Central University of Technology, Free State

THE DETECTION OF p53 AND CYCLIN D1 IN CHRONIC MYELOGENOUS LEUKAEMIA PATIENTS USING FLUORESCENCE *IN SITU* HYBRIDISATION

DEDRé OLIVIER

Dissertation submitted in fulfilment of the requirements of the Degree

MAGISTER TECHNOLOGIAE:
BIOMEDICAL TECHNOLOGY

in the

School of Health Technology
Faculty of Health and Environmental Sciences

at the

Technikon Free State

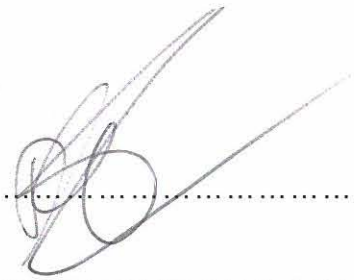
Supervisors: Prof GHJ Pretorius (DSc)
Dr WMJ vd Heever (PhD)

BLOEMFONTEIN
December 2002

Central University of Technology, Free State

DECLARATION OF INDEPENDENT WORK

I, Dedré Olivier, do hereby declare that this research project submitted to the Technikon Free State for the degree MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work that has not been submitted before to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.



.....

SIGNATURE OF STUDENT

2002-12-06

DATE

Central University of Technology, Free State

CONTENTS

ACKNOWLEDGEMENTS

GLORIA IN EXCELSIS DEO

I would like to thank the following people and institutions who contributed to this study:

- My study leaders, Prof Oubaas Pretorius and Dr Elmien vd Heever for their patience, support and guidance during the study;
- The Department of Haematology and Cell Biology, University of the Free State for providing the equipment and resources to complete this study;
- Technikon Free State (School of Health Technology) for the financial support and the extra time to work at the University to complete this study;
- Ms Wendy McKay, Dr Marius Coetzee, Dr Engela le Roux and Ms Hannie Dercksen for their help;
- My family, friends and colleagues for their encouragement and support;
- My parents for their love, support and encouragement.

SUMMARY

Chronic myelogenous leukaemia (CML) is a myeloproliferative disorder that results from the neoplastic transformation of haematopoietic progenitor cells. It accounts for 15-20% of leukaemias in adults and occurs with an incidence of 1-2 cases per 100 000 population, and the patients have a median survival period of 3-4 years from diagnosis.

Knowledge of normal haematopoiesis has increased the understanding of how specific perturbations may lead to the development of a transformed or malignant phenotype that is now clinically recognized as leukaemia. The discovery of oncogenes initiated a laboratory effort where the focus was to elucidate as yet undescribed oncogenes and to understand how single allele mutations could act dominantly in the "gain of function" ability to transform cells and cause tumours. These investigations obscured the existence of another set of genes which also appeared to be permissive of tumour formation. In contrast to the oncogenes, these genes behaved in a recessive manner so that "loss of function" as opposed to "gain of function", resulted in tumour formation. These genes therefore appeared to behave as tumour suppressor genes.

The role of tumour suppressor genes is to prevent tissue overgrowth, nullify cells with damaged genomes, and metastasis. The structure and expression of the p53 gene is altered in about 25% of myeloid blast crisis of CML, whereas chronic

phase CML rarely has detectable p53 alterations, suggesting that mutations in the p53 gene might be involved in the evolution of some cases of blast crisis.

Oncogenes are genes that have the ability to transform normal cells into cancer cells. Not all D-type cyclins are expressed in each tissue, suggesting that their function may be linked to the specific tissues in which they are expressed. Increased expression of cyclin D1 can play a critical role in tumour development and in maintenance of the malignant phenotype, thus over-expression of cyclin D1 can produce complex effects on various cellular functions involved in growth control and cell cycle progression.

Fluorescence *in situ* hybridization (FISH) allows the detection of numerical aberrations in interphase cell nuclei and provides a simple, fast and reliable means to assess genetic instability in cancer cells.

Significant p53 loss of allele was detected in 6 out of 25 samples, indicating that the p53 tumour suppressor gene can be involved in the progression of CML from chronic phase to blastic phase. Cyclin D1 amplification was not detected in any of the samples investigated by FISH, indicating that cyclin D1 is not expressed in cells of the lymphoid and myeloid lineages.

Keywords: chronic myelogenous leukaemia, p53, cyclin D1, fluorescence *in situ* hybridization

OPSOMMING

Kroniese miëloïede leukemie is 'n miëloproliferatiewe siekte wat ontstaan a.g.v. die neoplastiese transformasie van hematologiese voorloperselle. Dit verteenwoordig 15-20% van leukemieë in volwassenes, met 'n insidensie van 1-2 gevalle per 100 000 bevolking. Die pasiënte het 'n mediaan oorleweringsperiode van 3-4 jaar vanaf diagnose.

Kennis van normale hematopoïese het groter begrip meegebring aangaande hoe spesifieke afwykings kan lei tot die ontwikkeling van 'n getransformeerde of maligne fenotipe wat klinies herken word as leukemie. Met die ontdekking van onkogene is 'n laboratoriumpoging geïnisieer waar die fokus toegespits was om voorheen onomskryfde onkogene te verduidelik/verklaar, en om te verstaan hoe enkel alleel mutasies dominant kon optree met 'n "funksietoename" vermoë, om selle te verander en tumore te veroorsaak. Hierdie ondersoeke het egter die bestaan verberg van 'n ander stel gene wat klaarblyklik ook tumorformasie laat plaasvind het. In teenstelling met die onkogene, het hierdie gene egter resessief opgetree sodat "funksieverlies" en nie "funksietoename" nie, tumorformasie veroorsaak het. Derhalwe het hierdie gene skynbaar opgetree as tumoronderdrukkende gene.

Die rol van tumoronderdrukkende gene is om abnormale weefsel proliferasie, vernietiging van beskadigde genome, en metastase te voorkom. Die struktuur en uitdrukking van die p53 geen is verander in ongeveer 25% van miëloïede

blastselkrisisse, waar kroniese fase selde opspoorbare p53 veranderings toon, wat dui daarop dat die muteerde p53 geen waarskynlik betrokke is in die evolusie van blastselkrisisse.

Onkogene is gene wat die vermoë het om normale selle te transformeer. Nie alle D-tipe sikliene word uitgedruk in alle weefseltipes nie, wat dui daarop dat hul funksie waarskynlik gekoppel kan word aan die spesifieke weefsel waarin hulle uitgedruk word. Die oormatige uitdrukking van siklien D1 kan 'n kritieke rol speel in tumorontwikkeling en die handhawing daarvan, derhalwe kan oormatige uitdrukking van siklien D1 komplekse gevolge veroorsaak wat betrokke is by groeibeheer en selsiklusprogressie.

Fluoresensie *in situ* hibridisasie (FISH) maak dit moontlik om numeriese afwykings in interfase selkern op te spoor en is 'n eenvoudige, vinnige en betroubare manier om genetiese onstabiliteit in kanker selle te bepaal.

Betekenisvolle p53 alleel verlies was gevind in 6 uit 25 monsters, wat aandui dat die p53 tumoronderdrukkende geen betrokke kan wees in die vooruitgang van kroniese fase na blastfase. Siklien D1 amplifikasie is nie gevind in enige monsters met die FISH ondersoek nie, wat aandui dat siklien D1 nie amplifikasie word in selle van die limfoïede en miëloïede sellyne nie.

Sleutelwoorde: kroniese miëloïede leukemia, p53, siklien D1, flouresensie *in situ* hibridisasie

Central University of Technology, Free State

ABBREVIATIONS

/L	per litre
µl	microlitre
ABL	Abelson
ALL	acute lymphocytic leukaemia
AML	acute myelogenous leukaemia
AP	accelerated phase
BCR	breakpoint cluster region
BP	blastic phase
CDK	cyclin dependent kinase
CEP	chromosome enumeration probe
CLL	chronic lymphocytic leukaemia
CML	chronic myelogenous leukaemia
c-myb	avian myeloblastosis virus oncogene
CP	chronic phase
DAPI	4'6-diamidino-2-phenylindole
DMP	dentin matrix protein
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EBV	Ebstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
FISH	fluorescence *in situ* hybridization
G	gap

G_1	first gap (cell cycle)
G_2	second gap (cell cycle)
GTP	guanosine triphosphate
HD	Hodgkin's disease
i(17q)	Isochromosome 17q
kD	kilo Dalton
LFS	Li-Fraumeni syndrome
LOA	loss of allele
LSI	locus specific identifier
M phase	mitosis phase (cell cycle)
MDS	myelodysplastic syndrome
ml	milliliter
MM	multiple myeloma
mRNA	messenger ribonucleic acid
MyoD	myogenic determination factor
NF-1	neurofibromin-1
NP-40	Nonidet P-40
PCR	polymerase chain reaction
Ph	Philadelphia
RAS	rennin-angiotensin system
RB	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
RS	Reed-Sternberg
S phase	synthesis (cell cycle)

SG	spectrum green
SO	spectrum orange
src	steroid receptor co-activator
SSC	saline sodium citrate (buffer)
STAT	signal transducers and activators of transcription
SV40	simian vacualating virus
T antigen	target antigen
T/ALL	transformed to ALL
T/myel	transformed to myelofibrosis
v/v	volume per volume

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Chronic myelogenous leukaemia (CML) is a malignancy of the human haematopoietic stem cell characterised by a reciprocal translocation between chromosomes 9 and 22, termed the Philadelphia chromosome (Ph), resulting in the formation of a hybrid BCR/ABL (Abelson breakpoint cluster region) gene on chromosome 22 (Kantarjian *et al.*, 1985; Rowley, 1990; De Klein *et al.*, 1982). In its chronic phase, CML is characterised by an abnormal, unregulated expansion of myeloid, erythroid and megakaryocytic progenitors in the marrow. One of the most obvious characteristics of CML is the expansion of myeloid progenitors and precursors, which mature relatively normally. Increases in cell number can be the result of decreased cell death, increased production of cells, or both (Daley *et al.*, 1990).

Although the Ph chromosome, which generates $p210^{BCR/ABL}$, is an unique chromosomal abnormality in the chronic phase, additional chromosomal abnormalities are frequently detected in the blast crisis, suggesting that superimposed genetic events are responsible for disease progression (Honda *et al.*, 2000)

The p53 gene has been mapped to the short arm of chromosome 17 and encodes a 53 kiloDalton (kD) nuclear phosphoprotein involved in the control of cell proliferation (Isobe *et al.*, 1986; Lane and Benchimol, 1990). It plays a critical role in cellular proliferation and regulation of the cell cycle (Lane and Benchimol, 1990). It is frequently altered by mutations, rearrangements or deletions which contribute to the genesis or progression of a wide variety of human cancers (Cheng *et al.*, 1992). Inactivation of the p53 gene has been demonstrated in human cancers, such as colorectal and lung cancers, and leukaemias (Sugimoto *et al.*, 1992; Cheng *et al.*, 1992). Loss or mutation of the tumour suppressor gene, p53, is one of the most frequent secondary mutations in CML blast crisis. The transition between chronic phase and blast crisis is associated with increased resistance to apoptosis correlating with poor prognosis (Di-Bacco *et al.*, 2000).

D-type cyclins are important cell cycle regulators that promote cellular proliferation in response to growth factors by inactivation of the retinoblastoma protein (RB). Cyclin D1 has been shown to be over-expressed in several cancer types and to act as an oncogene in breast cancers. Signal transducers and activators of transcription (STAT) are a family of transcription factors that were originally identified as mediators of cytokine-induced gene expression. STAT5 also plays a major role in cellular transformation by the BCR/ABL oncogene (De-Groot *et al.*, 2000). As D-type cyclins are rate limiting for progression into synthesis (S) phase, the level at which they accumulate must be carefully regulated. Several mechanisms leading to over-expression of cyclin D1 have been reported including amplification, translocation and stabilisation of the

messenger ribonucleic acid (mRNA) (Russel *et al.*, 1999). Furthermore, over-expression of cyclin D1, similar to mutation of p53, appears to lead to genome instability (Zhou *et al.*, 1996).

The use of fluorescence *in situ* hybridisation (FISH) allows the detection of chromosomal amplifications, deletions or translocations at a single-cell level in dividing and resting cells (Panayiotidis and Kotsi, 1999).

The aim of this study was to detect the presence of p53 and cyclin D1 by means of FISH in patients with CML and to correlate the expression status of the genes with the clinical status of the patients.

CHAPTER 2

CHRONIC MYELOGENOUS LEUKAEMIA

LITERATURE REVIEW

2.1. INTRODUCTION

CML is a myeloproliferative disorder that results from the neoplastic transformation of haematopoietic progenitor cells and affects myeloid, monocytic, megakaryocytic, lymphoid and erythroid lineages. CML accounts for 15-20% of leukaemias in adults and occurs with an incidence of 1-2 cases per 100 000 population. CML occurs more frequently in males than in females (ratio of 1.3 to 1). Incidence increases with age, and the median age at presentation is between 45 and 55 years (Kantarjian *et al.*, 1993). However, the disease can occur in all age groups and there is an increasing proportion of younger patients (Faderl *et al.*, 1999a).

CML usually refers to a disease characterized by insidious onset with gradually increasing neutrophil leukocytosis, a gradual increase in spleen size and the eventual development of anaemia severe enough to demand specific treatment (Moossa *et al.*, 1991).

In most cases, cytotoxic drugs can suppress the proliferation of the haematopoietic cells for 1 to 4 years, but eventually disease progression or transition to acute leukaemia develops in most of the patients. During the terminal months of the disease, therapeutic agents are ineffective. Death results from inanition, organ infiltration by leukaemic cells, infection or haemorrhage (Williams *et al.*, 1986).

2.2 CLINICAL COURSE, PROGNOSIS AND CAUSATIVE FACTORS

2.2.1 CLINICAL COURSE

CML typically follows a biphasic or triphasic course. A chronic phase (CP), of variable length, precedes an accelerated phase (AP), which is often followed by a blastic phase (BP) (Kantarjian *et al.*, 1993). In most patients (85%), the disease is diagnosed in the chronic phase. Accelerated and blastic phase presentations occur in only 5-10% of patients (Savage *et al.*, 1997). The clinical course of CML varies from patient to patient and the median survival period of 3-4 years from diagnosis, is a near universal finding (Knowles, 1992).

2.2.1.1 Chronic phase

The signs and symptoms of CML develop insidiously at first, but tend to become continuously worse if treatment is not given to the patient (Williams *et al.*, 1986). CP is indolent and as much as 50% of patients in this stage have no symptoms and are only diagnosed by routine blood testing (Kantarjian *et al.*, 1993). A great majority of patients are diagnosed when the disease is still in uncomplicated CP (Moossa *et al.*, 1991). Marked leukocytosis, thrombocytosis and anaemia are

common laboratory features at presentation (Savage *et al.*, 1997). Granulocytes are present in all stages of maturation. The bone marrow of CML patients is usually hypercellular and may reveal reticulin fibrosis, especially with disease progression (Cortes *et al.*, 1996).

Among those patients who have symptoms, anorexia, fatigue, weight loss, early satiety, abdominal fullness, left upper quadrant discomfort, bleeding and sweats are encountered most frequently (Cortes *et al.*, 1996). Because the granulocytes have only mild functional abnormalities, infections are uncommon during CP. The duration of the CP is about 3 years and the survival for patients with CML is about 3,5 - 4 years (Knowles, 1992).

In the rare patient who presents with a very high white blood cell count, symptoms of hyperviscosity may occur; these include visual disturbances due to retinal hemorrhage, headaches, tinnitus, stupor and priapism. Splenomegaly is revealed in 50% of patients with CML and hepatomegaly in a lesser percentage (Cortes *et al.*, 1996).

2.2.1.2 Accelerated phase

CML invariably transforms and becomes refractory to therapy with agents such as hydroxyurea and busulfan. It then enters the accelerated phase that is characterized by basophilia and increases in peripheral blood blast and promyelocyte counts. The definition of the accelerated phase is vague and relies on several generally accepted clinical and laboratory criteria (Kantarjian *et al.*, 1988). Some of these criteria are: peripheral blood blasts of 15% or more,

peripheral blood blasts and promyelocytes of 30% or more, peripheral blood basophils of 20% or more, platelet count of 100 x 10^9 per liter (/L) or less (unrelated to therapy) and clonal evolution (Kantarjian *et al.*, 1993). A finding of 15-29% marrow blasts or of 30% promyelocytes and blasts in the bone marrow, is sufficient evidence for a diagnosis of the accelerated phase (Knowles, 1992).

2.2.1.3 Blastic phase

In about 75% of CML patients, the accelerated phase is followed (after 3 to 18 months) by the blastic phase. This phase resembles acute leukaemia and causes the death of the patients within 3 to 6 months. One-fourth of patients develop the blastic phase without an intervening accelerated phase (Kantarjian *et al.*, 1988).

In most patients, the disease will enter a phase in which blast cells or blast cells plus promyelocytes constitute 30% or more of the cells in the blood and/or bone marrow (Moossa *et al.*, 1991). In one-third of cases, blasts are characterized by lymphoid morphology and expression of lymphoid markers, such as terminal deoxynucleotidase. The remaining two-thirds of the patients have either the acute myeloblastic leukaemia or the acute undifferentiated leukaemia phenotype and form a heterogeneous group (Griffin *et al.*, 1983).

