CORRELATION OF THE D-TYPE CYCLINS WITH CLINICAL FEATURES AND SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

BY

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BY

JAMES T. PAUL

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

It has been reported that the gap 1 (G_1) cyclins can be increased in chronic lymphocytic leukemia (CLL) and may be important in the pathogenesis of this disease. In the present study, we have evaluated the D cyclins in 43 patients with classical CD19+/CD5+ CLL and correlated these findings with clinical features and survival. The RNase protection assay (RPA) was used to compare the messenger RNA (mRNA) expression levels of the cyclins in the leukemic cells of these patients with normal peripheral blood B and T cells and cord blood B cells. The mRNA levels of cyclins D1, D2, and D3 were 1.8-, 4.1- and 2.8-fold higher in the T cells compared to the peripheral or cord blood B cells. In CLL cells, the levels of cyclin D1 and D3 mRNA were significantly higher than in normal control T or B cells. In contrast, the levels of cyclin D2 mRNA were similar in CLL cells and normal B cells. The cyclin D1 and D3 mRNA levels correlated negatively with Rai stage, and early stage patients had the highest levels of cyclin D1 ($p=0.0004$) and D3 ($p=0.023$) mRNA. Additionally, there was a strong negative correlation with cyclin D1 ($p=0.043$) and D3 ($p=0.015$) mRNA levels and duration of disease. In contrast, the lymphocyte doubling time correlated positively with cyclin D1 ($p=0.003$) and D3 ($p=0.003$) mRNA expression. Univariate analysis demonstrated an inverse correlation between cyclin D1, and to a lesser extent cyclin D3, mRNA levels and survival, but the significance was lost when multivariate analysis included Rai stage, lymphocyte doubling time, duration of disease, lymphocyte count and age. The multivariate analysis showed that the most important prognostic indicators were Rai stage and lymphocyte doubling time and that cyclin D1, and D3 were markers for these clinical parameters. To assess the cause of the increased cyclin D1 and D3 mRNA levels in CLL, Southern blot analysis was carried out on all patients. Chromosomal amplification was not observed through Southern blotting although structural changes were seen for *cyclin D1* in 1 patient, *cyclin D2* in 1 patient, and *cyclin D3* in 2 patients.

In conclusion, cyclin D1, and to a less extent cyclin D3, mRNA levels are increased in CLL cells compared to normal B cells. High levels of these cyclins are predictive of a less aggressive clinical course.

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LIST OF ABBREVIATIONS

κ	kappa
λ	lambda
μg	microgram
μL	microlitre
μm	micrometer
μM	micromolar
[α-^{32}P]CTP	cytidine 5'-[α-^{32}P]triphosphate
[α-^{32}P]UTP	uridine 5'-[α-^{32}P]triphosphate
AET	2-aminoethylisothiouronium bromide
amp	ampicillin
ATM	ataxia telangiectasia
BMT	bone marrow transplant
bp	base pair
$CaCl_2$	calcium chloride
CBC	circulation blood cell
cc	cubic centimetre
CD	cluster differentiation
CdA	2-chlorodeoxyadenosine
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complimentary DNA
CGH	comparative genomic hybridization
CLB	chlorambucil
CLL	chronic lymphocytic leukemia
cm	centimetres
CO_2	carbon dioxide
CPM	counts per minute
Cyclo	cyclophosphamide
CYL	cyclin-like
D	diversity
ddH_2O	distilled deionized water
DEL	deletion
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide

FBS	fetal bovine serum
FISH	fluorescent *in situ* hybridization
Flu	fludarabine
GC	germinal centre
GITC	guanidine thiocyanate
GSK-3β	glycogen synthase kinase-3β
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HS	horse serum
IgH	immunoglobulin heavy chain
INK4	inhibit cdk4
J	joining
kb	kilobase
KCl	potassium chloride
KCO_3	potassium carbonate
$KCOOCH_3$	potassium acetate
KDa	kiloDalton
KH_2PO_4	potassium phosphate
L	litre
LB	Luria Bertani
LiCl	lithium chloride
M	mitosis
MCL	mantle cell lymphoma
MCS	multicloning site
MEK	mitogen-activated protein kinase
mg	milligrams
$MgCl_2$	magnesium chloride
mL	millilitre
mm	millimetres
mRNA	messenger RNA
Na_2HPO_4	disodium phosphate
$Na_3C_6H_5O_7$	sodium citrate
NaCl	sodium chloride
$NaCOOCH_3$	sodium acetate
NaOH	sodium hydroxide
NH_4Cl	ammonium chloride
NH_4COOCH_3	ammonium acetate
nm	nanometers
O.D.	optical density
^oC	degrees Celsius
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PRAD1	parathyroid

Pred	prednisone
Rb	retinoblastoma
RE	restriction endonuclease
RNA	ribonucleic acid
RNase A	ribonuclease A
RPA	RNase protection assay
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
S	synthesis
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SE	standard error
SRBC	sheep red blood cell
SSC	sodium chloride sodium citrate
TBE	Tris-borate-EDTA buffer
TCM	Tris-$CaCl_2$-$MgCl_2$ buffer
TE	Tris-EDTA buffer
TGE	Tris-glucose-EDTA buffer
TIF	tagged image file
TPA	12-0-tetradecanoylphorbol 13-acetate
TRIS	Tris(hydroxymethyl)aminomethane
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	variable
v/v	volume/volume
w/v	weight/volume
x g	times gravity
Thr-286	threonine-286
G3PDH	glyceraldehyde-3 phosphate dehydrogenase
G_0	gap 0
MDM-2	murine double minute 2
G_1	gap 1
DCF	2'-deoxycoformycin
G_2	gap 2
$Absorbance_{260}$	absorbance at 260 nm
$Absorbance_{280}$	absorbance at 280 nm

1. INTRODUCTION

1.1 FOCUS OF STUDY

The focus of this work is to evaluate the G_1 cell cycle regulators, the D cyclins, as prognostic markers in the hematological malignancy CLL. As with any disease, the discovery of markers that can predict disease aggressiveness and clinical course of a patient is worth further investigation. The ability to predict a patient's disease aggressiveness allows the clinician to tailor treatment regimens that are most beneficial to the patient. In addition, the discovery of new prognostic indicators may provide insight into the underlying mechanisms of the disease and aid the basic scientist in the development of new and novel treatments.

While the investigation of the D cyclin cell regulators in CLL is not new this is the first to correlate the D cyclin messenger RNA (mRNA) expression with patient characteristics: Rai stage; lymphocyte count, and lymphocyte doubling time in conjunction with patient survival. In addition, the wide spectrum of patients collected allows comparison between different stages of disease.

First, CLL will be introduced beginning with the clinical features, etiology, current treatment options, and current prognostic indicators. In addition, the field of genetics as it pertains to CLL and the development of new technologies that allow the study of this disease will be explored. Through studies of CLL genetics it has been shown that the immunoglobulin gene status in CLL is important in stratifying the disease into two

groups with distinct clinical course according to presence or absence of mutations in the variable chain genes. As such, an overview of normal B lymphocyte development, maturation, and immunoglobulin rearrangement will be discussed to allow an understanding of their relevance in CLL. As will be later presented, we postulate that these differences in immunoglobulin gene rearrangements may be related to the altered D cyclin mRNA expression. The focus will then shift briefly to introduce the basics of the eukaryotic cell cycle and outline some of the important regulatory stages. However, only the G_1 phase of the cell cycle will be discussed as it is this stage of the cell cycle in which the D cyclins exert their effects.

The D cyclins and their involvement in other cancers will be outlined, and the possible mechanisms which lead to their altered expression. Having an understanding of the normal function of the D cyclins in the cell cycle will help understand why their expression in CLL may be an important prognostic indicator. The final section will bring together the clinical features of CLL and the basic science of D cyclin function presented in the latter section. This will create a foundation allowing discussion of the altered D cyclin expression in CLL.

1.2 CHRONIC LYMPHOCYTIC LEUKEMIA

1.2.1 Clinical Features

There are approximately 1,200 new cases of CLL each year in Canada (1). CLL is the most common leukemia in North America and accounts for approximately 30% of all new cases (1). The disease primarily effects the middle aged and elderly, with the

median age being 55 years, and with a slight male predominance (2). The disease is characterized by the accumulation of mature-appearing cluster differentiation (CD) 5+ B lymphocytes (CD19+, CD20+) in the peripheral blood, marrow, spleen, and lymph nodes (Figure 1) (2). The disease is usually detected through a routine circulating blood cell (CBC) count, and diagnosis confirmed through demonstration of a persistent peripheral lymphocyte count of $>5 \times 10^9$/litre (L), and that the cell morphology is typical for CLL and that by immunophenotyping the cells are CD5+, CD19+. Other presenting features include lymphadenopathy or fatigue related to anemia (2).

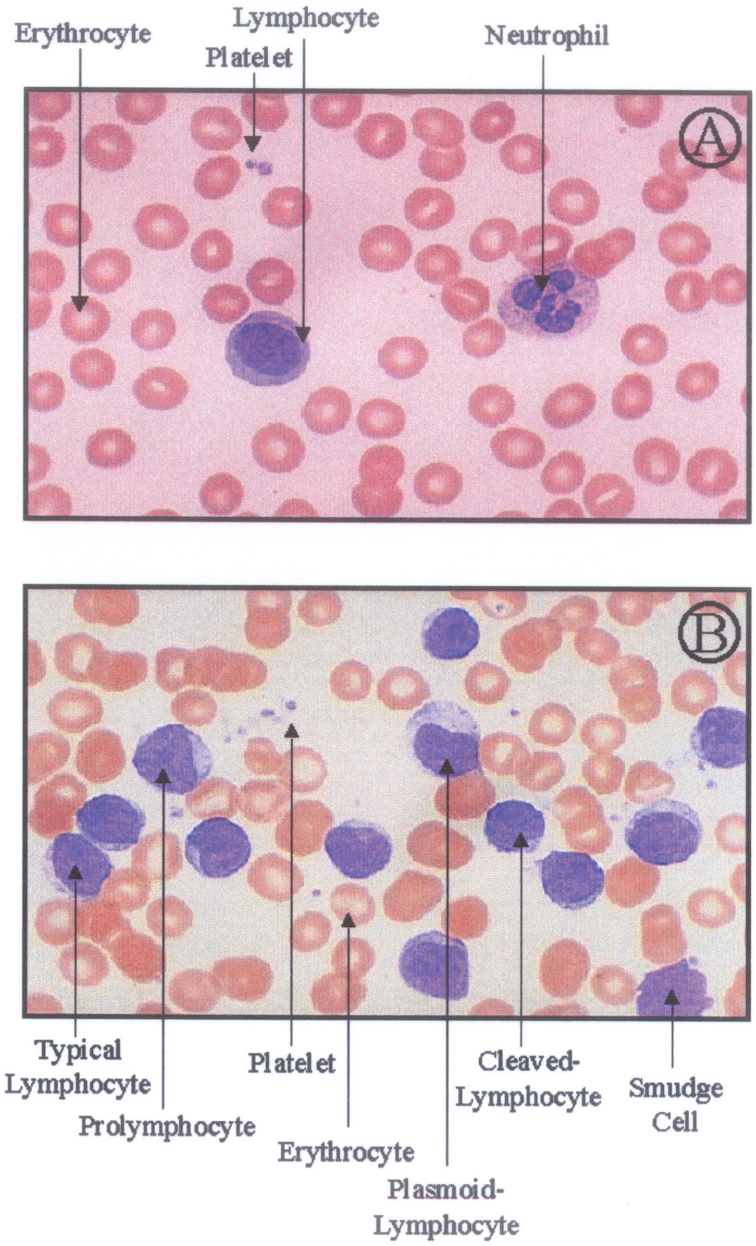


Figure 1: Normal and chronic lymphocytic leukemia peripheral blood smear.

Morphology and lymphocyte number comparison between normal and CLL peripheral blood. Panel A: normal peripheral blood smear with arrows indicating erythrocytes, typical lymphocytes, neutrophils, and platelets. Panel B: CLL peripheral blood smear with arrows indicating erythrocytes, prolymphocytes, plasmoid-lymphocytes, cleaved lymphocytes, smudge cells and increased numbers of mature appearing typical lymphocytes.

1.2.2 Etiology

The cause of CLL remains unknown, however, there is increasing evidence of environmental, and genetic factors. It has been observed that there is an increased incidence in farmers, rubber manufacturing workers, asbestos workers and tire repair workers, perhaps related to the chemicals to which many of these workers are exposed (3). However, studies trying to identify such a causative environmental agent have been unsuccessful (4). While most cases of CLL are sporadic there have been reports of multiple cases of CLL in a single family (5). There is strong evidence for a relationship between CLL and the risk for other hematological malignancies and other maladies such as autoimmune diseases (6). In many of these cases genetic anticipation, the phenomenon of earlier and more severe phenotypes in successive generations, has been observed where the age of onset was reduced by approximately 20 years between generations (5, 7). Identification of genetic mechanisms to account for the anticipation seen in CLL have been unsuccessful but have included: intergenerational inheritance of proto-oncogenes and tumor suppressor genes that are initiated by a defect in a DNA repair gene(s); inherited abnormalities of telomeres; or perhaps the uncontrolled expansion of a trinucleotide repeats as seen in fragile X syndrome (8). However as of yet, none of these mechanisms have been confirmed and no predisposition genes for CLL have been identified.

1.2.3 Treatment

Even with the development of novel chemotherapeutic agents CLL remains an incurable B cell malignancy. Prognosis can vary substantially between different patients,

depending on clinical stage and disease features such as lymphocyte doubling time, and patient age. Staging in CLL continues to play an important role in deciding whether to initiate therapy as early stage patients generally do not require treatment until their disease begins to progress. Patients with progressive or advanced stage disease are initially treated with the alkylating agent chlorambucil (CLB) or with the nucleoside analogs fludarabine, 2'-deoxycoformycin (DCF), or 2-chlorodeoxyadenosine (CdA). While dependent on stage of presentation, patients can generally live symptom free while on chemotherapy or when their disease is in remission. Unfortunately, after initial response to chemotherapy patients generally become refractory to treatment, and die from secondary infections. While it remains largely unknown why patients eventually become refractory to treatment, mutation of the tumor suppressor gene, *p53*, occurs in approximately 10% of patients and causes multidrug resistance (9).

Autologous transplants following high dose chemotherapy and stem cell rescue have been attempted in CLL. However only a small number of patients have shown complete clinical responses, presumably because of the high probability that stem cell collections are contaminated with CLL cells (10). This prompts the search for more effective purging techniques to remove leukemic cells from stem cell populations. Allogeneic stem cell transplants have also been performed in patients with relapsed and refractory disease, but are generally reserved for young patients who typically have aggressive disease. It is argued that allogeneic transplants result in a beneficial graft-versus-leukemia response in which donor lymphocytes reject leukemia cells (11). Few clinical

studies of allogeneic transplants have been completed due to a high treatment-related morbidity and mortality (12).

Most recently, the use of monoclonal antibodies, either alone or conjugated to toxins or radioisotopes have been investigated. Currently, two monoclonal based therapies, anti-CD20 (Rituximab) and anti-CD52 (Campath-1H), which are currently used in follicular lymphoma are the most promising (13). However, CLL cells express almost 10-fold less CD20 than most lymphoma cells, and therefore treatment with Rituximab may be less effective in CLL than for the lymphomas (14). Conversely, CD52 is expressed on most lymphocytes, including CLL cells, suggesting that Campath-1H treatment may be best in patients who are refractory to traditional chemotherapy agents. To date there is little data on the long-term survival in patients treated with either of these monoclonal antibodies. However, early results indicate Campath-1H is showing the most clinical activity in previously treated patients (15).

1.2.4 Prognostic Indicators

CLL is a highly heterogeneous disease, with some patients never affected by their leukemia while in others the disease will progress and cause death in 1 to 2 years (2). Prognosis has been assessed by clinical staging, using either the Rai (16) or Binet (17) staging systems. In North America the Rai staging system is the most commonly used, and is outlined in Table 1. However, even within each of the stages there is considerable variation in prognosis; for example, 25% of patients with initial Rai stage 0 disease will progress and the remaining 75% will remain stable and not progress. The lymphocyte

doubling time can also be used to assist in predicting which patients will progress (18); those patients with a lymphocyte doubling time of <12 months have a poor prognosis and will require therapy while those patients with a longer doubling time have a good prognosis. Lymphocyte doubling time is currently the most commonly used prognostic indicator.

Table 1: Rai classification system for chronic lymphocytic leukemia.

Stage	Modified Stage	Description*	Median Survival (Years)
0	Low-risk	Lymphocytosis	>10
I	Intermediate-risk	Lymphocytosis + lymphadenopathy	>8
II	Intermediate-risk	Lymphocytosis + splenomegaly +/- lymphadenopathy	6
III	High-risk	Lymphocytosis + anemia +/- lymphadenopathy or splenomegaly	2
IV	High-risk	Lymphocytosis + thrombocytopenia +/-anemia +/-splenomegaly +/- lymphadenopathy	2

*Lymphocytosis, lymphocyte count >5 x 10^9/litre (L) for >4 weeks; anemia, hemoglobin <11g/dl, and thrombocytopenia, platelets <100 x 10^9/L (16).

Genetic abnormalities are found in 50% of cases of CLL by cytogenetic analysis. However, as CLL cells are non-cycling, and are refractory to mitogens, metaphase analysis has been difficult. The advent of new technologies has allowed a more in-depth study of the genetics of CLL, and has renewed interest in identifying chromosomal aberrations that could pinpoint subgroups of patients who have different prognoses. The most common chromosomal abnormalities are: trisomy 12, and deletions of 6q, 11q, and 13q. All these abnormalities have been shown to serve as possible prognostic indicators

(19). Trisomy 12 is associated with atypical lymphocyte morphology, higher proliferative capacity, and a poor prognosis. Patients with 13q14 abnormalities typically have benign disease, and survive as long as their age and sex matched controls (19). However, patients with deletions of 6q tend to have aggressive disease (14, 20). Deletions of 11q are most commonly seen in younger patients, with these patients tending to have aggressive disease with more rapid progression than those without the deletion (14, 20). While not the most common genetic abnormality, patients with deletions of chromosome 17p, particularly 17p13, have a poor prognosis and are refractory to treatment (4). The identification of these abnormalities in CLL and some possible candidate genes involved are discussed in Section 1.2.5.

More recently, the search for new prognostic markers has focused on other aspects of the genetics of CLL rather than simply specific chromosomal aberrations. Some of these new interests have included telomere length, telomerase enzyme activity, and the mutation status of the immunoglobulin genes in patients. The strong association of telomerase reactivation, cellular immortalization, and cancer has led to the finding that CLL patients who had short telomere length and high telomerase enzyme activity had a poor prognosis (21). The long-term significance in larger studies remains to be determined (21). Recently, considerable interest has been generated in the study of the mutation status of the variable portion of the immunoglobulin heavy (IgH) genes in CLL cells. It appears that CLL cells can be segregated into two groups that differ in the extent to which the IgH variable genes in each have undergone somatic mutation (22, 23, 24,

25). This early work has found those who have mutated IgH chain genes are CD38- while those who are not mutated are CD38+. Correlative investigations with this CD marker have found that that approximately 75% of Rai stage 0 patients are CD38- with benign disease, with the remaining 25% of Rai 0 patients having CD38+ CLL have an aggressive clinical course (26). This identification of two fundamentally different types of CLL may explain why there is such heterogeneity in prognosis in CLL (22, 23, 24, 25). To allow a better understanding of immunoglobulin mutation analysis further discussion will be presented in Section 1.2.6.

1.2.5 Genetics and Cytogenetics

Classical cytogenetics have been difficult to carry out in CLL as the malignant CD5+ CD19+ cells are refractory to mitogens and metaphases are infrequent. In addition, as unfractionated peripheral blood cells are commonly used for cytogenetic studies, the dividing cells are frequently normal B and T lymphocytes (2). The use of B cell activators such as lipopolysaccharides, and 12-O-tetradecanoylphorbol-13-acetate (TPA) have improved the identification of specific abnormalities in CLL. However, the use of mitogens to stimulate cells to enter the cell cycle has met criticism since this approach may result in the selection of specific sub-populations of cells. The development of new more powerful and versatile technologies such as fluorescent *in situ* hybridization (FISH) has helped by allowing the study of interphase cells. Much of the data obtained through conventional cytogenetics has compared favorably with the more recent FISH data. Nevertheless, FISH has allowed the detection of microdeletions and translocations never before identified using conventional cytogenetics (27). While cytogenetics in CLL has

been simplified with the use of FISH (28, 29), interphase FISH depends on the preknowledge of candidate regions and on the availability of suitable DNA probes. Notwithstanding, interphase FISH has demonstrated that the number of chromosomal abnormalities increases over time, thereby confirming clonal evolution in CLL, with prognosis worse in patients having multiple abnormalities (30, 31). Of the 50% of patients identified with an abnormal karyotype, one-half of these will have a single chromosome abnormality, one-quarter will have two abnormalities, and the remainder have a complex pattern of abnormalities (32).

Development of alternative techniques, such as comparative genomic hybridization (CGH), has allowed screening of large populations of cells for areas of gene amplification and deletion in CLL. Initial studies using CGH by Bentz *et al.* (33) identified abnormities in 70% of patients with one-third of these having amplifications of all or part of chromosome 12. In addition, amplifications at 12q23-24, 12q13-22, and 12q13-15 have also been observed by FISH in one patient with CLL (34). Later studies by Werner *et al.* (35) using CGH, Southern blot analysis and FISH identified a patient with genomic amplification at 12p11-12 and 12p13, with the latter being the site of the *cyclin D2* gene.

