

Translocation t(14;18) and gain of chromosome 18/*BCL2*: effects on *BCL2* expression and apoptosis in B-cell non-Hodgkin's lymphomas

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Gain of chromosome 18q and translocation t(14;18) are frequently found in B-cell non-Hodgkin's lymphomas (B-NHL). Increased *BCL2* transcription and *BCL2* protein expression have been suggested to be the result of the gain. We utilized FISH, PCR and array CGH to study *BCL2* and chromosome 18 copy number changes and rearrangements in 93 cases of B-NHL. *BCL2* protein was expressed in >75% of the tumor cells in 92% of the cases by immunohistochemistry. Gain of *BCL2* was associated with a 25% increase in *BCL2* expression levels (immunoblotting), whereas t(14;18) resulted in a 55% increase in *BCL2* levels compared to cases without *BCL2* alterations. The tumor cell (spontaneous) apoptotic fractions were similar for the cases with different *BCL2* genotypes. However, the normal cell apoptotic fractions were higher for the tumors with t(14;18) compared to the tumors without *BCL2* alterations, while the tumors with gain of *BCL2* only showed intermediate levels. Low-level gains of parts of chromosome 18 were found in 14 of the 38 B-NHL cases with t(14;18), with a consensus region 18pter-q21.33 that did not include the *BCL2* gene. The 11 cases with 18q gain only showed a consensus region encompassing 18q21.2–18q21.32 and 18q21.33, which contain *PMAIP1/MALT1* and *BCL2*, respectively.

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Introduction

Translocation of the *BCL2* gene (18q21.3) to the *IGH* transcriptional enhancer, as a result of the t(14;18)(q32;q21), causes constitutive overexpression of the antiapoptotic *BCL2* protein.¹ Translocation t(14;18) is the primary tumorigenic event in most follicular lymphomas (FL) and some diffuse large B-cell lymphomas (DLBCL).^{2–4} Increased *BCL2* levels confer extended survival to B-cells, and may thus cause accumulation of cells likely to be targets for additional oncogenic events and transformation.^{5,6}

Gain of chromosome 18 material has been detected frequently in non-Hodgkin's lymphomas (NHL), lymphomas with t(14;18) included.^{7–18} It has been suggested that *BCL2* could be the target gene in the DLBCL cases with gain of 18q.^{19,20} *BCL2* protein expression was enhanced in the tumors with either t(14;18) or *BCL2* amplification; the latter two aberrations appeared to be exclusive.¹⁹ This is in agreement with the study of Horsman *et al*,¹⁷ showing that der(18)t(14;18),

not containing the *BCL2* coding sequences, was frequently gained in FL cases with *BCL2* rearrangement. In contrast, the *BCL2* gene was found to be included in the 18q amplica in lymphomas with t(14;18).²¹ The proto-oncogene *MALT1*, also involved in the t(11;18)(q21;q21) translocation,²² is another possible target in some B-cell non-Hodgkin's lymphomas (B-NHL) with 18q21 gain.²⁰ The expression of *PMAIP1* at locus 18q21.3 was upregulated in transformed FL with 18q21 gain,²¹ as well as in some cases of DLBCL.²³

We have studied the impact of *BCL2* genotypes on phenotypes such as *BCL2* expression, apoptosis, proliferation and survival in 93 cases of B-NHL. Copy number alterations on chromosome 18 were also assessed by array CGH and FISH.

Patients and methods

Tumor samples

Cell suspensions in DMSO prepared from 93 individual lymph node biopsies, histologically and immunologically typed as B-NHL, were included in this study. Preparation of the cell-suspensions, as well as the clinical characteristics and treatment of the patients, has been described in previous studies.^{15,24,25} Table 1 gives the classification of lymphomas according to WHO, as well as previously published data for gains and losses of chromosome 18 by conventional CGH, DNA index and malignant/normal cell apoptotic fractions.^{15,24}

Detection of t(14;18) by conventional and long-range PCR

The oligonucleotide primer for the 150-bp MBR was 5'-AAC TCT GTG GCA TTA TTG-3' and for the mcr 5'-GAC TCC TTT ACG TGC TGG TAC-3'. The primers were combined with a consensus *IGH* joining region primer: 5'-ACC TGA GGA GAC GGT GAC C-3' in the conventional PCR experiments. Long-range PCR, as described by Akasaka *et al*,²⁶ was applied to detect translocations outside the MBR and mcr as well. A primer hybridizing to a sequence 5' of the MBR (5'-CAC AAG TGA AGT CAA CAT GCC TGC CCC AAA CAA AT-3') was used together with a primer specific for the E μ region in the *IGH* gene (5'-CTA GGC CAG TCC TGC TGA CGC CGC ATC GGT GAT TC-3') for the detection of translocations with breakpoints in the MBR and 3'-MBR. 5'-mcr and mcr translocations were detected with a primer recognizing a sequence approximately 9.5 kbp 5' of the mcr (5'-GGT AGA GGT GAA TAC CCC AGG GCT GAG CAG GAA GG-3') and the E μ -primer as above. Cases with MBR or mcr translocations only detectable by long-range PCR,

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Table 1 BCL2 genotypes and phenotypes in 93 cases of B-NHL

