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MOLECULAR ANALYSIS IN BURKITT'S LYMPHOMA

Johnny Ndoni Mahlangu

A research report submitted to the Faculty of Health Sciences of the University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirement for the degree of Master of Medicine in Haematology

Johannesburg, 2007

DECLARATION

I, Johnny Ndoni Mahlangu, declare that this is my own work. It is submitted to the University of the Witwatersrand, Johannesburg, for the degree of Master of Medicine in Haematology. It has not been submitted before for any degree or examination at this or any other University.

I further declare that that this research was approved by the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, with clearance certificate number M00/05/12

_____

Johnny Ndoni Mahlangu

_____day of June, 2007

DEDICATION

This dissertation is dedicated to my

Wife Valeria and daughter Mbali

For their extreme patience and understanding

During the pursuit of this research

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

Mahlangu JN, Stevens WS, Stevens G

Long range PCR analysis for the t (8;14) translocation in Burkitt's lymphoma

3rd South African Haemopoietic Stem Cell Transplant Symposium.

Cape Town, 4 th-5th February 2005

Mahlangu JN, Stevens WS, Stevens G

Long range PCR analysis for the t (8;14) translocation Burkitt's lymphoma

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Long range PCR analysis for the t (8;14) translocation in Burkitt's lymphoma

44th Annual Congress of the Federation of South African Societies of Pathology, Stellenbosch, 4th-7th July 2004

ABSTRACT

Background: The t(8;14) translocation in Burkitt's lymphoma (BL) was the first non-random cytogenetic lesion to be described in lymphoproliferative disorders. This lesion occurs in 75-85% of all BL cases. However, the breakpoints in this cytogenetic lesion are very variable and far apart such that the t(8;14) translocation is not always amenable to standard polymerase chain reaction analysis. This is mainly due to the inability of the *Thermus aquaticus (Taq)* polymerase enzyme to synthesize long DNA products. Long range polymerase chain reaction (LD-PCR) with a high fidelity polymerase enzyme mix capable of longer PCR product synthesis has recently become available. In early studies, LD-PCR appeared to be capable of amplifying the t(8;14) translocation in the majority of published sporadic Burkitt's lymphoma analyses. The utility of t(8;14) translocation LD-PCR for routine use in the diagnosis of BL in our setting has not yet been studied. The aim of this study was to establish and optimize the t(8;14) LD-PCR technique and to apply it in the retrospective analysis of all BL diagnosed in the University of the Witwatersrand teaching hospitals in a ten year period from January 1994 to December 2003.

Materials and methods: High molecular weight non-degraded DNA was extracted from control cell lines as well as stored, unstained bone marrow slides remaining after routine diagnostic workup of previously identified Burkitt's lymphoma patients. Three hundred nanograms of patient and control DNA were amplified with the LD-PCR high fidelity polymerase enzyme mix under reaction conditions which were optimized using the tissue plasminogen

activator (tPA) gene as well as known Burkitt's lymphoma cell lines as controls. Each control and patient DNA sample was amplified with tPA primers as well as four pairs of MYC/IgH primer sets. The resulting amplicons were size fractionated on an agarose gel and visualized with ethidium bromide under ultraviolet (UV) light. The fractionated DNA fragment sizes were compared to those of the t(8;14) translocation positive controls, tPA controls and known DNA molecular weight markers.

Results: One hundred and ten Burkitt's lymphoma diagnoses were made in the three teaching hospitals of the University of the Witwatersrand from January 1994 to December 2003. Bone marrow involvement by BL was present in 84 of these cases. Archival bone marrow slides were available in 74 of the 84 BL patients. Intact high molecular DNA on which the t(8;14) LD-PCR analysis could be performed was present in 41 of the 74 BL patients. In the presence of appropriate controls, an t(8;14) translocation specific product was demonstrable by t(8;14) LD-PCR analysis in only 6 of 41 BL patients.

Conclusion: In this t(8;14) LD-PCR retrospective analysis of a large number known Burkitt's lymphomas, the diagnostic yield in carefully selected patients was extremely poor. With five primer pairs required per BL sample analysis, this technique was found too labour intensive and costly in our hands making it unsuitable for routine diagnostic use. The reasons for the poor diagnostic yield remains unclear and may need to be explored in future studies. Emerging alternative techniques for the diagnosis of BL such as fluorescence in situ hybridization and microarray gene expression analyses may prove to be better diagnostic tools than LD-PCR in its current form.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AIDS-BL	AIDS-related Burkitt's lymphoma
ATCC	American Type Culture Collection, Maryland, USA
BL	Burkitt's lymphoma
BLL	Burkitt-like lymphoma
CA-46	Burkitt lymphoma cell line with t(8;14) translocation
cMYC	Cellular myelocytomatosis oncogene
DLCL	Diffuse Large Cell Lymphoma
DNA	Deoxyribonucleic acid
eBL	Endemic Burkitt's lymphoma
EBV	Epstein Barr virus
FCS	Fetal calf serum
FISH	Fluorescent in situ hybridization
HIV	Human immunodeficiency virus
HIV-BL	HIV-related Burkitt's lymphoma, same as AIDS-BL
HTLV-1	Human T-cell leukemia/lymphoma virus type 1
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
LD-PCR	Long distance polymerase chain reaction
NHL	Non-Hodgkin's lymphoma
PCR	Polymerase chain reaction
Pwo	*Pyroccocus woesei* DNA polymerase used in LD-PCR
REAL	Revised European American Lymphoma classification
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute, culture medium
sBL	Sporadic Burkitt's lymphoma
SDS	Sodium dodecyl sulphate
SMIG	surface membrane immunoglobulin
TAE	Tris Acetic acid EDTA buffer
Taq	*Thermus aquaticus* DNA polymerase enzyme
TE	Tris EDTA buffer
tPA	Tissue plasminogen activator
UV	Ultraviolet light
WHO	World Health Organization

CHAPTER 1

1.0 OVERVIEW OF BURKITT'S LYMPHOMA AND LITERATURE REVIEW.

1.1 General introduction to Burkitt's lymphoma.

Burkitt's lymphoma (BL) is a B-cell Non-Hodgkin's lymphoma (B-NHL) characterized clinically by extranodal disease presentation with rapid progression, morphologically by small, non-cleaved lymphoid malignant cells and genetically by a non-random translocation involving the c-myc gene and immunoglobulin heavy chain or light chain gene loci (Harris, Jaffe et al. 1994; Harris, Jaffe et al. 2000). This high grade B-cell NHL was first described by Dennis Burkitt in 1958 as a tumor with predilection for the jaw and with high frequency in the equatorial Africa region (Burkitt 1958). It is now well established and accepted that BL occurs frequently outside the geographic and anatomic locations of it's original description (Magrath 1991; Mwanda, Rochford et al. 2004).

The genetic hallmark of BL is exchange of chromosomal material between the myc gene on chromosome 8 and one of the immunoglobulin genes on chromosome 2, 14 or 22. This is a balanced translocation in which material is lost and gained by both chromosomes as schematically illustrated in Figures 1.1 and 1.6 below. The resulting fused DNA segments have 3'-3' and 5'-5' orientations and therefore biologically not functional. No fusion trnascript is generated from the chromosomal trnaslocation, the MYC/IgH rearrnagement can only be detected at genomic DNA level. Of the three possible translocations, namely, t(2;8), t(8;14) and t(8;22), the t(8;14) is the most common, occurring in more than 75% of all BL cases (Braziel, Arber et al. 2001; Burmeister, Schwartz et al. 2005; Dave, Fu et al. 2006).

Whilst its cytomorphology suggests a single homogenous entity, BL displays significant heterogeneity in its epidemiology, clinical phenotype and molecular genetic features. The clinical varieties of BL, which initially included only the endemic (eBL) and sporadic (sBL) forms, have been expanded in the newer classifications of lymphomas. The Revised European American Lymphoma (REAL) classification recognized at least three BL variants, namely, the endemic BL (eBL), the sporadic BL(sBL) and a provisional entity of Burkitt's-like lymphoma(BLL) (Harris, Jaffe et al. 1994). Burkitt-like lymphoma was largely a morphologic entity in contradistinction to eBL and sBL which are true clinico-epidemiological variants(Bouffet, Frappaz et al. 1991; Hecht and Aster 2000).

The REAL classification of lymphomas has now been superseded by the current World Health Organization (WHO) classification of haematolymphoid malignancies (Harris, Jaffe et al. 2000). In this classification, BL is categorized as a single clinico-pathological entity incorporating morphologic variants as well as clinical and genetic subtypes.

The morphologic variants include the classic BL morphology, the Burkitt's-like lymphoma and the BL with plasmacytoid differentiation associated with the acquired immunodeficiency syndrome (BL-AIDS). The morphologic differentiation of these entities is outlined in section 1.5 below.

The clinical and genetic subtypes include the clinico-epidemiologically defined entities of eBL, sBL and AIDS-related BL (AIDS-BL). Current research is directed towards distinguishing these entities further at molecular level. Early indications suggests that they have different pathogenetic mechanisms and molecular lesions (Bellan, Lazzi et al. 2003; Burmeister, Schwartz et al. 2005; Stein and Hummel 2007). Some of the

different locations of breakpoints in the various BL subtypes are illustrated in Figure 1.7 below. Juxtaposition of the IgH gene enhancer on chromosome 14 and c-myc gene on chromosome 8 drives the c-myc overexpression in BL.

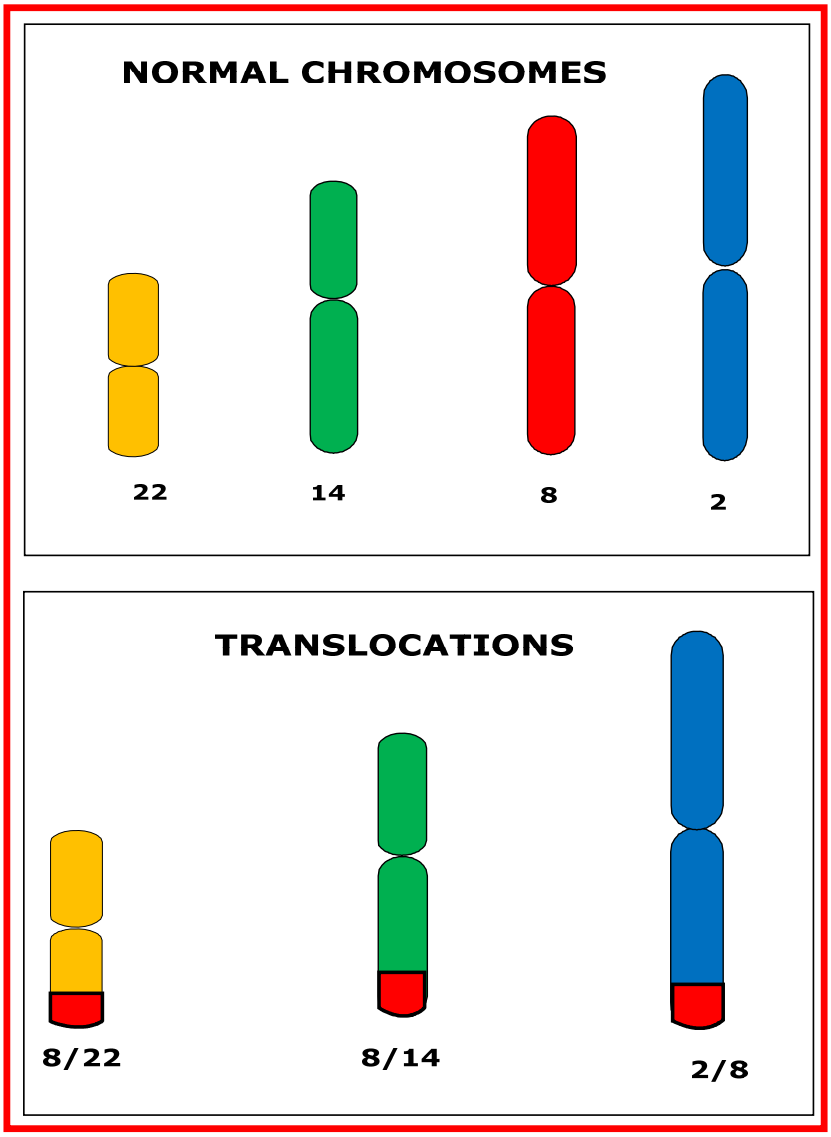


Figure 1.1 Chromosomal translocations in BL. Shown above are the three chromosomal translocations that may independently occur in Burkitt's lymphoma. In each case, the c-myc gene on chromosome 8 is translocated to the enhancer region of an immunoglobulin gene on any one of chromosome 2, 14, or 22. The distal part of these chromosomes is then reciprocally translocated to chromosome 8. The t(8;14) occurs in the majority of BL cases.

1.2 Overview of contribution of BL to pathology and oncology.

In the four decades since its first description by Dennis Burkitt, BL has had a significant influence on our understanding of the molecular basis of disease offering a number of innovative concepts linking molecular lesions with clinical phenotype. Recent molecular studies using microarray technology suggest that BL is probably going to continue to lead the way in linking molecular signatures with pathological entities(Dave, Fu et al. 2006; Hummel, Bentink et al. 2006). Some of these contributions are outlined in Table 1.1.

Table 1.1 Contribution of Burkitt's lymphoma to oncology and pathology.

Burkitt's Lymphoma Contribution	**Contributing Author(S) And Date**
1. The first human malignancy shown to be associated with a virus as a potential aetiologic agent	(Epstein, Barr et al. 1964)
2. The first tumor in which surface membrane immunoglobulins were described	(Clifford, Singh et al. 1967; Klein, Clifford et al. 1967)
3. The first human tumor potentially curable by chemotherapy alone	(Burkitt 1967)
4. The first lymphoma described to be associated with a non-random chromosomal translocation	(Manolov and Manolova 1972)
5. The first lymphoma shown to be associated with AIDS	(Chaganti, Jhanwar et al. 1983)
6. Probably the first lymphoproliferative disorder to have a microarray molecular classification	(Dave, Fu et al. 2006)

Burkitt's lymphoma was the first tumor in which surface membrane immunoglobulin(SMIG) was described and reported (Klein, Clifford et al. 1966; Klein, Clifford et al. 1967). Since this discovery, SMIG have become important targets used

in the diagnosis of B-cell malignancies. They are critical to our understanding of the pathophysiology of B-cell malignancies and provide a useful approach to their differential diagnoses (Holowiecki, Lutz et al. 1979; Naeim, Bergmann et al. 1979; Kuroyanagi and Kura 1981; Zucchetto, Sonego et al. 2005).

Burkitt's lymphoma was the first human malignancy shown to be associated with a virus as a potential aetiologic agent(Epstein, Barr et al. 1964; Epstein and Achong 1973; Epstein and Morgan 1983; Epstein 1988). Since then many viruses of the retrovirus and herpes virus families have been shown to be causally linked to a variety of human leukemias and lymphomas (Wang, Jeng et al. 2006). These included the human T-cell leukemia/ lymphoma virus type 1 (HTLV-1) and the Epstein Barr virus (EBV). HTLV-1 is causative agent for adult T-cell leukemia/lymphoma (Saxinger, Levine et al. 1985; Ambinder 1990; Ohshima, Suzumiya et al. 2002). The EBV is associated with Burkitt's lymphoma, lymphomas in immunocompromised people as well as Hodgkin's lymphoma (Ambinder 1990; Hirose, Sugai et al. 1999; Crawford 2001). The discovery of human herpes virus type 8 or Kaposi's Sarcoma herpes like virus (KSHV) has led to the identification of a range of virus-associated lymphoproliferative diseases (Ablashi, Easton et al. 1976; Hampl, Conrad et al. 1991). These include the body cavity based lymphomas as well as other lymphomas in the immunocompromised individuals (Nador, Cesarman et al. 1996; Gaidano, Pastore et al. 1997; Arguello, Sgarbanti et al. 2003).

Burkitt's lymphoma was the first NHL to be described in patients with acquired immunodeficiency syndrome (AIDS) (Chaganti, Jhanwar et al. 1983). Recent data suggest that hepatitis C virus infection is also associated with an increased incidence of lymphoma, whereas data relating to SV40 remains controversial (Franceschi,

Polesel et al. 2006; Lizardi-Cervera, Poo et al. 2006; Nieters, Kallinowski et al. 2006; Visco, Arcaini et al. 2006).

The first lymphoid tumor in which a non-random chromosomal translocation has been linked to be involved in the tumor pathogenesis was BL (Manolov and Manolova 1972; Zech, Haglund et al. 1976). To date a number of these non-random translocations have become indispensable tools in the diagnosis, grading, prognostication and therapeutic outcome evaluation (minimal residual disease) in B-cell and non-B-cell malignancies (Rabbitts 1983; Nowell and Croce 1986; Haluska and Croce 1987; Gauwerky and Croce 1993; Glassman, Hopwood et al. 2000).

BL was the first human tumor shown to be curable by chemotherapy alone (Burkitt 1967; Clifford, Singh et al. 1967; Rabbitts 1983; Nowell and Croce 1986; Haluska and Croce 1987; Gauwerky and Croce 1993; Glassman, Hopwood et al. 2000). Although most human malignancies remain incurable with chemotherapy alone, the BL experience has kept this treatment modality at the forefront of therapeutic intervention in malignant disorders. With this background, it is therefore not unreasonable to speculate that this B-NHL is likely to have considerable impact in our understanding of oncology and biomedical research in the years to come.

1.3 Clinico-epidemiological features of Burkitt's lymphoma.

1.3.1 General overview of clinico-epidemiological features.

In BL, epidemiology and clinical features are strongly linked and appear to have described through a number of phases in the last four decades. Three distinct clinico-epidemiologic patterns are seen in BL and these have evolved sequentially over time. They are underpinned by common morphologic and pathobiological features. Some of

the clinico-epidemiologic differences in the various subtypes of BL are summarized in Table 1.2 below.

The AIDS associated BL (AIDS-BL) is largely a disease of adults involving both nodal and extranodal sites (Diebold, Raphael et al. 1997; Davi, Delecluse et al. 1998; Spina, Tirelli et al. 1998; Spina, Simonelli et al. 2005). The eBL and sBL present primarily in childhood as extranodal disease with jaw involvement and abdominal presentations respectively (Sariban, Donahue et al. 1984; Magrath 1991). Bone marrow involvement at presentation occurs more frequently in the AIDS-BL and sBL subtypes compared to the eBL (Janus, Edwards et al. 1984; Magrath 1991; Diebold, Raphael et al. 1997; Davi, Delecluse et al. 1998). Central nervous system involvement occurs in up to a third of patients in all three subtypes of BL and is associated with poor outcome following chemotherapeutic intervention (Sariban, Edwards et al. 1983; Saurina, Ramirez de Arellano et al. 2001).

1.3.2 Clinico-epidemiological features of eBL.

In the first epidemiologic description in 1958, BL was thought to be a highly aggressive malignancy occurring exclusively in young African children and geographically confined to the equatorial Lymphoma belt (Burkitt 1969; Burkitt 1971). In this geographic region, which was defined as an area approximately 15˚ on either side of the equator, the incidence of eBL was high, ranging from 5 to 15 per 100 000 children younger than 6 years per year((Burkitt 1969). Generally the tumor did not occur in areas where the mean temperature at any given time was less than 16°C , the altitude was less than 1800m and annual rainfall less than $50cm^3$ (Burkitt 1969; Burkitt, Williams et al. 1969). These climatic and geographic descriptions may appear to have little relevance to our current understanding of eBL, but were pivotal

observations which incriminated a biological organism as a potential causative agent of BL. The association of EBV and BL was pursued and ultimately proven on the basis of these original important epidemiological observations(Nilsson and Ponten 1975; Kaplan, Goodenow et al. 1979).

Table 1.2 Clinico-epidemiological features of BL subtypes.

	Endemic BL (eBL)	**Sporadic BL (sBL)**	**AIDS related BL (AIDS-BL)**
Geographic distribution	Around Equatorial Africa	Away from equatorial Africa	Mirrors the HIV pandemic distribution
Incidence	5-15/100 000/yr	2-3/100 000/yr	25-35% of B-NHL
Age	Children>adults	Children>adults	Adults>children
Lymph node involvement	Rare	Rare	Common
Jaw involvement	Common	Uncommon	Uncommon
Abdominal Involvement	Common	More common than jaw	Common
Bone marrow involvement	10%	30%	30%
CNS involvement	20-30%	20-30%	20-30%

The annual incidence of eBL is 2.2 to 3.8 cases per 100 000 inhabitants and the peak age is 5-8 years in central Africa and 4-5 years in north Africa (Hecht and Aster 2000). In the early studies, BL was found to be commoner in boys with a male to female ratio of 2:1. This sex distribution has shown little change over time (Aboulola, Boukheloua et al. 1985; Ong, Xue et al. 2001; Mwanda 2004; Mwanda, Whalen et al. 2005).

