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

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Summary. In this report a follicular non-Hodgkin's lymphoma (NHL) carrying an unusual t(14;18) is described. This translocation most likely occurred during the V_H to D-J_H rearrangement process of the IgH locus. From the data combined with data from the literature we conclude that the chance for 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 indicated 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, influences the development of follicular NHL.

Keywords: NHL, *bcl-2*, t(14;18), IgH rearrangement, somatic mutation.

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 (Tsujimoto *et al*, 1984). The breakpoints on chromosome 18 cluster in mainly two cluster regions, i.e. the major breakpoint region (MBR) and the minor cluster region (MCR) (Cleary *et al*, 1986a, c; Tsujimoto *et al*, 1985a; Tsujimoto & Croce, 1986). The expression of *bcl-2* gene is elevated as a consequence of translocations involving this gene (Bertheas *et al*, 1991; Osada *et al*, 1989; Tsujimoto *et al*, 1985a).

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) gene segment are frequently involved. The presence of random nucleotides at the translocation junctions further supports this hypothesis (Bakhshi *et al*, 1987; Cotter *et al*, 1990; Tsujimoto *et al*, 1985b). Translocations as a result of ontogenetically later IgH gene rearrangements are rare and have been found until now confined only to the D to D-J_H rearrangement stage (Cotter *et al*, 1990; Seto *et al*, 1991; Wyatt *et al*, 1992). The t(2;18) and the t(18;22), which most likely occur during IgL rearrangements, have only occasionally been described in follicular lymphoma (Bertheas *et al*, 1991; Hillion *et al*, 1991; Osada *et al*, 1989).

Studies at the normal IgH and IgL alleles of lymphoma cells have provided an important insight into 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 (Bertoli *et al*, 1988), the lymphoma cells are monoclonal regarding their IgH and IgL chain rearrangements (Cleary *et al*, 1988). Within these genes, somatic mutations have been found (Cleary *et al*, 1986b; Kon *et al*, 1987; Levy *et al*, 1987, 1988). The somatic mutation pattern provides evidence for affinity selection for antigen prior to, and during clonal expansion (Bahler & Levy, 1992; Zelenetz *et al*, 1992). 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

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(Limpens *et al*, 1991), and the continuing accumulation of genetic defects in lymphoma subpopulations, eventually leading to a more dedifferentiated status (Yunis *et al*, 1987; Zelenetz *et al*, 1991). Also in the transgenic mice model with a deregulated *bcl-2* gene, only 11% of mice develop a monoclonal high-grade diffuse large-cell immunoblastic lymphoma after a relatively long latency period of 12–24 months (McDonnell & Korsmeyer, 1991).

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 the literature provides insight into molecular events occurring in normal early B-cell development as well as in the development of follicular lymphoma.

MATERIALS AND 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-positive 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 (Miller *et al*, 1988).

Oligonucleotides and polymerase chain reaction (PCR). Sense oligonucleotides used: MBR_2 (Meijerink *et al*, 1993); set of V_H family-specific oligonucleotides V_H1 to V_H6 (Deane & Norton, 1991); 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 (Meijerink *et al*, 1993); 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 (Meijerink *et al*, 1993): MBR_{18Q}: 5′-TGA TTT TGG CAG GAT AGC AGC ACA-3′. PCR conditions were as described before (Meijerink *et al*, 1993). 1 μg of genomic DNA was used as template, unless indicated otherwise.

End-labelling 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 4 M NH_4Ac, 5 μg yeast t-RNA and 250 μl of ethanol 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, U.S.A.). The concentration was determined by optical density. 5 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 (Denney & Weissman, 1990). The DNA was precipitated following a phenol extraction and a Sevag extraction, and dissolved in dH_2O.