Case	Lymphoma subtype (WHO)	DNA index ^a	CGH gain Chr. 18 ^a	PCR t(14;18)	FISH				IHC ^b BCL2	IB ^b BCL2	Apoptosis ^c	
					# red signals	# fusion red/green signals	# blue signals (cen 18 copy #)	BCL2 copy # (derived) ^d			Tumor cells	Normal cells
<i>t(14;18) positive</i>												
377/83	FL I/II	1.00	No	3'-MBR	3	2	2	2	> 75	1.69	0.2	0.6
364/86	FL I/II+SLL	1.02	18pter-q21	MBR	4	3	3	2	> 75	1.88	1.8	2.7
369/86	FL I	1.02	No	mcr	3	2	2	2		1.15	1.4	2.1
021/87	FL I/II	1.00	18pter-q21.1	MBR	4	3	3	2	> 75	1.66	0.6	2.0
241/87	FL I/II	1.02	No	mcr	3	2	2	2	> 75	1.00	1.3	4.0
345/87	FL II	1.00	18q	MBR	4	2	3	3	> 75	1.38	0.4	0.4
018/88	FL II	1.00	No	mcr	3	2			> 75	1.10	2.2	2.0
041/88	FL II	1.03	18q12.3-qter	MBR	4	2	3	3	> 75	1.42	0.9	1.9
046/88	FL II	0.97	No	MBR	3	2	2	2	> 75	1.37	0.3	
047/88	FL I/II	1.00	No	5'-mcr	3	2	2	2		1.29	0.7	1.6
176/88	FL II	1.04	18q12-q22	MBR	4	3	3	2	> 75	1.66	0.3	1.6
233/88	FL II	1.00	No	MBR	3	2	2	2		1.42	0.6	18.8
275/88	FL I/II	1.00	No	MBR	3	2	2	2	> 75	1.32	3.4	1.5
284/88	FL II	1.02	No	MBR	3	2	2	2	> 75	1.29	2.2	2.5
381/88	FL II	1.03	18	mcr	4	2	3	3	> 75		3.4	4.5
399/88	FL III	1.00	No	MBR	3	2	2	2	> 75	0.91	2.5	2.0
468/88	FL II	1.00	No	MBR	3	2	2	2	0	0.59	2.2	2.1
064/89	FL II	1.26	No	MBR	5	4	3	3	> 75	1.45	0.9	1.1
287/89	FL I	1.03	No	MBR	3	2	2	2	> 75	0.84	0.5	20.0
311/89	FL II	1.05	No	MBR	3	2	2	2	> 75	1.36	0.8	
521/89	FL II	1.00	No	5'-mcr	3	2	2	2	> 75	1.07	0.3	0.7
140/90	FL II	1.03	18	MBR					> 75	1.19	0.8	4.0
372/90	FL II	1.07	No	3'-MBR	3	2	2	2	> 75	0.68	3.3	3.2
377/90	FL II	1.06	No	5'-mcr	3	2	2	2	> 75	0.74	3.5	1.2
416/90	FL II	1.00	No	3'-MBR	3	2	2	2	> 75	0.98	2.7	5.4
581/90	FL II	1.22	18q12.3-q21	5'-mcr	4	3	3	2	> 75	1.19	1.8	1.2
635/90	FL I/II	1.00	No	3'-MBR	3	2	2	2		1.18	0.8	6.4
382/91	FL II	1.02	No	MBR	3	2	2	2	0	0.38	0.6	15.4
436/91	FL II	1.04	18,18pter-q21	MBR	5	4	4	2	> 75	1.54	1.3	12.4
489/91	FL I/II	1.03	No	0	3	2	2	2	> 75	1.30	1.6	8.0
021/92	FL II	1.02	No	MBR	3	2	2	2	> 75	0.87	2.8	1.2
103/92	FL II	1.00	No	MBR	3	2	2	2	> 75	1.20	0.5	4.1
130/92	FL I/II	1.03	18pter-q21	MBR	5	3	4	3	> 75	2.14	3.3	5.7
287/88	DLBCL	1.00	18cen-q22	mcr	4	3	3	2	> 75	1.46		
454/88	DLBCL	1.02	No	mcr	3	2	2	2	> 75	1.12	0.9	0.5
34/90	DLBCL+SLL	1.16	No	3'-MBR	3	0	3	3	> 75	2.22	1.2	2.3
472/90	DLBCL	1.16	18pter-q21	MBR	4	3	3	2	> 75	1.33	0.2	2.3
340/91	DLBCL	1.03	No	MBR	3	2	2	2	> 75	1.30	0.0	1.8
<i>BCL2 gain only</i>												
051/90	SLL	1.05	18q12.3-qter	0	4	0	2	4	> 75	1.09	0.8	0.6
154/88	MCL	0.95	No	0	3	0	2	3	> 75		0.1	1.0
265/88	MCL	1.00	18q12-qter	0	3	0	3	3	> 75	1.14	0.4	4.9
309/89	MCL	1.83	No	0	4	0	4	4	> 75	0.89	0.5	0.4
383/91	MCL	0.97	No	0	3	0	2	3	> 75	0.75	8.0	1.8
122/84	DLBCL	1.23	No	0	3	0	3	3	> 75	1.00	2.5	1.9
255/85	DLBCL	1.04	18q21-qter	0	3	0	3	3	> 75	1.09	0.6	0.2
131/89	DLBCL	2.23	18	0	6	0	5	6	> 75	1.02	0.9	0.8
525/89	DLBCL	1.07	18q21-qter	0	3	0	2	3	> 75	0.49	3.0	3.7
050/93	DLBCL	1.00	No	0	5	0	2	5		1.25	1.4	3.7
448/91	MZL	1.05	18	0	3	0	3	3	> 75	1.02	0.2	0.5
8/92	Unclass.	1.00	18	0	3	0	3	3		0.72	3.1	2.8
<i>No BCL2 alterations</i>												
577/90	FL II	1.97	No	0	4	0	4	4	5_15	0.24	7.6	7.6
533/91	FL II	1.00	No	0	2	0		2	> 75	0.64	1.8	1.3
300/92	FL II	1.00	No	0	2	0	2	2	> 75	0.74	8.8	0.9
140/83	SLL	1.00	No	0	2	0		2	> 75	0.97	2.1	0.5
086/85	SLL	1.00	No	0	2	0		2	> 75	1.07	1.5	0.6
320/88	SLL	1.00	No	0	2	0		2	> 75	0.81	0.7	0.7
325/88	SLL	1.00	No	0	2	0		2	> 75	1.31	0.8	0.4
416/88	SLL	1.03	No	0	2	0		2	> 75	0.74	0.2	0.1
470/88	SLL	1.02	No	0					> 75	0.88	3.4	1.6