The clinical features of eBL are distinct as it presents largely as a jaw mass with a frequency as high as 60% in some studies (Davies, Elmes et al. 1964; Ong, Xue et al. 2001; Mwanda 2004; Mwanda, Whalen et al. 2005). The precise cellular origin of the jaw mass is unclear, but typically there is involvement of the developing premolar and molar teeth. The maxillae are involved twice as often as the mandibles with orbital involvement relatively uncommon (Magrath 1991; Muwakkit, Razzouk et al. 2004; Mwanda 2004; Haralambieva, Boerma et al. 2005). Although not often emphasized, abdominal involvement in eBL is as common as jaw involvement, occurring in 58% of cases (Aboulola, Boukheloua et al. 1985). The retroperitoneum, mesentery and omentum are more commonly involved than the actual bowel. Other organs such as the kidneys, ovaries, pancreas and adrenal are also frequently involved (Janus, Edwards et al. 1984; Magrath, Janus et al. 1984). Bone marrow involvement is present in 12% of cases and central nervous system involvement in 30% of cases (Sariban, Edwards et al. 1983; Nkrumah, Neequaye et al. 1985). Peripheral lymph node involvement is very uncommon, comprising less than 1% of cases at clinical presentation (Magrath 1991; Mwanda 2004).

1.3.3 Clinico-epidemiological features of sBL.

Following the original description of eBL in Africa, several pathologists who were familiar with the eBL histology, started to recognize tumors which were histologically similar to eBL but occurred in Europe and North America (Dorfman 1965; O'Conor, Rappaport et al. 1965; Wright 1966). This sporadically occurring BL variant with distinct clinico-epidemiological features was therefore called sporadic BL (sBL).

The incidence of sBL is much lower than eBL, with reported rates of 2-3 per million of the population per year (Magrath 1997; Spina, Tirelli et al. 1998). Similar to eBL, sBL is a disease of children and is commoner in boys compared to girls.

Unlike eBL, sBL originates in Peyer's patches or mesenteric lymph nodes and then malignant lymphocytes will home into the follicular B cell zones of abdominal and peripheral lymph nodes. It is therefore not surprising that the most frequent clinical presentation is abdominal obstruction or discomfort as a result of a large abdominal mass (Levine, Connelly et al. 1985; Patton, McMillan et al. 1990; Carbone, Canzonieri et al. 2000). Jaw tumors characteristic of the majority of eBL cases, are present in only 15% of children with sBL (Patton, McMillan et al. 1990). Bone marrow involvement occurs early and is three fold commoner in sBL than eBL. Consequently, sBL has a greater propensity for leukemic dissemination. The leukemia associated with sBL is of the L3 morphology and is associated with a poor prognosis. Central nervous system involvement is common occurring in over 80% of children with a median survival of 6 months following treatment with conventional chemotherapy (Cairo, Sposto et al. 2003; Jacobsen and LaCasce 2006).

1.3.4 Clinico-epidemiological features of AIDS-BL.

In the era of the HIV pandemic, the frequency of several NHLs was noted to be increasing. Burkitt's lymphoma was the first type of B-NHL reported in HIV infected individuals in 1982(Doll and List 1982; Ziegler, Drew et al. 1982). Subsequent clinico-epidemiological studies, however, have demonstrated that BL is less common than diffuse large B cell non-Hodgkin's lymphoma and accounts for 30% of NHL associated with AIDS (Beral, Peterman et al. 1991). Burkitt's lymphoma associated with AIDS may be the first manifestation of the disease in a significant number of cases and this

has been shown to develop in the presence of relatively well preserved CD4 counts (Boyle, Swanson et al. 1990; Beral, Peterman et al. 1991; Roithmann, Tourani et al. 1991; Carbone, Gloghini et al. 1995). As HIV infected individuals live longer as a result of Highly active anti-retroviral therapy (HAART), the number of AIDS related NHLs , including BL, is expected to rise several fold.

The clinical presentation of AIDS-BL is often extranodal with gastrointestinal tract, bone marrow and liver involvement being common (Spina, Tirelli et al. 1998; Carbone and Gloghini 2005; Lim, Karim et al. 2005; Navarro and Kaplan 2006).

1.4 The Changing morphologic classification of Burkitt's lymphoma.

Our understanding of pathology and molecular biology of BL has been changing largely as a result of innovations and advances in diagnostic technology but also due to acquisition of new knowledge and molecular insight of pathology of lymphoproliferative disorders. Over the last four decades, the BL morphologic classification has evolved from simple morphologic and histopathologic descriptions to characterizations which include aspects of immunophenotypic, cytogenetic, and molecular analysis of the disease.

Changes in Burkitt's lymphoma classification are reflected in each of the major international classifications of lymphoproliferative disorders. The six major classifications included the Rappaport, the Lukes and Collins, the International Working Formulation, the Kiel, the Revised European American Lymphoma (REAL) and more recently the World Health Organization classification. The Burkitt's lymphoma description in each of the major classifications of lymphoproliferative

disorders is briefly outlined below. The changing descriptive terminology of BL in the various classifications are also summarized in table 1.3 below.

1.4.1 Rappaport classification.

In the original Rappaport classification of NHL of 1966, BLs were described as "undifferentiated cell type" because of their non-resemblance to either lymphoid or histiocytic differentiated cells (Rappaport 1966; Byrne 1977). In 1967, an expert committee commissioned by the WHO, convened to establish whether BL was a clinical syndrome, a clinicopathologic syndrome or a pathological entity. The committee established that BL was a separate pathological entity and suggested the term "malignant lymphoma, undifferentiated, Burkitt's type" (Carbone, Berard et al. 1969). The term "undifferentiated" was used to denote a morphological rather than an immunobiologic concept. This term had no immunological connotation at that stage as most immunological markers of differentiation were not yet identified. The Rappaport classification was superseded by the Lukes and Collins classification.

1.4.2 Lukes and Collins classification.

Lukes and Collins in their lymphoma classification of 1974, suggested that BL was of follicular centre origin and proposed the term "small non-cleaved lymphoma" to define this entity (Lukes and Collins 1974). At that stage it was well established that BL was of lymphoid lineage and not truly undifferentiated as previously suggested in the Rappaport classification. The small non-cleaved terminology first introduced in this classification, has remained the fundamental classic morphologic description for BL.

Table 1.3 BL description in the various lymphoma classifications†

LYMPHOMA CLASSIFICATION	BURKITT'S LYMPHOMA DESCRIPTION	YEAR OF DESCRIPTION
Rappaport classification	Malignant lymphoma, Undifferentiated, Burkitt type	1966
Lukes and Collins classification	Small non-cleaved lymphoma	1974
International working formulation classification	Small non-cleaved lymphoma, Burkitt's type	1982
Kiel classification	B-lymphoblastic lymphoma, small non-cleaved cell type	1988
Revised European American classification	Mature B-cell lymphoma, small non-cleaved, Burkitt	1994
World Health Organization	Mature B-cell lymphoma, classic Burkitt	2001

† Only descriptions of classic BL are included in this table. Atypical subtypes and variants excluded.

1.4.3 International working formulation (IWF) classification.

The International working formulation was a National Cancer Institute sponsored classification established largely for clinical usage (1982; Cohen 1983). In this classification, BL retained the term "small non-cleaved lymphoma" which was introduced in the Lukes and Collins classification. The small non-cleaved lymphoma (SNCL) group of the IWF included two main pathological variants: Burkitt's and non-Burkitt's lymphomas. The case was called Burkitt's type when it satisfied the WHO expert committee criteria.

1.4.4 Kiel Classification.

The original Kiel classification (Kruger, Grisar et al. 1981) included BL in the general B-lymphoblastic category whereas the updated version (Stansfeld, Diebold et al. 1988) put BL as a morphologically and immunophenotypically distinct entity. No changes in the morphologic description of BL were made in this classification.

1.4.5 Revised European American Classification (REAL).

The International Lymphoma Study Group proposed the REAL classification of lymphoid malignancies which took into account morphological, immunological, genetic and clinical features of malignant lymphoproliferative disorders(Harris, Jaffe et al. 1994). In this classification, classic BL as well as a provisional entity of Burkitt's- like lymphoma were recognized. The Burkitt's-like lymphoma category referred to non-Burkitt's type, small non-cleaved lymphoma with plasmablastic differentiation. This morphologic variant is recognized by its cellular heterogeneity, variation in nuclear size and presence of large nucleoli as illustrated in Figure 1.2 (Hui, Feller et al. 1988).

1.4.6 The World Health Organization (WHO) Classification.

The current WHO classification of lymphoid malignancies recognizes classical BL as well as BL variants (Harris, Jaffe et al. 2000). The variants include the BL with plasmablastic differentiation and atypical Burkitt's/Burkitt's-like variant which were included as a provisional entity in the REAL classification. The classical BL description of small non-cleaved cells has remained unchanged in this classification (Illustrated in Panel A of Figure 1.2).

1.5 Morphologic features of Burkitt's lymphoma.

As discussed, the main morphologic entities are classic BL, BL with plasmacytoid differentiation as well as atypical BL/ Burkitt's like lymphoma. These variants are illustrated in Figure 1.2 and described below.

1.5.1 Classic Burkitt's lymphoma.

The classic BL is a homogenous proliferation of medium-sized lymphoid cells with round, non-cleaved nuclei, multiple centrally located nucleoli and a clumped chromatin pattern (Berard 1985; Harris, Jaffe et al. 2000). They have deeply basophilic cytoplasm with abundant lipid vacuoles. On histological sections, the cells are monomorphic with multiple basophilic nucleoli and show a cohesive growth pattern. Histological sections show the typical "starry-sky" appearance due to the presence of numerous benign macrophages that have ingested apoptotic tumor cells. The nuclear size of the tumour cells is the same as that of admixed starry-sky histiocytes. Classical BL morphology is typically observed in the eBL and in a high proportion of sBL occurring in children (1969; Harris, Jaffe et al. 2000).

1.5.2 Burkitt's lymphoma with plasmacytoid differentiation.

Morphologically this variant is comprised of medium-sized cells with eccentric basophilic cytoplasm containing lipid vacuoles. The nucleus displays some degree of pleomorphism in shape and size and often has a single central nucleus (Harris, Jaffe et al. 1994). Plasmacytoid differentiation is more common in immunodeficiency states (Hui, Feller et al. 1988). Unlike classic BL which displays some degree of tumor

cell homogeneity, plasmacytoid differentiated BL shows tumor size heterogeneity with with variation in nuclear size and shape.

1.5.3 Atypical BL/ Burkitt's-like lymphoma.

This variant comprises medium sized cells with a greater degree of pleomorphism of size and shape as compared to the classical BL. Nucleoli are more prominent and fewer in number (Felman, Bryon et al. 1985; Harris, Jaffe et al. 1994). The tissue histological features are the same as those of classic BL (Raphael, Gentilhomme et al. 1991).

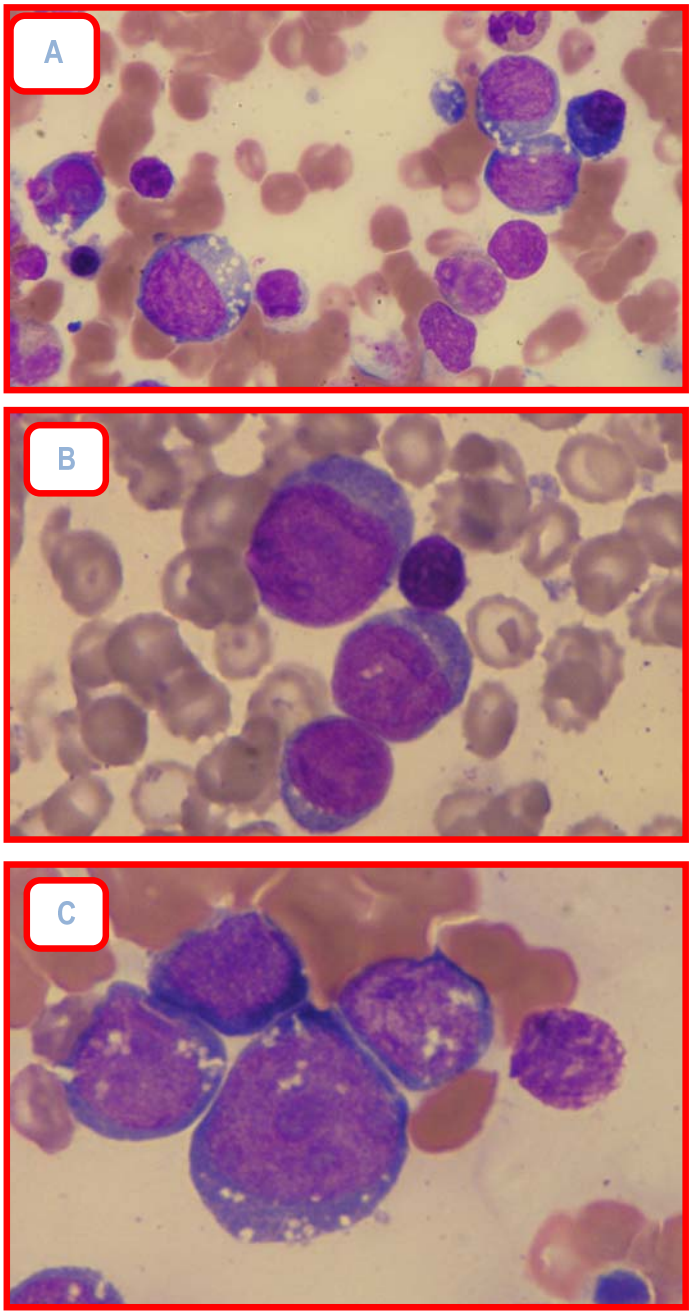


Figure 1.2 Morphologic variants of BL. Panel A shows the Classic BL morphology, Panel B is the plasmacytoid variant of BL and Panel C is Burkitt's-like variant. Please refer to section 1.5 in the text for description of these BL morphologic variants. Panel A is patient BL 4, panel B is patient BL12 and Panel C is patient BL 22(Please refer to results section, table 4.2 for further details on these patients)

1.6 Flow cytometric features of BL.

Surface marker analysis using flow cytometry has revolutionized and enhanced our understanding of many lymphoproliferative disorders including BL. Burkitt's lymphoma is now immunophenotypically well described with characteristic light scatter, phenotypic expression and DNA Ploidy features**.** The tumor cells express membrane IgM, light chain restriction and B-cell antigens. These immunophenotypic features are illustrated in Figures 1.3 and 1.4. In the routine diagnostic setting, these immunophenotypic findings are not pathognomic of BL but should always be interpreted in the context of the clinical presentation and morphologic findings of the patient.

1.6.1 Light scatter analysis.

BL can be identified on forward and side light scatter flow analysis as small to intermediate sized cells located in the lymphoid gate. These tumor cells are slightly larger and more complex than small mature lymphocytes (Magrath, Pizzo et al. 1980; Dive, Gregory et al. 1992; Nelson, Treaba et al. 2006). Burkitt's lymphoma with a leukemic phase may show extension of the tumor cell population towards the blast gate reflecting the tumor cell immaturity. This extension is shown in Panel A of Figure 1.3.

1.6.2 Phenotypic expression.

The postulated cell of origin for BL is the germinal centre B-cell (Hui, Feller et al. 1988; Jack, Barrans et al. 2005) and therefore BL express BCL-6. The diagnostic phenotype of BL has been defined as a combination of CD10 expression in

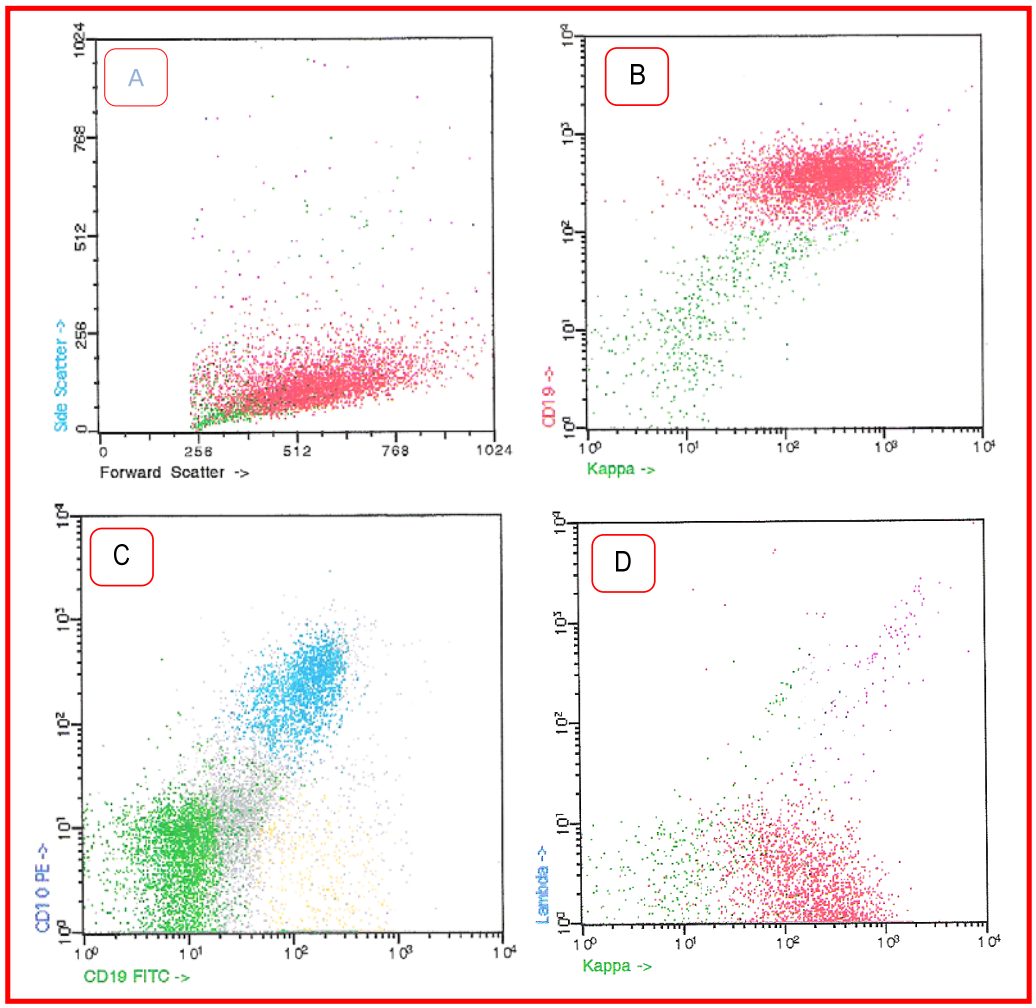


Figure 1.3 Flow cytometry features of BL.

Panel A shows the light scattergram of a bone marrow analysis with extensive BL tumor cells involvement (96%)(indicated by red dots). Note the variable size range of the tumor cells and a few residual T-cells shown in green dots. In Panel A there is virtually no residual normal haemopoiesis. Panel B is the analysis of a lymph node showing CD19 and Kappa light chain co-expression on BL tumor cells (red dots). Panel C demonstrates a bone marrow with 30% tumor cell involvement showing CD19/CD10 co-expression in blue. The green and grey dots are background T-cells and granulocytes respectively. Panel D shows analysis of a lymph node with BL tumor cells (red dots) with classic kappa light chain restriction. The flow cytometric data was acquired using a FACSCalibur flow cytometer with Paint-A-gate software (Becton Dickinson, Fullerton, CA, USA)

association with pan B-cell markers and light chain restriction. (Hui, Feller et al. 1988; Harris, Jaffe et al. 1994). The B-cell markers commonly demonstrable are CD19, CD20 and CD22. Burkitt's lymphoma does not express CD5, CD23 or BCL-2 which distinguishes it from Chronic Lymphocytic Leukemia and other NHLs. Expression of CD21 has been described in the endemic form but is largely absent from the sporadic cases (Magrath, Janus et al. 1984). Burkitt's lymphoma typically shows co-expression of CD10 and light chain expression. The differential diagnosis for this includes Follicular Cell lymphoma, B-Acute Lymphoblastic Lymphoma and Diffuse Large Cell Lymphoma. Ki-67, which is a cellular activation marker used in histopathology, is usually very high, present in nearly 100% of the BL tumor cells (Hui, Feller et al. 1988; Harris, Jaffe et al. 1994). This finding is consistent with the high grade nature of this tumor.

1.6.3 DNA Ploidy analysis.

In Burkitt's lymphoma, most of the tumor cells are active in cell cycle. It is therefore not surprising that the S-phase fraction is almost invariably very high, typically in the region of 20-40% (Christensson, Lindemalm et al. 1989; Pinto, Cabecadas et al. 2003). This feature is often critical in distinguishing this lymphoma from other high grade lymphomas such as Diffuse Large cell lymphoma which usually have low S-phase fractions (Pinto, Cabecadas et al. 2003). Most of the BL cells are diploid in chromosome composition although occasional aneuploid forms are seen (Wilson, Mian et al. 1987; Nelson, Treaba et al. 2006; Wu, Borowitz et al. 2006). An example of classic BL with high S-phase fraction and aneuploid DNA composition is shown in figure 1.4 below.

