The role of the *bcl-2* gene family in malignant hematopoiesis and their use for monitoring of disease

Eike P.P. Meijerink

The role of the *bcl-2* gene family in malignant hematopoiesis and their use for monitoring of disease

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Jules Peter Paul Meijerink

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Promotor
prof dr T J M de Witte

Co-promotores
dr E J B M Mensink
dr J M M Raemaekers

Manuscriptcommissie
prof dr P de Mulder
prof dr P Kluin (RUL)
prof dr E J J van Zoelen

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Jules Peter Paul Meijerink
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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Bax	Bcl-2 associated X gene
Bcl-2	B-cell lymphoma/leukemia-2 gene
BMT	bone marrow transplantation
C_H	immunoglobulin heavy chain constant gene segment
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
D_H	immunoglobulin heavy chain diversity gene segment
DLCL	diffuse large cell lymphoma
IgH	immunoglobulin heavy chain locus
J_H	immunoglobulin heavy chain joining gene segment
MBR	major breakpoint region
MCR	minor cluster region
MRD	minimal residual disease
NHL	non-Hodgkin's lymphoma
PCR	polymerase chain reaction
PML	promyelocytic leukemia
t(14;18)	chromosomal translocation between chromosome 14 and chromosome 18
VCR	variant cluster region
V_H	immunoglobulin heavy chain variable gene segment
WF	working formulation

CHAPTER 1

t(14;18), A JOURNEY TO ETERNITY

By Jules P.P. Meijerink

From the department of Hematology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands

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The t(14;18) is the most frequent chromosomal aberration observed in follicular non-Hodgkin's lymphoma (NHL), and less frequently in diffuse large cell lymphoma (DLCL). The t(14;18) does not affect overall survival in follicular NHL or DLCL. It provides an unique lymphoma specific marker that can be used to quantify residual disease during treatment, and may forward evaluation of new treatment protocols. The t(14;18) results in the deregulation of *bcl-2*, a proto-oncogene that protects against induction of programmed cell death (PCD). Nowadays, a whole family of *bcl-2* related genes has been identified and consist of members which can either protect or promote induction of PCD. The family members form protein dimers, and the relative abundance of specific dimers regulates the cellular sensitivity to death signals (the rheostat model). Aberrant expression of different family members in several types of malignancies alter the cellular rheostat and thereby promote cellular resistance to chemotherapy.

1.1 t(14;18) and non-Hodgkin's lymphoma

Specific chromosomal abnormalities are associated with certain types of malignancies. In follicular non-Hodgkin's lymphoma (NHL), a specific chromosomal translocation involving chromosome 14 and 18, the t(14;18)(q32,q21), is present in about 60% of the cases (Table 1A) The t(14,18) has also been found in 16% of the cases with *de novo* diffuse large cell NHL (DLCL) including immunoblastic (IB) NHL (Table 1B) The incidence of t(14;18) in follicular NHL or DLCL may differ in the populations investigated. The incidence of t(14;18) in follicular NHL and DLCL as determined by either cytogenetic analysis or molecular techniques like Southern blot or polymerase chain reaction (PCR) is only 38 and 9% in the Japanese population versus 62 and 18% in the rest of the world for follicular NHL and DLCL respectively[1-3].

The t(14;18) involves the immunoglobulin heavy chain (*IgH*) locus on chromosome 14q32 and the B cell leukemia/lymphoma-2 (*bcl-2*) gene on chromosome 18q21 (Figure 1). During the translocational event, the tip of chromosome 18 including the coding region of *bcl-2* is coupled to one of the IgH joining gene segments (J_H) on chromosome 14. This aberrant chromosome 14 is cytogenetically denoted as the $14q^+$ chromosome. In the reciprocal $18q^-$ chromosome, the tip of chromosome 14 including the IgH variable (V_H) and diversity (D_H) gene segments is coupled to the shortened chromosome 18, downstream of the *bcl-2* gene. The chromosomal breakpoints on chromosome 18 are clustered in two regions: a 150 bp region in the 3' untranslated region of *bcl-2* which is called the major breakpoint region (MBR) and a 500 bp region 20 kilobases downstream of *bcl-2* which is called the minor cluster region (MCR)[4-6]. In t(14;18) positive follicular NHLs, about 79% of the breakpoints on chromosome 18 occur within the MBR and about 21% occur in the MCR (Table 1A).

Detection of chromosomal abnormalities by karyotypic analysis or molecular biological techniques like Southern blot or PCR (Figure 2) are useful tools for detecting t(14;18) in tumor-biopsies

which contain a high percentage of malignant cells Cytogenetic analysis or molecular analysis both detect t(14;18) in an about equal number of cases in follicular NHL (Table 1A) and DLCL (Table 1B) The PCR technology provides an unique advantage in that it is extremely sensitive in comparison to karyotypic analysis or Southern blot[7]. The PCR technique may detect a single t(14,18), reflecting a single lymphoma cell, in a background of about one hundred thousand normal cells[8 9]. By PCR, it now becomes possible to monitor consecutive patient blood and bone marrow samples for minimal residual disease during treatment This will be further discussed in paragraph "Detection of minimal residual disease" and chapter 2-4[10-12]

Table 1A. Frequency of t(14;18) and BCL2 expression in follular NHL (working formulation type B,C, and D)

Country	Cyto-genetic	MBR	MCR	Total molecular	BCL2+	MBR+/BCL2+	Authors
USA	4/9	-	-	-	-	-	(13)
USA	29/48	-	-	-	-	-	(14 15)
USA	14/20	-	-	-	-	-	(16)
World	37/64	-	-	-	-	-	(17)
USA	-	9/18	2/18	12/18[a]	-	-	(18)
USA	-	21/36	11/36	32/36	-	-	(19)
USA	-	11/17	-	-	-	-	(20)
USA	10/13	21/31	-	-	-	-	(21)
USA	-	11/37	-	-	-	-	(22)
USA	-	NS	NS	13/20[a]	-	-	(23)
Japan	5/10	8/30	2/30	10/30	-	-	(1)
Japan	-	2/16	1/16	5/16[1a b]	-	-	(2)
UK-Denmark	-	18/51	3/51	21/51	-	-	(24)
UK	-	5/13	3/13	8/13	-	-	(25)
China	-	8/16	1/16	9/16	-	-	(26)
Germany	-	9/17	3/17	12/17	-	-	(27)
Germany	-	13/33	-	-	-	-	(28)
USA	-	56/88	18/88	74/88	-	-	(7)
Japan	6/15	5/11	0/11	5/11	-	-	(3)
Spain	3/5	3/5	0/5	3/5	-	-	(29)
UK	18/28	27/49	2/49	27/49	-	-	(30)
France	11/24	30/64	9/64	40/64[1a c]	60/64	37/60	(31)
USA	-	4/15	0/12	7/15[1]	15/16[d e]	7/15	(32)
UK-Denmark	-	NS	NS	27/61[1]	55/64	24/51	(33)
World (%)	137/236 (58 1%)	261/547 (47 7%)	55/426 (12 9%)	305/510 (59 8%)	130/144 (90 3%)	68/126 (54 0%)	

Table 1B. Frequency of t(14;18) and BCL2 expression in diffuse large cell NHL (working formulation type G and H)

Country	Cyto-genetic	MBR	MCR	Total molecular	BCL2+	MBR+/BCL2+	Authors
USA	7/29	-	-	-	-	-	(14 15)
World	9/78	-	-	-	-	-	(17)
USA	7/32	-	-	-	-	-	(16)
USA	2/13	8/20	-	-	-	-	(21)
USA	-	3/30	1/30	5/30	-	-	(19)
USA	-	4/26	-	-	-	-	(20)
USA	-	5/31	-	-	-	-	(22)
USA	-	NS	NS	10/54[a]	-	-	(23)
Japan	-	2/27	0/27	2/27	-	-	(2)
USA	35/205	8/81	0/81	10/81[a]	-	-	(34 35)
China	-	0/10	0/10	0/10	-	-	(26)
Netherld	-	NS	NS	13/52	-	-	(36)
Germany	-	2/47	-	-	-	-	(28)
Japan	4/44	2/20	0/20	2/20	-	-	(3)
Spain	1/5	1/5	0/5	1/5	-	-	(29)
USA	-	6/45	3/45	9/45	-	-	(37)
USA	-	3/14	0/14	4/14[a]	16/18[d f]	4/13[d]	(32)
Canada	-	15/83	-	-	55/83	2/15	(38)
Europe	-	-	-	-	57/83	-	(39)
World (%)	65/406 (16 0%)	59/439 (13 4%)	4/232 (1 7%)	56/338 (16 6%)	128/184 (69 6%)	6/28 (21 4%)	

Frequency of t(14,18), as detected by karyotypic analysis or molecular analysis by Southern blot or PCR, and BCL2 expression in follicular NHL **(A)** or diffuse large cell NHL **(B)** Abbreviations -, not determined, NS, not shown, [a]Includes *bcl-2* gene rearrangements as detected with a 5' *bcl-2* probe, [b]Includes one case with a *bcl-2* gene rearrangement in a region between the MBR and the MCR, [c]Includes two cases with *bcl-2* rearrangements detected with both the MBR and the VCR probe, [d]One follicular NHL case and two DLCL cases with barely detectable BCL2 staining are shown as BCL2 negative, [e]88% of cases (14/16) and [f]72% of cases (13/18) had intense BCL2 staining comparable with follicular mantle zone B cells

1.2 Variant translocations involving *bcl-2*

A number of translocations involving *bcl-2* but different from the classical t(14,18) have been observed in cases with follicular NHL and a case with lymphoblastic lymphoma In these cases, translocation breakpoints have been found in a 1 5 Kb region upstream and within the first exon of *bcl-2*. This region has been denoted VCR, for variant cluster region In one case, the breakpoint was mapped about 10 Kb upstream of the *bcl-2* locus within another transcriptionally active locus which is called follicular variant translocation gene (*fvt-1*)[40]. The IgH JH6 region was coupled to *bcl-2* in one case[41], but in most cases the kappa or lambda immunoglobulin light chain genes (IgL) are juxtaposed to *bcl-2* in a head-to-head orientation, resulting in the t(2,18)(p11;q21) or the t(18,22)(q21,q11)[2,42,43]. Based upon molecular analysis using Southern blot, the

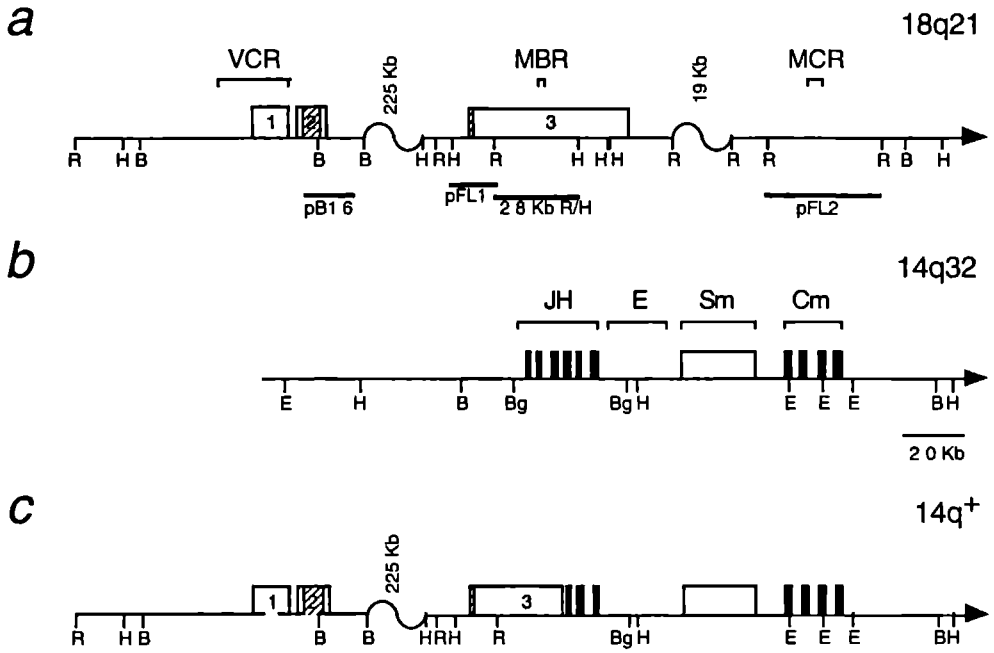


Figure 1. The *bcl-2* gene and the IgH gene are involved in the t(14,18)(q32,q21) **(A).** Schematic illustration of the *bcl-2* locus at chromosome 18q21 Exons are shown as stipled boxes The protein coding domains are shown as hatched boxes The three chromosomal breakpoint regions VCR, MBR and MCR are indicated as well as the DNA probes pB1 6, pFL-1, 2 8 KB R/H and pFL-2 which are most frequently used for Southern blot detection of *bcl-2* rearrangements Exon 2 and 3 are separated by an intron of 225 Kb The VCR is located upstream and within the first exon The MBR is located within the 3' untranslated region of the third exon The MCR is located 20 Kb downstream of the MBR **(B).** Schematic illustration of a part of the IgH locus at chromosome 14q32 The position of the germline JH gene segments, the IgH enhancer, the Cμ switch region and Cμ exons are indicated **(C).** Schematic illustration of an hypothetical 14q+ chromosome, in which the chromosomal breakpoints occured within the MBR of *bcl-2* and immediatly upstream of the JH4 gene segment of the IgH gene Restriction enzyme recognition sites are indicated with B for BamHI, Bg for BglII, H for HinDIII and R for EcoRI The relative positions of centromeres are indicated with arrowheads

t(2,18) and t(18,22) have been associated only recently with B cell chronic lymphocytic leukemia (B-CLL), and seem to occur in about 5% of the cases(Table 2). In the t(14,18), *bcl-2* is coupled to J_H6, J_H4 or less frequent to J_H5 or J_H3 on chromosome 14q+ ([53], and Meijerink, unpublished observation) In the reciprocal chromosome 18q-, a D_H gene segment is coupled to remaining *bcl-2* sequences[53-55] Random

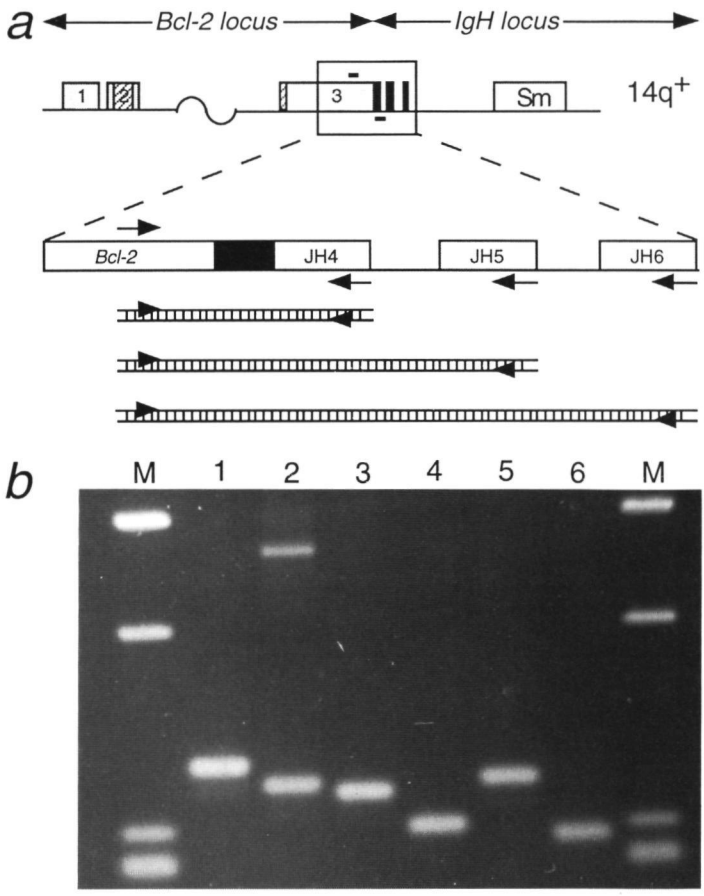


Figure 2. Detection of the 14q+ chromomal junction of the t(14;18) by PCR. **(A).** Schematic illustration of the 14q+ chromosome of an hypothetical t(14;18) translocation in which the chromosomal breakpoints occured within the MBR of *bcl-2* and immediately upstream of the JH4 gene segment of the IgH gene. In the enlarged fraction, the *bcl-2* sequences and IgH JH gene segments are indicated with stippled boxes. Random nucleotides at the chromosomal junction are shown as a black box. The *bcl-2* MBR primer and JH-concensus primer are shown as arrows. Three PCR products derived from the hypothetical 14q+ chromosomal junction are illustrated. **(B).** EtBr-staining of PCR products derived from the 14q+ chromosomal junction of six follicular NHL patients in a 2% agarose gel. Lane M: φX-174 HaeIII marker: marker bands of 872 bps, 603 bps, 310 bps and 281/271 bps are shown. Lane 1-6: PCR products from 6 follicular NHL patients. All 6 patients had a chromosomal breakpoint within the MBR of the *bcl-2* gene. For patient 1, 3-6, *bcl-2* was rearranged to the IgH JH6 gene segment. For patient 2, *bcl-2* was rearranged to the IgH JH4 gene segment. The *bcl-2*-JH4-JH5 PCR product of patient 2 is also visible.

Table 2. *Bcl-2* rearrangement and BCL2 expression in B-CLL

Translocation partner	VCR	MBR	MCR	Total molecular	BCL2+	Author
λ/κ	3/32	0/32	0/32	3/32	-	(44 46)
-	-	0/9	1/9	1/9	-	(25)
?/?/JH[a]	2/44	1/44	0/44	3/44	-	(47)
λ/?/JH	2/55[b]	1/55	0/55	3/55	-	(48)
λ/κ/JH	4/170[c]	0/170	3/170[d]	7/170	-	(49)
-	0/16	0/16	0/16	0/16	16/17[e f]	(50)
ND/κ/JH[g]	8/67[h]	3/71[h]	-	NS	71/71	(51)
-	-	-	-	-	3/3[i]	(32)
Total (%)	19/384 (4 9)	5/397 (1 3)	4/326 (1 2)	17/326 (5 2)	90/91 (98 9)	

NS, not shown, -, not determined, [a]Translocation partners for two cases with 5' *bcl-2* rearrangements were not detected, [b]Case with t(2,18) described in Tashiro *et al* (1992)[52], [c]Abundant BCL2 expression in two cases, [d]Breakpoints occuring upstream of MCR, [e]Includes four cases with BCL2 staining equivalent to normal PBLs, [f]70% (12/17) of cases had BCL2 staining equivalent or higher in comparison with t(14,18) carrying cell line RS11846, [g]Comigration of *bcl-2* allele with kappa light chain in four patients and IgH JH region in one patient, lambda light chain gene rearrangements were not investigated, [h]Includes two patients in which *bcl-2* rearrangements were detected with VCR and MBR probes, [i]BCL2 staining was less intense in comparison with follicular mantle zone B cells

nucleotides, denoted "N-nucleotides", are present at both translocational junctions, and resemble random nucleotides which are present in the V_H-D_H and D_H-J_H junctions and which are randomly inserted by terminal-deoxy transferase during the IgH assembly process It is now generally believed that the t(14,18) most likely results from a mistake during the IgH rearrangement process in the pre-B cell stage[5 54] In agreement with this, occasional t(14,18)s have been described which are the result of erroneous IgH rearrangements during either the D_H to D_H rearrangement process[55] and even the V_H to D_H-J_H rearrangement process (Chapter 5, [56]) Tsujimoto *et al* (1985) reported the presence of two opposite sets of immunoglobulin gene recombination signal-like sequences in the MBR of *bcl-2*, which resembles a pseudo D_H gene and may participate in the IgH recombination[57] However, about half of the translocations occuring in the MBR can not be explained by the presence of these signal-like sequences Alternatively, Wyatt *et al* (1992) suggested the involvement of prokaryotic recombination signal χ-related sequences in the translocation mechanism χ-like sequences are present in the MBR, V_H, D_H and J_H gene segments, and may be targets for illegitimate recombination by the VDJ recombinase complex[53]

Breakpoints in the IgL loci frequently involves IgL recombination signals, lending strength to the argument that these translocations may occur as mistakes during the IgL recombination process[2 40 52] However, no heptamer or nonamer signal-like sequences have been found in the region upstream of *bcl-2*

Adachi *et al*[46]demonstrated that breakpoints in the 5' flanking region of *bcl-2* occur in or near alternating purine-pyrimidine stretches. These nucleotide stretches can adopt an alternative DNA conformation, which is called "Z-DNA conformation". Z-DNA elements have been associated with numerous homologous recombination, insertions and deletions processes. No such stretches have been found in the MBR or MCR region of *bcl-2*. Therefore, the mechanisms involved in the genesis of the t(14,18) and the t(2,18) or t(18,22) may be different.

1.3 The clinical significance of t(14;18) in NHL

Chromosomal aberrations involving chromosome 2, 7 or 17 or chromosomal breaks in 1p32-36, 1q21-23, 6q21-25 are prognostic factors in follicular NHL or DLCL which are associated with decreased overall survival[23,34,58] The accumulation of several genetic hits during NHL progression leads to a further reduction of overall survival[34,58], probably due to dedifferentiation to a higher malignant state In one initial study involving 20 follicular NHL patients, a correlation was found between the presence of the t(14,18) and decreased overall survival. No such effect was found in many other studies involving follicular NHL or DLCL patients[33-35,37,39]. The t(14,18) has been correlated in several studies with a lower incidence of complete remission after chemotherapy and decreased disease-free survival in follicular NHL and DLCL[23 35 59 60] Since overall survival is not affected, these results indicate that relapses are responsive to further treatment. Paradoxically, one study correlated the identification of t(14,18) in DLCL at time of relapse with increased overall survival[34].

The chromosomal breakpoints in the t(14;18) cluster in small regions. The PCR is therefore an excellent method for easy, fast and extremely sensitive detection of residual lymphoma cells within blood or bone marrow samples of follicular NHL patients[8,9,55], and has been applied in several experimental as well as clinical studies[7 61,62]. Although t(14,18)s have been found at very low frequency in benigm hyperplastic lymph nodes and blood samples of normal blood donors (see section 2.3, [63-65]), the exact molecular composition of each t(14,18) is unique and can therefore be used as lymphoma specific marker in t(14,18) positive follicular NHL patients Monitoring of residual t(14;18) carrying lymphoma cells by PCR in bone marrow samples of patients who are in clinical complete remission and who had been pretreated with high-dose chemotherapy followed by ABMT, proved very useful for identifying patients with increased risk of relapse. Patients with residual lymphoma cells in the autograft after extensive immuno-purging had a significantly reduced disease-free survival when compared to patients in whom no residual lymphoma cells could be detected ($p<0.00001$). None of 58 patients without disease in bone marrow samples after ABMT relapsed during a median follow-up of about 2 years[59 60] In contrast, disease-free survival for 35 patients with apparent disease after ABMT was only 15 months. No lymphoma cells were detected in consecutive blood samples after transplantation in patients who had been transplanted with autologous marrow without evidence of residual disease Thirteen out of 14 patients who had been transplanted with autologous marrow

Table 3. *bcl-2* inhibits induction of cell death by various cytotoxic stimuli

Cell-types (cell line)	Cytotoxic stimuli	Drug-targets	Authors
pro-B (LyH7, FL5.12)	IL-3 deprivation		(72,80 84)
pro-myeloid (FDCP1)	GM-CSF deprivation		
mast cell (32D)	IL-4 deprivation		
T cell (CTLL2)	IL-2 deprivation		
kidney carcinoma (293)	Serum deprivation		
central neural (CSM14.1)	NGF deprivation		
sensory neurons	NT-3 deprivation		
	BDNF deprivation		
hypothalamic (GT17)	H_2O_2/OH radicals[a]	DNA. lipids, proteins	(74,81,85)
pro-B (FL5 12)	superoxide[b]		
T hybrydoma (2B4)	dexamethasone	glucocorticoid receptor	
central neural (CSM14.1)			
hepatoma (7316A)	hypoxia		(86)
pheochromocytoma (PC12)			
central neural (CSM14 1)	A23187	calcium ionophore	(81)
	t-BOOH	membrane peroxidation	
thymoma (WEHI7.2)	thapsigargin	calcium-pump (ER)	(87)
pre-B ALL	TA	glucocorticoid receptor	(88)
Burkitt lymphoma	5fluorodeoxyuridine	antimetabolite	(89)
	CB3717	thymidylate synthetase	
	ICI M247496	thymidylate synthetase	
T lymphoma (S49.1)	dexamethasone	glucocorticoid receptor	(90-96)
thymoma (WEHI7.2)	adriamycin	DNA intercallator	
pre-B ALL (697)	daunomycin	DNA intercallator	
B lymphoma (CH31)	etoposide	topoisomerase II	
neuroblastoma (Shep-1)	camptothecin	topoisomerase I	
fibroblast (GM701)	ionomycin	calcium ionophore	
pro-B (FL5.12)	methotrexate	DHR	
cervix squamous carcinoma	1βD-AraC	antimetabolite	
(HeLa)	vincristine	microtubuli	
	staurosporine	protein kinases	
	cyclophosphamide	DNA alkylator	
	nitrogen mustard	DNA alkylator	
pheochromocytoma (PC12)	enediyne-5	DNA damage	(97)
	cisplatin		
Chinese hamster ovary (CHO)	c-myc		(98,99)
fibroblast (Rat-1)			
mouse embryonal fibroblast	E1A		(100)
T lymphoma (J3D)	p53		(101,102)
myeloid leukemia (M1)			
(BHK)	Sindbis infection		(103)

[a]H_2O_2 and hydroxyl radical fomation due to glutathione depletion, [b]Superoxide formation due to menadione treatment, ActR I and II, Activin-A receptor type I and type II, BDNF, brain-derived neurotrophic factor; DHR, dihydrofolate reductase, NGF, nerve growth factor, NT-3, neurotrophin-3, TA, triamcinolone acetonide, *t*-BOOH, *tert*-butyl hydroperoxide

with PCR-detectable disease remained positive after transplantation[59 60]

Qualitative screening for residual t(14,18) positive lymphoma cells seems, therefore, sufficient to identify patients with increased risk for relapse However, residual t(14,18) positive lymphoma cells may remain detectable at low levels in follicular NHL patients who are in stable clinical remission[66] So qualitative screening may be insufficient for predicting relapse Accurate quantitative screening in consecutive samples may be a pre-requisite in order to predict progression or reoccurrence of disease (Chapter 2-4, [10-12]) Until recently, no curative treatment options were available for patients with follicular NHL. Now, new treatment protocols are emerging based upon high dose chemotherapy with autologous stem cell support, allogeneic stem cell transplantation, new purine analogues, interferon alpha or monoclonal antibodies Quantitative monitoring of t(14,18) carrying cells may have a major clinical impact on future evaluations of these treatment protocols

2.1 *bcl-2*, a new type of oncogene

The *bcl-2* gene is involved as an early genetic hit in the oncogenesis of the t(14,18)-positive follicular NHL It spans more than 230 Kb of DNA[67], and consists of three exons of which exon 2 and a small part of exon 3 encode for protein (Figure 1A) Exon 2 and 3 are separated by a large intron of about 225 Kb The MBR is located in the untranslated region of exon 3[68], and therefore the protein coding domain is maintained during the t(14,18) Dependent on splicing of intron 2, *bcl-2* encodes for two different mRNAs, Bcl-2α and Bcl-2β[69] of which only Bcl-2α seems to have biological relevance The BCL2 protein is a membrane protein and is located at the cytosolic site of the nuclear envelope, the endoplasmatic reticulum and the outer mitochondrial membrane probably near mitochondrial contact zones[70 71] *Bcl-2* is unique among proto-oncogenes, and it does not promote proliferation like *c-myc*, nor contributes to tumorigenesis upon loss-of-function like *p53* or retinoblastoma (*pRB*), but it prevents induction of cell death under cytotoxic conditions[72]. The *bcl-2* gene is able to prevent apoptosis induced by diverse cytotoxic conditions in various cell types ranging from growth factor deprivation, generation of intracellular reactive oxygen radicals, hypoxic conditions, many different chemotherapeutic agents and expression of cellular genes under suboptimal conditions (Table 3). *Bcl-2* can also protect against some forms of necrotic cell death[73 74]

The BCL2 protein is expressed in many different tissues BCL2 is expressed in early hematopoietic progenitor cells of all lineages BCL2 is also present in mature T-lymphocytes which reside in the thymic medulla, as well as in the long-lived IgM/IgD memory B cells in the germinal center follicle mantle zone of secondary lymphoid tissue. BCL2 is also present in the stem cells of differentiating epithelium like skin and intestine, in epithelial cells of hormonal responsive tissue like breast epithelium, thyroid gland epithelium, basal cells of prostate epithelium and acinar cells of pancreatic epithelium as well in neurons that are long-lived post-mitotic cells[75 76]

Bcl-2 knock-out mice have helped in determining the function of *bcl-2* These mice seem to have a normal embryonic development, but soon after birth they become retarded in growth, loose

pigmentation during the second hair follicle cycle, and acquire defects in the small intestine These mice ultimately die of renal failure due to severe polycystic kidney disease[77 79] B and T cell numbers are initially normal and both cell types demonstrate normal proliferative responses Massive cell death occurs in the lymphoid compartment soon after birth and results in lymphocytopenia[77 78] Although *bcl-2* seems not to be absolutely required for embryonal development, it is essential for long-term survival and maintenance of at least some cell types

2.2 *Bcl-2* expression during B cell development

During B cell development, pro-B cells in the bone marrow differentiate into naive B cells During differentiation, cells rearrange their IgH chain and IgL chain loci to encode for functional antibodies After functional rearrangements, pre-B cells differentiate into IgM^+/IgD^- immature B cells and become negatively selected for recognition of self-antigens After differentiation into mature naive B cells, cells leave the bone marrow compartment and enter the periphery During T cell dependent antibody responses, naive B cells undergo affinity maturation and enter germinal centers in the spleen or lymph nodes Here, naive B cells populate the dark zone of the germinal center, and are now called centroblasts Centroblasts rapidly divide, acquire somatic mutations in their rearranged IgH and IgL alleles and enter the light zone as non-dividing centrocytes At this site, centrocytes are tested for antibody affinity during intimate contact with antigen presenting cells Most centrocytes do not survive the selection process and die by apoptosis

Bcl-2 mRNA is expressed in most pro-B cells and in mature naive B cells, but it is temporarily down-regulated in the pre-B cell stage[104] In contrast, the *bcl-2* homologous gene *bcl-xl* becomes upregulated during the pre-B cell stage[105] Down- and upregulation of Bcl-2 and Bcl-xl may coincide with several selection processes First, selection for functional rearrangements of IgH and IgL loci may coincide with down-regulation of Bcl-2 and upregulation of Bcl-xl Second, negative selection for recognition of self-antigen may coincide with down-regulation of Bcl-xl and up-regulation of Bcl-2 Third, positive selection of centrocytes which express abundant Bcl-2 mRNA but no protein[76 106] *In-vitro* stimuli like αIgM, αCD23 plus rIL-1α, αCD40 or CD40-ligand, which mimic *in-vivo* selection stimuli induce BCL2 and $BCL\text{-}X_L$ upregulation in centrocytes or splenic B cells[105 107]

2.3 t(14;18) deregulates *bcl-2* gene expression

Due to the t(14,18), the *bcl-2* gene is in close proximity of the IgH enhancer This results in a continous high expression of the *bcl-2* gene[18 68 108 109] (Table 1A) Remarkably, the number of follicular NHL cases expressing BCL2 protein (about 90%) far exceeds the number of cases with detectable t(14,18) (about 60%) Only 54% of the cases expressing abundant BCL2 protein have detectable *bcl-2* rearrangements Since only some of these cases with t(14,18) may have remained undetectable by cytogenetic analysis or molecular analysis due to alternative breakpoints in the *bcl-2* locus, and since normal germinal center B cells

do not express BCL2 protein[75 76], it is apparent that additional mechanisms exist which results in a deregulated *bcl-2* gene expression