Table 1 Continued

Case	Lymphoma subtype (WHO)	DNA index ^a	CGH gain Chr. 18 ^a	PCR t(14;18)	FISH				IHC ^b BCL2	IB ^b BCL2	Apoptosis ^c	
					# red signals	# fusion red/green signals	# blue signals (cen 18 copy #)	BCL2 copy # (derived) ^d			Tumor cells	Normal cells
191/89	SLL	1.02	No	0	2	0		2	>75	0.79	0.2	
339/89	SLL	1.00	No	0	2	0		2	>75	0.77	1.1	2.1
445/89	SLL	1.02	No	0	2	0		2	>75	0.64	1.3	2.9
452/89	SLL	1.00	No	0	2	0	2	2	>75	1.08	0.4	0.3
159/90	SLL	1.04	No	0	2	0		2		0.71	1.1	1.9
571/90	SLL	1.00	No	0	2	0		2	>75	0.50	0.7	0.3
156/91	SLL	1.00	No	0	2	0		2	>75	0.70	0.4	2.1
244/91	SLL	1.00	No	0	2	0		2		1.00	1.2	0.3
462/91	SLL	1.00	No	0	2	0		2	>75	0.80	0.5	0.9
225/92	SLL	1.03	No	0	2	0		2	>75	0.63	0.0	0.0
315/92	SLL	1.00	No	0	2	0	2	2	>75	1.16	0.3	
538/92	SLL	1.00	No	0	2	0	2	2	>75	0.75	6.5	1.9
123/84	MCL	1.04	No	0	2	0	2	2	>75	1.00	0.7	8.7
358/87	MCL	1.94	No	0	4	0	4	4	>75	0.90	1.2	2.8
010/89	MCL	1.00	No	0	2	0		2	>75	0.69	2.7	14.4
129/89	MCL	1.02	No	0	2	0		2	>75	0.82	1.5	1.2
428/91	MCL	2.00	No	0	4	0	4	4	>75	0.98	0.4	0.3
037/92	MCL	1.00	No	0	2	0		2	>75	0.96	7.2	8.7
067/92	MCL	0.98	No	0	2	0		2	>75	0.95	8.4	6.0
245/92	MCL	1.03	No	0	2	0	2	2	>75	1.05	0.3	0.0
377/87	DLBCL	1.03	No	0	2	0		2	>75	0.58	0.9	3.5
399/87	DLBCL	1.06	No	0	2	0		2	>75	0.46	3.8	1.9
258/88	DLBCL	1.07	No	0	2	0		2	>75	0.52	2.6	1.3
438/89	DLBCL	1.00	No	0	2	0		2	>75	0.45	3.2	2.2
612/90	DLBCL	1.04	No	0	2	0		2	15_75	0.39	0.7	2.0
070/92	DLBCL	1.01	No	0	2	0		2	15_75	0.61	1.5	0.5
117/92	DLBCL	1.00	No	0	2	0	2	2	>75	0.89	0.8	
277/89	BL	1.04	No	0	2	0	2	2	5-15	0.14	6.7	15.3
458/88	BL-like	1.03	No	0	2	0	2	2	>75	0.98	1.6	9.1
42/92	BL-like	1.03	No	0	2	0	2	2	>75	0.25	21.5	4.9
315/88	MZL	1.00	No	0	2	0		2	>75	0.95	0.5	0.3
237/91	MZL	1.00	No	0	2	0		2	>75	0.87	0.5	0.0
214/88	Unclass	1.00	No	0	2	0		2	>75	0.63	2.9	2.4
Loss of <i>BCL2</i> :												
390/87	DLBCL	1.00	Loss 18q	0	1	0	2	1	0	0.31	7.7	2.2

Bold indicates high-level gain.

^aThe DNA indices (by flow cytometry) and conventional CGH results for these cases have been published.¹⁵

^bIHC = immunohistochemistry; IB = immunoblotting.

^cThe data for apoptosis were published previously.²⁴

^dThe *BCL2* copy number was derived as described in 'Patients and methods'.

and not by conventional PCR, were assumed to have breakpoints in the 3'-MBR or 5'-mcr, respectively. As a control for DNA integrity, a 10 560 bp germline sequence 5' of the mcr was amplified employing the primers 5'-GCG GCT ATT GAG CAC TGA AAT GTG ACT GAA ATG AC-3' and 5'-CAT CCA ACT GGC TTA GGA GGC AAG ACA GAA ACC TT-3'.

Translocation t(14;18) and BCL2/MALT1/PMAIP1/centromere 18 copy number analysis by FISH

A dual-color translocation probe (LSI *IGH* Spectrum Green/LSI *BCL2* Spectrum Orange) from Vysis (Downers Grove, IL, USA) was hybridized as previously described^{27,28} to detect t(14;18) and *BCL2* copy number. This probe set yields red (*BCL2* locus) and green (*IGH* locus) signals. The red and green signals are split and the two colors are colocalized if a translocation event has

occurred. The *BCL2* probe covers the *BCL2* gene and extends approximately 250 kbp 5' as well as 3' of the gene (http://www.vysis.com/ProbeMap_5235.asp). A blue fluorescent centromere 18 probe (CEP 18 Spectrum Aqua, VYSIS) was cohybridized with the t(14;18) probe set. The numbers of total red, cohybridized red/green and blue signals were assessed in a correlated manner.²⁷ der(14)t(14;18), and not the der(18)t(14;18), contains the *BCL2* coding sequences in the cases with breakpoints 3' of the *BCL2* coding sequences (assuming no other complicated rearrangements). The number of fusion signals was assumed to represent the total number of derivative chromosomes, while the number of blue centromere 18 signals was used to assess the total number of chromosome 18 and der(18)t(14;18). If there are no local amplica including the *BCL2* gene, an assumption that will be discussed in 'Results and discussion', the number of *BCL2* coding sequence copies in the cases with t(14;18) can be calculated as follows: #*BCL2*

coding sequences = $2 \times \# \text{red signals} - \# \text{red/green fusion signals} - \# \text{cen 18 signals}$.

FISH was also applied to examine the copy numbers of sequences corresponding to the RP11-108P20 (MALT1), RP11-103A19 (PMAIP1), RP11-46D1, RP11-563B11 and RP11-154H12 clones on chromosome 18, as well as the RP11-12F16 clone covering PAC820M16 at the telomere of chromosome 14 (BACPAC Resources, Oakland, CA, USA). The DNA was isolated, nick translated and labelled with Cy3-dUTP (Amersham Biosciences, Buckinghamshire, UK).²⁹ Hybridization was performed with 40 ng of labelled probe and 1 μg Cot-1 DNA (Invitrogen, Carlsbad, CA, USA) in 10 μl 50% formamide, $2 \times \text{SSC}$, 10% dextran sulfate.

Chromosome 18 copy number alterations examined by array CGH

The genomic microarrays printed covered the human genome at ~ 1 mbp resolution, and contained 77 BAC (RPC1-11 library³⁰) and one PAC clone for chromosome 18 (Wellcome Trust Sanger Institute, Cambridge, UK). The localization of each clone was obtained from Ensembl. DNA isolation, amplification by DOP-PCR and preparation of microarrays were carried out as described previously.³¹ The PCR products were arrayed in quadruplicate onto amine-binding slides (CodeLink, Amersham Biosciences). Two sets of duplicate spots were printed in separate areas of the array.

Sequence-verified human cDNA clones (approximately 40 000 clones) were purchased from Invitrogen. The cDNAs were amplified by PCR, purified, resuspended in $3 \times \text{SSC}$ and printed onto two Corning Gamma Amino Propyl Silane (GAPS) slides (Corning, NY, NY, USA) with each of the two slides consisting of 20 000 cDNA clones. The chromosomal position of the printed cDNAs was determined by BLAT analysis of the UCSC human genome browser (<http://genome.ucsc.edu/>).

DNA (1.5 μg) was digested, purified and labelled with Cy3-dCTP or Cy5-dCTP (Perkin Elmer, Boston, MA, USA) as described previously.³¹ Labelled tumor and sex-matched reference DNA were combined and ethanol precipitated together with 135 μg human Cot-1 DNA. The DNA and 400 μg yeast tRNA (Invitrogen) was dissolved in 108 μl 50% formamide, 10% dextran sulfate, 4% SDS, $2 \times \text{SSC}$ and denatured for 10 min at 70°C. Hybridization (48 h at 37°C) and washing was performed in an automated hybridization station, GeneTAC (Genomic Solutions/Perkin-Elmer).