1.7 Genetic and molecular features of BL.

1.7.1 Chromosomal abnormalities in BL.

All the clinico-epidemiological variants of BL, including the eBL, sBL, AIDS-BL and ALL L3 share a common genetic background. They are characterized by balanced chromosomal translocations involving the c-myc gene locus and one of the immunoglobulin chain loci. In 80% of BL, the translocation partner of c-myc is the immunoglobulin heavy chain (IgH) giving rise to the t(8;14)(q24;q32) translocation (Lai, Fenaux et al. 1989; Harris, Jaffe et al. 1994; Dave, Fu et al. 2006). The remaining 20% of BL involve c-myc and either the kappa light chain locus (in 15% of cases) or the lambda light chain locus (5%) (Bernheim, Berger et al. 1981) giving rise to the t(2;8)(q12;q24) and t(8;22)(q24;q11) variant translocations. The presence of t(8;14) or its variants are very useful in supporting a diagnosis of BL but are thought to be very poor predictors of disease outcome on their own (Glassman, Hopwood et al. 2000). It is only when these aberrations occur in the context of other additional chromosomal abnormalities that they are predictive of poor outcome (Glassman, Hopwood et al. 2000; Lones, Sanger et al. 2004).

Cytogenetic analysis is hampered in about 10% to 20% of the general specimens by a low mitotic index or poor quality of metaphase spread (Offit, Jhanwar et al. 1991; Siebert, Matthiesen et al. 1998). The scattering of breakpoints on 8q32 and 14q32 necessitates the use of multiple probes and sequence specific primers which render techniques such as Southern blotting time consuming and difficult to implement for routine diagnosis. FISH overcomes certain limitations posed by cytogenetics and molecular analysis and enables detection of chromosomal abnormalities independent of cycle status of the cells.

To detect chromosomal breakpoints by FISH, various approaches have been reported including the use of 1)chromosome library probes, 2) YACs, 3) chromosome specific centromeric probes and 4) coloured cosmid probes flanking the potential breakpoint(Lishner, Kenet et al. 1993; Tbakhi, Pettay et al. 1998; Glassman, Hopwood et al. 2000; Zunino, Viaggi et al. 2000; Szeles 2002).

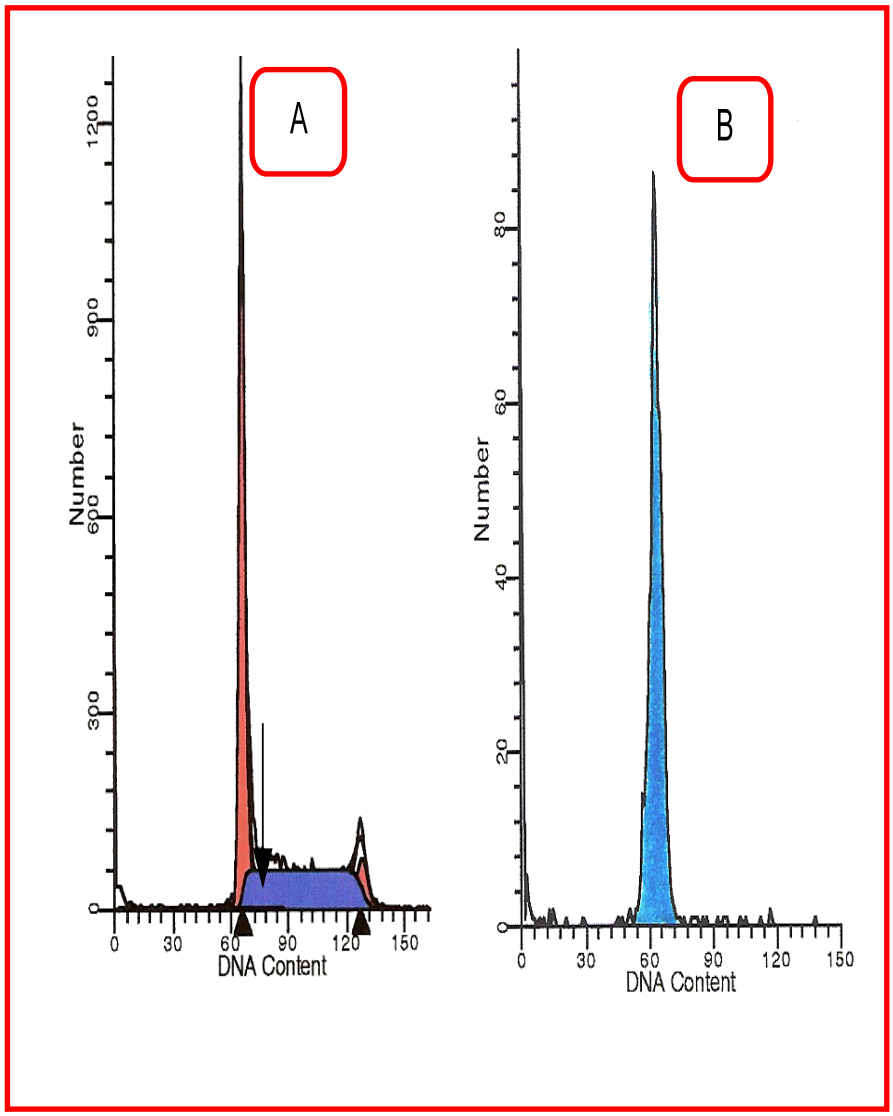


Figure 1.4 Burkitt's lymphoma DNA ploidy analysis. Panel A is a classic example of BL DNA ploidy with high S-phase fraction of 35% shown by the arrow. The first peak on the right in Panel A represents the diploid chromosome complement and the

second smaller peak is an aneuploid population. Panel B is normal Diploid DNA with no increase in S-phase or aneuploid DNA.

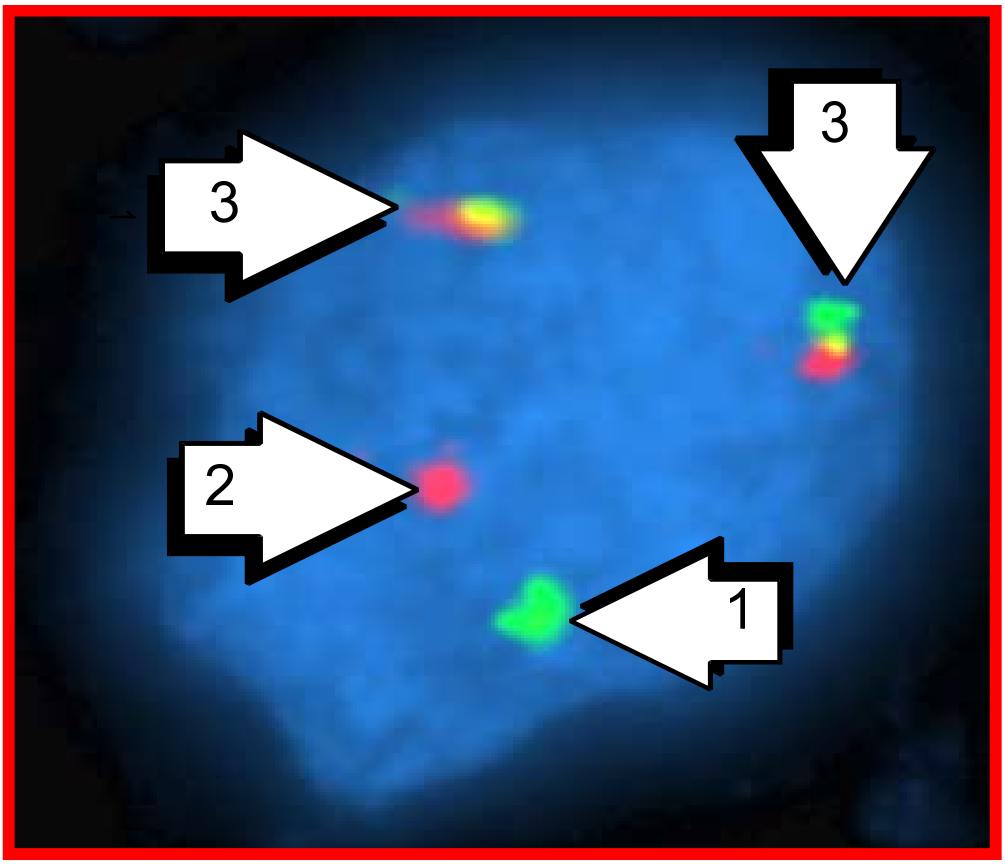


Figure 1.5 FISH analysis of the t(8;14) translocation in BL. The green signal (arrow 1) shows hybridization to the IgH gene. The red signal indicates hybridization to the myc gene(Arrow 2). The t(8;14) is represented by the joining of the two signals (yellow) as shown in the two arrows labeled 3. The presence of the two colocalizing signals are in keeping with the reciprocal translocation seen in BL (Picture courtesy of the NHLS Molecular Cytogenetics unit).

This is illustrated in Figure 1.5 which shows FISH analysis for the detection of the t(8;14) translocation using two different cosmid probes, one specific for the IgH gene and the other for the c-myc gene. The t(8;14) translocation is shown by the composite signal designated by a yellow colour and arrow numbered 3.

1.7.2 The pathology of t(8;14) translocation.

As discussed, this cytogenetic lesion was first described in 1972 and was the first translocation to be non-randomly associated with a lymphoid malignancy in 1976 (Manolov and Manolova 1972; Zech, Haglund et al. 1976). Figures 1.1 and 1.6 are schematic representations of the t (8;14) translocation. It is a balanced translocation mediated by recombinase enzymes in which the q24 locus on chromosome 8 is relocated to the long arm of chromosome 14 and the q32 locus on 14 is moved to the long arm of chromosome 8.

DNA sequence analyses have provided some insight into the timing and mechanism of the translocation event in the various subtypes of BL. In sBL and AIDS-BL with the t(8;14) translocation, the positions of the breakpoints in chromosome 14 suggest that they were created during attempted immunoglobulin class switching. This event is usually confined to germinal centre B cells, providing further support for a germinal centre B-cell origin of these forms of BL (Kaiser-McCaw, Epstein et al. 1977; Hecht and Aster 2000). In contrast, for eBL associated with t(8;14), the JH segments flanking the breakpoints usually have deletions and/or additions of base pairs that are characteristic of normal Ig VDJ segment rearrangement (Haluska and Croce 1987; Haluska, Tsujimoto et al. 1989).

Several models are plausible for the timing of t(8;14) formation in eBL. The re-arrangement of c-myc is an early event occurring in pre-B cells at the time of attempted VDJ recombination. The B cells then undergo maturation to germinal center cells and acquires additional genetic aberrations that collaborate to produce BL(Hikida, Mori et al. 1996; Han, Huang et al. 2006). In a second model, re-expression of RAG1 and RAG2 in germinal centres reactivates VDJ recombination and permits the t(8;14) to occur at this later stage of B-cell differentiation (Ma, Fisher et al. 1992; Ma, Pannicke et al. 2002; Raghavan, Swanson et al. 2005; Zou, Ma et al. 2007).

Very little is known about the mechanisms causing chromosomal breaks in c-myc. The breakpoints in c-myc occur in sites with no homology to VDJ recombination which suggest that they are unlikely to be dependent upon these activities(Gao, Smith et al. 1997).

Junctional sequences created by the t(8;14) translocation are in divergent orientation with the 5' end of the *c-myc* gene juxtaposed to the 5' end of the IgH gene (Basso, Frascella et al. 1999). As a consequence of this opposite orientation of the genes, no resultant functional fusion gene is formed. However, the functional consequence of the t (8;14) is a positioning of Ig enhancers in close proximity to the c-myc gene resulting in c-myc transcriptional dysregulation and consequent c-myc mRNA and protein overexpression. In addition, the regulatory sequences residing within c-myc are removed or mutated during the translocation resulting in increased c-myc activity (Hecht and Aster 2000). The mutated c-myc gene alters the amino acid sequence of the c-myc protein and this change can be detected by western blotting. The relative

positions of the breakpoints in the c-myc gene on chromosome 8 and IgH on chromosome 14 appear to result in the different phenotypes of the BL variants

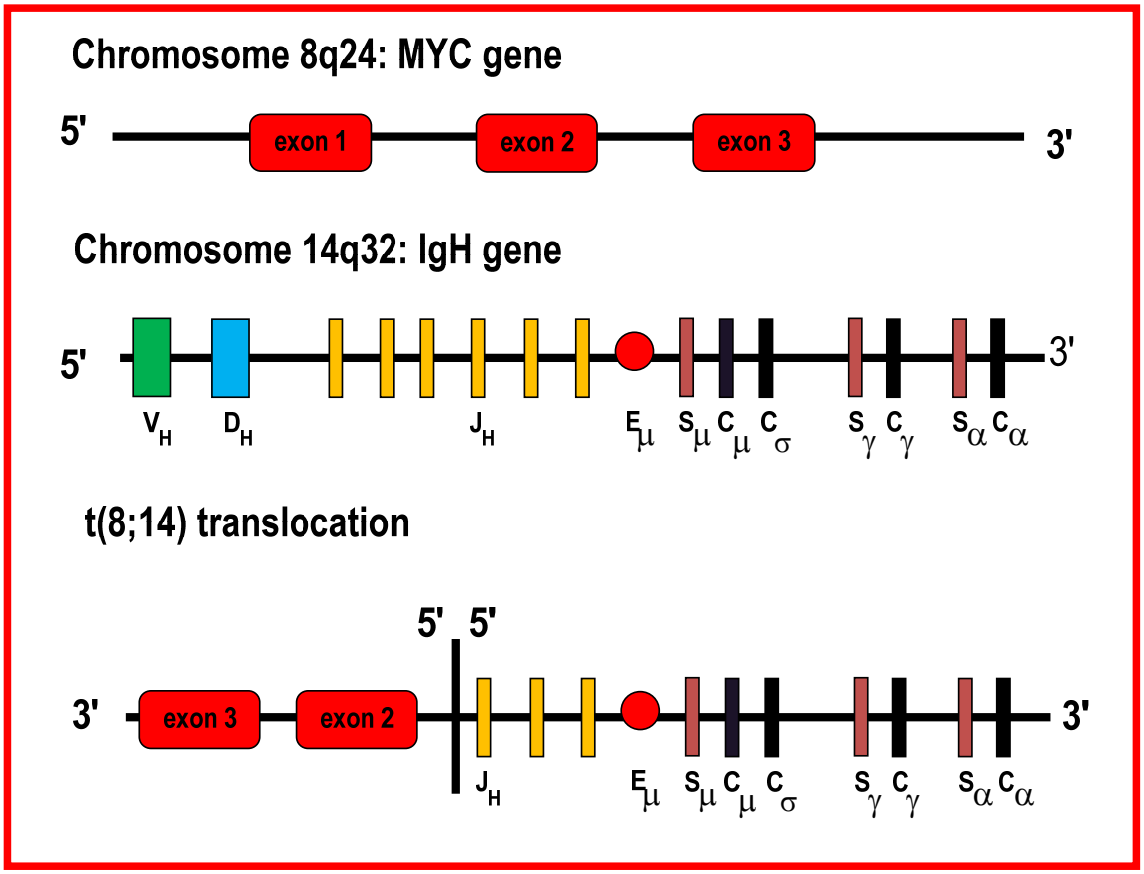
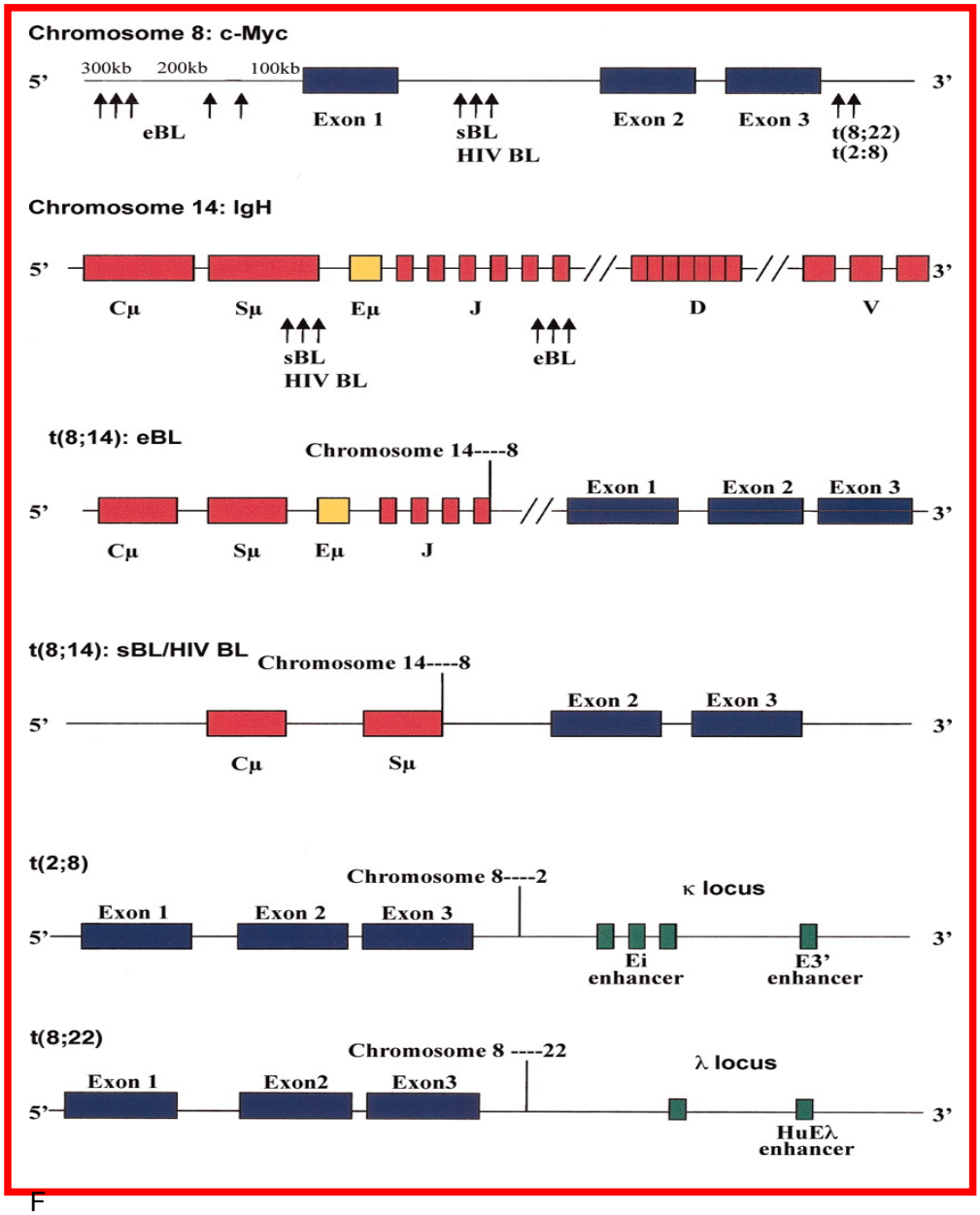


Figure 1.6 Schematic representation of the t(8;14) translocation. The breakpoints in the MYC gene usually occur at exon 1or intron 1 whilst those in the IgH gene are variable and could occur in the JH gene, switch regions or constant regions. The t(8;14) result in the 5'-5' orientation of the MYC and IgH gene segments with the consequent dysfunctional DNA segment (Pelicci, Knowles et al. 1986; Gutierrez, Bhatia et al. 1992).

In the eBL, the breakpoints on chromosome 8 occur more than 100kb 5' to the c-myc exon 1.. IgH breakpoint on chromosome 14 is within the joining region. These breakpoints seem to occur during the attempted VDJ recombination process critical to immunoglobulin rearrangement during B cell development. In the eBL, c-myc transcription is driven by the normal P1 and P2 c-myc enhancers. In the sBL and AIDS-BL the chromosome 8 breakpoints are between c-myc exon 1 and 2 and within the switch region on chromosome 14, resulting in a different molecular rearrangement and therefore different BL phenotypes

The breakpoint in the IgH gene occurs during attempted immunoglobulin switching. The translocation results in the loss of c-myc exon 1 and P1 and P2 promoters. C-myc transcription occurs from a cryptic promoter. In the BL variant translocations, the breakpoints on chromosome 8 are located 3' to the c-myc gene. The chromosome 2 and 22 breakpoints occur 5' to the kappa and lambda genes respectively (Neri, Barriga et al. 1988; Shiramizu, Barriga et al. 1991; Joos, Falk et al. 1992; Joos, Haluska et al. 1992)

The breakpoints characteristic of the different BL variants are shown by arrows on chromosomes 8 and 14 in Figure 1.7. The symbols Cμ, Sμ and Eμ represent the constant, switch and enhancer regions of the immunoglobulin heavy chain. The variable, diversity and joining regions of the immunoglobulin heavy chains are represented by V, D and J respectively. The BL variants are represented by sBL, eBL and HIV BL for sporadic, endemic and AIDS related BL respectively.