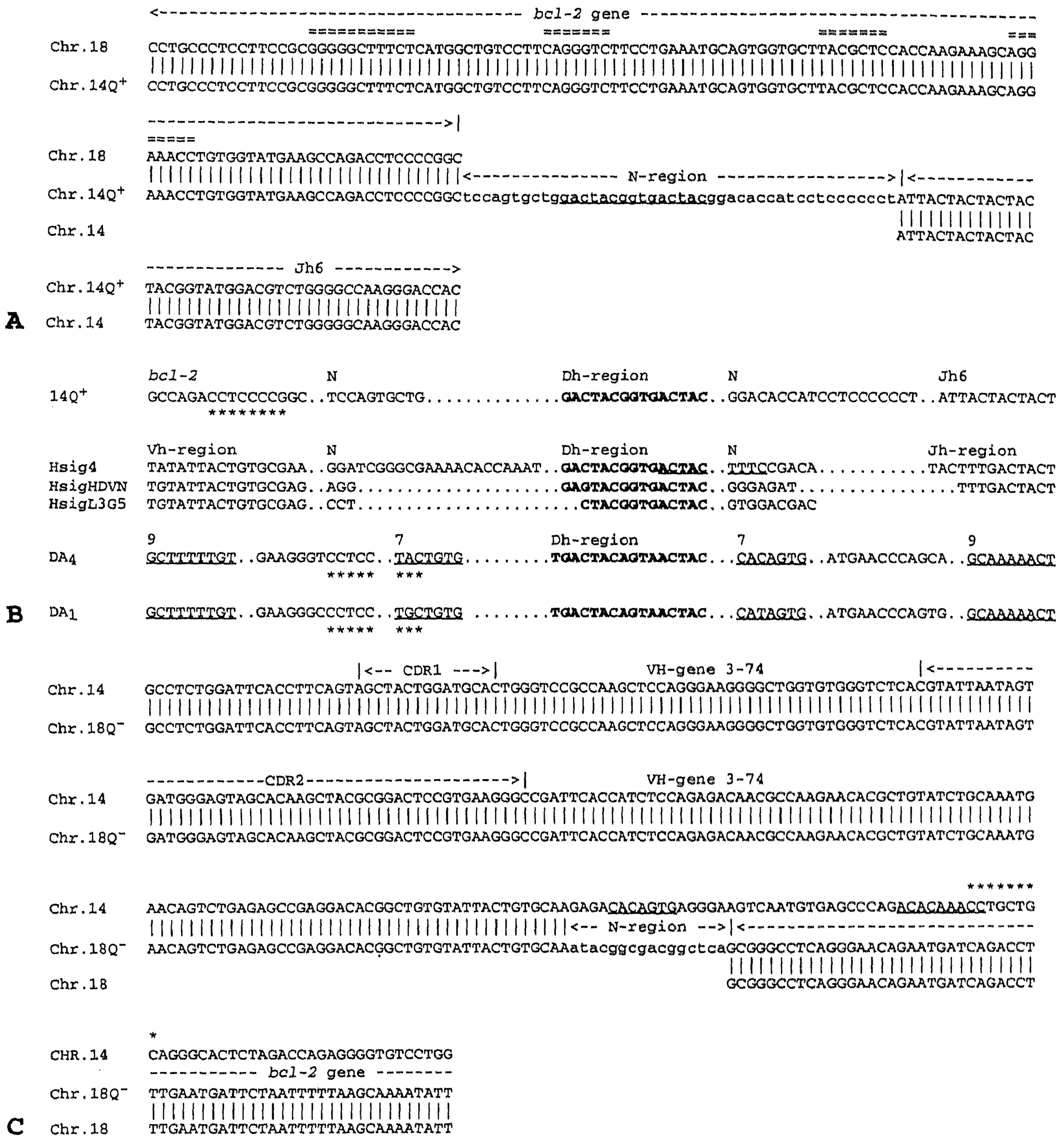
Digestion and dephosphorylation of vector DNA. A sample of 10 μg of pBluescribe KS^+ (Stratagene, La Jolla, Calif., U.S.A.) was digested with Sma-I (Gibco BRL) for 2·5 h at 30°C. The DNA was dephosphorylated using Calf Intestine Alkaline Phosphatase (CIP) (Amersham, Bucks., U.K.) according to a standard protocol (Sambrook *et al*, 1989). 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, Mass., U.S.A.) in a total volume of 20 μl at 14°C for 16 h. 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 bacterias (Gibco BRL). Transformation and Blue/White screening were performed according to standard protocols (Sambrook *et al*, 1989). 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 h. DNA was isolated from all bacterial cultures using the small-scale plasmid DNA isolation procedure (Sambrook *et al*, 1989).