Microarrays were scanned using an Agilent G2565BA scanner (Agilent Technologies, Palo Alto, CA, USA). The spots were segmented, local background was subtracted, and the fluorescence intensities and the intensity ratio of the two dyes were calculated for each spot. Further data processing, including filtering and normalization, was carried out using M-CGH, a MATLAB toolbox specifically designed for this purpose.³²

BCL2 protein analysis by immunohistochemistry (IHC) and immunoblotting (IB)

The protocols for IHC and IB, and the interpretation and evaluation of the data, have been described in detail.^{25,28,33,34} The anti-BCL2 antiserum C-15 (Santa Cruz Biotech, Santa Cruz, CA, USA) and a monoclonal anti-BCL2 antibody kindly provided by DY Mason (Oxford, UK) were used for IHC and IB, respectively.

Results and discussion

BCL2 genotype

PCR and long-range PCR were used to detect and characterize the translocation t(14;18) involving the *BCL2* and *IGH* loci (Figure 1a, Table 1). t(14;18) was found by PCR in 32 of 36 FL and in five of 18 DLBCL (Table 1). The *BCL2* breakpoints were within the MBR in 22 cases (59%) and within the mcr in six (16%) cases, that is, t(14;18) was also detected by standard PCR in these cases (standard PCR data not shown). The breakpoints in the remaining nine cases were distributed between these regions (3'-MBR, 5'-mcr) (Table 1). The t(14;18) translocations were also assessed by FISH (Figure 1b, Table 1). *BCL2* rearrangement results by PCR/long-range PCR and FISH showed concordance with the exception of two cases (Table 1; cases 489/91 and 34/90). Case 489/91 may have a t(14;18) involving the 5' region of *BCL2*, which will not be found by PCR with the primers employed in this work. Case 34/90 may have a more complex rearrangement, with, for example, insertion of a short fragment of the *IGH* gene containing the $\text{E}\mu$ enhancer between the MBR and the mcr.

The *BCL2* coding sequence copy number was derived from the FISH counts as described in 'Patients and methods'. Eight of the lymphomas with a translocation t(14;18) involving the *BCL2* locus had increased numbers of red signals (>3) and red/green signals (>2) compared to the majority of the cases with t(14;18) (Table 1; cases: 364/86, 21/87, 176/88, 287/88, 472/90, 581/90, 436/91, 130/92). However, in seven of these eight cases (excluding case 130/92), the number of centromere 18 signals was increased correspondingly (Table 1), indicating that der(18)t(14;18), and not der(14)t(14;18) containing the *BCL2* coding sequence, was gained (Figure 1b, case 21/87). Case 130/92 may additionally have gained an extra-chromosome 18. Three cases with t(14;18) (345/87, 41/88, 381/88) had four red and three blue signals, but only two red/green fusion signals, indicating that an extra copy of chromosome 18 was gained. Case 64/89 had a DNA index of 1.26, and by FISH this case had 5 red signals, four fusion signals and 3 centromere 18 signals. This result can be due to gain of both the translocation-derived chromosomes. Case 489/91 was suggested to have a t(14;18) 5' of the *BCL2* gene from the PCR and FISH results, and the *BCL2* coding sequences are found on der(18)t(14;18). However, this case showed the expected pattern for a balanced translocation (three red, two red/green and two blue), and the *BCL2* copy number was therefore 2.

In the cases without t(14;18), the number of red spots in the FISH experiments gives the *BCL2* copy number directly. Of 55 lymphoma cases, 12 without t(14;18) had gain of *BCL2* and/or chromosome 18 (five DLBCL, four MCL, one SLL, one MZL, and 1 unclassified B-NHL; Table 1, Figure 1b: case 265/88). Hence, *BCL2* was gained in the seven tumors without *BCL2* translocation that displayed 18q gain by conventional CGH, as well as in five others (122/84, 154/88, 309/89, 383/91, 50/93). The near-tetraploid cases 577/90, 358/87 and 428/91, which had no gain of chromosome 18 by conventional CGH, had four *BCL2*/centromere 18 copies (Table 1). The latter cases with no relative gain of *BCL2* compared to the DNA index were not included in the group with gain of *BCL2*. The suggested composition and number of chromosome 18 derived chromosomes will be discussed in more detail after presentation of the array CGH results (Figure 4, Table 2).

