 sponge pad, and the clear plate of the cassette. This creates the "sandwich" where the filter paper and sponge pad on either side of the cassette compact the nitrocellulose membrane and gel together to allow good contact and facilitate direct protein transfer. The final order (**Figure 2.7**) is important as the anionic proteins travel toward the red anode. Hence, the separating gel must be on the side of the black cathode and the nitrocellulose membrane on the side of the red/clear anode. Failure to do se results in protein from the gel being transferred onto the filter paper, in the opposite direction to the nitrocellulose membrane.

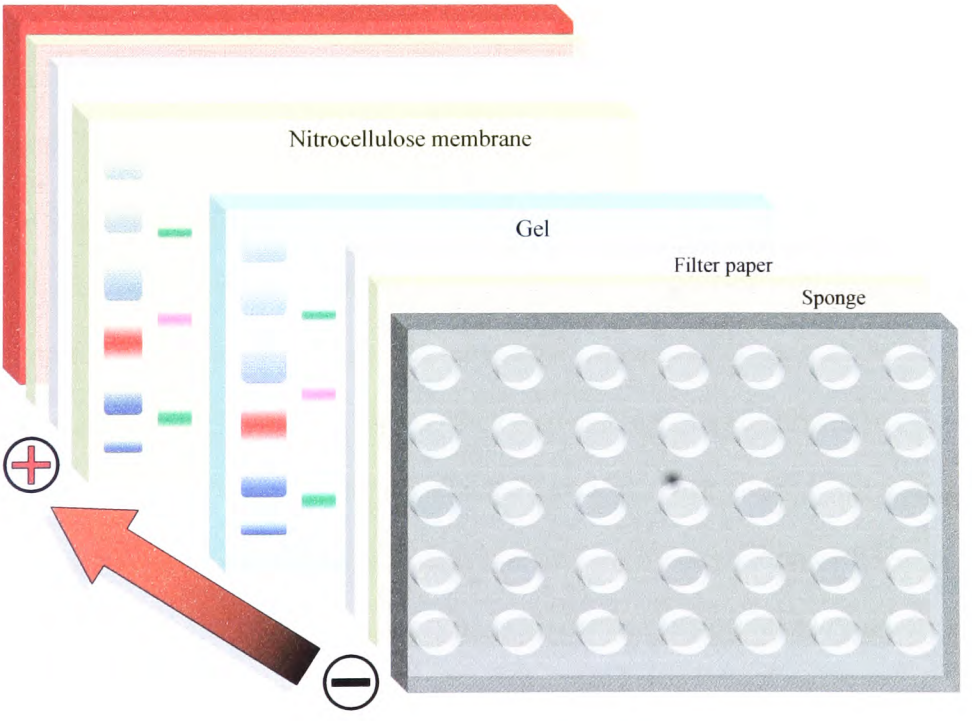
The "sandwich" cassette is placed in the tank, matching the anode and cathode terminals. A metallic stirrer is placed in placed at the bottom with an ice block between the 2 cassettes. The transfer is performed in a cold room (4°C) on a magnetic plate. The stirrer creates continuous buffer movement. Transfer was performed at either 100V for 1 hour at 4°C, or 30V overnight at 4°C. The ice block, cold transfer buffer and transfer in the cold room all aim to keep the temperature low during protein transfer process as the electrical conduction through the buffer could lead to increased temperature and protein degradation. Once the transfer was complete, the membrane was placed in a Petri dish with blocking buffer until ready for probing with primary, then secondary antibodies followed by ECL detection.

Figure 2.6: Electroblot protein transfer apparatus.



(www.plant.uoguelph.ca)

Figure 2.7: "Sandwich" cassette and direction of protein transfer



2.4.7 Blocking and Probing

The blocking buffer (**Appendix 7.5**) was used to reduce non-specific binding between the primary antibody and the membrane which background reading and increases band clarity. The following transfer/blocking schedules were used to maximise time usage:

a) Transfer 100V at 4C for 1 hour + block overnight at 4C

b) Transfer 30V at 4C overnight + block 1 hour at room temperature on shaker

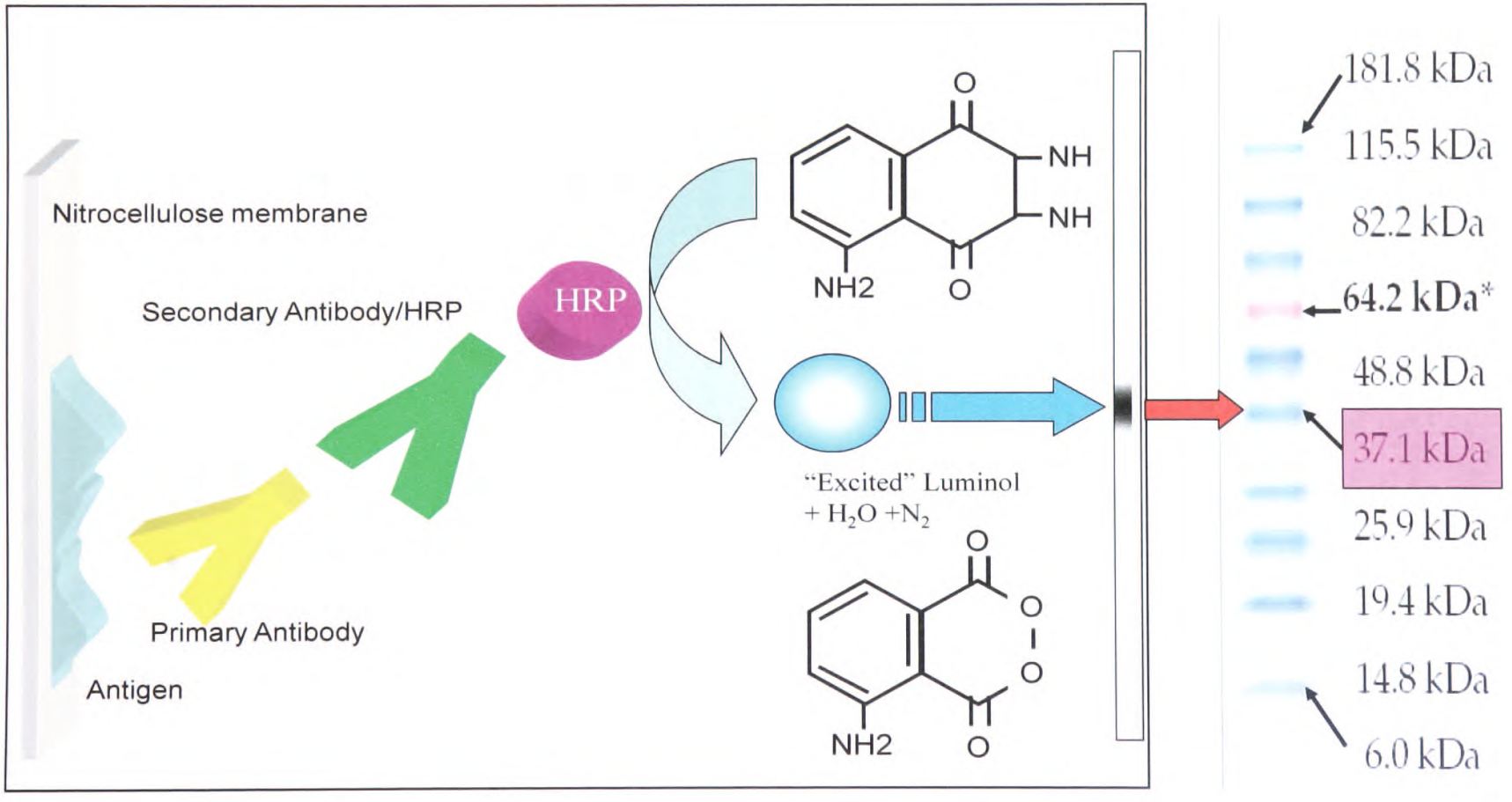
c) Transfer 100V at 4C for 1 hour + block 1 hour at room temperature on shaker. This was the least used schedule due to the length of time required to perform PAGE and Western Blot in the same day.

The membrane was removed from the blocking solution and placed in another Petri dish containing PBS-0.05% (v/v) Tween and washed for 3 cycles at 5 minutes per cycle. After each cycle, the PBS-Tween was emptied and replaced with fresh PBS-Tween. Once washed adequately, the membrane was incubated with a primary antibody in 1% Marvel in PBS and incubated for 1 hour at room temperature on a shaker. This was poured out and the membrane was washed again for 3 cycles for 5 minutes per cycle with PBS-Tween. The membrane was then incubated in a new Petri dish containing 1% Marvel in PBS with the secondary HRP-antibody for another hour on the shaker. The membrane was then similarly washed with fresh PBS-Tween for another 3 cycles, at 5 minutes per cycle. Once washed, the membrane was left in PBS to avoid dehydration while the detection reagents were prepared.

2.4.8 Enhanced Chemiluminescence (ECL)

The immobilised protein is indirectly bound to a secondary HRP-antibody which oxidises luminol to emit light which is captured on X–ray film (**Figure 2.8**) Light emission is magnified with "enhancing" agents, such as phenol in the Detection Solution. This technique is highly sensitive, being able to detect proteins concentrations of picogram (10^{-12}) / ml to femtogram (10^{-15}) / ml (Ornberg et al., 2005). The molecular weight of the protein is the determined by comparing its location to a pre-stained molecular weight ladder. ECL was performed in a dark room. An equal amount of Detection Reagent 1 and Detection Reagent 2 (Western Blotting Detection Reagents and Analysis System; Amersham, Biosciences UK) were mixed immediately before use and incubated with the membranes for 1 minute. The membrane was placed between 2 sheets of clear transparent film ensuring no air bubbles were trapped. The membrane was placed protein-side up, with an X–ray film on top and the cassette closed. The initial exposure time was 5 minutes but was varied (5 seconds to 60 minutes). Three trays were prepared - developer solution (Tray 1), acetic acid (Tray 2) and fixer solution (Tray 3). After 5 minutes, the X-ray film was removed and placed in Tray 1 until protein bands appeared (approximately 5 seconds to 5 minutes). The film was transferred to Tray 2 (30 seconds), and then rinsed in Tray 3 by continuous rocking (10 seconds to 1minute) until the background was transparent. This ensures the rinsing solution covers the film, and that the film does not adhere to the bottom of the tray which irreversibly imprints the film and obscures protein bands. The final molecular weight of the protein is determined by comparison with the membrane containing the pre-stained protein ladder.

Figure 2.8: Principles of ECL detection and comparison with protein ladder to determine molecular weight (e.g. 37.1 kDA)



3 Chapter 3: Anti-p53 auto-antibody in Cancer; Focus on Anti-p53 auto-antibody in Colorectal cancer

3.1 Introduction

The anti-p53 auto-antibody represents a humoral response to tumour associated antigens (TAA). This response is partly related to the underlying p53 mutation, but its long-term prognostic significance is unknown. The aim of this study is to investigate the incidence of anti-p53 auto-antibodies in all cancers in relation to p53 mutation rates, and then review the significance of anti-p53 auto-antibody in CRC.

3.2 Methodological Quality of Anti-53 auto-antibody studies

All published studies on anti-p53 auto-antibody (1979 – 2009) were retrospective or cross-sectional case control series. Sample sizes in individual studies were small with heterogeneous mix of different stage cancers (27-220 subjects tested, number of sero-positive patients 17-47). The largest single study was published by Tang et al with the largest sample size of 130 sero-positive patients in a cohort of 998 colorectal cancer patients (Tang et al., 2001). This included all stages thus leaving relatively small numbers of patients for stage-specific analysis. A non-systematic review had larger numbers but was more severely limited by heterogeneous population sample and different detection methods in individual studies (Soussi, 2000a). The primary outcome was not stated in most studies. All studies were retrospective case-comparisons and none were powered for survival outcomes.

3.3 Frequency of Anti-p53 auto-antibody in All Cancer

The reported frequency of anti-p53 auto-antibody in individual cancer studies vary significantly due to small sample sizes, stage bias (usually greater proportion of advanced stage tumours especially in earlier studies) and different detection methods used. In a landmark review, Soussi compiled results of 80 anti-p53 auto-antibody studies in 18 cancer types over a 20 year (1979 - 1999) period (Soussi, 2000a). The mean sero-positivity across all cancer types was 16.9% (1600 / 9489 patients, range 0-31%) compared with 1.45% (35 / 2404) in controls thus demonstrating remarkable specificity but poor sensitivity. Furthermore, half (17) the sero-positive control subjects were from a single study which reported an extra-ordinarily high control sero-positivity (24%, 17/70) which is in contrast to almost all other publications that consistently report <1% sero-positivity in healthy samples (Wollenberg et al., 1997). Taking this into account, anti-p53 auto-antibody specificity should be near 100% for malignancy. Anti-p53 auto-antibody is usually measured in patients' sera but has also been detected in ascitic fluid of patients with ovarian cancer (Abendstein et al., 2000, Angelopoulou et al., 1997), saliva of patients with oral cancer (Tavassoli et al., 1998) and in pleural effusions (12.5%) associated with lung, colon and pancreatic cancer (Munker et al., 1996). This thesis summarises results of all anti-p53 auto-antibody related publications in all cancers from 1999 - 2010, and combines this with results of Soussi's review (1979-1999) to provide the largest anti-p53 auto-antibody review to date, spanning 30 years (1979-2010) (**Table 3.1**). Salient conclusions of individual studies are also included.

Table 3.1: Cumulative reported frequencies of anti-p53 auto-antibody (anti-p53) in controls and individual cancers. Cancer types are listed in order of decreasing anti-p53 auto-antibody frequency. The reported studies within each cancer type are listed in reverse chronology.

Group	Author (Year)	Anti-p53 positive % (n)	Summary of Study and tumour type
Healthy/ Benign	(Suppiah et al., 2008)	0 (0/28)	Comparative study with colorectal carcinoma
	(Cai et al., 2008)	0 (0/30)	Comparative study with oesophageal carcinoma
	(Atta et al., 2008)	17.2 (5/29); 50 (13/26)[a]	Comparative study with hepatocellular carcinoma
	(Mattioni et al., 2007)	0 (0/64)	Comparative study with gastric carcinoma
	(Akere and Otegbayo, 2007)	8.9 (4/45)	Comparative study with hepatocellular carcinoma
	(Muller et al., 2006)	0 (0/57); 0 (0/379)[b]	Single study of anti-p53 in various cancers
	(Chang et al., 2005)	0 (0/40)	Comparative study with colorectal carcinoma
	(Fonseca et al., 2003)	0 (0/15)	Comparative study with glioma
	(Shimada et al., 2003)	6.3 (10/205)[a] ; 7 (13/189)[b]	Multi-institutional study of anti-p53 in various cancers
	(Neri et al., 2003)	0 (0/51)	Comparative study with lung carcinoma
	(Numa et al., 2001)	0 (0/9)	Comparative study with uterine, ovarian, cervical carcinoma
	(Mack et al., 2000)	2.2 (1/46)	Comparative study with SCLC
	(Chow et al., 2001)	3.6 (1/28)	Comparative study with head and neck carcinoma
	(Moch et al., 2001)	1.5 (2/130)	Comparative study with skin carcinoma (SCC/ BCC)
	(Hofele et al., 2002)	0 (0/80)	Comparative study with oral SCC
	(Hagiwara et al., 2000)	0 (0/13)	Comparative study with oesophageal carcinoma
	(Ralhan et al., 2000)	8 (4/50)	Comparative study with lung carcinoma
	(Bielicki et al., 1999)	0 (0/28)	Comparative study with colorectal carcinoma
	(Soussi, 2000a)	1.5 (35/2404)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**2.2 (88 / 3946)**	

Oesophageal	(Cai et al., 2008)	39.1 (18/46)	Correlates with advanced histological grade, stage, lymph node metastases and decreased tumour response following radiotherapy
	(Muller et al., 2006)	20 (10/50)	No correlation with stage or prognosis
	(Bergstrom et al., 2004)	73.8 (31/42)	No correlation with clinico-pathological parameters, tumour size or survival
	(Shimada et al., 2003)	29.9 (90/301)	Multi-institutional study of anti-p53 in various cancers
	(Kozlowski et al., 2001)	26.6 (20/75)	No correlation with stage, lymph node metastases or size.
	(Shimada et al., 2000)	40 (14/35)	Correlates with tumour p53 protein expression but not clinico-pathological parameters
	(Hagiwara et al., 2000)	28 (13/46)	Correlates with increased stage and tumour p53 protein expression but not prognosis
	(Ralhan et al., 2000)	60 (36/60)	Correlates with tumour p53 protein expression and missense mutations but not clinico-pathological parameters.
	(Soussi, 2000a)	31 (85/274)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**34.1 (317 / 929)**	
Head/Neck^	(Chow et al., 2001)	31 (23/75)	Correlates with nodal metastases but not prognosis
	(Shimada et al., 2003)	32.3 (10/31)	Multi-institutional study of anti-p53 in various cancers
	Total	**31.1 (33 / 106)**	
Oral	(Castelli et al., 2001)	18.7 (3/61); 69.2 (9/13)[c]	Serum anti-p53 is useful as a screening tool in pre-malignant lesions
	(Hofele et al., 2002)	18.6 (19/102)[d]; 50(12/24)[e]	Correlates with poor prognosis
	(Soussi, 2000a)	29.1 (309/1062)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**28.5 (343 / 1204)**	

Ovary	(Qiu et al., 2007)	39.1 (36/92)	Correlates with p53 expression, not clinico-pathological parameters
	(Shimada et al., 2003)	7.4 (2/27)	Multi-institutional study of anti-p53 in various cancers
	(Numa et al., 2001)	27 (8/30)	Correlates with p53 tumour expression and poor prognosis
	(Abendstein et al., 2000)	25 (28/113)	Correlation between serum and ascitic anti-p53. No correlation with stage
		19 (21/113)[f]	or grade. Anti-p53 in ascites associated with poor prognosis
	(Soussi, 2000a)	22 (140/635)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**23.9 (214 / 897)**	
Colorectal *(Detailed results in **Table 3.2**, **Table 3.7**)*	(Suppiah et al., 2008)	21.7 (20/92)	No correlation with stage or prognosis
	(Nozoe et al., 2007)	47.2 (17/36)	Correlates with advanced lymph node status and stage
	(Muller et al., 2006)	32 (63/197)[g]; 15.2 (7/46)[h]	No correlation with stage or prognosis
	(Chang et al., 2005)	28.1 (47/167)	p53 mutation, not anti-p53, correlates with poor prognosis
	(Lechpammer et al., 2004)	18.2 (40/220)	? Correlation with stage or prognosis in Dukes' A/B1 (Table 3.7)
	(Shimada et al., 2003)	23.9 (46/192)	Multi-institutional study of anti-p53 in various cancers
	(Forslund et al., 2001)	27.3 (24/88)	Correlates with p53 mutation
	(Tang et al., 2001)	13 (130/998)	Correlates with advanced lymph node involvement but not prognosis
			(Section 3.5.2.1: N - stage)
	(Broll et al., 2001)	15.4 (20/130)	No correlation with stage or prognosis
	(Takeda et al., 2001b) (Shiota	63 (17/27)	95% negative sero-conversion within 3 weeks post-surgery
	et al., 2000)	25.4 (18/71)	Correlates with advanced stage and poor prognosis
	(Bielicki et al., 1999)	20.7 (30/145)	? Correlation with Dukes'A →B
	(Soussi, 2000a)	24.7 (307/1244)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**21.5 (786 / 3653)**	

HCC	(Atta et al., 2008)	68.3 (28/41)	Correlates with advanced stage and shorter survival.
	(Akere and Otegbayo, 2007)	12.2 (5/41)	Correlates with increased Okuda stage
	(Muller et al., 2006)	23.8 (19/80)	Non-significant trend towards poor prognosis
	(Charuruks et al., 2001)	18.4 (26/141)	Correlates with stage but not tumour p53 protein expression
	(Tangkijvanich et al., 2000a)[a]	13.2 (16/121)	Preliminary report of Charuruks et al (2001). No correlation with severity, stage or prognosis. Survival too short for survival analysis (3 vs. 4 months)
	(Sitruk et al., 2000)	12 (19/159)	Correlates with multinodular, infiltrative tumour but not survival
	(Soussi, 2000a)	1.2 (82/387)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**20.1 (195 / 970)**	
Bladder	(Muller et al., 2006)	12.5 (3/24)	No correlation with prognosis
	(Watanabe et al., 2005)	27 (17/63)[i]	Correlates with higher grade, stage, lymph node metastases and tumour p53 protein expression, but not prognosis
	(Gumus et al., 2004b)	17.5 (14/80)	Correlates with tumour p53 protein expression and poor prognosis.
	(Gumus et al., 2004a)	33 (25/76)	Negative sero-conversion post-treatment (35%, 8/23) associated with good prognosis.
	(Shimada et al., 2003)	12.1 (4/33)	Multi-institutional study of anti-p53 in various cancers
	(Morita et al., 2000)	12 (12/100)	Correlates with stage, and p53 protein expression but not prognosis
	(Wunderlich et al., 2000)	12.5 (4/32)	Correlates with tumour protein p53 expression but not stage.
	(Soussi, 2000a)	27.6 (8/29)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**20.1 (88 / 437)**	

Lung	(Bergqvist et al., 2004)	16.6 (14/84)	No correlation with tumour volume. Correlates with survival in adenocarcinoma, but not SCC
	(Bergqvist et al., 2003)	20.7 (12/58)	No correlation with tumour volume or lymph node metastases
	(Neri et al., 2003)	6.7 (2/30)[j]; 16.7(8/48)[k]	No correlation with stage, histology or prognosis. Non-significant increased survival in LC but not MM
	(Cioffi et al., 2001)	32.1 (35/109)	Low sensitivity, but high specificity (100%) and accuracy (69%). Only 14% agreement with other tumour markers (CEA/TPA, CYFRA21-1, NSE.)
	(Zalcman et al., 2000)	20.6 (20/97)	Correlates with poor prognosis in limited stage SCLC, but not all SCLC
	(Mack et al., 2000)	11.1(4/35)[l]; NSCLC 13.3 (13/99)[m]	Correlates with stage and prognosis in NSCLC but not SCLC
	(Shimada et al., 2003)	14.4 (18/125)	Multi-institutional study of anti-p53 in various cancers
	(Soussi, 2000a)	17.1 (219/1282)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**17.5 (345 / 1967)**	
Uterus	(Numa et al., 2001)	12 (5/41)	No correlation with tumour p53 expression/prognosis (see Cervix, Ovary)
	(Shimada et al., 2003)	22.7 (5/22)	Multi-institutional study of anti-p53 in various cancers
	Total	**15.9 (10 / 63)**	
Cervix	(Numa et al., 2001)	14 (12/86)	No correlation with tumour p53 protein expression or prognosis
	(Shimada et al., 2003)	18.9 (10/53)	Multi-institutional study of anti-p53 in various cancers
	Total	**15.8 (22 / 139)**	

