Role of Cyclin D1 polymorphisms and protein over-expression in clinical outcomes of colorectal cancer

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By

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Dedicated

To

My wife, family and my parents

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Abbreviations

ADA	adenosine deaminase
APES	aminopropyltriehoxysilane
APC	Adenomatous polyposis coli
BMI	Body mass index
CCND1	Cyclin D1
CAK	CDK-activating kinase
CDK	cyclin dependent kinase
CI	confidence interval
CKI	cyclin dependent kinase inhibitors
CTNNB1	β-catenin
DAB	1, 3- diamino benezene
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
EB	elution buffer
EDTA	ethylene diaminoacitic acid
FAP	familial adenomatous polyposis
GSK-3beta	glycogen synthase kinase 3 beta
H&E	haematoxylin and eosin
hMLH	human Mut-L-Homologon-1
HNPCC	hereditary non-polyposis colorectal cancer
HR	hazards ratio
IMS	industrialised methylated spirit

OR	odds ratio
MMR	mismatch repair
PCR	polymerase chain reaction
PBS	Phosphate buffered saline
pRb	retinoblastoma protein
RFLP	restriction fragment length polymorphism
mRNA	messenger ribonucleic acid
RTPCR	real time – polymerase chain reaction
SCCHN	squamous cell carcinoma of head and neck
SNP	single nucleotide polymorphism
STAT	Signal transducer and activator of transcription 3

Aims and Objectives

- To examine the effects of CCND1 gene polymorphisms on clinical outcomes in patients with sporadic colorectal cancer.

- To examine Cyclin D1 protein over expression and clinical outcomes in colorectal cancer.

- To ascertain whether CCND1 gene polymorphisms have any influence over Cyclin D1 protein expression

- To investigate some of the major mechanistic pathways (β-catenin) involved in over expression of Cyclin D1 protein in patients with colorectal cancer

Abstract

Cyclin D1 is a key regulatory protein in the progression of G1-S phase of the cell cycle. Activated Cyclin D1 in turn activates S phase proteins and thereby leading to cell proliferation. Over expression of Cyclin D1 leads S phase cell cycle progression. There have been several individual studies that have tried to examine the pathways involved in Cyclin D1 protein expression and polymorphism in colorectal cancer and yet it lacks clarification. We examined the effects of CCND1 gene polymorphisms and their effect on clinical outcome on a cohort of about 634 sporadic colorectal cancer patients. We found that A/G^{870} polymorphism did not have any significant influence on clinical or survival outcomes. G/C^{1722} was associated with poor tumour grade (p=0.007, OR 2.17, 95% CI 1.23-3.83) and metastasis (p=0.022, OR 3.74, 95% CI 1.20-11.6). C/A^{1100} polymorphism was associated with left sided tumours (OR 3.66, P= 0.018, CI 1.25-10.76) and early stage disease (OR 0.40, p=0.012, CI 0.20-0.82). None of the polymorphisms significantly associated with Cyclin D1 over-expression. Over expression of Cyclin D1 protein was associated with early stage disease (OR 0.36, p=0.008, CI 0.17- 0.76), node negativity (OR 0.37, p=0.010, CI 0.17- 0.79) and improved survival (HR 0.54, p=0.006, CI 0.35-0.84). To investigate the mechanism underlying Cyclin D1 protein expression, we examined Cyclin D1 phosphorylation sites and found no mutations in these sites (n=27) (T286A, T156A). We then examined one of major pathways involved in regulation of Cyclin D1 expression in CRC namely β-catenin. Contrary to some studies, β-catenin expression was not significantly associated with Cyclin D1 over-expression. Cytoplasmic β-catenin expression was significantly associated with poor clinical outcome and β-catenin gene mutations were rare in colorectal cancer. The samples with Cyclin D1 and β-catenin over-expression

showed a significantly better 5-year survival (HR 0.43, p=0.019, CI 0.21- 0.87). From our study we infer that polymorphisms may not directly influence protein expression but were associated with tumour biology. The improved outcome observed with Cyclin D1 expression needs to be further elucidated. We also demonstrate that β-catenin protein may not be only factor for Cyclin D1 protein expression in colorectal cancer in contrast to some other studies. We conclude that Cyclin D1 regulation remains a complex pathway involving both upstream and downstream genes.

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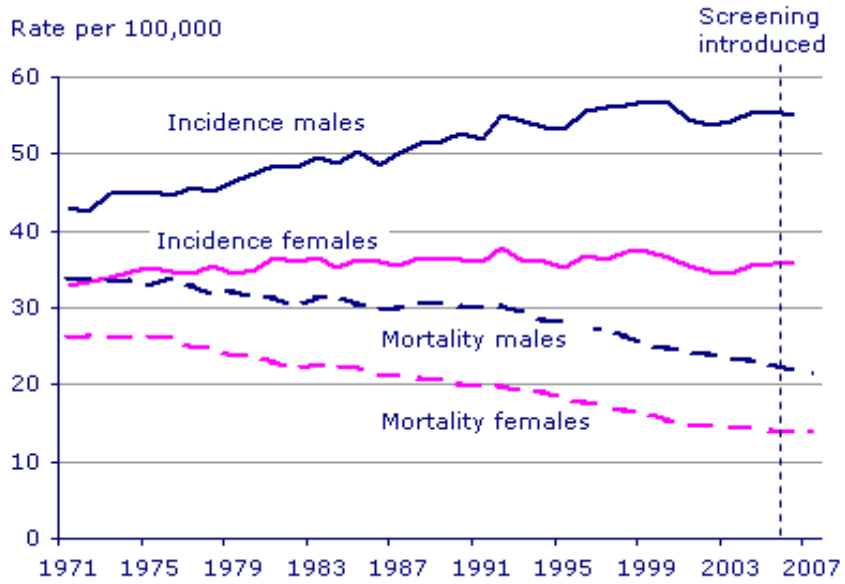
CHAPTER 1

INTRODUCTION

1.1 Epidemiology of colorectal cancer

Colorectal cancer (CRC) is an important public health problem across the globe. This is the second most cause of cancer related death in North America and Western Europe. Nearly a million new cases have been diagnosed worldwide each year and 500,000 deaths registered annually (IARC Cancer base No: 5, 2001). This disease is not only a major problem for the western countries since one-third of the new cases of CRC now occur outside industrialised countries. The numbers of new cases reported in US and United Kingdom per annum are more than 130,000 and 35,000 respectively. On average, over 16000 deaths occur each year due to CRC. (Office for National Statistics 2007). The incidence of colorectal cancer is on the increase in UK.

Figure-1.1: Colorectal cancer Incidence in England, 1971 – 2007. Source: Office for National Statistics, UK



In England and Wales, this number is increasing by 1% every year in men and staying the same in women. In the year 2000 alone there were an estimated 944,717 cases occurred worldwide. The incidence is higher in men than women (19.1 and 14.4 per 100,000 respectively) (British Medical Bulletin 2002).

In the UK, the survival rates have gradually improved over the past 25 years. This is largely due to education, improved diagnostic and treatment modalities (Cancer screening programme-UK). Approximately half of the patients diagnosed with CRC will be still alive in five years time.

1.2 Aetiology of colorectal cancer

At least 90% of colorectal cancers are said to develop from adenomatous polyps. The development of cancer is a multi-step process involving genetic mutations leading to loss of inhibitory control or activation of proto-oncogenes (Fearon *et al,* 1990). It has been suggested that at-least four to five genetic mutations are required for malignant tumour formation. Overall hereditary and environmental factors contribute to the development of CRC. Studies have clearly demonstrated the link between CRC and certain genetic disorders in subgroup analyses. The role of environmental influence remains multi-factorial and in sporadic CRC, some of the environmental causes still remain controversial.

1.2.1 Genetic conditions

The genetic component of CRC has been a rapidly developing area of research in recent years. Some 5% of colorectal cancers arise as a result of some form of genetic predisposition. Among the hereditary syndromes, there are two main conditions namely; Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), both of which have been investigated extensively over the years.

FAP is an autosomal-dominant disorder in which multiple adenomatous polyps develop in the colon withinevitable transformation into cancer. The incidence of FAP is approximately 1 in 8000 accounting for about 0.5% all CRCs' (Boyle *et al,* 2000). The genetic basis of FAP involves germ line mutation affecting the adenomatous polyposis coli gene in the chromosomal region 5q21 (Bodmer *et al,* 1987; Nilbert *et al,* 2008). The vast majority of APC mutations are 'nonsense' or 'frame-shift' mutations resulting in a truncated protein with abnormal function. The mutated adenomatous polyposis coli (APC) gene thus contributes to the development of the disease depending on the gene penetrance. These patients develop multiple colonic polyps occurring in their thousands throughout the colon. Mutations occurring at APC codons 1254 and 1464 causes profuse polyposis (>5000) and codon 1309 mutation was associated with early onset polyps in the thousands (Nilbert *et al,* 2008). These individuals may also develop extra-colonic lesions such as periampullary cancers, osteomas, medulloblastoma, hepatoblastoma and carcinoma of thyroid gland. The severity of involvement will depend on the extent of gene penetrance.

There are also many other documented polyposis syndromes such as Peutz Jegher's syndrome, familial juvenile polyposis and hereditary mixed polyposis syndrome. Peutz-Jegher's syndrome is due to a mutation involving *STK11/LKB1* gene (Launonen V, 2005). Juvenile polyposis syndrome is characterised by the early onset of polyposis of colon. The gene is called *SMAD4* accounts for more than 60% of cases (Alberici *et al,* 2008). All these disorders have identifiable gene mutations leading to polyps and subsequent cancer formation in not only the colon and rectum but also in extra colonic organs (e.g. thyroid, duodenum, and endometrium) (Fearnhead *et al,* 2002).

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is also an autosomal-dominant disorder in which patients develop multiple polyps in the colon. In this condition, mutations affect one of the following mismatch repair genes namely *hMLH1, hMSH2* and *hMSH6*. The defect can develop through germ line mutations or methylation of promoter regions (Poynter *et al,* 2008). Approximately 80% of the patients with these mutations will eventually develop colorectal cancer (Fearnhead *et al,* 2002). They also stand a significant chance developing synchronous and metachronous lesions. Patients have fewer polyps than FAP cases and the cancer occur more in the right and transverse colon as opposed to the left colon (Li *et al,* 2009), which is the case in sporadic and FAP cases. HNPCC patients also tend to develop cancers in extracolonic organs such as endometrium, ovary, hepato-biliary tract, skin and kidneys and it accounts for up to 6% of CRC cases (Boyle *et al,* 2000). Due to the complexity of this disease, The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (Hamilton SR, 1992) has defined the definition for this syndrome as follows:

1. Three relatives in a family must have histologically proven CRCs and one of those should be a first degree relative of the other two.
2. Familial Adenomatous Polyposis (FAP) should have been excluded
3. Two successive generations should be affected
4. One of the patients with colorectal cancer (CRC) should be below 50 years of age at the time of diagnosis

1.2.2 Diet and environmental factors

Overall it has been suggested that only around 5% of patients with this cancer have a familial predisposition and the rest appear to be sporadic in nature (Fearon *et al,* 1990;

Li *et al,* 2009). There have been several studies worldwide looking into the causative factors for this disease. The major influential factors seem to be mostly related to diet and environment.

Moderate physical activity appears to be associated with low risk of CRC (Thune *et al,* 1996). Furthermore a population-based cohort study from Norway, showed that people with high physical activity through their jobs or recreation, were associated with low colorectal cancer risk (Thune *et al,* 1996). This effect was associated even after correcting for confounding factors such as BMI and diet.

Increasing BMI has also been associated with the increased risk of developing adenomas and carcinomas (Giovannucci E, 1996). Diets that are high in saturated fats were associated withincreased risk whereas a high fibre diet is said lower it. However, the argument on dietary fibre in the protection CRC is still confusing due to conflicting results from various studies. (Willett *et al,* 1987; Tunys *et al,* 1987; Kune *et al,* 1987). Heavy cigarette smokers have a 3 fold elevated risk of developing this disease (Giovannucci E, 2001). Hormonal replacement therapy was associated with reduced risk of colon cancer (MacLennan *et al,* 1995).

1.3 Classification of colorectal cancer

1.3.1 Stages of colorectal cancer

There are several classifications that are available. Among those the most widely used system is the Dukes' classification (A, B and C) described by Cuthbert Dukes in 1932 for rectal cancer. This system was further modified by Astler-Coller to include a fourth stage (stage D). It is classified as follows:

Dukes A: Tumour confined to the mucosa and sub mucosa but no further

Dukes B: B1: Tumour penetrates into, but not through the muscularis propria of the bowel wall

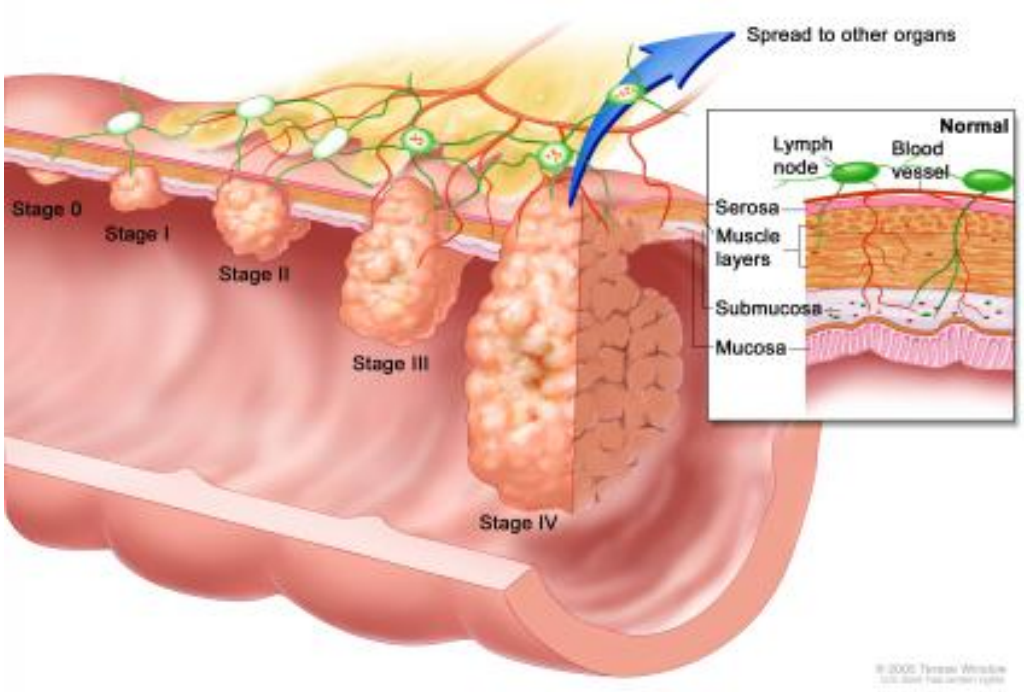
B2: Tumour penetrates into and through the muscularis layer

Dukes C1: Tumour penetrates into, but not through the serosa with the evidence of spread to the adjacent lymph nodes (close to colon).

Dukes C2: Tumour penetrates into and through the serosa with the evidence of spread to the regional lymph nodes

Dukes D: Tumour has spread beyond the confines of the lymph nodes namely organ metastasis.

Figure 1.2: Illustration of the stages of colon cancer (Source: www.cancer.umn.edu)



The modified Dukes staging is simple to use at the clinical setting. There are many other staging systems available to classify the colorectal cancer. One other system that is used by the pathologists internationally, is the TNM staging introduced by the American Joint Committee for Cancer (AJCC).

TNM staging as follows:

Tumour:

T1: Tumour invades sub mucosa

T2: Tumour invades muscularis propria

T3: Tumour invades through the muscularis propria into the serosa or into the pericolic or perirectal tissues

T4: Tumour directly invades other organs or structures, and/or perforates.

Node:

N0: no regional lymph node metastasis

N1: Metastasis in 1-3 regional lymph nodes

N2: Metastasis in 4 or more regional lymph nodes

Metastasis:

M0: No distant metastasis

M1: Distant metastasis present

The TNM staging gives a more detailed description of the level of involvement. For purposes of clinical usage, this has been arranged into four groups.

Stage I: T1N0M0; T2N0M0:

Cancer has begun to spread but still in the inner lining

Stage II: T3N0M0; T4N0M0:

Cancer has spread through the bowel wall and adjacent tissues but no nodal involvement

Stage III: any T, N1-2, M0:

Cancer has spread to the lymph nodes, but no distant organ metastasis

Stage IV: any T, any N, M1:

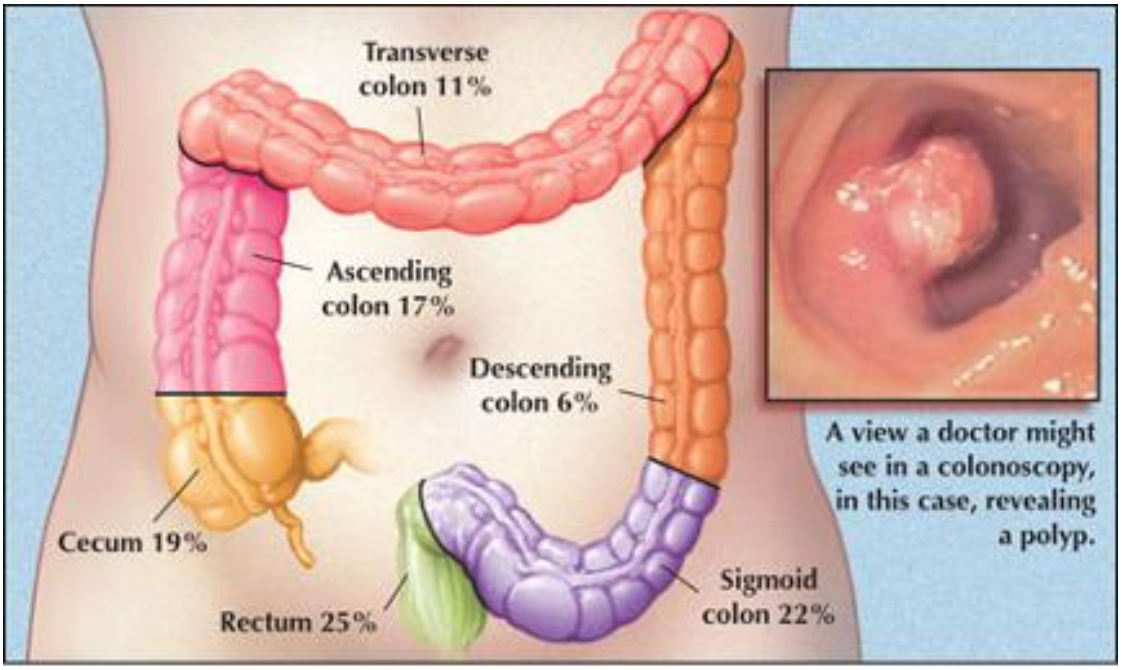
Distant organ involvement evident

The above system has been widely used in the clinical, pathological and radiological settings. This gives a more accurate picture of the cancer stage and helps clinicians to define appropriate treatment strategies.

1.3.2 Site distribution

The site of the tumour is also important as the tumour behaviour may vary depending on the site. Tumours that arise from caecum, ascending colon and up to two thirds of the transverse colon are commonly called ‘right-sided tumours. All other tumours that are distal to this point and up to the rectum are called ‘left-sided tumours’. Though this divide is an approximate one, in terms of clinical practice this conveys a lot of meaning. Right-sided tumours tend to be ulcerative, polypoid or fungating and the left-sided tumours are usually annular and constricting. The reason for these types of presentations is not clearly understood. Some argue that the tumour behaviour may be due to anatomical, embryological and physiological factors (Li *et al,* 2009).

Figure-1.3: Distribution of colorectal cancer according to the site. Source: www.sfchc.org



Cancer can occur anywhere in the colon, so it's important to have screening tests that examine the entire colon. Here, the percentages represent the incidence of colon cancer in the various parts of the colon.

1.4 Diagnosis

The clinical presentation of colorectal tumours can be insidious and the patient and sometimes the physicians easily dismiss some of the early symptoms. The symptoms can range from a non-specific vague abdominal pain to intestinal obstruction. The types of symptoms can vary according to the site and nature of the tumour itself. Left-sided tumours can present with alternating bowel habit, bowel obstruction, and/or bleeding per rectum. Right-sided tumours usually present with anaemia or a palpable mass. Weight loss can occur in these patients but it is less prominent in early stages of the disease and it is not related to the site of tumour. However these features are not always seen in every individual. Hence, a high index of suspicion is required to diagnose these tumours at their early stages.

The common pathway of investigating anyone with the lower gastrointestinal symptoms is a careful history and an initial clinical examination. The current guidelines for referral to colorectal outpatient are as follows:

1. Altered bowel habit for six weeks or more
2. Bleeding per rectum
3. Anaemia
4. Presence of abdominal mass

In the presence of one or more of the above symptoms, an urgent surgical referral is indicated. This is known as 'two week cancer waits' referral where patients must be seen within two weeks of the referral from the General practitioner (UK based criteria).

A clinical examination including digital examination of the rectum can palpate up to 75% of all the rectal tumours and detect 35% of large bowel tumours. Procto-sigmoidoscopy is an essential part of the examination.

Fibre-optic endoscopy (colonoscopy or flexible sigmoidoscopy) is the common mode of investigation performed to establish the diagnosis. Endoscopy has the advantage of direct visualisation and biopsy. Barium enema is another modality of investigating colonic pathology. However it is now mainly used to complement endoscopic test and may be used as a primary modality in patients with low index of suspicion of cancer. All the modalities of investigations have their failure rates. According to a recent survey (Tawn *et al,* 2005); a double contrast barium enema could miss up to 2.8% of the lesions. Another disadvantage of barium enema is lesions can often be misinterpreted and no histological information is possible.

Successfulness of endoscopic examination depends on the experience of the endoscopist. A colonoscopy is considered to be complete only when the operator has

reached caecum. Completion rates improve with experience (Harewood GC 2005). Completion rates over 90% are considered the minimum acceptable criteria for UK endoscopists (Ball JE 2004). This is also the recommendation from the Joint Advisory Group for endoscopy in this country (JAG committee). Newer technologies are growing rapidly. For instance, investigations such as MR Colonography are proving to be more accurate than conventional tests (Purkayastha *et al,* 2005).

Screening for colorectal cancers is now being introduced in United Kingdom. Following the results from two major pilot studies, the Department of Health has now rolled out the screening programme nationwide. People aged between 60 and 69 are invited to undergo faecal occult blood test (FOB). Those with the positive results would undergo colonoscopy. It has been estimated that 1.62/1000 of the tested population will have malignancy (NHS National Bowel Screening Programme 2006).

Once the diagnosis is established, the next step is to clinically stage the disease. This is accomplished by means of Computerised Tomography (CT scan) and where appropriate Magnetic Resonance imaging (MRI). CT scan of abdomen, pelvis and chest is nowadays used as a routine to stage colonic tumours. In the case of rectal tumours MRI is used to assess the extent of the tumour spread in deciding the appropriate preoperative management (Beets-Tan *et al,* 2005).

1.5 Treatment

Treatment modalities for colorectal cancer have improved over the years. This is largely due to specialisation in surgical techniques and technology. Due to specialisation, many centres are now doing high volume surgery and take on more complex procedures. It is believed that this has certainly improved the survival outcome in many cancers. This

statement is well supported by an audit conducted at Scotland between 1974-1979 and 1991-1995. (BMB 2002).

1.5.1 Surgery

For the early stage (Dukes A and B) cancers surgery generally remains the first line choice of management as this gives better survival outcomes (McArdle *et al,* 2005) . For advanced disease, surgery is still performed where feasible combined with adjuvant therapy. Surgery is also offered as a palliation to many-advanced disease cases in order to improve the quality of life (Cook *et al,* 2005). Surgical intervention is considered also in some patients with resectable liver or lung metastasis (Khatri *et al,* 2005). Many hospitals in this country offer surgery by laparoscopic method. It is a rapidly developing field and also recommended by National Institute for Health and Clinical of Excellence (TA105-NICE guidelines 2006).

1.5.2 Adjuvant therapy

Radiotherapy (DXT) and chemotherapy are used in both pre- and post operative periods. Preoperative radiotherapy aims to reduce the size of the tumour and makes it possible to achieve curative resection. Postoperative therapy is given to reduce recurrence of the tumour. The success of radiotherapy depends on the histological type of the tumour. It is known that squamous cell carcinomas especially in the ano-rectal region, respond well to radiotherapy whereas some adeno-carcinomas do not respond well. DXT is given pre-operatively along with chemotherapy in some rectal cancers. Studies have shown that preoperative chemo-radiotherapy improves outcome in a few defined group of patients (Hartley *et al,* 2005, Box *et al,* 2005).

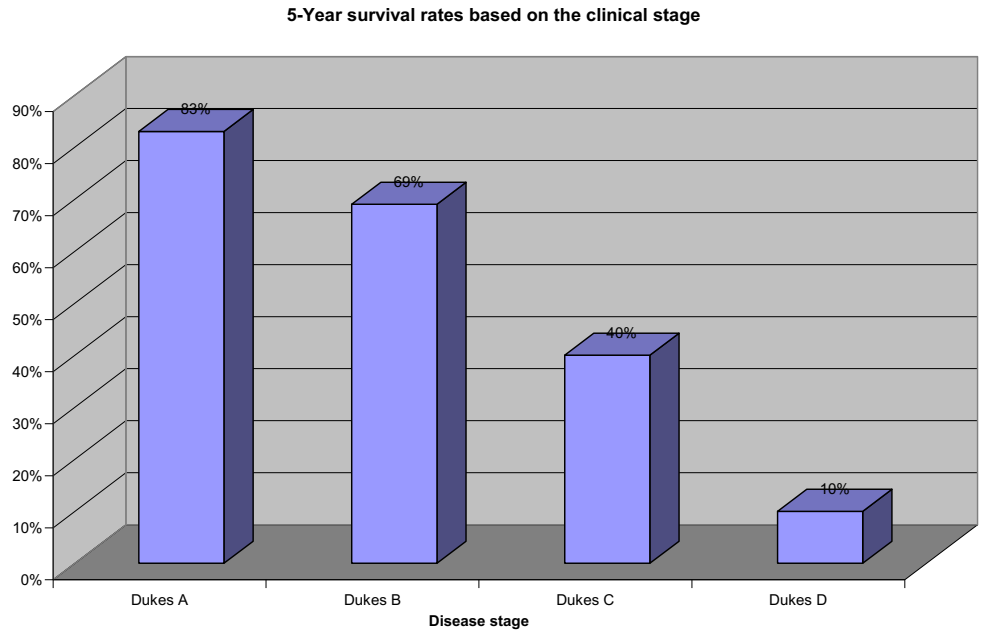
Considerable efforts are being made to improve adjuvant therapy in terms of modality and available types for CRC. Continuous research into this field to find new therapeutic

regimes is an on going process. There are numerous chemotherapeutic trials are being conducted in the UK and at international level in order to identify the effects of combination chemotherapy. For example, newer chemotherapeutic combinations (e.g. 5FU, Lucoverin, Cepecitabine, Oxaliplatin, Irinotecan) have shown some improvement in the disease-free survival including patients with metastatic disease (Bathe *et al,* 2004). With the help of newer chemotherapeutic agents, it is now possible to operate on certain patients with advanced stage CRC. The percentage of disease free survival are improving with the introduction of biological agents (e.g. Bevacizumab, Cetuximab etc.) along with other chemotherapy drugs though the rates of survival are only marginally improved (Van Custem E, 2007).

1.6 Survival in Colorectal Cancer

Five year survival rates for colorectal cancer according to the Dukes stage are as follows: Dukes A 83%, Dukes B 69%, Dukes C 40% and for Dukes D it's less than 10%. Current five year survival rates for colon cancer in England for men and women are 49.7% and 51.1% respectively. The survival rates have improved by 1.2% in comparison to 2004 statistics. (Office for National Statistics, UK, 2007).

Figure-1.4: Over all five year survival rates for colorectal cancer according to clinical stage (Illustration by author).



With the introduction of new radio- and chemotherapy techniques, it is possible to downgrade locally advanced tumours and render them surgically resectable tumours. This is particularly valuable for rectal tumours. Though this is only a small improvement, the implications of these results are invaluable and certainly will pave way for more effective therapeutic regimes in the future.

