The University of Hull

Telomerase: A Tumor Marker in The Peripheral Blood of Colorectal Cancer Patients

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by

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Abstract

Colorectal cancer (CRC) recurrence after curable resection is still high. This fact strongly suggests that the dissemination of circulating tumour cells (CTC) occurs early in the disease process. The first clinical results obtained suggest that CTC detection and enumeration can be used to estimate prognosis and may serve as an early marker to assess anti-tumour activity of a treatment.

Telomerase is an RNA-dependant DNA polymerase that synthesises telomeric DNA sequences on the tip of chromosomes and is widely considered to be one of the most abundant and common tumour markers. Telomerase consists of two components; one is the functional RNA (hTR), which acts as a template for telomeric DNA synthesis. The second component is human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, which is concomitantly associated with telomerase activation during carcinogenesis. Its expression in tumours has been very commonly described using reverse transcriptase-polymerase chain reaction (RT-PCR). Furthermore hTERT peptides were found to be immunogenic both *in vitro* and *in vivo* and hence may be a suitable target for novel cancer immunotherapy.

The aim of this study was to detect circulating tumour cells (CTC) in the peripheral blood of CRC patients using telomerase as a tumour marker. Telomerase presence and activity were measured using a combination of approaches including; ELISPOT assay to detect viable CTL that can recognise hTERT peptides in 30 patients with CRC. The results showed that 71% of pre-operative samples and 54% post-operative samples had CTL reaction towards hTERT peptides. The second approach was to study the enzyme activity using TeloTAGGG Telomerase TRAP assay. In this study 97 patients were recruited, and telomerase activity was positive in 11% of patients. Finally the RT-PCR approach used in this thesis failed to detect hTERT mRNA in serum samples as a result of long duration of samples storage. In conclusion: CTC detection in the peripheral blood is a rare event especially in none metastatic disease, There is an urgent need for standardised isolation and analysis techniques to be adopted thus allowing large-scale, appropriately controlled, multicenter trials to be undertaken on the most promising candidate marker.

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Abbreviations

APC	Adenomatous Polyposis Coli
Alpha 4 Gnt	Alpha 1,4 N-acetylglucosaminyl Transferase
AFP	Alpha-Fetoprotein
ALB	Albumin
Apo-A1	Apolipoprotein AI
APC	Antigen Presenting Cell
Anti-DIG-HRP	Polyclonal sheep antibody against Digoxigenin conjugated to Horseradish Peroxidase
Ag	Antigen
Bp	Base Pairs
B-HCG	Beta-subunit of Human Chorionic Gonadotropin
BSA	Bovine Serum Albumin
BM	Bone Marrow
CRC	Colorectal Cancer
COX	Cyclooxygenase
CTC	Circulating Tumour Cells
CEA	Carcino Embryonic Antigen
CK	Cytokeratins
C-MET	Hepatocyte Growth Factor Receptor
CEA	Carcino Embryonic Antigen
CEACAM5	Carcinoembryonic Antigen-related Cell Adhesion Molecule 5
CTL	Cytotoxic T lymphocytes
DPEP I	Dipeptidase I
DMSO	Dimethylsulphoxide
DIG	Digoxigenin
EGFR	Epidermal growth factor receptor
ECL	Electrochemiluminescence
ELISA	Enzyme Linked Immuno-Sorbent Assay

FAP	Familial Adenomatous Polyposis
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HRT	Hormonal Replacement Therapy
HNPCC	Hereditary Non-Polyposis Colon Cancer
HAT	Histone Acetyl Transferase
HDAC	Histone Acetyl Deacetylase
hMAM	Human Mamoglobulin
HER2/neu	Human Epidermal Growth Factor Receptor 2
hTERT	Human Telomerase Reverse Transcriptase
htR	Human Telomerase RNA
HiFCS	Heat Inactivated Foetal Calf Serum
HDL	High Density Lipoprotein
HEA	Human Epithelial Antigen
IGF-II	Insulin Like Growth Factor
IMS	Immunomagnetic Separation
IBD	Inflammatory Bowel Disease
MSI	Microsatellite Instability
MMR	Mismatch Repair
MZF-2	Myeloid-specific Zinc Finger Protein 2
MACS	Magnetic Affinity Cell Sorting
MUC-1	Membrane Associated Mucin 1
MAG-A3	Melanoma Associated Antigen
MAH	Membrane Array Hybridization
MAG-1	Malignant Antigen
MHC	Major Histocompatibility Complex
MPC	Magnetic Particle Concentrator
NPC	Nasopharyngeal Cancer
PKC	Protein Kinase C
PCR	Polymerase Chain Reaction
PCR-SSCP	Polymerase Chain Reaction-Single Strand Conformation Polymorphism
PTHrP	Parathyroid Hormone related Protein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PMA	Phorbol-12-Myristate-13-Acetate
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RER	Rough Endoplasmic Reticulum
SCCA	Squamous Cell Carcinoma Antigen
SFC	Spot Forming Cells
TNM	Tumour-Node-Metastasis
TGFβ	Transforming Growth Factor β
TNF	Tumour Necrosis Factor
TAA	Tumour Associated Antigens
TMB	Tetramethylbenzidine
TRAP	Telomeric Repeat Amplification Protocol
WHO	World Health Organisation

WT1 Wilms' Tumour 1 Tumour Suppressor Gene Product

Presentations and publications in support of this thesis

Full text manuscripts

Epithelial molecular markers in peripheral blood of colorectal cancer patients. G Khair, JRT Monson, J. Greenman. Dis Colon Rectum. 2007 Aug;50(8):1188-203.

Presentations

1. Determination of an anti-hTERT T cell response in colorectal cancer patients pre- and post-surgery. G Khair, G Hu, JRT Monson, J Greenman. Presented at the British association of surgical oncology, London, UK, November 2006

2. The prognostic value of circulating tumour cells in peripheral blood of colorectal cancer patients. G Khair, A Alabi, R Loveday, J Greenman, JRT Monson. The Society of Surgical Oncology, San Diego, USA, March 2006

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CHAPTER 1

INTRODUCTION

1.1 Colorectal Cancer (CRC)

1.1.1 Incidence

CRC is a common cancer worldwide with higher incidence rates in Western Europe and North America compared with Asia, Africa and South America, whereas intermediate rates prevail in Eastern Europe. Figure 1.1 shows the worldwide incidence of CRC.

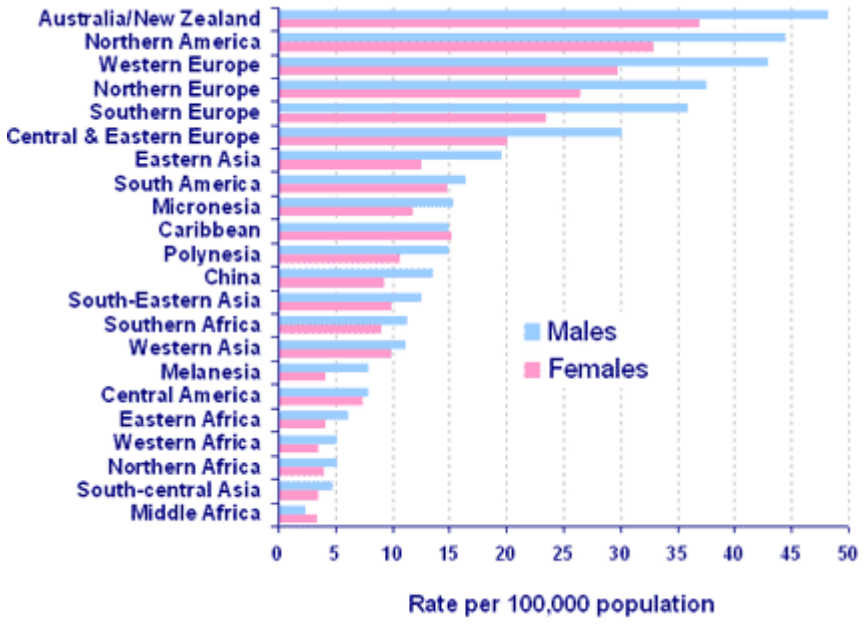


Figure 2.1 Incidence of CRC (Cancer Research UK 2007 website)

CRC is the most common malignancy in the UK after breast and lung. Around 100 new cases of CRC are diagnosed each day in the UK. In 2004 there were 36,109 new cases of large bowel cancer registered in the UK. It is the second most common cancer in women after breast cancer, accounting for 12% of all cancers and the third most common cancer in men after prostate and lung cancer accounting for 14% of all cancers. The incidence trends for men and women differ. Since the late 1970s male CRC incidence rates have increased by nearly 21% to almost 57 per 100,000 in 2000. Female rates have remained stable since the late 1970s at around 36 per 100,000 and mortality is declining at similar

rates for both genders (Coleman et al. 2004). CRC is more common in Scotland and Northern Ireland than in England and Wales (Cancer Research UK 2004).

1.1.2 Distribution

There are more cases of colon cancer than rectal cancer; around two-thirds (22,349) in the colon and one-third (13,760) in the rectum (Cancer Statistics registrations 2004). Half of colon cancer grows in the rectosigmoid colon, 25% in the right colon and 25% in the transverse colon, splenic flexure, descending colon and in the hepatic flexure (Fig 1.2).

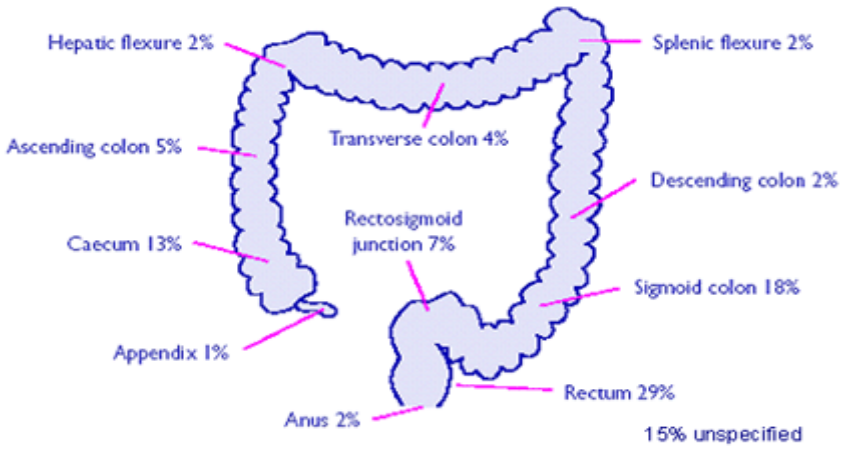


Figure 1.2 CRC distribution (Cancer Research UK website)

1.1.3 Mortality

In 2005 there were 16,092 deaths from CRC in the UK, comprising 10,308 from colon and 5,784 from rectal cancer (Office for National Statistics 2006) and the mortality rates were higher in men than women. Around 8,500 men and 7,600 women died of the disease. There have been significant improvements in 5-year survival over the last 30 years. For colon cancer the five year survival rate has increased from 22% to 47% for men and from 23% to 48% in women. Similarly, there have been improvements in the

five year survival rate of rectal cancer. For men 5-year survival increased from 25% to 47%, and for women, 27% to 51%. (Aaltonen et al. 1994).

1.1.4 Pathology

The vast majority (98%) of colorectal tumours are adenocarcinoma, the remainder being squamous cell carcinoma and carcinoids. Tumours of mesenchymal origin are relatively rare (Kumar 1994). The macroscopic appearances of CRC are classified into 4 types:

1) **Ulcer**: the ulcerative cancer which presents as a typical malignant ulcer with raised rolled edges, often with a necrotic base.
2) **Polypoid cancer**: a proliferative type of lesion which protrudes into the bowel lumen.
3) **Annular or stenotic cancer**: a circumferential lesion.
4) **Diffuse infiltrative cancer**: an extensive lesion infiltrating the bowel wall often for at least 5-8 cm.

1.1.5 Grading

Colorectal carcinoma varies in the degree of differentiation not only from tumour to tumour but also from area to area within the same tumour as they tend to be heterogeneous in their morphology. In 1999 the World Health Organisation (WHO) has classified the tumour grade into:

1) **Grade I:** poorly differentiated.
2) **Grade II:** moderately differentiated.
3) **Grade III**: well differentiated.

Approximately 60% of CRCs are moderately differentiated and the rest are equally divided into well and poorly differentiated.

1.1.6 Staging

The pathologic staging of CRC is based upon the extent of invasion of the tumour through the bowel wall, the involvement of regional lymph nodes and the presence of distant metastases. The Dukes' system was described in the early 1930s, originally solely for rectal tumours, and divides CRC into three groups as shown in Table 1.1 A more recent modification is the Astler-Coller system which was itself modified to include distant metastases (Table 1.2). Finally, the TNM (Tumour-Node-Metastasis) classification is a universal staging system which was adapted for a number of tumour types and is probably the most commonly used worldwide (Table 1.3). Table 1.4 enables equivalent stages in each system to be compared.

Table 1.1: Dukes' stage (Dukes 1932)

Dukes' Stage	Description
A	The tumour is confined to the bowel wall with no involvement of lymph nodes
B	The tumour extends through the bowel wall but with no involvement of lymph nodes
C	There is involvement of lymph nodes

Table 1.2: Astler-Coller staging (Astler and Coller 1954)

Astler-Coller	Description
A	Tumour limited to the bowel mucosa
B1	Tumour extends into the muscularis propria but not through it; nodes uninvolved
B2	Tumour penetrates through the muscularis mucosa; nodes uninvolved
C1	Tumour extends into the muscularis propria but not through it; with involved nodes
C2	Tumour penetrates through the muscularis mucosa; with involved nodes
D	Distant metastases

Table 1.3: TNM staging

T	**Tumour size**
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma insitu; intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propriety
T3	Tumour invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissue
T4	Tumour perforates the visceral peritoneum and/or directly invading other organs or tissues
N	**Lymph node involvement**
Nx	Regional nodes cannot be assessed
N0	No regional lymph nodes metastasis
N1	Metastasis in 1-3 regional lymph nodes
N2	Metastasis in 4 regional lymph nodes
N3	Involvement of any lymph node along named vascular trunk
M	**Metastases**
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

Table 1.4: Comparison of TNM group staging and Dukes'/modified Astler-Coller equivalents modified (Fleming et al. 1997)

TNM grouping stage	TNM stage	Dukes' stage	modified Astler-Coller
Tis, N0 M0	0	None	None
T1,N0, M0	I	A	A
T2, N0, M0		A	B1
T3, N0, M0	II	B	B2
T4, N0, M0		B	B3
Any T, N1, M0	III	C	C1
Any T, N2, M0		C	C2
Any T, N3, M0		C	C3
Any T, any N, M1	IV	None	D

1.2 Aetiology

The aetiology of CRC is multifactorial. And it is widely accepted that both environmental and genetic factors have a role in colorectal carcinogenesis.

1.2.1 Environmental factors

The international differences, migrants' data and recent rapid changes in incidence rates in Italy and Japan show that CRC is sensitive to environmental changes, e.g. Diet, medication, activity, etc. Dietary intake has been thought to play a role in the carcinogenesis of the colon and the rectum. In 1971 Burkitt was the first to popularize the theory that a high fibre diet reduces the intestinal transit time and thus decreases the exposure to potential carcinogens. Also that bulky stool has a dilution effect on the secondary bile acids which have a proliferative effect on the intestinal mucosa (Burkitt 1971). Other studies have subsequently suggested that CRC is at lower risk with higher intake of high fibre diet (Singh and Fraser 1998), and a high intake of vegetables and fruit decreased the risk of developing adenomatous polyps in men (Giovannucci et al. 1992).

However another study showed that there is no relation between high fibre diet and the risk of CRC in women (Fuchs et al. 1999).

Another dietary factor is Calcium and Vitamin D3, where calcium and vitamin D3 decrease the risk of CRC by binding to the secondary bile acids and fatty acids forming insoluble soaps therefore inhibiting their proliferative effect on intestinal mucosa (Newmark et al. 1984) (McMichael and Potter 1985). Folic acid depletion has been reported to play a role in colorectal carcinogenesis, a study undertaken by Freudenheim (428 colon and 372 rectal cancer cases) (Freudenheim et al. 1991) showed that increased folate intake is associated with lower risk of CRC. A second cohort study undertaken by Giovannucci assessed dietary intake for a 1-year period for a cohort of 47,931 U.S. males (Giovannucci et al. 1995) showed an increase risk of CRC with low folate and methionine intakes. Also the same study showed an increased risk of CRC with high alcohol intake. Other studies have shown that non-steroidal-anti-inflammatory-drugs (NSAIDS) such as aspirin have a protective role in CRC by suppressing Cyclooxygenase (COX) and regulating colon carcinoma-induced angiogenesis by two mechanisms: COX-2 can modulate production of angiogenic factors by colon cancer cells, while COX-1 regulates angiogenesis in endothelial cells. Inhibition of COX-2 produces effects on the proliferation and apoptosis (Barnes et al. 1998) and angiogenesis (Tsujii et al. 1998) of intestinal mucosa.

Other studies showed that hormonal factors have a protective role against CRC. A meta-analysis was performed on 18 epidemiologic studies of postmenopausal hormone therapy and CRC and found a 20% reduction [RR = 0.80, 95% confidence interval (CI), 0.74 to 0.86] in risk of colon cancer and a 19% decrease (RR = 0.81, 95% CI, 0.72 to 0.92) in the

risk of rectal cancer for postmenopausal women who had ever taken hormone therapy compared with women who never used hormones (Grodstein et al. 1999), however the relation of CRC with use of menopausal hormones was evaluated in the United States among 40,464 postmenopausal women, 41 to 80 years of age, who were followed for an average of 7.7 years. Long term use of menopausal hormones was not associated with risk of total CRCs or cancers of the colon or rectum. Recent hormone users, however, had a small but insignificant reduction in risk of CRC (RR = 0.78, CI = 0.55-1.1) (Troisi et al. 1997). Table 1.5 summarises the main findings regarding environmental factors with the molecular mechanisms by which they are thought to act (Potter 1999).

Table 1.5: Epidemiology and biology of CRC (Potter, JD) (Potter 1999)

Population risk factors	molecules
Family history	FAP → APC
	HNPCC→microsatellite instability pathway
	? Others
Meat and smoking	Nitrosamines and
	Heterocyclic amines
	→ ? APC mutation
	? K-ras mutation
Alcohol	acetaldehyde→ DNA damage
	Effects via reduced folate
Vegetables	Antioxidants→ reduced DNA damage
	Folate→ DNA integrity
	Fibre→ SCFAs→ apoptosis
Physical activity/low	Reduced growth stimulus
Body mass	Reduced transit time
NSAIDS	COX-2 inhibit
HRT	? Prevention of ER hypermethylation

SCFAs: short chain fatty acids
FAP: Familial Adenomatous Polyposis
APC: adenomatous Polyposis Coli
HRT: Hormonal Replacement Therapy

1.2.2 Familial and genetic factors

Family history is strongly associated with increased risk of CRC, 12-15% of colon cancer have a family history of colon cancer in a first degree relative (Potter et al. 1993), (Kerber et al. 1998). In 1976 Lovett was first to describe the association between the incidence of CRC and family history (Lovett 1976).

Studies have shown that proximal colon cancer is more associated with family history than distal colon (Lynch et al. 1993). Slattery (Slattery et al. 2003) collected data from two population-based case control studies of colon and rectal cancer: Cases were first primary colon cancer diagnosed between 1991 and 1994 (n = 1308 cases and 1544 controls) or rectal cancer diagnosed between 1997 and 2001 (n = 952 cases and 1205 controls). The results showed that family history of CRC was associated with the greatest risk among those diagnosed at age 50 or younger (OR: 2.09 95% CI: 0.94-4.65 for rectal tumours; OR: 3.00 95% CI: 0.98-9.20 for distal colon tumours; and OR: 7.88 95% CI: 2.62-23.7 for proximal colon tumours), while the effect of family history on rectal cancer was less significant (OR: 1.37 95% CI: 1.02-1.85). Table 1.6 represents the risk of having a family history of CRC.

Table 1.6: Degree of risk of developing CRC according to number of family members with the disease (Lovett 1976)

1 first degree relative	1/17
1 first degree relative, 1 second degree relative	1/12
1 first degree relative < 45 years old	1/10
2 parents	1/8.5
2 first degree relatives	1/6
3 first degree relatives	1/2

1.2.3 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominant genetic disorder first described by Lockhart-Mummery in 1925. It affects approximately 2 in 8,000 people (Bisgaard et al. 1994) and is associated with hundreds to thousands of adenomatous polyps in the colon and the rectum, usually manifested in the teens/early twenties, which inevitably develop into malignant polyps if not treated. FAP is caused by germline mutations in the adenomatous polyposis coli (APC) gene which was first identified in 1991 (Nishisho et al. 1991) and mapped to chromosome 5q (Groden et al. 1991). Somatic mutations of this gene are associated with tumourgenesis in 80% of colorectal tumours. APC encodes a large protein with multiple cellular functions and interactions, including roles in signal transduction, mediation of the intercellular adhesion, stabilization of the cytoskeleton and possibly of the regulation of the cell cycle and apoptosis (Fearnhead et al. 2001). However APC mutations are not solely responsible for all cases of FAP as intense screening for APC gene mutations were not found in 30% of classical FAP patients. In 2003 a biallelic germline mutation of the base –excision repair gene MYH was first reported (Lipton et al. 2003) (Sieber et al. 2003) which was found in the APC negative FAP patients. The germline MYH mutations predispose people to a recessive phenotype, multiple adenomas, or polyposis coli. For patients with about 15 or more colorectal adenomas especially if no germ-line APC mutation has been identified and the family history is compatible with recessive inheritance genetic testing of MYH is indicated for diagnosis and calculation of the level of risk in relatives.

1.2.4 Hereditary Non-Polyposis Colon Cancer (HNPCC)

HNPCC is an autosomal dominant syndrome (Lynch et al. 1985) , which accounts for approximately 2% of all CRC (Aaltonen et al. 1994) (Lynch et al. 1989). It is characterized by predominantly affecting the right side of the colon, with a tendency to arise in the younger age groups and possessing a strong hereditary trend. It also involves other cancer such as those of the endometrium, urinary tract, the biliary system and the small intestine (Lynch et al. 1989). The Amsterdam Criteria were set out to diagnose HNPCC (Vasen et al. 1991). However, extracolonic tumours were not taken into account and in order to improve sensitivity and specificity, newer, more detailed criteria were defined, namely the Amsterdam Criteria II (Vasen et al. 1999), the Bethesda (Rodriguez-Bigas et al. 1997) and the most recently Modified Bethesda Guidelines (Umar et al. 2004). These criteria are the basis upon which genetic counselling screening is offered to affected individuals and their families to determine the level of risk and offer appropriate family-screening. The latest criteria are shown in Table 1.7.

The clinical diagnosis can be confirmed by testing for germline mutations in a family of genes that are involved in DNA mismatch repair (MMR) (Liu et al. 1995) (Herman et al. 1998). The MMR system identifies and repairs errors that result from the activity of DNA polymerase during replication. The MMR system recognizes the mismatch, excises it and facilitates the resynthesis of the correct sequence (Kolodner et al. 1995).

Five mismatch repair genes have been known to be involved in HNPCC susceptibility (hMSH2, hMLH1, hPMS1, hPMS2 and hMSH6/GTBM) (Akiyama et al. 1997) (Miyaki et al. 1997) (Fishel et al. 1993). hMSH2 and hMLH1 germline mutations were found to be responsible for more than 90% of HNPCC families. (Lipton et al. 2004).

Table 1.7: The Revised Bethesda Guidelines for testing colorectal tumours for microsatellite instability (MSI) (Umar et al. 2004)

Tumours from individuals should be tested for MSI in the following situations:

1) Colorectal cancer diagnosed in a patient who is less than 50 years of age.
2) Presence of synchronous, metachronous colorectal, or other HNPCC associated tumours,* regardless of age.
3) Colorectal cancer with the MSI-H† histology‡ diagnosed in a patient who is less than 60 years of age.§
4) Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under age 50 years.
5) Colorectal cancer diagnosed in two or more first- or second-degree relatives with HNPCC-related tumours, regardless of age.

*Hereditary nonpolyposis CRC (HNPCC)-related tumours include Colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumours, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel.
†MSI-H _ microsatellite instability–high in tumours refers to changes in two or more of the five National Cancer Institute-recommended panels of microsatellite markers.
‡Presence of tumour infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern.
§There was no consensus among the Workshop participants on whether to include the age criteria in guideline 3 above; participants voted to keep less than 60 years of age in the guidelines.

1.3 Genetics of CRC

The evidence suggests that there are few pathways for a normal colonic cell to transform into a cancerous one. The adenoma –carcinoma sequence, was first described by Morson (Morson 1974).

APC is a tumour suppressor gene whose mutation usually occurs early in the adenoma-carcinoma sequence, germline mutations of APC being responsible for FAP. APC

mutations or allelic losses of 5q are seen in 40-80% of CRC and adenomas (Korinek et al. 1997). APC binds to a protein called β-catenin, and stimulates its phosphorylation. In cells with an APC mutation, this phosphorylation does not take place and β-catenin is activated. The intracellular location of β-catenin changes upon APC mutation; it migrates to the nucleus, interacts with transcription factors, and induces the expression of genes that stimulate cell growth.

Mutations in the APC or β-catenin genes results in failure of proper adhesion-migration of cells and transcription of proliferating signal (Polakis 1997) (Korinek et al. 1997) and are sufficient to initiate the growth of a small benign tumour but are not sufficient to make such tumours progress to more advanced forms. Several other pathways participate in this progression. One of these pathways involves transforming growth factor β (TGFβ), a small polypeptide hormone that negatively controls colon cell growth through regulation of transcription factors like SMAD4 (Kinzler and Vogelstein 1996).

K-*ras* is an oncogene whose mutation is also thought to occur early in the adenoma-carcinoma sequence. It encodes a protein involved in signal transduction within pathways critical for normal proliferation and differentiation. K-*ras* mutations occur in 35-42% of CRC and a similar proportion of large adenomas, but are less common in smaller adenomas, perhaps suggesting that K-*ras* mutation is not directly involved in initiating cancerous change but does facilitate subsequent growth.

The other pathway in CRC development is via the dysplasia- carcinoma sequence. In ulcerative colitis, patients have a 20 fold higher risk of developing CRC than normal individuals. It was found that most patients with ulcerative colitis have a mutation in the p53 gene resulting in dysplasia and carcinoma (Brentnall et al. 1994). The p53 gene is

found on chromosome 17p and either p53 mutation or 17p allelic loss or immuno-histochemical evidence of p53 overexpression has been found in 4-26% of adenomas, 50% of invasive foci within adenomas and 50-75% of CRC. P53 is inactivated not only in CRC but also in most other cancer types. Activation of the normal p53 gene inhibits cell growth by blocking the cell cycle and by stimulating cellular suicide (apoptosis). Kinzler's group has discovered several of the genes that mediate these effects. The $p21^{Waf1}$ and *14-3-3σ* genes control cell birth by regulating passage through various phases of the cell cycle. The *PUMA* gene is a powerful stimulator of apoptosis that is located in mitochondria and binds to homologous proteins, like BAX, that control cell death (Kinzler and Vogelstein 1996). Figure 1.3 demonstrates the key steps in the adenoma carcinoma sequence.

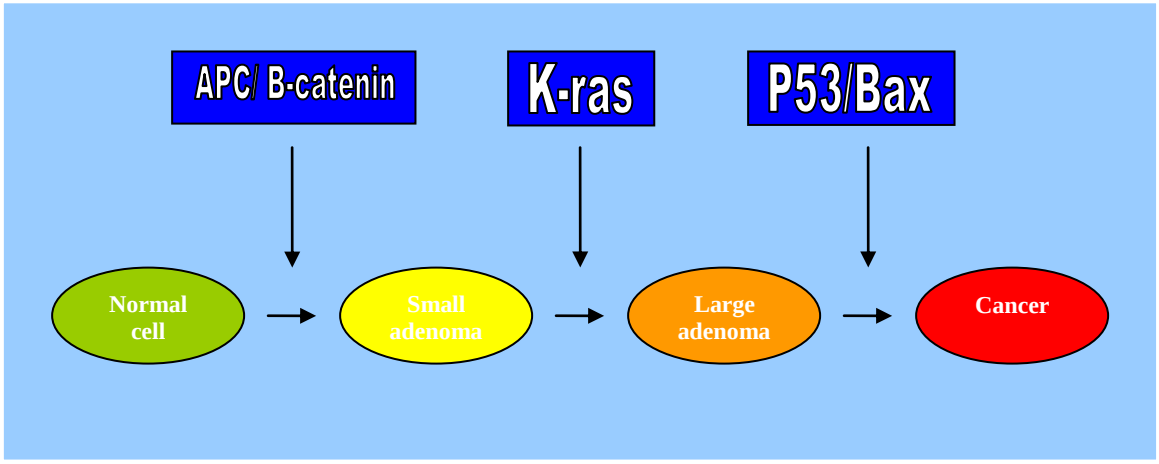


Figure 1.3 adenoma-carcinoma sequence

The final pathway involves mutations or hypermethylation of the MMR genes. (Kane et al. 1997) (Herman et al. 1998). This mutation results in microsatellite instability. Microsatellites are repetitive genetic loci which are relatively stable, when these loci become unstable as a result of insertion or deletion of repeated units, they become prone

to form small loop in DNA strand during DNA replication. These are normally repaired, but in the absence or mutated form of MMR genes, these loops become permanent and alleles will be formed in the next round of replication which lead to loss of growth and apoptosis control. Furthermore the mutation of MMR genes results in the inhibition of TGF receptor on the colonic epithelial cells (Markowitz et al. 1995) and BAX gene (Rampino et al.) which leads to loss of growth and apoptosis control. This hypothesis is supported by the fact that this tumour suppressor gene is absent in 90% of CRC with microsatellite instability and 10% of sporadic CRC (Grady et al. 1997).

1.4 Telomeres and telomerase

1.4.1 The end replication problem

Replication of chromosomes poses a special problem for cells. Watson and Olovnikov independently hypothesised that DNA should be progressively lost from the ends of chromosomes each time the cell divides (Olovnikov 1996).

The reason is that the traditional DNA polymerase cannot fully replicate the 3' end of the lagging strand of a linear molecule (Figure 1.4).