Some patients are still entirely free of symptoms; more often they feel non-specifically unwell. The duration of survival of patients in the BP is extremely variable: some will die within days of diagnosis despite optimal treatment, while

in others the disease will respond to treatment and survival may be prolonged for some months or even (rarely) for more than a year (Moossa *et al.*, 1991).

2.2.2 PROGNOSIS

The survival pattern of patients with CML has changed over the last decade. The median survival time of patients has doubled from 5 to 7 years, with up to 50% of patients alive at 5 years. This development is due to refinements in allogeneic stem-cell transplantations and growing expertise in the use of interferon-alfa, a biological agent that has been shown to suppress the leukaemic clone and to prolong survival in patients with CML (Faderl *et al.*, 1999b).

2.2.3 CAUSATIVE FACTORS

The causative factors of CML are unknown (Knowles, 1992). This disease occurs in every country of the world, furthermore, it is not known to be associated with any conventionally infectious virus or other agent (Moossa *et al.*, 1991). A higher than expected incidence of CML in patients given radiotherapy for ankylosing spondylitis, and atomic bomb survivors in Japan, has implicated ionising irradiation as a cause in some cases, but in most of the patients, no etiologic factors have been identified. A report on a relapse of CML in donor cells after an allogenic bone marrow transplantation suggested that some transmissible oncogenic agent or inducing factor might be responsible for reinducing the disease in the engrafted cells (Knowles, 1992).

2.3 CYTOGENETIC ABNORMALITIES

Assays for studying the growth and development of primitive haematopoietic cells *in vitro* have established the presence of numerous haematopoietic growth factors and cytokines; elucidated their cellular sources and their receptors, and showed how growth factor stimulated receptors transmit their message to the nucleus where the genes controlling differentiation and proliferation reside. The knowledge of normal haematopoiesis has increased the understanding of how specific perturbations in this process may lead to the development of a transformed or malignant phenotype that is now clinically recognized as leukaemia (Sawyers *et al.*, 1991).

Initially described by Nowell and Hungerford in 1960, the Ph chromosome in the leukaemic leukocytes of patients with CML, became the first chromosomal abnormality to be associated with a specific neoplastic disorder. The Ph chromosome results from a reciprocal translocation between the long arms of chromosome 9 and chromosome 22 (Nowell and Hungerford, 1960). The t(9;22)(q34;q11) translocation can be demonstrated in more than 90% of patients with CML. It is, however, also seen in up to 5% of children and 15-30% of adults with acute lymphocytic leukaemia (ALL), and in 2% of patients with acute myelogenous leukaemia (AML) who showed no evidence of a preceding CML phase (Kurzrock *et al.*, 1988). Cytogenetic analysis revealed the Ph chromosome in 90% of patients with CML. In the 10% of patients with CML in whom the Ph chromosome cannot be demonstrated by cytogenetic studies, molecular analysis revealed BCR/ABL fusion products in half of them (Guo *et al.*, 1991).

Evidence of a direct link between the expression of BCR/ABL fusion gene products and abnormal proliferation and malignant behaviour of haematopoietic progenitor cells came from experiments using *in vitro* and *in vivo* models for tumour development. *In vitro* bone marrow culture assays have shown that BCR/ABL causes factor-independent and leukemogenic cell growth in haematopoietic cell lines (Daley and Baltimore, 1988). During the chronic phase, myeloid cells containing BCR/ABL retain the capacity to differentiate normally. There is progressive loss of the capacity for terminal differentiation resulting in terminal blast crisis. An increase of the BCR/ABL mRNA expression in the leukaemic cell, in part due to duplication of the Ph chromosome, precedes the phenotypic transformation of the malignant clone (Gaiger *et al.*, 1995).

2.4 TUMOUR SUPPRESSOR GENES AND p53

The discovery of oncogenes initiated an intense laboratory effort where the focus was to elucidate as yet undescribed oncogenes and to understand how single allele mutations could act dominantly in the "gain of function" ability to transform cells and cause tumours in animal experiments. These investigations obscured the existence of another set of genes which, when perturbed, also appeared to be permissive of tumour formation. In contrast to the oncogenes, these genes behaved in a recessive manner so that "loss of function" as opposed to "gain of function", resulted in the tumour formation. These genes therefore appeared to behave as tumour suppressor genes. The verification of their existence included somatic cell hybridisation experiments in which tumour cells and normal cells were fused (Weinberg, 1991).

2.4.1 FUNCTION OF TUMOUR SUPPRESSOR GENES

The role of a tumour suppressor gene is to prevent tissue overgrowth, nullify cells with damaged genomes, and metastasis. These controlling functions, even in the presence of already severely damaged cells that are being driven by oncogenes, may be thwarted by a fully functional p53 protein (Hussain and Harris, 1998).

The p53 gene that was cloned from colon cancer cells is a well-known example of a suppressor gene whose loss of function has been associated with tumour function (Baker *et al.*, 1989). It appears that suppressor genes, like p53, regulate a cell's ability to progress through the cell cycle (Levine *et al.*, 1991). Mutations of p53 might be considered as relatively "late" leukemogenic events in comparison to germline mutations like loss of neurofibromin-1 (NF-1). NF-1 encodes a protein called neurofibromin, which acts as a guanosine triphosphate (GTPase) activating protein. Its loss in patients with neurofibromatosis type 1 leads to activation of renin-angiotensin system (RAS) and is strongly associated with the development of leukaemia or myeloproliferative syndromes (Shannon *et al.*, 1994).

2.4.2 p53

In a complex organism, somatic cells are under intermittent selection pressure for the emergence of mutants that can grow autonomously despite adverse conditions, and can survive environmental insults. Repeated rounds of mutation, selection and proliferation can lead to cancer, but no one change is common to all cancers, or is sufficient to cause autonomous proliferation and metastasis (Carson and Lois, 1995).

In addition to pertinent clinical data, new risk factors at the molecular and cellular level are the subjects of many ongoing studies. Prognostic and predictive factors in cancer can serve many purposes. They can be used to understand the natural history of cancer. To identify homogeneous patient populations, to characterize subsets of patients with a potentially favourable or unfavourable outcome, and to predict the success of therapy or to generate follow-up strategies (Volm *et al.*, 1998). The p53 tumour suppressor gene is considered to be one of the most important tumour suppressor genes (Porter *et al.*, 1992). The p53 gene has been studied in a large number of cancers and about 900 mutations have been found in more than 50 tumour types (Harris, 1993; Caron de Fromentel and Soussi, 1992).

2.4.2.1 Prevalence

Analysis of p53 mRNA levels suggests that the gene is expressed in all tissues of the body throughout development. High levels are seen in early embryonic development and in the testes of adults (Almon *et al.*, 1993). Immunohistochemical methods for detection of p53 have proved negative except in cases of testicular tissue where low levels of p53 specific staining have been seen. The explanation for this discrepancy lies in the very short half-life of p53 in normal tissues. The protein is constantly being produced but then very rapidly broken down so that its steady state level remains very low (Hall *et al.*, 1993). Wild-type p53 has a half-life of 6-20 minutes in normal cells, but the mutant form is more resistant to proteolysis, and therefore the half-life is longer, about 2-12 hours (Hinds *et al.*, 1990).

Mutant p53 protein is metabolically stable and exists longer in the nuclei of cancer cells than in wild-type, and various human malignancies such as breast, lung and colorectal cancers are frequently positive for the expression of p53 protein, whereas normal tissue is negative (Porter *et al.*, 1992; Bennett *et al.*, 1992).

2.4.2.2 Structure of the p53 protein

The nuclear phosphoprotein p53 was originally discovered in an extract of transformed cells that reacted with antiserum from animals with tumours that were induced by the simian vacualating virus (SV40) (Lane and Crawford, 1979). Little attention was paid to it until 1989 when point mutations in the p53 gene were detected in colorectal cancers (Culotta and Koshland, 1993).

The p53 tumour suppressor is a 393 amino acid nuclear phosphoprotein encoded by an 11 exon gene which is located on chromosome 17p13.1, Figure 2.1 (Lane, 1994; Benchimol *et al.*, 1985). p53 has a hydrophobic core flanked at the amino terminus and carboxy terminus by more highly charged head and tail domains. The amino terminal head region is acidic and it has the activity of a transcriptional transactivation domain when fused to other deoxyrinonucleic acid (DNA) binding domains, while the carboxy terminus is more basic and contains motifs involved in regulation of DNA binding activity and oligomerisation. The p53 protein contains multiple phosphorylation sites located at both the amino and carboxy termini of the molecule. The central region of the molecule is conformationally flexible and contains the binding site for SV40 large T antigen and the sequence-specific double stranded DNA (dsDNA) binding site (Lane, 1994).

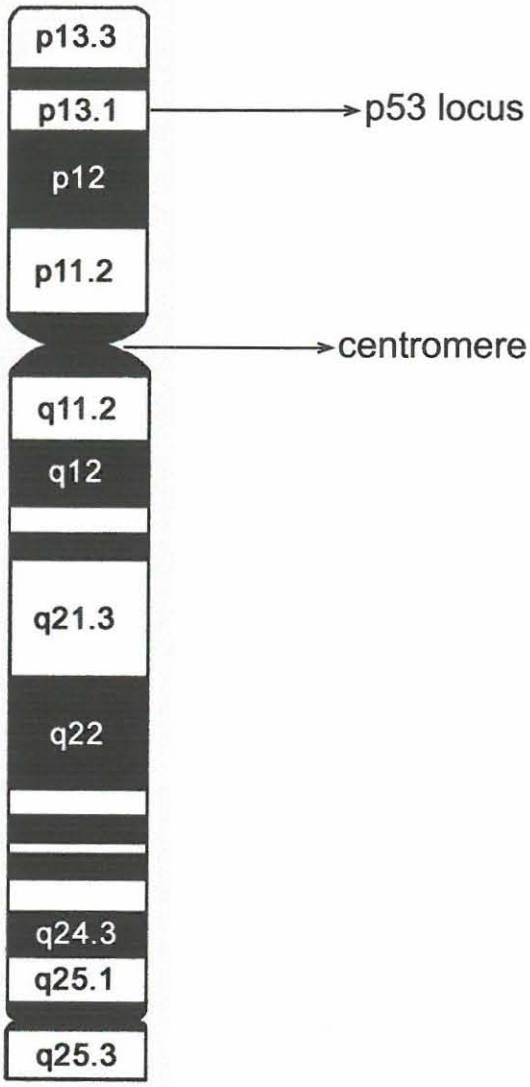


Figure 2.1 Chromosome 17 and p53 locus, p13.1 (www.vysis.com/0.asp?)

2.4.2.3 *Control and pathway of p53*

To be able to understand the role of p53 in normal cells, it is necessary to understand the basic concepts of the following: the cell cycle, oncogenes, tumour suppressor genes and transcription factors. Cell division consists of four phases called M, G_1, S and G_2. After mitosis (M phase) the cell enters the first gap phase (G_1). The next phase is that of DNA synthesis (S phase) and finally there is the second gap (G_2) phase before mitosis. During the cell cycle there are checkpoints that are able to regulate cell division (Hartwell and Kastan, 1994). The cell cycle will stop if certain criteria are not met (Tjian, 1995). It was shown that the G_1 growth arrest response to ionizing radiation of cells in culture was dependent on the presence of a wild-type p53 gene and was accompanied by an increase in the p53 protein level (Kuerbitz *et al.,* 1992). Therefore, because it regulates multiple components of the DNA damage control response and promotes cellular senescence, it has been called the "guardian of the genome" (Carson and Lois, 1995).

It was proposed that p53 act as a nodal point between stressful stimuli and the final fate of the cell, and that multiple events occur both upstream and downstream of p53 induction. Under normal circumstances, cells contain very low levels of p53 protein, but stressful stimuli trigger upstream events that lead to the activation or accumulation of p53, which in turn triggers the downstream pathway (Levine, 1997). Various forms of stress (e.g. heat shock, hypoxia, cytokines) can initiate the upstream pathway and it is likely that protein kinases which are activated by DNA damage and subsequently phosphorylate p53, may be responsible (Siliciano *et al.*, 1997). p53 is not required for normal

development but lack of p53 function confers an elevated risk of developing cancer. It thus seems to act as a tumour suppressor gene (Lane, 1994).

2.4.2.4 p53 mutations in human cancer

Malignant transformation is prevented by assuring that the DNA is accurately repaired before cell division by forcing the death of cells with excessive DNA damage and by placing limits on the replicative lifespan of most somatic cells (Carson and Lois, 1995). In most of the tumours that were examined, expression of wild-type p53 is lost because of a combination of genetic events that affect the maternal and the paternal alleles of the gene. The most common combination found in solid tumours is the complete loss of one allele of the p53 gene, which is usually the result of a large chromosomal deletion, combined with a point missense mutation in the other allele. The mutant protein is typically produced in large amounts by the tumour cell (Scheffner *et al.*, 1990, 1991).

It is widely accepted that p53 expression, which was detected by immunohistochemistry, is probably used as a surrogate for p53 mutation. Conversely, a stabilized p53 protein might not be mutant *in vitro*. The study of a possible correlation between chromosome 17, the p53 gene and p53 protein might be helpful for evaluating the significance of p53 expression in tumours (Li *et al.*, 1997).

2.4.2.5 Mutation by cancer type

The location and type of mutations in a specific sequence define a mutational spectrum. When the p53 mutations are grouped together, they identify several

codons at which exceptionally high numbers of tumour mutations are clustered (hot spots). When mutations are examined separately by cancer type, clear differences in spectra emerge, both with respect to the position of the hot spots, and with respect to the frequency of transitions (purine substituted for a purine or a pyrimidine for a pyrimidine) and transversions (purine is substituted for a pyrimidine or vice versa) (Hollstein *et al.*, 1991). The patterns of mutations in leukaemias and lymphomas are remarkably similar to that of colorectal tumours. Guanine to Thiamine transversions are uncommon, and Adenine:Thiamine to Guanine:Cytocine transitions predominate among substitutions at Adenine:Thiamine pairs (Gaidano *et al.*, 1991).

2.4.2.6 p53, DNA damage and apoptosis

The p53 gene is not necessary for cell growth and differentiation. Mice with disruptions of both copies of the p53 gene are usually normal at birth but they develop tumours at an early age because of an inadequate DNA damage-control response (Carson and Lois, 1995). In cells that contain a wild-type p53 gene, the accumulation of wild-type p53 results in a profound growth arrest and frequently to the rapid elimination of the cell by apoptosis. The signal is incompatible with continued survival and proliferation, but in a cell in which the p53 protein is inactivated, no such apoptotic signal is delivered. These cells continue to proliferate without resolving the defect that first led them to generate the signal for p53 stability; they are genetically unstable and send a continuous but futile signal to the p53 system (Lane, 1994; Carson and Ribeiro, 1993).

The contribution of p53 to apoptosis is not restricted to cells exposed to acute DNA damage or bearing certain activated oncogenes. Studies showed a role for p53 in mediating apoptosis in response to withdrawal of haematopoietic survival factors. A reduction in apoptotic death following factor deprivation was observed in haematopoietic progenitors from p53-null mice (Lotem and Sachs, 1993). This was also observed in leukaemic cells in which p53 was inactivated by either dominant-negative mutations or anti-sense oligonucleotides (Gottlieb *et al.*, 1994).

Chromosomal length is shortened by 50-60 nucleotide residues with each cell division, and when this shortening reaches a critical threshold value, apoptosis will ensue, even in cells without p53. Some cells will unfortunately withstand the crisis, and the survivors re-express the telomerase enzyme that prevents chromosome shortening and can thus grow continuously (Counter *et al.*, 1992).

The ability of the cell to survive after DNA damage depends on the p53 gene dosage. Epidermal cells with mutations in one copy of the p53 gene are slightly resistant to death by apoptosis (Ziegler and Jonason, 1994). Cells devoid of p53 do not properly recognize altered DNA and may begin mitosis before completion of chromosomal segregation and spindle formation (Cross and Sanchez, 1995). Replication of damaged DNA results in a high error frequency, giving rise to the gene amplifications or chromosomal translocations observed in many cancers. A normal p53 protein will monitor the fidelity of the cell cycle by preventing the replication errors that occur when damaged DNA is duplicated (Livingstone *et al.*, 1992).

2.4.2.7 *p53 in haematological malignancies*

Based on epidemiological and experimental evidence, leukaemia is mainly the result of an accumulation of mutations in critical target genes of haematopoietic cells. These mutations range from simple sequence substitutions to complex genomic rearrangements. In human leukaemia, point mutations are reported to affect proto-oncogenes like *c-fms*, *c-RAS,* and p53 (Bos, 1989; Ridge *et al.*, 1990; Slingerland *et al.*, 1991). Some of the most notable features of p53 alterations in haematopoietic malignancies are:

(a) Development of p53 mutations is often correlated with worsening or relapsing haematopoietic malignancy.

(b) Loss of the short arm of chromosome 17 is associated with a p53 mutation on the remaining allele in several haematopoietic malignancies.

(c) Hodgkin's disease (HD) has p53 positive Reed-Sternberg (RS) cells but accompanying lymphocytes, eosinophils and macrophages do not over-express p53, consistent with these cells being a reaction to the malignant process. Lymphocyte-predominant HD does not have p53-positive RS cells.

(d) B-cell lymphomas with p53 mutations often have avian myeloblastosis virus oncogene (*c-myb)* activation, but Epstein-Barr virus (EBV) infection does not appear to correlate with the presence of a p53 mutation.

Individuals with Li-Fraumeni syndrome (LFS) have a p53 mutation in their germline and have an increase incidence of leukaemias and lymphomas (Imamura *et al.*, 1994).