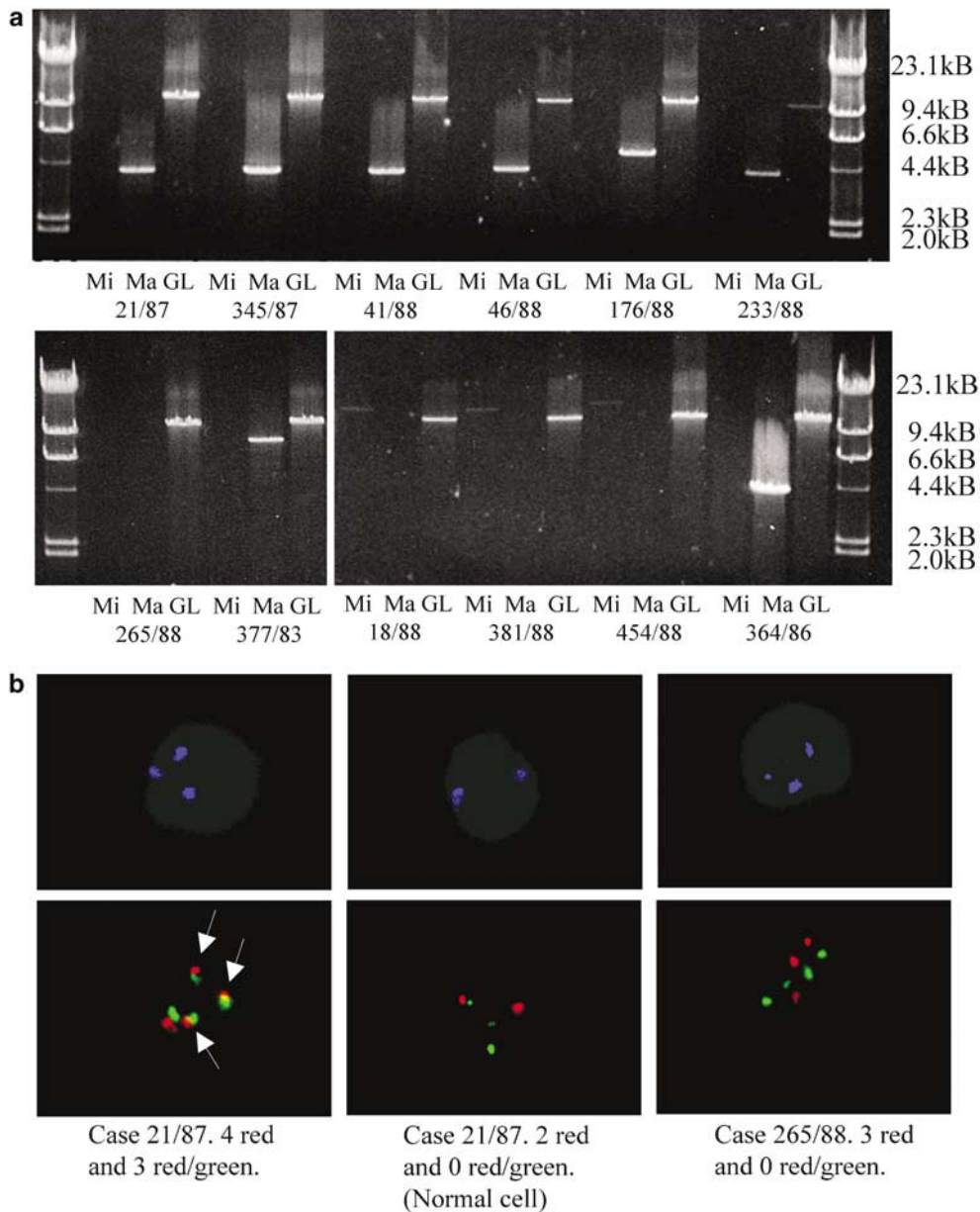


Figure 1 *BCL2* genotypes in B-cell NHL. (a) Long-range PCR was applied for the detection of t(14;18) translocations. PCR was performed with primers for t(14;18) with breakpoints on chromosome 18 within and 3' of the major breakpoint cluster region (Ma), or within and 5' of the minor breakpoint cluster region (Mi). The reverse primer was in the E μ region of the *IGH* gene. The germline product (GL) of length 10560bp was amplified with primers for a sequence located in the 3'-MBR region. (b) Detection of t(14;18) and *BCL2*/centromere 18 copy number by FISH. The *BCL2* probe was labelled in red, the *IGH* probe in green and the centromere 18 probe in blue. The upper panels show centromere 18 (blue) and background nuclear fluorescence (green), while the lower panels show *BCL2* (red) and *IGH* (green) for the same fields as shown in the upper panels. Arrows indicate colocalization (translocation).

BCL2 protein levels and dependence on *BCL2* genotype

IHC showed that more than 75% of the tumor cells expressed *BCL2* in 79 of 86 tumors examined (Table 1). Hence, *BCL2* expression is a property of the tumor cells in almost all B-NHL. IB could be used to assess the expression levels of *BCL2* (Figure 2a, Table 1), because of the high fraction of tumor cells in the samples (mean: 75%).¹⁵ The cases with both t(14;18) and *BCL2* gain had higher expression of *BCL2* (mean: 1.63 relative to p83) than the tumors with t(14;18) only (mean: 1.19; $P=0.008$). The latter cases had higher *BCL2* levels than the ones with *BCL2* gain only (mean: 0.96; $P=0.05$), which again had higher *BCL2*

levels than the tumors with no *BCL2* alterations (mean: 0.77; $P=0.03$; Figure 2b). Case 390/87 with loss of one *BCL2* allele was excluded from this analysis. Hence, gain of *BCL2*, in most cases one extra copy, was associated with a 25% increase in *BCL2* expression compared to the cases without *BCL2* alterations, whereas t(14;18) caused a 55% increase in *BCL2* expression. There was no difference in the expression of *BCL2* for the different translocation regions ($P=0.70$). The expression levels of *BCL2* were also compared in 17 cases of DLBCL. Case 34/90 had both t(14;18) and gain of *BCL2*, four cases showed t(14;18) only, five cases carried *BCL2* gain and seven cases had no *BCL2* alteration. The mean *BCL2* protein levels in these

Table 2 BAC array and FISH results for the 27 NHL cases with gains on chromosome 18

Case	Lymphoma subtype (WHO)	DNA index	BAC array CGH		FISH				Suggested number of chromosome 18 and derivatives
			Chromosome 18 gain (loss)	Chr14 qter gain (pac820m16)	cen 18 copy #	MALT1 copy #	PMAIP1 copy #	BCL2 copy #	
<i>t(14;18) positive</i>									
364/86	FL I/II+SLL	1.02	pter-ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
021/87	FL I/II	1.00	pter-ba215a20	-	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
345/87	FL II	1.00	pter-qter	-	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
041/88	FL II	1.03	pter-qter	+	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
176/88	FL II	1.04	pter-ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
381/88	FL II	1.03	pter-qter	-	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
064/89	FL II	1.26	pter-qter	-	3	3	3	3	1 × 18+2 × der(14)t(14;18)+2 × der(18)t(14;18)
140/90	FL II	1.03	pter-qter	-					2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
581/90	FL II	1.22	pter-ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
436/91	FL II	1.04	pter-ba215a20	+	4	4	4	2	1 × 18+1 × der(14)t(14;18)+3 × der(18)t(14;18)
130/92	FL I/II	1.03	pter-ba215a20	+	4	4	4	3	2 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
287/88	DLBCL	1.00	cen-ba215a20 (pter-cen)	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18) (loss 18p)
34/90	DLBCL+SLL	1.16	pter-qter	-	3	3	3	3	3 × 18 (complex rearrangement)
472/90	DLBCL	1.16	pter-ba215a20 (ba45a1-qter)	+	3		3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18) (loss 18q23)
<i>Gain only</i>									
051/90	SLL	1.05	ba25c13-qter	-	2	4	4	4	2 × 18 (gain 18q12.3-qter)
154/88	MCL	0.95	ba397a16-ba45a1 (ba169f17-qter)	-	2	3	3	3	2 × 18 (gain 18q21.2-q22.3(?), loss 18q22.3-qter)
265/88	MCL	1.00	cen-qter (pter-cen)	-	3	3	3	3	3 × 18 (loss 18p)
309/89	MCL	1.83	pter-qter	-	4			4	4 × 18
383/91	MCL	0.97	ba116k4-qter	-	2	3	3	3	2 × 18 (gain 18q21.2-qter(?))
122/84	DLBCL	1.23	pter-qter	-	3	3	3	3	3 × 18
255/85	DLBCL	1.04	pter-qter	-	3	3	3	3	3 × 18
390/87	DLBCL	1.00	ba296e23-ba178f10 (rest of 18)	-	2			1	1x18 (gain 18cen-q11.2)
131/89	DLBCL	2.23	pter-qter	-	5	6	6	6	5 × 18 (gain 18q(?))
525/89	DLBCL	1.07	ba116k4-qter	-	2	3	3	3	2 × 18 (gain 18q21.2-qter (?))
050/93	DLBCL	1.00	ba116k4-ba28f1	-	2	3	3	5	2 × 18 (gain 18q21.2-21.33)
448/91	MZL	1.05	pter-qter	-	3	3	3	3	3 × 18
8/92	Unclass.	1.00	pter-qter	-	3	3	3	3	3 × 18