Gastric	(Qiu et al., 2007)	31.1 (19/61)	Correlates with tumour size but not prognosis.
	(Mattioni et al., 2007)	15.3 (17/111)	Correlates with tumour p53 protein expression, prognosis and survival
	(Lawniczak et al., 2007)	22.5 (16/71)	Correlates with tumour type and age, but not stage or prognosis
	(Muller et al., 2006)	11.5 (14/122)	No correlation with prognosis
	(Shimada et al., 2003)	10.6 (13/123)	Multi-institutional study of anti-p53 in various cancers
	(Nakajima et al., 1999)	16 (13/81)	Correlates with lymph node metastases but not stage or prognosis
	(Maehara et al., 1999)	19.2 (23/120)	Correlates with increased stage and tumour p53 protein expression but not prognosis
	Soussi et al (2000)	14.1 (105/727)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**15.5 (220 / 1416)**	
Breast	(Muller et al., 2006)	34 (17/50)	Non-significant trend towards poor prognosis
	(Gao et al., 2005)	21.5 (31/144)	Correlates with stage, lymph node metastases, ER negative, c-erb-2 and tumour p53 protein expression
	(Shimada et al., 2003)	18.3 (13/71)	Multi-institutional study of anti-p53 in various cancers
	(Volkmann et al., 2002)	10.9 (18/165)	Poor concordance between recombinant/native p53 ELISA, immunoblot and immunofluoresence
	(Metcalfe et al., 2000)	15.4 (155/1006)	No correlation with stage and prognosis
	(Soussi, 2000a)	14.8 (296/2006)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**15.4 (530 / 3442)**	

Pancreas	(Muller et al., 2006)	22.7 (5/22)	Increase sensitivity in conjunction with CA19-9. No correlation with prognosis.
	(Shimada et al., 2003)	10.7 (3/28)	Multi-institutional study of anti-p53 in various cancers
	(Ohshio et al., 2002)	23.2 (19/82)	No correlation with tumour p53 expression or prognosis
	(Soussi, 2000a)	9.2 (60/650)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**11.1 (87 / 782)**	
Biliary tract^	(Limpaiboon et al., 2006)	12.2 (6/49)	Correlates with tumour p53 protein expression but not stage
	(Shimada et al., 2003)	16.7 (1/6)	Multi-institutional study of anti-p53 in various cancers
	(Tangkijvanich et al., 2000b)	7.3 (6/82)	Correlates with tumour p53 mutation
	Total	**9.5 (13 / 137)**	
Lymphoma	(Soussi, 2000a)	**7.7 (19 / 248)**	Literature review of anti-p53 in various cancers (1979-1999)
Glioma	(Fonseca et al., 2003)	20.8 (5/24)	No correlation with p53 protein but increased in patients<16 years
	(Shimada et al., 2003)	6.5 (2/31)	Multi-institutional study of anti-p53 in various cancers
	(Soussi, 2000a)	4.2 (6/144)	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**6.5 (13 / 199)**	
Haematological	(Shimada et al., 2003)	6.3 (32/364)[n]	Multi-institutional study of anti-p53 in various cancers
	(Soussi, 2000a)	3.3 (14/428)[o]	Literature review of anti-p53 in various cancers (1979-1999)
	Total	**5.8 (46 / 792)**	

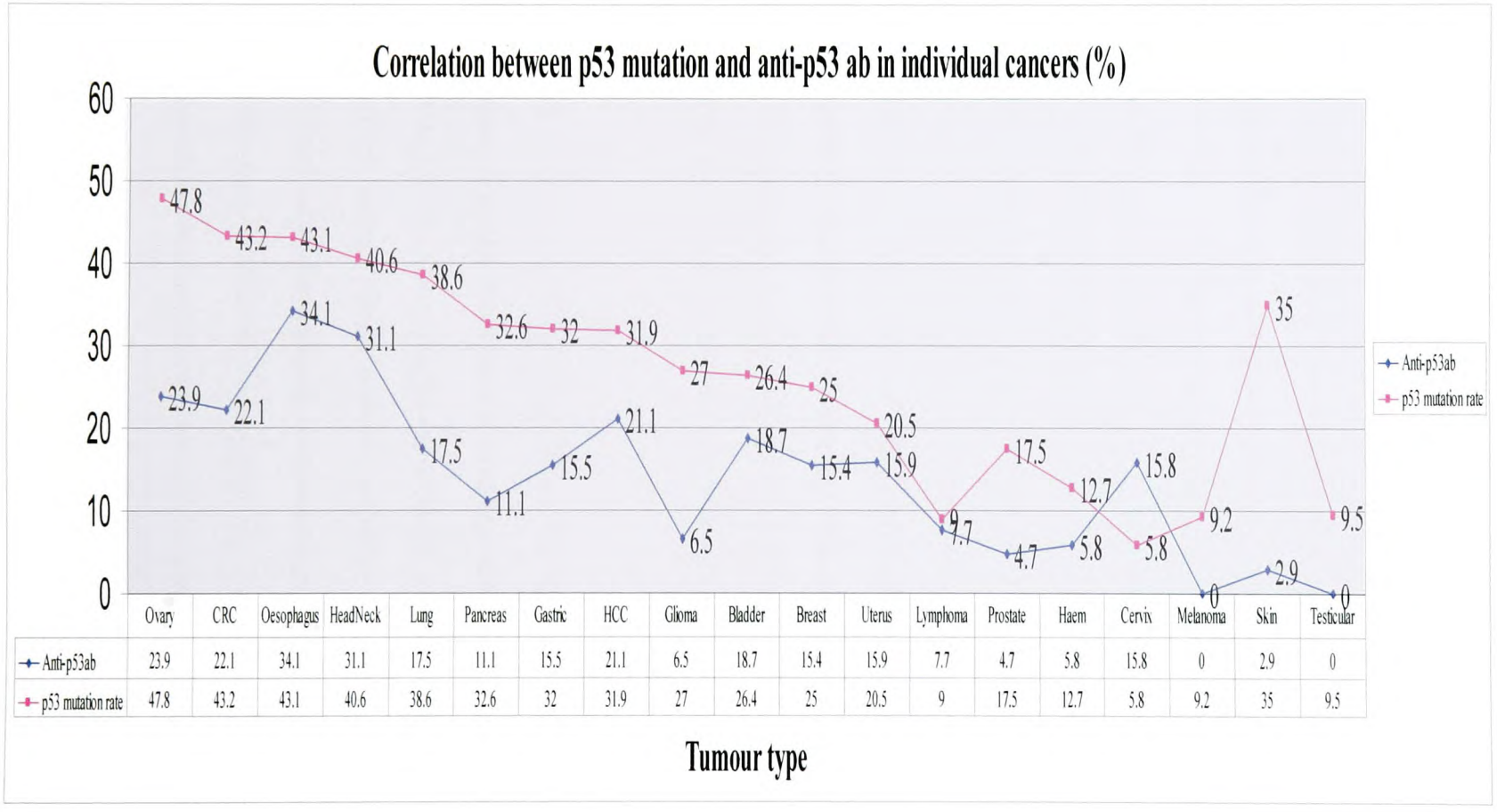
Prostate	(Shimada et al., 2003) (Soussi, 2000a) **Total**	17.4 (4/23) 2.7 (4/148) **4.7 (8 / 171)**	Multi-institutional study of anti-p53 in various cancers Literature review of anti-p53 in various cancers (1979-1999)
Skin	(Moch et al., 2001)	**2.9 (3 / 105)**	No difference between controls and patients. Increased in aggressive SCC (8%) vs. slow-growing BCC (1.5%)
Testicular	(Soussi, 2000a)	**0 (0 / 144)**	Literature review of anti-p53 in various cancers (1979-1999)
Melanoma	(Soussi, 2000a)	**0 (0 / 58)**	Literature review of anti-p53 in various cancers (1979-1999)
Total		**18.4 (3292 / 17859)**	All cancers (1979-2009)

Squamous cell carcinoma (SCC); basal cell carcinoma (BCC), hepatocellular carcinoma (HCC), carcino-embryonic antigen (CEA). Tissue polypeptide antigen (TPA), CYFRA21-1, Neurone-specific enolase (NSE), Oestrogen receptor (ER), c-erb-2. [a] Cirrhosis; [b] benign disease; [c] oral pre-malignant lesions-excluded from calculation; [d] primary carcinoma; [e] secondary/recurrent carcinoma; [f] ascitic titre, not included in calculation of serum titres; [g] colon; [h] rectum; [I] upper renal tract tumours, excluded from anti-p53 titres in bladder carcinoma; [j] malignant mesothelioma (MM); [k] lung carcinoma (LC); [l] small cell lung carcinoma (SCLC); [m] non-small cell lung carcinoma (NSCLC); [n] myeloma; [o] leukaemia; ^ tumour type not specified. [α] excluded as is preliminary report of the same cohort (duplicate) reported in Charuruks et al., 2001.

3.4 Anti-p53 auto-antibody and p53 mutation in Cancer

The International Agency for Research on Cancer (IARC) TP53 Mutation Database compiles information on p53 mutations in various cancers reported in all published literature and from other databases. This is the most authoritative report on p53 mutation rate in various cancers (IARC, 2008). The 30-year cumulative anti-p53 auto-antibody frequencies calculated in this thesis was plotted against the p53 mutation rate reported by the IARC to ascertain the relationship between p53 mutation and anti-p53 auto-antibodies (**Figure 3.1**). The graph shows partial correlation (R^2: 0.49, correlation 0.699) between auto-antibody and p53 mutation. Cancers with the highest p53 mutation rate such as lung (39%), colorectal (43%) and oesophageal (43%) also demonstrate highest anti-p53 auto-antibody rates of 18%, 22% and 34% respectively (Soussi, 2000a, Shimada et al., 2003). Conversely, hepatoma, melanoma and testicular carcinoma with the lowest mutation rate also have the lowest anti-p53 auto-antibody rates (<1%) (Soussi, 2000a). The two obvious exceptions are gliomas and skin cancers. Proposed reasons for this are poor brain antigenicity, poor antigen-presentation across the blood-brain barrier, and use of immuno-suppressive steroids (dexamethasone) in the majority of gliomas (Soussi, 2000a, Weller et al., 1998). Similar arguments about antigen presentation and auto-antibody production are made for skin cancer (Moch et al., 2001). Only one study on 5 sero-positive patients with glioma has been published since and is unable to provide further information (Fonseca et al., 2003). In summary, anti-p53 auto-antibody has average (35%) to poor (1%) sensitivity depending on cancer type but is 95 - 100% specificity for any malignancy which may provide clinical relevance. This is critically evaluated in this thesis.

Figure 3.1: p53 mutations (IARC,2008) and anti-p53 autoantibody incidence (Suppiah, 2010) in individual cancer types. (R^2 0.49, Correlation 0.699)



	Ovary	CRC	Oesophagus	HeadNeck	Lung	Pancreas	Gastric	HCC	Glioma	Bladder	Breast	Uterus	Lymphoma	Prostate	Haem	Cervix	Melanoma	Skin	Testicular
Anti-p53ab	23.9	22.1	34.1	31.1	17.5	11.1	15.5	21.1	6.5	18.7	15.4	15.9	7.7	4.7	5.8	15.8	0	2.9	0
p53 mutation rate	47.8	43.2	43.1	40.6	38.6	32.6	32	31.9	27	26.4	25	20.5	9	17.5	12.7	5.8	9.2	35	9.5

3.5 Anti-p53 Auto-antibody In Colorectal Cancer

CRC has among the highest anti-p53 auto-antibody sero-positivity rates due to high frequency of p53 mutation. The variables rates reported pre-1999 report (**Table 3.1**) are probably due to lack of methodology standardisation; 8 "in-house" ELISA, 1 WB, 1 immuno-precipitation, 1 using all 3 methods (Soussi, 2000a). Ten of these 11 studies report a consistent sero-positivity rate between 12.5% and 32%. The remaining study, which was also the only study to use WB only, reported a much higher 68% sero-positivity thus demonstrating the importance of the detection method (Shibata et al., 1996).

Commercial ELISA kits have been developed since and results of ELISA-only studies are shown (**Table 3.2**). Mean sero-positivity is 19.9% (479/2409) with individual studies reporting between 13% - 27%, except two studies which report inconsistently high rates of 47% and 63% (Nozoe et al., 2007, Takeda et al., 2001b). Both studies used the same ELISA (Pharmacell, France) and the high sero-positivity (2.6%, 1/38) in controls suggest a lower cut-off value for auto-antibody detection may have been used (Takeda et al., 2001b). The same authors, using the same ELISA, also reported an unusually high sero-positivity of 40% (14/35) in superficial oesophageal carcinoma compared with the other reports of 20 - 30% (Shimada et al., 2000). Interestingly, a later study by the same authors, but using a different ELISA kit (anti-p53 EIA Kit II, MESACUP), in a similar Japanese cohort reported a more consistent sero-positivity of 24% in CRC and 30% in oesophageal cancer, again highlighting problems with quantification, even with commercially available ELISA (Shimada et al., 2003).

Table 3.2: Summary of all ELISA studies on anti-p53 auto-antibody (anti-p53) in colorectal cancer (CRC).

Author (Year)	Method and manufacturer	Samples, % (n)	Follow-up	Key findings
(Suppiah et al., 2008)	ELISA (p53 ELISA^{PLUS}, Calbiochem, Darmstadt, Germany)	21.7 (20/92); 0 (0/20)[a] 0 (0/8)[b]	Median 97 months	No correlation with tumour stage, differentiation or location. Multivariate analysis show only Stage (Dukes' and TNM) to be independent prognostic factors
(Nozoe et al., 2007)	ELISA (Pharmacell, France)	47.2 (17/36)	Not stated	Antip53-ab (+) associated with greater lymphatic invasion (94.1%; 16/17 vs. 68.4%; 13/19), nodal involvement (70/6%; 12/17 vs. 17.6%; 3/17) and advanced stage (p=0.02). Anti-p53 frequency higher in p53 protein expressing tumours (74%; 14/19 vs. 18%; 3/17). Only 3 patients with Dukes' A CRC, all sero-negative
(Muller et al., 2006)	Immunoblot	Colon 32 (63/197); Rectum 15.2 (7/46); 0 (0/57)[a] 0 (0/379)[b]	CRC patients enrolled into trial with 5 year follow-up	No correlation with clinico-pathological parameters or prognosis. Trend toward higher anti-p53 sero-positivity in N2/3 disease, poor differentiation and metastases. There were no patients with Dukes' A in this study. Anti-p53 independent of CEA and CA19-9 with 16% information gain. This is the only study to report negative to positive sero-conversion (3.6%, 11/303)

(Chang et al., 2005)	ELISA (p53-AK, Dianova, Hamburg, Germany)	28.1 47/167); 0 (0/40)[a]	Median 36.3 months (4-58)	Anti-p53 correlates with p53 mutation (43% vs. 18%) but not tumour p53 expression, clinico-pathological features or prognosis. p53 mutations, advanced stage and pre-operative CEA>5ng/ml were independent prognostic factors (in that order). p53 mutation strongly associated with advanced stage and poor differentiation
(Lechpammer et al., 2004)	ELISA (ELISAPLUS Oncogene Research Products, Cambridge, USA)	18.2 (40/220); 0 (0/42)[a]	40 patients up to 20 weeks; 8 patients up to 48 weeks	Anti-p53 had higher tumour p53 expression (70% vs. 52%). Anti-p53 frequency shows highest increase in Dukes' A (0%, 0/28) →Dukes' B: (24%, 21/87) but no increase in progression to Dukes' C (18%,19/105). No correlation with overall tumour grade or metastases. Anti-p53 reflects tumour load following surgery, during chemotherapy and with disease recurrence.(**Table 3.6**)
(Shimada et al., 2003)	ELISA (Anti-p53 EIA Kit II, MESACUP anti-p53 Test; MBL; Nagoya, Japan)	23.9 (46/192); 4.9 (10/205)[a]; 6.9 (13/189)[b]	Not reported	Validation study for MESACUP ELISA using prevalence of anti-p53 in various cancers. Good intra- and inter-assay coefficient of variation of 1.85-2.37% and 0.3-3.2% respectively. Demonstrates stability of anti-p53 titres at room temperature for 7 days and following 10 freeze-thaw cycles. No comment on correlation with clinico-pathological parameters or prognosis.
(Forslund et al., 2001)	ELISA (Dianova, Hamburg, Germany)	27 (24/88)	Not reported	Cross-sectional study on relationship between p53 mutations and anti-p53 presence. Frequency of p53 mutation higher in anti-p53 sero-positive group (92%, 22/64 vs. 34%, 22/64) Correlation with clinico-pathological and survival parameters not reported

(Tang et al., 2001)	ELISA (Calbiochem-Novabiochem, Darmstadt, Germany)	13 (130/998); 1 (2/211)[c]	Not reported	Anti-p53 sero-positivity increases in progression from N2→N3 (2.9% to 10.6%); but not N0→N1 (11.7% to 12.3%), N1→N2 (12.3% to 10.6%) or M0→M1 (12% to 17%). No correlation with CEA, overall TNM stage or metastases. Anti-p53 associated with shorter survival in uni- but not multi-variate analysis. Largest study on anti-p53 in CRC.
(Broll et al., 2001)	ELISA (P53-autoantikorpfer ELISA, Dianova, Hamburg, Germany)	15 (20/130); 0 (0/44)[a]	Median 25.5	Anti-p53 positive predictive value of 100%, but accuracy 37% and negative predictive value 29% due to poor sensitivity (15%). Anti-p53 correlated with p53 expression ($p<0.05$), but not TNM stage, grade or location (exact numbers not shown). Approximately 70% of series Stage I/II CRC
(Takeda et al., 2001b)	Anti-p53 EIA (PharmaCell, Paris, France)	63 (17/27); 2.6 (1/38)[c]	Up to 2 years Median not reported	Anti-p53 correlates with p53 protein expression and independent of CEA and CA-19-9. Sero-conversion in 94% (16/17) within 3 weeks of endoscopic resection. No correlation with clinico-pathological parameters or prognosis/recurrence as all patients had early superficial CRC (23 mucosal, 4 submucosal invasion). This study reports exceptionally high anti-p53, especially considering very early CRC
(Takeda et al., 2001a)	ELISA (Anti-p53-EIA kit, Pharmacell, Paris, France)	40 patients with anti-p53 ab from previous studies	Up to 29 months	No correlation between post-operative anti-p53 sero-positivity and histological (depth, lymphatic or venous invasion) or clinico-pathological features of lymph node or liver metastases. High (96%; 27/28) sero-conversion in patients with complete tumour resection. No sero-conversion in patients with residual disease.

(Shiota et al., 2000) (2000)	ELISA (GIF, Munster, Germany)	25 (18/71); 6 (1/18)[c]	Not stated, median survival 56 months antip53 ab negative	Anti-p53 correlates with TNM stage (Stage I-IIIb: 9%, 4/45 vs. IV: 56%, 14/25), Dukes' stage (A-C: 9%, 4/45 vs. D: 56%, 14/25), CEA, CA19-9 and tumour p53 protein expression. Anti-p53 associated with shorted survival (56 months vs. 20 months) and is weak poor prognostic indicator. Anti-p53 prognostic significance secondary to other factors, including weak factors e.g. CEA and CA19-9. Only small number of Stage I-IIIb patients.
(Bielicki et al., 1999)	ELISA (Dianova, Hamburg, Germany)	21 (30/145); 0 (0/20)[b]; 0 (0/8)[c]	Not stated. Cross sectional study	No correlation with Dukes' Stage (A/B: 22%, 16/73 vs. C/D 19% 14/72), size, location, CEA. Highest increase in anti-p53 frequency from Dukes' A (0%, 0/6) to Dukes B1 (28%, 5/18) but no further difference in progression to Dukes' C (19%, 7/36). Only 6 Dukes' A patients in study, all sero-negative
(Soussi, 2000a)	ELISA/WB/IP	24.7 (307/1244)	ELISA/WB/IP	Review combining all studies with different methodologies from 1979-1999. Range of sero-positivity (12.5-68% in 11 studies)
Total (1999 – 2009)		**19.9 (479/2409)**		All modern studies (1999 onwards) using commercial ELISA only, with one exception using Immunoblot (Muller et al, 2006)
Thesis Total (1979 – 2009)		**21.5 (786/3653)**		All studies on anti-p53 in CRC (1979-2009)

Studies prior to 1999 used individual different methodology and not included (see below). Enzyme-linked immunosorbency assay (ELISA); Western Blot (WB); Immunoprecipitation (IP) [a] healthy, [b] benign disease, [c] adenoma. The study by Muller et al (2006) was included despite suing immunoblot technique as it was a recent study with relatively large sample size, and also reported the highest sero-conversion rates (**Section 3.5.4).**

3.5.1 Anti-p53 Auto-antibody in Diagnosis and Screening

Cancer screening tools are used where early detection and intervention leads to improved outcome, such as CRC where Dukes' A CRC has 95-100% 5-year cancer specific survival compared with 5% in Dukes' D. The ideal screening tool is 100% sensitive and specific, cost-effective, safe and acceptable but no such test exists. Colonoscopy is the current gold-standard diagnostic tool but is unsuitable for general population screening as it is painful, expensive and is associated with life-threatening complications such as colonic perforation (0.03 - 0.3%) and haemorrhage (0.6%) (Nelson et al., 2002, Lieberman et al., 2000). The potential benefits on serum anti-p53 auto-antibody testing are that it serum assays are not subject to tumour sampling errors and are quicker, cheaper, easier and far less traumatic, thus making it more acceptable to the general population and hence more repeatable. The auto-antibody itself is remarkably stable, showing no significant changes in titres when stored at room temperature for up to 7 days, or at stored at -80°C for 3 years (Angelopoulou et al., 1997). Furthermore, repeated freeze-thaw cycles (up to 10 cycles) have minimal or no effect on serum levels as the immunoglobulins are generally very robust (Shimada et al., 2003). Results are reproducible using commercially available ELISA with reported intra- and inter-assay coefficient of variation of 1.85-2.37% and 0.3-3.32% respectively (MESACUP anti-p53 Test; Medical and Biological Laboratories, MBL, Nagoya, Japan) (Shimada et al., 2003). Finally, anti-p53 auto-antibody appears to be independent of other conventional CRC tumour markers such as CEA which means it could detect CRC in CEA negative patients (**Section 3.5.3**). Despite these apparent benefits of serum testing and the auto-antibody properties, the low sensitivity

and lack of specificity for CRC makes it unsuitable for diagnosis in general population. Selective screening in high-risk groups (e.g. IBD) instead may increase utility of anti-53 auto-antibody, which is discussed next.

p53 mutation has been reported to occur earlier, and APC/kRAS mutations are not as consistent as in sporadic CRC suggesting different mechanism carcinogenesis in UC-CRC. (Rosman-Urbach et al., 2004, Brentnall et al., 1994). As p53 mutations occur earlier and are partly induce anti-p53 production, 2 studies have investigated anti-p53 auto-antibody in UC-CRC. The first study reported anti-p53 in sporadic CRC (52%, 43/82), UC (15%, 43/286) (Yoshizawa et al., 2007). The 52% anti-p53 positivity in sporadic CRC is higher than other reported studies and already suggests an over-estimation or a lower cut-off point used in the method. Thus, the 15% anti-p53 in UC would also likely be an over-estimation (as the same ELISA was used) suggesting even lower true sensitivity in all UC, regardless of presence of dysplasia or CRC. Next, only 13 patients with UC had CRC / dysplasia. Only 5 of the 13 were anti-p53 positive giving a false negative rate of 62% (8/13) for dysplasia/CRC. The positive predictive value was 14% as only 6 of the 43 anti-p53 positive-UC patients had dysplasia/CRC. None of the remaining anti-p53 positive UC patients (n=37) went on to develop CRC detected by colonoscopy or barium enema at the time of publication - minimum 2 year follow-up, with the patient cohort ranging from 2002-2005 and report published in 2007). The second study by Cioffi et al emphasised the benefits of serum testing but the results similarly showed only 9.3% (9/97) anti-p53 positivity in UC (Cioffi et al., 2004) again demonstrating a sensitivity which is far too low for clinical use.

