TRANSLOCATION (14;18) AS A MARKER FOR DETECTION OF MINIMAL DISEASE IN FOLLICULAR NON-HODGKIN'S LYMPHOMA.

TRANSLOCATIE (14;18) ALS EEN MERKER
VOOR DE DETECTIE VAN MINIMALE ZIEKTE
BIJ PATIENTEN MET EEN FOLLICULAIR NON-HODGKIN'S LYMFOOM.

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CONTENTS

ABBREVIATIONS

ABMT	autologuos bone marrow transplantation
ALL	acute lymphoblastic leukemia
BCL-2	B-cell lymphoma 2
BM	bone marrow
BSA	bovine serum albumine
C	constant
cb	centroblastic
cc	centrocytic
CCR	clinical complete remission
CD	cluster of differentiation
cDNA	copy DNA
CED	clinical evidence of disease
c-Ig	cytoplasmic immunoglobulin
CML	chronic myeloid leukemia
CVP	Cyclophosphamide Vincristine Prednisone
D	diversity
DNA	deoxyribonucleic acid
GM-CSF	granulocyte/macrophage colony-stimulating factor
FISH	flourescent *in situ* hybridization
FITC	flourescein isothiocyanate
α-IFn	interferon-α
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
Im	immunological marker analysis
J	joining
kb	kilo base
kDa	kilo Dalton
LN	lymph node
mbr	major breakpoint region
mcr	minor breakpoint region
Mo	morphological examination
MRD	minimal residual disease
mRNA	messenger RNA
NCED	no clinical evidence of disease

NHL	non-Hodgkin's lymphoma
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph	Philadelphia
RNA	ribonucleic acid
RT	radiotherapy
RT-PCR	reverse transcriptase mediated PCR
S	switch
s-Ig	surface immunoglobulin
t(14;18)	chromosomal translocation (14;18)
TdT	terminal deoxynucleotidyl transferase
TRITC	tetramethylrhodamine isothiocyanate
V	variable

CHAPTER 1

Introduction

1. Non-Hodgkin's Lymphomas.

1.1 Clinical and phenotypical aspects of non-Hodgkin's lymphomas.

Non-Hodgkin's lymphomas (NHL) represent a diverse group of clinical and pathological tumors and are the most common hematological malignancies in Europe and the USA (1,2). In the Netherlands approximately 1800 patients are affected each year (2). NHL may originate in B- or T- lymphocytes but are usually B-lymphocytes (3,4). The NHL cells contain the phenotypic properties of the normal cellular counterparts with respect to the type (B- or T- lymphocytes) and their differentiation stage. The similarities between NHL cells and normal lymphocytes are the basis for the immunophenotypical classification of NHL, e.g., the Kiel classification (5).

Follicular NHL, one subtype of NHL, accounts for approximately 20% of all NHL and is subject of investigation in this thesis (2). Follicular NHL is a B-cell malignancy and consists of monoclonal B-lymphocytes. The cell types of follicular NHL resemble the cells in the secondary germinal center of lymph nodes and tonsils, small cleaved cells (centrocytes) and large cells (centroblasts) (4,5). The neoplastic cells preferentially home to the germinal centers and may show the same follicular structure. The structure in follicular NHL and normal tonsils is maintained by dendritic reticulum cells. Loss of these cells in follicular NHL may lead to a more diffuse growth pattern and transformation to a more malignant NHL (for review see 6,7). Follicular NHL has a relatively indolent course of disease and is therefore classified as low grade malignant NHL. It is seen predominantly in patients aged above 55 years (1). At diagnosis, only 10% to 20% of the patients present with stage I or stage II disease (8), which means that the disease is localized to one or two lymph node(s) stations either above or below the diaphragm. These patients are usually treated by involved field radiotherapy alone, resulting in longstanding clinical complete remissions and even cure (9-11). Most patients (80% to 90%) present with disseminated disease (stages III and IV). The disease can then be detected in lymph nodes, peripheral blood, bone marrow or other organs. These patients are either treated with mild chemotherapy immediately at diagnosis or treatment may be postponed until disease progression ("wait and watch") (12,13). Although most patients initially respond to therapy, at some point the disease will relapse and in the end, usually several years after diagnosis, most patients do succumb from the disease. In patients with follicular NHL, accurate staging at diagnosis and the detection of small numbers of NHL cells during and after treatment may be important in the therapeutic management of the disease. In particular when new methods to detect minimal (residual) disease become available. A variety of methods to

detect malignant cells exists and will be discussed in more detail in section 3 paragraph 1 of this chapter.

1.2 Translocations in non-Hodgkin's lymphomas.

Numerous hematologic malignancies are characterized by chromosomal abnormalities (for review see 14). The most common chromosomal translocations in B-cell NHL are the t(14;18)(q32;q21) which activates the B-cell lymphoma-2 (BCL-2) oncogene, and the t(8;14)(q24;q32) in which c-MYC is the target gene (7,14). Less common 14q32-associated translocations are t(2;14)(p13;q32) (15), t(3;14)(q27;q32) (16) t(10;14)(q24;q32) (17), t(11;14)(q13;q32) (18,19), t(14;17)(q32;q23), t(14;19)(q32;q13) (20) and t(14;22)(q32;q11). Other chromosomal translocations are t(2;5)(p23;q35) (21), t(2;18)(p12;q21) (22,23), t(3;22)(q27;q11) (24,25) and t(2;3)(p12;q27) (16). Identified genes which are found in these translocations will be presented in Table 1 section 3 paragraph 2.

One of the most common chromosomal translocations in B-cell NHL, the t(14;18)(q32;q21), is typically associated with follicular NHL and the subject of investigation in this thesis. The translocation is found in 60% to 85% of these NHL (26-32). The incidence of t(14;18) in follicular NHL is dependent on the technique used. The detection of t(14;18) is enhanced, in comparison to polymerase chain reaction (PCR) and cytogenetic analysis, by pulsed-field gel electrophoresis (32). The t(14;18) is also found in 20% to 30% of diffuse large cell NHL (33,34). Thus, t(14;18) may be used as a marker in follicular and diffuse large cell NHL. The molecular organization of the chromosomal translocation and the function of the BCL-2 gene have been subject of intensive research in recent years. This will be discussed in more detail in the section 2 of this chapter.

2. The chromosome translocation t(14;18).

2.1 The BCL-2 gene and encoded proteins.

The BCL-2 gene, involved in the chromosomal translocation t(14;18), is located on chromosome 18 band q21 and has a three exon structure (Figure 1: chromosome 18). The first exon is not translated (35). The relatively large intron 2 exists of 225 kilobases (kb)(36). Three BCL-2 transcripts, 3.5, 5.5 and 8.5 kb long, have been identified (37). All transcripts contain exon 2 sequences, but the 5.5 and 8.5 kb transcripts contain also exon 3 sequences. These last two transcripts use different polyadenylation sites.

Two BCL-2 protein products have been identified, the 26 kilodalton (kDa) BCL-2α derived from the 8.5 or 5.5 kb transcripts and the 22 kDa BCL-2ß derived from the 3.5 kb transcript (37,38). These proteins differ in their carboxy terminus as a result of alternative splice site selection (37,38). The 26 kDa BCL-2α protein has a hydrophobic carboxy terminal membrane spanning domain and is located in the outer mitochondrial membrane (39). The 22 kDa BCL-2ß protein lacks the hydrophobic membrane spanning domain and is therefore not membrane associated (40).

2.2 The immunoglobulin heavy chain gene.

During differentiation of the hematopoietic stem cell into a B-lymphocyte, the immunoglobulin genes rearrange in an ordered manner. The rearrangement of the different segments of the immunoglobulin heavy (IgH) chain gene occurs first (41-45). The IgH gene is located on chromosome 14 (q32) (Figure 1: chromosome 14). Rearrangement of different segments of this gene may result in a functional heavy chain of the immunoglobulin. Antigen variability is created following the assembly of the variable (V), the diversity (D) and joining (J) segments of the IgH gene and the addition of several bases during this assembly (46,47). There are 100 to 200 V segments clustered in 7 families (48-50), at least 24 D segments clustered in 9 families (51-54) and 6 J segments (55). The first event of the IgH rearrangement is the D-J joining and subsequently the joining of one V segment to this complex. These rearrangement processes are mediated by a sequence specific recombination mechanism (56). The constant (C) region segments of the IgH gene are located downstream of the J segment and encode five different immunoglobulin classes (IgM, IgD, IgG, IgA and IgE). Upstream of most C segments a switch (S) segment is found, which plays a role in a late event in the generation of a functional IgH molecule, the class switch (57,58). The IgH gene is involved in the t(14;18).

2.3 Molecular characteristics of the translocation t(14;18).

In the translocation t(14;18), either the J or D segment of the IgH gene (14q32) is juxtaposed to the BCL-2 gene (18q21) (26,30,31,59-61) (Figure 1: translocation t(14;18)). This places BCL-2 in the same transcriptional orientation as the IgH locus. Breakpoints on chromosome 14 are usually located just 5' of one of the J segments, predominantly J3-J6 (62). Two breakpoint clusters have been identified within the BCL-2 gene, the major breakpoint region (mbr) spanning 150 base pairs (bp) (30,31,60,63) and the minor cluster region (mcr) spanning 500 bp (64-66). The molecular characteristics of both the mbr and mcr translocations allow the application of the highly sensitive and specific polymerase chain reaction, PCR, an *in vitro* DNA

amplification technique (63,66,67, chapters 2, 3 and 4). The size of the t(14;18) specific PCR fragments depend on the localization of the breakpoints on chromosomes 14 and 18. Therefore, the size of these fragments are usually different in individual patients. The majority (60% to 70%) of the breakpoints are in the mbr, located in the non-coding region of exon 3. 20% to 30% of the breakpoints are located in the mcr, at least 20 kb downstream from the mbr and outside the BCL-2 gene (64,66). In a minority of the patients the breakpoint is located 5' of the BCL-2 gene (64).

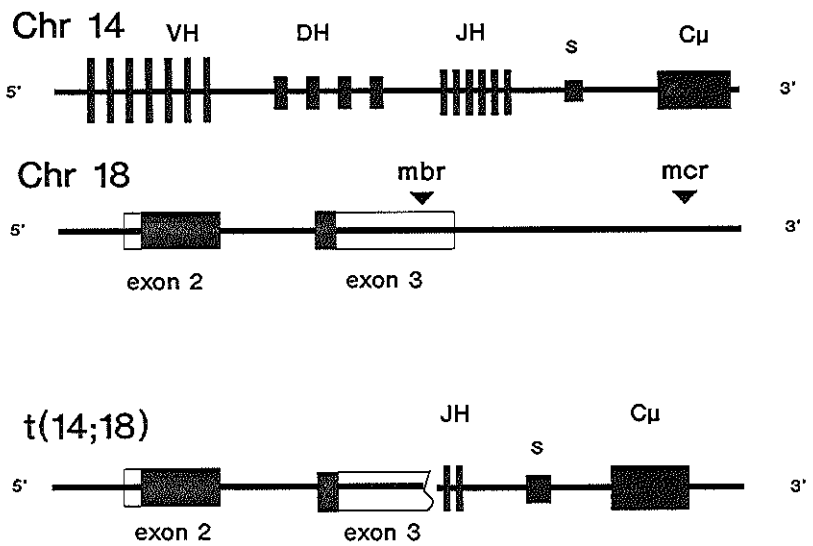

Figure 1: Schematical representation of the genomic organization of the chromosomes 14, 18 and the translocation t(14;18). The variable (V), diversity (D), joining (J), switch (S) and constant (C) segments of the immunoglobulin heavy chain gene are located on chromosome 14. The oncogene BCL-2 is located on chromosome 18. The two coding exons (2 and 3) and the breakpoint regions (mbr and mcr) are designated. The breakpoints of the t(14;18) are in this figure located just 5' of the JH5 segment and in the mbr of the BCL-2 gene (not drawn at scale).

2.4 Possible recombination mechanisms in the translocation t(14;18).

The clustering of the breakpoints, especially in the mbr, suggests a sequence specific recombination mechanism. Heptamer / spacer / nonamer recognition sequences are involved in the recombination of the VDJ or VJ segments of the IgH and T-cell receptor genes (56). Such sequences have also been reported to be located near the mbr of the

BCL-2 gene, although there are some sequence differences (68). These recombination signals can account for the aberrant joining of the BCL-2 gene to one of the J segments. Therefore, t(14;18) is thought to occur as a mistake in VDJ joining (68).

2.5 FUNCTION OF BCL-2.

The translocation t(14;18) places the BCL-2 gene under control of IgH regulatory sequences, inducing high expression levels of the BCL-2-Ig fusion RNA (35,64,69,70) and the normal 26 kDa BCL-2α protein (70). The function of BCL-2 has been elucidated in part by hematopoietic cell lines studies. BCL-2 cDNA has been transfected into interleukin-2 (IL-2), IL-3, IL-4, IL-6 and granulocyte/macrophage colony-stimulating factor (GM-CSF) dependent hematopoietic cell lines (71-73). Deprivation of the essential growth factor normally induces programmed cell death (apoptosis) in these growth factor dependent cell lines. In cell lines transfected with BCL-2, enhanced cell survival has been reported, i.e., prevention of programmed cell death following withdrawal of IL-3, IL-4 and GM-CSF. In an IL-3 dependent multipotent hematopoietic cell line this increased cell survival is accompanied by multilineage differentiation in the absence of IL-3 (73). The capacity of BCL-2 to inhibit cell death has also been reported for gamma irradiation induced apoptosis. Gamma irradiation causes the production of oxygen free radicals (39). Oxygen free radicals induce damage to DNA, proteins and lipids (39). BCL-2 functions in an antioxidant pathway to prevent cellular damage, especially damage caused by lipid peroxidation (39).

The BCL-2 gene is the first described oncogene which interferes with programmed cell death in mammalian cells. Recently, several genes with BCL-2 related sequences have been identified (74-79). One of those genes, the BCL-x gene has a role in both positive and negative regulation of apoptosis in a growth factor dependent cell line. Two protein products of this gene BCL-x_s and BCL-x_l with opposite functions have been identified (75,76). BCL-x_l is similar in size, structure and function to BCL-2. BCL-x_s differs from BCL-x_l because it lacks the sequences that display greatest similarity between BCL-x_l and BCL-2. BCL-x_s encodes a protein that inhibits the ability of BCL-2 to prevent apoptosis. Also a gene of a BCL-2 associated protein, Bax, has been identified (75,77). The 21 kDa Bax protein shows extensive amino acid homology with BCL-2. Overexpression of Bax neutralizes BCL-2 and accelerates apoptotic cell death induced by IL-3 deprivation in a IL-3 dependent cell line (75,77).

Functional aspects of BCL-2 gene expression have also been studied in transgenic mice. The BCL-2-Ig fusion gene of the chromosomal translocation t(14;18) has been used to

create BCL-2-Ig transgenic mice and the effect of t(14;18) was studied during development. Transgenic mice showing overproduction of BCL-2 had a long-term persistence of immunoglobulin secreting cells and an extended lifetime of memory B-cells (80). These transgenic mice developed lymphadenopathies infiltrated with polyclonal mature B cells (81). After a long latency period progression from a polyclonal to a monoclonal B-cell population and transformation to clinical lymphoma was apparent in these transgenic mice (82). Progression towards the malignant NHL was associated with additional c-*myc* rearrangements (82,83).

Despite widespread embryonic expression of the BCL-2 protein, transgenic mice carrying two inactivated BCL-2 genes develop normally but display polycystic kidney disease and hypopigmented hair (84,85). Lymphocyte differentiation is initially normal, but no stable immune system is generated after birth (84,85). The presence of polycystic kidney disease and hypopigmentation of hair in these mice, pathologies which are probably related the redox state of cells, is in concordance with the role for BCL-2 in an antioxidant pathway (39,85).

2.6 BCL-2 PROTEIN EXPRESSION IN NON-HODGKIN'S LYMPHOMAS.

Expression of BCL-2 protein has been reported in normal lymphoid cells and a variety of lymphoproliferative disorders (70,86). The BCL-2 protein has been demonstrated in precursor cells of all hematopoietic lineages, activated- and memory- B-cells, and plasma- and mantle zone- B-cells of follicle centers in human tonsils (70,86-89). In the follicle centers of human tonsils the BCL-2 protein could not be demonstrated (70,87,90 chapter 3). In follicular NHL carrying the t(14;18), the BCL-2 protein is always found (70,90,91, chapter 3). In addition, in follicular NHL without the translocation an equally intense BCL-2 protein expression has been demonstrated (70,92 chapter 3). In other histological subtypes of NHL, t(14;18) and BCL-2 protein expression may be present (70,87,93,94). In diffuse NHL t(14;18) may occur in 20% to 30% of the lymphomas (33,34). BCL-2 expression has been reported in a variable proportion (20% to 80%) of diffuse NHL and is not restricted to diffuse NHL carrying the t(14;18) (86,90,91,93). NHL of the gastro-intestinal tract, a neoplasm in which the t(14;18) occurs infrequently, may express the BCL-2 protein in 50% to 75% of the lymphomas (93,94). In lymphomas of mucosa-associated lymphoid tissue (MALT), presence of the BCL-2 protein and absence of t(14;18) has been reported (87). These findings suggest that there may be a role for BCL-2 in the development of NHL in general and follicular NHL in particular.

3. Detection of minimal (residual) disease.

3.1 Different methods for the detection of malignant cells.

Detection of small numbers of malignant cells is potentially useful for i) staging of the disease at initial diagnosis, ii) monitoring of the disease during and after therapy and iii) early detection of relapse. Minimal residual disease (MRD) is defined as the few malignant cells which have survived initial remission-induction chemotherapy (95). The persistence of these cells becomes apparent because they may cause relapse following variable intervals (95). There are several methods to detect small numbers of malignant cells in patients with hematological malignancies. The possibilities and limitations of methods to detect MRD depend on specificity (malignant cells have to be discriminated from normal cells) and sensitivity (detection limit). Many of the conventional techniques, such as morphological examination and immunological marker analysis are not specific for malignant cells and relatively insensitive. Certain cytogenetic and DNA techniques are highly specific but insufficiently sensitive. Recently, a new specific and sensitive DNA technique has been developed which may be used for the detection of MRD. Here, we summarize the methods that are currently used for the detection of minimal neoplastic disease:

a) Detection of malignant cells by morphological examination is insensitive and not specific. In order to identify malignant cells, at least 5% of the cell population must be morphologically distinguishable as malignant cells (96). Therefore, this method has limited value for the detection of MRD.

b) Immunological marker analysis is more sensitive than morphological examination. Malignant lymphoid cells express the same markers as their normal counterparts. Expression of unusual immunological markers is rarely seen. Therefore, lymphoid cells positive for certain immunological markers or marker combinations may indicate malignancy either when these cells are present at a greater frequency than normal or when they are present outside their normal homing areas. Specificity is dependent on the choice of immunological markers. Demonstration of a monoclonal B-cell population by double fluorescent staining for both the immunoglobulin heavy and light chains may be used in NHL. With this method a sensitivity of 0.1-1% can be reached (97,98, chapter 2). The greatest sensitivity of immunological marker analysis (0.01-0.001%) has been demonstrated by double immunofluoresence staining in T-cell acute lympoblastic leukemia using T-cell specific markers in combination with terminal deoxynucleotidyl transferase (TdT) (99,100). The normal counterparts of these double positive cells are rare in bone marrow and peripheral blood (100).

Detection of double positive cells in peripheral blood or bone marrow in those diseases, in which the combination of markers is rare in normal conditions, provides a very sensitive and specific method for the study of MRD.

c) Cytogenetic analysis using banding techniques can detect chromosomal abnormalities unique of the malignancy (101). With this technique only a limited number of cells in metaphasis (usually less than 40) can be evaluated. Although this technique is highly specific for the malignancy, the small cell sample size which can be evaluated reduces the applicability of this technique for the detection of MRD (101).

d) Fluorescent *in situ* hybridization (FISH) techniques can be used to detect chromosomal abnormalities such as the t(9;22) (102), the t(8;14) and t(14;18) (103), the t(1;7) (104) and translocations involving the chromosome 11q23 region (105). For t(9;22) two-colour FISH was carried out with probes specific for the Abelson oncogene of chromosome 9 and the BCR gene of chromosome 22 (102,106,107). This technique is highly specific for the malignancy and it can be applied to all cells of a population. However, the sensitivity of this technique is limited by the number of cells which can be evaluated and the statistical chance of overlapping signals.

e) Southern blot analysis can be used to detect chromosomal abnormalities, i.e. chromosomal translocations, and rearrangements of the antigen receptor genes of the B- and T- lymphocytes. Both chromosomal abnormalities and rearrangements of the antigen receptor genes are specific of the malignant clone. The sensitivity is about 5% (108-111).

f) The PCR is a technique in which DNA sequences are amplified *in vitro* using specific synthetic oligonucleotides (67). With these oligonucleotides a DNA sequence is defined, unique and specific of the malignant cell population. For instance, the detection of the chromosomal breakpoint regions, i.e., the translocation t(14;18), and rearrangement of the antigen receptor gene, representing the malignant cells may be used for detection of minimal numbers of neoplastic cells (100). With this technique a detection limit of 1 malignant cell among 10^5-10^6 normal cells has been reported (67,100). The specificity and the sensitivity make this technique promising for the detection of MRD.

g) Combining the high sensitivity of PCR with the cell localizing ability of *in situ* hybridization, *in situ* PCR, has been described for the detection of a variety of targets. Several RNA viruses and many human mRNAs have been detected using *in situ* reverse transcriptase mediated PCR (RT-PCR) (112,113). Application of this technique reveals that the frequency of virus infected mononuclear blood

cells is at least 10 times higher than previously described (112). This technique is also potentially promising for the detection of MRD.

3.2 Applicability of PCR in hematological malignancies.

The chromosomal translocation t(9;22)(q34;q11), which is seen in 95% of patients with chronic myeloid leukemia (CML) and in 30% to 40% of the adult acute lymphoblastic leukemia (ALL) patients, has been extensively studied by cytogenetic analysis and recombinant DNA techniques (100,106,107). In this translocation the 5' sequences of a gene designated BCR are fused to the 3' sequences of the Abelson oncogene (106,107). The translocation t(9;22) (also designated as the Philadelphia (Ph) chromosome) is used to study MRD. CML patients have been studied for the presence of t(9;22) after allogeneic bone marrow transplantation. In the majority of these patients t(9;22) is not detectable by cytogenetic analysis but with RT-PCR the t(9;22) can be demonstrated (114-119). Most patients show PCR detectable t(9;22)-positive cells during the first 6 months after transplantation (114,115). PCR-positivity for a longer period may indicate that these patients have not been cured (114-117). In T-cell depleted bone marrow transplantations more patients remain t(9;22) positive and this appears to correlate with the greater relapse frequency following T-cell modified transplants (115). In one study the t(9;22) has been demonstrated by PCR in approximately 55% of patients with adult common ALL. Detection of this translocation is correlated with remission duration and poor survival (116,118). The correlation between clinical outcome and PCR results compared to chromosomal analysis for t(9;22) is still under investigation (116). Chromosomal translocations which may be used as targets for PCR and for the study of MRD, are summarized in Table 1. Studies concerning the detection of MRD using PCR have been described for t(6;9) (121), t(8;21) (123-126), t(15;17) (130-132) and t(4;11)(q21;q23) (140,141). From these studies it is concluded that application of PCR in the *in vitro* amplification of the chromosomal breakpoint regions enhances the sensitivity by which MRD can be detected.

In NHL, especially follicular NHL, the chromosomal translocation t(14;18) is generally considered as a marker for NHL cells. The breakpoint of this translocation has been used for PCR detection of MRD in NHL patients in several studies (63,66,142-145, Chapters 2, 3 and 4). It has become apparent that t(14;18) should be utilized with caution as a marker for residual NHL cells because t(14;18) may also be identified among non-malignant lymphoid cells. The occurrence of t(14;18) not related to any malignancy has been reported in approximately 50% of the benign lymphoid tissues

Table 1: Chromosomal translocations in myeloid and lymphoid malignancies used for detection of minimal residual disease with PCR.

Chromosomal translocation	Disease	Genes	Target	References
t(6;9)(p23;q34)	acute myeloblastic leukemia	DEK, CAN	mRNA	14,120,121
t(8;21)(q22;q22)	acute myeloid leukemia	AML1, ETO	mRNA	14,122-126
t(9;22)(q34;q11)	chronic myelocytic leukemia, acute lymphoblastic leukemia	BCR, ABL	mRNA	14,106,107,114-119
t(15;17)(q22;q21)	acute promyelocytic leukemia	PML, RARα	mRNA	14,127-132
t(1;19)(q23;p13)	pre-B acute lymphoblastic leukemia	E2A, PBX1	mRNA	14,133-136
t(2;14)(p13;q32)	chronic lymphocytic leukemia	REL, IgH	DNA*	14,15
t(2;18)(p12;q21)	chronic lymphocytic leukemia, non-Hodgkin's lymphoma	Ig kappa, BCL-2	DNA*	14,22,23
t(4;11)(q21;q23)	childhood acute lymphoblastic leukemia	MLL, AF-4	mRNA	14,137
t(8;14)(q24;q32)	Burkitt's lymphoma	MYC, IgH	DNA*	14,138
t(10;14)(q24;q32)	B-cell non-Hodgkin's lymphoma	LYT10, IgH	mRNA	17
t(11;14)(q13;q32)	mantle cell lymphoma	BCL-1, IgH	DNA	14,139-142
t(14;18)(q32;q21)	B-cell non-Hodgkin's lymphoma	IgH, BCL-2	DNA	14,63,66,143-146
t(14;19)(q32;q13)	chronic lymphocytic leukemia, non-Hodgkin's lymphoma	IgH, BCL-3	DNA*	14,120

* PCR is possible if the breakpoint in each patient is cloned and characterized.

with follicular hyperplasia (146,147). In these conditions the frequency of t(14;18)-positive cells is extremely low and has been estimated at approximately one t(14;18)-positive cell per 10^5 lymphoid cells (146,147). These t(14;18)-positive cells in non-neoplastic tissue can be detected by PCR only. In order to search for residual t(14;18)-positive NHL cells and at the same time exclude the possibility that the t(14;18)-positive cells are not related to lymphoma, one would at least need the diagnostic lymph node biopsy from the patient, prior to treatment, to confirm that the t(14;18)-positive cells found in peripheral blood or bone marow are identical to the t(14;18)-positive cells of the lymph node biopsy. Studies related to this issue are reported in chapters 3 and 4.

In 20% to 30% of diffuse NHL the t(14;18) may also be used as a marker to study MRD. The study of MRD in diffuse NHL seems to be more clinical relevant than in follicular lymphoma. The reason for this is the cure rate of approximately 40% of the patients after conventional chemotherapy. For NHL and lymphoid malignancies lacking a characteristic translocation, the rearrangement of the antigen receptor genes may be used as a marker for the malignant cells. Rearrangement of the VDJ segments of the IgH gene and the addition of bases during the assembly, result in the generation of a highly specific and unique sequence for a single B-lymphocyte clone (44-56,148-157).

4. Aim of the study.

This study is concerned with the detection of small numbers of malignant cells in B-cell NHL, especially follicular NHL, using the translocation t(14;18) as a target for PCR. The possibilities of PCR in the detection of MRD are compared to those of other techniques (chapter 2). In chapters 3 and 4 t(14;18) has been used as marker for lymphoma cells. The clinical significance of the presence of residual t(14;18)-positive cells in peripheral blood and bone marrow has been studied in patients treated for localized and disseminated disease. The presence of t(14;18) and the BCL-2 protein was investigated in a different subtype of NHL, i.e., NHL with testicular localization (chapter 5). This has been studied in different subtypes of NHL, but had to our knowledge not been studied in NHL with testicular localization. Finally we have generated a new NHL cell line containing the chromosomal translocations t(8;14) and t(14;18). This cell line represents an addition to the few cell lines with the translocations t(8;14) and t(14;18) that are currently available (chapter 6). The availability of these cell lines may facilitate further studies aimed at the role of these translocations and thereby the absence of functional immunoglobulins in deregulating cell growth and maturation in NHL.

References.