1.7 Genetics of Colorectal Cancer

Tumour development involves two major genetic pathways. These are proto-oncogenes and tumour suppressor genes. Proto-oncogenes encode proteins that are required for normal cell cycle control. They become oncogenes by mutation or increased expression. A mutation involving proto oncogene would result in a change in the structure and consequently an increased protein activity. There would be also loss of regulation. As a consequence, there is increased protein availability for prolonged periods leading to abnormal cell cycle regulation. In their constitutively active form, oncogenes stimulate

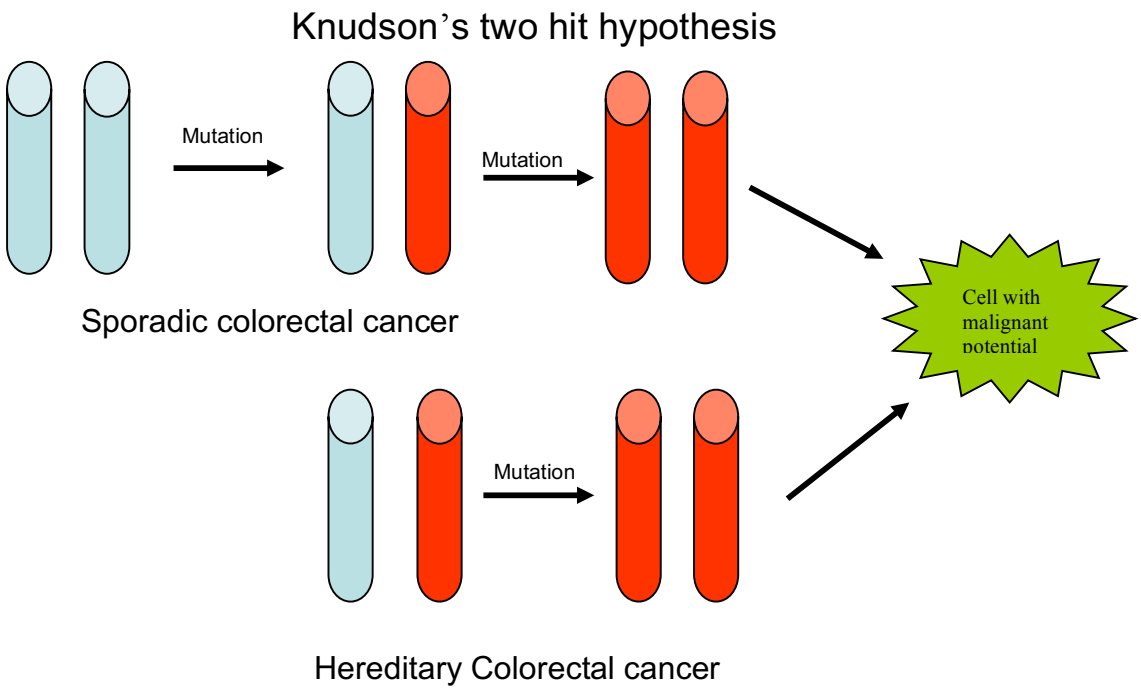
various genes that are involved in cell cycle progression. They do this by several mechanisms namely (Carlo *et al* 2008) gene amplification, gene duplication, mutations and insertions or deletions. Oncogenes exert their influence by a dominant effect i.e. only one allele is required for the transformation. Examples of proto oncogenes include RAS, MYC, ERK and WNT. *RAS* gene has been investigated extensively by several research groups. There are few different genes identified within this *RAS* gene family (e.g. *K-ras, N-ras* etc.). Frequent K-*ras* gene mutations have been observed (codon 12 and 13) in colorectal cancers. In some studies (Boss *et al,* 1987) involving colorectal tumours, *ras* gene mutations were found in approximately 50% of the cases and a similar amount of mutations were found in large adenomas (Farr, 1988). Downstream effectors of Ras include Raf, MEK, MEKK, MAPK, ERK, most of which in turn regulate genes that mediate cell proliferation.

Somatic activation of these genes can be achieved by many different mechanisms. The most frequent one is point mutation. Other pathways are gene rearrangements and gene amplifications whereby activation of oncogenes is achieved (Fearon ER *et al,* 1990). The presence of an amplified oncogene has been reported in epithelial cancers including colorectal cancer. Some of the examples include c-myc, c-myb, Cyclin D1 etc (Finlay *et al,* 1989).

Tumour suppressor genes also exert their influence on the cell cycle by an inhibitory effect. Transformation and subsequent cell proliferation are due to the loss of these suppressor gene functions by several mechanisms. Examples of tumour suppressor genes include Retinoblastoma protein, P53, PTEN and and APC. This is list not exhaustive. Homozygous loss of p53 is found in about two thirds of colorectal cancer s and about half of lung cancers (Sherr CJ 2004). Tumour suppressor genes follow the

'two hit hypothesis'. According to Knudson's two hit hypothesis (Knudson, 1985), tumour suppressor gene loss of function of the both alleles are required thus making it a recessive event. Inactivation of tumour suppressor genes often occurs as a result of mutation of one allele and loss of the remaining normal allele.

Figure-1.5: Knudson's two hit hypothesis (Author's version)



Allelic losses in chromosome 5q had been observed in about 20-50% of the colorectal carcinomas and in 30% of colorectal adenomas (Fearnhead *et al,* 2002). The loss of a large area of chromosome 17p has been seen in about 75% colorectal carcinomas (Vogelstein *et al,* 1988). This chromosomal area contains the *p53* gene which encodes an inhibitory protein controlling the transcription of several genes involved in cell cycle regulation and indirectly influencing Cyclin D1 (Baker *et al,* 1989). Another region of common allelic loss in colorectal tumours is at chromosome 18q. This has been noted in more than 70% of colorectal cancers and in more than 50% of colonic adenomas (Vogelstein *et al,* 1988). Apart from these there are several other chromosomal losses

also have been reported as well. The extent of the chromosomal losses can range from 1q to 22q. It has been estimated that median chromosomal loss ranges from 4 -5 per tumour (Vogelstein *et al,* 1988, Kern *et al,* 1989)

In sporadic tumours, according to the recessive hypothesis, two genetic events are needed to execute a phenotypic change. This can be through any of the mechanisms as discussed above. In the case of familial syndromes one copy of the tumour suppressor gene was inactivated which is unique for each disorder. However this rule may not affect all the suppressor genes. In some genes one mutation is enough to produce a phenotypic effect in the cell. It is also known that the level of expression of the suppressor gene may also play an important role in tumourigenesis (Fearon ER *et al,* 1990). These chromosomal changes can also occur at multiple levels (i.e. multiple allelic losses). Overall the exact mechanisms of carcinogenesis are the end result of many complex and multi-step processes.

1.8 General Overview of Colorectal Cancer Pathways

Colorectal cancer pathway remains complex and still needs to be further evaluated. The earlier sections described various proteins and genes and their functions individually. However these genetic changes must induce certain phenotypic changes in the cell in order to create malignant transformation. One such change would be impairment of normal DNA repair mechanisms within the cell leading to a state of genetic instability. Other phenotypes include ability to metastasise, to induce angiogenesis, ability to evade apoptosis and resistance to growth inhibition (Hahn and Weinberg, 2002). Among several hypothesised pathways, two are recognised in colorectal cancer namely chromosomal (CIN) and micro-satellite instability (MSI) pathways.

1.8.1 Chromosomal instability (CIN)

In this pathway there is loss or gain of chromosomes, rearrangements, and a loss of heterozygosity. These changes occur at a much faster rate than the normal cells. It has been said that approximately 60 – 80% of colorectal cancers show CIN changes (Tong R *et al,* 2004). Adenomatous polyposis coli (APC) gene is an example for this pathway. Truncal mutation in APC gene is one of the early genetic events in carcinoma development (Macleod K, 2000). This prevents the metabolic degradation of cytoplasmic β-catenin via the ubiquitin pathway. Thus the stabilised β-catenin migrates into the nucleus coupled with the activated transcriptional factors (Tcf/Lef1) leading to eventual activation of the target genes (Willett *et al,* 1998). Though the end result is the same as Wnt signal pathway, however there is one exception. The cells that go through Wnt pathway are prone to apoptosis whereas APC mutation makes the cells resistant to apoptotic process (Macleod K, 2000). It is believed that APC mutation seems to influence the apoptotic genes through regulatory genes in a way that reduces the apoptosis and promotes mitosis. E.g. Activation of Survivin, a member of IAP (inhibitor of apoptosis) family by Tcf/Lef1 factors.

1.8.2 Microsatellite instability (MSI)

Microsatellite instability is another mechanism where there is defective repair of mismatches that occur in the DNA. Around 10 - 15% of sporadic colorectal cancers are believed to arise this way. It also is a pathway in people with HNPCC. Mutations occurring in mismatch repair genes such as *hMLH1, hMSH2, hMSH6* results in frame shift mutations in many cancer associated genes (Macleod K, 2000). Mismatch repair genes lose expression through silencing of their promoter by methylation of *hMLH1* mainly. These promoters have many areas of CpG islands where an aberrant methylation occurs which is driven by an enzyme called DNMT1. Many genes are

silenced this way including *hMLH1, p14* and O-6-methylglutamine-DNA methyltransferase (*MGMT* - a DNA repair enzyme). Tumours that arise through MSI pathway commonly occur in the right colon (Watson AJ, 2004). In a recent study (Scholtka *et al,* 2009), MSI was shown to occur in most sporadic colonic cancers in association with mutations involving *APC, KRAS, and β catenin*.

1.8.3 Apoptosis

Apoptosis is a form of cell death that appears to be an energy dependent and programmed event. (Kastan MB, 1997). Apoptosis induces a series of cellular changes namely nuclear condensation, fragmentation, cell shrinkage and relative sparing of cell membrane and internal organelles. This occurs in response to a variety of stimuli such as irradiation, chemotherapy, viral infection, withdrawal of certain types of hormones etc. Some types of food constituents that are implicated epidemiologically in colorectal cancer prevention have been shown to enhance apoptosis following DNA damage. Examples include butyrate, flavonoids and glucosinate breakdown products from brassicas (Watson AJ, 2004).

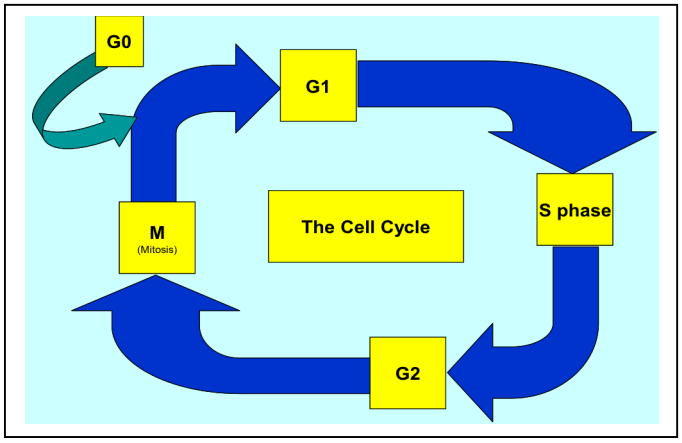
A number of tumour suppressor genes have been shown to induce apoptosis apart from the *p53* gene. *PTEN, APC* and *PML* are some of the other tumour suppressor genes that are capable of inducing the apoptotic process (Macleod K, 2000). These genes induce apoptosis through pro-apoptotic regulators such as *BAX, Apaf-1* and *caspase-9*. In contrast apoptosis is antagonised by *Bcl-2* and *Bcl-w*. (Merritt *et al,* 1994 and Pritchard *et al,* 1999). Under normal circumstances the epithelial cells have the tendency to undergo apoptosis following DNA damage. This process is said to occur when there are conflicting regulatory signals that are active in the cell or when there is a blockage of the extra cellular survival signals (Kastan MB, 1997). Mutations involving these

genes particularly *p53* could affect the inhibitory signals governing downstream genes namely *CCND1* gene and thereby the cell proliferation. Mutations of *APC* gene could affect the apoptotic process through β-catenin/Tcf and phosphorylation of Cyclin D1 by GSK3-beta involved in protein degradation.

1.9 Cell Cycle Regulation

The molecular events that happen during the cell division are complex. Under normal circumstances these events are well coordinated and are executed in an orderly fashion.

Figure-1.6: Different stages of the cell cycle pathway (Author's version)



For the purposes of understanding, the different phases of cell cycle have been divided into specific areas. Each step has important functions and well controlled by many growth stimulating and inhibitory factors. Once activated, the cascade of events generally progressed on to the next phase of the cell cycle. Hence, this area caught the attention of the scientists worldwide. Considerable amount of research has been done in this particular subject to understand the pathways and their control. Though we have some understanding, the whole mechanistic path is not yet fully understood.

1.9.1 G0 phase

After the cell division, the parent cells remain in this phase until a signal from growth stimulating factors. Once stimulated, this initiates a chain of events which leads to the progression of the cell cycle from G0 to the next phase (Sherr CJ *et al,* 1994, Roberts JM, 2004).

1.9.2 G1 to S phase transition

Cellular events that occur in this phase of the cell cycle, prepares the cell for DNA synthesis. We have better understanding about the controlling features of this phase of the cell cycle. In the middle of G1-S transition, lies the restriction point (R). This was originally defined as the discreet time point in mid to late G1 phase at which cells commit to entering S phase (Sherr CJ, 1994 and 2000). This 'R' point divides the G1-S phase into two components namely pre and post 'R' stage. Mitogenic signals are required in the pre R stage in order to initiate the preparation for the DNA synthesis (Assoian RK, 2008). Once this is initiated, the growth factors are no longer required to complete the transition. The cell initiates the DNA synthesis approximately 1 to 3 hours after passing the 'restriction point'. The key factors that are involved in the process will be discussed in detail in the following paragraphs.

1.9.3 DNA synthesis

The events that control this part of the cell cycle have yet to be fully evaluated. The cell starts copying the entire DNA. During this process, a staggering 3 billion bases of human genome are made within a few hours. The DNA polymerases help to make the DNA copies. However there are imperfections within the sequence. It is estimated that one incorrect nucleotide is incorporated for every 10^5 to 10^6 nucleotides. This would in theory produce 1000 to 10000 mutations in each cell. These DNA replication programs

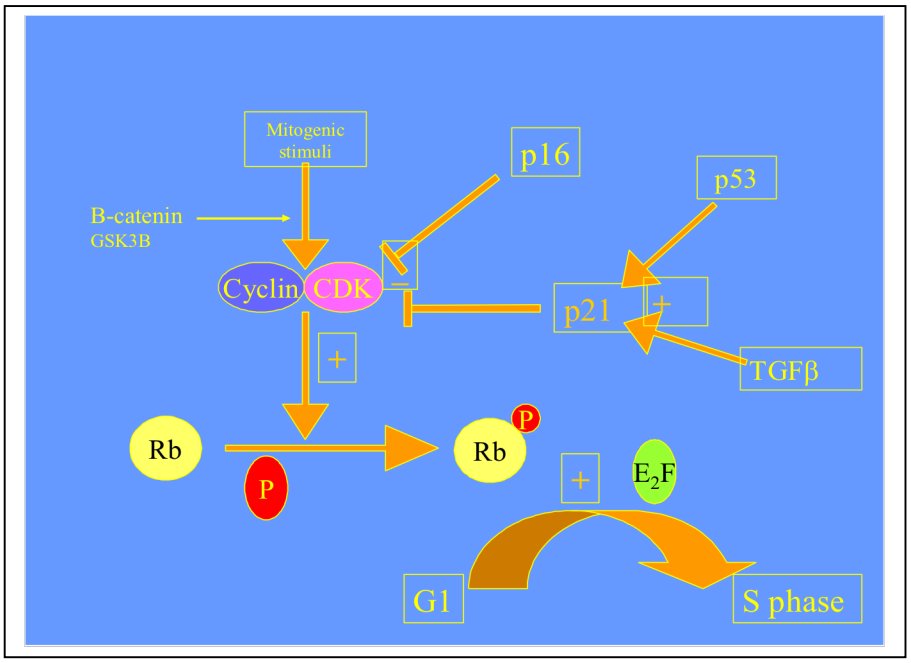
have incorporated ‘check’ mechanisms in order to reduce the error rates (Fearnhead *et al,* 2002). There are also DNA repair processes that happens within the cell to repair the DNA errors. It is believed that any deficiencies that compromises these repair mechanisms can eventually lead to cancer development.

G2 is the second gap phase during which the cell prepares for the process of division. During the M stage mitosis occurs. In this stage the newly replicated chromosomes segregate into nuclei (Johnson DG, 1999). This is followed by cytokinesis to form two daughter cells. This part of the cell cycle is not discussed in detail since my research work mainly involves G1 to S phase of the cycle.

1.10 Mechanisms Implicated in G1 to S phase Regulation

The stages of the cell division are very closely controlled and executed processes. Successful cell division is dependent on various groups of proteins that are collectively known as the cell cycle regulators. The functions of these proteins differ. Some of the proteins stimulate cell division while others exert an inhibitory effect. A protein that has a stimulatory effect on the cell cycle requires an external source of appropriate growth stimulus. These regulators also are normally kept under control by arrays of substances called ‘inhibitory proteins’. In the following pages each of these important cell cycle regulators are described in some detai

Figure-1.7: Illustration of the regulators that are involved in G1 to S phase of the cell cycle (Authors version).



1.10.1 Cyclins

Among the various group of proteins, cyclins are one of the most important groups of proteins. Vast amount of research has been done on cyclins over the years. Though we understand some of many of their roles in the cell division, the complete patho-physiological pathway is yet to be fully evaluated.

1.10.1 Cyclins

Cyclins are a group of proteins that share the homology in a ~ 100 amino acid region called the 'cyclin box'. This is a domain that binds and activates CDKs. At least 15 different types of cyclins have been identified (A, B1, B2, D1, D2, D3, E, F, G1, G2, H, I, K, T1 and T2). However, not all the cyclins are involved in regulating the cell cycle. Some types of cyclins take part in functions such as regulation of transcription, DNA repair, apoptosis etc (Johnson DG, 1999). For instance, cyclin C, T and H along with their appropriate CDK partners, participate in basal transcriptional activity.

1.10.2 D type cyclins

Among many cyclins, the 'D' type cyclins play an important role in G1-S phase progression. They are the first type to be induced as the cells are stimulated from G0 phase to enter the cycle (Sherr CJ, 1994). D type cyclins combine and activate CDK4 and CDK6. The primary target for D type cyclin kinases is the retinoblastoma protein (Rb). The combined CCND1 and CDK complex then phosphorylates the Rb protein. It acts through the 'restriction point'. However, we do not have the exact location of this point. Once initiated, a cascade of events that occur, trigger S phase proteins (Sherr CJ, 1994).

1.10.3 Other cyclins

Cyclin E is induced after the release of the S phase proteins. Cyclin E combines with CDK2 and this active complex is required for transition from G1 to S phase. This complex helps to maintain Rb protein in a hyperphosphorylated state (Holland TA *et al,* 2001).

Cyclin A is part regulated by E2F family of proteins and accumulates at G1-S phase transition and persists through S phase. In the early stages cyclin A associates with CDK2 and in the late stage of the S phase, it associates with CDK1. Cyclin A also accumulates in areas of DNA replication suggesting that it may play a part in DNA synthesis or may be preventing excess DNA replication (Johnson DG, 1999).

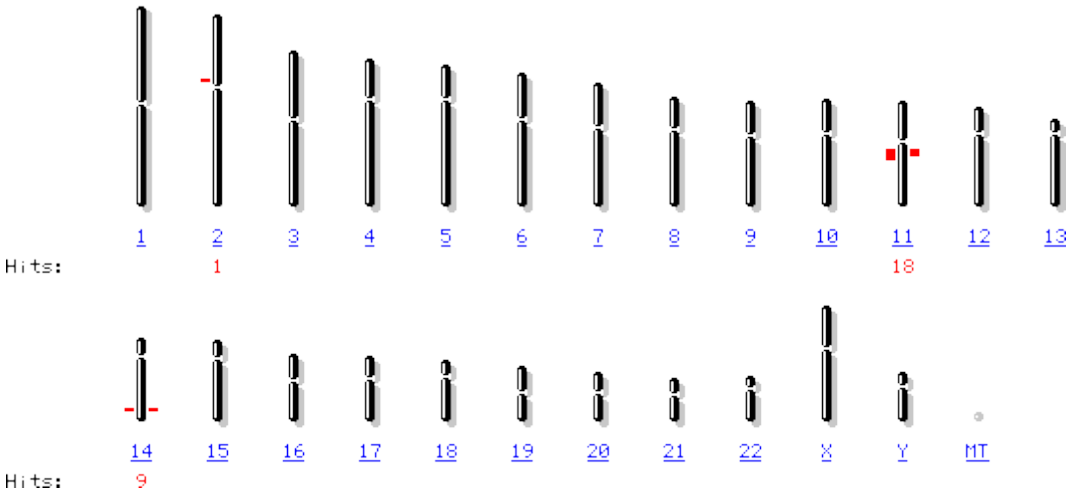
The activity of cyclins is not just confined to G1/S phase alone. They continue to exert their effect in G2 and M phases of the cell cycle as well. Mitosis is regulated by cyclins A, B1 and B2. These cyclins along with CDK1 phosphorylate proteins such as laminutes, histone H1 etc. These proteins are possible components of mitotic spindle (Arellano M *et al,* 1997).

1.10.4 Cyclin D1

The Cyclin D1 gene is located on chromosome 11q13 and it is otherwise known as *CCND1*. There are also other names such as '*PRAD-1*' as in parathyroid tumours and *BCL-1* in B-cell malignancies (Bartkova J *et al,* 1994). This gene has five exons and an UTR region and this has become a subject of interest among some scientists during the recent years. Genetic alterations of *CCND1* gene have been studied both *in vivo* and *in vitro*. Among those, polymorphisms, gene rearrangements and gene amplifications have gained importance. Betticher *et al* 1995, showed that a polymorphism (A870G) in exon 4 could produce two different transcripts (Tra and Trb) through splicing. It is argued that one of these transcripts affects the degradation of Cyclin D1 thereby

increasing its availability in the cell. *CCND1* gene activation or over-expression has been described in many epithelial cancers including colon (Holland T *et al,* 2001, Palmqvist *et al,* 1998, Maeda *et al,* 1998, McKay *et al,* 2000), head and neck (Jares *et al,* 1994), lung (Betticher *et al,* 1996) and endometrial cancer (Ashton KA *et al,* 2008).

Figure-1.8: Human Genome view and the position of Cyclin D1 gene at chromosome 11. (Reprinted from NCBI genome bank-see acknowledgement)



1.10.5 Cyclin-dependent kinases (CDK)

The cyclin box binds members of protein kinases that have the defining property of requiring a cyclin partner for their activation. Hence they are called cyclin-dependent kinases (CDKs). It is believed that the cyclin binding has two purposes. First it re-orientates bound ATP in such a way so that the γ-β phosphate bond becomes susceptible nucleophilic attack from substrate hydroxyl group. Secondly, it helps to displace a region of the CDK, the 'T' loop, which partly blocks the substrate-binding site (Pines J, 1995).

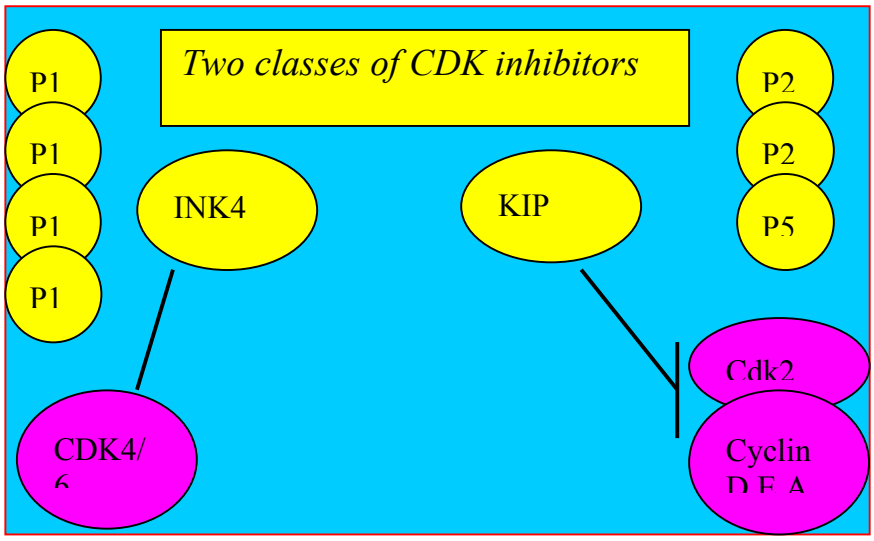
There are several CDKs that have been identified (CDK1 to CDK 9). Binding between various cyclin and CDK groups differ. It is argued that binding depends on substrate specificity. This is important in defining specific roles for each of the cyclin-cdk

complexes (Pines J, 1995 and Sherr CJ, 2000). Cyclins can directly bind the substrate or localise the CDKs to a sub cellular area where the substrate can be found. Some cyclins bind to a variety of CDKs and vice versa. Among those CDk2, CDK4, CDK5 and CDK6 are important since they bind to D type cyclins. Of these CDK4 is important because it plays an important role in G1/S phase of the cycle regulation. CDK4 is under down regulation by a group of CDK inhibitors e.g. INK4 family of proteins under normal circumstances (Alt JR *et al,* 2002). The roles of CDK inhibitors are discussed below.

1.10.6 Cyclin Dependent Kinase (CDK) Inhibitors

The actions of cyclins and CDKs are controlled by two groups of proteins namely Cip/Kip family and INK4 family. The former group act on cyclin/cdk complexes and the latter specifically interact on CDK4 and CDK6 but not on other CDKs.

Figure-1.9: Diagrammatic illustration of two major groups of CDK inhibitors (Author's version).



1.10.5a Cip/Kip family

The first and perhaps one of the most important proteins to be isolated was *p21*$^{Cip1/WAF1/SD11/CAP20/mda-6.}$ The number of aliases indicates the different methods and different groups that were used to identify *p21* (Palmero I 1996). This gene is

frequently over-expressed in colorectal cancers (Holland TA *et al,* 2001). The *p21* promoter gene has a p53-binding site, which allows transcriptional activation of this gene. Once activated it performs the following functions: 1. Inhibition of various cyclin/cdk complexes 2. Inhibition of DNA synthesis through PCNA binding (LaBaer J *et al,* 1997). Cells that lack p21 lose their ability to arrest at G1 phase which indicates the importance of this gene's role in the DNA repair process. The other members include $p27^{KIP}$ and $p57^{KIP2}$ that also exert their action on cyclin/cdk complexes (LaBaer J *et al,* 1997).

1.10.5b INK4 family

This consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These proteins specifically interact with CDK4 and 6 and prevent the binding with the D-type cyclins. INK4 proteins can also inhibit the activity of *CCND1/CDK4* and *CCND1/CDK6* pre-formed complexes. In many tumours *p16* appears to be inactivated by mechanisms such as deletion (Hall and Peters, 1996), point mutation and hypermethylation (Palmero I, 1996). Thus CDK inhibitors have an important role in the control of cell proliferation. This inhibitory control, we believe, is what keeps the CDK under negative regulation and thereby cell cycle progression (Sherr CJ, 1999).

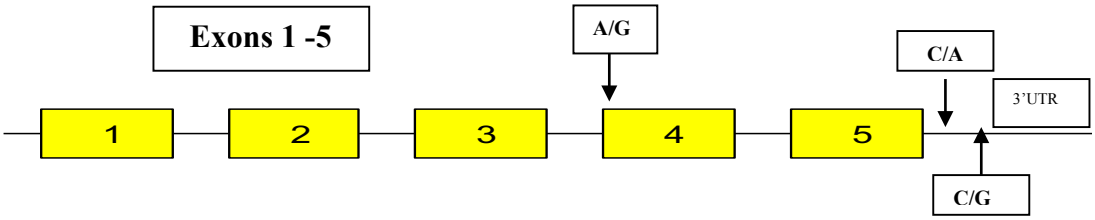
1.11 Regulation of Cyclin D1

1.11.1 CCND1 Gene Polymorphisms

CCND1 gene is frequently polymorphic and is known to be associated with the outcome in many epithelial cancers. The A-G polymorphism at nucleotide 870 position (g.A870G) has been investigated widely including colorectal cancer (McKay J, 1998, and Porter *et al,* 2002). It was the 'A' allele that was associated with poor outcome such as high grade tumour, reduced disease-free survival, etc. Over representation of the 'A'

allele was seen in familial non-HNPCC patients and sporadic colorectal cancer (Porter *et al,* 2002). In non-small cell lung cancer and squamous cell carcinoma of head and neck, the genotype was associated with survival rates. The 'G'

Figure-1.10: Illustration showing Cyclin D1 gene and the polymorphic sites namely A/G^{870} at exon-4 and C/A^{1100} and C/G^{1722} in 3'UTR region (Author's version).



allele was associated with better prognosis in patients with non small cell lung cancer (Betticher *et al,* 1995). The same genotype was associated with reduced disease free interval in pharyngeal and laryngeal cancers (Matthias *et al,* 1998). 'A' allele was also shown to be associated with prostate (Wang *et al,* 2003), bladder cancer (Wang *et al,* 2002) and endometrial cancer (Ashton *et al,* 2008). The other polymorphisms in the 3'UTR region have not been studied. Our group have published the effect of C/G at 1722 (g.C1722G) in head and neck cancers in our centre (Holley SL, 2001).