DNA consists of two strands of nucleic acid subunits. The "direction" of a strand of DNA is determined by how these nucleic acid subunits are attached and the two strands run antiparallel to one another. This means that one strand runs 5'-3' while the complimentary strand runs 3'-5'. During replication the DNA helix seperates into two strands. DNA polymerase synthesises the complementary leading and lagging strands of new DNA that combine with the parent strands to form two daughter copies of the DNA. DNA polymerase can only make DNA in a 5'-3' direction and it needs RNA primers which attach to the DNA strand thus giving the DNA polymerase a place to start. Thus

the result is one strand that is synthesised as a continuous piece of DNA (leading strand) and another that is synthesized in small, discontinuous pieces (lagging strand). This leaves a gap at the 3' end of the lagging strand which cannot be replicated and results in the telomere becoming shorter, by ~50-200 base pairs (bp), with each successive cell division. This would mean the loss of the genetic material each time the cell divides and hence loss of function and eventually cell death. But this does not happen; the reason is the presence of telomeres.

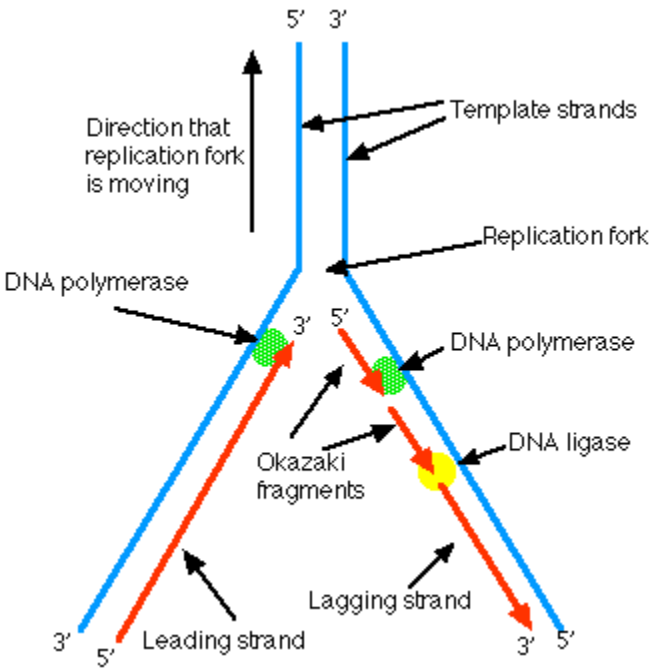


Fig.1.4 End replication problem (www.uic.edu/.../ReplicationFork.gif)

1.4.2 Telomeres

Telomeres are the terminal ends of each chromosome and consist of repetitive tandems of the sequence TTAGGG (Fig 1.5). Telomeres do not carry any genetic information; they function as a "cap" that protects the DNA chromosome from loss, therefore preserving

the genetic integrity of the chromosomes, preventing its degradation and end to end fusion.

The end of the telomere terminates with a single stranded 3' overhang of variable length. (Wright et al. 1997) (McElligott and Wellinger 1997) (Makarov et al. 1997). In addition to these repeated sequences, a number of proteins are attached to telomere DNA strands. These are telomeric repeat factors 1 (TRF1) and 2 (TRF2), which help to maintain the telomeric stability and regulate telomere length. They are known to specifically recognise the TTAGGG repeat sequence (Broccoli et al. 1997) (Bilaud et al. 1997). TRF1 regulates telomere length by inhibiting telomere elongation once telomeres reach a critical size by inhibiting the action of telomerase at the ends of individual telomeres (van Steensel and de Lange 1997). While TRF2 suppresses end to end fusion between chromosomes by maintaining the correct structure at telomere termini and hence serves to stabilize chromosome ends (van Steensel et al. 1998).

As a result of the end replication problem mentioned earlier, telomeric sequence of the lagging strand becomes shorter with each time the DNA replicates. Consistent with this hypothesis, telomere shortening was observed with progressive cell divisions *in vitro* and with increasing age *in vivo* (Harley et al. 1990) (Hastie et al. 1990) (Lindsey et al. 1991) (de Lange et al. 1990).

Telomere shortening occurs in different types of human tissues and organs including; dermal fibroblasts (Allsopp et al. 1995), mucosal keratinocytes (Kang et al. 2002), peripheral blood cells (Cawthon et al. 2003), gastrointestinal epithelial cells (Furugori et al. 2000), adrenocortical cells (Yang et al. 2001), renal cortex (Melk et al. 2000), liver

(Takubo et al. 2000) and spleen (Takubo et al. 2002). Most of these cells are mitotically active and hence indicate the effect of cell division on telomere length during ageing. Conversely it was also found that telomeres length is stable in tissues that do not have mitotic activity such as the heart and the brain tissues (Takubo et al. 2002).

Telomeres shorten during each cell division by 5-200 bp. Telomere length in human is 5-10 kbp and hence the proliferative capacity of primary human cells in tissue cultures is limited to 50-70 population doublings (Harley et al. 1990).

Telomeres shorten to a point where further division lead to cell senescence, this phase is known as phase 1 or M1 (Hara et al. 1991) (Wright et al. 1989). It is a process in which the cell is viable, but loses the ability to replicate; this phase can be bypassed by the mutation of certain tumour suppressor genes such as p53, p16 and rb genes. (Shay et al. 1991). Further shortening of the telomere length leads to the M2 crises phase. (Counter 1996) (Shay and Wright 1989), where further shortening leads to apoptosis and cell death. Rarely some cells manage to overcome these crises by the activation of the telomerase enzyme, in this case the cell becomes immortal or transforms into a cancerous cell.

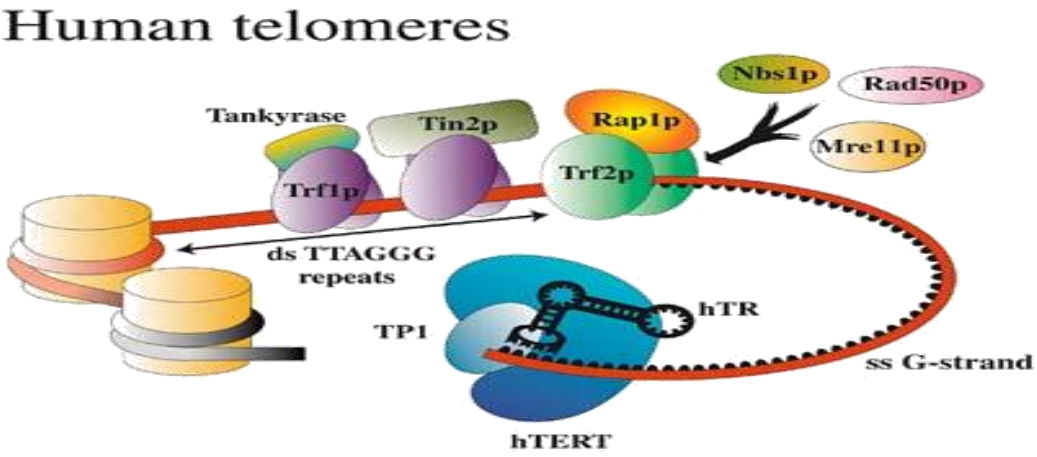


Fig. 1.5 Human telomere

1.4.3 Telomeres and ageing

While telomere shortening has been linked to the ageing process, it is not yet known whether shorter telomeres are just a sign of ageing or actually contribute to ageing. Richard Cawthon and colleagues found shorter telomeres are associated with shorter lives (Cawthon et al. 2003). Among 143 unrelated individuals older than 60, those with shorter telomeres were three times more likely to die from heart disease and eight times more likely to die from infectious diseases. Also Cawthon found that when people are divided into two groups based on telomere lengths, the group with longer telomeres live an average of five years more than those with shorter telomeres.

1.4.4 Telomerase

Telomerase is an RNA-dependant DNA polymerase that syntheses telomeric DNA sequences and almost universally provides the molecular basis for unlimited cellular proliferation. Telomerase is present in germline cells at a level which allows constant maintenance of the telomere length. It is absent in most normal somatic cells, but present in low levels in basal layer of the skin (Harle-Bachor and Boukamp 1996), peripheral blood mononuclear cells (PMNC) (Counter et al. 1995), activated lymphocytes (Weng et al. 1996) and intestinal crypts (Hiyama et al. 1996). The high turnover of these tissues implies the need for maintenance of telomere length to allow continues proliferation. Telomerase was first discovered in *Tetrahymena thermophila* in 1985 (Greider and Blackburn 1985). Telomerase consists of two components; one is the functional human telomerase RNA (hTR), which acts as a template for telomeric DNA synthesis (Feng et al. 1995). hTR is expressed in all tissues regardless of telomerase activity with cancer cells having a five-fold higher expression than normal cells (Kilian et al. 1997) (Lingner

et al. 1997). hTR is transcribed by RNA polymerase II and is processed at the 3' end to produce a mature transcripts of 451 nucleotides (Feng et al. 1995).

The second component of telomerase is the human telomerase reverse transcriptase molecule (hTERT), which is the catalytic subunit of telomerase (Yi et al. 1999), it is concomitantly associated with telomerase activation during cellular immortalization and carcinogenesis (Nakamura et al. 1997) (Meyerson et al. 1997) (Harrington et al. 1997).

The mode of action of telomerase was first described by Greider and Blackburn (Greider and Blackburn 1989) who observed that telomerase recognizes the G-rich strand of an existing telomere repeat sequence and elongates it in the 5' to 3' direction by adding single stranded TTAGGG repeats to the end of the chromosome. The telomerase complex is a RNA-dependant enzyme that uses the 3' nucleotide overhang as a template for telomerase mediated elongation of the telomere (Zakian 1996). The 3' end of the template region of the hTR anneals to the 3'overhang of the telomeric DNA then adds one DNA nucleotide at a time until full telomeric subunit is formed (Miller and Collins 2002). When the subunit is complete, the enzyme slides to a new end and starts the same process again (Figure 1.6).

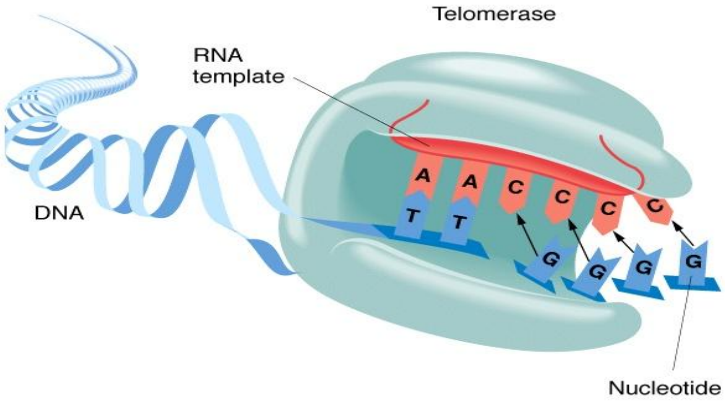


Figure 1.6 Telomerase structure (www.uic.edu/.../bios100/f05pm/telomerase.jpg)

1.5 Telomerase regulation

Telomerase regulation occurs at different levels including; hTERT transcription, hTERT splicing, epigenetic regulation and post-translational. Of all components of the telomerase complex hTERT plays the most important role in regulation.

1.5.1 Transcriptional regulation of hTERT

hTERT expression has been found to be up regulated in most precancerous lesions and human cancers studied including gastric (Jong et al. 1999), ovarian (Kyo et al. 1999), urinary bladder (Suzuki et al. 1999), cervical (Wisman et al. 2000) and skin (Wu et al. 1999) tumours, with expression being associated with telomerase activity (Weinrich et al. 1997). hTERT is encoded by hTERT gene located on the short arm of chromosome 5 (15p15.33) (Meyerson et al. 1997) (Bryce et al. 2000), it consists of 16 exons and 15 introns and extends over 40 kB (Cong et al. 1999) (Wick et al. 1999).

Transient transfection experiments showed that hTERT promoter is inactive in normal cells but active in immortal cells (Bryce et al. 2000) (Takakura et al. 1999). This promoter was found to be GC rich and lacking the TATA box. The proximal part of this promoter is responsible for the majority of transcriptional activity. This promoter has multiple binding sites such as E boxes and SP1 binding sites; some factors are involved in the activation of hTERT promoter by binding to these binding sites. One of these factors is c-myc, which is an oncogene that promotes growth, proliferation and apoptosis (Grandori et al. 2000). It has been shown that the c-myc gene induces hTERT expression and telomerase activity by binding to the E box on the promoter site (Wu et al. 1999). SP1 is another protein that has a role in the regulation of hTERT transcription where it binds to its binding site inducing hTERT expression (Kyo et al. 2000). Furthermore SP1

co-operates with c-myc to activate hTERT transcription. However these two factors are not enough to explain cancer specific telomerase activation since some tumours were found to have telomerase activity by up-regulation of hTERT transcription independent of c-myc and SP1 effect (Oh et al. 2001) (Gewin and Galloway 2001).

Some hormones have also been found to have a role in the regulation of hTERT transcription, including the steroid sex hormones. It was found that sequence analysis of hTERT promoters revealed oestrogen response elements. This binding of oestrogen to these sites was shown to induce the transcription of hTERT expression (Kyo et al. 1999a) (Kyo et al. 1999b) (Misiti et al. 2000). Also it was found that hTERT expression can be induced indirectly by inducing c-myc expression in oestrogen receptor positive cells (Kyo et al. 1999b).

Progesterone has a complex role in hTERT transcription, it initially activates transcription by inducing hTERT promoters (Wang et al. 2000), and later exhibits a negative effect by inducing p21 expression, the over-expression of p21 leads to repression of hTERT expression in progesterone positive cells (Mitchell et al. 1999a) (Mitchell et al. 1999b) (Belair et al. 1997).

1.5.2 hTERT transcription repressors

Telomerase repressors may exist in normal cells, which contribute to the silencing of telomerase activity. It was found that transfer of certain human chromosomes into cancer cells results in the repression of hTERT expression and down regulation of telomerase activity; i.e. the transfer of chromosome 3 into renal cancer and breast cancer cell lines resulted in hTERT mRNA repression and shortening of telomere length (Oshimura and Barrett 1997).

Few telomerase repressors have been clearly identified to date, one of these repressors is however the myeloid specific zinc finger protein 2 (MZF-2). This protein has an inhibitory effect on hTERT transcription through binding to its sites on hTERT promoter. Another repressor is the Wilms' tumour 1 suppressor gene product (WT1).
Its over-expression inhibits telomerase. However, WT1 gene is expressed in limited cell types such as the kidney spleen and gonads, which makes its potential role in hTERT regulation limited to these tissues.
P53 over-expression also has a role in hTERT regulation. It interacts with SP1 and prevents it from binding to hTERT promoter *in vitro* hence inhibits hTERT expression (Xu et al. 2000). It was found that over-expression of p53 in head and neck squamous cell carcinoma was associated with inhibition of telomerase activity (Kanaya et al. 2000) (Fuxe et al. 2000) (Henderson et al. 2000).

1.5.3 hTERT splicing

Variant splicing forms several transcripts which are detected in human body (Ulaner et al. 1998) (Ulaner et al. 2000). The function of these transcripts is still unknown and only the full-length transcripts function as the catalytic subunit of telomerase.
These transcripts include α transcript, which lacks a small region at the beginning of exons 6, β transcript which lacks exons 7 and 8, and α-β which lacks both areas.1.5.4

1.5.4 Epigenetic regulation of hTERT transcription.

DNA methylation is important for the development of mammals. Methylation of proto-oncogenes and tumour suppressor genes is associated with human cancer (Jones and Takai 2001).

It has been shown that treatment with DNA methylation inhibitor 5-azacytidine induces hTERT transcription in two telomerase-negative cell lines (Devereux et al. 1999) (Dessain et al. 2000). Also the degree of methylation in the hTERT promoter and telomerase activity in B-cell lymphocytic leukaemia has been shown to be inversely correlated (Bechter et al. 2002).

Transcription is influenced by chromatin structure as well as post-transcriptional modification of histones, and has been implicated in the physiological control of chromatin structure (Stein and Schultz 2000). Histone acetylation is catalyzed by histone acetyl transferase (HAT) and deacetylase (HDAC). Several transcriptional factors such as Mad can repress transcription by recruiting HDAC to specific sites in certain promoters (Laherty et al. 1997) (Heinzel et al. 1997), also HDAC mediates transcriptional repression via the SP1 binding sites (Doetzlhofer et al. 1999).

1.5.5 Post-translational regulation of hTERT

Normal ovarian tissues and uterine leiomyoma cells have no telomerase activity despite the fact that both express hTR and hTERT mRNA (Ulaner et al. 2000). Also hTERT mRNA is present in T and B lymphocytes. This suggests that the expression of hTERT is not always sufficient to produce telomerase activity and that posttranslational modification of hTERT may play an important role in regulating telomerase activity. Data indicate that hTERT phosphorylation has a role in determining telomerase activity. It was found that PMNC telomerase activity is enhanced by protein kinase C (PKC) and the use of phosphatase 2A inhibits telomerase activity by inhibiting hTERT phosphorylation (Li et al. 1997).

Also interesting data from Liu *et al* (Liu et al. 2001) showed that induction of telomerase activity during the activation of resting CD4 T cells was independent of hTERT protein increase. Instead, hTERT is phosphorylated and translocated from the cytoplasm to the nucleus, indicating that phosphorylation has an important role in the regulation of telomerase activity.

1.6 Telomerase and cancer

One of the most important differences between normal cells and tumour cells is that the former exhibit cellular senescence whereas tumour cells continue to proliferate indefinitely.

As mentioned earlier as the cell divides it reaches a stage where further cell division leads to critical shortening of telomere length, reaching the crisis phase (M2), at this point the cell either enters senescence or commences expression of telomerase and becomes a telomerase positive, immortal tumour cell. Telomerase maintains the telomere length allowing indefinite cell division and malignant transformation (Fossel 1998). It assures cancers cells immortality, which allows the accumulation of mutations required for cells to become malignant (Holt et al. 1996).

Although telomerase has an important role in tumourgenesis, it has been established that ectopic expression of telomerase in normal human fibroblasts is sufficient for their immortalization but is not associated with malignant transformation (Morales et al. 1999). This means that many cell types need hTERT plus other factors to produce immortality. It was found that inactivation of p53/p21 or pRb/p16 pathways play a role together with telomerase in tumourgenesis for some cell types, such as mammary epithelial cells and keratinocytes. hTERT have also been shown to collaborate with viral

oncoproteins, such as SV40LT (which inactivates p53 and pRb) or human papillomavirus 16 (HPV16) E6 and E7 proteins which inactivate pRb, to escape from senescence (Morales et al. 1999).

Most human cancers have shortened telomeres and express high levels of telomerase (Kim et al. 1994) (Shay et al. 1997). Owing to the strong association between telomerase and cancers, telomerase and its two components; hTR and hTERT have been the focus of intensive studies in many tumours as potential diagnostic markers or therapeutic markers. Researchers in the last decade have been trying to use different ways to measure telomerase tumour cells in both tissue and blood samples. Various studies have evaluated telomerase and its subunits as a tumour marker in CRC; Yan and colleagues (Yan et al. 1999) studied the correlation of telomerase activity and the expression of hTERT subunit in 25 adenocarcinoma tissue samples, 30 adenomas and 15 samples of normal mucosa. Telomerase activity and hTERT expression were measured using telomerase repeat amplification protocol (TRAP) assay and RT-PCR analysis, and it was found that hTERT mRNA was expressed in all cancer samples and, 12/13 telomerase-positive adenoma samples, but was never detected in the telomerase negative colorectal tissues. The authors concluded that hTERT expression correlates with telomerase activity. Another study used the same techniques to detect telomerase activity and hTERT mRNA in 140 cancer tissues, 140 normal adjacent mucosa and 20 adenomas. They found that the median value of hTERT expression and telomerase activity were higher in cancer tissues than adenomas and normal samples, and that hTERT expression significantly correlated with telomerase activity in cancer and adenoma samples ($p= 0.031$, $p= 0.021$) respectively (Niiyama et al. 2001).

Furthermore Gertler and colleagues assessed the correlation betwee hTERT mRNA and the disease histology by studying CRC samples from 57 patients utilizing real time RT-PCR. They found that the expression of hTERT in cancer samples and corresponding adjacent mucosa was higher in advanced disease (Gertler et al. 2002).

Telomerase activity has been detected in different types of human cancers. In his review Shay *et al* has summarised data on telomerase activity in various types of human cancers (Shay and Bacchetti 1997).

1.7 Circulating Tumour Cells (CTC)

Treatment of solid tumours is still insufficient in a great majority of cases in part due to the development of metastases. Lymphatic and haematogenous pathways are the most important pathways for tumour spread. Even in localized diseases the relapse rate is still high, this suggests that undetected occult tumour cells and micrometastases in localized disease play a crucial role in the process of metastases.

Metastases are an important factor that regulates the prognosis and cancer outcome. In the process of metastases, tumour cells shed from the primary tumour, spread through the blood stream, bind to the vascular endothelium, spread into the extracellular space, escape the host defence mechanisms and finally form a secondary tumour.

In 1869 Ashworth, reported in Ghossein (Ghossein and Bhattacharya 2000) was the first to describe CTC when he discovered cells in the blood stream similar to those in the tumour at post mortem. CTC are a potential cause of disease relapse particularly following surgery, therefore the presence or absence of CTC has been considered by some to be an important prognostic factor pre and/or post-operatively, and an indicator for the decision concerning adjuvant treatment and follow up (Hardingham et al. 1995).

However, this idea has been questioned as the majority of CTC shed from solid tumours do not survive in the blood and only approximately 1% live long enough to form distant metastases (Fidler 1970). Also, the reliability of using CTC has been debated from a methodological perspective because it has been reported that cells are only shed intermittently and often circulate in clumps, both factors can make accurate detection problematic (Ghossein et al. 1995).

1.8 CTC enrichment

As mentioned above one of the main limitations in the detection of CTC is that the number in the circulation can be very low. In order to solve this problem a variety of enrichment methods have been employed. Immunomagnetic separation has been one of the most widely-used methods. This technique has 3 main variations; MACS (magnetic affinity cell sorting) systems, magnetic beads and ferro fluid based system (Ring et al. 2004). The first two methods use specific antibodies linked to magnetic beads that bind tumour-expressed molecules and allow isolation by magnetism. There are different ways these reagents can be used, either for positive selection, where the antibodies interact directly with circulating epithelial cells and allow their separation, e.g. CEA 125 and Ber-EP4, or negative selection, where the beads remove all the *unwanted* cells from the mixture, leaving the desired cells in solution unmodified, e.g. CD45 beads with a pan-leucocyte marker can be used to remove white blood cells from any tumour cells of epithelial origin in blood. Figure 1.7 diagrammatically illustrates both positive and negative methods of isolation.

The third variation, the ferro fluid based system, is simply a modification of that described above and uses epithelial cell adhesion molecules, rather than antibodies,

coupled to colloids of 1nm ferro fluid, which can bind to cells and be magnetically separated (Racila et al. 1998).

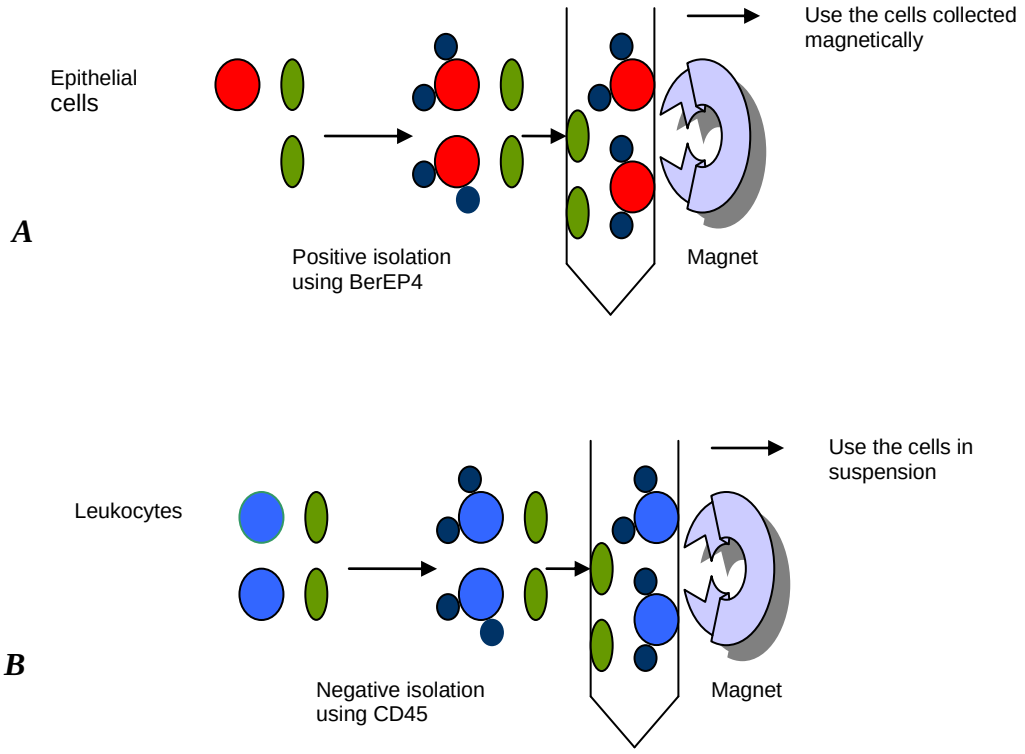


Figure 1.7 Positive (A) and negative (B) isolation of desired cell populations

1.9 CTC in blood and CRC

The relatively poor overall survival rate of CRC strongly supports that dissemination of CTC is thought to be early in the disease process and emphasise the need of finding new tumour markers valuable in the early detection and as a marker of prognosis and response to treatment.

The introduction of early lymphovascular ligation before manipulation of the tumour in surgery (non touch isolation) was first introduced by Barnes (Barnes 1952) as a way to reduce the incidence of tumour cell dissemination and metastasis development. It was

found that non touch isolation reduces the dissemination of CTC. In the study done by Hayasha 18/27 CRCs samples had mutations for K-ras and p53. Blood samples were then collected of the portal vein before, during, and after manipulation of tumours. Eleven out of eighteen were operated on in a conventional resection technique and 7 were in the no-touch isolation technique. In 8 /11 (73%) conventional resection technique cases the same genetic alteration of the primary tumour in the portal blood during operation were identified, whereas only 1 patient (14%) in the no-touch isolation technique group had a positive result. (Hayashi et al. 1999). Other studies has reinforced Hayashi results (Weitz et al. 1998) (Sales et al. 1999) but only one study showed that surgical manipulation has no role in CTC dissemination at the time of surgery, where sixteen patients underwent resection for primary CRC and two patients with irresectable tumours were studied, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect carcino embryonic antigen (CEA) in tumour biopsy specimens and blood samples obtained from the antecubital vein before and after surgery and from the main drainage vein of the tumour when the tumour had been excised; CEA was positive in all tissue samples. In two patients CEA was detected in antecubital vein blood before surgery and in one of them also after surgery. Only in one patient (Dukes C) CEA was detected from the main drainage vein of the tumour. (Garcia-Olmo et al. 1999).

The major obstacle of using blood samples to detect CTC was the presence of activated lymphocytes, which normally express high levels of telomerase, so in order to escape this problem different studies have used immunomagnetic separation techniques to separate CTC from activated lymphocytes. BerEP4 monoclonal antibody that recognizes an

epitope on the protein moiety of glycopeptides (34 and 39 kDa) expressed at the surface of epithelial cells in normal and malignant tissues (Latza et al. 1990).

Few studies have been done on colorectal and breast cancers. Loveday and colleagues did a study using BerEP4 antibody in immunomagnetic separation to separate tumour cells from pre and post-operative blood samples of 35 CRC patients, and tried to detect telomerase activity in CTC (Loveday et al. 2004).

Another study tried to detect telomerase activity in portal and peripheral blood samples of CRC patients, they found that telomerase activity was higher in portal than in peripheral blood samples, and that telomerase activity is more expressed in patients with liver metastasis, which suggests that the presence of telomerase activity in portal blood samples reflects the existence of liver metastasis of CRC (Nozawa et al. 2003).

Another study used immunomagnetic separation and TRAP-ELISA to detect telomerase activity in circulating tumour cells and non small cell carcinoma of the lung, this study found that 73% patients with stage IIIB or IV non small cell carcinoma of the lung had telomerase activity and in 72% of stage C or D colon cancer detected telomerase activity (Gauthier et al. 2001). This study did not look at the prognostic value of telomerase in circulating tumour cells since it was only looking at telomerase activity in pre-operative samples.

Also telomerase activity has been detected in CTC of breast cancer patients, where one study showed that 84% of patients with metastatic breast cancer have positive telomerase activity in their blood samples (Soria et al. 1999).

1.10 Methods of CTC detection

Over the past twenty years numerous groups have attempted to detect CTC, both with and without enrichment, in patients with solid malignancies, and to establish their clinical significance using a wide range of different techniques. The two main analysis techniques that have been commonly used are: cytometry and nucleic acid based assays, both of which have advantages and disadvantages.

1.10.1 Cytometric methodology

In both flow and immunocytochemical techniques monoclonal antibodies against various epithelial specific antigens are used to detect, and often enumerate, the tumour cells. The monoclonal antibodies label the tumour cells that can be subsequently visualized (directly or indirectly) fluorescently or colorimetrically (Cote et al. 1999). Both technologies are robust and have been demonstrated to work in model *in vitro* systems. A diverse repertoire of antigens has been investigated; by far the most common of these being the intracellular cytokeratins (CK). It is generally accepted that the detection limit for cytometric methodology is 1 specific tumour cell amongst 1,000,000 irrelevant cells when one or more different monoclonal antibodies against CKs have been used (Racila et al. 1998) (Pantel et al. 1993). However, there are two main disadvantages of using cytometric techniques; first, the sensitivity to detect micrometastases by these techniques has been questioned due to the limited amount of screened cells and secondly the lack of methodological standardization. Together these problems are thought to be the reason for many discrepancies in the immunocytochemical data from different studies (Braun and Pantel 1999) (Muller and Schlimok 2000), with false positive rates ranging from below 1% to over 80% having been reported.

1.10.2 Nucleic acid based methodology

This method relies heavily on the polymerase chain reaction (PCR) for the detection and molecular characterization of CTC. The use of nucleic acid based methodology gives at least a ten-fold improvement in the sensitivity of detecting CTC allowing the identification of 1 cell in a background of 10,000,000 unwanted cells (Pelkey et al. 1996)- (Castells et al. 1998). Both DNA and RNA molecular markers have been used for the detection of CTC and these will be discussed individually. The major disadvantage of circulating DNA material is that it can be released by dying tumour cells and remains in the circulation because of its relatively high stability in comparison to RNA. Therefore, the presence of DNA markers does not necessarily indicate the presence of viable CTC but is indicative of a tumour burden (Zehentner 2002).