The anti-p53 auto-antibody has not been investigated in other high-risk groups such as inherited familial syndromes but can be expected to have even less sensitivity as majority of the familial syndromes are thought via MMR defects, rather than the CIN mechanisms of the adenoma-carcinoma sequence in sporadic CRC, and hence less likely to possess p53 mutation and mount an auto-antibody response.

In conclusion, whilst serum testing using an appropriate antibody marker is appealing, the anti-p53 auto-antibody has too low sensitivity, specificity, positive and negative predictive, and hence is of no value in screening for CRC in general or high-risk populations.

3.5.2 Anti-p53 Auto-Antibody and Clinico-pathological Parameters of CRC

p53 mutation occurs in the final stages of the adenoma-carcinoma sequence. Hence, anti-p53 auto-antibody production is unlikely to occur in early pre-invasive lesions where p53 mutations have not yet manifested (Fearon and Vogelstein, 1990). This is supported by the largest study which reports only 1% (2/211) sero-positivity in adenomas. Furthermore, microfoci of invasive cancer within these adenomas cannot be excluded, and could account for this sero-positivity (Tang et al., 2001). Sero-positivity rates increased modestly to 6% in carcinoma-in-situ, which would be expected of tumours at the end of the adenoma-carcinoma sequence and hence, greater proportion of p53 mutation. This is consistent with postulation that frequency of anti-p53 auto-antibody is partly related to that of p53 mutation.

Correspondingly, sero-positivity rates should then increase further with greater tumour progression (e.g. Dukes A to Dukes' D) but only two studies reported 0%

(0 / 28) sero-positivity in Dukes A which increased to 9.6% (21 / 87) in Dukes' B, which was similar to Dukes' C sero-positivity of 8.6% (19 / 105) (Lechpammer et al., 2004) and Bielicki et al reported 0% sero-positivity in Dukes' A which increased to 28% Dukes' B1 and 22% Dukes' B2, which was again similar to sero-positivity in Dukes C (19%) (Bielicki et al., 1999). Both studies are based on absence of anti-p53 auto-antibody in a small group of early CRC patients. A much larger study using 998 patients reported no difference in progression from Dukes' A to B or with any stage progression, which was also confirmed by the majority of publications. This suggests that anti-p53 auto-antibody production is stimulated early in post-dysplasia (progression from carcinoma-in-situ→ Dukes' A) which is consistent with p53 mutation as a late event in malignant transformation. The humoral response is not induced by T-stage invasion beyond this (e.g. T2→T4).

3.5.2.1 N - stage

The majority of studies report no difference in sero-positivity rates between Dukes' B and C suggesting nodal involvement does not affect anti-p53 auto-antibody presence. However, the largest study reported increased anti-p53 auto-antibody frequency in N0-N2 *vs.* N3 disease (N1: 1 - 3 pericolic nodes; N2: 4 or more regional nodes; N3: 10 or more regional nodes or systemic nodal metastases) (Tang et al., 2001). No significant differences in sero-positivity were observed between node negative N0 (11.7%), early N1 (12.3%) or N2 (10.6%) disease (Tang et al., 2001).The difference in sero-positivity between N0-2 and N3 could be due to the dilution effect of the large number of sero-negative patients with N0 disease (n = 407 / 506; 88%) in the combined N0-2 group. Secondly, the increased auto-antibody frequency was

observed only in the "high" antibody titre group which was defined as patients with serum titres above that of the highest titre in the 611 controls subjects, which was 76U. However, up to 2% control subjects will possess the anti-p53 auto-antibody as calculated in this thesis using data from historical studies (**Table 3.1**). As a result, this "high" antibody group is a biased selected patient population who possess the highest anti-p53 auto-antibody titres - but does not include all sero-positive patients. In fact, the majority of sero-positive patients were excluded in this analysis (**Table 3.3**). The "low" group which constitutes the majority of anti-p53 positive patients in this study actually demonstrate no significant increase in anti-p53 auto-antibody with nodal involvement.

Table 3.3: Anti-p53 auto-antibody sero-positive rates (%) in each N-stage

N stage (n)	Negative (<10 U)	Low (10 – 76U)	High (> 76U)
N 0 (506)	88 %	9 %	2 %
N 1 (252)	88 %	8 %	5 %
N 2 (132)	89 %	7 %	4 %
N 3 (70)	77 %	7 %	16 %

Data from (Tang et al., 2001). Red box indicates highly select group used for analysis of N-stage. Yellow column indicates patients selectively excluded from analysis

The data from Tang et al was re-examined to obtain a more valid conclusion. Firstly the "low" and "high" groups were combined into a "positive" group. This avoided exclusion of the large number of sero-positive patients (yellow column in **Table 3.3**). Secondly, the nodal involvement was collapsed into node "negative" and node "positive" group which is more clinically relevant than individual N1/2/3 stages. The

resulting comparison showed no difference between anti-p53 auto-antibody and N-stage (**Table 3.4**). Statistical significance could not be calculated as the sample numbers were calculated from % sero-positivity reported in the original publication.

Table 3.4: Reclassification of nodal stage and anti-p53 auto-antibody status

	Anti-p53 Negative % (n)	Anti-p53 Positive % (n)
Node negative (506)	88 % (447)	12 % (59)
Node positive (454)	87 % (393)	14% (61)

(Tang et al., 2001). Exact patient numbers calculated from % reported

In summary, despite the reported increase in anti-p53 auto-antibody frequency observed with N3 disease in this study, there is no correlation between overall N – stage and anti-p53 auto-antibody status.

3.5.2.2 M-stage

The concept that early invasion may stimulate anti-p53 auto-antibody production by increasing likelihood of antigen presentation would suggest that haematogenous metastatic spread (M1) should also trigger anti-p53 auto-antibody production. However, almost all studies report no increase in sero-positivity rates with M1 disease (Suppiah et al., 2008, Muller et al., 2006, Lechpammer et al., 2004, Bielicki et al., 1999, Chang et al., 2005). Only one study reported increased anti-p53 auto-antibody with M1 CRC but there were substantial discrepancies within this study with low sero-positivity in small Stage I-III (9%, 4/45) group and high sero-positivity rates in Stage IV CRC (56%, 14/25) which is inconsistent with other studies (Shiota et al., 2001). This association with M1 also led this to be the only study to report anti-p53 to

correlate with CEA levels (section 3.5.3) and display independent prognostic significance (Section 3.5.5)

The most probable explanation for the absence of an increased anti-p53 auto-antibody response with distant metastases is that there have already been sufficient opportunities for antigen-presentation, either via local tumour invasion, microscopic haematogenous tumour cell dissemination, and/or lymph node invasion. The absence of a humoral response may no longer be dependant on antigen-presentation, but rather on patient specific biological factors which include (a) tumour factors e.g. p53 mutation type and presence of co-factors required for antigen presentation; and (b) patient immunity-specific factors such as MHC expression required to mount a response. This may also explain why patients with same cancer type and same p53 mutation type do not mount the same anti-p53 response, and also absence of positive sero-conversion despite disease progression in anti-p53 negative patients.

In conclusion, anti-p53 auto-antibody production may be induced in the final stages of malignant transformation where p53 mutation rate increases, in keeping with p53 mutation being one of the final stages of the adenoma-carcinoma sequence. The minority of studies reporting increased anti-p53 auto-antibody production with local or nodal invasion are poor quality with conclusions drawn from selective group analysis. Anti-p53 auto-antibody is not associated with CRC stage.

3.5.3 Anti-p53 auto-antibody and Carcino-Embryonic Antigen (CEA)

Carcino-Embryonic Antigen (CEA) is the most common serum tumour marker used in CRC. It is a 180kDa serum glycoprotein which is present at low levels in

normal cells but over-expressed in adenocarcinoma, especially of the colon, rectum, breast and lung (Thomas et al., 1990). Pre-operative CEA presence has been associated with aggressive CRC and poor prognosis (Park et al., 2009, Goldstein and Mitchell, 2005). CEA has also been used as an adjunct in CRC screening, monitoring for disease recurrence following resection, or as part of tumour marker panel for metastases of unknown primary origin. CEA has high specificity which is reduced due to false elevations in smokers, inflammatory diseases, cirrhoses, obstructive jaundice, gastric ulcers, emphysema, diabetes and collagen vascular diseases (Wilson et al., 1999, Begent, 1984).

CEA use is not recommended in screening, or use in isolation in detection of recurrence following surgery, due to its poor sensitivity (Tan et al., 2009, Hara et al., 2008). The most recent large study reported mean pre-operative CEA sensitivity of 20.6% (261 / 1263), which decreased further with early stage CRC - Stage I (6.6%, 14 / 212), Stage II (21.4%, 110 / 514) and Stage III (25.5%, 137 / 537) (Park et al., 2009). Although CEA sensitivity can be modulated by changing the cut-off values for "positivity" , a utility study showed this sensitivity remained low despite variations in the cut-off value used (Park et al., 2005) (Tan et al., 2009). Despite this, CEA remains the most reliable serum tumour marker in CRC. The American Society of Surgical Oncology (ASCO) guidelines suggest serial CEA measurements every 3 months in Stage II / III CRC for at least 3 years following diagnosis, and during treatment of metastatic disease surgery (ASCO, 2006). Sceptics remain unconvinced of the cost-effectiveness of this approach in screening or post-operative surveillance; or its ability to alter CRC outcomes significantly.

Tumour markers used in conjunction with CEA could increase the efficacy of serum testing in selected populations. Most studies report no correlation between CEA and anti-p53 auto-antibodies (Tang et al., 2001, Hammel et al., 1997, Bielicki et al., 1999) **(Table 3.5)**. Only two studies reported a correlation between the two markers, the first study which used a small and poorly distributed sample size of 18 anti-p53 auto-antibody positive patients - 14 of whom were Stage IV and only 4 were Stage I-IIIb (Shiota et al., 2000). The second study reported 78% anti-p53 auto-antibody positivity in CEA-positive patients which was significantly higher than 56% sero-positivity in patients with normal CEA. However, the 56% sero-positivity is exceptionally high and probably attributable to methodological error which relied on WB, not ELISA (Shibata et al., 1996). Hence the discrepant results caused by the different methodology in one study, and atypical sample distribution in both studies lead to association between the 2 markers. It is generally accepted that anti-p53 auto-antibody is independent of CEA. Its potential role is discussed.

Table 3.5: (a) Prevalence of anti-p53 auto-antibody and CEA in studies reporting the presence of both tumour markers in CRC; (b) Combined CEA and anti-p53 auto-antibody rates from all studies reporting the presence of both markers

(a)	CEA (%)	Anti-p53 (%)
(Tang et al., 2001)	408 / 943 (43.3)	130 / 998 (13.0)
(Shibata et al., 1996)	23 / 47 (48.9)	32 / 47 (68.0)
(Bielicki et al., 1999)	46 / 148 (31.1)	29 / 148 (19.6)
(Hammel et al., 1997)	20 / 54 (37.0)	14 / 54 (25.9)
Overall	**497 / 1192 (41.7)**	**204 / 1247 (16.4)**

(b) Total, n= 1192	CEA normal	CEA elevated
Anti-p53 ab present	112 (9.4 %)	90 (7.6 %)
Anti- p53 ab absent	584 (48.9 %)	406 (34.1 %)

Table 3.5 (b) suggests that anti-p53 is present in 9.4% of CRC patients with normal CEA i.e. "information gain". The combined sensitivity of anti-p53 and CEA (51%, as CEA and anti-p53 are absent in 48.9%) exceeds that of CEA alone (42%) and anti-p53 alone (17%). This suggests an information gain of 9% and 34%. Muller et al was the only study to report information gain rates and similarly confirmed increased sensitivity when anti-p53 auto-antibody was combined with CEA. Sensitivity increased from 55% to 71% in colon cancer and 78% to 83% in 46 rectal cancers (Muller et al., 2006). The estimated "information gain" was 16% which is consistent with that calculated in this thesis using data from all published studies.

This could be perceived as substantial "information gain" but must display appreciable clinical benefit if it is to be used. CRC has an overall low prevalence in the general population and hence screening detection rates will remain low despite the use of both markers, especially with poor sensitivity in a low prevalence disease. Also, both tumour markers have preponderance towards Stage II & III CRC and are most likely detect these CRC stages but are unable to detect early (stage I) CRC which would increase long-term survival. As such, although anti-p53 auto-antibody presence is independent of CEA with some "information gain", both markers used either in isolation, or in combination, are unlikely to alter clinical outcomes.

3.5.4 Anti-p53 Auto-antibody and Monitoring for Recurrence or Metastases

Serum anti-p53 auto-antibody titres are reported to be dependant on tumour load, with elevations in serum titres corresponding with disease recurrence / progression and decreased titres observed following surgery and / or chemotherapy. This trend has also been observed in lung, ovarian and breast cancer (Zalcman et al., 1998, Angelopoulou et al., 1994). However, all reports of variation in anti-p53 auto-antibodies titre with tumour load are highly selected case observations within larger studies. This is because no study aimed to investigate the temporal relationship between anti-p53 auto-antibodies and tumour load, the retrospective nature which limits serum samples available and significant selection bias. Patients examined sequentially for anti-p53 auto-antibody were those with ongoing treatment or disease progression and hence bias towards advanced CRC which were kept under intense surveillance. Almost all patients still demonstrate decrease in serum titres post-

surgery and during chemotherapy. Increases in auto-antibody during chemotherapy were associated with metastases (Angelopoulou et al., 1997, Polge et al., 1998). The results of these reports are summarised in **Table 3.6.**

Anti-p53 auto-antibody is an IgG class antibody. IgG antibody production occurs via an early primary response associated with MHC II – T-cell helper response (after the initial antigen presentation leads to IgM antibody production) and a later secondary response where matured plasma cells are able to produce the specific anti-p53 IgG without the need for direct antigen presentation. Takeda et al reported the highest negative sero-conversion rate (94%, 16 / 17) in a group of patients with superficial (mucosal and submucosal) CRC treated with endoscopic resection. This sero-conversion rate is higher than those reported by other studies which report a significant decrease in anti-p53 auto-antibody titres but not complete sero-conversion to anti-p53 auto-antibody negative status (Lechpammer et al., 2004, Hammel et al., 1997, Angelopoulou et al., 1997). The observation by Takeda et al. is likely attributable to the early stage CRC population where the smaller mutant p53 load may not have adequately stimulated the humoral system sufficiently to produce a prolonged sustained immune response following removal, as opposed to other studies using advanced CRC and reporting decreased but persistent anti-p53 auto-antibody response (Takeda et al., 2001b, Lechpammer et al., 2004, Polge et al., 1998, Angelopoulou et al., 1997). Decreasing titres are observed following treatment, possibly secondary to decreased tumour load but can also be caused by concurrent immuno-suppressive chemotherapy, which can continue to suppress IgG response after treatment cessation. Finally, the IgG response can persist for >20 years and has

not considered a suitable marker for disease diagnosis or eradication in certain infective conditions e.g. Lyme disease (Kalish et al., 2001). The clinical relevance of this is that the anti-p53 IgG antibody response may in itself be unreliable, in addition to poor sensitivity and specificity for CRC.

The cost-efficacy is considered next. Assuming 20% sero-positivity at diagnosis, 3 monthly titres over 3 years (as per CEA recommendation), and a very optimistic 3.6% positive sero-conversion (only 1 study reports positive sero-conversion) would yield 2.8 positives out of 1200 samples (100 patients x 4 samples per year x 3 years) at a cost of £11,200 (approx £373 per ELISA, 30 ELISA kits required). Next, anti-p53 is not specific to CRC (Section 3.5.1). Thirdly, anti-p53 is itself an IgG which could be suppressed even long after chemotherapy, resulting in further decrease in sensitivity with increased false negatives. Hence, anti-p53 auto-antibody use is not justifiable in screening all CRC patients.

An alternative strategy would be to screen all CRC patients for anti-p53 auto-antibody at diagnosis and perform serial measurements only in those positive at diagnosis. Patients with rising titres may benefit from more intensive surveillance or expedited tests for potential disease recurrence. However, this would still involve testing all patients at diagnosis and also the confounding effect of chemotherapy which is increasingly used. A cost efficacy study on 50 post-resection CRC patients not requiring chemotherapy, would require a conservative estimate of 500 new CRC patients at initial screening (£4,660), to obtain 100 CRC anti-p53 positive patients. Quarterly titres in a 5-year surveillance program would cost approximately a further £18,600 (2000 samples). Again, the isolated increase of anti-p53 auto-antibody

without corroborating clinical or radiological evidence will not alter clinical management. Finally, even if this cost was sustainable, anti-p53 would still be insufficiently specific, even in this highly selected population. It becomes obvious that none of the strategies are likely to be cost-effective or clinically justifiable.

In summary, based on the information available on small number of patients, anti-p53 auto-antibody is a poor marker of CRC recurrence. Various strategies in utilising anti-p53 auto-antibody in CRC surveillance have been discussed in this chapter, alongside corresponding cost-efficacy approximates, but none of these approaches are justifiable at present due to the poor sensitivity and specificity and variability of the IgG response of the anti-p53 auto-antibody itself

Table 3.6: Follow-up anti-p53 autoantibody (anti-p53) measurements in patients with colorectal cancer (CRC) and sero-conversion rates.

Study (Year)	Patients, Method	Follow-up	Findings
(Muller et al., 2006)	303 patients, 197 colon, 46 rectal Immunoblot	Median 6 months	All cancers: 3.6% (11/303) sero(-)→ (+); 3.6% (11/303) sero(+)→ (-); Total 7.2% (22/303) sero-conversion. Colon cancer: 3% (4/137) sero(-)→ (+); 3.6% (5/137) sero(+)→ (-); Total 6.6% (9/137) sero-conversion. Rectal cancer: 6.5% (2/31) sero(-)→ (+); 3.2% (2/31) sero(+)→ (-); Total 12.9% (4/31) sero-conversion
(Lechpammer et al., 2004)	32 , ELISA (Oncogene, Research Products, Cambridge, USA)	Up to 20 weeks; 8 patients - 48 weeks	Non-significant decrease at 4 weeks (pre-first cycle chemo) and significant decrease at 12 weeks post-surgery. Significant decreases during chemotherapy and 2 patients with anti-p53 increase at 12 weeks (during chemotherapy) developed recurrence. 8 patients with extended follow-up: 7/8 had decreased anti-p53 with no recurrence. 1/8 anti-p53 decrease post-surgery/ chemotherapy but increased at 12 weeks corresponding with liver metastases. Anti-p53 fluctuates in response to tumour load but does not disappear. Anti-p53 levels reflects tumour load even during chemotherapy
(Takeda et al., 2001a)	30 CUR A, 5 CUR B, 5 CUR C), (Anti-p53 EIA, Pharmacell)	Median 26 months (13-144)	CUR A (n=30): 28/30 sero(+)→(-) in 6 months; 2 no sero-conversion: 1 recurrence. CUR B (n=5): 2 sero(+)→(-) no recurrence. 3 no sero-conversion, 2 had metastases. CUR C: No sero-conversion. Correlation between post-operative negative conversion and operative curability.

(Takeda et al., 2001b)	17 mucosal / submucosal, ELISA (Anti-p53 EIA, Pharmacell, France)	Up to 2 years	94%, 16/17 sero(+)→(-) within 3 weeks post-surgery. No recurrences as early stage tumours and hence not able to comment on anti-p53 and recurrence rates
(Polge et al., 1998)	10, ELISA (Dianova, Hamburg, Germany)	Up to 6 months	8 followed-up: 5/8 remained sero(+) post-operatively. All developed metastases. 3/8 decreased anti-p53 titres. No metastases or recurrence. Anti-p53 titres decreased within 1 month of surgery/chemotherapy but no sero-conversion to anti-p53(-).
(Angelopoulou et al., 1997)	6, "In house" immunofluorometric assay	Up to 17 months	Anti p53 decreases with surgery/ chemotherapy but persists at low levels. Anti-p53 increases with recurrence. Anti-p53 reflects tumour load more sensitively than CEA (n=5) and in non-CEA producing tumour (n=1).
(Hammel et al., 1997)	12, "In house" ELISA	Up to 20 months	Anti-p53 in 5/8 patients decrease by >25% within 1 month. At 1 year, 3 with normal anti-p53 levels and 3 with substantial decrease in anti-p53 remain disease-free. 2 patients with post-operative increased anti-p53: 1 developed recurrence and 1 developed metastases. Anti-p53 decreased again following surgery in both patients. CEA and CA19-9 were normal in both cases.

Sero(-), Sero-negative; Sero(+), Sero-positive; CUR A: No residual tumour macroscopically; CUR B: No residual tumour but not as evaluable as CUR A; CUR C: definite residual tumour

Section 3.5.5: Anti-p53 auto-antibody and prognosis in CRC

p53 mutations have been associated with poor prognosis, possibly due to chemo-resistance against p53-dependant chemotherapy (e.g. 5 – fluorouracil) although this is not consistently reported (IARC, 2008, Soussi, 2008, Soussi, 2000b). As anti-p53 auto-antibody presence is partly associated with p53 mutations and serum testing is easier than DNA sequencing, attempts were made to assess the prognostic significance of anti-p53 auto-antibody. The majority of studies of anti-p53 auto-antibody in CRC report no independent prognostic value (**Table 3.7**). The minority of studies which report adverse prognostic significance of anti-p53 in CRC should be interpreted with caution (Shiota et al., 2000, Kressner et al., 1998, Houbiers et al., 1995).

The first of these studies associated anti-p53 auto-antibody with decreased overall and disease-free survival, but only in selective analysis of Duke's A and B1 CRC (Houbiers et al., 1995). This prognostic significance was not maintained in multivariate analysis of all Dukes stages. Similarly, adverse prognostic significance was seen in univariate but again lost in multivariate analysis in the second study (Kressner et al., 1998). The third study by Tang et al associated the auto-antibody sero-positivity with N3 CRC (Tang et al., 2001). However, the N3 stage (regional and central lymph node involvement) in this study should be classified M1 as discussed previously (**Section 1.1.7.1.2**), in which case, anti-p53 auto-antibody was in fact associated with metastatic disease. Even so, univariate prognostic significance was lost in multivariate analysis. Only one study reports an independent anti-p53 auto-antibody prognostic significance and is discussed next.