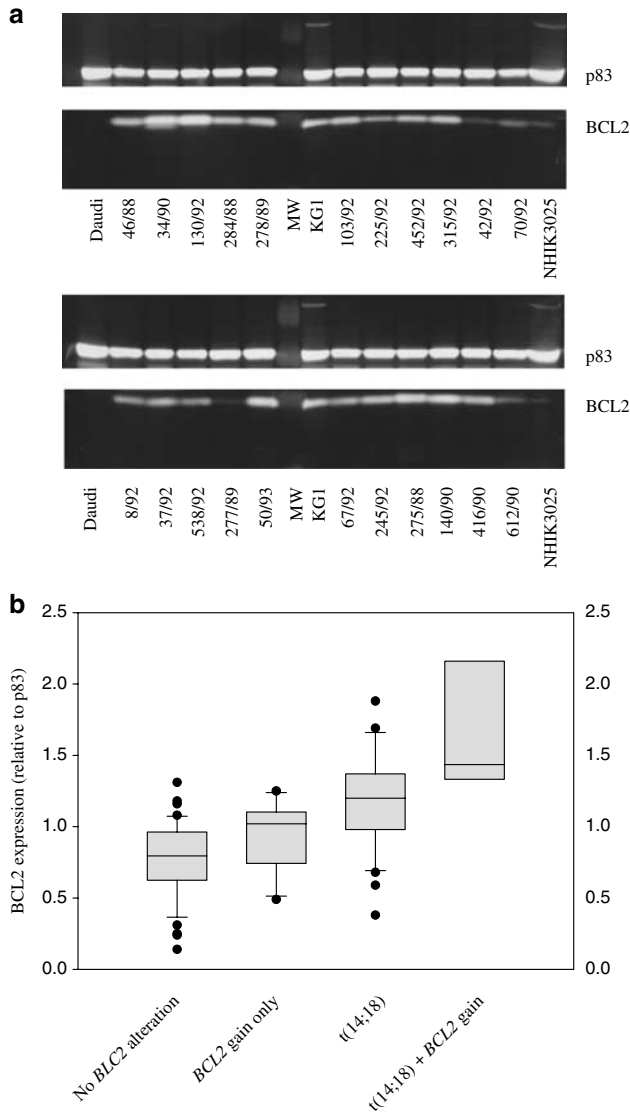


Figure 2 BCL2 protein analysis and dependence on *BCL2* genotype. (a) BCL2 and p83 protein levels were assessed by IB. p83 is an abundantly expressed nuclear protein used as a control for loading. The cell lines KG1 and Daudi served as positive and negative controls, respectively. (b) BCL2 expression according to *BCL2* genotype.

groups of DLBCL cases were 2.22, 1.30, 0.97 and 0.56, respectively, which was similar to the trend observed for the whole material. Hence, the gene dosage effect on BCL2 expression was less pronounced compared to the effect of t(14;18) in this study than in a previous one,¹⁹ where similar BCL2 protein levels were measured in DLBCL with *BCL2* amplification and t(14,18), respectively.

Cellular and clinical phenotypes and dependence on *BCL2* genotype and phenotype

There was a highly significant negative correlation between the expression of BCL2 and levels of spontaneous apoptosis in the tumor cells ($P < 0.001$) (Figure 3a). The correlation was not very strong ($r^2 = 0.11$), probably reflecting the fact that mediators

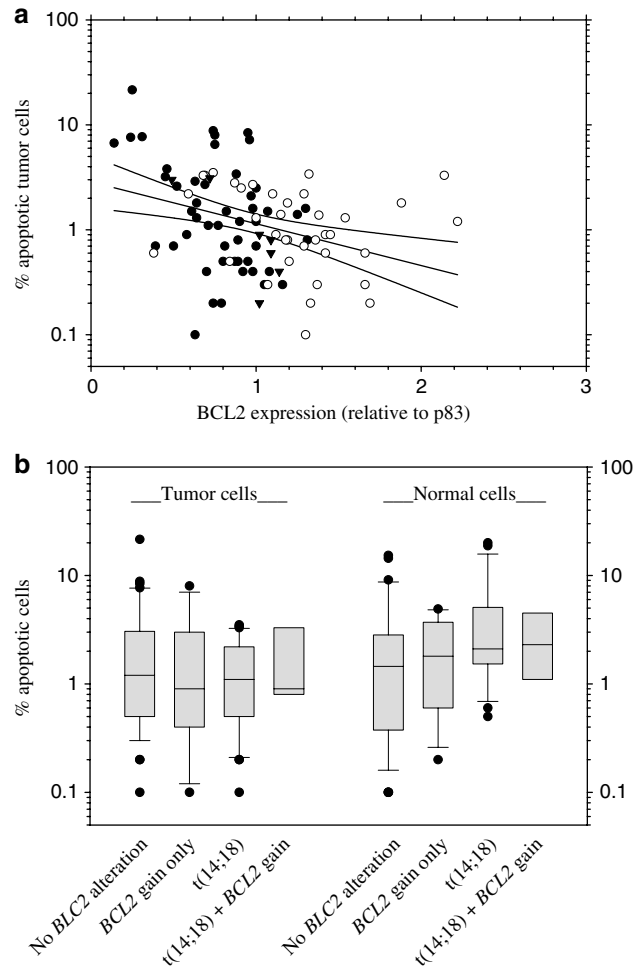


Figure 3 Apoptotic fractions correlated with BCL2 expression and dependence on *BCL2* genotype. (a) There was a negative correlation between apoptosis levels²⁴ and BCL2 protein levels (Figure 2a) ($P < 0.001$, $r^2 = 0.11$). ● = cases with no *BCL2* alteration; ○ = cases with t(14;18); and ▼ = cases with *BCL2* gain only. The regression line and the 95% confidence interval are also shown. (b) The apoptotic fractions of tumor cells and normal cells according to *BCL2* genotype.