Sequencing of clones. Insert DNA from each recombinant clone was asymmetrically amplified using the V_H3 and NM13.5 oligonucleotides. Asymmetric DNA templates were sequenced using end-labelled V_H3, $J_H$con or J_H6-intron oligonucleotides. The asymmetric amplification reaction and sequencing reaction were performed as described (Innis *et al*, 1988) 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. 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). 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 (Jukes & King, 1979). For the first mutation analysis the expected numbers of replacement and silent mutations in the rearranged VDJ region (bp 381–683; Fig 2) in germline configuration was calculated by summing all possible replacement substitutions for each codon and dividing by the total number of potential substitutions (replacement and silent) for all codons. This resulted in $0{\cdot}78n$ and $0{\cdot}22n$ respectively for n random mutations. The second mutation analysis was performed on the V_H gene segment only (bp 381–602). For this region, the expected numbers of replacement and silent mutations are $0{\cdot}79n$ and $0{\cdot}21n$ 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 $R_{CDRs} = 0{\cdot}38R$, $R_{FWRs} = 0{\cdot}62R$, $S_{CDRs} = 0{\cdot}38S$, $S_{FWRs} = 0{\cdot}62S$. For the second mutation analysis the $R_{CDRs} = 0{\cdot}30R$, $R_{FWRs} = 0{\cdot}70R$, $S_{CDRs} = 0{\cdot}30S$ and $S_{FWRs} = 0{\cdot}70S$. The p of kR mutations in the CDRs for n mutations was calculated using a binomial mutation model $p = [N!/k!(N-k)!]q^k(1-q)^{N-k}$, where q is the chance for a replacement mutation in the CDRs and $N = n_{(observed)} + R_{FWRs}$ (Schlomchik *et al*, 1987a). For the first mutation analysis $q = 0{\cdot}78 \times 0{\cdot}38 = 0{\cdot}30$ and for the second mutation analysis $q = 0{\cdot}79 \times 0{\cdot}30 = 0{\cdot}24$.

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 (Fig 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 (Stewart *et al*, 1993). 14/15 nucleotides are present in the CDR3 region of Hsighdvn, and 13/15 nucleotides are present in the CDR3 region of HsigL3G5 (Fig 1B). We conclude that this region represents an IgH D gene segment, which is most homologous to the germline IgH D_{A4} or D_{A1} segment (Ichihara *et al*, 1988).

Since it is suggested that the t(14;18) occurs as an error during the V(D)J gene rearrangement process in the Ig 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 member to the 3′ untranslated region of the *bcl-2* gene (Fig 1C) with insertion of random nucleotides. The consensus sequence segment was completely homologous to the V_H germline gene 3-74 [DA-8; (Cook *et al*, 1994) and DP53 (Tomlinson *et al*, 1992)].

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 (Tsujimoto *et al*, 1985b) (Fig 1). Regions homologous to the χ 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 (Figs 1B–C) and a region within the first FWR of this gene was completely homologous to the χ consensus sequence (Tomlinson *et al*, 1992).

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 30–40% of IgM/K-positive follicular lymphoma cells, and DNA from the blood sample were used as a template for the amplification of rearranged IgH VDJ gene segments of the lymphoma cells. Using all V_H family-specific oligonucleotides (Deane & Norton, 1991) 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 (Matsuda *et al*, 1993). The PCR fragment was cloned, and individual clones were used for mutation analyses.