Most of the chromosomal abnormalities described in CLL represent either deletions or alterations in chromosome number, and more specifically involve trisomy 12, and deletions of 6q, 11q, and 13q. Genomic amplification of chromosomal subregions in

CLL are rare and while extrachromosomal DNA and homogeneously staining regions are the most common form of gene amplification only a few cases of CLL with these abnormalities have been reported (36, 37). Translocations can be observed in atypical CLL which is generally associated with atypical lymphocyte morphology and aggressive disease, but whether these cases are true CLL remains unclear. For example, the t(11;14)(q13;q32) resulting in the overexpression of cyclin D1 in a small number of patients with atypical CLL has been observed using FISH and Southern blotting (38, 39, 40); however, this translocation is more typically involved in mantle cell lymphoma (MCL) (41). In addition, using FISH analysis some CLL cases have been described as having a t(14;18)(q32;q21) resulting in the overexpression of anti-apoptotic protein bcl-2 (39); however, this translocation is usually a marker for the follicular lymphomas. Although the pathogenic significance of trisomy 12 is unknown, trisomy 12 has been correlated with atypical lymphocyte morphology, higher proliferative activity, and poor prognosis. The biological significance of trisomy 12 is poorly understood, although as mentioned previously it has been suggested amplification of the genes on chromosome 12 may lead to a more aggressive phenotype. Cyclin D2, an important cell cycle regulator of the G_1 phase of the cell cycle, is located at 12p13, and its amplification may account for the high proliferative activity seen in patients with trisomy 12. In addition, amplification of the *murine double minute 2* (*MDM-2*) gene located at 12q13-15 has been implicated in CLL (22, 42). The MDM-2 oncoprotein plays an important role in the autoregulatory feedback loop with the tumor suppressor p53. Amplification of *MDM-2* leads to the overexpression of the protein that inactivates p53, and thereby inactivates an

important cell cycle checkpoint producing a similar effect as a *p53* mutation (43). Chromosome 13 is the chromosome most commonly involved in structural aberrations. In cases where metaphase analysis has been possible, 50% of patients have been identified as carrying a 13q14 deletion suggesting a possible tumor suppressor gene at this site (28, 29, 44, 45, 46). Twenty-percent of patients have a deletion of 11q22.3-q23.1 which is the site of the ataxia telangiectasia (*ATM*) gene, and these patients typically have marked lymphadenopathy, are younger and have short survival (47). Interestingly, it has recently been shown that one-third of patients have a mutation at the *ATM* locus, which results in reduced levels of the ATM protein and shortened survival (48, 49, 50). Ten-percent of patients have deletions of 17p13, resulting in the deletion of the *p53* gene with these patients having aggressive, and drug resistant disease (51, 52).

1.2.6 The Development of the B Lymphocyte

The function of the B lymphocyte is the production of antibodies against external antigens. B lymphocytes are produced in the bone marrow by CD34+ stem cells. Millions of B lymphocytes are produced daily with only a subpopulation of these ever emerging from the bone marrow. Of the B lymphocytes that do emerge from the marrow some never encounter antigen and soon undergo programmed cell death (apoptosis); however others do encounter antigen and become long-lived memory B cells and/or evolve into immunoglobulin-secreting plasma cells.

After initial production in the bone marrow the B lymphocytes migrate to the peripheral lymphoid organs where there is an endless cycling of B lymphocytes through the follicles

of the peripheral lymphoid organs (lymph nodes, spleen, gut), peripheral blood and lymphatic system (Figure 2). Here antigen stimulation starts their proliferation and maturation to mature B cells and plasma cells producing specific antibodies. In the bone marrow, initial lymphocyte development depends on random rearrangements of the heavy and light chain genes through a process called V(D)J recombination (Figure 3). In this process the gene segments between the rearranging gene elements are deleted or inverted. The heavy chain genes are assembled from variable (V), diversity (D), and joining (J) segments on chromosome 14. Short random DNA sequences ranging in length of 5 to 20 nucleotides are inserted between the D and J segments and between the V and D-J segments. As there are many different V, D, and J segments and there is a random insert of nucleotides each B cell generates a unique pair of genes for its heavy-chain variable region. The variable portions of the light-chains are assembled in a similar fashion except the resulting V-J segments are much shorter as there are no D segments involved. Cells which have completed light-chain variable region and heavy-chain variable region gene rearrangements which are out-of-frame V(D)J rearrangements are removed through apoptosis.

Before newly rearranged B cells leave the bone marrow they are called immature B cells. Upon migration from the bone marrow to the germinal centres (GC) of the peripheral lymphoid organs and before antigen stimulation they are called mature, naïve B cells (Figure 2). When naïve B cells recognize antigen they collect in the GC of the secondary lymphoid organs where they are now called a short-lived plasma B cell. This interaction

with antigen stimulates the process of proliferation and maturation to memory B cells and long-lived plasma cells. However, before this can begin the genomic DNA of the short-lived B cell is subject to three processes: somatic hypermutation; class switching, and sometimes receptor editing, which all serve to "fine tune" antibody production and the production of highly specific antibodies (53). As this process is random, these mutations may also result in the production of reduced affinity antibodies, if this occurs, the cells are eliminated by apoptosis.

The key to this entire process is that these unique gene rearrangements identify each B cell as individual, and can serve as clonal markers. It is the presence of a clonal population of these B cells that are an essential for analysis of B-cell lymphomas. In addition, expression of an antigen receptor by mature B cells is critical for survival; cells that have induced deletion of their receptor undergo apoptosis. While receptor expression plays an important role for B cell survival, receptor specificity also plays an equally important role in survival. B cells which express autoreactive receptors are either removed by apoptosis or their receptors are edited through a process termed secondary V(D)J rearrangements.

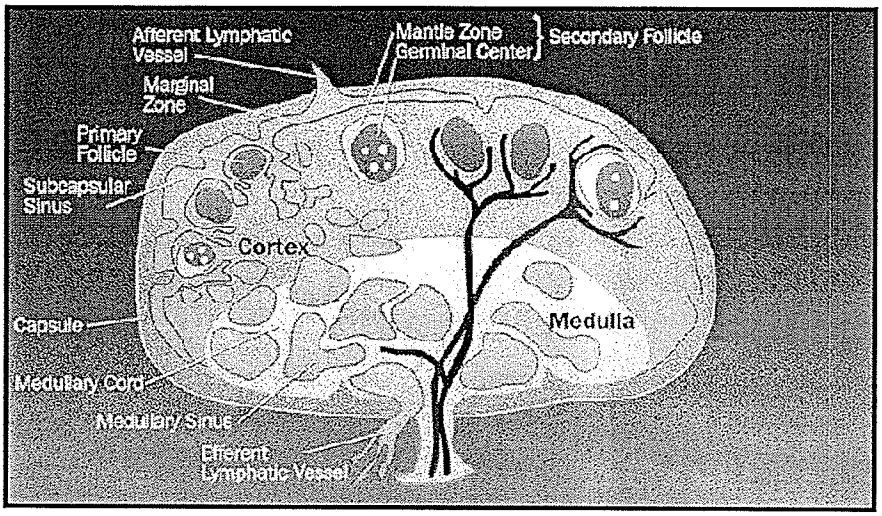


Figure 2: Schematic representation of a human lymph node.

Basic structure of a human lymph node consisting of a B cell area (cortex), a T cell area (paracortex-not shown) and a central medulla, which contains a mix of B cells, T cells, plasma cells and macrophages. B cells are found in unstimulated primary follicles, and in antigen-stimulated secondary follicles with germinal centers (adapted from Gascoyne (54)).

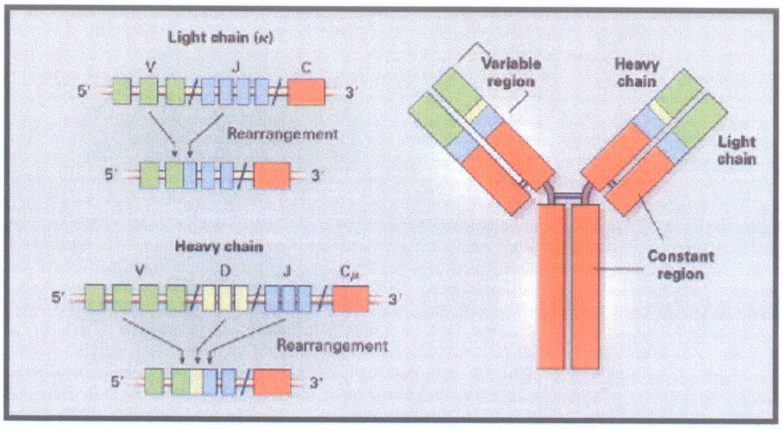


Figure 3: Schematic representation of immunoglobulin rearrangement.

The Y-shaped antibody is composed of two light and two heavy chains linked together by di-sulfide bridges. Much of the antibody is the same for all antibodies of the same immunoglobulin class and is termed the constant region (C). The remaining regions are called the variable regions (V), and are made up of unique amino acid sequences and create two identical antigen-binding sites. These sites are different in each specific type of antibody. The variable- region genes are assembled through somatic mutation of the V, diversity (D), and joining (J) segment for the heavy chains, and the V and J segments for the light chains. DNA located between the rearranging gene elements is deleted from the chromosome, or in some instances inverted (adapted from Küppers *et al.* (53)).

1.2.7 Immunoglobulin Heavy Chain Gene Mutations in CLL

There has been growing interest in the analysis of the rearranged IgH chain genes in the B cell lymphomas. These genes identify the stage of B cell development, and indicate if the B cell has participated in a GC reaction (ie. pre-GC or post-GC). To determine if a cell has undergone rearrangement the IgH genes are sequenced and compared to the variable segments in the germ line configuration. B cells which have not participated in a GC reaction do not differ in their sequence from germ line variable heavy chain. Conversely, B cells which have participated in an GC reaction have mutated variable heavy gene segments in comparison to the germ line variable heavy gene segments. The study of cells from B cell lymphomas suggests that malignant transformation often occurs or is initiated in GC B cells. It has been hypothesised that the rapid clonal expansion of cells, in addition to the DNA strand breaks which are required for somatic hypermutation switching, represents a risky scenario ideal for malignant transformation. This is further confirmed by the observation that T lymphocytes which do not undergo somatic hypermutation or class switching, account for only one-tenth to one-twentieth as many lymphomas as B cells (53).

Initial investigations of the mutation status in CLL B cells failed to identify somatic immunoglobulin mutations suggesting that CLL was a malignancy of mature, naïve B cells which are pre-GC (55). However, recent comprehensive sequencing of the immunoglobulin variable heavy chain segments has identified two subclasses of patients; those who did not have somatic immunoglobulin mutations (pre-GC) and others, which

have somatic immunoglobulin mutations (post-GC). Each class of patients has a very different clinical courses and prognosis. This finding has suggested that there may be (at least) two different subtypes of CLL. The CLL cells from approximately 50% of patients show somatic hypermutations of the variable portion of the IgH chain genes, indicating that these cells have been exposed to antigens in the GC of the lymphoid follicle (post-GC) (Figure 2) (23, 26). Leukemia cells from the remaining 50% of patients do not show hypermutations indicating they are mature, naïve B cells (pre-GC). Concomitantly, it has been shown by Damle *et al.* (25) that CLL cells which have undergone somatic hypermutations are CD38-, whereas naïve CLL cells are CD38+. Cytogenetics have also found that patients with the 13q14 deletion interestingly have CD38- CLL (56). In contrast, the 15% of patients with trisomy 12 usually have CD38+ disease, aggressive disease and the leukemia cells have atypical morphology (56, 57). Therefore, measuring the CD38 status may permit differentiation of the two types of CLL and may serve as a prognostic measure. However, the evaluation of immunoglobulin rearrangement remains technically difficult as indicated by the initial conflicting results (55) and therefore the findings remain controversial. The long-term significance of immunoglobulin rearrangement analysis still needs to be investigated.

1.2.8 Cell Surface Phenotype in CLL

The stage an individual B cell is at in development can be determined through analysis of individual cell surface phenotype. The CD5+ antigen is expressed by most T cells, and a subset of B cells. CD5+ B cells constitute a large percentage of B cells in umbilical cord blood, and the mantle zone of lymphoid tissue in adult humans (Figure 2). CD5+ cells

are also expressed in tumour B cells in CLL patients, and is believed that CD5 expression may reflect their site of origin which is thought to be the mantle zone of the lymph node. CLL cells are typically CD5+, CD19+, CD22-, CD23+, and FMC7- with sparse IgM and IgD immunoglobulin expression. It is this distinctive characteristic of a large percentage of B cells having the co-expression of T cell marker CD5 that is a hallmark of CLL (2).

1.2.9 Defect(s) of Apoptosis in CLL

It has long been appreciated that the CLL cell is a long-lived cell and although difficult to prove it has been assumed that this is related to a defect in apoptosis. This assumption is based on the observation that the majority of CLL cells are non-proliferating, non-cycling cells and long-lived (2, 23). As CLL cells are non-cycling much attention has focused on studying the important regulators of the cell cycle, and more specifically the G_1 D cyclins and their inhibitors. In addition, the longevity of these cells has been attributed to a defect(s) in apoptosis, possibly caused by the high levels of the anti-apoptotic protein bcl-2 seen in this disease (58, 59, 60). Further support for this hypothesis has recently been made with the discovery that CLL cells rapidly die *in vitro* when bcl-2 expression is down-regulated using antisense bcl-2 oligonucleotides (61). Alternatively, the reduction in apoptosis in CLL has been related to increased levels of basic fibroblast growth factor (62), cyclin D2 (63), nitric oxide synthase (64) or $p27^{KIP1}$ (136) expression, which are commonly observed in these cells. However, to-date, no single unifying hypothesis can explain how the levels of all these proteins are increased in CLL, although it has been hypothesized that it may be attributed to gene methylation (58), chromosomal

rearrangements (65), alterations in other cellular regulatory pathways, or alterations in the cellular macro-environment.

1.3 THE EUKARYOTIC CELL CYCLE

All eukaryotic cells have a well controlled cell cycle that can be divided into two broad stages; interphase and the mitotic (M) phase (Figure 4). Successive cell divisions during the M phase alternates with a much longer interphase, usually accounting for 90% of the time that elapses during each cell cycle (18 to 24 hours). During interphase the cell grows and copies its chromosomes in preparation for cell division. Interphase is comprised of three sub-stages called gap 1 (G_1); DNA synthesis (S), and gap 2 (G_2). An increase in cellular proteins, and cytoplasmic organelles occurs in all stages with chromosome division only occurring during M phase.

The timing and rate of cell division is critical to normal growth, development, and maintenance of the cell. The precise sequence of events required for successful cell division depends on the completion of each stage before the cell can progress to the next stage. Each stage is controlled by a unique complex(es) of proteins comprised of activators, called cyclins, and an appropriate catalytic subunit called a cyclin dependent kinase (CDK). The abundance of the various cyclins oscillates throughout the cell cycle in response to growth signals that reach the interior of the cell (66), with the CDK abundance remaining relatively constant throughout the cell cycle. Each stage of the cell cycle is regulated by a unique cyclin/CDK complex. Cyclin activity at each stage is dependent on complex formation with an appropriate CDK binding partner. Upon

formation of an appropriate cyclin/CDK complex the CDK portion phosphorylates downstream regulators allowing progression through that stage of the cell cycle. The G_1 phase is a key period in the control of the cell cycle, and it is this crucial checkpoint located just prior to S phase that serves as a point of evaluation to determine if the internal and external signals which include cell size, DNA content, and the presence of external growth factors are favorable for a dividing cell. If the conditions are unfavorable, the cell may exit from the cell cycle into a non-dividing state called Gap 0 (G_0) phase.

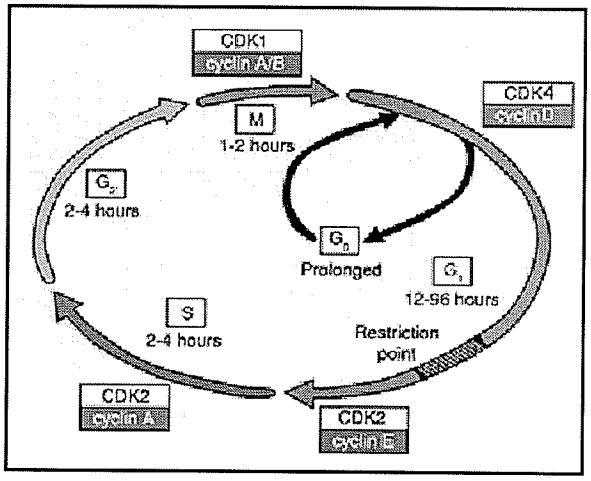


Figure 4: Schematic of the eukarytoic cell cycle.

In a dividing cell, the mitotic (M) phase alternatives with interphase. The first part of interphase is called gap1 (G_1), followed by DNA synthesis (S) phase, during which the chromosomes replicate, the last part of interphase is called gap 2 (G_2). Next, mitosis divides the nucleus and its chromosomes. Each phase of the cell cycle is controlled by complex formation of different proteins. Two key proteins in this complex formation are the cyclins and cyclin dependent kinases (CDK) (adapted from Israels and Israels (67)).

The more recent discovery of an additional family of cell cycle regulating proteins called the cyclin dependent kinase inhibitors (CDKI) has added not only another level of complexity, but also allows a finer level of cell cycle control. As their name suggests these proteins function to inhibit cell cycle progression; however, there is additional evidence suggesting that some of these proteins may also play a role in cyclin D-CDK complex formation and activation (124). While it is known that the CDKI mediate their effects through direct interactions with cyclin/CDK complexes how such dual roles are played remains unknown.

As the focus of this work is the D cyclins, the G_1 phase of the cell cycle will now be discussed in more detail.

1.3.1 The G_1 Phase of the Eukaryotic Cell Cycle

Two important eukaryotic cell cycle regulatory points are the G_1/S and the G_2/M phase checkpoints. During the G_1 phase cells are responsive to extracellular cues that ultimately determine whether the cell will replicate DNA, divide or exit the cell cycle into the quiescent stage, G_0. *In vitro* studies have shown that under conditions of stress, such as growth factor withdrawal, cells enter this non-cycling state, G_0 (152). Cells that enter G_0 can be stimulated *in vitro* to reenter the cell cycle through growth factor or mitogen stimulation (152). In mammalian cells the G_1 phase is regulated by two distinct families of cell cycle regulating proteins. The D cyclins, D1, D2, and D3 control the largest portion of the G_1 phase, with the E cyclins, E1, and E2 playing a role at the end of the G_1 phase.

The D cyclins sensitivity to external stimuli suggests that they may play a role in transduction of mitogenic stimuli from external receptors located on the membrane to the internal compartment of the cell. How this signal is transferred from an external receptor to the cyclin protein remains poorly understood. However, attempts to elucidate this pathway have most commonly used the cyclin D1 protein, and as a result is the most widely studied and best understood D cyclin. Although most investigations have focused on the cyclin D1/retinoblastoma (Rb) pathway it is believed that cyclin D2 and D3 operate via similar pathways and mechanisms.

The human G_1 D cyclins were first identified through their ability to overcome *CLN* deficiency in *Saccharomyces cerevisiae* (*S. cerevisiae*) (68, 69). Shortly after their identification in yeast Matsushime *et al.* (70) identified the cyclin-like (CYL) mouse homologues *CYL1*, *CYL2* and *CYL3*. Protein conformational studies using the CYL1 protein screening for human homologues identified the human *parathyroid* (*PRAD1*) gene that is overexpressed and chromosomally rearranged in some forms of benign parathyroid tumors (71, 72, 137). Shortly after this finding all three human homologues *cyclin D1*, *D2*, and *D3* were identified (73, 74), with *PRAD1* being *cyclin D1*.

1.3.1.1 The Role of the D cyclins in the Eukaryotic Cell Cycle

The three D cyclins, *D1*, *D2*, and *D3*, also known as *CCND1*, *CCND2* and *CCND3*, play an important role in regulating the early phase of the G_1 phase of the cell cycle. The D cyclin members have closely related sequences that are more closely related between

species (human and mouse) than with each other. Each of the D cyclins shares an average of 57% identity over their coding region, and 78% within the cyclin box (73). Aligned sequence analysis between the nucleotide coding regions of the human and mouse D cyclins genes has identified a 94%, 89% and 90% homology between *cyclin D1*, *D2*, and *D3*, respectively (73, 74). At the amino acid level, there is 94% homology between human and mouse for both *cyclin D2* and *D3* (74). Initial investigations have suggested that the D cyclins may have overlapping roles and expression patterns (70); however, more recent studies have suggested that their function and expression is more likely organ and tissue specific (75, 76).

During G_1, the D cyclins accumulate and assemble with their CDK4 or CDK6 partner. The CDK4 and CDK6 proteins are relatively long-lived proteins, when compared to the D cyclins, with their levels being stable throughout the entire cell cycle. Conversely, the uncomplexed D cyclins are unstable and have an uncomplexed half-life of approximately 30 minutes (77). D cyclin induction, synthesis and assembly with their CDK4/6 partners all depend upon mitogenic signaling. Upon D cyclin-CDK4/6 complex formation site-specific phosphorylation of the Rb family of pocket proteins (pRb, p107, and p130) occurs. This phosphorylation of Rb in the mid to late G_1 phase by cyclin D-CDK complexes results in the dissociation of the pRb-E2F complex. This dissociation allows the transcription of E2F target genes and entry into S phase. Rb normally functions to constrain growth and in its absence, cells lose the ability to shut down their proliferation in response to physiological signals. Evidence for this critical link between the D cyclins

and Rb has been demonstrated through microinjection studies where the D cyclins were inactivated (78). Inactivation of the D cyclins caused the cells with wild-type Rb to arrest in G_1 but this was not observed in cells that lacked functional Rb (79). This may be partly related to the finding that cells deficient in Rb have been shown to lack the ability to form cyclin D-CDK4 complexes (80). The effects of overexpression of the Rb pocket proteins triggers cell cycle arrest, but can be overcome by a subsequent increase in G_1 CDK activity.

1.3.1.1.1 Protein Stability and Cellular Localization of the D Cyclins

Any mechanism of cell cycle control that involves protein/protein interactions requires the ability to activate and inactive the appropriate proteins. Functional domain analysis to help determine the mechanism regulating D cyclin stability has identified a proline-directed phosphorylation site on each of the D cyclins. This site, threonine-286 (thr-286), is located near the C-terminus of the D cyclin protein. It is believed that this site may play an important role regulating the D cyclin activity and half-life through targeting the protein for proteasomal degradation and localization of the protein to specific areas of the cell. As indicated previously, the D cyclins have a short protein half-life (approximately 30 minutes) when bound to their appropriate CDK partner, with their levels declining rapidly after the removal of external stimuli (77). Mutation studies in mouse fibroblasts have shown that replacement of thr-286 with alanine prevents phosphorylation and subsequent polyubiquitination of the cyclin D1 protein, resulting in a markedly stable modified D cyclin with a half-life of 3.5 hours (77). Unphosphorylated cyclin D1 accumulates in the nucleus throughout the G_1 phase, but when phosphorylated relocalizes

in an inactive state in the cytoplasm for the remainder of interphase (81). It is thought that glycogen synthase kinase-3β (GSK-3β) mediates this cyclin D1 phosphorylation, and it is believed that upon migration to the cytoplasm cyclin D1 is degraded via proteasomal degradation pathways (81). While not fully understood, it is believed GSK-3β activation is modulated via a pathway involving Ras, phosphatidylinositol 3-kinase, and protein kinase B/Akt (82).

1.3.1.1.2 The Human *Cyclin D1* Gene

The *cyclin D1/bcl-1/PRAD1/CCND1* gene is located on chromosome 11q13 and encodes for a 36 kiloDalton (KDa) protein. The human *cyclin D1* gene is approximately 15-kilobase (kb), and is composed of 4 introns, and 5 exons (83, 84). It is most commonly reported that cyclin D1 mRNA is composed of two transcripts, 4.4-kb and 1.7-kb, although splice variants have been identified in some lymphoproliferative disorders (85, 97). These disorders involve a large heterogenous group of malignancies that include CLL (86).