The significance of the presence of polymorphism(s) within a gene is yet to be fully evaluated. In the A/G polymorphism, substitution at nucleotide 870 in the exon 4 region leads to splicing that which gives two transcripts namely, *transcript a* and *transcript b*.(Betticher *et al,* 1995). The 'A' allele is associated with transcript 'b' which reads into intron 4, whereas G allele is associated with both transcript 'a' (exon 5 splicing) and 'b' (Betticher *et al,* 1995). Both these transcripts encode a protein that has the amino acids 55-161, which is believed to play an important role in Cyclin D1 function. Many studies have shown functional differences between the proteins (Holley *et al*, 2001). As

a result of alternate splicing transcript 'b' encodes a shorter 43 amino acid sequence (reading into intron 4 and skipping exon 5) at its carboxy-terminus instead of 55 amino acids. It is believed that this amino acid change at the carboxy-terminus could lead to a longer half-life of Cyclin D1 protein (Betticher *et al,* 1995). Such a prolonged presence of Cyclin D1 could potentially lead to G1/S phase progression thereby causing cell proliferation in cancers. There are studies that examined the association between A/G^{870} polymorphism and Cyclin D1 over-expression in many epithelial cancers (e.g. McKay J *et al,* 1998 in colorectal cancer). These have failed to show a definitive link between the polymorphic change and protein over-expression.

There are other polymorphisms within *CCND1* gene particularly in the 3'UTR region. In our centre, we examined two polymorphisms in the 3'UTR region. One such polymorphism was found at nucleotide 1722 (C/G) region, but the effects and influence have not been widely investigated. The other polymorphism was identified at nucleotide 1100 (C/A). This was identified by one of our fellow research colleague Dr Holley SL. Then our group studied its influence on the clinical outcome in colorectal cancer patients using the blood DNA. There is ongoing work within our centre examining the 3-UTR region to look for further polymorphic sites.

1.11.2 Cyclin D1 protein over expression

Cyclin D1 over expression has been studied in several epithelial cancers Dhar *et al*, 1999, Holland *et al*, 2001 Nakamura *et al*, 1994, Betticher *et al*, 1996 and Gillett *et al*, 1994). In many studies, Cyclin D1 expression within the nucleus has been examined in some detail. But cytoplasmic localisation has also been noted in some studies (Nakamura *et al*, 1994). There are several mechanisms that are implicated in the accumulation of Cyclin D1 protein in the nucleus. These include gene rearrangement

through amplification (Gillett *et al*, 1994) or through mutations involving the upstream or the down stream regulators of Cyclin D1.

Interestingly, the clinical outcomes of Cyclin D1 protein expression are very variable. For instance, Dhar *et al* 1999) showed that there no impact on survival in ovarian cancers. On the other hand, Holland *et al* (2001) showed that there are survival advantages in sporadic colorectal cancers when this protein was over expressed in the nucleus. In breast cancers, Cyclin D1 over expression was associated with good prognosis (Betticher *et al*, 1996).

These studies show that the end point was predominant nuclear or cytoplasmic localisation of Cyclin D1 affecting clinical outcome in some cancers. In some types of epithelial cancers there were no impacts on survival. Most of these studies mainly examined the possible mechanisms leading to over expression of Cyclin D1 protein. However, the exact mechanistic pathways and the effects of variable outcome with Cyclin D1 over expression depending on the site of expression (i.e nuclear or cytoplasmic) have not been fully elucidated.

1.11.3 *CCND1* Gene Phosphorylation

Cyclin D1 protein normally has half-life of 20-25 minutes. It undergoes phosphorylation at specific *CCND1* gene phosphorylation sites and subsequently degraded by the ubiquitin-proteosomal pathway. This system exists in the cell cytoplasm. This process involves a series of reactions in which ubiquitin is activated by an activating enzyme (E1) and then transferred to substrates by ubiquitin-conjugating enzyme (E2, UBC) or indirectly activated by ubiquitin ligases. These poly-ubiquitinated substrates are then degraded by 26S proteosome. The process of

polyubiquitinisation can be affected by mutational changes occurring in the phosphorylation sites at least *in vitro.*

There are several phosphorylation sites identified within Cyclin D1. They are usually between codon 156 to codon 299. In vitro studies have demonstrated that the substitution of alanine in place of the threonine residue at sites 286 and 156 have resulted in inhibition of Cyclin D1 phosphorylation (Diehl *et al,* 1997). This effect has not been studied in vivo and especially in colorectal cancer. In theory, if the phosphorylation is blocked it will lead to the accumulation Cyclin D1 protein in the cell. Substitution of alanine in place of threonine at codon 288 also has shown to reduce the polyubiquitinisation of Cyclin D1 (Zou *et al,* 2004).

1.11.4 Cyclin D1 Gene Amplification

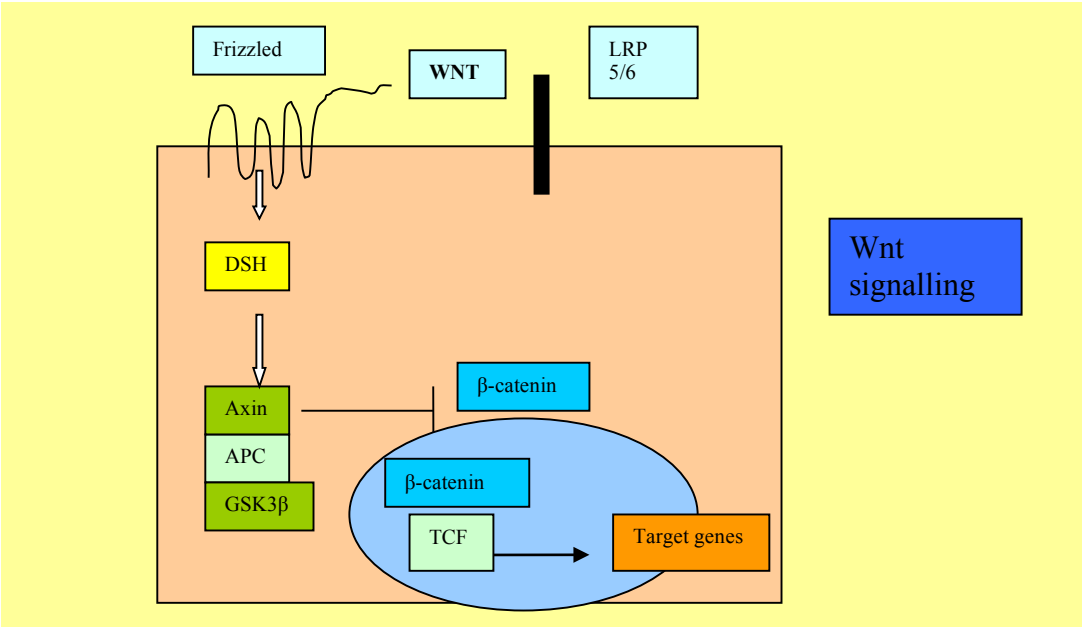
Chromosomal loss, rearrangements and amplification have been associated with many types of cancer. Rearrangement of Cyclin D1 gene locus resulting protein over-expression has been shown in malignancies (Dhar KK *et al,* 1999). Gene amplification at chromosome 11q13 has been studied and found in oesophageal (50%), breast (15%), bladder (21%) and lung cancers (13%) (Ormandy CJ *et al,* 2003). Amplification of this chromosome contributes to several genes including *CCND1* (Huang *et al,* 2002). Other genes include *FGF1, FGF4 and EMSI. CCND1* amplification through this pathway is another means where Cyclin D1 could accumulate within the cell. The frequency of occurrence the *CCND1* gene amplification in human malignancies seems variable. It's relatively frequent in oesophageal (Jiang *et al,* 1992) and breast cancers (Buckley *et al,* 1993, Ormandy *et al,* 2003) but is uncommon in ovarian (Dhar *et al,* 1999) and metastatic thyroid cancers (Khoo *et al,* 2002).

1.12 Other Important Genes Involved in the Cell Cycle Regulation

1.12.1 β-catenin (CTNNB1)

β-catenin (CTNNB1) is an important multi-functional protein in cell adhesion and signal transduction. It plays a pivotal role in cell adhesion by linking the cytoplasmic domain of cadherins to α-catenin which anchors the adhesion complex to the cytoskeleton (Tetsu *et al,* 1999).The cadherin-catenin complex is a target for the regulatory signals that control the cell adhesion and motility (Willert *et al,* 1998). β-catenin gene comprises of 16 exons. Exon-3 of this gene is frequently implicated in mutation and has been studied in several epithelial cancers. Exon 3 of β-catenin gene has the phosphorylation sites required for the protein degradation. These sites are frequently found to be mutated in epithelial cancers. The proteosomal pathway then degrades the phosphorylated protein through polyubiquitinisation (Palacios 1998, El-Bahrawy *et al,* 2002, Sparks *et al,* 1998). Wnt signal pathway has been shown in the figure below.

Figure 1.11 Wnt signalling pathway(Author's version)



Upon Wnt binding, axin, a scaffold protein translocates to the membrane where it interacts with LRP (Lipoprotein related protein). It has also been suggested that abnormalities of Wnt-pathway leading to defective metabolism of the Frizzled receptor proteins can also impair the metabolism of β-catenin. Destabilisation of axin or LRPs due to mutations leads to impaired cytoplasmic degradation of β-catenin and thereby accumulation in the cell. As a signalling molecule, β-catenin can activate gene transcription by forming a complex called the T-cell factor/lymphoid-enhancing factor (Tcf/Lef), a family of DNA binding proteins. This complex, upon entering the nucleus acts as an oncogene and induces transcription of *c-myc* and *CCND1* gene. In the absence of growth signals cytoplasmic β-catenin is phosphorylated by GSK3-β, a serine-threonine kinase and subsequently degraded by the ubiquitin-proteosomal pathway. Mutations within the GSK3-β can lead to impaired phosphorylation and the β-catenin metabolism.

The APC gene acts as scaffolding during the process of β-catenin degradation. Mutations occurring in the APC gene impair the β-catenin phosphorylation at the site of exon-3 of β-catenin. This leads to β-catenin accumulation within the cytoplasm. GSK3-β is needed can all lead to loss of phosphorylation of this protein and its subsequent accumulation in the cell (Tetsu *et al,* 1999).

The involvement of β-catenin as an oncogene has been investigated in many epithelial tumours (Fujimoto *et al,* 1997, El-Bahrawy *et al,* 2002, Joo *et al,* 2003). In colorectal cancer, it is has been postulated that β-catenin up-regulation is an early event. There are studies that demonstrate the relationship between mutations occurring in exon-3 of β-catenin and the intracellular accumulation of β-catenin. However, the effects of sub-

cellular localisation of β-catenin on genetic susceptibility and clinical outcome remains to be further evaluated.

1.12.2 Adenomatous Polyposis Coli (APC) gene

This is an important gene that is involved in the β-catenin metabolism. APC is a large gene and has a '15 aa repeat' region. This region acts as scaffolding for the β-catenin gene during complex forming process with other molecules such as axin. The so called 'mutation cluster region' (MCR) is where more than 70% of the mutations of APC occur. Mutation involving this region of the APC gene leads to impairment of β-catenin degradation and thereby it's cellular accumulation. β-catenin is then transported to nucleus by transcriptional factors (Tcf/Lef) and acts on the target genes such as *c-myc* and *CCND1* gene. It should also be noted that the APC pathway renders the cell resistant to apoptosis through complex pathway. (Kohler EM *et al,* 2008).

There are number of pathways by which the cell cycle can be affected. Cyclin D1 is considered to be the key regulatory gene involved in the G1 to S phase progression. There are several studies in many epithelial cancers conducted widely across the world in order to understand the pathways involved in the regulation of Cyclin D1 gene. Most studies examine the associations or mechanistic pathways that relate to Cyclin D1 in isolation. For instance there are several studies that investigated the polymorphisms within Cyclin D1, but there are fewer studies which attempt to investigate the effects of polymorphism in relation to the Cyclin D1 protein expression. Hence we still do not have a precise understanding of the Cyclin D1 related pathways. Our project aims to examine the major pathways that are involved Cyclin D1 regulation and tries to present a clearer understanding of the underlying mechanisms.

1.12.3 *RAS* gene

The *ras* family of genes serve as key effectors of growth factor-induced differentiation, proliferation, death, shape, and motility. These genes encode for GTPases that act as molecular switches. Ras activity is regulated by factors such as guanine nucleotide exchange factor (GEFs) which in turn affects many molecules including *Raf, PI3K, RalGDS*, and *AF6*. The exact mechanism of ras induced colorectal cancer is still unclear. It is believed to act via *Ras→Erk* pathway involving Erk1/2 phosphorylation. Cyclin D1 over-expression is regulated at the transcriptional level by signaling through *Ras3Erk* and would be predicted to be decreased after the inhibition of Erk1/2 phosphorylation.

There are three isoforms of ras oncogene available namely Kirstein (Ki), Harvey (Ha) and Neuroblastoma (N) (Ross PJ *et al,* 2001). Ki-ras is the most frequently mutated form of ras in human cancers. N-type is some times associated with haematological malignancies and Ha-ras is shown to be associated with gastric and bladder carcinomas. It has also been shown in animal studies that *ras* mutations induced over expression of Cyclin D1 in the rat intestine cells (Filmus J *et al,* 1994). K-*ras* mutations can occur up to 40% of the colorectal cancers. The codons that are frequently affected by point mutation are 12, 13 and 61 (Farr *et al,* 1988). Mutations involving K-ras are frequently associated with advanced tumour stage (T3/T4) (Schimanski C.C *et al,* 1999). Female preponderance was noted in patients with non-small cell lung cancers (Nelson *et al,* 1999).

1.12.4 Retinoblastoma protein (pRb) and S phase proteins

The retinoblastoma protein is the target for the activated cyclin-CDK complex. The activated complex phosphorylates the pRb protein. This cancels the growth-repressive

function of the pRb protein. This in turn releases the so-called 'S' phase proteins. These are family of proteins collectively called the 'E2F' family (Sherr CJ, 2000, Watson, 2004). These E2Fs in the activated form, triggers a battery of genes that regulate DNA metabolism. The E2F family also induce cyclin E and A genes. Cyclin E enters into a complex with its partner CDK2 and collaborates with other CDKs to complete the Rb phosphorylation (Grana *et al*, 1995). This shift in Rb phosphorylation from mitogen dependent cyclin D-CDK4/6 to mitogen independent cyclin E-CDK2 accounts in part for the loss of dependency on extra-cellular growth factors at the restriction point. Figure 1-7 gives the schematic representation of the events that occur through G1 to S phase.

1.12.5 E-cadherin

E-cadherin is an adhesion molecule involved in the process of cancer metastasis (Fujimoto *et al*, 1997). This is a trans-membrane glycoprotein. Downregulation of E-cadherin leads to dedifferentiation and lead to tumour progression. Germline mutations and promoter CpG hypermethylation are the two main mechanisms by which this molecule is over expressed and promotes carcinogenesis (Chan *et al,* 2003). This binds to many proteins particularly the catenins. This is a vital mechanism for cell adhesion. In vitro studies (Orsulic *et al* 1999) have demonstrated that loss of E-cadherin leads to accumulation of β-catenin and in turn transported into the nucleus through LEF pathway. Though there are no proven direct influence on Cyclin D1 gene regulation, E-cadherin mutation can potentially affect the catenin and LEF pathway and thereby intranuclear accumulation of β-catenin and thereby affecting the target gene such as Cyclin D1.

CHAPTER 2

METHODOLOGY

2.1 Patient Selection

During the study period, all patients with were recruited from the Department of Surgery, University Hospital of North Staffordshire, Stoke-on-Trent. Ethical approval was obtained by our Research Department in order to facilitate the study. Patients were approached during the hospital stay and consented prior to surgery for them to be included in the study. They were given an information sheet regarding the research and their queries were answered prior to consent. All the general surgical consultants were approached and their approval for involving their patients was obtained. The hospital pathology department was also contacted and their input for advice on the technical aspects of research including blood sample sorting and tissue blocks were taken.

Inclusion criteria:

Patients those who had given their consent for the study.

Patients with histologically proven colorectal cancer.

Age over 18 years.

Exclusion criteria:

Family history of colorectal cancer

Patients who received pre operative radio and/or chemotherapy.

Ethnic minority groups in whom the disease behaviour is not clearly known.

Those who refused consent.

The laboratory work was carried out within the hospital campus at the Institute for Science and Technology in Medicine. Research work on colorectal cancer has been going on for sometime within our group. As a result of this we had accumulated a cohort of over 634 patients. Therefore, for this thesis, data is presented based on this cohort, though DNA was not available on all samples.

2.2 Sample Collection

2.2.1 Tissue

Blood:

Blood samples were collected in three EDTA tubes when the patients were having blood tests as a part of their preoperative phase. Once these samples reached the haematology laboratory, they were then isolated and given a separate research identification number in order to anonymise patient identity. From here onwards, only the research numbers were used during experiments and for data analysis. These samples were then stored in the –20 C freezer for DNA extraction.

Tumour:

Tumour samples in paraffin embedded blocks were collected from the pathology department. We collected these samples with the help of our expert consultant histopathologists (Dr J Elder and Dr V Smith) from the Department of Histopathology, University Hospital of North Staffordshire. They also provided guidance in preparing samples and were involved in immunohistochemistry procedure (grading the slides).

The tumour samples were given a unique research identification number for study purposes and to anonymise patient identity. The types of sample included tissue from the core of the tumour, margins of tumour tissue with adjacent normal mucosa and normal mucosa only samples. The core tumour tissue was mainly used for DNA extraction. Those tumour samples with part of normal mucosa present were mainly used in immunohistochemistry and so was the pure mucosal mucosa (as controls). These blocks were cut in either 5μm or 10μm sections and mounted on APES coated slides.

These slides were stored in a dust free container at room temperature for the purposes of DNA extraction (10μm) and immunochemistry (5μm).

2.2.2 Data

Patient demographic data such as age, sex, geographical distribution, diagnosis, site of tumour and the nature of treatment were collected from the patient medical notes. Histology data included the type of specimen, stage and size of the tumour, differentiation, lymph node involvement, vascular invasion, host lymphocyte reaction and finally the extent and Dukes' stage, were collected from the histology records. All follow up data, radiological investigations that contributed to staging and death were recorded from the hospital HISS system. Survival data were collected from the medical records and data was kept up to date on a regular basis.

2.3 Data Handling, Anonymization and Storage

Data were stored in a computer with password protection and not connected to the internet. Only the research team would have access to it. The patients' hospital identification details were masked as they were given a research identification number. Therefore patient confidentiality was maintained at all times.

Microsoft access 2000 was used as the main database program and all our data were handled using this program. The clinical and biological variables (e.g. clinical parameters, polymorphism genotypes etc) were allocated suitable codes by using the Visual Basic Program in order render them suitable for statistical analysis. Once the data has been coded, they were converted into 'txt' files in order to be used in statistical programs.

2.4 **Statistical Analysis**

The statistics package called Stata version 5.0 (Texas corporation) was used for the entire study to carry out the analyses. The data were initially analysed by the researcher and was counterchecked and validated by Professor PW Jones at Keele University (Department of Mathematics).

Experimental variables

All data following experimental work were entered on to Microsoft excel spreadsheet with the laboratory identity numbers. The alleles were also then coded individually. For example, the A/G^{870} polymorphism alleles were coded in the following way: AA=1 AG=2 and GG=3. This same process was adopted for all other polymorphisms analysis. The results from the protein expression study were coded in the numerical sequence in Excel spreadsheet. Once these data have verified, then they were entered on to the Microsoft Access 2000 database in a password protected computer.

Clinical variables

The following were data were maintained in our database: patient demographics, stage of the disease, tumour site, type of surgery, adjuvant treatment, histological details, information regarding metastasis and the length of survival. There were again coded in appropriate numerical sequence (e.g. Tumour grade: well differentiated-1, moderately differentiated-2 and poorly differentiated-3).

These data then had to be linked within the database by using the Visual Basic program. The required coded information prior to the analysis was then imported in Excel file format and was used in the Stata statistical program.

Polymorphism and protein expression were individually examined for outcome measures against the clinical variables, tumour biology and demographics, using Pearson's X^2test. A 'p' value of less than 0.05 was considered as significant. Any variable that obtained statistical significance, were further analysed using multiple regression analysis. From logistical regression analysis, we obtained odds ratio, 'p' value and confidence interval for expression and polymorphism work. Confounding factors (mainly age and gender) were corrected during the analyses. We also examined the combined influence of the alleles of the different polymorphisms within Cyclin D1 using the X^2test. We analysed the relationship between the Cyclin D1 polymorphisms and protein expression in similar way as above. Finally, we also looked at the effects of β-catenin protein expression over Cyclin D1 protein expression from the results derived from the immunohistochemistry results.

Survival data were calculated using Cox's proportional hazard model demonstrated using the Kaplan-Meier survival curve.

2.5 Blood DNA Extraction

Blood DNA extraction was carried out using the Nucleon genomic DNA extraction kit (Anachem, Luton, UK). Reagent A (4X) from the DNA extraction kit was added to the defrosted blood (4mL) and mixed at room temperature for 10 min in a rotary mixer. The samples were then centrifuged at 13000 rpm for 4 min. This process was to prepare the cells. The supernatant fluid was discarded. 1mL of reagent B from the DNA extraction was added to each pellet in order to carry out cell lysis and vortexed to re-suspend the pellet. Then 250uL of 5M sodium per-chlorate was added to the tubes and mixed on a rotary mixer for 15 min. Samples were then incubated at 65°C for 25 minutes, mixing occasionally.

The final step in this process was the DNA extraction. Samples were cooled on ice and 2mls of chloroform was added to each tube. After mixing the tube for 10 minutes at room temperature they were centrifuged at 13000g for 4 minutes. The upper layer of each sample was then transferred into 1.5µl microtubes and centrifuged at 13000 rpm for 4 minutes. Then 150µl of Silica suspension was added to each tube and centrifuged at 13000rpm for 6 minutes at room temperature. The upper layer each sample was transferred into clean tubes. Two volumes of cold absolute ethanol was added and mixed several times by hand. The DNA was simply hooked out and resuspended in sterile water. The extracted material was then stored at 4°C for future use.

2.6 The Polymerase Chain Reaction

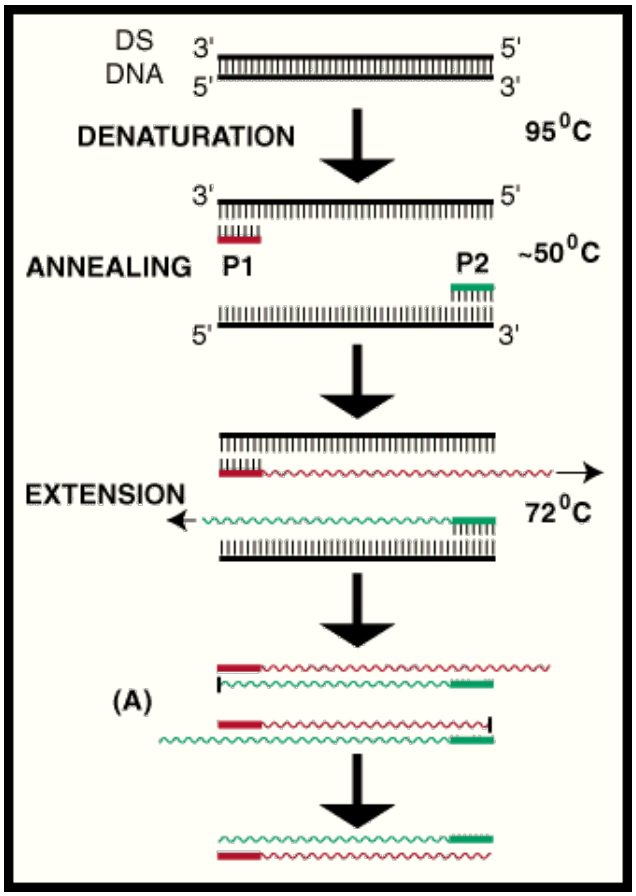
The polymerase chain reaction (PCR) is a sensitive and invaluable method used in the amplification of segments of nucleic acid sequences. There are different types of PCR based methods such as multiplex, ARMS and RT-PCR. The specific type of PCR method selection will depend on the type of DNA segments that are to be amplified as well as on the purpose for which the DNA will be used.

Amplification of the target DNA is achieved with two small sequences of nucleic acids called sense and anti-sense primers. They are designed in such a way that they bind to the specific complementary sequences of the DNA during the amplification process. To catalyse the replication process of DNA, a thermostable DNA polymerase (*Taq*) (Themus aquaticus) and the four deoxyribonucleic acids (dNTPs) are also added to the reaction mixture.

This mixture along with the DNA (blood or tumour) is incubated in a PCR machine otherwise called ‘thermocyclers’ and amplified using specific reaction conditions. The

types of reaction conditions are based up on the primer type, quality of the DNA and *Taq* polymerase used.

Figure 2-1: Illustration of the process of polymerase chain reaction (Source:www.flmnh.ufl.edu)



2.6.1 Primers

It's essential to have a good primer design in order to amplify a copy of successfully by PCR. The primers that are designed would complement the opposite strands of the DNA sequence. The length of any primer can vary from 15-30 bases. Generally longer the

length of the primer, better the specificity. The annealing temperature would depend up on the number and type of DNA bases in the primer, which should be 1- 5°C lower than the lowest primer melting temperature. The following equation was used to estimate the approximate annealing temperature when we designed the primers.

Tm = 4°C for each G/C base and 2°C for each A/T base.

A primer composition should ideally contain 50-60% G/C bases in order to bring down the annealing temperatures.

2.6.2 Basis of the PCR reaction

The following steps are an overview of the basic principles of a PCR method. These steps are described in detail for each individual polymorphism PCR process later. The first step is denaturing of the DNA template at temperatures usually between 94° – 96°C. Then the primers would bind to the complementary sequence at the determined site at certain temperatures and this process called annealing. The temperature setting for the annealing would depend on the primer design (usually 52°C -56°C). *Taq* DNA polymerase synthesises a complementary strand in the 5 to 3 direction. This process is continued for about 30 –36 cycles to obtain adequate amounts of amplified DNA segment. The final step is to ensure that the entire DNA strand is in double stranded formation.

2.6.3 Detection of *CCND1* gene polymorphisms

Cyclin D1 gene is frequently polymorphic and has five exons and an untranslated region. Polymorphism at nucleotide 870(A/G) position is the most commonly investigated polymorphism in many epithelial cancers. In our centre, we have also identified two other polymorphisms in the untranslated region of *CCND1* gene, one at nucleotide 1100 (C to A change) and the other at nucleotide 1722 (G to C change). The

effects of these polymorphisms have never been evaluated before. Therefore, we analysed these three polymorphisms in blood DNA samples from patients with colorectal cancer. The above mentioned principles were followed in conducting PCR, primer design and RFLP assay. The reaction times and mixtures for each of the polymorphisms described below.

2.6.4 Restriction Fragment Length Polymorphism (RFLP)

A polymorphism is a single base pair change that results in variations within the restriction fragment length. Thus in a given DNA segment this property can be used to cut them at their restriction sites using the commercially available enzymes. The result is that process would produce different lengths of DNA depending on the allelic polymorphism. Thus a person with homozygous allele would have a 'cutting' or 'non cutting' alleles that would be seen as a single band during an agarose gel electrophoresis method. In the case of a heterozygous sample, the gel pattern would have two bands since the sample would contain one allele each. The examples of a RFLP assay are given later in the chapter.

