Brief report

Intronic *BCL-6* mutations are preferentially targeted to the translocated allele in t(3;14)(q27;q32) non-Hodgkin B-cell lymphoma

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Translocations and somatic mutations are common genetic alterations of the *BCL-6* gene on chromosome 3q27 in B-cell lymphoma, with implications for lymphomagenesis. The 2 events may have linked origins and can influence juxtaposed loci. To evaluate this further, we compared mutations occurring within the major mutation cluster region of the translocated and untranslocated *BCL-6* alleles in 7 t(3;14)(q27;14q32) lymphomas. In 6 of 7 cases, the translocated allele revealed significantly higher mutations (mean, 5.8×10^{-2} bp^{-1}) than did the untranslocated allele (mean, 5.3×10^{-3} bp^{-1}; $P < .01$). The increase mapped to der(14q32), which retains the *BCL-6* promoter and is transcriptionally active, as revealed by fusion transcripts and ongoing somatic mutations, absent in the der(3q27) region. These results indicate that enhanced mutational activity at the translocated allele may be a consequence of loss of *cis* regulatory elements or gain of *IgH* enhancer elements. Junctional sequences indicate translocation origins from earlier *BCL-6* mutations and switch recombinase events. (Blood. 2003;102: 1872-1876)

Introduction

The *BCL-6* gene on chromosome 3q27 encodes a potent transcriptional repressor that regulates normal germinal center (GC) formation and function[1] and is suggested as an important factor in lymphomagenesis.[2]

Rearrangements of this locus are common events in diffuse large B-cell lymphoma (DLBCL)[3,4] and include chromosomal translocations mapping within the first noncoding *BCL-6* exon and the first intron.[4,5] A functional consequence is that regulatory and coding sequences on partner chromosomes are juxtaposed, generating a potential for aberrant gene expression on both rearranged partners.[6,7] A second type of genetic alteration targeting this locus involves somatic mutations, which map to a region of approximately 2 kilobase (kb) downstream of the initiation site, encompassing the major mutation cluster (MMC).[8,9] These can potentially dysregulate *BCL-6* expression.[10,11]

There is some evidence to suggest that the 2 somatic events, that is, somatic mutations and translocations, may be related. A 120–base pair (bp) breakpoint hypercluster region located within the MMC[12] displays a high *BCL-6* mutational rate in lymphoma, with up to 35% of identifiable somatic mutations occurring in this region.[13] Additional mechanisms may be involved. In t(3;14)(q27;q32) lymphomas, translocations usually involve the switch regions (S_H) of the *IgH* locus, suggesting a role for the switch recombinase.[12] This region also contains enhancer elements located 3′ of the Cμ gene in *IgH* that affect somatic mutations in *V* genes.[14]

To assess the role of surrounding genetic elements on somatic mutation, we investigated the effects of the t(3;14) translocation on *BCL-6* mutations by contrasting the untranslocated and translocated alleles in individual lymphoma cases. Junctional sequences were also analyzed to identify mechanisms likely to play a role in translocation origins.

Study design

Patient material

We analyzed 7 lymphomas: 5 DLBCLs with more than 80% tumor cells and 2 follicular lymphomas (FLs) with more than 60% tumor cells, classified by World Health Organization criteria,[15] each bearing the t(3;14) (3q27;14q32) translocation detected by conventional cytogenetic methods, with *BCL-6* gene rearrangement confirmed by Southern blot analysis.[2]

Molecular analysis of *BCL-6*

To amplify the *IgH*/*BCL-6* junctions, primers were designed according to the molecular anatomy of the t(3;14)(q27;q32) translocation described recently.[12] To amplify the untranslocated allele, primers were designed to span the breakpoints (Figure 1). The polymerase chain reactions (PCRs) were performed using established conditions,[16] and each product was cloned for sequence analysis. Identical amounts of DNA template ensured a comparable *Taq* polymerase rate error for both alleles, confirmed by repeated PCR analysis. In 4 of 4 cases with available RNA, fusion transcripts were detected by reverse transcriptase–PCR (RT-PCR) as previously described.[7,17]