A similar discordance between BCL2 protein expression and the presence of translocations involving *bcl-2* exists for DLCL and B-CLL About 70% of DLCL cases express abundant BCL2, but only 21% of BCL2 positive cases have a rearranged *bcl-2* gene (Table 1B) About all B-CLL cases express abundant BCL2 protein (Table 2) In 70% of these cases expression of BCL2 is equivalent or even higher compared to the BCL2 expression of a t(14,18) carrying follicular NHL cell line[50] Since only 5% of B-CLL cases have a *bcl-2* gene rearrangement, it is unclear if high BCL2 protein expression is of pathological origin B-CLL is supposed to be the malignant counterpart of normal mantle zone B cells, and high BCL2 protein expression in B-CLL could therefore be a mere reflection of long-lived memory B cell origin

Since most cases of follicular NHL have a t(14,18) (Table 1A) and since enforced *bcl-2* expression in transgenic mice seems oncogenic[110], it seems logical that deregulated *bcl-2* expression is an important early step in the pathogenesis of follicular lymphoma Similar translocations are also found in benign hyperplastic lymph nodes[63] and blood samples of healthy blood donors at very low frequency for up to several years[64 65], and indicates that neoplastic development requires additional genetic hits The nature of these genetic defects and the developmental stage in which they become apparent remains elusive Antigen specificity and selection processes may play an important role in the pathogenesis of follicular NHL Follicular NHL cells are frozen in the centroblastic/centrocytic differentiation stage in which the somatic mutation machinery remains active[111-114] The somatic mutation profiles of follicular NHL cells provide evidence that these neoplastic cells remain under continous affinity selection[115 116], so antigen-driven proliferation processes are most likely involved in the maintenance of the malignant cell population (Chapter 5, [56])

Bcl-2 transgenic mice are excellent tools to demonstrate the tumorigenicity of deregulated *bcl-2* gene expression In these mice, non-cycling polyclonal follicular center B cells accumulate, resulting in large splenic and nodular follicular center areas. B cell maturation is not inhibited and immunoblasts and plasma cells are present Splenic B cells have a prolonged livespan, but *in-vitro* activation seems unaffected[117,118]. Another *Bcl-2* transgenic mice model demonstrates increased B cell numbers of all developmental stages without evidence of follicular hyperplasia[119], and probably reflects differences in the genetic background or *bcl-2* expression cassette in both transgenic lines *Bcl-2* overexpression in the B cell lineage prolongs antibody responses upon antigenic challence[119], and prolongs B cell memory[120] After a relatively long latency period, 11% of these mice develop a monoclonal high-grade diffuse large cell immunoblastic lymphoma[110] This clearly demonstrates that t(14,18)s may occasionally occur in every individual, but these cells disappear over time or are specifically eliminated by the immune system[64 65] In some unfortunate cases however, these cells acquire secondary genetic defects which enable these cells to escape from immune surveillance and to exploit their neoplastic appearance fully resulting in follicular NHL

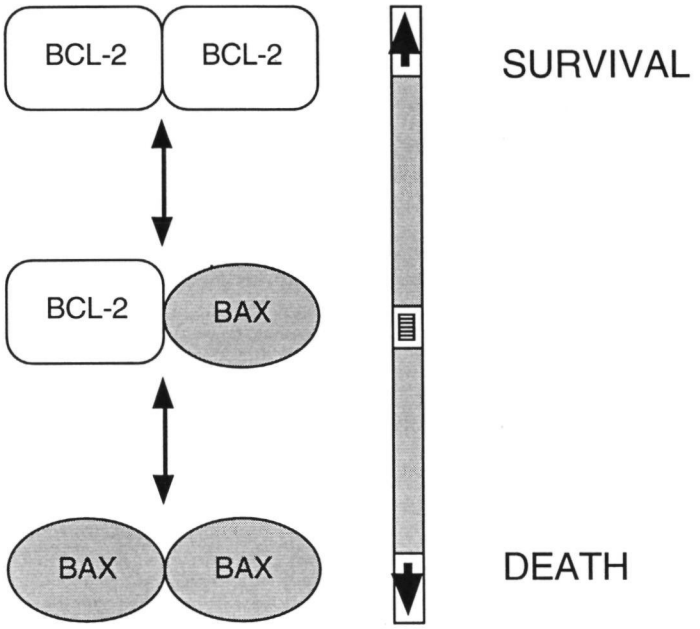


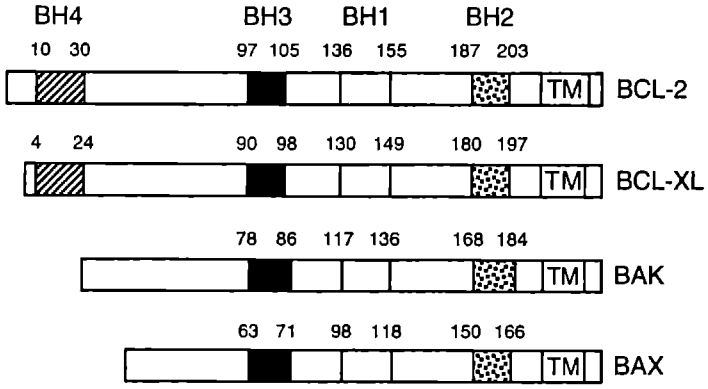
Figure 3. The rheostat model according to Oltvai *et al* (1993)[130].

3.1 The *bcl-2* gene family

Many different evolutionary conserved proteins have been discovered which are homologous to BCL2 and are recognized as the *bcl-2* gene family. Family members share at least two regions of homology with BCL2 and are called BCL2 homology domains 1 and 2 (BH1, BH2). Some family members like *bcl-xl*[121], *bcl-w*[122], *mcl-1*[123,124], *a1/bfl-1*[125-127] and *nr-13*[128,129] provide protection against cell death-inducing stimuli. Other family members like *bax*[130], *bak*[131-133], *bad*[134] and *bcl-xs*[121] have an opposite effect and promote cell death.

Bcl-2 family members form protein dimers[130,135,136], and the formation of specific dimers function as a cellular rheostat to determine the cellular fate following cell death signals[137]. In this rheostat model, relative abundance of cell death promotors like BAX would favour BAX homodimer formation and thereby promote cell death. Relative abundance of survival proteins like BCL2 would favor formation of BCL2/BAX heterodimers or BCL2 homodimers and thereby prevent induction of cell death. Factors like BAD and BCL-X_S compete for binding to cell death inhibiting molecules like BCL-X_L or BCL2, thereby displacing BAX and facilitating BAX homodimerization and induction of cell death[121,134] (Figure 3).

a



b

	BH4	BH3	BH1	BH2
BCL2	NREIVMKYIHYKLSQRGYEW	PVVHLALRQAGDDFSRRY	VVEELFRDGV-NWGRIVAFFE--FGG	TWIQDN-GGWDAFVELYG
BCLXL	NRELVVDFLSYKLSQKGYSW	AAVKQALREAGDEFELRY	VVNELFRDGV-NWGRIVAFFS--FGG	PWIQEN-GGWDTFVELYG
BCLW	TRALVADFVGYKLRQKGYVC	DPLHQAMRAAGDEFETRF	VSDELFQGGP-NWGRLVAFFV--FGA	DWIHSS-GGWAEFTALYG
BFL1	MTDCEFGYI-YRLAQDYLQC	CVLQIPQPGSGPSKTSRV	VMEKEFEDGIINWGRIVTIFA--FEG	EWIRQN-GGQENGFVKKF
NR13	TALLLEDYFQH--RAGGAAL	SATAAELRRAAAELERRE	VAAQLETDGGLNWGRLLALVV--FRG	EWMEEH-GGWDGFCRFFG
MCL1	FGLKRNAVIGLNLYCGGAGL	RKALETLRRVGDGVQRNH	VMIHVFSDGVTNWGRIVTLIS--FGA	DWLVKQR-GWDGFVEFFH
BAK		GQVGRQLAIIGDDINR	IATSLFESGI-NWGRVVALLG--FGY	RWIAQ-RGGWVAALNLGN
BAX		KKLSECLKRIGDELDS	VAADMFSDGNFNWGRVVALFY--FAS	GWIQDQ-GGWDGLLSYFG
BAD			RSAPPNLWAAQRYGRELRRMSDEFEG	GWTRIIQSWWDRNLGKGG

Figure 4. Conservation of protein domains among *bcl-2* gene family members **(A).** Protein domains BH1-4 are conserved in family members with anti-apoptotic activity, whereas only BH1-3 are conserved in members with pro-apoptotic activity (B) Alignment of homology domains (grey box residues) among different family members Conserved amino acid residues are shown in bold capitals Large arrows refer to amino acid residues in BH3 of BAK which interact with residues (small arrows) in the hydrophobic pocket (BH1-3) of BCL-XL [149]

3.2 BCL2 homology domains involved in regulation of apoptosis

Protein alignment of several family members revealed at least four domains of homology, and are denoted BH1 to BH4 (Figure 4). These domains are involved in protein dimerization and cell death regulation, although the contribution of the domains is different within different family members BH1 to BH3 are conserved in all family members except BAD, whereas BH4 is only conserved in family members with anti-apoptotic activity. BH1 and BH2 are critical for the cell death protecting function of BCL2 and BCL-X_L Specific amino acid substitutions in BH1 and BH2 result in loss-of anti-apoptotic activity as well as loss-of binding to BAX. Mutant nonfunctional BCL2 still dimerizes with wildtype BCL2, suggesting that BCL2 and BCL-X_L must dimerize with BAX to display their anti-apoptotic activity[135 138] However, the amino acid residues within BH1 and BH2 that are involved in maintaining the anti-apoptotic activity of BCL2 and BCL-X_L are partially different. Moreover, the cell death protective function of BCL-X_L has been demonstrated in absence of heterodimerization with BAX or BAK[103].

The BH3 domain seems to be crucial for pro-apoptotic molecules such as BAK and BAX In BAK, BH3 is sufficient for promoting death as well as dimerization with BCL-X_L[139] BAX homodimerization has been demonstrated to occur by BH3 to BH3 interaction[140] Deletion of BH3 renders both BAX and BAK non-functional[139 140] Moreover, a BH3 homologous domain has also been identified in pro-apoptotic molecules BIK/NBK[141,142], BID[143] and HRK[144], which are distantly related to the *bcl-2* gene family Therefore, BH3 may encode a new critical death domain Consistent with this, BCL2 can be converted into a pro-apoptotic molecule upon exchange of the BH3 domain with the equivalent domain of BAX[145].

Although the BH3 seems to be sufficient for homodimerization of BAX and for dimerization of BAX or BAK with BCL-X_L, BCL2 homodimerization may involve all four homology domains BCL2 probably forms homodimers by interaction of the N-terminal portion of the protein which includes BH4 with the C-terminal portion of the BCL2 partner protein which includes BH3, BH1 and BH2[136 146] All four homology domains as well as the spacing between BH3, BH1 and BH2 are vital for cell death protective function[95 147] BH4 seems not to be involved in heterodimerization with BAX[140 146] Based upon studies using deletion mutants, the involvement of the homology domains in the formation of specific protein dimers may be different among family members, as summarized in Figure 5

The three dimensional structure of BCL-X_L consists of two central hydrophobic α-helixes (α5 and α6), that are flanked by five amphiphatic α-helixes The three conserved domains BH1 (part of α4, α5),

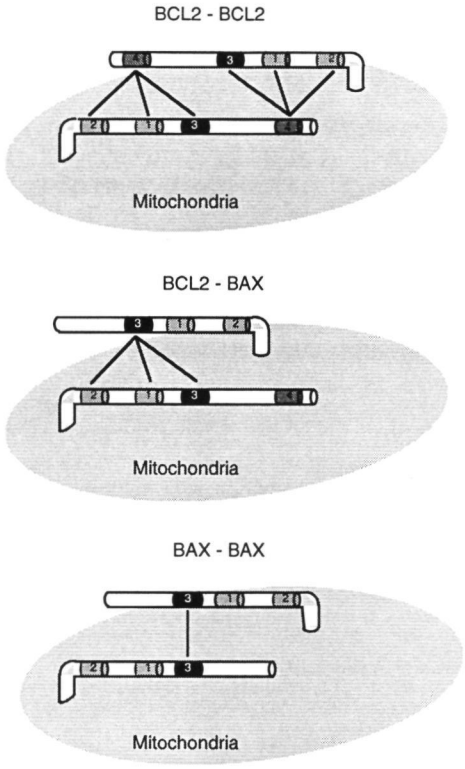


Figure 5. Hypothetical proteins interactions in BCL2 homodimers, BCL2-BAX heterodimers and BAX homodimers. The different homology domains are indicated, and interactions are visualized by lines.

BH2 (part of α6, α7) and BH3 (α2) are in close spatial proximity, and form a hydrophobic cleft[148]. Investigating the BAK-BCL-X_L interteraction, a synthetic peptide related to the BH3 region of BAK was able to bind in the hydrophobic cleft of BCL-X_L. The interaction was mediated by hydrophobic and electrostatical interactions between specific residues of the BH3 region from BAK and residues from the hydrophobic cleft of BCL-X_L. Furthermore, this peptide could inhibit the interaction between wildtype BAK and BCL-X_L through competition[149]. These data are compatible with the model as proposed in Figure 5, and the pro-apoptotic activity and protein interactions of BAK or BAX seems solely dependent on the BH3 domain. All four homology domains in BCL2 and BCL-X_L are required for anti-apoptotic activity and participation in protein dimers, and is probably dependent of forming a hydrophobic cleft.

We recently found mutations in *bax* in about 21% of human malignant hematopoietic lines (Chapter 6 and 7, [150 151]) Several cell lines contained a deletion or an insertion in an identical deoxyguanosine(G8) tract, resulting in truncation of BAX Identical mutations have recently been decribed in about 50% of colorectal cancers of the microsatelite mutator phenotype[152] Three different point mutations, G11E, G67R and G108V, were found in independent lines BAX^{G67R} and BAX^{G108V} were tested for function, and demonstrated altered protein dimerization and loss-of pro apoptotic activity As a consequence of the BAX^{G67R} mutation, a positive charged Arginine residue is introduced in the hydrophobic site of the BH3 domain Both neighboring residues, Ile^{66} and Asp^{68}, are involved in the binding of BAK to BCL-X_L[149] The G67R mutation may interfere with interactions of these residues with the hydrophobic pocket of BCL2 or BCL-X_L Strikingly, this BAX mutant is indeed unable to form heterodimers with BCL-2 or BCL-X_L, but can still form homodimers[151] Although BH1 of BAK or BAX does not seem to be involved in pro-apoptotic function or protein interactions[139 140], the BH1 BAX^{G108V} mutation resulted in a non functional protein that was unable to form homodimers, although heterodimerization seemed unaffected Sattler *et al* (1997) suggested that binding of BAK to BCL-X_L necessitates a conformational change in BAK in order to expose the BH3 domain to the protein surface[149] The BAX^{G108V} mutation may interfere with such a conformational change of BAX, at least for homodimerization Whatever residues are involved in protein dimerization, both mutations demonstrate that hetero- and homodimerization of BAX may involve different amino acid residues of the BH3 domain BH1 may directly be involved in protein interactions or is important for protein conformation

4. *Bcl-2* gene family and resistance to chemotherapy

Traditionally, it has been hypothesized that chemotherapeutic agents are effective in eliminating proliferating cells, but from emerging data it now becomes clear that at least some of these compounds function by inducing apoptosis Many genes are involved in the regulation of cell death pathways, and mutations in these genes can contribute to cellular resistance to chemotherapeutic regiments and radiation One of these genes is the tumor-suppressor gene *p53* This is one of the most frequently mutated genes in human cancer[153 154] In experimental systems, *p53*-deficiency has been associated with reduced death under suboptimal growth-conditions[155], treatment with chemotherapeutic compounds or gamma-radiation[156-160] Within hematopoietic malignancies, mutations in *p53* have been associated with resistance to treatment and reduced survival in AML, MDS and B-CLL[161 162] The mechanism by which P53 induces apoptosis remains enigmatic, but recently *bax* has been identified to be specifically upregulated by P53[102 163], and may participate in the *p53*-dependent death pathway P53-induced apoptosis is reduced but not blocked in the absence of *bax*[164 165], indicating that *bax* participates in the p53-dependent death pathway but that other death effectors are involved as well Furthermore, *bax*-deficiency promotes cellular transformation indicating that *bax* can act as a tumor-suppressor gene as well[164 165] Reduced BAX levels and resistance to cisplatin induced apoptosis may be related to absence of functional *p53* in progressive ovarian carcinomas[166]

Decreased BAX expression correlates with poor prognosis and shortened overall survival in women with metastatic breast cancer[167], but is not correlated with p53 status[168] Enforced BAX expression in malignant breast cancer lines restores the sensitivity to serum deprivation and reduces tumorigenicity in scid mice[168]

The *bcl-2* gene is one of the best studied family members in relation with resistance to chemotherapy It protects against various cytotoxic compounds with different mechanisms of action (Table 3) BCL2 does not inhibit early effects of cytotoxic compounds such as suppression of proliferation or breaks in chromosomal DNA Neighter does it affect DNA repair BCL2 inhibits the onset of a distal death pathway, leading to a reduction of apoptosis and the characteristic chromosomal DNA breakdown[89 92] Since DNA damage by cytotoxic drugs is not inhibited and cells continue to proliferate after drug removal[89 94 169], BCL2 expression promotes selection of cells with additional genetic hits and therefore contributes to dedifferentiation of disease BCL2 overexpression also indirectly promotes chemotherapy resistance by increasing the intracellular GSH levels[74], and shifts the cellular redox-potantial to a more reduced state High GSH levels detoxify a number of chemotherapeutic compounds by reducing free-radical intermediates Similar to BCL2, BCL-X_L can protect against cell death induced by various chemotherapeutic compounds[169 170] However, the ineffectiveness of *bcl-2* but not *bcl-xl* expression in repressing apoptosis in a murine B cell line suggests that both genes function in independent pathways[171]

5. Concluding remarks

The proto-oncogene *bcl-2* is involved in the pathogenesis of follicular NHL and probably in other diseases Overexpression of BCL2 due to the t(14,18) in follicular NHL is not correlated with overall survival, but the t(14,18) provides at least a disease-specific marker which can be used for monitoring of residual disease This may not only allow individualization of therapy, but may be especially helpful in determining the effectiveness of future treatment protocols *Bcl-2* represents a type of proto-oncogene that promotes cellular viability under circumstances which normally would lead to apoptosis Nowadays, a whole family of *bcl-2* related genes has been identified and consists of anti-apoptotic members like *bcl-2* and pro-apoptotic members like *bax* Proteins of this family form protein dimers The viability of a cell is determined by the relative abundance of specific dimers as explained by the rheostat model Aberrant expression or reduced expression of some family members contribute to cellular resistance towards chemotherapy Although much is known about the influence of independent members on the outcome of chemotherapy, future research should be focussed on the expression pattern of the entire family as a whole in relation to chemotherapy resistance Furthermore, a better understanding of the involvement of different homology domains in the formation of specific dimers and outcome on cell survival may provide potential targets for the development of new therapeutic regiments

6. Aims of this thesis

The cytogenetic abnormality t(14,18) is present in about 60% of follicular NHL patients The research leading to this thesis was aimed at the development of a sensitive assay This assay may allow accurate quantitation of

residual malignant cells in follicular NHL patients during and after therapy For this, we developed a method based upon the polymerase chain reaction The method was demonstrated on consecutive peripheral blood samples of several follicular lymphoma patients during treatment This method and it's application is described in Chapters 2-4.

In an attempt to identify disease-specific molecular markers, we investigated a case of follicular non-Hodgkin's lymphoma harbouring an exceptional t(14,18) (Chapter 5). Furthermore, we discovered the presence of mutations in the pro-apoptotic *bax* gene in several malignant hematopoietic lines (Chapter 6) Research was aimed to investigate the extend of mutations within different hematopoietic diseases by analyzing a panel of cell lines and primary patient samples Since some of these mutations were localized in evolutionary conserved domeins, we investigated the consequences of these mutations for pro-apoptotic function of BAX (Chapter 7) In Chapter 8, we reviewed cellulair mechanisms by which the activity of *bcl-2* gene family members may be modulated as well as and potential mechanisms by which *bcl-2* gene family members may regulate programmed cell death

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CHAPTER 2

Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) by competitive polymerase chain reaction

By Jules P.P. Meijerink, Toon F.C.M. Smetsers, John M.M. Raemaekers, M. José J.T. Bogman,* Theo J.M. de Witte, Ewald J.B.M. Mensink

*From the department of Hematology, and the *department of Pathology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands*

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A competitive polymerase chain reaction (PCR) technique was developed to quantify residual malignant cells in peripheral blood and bone marrow samples of patients with low-grade follicular non-Hodgkin's lymphoma carrying a translocation between chromosomes 14 and 18. Artificial translocation segments were constructed imitating a translocation between chromosome 14 and 18. These artificial translocation segments were used as competitor molecules in the quantitative PCR. Serial dilutions of a known amount of patient-derived translocation segments were coamplified with a fixed number of competitor molecules in order to construct a patient specific calibration curve. Patient samples were coamplified with an equal number of competitor molecules and the number of t(14;18) translocations within the samples was calculated by comparison with the calibration curve. The method was demonstrated on samples of four follicular non-Hodgkin's lymphoma (NHL) patients. In a patient, transplanted with allogeneic bone marrow, declining numbers of residual lymphoma cells were observed. We conclude that the method is accurate, relatively fast and the general principle of this method can be applied to all malignancies with characteristic abnormalities on DNA or RNA level that are detectable by PCR.

Characteristic chromosomal abnormalities occur in several human neoplasms[1,2] providing tumour-specific markers that can be used to monitor residual malignant disease. A reciprocal translocation, t(14,18) between chromosome 14 with chromosome 18 is present in most low-grade follicular non-Hodgkin's lymphomas (NHL) depending upon the population under investigation[3]. The B-cell lymphoma/Leukaemia 2 (*bcl-2*) proto-oncogene locus on chromosome 18q21 is coupled to the heavy-chain of the immunoglobulin locus (IgH) on chromosome 14q32 by a process which frequently involves an IgH joining-segment (J_H). N-nucleotides are inserted between the two fusion partners, resulting in a DNA pattern that is patient specific[4,5]. The breakpoints on chromosome 18 cluster within two small regions on the DNA, the major breakpoint region (MBR) located in the 3' untranslated part of the *bcl-2* gene, while the minor cluster region (MCR) is located 20 Kb downstream[6,7].

The polymerase chain reaction (PCR)[8,9] is a fast and sensitive assay capable of detecting the presence of as little as one malignant cell out of 10^5 normal cells[10]. The technique therefore seemed well suited to investigate the influence of treatment on the number of malignant cells in the peripheral blood as well as to monitore the efficiency of purging of autologous bone marrow for autologous bone marrow transplantation provided that an accurate quantitative method can be developed.

To this end, the t(14,18) breakpoint region of cell line SU-DHL-6 was subcloned and modified. PCR product of this artificial *bcl-2*/IgH translocation was used as competitor DNA. PCR product of the patient t(14,18) was necessary as patient-derived translocation segments for the construction of a patient specific calibration curve. For analysis of patient samples by our competitive PCR assay, first, a patient specific calibration curve was constructed by coamplification of serial dilutions as of known amount of patient-derived

translocation segments with a fixed number of competitor molecules The PCR product ratio was logarithmically plotted against the initial number of patient-derived translocation segments Second, patient samples DNA was coamplified in the presence of the same number of competitor molecules and the ratio between final products was determined Third, the ratio of each patient sample was compared with the patient specific calibration curve in order to calculate the number of t(14,18), which reflects the number of lymphoma cells. Samples of four patients with follicular NHL were determined to demonstrate and evaluate this technique.

Materials & Methods

Patients. Patient 1 was diagnosed as having a stage I, low-grade follicular centroblastic-centrocytic (CB-CC) NHL in September 1990. Bone marrow histology showed no evidence of disease and a complete remission (CR) was obtained after locoregional radiotherapy However she developed a slow, progressive cervical lymphadenopathy in February 1991 and tumour cells were found in her bone marrow. Despite treatment with chlorambucil from August 1991 to April 1992, residual tumour was present in lymph nodes and bone marrow after cessation of therapy.

Patient 2 was diagnosed as having stage IV, follicular CB-CC NHL with bone marrow involvement in April 1988 CR was induced following doxorubicin containing therapy which lasted until February 1990 when he relapsed. Treatment with chlorambucil achieved a complete clinical remission in October 1990, but lymphoma cells carrying t(14;18) were still detectable in the bone marrow He was transplanted in October 1990 with marrow from a HLA-identical, MLC non-reactive brother according to the ongoing protocol[11] and is now in stable remission

Patient 3 had a diagnosis of follicular CB-CC NHL in 1980 and was treated with a variety of chemotherapeutic regimens for multiple relapses until June 1991 when a relapse occurred in a lymph node Acute myeloid leukemia (AML) was also diagnosed and the patient died 3 months later following palliative treatment

A partial remission of stage IIA low-grade follicular CB-CC NHL was achieved in patient 4 in 1987 after a course of chemoradiotherapy In January 1988 progressive disease was treated with chlorambucil. However, by February 1990 abdominal involvement became apparent for which radiotherapy was given Eleven month later hypercalcaemia developed and diffuse centroblastic NHL was diagnosed with histological dedifferentiation culminating in death in May 1991.

Sample collection. Mononuclear cells were isolated from blood and bone marrow samples by density centrifugation on 1.085 g/ml Percoll (Pharmacia, Uppsala, Sweden), washed and stored as pellets at -80°C Fresh lymph node tissue was obtained by biopsy, pressed gently through a 70 µm nylon filter, washed and cryopreserved with 10% dimethylsulphoxide (DMSO) by means of a computerized freezing programme that maintened the functional and growth potential of the cells[12].

DNA isolation DNA was isolated from blood, bone marrow and lymph node samples as previously described by Miller *et al* (1988)[13] After determining the concentration, samples were stored at 4°C

Polymerase chain reaction Amplification was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, USA) in presence of 0 001% gelatine, 50 mM KCl, 20 mM Tris-HCl (pH=8 4), 2 0 mM $MgCl_2$, 500 mM of dATP, dTTP, dGTP and dCTP, 300 pmol/ml of oligonucleotides and 25 U/ml *Taq* polymerase (Gibco BRL) in a total volume of 100 µl Reactions were overlaid with 100 µl of mineral oil (Sigma) Amplification started with an initial denaturation step at 94°C for 5 min followed by several cycles at 94°C for 1 5 min, 55°C-58°C for 2 min, and 72°C for 2 min After the last cycle, extension phase was prolonged for 10 min at 72°C, and the reaction was cooled to 4°C

Oligonucleotides Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems model 391 EP, Warrington, UK) Oligonucleotides were deprotected, purified by two ethanol precipitations, and dissolved in diethylpyrocarbonate (DEPC) treated water Concentration was measured by optical density Sense oligonucleotides used MBR1 5'-TTA GAG AGT TGC TTT ACG TG-3', MBR2 5' TCC CTT TGA CCT TGT TTC TTG A, MBR3-XhoI 5'-CCC TCG AGG AGC TTT GTT TCA ACC AAG TC-3 , AN2S 5' GTC ACC GAT ATC GAA TTC CTG CAG G Antisense oligonucleotides used JH-CON 5'-ACC TGA GGA GAC GGT GAC C-3', JH-HindIII 5'-TCA AGC TTA CCT GAG GAG ACG GTG ACC-3', JH6-intron 5' CGC AGG AAA CCC CAC AGG CA-3', AN2A 5'-GTG ACC CTG CAG GAA TTC GAT ATC G-3'

Preparation of artificial bcl-2/IgH translocation segments competitor molecules 1 µg of genomic DNA of cell line SU-DHL-6 containing a t(14,18) was amplified for 35 cycles with oligonucleotides MBR2 and JH HindIII and an annealing temperature of 58°C The fragment was digested with NsiI and HindIII and ligated into PstI and HindIII digested Bluescribe KS^+ vector (Stratagene®, La Jolla, USA) Vector pAN2 2 was constructed by ligating annealed complementary oligonucleotides AN2A and AN2S into the BstEII site Direct sequencing revealed that vector pAN2 2 contained two oligonucleotide AN2A-AN2S duplexes in the insert The construction of the vector is illustrated in Figure 1 Ten reactions containing 20 ng DNA of pAN2 2 were amplified for 35 cycles with oligonucleotides MBR1 and JH-CON and an annealing temperature of 55°C PCR product was separated from remaining oligonucleotides on a 2% agarose gel, excised, and eluted by Biotrap (Schleicher & Schuell) DNA was precipitated, dissolved in 800 µl H_2O and the concentration was determined From this, the number of artificial *bcl-2*/IgH translocation segments was calculated for use as competitor DNA in a competitive PCR

Preparation of patient-derived translocation segments Several reactions containing 1 µg patient lymph node DNA were amplified for 35 cycles with oligonucleotides MBR3-XhoI and JH6 intron and an annealing temperature of 55°C PCR product separation from remaining oligonucleotides, determination of the

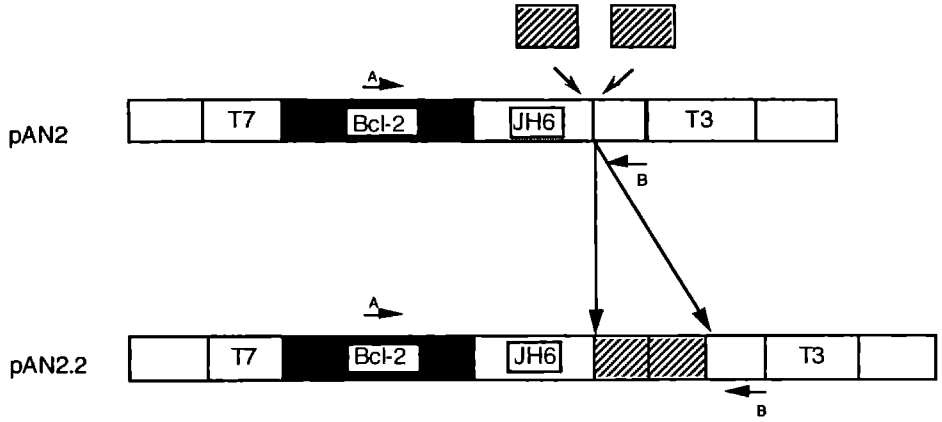

Figure 1. Schematic illustration of vector pAN2.2. The translocation region of cell line SU-DHL-6 (black and grey boxes) is cloned into vector Bluescribe K/S+ (white boxes). Two heteroduplexes of primers AN2A and AN2S (hatched box) are cloned into the BstEII site of the JH6 region. The MBR1 primer-site and the JH-CON primer-site are indicated by A & B respectively. PCR with this primer-pair results in a 272 bp product for the pAN2.2 vector.

concentration and subsequential calculation of the number of patient-derived translocation segments was as described for the preparation of competitor molecules.

Assay procedure. The competitive PCR assay is outlined in Figure 2. Amplification was performed for 55 cycles with oligonucleotides MBR1 and JH-CON in addition of 1.85 MBq/ml [α-^{32}P]dCTP (Amersham, 111 TBq/mmol; 370 MBq/ml).

Patient sample reaction. 1 µg of patient sample DNA was coamplified with a fixed number of competitor molecules.

Serial dilution reactions. Serial dilutions of patient-derived translocation segments were coamplified with a fixed number of the competitor molecules in addition of 1 µg K562 DNA (which contains no t(14;18)). Amplification of 1 µg K562 DNA served as a negative control to check for contamination.