2.6.5 Agarose Gel Electrophoresis

The gel was prepared using powdered agarose dissolved in a buffer called TBE (0.5%) solution. (See appendix) The percentage of gel concentration would depend on the size of the DNA fragment. If the DNA fragment was small then a higher concentration of the agarose gel would be used. For DNA purification, a low concentration of the agarose gel (0.8-1.4%) was prepared. For the RFLP polymorphism studies, we used 2 – 3% agarose gel as a standard. For the visualisation of the bands ethidium bromide would be added in small quantities (3- 5μl/10mg/ml stock) while preparing the gel. A

molecular marker is run in a separate well (∅ 174 *Hae III* or *Hinf I* Marker) to determine the size of the DNA fragment. A DNA marker such as *Hinf I,* would contain different sizes of DNAs and would be readily visualised during gel electrophoresis. The volume of the PCR product or the digest can be between 10 and 25 µl. The samples were run for 90 minutes at 150 volts in 0.5% TBE as a running buffer. The resulting products were visualised on an UV transilluminator and images were obtained using a mounted camera system.

2.7 PCR-RFLP - Genotyping of *CCND1* gene Polymorphisms

2.7.1 Genotyping of $^{A/G870}$ and G/C^{1722} polymorphisms

The host genotype frequencies for both $^{A/G870}$ and G/C^{1722} polymorphisms were detected by using PCR-RFLP assay. Peripheral blood DNA was used to identify these genotypes.

Reaction mix: Reactions were performed in 50µl solution containing 0.1µg of blood DNA, 0.5µg of each primer (New England Biolabs, UK), 1× *Taq* polymerase buffer and 0.2 units of *Taq* polymerase (Promega, Dorset, UK). The primer sequence used for these two polymorphisms is given in Table 1.

The reaction conditions were as follows: The samples were initially denaturated at 94°C for 2 minutes followed by 34 cycles at 55°C for 60 seconds, 72°C for 60 seconds, and 94°C for 60 seconds and a final extension step at 72°C for 3minutes.

RFLP assay for G/C^{1722} and A/G^{870} polymorphisms: PCR products were digested using the following enzymes and incubated at 37 C for 4 hours. *Scrf I*(New England Biolabs, UK) enzyme was used for A/G^{870} polymorphism and *Hae III* (New England Biolabs, UK) for G/C^{1722}. 10µl of the digested product containing 3 µl of 'tracking dye

containing a DNA marker (ϕ174 DNA/*Hae III* marker)' were run in a 3% agarose gel containing ethidium bromide (see appendix for composition of tracking dye).The gels were run at 150 volts between 60 and 70 minutes to allow separation of the products. The detail of agarose gel electrophoresis is described in the following chapters.

Figure 2-2a: RFLP analysis of G/C^{1722} polymorphism on host blood DNA on agarose gel electrophoresis. Homozygotes for this polymorphism CC/GG appear as single band and heterozygote GC as two bands. The size of the product was compared against ϕ174 DNA/Hae III marker.

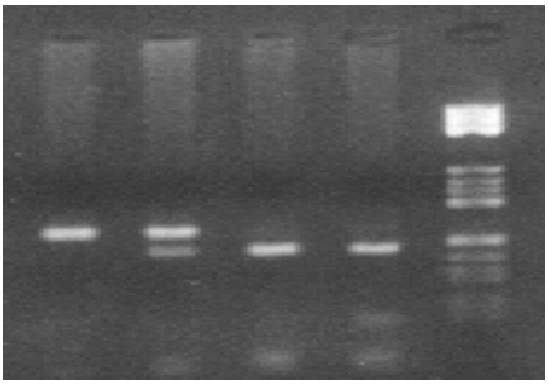
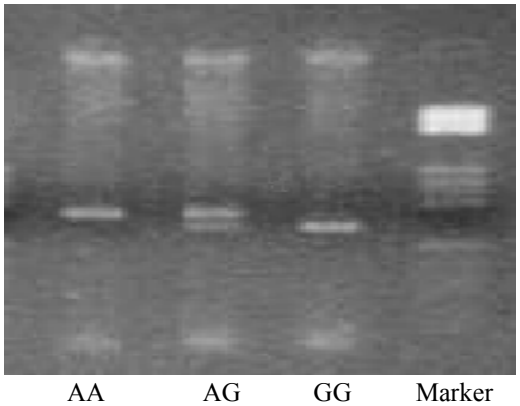


Figure 2-2b: RFLP analysis of A/G^{870} polymorphism on host blood DNA on agarose gel electrophoresis. *Homozygote for this polymorphism AA/GG appear as single band and heterozygote AC as two bands. The size of the product was compared against ϕ174 DNA/Hae III marker(size rRange 72-1353bp).*



2.7.2 ARMS-PCR assay for C/A^{1100} polymorphism

During ARMS-PCR we used two sets primers and amplified two alleles in one PCR reaction. This is based upon the enzymes specificity for the 3'OH group present on the 3 end of the primer. This is a useful tool for differentiation between genotypes of a given SNP. Each of the primers would only bind to specific SNP locations. Therefore two primers specific for the each nucleotide can be synthesised. We used this type of assay to identify the genotype frequencies for C/A^{1100} polymorphism due to lack of adequate cleaving enzymes and hence RFLP could not be performed.

Peripheral blood DNA was used to identify genotypes. PCR reactions were performed in 50µl total volume with 0.1µg of template DNA, 0.2µM of each primer (QIAGEN, UK), 250mM dNTPs (New England Biolabs, UK) 1× *Taq* polymerase buffer and 0.2 units of *Taq* polymerase (Promega, Dorset, UK). The primer sequences used for these polymorphisms are given in Table 2-1.

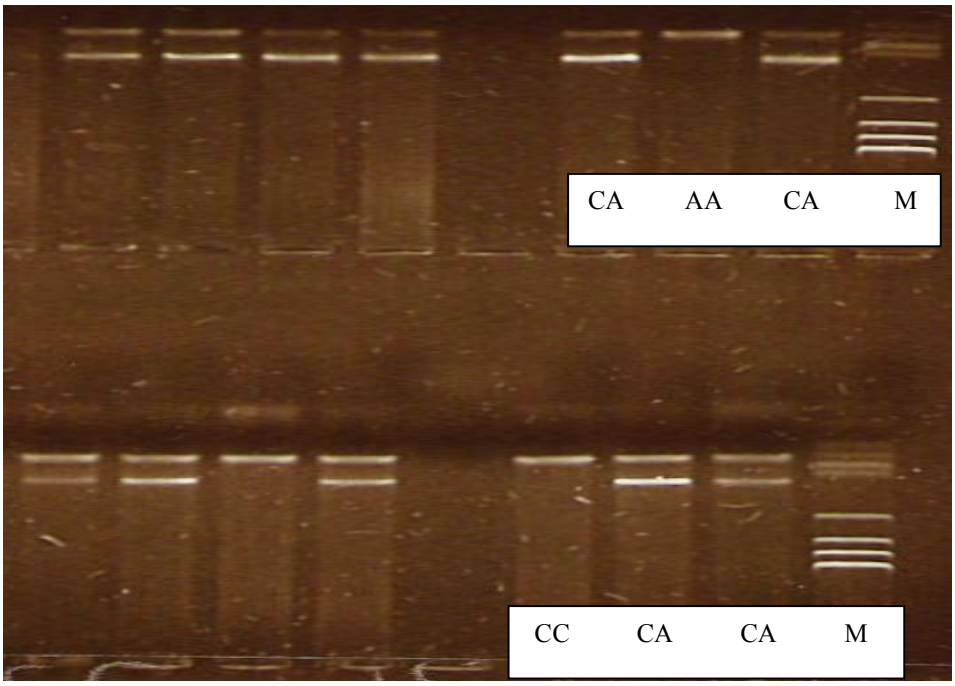
The reaction conditions were as follows: Initial denaturation at 94°C for 2 minutes. A touch-down PCR method was employed: 12 cycles at 66°C 45 seconds, 72°C for 60 seconds, 94°C for 45 seconds, 12 cycles at 65°C for 45 seconds, 72°C for 60 seconds 94°C for 45 seconds and 12 cycles at 64°C for 45 seconds, 72°C for 60 seconds, and 94°C for 45 seconds and a final extension step at 72°C for 3 minutes. The PCR products were visualised in a 2% agarose gel containing ethidium bromide (5µg/ml).

Table 2-1 displays the primer sequences used for CCND1gene polymorphisms

Primers for A/G^{870} and G/C^{1722} polymorphisms were supplied from MWG Biotech, UK and the primers for C/A^{1100} polymorphism was obtained from Qiogen, UK.

SNP	Primer sequence	Annealing Temp.
A870G	Cyc26 (Forward) 5'-GTG AAG TTC ATT TCC AAT CCG C-3' Cyc27 (Reverse) 5'-GGG ACA TCA CCC TCA CTT AC-3'	55°C
G1722C	Cyc5 (Forward) 5'-CTC TTG GTT ACA GTA GCG TAG C-3' Cyc7 (Reverse) 5'-ATC GTA GGA GTG GGA CAG GT-3'	55°C
A1100C	Forward: 5'- TCC GGG TCA TGG CAC CTG GGA AG – 3' Reverse (A allele) 5' – TCC GGA GAG GAG GGA CTG TCA GT-3' Reverse(C allele) 5' – TCC GGA GAG GAG GGA CTG TCA GG-3'	64-66°C

Figure 2-2c: RFLP analysis of A/G^{1100} polymorphism on host blood DNA on agarose gel electrophoresis. CC or AA genotypes were amplified when the DNA contains only those homozygote types. In case heterozygote DNA, both alleles were amplified.



2.8 Immunohistochemistry

This is a widely used method both in clinical situations and in the research units in order to identify many different proteins within the cell that frequently accumulate during pathological states such as cancer. There are two different types of immunochemistry namely *direct* method and an *indirect* method. We employed the *indirect* method as this is more sensitive and also the most widely practiced one. The advantage of this method is that it allows the visualisation of protein while keeping the cellular structure relatively intact.

The technique as follows: Initially the antigen should be precipitated. This can be achieved either by heating or by chemical means. The process of heat precipitation is discussed later in the technique. Then the unlabelled primary antibody is added and this binds to the target antigen in the tissue. The secondary labelled antibody is added and this reacts with the primary antibody. This secondary antibody must be raised against the IgG of the animal species in which the primary antibody is derived (e.g rabbits). This material is then incubated with Biotin/Avidin in order to enhance the amplification. Finally a chromogen and a counter-stain are added for visualisation.

Though this method was suitable for examining protein expression and its sub-cellular localisation, it is semi-quantitative. A scoring system was used to semi quantify expression status. In this study, the following scoring system was used to record expression which was dependent on the percentage of cells that contain the protein of interest and counting them in approximately every 200 cells per slide. The estimation was always done in the area of highest staining. The percentage of cells that stained was divided in to four groups:

Less than 10% - negative (0)

10 – 50% -weak staining (+1)

50 – 75% - moderate staining (+2)

75% & above – Strong staining (+3)

Another difficulty in these types of scoring system is the inter-observer variations. Hence all the slides that were once examined and scored by the researcher were independently scored by a qualified consultant pathologist (Dr J E and Dr V S). The samples that were considered inadequate were repeated. If they were still remained inadequate to grade, then they were excluded from the study. The other group of slides that were excluded from the study were those samples an agreement could not be reached between the two examiners.

2.8.1 Preparation of samples

Paraffin embedded sections were obtained from the pathology department, University Hospital of North Staffordshire and a separate research number was allocated. Sections were cut using a microtome, 5μm thickness slides were cut and laid in a water bath at 55˚C to remove the creases. Then they were mounted on to APES (aminopropyltriehoxysilane) coated slides and incubated for 2 hours in an oven at 37˚ C. Then the slides were stored in a dust free storage at room temperature.

Method for coating APES on glass slides

All the slides were initially washed with distilled water to remove any gross dust particles. Slides were then washed withindustrialised methylated spirit (IMS) twice for 5 minutes. A fresh solution of 2% APES (aminopropyltriehoxysilane) in acetone was prepared and the slides were coated for 5 minutes. These were washed in acetone for 60 seconds. Then the slides were washed twice in distilled water. All the treated slides were placed in a rack and were allowed to dry at room temperature overnight and stored in a dust free storage environment.

2.8.2 Haematoxylin and Eosin staining (H & E stain)

All the samples that were prepared were routinely stained for H & E staining. This serves two purposes. Firstly it identifies the tumour bearing area so that this area alone to be used as a template for other slides and also this tissue can be scrapped out for DNA extraction. Secondly it helps the examiner to focus the tumour area while scoring the slides for protein expression.

The method of H&E staining as follows:

The tissue containing slides were de-waxed in xylene for 10 minutes. Then the tissues were rehydrated in a graded series of alcohol (80%, 90%, and 100%) for 60 seconds followed by in distilled water for 30 seconds. Then these sections were stained in Gills No 2 haematoxylin for 8 minutes. After rinsing in running tap water, they were dipped in 2% sodium hydrogen sulphate and then dipped into 3% acid/alcohol for 5-10 seconds. Then an eosin yellow was added to the tissues and left for 3 minutes.

After washing, the slides are dehydrated with the graded series of alcohol and xylene

Finally a cover slip added by fixing with histomount.

2.8.3 Technique of immunohistochemistry

Tissues sections that were prepared as described in the earlier sections were placed in a metal rack and the research numbers were noted. The date of the procedure was added to the slides. For each set of the reaction a positive and a negative control slide was added. In some sets normal mucosa adjacent to the tumour acted as positive control.

All sections were treated with xylene for 10 minutes in order to remove the paraffin.

The slides were then rehydrated with a series graded alcohol (100%, 90% and 80% IMS respectively for 20 seconds each).

The next step was to block the peroxidase activity with 3% hydrogen peroxide in alcohol (70% IMS). This solution was prepared freshly for every set of slides.

For antigen retrieval, the precipitation of protein was achieved by heat method using the microwave oven (750watts) for approximately 20 minutes (4 X 5minute intervals). The two commonly used buffered solutions in our research were 1mM EDTA at pH 8.0 for β-catenin protein and 1mM citrate buffer at pH 6.0 for Cyclin D1 protein.

The slides are then washed with phosphate buffered saline at pH7.2 and were mounted onto a Shandon Sequenza (Shandon Scientific Limited, UK) cover slide and racked. Further steps in the procedure were carried out with slides in the Shandon rack.

The serum, secondary antibody and avidin/streptavidin solutions were prepared from Universal Vectastain Elite Kit (Vector Laboratories, UK) strictly following their recommendations. Diluted normal serum was added to the slides and was incubated at room temperature for 20 minutes. The primary monoclonal antibody (dilutions varied depending upon the type of antibody) was then added except the negative control and incubated for one hour at room temperature. The slides were washed with phosphate buffered saline (PBS) for 5 minutes. The biotinylated secondary antibody was added to the samples and incubated for 3o minutes for the target antigen binding. The slides were washed with PBS and Avidin/Biotin solution was added and the incubation was continued for further 30 minutes. After the PBS wash for 5 minutes, chromogen colour development was achieved by the addition of 1,3- diaminobenzedine (DAB) (Sigma Chemicals, UK) and 2mg/ml of urea H_2O_2 and was left to stand for 3 –7 minutes. Sections were washed with PBS and then counterstaining was carried out using Gills No: 2 Haematoxylin solution for 30 seconds. After washing with tap water and ‘bluing’ was done with 2% sodium hydrogen carbonate solution.

Finally the sections were dehydrated with the graded series of alcohol and Xylene. Then mounting was done with immunomount (Raymond Lamb, UK).

2.8.4 Cyclin D1 immunochemistry

Immunochemistry was performed using Cyclin D1 monoclonal antibody 1:25 dilution (Novocastra, UK) and the procedure was carried out as above. 1mM EDTA (pH 8.0) was used as a buffer solution during antigen retrieval. For positive controls, colonic sample with the known immunostaining status was used. Normal colonic mucosa or part of the normal mucosa was used as a negative control. Grading of the over-expression was done as described earlier. Nuclear grading of any strength and cytoplasmic staining except weak staining (<10%) was considered positive for over-expression.

2.8.5 β-catenin immunohistochemistry

The β-catenin primary monoclonal antibody 1:100 dilution (Novocastra, UK) was used at room temperature. 1mM citrated buffer solution at pH 6.0 was used for antigen retrieval. Any nuclear positivity was considered to be a significant over-expression. Those samples showing cytoplasmic over-expression 50% or more was taken as significant. This is because some weak staining of the cytoplasm frequently occurs in the normal mucosa as well.

2.9 β-catenin (*CTNNB1*) Gene Sequencing

2.9.1 Sample preparation

β-catenin has 16 exons and mutations are frequent in exon - 3 and hence this particular region was initially amplified by a PCR method. Amplification was carried out in two stages. Tumour DNA samples were used for amplification. The first PCR was performed using outside primers for 30 cycles. This is followed by a set of nested

primers were used further amplify the segment since the quality of the tumour DNA material obtained from paraffin sections were inferior in comparison with blood DNA.

Table 2-2: Primer sequence used for the amplification β-catenin tumour DNA.

Betacatenin primers
Outside primers: F1BC: AGC TGA TTT GAT GGA GTT GG R1BC: ACC AGC TAC TTG TTC TTG AG Inside primers: F2BC: CCA ATC TAC TAA TGC TAA TAC T R2BC: CTG CAT TCT GAC TTT CAG TAA G Supplied by: MWG Biotech, UK.

Reaction condition for both PCR as follows:

The mixture was initial denaturated at 94°C for 2 minutes followed by 30 cycles of 55°C for 45 seconds, 72°C for 45 seconds and 94°C for 45 seconds then followed by a final extension step of 72°C for 5 minutes.

The reaction mixture as follows:

PCR reactions were performed in 50µl total volume with 3µl of template DNA, 0.5µM of each outside primer for β-catenin (QIAGEN, UK) 250µM dNTPs (New England Biolabs, UK) 1× *Taq* polymerase buffer and 0.2 units of *Taq* polymerase (Promega, Dorset, UK) to amplify exon 3. Then 3µl of this sample was used in the second reaction using the nesting primers for further amplification. The details of the primer sequence are given below. The final PCR product was visualised in a 1.75% agarose gel containing ethidium bromide (5µg/ml) and the products were compared against (phi174) DNA/Hae III marker. After confirmation a Qiagen gene cleaning kit cleaned these PCR products as described below.

2.9.2 QIAquick PCR purification (QIAGEN, UK)

This tool is designed to purify single or double stranded DNA segments from PCR or other enzyme reactions. The product pack contains a number of QIAquick spin column tubes (with membrane) as well as solutions buffer PB, buffer PE (95-100% ethanol diluted) and buffer EB (10mM Tris.Cl, pH 8.5).

Procedure:

At first, 50µl of PCR product and 250 µl of buffer PB (1:5 ratio) were added mixed together. The mixture was then placed in a QIAquick spin column attached to a 2ml clean collection tube (provided). The sample was then centrifuged for 60 seconds in order to bind the DNA and the filtered material was discarded. Then 500µl of buffer PE was added to the spin column and centrifuged for another 60 seconds. Then the column was placed in the same collection tube and was centrifuged for additional 60 seconds to remove any residual ethanol. To elute the DNA, a QIAquick spin column was placed in a clean 1.5ml micro-centrifuge tube and 50 µl of the buffer EB was added to the centre of the column. This was then centrifuged for 60 seconds and the purified DNA product was used for future gene sequencing.

2.9.3 Gene sequencing method

The purified DNA products were visualised in 1.5% agarose gel containing ethidium bromide (5µg/ml) for confirmation. These PCR were then mixed with dye terminator solutions (Sequencing Kit RR-100, Applied Biosystems, Warrington, UK) and further amplified as described below:

To a 5 µl of the PCR product, 2.0 µl of the terminator dye solution, 1.0 µl of 5× buffer, 1.0 µl of forward primer (used for β-catenin PCR reaction) and 6.0 µl of deionised water were added (20µl volume in total). Then the sample was vortexed. The reaction mix

was then placed in GeneAmp2400 themocycler and 25 cycles were carried out (96°C for 10 seconds, 50°C for 5 seconds and then 60°C for 4 minutes). This was followed by rapid cooling to 4°C and until ready to purify the samples.

2.9.4 Purification (Removal of excess terminator dye)

The amplified sample (20µl volume) was placed in a 1.5ml centrifuge tube and 80µl of 80% isopropanol was added. The sample was then vortexed well and placed in ice for 10 minutes in order to precipitate the extension products. The precipitate was then centrifuged at 13000 rpm for 20 minutes. The supernatant was carefully aspirated and discarded. The remaining pellet was washed with 250µl of 75% isopropanol and vortexed to mix well. A further 15 minutes of centrifuge (13000 rpm) was carried out and supernatant fluid was carefully discarded. Any remaining liquid covering the pellet was dried at room temperature taking care not to over dry it.

2.9.5 Electrophoresis on the ABI Prism 310 sequencer

The pellet that was prepared as described above was resuspended in 12µl of Template suppression Agent (Applied Biosystems, Warrington, UK) and then vortexed well.

The sample was then heated at 95°C for 2 minutes to denature and then cooled immediately and kept on ice until ready for use (normally 10-15 minutes).

Finally the sample was in transferred in a suitable sterile container and loaded into the analyser and the graphical information was obtained for mutation analysis.

2.10 Analysis of Cyclin D1 Phosphorylation sites (T286A and T156A)

Cyclin D1 turnover is governed by ubiquitinisation and proteosomal degradation, which are positively regulated by Cyclin D1 phosphorylation. Phosphorylation can be impaired by changes that occur in codons that are involved in the process. In vitro studies have shown that threonine to alanine change at 286 and 156 causes a blockade

in the Cyclin D1 phosphorylation and its subsequent degradation by the ubiquitin pathway (Diehl JA *et al,* 1997). We analysed the Cyclin D1 phosphorylation sites in three groups of tumour DNA samples namely those samples that over-expressed Cyclin D1 protein in the nucleus, second group was those that were found in the cytoplasm and the third group was with no significant Cyclin D1 over-expression.

PCR method

PCR reactions were performed in a 50µl total volume with 3µl of template DNA, 0.5µM of each outside primer for codon 286 (QIAGEN, UK) 0.25µM dNTPs (New England Biolabs, UK) 1× Taq polymerase buffer and 0.2 units of Taq polymerase (Promega, Dorset, UK) for amplification. Then 3µl of this sample was used in the second reaction using the nesting primers for further amplification. The details of the primer sequence are given below. The final PCR product was run 3% agarose gel containing ethidium bromide and visualised products were compared against (ϕ174 DNA/Hae III marker. The same reaction method was also adopted for codon 156 as well.

The reaction as follows: The following reaction was used for outside and nested primer reaction. Initial denaturation at 94°C for 2 minutes followed by 30 cycles at 57°C for 60 seconds, 72°C for 60 seconds, and 94°C for 60 seconds and a final extension step at 72°C for 5 minutes. The digests were visualised in a 3% agarose gel containing ethidium bromide (5µg/ml). ϕ174 DNA (Hae III) marker was used to compare the amplified products.

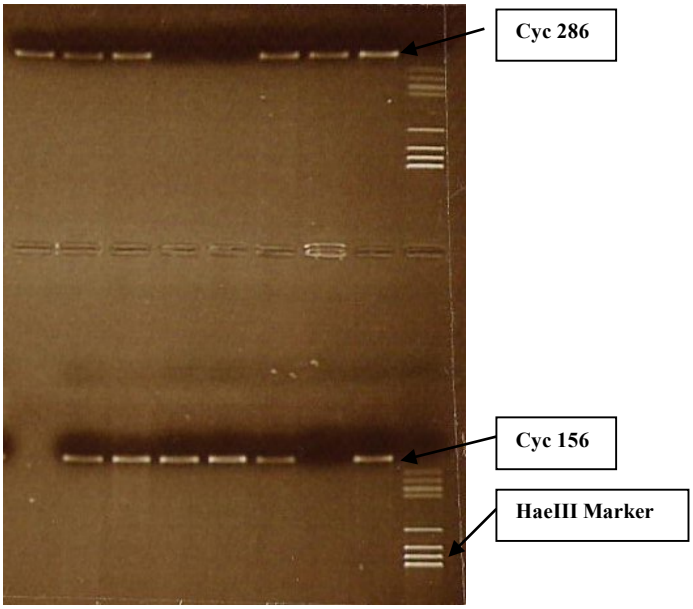
Once the PCR products were confirmed by agarose gel method, the remaining products were purified using QIAGEN clean kit, (UK) by the method outlined in previous

chapters. These products were then sent to QIAGEN laboratories (UK) in this country for DNA sequencing analysis.

Table 2-3: Primer sequences for the amplification of Cyclin D1 tumour DNAs for codons 286 and 156.

Outside primers for codon 286: Cyc 286 F: 5'-TGG AGT CAA GCC TGC GCC AGG CC-3' Cyc 286 R: 5'-CCC TTC TGG TAT CAA AAT GCT CCG GA-3'
Inside primers for codon 286: Cyc 286 Fn: 5'-CAG AAC ATG GAC CCC CAA GGC-3' Cyc 286 Rn: 5'-GAG GGA CTG TCA GTG GAG CAC CTG-3'
Outside primers for codon 156: Cyc 156 F: 5' - ATG GAG CTG CTC CTG GTG AAC AAG - 3' Cyc 156 R: 5' - CGC GTG TTT GCG GAT GAT CTG TTT G - 3'
Inside primers for codon156: Cyc 156 Fn: 5' - CTG GTG AAC AAG CTC AAG TGG AAC - 3' Cyc 156 Rn: 5' - GAT GAT CTG TTT GTT CTC CTC CGC C - 3'
Supplied by: Qiagen, UK

Figure 2-3: Agarose gel electrophoresis of PCR amplification of Cyclin D1phosphorylation sites at codons 286 and 156. The tumour samples were amplified and visualised in agarose gel along and was used as Hae III marker.



CHAPTER 3

RESULTS

Introduction

The results for whole of the research work is described under separate heading as outlined in the index pages. The distribution of patient demographics and the clinical parameters are given at the beginning of this chapter. Then the results of polymorphisms of *CCND1* gene are presented. This is followed by the results that show their relation to Cyclin D1 protein over-expression. We then presented the results of Cyclin D1 protein expression. Then results for β-catenin protein over-expression presented as an individual chapter and we analysed the relationship between Cyclin D1 and β-catenin protein expression. All the parameters were analysed using Pearson's Chi2 test. Any values that showed significance or clinically thought to be important or interesting were further analysed by multiple regression studies. The confounding factors such as age and sex were corrected where possible while using regression analysis. Subgroup analysis was carried out if a result was perceived to have any clinical significance. At the same time subgroup analysis was omitted if the numbers were too small to draw any meaningful conclusions. The survival data was calculated using Cox's proportional hazard model. All values were plotted in a Kaplan-Meier survival curve and their significance was noted.

3.1 General characteristics of the cohort of patients with colorectal cancer

Patient demographics and clinico-pathological data were collected by methods discussed in the methodology chapter. Many of such parameters are shown in table 3.0. The objective of the baseline analysis of the whole cohort was to show that our results were comparable with those in literature. About 65% of the patients were more than 65 years old and just over a half of the population were males (57%). We chose 65 years age the cut off since this is the retirement age in this country. Therefore we decided that

we would use this as a dividing point. However in clinical practice, the decisions are based on co-morbidities and the general fitness of a patient rather than age alone.