Table 3.7: Prognostic effect of anti p53 auto-antibody (anti-p53) in CRC. Hepatocellular carcinoma (HCC)

Study (Year)	% (n)	Follow-up	Findings
(Suppiah et al., 2008)	21.7 (20 / 92)	Median 97m	No difference in overall survival (62 vs. 60 months) or disease-free survival (73 vs. 82 months)
(Muller et al., 2006)	28.8 (70 / 243)	5-year trial protocol	No survival difference with anti-p53 in CRC and other cancers. Trend towards decreased survival in anti-p53 positive patients with HCC and breast carcinoma
(Tang et al., 2001)	13 (130 / 998)	Recruitment 1995–2000	Anti-p53 associated with decreased survival in univariate analysis but not multivariate analysis. Anti-p53 associated with advanced nodal disease (Stage N2→N3) and metastases (M1)
(Chang et al., 2005)	28.(147 / 167)	Median 36.3 months (22 – 85)	P53 mutation associated with poor differentiation and advanced stage. Multivariate analysis shows p53 mutation most significant survival predictor, followed by CRC stage. No prognostic significance of p53 protein expression or anti-p53
(Shiota et al., 2000)	25 (18 / 71)	Not stated	Anti-p53 associated with shorter overall survival (20 vs. 56 months) but highly significant association with metastases (M1). Cox regression showed prognostic significance with liver metastases, TNM stage, Dukes stage, Ca19-9 and anti-p53.(in that order).
(Kressner et al., 1998)	32.1 (59 / 184)	Median 6 years	Anti-p53 associated with decreased survival in univariate, but not multivariate analysis. Anti-p53 is independent prognostic indicator in Dukes' A – C with curative surgery (i.e. when metastases excluded)
(Houbiers et al., 1995)	25.5 (65 / 255)	36 months	Anti-p53 associated with reduced overall (75% vs. 88%) and disease-free survival (56% vs. 64%) at 3 years in subgroup analysis of Dukes' A and B1. No difference in overall survival (61 vs. 68%) or disease-free survival (51% vs. 58%) when all stages included

The final study reported significantly shorted median OS in anti-p53 auto-antibody positive patients (20 vs. 56 months). Stage IV CRC had uncharacteristically high sero-positivity rates thus strongly associating anti-p53 auto-antibody with metastatic disease. This was confirmed by the poor median survival of 20 months in the anti-p53 sero-positive group which contrasts with other studies reporting median 5-year survival rates of > 50% in anti-p53 auto-antibody positive patients (Muller et al., 2006, Suppiah et al., 2008, Tang et al., 2001). The anti-p53 auto-antibody bias towards Stage IV disease led to the auto-antibody maintaining prognostic significance in the Cox regression model. However, its significance was still secondary to a multitude of other well established prognostic factors such as Duke's stage, liver metastases, tumour stage; and even lesser established factors such as CEA (Shiota et al., 2000). Incredibly, the anti-p53 auto-antibody in this study had lesser prognostic significance than CA19-9, a pancreatic tumour marker which is not even recommended for screening or surveillance purposes in pancreatic cancer. Hence this study should be disregarded (ASCO, 2006).

In summary, anti-p53 auto-antibody does not display independent prognostic value. Three studies report significance but only in univariate analysis due to association with stronger prognostic indicators, which was always lost in multivariate analysis. The only study to report independent significance should be disregarded. The auto-antibody on its own is most likely a para-neoplastic phenomenon which may accompany advanced tumours, but does not add any independent prognostic value.

3.6 Conclusion

The anti-p53 auto-antibody represents the end-point of a complex multi-factorial humoral response to the accumulation of p53 protein, which is usually a result of mutation, but can also occur through a variety of other pathways. It has low (13 – 32%) sensitivity, near 100% specificity for all cancer, but does not predict for CRC alone. Sero-positivity rates may increase in early post-dysplasia but does not correlate with local, nodal involvement of distal spread. It is not sufficiently consistent to form a separate stage classification. Similarly, low sensitivity, poor CRC-specificity and the variability of an IgG response makes it unsuitable for diagnosis, screening (general of high-risk populations) and post-resection surveillance. The sporadic association with advanced disease in flawed minor studies suggest adverse prognostic significance, which is always lost in multivariate analysis. Anti-p53 auto-antibody does not display independent prognostic significance.

4 Chapter 4: Anti-p53 Auto-antibody Results and Discussion

4.1 Introduction

The aim of this thesis was to investigate the long-term prognostic significance of anti-p53 auto-antibody. This was ensured by setting rigid inclusion criteria of samples with minimum 5-year follow-up which significantly limited the number of serum samples available for analysis. Measurement of anti-p53 auto-antibody in plasma samples could increase sample size but the accuracy of plasma titres has never been reported. Hence, a study of comparability between anti-p53 auto-antibodies in serum and plasma was firstly undertaken (**Section 4.2**), followed by results of the long-term prognostic significance of anti-p53 auto-antibody (**Section 4.3**).

4.2 Anti-p53 auto-antibody in serum and plasma

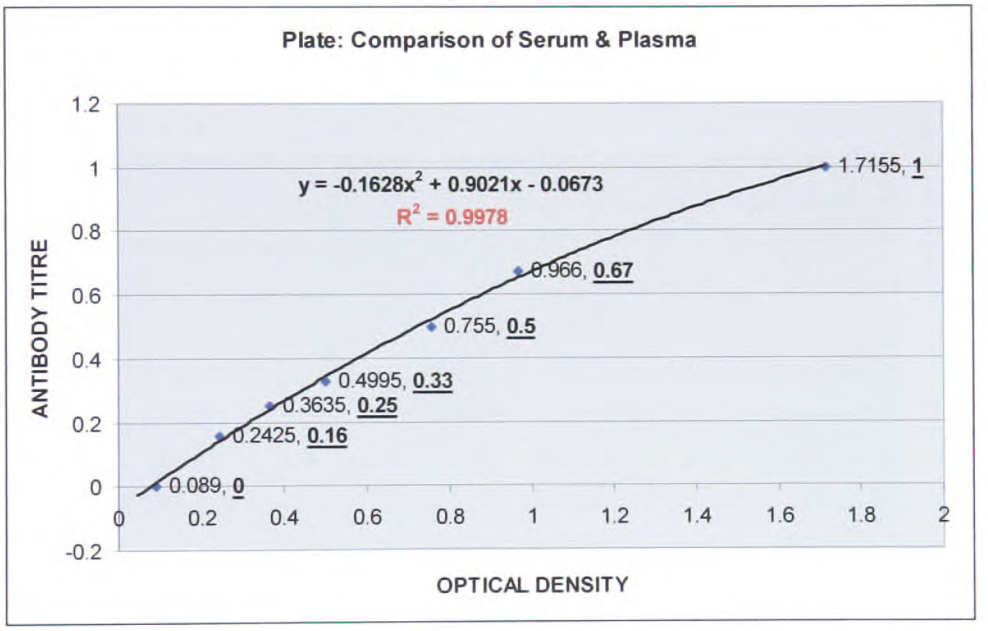
Serum is a processed liquid component of blood which retains the soluble components of blood (e.g. electrolytes, immunoglobulins) but without cells or clotting factors. Plasma is another liquid component of blood, which similarly contains electrolytes and hormones but in addition, also contains clotting and tissue factors. Anti-p53 auto-antibody has always been measured in serum. Eighteen "paired" plasma-serum samples were available. As plasma anti-p53 auto-antibody titres had not been reported before, and the ELISA manufacturer recommended serum for anti-p53 auto-antibody measurement, plasma anti-p53 auto-antibody titres required validation first.

4.2.1 Validation ELISA Technique

The ELISA methodology for plasma validation was demonstrated by

- High R^2 (0.9978) indicating near perfect reliability (**Figure 4.1**).
- CoV OD_{450nm} < 20% in 41 out of 48 (>85%) duplicate assays indicating good technique.

Figure 4.1: Calibration Curve for ELISA comparing serum and plasma titres of anti-p53 auto-antibody; Optical density (*x*-axis) = OD_{450nm}

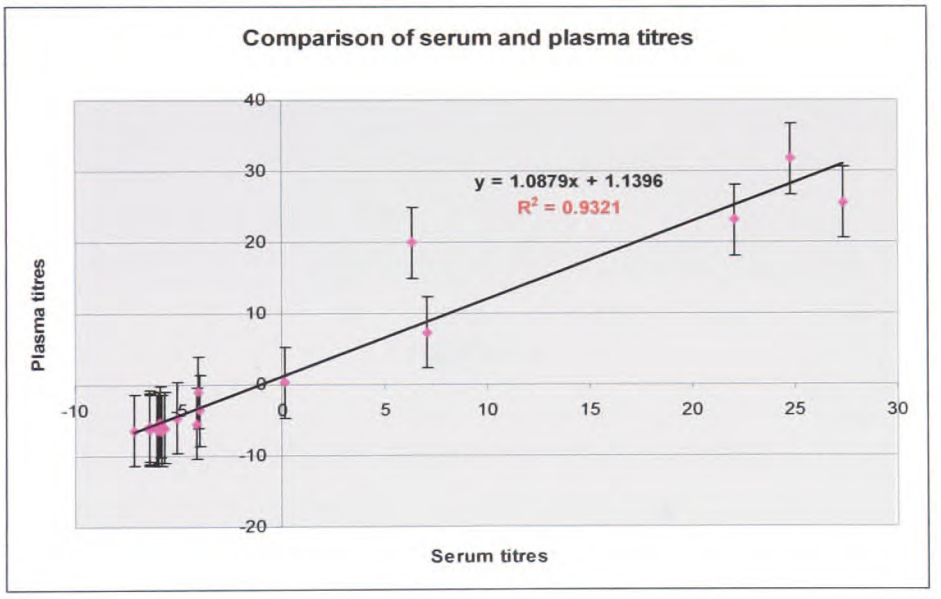


4.2.2 Comparison of serum and plasma anti-p53 auto-antibody

The mean OD_{450nm} of the negative control standard = 0.0895. Samples with OD_{450nm} >0.0895 were labelled positive, and those below this were labelled negative. Anti-p53 auto-antibody was present in 5 patients. There was perfect agreement in anti-p53 auto-antibody presence between plasma and serum samples in qualitative assessment.

The CoV comparing the mean plasma and serum titres in each patient was < 20% in 85% (17 / 20) which suggested good agreement between serum and plasma titres in qualitative analysis. Quantitative analysis by linear regression ($R^2 = 0.9321$) also concluded good correlation between serum and plasma titres. However, when graphically displayed, sero-positive samples (top right quadrant) were quite disparately away from the "line-of-best-fit" (**Figure 4.2**) which suggested the high R^2 value may be biased by the large cluster of negative samples (bottom left quadrant). The positive samples were then analysed separately.

Figure 4.2: Liner regression of serum and plasma antibody titres



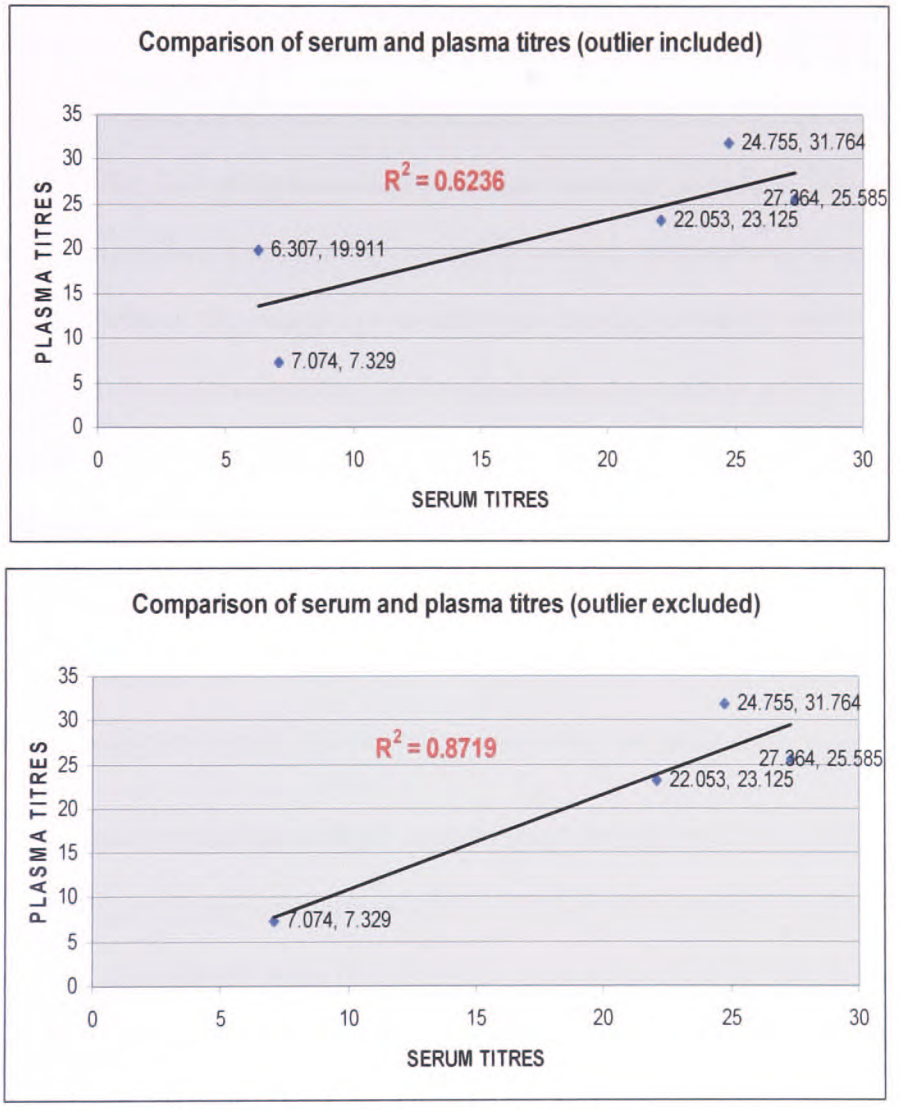
4.2.3 Analysis of Sero-positive samples only

The mean CoV was poor (20%) between plasma and serum antibody titres and caused by a single outlier (Patient 164) and to a lesser extent, Patient 145 (**Table 4.1**). CoV decreased to 7% and R^2 increased from 0.624 to 0.872 when Patient 164 was excluded (**Figure 4.3**). This discrepancy and the exclusion of plasma samples are discussed.

Table 4.1: CoV between serum and plasma auto-antibody titre in positive patients

Patient	Mean Serum	Mean Plasma	CoV
143	22.053	23.125	0.033557
164	6.307	19.911	0.733807
145	24.755	31.764	0.175379
141	7.074	7.329	0.025038
169	27.364	25.585	0.047515

Figure 4.3: Linear regression with and without outlier (Patient 164)



Data labels indicate (serum titre, plasma titre)

4.2.4 Discussion

The R^2 comparing plasma and serum anti-p53 auto-antibody titres were the weakest in this thesis and may be attributed to small number (5) of patients. However, similar sample sizes of 7 calibrators were used to plot other ELISA calibration curves in this thesis, all of which $R^2 > 0.98$ (**Section 1.1**) thus proving good R^2 is obtainable despite small samples.

The discrepancy between serum and plasma titres may be a true result or methodological error. Methodological error would be shown by high CoV in duplicate assays. The CoV of duplicate serum (0%) and duplicate plasma (0.3%) shows nearly identical antibody titres making this highly unlikely attributable to methodological error. Similarly, the majority of the duplicate samples assayed in this ELISA also had CoV < 10%, again supporting good methodological technique. Finally, other studies report CoV < 3% in ELISA, albeit in serum samples only, suggesting reliable measurements with ELISA (Shimada et al., 2003). Hence, the discrepancy between plasma and serum auto-antibody titres most probably represents a true result. The most likely cause of the higher anti-p53 auto-antibody readings in plasma would be non-specific binding by plasma components which are not present in serum.

The inclusion of plasma samples could increase the cohort size but with potential introduction of substantial error (CoV 17 - 73% in 40% patients). As the sample size was already relatively large (92) with only modest benefit from plasma inclusion, plasma anti-p53 auto-antibody measurements were excluded from prognostic studies of anti-p53 auto-antibody in CRC.

4.3 Results: Prognostic Significance of anti-p53 auto-antibody

Anti-p53 auto-antibody was measured (**Section 2.2**), the ELISA technique assessed (**Section 4.3.1**) and the association between anti-p53 auto-antibody and clinical parameters (**Section 4.3**) and prognosis (**Section 4.4**) was evaluated.

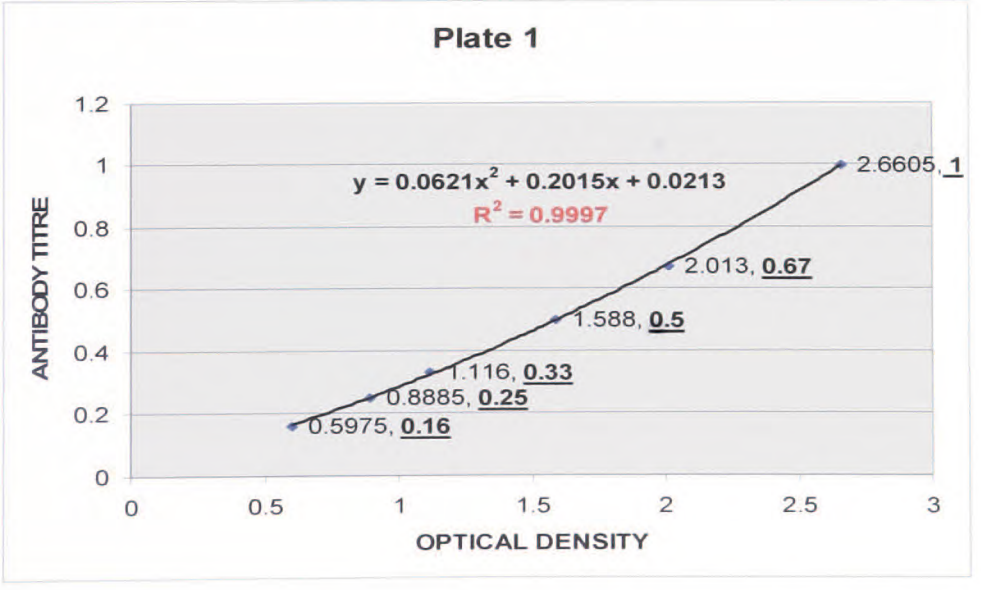
4.3.1 ELISA Validation

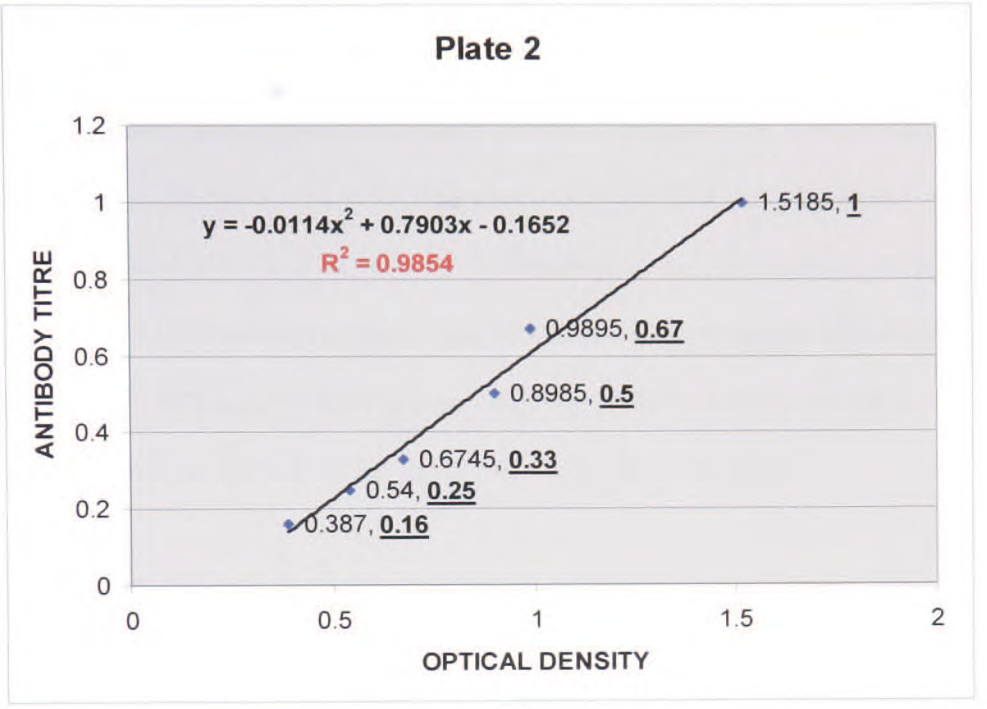
Serum samples were labelled "positive" or "negative" (**Table 4.2**). "Critical" samples were re-assayed. Sera were not available for repeat assay in 3 "critical" patients and these were labelled negative. The R^2 demonstrates good technique. Plate 1 & Plate 3 display $R^2 \sim 1.0$ suggesting near perfect correlation (**Figure 4.4**).

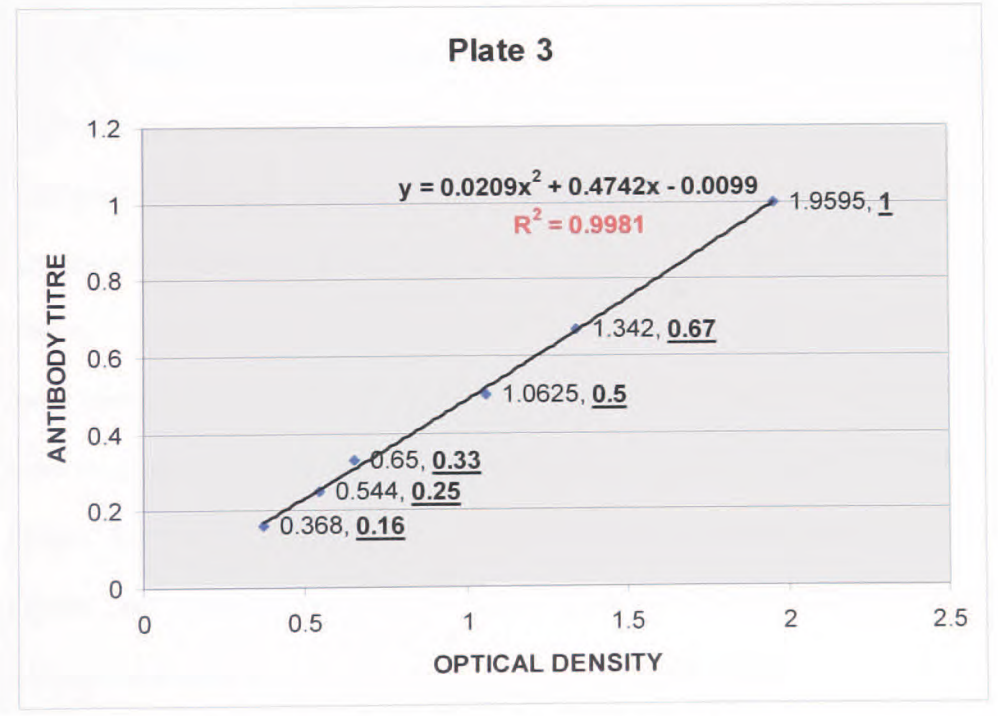
Table 4.2: OD_{450nm} criteria used for labelling serum samples

	"Positive"	"Negative"	"Critical"
Plate 1	> 0.253	< 0.212	0.212 – 0.253
Plate 2	> 0.073	< 0.061	0.060 – 0.073
Plate 3	> 0.215	< 0.179	0.179 – 0.215

Figure 4.4: Calibration curve and R^2 in all ELISA plates







Data labels indicate (Calibrator OD_{450nm}, **calibrator dilution**)

4.3.2 Anti-p53 auto-antibody in patients *vs.* controls

The control group comprised 8 healthy subjects (6 male, 2 female) and 20 patients (11 male, 9 female) with benign neurological disease (**Appendix 7.9**). There were 17 males and 11 females (1.6:1 ratio) with a mean age of 67.5 (63.5 -72.8) years. The patient group consisted of 58 male and 34 female patients (1.7:1) with mean age of 70 years (32 - 91). There was no significant difference in age or gender between the two groups. Anti-p53 auto-antibody was significantly higher in the patient group, 21.7% (20 / 92), compared to 0% (0/20) in the control group ($p = 0.008$).