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Figure 1.7 Schematic representation of the translocation breakpoints in BL. In sBL and HIV-BL the breakpoints on chromosome 8 are within intron 1. In eBL breakpoints occur 5' to the c-myc gene. On chromosome 14, the breakpoints are in the Sμ region with sBL and HIV-BL and in the J region with the eBL (Illustration from Blum et al 2004).

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1.7.3 The biological consequences of c-myc dysregulation.

c-Myc plays a central role in a number of cellular processes. It appears to enhance apoptosis, cell cycle progression and telomerase activity (Marcu, Bossone et al. 1992; Mateyak, Obaya et al. 1999). c-Myc downregulates differentiation and cell adhesion (Langdon, Harris et al. 1986; Felsher and Bishop 1999; Felsher and Bishop 1999). The net c-myc effect of myc over expression is uncontrolled cell proliferation with associated genomic instability and cell immortalization. These findings have been summarized graphically in figure 1.8.

1.7.4 Clonality and immunoglobulin analysis in BL

The distinction between neoplastic and reactive disorders in the diagnosis of lymphomas still presents a challenge to pathologists. In many instances the combination of morphology and immunophenotype findings can be difficult to interpret (Delecluse, Raphael et al. 1993; Fodinger, Winkler et al. 1999). Demonstrating clonality by the detection of rearrangement of the immunoglobulin (Ig) gene provides a strong argument for differentiating neoplastic from reactive lymphoproliferative disorders(Hecht and Kaiser-McCaw 1981; Hecht and Aster 2000).

During B-cell ontogeny, a series of double stranded DNA breaks occur to produce a functional Ig gene(Vanasse, Concannon et al. 1999; Willis and Dyer 2000). Errors occurring during these events as well as defects in other DNA repair genes may predispose B-cells to Ig loci translocations associated with certain specific lymphoid neoplasms. The principal consequences of Ig translocations are deregulated

expression and in some instances, mutation of the translocated genes. Therefore studying immunoglobulin gene rearrangement can be an important tool for studying

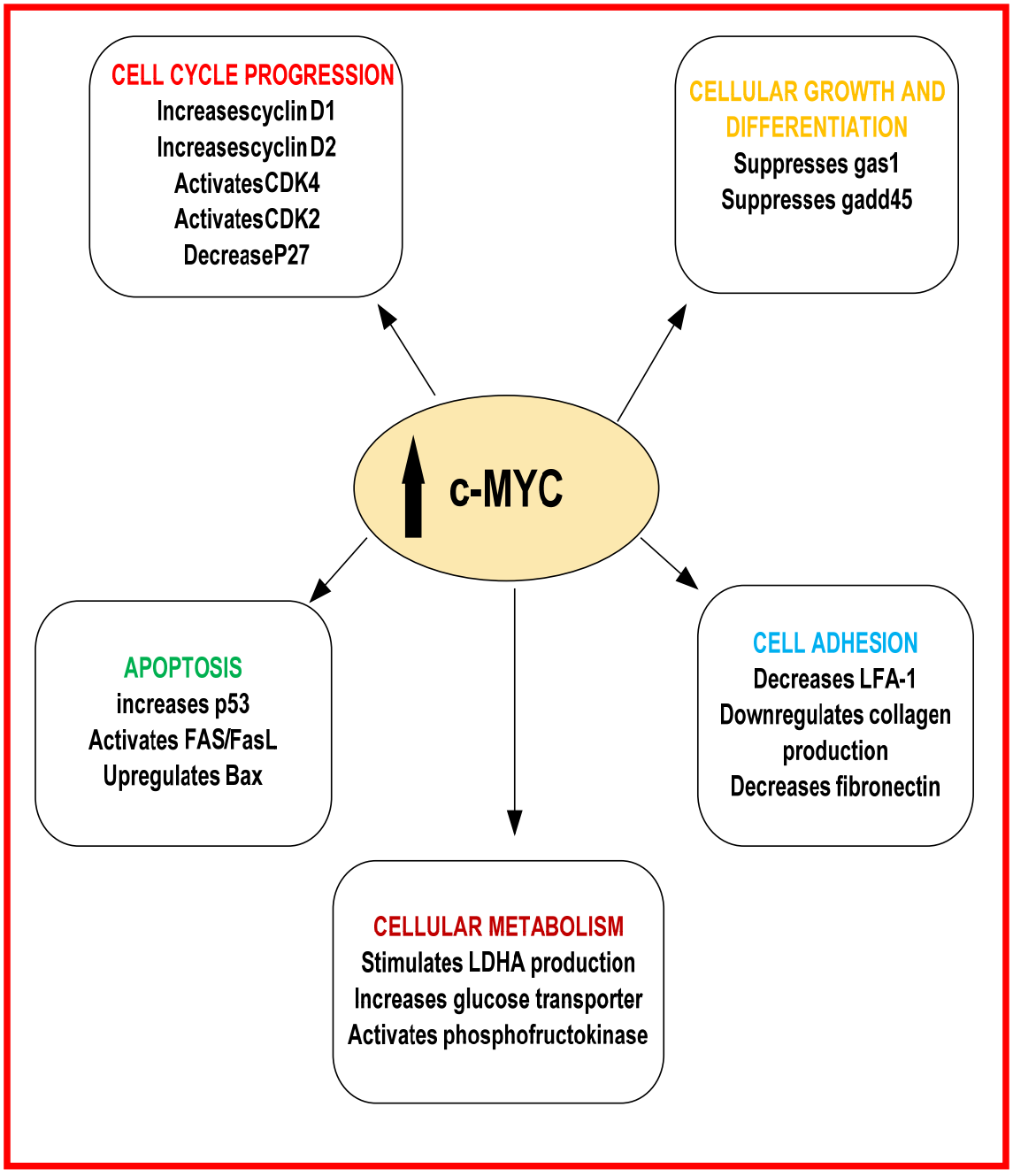


Figure 1.8 Cell biological consequences of c-MYC. (Modified from Blum et al 2004)

B-cell clonality. In the case of BL, demonstration of clonal Ig gene rearrangement or light chain restriction by flow cytometric analysis is an important step in confirming clonality.

Southern blotting is the established method detecting Ig gene rearrangement. The limitations of this technique are it's poor sensitivity and being time consuming and labour intensive. Clonality analysis of lymphoid cells using PCR to amplify the VDJ Junctional region of the Ig heavy chain gene can circumvent some of the disadvantages of the Southern blotting. Experience with application of PCR for Ig heavy gene rearrangement has been limited owing to the limitation of the spread of the breakpoints involved in BL translocations (Akasaka, Muramatsu et al. 1996; Armes, Southey et al. 1996).

1.7.5 Microarray analysis in Burkitt's lymphoma.

Molecular analysis of BL has recently been enhanced by the availability and application of microarray technology to lymphoma diagnosis. Although experience with BL gene expression profiling is still limited, recently published studies suggest that microarray technology using the BL molecular signature is potentially superior to morphology, cytogenetics and immunophenotypic analysis at accurately identifying and differentiating BL from other categories of diffuse large cell lymphomas. (Dave, Fu et al. 2006; Hummel, Bentink et al. 2006). This molecular classification is able to identify the morphologically classical as well as atypical forms of BL (Hummel, Bentink et al. 2006).

It is now widely accepted that correct identification of BL with atypical morphology is particularly challenging in the background of HIV infection (Diebold, Raphael et al. 1997; Cabalo, Wilkinson et al. 2002; Cheung 2004). Although there are no studies done on HIV infected patients as yet, the BL molecular signature used in microarray analysis is unlikely to be affected by HIV infection and may prove to be a useful tool in addressing this diagnostic dilemma.

The BL molecular signature also allows identification of a subset of this BL tumour without FISH or karyotype demonstrabl immunoglobulin-myc BL translocation (Dave, Fu et al. 2006). Using currently available non-microarray diagnostic tools, these lymphomas would have been misclassified and mismanaged as non-Burkitt's lymphomas.

One of the critical advances of this type of molecular classification is its ability to accurately differentiate BL from Diffuse Large Cell Lymphomas (DLCL) where morphological features may be identical (Hummel, Bentink et al. 2006). Burkitt's lymphoma and DLCL require different therapeutic approaches necessitating correct identification of these lymphomas. In a recent microarray analysis of lymphomas, a number of morphologically characterized DLCLs had the typical gene-expression profile of BL (Dave, Fu et al. 2006)

Lymphomas with morphologic features that are intermediate between those of BL and Diffuse Large Cell Lymphoma have been termed non-Burkitt or Burkitt-like lymphomas. In the WHO classification Burkitt-like lymphoma is reserved for those entities with immunophenotypic and genetic criteria characticstic of BL with atypical morphologic features (Harris, Jaffe et al. 2000; Harris and Horning 2006). Preliminary

data suggest that it would be possible to accurately classify these atypical BL using gene expression profiling (Hummel, Bentink et al. 2006).

EBV specific microarray chips have also become recently available (Gomez-Curet, Perkins et al. 2006; Hummel, Bentink et al. 2006; Li, Chen et al. 2006). EBV microarray analysis is likely to enhance our understanding of the role this virus plays in the aetiology, pathogenesis and prognosis of BL and it's subtypes..

The great impact of microarray analysis in BL is thus likely to be its use as a diagnostic tool to determine the type of therapeutic intervention required to treat the lymphoma. There is already evidence to indicate that patients with a molecular diagnosis of BL who are treated with aggressive chemotherapeutic regimens have better outcomes than those who received standard chemotherapy (Dave, Fu et al. 2006).

CHAPTER 2

2.0 DIAGNOSTIC CHALLENGES IN BURKITT'S LYMPHOMA.

2.1 Clinical diagnostic challenges in BL.

The three clinical variants of BL described in the previous chapter would appear to be fairly distinct clinico-epidemiological entities. However, there is some considerable overlap in their clinical features and epidemiological characteristics(Blum, Lozanski et al. 2004).

Although jaw mass presentation is very characteristic of eBL, occurring in up to 72% in some eBL series (Wang, Strasnick et al. 1992; Carbone, Canzonieri et al. 2000; Mwanda, Orem et al. 2005), it is not exclusive to endemic areas. Jaw mass presentation of sBL and AIDS-BL occur frequently outside the BL endemic areas. These clinical presentations have been extensively reported both as case studies and case series (Mukerji and Hilfer 1993; Jan, Vora et al. 2005; Ugar, Bozkaya et al. 2006). In the Muwakkit sBL series, 17% of cases presented with jaw masses (Muwakkit, Razzouk et al. 2004) which is high for this study done in a non-endemic region.

This clinical resemblance of eBL by sBL and AIDS-BL may be partly a consequence of population migrations and blurring of boundaries between endemic and non-endemic geographic locations. This has significantly reduced the clinical utility of jaw mass presentation in differentiating the various clinico-epidemiological variants. It is quite clear therefore, that neither the geographic location of the patient nor the anatomic location of the tumor are reliable clinical indicators for differentiating the eBL from the sBL.

It is now well established that HIV infection mimics and often masks the classical clinical features of many diseases, including lymphomas (Whitworth, Birchall et al. 1993; Clunie and Macleish 1994; Druillennec, Dong et al. 1999). A patient with BL and persistent generalized lymphadenopathy or lymphadenopathy secondary to infection may be clinically indistinguishable from a lymphoma with nodal presentation (Tirelli, Vaccher et al. 1987; Zagonel, Tirelli et al. 1988; Tirelli, Vaccher et al. 1989; Aguilar Ligorit, Guix Garcia et al. 1990; Peter, Boyle et al. 1994; Bhargava, Chang et al. 2006). Distinguishing AIDS-related BL from infections or other lymphomas in the setting of HIV remains a challenge which can lead to incorrect diagnostic evaluation and therapeutic interventions.

2.2 Morphological diagnostic challenges in BL.

Although BL cytomorphology suggests a single homogenous entity, there is a significant degree of tumor morphological variation occurring as a result of intrinsic and extrinsic factors.

Intrinsic nuclear pleomorphism is well described in BL and is characteristic of the Plasmacytoid differentiated and atypical/Burkitt's-like variants (Harris, Jaffe et al. 1994; Harris, Jaffe et al. 2000). In the absence of extrinsic modifying factors, extreme nuclear pleomorphism is associated with atypical BL whilst mild to moderate pleomorphism is characteristic of the plasmacytoid morphologic variant.

Burkitt's lymphoma morphology is often poorly discernable in the setting of extrinsic factors such as infections, drugs, toxins and concomitant other pathology. HIV infection is frequently associated with dysplastic cytomorphologic changes which are known to complicate diagnostic assessments (Marche, Tabbara et al. 1990; Sham and Bennett 1992). Significant dysplasia is also associated with exposure to a number

of toxins and drugs. The classic examples are steroids and chemotherapeutic agents which are also important in the therapeutic management of lymphomas (Irvine, Magill et al. 1998). Morphologic differentiation of the various BL variants is often very challenging even in the hands of skilled morphologists. Therefore, establishing a definitive morphological diagnosis of BL in the presence of these factors is often challenging and sometimes impossible.

Morphologic differentiation of leukemic BL from Acute Lymphoblastic Leukemia L3 subtype poses a special challenge. Distinct management protocols dictate that these entities be differentiated. Morphologic features, age of presentation, and sites of involvement of these two diseases are often indistinguishable (Sham and Bennett 1992; Velangi, Reid et al. 2002). Distinction between them is ultimately reliant on flow cytometry, cytogenetics and molecular analyses (Harris, Jaffe et al. 2000).

2.3 Cytogenetic diagnostic challenges in BL.

Traditional karyotypic analysis is unsuccessful in approximately 10-20% of blood and bone marrow specimens due to a low mitotic index of cells or poor quality of the metaphase spread (Offit and Chaganti 1991; Offit, Jhanwar et al. 1991; Offit, Wong et al. 1991; Siebert, Matthiesen et al. 1998). In BL, which is a high grade lymphoma the cytogenetic yield is expected to be significantly better. However, there is currently no published data to support this expectation.

The second limitation of conventional cytogenetics for the analysis of BL is the requirement for fresh, viable cellular specimens. Archival material and old specimens are unsuitable for analysis by this technique. In recent studies, cytogenetic variants such as fluorescence in-situ hybridization (FISH) have been shown to be suitable alternatives (Siebert, Matthiesen et al. 1998).

2.4 Southern blot analysis in BL.

Southern analysis was the first molecular tool used to elucidate the molecular pathology of many haematologic malignancies including the translocations in BL (Bernard, Cory et al. 1983). Until recently, it has been considered the gold standard in BL molecular analysis (Vital, Merlio et al. 1992). Although the impact of using this technique was significant in our early understanding of the BL molecular pathophysiology, it has a number of disadvantages which make it unsuitable for routine diagnostic use. These include the extremely slow turnaround times, high level of technical expertise required, exposure to radioactivity, very low sensitivity and the need to use multiple probes which increases the cost significantly (Langerak, Szczepanski et al. 1997). Consequently, southern blotting has been largely replaced by the cheaper, faster and sensitive polymerase chain reaction (PCR) approaches.

2.5 Polymerase chain reaction (PCR) analysis in BL.

Since its invention, PCR has become the molecular tool of choice for diagnostic investigation, detection of prognostic markers, disease monitoring and minimal residual disease detection of many haematological malignancies and infections. PCR is highly sensitive with fast turnaround times making it suitable for routine diagnostic applications (Buchbinder, Josephs et al. 1988; Lo and Chan 2006; Rumsby 2006).

Whilst PCR is currently extensively used in the routine diagnosis of many lymphoproliferative disorders (Williams, Zukerberg et al. 1995; Au, Horsman et al. 2002; Jiang, Medeiros et al. 2002; Janz 2006; Lo and Chan 2006; Rumsby 2006), it has not been readily available for routine detection of translocations in BL for two reasons. Firstly, BL is very heterogeneous at a molecular level, breakpoints in the

t(8;14) translocation are distributed over a large region yielding products that are too large to a synthesize by standard Taq DNA polymerase amplification. The standard Taq polymerase can only amplify targets up to 3 kilobases long (Akasaka, Muramatsu et al. 1996). Secondly, the breakpoints within the IgH gene sometimes occur within the switch region made up of repeat sequences for which unique primers are often difficult to design (Blum, Lozanski et al. 2004; Ferry 2006).

2.6 Long distance polymerase chain reaction (LD PCR).

Recently, the DNA polymerases have been improved for effective amplification of longer targets from human genomic DNA. These polymerase improvements and optimization were first described by Cheng and colleagues in 1994 (Cheng, Fockler et al. 1994). The DNA polymerases used in LD-PCR have identical thermostable and polymerase properties as the standard Taq DNA polymerase. They differ from Taq by possessing the 3'-5' exonuclease or "proof reading" activity. The exonuclease activity prevents mis-incorporation of nucleotides and therefore improves the efficacy of long segment amplification.

Since the original description, a number of these DNA polymerases with exonuclease activity have been described, optimized and commercialized. These include the *Pwo/Taq* DNA polymerase mixture supplied by Boerhinger Mannheim (Hinnisdaels, Del-Favero et al. 1996) and *Tgo*/*Taq* DNA polymerase enzymes mix supplied by Roche (Hopfner, Eichinger et al. 1999). Optimization of these polymerase mixtures has been accomplished by increasing the pH, adding glycerol and dimethysulphoxide (DMSO), decreasing denaturation times and increasing the extension time. Under these optimized conditions, they are capable of synthesizing DNA fragments up to 23Kb long (Cheng, Chang et al. 1994; Cheng, Fockler et al. 1994).

Preliminary studies suggest that the LD-PCR enzyme mixture can be used for the detection of translocations in a variety of B-cell lymphoproliferative disorders(Akasaka, Muramatsu et al. 1996; Akasaka, Ohno et al. 1997). In these settings, LD-PCR has been shown to be highly sensitive, with a detection limit of 1 in 1000 malignant cells with ethidium bromide staining and 1 in 10 000 with radioactive detection. These data suggest that LD-PCR is a sensitive technique suitable for minimal residual disease detection.

The specific application of the optimized LD-PCR in the detection of the t(8;14) translocation in BL suggests that it is fast, highly sensitive and specific (Akasaka, Ohno et al. 1997; zur Stadt, Reiter et al. 1997; Basso, Frascella et al. 1999; Mussolin, Basso et al. 2003). There are currently three published studies on the utility of LD PCR for the diagnosis of BL and findings have been summarized in Table 2.1 below.

Table 2.1 Summary of published LD-PCR studies in BL.

Publication	**Number of BL analyzed**	**Source of DNA**	**Age of patients**	**Proportion diagnosed with LD_PCR**
Zur Stadt et al 1999	20	Bone marrow	Pediatric	11/20(55%)
Basso et al 1999	15	Bone marrow	Pediatric	13/15(87%)
Mussolin et al 2003	78	Bone marrow	Pediatric	52/78(67%)

These studies have been conducted entirely in the paediatric age group in the setting largely of sBL. To date, no LD-PCR analysis of adult patients, patients with eBL or AIDS-BL have been published. All studies have been done on bone marrow aspirate and biopsy specimens and there was bone marrow involvement in all cases included. The proportion of known BL identified by LD-PCR was variable, ranging from 55-87%.

Despite the specificity and sensitivity of t(8;14) translocation PCR, it is noteworthy that this cytogenetic lesion could not be demonstrated in a significant number of known BL cases. Theoretically, the reasons for these are numerous and would include the variant of BL studied, the specificities and number of primers used in the PCR analysis and extent of bone marrow involvement.

2.7 Rationale for the current study.

The HIV pandemic has had its greatest effect in sub-Saharan Africa, but there is little published data available to support an increase in NHL in the region. The projected frequency of NHLs is expected to increase even further as the life expectancy of HIV-infected individuals is prolonged by novel antiretroviral therapeutic strategies (Pluda and Yarchoan 1990; Pluda, Yarchoan et al. 1990; Beral, Peterman et al. 1991).

The two common AIDS-related NHLs are BL and diffuse large cell lymphoma (DLCL) of B-cell origin. It is pertinent to distinguish these NHL as patient management approaches and treatment outcomes differ significantly.(Lim, Karim et al. 2005). For example, Burkitt's lymphoma, as opposed to DLCL, frequently presents with extranodal disease, with CNS involvement requiring intense, aggressive chemotherapy including intrathecal treatment. Upto 30% of cases of DLCL show 3q27 region abnormalities and 20-30% have translocation of the BCL2 gene (Harris, Jaffe et at 2000).

In the setting of HIV, morphological distinction between these two NHL can be very challenging. The hallmark of BL and important distinguishing feature is the associated t(8;14) translocation seen in the majority of individuals with this neoplasm. Diffuse

large cell lymphoma is not associated with a specific recurrent chromosomal translocation.

Although the t(8;14) translocation was the first non-random abnormality to be described in lymphoid malignancies and occurs in 80% of BL, it has not been possible to use this as a diagnostic tool for BL prior to introduction of LD-PCR technique. The recent availability of this technique has made it possible to characterize BL in our setting which is unique in several ways. Johannesburg is a cosmopolitan city which is often the first port of call for African migrants seeking medical attention. Although the predominant BL epidemiologic variant in South Africa should be sBL, this has been significantly altered by perennial population migrations from central and west Africa to Johannesburg. When this migration mix is superimposed on the current HIV epidemic, all three BL subtypes would theoretically be expected in Johannesburg. There are currently no studies done to characterize the clinico-epidemiology of BL in this region.