1. Devesa SS and Fears T. Non-Hodgkin's lymphoma time trends: United States and international data. Cancer Res (suppl) 52: 5432-5440, 1992.
2. Incidence of cancer in the Netherlands: first report of the Netherlands Cancer registry. ISBN-number: 90-72175-06-9. Utrecht, 1990.
3. Jaffe ES. The role of immunophenotypic markers in classification of non-Hodgkin's lymphomas. Semin Oncol 17: 11-19, 1990.
4. Freedman AS and Nadler LM. Immunologic markers in non-Hodgkin's lymphoma. Hematol Oncol Clin North Am 5: 871-889, 1991.
5. Stansfeld AG, Diebold J, Noel H, Kapanci Y, Rilke F, Kelenyi G, Sundstrom C, Lennert K, van Unnik JAM, Mioduszewska O and Wright DH. Updated Kiel classification for lymphomas. The Lancet i: 292-293, 1988.
6. Jaffe ES, Raffeld M, Mediros LJ and Stetler-Stevenson M. An overview of the classification of non-Hodgkin's lymphomas: An integration of morphological and phenotypical concepts. Cancer Res (suppl) 52: 5447-5452, 1992.
7. Potter M. Pathogenetic mechanisms in B-cell non-Hodgkin's lymphomas in humans. Cancer Res (suppl) 52: 5522-5528, 1992.
8. Carbone PP, Kaplan HS, Musshoff K, Smithers DW and Tubiana M. Report of the Committee on Hodgkin's disease staging classification. Cancer Res 31: 1860-1861, 1971.
9. Gallagher CJ, Gregory WM and Jones AW: Follicular lymphoma: Prognostic factors for response and survival. J Clin Oncol 4: 1470-1480, 1986.
10. Gallagher CJ and Lister TA: Follicular non-Hodgkin's lymphoma. Bailliere's Clinical Hematology, international practice and research 1: 141-155, 1987.
11. Simon R, Durrleman S, Hoppe RT, Bonadonna G, Bloomfield CD, Rudders RA, Cheson BD and Berard CW: The non-Hodgkin lymphoma pathologic classification project: Long-term follow-up of 1153 patients with non-Hodgkin lymphomas. Annals of Internal Medicine 109: 939-945, 1988.
12. Horning SJ and Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphomas. N Engl J Med 311: 1471-1475, 1984.
13. Horning SJ. Treatment approaches to the low-grade lymphomas. Blood 83: 881-884, 1994.
14. Berger R. The cytogenetics of haematological malignancies. Bailliere's Clinical Hematology, the molecular genetics of haematological malignanacy 5: 791-814, 1992.
15. Lu D, Thompson JD, Gorski GK, Rice RR, Mayer MG and Yunis JJ. Alterations at the REL locus in human lymphoma. Oncogene 6: 1235-1241, 1991.
16. Bastard C, Tilly H, Lenormand B, Bigorgne C, Boulet D, Kunlin A, Monconduit M and Piguet H. Translocations involving band 3q27 and Ig gene regions in non-Hodgkin's lymphoma. Blood 79: 2527-2531, 1992.
17. Neri A, Chang C-C, Lombardi L, Salina M, Corradini P, Maiolo A, Changanti RSK and Dalla-Favera RB. B-cell lymphoma-associated chromosomal translocation involves candidate oncogene LYT-10, homologous to NF-kappaB p50. Cell 67: 1075-1087, 1991.
18. Withers DA, Harvey RC, Faust JB, Melnyk O, Carey K and Meeker TC. Characterization of a candidate BCL-1 gene. Mol Cell Biol 11: 4846-4853, 1991.
19. Williams ME, Meeker TC and Swerdlow SH. Rearrangement of the chromosome 11 BCL-1 locus in centrocytic lymphoma: Analysis with multiple breakpoint probes. Blood 78: 493-498, 1991.
20. Ohno H, Takimoto G and McKeithan TW. The candidate proto-oncogene BCL-3 is related to genes implicated in cell lineage determination and cell cycle control. Cell 60: 991-997,1990.
21. Mason DY, Bastard C, Rimock R, Dastudue N, Huret LJ, Kristofferson U, Magaud JP, Nezelof C, Tilly H, Vannier JP, Hemet J and Warnke R. CD30-positive large cell lymphomas ("Ki-1 lymphomas") are associated with a chromosomal translocation involving 5q35. Br J Haematol 74: 161-168, 1990.
22. Hillion J, Mecucci C, Aventin A, leroux D, Wlodarska I, van den Berghe H and Larsen C-J. A variant translocation t(2;18) in follicular lymphoma involves the 5' of BCL-2 and Ig kappa light chain gene. Oncogene 6: 169-172, 1991.
23. Rimock R, Gadoux M, Bertheas M-F, Berger F, Garoscio, Deleage G, Germain D and Magaud J-P. FVT-1, a novel human transcription unit affected by variant translocation t(2;18)(p11;q21) of follicular lymphoma. Blood 81: 136-142, 1993.
24. Leroux D, Stul M, Sotto JJ, Bastard C, Couderc P, Bensa JC, Monteil M, Cassiman JJ and Jalbert P. Translocation t(3;22)(q27;q11) in three patients with diffuse large cell lymphoma. Leukemia 4: 373-376, 1990.
25. Offit K, Jhanwar S, Ebrahim SAD, Filippa D, Clarckson BD and Chaganti RSK. t(3;22)(q27;q11): A

novel translocation associated with diffuse non-Hodgkin's lymphoma. Blood 74: 1876-1879, 1989.
26. Weiss LM, Warnke RA, Sklar J and Cleary ML. Molecular analysis of the t(14;18) chromosome translocation in malignant lymphomas. N Engl J Med 317: 1185-1189, 1987.
27. Rowley JD. Chromosome studies in the Non-Hodgkin's Lymphomas: The role of the 14;18 translocation. J Clin Oncol 6: 919-925, 1988.
28. Pezzella F, Ralfkiaer E, Gatter KC and Mason DY. The 14;18 translocation in European cases of follicular lymphoma: Comparison of Southern blotting and the polymerase chain reaction. Br J Haematol 76: 58-64, 1990.
29. Griesser H and Lennert K. Bcl-2 rearrangements in malignant B cell lymphomas. Br J Haematol 76: 142-143, 1990.
30. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around Jh on chromosome 14 and near a transcriptional unit on 18. Cell 41: 889-906, 1985.
31. Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. USA 82: 7439-7443, 1985.
32. Zelenetz AD, Chu G, Galili N, Bangs CD, Horning SJ, Donlon TA, Cleray ML and Levy R. Enhanced detection of the t(14;180translocation in malignant lymphoma using pulsed-field gel electroforeses. Blood 78: 1552-1560, 1990.
33. Aisenberg AC, Wilkes BM and Jacobson JO. The BCL-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 71: 969-972, 1988.
WB, Seto M, Boutain B, Goldman P and Korsmeyer SJ. Expression of BCL-2 and BCL2-Ig fusion transcripts in normal and neoplastic cells. J Clin Invest 80: 1512-1515, 1987.
34. Offit K, Kodura PRK, Hollis R, Filippa D, Jhanwar SC, Clarckson BC and Chaganti RSK. 18q21 rearrangement in diffuse large cell lymphoma: Incidence and clinical significance. Br J Haematol 72: 178-183, 1989.
35. Seto M, Jaeger U, Hocket RD, Graininger W, Bennett S, Goldman P and Korsmeyer SJ. Alternative promotors and exons, somatic mutations and deregulation of the BCL-2-Ig fusion gene in lymphoma. EMBO J 7: 123-131, 1988.
36. Silverman GA, Green ED, Young RL, Jockel JL, Domer PH and Koresmeyer SJ. Meiotic recombination between yeast artificial chromosomes yields a single clone containing the entire BCL-2 proto-oncogene. Proc Natl Acad Sci USA 87: 9913-9917, 1990.
37. Tsujimoto Y and Croce C. Analysis of the stucture, transcripts and protein products of BCL-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83: 5214-5218, 1986.
38. Tsujimoto Y, Ikegaki N and Croce CM. Characterization of the protein product of BCL-2, the gene involved in human follicular lymphoma. Oncogene 2: 3-7, 1987.
39. Hockenbery D, Oltvai ZN, Yin X-M, Milliman CL and Korsmeyer SJ. BCL-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75: 241-251, 1993.
40. Hockenbery D, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ. BCL-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348: 334-336, 1990.
41. Tonegawa SS. Somatic generation of antibody diversity. Nature 302: 575-581, 1983.
42. Yancopoulos GD and Alt FW. Regulation of the assembly and expression of variable region genes. Ann Rev Immunol 4: 339-368, 1986.
43. Waldmann TA. The rearrangement of immunoglobulin and T-cell receptor genes in human lymphoproliferative disorders. Adv Immunol 40: 247-321, 1987.
44. Honjo T. Immunoglobulin genes. Ann Rev Immunol 1: 449-528, 1983.
45. Sanz J. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. J Immunol 147: 1720-1729, 1991.
46. Alt FW and Baltimore D. Joining of immunoglobulin heavy chain gene segments: Implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 79: 4118-4122, 1982.
47. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW and Baltimore D. Insertions of N regions into heavy-chain genes is correlated with expression of terminal denucleotidyl transferase in B cells. Nature 311: 752-755, 1984.
48. Berman JE, Melissa SJ, Pollock R, Smith CL, Suk H, Heinke B, Kowal C, Sarti M, Chess L, Cantor CR and Alt FW. Content and organization of human Ig VH locus: Definition of three new VH families and linkage to the IgH locus. EMBO J 7: 727-738, 1988.
49. Shin EK, Matsuda F, Nagaoka H, Fukita Y, Imai T, Yokoyama K, Soeda E and Honjo T. Physical map of the 3' region of the human immunoglobulin heavy chain locus: Clustering of autoantibody-related viriable segments in one haplotype. EMBO J 10: 3641-3645, 1991.

50. Kirkham PM, Mortari F, Newton JA and Schroeder W Jr. Immunoglobulin VH clan and family identity predicts variable domain structure and may influence antigen binding. EMBO J 11: 603-609, 1992.
51. Siebenlist U, Ravetch JV, Korsmeyer SJ, Waldmann T and Leder P. Human immunoglobulin D segments encoded in tandem multigenic families. Nature 294: 631-635, 1981.
52. Matsuda F, Lee KH, Nakai S, Sato T, Kodaira M, Zong SQ, Fukuhara S and Honjo T. Dispended localization of D segments in the human immunoglobulin heavy chain locus. EMBO J 7: 1047-1051, 1988.
53. Ichihara Y, Matsuoka H and Kurosawa Y. organization of human immunoglobulin heavy chain diversity gene loci. EMBO J 7: 4141-4150, 1988.
54. Bulerwela L, Albertson DG, Sherrington P, Rabbits PH, Spurr W and Rabbits TH. The use of chromosomal translocations to study human immunoglobulin gene organization: Mapping DH segments within 35 kb of the Cμ gene identification of a new DH locus. EMBO J 8: 2003-2010, 1989.
55. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T and Leder P. Structure of the human immunoglobulin μ locus: Characterization of embryonic and rearranged J and D genes. Cell 27: 583-591, 1981.
56. Akira S, Okazaki K and Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 238: 381-384, 1987.
57. Shimizu A and Honjo T. Immunoglobulin class switching. Cell 36:801-803, 1984.
58. Krawinkel U and Radbruch A. Immunoglobulin heavy chain class switching. In: Calabi F and Neuberger MS, eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier scienc publishers: 135-151, 1987.
59. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC and Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226: 1097-1099, 1984.
60. Cleary ML, Smith SD and Sklar J. Cloning and structural analysis of cDNAs for BCL-2 and a hybrid BCL-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47: 19-28, 1986.
61. Wyatt RT, Rudders RA, Zelenetz A, Delellis RA and Krontiris TG. BCL-2 oncogene translocation is mediated by a chi-like consensus. J Exp Med 175: 1575-1588, 1992.
62. Cotter FE, Price C, Meerabux J, Zucca E and Young BD. Direct sequence analysis of 14q+ and 18q- chromosome junctions at the MBR and MCR revealing clustering within the MBR in follicular lymphoma. Annals of Oncology 2: 93-98, 1991.
63. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM and Stass SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 227: 175-178, 1987.
64. Tsujimoto Y, Bashir MM, Givol J, Cossman J, Jaffe E and Croce CM. DNA rearrangements in human follicular lymphoma can involve the 5' or the 3' region of the BCL-2 gene. Proc Nat Acad Sci USA 84: 1329-1331, 1987.
65. Cleary ML, Galili N and Sklar J. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 164: 315-320, 1986.
66. Ngan B-O, Nourse J and Cleary ML. Detection of chromosomal translocation t(14;18) within the minor cluster region of BCL-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. Blood 73: 1759-1762, 1989.
67. Mullis KB and Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in Enzymology 155: 335-350, 1987.
68. Tsujimoto Y, Gorham J, Cossman J, Jaffe E and Croce CM. The t(14;18) chromosome translocations involved in B-cell neoplasms results from mistakes in VDJ joining. Science 229: 1390-1393, 1985.
69. Graigninger WB, Seto M, Boutain B, Goldman P and Korsmeyer SJ. Expression of BCL-2 and BCL-2-Ig fusion transcripts in normal and neoplastic cells. J clin Invest 80: 1512-1515, 1987.
70. Pezzella F, Tse AG, Cordell JL, Pulford KA and Mason DY. Expression of the BCL-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137: 225-232, 1990.
71. Nunez G, London L, Hockenbery D, Alexander M, McKearn JP and Korsmeyer SJ. Deregulated BCL-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immun 144: 3602-3610, 1990.
72. Vaux DL, Cory S and Adams JM. BCL-2 gene promotes hemopoetic cell survival and cooperates with c-myc to immortalize pre-B-cells. Nature 335: 440-442, 1988.
73. Fairbairn LJ, Cowling GJ, Reipert BM and Dexter TM. Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74: 823-832, 1993.

74. Williams GT. Programmed cell death: Apoptosis and oncogenesis. Cell 65, 1097-1098, 1991.
75. Williams GT and Smith CA. Molecular regulation of apoptosis: Genetic controls on cell death. Cell 74: 777-780, 1993.
76. Boise LH, Gonzalez-Carcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G and Thompson CB. BCL-x, a BCL-2 related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597-608, 1993.
77. Oltvai ZN, Milliman CL and Korsmeyer SJ. BCL-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619, 1993.
78. Hengartner MO, Ellis RE, and Horvitz HR. *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. Nature 356: 494-499, 1992.
79. Kozopas KM, Yang T, Buchan HL, Zhou P and Craig RW. MCL-1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL-2. Proc Natl Acad Sci USA 90: 3516-3520, 1993.
80. Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM and Korsmeyer SJ. BCL-2 maintains B cell memory. Nature 353: 71-73, 1991.
81. McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP and Korsmeyer SJ. BCL-2-immunoglobulin transgenic mice demonstrate extended B-cell survival and follicular lymphoproliferation. Cell 57: 79-88, 1989.
82. McDonnell and Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature 349: 254-256, 1991.
83. Strasser A, Harris AW, Bath ML and Cory S. Novel primitive lymphoid tumors induced in transgenic mice by cooperation between MYC and BCL-2. Nature 348: 331-333, 1990.
84. Nakayama K-I, Nakayama K, Negishi I, Kuida K, Shinkai Y, Louie MC, Fields LE, Lucas PJ, Stewart V, Alt FW and Loh D. Disappearance of the lymphoid system in BCL-2 homozygous mutant chimeric mice. Science 261: 1584-1588, 1993.
85. Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ. BCL-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229-240, 1993.
86. Zutter M, Hockenbery D, Silverman GA and Korsmeyer SJ. Immunolocalization of the BCL-2 protein within hematopoietic neoplasms. Blood 78: 1062-1068, 1991.
87. Isaacson PG, Wotherspoon AC, Diss TC and Pan XL. BCL-2 expression in lymphomas. The Lancet 337; 175-176, 1991.
88. Pettersson M, Jernberg-Wiklund H, Larsson L-G, Sundstrom C, Givol I, Tsujimoto Y and Nilsson K. Expression of the BCL-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood 79:495-502, 1992.
89. Hockenbery DM, Zutter M, Hickey W, Nahm M and Korsmeyer SJ. BCL-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88: 6961-6965, 1991.
90. Ngan B-Y, Chen-Levy Z, Weiss LM, Warnke RA and Cleary ML. Expression in non-Hodgkin's lymphoma of the BCL-2 protein associated with the t(14;18) chromosomal translocation. N Engl J Med 318: 1638-1644, 1988.
91. Gaulard P, d'Agay M-F, Peuchmaur M, Brousse N, Gisselbrecht C, Solal-Celigny P, Diebold J and Mason DY. Expression of the BCL-2 gene product in follicular lymphoma. Am J Pathol 140: 1089-1095, 1992.
92. Pezzella F, Gatter KC, Mason DY, Bastard C, Duval C, Krajewski A, Turner GE, Ross FM, Clark H, Jones DB, Leroux D and Le Marc'Hadour F. BCL-2 protein expression in follicular lymphomas in absence of 14;18 translocation. The Lancet 336: 1510-1511, 1990.
93. Kondo E, Nakamura S, Onoue H, Matsuo Y, Yoshino T, Aoki H, Hayashi K, Takahashi K, Minowada J, Nomura S and Akagi T. Detection of BCL-2 protein and BCL-2 messenger RNA in normal and neoplastic lymphoid tissues by immunohistochemistry and *in situ* hybridization. Blood 80: 2044-2051, 1992.
94. LeBurn DP, Kamel OW, Cleary ML, Dorfman RF and Warnke RA. Follicular lymphomas of the gastrointestinal tract: Pathological features in 31 cases and BCL-2 oncogenic protein expression. Am J Pathol 140: 1327-1335, 1992.
95. Hagenbeek A. Minimal residual disease in leukemia: State of the art 1991. Leukemia 6 (suppl 2): 12-21, 1992.
96. Hagenbeek A and Martens ACM. Detection of minimal residual disease in acute leukemia: Possibilities and limitations. Eur J Cancer Clin Oncol 21: 389-395, 1985.
97. Ault KA. Detection of small numbers of monoclonal B-lymphocytes in the blood of patients with lymphoma. N Engl J Med 300: 1401-1405, 1979.
98. Smith BR, Weinberg DS, Robert NJ, Towle M, Luther E, Pinkus GS and Ault KA. Circulating

monoclonal B-lymphocytes in non-Hodgkin's lymphoma. N Engl J Med 311: 1476-1481, 1984.
99. van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hahlen K and van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: Possibilities and limitations. In: Hagenbeek A and Lowenberg B, Minimal residual disease in acute leukemia: 113-133, 1986.
100. van Dongen JJM, Breit TM, Adriaansen HJ, Beishuizen A and Hooijkaas H. Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. Leukemia 6: 47-59, 1992.
101. Hagemeijer A, Smit EME and Bootsma D. Improved identification of chromosomes of leukemic cells in methotrexate-treated cultures. Cytogenet Cell Genet 23: 208-212, 1979.
102. Tkachuk DC, Westbrook CA, Andreeff M, Donlon TA, Cleary ML, Suryanarayan K, Homge M, Redner A, Gray J and Pinkel D. Detection of BCR-ABL fusion in chronic myelogenous leukemia by *in situ* hybridization. Science 250: 559-562, 1990.
103. Ooteghem RBC, Smit EME, Beishuizen A, Lambrechts AC, v.d. Blij-Philipsen M, Smilde TJ and Hagemeijer A. A new B-cell line showing a complex translocation (8;14;18) and BCL-2 rearrangement. Cancer Genet Cytogenet in press.
104. Kibbelaar RE, van Kamp H, Dreef EJ, de Groot-Swings G, Kluin-Nelemans JC, Beverstock GC, Fibbe WE and Kluin PhM. Combined immunophenotyping and DNA *in situ* hybridization to study lineage involvement in patients with myelodysplastic syndromes. Blood 79: 1823-1828, 1992.
105. Kearney L, Bower M, Gibbons B, Das S, Chaplin T, Nacheva E, Chessells JM, Reeves B, Riley JH, Lister TA and Young BD. Chromosome 11q23 translocations in both infant and adult acute leukemias are detected by *in situ* hybridization with a yeast artificial chromosome. Blood 80: 1659-1665, 1992.
106. Heisterkamp N, Stam K, Groffen J, de Klein A and Grosveld G. Structural organization of the BCR gene and its role in the Phi translocation. Nature 315:311-324, 1984.
107. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR and Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, BCR, on chromosome 22. Cell 36: 93-99, 1984.
108. Arnold A, Cossman J, Bakshi A, Jaffe ES, Waldman TA and Korsmeyer SJ. Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J of Med 309: 1593-1599, 1983.
109. Wright JJ, Poplack DM, Bakshi A, Reaman G, Cole D, Jensen JP and Korsmeyer SJ. Gene rearrangements as markers of clonal variation and minimal residual disease in acute lymphoblastic leukemia. J Clin Oncol 5: 735-741, 1987.
110. Zehnbauer BA, Pardoll DM, Burke Ph, Graham ML and Vogelstein B. Immunoglobulin gene rearrangements in remission bone marrow specimens from patients with acute lymphoblastic leukemia. Blood 67: 835-838, 1986.
111. Sambrook J, Fritsch EF and Maniatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, 1989.
112. Bagasra O, Hauptman SP, Lischner HW, Sachs M and Pomeranrz RJ. Detection of human immunodeficiency virus type 1 provirus in mononuclear cells by *in situ* polymerase chain reaction. N Engl J Med 326: 1385-1391, 1992.
113. Nuovo GJ, Gallery F and MacConnell P. Importance of different variables for optimizing *in situ* detection of PCR-amplified DNA. Amplifications: A forum for PCR users 11: 4-6, 1993.
114. Hughes TP, Morgan GJ, Matriat P and Goldman JM. Detection of residual leukemia after bone marrow transplantation for chronic myeloid leukemia: Role of the polymerase chain reaction in predicting relapse. Blood 77: 874-878, 1991.
115. Bartram CR, Janssen JWG, Schmidberger M, Lyons J and Arnold R. Minimal residual leukemia in chronic myeloid leukemia patients after T-cell depleted bone marrow transplantation. The Lancet i: 1260, 1989.
116. Morgan GJ and Wiedemann LM. The clinical application of molecular techniques in Philadelphia-positive leukemia. Br J Haematol 80: 1-5, 1992.
117. Lee M, Khouri I, Champlin R, Kantarjian H, Talpaz M, Trujillo J, Freireich E, Deisseroth A and Stass S. Detection of minimal residual disease by polymerase chain reaction of BCR/ABL transcripts in chronic myelogenous leukemia following allogeneic bone marrow transplantation. BR J Haematol 82: 708-714, 1992.
118. Maurer J, Janssen JWG, Thiel E, van Denderen J, Ludwig W-D, Aydemir U, Heinze B, Fonatsch C, Harbott J, Reiter A, Riehm H, Hoeltzer D and Bartram CR. Detection of chimeric BCR-ABL genes in acute lymphoblastic leukemia by the polymerase chain reaction. The Lancet i: 1055-1058, 1991.
119. Roth MS, Antin JH, Ash R, Terry VH, Gotlieb M , Silver SM and Ginsburg D. Prognostic significance of Philadelphia chromosome-positive cells detected by the polymerase chain reaction

after allogeneic bone marrow transplant for chronic myelogenous leukemia. Blood 79: 276-282, 1992.
120. von Lindern M, Poutska A, Lerach H and Grosveld G. The (6;9) chromosome translocation associated with a specific subtype of non-lymphocytic leukemia leads to aberrant transcription of a target gene on 9q34. Mol Cell Biol 10: 4016-4026, 1990.
121. Soekarman D, von Lindern M, Daenen S, de Jong B, Fonatsch C, Heinze B, Bartram C, Hagemeijer A and Grosveld G. The translocation (6;9)(p23;q34) shows consistent rearrangement of two genes and defines a myeloproloferative disorder with specific clinical features. Blood 79: 2990-2997, 1992.
122. Kita K, Nakase K, Miwa H, Masura M, Nishii K, Morita N, Takaura, N, Otsuji A, Shirakawa S, Ueda T, Nasu K, Kyo T, Dohy H and Kamada N. Phenotypical characteristics of acute myelocytic leukemia associated with the t(8;21)(q22;q22) chromosomal abnormality: Frequent expression of immature B-cell antigen CD19 together with stem cell antigen CD34. Blood 80: 470-477, 1992.
123. Nuciflora G, Birn DJ, Erickson P, Gao J, LeBeau M, Drabbin HA and Rowley J. Detection of DNA rearrangements in the AML 1 and ETO loci and of an AML/ETO fusion mRNA in a patient with t(8;21) acute myeloid leukemia. Blood 81: 883-888, 1993.
124. Kusec R, Laczika K, Knobl P, Friedl J, Grenix H, Linkesch W, Schwarzinger I, Mitterbauer G, Purtscher B, Haas OA, Lechner K and Jaeger U. AML1/ETO fusion mRNA can be detected in remission blood samples of all patients with t(8;21) acute myeloid leukemia after chemotherapy or autologous bone marrow transplantation. Leukemia 8: 735-739, 1994.
125. Zhang T, Hillion J, Tong J-H, Cao Q, Chen S-J, Berger R and Chen Z. AML-1 gene rearrangement and AML1-ETO gene expression as molecular markers af acute myeloblastic leukemia with t(8;21). Leukemia 8: 729-734, 1994.
126. Maryama F, Stass SA, Estey EH, Cork A, Hirano M, Ino T, Freireich EJ, Yang P and Chang K-S. Detection of AML/ETO fusion transcript as a diagnostic tool for diagnosing t(8;21) positive acute myelogenous leukemia. Leukemia 8: 40-45, 1994.
127. Borrow J, Goddard AD, Sheer D and Solomon E. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 249: 1577-1580, 1990.
128. Longo L, Pandolfi PP, Biondi A, Rambaldi A, Mencarelli A, Lo Coco F, Diverio D, Pegoraro L, Avanzi G, Tabilio A, Zangrilli D, Alcalay M, Donti E, Grignani F and Pelicci PG. Rearrangements and aberrant expression of the retinoic acid receptor alpha gene in acute promyelocytic leukemia. J Exp Med 172: 1571-1575, 1990.
129. de The H, Chomienne C, Lanotte M, Degos L and Dejean A. The (15;17) translocation of acute promyelocytic leukemia fuses the retinoic receptor alpha gene to a novel transcribed locus. Nature 347: 558-561, 1990.
130. Chang K-S, Lu J, Wang G, Trujilo JM, Estey E, Cork A, Chu D-T, Freireich EJ and Stass SA. The t(15;17) breakpoint in acute promyelocytic leukemia clusters within two different sites of the MYL gene: targets for the detection of minimal residual disease by the polymerase chain reaction. Blood 79: 554-558, 1992.
131. Biondi A, Rambaldi A, Pandolfi PP, Rossi V, Giudici G, Alcalay M, Lo Coco F, Diverio D, Pogliani EM, Lanzi EM, Mandelli F, Masera G, Barbui T and Pelicci PG. Molecular monitoring of the MYL/retinoic acid receptor-α fusion gene in acute promyelocytic leukemia by polymerase chain reaction. Blood 80: 492-497, 1992.
132. Laczika K, Mitterbauer G, Korninger L, Knobl P, Schwarzinger I, Kaiotis S, Haas OA, Kyrle PA, Pont J, Oehler L, Purtscher B, Thalhammer F, Lechner K and Jaeger U. Rapid achievement of PML-RARα polymerase chain reaction (PCR)-negativety by combined treatment with all-trans-retinoic acid and chemotherapy in acute promyelocytic leukemia: a pilot study. Leukemia 8: 1-5, 1994.
133. Mellentin JD, Murre C, Donlon TA, McCaw PS, Smith SD, Carroll AJ, McDonald ME, Baltimore D and Cleary ML. The gene for enhancer binding protien E12/E47 lies at the t(1;19) breakpoint in acute leukemias. Science 246: 379-382, 1989.
134. Kamps MP, Look AT and Baltimore D. The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes and Development 5: 358-368, 1991.
135. Privitera E, Kamps MP, Hayashi Y, Inaba T, Shapiro L, Raimondi SC, Behm F, Hendershot L, Carroll AJ, Baltimore D and Look AT. Different molecular consequences of the 1;19 chromosomal translocation in childhood B-cell precursor acute lymphoblastic leukemia. Blood 79: 1781-1788, 1992.
136. Privitera E, Luciano A, Ronchetti D, Arico M, Santostasi T, Basso G and Biondi A. Molecular variants of the 1;19 chromosomal translocation in pediatric acute leymphoblastic leukemia (ALL). Leukemia 8: 554-559, 1994.
137. Downing JR, Head DR, Raimondi SC, Carroll AJ, Curcio-Brint AM, Motroni TA, Hulshof MG, Pullen

J and Domer PH. The der(11)-encoded MLL/AF-4 fusion transcript is consistently detected in t(4;11)(q21;q23)-containing acute lymphoblastic leukemia. Blood 83: 330-335, 1994.
138. Shiramizu B and Magrath I. Localization of breakpoints by polymerase chain reactions in Burkitt's lymphoma with 8;14 translocations. Blood 75: 1848-1852, 1990.
139. Tsujimoto Y, Yunis J, Overato-Showe L, Euhson J , Powell PC and Croce CM. Molecular cloning of the chromosomal breakpoint of B cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224: 1403-1406, 1984.
140. Rimokh R, Berger F, Delsol G, Digonnet I, Rouault JP, Tigaud JD, Gadoux M, Coiffier B, Bryon PA and Magaud JP. Detection of the chromosomal translocation t(11;14) by polymerase chain reaction in mantle cell lymphomas. Blood 83: 1871-1875, 1994.
141. Molot RJ, Meeker TC, Wittwer CT, Perkins SL, Segal GH, Masih AS, Braylan RC and Kjeldsberg CR. Antigen expression and polymerase chain reaction amplification of mantle cell lymphomas. Blood 83: 1626-1631, 1994.
142. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC and Korsmeyer SJ. Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 85; 4869-4873, 1985.
143. Stetler-Stevenson M, Raffeld M, Cohen P and Cossman J. Detection of occult follicular lymphoma by specific DNA amplification. Blood 72: 1822-1825, 1988.
144. Gribben JG, Freedman AS, Neuberg D, Roy DC, Blake KW, Woo SD, Grossbard ML, Rabinowe SN, Coral F, Freeman GJ, Ritz J and Nadler LM. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. N Engl J Med 325: 1525-1533, 1991.
145. Price CGA, Meerabaux J, Murtagh S, Cotter FE, Rohatiner AZS, Young BD and Lister TA. The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 9: 1527-1532, 1991.
146. Limpens J, de Jong D, van Krieken JHJM, Price CGA, Young BD, van Ommen G-JB and Kluin PLM. BCL-2/JH rearrangement in benign lymphoid tissues with follicular hyperplasia. Oncogene 6: 2271-2276, 1991.
147. Aster JC, Kobayashi Y, Shiota M, Mori S and Sklar J. Detection of the t(14;18)at similar frequencies in hyperplastic lymphoid tissues from American and Japanese patients. Am J Pathol 141: 291-299, 1992.
148. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, Tsujimoto Y, Caton AJ and Rovera G. Detection of minimal disease in hematopoietic malignancies of the B lineage by using third-complementarity-determining region (CDR III) specific probes. Proc Natl Acad Sci USA 86: 5123-5127, 1989.
149. Yamada M, Wasserman R, Lange B, Reichard BA, Wormer RB and Rovera G. Minimal residual disease in childhood B-lineage lymphoblastic leukemia. N Engl J Med 323: 448-455, 1990.
150. McCarthy KP, Sloane JP and Wiedeman LM. Rapid method for distinguishing clonal from polyclonal B-cell populations in surgical biopsy specimens. J Clin Pathol 43: 429-432, 1990.
151. Trainor KJ, Brisco MJ, Story CJ and Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood 75: 2220-2222, 1990.
152. Deane M and Norton JD. Detection of immunoglobulin gene rearrangements in B-lymphoid malignancies by polymerase chain reaction gene amplification. Br J Haematol 74: 251-256, 1990.
153. Billadeau D, Blackstadt M, Greipp P, Kyle RA, Oken MM, Kay N and van Ness B. Analysis of B-lymphoid malignancies using allele specific polymerase chain reaction: a technique for sequential quantification of residual disease. Blood 78: 3021-3029, 1991.
154. Ben-Ezra J. Variable rate detection of immunoglobulin heavy chain V-D-J rearrangement by PCR : A systemic study of 41 B-cell non-Hodgkin's lymphomas and leukemias. Leuk. Lymphoma 7: 289-295, 1992.
155. Diss TC, Peng H, Wotherspoon AC, Isaacson PG and Pan L. Detection of monoclonality in low-grade B-cell lymphomas using the polymerase chain reaction is dependent on primer selection and lymphoma type. J Pathol 169: 291-295, 1993.
156. Reed TJ, Reid A, Wallberg K, O'Leary TJ and Frizzera G. Determination of B-cell clonality in paraffin-embedded lymph nodes using the polymerase chain reaction. Diagn Mol Pathol 2: 42-49, 1993.
157. Slack DN, McCarthy, Wiedemann LM and Sloane JP. Evaluation of sensitivity, specificity, and reproducability of an optimized method for detecting clonal rearrangements of immunoglobulin and T-cell receptor genes in formalin-fixed, paraffin-embedded sections. Diagn Mol Pathol 2: 223-232, 1993.

CHAPTER 2

Detection of residual disease in translocation (14;18) positive non-Hodgkin's lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods.

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Detection of Residual Disease in Translocation (14;18) Positive Non-Hodgkin's Lymphoma, Using the Polymerase Chain Reaction: A Comparison With Conventional Staging Methods

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This paper reports the detection of residual lymphoma cells in blood and bone marrow samples from patients with translocation (t) (14;18) positive non-Hodgkin's lymphoma by the polymerase chain reaction (PCR) compared with conventional staging techniques. In 15 of 22 samples, in which no lymphoma cells could be detected by morphological examination, t(14;18) positive cells were detected by PCR. In 13 of 21 samples, in which a monoclonal B-cell population was not detectable by immunological marker analysis, PCR was positive. The clinical status (physical examination, imaging techniques, leucocyte count, and occasionally morphology and immunological marker analysis) was documented in 30 patients at the time of PCR analysis. In three of 19 patients with clinical evidence of disease, circulating t(14;18) positive cells were not detectable by PCR. Five of 11 patients in clinical remission from 7 to 47 months, showed t(14;18) positive cells in the blood. Our data show that PCR analysis in t(14;18) positive non-Hodgkin's lymphoma offers a powerful tool in the study of residual disease.