1.3.1.1.2.1 Overexpression of Cyclin D1

In 1994 the first cyclin D1 overexpressing transgenic mouse was generated allowing the study and identification of events following cyclin D1 overexpression. The lymphocytes in these mice were found to have normal cell cycle activity, growth size and mitogen responsiveness (87, 88, 89). The mice did not develop lymphomas but a small number developed mammary carcinoma (90). However upon co-expression of cyclin D1 and the proto-oncogene c-myc there was a strong cooperative effect resulting in the development of clonal pre-B and B-cell lymphomas. These findings strengthened the idea that the

cyclin D1 gene may act as a proto-oncogene, and suggested a link between disturbances in cell cycle regulation to the development of B- and T-lymphomas. Furthermore, these findings indicated cyclin D1's activity was dependent not only on a specific partner, but also specific cell types. Additional cyclin D1 overexpression studies using established immortal murine fibroblasts found that the cells remained contact inhibited, required serum for growth, and would not grow in soft agar (78). These findings suggested that overexpression of wild cyclin D1 alone was insufficient to promote neoplastic transformation without some cooperating mutations and confirmed the data obtained using transgenic mice. In contrast, experiments using immortal rat fibroblasts overexpressing cyclin D1 identified cells with a shortened G_1 phase that were able to grow in soft agar (91). These differences in results between different cell lines has been attributed to required genetic changes that allow the establishment of an immortalized clone resulting in the establishment of a cell line. It is believed that these random changes, which are akin to the co-expression studies using c-myc in the transgenic mice, demonstrate that overexpression of cyclin D1 alone is insufficient to promote neoplastic transformation. In 1995, after the development of the overexpressing cyclin D1 transgenic mouse, the first *cyclin D1* knockout (D1 -/-) mouse was generated (92). These mice developed normally with the exception of decreased body size, reduced viability, neurological impairment and a reduction in cell number in the retina and breast epithelium in pregnant mice (92). These results further suggest that specific tissues may acquire malignant tendencies by specific genes during their normal development (92).

1.3.1.1.2.2 Mechanisms of Cyclin D1 Deregulation and Cancer

Cyclin D1 is most commonly reported overexpressed in a subset of parathyroid adenomas as a result of a chromosomal inversion, inv(11)(p15;q13). This chromosomal inversion results in the *cyclin D1* gene being placed under the control of the *PRAD1* gene promoter and consequently cyclin D1 is highly overexpressed both at the mRNA and protein levels. In B cell malignancies the characteristic t(11;14)(q13;q32) is the most common form of *cyclin D1* deregulation and is viewed as a key step in the development of the lymphoma. Overexpression of cyclin D1 as a result of a translocation is most commonly due to *cyclin D1* being translocated to the IgH chain locus on chromosome 14q32 yielding t(11;14)(q13;q32) (94). However, translocation variations of the *cyclin D1* gene to the immunoglobulin lambda (λ) light chain gene site on chromosome 22 have also been reported (93). Detection of these translocations can be difficult because the breakpoint can vary from 15-kb to more than 400-kb making tests such as the polymerase chain reaction (PCR), Southern blotting, and FISH unable to detect all translocations. The presence of a *cyclin D1* translocation is characteristic of some B cell malignancies, most notably mantle cell lymphoma (MCL) (94). It is also seen in other lymphoproliferative disorders including CLL, multiple myeloma, splenic marginal zone lymphoma, and hairy cell leukemia (95, 96, 97, 98). Cyclin D1 has also been found overexpressed in some cases of breast (99, 100), esophageal (101), pancreatic (102), and head and neck squamous cell carcinomas (103). While translocations (95, 104, 105, 106) are the most commonly reported mechanism responsible for the aberrant expression of cyclin D1, alternative mechanisms may include: genomic amplification (99, 101);

enhanced protein stability (107), and transcriptional/post-transcriptional mechanisms (75).

1.3.1.1.3 The Human *Cyclin D2* Gene

The *cyclin D2/CCND2* gene is located on chromosome 12p13 and encodes for a 35 KDa protein; however, its gene structure and size have not been reported. The *cyclin D2* gene encodes for a single 7.0-kb transcript (63) but the length varies according to the cell type (63, 74, 108). Like *cyclin D1*, *cyclin D2* is considered to be a proto-oncogene because the immortalization of primary B lymphocytes by the Epstein-Barr virus (EBV) is accompanied by transcriptional activation of cyclin D2 (109). Transgenic mouse models overexpressing cyclin D2 have only investigated the effect of cyclin D2 on growth and differentiation in epithelial tissues, with no report on the effect on lymphoid cells (110). *Cyclin D2* knockout (-/-) mouse studies have found only specific cells in the ovaries and testes are affected (111). In addition, *in vitro* cell culture analysis of *cyclin D2* knockout (-/-) cells has found that cyclin D3 can play a compensatory role for the lack of cyclin D2 protein (112).

1.3.1.1.3.1 Mechanisms of Cyclin D2 Deregulation and Cancer

Cyclin D2 has been reported to be overexpressed in gastric cancer, male germ cell tumors (113) and a variety of B-lymphoproliferative disorders including CLL (63, 114). Mechanisms to account for this aberrant expression are less well characterized than for cyclin D1. However, it has been suggested that gene amplification (39, 41, 113), enhanced protein stability (107) or post-transcriptional mechanisms may play a role (86).

Loss of cyclin D2 expression has been reported in breast cancer, and is thought to be related to promoter hypermethylation (115).

1.3.1.1.4 The Human *Cyclin D3* Gene

The *cyclin D3/CCND3* gene is located on chromosome 6p21 and encodes for a dimeric 31 and 34 KDa protein; however, like *cyclin D2*, the gene structure and size of *cyclin D3* have not been reported. The *cyclin D3* gene encodes for a single 2.2-kb transcript (74). However, unlike cyclin D1 or cyclin D2, cyclin D3 is not considered to be a proto-oncogene, and is the most poorly understood of the three D cyclins (98). As with cyclin D2, transgenic mouse models overexpressing cyclin D3 have only investigated cyclin D3 effects on growth and differentiation in epithelial tissues, with no report to-date on the effect on the lymphoid cell compartment (110). However, in immortalized fibroblasts overexpressing cyclin D3 there is a delay in G_1/S transition (116). Transgenic mouse and lymphoid cell line *cyclin D3* knockout (-/-) studies have not yet been reported.

1.3.1.1.4.1 Mechanisms of Cyclin D3 Deregulation and Cancer

There are few reports on aberrant cyclin D3 expression in cancer. However, cyclin D3 has been observed to be overexpressed in pancreatic cancer (117), multiple myeloma (118) and in both B and T cell lymphoproliferative disorders (119). Mechanisms accounting for the altered cyclin D3 expression are few, however, a recent report studying multiple myeloma has identified a translocation involving the *cyclin D3* gene and the immunoglobulin locus (118).

1.3.1.2 The Role of the Cell Cycle Inhibitors in the Eukaryotic Cell Cycle

Complex assembly involving the D cyclins and their catalytic partners CDK4/CDK6 is not an automatic process and is dependent on a variety of conditions that include D cyclin synthesis, stability, and cellular localization (120). It has only been recently discovered that actual regulatory proteins may play an important role in the assembly process. These proteins, the CDKIs, are believed to function both as negative regulators of the cell cycle and as cyclin D-CDK4/6 activators. How these two processes are differentially regulated is unknown, but is believed to be dependent on the CDKI involved. The CDKIs members can be divided into two broad categories, the INhibit cdK4 (INK4) family, which are specific inhibitors, and the CIP/KIP family which are broad inhibitors.

1.3.1.2.1 INK4 Family

The INK4 family of CDKIs is comprised of four members: $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$. Each member of this family specifically inhibits CDK4 or CDK6 by competing with the D cyclins for the same binding region on the CDK protein. This mechanism of binding allows a fine level of control of D cyclin activity through competitive binding to their target CDKs. Increasing cellular levels of an INK4 inhibitor prevents D cyclin binding to the CDK protein, and antagonizes the phosphorylation of the Rb protein, preventing cell cycle exit from G_1, with any uncomplexed D cyclin is quickly degraded by proteasomes. The data from INK4 mouse knockout models is minimal but suggests that disabling a single INK4 family member does not particularly increase the rate of tumor development in mice (121). In contrast, analysis of human cancers has indicated an increased loss of function mutations in the *INK4* genes. Analysis of patients

with non-Hodgkin's lymphomas of B-cell origin identified genetic alterations in $p16^{INK4A}$ in some patients (122). In multiple myeloma, hypermethylation and inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ genes has been reported (123).

1.3.1.2.2 CIP/KIP Family

The CIP/KIP family of CDKIs is comprised of three family members $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. It is believed these CDKIs are broad-spectrum inhibitors affecting the formation of many cyclin-CDK complexes, in all stages of the cell cycle. Interaction of the CIP/KIP proteins with a variety of cyclin-CDK complexes occurs through a conserved N-terminal domain that contains both cyclin and CDK binding sites.

In vitro studies have shown that overexpression of $p27^{KIP1}$ prevents cell passage through G_1/S, indicating that $p27^{KIP1}$ may play a critical role in cell cycle exit (136). The levels of the $p27^{KIP1}$ levels are maximal in G_1, and decline as cyclin E accumulates towards the latter part of G_1. Recently, Cheng *et al.* (124) has shown that $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are often found in complexes with active cyclin D-CDK4/6. *In vitro* loss of expression studies of both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in cell lines found decreased steady-state levels of active cyclin D-CDK4 complexes. In addition, the turnover rate of the cyclin D-CDK4 complexes was increased in those cells with loss of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Reintroduction of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ into these cells increased the stability of D cyclin protein and the formation of cyclin D-CDK complexes. However, D cyclin complexes not associated with $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ have a shorter half-life of approximately 20 minutes versus 4 hours (81, 124). Through these findings and others it

has been suggested that the CIP/KIP family of CDKI may serve as multifunctional proteins that not only act solely as cell cycle inhibitors, but also play important roles in: regulating cell cycle progression; cyclin D-CDK4 complex assembly; cyclin D-CDK4 complex stability, and D cyclin nuclear localization (81, 124, 125). It has been hypothesized that $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ prevents phosphorylation of the critical thr-286 residue on the D cyclin proteins therefore protecting them from degradation by proteasomes (124).

Studies investigating $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ inhibitor susceptibility found CDK4/6 isolated from mammalian cells were less susceptible to $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ mediated inhibition than cyclin E-CDK2. It has been hypothesized that sequestering of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ by cyclin D-CDK4/6 relieves the cell cycle restraint allowing active cyclin E-CDK2 complexes to propel the cell through the S-phase of the cell cycle. This view of the CIP/KIP CDKI represents them as activators of CDK4 and inhibitors of CDK2 (124). It is believed cyclin D-CDK4/6 complexes may act as a "mop" for $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ allowing the activated cyclin E-CDK2 complexes to phosphorylate $p27^{KIP1}$. Phosphorylation of $p27^{KIP1}$ converts it to a form recognized by ubiquitin ligases and results in its destruction by proteasomes (126).

Much less is known about the role of $p57^{KIP2}$ as an inhibitor, but structural differences to the better known $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ members include a long insert of proline/alanine-rich or acidic motifs following the conserved CDKI domain. In addition,

the expression of $p57^{KIP2}$ is more restricted than that of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, as cells expressing $p57^{KIP2}$ are generally terminally differentiated, signifying a role for $p57^{KIP2}$ in the decision to exit the cell cycle for differentiation (127).

There is increasing evidence to suggesting that INK4 and CIP/KIP proteins may work closely together to regulate the cell cycle. Currently, it is believed that the INK4 proteins sequester the CDK4/6 proteins releasing the CIP/KIP protein from the cyclin D-CDK4/6 complexes. This allows the CIP/KIP proteins to inhibit cyclin E-CDK2 activity and ensures cell cycle arrest. If the cyclin E-CDK2 complex is not inactivated it is able to phosphorylate $p27^{KIP1}$ resulting in $p27^{KIP1}$ destruction.

1.4 THE D CYCLINS IN CLL

A large number of hematological malignancies have alterations in the Rb cell cycle pathway. As a result, the search for pharmacological agents that may act as inhibitors of this pathway has generated great interest. Recently, the CDKI flavone anticancer agent, flavopiridol, has been identified as being a potent inhibitor of this Rb pathway. Flavopiridol interacts within the binding pocket of CDK thereby preventing association with the D cyclins (128). *In vitro* experiments treating CLL cells with flavopiridol has found that it is highly toxic suggesting the D cyclin/Rb pathway plays an important survival influence in CLL cells (129). Analysis of cyclin D1, cyclin D3 and cyclin E expression has found that expression decreases after flavopiridol treatment (128).

Of all the D cyclins studied in the hematological malignances, cyclin D1 has generated the most interest. This is partly related to the fact that cyclin D1 was the first D cyclin characterized and is also related to its role in the causation of parathyroid adenomas and MCL. In these two disorders overexpression of cyclin D1 is characteristically observed as a result of t(11;14). This translocation results in *cyclin D1* being translocated to the IgH chain locus on chromosome 14q32 yielding t(11;14)(q13;q32) (94). While perturbed cyclin D1 expression is most noted in MCL, Suzuki *et al.* (98) has shown that it is increased in a subset of CLL patients (98).

Cyclin D2 has been found to be overexpressed at the mRNA (63) and protein (35, 98) levels in some patients with CLL. The extent of altered cyclin D2 expression in CLL remains controversial (98). The mechanisms responsible for increased cyclin D2 expression is also unknown, but may be related to amplification of the *cyclin D2* locus on 12p13 (33, 35) or to trisomy 12 that occurs in 15% of patients (28, 29, 57). It has been suggested that overexpression of cyclin D2 may account for a number of features typically seen in CLL. For example, it has recently been shown that elevated cyclin D2 expression prevents cells in culture from entering S phase, and may explain why CLL cells remain in G_0 (130). In addition, overexpression of cyclin D2 has been reported to reduce apoptosis (131) and differentiation (132).

There are few studies related to cyclin D3. However, it is reported that cyclin D3 levels are reduced in malignancies overexpressing cyclin D1 or cyclin D2 (98). The data

available regarding cyclin D3 expression in CLL is highly variable. For example, some studies have failed to detect cyclin D3 overexpression at the mRNA (63) or protein (114, 119) level in CLL. Whereas others have reported increased cyclin D3 mRNA (86, 98) and protein (133). The mechanism responsible for altered cyclin D3 expression is unknown but it has been suggested to be related to gene amplification. However, to date, chromosome 6p has not been identified in CLL as a region involved in amplification (33, 35).

The exact role the D cyclins may play in CLL remains unknown. However, the D cyclins may have a role in disease initiation, and facilitate tumor progression, transformation and drug resistance. Continued overexpression of the D cyclins may permit the development of new chromosomal changes, as has been observed *in vitro* through cyclin D1 overexpression studies (134). Support for a role of the D cyclins in the pathogenesis of CLL is further strengthened through the observation that similar chromosomal abnormalities are seen in CLL as in MCL where there is constitutive overexpression of cyclin D1 (135).

Of the CDKIs, $p27^{KIP1}$ is the most extensively studied in hematological malignancies. In normal T lymphocytes, $p27^{KIP1}$, is easily detectable and the levels decrease with cell cycle progression (136). The expression of the $p27^{KIP1}$ protein in CLL is highly variable with the highest levels being observed in patient with advanced stage; however, whether it adds additional prognostic information in addition to clinical staging is unclear (136).

Interestingly in this study the $p27^{KIP1}$ mRNA levels were not consistent with the protein levels suggesting that $p27^{KIP1}$ regulation is post-transcriptional (126).

2. SUMMARY

In summary, although the vast majority of leukemic cells in CLL are non-proliferating, it has been reported that CLL cells may have increased levels of cyclin D1, D2 or D3, although the clinical significance of these findings are unknown. In the present study I have evaluated the expression of the three D cyclins in 43 patients with CLL, and determined whether their increased expression could be relative to chromosomal rearrangements or amplification and whether alterations in their expression correlate with clinical features and/or survival.

3. MATERIALS AND METHODS

3.1 REAGENTS

All laboratory chemicals were purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Ltd., Oakville, Ontario, Canada) unless otherwise noted. All solutions, buffers and bacterial media were prepared using distilled deionized water (ddH_2O) and sterilized by autoclaving prior to use. All reagents and solutions required for RNA work were treated overnight with 0.2% diethyl pyrocarbonate (DEPC) with constant stirring at room temperature (RT) to inactivate RNases. After overnight treatment, DEPC containing solutions were autoclaved to remove traces of DEPC. Tris(hydroxymethyl)aminomethane (Tris) based solutions were not directly treated with DEPC, and if required were made using DEPC treated ddH_2O.

3.2 COMPLEMENTRY DNAS (cDNAS)

The human *cyclins D1*, *D2* and *D3* cDNA were a gift from Dr. Andrew Arnold (The School of Medicine at the University of Connecticut Health Centre, Farmington, Connecticut, USA) (137, 138) (Table 2, Figure 5). While cyclin D2 was provided by Dr. Andrew Arnold it was transferred with permission from its originator Dr. Gordon Peters (Imperial Cancer Research Fund, London, United Kingdom). The glyceraldehyde-3 phosphate dehydrogenase (*G3PDH*) cDNA, Cat#9805-1, was purchased as a prepared 1.1-kb cDNA from Clontech Laboratories Inc. (Palo Alto, California, USA) (139, 140) (Table 2).

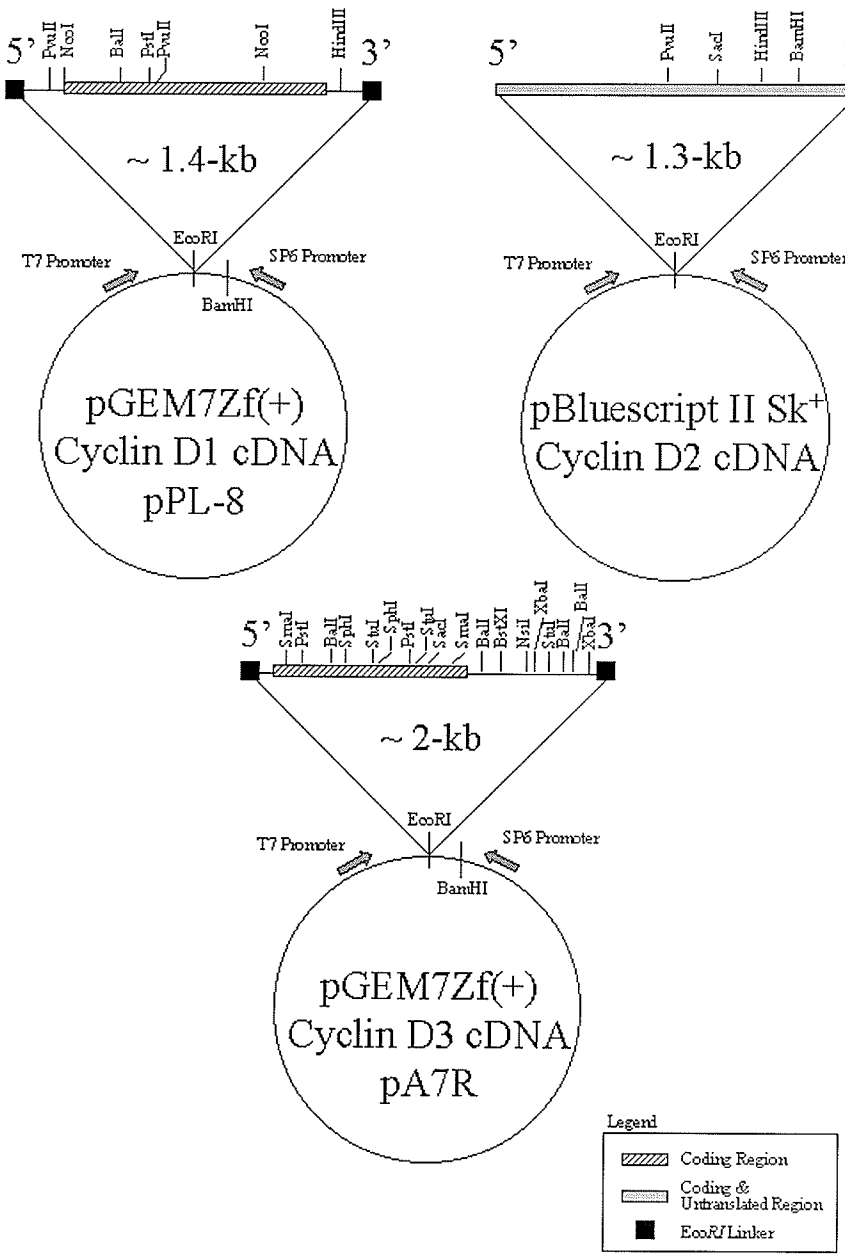


Figure 5: Schematic representation of *cyclin D1*, *D2*, and *D3* cDNAs.

Table 2: Summary of cDNA probes used for Southern blotting.

Gene Name	Clone Name	Type Probe/Size	Chromosomal Location	Source/Reference
Cyclin D1	pPL-8	Human cDNA, ~1.4-kb, with linkers added, subcloned into Eco*RI* multi-cloning site (MCS) of the pGEM7Zf(+) plasmid. Insert name λP1-4.	11q13	Dr. Andrew Arnold (137).
Cyclin D2	None Provided	Human cDNA, ~1.3-kb, subcloned into the Eco*RI* MCS of the pBluescript II Sk+ plasmid.	12p13	Dr. Gordon Peters (None Provided).
Cyclin D3	pA7R	Human cDNA, ~2-kb, subcloned into the Eco*RI* MCS of the pGEM7f(+) plasmid. Insert missing a small region of the 5' untranslated region (UTR).	6p21	Dr. Andrew Arnold (138).
G3PDH	None Provided (Cat # 9805-1)	Human cDNA, 1.1-kb in length consisting of a 1.0-kb cDNA fragment attached to a T7 RNA polymerase promoter. Probe covers region from nucleotide 71 to 1053.	12p13	ClonTech Laboratories, Inc., Palo Alto, California, USA (139, 140).

3.3 GROWTH MEDIA

3.3.1 Bacterial Media

Commercially available pre-mixed powder for Luria Bertani (LB) (Difco Laboratories, Detroit, MI, USA) broth was prepared according to manufacturer instructions and pH adjusted to 7.0 using concentrated hydrochloric acid (HCl). For LB agar, 1.0% weight/volume (w/v) Bacto-agar (Difco Laboratories) was added prior to autoclaving. After autoclaving LB agar was cooled in a 55 degrees Celsius (°C) Jeio Tech shaking water bath, model#BS-10 (Jeio Tech Co., Ltd., Kimpo-city, Kyounggi-do, Republic of Korea). If required, 100 micrograms (μg)/millitre (mL) ampicillin (amp) was added prior to pouring into 100 x 15 millimetre (mm) plastic petri dishes (Fisher Scientific Company, Nepean, Ontario, Canada). The plates were allowed to set overnight, and were stored inverted at 4°C until needed. Liquid LB was stored at RT and, if required, 100 μg/mL amp was added immediately prior use. SOC medium used for bacterial transformations was purchased (Gibco BRL Life Technologies, Gaithersburg, MD, USA).