Previous molecular studies have demonstrated that the t(8;14) translocation breakpoints occur at different sites in the eBL and sBL (Pelicci, Knowles et al. 1986). As illustrated in Figure 1, the breakpoints in sBL and AID-BL are clustered within the first intron whereas in eBL they occur 5' to the first exon on chromosome 8. On chromosome 14, the eBL breakpoints are found in the joining region and those for the sBL and AIDS-BL are located in the switch region of the IgH. With the t(8;14) translocation LD-PCR, it is possible to characterize the various BL variants based on the PCR product size. This molecular analysis of BL subtypes has not yet done across all age groups in our cosmopolitan population with high HIV prevalence.

2.9 Aims of the current study.

The aims of this study are

- To establish and optimize the LD-PCR assay for the t(8;14) translocation using well characterized BL cell lines
- To apply this LD-PCR technique to retrospectively analyze the t(8;14) translocation in adult and pediatric Burkitt's lymphomas previously diagnosed in Johannesburg during a ten year period from January 1994 to December 2003.

CHAPTER 3

3.0 MATERIALS AND METHODS.

3.1 Selection of patients for the study.

Subjects for this study were paediatric and adult patients with a diagnosis of Burkitt's lymphoma made at the academic teaching hospitals affiliated to the University of the Witwatersrand, Johannesburg. These hospitals included the Johannesburg Hospital, Chris Hani Baragwanath Hospital and the Helen Joseph Hospital. The diagnosis was made by the treating doctors on the basis of a combination of clinical, morphological, cytogenetic and flow cytometric criteria. The diagnostic criteria are well established and some of them are summarized in Table 3.1 below (Harris 1967; Magrath 1991; Harris, Jaffe et al. 1994; Harris, Jaffe et al. 2000; Mwanda 2004; Haralambieva, Boerma et al. 2005).

Only subjects with an established and confirmed diagnosis of BL with bone marrow involvement were considered for inclusion in this study. Subjects with ALL-L3 were carefully identified by morphology, flow cytometry and clinical features and not considered for the study. Those patients with a diagnosis of BL made on tissue biopsy without bone marrow involvement or tissue cells available for flow cytometric analysis were also identified and excluded.

In this retrospective analysis, any patient with a diagnosis of BL but no bone marrow material left from the routine diagnostic procedure were not included in the study. There was no bone marrow procedure done specifically to obtain material for this research.

Table 3.1 Criteria for diagnosis of Burkitt's lymphoma†

DIAGNOSTIC CATEGORY	DIAGNOSTIC FEATURES
Clinical features	• B-symptoms • Tumor mass (Jaw, abdominal etc) • Organomegaly (spleen ,liver etc) • Clinical features of bone marrow failure • Organ failure secondary to tumor lysis • CNS symptomatology
Bone Marrow Aspirate or Peripheral blood Morphological features	• Abnormal lymphoid infiltrate/lymphoid blasts • Typical/atypical L3 morphology • Displacement of normal haemopoiesis
Tissue histology features (Trephine biopsy , lymph node , mass biopsy)	• Medium size lymphoid haemopoietic infiltrate • Starry sky appearance • Displacement / disruption of normal structure
Flow cytometric immunophenotypic features	• Abnormal lymphoid infiltrate • Light chain restriction • Pan B cell marker expression • High S-phase fraction on DNA Ploidy analysis • Aneuploidy • Aberrant markers
Cytogenetics features	• t(8;14) translocation • t(8;22) translocation • t(2;8) translocation • Other cytogenetic abnormalities

† Modified and expanded from the WHO Classification of haematolymphoid malignancies (Harris, Jaffe et al. 2000)

3.2 Patient DNA material.

Tissue available for analysis included the following:

- bone marrow aspirate smears,
- trephine imprint smears,
- cell suspensions remaining after the aspirate sample was used for flow cytometric analysis.

The aspirate and trephine imprint slides were the unfixed archival excess remaining after the initial diagnostic workup. Although theoretically there should be no difference between the use of stained or unstained slides (Anderegg, Schluter et al. 1998), only unstained slides were used as patient DNA source in this research. These slides were stored at ambient laboratory temperature (22°C) for varying time periods ranging from two weeks to 10 years prior to use in the study. Cells remaining after routine flow cytometric analysis were also stored at -20°C for varying periods prior to use.

3.3 Human ethic clearance.

Permission to use patient material for this study was sought from the institutional protocol review committee prior to study initiation. Ethics approval was granted by the University of the Witwatersrand Human Research Ethics Committee, clearance certificate number M00/05/12, which is included as appendix A of this thesis

3.4 Control DNA material.

Positive and negative controls were included with each patient PCR analysis performed for the t(8;14) translocation. Control DNA was extracted from well characterized human Burkitt's' lymphoma cell lines obtained from the American Type Culture Collection (ATCC, Maryland, United States of America).

The t(8;14) translocation positive Burkitt's lymphoma cell line used was the ATCC CA46 repository originally described by Magrath and colleagues in 1980 (Magrath, Lee et al. 1980). It is a human derived cell line obtained from a patient with Burkitt's lymphoma. This cell line had retained its original characteristics with serial propagation in culture.

The DAUDI cell line DNA, derived from the Burkitt's lymphoma tumor without t(8;14), translocation was used as the negative control. The DAUDI line was the derivative of that originally described by Klein and Klein in 1968 (Klein and Klein 1968).

Table 3.2 below describes some of the pertinent features of the DAUDI and CA46 cell lines. Both control cell lines were supplied as lyophilized pellets requiring suspension and tissue culture propagation prior to DNA extraction.

3.5 Culture of the cell lines.

3.5.1 Cell culture.

The lyophilized and frozen cell lines were received in sealed sterile ampoules. They were rapidly thawed for 60 seconds in a 37ºC waterbath and subsequently immersed in 70% ethanol. The entire ampoule volume of 1ml containing $6.8x10^6$ cells was suspended in 10ml of freshly prepared RPMI 1640 culture medium (Highveld biologicals, South Africa) containing 20% fetal calf serum (FCS, Highveld Biologicals, South Africa), 100µg/ml Penicillin G (Novo Nordisk, SA)), 100µg/ml Streptomycin,Novo Nordisk, SA) and 100µg/ml Fungizone (Brystol-Meyers-Squib, SA) as previously described(Perlman 1979; Prockop 1997).

The culture medium was renewed by washing cells in fresh RPMI within the first 12 hours of initial culture to remove the cryoprotective dimethy sulphoxide (DMSO). In subsequent cultures, cells were seeded at a density of $5x10^6$ cells /ml of RPMI 1640 culture medium and the growth medium was renewed every 2-3 days or sooner if the cell growth was too rapid. Standard aseptic culture techniques were observed and any contaminated cultures were immediately discarded and the culture process re-initiated from clean stock.

Table 3.2 Summary of pertinent features of control BL cell lines†

DESCRIPTION	**CA-46 cell line**	**DAUDI cell line**
ATCC number	CRL 1648	CCL0213
Source Organism	*Homo sapiens*	*Homo sapiens*
Depositor	IT Magrath	G Klein
Tissue	Burkitt's lymphoma	Burkitt's lymphoma
Receptors expressed	Complement (12%)	Complement, Fc of IgG
Morphology	Lymphoblast	Lymphoblast
Source	**Organ type:** peripheral blood **Cell type**: B lymphocyte **Disease:** Burkitt's lymphoma	**Organ:** peripheral blood **Cell type**: B lymphoblast **Disease:** Burkitt's lymphoma
Karyotype	46,XY, dup1(q21-32), t(8;14) (8pter --> 8q23 :: 14q32 --> 14qter;14pter -->14q32: 8q23 --> 8qter), 13q+, +16	Normal male
Propagation	**ATCC propagation medium**: RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, fetal bovine serum, 20% **Temperature:** 37.0°C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%	**ATCC propagation medium**: RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, fetal bovine serum, 10% **Temperature:** 37.0°C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%

†Information extracted from the ATCC cell line catalogue(ATCC, Maryland, USA)

3.5.2 Cell counting and viability check.

Five microliters of the cultured cell suspension was diluted 1 in 20 in Turk's white cell counting fluid containing 1% v/v Glacial acetic acid and 0.5% Gentian blue in distilled water (Rebulla and Dzik 1994).

Suspended cells were counted using standard procedure on the Neubauer counting haemocytometer with improved rulings (VWP Scientific, Philadelphia, PA, USA). Cell viability was expressed as percentage of live cells to sum total of dead and alive cells. Cultures with viability of more than 80% were deemed suitable for subsequent subculturing and for use as source control DNA.

3.5.3 Cell harvesting and storage of cultured cells.

A cryopreservation solution containing 10% DMSO in neat Fetal calf serum(FCS) was prepared using an aseptic technique. The stock culture cells were counted and then suspended in this solution at a density of $1x10^6$ cells per milliliter in sterile cryotubes. These cryotubes were snap frozen in liquid nitrogen and then stored at -70ºC. The cryopreserved Burkitt's lymphoma cells were kept indefinitely as the propagatable cell stock.

3.6 Cytogenetic analysis of the cell lines.

Confirmation of the karyotype of the two cell lines was carried out as previously described (Hozier and Lindquist 1980; Swansbury 2003). Pertinent steps in this karyotype confirmation process are outlined below.

3.6.1 Culture initiation.

The cultured cell suspension was carefully decanted into a 15ml polypropylene tube and centrifuged at 1500rpm at room temperature for 8 minutes. Using a Pasteur pipette, the supernatant was carefully removed from the cell pellet and discarded leaving only 0.5ml of medium in which the cell pellet was resuspended. Five milliliters of a hypotonic solution (0.568g% KCl in sterile deionized water) was added to the cell suspension and mixed. Fifty microlitres of clocemid was added. The suspension was mixed well and incubated at 37ºC for 25 minutes.

3.6.2 Cell harvest fixation.

After 25 minutes of incubation, 3 drops of cold ethanol-acetic acid fixative (3:1 mixture) was added drop by drop into the cell suspension using a Pasteur pipette and mixed well. The suspension was centrifuged at 1500rpm for 10 minutes. The supernatant was removed and discarded leaving a small volume of fluid in which the pellet was resuspended. A further 5ml of the ethanol-acetic acid fixative was added slowly. The cell pellet was resuspended and centrifuged at 1500 rpm for 10 minutes. This fixation process was repeated until the supernatant was clear. If not analyzed immediately, the suspension was stored at -20ºC until slides were made.

3.6.3 Preparation of slides.

The stored cell suspensions were allowed to stand at room temperature for 15 minutes. New, factory pre-cleaned, frosted slides were used. The chromosome separation seems to work best if the slides are chilled in the freezer first. Slides were placed side by side on paper toweling with no separation between them. The entire

content of the centrifuge tube was withdrawn into a Pasteur pipette. From a height of about 18 inches, two or three drops of fluid were dropped onto each slide. Slides were allowed to dry thoroughly at room temperature. Occasionally slides were placed in an incubator at 37ºC overnight to complete the drying process. The dried slides were stained with Giemsa for 7-10 minutes. Excess Giemsa was rinsed off with distilled water and slides air dried.

Dried slides are mounted with DPX (glue) under a coverslip. The slide's appearance was assessed under the microscope and recorded. Once the chromosomes have been prepared and mounted upon slides they are ready for analysis.

3.7 Patient DNA extraction.

Unstained and unfixed bone marrow aspirate smear slides from previously diagnosed Burkitt's' lymphoma patients were the source material for DNA extraction.

Extraction was carried out using the method of Anderegg and colleagues (Anderegg, Schluter et al. 1998). Marrow was scraped off the glass slides with a sterile scalpel blade into a sterile 2ml Eppendorf tube. The scraped marrow sample was then suspended thoroughly in 300µl of lysis buffer with pH 8.8 containing 0.1M Tris/EDTA, and 1% sodium dodecyl sulphate (SDS) . Protein was digested with 30ng Proteinase K (Amersham, South Africa) at 56ºC for 60 minutes. RNA was removed by adding 6µg of RNAse A (Amersham, South Africa) and the tube inverted at least 25 times before it was incubated in a heated block at 37ºC for 15-60 minutes. The suspension was then cooled back to room temperature. Protein was then precipitated by adding 100µl of protein precipitating solution (Gentra Systems, Mineapolis, MN, USA. The tubes were mixed on a whirl mixer for 20 seconds and incubated on ice for 5 minutes. The protein

precipitate was then pelleted by centrifugation at 12 000g for 3minutes. The supernatant containing DNA was decanted into a clean sterile 2ml epppendorf tube. The suspended DNA was precipitated with 300µl of ice cold isopropanol. Where the DNA yield was visually perceived to be poor, it was improved by the addition of 20ng glycogen into the tube. The tube was inverted 50 times and the DNA pelleted by centrifugation at 12 000g for 5 minutes. The DNA precipitate was washed with 300µl of ice cold ethanol. DNA was finally suspended in a suitable volume of TE buffer, at pH 7.5. Suspension was facilitated by subsequent incubation at 56ºC for up to 60 minutes with gentle shaking.

3.8 Control DNA extraction.

Cells suspended in RPMI culture medium were pelleted by centrifugation at 5 000g for 2 minutes. The cell pellet was transferred to a clean sterile 2ml Eppendorf tube. The cells were lysed and subsequently treated as described in the Andregg et al method described in section 3.7 above.

3.8.1 DNA quantification and purity check.

A sample of suspended DNA was diluted in TE buffer (10mM Tris, 1mM EDTA) to give an optimal density reading of less than one. The absorbance of the DNA sample was measured at 260nm and 280nm in an appropriately calibrated UV Spectrophotometer (Beckman DU6, GMI Inc, Minnesota, USA). The DNA concentration in the original tube was calculated using standard conversion factors. The ratio of DNA absorbance at 260/280 was calculated and used as measure of DNA purity. The DNA was stored at -20°C until further analysis.

3.8.2 DNA agarose gel fractionation.

A sample of DNA was mixed with sample loading solution and run on an 0.8% Agarose gel containing 0.5mg/ml Ethidium bromide. The resultant electrophoretogram was viewed and photographed under ultraviolet light. The size of DNA product was compared to known lambda DNA II size markers (Roche diagnostics, USA). In addition, each DNA sample was carefully analyzed for evidence of degradation. Degraded DNA and DNA of low molecular weight were excluded from subsequent PCR analysis.

Table 3.3 PCR master mixture preparation.

Mixture components	Final volume	Final concentration
Master Mixture1		
• Double distilled H_2O	to final volume of 25µl	-
• dNTP mixture†	2.5µl	350µM each
• Forward primer	4µl	60pmol
• Reverse primer	4µl	60pmol
• Template DNA	4ul	300ng
Master Mixture 2		
• Sterile H20	to final volume of 25µl	-
• Buffer 3	5µl	1.75mM
• Enzyme mixture‡	0.75µl	2.5U/reaction

†Contains equal proportion of dATP, dCTP, dGTP and dTTP
‡Taq/ Pwo DNA polymerase enzyme mix

3.9 PCR master mixture preparation.

Two PCR master mixtures comprising PCR reagents shown in table 3.3 were prepared as per Roche Expand Long template PCR System protocol (Catalogue number 1681842, Roche Diagnostics, USA). These were added in the order shown in

table 3.3 into a 200µl thin walled PCR tube. The master mixtures were then pooled and briefly vortexed before being put in a thermal cycler for amplification.

3.10 tPA LD-PCR analysis of patient DNA.

To establish the suitability of patient DNA for LD-PCR use, each patient DNA was first amplified with the tPA primer set as a control measure. The tPA primers were purchased from Roche Diagnostics and have been optimized for use with the Expand Long Template LD PCR system (Catalogue number 1691014, Roche Diagnostics, USA). The primer set properties are shown in Table 3.4. Primer preparation was done as described in table 3.3. The tPA LD-PCR analytical conditions were exactly the same as those for the t(8;14) and are described in table 3.6 below.

Multiplex LD-PCR reactions were attempted in which the tPA and t(8;14) amplification reactions were done in the same tube. In most instances this approach proved to be problematic with inconsistent results.

Table 3.4 tPA LD-PCR primer properties.

Primer name	Primer sequence	Annealing temperature
tPa forward 7	5'-GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C-3'	65°C
tPa reverse 10	5'-GAT GCG AAA CTG AGG CTG GCT GTA CTG TCT C-3'	65°C

3.11 t(8;14) LD-PCR analysis of patient DNA.

Each LD-PCR run included a blank sample with only reagents but no DNA material, CA-46 control DNA and DAUDI control DNA. These controls were run with four separate primer pairs, namely, MYC-Cα, MYC-Cμ, MYC-Cγ and MYC-JH primer pairs. The MYC oligonucleotide was the forward primer and one of the four IgH oligonucleotides was the reverse primer. The primer properties are described in Figure 3.5 below

DNA amplification was carried out in a Thermal Cycler 9600 (Perkin Elmer, Norwalk, CT). The reaction conditions were those described by Basso and colleagues (Basso, Frascella et al. 1999) and are outlined in table 3.6 below. Both the t(8;14) positive and negative BL DNA were included as controls in each LD-PCR analysis.

Table 3.5 t(8;14) LD-PCR primer properties.

Primer name	Primer Composition	Primer Length	Primer Tm
MYC	5'-ACA GTC CTG GAT GAT GAT GTT TTT GAT GAA GGT CT -3'	35-mer	67.1°C
Cμ	5'-TGC TGC TGA TGT CAG AGT TGT TCT TGT ATT TCC AG - 3'	35-mer	68.3°C
Cγ	5'-AGG GCA CGG TCA CCA CGC TGC TGA GGG AGT AGA GT-3'	35-mer	>75°C
Cα	5'-TCG TGT AGT GCT TCA CGT GGC ATG TCA CGG ACT TG-3'	35-mer	73.0°C
JH	5'-ACC TGA GGA GAC CAG GGT GAC GGT-3'	24-mer	67.8°C

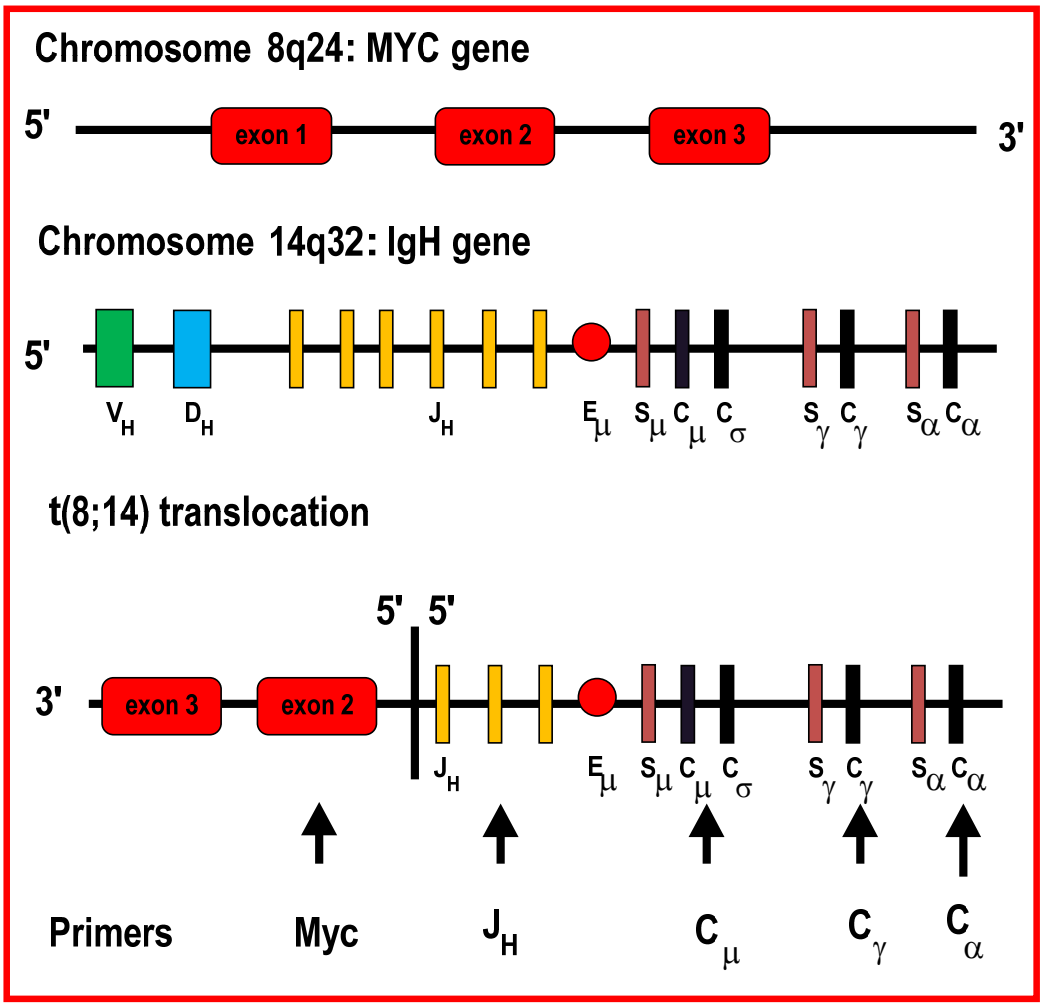


Figure 3.1 Schematic representation of the t(8;14) with primer positions. The breakpoints in the MYC gene usually occur at exon 1or intron 1 whilst those in the IgH gene are variable and could occur in the JH gene, switch regions or constant regions. The t(8;14) result in the 5'-5' orientation of the MYC and IgH gene segments with the consequent dysfunctional DNA segment. The positions of the five primers are shown by arrows. The upstream MYC gene primer was alternative paired with one of the four IgH primers (Modified from Hecht and Asler 2000)

Table 3.6 LD-PCR analytical conditions.