4.3.3 Anti-p53 auto-antibody and Clinico-pathological Features of CRC

Anti-p53 auto-antibody was present in 22% (20/92). The overall mean patient age was 70 years (32 – 91) and hence age-specific analysis was divided to patients < 70 years and > 70 years of age. Anti-p53 auto-antibody was not associated with individual Dukes' stage or TNM stages, T - stage, N - stage, M - stage, differentiation or mucinous morphology (**Table 4.3**). Individual groups within a variable were then combined to form larger groups, for example, Dukes' A combined with Dukes' B, and Dukes C combined with Dukes' D (Dukes A/B *vs.* C/D) to obtain larger groups and more robust analysis. These groups could be statistically rationally combined as there were no significant differences (χ^2) in anti-p53 auto-antibody frequency between each groups, for example Dukes' A and B could be combined as sero-positivity rates were similar (20% and 27% respectively). Again, there was no association between anti-p53 auto-antibody and these new combined groups (**Table 4.3**b)

Serum anti-p53 auto-antibody titres were calculated but preliminary analysis showed substantial titre variation within a small sample (Range: 5.2 – 134.2 IU/100µl in 20 patients, **Table 4.3a**). There was no association with stage but the numbers were too small (e.g. 1 patient in Dukes D) for further analysis. Mean titres in each group were Dukes' A (15.9 IU/100µl), Dukes' B (43.4 IU/100µl), Dukes' C1 (32.4 IU/100µl), Dukes' C2 (19.2 IU/100µl), overall Dukes' C (29.5 IU/100µl, 265.1/9) and 1 patient in Dukes' D (7.9 IU/100µl). Hence, further analysis was performed using categorical "positive/negative" rather than numerical values.

Anti-p53 titre (IU/100µl)	Dukes' Stage
5.2	C1
7	C1
7.9	D
8.6	A
9.2	C1
10.2	B
10.5	B
14	A
14.4	C1
18.6	C2
19.2	B
19.8	C2
25	A
28.9	C1
35.2	B
38.1	C1
43.5	B
50.8	B
123.9	C1
134.2	B

Table 4.3a: Increasing serum titres with corresponding coloured Dukes' stage

Table 4.3b: Anti-p53 antibody (-) negative and (+) positive; and clinical parameters

Parameter		n	Anti-p53 (-); n (%)	Anti-p53(+); n (%)	p
Age	< 70	44	33 (75)	11 (25)	**0.750**
	> 70	48	39 (18.8)	9 (18.8)	
Sex	Male	58	46 (79.3)	12 (20.7)	**0.468**
	Female	34	26 (76.5)	8 (23.5)	
Dukes'	A	11	8 (72.3)	3 (27.3)	-
	B	35	28 (80)	7 (20)	-
	C1	35	28 (80)	7 (20)	-
	C2	7	5 (71.4)	2 (28.6)	-
	C1/2	42	33 (78.6)	9 (21.4)	-
	D	4	3 (75)	1 (25)	-
	A/B	46	36 (78.3)	10 (21.7)	**1.0**
	C/D	46	36 (78.3)	10 (21.7)	
Stage	Stage I	5	4 (80)	1 (20)	-
	Stage II	41	32 (78)	9 (22)	-
	Stage III	41	33 (78.6)	9 (22)	-
	Stage IV	4	3 (75)	1 (25)	-
	Stage I / II	46	36 (78.3)	10 (21.7)	**1.0**
	Stage III / IV	46	36 (78.3)	10 (21.7)	
T-stage	T1	6	5 (83.3)	1 (14.3)	-
	T2	7	5 (71.4)	2 (28.6)	-
	T3	67	50 (75.8)	16 (23.9)	-
	T4	12	11 (91.7)	1 (8.3)	-
	T 1/2	13	10 (76.9)	3 (23.1)	**0.900**
	T 3/4	79	62 (78.5)	17 (21.5)	
N-stage	N0	47	37 (78.7)	10 (21.3)	-
	N1	33	26 (78.8)	7 (21.2)	-
	N2	12	9 (75)	3 (25)	-
	N0	47	37 (78.7)	10 (21.3)	**0.912**
	N1/2	45	35 (77.8)	10 (22.2)	
M-stage	M0	88	69 (78.4)	19 (21.6)	**0.632** †
	M1	4	3 (75)	1 (25)	
Site	Proximal	24	22 (91.7)	2 (8.3)	
	Distal	28	21 (75)	7 (25)	**0.175**
	Rectum	40	29 (72.5)	11 (27.5)	
Diff	Well	11	8 (72.7)	3 (27.3)	-
	Moderate	44	32 (72.7)	12 (27.3)	-
	Poor	37	32 (86.5)	5 (13.5)	-
	Well/Mod	55	40 (72.7)	15 (27.3)	**0.117**
	Poor	37	32 (86.5)	5 (13.5)	
Mucinous		11	8 (72.7)	3 (27.3)	0.699 †
Non-mucinous		81	64 (79)	17 (21)	

Diff, Differentiation; Mod, Moderate; p-value χ^2 test or † Fisher's Exact (n<5)

4.4 Anti-p53 auto-antibody and Survival

Survival analysis was performed by examining the follow-up period distribution (**Section 4.4.1**), patients' final status (**Section 4.4.2**) and overall and disease-free survival (**Section 4.4.3**)

4.4.1 Kolmogrov-Smirnov analysis of follow-up duration

The mean length of follow up was 45.3 months in the anti-p53 auto-antibody positive group and 50.8 months in the negative group ($p = 0.411$). The statistical tests to be used are determined by normality of follow-up distribution. The follow-up duration demonstrated significant ($p=0.015$) skew (**Figure 4.5 & Figure 4.6**). Therefore the choice of statistical tests for survival analysis was limited to non-parametric tests.

4.4.2 Final Disease Status

The median time from date of diagnosis to end of study period (1 October 2006) was 97 months (IQR: 91-102). The patients' status at end of study period is shown in **Table 4.4**.

Table 4.4: Patient status by anti-53 auto-antibody presence. (%) are expressed as proportion within each column.

Status	Anti-p53 Negative n = 72 (%)	Anti-p53 Positive n = 20 (%)	All patients n (%)
Not known/moved	2 (2.8)	1 (5.0)	3 (3.3)
Alive, Disease free	26 (36.1)	6 (30.0)	32 (34.8)
Alive, with Disease	0	0	0
Died of Disease	24 (33.3)	9 (45.0)	33 (35.9)
Died, other cause	20 (27.8)	4 (20.0)	24 (26.1)
Total	72 (100)	20 (100)	92 (100)

Figure 4.5: Overall Length of Follow-up Distribution

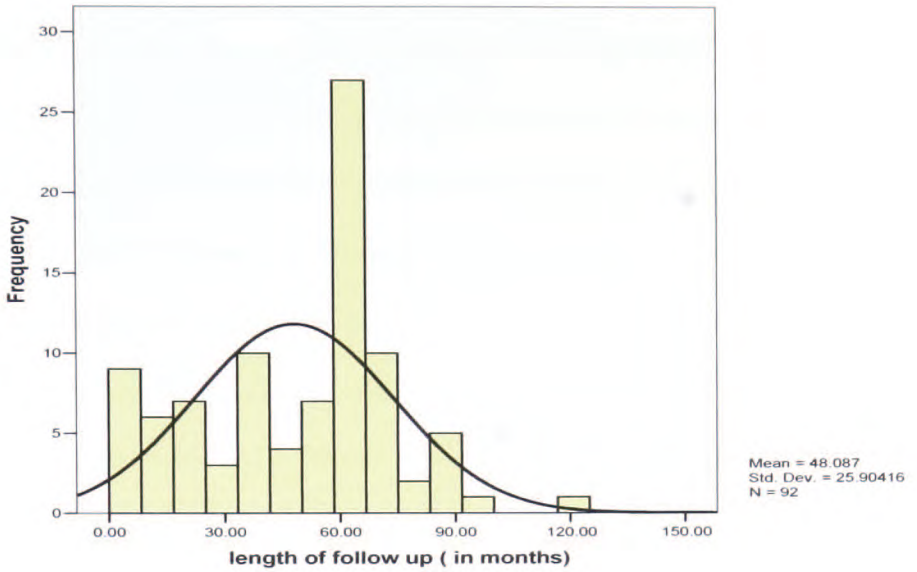
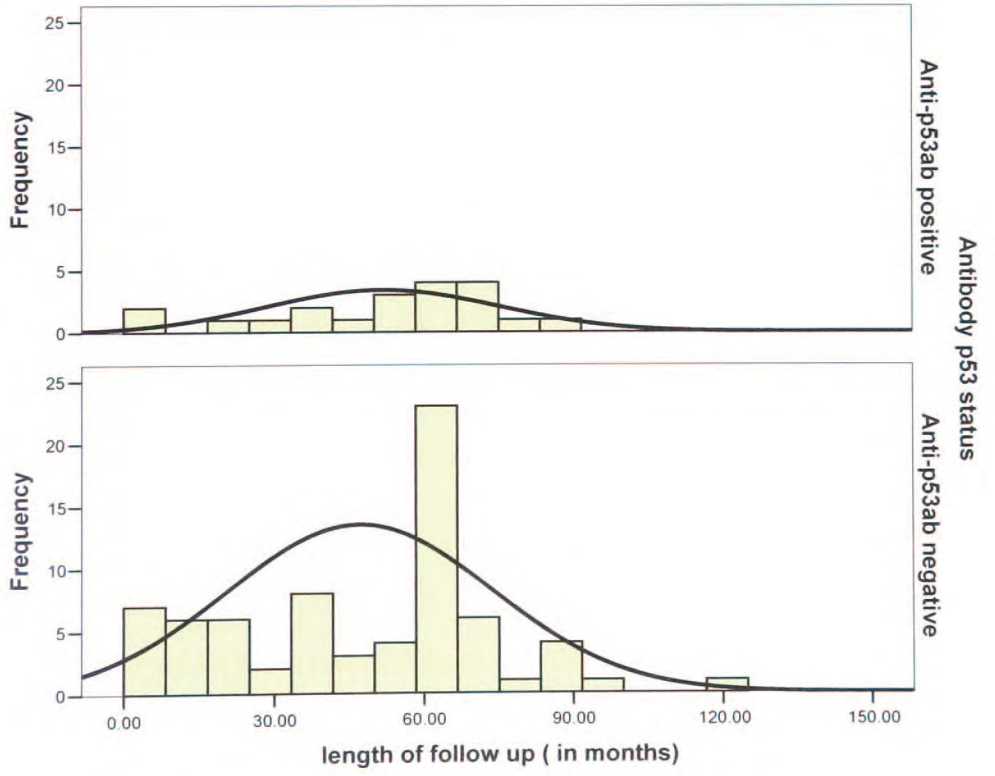


Figure 4.6: Follow-up distribution by anti-p53 auto-antibody status



4.4.3 Overall Survival and Disease Free Analysis

Median overall survival (OS) in all patients was 62 months (95% CI: 57.6 - 66.4). Thirty two patients were alive at the end of the study. Three patients who had moved away were excluded. Of the 57 patients who died, 33 died of the disease and 24 died of other causes. Dukes' / TNM stage, T-stage, N-Stage, M-stage and differentiation were selected for survival analysis to confirm consistency with current literature and hence increase validity of this cohort. The survival curve for each variable in OS is shown in **Appendix 7.13**. OS uni- and multi-variate analysis is summarised in **Table 4.5**. Dukes' stage (C/D), T-stage (T3/4), Node positive disease, metastases at presentation and tumour location were associated with decreased OS in univariate analysis. Dukes' / TNM stage was significant in multi-variate model. Anti-p53 auto-antibody was not associated with OS in uni- or multi-variate analysis.

The median disease-free survival (DFS) of 73 months (95% CI: 60.0 – 86.0) was greater than the median OS of 62 months. At the end of follow-up, 24 patients had died of non-cancer related death leaving 68 patients for disease-specific survival analysis. The DFS survival curves are shown in **Appendix 7.14**. DFS in univariate and multi-variate analysis is summarised in **Table 4.6**. Dukes' stage (C/D), T-stage (T3/4), Node positive disease, metastases at presentation was highly associated with decreased DFS, even more so than in OS analysis. Dukes' / TNM stage was the only independent prognostic factor in DFS. Poor differentiation displayed a non-significant toward decreased DFS which is discussed later **(Section 4.4.5)**. Anti-p53 auto-antibody was not associated with DFS in uni- or multi-variate analysis.

Table 4.5: Median overall survival (in months) and prognostic significance of clinico-pathological parameters

Parameter	Variable	Median (95% CI)	†p	*p
Anti-p53 Antibody	Positive Negative	62 (43.8 – 80.3) 60 (52.9 – 67.1)	0.980	**0.296**
Dukes' Stage	A / B C / D	73 (58 – 88) 48 (30.9 – 65.1)	**0.005**	NI
TNM Stage	I / II III / IV	73 (58 – 88) 48 (30.9 – 65.1)	**0.005**	**0.001**
T stage	T1 / 2 T3 / 4	60 (51.1 – 68.9) 62 (57.2 – 66.8)	0.994	0.278
N stage	Node negative Node positive	73 (58.0 – 88.0) 51 (34.5 – 67.5)	**0.009**	NI
M stage	M0 M1	62 (53.9 – 70.1) 7 (0.0 – 34.4)	**0.021**	NI
Site	Rectum Left Right	60 (50.2 – 69.8) 72 (54.9 – 89.1) 48.0 (21.2 – 74.2)	**0.036**	NI
Differentiation	Well/Moderate Poor	62 (50.4 – 73.6) 60 (35.2 – 84.8)	0.191	0.258

† Univariate analysis using log-rank test. * Cox-regression using 4 covariates (anti-p53 autoantibody, T stage, TNM stage and poor differentiation); NI – not included in Cox regression model

Table 4.6: Median disease-free survival (in months) and prognostic significance of clinico-pathological parameters.

Parameter	Variable	Median (95% CI)	†p	*p
Anti-p53 Antibody	Positive Negative	73 (49-97) 82 (52-112)	0.874	**0.112**
Dukes' Stage	A / B C / D	see below 54 (36-60)	**0.000**	NI
TNM Stage	I / II III / IV	see below 54 (36-72)	**0.005**	**0.001**
T stage	T1 / 2 T3 / 4	(NA) 73 (62-84)	0.475	0.856
N stage	Node negative Node positive	see below 54 (36 – 72)	**0.000**	NI
M stage	M0 M1	74 (56-92) 7 (0-34)	**0.001**	NI
Site	Rectum Left Right	73 (54 – 92) 74 (69 – 79) 82 (NA)	0.419	NI
Differentiation	Well/Moderate Poor	74 (56-92) 62 (49-75)	0.376	0.208

Median disease-free survival in Dukes' A/B and N0 cannot be calculated due to insufficient events (disease recurrence/cancer death) as reflected in highly significant p-value. † Univariate analysis using log-rank test. * Cox-regression using 4 covariates (anti-p53 autoantibody, T stage, Dukes' stage, poor differentiation); NI – not included

4.4.4 Anti-p53 auto-antibody and disease progression

Anti-p53 auto-antibody was tested for ability to predict disease progression, defined as local recurrence or post-operative metastases. Adjuvant chemotherapy may influence local recurrence and systemic metastases. Hence chemotherapy provision in each anti-p53 auto-antibody group was compared first. Forty six patients with Stage III / IV were offered chemotherapy. Twenty seven received chemotherapy and 19 patients not receive chemotherapy due to refusal or significant co-morbidity. There as no difference in chemotherapy use between the anti anti-p53 auto-antibody positive (25%, 5/20) and auto-antibody negative group (30.5%, 22/72).

4.4.4.1 Local recurrence

Anti-p53 auto-antibody positive patients had lower local recurrence rate (5% *vs.* 10%) which was not statistically significant (p = 0.479) (**Table 4.7**). The proportion of patients with the anti-p53 auto-antibody who developed local recurrence (13%; 1 / 8) was also similar to those without the auto-antibody who developed local recurrence (23%, 19/81). Hence, anti-p53 auto-antibody was not associated with local recurrence.

Table 4.7: Anti-p53 auto-antibody status and subsequent local recurrence.

	Anti-p53 Negative	Anti-p53 Positive
No LR	62	19
LR	7	1
Total	69	20
%	10% (7 / 69)	5% (1 / 20)

LR, local recurrence, (χ^2 test, p=NS)

4.4.4.2 Post-operative distant metastases

Anti-p53 auto-antibody presence was tested for ability to predict post-operative metastases. Firstly, anti-p53 auto-antibody frequency was similar in patients with metastases and those without metastases at diagnosis (M-stage, **Section 4.3.3**). Secondly, anti-p53 auto-antibody was not associated with post-operative metastases which occurred in 15.8% (3 / 19) anti-p53 auto-antibody positive patients compared to 19.7% (13 / 66) anti-p53 auto-antibody negative patients (**Table 4.8**). Finally, the overall incidence of metastases (pre- and post-operative metastases) was similar in the anti-p53 auto-antibody positive group (20%, 4 / 20) and the anti-p53 auto-antibody negative group (23%, 16 / 69) (**Table 4.8**). Anti-p53 auto-antibody was not associated with metastases at diagnosis or long-term incidence of metastases. Anti-p53 auto-antibody was also not able to predict subsequent development of metastases.

Table 4.8: Anti-p53 auto-antibody association and metastases

	Anti-p53 Negative	Anti-p53 Positive	Total	Total patients
Pre met	3	1	4	92
Post met	13	3	16	89 *
Total	23% (16 / 69)	20% (4 / 20)	23% (20 / 89)	

Pre met, pre-operative metastases; Post met, Post-operative metastases; Total, total number of patients with metastases. * indicates 3 patients who were lost from final analysis (χ^2 test, p=NS).

4.4.5 Poor Differentiation and Metastases

Poor differentiation has been associated with more aggressive CRC (**Section 1.1.7.2**). Poor differentiation had no prognostic significance in multivariate analysis in this thesis. However, poor differentiation was associated with higher rates of post-operative metastases which was not statistically significant (p = 0.212) (**Table 4.9**).

Table 4.9: Differentiation (Diff.) and post-operative metastases (pM) (χ^2 test, p=NS)

	Well / Mod	Poor	Total	% with Poor Diff.
pM 0	41	22	63	35% (22 / 63)
pM 1	11	11	22	50% (11 / 22)
Total % with pM	21% (11 / 53)	31% (11 / 36)		

Poor differentiation was then compared with overall incidence of metastases (pre- and post-operative metastases). Poor differentiation was again associated with higher overall rate of metastases (39% *vs.* 23%) (**Table 4.10**). This did not achieve statistical significance at 95% confidence interval (p=0.098) but was significant at the 90% confidence interval.

Table 4.10: CRC differentiation (Diff) and overall metastases (M), (χ^2 test, p=NS)

	Well / Mod	Poor	Total	% with Poor Diff.
M 0	41	22	63	35% (22 / 63)
M 1	12	14	26	46% (12 / 26)
Total % with M	23% (12 / 53)	39% (14 / 36)		

4.5 Discussion : Confirming validity of thesis cohort and results

Table 4.11 schematically compares this thesis results with all ELISA reports of anti-p53 auto-antibody in CRC. The study by Muller et al used immunoblot but was included as this was a recent, large study. A summary comparison with % agreement with other studies is performed below to confirm validity of the thesis cohort,

Age and gender: Anti-p53 auto-antibody was not associated with age or gender in this thesis, which is consistent with all (100%) other published reports.

Dukes' stage: Anti-p53 was not associated with Dukes' stage in this thesis, which is similar to 4 out of 7 (57%) studies (**Section 4.5.1**)

TNM stage: Anti-p53 was not associated with TNM stage in this thesis, which is similar to 4 out of 6 (66%) studies (**Section 4.5.1**)

T stage: Anti-p53 was not associated with T-stage in this thesis, which is consistent with all 3 (100%) of the other studies reporting this (**Section 4.5.1**)

N-stage: Anti-p53 was not associated with nodal involvement in this thesis, which is consistent with 5 out of 8 (63%) studies (**Section 4.5.1**)

M-stage: Anti-p53 auto-antibody was not associated with metastases in this thesis, which is consistent with 5 out of 7 (71%) studies. (**Section 4.5.1**)

Differentiation: Anti-p53 auto-antibody was not associated with poor differentiation which is consistent with 6 out of 7 (86%) studies (**Section 4.5.1**)

Location: Anti-p53 auto-antibody was not associated with location, which is consistent with 6 out of 7 (86%) studies (**Section 4.5.1**)

Hence, this patient cohort and anti-p53 association with clinico-pathological factors is consistent with current evidence. This strengthens the data validity of this thesis cohort in long-term survival analysis of anti-p53 auto-antibody in CRC (**Chapter 4**).

Table 4.11: Association between anti-p53 auto-antibody and clinico-pathological parameters of CRC in all published ELISA studies, except Muller et al (immunoblot).

Author (Year)	Age	Gender	Dukes'	TNM	T	N	M	Location	Diff.	Others	Prognosis
Current (2010)	X	X	X A/B *vs.* C/D	X I / II *vs.* III/IV	X T1/2 *vs.* T3/4	X N0 *vs.* N1	X M0 *vs.* M1	X Colon *vs.* Rectum	X Well/Mod *vs.* Poor	X mucin	X
Nozoe (2007)	X	X	√ A=0/3	√ T1/2 *vs.* T3/4	X	√ N0 *vs.* N1	NR	X	X Well *vs.* Mod *vs.* Poor	√ lymphatic invasion	NR
Muller (2006)	X	X	X A=0	X	NR	X N2/N3(NS)	X M1(NS)	NR	X Well *vs.* Mod. *Vs.* Poor (NS)		NR
Chang (2005)	NR	X	NR	X	NR	NR	NR	X	√ Well *vs.* Mod *vs.* Poor	X Mucin (NS)	X
Lechpammer (2004)	X	X	√ A =0/28 A *vs.* B A *vs.* C	NR	NR	√ A *vs.* C **X** B *vs.* C	X	NR	X Well *vs.* Mod *vs.* Poor		NR

Broll (2001)	**NR**	**NR**	**X**	**X**	**NR**	**NR**	**NR**	**X**	**X**	$>10cm^3$ (NS)	**X**
Tang (2001)	**X**	**X**	**NR**	**X** I&II *vs.* III *vs.*IV	**X**	√ >10 nodes X <10 nodes	√ M0 *vs.*M1	√ Left, rectum *vs.* right	**X**	**X** mucin	√ Univariate only
Shiota (2000)	**X**	**X**	√ A-C *vs.* D	√ I-III *vs.* IV	**NR**	**X** A/B *vs.* C/D	√ I-III *vs.* IV	**X**	**X**	$>50cm^3$, CEA, Ca19-9 NS	√ Multivariate
Bielicki (1999)	**X**	**X**	**X** A *vs.* B/C/D A/B *vs.*C/D	**NR**	**NR**	**X**	**X**	**X**	**NR**	>50mm diameter NS	**NR**
Houbiers (1995)	**X**	**X**	**X**	**NR**	**NR**	**X**	**X**	**NR**	√ Well *vs.*Mod *vs.* Poor	√ shape, vascular invasion	√ A & B1 only Univariate
Author (Year)	**Age**	**Gender**	**Dukes'**	**TNM**	**T**	**N**	**M**	**Location**	**Diff.**	**Others**	**Prognosis**

The yellow cells indicate significant association between anti-p53 auto-antibody and a clinico-pathological parameter in that study. The smaller font indicates the groups compared in the study. (X) No association; (√) Significant association. (NS) Increased trend but not Significant; (NR) Not reported; (Diff) differentiation; (Mod) moderate; (mucin) mucinous CRC

4.5.1 Anti-p53 auto-antibody and Clinico-pathological features of CRC

The anti-p53 auto-antibody frequency in this thesis (21.7%) is consistent with the mean 22% frequency from all studies over the last 30 years (**Table 3.1**) and 19% in ELISA only studies (**Table 3.2**). Two ELISA studies reported an unusually high frequency of 63% and 47% but the discrepant results in these studies have been discussed (Nozoe et al., 2007, Takeda et al., 2001b) (**Section 3.5.2**). The age and gender distribution was similar to other studies (and that expected of a CRC population) with no differences in anti-p53 auto-antibody frequency which is also consistent with other reports. The stage distribution was consistent with other studies which similarly demonstrate smaller proportion of Dukes' A & D stages with majority of patients in Dukes' B & C. This contrasts with CRC population trends of up to 30% Dukes' D at diagnosis (Andreoni et al., 2007). The discrepancy between study population and general CRC population is due to a significant proportions of Dukes' D patients presenting with gastrointestinal obstruction / perforation which requires emergency surgery, and hence are not included in trials. Another substantial proportion of Dukes' D patients are diagnosed electively and receive palliation only including chemotherapy and palliative surgery, but not curative surgery. In this study, only Dukes' D CRC, operated on electively and with curative intent were included resulting in a small proportion of highly selected Dukes' D patients (4%), compared to the CRC population trends of 30%.