All the results were initially examined with Pearson's X^2 test. If a particular variable showed statistical significance, then these variables were further analysed with regression method and correlated with clinical significance. All the parameters were also analysed with Cox proportional hazard model. Gender (HR 0.96, P=0.76 CI 0.77-1.20) did not influence the survival. Those who were over 65 years of age did have a slightly higher rate of mortality (HR 1.55, p=0.0001, CI 1.21-1.98) (Fig 3.1). The site of the tumour did not have any effect on the survival rate. Tumour site also was not significantly associated with alteration in survival (HR 1.24, p=0.12, CI 0.93-1.65). Advanced Dukes' stage (Fig 3.2) (HR 10.29, p=0.0001, CI 5.73-18.48), high grade tumour (HR 2.55, p=0.0001, CI 1.69-3.85), advanced 't' stage ((HR 2.04, p=0.023, CI 1.10-3.77), nodal invasion (HR 4.13, p=0.0001, CI 3.03-5.63) and metastases (HR 4.68, p=0.0001, CI 3.67-5.97) unsurprisingly were significantly associated with reduced over all survival rates in this cohort of patients. Other parameters that influenced survival were vascular invasion (HR 2.26, p=0.0001, CI 1.76-2.89) and liver metastases (HR 4.75, p=0.0001, CI 3.62-6.23). It was of note that host lymphocytic reaction (HR 0.58, p=0.001, CI 0.43-0.79) was shown have a significant protective effect in patients with colorectal cancer. The data shown above reflects the nature of the study group and appear to follow the general history and fate of the disease.

Table 3.0: shows the values of clinical variables for the whole colorectal cancer patients.

Variables		No of patients	Percentage	
Gender	Male	362	(56.83)	
	Female	275	(43.17)	
Age*	<65yrs	219	(34.49)	
	>65yrs	416	(65.51)	(HR=1.55)
Dukes stage*	A	58	(9.51)	
	B	256	(41.97)	
	C	215	(35.25)	
	D	81	(13.28)	(HR=10.29)
T stage	1	28	(4.71)	
	2	74	(12.44)	
	3	279	(46.89)	
	4	214	(35.97)	
Node state	Negative	325	(55.65)	
	Immediate	164	(28.08)	
	Distant	95	(16.27)	
Tumour site	Right	140	(22.51)	
	Left	482	(77.49)	
Metastasis*	Yes	128	(20.09)	
	No	509	(79.91)	(HR=4.75)
Vascular Invasion*	Yes	178	(32.96)	
	No	362	(67.04)	(HR=2.26)
Tumour margins	I	222	(58.73)	
	P	156	(41.27)	
Host lymphocyte Reaction*	Yes	226	(57.65)	
	No	166	(42.35)	(HR=0.58)

All parameters have been analysed by Chi^2 test. *Any significant results were further analysed by Cox proportional hazard analysis

Figure 3.1: Kaplan-Meier survival curve for the whole cohort of colorectal cancer patients based on the age groups (HR 1.55, p=0.0001, CI 1.21-1.98)

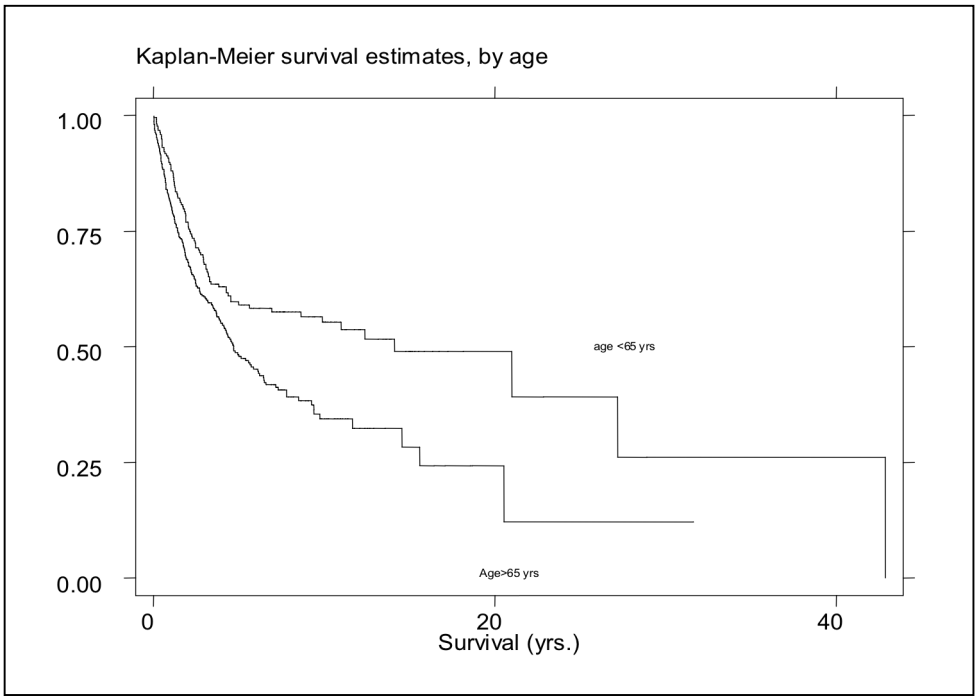
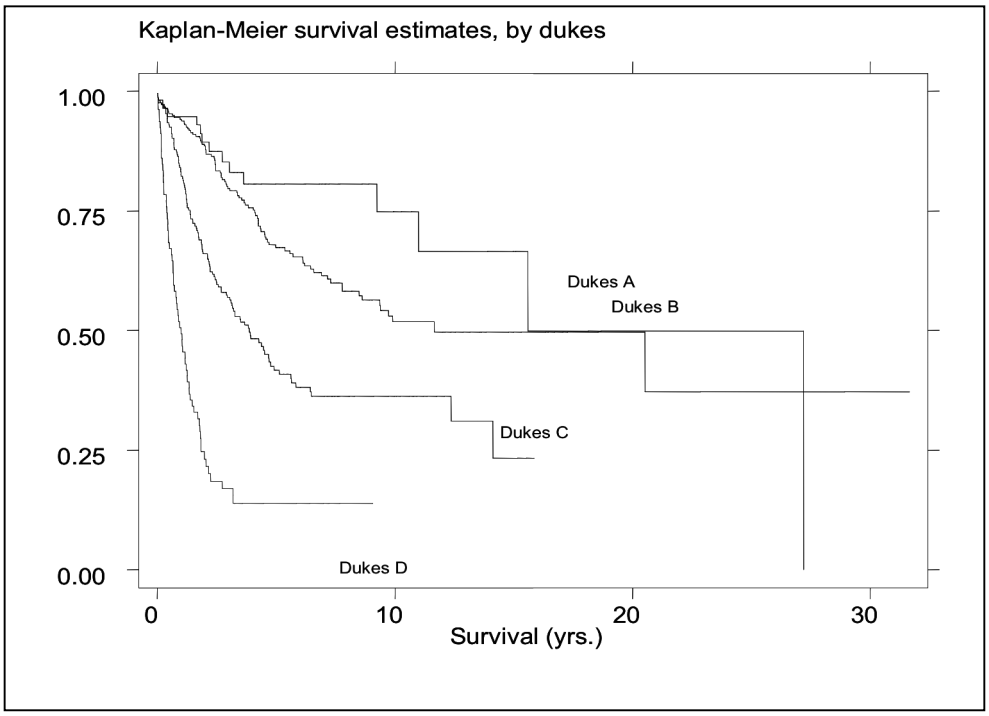


Figure 3.2: Kaplan-Meier survival curve for the whole cohort of colorectal cancer patients based on the Dukes stage (HR 10.29, p=0.0001, CI 5.73-18.48)



3.2 CCND1 Polymorphisms

We investigated three polymorphisms namely A/G^{870}, G/C^{1722} and C/A^{1100} within Cyclin D1 gene. The effects of these polymorphisms are discussed individually. We also analysed their 'combined' effects on the outcome in patients with colorectal cancer.

3.2.1 Distribution of allele frequencies of *CCND1* polymorphism

Table 3.2 shows the distribution of genotype frequencies and percentage distribution. Their allelic frequency distribution was calculated with Hardy-Weinburg equilibrium. All groups obeyed Hardy-Weinburg equilibrium. The allelic frequencies for A/G^{870} polymorphism were (A, 0.46/G, 0.54). Similarly, the allelic frequencies for G/C^{1722} polymorphism were (C, 0.39/G, 0.61). These were similar to those previously published studies (Matthias *et al,* 1998 and from our group Holley *et al,* 2001). Cases genotyped for the *CCND1* C/A^{1100} polymorphism also conformed to Hardy-Weinberg equilibrium with the following allele frequencies C^{1100} (0.45) and A^{1100} (0.55). Significant linkage disequilibrium was demonstrated between *CCND1*1 C/G^{1722} and A/G^{870} alleles, thus 51 of 64 (79.7%) of individuals with *CCND1* GG^{1722} were also *CCND1* AA^{870} (p=0.0001). Significant linkage disequilibrium was also demonstrated between *CCND1*A/G^{870} and C/A^{1100} alleles, thus 36 of 47 (76.6%) of individuals with *CCND1*AA^{870} were also *CCND1*AA^{1100} (p=0.0001).

Table 3.1: Distribution of genotype frequencies of CCND1 polymorphisms.

Polymorphism	Genotype	Frequency n(%)
A/G^{870}	AA	87(22.48%)
	AG	180(46.51%)
	GG	120(31.01%)
G/C^{1722}	CC	58(17.85%)
	GC	140(43.08%)
	GG	127(39.08%)
C/A^{1100}	CC	48(18.68%)
	CA	133(51.75%)
	AA	76(29.57%)

Table 3.2: Significant linkage disequilibrium between CCND1 A/G^{870} and CCND1 G/C^{1722} genotypes.

	CCND1 G/C^{1722} genotypes n(%)		
CCND1A/G^{870} genotypes	CC	C/G	G/G
AA	4 (6.25)	9 (14.06)	51 (79.69)
A/G	19 (13.01)	77 (52.74)	50 (34.25)
GG	33 (35.87)	44 (47.83)	15 (16.30)

$\chi^2_{(4)}$= 79.4, p≤0.0001

Table 3.3: Significant linkage disequilibrium between CCND1 A/G^{870} and CCND1 C/A^{1100} genotypes

	CCND1 CA^{1100} genotypes		n(%)
CCND1 A/G^{870} genotypes	AA	AC	CC
AA	36 (76.60)	8 (17.02)	3 (6.38)
A/G	13 (15.48)	63 (75.00)	8 (9.52)
GG	3 (5.88)	26 (50.98)	22 (43.14)

$\chi^2_{(4)}$= 95.8, p≤0.0001

Table 3.4: Significant linkage disequilibrium between CCND1 G/C^{1722} and CCND1 C/A^{1100} genotypes.

	CCND1 C/A^{1100} genotypes		n(%)
CCND1 G/C^{1722} genotypes	AA	AC	CC
CC	3 (10.71)	11 (39.29)	14 (50.0)
CG	6 (7.89)	55 (72.37)	15 (19.74)
GG	43 (59.72)	26 (36.11)	3 (4.17)

$\chi^2_{(4)}$= 71.4529, p≤0.0001

3.2.2 Association of CCND1 A/G^{870} genotypes with prognostic indicators (Table 3.5).

The mean age for this group was 68.40 years (range 31-92 years). In addition to general analysis, the effects of each allele 'A' and 'G' were examined by comparing against the other (e.g. for A allele - AA genotype was compared against AG/GG and vice versa). There were significant associations found with the demographic parameters (gender p=0.92, age p=0.96 X^2 test). The other variables that were examined also did not show any significant associations (tumour differentiation p=0.79, Dukes' stage p=0.69, nodal invasion p=0.43, metastases p=0.53, vascular invasion p=0.18, tumour site p=0.39, host lymphocyte reaction p=0.48 X^2 test). However, a non-significant trend was observed between CCND1 AA^{870} and presence of vascular invasion (Armitage trend test p=0.09).

3.2.3 Association of CCND1 G/C^{1722} genotypes with prognostic indicators (Table 3.5).

The CCND1 C/G^{1722} polymorphism proved to be more interesting. The mean age group was 66.29 years (range 30-90 years). There were no significant associations found with age and gender (p=0.19). No associations were observed with tumour stage, nodes, metastases, host lymphocyte reaction or tumour margins. However, using CCND1 CC/CG^{1722} as a reference a significant association was observed between CCND1 GG^{1722} and poorly differentiated tumours (p=0.007, OR 2.17, 95% CI 1.23-3.83). We also observed a non-significant trend between CCND1 GG^{1722} and later staged (Dukes') tumours. An association was also observed between CCND1 GG^{1722} and vascular invasion (using CCND1 CC/CG^{1722} as a reference p=0.046, OR 1.80, 95% CI 1.01-3.19). Conversely, when liver metastases excluded from the cohort, a significant association was observed between CCND1 CC^{1722} and the metastases at other sites (using CCND1 GG/CG^{1722} as a reference p=0.022, OR 3.74, 95% CI 1.20-11.6).

3.2.4 Association of CCND1 C/A^{1100} genotypes with prognostic indicators (Table 3.5).

This polymorphism exhibited some associations with tumour prognostic indicators. There were no significant associations found with age or gender. However, CC^{1100} genotype (using CA/AA^{1100} as reference) was significantly associated with left sided tumours. (OR 3.66, P= 0.018, CI 1.25-10.76). CC^{1100} genotype patients were less likely to be associated with advanced Dukes stage (OR 0.40, p=0.012, CI 0.20-0.82) and also were significantly associated with node negativity (OR 0.38 p=0.01, CI 0.18-0.79). When the AA^{1100} genotype (using CC/CA^{1100} as reference) was compared against vascular invasion it showed a positive significance level (OR 2.10 p=0.05 CI 0.98 4.53).

3.2.5 CCND1 polymorphisms and impact on survival

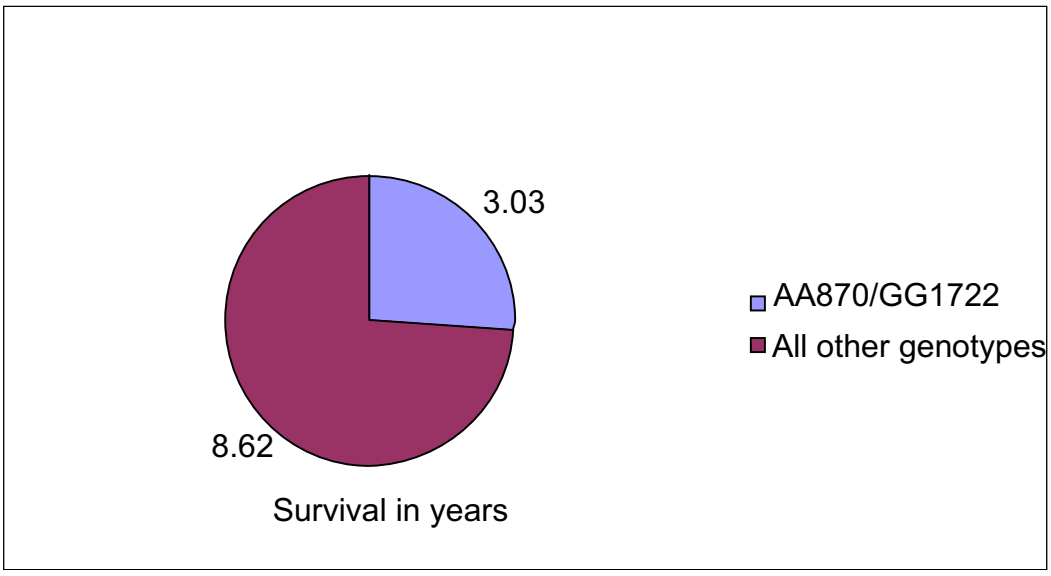
All three polymorphisms did not make any influence on the survival rates in this cohort of patients except G/C^{1722} polymorphism. There was an increase in the hazard ratio was observed accounting for patients who had vascular invasion and the CCND1 CC^{1722} genotype (p=0.003, HR 4.56, 95% CI 1.68-12.33.

3.3 Genotype interaction between the genotypes of CCND1 polymorphisms

Since the 'A' allele of AG^{870} polymorphism and the 'G' allele of GC^{1722} polymorphism showed some associations with tumour biology on individual analysis, we wanted look the effects of combining the genotypes between these two polymorphisms i.e. AA^{870}/GG^{1722} versus other genotypes. However this was not a haplotype analysis. A total of 302 patients with colorectal cancer (blood DNA) were analysed by PCR and had genotype results for each of the two polymorphisms available. Significant linkage disequilibrium was noted between these polymorphisms (X^2 test- p=0.0001). 16.88% (51/302) of cases had A^{870}/G^{1722} alleles in Cyclin D1 gene. Logistic regression analysis

showed a significant association between AA^{870}/GG^{1722} and vascular invasion (OR=2.94 p=0.003 CI=1.45-5.95). The median survival rates for AA^{870}/GG^{1722} versus other genotypes combined were 3.03 and 8.62 respectively (Fig 3.3). The overall 5-year survival rates for AA^{870}/GG^{1722} versus other genotypes were 41% and 59% respectively. However the 'p' value failed to achieve significance level (p=0.13). This type of analysis was also applied to the third polymorphism C/A^{1100} in CCND1 gene. This did not show any significant survival rates when compared with the genotypes of the other two polymorphisms. Further subgroup analysis of was not carried out due to smaller numbers of patients available with C/A^{1100} genotypes.

Figure 3.3: Pie chart showing the outcome of survival between patients with AA^{870}/GG^{1722} genotypes in comparison with all other genotypes (comparison between A/G^{870} and G/C^{1722} polymorphism)



3.4 Haplotype analysis of CCND1 polymorphisms

We then wanted to analyse the 'true' effect of each of these alleles with clinical outcome by haplotype analysis. The alleles were grouped into the following: A A^{870}/C^{1722}, A^{870}/G^{1722}, G^{870}/C^{1722} and G^{870}/G^{1722}. We did not carry out haplotype analysis for third polymorphism (C/A^{1100}), once again due to the smaller numbers.

A^{870}/G^{1722} alleles were significantly associated with vascular invasion (OR=1.98 p=0.052 CI 0.99-3.08). On the other hand, G^{870}/C^{1722} (OR 0.48 p=0.04 CI 0.24-0.97) and G^{870}/G^{1722} (OR 0.46 p=0.032 CI 0.23-0.93) were significantly protective for vascular invasion.

G^{870}/C^{1722} was found to have significant protective effect against poorly differentiated disease (OR 0.50 p=0.05 CI 0.25-0.99).

A^{870}/C^{1722} allele was associated (nearing significant level) with metastasis (OR 2.24 p=0.06 CI 0.97-5.19). There was no significant influence on the survival noted with any of these groups.

C/A^{1100}genotype:

The homozygtes with 'A' allele were more likely to be females (χ^2 test p=0.016) (OR=1.93 p=0.017 CI= 1.12-3.33) and were significantly associated with vascular invasion (χ^2 test p=0.013) (OR=2.13 p=0.012 CI= 1.18-3.84).

The homozygotes with 'C' allele were significantly associated with left sided tumours (χ^2 test p=0.010) (OR=3.75 p=0.016 CI= 1.28-10.98). These patients were significantly associated with early stage disease (χ^2 test p=0.008) (OR=2.45 p=0.010 CI=1.23-4.87) and were apparntly protected against nodal invasion (χ^2 test p=0.009) (OR=0.39 p=0.013 CI= 0.204-0.823). Using the Cox's proportional hazards model, we found no significant influence on the survival. This 'apparent' effect on nodal invasion had failed to influence the survival rates.

Table 3.5: The frequency of CCND1 genotype according to clinical characteristics, in colorectal cancer patients.

	Cyclin D1 A/G^{870}			Cyclin D1 C/G^{1722}			Cyclin D1 C/A^{1100}		
	AA^{870}	**AG^{870}**	**GG^{870}**	**CC^{1722}**	**CG^{1722}**	**GG^{1722}**	**AA^{1100}**	**CA^{1100}**	**CC^{1100}**
Sex									
Male	50 (22.1)	107 (47.4)	69 (30.5)	35 (19.0)	85 (46.2)	64 (34.8)	22(18.49)	71(59.66)	26(21.85)
Female	37 (23.0)	73 (45.3)	51 (31.7)	23 (16.3)	55 (39.0)	63 (44.7)	16(19.75)	34(41.98)	31(38.27)
Tumour Differentiation†									
Well	6 (21.4)	13 (46.4)	9 (32.1)	4 (16.7)	11 (45.8)	9 (37.5)	4 (5.63)	7 (5.38)	5 (10.87)
Moderate	56 (21.5)	121 (46.5)	83 (31.9)	40 (18.4)	104 (47.9)	73 (33.6)	47(66.20)	97 (74.62)	34 (73.91)
Poor	19 (25.3)	33 (44.0)	23 (30.7)	12 (18.8)	19 (29.7)	33 (51.6)	20(28.17)	18 (20.00)	7 (15.22)
TNM Classification									
Direct Local Invasion									
T1	1 (6.7)	9 (60.0)	5 (33.3)	1 (7.7)	5 (38.5)	7 (53.9)	1 (1.52)	7 (5.60)	3 (6.98)
T2	11 (26.8)	16 (39.0)	14 (34.2)	6 (17.6)	11 (32.4)	17 (50.0)	7 (10.61)	13(10.40)	6 (13.98)
T3	40 (21.7)	84 (45.6)	60 (32.6)	28 (17.4)	80 (49.7)	53 (32.9)	34(51.52)	69(55.20)	24(55.81)
T4	27 (23.5)	56 (48.7)	32 (27.8)	20 (21.3)	37 (39.4)	37 (39.4)	24(36.35)	36(28.80)	10(23.26)
Lymph Nodes∞									
N0	41 (20.3)	93 (46.0)	68 (33.7)	32 (18.9)	78 (46.2)	59 (34.9)	8 (19.05)	4 (9.52)	30(71.43)
N1	18 (19.4)	46 (49.5)	29 (31.2)	14 (16.9)	35 (42.2)	34 (41.0)	18(14.40)	44(35.20)	63(50.40)
N2	17 (32.7)	21 (40.4)	14 (26.9)	9 (19.2)	18 (39.3)	20 (42.6)	16(25.00)	18(28.12)	30(40.88)
Metastases*									
M0	67 (21.9)	140 (45.8)	99 (32.4)	44 (16.7)	112 (42.6)	107 (40.7)	61(80.26)	101(75.94)	36(75.00)
M1	20 (24.7)	40 (49.4)	21 (25.9)	14 (22.6)	28 (45.2)	20 (32.3)	15(19.74)	32(24.06)	12(25.00)
Tumour Margin									
Pushing	23 (23.0)	42 (42.0)	35 (35.0)	14 (15.7)	38 (42.7)	37 (41.6)	17 (28.3)	31 (51.7)	12 (20.0)
Infiltrating	31 (21.7)	67 (46.9)	45 (31.5)	25 (19.5)	60 (46.9)	43 (33.6)	21 (28.8)	42 (57.5)	10 (13.7)
Modified Dukes' Stage									
A	4 (11.8)	19 (55.9)	11 (32.4)	6 (20.7)	11 (37.9)	12 (41.4)	6 (8.96)	14(10.77)	8 (18.18)
B	33 (20.6)	73 (45.6)	54 (33.8)	25 (19.1)	62 (47.3)	44 (33.6)	23(34.33)	43(33.08)	21(47.73))
C	28 (23.7)	55 (46.6)	35 (29.7)	18 (16.5)	45 (41.3)	46 (42.2)	28(41.79)	55(42.31)	8 (18.18)
D	14 (27.5)	22 (43.1)	15 (29.4)	7 (18.4)	19 (50.0)	12 (31.6)	10(14.93)	18(13.85)	7 (15.91)

Vascular Invasion‡									
Yes	25 (30.9)	33 (40.7)	23 (28.4)	12 (16.7)	27 (37.5)	33 (45.8)	33(47.83)	39 (31.97)	11(26.83)
No	39 (20.7)	83 (44.2)	66 (35.1)	30 (18.8)	78 (48.8)	52 (32.5)	36(52.17)	83(68.03)	30(73.17)
Host Lymphocyte Reaction									
Yes	26 (20.0)	59 (45.4)	45 (34.6)	18 (15.5)	53 (45.7)	45 (38.8)	15 (22.4)	40 (59.7)	12 (17.9)
No	31 (26.3)	51 (43.2)	36 (30.5)	21 (20.4)	46 (44.7)	36 (35.0)	24 (35.8)	32 (47.8)	11 (16.4)
Tumour Site (Right Vs Left)									
Right side	NA	NA	NA	NA	NA	NA	15(20.27)	40(30.53)	4 (8.89)
Left side	NA	NA	NA	NA	NA	NA	59(79.73)	91(69.47)	41(91.11)

All the above parameters shown in numbers and the percentages within brackets and CHI^2 test were used. The variables that showed significant 'p' value were further analysed by logistic regression. † Using logistic regression analysis a significant association was observed between *CCN D1* GG^{1722} and poorly differentiated tumours (p=0.007, OR 2.17, 95% CI 1.23-3.83).

∞Using logistic regression analysis a significant association existed between fewer patients with the *CCN D1* CC^{1100} genotype and presence of nodes at presentation (using CCND1 AA/AC^{1100} as a reference p=0.011, OR 0.19, 95% CI 0.05-0.69). NA-not available.

3.5 Cyclin D1 protein expression

Immunohistochemistry was performed on 176 patients' tumour tissue with proven colorectal cancer. Table 3.6 shows the distribution between clinical variables and the tumour expression of Cyclin D1. Tables 3.7 & 3.8 display the over-expression status for the same variables for Cyclin D1 protein nuclear and cytoplasmic over-expression.

3.5.1 CyclinD1 overall protein expression

115 out of 176 (65.3%) patients were found to have over expression of Cyclin D1 either in the nucleus, cytoplasm or both. No expression of Cyclin D1 was found in 61/176 (34.7%) tumour samples. Nuclear expression was found in 34/115 (29.6%) samples. Expression exclusive to cytoplasm alone was seen in 75/115 (65.2%) patients. Only 6/115 (5.2%) patients were positive for both cytoplasmic and nuclear expression.

There were no significant associations with patient demographics such as age and gender. Those who over-expressed Cyclin D1 were less likely to have significant metastatic disease (P = 0.03, X^2 *test*) (OR 0.42, p=0.028, CI 0.20- 0.91). It was also shown that metastases to other sites (excluding liver) were significantly less frequent in patients with Cyclin D1 over-expression (P = 0.0001, X^2 *test*) (OR 0.05, p=0.0001, CI 0.01- 0.25). These patients were significantly associated with pushing tumour margins in comparison to invasive margins (P = 0.04, X^2 *test*) (OR 0.49, p=0.049, CI 0.24-0.99).

3.5.2 Cyclin D1 nuclear over-expression

Subgroup analysis of nuclear over-expression was done to examine the influence of this protein according to the site of localisation.

Nuclear over-expression was significantly associated with early dukes stage tumours (P= 0.006, X^2 *test*) (OR 0.36, p=0.008, CI 0.17- 0.76) and node negativity (P= 0.008, X^2 *test*) (OR 0.37, p=0.010, CI 0.17- 0.79). Once again nuclear over-expression was less likely to be associated with distant metastasis to other sites except liver (P= 0.025,

X^2 *test*) (OR 0.27, p=0.043, CI 0.079- 0.96). Metastasis to liver was also less in these patients though the result was statistically not significant (P= 0.058, X^2 *test*) (OR 0.26, p=0.09, CI 0.05- 1.23)

3.5.3 Cyclin D1 cytoplasmic over-expression

Patients with strong cytoplasmic over-expression had fewer propensities for metastases to distant sites (P= 0.03, X^2 *test*) (OR 0.23, p=0.038, CI 0.06- 0.92). These patients showed significant host lymphocyte reaction (P= 0.006, X^2 *test*) (OR 0.36, p=0.004, CI 0.18- 0.72). These cases were also significantly associated with pushing tumour margins in comparison to invasive margins (P = 0.036, X^2 *test*) (OR 0.47, p=0.035, CI 0.23-0.94). A non significant association was found with early Dukes stage disease (P = 0.071, X^2 *test*).

3.5.4 Cyclin D1 expression and survival analysis

Survival analysis was calculated using the Cox proportional hazard model. There was a non-significant association between nuclear cyclin expression and the survival (HR 0.59, p=0.091, CI 0.31- 1.08) (figure-3.6). Exclusive cytoplasmic expression was not significantly associated with survival (HR 0.73, p=0.1, CI 0.46-1.16) (figure-3.7). However the overall (i.e. both cytoplasm and nuclear expression combined) survival was significantly associated with survival rates (HR 0.54, p=0.006, CI 0.35-0.84) (figure-3.5).

Figure 3.4 (a&b) showing the immunohistochemistry staining for Cyclin D1 protein in colorectal cancer tumour samples.

Figure 3.4a: Cytoplasmic expression of CyclinD1 protein (High power).