No of cycles	Process	Temperature & duration
1 x	Template denaturation	94°C for 2min
10 x	Denaturation Annealing Elongation	94°C for 10 secs 65°C for 30 secs 68°C for 4min
20 x	Cycle 1 Denaturation Annealing Extension Cycle 2-20	94°C for 10secs 65°C for 30secs 68°C for 4min Denaturation and annealing the same as above, extension extended by 20secs/cycle
1 x	Final extension	68°C for 10 min
Cooling	Cooling	4°C until removed from cycler

3.12 LD-PCR product quality check.

A 0.8% Agarose gel containing 0.5 x TAE buffer and 0.5µg/ml Ethidium bromide was prepared as shown in table 3.7 below. Briefly, agarose was weighed in a weighing boat and transferred into a 250ml conical flask. An appropriate volume stock TAE buffer was added and the volume made up to required amount with water. Agarose was dissolved in a microwave and then cooled down to 50°C. Ethidium bromide was added and the gel was poured into the minigel system and allowed to set at room temperature for an hour.

Five microlitres of the PCR amplicon and molecular weight markers was sampled into an Eppendorf tube and mixed with sample loading buffer containing 25% Ficoll 400, 0.25% Bromophenol blue and 0.25% Xylene Cyanol. The mixture was loaded into the agarose gel.

Electrophoresis was carried out using a gel running buffer (1 xTAE buffer) containing 0.5ug/ul Ethidium bromide at 50-100volts.

Table 3.7 Agarose gel preparation.

Stock component	**50ml final Volume**	**100ml final volume**
Agarose powder (g) (molecular biology grade)	0.4g	0.8g
Stock 50XTAE buffer (2M Tris acetate + 0.05M EDTA)	500µl	1000µl
Stock Ethidium bromide 10mg/ml	2.5µl	5µl
Double distilled sterile water	To 50ml	To 100ml

4. CHAPTER 4

4.0 RESULTS

4.1 Study population selection.

The study population was selected according to inclusion criteria defined in section 3.1 above and the selection process results are depicted graphically in Figure 4.1 below. Between January 1994 and December 2003 there were 110 patients diagnosed with BL in the three teaching hospitals in Johannesburg. Eighty four (76%) of these patients had BL with bone marrow involvement whilst 26 individuals presented with other organ system involvement with no demonstrable tumor cells in the bone marrow. Archival bone marrow smear slides remaining after routine diagnosis were available in 74 of 84 (88%) identified BL patients. The 10 cases excluded were mainly as a result of all marrow slides being used up during the routine diagnostic workup with no remaining archival material. DNA extraction was performed in the 74 BL available patients with slides. Extracted DNA was either of extremely poor quality or quantitatively very small or absent in 23 of the 74 cases (31%). These cases were therefore not suitable for further analysis leaving only 41 cases that could be analyzed with LD-PCR.

4.2 Study population demographics.

The demographics of the study population are shown in Figure 4.1 below. The mean age of the 41 patients included in the study was 21.4 years with a range from 3-60 years. In the selected study population there was a slight predominance of males comprising 23 subjects (56%). Three quarters of study population comprise black subjects with only a third of patients being white. In terms of traditional divisions of care of patients using 15 years as a cut off, approximately an equal

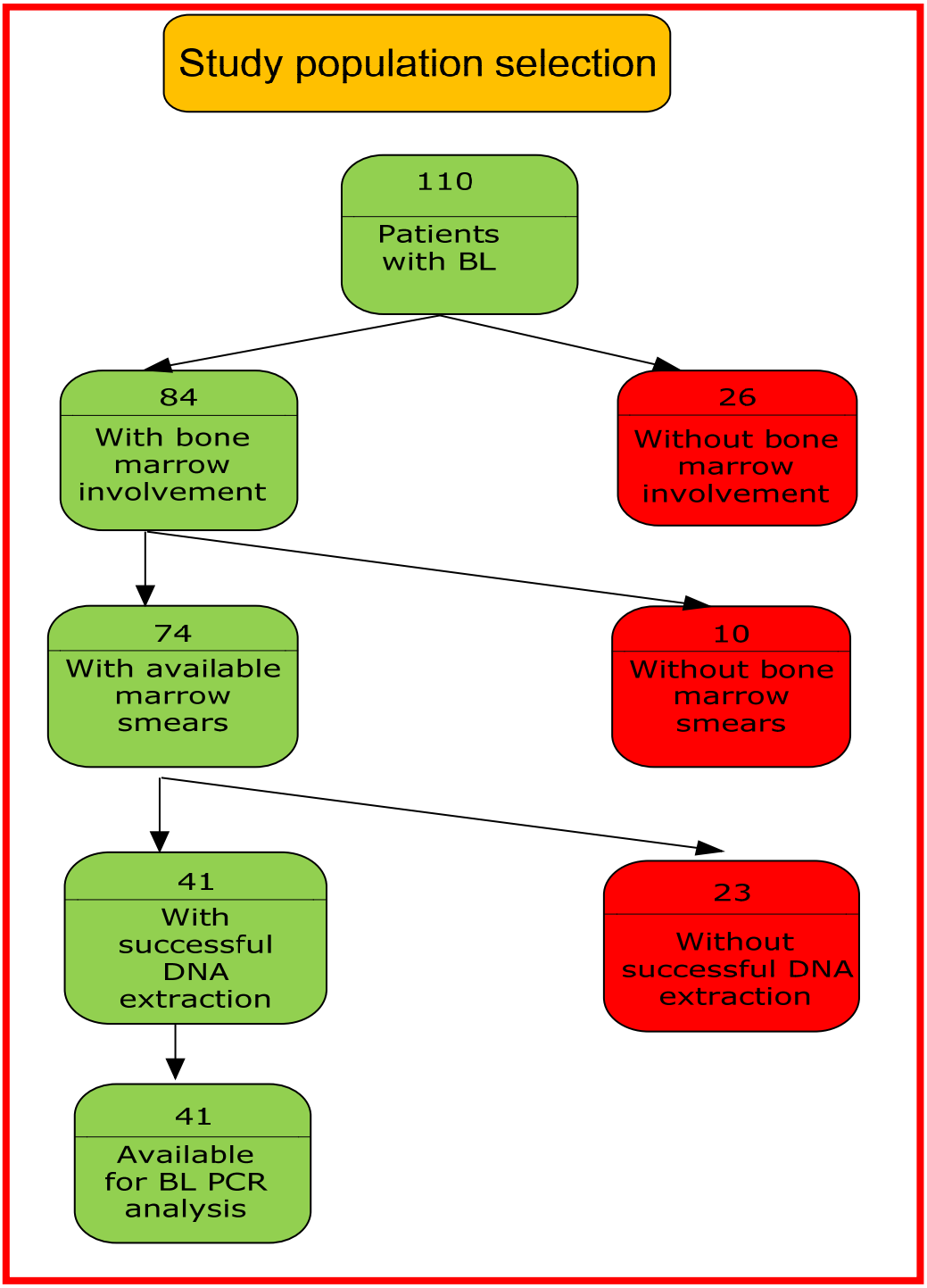


Figure 4.1 Study population selection

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Table 4.1 Study population demographics.

Patient number	Age (years)	Race	Sex
BL1	9	White	Male
BL2	19	White	Male
BL3	13	White	Male
BL4	32	Black	Female
BL5	8	Black	Female
BL6	45	Black	Male
BL7	36	White	Male
BL8	6	White	Male
BL9	6	Black	Female
BL10	24	Black	Female
BL11	60	Black	Male
BL12	32	Black	Male
BL13	28	Black	Female
BL14	22	Black	Male
BL15	51	Black	Male
BL16	6	Black	Female
BL17	25	Black	Female
BL18	58	Black	Male
BL19	9	Black	Female
BL20	10	Black	Male
BL21	14	Black	Male
BL22	8	Black	Female
BL23	32	Black	Male
BL24	6	White	Female
BL25	11	Black	Male
BL26	23	Black	Female
BL27	32	White	Male
BL28	25	Black	Female
BL29	22	Black	Female
BL30	47	Black	Male
BL31	50	Black	Male
BL32	35	Black	Female
BL33	23	Black	Female
BL34	4	White	Female
BL35	7	White	Male
BL36	8	Black	Female
BL37	8	Black	Male
BL38	11	White	Male
BL39	5	Black	Female
BL40	6	Black	Male
BL41	3	Black	Male

number of patients were looked after by adult physicians and paediatricians (21 versus 20 patients respectively)

Patients were analyzed in the sequence in which they were identified and patient names in this table have been replaced with numbers to maintain confidentiality.

4.3 Study population baseline laboratory parameters.

The baseline laboratory parameters for patients in this study are shown in table 4.2 below. The majority (29 of 41)(70%) of study participants were HIV negative by standard HIV Elisa tests and only 12 were HIV positive. The viral loads and CD4 counts of the 12 HIV positive patients were unfortunately not consistently available in this retrospective analysis, with some patients having been tested for CD4 count or viral load but not both. By definition, all HIV positive patients had AIDS as per CDC criteria (Keou, Belec et al. 1992; Vella, Chiesi et al. 1994).

The extent of bone marrow involvement, as measured by flow cytometric analysis, ranged from 4-97% with a mean of 57.9%. These figures correlated well with those obtained by morphologic examination. In general, bone marrow involvement evaluated by morphologic examination was slightly higher than the flow cytometric estimate on the same patient. This was largely due to Ficoll processing of specimen required for flow cytometry specimen preparation which resulted in some cell loss. There were no cases demonstrating diagnostic discrepancy between morphology and immunophenotypic analysis.

Table 4.2 Baseline laboratory parameters of study population.

Patient number	HIV status	% BM involvement	Immunoglobulin Light chain	CD19	CD10	% S-phase
BL1	Neg	90.0	Kappa	90.0	90.0	ND
BL2	Neg	80.0	Kappa	80.0	80.0	ND
BL3	Neg	19.0	Kappa	26.0	46.0	ND
BL4	Neg	85.0	Kappa	85.0	85.0	35.0
BL5	Neg	91.0	Kappa	94.0	94.0	35.0
BL6	Neg	90.0	Lambda	95.0	95.0	40.0
BL7	Neg	80.0	Kappa	80.0	95.0	40.0
BL8	Pos	34.0	Kappa	76.0	76.0	27.0
BL9	Neg	35.0	Lambda	35.0	35.0	ND
BL10	Pos	4.0	Lambda	16.0	16.0	ND
BL11	Pos	94.0	Kappa	94.0	94.0	ND
BL12	Neg	5.0	Kappa	5.0	5.0	ND
BL13	Pos	7.0	Lambda	5.0	5.0	ND
BL14	Neg	9.0	Kappa	9.0	9.0	ND
BL15	Neg	9.0	Lambda	10.0	10.0	ND
BL16	Pos	4.0	Kappa	5.0	5.0	ND
BL17	Pos	30.0	Lambda	38.0	33.0	25.0
BL18	Pos	77.0	Kappa	78.0	77.0	ND
BL19	Pos	76.0	Lambda	76.0	76.0	43.0
BL20	Neg	49.0	Lambda	56.0	56.0	35.0
BL21	Neg	6.0	Kappa	6.0	7.0	ND
BL22	Neg	8.0	Kappa	7.0	7.0	ND
BL23	Pos	45.0	Lambda	66.0	55.0	ND
BL24	Neg	65.0	Kappa	65.0	67.0	34.0
BL25	Neg	80.0	Kappa	80.0	80.0	22.0
BL26	Neg	65.0	Lambda	65.0	65.0	ND
BL27	Neg	74.0	Kappa	74.0	74.0	54.0
BL28	Pos	25.0	Kappa	25.0	25.0	ND
BL29	Pos	80.0	Lambda	80.0	80.0	35.0
BL30	Pos	82.0	Kappa	80.0	80.0	ND
BL31	Neg	91.0	Kappa	91.0	91.0	50.0
BL32	Neg	88.0	Kappa	88.0	88.0	44.0
BL33	Neg	80.0	Kappa	80.0	10.0	ND
BL34	Neg	54.0	Kappa	55.0	55.0	ND
BL35	Neg	91.0	Kappa	91.0	91.0	40.0
BL36	Neg	90.0	Kappa	90.0	90.0	ND
BL37	Neg	91.0	Lambda	91.0	91.0	ND
BL38	Neg	90.0	Kappa	90.0	90.0	ND
BL39	Neg	24.0	Kappa	25.0	25.0	ND
BL40	Neg	97.0	Kappa	95.0	95.0	ND
BL41	Neg	80.0	Kappa	80.0	80.0	ND

Key: Neg= negative , Pos= positive, ND= not done

Table 4.3 Cytogenetic findings of study population

Patient number	Cytogenetics for t(8;14)	Other aberrations
BL1	Neg	
BL2	Pos	Trisomy 12
BL3	Neg	
BL4	ND	
BL5	NG	
BL6	NG	
BL7	NG	
BL8	Neg	
BL9	Pos	xxy
BL10	NG	
BL11	NG	
BL12	NG	
BL13	NG	
BL14	Neg	
BL15	Pos	
BL16	NG	
BL17	NG	
BL18	NG	
BL19	NG	
BL20	NG	
BL21	Neg	
BL22	ND	
BL23	NG	
BL24	NG	
BL25	Neg	
BL26	Pos	
BL27	NG	
BL28	NG	
BL29	NG	
BL30	Neg	
BL31	Neg	
BL32	Pos	
BL33	NG	
BL34	Neg	
BL35	NG	
BL36	NG	
BL37	NG	
BL38	NG	
BL39	NG	
BL40	NG	
BL41	NG	

Key: Neg= negative , Pos= positive, ND= not done, NG=No growth

On immunophenotypic analysis, all BL cases demonstrated light chain restriction with the vast majority(71%) being Kappa rather than lambda light chain restricted. All BL tumors expressed CD10 and CD19 as well as other B-cell markers

Aberrant marker expression by BL tumors was not reported. The majority (26 of 41) of the BL samples had no S-phase fraction analysis done. The s-phase fraction in the 14 samples where it was performed was consistently above 20%. This finding is in keeping with the high grade nature of BL. All BL tumor cells in this study population were diploid by flow cytometric analysis with only two patients showing an additional aneuploid population.

Although karyotype analysis was attempted in all samples, this analysis was largely unsuccessful due to consistent poor culture yield(24/41)(58%). The cytogenetics results are shown in table 4.3. Only five of the 41 patients in the study had the t(8;14) translocation demonstrable by cytogenetics.

4.4 Burkitt's lymphoma cell line culture results.

The cell line culture was successful with no modification to the ATCC recommended culture conditions. At least 80% cell viability was consistently attained in every analysis of culture undertaken.

Table 4.4 below shows the typical viabilities of four cell line cultures done during the course of this study.

Table 4.4 Burkitt's lymphoma cell line viabilities**.**

Culture number	Cell line	Viability
1	Daudi	88%
2	CA46	90%
3	Daudi	80%
4	CA46	85%

4.5 Culture storage and propagation.

Cultures that were stored in DMSO were successfully propagated at various time intervals. The viability and culture behavior of these DMSO cryopreserved cell lines was not different from those of the original culture.

4.6 Cytogenetic analysis of the Burkitt's lymphoma cell lines.

Table 4.5 below shows the results of confirmation karyotypic analysis of the two Burkitt lymphoma cell lines. The corresponding cell line chromosome analyses are illustrated in figures 4.2 and 4.3. The Daudi cell line had a normal chromosome complement as expected and the CA-46 cell line had the t(8;14) translocation depicted by arrows in Figure 4.3

These cytogenetic results were reproducible over time indicating the stability of the cell lines

Table 4.5 Burkitt's lymphoma cell karyotypic findings.

Cell line	Cytogenetic karyotypic findings
Daudi original cell line	46, XY - normal male.
Daudi subcultured cell line	46, XY - normal male
CA46 original cell line	46, XY, t(8;14) - BL male
CA-46 subcultured cell line	46, XY, t(8;14)- BL male

4.7 Control DNA purity and yield.

Significant amounts of DNA was extracted from control cell lines using the Anderegg method described in the previous chapter. The typical yield of the various extractions from 20 million cells is illustrated in Table 4.6 below. This DNA was of acceptable quality with minimal protein contamination as illustrated by the 260/280 absorbance ratio. The integrity, high molecular weight and RNA content of the extracted DNA was confirmed by running a 5µl sample on agarose gel containing ethidium bromide. This control DNA check is illustrated in Figure 4.4.

4.8 Patient DNA extraction and quantification.

As illustrated in Figure 4.1, DNA extraction was performed in 74 BL patients with bone marrow involvement. Extracted DNA was quantified and its protein contamination assessed by measuring the ratio of sample absorbance at 260nm and 280nm. In 41 of the 70 extractions, DNA was of high quality showing minimal degradation and RNA contamination. This DNA was therefore deemed suitable for subsequent LD-PCR analysis. The successful DNA extraction and quantification is shown in Table 4.6 below.

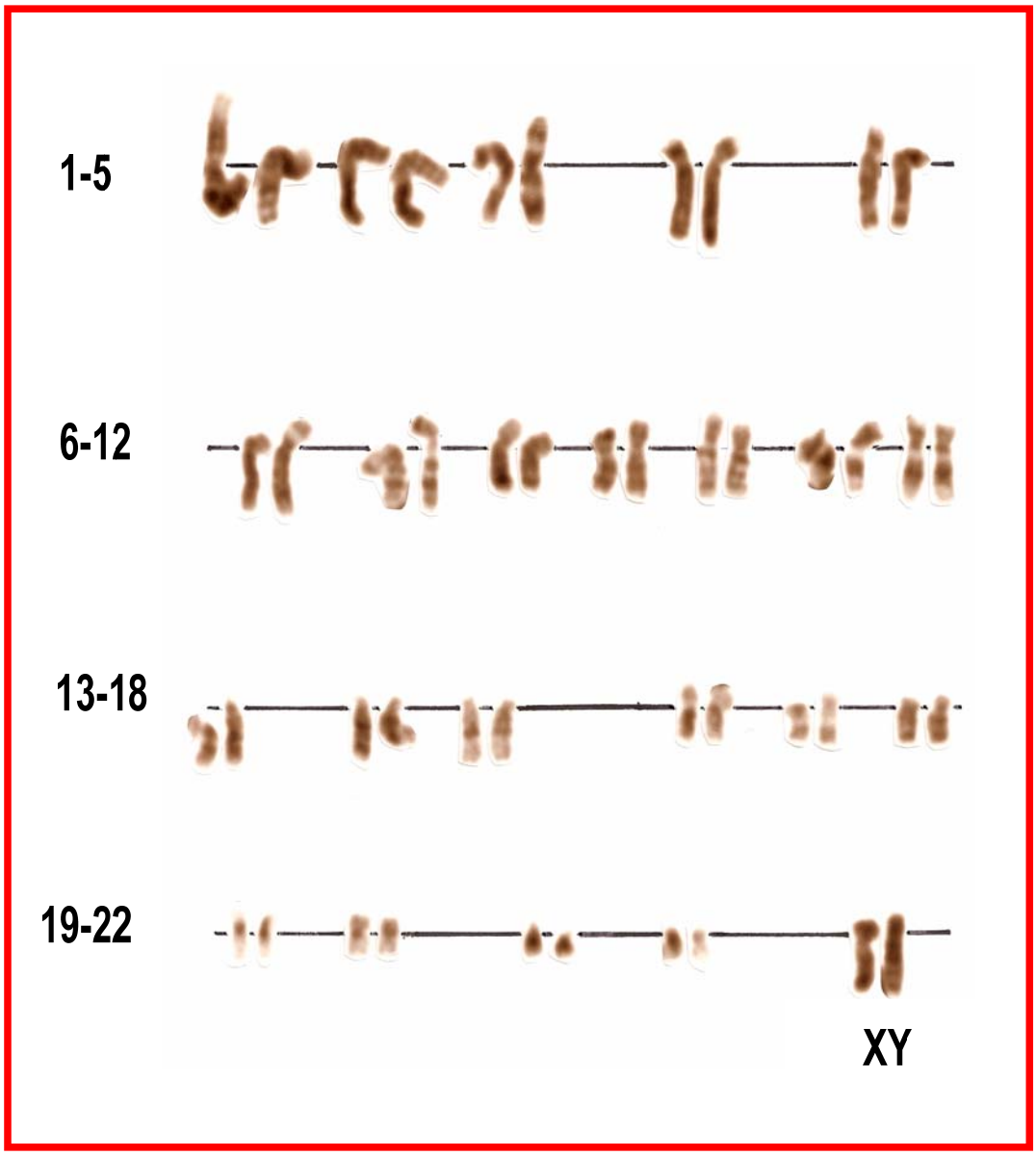


Figure 4.2 Giemsa stained chromosomes of the Daudi cell line. This karyotype is consistent with normal chromosome number and structure expected in the Daudi cell line. Chromosomes 8 and 14 were normal in this karyotype analysis.