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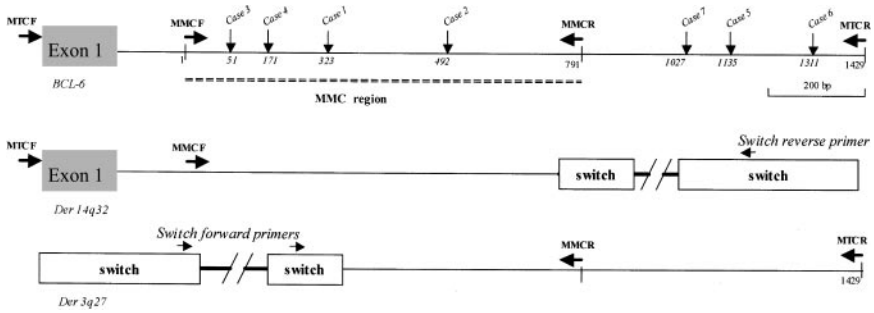

Figure 1. Schematic representation of the first *BCL-6* intron and derivative chromosomes after t(3;14) translocation. Breakpoints on der(3q27) are indicated (vertical arrows) for each case and located according to the MMC germ line sequence (GenBank accession no. AF191831). MTC or MMC forward (F) and reverse (R) primers, previously designed,[9,16] were used to amplify the junction site and the translocated allele in combination with Switch μ or Switch γ primers (Sμ 01F 5′-ggcaatgagatggctttagctg, Sμ 02F 5′-tgggctgagcaggttgtac, Sμ 01R 5′-tgccatgtccttccagttcatcc, SγF 5′-gggcagaatggtcataat, SγR 5′-atgttctcccctgttccctgag). To exclusively amplify the untranslocated allele, MTC or MMC primers spanning the breakpoints were used under PCR conditions identical to those used for amplification of the *BCL6-IgH* junctions.

Statistical analysis

The frequency of mutations occurring between the translocated and the untranslocated allele were compared using the χ^2 test or the Fisher exact test.

Results and discussion

In 7 of 7 cases, both the translocated and untranslocated alleles were amplified. Of the 7 t(3;14) translocations, reciprocal breakpoint regions were identified in 6 cases (Figures 1-2), with der(14) not being found in 1 case (Table 1).

BCL-6 mutations were analyzed in each region. Polymorphic mutations (397G>C, 502G>A, or 520delT) allowed intra-allelic discrimination. Clonal mutations were observed. Mutations included point mutations and short additions and deletions (5 of 7 cases), biallelic mutations, and identical, recurrent mutations between cases (Table 1).

Notably, a significant imbalance in the *BCL-6* mutational load was observed between the translocated (mean, 5.8×10^{-2} bp^{-1}) and the untranslocated (mean, 1.5×10^{-3} bp^{-1}) allele in 6 of 7 cases ($P < .01$; Table 1 and Figure 2A). Mutations in *BCL-6* can occur in the absence or presence of chromosomal rearrangements,[8] but the influence of such events on mutational activity has not been delineated. These data show that in *BCL-6*, a dramatic increase in mutational rate can occur in t(3;14) lymphoma, which is preferentially targeted to the translocated allele. The data demonstrate clear differences in the level of *BCL-6* mutations between the translocated and untranslocated alleles. This suggests that mechanistic constraints that regulate the level of mutational activity at the