1.10.3 DNA markers

Genomic mutations in K-ras or p53, two of the commonest genetic abnormalities in CRC, have been reported to be useful for the detection of tumour cells circulating in the lymphatics of colorectal and non-small cell carcinoma of the lung (Hayashi et al. 1995) (Hashimoto et al. 2000). A recent study done by Hsieh and colleagues (Hsieh et al. 2005) tried to identify the prognostic values of K-ras, p53 and adenomatous polyposis coli (APC) gene mutations in tumours and sera of 118 CRC patients using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, followed by direct DNA sequencing of the amplified genomic DNA. Subsequently, serum molecular markers were correlated to the patients' clinicopathological features and risk of metastases and recurrence. This study showed that both serum APC and p53 presence closely correlated with lymph node metastasis and TNM stage (both $P < 0.05$).

Moreover, the presence of one or more serum markers was associated with depth of tumour invasion ($P = 0.03$), lymph node metastasis ($P < 0.001$), and TNM stage ($P < 0.001$). In addition, postoperative metastasis and recurrence rates were higher in patients positive for one or more serum markers, compared with those patients who were negative for all three ($P < 0.001$). APC and K-ras markers were more frequently observed in patients with metastasis (both $P < 0.05$), while p53 was commonly detected in the cases of peritoneal metastasis ($P = 0.004$). These recent findings, from a relatively large patient cohort, suggest that serum molecular markers are potentially useful markers for CRC prognosis.

Also, Hibi *et al* (Hibi et al. 1998) characterised the genetic alterations in serum DNA obtained from 44 CRC patients using an oligonucleotide-mediated mismatch ligation assay. Among the 16 cases with K-ras gene mutations in these tumours, the same mutation was detected in three paired serum samples simultaneously. In the 10 tumours with a p53 mutation an identical mutation was detected in DNA from serum samples of seven. Comparison of the molecular analysis with clinical diagnosis of these patients revealed that five of these seven patients had early stage tumours ($P = 0.01$). Taken in combination, either a K-ras or p53 mutation was detected in serum from 40% of the patients (n=10) whose primary tumours contained a mutation, and in 23% of all the 44 patients with CRC. It was concluded that the frequent detection of p53 mutation in the serum of patients with early stage tumours suggests a possible use as a marker for CRC monitoring; however the relatively small cohort size requires further confirmatory work.

Hardingham and colleagues also studied K-ras mutation and found that the presence of mutations on codon 12 of K-ras in CTC was predictive of decreased free survival and

increased the risk of relapse (Hardingham et al. 1995). The study investigated the presence of tumour cells in perioperative peripheral blood samples in a cohort of twenty-seven patients. Immunomagnetic beads, labelled with Ber- EP4, were used to harvest epithelial cells from blood and K-ras mutations were identified by PCR. Circulating K-ras mutant cells were detected in 9 of 27 patients; seven of whom died as a result of recurrent or metastatic disease after a median of 14 months, whereas mutant cells were not detected in 18 patients, and 16 of 18 remained disease free after a median follow-up of 16 months. This study indicates that detection of circulating tumour cells perioperatively by immunobead-PCR can provide a sensitive prognostic marker for recurrent and metastatic CRC. Additional, well-controlled, studies of this type are urgently needed to verify these encouraging findings.

1.10.4 RNA markers

The fact that the presence of circulating DNA cannot be used to definitely confirm the presence of viable cancer cells because of its relatively high stability, researchers have turned to mRNA as it has a shorter half-life once released from tumour cells (Zehentner 2002). The most common hypothesis for the presence of circulating mRNA is from cell lysis of circulating tumour cells, micrometastases shed by the tumour, the induction of tumour necrosis, apoptosis and active release (Anker et al. 1999).

Initially there was a concern that mRNA would be too labile however, it was successfully detected in various malignancies. Kopreski *et al* was the first to detect tyrosinase mRNA in the plasma of malignant melanoma patients using reverse transcriptase –PCR (RT-PCR) (Kopreski et al. 1999). Subsequently mRNA of various genes has been widely used to detect CTC in different types of solid tumours. Table 1.8 shows all the studies on

tumours of gastrointestinal tract to date and compares these with the studies on breast cancer; the single most studied tumour type.

Table 1.8: mRNA markers of breast and gastrointestinal tumours

	BREAST			
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
Maspin	30	Nested RT-PCR	38% in post CT samples	(Sabbatini et al. 2000)
PTHRP	30	RT-PCR	30%	(Wulf et al. 1997)
MUC-1	122	RT-PCR & southern blot	11% primary cancer 24% in mets	(de Cremoux et al. 2000)
hTERT	18	RT-PCR	25%	(Chen et al. 2000)
B-HCG	119	RT-PCR & southern blot	8%	(Hoon et al. 1996)
hMAM	114	Nested RT-PCR	25%	(Zach et al. 1999)
hMAM	78	Nested RT-PCR	24%	(Silva, A. L. et al. 2002)
hMAM	98	Real time-PCR	11%	(Suchy et al. 2000)
hMAM	60	Nested RT-PCR	9% with no mets 24% with mets	(Bossolasco et al. 2002)
hMAM	33	RT-PCR	54%	(Lin et al. 2003)
HER2/neu	17	Semi nested RT-PCR	59%	(Wasserman et al. 1999)
MAG	16	RT-PCR & ECL	25%	(Miyashiro et al. 2001)
CK19	60	Nested RT-PCR	13% in primary cancer 54% in mets	(Slade et al. 1999)
CK19	78	RT-PCR	25%	(Schoenfeld et al. 1997)

For abbreviations see page 42

	BREAST (continue)			
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
CK19	53	Nested RT-PCR	43% before CT 14% after CT	(Manhani et al. 2001)
CK19	34	Nested RT-PCR	21%	(Datta et al. 1994)
CK19	109	Nested RT-PCR	30% with no node disease 36% with node disease 71% with mets	(Kahn et al. 2000)
CK19		Real time-PCR	30% with no mets > 70% with mets	(Aerts et al. 2001)
CK19	148	Nested RT-PCR	30% with no mets 52% with mets	(Stathopoulou et al. 2002)
CK19	161	Nested RT-PCR	27% after CT	(Xenidis et al. 2003)
CK19	77 with no mets 47 with mets	Nested RT-PCR	31% in early before CT 7% in early before CT 40% with mets before CT 20% with mets after CT	(Stathopoulou et al. 2003)
CK19 CEA Maspin		RT-PCR	70% 63% 22%	(Lopez-Guerrero et al. 1999)
CK19 hMAM	45	RT-PCR	49% 60%	(Silva et al. 2001)
B-HCG C-MET Mag-A3 alpha 4 Gnt	65	Nested RT-PCR	2% for 4 markers 3% for 3 markers 15% for 2 markers 49% for 1 marker	(Taback et al. 2001)
Maspin	29	Nested RT-PCR	20% with mets	(Luppi et al. 1996)
hMAM CK19 EGFR	133 invasive cancer	Nested RT-PCR	8% 48% 10%	(Grunewald et al. 2000)
CK19 EGFR CK20	20	Nested RT-PCR & southern blot	90% 5% 0%	(Gradilone et al. 2003)
CK19 CK20 B-HCG	72	RT-PCR	10% 3% 13%	(Hu and Chow 2001)

	OESOPHAGEAL & OROPHARYNGEAL			
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
CEA & CK20	28	Real time-PCR	CEA 25% CK20 105	(Ito et al. 2004)
SCCA	70	RT-PCR	33%	(Kaganoi et al. 2004)
CEA	54	RT-PCR	57%	(Nakashima et al. 2003)
CK19	40 NPC	Nested RT-PCR	25% early stage 40% advanced stage 75% distant mets	(Lin et al. 2000)
	GASTRIC			
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
CK19, CK20	52	RT-PCR	10%	(Majima et al. 2000)
CK20	47	RT-PCR	43%	(Zhang et al. 2003)
CK19 hTERT CEA MUC1	64	MAH		(Wu et al. 2006)
c-MET MUC1	52	RT-PCR	62% 71%	(Uen et al. 2006)
alpha 4 Gnt	37		62%	(Shimizu et al. 2003)
CEA	59	Real time -PCR	46%	(Ikeguchi and Kaibara 2005)
CEA	57	RT-PCR	37%	(Miyazono et al. 2001)
CEA	46	RT-PCR	can identify risk of recurrence	(Seo et al. 2005)
CK20	70	RT-PCR	40%	(Illert et al. 2005)
CEA	36	RT-PCR	22%	(Nishida et al. 2000)
CK	34	RT-PCR	21%	(Yeh et al. 1998)

LIVER				
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
AFP	38	Real time-PCR	24%	(Morimoto et al. 2005)
AFP & MAG-1	65	Nested RT-PCR	42% for MAG-1 54% for AFP	(Yang et al. 2005)
IGF-II	111	RT-PCR	34%	(Dong et al. 2005)
AFP	50	RT-PCR	40%	(Cillo et al. 2004)
AFP	93	RT-PCR	53%	(Liu et al. 1998)
AFP	30	RT-PCR	95%	(Jiang et al. 1997)
ALB	62	RT-PCR	63%	(Muller et al. 1997)
AFP	64	RT-PCR	36%	(Komeda et al. 1995)
AFP	33	RT-PCR	52%	(Matsumura et al. 1994)
ALB	27	RT-PCR	43%	(Hillaire et al. 1994)
BILIARY & PANCREAS				
mRNA marker	**Cohort size**	**Method**	**Positive results**	**Ref**
alpha 4 Gnt	55	Quantitative RT-PCR	76%	(Ishizone et al. 2006)
CEA	67	RT-PCR	48%	(Uchikura et al. 2002)
CEA	40	Nested RT-PCR	42% in bile duct cancer 62% in pancreatic cancer	(Miyazono et al. 1999)

RT-PCR, Reverse transcriptase polymerase chain reaction; ECL, Electrochemiluminescence; PTHrP, Parathyroid hormone related protein; hMAM, Human mamoglobulin; HER2/neu, human epidermal growth factor receptor 2; hTERT, Human telomerase reverse transcriptase; B-HCG, Beta-subunit of human chorionic gonadotropin; MUC-1, Membrane associated mucin 1; MAG-A3, Melanoma associated antigen; Alpha 4 Gnt, alpha 1,4 N-acetylglucosaminyltransferase; SCCA, Squamous cell carcinoma antigen; NPC, Nasopharyngeal cancer; CK, Cytokeratins; CEA, Carcino embryonic antigen; MAH, Membrane array hybridization; C-MET, Hepatocyte growth factor receptor; Mets, Metastases; AFP, Alpha-fetoprotein; MAG-1, Malignant antigen; IGF-II; Insulin like growth factor; ALB, Albumin.

RT-PCR has been used to detect disseminated tumour cells in the peripheral blood, bone marrow, and peritoneal lavage of CRC patients by detecting the mRNA of various tumour and epithelial markers, and prognostic value has been reported (Schott et al. 1998). As said previously this technique has the ability to detect at best 1 cancer cell in 10,000,000 unwanted cells (Castells et al. 1998) (Mori et al. 1996) (Nakamori et al.

1997). Despite its wide use in detecting cancer cells RT-PCR has the disadvantage of not being able to quantify marker expression, it simply gives negative/positive discrimination. This has led researchers to use real time PCR, which allows the quantification of low level background transcription and the definition of cut off values for marker expression, hence improving sensitivity (Bustin et al. 1999) (Straub et al. 2001). Although an extremely powerful and reliable technique any PCR-based technique still has the disadvantage of potentially being over-sensitive, in that it can detect minimal amounts of mRNA expression in a non tissue specific manner (illegitimate transcription) meaning the technique is usually highly sensitive but insufficiently specific (Solmi et al. 2004).

The most commonly used RT-PCR targets in CRC studies are human telomerase reverse transcriptase (hTERT), cytokeratins (CK), carcino-embryonic antigen (CEA), epidermal growth factor receptor (EGFR), as well as a number of rare markers such as Dipeptidase I (DPEP I) Apolipoprotein AI (Apo-A1). The merits and limitations of each of these markers will be considered in turn.

1.10.5 hTERT mRNA

hTERT expression is associated with telomerase activation during cellular immortalisation and carcinogenesis as discussed in section 1.4.4. Its expression has been detected in serum samples from 70/78 patients (90%) with hepatocellular carcinoma (Miura, N. et al. 2003) and in four serum samples out of 16 patients (25%) with breast cancer (Chen et al. 2000) using RT-PCR.

hTERT expression was also detected intracellularly in the peripheral blood of 18/65 (46%) patients with gastric cancer using red blood cell (RBC) lysis followed by RT-PCR

(Shin et al. 2002), and detected in 29/55 (53%) patients with hepatocellular carcinoma using immunomagnetic separation technique to isolate CTC followed by RT-PCR (Waguri et al. 2003).

hTERT mRNA has been detected in the plasma samples of CRC patients (Lledo et al. 2004). Peripheral blood samples of 50 patients were collected pre operatively and real time PCR was used to detect the expression of hTERT, it was found that 82% of CRC patients had hTERT mRNA expression values higher than the maximum values of normal control and the sensitivity and the specificity of the assay were 98% and 64% respectively. The authors concluded however that, "hTERT expression did not correlate with the disease stage nor with the serum levels of CEA". The fact that plasma samples alone were used in this study, without an attempt to first isolate tumour cells by means of immunomagnetic separation, could explain the high level of detection of hTERT expression, as transcription of telomerase from non-tumour sources such as activated lymphocytes would have been possible.

1.10.6 CK

CK are intermediate filament keratins found in epithelial tissue. There are two main types: the acidic type I CKs and the basic or neutral type II CKs. These molecules are expressed by all epithelial cells and are part of the cell architecture; the most commonly used CKs for detection of CTC are CK19 and CK20 which are expressed in the gastrointestinal epithelium, urothelium, merkel cells and tumours derived from these tissues (Weitz et al. 1999).

Various studies have managed successfully to detect CK markers intracellularly and extracellularly. Vlems evaluated the use of CK 20 intracellularly in CRC patients using

RT-PCR on mononuclear cells collected from the peripheral blood and bone marrow and found that it was expressed in 9/30 blood and in 9/19 bone marrow samples (Vlems et al. 2002); but was also found to be expressed in 10/47 of healthy, non cancer individuals. This study has been corroborated by a number of other studies (Bustin et al. 1999) (Champelovier et al. 1999). A recent study done by Guo and co-workers (Guo et al. 2005) detected CK20 mRNA intracellularly in 72.5-82.5% in CTC of 40 CRC patients using a combination of positive and negative immunomagnetic isolation and real time PCR. They demonstrated that CK20 mRNA presence in CTC correlates significantly with the tumour size and lymphatic and hepatic invasion, but not the grade of differentiation. A study by Wong and colleagues, (Wong et al. 2001) evaluated the use of CK19 expression in a cohort of 33 CRC patients and 26 normal controls, by separating PBMC containing any CTC and extracting RNA from the cells in a one step method. A standard curve was made using different concentrations of PBMC spiked with the SK-BR-3 cell line followed by a semi-quantitative RT- PCR and Southern blot analysis. This study found that CK19 was expressed in 64% of CRC patients and 19% of controls and that the upper limit of CK mRNA among controls was exceeded by 14 patients, 12 of which (86%) developed metastases or recurrence. It was concluded that CK19 expression correlates with disease stage and CEA serum levels. Other studies have explained the expression of CK19 in healthy, non cancer, patients by the fact that blood samples might have been contaminated by epidermal cells during venepuncture leading to the illegitimate transcription of CK19 in the epidermal cells (Dingemans et al. 1997) (Castells et al. 1998) (Krismann et al. 1995), such technical difficulties are hard to overcome.

Some studies have used both CK19 and CK20 expression in an attempt to increase the specificity of the prognostic markers. Hardingham and colleagues (Hardingham et al. 2000) used both markers to detect CTC from blood samples of 94 CRC patients and 64 patients with adenomas and inflammatory bowel disease, using immunomagnetic separation and RT-PCR. They found that 20% of CRC patients had CK19 and CK20 expressed in CTC, whereas 10% of patients with benign adenoma had CTC expressing CK19 and CK20 and only 2% of patients with benign inflammatory bowel disease were positive. It was concluded that the presence of CK19/CK20 positive CTC was associated with a reduction in the disease free survival period.

Another study investigated the prognostic value of CK in CRC using a MACS system, followed by immunocytochemistry, to detect CTC expressing CK in the bone marrow. Of the 51 patients with CRC, 65% showed positive expression of CK and the presence of these cells correlated significantly with tumour stage, tumour extension and the clinically used tumour marker CA 19-9 (Weihrauch et al. 2002). Conversely, in a small study of patients with CRC (n=12) Gradilone and colleagues (Gradilone et al. 2003), using RT-PCR and Southern blot analysis for CK19 and CK20 in peripheral blood, claimed that both markers had no prognostic value after a follow up of 40 months. It is difficult to explain such discrepancies, although the small size of the study cohorts is likely to be very influential, which are exemplified by many other studies. See Table 1.9 for a summary of all the work which has investigated the prognostic value of CK in CRC patients. The main limitation of using CK as a tumour marker, despite some encouraging results, is the fact that normal controls commonly give positive results, and so this has encouraged researchers to look for better markers.

Table 1.9: CK as a marker in the peripheral blood of CRC patients

Cohort Size	Marker	Sample	Method	Positive results	Correlation	Ref.
30	CK20	PBMC	RT-PCR	9/30 in blood 9/19 in BM	No	(Vlems et al. 2002)
33	CK19	PBMC	Semiquantitive RT-PCR & Southern blot	64%	Yes	(Wong et al. 2001)
58	CK20	PBMC	RT-PCR	78% in BM 74% in portal vein 69% in peripheral blood	Yes	(Miura, M. et al. 2003)
40 27 CRC	CK20	PBMC	IMS & real time PCR	73%-83%	Yes	(Guo et al. 2005)
21 Controls	CK19+ ck20 & GCC	PBMC	RT-PCR	30% CK19 100% ck20 80% GCC	GCC a good marker CK not a good marker	(Bustin et al. 1999)
94 CRC 64 Benign	CK19+CK20	Whole blood	IMS+RT-PCR	20% in CRC 10% in adenoma 12% in IBD	Yes	(Hardingham et al. 2000)
26	CK8+CK19 CK20	Whole blood	IMS+ nested RT-PCR	12/23 patients	No	(Silva, J. M. et al. 2002)
100	CK20	Whole blood	RBC lysis + RT-PCR	34/100 16 with no mets	Yes	(Guller et al. 2002)
53	CK19	Plasma	Nested RT-PCR	74%	Yes	(Hampton et al. 2002)
129 CRC 58 Controls	CK20	Serum	Real time PCR	88% CRC 84% controls	No	(Wharton et al. 1999)
12 CRC 35 Controls	CK19+CK20	Serum	RT-PCR + southern blot	75% CK19 8% ck20	No	(Gradilone et al. 2003)

CK, Cytokeratins; PBMC, Peripheral mononuclear cells; BM, bone marrow: IMS, Immunomagnetic separation; RT-PCR, reverse transcriptase-polymerase chain reaction; IC, intracellular; EC, extracellular; RBC, red blood cells.

1.10.7 CEA

CEA is a glycoprotein involved in cell adhesion. It was first identified in 1965 in human colon cancer tissue extracts, and it was later found that serum from individuals with CRC and other carcinomas had raised levels of CEA compared with healthy individuals. CEA and related genes make up the CEA family which belong to the immunoglobulin superfamily. In humans there are 29 genes, 18 of which are normally expressed by columnar epithelial cells and goblet cells of the colon, mucous neck cells and pyloric mucous cells of the stomach, as well as various specialised squamous and secretory epithelium (Hammarstrom 1999).

Because of the observed over-expression of CEA protein in CRC, the mRNA has been used as a marker to detect CTC (Berinstein 2002). In summary, most available data on CRC indicates that the presence of CTC detected by CEA mRNA correlates with the presence of distant metastases (Jonas et al. 1996) and hence, as expected, associated with poor prognosis (Mori et al. 1998). However CEA mRNA was also detected in patients with inflammatory disease and in normal controls reducing its specificity as a tumour marker per sé in CRC. A recent study (Douard et al. 2005) developed a new multiplex RT-PCR assay for CTC detection based on the expression of carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5; formerly CEA) and CEACAM7 (formerly CGM2) in 84 CRC patients, 32 non-CRC patients and 41 healthy individuals. Positive immunomagnetic separation was applied to blood samples before RT-PCR. RT-PCR assay was negative for all the 41 healthy individuals and the 32 non-CRC patients. The test was positive in 53/84 (63%) of the CRC patients for CEACAM5 and/or CEACAM7, and 32/84 (38%) were positive for both markers. When the CRC patients were sub-divided in terms of stage, it was seen that 80% of stage III-IV cases (36/45) were positive

for one of the two markers, in contrast only to 44% of cases (17/39) for the early stage (I-II) patients. Further larger studies using a similar methodology, perhaps including additional markers, are eagerly awaited. A full list of all the studies to date that have investigated CEA mRNA, in CRC is shown in Table 1.10

In a very recent study Lloyd and her co-workers utilized multiple markers including CEA and CK20 to detect CTC in peripheral blood and peritoneal lavage using immuno-bead RT-PCR. They reported that the presence of CTC in the peritoneal lavage, post bowel manipulation, correlated significantly with disease prognosis ($P = 0.002$), and suggested that lavage sampling is a more appropriate medium in which to detect CTC than peripheral blood (Lloyd et al. 2006).

Table 1.10: Carcinoembryonic antigen as a molecular marker in CRC

Cohort Size	Sample	Method	Positive results	Correlate with Disease's stage	Ref.
36	PBMC	Real time PCR	More in Duke's D	Yes	(Miura, M. et al. 2003)
53	Plasma	Nested RT-PCR	32%	Yes	(Silva, J. M. et al. 2002)
39	PBMC	Real time PCR	6/39 in pre-op samples 10/39 in post-op samples		(Guller et al. 2002)
95 CRC 9 IBD	whole blood	RT-PCR	41% in CRC 55.6% in IBD	Yes	(Castells et al. 1998)
32	whole blood	Real time PCR	16%	No	(Hampton et al. 2002)
51 CRC 8 benign polyps	PBMC	Nested RT-PCR	67% in CRC Negative in benign polyps	Yes	(Guadagni et al. 2001)
100	whole blood	RBC lysis + RT-PCR	48%		(Wharton et al. 1999)
129 CRC 58 controls	Plasma	Real time PCR	86% in CRC 83.7% in controls		(Schuster et al. 2004)
33 CRC 26 controls	PBMC	Semiquantitive RT-PCR & southern blot	88% in CRC 92% controls		(Wong et al. 2001)
19	PBMC	RT-PCR	21.1% in pre-op samples 52.6% on follow up	No	(Noh et al. 2001)
84 CRC 32 BENIGN 41controls	PBMC	IMS + RT-PCR for CEACAM5 + CEACAM7	CEACAM5 53/84 CEACAM7 32/84		(Douard et al. 2005)

CRC, Colorectal cancer; IBD, inflammatory bowel disease; PBMC, Peripheral mononuclear cells; RT-PCR, Reverse transcriptase-polymerase chain reaction; CEACAM, carcinoembryonic antigen related cell adhesion molecule.

1.10.8 Epidermal growth factor receptor (EGFR)

EGFR are receptors for the epidermal growth factor which plays an important role in the regulation of cell growth and proliferation. The binding of EGF to its receptor causes activation of the intrinsic protein kinase activity that in turn stimulates DNA synthesis and cell proliferation (Carpenter and Cohen 1990). Mutation involving EGFR can lead to its constant activation and result in the uncontrolled cell proliferation; a hallmark of cancer (Lynch et al. 2004). As a tumour marker EGFR has the advantage of being expressed in a wide range of epithelial tumours (Khazaie et al. 1993), being rarely expressed by haemopoietic cells (Hildebrandt et al. 1997) and a number of high specificity antibodies are available, as these are being tested as potential targeted therapies (Baselga and Mendelsohn 1994) (Naruse et al. 2002) .

EGFR is over-expressed in 50-70% of primary colon, breast and lung cancer tissue samples (Salomon et al. 1995). EGFR has been detected in CTC in 22% of metastatic breast cancer patients (n=37), but not in localised disease or normal controls (Leitzel et al. 1998), and was found to be expressed in CTC in 74% of blood samples of bladder cancer patients (n= 27, n=16 respectively), but not in healthy controls (Gazzaniga et al. 2001a) (Gazzaniga et al. 2001b)

EGFR expression has been found to be positive in 12.5%, 30%, 18% (n=16 , n=10 , n=11) of advanced CRC, non small cell lung cancer and pancreatic cancer respectively (Clarke et al. 2003). In this study blood samples were collected from patients with advanced stage cancers and samples were mixed with red blood cell lysis buffer and then tumour markers were detected using real time PCR. The integrity of the RNA was

confirmed using human beta2 microglobulin primers. In a similar study De Luca (De Luca et al. 2000) examined the expression of EGFR in 91 patients with colon, breast and lung cancer. They isolated CTC mixing peripheral blood sample with 5% (w/v) dextran saline and then EGFR was detected using reverse transcriptase and Southern blotting. EGFR was expressed in 17/30 (57%) of stage IV lung cancer, 11/23 (48%) of stage IV breast cancer and in 16/38 (59%) of Duke's D colon cancer, it was found that 10.5% of healthy controls had a positive expression of EGFR as a result of illegitimate transcription of EGFR in leucocytes. Also in this study EGFR sensitivity and specificity were compared with CEA and CK 19 mRNA; it was found that 5/11 (45%) and 3/11 (27%) of patients expressed CEA mRNA and CK mRNA respectively and that 20% and 10% of normal controls expressed CEA mRNA and CK mRNA respectively.

The above studies have suggested that EGFR has a role in tumourigenesis, however, like that of hTERT, it is detected in the blood sample of patients with advanced disease and it could result in some false positive results, as a result of the illegitimate transcription.

1.10.9 Dipeptidase I (DPEP I)

DPEP I is a zinc dependent metallopeptidase that hydrolyses a variety of dipeptides and is involved in glutathione metabolism (Kozak and Tate 1982). DPEP I was identified as a tumour suppressor gene as a result of its decreased expression in Wilm's tumour (Austruy et al. 1993), however, its exact role is still unknown. One idea is that the enzyme is involved in the degradation of the extracellular matrix component that would facilitate the migration of tumour cells (McIver et al. 2004).

Using immunomagnetic isolation CTC were separated and real time PCR for DPEP I showed that the molecules was over expressed by ≥ 2 fold in colon tumours, compared

with normal adjacent mucosa in 82% of patients, also it was detected in peritoneal lavage and blood samples of 15/38 (39%) patients with resectable CRC, but was absent in blood samples of healthy controls (McIver et al. 2004). This study demonstrated some encouraging results showing that DPEP I was detectable in cancer patients, but not in normal controls. Because the study was a small scale pilot, it needs to be reassessed with a larger group of patients; as this remains the only study to utilize DPEP I as a tumour marker. Its applicability to other tumours also needs to be assessed.

1.10.10 Apolipoprotein AI (Apo-A1)

Apolipoprotein AI is the major protein moiety of high density lipoprotein (HDL); it is mainly produced in the liver and the intestines (Brewer et al. 1978), but is also produced *in vitro* by some differentiated cell lines established from human colorectal tumours (Reisher et al. 1993).

Normanno and colleagues (Normanno et al. 1998) attempted to detect disseminated tumour cells in peripheral blood in 20 CRC patients using Apo-A1 mRNA. Blood samples were collected pre- and immediately post-operatively and CTC were isolated by mixing the blood sample with 5% (w/v) dextran. RNA was extracted and then northern blot and RT-PCR were used to detect expression of APO- A1m RNA. No normal controls or patients with resected cancer were found to have APO-A1 expressed in peripheral blood, but two out of 10 patients with metastatic disease were positive. Despite the small numbers this study suggests that their RT-PCR technique is highly sensitive and specific, since they could detect up to 10 tumour cells in 5 ml of peripheral blood (one tumour cell in a background of 2.5×10^6).

1.11 Telomerase and cancer Immunology

1.11.1 The role of Cytotoxic T lymphocytes (CTL)

In the last few decades efforts have been made to understand the role of immune surveillance mechanisms against cancer cells in order to develop effective anti tumour immunotherapy.

The immune response against cancer cells is a complex one and involves the interaction of different cells and their products. However CTL play the major role in cancer immunity (Greenberg 1991) (Robins 1986). CTL are $CD8^+$ and therefore are class I Major Histocompatibility Complex (MHC) restricted in terms of cytotoxicity. MHCI molecules are glycoproteins expressed on the surface of all nucleated cells; in human there are 3 principal forms of MHCI: HLA-A, HLA-B and HLA-C, these molecules are involved in antigen presentation to CTL. Antigen peptides bind to the α-chain of the MHC molecule to form a stable MHC-peptide complex which exits the rough endoplasmic reticulum (RER) to the surface of the Antigen Presenting Cell (APC) via Golgi apparatus which is then recognized by $CD8^+$ cells. Once CTL are activated they perform their cytolytic activity by releasing perforin and proteases. Perforin forms pores in the tumour cell membrane through which proteases enter the tumour cell stimulate apoptosis, and fragment the tumour DNA (Atkinson and Bleackley 1995).

An alternative way by which CTL respond against tumour cells is the Fas-FasL interaction. FasL is part of the tumour necrosis factor receptor family (TNF) that binds to Fas on the membrane of tumour cells inducing cell death (Ishiwatari-Hayasaka et al. 1997). Some CTL also have Fas on their cell membranes which means potential self destruction of CTL as a tumour escape mechanism (O'Connell et al. 1996), also this interaction has been “accused" of facilitating local tumour invasion by inducing apoptosis of hepatocytes adjacent to tumour margins (Yoong et al. 1999).