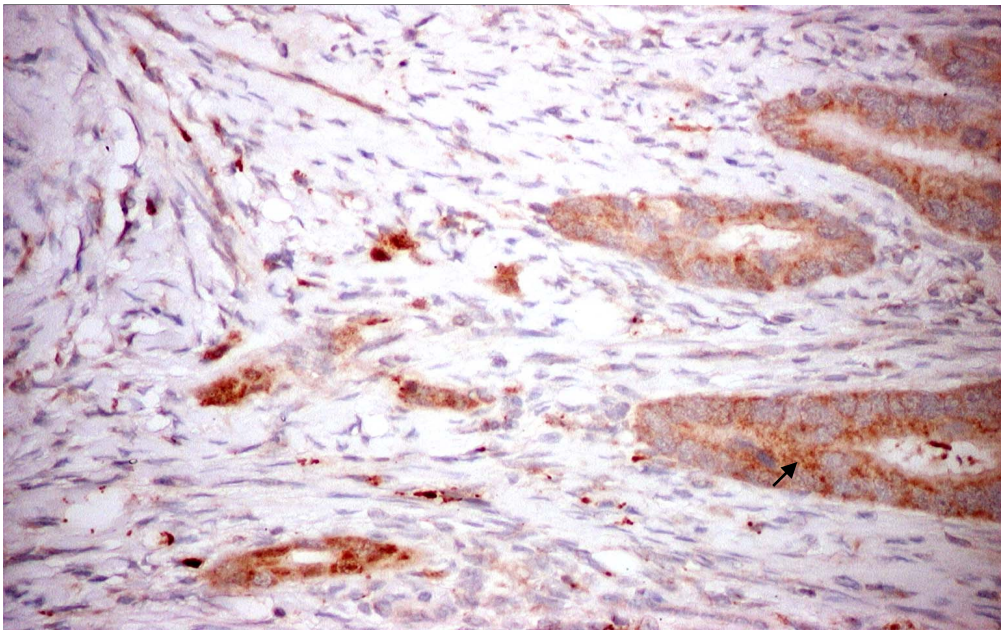
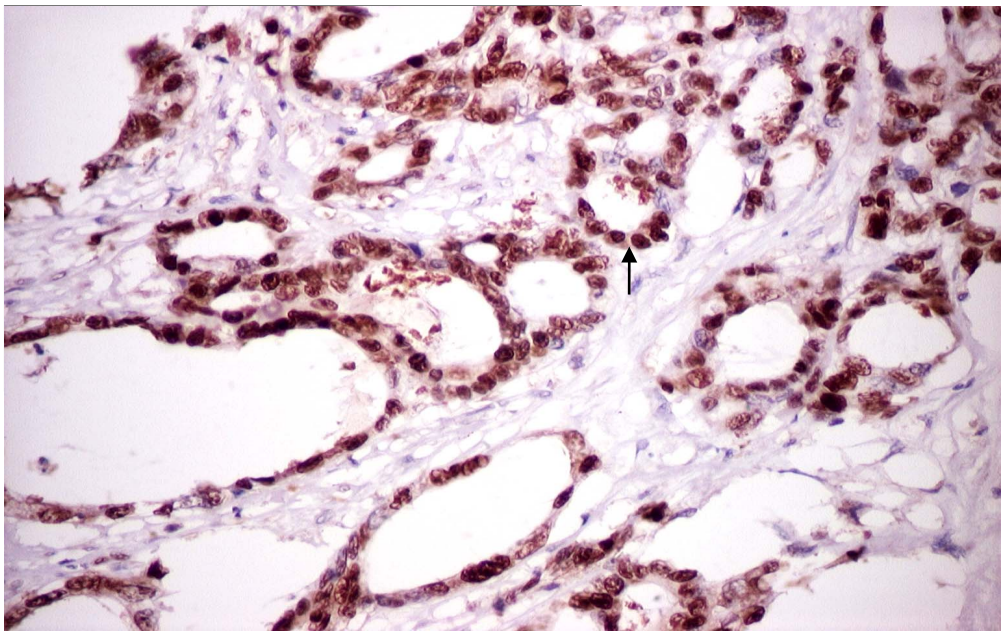


Figure 3.4b: Nuclear staining of Cyclin D1 protein (High power).

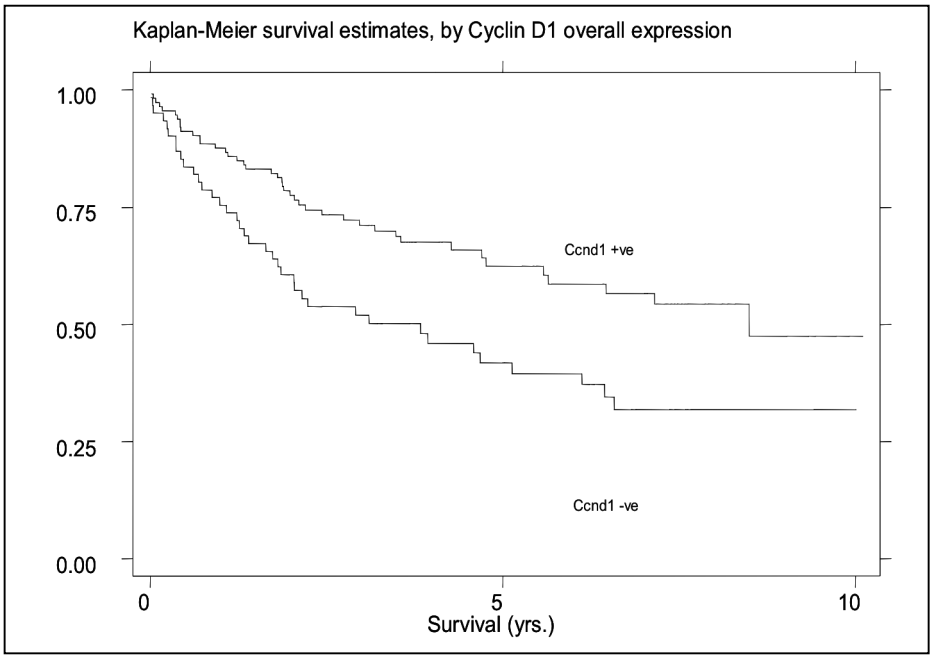


The arrow indicating the dark brown material (5a: Cytoplasmic -Grade 2 and 5b-Nuclear – Grade 3) represents the protein accumulation in these samples.

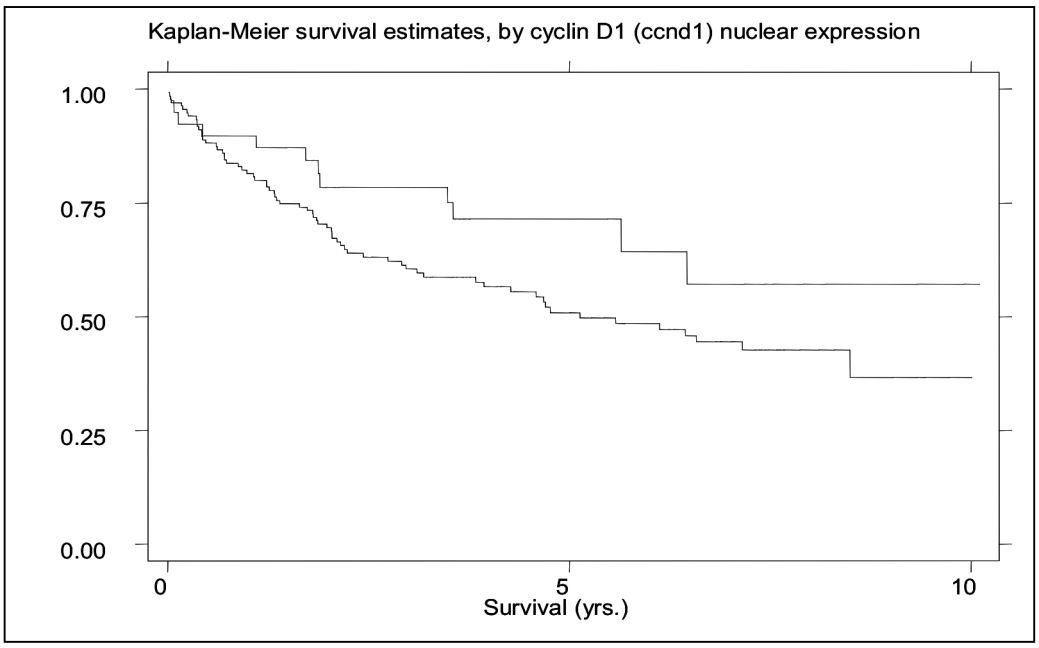
3.5.5 Analysis of influence of CCND1 polymorphisms and Cyclin D1 protein over-expression

We compared all genotypes of CCND1 polymorphisms (A/G^{870}, G/C^{1722} and C/A^{1100}) against Cyclin D1 protein expression using Pearson's chi square test and logistic regression analysis. There were 175 samples available to compare between these three polymorphisms and protein expression. Comparisons were made between individual genotypes and over all expression status. Then the Cyclin D1 genotypes were also compared against cytoplasmic and nuclear expression. There were no significant associations between A/G^{870} genotypes and Cyclin D1 protein over expression. There were no significant associations found with G/C^{1722} and C/A^{1100} polymorphisms and Cyclin D1 protein over expression. We did not find any influence on the survival rates either.

Figure 3.5: Kaplan-Meier curve Cyclin D1 over all protein expression



Figures 3.6 shows Kaplan-Meier survival curves for Cyclin D1 nuclear protein over-expression



Figures 3.7 shows Kaplan-Meier survival curves for Cyclin D1 cytoplasmic protein over-expression

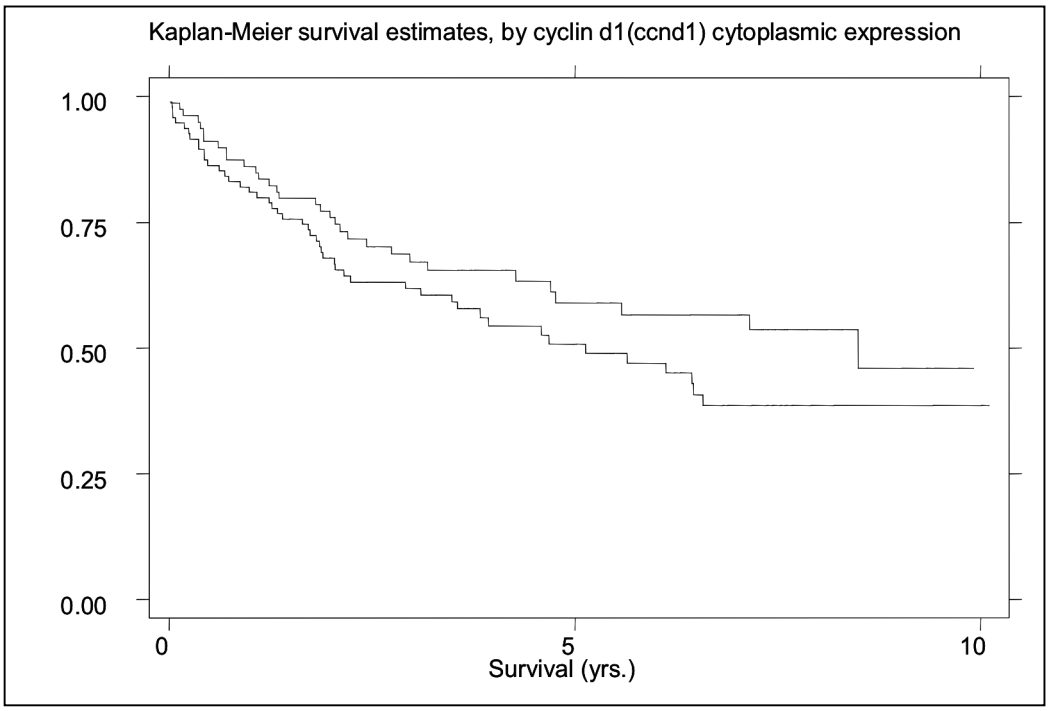


Table 3.6 shows the distribution of clinical variables - Cyclin D1 protein overall expression versus no expression in colorectal cancer patients

Variables	Gender		Age		Tumour differentiation			Tumour site		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
CyclinD1 Expression Present	62(53.91)	53(46.09)	3631.30)	79(68.70)	14(12.17)	75(65.22)	26(22.61)	25(22.73)	85(25.77)	9(7.89)	45(39.47)	46(40.35)	14(12.28)
CyclinD1 Expression Absent	33(54.10)	28(45.90)	17(27.87)	44(72.13)	14(22.95)	35(57.38)	12(19.67)	14(23.33)	46(76.67)	6(9.84)	23(37.70)	24(39.34)	8(13.11)

Variables continued

Variables	Vascular Invasion		Lymph node Involvement			Metastasis		Liver metastasis		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
CyclinD1 Expression Present	43(38.74)	68(61.26)	57(50.44)	32(28.32)	24(21.24)	18(15.65)	97(84.35)	15(18.07)	68(81.93)	45(45.92)	53(54.08)
CyclinD1 Expression Absent	21(37.50)	35(62.50)	29(47.54)	19(31.15)	13(21.31)	18(29.51)	43(70.49)	12(22.64)	41(77.36)	29(59.18)	20(40.82)

Table 3.7 shows the distribution of variables - Cyclin D1 protein nuclear expression versus no expression in colorectal cancer patients

Variables	Gender		Age		Tumour differentiation			Tumour site		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
CyclinD1 nuclear Expression Present	19(48.72)	20(51.28)	9(23.08)	30(76.92)	5(12.82)	24(61.54)	10(25.64)	7(19.44)	29(80.56)	5(12.82)	21(53.85)	11(28.21)	2(5.13)
CyclinD1 nuclear Expression Absent	76(55.47()	61(44.53)	44(32.12)	93(67.88)	23(16.79)	86(62.77)	28(20.44)	32(23.88)	102(76.12)	10(7.35)	47(34.56)	59(43.38)	20(14.71)

Variables continued

Variables	Vascular Invasion		Lymph node Involvement			Metastasis		Liver metastasis		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
CyclinD1 nuclear Expression Present	12(30.77)	27(69.23)	26(68.42)	6(15.79)	6(15.79)	3(7.69)	36(92.31)	2(7.14)	26(92.86)	20(58.82)	14(41.18)
CyclinD1 nuclear Expression Absent	52(40.62)	76(59.38)	60(44.12)	45(33.09)	31(22.79)	33(24.09)	104(75.91)	25(23.15)	83(76.85)	54(47.79)	59(52.21)

Table 3.8 shows the distribution of variables - Cyclin D1 protein cytoplasmic expression versus no expression in colorectal cancer patients

Variables	Gender		Age		Tumour differentiation			Tumour site		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
CyclinD1 cytoplasmic Expression Present	48(59.26)	33(40.74)	29(35.80)	52(64.20)	9(11.11)	55(67.90)	17(20.99)	18(23.08)	60(76.92)	6(7.50)	26(32.50)	36(45.00)	12(15.00)
CyclinD1 cytoplasmic Expression Absent	47(49.47)	48(50.53)	24(25.26)	71(74.74)	19(20.00)	55(57.89)	21(22.11)	21(22.83)	71(77.17)	9(9.47)	42(44.21)	34(35.79)	10(10.53)

Variables continued

Variables	Vascular Invasion		Lymph node Involvement			Metastasis		Liver metastasis		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
CyclinD1 cytoplasmic Expression Present	32(41.56)	45(58.44)	35(43.75)	26(32.50)	19(23.75)	16(19.75)	65(80.25)	13(22.03)	46(77.97)	26(38.24)	42(61.74)
CyclinD1 cytoplasmic Expression Absent	32(35.56)	58(64.44)	51(54.26)	25(26.60)	18(19.15)	20(21.05)	75(78.95)	14(18.18)	63(81.82)	48(60.76)	31(39.24)

3.6 β-catenin (*CTNNB1*)

3.6.1 Mutational analysis

Exon-3 of β-catenin gene was screened for mutations by a method outlined above on 77 tumour DNA samples. All the sequenced samples were meticulously checked for mutation in exon-3 region. Any DNA that showed doubtful changes in the electrophoretic sequence was repeated once again. Mutations were infrequent in our cohort of patients. There were 2 mutations found out of 77 samples (0.03%) (figure 3.8 and 3.9). These were at Codon 61 CAA-CTA and codon 36 CAT-CAG. The mutational change at codon 61 has never been previously reported. We also checked blood DNA sample (once) of the same patient was by DNA sequencing method to ensure that this was not a polymorphism. All the samples have had their β-catenin over-expression status determined. Both these samples with mutations over-expressed β-catenin in the nucleus. All the sequenced traces were shown to the scientific supervisor and counter checked.

Figure 3.8 shows the mutation at codon 61 T to A at exon-3 of β-catenin gene.
Aminoacid change CAA – CTA (Gln – Leu)

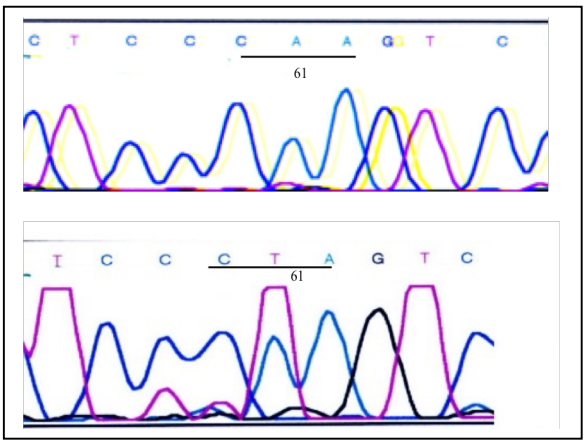
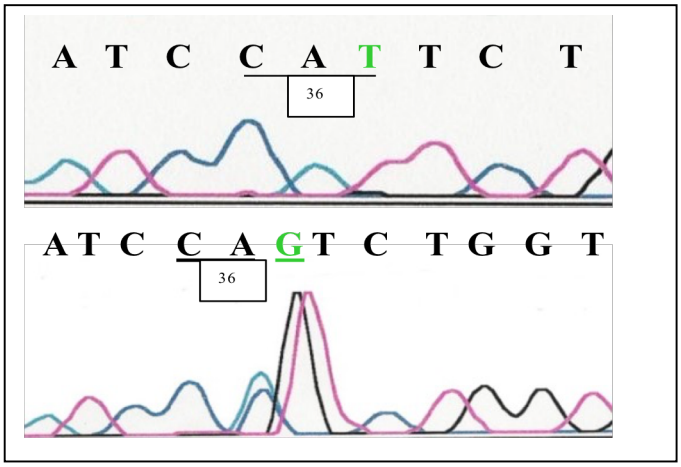


Figure 3.9: Mutation at codon 36 (CAT – CAG) at exon 3 of β-catenin gene
Amino acid chang : His – Gln.



3.6.2: β-catenin protein over-expression

Table 3.9 below shows the distribution of β-catenin over-expression status in CRC patients.

β-catenin Over-expression (n=176)	No. of samples (%)
Nuclear positivity	61(61.0)
Cytoplasmic positivity	51(67.1)
Both (nuclear & cytoplasm) positive	39(39.0)
Both negative	25(32.9)

Table 3.10 below shows the distribution between β-catenin over-expression and the clinical parameters. In tables 3.11 & 3.12, the distribution of variables among nuclear and cytoplasmic over-expressing samples respectively.

Figure 3.10 (a, b & c) shows the immunohistochemistry of β-catenin protein in colorectal cancer samples.
Figure 3.10a shows a faint staining pattern for β-catenin protein which is usually seen in most normal tissues. Figure 3.10b shows intense cytoplasmic staining (Grade 3) and figure 3.10c with nuclear accumulation of β-catenin (Grade 3) indicated by the arrow point.

Figure 3.10a: Normal colonic mucosa.

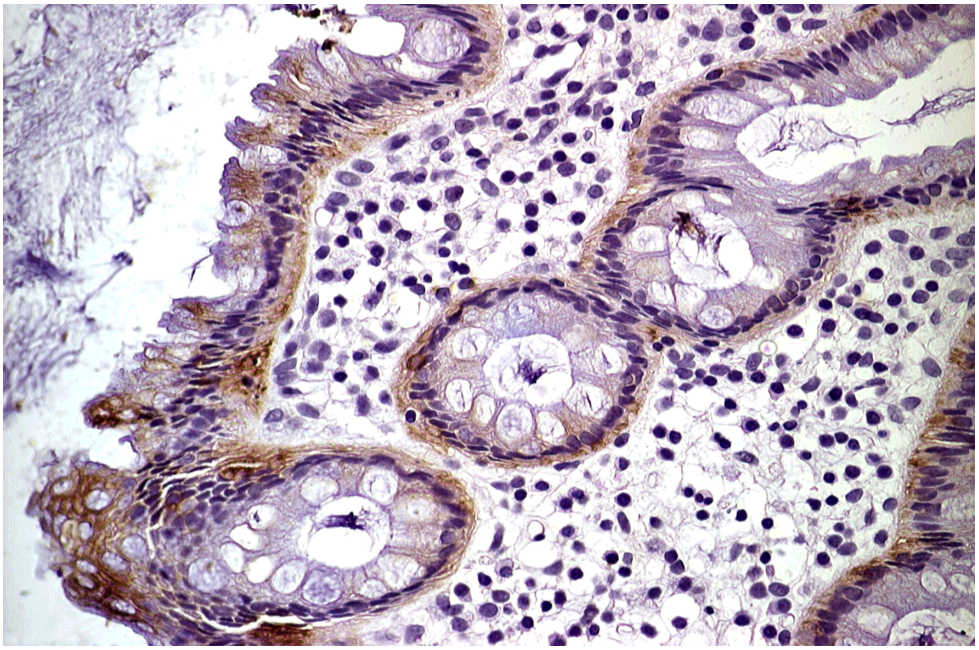


Figure 3.10b: Cytoplasmic β-catenin expression (High power). The arrow indicates the dark staining of protein within the nucleus.
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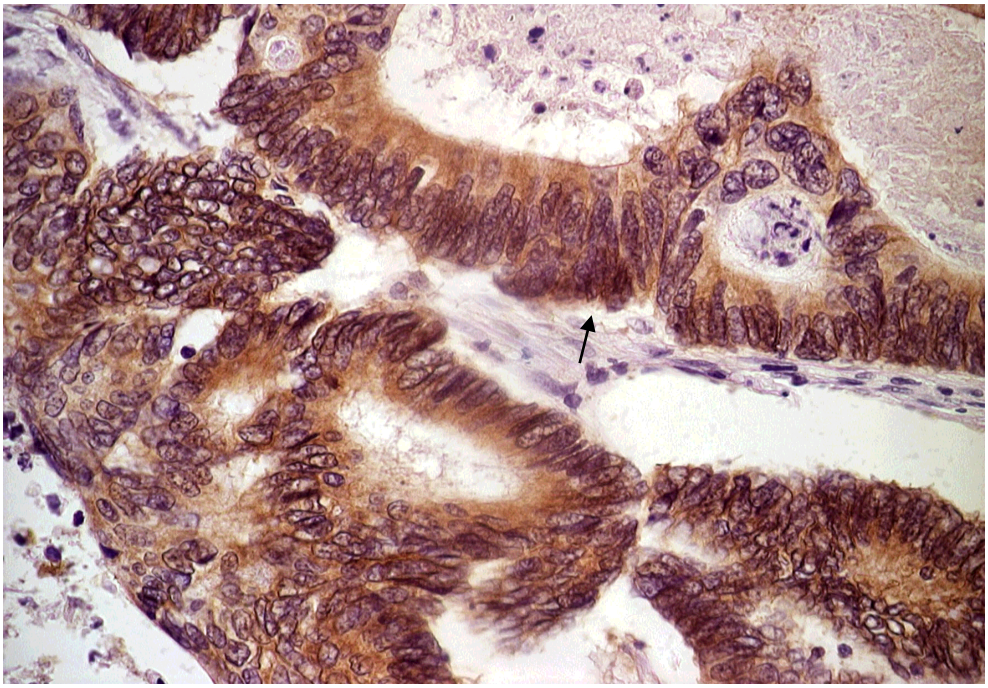

Figure 3.10c: Nuclear β-catenin expression (High power). The arrow indicates the dark staining of protein within the nucleus.

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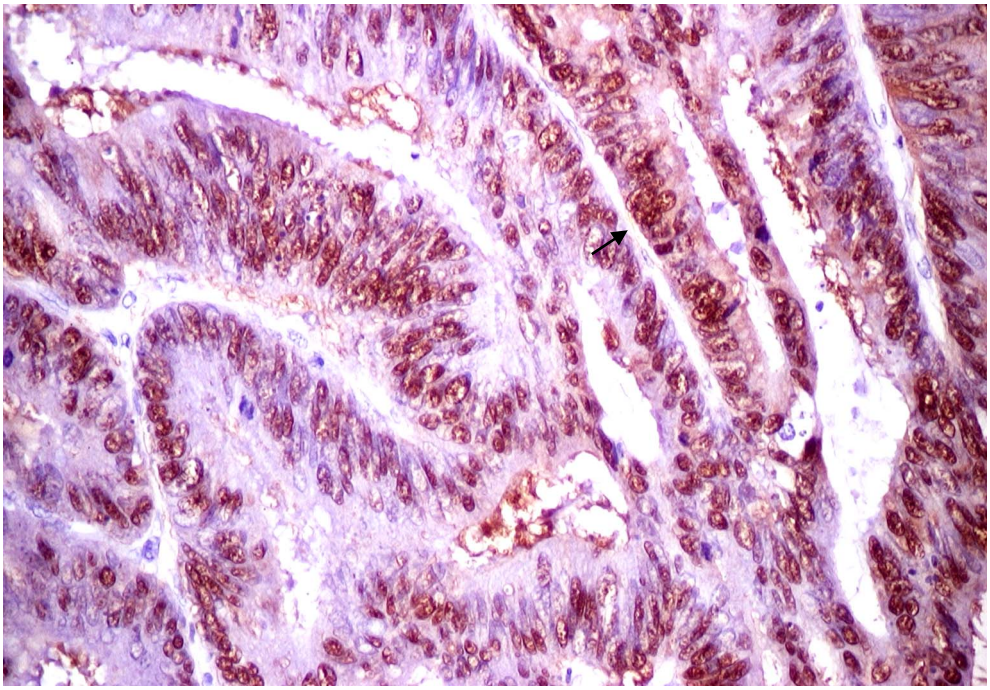


β-catenin overall expression was significantly associated with male gender (OR 2.88, p=0.021, CI 1.17-7.10) (table-3.10). There were no significant associations between age, tumour grade or stage of the disease. A non-significant association was found with patients showing liver metastases (n=136) (p=0.071). There were no significant associations found with histological variables such as vascular invasion, host lymphocyte reaction, site of the tumour (Right colon i.e. caecum to mid transverse colon Vs Left colon i.e. mid transverse colon to sigmoid colon), resection margins etc. Survival rate showed non-significant association with β-catenin over-expression (HR 1.92, p=0.09, CI 0.88-4.12) (table-3.10).

Nuclear over-expression of β-catenin showed a non-significant association with well-differentiated tumours (p=0.09 χ^2 test). There were no significant associations

between age, gender, histology and stage of the disease. There was no significant association with the survival rates. Nuclear staining was consistently noted at the invasive edge of the tumour samples (table-3.11).

Significant cytoplasmic over-expression associated with male gender (OR 1.82, p=0.053, CI 0.99-3.29). Cytoplasmic over-expression was also significantly associated with nodal involvement (p=0.05 χ^2 test). On further analysis, cytoplasmic over-expression tends to be involved with distant (N2) nodal metastasis than immediate nodal involvement (N1) (OR 0.43 p=0.021, CI 0.21-0.88). Cytoplasmic over-expression was also significantly associated with liver metastasis (OR 2.54, p=0.039, CI 1.04-6.15). There was non-significant association with survival (HR 1.18, p=0.09, CI 0.96-1.45) (table-3.12).

Those with β-catenin in the nucleus as well as cytoplasm (n=39) did not show any significant association with clinical parameters. The samples (n=25) that had no strong over-expression of β-catenin had no significant effects on the clinical outcome.