CML mostly evolves from a chronic, relatively indolent disease to a more aggressive leukaemia whose progressive stages are accelerated phase and blast crisis (Muehleck *et al.*, 1984). The blast cells of the aggressive phases may be of several types, most commonly myeloid, less commonly lymphoid, and rarely other phenotypes, suggesting that different molecular events induce different forms of disease (Liu *et al.*, 1988). The structure and expression of the p53 gene is altered in about 25% of myeloid BC of CML, whereas CP CML cells rarely have detectable p53 alterations, suggesting that the p53 gene might be involved in the evolution of some cases of BC (Ahuja *et al.*, 1989 a,b).

Several features of CML and p53 are the following:

(a) The p53 alterations almost always occur in myeloid and not lymphoid blast crisis (Nakai *et al.*, 1992).

(b) The p53 mutations are associated most frequently with samples in which one of the short arms of chromosome 17 (17p) has been lost, usually through the formation of either an isochromosome 17q (i(17q)) or unbalanced translocation. The i(17q) chromosome occurs in about 30% of cases of myeloid blast crisis of CML and about 40% of these have p53 mutations on the remaining p53 allele. Loss of 17p (containing p53) may precede the p53 mutation of the remaining allele in CML patients, whereas the p53 mutations in colorectal and breast tumours occur on one p53 allele and the remaining normal p53 allele is lost. These observations emphasize the strong selection for complete loss of p53 function in the process of carcinogenesis (Nakai *et al.*, 1992).

(c) CML is analogous to osteosarcoma as far as the p53 gene can be altered by either point mutations or major DNA rearrangements (Nakai *et al.*, 1992).

(d) Evidence suggests that a p53 mutation in the CML clone can result in disease transformation to myeloid blast crisis (Foti *et al.*, 1991). When wild-type p53 is transfected and stably expressed in the p53 null CML erythroblastic cell line K562, growth of the cells slows and they undergo partial differentiation suggesting an involvement of wild-type p53 in the differentiation processes (Feinstein *et al.*, 1992).

Some of the haematopoietic malignancies in which p53 mutations are associated with the progression of disease are: evolution from a follicular to high-grade lymphoma; evolution from chronic lymphocytic leukaemia (CLL) to high-grade Richter's-type lymphoma; progression to a refractory phase of multiple myeloma (MM); development of relapsed B- or T-ALL; evolution from myelodysplastic syndrome (MDS) to acute myelogenous leukaemia, and evolution from chronic phase to myeloid blast crisis of CML (Imamura *et al.*, 1994). Evolution of CML and MDS to myeloid blast crisis and AML respectively, have been associated with loss of the short arm of chromosome 17 and the mutation of the remaining p53 allele. Several of the other lymphoproliferative disorders such as CLL and follicular lymphoma can acquire a p53 alteration as they progress to a more malignant phenotype. p53 mutations have also been associated with progression of solid malignancies such as the transition from benign adenoma to malignant colon carcinoma (Baker *et al.*, 1989).

2.5 ONCOGENES AND CYCLIN D1

Activation of proto-oncogenes and loss of tumour suppressor genes are genetic changes associated with carcinogenesis. Proto-oncogenes are normal cellular genes that control cell proliferation, differentiation and apoptosis. Almost all proto-oncogenes encode a protein component of the signal transduction cascade. This integrated, multi-process system is responsible for the specific transmission of extracellular signals to the nucleus, and this process regulates gene transcription with respect to replication (Cobb, 1999). When proto-oncogenes are activated, they are called oncogenes. Oncogenes exert a positive driving force for cell growth by their failure to desist in response to the absence of stimulation (Krontiris, 1995).

2.5.1 The role of oncogenes in regulating cell growth and malignant transformation

Orderly cell growth requires the careful orchestration of events that occur first at the cell membrane and then progress through the cytoplasm and into the nucleus. There the genes that are required to carry out the command of the stimulus, initiated at the membrane, must be activated. This chain of command requires many interacting elements, including the signal initiator, which is often a growth factor; the growth factor's receptor; a signal transducing apparatus, often consisting of membrane tyrosine kinases; GTP binding (RAS family) nuclear transcription factors, and other yet undefined elements (Dang, 1991).

Given that oncogenes encode products that are highly homologous to growth factors, growth factor receptors or elements of the signal transduction apparatus, it seems reasonable that altered expression or activation of these genes or their products could play a role in carcinogenesis. Examples of this include chromosomal translocations leading to structural alterations of the gene; the acquisition of point mutations in coding sequence; retroviral insertion disrupting promoter element or coding function; deletions and amplifications, and/or over-expression (Bishop, 1987, 1991). Any of these mechanisms could induce a critical change in the gene leading to its activation. This activation in turn will result in production of a protein with aberrant function as in the case of RAS-type mutations, which leave a critical signal transducing protein in a permanently "on" position (Bos, 1989). Amplification of an affected gene, resulting in inappropriate expression or over-expression, may also be capable of transforming and may contribute directly to the expression of the malignant phenotype (Henderson *et al.*, 1991). Currently over fifty oncogenes have been identified in human cancers (Haber and Fearon, 1998).

2.5.2 Clinical relevance of oncogenes

The ability to detect and understand the function of oncogenes also has clinical implications. These become obvious when the following is considered: diagnostic tools, monitoring of cancer therapy, prediction of prognosis and therapy (Bishop, 1991; Gullick and Sikora, 1990). Consistently recurring karyotypic abnormalities are found in many haematologic and solid tumours. These abnormalities include chromosomal re-arrangements as well as the gain or loss of whole chromosomes or chromosome segments (Nowell and Hungerford,

1960). A good example is found in CML where the reciprocal translocation between chromosomes 9 and 22 in which ABL (9q34) joins to the breakpoint cluster region (BCR) on 22q11 to form a new fusion gene and an 8.5-kb chimeric mRNA is transcribed (Kantarjian *et al.*, 1994).

Another example is the RAS mutations commonly seen in cases of AML and MDS. This aberration might be useful to detect the early stage of leukaemia and to monitor the effect of chemotherapy. Due to the fact that patients with MDS with RAS mutations have a higher possibility of progressing to AML and have poorer prognosis, this oncogene aberration might be useful to predict the prognosis (Bos, 1989).

2.5.3 Cyclins and cell cycle control

The G_0/S phase transition in higher eukariotic cells is controlled by the assembly of G_1 cyclins, cyclins C, D1-3 and E, with their cyclin dependent kinase (CDK) partners, CDK2 and CDK4 (Draetta, 1994; Sherr, 1994 and 1995). The cyclins are divided into three groups based on the phases of the cell cycle in which they act: G_1 cyclins (C, D1, D2, D3 and E cyclins), an S phase cyclin (A) and G_2/M cyclins (B1, B2 and A cyclins) (Draetta, 1994; Hunter and Pines, 1994; Sherr, 1994).

The G_1 cyclins were independently isolated by their ability to complement conditionally defective G_1 cyclins in yeast (C, D1 and E cyclins) and as early growth factor-induced genes (D1, D2 and D3 cyclins) (Lew *et al.*, 1991; Matsushime *et al.*, 1991). Cyclin B participates in the regulation of the G_2/M

transition by its association with $p34^{cdc2}$, whereas cyclin A appears to be essential for the completion of S phase entry into G_2 phase in complexes with both $p34^{cdc2}$ and CDK2 (Rosenblatt *et al.*, 1992; Walker and Maller, 1991; Roy *et al.*, 1991; Solomon *et al.*, 1990). In contrast, the complexes formed between D-type cyclins and CDK4 and CDK6 integrate growth factor signals and the cell cycle, allowing cells to progress normally through G_1 phase (Kato *et al.*, 1993; Meyerson and Harlow, 1994). With the genetic alterations that occur in their pathway during oncogenesis, it appears to involve many of its components, including the D-type cyclins, cyclin-dependent protein kinases and cyclin-dependent kinase inhibitors (Lathi *et al.*, 1997).

2.5.4 D-type cyclins

Unlike other G_1 cyclins (e.g. cyclin C and cyclin E), the D-type cyclins are highly homologous to one another and their expression overlaps during G_1 phase, thus suggesting that this group of cyclins may be somewhat redundant in their function (Khatib *et al.*, 1993). It must, however, be noted that not all D-type cyclins are expressed in each tissue, suggesting that their function may be linked to the specific tissues in which they are expressed. This particular cell cycle pathway is subject to a number of alterations during tumourigenesis, presumably due to its importance in response to mitogenic stimulation (Parry *et al.*, 1995; Koh *et al.*, 1995; Medema *et al.*, 1995).

It was established that each of the D-type cyclins contain a functional retinoblastoma (RB)-binding motif and that these cyclins are induced in response

to mitogens in a cell lineage-specific manner (Sherr, 1994). Cyclins D2 and D3 preferentially bind to RB, whereas cyclin D1·RB complexes are less stable (Ewen *et al.*, 1993; Dowdy *et al.*, 1993). Microinjection of cyclin D1 antibodies and/or antisense oligonucleotides into normal human diploid fibroblasts, NIH3T3 and Rat-2 cells, revealed that a sub-group of these cells could not enter the S-phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993). The ability of the microinjected cells to pass the restriction point and begin DNA replication was directly related to the time that elapsed between the re-addition of serum and microinjection of cyclin D1 antibodies or antisense oligonucleotides. By decreasing the level of functional cyclin D1 proteins prior to the restriction point in late G_1 phase, the ability of these cells to progress through the cell cycle was impaired. These agents seem to have no effect on the cell cycle once cells have passed the restriction point late in the G_1 phase of the cell cycle. It was also shown that ectopic expression of cyclin D1 inhibits myogenic determination factor (MyoD) and myogenin-mediated skeletal muscle differentiation indirectly. Experiments to determine the role of cyclin D1 in muscle differentiation were designed to determine whether MyoD/myogenin and/or RB were targets of cyclin D1 CDK phosphorylation. Mutation of CDK phosphorylation sites in myogenin had no effect on the ability of cyclin D1 to inhibit differentiation. Ectopic MyoD expression in fibroblasts can induce muscle-specific gene expression, as long as wild-type RB is expressed (Skapek *et al.*, 1996). In RB -/- fibroblasts expressing MyoD and a mutated RB (which cannot be hyperphosphorylated), ectopic cyclin D1 expression continues to inhibit muscle-specific gene expression, whereas ectopically expressed cyclins A and E have no effect. This suggests that the initiation of muscle-specific gene

expression, can be blocked by two distinct pathways, one of which is dependent on RB hyperphosphorylation and the other not (Lahti *et al.*, 1997).

2.5.5 Cyclin D1 in tumourigenesis

The D-type cyclins play a major role in tumour development and progression. Their over-expression due to chromosomal translocation, DNA amplification, retroviral integration or gene mutation contributes to the development of many tumour types (Musgrove *et al.*, 1994; Bartkova *et al.*, 1995; Lukas *et al.*, 1996).

Regulation of the vertebrate cell cycle requires the periodic formation, activation and inactivation of unique protein kinase complexes that consist of cyclin subunits, and cell cycle-dependent fluctuations in the levels of many of the cyclin proteins are thought to contribute, at least in part, to the activation of these enzymes (Maller, 1991; Nurse, 1990; Sherr, 1994). The cyclins are required to regulate many of the $p34^{cdc2}$-related cyclin-dependent protein kinases which have diverse functions prominently linked to the control of cell division (Devoto *et al.*, 1992; Gabrielli *et al.*, 1992; Meyerson *et al.*, 1992).

Cyclin D1 was implicated in tumourigenesis as it was found amplified or over-expressed in parathyroid, stomach, breast, head and neck tumours (Motokura and Arnold, 1993a). Among the G_1 cyclins, cyclin D1 appears to be most strongly implicated in human tumourigenesis (Motokura and Arnold, 1993b). This protein is encoded by the CCND1 gene (also known as *bcl-1* or *PRAD1*) on chromosome 11q13 Figure 2.2 (Baldin *et al.*, 1993). The 11q13 region harbours several genes that could play a role in tumourigenesis, including the proto-oncogenes INT2,

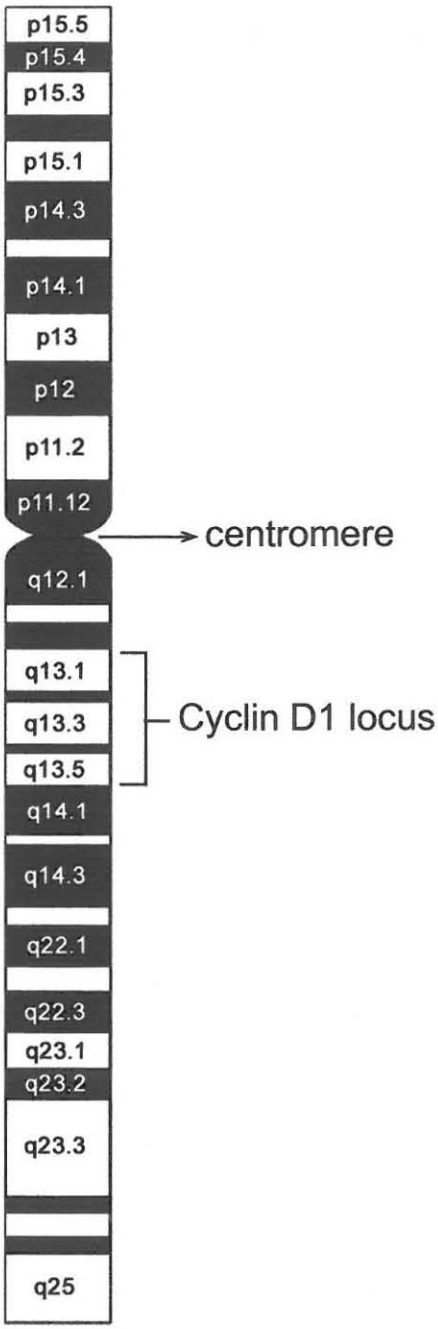


Figure 2.2 Chromosome 11 and cyclin D1 locus, 11q13 (www.vysis.com/0.asp?)

HST1 and EMS1, which encodes a putative steroid receptor co-activator (src) kinase substrate (Lammie and Peters, 1991; Schuuring, *et al.*, 1993). It was shown that a moderate increase in the level of cyclin D1 can promote cellular proliferation by accelerating the G_1 transition, while micro-injection of D1 antisense ribonucleic acid (RNA) or antibodies can prevent S phase entry (Quelle *et al.*, 1993; Reznitsky *et al.*, 1994; Baldin *et al.*, 1993). However, the induction of high levels of cyclin D1 at the G_1/S border can functionally antagonize cellular proliferation by preventing S phase entry and this suggested that the physiological effect of cyclin D1 may depend on the timing and the levels to which it is induced (Quelle *et al.*, 1993; Baldin *et al.*, 1993). Evidence pointed to a direct role for D-type cyclins in transcriptional activation and repression. For example, it was shown that cyclin D1 inhibits transcriptional activation by the dentin matrix protein 1 (DMP) transcription factor (Inoue and Sherr, 1998). Conversely, a multitude of evidence showed that cyclin D1 binds the estrogen receptor and activates its transactivation potential in a ligand-independent manner (Neuman *et al.*, 1997; Zwijsen *et al.*, 1997). In both of these cases, the influence of cyclin D1 on transcription have been shown to occur independently of its role in cell cycle progression, thus defining a novel function for the D-type cyclins as transcriptional regulators (Knudsen *et al.*, 1999).

Another regulatory mechanism involves the binding of specific inhibitory proteins ($p21^{WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$) to CDK complexes or the binding of specific inhibitory proteins directly to CDK4 (p15, $p16^{INK1}$, p18 and p19) (Gu *et al.*, 1993; Serrano *et al.*, 1993; Hannon and Beach, 1994). There is now enough evidence to indicate that disturbances in specific cyclins, CDKs, or the above mentioned

inhibitory proteins do play an important role in several types of human cancer (Weinstein and Zhou, 1996). The most frequent abnormalities relate to cyclin D1. It is rearranged and over-expressed in parathyroid tumours and centrocytic lymphomas and is amplified and/or over-expressed in a major fraction of human tumours of the breast and esophagus, squamous carcinomas of the neck and head as well as various other types of cancer (Draetta, 1994; Hunter and Pines, 1994; Motokura *et al.*, 1991; Jiang *et al.*, 1993b). It was observed that ectopic over-expression of cyclin D1 in fibroblast cultures not only shortens the G_1 phase of the cell cycle, but also enhances cell growth, cell transformation and tumourigenicity (Jiang *et al.*, 1993a).

Over-expression of cyclin D1 in rat fibroblasts enhanced their growth and tumourigenicity (Jiang *et al.*, 1993a). Cyclin D1 collaborated with an activated *RAS* oncogene or a defective adenovirus E1A oncogene to increase the transformation of the primary rodent fibroblast (Lovec, 1994; Hinds *et al.*, 1994). It was demonstrated that the introduction of an antisense cyclin D1 DNA sequence into a human esophageal carcinoma cell line, in which the cyclin D1 gene is amplified and over-expressed, reduced the level of expression of this protein and caused reversion of the malignant phenotype (Zhou *et al.*, 1996). These results suggest that increased expression of cyclin D1 can play a critical role in tumour development and in maintenance of the malignant phenotype, thus, over-expression of cyclin D1 can produce complex effects on various cellular functions involved in growth control and cell cycle progression (Imoto *et al.*, 1997). Transgenic mice carrying a Maloney Mammary Tumour Virus-cyclin D1, displayed mammary epithelial hyperplasia and developed mammary

carcinomas (Wang *et al.*, 1994). Immunohistochemical detection of cyclin D1 in a series of human breast cancers has led to the identification of a subset of carcinomas in which there is increased expression of cyclin D1 gene in the absence of gene amplification (Gillet *et al.*, 1994). The increased expression of cyclin D1 mRNA and protein in the absence of cyclin D1 gene amplification, has also been seen in human colon carcinomas (Arber *et al.*, 1996.) Thus, mechanisms other than gene amplification and chromosomal rearrangement can lead to increased levels of the cyclin D1 protein in certain human cancers (Arber *et al.*, 1996).