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 Fig 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

Fig 1. (A) Comparison of the $14Q^+$ chromosomal junction to the MBR of the *bcl-2* gene (Cleary *et al*, 1986c) and the J_H region of the IgH locus (Ravetch *et al*, 1981). The N-region is shown in small characters. Underlined regions shares homology with human D regions. Heptamer–nonamer signal and signal-like sequences as suggested by Tsujimoto *et al* (1985b) are indicated by double lines. (B) Comparison of the N-region from the $14Q^+$ chromosomal junction to germline D segments D_{A4} and D_{A1} (Ichihara *et al*, 1988), and somatic D segments. The somatic D segments were deduced from VDJ sequences Hsig4 (Stewart *et al*, 1993), HsigI3g5 (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 χ consensus sequence are marked by asterisks. (C) Comparison of the $18Q^-$ chromosomal junction to germline sequences of the IgH V_H3-74 gene segment (Cook *et al*, 1994) and the MBR of the *bcl-2* gene (Cleary *et al*, 1986c). The N-region is shown in lower case. The CDR1 and CDR2 regions are indicated according to Kabat *et al* (1987). The signal heptamer and nonamer sequences are underlined. Regions sharing homology to the χ consensus sequence are marked by asterisks.

homologous mapped germline gene V_H3-7 (Matsuda *et al*, 1993). The leader intron (bp 197–299) was completely homologous to germline V_H3-48 gene segment. The D part of the VDJ rearrangement (bp 610–630) was 85% homologous to germline $D_{XP'1}$ gene segment (Ichihara *et al*, 1988). For subsequent analyses, we assumed that the productively rearranged VDJ gene segments in the lymphoma cells were composed of germline counterparts V_H3-48 (Matsuda *et al*, 1993), $D_{XP'1}$ (Ichihara *et al*, 1988) and J_H6 (Ravetch *et al*, 1981).

The consensus VDJ sequence (bp 1–713; Fig 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. 12 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 11 replacement mutations at positions 400, 406, 456, 474, 475, 597, 615, 619, 628, 636, 661 and two silent mutations at positions 446 and 467 by comparison with the germline genes. A C-residue at position 656 was also present in the J_H6 region of the $14Q^+$ chromosome (Fig 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 positions 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 subpopulations 1 and 2 shared 13 and 15 mutations respectively compared with their germline counterparts. These mutations reflect somatic mutations acquired during clonal evolution of both lymphoma populations. These mutations were used in two mutation analyses (Schlomchik *et al*, 1987a, b). Since the germline counterpart of the D gene segment was most likely $D_{XP'1}$, and since not all germline D genes have been identified (Ichihara *et al*, 1988), we devided the analysis in two parts. In one part we analysed the mutations present in the entire VDJ gene (Table IA; region bp 381–683). In the other part we analysed the mutations in the V_H gene segment only (Table IB; region bp 381–602). The distribution of replacement and silent mutations as found in the CDRs and FWRs are summarized in Table IA and IB 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 and 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 ($k = 5$, $n = 10$, $N = 13$).

```
                                                                                                1                  18
Vh3-48                                                                                          a.................c.
Consensus                                                                                       ctatagtaggagatatgc

                19                                                                                                 108
Vh3-48          ..a....n.........................c.....................................................................
Consensus       aagtagggccctccctctactgatgaaaa-ccaacccaaccctgaccctgcagctctcagagaggtgccttagccctggattccaaggca

                109                                                                                                198
                                                        >|<                    leader                      >|<
Vh3-48          ........................a.....................G.....................................................
Consensus       tttccacttggtgatcagcactgtacacagaggactcaccATGGACTTGGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTga

                199                                                                                                288
                                                                leader-intron
Vh3-48          ................................................................................................
Consensus       ttcatggaaaactagagagatttagtgtgtgtggatatgagtgagagaaacagtggatatgtgtggcagtttctgaccttggtgtctctt

                289                                                                                                378
                          >|<leader>|1<                                        FWR-1
Vh3-48          ..........................................................G.....................................
Consensus       tgtttgcaggtGTCCAGTGTGAGGTGCAGCTGGTGGAGTCTGGGGGAGCCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAG
```