3.3.2 Tissue Culture Media

RPMI 1640 containing L-glutamine was made according to manufacturer's instructions (Gibco BRL Life Technologies), supplemented with 100 units/mL of penicillin-G and 54.5 units/mL of streptomycin sulfate. The pH was adjusted between 7.0 to 7.5 using concentrated HCl. All cell culture media was prepared using ddH_2O and filter sterilized using a 0.2 micrometre (μm) VacuCap® 90 filter unit (Gelman Laboratory, Ann Arbor, Michigan, USA) into autoclaved sterile glass bottles. Media was stored in the dark at 4°C until required.

3.4 COLLECTION OF NORMAL CONTROL PERIPHERAL BLOOD SAMPLES

Normal donor blood samples were collected from healthy normal volunteers using 50 cubic centimetre (cc) syringes coupled to a Vacutainer brand blood collection set with a ¾ inch 21-gauge needle and 7 inch tubing (Becton Dickenson and Company, Franklin Lakes, New Jersey, USA). Blood was transferred from the 50 cc syringes into 16 x 100 mm Vacutainers containing 143 USP of sodium heparin (Becton Dickenson and Company) and mixed gently by inversion to dispense heparin and prevent clotting of blood. Donors ranged from 24 to 49 years of age, all donors were male. All normal donors were healthy at the time of donation and had lymphocyte counts $<5.0 \times 10^9$ cells/L.

3.5 COLLECTION OF UMBILICAL CORD BLOOD SAMPLES

Cord blood samples were collected using 50 cc syringes coupled to an 18 gauge needle from the umbilical cord shortly after baby delivery in the Maternity Ward of the Woman's Pavilion in the Health Science Centre (Winnipeg, Manitoba, Canada). Blood was transferred into 16 x 100 mm Vacutainers containing 143 USP of sodium heparin, and mixed gently by inversion to dispense heparin and prevent clotting of blood.

3.6 PATIENT RECRUITMENT AND COLLECTION OF CLL PERIPHERAL BLOOD SAMPLES

Peripheral blood from patients with B cell CD5+ CLL were collected by venipuncture into 16 x 100 mm Vacutainers containing 143 USP of sodium heparin by the staff in The Department of Haematology in CancerCare Manitoba (Winnipeg, Manitoba, Canada). Approximately 60 cc of blood was collected per patient but volumes varied depending on

patient lymphocyte count. All patients were under the care of Drs. James B. Johnston or Lyonel G. Israels in the Section of Haematology/Oncology in CancerCare Manitoba. To be eligible for the study all patients were required to provide informed consent, be untreated or to have been off treatment for at least one month. The diagnosis of CLL was made by demonstrating patients with a persistent peripheral lymphocyte count of >5 x 10^9/L and that the cell morphology was typical for CLL and that by immunophenotyping the cells were CD5+, CD19+, and usually CD23+ (Figure 1). Immunophenotyping was carried out by flow cytometry in the Hematopatholgy Laboratory in the Health Science Centre.

3.7 TISSUE CULTURE CELL LINES

The non-Epstein-Barr virus (EBV) transformed WSU-CLL cell line with a doubling time of 24 hours was grown in RPMI 1640 supplemented with 10% volume/volume (v/v) fetal bovine serum (FBS) (Gibco BRL Life Technologies) and 2 millimolar (mM) L-glutamine (141). Cells were cultured in a humidified 5 percent (%) carbon dioxide (CO_2) Napco® E series, model 5200, CO_2 incubator (Precision Scientific, Winchester, Virginia, USA) at 37°C, and were split every two days.

3.8 PERIPHERAL BLOOD LYMPHOCYTE SEPARATION

Blood was centrifuged at 350 times gravity (x g) for 10 minutes at RT in a bench-top Damon/IEC, model#HN-SII, centrifuge (Needham Heights, Massachusetts, USA). Using a sterile pasteur pipette the suspended white mononuclear cell layer containing lymphocytes, monocytes, neutrophils, and contaminating red blood cells were transferred from each Vacutainer to a 50 mL Corning™ tube (Corning Incorporated, Acton,

Massachusetts, USA) containing 5 to 10 mL of 1X Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1% FBS. DPBS was purchased commercially (Gibco BRL Life Technologies). If monocyte depletion was required, the plasma layer was transferred to a 50 mL Corning™ tube and saved until needed. Due to the small volume obtained, umbilical cord blood was not initially centrifuged, but was diluted 1/5 with 1X DPBS supplemented with 1% FBS in a 50 mL Corning™ tube and processed as follows. The diluted mixture of mononuclear cells (or diluted cord blood) was gently layered upon a 5 mL cushion of Ficoll-Paque™ (Amersham Pharmacia Biotech AB, Baie d'Urfe, Quebec, Canada) in a 13 mL Corning™ tube. The Ficoll-Paque™ density gradient was centrifuged at 350 x g for 30 minutes at RT to separate the lymphocytes and monocytes which remain at the interphase from the neutrophils and contaminating red blood cells that pass through the Ficoll-Paque™ (142). Using a sterile pasteur pipette, the suspended "buffy-coat" interphase layer containing the lymphocytes and monocytes was removed and transferred to a sterile 50 mL Corning™ tube. The tube was centrifuged at 350 x g for 10 minutes at RT to pellet the cells, and the supernatant discarded. The cell pellet was resuspended in 10 mL of ACK lysis (0.15 Molar (M) ammonium chloride (NH_4Cl), 7.2 mM potassium carbonate (KCO_3), 0.11 mM ethylenediaminetetraacetic acid (EDTA)) buffer to lyse any residual red blood cell contamination and incubated at RT for 10 minutes. The tube was centrifuged at 350 x g and the supernatant discarded. The cells were resuspended in the desired buffer, as outlined Section 3.8.3. If the patient's peripheral blood lymphocyte count was less than 30 x 10^9/L the sub-populations of cells

were separated as outlined in Sections 3.8.1 and 3.8.2 to obtain a pure population of CLL cells.

Normal peripheral blood samples were monocyte depleted as in Section 3.8.2, and B and T cells were separated using CD19 antibodies coupled to magnetic Dynabeads® (Dynal Biotech, Oslo, Norway), as described by the manufacturer. Dynabeads® were only used for normal samples due to the small numbers of cells.

3.8.1 B and T Lymphocyte Cell Depletion

3.8.1.1 Sheep Red Blood Cell (SRBC) Sensitization

Two mL of SRBCs (Provided as a 15% stock from National Biological Laboratories Ltd., Dugald, Manitoba, Canada) were transferred to a 50 mL Corning™ tube, and cells centrifuged at 350 x g for 10 minutes at RT. The cells were washed 3 times with 10 mL of 1X phosphate buffered saline, pH 7.4, (PBS) (137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8.0 mM disodium phosphate (Na_2HPO_4), 1.5 mM potassium phosphate (KH_2PO_4)), and centrifuged at 350 x g for 10 minutes at RT between washes. The final washed pellet was resuspended in 15 mL of freshly prepared 0.14 M 2-aminoethylisothiouronium bromide (AET), pH 9.0, and incubated for 15 minutes at 37°C. The cells were washed 4 times with 10 mL of 1X PBS, and centrifuged at 350 x g for 10 minutes at RT between washes. The final washed pellet was resuspended in 30 mL of Hanks' Balanced Salt Solution (HBSS) to yield a final concentration of 3×10^6 cells/mL. HBSS was purchased commercially (Gibco BRL Life

Technologies). Cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter Inc., Mississauga, Ontario, Canada).

3.8.1.2 SRBC Rosetting

Separated lymphocytes were resuspended in HBSS if not already done so at a concentration of 3 x 10^6 cells/mL. The cell suspension was mixed with an equal volume of AET-SRBCs and incubated at 37°C for 60 minutes. The concentration can be changed as long as the ratio of SRBCs to lymphocytes remains 1:10. The suspension was mixed by inversion, and Ficoll-Paque™ density gradient centrifugation was performed as outlined in Section 3.8. Using a sterile pasteur pipette the B cells and monocytes in the suspended "buffy-coat" interphase layer were transferred to a sterile 50 mL Corning™ tube (Section 3.8.1.2.1). If required, a second Ficoll-Paque™ density gradient centrifugation was completed if there was red blood cell contamination in the interphase layer. Using a sterile pasteur pipette the T cells which are bound to the SRBC pellet were transferred to a sterile 50 mL Corning™ tube (Section 3.8.1.2.2).

3.8.1.2.1 Separation of B Lymphocytes

The 50 mL Corning™ tube containing the B cells and monocytes was centrifuged at 350 x g for 10 minutes at RT, and the supernatant discarded. To deplete the monocytes and obtain a pure B cell population see Section 3.8.2. Otherwise, the cells were resuspened in the desired buffer as outlined in Section 3.8.3.

3.8.1.2.2 Separation of T Lymphocytes

The 50 mL Corning™ tube containing the T cells and SRBC was centrifuged at 350 x g for 10 minutes at RT, and the supernatant discarded. The SRBC were lysed with 10 mL

of ACK lysis buffer for 10 minutes at RT as outlined Section 3.8. The tube was centrifuged at 350 x g for 10 minutes at RT, and supernatant discarded. The cell pellet was washed with 10 mL of 1X DPBS supplemented with 1% FBS, and centrifuged at 350 x g for 10 minutes at RT. The supernatant was discarded, and the purified T cells were resuspended in the desired buffer, as outlined in Section 3.8.3.

3.8.2 Monocyte Cell Depletion

3.8.2.1 Fibronectin Flask Preparation

Five mL of autoclaved, molten 2% gelatin was added to the bottom of a Corning™ T-75 flask. After gelatin solidification, the flasks were incubated at 37°C for 2 hours. Excess gelatin was aspirated off and the flask allowed to dry. The plasma collected in Section 3.8 was centrifuged at 350 x g for 10 minutes at RT. The platelet-free supernatant was transferred to a sterile 50 mL Corning™ tube. Five mL of the now platelet-free plasma was transferred to the gelatin coated flasks. The flasks were incubated at 37°C for 30 to 60 minutes to allow the fibronectin in the plasma to bind to the gelatin coating in the flask. After incubation the plasma was aspirated off and the flask gently washed with 10 mL of calcium- and magnesium-free HBSS.

3.8.2.2 Fibronectin Depletion of Monocytes

The cells were resuspended in RPMI 1640 supplemented with 20% Horse Serum (HS) at a concentration of 2 x 10^6 cells/mL. To each flask a maximum of 10 mL of the cell suspension containing B lymphocytes and monocytes was added to each flask and incubated at 37°C for 40 minutes. This incubation period allows the monocytes to bind to the fibronectin leaving the lymphocytes free in suspension. The media was removed and

transferred to a sterile 50 mL Corning™ tube, and the flask gently washed 3 times with 10 mL aliquots of prewarmed 37°C RPMI 1640. The washes were combined into the original non-adherent cell sample. Tubes were centrifuged at 350 x g, for 10 minutes at RT and the supernatant discarded.

3.8.3 Storage of Isolated Cell Populations

After individual cell populations were separated according to Section 3.8 each cell population was washed with an aliquot of 1X DPBS supplemented with 1% FBS, centrifuged at 350 x g for 10 minutes at RT, and supernatant discarded. Cells were resuspended in a suitable volume of 1X DPBS to yield approximately 1.0 to 2.0 x 10^8 cells/mL and were counted using a Z2 Coulter Counter.

3.8.3.1 Storage for DNA and Protein Isolation

An appropriate volume of cells was transferred to sterile 1.5 mL Eppendorf™ (Brinkman Instruments Inc., Mississauga, Ontario, Canada) tubes and centrifuged at 1,310 x g in a microfuge, model 5415 (Brinkman Instruments Inc.) for 10 minutes at RT. The supernatant was removed and the cells were stored as a dry pellet in 1.5 mL Eppendorf™ tubes at –80°C. Cell numbers generally ranged from 0.5 to 3.0 x 10^8 cells/tube but were highly dependent on initial cell counts and cell yield.

3.8.3.2 Storage for Total RNA Isolation

An appropriate volume of cells was transferred to sterile 1.5 mL Eppendorf™ tubes and centrifuged at 1,310 x g in a microfuge for 10 minutes at RT. The supernatant was removed and the cells were lysed in 500 microlitres (μL) 4 M guanidine thiocyanate (GITC) (4 M GITC, 25 mM sodium citrate ($Na_3C_6H_5O_7$), 0.5% sarcosine with 100 mM

of freshly added β–mercaptoethanol) solution. Cells were sheared using a 1 mL syringe with a 21-gauge needle and stored at –80°C as a lysed mixture.

3.9 DNA EXTRACTION FROM FROZEN SAMPLES

Five x 10^7 cells were lysed in 25 mL genomic lysis buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, pH 8, 0.2% sodium dodecyl sulfate (SDS)) in a 50 mL polypropylene Corning™ tube. To digest RNA, 50 µg/mL Ribonuclease A (RNase A) was added and incubated at 37°C for 30 to 60 minutes. The RNase was inactivated and the protein digested by the addition of 100 µg/mL proteinase K. The tube was inverted to mix, and incubated overnight at 37°C. The next day, the genomic DNA was precipitated by addition of 1/10 volume 3 M ammonium acetate (NH_4COOCH_3), pH 4.8, and 2.5 volumes absolute ethanol, vortexed and placed at –20°C overnight. The next day, the tube was centrifuged at 10800 x g in a Sorvall RC5C centrifuge (Kendro Laboratory Products, Newtown, Connecticut, USA) with a GS-3 Sorvall rotor (Kendro Laboratory Products), and the supernatant was discarded. The pellet was dissolved in 1 to 2 mL of Tris-EDTA (TE) (10 mM Tris, pH 7.4, 1.0 mM EDTA, pH 8.0) buffer and, if required, agitated at 37°C to help dissolve the pellet. The dissolved DNA was transferred in 500 µL aliquots into sterile 1.5 mL Eppendorf™ tubes. The genomic DNA was extracted with 500 µL of a freshly made phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) mixture. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT. The aqueous top layer was carefully transferred to a sterile 1.5 mL Eppendorf™ tube avoiding the white protein interphase layer. The extraction was repeated until the aqueous layer was clear. Residual phenol was removed with a final

400 µL chloroform wash. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT and the upper aqueous phase transferred to a sterile 1.5 mL Eppendorf™ tube. The DNA was precipitated with a 1/10 volume of 3 M NH_4COOCH_3, pH 4.8, and 2.5 volumes of absolute ethanol at –20°C overnight. The next day, the DNA was pelleted by centrifugation at 13,800 x g for 30 minutes at 4°C. The pellet was washed with 70% absolute ethanol (to remove residual salts) and centrifuged at 13,800 x g for 30 minutes at 4°C, and the 70% ethanol wash discarded. The DNA pellet was air-dried, resuspended in ~1 mL TE and, if required, agitated at 37°C to help dissolve the pellet. DNA concentration was determined by measuring the absorbance at 260 nanometres (nm) using a 1.0 mL quartz cuvette in a Cary 1 spectrophotometer (Varian, Mississauga, Ontario, Canada). Where an absorbance reading of 1.0 at 260 nm equals a DNA concentration of 50 µg/mL. The yield was calculated as follows: $Absorbance_{260}$ x 50 µg/mL x dilution factor. Purity of DNA preparations was estimated by measuring the ratio of $Absorbance_{260}$ to Absorbance at 280 nm ($Absorbance_{280}$). DNA preparations having a ratio of 1.8 to 2.0 are considered to be suitable for further use. DNA samples were stored at 4°C until required.

3.10 RNA EXTRACTION FROM FROZEN SAMPLES

Note: RNase and DNase free barrier pipette tips were used in all steps of RNA isolation and manipulation to help decrease the probability of aerosol cross-contamination of RNA and/or RNases.

3.10.1 Guanidine Thiocyanate (GITC) Method

Prior to RNA isolation the samples were removed from –80°C and allowed to thaw at RT. RNA was isolated as described with some modifications (143). Samples were resuspended using a 1 mL syringe with a 21-gauge needle. If required, samples volumes were adjusted using GITC solution to achieve a final concentration of 1.0×10^8 cells/mL with a maximum volume of 500 µL per 1.5 mL Eppendorf™ tube. One-tenth volume of 2 M sodium acetate ($NaCOOCH_3$), pH 4.0, 1 volume of water-saturated acid based phenol, pH 4.0, and a 1/5 volume of chloroform:isoamyl alcohol (50:1) was added to each tube. Each tube was mixed after the addition of each reagent, and then vortexed until a white emulsion formed, and incubated on ice for 15 minutes. The tubes were centrifuged at 13,800 x g for 20 minutes at 4°C. The aqueous top layer was carefully transferred to a sterile 1.5 mL Eppendorf™ tube avoiding the white protein interphase layer. Two-hundred µL of fresh GITC solution was added to each tube to increase sample volume and help minimize RNA loss during subsequent phenol/chloroform extractions. To remove residual proteins, the acid based phenol:chloroform:isolamyl alcohol extraction was repeated without additional 2 M $NaCOOCH_3$. One volume of water-saturated acid based phenol, pH 4.0, 1/5 volume of chloroform:isoamyl alcohol (50:1) was added to each tube. Each tube was mixed after the addition of each reagent, and then vortexed until a white emulsion formed, and incubated on ice for 15 minutes. The tubes were centrifuged at 13,800 x g for 20 minutes at 4°C. The aqueous layer was carefully transferred to a sterile 1.5 mL Eppendorf™ tube avoiding the white protein interphase layer. An equal volume (normally 400 µL) of 2-butanol (isopronanol) was

added to each tube, vortexed and placed at –20°C overnight to precipitate the RNA. The next day, the tubes were centrifuged at 13,800 x g for 30 minutes at 4°C and supernatant discarded. The pellet was resuspended in 600 µL of 4 M GITC solution. The tubes were vortexed and left at RT for 1 to 2 hours to allow the pellet to dissolve. An equal volume of 2-butanol was added to each tube, vortexed and placed at –20°C overnight to precipitate the RNA. The next day, the tubes were centrifuged at 13,800 x g for 30 minutes at 4°C and the supernatant discarded. Five-hundred µL of 70% ethanol was added to each tube to wash the pellet. The tubes were centrifuged at 13,800 x g for 15 minutes at 4°C and the 70% ethanol wash discarded. The pellets were allowed to air dry for 20 minutes and the pellets dissolved in 50 µL of RNase-free ddH_2O. RNA samples were heated at 65°C for 10 minutes, if required, to dissolve the RNA. A 5 µL aliquot of RNA was diluted 1/200 in RNase-free TE and quantified using the RiboGreen® RNA quantitation kit, Cat#R-11490 (Molecular Probes, Eugene, Oregon, USA) as described by the manufacturer. Samples were read using a Molecular Dynamics Storm 860 system (Amersham Pharmacia Biotech AB) as described by the manufacturer. RNA was stored in RNase-free ddH_2O at –80°C until required.

3.10.2 TRIzol® Method

Two previously separated stored samples: umbilical cord blood, and CLL patient sample #9 had been stored in TRIzol® (Gibco BRL Life Technologies) reagent at –80°C. RNA was isolated from these samples using the TRIzol® based method as described by the manufacturer (Gibco BRL Life Technologies).

3.11 PREPARATION OF *CYCLIN D1*, *D2* AND *D3* CDNAS

3.11.1 Preparation of Chemically Competent *Escherichia coli* (*E.coli*)

Using a sterile toothpick 2 mL of LB was inoculated with stock DH5α *E. coli* and grown overnight at 37°C in a New Brunswick, model G25, shaking incubator (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). The next day, a 50 mL culture of LB was inoculated using the overnight culture and incubated at 37°C in a shaking incubator until an optical density (O.D.) of 0.75 at 650 nm or 0.5 to 0.6 at 540 nm was reached. Once this density was obtained the culture was transferred into a sterile 50 mL Corning™ tube and incubated on ice for 25 minutes. The bacteria were centrifuged at 2,000 x g for 5 minutes at 4°C in a Sorvall RC5C centrifuge. The supernatant was discarded and the bacteria resuspended in 12.5 mL of 50 mM calcium chloride ($CaCl_2$) and incubated on ice for 1 hour. The suspension was centrifuged at 1,310 x g for 5 minutes at 4°C, and the supernatant discarded. The competent bacteria were resuspended in 600 μL of 50 mM $CaCl_2$, 1.4 mL 87% glycerol and 3 mL ddH_2O, and aliquoted in 125 μL volumes into sterile 1.5 mL Eppendorf™ tubes, and stored at –80°C until required.

3.11.2 cDNA Transformation of Competent *E. coli*

The human *cyclin D1*, *D2* and *D3* cDNA containing plasmids were transformed into *E. coli*. One-half to 1.0 μg of plasmid DNA in a volume of 10 μL of TE was aliquoted into an 1.5 mL Eppendorf™ tube and placed on ice. An additional 40 μL TE, along with 20 μL Tris-$CaCl_2$-magnesium chloride ($MgCl_2$) (TCM) (0.1 M Tris, pH 7.5, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$) buffer, and 125 μL of competent DH5α *E. coli* bacteria were added to the plasmid DNA, mixed, and incubated on ice for 20 minutes. The bacteria were heat

shocked for exactly 2 minutes at 41°C, and allowed to cool to RT for 10 minutes. One mL of SOC medium was added to the transformed bacteria, and incubated in a shaking incubator at 37°C for a minimum of 1 hour up to a maximum of 4 hours. Aliquots of 100 µL and 200 µL of transformed *E. coli* bacteria were plated on LB petri-plates supplemented with 100 µg/mL amp, spread using a sterile bent glass rod, and incubated inverted for 12 to 15 hours at 37°C. Plates were stored inverted at 4°C.