INTRODUCTION

Non-Hodgkin's lymphoma (NHL), in contrast to leukaemias, is a primary localized disease, which may secondarily spread to the blood and bone marrow. For the detection of minimal disease at staging and after treatment, various complementary techniques, including imaging of lymph nodes and detection of lymphoma cells in blood and bone marrow by morphology and immunological marker analysis, must be relied upon. The *in vitro* amplification of DNA sequences by the polymerase chain reaction (PCR) has opened new perspectives for a very sensitive detection of lymphoma cells. Target sequences that are unique for lymphoma cells are provided by chromosomal translocations and antigen receptor rearrangements. The reciprocal translocation between chromosomes 14 and 18 [t(14;18)] is the most common chromosomal abnormality found in B-NHL. Although t(14;18) is seen in various histological subtypes of NHL, it predominates in lymphomas with a follicular growth pattern. Both cytogenetic and molecular studies report the occurrence of the t(14;18) in 50–80% of follicular NHL and in up to 30% of diffuse NHL (1–5). In this translocation the joining (JH) segment of the immunoglobulin heavy chain (IGH) gene, located on the long arm of chromosome 14 (band q32), is transferred to the BCL2 gene on the long arm of chromosome 18 (band q21) (6–10). This translocation creates a DNA sequence which is unique for the lymphoma cells: a hybrid BCL2/JH sequence (6,8,11,12). Two breakpoint clusters are identified within the BCL2 gene (13). The major breakpoint region (mbr) is involved in 50–60% of the translocations and spans only 150 bases (8,10,14). The minor cluster region (mcr) is found in 25–30% of the translocations and involves a breakpoint region of 500 bases (1,13,15). In a minority of patients with t(14;18) positive lymphoma the breakpoint is located 5′ of the BCL2 gene (16). The frequency of this translocation and the molecular characteristics of both the mbr and the mcr allow the application of the PCR to detect lymphoma cells in a substantial number of NHL patients.

Here we report the results of the PCR analysis of blood or bone marrow samples for the detection of residual lymphoma cells compared with morphology and immunological marker analysis in patients with clinical evidence of disease (CED) or in clinical complete remission (CCR).

MATERIALS AND METHODS

Patients

Lymph node biopsies, blood and bone marrow samples from 79 patients with NHL were collected at diagnosis or during and after treatment. Staging of the patients (17) was performed following standard procedures, including physical examination, CT scan of the thorax and abdomen, and morphological and immunological analyses of blood and bone marrow. The distribution of the different types of NHL according to the Working Formulation for NHL (18) [Kiel Classification (19)] was: 56 follicular mixed small and large cells [follicular centroblastic centrocytic (cb/cc)], six diffuse mixed small and large cells (diffuse cb/cc), four diffuse small cleaved (diffuse cc), and two diffuse large cleaved cell (diffuse cb), eight small lymphoplasmacytoid (immunocytoma), two lymphoblastic (lymphoblastic), and one small lymphocytic (lymphocytic). The patients were treated, if the grade of malignancy of the lymphoma was low, with cyclophosphamide, vincristine and prednisone (CVP), and if the grade of malignancy was intermediate or high, with these cytostatic drugs combined with adriamycin (CHOP).

DNA Extraction

Two portions of white blood cells were isolated from 20 ml of heparinised blood or 2–5 ml of bone marrow using an erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4), and stored in liquid nitrogen. High molecular weight DNA was prepared from these isolates and from frozen lymph node biopsies and cells from the cell line SU-DHL-6 (a gift of Dr K. Thielemans) carrying a mbr t(14;18) (20), according to standard protocols (21,22). If frozen tissue was not available, DNA was isolated from paraffin embedded lymphoma biopsies. One 8 μm section was deparaffinated by xylene (2 × 5 min), washed with 96 and 70% ethanol, and dried. The section was

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resuspended in 100 μl of water and heated to 95°C for 10 min; 1–10 μl were used for amplification by PCR.

Southern Blot Analysis

Ten micrograms of chromosomal DNA, extracted from the lymph node biopsies on which the diagnosis was made, were digested with 20–30 U of the restriction enzyme HindIII under the conditions recommended by the suppliers. DNA was size-fractionated on 0.8% agarose gels and transferred to nylon membranes (Hybond N^+, Amersham). Hybridization was performed according to standard procedures, using ^{32}P-labeled DNA probes radiolabeled by the hexamer primer method (21). The following probes were used: a JH probe, specific for the joining regions (JH3-JH6) of the IGH gene (23), a BCL2 probe specific for the mbr-t(14;18), a gift of Dr Y. Tsujimoto (7), and the mcr-t(14;18), a gift of Dr M.L. Cleary (15).

Polymerase Chain Reaction

For PCR analysis (24) of the mbr t(14;18), two primers were defined: a BCL2 primer (5'-GGTGGTTTGACCTTTAGA-3') and a JH consensus primer (5'-TGAGGAGACGGTGACC-3') (synthesized by Pharmacia Nederland, Woerden, The Netherlands) (6,8,14,25,26). For the mcr t(14;18) a BCL2 primer (5'-CAGTCTCTGGGGAGGA-3') was combined with the same JH primer (27). DNA (0.5 μg) in a reaction volume of 25 μl was subjected to a 30 cycle PCR amplification using an automated Perkin-Elmer/Cetus DNA Thermal Cycler, Taq DNA polymerase (Perkin-Elmer/Cetus Nederland, Gouda, The Netherlands: 0.25 U; Promega Cooperation Leiden, The Netherlands: 0.20 U) and the recommended buffer conditions. The optimum cycle conditions were: denaturing at 94°C for 7 min in the first cycle and 1 min for the 29 subsequent cycles, annealing at 58°C (mbr) or 56°C (mcr) for 2 min and extension for 2 min at 72°C, followed by a final extension of 7 min.

One fifth of the amplified samples was analysed on a 1.2% agarose gel, followed by Southern blotting. Ethidium bromide staining occasionally showed a visible band. Hybridization with the corresponding BCL2 probe was often necessary to detect the amplified product. As a control to DNA quality, all samples were subjected to a 25 cycle PCR amplification of the interleukin-3 (IL-3) gene (primers: 5'-GAGGTTCCATGTCAGATA-3' and 5'-CCTCACCTAGAACTGCCT-3') (28). The cycle conditions were identical to the conditions used for the mbr t(14;18) and resulted in a 750 bp IL-3 fragment detectable upon ethidium bromide staining. Samples were excluded from evaluation in this study when IL-3 amplification was not detected.

In each experiment DNA from various dilutions (10^{-2}–10^{-6}) of SU-DHL-6 cells in normal mononuclear blood cells was tested as a positive control. Routinely, the 10^{-2} dilution gave a band, visible after ethidium bromide staining; the 10^{-5} dilution was positive following hybridization. As negative controls, all reaction components without template DNA and all reaction components with DNA isolated from normal mononuclear donor cells were tested. Each DNA sample was analysed for breakpoint amplification in at least two independent experiments. Samples were considered to be positive when the amplified fragment hybridized with the mbr t(14;18) or mcr t(14;18) BCL2 probes. In most samples amplification could be confirmed by hybridization with the JH probe. In the absence of specific amplification, a patient sample was evaluated as negative only when t(14;18) specific amplification was successful in the 10^{-5} dilution of SU-DHL-6. If positive PCR data were not confirmed by other parameters (morphological or immunological analysis or clinical status) a second, independently isolated DNA sample was tested at least four-fold.

Morphological Analysis

Blood and bone marrow smears were prepared following routine methods and stained with May–Grünwald–Giemsa for microscopic examination. Leucocyte concentrates from peripheral blood samples were made, when the leucocyte count was below 10×10^9/l, by using the pellet after centrifugation of the buffy coat, which results after sedimentation for 1 h at 37°C of anticoagulated blood. Smears, made from these pellets, were screened for the presence of cells with abnormal morphology.

Immunological Marker Analysis

Mononuclear cells from blood and bone marrow were isolated by Ficoll–Isopaque density gradient centrifugation of anticoagulated samples. Surface marker analysis was performed by indirect immunofluoresence with the monoclonal antibodies CD19, CD20, CD3, CD4, CD8 and HLA-DR (Becton Dickinson Sunnyvale, CA, USA), CD14 (Coulter Clone, Hialeah, FL, UK), and CD15 (Dr W. Knapp, Vienna, Austria) (29) and FITC-conjugated goat anti-mouse immunoglobulin serum (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) as a second step. Direct immunofluoresence was performed with FITC-conjugated goat anti-human IgM, IgD, IgG, IgA and lambda serum and TRITC-conjugated goat anti-human kappa and lambda serum (Nordic Immunological Laboratories Tilburg, The Netherlands). Double staining was performed with TRITC-conjugated kappa plus FITC-conjugated lambda or with the FITC-conjugated heavy chain expressed by the malignant B-cell (mostly IgM) plus TRITC-conjugated kappa or lambda. The preparations were read by microscope. Kappa and lambda ratios were determined by counting kappa or lambda positivity of 200 cells positive for the appropriate heavy chain. A B-cell population was considered to be monoclonal when more than 95% expressed kappa or lambda. With this procedure a monoclonal B-cell population of 0.1–1% in a mononuclear cell fraction after Ficoll-Isopaque centrifugation can be detected.

RESULTS

Incidence of t(14;18) in NHL

Frozen lymph node biopsies were available and were investigated for the presence of an mbr or mcr t(14;18) using both Southern blot and PCR analysis (Table 1) for 25 of the 79 NHL patients in this study. In 11 of 19 biopsies from patients with follicular NHL, a t(14;18) was detected with both techniques, 10 having mbr t(14;18) and one mcr t(14;18) positive cells. From the six patients with non-follicular types of NHL, one lymph node (diffuse cb/cc) showed mbr t(14;18) positive cells. Twelve paraffin embedded lymph node biopsies from follicular NHL patients were investigated using PCR. Nine biopsies were evaluable, four showing mbr t(14;18) positive cells and none mcr t(14;18)

Table 1. Analysis of Frozen Lymph Node Biopsies and Paraffin Embedded Lymphoma Tissue for the Presence of a mbr or mcr t(14;18) in Follicular NHL and in Other Types of NHL (Three Diffuse cb/cc, One Diffuse cb, One Lymphoblastic and One Lymphocytic NHL). Paraffin Embedded Material was Only Included When PCR of the IL-3 Gene was Positive.

Type of NHL	No. of Patients	mbr	mcr	Percentage positive
Follicular CB/CC	28	14	1	54
Other types	6	1	0	17

positive cells. The overall incidence of t(14;18) in follicular NHL found in our patient group was 54%.

Detection of Residual Lymphoma Cells by PCR

Blood (138 samples), bone marrow (19 samples) or pleural effusion (two samples) from 74 NHL patients were investigated for the occurrence of mbr or mcr t(14;18) positive cells using PCR (Table 2). Nineteen of the 52 patients with follicular NHL showed on one or more occasions t(14;18) positive cells; 17 were positive for the mbr t(14;18) and two for the mcr t(14;18). In six patients whose lymph node biopsies were positive for the mbr t(14;18), no mbr t(14;18) positive cells could be detected in blood or bone marrow (Table 2). From 22 patients with other types of NHL, six patients were positive for t(14;18) on one or more occasions, five for the mbr t(14;18) and one for the mcr t(14;18) (Table 2). From these six patients five had diffuse centroblastic, centrocytic, or mixed NHL. Seventeen patients, negative for t(14;18) in the lymph node biopsy, did not show specific amplification products, indicating the specificity of the reaction and the absence of false positive reactions.

An example of a PCR analysis is presented in Figure 1. DNA samples prepared from a blood specimen and from various dilutions of SU-DHL-6 cells (30) in normal cells were subjected to PCR amplification and hybridization analysis. A fragment of approximately 235 base pairs (bp) hybridizing with both BCL2 and JH probes is found in up to 10^{-5} dilutions of SU-DHL-6 cells. The reduced sensitivity of the JH probe is explained by the short JH homologous fragment (64 bp) in the product (8). Analysis of two independently prepared DNA samples from a single blood sample from the patient showed amplification of an approximately 200 bp fragment with homology to both BCL2 and JH (Figure 1 lanes 1–4). The negative result in lane 4 is explained by the low frequency of mbr t(14;18) positive cells in the blood sample. Owing to the reduced sensitivity of the JH probe and the presence of large (> 500 bp) non-specific amplification products, the BCL2 probe has been preferentially used for screening purposes.

Comparison of PCR With Morphology and Immunological Marker Analysis

From patients with t(14;18) positive NHL the PCR data from blood or bone marrow were compared with the results of morphological examination and immunological marker analysis (Table 3). The samples were taken during treatment, at follow-up and, occasionally, at suspicion of relapse. In 15 of 22 samples, in which morphology revealed no abnormal cells, t(14;18) positive cells were detected. In five samples both PCR and morphology were positive. In 13 of 21 samples, in which immunological marker analysis was negative, PCR analysis showed t(14;18) positive amplification (Table 3). Five samples, including two pleural effusions, were both positive with PCR and immunological marker analysis. The PCR data were not negative in samples which were either morphologically or immunologically positive.

Table 2. PCR Analysis of Blood, Bone Marrow, or Pleural Effusion for t(14;18) Positive Cells. Patients Were Grouped According to t(14;18) Status of the (Primary) Lymph Node Biopsy. If Biopsy Material was not Available, Tumors Were Classified as Unknown.

Lymph Node Characteristics	No. of Patients Tested	No. of Patients PCR Positive
Follicular CB/CC (n = 52)		
t(14;18) positive	12	6
t(14;18) unknown	28	13
t(14;18) negative	12	0
Others (n = 22)		
t(14;18) Positive	0	0
t(14;18) Unknown	17	6
t(14;18) Negative	5	0

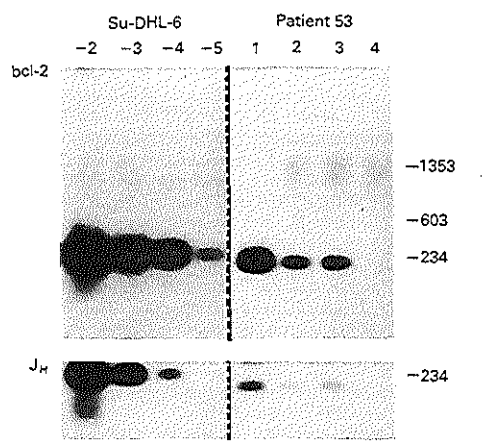

Figure 1. PCR analysis of various dilutions from SU-DHL-6 cells (10^{-2}–10^{-5}) and from a blood sample of patient 53 (lanes 1–4). Lane 1, the result of the amplification from the first extracted DNA sample; lanes 2–4, from the second sample. Blots were hybridized to both the BCL2 and JH probe, washed in 3 × SSC + 0.5% SDS at 65°C. At the right, the size marker PhiX DNA digested with HaeIII is shown.

Table 3. PCR Analysis of Blood, Bone Marrow, and Pleural Effusion From Patients With a t(14;18) Positive NHL Were Compared With the Staging Results (Number of Patients Between Brackets). M = Morphological Examination, I = Immunological Marker Analysis, CED = Clinical Evidence of Disease, CCR = Clinical Complete Remission.

PCR Results	M⁻	M⁺	I⁻	I⁺	CED	CCR
PCR⁺	15 (13)	5 (4)	13 (11)	5 (4)	27 (16)	11 (5)
PCR⁻	7 (5)	0	8 (6)	0	6 (3)	7 (6)

Comparison of PCR and Clinical Status

In 30 patients with t(14;18) positive NHL, PCR data from blood or bone marrow samples were compared

with the clinical status: 19 patients had CED by physical examination, imaging techniques, or had lymphoma cells in the blood or bone marrow by morphological or immunological analysis and 11 patients were in CCR, based on these examinations (Table 3). Different patterns were seen. In 16 patients with CED, including the four patients with morphological and immunological positive samples, t(14;18) positive cells were detectable. In three patients with t(14;18) positive NHL, all follicular, who had CED only in the lymph nodes, no t(14;18) positive cells were detectable by PCR in the blood or bone marrow. These patients will be discussed in more detail.

Patient A was a 47-year-old woman with follicular, predominantly centrocytic, lymphoma stage III[a]. She showed a good partial remission after eight courses of CVP. The PCR analysis performed on blood samples taken after six and eight courses showed the presence of t(14;18) positive cells. In the bone marrow taken after eight courses, no t(14;18) positive cells were found. Local radiotherapy was given on the upper abdomen, the only residual mass. Interferon-α maintenance treatment (3×10^6 IU three times a week) was given and further regression of the abdominal mass was noticed during the next few months. PCR analysis after 5 and 8 months no longer revealed t(14;18) positive cells in the blood.

Patient B was a 63-year-old women, in whom the diagnosis of NHL follicular cb/cc stage IVa with blood and bone marrow localization was made 10 years ago. She was treated several times with CVP courses and local irradiation. A search for t(14;18) positive cells in the blood was performed when she was receiving CVP treatment and pathological abdominal lymph nodes were the only clinical manifestation of the disease. No t(14;18) positive cells were detected by PCR. The blood and bone marrow remained morphologically and immunologically negative, whereas in a pleural effusion, which was morphologically and immunologically positive for lymphoma cells, t(14;18) positive cells could be detected.

Patient C was a 60-year-old woman with follicular NHL stage III[a], who showed a CCR after eight CVP courses, but relapsed 10 months after treatment. A lymph node biopsy, taken at this relapse, was t(14;18) positive. CVP was restarted after progression on chlorambucil treatment. In a blood sample taken during CVP treatment, when small cervical and mediastinal lymph nodes were present, the PCR results were negative and remained negative 2 months after treatment and in second CCR.

From the 11 patients in CCR, all of whom had follicular lymphoma, five patients showed t(14;18) positive cells in the circulation. The longest period of CCR after discontinuation of treatment until sampling of blood or bone marrow in these patients was 7, 7, 26, 46, and 47 months. The longest period between two positive observations in the three patients who were tested more than once was 4, 7, and 14 months. In the other six patients, tested in CCR, no t(14;18) positive cells were detectable in the blood. They were tested 2, 5, 9, 35, 38, and 90 months after treatment.

DISCUSSION

In this study we used the sensitive PCR technique for the detection of t(14;18) positive lymphoma cells in blood and bone marrow from a large series of NHL patients. We show that it is a useful addition to the conventional staging investigations (10,26,31). The detection of a minor cell population by PCR is determined by the sensitivity of the technique, the sample error and the efficacy of the reaction. The sensitivity is limited by the maximum amount of DNA (up to 0.5 μg, corresponding to the DNA content of 100 000 cells) that can be analysed per reaction. If the number of t(14;18) positive cells in the sample is around the detection limit, variability of the DNA sampling may affect the result of the PCR; sometimes a sample may give negative results (Figure 1, lane 4), whereas others, drawn from the same DNA preparation, show specific amplification (Figure 1, lanes 1–3). By enlarging the number of independent PCR experiments, the sensitivity may be slightly increased. The efficacy of the reaction is determined by the quality of the DNA, in this study monitored by concomitant amplification of the IL-3 gene, and the reaction conditions, monitored by testing different dilutions of the t(14;18) positive cell line SU-DHL-6 in normal cells in the same experiment. Another limitation may be introduced by the heterogeneous distribution of lymphoma cells within a body compartment. Such a sampling error is less likely to occur in blood than in a bone marrow specimen.

PCR analyses of lymph node biopsies showed approximately a 50% incidence of the t(14;18) in follicular NHL in our patient series. This frequency is relatively low compared with American studies (1,2) but in agreement with results of another European study (3). This discrepancy might be explained by variations in the definition for follicular growth pattern in NHL between pathologists or ethnological differences (3). The incidence of t(14;18) in diffuse types of NHL seems to be more frequent in diffuse lymphomas with centroblastic and/or centrocytic components (4). There is some evidence that these diffuse lymphomas originate from follicular lymphomas which underwent transformation to a higher malignancy grade (32–35).

Comparison of the results obtained by PCR and by morphology or immunological marker analysis showed two general patterns. Firstly, PCR results were in concordance with the conventional analyses, both either positive or negative. In patients with t(14;18) negative lymph node biopsies no circulating t(14;18) positive cells were found in blood or bone marrow, indicating a lack of false positive results after t(14;18) amplification and the absence of circulating t(14;18) positive cells not related to malignant lymphoma (36). Secondly, positive PCR results were seen in otherwise negative samples, which indicates the greater sensitivity of the first (14,25,30). Comparison of the clinical status of the patient and PCR results showed concordant data in most, but discordance was seen in both directions. In five patients with follicular NHL in CCR we found t(14;18) positive cells in the blood up to 47 months after

discontinuation of treatment. This means that, if a leucocyte count of $6 \times 10^9/l$ is assumed, and with a detection limit of one lymphoma cell in 100 000 normal cells, more than 300 000 lymphoma cells are present in the circulation for several years without evidence of clinical relapse. On the other hand, three patients with t(14;18) positive follicular lymphoma and CED in the lymph nodes showed no t(14;18) positive cells in blood or bone marrow. Circulating t(14;18) positive cells were apparently absent or below the detection limit in these patients. This finding may be explained by either a low propensity of the lymphoma cells in some patients to spread into the circulation, or may be the result of treatment (31). Two patients (B and C) were tested during chemotherapy; one patient (A) was receiving interferon-α maintenance treatment.

The clinical relevance of the presence of several thousand lymphoma cells in the blood of patients in CCR, especially in patients with t(14;18) positive follicular NHL, has to be studied further by a longer follow-up (none of the patients in this study in CCR with detectable lymphoma cells in the blood, as yet, has had a relapse). Also an accurate quantification procedure of the PCR is required to establish progression of minimal disease in time. Various procedures have been described for semi-quantitative analysis (37,38), but serious problems have to be resolved to establish a reliable protocol. The clinical meaning of residual lymphoma cells in the blood with respect to remission duration may be different for the different types of NHL.

As half of the patients with follicular NHL lack the t(14;18) marker, and the incidence is lower in other types of NHL, application of the PCR to t(14;18) breakpoint sequences is possible in a restricted number of the NHL patients. Amplification of the hypervariable DNA sequences of the VDJ segment of the rearranged immunoglobuline heavy chain gene, which provides a specific tumor marker, may allow a study of the clinical significance of residual disease in patients who lack this translocation, especially those with intermediate or high grade malignant NHL (39,40).

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REFERENCES

1. Weiss LM, Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t(14;18) chromosome translocation in malignant lymphomas. N Engl J Med **1987**; 317:1185–1189.
2. Rowley JD. Chromosome studies in the non-Hodgkin's lymphomas: the role of the 14;18 translocation. J Clin Oncol **1988**;6:919–925.
3. Pezzella F, Ralfkiaer E, Gatter KC, Mason DY. The 14:18 translocation in European cases of follicular lymphoma: comparison of Southern blotting and the polymerase chain reaction. Br J Haematol **1990**;76:58–64.
4. Griesser H, Lennert K. Bcl-2 gene rearrangements in malignant B cell lymphomas. Br J Haematol **1990**;76:142–143.
5. Aisenberg AC, Wilkes BM, Jacobson JO. The bcl-2 gene is rearranged in many diffuse B-cell lymphomas. Blood **1988**;71:969–972.
6. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. Cell **1981**;27:583–591.
7. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science **1984**;226:1097–1099.
8. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around Jh on chromosome 14 and near a transcriptional unit on 18. Cell **1985**;41:889–906.
9. Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transciptionally active locus on chromosome 18. Proc Natl Acad Sci USA **1985**;82:7439–7443.
10. Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell **1986**;47:19–28.
11. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science **1985**;229:1390–1393.
12. Cotter F, Price C, Zucca E, Young BD. Direct sequence analysis of the 14q+ and 18q− junctions in follicular lymphoma. Blood **1990**;76:131–135.
13. Tsujimoto Y, Bashir MM, Givol J, Cossman J, Jaffe E, Croce CM. DNA rearrangements in human follicular lymphoma can involve the 5′ or the 3′ region of the bcl-2 gene. Proc Natl Acad Sci USA **1987**;84:1329–1331.
14. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science **1987**;237:175–178.
15. Cleary ML, Galili N, Sklar J. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med **1986**;164:315–320.
16. Adachi M, Tefferi A, Greipp PR, Kipps TJ, Tsujimoto Y. Preferential linkage of bcl-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. J Exp Med **1990**;171:559–564.
17. National Cancer Institute Sponsored Study of Classifications of Non-Hodgkin's Lymphomas. Summary and Description of a Working Formulation for Clinical Usage. Cancer **1982**;49:2112–2135.
18. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M. Report of the Committee on Hodgkin's disease staging classification. Cancer Res **1971**; 31:1860–1861.
19. Stansfeld AG, Diebold J, Noel H, Kapanci Y, Rilke F, Kelényi G, Sundstrom C, Lennert K, van Unnik JAM, Mioduszewska O, Wright DH. Updated Kiel classification for lymphomas. Lancet **1988**;i:292–293.
20. Epstein AL, Levy R, Kim H, Henle W, Henle G, Kaplan HS. Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines. Cancer **1978**;42:2379–2391.

21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, 1989.
22. Miller SA, Dykes DD, Polesky HF. Simple and efficient method for isolating high molecular weight DNA from mammalian sperm. Nucleic Acid Res. **1988**;16:1215.
23. Takahashi N, Nakai S, Honjo T. Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acid Res. **1980**;8:5983–5991.
24. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol **1987**;155:335–350.
25. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ. Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA **1988**:85:4869–4873.
26. Stetler-Stevenson M, Raffeld M, Cohen P, Cossman J. Detection of occult follicular lymphoma by specific DNA amplification. Blood **1988**;72:1822–1825.
27. Ngan B-O, Nourse J, Cleary ML. Detection of chromosomal translocation t(14;18) within the minor cluster region of bcl-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. Blood **1989**;73:1759–1762.
28. Burger H, van Leen RW, Dorssers LCJ, Persoon NLM, Lemson PJ, Wagemaker G. Species specificity of human interleukin-3 demonstrated by cloning and expression of the homologous Rhesus monkey (*Macaca mulatta*) gene. Blood **1990**;76:2229–2234.
29. Knapp W, Dörken B, Rieber P, Schmidt RE, Stein H, von dem Borne AEGKr. CD antigens 1989. Blood **1989**;74:1448–1450.
30. Negrin RS, Kiem H-P, Schmidt-Wolf IGH, Blume KG, Cleary ML. Use of the polymerase chain reaction to monitor the effectiveness of ex vivo tumor cell purging. Blood **1991**;77:654–660.
31. Cunningham D, Hickish T, Rosin RD, Sauven P, Baron JH, Farrell PJ, Isaacson P. Polymerase chain reaction for detection of dissemination in gastric lymphoma. Lancet **1989**;i:695–697.
32. Offit K, Wong G, Filippa DA, Tao Y, Chaganti RSK. Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations. Blood **1991**;77:1508–1515.
33. Offit K, Jhanwar SC, Ladanyi M, Filippa DA, Chaganti RSK. Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: correlations between recurrent aberrations, histology and exposure to cytotoxic treatment. Genes Chrom Cancer **1991**;3:189–201.
34. Hubbard SM, Chabner BA, DeVita VT Jr, Simon R, Berard CW, Jones RB, Garvin A, Canellos GP, Osborne CK, Young RC. Histologic progression in non-Hodgkin's lymphoma. Blood **1982**;59:258–264.
35. De Jong D, Voetdijk BMH, Beverstock GC, van Ommen GJB, Willemze R, Kluin PM. Activation of the c-myc oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. N Engl J Med **1988**:318:1373–1378.
36. Limpens J, De Jong D, Van Krieken JHJM, Price CGA, Young BD, van Ommen G-JB, Kluin PLM. BCL-2/JH rearrangement in benign lymphoid tissues with follicular hyperplasia. Oncogene (in press).
37. Pang S, Koyanagi Y, Miles S, Wiley C, Vinters HV, Chen ISY. High levels of unintegrated HIV-I DNA in brain tissue of AIDS dementia patients. Nature **1990**;343:85–89.
38. Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci USA **1990**;87:2725–2729.
39. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, Tsujimoto Y, Caton AJ, Rovera G. Detection of minimal disease in haematopoetic malignancies of the B-cell lineage by using third-complementary-determining region (CDR-III) specific probes. Proc Natl Acad Sci USA **1989**:86:5123–5127.
40. Deane M, Norton JD. Detection of immunoglobulin gene rearrangement in B lymphoid malignancies by polymerase chain reaction gene amplification. Br J Haematol **1990**:74:251–256.

CHAPTER 3

Translocation (14;18)-positive cells are present in the circulation of the majority of patients with localized (stage I and II) follicular non-Hodgkin's Lymphoma.

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Translocation (14;18)-Positive Cells Are Present in the Circulation of the Majority of Patients With Localized (Stage I and II) Follicular Non-Hodgkin's Lymphoma

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Stage I and II follicular non-Hodgkin's lymphoma (NHL) is clinically defined as a localized disease. To study the possibility that this disease is in fact disseminated, we used the sensitive polymerase chain reaction (PCR) method using translocation (14;18) as marker. Samples from 21 patients who were clinically diagnosed with stage I or II follicular NHL were analyzed for the presence of t(14;18)-positive cells using PCR. We analyzed (1) the diagnostic lymph node biopsy and (2) the peripheral blood or bone marrow samples from these patients. Translocation (14;18) cells were detected in the diagnostic lymph node biopsies of 12 patients. In 9 of these patients, t(14;18)-positive cells were detected in peripheral blood and/or bone marrow samples at diagnosis and/or after therapy. Thus, in 75% of the follicular NHL patients carrying the t(14;18) as a marker for lymphoma cells, t(14;18)-positive cells were detected in peripheral blood and bone marrow at diagnosis and after therapy. Our results show that t(14;18)-positive cells can be detected in the circulation of patients with stage I and II follicular NHL, indicating that, although diagnosed as localized, the disease is disseminated.

FOLLICULAR LYMPHOMA is the most common type of non-Hodgkin's lymphoma (NHL) in the United States and Europe. The majority of these patients present with disseminated disease (stage III and IV). After appropriate staging only 10% to 20% of the patients will be diagnosed as stage I or II, implying localized disease.[1-3] These patients are usually treated with involved field radiotherapy (RT) alone, resulting in longstanding clinical complete remissions (CCR) and even cure. Relapse-free survival for patients with stage I or II follicular NHL is 83%, with a median follow-up of 6 years[2,4]; a 14-year survival probability of 72% for stage I and of 37% for stage II has been reported.[5]

In follicular NHL the reciprocal translocation between the long arms of chromosomes 14 and 18 [t(14;18)] is found in 55% to 70% of the patients in Europe.[3,6,7] In American studies, an incidence of 70% to 95% is observed.[8,9] As a result of this translocation, the joining (JH) region of the Ig heavy chain (IgH) gene (14q21) is juxtaposed to the BCL-2 gene (18q32).[10-16] Two breakpoint clusters are located within the non-coding part of the BCL-2 gene, the major breakpoint region (mbr) and the minor cluster region (mcr). The mbr is involved in approximately 60% of the translocations and spans only 150 bases.[12,14,17,18] The mcr is found less frequently and involves a breakpoint region of 500 bases.[8,19,20] In neither the peripheral blood of healthy donors nor in the peripheral blood and/or bone marrow from patients with t(14;18)-negative NHL, could t(14;18)-positive cells be detected.[3] The t(14;18) therefore provides a unique marker for the lymphoma cells and a suitable target for the polymerase chain reaction (PCR).

We present a retrospective study in which the presence of t(14;18)-positive cells is demonstrated in peripheral blood and/or bone marrow from stage I or II follicular NHL patients. Our results show that in the majority of these patients t(14;18)-positive cells are present in the circulation both at diagnosis and after therapy. These results indicate that stage I and II follicular NHL is often not a localized but a disseminated disease.