1.11.2 Telomerase and tumour associated antigens (TAA)

TAA are proteins aberrantly and overly expressed by tumour cells. TAA can be classified into humoral and cellular ($CD4^+$ and $CD8^+$) responses according to the type of immune response they elicit. Many clinical studies have tried to identify one or more TAA that could have a role in future cancer immunotherapy but unfortunately most of those TAA described to date are restricted in expression to a few tumour types, therefore the concept of “epitope deduction” was used, in which some gene products with selective tumour expression were deducted and then scanned for peptides that match MHC binding motifs (Schultze and Vonderheide 2001) (Vonderheide 2002), the above description was used in order to identify a universal TAA which desirably should possess the following features:

1) Expressed by most human tumours but absent or rare in normal tissue.
2) Plays a universal role in tumourgenesis.
3) Includes peptide sequences that bind to MHC molecules.
4) Be recognized by the T cell repertoire in an MHC restricted manner.
5) Has the potential to be a major factor in future cancer immunotherapy.

Telomerase and its catalytic subunit have been proposed as a universal tumour antigen since it has all of the above features. Vonderheide and colleagues described the first immunogenic peptides from hTERT. This peptide designated as -I540 (ILAKFLHWL) - was found within the middle of the deduced amino acid sequence of hTERT, roughly 70 amino acids to the amino terminus of the first reverse transcriptase motif. This peptide binds to CTL in a MHC class I (HLA-A2) restricted fashion (Vonderheide et al. 1999), and demonstrated that CTL were induced in 70% of individuals specific to I540 epitope *in vitro*. These CTL lines killed a wide range of tumour cell line and primary tumours in a peptide-specific MHC-restricted fashion.

Using the same method Meniv *et al*. managed to identify another hTERT epitope -P865- (RLVDFLLV). In their experiments PMNC from prostate cancer patients and normal individuals were used. Positive HLA-A2 samples were pulsed with P540 and P 865 hTERT peptides and their result showed that 9/10 blood samples from donors developed CTL activity for P540 with 7 having activity for P865 after *in vitro* immunization. Furthermore CTL had lytic activity in 3 out of 4 patients with prostate cancer, moreover *in vivo* immunization of HLA-A2 transgenic mice generated a specific CTL response against both hTERT peptides (Minev et al. 2000).

Using the method of epitope deduction, Vonderheide groups screened HLA-A3-restricted peptide epitopes and identified K973 (KLFGVLRLK) which was subsequently tested for immunogenicity on human *in vitro* T-cell system. This epitope generated specific CTL from HLA-A3+ cancer patients and healthy individuals. These CTL lysed tumours from multiple histologies in an MHC-restricted fashion, suggesting that the epitope is naturally processed and presented by tumours. (Vonderheide et al. 2001).

Another study identified new hTERT-derived peptides carrying motifs for HLA-A24 (Arai et al. 2001). Two of the 5 peptides tested, VYAETKHFL and VYGFVRACL, were capable of generating HLA-A24-restricted CTL and demonstrated cytotoxicity against leukemia cells.

Cryptic epitopes that have a low affinity for HLA were also identified. Their affinity was increased using the P1Y heteroclitic peptide approach where peptides were mutated by tyrosine substitution at position one of the peptides in order to increase their affinity to bind to MHC. Two hTERT (hTERT 572 and hTERT 988)-derived low affinity peptides exhibited strong affinity for HLA-A2 and stimulated specific CTL from healthy donor PBMC. These CTL were able to lyse myeloma, melanoma and ovarian carcinoma cell lines. Also *in vivo* studies showed that HLA-A2 transgenic mice vaccinated with those two peptides generated CTL that specifically lysed antigen (Ag)-expressing tumour cells, thus recognizing the cognate endogenous Ag. This study suggests that low affinity epitopes could be used for cancer immunotherapy (Scardino et al. 2002). Another study confirmed that the low affinity peptide (P572) was processed and presented by HLA -A2 in tumour cells, and is recognized by specific CTL (Hernandez et al. 2002).

Based on the above studies a few clinical experiments were done on colorectal and breast cancer patients. Titu *et al* studied the T-cell response against two HLA-A2-specific epitopes of hTERT (P540 and P865) in 37 CRC patients and 12 normal controls using an interferon gamma (IFN-γ) ELISPOT assay (Titu et al. 2004). CTL recognized P540 HLA-A2 in 5 (13%) and P865 HLA-A2 in 4 (11%) of CRC patients. Two (5%) patients possessed T-cells that recognized both these peptides. No relationship between cancer stage and the presence of specific CTL against hTERT was observed although the

number of the patients was small. None of the normal controls possessed T-cells capable of recognizing the hTERT or the epitopes. The results of this study demonstrate that CTL active against hTERT are present in 20% of CRC patients, the authors suggested that this could be due to an error in protesome processing (Ayyoub et al. 2002), lack of co-stimulatory molecules (Greenfield et al. 1998) or anergy and resistance to activation in ELISPOT assay (Hermans et al. 1998).

In a similar manner Amaranath and colleagues investigated the specific $CD8^+$ CTL response in a cohort of 45 primary breast cancer patients and 44 normal controls against 3 hTERT peptides (I540, R865 and K973). In this study CTL recognized HLA-A2 P540 and P865 epitopes in 13/17 (76%) and 8/17 (47%) respectively, CTL recognized both HLA-A2 epitopes in 8/17 (47%), CTL recognized HLA-A3 K973 epitope in 5/7 (71%) patients. In contrast, 38% of normal healthy female controls responded against a single peptide only (Amarnath et al. 2004).

1.12 Aim of thesis

Finally the aim of this thesis was to detect CTC in the peripheral blood of CRC patients using telomerase as a tumour marker and correlate the findings with clinicopathological status of the patients. Telomerase presence and activity was measured using a combination of approaches including; ELISPOT assay to detect CTL that can recognize hTERT epitopes, the TeloTAGGG™ Telomerase TRAP assay to study the enzyme activity, and finally RT-PCR to measure hTERT mRNA in serum.

These techniques have increasing sensitivity, with the RT-PCR approach being expected to be the most sensitive.

CHAPTER 2
METHODS AND MATERIALS

In this chapter the general methods and materials for the following experiments conducted in this thesis will be described.

2.1 Study approval

This study received ethical approval from the Hull and East Riding Local Research Ethics Committee (Ref: LREC/12/00/232) and NHS approval from the Hull and East Yorkshire NHS Hospitals Trust Research and Development department (Ref: ELSY 2654).

2.1.1 Patient recruitment

Venous blood (30ml for PBMC and 10ml for CTC preparation) was initially taken from 105 CRC patients. In addition healthy volunteers of similar age were recruited in this study as normal controls. Written informed consent form was obtained from all patients and normal controls.

The blood samples were obtained by phlebotomy under aseptic technique. Venous blood was aspirated into a 60ml Luer-lock syringe pre-coated with 5000 I.U. (1ml) of sodium heparin, maintained at room temperature and then processed in the laboratory within a maximum of 2 hours.

2.1.2 Aseptic technique

All aseptic techniques were performed in a class II microbiological safety cabinet (Walker Safety Cabinets, Glossup, UK) fitted with a UV sterilizing lamp. All glassware

and heat-stable solutions were autoclaved prior to use at 121^{o}C for 15 minutes, whilst heat-labile solutions were sterilized by passage through a 0.2 Micron syringe filter (Sartorius AG, Goettingen, Germany). All disposable equipment and solutions were of sterile tissue culture grade, and all chemicals used were of molecular grade.

2.1.3 Selection criteria

Patients were selected according to the following criteria:

- Histological evidence of colonic or rectal adenocarcinoma.
- Age > 18 years.
- Patient able to provide written, informed, consent.

Patients were excluded according to the following criteria:

- Patients with other than benign or malignant tumours.
- Underlying immunodeficiency disorder or immunodeficiency state.
- Active autoimmune disorder.
- Patients unwilling or unable to give written informed consent.
- Concurrent chemotherapy or radiotherapy

2.1.4 Study protocol

Blood samples were obtained from CRC patients at multiple time points. The initial sample was obtained pre-operatively at the time of recruitment. Two further samples were then obtained in the post-operative period at between four and eight weeks following surgery. If the patient did not go on to receive post-operative, adjuvant therapy then their participation in the study finished at this point. Those patients who received

adjuvant treatment were requested to provide samples approximately every four weeks during the course of their treatment. A flow chart in figure 2.1 summarises the study protocol.

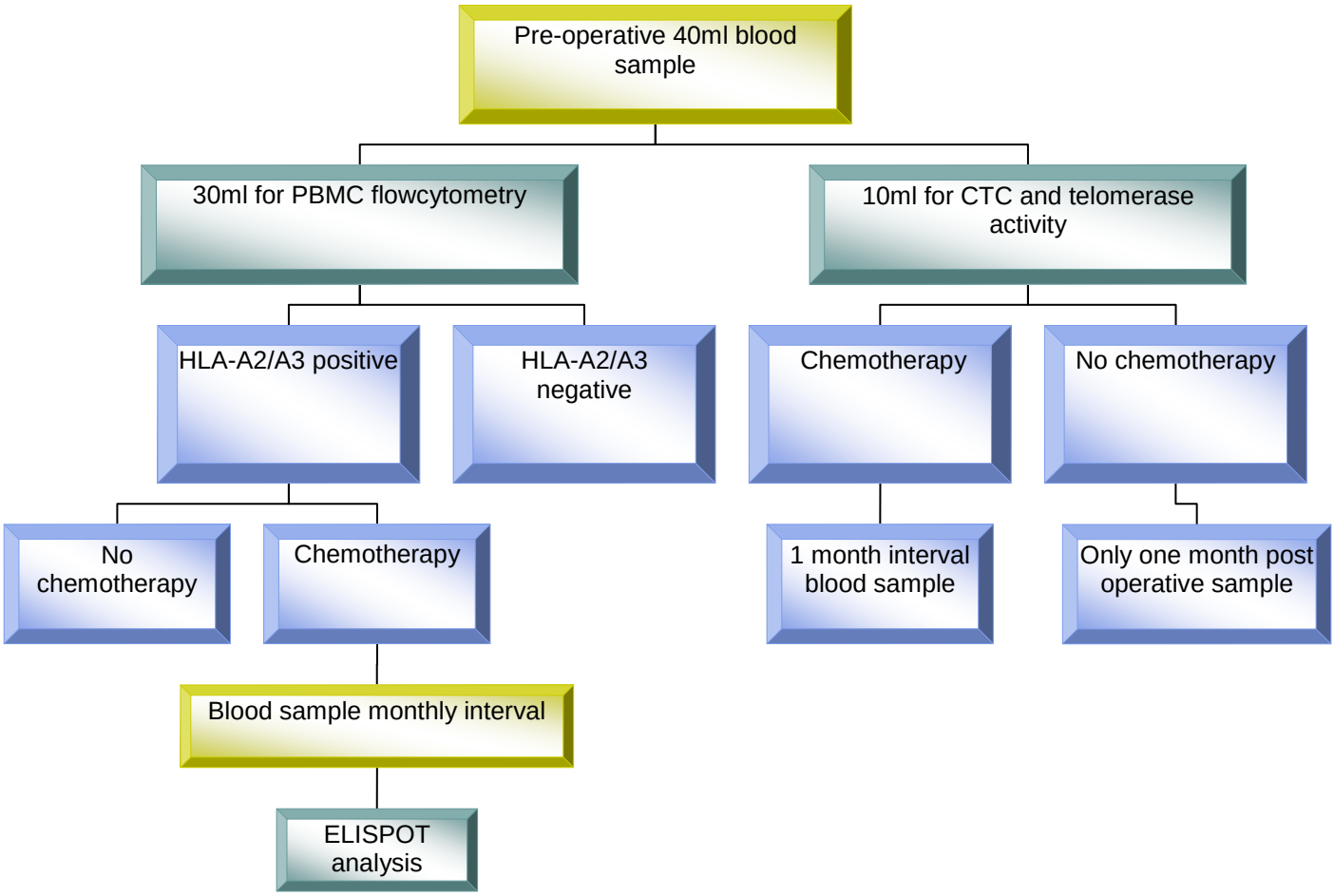


Figure 2.1 Study protocol

CTC: circulating tumour cells

2.2 Labelling Dynabeads with BerEP4

From the stock suspension 500µl (2 x 10^8 beads) was removed and placed in a 1.5ml microcentrifuge tube. This was then placed in a Dynal Magnetic Particle Concentrator (MPC) for 1 minute before pipetting off the supernatant. The tube was removed from the MPC and the beads resuspended in 500µl PBS-0.1% BSA and the washing step repeated to ensure the removal of the azide preservative. Following this 100µl (32µg) mouse monoclonal anti-human epithelial antigen clone Ber-EP4 antibody (Dako, Ely, UK) was added and the tube rotated at 4˚C for 30 minutes.

2.3 Preparation of CTC

The 10 ml of venous blood was mixed with an equal amount of phosphate buffered saline (PBS), pH 7.4, and then layered onto Ficoll-Hypaque (Histopaque, Sigma, Poole, UK). Blood/PBS mixture (20ml) was gently layered onto Histopaque (20ml) in a 50ml polypropylene tube (Greiner, Stonehouse, UK). The tube was subsequently centrifuged at 400*g* for 30 minutes at 4^{o}C. PBMC were isolated from the interface between Histopaque and plasma, and collected into a polypropylene tube.

2.3.1 Immunomagnetic separation (IMS) and CTC preservation

The isolated PBMC were then washed with an equal amount of PBS and centrifuged at 400*g* for 5 minutes. After centrifugation the PBMC were resuspended in 500µl PBS-1% w/v bovine serum albumin (BSA) and 12µl of 5x10^6 pre-washed immunomagnetic Dynabeads.

The mixture was then incubated with rotation at 4^{o}C for 30 minutes in the dark room. After this incubation the beads and the epithelial cells were harvested by placing the tube against a magnet (Dynal). Cells were washed 3 times in PBS-1% BSA, the supernatant

was discarded, and cells were resuspended at a concentration of 1-2x10^7 cells/ml in heat inactivated foetal calf serum (HiFCS) with 10% (v/v) dimethylsulphoxide (DMSO) as cryopreservative. The cells were finally transferred into 1ml cryotubes (Nunc) and incubated overnight at -80^{o}C (cooling rate 1^{o}C/minute). The following morning the cryotubes were transferred for long term storage in liquid nitrogen until further use.

2.4 Telomerase PCR ELISA

Telomerase activity was assessed using TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Sussex, UK), all reagents were supplied in the kit. The manufacturers' protocol was followed throughout as detailed below.

2.4.1 Preparation of cell lysates

Cells were thawed on ice, transferred to 1.5 ml polypropylene tubes and then centrifuged at 2000*g* for 10 minutes to pellet the cell-bead complexes from section 2.3.1. Supernatant was removed, and then the pellet was washed with 1ml of PBS and the mixture was centrifuged again for 10 minutes to ensure that the cell bead complexes are all collected. And after removing the supernatant cells were resuspended in 100µl lysis buffer from the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit and incubated for 30 minutes on ice. Cell debris were removed by centrifugation (13000*g* for 20 minutes, at 4^{o}C), and then 80-90µl of supernatant was removed, taking care not to transfer any cellular debris, and then stored at -80^{o}C until analysis.

2.4.2 Protein assay of lysates

The protein concentration of lysates was determined using Coomassie Plus kit (Pierce, Rockford, IL, USA) (Table 2.1). The contents of one BSA standard and PBS as a diluent were used to prepare a set of protein standards.

Table 2.1: Preparation of diluted BSA standards

Vial	Volume of diluent	Volume and source of BSA	Final BSA concentration
A	0	300µl of stock	2,000µg/ml
B	125µl	375µl of stock	1,500µg/ml
C	325µl	325µl of stock	1,000µg/ml
D	175µl	175µl of vial B dilution	750µg/ml
E	325µl	325µl of vial C dilution	500µg/ml
F	325µl	325µl of vial E dilution	250µg/ml
G	325µl	325µl of vial F dilution	125µg/ml
H	400µl	100µl of vial G dilution	25µg/ml
I	400µl	0	0

Standards and cell lysates (10µl) were added into the appropriate microplate wells, 300µl of Coomassie Plus reagent was added to each well and the mixture was put on a shaker for 30 seconds. The plate was then incubated for 10 minutes at room temperature after which the absorbance at 595nm was read using a plate reader (Anthos 2010, LabTech, UK). A standard curve was prepared by plotting the average blank-corrected 595nm measurement for each BSA standard. The standard curve was used to determine the protein concentration of each lysate sample. See figure 2.2 for an example.

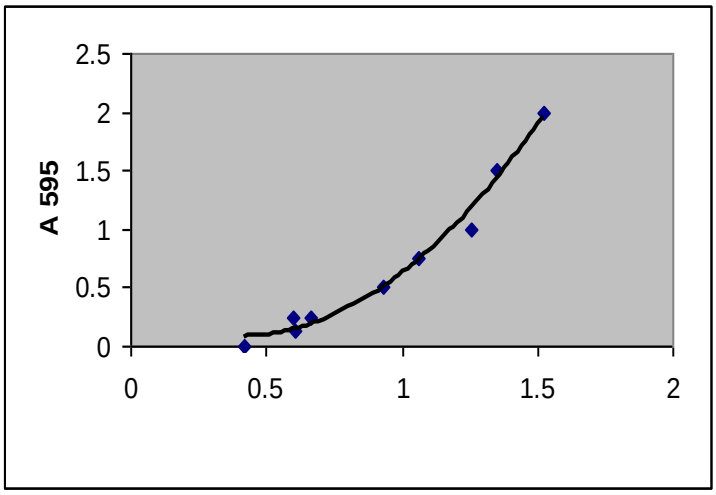


Figure 2.2: Protein standard curve example

2.4.3 PCR condition

For each sample and control template a master mix consisting of 25µl of reaction mixture and 5µl of internal standard were prepared per each tube. Master mix (30µl) was transferred into a microtitre plate per PCR reaction. Cell lysates (3µl) and 1µl of control template control (high and low) were added to each well. Nuclease-free water was added to each well to make a total volume of 50µl. Two sets of aliquots of the above were prepared; one was treated with heat (85^{o}C for 10 minutes) in order to inactivate the samples for use as a negative control, and one was the test. Then the relevant tubes were subjected to thermal cycling (Techne Progene, UK) according to the following protocol:

1) Primer elongation for 30 minutes at 25^{o}C
2) Telomerase inactivation for 5 minutes at 94^{o}C
3) Amplification (30 seconds at 94^{o}C, 30 seconds at 50^{o}C, 90 seconds at 72^{o}C) x 30 cycles and 10 minutes at 72^{o}C

2.4.4 Hybridization and ELISA

Following PCR, two aliquots of amplification products (2.5µl) were denatured at room temperature for 10 minutes with 10µ denaturation reagent. The denatured hybridization products were then hybridized separately to one of two digoxigenin labelled detection probes, either specific for telomeric repeats (hybridization buffet T) or the internal standard (IS buffer), mixed briefly and then added to streptavidin coated microtitre plate. The plate was covered and incubated at 37^{o}C on a shaker (300rpm) for 2 hours. Hybridization solutions were removed and the wells washed three times with washing buffer. The anti-DIG-HRP antibody (polyclonal sheep antibody against digoxigenin conjugated to horseradish peroxidase) conjugate working solution (100µ) was added and incubated with shaking at room temperature for 30 minutes. The solution was removed and the wells were washed five times with a solution buffer. Tetramethylbenzidine (TMB) substrate (100µl) was added; the plate was covered with foil and incubated on shaker at room temperature for 10-20 minutes (until colours developed). A 100µl of stop reagent that contains < 5% of sulphuric acid was added and the absorbance of samples measured (450nm-690nm) on an Anthos plate reader (Lab Tech, UK). The mean of the absorbance readings of the negative samples were subtracted from the absorbance readings of the test samples. Samples were regarded as positive if the difference in absorbance was higher than twice the background activity, as recommended by the manufacturer's protocol.

2.5.1 Preparation of PBMC

PBMC were prepared and stored using the methods and materials mentioned in section 2.3 and 2.3.1using 30 ml of venous blood.

2.5.2 Cell counting and viability testing

Trypan blue (0.1%w/v, 10µl) was added to 10µl of PBMC suspension and the concentration of cells in solution determined in duplicate using a haemocytometer (Improved Neubauer, Weber, UK). Viable cells exclude the dye, whereas dead cells take it up through their disrupted membranes. A systematic method of counting was used in order to ensure accuracy and precision and to avoid re-counting the same cell. The 25 large squares in the counting chamber were counted starting from left to right and then across and back, in a snake-like fashion. Each of the large squares, made up of 25 smaller squares, were counted in the same way as the large squares. A manual clicker counter was used to enumerate the cells. A minimum count of 100 viable cells was needed in order to ensure that the cells could be fixed to a concentration of $2x10^6$cells/ml. The concentration of cells was calculated by: number of counted cells x 10^4 x dilution factor. When calculating cell numbers for subsequent assay only viable cell numbers were used for HLA-A2 and HLA-A3 typing.

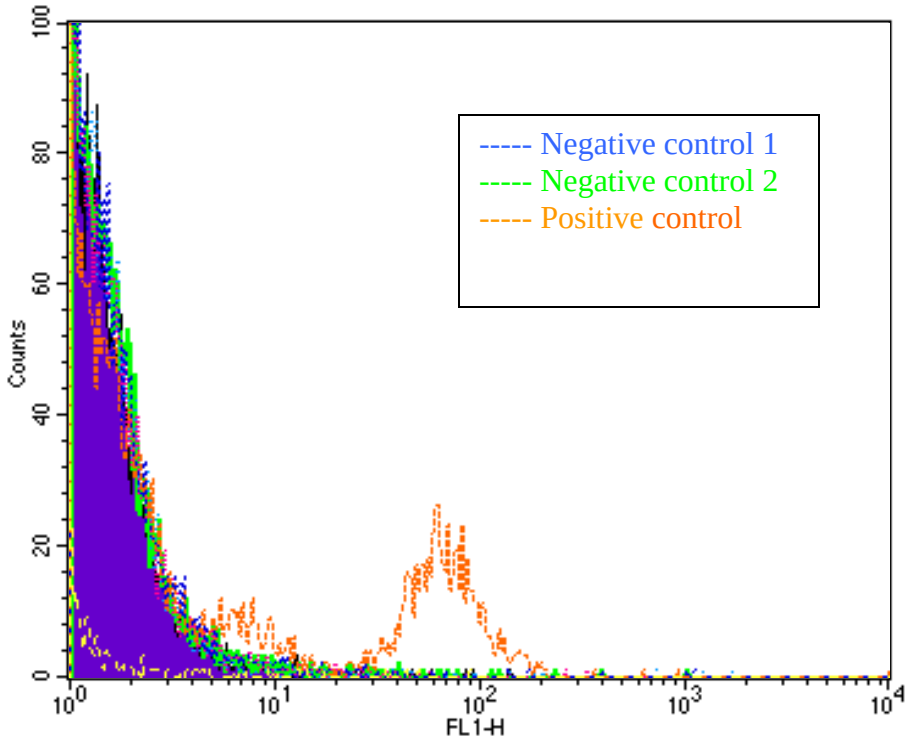
2.5.3 HLA-A2 and HLA-A3 typing of PBMC

The HLA typing of the patients was determined by flowcytometry (FACS). PBMC were incubated with mouse anti-human HLA-A2 mAb, mouse anti human HLA-A3 mAb, and two irrelevant mouse mAb; Ig1-RPE/IgG1-FIT (as negative controls), and CD3-RPE/CD4-FITC (as positive control), in order to confirm the interpretation of the fluorescence histograms. The cells were incubated with the primary antibodies at 4^oC, after which the excess antibody was washed away with 500µl of PBS-BSA-N_3 solution (PBS with 0.25% w/v BSA, and 10mM sodium azide) and then the cells centrifuged (400*g* for 5 minutes at 4^oC). The supernatant was discarded and 50µl/tube of secondary

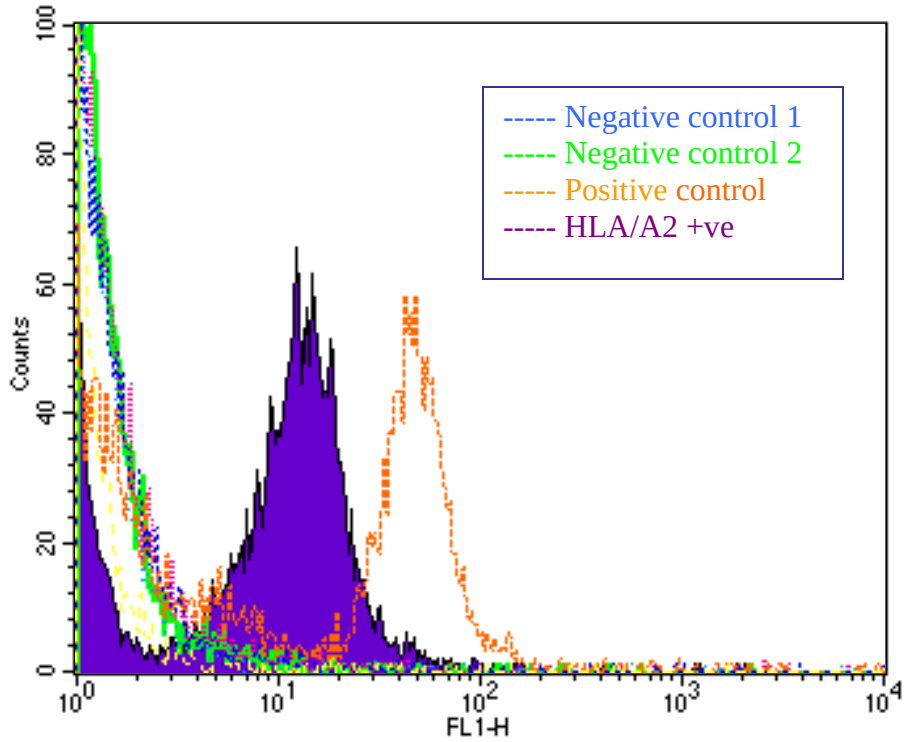
antibodies ($F(ab')_2$ rabbit anti-mouse IgG:FITC, 1mg/ml stock – 1/100 dilution - 50µl (Serotec) was added. Two other controls were established, represented by tubes to which only the secondary antibody or cells only were added, respectively. The cells were then incubated for another 30 minutes at 4^oC. Unbound antibody was washed from the cells as before with PBS-BSA-N_3 and the cell pellets were finally re-suspended in 400µl of this buffer.

To measure the proportion of labelled cells within a population, a flow cytometer (FACSCalibur, BD Biosciences) was used, and data analyzed using CELL Quest® software (BD Biosciences).

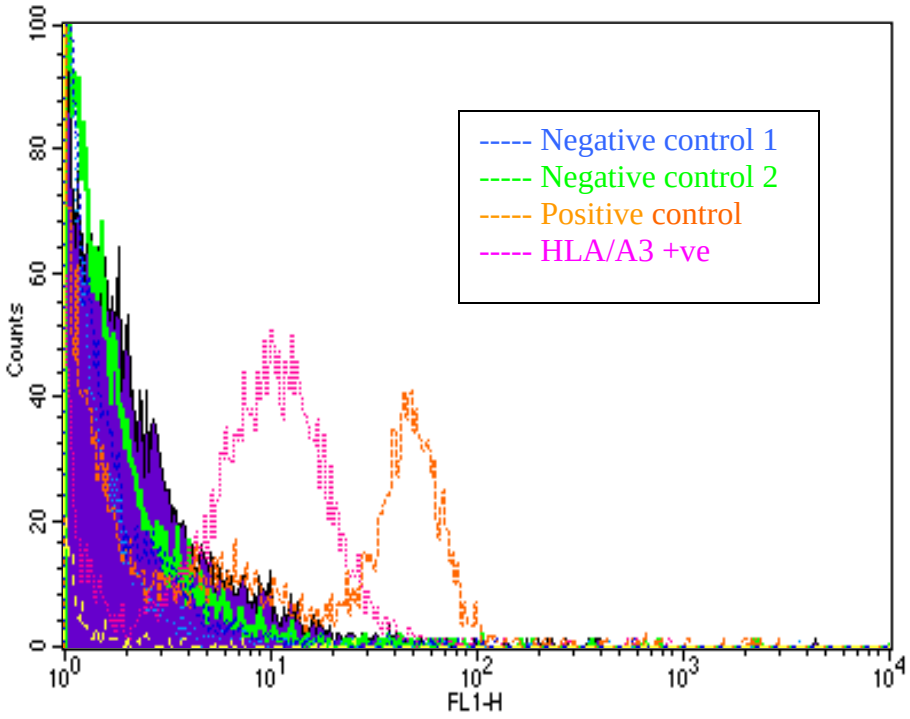
Only patients who reacted positively with both anti HLA-A2 and/or HLA-A3 were considered to be HLA-$A2^+/A3^+$ and their PBMC was used for subsequent analysis of anti hTERT $CD8^+$ T cells. Figure 2.3 illustrates HLA typing by flow-cytometry.



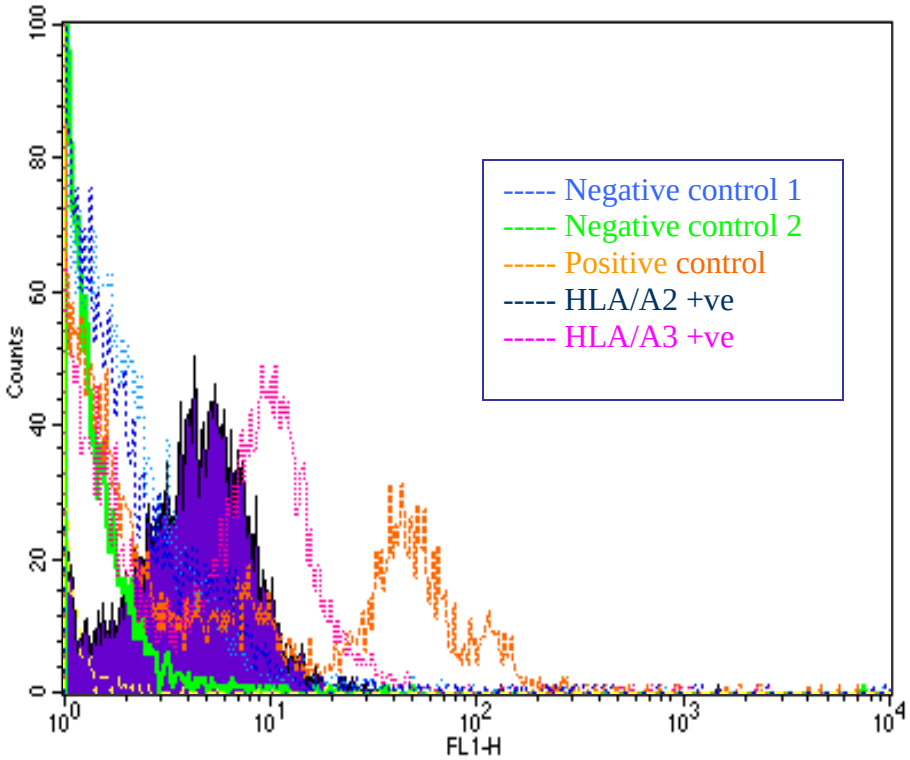
1) HLA-A2/A3 negative



2) HLA-A2 positive



3) HLA-A3 positive



4) HLA-A2/A3 positive

Figure 2.3 examples of HLA typing on flow cytometry.