There was no association between anti-p53 auto-antibody and CRC stage which is consistent with most reports. The four studies reporting a correlation between anti-p53 auto-antibody and advanced stage is due to low numbers of Dukes A CRC (0/3) (Nozoe et al., 2007), or unusually low sero-positivity in early CRC (0/28)

(Lechpammer et al., 2004) or biased classification (Dukes A-C *vs.* D) (Shiota et al., 2000). The fourth, and the largest anti-p53 auto-antibody in CRC study by Tang et al, reported increased anti-p53 auto-antibody with N0-N2 *vs.* N3 disease progression, a conclusion again based on biased selective group analysis, which when corrected for, showed no association between anti-p53 auto-antibody and TNM Stage (Tang et al., 2001) (**Section 3.5.2.1**). Hence, anti-p53 is not associated with overall Dukes' or UICC TNM CRC stage.

4.5.2 Anti-p53 auto-antibody and prognosis

The reported prognostic significance of anti-p53 auto-antibody in CRC was discussed (**Section 4.4.5**) and results of this thesis were compared to other studies (**Table 4.11**). This table shows that anti-p53 auto-antibody only displays prognostic value when anti-p53 is associated with other poor prognostic indicators within the study.

4.5.2.1 Overall and Disease-Free Survival

The median 97 month follow-up for survival analysis is the longest published follow-up period to date and reflects the primary objective of this thesis and the pre-set inclusion criteria of minimum 5 year follow-up. The closest published follow-up period to this was median 36 months (Chang et al., 2005, Houbiers et al., 1995). Univariate analysis showed advanced Dukes' C&D, or Stage III & IV, nodal involvement (N1 / 2), metastasis at diagnosis (M1) to be poor prognostic indicators in overall survival (OS) (**Table 4.5**). This is consistent with published evidence and strengthens the data validity of this cohort. Anti-p53 auto-antibody did not predict OS.

There smaller sample size (68) for DFS analysis is expected in a population with median age 70 years, co-morbidity, cancer diagnosis, major surgery and median follow-up of 97 months. No patients "with disease" were alive at the end of this study due to the natural history of CRC. Patients with metastases at presentation, or those with recurrence are unlikely to survive this follow-up period. The rate of attrition in this and many other survival graphs beyond 50 months follow- up increases (**Appendix 7.13**), most likely due to co-morbidity in this already elderly population.

The overall median DFS was 73 months. Univariate analysis showed advanced Dukes' stage and TNM stage, nodal involvement and metastases to be poor prognostic indicators (**Table 4.6**) which is consistent with other studies. Only four factors were selected for inclusion into the Cox-regression model to reduce over-analysis - anti-p53 auto-antibody (the study objective), and Dukes' stage, T – stage and CRC differentiation. TNM stage is equivalent to Dukes' stage and its inclusion would dilute any prognostic significance of anti-p53 auto-antibody. Similarly, N- and M- stage were already categorised within Dukes' stage and would also have diluted any subtle prognostic significance. Of the remaining factors (differentiation and CRC site), poor differentiation was selected as it had stronger adverse prognostic significance than location. Multivariate DFS analysis showed only Dukes' stage to be an independent prognostic indicator which is consistent with all reports.

A few studies report anti-p53 auto-antibody prognostic significance in univariate analysis which is always lost in multivariate analysis supporting the observation that this is due to anti-p53 auto-antibody association with adverse clinico-pathological parameters (Shiota et al., 2000, Kressner et al., 1998, Houbiers et al., 1995). The only study to report independent anti-p53 auto-antibody prognostic significance in multi-variate analysis linked anti-p53 auto-antibody extremely strongly with metastatic disease and should be disregarded. Anti-p53 auto-antibody significance was secondary to a vast array of traditional prognostic factors (e.g. stage). Incredibly, anti-p53 auto-antibody even had less prognostic value than CA 19-9, a pancreatic tumour marker which is not recommended for used in CRC or American and European pancreatic cancer guidelines (Locker et al., 2006, Duffy et al., 2009).

4.5.2.2 Local Recurrence and Metastases

Subtle prognostic indicators (e.g. differentiation) can be over-shadowed by the influence of stronger factors in multi-variate analysis. Poor differentiation did not display prognostic significance in this thesis. Interestingly, when stage was excluded, poor differentiation was able to predicted post-operative metastases at the 90% CI, but not 95% CI (**Section 4.4.5**). This may explain the divergence in the DFS curves for differentiation (**Appendix 7.14**). Poor differentiation had significantly lower DFS in up to years following diagnosis which is the period when metastases most likely occurs. However, the DFS curves converge beyond 5 years when metastases are rare. Poor differentiation may not display prognostic significance as it was a weak prognostic indicator and its effect was masked by incorporation of Dukes' D or M1 status into survival analysis. As this supported the notion than subtle prognostic effects can be masked by strong prognostic indicators, anti-p53 auto-antibody was examined independently for ability to predict local recurrence or metastases. Anti-p53 auto-antibody again did not predict disease progression (**Section 4.4.4**)

4.6 Summary

Table 4.11 compares reported associations between clinico-pathological parameters, prognosis and anti-p53 auto-antibody in individual studies. It is clear that anti-p53 auto-antibody only displays prognostic significance if there is a correlation between the auto-antibody and other adverse prognostic indicators within the study. No study to date reports an independent adverse prognostic significance without the auto-antibody being associated with other prognostic indicators.

The true prognostic significance of a survival variable can only be established in an adequately powered trial. Assuming 50% survival at 5 years for all Dukes' stages, an average of 1500 patients per group would be required (assuming no drop-out) to detect a 5% difference. i.e. 3000 anti-p53 auto-antibody positive patients (two-tailed analysis, α 0.05; β 0.2). The sero-positivity is approximately 20% in CRC patients, thus requiring 15,000 patients, with 0% drop-out for 5 years, to produce the 3,000 patients for analysis. Based on conclusions of previous studies and the findings of this thesis, a trial of this magnitude is clinically and logistically unfeasible.

The majority of prognostic studies report short to medium term follow-up. This is the first study to confirm anti-p53 auto-antibody has no prognostic significance with extended follow-up into the long-term. Anti-p53 auto-antibody is a para-neoplastic phenomenon which occasionally accompanies advanced tumours but does not independently possess short or long-term prognostic value.

5 Chapter 5: Optimisation of a Method to Detect Anti-hTERT auto-antibody

5.1 Introduction

The importance of p53 inactivation and hTERT up-regulation in the "2-hit" hypothesis in overcoming M1 (p53) and M2 (hTERT) mortality barriers for cancer cells to attain immortality has been discussed (**Section 1.3.4**). The humoral response to p53 inactivation, commonly regarded the "first hit", was demonstrated earlier in this thesis. The humoral response to hTERT re-activation, the "second-hit", has only been detected in one study which used recombinant hTERT as the target antigen in a cohort of patients with hepatocellular carcinoma (Masutomi et al., 2002). There have been no further publications since on the isolation of hTERT or the detection of anti-hTERT auto-antibody.

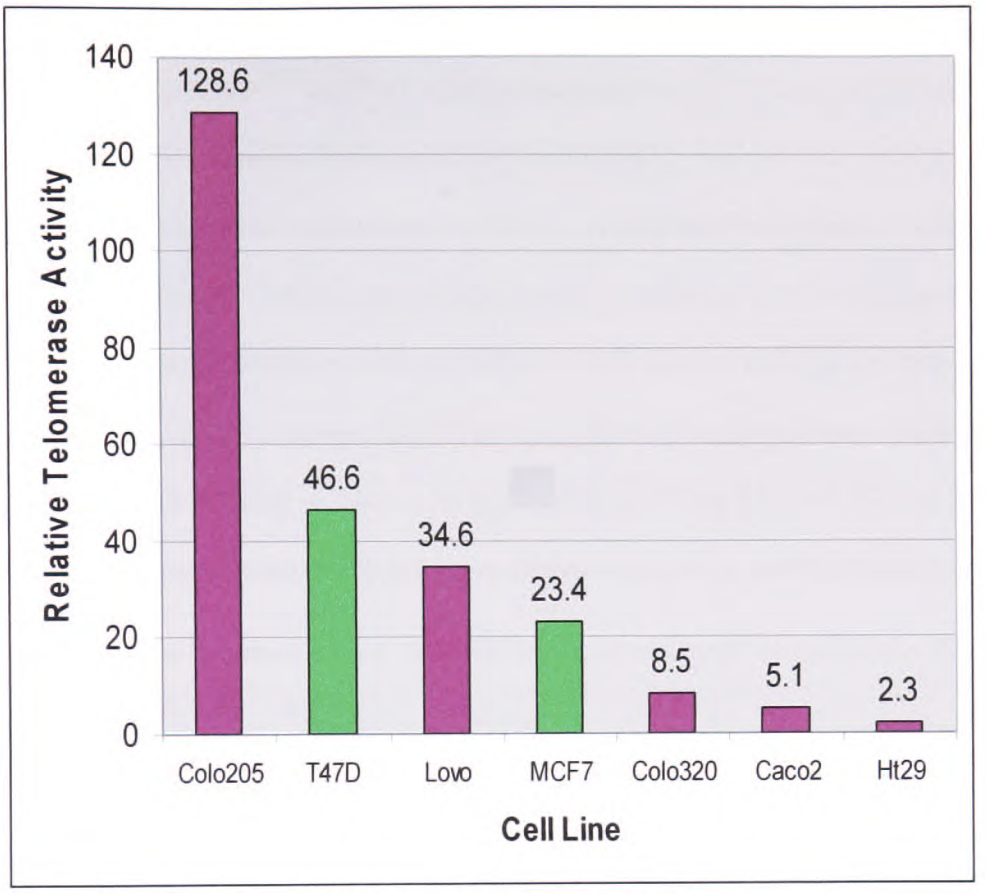
The aim of the thesis was to develop an alternative method to detect the anti-hTERT auto-antibody response. The anti-hTERT auto-antibody could be correlated with the anti-p53 auto-antibody to investigate the significance of the combined presence of both humoral responses to these two key events in CRC carcinogenesis. The initial objective was to develop a method of isolating hTERT which was more accessible than molecular recombinant technology. The isolated hTERT would be used to detect anti-hTERT antibody in Western Blot (WB), and later developed into a screening tool using ELISA. The processes of optimisation along with suggestions for improvements are discussed in this chapter.

5.2 Selection of Cancer Cells based on Telomerase Activity

Telomerase activity (TA) is reactivated in cancer by hTERT re-expression. Telomerase activity is required for cancer cell immortality (Shay and Wright, 2000, Hahn et al., 1999, Kim et al., 1994). Several cancer cell lines were examined for telomerase activity using Telomerase Repeat Amplification Protocol (TRAP) in spectro-photometric ELISA. (TeloTAGGG Telomerase PCR-ELISA^{PLUS}, Roche Diagnostics Ltd., West Sussex, UK) (**Figure 5.1**) (Amarnath, 2004). Detailed information of individual cancer cell lines is shown in **Appendix 7.3.**

Cancer cell lines were initially selected based on telomerase activity rather than cell of origin (breast or colon) for two reasons. Firstly, cells with the highest telomerase activity are likely to contain the largest amount of hTERT which maximizes the chance of hTERT detection. Secondly, several cell lines with different levels of TA could be included to determine if anti-hTERT levels corresponded with levels of telomerase activity. These selected cancer cell lines were subdivided to high (Colo205, T47D), moderate (LoVo, MCF7) and low TA cells (Colo320, Caco). However, early experiments were unable to detect any hTERT, possibly due to insufficient hTERT quantities. Hence subsequent experiments used only Colo205 cell lysates which had the highest TA and theoretically, the highest hTERT load. Colo205 was also an adenocarcinoma of colorectal origin.

Figure 5.1: Telomerase activity in cancer cell lines (Amarnath, 2004).



Purple column (colon origin); Green column (Breast origin)

High activity: Colo205, T47D

Moderate activity: Lovo, MCF7

Low activity: Colo320, Caco, Ht29

Details of cancer cell origin listed in **Appendix 7.3**

5.3 Western Blot of Streptavidin Immuno-affinity Column Elute

The cancer cell lysates (5×10^6 - 1×10^8 cell equivalents / 40µl) were passed down the streptavidin immuno-affinity column (**Section 2.4.3**). The elute was used in WB with 5% PAGE, native and reducing conditions and rabbit anti-telomerase (ab32020, Abcam, **Appendix 7.7**) based on preliminary data detecting hTERT in circulating tumour cells in the laboratory (Foster, 2005). hTERT was not detected and WB conditions were varied substantially by changing the acrylamide content, native *vs.* non-native and reducing *vs.* non- reducing conditions (**Table 5.1**). All WB and ECL were also repeated twice to minimise non-detection caused by methodological error.

Table 5.1: WB conditions. Each WB was repeated twice with multiple ECL exposure

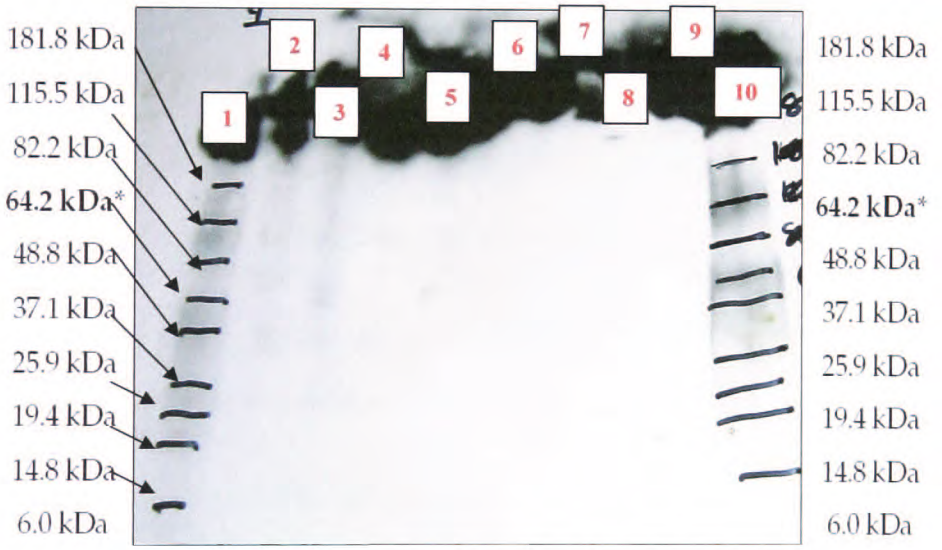
Acrylamide %	Native	Reducing	ECL
5%	Native	Reducing	If no detection after 5 minutes, ECL repeated with 20 minute exposure, 40 minute exposure and 60 minute exposure
		Non reducing	
	Non-native	Reducing	
		Non reducing	
10%	Native	Reducing	
		Non reducing	
	Non-native	Reducing	
		Non reducing	
15%	Native	Reducing	
		Non reducing	
	Non-native	Reducing	
		Non reducing	

hTERT was not detected despite the all the conditions above, performed in duplicate. It was possible the elute did not contain hTERT due to the harsh (pH 2.2) elution conditions used in the column which could also have denatured hTERT.

5.4 Western Blot on Cancer Cell Lysate

The next WB were performed directly on cell lysates using conditions in **Table 5.1**. This avoided column-related factors and elution conditions. TERT was not detected. The WB methodology was then examined in a step-wise manner which were performed entirely on Colo205 cell lysate to increase hTERT concentrations. hTERT was not detected. The various cell lysate concentrations were increased to maximise the amount of hTERT. hTERT was still not detected despite maximum possible lysate concentrations evidenced by lysate immobility in the wells (**Figure 5.2**).

Figure 5.2: Protein migration with maximal cell lysate concentrations



Lane 1&10: Benchmark™ Protein Ladder (labels and molecular weights shown on either side of figure); Lane 2&3: Colo205 ($1x10^8$ ceq); Lane 4&5: Colo205 ($1x\ 10^7$ ceq); Lane 6&7: MCF7 ($1x10^8$ ceq); Lane 8&9: Colo320 ($1x10^8$ ceq)

HeLa nuclear extract was used to increase hTERT in further WB (**Table 5.1**). hTERT was not identified. The reasons were narrowed down to the primary antibody (**Section 5.7**) or the WB, which was examined (**Section 5.5**)

5.5 Precast Gels and Protein Separation Process

The Pierce Precast Gels were available after the initial WB. The 4% - 20% precast gel were used as hTERT (Mw: 120 – 127) should be adequately separated based on manufacturers' information. Coomassie stain on "in-house" and precast gels showed similar resolution in both gels. The precast gels demonstrated significant advantages over "in-house" preparation gels. These were:

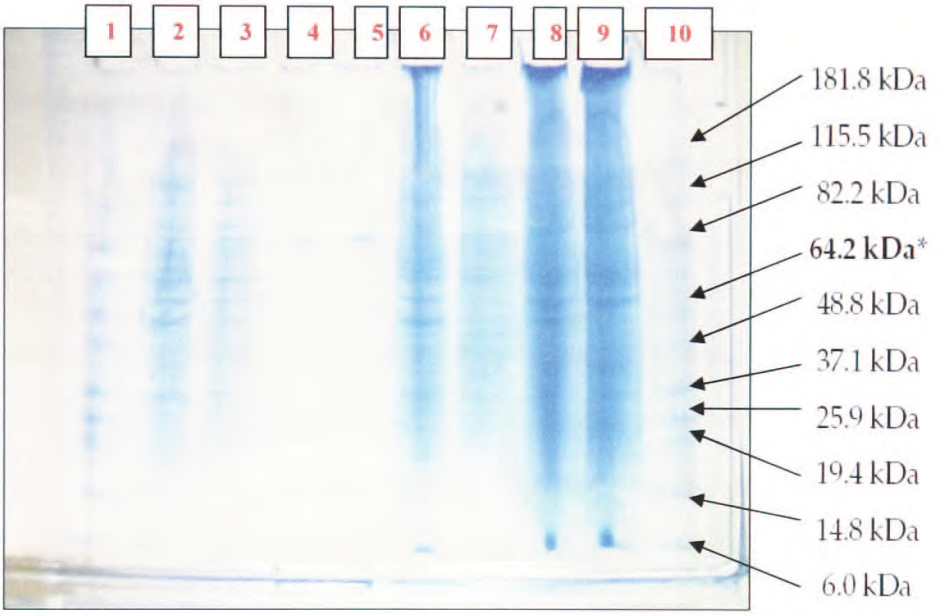
1. avoidance of technical problems e.g. gel not setting/ gel distortion or spillage during polymerisation/ setting apparatus problems
2. decreased electrophoresis time of 45 minutes compared with 60-90 minutes for in-house gels
3. reliability and robustness - precast gel cassettes were less likely to split/separate during PAGE and gel recovery
4. availability – precast gels can be stored at 4°C with 18-month shelf life, and is ready to use immediately
5. no time required for gel preparation or waiting for polymerisation, thus allowing more experiments to be conducted in a day

The precast gels were used in all remaining WB and after the protein separation had been validated by Coomassie staining.

5.5.1.1 Coomassie stain validation of Precast Gels

The Coomassie stain (**Figure 5.3**) demonstrated the adequacy of the PAGE separation. Colo205 cell lysate was separated on the precast gel, and then incubated with Coomassie stain, the washed in distilled H_20.

Figure 5.3: Coomassie stain of cell lysates in precast gel



Lane [1&10]: Benchmark™ Prestained Protein Ladder.

Lane [2 – 9]: Duplicate Colo205 lanes of 5 x 10^6 ceq.

Lane [2 & 3] 1:200dilution;

Lane [4 & 5] 1:500 dilution,

Lane [6 &7] 1:50 dilution

Lane [8 & 9] Neat cell lysate

5.6 Validity of the WB method

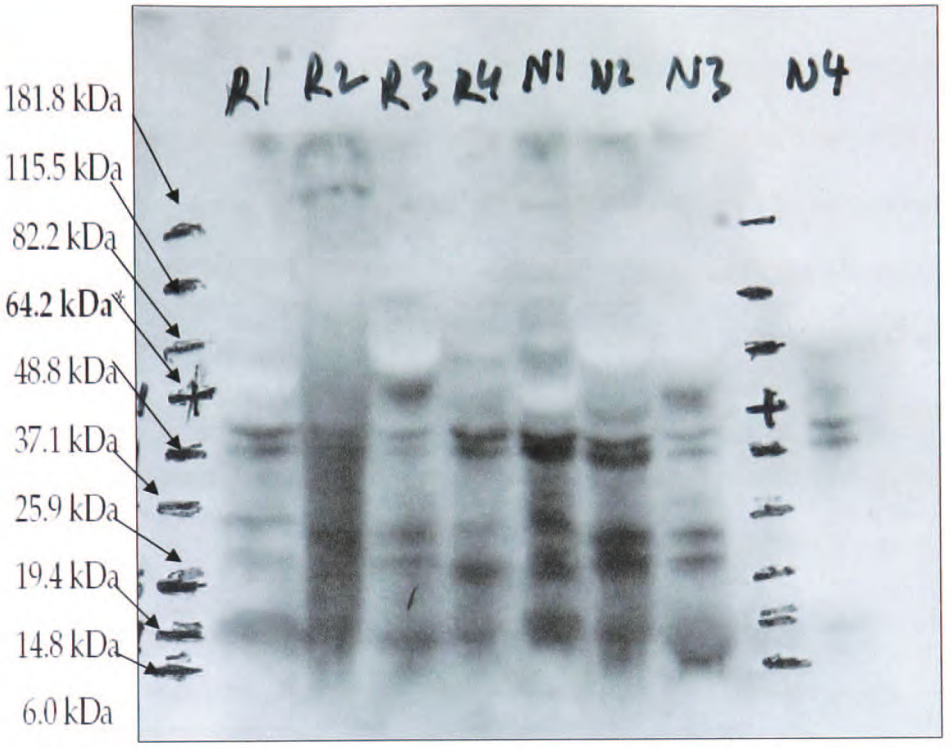
The WB process was tested to confirm a working WB methodology. Several conditions were stipulated to ensure a WB as close as possible to those used in attempts to isolate hTERT.

Firstly, an intra -nuclear protein was required as hTERT is intranuclear and hTERT absence may be caused by harsh cell lysis conditions required to break the nucleus which may also denature the protein. Alternatively, insufficient lysis of the nucleus could have resulted in the nucleus and its associated proteins being discarded in the supernatant.