MATERIALS AND METHODS

Patients. Twenty-one patients initially included in this study were diagnosed with follicular centroblastic centrocytic NHL according to the Kiel classification.[21] The slides of the lymph node biopsies were reviewed by a panel of pathologists. Staging of these patients was performed according to the Ann Arbor classification.[22] The staging procedures included physical examination, CT scan of thorax and abdomen, morphologic examination, and immunologic marker analysis of peripheral blood and bone marrow aspirates.[3] These procedures were repeated after therapy and at regular intervals during follow-up. For patients in CCR, neither morphologic examination nor immunologic marker analysis showed the presence of lymphoma cells in the peripheral blood or bone marrow. Most patients were treated with surgical excision or were involved in field radiotherapy, some in combination with chlorambucil. All patients had given informed consent to donate peripheral blood and bone marrow.

DNA extraction and analysis. High molecular weight DNA was prepared from frozen lymph node biopsies and fresh peripheral blood and/or bone marrow cells according to previous described methods.[3] Frozen lymph node biopsies were available from nine patients and were subjected to Southern blot and PCR analysis.[3] For hybridization the following probes were used: a JH probe, specific for the joining regions (JH3-JH6) of the IGH gene;[23] a BCL-2 probe specific for the mbr t(14;18), gift from Y. Tsujimoto;[11] and the pFL-2 probe specific for the mcr t(14;18), gift from M.L. Cleary.[19] Paraffin embedded lymph node biopsies were analyzed by PCR in 12 patients. DNA from paraffin embedded lymphoma biopsies was isolated as follows: Two 6 μm sections were deparaffinated by xylene (twice for 5 minutes), washed with 96% and 70% ethanol, and dried. The sections were incubated for 12 to 18 hours at 37°C in 200 μL tissue preparation mixture (0.75 × PCR buffer [Promega Cooperation, Leiden, The Netherlands], 0.5% Tween-20, and 200 μg/mL proteinase K). The samples were heated to 95°C for 8 minutes, extracted with phenol/chloroform, and precipitated with sodium acetate and ethanol. The DNA was resuspended in 100

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μL 10 mmol/L Tris-HCl (pH 7.4); 1 to 10 μL was used for the in vitro amplification by PCR.

In vitro DNA amplification by PCR. Two different PCR strategies were used to detect mbr t(14;18)-positive cells in peripheral blood and bone marrow from stage I and II NHL patients. For one-step PCR, 1 μL of DNA in a reaction volume of 50 μL was subjected to 30 cycles of PCR amplification using an automated Perkin-Elmer/Cetus DNA Thermal Cycler (Gouda, The Netherlands). Taq DNA polymerase (Promega; 0.40 U), and the recommended buffer conditions. Thirty-five to 40 pmol of JH16 and BCL18 in a volume of 50 μL was used (Table 1). Primers were synthesized by Pharmacia Nederland BV (Woerden, the Netherlands). The optimal cycle conditions were 94°C for 4 minutes; subsequently 30 cycles of 1 minute at 94°C, 2 minutes at 58°C, 2 minutes at 72°C, followed by a final extension of 7 minutes at 72°C.[3] To generate ethidium bromide visible products 1/50 of the amplified product was reamplified in 20 cycles using 35 to 40 pmol of "nested" JH20 and BCL18 in a reaction volume of 50 μL. The nested JH20/BCL18 amplified products were 13 bp shorter than the PCR product generated after amplification with JH16 and BCL18.[10] (Table 2). For two-step PCR, 50 μL PCR reaction mixture included 0.5 U of Taq DNA polymerase, 0.35 to 0.40 pmol of the primers JH16 and BCL20, and 35 to 40 pmol of the primers JH14 and BCL18 (Table 1). The cycle profile was denaturing for 5 minutes at 94°C in the first cycle and for 45 seconds in the subsequent 39 cycles. In the first 10 cycles, annealing was performed at 65°C for 2 minutes, permitting only primers JH16 and BCL20 (present in a limiting amount of 0.35 to 0.40 pmol) to hybridize to the target DNA. In the next 30 cycles annealing was at 54°C for 2 minutes allowing primers JH14 and BCL18 to hybridize. Extension of the annealed primers was at 72°C for 2 minutes, followed by a final extension at 72°C for 7 minutes. For the detection of mcr t(14;18)-positive cells primers JH16 and BCLMC12 were used under the same conditions as described for the detection of mbr t(14;18)-positive cells in the one step PCR (Table 1).[19] One fifth of the amplified DNA was analyzed on a 1.2% agarose gel followed by Southern blot analysis with the BCL-2 or pFL-2 probe.[3]

As a control to DNA quality, a 750-bp fragment of the interleukin-3 (IL-3) gene was amplified in 25 cycles, using primers IL-3/2 and IL-3/9 ([3] (Table 1). Provided that this IL-3 fragment was visible on ethidium bromide staining, results of the t(14;18) PCR were evaluated. For the mbr t(14;18) amplification, DNA extracted from SU-DHL-6 cells diluted in normal mononuclear blood cells (up to 10^{-6} dilution) was used as a positive control. With the one-step PCR the 10^{-2} dilution of SU-DHL-6 resulted in a ethidium bromide band visible on gel, and the 10^{-5} dilution of SU-DHL-6 was positive following hybridization with the BCL-2 probe. With the two step PCR the 10^{-4} dilution of SU-DHL-6 cells was positive after ethidium bromide staining, and the presence of mbr t(14;18)-positive cells in the 10^{-5} dilution was confirmed by hybridization. For the mcr t(14;18) PCR, DNA from an mcr t(14;18)-positive lymph node biopsy was diluted in DNA from normal mononuclear blood cells. Up to the 10^{-5} dilution hybridization was seen with the pFL-2 probe. The detection limit of both mbr- and mcr-t(14;18)-positive cells was estimated to be approximately 1 t(14;18)-positive cell out of 100,000 normal cells. Each DNA sample was analyzed for breakpoint amplification in at least two independent experiments. From the isolated DNA samples, at least two separate dilutions were made from a stock DNA sample and tested with PCR. Samples were considered to be positive when an amplified fragment hybridized with the BCL-2 or pFL-2 probe. Only those experiments were evaluated in which the positive control was successfully amplified up to a 10^{-5} dilution.

Table 1. Code and Sequence of Oligonucleotides Used for PCR Analysis

Code of Primer	5' → 3' Sequence of Primer
JH14	AGGAGACGGTGACC
JH16	TGAGGAGACGGTGACC
JH20	ACCAGGGTTCCTTGGCCCCA
BCL18	GGTGGTTTGACCTTTAGA
BCL20	TAAAAATCCAGATGGCAAAT
BCLMC12	TTGATGGCTTTGCTGAGA
IL-3/2	GAGGTTCCATGTCAGATA
IL-3/9	CCTCACCTAGAACTGCCT
IL-3/11	CATCGATCATGTTAGAGC

The quality control for DNA isolated from paraffin embedded tissue is slightly different. We used the same cycle profile for the SU-DHL-6 dilutions to establish the sensitivity of each individual experiment. DNA from paraffin embedded tissue was amplified in 40 cycles using the primers IL-3/2 and IL-3/11, resulting in a 300-bp fragment visible on ethidium bromide staining. To amplify this IL-3 fragment, several DNA dilutions of paraffin embedded tissue were tested. In most samples, no 750-bp IL-3 fragment could be amplified with the primers IL-3/2 and IL-3/9. If t(14;18)-specific PCR products could not be detected by hybridization in three independent amplification experiments, than the diagnostic lymph node biopsy was considered to be negative for t(14;18).

Sequence analysis. Sequence analysis by the dideoxy chain termination method was performed on PCR product of patient ME. DNA was amplified with the BCL20 and JH16 primers. An approximately 1,050-bp fragment was detected on ethidium bromide staining. This fragment was isolated out of the gel, reamplified using BCL18 and JH16, and cloned into the *Sma* I site of PTZ18R. The sequence was analyzed using the Pharmacia T7 sequence kit.

RESULTS

Definition of the patients used in this study. Twenty-one stage I and stage II follicular NHL patients (Table 3) were studied for the presence of t(14;18)-positive cells in (1) the diagnostic lymph node biopsy and (2) peripheral blood and bone marrow at diagnosis and during follow-up after therapy. Immunocytochemistry confirmed monoclonality of the diagnostic lymph node biopsy in 13 patients using κ and λ light chain antibodies (Table 3). The remaining eight biopsies, all paraffin-embedded, were studied using an antibody directed against BCL-2.[24] All these biopsies were positive for BCL-2 protein expression in the follicles (Table 3). In a reactive lymph node biopsy the follicle centers were negative for BCL-2 protein expression in contrast to the mantel zone, where BCL-2 protein expression was detected. The t(14;18) was detected by PCR using two specific primer sets, defining either the mbr or the mcr breakpoint of t(14;18) (Table 1). Using the mbr specific primer set we detected an amplified product of 100 to 300 bp in the majority of the t(14;18)-positive patients (Fig 1). Translocation (14;18)-positive cells were identified in 13 patients, either in the diagnostic lymph node biopsy or in peripheral blood and bone marrow samples. In 12 of those patients, t(14;18) was demonstrated in the diagnostic lymph node biopsies on frozen tissue (n = 5) using both Southern analysis and PCR analysis or on paraffin-embedded tissue (n = 7) using PCR only (Table 3).

Table 2. Clinical Characteristics and PCR Results From Patients With t(14;18)-Positive Stage I and II Follicular NHL at Diagnosis and During Follow-up

Patient	Breakpoint/Size	Follow-up (in months)	Sample	Im	Mo	PCR t(14;18)	Clinical Status	Therapy
BA	MBR/200	0	LN	+	+	Positive	I	
		0	PB	–	–	–		
BD	MBR/180	0	LN	+	+	Positive	I	RT
		0	BM	–	–	+		
		+3	PB	NT	NT	+	CCR	
BM	MBR/230	0	LN	+	+	Positive	I	Surgery
		0	PB	–	–	–		
		0	BM	–	–	+		
DH	MBR/190	0	LN	+	+	Positive	I	RT + chem
		+156	PB	NT	–	–	CCR	
		+168	PB	NT	–	–	CCR	
DL	MBR/280	0	LN	+	+	Positive	I	RT
		0	PB	–	–	–		
		+4	PB	NT	NT	+	CCR	
DO	MBR/120	0	LN	+	+	Positive	I	RT
		0	PB	–	–	+		
		0	BM	–	–	+		
		+4	PB	NT	NT	+	CCR	
		+7	PB	NT	NT	+	CCR	
		+13	PB	–	–	–	CCR	
		+17	PB	NT	–	+	CCR	
		+20	PB	NT	NT	–	CCR	
		+23	PB	–	–	+	CCR	
IL	MBR/180	0	LN	+	+	Positive	I	RT
		0	BM	–	NT	+		
ME	MBR/1000	0	LN	+	+	–	I	Surgery
		+32	PB	NT	NT	+++	CCR	
		+44	PB	NT	–	+++	CCR	
		+51	PB	NT	NT	+++	CCR	
		+55 = 0′	PB	–	NT	+++	Relapse	RT
		+55 = 0′	BM	–	–	+++		
		+6′	PB	NT	NT	+++	NC	
QO	MCR/600	0	LN	+	+	Positive	I	RT
		0	PB	–	–	+		
		+4	PB	NT	NT	–	CCR	
SW	MBR/300	0					I	RT
		+56 = 0′	BM	+	–	++	Relapse	No
		+4′	PB	NT	NT	++	NC	
		+6′	PB	NT	–	++	NC	
		+10′	PB	NT	NT	++	NC	
		+16′	LN	NT	–	Positive	NC	Surgery
TE	MBR/160	0	LN	+	+	Positive	I	RT
		+24	PB	–	–	–	CCR	
TH	MBR/200	0					II	RT + chem
		+96	PB	NT	NT	+	CCR	
		+100 = 0′	LN	+	+	Positive	Relapse	Surgery
		+7′	PB	NT	NT	+	CCR	
		+10′	PB	NT	NT	–	CCR	
		+19′	PB	NT	NT	+	CCR	
WI	MBR/220	0	LN	+	+	Positive	II	RT
		0	PB	–	–	+		
		0	BM	–	–	+		
		+7	PB	NT	NT	–	CCR	
		+10	PB	–	–	+	CCR	
		+10	BM	–	–	–	CCR	

Translocation (14;18) results are presented as follows: diagnostic lymph node (positive); ethidium bromide visible bands (+++); no ethidium bromide visible band but strong signal after hybridization (++); signal at threshold of detection after hybridization (+).

Abbreviations: ′, months after local relapse; BM, bone marrow; CCR, clinical complete remission; chem, chemotherapy; Im, immunologic marker analysis; LN, lymph node; Mo, morphologic examination; NC, no charge; NT, not tested; PB, peripheral blood; RT, radiotherapy.

Table 3. Details of Follicular Centroblastic Centrocytic NHL Patients and Their Lymph Node Biopsies

Patient	Age (yr)	Sex	Stage	Localization	Frozen or Paraffin	t(14;18)	Immunology BCL-2	Immunology
BA	77	M	I	Axilla left	P	+	+	NE
BD	61	F	I	Supraclavicular left	F	+		κ
BM	75	F	I	Cutaneous forehead	F	+		κ
DB	56	M	II	Orbita left and right	F	a		κ
DH	65	M	I	Supraclavicular left	P	+	+	NE
DL	45	M	I	Supraclavicular left	P	+	+	λ
DO	54	M	I	Supraclavicular left	F	+		κ
IL	45	M	I	Parotis right	P	+	+	NE
KH	78	M	I	Supraclavicular right	F	−		κ
KO	63	M	I	Submandibular right	P	−	+	NE
KV	41	M	I	Submandibular left	F	−		κ
LB	25	F	I	Axilla right	P	−	+	λ
LW	71	M	II	Orbita and cervical left	P	−		λ
ME	58	F	I	Groin left	P	−	+	NE
PD	62	F	I	Axilla left	P	−	+	NE
QO	60	F	I	Supraclavicular left	F	+		κ
SW	66	F	I	Axilla right	P	+		κ
TE	51	M	I	Parotis right	P	+	+	NE
TH	52	F	II	Submandibular and axilla left	F	+		λ
VR	58	F	I	Thorax wall	F	−		κ
WI	58	M	II	Cervical right and left	P	+	+	NE

Characteristics of patients and the localization and stage of the disease are designated. From the diagnostic lymph node either frozen (F) or paraffin (P) embedded tissue was available and analyzed for t(14;18). Detection of t(14;18) by Southern analysis on frozen tissue or by PCR on paraffin embedded tissue (+); absence of t(14;18) (−); detection of t(14;18) on frozen tissue using PCR, which could not be confirmed by Southern analysis (a). Immunocytochemistry using the antibodies directed against the κ and λ Ig light chains was performed on frozen tissue to confirm monoclonality (κ or λ) at diagnosis. On paraffin-embedded tissue monoclonality could not be evaluated using κ and λ antibodies (NE). BCL-2 protein expression was detected in all the paraffin embedded tissues studied using an antibody BCL-2 100α (+).

In 1 patient (ME), t(14;18)-positive cells were detected in peripheral blood and bone marrow during follow-up on different occasions, but could not be confirmed in the paraffin-embedded diagnostic lymph node biopsy. The reason for this discrepancy could be that the amplification product was large (approximately 1,000 bp), in comparison to the usual 100- to 300-bp fragments. DNA isolated from paraffin-embedded tissue is often of relatively poor quality and does not allow amplification of large fragments. The fact that we could amplify the control 300-bp IL-3 fragment, but not the 750-bp IL-3 fragment, indeed showed that the paraffin-derived DNA from this patient was in poor condition. Se-

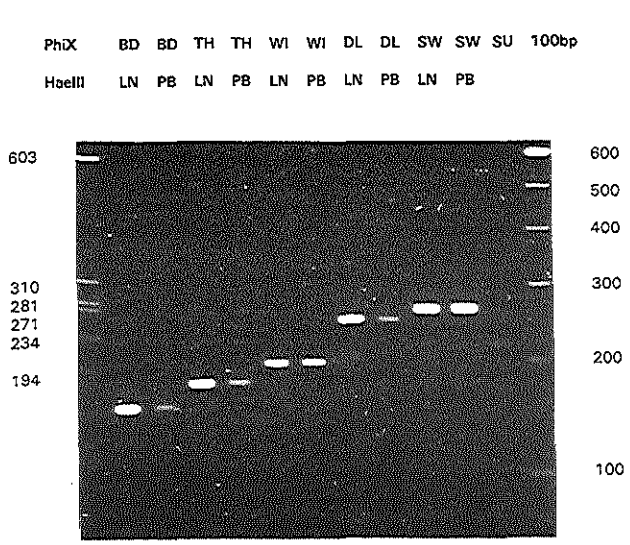

Fig 1. t(14;18)-positive cells from lymph node biopsy and circulation yield identical PCR fragments. t(14;18)-specific PCR products were generated after reamplification of JH16/BCL18 products using the nested JH20 and BCL18 primers. Samples from the diagnostic lymph node biopsy (LN) and peripheral blood (PB) were compared. The results from 5 patients are shown. In addition the 10^{-5} dilution of SU-DHL-6 (SU) is shown. At the left, the size marker *Phi*X X *Hae*III and at the right a 100-bp size ladder is shown. The estimated size of the PCR products is 160, 180, 200, 260, and 280 bp, respectively; the size of the SU-DHL-6 fragment is 222 bp.[11]

quence analysis of the 1,000-bp PCR product showed that the fragment consisted of 951 bp homologous to the BCL-2 gene plus 52 bp homologous to the JH6 sequence. The breakpoint on chromosome 18 is exceptional in that it is 800 bp downstream of the mbr. So far, only breakpoints upstream of the mbr are described.[16,25] The PCR product that we detected in peripheral blood and bone marrow from patient ME is thus derived from the junction between the BCL-2 and JH gene. Therefore, we include this patient in our analysis of the 13 patients with t(14;18)-positive NHL. Six of the 21 patients were negative for t(14;18) in the diagnostic lymph node biopsy, and in peripheral blood and bone marrow at diagnosis and/or after therapy. Two of the 21 patients (DB and KO) were excluded from evaluation in this study for different reasons. In the frozen lymph node biopsy from patient DB, t(14;18)-positive cells were identified only by PCR. Southern analysis showed one rearranged Ig heavy chain gene and no translocated BCL-2 gene. It was concluded that these t(14;18)-positive cells did not represent the monoclonal B-cell population. One peripheral blood sample from patient KO contained low numbers of t(14;18)-positive cells 11 years after diagnosis. The presence of this translocation could not be confirmed in the paraffin-embedded diagnostic lymph node biopsy, probably due to inferior quality of the DNA isolated.

In conclusion, samples from 13 of 21 patients that were analyzed contained t(14;18)-positive cells. Clinical characteristics and PCR results from patients with t(14;18)-positive NHL are summarized in Table 2 and will be discussed in more detail in the next sections.

Presence of t(14;18)-positive cells in peripheral blood and/or bone marrow at diagnosis. From the group of 12 patients with t(14;18)-positive cells in the diagnostic lymph node biopsy, we collected peripheral blood and/or bone marrow samples at diagnosis from 8 of them. Of six patients from whom we had peripheral blood, 3 were positive for t(14;18) cells (DO, QO and WI [Table 2]). Of 5 patients from whom we had bone marrow, all 5 were positive for t(14;18) cells (BD, BM, DO, IL, and WI). In patients DO and WI, both peripheral blood and bone marrow contained t(14;18)-positive cells. Thus, in 6 of the 8 patients analyzed, t(14;18)-positive cells were detected in the circulation at diagnosis.

Presence of t(14;18)-positive cells in peripheral blood and/or bone marrow after therapy. From the group of 13 patients with t(14;18)-positive NHL, we collected peripheral blood and/or bone marrow after therapy from 10 of them. Of 10 patients from whom we had peripheral blood, 7 were positive for t(14;18) cells (BD, DL, DO, ME, SW, TH, and WI [Table 2]). Of 3 patients (ME, SW, and WI) from whom we had bone marrow, 2 were positive for t(14;18) cells (ME and SW). Both peripheral blood and bone marrow from these 2 patients contained t(14;18)-positive cells. In the bone marrow from patient WI, no t(14;18)-positive cells were detected, but the peripheral blood taken at the same time contained t(14;18)-positive cells. Thus, in 7 of the 10 patients analyzed, t(14;18)-positive cells were detected in the circulation after therapy.

Translocation (14;18)-positive cells from lymph node biopsy and circulation yield identical PCR products. To exclude the possibility that the t(14;18)-positive cells from the diagnostic lymph node biopsy and from the circulation contained a different t(14;18) breakpoint, we compared their PCR products. In all 9 patients who had t(14;18)-positive cells in the diagnostic lymph node biopsy and in the circulation, we found amplified DNA fragments of identical size. This is shown for 5 patients in Fig 1 and for 1 additional patient in Fig 2. The difference in size (100 to 300 bp) of the nested PCR products among 5 different patients is also illustrated in Fig 1.

Clinical course in patients with follicular NHL. The total number of patients with t(14;18)-positive cells in the circulation, either at diagnosis or after therapy is 10 of the 13 patients with t(14;18)-positive NHL (Table 2). We evaluated the clinical course of these 10 patients. Three of these patients relapsed 55, 60, and 100 months after initial therapy; the mean duration of the CCR being 72 months. Five of these patients are in ongoing CCR, with a mean follow-up of 9 months (range, 3 to 23 months). For the remaining 2 patients, no follow-up samples were available. Of the 3 patients with t(14;18)-positive NHL but without t(14;18)-positive cells in the circulation, 2 patients were in CCR for 24 and 172 months, respectively.

Three of the 6 patients with t(14;18)-negative NHL were in CCR at the moment of evaluation, with a follow-up of 3, 15, and 23 months, respectively. Of the other 3 patients, no follow-up samples were available. One patient (DO) with a relatively long follow-up and circulating lymphoma cells will be discussed in more detail to illustrate the course of the disease. mbr t(14;18)-positive cells could be detected in the diagnostic lymph node biopsy and in the peripheral blood and bone marrow taken at diagnosis (Fig 2). While the patient was in CCR, faint t(14;18)-specific bands hybridizing with BCL-2 were detected in the peripheral blood 4, 7, 17, and 23 months after local radiotherapy. This indicates a low amount of circulating t(14;18)-positive cells. In the peripheral blood samples taken after 13 and 20 months, no mbr t(14;18)-positive cells could be detected (Table 2). In Fig 2, the results of one experiment are shown. The peripheral

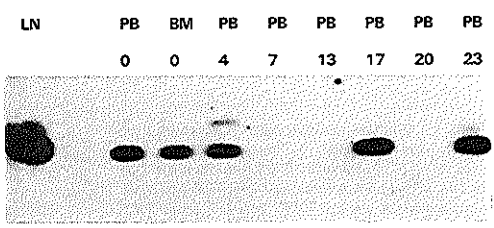

Fig 2. The course of the follicular NHL in 1 patient as indicated by t(14;18)-specific PCR fragments. t(14;18) PCR fragments were generated from the diagnostic LN, PB, and bone marrow (BM) from patient DO at diagnosis and at various time points (indicated in months) after completion of therapy. The 120-bp t(14;18)-specific fragments were visualized by hybridization with the BCL-2 probe. For LN 1/50 of the amplified product and for PB and BM 1/5 of the amplified product was loaded.

blood samples taken after 7, 13, and 20 months contained no detectable t(14;18)-positive cells. However, in two additional experiments, we could detect t(14;18)-positive cells in peripheral blood taken after 7 months. These results indicate that the amount of circulating t(14;18)-positive cells is apparently at the threshold of the detection.

DISCUSSION

In this report we described the presence of t(14;18)-positive cells in the circulation of patients with localized (stage I and II) t(14;18)-positive follicular NHL. Translocation (14;18)-positive cells in the diagnostic lymph node biopsy were detected with an overall incidence of t(14;18) in 63% of the patients (12 of 19). In the group of patients with t(14;18)-positive NHL, we detected t(14;18)-positive cells in the circulation at diagnosis and after therapy, with an incidence of about 77%. The fragment sizes of the amplified products from peripheral blood and/or bone marrow samples were identical to the fragment sizes of the t(14;18)-specific products in the corresponding lymph node biopsies (Figs 1 and 2). The circulating t(14;18)-positive cells are therefore most probably lymphoma cells, identical to the t(14;18)-positive cells in the diagnostic lymph node biopsy. We conclude from these results that stage I and II t(14;18)-positive follicular NHL, diagnosed as localized disease according to conventional staging techniques, is already disseminated at diagnosis. Furthermore, therapy does not eradicate the circulating t(14;18)-positive cells.

The sensitivity of the PCR method for detecting t(14;18)-positive cells implies that about 300,000 t(14;18)-positive cells are present in the circulation of patients with a normal leukocyte count for long periods of time.[3,26] This evokes the question about what the presence of t(14;18)-positive cells in the circulation of these stage I and II follicular NHL patients means with respect to the risk of relapse. These patients usually have a favorable prognosis. In this study 3 (ME, SW, and TH) of the 10 patients with t(14;18)-positive cells in the circulation had a clinical relapse. In 2 of them (ME and SW), a high frequency of circulating t(14;18)-positive cells was detected. In the other patient (TH), the frequency of circulating t(14;18)-positive cells remained near the detection limit of PCR analysis. The frequency of circulating t(14;18)-positive cells in all patients in continuing CCR was near the threshold of detection by PCR (Fig 2). The small group of patients analyzed, does not allow us to make a connection between the presence of t(14;18)-positive cells in circulation and the prognoses of these patients. A longer follow-up period and refinement of the quantification procedure of the PCR are necessary to address this question.

The biologic relevance of t(14;18)-positive cells in the circulation of patients with follicular lymphoma is unclear. Two mechanisms could be responsible for this phenomenon. The high expression of the BCL-2 oncogene, present in t(14;18)-positive cells may prevent a slowly expanding clone from apoptosis.[27] If so, it is expected that every patient with t(14;18)-positive cells will eventually relapse. The other possibility is that the t(14;18) marks only a premalignant status. In that case, an additional, so far unknown event, may transform one of these cells to malignancy resulting in a local manifestation of the disease. Local therapy may eradicate the malignant clone in stage I and II NHL and reduce the disease to a premalignant status. In this case, patients may be cured not withstanding the presence of t(14;18)-positive cells. In favor of this second possibility is the finding that low numbers of t(14;18)-positive cells have been detected by PCR in benign follicular hyperplasia.[28] Similarly, the low numbers of t(14;18)-positive cells detected in the frozen lymph node biopsy from patient DB most probably represent premalignant t(14;18)-carrying cells.

Our study provides strong evidence that stage I and II follicular NHL are usually disseminated diseases. However, at present we do not know the significance of the presence of t(14;18)-positive cells in circulation in stage I and II follicular NHL patients with regard to the risk of clinical relapse.

ACKNOWLEDGMENT

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REFERENCES

1. Mead GM: Clinical aspects of early stage non-Hodgkin's lymphoma. Br J Cancer 61:7, 1990

2. Gallagher CJ, Gregory WM, Jones AW: Follicular lymphoma: Prognostic factors for response and survival. J Clin Oncol 4:1470, 1986

3. Lambrechts AC, de Ruiter PE, Dorssers LCJ, van't Veer MB: Detection of residual disease in translocation (14;18) positive non-Hodgkin's lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods. Leukemia 6:29, 1992

4. Gallagher CJ, Lister TA: Follicular non-Hodgkin's lymphoma. Bailliere's Clin Hematol 1:141, 1987

5. Simon R, Durrleman S, Hoppe RT, Bonadonna G, Bloomfield CD, Rudders RA, Cheson BD, Berard CW: The non-Hodgkin's lymphoma pathologic classification project: Long-term follow-up of 1153 patients with non-Hodgkin's lymphomas. Ann Intern Med 109:939, 1988

6. Clark HM, Jones DB, Wright DH: Cytogenetic and molecular studies of t(14;18) and t(14;19) in nodal and extranodal B-cell lymphoma. J Pathol 166:129, 1992

7. de Jong D, Voetdijk BHM, van Ommen GLB, Kluin-Nelemans JC, Beverstock GC, Kluin PhM: Translocation t(14;18) in B-cell lymphomas as a cause for defective immunoglobulin production. J Exp Med 169:613, 1989

8. Weiss LM, Warnke RA, Sklar J, Cleary ML: Molecular analysis of the t(14;18) chromosome translocation in malignant lymphomas. N Engl J Med 317:1185, 1987

9. Rowley JD: Chromosome studies in the non-Hodgkin's lymphomas: The role of the 14;18 translocation. J Clin Oncol 6:919, 1988

10. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P: Structure of the human immunoglobulin μ locus: Characterization of embryonic and rearranged J and D genes. Cell 27:583, 1981

11. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC, Croce CM: Cloning of the chromosome break-point of neoplastic B cells with the t(14:18) chromosome translocation. Science 226:1097, 1984

12. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ: Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41:889, 1985

13. Cleary ML, Sklar J: Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82:7439, 1985

14. Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19, 1986

15. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM: The t(14;18) chromosome translocations involved in B-cell neoplasms results from mistakes in VDJ joining. Science 229:1390, 1985

16. Cotter FE, Price C, Zucca E, Young BD: Direct sequence analysis of the 14q+ and 18q− junctions in follicular lymphoma. Blood 76:131, 1990

17. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA: Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 227:175, 1987

18. Cotter FE, Price C, Meerabux J, Zucca E, Young BD: Direct sequence analysis of 14q+ and 18q− chromosome junctions at the MBR and MCR revealing clustering within the MBR in follicular lymphoma. Ann Oncol 2:93, 1991

19. Cleary ML, Galili N, Sklar J: Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 164:315, 1986

20. Ngan B-O, Nourse J, Cleary ML: Detection of chromosomal translocation t(14;18) within the minor cluster region of bcl-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. Blood 73:1759, 1989

21. Stansfeld AG, Diebold J, Noel H, Kapanci Y, Rilke F, Kelenyi G, Sundstrom C, Lennert K, van Unnik JAM, Mioduszewska O, Wright DH: Updated Kiel classification for lymphomas. Lancet 1:292, 1988

22. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M: Report of the Committee on Hodgkin's Disease Staging Classification. Cancer Res 31:1860, 1971

23. Takahashi N, Nakai S, Honjo T: Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acids Res 8:5983, 1980

24. Pezzella F, Tse AG, Cordell JL, Pulford KA, Mason DY: Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225, 1990

25. Tsujimoto Y, Bashir MM, Givol J, Cossman J, Jaffe E, Croce CM: DNA rearrangements in human follicular lymphoma can involve the 5′ or the 3′ region of the bcl-2 gene. Proc Natl Acad Sci USA 84:1329, 1987

26. Price CGA, Meerabaux J, Murtagh S, Cotter FE, Rohatiner AZS, Young BD, Lister TA: The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 9:1527, 1991

27. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: BCL-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334, 1990

28. Limpens J, de Jong D, van Krieken JHJM, Price CGA, Young BD, van Ommen G-JB, Kluin PLM: BCL-2/JH rearrangement in benign lymphoid tissues with follicular hyperplasia. Oncogene 6:2271, 1991

CHAPTER 4

Clinical significance of t(14;18)-positive cells in the circulation of patients with stage III or IV follicular non-Hodgkin's lymphoma during first remission.

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Journal of Clinical Oncology in press.

SUMMARY

Purpose: To evaluate polymerase chain reaction (PCR) analysis as a method for the detection of circulating lymphoma cells in patients with stage III and IV t(14;18)-positive follicular non-Hodgkin's lymphoma (NHL) in first remission in a longitudinal prospective study.

Patients and Methods: Peripheral blood or bone marrow from eight patients with stage III and IV t(14;18)-positive NHL was studied using PCR to detect the presence of t(14;18)-positive cells in the circulation at different time points during first remission.

Results: In four of six patients with no clinical evidence of disease (NCED), t(14;18)-positive cells were detectable in the circulation. In one of two patients with clinical evidence of disease (CED), no t(14;18)-positive cells were found at the four different occasions tested during first remission. First-remission duration ranged from 17 to 81^{+} months. The duration from the first PCR determination in remission until first relapse or the end of the observation period ranged from 10 to 37^{+} months.