3.11.3 Small Scale Extraction of Human *Cyclin D1*, *D2* and *D3* cDNAs

To ensure the correct D cyclins were isolated from transformed *E. coli* colonies a small scale plasmid purification was carried out following the alkali method with some modification (144). Using sterile toothpicks cyclin D1, D2, and D3 containing *E. coli* transformants from LB petri-plates supplemented with 100 µg/mL amp (Section 3.11.2) were inoculated into 2 mL of LB supplemented with 100 µg/mL amp and incubated in a shaking 37°C incubator. The next day, the overnight cultures were transferred to sterile 1.5 mL Eppendorf™ tubes and centrifuged at 13,800 x g for 60 seconds at RT. The supernatant was discarded, and pellet resuspended in 100 µL cold Tris-glucose-EDTA (TGE) (1 M Tris, pH 8.0, 100 mM glucose, 0.5 M EDTA, pH 8.0) buffer. The cells were lysed with 200 µL of freshly prepared lysis (1% SDS, 0.2 M sodium hydroxide (NaOH)) buffer, vortexed, and placed on ice for 5 minutes. To precipitate bacterial chromosomal DNA, RNA and protein, 50 µL of 3 M cold potassium acetate ($KCOOCH_3$), pH 5.2, was added, vortexed, and placed on ice for 5 minutes. The tube was centrifuged at 13,800 x g for 5 minutes at 4°C. While avoiding the white precipitate the supernatant was transferred to a sterile 1.5 mL Eppendorf™ tube and treated with 4 µL RNase A (10

milligrams (mg)/mL) for 30 minutes at 37°C. The treated sample was extracted with 500 µL of a freshly made phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) mixture. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT and the upper aqueous phase carefully transferred to a sterile 1.5 mL Eppendorf™ tube. Residual phenol was removed with a final 400 µL chloroform wash. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT and the upper aqueous phase transfer to a sterile 1.5 mL Eppendorf™ tube. The plasmid DNA was precipitated with 1/10 volume 3.0 M NH_4COOCH_3, pH 4.8, and 2.5 volumes of absolute ethanol. The tube was vortexed and placed at –80°C for 20 minutes to precipitate plasmid DNA. The precipitated plasmid DNA was pelleted by centrifugation at 13,800 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet washed with 200 µL of 70% ethanol. The tube was centrifuged at 13,800 x g for 30 minutes at 4°C, and the 70% ethanol wash discarded. The pellet was air dried for 20 minutes and dissolved in 50 µL of sterile TE and, if required, agitated at 37 °C to help dissolve the pellet. All plasmid preparations were stored at 4°C until required.

To confirm the identity of *cyclin D1*, *D2*, and *D3* cDNA plasmids, restriction endonuclease (RE) analysis using Eco*RI* was performed to ensure the correct insert size was released. The restriction fragments were compared to the expected size references provided by Dr. Andrew Arnold, the originator of the plasmids. The RE digestions were carried out using 5 µl plasmid DNA, 1.5 µL 10X manufacturer supplied buffer (Roche Diagnostics, Laval, Quebec, Canada), 8.0 µL ddH_2O and 0.5 µL Eco*RI* enzyme (40

units/μl, Roche Diagnostics). The samples were incubated at 37°C for 1 hour after which 2 μL of 6X loading dye (0.25% bromophenol blue, 0.25% xylene-cyanol, 15% ficoll) added to each sample. The samples were loaded into a 1% agarose LE (Roche Molecular Diagnostics) gel containing 0.5 mg/mL ethidium bromide (EtBr). In addition to digested plasmid DNA, 2.5 μg lambda DNA-Hind*III* ladder (Amersham Pharmacia Biotech AB.) was loaded flanking plasmid DNA to allow fragment size comparison. The agarose gel was electrophoresed in a Mini-Sub-Cell GT (Bio-Rad, Mississauga, Ontario, Canada) at 3 volts/centimetre (cm) for 2 hours in 1X Tris-borate-EDTA (TBE) running buffer. Gels were viewed using a Chromato-VUE® transilluminator, model#TL-33 (Ultra-Violet Products Inc., San Gabriel, California, USA) and documented using a Polaroid MP-4 land camera (Polaroid Corporation, Cambridge, Massachusetts, USA) containing Polapan 667, ISO 3000/36° (Polaroid Corporation) instant film.

3.11.4 Large Scale Extraction of Human *Cyclin D1*, *D2* and *D3* cDNAs

One clone for each cDNA that contained the correctly sized cDNA was selected for large scale plasmid preparation (145). A 2.0 mL starter culture of LB broth supplemented with 100 μg/mL amp was inoculated from the remaining mini-preparation cultures (Section 3.11.3) and grown for 6 hours in a shaking 37°C incubator. The 6 hour starter cultures were added to 500 mL of prewarmed 37°C LB broth supplemented with 100 μg/mL amp and grown overnight in a shaking 37°C incubator. The 500 mL overnight culture was transferred into sterile 500 mL centrifuge bottles and centrifuged at 2070 x g for 15 minutes at 4°C. Supernatant was discarded and the pellet resuspended in 16 mL of solution I (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0). To ensure

complete lysis 2 mL of lysoszyme solution (10 mg/mL lysoszyme, 10 mM Tris, pH 8.0) was added and mixed well by shaking, and incubated on ice for 10 minutes. The genomic DNA, protein and large molecular weight RNA was precipitated by addition of 40 mL of freshly prepared Solution II (200 mM NaOH, 1% w/v SDS), and mixed well by shaking. The resulting white flocculent precipitate was pelleted by centrifugation in a Sorvall RC5C centrifuge at 2,700 x g for 25 minutes at 4°C, the rotor was allowed to come to rest without the brake. The supernatant was slowly decanted through 2 to 3 layers of sterile cheesecloth into a sterile 500 mL centrifuge bottle and 50 mL of isopropanol was added, mixed, and allowed to stand at RT for 10 minutes. Bottles were centrifuged at 4220 x g for 15 minutes at RT. The supernatant was discarded and the pellet gently washed with 50 mL 70% absolute ethanol. The 70% ethanol wash was discarded and the pellet was allowed to air dry for 15 to 20 minutes. The pellet was dissolved in 3 mL of TE and if necessary, the pellet was heated to 37°C to help dissolve the pellet. Five hundred µL aliquots of the dissolved nucleic acids were transferred into sterile 1.5 mL Eppendorf™ tubes. High molecular weight RNA was precipitated by the addition of 500 µL volumes of –20°C 5 M lithium chloride (LiCl) to each tube, mixed well, and centrifuged at 13,800 x g for 10 minutes at 4°C (145). The supernatant was transferred to fresh, sterile 1.5 mL Eppendorf™ tubes. Aliquots of 500 µL of isopropanol were added to each tube, mixed well, and incubated at RT for 5 minutes. Precipitated samples were centrifuged at 13,800 x g for 10 minutes at RT, and the supernatant discarded. The pellet was washed with 500 µL of 70% absolute ethanol. The 70% ethanol wash was discarded and the pellet allowed to air dry. All pellets were

dissolved in a total of 500 μL TE buffer, pH 8.0, and pooled into one 1.5 mL Eppendorf™ tube. RNA was digested by addition of 4 μL of RNase (10 mg/mL) and incubated at 37°C for 30 minutes. Five-hundred μL of polyethylene glycol (PEG) (1.6 M NaCl, and 13% PEG 8000) solution was added to each tube, and mixed well. The tubes were centrifuged at 13,800 x g for 5 minutes at 4°C, and the supernatant discarded. The pellet was dissolved in 570 μL of TE. The treated sample was extracted with 570 μL of a freshly made phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) mixture. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT and the upper aqueous phase carefully transferred to a sterile 1.5 mL Eppendorf™ tube. Residual phenol was removed with a final 400 μL chloroform wash. Following a quick vortex the mixture was centrifuged at 13,800 x g for 5 minutes at RT and the upper aqueous phase transfer to a sterile 1.5 mL Eppendorf™ tube. Aliquots of 225 μL TE were transferred to sterile 1.5 mL Eppendorf™ tubes and precipitated with 56 μL of 10 M NH_4COOCH_3 and 562 μL of absolute ethanol, mixed, and left at RT for 10 minutes. The precipitated plasmid DNA was centrifuged at 13,800 x g for 30 minutes at 4°C. The supernatant was discarded and 500 μL of –20°C 70% absolute ethanol was added to wash the pellet. The tube was gently mixed by inversion and centrifuged at 13,800 x g for 5 minutes at 4°C, and the 70% ethanol wash discarded. The pellet was air-dried, resuspended in 250 μL of TE and, if required, agitated at 37°C to help dissolve the pellet. Plasmid concentration was determined from the $Absorbacnce_{260}$ measurement using a 1.0 mL quartz cuvette as outlined in Section 3.9.

3.11.5 cDNA Fragment Isolation from Plasmid

The RE digestions were carried out in sterile 1.5 mL Eppendorf™ tubes using 15 μg plasmid DNA (Section 3.11.4), 2.5 μL 10X manufacturer supplied buffer, and the required volume of ddH_2O to bring the final volume to 23 μL. Two μL of Eco*RI* enzyme (40 units/μL) were added to the mixture, mixed gently, and incubated at 37°C overnight. After overnight incubation, 5 μL of 6X loading dye was added to each sample. The samples were loaded into a 1% agarose LE gel containing 0.5 mg/mL EtBr. In addition to digested plasmid DNA, 2.5 μg lambda DNA-Hind*III* ladder was loaded flanking plasmid DNA to allow fragment size comparison. The agarose gel was electrophoresed in a Mini-Sub-Cell GT at 3 volts/cm in 1X TBE running buffer until bands were sufficiently separated when viewed using the long-wave ultra-violet (UV) setting on an hand-held transilluminator Model UVGL-25 Mineralight® lamp (Ultra-Violet Products Inc.). Using a scalpel and the hand-held UV transilluminator the desired band was excised and placed in a GenElute™ agarose spin column Cat#5-6500 (Sigma-Aldrich Ltd.) and processed as described by the manufacturer. cDNA yield was estimated by electrophoresis of various aliquots of isolated band, and compared to the known concentration of lambda DNA-Hind*III* ladder.

3.12 SOUTHERN ANALYSIS

3.12.1 Restriction Enzyme Digestion and Electrophoresis of Genomic DNA

The RE digestions were carried out in sterile 1.5 mL Eppendorf™ tubes using 10 μg of genomic DNA (Section 3.9), 3.5 μL 10X manufacturer supplied buffer (Roche Diagnostics), and the required volume of ddH_2O to bring the final volume to 33.0 μL.

Two μL of the respective RE Eco*RI*, Hind*III* or Pst*I* (40 units/μL, Roche Diagnostics) was added to the mixture, mixed gently, and incubated at 37°C overnight. The next day, digestions were boosted with an additional 1 μL (40 units/μL) of the respective enzyme and incubated for an additional 3 hour period at 37°C to ensure complete digestion. Five μL of Blue Juice™ (Invitrogen Canada Inc., Burlington, Ontario, Canada) was added to each sample. The samples were loaded into a 1% agarose LE gel containing 0.5 mg/mL EtBr. In addition to digested plasmid DNA, 2.5 μg lambda DNA-Hind*III* ladder was loaded flanking the digested genomic DNA to allow evaluation of electrophoresis. The agarose gel was electrophoresed in a Sub-Cell GT (Bio-Rad) at 1 volt/cm in 1X TBE running buffer overnight until the 2,322 base pair (bp) lambda DNA-Hind*III* ladder band had migrated approximately 7 cm from the well. The agarose gel was viewed using Chromato-VUE® transilluminator, model#TL-33, and documented using a Polaroid MP-4 land camera containing Polapan 667, ISO 3000/36° instant film.

3.12.2 Capillary Transfer of Electrophoresed DNA to Nylon Membrane

After documentation the gel was submerged in freshly prepared 0.25 M HCl and incubated on an oscillating shaker for 15 minutes. This step, also referred to as acid hydrolysis results in the cleavage of DNA fragments. This does not alter fragment location on the gel, rather, it creates smaller DNA fragments which transfer to the membrane more efficiently than large fragments of DNA. The HCl solution was discarded and the gel rinsed twice in distilled water (dH_2O) to remove excess HCl. The gel was submerged in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and incubated on an oscillating shaker for 15 minutes, and repeated. The denaturing solution was

discarded and the gel rinsed twice in dH_2O to remove excess denaturing solution. The gel was then submerged in neutralization solution (0.5 M Tris, 1.5 M NaCl, pH 7.4) and incubated on an oscillating shaker for 15 minutes, and repeated. The neutralization solution was discarded and the gel rinsed twice in dH_2O to remove excess neutralization solution. The DNA was transferred from the agarose gel to Hybond XL membrane (Amersham Pharmacia Biotech. AB) by capillary action in 10X sodium chloride sodium citrate (SSC), pH 7.0, (1.5 M NaCl, 0.15 M $Na_3C_6H_5O_7$) buffer for 48 hours, with additiona 10X SSC added as required. Following transfer of DNA the membrane was removed from the gel and cross-linked by baking in gel-dryer (BioRad) at 80°C for 2 hours under vacuum and further autocrosslinked in a UV Stratalinker® 1800 (Stratagene, La Jolla, California, USA). Complete transfer of DNA was confirmed by viewing the gel on the Chromato-VUE® transilluminator Model#TL-33.

3.12.3 Cytidine 5'-[α-^{32}P]triphosphate (CTP-[α-^{32}P])Labeled cDNA Preparation

Twenty-five to 50 ng of appropriate cDNA (*cyclin D1*, *D2*, *D3* or *G3PDH*) was labeled with CTP-[α-^{32}P] (Amersham Pharmacia Biotech AB) using the Amersham Megaprime™ DNA labeling system, Cat#RPN1607 (Amersham Pharmacia Biotech AB) as described by the manufacturer. Unincorporated nucleotides and excess [α-^{32}P]CTP was removed using a Bio-Spin® chromatography column (Bio-Rad, Mississauga, Ontario, Canada) as described by the manufacturer. Probe labeling efficiency was determined by measuring 1 and 3 μL aliquots of the labeled probe using a Beckman scintillation counter, model LS3801 (Beckman Coulter Inc.).

3.12.3.1 Hybridization of Labeled cDNA to Membrane

Hybridization was carried out according to standard formamide methods (146). The baked membrane was moistened with 2X SSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7$) and placed in a 30 cm Wheaton glass hybridization tube (Robbins Scientific, Sunnyvale, California, USA), with up to two membranes per tube. Thirty mL of hybridization cocktail, pH 7.0, (50% formamide, 1X Denhardts (0.5% SDS, 7.5% 500,000 molecular weight dextran sulfate), 5X SSC (0.75 M NaCl, 0.075 M $Na_3C_6H_5O_7$), 80 μg/mL of sheared, and denatured herring sperm DNA) was added to the hybridization tube and pre-hybridized in a Robbines rotating micro hybridization incubator, model 2000 (Robbins Scientific) at 42°C for a minimum of 3 hours. The appropriate amount of quantified labeled probe (Section 3.12.3) was transferred into a sterile 1.5 mL screw-cap Eppendorf™ tube containing 1.2 mL 10 mg/mL of sheared herring sperm to yield a final concentration of 2 x 10^6 dpm/mL after addition to the 30 mL hybridization buffer. The probe and herring sperm mixture was denatured by boiling for 10 minutes, incubated on ice for 1 minute and placed at 68°C for 10 minutes before adding to the bottom of the hybridization tube. The membrane was incubated overnight at 42°C in the rotating hybridization oven for a minimum of 24 hours. The next day, the membrane was washed in Buffer 1 (2X SSC, 0.1% SDS) on an oscillating shaker for 15 minutes at RT, and repeated. The membrane was then washed in Buffer 2 (1X SSC (0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7$, 0.1% SDS) on an oscillating shaker for 15 minutes at RT, and repeated. The membrane was then washed in pre-warmed 65°C Buffer 3 (0.5X (0.075 M NaCl, 0.0075 M $Na_3C_6H_5O_7$, 0.1% SDS) on an oscillating shaker for 15 minutes at RT. The moist

membrane was heat-sealed in a Kapak plastic bag, stock #408 (Kapak Corporation, Minneapolis, Minnesota, USA), and exposed to Amersham MP film (Amersham Pharmacia Biotech AB) at -80°C for suitable period of time, and developed using a AGFA, model CP1000 (AGFA Inc, Toronto, Ontario Canada) automatic film developer.

3.12.4 Removal of Labeled cDNA from Membrane

Hyrbidized radiolabeled probe was removed by adding boiling stripping-buffer (0.01% SDS, 0.01X SSC (1.5 mM NaCl, 0.15 mM $Na_3C_6H_5O_7$) to the membrane and agitated on an oscillating shaker, Red Rotor, Model PR70-115V (Hoefer Scientific Instruments, San Francisco, California, USA) for 10 to 15 minutes. Subsequent washes with fresh boiling stripping buffer were performed, if required; the approximate efficiency of stripping was determined using a geiger counter, although complete stripping was ensured by exposing membrane to Amersham MP film -80°C overnight, and developed using a AGFA, model CP1000 automatic film developer.

3.12.4.1.1 Analysis of Hybridized Labeled cDNA to Membrane for Gene Rearrangements and Amplification

3.12.4.1.1.1 Cyclin D1, D2, and D3 Gene Rearrangement Analysis

To identify gene rearrangements the restriction fragment length profile for each RE in each CLL sample was compared to the restriction fragment profile obtained for normal samples. Possible rearrangements were marked, and compared to different exposures of the same hybridization to ensure the aberration noted was not an artifact of under- or over-exposure of the autoradiograms.

3.12.4.1.1.2 Cyclin D1, D2, and D3 Gene Amplification Analysis

Using a ScanJet IIcx/T (Hewlett Packard, Montreal, Quebec, Canada) autoradiograms were scanned and saved in tagged image file (TIF) format. The scanned images were then converted to GEL format and analyzed using ImageQuant version 5.1 software (Molecular Dynamics, Amersham, Pharmacia Biotech AB). The quantity of each *cyclin D1*, *D2* and *D3* gene was determined by measuring the intensity of a constant band in each RE digestion, using the "volume function". Background correction was completed using the "local average" setting. To correct for loading and to determine if amplification was present a constant restriction fragment was identified for each D cyclin and G3PDH digestion. The D cyclin:G3PDH ratio was then calculated using Microsoft® Excel version 2000 software (Microsoft Corporation, Redmond, Washington, USA). Variation between hybridization conditions on individual Southern blots was corrected using a constant sample which was loaded on each Southern. Statistical analysis of the calculated D cyclin:G3PDH ratios investigating gene amplification was performed by Ms. Mary Cheang at the Statistical Consulting Unit at the University of Manitoba (Winnipeg, Manitoba, Canada) using SAS software (SAS Institute Inc., Cary, North Carolina).

3.13 RNASE PROTECTION ASSAY

A RiboQuant™ custom human template set, Cat#559660-16667 (Pharmingen, San Diego, California, USA) was created containing the templates p130; Rb; p107; Cyclin E; Cyclin D1; Cyclin D2; Cyclin D3, and the controls L32 and G3PDH. Using uridine 5'-[α-^{32}P]triphosphate triethylammonium salt ([α-^{32}p]UTP) labeled templates were

synthesized as described by the manufacturer (Pharmingen). Probe labeling efficiency was determined by measuring duplicate 1 μL aliquots of the labeled template probe using a Beckman scintillation counter, model LS3801. Three x 10^5 counts per minute (cpm) of labeled template were incubated with 2 to 15 μg cell line RNA; 15 μg normal B cell RNA; 15 μg normal T cell RNA; 15 μg cord blood RNA, or 15 μg patient RNA as described by the manufacturer (Pharmingen). Denatured samples and a 3000-5000 cpm/lane dilution of the undenatured probe (to serve as a reference) were loaded into the 5 mm wells of a 0.4 mm 5% acrylamide, 0.5X TBE gel pre-warmed to 40 to 50°C. The gel was run using a Sequi-GEN™ sequencing gel apparatus (Bio-Rad) at 40 watts constant power. The gel was run until the leading edge of the front dye, Bromophenol Blue reached 30 cm from the well. The apparatus was dismantled and the gel placed on a piece of 3MM Whatman chromatography paper (Whatman Inc., Clifton, New Jersey, USA) and dried in a gel-dryer at 80°C for 2 hours. The dried gel was exposed to Amersham MP film at –80°C for suitable period of time for documentation, and developed using a AGFA, model CP1000, automatic film developer. In addition, dried gels were also exposed to a Molecular Dynamics phosphoimager screens (Amersham Pharmacia Biotech AB) and the image captured using a Molecular Dynamics Storm 860 System as described by the manufacturer to allow quantitation of RNA expression.

3.13.1 RNA Expression Analysis of Cyclin D1, D2, and D3

Using ImageQuant version 5.1 software the quantity of each cyclin D1, D2 and D3 protected species in the original RNA sample was determined based on the intensity of the appropriately-sized, protected probe fragment using the volume function.

Background correction was completed using the "local average" setting. To correct for loading and to determine expression for individual RNA species a ratio for each D cyclin:G3PDH was determined using Microsoft® Excel version 2000. Statistical analysis of the calculated D cyclin:G3DPH ratios and patient characteristics was performed by Ms. Mary Cheang in the Statistical Consulting Unit at the University of Manitoba using SAS software.

4. PROJECT HYPOTHESIS AND AIMS

Hypothesis

1. The RNA expression of the D cyclins may be altered in CLL as a result of gene amplification, or alterations.

2. The RNA level of the D cyclins may reflect the underlying growth characteristics of the CLL cell and may change with disease duration, stage, lymphocyte doubling time and patient survival.

Aims

1. To determine if the levels of the D cyclins are altered in CLL and whether these changes can be related to gene amplification, or alteration.

2. To determine if the levels of the D cyclins correlate with disease duration, stage, lymphocyte doubling time or patient survival.

5. RESULTS

5.1 CLL PATIENT COHORT

Peripheral blood samples were obtained from 43 patients with confirmed CLL. Sufficient blood was collected to allow DNA analysis in all 43 patients, and RNA analysis in 38 patients (Section 3). Clinical charts of all patients were reviewed and the following data recorded: lymphocyte count; clinical stage according to Rai staging (16); age at diagnosis; lymphocyte doubling time (defined as the time needed to double the lymphocyte count); duration of disease at sample collection; treatment history, and total duration of disease until death or study end date (Table 1 and Table 3). The diagnosis of CLL was made by demonstrating patients had a persistent peripheral lymphocyte count of $>5 \times 10^9$/L, the cell morphology was typical for CLL, and that by immunophenotyping the cells were CD5+, CD19+, and usually CD23+ (Figure 1). Of the 43 patients, 32 were male and 11 were female. Rai staging ranged from 0 to IV with 9 patients being Rai 0, 8 Rai stage I, 9 Rai stage II, 5 Rai stage III and 12 Rai stage IV disease. Patient staging was determined at the time of sample collection. The average age at diagnosis was 67 with a range of 41 to 83 years of age. Whenever possible only patients with lymphocyte counts greater than 30×10^9 cells/L at time of collection were studied to help ensure the majority of B cells collected were CLL cells (147). Patient lymphocyte counts ranged from 2 to 388×10^9 cells/L, with an average lymphocyte count of 77×10^9 cells/L. Twenty-seven patients had previously required chemotherapy and/or radiation treatment prior to sample study. Of patients treated, it was required that they be off treatment for a minimum of 1 month prior to sample collection. Follow-up following sample collection

varied from 1 to 75 months (mean=22, median=14). At study completion 19 patients (44%) had died.