Analysis and quantitation of PCR products. 5 µl of ten fold diluted PCR product in formamide dye (20 mM NaOH, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, 10 mM EDTA (pH=8.0) in formamide) was applied to a 7% polyacrylamide (PAA) (acrylamide:bisacrylamide=24:1), 7 M Urea gel in half TBE buffer. The gel was exposed to X-ray film (Kodak) and autoradiograms were analysed by densitometric scanning on a LKB laser densitometer. Peak intensities were determined using Gelscan® XL software. Ratios of the band intensities of the serial dilution reactions were plotted logarithmically against the initial number of patient-derived translocation segments used. A regression curve was constructed for calibrating the number of

t(14,18) translocation present in each patient sample

Sequencing Sequencing of PCR product was performed as described by Innis *et al* (1988)[14] with minor modifications

Results

Results obtained by the present assay and clinical data for four different follicular NHL patients are summarized in Table 1 The blood sample of the first patient contained detectable numbers of t(14,18) at time of diagnosis Five months later she relapsed with a 17-fold increase of t(14,18) in her blood After treatment she remained in stable relapse and three consecutive blood samples contained virtually equal numbers of translocations T(14,18) was detected in a lymph node sample obtained at the time of relapse in the second patient and also in a blood sample taken 6 months later Complete clinical remission was achieved after 8 months chemotherapy allowing him to receive an allogeneic BMT He remained clinically well even though translocations were still detectable in consecutive blood samples obtained after BMT which declined gradually until finally becoming negative 17 months after BMT The third patient had a relapse of follicular NHL as well as AML for which palliative treatment was given The lymph node sample contained t(14,18) and both blood and bone marrow samples were only slightly positive Three months later his disease progressed with a slight increase in the number of translocations at which point he died Progressive disease was manifested in the fourth patient who had a positive lymph node sample Few translocations were detected even after diffuse centroblastic NHL had been diagnosed and the patient died after 4 months palliative treatment

The competitive PCR assay is demonstrated on several patient 2 samples using different conditions (Figure 3) Amplification of patient 2-derived translocation segments and different samples of this patient resulted in a 184 bp PCR product, and amplification of competitor molecules resulted in a 272 bp PCR product The correlation coefficients of the calibration curves of Figures 3A, 3B & 3C were R= 0 999, R= 0 990 and R= 0 934 respectively Negative controls showed no evidence of contamination (not shown) Amplification of the blood sample of February 1990 (lane 17, Figures 3A, 3B & 3C) was completely inhibited Using 1000 competitor molecules and between 100 to 10000 patient 2-derived translocation segments (Figure 3A), the product ratio for the diluted sample from the lymph node of January 1990 (lane 16) and the next available blood sample of July 1990 (lane 18) was brought within the range for calibration The other blood and bone marrow samples contained too few translocations to estimate This was overcome by lowering the number of competitor molecules to 100, and therefore the competition between competitor and patient translocations bringing the ratio of the bone marrow sample of April 1991 (lane 19) and that of the blood of August 1991 (lane 20) within range The blood sample of October 1991 (lane 21) and the bone

Figure 2. Schematic illustration of the quantitative PCR method as described in Materials & Methods A MBR-1 primer-site, B JH-CON primer-site, C MBR3-XHOI primer-site, D JH6-intron primer-site

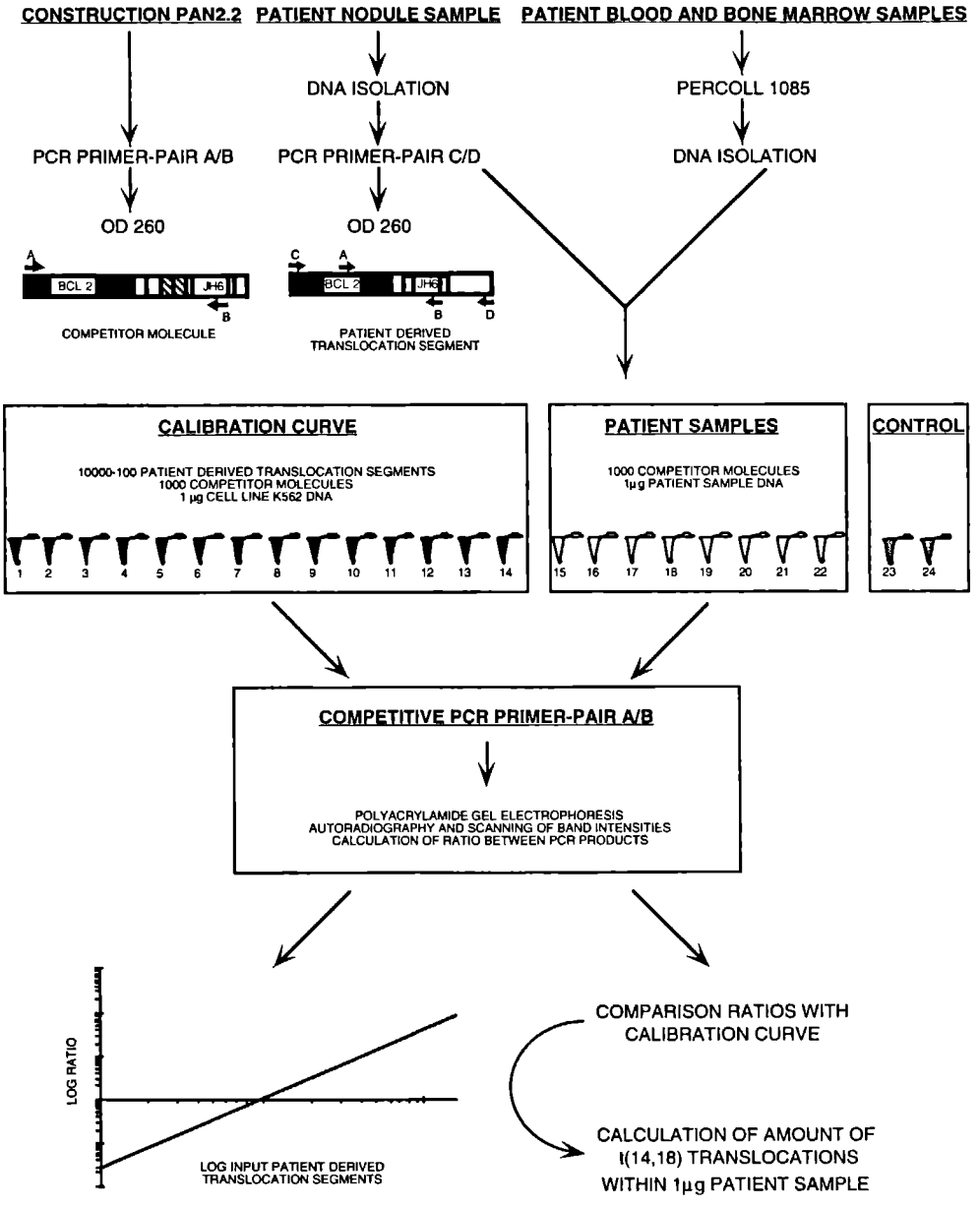
CONSTRUCTION PAN2.2
PATIENT NODULE SAMPLE
PATIENT BLOOD AND BONE MARROW SAMPLES
DNA ISOLATION
PERCOLL 1085
PCR PRIMER-PAIR A/B
PCR PRIMER-PAIR C/D
DNA ISOLATION
OD 260
OD 260
A
BCL 2
JH6
B
COMPETITOR MOLECULE
C
A
BCL 2
JH6
B
D
PATIENT DERIVED TRANSLOCATION SEGMENT
CALIBRATION CURVE
10000-100 PATIENT DERIVED TRANSLOCATION SEGMENTS
1000 COMPETITOR MOLECULES
1 µg CELL LINE K562 DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PATIENT SAMPLES
1000 COMPETITOR MOLECULES
1µg PATIENT SAMPLE DNA
15 16 17 18 19 20 21 22
CONTROL
23 24
COMPETITIVE PCR PRIMER-PAIR A/B
POLYACRYLAMIDE GEL ELECTROPHORESIS
AUTORADIOGRAPHY AND SCANNING OF BAND INTENSITIES
CALCULATION OF RATIO BETWEEN PCR PRODUCTS
LOG RATIO
LOG INPUT PATIENT DERIVED TRANSLOCATION SEGMENTS
COMPARISON RATIOS WITH CALIBRATION CURVE
CALCULATION OF AMOUNT OF t(14,18) TRANSLOCATIONS WITHIN 1µg PATIENT SAMPLE

Table 1 Residual disease in four follicular NHL patients during treatment

Case no.	Age/ Sex	Date	Diagnosis	Clinical stage	Treatment	Tissue	Histology	#t(14;18)/1μg DNA (¥)
1	52 / F	Sep-90	CB-CC NHL	IIA	Radiotherapy	BM[1]	Negative	ND
						Blood		128 ± 12
		Sep-90/ Feb-91	CR					
		Feb-91	Relapse			Blood		2169 ± 95
		Jun-91	Progression			Blood		857 ± 39
		Aug-91	Progression		Chlorambucil	LN	Positive	Positive
						Blood		789 ± 37
		Okt-91	no CR			Blood		823 ± 37
		Jan-92	no CR			Blood		686 ± 33
2	31 / M	Apr-88	CB-CC NHL	IVA	CHOP	BM[1]	Positive	ND
		May-88/ Feb-90	CR					
		Jan-90				LN	Positive	Positive
		Feb-90	Relapse		Chlorambucil	Blood		
		Jul-90			Chlorambucil	Blood		1989 ± 44
		Okt-90	CR		BMT	BM[1]	Positive	ND
		Apr-91	CR			BM[2]		75 ± 5
		Aug-91	CR			Blood		54 ± 4
		Okt-91	CR			Blood		27 ± 4
						BM[2]		11 ± 2
		Mar-92	CR			Blood		0
3	61 / M	Jun-91	Relapse CB-CC NHL		Palliative	LN	Positive	Positive
			AML		Treatment	Blood		28 ± 4
						BM[1]		44 ± 6
		Sep-91	progression †			Blood		99 ± 10
4	56 / M	Jan-87	CB-CC NHL	IIA	Radiotherapy			
		Jan-88	Progression		Chlorambucil			
		Jan-90				LN		Positive
		Feb-90	Progression					
		May-90	Progression			Blood		3 ± 1
		Jan-91	DCB NHL		Palliative			
		Apr-91	Progression		Treatment	Blood		10 ± 2
		May-91	†					

Legend to Table 1 Abbreviations, ND, not determined, BM^1, bone marrow biopty, BM^2, bone marrow aspirate LN, lymph node, CHOP, Cyclophosphamide, Doxorubicin (=Adriamycin), Vincristine, Prednisone, CB-CC, Centroblastic centrocytic, DCB, diffuse centroblastic † Death of patient, ¥Amount of follicular lymphoma cells within 1 µg DNA of nucleated cells after Percoll gradient density centrifugation Lymph node samples with detectable translocations were indicated as positive, since cells are not distributed homogenously and only a small part of the node was used for DNA isolation

marrow sample of October 1991 (lane 22) still contained too few translocations for optimal amplification Further reduction to 10 competitor molecules (Figure 3C) permitted estimation of the number of t(14,18) in these samples (lane 21 & 22) but led to all previous samples being beyond the range for calibration Non-specific PCR fragments also became apparent although these had little effect on the correlation coefficient of the calibration curve

Discussion

PCR appears suitable for the detection of minimal residual disease[8 15], but small variations in reaction conditions can lead to large differences in product yield necessitating the use of an internal standard which corrects for any non-specific inhibition of the reaction and for false negativity For example, the amplification of the peripheral blood sample of February 1990 from patient 2 (lane 17, Figures 3A, 3B & 3C) was completely inhibited by an unknown factor within the sample DNA which was confirmed by the fact that amplification of the competitor molecules was also inhibited The internal standard used in the present study was amplified with the same primer-pair as the target DNA, for equal efficiency of amplification, a principle also employed by Wang *et al* (1989)[16]

The PCR method employed in the present study is able to detect a single translocation within 1 µg genomic DNA, as is demonstrated by the serial dilution of patient-derived translocation segments (Figure 3C) Amplification occurs for 55 amplification cycles to ensure optimal competition for available enzyme, nucleotides and primer molecules, resulting in non-specific PCR fragments when very low amount of specific template is present at the beginning of the reaction (Figure 3C) However, this background has little effect on the correlation coefficient of the calibration curve Gilliland *et al* (1990)[17] and Thompson *et al* (1992)[18] both added a variable number of competitor molecules to a constant amount of patient sample and whenever the product yield for competitor and target molecules was the same, they assumed that the initial target number is equal to the number of competitor molecules added The competitor as used in their method was identical to the target molecule, except for a few mutations lying outside the primer recognition sites, resulting in an identical efficiency of amplification However, in their method it was necessary to digest the product of the competitor before relative product yield could be compared, lowering the accuracy of quantitation Moreover, large number of reactions are required in order to determine the equilibrium for each patient sample

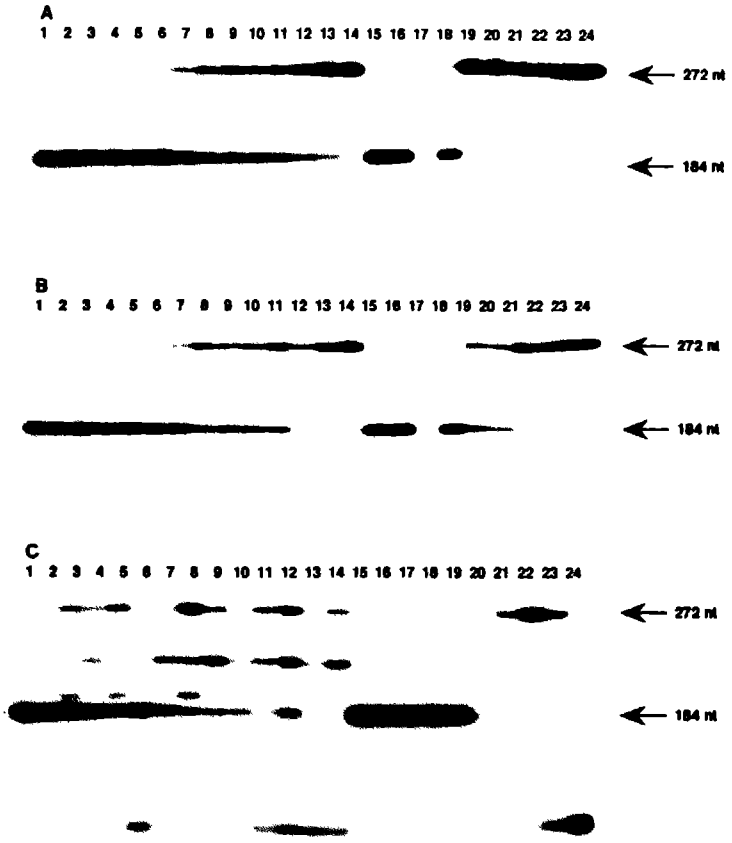

Figure 3. Autoradiograms for three competitive PCRs on several patient 2 samples. 272 nt: Competitor fragment. 184 nt: Patient 2 fragment. **(A)** Initial amount of competitor molecules in all lanes was 1000 molecules. Initial amount of patient 2-derived translocation segments in the serial dilution (lane 1-14) was 10000, 8000, 6000, 5000, 4000, 3000, 2000, 1000, 900, 800, 600, 400, 200 and 100 molecules respectively in addition of 1 µg K562 DNA. Initial amount of separate patient 2 samples was 0.1 µg and 0.01 µg DNA of lymph node of January 1990 (lane 15 & 16), 1 µg DNA of separate blood samples of February 1990, July 1990, August 1991 and October 1991 (lane 17, 18, 20 & 21) or bone

Legend to Figure 3 continued. marrow samples of Apnl 1990 and October 1991 (lane 19 & 22) Lane 24 contained 1 µg K562 DNA. **(B)** Initial amount of competitor molecules in all lanes was 100 molecules Initial amount of patient 2-derived translocation segments in the serial dilution (lane 1-14) was ten fold decreased as described in A in addition of 1 µg K562 DNA Initial amount of separate patient 2 samples (lane 15-22) or K562 DNA (lane 24) was as described in A **(C)** Initial amount of competitor molecules in all lanes was 10 molecules Initial amount of patient 2-derived translocation segments in the serial dilution (lane 1-14) was one hundred fold decreased as described in A, in addition of 1 µg K562 DNA Initial amount of separate patient 2 samples (lane 15-22) or K562 DNA (lane 24) was as described in A

By varying the initial number of patient-derived translocation segments in the coamplification reaction with a fixed number of competitor molecules, a patient specific calibration curve can be constructed from which the number of translocations in a patient sample can be estimated This obviates the need for determining equilibrium in product yield and thereby reducing the number of reactions required Furthermore, no corrections are necessary for any variations in the efficiency of amplification as the result of differences in product length or mutations within the primer recognition sites between competitor and target molecules Tanaka *et al* (1992)[19] detected mutations in the second exon of the *bcl-2* gene in several follicular lymphoma patients It is not known whether mutations also accumulate within the translocation region during the course of follicular lymphoma, but it seems unlikely that sporadic mutations would lead to differences in amplification efficiency between samples of the same patient as long as the primer recognition sites remain untouched Consequently, the assay as described in this report is relatively fast and, once a serial dilution of patient-derived translocation segments is prepared, large numbers of different samples from the same patient can be processed simultaneously

The correlation coefficients for all calibrations curves exceeded 0 93, indicating a high degree of accuracy The current PCR method was employed on DNA isolated from nucleated cells following density centrifugation The number of t(14,18) translocations reflects the number of lymphoma cells, it becomes possible to estimate their number on the assumption that 1 µg DNA is approximately equivalent to 150000 nucleated cells Therefore, it should be possible to monitor the course of the disease in patients receiving an allogeneic or autologous BMT as illustrated by patient 2 The method can also be extended to other malignancies where the PCR technique can detect alterations on DNA or cDNA level, like the t(9,22) in chronic myeloid leukemia (CML)[15], the t(15,17) in acute promyelocytic leukemia (APL)[20], and the tald deletion at chromosome 1[21]

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CHAPTER 3

Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) in a patient before and after allogeneic bone marrow transplantation

By Jules P.P. Meijerink, Gerard J. Goverde, Toon F.C.M. Smetsers, John M.M. Raemaekers, M. José J.T. Bogman,* Theo J.M. de Witte & Ewald J.B.M. Mensink

*From the department of Hematology, and the *department of Pathology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands*

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A competitive PCR method was developed, enabling accurate quantitation of residual lymphoma cells carrying the t(14;18) in blood and bone marrow samples of follicular non-Hodgkin's lymphoma (NHL) patients during treatment. A patient with residual lymphoma cells received an allogeneic bone marrow transplantation (BMT). After BMT, the patient is in continuing clinical complete remission. Using the competitive PCR and two-step PCR method, we were able to demonstrate a gradual decline in the number of lymphoma cells within consecutive patient blood and bone marrow samples after BMT. The competitive PCR is a suitable method for the detection of minimal residual disease. Further research might reveal the clinical relevance of data obtained by this method.

The chromosomal translocation t(14,18), frequently found in follicular non-Hodgkin's lymphoma and to a lesser extend in diffuse B-cell lymphoma, juxtaposes the *bcl-2* gene on chromosome 18 to an immunoglobulin heavy chain joining segment on chromosome 14[1]. Since most breakpoints on chromosome 18 are clustered within two small regions, e g the major breakpoint region (MBR) and the minor cluster region (MCR), the polymerase chain reaction (PCR) seems to be a suitable method for sensitive detection of lymphoma cells[2-4] Recently, we developed a competitive PCR assay, by which we are able to detect and quantify residual lymphoma cells in patient blood and bone marrow samples during the course of treatment[5].

Patients & Methods

Patient In April 1988, a thirty year old male was diagnosed as having a stage IV low-grade follicular centroblastic-centrocytic (CB-CC) non-Hodgkin's lymphoma (NHL) Doxorubicin containing chemotherapy induced partial remission (PR) After cessation of treatment, progressive lymphadenopathy was observed, and a biopsy of a representative lymph node showed similar histology without signs of dedifferentiation in January 1990 Treatment with chlorambucil induced a PR In October 1990, the patient received an allogeneic bone marrow transplantation (BMT) with marrow of a HLA-identical, MLC non-reactive brother, according to the ongoing protocol[6] The patient achieved complete remission (CR), which is persisting ever since, twenty-eight months after BMT.

Sample collection Isolation of mononuclear cells from bone marrow and blood samples was performed using density centrifugation, as previously described[5].

Competitive PCR The competitive PCR method applied on several samples of this patient and subsequent analysis of reaction products was performed as has been previously described[5]

Two-step PCR The two-step PCR was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, USA) on 1 µg of genomic DNA of several patient samples in presence of 0 001% gelatine, 50 mM KCl, 20 mM Tris-HCl (pH=8 4), 2 0 mM $MgCl_2$, 500 µM of dATP, dTTP, dCTP and dGTP, 300 pmol/ml of MBR2 (5'-TCC CTT TGA CCT TGT TTC TTG A-3') and JH-CON (5'-ACC TGA GGA GAC GGT GAC C-3')

oligonucleotides and 2 5 Units of *Taq* polymerase (Gibco BRL) in a total volume of 50 µl The reactions were overlaid with 100 µl mineral oil (Sigma) Amplification started with an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 5 min, 58°C for 2 min and 72°C for 2 min After the last cycle, the extension phase was prolonged at 72°C for 10 min, and the reactions were cooled to 4°C After the first round of amplification, the reaction volume was increased to 100 µl by addition of gelatine, KCl, Tris-HCl (pH=8 4), $MgCl_2$, dNTP's, and oligonucleotides to the same final concentration 2 5 Units of *Taq* polymerase were added, and the reactions were amplified for another 35 cycles

Analysis of PCR products 10 µl of PCR product was applied to a 2% agarose gel in 1x TAE buffer The products were transfered to a Hybond™ N^+ (Amersham) filter, using the DNA alkali blotting procedure according to the manufacturer The filter was hybridised with ^{32}P-end-labeled internal oligonucleotide MBR-INT (5'-TTT CAA CAC AGA CCC ACC CAG AGC C-3') After washing, the filter was exposed to X-ray film (Kodak)

Results

The amount of lymphoma cells carrying t(14,18) in 1 µg DNA of several consecutive bone marrow and blood samples, as previously determined by our competitive PCR assay[5], is summarized in Table 1 A blood sample taken prior to BMT, when the patient was clinically in PR, contained a considerable amount of lymphoma cells After BMT, when the patient was in CR, we were able to demonstrate a decline in the number of lymphoma cells in consecutive blood or bone marrow samples Using our competitive PCR assay, which has a detection limit of one t(14,18) in 1 µg DNA (approximately 150000 nucleated cells), we were unable to detect this chromosomal translocation in a blood sample, taken seventeen months after BMT

For measurement at a lower detection limit, we performed a two step PCR method on DNA of the last four samples of this patient One reaction was positive for the patient specific t(14,18) of 12 separately amplified reactions, when DNA of the blood sample of March 1992 or the bone marrow sample of February 1993 was used as template No t(14,18) was detected in the DNA of the bone marrow sample of October 1992 or the blood sample of February 1993

Discussion

The PCR is a very sensitive method to detect malignant cells carrying t(14,18) in blood and bone marrow samples of follicular lymphoma patients, at all stages of disease, before and after treatment[2 3 7] Since the t(14,18) has also been found in lymphoid tissue of healthy individuals with follicular hyperplasia[4], as well as in patients with advanced stage of disease who are in remission for several years[8], it is difficult to draw conclusions on the clinical relevance of detecting residual lymphoma cells in a single patient sample However, accurate quantitation of residual disease in consecutive samples may reveal prognostic significance[9] We are currently investigating a possible correlation between competitive PCR data of multiple samples and clinical data from a larger set of patients, during their course of treatment

Table 1. Residual disease in a patient with follicular NHL before and after BMT

Date	Diagnosis	Treatment	Tissue	Histology	No. t(14;18)/1μg DNA by competitive PCR	No. t(14;18)/1μg DNA by two-step PCR
apr-88	CB-CC NHL	CHOP	BM[a]	Positive	ND	
May-88/ Jan-90	PR					
Jan-90	Relapse		LN	Positive	Positive	
Feb-90		Chlorambucil	Blood			
Jul-90	PR	Chlorambucil	Blood		1989 ± 44	
Oct-90	PR	BMT	BM[a]	Positive	ND	
Apr-91	CR		BM[b]		75 ± 5	
Aug-91	CR		Blood		54 ± 4	
Oct-91	CR		Blood		27 ± 4	
			BM[b]		11 ± 2	
Mar-92	CR		Blood		0	≤0 083
Oct-92	CR		BM[b]		ND	0
Feb-93	CR		BM[b]		ND	≤0 083
			Blood		ND	0

Abbreviations ND, not determined, BM[a], bone marrow biopty, BM[b], bone marrow aspirate, LN, lymph node, PR, partial remission, CR, complete remission, CHOP, cyclophosphamide, doxorubicin (=adriamycin), vincristine, prednisone

The data of the patient demonstrated in this article, may encourage such a correlation Using our competitive PCR method and two-step PCR assay, we were able to detect a gradually declining number of lymphoma cells after allogeneic BMT, when the patient was clinically in CR This decrease in lymphoma cells might result from a Graft-versus-Lymphoma activity, and may be similar to the Graft-versus-Leukemia effect as has recently been reviewed for patients with acute or chronic leukemias[10] Alternatively, we cannot exclude the possibility that these cells have lost their proliferative capacity as a consequence of treatment, and slowly disappear over time

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CHAPTER 4

Quantitative analysis of DNA aberrations amplified by competitive polymerase chain reaction using capillary electrophoresis

by Aldy W.H.M. Kuypers, Jules P.P. Meijerink, Toon F.C.M. Smetsers, Peter C.M. Linssen & Ewald J.B.M. Mensink

From the department of Hematology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands

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We compared the use of capillary electrophoresis (CE) in a polymer network with the use of slab gel electrophoresis for the quantitative analysis of polymerase chain reaction (PCR) amplified DNA samples. We quantified residual lymphoma cells carrying a translocation between chromosomes 14 and 18, in consecutive patient peripheral blood samples that were amplified by competitive PCR. For CE analysis we used a 4% linear polyacrylamide network. Results show that the calculated number of translocations in patient samples using both analyses were comparable. We conclude that CE is a sensitive, non-radioactive, fast and accurate method for quantitation of competitive PCR products.

Tumour specific markers can be used to monitor residual disease in patients during treatment One of these markers is the reciprocal chromosomal translocation t(14,18), frequently found in follicular lymphomas In this translocation, the *bcl*-2 gene on chromosome 18 is coupled to the immunoglobulin heavy-chain locus on chromosome 14 Random nucleotides are inserted between the two genes providing a patient specific DNA pattern[1] The polymerase chain reaction (PCR) is a sensitive method to detect the presence of lymphoma cells carrying t(14,18) by amplification of the translocation breakpoint[2] Since small variations in reaction conditions can lead to large differences in product yield as a result of exponential amplification, an internal standard is needed for accurate quantitation This internal standard has to be coamplified in the same reaction as the target DNA using the same primers for equal amplification efficiency

For this purpose, a competitive PCR has been developed[3] Serial dilutions of a known number of patient-derived translocation molecules are coamplified with a fixed number of competitor molecules These competitor molecules serve as an internal standard Logarithmic plotting of the ratios of the two PCR products against the initial number of patient specific translocation molecules results in a patient specific calibration curve By comparison of the ratio of PCR products from an unknown number of patient translocation molecules which has been coamplified with the same number of competitor molecules with the calibration curve, the unknown number of translocations in the patient samples can be calculated This way, the residual disease of a patient with a non-Hodgkin's lymphoma (NHL) could be monitored after allogeneic bone marrow transplantation[4] In this procedure, the competitive PCR products were analysed on conventional slab gels using radio-activity for detection

The fast development of capillary electrophoresis (CE) prompted us to investigate whether this time consuming and laborious slab gel procedure could be replaced by automated CE analysis Recently a quantitative analysis using capillary electrophoresis was described by Lu *et al* (1994)[5] However, they analyzed products of a non-competitive PCR, e g without the use of an internal standard We analyzed competitive PCR products of consecutive samples from a patient with lymphoma cells carrying t(14,18) using capillary electrophoresis as well as the traditional slab gel electrophoresis The unknown numbers of translocations in the patient samples were determined and compared using the two methods

Materials & Methods

DNA isolation DNA was isolated[6] from a lymph node sample of patient 1 and from a lymph node sample and consecutive peripheral blood samples of patient 2 All samples contained lymphoma cells carrying the t(14,18)

Oligonucleotides Oligonucleotides were synthesised on a 391A DNA synthesiser (Applied Biosystems, Warrington, UK) The oligonucleotides used for the *bcl-2* region were MBR3-Xhol, 5'-CCC TCG AGG AGC TTT GTT TCA ACC AAG TC-3' (position 2752 for the 7th nucleotide in Gen EMBL acc M14745) and MBR2, 5'-TCC CTT TGA CCT TGT TTC TTG A-3' (position 2826 in GenEMBL acc M14745) Oligonucleotides used for the immunoglobulin heavy-chain region were JH-CON, 5'-ACC TGA GGA GAC GGT GAC C-3' and JH-HindIII, 5'-TCA AGC TTA CCT GAG GAG ACG GTG ACC-3' (identical to JH-CON but contains 8 additional bases at the 5' site)[2].