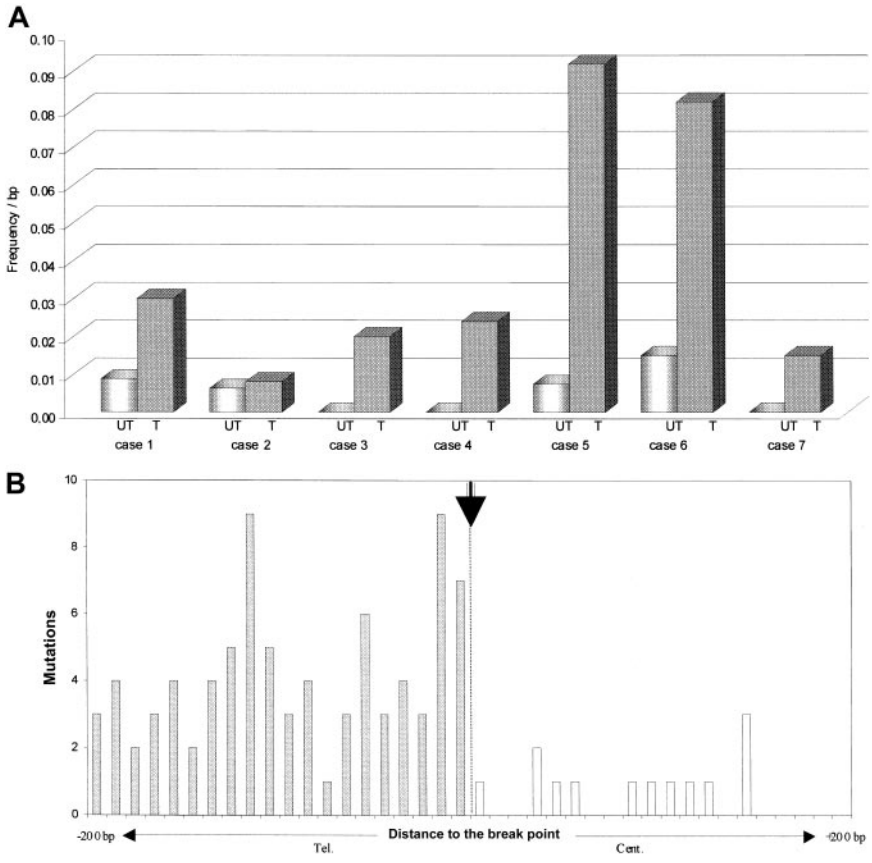

Figure 2. Distribution of the BCL-6 somatic mutations in both alleles of t(3;14) lymphoma. (A) Frequency of mutations in the *BCL-6* major mutation cluster region observed within the translocated (T) and the untranslocated (UT) allele. (B) Mutations in *BCL-6* first intron relative to junction sites in 6 lymphomas with t(3;14) translocation. The figure is a composite and shows mutations in *BCL-6* in der(14q32) (shaded bars) and in der(3q27) (open bars). Mutational frequency in the *BCL-6* locus permitted a direct comparison of rates for all derivative chromosomes.

Table 1. Clonal mutations observed in the major mutation cluster region of the first *BCL-6* intron in 7 cases of lymphoma with t(3;14) translocation