Conclusion: In patients with stage III and IV t(14;18)-positive follicular NHL treated with conventional remission induction therapy, the presence or absence of t(14;18)-positive cells in the circulation shows no obvious correlation with the clinical remission status and the remission duration.

INTRODUCTION

Follicular non-Hodgkin's lymphoma (NHL) accounts for approximately 45% of all NHLs (1,2). In 60% to 85% of patients, it is characterized by a specific chromosomal translocation between chromosomes 14q32 and 18q21 [t(14;18)] (3-7). As a result of this translocation, the joining (JH) region of the immunoglobulin heavy chain (IgH) gene (14q32) is juxtaposed to the BCL-2 gene (18q21) and a hybrid BCL2/JH sequence, unique for the NHL cells, is created (8-12). Two breakpoint clusters are located within the 3' non-coding part of the BCL-2 gene, the major breakpoint region (mbr) and the minor cluster region (mcr)(13). The mbr is involved in 60% to 70% of the translocations and spans only 150 bases (10,14-16). A mcr breakpoint is found in 20% to 30% of the translocations and involves a breakpoint region of 500 bases (6,13,17). Thus, the t(14;18) provides a unique marker for the NHL cells and a suitable target for the sensitive *in vitro* DNA amplification technique, the polymerase chain reaction (PCR)(18).

Follicular NHL is classified as a low-grade malignant NHL and has a relatively indolent course (19). The majority of the patients (range, 80% to 90%) present with

disseminated disease that is restricted to lymph nodes (stage III) or also includes peripheral blood, bone marrow and organ localization (stage IV)(20). These patients are usually treated with chemotherapy. Most patients respond to therapy and about half of the patients achieve a clinical complete remission (21,22). The median first-remission duration for stage III and IV follicular NHL patients is reported in different studies to range from 18 to 28 months (21). In addition to conventional techniques such as imaging of lymph nodes and detection of NHL cells in the circulation (peripheral blood and bone marrow) by morphological examination and immunological marker analysis, PCR on the breakpoint regions of t(14;18) is used for the detection of minimal disease (3,23,24).

We report here the results of PCR analysis on peripheral blood and/or bone marrow cells in a longitudinal study, to evaluate this technique as a method for detection of minimal residual disease in patients with stage III and IV t(14;18)-positive follicular NHL in first remission.

MATERIALS AND METHODS

Patients: All patients in this study were diagnosed with stage III and IV (20) follicular centroblastic centrocytic NHL (25). The slides of the lymph node biopsies were reviewed by a panel of pathologists. Only those patients were included in whom the lymph node biopsy at diagnosis contained t(14;18)-positive NHL cells. The staging procedures included physical examination, computed tomographic scan of thorax and abdomen, morphological examination, and immunological marker analysis of peripheral blood and bone marrow (3). These procedures were repeated after therapy and at regular intervals during the follow-up period. All patients were initially treated with 8 courses of combination chemotherapy (cyclophosphamide, vincristine and prednisone (CVP)) every four weeks (according to EORTC trial 20586). This initial chemotherapy was aimed at the highest reduction of disease. After chemotherapy, radiotherapy was administered to residual enlarged lymph node masses if present. Patients were then randomized for treatment with interferon-α or no further treatment. All patients had given informed consent to donate peripheral blood and bone marrow.

DNA extraction and analysis: DNA extraction, Southern blotting, in vitro DNA amplification by PCR, and analysis of PCR products was performed as described previously (3,23). A frozen lymph node biopsy was available from one patient, and DNA from this biopsy was subjected to Southern blotting and PCR analysis (3). Paraffin-embedded lymph node biopsies were available from seven patients, and serial dilutions of the DNA isolated from these biopsies were subjected to PCR analysis. To evaluate the efficacy of PCR experiments, amplification of the mbr t(14;18)-positive SU-DHL-6 cell line cells serially diluted in normal mononuclear blood cells was performed. The experiments were evaluated if the 10^{-5} dilution was positive after 30 cycles. The quality of high-molecular weight DNA isolated, was

established by amplification of a 750 base pair (bp) fragment of the interleukin-3 (IL-3) gene in 25 cycles for frozen tissue. This amplified product was visible upon ethidium bromide staining, indicating large amounts of amplified products (3,23). The quality of DNA isolated from paraffin-embedded tissue was reduced, as demonstrated by the results of the amplification of a 300-bp and 750-bp fragment of the IL-3 gene in 40 cycles (3,23). In most samples, the 300-bp IL-3 fragment was detectable upon hybridization, whereas the 750-bp IL-3 fragment was absent.

RESULTS

Eight patients with t(14;18)-positive follicular NHL were evaluated for the presence of t(14;18)-positive cells in the circulation (peripheral blood and bone marrow) during first remission. In Table 1 lists data concerning the remission status and duration of these patients, as well as PCR results.

Presence of t(14;18)-positive cells during first clinical remission.
After initial treatment, six patients reached a complete remission and showed no clinical evidence of disease (NCED) and 2 patients responded partially to treatment and had clinical evidence of disease (CED). In the circulation of four patients with NCED, t(14;18)-positive cells were detected; in the other two patients, PCR was negative during first remission. In patients with CED, one patient showed no t(14;18)-positive cells in the peripheral blood on all occasions tested, and the other patient had positive PCR's during first remission. Comparison of clinical status at the date of sampling with PCR data showed the following results: t(14;18)-positive cells were detected in 15 samples, while there was NCED in 13; and no t(14;18)-positive cells were detected in 18 samples, while there was CED in four.

Presence of t(14;18)-positive cells with respect to duration of first clinical remission.
The remission duration is calculated for all patients 8 months from diagnosis (eight courses of CVP every 4 weeks plus or minus radiotherapy) until progression or the end of the observation period. The first-remission durations for three patients who relapsed during the observation period were 17, 17 and 78 months. The first-remission durations of the 5 patients in ongoing remission was 22, 56, 57, 61 and 81 months. For these eight patients we also calculated the duration from the first PCR determination to clinical relapse or the last PCR determination. For the three patients with clinical relapse, the durations of PCR follow-up were 10, 17 and 34 months. For the five patients in ongoing first remission the durations of PCR follow-up were 20, 27,

33, 34 and 37 months.

Circulating t(14;18)-positive cells in individual patients.

In the individual patients, we found a tendency that t(14;18)-positive cells were either present or absent in the circulation. This is not only true for the six patients with NCED who nearly always showed a positive PCR (patient no. 2, three of three; 4, five of five; 5, two of three; and 7, three of five) or a negative PCR (patient no. 1, six of six; and 6, five of five), but also for the two patients with CED, with patient no. 3 being positive (two of two) and patient no. 8 being negative (four of four). Of the three patients who relapsed during the observation period, one was already positive during clinical complete remission (no. 4), one remained negative (no. 8), and one showed both positive and negative results during remission and was not tested after relapse (no. 7). In patient no. 7, t(14;18)-positive cells were detected 48 and 62 months after diagnosis; at 68 and 78 months, the amount of circulating t(14;18)-positive cells was below the detection level, but reappeared with slightly increased frequency at 82 months after diagnosis. Eighty-five months after diagnosis, a clinical relapse became evident. Figure 1 shows one PCR experiment in which, in contrast to other experiments, no amplification product was seen at 62 months. In patient no. 3, t(14;18)-positive cells were detected in all samples, except for the sample just taken after completion of chemotherapy.

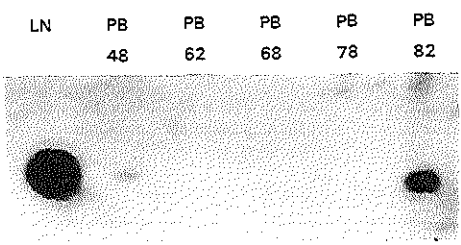

Figure 1:
PCR experiment of patient no. 7. t(14;18)-positive cells were detected in the lymph node (LN) and peripheral blood (PB) samples (48 and 82 months after diagnosis). The amplified fragment is approximately 200-bp.

DISCUSSION

In this longitudinal study, we used amplification of the t(14;18) by PCR to detect minimal residual disease in patients with stage III and IV t(14;18)-positive follicular NHL during first remission. In these patients, initial treatment was given in conventional dosages with the aim to reduce the disease as much as possible.

Table 1: Clinical characteristics and PCR results of patients with t(14;18)-positive follicular NHL at diagnosis and during follow-up.

Patient no.	Date of Diagnosis	Stage	Follow-up (months)	Sample	t(14;18) PCR (fragment size)	Clinical Status	Clinical Remission (months)	Therapy	Im	Mo
1	11/87	IV	0	LN (p)	+ + + (160 bp)			8xCVP, IFN-α	IgM kappa	
			27	PB	-	NCED			ND	ND
			33	PB	-	NCED			ND	ND
			40	PB	-	NCED			ND	ND
			52	PB	-	NCED			ND	ND
			64	PB	-	NCED	56		-	-
				BM	-				-	-
2	6/87	IV	0	LN (p)	+ + + (220 bp)			8xCVP, RT, IFN-α	ND	
			42	PB	+	NCED			-	ND
			55	PB	+	NCED			ND	ND
			69	PB	+	NCED	61		ND	ND
3	9/90	III	0	LN (p)	+ + + (150 bp)				ND	
				PB	+				ND	ND
			1	PB	+	CED		1xCVP	-	-
			5	PB	+	CED		5xCVP	ND	-
			8	PB	-	CED		8xCVP	-	-
			10	PB	+	CED		RT	ND	ND
			30	PB	+	CED	22		ND	ND
4	2/89	IV	0	LN (p)	+ + + (235 bp)			8xCVP	IgM kappa	
			8	PB	+	NCED		8xCVP	ND	-
			9	PB	+	NCED			-	-
				BM	+	NCED			-	-
			11	PB	+	NCED			ND	ND
			15	PB	+	NCED			ND	ND
			26	PB	+	NCED	17		-	ND
			31	PB	+	CED, relapse		Surgery	+	+
			43	PB	+	CED			+	+
				BM	+				ND	-
5	7/85	III	0	LN (p)	+ + + (230 bp)			8xCVP, RT	ND	
			55	PB	+	NCED			ND	ND
			75	PB	+	NCED			ND	ND
			89	PB	-	NCED	81		ND	ND

6	8/87	III	0	LN (p)	+ + + (120 bp)			8xCVP, RT	IgM lambda	
			32	PB	-	NCED			ND	ND
			45	PB	-	NCED			ND	ND
			52	PB	-	NCED			ND	ND
			59	PB	-	NCED			ND	ND
			65	PB	-	NCED	57		ND	ND
7	3/86	III	0	LN (f)	+ + + (200 bp)			8xCVP, RT, IFN-α	IgM kappa	
			48	PB	+	NCED			ND	ND
			62	PB	+	NCED			ND	ND
			68	PB	-	NCED			ND	ND
			78	PB	-	NCED			ND	ND
			82	PB	+	NCED	78		ND	ND
			85			CED, nodal relapse		Expectative		
8	6/89	III	0	LN (p)	+ + + (200 bp)			8xCVP, RT, IFN-α	ND	
			2	PB	-	CED		4xCVP	ND	ND
			5	PB	-	CED		7xCVP	ND	ND
			7	PB	-	CED			ND	ND
				BM	-	CED			ND	ND
			15	PB	-	CED		IFN-α	ND	ND
			18	PB	-	CED		IFN-α	ND	ND
			21	PB	-	CED		IFN-α	ND	ND
			25	PB	-	CED	17	IFN-α	ND	ND
			33	PB	-	CED, progression		Chlorambucil	ND	ND
			39	PB	-	CED			ND	ND

Abbreviations: LN lymph node: (p) paraffin
(f) frozen
PB peripheral blood
BM bone marrow

CED clinical evidence of disease
NCED no clinical evidence of disease

CVP cyclophosphamide, vincristine and prednisone
IFN-α interferon-α
RT radiotherapy

Im immunological marker analysis
Mo morphological examination
ND not determined

+ + + PCR positive on diagnostic lymph node biopsy
+ PCR positive upon hybridization

A method for the detection of minimal disease can be useful for two reasons: to establish the clinical extension of disease at diagnosis and after treatment and to predict clinical relapse. In such a sensitive method as PCR, one may expect positive reactions not only in patients with CED, but also in patients with NCED. We and others have shown that the latter is the case (3,23,24). Also in this study, in four of six patients with NCED, t(14;18)-positive cells remained detectable. In two of four patients, negative reactions were found in one of three and two of five time points. This indicates that the number of circulating t(14;18)-positive cells in these patients fluctuated around the detection level of PCR. The finding of t(14;18)-positive cells in the circulation of patients with NCED is in agreement with observations in patients with localized disease (stage I and II), in whom we and others found circulating t(14;18)-positive cells both at diagnosis and after local radiotherapy in a high percentage of the patients (23,26). In two patients with NCED, the PCR was negative, which means that either no t(14;18)-positive cells were present or the frequency of these cells in the circulation was below the detection level. However, here we describe also a patient with CED who repeatedly showed negative reactions. It is unlikely that interferon-α treatment in this patient caused the negative PCR results, because these results remained negative during nodal progression and after cessation of treatment with interferon-α. Also, shortly after the end of chemotherapy treatment, a negative reaction was found in patient no. 3, although nodal disease was clinically evident. Therefore, negative findings may occur not withstanding the presence of nodal disease, and these findings do not exclude the possibility of relapse or progression.

Our data also indicate that there is no obvious correlation between PCR-detectable t(14;18)-positive cells and the duration of first remission in patients with stage III and IV follicular NHL. Clinical remission duration of PCR-positive patients ranged from 17 to 81$^+$ months, and of PCR negative patients from 17 to 65$^+$ months. This is in agreement with the observation that circulating t(14;18)-positive cells are also detectable in stage I and II patients for long times and may be explained by either the indolent course of disease or the assumption that the presence of t(14;18) may represent a premalignant status of the cell (23,24,26). The two patients with NCED and without PCR-detectable t(14;18)-positive cells in the circulation showed an ongoing clinical complete remission. Further studies and longer follow-up durations may show that, in those few patients, a negative PCR finding has some prognostic value.

So, our study shows that PCR for the detection of minimal residual disease in patients treated as described here is not clinically helpful. Besides the reasons mentioned here, one may also argue that spread of lymphoma cells in the circulation is not solely dependent on the number of lymphoma cells present in the body, but is a

characteristic of the individual lymphoma. In this study, we show a tendency that the number of circulating cells in a patient is rather constant, mostly either positive or negative during the observation period. In this respect, patient no. 8 is of importance, as she never showed a positive PCR reaction, not even at the time of clinical progression. If PCR shows positive results, then the number of circulating t(14;18)-positive cells is just above the detection level. In the two PCR positive patients who relapsed, no or a small increase of the intensity of the hybridizing bands was found. More precise quantitative methods are necessary to evaluate whether clinical relapse is accompanied by an increase in the number of circulating t(14;18)-positive cells (27). However, the factors that influence recirculation of lymphoma cells are largely unknown.

A correlation between the presence of circulating t(14;18)-positive cells with the prediction of impending relapse has been reported for patients treated with bone marrow ablative therapy followed by autologous bone marrow transplantation. Disease-free survival was markedly increased in patients without PCR-detectable t(14;18)-positive cells in the graft (28-30). Disease-free survival was also markedly increased when no PCR-detectable t(14;18)-positive cells were found in the bone marrow after bone marrow transplantation (30). The patients who relapsed had PCR-detectable t(14;18)-positive cells in their bone marrow before clinical relapse occurred (30).

Thus far, PCR monitoring of patients with t(14;18)-positive follicular NHL treated by conventional therapy is questionable in the prediction of clinical outcome. However, monitoring seems to be highly justified in patients treated with bone marrow transplantation or other future treatment modalities aimed at cure.

REFERENCES

1. Devesa SS and Fears T. Non-Hodgkin's lymphoma time trends: United States and international data. Cancer Res (suppl) 52: 5432-5440, 1992.
2. Rosenberg S, Berard C, Brown B, Burke J, Dorfman R, Glatstein E, Hoppe R and Simon R. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas. Summary and description of a working formulation for clinical usage. Cancer 49: 2112-2135, 1982.
3. Lambrechts AC, de Ruiter PE, Dorssers LCJ and van 't Veer MB. Detection of residual disease in translocation (14;18) positive non-Hodgkin's lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods. Leukemia 6: 29-34, 1992.
4. Clark HM, Jones DB and Wright DH. Cytogenetic and molecular studies of t(14;18) and t(14;19) in nodal and extranodal B-cell lymphoma. J Pathol 166: 129-134, 1992.
5. de Jong D, Voetdijk BHM, van Ommen GLB, Kluin-Nelemans JC, Beverstock GC and Kluin PhM. Translocation t(14;18) in B-cell lymphomas as a cause for defective immunoglobulin production. J Exp Med 169: 613-624, 1989.
6. Weiss LM, Warnke RA, Sklar J and Cleary ML. Molecular analysis of the t(14;18) chromosome translocation in malignant lymphomas. N Engl J Med 317: 1185-1189, 1987.
7. Rowley JD. Chromosome studies in the Non-Hodgkin's lymphomas: The role of the 14;18 translocation. J Clin Oncol 6: 919-925, 1988.
8. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T and Leder P. Structure of the human immunoglobulin μ locus: Characterization of embryonic and rearranged J and D genes. Cell 27:

583-591, 1981.
9. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC and Croce CM. Cloning of the chromosome breakpoint of neoplastic B-cells with the t(14;18) chromosome translocation. Science 226: 1097-1099, 1984.
10. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41: 889-906, 1985.
11. Tsujimoto Y, Gorham J, Cossman J, Jaffe E and Croce CM. The t(14;18) chromosome translocations involved in B-cell neoplasms results from mistakes in VDJ joining. Science 229: 1390-1393, 1985.
12. Cotter F, Price C, Zucca E and Young BD. Direct sequence analysis of the 14q+ and 18q- junctions in follicular lymphoma. Blood 76: 131-135, 1990.
13. Tsujimoto Y, Bashir MM, Givol J, Cossman J, Jaffe E and Croce CM. DNA rearrangements in human follicular lymphoma can involve the 5' or the 3' region of the BCL-2 gene. Proc Nat Acad Sci USA 84: 1329-1331, 1987.
14. Cleary ML, Smith SD and Sklar J. Cloning and structural analysis of cDNAs for BCL-2 and a hybrid BCL-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47: 19-28, 1986.
15. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM and Stass SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 227: 175-178, 1987.
16. Cotter FE, Price C, Meerabux J, Zucca E and Young BD. Direct sequence analysis of 14q+ and 18q- chromosome junctions at the MBR and MCR revealing clustering within the MBR in follicular lymphoma. Annals of Oncology 2: 93-98, 1991.
17. Cleary ML, Galili N and Sklar J. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 164: 315-320, 1986.
18. Mullis KB and Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in Enzymology 155: 335-350, 1987.
19. Gallagher CJ, Gregory WM and Jones AW. Follicular lymphoma: Prognostic factors for response and survival. J Clin Oncol 4: 1470-1480, 1986.
20. Carbone PP, Kaplan HS, Musshoff K, Smithers DW and Tubiana M. Report of the Committee on Hodgkin's disease staging classification. Cancer Res 31: 1860-1861, 1971.
21. Gallagher CJ and Lister TA. Follicular non-Hodgkin's lymphoma. Bailliere's Clinical Hematology, the lymphomas 1: 141-155, 1987.
22. Lister TA. The management of follicular lymphoma. Annals of Oncology 2: 131-135, 1991.
23. Lambrechts AC, Hupkes PE, Dorssers LCJ and van 't Veer MB. Translocation (14;18) positive cells are present in the circulation of the majority of patients with localized (stage I and II) follicular non-Hodgkin's lymphoma. Blood 82: 2510-2516, 1993.
24. Price CGA, Meerabaux J, Murtagh S, Cotter FE, Rohatiner AZS, Young BD and Lister TA. The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 9: 1527-1532, 1991.
25. Stansfeld AG, Diebold J, Noel H, Kapanci Y, Rilke F, Kelenyi G, Sundstrom C, Lennert K, van Unnik JAM, Mioduszewska O and Wright DH. Updated Kiel classification for lymphomas. The Lancet i: 292-293, 1988.
26. Finke J, Slanina J, Lange W and Dolken G. Persistence of circulating t(14;18)-positive cells in long term remission after radiation therapy for localized-stage follicular NHL. J Clin Oncol 11: 1668-1673, 1993.
27. Meijerink JPP, Smetsers TFCM, Raemaekers JMM, Bogman MJJT, de Witte T and Mensink EJBM. Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) by competive polymerase chain reaction. Br J Haematol 84: 250-256, 1993.
28. Gribben JG, Freedman AS, Neuberg D, Roy DC, Blake KW, Woo SD, Grossbard ML, Rabinowe SN, Coral F, Freeman GJ, Ritz J and Nadler LM. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. N Engl J Med 325: 1525-1533, 1991.
29. Gribben JG, Saporito L, Barber M, Blake KW, Edwards RM, Griffin JD, Freedman AS and Nadler LM. Bone marrow of non-Hodgkin's lymphoma patients with a BCL-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. Blood 80: 1083-1089, 1992.
30. Gribben JG, Neuberg D, Freedman AS, Gimmi CD, Pesek KW, Barber M, Saporito L, Woo SD, Coral F, Spector N, Rabinowe SN, Groosbard ML, Ritz J and Nadler LM. Detection by polymerase chain reaction of residual cells with the BCL-2 translocation is associated with increased risks of relapse after autologous bone marrow transplantation for B-cell lymphoma. Blood 81: 3449-3457, 1993.

CHAPTER 5

Lymphomas with testicular localization show a consistent BCL-2 expression without a translocation (14;18): A molecular and immunohistochemical study.

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SUMMARY

The presence of the BCL-2 protein was studied in nine non-Hodgkin's lymphomas (NHL) with testicular localization. A consistent presence of the BCL-2 protein was found. The chromosomal translocation (14;18) was seen neither by cytogenetic analysis (n=4) nor by polymerase chain reaction amplification and Southern blot analysis (n=9). Therefore, this translocation is not responsible for the presence of the BCL-2 protein in NHL with testicular localization. We suggest that the presence of the BCL-2 protein in these lymphomas is related to the differentiation stage of the B-lymphocytes and/or may play a role in the pathogenesis of these lymphomas. The consistent finding of the BCL-2 protein in lymphomas with testicular localization may support the clinical observation that these lymphomas are a separate entity.

INTRODUCTION

Non-Hodgkin's lymphomas (NHL) localized in the testis are extremely rare. They may occur as a primary manifestation or in the context of dissemination of nodal NHL (secondary testicular NHL) (1-4). Thus far, there are no criteria to discriminate between these two. These NHL of the testis are usually diffuse large cell NHL according to the Working Formulation for lymphomas (1-4). The prognosis of NHL with testicular localization is poor, most patients die of disseminated NHL within two years. In addition, they show a specific pattern of metastases, e.g., extranodal sites such as the upper airways, central nervous system and bones are especially involved (2,3).

The chromosomal translocation between the chromosomes 14 and 18 [t(14q32;18q21)] is found in 60% to 85% of the follicular NHL and 20% to 30% of the diffuse large cell NHL (5-10). Due to the t(14;18) the B-cell lymphoma 2 (BCL-2) gene located on the long arm of chromosome 18 is juxtaposed to the joining (JH) region of the immunoglobulin heavy chain (IgH) gene located on the long arm of chromosome 14 (11-14). This results in an enhanced expression of the BCL-2 gene which subsequently results in a disturbed programmed cell death (PCD), i.e., a prolonged cell survival (15-19).

In follicular NHL, diffuse large cell NHL and NHL of the gastrointestinal tract the BCL-2 protein is found using immunohistochemistry (20-25). Presence of the BCL-2 protein is also reported in normal lymphoid cells and is demonstrated in precursor cells of all hematopoietic lineages, memory B-cells and plasma- and mantle zone- B-cells (26-28). In nearly all follicular NHL, independent of the presence of the t(14;18), the BCL-2 protein is present (20,21,23). In 22% to 80% of the diffuse large cell NHL the BCL-2

protein is found, also not restricted to those with the t(14;18) (20-22,24). NHL of the gastrointestinal tract, neoplasms in which t(14;18) occurs infrequently, show presence of the BCL-2 protein in about 50% of the cases (24,25). Thus the BCL-2 protein is found in normal lymphoid cells and in many different histological subtypes of NHL independent of the presence of the t(14;18).

In the context that NHL with testicular localization show a separate clinical identity among NHL, we investigated the presence of the t(14;18) by combining cytogenetic analysis (n=4), polymerase chain reaction (PCR) analysis and Southern blotting analysis (n=9). The presence of the BCL-2 protein was studied using immunohistochemistry on frozen tissue sections (n=9).

MATERIALS AND METHODS

Samples: Tumor samples were collected in the Netherlands from 9 patients with NHL localized in the testis. The tumors were classified according to the Working Formulation for lymphomas by a panel of pathologists (29). Staging was according to the Ann Arbor classification (30). Representative frozen tissue sections were used for immunohistochemical and immunofluorescence analysis (9).

Immunohistochemistry: Frozen tissue sections of 4 micron were fixed in acetone (100%) for 10 minutes. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in PBS, containing 0.2% BSA. Expression of the BCL-2 protein was studied using a mouse monoclonal antibody BCL-2 100α (kindly provided by Drs. F. Pezzella and D.Y. Mason (21). Visualization was performed using an indirect peroxidase assay with a streptavidin-biotin conjugated goat anti-mouse immunoglobulin antibody as a second step. Hyperplastic lymph nodes were used as positive controls.

Immunofluorescence: Surface marker analysis was performed by direct immunofluorescence with FITC-conjugated goat anti-human IgM, IgD, IgG, IgA and lambda serum and TRITC-conjugated goat anti-human kappa and lambda serum (Nordic Immunological Laboratories Tilburg, The Netherlands). Double staining was performed with TRITC-conjugated goat anti-human kappa plus FITC-conjugated goat anti-human lambda or with the FITC-conjugated antibody against the heavy chain expressed by the malignant B-cell plus TRITC-conjugated anti-kappa or anti-lambda.

Cytogenetic analysis: Cytogenetic analysis was performed on metaphase spread chromosomes of four cases according to standard procedures (31). The chromosomes were identified with G banding (GTG banding) and described according to ISCN 1991 (32).

DNA extraction and Polymerase Chain Reaction analysis: High molecular weight DNA was isolated from frozen tissue sections of all NHL with testicular localization. DNA was digested with *Hind* III and analyzed for t(14;18) and immunoglobulin heavy chain (IgH) rearrangement using Southern blotting and probing with BCL-2 (12) and the joining region of

Table 1: Summary of the age of the patients, localization and stage of the disease at diagnosis, response to initial treatment, the localization of metastasis at relapse and data on histology of the nine NHL with testicular localization. Further, we present the results of Southern blot analysis for IgH rearrangement, polymerase chain reaction and Southern analysis for the detection of t(14;18). Also, the immunohistochemical detection of the BCL-2 protein and the immunofluorescence detection of immunoglobulin heavy chain and light chain proteins are presented.

Case	Age in years	Stage	Localization at diagnosis[1]	Response to treatment[2]	Localization at relapse or after progression[3]	Histology[4]	Southern[5]	PCR t(14;18)	BCL-2 protein	IgH[6] protein	IgL[7] protein
1	52	I	testis L	CCR	spleen, liver, kidney, bone marrow	LBC	G/R	-	+	IgM	kappa
2	78	I	testis L	progression	skin	LBC	G	-	+	IgM	kappa
3	74	II	testis R, lymph nodes para-aortal	CCR	CNS	LBC, PC	G/R	-	+	IgM	lambda
4	56	II	testis R, lymph nodes para-iliacal and para-aortal	CCR		LBC, IB	G/R	-	+	IgM	kappa
5	83	II	testis R, lymph nodes vena cava inferior and para-aortal	progression	Waldeijer ring, lung, sub-mandibular, supraclavicular	LBC, PC	G/R	-	+	IgM	kappa
6	40	I	testis L	CCR	CNS	LBC, IB	G/R	-	+	IgM	kappa
7	64	I	testis L	CCR		LBC	G/R	-	+	IgM	lambda
8	66	I	testis		lost for follow-up	LBC, PC	G/R	-	+	IgM	kappa
9	81	I	testis R	CCR		LBC, IB	G/R	-	+	IgM	lambda

1 L = left, R = right
2 CCR = clinical complete remission
3 CNS = central nervous system
4 LBC = large B-cell lymphoma, PC = polymorph centroblastic, IB = immunoblastic
5 G = germ line; R = rearranged
6 IgH = immunoglobulin heavy chain protein
7 IgL = immunoglobulin light chain protein

IgH allele (33). In addition, the polymerase chain reaction was used to detect small amounts of t(14;18)-positive cells. PCR primers were designed for the major breakpoint region (mbr (5'GGTGGTTTGACCTTTAGA 3')) of the BCL-2 gene and the consensus region of the JH genes (5' TGAGGAGACGGTGACC 3')(9). As a control to DNA quality the interleukin-3 gene of the DNA was amplified in 25 cycles, as described (9). For the detection of t(14;18) a total of 0.5-1.0 μg of DNA in a reaction volume of 25-50 μl was subjected to 30 cycles of PCR amplification using an automated Perkin-Elmer/Cetus DNA Thermal Cycler (Gouda, the Netherlands). In each experiment positive and negative controls were included. As positive controls different dilutions of DNA from a mbr t(14;18) positive cell line (SU-DHL-6) or DNA from a mcr t(14;18) positive lymph node biopsy was used. Amplified samples were analyzed as described using the BCL-2 and JH probes (9).

RESULTS

The age of the patient, the localization and the stage of disease at diagnosis, response to treatment, the localization of metastasis at relapse and the histological subtype of the non-Hodgkin's lymphoma localized in the testis are summarized in Table 1. Additional data concerning the results of Southern blotting analysis for IgH rearrangements and PCR for t(14;18) and the screening for BCL-2, immunoglobulin heavy (IgH) and light chain (IgL) proteins are also presented in Table 1.

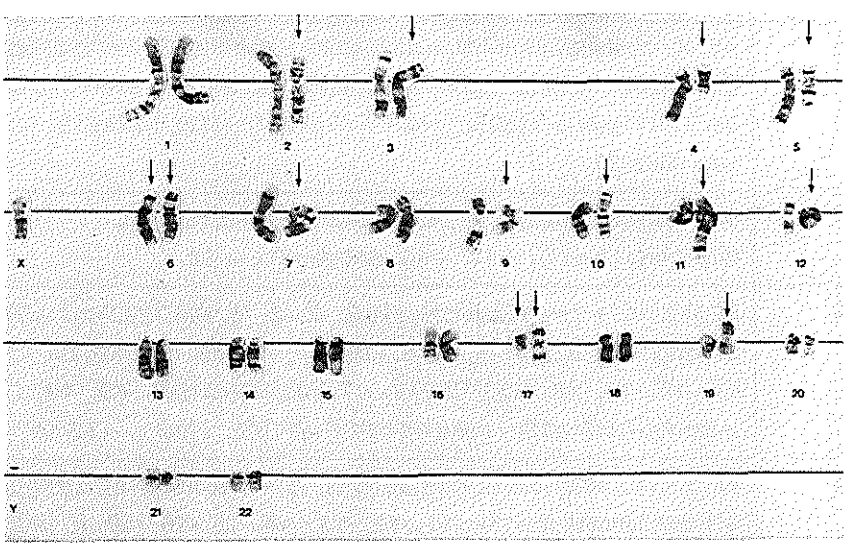

Figure 1: A representative karyotype of one non-Hodgkin's lymphoma with testicular localization (case 3, Table 1 and Table 2).