2.5.4. ELISpot Assay

The ELISpot assay assesses CTL responses against specific peptides expressed by host MHC; it was performed using an IFN-γ ELISpot kit according to the manufacturer's instructions (R&D systems, UK). If CTL react against the tumour-derived peptide the CD8 positive cells will usually release IFN-γ that can be detected with immobilised antibodies. The multiscreen-HA sterile 96 well filtration plate (ELISpot plate) (Millipore, CA, USA) used allows for a maximum of seven samples to be run on a single plate. All the techniques were carried out in sterile conditions, in a class II microbiological safety cabinet.

2.5.4.1 Day 1

For each assay, up to seven PBMC samples were selected and removed from the liquid nitrogen. Attention was taken to ensure that all the samples had the same HLA status so that they could all be put onto the same plate with the same peptides. The PBMC samples were thawed in a water bath at 37°C for a few minutes. Each sample was carefully mixed and transferred into RPMI 1640 culture medium (10ml) and centrifuged at 400*g* for 5 minutes to remove freeze media. The supernatant was discarded and the pellet re-suspended in 1ml CTL medium. The samples were incubated at 37°C until ready to be used. The number of viable cells present in the sample was determined by counting the number of viable PBMC using the method in section 2.5.2. Samples with a cell count of 100 or more were used and samples with a significantly low number of cells were discarded. The samples that had between 70-99 cells were kept and if there was another sample stored in the liquid nitrogen, it was prepared in the same way, counted, and combined to increase the number of cells to make up at least 100. In the case when two

samples were mixed together, these were centrifuged again (400*g* for 3 minutes) and resuspended in 1ml CTL medium. If the samples could not be used, more samples were defrosted in the same way, prepared and counted, until there were seven different patients' samples that could be used. The samples were adjusted to a concentration of $2x10^6$ cells/ml using CTL medium. Each sample was put into a well of a 6 well plate and put in a 37°C incubator overnight. Any spare wells with no cells were filled with RPMI, in order to create a humidified environment for the growth of the cells. Capture antibody (100µl, IFN-γ without biotin) was pipetted into each of the wells of the ELISpot plate and incubated in the fridge at 4°C overnight.

2.5.4.2 Day 2

The plate was washed six times by multichannel pipetting 200µl sterile PBS into the wells and discarding in virkon (2% w/v). After tapping dry, 150µl of blocking medium (a 15ml quantity of PBS-10% w/v BSA was made up and filter sterilised by passage through a 0.2µm filter, Sartorius AG, Goettingen, Germany) was added to each well and the plate incubated for two hours at 37°C. After discarding the blocking medium and tapping dry, 100µl of each cell sample from the 6 well plates was added to the ELISpot microtitre plate. Appropriate hTERT peptides (10µl of 100ng/ml, ProImmune, Oxford, UK) were then added to the wells according the pipetting scheme layout (Figure 2.4)

	1	2	3	4	5	6	7	8	9	10	11	12
A	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
B	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
C	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
D	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
E	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
F	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
G	hTERT-1	→	hTERT-2	→	Inf-A2	→	hTERT-A3	→	Inf-A3	→	PMA	Cells only
	IFN-γ	IFN-γ	IFN-γ							CTL	CTL	CTL

Figure 2.4: Schematic ELISpot plate layout

Note: CTL denotes CTL medium (a negative control set of wells)

Influenza peptides (10µl) and 5µl of Phorbol 12-myristate 13-acetate PMA were added to their respective wells. In addition, 100µl of recombinant IFN-γ (1ng/µl, Serotec, Oxford, UK) was added to three wells as an additional positive control. The presence of IFN-γ should always give a positive result. No peptides were added to some of the cells, leaving the cells in the well alone with the capture antibody, which will show a background level of IFN-γ response. Any response measured in these wells was deducted from the values obtained from the test peptides. CTL medium (100µl) was used as a negative control, and was added to three wells. The plate was incubated at 37°C overnight.

2.5.4.3 Day 3

The wells were washed six times using 200µl PBS-0.05% v/v Tween-20 (Sigma) and tapped dry. Mouse anti-human IgG1 IFNγ antibody (100µl) conjugated to biotin

(Mabtech AB, Nacka, Sweden) was added to each well, to detect bound IFNγ, and incubated overnight in the fridge at 4°C.

2.5.4.4 Day 4

PBS-0.05% Tween-20 (200µl) was used to wash the plate six times, as before. 100µl of streptavidin-alkaline phosphatase (Bio-Rad Laboratories, Hemel Hemstead, UK), diluted 1 in 1000 with PBS, was added to the wells and left in the dark at room temperature for two hours. The plate was washed six times as before using PBS-0.05% v/v Tween, alkaline phosphatase substrate was made up from a kit (Bio-Rad) by adding 100µl of solution A, 100µl of solution B and 400µl of buffer to 9.6ml distilled water. Then the alkaline phosphatase substrate solution (100µl) was added to the plate. The plate was left, covered, for 15-30 minutes until visible blue spots appeared. The remaining reagent in the plate discarded. The plate was then washed under running tap water and left to dry.

The plates were stored in a dark and dry place at room temperature until they were taken to be counted using the AID ELISpot automated plate reader at St James University Hospital, Leeds by Dr. L Madden. The analysis of the results was done using the AID ELISpot analysis software.

2.6 RT-PCR of serum samples

Serum samples were collected from 97 CRC patients, who underwent curative surgical resection. Blood samples were collected on three occasions; pre-operatively, one month and one year post operatively.

2.6.1 Serum preparation

The venous blood samples were kept in the fridge (4^{o}C) in an upright position for at least 30 minutes. After that the blood samples were centrifuged (3000*g*, for 10 minutes, at 4^{o}C). Then serum was extracted and stored at -80^{o}C until use.

2.6.2 RNA extraction

The manufacturer's protocol was followed throughout to extract serum RNA. (SV Total RNA Isolation System, Promega USA).

Serum samples were thawed on ice, and then 175µl of serum was transferred into a sterile 1.5 ml micro-centrifuge tube using RNase-free pipettes tips to reduce the chance of RNase contamination. The same amount of SV RNA lysis buffer was added to each sample and mixed gently by pippeting. Next 175µl of the mixture was added to 350µl of SV RNA dilution buffer and mixed gently by inverting the tube 3- 4 times; the tubes were then placed in a water bath at 70^{o}C for 3 minutes. Finally the tubes were centrifuged at 13000*g* at 20-25^{o}C to pellet cell protein debris.

The cleared lysate solution was transferred to a fresh micro-centrifuge tube and 200µl ethanol (95%) was added to each tube and mixed gently by pipetting 3-4 times. The mixture was then transferred to spin column assembly and the mixture was centrifuged at 13000g for one minute. After discarding the liquid from the spin basket 600µl of SV RNA wash solution was added to the spin column assembly, and then the columns were centrifuged at 13000*g* for one minute.

After that a 50µl of DNase incubation mix (40µl Yellow Core Buffer, 5µl 0.09M $MnCL_2$ and 5µl of DNase I enzyme) was added directly to the membrane of each spin column assembly and incubated for 15 minutes at room temperature. After the incubation period

200µl of SV DNA stop solution was added to each sample and then centrifuged at 13000g for one minute. Then 600µl of SV RNA wash solution was added and the spin column assembly centrifuged for a further one minute. The spin basket was then emptied and 250µl of SV RNA Wash Solution was added and the column was centrifuged for 2 minutes. Then the spin basket was put in a new elution tube and 100µl of nuclease-free water was added directly to the membrane of the spin basket and then the tube was centrifuged for 2 minutes to elicit the purified RNA which if not used immediately was stored at -80^{o}C.

2.6.3 RT-PCR condition

The QIAGEN oneStep RT-PCR protocol was followed thoroughly (QIAGEN, West Sussex). RNA extract, primer solutions, hTERT forward and reverse, (RL-hTERT-FOR: 5'-CGT GGT TTC TGT GTG GTG TC-3', RL-hTERT-REV: 5'-CCT TGT CGC CTG AGG AGT AG-3') (http://frodo.wi.mit.edu/), dNTP Mix, 5x QIAGEN oneStep RT-PCR Buffer and RNase free water were all thawed on ice. A master mix was made according to Table 2.2, and 45µl dispensed into PCR tubes.

Table 2.2: Master Mix concentrations

RNase-free water	29.75µl/reaction
RT-PCR Buffer	10.0µl/reaction
dNTP Mix	2.0µl/reaction
hTERT for	0.5µl/reaction
hTERT rev	0.5µl/reaction
RT-PCR enzyme mix	2.0µl/reaction
RNase inhibitor	0.25µl/reaction
Total Master Mix	45µl/reaction

Template RNA from patients, normal controls, or CRC cell lines (5µl) (CaCO2 & LoVo cell lines) and 5µl of nuclease-free water as a negative control were added to each individual PCR tube. The thermal cycler was pre-heated to 50^{o}C before the PCR tubes were placed in the thermal cycler. The following PCR conditions were applied (Table 2.3) and the PCR products were later run on an agarose gel (section 2.6.4).

Table 2.3: PCR conditions

RT-PCR steps	**Time**	**Temperature**
Reverse transcription	30 minutes	50^{o}C
Initial PCR activation step	15 minutes	95^{o}C
3-step cycling (40 cycles)		
Denaturation	1 minute	94^{o}C
Extension	1 minute	72^{o}C
Final extension	10 minutes	72^{o}C

2.6.4 3% agarose gel preparartion

Agarose (3g) was mixed with 100ml of 1xTBE (Tris base, boric acid and EDTA); the mixture was put in the autoclave to allow the 3% w/v gel to desolve. The gel solution was left to cool down, and after that 2µl of ethidium bromide was added to the gel. The gel was poured into a casting mould with a comb to form the wells and the gel was left to set.

2.6.5 PCR products loading

PCR product (10µl) were mixed with 2µl of 6x loading dye (BlueJuice™ Gel Loading Buffer, Invitrogen), and then the mixture and the DNA ladder (GeneRuler^{TM} DNA ladder, Invitrogen) were loaded in the agarose plate and left running on electrophoresis at 100 V for 45 minutes. The gel was photographed using a UV illuminator (Uvi Tech).

CHAPTER 3

Detection of specific cytotoxic T lymphocytes recognising hTERT using the ELISpot assay

In this chapter the results of the telomerase activity in peripheral blood of CRC patients using ELISpot assay will be discussed

3.1 Introduction

TAA are oncogenic proteins expressed by tumour cells. Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase and it is the rate limiting factor. Peptide epitopes from this molecule could be a used as universal TAA since it is expressed by most human tumours, plays a universal role in tumourgenesis and is recognized by the CD8 cytotoxic T lymphocytes (CTL) in an MHC restricted manner (see section 1.11.2)

The aim of this study was to detect CTL that can recognize hTERT epitopes.

3.2 Patient samples

Out of the 105 patients that were initially recruited, 34 were HLA-A2 or HLA-A3 positive; the remaining 71 patients were not eligible to be used in the ELISpot study. Some of the samples were found to be either dead or there were not enough viable cells ($2x10^5$cells/µl) required to perform the ELISpot, therefore, only 30 of the 34 patients were used. Table 3.1 shows the relevant patient details of the patients that were used for the ELISpot assay. The median age of the patients was 69 (53-83).

Although all of the patients had at least two samples taken: pre (sample 1) and post-operation (sample 2), not all the samples could be analysed, due to insufficient viable cells. Table 3.2 shows the patient and their samples that were used. 28 pre and 13 post-operational samples were tested, providing a cohort of 12 sample pairs that for analysis. A further 6 patients had more than 2 consecutive samples tested, allowing a small pilot study on follow-up with time.

Table 3.1: Patient details that were used for the ELISpot assay

Patient No.	Site of Cancer	Dukes Stage	Age	Date of Death	HLA Status
21	Rectum	C	57	n/a	A2+
26	Colon	C2	74	n/a	A2+
36	Colon	D	73	n/a	A2+
37	Colon	C2	83	n/a	A2+
40	Rectum	C	62	19/02/2005	A2+
42	Colon	C	67	n/a	A2+
43	Colon	C	58	n/a	A2+
44	Rectum	C2	77	n/a	A2+/A3+
53	Benign	Benign	75	26/02/2005	A2+
61	Rectum	C1	59	n/a	A3+
66	Colon	B	77	n/a	A2+
67	Rectum	C1	62	n/a	A2+
69	Rectum	C1	68	n/a	A2+
71	Colon	B	69	n/a	A3+
72	Colon	B	77	n/a	A2+
73	Benign	Benign	72	n/a	A2+
75	Rectum	C2	78	n/a	A2+
76	Colon	B	59	n/a	A2+
77	Rectum	C1	53	n/a	A2+
79	Rectum	A	78	n/a	A2+/A3+
80	Colon	B	80	n/a	A2+
90	Benign	Benign	80	02/05/2005	A2+
91	Colon	D	70	n/a	A3+
95	Rectum	C2	81	06/11/2005	A2+
97	Rectum	C	67	25/02/2006	A2+/A3+
98	Colon	C1	63	n/a	A2+/A3+
99	Colon	A	76	n/a	A2+
100	Benign	Benign	65	n/a	A2+/A3+
101	Rectum	C1	67	n/a	A2+/A3+
105	Rectum	B	64	n/a	A2+

Note: n/a=not applicable, as patient did not die during the course of the study

Table 3.2: Showing the ELISpot assays performed

Patient No.	Dukes Stage	Sample No.					
		1	2	3	4	5	6
21	C	■	■		■		■
26	C2		■				
36	D		■	■			
37	C2	■	■				
40	C	■					
42	C	■	■	■			
43	C	■		■	■		
44	C2	■		■	■		
53	Benign	■					
61	C1	■					
66	B	■	■	■			
67	C1	■	■				
69	C1	■	■	■			
71	B	■	■	■	■	■	
72	B	■	■				
73	Benign	■					
75	C2	■					
76	B	■					
77	C1	■					
79	A	■					
80	B	■					
90	Benign	■					
91	D	■	■				
95	C2	■					
97	C	■	■				
98	C1	■	■				
99	A	■					
100	Benign	■					
101	C1	■	■				
105	B	■					

Note: Black boxes indicate that the sample was used for the ELISpot assay

3.3 HLA status and CTL response against hTERT peptides in CRC patients

From the 30 patients that were investigated, 21 (70%) were HLA-A2 positive, 3 (10%) were HLA-A3 positive and 6 (20%) were HLA-A2 and A3 positive.

The immediate results from the practical work can be shown by observing the spots obtained on the ELISpot plates. The results obtained were variable, as Figure 3.1 shows that the size, intensity, and number of spots were different in each case. The spots were

then counted using an AID ELISpot automated plate reader (St James Hospital, Leeds) to give the final results as numbers.

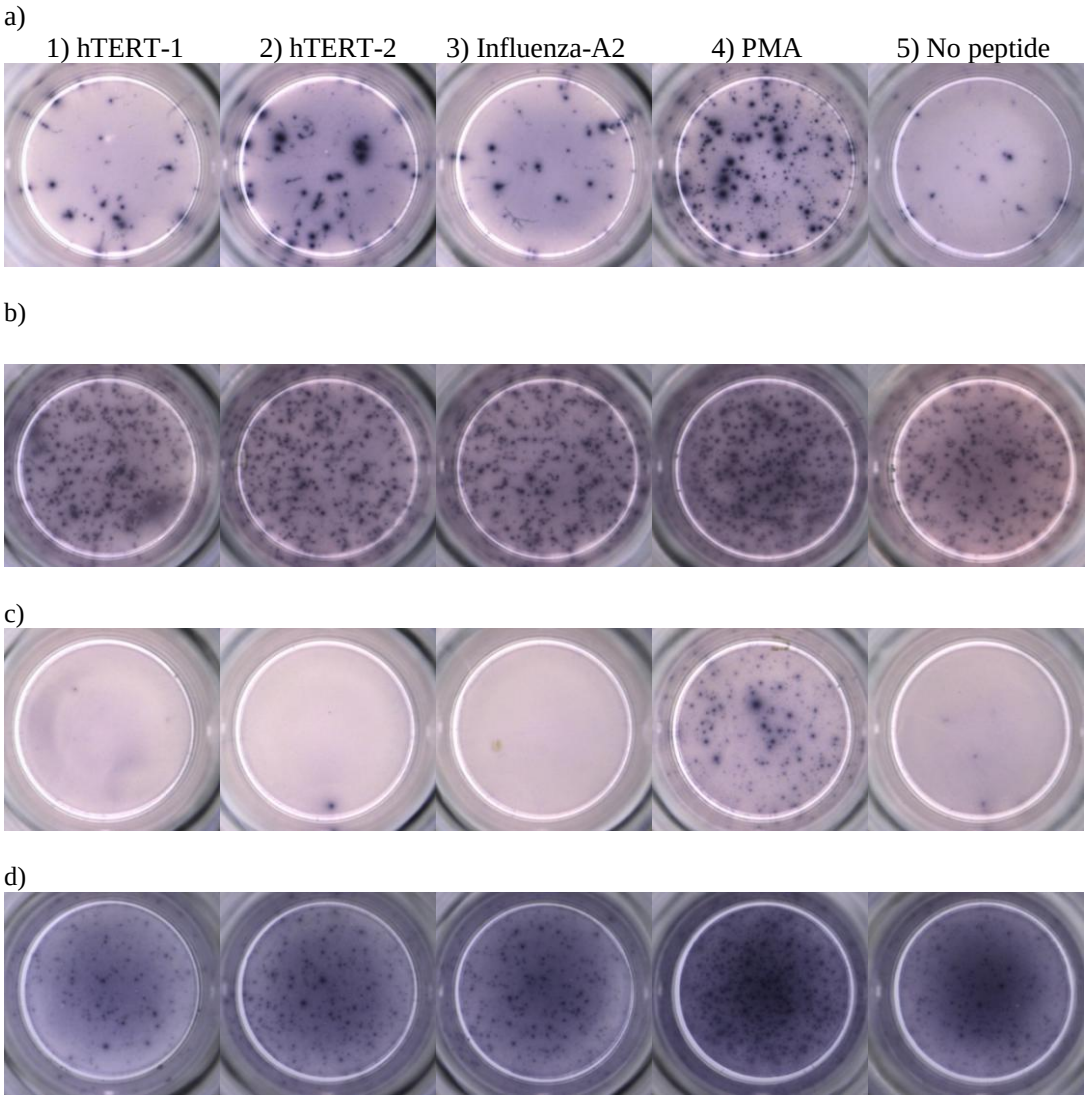


Figure 3.1 Examples of ELISpot results.

a) Good response
b) Good response, but high response with no peptide creates negative results
c) Poor response, but positive PMA.
d) Poor result due to background colouration (from prelimary work).

For all the data, the numbers of spots are recorded per $2x10^5$ PBMC. The specific number of spots was calculated by subtracting the number of spots present in the wells with no

peptide (negative control well). This gives the number of spot forming cells (SFC), which relates directly to the exact number of cells that responded. Where the number of spots became a negative number, the value was changed to zero, as it is not possible to have a negative number of spots. This method was previously used by (Amarnath et al. 2004)

Tables 3.3 and 3.4 show the average ELISpot results for the A2 and A3 positive patients respectively. The patients that are positive for A2 and A3 are recorded in both tables, and the results analysed independently, denoted as two samples.

The results obtained can be looked at on an individual basis, or may be compared in groups to establish patterns of responses, at a certain time-point, or over time. It is mainly the hTERT peptide response and influenza response that will be studied. Other factors that come into consideration will also be noted, such as the site and stage of cancer, and patient death.

Table 3.3: ELISpot results for A2 positive patients

Patient		Average number of SFC per well					Specific number of SFC per well			
Number	Sample	hTERT-1	hTERT-2	Inf A2	PMA	No peptide	hTERT-1	hTERT-2	Inf A2	PMA
21	2	8	4	7	16	1	7	3	6	15
21	2	59	69	55	171	24	35	45	31	147
21	4	194	213	187	269	203	0	10	0	66
21	6	88	90	87	193	81	7	9	6	112
26	2	15	8	3	37	6	9	2	0	31
36	2	0	1	3	1	2	0	0	1	0
36	3	10	3	1	116	2	8	1	0	114
37	1	56	38	45	185	42	14	0	3	143
37	2	1	3	4	9	5	0	0	0	4
40	1	7	0	1	12	5	2	0	0	7
42	1	0	0	0	38	0	0	0	0	38
42	2	9	4	11	25	1	8	3	10	24
42	3	5	2	7	5	1	4	1	6	4
43	1	1	2	1	69	2	0	0	0	67
43	3	26	17	6	21	2	24	15	4	19
43	4	1	2	1	14	1	0	1	0	13
44	1	5	3	0	5	1	4	2	0	4
44	3	90	100	97	329	100	0	0	0	229
44	4	27	39	30	87	32	0	7	0	55
53	1	5	1	2	12	1	4	0	1	11
66	1	25	27	31	287	19	6	8	12	268
66	2	26	29	20	153	22	4	7	0	131
66	3	123	59	47	183	35	88	24	12	148
67	1	186	164	174	205	120	66	44	54	85
69	1	5	2	0	12	3	2	0	0	9
71	1	95	101	77	148	81	14	20	0	67
72	1	91	80	82	246	55	36	25	27	191
72	2	12	20	18	111	21	0	0	0	90
73	1	100	89	87	182	78	22	11	9	104
75	1	58	50	66	305	56	2	0	10	249
76	1	174	186	170	240	169	5	17	1	71
77	1	242	238	233	260	204	38	34	29	56
79	1	25	12	19	368	8	17	4	11	360
80	1	0	1	0	65	1	0	0	0	64
90	1	0	0	2	5	4	0	0	0	1
95	1	3	5	11	32	13	0	0	0	19
97	1	10	31	16	118	58	0	0	0	60
97	2	3	3	1	12	1	2	2	0	11
98	1	13	10	7	5	9	4	1	0	0
98	2	13	21	17	17	3	10	18	14	14
99	1	1	8	8	5	7	0	1	1	0
100	1	97	112	147	166	109	0	3	38	57
105	1	1	0	0	8	0	1	0	0	8

Table 3.4: ELISpot results for A3 positive patients

Patient		Average number of SFC per well				Specific number of SFC per well		
Number	sample	hTERT A3	Inf A3	PMA	no peptide	hTERT A3	Inf A3	PMA
44	1	3	3	5	1	2	2	4
44	3	72	74	329	100	0	0	230
44	4	31	31	100	32	0	0	55
61	1	23	29	159	32	0	0	127
71	1	9	4	12	12	0	0	0
71	2	99	89	116	115	0	0	1
71	3	5	9	7	73	0	0	0
71	4	56	38	95	86	0	0	9
71	5	42	50	42	215	0	0	0
79	1	16	13	368	8	8	5	360
91	1	2	8	4	6	0	2	0
91	2	123	59	87	90	33	0	0
97	1	10	14	118	58	0	0	60
97	2	5	3	12	1	4	2	11
98	1	29	9	17	3	26	6	14
98	2	133	121	166	109	24	12	57
100	1	133	121	166	109	24	12	57
101	1	18	0	1	3	15	0	0
101	1	0	3	29	2	0	1	27
101	2	59	69	172	77	0	0	95

Twenty-eight HLA-A2 positive patient samples were stimulated with PMA of these 18 (64%) showed recognition of the hTERT-1 peptide, 19 (68%) responded to hTERT-2 peptide, and 16 (57%) recognised both peptides. Six of the 9 (67%) HLA-A3 positive samples recognised the hTERT A3 peptide**.**

The pattern of response to hTERT was also investigated by examining the results for samples that belong to the same patient pre-operatively and post-operatively. Where CTL that recognized hTERT were detected in 20/28 patients (71%) pre-operatively, and 7/13 (54%) analyzable post-operative samples showed a CTL response. Table 3.5 simplifies the results of tables 3.3 and 3.4.

Table 3.5: CTL response against hTERT in patients' samples

Patient No.	Dukes Stage	Sample No.					
		1	2	3	4	5	6
21	C	+	+		+		+
26	C2		+				
36	D		-	+			
37	C2	+	-				
40	C	+					
42	C	-	+	+			
43	C	-		+			
44	C2	+	-	+			
53	Benign	+					
61	C1	-					
66	B	+	+	+			
67	C1	+					
69	C1	+					
71	B	+	-	-	-	-	
72	B	+	-				
73	Benign	+					
75	C2	+					
76	B	+					
77	C1	+					
79	A	+					
80	B	-					
90	Benign	-					
91	D	-	+				
95	C2	-					
97	C	-	+				
98	C1	+	+				
99	A	+					
100	Benign	+					
101	C1	+					
105	B	+	-				

+: positive CTL response
-: negative CTL response

The above pre and post operative samples provided a cohort of 12 sample pairs. In this cohort 3 patients had CTL response in their pre and post operative samples, five patients had CTL response in the pre-operative sample but not in any of the post-operative samples and 4 patients developed CTL response in their post-operative samples but not in the pre-operative samples.

A further 6 patients had more than 2 consecutive samples tested, allowing a small pilot study on follow-up with time. In this group 5 patients in their last sample had CTL against hTERT peptides detected and one sample was negative. Four of these patients were Duke's C and 2 were Dukes' B, these samples were collected over a period of 4 months.

3.4 Repeated samples

In order to validate the assay, the ELISpot assay was repeated on the same samples but it was not possible to repeat the tests for all the samples as there was either insufficient time or there were insufficient cells from the patient. In the case of patient 21, sample 2, and patient 101, sample 1, where the only two samples that were repeated. The results are shown in Table 3.6 and 3.7.

Table 3.6: ELISpot results for a HLA-A2 positive patient- patient 22, sample 2

Patient		Specific number of SFC per well				Positive results			
Number	Sample	hTERT-1	hTERT-2	Inf A2	PMA	hTERT-1	hTERT-2	Inf A2	PMA
21	2	7	4	6	16	Y	Y	Y	Y
21	2	35	46	31	147	Y	Y	Y	Y

Table 3.7: ELISpot results for a HLA-A3 positive patient- patient 101, sample 1

Patient		Specific number of SFC per well			Positive results		
Number	Sample	hTERT A3	Inf A3	PMA	hTERT A3	Inf A3	PMA
101	1	16	0	0	Y	N	N
101	1	0	1	27	N	Y	Y

Even though the magnitude of the responses was not the same in the repeat, the results for patient 21 and 101 showed positive for both samples. Therefore this assay's validity is confirmed.

3.5 Averaged results

The results for the final ELISpot count, given in Tables 3.2 and 3.3, are calculated averages between several wells. For each sample, the test for each peptide was triplicated, and an average taken, to look at the variability of the result and to ensure a representative number. Table 3.8 shows a few examples of the original number of spots in the wells, as well as the calculated average. In the case of patients 76 and 77, the original numbers are very closely associated so, therefore, the average is a true reflection of the result. However, there are some samples, for example patient 40, where the numbers of spots are not so closely associated, giving a number that may be incorrect. The variability makes comparisons difficult and may be overcome by repeating the assay and taking more care over the procedure.

Table 3.8: Showing an example of the number of SPC counted per well and the average

Patient		hTERT-1				PMA			No peptide		
Number	Sample				Average			Average			Average
40	1	3	16	1	7	19	4	12	0	9	5
76	1	160	193	170	174	247	233	240	181	156	169
77	1	256	233	238	242	257	263	260	222	186	204

3.6 PMA stimulation and Influenza response

PMA is a positive control, as it causes a non-specific response in all viable cells. However, not all the results show an effective PMA stimulation, suggesting that the cells may be dead. The threshold for a positive response was taken from (Titu et al. 2004) to be 16 and above. In his study the most appropriate method was to calculate the mean number of spots counted in all irrelevant wells and add three standard deviations to obtain the threshold above which the number of spots counted in study wells can be considered

as being significant. More than 99% of the spots obtained in the irrelevant wells would be found below this threshold, and, consequently, it can be postulated that any number of spots obtained in the study wells above this threshold indicates a positive response to peptide stimulation. As a method of further verifying the results obtained, the frequencies of CTL active against an HLA-A2/A3-restricted epitope of the influenza a (IA) matrix protein were also quantified. The influenza response gives indication of the state of the patient's immune system, and because of the efficient vaccination of the human body against influenza it was used as a positive control for the assay in many studies (Scheibenbogen et al. 1997) (Vonderheide et al. 2001). A negative influenza response shows that the body had no or very little immune response. Table 3.9 shows patient sample responses to both PMA and influenza. Out of the 51 samples, 30 (59%) showed a positive response to PMA. The average number of SFC in response to PMA was 65. Twenty-three (45%) samples out of the 51 responded to influenza, and 16 (31%) showed a positive response to both PMA and influenza. Seven samples were viable but had no immune response. The average number of SPC in response to influenza was 5.

Table 3.9: Patient responses to PMA stimulation and influenza: number of SFC obtained with PMA and Influenza.

Patient No.	Sample No.	PMA	Above Threshold	Influenza	Response
21	2	81	+	18	+
21	4	66	+	0	-
21	6	112	+	6	+
26	2	31	+	0	-
36	3	114	+	0	-
36	2	0	-	1	+
37	1	143	+	3	+
37	2	4	-	0	-
40	1	7	-	0	-
42	1	38	+	0	-
42	2	24	+	10	+
42	3	4	-	6	+
43	1	67	+	0	-
43	3	19	+	4	+
43	4	13	-	0	-
44	1	4	-	0	-
44	3	229	+	0	-
44	4	55	+	0	-
53	1	11	-	1	+
61	1	127	+	0	-
66	1	268	+	12	+
66	2	131	+	0	-
66	3	148	+	12	+
67	1	85	+	54	+
69	1	9	-	0	-
71	1	0	-	0	-
71	2	1	-	0	-
71	3	0	-	0	-
71	4	9	-	0	-
71	5	0	-	0	-
72	1	191	+	27	+
72	2	90	+	0	-
73	1	104	+	9	+
75	1	249	+	10	+
76	1	71	+	1	+
77	1	56	+	29	+
79	1	360	+	13	+
80	1	64	+	0	-
90	1	1	-	0	-

Patient No.	Sample No.	PMA	Above Threshold	Influenza	Response
91	1	0	-	2	+
91	2	0	-	0	-
95	1	19	+	0	-
97	1	60	+	0	-
97	2	11	-	1	-
98	1	7	-	3	+
98	2	57	+	13	+
99	1	0	-	1	+
100	1	57	+	25	+
101	1	13	-	1	+
101	2	95	+	0	-
105	1	8	-	0	-

Note: Repeated results were averaged

3.7 Benign tumours

Four patients were found to have benign tumours. The ELISpot assay was still performed, as this information was revealed after the assay had been carried out, and provided an interesting contrast with malignant disease.