2.6 FLUORESCENCE *IN SITU* HYBRIDISATION

Chromosomal abnormalities are the hallmark of cancer cells. Recurring and highly consistent structural and numerical alterations have been identified in a large number of solid tumours, leukaemias and lymphomas. The identification of recurrent genetic alterations and the isolation of molecular markers have clinical applications in the diagnosis and the prognosis of neoplasia and in the detection of minimal residual disease that are essential for designing the most effective therapeutic approach (Popescu and Zimonjic, 1997).

The polymerase chain reaction (PCR) and fluorescence *in situ* hybridisation (FISH) are powerful techniques for the detection of genomic alterations. Virtually any chromosomal alterations, regardless of their complexity, can be resolved by the battery of FISH methods and DNA probes that are available. Combined chromosome banding, multifluor or spectral karyotyping and comparative

genomic hybridisation allow for the identification of structural and numerical alterations on a global basis; mapping of the DNA copy number of the entire tumour genome; complete derivation of complex rearrangements, and the localization of the breakpoints of translocations and deletions. Regions of recurrent alterations can be micro-dissected, amplified, microline libraries constructed and probes localized on extended chromosomes or chromatin fibres for construction of high-resolution physical maps that are critical for positional cloning and gene identification (Popescu and Zimonjic, 1997).

FISH enables the detection of structural and numerical chromosome aberrations in both metaphase spreads and interphase cell nuclei, and provides a simple, fast and reliable means to assess genetic instability in cancer (Gray and Pinkel, 1992; Kallioniemi *et al.*, 1992). FISH can detect genetic anomalies over a much greater dynamic size range than other techniques. The FISH reagents that are available can identify either entire chromosomes or regions smaller than a single gene (Fox *et al.*, 1995). For the enumeration of chromosome copy numbers in interphase nuclei in neoplasms, chromosome specific centromeric DNA probes can be used (Gray and Pinkel, 1992). In addition, interphase cells can be utilized, eliminating the need to cultivate cells and often permitting simple "touch" preparations to be used for biopsy samples (Fox *et al.*, 1995). This is a commonly used technique for the determination of gene and chromosome dosage. In tissue sections it allows precise histopathologic correlation of multiple foci of normal epithelium, premalignant lesions and carcinoma within a single sample, including a study of intratumoural heterogeneity (Qian *et al.*, 1999). FISH can also be a very effective method for the detection of homozygous chromosome deletions in primary

tumour tissues and the demonstration of homozygous deletion has been very useful in pinpointing tumour suppressor gene locations (Xiao *et al.*, 1995; Schofield and Fletcher, 1992).

2.6.1 Chromosomal translocations

Localization of genes in the human genome has been essential for molecular analysis of chromosomal alterations in cancer cells. Oncogenes have been found to be associated with cancer-specific translocations (Croce and Nowell, 1985). Lymphomas, leukaemias and an increasing number of sarcomas are characterized by specific chromosomal translocations and several general principles have emerged from molecular studies of recurrent translocations. These re-arrangements, which are frequently part of relatively simple karyotypes, particularly in lymphomas and leukaemias, lead to activation of proto-oncogenes or to the formation of new oncogenic chimeric genes. Both the gene fusion proteins and oncogene products are often transcriptional factors (Rabbits, 1994). FISH offers a way to identify chromosomal translocations on the whole karyotype by the use of combination multifluor detection or spectral analysis. Hybridisation with chromosome and single-copy gene probes and bi- and multicolour detection can characterize specific translocations. FISH with two chromosome probes permits the identification of complex re-arrangements (Zhang *et al.*, 1993).

2.6.2 Gene mapping

FISH is the most efficient and reproducible approach for the precise localization of single sequences within metaphase chromosomes. Along the chromosome length, the relative position of the hybridisation signal can be determined by

measurements on unbanded preparations or by indirect localization of G-banded chromosomes as described for isotopic *in situ* hybridisation (Popescu *et al.*, 1985). The resolution of gene mapping was significantly enhanced by the development of methods for direct visualization of the fluorescent signal on banded metaphase chromosomes, and procedures for induction of G-, R- or Q-banding have been successfully used to map single-copy genes. These procedures include the use of fluorescent dyes, combinations of heat or alkaline pH treatments or cohybridisation with Alu-PCR products (McNeil *et al.*, 1991; Lichter *et al.*, 1991). Popescu and Zimonjic (1997) reported on the use of a procedure that is based on contrast enhancement of 4'6-diamidino-2-phenylindole (DAPI)-banded chromosome images that permit the direct localization of the fluorescent signal on G-like bands.

2.6.3 FISH and high-resolution mapping

FISH is now increasingly used to construct high-density cytogenetic maps. Multicolour FISH metaphase chromosomes are feasible if the fluorescent signals are separated by at least one megabase (Trask, 1991). Further refinement of the interphase resolution of mapping can be achieved by alkaline-borate treatment that will increase the nuclear diameter fivefold (Yokota *et al.*, 1995).

2.6.4 DNA amplification

As in the case of other structural alterations, DNA amplification is a manifestation of genomic instability. The small chromatin structures that are referred to as double minutes, and abnormally banded or homogeneously stained regions, are cytological manifestations of DNA amplification. DNA amplification is commonly

associated with the acquisition of drug resistance and tumour progression, and it also occurs in the early stages of cell transformation, conferring a selective growth advantage to the cells. DNA amplification should be regarded as a critical alteration in cancer development because commonly it involves proto-oncogenes and persists over indefinite rounds of replication (Bishop, 1987). Amplification of single or multiple genes can be readily visualized and quantified by FISH. The level of gene amplification on a cell-by-cell basis may however vary considerably (Kallioniemi *et al.*, 1994).

2.6.5 RNA localization

FISH can be successfully applied to detect nuclear RNA. The direct visualization of viral and cellular RNA transcripts was demonstrated by an original method designed to optimize the preparation of interphase cells and the hybridisation conditions (Lawrence *et al.*, 1989). The ability to detect RNA transcripts to interphase nuclei, confers new potential applications for investigating disorders resulting from RNA-processing defects, screening for expressed sequences and detection of viral expression (Popescu and Zimonjic, 1997).

Central University of Technology, Free State

CHAPTER 3

METHODOLOGY

3.1 MATERIALS

Chemicals were bought from Merck and were of AR grade.

Ultra pure sterile water was used throughout.

VECTASHIELD Mounting medium with DAPI (4'6-diamidino-2-phenylindole) was bought from BDS.

Probes were bought from Vysis:

Locus specific identifier (LSI) Cyclin D1 (11q13) Spectrum Orange (SO) (300 kb) / Chromosome enumeration probe 11 (CEP 11) Spectrum Green (SG). Cyclin D1 probe was used to determine the copy number of the Cyclin D1 locus and as an enumerator probe for chromosome 11 in interphase nuclei (Part #: 32-191039).

LSI p53 (17p13.1) Spectrum Orange (130 kb). This probe was used to detect deletion or amplification of the p53 locus in interphase nuclei (Part #: 32-190008).

CEP 17 (17p11.1-q11,1) Spectrum Green was used as internal control to determine the copy number for chromosome 17 in interphase nuclei (Part #: 32-132017).

3.2 POPULATION AND SAMPLE SIZE

Twenty-one blood samples (heparin blood) and 4 archival bone marrow samples were obtained from the Department of Hematology, Universitas Hospital in Bloemfontein. No informed consent was obtained from patients as all the samples were treated anonymously. Samples were randomly collected from January 2001 to November 2001, at the time the samples were sent to the laboratory for FISH analysis for Ph chromosome and PCR analysis for the BCR/ABL breakpoint lesion. Inclusion criterion was the clinical and haematological diagnosis of CML.

A normal peripheral blood sample was used as an internal control to assess hybridisation efficiency.

3.2.1 *Patient characteristics*

Of the 25 CML samples, 11 were of female patients and 14 were of male patients. Two of the peripheral blood samples (8 and 10) were of the same patient sent in at different times.

The ages ranged from 8 to 81 years, with a mean age of 41.92 years (age for numbers 8 and 10 were calculated once). Nineteen of the patients were in chronic phase, 3 in accelerated phase, 1 in blastic phase, 1 transformed to ALL (T/ALL) and 1 transformed to myelofibrosis (T/myel).

Twenty-two of the samples were positive for Ph chromosome studies by FISH, 1 was non-conclusive, 1 was not performed and 1 was negative. Eleven of the

samples were positive for the BCR/ABL PCR translocation, 6 were negative and 8 were not performed. Table 3.1 summarises the patient characteristics.

3.3 SAMPLE PREPARATION

The Ficoll paque method was used to isolate leukocytes from the blood. Five millilitres (ml) of blood (heparin) was layered onto 5 ml Ficoll paque (Ficoll Paque, Pharmacia Biotech) in a 15 ml Falcon tube. It was then centrifuged at 1400 revolutions per minute (rpm) for 30 minutes in a Heraues bench centrifuge. The supernatant (Ficoll and plasma) were removed and the buffy layer was transferred to a clean tube. Five ml of phosphate buffered saline was added and centrifuged at 2500 rpm for 5 minutes. The supernatant was carefully removed without disturbing the pellet. Ten ml of prewarmed (37^0C) potassium chloride (75 millimolar) was added while mixing on a vortex mixer (the first ml was added drop by drop to prevent clumping of the cells) and the tube was incubated at 37^0C for 10-20 minutes. After centrifugation at 2500 rpm for 5 minutes the supernatant was removed, leaving a small amount for re-suspension. Seven ml of fresh cold fixative (3:1 Methanol:Acetic acid, volume per volume (v/v)) was added drop by drop (first ml) while mixing on a vortex mixer. After centrifugation at 2500 rpm for 5 min, the supernatant was removed and fresh fixative was added twice more. After the last centrifugation, fixative was added to make a slightly milky suspension. The cells were stored at 4^0C until use. Before use, the cells were centrifuged and re-suspended in fresh cold fixative twice before the slides were made.

Table 3.1 Characteristics of patients

Sample number	Age	Sex	Clinical course	Ph chromosome (FISH)	BCR/ABL (PCR)
1	35	F	CP	+	-
2	49	M	CP	Non-conclusive	+
3	48	F	CP	+	+
4	30	F	CP	+	Not performed
5	38	F	CP	+	+
6	8	F	CP	+	+
7	39	M	CP	+	Not performed
8	81	F	CP	+	-
9	56	M	CP	+	+
10	81	F	CP	+	-
11	16	M	BP	+	+
12	53	M	CP	+	Not performed
13	39	F	CP	+	Not performed
14	48	M	AP	+	+
15	57	M	CP	+	+
16	44	F	CP	+	+
17	25	M	T/ALL	+	-
18	48	M	CP	+	Not performed
19	67	F	CP	+	-
20	14	M	CP	+	Not performed
21	62	M	CP	+	+
22	19	M	AP	+	Not performed
23	44	M	T/myel	Not performed	Not performed
24	52	M	AP	-	-
25	34	F	CP	+	+

+: Positive, -: Negative
F: Female, M: Male
BCR/ABL: Translocation determined by PCR
Nrs. 1-21: peripheral blood
Nrs. 22-25: bone marrow

The bone marrow smears were left to soak for 40 minutes in fresh cold fixative (3:1 Methanol:Acetic acid, v/v) after which they were dried at room temperature and handled according to the Vysis protocol.

3.4 SLIDE PREPARATION

Slides were produced by dropping a single drop of the cell suspension onto a precleaned slide. When dry, the slides were observed by phase contrast microscopy to ensure that there were enough single cells for hybridisation. The number and spread of the cells were adjusted by adding more or less fixative at this stage. Two slides from each sample were prepared, one for p53 and one for Cyclin D1. Normal peripheral blood control slides were prepared at the same time to assess hybridisation efficiency for p53 and cyclin D1 respectively.

3.5 HYBRIDISATION WITH LSI/CEP PROBES

The hybridisation areas were marked with a diamond pen and the slides were immersed in the denaturation solution (70% formamide/2X saline sodium citrate (SSC) at 73° C for 5 minutes. Dehydration was performed in an ethanol series with one minute steps of 70%, 80% and 100% at room temperature (slides were kept in 100% ethanol until the probe was applied). The hybridisation mixtures for p53 (in combination with a probe specific for chromosome 17) and Cyclin D1 (in combination with a probe specific for chromosome 11) (fluorophore-labeled painting probes and blocking DNA in Tris– ethylenediaminetetraacetic acid (EDTA) buffer) were prepared according to the Vysis protocol (1998, Vysis, Inc.).

Seven microlitre (µl) of hybridisation buffer (dextran sulfate, formamide, SSC, pH7.0), 2 µl purified water and 1 µl of DNA probe (Cyclin D1/CEP11) was dispensed into a microcentrifuge tube at ambient temperature. For the preparation of the p53/CEP 17 only 1 µl of purified sterile water was used and 1 µl each of the probes for p53 and chromosome 17. The tube was centrifuged for 3 seconds, vortexed and centrifuged again. The slides were removed from the ethanol and dried at 50°C in a temperature controlled chamber. 10 µl of the probe mixture was applied on the nuclei spots, coverslipped and sealed with commercial glue. A Hybaid Omnigene with hybridisation block and humidified chamber was used to co-denature the sample and the probe. The temperature was first set at 85°C for one minute and then reduced to 37°C, and the slides were left to hybridise overnight. After hybridisation, the coverslips were carefully removed. Post hybridisation washes consisted of one rinse in 0.4X SSC/0.3% Nonidet P-40 (NP-40) at 73°C for 2 minutes and one rinse in 2X SSC/0.1% NP-40 at ambient temperature for 1 minute. The slides were removed and allowed to air dry in the dark at ambient temperature. The nuclei were counterstained with 10 µl of DAPI with antifade and coverslipped. Slides were stored at minus 20°C in the dark until scoring was performed (http://www.vysis.com/tech_sup_proto_lsi.asp#4).

3.6 VISUALIZATION AND SCORING OF RESULTS

Hybridisation sites were analyzed using a Nikon E400 fluorescence microscope equipped with appropriate filter sets (DAPI, FITC, Rhodamine) for visualizing spectrum green and orange fluorescence signals. The target area was scanned

using a low power objective to examine cell distribution. Cells were scored using a 100X oil objective, counting the number of signals for one probe colour within the nuclear boundary. Hybridisation signals were counted in 100 nuclei each for p53 and cyclin D1. For p53, cells were simultaneously scored, individually for the number of p53 and CEP 17 signals. For cyclin D1, cells were simultaneously scored, individually for the number of cyclin D1 and CEP 11 signals (signals appeared as either bright and compact oval shapes, split into two smaller but connected dots, or a stringy diffuse shape). The signal count for each cell was recorded and the scanning process continued until 100 nuclei were enumerated and the data collected.

Photos were taken using 400 ASA colour film. Photos representative of selected examples (p53 and cyclin D1) were taken and digitally enhanced.

The following criteria were applied for *in situ* hybridisation signals according to the manufacturer's protocols

- Cells that touched or overlapped were not counted.
- Diffuse signals were counted if they were separate from other signals.
- Split signals in very close proximity were counted as one.
- Signals connected by a strand of fluorescence were counted as one.
- Nuclei that were not intact, were not evaluated.
- Nuclei with overlapping signals of different colour were not evaluated.
- Non-specific hybridisation signals were not counted. Their lower intensity and different shapes recognized these signals.
- Nuclei with signals located on the periphery of the nucleus were not evaluated.

3.7 QUALITY CONTROL

Quality assurance certificates were issued with each Vysis set of probes showing that the lot number had been tested and found to comply with all the specifications.

- Hybridisation procedures were performed as indicated in the package insert provided with the probe product.
- Only four slides were washed at the same time to maintain accurate temperature of the wash buffers.
- To limit the degradation of the photo-sensitive fluorophores, all solutions containing fluorophores were handled in reduced light. All the steps that did not require light for manipulation, were performed in darkness.
- A calibrated thermometer was used for measuring the temperature of the solutions.
- Only slides with a good quality were scored. Factors taken into consideration were: even distribution of cells across the slide, concentration of the cells per field and clumping, and background or nuclear fluorescent "noise".
- Since the quality control guidelines were written for the *ProbeChek* control slide product, and none were available for p53 and cyclin D1, normal peripheral blood was used as control in order to monitor hybridisation efficiency. No quality parameters for p53 and cyclin D1 for the laboratory were available.