Of the four NHL in which cytogenetic analysis was performed, the representative karyotypes are summarized in Table 2. Besides multiple chromosomal abnormalities, no translocation t(14;18)(q32;q21) was detected. The karyotype of case 3 is presented in Figure 1. In addition, none of the NHL with testicular localization, showed evidence for the presence of t(14;18) by PCR and Southern blot analysis.

Immunological phenotyping of the NHL with testicular localization showed that all are B-cell NHL. Monoclonality of the B-cell population was demonstrated by the expression of either kappa or lambda in all biopsies. Monoclonality was confirmed by Southern blot analysis in eight of the nine NHL by the detection of a IgH gene rearrangement using a probe specific for the JH region. In case 2 monoclonality was not confirmed by the detection of a IgH gene rearrangement. This might be caused by the fact that the germ line and rearranged fragments were identical in size. The BCL-2 protein was consistently present in the cytoplasm of all lymphoma cells of the NHL with testicular localization. A representative example is given in Figure 2.

Table 2: Representative karyotypes of four non-Hodgkin's lymphomas localized in the testis (cases 3, 4, 5, 9, Table 1).

Case	Description
3	45, X, -Y, del(2)(p12p13), add(3)(q26.1), del(4)(q22), del(5)(q15q31), del(6)(p24), del(6)(q21q23), add(7)(q22), del(9)(p23), add(10)(p12), der(11)(11pter->11q25::11q25->11q22::?), r(12), i(17)(p10), i(17)(q10), add(19)(p13)
4	49, dup(X)(p21p22.2), -Y, del(6)(q23), del (6)(q15), +8, t(11;14)(p11;q11), +12, +13, del(14(q31), +18.
5	88, XXYY, +Y, +Y, add(1)(q31)x2, -2, -2, -5, add(7)(q21)x2, i(7)(q10), -8, add(8)(q24)x2, -9, -9, -9, -9, -10, -11, -11, del(11)(q22q23), -12, -14, -15, -16, -16, -17, -17, -17, -17, add(18)(q21)x2, -19, -19, +der(?)t(?;5)(?;q13), +der(?)t(?;17)(?;q21)x2, +mar1, +12mar.
9	88, XXYY, add(1)(p11), del(2)(p11.1), -3, -4, add(6)(q15), add(6)(q16)x2, +7, t(7;19)(q11.2;q13), -11, -12, -13, -13, -15, -15, -17, add(19)(p13.1), -20, -22, +mar1x2, +4mar.

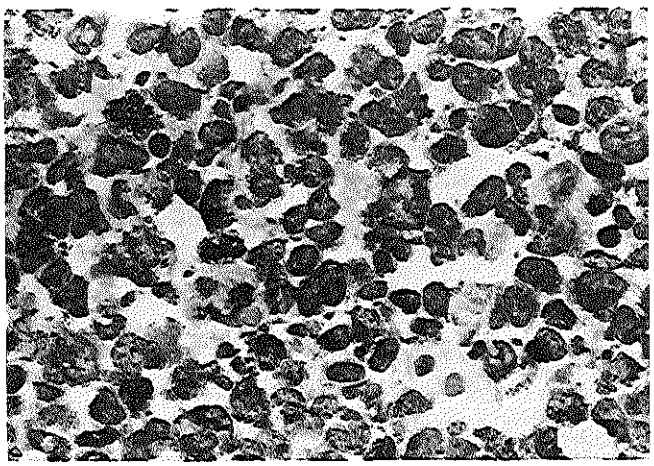

Figure 2: A representative example of the immunohistochemical detection of the BCL-2 protein on frozen tissue sections of a non-Hodgkin's lymphoma with testicular localization (case 3, Table 1).

DISCUSSION

NHL localized in the testis are high grade malignant NHL and usually of B-lymphocyte origin. They compromise approximately 1% of all lymphomas and have a specific clinical course of disease. They metastasize to uncommon sites for other lymphomas, for instance the central nervous system (2,3).

Cytogenetic analysis of four of the NHL with testicular localization revealed many different and complex chromosomal abnormalities but no chromosomal translocation (14;18) as found in other histological subtypes of NHL (5-10). In addition, we used PCR analysis, a technique able to detect one t(14;18)-positive cell out of 100,000 normal cells, to evaluate nine of the NHL with testicular localization for the presence of t(14;18)-positive cells. No t(14;18)-positive cells were detected in these NHL. The presence of the BCL-2 protein in different histological subtypes of NHL without the presence of t(14;18) was the reason to evaluate the involvement of the presence of the BCL-2 protein in NHL with testicular localization as well (24,25). While the BCL-2 protein was present in normal lymphoid cells, no BCL-2 protein was reported in normal testis (26-28). Our data show the presence of the BCL-2 protein in all of the NHL with testicular localization studied. Thus in all cases of NHL with testicular

localization we studied, the BCL-2 protein is consistently present without a t(14;18). In spite of the relatively small number of cases, this finding supports the clinical observation that NHL with testicular localization represent a separate entity. The presence of the BCL-2 protein may represent a differentiation stage of the B-lymphocyte or a functional subpopulation of B-lymphocytes that preferentially localize in extranodal sites, for example the testis, and metastasize to other extranodal sites possibly explaining the aggressive behavior of these NHL.

REFERENCES

1. Rappaport H. Tumors of the hematopoietic system. In: Atlas of tumor Pathology, section 3, fascicle 8: 9-14, 1966.
2. Doll DC and Weis RB. Malignant lymphomas of the testis. Am J Med 81: 515-524, 1986.
3. Hamilton CR and Horwich A. Rare tumours of the testis and paratesticlar tissues. Textbook of uncommon cancer: 225-248, 1988.
4. Martenson JA, Buskirk SJ, Ilstrup DM, Banks PM, Evans RG, Colgan JP and Earle JD. Pattern of failure in primary testicular non-Hodgkin's lymphoma. J Clin Oncol 6: 297-302, 1988.
5. Aisenberg AC, Wilkes BM and Jacobson JO. The BCL-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 71: 969-972, 1988.
6. Rowley JD. Chromosome studies in the Non-Hodgkin's lymphomas: The role of the 14;18 translocation. J Clin Oncol 6: 919-925, 1988.
7. Pezzella F, Ralfkiaer E, Gatter KC and Mason DY. The 14;18 translocation in European cases of follicular lymphoma: Comparison of Southern blotting and the polymerase chain reaction. Br J Haematol 76: 58-64, 1990.
8. Griesser H and Lennert K. BCL-2 rearrangements in malignant B-cell lymphomas. Br J Haematol 76: 142-143, 1990.
9. Lambrechts AC, de Ruiter PE, Dorssers LCJ and van 't Veer MB. Detection of residual lymphoma disease in translocation (14;18) positive non-Hodgkin's lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods. Leukemia 6: 29-34, 1992.
10. Limpens J, Beelen M, Kramer MHH, Stad R, Haverkort M, van Ommen GJB, van Krieken JHJM and Kluin PhM. Comparative studies on the detection of the t(14;18) translocation in frozen and formalin fixed tissues. Blood (suppl) 1875, 1992.
11. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC and Croce CM. Cloning of the chromosome breakpoint of neoplastic B-cells with the t(14;18) chromosome translocation. Science 226: 1097-1099, 1984.
12. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41: 889-906, 1985.
13. Cleary ML and Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82: 7439-7443, 1985.
14. Korsmeyer SJ. BCL-2 initiates a new catagory of oncogenes: Regulators of cell death. Blood 80: 879-886, 1992.
15. Vaux DL, Cory S and Adams JM. BCL-2 gene promotes heamopoetic cell survival and cooperates with c-myc to immortalize pre-B-cells. Nature 335: 440-442, 1988.
16. Hockenbery D, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ. BCL-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348: 334-336, 1990.
17. Nunez G, London L, Hockenbery D, Alexander M, McKearn JP and Korsmeyer SJ. Deregulated BCL-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immun 144: 3602-3610, 1990.
18. Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E and Rickinson A. Induction of BCL-2 expression by Epstein-Barr Virus latent membrane protein 1 protects infected B-cells from programmed cell death. Cell 65: 1107-1115, 1991.
19. Garcia I, Martinou I, Tsujimoto Y and Martinou J-C. Prevention of programmed cell death of sympathetic neurons by the BCL-2 proto-oncogene. Science 258: 302-305, 1992.

20. Ngan B-Y, Chen-Levy Z, Weiss LM, Warnke RA and Cleary ML. Expression in non-Hodgkin's lymphoma of the BCL-2 protein asscciated with the t(14;18) chromosomal translocation. N Engl J Med 318: 1638-1644, 1988.
21. Pezzella F, Tse AG, Cordell JL, Pulford KA, Mason DY. Expression of the BCL-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137: 225-232, 1990.
22. Zutter M, Hockenbery D, Silverman GA and Korsmeyer SJ. Immunolocalization of the BCL-2 protein within hematopoietic neoplasms. Blood 78: 1062-1068, 1991.
23. Gaulard P, d'Agay M-F, Peuchmaur M, Brousse N, Gisselbrecht C, Solal-Celigny P, Diebold J and Mason DY. Expression of the BCL-2 gene product in follicular lymphoma. Am J Pathol 140: 1089-1095, 1992.
24. Kondo E, Nakamura S, Onoue H, Matsuo Y, Yoshino T, Aoki H, Hayashi K, Takahashi K, Minowada J, Nomura S and Akagi T. Detection of BCL-2 protein and BCL-2 messenger RNA in normal and neoplastic lymphoid tissues by immunohistichemistry and in situ hybridization. Blood 80: 2044-2051, 1992.
25. LeBurn DP, Kamel OW, Cleary ML, Dorfman RF and Warnke RA. Follicular lymphomas of the gastrointestinal tract: Pathological features in 31 cases and BCL-2 oncogenic protein expression. Am. J. Pathol. 140: 1327-1335, 1992.
26. Hockenbery DM, Zutter M, Hickey W, Nahm M and Korsmeyer SJ. BCL-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88: 6961-6965, 1991.
27. Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM and Korsmeyer SJ. BCL-2 maintains B-cell memory. Nature 353: 71-73, 1991.
28. Pettersson M, Jernberg-Wiklund H, Larsson L-G, Sundstrom C, Givol I, Tsujimoto Y and Nilsson K. Expression of the BCL-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood 79: 495-502, 1992.
29. Rosenberg S, Berard C, Brown B, Burke J, Dorfman R, Glatstein E, Hoppe R and Simon R. National Cancer Institute sponsored study of classification of non-Hodgkin's lymphomas: Summary and description of a working formulation for clinical usage. Cancer 49: 2112-2135, 1982.
30. Carbone PP, Kaplan HS, Musshoff K, Smithers DW and Tubiana M. Report of the committee on Hodgkin's disease staging classification. Cancer Res 31: 1860-1861, 1971.
31. Gibas LM, Gibas Z and Sandberg AA. Technical aspects of cytogenetic analysis of solid tumors. Karyogram 10: 25-27, 1984.
32. ISCN (1991). Mittelman F and Karger S. Guidelines for cancer cytogenetics: Supplement to an international system for human cytogenetic nomenclature. Basel 1991.
33. Takahashi N, Nakai S, Honjo T. Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acid Res 8: 5983-5990, 1980.

CHAPTER 6

Genomic organization of the translocations (8;14) and (14;18) in a new lymphoma cell line.

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SUMMARY

We generated a new lymphoma cell line carrying the translocations (8;14) and (14;18) and studied the genomic organization and expression of the BCL-2 and *MYC* genes. PCR and Southern analysis showed that the breakpoints of t(14;18) were located in the mbr of the BCL-2 gene and just 5' of JH6 in the IgH locus. The breakpoints of the t(8;14) were located upstream of exon 2 in the non-coding region of the *MYC* gene and near the switch region of the IgH locus. Both IgH loci were involved in chromosomal translocations resulting in the absence of a functional B-cell receptor. Normal BCL-2 and truncated *MYC* transcripts were detected in these cells. The BCL-2 protein was expressed.

INTRODUCTION

Specific chromosomal translocations are consistently present in some hematological malignancies. The translocation (14;18)(q32;q21) is predominantly associated with follicular non-Hodgkin's lymphoma (NHL), and is found in 50-85% of these lymphomas (1-4). In diffuse NHL the frequency of t(14;18) is about 20% (4-6). As a result of this translocation, the oncogene BCL-2, located on chromosome 18, is placed near the joining region (JH) of the immunoglobulin heavy chain genes (IgH) located on chromosome 14 (4,7-9). This translocation occurs as an aberrant V-D-J heavy chain joining and is thought to be an early event in the development of malignancy. The translocation usually results in increased BCL-2 protein expression (2,10). The latter has been linked to the prevention of programmed cell death (apoptosis) (11).

The translocation (8;14)(q24;q32) is associated with 80% of the Burkitt lymphomas (BL) and has also been detected in other lymphomas, including diffuse large cell lymphomas (4,12). In t(8;14), the *MYC* oncogene located on chromosome 8 translocates with divergent transcriptional orientation to the IgH locus on chromosome 14. This translocation is thought to take place during heavy gene isotype switching (4,13). In the remaining 20% of BL, variant translocations involving the *MYC* gene and the kappa or lambda light chain constant region genes have been observed, resulting in respectively t(2;8)(p12;q24) and t(8;22)(q24;q11) (14,15). Also, these translocations usually result in an aberrant expression of the *MYC* oncogene (4,16).

Histological progression of follicular NHL towards a leukemia with immunologic characteristics of early pre-B-cells has been reported. These leukemic blast cells carry both the t(14;18) and the t(8;14) (17-19).

We present the cytogenetic analysis and genomic organization of a new cell line,

established from a bone marrow sample of a patient with an immunoblastic large cell lymphoma in which a t(14;18) and a t(8;14) coexist. Further, we analyzed the expression of both the BCL-2 and *MYC* oncogene.

MATERIAL AND METHODS

Case report: A 70 year old female patient (DS) presented with a 5 week history of tiredness, weight loss and fever. Physical examination and CT scanning showed no lymphadenopathy and no spleen or liver enlargement. The leucocyte count at diagnosis was 5.4 x 10^9/L with 47% lymphocytes and 1% blasts. The diagnosis of large cell immunoblastic B-cell lymphoma was based on morphological criteria and immunological phenotyping of peripheral blood and bone marrow cells (20,21). Clinical improvement of only short duration was achieved after a first chemotherapy course. Two months after diagnosis, the disease manifested in the central nervous system and the patient died 2 months later.

Immunological marker analysis: Immunological marker analysis was carried out on peripheral blood and bone marrow at diagnosis and at relapse. The marker analysis was performed by indirect immunofluorescence with the monoclonal antibodies CD19, CD20, CD3, CD22, CD37, CD45, HLA-DR (Beckton Dickinson Sunnyvale, CA, USA), CD38, CD13, CD33 (Coulter, Mijdrecht, The Netherlands), CD10, CD15, CDw65 (a gift from Dr. W. Knapp, Vienna, Austria) and CD2 (ITK diagnostics, Uithoorn, The Netherlands) as a first step and flourescein-isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin serum (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) as a second step (23). A polyclonal rabbit anti-terminal-deoxynucleotidyl-transferase (TdT) serum was used combined with a goat anti-rabbit serum as a second step (ITK diagnostics, Uithoorn, The Netherlands). Direct immunofluorescence was performed with FITC-conjugated goat anti-human IgM, IgD, IgG, IgA and lambda serum and tetramethylrhodamine-isothiocyanate (TRITC) conjugated anti-human kappa and lambda serum (Nordic Immunological Laboratories, Tilburg, the Netherlands).

Expression of the BCL-2 protein was analyzed using cytocentrifuged cell preparations, both unfixed and fixed by acetone (10 minutes). Cell preparations of the cell line (DS) derived from the bone marrow of patient DS and the cell lines SU-DHL-6 and Ros-50 (23), all carrying a t(14;18), were evaluated in an indirect immunoperoxidase assay using the BCL-2 100-α antibody (a gift from Drs. F. Pezzella and D.Y. Mason) (24) as the first step and a streptavidin-biotin conjugated goat anti-mouse immunoglobulin serum as a second step.

In vitro culture: Lymphoma cells were isolated from the bone marrow by Ficoll Isopaque (Nygaard, Oslo, Norway) centrifugation. The interface fraction containing the lymphoma cells was T cell depleted by E rosetting and Ficoll separation (25). Monocytes were removed by plastic adherence (25). The remaining cells (2 x 10^6) were placed in 10 ml serum free culture medium in a T25 flask (Greiner, Alphen aan de Rijn, The Netherlands) and incubated at 37°C in a humidified atmosphere of 5% CO_2. The cultures were replenished biweekly and cell

concentrations were adjusted to 2 x 10^5 cells/ml. A spontaneously proliferating cell line (DS) emerged from this culture, which has been propagated continuously for more than one year.

Cytogenetic analysis: Cytogenetic analysis was performed on a 24 hrs culture of bone marrow cells at diagnosis and on the cell line DS, according to standard procedures. The chromosomes were identified with R banding.

DNA extraction and Southern blot analysis: High molecular weight genomic DNA was prepared from the cell lines DS, SU-DHL-6 and an Epstein-Barr virus (EBV) transformed B cell line (EBV-B) according to standard protocols (26,27). Ten micrograms of DNA were digested with 20-30 units of restriction enzymes (Hind III, Hind III + Xba I, Hind III + Xho I, EcoR I, EcoR I + Xba I, EcoR I + Xho I and BamH I) under conditions recommended by the suppliers. DNA was size fractionated on 0.8% agarose gels and transferred to nylon membranes (Hybond N^+, Amersham, 's-Hertogenbosch, The Netherlands). Hybridization of the JH and Cμ region of the IgH gene, BCL-2 and c-*MYC* was performed according to standard procedures, using ^{32}P-labeled DNA probes radiolabeled by the hexamer primer method (26). The probes used were: a 2.8 kb EcoR I - Bgl II fragment containing part of the joining gene region of the IgH gene (JH3-JH6)(28), a 1.2 kb EcoR I fragment of the Cμ region (29), a 2.5 kb EcoR I fragment containing the constant kappa light chain region (30), a 1.4 kb Bgl II - Hind III fragment containing the constant lambda 2 light chain region (31) and a 2.8 kb EcoR I - Hind III BCL-2 fragment specific for the major breakpoint region (7). For c-*MYC* a 2 kb Pst I fragment 5' of exon 1 (*MYC*285), a 1 kb Pvu II fragment containing exon 1 (*MYC*256) and a 1.5 kb Cla I - EcoR I fragment specific for exon 3 (*MYC*285 and *MYC*256 are gifts from Dr. I.A. Liard Offringa, Leiden, the Netherlands).

Polymerase chain reaction: Polymerase chain reaction (PCR) analysis of the major breakpoint region (mbr) t(14;18) was performed as described using the JH consensus and BCL-2 primers (3). Also a JH6 specific primer (5' TCGGAACATGGTCCAG 3') was used in combination with the same BCL-2 primer (32). The JH consensus primer hybridizes with sequences in all six JH genes. The JH6 specific primer hybridizes with sequences located 122 base pairs (bp) downstream of the JH consensus hybridization site in JH6 (32).

RNA extraction and Northern blot analysis: RNA was isolated from the cell-lines DS, SU-DHL-6 and EBV-B and the original bone marrow sample from which the cell line DS was derived, using the standard guanidinium isothiocyanate, cesium chloride method (22). Ten μg of total RNA was loaded on a denaturing 1.2% agarose formaldehyde gel and blotted to nitrocellulose according to standard procedures (22). The probes used for BCL-2 and c-*MYC* hybridization were the same as mentioned above. Hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was performed to provide an internal standard for quantification of total mRNA (33). Expression levels for BCL-2 and c-*MYC* were estimated and normalized for GAPDH mRNA levels by scanning with a densitometer (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

Table 1: Immunophenotypic and genotypic status of the lymphoma cells of the patient and the cell line DS.

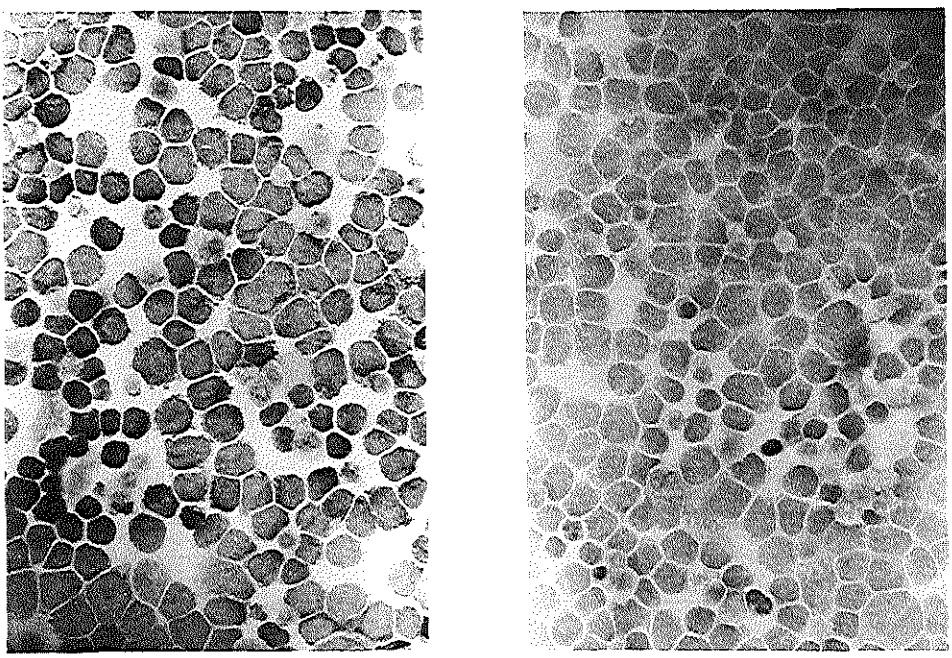
Immunological Marker	Patient Cells	Cell line
CD2	-	-
CD3	-	-
CD19	+	+
CD10	+	+
CD22	ND	-
CD20	-	-
CD37	ND	+
CD38	+	ND
CD45	ND	+
CD13	-	-
CD15	-	-
CD33	-	-
CDw65	-	-
s-IgH	-	-
c-IgH	-	-
s-Ig kappa	-	-
c-Ig kappa	-	-
s-Ig lambda	-	-
c-Ig lambda	-	-
HLA-DR	+	+
TdT	-	-
BCL-2	ND	+
C kappa genes	ND	deleted
C lambda genes	ND	rearranged

Abbreviations: ND = not determined
+ = positive
- = negative

RESULTS

Immunological marker analysis.

The cell line DS and the primary lymphoma cells express identical immunophenotypes (Table 1). The presence of the B-cell marker CD19 and the absence of T-cell (CD2, CD3) and myeloid (CD13, CD33) antigens is consistent with the B-cell origin of this tumor. The BCL-2 protein is expressed in the cell line DS (Figure 1).



1A 1B

Figure 1: Immunohistochemical detection of BCL-2 protein expression (A) and a negative control (B) on cytocentrifuged cell preparations of the cell line DS.

Cytogenetic analysis.

The bone marrow sample taken at diagnosis showed the following karyotype: 48, XX, t(1;6)(q12;q26), add (2)(q37), + del (7)(q21q32), t(8;14)(q24;q32), + inv (12)(p12.3q24), der (13) t(1;13)(q12;p12), t(14;18)(q32;q21), del (20)(p11p13), + ace. The cell line DS expresses the same cytogenetic abnormalities (Figure 2).

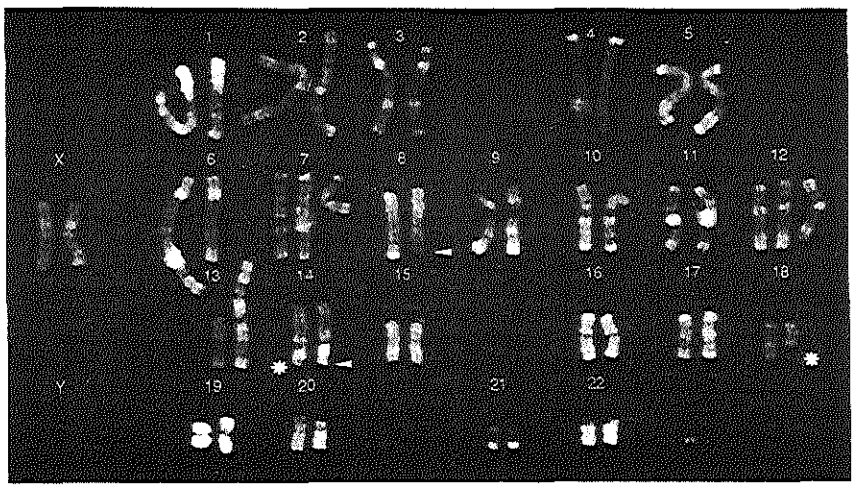


Figure 2: Karyogram of the large cell lymphoma cell line DS. Arrow heads indicate the derivative chromosomes of t(8;14). Asterisks indicate the derivatives of t(14;18). The karyotype is described according to ISCN 1991 (41).

Genomic organization of the breakpoints on chromosome 8,14 and 18.

PCR with the two mbr-t(14;18) primer sets (BCL-2/JH; BCL-2/JH6) gave rise to amplified, breakpoint specific products (Figure 3 lanes 4 and 5 respectively). The fragment size of the PCR product generated with a JH6 specific primer is 250 bp (Figure 3, lane 5), which indicates that the breakpoint is located just 5′ of the JH6 gene. The 120 bp size reduction of the PCR product generated using the JH common primer confirms the localization of the breakpoint within the JH gene (Figure 3, lane 4). The presence of the mbr-t(14;18) translocation in this cell line was also detected using restriction enzyme analysis and Southern blotting. BCL-2 hybridization of genomic DNA from the cell line DS, digested with Hind III, showed a germ line fragment of 4 kb and rearranged fragments of 3.5 kb and 7 kb representing the translocation and the reciprocal translocation (Figure 4 lane BCL). The 7 kb fragment also hybridized with the JH probe and represents most probably the reciprocal translocation (Figure 4 lanes BCL and JH).

Restriction enzyme analysis and Southern blotting revealed that the breakpoint on chromosome 8 was located between the Xho I site in exon 1 and the Xba I site in intron 1 (Figure 5, germ line chromosome 8 and Table 2). EcoR I + Xho I and Hind III + Xho I digested DNA from the cell line DS showed only germ line fragments after hybridization with *MYC*285 probe (Table 2). EcoR I + Xba I and Hind III + Xba I

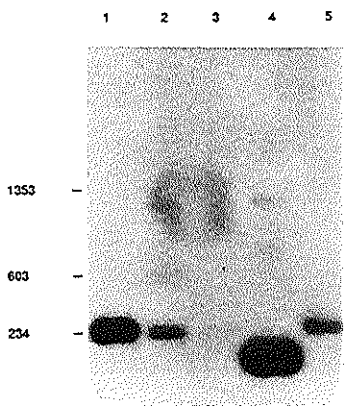


Figure 3:
PCR analysis of genomic DNA derived from the cell line DS (lane 4+5) and from serial dilutions of SU-DHL-6 cells in normal cells (lanes 1-3). In lane 5 the result from the amplification of genomic DNA from the cell line DS with the JH6 specific and the *BCL-2* primer is shown (250 bp). The amplified products generated with the JH consensus and the same *BCL-2* primer are presented in lane 4 (130 bp) for the cell line DS and in the lanes 1 to 3 for the SU-DHL-6 dilutions (10^{-3} to 10^{-5}) (235 bp). Blots were hybridized to both the *BCL-2* and JH probe. At the left, the position of the size marker PhiX DNA digested with Hae III is depicted (in base pairs).

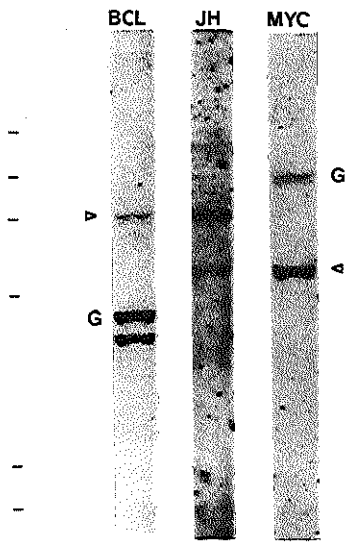


Figure 4:
Genomic DNA from the cell line DS digested with Hind III was hybridized with three different probes, *BCL-2* (lane BCL), JH3-JH6 (lane JH) and *MYC*285 (lane MYC). Translocations are marked by arrow heads, for t(14;18) see lane BCL and for t(8;14) see lane MYC. Germ line fragments are marked by G. The size marker indicated on the left correspond to the 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb fragments of lambda DNA digested with Hind III.

digested DS cell line DNA showed germ line fragments and translocated fragments of respectively 10 and 5.5 kb after hybridization with *MYC*285 and *MYC*256 probes (Table 2). Hybridization with the *MYC* exon 3 probe to EcoR I + Xba I and Hind III + Xba I digests showed only germ line fragments (Table 2). In EcoR I + Xho I and Hind III + Xho I digests germ line fragments and additional translocated fragments of approximately 7 kb and 9.5 kb were seen respectively (Table 2).

Hind III digests revealed a 5.5 kb and a 7 kb JH hybridizing fragment but no 11 kb germ line fragment indicating that both JH genes were non-germ line and were

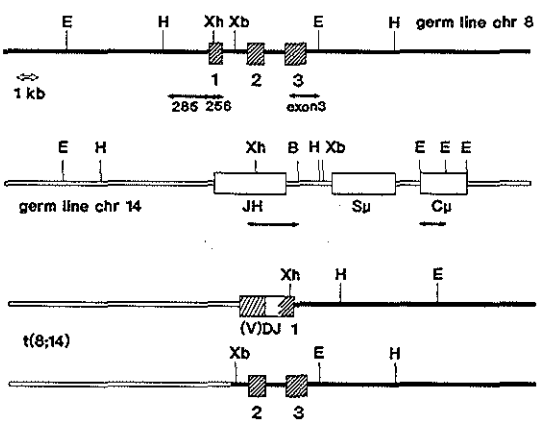


Figure 5:
Genomic organization of the germ line c-MYC (chromosome 8) and Ig heavy chain (chromosome 14) loci and the t(8;14). Both the translocation and the reciprocal translocation (8;14) are schematically presented. Restriction endonuclease sites as well as the different probes used (JH=JH3-JH6, C*μ*, *MYC*285, *MYC*256, *MYC* exon3) are shown. The restriction endonuclease sites: B = Bgl II, H = Hind III, E = EcoR I, X = Xho I and X = Xba I.

Table 2: Localization of the breakpoint on chromosome 8.

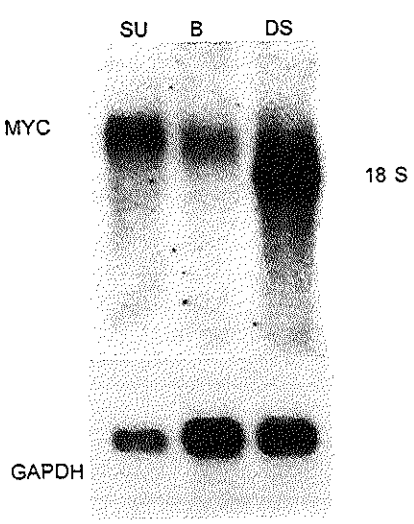
Digests	*MYC*285 probe fragment size	*MYC* exon 3 probe fragment size
EcoR I + Xho I	7 kb (germ line)	5 kb (germ line) + 7 kb
EcoR I + Xba I	8 kb (germ line) + 10 kb	4 kb (germ line)
Hind III + Xho I	2.5 kb (germ line)	8 kb (germ line) + 9.5 kb
Hind III + Xba I	3.5 kb (germ line) + 5.5 kb	7 kb (germ line)

either rearranged or involved in a translocation (Figure 4 lane JH). The 5.5 kb JH containing fragment is exclusively coupled to the 5' region of c-MYC as a result of the t(8;14) (Figure 4 lanes JH and MYC). The 7 kb JH containing fragment hybridized with the BCL-2 probe as a result of the t(14;18) (Figure 4 lanes JH and BCL). Cμ hybridizing fragments were not detected in the cell line DS, but were readily detected in the IgM expressing control cell lines, SU-DHL-6 and EBV-B, indicating that both Cμ loci were deleted in the cell line DS.

mRNA expression of the c-MYC and BCL-2 oncogene.