Preparation of patient-derived molecules Several polymerase chain reactions were amplified in a Perkin-Elmer Cetus thermocycler 480 (Norwalk, CT, USA) Each reaction contained 1 µg lymph node DNA of patient 1 or patient 2, 500 mM dNTP's, 30 pmol of oligonucleotides MBR3-Xhol and JH-HindIII and 2 5 U *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) in a total volume of 100 µl, overlaid with 80 µl mineral oil Amplification started with an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 1 5 min, at 55°C for 2 min and at 72°C for 2 min The PCR products were pooled, separated from oligonucleotides on a 2% agarose gel, excised and electro-eluted by Biotrap (Schleicher & Schuell, Dassel, Germany) After precipitation, the DNA was dissolved in 800 µl water The concentration was determined by optical density at OD260 nm and from this, the number of patient derived translocation molecules was calculated The translocation molecules of patient 1 were used as competitor molecules in the competitive PCR

Competitive Polymerase Chain Reaction The competitive PCR was performed as previously described[3] Serial dilutions of a known number of patient 2 derived translocation molecules were coamplified with a fixed number of patient 1 derived competitor molecules in addition of 1 µg K562 DNA (contains no t(14,18) and serves as a negative control) Amplification was performed with 30 pmol of oligonucleotides MBR2 and JH-CON for 55 cycles with an annealing temperature of 58°C Samples which were analysed using slab gel electrophoresis were amplified in the presence of 1 85 MBq/ml [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK) Other conditions were kept as described above 1 µg of several consecutive peripheral blood DNA samples of patient 2, with an unknown number of translocation molecules, were also coamplified with the same number of competitor molecules

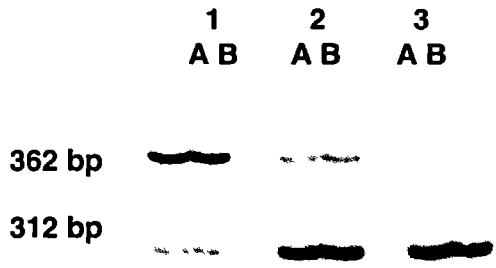
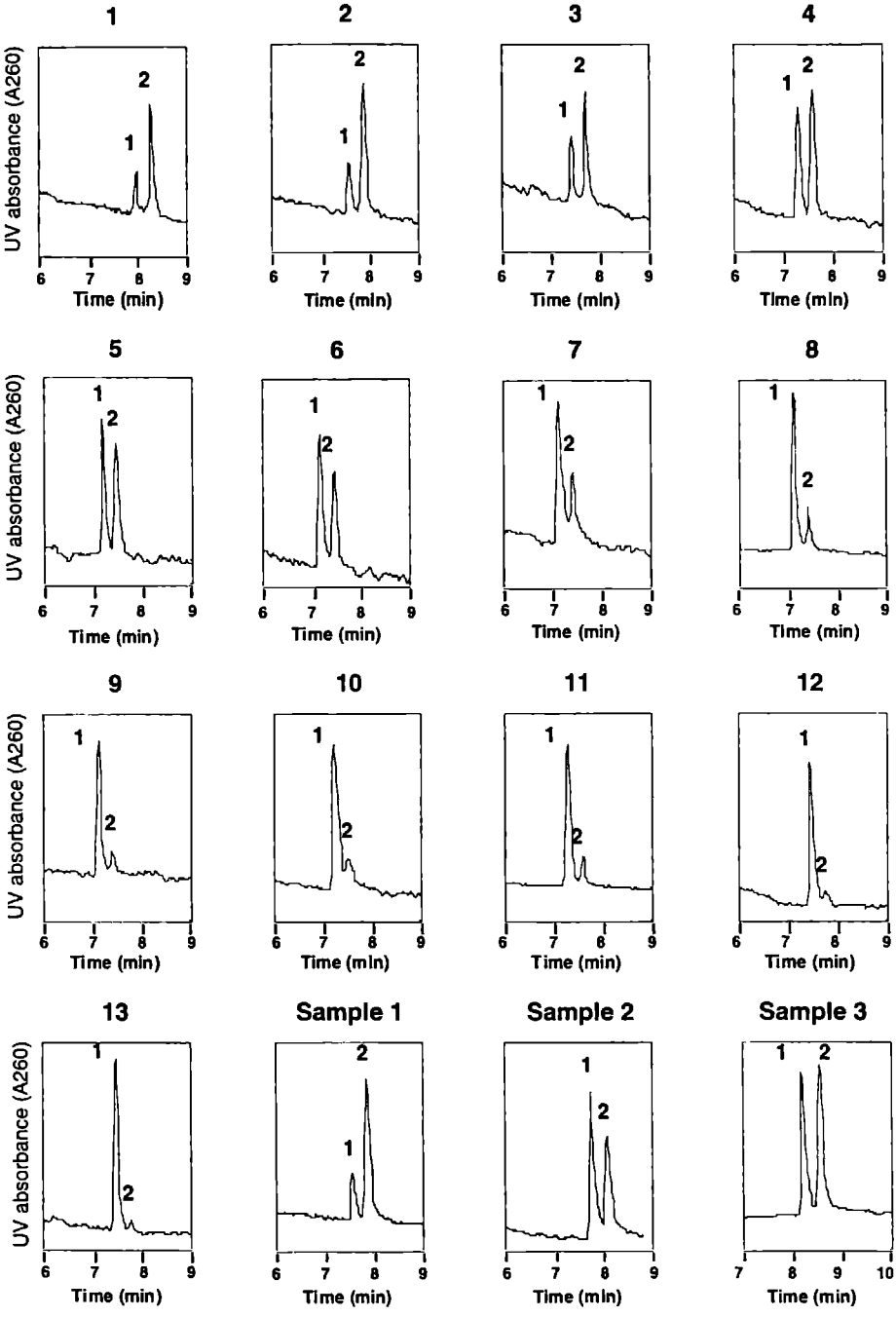


Figure 1 Autoradiogram of 3 different competitive PCR products before and after purification with the Magic PCR Preps DNA purification system Lane 1 1000 competitor molecules and 6000 patient derived translocation molecules before (b) and after (a) purification, lane 2 1000 competitor molecules and 1000 patient derived translocation molecules before (b) and after (a) purification, lane 3 1000 competitor molecules and 600 patient derived translocation molecules before (b) and after (a) purification

Capillary A fused silica capillary (310 mm (L^{eff}) x 0 075 mm I D) (SGE, Ringwood, Victoria, Australia) was precoated with 3-methacryloylpropylmethoxysilane (Sigma, St Louis, MO, USA) Additional coating was performed by purging with a 4% acrylamide (4% T, 0% C) solution containing 8 µl of 10% N,N,N',N'-tetramethylethylenediamine (TEMED) and 4 µl of 10% ammonium persulfate (APS) per ml of acrylamide solution [7] The acrylamide, TEMED and APS were all obtained from Bio Rad Laboratories Richmond, CA, USA The acrylamide solution in the capillary was placed under 15 bar, and allowed to polymerize for 18 hrs The filled capillary was mounted in an assembly cartridge and placed in a Bio-Rad BioFocus 3000 CE instrument (Bio-Rad, Hercules, CA, USA) After each run, the capillary was rinsed with water for 5 min and filled again with the polymerised 4% linear polyacrylamide by purging for 5 min at 8 bar

Analysis and quantitation of PCR products using Capillary Electrophoresis The competitive PCR products were purified with the Magic PCR Preps DNA purification system (Promega, Madison, WI, USA) and dissolved in 50 µl water All samples were injected electrokinetically at reversed polarity (cathode at the injection side) of 280 V/cm for 10 s and separated under constant voltage of 425 V/cm, in a TAE running buffer (40 mM Tris-acetate pH 8 3, 2 mM EDTA) UV absorbance was measured at 260 nm During the runs the carrousel was kept at 15°C and the capillary was kept at 25°C Using the BioFocus 3000 Integrator program (Bio-Rad), the areas under the curves (UAC) were determined Ratios of the AUC of the serial dilution reactions were

Legend to Figure 2 Electropherograms of analysis using capillary electrophoresis 1-13 as described for lane 1-13 in Fig 3, sample 1-3 as described for lane 17-19 in Fig 3 Peak 1 represents the 312 bp PCR product and peak 2 represents the 362 bp PCR product


1
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7
8
9
10
11
12
13
Sample 1
Sample 2
Sample 3
UV absorbance (A260)
Time (min)

plotted logarithmically against the initial number of known patient 2 derived translocation molecules in order to construct a patient specific calibration curve Using this calibration curve, the number of unknown t(14,18) translocation molecules in the patient 2 samples could be determined

Analysis and quantitation of PCR products using slab gel electrophoresis 5 µl of 10-fold diluted PCR product in formamide were loaded on a 7% PAA (acrylamide bisacrylamide=24 1), 7 M urea gel Separation was performed in a half TBE running buffer (45 mM Tris borate pH 8 3, 0 1 mM EDTA) The gel was exposed to an X-ray film (Kodak) and the autoradiogram was analyzed by densitometric scanning on a LKB laser densitometer Band intensities were determined using Gelscan™ XL software and the ratios were plotted logarithmically against the initial number of patient 2 derived translocation molecules

Results

We used a competitive PCR to quantify residual lymphoma cells carrying a t(14,18) Competitive PCR amplification of patient 1 derived translocation molecules resulted in a 362 bp PCR product Amplification of patient 2 derived translocation molecules resulted in a 312 bp PCR product The 362 bp fragments were used as internal standard (competitor molecules)

We analyzed the competitive PCR products by capillary electrophoresis as well as slab gel electrophoresis Because of electrokinetic injection, the samples analyzed by capillary electrophoresis were desalted prior to electrophoresis using the Magic PCR Preps DNA purification system To exclude that the

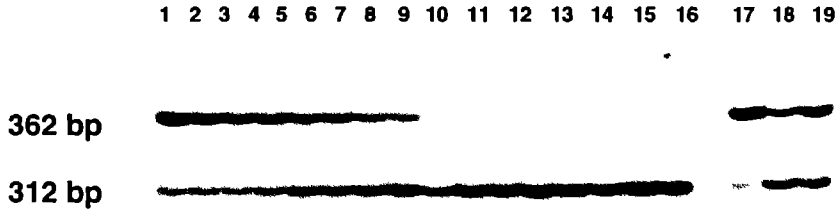

Figure 3. Autoradiogram of analysis using slab gel electrophoresis Lane 1-14 coamplification of serial dilutions of a known number of patient derived translocation molecules (10000, 8000, 6000, 5000, 4000, 3000, 2000, 1000 900, 800, 600, 400, 200 and 100) and 1000 competitor molecules in addition of 1 µg K562 DNA lane 15 control, 1000 competitor molecules, lane 16 control, 1000 competitor molecules and 1 µg K562 DNA, lane 17-19 coamplification of consecutive patient blood samples (resp Feb '91, Aug 91 and Jan 92) with an unknown number of translocation molecules and 1000 competitor molecules

Magic PCR Preps DNA purification system had a predilection for eighter the 312 bp or the 362 bp PCR product, we compared different radioactive samples before and after purification (Fig 1) Results from densitometric scanning of the band intensities showed no predilection for eighter of the PCR products (data not shown)

Figure 2 shows the quantitative analysis using capillary electrophoresis A patient specific calibration curve was constructed by plotting the ratio of UACs against the initial number of patient-derived translocation segments on logarithmic scale (R= 0 993) The corresponding equation is log(y) = 0 983 log(x) - 8 031, where y is the ratio and x is the initial number of known translocation molecules

Figure 3 shows the analysis using slab gel electrophoresis Scanning of the band intensities and plotting of the ratios against the initial number of known patient 2 derived translocation molecules resulted in a patient specific calibration curve with a correlation coefficient R= 0 970 The corresponding equation is log(y) = 1 220 log(x) - 9 895

The results of the calculated number of unknown translocation molecules in the consecutive samples of patient 2 using capillary electrophoresis and slab gel electrophoresis are summarized in Table 1

To check the reproducibility of CE analysis using electrokinetic injection, we divided a sample into three equal portions which were separately injected The standard deviation of the ratio of the AUC was 3 2% (data not shown)

Table 1. Calculation of an unkown number of translocation molecules in consecutive samples of patient 2, using the patient specific calibration curves

Sample	Date	#t(14;18)/µg DNA	#t(14;18)/µg DNA
		Slab gel	Capillary
1	Feb '91	9964±1566	12027±987
2	Aug '91	2854±274	3195±155
3	Jan '92	4084±444	5038±290

Discussion

The PCR is a sensitive method to determine the presence or absence of malignant cells carrying t(14,18) In the competitive PCR used in this study, translocation molecules from a patient were used as competitor molecules Because the length of the amplified t(14,18) breakpoint differs in each patient, the internal standard molecules can be distinguished from the target molecules by size This competitive PCR method can also be used to quantify other DNA or RNA molecules Celi *et al* (1993)[8] have recently described a rapid

and versatile method to synthesize internal standards which can be used as competitor molecules in competitive PCRs.

The products of the competitive PCR are usually analyzed using conventional slab gels. The preparation of these gels is time consuming, laborious and they can be used only once. We therefore used capillary electrophoresis in a polymer network. The preparing of the capillary is easy and it can be used for several consecutive automated analyses[9], since the capillary can be refilled. Another advantage of CE compared to slab gel electrophoresis is the detection. The detection of DNA molecules by UV absorption is direct and the data are stored in an electropherogram, which can be immediately used for evaluation. Detection of DNA molecules using radioactivity is hazardous, the gel has to be exposed which takes additional time and the band intensities have to be scanned after exposure to obtain information about the quantity. Scanning and subsequent computer analysis is time consuming and only a selective part of the band is scanned. This means that any irregularity in the band can lead to different densitometric results. Another disadvantage of radioactivity and autoradiography is the non-linear dose-response relationship, e.g. the band intensities on an autoradiogram can reach a maximum. If the intensity of one of the bands on the autoradiogram reaches a maximum, the ratio of the band intensities is disturbed. Detection using UV absorption doesn't have this problem because there is a linear relationship between the number of molecules and the area under the curve (UAC).

Previously described methods to analyze competitive PCR products are: counting the radioactivity present in bands excised from an agarose gel[10], or a polyacrylamide gel[11] or densitometric scanning of bands of a negative film of an ethidiumbromide stained agarose gel[12]. The first two methods require radioactivity and have the disadvantages of slab gel electrophoresis. Furthermore, excising bands is delicate. The third method is based upon scanning, which has disadvantages as previously discused.

Because samples analyzed using CE have to be run sequentially while samples analyzed using slab gel electrophoresis can bve run all at once, total analysis time might be disadvantage. In our study the analysis time for one sample was 20 min (10 min purging and 10 min running). It took about 6 hr to analyze all samples (n=16). The total electrophoresis time using slab gel electrophoresis is faster (2 hrs), but regarding the additional time needed to prepare appropriate exposures and scanning of band intensities, we conclude that analysis using CE is faster. Furthermore, the analysis using CE is fully automated.

Results show that the number of translocation molecules as determined by both strategies are within each others range. The callibration curves show that the analysis using capillary electrophoresis is comparable to the analysis using slab gel electrophoresis (R=0.993 vs. R=0.970). The standard deviation of the AUC ratios (3.2%), obtained by injecting a sample 3 times, provides sufficient accuracy for quantification of DNA or RNA molecules.

We conclude that CE is a non-radioactive, fast, accurate and sensitive method for the quantitation of competitive PCR products and since CE is automated it opens possibilities for genetic routine analysis.

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CHAPTER 5

New type of t(14;18) in a non-Hodgkin's lymphoma provides insight in molecular events in early B-cell differentiation

by Jules P.P. Meijerink, John M.M. Raemaekers and Ewald J.B.M. Mensink

From the department of Hematology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands

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In this report, a follicular non-Hodgkin's lymphoma (NHL) carrying an unusual t(14;18) is described. This translocation occurred most likely during the V_H to D-J_H rearrangement process of the IgH locus. From the data combined with data from literature, we conclude that the chance for the development of a t(14;18) decreases during progression of the immunoglobulin rearrangement process in the pre-B-cell ontogeny. This is probably due to decreased accessibility of the *bcl-2* locus by reduced transcription. We analysed the somatic mutation pattern of the productively rearranged IgH gene. Like other follicular lymphomas, somatic mutations were present in this gene and argued in favour of positive selection, probably for an antigen. We found no evidence for ongoing induction of somatic mutations during lymphoma development. We conclude that *bcl-2* gene deregulation but not the precise moment at which this occurs during the pre-B-cell stage is of influence on the development of follicular NHL.

Molecular studies at the 14Q+ chromosomal junctions from t(14;18) (q32,q21) carrying cell lines and follicular non-Hodgkin's lymphoma (NHL) samples revealed the rearrangement of the B-cell lymphoma/Leukaemia 2 gene (*bcl-2* gene) from chromosome 18 to the immunoglobulin heavy chain (IgH) joining (J_H) region on chromosome 14[1] The breakpoints on chromosome 18 cluster in mainly two cluster regions, i e the major breakpoint region (MBR) and the minor cluster region (MCR)[2-5]. The expression of *bcl-2* gene is elevated as a consequence of translocations involving this gene[4,6,7].

The t(14,18) most likely occurs during early IgH gene rearrangements at the pre-B cell stage and a J_H gene segment and a diversity (D_H) gene segment are frequently involved The presence of random nucleotides at the translocation junctions further supports this hypothesis[8-10] Translocations as a result of ontogenetically later IgH gene rearrangements are rare and are uptill now only confined to the D_H to D_H-J_H rearrangement stage[9,11,12]. The t(2;18) and the t(18,22), which most likely occur during IgL rearrangements, have only occasionally been described in follicular lymphoma[6,7,13].

Studies at the normal IgH and IgL alleles of lymphoma cells have provided important insight in the development of the malignant phenotype. Although an expanded oligoclonal $\mu^+\kappa^-$ pre-B-cell population in the bone marrow of a follicular lymphoma patient has been found[14], the lymphoma cells are monoclonal regarding their IgH and IgL chain rearrangements[15]. Within these genes, somatic mutations have been found[16-19]. The somatic mutation pattern provides evidence for affinity selection for antigen prior and during clonal expansion[20,21]. Although the *bcl-2* gene is deregulated in all lymphoma cells, this seems not to be sufficient for all cells to survive the selection process.

The development of a monoclonal follicular NHL population out of a polyclonal pre-neoplastic pre-B-cell population indicates that additional mutations besides antigen selection are necessary for the development of the malignant phenotype This is further supported by the finding of t(14,18)s in individuals with benign follicular hyperplasia[22], and the continuing accumulation of genetic defects in lymphoma subpopulations, eventually leading to a more dedifferentiated status[23,24]. Also in the transgenic mice model

with a deregulated *bcl-2* gene, only eleven percent of mice develop a monoclonal high-grade diffuse large-cell immunoblastic lymphoma after a relatively long latency period of twelve to twenty-four months[25]

In this report, we describe a follicular NHL carrying an unusual t(14,18) To investigate the moment of translocation in the pre-B-cell ontogeny, we performed a detailed molecular study involving the translocation junctions of the $14Q^+$ and the $18Q^-$ chromosomes To investigate the consequences of the deregulation of the *bcl-2* gene at a relative late pre-B-cell stage on the subsequent development of follicular lymphoma, mutation analyses of the normal IgH allele were performed The data presented here combined with data available in literature provide insight in molecular events occuring in normal early B-cell development as well as in the development of follicular lymphoma

Materials & Methods

Description of Patient A 43-year old male was diagnosed as having a follicular centroblastic-centrocytic (CB-CC) NHL of low-grade malignancy Immunological examination of a cervical lymph node showed the presence of 30-40% follicular IgM^+/K^+ lymphoma cells. This lymph node sample and a concurrent blood sample were used in the present study as the only material available

DNA isolation DNA was isolated from blood or lymph node samples according to a standard procedure[26]

Oligonucleotides and Polymerase Chain Reaction (PCR) Sense oligonucleotides used MBR_2[27], set of V_H family-specific oligonucleotides V_H1 to V_H6[28], V_H3-48x· 5'-TCC CCA CCC TAG AGC TTG CT-3', NM13 5 5'-CAG CTA TGA CCA TGA TTA CGC CAA G-3' Antisense oligonucleotides used $J_H$con[27], J_H3-intron 5'-GGC AGA AGG AAA GCC CAT CTT-3', J_H4-intron 5'-CCA AAA GTC ACA AAC CTC GAG T-3', J_H5-intron 5'-CTT TCT TTC CTG ACC TCC AAA A-3', J_H6-intron[27], MBR_{18Q} 5'-TGA TTT TGG CAG GAT AGC AGC ACA-3' PCR conditions were as described before[27] One µg of genomic DNA was used as template, unless indicated otherwise

End-labeling of oligonucleotides Oligonucleotides (50 pmol) were phosphorylated in the presence of 50 mM Tris-HCl (pH=7 5), 10 mM $MgCl_2$, 5 mM dithiotreitol (DTT), 0 1 mM spermidine, 92 5 MBq/ml [γ-^{32}P] ATP (Amersham, 110 TBq/mmol, 370 MBq/ml) and 20 U of T_4 polynucleotide kinase (Gibco BRL), in a total volume of 20 µl at 37°C for 45 min. After incubation, 25 µl 4M NH_4Ac, 5 µg yeast t-RNA and 250 µl of EtOH were added to the reaction sample, and the oligonucleotide was precipitated The precipitation procedure was repeated twice After precipitation, the oligonucleotide was dissolved in 10 mM Tris pH=8 0, 3 0 mM $MgCl_2$

Amplification of IgH VDJ gene segments Rearranged IgH VDJ gene segments were amplified using V_H family-specific primers in combination with the $J_H$con or J_H-intron primers at an annealing temperature of

55°C Genomic DNA (0 2 µg) from the blood or lymph node sample was used as template An aliquot of the PCR product, amplified with the V_H3 and J_H6-intron primers, was used for direct sequencing, and the remainder was purified using the Magic™ PCR Preps DNA purification system (Promega Corporation, Madison, USA) The concentration was determined by optical density Five pmol of PCR product was phosphorylated as described above in the presence of 2 0 mM ATP The DNA was precipitated following a phenol extraction and a Sevag (phenol chloroform, isoamylalcohol = 25 24 1) extraction, and dissolved The PCR product was subsequently treated by Klenow enzyme (Gibco BRL) to remove possible 3' non-template directed nucleotide additions, in the precence of 100 µM dNTPs for 60 min at 14°C[29] The DNA was precipitated following a phenol extraction and a Sevag extraction, and dissolved in dH_2O

Digestion and dephosphorylation of vector DNA A sample of 10 µg of pBluescribe KS+ (Stratagene, La Jolla, CA, USA) was digested with Sma-I (Gibco BRL) for 2 5 Hr at 30°C The DNA was dephosphorylated using Calf Intestine Alkaline Phosphatase (CIP) (Amersham, Buckinghamshire, UK) according to a standard protocol[30] The DNA was dissolved in dH_2O to a final concentration of 20 ng/µl

Ligation, transformation and screening for recombinant clones A sample of 100 ng of Sma-I digested and CIP-treated vector DNA and 1 µg of phosphorylated and klenow-treated PCR DNA was ligated using 800 U of T_4 DNA ligase (Biolabs, Beverly, MA, USA) in a total volume of 20 µl at 14°C for 16 Hr After ligation, the reaction volume was increased to 50 µl with dH_2O, and 25 µl was used for the transformation of 100 µl of competent *Escherichia coli* strain DH5α bacteries (Gibco BRL) Transformation and Blue/White screening were performed according to standard protocols[30] White colonies were used to inoculate 5 ml of Luria-Bertani medium containing 50 µg/ml of ampicillin, and incubated at 37°C for 16 Hr DNA was isolated from all bacterial cultures using the small-scale plasmid DNA isolation procedure[30]

Sequencing of clones Insert DNA from each recombinant clone was a-symmetrically amplified using the V_H3 and NM13 5 oligonucleotides A-symmetric DNA templates were sequenced using end-labeled V_H3, $J_H$con or J_H6-intron oligonucleotides The a-symmetric amplification reaction and sequencing reaction were performed as described[31] with minor modifications

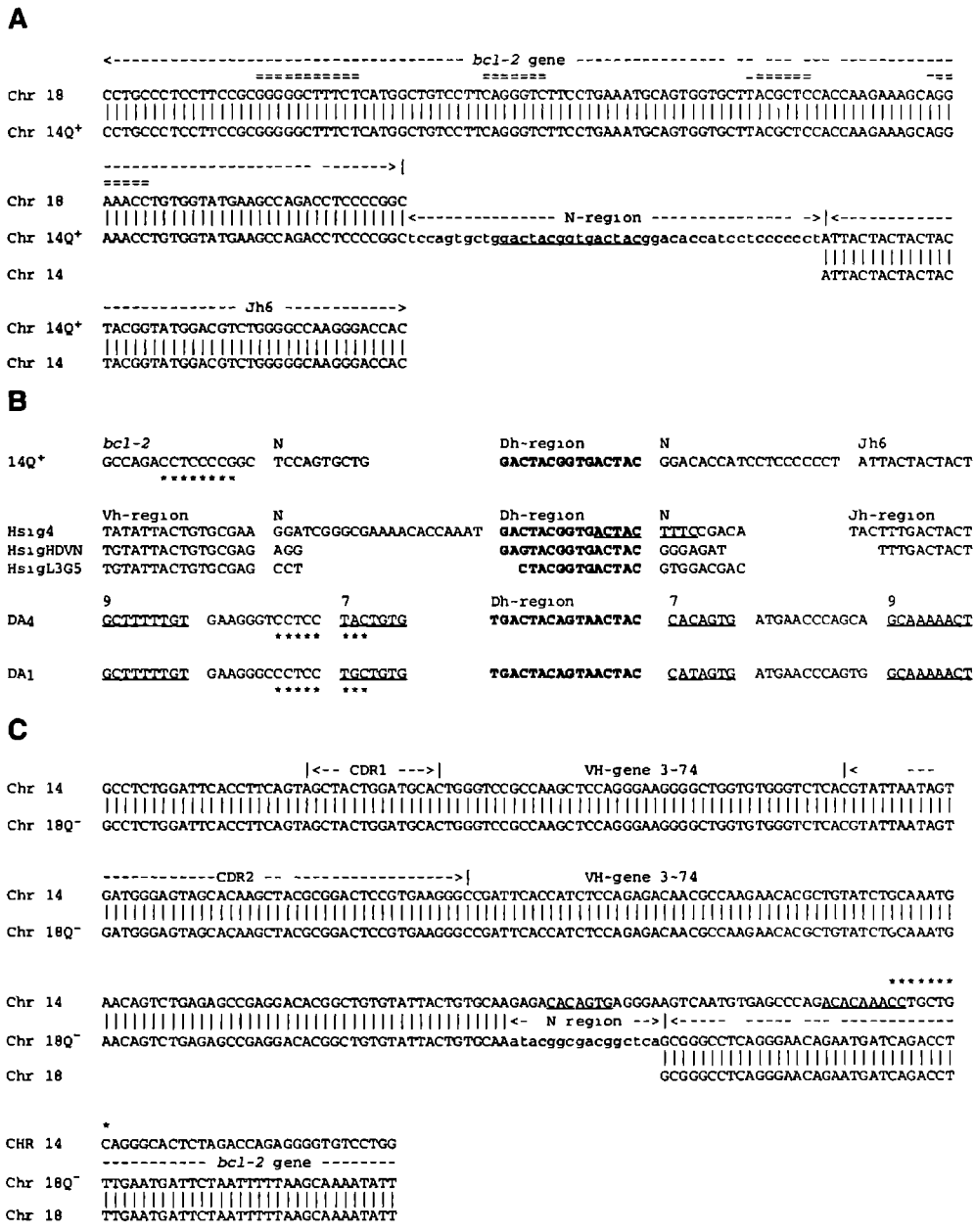
Somatic Mutation Analysis Somatic mutations in the framework regions (FWRs) and the hypervariable regions of the rearranged IgH VDJ gene segments in the lymphoma cells were analysed in comparison to germline candidates in two separate mutation analyses[32] The hypervariable regions correlates with the complementary determining regions (CDRs), i e the contact sites of the antibody with the antigen, and are indicated according to Kabat *et al* (1987)[33] The expected number of replacement (R) mutations (mutations which give rise to another amino acid residue) and silent (S) mutations (mutations which do not give rise to another amino acid residue) by a random mutation mechanism in a region which is not under selective

pressure is dependent on the composition of the codons[34] For the first mutation analysis, the expected numbers of replacement and silent mutations in the rearranged VDJ region (bp 381 to 683, Fig 2) in germline configuration was calculated by summing all possible replacement substitutions for each codon and deviding by the total number of potential substitutions (replacement and silent) for all codons This resulted in 0 78*n* and 0 22*n* respectively for *n* random mutations The second mutation analysis was performed on the V_H gene segment only (bp 381 to 602) For this region, the expected numbers of replacement and silent mutations are 0 79*n* and 0 21*n* respectively for *n* random mutations The distribution of replacement and silent mutations over the CDRs or FWRs in absence of selection is dependent of the relative size of CDRs and FWRs, assuming that all nucleotides have an equal probability to mutate For the first mutation analysis, the CDRs and FWRs corresponds to 38% and 62% of the total sequence respectively, and therefore R_{CDRs} = 0 38R, R_{FWRs} = 0 62R, S_{CDRs} = 0 38S, S_{FWRs} = 0 62S For the second mutation analysis the R_{CDRs} = 0 30R, R_{FWRs} = 0 70R, S_{CDRs} = 0 30S and S_{FWRs} = 0 70S The *p* of *k*R mutations in the CDRs for *n* mutations was calculated using a binomial mutation model $p = [N!/k!(N-k)!]q^k(1-q)^{N-k}$, whereby *q* is the chance for a replacement mutation in the CDRs and $N = n_{(observed)} + R_{FWRs}$[35] For the first mutation analysis q = 0 78 x 0 38 = 0 30 and for the second mutation analysis q = 0 79 x 0 30 = 0 24

Results

The 14Q+ chromosomal junction of the t(14,18) was detected in the blood sample DNA in a routine investigation using PCR With the MBR_2 and $J_H$con oligonucleotides, a 449 bp PCR fragment was generated and sequencing confirmed the origin of this fragment The fragment was partially homologous to the *bcl-2* gene on chromosome 18 as well as the IgH J_H6 gene segment on chromosome 14 (Figure 1A) Between both regions of homology, a region of 44 nucleotides was inserted Comparison of this region to sequences of human rearranged IgH VDJ genes in the GenEMBL Database Library revealed a 15 bp region which is also present in the CDR3 region of Hsig4[36] Fourteen out of 15 nucleotides are present in the CDR3 region of Hsighdvn, and 13 out of 15 nucleotides are present in the CDR3 region of HsigL3G5 (Figure 1B) We conclude that this region represents an IgH D_H gene segment, which is most homologous to the germline IgH D_{A4} or D_{A1} segment[37]

Since it is suggested that the t(14,18) occurs as an error during the V(D)J gene rearrangement process in the IgH genes, we tried to amplify the reciprocal 18Q- chromosomal junction making use of V_H family-specific oligonucleotides in combination with the MBR_{18Q} oligonucleotide (located downstream of the MBR of the *bcl-2* gene) Using the V_H3 oligonucleotide in combination with MBR_{18Q}, a PCR fragment was generated Sequencing of this PCR fragment confirmed the juxtaposition of a V_H3 family

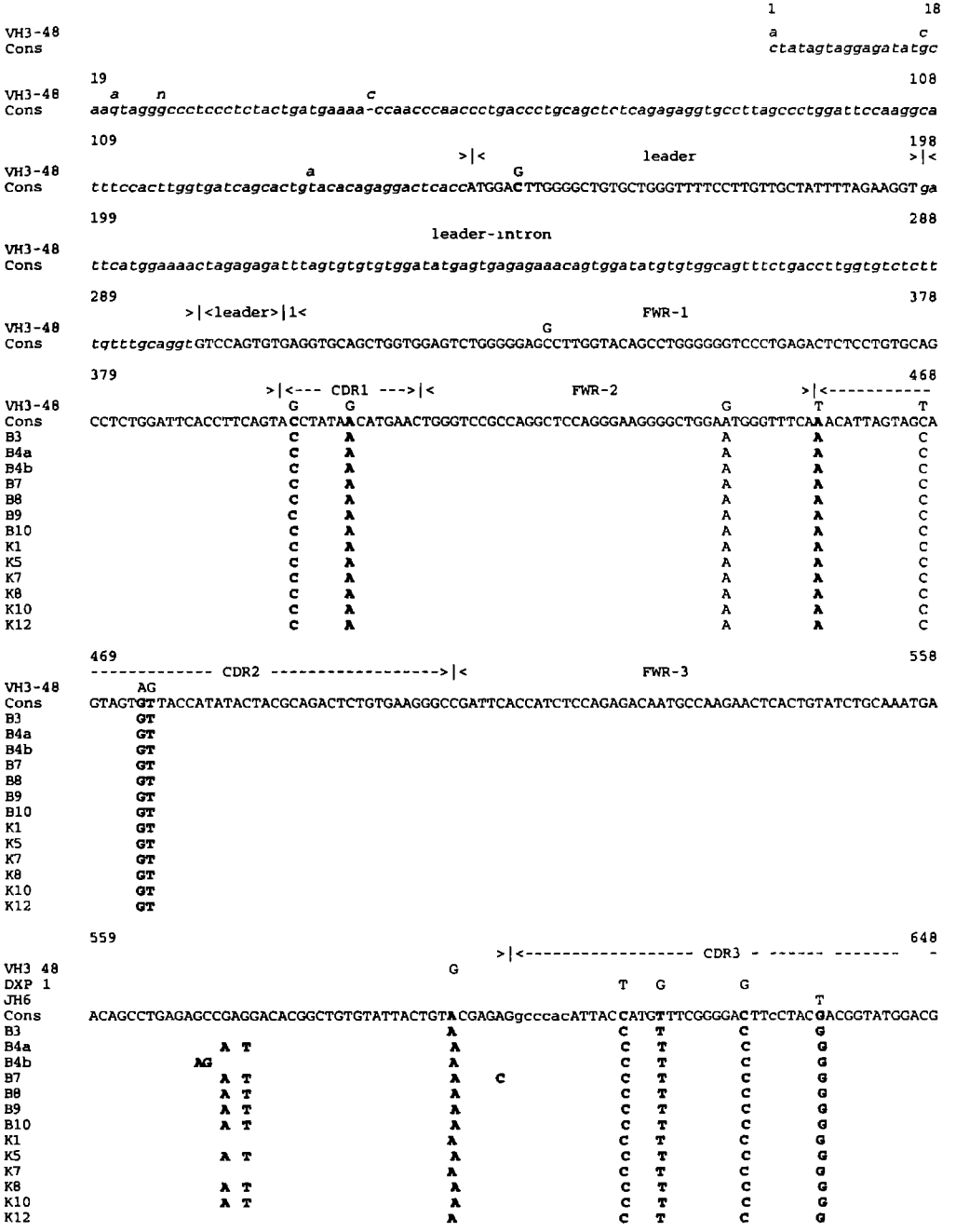

A
bcl-2 gene
Chr 18
Chr 14Q+
N-region
Jh6
Chr 14
B
bcl-2
N
Dh-region
Jh6
14Q+
GCCAGACCTCCCCGGC
TCCAGTGCTG
GACTACGGTGACTAC
GGACACCATCCTCCCCCCT
ATTACTACTACT
Vh-region
Jh-region
Hs1g4
Hs1gHDVN
Hs1gL3G5
TATATTACTGTGCGAA
GGATCGGGCGAAAACACCAAAT
TGTATTACTGTGCGAG
AGG
CCT
GACTACGGTGACTAC
TTTCCGACA
TACTTTGACTACT
GAGTACGGTGACTAC
GGGAGAT
TTTGACTACT
CTACGGTGACTAC
GTGGACGAC
DA4
GCTTTTTGT
GAAGGGTCCTCC
TACTGTG
TGACTACAGTAACTAC
CACAGTG
ATGAACCCAGCA
GCAAAAACT
DA1
GCTTTTTGT
GAAGGGCCCTCC
TGCTGTG
TGACTACAGTAACTAC
CATAGTG
ATGAACCCAGTG
GCAAAAACT
C
CDR1
VH-gene 3-74
Chr 14
Chr 18Q-
CDR2
N region
Chr 18
CHR 14
bcl-2 gene

member to the 3' untranslated region of the *bcl-2* gene (Figure 1C) with insertion of random nucleotides The consensus sequence segment was completely homologous to the V_H germline gene 3-74 [DA-8[38] and DP53[39]]