Fig 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 (Matsuda *et al*, 1993), $D_{XP'1}$ (Ichihara *et al*, 1988), J_H6 (Ravetch *et al*, 1981), or the consensus sequence are shown in capitals, and replacement mutations are shown in bold capitals. The N-regions are shown in lower case. The J_H6-intron region is shown by small characters in italic. The C-residue at position 656 represents allelic variation.

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           379                                                                                       468
                                   >|<--- CDR1 --->|<                FWR-2                   >|<-----------
Vh3-48     ......................G....G.......................................G.........T..........T.
Consensus  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...................................................................................
Consensus  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............
Consensus  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............

           649                                                       713
           ->|<              FWR4            >|<         Jh-intron
Jh6        .......G....G....................................................
Consensus  TCTGGGGCCAAGTGACCACGGTCACCGTCTCCTCAGgtaagaatggccactctagggcctttgtt
B3         ............T..........................................
B4a        ............T.............................................
B4b        ............T.............................................
B7         ............T.............................................
B8         ............T....................................................
B9         ............T....................................................
B10        ............T....................................................
K1         ............T....................................................
K5         ............T......................................g.............
K7         ............T......A.............................................
K8         ............T...........................................a........
K10        ............T...........................................a........
K12        ............T....................................................
```

Fig 2 (*continued*)

Table I. Distribution of shared mutations in the rearranged VDJ gene segments (A) or in the V_H region of the VDJ rearrangement of the lymphoma cells (B).

A

	FWR1 (partial)	FWR2	FWR3	FWR4	Total FWRs	Expected FWRs	CDR1	CDR2	CDR3	Total CDRs	Expected CRDs
Subpopulation 1											
Replacement	0	0	1	1	2	6	2	3	4	9	4
Silent	0	1	0	0	1	2	0	1	0	1	1
R/S	–	0	∞	∞	2	3	∞	3	∞	9	4
Subpopulation 2											
Replacement	0	0	3	1	4	7	2	3	4	9	5
Silent	0	1	0	0	1	2	0	1	0	1	1
R/S	–	0	∞	∞	4	3·5	∞	3	∞	9	5

B

	FWR1 (partial)	FWR2	FWR3	Total FWRs	Expected FWRs	CDR1	CDR2	Total CDRs	Expected CDRs
Subpopulation 1									
Replacement	0	0	1	1	5	2	3	5	2
Silent	0	1	0	1	1	0	1	1	0
R/S	–	0	∞	1	5	∞	3	5	∞
Subpopulation 2									
Replacement	0	0	3	3	6	2	3	5	2
Silent	0	1	0	1	1	0	1	1	1
R/S	–	0	∞	3	6	∞	3	5	2

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

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. Almost 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 down-regulated on mRNA level (Haury *et al*, 1993) as well as protein level (Merino *et al*, 1994) 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 aberrantly 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 χ-like sequences (Wyatt *et al*, 1992). 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 classic 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 (Tutter & Riblet, 1989), in D and J_H gene segments, and have also been found in the MBR of the *bcl-2* gene (Wyatt *et al*, 1992). Recently, proteins have been identified which bind to χ-like sequences present in the MBR of the *bcl-2* gene (Aoki *et al*, 1994; Jaeger *et al*, 1993). 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 occurrence 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 homo-