Case no. and diagnosis	Mutations located within the MMC or 3′ of the MMC	Intraclonal heterogeneity	No. of sequenced clones
1. DLBCL			
Untranslocated allele	119C>G; 123C>T; 265G>A; 378G>T; 423C>T; 519C>T; 709A>G	Yes	6
Translocated allele			
der(3q27)	345G>C; 347G>A; 349-350insG; 350T>G; 359A>G; 365G>C; 423C>G; 429C>G; 441G>A; 443G>A; 478T>G; 685C>T; 697G>A; 737delT; 741T>A; 780G>A	No	9
der(14q32)	Unknown*		
2. Grade 3 FL		Yes	9
Untranslocated allele	18T>C; 95T>A; 542T>C; 548C>T; 782A>T		
Translocated allele			
der(3q27)	No clonal mutation	No	6
der(14q32)	18T>C; 375G>A; 416G>A; 423C>G	Yes	7
3. DLBCL			
Untranslocated allele	No clonal mutation	No	18†
Translocated allele			
der(3q27)	51C>G; 87T>A; 90C>G; 100A>G; 107A>G; 136A>G; 142A>G; 170C>T; 196A>T; 252C>T; (387-393)del	No	9
der(14q32)	6G>A; 7C>A; 30T>C; 36T>C; 38T>A	Yes	6
4. DLBCL			
Untranslocated allele	No clonal mutation	No	18†
Translocated allele			
der(3q27)	**520delT**	No	6
der(14q32)	8T>G; 16A>T; 23T>C; 44T>G; 45C>A; 71A>C; 76T>C; 88C>A; 89T>A; 97T>A; 105T>G; 107T>A; 110T>G; 123C>G; 143T>C; 145C>T; 149A>C; 151-152insT, 153T>G	No	6
5. DLBCL			
Untranslocated allele	202A>T; 226A>G; 267T>G; 269T>A; 344A>T; 434G>T	No	4
Translocated allele			
der(3q27)‡	1249G>C; 1260G>C; 1284C>T; 1285C>T	No	4
der(14q32)	23T>C; 24A>G; 32A>G; 43T>C; 44T>G; 47G>C; 57A>G; 71A>G; 73G>C; 75T>A; 84G>C; 87T>A; 90A>G; 100A>G; 104A>C; 108A>C; 116A>G; 121A>C; 122G>C; 140A>G; 141A>G; 176delA ; 178G>T; 181delA; 183delA; 184G>T; 188G>A; 209T>G; 211G>C; 222G>A; 234G>C; 257T>G; 298A>C; 309G>A; 312T>A; 328G>A; 335G>C; 347G>C; 349T>G; 350T>G; 356G>C; 360T>C; 377G>A; 390 A>C; 393C>G; 394C>T; 402G>A; 421C>G; 431G>A; (433-442)del; 459C>G; 466T>G; 477G>A; 500T>G; 501T>C; 505A>C; 509G>A; 510C>G; 519C>T; 526G>A; 608G>A; 620G>C 623G>A; 632T>G; 659C>G; 692C>G; 694G>A; 732T>C; 734delG; 738T>C; 742G>A; 769G>A; 775A>G; 779A>G; 782A>T	No	9
6. DLBCL			
Untranslocated allele	46C>G; 84G>A; 142A>G; 193G>A; 211G>A; **502G>A**; **520delT**; 573T>C; 599T>C; 677T>A; 717A>G; 737T>G; 750T>G; 752T>A	Yes	8
Translocated allele			
der(3q27)‡	1314G>C	No	5
der(14q32)	25C>T; 26C>T; 84G>A; 100A>G; 106A>G; 108A>C; 119C>T; 121A>C; 122G>T; 139T>C; 173C>G; 191A>T; 195A>C; 197T>C; 200T>C; 217T>C; 261T>C; 275T>A; 279T>C; 328G>C; 329C>T; 334A>C; 337A>G; 345G>A; 350-351linsG; 400G>C; 423C>G; 424T>C; 425C>G; 445A>G; 478T>A; 479G>A; 486C>T; 487C>A; 488C>T; 499C>G; 503C>G; 507C>G; 508T>A; 509G>C; 512T>A; 524T>G; 541T>G; 545C>G; 555T>A; 561C>T; 574C>A; 579C>T; 614A>G; 617A>G; 650C>G; 671T>G; 677T>A; 679C>G; 697C>T; 718T>G; 720C>G; 727A>G; 728C>T; 735G>A; 741T>C; 761 61T>A; 763A>C; 766C>G	No	15
7. Grade 1 FL			
Untranslocated allele	No clonal mutation	No	13†
Translocated allele			
der(3q27)	No clonal mutation	No	4
der(14q32)	73G>C; 177G>C; 339G>C; **397G>C**; 441G>A; 445A>C; 475C>T; 504A>T; 549C>T; 613A>T; 633C>T; 735G>A; 736G>A	Yes	4

To analyze the MMC, sequences were aligned with the reported *BCL-6* germ line gene[9] (GenBank accession nos. AF 191831 and Z79581). The first nucleotide of the amplified *BCL-6* first intron corresponds to the first nucleotide of the MMCF primer, defining position +1. In each case, multiple clones were obtained from at least 2 independent PCRs using different *BCL-6* primers. Clonal mutations are observed in at least 2 independent clones. *Taq* error rate (1×10^{-3} bp^{-1}) was also calculated by amplification and cloning of *BCL-6* using an identical amount of monocyte DNA template not targeted for somatic mutation. MMC indicates major mutation cluster region; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma. Polymorphisms are shown in boldface type; biallelic mutations are underlined. del indicates deletion.

*In case 1, no PCR product was obtained using different *BCL-6* forward primers and switch reverse primers.

†In absence of clonal mutations, 1 or 2 mutations per clone may have resulted from *Taq* error or may represent unrelated cells.

‡In these cases, mutations were located 3′ of the MMC.