Figure 3.11 below shows the Kaplan-Meier survival curve for colorectal cancer patients with cytoplasmic β-catenin expression

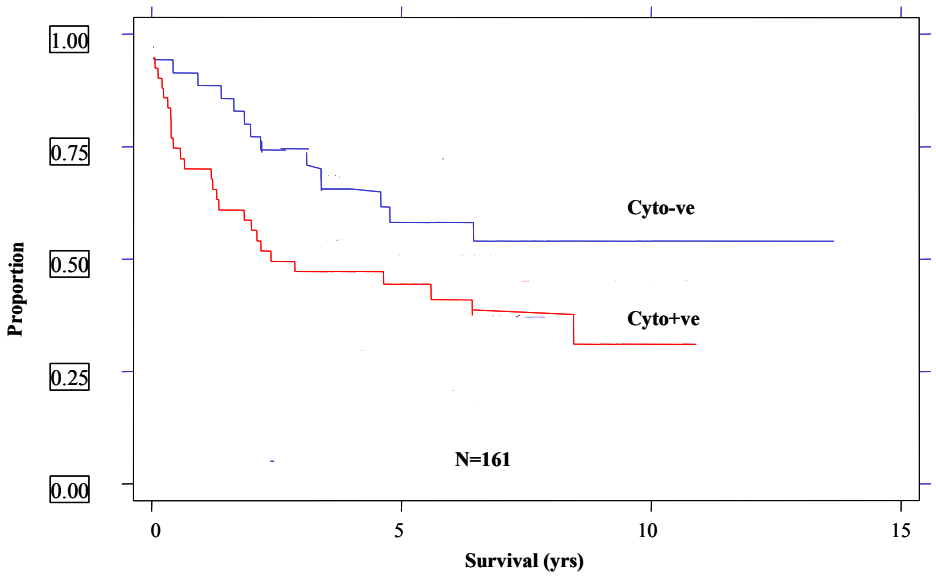


Figure 3.12 below shows the Kaplan-Meier survival curve for colorectal cancer patients with nuclear β-catenin expression

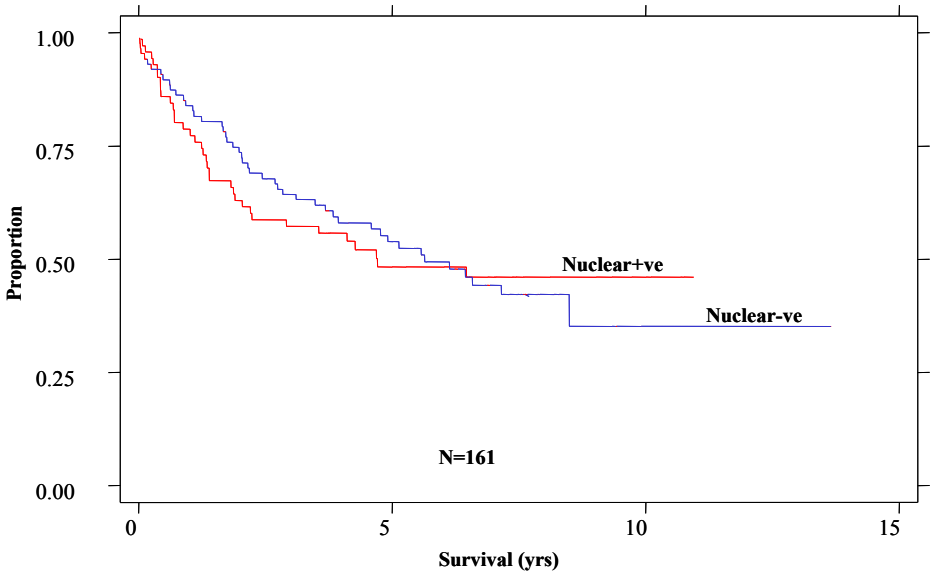


Table 3.10 shows the distribution of over all expression of β-catenin protein (BC) and non-expressers

Variables	Gender		Age		Tumour differentiation			Tumour site*		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
B-catenin Expression Present	87(57.62)	64(42.38)	44(83.02)	107(86.99)	25(89.29)	95(86.36)	31(81.58)	36(24.83)	109(75.17)	13(8.61)	60(39.74)	56(37.09)	22(14.57)	
B-catenin Expression Absent	8(32.00)	17(68.00)	9(16.98)	16(13.01)	3(10.71)	15(13.64)	7(18.42)	3(12.00)	22(88.00)	2(8.33)	8(33.33)	14(58.33)	0(0.00)	

Variables continued

Variables	Vascular Invasion*		Lymph node Involvement			Metastasis		Liver metastasis		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
B-catenin Expression Present	55(38.19)	89(61.87)	76(50.67)	42(28.00)	32(21.33)	33(21.85)	118(78.15)	26(22.41)	90(77.59)	62(48.82)	65(51.18)
B-catenin Expression Absent	9(39.13)	14(60.87)	10(41.67)	9(37.50)	5(20.83)	3(12.00)	22(88.00)	1(5.00)	19(95.00)	12(60.00)	8(40.00)

* Indicates that some clinical parameters had slightly decreasing numbers due to statistical package. These are due to incomplete filling of certain parameters during the analysis.

Table 3.11 shows the distribution of variables in β-catenin protein (BC) nuclear expression

Variables	Gender		Age		Tumour differentiation			Tumour site		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
BC nuclear Expression Present	59(59.00)	41(41.00)	34(34.00)	66(66.00)	20(20.00)	63(63.00)	17(17.00)	25(25.51)	73(74.49)	9(9.00)	37(37.00)	41(41.00)	13(13.00)
BC nuclear Expression Absent	36(47.37)	40(52.63)	19(25.00)	57(75.00)	8(10.53)	47(61.84)	21(27.63)	14(19.44)	58(80.56)	6(8.00)	31(41.33)	29(38.67)	9(12.00)

Variables continued

Variables	Vascular Invasion		Lymph node Involvement			Metastasis		Liver metastasis		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
BC nuclear Expression Present	33(34.02)	64(65.98)	48(48.00)	30(30.00)	21(21.00)	18(18.00)	82(82.00)	18(78.15	16(20.25)	63(79.75)	41(49.40)
BC nuclear Expression Absent	31(44.29)	39(55.71)	38(50.67)	21(28.00)	16(21.33)	18(23.68)	58(76.32)	1(19.30)	46(80.70)	32(50.00)	32(50.00)

Table 3.12 shows the distribution of variables in β-catenin protein (BC) cytoplasmic expression

Variables	Gender		Age		Tumour differentiation			Tumour site		Dukes stage			
	Males	Females	<65yrs	>65yrs	well	Moderate	Poor	Right	Left	A	B	C	D
BC cytoplasmic Expression Present	55(61.11)	35(38.89)	27(30.00)	63(70.00)	11(12.22)	57(63.33)	22(24.44)	21(25.00)	63(75.00)	9(10.00)	37(41.11)	28(31.11)	16(17.78)
BC cytoplasmic Expression Absent	40(46.51)	46(53.69)	26(30.23)	60(69.73)	17(19.77)	53(61.63)	16(18.60)	18(20.93)	68(79.07)	6(7.06)	31(36.47)	42(49.41)	6(7.06)

Variables continued

Variables	Vascular Invasion		Lymph node Involvement$_{\infty}$			Metastasis		Liver metastasis*		Host lymphocyte reaction	
	Present	Absent	N0	N1	N2	Present	Absent	Present	Absent	Present	Absent
BC cytoplasmic Expression Present	35(41.67)	49(58.33)	48(53.93)	18(20.22)	23(25.84)	23(25.56)	67(74.44)	18(27.27)	48(72.73)	35(46.05)	41(53.95)
BC cytoplasmic Expression Absent	29(34.94)	54(65.06)	38(44.71)	33(38.82)	14(16.47)	13(15.12)	73(84.88)	9(12.86)	61(87.14)	39(54.93)	32(45.07)

* Indicates p=0.05 for liver metastasis and$_{\infty}$ indicates p=0.04 for nodal involvement (Multiple regression analysis)

3.7 Analysis of the relationship between Cyclin D1 and β-catenin over-expression

We examined the influence of β-catenin over-expression against Cyclin D1 protein expression. Further analysis carried out to see whether there was any effect on the demographics, tumour biology and survival rates. Logistic regression analysis and Cox hazard model were used to compare the variables.

Table 3.13 below indicates the distribution of sample numbers between β-catenin and cyclind1 over-expression

β-catenin over-expression	Cyclin D1 over-expression	
	Yes	No
Yes	95 (54.59%)	54(31.04%)
No	18(10.34%)	7 (4.03%)

* p=0.42 chi^2 test

Out of one hundred and seventy four samples compared, 95 patients over-expressed both the proteins either in the cytoplasm, nucleus or both. However this had failed achieve any significance (p=0.42, X^2 test). There were 18 patients who over-expressed Cyclin D1 protein without β-catenin over-expression and a further 54 patients had β-catenin over-expression did not express Cyclin D1 protein. Only 19 samples over-expressed both β-catenin and Cyclin D1 in the nucleus. 24 out of 174 samples had over-expressed β-catenin in the cytoplasm, had also over-expressed Cyclin D1 in the nucleus. Those who over-expressed both β-catenin and Cyclin D1 were compared against clinical and histological parameters. There were no significant associations found with age (p=0.9, X^2 test) and gender (p=0.7, X^2 test). No significant associations found with

tumour grade (p=0.62, X^2 test), Dukes stage (p=0.63, X^2 test), metastasis (p=0.11, X^2 test), nodal invasion (p=0.33, X^2 test), and vascular invasion (p=0.91, X^2 test).

However those who had over-expression (either in cytoplasm or nucleus) of both Cyclin D1 and β-catenin protein had significantly better 5-year survival rates (p=0.021, X^2 test) (HR 0.43, p=0.019, CI 0.21- 0.87). This effect was persistent after correcting for several variables (age, tumour grade, Dukes stage, nodal status and metastasis). However the over all survival rates failed to achieve significance level (HR 0.66, p=0.067, CI 0.42-1.02) (figure-3.11).

Figure 3.13 – Kaplan-Meier survival curve for samples expressing both β-catenin and Cyclin D1 protein

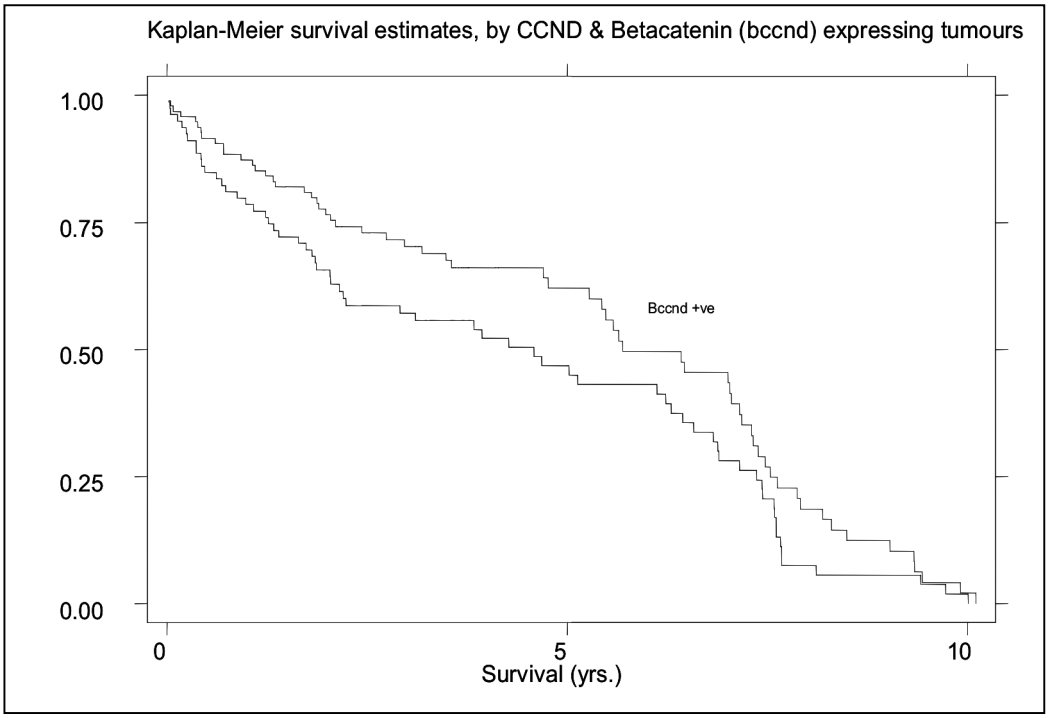


Table 3.14 shows the distribution of variables in patients with both B-catenin and Cyclin D1 over expression (BC & CCND)

Variables	Gender		Age		Tumour grade		
	Male	Female	<65yrs	>65 yrs	well	Moderate	poor
	A	B	C	D			
BC & CCND Expression Present	52(54.74)	43(45.76)	29(30.53)	66(69.47)	10(10.53)	63(66.32)	22(23.16)
BC & CCND Expression Absent	41(51.90)	38(48.10)	24(30.38)	55(69.62)	17(21.52)	46(58.23)	16(20.25)

Dukes stage				
A	B	C	D	
8(8.42)	40(42.11)	34(35.79)	13(13.68)	
7(8.97)	28(35.90)	35(44.87)	8(10.26)	

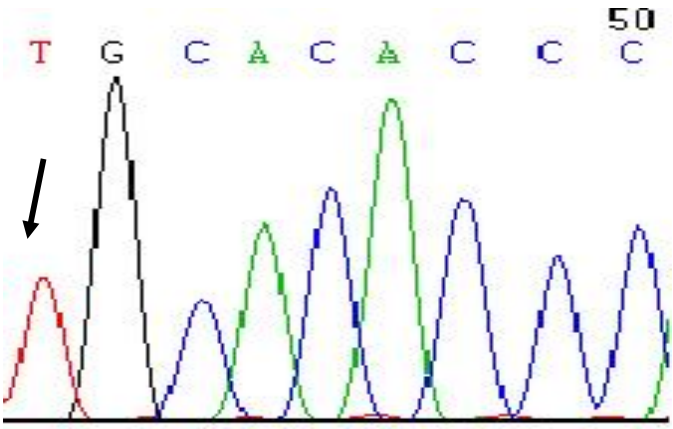
Variables Cont'd	Tumour site		Vascular invasion		Metastasis	
	Right	Left	Yes	No	Yes	No
N0	N1	N2	Yes	No		
BC & CCND Expression Present	22(24.44)	68(75.56)	36(39.13)	56(60.87)	15(15.79)	80(84.21)
BC & CCND Expression Absent	17(21.79)	61(78.21)	28(38.36)	45(61.64)	20(25.32)	59(74.68)

Nodal invasion			Host lymphocyte response	
N0	N1	N2	Yes	No
50(53.19)	23(24.47)	21(22.34)	37(45.12)	45(54.88)
35(44.87)	27(34.62)	16(20.51)	37(58.73)	26(41.27)

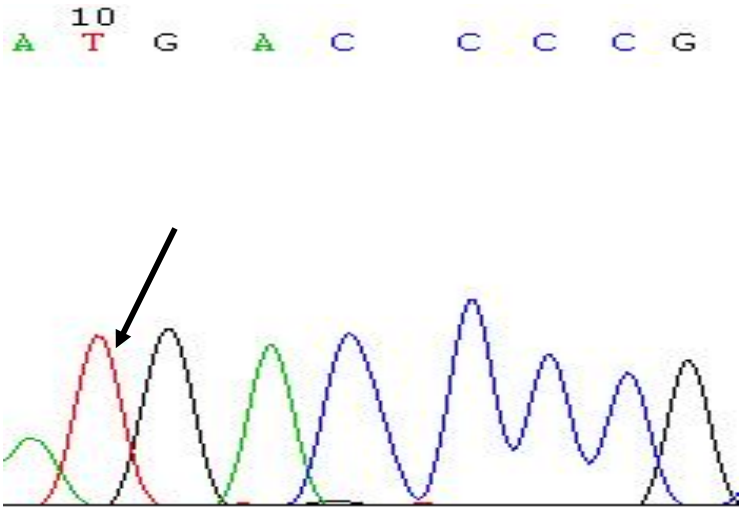
3.8 Cyclin D1 phosphorylation site analysis

A total of 27 samples were analysed for both codon 286 and codon 156. These samples were selected according to the expression status. Eight samples with nuclear expression of Cyclin D1, ten samples with cytoplasmic expression of Cyclin D1 and seven samples with no expression were selected. The DNA were amplified by the PCR method, prepared and were sent to QIAGEN (UK) Laboratory for sequencing. The selections of these samples were based upon the tumour DNA availability and also funding limitations to perform the sequencing analysis. Figures below show the pattern of amino acid distribution in each of the codons (286 and 156) if there was to be an amino acid change. (figures-3.14 & 3.15). None of the samples in our cohort showed any T to A change in the codons. The figures below show the amino acid change if there was to be a mutational change. However we did not find any amino acid change in our samples.

Figure 3.14 & 3.15 below shows the phosphorylation sites (but no actual changes in our series) at codon 286 and 156 respectively (author's own photos).



Arrow indicates the phosphorylation site at codon286 of Cyclin D1 gene where threonine is replaced with alanine (Figure 3.14)



Arrow indicates the phosphorylation site at codon156 of Cyclin D1 gene where threonine is replaced with alanine (Figure 3.15)

CHAPTER 4

DISCUSSION

Introduction

The *CCND1* gene has been a subject of interest to many researchers worldwide. This is evident from number of studies published so far, investigating various aspects of the gene and the pathways it is involved in. Despite the amount of evidence, we do not yet fully understand the mechanisms involved in the regulation of *CCND1* gene and thereby cell proliferation. In my work, I have made an attempt to evaluate polymorphisms within the *CCND1* gene and also investigated the mechanisms that are involved in the over-expression of Cyclin D1. At the beginning of this chapter, we discuss various polymorphisms and their effect on clinical parameters and outcome. Later on we discuss the importance of the β-catenin pathway, over-expression of β-catenin and Cyclin D1 and finally correlate our research findings with clinical outcomes and try to postulate the possible pathway for Cyclin D1 regulation.

4.1: Cyclin D1 (*CCND1*) polymorphisms

4.1.1 A/G^{870} polymorphism

The effect of A/G^{870} polymorphism has been investigated in several epithelial cancers including colorectal cancers (McKay *et al,* 2000, Porter *et al,* 2002, Wang *et al,* 2002 and 2003, Zhang *et al,* 2003). The effect of the each allele within this polymorphism was associated with different outcomes. 'A' allele was significantly associated withincreased susceptibility to cancer in squamous cell carcinoma of head and neck (Zheng *et al,* 2001), poorly differentiated tumours in hepatocellular carcinoma (Zhang *et al,* 2002) and endometrial cancer (Ashton *et al,* 2008). However, Holley *et al,* (2005) demonstrated that G^{870} allele of this polymorphism was associated withincreased susceptibility to oral squamous cell cancers. However McKay and *et al,* (2000) showed no significant associations between this polymorphism and clinical or tumour biological outcomes in colorectal cancer. In our study also there were no significant associations with neither 'A' or 'G' allele though the 'A' allele showed some non-significant association with the vascular invasion. Most of these papers concluded that the effects could be due to the effect of this A/G^{870} polymorphism on Cyclin D1 expression.

The mechanism by which A/G^{870} polymorphism exerts its influence remains unclear. Some believe that this may be due to the effect on splicing of CCND1 gene. It has been demonstrated that the Cyclin D1 A/G^{870} polymorphism modulates Cyclin D1 splicing of two transcripts termed transcript a (Cyclin D1^{tra}) and b (Cyclin D1^{trb}) [Betticher *et al,* 1995 and Knudsen KE *et al,* 2006]. Bala *et al,* (2001) observed the relative abundance of Cyclin D1^{tra} and Cyclin D1^{trb} transcripts could modify the age at onset of in HNPCC. They observed that the presence of Cyclin D1^{trb} was associated with an

increase likelihood of early onset of HNPCC compared to individuals with only Cyclin $D1^{tra}$. Functionally, we, and others have shown that the protein product from Cyclin $D1^{tra}$ (Cyclin $D1^{TRA}$) enhances adherent cellular proliferation (Holley *et al,* 2004). In contrast, Cyclin $D1^{TRB}$ has no effect on adherent monolayer proliferation but enhances the ability of cells to grow in an anchorage independent manner again a classic feature of malignant transformation (Holley *et al,* 2004). These growth strategies may contribute to tumourigenesis and invasion and thereby facilitating cancer progression (Friedl *et al*, 2004).

4.1.2 G/C^{1722} polymorphism

Interestingly this is the first report to comment that the CCND1 C/G^{1722} polymorphism is associated with tumourigenesis in colorectal cancer. A study from our centre previously showed the associations between the CC^{1722} genotype and poorly differentiated tumours and reduced disease free interval in squamous cell carcinoma of the head and neck (SCCHN) (Holley *et al,* 2001). Park *et al,* (2004), used seven polymorphisms in CCND1, (including CCND1 $^{A/G870}$ and CCND1 C/G^{1722}) and observed two major haplotypes but found that statistical analysis with two phenotypes revealed no significant associations. Sathyan *et al* (2008) showed that 'C' allele of this polymorphism was associated with poor survival outcome in oral cancers. Shih *at al* (2012) demonstrated that 'G' allele was associated with less risk of nasopharyngeal cancers.

In our study, the G^{1722} allele was significantly associated with poor tumour grade and vascular invasion whereas the 'C^{1722}' allele was associated with distant metastatic potential excluding liver metastasis. The effect of both alleles seems to have a worse effect on tumour biology and propagation. Though there was no impact on overall survival in this cohort of patients, survival rate was significantly affected those with

vascular invasion (HR 4.56). This may mean that the 'G' allele may be truly associated with poor outcome. It is less clear why the 'C' allele also predicts poor outcome. One possible explanation could be that the smaller sample size when we analysed the effects of distant metastasis ('C'). Future studies are needed to answer these questions.

4.1.3 C/A^{1100} polymorphism

This is the first report of associations between CCND1 $^{C/A1100}$ and any disease. This polymorphism was found in the 3'UTR region of CCND1 gene. Interestingly the Homozygotes with 'A' allele were more likely to be females and it was associated with vascular invasion. On the other hand, the homozygotes to 'C' allele were associated with left sided tumours, less nodal invasion and had early stage disease as determined by the Dukes' classification. It is the first time CCND1 polymorphism showed a significant association with gender and tumour site. This has some importance in clinical application because of its potential use as screening tool in patients with family history. Despite their influence on tumour biology and demographics, the survival rates remain unaffected as in other polymorphisms in our cohort. This effect may well be due to relatively smaller number.

Earlier we have shown that the CCND1 polymorphisms were associated few favourable and some unfavourable outcomes. If these polymorphisms do truly affect the outcome, one would expect to find some impact on survival. This was not the case in our studies. This raises the question as to whether these polymorphisms have a 'combined' influence on tumourigenesis. This was the reason why we looked the combined effects of these polymorphisms in our series.

4.1.4 Combined influence of CCND1 genotypes

Upon investigating additive polymorphic effects we observed that the AA^{870}/GG^{1722} combination was again associated with patients whose tumours showed vascular

invasion. Further interaction analysis demonstrated that this effect was probably driven by the CCND1 A/G^{870} polymorphism. Even though when the CCND1 A/G^{870} was examined on its own showed no significant associations, this may be due to the relatively smaller numbers of patients examined. On the other hand, it is possible that the true effect of 'A^{870}' allele may have been masked by factors such as allelic influence by some other polymorphisms within this gene. Poor prognosis has been associated with the CCND1 AA^{870} genotype in non-small cell lung cancer (Betticher *et al,* 1995). The CCND1 AA^{870} genotype has shown associations with forms of colorectal disease that result in severe morbidity and mortality in advanced colorectal cancer patients (Le Marchand *et al,* 2003).

Holley *et al,* (2001) from our own centre, observed that although CCND1 G^{870} and C^{1722} alleles were in linkage disequilibrium in their patients with squamous cell carcinoma of head and neck (SCCHN), their influence on tumour pathology and patient outcome was different. CCND1 GG^{870} was an independent marker for tumour recurrence in SCCHN, whereas CCND1 CC^{1722} was associated with tumour recurrence by a mechanism associated with tumour biology, mainly through differentiation. In our cohort CCND1 A/G^{870} was associated mainly with patients whose tumours showed (non-significant) vascular invasion and CCND1 C/G^{1722} alleles were associated with tumour differentiation. This seemed to be supported via the finding of the haplotype analysis which elucidated that the presence of the A^{870} allele in the CCND1 A^{870}/G^{1722} haplotype was associated with patients whose tumours showed vascular invasion, and that presence of the G^{870} allele in a haplotype with either of the CCND1 C/G^{1722} alleles was associated with fewer patients presenting with tumours showing vascular invasion. This may mean that 'A' allele of CCND1 A/G^{870} polymorphism may influence outcome only in combination with other genotypes.

The mechanism through which CCND1 alleles might exert an effect on colorectal tumourigenesis may be through the influence that these alleles have on Cyclin D1 over-expression. In some primary tumours and cell lines Cyclin D1 mRNA of 1.5, 2 and 3 kilo bases in size can be detected in addition to the normal 4.5-kb mRNA. The smaller transcripts result from deletions may be responsible for the short half-life of the wild type mRNA. Northern blot analyses confirm the steady state of Cyclin D1 transcripts is substantially increased in mantle cell lymphoma cells with complex rearrangements. In the recent years there have been some interest shown towards the untranslated region. It is believed mutations in this region could affect the structure of the Cyclin D1 and thereby altering its half-life (Rimokh *et al,* 1994, Conne *et al,* 2000). Cyclin D1 C/G^{1722} and A/C^{1100} may therefore have distinct functional effects in colorectal carcinoma.

Our study demonstrates that CCND1 polymorphisms CCND1 A/G^{870} and CCND1 C/G^{1722} affect tumour biology such as tumour differentiation status and the adverse parameter of vascular invasion of tumours. However the effect on vascular invasion is mainly seen when we combine the genotypes. We observed no impact of CCND1 polymorphism on patient survival. Haplotype analysis revealed that these polymorphisms may have an effect when they act in combination. From all of the above, we hypothesise that polymorphisms in Cyclin D1 may have impact tumour biology and possibly the survival outcome. Though our results appear to be very interesting, the numbers of individuals in our genotype groups were relatively low and, therefore, one has to be cautious in interpreting these results. Larger scale molecular epidemiological studies are required to both confirm associations found in this study and comprehensively investigate genotype interactions and haplotypes within Cyclin D1 in colorectal and other cancers.

4.2 Cyclin D1 Protein over-expression

Cyclin D1 over-expression has remained a topic of interest for many researchers. Various pathways have been linked to Cyclin D1 activation and its subsequent over-expression. In our cohort of colorectal cancer patients, the overall Cyclin D1 over expression was 65.34% whereas in another study that examined colorectal cancer it was 48% (Maeda *et al,* 1998) and Holland *et al,* (2001) showed 58.7% over expression. The rate of over expression also appears vary with type of epithelial cancer. In some studies Cyclin D1 over expression was shown to be as low as 34% in human breast carcinoma (Rey *et al,* 1998).

The reason for this variation in the level of expression among colorectal cancer studies may be explained by the number of patients in the study group, time period at which the sample was examined and also the area of the tumour where the sample was taken. Allowing for the above mentioned reasons, it is somewhat difficult to explain the variation among different epithelial cancers. It may be that the intensity and/or the type of mitogenic stimuli involved in the *ccnd/cdk* pathway are different. Another possible mechanism can be due to the defect within the Cyclin D1 gene such as gene amplification (Dhar *et al,* 1999) or errors within the phosphorylation site (T286A). Recently Pontano *et al,* (2008) suggested that genomic errors that occur in the degradation pathway involving $SCF^{Fbx\ 4\text{-}ABcrystallin}$ E3 ubiquitin ligase could lead to accumulation of Cyclin D1 in the cell.

Nuclear (29.6%) and cytoplasmic (65.2%) expression in our study were also comparable to other study groups (Holland *et al,* 2001). Some earlier studies did not included cytoplasmic staining (Maeda *et al,* 1998). On the other hand McKay *et al,* (2000) have commented mainly on cytoplasmic expression. Our study reports the effects of overall expression, absent as well as nuclear and cytoplasmic expression. We

believe that it is important to elucidate the effects of Cyclin D1 protein in all possible ways to try and obtain a clearer picture on associations and mechanisms which can potentially affect the tumour behaviour in human cancers.