A search for heptamer-nonamer-like Ig recombination signals on both sides of the breakpoint on chromosome 18 did not result in the identification of other signal-like sequences as described[10] (Figure 1) Regions homologous to the chi-consensus sequence (CC[T/A]CC[T/A]GC) are present in all germline gene segments involved in this translocation A region directly upstream of the breakpoint on chromosome 18 had a 5/8 match, a region directly upstream of the germline D_{A4} or D_{A1} gene segments had a 7/8 and 8/8 match respectively, a region downstream of the signal sequences of the germline V_H3 74 or DP53 gene segment had a 7/8 match (Figures 1B-C) and a region within the first FWR of this gene was completely homologous to the c consensus sequence[39]

The malignant lymphoma cells were further investigated by analysis of somatic mutations present in the productively rearranged IgH allele DNA derived from the lymph node sample, which was shown by immunochemistry to contain 30 to 40% of IgM+/K+ follicular lymphoma cells, and DNA from the blood sample were used as template for the amplification of rearranged IgH VDJ gene segments of the lymphoma cells Using all V_H family-specific oligonucleotides[28] in combination with J_H-intron specific oligonucleotides under stringent conditions, only the V_H3 oligonucleotide in combination with the J_H6-intron oligonucleotide generated a 388 bp PCR product for both tissue samples (data not shown) After sequencing, the V_H3 gene segment of this rearrangement was compared with sequences from the GenEMBL Database Library, and was most homologous to germline gene segment V_H3-48[40] The PCR fragment was cloned, and individual clones were used for mutation analyses

Legend to Figure 1 (A). Comparison of the 14Q+ chromosomal junction to the MBR of the *bcl 2* gene[2] and the J_H region of the IgH locus[41] The N-region is shown in small characters Underlined region shares homology with human D_H regions Heptamer-nonamer signal and signal-like sequences as suggested by Tsujimoto *et al* (1985b)[10] are indicated by double lines **(B)** Comparison of the N-region from the 14Q+ chromosomal junction to germline D_H segments D_{A4} and D_{A1} [37], and somatic D_H segments The somatic D_H segments were deduced from VDJ sequences Hsig4[36], Hsigl3g5 (GenEMBL accession L04332), and Hsighdvn (GenEMBL accession M65101) Gaps in sequences were introduced for clarity The underlined region in the Hsig4 sequence represent the D_{LR5} gene segment, as indicated by the authors The heptamer and nonamer sequences in the germline D_{A4} and D_{A1} gene segments are underlined Regions sharing homology to the c consensus sequence are marked by asterisks **(C)** Comparison of the 18Q- chromosomal junction to germline sequences of the IgH V_H3-74 gene segment[38] and the MBR of the *bcl-2* gene[2] The N-region is shown in lowercase The CDR1 and CDR2 regions are indicated according to Kabat *et al* (1987)[33] The signal heptamer and nonamer sequences are underlined Regions sharing homology to the χ consensus sequence are marked by asterisks


1 18
VH3-48 a c
Cons ctatagtaggagatatgc
19 108
VH3-48 a n c
Cons aagtagggccctccctctactgatgaaaa-ccaacccaaccctgaccctgcagctctcagagaggtgccttagccctggattccaaggca
109 198
>|< leader >|<
VH3-48 a G
Cons tttccacttggtgatcagcactgtacacagaggactcaccATGGACTTGGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTga
199 288
leader-intron
VH3-48
Cons ttcatggaaaactagagagatttagtgtgtgtggatatgagtgagagaaacagtggatatgtgtggcagtttctgaccttggtgtctctt
289 378
>|<leader>|1< FWR-1
VH3-48 G
Cons tgtttgcaggtGTCCAGTGTGAGGTGCAGCTGGTGGAGTCTGGGGGAGCCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAG
379 468
>|<--- CDR1 --->|< FWR-2 >|<----------
VH3-48 G G G T T
Cons CCTCTGGATTCACCTTCAGTACCTATAACATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAATGGGTTTCAAACATTAGTAGCA
B3 C A A A C
B4a C A A A C
B4b C A A A C
B7 C A A A C
B8 C A A A C
B9 C A A A C
B10 C A A A C
K1 C A A A C
K5 C A A A C
K7 C A A A C
K8 C A A A C
K10 C A A A C
K12 C A A A C
469 558
---------- CDR2 ------------------>|< FWR-3
VH3-48 AG
Cons GTAGTGTTACCATATACTACGCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACTCACTGTATCTGCAAATGA
B3 GT
B4a GT
B4b GT
B7 GT
B8 GT
B9 GT
B10 GT
K1 GT
K5 GT
K7 GT
K8 GT
K10 GT
K12 GT
559 648
>|<---------- CDR3 - ------ ------- -
VH3 48 G
DXP 1 T G G
JH6 T
Cons ACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTACGAGAGgcccacATTACCATGTTTCGGGGACTTcCTACGACGGTATGGACG
B3 A C T C G
B4a A T A C T C G
B4b AG A C T C G
B7 A T A C C T C G
B8 A T A C T C G
B9 A T A C T C G
B10 A T A C T C G
K1 A C T C G
K5 A T A C T C G
K7 A C T C G
K8 A T A C T C G
K10 A T A C T C G
K12 A C T C G

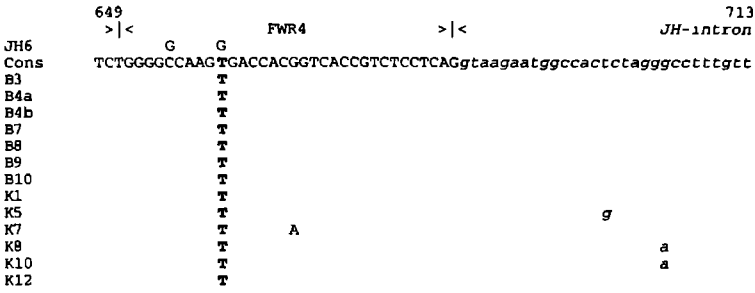


Figure 2. Comparison of the consensus sequence and individual clones to germline counterparts Only the complete consensus sequence is shown Identical nucleotides are given by dots Silent mutations compared to the germline counterparts V_H3-48[40] $D_{XP'1}$[37], J_H6[41] or the consensus sequence are shown in capitals, and replacement mutations are shown in bold capitals The N-regions are shown in lowercase The J_H6-intron region is shown by small characters in italic The C residue at position 656 represents allelic variation

For more accurate identification of the germline counterpart of the V_H3 gene segment present in the VDJ rearrangement, we tried to amplify this specific rearrangement with an oligonucleotide located upstream of the leader peptide (V_H3-48x) in combination with the J_H6 intron oligonucleotide The specific VDJ rearrangement was amplified using these oligonucleotides and sequenced (see consensus sequence in Figure 2) The V_H3 region of this VDJ rearrangement (bp 1-603), including upstream sequence and the leader intron, was 97 2% homologous to the mapped germline V_H3 48 gene segment and 92% homologous to the next most homologous mapped germline gene V_H3-7[40] The leader intron (bp 197-299) was completely homologous to germline V_H3-48 gene segment The D_H part of the VDJ rearrangement (bp 610-630) was 85% homologous to germline $D_{XP'1}$ gene segment[37] For subsequent analyses, we assumed that the productively rearranged VDJ gene segments in the lymphoma cells were composed of germline counterparts V_H3-48[40], $D_{XP'1}$[37] and J_H6[41]

The consensus VDJ sequence (bp 1-713, Figure 2) contained several mutations compared to their germline counterparts Five mutations and a deletion were present in the region upstream of the leader peptide The leader peptide itself contained one replacement mutation, whereas the leader intron was completely conserved Twelve replacement mutations and two silent mutations compared to the germline genes were present in the CDRs and FWRs

For mutation analysis, the 388 bp V_H3-48-$D_{XP'1}$-J_H6 PCR product was cloned, and individual clones originating from blood (B3, B4a, B4b, B7, B8, B9 and B10, Fig 2) or nodule sample DNA (K1, K5, K7, K8, K10 and K12, Fig 2) were sequenced The clones B3, B4b, K1, K7 and K12 shared eleven replacement mutations at position 400, 406, 456, 474, 475, 597, 615, 619, 628, 636, 661 and two silent

mutations at position 446 and 467 by comparison to the germline genes A C residue at position 656 was also present in the J_H6-region of the 14Q+ chromosome (Figure 1A), and may therefore reflect an allelic polymorphism rather than a mutation These clones represent lymphoma subpopulation 1 Clones B4a, B7, B8, B9, B10, K5, K8 and K10 all had two additional replacement mutations at position 573 and 575, and represent subpopulation 2 Besides these mutations shared by all members of the same subgroup, individual mutations and mutations shared by only two members of the same subgroup were present Clones K8 and K10 both had a common mutation in the J_H6-intron region at position 705 Clone B4b had two additional replacement mutations at position 570 and 571, clone B7 had a replacement mutation at position 602, clone K7 had a silent mutation at position 668 and clone K5 had a mutation within the J_H6-intron region at position 699

Lymphoma subpopulation 1 and 2 shared thirteen and fifteen mutations respectively compared to their germline counterparts These mutations reflect somatic mutations acquired during clonal evolution of both lymphoma populations These mutations were used in two mutation analyses[32 35] Since the germline counterpart of the D_H gene segment was most likely DXP'1, and since not all germline D genes have been identified[37], we devided the analysis in two parts In one part, we analysed the mutations present in the entire VDJ gene (Table 1A, region bp 381-683) In the other part, we analysed the mutations in the V_H gene segment only (Table 1B, region bp 381-602) The distribution of replacement and silent mutations as found in the CDRs and FWRs are summarized in Table 1A & 1B for both regions The expected distribution of replacement and silent mutations by a random mutation process was calculated for both subpopulations for both regions (see materials & methods) The distribution of mutations as found in the VDJ gene or the V_H gene segment differ from the expected pattern of mutations The $p_{(kR)}$ was calculated for both subpopulations in both regions analysed For the VDJ rearrangement the $p_{(9R)}$ in subpopulation 1 was 0 012 (k = 9, n = 13, N = 15), the $p_{(9R)}$ in subpopulation 2 was 0 05 (k = 9, n = 15, N = 19), For the V_H region the $p_{(5R)}$ in subpopulation 1 was 0 03 (k = 5, n = 8, N = 9), the $p_{(5R)}$ in subpopulation 2 was 0 11 (k = 5, n = 10, N = 13)

Legend to Table 1 (A) For n mutations, the expected number of replacement and silent mutations in the VDJ rearrangement of the lymphoma cells is $R_{exp} = 0\,78 \times n$; $S_{exp} = 0\,22 \times n$ The expected distribution of replacement and silent mutations within the FWRs and CDRs of the VDJ rearrangement is $R_{FWRs} = 0\,62 \times R$ $R_{CDRs} = 0\,38 \times R$, $S_{FWRs} = 0\,62 \times S$, $S_{CDRs} = 0\,38 \times S$ **(B)** For the V_H segment, the expected numbers of replacement and silent mutations are $R_{exp} = 0\,79 \times n$ $S_{exp} = 0\,21 \times n$ The expected distribution of replacement and silent mutations within the FWRs and CDRs of the V_H region are $R_{FWRs} = 0\,70 \times R$, $R_{CDRs} = 0\,30 \times R$, $S_{FWRs} = 0\,70 \times S$ $S_{CDRs} = 0\,30 \times S$ (See Materials and Methods)

Table 1A. Distribution of shared mutations in the rearranged VDJ gene segments of the lymphoma cells

	FWR1	FWR2	FWR3	FWR4	Total FWRs	Expected FWRs
Subpopulation 1						
Replacement	0	0	1	1	2	6
Silent	0	1	0	0	1	2
R/S	-	0	∞	∞	2	3
Subpopulation 2						
Replacement	0	0	3	1	4	7
Silent	0	1	0	0	1	2
R/S	-	0	∞	∞	4	3.5

	CDR1	CDR2	CDR3	Total CDRs	Expected CDRs
Subpopulation 1					
Replacement	2	3	4	9	4
Silent	0	1	0	1	1
R/S	∞	3	∞	9	4
Subpopulation 2					
Replacement	2	3	4	9	5
Silent	0	1	0	1	1
R/S	∞	3	∞	9	5

Table 1B. Distribution of shared mutations in the V_H region of the VDJ rearrangement of the lymphoma cells

	FWR1 (partial)	FWR2	FWR3	Total FWRs	Expected FWRs
Subpopulation 1					
Replacement	0	0	1	1	5
Silent	0	1	0	1	1
R/S	-	0	∞	1	5
Subpopulation 2					
Replacement	0	0	3	3	6
Silent	0	1	0	1	1
R/S	-	0	∞	3	6

	CDR1	CDR2	Total CDRs	Expected CDRs
Subpopulation 1				
Replacement	2	3	5	2
Silent	0	1	1	0
R/S	∞	3	5	∞
Subpopulation 2				
Replacement	2	3	5	2
Silent	0	1	1	1
R/S	∞	3	5	2

Discussion

In this study, we describe a variant t(14,18) which was detected in a follicular NHL patient It is the first demonstration of a t(14,18) as the result of an error during the V_H to D-J_H rearrangement process in a pre-B-cell Allmost all t(14,18)s occur during the IgH D to J_H rearrangement process and seldomly during other Ig rearrangement processes The reason for this phenomenon is not understood The Ig recombination machinery for IgH and subsequent IgL rearrangements remains active during the complete pre-B-cell stage and therefore both loci are highly accessible This is different for the *bcl-2* locus The *bcl-2* expression is downregulated on mRNA level[42] as well as protein level[43] upon entry of pro-B-cells at the pre-B-cell stage Therefore it seems likely that the reduced transcriptional rate of the *bcl-2* locus is accompagnied by a reduced accessibility, and therefore leads to a decreased incidence of t(14,18)s during later Ig rearrangement processes

Due to the temporary open chromatin structure of the *bcl 2* gene, the Ig recombination machinery may aberantly recognize target sequences on the *bcl-2* locus The translocation in our case can not simply be explained by the presence of heptamer and nonamer signal sequences as targets of the VDJ recombination complex, but more likely by chi-like (χ) sequences[12] The χ consensus sequences may act as primary recombination targets for distant Ig gene segments in an intermediate recombination process, which is further processed by the VDJ recombinase complex using the classical Ig recombination signals These χ-like sequences are especially conserved in the FWR1 of group III V_H families in mammals, to which the human V_H3 family belongs[44], in D_H and J_H gene segments, and have also been found in the MBR of the *bcl-2* gene[12] Recently, proteins have been identified which bind to χ-like sequences present in the MBR of the *bcl-2* gene[45 46] These sequences may have been involved in the generation of the t(14,18) in our case, and are present in all germline counterparts

To study the effect of the relatively late occurence of the t(14,18) in this case on lymphoma development, a mutation analysis was performed The V_H gene segment of the lymphoma specific VDJ rearrangement was most homologous (97 2%) to germline V_H3-48 gene segment, and only 92% homologous to the next most homologous V_H segment V_H3-7[40] Since the map of the human Ig V_H locus is now complete and almost all the sequences are known[38], it is reasonable to assume that V_H3-48 represents the germline counterpart in this case Based on homology, we assume that the germline counterpart of the D_H gene segment is Dxp'1[37] From amplification conditions as well as sequence information it is obvious that the germline J_H gene segment is J_H6[41]

From both mutation analyses, it is obvious that the amount of replacement mutations present in the CDRs of both lymphoma subpopulations exceeds the number of mutations expected in absence of selection As discussed by Schlomchik *et al* (1987b)[32], an R/S ratio higher than 2 9 indicates positive selection In both analyses, the R/S ratios for the CDRs of both subpopulations strongly argued in favour of positive selection An R/S ratio lower than 2 9 indicates negative selection, as is expected for the FWRs in order to maintain the function of the antibody From both mutation analyses it is obvious that a negative

selection pressure was acting on the FWRs of subpopulation 1 Although subpopulation 2 evolved from subpopulation 1, it is not clear whether a negative selective force was still maintained on the FWRs of subpopulation 2 Using the binomial model[35], it seems highly unlikely that the mutation pattern present in both lymphoma populations for both regions analyzed had occurred by chance

Analysis of the individual mutations provides insight in the most recent selection forces which have been acting on the lymphoma cells However, the insert of each clone is amplified by *Taq* DNA polymerase, and may contain unique mutations generated by this enzyme Although the amount of unique mutations exceeds the number expected to be induced by *Taq* polymerase[47], it is less than expected if the somatic mutation process was ongoing This frequency is estimated as 1 mutation in 1000 bps in each cell cycle[48], resulting in one mutation in the VDJ-region of almost every lymphoma cell Since we used 0 2 µg of genomic DNA in our PCR (which roughly equals $3x10^4$ cells) and since the amount of lymphoma cells within the lymph node biopt was 30% to 40%, it seems highly unlikely that five out of seven clones originating from the blood sample DNA and two out of six clones originating from the lymph node sample DNA did not contain unique somatic mutations if this process was still ongoing Furthermore, the position of these mutations does not argue in favour of positive selection We conclude that the somatic mutation process in the lymphoma cells was turned off, active at a very low level or active in a minor lymphoma population at time of presentation, which is in contrast with some other cases of follicular lymphoma[15 17 21]

In conclusion, translocations involving the *bcl-2* gene which occur at other stages of the Ig rearrangements than the IgH D_H to J_H gene rearrangement process are extremely rare in follicular lymphoma We propose that this correlates with decreased *bcl-2* transcription by pro-B cells upon entry in the pre-B-cell stage and a concomitant decreased accessibility of potential target sites within the *bcl-2* locus recognised by χ or the Ig recombination machinery Like other cases, the somatic mutation machinery had been active in this follicular lymphoma case We therefore conclude that *bcl 2* gene deregulation and not the precise moment of deregulation during the pre-B-cell stage is of influence on the subsequent development towards follicular lymphoma

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CHAPTER 6

Bax mutations in cell lines derived from hematological malignancies

by Jules P.P. Meijerink, Toon F.C.M. Smetsers, Annet W. Slöetjes, Ellen H.P. Linders, Ewald J.B.M. Mensink.

From the department of Hematology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands

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Many genes are involved in cell cycle control, DNA repair and induction of cell death. Alterations in these genes have been responsible for the development of cancer as well as for resistance to cancer therapy. Recently, an emerging family of *bcl-2* like genes has been identified that plays a role in the regulation of cell death. Its members are highly conserved in several domains which have been shown to be important for homodimerization or heterodimerization. The ratio between BAX/BCL2 heterodimers and BAX/BAX homodimers appears to be pivotal in the decision between life or death of a cell. We recently detected mutations in evolutionary highly conserved domains of the *bax* gene in cell lines derived from hematologic malignancies. Similar artificially generated mutations in other *bcl-2* like family members *bcl-2*, *bcl-xl*, or *ced-9* have been shown to alter their function. This suggests a role for *bax* mutations in the multistep pathogenesis of hematological malignancies.

Cell proliferation and cell death are two important processes involved in embryonal development, tissue homeostasis and regeneration One of the genes involved in the regulation of cell death is the human *bcl-2* gene, which can protect against cell death induced by a variety of death signals[1 2] Recently, an emerging family of related proteins has been identified[3]. The protein products of *bcl-2*[1], *bcl-xl*[4], *mcl-1*[5] can protect against apoptosis whereas the protein products *bax*[6], *bcl-xs*[4], *bad*[7] and *bak*[8 10] make cells more sensitive for apoptosis induction. The regulation of the cell death pathway by the *bcl-2* like family members is very conserved throughout evolution The human *bcl-2* gene can partially substitute for *ced-9* in nematode *Caenorhabditis elegans* in preventing developmental cell death in *ced-9* loss-of-function mutants[11].

The different members of the *bcl-2* like family can form homodimers or heterodimers with other members of the same family, possibly in a head-to-tail configuration[12] Three conserved domains, box 1 to 3 as defined by Hengartner and Horvitz (1994) [11], seem to be important for dimerization Mutation analysis of the two C-terminal homology domains, called *bcl-2* homology domain 1 (BH1, which equals box 2) and BH2 (which equals box 3), demonstrates that several amino acid residues are crucial for dimerization[13] For *bcl-2* and *bax* it has been demonstrated that their protein ratio determines the cells susceptibility for cell death following a death signal BAX/BAX homodimers renders a cell more vulnerable for apoptosis and BCL2/BAX heterodimers provides protection[6 13] The model is more complex· First, the *bax* gene encodes for several protein products of which BAXα and BAXβ seem to be the most important ones. BAXα is a membrane-bound protein whereas BAXβ is a cytoplasmic protein[6]. Second, other family members are also involved and complex interactions among these proteins may dictate complex set points unique to each cell[14]. Third, most family members are also expressed in a tissue specific manner, like *bcl-x* which seems to be predominantly expressed in neurons and thymocytes[4] Fourth, expression patterns of specific family members can be modulated by external signals[15], and therefore affect the specific set point within target cells

Upon genotoxic stress, an intracellular defense program is activated to facilitate repair prior to distribution of mutations over daughter cells. This damage response pathway is regulated by the *p53*-gene[16 17] Upon sustaining damage by chemotherapeutic agents or γ-radiation, the *p53* expression is upregulated by a post-transcriptional mechanism and immediately blocks the cell cycle at the G1 to S phase

transition[16] and facilitates repair. For this, the *p53* gene upregulates the expression of the *waf1/cip1* gene[18] and the *gadd45* gene[19] If DNA damage is too severe, P53 can induce cell death[20]

Induction of apoptosis in a *p53* dependent pathway is regulated by members of the *bcl-2* like family P53 induced apoptosis is preceded by a decrease of *bcl-2* gene expression and an increase of *bax* gene expression[21,22]. The P53 protein can directly modulate *bcl-2* and *bax* expression by a cis-acting *p53* negative response element located in the 5' untranslated region of the *bcl-2* gene[23] and a *p53* binding-site located in the promotor region of the *bax* gene[24].

Inactivation of the *p53*-dependent cell death pathway can contribute to resistance to therapy as well as to oncogenesis itself In nearly all human neoplasms, mutations have been found in the *p53* gene with concomitant loss of the normal allele[25 26]. Germ-line mutations in the *p53* tumor suppressor gene as found in families with Li-Fraumeni syndrome have been associated with increased risk for cancer development[27]. Mutations in the *p53* gene result in loss of the tumor suppressor activity and enhancement of oncogenic activity by dimerization of mutant P53 with wild-type P53 to form an inactive dimer or tetramer[28]. This lowers the DNA binding capacity of the P53 complex to target genes and subsequent transcriptional activation[29] In vivo experiments as well as correlative studies have shown that mutations in the *p53* gene also contribute to resistance to treatment[17,30,31].

Deregulation of downstream genes in the *p53* dependent cell death pathway can also contribute to oncogenesis and increased resistance to treatment In many cases of human follicular lymphoma, the *bcl-2* gene is overexpressed due to a translocational event As an effect of *bcl-2* overexpression, cells become less sensitive for induction of apoptosis and therefore have a longer lifespan with a simultaneous increased risk for the accumulation of secondary mutations[1] In mice, overexpression of *bcl-2* results in the expansion of a polyclonal B-cell compartment, and occasionally dedifferentiates into a high-grade monoclonal disease in which the *c-myc* gene is frequently rearranged[32] Overexpression of *bcl-2* has also been correlated with poor prognosis to therapy in patients with non-Hodgkin's lymphoma, acute myeloid leukemia and prostate cancer[2] Since BCL2 and BAX have opposite effects on induction of apoptosis following a death signal, it seems reasonable that mutations which disturb BAX function exert a similar effect on cells as BCL2 overexpression. This way, aberrations in the *bax* gene could be involved in the genesis of cancer. It also seems likely that these mutations contribute to enhanced resistance to chemotherapy.

In this report we describe the presence of *bax* mutations in cell lines derived from hematological malignancies. The frequency of the mutations is relatively high in the small number of cell lines tested. The potential role of these mutations in the pathogenesis of malignancies will be discussed

Materials & Methods

Cell lines Cell lines of different hematopoietic background were used in the present study and include three human T-ALL cell lines: CEM, Jurkat and HPB-ALL, a human Burkitt lymphoma cell line Daudi, and two human B-cell lines JM and KM3.

RNA-isolation RNA was isolated according to a method developed by Chirgwin *et al* (1979)[33]

Oligonucleotides Sense oligonucleotides used *bax*-exon 3 5'-GTC CAC CAA GAA GCT GAG CG-3, *bax*-exon 4 5-GCC CTT TTC TAC TTT GCC AGC-3' Antisense oligonucleotides used *bax* exon 5 5'-TCC AGC CCA ACA GCC GCT C 3', *bax*-intron 5 2 5'-GAC ACG TAA GGA AAA CGC ATT AT-3, *bax*-exon 6 5'-GCA CTC CCG CCA CAA AGA TG-3', *bax*-exon 6 2 5'-TCA GCC CAT CTT CTT CCA GAT-3' The C-terminal coding region of *baxα* or *baxβ* cDNA was amplified using *bax*-exon 3 primer in combination with *bax*-exon 6 2 primer or *bax*-intron 5 2 primer respectively

RT-PCR amplification 1 µg of total RNA was reverse transcripted in the presence of 40 mM Tris-HCl (pH = 8 3), 75 mM KCl, 3mM $MgCl_2$, 10 mM DTT, 625 mM of dATP, dTTP, dCTP and dGTP, 5 mg/ml oligo dT, 1000 U/ml RNAse inhibitor RNasin (Promega, Madison, USA), 10 000 U/ml Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Gibco BRL, Gaithersburg, USA) in a total volume of 20 µl Reactions were overlaid with 60 µl mineral oil (Sigma, USA), and were subsequently incubated at 20°C for 10 min, at 42°C for 45 min and at 95°C for 10 min Samples were amplified by polymerase chain reaction (PCR) in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, USA), in the presence of 0 001% gelatine, 65 mM KCl, 28 mM Tris-HCl (pH = 8 3), 2 6 mM $MgCl_2$, 500 mM of dATP, dTTP, dCTP and dGTP, 300 pmol/ml of oligonucleotides, and 25 U/ml *Taq* DNA polymerase (Gibco BRL) in a total volume of 100 µl Amplification started with an initial denaturation step at 94°C for 5 min, followed by thirty cycles at 94°C for 1 5 min, 59°C for 2 min and 72°C for 2 min After the last cycle, extension phase was prolonged for 10 min at 72°C, and the reactions were cooled to 4°C

Sequencing PCR products were sequenced according to the method developed by Innis *et al* (1988)[34], with minor modifications *Baxα* PCR product was sequenced using oligonucleotides *bax* exon 4, *bax*-exon 5 or *bax*-exon 6 *Baxβ* PCR product was sequenced using oligonucleotides *bax*-exon 4, *bax* exon 5 or *bax*-intron 5

Antibodies BCL2 antibody 124 (Dako, Glostrup, Denmark) was a mouse IgGI, kappa BAX antiserum P-19 (Santa Cruz Biotechnology, Santa Cruz, California, USA) is a polyclonal rabbit antiserum raised to a peptide corresponding to amino acids 43-61 of the mouse *bax* gene, but also reacts with human *bax* Horseradish peroxidase conjugated goat anti mouse IgGI (Southern Biotechnology Association Inc, Birmingham, USA) and peroxidase conjugated goat anti rabbit (Jackson Immuno Research, West Grove, USA) were used as secondary antibodies

SDS PAGE and immunoblotting procedure Cells were washed once with phosphate buffered saline (PBS) and resuspended at a concentration of 2 x 10^7 cells/ml in PBS Cells were boiled after addition of 2 x SDS sample buffer One µl of these extracts was applied to a 12 5 % polyacrylamide gel using the fast system

(Pharmacia, Upsulla, Sweden). After electrophoresis the samples were blotted by diffusion for 1 hr at 60°C onto a 0 45 µm nitro-cellulose membrane (Schleicher & Schuell, Dassel, Germany) in blotting buffer (25 mM Tris, 192 mM Glycine (pH 8 3), 20 % methanol) Blocking buffer for immunological detection was prepared by heating 0 5 % NIP 552 blocking reagent (Amersham, Buckinghamshire, England) for 1 hr at 65°C Blocking buffer was cleared by centrifugation and tween-20 was added to a final concentration of 0 05% The membrane was blocked for 1 hr at room temperature (RT) in blocking buffer The first antiserum was added to a final concentration of 2 µg/ml The membrane was washed three times in PBS containing 0 05 % tween 20 and incubated for 1 hr at RT in 3000 fold diluted secondary antibody in blocking buffer After 5 washes with PBS containing 0 05% tween-20, blots were developed using the ECL detection system (Amersham) and used for exposure on an x-ray film

Results & Discussion

mRNA of six hematopoietic cell lines were screened for mutations in the *bax* gene by an RT-PCR strategy We focussed on the C-terminal coding region of the *bax* gene in which the BH1 and BH2 domains are localized, and which are important for heterodimerization of BAX with BCL2 or BCL-X_L[7 13] The region containing the N-terminal homology domain Box 1[11] was not screened, although this region may also be important for dimerization of *bcl-2* like family members[12] We found three mutations in two out of six cell lines (33%) Since we didn't check the N-terminal coding region of the *bax* gene, the frequency may be underestimated

Cell line HPB-ALL, which was derived from a patient suffering T-cell acute lymphoblastic leukemia (T-ALL), expressed *bax* mRNA in which a G to A mutation (not shown) was present at position 199 (relative to the ATG start codon) as well as the normal allele This mutation leads to the substitution of a glycine residue by an arginine residue (G67R) Alignment of this region to other *bcl-2* like family members (Figure 1A) demonstrates that this glycine residue is very conserved throughout evolution This domain is located between the conserved Box 1 and Box 2 regions[11], and the functional significance of this region has not been determined A second C to T mutation was present in intron 5 at position 508 (not shown) in the same cell line The unmutated allele was also expressed on mRNA level Since this intron is alternatively spliced, this mutation only affects the BAXβ variant[6] By this mutation an arginine residue is substituted by a cystein residue It is not known if expression of BAXβ like BAXα renders a cell more vulnerable for induction of cell death, and therefore it is difficult to draw any conclusion regarding this mutation It is not known if both mutations occur in the same allele