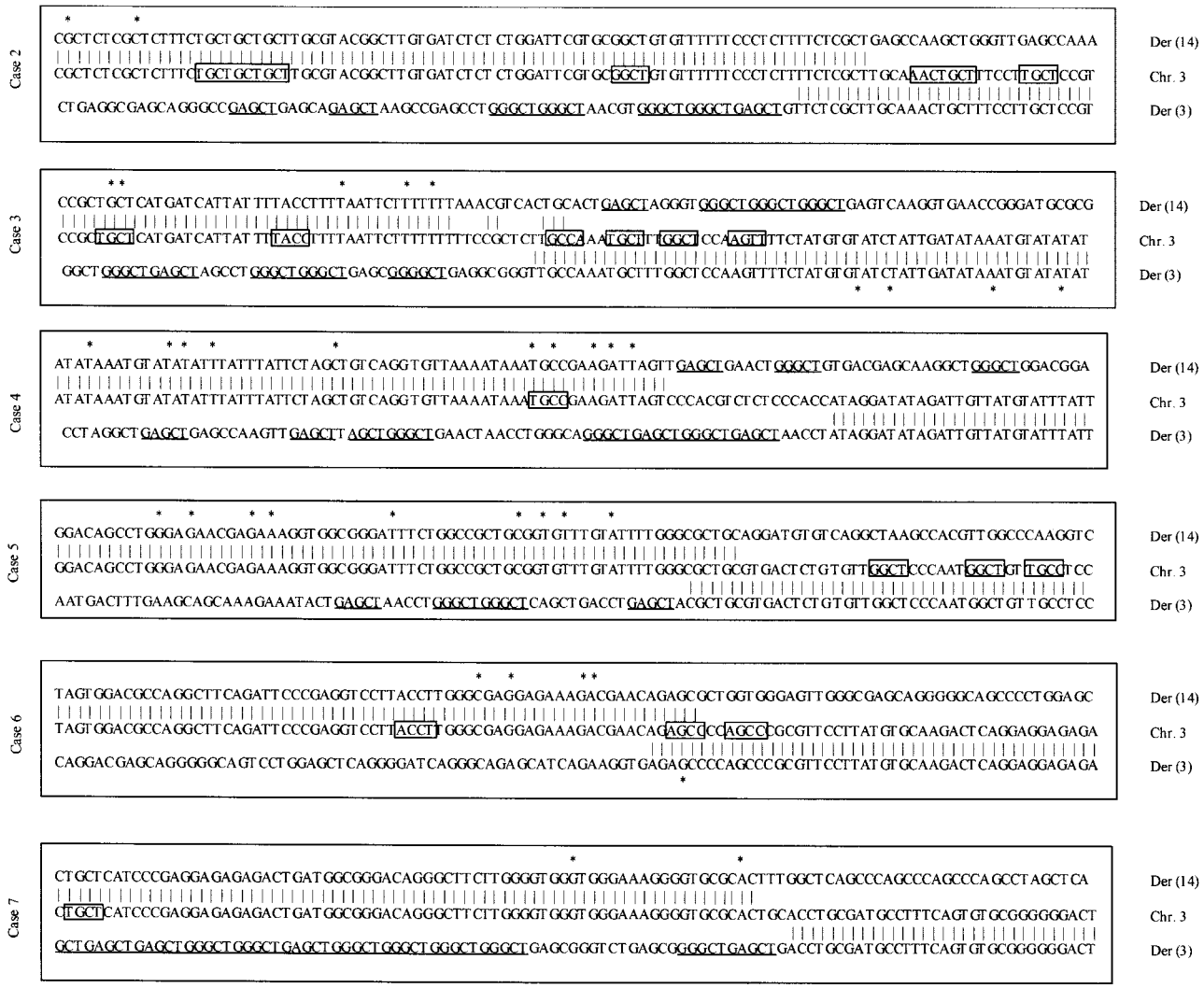

Figure 3. Nucleotide sequence analysis of the breakpoint junctions of 6 lymphomas with t(3;14) translocation. Sequence identities with the *BCL-6* germ line gene (GenBank accession nos. AF191831 and Z79581) are indicated by the vertical lines; asterisks indicate location of mutations. The S_H–BCL-6 junctions were analyzed by alignment to the closest switch (S_H) sequence (GenBank accession nos. X56795, D78345, AL122127). RGYW and WRCY motifs are boxed; pentameric repeat motifs of the Sμ region are underlined. In case 3, partial homology with the *BCL-6* germ line and the absence of homology with the Switch μ germ line sequence did not allow an exact location of the breakpoint. These sequences have been submitted to the GenBank database (GenBank accession nos. AJ557805 to AJ557817).