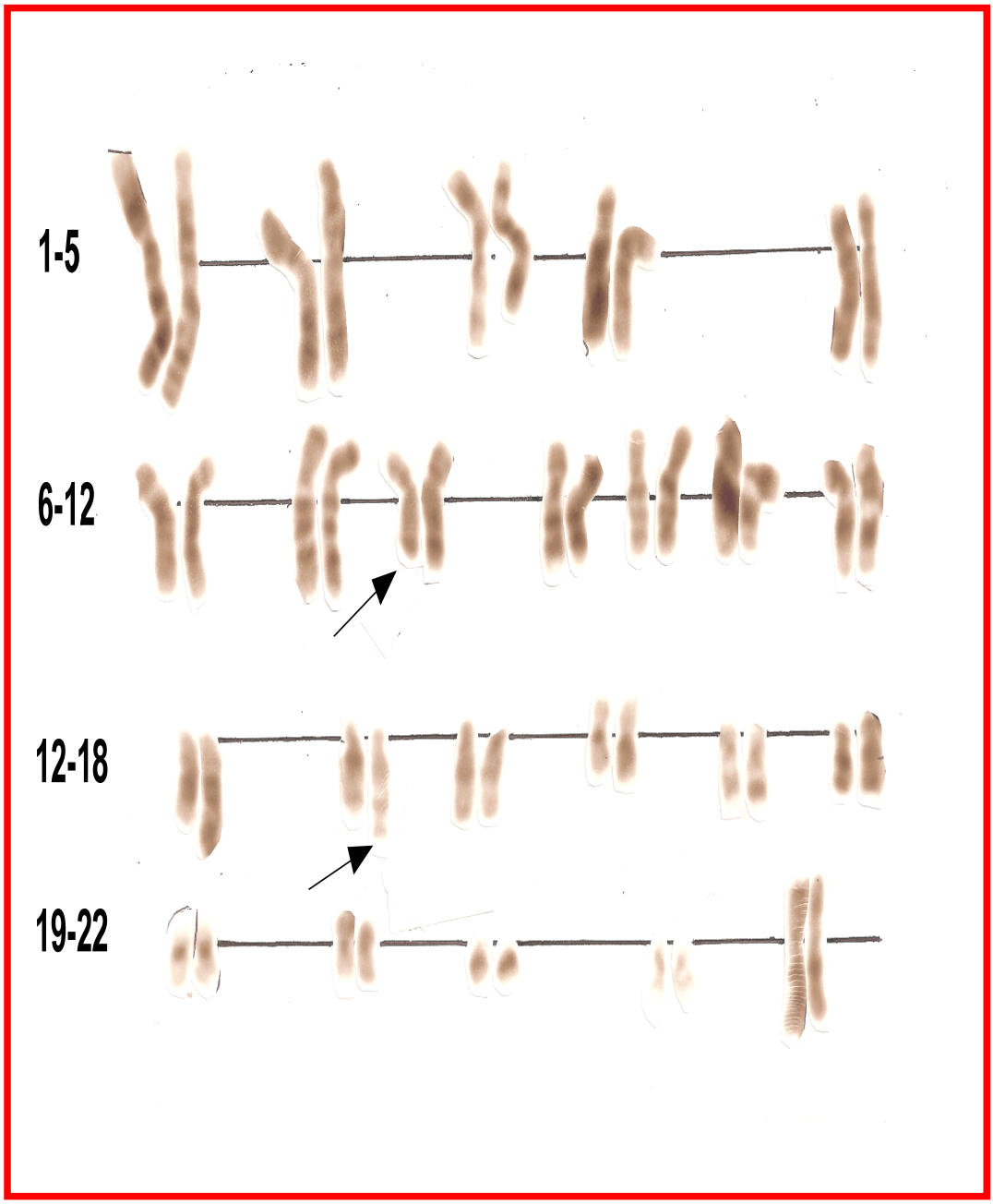


Figure 4.3 Giemsa stained chromosomes of the CA-46 cell line. This karyotype shows the classic t(8;14) translocation which was expected in this cell line (shown by arrows). There were no other abnormalities noticeable on this karyotype.

Table 4.6 Control DNA extraction results.

Cell line extractions	**Absorbance at 260nm**	**Absorbance at 280nm**	**Ratio of 260/280**	**DNA concentration** µg/µl
CA-46 extraction 1	0.110	0.044	2.5	5.5µg/µl
CA-46 extraction 2	0.320	0.173	1.83	16.0µg/µl
CA-46 extraction 3	0.118	0.063	1.87	5.9µg/µl.
DAUDI extraction 1	0.111	0.053	2.1	5.5µg/µl
DAUDI extraction 2	0.256	0.143	1.8	12.8µg/µl
DAUDI extraction 3	0.206	0.119	1.73	10.3µg/µl

Notes: Extractions 1, 2 and 3 of the various cell lines were carried out at various time intervals from approximately six million cultured cells. Ten microlitres suspended DNA was diluted in TE buffer and its absorbance measured at two wavelengths .Measurement at 260nm was for nucleic acids and measurement at 280nm was for protein contamination. The ratio of 260/280 represented the purity of the nucleic acid with an acceptable ratio in all extractions of more than 1.7. In patient samples an LD PCR product was possible even with a ratio of <1.7

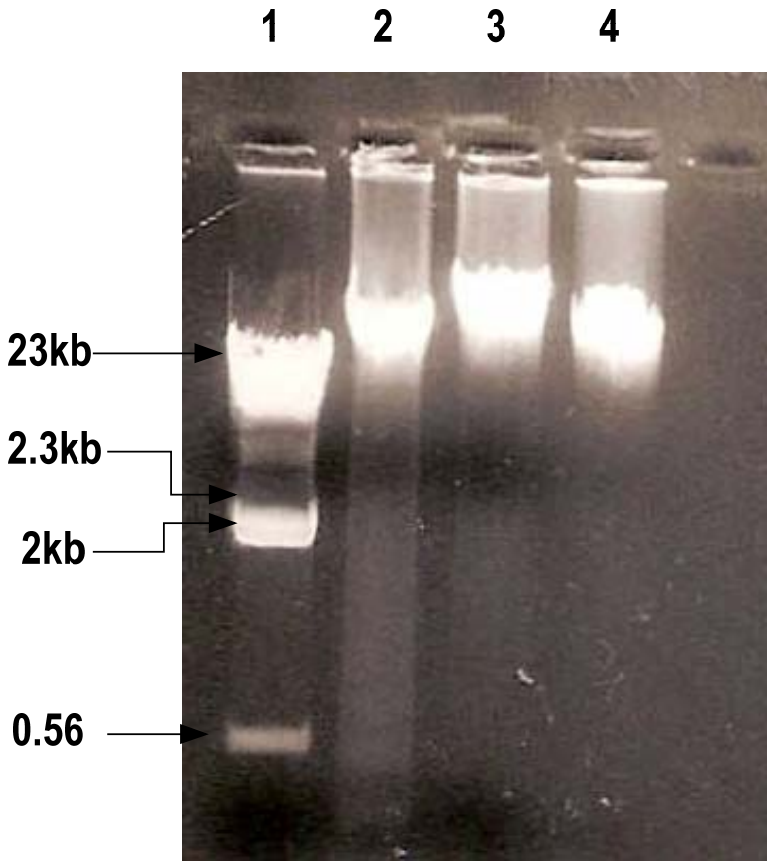


Figure 4.4 Agarose gel electrophoresis of control DNA. Five microlitres of control DNA suspended in TE buffer was run in 0.8% agarose gel containing 1xTAE buffer and 0.5μg/μl ethidium bromide. Lane 1 is λ DNA II molecular marker (Roche diagnostics). Lane 2 is healthy human volunteer control DNA. Lane 3 is the CA-46 control DNA. Lane 4 is Daudi control DNA. All control DNA was of high molecular weight suitable for PCR analysis with no evidence of significant degradation or RNA contamination.

4.9 Patient DNA quality check.

After quantification, 2µl of patient DNA was electrophoresed in a 0.8% agarose gel and photographed. Figure 4.5 shows examples of patient DNA that was intact and of high molecular weight suitable for subsequent PCR analysis as well as degraded DNA not used for subsequent analysis.

4.10 The tPA LD-PCR analysis of control DNA.

The CA-46 cell line was amplified using the tPA primers to check integrity and suitability of this control DNA for LD-PCR. The result of this analysis are shown in Figure 4.8 below. A single 4kb band was generated as an indication that the tPA gene in CA-46 was successfully amplified using LD-PCR. This analysis was also performed with the Daudi cell line which also showed successful amplification with similar fragment size.

4.11 The combined t(8;14) and tPA LD-PCR analyses of patient DNA

LD-PCR analysis using two primer sets was set up as described in table 3.3 and 3.6 above. The total reaction volumes and DNA amounts were adjusted accordingly. The result of this analysis is shown in Figure 4.6 below.

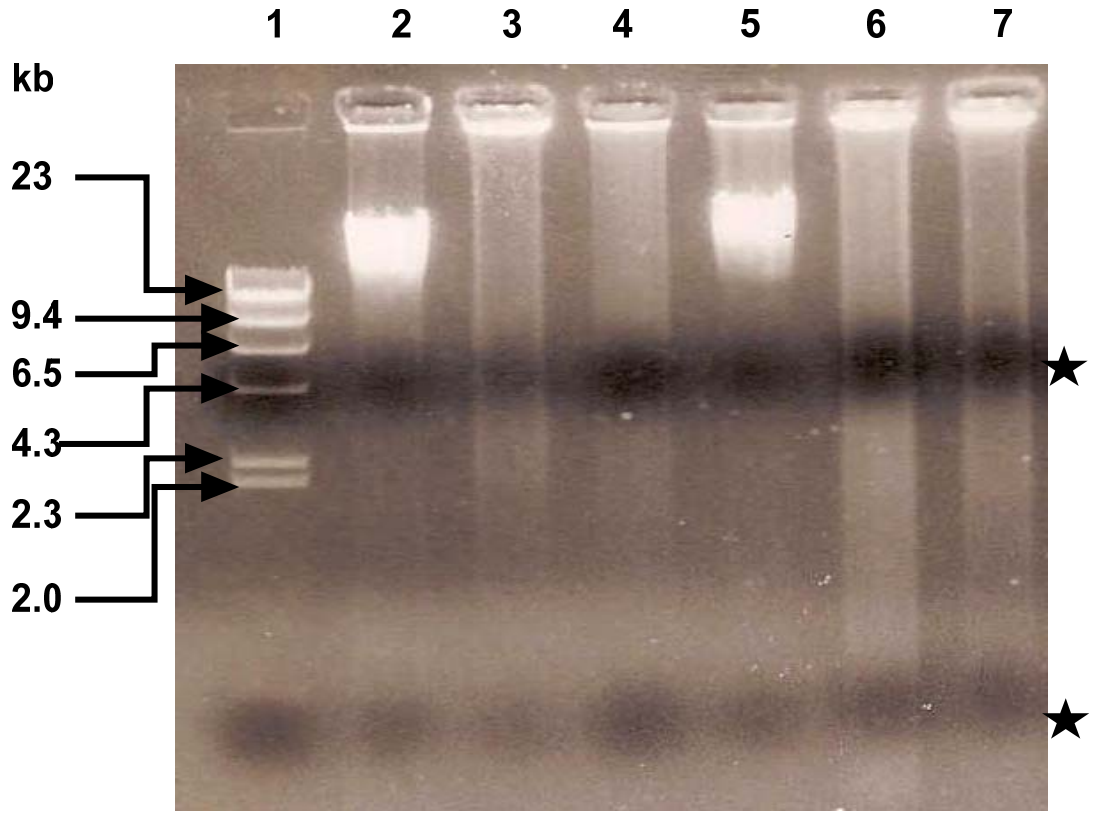


Figure 4.5 Agarose gel electrophoresis of patient DNA. Two microlitres of patient DNA dissolved in TE buffer was loaded on a 0.8% agarose gel containing 1xTAE buffer and 0.5mg/ml Ethidium bromide. Lane 1 is the λ DNA II molecular marker (Roche diagnostics, USA). Lanes 2 and 5 are examples of high molecular weight patient DNA from patients BL6 and BL22 respectively. Lanes 3, 4, 6 and 7 are examples of poor quality partially degraded patient DNA that was not included in the subsequent PCR analysis. The dark bands in the middle and bottom of the gel (Marked with black stars) are the sample loading buffer dyes.

Table 4.7 Patient DNA extraction and quantification.

Patient number	Absorbance at 260nm†	Absorbance at 280nm	Ratio 260/280	[DNA]‡ µg/µl
BL1	0.166	0.095	1.7	**83.0**
BL2	0.119	0.09	1.3	**59.5**
BL3	0.071	0.062	1.1	**35.5**
BL4	0.101	0.085	1.2	**50.5**
BL5	0.245	0.185	1.3	**122.5**
BL6	0.227	0.163	1.4	**113.5**
BL7	0.169	0.099	1.7	**84.5**
BL8	0.176	0.126	1.4	**88.0**
BL9	0.133	0.125	1.1	**66.5**
BL10	0.033	0.027	1.2	**16.5**
BL11	0.228	0.257	0.9	**114.0**
BL12	0.301	0.198	1.5	**150.5**
BL13	0.135	0.092	1.5	**67.5**
BL14	0.37	0.277	1.3	**185.0**
BL15	0.191	0.127	1.5	**95.5**
BL16	0.145	0.085	1.7	**72.5**
BL17	0.263	0.238	1.1	**131.5**
BL18	0.19	0.16	1.2	**95.0**
BL19	0.192	0.125	1.5	**96.0**
BL20	0.12	0.08	1.5	**60.0**
BL21	0.195	0.117	1.7	**97.5**
BL22	0.445	0.263	1.7	**222.5**
BL23	0.145	0.116	1.3	**72.5**
BL24	0.107	0.1	1.1	**53.5**
BL25	0.137	0.096	1.4	**68.5**
BL26	0.087	0.075	1.2	**43.5**
BL27	0.06	0.053	1.1	**30.0**
BL28	0.235	0.178	1.3	**117.5**
BL29	0.911	0.669	1.4	**455.5**
BL30	0.156	0.117	1.3	**78.0**
BL31	0.72	0.377	1.9	**360.0**
BL32	0.48	0.393	1.2	**240.0**
BL33	0.675	0.393	1.7	**337.5**
BL34	0.945	0.521	1.8	**472.5**
BL35	0.761	0.436	1.7	**761.0**
BL36	0.129	0.064	2.0	**64.5**
BL37	0.313	0.167	1.9	**156.5**
BL38	0.152	0.102	1.5	**76.0**
BL39	0.52	0.306	1.7	**260.0**
BL40	0.331	0.194	1.7	**331.0**
BL41	0.127	0.084	1.5	**127**

†**CONVERSION FACTOR** : 1OD at 260nm = 50µg/ml dsDNA
‡[DNA] is DNA concentration

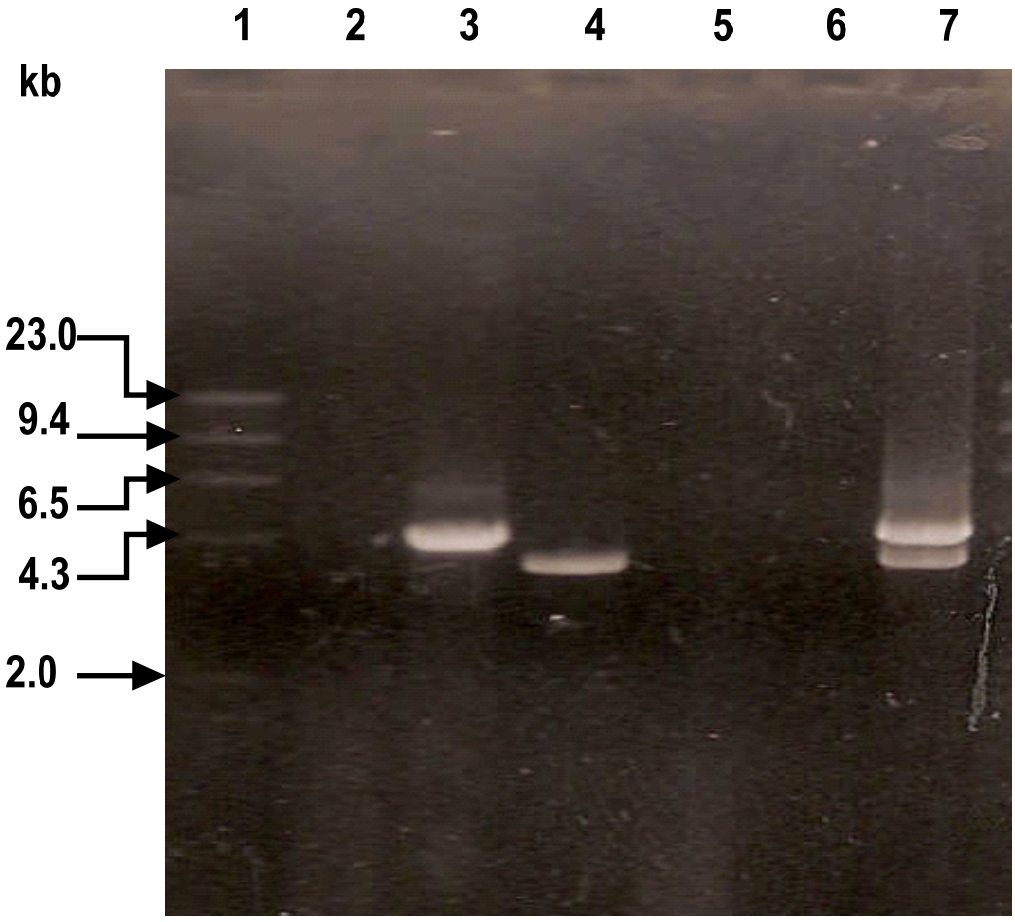


Figure 4.6 The tPA and t(8;14) LD-PCR analysis of CA-46. The CA-46 cell line DNA was amplified using the tPA and MYC-Cα primers in separate tubes(Lanes 3 and 4) and multiplexed in a single tube (Lane 7). The resultant amplicons were electrophoresed in a 0.8% agarose gel containing 1xTAE buffer and 0.5mg/ml ethidium bromide. Lane 1 is DNA molecular marker II (Roche Diagnostic, USA). Lane 2 is the reaction mixture without the Taq-PWO enzyme mixture. Lane 3 is CA-46 amplification using tPA primers. Lane 4 is CA-46 amplification using the MYC- Cα primer set. Lane 5 is the reaction mixture without DNA. Lane 6 is the reaction mixture without dNTPs. Lane 7 is LD-PCR using the tPA and MYC- Cα in the same tube.

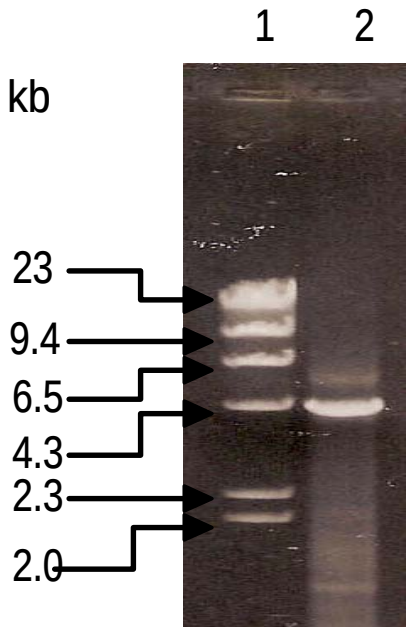


Figure 4.7 The tPA LD-PCR analysis of the CA-46 cell line. Two microlitre of DNA molecular marker II and LD-PCR amplicon was loaded on a 0.8% agarose gel containing 1xTAE buffer and 0.5mg/ml ethidium bromide. Lane 1 is the DNA molecular marker II showing the top six bands . Lane 2 is the CA-46 LD-PCR product using tPA primers. The amplicon fragment size was approximately 4kb which was as expected.