Secondly, the secondary antibody used required to be the same as that used in WB for hTERT. The two primary anti-hTERT antibodies used at this stage were goat anti-hTERT (Santa cruz, sc-7212) and rabbit anti-hTERT (Abcam, ab32020) and hence a secondary anti-goat or an anti-rabbit would be required.

A WB using the Gemini intra-nuclear protein was tested in WB as this fulfilled both the above conditions. The WB showed a clear band at 33kDa, with potential break-down products or non-specific binding occurring at 45kDa in glioblastoma samples **Figure 5.4** (Yousaf, 2010). The secondary antibody used in this WB (STAR54, Serotec, Sheep Anti-rabbit IgG: HRP, **Appendix 7.8**) was also the same secondary antibody used in WB for anti-hTERT.

Figure 5.4: WB for Gemini proteins in glioblastoma (Yousaf, 2010)



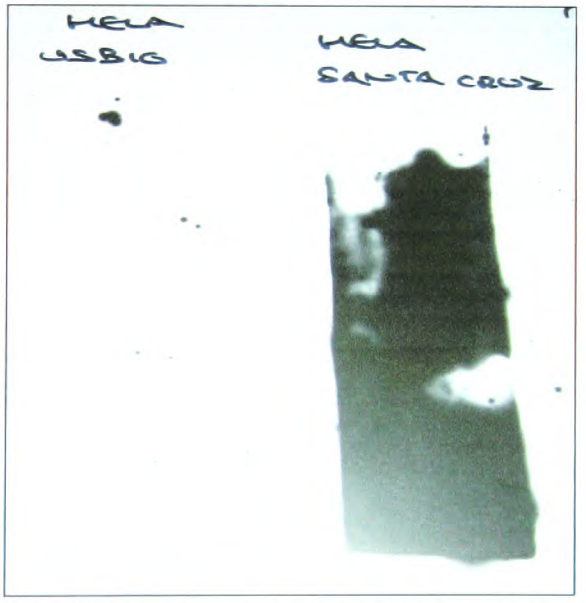
R1-R4: Glioblastoma samples; N1-N4, normal cell samples ($1x10^7$ ceq.)

This WB not only confirmed the validity of the WB method and ECL detection, but more importantly, also confirmed intra-nuclear proteins are detectable using this WB technique. Furthermore, it confirms that the secondary anti-rabbit antibody binds to the primary rabbit antibody. Additional experiments by Mr. Yousaf, a laboratory colleague, also showed that no reactivity was obtained with the secondary antibody alone (figure not shown) (Yousaf, 2010). This suggested that the most likely reason for negative WB was a defective primary anti-hTERT antibody.

5.7 Examination of the anti-hTERT antibodies

Preliminary work identified hTERT in circulating tumour cells using rabbit anti-hTERT (Ab32020, **Appendix 7.8**) which did not provide definite bands with all samples. The same antibody was used initially with varying cell lysate concentrations. No hTERT was detected. The company was contacted regarding the non-performance of this reagent but it had been withdrawn. No reason for the withdrawal was available. The WB (**Figure 5.4**) had already confirmed that intranuclear proteins extracted using the same lysis buffer could be detected. Three new primary anti-hTERT antibodies were tested (sc-7214, USBiotechnologies, ab5181) (**Figure 5.5**). HeLa extract was used as this was the manufacturer's control reagent for primary antibody.

Figure 5.5: Santa Cruz and US Biotechnologies with HeLa nuclear extract



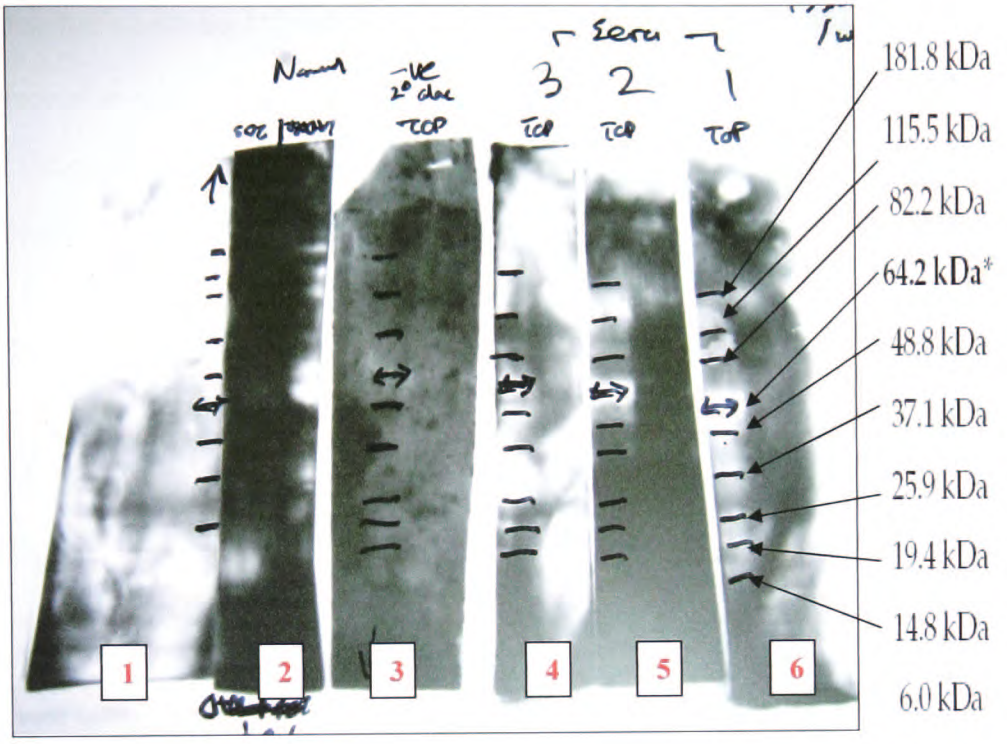
Primary antibodies: Santa Cruz, sc-7214 at 1:200 dilution; USBiotechnologies 1:100 dilution (initially 1:500);
Secondary antibodies at 1:5,000 (STAR54, Serotec) and 1:20,000 dilution (STAR88P, Serotec)

The Santa cruz (sc-7214) anti-hTERT showed large amounts of non-specific binding to the HeLa extract. In contrast, the USBiotechnologies (**Figure 5.5**) and Abcam (figure not shown) showed no binding to the HeLa nuclear extract. As previous WB with the other intranuclear proteins had already showed that the secondary antibodies bind specifically with minimal background staining (**Figure 5.4**), the problem was not with the WB technique or secondary antibodies, but with the primary anti-hTERT antibodies. The manufacturers were contacted again. The Santa Cruz and USBiotechnologies antibodies had again been withdrawn and replaced with the next generation of anti-hTERT antibodies. As the previous anti-hTERT antibodies from these companies had already been tested without success, a final attempt was made with Abcam anti-hTERT (ab 5181). Again, no hTERT was detected using this antibody with Colo205 cell lysate ($5x10^6$ ceq) or HeLa nuclear extract.

5.8 Human Sera in WB

Five different anti-hTERT antibodies (**Appendix 7.7**) had been tested, none of which displayed any specific protein-binding of the eluate from immuno-affinity column or the various cancer cell lysates or the HeLa nuclear extract. In a final attempt to obtain a positive control and demonstrate specific protein binding, patient and control sera were used (**Figure 5.6**). The postulation was that CRC patient sera may contain antibodies against a variety of TAA including hTERT; and WB bands detected in patients' but not control subjects' sera, suggests an antibody unique to CRC. This was a validation method used by Masutomi et al to demonstrate anti-hTERT. The recombinant hTERT on membrane showed a distinct band only with patient sera.

Figure 5.6: WB with serum and negative controls.



[1] Secondary-HRP only;

[2] Lysate, control sera, secondary HRP;

[3] Lysate, secondary-anti-human HRP;

[4-6] Lysate, patients sera (Patient 1, 2 & 3), secondary-HRP

The patients' sera contained a vast array of antibodies which bound non-specifically to Colo205 lysate. Despite modifying the blocking, washing and ECL conditions vastly, the results ranged from over-developed membranes which obscured the potential regions where hTERT would be expected to be present (figure not shown), to a gray non-specific background.

The next observation of interest was in the 2 negative controls [Membrane 1 & 3]. The first membrane [1] contained no cell lysate, primary antibody or serum and was incubated only with the secondary anti-human HRP. This displayed non-specific binding suggesting the secondary antibody could bind non-specifically, even to an empty membrane.

The second observation was in negative control [3] which consisted membrane with Colo205 lysate with the secondary anti-human antibody, but not primary anti-hTERT antibody or sera. This also showed general protein binding. This suggested the secondary anti-human would again bind directly to the Colo205 cell lysate without the requirement of primary antibody or patient sera. This may be expected as Colo205 is of human origin.

The conclusion from the early optimisation process was that the primary anti-hTERT antibodies were not sufficiently specific. This final experiment concludes that normal subject sera cannot be used to identify hTERT due to non-specificity; and a secondary species specific antibody (in this case, anti-human) also cannot be used to identify hTERT in cancer cell lysates using sera as there are far too many human-specific proteins in cancer cell lysate. A purified hTERT is required.

In summary, after 18 months and despite (a) all the varied WB conditions used, (b) performed in duplicate resulting in an excess of 100 membranes, (c) each with further multiple ECL exposures; and (d) having proven that the WB technique itself was viable for intranuclear protein detection and secondary antibody specificity, the optimisation process was stopped.

5.9 Discussion

The authors of the only other study on anti-hTERT auto-antibody produced hTERT using insect-expressed recombinant, initially in 2000, and only later detected serum anti-hTERT auto-antibody in 2002 (Masutomi et al., 2000, Masutomi et al., 2002). The time lag between the two studies and the authors' description of the difficulty in expressing and isolating hTERT with standard bacterial expression systems and eventual resort to baculovirus system demonstrates the complexity in obtaining pure hTERT even with advanced molecular and recombinant techniques. Furthermore, there have been no further reports of anti-hTERT since 2002 which again suggests hTERT production or detection was not viable at that stage. Hence one of the aims of this study was to isolate hTERT directly without the need for extensive molecular biology techniques, and establish an ELISA for anti-hTERT detection using hTERT isolated by standard laboratory techniques.

The optimisation was severely hampered by the inability to confirm hTERT presence by WB. This meant that it was not possible to confirm or refute if hTERT was isolated from the lysates. Although all the primary anti-hTERT antibodies tested were sold as suitable for WB, none worked in this current study and several had been withdrawn during this thesis period.

The immuno-affinity column and WB is a standard method of protein isolation and detection (Springer, 1996, Harlow and Lane, 1988). This was unsuccessful as either the hTERT was not present, or if present, it was not captured by the anti-hTERT. Assuming the primary antibodies were functional, then hTERT must indeed be

absent in the column eluate. The possible reasons for this are that hTERT was not bound when passed down the column, hTERT was denatured during the elution (pH 2.2) process, or hTERT levels were too low for detection, or a combination of 2 or more of these possibilities. These potential issues were addressed first, prior to addressing the potential issues with the primary antibody

In order to address this, firstly, the column was discarded and WB performed directly on the cell lysate to avoid potential column related factors such as non-capture of hTERT and the elution conditions. hTERT was not detected in WB of cell lysates. Secondly, the cell lysate concentrations were increased to the maximum possible, and then switched to HeLa nuclear extract to address the issue of potentially insufficient hTERT for detection. Again, hTERT was not detected.

As none of these solutions provided identifiable hTERT, the WB process itself required re-examination. A comparative WB was designed to test this WB ability to detect intranuclear proteins. This WB showed Gemini intranculear protein could be detected using this method. Furthermore, the secondary antibody was sufficiently specific. The results of these examinations led to the possibility of non-functioning anti-hTERT antibodies. This would also explain the lack of binding within the immune-affinity column, a usually reliable method of protein isolation. This was a major negative result and setback in the early work, but did help direct subsequent experiments and targeting cell lysates only with different primary and secondary antibodies. However at the end of the optimisation development, this could not be tested directly as there was no means of proving or disproving the presence of hTERT in any of the samples as all the primary antibodies were not sufficiently specific.

Other hTERT isolation methods were considered. Magnetic beads have been used to isolate antigens and had previously been used in this laboratory to isolate circulating tumour cells from blood of CRC patients (Thornton, 2003, Horgan and Shaw, 1995) (Khair, 2010). The magnetic beads required streptavidin coating, incubation with biotinylated anti-hTERT antibody and then incubation with cancer cell lysate. A magnetic field could isolate the microbead-streptavidin ↔anti-hTERT ↔ hTERT complex. However, the harsh elution conditions in the immuno-affinity column could also cause hTERT denaturation here. Alternative methods of enzymatic rather than chemical cleavage to break the biotinylated hTERT ↔ hTERT bond were explored but were not attempted as there was increasing evidence that the anti-hTERT primary antibodies were non-functioning. The development of alternative methods would be futile without a functioning anti-HTERT to capture or detect hTERT.

The authors (Masutomi et al., 2002) were contacted but the recombinant hTERT was not available. As large amounts would be required for WB optimisation and subsequent development of ELISA screening method, commercial laboratories had been contacted but were unable to help as this product is not available and custom production was prohibitively expensive. The idea of producing recombinant hTERT in this laboratory had also been explored. This was unfeasible due to time constraints and potential problems with Genetic Manipulation approval as recombinant hTERT could be considered a potential biohazard due to its key role in tumorigenesis. It became increasingly apparent in the final stages of this study that the recombinant hTERT was required in order to develop any further methodology as the anti-hTERT antibodies could not be relied upon.

5.10 Summary

In order to undertake the analysis proposed, it was essential that a proven source of hTERT is obtained, and recombinant technology seems the only realistic technology. However, the authors of the only study to isolate hTERT also describe difficulty in expressing and purifying hTERT even with this technology. Future studies should focus on development of technologies to obtain hTERT, and the raising of an antibody specific to hTERT. Only then can attempts at detecting hTERT be realistically attempted.

6 Chapter 6: Final Discussion

CRC is a common malignancy and a major contributor to cancer related death. It has multifactorial aetiology and multiple biomarkers are implicated in its carcinogenesis. The potential importance of the humoral response to tumour associated antigens, mainly p53 mutation and telomerase (hTERT) re-activation is discussed.

6.1 Limits of current therapy

Surgery is the traditional mainstay in CRC treatment. Advances in neo-adjuvant therapy and modern surgical techniques enable complete tumour resection with clear excision margins. Surgery has potentially reached the limit of its curative role in resection of the primary tumour. The main cause of cancer related-death is local recurrence (despite clear surgical margins) and distant metastases. These issues are addressed primarily by chemo- and chemo-radiotherapy treatments. Significant advancements have been made in these fields. Traditional 5-FU / Leucovorin (LV) treatments have improved disease-free and overall survival substantially by 35% and 22% respectively (O'Connell et al., 2005, Sargent et al., 2001, de Gramont et al., 2000). Additional therapy with oxaliplatin and irinotecan have led to further success in controlling disease (Goldberg et al., 2004, Hurwitz et al., 2004, Andre et al., 2004). The expanding knowledge of tumour molecular biology has led to the addition of immunotherapeutic agents such as EGFR, VEGF and COX-2 inhibitors which all aim to reduce disease recurrence and systemic metastases (Van Cutsem et al., 2008, Cassidy, 2007, Cunningham et al., 2004). However, this remains a blanket treatment

approach, and these treatments are associated with significant morbidity. Furthermore, recent advances now show non-efficacy of these treatments in certain CRC groups due to individual tumour biology (e.g. kRAS and PTEN mutations in EGFR and VEGF inhibition) and highlights the need for continuous research into identification of accurate biomarkers to guide new treatments and reduce disease progression. The humoral antibody response is specific to individual TAA in CRC and may provide benefit in this respect.

6.2 CRC stem cell theory

CRC stem cells are defined as the distinct subpopulation within a heterogeneous tumour that is able to self-renew and differentiate (Yeung et al., 2010, Clarke et al., 2006, Radtke and Clevers, 2005). The traditional cancer model suggests every cancer cell has the ability to progress indefinitely (Stochastic model). The stem cell model suggests that only a few cancer cells are the source of indefinite CRC replication (the "stem cell"). The vast majority of CRC cells arise from these stem cells and do not possess indefinite replicative capacity. Only a few select cells inherit the ability to become future cancer stem cells ("daughter cells") (Bonnet and Dick, 1997). It is though that this subpopulation, and not the rest of the non-stem cancer cells that drives tumour progression in CRC.

The implication of the cancer stem cell model on CRC treatment is that the excision of these cells cannot be assessed despite complete surgical excision as assessed by histopathological examination, thus leaving the possibility of residual cancer stem cells. These residual stem cells may be the driving force for local recurrence despite

complete tumour excision. Certain biomolecular markers have been identified to aid recognition of CRC stem cells but with limited success. The most recent systematic review on colorectal cancer stem cells reported CD-133 as a promising marker but whilst able to identify cells which initiate carcinogenesis was unable to identify CRC cells driving further progression (Yeung et al., 2010). Other markers under investigation include CD44, CD24, EPCAM groups, Wnt and FAP mutation (Haraguchi et al., 2008, Lang et al., 2004, Winter et al., 2003).

Telomerase activity is present at low levels in normal proliferating gastrointestinal crypt cells (Forsyth et al., 2002). It is likely that CRC stem cells arise from these normal self-renewing stem cells as part of the mortality barrier has already been overcome. Thus less mutation are required for carcinogenesis, compared to the normal non-proliferative cells which require more alterations to overcome the mortality barriers (Barker et al., 2009, Boman et al., 2008, Johnston et al., 2007). This increase in telomerase activity (by upregulation of hTERT) in progression from normal to CRC stem cell may be valuable tool in diagnosing CRC. The only study on anti-hTERT auto-antibody reported significant increase serum titres in progression from normal to pre-malignant (cirrhosis) and pre-malignant (chronic active hepatitis) to malignant (HCC). The anti-hTERT response is most likely in response to hTERT upregulation of hTERT suggesting that this response is sensitive to TAA which occur in the pre-malignant stage. This feature could be utilised in various roles in CRC such as screening, or serum testing for potential residual tumour despite histologically completely excised margins, or guiding local excision.

6.3 Screening

Serum anti-hTERT auto-antibodies could be used as an early screening tool to guide endoscopic surveillance. Substantial stage shift was observed in the initial CRC screening program with up to 40% Stage I / II CRC but yet survival benefit is not observed. This may be because of the lag-time required to observe survival differences (5 – 10 years). Anti-hTERT or anti-p53 auto-antibody can be tested more frequently and in the pre-malignant stage which may increase detection of early tumours. Patients positive for the auto-antibody but with no clinical evidence of CRC may benefit from regular screening endoscopy or tests for other occult malignancies.

6.4 Post-operative CRC

Monitoring of serum anti-hTERT titres following surgical resection may aid disease monitoring. Despite complete excision of low risk tumours, the cancer stem cell hypothesis suggests that stem cells may drive tumour progression and remain untreated. No biomolecular markers are able to accurately predict this. Persistently elevated anti-hTERT levels may suggest residual stem cells even if the cells are macro- or microscopically undetectable. The anti-hTERT response could be used to stratify risk of disease recurrence or metastases, and requirement for adjuvant chemo-immunotherapy in the immediate post-operative period. The sensitivity of anti-hTERT to tumour load requires further assessment as elevations in anti-hTERT auto-antibodies may also suggest the re-activation of TAA in preparation for disease recurrence or progression.

6.5 Local excision

There is paradigm shift in the definition of cancer treatment "success". The optimal aim of surgery is no longer lowest recurrence rates, but to balance surgical morbidity with post-operative quality of life (QOL). This is demonstrated by the mandatory inclusion of multiple QOL-related outcomes in all new CRC trials. Local excision has less morbidity but is hampered by high recurrence rates caused by lack of accurate radiological imaging and pathological staging despite addition of features such as mucinous production, signet cell, Kikuchi grade and tumour budding. The auto-antibody response may aid in predicting tumour aggression, or residual CRC, which would then be an indication for extensive radical surgery or adjuvant chemotherapy.

6.6 Final Conclusion

The humoral response is aimed at identifying the tumour-associated antigens and its presence as an early marker in carcinogenesis or disease recurrence. The anti-p53 auto-antibody in CRC is not associated with clinico-pathological or prognostic factors in CRC. Although limited subgroup analysis suggest anti-p53 is a marker of early disease recurrence and may guide screening strategies in high-risk groups, its sensitivity and specificity for CRC is low but may be used in conjunction with other markers as part of a tumour panel. Anti-hTERT may have similar uses. However, the main obstacle in assessing the anti-hTERT response is isolation of hTERT molecule itself and the development of a sufficiently specific antibody. The association between telomerase activity, hTERT expression and anti-hTERT in conjunction with CRC stem cells is an exciting future possibility.