The Burkitt lymphoma cell line Daudi, expressed *bax* mRNA containing a G to T mutation in BH1 on position 323, but also expressed the normal allele (Figure 2) By this mutation, a glycine residue is substituted by a valine residue This G108V mutation is located in BH1[13] (also defined as Box 2[11]) at a position which is completely conserved in all known *bcl-2* like family members (Figure 1B) Mutation analysis for the *bcl-2* gene[13] and the *bcl-xl* gene[7] clearly demonstrates that substitution of this particular glycine residue by glutamate or alanine respectively results in complete loss of heterodimerization capacity with

A

CED9	116	MRVMGTIFEKKHAENFE	132	(C elegans)
CED9	116	MRSLGTIFEKRHAEMFE	132	(C briggsae)
BCL2	91	LRQAGDEFSRRYQRDFA	107	(Chicken)
BCL2	94	LRRAGDDFSRRYRRDFA	110	(Mouse)
BCL2	97	LRQAGDDFSRRYRGDFA	113	(Human)
BAX	63	LKRIGDELDSN--MELQ	77	(Human)
BCL-XL/s	90	LREAGDEFELRYRRAFS	106	(Human)
BCLX	86	LRDAGDEFELRYRRAFS	102	(Chicken)
MCL1	213	LRRVGDGVQRNHETVFQ	229	(Human)
BAK	78	LAIIGDDINRRYDSEFQ	94	(Human)

Δ

B

LMW5-HL	76	ELFKDLI-NWGRICGFIV--FSA	95	(African Swine virus)
BHRF1	88	EIFHRGDPSLGRALAWMA--WCM	108	(Epstein Barr virus)
CED9	159	AQTDQCPMSYGRLIGLIS--FGG	179	(C elegans)
CED9	159	SSNTPCPMSYGRLIGLIS--FGG	179	(C briggsae)
BCL2	130	ELFRDGV-NWGRIVAFFE--FGG	149	(Chicken)
BCL2	130	ELFRDGV-NWVRIVAFFE--FGG	149	(Chicken*)
BCL2	133	ELFRDGV-NWGRIVAFFE--FGG	152	(Mouse)
BCL2	136	ELFRDGV-NWGRIVAFFE--FGG	155	(Human)
BAX	98	DMFSDGNFNWGRVVALFY--FAS	118	(Human)
BCL-XL	130	ELFRDGV-NWGRIVAFFS--FGG	149	(Human)
BCLX	125	ELFHDGV-NWGRIVAFFS--FGG	144	(Chicken)
MCL1	233	HVFSDGVTNWGRIVTLIS--FGA	253	(Human)
BAK	117	SLFESGI-NWGRVVALLG--FGY	136	(Human)
BAD	138	PPNLWAAQRYGRELRRMSDEFEG	160	(Mouse)
A1	77	KEFEDGIINWGRIVTIFA--FGG	97	(Mouse)

Δ

Figure 1. Alignment of BAX domains which were mutated in cell lines HPB-ALL and Daudi with other *bcl-2* like family members **(A).** Alignment of *C elegans* or *C briggsae* CED9[11], chicken BCL2[39], mouse BCL2[40], human BCL2[41], human BAX[6], human BCL-XS and BCL-XL[4], chicken BCL-X[4], MCL1[42] and BAK[9] in a domain which contains the BAXG67R mutation of cell line HPB-ALL (indicated by a triangle) The most conserved amino acid residues amoung the genes are indicated in bold lettertype **(B).** Alignment of the BH1 domain for *bcl-2* like genes as used in Figure 1A and extended with chicken BCL2 [35], mouse BAD[7], mouse A1[43], Epstein Barr virus BHRF1 and African swine virus LMW5-HL[3] The BAXG108V mutation of cell line Daudi is indicated by a triangle The most conserved amino acids are indicated in bold lettertype

BAX and concomitant loss of cell death repressor function. In another mutation analysis, the nematode *C. elegans* paradoxically demonstrates a gain-of-function of CED9 upon substitution of the same glycine residue by glutamate. Although BCL2 normally can substitute for CED9 in *C elegans*[11], this mutation renders BCL2 completely ineffective for rescue of CED9 loss-of-function mutations. Interestingly, the *bcl-2* gene isolated from a chicken B-cell lymphoma cDNA bank coded for a valine instead of a glycine residue a tthe same position[35], and it was speculated that this mutation contributed to lymphoma genesis[36]

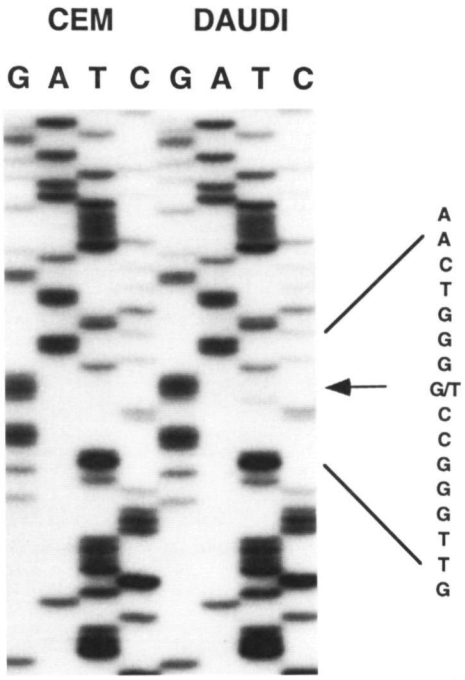

Figure 2. Sequence of the BH1 region of the *bax* gene from cell lines CEM and Daudi. The G to T mutation present in one of the *bax* alleles in the Daudi cell line results in the BAXG108V mutation.

Mutating this glycine residue has not only different effects in different proteins of the *bcl-2* like family members, but it's effect is also dependent on the nature of the amino acid substitution.

What are the consequences of mutations within the *bax* gene? We hypothesize that the mutation in the *bax* gene as found in the cell line Daudi is accompanied with a loss-of-function of BAX, since gain-of-function would lead to a dramatic increase of cell death. The protein data also argue in favour of this suggestion (Figure 3). Normal bone marrow cells as well as the cell lines CEM, Jurkat, JM, HPB-ALL and KM3 all express BAX as well as BCL2, although there are differences in the relative BAX/BCL2 ratio as measured by our assay. The situation is different in cell line Daudi, since we didn't detect any BCL2 expression whereas BAX expression seems to be abundant. Therefore, BAX homodimers probably will be abundant as well, and this cell line would be extremely sensitive for apoptosis induction by normal BAX function which is not the case. It would be thrilling to know in what way these mutations affect BAX function. Do they make BAX oncogenic by more effective heterodimerization with BCL2 or BCL-X_L or by less

effective homodimerization? Do they act in a dominant negative way on the normal allele by BAX(mutant)/BAX(wildtype) homodimerization and concomitant loss-of-function? Functional studies will be necessary to provide an answer.

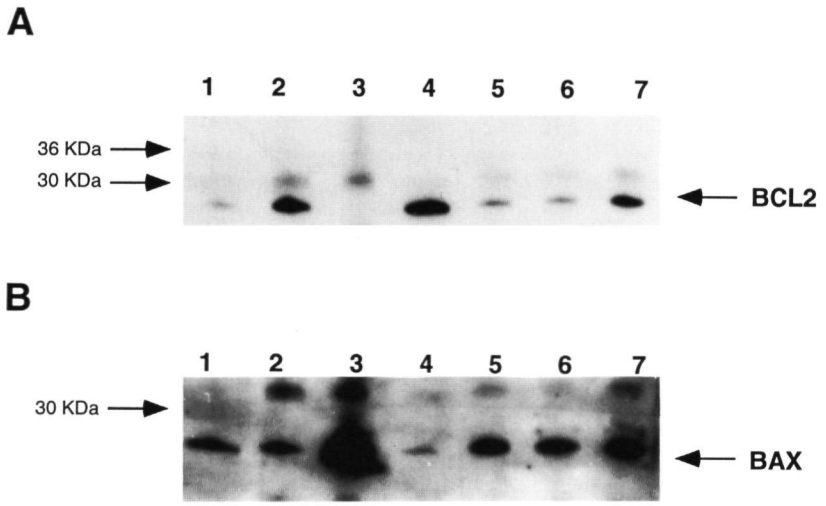

Figure 3. BCL2 and BAX detection by an immunoblot of cell lysates from 1: normal bone marrow cells; 2: HPB-ALL ; 3: Daudi; 4: KM3; 5:CEM; 6: Jurkat; 7: JM. The immunoblot was consecutively screened for **(A).** BCL2 with moab 124 (Dako) or **(B).** BAX with polyclonal P-19 antiserum (Santa Cruz Biotechnology).

The Daudi cell line is derived from a Burkitt lymphoma with a rearranged and overexpressed *c-myc* gene. Overexpresssion of *c-myc* induces apoptosis in fibroblasts under conditions which normally promote G1-arrest, and it was hypothesized that secondary defects in tumorigenesis affect the cell death pathway[37]. It has been demonstrated that ectopic expression of *bcl-2* inhibits apoptosis induced by deregulated *c-myc* expression[38]. Since BCL2 and BAX have opposite effects on the cells susceptibility to undergo cell death, it is clear that mutations which disrupt BAX function could contribute to Burkitt lymphoma genesis in an identical manner. This may have been the case in the patient from which the Daudi cell line is derived.

Overexpression of BCL2 as a first genetic defect seems to be sufficiently tumorigenic in a mice model[32]. Most human follicular lymphoma cases carry the t(14;18) in which the *bcl-2* gene is deregulated. Therefore, it is important to investigate whether mutations in the *bcl-2* like family member *bax*

also contribute to cancer development Mutations in the *p53* gene and defects in genes which are part of the *p53*-dependent apoptotic machinery like *bcl-2* provide increased resistance to chemotherapeutic agents as well as γ-radiation[2,17,26,30,31] It therefore seems reasonable that mutations negatively affecting BAX function also provide increased resistance to treatment.

In conclusion, we found mutations in the *bax* gene in about thirty percent of the human hematological cell lines tested Mutations in the conserved regions of the *bax* gene are probably associated with a loss-of-function of BAX, and may contribute as an oncogenic event to the pathogenesis of cancer as well as to increased resistance to treatment Thusfar these mutations have been found in hematological cell lines To demonstrate the frequency and importance of these mutations, it is necessary to screen a large panel of tumor samples derived from hematological malignancies as well as other types of cancer

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CHAPTER 7

Hematopoietic Malignancies Demonstrate Loss-of-Function Mutations of BAX

By Jules P.P. Meijerink, Ewald J.B.M. Mensink, Kuhn Wang, Thomas W. Sedlak, Annet W. Slöetjes, Theo J.M. de Witte & Stanley J. Korsmeyer

From the department of Hematology, University Hospital "St. Radboud" Nijmegen, Nijmegen, the Netherlands; and the division of Molecular Oncology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO.

The *Bcl-2* gene family regulates the suspectability to apoptotic cell death in many cell types during embryonic development and normal tissue homeostasis. Deregulated expression of anti-apoptotic BCL2 can be a primary aberration that promotes malignancy and also confers resistance to chemotherapeutic agents. Recently, studies of *bax*-deficient mice have indicated that the pro-apoptotic BAX molecule can function as a tumor suppressor. Consequently, we examined human hematopoietic malignancies and found that approximately 21% of lines possessed mutations in *bax*, perhaps most commonly in the Acute Lymphoblastic Leukemia subset. Approximately half were nucleotide insertions or deletions within a deoxyguanosine (G8) tract resulting in a proximal frame shift and loss of immunodetectable BAX protein. Other *bax* mutants bore single amino acid substitutions within BH1 and BH3 domains, demonstrated altered patterns of protein dimerization, and had lost death promoting capacity. Thus, mutations in the pro-apoptotic molecule *bax* which confer resistance to apoptosis are also found in malignancies.

The *Bcl-2* gene family regulates cellular responsiveness to a wide variety of death inducing stimuli, including growth factor deprivation, glucocorticoids, anti-receptor antibody, γ- and UV irradiation and chemotherapeutic agents[1,2]. Death antagonists including BCL2, BCL-X_L, MCL1 and A1 provide protection whereas death agonist members including BAX, BAK, BAD and BCL-X_S increase sensitivity to death inducing signals The ratio of death agonists to antagonists determines the suspectability to death stimuli[3] A prominent feature of the BCL2 gene family is their capability to form both homo- and heterodimers[3 5] Several conserved domains entitled BH1, BH2 and BH3 participate in the formation of various dimer pairs as well as the regulation of cell death[6-8] (Figure 1A). Recently, the multidimensional NMR and X-ray crystallographic structure of a BCL-X_L monomer indicated that BH1-3 correspond to α helixes which are closely juxaposed to form a hydrophobic pocket[9]. Mutational analysis has indicated the importance of BH1 and BH2 domains for the anti-apoptotic function in BCL2 and BCL-X_L as well as their binding to BAX.[5,6] The BH3 domain of BAK and BAX appear critical for promoting cell death and dimerization to BCL-X_L or BCL2[7,10]. Moreover, several distantly related pro-apoptotic molecules, BIK and BID, possess only a BH3 domain[11-13]. These lend strenght to the argument that BH3 represents the critical death domain Besides BH1, BH2 and BH3 domains, a fourth N-terminal α helical domain entitled BH4 is conserved between BCL2 and BCL-X_L, and is vital for death repressor function[14,15].

Deregulated expression of some *bcl-2* family members has been noted in several types of human malignancies and may affect clinical outcome. *Bcl-2* was discovered on chromosome 18 at the site of translocation with the immunoglobulin heavy chain locus (IgH) in follicular lymphoma. Increased expression of BCL2 in cases of non-Hodgkin's lymphoma, myeloid leukemia and prostate cancer has been associated with a poor prognosis[1,2,16]. Reduced *bax* RNA and protein expression has been noted in metastatic breast cancer[17,18], and correlates with a poor response to chemotherapy and shorter overall survival[18]. Elevated

bcl-2/bax RNA ratios have been associated with progression of disease in B-Chronic Lymphocytic Leukemia[19] and low-grade urinary bladder cancer [20]

Bax-deficient mice indicate that several normal developmental cell deaths depend on *Bax*[21] Moreover, neuronal cell death due to deprivation of neurotrophic factors proved to be dependent on *bax*[22] Induction of BAX expression can be sufficient to induce apoptosis and did not require an additional death stimulus[23] Induction of BAX results in the activation of Caspases,[23 24], and also triggers a mitochondrial dysfunction program [23 25] Finally, experimental models utilizing *Bax*-deficient mice argue that approximately half of certain p53-dependent cell deaths require BAX The removal of BAX substantially decreases apoptosis induced by a transgene expressing a truncated T antigen (TGT121), a previously documented p53-dependent death[26] Moreover, chemotherapeutic agents induce apoptosis in embryonic fibroblasts in a *p53* dependent manner Elimination of BAX prevents approximately half of those chemotherapy-induced deaths [27]

Recently, we noted that several cell lines of human hematopoietic malignancies bore mutations of the *bax* gene[28] Here, we extend this study to a larger panel of malignant hematopoietic lines Frameshift mutations eliminated the production of BAX These were focused in the same simple repeat sequence found to be mutated in some colon cancers with mutator phenotypes[29] Other substitution mutations within the BH1 and BH3 domains resulted in a loss of pro-apoptotic function and altered the dimerization capabilities of BAX

Materials & Methods

Amplification of bax and SSCP-gel analysis *Bax* mRNA from cell lines of human hematopoietic malignancies was amplified by RT PCR either unlabeled, or alternatively in the presence of 500 µM dATP, dTTP and dGTP, 125 µM dCTP and 100 µCi/ml [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol, 10 mCi/ml) The complete *Bax* coding region was covered by two partially overlapping PCR reactions (Figure 1B)[3] In one PCR reaction, *Bax* cDNA was amplified from exon 1 to the exon 4/5 boundary using forward primer A (5'-TGG ACG GGT CCG GGG AGC-3') and reverse primer B (5' GCA CAG GGC CTT GAG CAC C-3) In a second reaction, *Bax* cDNA was amplified from exon 4 through exon 6 using forward primer C (5 -GCC CTT TTC TAC TTT GCC AGC-3') and reverse primer D (5'- TCA GCC CAT CTT CTT CCA GAT 3') Products were analyzed by SSCP-PAGE For the SSCP analysis, non-radioactive PCR products were purified on Wizard™ PCR Preps columns (Promega) and dissolved in TE-buffer Five microliter of PCR product was mixed with an equal volume of loading buffer (20% EDTA, 20 mM NaOH, 0 05% xylene cyanol, 0 05% bromophenolblue in 96% formamide) and denatured for 10' at 95°C One microliter was separated on a 4 15% polyacrylamide gradient gel or a 12 5% polyacrylamide gel (Pharmacia LKB Phastsystem) Products were visualised by silver staining Alternatively, 2 µl of 10-fold diluted radioactive PCR products in SSCP loading buffer were separated on a 5% nondenaturating polyacrylamide gel (49 1), 5% glycerol in half TBE buffer The gel was exposed to X-ray films (Kodak)

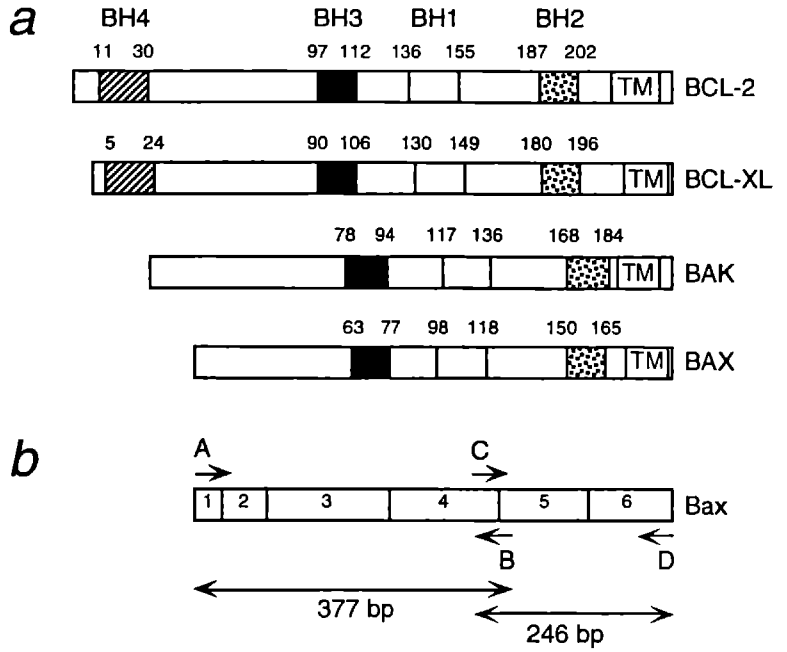

Figure 1 BCL2 homology (BH) domains within BCL2 family members **(A).** Schematic representation of BCL2, BCL-XL, BAK and BAX and the relative position of the BH1, BH2, BH3, BH4 domains and trans-membrane region TM **(B).** Schematic overview of *bax* open reading frame Exons are numbered BH1-3 and transmembrane domains are shaded Positions of PCR primers (A-D) are indicated PCR amplification using primer pair A, B results in a 377 bp product PCR amplification using primer pair C, D results in a 246 bp product

Immunoprecipitation Immunoprecipitation was performed as described[3] For immunoprecipitation, 50 mg/ml of 6C8 (Hamster anti-human BCL2 Moab[30]) or 12CA5 (Mouse anti-influenza virus hemagglutinin protein epitope moab[31]) was used

Preparation of constructs EcoRI sites, an upstream Kozak sequence and hemagglutinin influenza (HA) epitope tag were introduced by PCR using extended primers Germline *Bax* cDNA sequences and were cloned into PCRII using the TA-Cloning kit (Invitrogen), and confirmed by DNA sequencing Identical G67R and G108V mutations as found in Daudi and HPB-ALL respectively were introduced by site-directed mutagenesis (Clontech) Mutations were confirmed by sequencing *Bax* and mutant *Bax* cDNAs were cloned into pSFFV-LTR neo expression vector. For yeast two-hybrid experiments, cDNAs without their C-terminal signal-anchor sequence were cloned into the Gal4 activation domain (AD) vector pACTII, and the LexA DNA

binding domain (DB) vector pBTM116)[5] Extended primers were used to introduce a 5' EcoRI site and a 3' SalI site for cloning into pBTM116 or a 5' NcoI site and a 3' SmaI site for cloning into pACTII Constructs were confirmed by DNA sequencing The constructs for *Bcl-X*$_{L\Delta C19}$, *Bcl-2*$_{\Delta C22}$ in pACTII, and *Bcl-X*$_{L\Delta C19}$ in pBTM116 were previously described[5].

Transfection and Western Blots 20 µg DNA of Xba-1 linearized pSFFV-LTR neo vector expressing HA-*Bax*, HA-*Bax*^{G67R} or HA-*Bax*^{G108V} was transfected into FL5 12 cells or cotransfected with 1 µg of linearized pGK-hygro vector DNA into FL5 12-*Bcl-2* cells Stable transfectant clones were selected for neomycin resistance (1 mg/ml G418) or hygromycin resistance (1 mg/ml) Transfected clones were analyzed for HA-BAX, HA-BAX^{G67R} or HA-BAX^{G108V} expression by Western-blotting using polyclonal antiserum N20 (Santa Cruz, 1 500) as primary antibody and Goat-anti-Rabbit HRPO (Caltag Labs, 1 2000) as the secondary Ab BCL2 expression was detected by the antibody 6C8 (1 250), and a secondary Goat-anti-Hamster HRPO Ab (Caltag Labs, 1 2000) Immunoblots were developed by ECL (Amersham)

Yeast Two-Hybrid analysis cDNAs cloned in pBTM116 or pACTII were co-transformed into yeast strain L40 (*MATa trp1-901 leu2-3,112 ade2 his3-Δ200 LYS2 (lexAop)*$_4$*-HIS3 URA3 (lexA)*$_8$*-LacZ*[32] using the Lithium-Acetate method[33] After transformation, yeast cells were plated on selective media (His^+, Ura^-, Leu^-, Trp^-) and incubated at 30 °C After 2-4 days, yeast colonies were transferred to nitrocellulose filters, and incubated for 1' in liquid N_2 Filters were dried on whatmann paper and stained with 5-bromo-4 chloro-3-indolyl β-D-galactoside (X-Gal) substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-Mercaptoethanol, 1 mg/ml X-Gal, pH=7 0 for 1 to 12 hrs at 37°C

Results

Substitution mutations Twenty-nine cell lines derived from hematopoietic malignancies of various cell types were analyzed for mutations in *bax* by SSCP and sequencing analysis We found evidence for mutations in 7 cell lines (Table 1) The Burkitt Lymphoma (BL) cell line Daudi expressed a mutant *bax* allele which contained a G108V mutation and a wild-type allele This mutation substitutes the central glycine within the BH1 domain (Figure 1A) The Plasmacytoma (PC) cell line OPM1 expressed a mutant allele with a G11E mutation and a wild-type allele This glycine resides in the N terminus proximal to the first predicted α helix The Acute Lymphoblastic Leukemic cell line HPB-ALL expressed a G67R mutant allele as well as a wild-type allele This mutation alters the central glycine within the BH3 domain (Figure 1A) All three cell lines expressed BAX protein by Western analysis

Frameshift mutations SSCP analysis also noted a distinct, altered pattern common to four cell lines (KM3, CEM, Jurkat, JM*) (Table 1) DNA sequence analysis revealed the same single nucleotide deletion $(G)_7$ in a simple tract of 8 deoxyguanosine residues $(G)_8$ (nt 114-121) encompassing codons 38 to 41 of human *bax* (Table 1) This deletion was consistently detected in 3 or more independently amplified RT-

Table 1. Summary of mutation analysis

Cell-line	Lineage	Origin	SSCP	Sequence	Protein
Daudi	B	BL	mutation	G108V	+
Raji	B	BL	wt	wt	ND
Ramos	B	BL	wt	wt	ND
Rpmi6666	B	HD	wt	wt	ND
SUDHL6	B	FL	wt	wt	+
UM1	B	MM	wt	wt	ND
U266	B	MM	wt	wt	ND
Rpmi8226	B	MM	wt	wt	ND
IM-9	B	MM	wt	wt	ND
FRAVEL	B	MM	wt	wt	ND
OPM1	B	PC	mutation	G11E	+
HS-sultan	B	PC	wt	wt	ND
MC-CAR	B	PC	wt	wt	ND
ARH77	B	PCL	wt	wt	ND
KM3	B	B-ALL	mutation	del ($g^{114\text{-}121}$)§	-
CEM	T	T-ALL	mutation	del ($g^{114\text{-}121}$)§	-
Jurkat	T	T-ALL	2 mutations	del/ins ($g^{114\text{-}121}$)¥	-
JM*	T	T-ALL	2 mutations	del/ins ($g^{114\text{-}121}$)¥	-
HPB-ALL	T	T-ALL	mutation	G67R	+
GH1	T	T-ALL	wt	wt	ND
PEER	T	T-ALL	wt	wt	ND
HSB2	T	T-ALL	wt	wt	ND
MOLT4	T	T-ALL	wt	wt	ND
KCL22	M	CML	wt	wt	ND
KYO	M	CML	wt	wt	ND
K562	M	CML-E	wt	wt	+
BV173	M	CML-L	wt	wt	+
HL60	M	APL	wt	wt	ND
Kazumi	M	AML-M2	wt	wt	ND

The presence of mutations in cell lines of hematopoietic malignancies as determined by SSCP and sequence analysis Abbreviations BL, Burkitt's Lymphoma, B-ALL, B-cell Acute Lymphoblastic Leukemia, HD, Hodgkin's Disease, FL, Follicular Lymphoma, MM, Multiple Myeloma, PC, Plasmacytoma, PCL, Plasma Cell Leukemia, T-ALL, T-cell Acute Lymphoblastic Leukemia, CML, Chronic Myeloid Leukemia, CML-E, CML with blast crisis of Erythroid lineage, CML-L, CML with blast crisis of Lymphoid lineage, APL, Acute Promyelocytic Leukemia, AML-M2, Acute Myeloid Leukemia of M2 subtype, *JM derived from Jurkat line, §Deletion of 1 guanine residue in guanine stretch ($nt^{114\text{-}121}$), ¥Deletion of 1 guanine residue in guanine stretch ($nt^{114\text{-}121}$) on first allele and insertion of 1 guanine residue in guanine stretch ($nt^{114\text{-}121}$) on second allele RT-PCR results are based upon at least 2-3 independent SSCP or sequencing analysis

PCR products from each cell line DNA sequence of four or five independent clones from these RT-PCR products demonstrate the same $(G)_7$ deletion SSCP and DNA sequence analysis of the pre-B cell ALL, KM3 and the T cell ALL CEM only detected the $(G)_7$ deletion and no wild type allele indicating the mutation hemizygous or homozygous. PCR amplification of the 3rd exon of *bax* from the genomic DNA of KM3 also

failed to reveal a wild-type allele (not shown) The $(G)_7$ deletion frameshift generated a proximal stop codon Consistent with this no immunodetectable protein was observed in KM3 or CEM (Table 1)

The T cell ALL Jurkat and Jurkat derivate JM[34] both displayed an additional alteration on SSCP which upon DN sequencing proved to be a single nucleotide insertion $(G)_9$ in the same $(G)_8$ (nt 114-121) tract (Table I) Approximately half of the independent clones from the RT-PCR products of Jurkat and JM demonstrated the $(G)_9$ insertion while others the $(G)_7$ deletion The $(G)_9$ insertion frameshift also generates a proximal stop codon consistent with the lack of immunodetectable protein in Jurkat and JM cells (Table 1) and the presence of two mutated *bax* alleles.

Table 2. Yeast two hybrid interactions

	pACTII	BAX	G67R	G108V	BCL2	BCL-X_L
BAX	-	+	+	+	+	+
G67R	-	+	+++	ND	-	-
G108V	-	+	ND	-	++	++
BCL-X_L	-	+	-	+	ND	ND

Yeast two hybrid analysis of dimerization capacity of wildtype BAX versus BAX^{G67R} and BAX^{G108V} AD-fusion constructs are indicated accross the top lane control vector pACTII, $BAX_{\Delta}C19$ $BAX^{G67R}{}_{\Delta}C18$, $BAX^{G108V}{}_{\Delta}C18$ $BCL\text{-}2_{\Delta}C22$, $BCL\text{-}X_{L\Delta}C19$ DB-fusion constructs are indicated in the left hand column $BAX_{\Delta}C18$, $BAX^{G67R}{}_{\Delta}C18$, $BAX^{G108V}{}_{\Delta}C18$, $BCL\text{-}X_{L\Delta}C19$ Protein interactions as measured by X-gal substrate conversion are indicated as '-' for no interaction or "+" for clear positive Interactions that were enhanced are indicated as '++' or "+++" Interactions were tested in 2-4 independent experiments

Protein dimerization The BAX^{G67R} and BAX^{G108V} mutants found in HPB-ALL and Daudi reside in the conserved BH3 and BH1 domain respectively, and consequently were tested for dimerization capacity with BCL2 family members by means of yeast two hybrid analysis (Table 2) While the binding of BAX^{G67R} to wild-type BAX appeared unaffected, it demonstrated enhanced dimerization with itself, BAX^{G67R}. In contrast, the capacity to heterodimerize with BCL2 or BCL-X_L was lost BAX^{G108V} did not form homodimers with itself, BAX^{G108V}, but bound to wild-type BAX BAX^{G108V} demonstrated enhanced binding to BCL2 and BCL-X_L. Thus, both mutations demonstrated altered, yet very distinct dimerization characteristics.