MYC and BCL-2 mRNA expression was analyzed in the cell line DS and the bone marrow sample from which the cell line DS was derived. The *MYC* mRNA detected after hybridization with the exon 3 probe in the cell line DS was approximately 0.4 kb shorter than 2.4 kb *MYC* mRNA in the control cell lines SU-DHL-6 and EBV-B (Fig 6). The bone marrow sample from which the cell line DS was derived contained an identical 2.0 kb *MYC* mRNA (data not shown). Densitometric analysis showed a 4-fold increase in the quantity of *MYC* RNA in the cell line DS in comparison to the EBV-B cell line. No enhanced *MYC* expression was detected in comparison to the SU-DHL-6 cell line. In the cell line and bone marrow sample from DS two normal 8.5 and 5.5 kb BCL-2 mRNA transcripts were detected (data not shown).

Figure 6:
MYC mRNA expression, detected by hybridization with *MYC* exon 3 probe, of the cell lines SU-DHL-6 (SU), EBV-B (B) and DS. The lower panel shows the GAPDH control. At the right the location of 18 S ribosomal RNA is indicated.



DISCUSSION

We described a cell line (DS) with the translocations (8;14) and (14;18). Expression of B-cell specific markers (CD19, CD10, CD37) and the absence of TdT expression suggests that the immunophenotype of the cell line, which was identical with the original lymphoma, represents that of a relatively mature B-cell. Expression of other B-cell specific markers (CD20, CD22) could not be detected. Differential expression of B-cell specific antigens is seen in cell lines, non-Hodgkin's lymphomas and chronic lymphocytic leukemia (34). The relatively mature appearance of the B-cell line is further strengthened by the observation that the kappa genes were deleted and the lambda genes were rearranged which is a late event in B-cell development. No expression of surface- or cytoplasmic- immunoglobulin heavy and light chain proteins was present. Deregulation of IgH protein expression is in line with the cytogenetic data which showed that both chromosome bands 14q32 were involved in chromosomal translocations, namely t(8;14) and t(14;18) and that both IgH loci were possibly altered. Restriction enzyme- and PCR- analysis further confirmed that both the IgH loci were involved.

The breakpoints involved in the t(14;18) were localized by the primers used for PCR and occurred in the mbr region of the BCL-2 gene and just 5' of JH6. Restriction enzyme analysis of genomic DS DNA showed the translocation (3.5 kb fragment) as well as the reciprocal translocation (7 kb fragment) after hybridization with a BCL-2 probe. In contrast, comigration with JH could only be detected for the 7 kb fragment and not in the 3.5 kb fragment. This is most likely due to a lack of hybridization to a short JH homologous region. This is in agreement with the observation that sequences located downstream of the JH genes on chromosome 14, the Cμ genes, were deleted in the cell line DS. Likewise, the faint BCL-2 hybridization signal of the 7 kb fragment may be explained by a short mbr BCL-2 homologous region in the reciprocal translocation. BCL-2 mRNA expression was detected in the cell line and the bone marrow sample derived from patient DS (35). BCL-2 protein expression was detected in the cell line DS.

Deregulation of the *MYC* oncogene was caused by a t(8;14). One rearranged Hind III fragment hybridizing with probes specific for the 5' part of the *MYC* gene (exon 1) and the JH gene of the IgH locus was identified (Figure 4). Therefore, a Hind III site must be located just 5' of the JH gene which implies that rearrangement of at least the D-J genes and most probably V-D-J genes must have taken place. If the latter is the case then this (8;14) translocation most probably occurred during heavy chain isotype switching. The reciprocal translocation, containing the *MYC* coding exons 2 and 3,

Table 3: Characteristics of the cell lines with t(8;14) and t(14;18).

Cell Line	380	SU-DUL-5	Ros-50	DS
Origin	acute lympho-blastic leukemia	lymphoblastic lymphoma	acute lympho-blastic leukemia	immunoblastic lymphoma
Reference	38	37	23	
Translocation	t(8;14), t(14;18)	t(8;14;18)	t(8;14;18)	t(8;14), t(14;18)
t(8;14)	*MYC* germ line	5' of *MYC*	ND	exon 1 or intron 1 of *MYC*
t(14;18)	ND	mcr	mcr	mbr
CD19	ND	ND	+	+
CD10	+	ND	-	+
TdT	+	ND	-	-
s-IgH	-	ND	IgM	-
c-IgH	-	ND	-	-
s-IgL	-	ND	kappa	-
c-IgL	-	ND	-	-
BCL-2 mRNA	ND	-	ND	normal
MYC mRNA	ND	normal	ND	truncated
BCL-2 protein	ND	-	+	+
C kappa genes	deleted	ND	ND	deleted
C lambda genes	rearranged	ND	ND	rearranged

Abbreviations:
+ positive
- = negative
ND = not determined
mbr = major breakpoint region of the BCL-2 gene (8,9).
mcr = minor cluster region of the BCL-2 gene (40).

could not be linked to JH and Cμ sequences of the IgH locus (36). The IgH enhancer, located between the JH and switch (Sμ) region, could be present in the t(8;14) or the reciprocal t(8;14). This enhancer or other IgH regulatory elements are involved in the expression of the truncated *MYC* mRNA. The expression level of the truncated *MYC* mRNA was about the same as the expression level of normal *MYC* mRNA in the cell line SU-DHL-6 and increased in comparison to the EBV-B cell line. The truncated mRNA contained all coding sequences, thus expression of normal *MYC* protein(s) may be expected. In our case a deregulated *MYC* expression may have contributed to the malignant clinical behavior and the spontaneous growth *in vitro*.

Coexistence of t(8;14) and t(14;18) has previously been reported for 3 cell lines (23,37,38) and approximately 8 patients (for review see 23,39). The characteristics of these 3 cell lines (380, SU-DUL-5, ROS-50) and the cell line DS are presented in Table 3. In the cell lines, SU-DUL-5 and ROS 50, a three way translocation t(8;14;18) was found (Table 3)(37,23) Only in the cell lines DS and 380 the translocations (8;14) and (14;18) were detected (Table 3)(38). In agreement with our observation in the cell line DS, the cell line 380 did also not express surface and cytoplasmic immunoglobulins and thus no B-cell receptor. In the cell line DS the breakpoint on chromosome 8 was located within the *MYC* gene, whereas in the cell line 380 no rearrangement of the *MYC* gene was detected. Thus only in the cell line DS an aberrant *MYC* mRNA was detected.

In conclusion, our cell line is an addition to a scarce panel of cell lines with the translocations (8;14) and (14;18). These cell lines may be used for further studies aimed at the involvement of these translocations and the absence of a functional B-cell receptor in deregulating cell growth and maturation in lymphomas.

REFERENCES

1. LeBeau MM, Rowley JD. Cytogenetics. In Williams WJ, Beutler E, Erslev AJ and Lichtman MA. Haematology. New-York: McGraw-Hill, 78-89, 1990.
2. Tsujimoto Y, Gorham J, Cossman J, Jaffe E and Croce CM. The t(14;18) chromosome translocations involved in B-cell neoplasms results from mistakes in VDJ joining. Science 229: 1390-1393, 1985.
3. Lambrechts AC, de Ruiter PE, Dorssers LCJ and van 't Veer MB. Detection of residual disease in translocation (14;18) positive non-Hodgkin's lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods. Leukemia 6: 29-34, 1992.
4. McKeithan TW. Molecular biology of non-Hodgkin's Lymphomas. Seminars in Oncology 17: 30-42, 1990.
5. Bloomfield CD, Arthur DC, Frizzera G, Levine EG, Peterson BA and Gajl-Peczalska KJ. Nonrandom chromosome abnormalities in lymphoma. Cancer Res 43: 2975-2984, 1983.
6. Aisenberg AC, Wilkes BM and Jacobson JO. The BCL-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 71: 969-972, 1988.
7. Tsujimoto Y, Fingler LR, Yunis J, Nowell PC and Croce CM. Cloning of the chromosome breakpoint of neoplastic B-cells with the t(14;18) chromosome translocation. Science 226: 1097-1099, 1984.
8. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on

chromosome 14 and near a transcriptional unit on 18. Cell 41: 889-906, 1985.
9. Cleary ML and Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82: 7439-7443, 1985.
10. Bakhshi A, Wright JJ, Graininger W, Seto M, Cossman J, Jensen JP, Goldman P and Korsmeyer SJ. Mechanisms of the t(14;18) chromosomal translocation: Structural analysis of both derivate 14 and 18 reciprocal partners. Proc Natl Acad Sci USA 84: 2396-2400, 1987.
11. Hockenbery BM, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ. BCL-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348: 334-336, 1990.
12. Manolov G and Manolova Y. Marker band in one chromosome 14 from Burkitt's lymphomas. Nature 237: 33-34, 1972.
13. Haluska FG, Finver S, Tsujimoto Y and Croce CM. The t(8;14) chromosomal translocation occurring in B-cell malignancies results from mistakes in V-D-J joining. Nature 324: 158-161, 1986.
14. Croce CM, Thierfelder W, Erikson J, Nishikura K, Finan J, Lenoir GM and Nowell PC. Transcriptional activation of an unrearranged and untranslocated c-*MYC* oncogene by translocation of C lambda locus in Burkitt lymphoma. Proc Natl Acad Sci USA 80: 6922-6926, 1983.
15. Erikson J, Nishikura K, ar-Rushdi A, Finan J, Emanuel B, Lenoir G, Nowell PC and Croce CM. Translocation of an immunoglobulin kappa locus to a region 3' of an unrearranged c-*MYC* oncogene enhances c-*MYC* transcription. Proc Natl Acad Sci USA 80: 7581-7585, 1983.
16. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC and Croce CM. Human c-*MYC* oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 79: 7824-7827, 1982.
17. De Jong D, Voetdijk BMH, Beverstock GC, van Ommen GJB, Willemze R and Kluin PM. Activation of the c-*MYC* oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. N Engl J Med 318: 1373-1378, 1988.
18. Gauwerky CE, Haluska FG, Tsujimoto Y, Nowell PC and Croce CM. Evolution of B-cell malignancy: Pre-B-cell leukemia resulting from *MYC* activation in a B-cell neoplasm with a rearranged BCL-2 gene. Proc Natl Acad Sci USA 85: 8548-8552, 1988.
19. Gauwerky CE, Hoxie J, Nowell PC and Croce CM. Pre-B-cell leukemia with a t(8;14) and a t(14;18) translocation is preceded by follicular lymphoma. Oncogene 2: 431-435, 1988.
20. Rosenberg S, Berard C, Brown B, Burke J, Dorfman R, Glatstein E, Hoppe R and Simon R. National Cancer Institute sponsored study of classification of non-Hodgkin's lymphomas. Summary and description of a Working Formulation for clinical usage. Cancer 49: 2112-2135, 1982.
21. Weisenburger DD. Pathological classification of non-Hodgkin's lymphoma for epidemiological studies. Cancer Res (suppl) 52: 5456-5464, 1992.
22. Knapp W, Dörken B, Rieber P, Schmidt RE, Stein H and von dem Borne AEGKR. CD antigens 1989. Blood 74; 1448-1450, 1989.
23. Ooteghem RBC, Smit EME, Beishuizen A, Lambrechts AC, v.d. Blij-Philipsen M, Smilde TJ and Hagemeijer A. A new B-cell line showing a complex translocation (8;14;18) and BCL-2 rearrangement. Cancer Genet Cytogenet in press.
24. Pezella F, Tse A, Gordell J, Pulford KAF, Gatter KC and Mason DY. Expression of the bcl-2 oncogene protein is not specific for the t(14;18) translocation. Am J Pathol 137: 225-232, 1990.
25. Touw I, Pouwels K, van Agthoven T, van Gurp R, Budel L, Hoogerbrugge H, Delwel, R Goodwin R, Namen A and Löwenberg B. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 75: 2097-2101, 1990.
26. Sambrook J, Fritsch EF and Maniatis T. Molecular Cloning: A Laboratory Manual, second edition. Cold spring harbor laboratory press 1989.
27. Miller SA, Dykes DD and Polesky HF. Simple and efficient method for isolating high molecular weight DNA from mammalian sperm. Nucleic Acid Res 16: 1215, 1988.
28. Takahashi N, Nikai S and Honjo T. Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acid Res 8: 5983-5991, 1980.
29. Rabbitts TH, Forster A and Milstein CP. Human immunoglobulin heavy chain genes: Evolutionary comparison of Cμ, Cδ and Cτ genes and associated switch sequences. Nucleic Acid Res 9: 4509-4524, 1981.
30. Hieter PA, Max EE, Seidman JG, Maizel JV and Leder P. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 22: 197-207, 1980.
31. Hieter PA, Hollis GF, Korsmeyer SJ, Waldman TA and Leder P. Clustered arrangement of immunoglobulin lambda constant region genes in man. Nature 294: 536-540, 1981.
32. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T and Leder P. Structure of the human

immunoglobulin μ locus: Characterization of embryonic and rearranged J and D genes. Cell 27: 583-591, 1981.
33. Benham FJ, Hodgkinson S and Davies KE. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosomes defines a multigene family. EMBO J 3: 2635-2640, 1984.
34. Schwartz-Albiez R and Moldenhauer G. Immunochemistry and epitope analysis using CD10, CD19, CD20, CD21, CD23, CD24, CD37, CD38, CD39 and CD40 mAb. In Knapp W, Dörken B, Gilks WR, Rieber EP, Schimdt RE, Stein H and von dem Borne. Leucocyte Typing IV: White cell differentiation antigens. Oxford university press 1989.
35. Longo DL. What's the deal with follicular lymphomas? J Clin Oncol 11: 202-208, 1993.
36. Marcu KB, Bossone SA and Patel AJ. MYC function and regulation. Ann Rev Biochem 61: 809-860, 1992.
37. Kiem H-P, Nourse J, Saltman DL, Blume KG and Cleary ML. Concurrent activation of c-*MYC* and inactivation of BCL-2 by chromosomal translocation in a lymphoblastic lymphoma cell-line. Oncogene 5: 1815-1819, 1990.
38. Pegoraro L, Palumbo A, Erikson J, Falda M, Giovanazzo B, Emanuel BS, Rovera G, Nowell PC and Croce C. A 14;18 and 8;14 chromosome translocation in a cell line derived from an acute B-cell leukemia. Proc Natl Acad Sci USA 81: 7166-7170, 1984.
39. Brito-Babapulle V, Crawford A, Khokhar T, Laffan M, Matutes E, Fairhead S and Catovsky D. Translocations t(14;18) and t(8;14) with rearranged BCL-2 and c-*MYC* in a case presenting as B-ALL (L3). Leukemia 5: 83-87, 1991.
40. Cleary ML, Galili N and Sklar J. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 164: 315-320, 1986.
41. ISCN (1991). Mittelman F and Karger S. Guidelines for cancer cytogenetics: Supplement to an international system for human cytogenetic nomenclature. Basel 1991.

CHAPTER 7

Discussion and perspectives

1. Markers for the detection of minimal residual disease in patients with non-Hodgkin's lymphoma.

1.1 Clinical relevance of minimal disease detection in non-Hodgkin's lymphoma.

This thesis describes the application of the polymerase chain reaction (PCR) in the detection of minimal (residual) disease in patients with non-Hodgkin's lymphoma (NHL), especially follicular NHL characterized by the chromosomal translocation t(14;18) [t(14;18)]. This translocation provides a unique DNA sequence for these NHL cells and can be used as a target for PCR. Detection of small numbers of NHL cells is important for accurate staging of the disease at initial diagnosis and for monitoring the course of the disease during and after therapy (1). We investigated the significance of the presence of t(14;18)-positive cells in the peripheral blood and bone marrow of patients with follicular NHL with respect to the stage of disease at diagnosis and the remission status and remission duration after (initial) treatment.

1.2 The chromosomal translocation t(14;18) as marker for non-Hodgkin's lymphoma cells.

Even though the use of t(14;18) as a clonal marker for NHL and in the study of minimal residual disease (MRD) is very attractive, a number of limitations should be considered.

i) The translocation t(14;18) is found in 60% to 85% of follicular NHL (2-4) and in 20-30% of diffuse large cell NHL (5). In NHL the t(14;18) appears present in all lymphoma cells and can, therefore, be used as a clonal marker. Thus, the t(14;18) can be used as a marker in a substantial proportion of NHL patients.

ii) Further, t(14;18) can be found in other B-cell malignancies, including hairy cell leukemia (6), acute lymphoblastic leukemia (7,8) and multiple myeloma (9). In these cases t(14;18) has been demonstrated by cytogenetic analysis. In Hodgkin's disease t(14;18) has been demonstrated in 30% of the patients and is thought to occur in Reed-Sternberg cells or in polyclonal expanded B-cells (10). In Hodgkin's disease, however, t(14;18) is present in an extremely low proportion of the cells and can only be demonstrated with very sensitive techniques as PCR. The presence of t(14;18)-positive cells in Hodgkin's disease and in a variety of B-cell malignancies would suggest that t(14;18) is a relatively common event in B-cell development. In Hodgkin's disease t(14;18) is not a clonal marker and not suitable for detection of MRD. In the other cases t(14;18) is a clonal marker and may be used for the detection of MRD.

iii) In addition, t(14;18) may also appear in benign lymphoid disorders without progression to NHL (11-13). In benign lymphoid tissues the t(14;18)-positive cells are also present in extreme low numbers and can be detected with PCR only. In these

conditions approximately one t(14;18)-positive cell per 10^5 normal cells was estimated to be present. In these cases t(14;18) is not a clonal marker and not suitable for detection of MRD.

iiii) In peripheral blood and bone marrow more than one type of t(14;18) may be found, while the lymph node biopsy of the patient discloses only a single t(14;18) (14). As a matter of fact t(14;18)-positive cells not related to the original NHL are detected due to the extreme sensitivity of PCR. Therefore, the presence of t(14;18)-positive cells in peripheral blood and bone marrow from NHL patients per se is not sufficient to conclude that MRD is present. For example, a significant number of patients with follicular NHL (7 of 44 patients (14)) showed evidence of two coexisting t(14;18) with different breakpoints in peripheral blood samples taken in remission (14). In the lymph node biopsy from 5 of these 7 patients only one of the translocations could be demonstrated. Obviously, only the t(14;18) found in both the lymph node biopsy and in the peripheral blood of the patient can be used as a clonal marker for NHL. For this reason it is necessary to provide evidence that the t(14;18)-positive cells in peripheral blood and bone marrow are identical to the t(14;18)-positive cells in the lymph node biopsy by showing that the generated t(14;18)-specific PCR products have the same size (chapter 2 figure 1, chapter 3 figures 1 and 2, and chapter 4 figure 1). Another possibility to provide this evidence is to determine the DNA sequence of the breakpoint region. This DNA sequence is most probably unique to each translocation and therefore more specific than the size of the t(14;18)-specific PCR fragment. However, DNA sequencing is cumbersome and thus not attractive to apply on a routine basis.

In the studies described in this thesis we have taken these points into account. The study deals with the detection of t(14;18)-positive cells in peripheral blood and bone marrow from patients with follicular NHL as a minor lymphoma cell population. To reduce the possibility that these t(14;18)-positive cells were not related to follicular NHL, the t(14;18) was used as a marker only in patients whose diagnostic lymph node biopsies were proven to be positive for t(14;18). The size of the t(14;18)-specific PCR products from the diagnostic lymph node biopsy and from peripheral blood and bone marrow samples were identical in individual patients.

1.3 Clinical relevance of the presence of t(14;18)-positive cells in peripheral blood and bone marrow.

The use of PCR in the detection of MRD in t(14;18)-positive follicular NHL provides a 100 to 1,000 fold increase of the sensitivity of the detection of NHL cells in comparison to conventional techniques (chapter 2). We evaluated peripheral blood and

bone marrow negative for NHL cells according to conventional techniques, whereas t(14;18)-positive cells were detected with PCR (15-18, chapters 2, 3 and 4). It is, however, important to assess the clinical relevance of the presence of t(14;18)-positive cells detectable by PCR only, i.e. in different clinical stages at diagnosis and after therapy.

We evaluated the presence of t(14;18)-positive cells in patients with stages I and II disease. These patients are usually treated with involved field radiotherapy only. This treatment may result in longstanding clinical complete remissions and possibly even cure. The disease free survival for these patients is better than for patients with more advanced disease (19-22). Translocation t(14;18)-positive cells were frequently found in peripheral blood and bone marrow from patients with stages I and II follicular NHL at diagnosis and after therapy, whereas no clinical symptoms of disease were evident (15, chapter 3). Thus, the results obtained by evaluation of the presence of t(14;18)-positive cells in peripheral blood and bone marrow of these patients using PCR indicates that follicular NHL is often a disseminated disease regardless the clinical staging. Despite the presence of t(14;18)-positive cells in these patients, some of these patients reach longstanding clinical complete remissions and possibly even cure (chapter 3). It is possible that in these patients the PCR detectable t(14;18)-positive cells do not represent NHL cells. Instead they could represent pre-malignant cells carrying the translocation without additional cellular changes which are thought to be necessary for progressive malignancy (16,23). Apparently, PCR detection of t(14;18)-positive cells in peripheral blood and bone marrow of patients with stages I and II follicular NHL has no or limited clinical relevance.

We also evaluated the presence of t(14;18)-positive cells during remission in patients with stages III and IV follicular NHL, treated with conventional chemotherapy. Although most patients respond to treatment, at some point the disease recurs and the patients ultimately succumb from relapse of NHL (21,22). In peripheral blood of patients in (first) clinical complete remission t(14;18)-positive cells were frequently present, i.e., in approximately 55% to 85% of the patients (14,16-18, chapters 2 and 4). In patients with clinical signs of disease t(14;18)-positive cells were frequently, but not always, found in peripheral blood and bone marrow (17, chapters 2 and 4). Similar to the results in stage I and II patients, the presence or absence of t(14;18)-positive cells during remission in stage III and IV patients treated with conventional therapy does not correlate with the probability of relapse. This observation is in agreement with the report of Price *et. al.* that t(14;18)-positive cells are detected in the circulation of

patients in long term remission from follicular NHL using PCR (16). As stated in the previous section, these observations support the view that the event of the translocation itself may not be tumorigenic. Thus, PCR detection of t(14;18)-positive cells in peripheral blood and bone marrow of patients with stages III and IV follicular NHL has no or little clinical relevance.

Of particular interest are t(14;18) PCR results obtained in patients treated with autologous bone marrow transplantation (ABMT). PCR was used to detect t(14;18)-positive cells in bone marrow harvested for ABMT before and after ex-vivo purging (24-28). The presence of residual t(14;18)-positive cells has been demonstrated in nearly all autologous bone marrow transplants before ex-vivo purging (17,24,25,27). In 50% to 86% of the grafts residual t(14;18)-positive cells could still be demonstrated after ex-vivo purging (24,27). Contradictory results have been reported with respect to PCR detection of t(14;18)-positive cells and disease free survival (24,27,28). A significantly better disease free survival was reported in one study if the graft was negative for t(14;18) (24). In addition, the correlation between the presence or absence of residual t(14;18)-positive cells in the bone marrow after ABMT with disease free survival was studied (27,29). Again, contradicting results have been reported. A strong correlation between the absence of t(14;18)-positive cells and disease free survival was demonstrated. No relapse was seen in 58 patients with no PCR detectable t(14;18)-positive cells in the bone marrow after ABMT, while a relapse appeared in 25 of 35 patients with PCR detectable t(14;18)-positive cells consistently present in the bone marrow after ABMT (29). In another study no correlation could be demonstrated because in the bone marrow of all but one patient t(14;18)-positive cells were detected after bone marrow transplantation (27). These data indicate that confirmatory studies as well as longer follow-up are necessary to establish the prognostic value of PCR determination of t(14;18) in patients treated with bone marrow ablative therapy.

In conclusion, the usefulness of t(14;18) as a marker for the detection of MRD in patients with t(14;18)-positive follicular NHL may depend on the choice of therapy. The application of extraordinarily sensitive techniques for detection of residual lymphoma cells is particularly useful to assess the level of tumor cell elimination and distinguish between complete and near complete remissions. While our and other studies show that there is no correlation between the presence or absence of PCR detectable t(14;18)-positive cells in peripheral blood and bone marrow with the stage of disease, remission status and remission duration in patients treated with conventional therapies, in patients treated with high dose therapy and ABMT the presence of t(14;18)-positive

cells in the graft or in the bone marrow after ABMT may predict decreased disease free survival. It is possible that PCR for the detection of t(14;18)-positive cells may become a useful tool if patients with follicular NHL are treated with therapeutic modalities aimed at cure, e.g., bone marrow transplantation, new chemotherapeutic agents, interleukins, monoclonal antibodies or combination modalities. In these situations, precise quantitation of residual t(14;18)-positive cells by competitive PCR may contribute to a better understanding of the possible importance of the numbers of t(14;18)-positive cells in peripheral blood and bone marrow with respect to remission duration and the efficiency of different treatment approaches (30).

1.4 The rearrangement of the immunoglobulin heavy chain gene as a marker for non-Hodgkin's lymphoma cells.

As discussed in section 1.1, t(14;18) is not a clonal marker for 15% to 40% of follicular NHL. For follicular NHL without the t(14;18) and for B-cell malignancies in general rearranged alleles of the immunoglobulin heavy (IgH) and light (IgL) chain genes can be used as a clonal marker. Rearrangement of the variable (V), the diversity (D) and joining (J) segments of the IgH gene and rearrangement of the V and J segments of the IgL gene create a unique immunoglobulin protein specificity. Furthermore, the addition of several bases during the assembly between these segments augments the variability of the DNA sequences in the rearranged Ig gene (31,32). Using PCR, the V-D-J sequences of the rearranged IgH gene can be amplified *in vitro* by choosing primers homologous to known V and J common sequences (33,34). The PCR product generated in this way is unique for the (malignant) B-cell. This implies that the V-D-J sequence is patient specific, and can be used as a marker for NHL. For each malignant B-cell clone a specific probe has to be created which may subsequently be used to study MRD in that particular patient. IgH gene rearrangements have been detected successfully with PCR in acute lymphoblastic leukemias, chronic lymphocytic leukemias and multiple myelomas (34-43). Also the amount of neoplastic cells has been quantified using the rearranged IgH gene as a clonal marker (34,39-44). In analogy, the rearranged IgH gene may also be used for the detection of minimal residual disease in B-cell NHL. So far, only few studies have been published (34,35,45-49).

We investigated the applicability of the use of the rearranged IgH gene as a clonal marker in intermediate and high grade malignant B-cell NHL. In these lymphomas the t(14;18) is present in only 20% to 30% of the cases and detection of minimal disease seems to be more clinically relevant than in follicular NHL. About 80 percent of these patients, according to evaluation with conventional techniques, may attain clinical

complete remissions following conventional chemotherapy. Approximately half of these patients have recurrences of NHL within 2 years, the others appear cured. The possibility to improve the detection of residual NHL cells may be of importance for monitoring the response to therapy, establishing the quality of the remission and identifying patients with a high probability of relapse.

V-D-J PCR was applied on a series of intermediate and high grade malignant NHL (n=19). A distinct IgH gene rearrangement was detectable on Southern blots using a J probe. Amplification of the V-D-J sequences was attempted with V family-specific primers in combination with a consensus J primer. The V family-specific primers were chosen in the leader sequence or the framework 1 region of the IgH gene. The results of these initial experiments may be divided in three different patterns of generated PCR products:

a) A specific PCR product was obtained with a single V family-specific primer in 30 cycles in approximately 20% of the cases (n=4). With other V family-specific primers no significant PCR products were detected.

b) None of the V family-specific primers resulted in the production of a abundant PCR product at the standard number of cycles (n=10). In most reactions PCR products were detectable upon hybridization.

c) None of the V family-specific primers resulted in the generation of PCR products detectable upon hybridization at the standard cycles conditions although amplification of a different target (the interleukin-3 gene) was efficient (n=5).

In summary, our results so far have pointed at two technical problems: i) low recovery of specific PCR products after amplification of V-D-J sequences (36-39,45-48) and ii) difficulties in providing conclusive evidence that the generated markers were specific for the IgH gene rearrangement of the NHL.

ad i) The absence of a specific PCR product in approximately 80% of the cases strongly suggest that mismatches between the primer and the target sequences affect the efficiency of the amplification reaction (36,46,49,50). Variable success rates have been reported applying V-D-J PCR for different B-cell neoplasms (34,36,41,42,44,46). The highest success rate is reported for ALL and CLL and varies between 80% and 100% (34,36,41,42,44,46), whereas the success rate for lymphomas seems dependent on the histological subtype and varies between 17% to 67% (46,49). Low success rates were also attributed to primer mismatches in these reports. An additional complicating factor in NHL appears to be the presence of substantial amounts of oligo- or polyclonal B-cells in the lymph node biopsy. Despite their under-representation in the biopsy, they will contribute to the amplification reaction and even dominate the PCR

products if the IgH gene rearrangement is not efficiently amplified. Strategies to improve the recovery of the presence of the wanted target most likely have to deal with the primer mismatches and the presence of non-malignant B-cells. Possible modifications are:

a) Amplification of serial dilutions (up to 10^{-5}) of DNA. If the lymphoma cells constitute a substantial percentage of the total B-cell population, as is suggested by the Southern blot analysis, the polyclonal normal B-cells will then fall under a critical threshold and will not give rise to PCR products.

b) Additional primers may be selected for each V family in the conserved domains or even V consensus primers for the framework 2 and 3 regions of the IgH gene. Furthermore, to reduce the number of amplification reactions, the V family used in the IgH gene rearrangement of the NHL may be established by Southern blot analysis.

c) Target enrichment may be achieved by microdissection of lymphoma tissue (51). Alternatively, the IgH gene rearrangement may be isolated after size fractionation, using restriction endonucleases, from agarose gels and used as target for V-D-J sequence amplification. Its position in the gel is established by Southern blot analysis.

d) RNA may be isolated from the lymph node biopsy which can be subjected to RT-PCR using 3' primers specific for the constant region of the IgH gene. At the 5' end either V family specific primers or ligated primers may be used. The type of constant region attached to the variable domain can be determined by immunological marker analysis.

ad ii) The problems with the amplification of the V-D-J sequence of the IgH gene of intermediate and high grade malignant NHL obviate the need for absolute validation of the specificity of the generated patient-specific markers. This may be achieved by using highly labelled PCR product as a probe for detection of the IgH gene rearrangement on Southern blot.