BCL-6 locus can be overcome in this case by involving *IgH* regulator elements. Whether this mutational activity results in higher levels of *BCL-6* mutations as compared with lymphoma cases without the t(3;14) translocation lies outside the scope of this study, and large numbers of cases will clearly be required to reach statistical validity, for example, in correlating prognostic significance of *BCL-6* mutations in lymphoma.[18]

By comparing *BCL-6* mutations in both the derivative arms of the translocated allele, we obtained further insights into preferential targeting at this allele. Mutational activity in the translocated *BCL-6* allele was notably in excess in der(14q32) compared with der(3q27), with 74% of mutations associating with der(14q32) (Table 1 and Figure 2B). The *BCL-6* promoter is clearly transcriptionally active on der(14q32), as revealed by the presence of *BCL-6–IgH* fusion transcripts (2 of 4 cases; data not shown), and it is clear that transcription is a necessary prerequisite for somatic mutation.[19] This suggests that enhancer elements downstream of the Cμ gene in *IgH* or loss of putative silencer elements in 3q27 affect the *BCL-6* promoter and result in the high mutation rate observed in der(14q32). It reveals potential synergistic interaction between regulatory factors that control hypermutation at 2 different loci, *BCL-6* and *V* genes.

Furthermore, somatic mutations in *BCL-6* in der(14q32) were apparently ongoing, as revealed by intraclonal heterogeneity at this site, but this was not true in der(3q27) in the same cases (Table 1 and Figure 2B). This argues for the acquisition of mutations after the chromosomal translocation event. Continuing mutational activity was also observed in the untranslocated allele, indicating biallelic targeting. The relationship between mutations in *BCL-6* and *V* genes is not yet clear, but ongoing mutations are a feature of *V* genes in DLBCL or FL.[20] Translocation of der(14q32) to other mutationally susceptible loci can potentially have an impact on mutations in partner genes, as in t(8;14) Burkitt lymphoma[21] or in t(14;18) FL.[22]

The translocated breakpoints were identified. The MMC was directly involved at the junction site in 4 of 7 cases (cases 1-4), with 3 of 7 cases mapping downstream of MMC (Figure 1). Translocation mapped to Sμ in 6 cases and in 1 case was located in Sγ (Figure 3). In cases translocated to Sμ, a GAGCT or GGGCT pentameric repeat motif was identified at the breakpoint. Deletions

in Sμ were identified in 6 of 6 cases, consistent with known features of isotype switch.[23] *BCL-6* sequences at the der(3q27) and der(14q32) junctional regions showed short deletions or duplications (cases 2, 4, 5, and 6) or precise double-stranded DNA breaks (Figure 3). Overall, in 5 of 7 cases the breakpoint was within or in close proximity to RGYW/WRCY motifs in *BCL-6* (Figure 3).

Although switch events are involved in the translocated allele, we failed to find any homology with switch recognition motifs at the *BCL-6* junction site on 3q27. This suggests involvement of additional proteins in addition to the switch recombinase. *BCL-6* sequences at the junctional regions showed short deletions or duplications or precise double-stranded DNA breaks. Short-template insertions, sometimes with single substitutions, suggest a short-patch error-prone DNA synthesis. These features are consistent with a role for the hypermutation mechanism. A common enzyme, activation-induced cytidine deaminase, is required for *V* gene somatic mutation as well as switch recombination.[24] Whether this key enzyme is implicated here in t(3;14) lymphoma is as yet not known. Our data show that at least 2 separate mechanisms, somatic mutation and isotype switch, underlie this frequent and important translocation in lymphoma.