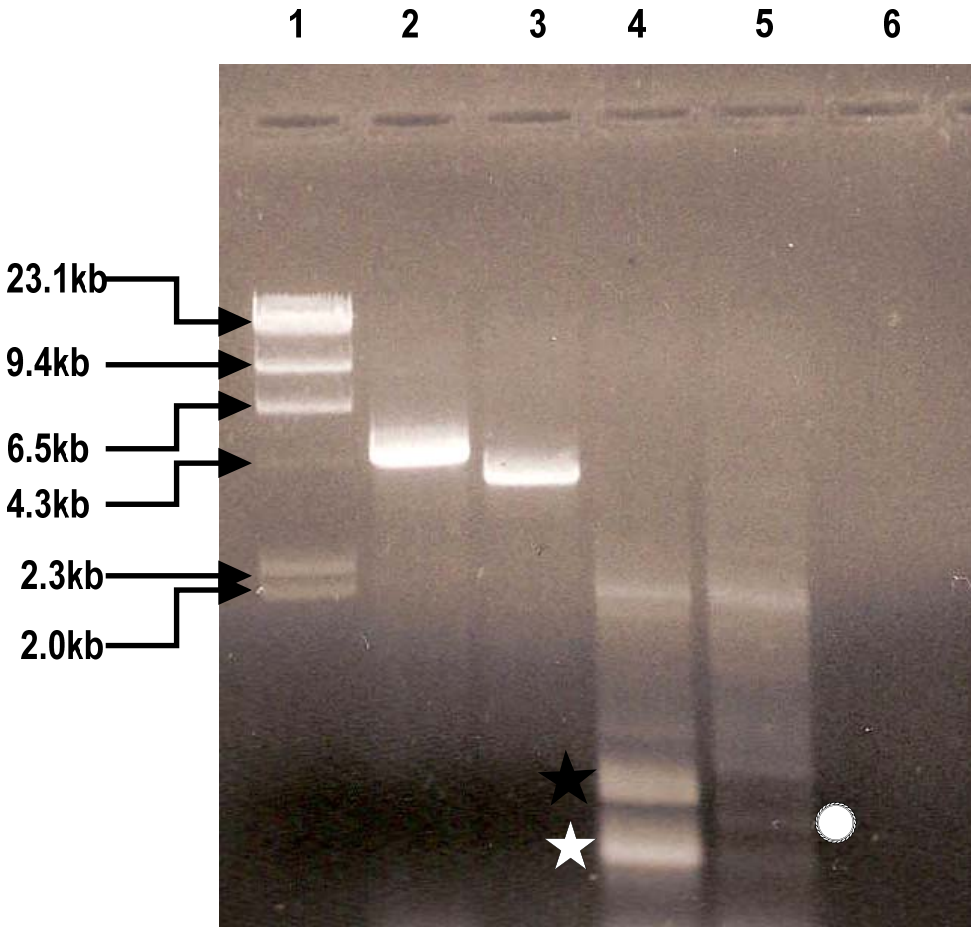


Figure 4.8 Agarose gel electrophoresis of positive and negative controls. Following long distance polymerase chain reaction, 2µl of amplicon sample was loaded on a 0.8% Agarose gel containing 1xTAE and 0.5mg/ml ethidium bromide. Electrophoresis was carried out at 100mA at ambient room temperature. Lane 1 is the Roche DNA molecular marker II. Lane 2 is the CA-46 LD-PCR amplicon using the MYC- Cα primer pair. Lane 3 is the CA-46 LD-PCR product using the MYC- Cγ primer pair. Lane 4 is the CA-46 LD_PCR product using the MYC- Cµ primer pair. The stars on lane 4 show two different bands using this primer pair. Lane 5 is the CA-46 LD-PCR product using MYC- J_H primer pair. The circle shows the band which was much more visible on the gel than it is on the current photograph. Lane 6 is DAUDI LD-PCR using the MYC- Cα primer pair. This primer pair yielded no product as expected.

4.12 The t(8;14) LD-PCR analysis of patient DNA.

Each patient LD-PCR analysis included positive and negative controls for the t(8;14) translocation. In addition to the tPA LD-PCR analysis, each patient t(8;14) LD-PCR was performed 4 times with each of the four MYC-IgH primer pairs.

Following amplification, 2µl of the LD-PCR product was run on an agarose gel and analyzed for DNA degradation and product size. Figure 3.1 illustrates the t(8;14) translocation and positions of primers used in this study. As can be seen in this illustration, the products of the various primer sets have different lengths and therefore can be easily distinguished. It can be deduced from this figure that , depending on the position of the breakpoints, the MYC-JH primer product should produce the shortest amplicon fragment size and the MYC-Cα product should be the longest fragment size.

The results of the t(8;14) translocation and tPA LD-PCR analysis of patient DNA are tabulated in Table 4.8. The tPA LD-PCR was positive in all 41 patients. In some instance, this positivity was confirmed on more than one occasion when repeat analysis was indicated.

Only 6 of the 41 BL patients were positive for the t(8;14) translocation using all four primer sets. Two of these involved the JH region and there were two in the Cµ region. One occurred in each of the Cα and Cγ regions. Attempts to correlate these patients PCR results with clinical outcomes were unsuccessful as 4 of the six patients had died and the archived clinical notes were incomplete in 3 of the six patients.

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Table 4.8 LD-PCR results using t(8;14) and tPA primers.

Patient number	tPA LD-PCR result	t(8;14) LD-PCR result	Region involved	t(8;14) LD-PCR fragment size (kb)
BL1	+	-		
BL2	+	-		
BL3	+	-		
BL4	+	-		
BL5	+	-		
BL6	+	+	J_H	1.0
BL7	+	-		
BL8	+	-		
BL9	+	-		
BL10	+	-		
BL11	+	-		
BL12	+	-		
BL13	+	-		
BL14	+	-		
BL15	+	-		
BL16	+	-		
BL17	+	-		
BL18	+	+	J_H	1.2
BL19	+	-		
BL20	+	-		
BL21	+	-		
BL22	+	-		
BL23	+	-		
BL24	+	-		
BL25	+	-		
BL26	+	+	Cμ	0.7; 1.5
BL27	+	-		
BL28	+	-		
BL29	+	-		
BL30	+	+	Cμ	0.7
BL31	+	+	Cγ	3.6
BL32	+	-		
BL33	+	-		
BL34	+	-		
BL35	+	-		
BL36	+	-		
BL37	+	-		
BL38	+	-		
BL39	+	-		
BL40	+	-		
BL41	+	+	Cα	4

CHAPTER 5

5.0 DISCUSSION AND CONCLUSION

5.1 General discussion.

The most frequent genetic alteration in BL, accounting for 75-85% of the total, is the t(8;14)(q24;q32) translocation in which the MYC gene is juxtaposed to the IgH on chromosome 14 in divergent orientation (Basso, Frascella et al. 1999; Mussolin, Basso et al. 2003). Whilst PCR has revolutionarized the diagnostic approach of many lymphoproliferative disorders, the lesions in BL have not been amenable to analysis by this technique. The major limitation has been the distance between the breakpoint in BL being far so apart such that they not amplifiable by using standard Taq polymerase enzyme which can amplify up to 3kb DNA segments. Recently, DNA polymerases with effective amplification of longer targets have been discovered (Hinnisdaels, Del-Favero et al. 1996). With these new high fidelity enzyme mix it is now possible to characterize the t(8;14) translocation in BL.

In this retrospective molecular analysis, LD-PCR was established and optimized for the t(8;14) translocation using the commercially available LD-PCR system and BL lymphoma cell lines. The t(8;14) LD-PCR was then applied to a retrospective analysis of BL cases diagnosed in a period of 10 years from 1994 to 2003.

5.2 Analysis of controls.

In the establishment of a new assay, the critical importance of including controls cannot be overemphasized.

Appropriate BL controls were sourced from two cell lines, namely, the CA-46 with the t(8;14) translocation (positive control) and the Daudi cell line without the t(8;14) translocation (negative). Culture of the two cell lines was reproducible and their growth and cytogenetic characteristics were confirmed. There were no additional chromosomal aberrations noted in the subcultured CA-46 and Daudi cell lines. It is noteworthy that apart from the t(8;14) translocation, the original CA-46 description from ATCC included other cytogenetic lesions such as 13q+, +16 and duplication of chromosome 1 which were absent in the cell line stock we obtained. The reasons for this are uncertain.

The optimization of the t(8;14) assay was performed using the BL cell line DNA. As shown in Figure 4.4, high molecular weight normal DNA was also extracted. Normal DNA would have been insufficient to use alone in the absence of a t(8;14) positive control. High molecular weight non-degraded DNA was obtained from both patient samples and control cell lines as shown in figures 4.4 and 4.5. Unlike standard PCR in which almost any size DNA segment can be amplified, it was important to have large DNA segments for longer product synthesis in this LD-PCR assay. Therefore, the validation of DNA integrity and quality check was one of the critical steps in the analysis.

As a result of this DNA quality concern, a large number of extracted DNA could not be used for subsequent analysis. Of the 74 BL patient samples in which DNA was extracted, only 41 passed this important validation step and were used for the subsequent LD-PCR assay. In the 23 BL DNA samples that could not be used, there were some that were completely degraded as shown in lanes 3, 4, 6 and 7 of figure 4.5. Most of this degradation would appear to have occurred during storage of the bone marrow slides rather than during sample processing in this assay. This was

confirmed by analysis of the pattern of degradation which showed that it was more common in older slides and less common in recently stored bone marrow slides. Although DNA is known to be stable, storage degradation was not completely unexpected as all slides were stored uncovered at room temperature.

In the LD-PCR assay setup, the tPA LD-PCR gene analysis was included as an internal control. This served two purposes, namely, to establish the integrity and suitability of DNA for the assay,to exclude inhibition and also to validate the LD-PCR assay. Any sample in which the tPA gene could not be amplified was therefore excluded. As illustrated in figure 4.8, the expected tPA LD-PCR product was approximately 4.7 kb in this analysis. This is well within the expected published range of 4-7kb. The tPA LD-PCR band had a slightly different electrophoretic mobility to the MYC-Cα primer product and therefore the tPA LD-PCR assay could be run in the same tube in a multiplexing reaction as shown in figure 4.7. For simplicity and cost saving, the tPA assay was done first in all samples and if successful, the same DNA sample and reaction conditions were repeated with the t(8;14) primers. Both the negative and positive control DNA sample were successfully amplified with the tPA primers. As illustrated in table 4.8 all patient DNA was also amplifiable with the tPA primers confirming the integrity of the DNA specimens and LD-PCR assay.

In addition to the above controls, each LD-PCR assay had a blank tube included with each run. The blank control had all the assay reagents except for the template DNA. This was mainly done to detect contamination and non-specific exogenous DNA amplification. The inclusion of blank analysis is shown in lane 5 of figure 4.6 During the patient and control LD-PCR analyses, no blank tube assay was ever reported positive. This is an indication that consistency and reproducibility of the assay conditions was maintained and exogenous DNA contamination was well controlled.

The ideal scenario would have been for each of the t(8;14) primers to have their own positive controls. The CA-46 cell line breakpoint was amplifiable with the MYC-Cα primer set. In the absence of suitable cell lines for the other three primer sets, it was felt that this would not be a problem in this retrospective analysis as all specimens available would be analyzed and positive patient DNA specimens would then be used as controls in future analyses.

5.3 The t(8;14) LD-PCR analysis.

Following appropriate selection and control analysis there were 41 BL patient DNA samples which could be evaluated by the t(8;14) LD-PCR technique. Five LD-PCR analysis were performed on each of these DNA samples using first the tPA primers and then each of the four t(8;14) primer sets. In effect 164 different LD-PCR assays were performed with t(8;14) primers on the 41 BL specimen. With repeat analysis and inclusion of controls, well over 300 LD-PCR analyses were performed in the analysis.

In the presence of appropriate controls, only 6 of the 41 t(8;14) LD-PCR analyses yielded a product confirming rearrangement of the BL derived patient DNA. Each of these positive analyses was repeated at least once and therefore this step ruled out the possibility of false positive results and/or DNA contamination. As shown in table 4.8, two of these amplifications were with the MYC-JH primers and another two with the MYC-Cμ primers. Amplification with primers MYC-Cγ and MYC-Cα yielded was positive for one patient each.

There was no obvious correlation of the t(8;14) LD-PCR positivity with age, gender, HIV status, extent of bone marrow involvement or quality of DNA.

In subject BL26, there was a correlation between the cytogenetic and PCR results which were both postive. Sublects BL 30 and BL 31 yielded a positive result on LD-PCR but failed to demonstrate t(8;14) on conventional cytoenetics. This discrepancy might reflect the limitations of cytognetics which is reliant on fresh material with vialbe cells. On the other hand there were four cases which were LD-PCR negative but positive for the t(8;14) on cytogenetics. The reasons for this discrepancy are not clear but could include variable breakpoints which are not amenable to PCR amplification.

The MYC-Cα primer product size was the largest as predicted from the schematic representation shown in figure 3.1. This MYC-Cα primer product size was comparable to that obtained with CA-46 cell line in other studies (Akasaka, Ohno et al. 1997; Basso, Frascella et al. 1999).

Although there were no positive controls, the MYC-Cγ, MYC-Cμ and MYC-JH product sizes were in line with the predicted sizes on the schematic diagram.

LD-PCR analysis of patient BL26 DNA yielded two products with the MYC-Cμ primer pair. The significance of these two bands is uncertain. They were reproducible on repeat analysis using a different sample of the same DNA with the same primer pair. Although also possible, the possibility of primer dimerization was considered highly unlikely. Ideally the specificity of these bands should have been confirmed on southern blot analysis using a myc-specific probe.

The majority of patients (35 out of 41) with BL lymphoma in this study were negative for the expected t(8;14) translocation. The reasons for this finding are not clear but a number of possibilities exist.

It is possible that for this cohort of BL all the breakpoints are far upstream exon 2 of the MYC gene such that they are missed by the exon 2 specific upstream primer. As shown in Figure 1.2 these would be possible if the epidemiologic variants were largely eBL as opposed to sBL or AIDS-BL. The fact that Johannesburg is not regarded as a BL endemic region makes hypothesis highly unlikely. Most of the patients studied were indigenous South Africans by first and second name criteria which make the alternative explanation due to migration also less likely.

Similarly, the breakpoints could be clustered far downstream of the immunoglobulin gene such that they are not picked up by the immunoglobulin primers used. Unlike the 5' end of the MYC gene in which many breakpoints up to 300kb have been characterized (Yano, Sander et al. 1993; Veronese, Ohta et al. 1995; Hoglund, Johansson et al. 1996; zur Stadt, Hoser et al. 1997; Felix and Jones 1998), breakpoints 3' to the IgH gene have all been consistently shown to occur within the switch regions of the various immunoglobulin constant region domains (Adams, Gerondakis et al. 1983; Bernard, Cory et al. 1983; Pelicci, Knowles et al. 1986; Haluska, Tsujimoto et al. 1988; Shiramizu and Magrath 1990; Shiramizu, Barriga et al. 1991)

It is possible that BL in South Africa do have a different breakpoint to those described in Europe or America. Gutiirrez et al analyzed chromosomal breakpoints in 39 cases from South America and shosed that the majority of breakpoints on chromosome 8 occureed in the immediate flankng region of c-myc. These breakpoint were 5' of the typical sporadic breakpoint in the first intron/exon region and 3" of the typical breakpoints described in eBL(Gutierrez, Bhatia et al. 1992)

The possibility that the low yield of t(8;14) could also be due to a technical problem was also considered. The primers with more than one positive result in the t(8;14) analysis are probably specific and functional. Those primers with just one positive result and no positive control make this possibility plausible. The large number of controls and consistently produced internal controls make the reagents and analytical process unlikely explanations. The template DNA in all instances was of high quality and integrity.

5.4 Limitations of the study.

The design of this study, being a retrospective analysis, was a major limitation. Although analysis from stored slides would be an advantage, this storage is associated with gradual deterioration of template DNA for analysis. A large number of slides had varying degrees of DNA degradation with up to 23 BL excluded on the basis of the poor quality of the DNA. In addition to poor quality, not all BL slides were available for analysis.

The fact that there were no positive controls for some the t(8;14) primer sets is also a limitation. Ideally all primer amplification products should be correlated with appropriate positive and negative controls. At the time of study initiation, cell lines with positive controls for the Cμ, Cγ and JH regions were not easily obtainable. Reliance on obtaining patient positive control is probably acceptable but not the best practice. Other studies such that by Basso etal (Basso, Frascella et al. 1999) have used cell lines which were positive for all the regions of the IgH as appropriate controls. These cell lines are not easily available and efforts to obtain at the time of the study proved fruitless.

Each analysis had to be done five times which made this LD-PCR particularly labour intensive. The impact would be particularly evident if one or two samples are analyzed as opposed to the 41 samples that were done at the same time in this study. Sequential analysis of the samples with different primer sets would be an alternative however this option impacts negatively on analytical turnaround time.

Ideally, positive samples should have been confirmed with sequencing or Southern blot analysis and hybridizaton to a MYC oligonucleotide probe. This would have conclusively confirmed the specificity of the amplicon fragments obtained with LD-PCR analysis. The analytical costs and labour intensive nature of southern blot analysis and sequencing precluded this essential step.

The biggest limitation by far is the inability of this assay in our hands to confirm the t(8;14) translocation in BL with an otherwise well established diagnosis. Whether this represents a variant of BL requiring a completely different primer set or due to any of the reasons discussed above remains uncertain. What is certain however is that this LD-PCR in its current form cannot be used for routine diagnosis of BL. By implication this excludes its utility in minimal residual disease detection and minimal disseminated disease detection.

The inability of LD-PCR to pick up the BL translocation is not unique to this study. None of the previous studies have been able to identify all known BL. In the three studies reviewed in chapter 1, the diagnostic sensitivity of LD-PCR was variable ranging from 55% to 87% (Shiramizu, Barriga et al. 1991; Akasaka, Ohno et al. 1997; zur Stadt, Reiter et al. 1997; Mussolin, Basso et al. 2003). A significant proportion of BLs could not be diagnosed using LD-PCR in these studies.

5.5 Alternative diagnostic modalities for BL.

The last study on t(8;14) LD-PCR was published in 2003 (Mussolin, Basso et al. 2003). The limited number of studies published and absence of further studies on the diagnostic utility of the t(8;14) LD-PCR in the face of growing popularity of the PCR technique could perhaps be an indirect indicator of the poor diagnostic utility of this approach. In the interim, a number of other diagnostic tools for the t(8;14) lesions are beginning to emerge and these include the Fluorescence in situ hybridization (FISH) and microarray gene profiling analyses.

Fluorescence in situ hybridization (FISH) analysis for the t(8;14) has become a popular tool for studying cytogenetic lesions in BL. The advantage of FISH in BL analysis is its ability to identify not only the t(8;14) but also the t(2;8) and t(8;22) translocations. In a recent analysis of fixed paraffin embedded tissue sections for t(8;14) , 100% of the translocations were identified by an automated FISH procedure. (Reichard, Hall et al. 2006). Although FISH lacks the sensitivity of PCR in the detection of minimal residual disease, this technique is likely to be the tool of choice for diagnosis of cytogenetic lesions in BL.

More recently, microarray gene profiling using BL molecular signatures has also become available. Although experience with BL gene profiling is still at its infancy and limited, evidence from recent studies suggest that BL microarray analysis could provide superior molecular subtyping of BLs compared to current morphologic and immunophenotypic approaches (Dave, Fu et al. 2006; Hummel, Bentink et al. 2006). The BL molecular signature is also useful in accurately distinguishing BL from DLCL, an important distinction to be made for planning therapeutic intervention in these conditions. The biggest advantage of the microarray approach is its ability to correctly

identify all BL including those with atypical morphology as well as those without demonstrable myc-immunoglobulin BL translocations (Dave, Fu et al. 2006; Hummel, Bentink et al. 2006). The latter is particularly important in the context of this LD-PCR study which failed to demonstrate the t(8;14) in the majority of known BLs. It is possible that the t(8;14) translocation may be inferior to the BL molecular signature for the diagnosis of all subtypes of BL.

5.6 CONCLUSION.

In this molecular analysis of BL, LD-PCR was established and optimized using t(8;14) positive and negative BL cell lines. Subsequent retrospective analysis of t(8;14) translocation resulted in an extremely poor yield in a sample of known BL. In our hands, t(8;14) LD-PCR performance is unacceptably poor, labour intensive and costly making it unsuitable for routine diagnostic use. Other diagnostic tools such as FISH and microarray analysis are starting to show superior diagnostic yields and could become the tools of choice for BL diagnosis, prognostication and therapeutic monitoring in future.

APPENDIX A: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Mahlangu

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M00/05/12

PROJECT Molecular Analysis In Burkitt's Lumphoma

INVESTIGATORS Dr JN Mahlangu

DEPARTMENT Molecular Medicine & Haema, SAIMR

DATE CONSIDERED 00/05/26

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 00/05/29 **CHAIRMAN**.....(Professor P E Cleaton-Jc

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr W Stevens
Dept of Molecular Medicine & Haema, SAIMR

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