We next examined whether these BAX mutants demonstrated altered dimerization within mammalian cells Stably transfected FL5 12-*Bcl2* clones expressing comparable amounts of hemagglutinin epitope-tagged (HA) molecules HA-BAX, HA-BAX^{G67R} or HA-BAX^{G108V} were generated (Figure 3A) When BCL2 was immunoprecipitated from lysates, it co-precipitated HA-BAX and the endogenous BAX (Figure 2A,

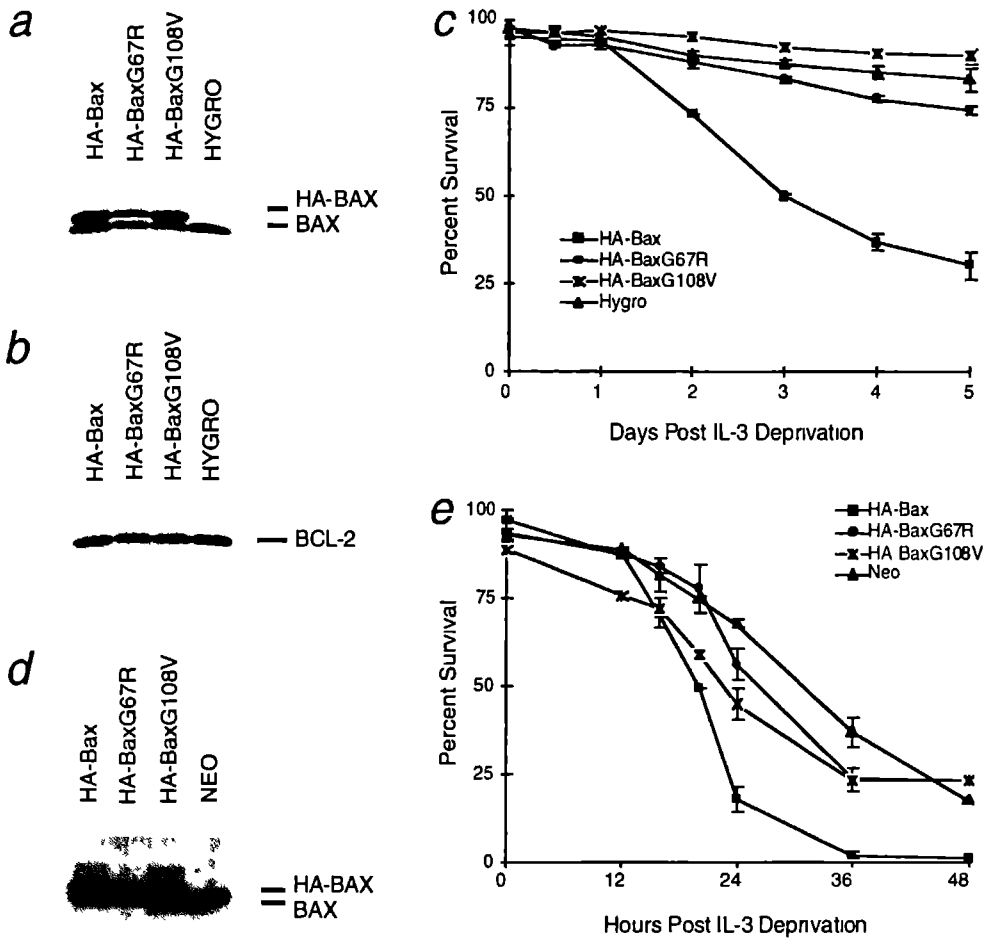

Figure 2 Altered dimenzation of BAX^{G67R} and BAX^{G108V} *in-vivo* compared to wildtype BAX **(A).** Immuno-precipitation of BCL2 from 0 25% NP-40 lysates of ^{35}S methionine/cysteine-labeled FL5 12-*bcl-2* cells (Hygro) or HA-BAX HA-BAX^{G67R} or HA-BAX^{G108V} stably transfected clones using anti-human BCL2 Moab 6C8 and SDS-PAGE[3] **(B).** Immuno-precipitation and SDS-PAGE analysis using anti-HA Moab 12CA5 from 0 25% NP-40 lysates of ^{35}S methionine/cysteine-labeled FL5 12-*bcl-2* cells (Hygro) or HA-BAX, HA-BAX^{G67R} or HA-BAX^{G108V} stably transfected clones

Lane 1) HA-BAX^{G108V} but not HA-BAX^{G67R} was also co-precipitated with BCL-2 confirming the interactions suggested in yeast two-hybrid (Figure 2A, Lane 2,3) A consistent higher ratio of HA-BAX^{G108V}/endogenous

Figure 3. BAX^{G67R} and BAX^{G108V} have lost cell death promoting activity. **(A).** Western blot analysis using anti-BAX polyclonal antiserum N20 upon lysates of FL5.12-*bcl-2* cells (Hygro) or HA-BAX, HA-BAX^{G67R} or HA-BAX^{G108V} stably transfected clones. **(B).** Western blot analysis of the same lysates as in (A) using anti-human BCL2 Moab 6C8. **(C).** Viability assay: Transfected clones described in (A) were deprived of IL-3 and viability was determined by trypan blue exclusion at 0, 0.5, 1, 2, 3, 4 and 5 days following IL-3 withdrawal and plotted as the mean percent survival +/- SEM. **(D).** Western blot analysis using anti-BAX polyclonal antiserum N20 upon lysates of FL5.12 cells (Neo) or HA-BAX, HA-BAX^{G67R} or HA-BAX^{G108V} stably transfected clones. **(E).** Viability assay: Transfected clones described in (D) were deprived of IL-3 and viability was determined by trypan blue exclusion at 0, 12, 16, 20, 24, 36 and 48 hrs following IL-3 deprivation and plotted as the mean percent survival +/- SEM.

BAX compared to HA-BAX/endogenous BAX in BCL2 immunoprecipitates is consistent with the enhanced binding of BAX^{G108V} noted in the yeast two-hybrid analysis (Table 2) In the reciprocal experiment, HA-BAX, $HA\text{-}BAX^{G67R}$ or $HA\text{-}BAX^{G108V}$ were immunoprecipitated with anti-HA Moab 12CA5 and all heterodimerized to endogenous BAX (Figure 2B, Lane 1-3) As before, HA-BAX, and $HA\text{-}BAX^{G108V}$ but not $HA\text{-}BAX^{G67R}$ co-precipitated BCL2 confirming the pattern of interactions within mammalian cells (Figure 2)

Functional analysis of Bax mutants To address the functional consequence of the BH1 and BH3 mutations they were assessed in an IL-3 deprivation assay using the FL5 12 line Clones of FL5 12 (Neo) or FL5 12-*Bcl-2* expressing comparable amounts of HA-BAX, $HA\text{-}BAX^{G67R}$, $HA\text{-}BAX^{G108V}$ were identified by immunoblots (Figure 3A, B and D) Addition of wild-type HA-BAX but not $HA\text{-}BAX^{G67R}$ or $HA\text{-}BAX^{G108V}$ was capable of promoting cell death in FL5 12-*Bcl-2* cells that were protected by BCL2 (Figure 3C) Similarly, $HA\text{-}BAX^{G67R}$ or $HA\text{-}BAX^{G108V}$ did not substantially alter the survival of native FL5 12 cells while wild-type HA-BAX clearly enhanced apoptosis (Figure 3E)

Discussion

Prolonged cell survival with resistance to apoptosis can be a primary oncogenic event Transgenic mice bearing a bcl-2-Ig minigene that recapitulated the t(14,18) found in human follicular lymphoma display B cell hyperplasia that progressed to gigh grade lymphoma[35] Evidence is emerging that a principal contribution from loss of p53 function is the elimination of a death pathway[36 37] Recent evidence suggests that BAX, a pro-apoptotic member of the BCL2 family can also qualify as a tumorsuppressor *Bax*-deficient mice display cellular expansions of neurons, lymphocytes, ovarian granulosa cells and spermatogonia reflecting the survival of cells that avoided developmental death[21] TGT121 transgenic mice which express a truncated T antigen that inhibits Rb but leaves p53 intact displayed an accelerated progression to malignancy upon a *bax*-deficient background[26] Of note, heterozygous *bax* (+/-) mice also displayed an earlier onset of malignancy suggesting that alteration of a single *bax* allele could be of functional significance An increase in focus formation was also documented in *bax* deficient versus wild-type fibroblasts when transfected with *ras* and *E1A*[27] These experimental models argue that *bax* can also be considered a tumor suppressor and that loss of this pro-apoptotic molecule promotes tumorigenesis

We found mutations in *bax* in approximately 21% (6 of 28 independent lines with confirmation of the mutation in Jurkat in its derivative JM) of human hematopoietic malignancy lines No *bax* alterations were noted in thirty-five normal individuals or in eight EBV transformed lymphoblastoid lines indicating that the observed alterations of *bax* are not common polymorphisms or associated with immortalization Approximately half of the mutations were frameshifts confined to a single mononucleotide $(G)_8$ tract (nt114-121) The existence of both insertions $(G)_9$ and deletions $(G)_7$ within the same leukemia (Jurkat and JM) favors a biallelic aberration and argues that the elimination of *bax* is a selective advantage Lack of immunodetectable BAX protein in other leukemias with $(G)_7$ deletions is compatible with a homozygous abnormality or perhaps loss of the second allele Recently, Rampino *et al* (1987) described the

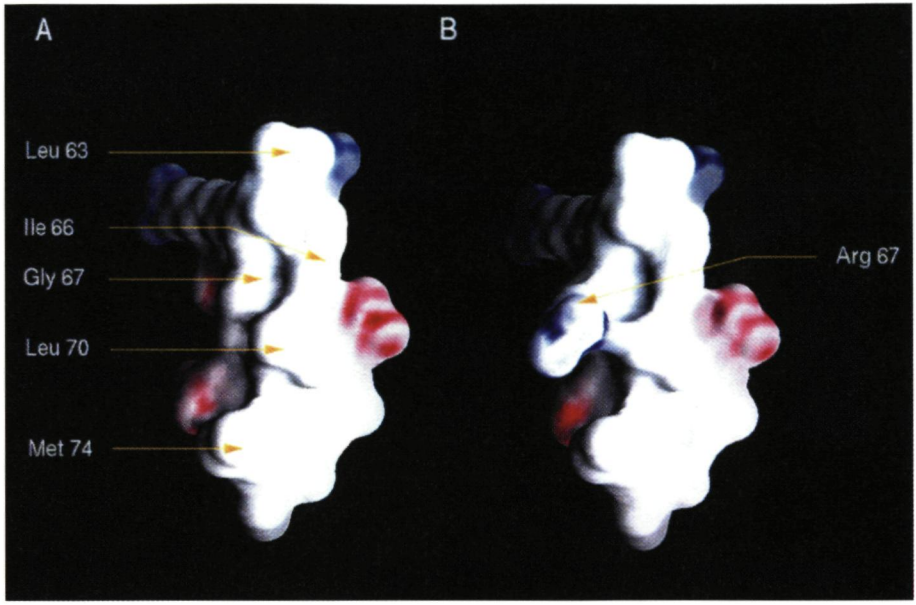

Figure 4. Three dimensional structure of BH3 region of BAX. Views of a modeled surface of the BH3 amphiphatic α-helix of BAX, calculated and displayed using GRASP (Muchmore *et al* (1996)). The G67R substitution as occurs in HPB-ALL is displayed at right. The surface is colored deep blue ($23K_bT$) in the most negative, with linear interpolation for values in-between. The model was generated using the protein building module (BUILDER) of INSIGHT II (Biosyn, San Diego) and minimized using DISCOVER, the forefield simulation mode.

presence of frameshift mutations in the identical $(G)_8$ tract of *bax* in about 50% of human colon carcinomas with the microsatelite mutator phenotype (MMP)[29]. Some sporadic cancers and almost all cancers associated with the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) accumulate mutations in microsatelites of nucleotide repeats due to defects in the human DNA mismatch repair genes including *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*[38]. This raises the possibility that a subset of Acute Lymphoblastic Leukemia may have a mutator phenotype.

In addition to the frameshift mutations we found missence mutations including BAX^{G67R} in the BH3 and BAX^{G108V} in the BH1 domain. Both mutations demonstrated abnormal dimerization characteristics, but were nearly opposite in their pattern. This argues that the death agonist activity of BAX may not strictly correlate with the capacity to form any single set of homo or heterodimer pairs. Molecular modeling of the BH3 α2-helix revealed a classic amphiphatic α-helix. The G67R mutation would introduce a charged residue onto the hydrophobic face of this hellix (Figure 4). NMR analysis of wild-type and mutant

peptides of the BH3 α2-helix of BAK indicated critical interactions with BCL-X_L through both hydrophobic and electrostatical interactions[39]. The substitution of the central glycine in this α2-helix to arginine noted here has a much greater effect than an alanine substitution analyzed for BIK[39] The impact of the G67R muation in BAX provides additional evidence that BH3 domains are critical for pro-apoptotic molecules

The G108V substitution in the BH1 α5-helix of BAX also resulted in the loss of pro-apoptotic activity While eliminating mutant/mutant dimerization, it if anything enhanced heterodimerization with BCL2 and BCL-X_L. Previous substitution of this central glycine to alanine in BCL2 (G145A) and in BCL-X_L (G159A) eliminated both their heterodimerization with BAX and their anti-apoptotic activity[5,6] However, the comparable substitution in BAX (G108A) had no effect (unpublished observations) Thus the strong effect of the G108V substitution was somewhat unexpected. The G108 residue resides in the long hydrophobic α5-helix felt to be part of the transmembrane helical cores responsible for the ion channel activity of BCL-X_L[40] and in similar approaches also for BAX[41 42]. This provides an alternative role for this residue beyond protein interaction.

but upon substitution into valine (BAX^{G108V}) results in loss-of homodimerization as well as death agonistic function. So glycine^{108} in BH1 of BAX preserves an evolutionary conserved site important for function and dimerization. Glycine^{67} (BH3) of BAX is also critical for maintaing cell death promoting function and dimerization with at least BCL-2 and BCL-X_L. Glycine 67 is positioned within the hydrophobic site of BH3 (Fig 4) and substitution into arginine may introduce a positive charge into this region

Oncogenes which promote proliferation contribute to cancer through gain-of-function alterations, while growth inhibitory tumor suppressor genes contribute principally through loss-of-function mutations The gain-of-function alteration of the *bcl-2-Ig* translocation overexpressed the anti-apoptotic molecule BCL2 in follicular lymphoma. This suggested that pro-apoptotic molecules could contribute to oncogenesis by loss-of-function mutations The discovery of *bax* muatations in a subset of colon carcinomas and in hematopoietic malignancies here provide such evidence This adds evidence in human tumors to the prospective experiments utilizing *bax*-deficient mice. The loss of BAX function would confer resistence to programmed cell death within hematopoietic cells and could contribute to malignancy in several ways Extended cell survival and resistance to apoptosis would enable cells to withstand additional genetic alterations In this contex t loss of BAX function could be a primary oncogenic abberation for which the *bax*-deficient mice provide evidence. *Bax* mutations could also contribute to tumor progression or the establishment of cell lines. Finally, chemotherapy could have selected for the loss of BAX as BAX deficiency would confer chemoresistence.

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CHAPTER 8

The *BCL-2* GENE FAMILY, TO LIVE OR LET DIE

Introduction

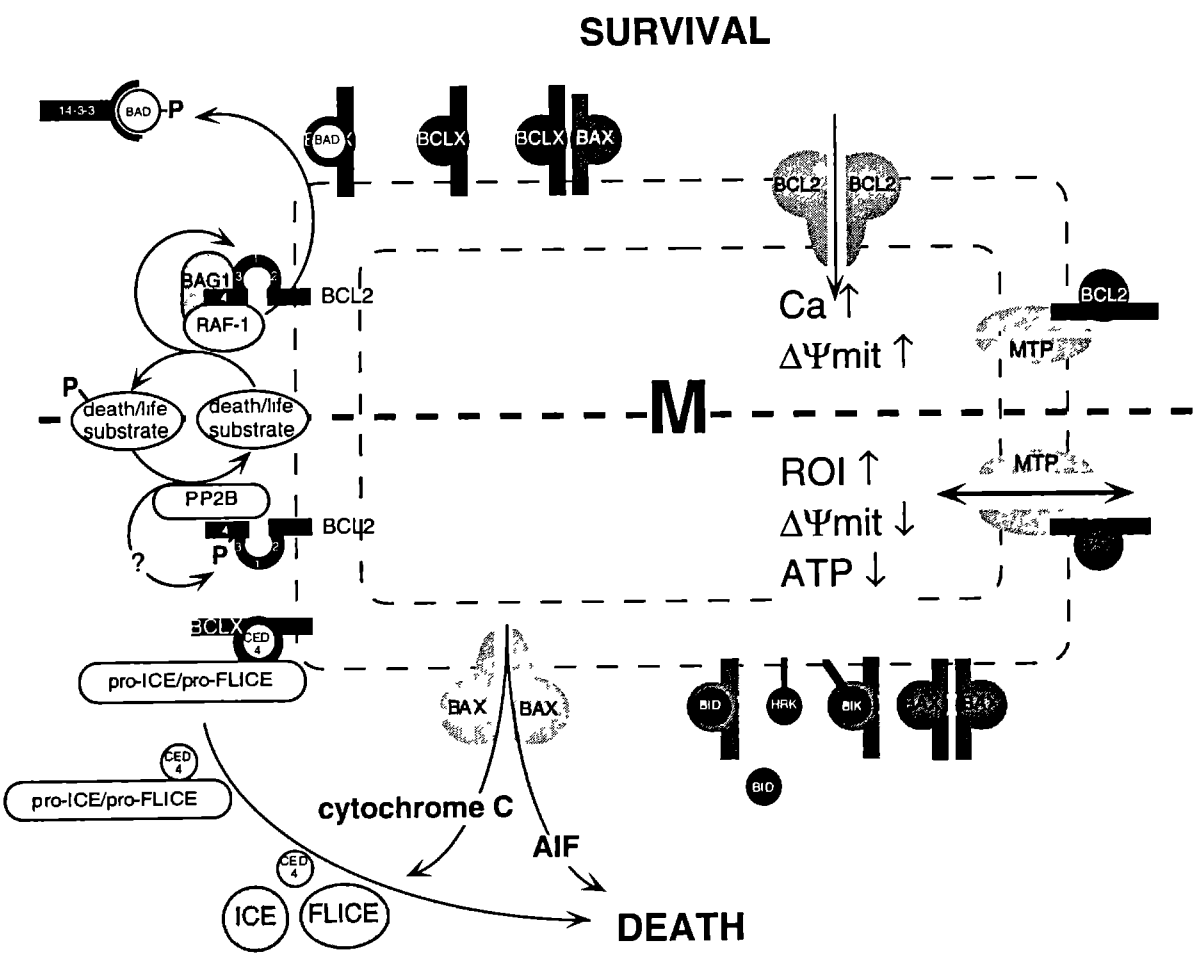
Apoptosis is a genetically encoded program which causes cells to die following a characteristic morphological pattern of membrane blebbing, cell shrinkage, chromatine condensation and breakdown, formation of apoptotic bodies and phagocytosis by macrophages Apoptosis occurs throughout live and is crucial during embryonal development, tissue homeostasis, immunological response and aging Deregulation of this proces contributes to autoimmune diseases, cancer, neurodegenerative diseases and AIDS [1]

The *bcl-2* gene family is one of the most pronounced regulators of programmed cell death (PCD). It consists of several members of which some members protect against the onset of PCD (*bcl-2*, *bcl-xl*, *mcl-1*, *a1*, *bcl-w*, *nr-13*, *bfl-1*) while others promote PCD under suboptimal or cytotoxic conditions (*bax*, *bak*, *bad*, *bcl-xs*)[2] All family members except *bad* are expressed as membrane bound proteins on the mitochondrial outer membrane proximate to inner and outer membrane contact sites. At least BCL2 is also present on the endoplasmatic reticular and nuclear outer membranes The family members form protein dimers through interactions between conserved domains[2-4]. Cellular responsiveness to cell death signals is regulated by the relative abundance of specific dimers and determines a specific cellular treshold (also called the cellular rheostat[5] (reviewed in [4 6,7]).

Ever since the discovery of *bcl-2* as the first member of this still emerging family of related genes, one of the most interesting issues has still not been solved: How does the *bcl-2* like family regulate cell death? Many different mechanisms by which *bcl-2* and other family members regulate PCD have been proposed (summarized in Figure 1), and will be reviewed in this paper.

1. Scavengers of intracellular free radicals

Reactive oxygen intermediates (ROI) are produced as natural spin-off during oxidative phosphorylation in mitochondria, and are normally detoxified by superoxide dismutase and oxidation of mitochondrial glutathion to prevent lipid and protein damage. Extensive production of ROIs may occur passively during apoptosis as a result of mitochondrial dysfunction or may be produced actively as a mechanism to execute apoptosis In support of this latter hypothesis, ROI scavenger N-acetylcysteine as well as overexpression of gluthatione peroxidase both protect against PCD of IL-3 dependent cells following IL-3 deprivation[8] Since *bcl-2* can protect against PCD as a result of many different cytotoxic conditions including treatment with peroxides or depletion of mitochondrial glutathion[8,9] and since BCL2 does not interfere with ROI production, BCL2 may reduce the destructive effects of ROIs probably by functioning as a direct radical scavenger[8] This explanation however may not suffice, and *bcl-2* and *bcl-xl* are also protective against various cytotoxic stimuli under hypoxic conditions during which ROIs are not produced[10-12]. Alternatively, ROIs may promote the nuclear translocation of transcription factor NF-κβ as an alternative mechanism to induce PCD Nuclear translocation of NF-κβ may activate a specific death gene. *Bcl-2* may inhibit PCD by interfering with the nuclear translocation of NF-κβ directly[13] or indirectly[14]. On the other hand, apoptosis can be observed in most cell types in the presence of protein synthesis inhibitor cycloheximide, so cells may allready express all constituents for PCD in a constitutive manner[15].


SURVIVAL
14-3-3
BAD
P
BAD
BCLX
BCLX
BAX
BCL2
BCL2
Ca↑
ΔΨmit ↑
BAG1
RAF-1
BCL2
MTP
P
death/life substrate
death/life substrate
M
ROI ↑
ΔΨmit ↓
ATP ↓
MTP
PP2B
BCL2
?
P
BCLX
CED 4
pro-ICE/pro-FLICE
BAX
BAX
BID
HRK
BIK
BID
CED 4
pro-ICE/pro-FLICE
cytochrome C
AIF
CED 4
ICE
FLICE
DEATH

2. Regulators of intracellular calcium balance

The intracellular calcium balance is regulated by storage and release from intracellular organelles like the endoplasmatic reticulum (ER) and influx through voltage independent calcium-channels in the plasma membrane. Following cellular activation, cytoplasmic calcium levels are temporarily increased through depletion of stores and influx of extracellular calcium. Calcium fluxes have also been observed during cell-kill by cytotoxic T cells and apoptosis of self-reactive thymocytes, so calcium may play a role in cell death as well[16]. Transient sequestering of calcium in mitochondria may be a mechanism to regulate mitochondrial function[17,18], or may coordinate cytoplasmic calcium fluxes[19]. In several studies, it was suggested that BCL2 inhibits cell death by regulating the intracellular calcium fluxes During growth factor deprivation of IL-3 dependent cells, intracellular calcium is repartitioned from cytoplasm and ER to mitochondria prior to the onset of apoptosis. BCL2 is able to inhibit or reduce calcium repartitioning[20]. In agreement with this, BCL2 may inhibit the release of calcium from the ER[21].

If BCL2 is indeed able to inhibit programmed cell death by controlling cellular calcium fluxes, overexpression of BCL2 could reduce or inhibit other calcium-dependent processes like lymphocyte activation by antigens. This has not been observed, on the contrary, *lck-bcl-2* transgenic mice have an increased rather than a decreased T cell response towards superantigens[22,23] Recently, BCL2 has been implicated in the regulation of mitochondrial permeability transition (section 3), and *bcl-2* associated calcium effects may more likely be a consequence rather than a direct site of regulation. Opening of the mitochondrial voltage dependent membrane pore is facilitated by high mitochondrial calcium levels[24,25] and by reduced mitochondrial calcium efflux[26]. *Bcl-2* may regulate the mitochondrial transition pore directly (discussed in section 3), inhibiting calcium entry in mitochondria in order to prevent permeability transition in some models[20,21], or promoting maximum calcium influx without inducing mitochondrial permeability transition and respiratory damage in other models[27]. Alternatively, BCL2 may be a membrane pore itself (discussed in section 3), optimizing mitochondrial calcium efflux during suboptimal conditions in order to prevent mitochondrial permeability transition Furthermore, *bcl-2* expressing cells may exhibit a higher mitochondrial membrane potential[28]. High membrane potentials further reduce the probability of mitochondrial permeability transition[29], and as a result may facilitate higher calcium levels[19].

Legend to Figure 1. Summary of various mechanisms as proposed for the *bcl-2* gene family in order to signal for live or death Decision between live or death may depend on critical protein interactions, phosphorylation or dephosphorylation events of a death or survival substrate, regulation of ion or protein transport across mitochondrial membranes or regulation of cell cycle.

3. Regulators of mitochondrial permeability transition

The mitochondrial permeability transition pore is an non-specific high conductance channel in the mitochondrial inner membrane, and may be identical to the mitochondrial megachannel as found by patchclamp techniques. Opening of the pore results in the release of solutes up to 1 5 kDa in size, in the collapse of the proton electrochemical gradient and in the cessation of ATP production[30,31]. In a cell-free system, a strict correlation was found between reduction of the mitochondrial membrane potential ($\Delta\psi_{mit}$) due to mitochondrial permeability transition and induction of nuclear apoptosis[32] Loss-of $\Delta\psi_{mit}$ as a result of treatment with various cytotoxic drugs is an early event in apoptosis as well as necrotic processes and precedes other apoptotic features like uncoupling of electron transport from ATP production, genomic DNA degradation, alterations of nuclear morphology, changes in membrane integrity and the formation of apoptotic bodies[33-36]. These latter apoptotic features may also depend on ROI formation, and are reduced by conditions under which production of ROIs is inhibited[36].

Loss-of $\Delta\psi_{mit}$ can be inhibited by overexpression of *bcl-2* or *bcl-xl* but not by cytokine response modifier A (*CrmA*) or other specific cysteine protease inhibitors Permeability transition due to pore opening may therefore occur independently of caspases and probably precedes activation of caspases in the apoptotic cascade. *Bcl-2* or *bcl-xl* does not protect against loss-of $\Delta\psi_{mit}$ during Fas-mediated apoptosis[37-39] or TNF-mediated apoptosis[40], arguing that the *bcl-2* gene family and the *fas/tnf-α* superfamily participate in independent death pathways. Bongkrekic acid (BA), which is a specific antagonist of mitochondrial permeability transition, inhibits loss-of $\Delta\psi_{mit}$ and nuclear apoptosis induced by several compounds in the cell-free system[32]. BA is able to inhibit induction of apoptosis in thymocytes by various cytotoxic stimuli. BCL2 and BA inhibit permeability transition induced by identical compounds, raising the possibility that BCL2 inhibits mitochondrial permeability transition by interacting with the adenine nucleotide translocator (ANT) as constituent of the transition pore[32].

Recently, the three dimensial structure of BCL-X_L was resolved by X-ray crystallography and nuclear magnetic resonance. BCL-X_L consists of two antiparallel hydrophobic α-helixes surrounded by five amphipathic helixes. The BH1, BH2 and BH3 domains are organized in close spatial proximity and form a hydrophobic pocket that can function as a docking-site for other family members. The position of the N-terminal BH4 domain is variable due to the presence of a flexible loop between BH4 and BH3 which is designated the loop domain and comprises a serine phosphorylation site[41,42] Since other BCL2 like proteins share conserved domains, they may adopt a similar conformation The structure of BCL-X_L resembles the pore-forming domains of bacterial toxins like diphtheria toxin or colicins A and E1, which form membrane-pores upon dimerization[41]. Likewise, BCL-X_L and BCL2 are able to form cation-specific channels under physiological pH. Several channels with multiple conduction states are observed that are probably the result of different oligomeric states of BCL2 and BCL-X_L in membranes[43,44]. In analogy to diphtheria toxin which translocates the ADP ribosylating A-subunit into the cytosol through the formation of a membrane pore[45], *bcl-2* family members may control cell death by transporting ions or proteins over the mitochondrial membrane.

During apoptosis, several mitochondrial factors are released into the cytosol. In a cell-free system based upon cytosolic fractions, Liu *et al* (1996) identified two factors which are stimulated by the addition of dATP and activate the cysteine protease CPP32 and nuclear apoptosis. One of these factors denoted Apaf-2 was identified as cytochrome C, and is released from the mitochondrial intermembrane space upon induction of apoptosis[46]. The identity of second factor denoted Apaf-1 is still unknown, but is probably the same factor designated AIF (for apoptosis inducing factor) as recently identified by Susin *et al* (1996). This cysteine protease of about 50 kDa resides under normal conditions in the mitochondrial intermembrane space. AIF is different from other cysteine proteases like ICE or CPP32 since it does not degrade CPP32 specific substrates like polyadenosine diphosphate-ribose polymerase (PARP) and Lamin A/B and it is not inactivated by ICE or CPP32 specific inhibitors. AIF triggers genomic DNA degradation and nuclear apoptotic morphology[47].

It is attractive to speculate that the *bcl-2* gene family regulates the release of mitochondrial factors as a mechanism to regulate PCD. BCL2 is able to inhibit or to retard the release of holocytochrome C and AIF from the mitochondria upon induction of apoptosis. In contrast to AIF which is released upon collapse of $\Delta\psi_{mit}$, release of holocytochrome C from the mitochondria to the cytosol is a relatively early event during apoptosis. It probably precedes loss-of $\Delta\psi_{mit}$, activation of caspases like CPP32, cleavage of protease specific substrates and fragmentation of DNA[48,49]. BCL2 and its family members may not only be involved in the regulation of mitochondrial permeability transition pore opening or release of mitochondrial death factors, they may also function by recruiting various cytoplasmic proteins to the mitochondrial environment.

4a. Docking sites for cytoplasmic proteins

The *bcl-2* gene family may regulate PCD by the formation of specific homodimers and heterodimers A number of genes not belonging to the *bcl-2* gene family have been identified that can interact with *bcl-2* gene family members at the protein level. Among these genes is the protein kinase *raf-1* (for Ras-activated factor-1)[50], the phosphatase-2B calcineurin[51], *bag-1* (for BCL2 associated athanogene-1[52]), *ced-4* (for cell death gene-4[53-55]), *bik/nbk* (for BCL2 interactive killer or natural born killer)[56,57], *bid* (for BH3 interacting domain death agonist)[58], *hrk* (for harikiri)[59], *bbp/53bp2* (for BCL2 binding protein or p53 binding protein 2[60]) and three uncharacterized cDNAs, *nip-1*, *nip-2* and *nip-3* (for E1B 19K interacting proteins[61]). Some of these genes may be involved in the modulation of the anti-apoptotic or pro-apoptotic activity of *bcl-2* gene family members, while others may be directly involved in cell survival or death.

BCL2 can bind to RAF1[50,62], and may be dependent on cell-type[63]. The BCL2-RAF1 interaction is probably facilitated by a third protein denoted BAG1, and may result in the formation of a tri-molecular complex. BAG1 is unique among cellular proteins in that it not only binds to BCL2[52], the hepatocyte growth factor (HGF) receptor, the platelet-derived growth factor (PDGF)[64] and RAF1[65], it also potentiates the anti-apoptotic effect of BCL2, HGF or PDGF during drug-induced apoptosis as well as the phosphorylation of RAF1 substrate MAPK/ERK kinase (MEK) Both RAF1 and BAG1 bind to the BH4 domain of BCL2. In a mitochondrial environment, RAF1 is able to enhance the anti-apoptotic effect of BCL2[50].