References

1. Allman D, Jain A, Dent A, et al. BCL-6 expression during B-cell activation. Blood. 1996;87:5257-5268.
2. Dent AL, Vasanwala FH, Toney LM. Regulation of gene expression by the proto-oncogene BCL-6. Crit Rev Oncol Hematol. 2002;41:1-9.
3. Butler M, Corbally N, Dervan PA, Carney DN. BCL-6 and other genomic alterations in non-Hodgkin's lymphoma (NHL). Br J Cancer. 1997; 75:1641-1645.
4. Bastard C, Deweindt C, Kerckaert JP, et al. LAZ3 rearrangements in non-Hodgkin's lymphoma: correlation with histology, immunophenotype, karyotype, and clinical outcome in 217 patients. Blood. 1994;83:2423-2427.
5. Akasaka H, Akasaka T, Kurata M, et al. Molecular anatomy of BCL6 translocations revealed by long-distance polymerase chain reaction-based assays. Cancer Res. 2000;60:2335-2341.
6. Ye BH, Chaganti S, Chang CC, et al. Chromosomal translocations cause deregulated BCL6 expression by promoter substitution in B cell lymphoma. EMBO J. 1995;14:6209-6217.
7. Kaneita Y, Yoshida S, Ishiguro N, et al. Detection of reciprocal fusion 5′-BCL6/partner-3′ transcripts in lymphomas exhibiting reciprocal BCL6 translocations. Br J Haematol. 2001;113:803-806.
8. Migliazza A, Martinotti S, Chen W, et al. Frequent somatic hypermutation of the 5′ noncoding region of the BCL6 gene in B-cell lymphoma. Proc Natl Acad Sci U S A. 1995;92:12520-12524.
9. Lossos IS, Levy R. Mutation analysis of the 5′ noncoding regulatory region of the BCL-6 gene in non-Hodgkin lymphoma: evidence for recurrent mutations and intraclonal heterogeneity. Blood. 2000;95:1400-1405.
10. Artiga MJ, Saez AI, Romero C, et al. A short mutational hot spot in the first intron of BCL-6 is associated with increased BCL-6 expression and with longer overall survival in large B-cell lymphomas. Am J Pathol. 2002;160:1371-1380.
11. Pasqualucci L, Migliazza A, Basso K, et al. Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. Blood. 2003;101:2914-2923.
12. Akasaka H, Akasaka T, Kurata M, et al. Molecular anatomy of BCL6 translocations revealed by long-distance polymerase chain reaction-based assays. Cancer Res. 2000;60:2335-2341.
13. Lossos IS, Jacobs Y, Cleary ML, Levy R. Re: Akasaka, H., et al., Molecular anatomy of BCL6 translocations revealed by long-distance polymerase chain reaction-based assays [letter]. Cancer Res. 2001;61:7363-7364.
14. Jacobs H, Bross L. Towards an understanding of somatic hypermutation. Curr Opin Immunol. 2001;13:208-218.
15. Jaffe E, Harris N, Stein H, Vardiman JE. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyons, France: IARC Press; 2001.
16. Jardin F, Gaulard P, Buchonnet G, et al. Follicular lymphoma without t(14;18) and with BCL-6 rearrangement: a lymphoma subtype with distinct pathological, molecular and clinical characteristics. Leukemia. 2002;16:2309-2317.
17. Kawamata N, Nakamura Y, Miki T, et al. Detection of chimaeric transcripts of the immunoglobulin heavy chain and BCL6 genes by reverse-transcriptase polymerase chain reaction in B-cell non-Hodgkin's lymphomas. Br J Haematol. 1998; 100:484-489.
18. Vitolo U, Botto B, Capello D, et al. Point mutations of the BCL-6 gene: clinical and prognostic correlation in B-diffuse large cell lymphoma. Leukemia. 2002;16:268-275.
19. Zan H, Li Z, Yamaji K, et al. B cell receptor engagement and T cell contact induce Bcl-6 somatic hypermutation in human B cells: identity with Ig hypermutation. J Immunol. 2000;165:830-839.
20. Ottensmeier CH, Thompsett AR, Zhu D, et al. Analysis of VH genes in follicular and diffuse lymphoma shows ongoing somatic mutation and multiple isotype transcripts in early disease with changes during disease progression. Blood. 1998;91:4292-4299.
21. Rabbitts TH, Forster A, Hamlyn P, Baer R. Effect of somatic mutation within translocated c-myc genes in Burkitt's lymphoma. Nature. 1984;309: 592-597.
22. Tanaka S, Louie DC, Kant JA, Reed JC. Frequent incidence of somatic mutations in translocated BCL2 oncogenes of non-Hodgkin's lymphomas. Blood. 1992;79:229-237.
23. Zhang K, Cheah HK, Saxon A. Secondary deletional recombination of rearranged switch region in Ig isotype-switched B cells. A mechanism for isotype stabilization. J Immunol. 1995;154:2237-2247.
24. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-563.