Central University of Technology, Free State

CHAPTER 4

RESULTS

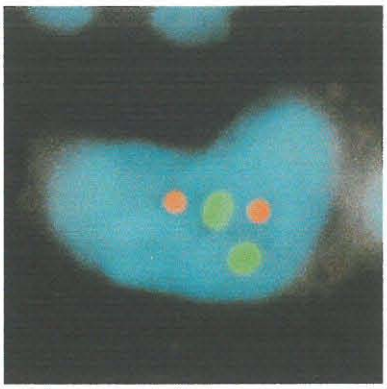
p53 signals were counted for 100 interphase nuclei per sample slide. Staining quality assurance controls, consisting of a normal peripheral blood sample, were included for each probe set. Since there were nuclei that failed to react with the probes (caused by inadequate hybridization or chromatin loss), cells were scored only when at least one bright orange signal for p53 or one green signal for chromosome 17 was present. The total number of cells (per sample) with hybridisation signals for p53 was compared to the total number of cells with signals for chromosome 17 in order to determine the presence of amplification or deletion. LSI SO p53 DNA probe hybridised to band 17p13.1 of human chromosome 17, while the CEP SG 17 DNA probe hybridised to the centromere (band region 17p11.1-q11.1, locus D17Z1) of chromosome 17.

Allelic loss is defined as fewer p53 signals per cell than chromosome 17 centromere signals in the same cell, with the exact threshold being open to debate. A cut-off rate was established as 15% of cells analyzed, and the loss of allele (LOA) rate was considered significant when it was higher than 15% (Smith *et al.*, 2000; Stuppia *et al.*, 1997). However, Lazaridou *et al.* (2002) arrived at a figure of 12 ± 3% and for this study a cut-off rate of 9% was adopted (Lazaridou *et al.*, 2000).

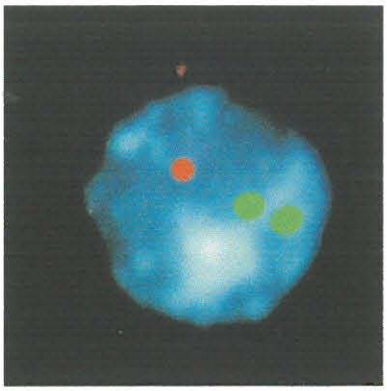
FISH was carried out on 18 Ph^- samples in chronic phase, 1 non-conclusive Ph sample in chronic phase, 1 Ph^+ sample in blastic phase, 2 Ph^+ samples in accelerated phase, 1 Ph^- sample in accelerated phase, 1 Ph^+ sample which transformed to ALL and 1 not-performed Ph sample which transformed to myelofibrosis. One of the bone marrow samples, Nr 23, failed to react with either the chromosome 17 or the p53 probe, thus the results of this sample were not included. Significant p53 amplification was not observed in any of the remaining 24 samples as only a few nuclei in a small number of samples (8) had 3 or more signals.

Eleven (46%) of the 24 samples showed nuclei with no signals for p53, 23 (96%) samples showed nuclei with one signal, 23 (96%) samples showed nuclei with two signals and 8 (35%) samples showed nuclei with 3 signals. None of the samples showed the presence of more than three signals per nucleus. Figure 4.1 shows representative photographs of p53 hybridisation.

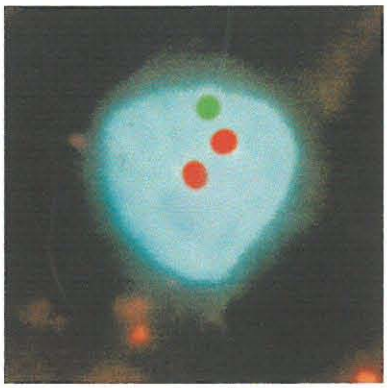
Eighteen of the samples showed an LOA rate of <9%, meaning no significant loss of p53. Six of the samples displayed an LOA of >9%: samples 4, 7, 8, 10, 12 and 24 showed an LOA of 44%, 29%, 10%, 10%, 56% and 100% respectively. Samples 4, 7, 8, 10 and 12 (peripheral blood) were in CP, whereas sample 24 was in AP (bone marrow). For samples 4, 7, 8, 10 and 12 the Ph chromosome studies were positive, whereas in sample 24 the Ph chromosome was negative. For samples 4, 7, 12 the BCR/ABL PCR was not performed, and the BCR/ABL PCR of samples 8, 10 and 24 were negative. It must be noted that samples 8 and 10 were those of the same patient sent in at different times. Twelve of the



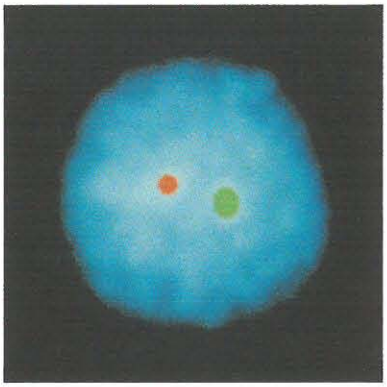
Nucleus with 2 signals for chromosome 17 (green) and 2 signals for p53 (orange)



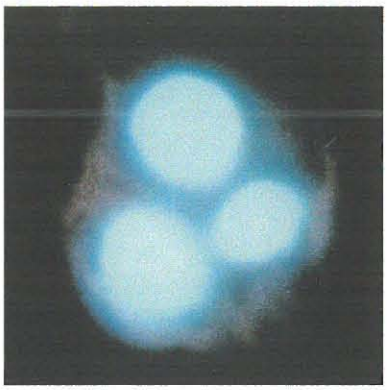
Nucleus with 2 signals for chromosome 17 (green) and 1 signal for p53 (orange)



Nucleus with 1 signal for chromosome 17 (green) and 2 signals for p53 (orange)



Nucleus with 1 signal for chromosome 17 (green) and 1 signal for p53 (orange)



Nuclei that failed to react with either probe

Figure 4.1 Representative photographs of p53 hybridisation

samples showed no LOA. Sample 11 in BP showed an LOA of only 2%, and samples 14 and 22 in AP showed no LOA, whereas sample 24 in AP showed an LOA of 100%. Sample 17, which transformed to ALL, showed no LOA.

For chromosome 17 copy numbers, 24 (100%) of the samples showed one signal, 24 (100%) samples showed two signals and 1 (4%) sample showed 3 signals for p53. None of the samples showed more than 3 copies. Table 4.1 shows the relationship between number of signals for chromosome 17 and p53 in the samples by FISH.

No significant p53 amplification or deletion was detected in any of the 900 control nuclei counted to assess hybridisation efficiency (Table 4.2). Twenty-nine (3%) of the 900 nuclei counted showed 1 signal for p53, 862 (96%) showed 2 signals and 9 (1%) showed 3 signals. None of the control nuclei counted showed more than 3 signals for p53. No extra copy numbers for chromosome 17 were counted in any of the 900 control nuclei. Thirteen (1%) of the 900 nuclei counted showed 1 signal and 887 (99%) showed two signals for chromosome 17.

Table 4.1 Relationship between number of signals for [illegible] p53 by FISH

Sample number	Clinical course	Ph status	p53 number of nuclei with this number of signals					p53 % LOA	Chromosome 17 number of nuclei with this number of signals				
			0	1	2	3	>3		0	1	2	3	>3
1	CP	+	6	31	62	1	0	6	0	29	71	0	0
2	CP	NC	0	25	75	0	0	0	0	27	73	0	0
3	CP	+	0	52	48	0	0	0	0	48	52	0	0
4	CP	+	44	48	8	0	0	44	0	76	24	0	0
5	CP	+	0	46	54	0	0	0	0	49	51	0	0
6	CP	+	2	55	42	1	0	2	0	56	44	0	0
7	CP	+	29	49	22	0	0	29	0	61	39	0	0
8	CP	+	10	49	41	0	0	10	0	40	59	1	0
9	CP	+	0	44	56	0	0	0	0	41	59	0	0
10	CP	+	10	49	41	0	0	10	0	42	58	0	0
11	BP	+	2	81	16	1	0	2	0	64	36	0	0
12	CP	+	56	38	6	0	0	56	0	52	48	0	0
13	CP	+	8	30	60	2	0	8	0	36	64	0	0
14	AP	+	0	10	89	1	0	0	0	8	91	0	0
15	CP	+	0	20	79	1	0	0	0	20	80	0	0
16	CP	+	0	29	71	0	0	0	0	18	82	0	0
17	T/ALL	+	0	2	98	0	0	0	0	1	99	0	0
18	CP	+	0	24	76	0	0	0	0	2	98	0	0
19	CP	+	6	3	91	0	0	6	0	35	65	0	0
20	CP	+	0	13	86	1	0	0	0	9	91	0	0
21	CP	+	2	12	85	1	0	2	0	5	95	0	0
22	AP	+	0	1	99	0	0	0	0	1	99	0	0
23	T/Myel	NP	0	0	0	0	0	NC	0	0	0	0	0
24	AP	-	100	0	0	0	0	100	0	2	98	0	0
25	CP	+	0	7	93	0	0	0	0	8	92	0	0

Nrs. 1-21: peripheral blood; Nrs. 22-25: bone marrow; NC: non-conclusive; NP: not-performed

Table 4.2 Relationship between number of signals for chromosome 17 and p53 in 900 normal peripheral blood leukocytes

Count per 100 nuclei	p53					Chromosome 17				
	# of nuclei with 0 signals	# of nuclei with 1 signal	# of nuclei with 2 signals	# of nuclei with 3 signals	# of nuclei with >3 signals	# of nuclei with 0 signals	# of nuclei with 1 signal	# of nuclei with 2 signals	# of nuclei with 3 signals	# of nuclei with >3 signals
1	0	2	97	1	0	0	1	99	0	0
2	0	3	95	2	0	0	3	97	0	0
3	0	5	93	2	0	0	2	98	0	0
4	0	3	97	0	0	0	1	99	0	0
5	0	4	95	1	0	0	4	96	0	0
6	0	4	95	1	0	0	1	99	0	0
7	0	3	95	2	0	0	0	100	0	0
8	0	2	98	0	0	0	0	100	0	0
9	0	3	97	0	0	0	1	99	0	0

Cyclin D1 signals were counted for 100 interphase nuclei per sample slide. Staining quality assurance controls, consisting of a normal peripheral blood sample, were included for each probe set. Since there were nuclei that failed to react with the probes, cells were scored only when at least one bright orange signal for cyclin D1 and/or one bright green signal for chromosome 11 was present. The total number of cells (per sample) with hybridisation signals for cyclin D1 was compared to the total number of cells with signals for chromosome 11 in order to determine the presence of amplification or deletion. LSI cyclin D1 SO / CEP 11 SG dual colour DNA probe hybridised to band 11q13, and to the centromere, band region 11p11.11-q11, locus D11Z1 of human chromosome 11, respectively.

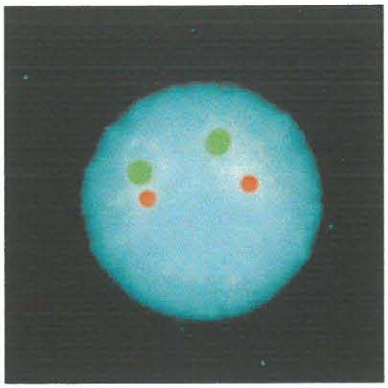
The criteria for cyclin D1 gene amplification in interphase nuclei and metaphase chromosomes are not well established in the literature, nor is there consensus as to what should be called amplification. The definition of gene amplification is thus more or less subjective. In this instance amplification was defined as more than two foci of cyclin D1 hybridisation per nucleus (Sheyn *et al.*, 1997; Suzuki *et al.*, 1998; Hoechtlen-Vollmar *et al.*, 2000). The significance of LOA for cyclin D1 could not be established from the literature.

Cyclin D1 amplification was not observed in any of the 25 CML samples examined. Thirteen (52%) of the 25 samples showed the presence of nuclei with no signals, 23 samples (92%) showed nuclei with one signal and all 25 (100%) samples showed the presence of nuclei with two signals. None of the samples showed the presence of more than two signals for cyclin D1 per nucleus. Figure

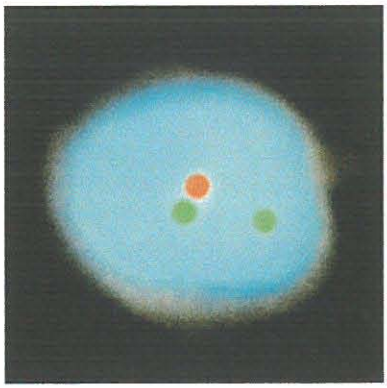
4.2 shows representative photographs of cyclin D1 hybridisation. However, although no amplification was detected, LOA for cyclin D1 was detected in 13 of the 25 samples. Samples 14, 22 and 24 in AP showed an LOA of 0%, 75% and 96% respectively. Sample 11 in BP showed an LOA of 45%, sample 17 which transformed to ALL showed a 2% LOA and sample 23 which transformed to myelofibrosis showed an LOA of 0%. The percentage LOA between samples in CP varied from 0-61%.

No extra copies of chromosome 11 were found in any of the samples. One sample (4%) showed the absence of chromosome 11 in 30% of the nuclei counted, 24 (96%) samples showed one signal for chromosome 11 and 25 (100%) samples showed the presence of 2 signals for chromosome 11. Table 4.3 shows the relationship between the number of signals for chromosome 11 and cyclin D1 in the samples by FISH.

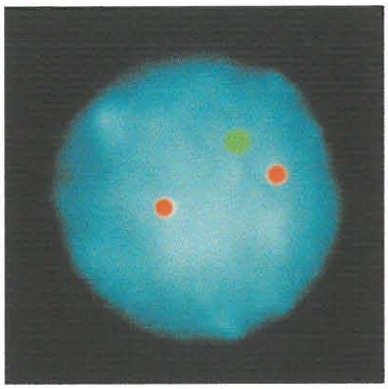
Amplification of cyclin D1 was not detected in any of the 900 control nuclei counted to assess hybridisation efficiency (Table 4.4). Thirty-four (4%) of the 900 nuclei counted showed 1 signal, 859 (95%) showed 2 signals and 7 (1%) showed 3 signals. No significant amplification or deletion of chromosome 11 copy numbers was detected. In the 900 control nuclei counted, 6 (1%) nuclei showed 3 signals for chromosome 11, 57 (6%) nuclei showed one signal, and 837 (93%) nuclei showed two signals.



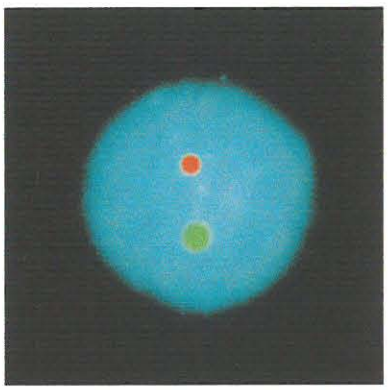
Nucleus with 2 signals for chromosome 11 (green) and 2 signals for cyclin D1 (orange)



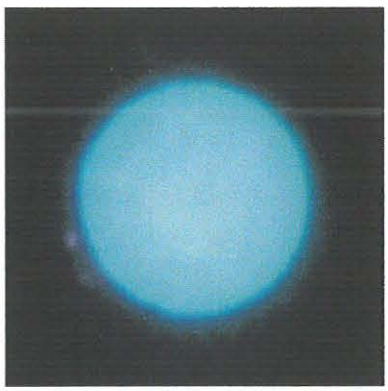
Nucleus with 2 signals for chromosome 11 (green) and 1 signal for cyclin D1 (orange)



Nucleus with 1 signal for chromosome 11 (green) and 2 signals for cyclin D1 (orange)



Nucleus with 1 signal for chromosome 11 (green) and 1 signal for cyclin D1 (orange)



Nucleus that failed to react with either probe

Figure 4.2 Representative photographs of cyclin D1 hybridisation

Table 4.3 Relationship between number of signals for chromosome 11 and cyclin D1 by FISH

Sample number	Clinical course	Ph status	Cyclin D1 number of nuclei with this number of signals					Cyclin D1 % LOA	Chromosome 11 number of nuclei with this number of signals				
			0	1	2	3	>3		0	1	2	3	>3
1	CP	+	0	23	77	0	0	0	0	28	72	0	0
2	CP	NC	0	15	85	0	0	0	0	15	85	0	0
3	CP	+	11	26	62	0	0	11	0	28	72	0	0
4	CP	+	26	38	36	0	0	26	30	50	20	0	0
5	CP	+	8	25	66	0	0	8	0	26	74	0	0
6	CP	+	2	25	73	0	0	2	0	27	73	0	0
7	CP	+	16	38	46	0	0	16	0	45	55	0	0
8	CP	+	0	5	95	0	0	0	0	5	95	0	0
9	CP	+	4	47	49	0	0	4	0	54	46	0	0
10	CP	+	3	20	77	0	0	3	0	19	81	0	0
11	BP	+	45	40	14	0	0	45	0	72	28	0	0
12	CP	+	61	34	5	0	0	61	0	65	35	0	0
13	CP	+	8	27	65	0	0	8	0	38	62	0	0
14	AP	+	0	0	100	0	0	0	0	5	95	0	0
15	CP	+	0	15	85	0	0	0	0	15	85	0	0
16	CP	+	0	22	78	0	0	0	0	23	77	0	0
17	T/ALL	+	2	10	88	0	0	2	0	10	90	0	0
18	CP	+	0	41	59	0	0	0	0	46	54	0	0
19	CP	+	0	1	99	0	0	0	0	3	97	0	0
20	CP	+	0	0	100	0	0	0	0	0	100	0	0
21	CP	+	0	2	98	0	0	0	0	1	99	0	0
22	AP	+	75	4	21	0	0	75	0	1	99	0	0
23	T/Myel	NP	0	7	93	0	0	0	0	7	93	0	0
24	AP	-	96	1	3	0	0	96	0	9	91	0	0
25	CP	+	0	16	84	0	0	0	0	14	86	0	0

Nrs. 1-21: peripheral blood
Nrs. 22-25: bone marrow
NC: non-conclusive
NP: not-performed

Table 4.4 Relationship between number of signals for chromosome 11 and cyclin D1 in 900 normal peripheral blood leukocytes

Count per 100 nuclei	Cyclin D1					Chromosome 11				
	# of nuclei with 0 signals	# of nuclei with 1 signal	# of nuclei with 2 signals	# of nuclei with 3 signals	# of nuclei with >3 signals	# of nuclei with 0 signals	# of nuclei with 1 signal	# of nuclei with 2 signals	# of nuclei with 3 signals	# of nuclei with >3 signals
1	0	7	92	1	0	0	5	95	0	0
2	0	4	96	0	0	0	8	90	2	0
3	0	4	96	0	0	0	4	96	0	0
4	0	3	97	0	0	0	3	97	0	0
5	0	1	99	0	0	0	3	95	2	0
6	0	7	91	2	0	0	9	91	0	0
7	0	2	96	2	0	0	7	92	1	0
8	0	1	98	1	0	0	9	91	0	0
9	0	5	94	1	0	0	9	90	1	0

COMPARATIVE

In general, the patient group studied here showed more abnormality for chromosome 17/p53 than for chromosome 11/cyclin D1. In the 6 samples that showed LOA >9% for p53, cyclin D1 LOA was observed in five of the six samples, whereas samples 4, 7, 8, 10, 12 and 24 showed cyclin D1 deletion in 26%, 16%, 0%, 3%, 61% and 96% of the nuclei respectively (Table 4.5). In samples 3, 5, 6, 9, 10, 11, 13, 17 and 22 where cyclin D1 LOA was observed, only sample 10 showed a significant p53 LOA of 10%. In samples 4, 7, 12, and 24, high percentages of LOA were detected both for p53 and cyclin D1. Sample 23 that failed to react with the p53 and chromosome 17 probes, did however react with cyclin D1, but no amplification or deletion was detected for cyclin D1. It must be noted that in sample four, 30% of the nuclei failed to react with the probe for chromosome 11, whereas 26% of nuclei failed to react with the probe for cyclin D1.