Patients 53 and 90 did not show a positive response to PMA, so their data is not valid. Patient 73 showed good PMA stimulation, both hTERT peptides responded, and influenza caused a response. Patient 100 showed almost no response to the A2 hTERT peptides, but responded highly to hTERT A3 (see Table 3.3 & 3.4).

3.8 Site and stage of cancer

Out of all the patients used for the study, half originated in the colon and half in the rectum (excluding the 4 benign patients). Tables 3.10 and 3.11 show the results grouped according to their cancer origin.

For the samples from the colon, all except for 3 samples (67%) responded to hTERT. About 60% of the rectal cancer patients responded to hTERT.

Table 3.10: Patients samples with tumours originating in the colon

Patient		Specific number of SFC per well				
Number	Sample	hTERT-1	hTERT-2	hTERT A3	PMA	Influenza
26	2	9	2	n/a	31	0
36	3	8	1	n/a	114	0
37	1	14	0	n/a	143	3
42	1	0	0	n/a	38	0
42	2	8	3	n/a	24	10
43	1	0	0	n/a	67	0
43	3	24	15	n/a	19	4
66	1	6	8	n/a	268	12
66	2	4	7	n/a	131	0
66	3	88	24	n/a	148	12
72	1	36	25	n/a	191	27
72	2	0	0	n/a	90	0
76	1	5	17	n/a	71	1
80	1	0	0	n/a	64	0
98	2	10	18	24	57	13

Table 3.11: Patient samples with the tumour originating in the rectum

Patient		Specific number of SFC per well				
Number	Sample	hTERT-1	hTERT-2	hTERT A3	PMA	Influenza
21	2	21	24	n/a	81	18
21	4	0	10	n/a	66	0
21	6	7	9	n/a	112	6
44	3	0	0	0	229	0
44	4	0	7	0	55	0
61	1	n/a	n/a	0	127	0
67	1	66	44	n/a	85	54
75	1	2	0	n/a	249	10
77	1	38	34	n/a	56	29
79	1	17	4	8	360	13
95	1	0	0	n/a	19	0
97	1	2	2	4	60	0
101	2	n/a	n/a	0	95	0

Putting the site of cancer aside, 2 (8%) patients' had Dukes' A cancer, 6 (23%) had Dukes' B, 16 (61%) Dukes' C (including Dukes' C1 and C2), and 2 (8%) had Dukes' D.

3.8 Deceased patients

Table 3.12 shows the relevant details and results of the 5 patients who died. Two of the patients who died had benign tumours. Both died within 3 months of their operation. Their average age was 78, suggesting that that may have died due to operation stress rather than the tumour per se. The other three patients had CRC in the rectum. Their CRC were all at a fairly late stage: Dukes' C2.

The hTERT responses were consistently low and all the patients had no or a very low influenza response, indicating that the immune system was not functioning normally. This could have been one reason for the cause of death. A faster rate of disease progression than otherwise would be normal may have caused death, due to many other factors.

Table 3.12: Patient details of patients who died during the course of the study

Patient Number	Site of Cancer	Dukes Stage	Age	Specific number of SFC per well				
				hTERT-1	hTERT-2	hTERT A3	PMA	Influenza
40	Rectum	C	62	2	0	n/a	7	0
53	Benign	Benign	75	4	0	n/a	11	1
90	Benign	Benign	80	0	0	n/a	1	0
95	Rectum	C2	81	0	0	n/a	19	0
97	Rectum	C	67	2	2	2	35	0

3.9 Conclusion

In this thesis 2 peptides were used that react to CTL in an HLA-A2 and HLA-A3 restricted manner.

Thirty patients in this study were HLA-A2/A3 positive and were having chemotherapy and hence were studied for CTL reaction. Twenty eight pre-operative samples and thirteen post-operative samples were analysable, 71% of pre-operative samples and 54%

post-operative samples had CTL reaction towards hTERT peptides whether in an HLA-A2, HLA-A3 or HLA2/A3 restricted manner.

The results of this thesis seems to be higher than Titu *et al* results, who studied the T-cell response against two HLA-A2 specific peptides of hTERT (I540 and P865) in 37 CRC patients and 12 normal controls (Titu et al. 2004). The results of their study demonstrate that CTL active against hTERT are present in only 20% of CRC patients. The discrepancy in the results of both studies could be due to the fact that in our study samples were considered positive only if the value SFC was ≥ 1. Whereas in Titu *et al* study the samples were considered positive only when the number of the spots with peptides were ≥ 16 spots which is the threshold level from the cells with no peptides. Furthermore in our study we added another hTERT which have contributed for the higher results.

The results in this thesis were closer to Amarnath *et al* (Amarnath et al. 2004) results who investigated CTL response in breast cancer patients. The same methods and peptides were used in their study and CTL recognized HLA-A2 P540 and P865 peptides in 76% and 47% respectively, CTL recognized both HLA-A2 peptides in 47%, CTL recognized HLA-A3 K973 peptides in 71% patients.

In this study the responses to both PMA and influenza were analysed. Out of the 51 samples, 30 (59%) showed a positive response to PMA. The average number of SFC in response to PMA was 65.

Twenty three (45%) samples out of the 51 responded to influenza, and 16 (31%) showed a positive response to both PMA and influenza. 7 samples were viable but had no immune response. The average number of SPC in response to influenza was 5. These

results were more or less similar to results of many studies (Vonderheide et al. 2001) (Titu et al. 2004) (Amarnath et al. 2004) (Scheibenbogen et al. 1997) studies.

Although the experimental methods in this chapter were used by other authors and they produced reliable generic methodology for the study of the CTL immune response against any peptide antigens, in our study there was variability in the results were the size, intensity, and number of spots were different in each case as shown in figure 3.1, which could be due to contamination making our results unreliable.

So far all studies conducted agree that CTL precursors capable of recognizing hTERT peptides can be found or expanded *in vitro* in cancer patients but none of them have studied the effect of these CTL cells on tumours' development and progression.

CHAPTER 4
Detection of hTERT mRNA in serum samples of CRC patients

In this chapter the results of the hTERT mRNA detection serum samples of CRC patients using RT-PCR will be discussed

4.1 Introduction

The expression of hTERT in blood, reported in only five previous studies, has been detected in: 90% (70/78) of serum samples of patients with hepatocellular carcinoma (Miura, N. et al. 2003), 25 % (4/16) of serum samples of patients with breast cancer (Chen et al. 2000) using RT-PCR, 46% (30/65) of the peripheral blood of patients with gastric cancer using red blood cell lysis followed by RT-PCR (Shin et al. 2002), and in 53% (29/55) of patients with hepatocellular carcinoma using immunomagnetic separation technique to isolate CTC followed by RT-PCR (Waguri et al. 2003).

Finally hTERT mRNA has been detected in the plasma samples of CRC patients (Lledo et al. 2004) with 82% (41/50) of these patients having hTERT mRNA expression values higher than the maximum values of normal control using real time PCR.

In this study the aim was to detect the expression of hTERT mRNA in serum samples of CRC patients who underwent curative resection and to determine the correlation between hTERT expression, disease stage and patient prognosis.

4.2 Detection of serum hTERT mRNA

Ninety seven patients were recruited prospectively and serum blood samples were collected pre-surgery, one month and one year post operatively. The serum samples were aliquoted and stored at -80^{O}C until use. The mean age for the patients was 71 (43-90 years). Forty six percent of the patients were Duke's C, 26% Duke's B, 20% Duke's A and 7% were Duke's D. The mortality rate in this study was 17%. The demographic features of the patients recruited are shown in Appendix 4.1.

4.2.1 Preliminary work

In the first experiment of serum hTERT mRNA expression, 5 patients' samples were used to extract RNA. Purified water (ddH_2O) and a sample from a normal volunteer were used as negative controls. The RT-PCR reaction was prepared using the following primers: (http://frodo.wi.mit.edu/), with TERT PCR product size of 4015 bp.

1) RL-hTERT-FOR: 5'-CGT GGT TTC TGT GTG GTG TC-3'
2) RL-hTERT-REV: 5'-CCT TGT CGC CTG AGG AGT AG-3'
3) GAPDH-FOR: 5'-ACC ACA GTC CAT GCC ATC AC-3'
4) GAPDH-REV: 5'-TCC ACC ACC CTG TTG CTG TA-3'

The RT-PCR reaction steps were followed as detailed in section 2.6.3 in the methods chapter, but initially 35 steps were used for denaturation and extension. In this reaction hTERT and GAPDH expression should show bands of 214 bp and 450 bp respectively. The results of this experiment were all negative even using the GAPDH primers (Figure 4.1). The same experiment was repeated 3 times using the same samples and all were negative.

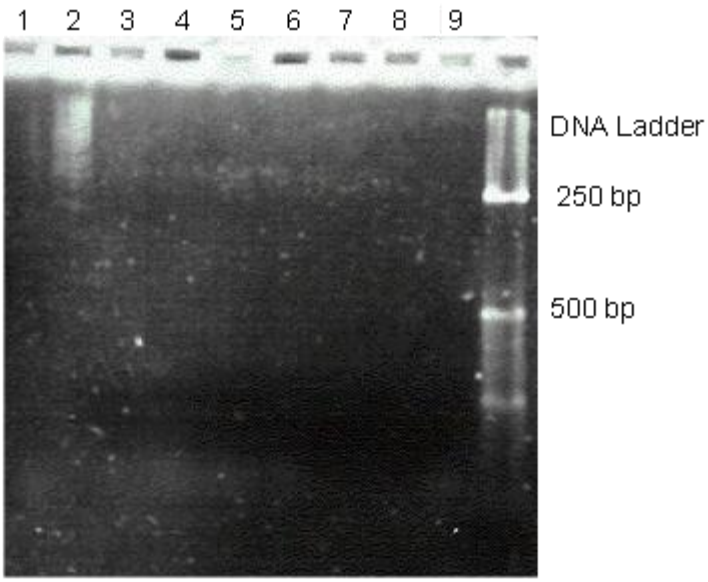


Figure 4.1 A 1.2% w/v agarose loading gel showing no PCR bands in any tube.
N.B. 100 bp ladder was used (GeneRuler^{TM} DNA ladder, Invitrogen)

Tube 1: ddH_2O + GAPDH primers (negative control)
Tube 2: normal volunteer + GAPDH primers
Tube 3: normal volunteer + RL-hTERT primers
Tube 4: ddH_2O + RL-hTERT primers (negative control)
Tube 5: normal volunteer + RL-hTERT primers (negative control)
Tube 6: Sample 92a + RL-hTERT primers
Tube 7: Sample 90a + RL-hTERT primers
Tube 8: Sample 87a + RL-hTERT primers
Tube 9: Sample 50c + RL-hTERT primers
Note: a: pre- operative sample, b: 1 month post operative sample and c: one year post operative sample.

4.2.2 RT-PCR verification

As a consequence of the negative results in the GAPDH controls it was decided to use an additional house keeping gene and two cancer cell lines that are known to express hTERT mRNA (positive controls) in order to check the integrity of the RT-PCR reaction. The two cancer cell lines were CACO2 (colon cancer cell line) and MCF-7 (breast cancer cell line).

Initially the integrity of the cancer cell lines was checked by measuring the RNA concentration by measuring the absorbance of each sample at 260 nm by spectrophotometry. RNA preparations from CACO2 and MCF-7 cancer cell line showed sufficient RNA concentrations (10 ng/µl and 70 ng/µl respectively), that subsequently produce a positive RT-PCR reaction since the QIAGEN RT-PCR kit provides highly efficient sensitive reverse transcriptase of any RNA quantity from 1pg to 2 µg.
In this experiment the RT-PCR was optimised to allow the detection of the control genes using both cancer cell lines (Figure 4.2). This experiment was repeated again and the same positive results were obtained.

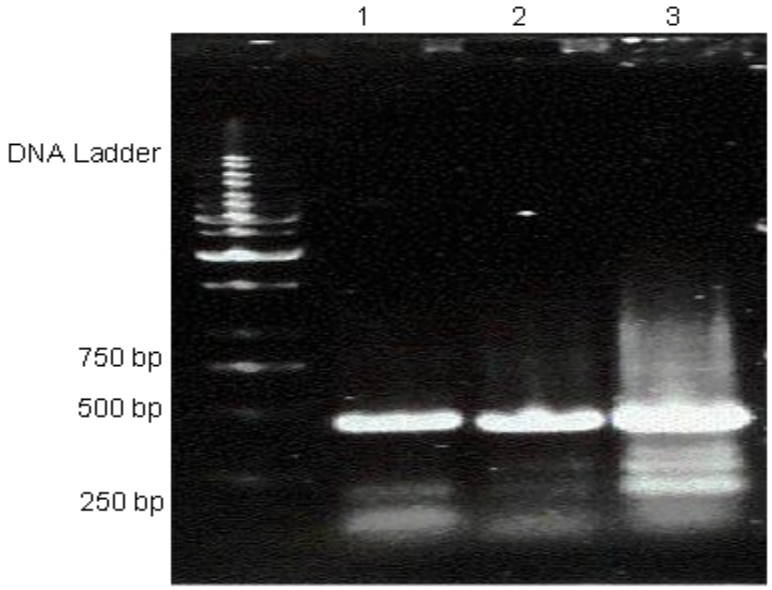


Figure 4.2 The bright bands at approximately 450 bp demonstrate the positive expression of the cancer cell lines of GAPDH and ß-actin genes.

Tube 1: CACO2 + GAPDH
Tube 2: CACO2 + ß-actin
Tube 3: MCF-7 + GAPDH

4.2.3 RT-PCR for patient samples

Having demonstrated that the cell lines obtain sufficient mRNA and the RT-PCR reaction can detect both control genes, these samples together with a number of patients' samples were tested for house keeping genes and hTERT expression.

The RNA concentration and purity for patients' samples were measured to check the samples integrity using the spectrophotometer where the purity ratio, according to the manufacturer protocol, should be within the following range (1.9-2.1 in 10 mM Tris.CL, pH 7.5). Then their concentration and purity were compared to CACO2 cell line RNA concentration and purity that had positive results for GAPDH and ß-actin. The results are shown in Table 4.1:

Table 4.1: RNA concentration and purity

Sample no.	Conc. ng/µl	Purity ratio
50c	7	1.19
87a	93	0.8
87b	14	1.2
90a	14	1.2
92a	15	1.18
Normal control	11	1.18
CACO2 cell line	32	1.05

The above table shows that RNA concentration are lower than the CACO2 cell line except sample 87a, and shows that the patients samples and the CACO2 cell line all have low RNA purity. Although the above shows low RNA concentrations and purity it was

decided to use the same samples for the next RT-PCR using the same conditions as previously used successfully.

In this experiment the 1.2 % w/v agarose gel demonstrated faint bands in samples 8 (87a) and 9 (50c) at approximately 215 bp which is the correct-sized band for hTERT mRNA expression which could be explained by the insufficient RNA concentration in patients' samples, its low purity or a combination of both factors (Figure 4.3).

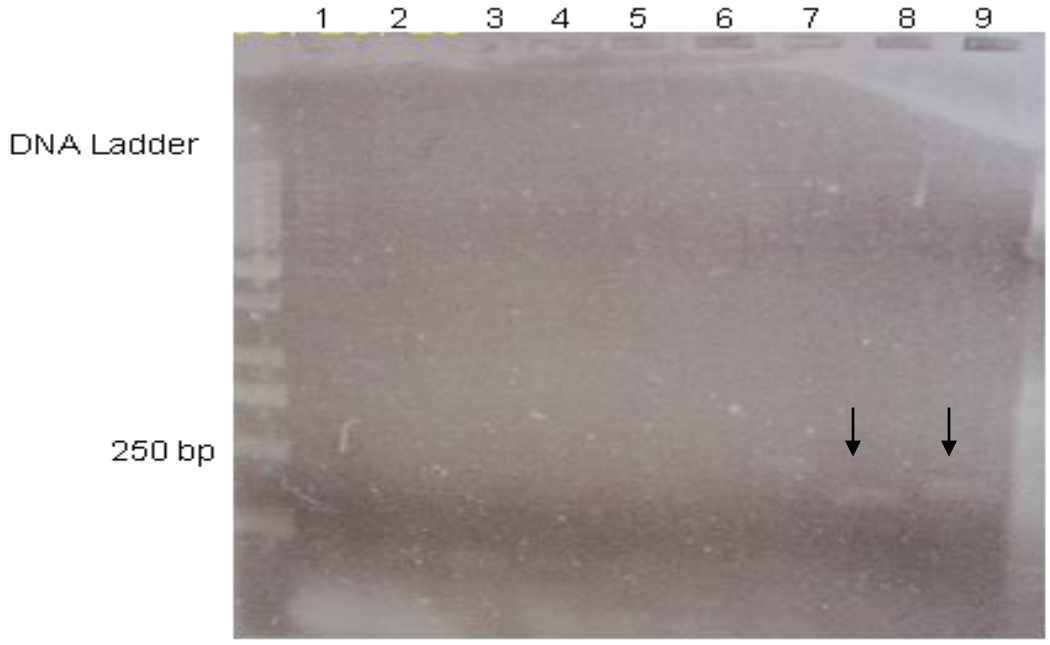


Figure 4.3: 1.2% w/v agarose loading gel showing faint PCR bands in tubes 8 & 9

Tube 1: ddH_2O + GAPDH primers
Tube 2: normal volunteer + GAPDH primers
Tube 3: ddH_2O + LR-hTERT primers
Tube 4: normal volunteer + LR-hTERT primers
Tube 5: Sample 92a + LR-hTERT primers
Tube 6: Sample 90a + LR-hTERT primers
Tube 7: Sample 87b + LR-hTERT primers
Tube 8: Sample 87a + LR-hTERT primers
Tube 9: Sample 50c + LR-hTERT primers

As the RNA concentration and purity in the samples used were low, it was decided to increase the number of RT-PCR cycles in this experiment from 35 to 40 cycles in order to

get a higher yield of RT-PCR products. The previous experiment was repeated using the same samples as two of these samples demonstrated positive hTERT bands, in addition GAPDH primers and CACO2 cell line were used (Figure 4.4).

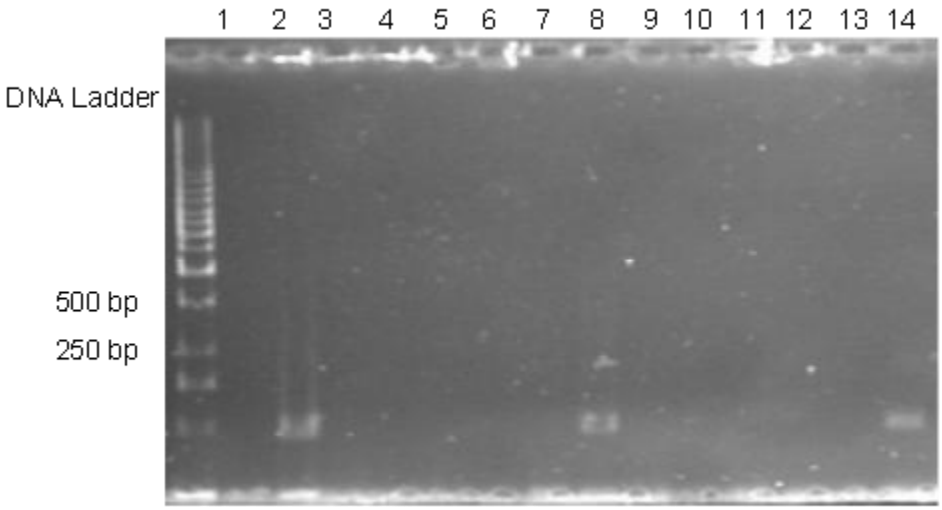


Figure 4.4 RT-PCR increased to 40 cycles to get a higher yield of RT-PCR products

Tube 1: Sample 50c + LR-hTERT primers
Tube 2: Sample 50c + GAPDH primers
Tube 3: Sample 87a + LR-hTERT primers
Tube 4: Sample 87a + GAPDH primers
Tube 5: Sample 87b + LR-hTERT primers
Tube 6: Sample 87b + GAPDH primers
Tube 7: Sample 90a + LR-hTERT primers
Tube 8: Sample 90a + GAPDH primers
Tube 9: Sample 92a + LR-hTERT primers
Tube 10: Sample 92a + GAPDH primers
Tube 11: normal control + LR-hTERT primers
Tube 12: normal control + GAPDH primers
Tube 13: CACO2 + LR-hTERT primers
Tube 14: CACO2 + GAPDH primers

Figure 4.4 showed that samples 2, 8 and 14 had expression of RT-PCR products when GAPDH primers were used in two patients samples and CACO2 cell line (at band 450

bp), but none of the samples expressed hTERT mRNA which could be due to the insufficient mRNA concentration in the samples.

As the result of the above it was decided to extract RNA from new patients' serum samples and use them as fresh in order to avoid any RNA degradation and use new LR-hTERT primers and hTERT primers that were used in Chen *et al* and Lledo *et al* studies (Chen et al. 2000) (Lledo et al. 2004) in order to compare them to the LR-hTERT primers, these new hTERT are:

CHEN-hTERT FOR: 5'-TGA CAC CTC ACC TCA CCC-3'

CHEN-hTERT REV: 5'-CAC TG CTT CCG CAA GTT CAC-3'

LLEDO-hTERT FOR: 5'-ACC GTC TGC GTG AGG AGA TC-3'

LLEDO-hTERT REV: 5'-CCG GTA GAA AAA AGA GCC TGT TC -3'

Furthermore new ddH_2O and GAPDH primers were used to avoid contamination.

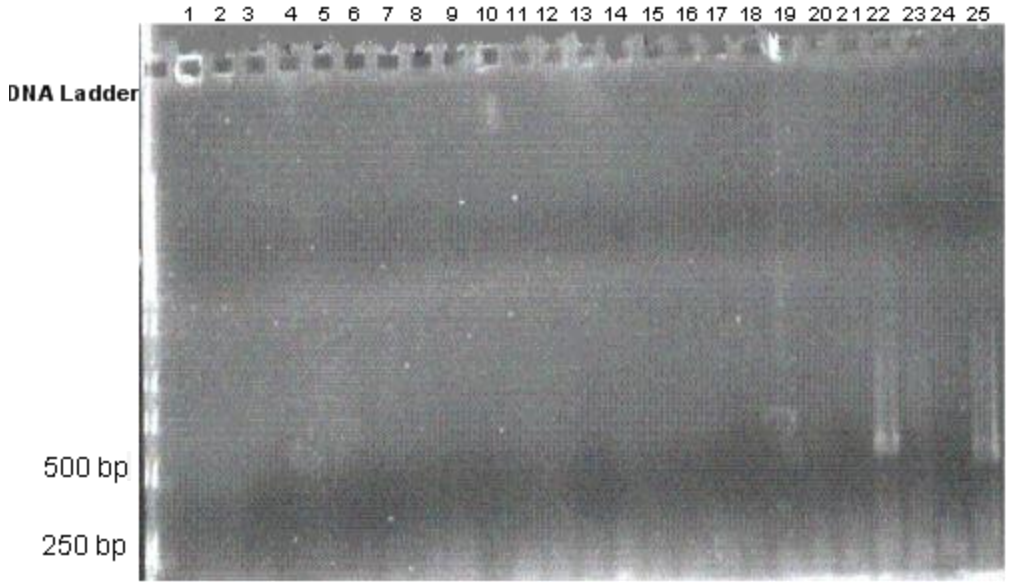


Figure 4.5 There are two faint bands at around 450 bp in columns 22 and 25.

Tube 1: Sample 9a + LR-hTERT primers
Tube 2: Sample 9b + LR-hTERT primers
Tube 3: Sample 11a + LR-hTERT primers
Tube 4: Sample 11b + LR-hTERT primers
Tube 5: Sample 19a + LR-hTERT primers
Tube 6: Sample 19b+ LR-hTERT primers
Tube 7: ddH_2O + LR-hTERT primers
Tube 8: 9a + GAPDH primers
Tube 9: 9b + GAPDH primers
Tube 10: 11a + GAPDH primers
Tube 11: 11b + GAPDH primers
Tube 12: 19a + GAPDH primers
Tube 13: 19b+ GAPDH primers
Tube 14: ddH_2O + GAPDH primer
Tube 15: 9a + CHEN-hTERT primers
Tube 16: 9b + CHEN-hTERT primers
Tube 17: 9a + LLEDO-hTERT primers
Tube 18: 9b + LLEDO-hTERT primers
Tube 19: 9a + ß-actin primers
Tube 20: 9b + ß-actin primers
Tube 21: CACO2 + LR-hTERT primers
Tube 22: CACO2 + GAPDH primers
Tube 23: CACO2 + CHEN-hTERT primers
Tube 24: CACO2 + LLEDO-hTERT primers
Tube 25: CACO2 + ß-actin primers

This experiment again has failed to demonstrate hTERT expression. There were 2 samples, only positive with ß-actin and GAPDH primers used (samples 22 and 25). Furthermore the gel was difficult to analyse, as it was difficult to differentiate between the bands that were created by hTERT expression or the bands of the starting template, in order to over come this obstacle 3% w/v agarose gel was used instead of the 1.2% w/v agarose. The 3% w/v gel has the ability to give wider spaces between bands of a small

size and hence can differentiate between the bands created by hTERT expression or the starting template.

The following RT-PCR (Figure 4.6) was performed using two colon cancer cell lines with the available hTERT primers and 3% w/v agarose gel used for electrophoresis.

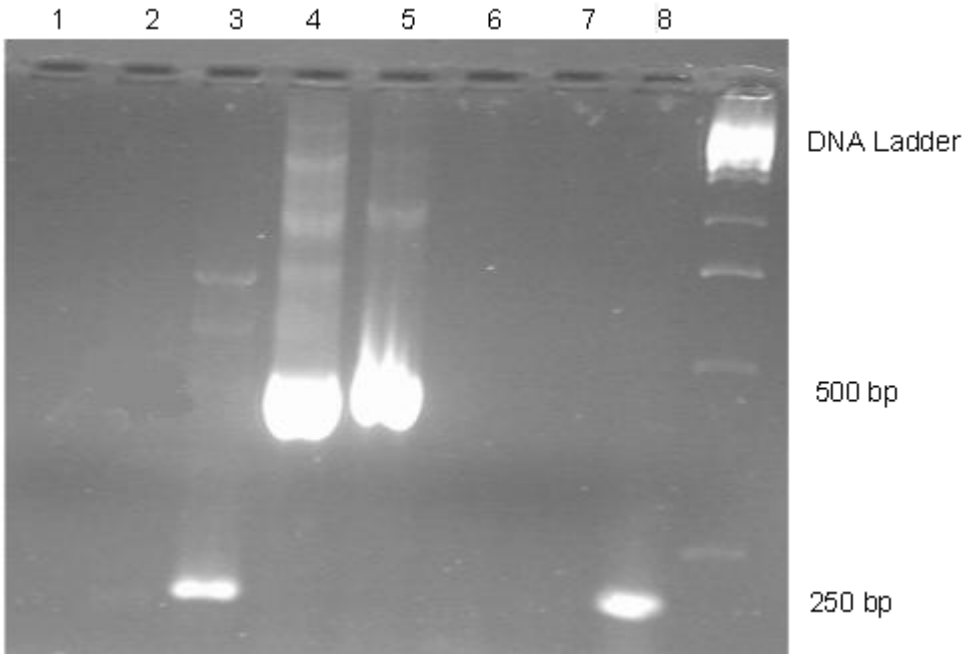


Figure 4.6 Demonstrates bright bands at 450 bp and 214 bp

Tube 1: ddH_2O + LR-hTERT primers
Tube 2: ddH_2O + GAPDH primers
Tube 3: CACO2 + LR-hTERT primers
Tube 4: LoVo + ß-actin primers
Tube 5: LoVo + GAPDH primers
Tube 6: LoVo + LLEDO-hTERT primers
Tube 7: LoVo + CHEN-hTERT primers
Tube 8: LoVo + LR-hTERT primers

From this experiment it was concluded that Both LoVo and CACO2 have expressed hTERT mRNA (band 214 bp) when the LR-hTERT primers were used but not CHEN-hTERT and LLEDO-hTERT primers, and both LoVo and CACO2 produced a bright band at around 450 bp when the ß-actin and GAPDH primers were used. This experiment was repeated on two occasions and was successful.

Having succeeded in detecting hTERT mRNA using the appropriate cancer cell lines and hTERT primers, the experiment was repeated applying the same conditions using patients' serum samples from which the RNA was freshly extracted (Figure 4.7).