Destabilization of the complex by phosphorylation of RAF1 and BCL2 upon taxol treatment result in loss-of anti-apoptotic function[62 66] Recruitement of RAF1 may serve several functions First, RAF1 may modulate the activity of some *bcl-2* gene family members such as BAD[50 67] Second, RAF1 may also be involved in the activation of critical death or survival effectors Since BCL2 is also able to recruit phosphatase-2B Calcineurin to the mitochondrial environment[51], such downstream effectors are subjected to extensive phosphorylation and dephosphorylation events

The downstream effectors in the cell death cascade may involve members of a new *bcl-2*-related gene family One of these members denoted *bik/nbk* binds to BCL2 family members except BAX BIK/NBK contains a BH3 domain, but shares no further domains of homology with the *bcl-2* gene family[56 57] Recently, two other members denoted *bid* and *hrk* have been identified[58 59] Their general expression in most cell types is consistent with their possible function as downstream effectors in the cell death or survival pathway, although the expression of *hrk* is limited to bone marrow and spleen Unlike the other two genes, *Bid* has no putitive membrane-spanning domain *Bik/nbk*, *bid* or *hrk* induce apoptosis upon transient transfection that can be blocked by BCL2 or BCL-X_L[56 59] At present it is unclear how these proteins signal for death These proteins may resemble BAX and BAK in signalling for death[56 57 59], or act as specific death substrates when bound to pro-apoptotic *bcl-2* gene family members[58]

4b. Regulators of Caspase activation

Other protein interactions by which the *bcl-2* gene family may regulate cell viability has been provided by the nematode *Caenorhabditis elegans* model Three genes denoted *ced-9*, *ced-3* and *ced 4* have been identified so far that are essential for developmental cell death *Ced 9* is the nematodian homologue of the human *bcl-2* gene[68], whereas *ced-3* and *ced 4* are able to activate apoptosis *Ced-3* is homologous to IL 1β - converting enzyme *ice* (caspase-1[69 70]), but no human homologue of the *ced-4* gene have been identified so far The *ced-9* gene is able to inhibit the function of *ced-3* and *ced-4*, suggesting that *ced 9* functions upstream of *ced-3* and *ced-4* [71] For this, CED9 and BCL-X_L may bind to CED4 directly Mutations in CED9 and BCL-X_L which result in a loss-of-function phenotype also result in loss-of-binding to CED4 The binding site for CED4 involves the BH1-3 domains of CED9 or BCL-X_L, and may require a hydrophobic pocket conformation[53-55] Since CED4 binds to CED3, a tri-molecular complex can be formed This way, BCL-X_L is able to recruit pro-ICE (caspase-1) and pro-FLICE (caspase-9) but not pro-Yama (caspase-3) or pro-Mch2 (caspase-6) to mitochondria, thereby inhibiting the activation of caspases by CED4[55] BAX may signal for death by competing with CED4 for binding to BCL-X_L, allowing the CED4/caspase complex to enter the cytosol and to activate the protease by CED4

Different death pathways converge to the activation of caspases and it is now generally accepted that caspases are involved in the executioner-phase of the cell death cascade However, recently it was demonstrated that cells can still be induced to die by apoptosis in the presence of cysteine protease inhibitors following expression of specific oncogenes, DNA damage or lethal expression of BAK or BAX but not upon ligation of Fas/Apo-1[72 73] After the induction of a cytotoxic stimulus, cells retain functional

mitochondria and intact cell membranes, do not demonstrate chromatine condensation, DNA fragmentation or degradation of protease substrates like nuclear PARP (poly(ADP ribose) polymerase) and cytoplasmic D4-GDI (GDP dissociation inhibitor for the rho family) in the presence of cysteine protease inhibitor ZVAD-fmk for at least 48 hours after onset of membrane blebbing[73]. ZVAD-fmk treated cells finally die after several days displaying an apoptotic phenotype although chromatine condensation, DNA fragmentation and cleavage of protease substrates may be reduced[73]. Therefore, caspases are important for a fast and irreversible execution of cell death, but the actual decision between live and death precedes the activation of caspases.

5. Regulators of cell cycle

Tissue homeostasis demands a balance between cell renewal (cell division) and cell death (apoptosis), but at present it is unclear how these processes are connected Such a connection seems logical since active cycling cells are especially sensitive to undergo apoptosis by cytotoxic compounds. The recent observation that *bcl-2* family members can affect cell cycle entry may provide new insight in the communication between these processes. By making use of cell line models and transgenic mice models[74 77], several groups found that *bcl-2*, *bcl-xl* or *E1B-19K* retard cell cycle entry from G0 phase and/or prolong the G1 cell cycle phase This results in reduced numbers of cells in S or G2/M phase and in a prolongation of total cell cycle time *Bcl-2* mutants which are incapable to promote survival[78] are unable to affect entry of cells from the quiescent stage into the cell cycle[75]. So domains which are required for regulation of cell death are also involved in regulation of cell cycle Moreover, *bax* transgenic mice display an increased percentage of thymocytes in S phase[79] and cell cycle regulation may therefore not be regulated by a single *bcl-2* family member, but may be dictated by the relative abundance of specific dimers.

How does the *bcl-2* family interact with the cell cycle machinery? A direct interaction has not been proven yet, but the effects of *bcl-2* on cell cycle appears to correlate with a hypophosphorylated pRB status[74] Hypophosphorylated pRB may be related with persistent cyclin/Cdk2-inhibitor $p27^{Kip1}$ expression during *in-vitro* T cell activation[76] Likewise, increased cell cycle entry of *Bax*-transgenic thymocytes during *in-vitro* activation has been correlated with faster decrease of $p27^{Kip1}$ levels[79]. The $p21^{Waf1}$ expression level is not affected The mechanism by which the *bcl-2* family regulates the levels of $p27^{Kip1}$ is unclear, but may involve activation of a specific caspases. BCL2 also reduces nuclear translocation of nuclear factor of activated T cells (NFAT)[76], by recruiting calcineurin to the mitochondria[51] resulting in reduced IL-2, IL-3 and GM-CSF production However, addition of exogenous IL-2 has no compensatory effect on reduced cell cycle entry by BCL2[76].

Concluding remarks

Many components of the cell death cascade have been identified during the last years, and their relative position in the cascade is better understood. The *bcl-2* gene family is an important point of convergence of the cellular pathways that are intermingled with a decision between live and death Although several different mechanisms have been proposed by which the *bcl-2* gene family signals for live or death, none of those have

been indisputably proven yet At present it is still unclear if signaling by the *bcl-2* gene family requires a critical protein interaction or involves the regulation of cellular processes like ion or protein transport from mitochondria (and ER) to cytoplasm and vice versa Future research to provide answers about this still enigmatic issue will require close scientific cooperation between the molecular biology research field and the physiology research field.

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SUMMARY

Summary of this thesis

The t(14;18)(q32;q21) is found in the majority of follicular lymphoma patients. This translocation has been recognized as one of the critical steps in the development towards malignancy (reviewed in **Chapter 1**). Cloning of the chromosomal breakpoint regions resulted in the discovery of the *bcl-2* gene, a new type of proto-oncogene that is able to regulate the lifespan of a cell. The discovery of this gene led to a new concept of cancer development. One started to realize that cancer was not only the result of uncontrolled cellular proliferation but it may also result from neglect of programmed cell death.

A whole family of *bcl-2* related genes has been identified, consisting of members which protect against cell death like *bcl-2*, but also of genes which promote programmed cell death, such as the *bax* gene. Although the *bcl-2* gene is the only gene from this family that has been correlated with the pathogenesis of disease until now, deregulated expression of at least some members have been correlated with increased cellular resistance of some cancer types to chemotherapy and γ-radiation.

The t(14;18) is a tumor specific marker, and it provides an excellent opportunity to monitor residual tumor burden during the course of disease, provided that accurate quantitation is feasible. Moreover, quantitation of residual disease may be very helpful in designing effective treatment strategies in future. For this purpose, we developed a quantitative method based upon the polymerase chain (**Chapter 2**). The polymerase chain reaction is extremely sensitive, and the detection of a single t(14,18) which is equivalent to one lymphoma cell in a background of about 150,000 normal cells is feasible. A major advantage of the quantitative method is that the amplification of each sample is internally checked for amplification fidelity by the application of an internal standard. Using the competitive assay (**Chapter 3**), declining numbers of residual lymphoma cells were detected in consecutive blood samples of a follicular non-Hodgkin's lymphoma patient after allogeneic bone marrow transplantation. This progressive decline may reflect specific immunity of the donor graft to residual lymphoma cells, e.g. graft-versus-lymphoma activity. The method was further improved by the coupling of polymerase chain reaction technology with capillary electrophoresis (**Chapter 4**). This enabled non-radioactive and fully automated analysis of assay products.

In **Chapter 5**, we described the cloning of both translocational junction regions of an unusual t(14,18) which was identified in a follilicular non-Hodgkin's lymphoma patient. We concluded that this translocation was the result of an aberrant rearrangement during the immunoglobulin heavy chain rearrangement process in the pre-B cell stage that had happened during an attempted V_H to D_H-J_H rearrangement. Since the majority of t(14,18)s occur as mistakes during relatively earlier immunoglobulin rearrangement processes, we studied the pathogenesis of this lymphoma case by analyzing the somatic mutation pattern in the functionally rearranged immunoglobulin heavy chain. Like other follicular lymphoma cases, somatic mutations were present providing evidence for antigen-driven selection of lymphoma cells. The rarity of t(14;18)s during relatively later immunoglobulin heavy chain rearrangement processes compared

to the D_H to J_H joining process may coincide with reduced transcription rate and accessibility of the *bcl-2* locus during the pre-pre-B cell stage

In **Chapter 6**, we reported the presence of point mutations in the pro-apoptotic *bcl 2* family member *bax* in several malignant hematopoietic lines One mutation (G108V) was localized in an evolutionary conserved domain previously denoted as BCL2 homology domain 1 (BH1) Equivalent mutations in *bcl-2* or *bcl-x* resulted in complete loss of anti-apoptotic function, so an equivalent mutation in *bax* may have major implications for function Protein alignment of several *bcl-2* family members predicted that another point mutation (G67R) was also localized in a region with extensive protein conservation This region has recently been identified as a critical death domain of *bcl-2* family members with pro-apoptotic activity and denoted BH3 Furthermore, we observed clustering of somatic frameshift mutations in a single mononucleotide stretch consisting of eight deoxyguanosine residues in at least three independent malignant cell lines (**Chapter 7**) Clustering of identical frameshift mutations have recently been described in patients with colorectal cancer, and is associated with the microsatelite mutator phenotype Functional analyses of two *bax* mutants having a mutation in the BH1 (G108V) or BH3 (G67R) region respectively, demonstrated that both mutations were associated with altered protein binding and loss of pro-apoptotic function of mutant BAX

In **chapter 8**, we reviewed the cellular mechanisms by which the *bcl-2* gene family may regulate cell death Several different mechanisms have been proposed like scavenging of intracellular reactive oxygen radicals, control of intracellular calcium balance, regulation of mitochondrial permeability transition by a direct interacting with the mitochondrial transition pore or by serving as membrane pore, binding of intracellular proteins which are involved in signal transduction or caspase activation, or by regulation of cell-cycle

Future research perspectives

Future research will be directed to clarify the relation between mutations in *bax* and cellular resistance to chemotherapy For this purpose, we shall test wildtype *bax* and mutant *bax* transfected cell lines for sensitivity to chemotherapeutic regiments Wildtype and mutant *bax*-transgenic mice are currently being developed, and will be used in *in-vivo* experiments for testing cellular sensitivity to chemotherapeutic regiments These mice will be monitored for tumour development in a longterm cohort study to further investigate a potential relationship between loss-of-function mutations in the pro-apoptotic gene *bax* and cancer development

Both loss-of-function mutants of BAX as described in chapter 7 provides a powerful model to investigate the mechanism by which BAX regulates cell death We shall attempt to identify downstream effectors in the cell death cascade For this, we are currently performing a differential screening of an expression library by means of the yeast-two-hybrid technique Furthermore, wildtype and mutant *bax* transfectants will be used to investigate a direct role of *bax* in the regulation of mitochondrial permeability transition

Research has already been directed to optimize the quantitation of residual lymphoma cells during treatment of follicular lymphoma patients by introduction of recently developed Taqman chemistry This will result in a faster and almost fully automated quantitation assay, in which contaminations are less likely to occur This method will be used to monitor residual tumor burden in patients with follicular non-Hodgkin's lymphoma which have been treated according to various experimental treatment protocols

Samenvatting

De chromosomale translocatie t(14,18)(q32,q21) is in de meeste folliculair non-Hodgkin's lymfoom patienten aanwezig Deze translocatie wordt gezien als één van de belangrijkste stappen in het ontstaan van deze ziekte (zie overzicht in **Hoofdstuk 1**) Het *bcl-2* gen is gevonden door klonering van de chromosomale breekpunten van de translocatie Het bleek een nieuw type proto-oncogen, en is van belang voor regulatie van geprogrammeerde celdood Celdood is een actief cellulair proces, waarbij de cel dood gaat middels een karakteristiek morfologisch patroon dat ook wel "apoptose" genoemd wordt De ontdekking van dit gen heeft geleid tot een kompleet nieuw inzicht in het ontstaan van kanker Tot nu toe zag men kanker als het resultaat van ongecontroleerde celgroei, maar nu bleek kanker ook te kunnen ontstaan door een defect in het celdood programma waardoor de getransformeerde cel een verlengde levensduur krijgt

Tegenwoordig zijn er meerdere genen bekend die verwant zijn aan *bcl-2*, en vormen tesamen de *bcl-2* gen familie Deze familie betsaat uit genen die de cel kunnen beschermen tegen de inductie van het celdood programma zoals het *bcl-2* gen, maar ook uit genen die de inductie van dit programma juist stimuleren, zoals bijvoorbeeld het *bax* gen Het *bcl-2* gen is tot nu toe het enige gen uit de genfamilie dat gekoppeld is met een ziektebeeld Echter, de expressie van *bcl-2* of andere familieleden kunnen in sommige kankertypen verhoogd zijn en aanleiding geven tot een resistentie tegen chemotherapie of bestraling

De t(14,18) is een lymfoomcel-specifieke genafwijking Hierdoor is het mogelijk om residuale lymfoom cellen aan te tonen en te kwantificeren tijdens de behandeling van het ziekteproces Nauwkeurige kwantificering van residuale kanker cellen is belangrijk voor het bepalen van de effectiviteit van nieuwe behandelings-strategieën **Hoofdstuk 2** beschrijft een kwantitatieve methode, die gebaseerd is op de polymerase kettingreaktie Deze methode kan residuale lymfoomcellen kwantificeren in bloedmonsters van lymfoom patienten De polymerase ketting reaktie is een specifieke en zeer gevoelige methode, waarmee een enkele t(14,18) overeenkomend met één lymfoomcel in een achtergrond van 150 000 normale cellen aangetoond kan worden De toepassing van een interne standaard om het verloop van de reaktie te kontroleren biedt een belangrijk voordeel Deze assay toonde een langzame afname aan van residuale lymfoomcellen in het bloed van een patient met een follicular non-Hodgkin's lymfoom patient na allogene beenmergtransplantatie (**Hoofdstuk 3**) Deze afname was mogelijk het gevolg van specifieke immuniteit van donorcellen tegen residuale lymfoomcellen, en wordt dan ook "graft-versus-lymphoma" activiteit genoemd De methode werd verder verbeterd door de toepassing van capillaire electroforese voor de analyse van de reaktieprodukten (**Hoofdstuk 4**), waardoor het mogelijk is om de reaktieprodukten zonder toepassing van radionucliden bijna volledig geautomatiseerd te analyseren

De klonering en analyse van beide translocatie breekpunten van een zeldzame t(14,18) van een patient met een follicular non-Hodgkin's lymfoom, is beschreven in **Hoofdstuk 5** De translocatie is waarschijnlijk het resultaat van een foutieve rangschikking van gensegmenten tijdens het immunoglobuline

zware keten rangschikkingsproces, mogelijk tentijde van een V_H naar D_H-J_H gensegment koppeling Vrijwel alle t(14,18)s zijn het gevolg van fouten in relatief vroegere gensegment rangschikkingsprocessen Daarom bestudeerde wij dit lymfoom door analyse van somatische mutaties in het functionele zware keten immunoglobuline-gen Het somatische mutatiepatroon vormde een bewijs voor antigeen-selectie tijdens de ontwikkeling tot folliculair non-Hodgkin's lymfoom Het sporadische voorkomen van t(14,18)s tengevolge van relatief late immunoglobuline zware keten rangschikkingen hangt mogelijk samen met verminderde transcriptie en daardoor mindere toegankelijkheid van het *bcl-2* locus in het pre-B cell stadium

In diverse kwaadaardige hematopoietische cellijnen werden puntmutaties in het pro-apoptotische *bax* gen aangetoond, zoals beschreven in **Hoofdstuk 6** Een van de puntmutaties, de G108V-mutatie zoals aanwezig in de cellijn Daudi, is gelegen in een evolutionair geconserveerd domein dat het "BCL2 homologie domein 1 (BH1)" genoemd wordt De G108V mutatie in *bax* heeft mogelijk consequenties voor de pro-apoptotische activiteit van dit gen Equivalente mutaties in het *bcl-2* of *bcl x* gen leiden tot een verlies van anti-apoptotische activiteit Een andere puntmutatie, de G67R mutatie zoals aanwezig in de cellijn HPB-ALL, is gelegen in een andere geconserveerd domein dat het BH3 domein genoemd wordt Recentelijk is aangetoond dat BH3 van cruciaal belang is voor de activiteit van BAX In drie cellijnen van acute lymfatische leukemie origine werden frameshift mutaties aangetoond die geklusterd waren in een mononucleotide stretch bestaande uit acht opeenvolgende deoxyguanosine residuën (**Hoofdstuk 7**) Recentelijk zijn identieke frameshift mutaties in het *bax* gen aangetoond in patienten met dikke darmkanker Beide *bax* mutaties, G67R en G108V, zijn getest op functionaliteit Beide mutaties bleken te leiden tot veranderde eiwit dimerisaties en verlies van pro-apoptotische activiteit

Hoofdstuk 8 is een samenvatting van verschillende mechanismen waarmee de *bcl-2* gen-familie de celdood kan reguleren, zoals binding van reaktieve zuurstof radicalen, regulatie van de intracellulaire calcium balans, regulatie van mitochondriale permeabiliteits transitie door een direkt interaktie met de mitochondriale transitie porie of door porie-vormende activiteit van de *bcl-2* gen familie, door binding van cellulaire eiwitten die betrokken zijn bij de signaal-transduktie en activatie van caspases, of door regulatie van de cel cyclus

Dankwoord - Epilogue

Het "dankwoord" is een speciaal onderdeel van vele proefschriften. Meestal is het een verzameling van namen van collega's die op een of andere wijze hebben bijgedragen aan het ontstaan van het proefschrift U zou misschien geneigd zijn te zeggen dat het daardoor een verplicht en saai onderdeel is Naar mijn mening is echter niets minder waar. Iedere keer wanneer een nieuw proefschrift in mijn omgeving wordt uitgedeeld, zie ik bijna iedereen als eerste bladeren in dit onderdeel, het dankwoord daarmee tot het meest gelezen deel makende. Wat zegt U? Dat de rest nog minder gelezen wordt iets zegt over de mate van aantrekkelijkheid? Geen commentaar!

Als eerste wil ik graag mijn promotor, prof. dr T. de Witte, bedanken voor het mogelijk maken van mijn promotie-plaats. Theo, bedankt voor jouw steun en vertrouwen Ik ben echter nog steeds van mening dat een goed onderzoeker een zekere mate van naiviteit nodig heeft t a v zijn of haar experimenten Veel dank ook aan mijn beide co-promotoren Ewald Mensink en John Raemaekers Ewald, met name jij bent als begeleider het meest direkt betrokken geweest bij mijn werk Met groot plezier denk ik niet alleen terug aan onze gezamenlijke wetenschappelijke inspanningen, maar ook aan de "gezellige ambiance" die je wist te creeren (de tempelier, de hond van de buren, gezang in de trein) waarin vele wetenschappelijke ideeen hun oorsprong vonden John, jij bedankt voor het clinische aspect binnen het onderzoek, en de vele inspanningen die jij hebt gedaan voor het leggen van goede samenwerkingscontacten met pathologie (José Bogman), cytogenetica (Ad Geurts van Kessel) alsook de farmaceutische industrie (Schering-Plough, Robert Alferink, bedankt!) Veel dank ook verschuldigd aan mijn direkt betrokken AIO-collega's Toon Smetsers, Peter Willems (Peewee) en Aldy Kuypers. We mogen elkaar niet altijd begrepen hebben, een levendige en enerverende club was het wel Bedankt ook de mensen die direkt betrokken zijn geweest bij mijn projekten: Ruth Knops, Arjen Nooteboom, Helger Yntema, Annet Sloetjes, Evelien Tonnissen, Agnes Zoetbrood, Gerard Goverde, Louis van de Locht, Ellen Stevens-Linders, Peter Donnely en Aniek de Graaf alsook de overige mensen van de DNA-club Verder wil ik alle medewerkers van de afdeling Hematologie alsook van het Centraal Hematologisch Laboratorium bedanken voor deze fijne jaren van samenwerking

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Tenslotte Nicole, bedankt voor al die jaren van ondersteuning en eindeloos geduld die het mogelijk maakte om dit proefschrift tot een goed einde te brengen

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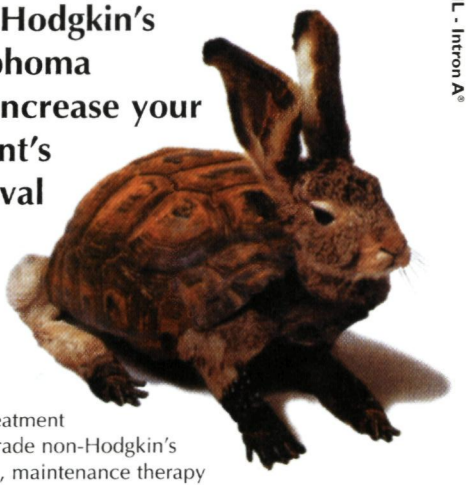
Jules Peter Paul Meijerink werd op 24 december 1964 geboren te Hoeven. Na afronding van het Atheneum aan de Pius X college te Almelo in 1984, werd in september van hetzelfde jaar begonnen met een Biologie-studie aan de faculteit Wiskunde en Natuurwetenschappen van de Katholieke Universiteit te Nijmegen. In het kader van deze studie werd een hoofdvakstage medische-biologie doorlopen bij de afdeling Hematologie (toenmalig hoofd: prof. dr. C. Haanen) van het academisch ziekenhuis "St Radboud" te Nijmegen onder begeleiding van dr F. Preijers. Een tweede hoofdvakstage biochemische-biologie werd gedaan op de afdeling Genetica (hoofd: prof dr. W. Hennig) van de Katholieke Universiteit te Nijmegen In september 1990 werd het doctoraal examen Biologie behaald. In november 1990 werd aangevangen met een promotie-onderzoek aan de afdeling Hematologie (hoofd: prof dr. T de Witte) van het academisch ziekenhuis "St Radboud" te Nijmegen. Onder begeleiding van dr E. Mensink werd onderzoek gedaan zoals beschreven in deze dissertatie In het kader van een samenwerkingsverband werkte de auteur van augustus 1995 tot april 1996 op de afdeling Moleculaire Oncology (hoofd prof. S J. Korsmeyer) van het Howard Hughes Medical Institute van de Washington University School of Medicine te Saint Louis (MO, USA) Op 25 juni 1996 trad hij in het huwelijk met Nicole van de Staak Vanaf mei 1997 is hij als post-doc werkzaam op het Centraal Hematologisch Laboratorium (hoofd: dr. E.J.B M. Mensink) van het academisch ziekenhuis "St Radboud" te Nijmegen.

Slow the Progression of Non-Hodgkin's Lymphoma and Increase your Patient's Survival



In the treatment of low grade non-Hodgkin's lyphoma, maintenance therapy with Intron A can significantly increase your patient's disease free survival and overall survival[1,2,3,4] and ensure a better quality of life.[1]

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NHL - Intron A®

NAAM VAN HET GENEESMIDDEL: Intron A® Injection (interferon alfa-2b) Poeder voor injectievloeistof, Injectievloeistof (voor subcutane, intramusculaire en intralaesionale toediening) **KWALITATIEVE EN KWANTITATIEVE SAMENSTELLING:** Poeder voor injectievloeistof : Elke injectieflacon Intron A Injection, poeder voor injectievloeistof, bevat 3; 5; 10 of 30 miljoen I.E. interferon alfa-2b. Injectievloeistof : Elke injectieflacon Intron A Injection, injectievloeistof, bevat 10 of 25 miljoen I.E. interferon alfa-2b. **FARMACEUTISCHE VORM:** Poeder voor injectievloeistof; Injectievloeistof. **THERAPEUTISCHE INDICATIES:** Haarcelleukemie, cutaan T-cel lymfoma, oppervlakkige en nodulolceratieve basale celcarcinomen bij patiënten waarvoor een chirurgische behandeling of bestralingstherapie niet geschikt is. Condyloma acuminata bij patiënten die onvoldoende reageren op een andere behandeling. Chronische actieve hepatitis B bij volwassen patiënten, waarbij virale replicatie werd aangetoond door middel van bv. HBV-DNA, DNA polymerase of HBeAg. Chronische hepatitis C/non-A, non-B bij volwassen patiënten met verhoogde leverenzymwaarden en zonder leverdecompensatie ter onderdrukking van de ziekteactiviteit. Studies bij deze patiënten tonen aan dat de behandeling met Intron A Injection normalisatie van de SGPT/ALT-spiegel kan teweegbrengen, klaring van het serum HCV-RNA en verbetering van de leverhistologie. Recent klinisch onderzoek bij patiënten behandeld met Intron A Injection gedurende 12 - 18 maanden toont aan dat een groter deel van de patiënten een langdurige verbetering vertoont na langdurige behandeling in vergelijking met een behandeling van 6 maanden. Sarcoom van Kaposi bij patiënten met AIDS zonder voorgeschiedenis van opportunistische infecties. Multipel myeloom als onderhoudsbehandeling bij patiënten die een objectieve remissie hebben bereikt na een inductiebehandeling. Non-Hodgkin lymfoom : Als adjuvanstherapie in combinatie met een geschikte chemotherapie zoals een CHOP-kuur bij de behandeling van follicular lymfoom met een hoge tumorlast (Stadium III of IV). **CONTRA-INDICATIES :** Bij patiënten die overgevoelig zijn voor interferon alfa-2b of voor één van de andere bestanddelen van Intron A Injection. Reeds bestaande ernstige hartaandoeningen; ernstige stoornis van de nier- of leverfunctie; epilepsie en/of aangetaste functie van het centraal zenuwstelsel (zie 4.4. Speciale Waarschuwingen); chronische hepatitis met gevorderde gedecompenseerde aandoening van de lever of levercirrose; chronische hepatitis bij patiënten die recentelijk behandeld werden of onder behandeling zijn met immunosuppressiva, met uitzondering van "steroid withdrawal" op korte termijn; auto-immuun hepatitis, voorgeschiedenis van auto-immune aandoeningen, ontvangers van een transplantaat onder immunosuppressie ; reeds bestaande schildklieraandoening, tenzij deze aandoening onder controle is met een conventionele behandeling. **SPECIALE WAARSCHUWINGEN EN BIJZONDERE VOORZORGEN BIJ GEBRUIK:** Intron A Injection moet met voorzichtigheid gebruikt worden bij patiënten met een verzwakte klinische conditie, zoals bij een voorgeschiedenis van longaandoening (bv. chronische obstructieve longaandoening) of diabetes mellitus, vatbaar voor ketoacidose. Voorzichtigheid is ook noodzakelijk bij patiënten met stollingsstoornissen (bv. thrombophlebitis, longembolie) of ernstige myelosuppressie. Elke patiënt die gedurende een behandeling met Intron A Injection een afwijking van de leverfunctie vertoont moet nauwkeurig gevolgd worden en de behandeling moet worden stopgezet indien de symptomen verergeren. Patiënten met chronische hepatitis B waarbij een verminderde leversynthesefunctie werd vastgesteld, zoals verminderde albuminespiegels of verlengde protrombinetijd en die nochtans voldoen aan de criteria voor behandeling, kunnen een verhoogd risico op decompensatie hebben indien een plotse verhoging van aminotransferases optreedt tijdens de behandeling. Hypotensie kan tijdens de behandeling met Intron A Injection of tot twee dagen na de therapie voorkomen. Behandeling kan noodzakelijk zijn. Voldoende vloeistof moet worden toegediend aan patiënten die behandeld worden met Intron A Injection daar bij enkele patiënten hypotensie, gerelateerd aan vochtdepletie, is waargenomen. Vochttoediening kan dus noodzakelijk zijn. Patiënten met een ziektegeschiedenis van congestief hartfalen, myocardinfarct en/of voorgaande of bestaande ritmestoornissen die met Intron A Injection behandeld worden, vereisen een nauwgezette controle. Bij patiënten met een reeds bestaande hartafwijking en/of diegenen die in een gevorderd stadium van kanker zijn, dient een ECG routinematig gemaakt te worden, zowel vóór als tijdens de behandeling. Hartritmestoornissen (vooral supraventriculair) reageren gewoonlijk op conventionele therapie, maar het kan noodzakelijk zijn de behandeling met Intron A Injection te stoppen. **BIJWERKINGEN:** Systemische Toediening De meest voorkomende bijwerkingen zijn koorts, vermoeidheid, hoofdpijn en spierpijn. Koorts en vermoeidheid waren reversibel binnen 72 uur na onderbreking of stopzetting van de behandeling. Deze verschijnselen zijn dosisgebonden. In de hepatitisgroep waren deze effecten gering tot matig. Andere frequent voorkomende bijwerkingen zijn rigor, anorexie en misselijkheid. Minder frequent voorkomende bijwerkingen zijn braken, diarree, gewrichtspijn, asthenie, slaperigheid, duizeligheid, droge mond, haaruitval, griepachtige verschijnselen, rugpijn neerslachtigheid, malaise, pijn, toegenomen transpiratie, smaakverandering, prikkelbaarheid, slapeloosheid, verwarring, verminderd concentratievermogen en hypotensie. Zelden waargenomen bijwerkingen zijn buikpijn, rash, zenuwachtigheid, reacties op de injectieplaats, paresthesie, herpes simplex, pruritus, oogpijn, angstgevoelens, neusbloeding, hoesten, faryngitis, longinfiltratie, pneumonitis, pneumonie, verminderd bewustzijn, gewichtsverlies, gelaatsoedeem, dyspnoea, dyspepsie, tachycardie, hypertensie, verhoogde eetlust, verminderd libido, verlaagde gevoeligheid, smaakperversie, dunne ontlasting, tandvleesbloeding, neuropathie en polyneuropathie. In zeldzame gevallen kwamen hyper- en hypothyroïdie voor. Levertoxiciteit met fatale afloop werd zelden waargenomen. UR Januari 1995.

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Arrangementen

Zakenbesprekingen

Stellingen

behorende bij het proefschrift:

The role of the *bcl-2* gene family in malignant hematopoiesis and their use for monitoring of disease

door J.P.P. Meijerink

17 december 1997

1. Door middel van de polymerase ketting reaktie is het mogelijk om residuale lymfoom cellen met een chromosomale translocatie tussen chromosoom 14 en 18, de t(14,18), in follikulaire non-Hodgkin lymfoom patienten te kwantificeren tijdens het ziekte verloop met een gevoeligheid van tenminste 1 lymfoomcel op de 150 000 normale cellen
   Dit proefschrift

2. Het is aannemelijk dat het gemeten aantal lymfoomcellen met een t(14,18) in het bloed van follikulaire non-Hodgkin lymfoom patienten tijdens het ziekteproces een goede maat is voor ziekte-activiteit
   Dit proefschrift

3. De kans op het ontstaan van een t(14,18) tengevolge van een foutieve koppeling tijdens het immunoglobuline zware keten rangschikkingsproces neemt af naarmate dit proces verder gevorderd is.
   Dit proefschrift

4. Ongeveer 20% van de hematopoietische cellijnen van maligne origine bevat een mutatie in het pro-apoptotische *bax* gen, waardoor een niet-functioneel BAX eiwit gevormd wordt.
   Dit proefschrift

5. Het voorkomen van identieke insertie/deletie mutaties in een monodeoxyguanosine stretch in het *bax* gen van cellijnen van acute lymfatische leukemie (ALL) origine zoals ook gevonden bij 50% van de colon kanker patienten met het microsatelliet mutator fenotype (MMP+) suggereert het bestaan van een MMP+ ALL subpopulatie.
Rampino *et al*, Science, 14 februari 1997,en dit proefschrift

6. Het BH1 domein van BAX is in tegenstelling tot het BH1 domein van BAK wel degelijk van belang voor de pro-apoptotische activiteit van BAX.
Dit proefschrift

7. Het ontrafelen van het werkingsmechanisme waarmee de *bcl-2* gen familie celdood reguleert, zal leiden tot de ontwikkeling van nieuwe therapeutische behandelingen van kanker.

8. Succes in de wetenschap is mede afhankelijk van de naïviteit van de onderzoeker.

9. Iedereen rondt in het leven zijn of haar eigen Kaap Hoorn.
Henk van de Velde (solozeiler)

10. De verhoging van de accijns op benzine ter verbetering van de infrastructuur van het Nederlandse wegenstelsel zou beter aangewend kunnen worden voor het subsidiëren van het openbaar vervoer, waardoor het fileprobleem effectiever benaderd wordt.

11. Fight like a brave, don't be a slave,
No one can tell you, you have got to be afraid.
Red Hot Chili Peppers

12. Nil volentibus arduum (niets is te moeilijk voor hen die willen).

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