7 Appendix

7.1 Commonly Used Reagents

Ammonium Persulphate (APS); Melford Lab Ltd, Ipswich, Suffolk

Bicine; Melford Lab Ltd, Ipswich, Suffolk

Glycine; Fisher Scientific, Loughborough, Leicestershire

HEPES; Melford Lab Ltd, Ipswich, Suffolk

Sodium dodecyl sulphate (SDS); Melford Lab Ltd, Ipswich, Suffolk

Tris Base; Melford Lab Ltd, Ipswich, Suffolk

Phospate Buffered Saline (PBS); Sigma-Aldrich, St. Louis, MO, USA

Methanol; Department of Chemistry, University of Hull

TEMED; Sigma-Aldrich, St. Louis, MO, USA

2β Mercaptoethanol; Sigma-Aldrich, St. Louis, MO, USA

Butanol; BDH Ltd., Poole, UK

Amersham ECL Plus™ Western Blotting Detection Reagents; GE Healthcare, Buckinghamshire, UK

7.2 Cell Lysate Preparation: Lysis Buffer

Lysis Buffer
10mM Tris Hcl, pH 7.4
1% (w/v) NP-40 Iqepal A – 630 (Sigma-Aldritch, Poole, UK)
150 mM NaCl
1 mM EDTA
1:200 Protease Inhibitor Cocktail Set III (Calbiochem, Merck4Biosciences, Darmstadt, Germany)

7.3 Cell Lysate Preparation: Cancer Cell Lines

Cell Line	Line	ATCC No.	Origin and Description
MCF 7	Breast	HTB - 22	69 year female Caucasian, adenocarcinoma, pleural effusion, ER+,
T47D	Breast	HTB - 133	54 year female, adenoarcinoma, pleural effusion ER, PG, AR, calcitonin, GC, Prolactin +
Caco-2	Colon	HTB - 37	Monolayer heterogeneous adenocarcinoma, EGF+
LoVo	Colon	CCL - 229	56 year male, Dukes' C, Grade 4, homogenous adenocarcinoma, left supraclavicular nodule, myc, myb, ras, fos, p53 +
Ht29	Colon	HTB - 38	44 year female Caucasian adenocarcinoma, mucinous, CEA+; myc, ras, myb, fos, p53 +
COLO320	Colon	CCL - 220.1	55 year female Caucasian, Dukes' C, adenocarcinoma, CEA
Colo205	Colon	CCL - 222	70 year male Caucasian, Dukes' D, adenocarcinoma, ascitic fluid, CEA+, IL-10+

ATCC, American Type Culture Collection (Manassas, VA, USA)

7.4 Western Blot: PAGE Gel Recipes

	Reducing	Native
4% Stacking Gel		
Acrylamide	1.3 ml	1.3 ml
1M Tris Base; pH 6.8	1.25 ml	1.25 ml
SDS (w/v) 10%	0.1 ml	X
dH2O	7.4 ml	7.4 ml
TEMED	20 µl	20 µl
APS (w/v) 10%	50 µl	50 µl
10% Separating Gel		
Acrylamide	2.5 ml	3.33 ml
Tris HCl, 1.5M; pH 8.8	2.5 ml	2.5 ml
SDS (w/v) 10%	0.1 ml	X
dH2O	4 ml	4 ml
TEMED	10 µl	10 µl
APS (w/v) 10%	50 µl	50 µl

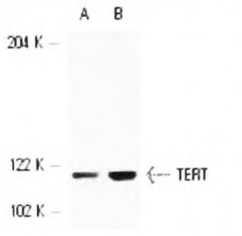
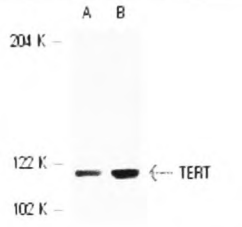
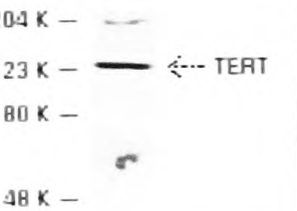
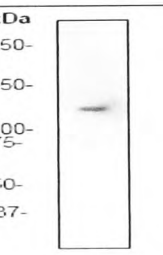
7.5 Western Blot: "In-house" Buffer Recipes

	Reducing	Native
Sample Buffer		
Tris HCl, 1M; pH 6.8	13 ml	13 ml
SDS (w/v) 20%	6.5 ml	X
Glycerol	5.2 ml	5.2 ml
Bromophenol blue (w/v) 0.5%	0.26 ml	0.26 ml
Running Buffer		
Tris Base (Dry)	3 g	3 g
Glycine (Dry)	14.4 g	14.4 g
SDS (w/v) 10%	10 ml	X
dH2O	Make up to 1L	Make up to 1L
Transfer Buffer		
Tris Base (Dry)	3 g	3 g
Glycine (Dry)	14.4 g	14.4 g
Methanol	200 ml	200 ml
dH2O	Make up to 1L	Make up to 1L

7.6 Western Blot: PreciseTM Precast Gel Buffers Recipes

Sample Buffer	
Tris HCl, 0.5M	2.5 ml
SDS (w/v) 10%	4 ml
Glycerol	2 ml
Bromophenol blue, 0.1% (w/v)	1 ml
2-β Mercaptoethanol, 2-5% (w/v)	
dH2O	10 ml
TRIS-HEPES-SDS Running Buffer	
Tris Base	12.1 g
HEPES	23.8 g
SDS	1 g
ddH2O	Make up to 1L
Transfer Buffer	
Tris Base	3 g
Bicine	4. 08 g
Methanol	100 ml
ddH2O	Make up to 1L

7.7 Primary Antibody List

Antibody	Reactivity	Dilutions	Control
Biotinylated anti-hTERT (H-231): sc - 7212 (Santa Cruz, Biotechnology, Autogen Bioclear UK Ltd., Wiltshire, UK)	Mouse, Rat, Human	1:100 – 1:5,000	
Rabbit Anti-hTERT (H-231): sc-7212 (Santa Cruz, Biotechnology, Autogen Bioclear UK Ltd., Wiltshire, UK)	Mouse, Rat, Human	1:100 – 1:5,000	
Goat Anti-hTERT (L-20): sc-7214 (Santa Cruz, Biotechnology, Autogen Bioclear UK Ltd., Wiltshire, UK)	Human	1:100 – 1:5,000	
Rabbit Telomerase antibody [Y182] (ab32020; Abcam plc, Cambridge, UK)	Human	1:100 – 1:1000	
Mouse Telomerase antibody [2C4] (ab5181; Abcam plc, Cambridge, UK)	Human	1:200 – 1:1000	
Rabbit Anti- Telomerase, CT, T2399-15B (USBiological, Massachusetts, USA)	Human	1:200 – 1:1000	No image provided

7.8 Secondary Antibody List

Antibody	Reactivity	Dilutions Used
Mouse Anti-Human (MCA515G; Serotec Ltd, Oxford, UK)	Human IgG2	1:5,000 – 1:20,000
Mouse Anti-Human IgG (Fc):HRP (MCA647P, Serotec Ltd, Oxford, UK)	Human IgG Fc	1:1,000 – 1:15,000
Rabbit Anti-Mouse IgG:HRP. (STAR13B; Serotec Ltd, Oxford, UK)	Mouse and rat IgG	1:2,000 – 1:15,000
Sheep Anti-Rabbit IgG:HRP (STAR54; Serotec Ltd, Oxford, UK)	Rabbit IgG	1:5,000 – 1:20,000
Donkey Anti-Sheep IgG:HRP (STAR88P, Serotec Ltd, Oxford, UK)	Sheep IgG	1:5,000 – 1:20,000

7.9 Control Sample Characteristics

No	Label	Sex	Age	Status
1	Con1	M	62	Healthy
2	Con2	M	67	Healthy
3	Con3	M	75	Healthy
4	Con4	F	65	Healthy
5	Con5	M	65	Healthy
6	Con6	F	72	Healthy
7	V1A	M	71	Healthy
8	V2A	M	67	Healthy
9	r11	M	73	Benign Disease
10	r12	F	56	Benign Disease
11	r13	M	63	Benign Disease
12	r16	M	67	Benign Disease
13	r18	M	73	Benign Disease
14	r19	M	70	Benign Disease
15	r21	F	59	Benign Disease
16	r22	M	77	Benign Disease
17	r23a	M	67	Benign Disease
18	r24	F	65	Benign Disease
19	r27	M	61	Benign Disease
20	r28	F	72	Benign Disease
21	r29	F	61	Benign Disease
22	r31	F	69	Benign Disease
23	r32	F	73	Benign Disease
24	r33	M	85	Benign Disease
25	r35	M	69	Benign Disease
26	r37	F	68	Benign Disease
27	r40	M	76	Benign Disease
28	r44	F	53	Benign Disease

7.10 Plate 1: OD_{450nm} and CoV

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.688	2.663	0.721	0.748	0.112	0.151	0.069	0.082	0.064	0.09	0.245	0.238
Mean	2.6755		0.7345		0.1315		0.0755		0.077		0.2415	
SD	0.0177		0.0191		0.0276		0.0092		0.0184		0.0049	
CoV	0.007		0.026		0.210		0.122		**0.239**		0.020	
B	1.995	2.061	0.091	0.027	0.054	0.056	0.059	0.062	0.214	0.226	0.038	0.038
Mean	2.028		0.0590		0.0550		0.0605		0.2200		0.0380	
SD	0.0467		0.0453		0.0014		0.0021		0.0085		0.0000	
CoV	0.023		**0.767**		0.026		0.035		0.039		0.000	
C	1.508	1.698	0.094	0.091	0.95	0.399	1.187	1.241	0.109	0.069	0.073	0.077
Mean	1.603		0.0925		0.6745		1.214		0.089		0.075	
SD	0.1344		0.0021		0.3896		0.0382		0.0283		0.0028	
CoV	0.084		0.023		**0.578**		0.031		**0.318**		0.038	
D	1.09	1.172	0.106	0.073	0.138	0.164	0.038	0.038	0.058	0.061	0.147	0.142
Mean	1.131		0.0895		0.151		0.038		0.0595		0.1445	
SD	0.0580		0.0233		0.0184		0.0000		0.0021		0.0035	
CoV	0.051		**0.261**		0.122		0.000		0.036		0.024	
E	0.914	0.893	0.373	0.4	0.129	0.165	0.492	0.556	0.073	0.092	0.113	0.077
Mean	0.9035		0.3865		0.147		0.524		0.0825		0.095	
SD	0.0148		0.0191		0.0255		0.0453		0.0134		0.0255	
CoV	0.016		0.049		0.173		0.086		0.163		**0.268**	
F	0.602	0.623	0.174	0.164	0.11	0.167	0.076	0.076	0.089	0.075	0.134	0.152
Mean	0.6125		0.169		0.1385		0.076		0.082		0.143	
SD	0.0148		0.0071		0.0403		0.0000		0.0099		0.0127	
CoV	0.024		0.042		**0.291**		0.000		0.121		0.089	
G	0.229	0.223	0.072	0.075	0.107	0.106	0.137	0.117	1.313	1.276	0.093	0.069
Mean	0.226		0.0735		0.1065		0.127		1.2945		0.081	
SD	0.0042		0.0021		0.0007		0.0141		0.0262		0.0170	
CoV	0.019		0.029		0.007		0.111		0.020		**0.210**	
H	0.019	0.011	0.35	0.318	0.101	0.232	0.051	0.082	0.293	0.268	0.165	0.1
Mean	0.015		0.334		0.1665		0.0665		0.2805		0.1325	
SD	0.0057		0.0226		0.0926		0.0219		0.0177		0.0460	
CoV	**0.377**		0.068		**0.556**		**0.330**		0.063		**0.347**	

The rows below each reading indicate the mean, standard deviation (SD) and the Co-efficient of Variation (CoV), defined as the ratio of the SD to the Mean (SD / M). The red shaded cells indicate CoV > 20% in OD_{450} of duplicate samples. Samples were unable to be re-assayed due to limited availability.

7.11 Plate 2: OD_{450nm} and CoV

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.409	1.717	0.114	0.127	0.106	0.135	0.211	0.166	0.157	0.132	0.112	0.099
Mean	1.563		0.1205		0.1205		0.1885		0.1445		0.1055	
SD	0.2178		0.0092		0.0205		0.0318		0.0177		0.0092	
CoV	0.139		0.076		0.170		0.169		0.122		0.087	
B	1.016	1.052	0.091	0.118	0.106	0.143	0.549	0.6	0.116	0.1	0.009	0.145
Mean	1.0340		0.1045		0.1245		0.5745		0.1080		0.0770	
SD	0.0255		0.0191		0.0262		0.0361		0.0113		0.0962	
CoV	0.025		0.183		**0.210**		0.063		0.105		**1.249**	
C	0.858	1.028	0.082	0.077	0.798	1.017	0.104	0.094	0.107	0.08	0.109	0.103
Mean	0.943		0.0795		0.9075		0.099		0.0935		0.106	
SD	0.1202		0.0035		0.1549		0.0071		0.0191		0.0042	
CoV	0.127		0.044		0.171		0.071		**0.204**		0.040	
D	0.662	0.776	0.103	0.097	0.304	0.254	0.506	0.492	0.071	0.072	0.082	0.097
Mean	0.719		0.1		0.279		0.499		0.0715		0.0895	
SD	0.0806		0.0042		0.0354		0.0099		0.0007		0.0106	
CoV	0.112		0.042		0.127		0.020		0.010		0.119	
E	0.581	0.588	0.113	0.101	0.0085	0.129	0.1	0.096	0.057	0.057	0.099	0.079
Mean	0.5845		0.107		0.06875		0.098		0.057		0.089	
SD	0.0049		0.0085		0.0852		0.0028		0.0000		0.0141	
CoV	0.008		0.079		**1.239**		0.029		0.000		0.159	
F	0.401	0.462	0.086	0.062	0.067	0.073	0.111	0.074	0.076	0.069	0.084	0.114
Mean	0.4315		0.074		0.07		0.0925		0.0725		0.099	
SD	0.0431		0.0170		0.0042		0.0262		0.0049		0.0212	
CoV	0.100		**0.229**		0.061		**0.283**		0.068		**0.214**	
G	0.111	0.099	0.091	0.078	0.092	0.107	0.126	0.109	0.084	0.091	0.072	0.083
Mean	0.105		0.0845		0.0995		0.1175		0.0875		0.0775	
SD	0.0085		0.0092		0.0106		0.0120		0.0049		0.0078	
CoV	0.081		0.109		0.107		0.102		0.057		0.100	
H	0.043	0.046	0.082	0.086	0.096	0.105	0.106	0.107	0.066	0.063	0.091	0.079
Mean	0.0445		0.084		0.1005		0.1065		0.0645		0.085	
SD	0.0021		0.0028		0.0064		0.0007		0.0021		0.0085	
CoV	0.048		0.034		0.063		0.007		0.033		0.100	

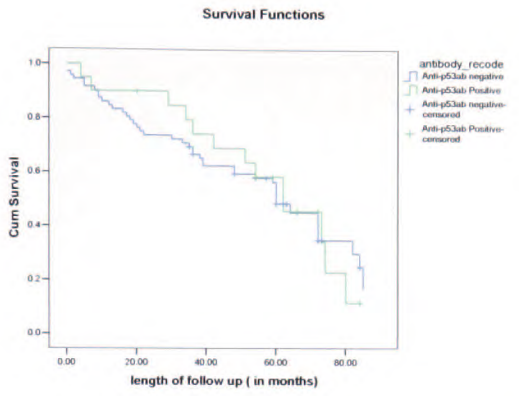
The rows below each reading indicate the mean, standard deviation (SD) and the Co-efficient of Variation (CoV), defined as the ratio of the SD to the Mean (SD / M). The red shaded cells indicate CoV > 20% in OD_{450} of duplicate samples. Samples were unable to be re-assayed due to limited availability.

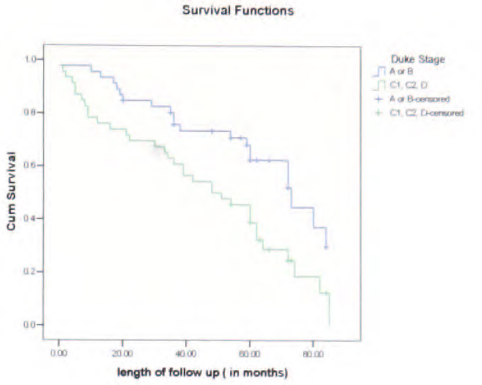
7.12 Plate 3: OD_{450nm} and CoV

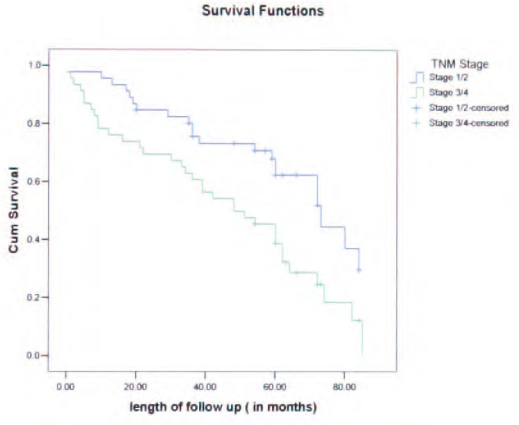
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.966	2.016	0.081	0.062	0.087	0.102	0.145	0.139	0.116	0.186	0.142	0.134
M ean	1.991		0.0715		0.0945		0.142		0.151		0.138	
SD	0.0354		0.0134		0.0106		0.0042		0.0495		0.0057	
CoV	0.018		0.188		0.112		0.030		**0.328**		0.041	
B	1.343	1.404	0.069	0.077	0.064	0.066	0.086	0.064	0.076	0.149	0.074	0.06
Mean	1.3735		0.0730		0.0650		0.0750		0.1125		0.0670	
SD	0.0431		0.0057		0.0014		0.0156		0.0516		0.0099	
CoV	0.031		0.077		0.022		**0.207**		**0.459**		0.148	
C	1.038	1.15	0.112	0.095	0.124	0.094	0.062	0.059	0.043	0.054	0.079	0.068
Mean	1.094		0.1035		0.109		0.0605		0.0485		0.0735	
SD	0.0792		0.0120		0.0212		0.0021		0.0078		0.0078	
CoV	0.072		0.116		0.195		0.035		0.160		0.106	
D	0.671	0.692	0.674	0.618	2.684	2.501	0.068	0.061	0.086	0.056	0.115	0.11
Mean	0.6815		0.646		2.5925		0.0645		0.071		0.1125	
SD	0.0148		0.0396		0.1294		0.0049		0.0212		0.0035	
CoV	0.022		0.061		0.050		0.077		**0.299**		0.031	
E	0.534	0.617	0.076	0.084	0.061	0.063	0.154	0.145	0.951	0.915	0.076	0.057
Mean	0.5755		0.08		0.062		0.1495		0.933		0.0665	
SD	0.0587		0.0057		0.0014		0.0064		0.0255		0.0134	
CoV	0.102		0.071		0.023		0.043		0.027		**0.202**	
F	0.396	0.403	0.082	0.074	0.066	0.074	0.074	0.081	0.052	0.057	0.095	0.114
Mean	0.3995		0.078		0.07		0.0775		0.0545		0.1045	
SD	0.0049		0.0057		0.0057		0.0049		0.0035		0.0134	
CoV	0.012		0.073		0.081		0.064		0.065		0.129	
G	0.201	0.22	2.389	2.439	0.046	0.09	0.222	0.166	0.107	0.122	0.069	0.079
Mean	0.2105		2.414		0.068		0.194		0.1145		0.074	
SD	0.0134		0.0354		0.0311		0.0396		0.0106		0.0071	
CoV	0.064		0.015		**0.458**		**0.204**		0.093		0.096	
H	0.037	0.026	0.094	0.087	0.098	0.102	0.048	0.06	0.047	0.05	0.111	0.129
Mean	0.0315		0.0905		0.1		0.054		0.0485		0.12	
SD	0.0078		0.0049		0.0028		0.0085		0.0021		0.0127	
CoV	**0.247**		0.055		0.028		0.157		0.044		0.106	

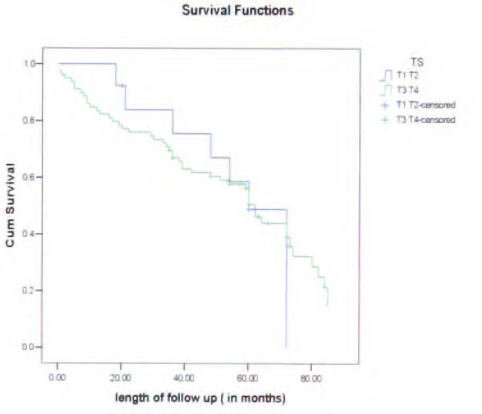
The rows below each reading indicate the mean, standard deviation (SD) and the Co-efficient of Variation (CoV), defined as the ratio of the SD to the Mean (SD / M). The red shaded cells indicate CoV > 20% in OD_{450} of duplicate samples. Samples were unable to be re-assayed due to limited availability.

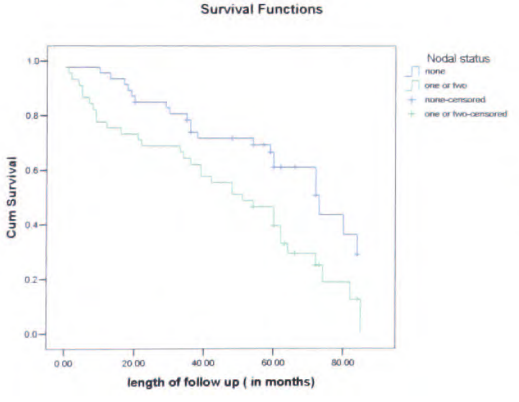
7.13 Kaplan Meier Survival Curve : Overall Survival

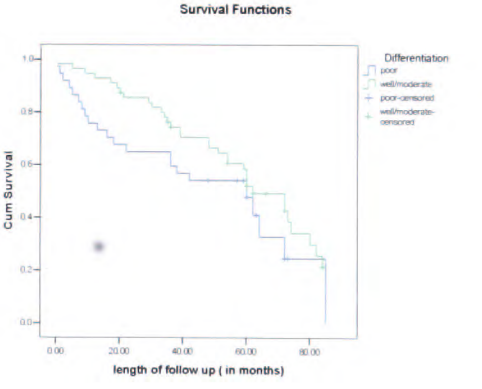




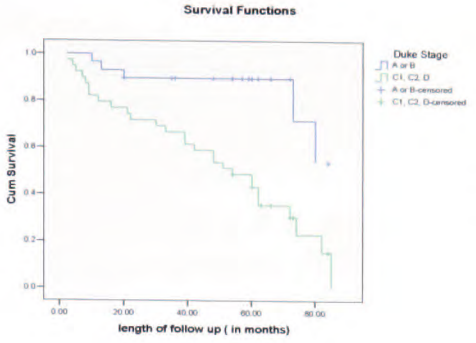


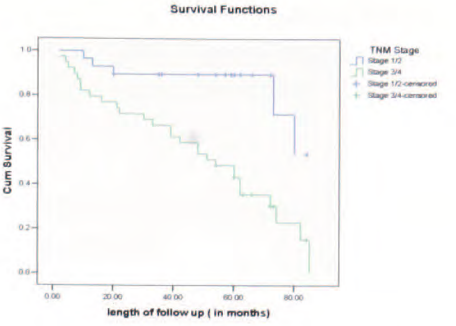


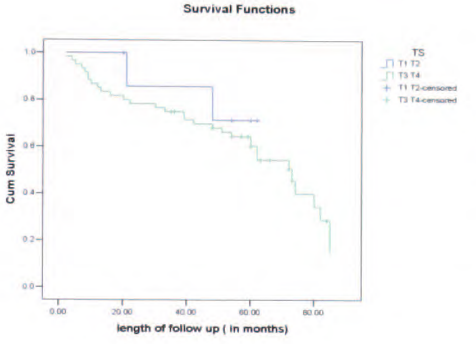


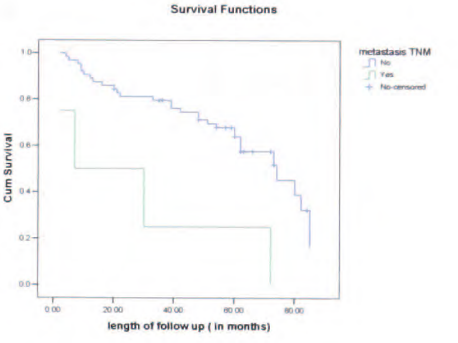


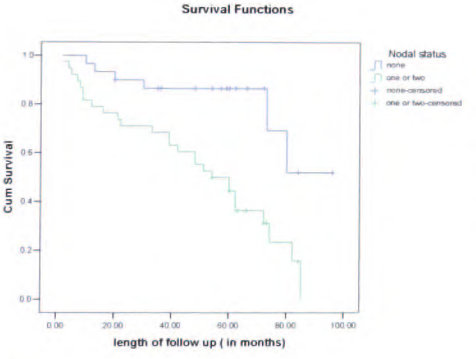
7.14 Kaplan-Meier Survival Curves : Disease Free Survival

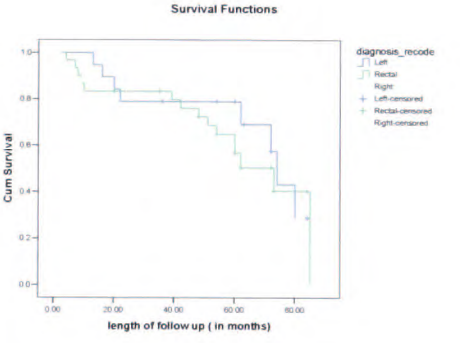


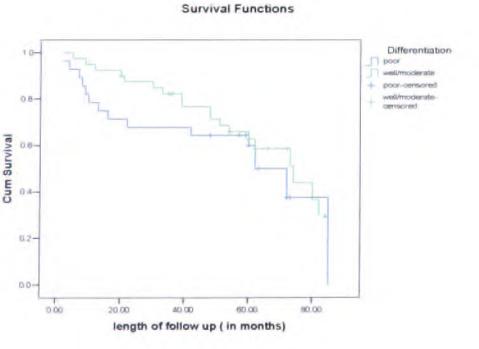












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