logous (97·2%) to germline V_H3-48 gene segment, and only 92% homologous to the next most homologous V_H segment V_H3-7 (Matsuda *et al*, 1993). Since the map of the human Ig V_H locus is now complete and almost all the sequences are known (Cook *et al*, 1994), 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 gene segment is Dxp'1 (Ichihara *et al*, 1988). From amplification conditions as well as sequence information it is obvious that the germline J_H gene segment is J_H6 (Ravetch *et al*, 1981).

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 the absence of selection. As discussed by Schlomchik *et al* (1987b), an R/S ratio $> 2{\cdot}9$ indicates positive selection. In both analyses the R/S ratios for the CDRs of both subpopulations strongly indicated positive selection. An R/S ratio $< 2{\cdot}9$ 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 (Schlomchik *et al*, 1987a), it seems highly unlikely that the mutation pattern present in both lymphoma populations for both regions analysed 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 (Bakkus *et al*, 1992), 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 (Berek, 1992), resulting in one mutation in the VDJ region of almost every lymphoma cell. Since we used $0{\cdot}2\ \mu$g of genomic DNA in our PCR (which roughly equals 3×10^4 cells) and since the amount of lymphoma cells within the lymph node biopt was 30–40%, 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. The position of these mutations also does not suggest 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 the time of presentation, which is in contrast with some other cases of follicular lymphoma (Cleary *et al*, 1986b, 1988; Kon *et al*, 1987; Zelenetz *et al*, 1992).

In conclusion, translocations involving the *bcl-2* gene which occur at other stages of the Ig rearrangements than the IgH D 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 recognized 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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REFERENCES

Aoki, K., Nakahara, K., Ikegawa, C., Seto, M., Takahashi, T., Minowada, J., Strominger, J.L., Maziarz, R.T. & Kasai, M. (1994) Nuclear proteins binding to a novel target sequence within the recombination hotspot regions of *bcl-2* and the immunoglobulin D_H gene family. *Oncogene*, **9**, 1109–1115.

Bahler, D.W. & Levy, R. (1992) Clonal evolution of a follicular lymphoma: evidence for antigen selection. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 6770–6774.

Bakhshi, A., Wright, J.J., Graninger, W., Seto, M., Owens, J., Cossman, J., Jensen, J.P., Goldman, P. & Korsmeyer, S.J. (1987) Mechanism of the t(14;18) chromosomal translocation: structural analysis of both derivative 14 and 18 reciprocal partners. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 2396–2400.

Bakkus, M.H.C., Heirman, C., Van Riet, I., Van Camp, B. & Thielemans, K. (1992) Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. *Blood*, **80**, 2326–2335.

Berek, C. (1992) The development of B cells and the B-cell repertoir in the microenvironment of the germinal center. *Immunology Review*, **126**, 5–19.

Bertheas, M.F., Rimokh, R., Berger, F., Gaucherand, M., Machado, P., Vasselon, C., Calmardoriol, P., Jaubert, J., Guyotat, D. & Magaud, J.P. (1991) Molecular study of a variant translocation t(2;18)(p11;21) in a follicular lymphoma. *British Journal of Haematology*, **78**, 132–134.

Bertoli, L.F., Kubagawa, H., Borzillo, G.V., Burrows, P.D., Schreeder, M.T., Carroll, A.J. & Cooper, M.D. (1988) Bone marrow origin of a B-cell lymphoma. *Blood*, **72**, 94–101.