Normal peripheral blood donors ranged from 24 to 49 years of age, all donors were male. All normal donors were healthy at the time of donation and had lymphocyte counts <5.0 x 10^9 cells/L. All blood was combined to create a single homogenous blood population prior to cell separation (Section 3). Umbilical cord blood samples were also combined prior to cell separation (Section 3).

Table 3: Chronic lymphocytic leukemia patient cohort.

Patient	Rai stage	Sex*	Age† (years)	Lymphocytes† (10^9/Litre)	Doubling time†,‡ (months)	Disease duration† (months)	Duration from sample collection to death or study end date§ (months)	Disease duration from diagnosis to death or study end date§ (months)	Treatment before sample collection**	Sample Analyzed
1	O	M	67	30	13	35	Died, 54	89	Flu, CLB, X-Ray	RNA, DNA
2	O	F	76	32	18	30	13	44	-	RNA, DNA
3	O	M	68	58	40	115	75	190	-	RNA, DNA
4	O	F	73	68	58	99	39	137	-	RNA, DNA
5	O	F	70	51	18	143	5	149	-	RNA, DNA
6	O	F	45	38	13	12	12	24	-	RNA, DNA
7	O	M	53	22	6	83	12	96	CLB, FLU, BMT	RNA, DNA
8	O	F	67	25	22	152	14	166	CLB	RNA, DNA
9	O	M	53	89	8	19	31	50	-	RNA, DNA
10	I	M	59	40	21	46	10	57	-	RNA, DNA
11	I	M	65	65	NA	34	Died, 1	35	CLB	DNA
12	I	M	48	51	10	67	43	110	Flu	RNA, DNA
13	I	F	66	64	32	46	2	48	-	DNA
14	I	M	69	29	5	20	Died, NOF	NOF	-	DNA
15	I	M	65	39	Stable	16	12	28	-	RNA, DNA
16	I	M	67	53	6	14	12	26	CLB, Flu	RNA, DNA
17	I	M	71	28	Stable	10	37	48	-	RNA, DNA
18	II	M	67	73	8	72	53	125	CLB	RNA, DNA
19	II	M	46	143	15	46	39	85	-	RNA, DNA
20	II	M	64	56	15	45	Died, 33	77	CLB, Pred, Cyclo	RNA, DNA
21	II	M	79	28	7	133	14	147	CLB, CdA	RNA, DNA
22	II	F	83	116	Stable	212	34	246	CLB	RNA, DNA
23	II	M	53	69	2	78	1	79	CLB, P, Flu	RNA, DNA
24	II	F	78	100	2	161	33	193	CLB	RNA, DNA

Patient	Rai stage	Sex*	Age† (years)	Lymphocytes† (10^9/Litre)	Doubling time†,‡ (months)	Disease duration† (months)	Duration from sample collection to death or study end date§ (months)	Disease duration from diagnosis to death or study end date§ (months)	Treatment before sample collection**	Sample Analyzed
25	II	M	63	130	2	188	17	206	CLB, Flu, X-ray	DNA
26	II	M	67	61	2	37	Died, 27	64	CLB	RNA, DNA
27	III	M	75	95	2	66	Died, 8	74	CLB, Pred	RNA, DNA
28	III	F	71	128	2, P	103	Died, 14	117	CLB, Pred, Flu, X-Ray	RNA, DNA
29	III	M	69	42	15	83	11	94	CLB	RNA, DNA
30	III	F	78	2	Stable	109	Died, 48	156	CLB, Pred	DNA
31	III	M	66	388	NA	0	Died, 6	6	-	DNA
32	IV	M	83	45	3	1	Died, 20	21	-	RNA, DNA
33	IV	M	72	43	Stable	34	Died, 34	69	CLB, Flu	RNA, DNA
34	IV	M	73	54	5	56	Died, 27	83	P	RNA, DNA
35	IV	M	72	73	48	153	13	166	CLB	RNA, DNA
36	IV	M	70	168	2	121	Died, 7	127	CLB, Flu, X-Ray	RNA, DNA
37	IV	M	70	66	2	174	Died, 16	190	CLB, Flu, X-Ray	RNA, DNA
38	IV	M	55	210	1	96	Died, 7	103	CLB, Pred, Flu	RNA, DNA
39	IV	M	73	40	Stable, P	238	Died, 4	241	CLB, Pred, dCF	RNA, DNA
40	IV	M	75	37	11	37	15	52	CLB	RNA, DNA
41	IV	F	41	112	12	9	Died, 13	22	-	RNA, DNA
42	IV	M	76	86	2	92	Died, 1	93	CLB, Pred, Flu	RNA, DNA
43	IV	M	79	18	3	25	Died, 52	76	CLB	RNA, DNA

Notes:

* M, male; F, female.

† at time of sample collection.

‡ NA, not available; Stable, doubling time over 12 months, unable to calculate exact doubling time; P, on long-term, low-dose prednisone (Pred) at time of sample collection.

§ NOF, not on file.

** X-ray, splenic irradiation; CLB, chlorambucil; Cyclo, cyclophosphamide; Pre, prednisone; Flu, fludarabine, and BMT, bone marrow transplant.

5.2 CLINICAL CHARACTERISTICS, DISEASE STATUS, AND PROGRESSION IN CLL PATIENTS

There was no statistically significant correlation between patient sex or age on patient survival. However, there was a correlation between Rai stage and patient survival. Patients with early stage CLL had a statistically significant longer survival than late stage CLL patients according to the Kaplan-Meyer graph and the Log-Rank test ($p=0.0009$) (Figure 6). While not statistically significant, there was a trend that patients on chemotherapy had a poorer survival than those not on chemotherapy according to the Log-Rank test ($p=0.057$). This finding relates to the fact that in general, only those patients with advanced Rai stage received chemotherapy. Lymphocyte count, and doubling time were significant indicators of patient survival according to the univariate Wilcoxon test ($p=0.021$, and 0.003, respectively). Those patients with a lymphocyte doubling time of less than 12 months had a shorter survival than those with a longer lymphocyte doubling time. Covariant analysis of lymphocyte count and doubling time versus survival were also significant, according to the Wilcoxon test ($p=0.003$, and 0.048, respectively).

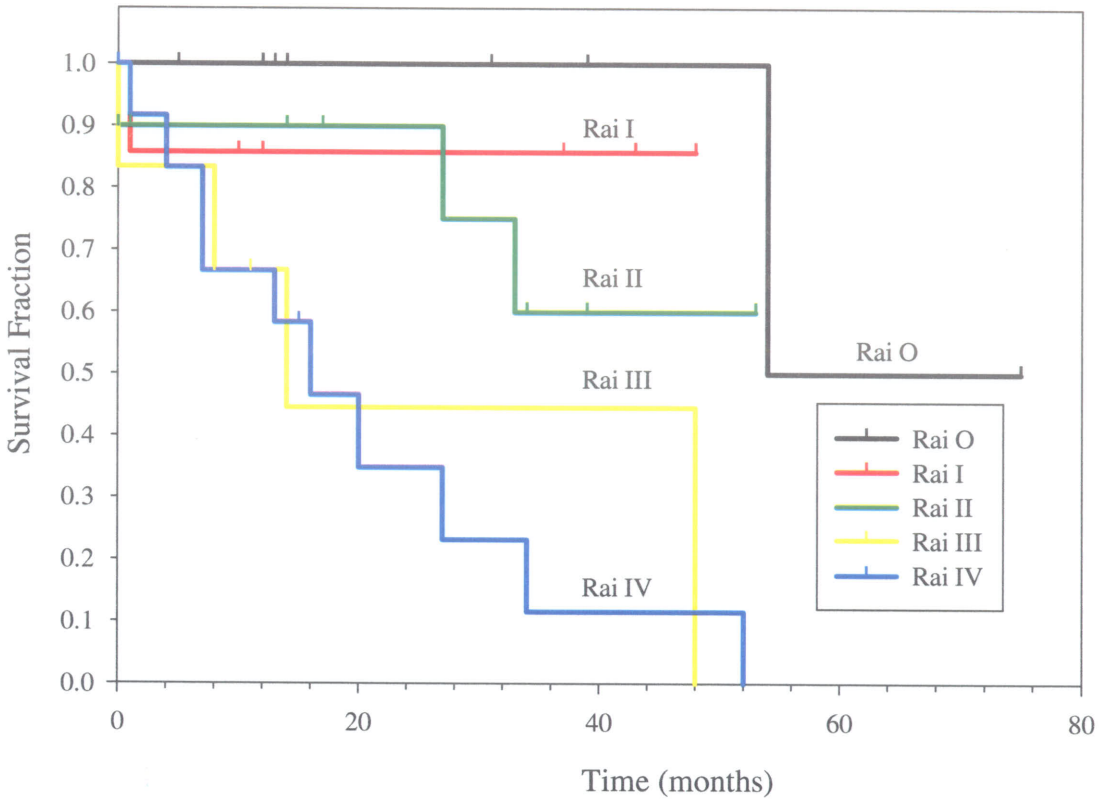


Figure 6: Kaplan-Meier survival curve in chronic lymphocytic leukemia patients.
Duration of survival based on time of sample collection to date of death according to Rai stage (16).

5.3 EXPRESSION OF CYCLIN D1, D2, AND D3 RNA IN NORMAL LYMPHOCYTES

RNA expression was evaluated using a custom designed template kit for the RNase Protection Assay (RPA) (Section 3.13). This assay is highly sensitive allowing the simultaneous expression of the three D cyclins and two housekeeping genes. In initial studies to evaluate this technique we measured the D cyclin levels in WSU-CLL and HeLa cell lines. There was less than 1.6-fold variability in the results of the different D

cyclins on three different occasions, indicating reproducibility. In addition, relative expression of the D cyclins compared to the housekeeping genes was found to be remarkable consistent, on three separate occasions.

Using this assay it was determined normal non-stimulated primary peripheral blood B lymphocytes expressed relative D2 > D3 > D1 (0.9, 0.2, 0.02) mRNA levels (Figure 7). Normal non-stimulated primary peripheral blood T lymphocytes expressed relative D2 >> D3 > D1 (3.6, 0.5, 0.04) mRNA levels (Figure 7). The D cyclin expression was 1.8-, 4.1-, and 2.8-fold higher in T lymphocytes compared to B lymphocytes for cyclin D1, cyclin D2 and cyclin D3 mRNA, respectively (Table 4). Normal non-stimulated primary umbilical cord blood lymphocytes expressed relative D2 $\approx$ D3 > D1 (0.7, 0.7, 0.03) mRNA levels (Table 4, Figure 7).

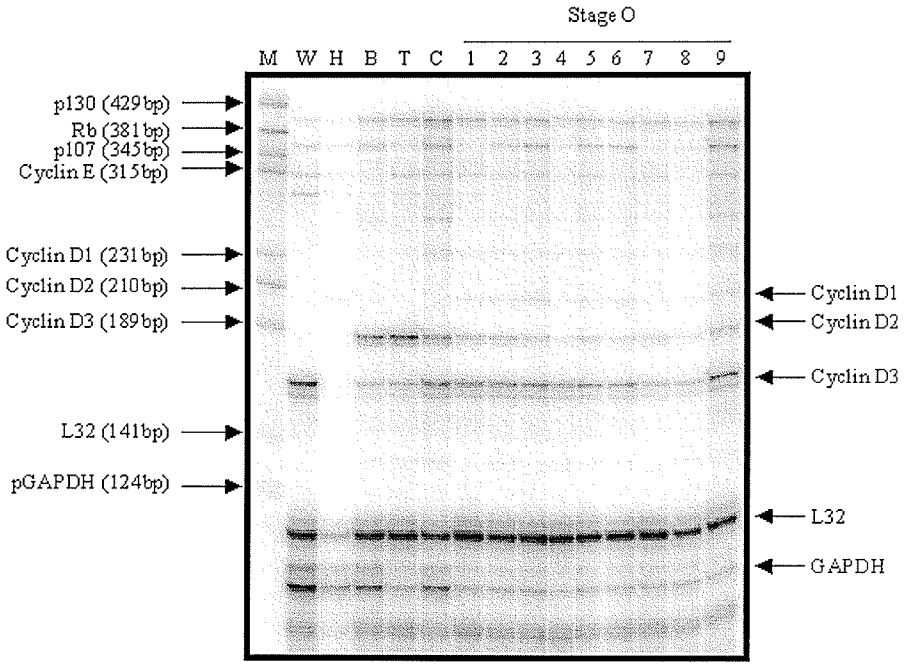


Figure 7: RNase protection assay analysis for cyclin D1, D2, and D3 mRNA in Rai stage 0 chronic lymphocytic leukemia patients.

Total RNA was isolated from WSU-CLL cell line, normal B lymphocytes, normal T lymphocytes, cord blood and CLL patients. Total RNA from WSU-CLL cell line, HeLa cell line (manufacturer supplied, Pharmingen, San Diego, California, USA), normal B lymphocytes, normal T lymphocytes, cord blood, and CLL patients was incubated with a radiolabeled probe set provided by manufacturer (Assay Cat#559660-1117, Pharmingen) overnight, electrophoresed at 40 watts constant power until leading dye band migrated 30 centimeters, dried and exposed to film. RNA samples examined were template probe set (lane M); CLL cell line WSU-CLL (lane W); fibroblast cell line HeLa (lane H); normal B lymphocytes (lane B); normal T lymphocytes (lane T); umbilical cord blood CD5+/CD19+ B lymphocytes (lane C), and CLL sample B lymphocytes (lanes 1 to 9).

Table 4: RNA expression levels of cyclin D1, D2, and D3 in normal B and T lymphocytes, and umbilical cord blood.

Cell Type*	Cyclin D1†	Cyclin D2†	Cyclin D3†	Cumulative D cyclin expression‡
B Lymphocytes	1.0	1.0	1.0	3.0
T Lymphocytes	1.8	4.1	2.8	8.8
Umbilical Cord Blood	1.3	0.8	3.5	5.6

Notes:
* All cells were non-stimulated.
† D cyclin expression based on the value for each cell type divided by the non-stimulated, primary peripheral B lymphocyte expression.
‡ Cumulative D cyclin expression is the sum of values obtained for mRNA expression for cyclin D1, D2, D3.

5.4 EXPRESSION OF CYCLIN D1, D2, AND D3 RNA IN CLL PATIENTS

Of the 43 patient samples, sufficient material was collected to study D cyclin mRNA expression in 37 patients. RNA expression was evaluated using a custom designed template kit for the RPA (Table 5, Figure 7, Figure 8, and Figure 9) (Section 3.13). In the 37 CLL samples the level of cyclin D1 mRNA expression was highly variable, with a ratio to normal peripheral B lymphocytes ranging from 0.3 to 20.1 (mean, 4.57 ± 0.638 Standard Error (S.E.)). Cyclin D2 mRNA expression was the least variable of all three D cyclins with a ratio to normal peripheral B lymphocytes ranging from 0 to 1.4 (mean, 0.51 ± 0.048 S.E.). Cyclin D3 mRNA expression was also highly variable among samples, with a ratio to normal peripheral B lymphocytes ranging from 1.7 to 15.6 (mean, 5.78 ± 0.480 S.E.).

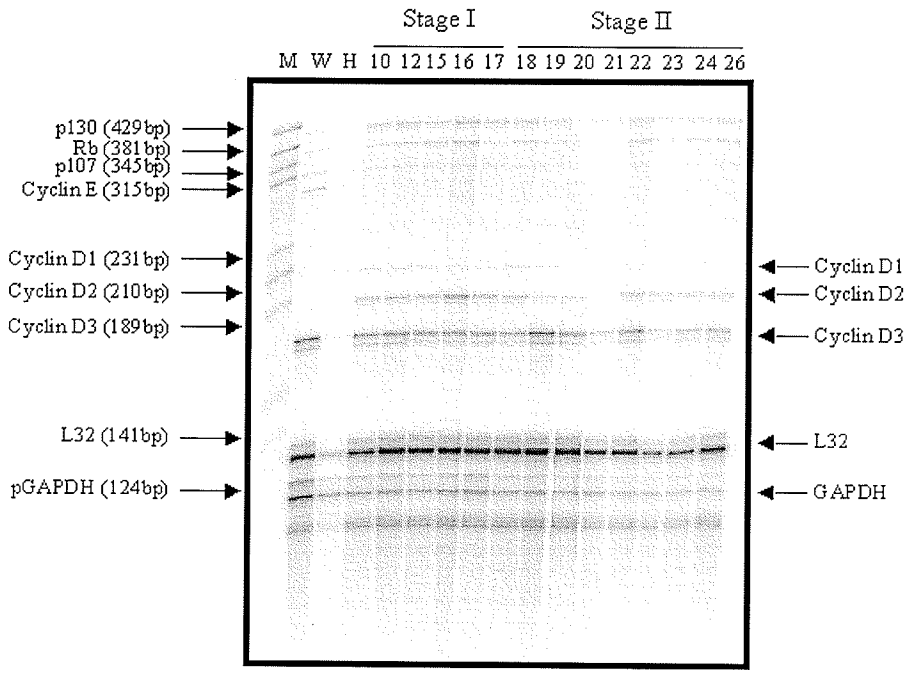


Figure 8: RNase protection assay analysis for cyclin D1, D2, and D3 mRNA in Rai stage I and II chronic lymphocytic leukemia patients.

Total RNA was isolated from WSU-CLL cell line, and CLL patients. Total RNA from WSU-CLL cell line, HeLa cell line (manufacturer supplied, Pharmingen, San Diego, California, USA), and CLL patients was incubated with a radiolabeled probe set provided by manufacturer (Assay Cat#559660-1117, Pharmingen) overnight, electrophoresed at 40 watts constant power until leading dye band migrated 30 centimeters, dried and exposed to film. RNA samples examined were template probe set (lane M); CLL cell line WSU-CLL (lane W); fibroblast cell line HeLa (lane H), and CLL sample B lymphocytes (lanes 10, 12, 15 to 26).

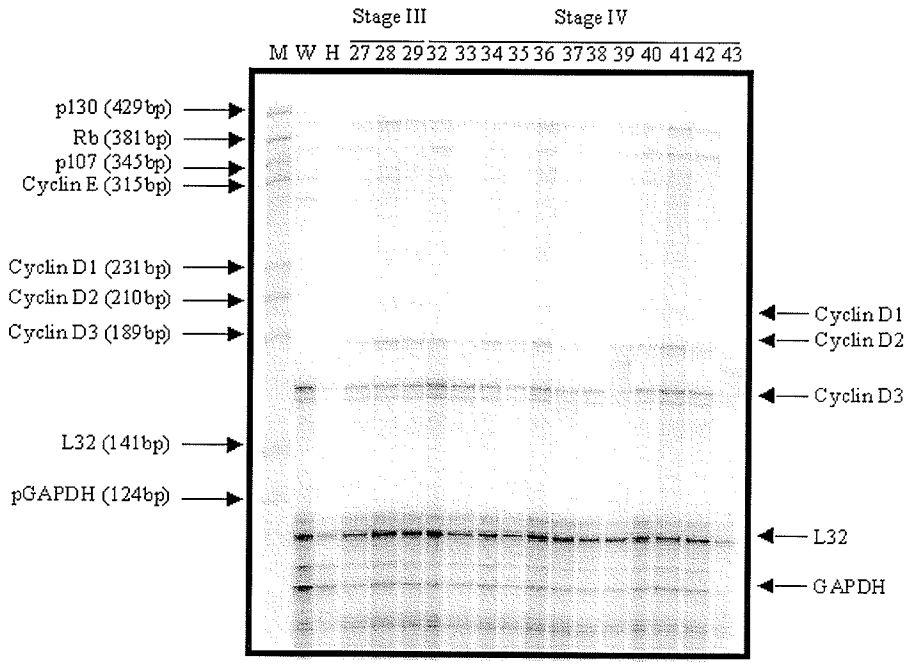


Figure 9: RNase protection assay analysis for cyclin D1, D2, and D3 mRNA in Rai stage III and IV chronic lymphocytic leukemia patients.

Total RNA was isolated from WSU-CLL cell line, and CLL patients. Total RNA from WSU-CLL cell line, HeLa cell line (manufacturer supplied, Pharmingen, San Diego, California, USA), and CLL patients was incubated with a radiolabeled probe set provided by manufacturer (Assay Cat#559660-1117, Pharmingen) overnight, electrophoresed at 40 watts constant power until leading dye band migrated 30 centimeters, dried and exposed to film. RNA samples examined were template probe set (lane M); CLL cell line WSU-CLL (lane W); fibroblast cell line HeLa (lane H), and CLL sample B lymphocytes (lanes 27 to 29, 32 to 43).

Table 5: *Cyclin D1*, *D2*, and *D3* gene rearrangement, and mRNA expression levels in chronic lymphocytic leukemia patients.

Patient	D cyclin rearrangement by Southern blot hybridization*			D cyclin mRNA expression by RNase protection assay† (fold)			Cumulative D cyclin mRNA expression‡
	D1	D2	D3	D1	D2	D3	
1	-	-	-	7.5	0.9	8.1	16.6
2	-	-	add 3.0-kb, Pst*I*	8.0	1.0	8.7	17.7
3	-	-	-	13.5	0.6	7.7	21.9
4	-	add 22-kb, Hind*III*	-	4.4	0.3	9.7	14.4
5	-	-	-	10.7	0.6	7.3	18.7
6	-	-	-	11.0	0.5	7.5	19.0
7	-	-	-	3.5	0.5	3.4	7.3
8	-	-	-	2.9	0.3	3.5	6.7
9	-	-	-	20.1	1.4	15.6	37.1
10	-	-	-	6.5	0.5	4.8	11.8
11	-	-	add 2.3-kb, Eco*RI*	sna	sna	sna	-
12	-	-	-	4.5	0.5	5.1	10.1
13	-	-	-	sna	sna	sna	-
14	-	-	-	sna	sna	sna	-
15	-	-	-	5.5	0.5	5.5	11.6
16	-	-	-	5.9	1.2	5.1	12.1
17	-	-	-	2.5	0.4	3.4	6.2
18	-	-	-	5.4	0.5	3.2	9.2
19	-	-	-	2.8	0.2	8.6	11.6
20	-	-	-	3.6	0.8	9.8	14.2
21	-	-	-	0.3	0.0	2.4	2.7
22	-	-	-	3.4	0.5	8.6	12.5
23	-	-	-	1.1	0.8	2.4	4.3
24	-	-	-	0.6	0.5	4.6	5.7
25	-	-	-	sna	sna	sna	-
26	-	-	-	2.3	0.5	5.0	7.8
27	-	-	-	1.6	0.2	4.4	6.1
28	-	-	-	2.6	0.5	2.4	5.5
29	-	-	-	4.4	0.4	3.5	8.2
30	-	-	-	sna	sna	sna	-

	D cyclin rearrangement by Southern blot hybridization*			D cyclin mRNA expression by RNase protection assay† (fold)			Cumulative D cyclin mRNA expression‡
31	-	-	-	sna	sna	sna	-
32	add 2.3-kb, Hind*III*	-	-	1.0	0.1	8.3	9.4
33	-	-	-	3.0	0.4	3.8	7.2
34	-	-	-	4.8	0.7	5.8	11.3
35	-	-	-	3.0	0.4	3.1	6.5
36	-	-	-	2.4	0.1	2.6	5.1
37	-	-	-	0.8	0.1	3.8	4.8
38	-	-	-	2.3	0.5	2.0	4.9
39	-	-	-	3.2	0.4	1.7	5.3
40	-	-	-	4.2	0.7	6.0	10.9
41	-	-	-	1.8	0.2	3.2	5.2
42	-	-	-	4.1	0.5	6.7	11.3
43	-	-	-	4.2	0.5	9.1	13.9

Notes:

* - denotes "no rearrangement detected" for *cyclin D1*, *D2*, or *D3* genes; add denotes "additional restriction fragment".