other than BCL2 can regulate apoptosis in transformed B cells.^{35–37} However, the median tumor apoptotic fractions in the cases with both t(14;18) and *BCL2* gain (0.9%), t(14;18) (1.1%), *BCL2* gain (0.9%) and no *BCL2* alterations (1.2%) were not different ($P = 0.86$; Figure 3b). Surprisingly, levels of spontaneous apoptosis in the normal cells in the t(14;18)-positive tumors without (2.1%) and with (2.3%) *BCL2* gain were higher than the corresponding tumor cell apoptotic fractions ($P = 0.002$ for the cases with t(14;18); Figure 3b). No difference in tumor and normal cell apoptotic fractions was found for tumors with *BCL2* gain only and no *BCL2* alterations ($P = 0.86$ and $= 0.96$, respectively). Furthermore, the normal cells of t(14;18)-positive tumors showed increased apoptotic fractions compared to the normal cells in tumors with no *BCL2* alterations (median: 2.1 and 1.45%, respectively; $P = 0.02$). The normal cells in the tumors with *BCL2* gain only showed intermediate apoptotic fractions (median: 1.8%). A mean of 23% (\pm s.d. = 11%)/1.2% (\pm 1.2%), 17% (\pm 11%)/2.2% (\pm 1.8), and 23% (\pm 14%)/1.5% (\pm 2.4%) of the cells in the suspensions were T lymphocytes/monocytes in the cases with t(14;18)

($N=36$), with *BCL2* gain only ($N=11$) and without *BCL2* alterations ($N=43$), respectively, as assessed by immunofluorescence. Since the average tumor cell fraction was 75%,¹⁵ this means that the large majority of normal cells in the samples were T-lymphocytes. It may be speculated that disruption of an apoptotic pathway (increased *BCL2* levels) in the tumor cells may cause the release of proapoptotic mediators causing cell death in normal T-lymphocytes in the affected lymph node.

The median tumor cell S-phase fraction was higher in the B-NHL with *BCL2* gain only (6.2%), compared to the cases with t(14;18) (1.1%; $P<0.001$), and without *BCL2* alterations (1.3%; $P=0.02$). The normal cell S-phase fractions were not different in the lymphomas with various *BCL2* genotypes (0.9–1.5%; $P>0.17$).

Patients with lymphomas with gain of *BCL2* only had a poor prognosis (relative risk: 2.9, $P=0.002$), in agreement with the results of Bea *et al.*³⁸ Cox multivariate survival analysis was employed to assess the prognostic value of *BCL2* gain when entered together with the prognostic parameters found in an earlier study (TP53 aberrations, 11q21–23.1 deletions, 6p gain, IPI).¹⁵ The results showed that *BCL2* gain only had no independent prognostic value ($P=0.09$), probably due to covariation with the (stronger) prognostic parameters 6p gain ($P=0.007$), and TP53 aberrations ($P=0.05$). The six patients with lymphomas with concomitant *BCL2* rearrangement and amplification, as well as the others with t(14;18) only, survived slightly longer than the remaining patients, but the differences were not significant ($P>0.29$).

Our previous study showed that spontaneous apoptosis in the tumor cells has no prognostic value in this tumor material.³⁹ The apoptotic fraction of the normal cells did not have any prognostic value for the whole group of patients ($P=0.91$, cutoff at median = 1.9%), or for the patients with FL ($P=0.62$). The gene expression patterns in the normal cells in FL have prognostic value,⁴⁰ however, the majority of the genes with prognostic value in this study were not apoptosis associated.

Assessment of DNA copy number changes on chromosome 18 by array CGH and FISH

Array CGH was applied to define amplified regions on chromosome 18 in more detail (Figure 4, Table 2, web Table: radium.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls). The observed ratios in microarray experiments will generally depend on the linearity and dynamic range of the assay, as well as on the fraction of normal cells in the sample, tumor heterogeneity and the DNA index of the tumor. We used the near-diploid DLBCL cell line U698 to test the dynamics of our array CGH platforms. U698 has a narrow amplicon (~1 mbp) at chromosome 6p, which was observed by both BAC and cDNA array CGH. The expected ratio of approximately 10 was observed with one probe on the BAC array (20 copies of the 6p locus by FISH), whereas the ratio at this locus was only two when U698 cells were examined by cDNA array CGH with several more densely spaced probes. Thus, there was not a one-to-one relationship between copy number and ratio in the cDNA array experiments. There was also noise in the data, and the sensitivity for detection of copy number aberrations was much lower for cDNA arrays compared to BAC arrays. The primary tumors also contained an average of 25% normal cells,¹⁵ which tended to bring the ratios towards unity. Hence, the ratios in the BAC array CGH experiments were expected to be somewhat lower than predicted from the copy numbers in the case of gains. No sharp high-level amplifications were

detected by BAC array CGH in any of the 93 tumors, and the \log_2 ratios varied between -1.05 and $+0.92$ (web Table: radium.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls).

The spacing between the probes on the BAC array was approximately 1 mbp. We therefore employed cDNA arrays with a much closer spacing of the probes (~100 kbp) to reveal any high-level amplification of small regions that could have been missed with the BAC arrays. No gains were detected on chromosome 18 by cDNA array CGH in any of the tumors listed in Table 2, indicating that there were no high-level amplification of regions of size larger than ~100 kbp. FISH showed that one to two extra copies of *MALT1* and *PMAIP1* were gained compared to the average copy number of the tumors in Table 2, in agreement with the low-level gains observed by BAC array CGH.

Seven of the tumors with t(14;18) had gains that extended from 18pter to clone ba215a20 at 18q21.33, thus defining the consensus region of gain (defined by at least three tumors). Case 287/88 had gain from the centromere to clone ba215a20. Clone ba215a20 is the closest clone (in this study) centromeric of *BCL2*, and since the segment recognized by the 14qter probe pac820m16 was gained in seven of these eight tumors (Figure 4, Table 2; none of these tumors had any other gains on chromosome 14), it is strongly suggested that one extra copy of der(18)t(14;18) was gained in cases 364/86, 21/87, 176/88, 472/90 and 581/90. Case 472/90 showed loss from ba45a1 to 18qter, indicating that either the der(14)t(14;18) or the untranslocated chromosome 18 had lost this region. Case 287/88 may have gained an extra copy of a der(18)t(14;18) lacking the p-arm, or the p-arm may have been lost from the untranslocated chromosome 18 and 1 of the der(18)t(14;18). Case 436/91 had probably gained two extra copies of der(18)t(14;18), while case 130/92 may have gained one copy of der(18)t(14;18) and one of chromosome 18. These results are in agreement with the study of Horsman *et al.*¹⁷ which showed that der(18)t(14;18) was frequently gained in FL cases with *BCL2* rearrangement. The six other cases with chromosome 18 gain and *BCL2* rearrangement showed gain of the entire chromosome 18. There were no indications of local amplica in the tumors with t(14;18), also justifying the calculation of *BCL2* copy numbers performed in Table 1.