Cleary, M.L., Galili, N. & Sklar, J. (1986a) Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. *Journal of Experimental Medicine*, **164**, 315–320.

Cleary, M.L., Galili, N., Trela, M., Levy, R. & Sklar, J. (1988) Single cell origin of bigenotypic and biphenotypic B cell proliferations in

human follicular lymphomas. *Journal of Experimental Medicine*, 167, 582–597.

Cleary, M.L., Meeker, T.C., Levy, S., Lee, E., Trela, M., Sklar, J. & Levy, R. (1986b) Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell*, 44, 97–106.

Cleary, M.L., Smith, S.D. & Sklar, J. (1986c) Cloning and structural analysis of cDNAs for *bcl-2* and a hybrid *bcl-2*/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell*, 47, 19–28.

Cook, G.P., Tomlinson, I.M., Walter, G., Riethman, H., Carter, N.P., Buluwela, L., Winter, G. & Rabbitts, T.H. (1994) A map of the human immunoglobulin V_H locus completed by analysis of the telomeric region of chromosome 14q. *Nature Genetics*, 7, 162–168.

Cotter, F., Price, C., Zucca, E. & Young, B.D. (1990) Direct sequence analysis of the 14q+ and 18q− chromosome junctions in follicular lymphoma. *Blood*, 76, 131–135.

Deane, M. & Norton, J.D. (1991) Immunoglobulin gene 'fingerprinting': an approach to analysis of B lymphoid clonality in lymphoproliferative disorders. *British Journal of Haematology*, 77, 274–281.

Denney, D. & Weissman, I. (1990) DNA generated by polymerase chain reaction using *Taq* DNA polymerase has non-template nucleotide additions: implications for cloning PCR products. *Amplifications*, 4, 25–26.

Haury, M., Freitas, A., Hermitte, V., Coutinho, A. & Hibner, U. (1993) The physiology of *bcl-2* expression in murine B-lymphocytes. *Oncogene*, 8, 1257–1262.

Hillion, J., Mecucci, C., Aventin, A., Leroux, D., Wlodarska, I., Van-Den-Berghe, H. & Larsen, C.J. (1991) A variant translocation t(2;18) in follicular lymphoma involves the 5′ end of *bcl-2* and Ig kappa light chain gene. *Oncogene*, 6, 169–172.

Ichihara, Y., Matsuoka, H. & Kurosawa, Y. (1988) Organization of human immunoglobulin heavy chain diversity gene loci. *EMBO Journal*, 7, 4141–4150.

Innis, M.A., Myambo, K.B., Gelfand, D.H. & Brow, M.A. (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 9436–9440.

Jaeger, U., Purtscher, B., Delle Karth, G., Knapp, S., Mannhalter, C. & Lechner, K. (1993) Mechanism of the chromosomal translocation t(14;18) in lymphoma: detection of a 45-Kd breakpoint binding protein. *Blood*, 81, 1833–1840.

Jukes, T.H. & King, J.L. (1979) Evolutionary nucleotide replacements in DNA. *Nature*, 281, 605–606.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M. & Gottsman, K.S. (1987) *Sequences of Proteins of Immunological Interest*. U.S. Public Health Service, Bethesda, Md.

Kon, S., Levy, S. & Levy, R. (1987) Retention of an idiotypic determinant in a human B-cell lymphoma undergoing immunoglobulin variable-region mutation. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 5053–5057.

Levy, R., Levy, S., Cleary, M.L., Carroll, W., Kon, S., Bird, J. & Sklar, J. (1987) Somatic mutation in human B-cell tumors. *Immunology Reviews*, 96, 43–58.

Levy, S., Mendel, E., Kon, S., Avnur, Z. & Levy, R. (1988) Mutational hot spots in the Ig V region genes of human follicular lymphomas. *Journal of Experimental Medicine*, 168, 475–489.

Limpens, J., de Jong, D., van Krieken, J.H.J.M., Price, C.G.A., Young, B.D., van Ommen, G.J.B. & Kluin, P.M. (1991) *Bcl-2*/J_H rearrangements in benign lymphoid tissues with follicular hyperplasia. *Oncogene*, 6, 2271–2276.

Matsuda, F., Shin, E.K., Nagaoka, H., Matsumura, R., Haino, M., Fukita, Y., Taka-ishi, S., Imai, T., Riley, J.H., Anand, R., Soeda, E. & Honjo, T. (1993) Structure and physical map of 64 variable segments in the 3′ 0.8-megabase region of the human immunoglobulin heavy-chain locus. *Nature Genetics*, 3, 88–94.