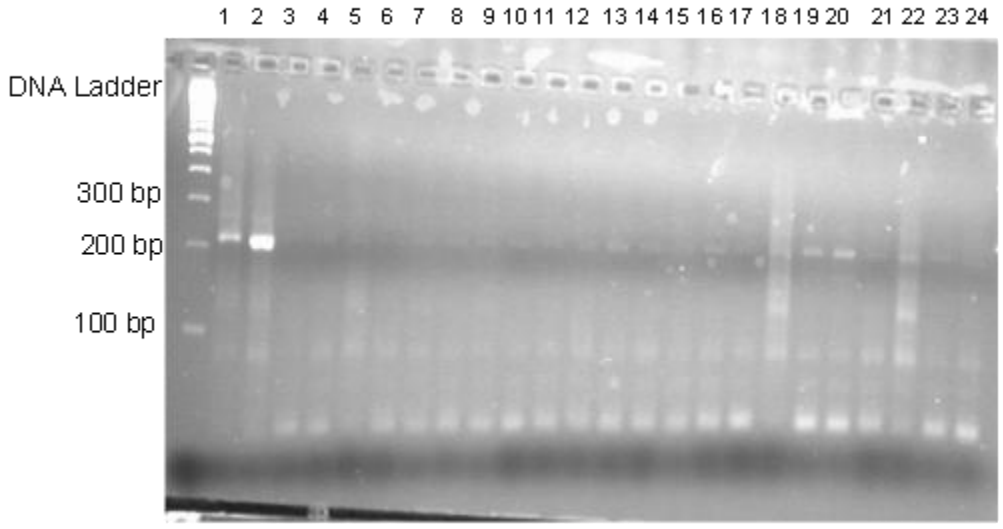


Figure 4.7 RT-PCR using patients' serum samples

Tube 1: LoVo 10% + LR-hTERT primers
Tube 2: LoVo + LR-hTERT primers
Tube 3: ddH_2O + LR-hTERT primers
Tube 4: normal control + LR-hTERT primers
Tube 5: Sample 85b + LR-hTERT primers
Tube 6: Sample 84a + LR-hTERT primers
Tube 7: Sample 83a + LR-hTERT primers
Tube 8: Sample 82 a + LR-hTERT primers
Tube 9: Sample 81b + LR-hTERT primers
Tube 10: Sample 81a + LR-hTERT primers
Tube 11: Sample 80b + LR-hTERT primers
Tube 12: Sample 80a + LR-hTERT primers
Tube 13: Sample 79a + LR-hTERT primers
Tube 14: Sample 78a + LR-hTERT primers
Tube 15: Sample 77a + LR-hTERT primers
Tube 16: Sample 76b + LR-hTERT primers
Tube 17: Sample 76a + LR-hTERT primers
Tube 18: Sample 75b + LR-hTERT primers
Tube 19: Sample 74a + LR-hTERT primers
Tube 20: Sample 73b + LR-hTERT primers
Tube 21: Sample 72b+ LR-hTERT primers
Tube 22: Sample 72a + LR-hTERT primers
Tube 23: Sample 71b + LR-hTERT primers
Tube 24: Sample 71a + LR-hTERT primers

The results (Figure 4.7) of this experiment were very encouraging, as LoVo cancer cell line expressed hTERT mRNA and Samples 19,20,22,23, and 13 may have expressed hTERT mRNA.

In order to check the integrity of the results the experiment was repeated on the positive samples (Figure 4.8).

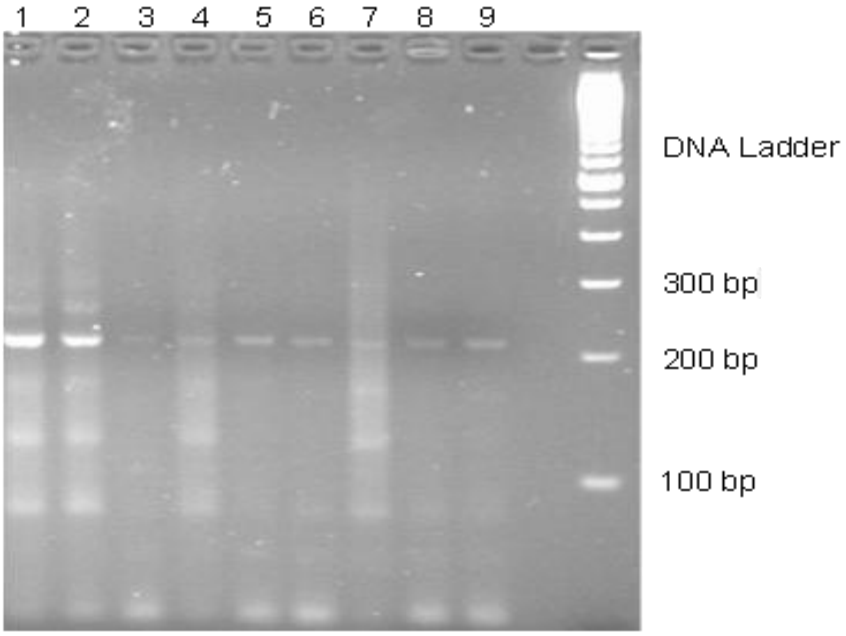


Figure 4.8 All patients' samples expressed hTERT mRNA

Tube 1: CACO2 + LR-hTERT
Tube 2: LoVo + LR-hTERT primers
Tube 3: ddH_2O + LR-hTERT primers
Tube 4: Sample 72a + LR-hTERT primers
Tube 5: Sample 73b + LR-hTERT primers
Tube 6: Sample 74a + LR-hTERT primers
Tube 7: Sample 75b + LR-hTERT primers
Tube 8: Sample 76b + LR-hTERT primers
Tube 9: Sample 79a + LR-hTERT primers

Based upon the encouraging results above, RNA was extracted from 42 new samples of patients' serum and using the same RT-PCR conditions it could not give any positive results, in fact the products were smeared on the 3% w/v agarose gel with no obvious

explanation (Figure 4.9), the only possibility was water contamination, the water was replaced with a new one but still the results were negative, the experiment was repeated many time on the new samples but never managed to get positive results again. As a result of continuous inconsistency of the experiment and lack of positive RT-PCR on serum samples it was decided not to pursue this approach.

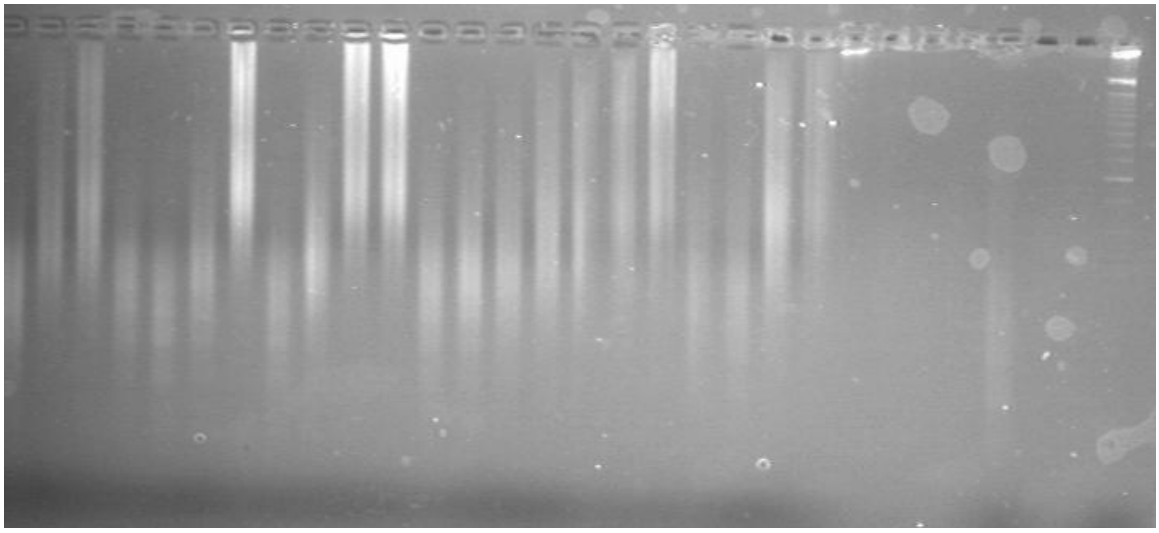


Figure 4.9 3% w/v agarose gel with smeared products

4.3 Conclusion

This study failed to find a standard reliable technique that could detect hTERT mRNA in serum samples of CRC as hTERT mRNA was only detected in 6 samples out of all other samples. Such approach has been reported previously and the discrepancy could be for a number of reasons;

1) The first reason could be the source of samples, where in this study serum samples of CRC patients were used. Two studies used RT-PCR on serum samples, the first on breast cancer patients (Chen et al. 2000) and the other using hepatocellular cancer (Miura, N. et

al. 2003), both studies managed to produce positive results using the same methods as applied in this thesis, but both studies have not commented on the duration of sample storage, hTERT expression was also detected intracellularly in the peripheral blood in 46% of patients with gastric cancer using red blood cell (RBC) lysis followed by RT-PCR (Shin et al. 2002), and detected in 29/55 (53 percent) patients with hepatocellular carcinoma using immunomagnetic separation technique to isolate CTC followed by RT-PCR (Waguri et al. 2003).

Furthermore hTERT mRNA has been detected in the plasma samples of CRC patients.(Lledo et al. 2004) peripheral blood samples of 50 patients using real time PCR, it was found that 82% of CRC patients had hTERT mRNA expression values higher than the maximum values of normal control and the sensitivity and the specificity of the assay were 98% and 64% respectively. The fact that plasma samples alone were used in this study, without an attempt to first isolate tumour cells by means of immunomagnetic separation, could explain the high level of detection of hTERT expression, as transcription of telomerase from non-tumour sources such as activated lymphocytes would have been possible.

In 2007 Uen *et al* used a (Uen et al. 2007) Membrane-arrays consisting of a panel of mRNA markers that included hTERT, CK19, CK20, and CEA mRNA were used to detect CTCs in the peripheral blood of 194 stage II CRC patients who underwent potentially curative resection. Digoxigenin (DIG)-labelled cDNA were amplified by RT-PCR from the peripheral blood samples, which were then hybridized to the membrane-array. All patients were followed up regularly, and their outcomes were investigated completely. Fifty-three of 194 (27.3%) stage II patients were detected with the expression

of all 4 mRNA markers using the membrane-array method. After a median follow up of 40 months, 56 of 194 (28.9%) developed recurrence/metastases postoperatively. Univariately, postoperative relapse was significantly correlated with the depth of invasion ($P < 0.001$), the presence of vascular invasion ($P < 0.001$), the presence of perineural invasion ($P = 0.048$), the expression of all 4 mRNA markers ($P < 0.001$), and the number of examined lymph nodes ($P = 0.031$). Meanwhile, using a multivariate logistic regression analysis, T4 depth of tumour invasion ($P = 0.013$), the presence of vascular invasion ($P = 0.032$), and the expression of all 4 mRNA markers ($P < 0.001$) were demonstrated to be independent predictors for postoperative relapse. Combination of the depth of tumour invasion, vascular invasion, and all 4 mRNA markers as predictors of postoperative relapse showed that patients with any 1 positive predictor had a hazard ratio of about 27-fold to develop postoperative relapse ($P < 0.001$; 95% CI = 11.42-64.40). The interval between the detection of all 4 positive molecular markers and subsequently developed postoperative relapse ranged from 4 to 10 months (median: 7 months). Furthermore, the expression of all 4 mRNA markers in all stage II CRC patients, or either stage II colon or rectal cancer patients were strongly correlated with poorer relapse-free survival rates by survival analyses (all $P < 0.001$). This study concluded that the constructed membrane-array method for the detection of CTCs is a potential auxiliary tool to conventional clinicopathological variables for the prediction of postoperative relapse in stage II CRC patients who have undergone curative resection. This study is the first one to study the prognostic value of hTERT mRNA as a tumour marker as the patients were followed up over a period of 40 months.

2) The second explanation is the duration of storage; the samples in this study were stored at a temperature of -80^{C} for a period ranging between few days to few months; the long duration of the samples could have degraded the RNA present in the samples.
The third explanation for the discrepancy in the results, are the conditions of RT-PCR; i.e. single or multiplex, number of PCR cycles and if the PCR is standard, nested or real time. RT-PCR is a very sensitive technique that could give variable results depending on the conditions applied, the previous studies used different condition, hence the discrepancy in their results, although it is hard to compare between their results as each study dealt with cancers of different origins.

At the end of this chapter it is recommended that there is an urgent need to define a standard technique, not requiring excessive time and labour input, which can be utilised by different groups to undertake large, well-controlled, randomized studies on the markers with most promise.

CHAPTER 5

Detection of circulating tumour cells in the peripheral blood of CRC patients

This chapter describes the use of the TeloTAGGG PCR-ELISA to identify circulating colorectal tumour cells

5.1 Introduction

Nearly half of all CRC patients who undergo curative resection relapse as a result of occult metastases (the presence of CTC). In 1869 Ashworth as cited in Ghossein and Bhattacharya (Ghossein and Bhattacharya 2000) was the first to describe CTC. CTC are a potential cause of metastases and subsequently disease relapse particularly following surgery; therefore the presence or absence of CTC could be an important prognostic factor, and an indicator for the decision concerning adjuvant treatment and follow-up.(Hardingham et al. 1995)

Telomerase has been used as a diagnostic marker to detect CTC in tissue, bone marrow and blood samples (section 1.6). It has the advantage of being both highly cancer specific and an indicator for the detection of viable tumour cells.

The aims of this study were to:

1) Determine the prognostic value of telomerase activity in CTC in a previous cohort of patients with CRC.
2) Assess the validity of using the TeloTAGGG™ Telomerase PCR ELISA to identify CTC.

5.2 Prognostic value of telomerase activity in CTC

A previous retrospective study was undertaken on a cohort of 34 patients who underwent curative resection for CRC in Hull. Peripheral blood samples were taken pre- and 1 week post operatively from all patients and from 10 healthy normal controls with the same age and sex-match. A mononuclear cell preparation was made by density centrifugation, epithelial tumour cells were isolated using Dynabeads coupled with Ber-EP4 and telomerase activity was assessed using TeloTAGGG PCR-ELISA assay as mentioned in

the methods and material chapter section 2.3 and 2.4. The study was carried out by Dr R Loveday (University of Hull) (Loveday et al. 2004) in order to detect the presence of CTC via telomerase activity in the pre and post operative samples and to determine its diagnostic value.

In this thesis the same patients were followed up over a period of time and the mortality and recurrence rates of those patients were correlated with presence of CTC as assessed by telomerase activity.

5.2.1 Results

Thirty four patients underwent a surgical resection for CRC. The clinicopathological data and telomerase status are shown in Table 5.1.

In this study there were 22 males (65%) and 12 (35%) females, the mean age was 64 (range 30-81). Half of the tumours were located in the rectum and the remaining cases were distributed throughout the colon. In this study 15% of the tumours were Dukes A, 35% Dukes B, 29% Dukes C and 21% Dukes D. The mean time from diagnosis to last follow up was 47.5 months. Telomerase activity was detected in 32% of the pre-operative samples and 56% of the post-operative samples and was not detected in any of the healthy control samples. CTC presence did not correlate to the tumour site or Dukes stage. Seventeen (50%) patients developed recurrence or metastases with a mean time of 18.2 months. Three patients out of 11 (27%) were positive for CTC in their pre-operative sample, and 8 out of 19 (42%) with positive CTC in their post-operative samples died as a result of metastases.

Table 5.1: Patients' clinicopathological data

Patient No.	Tumour Site	Dukes	Follow up	Recurrence	Pre-op CTC	Post-op CTC
1	Rectum	A	58	No	Positive	Positive
2	Rectum	A	48	Yes	Negative	Negative
3	Rectum	A	68	No	Positive	Negative
4	Sigmoid	A	50	No	Negative	Positive
5	Rectum	A	50	Yes	Negative	Positive
6	Rectum	B	57	Yes	Positive	Positive
7	Transverse colon	B	55	No	Negative	Negative
8	Rectum	B	15	Yes	Positive	Positive
9	Sigmoid	B	42	Yes	Positive	Negative
10	Descending colon	B	50	No	Positive	Positive
11	Sigmoid	B	51	Yes	Negative	Positive
12	Rectum	B	53	No	Negative	Negative
13	Sigmoid	B	53	No	Negative	Negative
14	Sigmoid	B	54	No	Negative	Positive
15	Sigmoid	B	53	No	Negative	Negative
16	Sigmoid	B	50	No	Negative	Negative
17	Sigmoid	B	49	No	Positive	Positive
18	Rectum	C	58	No	Positive	Positive
19	Sigmoid	C	58	No	Negative	Positive
20	Rectum	C	67	No	Positive	Positive
21	Rectum	C	47	Yes	Negative	Negative
22	Sigmoid	C	53	Yes	Positive	Negative
23	Sigmoid	C	59	No	Negative	Positive
24	Rectum	C	56	Yes	Negative	Positive
25	Rectum	C	48	No	Negative	Positive
26	Rectum	C	57	Yes	Negative	Negative
27	Rectum	C	53	No	Negative	Positive
28	Rectum	D	56	Yes	Negative	Positive
29	Rectum	D	31	Yes	Negative	Negative
30	Sigmoid	D	29	Yes	Negative	Negative
31	Sigmoid	D	21	Yes	Negative	Negative
32	Rectum	D	6	Yes	Negative	Negative
33	Transverse colon	D	8	Yes	Negative	Positive
34	Sigmoid	D	53	Yes	Positive	Positive

M: male, F: female (Loveday et al. 2004)

Five out of 11 patients (45%) with positive CTC in their pre-operative samples and 8 out of 19 patients (42%) with positive CTC in their post operative samples developed recurrence or metastases. Eight (23.5%) patients had CTC detected in both pre and pos-

operative samples; 3 of which developed recurrence or metastases and 2 died of metastatic disease.

The above results showed no significant statistical correlation between the presence of CTC in peripheral blood and recurrence or mortality rates.

5.2.2 Conclusion

This study has demonstrated that the use of immunomagnetic separation of the tumour cells from the PBMC, and the use of the TeloTAGGG PCR-ELISA assay for the detection of CTC and telomerase activity in the peripheral blood of CRC patients to be specific where 32% of pre-operative samples and 56% post-operative samples were positive. However this small cohort study has failed to demonstrate any correlation between the presence of CTC and the disease stage. The presence of CTC in peripheral blood was shown to have no prognostic value after a median follow up of 47.5 months. Based on the result of this small cohort study it was decided to perform a prospective study on a larger scale in order to confirm the findings.

5.3 Detection of Telomerase activity in circulating epithelial cells by Telomerase PCR ELISA

5.3.1 Methods

A prospective study was undertaken on a cohort of 98 patients who underwent curative resection for CRC. Peripheral blood samples were taken pre- and 4-6 weeks post operatively and if the patients were receiving chemotherapy further blood samples were obtained on a monthly interval. Methods and materials described in section 2.3 and 2.4 were used for cell isolation and telomerase activation.

5.3.2 The conception of TeloTAGGG Telomerase PCR ELISA

1) Telomeric Repeat Amplification Protocol assay (TRAP)

A biotin-labelled synthetic P1-TS-primer and a 216 bp internal standard (IS) are provided in the kit. The IS is an internal amplification control that allows detection of *Taq* DNA polymerase inhibitors. It has been reported that some tissue samples contain inhibitors of *Taq* DNA polymerase (Wright et al. 1995), thus giving false-negative results when analysed for telomerase activity. Therefore an IS has been used in order to identify false negative tumour samples.

In this first step if telomerase is present in patients' samples the enzyme will add telomeric repeats (TTAGGG) to the 3'-end of the biotin-labeled synthetic P1-TS-primer. The elongation products as well as the IS are subsequently amplified by PCR using the primers P1-TS and the anchor-primer P2 to allow highly sensitive detection of telomerase activity.

To confirm the specificity of product formation in the TRAP assay, samples were split into two groups. One group was treated with heat for 10 min at 85°C to inactivate telomerase protein prior to the TRAP assay.

2) ELISA (Enzyme Linked Immuno-Sorbent Assay) (see section 2.4):

Briefly the PCR products were denatured and hybridized separately to digoxigenin-(DIG)-labeled detection probes, specific for the telomeric repeats (P3-T) and for the (IS) (P3-Std), respectively. The resulting products were then immobilized via the biotin label to a streptavidin-coated microplate, and subsequently detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (Anti-DIG-HRP). A peroxidase

substrate TMB (3,3',5,5'-tetramethylbenzidine)is then added to the plate which induces a colour change that can be measured on a plate reader A_{450nm}-A_{690nm}.

5.3.3 Data analysis

Negative Control

Controls should be considered as negative only when their values are less than $0.1A_{450nm}$-$A_{690nm\ units}$ when analyzed with the telomerase specific detection probe (P3-T). If the telomerase-specific values are higher than that, the entire experiment —including the TRAP reaction— should be repeated with prolonged heat.

Positive Control

The values for low and high controls should be in the range of 0.2–0.5 and 2.0–4.0 absorbance unit (after 10 min substrate reaction) respectively as set by the manufacturer protocol.

Samples

Samples are to be considered as telomerase-positive if the difference in absorbance (ΔA) between the negative controls and samples is higher than the twofold background activity (background activity = value of negative control or heat-treated sample).

5.3.4 Results

The full details of the patients' clinicopathological status were shown previously in chapter 4 (Table 4.1 chapter 4).

The patients' samples, positive and negative controls were arranged in the ELISA plate in the following order as shown in Figure 5.1

	1 S + TB	2 S + TB	3 S/S + TB	4 S + TB	5 S + TB	6 S + IS	7 S + IS	8 S + IS
A	H + TB	S + TB	S +TB	S + TB	S + TB	H + IS	S + IS	S + IS
B	L + TB	S + TB	S +TB	S + TB	S + TB	L + IS	S + IS	S + IS
C	LB + TB	S + TB	S +TB	S + TB	S + TB	LB + IS	S + IS	S + IS
D	S + TB	S + TB	S +TB	S + TB	S + TB	S + IS	S + IS	S + IS
E	S + TB	S + TB	S +TB	S + TB	S + TB	S + IS	S + IS	S + IS
F	S + TB	S + TB	S + TB	S + TB	S + TB	S + IS	S + IS	
G	S + TB	S + TB	S + TB	S + TB	S + TB	S + IS	S + IS	
H	S + TB	S + TB	S + TB	S + TB	S + TB	S + IS	S + IS	

Figure 5.1 Samples distribution on ELISA plate

H: High positive control
L: Low positive control
LB: Lysis buffer
TB: Telomerase buffer
S: heat treated samples
S: None heat treated samples
IS: Internal standard

In the experiment PBMC lysates were extracted from patients' samples, and were kept frozen before they were used for the TeloTAGGG assay. The protein concentration was determined using Coomassie Plus kit (section 2.4.2).

The study protocol set the level of protein content in the sample lysates to be 0.2µg/µl hence the samples were then standardised to 0.2µg/µl by dilution with lysis buffer. The manufacturer's recommendation for the TeloTAGGG assay is 0.5-10µg total protein per tube. Table 5.2 shows that some samples had sufficient protein concentration for TeloTAGGG assay with exception of samples highlighted in red. The low protein concentration in some samples could be due to its relatively low cell numbers in the CTC

preparation during storage in liquid nitrogen before extracting the cell lysates and/or due to the fact that the cell lysates were frozen for more than 4-6 weeks before using them for TeloTAGGG assay and hence some protein may have been lost.

Table 5.2: Protein concentration of cell lysates

Sample number	Sample Concentration µg/µl
19#5	4.2
19#6	0.1
21#5	0.1
21#6	3.1
28#2	2.8
31#2	3.6
34#2	3.5
36#3	2
39#2	0.3
41#2	3.2
44#2	0.1
45#2	0.6
53#1	3
54#2	0.1
56#1	4
57#3	0.1
62#1	0.2
63#1	2.6

N.B. The first number indicates the patient number and the #x indicates the number of sample, for an example 19#6 means; blood sample number 6 from patient number 19

Table 5.3 shows the results of the TeloTAGGG assay, where the high and low controls were positive when telomerase and internal standard (TB, IS) buffers were used in fact both were higher than expected as the values for low and high controls should be in the range of 0.2–0.5 and 2.0–4.0 respectively as set by the manufacturer protocol.

Furthermore the lysis buffer tube was negative indicating that there was no contamination.

Looking at the samples inactivated with heat (negative controls) most of them had negative results with the exception of the samples highlighted in red; this could be as a result of insufficient inactivation with heat or could represent some sort of contamination hence the associated results should be considered carefully. Most of the IS (the positive controls) were significantly positive and were 2 fold higher than that of the negative controls (lysis buffer and heat treated samples) with the exception of the samples highlighted in gray, this could be due to the presence of telomerase inhibitors in these samples. In the TB samples only sample 19#5 was positive where it was 2 fold higher than the negative control.

Table 5.3: An example TeloTAGGG assay

	C+ IS	C + TB	Sample no.	HT + TB	NHT + TB	NHT + IS
H	2.599	2.93	19#5	0.046	0.222	2.577
L	1.657	1.893	19#6	0.066	0.036	2.682
LS	0.04	0.056	21#5	0.075	0.052	3.039
			21#6	0.074	0.049	2.52
			28#2	0.045	0.051	0.054
			31#2	0.49	0.041	0.048
			34#2	0.065	0.067	1.832
			36#3	0.083	0.058	2.344
			39#2	0.139	0.059	2.702
			41#2	0.046	0.045	0.055
			44#2	0.081	0.059	2.951
			45#2	0.114	0.045	2.82
			53#1	0.08	0.07	2.325
			54#2	0.086	0.059	2.814
			56#1	0.124	0.083	2.179
			57#3	0.143	0.057	3.11
			62#1	0.051	0.055	2.837
			63#1	0.05	0.064	2.828

H: High
L: Low
LS: Lysis buffer
C: Control
IS: Internal standard
HT: Heat treated
TB: Telomerase buffer
NHT: None heat treated

The same experiment was repeated using another set of 18 samples, where the cell lysates were more than 4-6 weeks old. Some samples had a very low protein concentration with the exception of few samples highlighted in pink which meant that the samples were used undiluted for the PCR reaction. Tables 5.4 and 5.5 demonstrate the results of these experiments.

Table 5.4: Protein concentrations

Sample number	Sample Concentration µg/µl
19#4	0.16
21#2	0.1
21#4	0.07
23#2	0.16
24#2	0.09
35#1	0.18
35#2	3.4
35#3	0.3
36#1	0.7
36#2	0.7
36#3	3.4
41#3	2.8
46#1	0.08
46#2	3.1
46#3	0.1
47#1	0.3
47#2	0.8
47#3	0.25

Table 5.5: TeloTAGGG assay

	C + IS	C + TB	Sample no.	HT + TB	NHT + TB	NHT + IS
H	1.35	0.3	19#4	0.038	0.04	2.85
L	1.12	0.08	21#2	0.062	0.056	3.13
LB	0.9	0.04	21#4	0.042	0.04	4.95
			23#2	0.041	0.043	2.52
			24#2	0.04	0.047	3.12
			35#1	0.034	0.037	0.7
			35#2	0.035	0.041	0.045
			35#3	0.036	0.032	1.91
			36#1	0.036	0.039	0.399
			36#2	0.028	0.041	0.157
			36#3	0.037	0.041	0.587
			41#3	0.033	0.043	0.033
			46#1	0.039	0.042	2.39
			46#2	0.04	0.042	0.042
			46#3	0.035	0.039	1.61
			47#1	0.039	0.042	1
			47#2	0.039	0.036	0.503
			47#3	0.038	0.062	0.037

H: High
L: Low
LS: Lysis buffer
C: Control
IS: Internal standard
HT: Heat treated
TB: Telomerase buffer
NHT: None heat treated

Table 5.5 demonstrates that the standard positive high and low controls were positive, again the low control is higher than it should be according to the manufacturer's instructions, which could be due to contamination. All of the samples cell lysates used with TB were negative and some of the cell lysates when used with the IS showed

negative results. In this experiment increasing the protein content of the sample lysates did not increase the ability of detecting CTC using the TeloTAGGG assay.

In the next step it was decided to extract the cell lysates and use them for TeloTAGGG assay immediately without any storage period in order to prevent any potential protein degradation.

Table 5.6: TeloTAGGG assay

	C + IS	C + TB	Sample no.	HT + TB	NHT + TB	NHT + IS
H	3.330	2.8	44#1	0.091	0.052	2.156
L	2.835	1.58	67#1	0.085	0.078	1.797
LB	0.04	0.032	67#2	0.061	0.072	1.952
			68#1	0.065	0.055	2.134
			68#2	0.083	0.048	2.133
			69#1	0.132	0.047	2.695
			69#2	0.154	0.043	2.498
			70#1	0.138	0.039	2.309
			71#1	0.077	0.059	1.141
			72#1	0.083	0.044	1.367
			73#1	0.068	0.043	1.511
			74#1	0.066	0.481	1.64
			75#1	0.073	0.418	1.402
			76#1	0.12	0.318	2.528
			77#1	0.108	0.254	1.741
			78#1	0.176	0.169	1.477
			79#1	0.147	0.181	1.633
			80#1	0.056	0.062	0.674

H: High
L: Low
LS: Lysis buffer
C: Control
IS: Internal standard
HT: Heat treated
TB: Telomerase buffer
NHT: None heat treated

Four patients' samples with acceptable protein concentrations, according to the protocol, when used with TB, showed positive results.

Two samples were higher than their negative controls by 2 folds (blue colour) and the remaining (highlighted in red) where only 2-3 times higher than there negative controls (0.318/0.120), (0.254/0.108) (Table 5.6).

To verify the results 2 further TeloTAGGG assay were undertaken using freshly extracted cell lysates: 4 samples out of 35 were telomerase positive. The positive and negative controls on the plates all gave the appropriate values. After these two experiments 3 further Telo*TAGGG* kits were used on freshly extracted cell lysates from 46 samples, all of the 3 assays failed to detect any telomerase activity. When the positive standard controls and the cell lysates used with IS buffers were studied none of them showed any positive results which raise up the suspicion of a technical failure of the kit and and as a result the experiment was ended.

Although the aim of this experiment was to correlate the telomerase activity in the CTC samples, stage of the disease and its prognosis. It was difficult in this study to make any correlation as there were only few positive results and not possible to analyse all samples as a result of the faulty batches used later in the experiments. The majority of the patients analysed were in the Dukes' C stage and only one patient has Dukes' A Table 5.7 shows the clinicopathological status of the patients with the positive results.

Table 5.7: Patients with positive telomerase activity

NO	SEX	AGE	DUKE	METS	DEATH
14	M	80	A	N	N
16	F	72	C	N	N
19	M	64	C	N	N
23	F	53	D	N	N
24	F	59	D	LIVER	N
71	M	47	C	N	N
74	F	64	C	N	N
75	M	56	D	N	N
76	M	61	B	N	N
77	M	72	D	LIVER	N
79	F	78	B	N	N
84	M	68	C	N	N
92	F	72	B	N	N
93	F	69	C	N	N

5.4 Summary

In this chapter the aim was to detect telomerase activity using TeloTAGGG assay, in total assays were performed on independent 132 samples, fourteen samples showed positive telomerase activity (11%). In this study 36 samples, in which the cell lysates had been stored for ≥ 4-6 weeks before use, were assayed with only one positive result (3%). A further 71 samples were analysed immediately after cell lysing and 9 (13%) were found to be positive for telomerase activity. One of these assays was performed in collaboration with a colleague (Mr. D Beral) working in the same laboratory, where telomerase activity was detected in 4 out of 25 samples tested, the cell lysates used in this assay were stored for less than 4 weeks and when the assay was repeated 2 and 4 weeks after the first experiment the same samples gave negative results. In fact in this study it was not

possible to get positive results on repeated samples, which strongly suggests that storage leads to the loss of telomerase activity.