One would expect a correlation between chromosome abnormality and disease stage. In this study, however, the correlation is not that clear. Nineteen of the samples are in CP and samples 11, 14, 17, 22, 23 and 24 are in advanced stages of the disease, yet their results do not reflect this. This clearly shows that other genes are involved as well and that the progression of this disease is very complex.

Table 4.5 Comparison of %LOA between p53 and cyclin D1

Sample number	Clinical course	Ph status	P53: % LOA	Cyclin D1: % LOA
1	CP	+	6	0
2	CP	NC	0	0
3	CP	+	0	11
4	CP	+	44	26
5	CP	+	0	8
6	CP	+	2	2
7	CP	+	29	16
8	CP	+	10	0
9	CP	+	0	4
10	CP	+	10	3
11	BP	+	2	45
12	CP	+	56	61
13	CP	+	8	8
14	AP	+	0	0
15	CP	+	0	0
16	CP	+	0	0
17	T/ALL	+	0	2
18	CP	+	0	0
19	CP	+	6	0
20	CP	+	0	0
21	CP	+	2	0
22	AP	+	0	75
23	T/Myel	NP	NC	0
24	AP	-	100	96
25	CP	+	0	0

Nrs 1-21: peripheral blood
Nrs 22-25: bone marrow
NC: non-conclusive
NP: not-performed

CHAPTER 5

DISCUSSION

Despite the multitude of studies on p53, there are considerable differences in the methods of detection and interpretation criteria. A major problem in drawing conclusions from this study, is the difficulty in comparing malignant cells from the different clinical courses, due to the uneven distribution of the different population sizes and the fact that no control cell lines were used. However, comparative deletion studies between control cell lines and leukaemic patient cells are a problem because it is difficult to obtain control cell line samples as normal counterparts of leukaemic cells. Sugimoto *et al*. (1992) suggested that mutation or inactivation of the p53 gene, unlike in fresh myeloid leukaemia cells, is common in myeloid leukaemia cell lines because loss of normal function of this gene helps them acquire long-term proliferation potential *in vitro*. Because cell lines are frequently used in experimental studies as prototype malignant cells that grow *in vivo*, it is important to know whether abnormalities in critical cancer-associated genes, such as p53 observed in cancer cell lines, indeed reflect the lesion present in the original malignant cells growing *in vivo* (Sen *et al*, 1995).

p53 LOA >9% was detected in 5 chronic phase samples (Ph^+ / peripheral blood) and in one accelerated phase sample (Ph^- / bone marrow). The absence of the Ph chromosome (sample 24) is a major poor-prognosis feature translating into a median survival time of 9 to 14 months compared with 3 to 4 years for Ph chromosome positive disease (Gomez *et al*., 1981). p53 deletions and/or

mutations have been described in 10-25% of the cases of CML, especially those with a myeloid phenotype, but were rare during the chronic phase (5%) (Imamura *et al.*, 1994). No LOA >9% was detected in any of the other samples despite the clinical course. The results of this study correlate with those done by Stuppia *et al.*, (1997) who showed a significant LOA >15% in 3 out of 12 (25%) patients in chronic phase and an LOA <15% in 5 out of 13 (39%) patients in blastic phase. Eight out of 13 patients did, however, show an LOA of >15% in BP. It is thus possible that the samples in the present study that showed LOA >9%, was analysed during a late chronic phase, just preceding blastic crisis, indicating that p53 tumour suppressor genes may be involved in the progression of 6 out of 24 cases of CML. Several investigators have shown that loss of the normal function of p53 plays a significant role in the malignant transformation process. Inactivation of functional alterations of both alleles of the gene through deletions, insertions and point mutations have been shown in various types of human malignancies, including those of haematopoietic origin (Honda *et al.*, 2000; Stuppia *et al.*, 1997). Among myeloid leukaemia cells, although gross structural alterations and point mutations have both been reported, their frequency remains a subject of controversy (Kelman *et al.*, 1989).

Changes in the p53 gene may also be involved in the rare megakaryocytic blast crisis variant, but evidence was against its involvement in lymphoid blastic transformation of in Ph^+ ALL (Ahuja *et al.* 1991). In studies done by Stuppia *et al.*, (1997) 5 out of 13 patients without a significant LOA rate had a lymphoid BC, and showed a persistence of apoptotic processes although at a lower rate than compared to CP. p53 rearrangements and the related suppression of apoptosis

seem to be specifically associated with a myeloid evolution of the disease. This would explain the results of sample 17 which transformed to ALL and where no apparent deletion or amplification was detected. Molecular events other than those involving the p53 gene are thus likely to be responsible for the transformation of CML to Ph^+ ALL. Since CML evolution occurs toward two distinct forms, namely a myeloid or a lymphoid acute phase, different genetic alterations might thus lead to the distinct blast crisis phenotypes (Iolascon *et al.*, 1998).

In the present study, factors such as therapy, disease evolution, possible transplants and survival were not taken into consideration, nor was any analysis done to determine mutations of p53 or the possible actions of cytokines. Another important point is the relationship between the p53 rearrangement and the phenotype of the blastic phase. A larger group of samples studied in a prospective manner during the duration of disease progress from time of diagnosis to fatal blast crisis, will better clarify these issues. FISH with chromosome- and/or locus-specific DNA probes allows one to obtain cytogenetic information from interphase cells and is a powerful tool for monitoring the expansion of the leukaemic clone with p53 LOA. Furthermore, by using FISH, it was easy to determine whether a locus is lost. It would be advisable, however, that in the instance where bone marrow examples are examined, the samples be collected in a heparinised syringe and that the mononuclear bone marrow cells be separated by density gradient centrifugation over Ficoll-Hypaque (Drach *et al.*, 1998; Sankar *et al.*, 1998). Using this method one can more readily control the distribution of the cells on the slides, avoiding overlapping and layering of cells,

and eliminating unnecessary background material, which causes technical difficulty when scoring the results.

Isochromosome 17q (i(17q)) is the most common isochromosome in haematologic malignancies and has been described as a primary and a secondary aberration. i(17q) is a frequent secondary chromosomal aberration in the accelerated phase or blast crisis of CML, indicating that this abnormality also plays an important role in disease progression (Mitelman, 1993). As a consequence of the i(17q) formation, one copy of this gene will be lost, an event that could unmask the effects of an existing mutation in the other allele. Given the frequent occurrence of i(17q) in haematologic malignancies, and the fact that i(17q) is sometimes found as the sole karyotypic abnormality, suggesting a possible role as a primary genetic alteration in leukemogenesis, Fioretos *et al.* (1999) studied its role in haematologic neoplasms by FISH. Because i(17q) formation results in loss of 17p material, potentially unmasking the effect of a tumour suppressor gene, they also studied the occurrence of p53 mutations by sequencing the entire coding region. No coding p53 mutations were identified in any of the cases investigated, hence the formation of an i(17q) does not serve to uncover the effect of an already existing p53 mutation in the normal appearing chromosome 17. They also did not detect any clear-cut differences between the localization of the chromosomal breakpoints and the type of haematologic malignancy in their studies, suggesting that 17p harbours a "promiscuous" gene which, if structurally rearranged or otherwise deregulated, is pathogenetically important in a wide variety of neoplasms (Fioretos *et al.*, 1999). In this study the presence of i(17q) was not detected by interphase FISH, so it may be present but

undetected. By using dual colour FISH with specific probes for the centromeric region of chromosome 17 and for the p53 gene, it is, however, possible to determine simultaneously the number of chromosome 17 and detect regional loss of 17p13 in metaphase chromosomes. It is a simple method, requiring very little material (Kobayashi *et al.*, 1996).

The high sensitivity of FISH was also exploited by Sankar *et al.* (1998) to detect the loss of various loci on 17p13, among others p53 (17p13.1), cCI17-732 (17p13.1) D17S34 (17p13.1), D17S379 (17p13.3) and cCI17-636 (17p13.2). Their results indicated that FISH was accurate and a relatively easy method, and technical errors occurring during PCR can be avoided. FISH is therefore an appropriate method for performing deletion mapping in leukaemia patients. Furthermore, their results suggested that there may be new oncogenes located on the commonly deleted region (17p) that may have an important role in the progression of leukaemia (Sankar *et al.*, 1998). Loss of p53 hybridisation signals is predominantly due to an interstitial deletion that is presumably to small to be detected on metaphase chromosomes, thus increasing the evidence that FISH is a more sensitive method than banding analysis to detect deletions of narrow chromosomal regions (Drach *et al.*, 1998).

Cyclin D1 amplification by FISH was not detected in any of the peripheral blood samples or the bone marrows. These results are in concurrence with those found by other researchers, albeit by other methods. The expression of the three types of D-cyclins that have been identified, is tissue and cell-type specific. Cyclin D1 is mainly expressed by epithelial and mesenchymal cells, but not in cells of

lymphoid and myeloid lineages, whereas cyclins D2 and D3 are expressed by myelomonocytic and lymphoid cells (Sonoki *et al.*, 1999; Ott *et al.*, 1997). Della Ragione *et al.* (1997) analyzed the G_1 phase proteic machinery, including cyclin D types, CDKs and CDK inhibitors, of cell populations obtained at different stages of haematopoietic cell lineage. Five cellular phenotypes were studied as representatives of distinct differentiation pathways. Their results indicated that al the cellular preparations express cyclin D2 and D3, but not cyclin D1. Ajchenbaum *et al* (1993) observed the induction of cyclin D2 and D3, but not cyclin D1, in normal murine T cells. In studies with 24 different transformed human haematopoietic cell lines, cyclin D3 was found to be expressed in both myeloid and lymphoid cell lines, whereas cyclin D2 was expressed in a small fraction, without regard to whether the line was myeloid, T cell or B cell in origin. The significance of cyclin D1 in human myeloproliferative disease needs clarification since cyclin D1, which is the major cyclin D occurring in mesenchymal tissues, is not normally expressed in human haematopoietic cells, and mice with cyclin D1 knock-outs do not have clear defects in haematopoiesis (Della Ragione *et al.*, 1997; Ajchenbaum *et al.*, 1993; Sicinski *et al.*, 1995). Cyclins D2 and D3 are, however, expressed in human haematopoietic cells and are good candidates as BCR/ABL target molecules (Gesbert *et al.*, 2000).

The 11q13 region of chromosome 11 is amplified in several types of human carcinoma and harbours several genes that could play a role in tumourigenesis, including the proto-oncogenes INT2, HST1 and EMS1 (Lammie and Peters, 1991). Since the amplicon is quite extensive, these other candidate oncogenes could be co-amplified. The role of these genes on the 11q13 region was not

investigated in this study, thus their role in tumourigenesis of CML was not established.

In the present study the 11q13 amplification of cyclin D1 in peripheral blood cells and bone marrow from patients, with various stages of CML, were investigated. Although this assay assesses only chromosome 11 and cyclin D1, the ease and convenience of this method are very attractive. Although no amplification was observed in this study, FISH was demonstrated by others to be a suitable method for the detection of 11q13 amplification in squamous cell carcinoma of the head and neck, esophageal squamous cell carcinomas, mantle cell lymphomas, and ovarian carcinomas, (Alavi *et al.*, 1999; Sheyn *et al.*, 1997; Katz *et al.*, 2000; Diebold *et al.*, 2000). FISH allows for the precise localization of the amplified gene within the cells and it allows the detection interphase chromosomal rearrangements using non radio-active probes (Sheyn *et al.*, 1997; Devilee *et al.*, 1988). Furthermore, FISH does not require purified DNA and results are thus not affected by degradation. Multicoloured probes can be used simultaneously, thereby increasing the significance of the observation, and results are available in 24 hours (Alavi *et al.*, 1999). Dual labelling and hybridisation with multiple probes allows localization of the gene to its specific chromosome. It was also shown that FISH analysis can be readily applied to formalin-fixed, paraffin-embedded tissues. FISH analysis is therefore well suited for use in studies using archival tissues (Sheyn *et al.*, 1997). However, a drawback of this method is that the probe which is specific for the 11q13 breakpoint, may not be useful for detecting the t(11;14)(q13;q32) chromosomal translocation, and, in addition, the 11q13

locus may be translocated with sites other than 14q32, resulting in a fusion product that is not the 1(11;14) (Katz *et al.*, 2000).

Similar negative amplification results have been reported in anal carcinomas, and it was suggested that cyclin D1 alterations do not play a significant role in tumourigenesis in the anal region (Sheyn *et al.*, 1997). Katz *et al.*, (2000) also showed that B-cell non-Hodgkin lymphomas rarely have a substantial number of cells with more than two 11q13 signals. Immunohistochemical examination of cases of AML, ALL, T-cell ALL and MDS also showed no cyclin D1 expression, suggesting that cyclin D1 might not be involved in the pathogenesis of the leukaemia despite the cytogenetic involvement of 11q13 (Wong, 1999). The involvement of 11q13 in a diversity of chromosomal rearrangements in myeloid and lymphoid neoplasia is intriguing. There is a possibility of a variety of breakpoint involvement in 11q13 abnormalities which are indistinguishable at microscopic level. On the other hand, the underlying molecular changes may be similar, despite the fact that different chromosomes are involved in rearrangement with 11q13, as in the case of 11q23 (Kwong *et al.*, 1995). Clearly, further molecular studies are required to clarify this issue.

The results of this study require a re-examination of the involvement of cyclin D1 in leukocyte proliferation as it did not endorse the diagnostic value of cyclin D1 amplification. Cyclin D1 over-expression, as a result of amplifications and/or re-arrangements, is an important contributing factor to oncogenesis although its pathological significance in CML is still unclear. However, the high expression of cyclin D1 in certain myeloid leukaemias was identified to reflect their proliferative

activity and not to represent the oncogenic over-expression (Suzuki *et al.*, 2001). Because cyclin D1 expression is not seen in normal haematopoietic cells, the expression of cyclin D1 in lymphomas other than MCL may also indicate that cyclin D1 is an ubiquitous oncogene (Aguilera *et al.*, 1998). The functional role of STAT5 and cyclin D1 in haematopoiesis might be redundant and replaced sufficiently by other signalling molecules and other D-type cyclins (Matsumura *et al.*, 1999). Thus, analysis of normal counterparts for regulation of the D-type cyclins, especially cyclin D2 and D3, is needed to understand the significance of the apparent promiscuous usage of D-type cyclins in haematopoietic cells.

In this study no correlation could be made between p53 LOA and over-expression of cyclin D1 in the various stages of CML. It was, however, demonstrated in studies by Opitz *et al.*, (2001) that over-expression of cyclin D1 and p53 inactivation led to the immortalization of oral keratinocytes. It was also shown than cyclin D1 protein accumulates to significantly higher levels in murine fibroblasts in which wild-type p53 is induced, and it provided evidence that increased cyclin D1 protein appears necessary, in concert with waf1/p21, for the induction of a proper G_1 arrest as mediated by wild-type p53 (Del Sal *et al.*, 1996).

Despite all the information on CML, one can only quote Deininger *et al.* (2000) in asking: "Why is there a predominantly myeloid expansion when all 3 lineages carry the BCR/ABL translocation? What is the biologic basis for the extraordinary variability in the clinical course of a disease that appears to carry just a single genetic lesion? What is the molecular basis for the genomic instability that we

see clinically as relentless progression to blast crisis?" It is clear that cellular processes rely on integrated networks and not on unidirectional pathways. Only in this way can the cell achieve the flexibility required to respond to the various stimuli within a multicellular organism. To understand the extraordinary complexity of CML, it is therefore necessary to undertake the demanding task of studying signal transduction in primary progenitor cells.