McDonnell, T.J. & Korsmeyer, S.J. (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature*, 349, 254–256.

Meijerink, J.P.P., Smetsers, T.F.C.M., Raemaekers, J.M.M., Bogman, M.J.J.T., Dewitte, T. & Mensink, E.J.B.M. (1993) Quantitation of follicular non-Hodgkin's lymphoma cells carrying t(14;18) by competitive polymerase chain reaction. *British Journal of Haematology*, 84, 250–256.

Merino, R., Ding, L., Veis, D.J., Korsmeyer, S.J. & Nunez, G. (1994) Developmental regulation of the *bcl-2* protein and susceptibility to cell death in B lymphocytes. *EMBO Journal*, 13, 683–691.

Miller, S.A., Dykes, D.D. & Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, 16, 1215.

Osada, H., Seto, M., Ueda, R., Emi, N., Takagi, N., Obata, Y., Suchi, T. & Takahashi, T. (1989) *Bcl-2* gene rearrangement analysis in Japanese B cell lymphoma: novel *bcl-2* recombination with immunoglobulin kappa chain gene. *Japanese Journal of Cancer Research*, 80, 711–715.

Ravetch, J.V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. (1981) Structure of human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. *Cell*, 27, 583–591.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Schlomchik, M.J., Aucoin, A.H., Pisetsky, D.S. & Weigert, M.G. (1987a) Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 9150–9154.

Schlomchik, M.J., Marshak-Rothstein, A., Wolfowicz, C.B., Rothstein, T.L. & Weigert, M.G. (1987b) The role of clonal selection and somatic mutation in autoimmunity. *Nature*, 328, 805–811.

Seto, M., Osada, H., Ueda, R., Ito, C., Iwaki, O., Oyama, A., Suchi, T. & Takahashi, T. (1991) *Bcl-2* translocation in Japanese B cell lymphoma: novel *bcl-2* translocation with immunoglobulin heavy chain diversity segment. *Japanese Journal of Cancer Research*, 82, 65–71.

Stewart, A.K., Huang, C., Stollar, B.D. & Schwartz, R.S. (1993) High-frequency representation of single V_H gene in the expressed human B cell repertoires. *Journal of Experimental Medicine*, 177, 409–418.

Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B. & Winter, G. (1992) The repertoire of human germline V_H sequences reveals about fifty groups of V_H segments with different hypervariable loops. *Journal of Molecular Biology*, 227, 776–798.

Tsujimoto, Y., Cossman, J., Jaffe, E. & Croce, C.M. (1985a) Involvement of the *bcl-2* gene in human follicular lymphoma. *Science*, 228, 1440–1443.

Tsujimoto, Y. & Croce, C.M. (1986) Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*, 83, 5214–5218.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. & Croce, C.M. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science*, 226, 1097–1099.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. & Croce, C.M. (1985b) The t(14;18) chromosome translocations involved in

B-cell neoplasms result from mistakes in VDJ joining. *Science*, **229**, 1390–1393.

Tutter, A. & Riblet, R. (1989) Conservation of an immunoglobulin variable-region gene family indicates a specific, noncoding function. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 7460–7464.

Wyatt, R.T., Rudders, R.A., Zelenetz, A., Delellis, R.A. & Krontiris, T.G. (1992) *Bcl-2* oncogene translocation is mediated by a chi-like consensus. *Journal of Experimental Medicine*, **175**, 1575–1588.

Yunis, J.J., Frizzera, G., Oken, M.M., McKenna, J., Theologides, A. & Arnesen, M. (1987) Multiple recurrent genomic defects in follicular lymphoma: a possible model for cancer. *New England Journal of Medicine*, **316**, 79–84.

Zelenetz, A.D., Chen, T.T. & Levy, R. (1991) Histologic transformation of follicular lymphoma to diffuse lymphoma represents tumor progression by a single malignant B cell. *Journal of Experimental Medicine*, **173**, 197–207.

Zelenetz, A.D., Chen, T.T. & Levy, R. (1992) Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection. *Journal of Experimental Medicine*, **176**, 1137–1148.