The solution of these technical problems is a prerequisite for investigating the significance of these patient-specific probes as clonal markers in the detection of minimal residual disease in intermediate and high grade malignant NHL in a prospective study. Even then, the possibility exists that with the generated patient-specific clonal marker, residual NHL cells may be missed because of clonal selection of the tumor. NHL cells may be in the stage of differentiation in which hypermutation of the Ig alleles takes place to select cells that make antibodies with a higher avidity. This phenomenon is known to occur in follicular NHL and may also take place in other histological subtypes of NHL (50,52-55). To overcome the problem of clonal selection, characterization of the NHL clone by more than one clone-specific marker, for instance the rearranged immunoglobulin heavy and light chain alleles or t(14;18), which is

present in 20% to 30% of these NHL, may be used (5,56,57). In spite of the problems mentioned in generating patient-specific clonal markers with PCR in intermediate and high grade malignant NHL, the possibility to use these markers in the study of minimal residual disease in B-cell neoplasms and the clinical relevance justifies further investigations.

References.

1. Negrin RS and Blume KG. The use of the polymerase chain reaction for the detection of minimal residual malignant disease. Blood 78: 255-258, 1991.
2. Rowley JD. Chromosome studies in the Non-Hodgkin's Lymphomas: The role of the 14;18 translocation. J Clin Oncol 6: 919-925, 1988.
3. Pezzella F, Ralfkiaer E, Gatter KC and Mason DY. The 14;18 translocation in European cases of follicular lymphoma: Comparison of Southern blotting and the polymerase chain reaction. Br J Haematol 76: 58-64, 1990.
4. Griesser H and Lennert K. BCL-2 rearrangements in malignant B-cell lymphomas. Br J Haematol 76: 142-143, 1990.
5. Aisenberg AC, Wilkes BM and Jacobson JO. The BCL-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 71: 969-972, 1988.
6. Brito-Babapulle V, Pittman S, Melo JV, Parreira L and Catovsky D. The 14q+ marker in hairy cell leukemia: A cytogenetic study of 15 cases. Leukemia Res 10: 131-138, 1986.
7. Mufti GJ, Hamblin Tj, Oscier DG and Johnson S. Common ALL with Pre-B-cell features showing (8;14) and (14;18) chromosome translocations. Blood 62: 1142-1146, 1983.
8. Brito-Babapulle V, Crawford A, Khokhar T, Laffan M, Matutes E, Fairhead S and Catovsky D. Translocations t(14;18) and t(8;14) with rearranged BCL-2 and c-MYC in a case presenting as B-ALL (L3). Leukemia 5: 83-87, 1991.
9. Nishida K, Taniwaki M, Misawa S, Abe T, Takino T and Fuji H. Translocation (14;18) found in a patient with multiple myeloma. Cancer Genet Cytogenet 25: 375-377, 1987.
10. Stetler-Stevenson M, Crush-Stanton S and Cossman J. Involvement of the BCL-2 gene in Hodgkin's disease. J Natl Cancer Inst 2: 855-858, 1990.
11. Grace J, Hali BS, Lew M, Singh S, Pittman S, Palmar A, Zbroja R, Wass J and Vincent PC. Cytogenetic abnormalities in benign lymphoid hyperplasia: A dual parameter study using chromosome analysis and flow cytometry. Int J Cancer 44: 959-964, 1989.
12. Limpens J, de Jong D, van Krieken JHJM, Price CGA, Young BD, van Ommen G-JB and Kluin PLM. BCL-2/JH rearrangement in benign lymphoid tissues with follicular hyperplasia. Oncogene 6: 2271-2276, 1991.
13 Aster JC, Kobayashi Y, Shiota M, Mori S and Sklar J. Detection of the t(14;18) at similar frequencies in hyperplastic lymphoid tissues from American and Japanese patients. Am J Pathol 141: 291-299, 1992.
14. Price CGA, Tuszynski A, Watt SM, Murdoch SJ, Lister TA and Young BD. Detection of additional JH/BCL-2 translocations in follicular lymphoma. Leukemia 5: 548-554, 1991.
15. Finke J, Slanina J, Lange W and Dolken G. Persistence of circulating t(14;18)-positive cells in long-term remission after radiation therapy for localized-stage follicular NHL. J Clin Oncol 11: 1668-1673, 1993.
16. Price CGA, Meerabaux J, Murtagh S, Cotter FE, Rohatiner AZS, Young BD and Lister TA. The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 9: 1527-1532, 1991.
17. Gribben JG, Freedman AS, Woo SD, Blake K, Shu RS, Freeman G, Longtine JA, Pinkus GS and Nadler LM. All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of BCL-2 have residual cells containing the BCL-2 rearrangement at evaluation and after treatment. Blood 78: 3275-3280, 1991.
18. Lee KA, Goepel JR, Winfield DA, Hancock BW and Goyns MH. Investigation of BCL-2 gene rearrangements in a United Kingdom series of low and High grade non-Hodgkin's lymphomas. Leuk Lymphoma 11: 91-98, 1993.
19. Gallagher CJ, Gregory WM and Jones AW. Follicular lymphoma: Prognostic factors for response and survival. J Clin Oncol 4: 1470-1480, 1986.

20. Gallagher CJ and Lister TA. Follicular non-Hodgkin's Lymphoma. Bailliere's Clinical Hematology, the lymphomas 1: 141-155, 1987.
21. Simon R, Durrleman S, Hoppe RT, Bonadonna G, Bloomfield CD, Rudders RA, Cheson BD and Berard CW. The non-Hodgkin's lymphoma pathologic classification project: Long-term follow-up of 1153 patients with non-Hodgkin lymphomas. Annals of Internal Medicine 109: 939-945, 1988.
22. Lister TA. The management of follicular lymphoma. Annals of Oncology 2: 131-135, 1991.
23. Gribben JG. The attainment of molecular remission: A worthwhile goal? J Clin Oncol 12: 1532-1534, 1994.
24. Gribben JG, Freedman AS, Neuberg D, Roy DC, Blake KW, Woo SD, Grossbard ML, Rabinowe SN, Coral F, Freeman GJ, Ritz J and Nadler LM. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. N Engl J Med 325: 1525-1533, 1991.
25. Gribben JG, Saporito L, Barber M, Blake KW, Edwards RM, Griffin JD, Freedman AS and Nadler LM. Bone marrow of non-Hodgkin's lymphoma patients with a BCL-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. Blood 80: 1083-1089, 1992.
26. Negrin RS, Kiem H-P, Schmidt-Wolf IGH, Blume KG and Cleary ML. Use of the polymerase chain reaction to monitor the effectiveness of ex-vivo tumor cell purging. Blood 77: 654-660, 1991.
27. Johnson PWM, Price CGA, Smith T, Cotter FE, Meerabux J, Rohatiner AZS, Young BD and Lister TA. Detection of cells bearing the t(14;18) translocation following myeloablative treatment for autologous bone marrow transplantation for follicular NHL. J Clin Oncol 12: 798-805, 1994.
28. Freedman A, Gribben J, Rabinowe S, Neuberg D, Mauch P, Spector N, Anderson K, Soiffer R, Pesek K, Robertson M, Whelan M, Ritz J and Nadler L. Autologous bone marrow transplantation in advanced low grade B-cell non-Hodgkin's lymphoma in first remission. Abstact 1313, American Society of Hematology meeting 1993.
29. Gribben JG, Neuberg D, Freedman AS, Gimmi CD, Pesek KW, Barber M, Saporito L, Woo SD, Coral F, Spector N, Rabinowe SN, Groosbard ML, Ritz J and Nadler LM. Detection by polymerase chain reaction of residual cells with the BCL-2 translocation is associated with increased risks of relapse after autologous bone marrow transplantation for B-cell lymphoma. Blood 81: 3449-3457, 1993.
30. Meijerink JPP, Smetsers TFCM, Raemaekers JMM, Bogman MJJT, de Witte T and Mensink EJBM. Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) by competitive polymerase chain reaction. Br J Haematol 84: 250-256, 1993.
31. Alt FW and Baltimore D. Joining of immunoglobulin heavy chain gene segments: Implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 79: 4118-4122, 1982.
32. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW and Baltimore D. Insertions of N regions into heavy-chain genes is correlated with expression of terminal denucleotidyl transferase in B-cells. Nature 311: 752-755, 1984.
33. Schutte MEM, Ebeling SB, Akkermans-Koolhaas KE, Gmelig-Meyling FHJ and Logtenberg T. Antibody specificity and immunoglobulin VH gene utilization of human monoclonal CD5 B-cell lines. Eur J Immunol 21: 1115-1121, 1991.
34. Deane M and Norton JD. Detection of immunoglobulin gene rearrangements in B-lymphoid malignancies by polymerase chain reaction gene amplification. Br J Haematol 74: 251-256, 1990.
35. Deane M and Norton JD. Immunoglobulin heavy chain variable region family usage is independent of tumor cell phenotype in human B-lineage leukemias. Eur J Immunol 20: 2209-2217, 1990.
36. Deane M, McCarthy KP, Wiedemann LM and Norton JD. An improved method for detection of B-lymphoid clonality by polymerase chain reaction. Leukemia 5: 726-730, 1991.
37. McCarthy KP, Sloane JP and Wiedeman LM. Rapid method for distinguishing clonal from polyclonal B-cell populations in surgical biopsy specimens. J Clin Pathol 43: 429-432, 1990.
38. Trainor KJ, Brisco MJ, Story CJ and Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood 75: 2220-2222, 1990.
39. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, Tsujimoto Y, Caton AJ and Rovera G. Detection of minimal disease in hematopoietic malignancies of the B-lineage by using third-complementarity-determining region (CDR III) specific probes. Proc Natl Acad Sci USA 86: 5123-5127, 1989.
40. Yamada M, Wasserman R, Lange B, Reichard BA, Wormer RB and Rovera G. Minimal residual disease in childhood B-lineage lymphoblastic leukemia. N Engl J Med 323: 448-455, 1990.
41. Brisco MJ, Condon J, Sykes PJ, Neoh SH and Morley AA. Detection and qantitation of neoplastic cells in acute lymphoblastic leukemia, by use of the polymerase chain reaction. Br J Haematol 79: 211-217, 1991.

42. Billadeau D, Blackstadt M, Greipp P, Kyle RA, Oken MM, Kay N and van Ness B. Analysis of B-lymphoid malignancies using allele specific polymerase chain reaction: a technique for sequential quantitation of residual disease. Blood 78: 3021-3029, 1991.
43. Billadeau D, Quam L, Thomas W, Kay N, Greipp P, Kyle RA, Oken MM and van Ness B. Detection and quantitation of malignant cells in peripheral blood of multiple myeloma patients. Blood 80: 1818-1824, 1992.
44. Nizet Y, Martiat P, Vaerman JL, Philippe M, Wildmann C, Staelens JP, Cornu G, Ferrant A, Michaux JL and Sokal G. Follow-up of residual disease (MRD) in B-lineage acute leukemias using a simplified PCR strategy: Evolution of MRD rather than its detection is correlated with clinical outcome. Br J Haematol 79: 205-210, 1991.
45. Wan JH, Trainor KJ, Brisco MJ and Morley AA. Monoclonality in B-cell lymphoma detected in paraffin-wax-embedded sections using the polymerase chain reaction. J Clin Pathol 43: 888-890, 1990.
46. Ben-Ezra J. Variable rate detection of immunoglobulin heavy chain V-D-J rearrangement by PCR: A systemic study of 41 B-cell non-Hodgkin's lymphomas and leukemias. Leuk Lymphoma 7: 289-295, 1992.
47. Diss TC, Peng H, Wotherspoon AC, Isaacson PG and Pan L. Detection of monoclonality in low-grade B-cell lymphomas using the polymerase chain reaction is dependent on primer selection and lymphoma type. J Pathol 169: 291-295, 1993.
48. Reed TJ, Reid A, Wallberg K, O'Leary TJ and Frizzera G. Determination of B-cell clonality in paraffin-embedded lymph nodes using the polymerase chain reaction. Diagn Mol Pathol 2: 42-49, 1993.
49. Slack DN, McCarthy, Wiedemann LM and Sloane JP. Evaluation of sensitivity, specificity and reproducability of an optimized method for detecting clonal rearrangements of immunoglobulin and T-cell receptor genes in formalin-fixed, paraffin-embedded sections. Diagn Mol Pathol 2: 223-232, 1993.
50. Cleary ML, Meeker TC, Levy S, Trela M, Sklar J and Levy R. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B-cell lymphoma. Cell 44: 97-106, 1986.
51. Kuppers R, Zhao M, Hansmann M-L and Rajewsky K. Tracing B-cell development in human germinal centres by molecular analysis of single cells picked from histological sections. EMBO J 12: 4955-4967, 1993.
52. Bahler DW and Levy R. Clonal evolution of follicular lymphoma: Evidence for antigen selection. Proc Natl Acad Sci USA 89: 6770-6774, 1992.
53. Bahler DW, Zelenetz AD, Chen TT and Levy R. Antigen selection in human lymphomagenesis. Cancer Res (suppl) 52: 5547-5551, 1992.
54. Zelenetz AD, Chen TT and Levy R. Clonal expansion in follicular lymphomas occurs subsequent to antigen selection. J Exp Med 176: 1137-1148, 1992.
55. Schutte MEM, Ebeling SB, Akkermans-Koolhaas KE, Gmelig-Meyling FHJ and Logtenberg T. Immunoglobulin VL gene expression in human CD5 B cell lines: Evidence for somatic mutations in the CD5 subset. Thesis 1992, Utrecht, The Netherlands.
56. Cannell PK, Foroni L and Hoffbrand AV. Variable kappa gene usage in lymphoproliverative disorders. Exp Hematol 21: 1160, 1993.
57. Offit K, Kodura PRK, Hollis R, Filippa D, Jhanwar SC, Clarckson BC and Chaganti RSK. 18q21 rearrangement in diffuse large cell lymphoma: Incidence and clinical significance. Br J Haematol 72: 178-183, 1989.

SUMMARY

In this thesis a new method to detect low numbers of t(14;18)-positive cells, the polymerase chain reaction (PCR), is used to evaluate the presence of t(14;18)-positive cells in peripheral blood or bone marrow of patients with t(14;18)-positive follicular non-Hodgkin's lymphoma (NHL) in staging at diagnosis and in monitoring the response to therapy.

In chapter 1, we describe clinical and phenotypical aspects of follicular NHL, the most common type of NHL. This type of NHL is associated with a specific chromosomal translocation, the translocation between chromosomes 14q32 and 18q21 [t(14;18)]. The molecular characteristics of the translocation allow the application of the sensitive PCR, an *in vitro* DNA amplification technique, for the detection of minimal (residual) disease with greater sensitivity in comparison to more conventional techniques such as imaging techniques, cytogenetic analysis, morphological examination and immunological marker analysis. Application of PCR increases the sensitivity with which lymphoma cells can be detected approximately 100 to 1000 times. This may be of importance in staging the disease at diagnosis and monitoring the disease after therapy.

In chapter 2 we used the PCR technique to evaluate the presence of t(14;18)-positive cells in peripheral blood or bone marrow from patients with predominantly follicular centroblastic centrocytic NHL. The PCR results were compared with the results of two other techniques which were used to detect NHL cells, immunological marker analysis and morphologic examination. The PCR results were also combined with the clinical status of the patient (stage at diagnosis and remission status after therapy) which was established by imaging techniques (X-ray photography and CT scanning). Translocation (14;18)-positive cells were demonstrated in peripheral blood or bone marrow using PCR whereas with immunological marker analysis and morphlogical examination no NHL could be detected. In addition, t(14;18)-positive cells were detected in peripheral blood or bone marrow of patients without clinical evidence of disease after therapy. Therefore, PCR appears to be a powerful technique to study minimal residual disease.

In chapter 3 we used the PCR technique to study the presence of t(14;18)-positive cells in peripheral blood or bone marrow of patients with localized (stages I and II) t(14;18)-positive follicular NHL. We found t(14;18)-positive cells in peripheral blood or bone marrow in 75% of these patients. Local therapy does not eradicate these t(14;18)-positive cells in most of the patients. These results support the notion that

follicular NHL is in general a disseminated disease regardless clinical staging.

Chapter 4 describes a longitudinal study to investigate the presence of t(14;18)-positive cells in peripheral blood or bone marrow of patients with disseminated (stages III and IV) t(14;18)-positive follicular NHL during first remission obtained by conventional therapy. We found no obvious correlation between the presence or absence of t(14;18)-positive cells in peripheral blood or bone marrow and i) the remission status and ii) the remission duration. Notably, there was a tendency that PCR detectable t(14;18)-positive cells were either continuously present or absent in the peripheral blood or bone marrow during remission in individual patients.

In chapter 5 we investigated characteristics of lymphoma cells in patients with NHL localized in the testis. These NHL are relatively rare. NHL may disseminate to the testis or testicular NHL may occur as a primary manifestation. NHL with testicular localization metastasizes preferentially to specific sites. We investigated whether t(14;18) was present in this type of NHL. To detect the t(14;18) we used cytogenetic analysis, Southern blotting and PCR analysis. The BCL-2 gene is involved in t(14;18) and therefore we used immunohistochemistry to investigate the presence of the BCL-2 protein. We found that the BCL-2 protein was present in all lymphomas tested, but that t(14;18) was absent in all cases. We suggest that the presence of the BCL-2 protein in these lymphomas is related to the differentiation stage of the B-lymphocyte and/or may play a role in the pathogenesis of these lymphomas. The consistent presence of the BCL-2 protein without a t(14;18) may support the clinical observation that these NHL are a separate entity.

In chapter 6 we studied the genomic organization in a newly generated cell line containing the chromosomal translocations (8;14) and (14;18). We established the localization of the breakpoints on chromosome 8, 14 and 18. Both immunoglobulin heavy chain (IgH) genes located on chromosome 14 were disrupted by these two translocations. The breakpoint on chromosome 18 was in the major breakpoint region of the BCL-2 gene. The breakpoint on chromosome 8 was located in exon 1 or intron 1 of the MYC gene. The BCL-2 protein and normal BCL-2 mRNA transcripts were detected in the cell line. In contrast, the MYC mRNA was approximately 0.4 kb shorter than usual. As can be expected the cells did not express immunoglobulins in the cytoplasm or on the cell surface. The cell line may be useful for further studies aimed at the involvement of these translocations in deregulating cell growth and maturation.

In chapter 7 the clinical value of the detection of t(14;18)-positive cells in the peripheral blood or bone marrow by PCR in patients with t(14;18)-positive NHL is discussed. The detection of t(14;18)-positive cells by PCR is only indicative for the presence of lymphoma cells if has been established that t(14;18) is a clonal marker for the lymphoma. The clinical value of the detection of t(14;18)-positive cells by PCR is limited in patients treated with conventional therapy. In these patients we did not found a correlation between the presence of t(14;18)-positive cells in peripheral blood or bone marrow and i) the stage of disease, ii) remission status and iii) remission duration. However, in patients treated with high dose therapy and ABMT, the presence of t(14;18)-positive cells in the bone marrow after transplantation may possibly predict relapse. Thus, the PCR method can be useful to monitor the disease when therapy modalities are aimed at cure. To extent the applicability of PCR based detection methods, we started to use the unique immunoglobulin heavy chain gene rearrangement in NHL as a patient-specific clonal marker for the malignant cells. In spite of technical problems in generating patient-specific clonal markers by PCR, the possibilities to use these markers in the study of minimal (residual) disease in B-cell neoplasms and their clinical relevance, especially in patients with intermediate and high grade malignant lymphomas, justifies further investigations.

SAMENVATTING

In dit proefschrift maken wij gebruik van een nieuwe methode om kleine aantallen t(14;18)-positieve cellen te kunnen detecteren, namelijk de polymerase ketting reactie (PCR). Wij evalueerden de aanwezigheid van t(14;18)-positieve cellen in het bloed of beenmerg van patienten met een t(14;18)-positief folliculair non-Hodgkin lymfoom (NHL) tijdens stagering van de ziekte bij diagnose en tijdens het verloop van deze ziekte na behandeling.

In hoofdstuk 1 beschrijven wij klinische en fenotypische aspecten van het meest voorkomende NHL namelijk het folliculaire NHL. Dit type NHL is geassocieerd met een specifieke chromosoom translocatie, namelijk een translocatie tussen de chromosomen 14q32 en 18q21 [t(14;18)]. De moleculaire karakteristieken van deze translocatie maken de toepassing van de gevoelige PCR techniek, een *in vitro* DNA amplificatie techniek, mogelijk. Door toepassing van PCR kan minimale ziekte met een grotere gevoeligheid ten opzichte van de meer conventionele technieken zoals beeldtechnieken, cytogenetische, morfologische en immunologische technieken opgespoord worden. De gevoeligheid waarmee lymfoom cellen opgespoord kunnen worden, is door toepassing van de PCR techniek met een factor 100 tot 1000 toegenomen. Dit kan van belang zijn voor de stagering van de ziekte bij diagnose en voor het vervolgen van de ziekte na behandeling.

In hoofdstuk 2 gebruikten wij de PCR techniek om de aanwezigheid van t(14;18)-positieve cellen in het bloed of beenmerg van voornamelijk patienten met een folliculair centroblastisch centrocytisch NHL te evalueren. De PCR resultaten werden vergeleken met de resultaten verkregen door immunologische en morfologische analyse van het bloed of beenmerg. De PCR resultaten werden tevens gecombineerd met de klinische status van de patient zoals vastgesteld door middel van röntgenfoto's en CT scans. Translocatie (14;18)-positieve cellen werden met behulp van de PCR techniek gevonden in het bloed en beenmerg terwijl er geen lymfoom cellen aangetoond konden worden met behulp van immunologische en morfologische analyses. In het bloed en beenmerg van patienten zonder klinisch aantoonbare ziekte werden ook t(14;18)-positieve cellen aangetoond. Dus de PCR techniek is een krachtige techniek waarmee minimale ziekte bestudeerd kan worden.

In hoofdstuk 3 hebben we de PCR techniek gebruikt om de aanwezigheid van t(14;18)-positieve cellen in bloed of beenmerg van patienten met een gelokaliseerd (stadia I en II)

t(14;18)-positief folliculair NHL te bestuderen. Wij toonden t(14;18)-positieve cellen aan in het bloed of beenmerg van 75% van deze patienten. In de meeste patienten werden deze t(14;18)-positieve cellen door lokale therapie niet uitgeroeid. Dus in een groot deel van de patienten met een folliculair NHL werden t(14;18)-positieve cellen gevonden in het bloed en beenmerg, onafhankelijk van het stadium van de ziekte.

Hoofdstuk 4 beschrijft een longitudinale studie waarin de aanwezigheid van lymfoma cellen in bloed of beenmerg van patienten met een uitgezaaid (stadia III en IV) t(14;18)-positief folliculair NHL tijdens de eerste remissie na conventionele behandeling bestudeerd werd. Wij vonden geen duidelijke relatie tussen de aanwezigheid of afwezigheid van t(14;18)-positieve cellen in bloed of beenmerg en i) de mate van de remissie en ii) de duur van de remissie. Opmerkelijk is de tendens dat de met behulp van PCR aan te tonen t(14;18)-positieve cellen continue aanwezig of afwezig waren in bloed of beenmerg tijdens remissie bij individuele patienten.

In hoofdstuk 5 werden de karakteristieken van NHL met testiculaire localisatie bestudeerd. Deze NHL zijn relatief zeldzaam. Deze NHL kunnen zich verspreiden naar de testis of ontstaan primair in de testis. Testiculaire NHL verspreiden met voorkeur naar specifieke plaatsen. Wij onderzochten of t(14;18) aangetoond kon worden in dit type NHL. Om t(14;18) aan te tonen hebben we gebruik gemaakt van cytogenetica, Southern blot en PCR analyses. Het BCL-2 gen is betrokken in de t(14;18) en daarom hebben wij immunohistochemie gebruikt om de aanwezigheid van het BCL-2 eiwit aan te tonen. Het BCL-2 eiwit kon aangetoond worden in alle geteste lymfomen, dit in tegenstelling tot de t(14;18). Wij denken dat de aanwezigheid van het BCL-2 eiwit in deze lymfomen gerelateerd is aan het ontwikkelingsstadium van de B-lymfocyt en/of belangrijk is in de pathogenese van deze lymfomen. De aanwezigheid van het BCL-2 eiwit en de afwezigheid van de t(14;18) ondersteunen de klinische aanwijzingen dat deze lymfomen een aparte identiteit bezitten.

In hoofdstuk 6 bestudeerden wij de genomische organisatie van een nieuw gegenereerde cellijn met de chromosoom translocaties (8;14) en (14;18). We bepaalden de localisatie van de breukpunten op chromosoom 8, 14 en 18. Beide zware immunoglobuline keten genen, gelegen op chromosoom 14, waren onderbroken door deze twee translocaties. Het breekpunt op chromosoom 18 was gelegen in een specifiek breekpunt gebied van het BCL-2 gen. Het breekpunt op chromosoom 8 was gelegen in exon 1 of intron 1 van het MYC gen. Het BCL-2 eiwit en de normale BCL-2 mRNA transcripten werden gevonden in deze cellijn. Het MYC mRNA was ongeveer 0,4

kb korter dan normaal. Doordat beide zware immunoglobuline keten genen betrokken waren in de translokaties werd geen expressie van immunoglobulines in het cytoplasma en op de celwand gevonden. De cellijn kan een bijdrage leveren aan het verbeteren van het inzicht in de rol van deze translocaties bij de deregulering van celgroei en maturatie.

In hoofdstuk 7 wordt het klinische belang van het aantonen van kleine aantallen t(14;18)-positieve cellen met behulp van PCR in patienten met een t(14;18)-positief NHL bediscuseerd. Het opsporen van t(14;18)-positieve cellen met behulp van PCR wijst alleen op de aanwezigheid van lymfoom cellen als de t(14;18) een klonale merker is voor het lymfoom. De klinische waarde van het aantonen van kleine aantallen t(14;18)-positieve cellen bij patienten behandeld met conventionele therapie is beperkt. Bij deze patienten werd geen relatie gevonden tussen de aanwezigheid van t(14;18)-positieve cellen in het bloed of beenmerg en i) het stadium van de ziekte, ii) de mate van de remissie en iii) de duur van de remissie. Echter bij patienten behandeld met een beenmergtransplantatie is mogelijk wel een relatie gevonden tussen de aanwezigheid van t(14;18)-positieve cellen in het beenmerg na transplantatie en de voorspelling van een recidief. Vanwege deze mogelijke voorspellende waarde is PCR vooral belangrijk voor het vervolgen van de ziekte als de behandeling gericht is op genezing. Om PCR detectie methoden algemener te kunnen toepassen zijn wij gestart met het gebruiken van het unieke zware immunoglobuline keten gen als een patient-specifieke klonale merker voor maligne cellen. Ondanks technische problemen is verder onderzoek gerechtvaardigd door de mogelijkhden die deze merkers kunnen hebben voor het onderzoek naar minimale ziekte in B-cel maligniteiten, met name non-Hodgkin's lymfomen van de intermediaire en hoge maligniteits graad en hun klinische relevantie.

CURRICULUM VITAE

Aleida Cornelia Lambrechts werd op 8 september 1964 te Amersfoort geboren. In 1982 behaalde zij het diploma VWO-B aan de openbare scholengemeenschap "de Amersfoortse Berg". In datzelfde jaar werd begonnen met de studie biologie aan de Rijksuniversiteit te Utrecht. Het doctoraal examen met als hoofdvak "Fysiologische Chemie" (Dr. J.W.M. Höppener en Prof. Dr. H.S. Jansz) en als bijvakken "Moleculaire Neurobiologie" (Dr. M. van den Buuse en Prof. Dr. W. de Jong) en "Ontwikkelingsbiologie" (Dr. B.C. Tilly en Prof. Dr. S. W. de Laat) werd in 1988 afgelegd. Van 16 mei 1989 tot 16 mei 1993 was zij aangesteld als wetenschappelijk medewerkster aan de Dr. Daniel den Hoed Kliniek op een door de Nederlands Kankerbestrijding gefinancieerd project. Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van Dr. M.B. van 't Veer, internist, hematoloog en Dr. Ir. L.C.J. Dorssers, hoofd afdeling moleculaire biologie.

BIBLIOGRAPHY

1. A.C. Lambrechts, P.E. de Ruiter, L.C.J. Dorssers and M.B. van 't Veer. (1992). Detection of residual disease in translocation (14;18) positive non-Hodgkin's Lymphoma, using the polymerase chain reaction: A comparison with conventional staging methods. **Leukemia** 6, 29-34.

2. A.C. Lambrechts, P.E. Hupkes, L.C.J. Dorssers and M.B. van 't Veer. (1993). Translocation (14;18) positive cells are present in the circulation of the majority of patients with localized (stage I and II) follicular non-Hodgkin's Lymphoma. **Blood** 82: 2510-2516.

3. A.C. Lambrechts, P.E. Hupkes, L.C.J. Dorssers and M.B. van 't Veer. (1994). Clinical significance of translocation (14;18)-positive cells in the circulation of patients with stage III or IV follicular non-Hodgkin's lymphoma during first remission. **Journal of Clinical Oncology** in press.

4. A.C. Lambrechts, L.H.J. Looijenga, M.B. van 't Veer, B. de Jong and J.W. Oosterhuis. (1994). Lymphomas with testicular localization show a consistent BCL-2 expression without a translocation (14;18): A molecular and immunohistochemical study. Submitted for publication.

5. A.C. Lambrechts, P.E. Hupkes, L.C.J. Dorssers, A. Hagemeijer, M.B. van 't Veer and I.P. Touw. (1994). Genomic organization of translocations (8;14) and (14;18) in a new Lymphoma cell line. **Leukemia** in press.

6. R.B.C. van Ooteghem, E.M.E. Smit, A. Beishuizen, A.C. Lambrechts, M. van de Blij-Philipsen, T.J. Smilde and A. Hagemeijer. (1994). A new B-cell line showing a complex translocation (8;14;18) and BCL-2 rearrangement. **Cancer Genetics Cytogenetics** in press.

7. B.C. Tilly, L.G.J. Tertoolen, A.C. Lambrechts, R. Remorie, S.W. de Laat and W.H. Moolenaar. (1990). Histamine-H1-receptor-mediated phosphoinositide hydrolysis, Ca^{2+} signalling and membrane-potential oscillations in human HeLa carcinoma cells. **Biochemical Journal** 266, 235-243.

8. B.C. Tilly, A.C. Lambrechts, L.G.J. Tertoolen, S.W. de Laat and W.H. Moole-

naar. Regulation of phosphoinositide hydrolysis induced by histamine and guanine nucleotides in human Hela carcinoma cells. Cacium and pH dependence and inhibitory role of protein kinase C. (1990). **Febs Letters** 265, 80-86.

9. A.C. Lambrechts and B.C. Tilly. Histamine a growth factor acting through inositol lipid hydrolyses. Netherlands Journal of Zoology, Proceedings van de Nederlandse Dierkundige Vereniging, 29 april 1988.

10. A.P. Otte, IJ.M. Kramer, M. Mannesse, C. Lambrechts and A.J. Durston. Characterization of protein kinase C in early *Xenopus* embryogenesis (1990). **Development** 110, 461-470.

11. J.W.M. Höppener, S. Mosselman, P.J.M. Roholl, A.C. Lambrechts, R.J.C. Slebos, P. de Pagter-Holthuizen, C.J.M. Lips, H.S. Jansz and J.S. Sussenbach. (1988). Expression of insulin-like growthfactor-I and -II genes in human smooth muscle tumors. **EMBO Journal** 7, 1379-1385.

12. M. van den Buuse, A.C. Lambrechts and W. de Jong. Pressor effects of electrical stimulation of the rat ventral tegmental A10 dopamine system. Dopamine 1987. Dopamine systems and their regulation. Satellite to IUPHAR congress of Pharmacology.

13. W. de Jong , A.C. Lambrechts and M. van den Buuse. (1986). Blood pressure effects of dopamine and bromocriptine in concious spontaneously hypertensive rats (SHR). **British Journal of Pharmacology** 89, 688.