Central University of Technology, Free State

CHAPTER 6

REFERENCES

Aguilera NSI, Bijwaard KE, Duncan B, Krafft AE, Chu W-S, Abbondanzo SL, Lichy JH and Taubenberger JK, 1998. Differential expression of cyclin D1 in mantle cell lymphoma and other non-Hodgkin's lymphomas. *Am J Path*, **153**, 1969-1976.

Ahuja HG, Bar-Eli M, Advani SH, Benchimol S and Cline, MJ, 1989a. Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukaemia. *Proc Natl Acad Sci USA*, **86**, 6783-6787.

Ahuja HG, Bar-Eli M, Clarke P, Snyder D, Foreman S, Goldman J and Cline MJ, 1989b. p53 gene alterations in blast crisis of chronic myelogenous leukemia. *Cancer Cells (Cold Spring Harbor)*, **7**, 117-120.

Ahuja H, Bar-Eli M, Arlin Z, Advani S, Allen SL, Goldman J, Snyder D, Foti A and Cline M, 1991. The spectrum of molecular alterations in the evolution of chronic myelocytic leukaemia. *J Clin Invest*, **87**, 2042-2047.

Ajchenbaum F, Ando K, DeCaprio JA and Griffin JD, 1993. Independent regulation of human D-type cyclin gene expression during G_1 phase in primary human T lymphocytes. *J Biol Chem*, **268**, 4113-4119.

Alavi S, Namazie A, Calcaterra TC and Wang MB, 1999. Clinical application of Fluorescence *in situ* Hybridization for chromosome 11q13 analysis in head and neck cancer. *Laryngoscope*, **109**, 874-879.

Almon E, Goldfinger N, Kapon A, Schwartz D, Levine AJ and Rotter V, 1993. Testicular tissue-specific expression of the p53 suppressor gene. *Dev Biol*, **156**, 107-116.

Arber N, Hibshoosh H and Moss SF, 1996. Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. *Gastroenterology*, **110**, 669-674.

Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, van Tuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R and Vogelstein B, 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*, **244**, 217-221.

Baldin V, Lukas J, Marcote MJ, Pagano M and Draetta G, 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev*, **7**, 812-821.

Bartkova J, Lukas J, Strauss M and Bartek J, 1995. Cyclin D1 aberrantly accumulates in malignancies of diverse histogenesis. *Oncogene*, **10**, 775-778.

Benchimol S, Lamb P, Crawford LV, Sheer D, Shows TB, Bruns GA and Peacock J, 1985. Transformation associated p53 protein is encoded by a gene on human chromosome 17. *Somat Cell Molec Genet*, **11**, 505-510.

Bennett WP, Hollstein MC, Metcalf RA, Welsh JA, He A, Zhu SM and Kusters I, 1992. p53 mutation and protein accumulation during multistage human esophageal carcinogenesis. *Cancer Res*, **52**, 6092-6097.

Bishop JM, 1987. The molecular genetics of cancer. *Science*, **235**, 305-311.

Bishop JM, 1991. Molecular themes in oncogenesis. *Cell*, **64**, 235-248.

Bos JL, 1989. Ras oncogenes in human cancer: review. *Cancer Res*, **49,** 4682-4689.

Caron de Fromentel C and Soussi T, 1992. TP53 tumor suppressor gene: A model for investigating human mutagenesis. *Genes Chromosome Cancer*, **4**, 1-15.

Carson DA and Ribeiro JM, 1993. Apoptosis and disease. *Lancet*, **341**, 1251-1254.

Carson DA and Lois A, 1995. Cancer progression and p53. *Lancet*, **346**, 1009-1011.

Cheng J, Yee JK, Yeargin J, Friedmann T and Haas M, 1992. Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. *Cancer Res*, **52**, 222-226.

Cobb MH, 1999. MAP kinase pathways. *Prog Biophys Mol Biol*, **71**, 479-500.

Cortes J, Talpaz M and Kantarjian HM, 1996. Chronic myelogenous leukemia: A review. *Am J Med*, **100**, 555-570.

Counter MD, Avilion AA, Le Feuvre CE, Steward NG, Greider CW, Harley CB and Bachetti S, 1992. Telomere shortening associated with chromosomal instability is arrested in immortal cells which express telomerase activity. *EMBO J*, **11**, 1921-1929.

Croce CM and Nowell PC, 1985. Molecular basis of B-cell neoplasia. *Blood*, **65**, 1-7.

Cross SM and Sanchez CA, 1995. A p53 dependent mouse spindle checkpoint. *Science*, **267**, 1353-1356.

Culotta E and Koshland DE, 1993. p53 sweeps through cancer research. *Science,* **262,** 1958-1961.

Daley GQ and Baltimore D, 1988. Transformation of an interleukin-4 dependent cell line by the chronic myelogenous leukemia-specific p210 bcr/abl protein. *Proc Natl Acad Sci USA*, **85**, 9312-9316.

Daley GQ, van Etten RA and Baltimore D, 1990. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*, **247**, 825-829.

Dang CV, 1991. c-Myc oncoprotein function. *Biochem Biophys Acta*, **1072**, 103-113.

De Groot RP, Raaijmakers JA, Lammers JW and Koenderman L, 2000. STAT5-Dependent Cyclin D and Bcl-xL expression in Bcr-Abl-transformed cells. *Mol-Cell-Biol-Res-Commun*, **3(5)**, 299-305.

De Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J and Stephenson JR, 1982. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*, **300**, 765-767.

Deininger MWN, Goldman JM and Melo JV, 2000. The molecular biology of chronic myeloid leukemia. *Blood*, **96**, 3343-3356.

Della Ragione F, Borriello A, Mastropietro S, Della Pietra V, Monno F, Gabutti V, Locatelli F, Bonsi L, Bagnara GP and Iolascon A, 1997. Expression of G1-phase

cell cycle genes during hematopoietic lineage. *Biochem Biophys Res Commun*, **231**, 73-76.

Del Sal G, Murphy M, Ruaro EM, Lazarevic D, Levine AJ and Schneider C, 1996. Cyclin D1 and p21/waf1 are both involved in p53 growth suppression. *Oncogene*, **12**, 177-185.

Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AH, Willard HF, Pearson PL and Cornelisse CJ, 1988. Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. *Cancer Res*, **48**, 5825-5830.

Devoto SH, Mudryj M, Pines J, Hunter T and Nevins JR, 1992. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. *Cell*, **68**, 167-176.

Di-Bacco A, Keeshan K, McKenna SL and Cotter TG, 2000. Molecular abnormalities in chronic myeloid leukemia: deregulation of cell growth and apaptosis. *Oncologist*, **5,** 405-415.

Diebold J, Mösinger K, Peiro G, Pannekamp U, Kaltz C, Baretton GB, Meier W and Löhrs U, 2000. 20q13 and cyclin D1 in ovarian carcinomas. Analysis by fluorescence *in situ* hybridization. *J Pathol*, **190**, 564-571.

Dowdy SF, Hinds PW, Louis K, Reed SI, Arnold A and Weinberg RA, 1993. Physical interaction of the retinoblastoma protein with human D cyclins. *Cell*, **73**, 499-511.

Drach J, Ackermann J, Fritz E, Krömer E, Schuster R, Gisslinger H, DeSantis M, Zojer N, Fiegl M, Roka S, Schuster J, Heinz R, Ludwig H and Huber H, 1998. Presence of a p53 gene deletion in patients with multiple myeloma predicts for short survival after conventional-dose chemotherapy. *Blood*, **92**, 802-809.

Draetta G, 1994. Mammalian G1 cyclins. *Curr Opin Cell Biol*, **6**, 842-846.

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato JY and Livingston DM, 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*, **73**, 487-497.

Faderl S, Talpaz M, Estrov Z and Kantarjian HM, 1999a. Chronic myelogenous leukemia: Biology and therapy. *Ann Intern Med*, **131**, 207-219.

Faderl S, Kantarjian HM and Talpaz M, 1999b. Chronic Myelogenous Leukemia: Update on Biology and Treatment. *Oncology*, **13(2)**, 169-184.

Feinstein E, Gale RB, Reed J and Cananni E, 1992. Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene*, **7**, 1853-1857.

Fioretos T, Strömbeck B, Sandberg T, Johansson B, Billström R, Borg A, Nilsson PG, Van Den Berghe H, Hagemeijer A, Mitalman F and Höglund M, 1999. Isochromosome 17q in blast crisis of chronic myeloid leukemia and in other hematologic malignancies is the result of clustered breakpoints in 17p11 and is not associated with coding TP53 mutations. *Blood*, **94 (1)**, 225-232.

Foti A, Ahuja HG, Allen SL, Koduru P, Schuster MW, Schulman P, Bar-Eli M and Cline MJ, 1991. Correlation between molecular and clinical events in the evolution of chronic myelocytic leukemia to blast crisis. *Blood*, **77**, 2441-2444.

Fox JL, Hsu P, Legator MS, Morrison LE and Seelig SA, 1995. Fluorescence *in situ* Hybridization: Powerful molecular tool for cancer prognosis. *Clin Chem*, **41**, 1554-1559.

Gabrielli BG, Roy LM, Gautier J, Philippe M and Maller JL, 1992. A cdc2-related kinase oscillates in the cell cycle independently of cyclins G2/M and cdc2. *J Biol Chem*, **267**, 1969-1975.

Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM and Della-Favera R, 1991. p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA,* **88**, 5413-5417.

Gaiger A, Henn T, Horth E, Geissler K, Mitterbauer G, Maier-Dobersberger T, Greinix H, Mannhalter C, Haas OA, Lechner K, *et al.*, 1995. Increase of bcr-abl

chimeric mRNA expression in tumor cells of patients with chronic myeloid leukemia precedes disease progression. *Blood*, **86**, 2371-2378.

Gesbert F, Sellers WR, Signoretti S, Loda M and Griffin JD, 2000. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ through the phosphatidylinositol 3-kinase/AKT pathway. *J Biol Chem*, **275**, 39223-39230.

Gillet C, Fantl V and Smith R, 1994. Amplification and over-expression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res*, **54**, 1812-1817.

Gomes GA, Sokal JE and Walsh D, 1981. Prognostic features at diagnosis of chronic myelocytic leukemia. *Cancer*, **47**, 2470-2475.

Gottlieb E, Haffner R, Von Ruden T, Wagner EF and Oren M, 1994. Downregulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. *EMBO J*, **13**, 1368-1374.

Gray JW and Pinkel D, 1992. Molecular cytogenetics in human cancer diagnosis. *Cancer*, **69**, 1536-1542.

Griffin JD, Todd RF, Ritz J, Nadler LM, Canellos GP, Rosenthal D, Gallivan M, Beveridge RP, Weinstein H, Karp D and Schlossman SF, 1983. Differentiation patterns in the blastic phase of chronic myeloid leukemia. *Blood*, **61**, 85-91.

Gu Y, Turck CW and Morgan DO, 1993. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature*, **366**,707-710.

Gullick W and Sikora K, 1990. Oncogenes as clinical tools. In: Carney D, Sikora K, eds. *Genes and cancer*. New York: Wiley, 11-18.

Guo JQ, Wang JYG and Arlinghaus RB, 1991. Detection of BCR-ABL proteins in blood cells of benign phase chronic myelogenous leukemia patients. *Cancer Res*, **51**, 3048-3051.

Haber DA and Fearon ER, 1998. The promise of cancer genetics. *Lancet*, **351**, 1-8.

Hall PA, McKee PH, Menege HD, Dover R and Lane DP, 1993. High levels of p53 protein in UV-irradiated normal human skin. *Oncogene*, **8**, 203-207.

Hannon GJ and Beach D, 1994. P15 (INK4B) is a potential effector of TGF induced cell cycle arrest. *Nature*, **371**, 257-261.

Harris CC, 1993. p53: At the crossroads of molecular carcinogenesis and risk assessment. *Science*, **262**, 1980-1981.

Hartwell LH and Kastan MB, 1994. Cell cycle control and cancer. *Science*, **266**, 1821-1828.

Henderson BE, Ross RK and Pike MC, 1991. Toward the primary prevention of cancer. *Science*, **25**, 1131-1138.

Hinds PW, Finlay CA, Quartin RS, Baker SJ, Fearon ER, Vogelstein B and Levine AJ, 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. *Cell Growth and Differentiation*, **1**, 571-580.

Hinds PW, Dowdy SF, Eaton EN, Arnold A and Weinberg RA, 1994. Function of a human cyclin gene as an oncogene. *Proc Natl Acad Sci USA*, **91**, 709-713.

Hoechtlen-Vollmar W, Menzel G, Wick M, Seidel D, Bartl R and Lamerz R, 2000. Amplification of cyclin D1 gene in multiple myeloma: Clinical and prognostic relevance. *Br J Haematol*, **109**, 30-39.

Hollstein MC, Sidransky D, Vogelstein B and Harris CC, 1991. p53 mutations in human cancers. *Science*, **253**, 49-53.

Honda H, Ushijima T, Wakazono K, Oda H, Tanaka Y, Aizawa S, Ishikawa T, Yazaki Y, and Hirai H, 2000. Acquired loss of p53 induces blastic transformation in $p210^{bcr/abl}$-expressing hematopoietic cells: a transgenic study for blast crisis of human CML. *Blood,* **95**, 1141-1150.

http://www.vysis.com/0.asp?Page=ChromoSearch&View=Body&ChromoNumber=17

http://www.vysis.com/0.asp?Page=ChromoSearch&View=Body&ChromoNumber=11

http://www.vysis.com/tech_sup_proto_lsi.asp#4

Hunter T and Pines J, 1994. Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. *Cell*, **79**, 573-582.

Hussain SP and Harris CC, 1998. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res*, **58**, 4023-4037.

Imamura J, Miyoshi I and Koeffler HP, 1994. p53 in Hematologic malignancies. *Blood*, **84**, 2412-2421.

Imoto M, Doki Y, Jiang W, Han EK and Weinstein IB, 1997. Effects of Cyclin D1 over-expression of G1 progression-related events. *Exp Cell Res*, **236**, 173-180.

Inoue K and Sherr CJ, 1998. Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent kinase independent mechanism. *Mol Cell Biol*, **18**, 1590-1600.

Iolascon A, Della Ragione F, Giordani L, Serra A, Saglio G and Faienza MF, 1998. Expression of cell cycle regulatory genes in chronic myelogenous leukemia. *Haematologica*, **83**, 771-777.

Isobe M, Emanuel B, Givol D, Oren M and Croce C, 1986. Localization of gene for human p53 tumor antigen to band 17pq13. *Nature*, **320**, 84-85.

Jiang W, Kahn SM, Zhou P, Zhang YJ, Cacace AM, Infance AS, Doi Y, Santella RM and Weinstein IB, 1993a. Over-expression of cyclin D1 in rat fibroblast causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene*, **8,** 3447-3457.

Jiang W, Zhang YJ, Kahn S, Hollstein M, Santella R, Lu SH, Harris CC, Montesano R and Weinstein IB, 1993b. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc Natl Acad Sci USA,* **90**, 9026-9030.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray J, Waldman F and Pinkel D, 1992. Comparative genomic hybridization for molecular cytogenetics analysis of solid tumors. *Science*, **258,** 818-821.

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stooke T, Chen L, Smith HS, Pinkel D, Gray JW and Waldman FM, 1994. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA*, **91**, 2156-2160.

Kantarjian HM, Smith TL McCredie KB, Keating MJ, Walters RS, Talpaz M, Hester JP, Bligham G, Gehan E and Freireich EJ, 1985. Chronic myelogenous leukemia: a multivariate analysis of the associations of patient characteristics and therapy with survival. *Blood,* **66,** 1326-1337.

Kantarjian H, Dixon D, Keating MJ, Talpaz M, Walters RS, McCredie KB and Freireich EJ, 1988. Characteristics of accelerated disease in chronic myelogenous leukemia. *Cancer*, **61**, 1441-1445.

Kantarjian HM, Deisseroth A, Kurzrock R, Estrov Z and Talpaz M, 1993. Chronic myelogenous leukemia. A concise update. *Blood*, **82**, 691-703.

Kantarjian H, Talpaz M, Estey E, Ku S and Kurzrock R, 1994. What is the contribution of molecular studies to the diagnosis of BCR-ABL-positive disease in adult acute leukemia. *Am J Med*, **96**, 133-137.

Kato JY, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ, 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinaseCDK4. *Genes Dev*, **7**, 331-342.

Katz RL, Caraway NP, Gu J, Jiang F, Pasco-Miller LA, Glassman AB, Luthra R, Hayes KJ, Romaguera JE, Cabanillas FF and Mederros LJ, 2000. Detection of chromosome 11q13 breakpoints by interphase fluorescence *in situ* hybridization. *Am J Clin Pathol*, **114**, 248-257.

Kelman Z, Prokocimer M, Peller S, Kahn Y, Rechavi G, Manor Y, Cohen A and Rotter V, 1989. Rearrangements in the p53 gene in Philadelphia chromosome positive chronic myelogenous leukemia. *Blood,* **74**, 2318-2324.

Khatib ZA, Matsushime H, Valentine M, Shapiro DN, Sherr CJ and Look AT, 1993. Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. *Cancer Res*, **53**, 5535-5541.

Knowles DM, 1992. Neoplastic Hematopathology, Williams & Wilkins. Chapter 47, 1405-1417.