5.5 Conclusions and recommendations

In this experiment telomerase activity was detected in CTC however some difficulties were encountered using the above kit, and this experiment has raised a number of questions.

5.5.1 The validity of IMS technique

The efficiency of IMS technique in isolating CTC from PBMC has been checked by a colleague (Mr. D. Beral) who clearly demonstrated the ability of Dynabeads covered with BerEP4 antibody to isolate BerEP4 expressing cells. Cancer cell that express BerEP4 and others that do not express BerEP4 were mixed with Dynabeads covered with BerEP4 antibody and this was observed under the microscope. The pictures showed that the cell lines that express BerEP4 were attached to the Dynabeads coated with the relevant antibody (Figure 5.2 & 5.3)

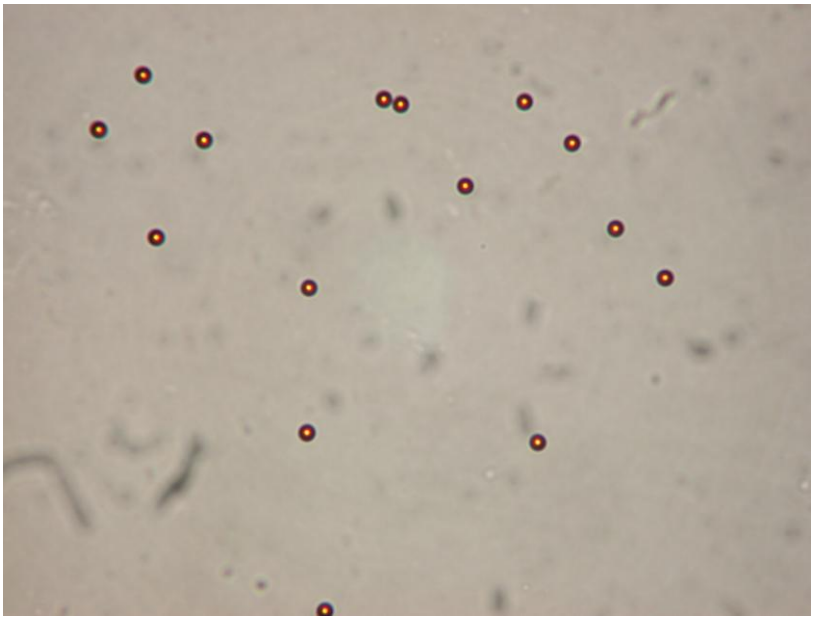


Figure 5.2 BerEP4-Dynabeads with no cells attached after mixing with BerEP4-negative cell line (CaCO-2) and extraction with magnet. Provided by D. Beral

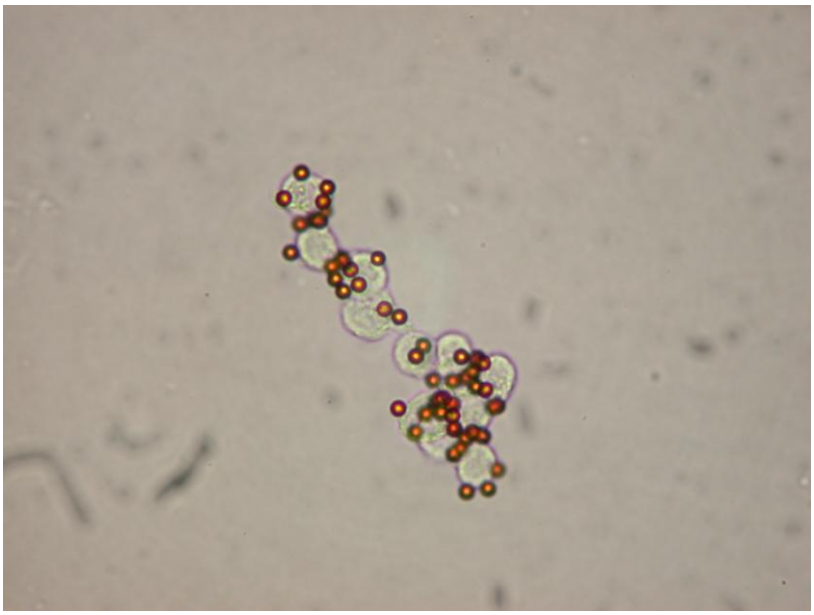


Figure 5.3 BerEP4-Dynabeads attached to cells after mixing with a BerEP4-positive cell line (MCF-7) and extraction using the magnet. Provided by D. Beral

5.5.2 Protein concentrations of cell lysates used in TeloTAGGG assay

The study protocol set the level of protein content in the sample lysates to 0.2µg/µl using the modified Bradford assay. The manufacturer's recommendation for the TeloTAGGG assay was 0.5-10µg total protein per tube, therefore with 3µl per reaction tube there was 0.6µg total protein per tube. This level was deliberately chosen to be at the lower end of the recommended range in order to reduce the risk of false positive results and had been used previously within this laboratory (Loveday et al. 2004). After initial assays on study cohort samples it became apparent that very few samples were giving positive results. It was hypothesised that the protein content may have been set too low. To test this, undiluted samples were used in order to increase the protein content in the lysates but none of the samples gave positive results for telomerase thus not supporting the hypothesis that the protein content was set too low.

5.5.3 The duration of samples storage

In this experiment 36 samples in which the cell lysates had been stored for ≥ 4-6 weeks before use were assayed with only one positive result (3%). A further 71 samples were analysed immediately after cell lysing and 9 (13%) were found to be positive for telomerase activity. In a previous experiments performed by Mr. Beral the 4/25 positive samples failed to reproduce positive results after storage for an additional 2 and 4 weeks. Together these experiments strongly suggest that the fresher the cell lysates the more likely they are to produce positive results. As it seems that there is a degree of protein degradation during the period of cell lysates storage. This conclusion is supported when the data was compared to Loveday study (Loveday et al. 2004), as in this study the

isolated CTC were lysed only few days after storage and TRAP assay was performed on the samples within 2-4 weeks of storing the cell lysates.

Furthermore it was hypothesised that the longer the CTC are frozen in liquid nitrogen the higher the chance of protein degradation, hence the lesser the chance of telomerase detection. To investigate this hypothesis the samples were divided into two groups. The first group contained the patients with positive telomerase results and the second group was the one with negative results. The time between CTC isolation and their lysis was determined for each group. It was shown that the samples with the positive telomerase were stored for an average period of 28 days (range 2-60) whereas the negative telomerase group was stored for an average of 73 days (range 1-275), fully supporting the above hypothesis. Furthermore the results of Gauthier *et al* study support this hypothesis (Gauthier et al. 2001) where 8/11 (72%) of Duke's A patients were telomerase positive, in this study the samples were lysed and stored without a period of storage to the isolated epithelial cells.

5.5.4 The validity of the TeloTAGGG kits

The manufacturers were contacted for advice regarding the persistently negative results in the last batch to see if any systematic error in performing the assay could be identified, despite having followed the manufacturers' instructions. No such error was able to be identified, however it was noted that the kit was subsequently withdrawn from the market, with no explanation.

In conclusion isolated CTC should be stored in liquid nitrogen for only a short period of time before extracting cell lysates and the cell lysates should be prepared for assay

immediately before performing the assay as long as there is no faulty technique with the assay used.

CHAPTER 6
Discussion

In this chapter the wider implications of the work reported in this thesis will be discussed

6.1 CTL response against tumour cells

The catalytic subunit of telomerase (hTERT) has been widely considered to be a universal tumour antigen and has the potential to be a major factor in future cancer immunotherapy since it is expressed by most human tumours but is absent in the majority of normal tissues. It also plays a universal role in tumourgenesis, and its peptide sequences have been recognized by the T cell repertoire in an MHC restricted manner. Seven synthetic peptides derived from hTERT that induce specific CTL response have been investigated to date; four of these peptides react with CTL in an HLA-A2 restricted manner (Vonderheide et al. 1999) (Scardino et al. 2002); a fifth peptide acts in an HLA-A3 restricted manner (Vonderheide et al. 2001) and the final two peptides bind in an HLA-A24 restricted manner (Arai et al. 2001).

In this thesis 2 peptides were used [hTERT (I540 and P865)] that react with CTL in an HLA-A2 restricted manner and hTERT (K973) that acts in an HLA-A3 restricted manner.

Thirty patients with CRC were studied for CTL reaction. The results showed that 70% of the patients were HLA-A2 positive, 10% HLA-A3 positive and 20% were HLA2/A3 positive. Twenty eight pre-operative samples and thirteen post-operative samples were analysable, 71% of pre-operative samples and 61% post-operative samples had CTL reaction towards hTERT peptides whether in an HLA-A2, HLA-A3 or HLA2/A3 restricted manner.

On analysis the results appear to be higher than the results obtained by Titu *et al* who studied the T-cell response against the same two HLA-A2 specific peptides of hTERT (I540 and P865) in 37 CRC patients and 12 normal controls (Titu et al. 2004). Their

results demonstrate that CTL active against hTERT are present in only 20% of CRC patients. There are two explanations for the discrepancy in the results of both studies; the first explanation is that in our study the specific number of spots was calculated by subtracting the number of cells present in the well with no peptide (negative control well) from the number of cells present with peptides, and samples were considered positive only if the value was ≥ 1. Whereas in the study of Titu and colleagues the samples were considered positive only when the number of the spots with peptides were ≥ 16 spots which is the threshold level calculated from the cells with no peptides. In his study the mean and the standard deviation was calculated for the number of spots in the control wells with no peptides, and a threshold level was set by adding 3 standard deviations to the mean, his method was previously used by Pittet *et al*. (Pittet et al. 1999). The second explanation is that in this study another hTERT peptide was added which might have contributed for the higher results.

Our results were more similar to Amarnath *et al* (Amarnath et al. 2004) results who investigated the specific $CD8^+$ CTL response in primary breast cancer patients. In her study of 45 patients using the same methods and peptides as utilised in this thesis, CTL recognized HLA-A2 P540 and P865 peptides in 76% and 47% respectively, CTL recognized both HLA-A2 peptides in 47% of patients and CTL recognized HLA-A3 K973 peptides in 71% patients.

Furthermore our results appear to contradict those of Vonderheide *et al*. (Vonderheide et al. 2001) who were unable to detect a CTL response against hTERT-P540 peptide in freshly isolated PBMC from either cancer or normal controls. This could be due to a number of reasons; firstly in their experiments they analysed samples from 8 patients

only which make it possible to have missed the ones with circulating CTL; secondly the patients analysed were of various histologies (multiple myeloma, melanoma, lymphoma and prostate cancer) and none of them had CRC which, makes it difficult to compare directly between the two studies; and finally in their study only one hTERT peptide was used compared with 3 peptides in the present work.

6.1.1 Clinical trials of hTERT immunotherapy and future work

Multiple phase 1 clinical trials of hTERT immunotherapy have already been conducted in patients with advanced cancer, each of which test the hypothesis that hTERT-specific vaccination can overcome immunological tolerance and trigger anti-tumour T cell responses *in vivo*. Main findings include the induction of hTERT-specific T cells in the absence of toxicity. Objective clinical responses have been rarely reported, but no study yet has been statistically powered to measure this endpoint adequately.

Vonderheide and colleagues in 2004 has taken the first step into applying the above laboratory results into clinical practice when they performed a Phase I clinical trial to evaluate the clinical and immunological impact of vaccinating 5 patients with hormone refractory metastatic prostate cancer and 2 patients with metastatic chemotherapy resistant breast cancer with the HLA-A2-restricted hTERT I540 peptide presented with dendritic cells (Vonderheide et al. 2004). hTERT-specific T lymphocytes were induced in 4 of 7 patients with both cancers after vaccination with dendritic cells pulsed with hTERT peptide. Tetramer-guided high-speed sorting and polyclonal expansion achieved highly enriched populations of hTERT-specific cells that killed tumour cells in an MHC-restricted fashion. Partial tumour regression in 1 patient was associated with the induction of CD8+ tumour infiltrating lymphocytes. These results demonstrate the immunological

feasibility of vaccinating patients against telomerase and provide rationale for targeting self-antigens with critical roles in oncogenesis.

In another clinical trial, 19 HLA-A2 positive women with metastatic breast cancer were vaccinated subcutaneously with hTERT I540 peptide emulsified in Montanide adjuvant and administered with granulocyte macrophage colony stimulating factor (GM-CSF) up to eight times (Domchek et al. 2006). Based on *in vitro* analyses performed on peripheral blood obtained before and after treatment, 68% of patients were found to have responded immunologically. Tumour-infiltrating lymphocytes (TIL) were evident after, but not before vaccination, with 4-13% of post-vaccine CD8+ TIL specific for hTERT I540. Induction of TIL manifested clinically with tumour-site pain and pruritis and pathologically with marked tumour necrosis. Peripheral blood hTERT-specific CD8+ T cells were also induced and shown *in vitro* to proliferate, produce IFN-γ, and lyse tumours. An exploratory landmark analysis revealed an association between an hTERT-specific CD8+ T cell immune response and overall survival in these patients. Interestingly, these clinical and immunological findings were not observed in an earlier study where 14 patients with metastatic cancers (melanoma, renal, colon cancers) and were injected with hTERT I540 in Montanide without GMCSF. Although induction of I540-specific CTL was observed in 50% of patients without toxicity, CTL isolated *ex vivo* failed to lyse tumours endogenously expressed telomerase and no clinical benefit was observed (Parkhurst et al. 2004). With the goal of inducing both CD4 and CD8 T cells responses in patients, another trial enrolled 26 patients with non-small cell lung cancer who were repeatedly injected with 2 hTERT peptides; I540 (HLA-A2 peptide) and E611 (DR, DP and DQ peptide) and GM-CSF (Brunsvig et al. 2006). Treatment was well

tolerated and immune responses against E611 were detected in 46% of evaluable patients. A complete tumour response was observed in one patient who developed an immune response following treatment. In a second trial at the same institution, patients with non-resectable pancreatic cancer were immunised with E611 peptide alone, with GM-CSF, with specificT cell responses detected in 63% of patients (Bernhardt et al. 2006.) Median survival for immune responders was significantly higher than that for non-responders.

To test the immunogenicity of low-affinity hTERT peptides, two trials enrolled HLA-A2 positive patients. Patients were given subcutaneous injections of 572Y modified peptide in Montanide adjuvant. Nineteen patients with chemotherapy refractory and progressing malignant tumours advanced were treated in a phase I trial (Mavroudis et al. 2006), and 22 patients with advanced non-small cell lung cancer were treated in a follow up expanded safety trial Peptide specific T cell responses were observed in the majority of patients without toxicity. In the second trial, estimated overall survival was 30 months for immunological responders vs. 4 months for non-responders (Bolonaki et al. 2007). Another trial testing both I540 and 572Y peptides loaded onto autologous B cells as a vaccine was tested in 15 patients with advanced prostate cancer. Again, immune responses were observed without toxicity (Cortez-Gonzalez and Zanetti 2007).

Finally, in the first clinical trial evaluating immunotherapy with full-length hTERT (Su et al. 2005), 20 patients with metastatic prostate cancer were administered autologous dendritic cells transfected with mRNA encoding hTERT with or without the inclusion of a chimeric construct of lysosome-associated membrane protein-1 (LAMP). Induction of hTERT-specific CD8 T cells was observed in all but one patient, with up to 1.8% of CD8+ T cells exhibiting hTERT specificity. Antigen-specific immune measurements

were higher in patients immunised with chimeric LAMP hTERT. Treatment was associated with an increase in the prostate-specific antigen doubling time and molecular clearance of circulating micrometastases.

The above published reports of hTERT vaccination offer preliminary evidence to support the notion that hTERT can function as a TAA target for novel vaccines. If, in further studies with second-generation vaccines, optimal immunity can be successfully elicited in cancer patients without the induction of severe autoimmunity, hTERT clearly becomes a prime candidate for a widely applicable cancer vaccine.

6.2 Telomerase and CTC

Despite the modest improvements in patient survival from CRC in the last few decades, the overall five-year survival rate remains at 40-45 %. Surgical resection is the mainstay of treatment for CRC, but nearly half of all patients who undergo a potentially curative resection will relapse because of undetected micrometastases. The fact that the overall survival rate remains poor, strongly suggests that the dissemination of these cells occurs early in the disease process and emphasizes the need for finding feasible diagnostic methods with sufficient sensitivity and specificity.

In this thesis two well described techniques in the literature were used to detect CTC in the peripheral blood of CRC patients utilising telomerase activity and its catalytic subunit. In the first experiment peripheral blood samples were collected from 105 patients with curative CRC before and after surgery and CTC were isolated from the PBMC using the immunomagnetic separation technique and a TeloTAGGG Telomerase PCR ELISA^{PLUS} assay was done on the isolated cell lysates in order to detect telomerase

activity. In the second experiment serum samples of CRC patients were used to detect the expression of hTERT mRNA utilising RT-PCR technique.

In the first telomerase assay performed only 14 samples demonstrated telomerase activity, these 14 samples were either freshly prepared or frozen for less than 4 weeks compared with the remaining negative samples. It was concluded that the longer the samples are kept frozen the higher the chance of protein degradation. In this experiment a correlation between the disease stage and the presence of telomerase activity or its prognostic value could not be studied as the reliability of the test could not be confirmed. Furthermore, it was not possible to find a standard reliable technique which could detect hTERT mRNA in serum samples of CRC patients as hTERT mRNA was only detected in 6 samples out of all other samples. This could be again as a result of the long duration of samples storage.

The expression of hTERT in peripheral blood samples was reported in previous studies, where it has been detected in blood samples of patients with hepatocellular carcinoma (Miura, N. et al. 2003) (Waguri et al. 2003), breast cancer (Chen et al. 2000), gastric cancer (Shin et al. 2002), and CRC patients (Lledo et al. 2004) .

Furthermore recently (Uen et al. 2007) was the first to report the prognostic value of hTERT mRNA as a tumour marker where the patients were followed up over a period of 40 months. A Membrane-arrays technique was used consisting of a panel of mRNA markers that included hTERT, CK19, CK20, and CEA mRNA to detect CTCs in the peripheral blood of 194 stage II CRC patients who underwent potentially curative resection. Fifty-three of 194 (27.3%) stage II patients were detected with the expression

of all 4 mRNA markers. After a median follow up of 40 months, 56 of 194 (28.9%) developed recurrence/metastases postoperatively.

6.3 Conclusion

6.3.1 Technical issues in CTC detection

CTC detection is all about rare event detection. In metastatic cancer patients, CTC in the peripheral circulation occur at an estimated number of one CTC per $1X10^{5–7}$ peripheral blood mononuclear cells. Therefore, most assays apply an enrichment step to increase the sensitivity.

Another major problem in the routine and automated detection of CTC arises from the illegitimate expression of the epithelial cell-related markers and the lack of tumour cell specific markers. As the detection of mRNA markers is especially difficult due to the sensitivity of mRNA to preparation procedures, the exploration of new marker or markers is required.

Furthermore, tumour cells may lose epithelial features during dissemination through a process called epithelial-to-mesenchymal transition (EMT). EMT renders tumour cells to become less rigid and more motile thereby facilitating their invasion of stromal tissue. (Willipinski-Stapelfeldt et al. 2005) (Yang et al. 2006). This process is characterised by the loss of cell-to-cell adhesion, increased expression of mesenchymal markers, and loss of several epithelial markers. Since the epithelial markers that get lost during EMT may include markers used for CTC detection, underestimation of the actual CTC number may occur.

6.3.2 Clinical application of CTC detection in cancer prognosis and treatment

The detection of CTC for CRC in early diagnosis, staging and prognosis is a long term dream of researchers and clinicians. As the molecular biology tools became widely applicable in the last 15 years, significant achievements were observed.

Initial results obtained from clinical studies strongly suggest that CTC detection and enumeration has indeed prognostic value and can serve as an early marker to assess anti-tumour activity of cancer treatment.

A recent study in patients with CRC identified CTC by RT-PCR positivity for both CK-20 and CEA mRNA. A Hundred and ninety-six patients with curative CRC undergoing primary tumour resection were observed over a median follow-up of 1393 days, they concluded that the presence of CTC within 24 hours after resection was predictive for relapse, in particular when combined with lymph node status (Allen-Mersh et al. 2007).

Concerning metastatic disease, all studies published so far in which CTC were identified through PCR-based methods included numbers of patients too small to draw firm conclusions. By contrast, data from studies recruiting large numbers of metastatic patients are available using the CellSearch TM Epithelial Cell Kit/CellSpotter TM Analyzer. In this method, CTC were isolated from whole blood by IMS using ferrofluids coated with epithelial cell adhesion molecule (EpCam) specific antibodies. Subsequently, the isolated cells were stained with fluorescent monoclonal antibodies for epithelial cells (CK 8, 18, 19), leucocytes (CD45) and a nuclear staining dye, and thereafter enumerated using a semi-automated fluorescence microscope. CTC were defined as nuclear cells, expressing CK and lacking CD45 expression (Allard et al. 2004). With this procedure it

was demonstrated that only one out of 344 (0.3%) healthy and non-malignant subjects had ≥2 CTC per 7.5 ml whole blood, while approximately 60–70% of metastatic breast cancer patients have ≥2 CTC (Riethdorf et al. 2007) (Cristofanilli et al. 2004). In a prospective, multicentre study including 177 metastatic breast cancer patients, a high CTC number at baseline, defined as ≥5 CTC per 7.5 ml whole blood, was an independent predictor for a poor outcome in terms of progression-free (2.7 versus 7.0 months) and overall survival (10.1 versus >18 months) (Cristofanilli et al. 2004). Also, at each examined time point after treatment initiation, from 3 weeks onwards, a high CTC count was able to distinguish patients with an unfavourable clinical outcome. In addition, patients who initially had an increased number of CTC but a low number after the first administration of systemic treatment had a better outcome in terms of progression-free survival and overall survival compared with patients in whom the number of CTC remained elevated (Cristofanilli et al. 2005) (Hayes et al. 2006). This strongly suggests that CTC enumeration can serve as an early surrogate marker for response assessment. The feasibility of CTC enumeration to guide treatment was further supported by the observation that in patients without progression at radiological assessment those patients with a high CTC count had a poorer overall survival than patients with a low count. Additionally, the inter-reader variability for determining a response on the basis of CTC enumeration was smaller than when using radiological imaging (Budd et al. 2006), which suggests that CTC enumeration outperforms radiological assessments for response evaluation.

Using the assay mentioned earlier, similar results were recently presented in a multi-centre study comprising 413 patients with metastatic colorectal carcinoma (Meropol et al.

2007). Patients with ≥3 CTC per 7.5 ml blood at base-line fared more poorly than patients with less CTC in terms of median progression-free and overall survival; 4.5 versus 7.9 months and 9.4 versus 18.5 months, respectively. Additionally, a decrease in CTC levels 3–5 weeks following treatment initiation was associated with improved progression-free and overall survival compared to those patients who remained at high CTC levels.

Also, in patients with hormone-refractory prostate cancer showing prostate specific antigen (PSA)-progression and who started chemotherapy, CTC enumeration showed encouraging results (Moreno et al. 2007). Using a cut-off level of ≥5 CTC per 7.5 ml blood, patients with a low level at base line (43% of the 240 patients) had a better median overall survival of 21.4 months compared to patients with an elevated count (10.7 months). Similar to the studies performed in breast and CRC, a CTC decrease to low levels at all time-points assessed was predictive for improved survival, from 2 to 5 weeks after starting therapy onwards.

6.3.3 Future work

Determination of the significance of micrometastatic cell in the peripheral blood of Dukes stage B under chemotherapy is still missing. Currently, there are several methods available for CTC detection, some of which in clinical studies have been shown to be associated with clinical outcome. However, confirmation of the results, which look promising at first glance indeed, is required prior to widespread use and having clinical impact. In particular, the observation that CTC levels during treatment may serve as marker for early assessment of anti-tumour activity is intriguing, but whether this should have therapeutic consequences is currently unknown. To solve this question, randomised

studies would be required in which patients exhibiting a high CTC count after starting a certain treatment are randomly allocated to receive an alternative treatment directly or to continue the same systemic therapy and only after radiological progression to be switched to the alternative therapy. Such a study can determine whether early switching to another type of therapy on the basis of CTC enumeration yields a better outcome.

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Appendix 4.1: Patients' data

No.	Sex	Age	Duke	Site	Operation	Complications	Met
1	M	80	C	Rectum	APR	N	N
2	M	59	C	Rectum	AR	N	N
3	M	74	A	Rt colon	AR	N	N
4	M	71	B	Rt colon	Rt hemicolectomy	N	N
5	M	73	B	Sigmoid	AR	N	N
6	M	89	A	Sigmoid	AR	N	N
7	M	84	A	Rectum	AR	Wound infection	N
8	F	49	B	Lt colon	Total colectomy	N	N
9	F	85	B	Rectum	AR	N	N
10	M	71	A	Sigmoid	AR	Wound infection	N
11	M	60	C	Rectum	AR	N	N
12	F	75	C	Rectum	AR	N	N
13	M	86	D	Rectum	AR	N	Lung
14	M	80	A	Rectum	AR	N	N
15	F	68	C	Rectum	Total colectomy	N	N
16	F	72	C	Rectum	APR	N	N
17	M	66	B	Sigmoid	AR	N	N
18	M	43	C	Rectum	APR	Small bowel obstruction	N
19	M	64	C	Sigmoid	Lt hemicolectomy	N	N
20	M	79	C	Rectum	Hartmanns	N	N
21	M	70	C	Rectum	Lt hemicolectomy	N	N
22	M	74	C	Sigmoid	AR	N	N
23	F	53	D	Sigmoid	AR	N	Liver
24	F	59	D	Rectum	AR	N	Liver
25	M	62	A	Sigmoid	AR	N	N
26	F	75	C	Sigmoid	Total colectomy	N	N
27	F	78	C	Caecum	Rt hemicolectomy	N	N
28	M	78	C	Lt colon	AR	N	N
29	F	67	B	Rectum	APR	N	N
30	F	71	B	Rectum	AR	N	N
31	M	87	C	Rt colon	Rt hemicolectomy	N	N
32	M	83	B	Rt colon	Rt hemicolectomy	N	N
33	M	79	B	Rectum	AR	N	N
34	F	83	A	Sigmoid	AR	N	N
35	F	88	A	Rectum	AR	AL	N
36	M	75	D	Sigmoid	AR	N	N
37	M	81	C	Rectum	Colostomy	N	N
38	F	90	B	Rectum	APR	N	N
39	F	54	C	A colon	Rt hemicolectomy	N	N
40	F	81	C	Rectum	APR	Pancreatitis	N
41	F	56	B	Caecum	Rt hemicolectomy	N	N
42	M	78	B	Rectum	Total colectomy	N	N
43	M	87	C	Rt colon	Rt hemicolectomy	N	N
44	M	64	B	Rectum	Hartmanns	Wound infection	N
45	F	68	C	Rectum	AR	N	N
46	F	72	A	Rectum	AR	N	N

No.	Sex	Age	Duke	Site	Operation	Complications	Met
47	F	69	C	Sigmoid	Sigmoid colectomy	N	N
48	F	71	C	Caecum	Total colectomy	N	Liver
49	F	81	C	Rectum	Hartmanns	N	N
50	F	60	C	Lt colon	Lt hemicolectomy	N	N
51	F	85	C	Rt colon	Lt hemicolectomy	N	N
52	M	83	C	Caecum	Rt hemicolectomy	N	N
53	M	55	C	Rectum	AR	N	N
54	M	60	B	Rt colon	Lt hemicolectomy	N	N
55	F	77	A	Sigmoid	AR	N	N
56	F	62	C	Rectum	APR	N	N
57	M	64	C	Rectum	AR + LI	N	N
58	M	76	C	Sigmoid	AR	Colocutanous fistula	N
59	M	74	A	Rectum	AR	N	N
60	F	56	C	Sigmoid		N	N
					LT colectomy and		
					liver resection		
61	F	74	A	Rectum	AR	N	N
62	F	82	A	Rectum	AR	N	N
63	F	71	B	Sigmoid	AR	N	N
64	F	73	D	Rectum	AR	N	N
65	F	51	C	Rectum	AR	N	N
66	M	70	C	Rectum	AR	N	N
67	M	82	D	Rectum	AR	Fluid collection	Liver
68	F	84	B	Sigmoid	Sigmoid colectomy	N	N
69	F	75	C	Sigmoid	AR	AL	N
70	F	63	A	Sigmoid	AR	N	N
71	M	47	C	T colon	Subtotal colectomy	N	N
72	M	57	A	Rectum	AR	AL	N
73	M	52	C	Rectum	AR	N	N
74	F	64	C	Rectum	AR	N	N
75	M	56	D	Sigmoid	Total colectomy	N	N
76	M	61	B	Caecum	Total colectomy	Stenosis	N
77	M	72	D	Rt colon	Rt hemicolectomy	N	N
78	M	79	B	Caecum	Rt hemicolectomy	N	N
79	F	78	B	Rt colon	Rt hemicolectomy	N	N
80	M	60	A	Rectum	AR	N	N
81	M	76	B	Rectum	AR	Pneumonia	N
82	M	82	C	Rectum	AR	N	N
83	M	71	C	T Colon	Extended Rt hemicolectomy	N	N
84	M	68	C	Rectum	AR	N	N
85	M	70	B	T colon	AR	N	N
86	F	69	A	Rt colon	Rt hemicolectomy	N	N
87	F	73	B	Rectum	AR	N	N
88	M	64	C	Rectum	AR	N	Liver
89	F	66	A	Rt colon	Rt hemicolectomy	N	N
90	F	53	C	Rectum	AR	N	N

No.	Sex	Age	Duke	Site	Operation	Complications	Met
91	M	79	C	Rectum	AR	N	N
92	F	72	B	Sigmoid	AR	N	N
93	F	69	C	Sigmoid	AR	N	N
94	M	77	B	Sigmoid	AR	N	N
95	M	85	C	T colon	Lt hemicolectomy	N	N
96	F	78	B	Rt colon	Rt hemicolectomy	N	N
97	F	73	A	Lt colon	Extended Rt hemicolectomy	N	N

F: Female
M: Male
Lt: Left
Rt: Right
T: Transverse
A: Ascending
AR: Anterior resection
LI: Loop ileostomy
APR: Abdomino-perineal resection
AL: Anastomotic leak
Met: Metastases