In our cohort, overall expression was not associated with age, gender. There was no significant association with right or left sided tumours although two studies noted the prevalence of Cyclin D1 expression in right sided tumours (Holland *et al,* 2001, Palmqvist *et al,* 1998). Overall Cyclin D1 positivity was related less metastatic potential ($p=0.03$) and less propensity for distant metastases ($p=0.0001$). Interestingly exclusive nuclear expression was associated with early Dukes' stage and node negative tumours. Samples with exclusive cytoplasmic expression showed significant host lymphocyte reaction. On the other hand, in papillary thyroid micro-carcinomas, Cyclin D1 over-expression was significantly associated with lymph node metastasis (Khoo *et al,* 2002). In human hepato-cellular carcinomas, cytoplasmic D1 expression was significantly associated tumour thrombus in portal vein and intra hepatic metastasis (Sato *et al,* 1999). The exact reason for these variations in the outcome of Cyclin D1 protein expression remains unclear. One explanation would be that there may be several cross link pathways involved in different epithelial cancers and may independently alter the tumour biology regardless of Cyclin D1 status.

It appears that nuclear Cyclin D1 expression had less aggressive tumour biology and so as the cytoplasmic Cyclin D1 over-expression. Irrespective of the Cyclin D1 protein localisation these patients seem to have demonstrable 'protective' effect. The influence of cytoplasmic Cyclin D1 on host lymphocyte response has never been shown in any studies. It believed in general that tumours with host lymphocyte reaction (Buckowitz *et al,* 2005, Romano *et al,* 2004) would do better than those without. It may be that patients with cytoplasmic D1 are protected against disease progression because they

evoke a better host immune response. If this were true, this would have significant impact on deciding adjuvant therapy in colorectal cancer patients.

There aren't many studies that examined the effect of Cyclin D1 expression on survival. In a previous study from our centre (Holland *et al,* 2001) it was shown that both nuclear and cytoplasmic Cyclin D1 over-expression were associated with improved survival rates. Holland's study, showed low hazard ratios (Nuclear D1- HR 0.53, Cytoplasmic D1- HR 0.73) indicating the better survival outcome although the individual (cytoplasm or nuclear Cyclin D1 protein) 'p' values have failed to reach any significance level. On the other hand, the overall survival rates remain significantly high (p=0.006). This is probably through the influence of Cyclin D1 protein on tumour biology.

In our study, we did not analyse the outcome of patients expressing Cyclin D1 protein in cytoplasm and nucleus since the numbers were too low for subgroup analysis. From the findings that were mentioned thus for, we expect patients with both nuclear and cytoplasmic Cyclin D1 protein would have a favourable clinical outcome. We do not know the underlying mechanism for this effect. It may involve one or more of the cross pathways between Cyclin D1 and other factors such as *DACH1*(Wu K *et al,* 2006).

4.3 CCND1 polymorphisms and their influence on Cyclin D1 protein expression

The influence of polymorphisms on protein expression has been an area of interest in many genes including CCND1 gene. There are some associational studies that examined the influence of A/G^{870} polymorphism on Cyclin D1 protein over-expression and had found no significant associations (McKay *et al,* 1997). This polymorphism has gained significant attention because of the fact that it alters splicing in exon 4 of CCND1 gene giving rise to transcript A and B (Betticher *et al,* 1995). These transcripts have different half-life. This could potentially affect how the Cyclin D1 protein is degraded within the cell. It has been said that transcript B has a longer half-life and, therefore, could remain in the cell for longer periods. This would mean that this protein could continue to promote the (Sherr, 2000) phosphorylation of the retinoblastoma protein and thereby contributing to the cell proliferation.

In many studies it was shown that A/G^{870} polymorphism was not significantly associated with Cyclin D1 protein over-expression. This was also case in our study as well. This raises several questions on the nature and mode of action of these transcripts. It may be that the polymorphism could be responsible for the production of these two transcripts. But the influence of the transcripts on the accumulation of the Cyclin D1 protein within the cell remains to be further studied. It may that there are other factors involved in the regulation of Cyclin D1 protein expression along with transcripts.

We also for the first time showed the effects of two other polymorphisms (G/C^{1722} and C/A^{1100}) that exist in the 3'UTR region of the CCND1 gene. These polymorphisms showed some associations with tumour biology and progression of the disease. However, they had failed to demonstrate any significant impact on the protein

expression. These results indicate that polymorphisms do not appear to have direct demonstrable effect on protein expression.

We demonstrated in the earlier chapters that CCND1 genotypes could effect the tumour behaviour in combination. This was shown by the haplotype analysis. These results suggest that polymorphisms could function together as a unit and alter the expression status. We did not compare Cyclin D1 expression with haplotypes due to small numbers available for the subgroup analysis. If we assume that genotypic interactions influenced gene expression, then this theory could explain the relationship between A/G^{870} polymorphism and the transcript *b* and their effects (Betticher *et al,* 1995). That is to say that A/G^{870} polymorphic alleles though they alter splicing, the effects of the transcripts may be through the additional effect of other polymorphic alleles within CCND1 gene (Knudsen KE *et al,* 2006).

4.4 Cyclin D1 phosphorylation sites

Cyclin D1 phosphorylation has been under investigation *in vitro* by some investigators in the last few years. Diehl *et al,* (1997) worked on some of the phosphorylation sites namely codon 286 and codon 156 (Diehl AJ *et al,* 1997). In their *in vitro* studies, they had showed, by replacing threonine to alanine at these codons prevented phosphorylation of Cyclin D1 in the cell and thereby its accumulation. This prompted further investigations by one or to groups. However, these works were done mostly *in vitro* and mouse models (*in vivo*) (Germain *et al,* 2000, Guo *et al,* 2005).

We wanted find out whether the mutational change in phosphorylation sites led to the over-expression of Cyclin D1 in human malignancies. There were no such mutational changes noted in the small number of patients in our cohort.

The mechanism of Cyclin D1 accumulation in the cell occurring as a result of phosphorylation site mutation is far from clear. Mutation at these sites leading to

replacement of the amino acid threonine to alanine interfered with polyubiquitinisation of Cyclin D1 protein. This resulted in prolongation of Cyclin D1-CDK complex half-life up to 3.5 hours (Normal half-life is <30 minutes). It is argued that this could lead to hyper-phosphorylation of retinoblastoma protein and thereby cell proliferation. However this has not been studied in any epithelial cancers.

Another investigation by Diehl *et al,* (1997) was on codon 156. This work showed that 156A mutational change could lead to deficient phosphorylation of Cyclin D1. They also suggested that threonine to alanine change could effect the transport of Cyclin D1 in to nucleus. Some mutational changes at codon 156 (i.e.: T156A, T156E) affects the phosphorylation by CDK-activating kinase (CAK) leading to inactive Cyclin D1-CDK4 complexes in the cytoplasm. CAK is not required for the transportation of these complexes in to the nucleus. Moreover the Cyclin D1-CDK4 complex is resistant to ubiquitinisation (Diehl *et al,* 1997). This may be the reason for cytoplasmic over expression of Cyclin D1 seen in some epithelial cancers with no significant clinical associations. Although we used small number of samples in our study, the results indicate that mechanism may be less common in colorectal cancers. Larger scale studies are needed to answer this question.

4.5 β-catenin mutations

β-catenin is a gene that has sixteen exons. Most of the studies to date have studied the exon 3 region where there is an 'armadillo repeat region'. This is the region where most of the mutation occurs and that's why there has been interest. There are no documented elaborate studies involving other exons within the β-catenin gene. Exon 3 of the β-catenin mutations have been investigated in many epithelial cancers (Palacios J 1998, Sparks *et al,* 1998, Park *et al,* 1999). The frequency of these mutations varies with the type of epithelial tumour. In hepatoblastomas, β-catenin mutations were very frequent (50-90%) (Armengol C *et al,* 2009). On the other hand, in colorectal cancer the mutations were less common (Kitaeva *et al,* 1997); they found 2 mutations out of 92 colorectal cancer samples. We screened exon 3 of the β-catenin for mutations and found 2 out of 77 samples. Mutations can occur in any of the aminoacids in the 'armidello' repeat region that codes for phosphorylation (e.g codon 37 TCT to TTT, codon 41 ACC to GCC). The heterozygous mutation we found at codon 61 has not been reported previously. This mutation changed the amino acid from *GLN to LEU*. The two samples with codon 61 mutations both over-expressed β-catenin in their nucleus. This once again indicates the importance of integrity of the phosphorylation sites' in exon 3 and its important role in the regulation of β-catenin degradation *in vivo*. However the vast majority of the samples that over expressed β-catenin whether in the nucleus or in the cytoplasm did not exhibit any mutational changes in exon 3. There are several possible explanations for this phenomenon: mutations involving APC gene at the β-catenin scaffolding site interferes with the phosphorylation of β-catenin (Polakis P 1999), mutations involving GSK 3-β can also lead to improper β-catenin degradation. These pathways in conjunction with β-catenin expression appear to result in a complex pathway that requires further work. For the purposes of our study and within the time

constraints available, we restricted the investigations to the mutational analysis of β-catenin alone.

4.6 β-catenin protein over-expression

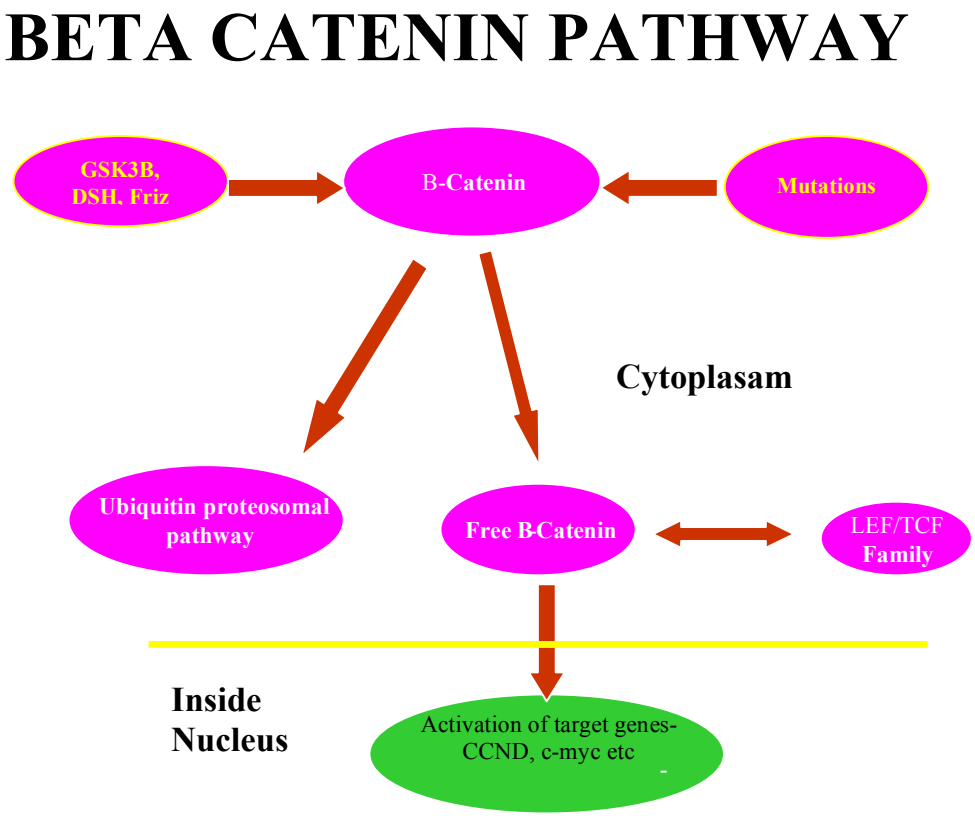
β-catenin over-expression is a common feature in colorectal cancer. The amount of expression ranges from 70-90% based on previously reported studies (El-Bahrawy *et al,* 2002, Yanagisawa N *et al,* 2001). In our study, 86.4% of the patients were found to over-express β-catenin in the nucleus, cytoplasm or both. Nuclear expression was significantly associated well-differentiated tumours and early stage disease. Similar finding had also been reported in endometrioid ovarian cancer (Palacios J 1998). However in hepatocellular carcinoma (Nhieu J *et al,* 1999, Lee *et al,* 2003), nuclear β-catenin was associated with aggressive tumour behaviour. The exact pathophysiology for the phenomenon is unclear. One common explanation would be tissue specificity, which may be an important factor in β-catenin expression. The other possibility would be tumour progression through E-cadherin mediated pathway.

β-catenin expression, particularly the cytoplasmic expression was significantly associated with male gender in our cohort of patients. This finding has never been shown in any other study previously. Is also has to be noted that patients with cytoplasmic over-expression did have metastatic potential to liver and lymph nodes. However this group of patients showed a non significant association with survival. These findings suggest that cytoplasmic expression may play a role in tumour progression and promote metastasis. Hence the β-catenin could potentially be used as a prognostic marker in colorectal cancer.

The reason for the poor outcome in patients with cytoplasmic expression warrants further investigation. The role of excess cytoplasmic β-catenin protein can be due to several reasons. Perhaps the most important one would be its transportation in to the

nucleus by the transcriptional factors (TCF and LEF). The cytoplasmic protein is resistant to phosphorylation and therefore degradation by the proteosomal pathway (figure-4.1). This could be due to mutations in exon 3 of β-catenin gene, mutations occurring in the mutation cluster region of APC gene or any of the upstream genes in the Wnt signal pathway. It is known that the cells with β-catenin accumulation as a result of APC gene mutation can be resistant to apoptosis (Macleod, 2000). This may contribute to the ongoing tumour progression. From our study it appears that cytoplasmic protein does have an active role in metastasis. The may be achieved through the alteration of cell adhesion properties, as β-catenin is a submembrane protein. This pathway certainly requires investigation because this has implications on factors such as adjuvant therapy and disease free survival.

Figure 4.1 below shows the mechanistic pathway of β-catenin in tumourigenesis (Authors version)



4.7 Influence of β-catenin on Cyclin D1 protein expression

We compared the expression status of both β-catenin and Cyclin D1 protein expression in colorectal cancer patients. In our study, just over 50% (95/176) of the tumour samples had significant expression of both proteins. This value failed to reach any statistical significance. Wong *et al,* (1999) showed that β-catenin expression was significantly associated with Cyclin D1 expression although the study group was small (n=53). It was also stated that only the samples that over expressed in the nucleus had over expressed Cyclin D1. In our study, there were 31% of the samples that over-expressed β-catenin did not have significant Cyclin D1 expression. A small number of samples expressed Cyclin D1 in the cell without significant β-catenin expression.

Cyclin D1 is one of the target genes for activated nuclear β-catenin. This pathway is said to be one of the important pathway for CCND1 gene activation. (Brabletz *et al,* 2001). If this were the case, our cohort also would have shown the same result showing significant association between β-catenin and Cyclin D1. Our findings on the other hand showed no significant associations between these two genes. To support this argument, a recent study by Lee *et al,* (2003) showed no correlation between β-catenin expression and Cyclin D1 expression in hepato-cellular carcinoma.

In our series there were cases expressing β-catenin but without Cyclin D1 expression. This suggests that nuclear transport of β-catenin could be regulated by the factors that influence LEF/TCF pathway. In the case of β-catenin in the nucleus but with out Cyclin D1 expression raises the question whether the nuclear β-catenin need additional factors in order to activate Cyclin D1. It is also to be noted that there were samples with out β-catenin expression that showed D1 expression. This may be due to mechanisms other than β-catenin that involves Cyclin D1 protein expression. Our study indicates Cyclin

D1 expression is governed by more than one mechanistic pathway. Further studies are needed to elucidate pathways that are involved in this complex process.

The clinical parameters were analysed on patients those expressed both β-catenin and Cyclin D1. There were no significant associations found with the variables. Interestingly these groups of patients (those with β-catenin and Cyclin D1 expression) had a better survival rates in the first five years. This effect failed to remain significantly better after five years. We believe that this effect may be due to the presence of Cyclin D1 protein than β-catenin.

4.8 Other factors that could potentially influence Cyclin D1 regulation

In the previous chapters we have discussed about the major pathways such as β-catenin that are involved in the regulation of Cyclin D1. There have in vivo and in vitro studies that attempt to identify more and more substances involved or potentially could interfere with Cyclin D1 regulation. Some of the other genetic pathways that could affect the Cyclin D1 regulation are discussed below.

4.8.1 Cyclin D1 gene amplification

Amplification of CCND1 gene has been suggested as a possible cause for protein over-expression in colorectal cancer. There are studies that looked at the protein levels in relation to CCND1 amplification (Dhar *et al,* 1999, Khoo *et al,* 2002). Many of these quoted studies did not find significant correlation between amplification and Cyclin D1 protein expression. Gillett and *et al,* (1994) showed that despite the CCND1 amplification in breast tumours, protein expression was found to be weaker in their study group. They argued that the amplified/rearranged CCND1 allele may have had reduced transcriptional activity. These findings suggest that CCND1 gene amplification may not be an important pathway in Cyclin D1 protein expression.

The exact mechanism of amplification of 11q13 loci and hence Cyclin D1 is not clearly defined (Khoo *et al,* 2002). Several studies have been attempted to evaluate this chromosomal segment. Researchers have used techniques such as fluorescence in situ hybridisation (FISH), mapping techniques and Southern blot analysis in order to mar the 11q13 amplicon core. In one study, a new segment was identified and this was situated close CCND1 gene. The so-called *TSOA1* (tumour amplified and over-expressed sequence1) is frequently amplified and over-expressed in tumour cell lines. It was suggested that this could be a driving force for the amplification of 11q13 amplicon or at-least one of the factors responsible (Huang *et al,* 2002). It would be interesting to study *TSOA1* along with CCND1 to see which one of them influence the other if they do so.

4.8.2 *Kras* mutations

Kras mutations have been studied over the past two decades. Approximately 40% of the carcinomas can exhibit *ras* mutations (Yamazaki eta al 2003). Most of these mutations occur in codon 12 and 13. Cyclin D1 expression can occur through ras signalling pathway. Some animal and in vitro studies have shown Cyclin D1 expression is stimulated through mitogen activated protein kinase (MAPK). It also has been stated that *MAPK* pathway can independently regulate protein expression (Albanesse *et al,* 1995, Arber *et al,* 1996).

4.8.3 Other mechanistic factors

*STAT*s (signal transducers and activators of transcription): *STAT*s are a family of transcriptional factors that are activated in response to cytokine and growth factor stimulus. There are seven different types of *STAT*s identified in mammalian species. Among those *STAT 3* has been found to have oncogenic properties. *STAT 3* not only have cellular growth potential but also have the ability to interfere with apoptotic

pathways (Ma *et al,* 2003). In colorectal cancers, *STAT 3* was over expressed along with Cyclin D1 protein (Ma *et al,* 2003). It is hypothesised that *STAT 3* could affect the down stream genes through its transcriptional activity. The exact pathway is not clearly understood.

Egr-1/PTEN: *Egr-1* (early growth response-1) is a transcriptional factor that is involved in the regulation certain oncogenes and tumour suppressor genes. A good example would be its action on *PTEN* which is a tumour suppressor gene (Baron *et al,* 2005). It has the ability to induce apoptotic factors and thereby influencing the cell proliferation. Other important gene that is affected by *Egr-1* it would also affect *p53* as well. We know that Cyclin D1 is negatively regulated by *p53* gene and hence a check on the cell cycle division. Impairment of *p53* regulatory mechanism could up regulate Cyclin D1 in the cell and contribute tumourigenesis.

These are some of the newer proteins that have linked to the Cyclin D1 protein regulation. The list of these types of proteins is growing rapidly. This means the mechanisms of regulation appears to be lot more complex than previously believed.

Summary

Our study demonstrates association of polymorphisms with clinical outcome in patients with colorectal cancer. For the first time we showed the influence of polymorphisms in the 3' UTR region (G/C^{1722}, C/A^{1100}). We also have shown that there is some interaction between the polymorphic alleles of CCND1 gene and tumour biology. However, these findings need to be further elucidated. There were no significant associations between the CCND1 genotypes and survival and the Cyclin D1 protein expression.

The patients with Cyclin D1 over expression irrespective of its localisation in the cell had significantly good prognostic factors in terms of tumour stage and metastatic potential. They also had significant survival advantage. One possible mechanism is through the induction of apoptotic pathways. However, it is questionable whether both nuclear and cytoplasmic localisation of Cyclin D1 induces apoptosis through the same pathway.

However, we examined the mechanisms of Cyclin D1 expression in order to identify a clearer understanding. β-catenin was considered to be a major pathway in the regulation of Cyclin D1 through the transcriptional activity and its subsequent nuclear translocation (Wong *et al,* 2002). Our study did not show any significant association between β-catenin and Cyclin D1 expression. It is to be noted that our study has examined greater numbers of patients than any other study of this kind. We also examined the clinical outcome and found that those expressing both proteins (β-catenin and Cyclin D1) had significantly better survival rates. Surprisingly this effect failed to remain significant after five years. We think that the effect on survival may be through Cyclin D1 itself rather than β-catenin.

Cyclin D1 phosphorylation sites did not seem to have any impact on the expression of Cyclin D1 protein in human cases whereas this was shown to be happening in vitro cell line studies (Diehl *et al,* 1997). We did not find any mutational changes in these sites. We conclude that Cyclin D1 regulation seems to be rarely affected by these changes in human cancers or at least this is yet to be proved beyond any doubt. Gene amplification may be another mechanism where Cyclin D1 can be accumulated in the cell. However this pathway appears to be less important in some cancers (Dhar *et al,* 1999 – ovarian cancer) including colorectal cancers.

The findings from our study demonstrate that Cyclin D1 regulation is a complex process and may require more than one pathway's involvement in order to achieve tumourigenesis and progression. This could mean the involvement of both up stream and down stream regulators of genetic pathways involved in Cyclin D1 regulation. We suggest that the future work should focus on simultaneous examination of β-catenin and the genes that are implicated in the alternative pathway using technology such as microarray analysis.

Appendix

Buffers for DNA extraction

Reagent A

10mM Tris-Hcl

320mM Sucrose

5mM Magnesium Chloride (MgCl2)

1 % Triton-X

Reagent B

40mM Tris-HCl

60mM EDTA

150mM Sodium Chloride

2 % SDS detergent to break cell membrane

Running buffer for gel electrophoresis (Tris-borate (TBE) pH 8.4)

0.5 x 0.045M Tris-borate

0.001M EDTA

Tracking Dye

8ml blue dye

2ml of phi174 marker

Buffers for immunohistochemistry

Antigen retrieval buffer pH 8.0

1mM EDTA

1mM buffer solution at pH 6.0 (using citrate)

Abstracts:

Polymorphisms within Cyclin D1 gene influence outcome in patients with sporadic colorectal carcinoma

Sivakumar R, Holley SL, Smith V, Deakin M, Hall C, Jones PW, Fryer AA, Elder JB, Hoban PR.

Introduction: Cyclin D1 is a key regulatory protein in cell cycle progression during G1 to S phase. Polymorphisms within Cyclin D1 have been associated with outcome in epithelial cancers of head and neck and prostate.
Aim: we investigated, for the first time, the combined effects of two commonly occurring polymorphisms, A/G^{870} (Exon 4) and G/C^{1722} (3'UTR), within the Cyclin D1 gene in sporadic colorectal cancer patients.
Method: Using peripheral blood DNA, both polymorphisms were analysed by PCR-RFLP assay in patients and controls. The variables were analysed by logistic regression and Cox's proportional hazard model, correcting for confounding factors.
Results: The number of patients and controls for A/G^{870} and G/C^{1722} polymorphisms were n=387/n=148 and n=325/n=112 respectively. Allele frequencies were similar between patients and controls. There were no significant associations found with age and gender in both polymorphisms. In G/C^{1722} polymorphism, GG genotype was significantly associated with poorly differentiated disease (OR=2.23, p=0.01, CI=1.20-4.11). Paradoxically, CC genotype was significantly associated with metastasis to other sites (OR=3.63, p=0.02, CI=1.20-10.92). In $^{A/G870}$ polymorphism, AA genotype was associated with recurrence (OR=2.35, p=0.06, CI=0.96-5.75).
Combining AA^{870} and GG^{1722} from each of the polymorphisms, we found significant association with vascular invasion (OR=2.94, p=0.003, CI=1.45-5.95) and recurrence with reduced survival rate (HR=2.74, p=0.04, CI=1.04-7.18). The median survival rates for AA^{870} /GC^{1722} versus all other genotypes were 3.03 and 8.62 years respectively.
Conclusion: Both polymorphisms, together, influenced tumour biology and survival significantly. These data cast a new light on the mechanisms affecting outcome in sporadic colorectal cancer.

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3'UTR polymorphism within the Cyclin D1 gene influences the genetic susceptibility and clinical outcome in sporadic colorectal cancer

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Background:
We investigated the association of a new polymorphism $^{C/A1100}$ in 3'UTR region, within the CyclinD1 gene with outcome in patients with sporadic colorectal cancer.

Methods:
A total of 257 patient samples were analysed using the peripheral blood DNA. The polymorphism was typified by ARMS-PCR assay and further confirmed by WAVE analysis. The results were compared against susceptibility, clinical parameters and survival.

Results:
The homozygtes with 'A' allele more likely to be females (χ^2 test p=0.016) (OR=1.93 p=0.017 CI= 1.12-3.33) and were significantly associated with vascular invasion (χ^2 test p=0.013) (OR=2.13 p=0.012 CI= 1.18-3.84).
The homozygotes with 'C' allele were significantly associated with left sided tumours (χ^2 test p=0.010) (OR=3.75 p=0.016 CI= 1.28-10.98). These patients were significantly associated with early stage disease (χ^2 test p=0.008) (OR=2.45 p=0.010 CI=1.23-4.87) and were protected against nodal invasion (χ^2 test p=0.009) (OR=0.39 p=0.013 CI= 0.204-0.823)
Using the Cox's proportional hazards model, we found no significant influence on the survival.

Conclusion:
This is the first study on 3'UTR polymorphism in colorectal cancer. Our study demonstrates that this new found polymorphism within Cyclin D1 was associated with the genetic suscepitibility and tumour biology. Further evaluation needs to be done in terms of polymorphic interactions within Cyclin D1 gene and the mechanisms by which it exerts its influence since this polymorphism can potentially be used a clinical marker in colorectal cancer.

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Sub cellular localisation of Betacatenin is associated with clinical outcome in Sporadic Colorectal Cancer

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Background: Betacatenin is an important membranous protein involved in cell adhesion[1]. Its expression is frequently up regulated in many epithelial tumours[2]. In colorectal cancer, deregulated betacatenin expression is thought to be an early event[3]. However the localisation of betacatenin expression within the tumour cell appears to vary according to the extent of tumour progression[4]. The influence of betacatenin subcellular localsiation on clinical outcome in patients with colorectal cancer has not been studied.

Aim: The objective of our study was to classify betacatenin expression and determine the effects of altered subcellular localisation on clinical outcome in patients with sporadic colorectal cancer.

Method: A total of 161 patient samples with proven colorectal cancer were studied. Histological sections (4mm thickness) were made from paraffin embedded tumour specimens. Immunohistochemistry was carried out using the streptavidin-biotin complex indirect immunoperoxidase method For negative controls, normal colonic mucosa was used and grading of the slides was performed as described previously2. Statistical analyses were carried out using Stata(version 5) statistical package

Results:

• There were no significant associations between betacatenin expression and patient age or gender.

• Nuclear expression of betacatenin was significantly associated with well-differentiated tumours (OR=3.14 p=0.014 CI= 1.25-7.87) and early stage disease (OR=2.77 p=0.017 CI= 1.20-6.43).

• Strong cytoplasmic expression was significantly associated withincreased nodal involvement (p=0.05 χ^2 test) and liver metastasis (p=0.05 χ^2 test) and a non-significant association withincreased metastasis (p=0.08 χ^2 test).

• Using Cox's proportional hazard model, we found a significant association between strong cytoplasmic expression of betacatenin in patients with advanced Dukes stage disease and reduced survival (HR=1.59 p=0.04 CI= 1.01-2.50).

• Those with beta-catenin in both the nucleus and cytoplasm, did not show any significant association with clinical parameters.

Conclusion: Our data suggests that subcellular distribution of betacatenin had a differential effect on colorectal tumour progression. Thus cytoplasmic expression of betacatenin was associated with advanced tumour progression in contrast nuclear expression was associated with less progressive disease. We hypothesise that betacatenin influences tumour progression by more than one mechanism through altered subcellular localisation.

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