Knudsen KE, Cavenee WK and Arden KC, 1999. D-Type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. *Cancer Res*, **59**, 2297-2301.

Kobayashi M, Kawashima A, Mai M and Ooi A, 1996. Analysis of Chromosome 17p13 (p53 locus) Alterations in gastric carcinoma cells by dual-colour fluorescence *in situ* hybridization. *Am J Path,* **149**, 1575-1584.

Koh J, Enders GH, Dynlacht BD and Harlow E, 1995. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature*, **375**, 506-510.

Krontiris TG, 1995. Oncogenes. *N Eng J Med*, **333**, 303-306.

Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB, 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA*, **89**, 7491-7495.

Kurzrock R, Gutterman JU and Talpaz M, 1988. The molecular genetics of Philadelphia chromosome-positive leukemias. *N Engl J Med*, **319**, 990-998.

Kwong YL, Tso SC, Wong KF, Tang JKC and Chan TK, 1995. Translocational rearrangement of 11q23 in acute monoblastic leukemia. *Cancer Genet Cytogenet*, **82**, 76-79.

Lahti JM, Li H and Kidd VJ, 1997. Elimination of Cyclin D1 in vertebrate cells leads to an altered cell cycle phenotype, which is rescued by over-expression of murine Cyclins D1, D2 or D3 but not by a mutant Cyclin D1. *J Biol Chem*, **272,** 10859-10869.

Lammie G and Peters G, 1991. Chromosome 11q13 abnormalities in human cancer. *Cancer Cells*, **3**, 413-420.

Lane DP and Crawford LV, 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (Lond.)*, **278**, 261-263.

Lane DP and Benchimol S, 1990. p53 oncogene or anti-oncogene? *Genes Dev,* **4**, 1-8.

Lane DP, 1994. On the expression of the p53 protein in human cancer. *Mol Biol Rep*, **19**, 23-29.

Lawrence JB, Singer RH and Marselle LM, 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell*, **57**, 493-502.

Lazaridou A, Miraxtsi C, Korantzis J, Eleftheriadis N and Christakis JI, 2000. Simultaneous detection of BCL-2 protein, trisonomy 12 retinoblastoma and p53 monoallelic Gene. *Leuk Lymphoma*, **36**, 503-512.

Levine AJ, 1997. p53, the cellular gatekeeper for growth and cell division. *Cell*, **88**, 323-327.

Levine AJ, Momand J and Finlay CA, 1991. The p53 tumor suppressor gene. *Nature*, **351**, 453-456.

Lew DJ, Dulic V and Reed SI, 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell,* **66**, 1197-1206.

Li A, Tsuji T, Wen S, Mimura Y, Sasaki K and Shinozaki F, 1997. Detection of numeric abnormalities of chromosome 17 and p53 deletions by fluorescence *in situ* hybridization (FISH) in pleomorphic adenomas and carsinomas in pleomorphic adenoma. *Cancer*, **79**, 2314-2319.

Lichter P, Boyle AL, Cremer T and Ward DC, 1991. Analysis of genes and chromosomes by nonisotopic *in situ* hybridization. *GATA*, **8**, 24-35.

Liu E, Hielle B and Bishop JM, 1988. Transforming genes in chronic myelogenous leukemia. *Proc Natl Acad Sci USA*, **85**, 1952-1956.

Livingstone LR, White A, Sprouse J, Livanos E, Jacks T and Tisty T, 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923-935.

Lotem J and Sachs L, 1993. Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. *Blood*, **82**, 1092-1096.

Lovec H, 1994. Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. *Oncogene*, **9**, 323-326.

Lukas J, Aagaard L, Strauss M and Bartek J, 1996. Oncogenic aberrations of p16 $^{INK/CDKN2}$ and cyclin D1 cooperate to deregulate G1 control. *Cancer Res*, **55**, 4818-4823.

Maller JL, 1991. Mitotic control. *Curr Opin Cell Biol*, **3**, 269-275.

Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, Downward J, Pestell RG and Kanakura Y, 1999. Transcriptional regulation of the cyclin D1 promotor by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J*, **18**, 1367-1377.

Matsushime H, Roussel MF, Ashmun RA and Sherr CJ, 1991. Colony stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell,* **65**, 701-713.

McNeil JA, Villnave-Johnson CA, Karter KC, Singer RH and Bentley LJ, 1991. Localizing DNA and RNA within nuclei and chromosomes by fluorescence *in situ* hybridization. *GATA*, **8**, 41-58.

Medema RH, Herrera RE, Lam F and Weinberg RA, 1995. Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci USA*, **92,** 6289-6293.

Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E and Tsai LH, 1992. A family of human cdc2-related protein kinases. *EMBO J*, **11,** 2909-2917.

Meyerson M and Harlow E, 1994. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol*, **14**, 2077-2086.

Mitelman F, 1993. The cytogenetic scenario of chronic myeloid leukemia. *Leuk Lymphoma*, **1**, 11-15.

Moossa AR, Schimpff SC and Robson MC, 1991 Comprehensive textbook of Oncology, Volume two, 2nd Edition. Williams & Wilkins. Chapter 118, 1212-1217.

Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM and Arnold A, 1991. A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature*, **350**, 512-515.

Motokura T and Arnold A, 1993a. Cyclin D and oncogenesis. *Curr Opin Genet Dev*, **3**, 5-10.

Motokura T and Arnold A, 1993b. Cyclins and oncogenesis. *Biochem Biophy Acta*, **1155**, 63-78.

Muehleck SD, McKenna RW, Arthur DC, Parkin JL and Brunning RD, 1984. Transformation of chronic myelogenous leukemia: clinical, morphologic and cytogenetic features. *J Clin Pathol*, **82**, 1-14.

Musgrove EA, Lee CSL, Buckley MF and Sutherland RL, 1994. Cyclin D1 induction in breast cancer shortens G_1 and is sufficient for cells arrested in G_1 to complete the cell cycle. *Proc Natl Acad Sci USA*, **91**, 8022-8026.

Nakai H, Misawa S, Toguchida J, Yandel DW and Ishizaki K, 1992. Frequent p53 gene mutations in blast crisis of chronic myelogenous leukemia, especially in myeloid crisis harboring loss of a chromosome 17p. *Cancer Res*, **52**, 6588-6593.

Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M and Ewen ME, 1997. Cyclin D1 stimulation of estrogen

receptor transcriptional activity independent of cdk4. *Mol. Cell Biol*, **17**, 5338-5347.

Nowell PC and Hungerford DA, 1960. Chromosome studies on normal and leukemia human leukocytes. *J Natl Cancer Inst*, **25**, 85-109.

Nurse P, 1990. Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503-508.

Opitz OG, Suliman Y, Hahn WC, Harada H, Blum HE and Rustgi AK, 2001. Cyclin D1 over-expression and p53 inactivation immortalize primary oral keratinocytes by a telomerase-independent mechanism. *J Clin Invest*, **108**, 725-732.

Ott MM, Bartkova J, Bartek J, Dürr A, Ott G, Müller-Hermelink HK and Kreipe H, 1997. Cyclin D1 expression in mantle cell lymphoma is accompanied by downregulation of cyclin D3 and is not related to the proliferative activity. *Blood*, **8**, 3154-3159.

Panayiotidis P and Kotsi P, 1999. Genetics of small lymphocyte disorders. *Semin-Hematol*, **36(2)**, 171-177.

Parry D, Bates S, Mann DJ and Peters G, 1995. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. *EMBO J*, **14**, 503-511.

Popescu NC, Amsbaugh SC, Swan DC and DiPaolo JA, 1985. Induction of chromosome banding by trypsin/EDTA for gene mapping by *in situ* hybridization. *Cytogenet Cell Genet*, **39**, 73-74.

Popescu NC and Zimonjic DB, 1997. Molecular Cytogenetic Characterization of Cancer Cell Alterations. *Cancer Genet Cytogenet*, **93**, 10-21.

Porter LP, Gown AM, Kramp SG and Coltrera MD, 1992. Widespread p53 overexpression in human malignant tumors. *Am J Pathol,* **140,** 145-153.

Qian J, Jenkins RB and Bostwick DG, 1999. Genetic and Chromosomal alterations in prostatic intraepithelial neoplasia and carcinoma detected by Fluorescence *in situ* Hybridization. *Eur Urol*, **35**, 479-483.

Quelle D, Ashmun RA, Shurtleff S, Kato J, Bar-Sagi D, Roussel MF and Sherr CJ, 1993. Over-expression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev*, **7,** 1559-1571.

Rabbits TH, 1994. Chromosomal translocations in human cancers. *Nature,* **372**, 143-149.

Resnitzky D, Gossen M, Bujard H and Reed SI, 1994. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol*, **14**, 1669-1679.

Ridge SA, Worwood M, Oscier D, Jacobs A and Padua RA, 1990. FMS mutations in myelodysplastic, leukemic, and normal subjects. *Proc Natl Acad Sci USA*, **87**, 1377-1380.

Rosenblatt J, Gu Y and Morgan DO, 1992. Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. *Proc Natl Acad Sci USA*, **89**, 2824-2828.

Rowley JD, 1990. The Philadelphia chromosome translocation: a paradigm for understanding leukemia. *Cancer,* **65**, 2178-2182.

Roy LM, Swenson KI, Walker DH, Gabrielli BG, Li RS, Piwnica-Worms H and Maller JL, 1991. Activation of p34cdc2 kinase by cyclin A. *J Cell Biol*, **113**, 507-514.

Russel A, Thompson MA, Hendley J, Trute L, Armes J and Germain D, 1999. Cyclin D1 and D3 associate with the SCF complex and are coordinately elevated in breast cancer. *Oncogene,* **18**, 1983-1991.

Sankar M, Tanaka K, Kumaravel TS, Arif M, Shintani T, Yagi S, Kyo T, Dohy H and Kamada N, 1998. Identification of a commonly deleted region at 17p13.3 in leukemia and lymphoma associated with 17p abnormality. *Leukemia*, **12**, 510-516.

Savage DG, Szydlo RM and Goldman JM, 1997. Clinical features at diagnosis in 430 patients with chronic myeloid leukemia seen at a referral center over a 16-year period. *Br J Haematol*, **96**, 111-116.

Sawyers CL, Denny CT and Witte ON, 1991. Leukemia and the disruption of normal hematopoiesis. *Cell*, **64**, 337-350.

Scheffner M, Werness BA, Hulbregtse JM, Levine AJ and Howley PM, 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129-1136.

Scheffner M, Munger K, Byrne JC and Howley PM, 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci*, **88**, 5523-5527.

Schofield DE and Fletcher JA, 1992. Trisomy 12 in pediatric granulosa-stromal cell tumors: demonstration by a modified method of fluorescence *in situ* hybridization on paraffin embedded material. *Am J Pathol*, **141**, 1265-1269.

Schuuring E, Verhoeven E, Litvinov S and Michlides RJAM, 1993. The product of the EMS1 gene, amplified and over-expressed in human carcinomas, is homogeneous to a *v-src* substrate and is located in cell-substratum contact site. *Mol Cell Biol*, **13**, 2891-2898.

Sen S, Zhou H, Andersson BS, Cork A, Freireich EJ and Stass SA, 1995. p53 Gene mutations with chromosome 17 abnormalities in chronic myelogenous leukemia blast crisis patients persist in long-term cell lines but may be acquired in acute myeloid leukemia cells *in vitro*. *Cancer Genet Cytogenet*, **82**, 35-40.

Serrano M, Hannon GJ and Beach D, 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704-707.

Shannon KM, O'Connell P, Martin GA, Paderange D, Olson K, Dinndorf P and McCormick F, 1994. Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med*, **330**, 597-601.

Sherr CJ, 1994. G1 phase progression: Cycling on cue. *Cell*, **79**, 551-555.

Sherr CJ, 1995. D-type cyclins. *Trends Biochem Sci*, **20**, 187-190.

Sheyn I, Noffsinger AE, Heffelfinger S, Davis B, Miller BS and Fenoglio-Preiser CM, 1997. Amplification and expression of the cyclin D1 gene in anal and esophageal squamous cell carcinomas. *Hum Path*, **28**, 270-276.

Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Garner H, Haslam SZ, Bronson RT, Elledge SJ and Weinberg RA, 1995. Cyclin D1 provides a link between development and oncogenessis in the retina and breast. *Cell*, **82**, 621-630.

Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E and Kastan MB, 1997. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev*, **11**, 3471-3481.

Skapek SX, Rhee J, Kim PS, Novitch BG and Lassar AB, 1996. Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol Cell Biol*, **16**, 7043-7053.

Slingerland JM, Minden MD and Benchimol S, 1991. Mutation of the p53 gene in human acute myelogenous leukemia. *Blood*, **77**, 1500-1507.

Smith CA, Pollice AA, Gu L-P, Brown KA, Singh SG, Janocko LE, Johnson R, Julian T, Hyams D, Wolmark N, Sweeney L, Silverman JF and Shackney SE, 2000. Correlations among p53, Her-2/neu, and ras overexpression and aneuploidy by multiparameter flow cytometry in human breast cancer: Evidence for a common phenotypic evolutionary pattern in infiltrating ductal carcinomas. *Clin Can Res*, **6**, 112-126.

Solomon MJ, Glotzer M, Lee TH, Philippe M and Kirschner MW, 1990. Cyclin activation of p34cdc2. *Cell*, **63**, 1013-1024.

Sonoki T, Hata H, Kuribayashi N, Yoshida M, Harada N, Nagasaki A, Kimura T, Matsuno F, Mitsuya H and Matsuzaki H, 1999. Expression of PRAD1/cyclin D1 plasma cell malignancy: incidence and prognostic aspects. *Br J Haemat*, **104**, 614-617.

Stuppia L, Calabrese G, Peila R, Guanciali-Franchi P, Morizio E, Spadano A and Palka G, 1997. p53 Loss and point mutations are associated with suppression of apoptosis and progression of CML into myeloid blastic crisis. *Cancer Genet Cytogenet*, **98**, 28-35.

Sugimoto K, Toyshima H, Sakai R, Miyagawa K, Kagiwara K, Ishikawa F, Takau F, Yagaki Y and Hirai H, 1992. Frequent mutations in the p53 gene in human myeloid leukemia cell lines. *Blood*, **79**, 2378-2383.

Suzuki H, Fujioka Y and Nagashima K, 1998. Cyclin D1 gene amplification and p16 gene deletion in patients with esophageal carcinosarcoma. *Diag Mol Path*, **7(5)** 253-259.

Suzuki R, Takemura K, Tsutsumi M, Nakamura S, Hamajima N and Seto M, 2001. Detection of cyclin D1 over-expression by real-time reverse-transcriptase-mediated quantitative polymerase chain reaction for the diagnosis of mantle cell lymphoma. *Am J Path*, **159**, 425-429.

Tjian R, 1995. Molecular machines that control genes. *Sci Am*, **72**, 54-61.

Trask BJ, 1991. Fluorescence *in situ* hybridization: Application in cytogenetics and gene mapping. *Trends Genet*, **7**, 149-154.

Volm M, Koomägi R and Rittgen G, 1998. Clinical implications of cyclins, cyclin-dependent kinases, RB and E2F1 in squamous-cell lung carcinoma. *Int J Cancer (Pred.Oncol.)*, **79**, 294-299.

Walker DH and Maller JL, 1991. Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature*, **354**, 314-317.

Wang TC, Cardiff RD and Zukerberg L, 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature*, **369,** 669-671.

Weinberg RA, 1991. Tumor suppressor genes. *Science*, **254**, 1138-1146.

Weinstein IB and Zhou P, 1996. Encyclopedia of Cancer, Academic Press, San Diego, CA. Vol.1, 256-267.

Williams WJ, Beutler E, Erslev AJ and Lichtman MA, 1986. Hematology, 3rd Edition, McGraw-Hill Inc. Chapter 24, 196-207.

Wong KF, 1999. 11q13 is a cytogenetically promiscuous site in hematologic malignancies. *Cancer Genet Cytogenet*, **113**, 93-95.

Xiao S, Renshaw AA, Cibas ES, Hudson TJ and Fletcher JA, 1995. Novel fluorescence *in situ* hybridization approaches in solid tumors: characterization of frozen samples, touch preparation, and cytological preparations. *Am J Pathol*, **147,** 896-904.

Yokota H, van den Eng G, Mostert M and Trask B, 1995. Treatment of cells with alkaline borate buffer extends the capability of interphase FISH mapping. *Genomics*, **5**, 485-491.

Zhang J, Meltzer P, Jenkins R, Guan XY and Trent JM, 1993. Application of chromosome microdissection probes for elucidation of BCR-ABL fusion and variant Philadelphia chromosome translocations in chronic myelogenous leukemia. *Blood*, **12**, 3365-3371.

Zhou P, Jiang W, Weghorst CM and Weinstein IB, 1996. Over-expression of cyclin D1 enhances gene amplification. *Cancer Res,* **56**, 36.

Zhou P, Jiang W, Zhang YJ, Kahn SM, Schieren I, Santella RM and Weinstein IB, 1996. Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. *Oncogene*, **11**, 571-580.

Ziegler A and Jonason AS, 1994. Sunburn and p53 in the onset of skin cancer. *Nature*, **372**, 773-776.

Zwijsen RML, Wientjens E, Klompmaker R, van der Sman J, Bernards R and Michalides RJAM, 1997. CDK-independent activation of estrogen receptor by cyclin D1. *Cell*, **88**, 405-415.