† sna denotes "sample not available for analysis"; D cyclin expression was calculated by dividing D cyclin level by G3PDH and then dividing by non-stimulated, primary peripheral B lymphocyte expression to yield an expression relative to B lymphocytes.

‡ Cumulative D cyclin expression is the sum of values obtained for mRNA expression for cyclin D1, D2, and D3.

5.5 CORRELATION BETWEEN CYCLIN D1, D2, AND D3 RNA EXPRESSION AND CLINICAL DATA IN CLL PATIENTS

Univariate analysis of cumulative D cyclin mRNA expression identified a strong negative statistically significant correlation with Rai stage using the general linear model ($p=<0.0001$). Patients with Rai stage 0 disease had higher levels of the D cyclins than those patients with Rai stage IV disease (Figure 10). In addition, analysis of the individual D cyclins found a strong negative correlation with Rai stage. Early stage patients had the highest levels of cyclin D1 ($p=0.0004$) and D3 ($p=0.023$) mRNA according to the Spearman correlation test (Table 6, Figure 11, and Figure 12). In contrast, cyclin D2 mRNA expression was not found to correlate with Rai stage (Figure 13). In addition, there was a strong negative correlation with cyclin D1 ($p=0.043$) and D3 ($p=0.015$) mRNA levels and duration of disease, according to the Spearman correlation test. In contrast, the lymphocyte doubling time correlated positively with cyclin D1 ($p=0.003$) and D3 ($p=0.003$) mRNA expression, according to the Spearman correlation test. Thus patients with a short lymphocyte doubling time (<12 months) exhibited lower cyclin D1 and D3 mRNA expression than patients with a more prolonged lymphocyte doubling time. Univariate analysis demonstrated an inverse correlation between cyclin D1, and to a lesser extent cyclin D3, mRNA levels and survival. However, the significance was lost when multivariate analysis included Rai stage, lymphocyte doubling time, duration of disease, lymphocyte count and age. With multivariate analysis the most important prognostic markers were Rai stage and lymphocyte doubling time.

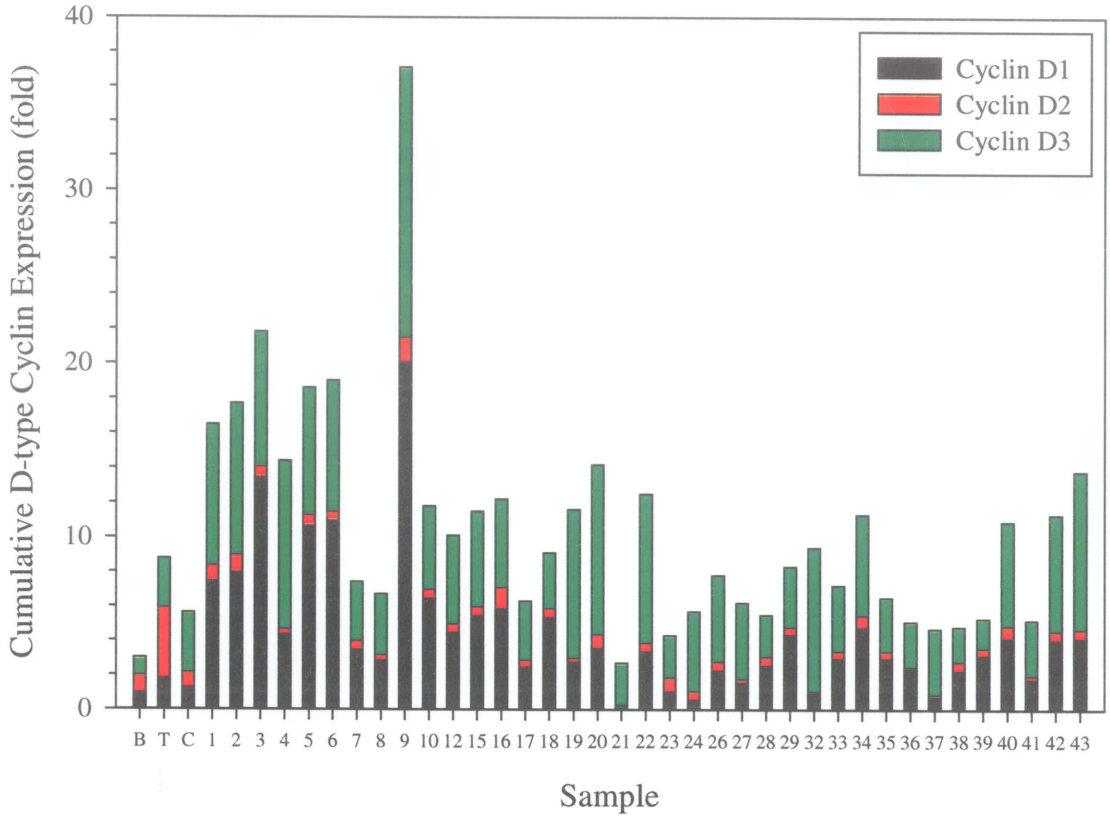


Figure 10: Graph of cumulative D cyclin expression in chronic lymphocytic leukemia patients.

Cumulative D cyclin expression was calculated by adding the individual mRNA expression levels of cyclin D1, D2, and D3 determined using the RNase protection assay as outlined in Section 3.13. RNA loading was corrected by dividing each D cyclin value by G3PDH as outlined in Section 3.13.1. This value was then divided by the non-stimulated primary peripheral B lymphocyte expression value to yield an expression relative to B lymphocytes. RNA samples examined were: normal B lymphocytes (B); normal T lymphocytes (T); umbilical cord blood (C), and CLL samples (Rai stage 0: samples 1-9, Rai stage I: samples 10,12,15-17, Rai stage II: samples 18-24, 26, Rai stage III: samples 27-29, and Rai stage IV: samples 32-43).

Table 6: Cellular levels of indicated D cyclin mRNA levels in chronic lymphocytic leukemia patients.

RNA expression was determined using the RNase protection assay as outlined in Section 3.13. RNA loading was corrected by dividing the cyclin D1 value by G3PDH as outlined in Section 3.13.1. This value was then divided by the non-stimulated primary peripheral B lymphocyte expression value to yield an expression relative to B lymphocytes. Values are grouped according to Rai stage (16).

Stage		D1	D2	D3
O	Maximum	20.1	1.4	15.6
	Minimum	2.9	0.3	3.4
	Mean	9.1	0.7	7.9
	Median	8.0	0.6	7.7
I	Maximum	6.5	1.2	5.5
	Minimum	2.5	0.4	3.4
	Mean	5.0	0.6	4.8
	Median	5.5	0.5	5.1
II	Maximum	5.4	0.8	9.8
	Minimum	0.3	0.0	2.4
	Mean	2.4	0.5	5.6
	Median	2.6	0.5	4.8
III	Maximum	4.4	0.5	4.4
	Minimum	1.6	0.2	2.4
	Mean	2.9	0.4	3.4
	Median	2.6	0.4	3.5
IV	Maximum	4.8	0.7	9.1
	Minimum	0.8	0.1	1.7
	Mean	2.9	0.4	4.7
	Median	3.0	0.4	3.8

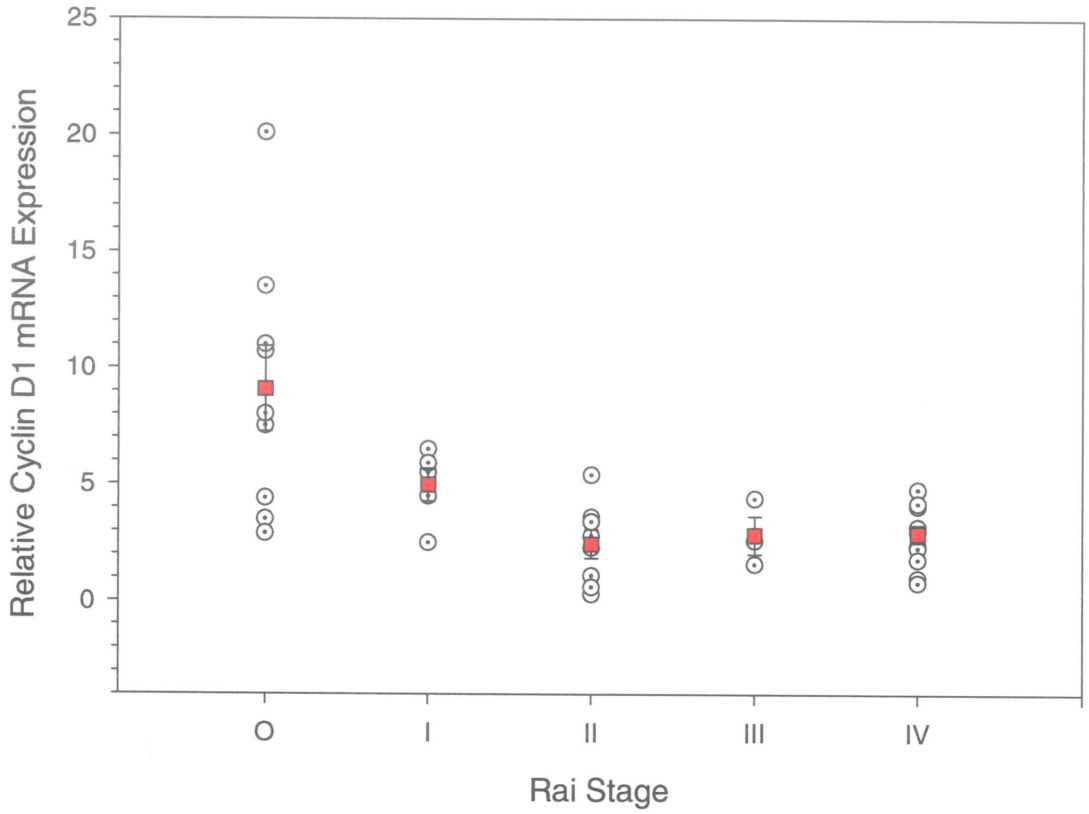


Figure 11: Comparison of the cellular levels of cyclin D1 mRNA from patients with Rai stage 0, I, II, III, and IV chronic lymphocytic leukemia to normal, non-stimulated primary peripheral B lymphocytes.

RNA expression was determined using the RNase protection assay as outlined in Section 3.13. RNA loading was corrected by dividing the cyclin D1 value by G3PDH as outlined in Section 3.13.1. This value was then divided by the non-stimulated primary peripheral B lymphocyte expression value to yield an expression relative to B lymphocytes. The samples (O) where then graphed according to Rai stage and the mean (■) levels ± S.E. for each Rai stage was calculated and shown (16).

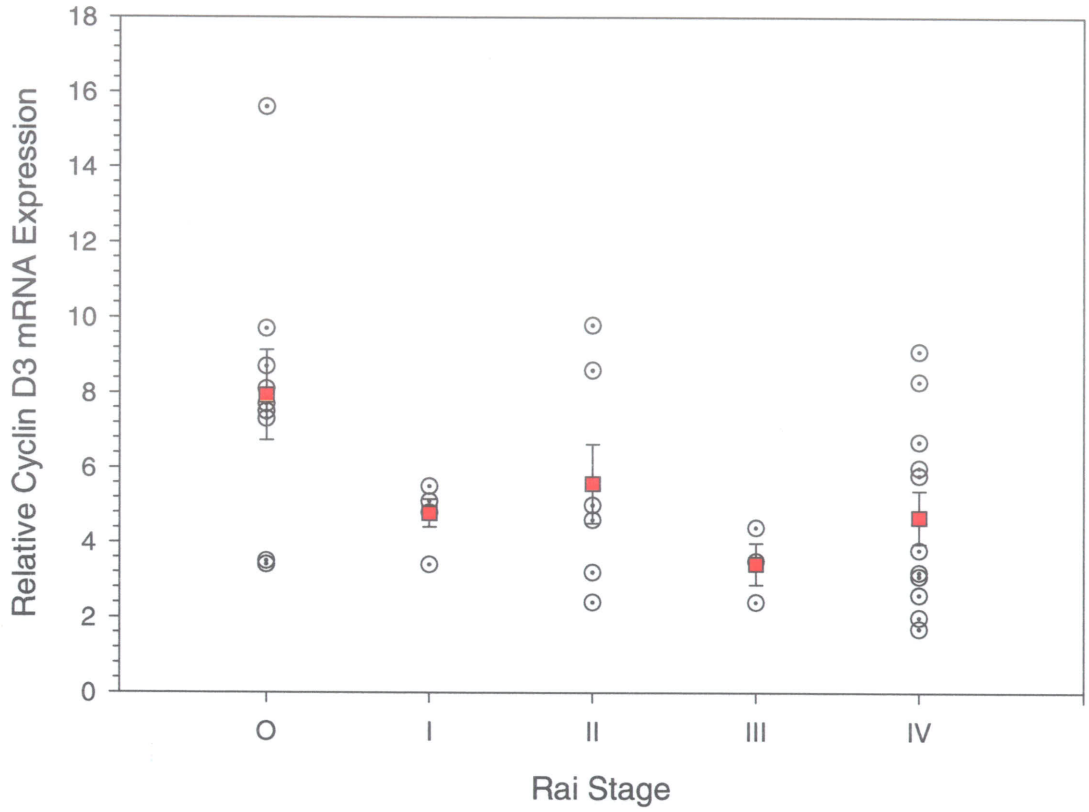


Figure 12: Comparison of the cellular levels of cyclin D3 mRNA from patients with Rai stage 0, I, II, III, and IV chronic lymphocytic leukemia to normal, non-stimulated primary peripheral B lymphocytes.

RNA expression was determined using the RNase protection assay as outlined in Section 3.13. RNA loading was corrected by dividing the cyclin D3 value by G3PDH as outlined in Section 3.13.1. This value was then divided by the non-stimulated primary peripheral B lymphocyte expression value to yield an expression relative to B lymphocytes. The samples (O) where then graphed according to Rai stage and the mean (■) levels ± S.E. for each Rai stage was calculated and shown (16).

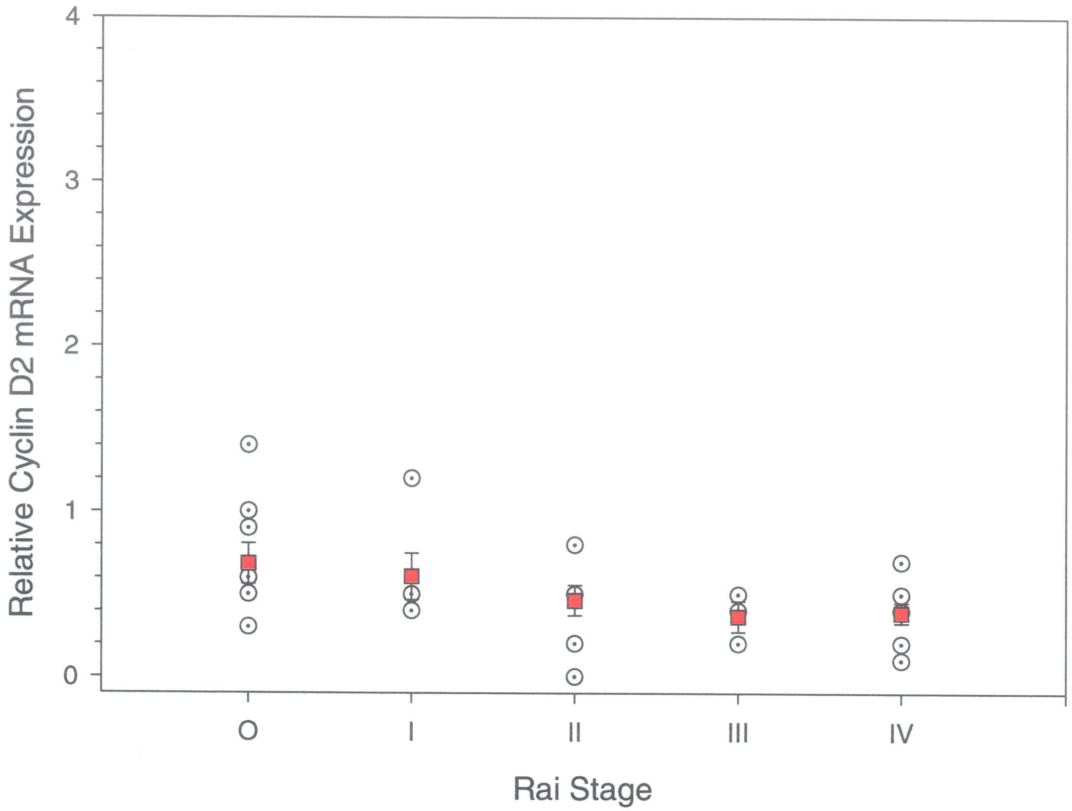


Figure 13: Comparison of the cellular levels of cyclin D2 mRNA from patients with Rai stage 0, I, II, III, and IV chronic lymphocytic leukemia to normal, non-stimulated primary peripheral B lymphocytes.

RNA expression was determined using the RNase protection assay as outlined in Section 3.13. RNA loading was corrected by dividing the cyclin D2 value by G3PDH as outlined in Section 3.13.1. This value was then divided by the non-stimulated primary peripheral B lymphocyte expression value to yield an expression relative to B lymphocytes. The samples (O) where then graphed according to Rai stage and the mean (■) levels ± S.E. for each Rai stage was calculated and shown (16).

5.6 *CYCLIN D1*, *D2*, AND *D3* GENE STRUCTURE ANALYSIS IN CLL PATIENTS

Southern blot analysis was carried out in an attempt to detect gene rearrangements which may be responsible for the altered D cyclin expression observed in CLL patients. Patient genomic DNA was digested with the restriction enzymes Eco*RI*, Hind*III* and Pst*I*. Restriction enzyme selection was based on previous reports in the literature related to the likelihood detecting a gene rearrangement (97, 156). After digestion and electrophoresis the DNA was Southern blotted onto membrane, and sequentially hybridized with *Cyclin D1*, *D2*, *D3* and *G3PDH* cDNAs. Membranes were stripped between hybridizations. The restriction fragment pattern obtained for each D cyclin hybridization was compared to normal B genomic DNA. As there is significant homology between the D cyclins the restriction fragment lengths obtained for each D cyclin was compared to previous reports in the literature. It was assumed that if the fragment pattern for normal lymphocytes digested with Eco*RI* compared well to those in the literature, or if the hybridization pattern was different between the other D cyclins, that the hybridization conditions were optimal for the all restriction enzymes used and that there was minimal or no cross-hybridization. Table 7 outlines the expected and obtained fragment lengths for normal lymphocyte DNA for each D cyclin when digested with Eco*RI* and hybridized with the indicated D-type cDNA. Any alteration seen in the patient hybridization banding pattern compared to normal lymphocyte genomic DNA was considered a gene alteration (Table 5).

Table 7: Comparison of restriction fragment lengths obtained to those reported in the literature for the D cyclins.

Gene, Enzyme used	Restriction fragment lengths reported in literature	Source of DNA	Reference	Our results
Cyclin D1, Eco*RI*	4.1-kb, 2.3-kb, 2.1-kb 4.0-kb, 2.2-kb, 2.0-kb	Human, placenta Human, breast cancer tumors/cell lines	(97) (99, 148)	4.0-kb, 2.3-kb, 2.0-kb
Cyclin D2, Eco*RI*	15-kb, 10-kb, 4.5-kb	Human, breast cancer tumors/cell lines	(99,148)	15-kb, 9.0-kb, 4.0-kb
Cyclin D3, Eco*RI*	18-kb, 10-kb, 4.5-kb, 1.7-kb	Human, breast cancer tumor/cell lines	(99, 148)	23-kb, 9.4-kb, 2.0-kb

CLL sample #32, Rai Stage IV, was identified to have a rearrangement in the *cyclin D1* gene (Figure 14) through the presence of an extra 2.3-kb fragment present in the Hind*III* digested sample. This rearrangement was not visible using the other restriction enzymes: Eco*RI* or Pst*I*.

CLL sample #4, Rai Stage 0, was identified to have a rearrangement in the *cyclin D2* gene (Figure 15) through the presence of an extra 22-kb fragment present in the Hind*III* digested sample. This sizing is only approximate as the fragment was very large and it is

difficult for agarose gels to resolve large base pair fragments. This rearrangement was not visible using the other restriction enzymes: Eco*RI* or Pst*I*.

CLL samples #2 and #11, Rai Stage 0 and I respectively, were identified as having a rearrangement in their *cyclin D3* genes (Figure 16). For sample #2 this was noted by the presence of an extra 3.0-kb band in the Pst*I* digested sample, and for sample #11 there was an extra 2.3-kb band in the Eco*RI* digested sample. In neither case was this rearrangement visible with the other restriction enzymes Eco*RI* and Hind*III* for sample #2 and Pst*I* and Hind*III* for sample #11.

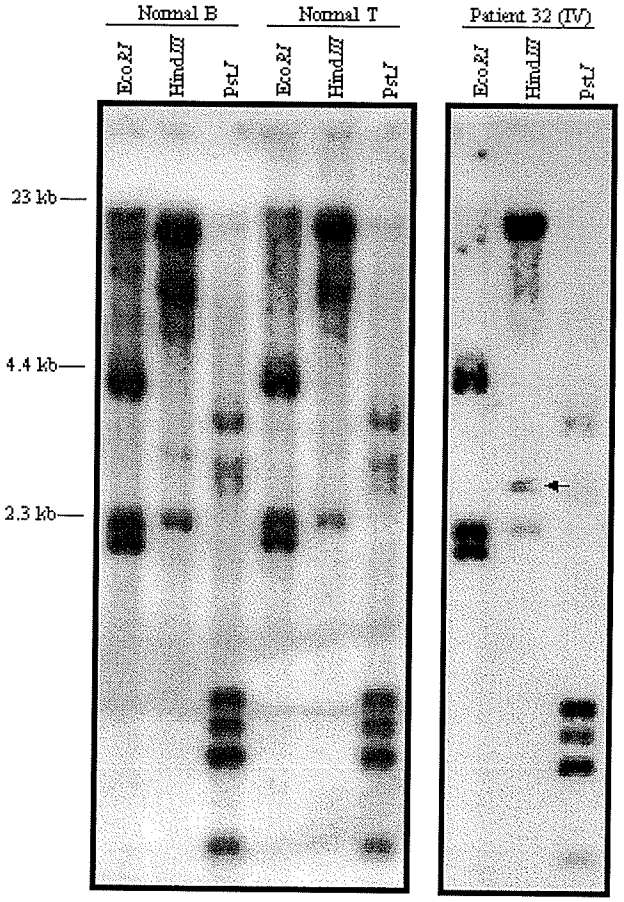


Figure 14: Amplification and rearrangement analysis of the *cyclin D1* gene in chronic lymphocytic leukemia patients.