Six of the 12 tumors with *BCL2* gain, but without translocation had gains extending from 18pter to 18qter, suggesting that an extra copy of the whole chromosome 18 was gained in these cases. Case 390/87, with loss of *BCL2*, had a narrow amplicon extending from the centromere to ba17i14. The other lymphomas with 18q gain only defined the consensus region to be 18q21.2 (ba116k4)–18qter (Figure 4). In case 50/93, the ratio was increased for clone ba28f1, which was in agreement with the five copies detected by FISH for this tumor. However, there was a clear decrease in ratio telomeric of ba28f1 also in cases 154/88, 383/91 and 525/89 (Figure 4). Also, the ratios appeared to be lower between ba350k6 and ba28f1 in cases 154/88, 383/91, 525/89 and 50/93. If these fine structures in the amplica were taken into consideration, the consensus regions of gain were 18q21.2 (ba116k4)–18q21.32 (ba350k6) and 18q21.33 (ba28f1).

Although the level of amplification was lower in the present study employing primary tumor samples, we found the same regions of gain as Sanchez-Izquierdo *et al.*²⁰ centered on the *BCL2* and *MALT1/PMAIP1* loci. However, while the cell lines of MCL and DLBCL origin showed preferential amplification of the region containing the *BCL2* locus,²⁰ we found coamplification of these regions in the primary MCL cases 154/88 and 383/91, and the DLBCL cases 525/89 and 50/93. The high-level gains

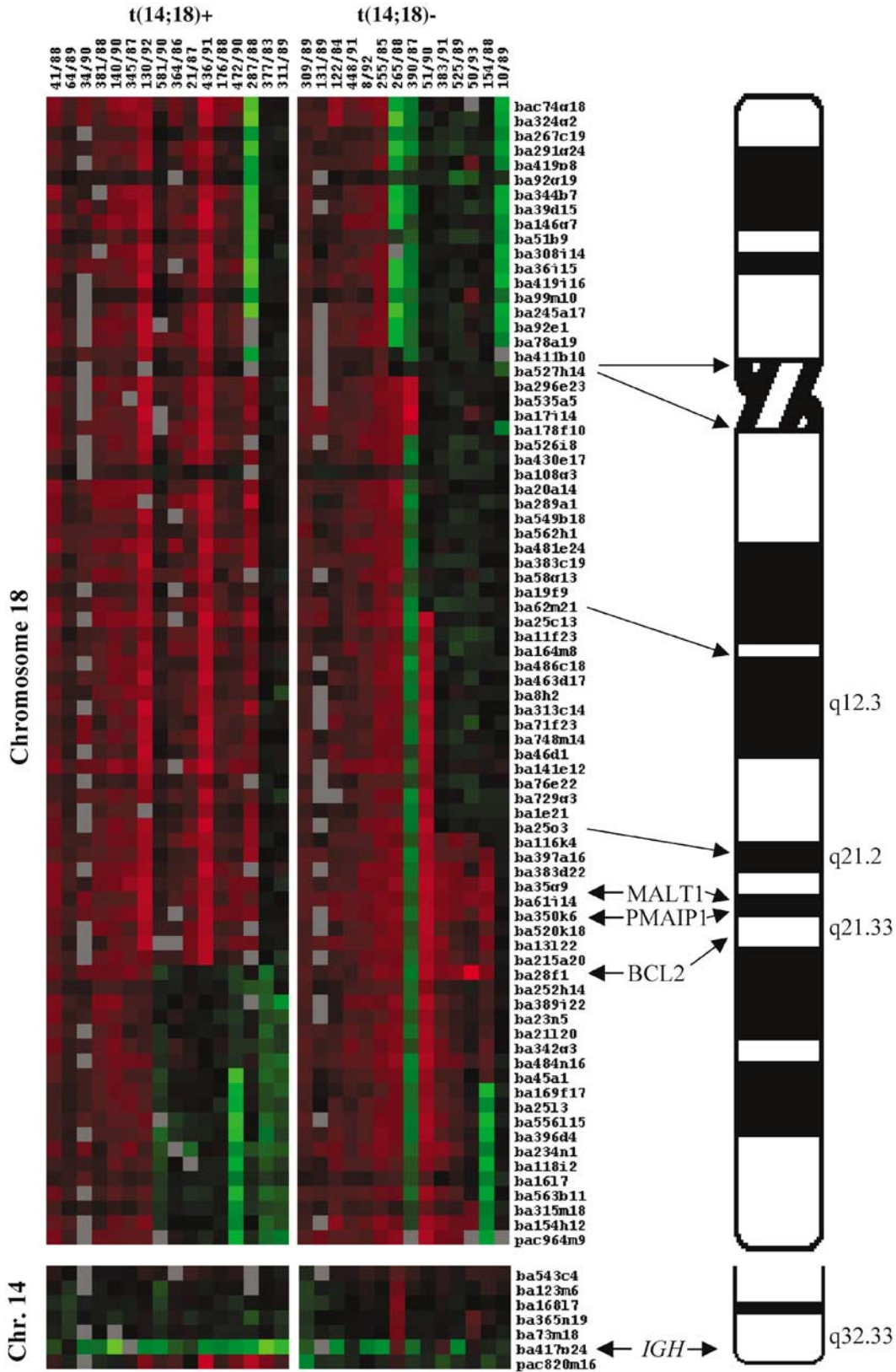


Figure 4 Chromosome 18 copy number changes in B-NHL. BAC arrays covering chromosome 18 with a resolution of approximately 1 mbp (78 clones) were used to assess DNA copy number changes in 93 cases of B-NHL. The 30 cases with alterations on chromosome 18 are shown. The copy number alterations of the seven clones most telomeric on chromosome 14q are also shown. DNA gains and losses are colored in red and green, respectively. The most intense red and green colors represent \log_2 ratios of >0.72 and <-0.72 , respectively. The ratios varied between -1.05 and $+0.92$.

may be more frequent in B-cell lymphoma lines than in primary tumors,²⁰ in agreement with the results presented here and in another study.¹⁹

Interestingly, the majority of tumors with *BCL2* alterations (Figure 4), as well as the ones without (radius.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls), showed selective loss of the *IGH* probe ba417p24, covering the region between the D-segments and C α 3. This loss was probably the result of V(D)J, and, in some cases, class switch rearrangements.

Conclusions

In this study, we have shown that whole or partial gains of chromosome 18 are common in B-NHL, both with and without t(14;18). There appears to be a *BCL2*-collaborating gene in the region 18pter-q21.33 (excluding *BCL2*), as this was the consensus region of gain for the cases with t(14;18). The low level gain of *BCL2* in primary tumors led to elevated BCL2 levels in the t(14;18)-negative cases. Increased BCL2 expression was associated with increased apoptosis in the normal cells in the tumors. The region 18q21.2–18q21.32 was amplified together with the region containing *BCL2* in the t(14;18)-negative tumors, indicating that the increased expression of another gene in this region, together with the increased BCL2 expression, is of importance in lymphoma development.

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