Genomic DNA was isolated from normal B lymphocytes, normal T lymphocytes, and CD5+/CD19+ B lymphocytes from chronic lymphocytic leukemia patients. Ten micrograms of DNA was digested with indicated restriction enzymes, electrophoresed overnight at 2 volts/cm, Southern blotted and hybridized with a radiolabeled human *cyclin D1* cDNA (Table 2). Genomic rearrangements are indicated with a small black arrow (←).

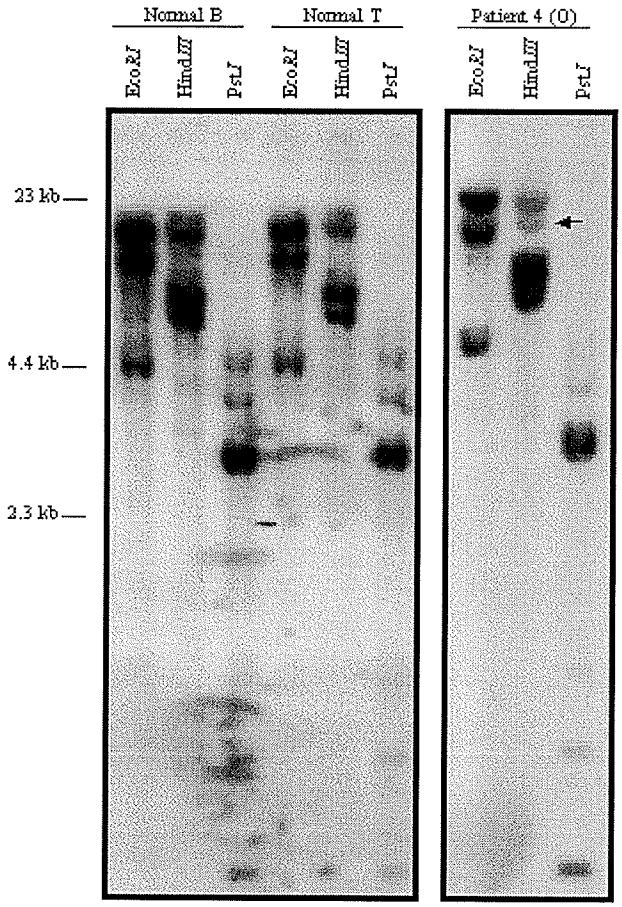


Figure 15: Amplification and rearrangement analysis of the *cyclin D2* gene in chronic lymphocytic leukemia patients.

Genomic DNA was isolated from normal B lymphocytes, normal T lymphocytes, and CD5+/CD19+ B lymphocytes from chronic lymphocytic leukemia patients. Ten micrograms of DNA was digested with indicated restriction enzymes, electrophoresed overnight at 2 volts/cm, Southern blotted and hybridized with a radiolabeled human *cyclin D2* cDNA (Table 2). Genomic rearrangements are indicated with a small black arrow (←).

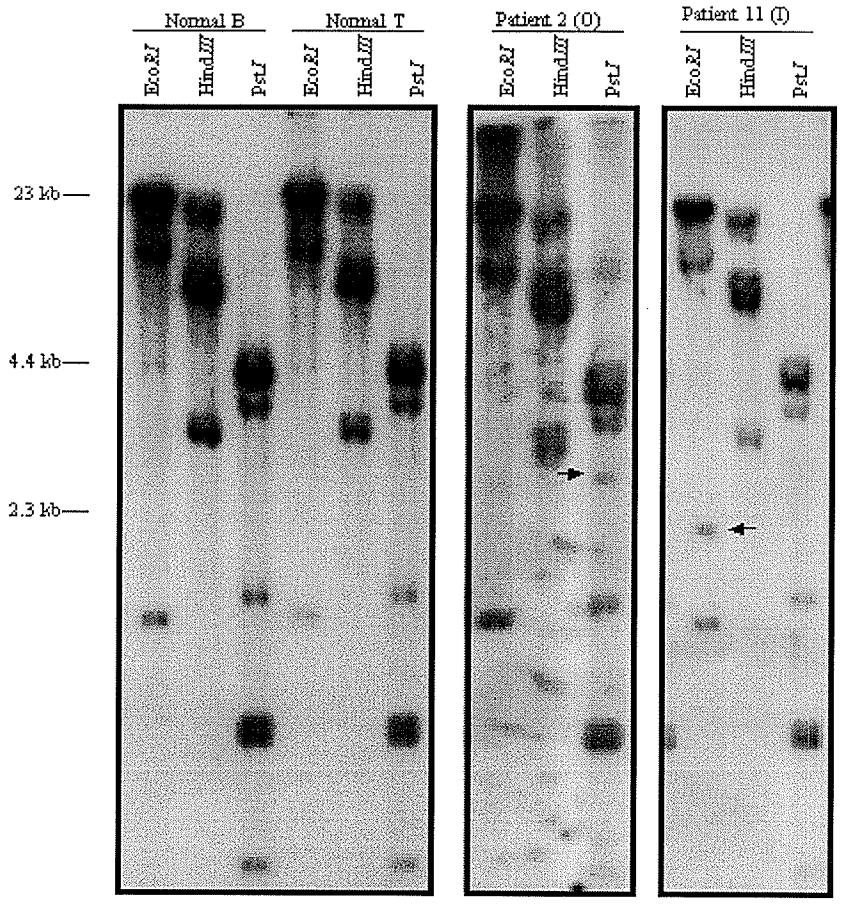


Figure 16: Amplification and rearrangement analysis of the *cyclin D3* gene in chronic lymphocytic leukemia patients.

Genomic DNA was isolated from normal B lymphocytes, normal T lymphocytes, and CD5+/CD19+ B lymphocytes from chronic lymphocytic leukemia patients. Ten micrograms of DNA was digested with indicated restriction enzymes, electrophoresed overnight at 2 volts/cm, Southern blotted and hybridized with a radiolabeled human *cyclin D3* cDNA (Table 2). Genomic rearrangements are indicated with a small black arrow (←).

5.7 *CYCLIN D1*, *D2*, AND *D3* GENE AMPLIFICATION ANALYSIS IN CLL PATIENTS

As gene rearrangements were detected only in a small subset of CLL patients, gene amplification analysis was carried out on the Southern blots in an attempt to detect alterations in the genomic levels of the D cyclins (gel images not shown). Sample genomic DNA loading inconsistencies and variability between Southern blot hybridizations were corrected through the calculation of a cyclin:G3PDH ratio for each D cyclin (Section 3.12.4.1.1.2). Statistical analysis was performed comparing the obtained ratios to those obtained for normal B lymphocytes. However, no amplification of *cyclin D1*, *cyclin D2*, or *cyclin D3* was identified.

6. DISCUSSION

In this study we have carried out a complete analysis of the D cyclins in the common lymphoproliferative disorder, CLL. Previous studies have evaluated the D cyclins in CLL, but the present study differs in that: a) a relatively large number of CLL patients were studied with sufficient follow-up to allow a statistical correlation between D cyclin mRNA levels and stage; b) considerable care was taken to ensure the purity of the isolated cell populations, and c) the use of the RPA technique allowed concomitant measurement of all the D cyclins. Previous investigations typically evaluated the D cyclins in a variety of lymphoproliferative disorders, which included a small number of patients with CLL (86, 98, 114, 119, 133, 149, 150). The lymphoproliferative disorders, of which CLL is a member, are malignancies of lymphoid tissue and can be separated by morphology, phenotype, molecular genetics and clinical course. Not unexpectedly, the D cyclin expression profile varies significantly between these disorders (150). In addition, these previous studies typically evaluated only one or two of the D cyclins leaving an incomplete picture of the expression profile of the D cyclins (114, 119, 133, 149). Finally, our study differed in that we used the RPA technique to detect D cyclin mRNA expression while previous studies have used either northern blotting or the reverse-transcriptase polymerase chain reaction (RT-PCR). One advantage of RPA over these techniques is that all the D cyclins and the two housekeeping genes can be concomitantly measured in a large number of patients and controls. This eliminates the difficulties and variability that can occur with membrane stripping that is required for northern blotting,

and the concern of DNA contamination that will influence results with RT-PCR. Secondly, the RPA is highly sensitive, allowing the detection of very low levels of D cyclin mRNA, which permits accurate comparisons of expression in different cell types, and can provide useful information even with partially degraded RNA samples. Thirdly, smaller quantities of isolated RNA can be analysed using the RPA technique than by northern blotting. This is especially important when working with samples from clinical patient samples which may be limited. Finally, and most importantly, as we have demonstrated in the WSU-CLL and HeLa cell lines, the RPA technique is highly reproducible, especially when used in conjunction with a storage phospor screen which is more sensitive than traditional enhanced x-ray film autoradiography techniques (151).

For comparative purposes, the D cyclin levels were initially measured in peripheral blood B and T cells and in cord blood lymphocytes. As only 5% of peripheral blood B cells are CD5+, cord blood lymphocytes were also evaluated. The majority of these cells are CD5+/CD19+ and are phenotypically similar to CLL cells. The normal counterpart of the CLL cell is the mantle zone lymphocyte, which is also CD5+/CD19+. However, normal lymph nodes are usually too small to detect or biopsy. Tonsils also contain mantle zone lymphocytes, but tonsils obtained following tonsillectomies are invariably infected, which alters cell characteristics. Using the RPA assay, it was determined that peripheral and cord blood B lymphocytes had similar mRNA expressions of the D cyclins except for cyclin D3 which had slightly higher levels in cord blood, with the relative levels of the individual cyclins being D2 > D3 > D1. Compared to B cells, peripheral

blood T cells expressed 1.8-, 4.1- and 2.8-fold higher levels of cyclins D1, D2 and D3 mRNA, respectively. These findings indicate fundamental differences between B and T cells and confirm the tissue specificity of D cyclin expression. While this is the first report of D cyclin mRNA expression in cord blood lymphocytes, the measured mRNA levels in normal B and T cells are at odds with previous reports using different techniques to detect mRNA expression. Delmer *et al.* (63) using northern blotting reported undetectable levels of cyclin D1 and D3, and very low levels of cyclin D2 mRNA in peripheral blood B lymphocytes. Using a similar technique, Ajchenbaum *et al.* (152) found that peripheral blood T cells had low levels of cyclin D2 and undetectable levels of cyclins D1 and D3 mRNA. Conversely, Suzuki *et al.* (98) and others (153) reported predominant cyclin D3 expression, lower levels of cyclin D2 and no detectable expression of cyclin D1 mRNA in peripheral blood T cells. This variation in results may be related to the purity of cell samples, duration of time the cells incubated prior to RNA extraction and the technique used for D cyclin analysis. As we have demonstrated, there are significant differences in the expression of the D cyclins between B and T cells, and it is likely that the levels will be different again in monocytes. Thus, any contamination of the cell population by other cell types will influence the D cyclin results. Secondly, a recent study has shown that differences in cell manipulation may contribute to significant changes in D cyclin expression, where it was found that up to a 3.7-fold change in individual D cyclin mRNA levels occurred when cells were cultured prior to analysis (154). Subsequent culturing of B and T cells after isolation resulted in a decrease in expression of cyclins D1 and D3 mRNA (cyclin D2 was not evaluated) (154). Finally, as

previously discussed the levels of the D cyclin mRNA levels may vary according to the technique used and differences in sensitivity.

Although there have been a number of reports evaluating D cyclin expression in CLL, controversy remains as to which cyclin is primarily effected. The first published report of D cyclin overexpression in CLL was made by Delmer *et al.* (63), who demonstrated using northern blotting that cyclin D2 mRNA was overexpressed in a large proportion of CLL patients, but surprisingly cyclin D1 and D3 mRNA expressions were undetectable. In contrast, Suzuki *et al.* (98) used northern blotting and Sola *et al.* (86) used RT-PCR and showed that cyclin D2 and D3 mRNA expression was increased in CLL patients, and cyclin D1 was elevated in a subgroup of patients with atypical CLL. Accounting for the differences in findings by northern blotting remain unclear but may be related to the cDNA probes used. Previous investigations have used cDNA probes of murine origin when studying human samples, perhaps accounting for the differences in results (63). Suzuki *et al.* (98) investigated D cyclin mRNA expression in normal B and T lymphocytes but did not compare these expression levels to those detected in CLL. In addition, Sola *et al.* (86) did not evaluate D cyclin expression in normal B and T lymphocytes, and it remains unknown in both these studies (86, 98) whether the D cyclin mRNA levels measured in these CLL cells differed significantly from those in normal B and T lymphocytes. Delmer *et al.* (63) may have had contaminating T cells, which express higher levels of cyclin D2 than B cells, accounting for the seemingly elevate cyclin D2 mRNA level seen in CLL. In our study, we have shown that the cyclin D1

mRNA expression in CLL cells was two-fold greater than normal B cells in 30 of 37 (mean, 4.6-fold; median, 3.5-fold) patients, while cyclin D3 mRNA expression was two-fold greater than normal B cells in 36 of 37 patients (mean, 5.6-fold; median, 5.0-fold). However, unlike Delmer *et al.* (63), we did not see an increase in cyclin D2 expression in our CLL samples. Although cyclin D3 mRNA expression was more consistently increased in the CLL samples, there was a greater variation in cyclin D1 mRNA expression and a more statistically significant correlation with clinical features. Still, both cyclin D1 and D3 mRNA levels were higher in patients with Rai stage 0 and long lymphocyte doubling times, and correlated inversely with Rai stage and survival. However, multivariate analysis for survival showed that the predictive value for cyclin D1 and D3 mRNA was primarily related to its correlative relationship with Rai stage and lymphocyte doubling time. A larger number of patients is required to determine whether cyclin D1 and D3 mRNA expression can predict progression with specific Rai stages.

To identify the mechanism responsible for the perturbed D cyclin mRNA levels in CLL we examined the CLL cells for genomic amplification and rearrangements of the three D cyclin genes. Genomic alterations of these genes are the most commonly reported mechanisms responsible for altered D cyclin mRNA levels. Some of these mechanisms may include the characteristic (11;14) translocation (85, 94), deletions (98), polymorphisms (155), promoter disruptions (156) or gene amplifications (34, 35). The (11;14) translocation is the best understood mechanism resulting in the *cyclin D1* on chromosome 11 coming under the control of the IgH gene promoter on chromosome 14.

This translocation is universally observed in MCL and is a critical diagnostic feature of this disease. Southern blotting is the standard technique to detect gene rearrangements, translocations and amplifications (99), and was thus used in the present study. However, it is limited in that nucleotide substitutions, deletions and additions can only be detected if the region affected is contained within the recognition sequence of the RE used and if the restriction fragment changes obtained are large enough to be resolved on an agarose gel. In general, Southern blotting is incapable of detecting rearrangements located more than several kilobases away from the particular probe used and deletions of less than 50 base pairs may be missed due to their small size (97). In this study, we used three common RE that had previously been used to detect D cyclin translocations (97, 156). Despite this precaution, of the forty-three patients analysed only four had identifiable D cyclin gene rearrangements (Table 5). *Cyclin D1* was rearranged in one patient (Figure 14), *cyclin D2* in one patient (Figure 15), and *cyclin D3* in two patients (Figure 16). None of these patients had rearrangements of more than one D cyclin. In addition the extra fragments are of lighter intensity other normal occurring fragments on the autoradiograph film suggesting the rearrangements may only be present in a small set of a patient's CLL cells, or that there may have been incomplete digestion of the DNA sample. While these rearrangements are clearly identifiable, further studies are required to determine if these rearrangements have occurred within one chromosome, between chromosomes, or are naturally occurring polymorphisms. Techniques allowing the study of individual cells and chromosomes such as FISH could now be used to more accurately determine the type of rearrangement that has occurred. In patients having an identifiable

D cyclin gene rearrangement there was no direct correlation with the presence of a rearrangement and a specific increase or decrease in that particular D cyclin mRNA. However, it is possible that these rearrangements may have produced RNA spliced variants that would not be detected by the RPA technique. More sophisticated studies are required to detect gene rearrangements as a cause of the perturbed D cyclin mRNA levels. Identifying the exact alteration (translocation, nucleotide substitution, deletion, or insertion) and region of the gene affected (promoter, regulatory element, coding region, or non-coding region) is important, as some alterations may have no affect (silent mutation) on gene function, and/or RNA (or protein) function. This is particularly true if the alterations are simple nucleotide substitutions, which may be naturally occurring polymorphisms.

Alternatively, it has been demonstrated that gene amplification may cause an increase in the D cyclin levels, and this has been observed for cyclin D2 in colon carcinoma cells (108) and for cyclin D1 in multiple myeloma (157). To determine if gene amplification could explain the increased levels of cyclin D1 and D3 mRNA levels in our patients, densitometry analysis of the Southern blot autoradiographs was performed. However, although no amplification of the D cyclin genes was noted using this technique, it should be emphasized that Southern blotting will only detect large genomic amplification. More sensitive techniques, such as FISH, can detect chromosomal and extrachromosomal gene amplification in individual cells that is not detectable by Southern blotting.

Other mechanisms rather than genomic may be responsible for the altered D cyclin mRNA levels in CLL. For example, it has been demonstrated in breast cancer that methylation changes of the cyclin D2 promoter results in loss of cyclin D2 expression (115). Therefore, alterations in the methylation pattern of the promoters controlling the D cyclins may result in perturbed levels of expression. Alternatively, alterations in the transcription factors that regulate D cyclin gene transcription, or an increase in mRNA stability could also result in an increase in individual D cyclin mRNA levels.

Although not evaluated in this study, it would be expected that alterations in the D cyclin mRNA levels would be paralleled by changes in the cyclin D protein levels. Ott *et al.* (133) has demonstrated by immunohistochemistry that cyclins D2 and D3 proteins were increased in CLL. However, this increase was only observed in 9% of the leukemic cells. In contrast, Levy *et al.* (149) found that the changes in cyclin D1 mRNA and protein levels were similar and this may also be true for cyclin D2 and D3 mRNA expression. However, using western blot analysis others have not observed an increase in cyclin D3 protein despite increases in cyclin D3 mRNA in CLL (63, 86, 150). Differences in the correlation between mRNA and protein expression are likely technical, as there is presently considerable variation in the quality of purchased cyclin D antibodies. Our group has used a variety of commercially available antibodies to measure D cyclin protein levels in both positive control cells lines and CLL cells. Unfortunately, the results varied according to the batch of antibody and were considered unreliable (unpublished results).

Furthermore, even if the cyclin D1 and D3 protein levels are increased in CLL it remains unknown if they are functionally active. Evaluation of the phosphorylation status of amino acid residue thr-286 on the D cyclins would determine if the D cyclins are active, as it is this residue that is responsible for control of degradation and cellular localization. Alternatively, if the D cyclins are found predominately in the cytoplasm it suggests they are functionally inactive as active D cyclin is located in the nucleus. In addition, the D cyclins are active only when bound to their CDK4/6 partner and this complex can be inhibited by the CDKIs. Therefore reduced levels of the CDK4/6 or increased levels of the CDKIs could prevent elevated D cyclins from being functional. It is unknown what the levels of CDK4/6 are in CLL, but the CDKI, $p27^{KIP1}$, has been demonstrated to be overexpressed in CLL, particularly in patients with advanced disease (136). *In vitro* studies have shown that overexpression of $p27^{KIP1}$ prevents passage through G_1/S (158) but it is unknown whether the elevated levels of the D cyclins are responsible for the increase in $p27^{KIP1}$, or if the increased $p27^{KIP1}$ has led to a compensatory increase in the D cyclins. Further studies should investigate D cyclin and $p27^{KIP1}$ expression simultaneously in CLL.

Whether the D cyclins are non-specifically increased in CLL and whether they have a biological function in this leukemia is unknown. Likewise it is unclear why the highest levels of cyclin D1, and to a lesser extent D3 mRNA are seen in patients with early stage disease. It has recently been suggested that there are two types of CLL, those in which

the leukemic cells have undergone somatic hypermutations of the variable portion of the immunoglobulin gene, and those which have not undergone hypermutations (22, 23, 24, 25). Patients with somatic hypermutations usually have Rai stage 0 disease and a more prolonged survival than those without hypermutations. Thus, it is possible that the level of D cyclin is related to the type of CLL. Whether the elevated levels of the D cyclins influence cell biology is unknown.

If the D type cyclins are functionally active it seems counter-intuitive to think that the highest levels of the D cyclins are seen in early stage patients, as it is expected that increased cyclin levels would promote cell cycle progression. As a result, it would be predicted that patients with advanced and aggressive disease would have the highest levels of the D cyclins. However, the fundamental abnormality in CLL is a defect in apoptosis (159), not an abnormality in proliferation, and it is possible that the abnormal D cyclin expression is also influencing cell survival. It has been reported by Bartkova *et al.* (160) that a cyclin D3 abundance occurs in proliferating cells reaching a maximum level as cells become quiescent at advanced stages of differentiation. As the CLL cells in patients with early stage disease have the highest levels of cyclins D1 and D3, this suggests that these cells are more differentiated than those in patients with advanced stage disease. This finding would be in keeping with the observation that the majority of CLL cells in Rai stage 0 patients have undergone somatic hypermutations of the variable portion of the immunoglobulin gene; these cells are more differentiated than the cells in patients without hypermutations which predominate in Rai stages III/IV.

In conclusion, we have demonstrated by the RPA technique that cyclin D1 and D3 mRNA expression levels are higher in CLL cells than in normal peripheral or cord blood B cells. By Southern blotting, we have identified D cyclin gene rearrangements in several of these patients, but the cause of the altered D cyclin expression in the majority of cases remains unknown. Southern blotting failed to identify amplification of the *D cyclin* genes; however, this is a relatively crude measure and further studies using FISH are required to rule out chromosomal or extrachromosomal amplification of these genes. The altered D cyclin levels are of clinical significance, in that the highest levels are seen in patients with early stage disease who have the best prognosis. However, whether the levels of the D cyclins reflect differences in the two types of CLL, and whether the altered levels influence the tumor cell biology requires further study.

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